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S 1 - GERMPLASM AND GENETIC RESOURCES

Variety of Barley Crops in the Republic of Yemen

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1 - Preface

At the beginning of this paper, I'd like to thank the organizers of this conference for inviting me to participate and for giving me the chance of attending, especially Prof. Dr. Oldrich Chloupek who was my colleague in faculty of agriculture in Brno and he is now the chairman of the department of crops, faculty of agronomy- MENDELOVA ZEMEDELSKA A LESMICKA UNIVERSITA.

There is no doubt that the agriculture has its own importance all over the world, and it is the main pillar to which many countries pay of all attention in as much as it is the main support for human to continue living. Since this conference is focusing mainly on the barley crop, and specifically on the genetic side by which we can finally get a new kind of crop with a high quality and productivity.

Before presenting my brief paper, I'd like to talk briefly about my country and its geographical position on earth, perhaps many of you know a little about my country, though today the world becomes a small village due to the development of telecommunications and transportation means.

Republic of Yemen is located in the southwest of Arab peninsula, it is distinguished with its excellent position, it overlooks on the Red Sea to the west, which separates Yemen from Africa. The Kingdom of Saudi Arabia borders it from the north, the Arabian Sea and Oman to the east and to the south is the Arabian Sea.

Yemen can be divided into four sections they are as follows:

1- The plain area along the Red Sea, in these areas, the temperature is high all over the year.
2- The central area which is a chain of mountains, some of them rises to more than 200 m above the sea level, between these mountains lie many valleys and farmlands, the rain there falls heavily in some seasons for which the farmers can depend for farming.
3- The eastern area which is a separate mountains, through them there are many valleys and fields.
4- The area in the far east, which is part of Al Rub Alkhali desert.

The total area of Yemen is around 555,000 square Km, and the population is nearly 20 millions, 65% of them work in the agriculture sector, the rest works in trading, industry and vocational careers.

Republic of Yemen has relations with many countries all over the world, it exports to them oil and gas but in a limited quantity. In addition to coffee, leather, fish, fruits and vegetables which are exported to the neighboring countries and others. Yemen imports many things from all the countries. These imports reach to 80% in comparison to the exports.

2 – The Objectives

The purpose of this paper is to transfer a complete picture of planting the barely crop in our country and how it is cultivated, also to present the different kinds of the genetically improved crop and areas of the cultivated land in some governorates.

Growing the barely crop in Yemen is limited because few people come to buy the crop, it is not used as a bread any more except in some cases. Nowadays people would like to buy the wheat instead that is widely used in the bread, cake, desserts…etc. Due to the large imports from the USA, Canada and Australia, Yemeni farmers grow a small amount of barley and wheat in their fields.

Look at the table below which shows the areas of barley crop by Hectare and production in ton.

Area/ha and production/ton table

Years					Area/ha Production/ton	Governments
2002	2001	2000	1999	1998		
18843	19666	18116	18116	23240	Area/ha	Sana'a
13617	15721	14697	16331	24197	Product./ton	
2553	2722	2409	2409	2188	Area/ha	Ibb
4121	4918	4771	4337	3907	Product./ton	
2633	2731	2683	2712	2851	Area/ha	Sadah
3115	3411	3312	3400	3763	Product./ton	
2204	2360	2115	2001	2116	Area/ha	Al Jawf
26792	2813	2711	2510	2749	Product./ton	
26792	27479	25323	25238	84637	Area/ha	Total
23057	26863	25441	26578	34616	Production/ton	

References: General Department of Agricultural Static/Ministry of Agricultural and Irrigation. 2000 - Year Book.

I'd like to apologize for not presenting a complete paper about barley and the work related to the genetic side of it due to the scarcity of researches on this field, but I have known that the general authority for researches in Dhamar governorate tested four kinds of barley brought from outside which give high production better than the local one. They began expanding farming these kinds by distributing their seeds to the local farmers.

These four kinds are:

1- Arafat
2- Bitcher
3- Mabser – Dhamar
4- Jahrani

And there is a local kind called (Sagleh). There is no data available on the detailed contents that features the local kind.

It has been noted that Yemeni people are not interested in growing barely since it is not baked as bread. It is used only as a syrup in the wedding ceremony and the Qat chewing sessions, but it is mostly used as a fodder for animals. In addition, it is not used as a bear drink as it is happening in Europe because it is prohibited as Yemen is an Islamic country.

3 – Summary

As I mentioned before the growing of barley is limited since the Yemeni farmer does not like to expand it because it is sold in a small amount in the Yemeni market and not be used as a bread.

Nowadays, Yemeni family greatly turn to grow wheat because the increasing demand in the local market, it is much consumed as a bread cakes, biscuit …etc.

Because of the large demand for wheat by consumers, the government has to import it from abroad in hard currency. It imports about 1,126,531 tons with the cost of 150,000,000 $ that means 133$ for each ton.

It is mainly imported from the USA, Canada and Australia and sometimes as a grant.

Now, the government is working hard to expand planting the wheat by encouraging the farmers to grow it. The aim of the government is to reach the self-sufficiency and to reduce the imports. Currently, the ministry of agriculture is supporting the farmers through its local offices in making extension services on how to choose the suitable soil and the proper climate for wheat and by easing the use of the modern techniques which will help to increase the production in quality and quantity as well.

Sources: the central organization for statistics.

Republic of Yemen – Ministry of Agriculture & Irrigation
Agricultural Research & Extension
Authority (AREA) – Head Quarter

4 – Recommendations

I'd like to suggest to make a team from the participants in the conference to have a visit to Yemen to be familiar with the circumstances of this amazing country which was unknown to many countries before the revolutions of 26th Sept. 1962 and 14th Oct. 1963. These revolutions made a large turnover in my country and lead Yemen to be opened to all world Often it was completely closed state where the life was like in the medieval ages.

The visitors will see the wonderful and astonishing habits and traditions as well as behaviors of the Yemeni people.

Furthermore, they will find out a rare plants especially in Socotra Island which is near to Hadramout governorate to the south of Yemen. Also they will notice the deferent climates despite the short distances between them. There is nothing like Yemen anywhere in the world. When you drive for two hours you will find the climate is completely different, therefore, there can be found a different kind of crops though they are close to each other. Also there are many special agricultural crops particularly fruits and vegetables and due to the variety of the climate you will find these crops all over the year.

One of the special characteristics is its delicious taste for example:

The grape has many different kinds. It is different to find grape like it. It has a high percentage of sugar and it is proverbial in the Arab world. Beside that it can be found for six months in the market while the other crops often are yield twice a year, while the fodder crops in some parts is existed all over the year.

I kindly invite you to come to this interesting country and you will find the truth yourself, and I will be available with any visitor from you.

Look at the attached photos. (Pictures) from Yemen.

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Matching Soil, Water and Genotype for Barley Cultivation in Kuwait

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Abstract
In 1996 a government master plan was approved for the greening and agriculture of Kuwait. Barley is among 50 prioritised plants. A general survey of all Kuwait soils identified potential sites for cultivation, greening and other uses. Wafra was for selected as a potential cultivation site and the soil was investigated further using USDA standard tests. The ground water at Wafra was brackish, but had potential for irrigation of salt tolerant crops such as barley. Large field trials, irrigated with local ground water and imported fresh (desalinated) water, were set up over two seasons. A wide range of genotypes (141) were tested and included: 1) control recommended salt tolerant cultivars; and various 2) wild barleys; 3) semi-dwarfs and 4) parental lines. Various agronomic traits were investigated, the most important being plant weight (barley is used mainly for fodder). The two control genotypes performed well in brackish and fresh water irrigation. A list of the best performers in both treatments contained representatives from all four groups. Semi-dwarfs (*ari-e*. mutants) were notable by their performance in brackish irrigation. Sustainable barley cultivation is feasible in Kuwait using selected soils, local ground water, soil rejuvenation, water table control and selected genotypes.
Keywords: Kuwait; barley; soil; water; salinity

Introduction

Kuwait is a desert country and farming takes up less than 17,000 hectares (3% of the land area). Farming is confined to the Abdali region in the North and the Wafra region in the south. Brackish water is used for irrigation in these areas and can lead to rising water tables and secondary soil salinity. In Abdali the use of ground water caused a build up of salts and sodicity (AL-RASHAD & AL-GHAWAS 1999). At Wafra salinity increases with increasing depth (AL-RASHED & AL-SENAFY 1995). The combination of aridity and salinity in Kuwait limits the range of crops.

Barley (*Hordeum vulgare*) is an important forage crop in Kuwait, but the lack of local production necessitates expensive import. The Public Authority for Agriculture and Fish Resources (PAAFR) of Kuwait has surveyed several thousand of hectares with a view of expanding barley cultivation in Kuwait. Barley is also is the most drought and salt tolerant of the small grain cereals and included in the list of priority crops in the "Kuwait Greenery National Plan" (KISR 1996) and in the Agriculture Master Plan of the State of Kuwait (KISR 1997). The work programme was therefore set up to survey and match soils, water and genotypes for barley cultivation in Kuwait.

Material and Methods

Test Site
Following a national soil survey (OMAR *et al*. 2001) a barley cultivation test site was selected at Wafra. The Wafra soils are loose and susceptible to wind erosion and a protected site, surrounded on three sides by tamarix trees, was chosen. The deep-rooted trees provided an additional advantage in that they prevented a rise in the water table, a hazard in irrigation schemes.

Soil Survey
Physical and chemical properties were evaluated in the field and laboratory using USDA standards. The soil was analysed before and after each field trial. The soil type was defined as Muslan, but importantly was non-saline down to a depth of 100-150 cm. In general the soil was non-gypsiferous, calcareous and non-sodic with a very low clay and organic matter content and with a low water storage capacity. A more detailed soil description is given in Al-MENAIE (2003).

Water Analysis
Salinity of the ground water at Wafra was measured by electrical conductivity (6.75 dSm^{-1}) and classed as S1 (low sodium adsorption ratio) and C4 (high salinity) (RICHARDS 1954). This brackish water was saturated with Ca and SO_4. The analysis indicated that Al-Wafra water could be used in conjunction with permeable soils and moderately salt tolerant crops such as barley. More details of the water analysis are given in AL-MENAIE (2003).

Genotype Survey
A wide range of barley germplasm was surveyed for field testing in 1999/2000 and included the groups:
1. Recommended lines for the regions, ICARDA recommended the salt tolerant cultivars Gustoe and California Mariout.
2. Wild barley lines from the "Fertile Crescent" (primary centre of diversity of *H. spontaneum*), previously tested for salt tolerance (PAKNIYAT *et al*. 1997).
3. Semi-dwarf mutant lines, previously assessed for salt tolerance (FORSTER 2001).
4. Parental lines of genetic mapping populations, including cultivars, breeding lines and landrace material.

In the second field trial (2000/2001) the best and worst lines were re-tested along with F_1 hybrids from selected crosses.
Over 140 genotypes were tested over two seasons. Two treatments were applied: fresh water irrigated and ground water irrigated, applied at the surface. It was important that these treatments did not overlap and were separated 1.5 m in a split plot experimental design. In the first year 141 genotypes were tested. Seed were sown in parallel rows with one replicate per genotype occupying 1.25 m of a row in a bed. Each replicate row consisted of 5 seed spaced at 25 cm intervals. Each treatment plot was composed of 3 beds, and each bed consisted of all (randomised) genotypes. After harvest salt was flushed from the soil with fresh water. The second field trial consisted of 54 entries.
Data were collected on: plant weight, number of seed/spike, number of seed/plant, spike weight, survival, number of tillers, height of main stem and tillers.

Results

Because of the large data sets (>10,000 data points), only an overview is presented here. Over all yields in brackish water were reduced by about 50%, but there were strong genotype and genotype*treatment interactions for all traits in both trials. Table 1 gives a summary of the first trial results, because biomass was considered the most important trait for Kuwait, the genotypes have been ranked according to plant weight. Relative performance was calculated as: $\frac{\text{Control} - \text{Stress}}{\text{Control}} \times 100$

Table 1. First year overall rankings for relative performance of the top 10 and worst 10 genotypes arranged according to plant weight

Genotypes (*Group)	Plant weight	No. of seed/ spike	No. of seed/ plant	Spike weight	Survival	No. of spikes	No. of tillers	Height of the main stem	Mean height of tillers	Overall ranking
Golden Promise[3]	1	58	20	10	16	31	38	107	18	3
Tab 37[2]	2	108	120	94	108	85	67	92	49	95
*ari-e.*228[3]	3	40	16	24	77	35	43	115	61	16
*ari-e.*1[3]	4	53	22	46	110	25	26	105	56	29
*ari-e.*156[3]	5	70	38	40	3	41	37	82	104	19
Tab 45[2]	6	22	99	9	141	116	107	90	112	84
Foma[3]	7	49	27	39	39	62	64	73	71	22
Gustoe[1]	8	44	15	53	137	29	36	117	82	47
Maythorpe[4]	9	23	10	14	61	26	30	61	37	2
California Mariot [1]	10	28	13	33	105	44	48	87	119	37
I-15[2]	132	111	85	8	94	78	74	72	52	89
T-20[2]	133	59	71	132	100	76	72	140	140	130
Gadot[2]	134	69	116	106	40	121	119	52	121	126
I-6[2]	135	99	48	67	22	22	14	129	128	73
NL 5/1[2]	136	79	82	122	23	86	86	35	31	80
T-17[2]	137	133	134	65	15	118	112	66	93	124
NL 5/2[2]	138	45	55	126	73	74	81	78	43	92
I-1[2]	139	120	64	83	34	24	25	119	103	91
T-7[2]	140	86	94	125	26	95	93	125	115	128
I-2[2]	141	68	124	72	78	125	125	123	125	137

* [1]Recommended, [2]wild, [3]mutant , [4]parental genotype

Similar results were obtained in Year 2 with Golden Promise, *ari.e.*228, Foma, Gustoe and Maythorpe being in the top 10 in terms of overall relative performance (California Mariout was just outside the top 10, twelfth), and with the worst all being wild barley lines (AL-MENAIE 2003).

Discussion

Taking both years into account, promising genotypes could be identified for fresh and brackish water irrigation in Kuwait, Table 2.

Table 2. Promising genotypes for biomass production

Fresh water irrigation	Brackish water irrigation
Gustoe[1]	Gustoe[1]
California Mariout[1]	California Mariout[1]
Derkado[4]	Golden Promise[3]
Bonus[4]	Derkado[4]
Maythorpe[4]	ER/Apm[4]
26-35[2]	*ari-e*.228[3]
7-168[2]	B83-12/21/5[4]
ER/Apm[4]	*ari-e*.156[3]
NM6/5[2]	Bonus[4]
22-30[2]	Foma[4]

Genotypes from all four groups possessed useful traits and some interesting contrasts were revealed between the best genotypes in fresh water irrigation compared to brackish. For example four of the top ten lines in the brackish treatment carry semi-dwarf alleles at the *Ari-e*. locus (Golden Promise, B83012/21/5, *ari-e*.228 and *ari-e*.156). The salt tolerance of these lines has been noted in previous studies (FORSTER 2001; PAKNIYAT *et al*. 1997a, 1997b). Furthermore, studies by Al-MENAIE (2003) showed that ari-e. genotypes had short roots and this may have an additional advantage at Wafra in not penetrating into the saline soil zone at 100-150 cm depth.. There has been no breeding of barley for Kuwait and the potential for improvement is great. Traditional breeding (crossing the best with the best) is one route forward as recommended cultivars, California Mariout and Gustoe, showed good performances. These two cultivars have been crossed together and doubled haploids (DHs) have been produced from the F_1 for further studies. Landrace and wild barley genotypes, as in other studies (CECCARRELLI *et al*. 1998; ELLIS *et al*. 2000; FORSTER *et al*. 2004), were also identified for breeding and genetic studies of abiotic stress tolerance. Selected genotypes have been crossed onto California Mariout and Gustoe and DH lines produced. These populations have been developed to study the genetics controls of performance as well as to initiate a barely breeding programme in Kuwait.

Irrigation with brackish water is of concern. In this study we found that brackish water irrigation had positive and negative effects on soil properties, but analysis after each trial showed that soil rejuvenation was possible by flushing out salts with fresh water. The site benefited from the presence of tamarix trees in maintaining the level of the water table and reducing wind damage. The work demonstrates that barley production can be expanded in Kuwait, but an integrated approach is necessary in matching soils, water, genotype and ecology.

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SSR-Based Genetic Diversity Assessment among Tunisian Winter Barley and Relationship with Morphological Traits

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Abstract

For studying genetic diversity caused by selection for adaptation and end-use, 17 microsatellites (SSR), representative of the barley genome, were used in 26 barley (*Hordeum vulgare* L.) accessions and cultivars in Tunisia. The accessions/cultivars originate from different geographic regions and are of different end-use. For the 15 polymorphic SSR, the mean number of alleles per locus was 3.6 and the average polymorphism information content was 0.45. Cluster analysis based on SSR data and on morphological data clearly differentiate the genotypes according to their type (local landraces vs. varieties), row-number and end-use. The correlation between both diversity measures was highly significant (r=0.25, $P<10^{-5}$) and the correspondence between the clustering based on SSR and morphological data was relatively good. Our results show the large genetic diversity of the Tunisian barley cultivars and the association of this diversity with adaptation traits.

Introduction

In Tunisia, barley is the most important cereal crop after wheat (*Triticum aestivum*) and occupies one 38% of the cereal cultivated area. Prior to national barley breeding programs, which started in the 1960's, cultivated barley represented essentially winter-type landraces. Despite their low productivity, they still constitute 40% of the present barley cultivated area.

Since the creation of barley breeding centres in Tunisia, new cultivars were developed by (i) selection from local populations, (ii) introduction of new varieties and (iii) crossing and selection for yield. Several high-yielding winter barley cultivars of two and six-row type were introduced from Algeria, France, Denmark, Australia, USA and from the International Centre for Agricultural Research in the Dry Areas (ICARDA) in Syria. However, the cultivation of two-row barley for brewing were rapidly replaced by six-row cultivars, mainly because of farmers' preference and agricultural practices, that were not relevant for two-row barley. Currently, the most interesting cultivars are 'Martin' and 'Rihane-03' introduced, respectively, from Algeria in 1931 and from ICARDA in 1986, which occupy about 20% and 60% of barley cultivated area, respectively.

Molecular markers that reveal polymorphism at the DNA level have been shown to be a very powerful tool for genotype characterisation and estimation of genetic diversity. Among these, microsatellite or SSR (simple sequence repeats) markers were showed to have a high potential for identification and estimation of genetic diversity of barley germplasm collection (SAGHAI-MAROOF *et al.* 1994; RUSSEL *et al.* 1997b; STRUSS & PLIESKE 1998; PILLEN *et al.* 2000). The fact that microsatellite sequences were shown to be more frequent in transcribed regions (MORGANTE *et al.* 2002) and that microsatellite markers are under the influence of natural selection in barley and wheat (SAGHAI–MAROOF *et al.* 1994; STACHEL *et al.* 2000) constitutes the advantage of SSR markers to assess the relationship between DNA polymorphism based on microsatellite markers and adaptation of barley accessions to particular

environments.

To date no information is available i genetic variation of Tunisan barly germplasm at the molecular level. The objectives of the present study were to use the microsatellite markers, described by SAGHAI-MAROOF *et al.* (1994) and LIU *et al.* (1996) and which are distributed all over the barley genome, for characterisation of Tunisian barley germplasm including two-row and six-row winter barley accessions and estimation of the genetic diversity. Barley germplasm was also characterised for morphological and agronomic traits and relationship between genetic similarities based on SSR markers and euclidian distances based on some morphological and agronomic traits is described.

Material and Methods

Plant Material and DNA isolation

Twenty six Tunisian winter barley cultivars /landraces of diverse geographic origin, end-use and row type were used in this study (Table1).

SSR Markers and Protocol

Seventeen SSR markers from the commercially available Mappair primer sets developed by Research Genetics Inc. (Huntsville, USA) (Table 2). They provided at least one marker per chromosome, however there were four markers on chromosomes 6 and 7. PCR amplification was performed with 20 ng DNA in 15 µl volume reaction according to the protocol developed by LIU *et al.* (1996). The amplification products were resolved on 6% polyacrylamide gels (PAGE) followed by silver-staining according to the protocol described by PILLEN *et al.* (2000).

Morphological and Agronomic Traits

Twelve traits were scored on the 26 barley lines. All traits, except the number of rows in the spike (NR), were evaluated using one-row plot of 1.5 m and three replicates. These include morphological traits (NL, LI, HMB, LB), earliness and maturity traits (FL, PhM, EP, DRG) and yield components (NGE, PMG). All traits were standardised before analysis.

Data Collection and Analysis

Marker Polymorphism

To measure the informativeness of the markers, the polymorphism information content (PIC) for each SSR was calculated according to the formula: $PIC= 1- (\Sigma p_i^2)$, where i is the total number of alleles detected for an SSR marker and p_i is the frequency of the ith allele in the set of 26 barley cultivars investigated. Null allele is considered as one of the series of multiple allele and is included in the computation of PIC values. PIC is also an estimate of the discriminatory power of an SSR marker locus.

Genetic Similarity Estimation and Cluster Analysis

Each SSR band was scored as present (1), absent (0) or as a missing observation for the different cultivars. Genetic similarity (gs) between two cultivars i and j was estimated following NEI & LI (1979). Based on the genetic similarity matrix (denoted GS), UPGMA cluster analysis were used to assess pattern of diversity among the barley entries. To test the goodness of fit of this clustering to the genetic similarity data, the cophenetic correlation, r_c, that is the correlation between the matrix of gs_{ij} and the matrix of 'cophenetic' similarity value (computed from the tree matrix), was calculated. All calculations were performed using the NTSYS-pc version 2.1 software (ROHLF 2000).

Analysis of Morphological Traits

A principal component analysis (PCA) was performed on observed morphological traits after standardisation All traits were standardised by subtracting the mean value and dividing by the standard deviation; this allows to remove scale effects before calculating Euclidian distances. Based on standardised trait values, euclidian distances (md_{ij}) between the lines were calculated. Morphological similarities (ms_{ij}) were also calculated as ($1-md_{ij}$). Matrix of these values is denoted MS. Using the matrix (denoted MD) of euclidian distances, an UPGMA cluster analysis was performed producing a second dendrogram depicting relationships among cultivars relative to their morphological characteristics. As for genetic similarity, the cophenetic correlation was calculated to measure the quality of the clustering with regard to the original data.

Comparison of Marker and Morphological Data

Simple (r) and Spearman rank correlation (rs) coefficients between the 325 values of genetic similarities (gsij) and morphological similarities (msij) were calculated. P-values for these coefficients were calculated based on their respective asymptotic distributions (Kendall & Stuart, 1979).Correspondence between the two similarity matrices GS and MS (matrix of msij values) as well as between their corresponding cophenetic matrices was tested with the Mantel Z statistic (Mantel 1967). Significance of Z was determined by comparing the observed Z values with a critical Z value obtained by calculating Z for one matrix with 1000 permuted variants of the second matrix. All computations were performed with appropriate procedures of the NTSYS-pc version 2.1 software (Rohlf 2000).

Results and Discussion

Characteristics of SSR Markers

Seventeen microsatellite markers dispersed across the genome were used to test the genetic diversity of 26 landraces/cultivars. Fifteen SSR markers generated polymorphic patterns and two (HVDHN9 and HVM64) gave a monomorphic pattern, yielding a polymorphism rate of 88.2%. A total of 55 alleles was detected by 15 markers. The number of alleles per locus varied from 2 to 7, with a mean of 3.6 alleles/locus (Table 2). The mean PIC for the 15 SSR markers was 0.45, with values ranging from about 0.068 for HVM11 to 0.78 for HVM74. This result confirms that SSR markers are highly informative compared to the value (0.38) obtained by Pillen et al. (2000) who used 22 microsatellite markers and a set of 28 mainly German barley cultivars and two wild forms.

Genetic Diversity Levels

The average GS among the barley lines was 0.58 with values ranging from 0.31 between ‘Roho’ and the accession collected from Bizerte and 0.94 between ‘Cowra’ and Tunis. The average GS among the OLT accessions is significantly larger (0.70), ranging from 0.47 between OLTGabes2 and OLTGabes9 and 0.87 between OLTTeboulba and OLTGuellala.

Based on GS values, we attempted the identification of the accessions collected from different areas in Tunisia by examining the most similar known cultivar (having the highest genetic similarity). The most similar cultivars/landraces to the accessions collected from the regions Tunis, Bizerte, Ariana and Dinar, were, respectively, ‘Cowra’, OLT Gabes8, and ‘OrgeBlanche’. These relationships were supported by similarity of morphological traits of the spike. For example, the genotype Tunis and the cultivar ‘Cowra’ which have high genetic similarity value, have also dense spike, and yellow awn and glume.

Genetic Diversity Pattern

UPGMA cluster analysis of SSR genetic similarity matrix resulted in the phenogram in Fig.1, which has quite a good fit to the GS matrix (r_{cs}= 0.77). Four groups can be distinguished by truncating the dendrogram at gs value of 0.55. The major group (denoted group I) consists of 20 genotypes and includes all OLT landraces, cultivars derived from local landraces ('OrgeBlanche'), the accessions collected from Tunis, Dinar, Ariana and Bizerte as well as introduced cultivars ('Rihane-03', 'Hor1259', 'Cowra'). The position of cultivars 'Rihane-03' and 'Hor1259' within the OLT group is consistent with their origin; they are both derived from a cross involving Atlas (As) as a parent genome that is a local landrace collected from the Atlas mountains of North Africa. Several local accessions were collected by Australian breeders at the beginning of the nineteen century of which 55 were catalogued in ICARDA. In this group I microsatellite markers used were also able to discriminate between all the OLT landraces even when they were originated from the same region (four from Oasis Gabes) and have the same common name (Sfira). This high discrimination between closely related landraces is also the result of the large heterogeneity of local landraces, which are defined in Tunisia as geographically based populations. Each population has typical characteristics of the grain appreciated by farmers and local inhabitants for food and tasting properties (MEDIMAGH, pers. Comm.). GRANDO *et al.* (2000) mentioned that considerable heterogeneity exists both between landraces collected in different farmers fields (if designated by the same name) and between individual plants within the same farmers field for several plant characteristics.

In group I, a subgroup consisting of 'Hor1259' together with 'OrgeBlanche' and Ariana corresponds to a homogeneous Group of fodder barley cultivars.

Another group (Group III) includes all three two-row barley cultivars ('Faiez', 'Taj', 'Roho'). These results are consistent with those obtained by several authors who used RFLP, RAPD and AFLP markers (GRANER *et al.* 1994; MELCHINGER *et al.* 1994; RUSSELL *et al.* 1997a). 'Martin' and 'Manel' are clustered together and form a separate group (group II). The cultivar 'Mumtez' is well separated from the other groups and forms Group IV.

Morphological Analyses

In the principal component analysis (PCA), the first four principal components (having eigenvalues > 1) explained about 87% of the variation. The first two axes explaining about 58.4% of the variation, were, respectively, linked to variables related to yield and maturity (correlated negatively with EP, PhM and PMG and positively with HMB and NGE, (r> |0.7|) and to earliness (positively correlated with NR and Fl, r>0.8). The third axis, explaining 17.9% was correlated positively with NF (r=0.79) and negatively with LEN (r=-0.95).

Based on the projection of varieties in the first principal plan (Fig. 2A) one can distinguish four groups. The first group in the lower-left part of Fig. 2A comprises 'Faiz', 'Roho' and 'Taj', which are the two-row early barley commercial varieties introduced in the end of the seventies and mainly characterized by high PMG and small NGE. On the opposite side, a second group is composed by the accession collected from Ariana, 'OrgeBlanche' and 'Hor1259', which have naked grain and are fodder barley characterised by their tall straw (high HMB), late ear emergence (high EP) and high leaf area (high NF). Note that these two groups are also clearly distinguished in the second principal plan of axes 1 and 3 (Fig. 2B).

The central group in Fig. 2A could be split into two groups: first one composed of varieties 'Martin', 'Cowra', the accessions collected from Bizerte and Tunis, and Safra, and the second composed of all the remaining varieties, i.e., all the OLTs plus 'Manel', 'Rihane-03', 'Mumtez' and the accession collected Dinar. However, when examining the second principal plan, one sees (Fig. 2B) that 'Manel', 'Rihane-03', and 'Mumtez', which are six-row high-yielding commercial cultivars could now be considered as a separate group whereas the group of the five cultivars/landraces 'Martin', 'Cowra', Safra, Tunis Bizerte and is now spread and partly mixed with the OLT group. This gives further homogeneity of OLT group, whereas the nine remaining genotypes ('Martin', Bizerte, Tunis, 'Cowra', Safra, 'Manel', 'Rihane-03', 'Mumtez' and Dinar) may be considered as a heterogeneous group including both old cultivars used since the 1930s ('Cowra', Tunis, 'Martin', Safra, Dinar) and registered commercial varieties used since the end of the 1980s ('Manel', 'Rihane-03', 'Mumtez').

Comparison of SSR and Morphological Traits-Based Diversity Estimates

Correlations between GS and MS values were significant (r=0.25, $P<10^{-5}$; r_s=0.22 $P<10^{-3}$). The Mantel Z test statistic was also significant between GS and MS matrices (Z=2.43; P=0.006) as well as between the cophenetic matrices of GS and MS (Z_c=2.88; P=0.002). These results indicate a good correspondence between the two similarity measures. The relatively high correlation between morphological distances and genetic similarity based on SSR markers is revealed by a similar grouping of varieties; in fact, three consistent groups were found in both classifications (SSR and morphological-based): the two-row group ('Faiez', 'Roho' and 'Taj'), the naked fodder group (Ariana, 'Hor1259' and 'Orge blanche') and the OLT group. The other varieties (including landraces and commercial cultivars) are so diverse with respect to their genetic background that no consistent relationship could be found. When these varieties were removed and the remaining were re-analysed, we obtained an even better correspondence between SSR and morphological similarities (Z=4.47, P<0.0001; Z_c=4.85, P<0.0001).

In this study the microsatellites markers proved to be valuable in genotyping barley accessions. The finding of relationship between SSR based polymorphism and morphological traits showed that SSR markers are powerful to examine functional diversity. A broader study with a large collection of well characterised accessions collected from several areas of Tunisia will be realised using EST-SSR markers to examine genetic diversity in relation to adaptive variation.

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Table 1. Genotypes used in SSR and morphological analyses, their pedigree (when available), their breeding institute (for cultivars), end use and region of origin

Barley	Number	Breeder/ Country	End use
Landraces	12	Tunisia	Food
Collected accessions	4	Tunisia	
Six-row varieties	7	International and national breeding centers	Food and fodder
Two-row varieties	3	ICARDA, Riso laboratory Denmark, West Institue /USA	Brewing

Table 2. Characteristics of SSR markers used with the number of alleles and polymorphism information content calculated over a set of 26 barley genotypes

Marker	Repeat pattern	Chromosomal location	Number of alleles	PIC	Number of null alleles
HVM9	(TCT)5	3	2	0.45	0
HVM11	(GGA) 3	6	3	0.07	1
HVM20	(GA)19	5	4	0.54	0
HVM30	(CA)8	7	3	0.54	4
HVM31	(AC)9	6	2	0.07	0
HVM33	(CA)7	3	5	0.56	1
HVM40	(GA)6(GT)4	4	4	0.47	0
HVM43	(CA)9	5	6	0.67	0
HVM64	(GA)4(GT)7	6	1	0.00	0
HVM74	(GA)13	6	7	0.78	2
HVM77	(CA)7	4	4	0.66	2
HVCMA[C]	(AT)9	1	2	0.26	0
HVBKASI[§]	(C)10,(A)11	2	2	0.56	0
WMS6	(GA)40	4	3	0.53	7
HVLEU[§]	(ATTT)4	7	4	0.45	2
HVDHN7[§]	(AAC)5	7	4	0.20	23
HVDHN9[§]	(AC)6	7	1	0.00	0
Average			3.6	0.45	2.8

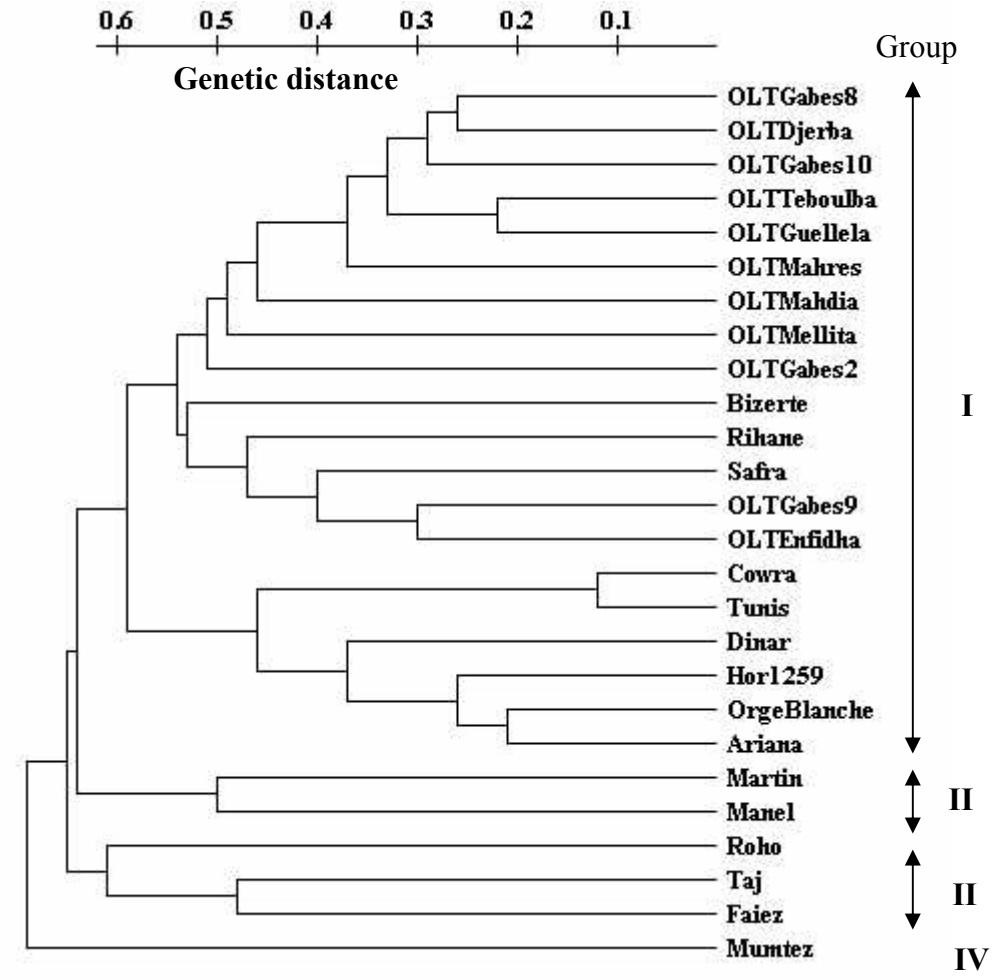

Fig. 1 Dendrogram resulting from an UPGMA cluster analysis of 26 barley genotypes and based on data of 15 microsatellite primer pairs

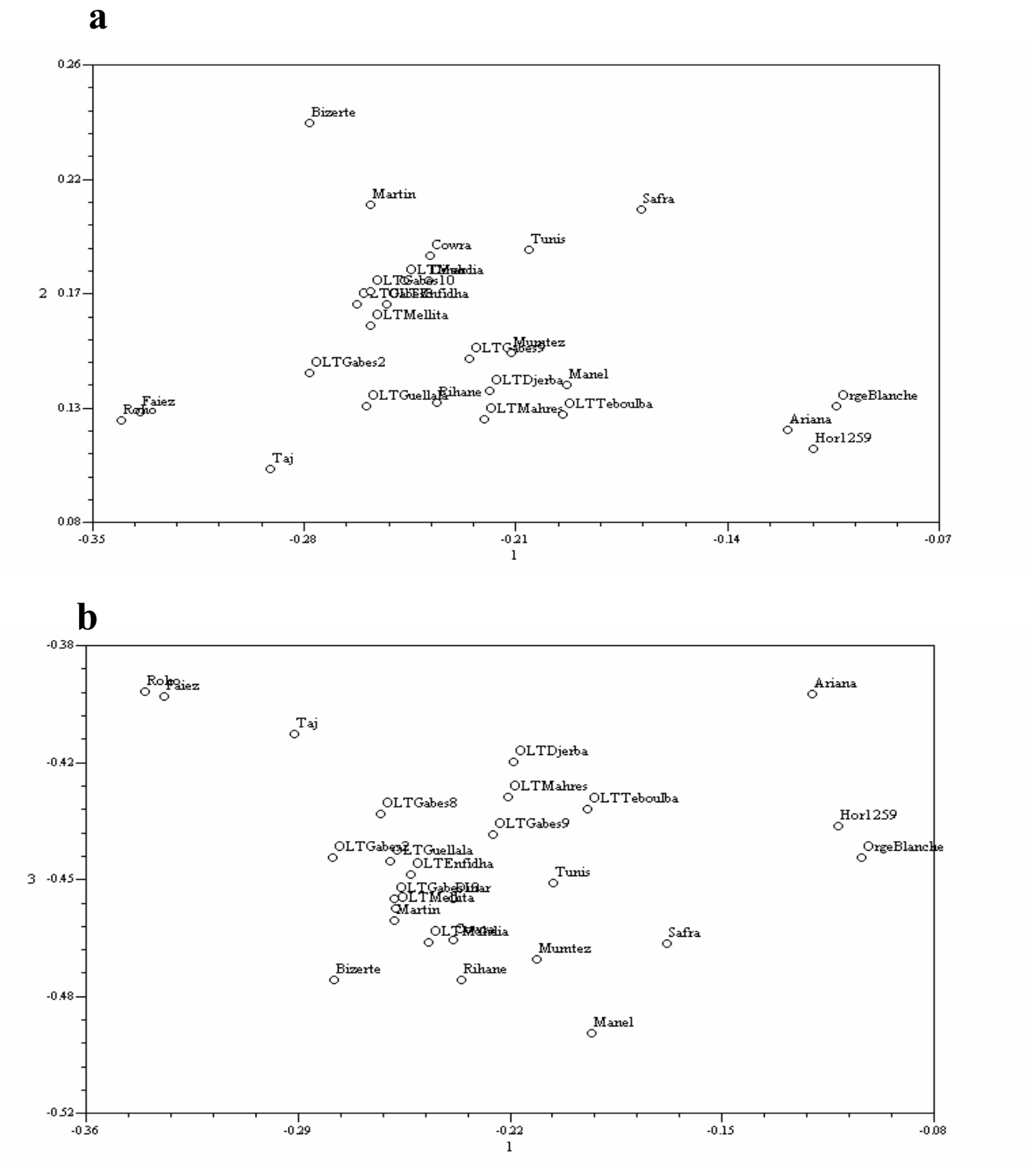

Fig. 2 Principal component analysis (PCA) two-dimensional plots of 26 barley genotypes based on morphological trait values. A. The first principal plan (PC1-PC2) B. the second principal plan (PC1-PC3).

Chances and Problems of Using Wild Genotypes for the Improvement of Quantitative Traits - A Case Study Using Barley

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Abstract
Decreasing genetic variability in modern cultivars may become a problem in the near future. The utilization of exotic genotypes is a possibility of broadening the genetic base, but presents many problems if quantitative traits are to be improved. A large number of suitable accessions exist in the gene banks, but how can a promising genotype be chosen without first undergoing a long and complicated back cross procedure?
To answer some of the many questions that arise when dealing with crosses between wild genotypes and modern cultivars ten parents were chosen from each group and 30 *Hordeum vulgare* x *Hordeum spontaneum* crosses were established in four back cross generations. A number of quantitative traits of the progeny as well as the cultivars and wild barley accessions used as parents were evaluated in field trials.
Means and variances of the progeny in different backcross generations were compared to the theoretical expectations of various traits. The general and specific combining ability of the modern cultivars and the wild barley were analysed. The potential for positive transgression for the trait yield was examined. The feasibility of predicting which *H. spontaneum* accessions will contribute most to an improvement of traits and the consequences for the choice of an optimal breeding methodology are discussed.
Keywords: *H. spontaneum*; quantitative traits

Introduction

Genetic resources are growing more and more important as the genetic variability of our modern cultivars decreases. Until now exotic material has only been used to improve traits influenced by either one or only a few genes. However wild forms have the potential to improve quantitative traits as well (SCHACHT 1998, ZELLER 1998).
To the present date a plant breeder does not have the knowledge to choose appropriate parents from the exotic material.
Studies using AB-QTL analysis can speed up the breeding process only if few wild accessions are used as parents as the high costs and huge amount of work make the analysis of many wild accessions nearly impossible.
In this study the inheritance of quantitative traits in "wide" crosses are examined using barley backcrosses between *H. vulgare* and *H. spontaneum* as a model.

Material and Methods

Ten randomly chosen *Hordeum vulgare* varieties were crossed with ten randomly chosen *Hordeum spontaneum* accessions using an incomplete factorial mating design. A number of backcross generations (BC_1 – BC_4) were then produced by crossing the descendents with their respective *H. vulgare* parents. In each backcross generation up to 28 lines were derived from each cross. The derived lines were selfed for at least three generations.
These lines were then evaluated in field trials with their parents for two years. A number of quantitative traits were studied, e.g. grain yield or height.

The resulting data was analysed by comparing the means for various quantitative traits of the progeny with the theoretical expectations that were calculated using the model developed by COX (1984). In addition the general and specific combining ability were examined. The estimation of variance components was computed in order to investigate the relative significance of the general and specific combining abilities.
Another interesting aspect was the ability of the *H. spontaneum* accessions to improve the performance of the modern varieties used as parents. To find out more about the potential of this exotic material the number of progeny with grain yields higher by at least two standard deviations than those of their respective *H. vulgare* parents was computed.

Results and Discussion
The phenotypes of the *H. vulgare* cultivars and the *H. spontaneum* accessions differ strongly for nearly all traits, as is shown in Figure 1. Especially traits that have been subjected to intensive breeding, e.g. grain yield, show large differences. *H. spontaneum* has many characteristics common to wild plants, e.g. seed shattering.

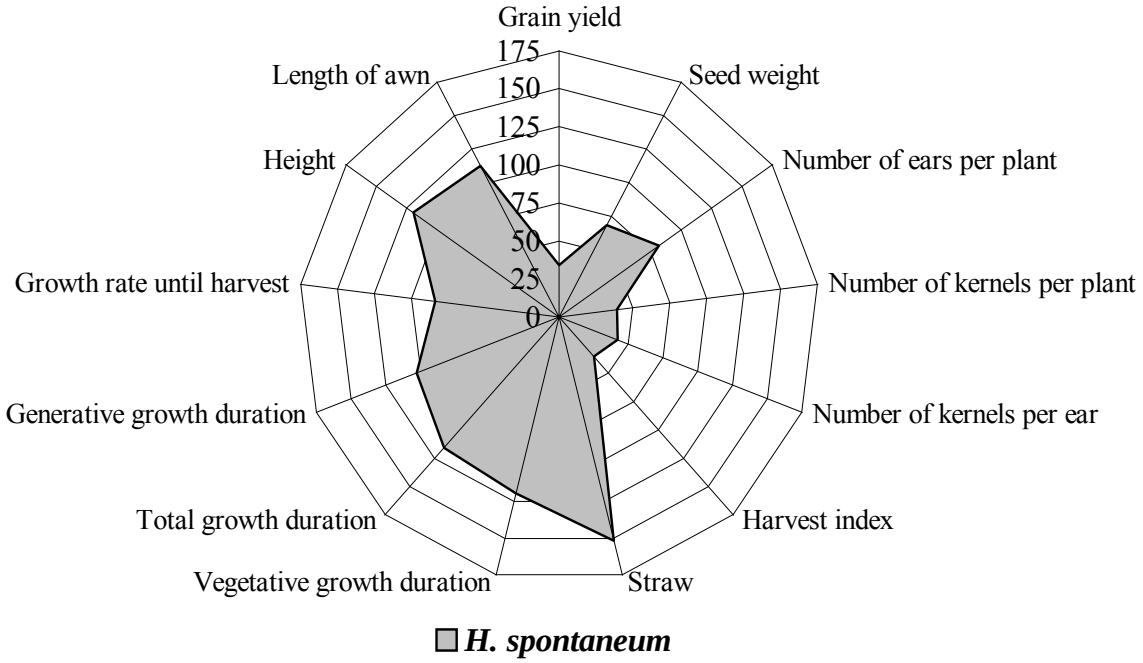

Figure 1: Traits of *H. spontaneum* depicted relative to the mean of the *H. vulgare* cultivars (mean of the *H. vulgare* = 100%)

In Figure 2 the theoretical expectations based on the model by COX (1984) and the empirical data of the various quantitative traits are shown. With the exception of the number of kernels per ear most traits approach the theoretical expectation in later generations as the amount of *H. vulgare* alleles increase. Choosing suitable parents in early backcross generations using the model developed by COX (1984) presents problems. The empirical values and the theoretical expectations do not correspond well in early generations.
The trait grain yield, which is of agronomic importance, fits the model better than a number of other traits. A possible explanation may be that the model takes only additive effects into account and neglects epistatic and dominant effects. Grain yield, a trait which is influenced by numerous genes, seems to be governed mainly by additive effects.

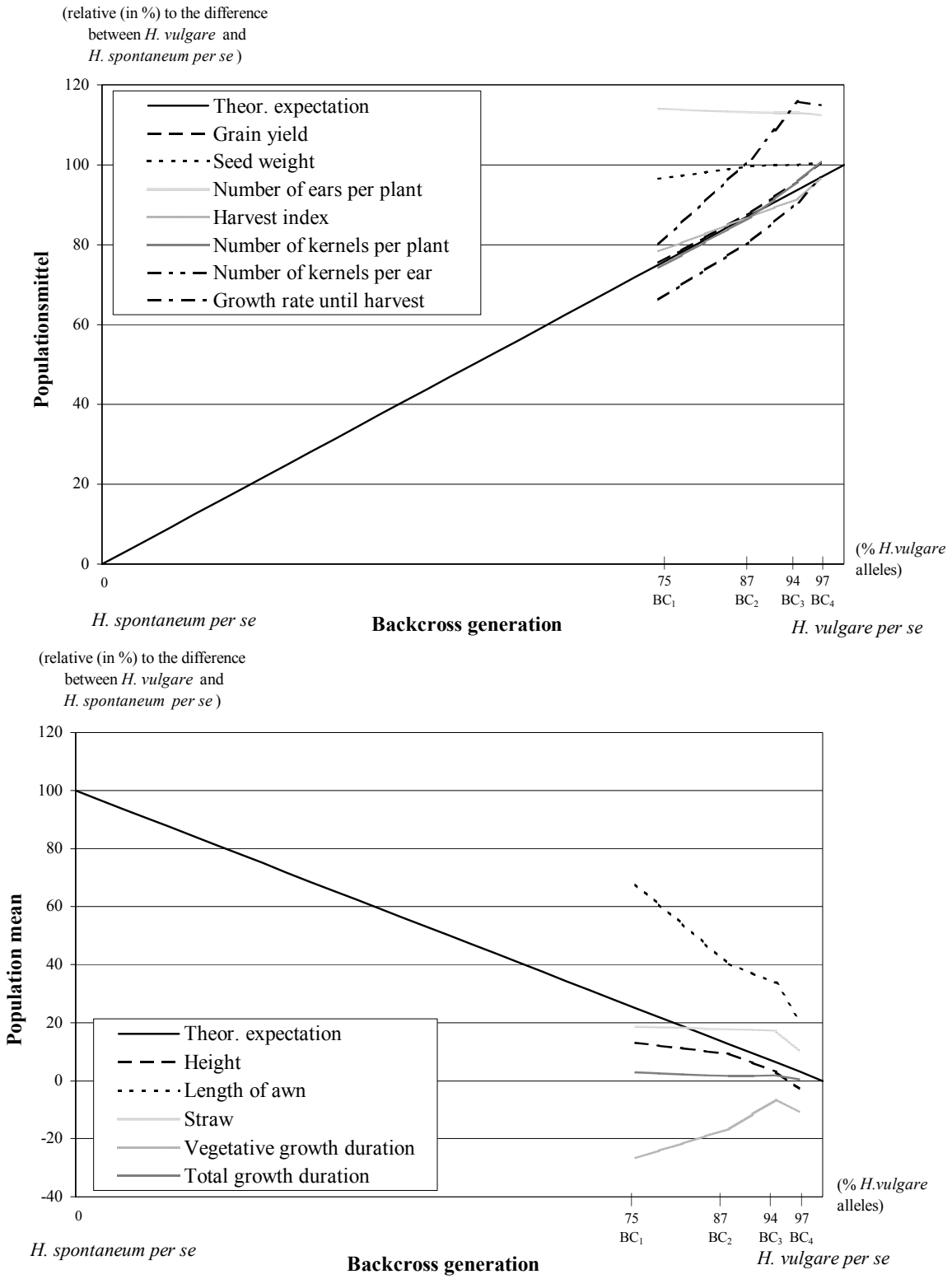

Figure 2: Population mean of four backcross generations (BC_1 to BC_4) for various quantitative traits for the progeny of thirty crosses in an incomplete mating design with *H. vulgare*H. spontaneum* in relation to the difference between the performance of the parents and the resulting theoretical expectations based on field trials.

The statistical analysis of the deviations of the empirical values from the theoretical expectations show significant deviations for a number of traits (Tab. 1): seed weight, kernels per ear, height, vegetative growth duration, generative growth duration and total growth duration. In later generations the number of significant deviations is smaller then in early generations. Possibly the effects due to the *H. spontaneum* accessions, that are neither adapted to our climate nor to intensive agricultural systems, are countered by the increase of alleles of the modern cultivars.
A further explanation is that the model developed by Cox is based on the performance of the respective parents. As the evaluation of the *H. spontaneum* accessions is highly problematic in field trials, partially due seed shattering, but also because there is no synchronization of flowering time or ripening, one of the parameters that form the basis of calculation of the theoretical model must be viewed with caution.

Tab. 1: Means of the backcross generations for various traits for the incomplete factorial compared to the mean performance of the parents and the theoretical expectations based on the data from field trials.

		BC_1		BC_2		BC_3		BC_4		
	H. spontaneum									*H. vulgare*
Grain yield	1,27	4,33		4,84		5,16		5,35		5,32
Seed weight	26,05	43,2	↑	43,7	↑	43,8		43,9		43,8
Ears per plant	3,87	5,72		5,71		5,71		5,70		5,50
Kernels per plant	36	100		111		118		122		122
Kernels per ear	8,2	17,5	↓	19,6	↓	21,0	↓	21,8	↓	22,3
Straw	8,18	5,75		5,72		5,71		5,50		5,19
Harvest index	0,11	0,39		0,42		0,44		0,46		0,47
Height	98,9	82,7	↓	82,0		80,9		79,8		80,3
Length of awn	11,1	10,8		10,4		10,4		10,2		10,0
Vegetative growth duration	1018	744	↓	766	↓	788	↓	779	↓	802
Generative growth duration	396	467	↑	445	↑	421		428		403
Total growth duration	1403	1210	↓	1208	↓	1208	↓	1205		1205

General and Specific Combining Ability
The general and specific combining abilities were calculated and the variance components were estimated separately for each backcross generation as the definition of the combining abilities does not take the different allele frequencies into account. Information on the influence of the parents respectively of specific crosses can be helpful in developing suitable breeding strategies.
In the current study the estimation of the variance components proved that the general combining ability (GCA) of the *H. vulgare* parents had the biggest influence for nearly all traits in nearly every backcross generation. The general combining ability of the *H. spontaneum* accessions did not play an important role in any case.
For a number of traits the specific combining ability played a major role in some of the backcross generations, mainly the early ones. The grain yield was an exception, in this case the specific combining ability (SCA) had the biggest influence in all generations except the BC_3. In the BC_1 straw, length of awn, ears per plant, kernels per ear, kernels per plant, vegetative and generative growth duration and in the BC_2 kernels per plant were the traits influenced mainly by the SCA.

The influence of the specific combining ability especially in early backcross generations explains the problems that arise if markers for quantitative traits are used in different populations. The effects often cannot be reproduced as they do not exist anymore in a different cross.

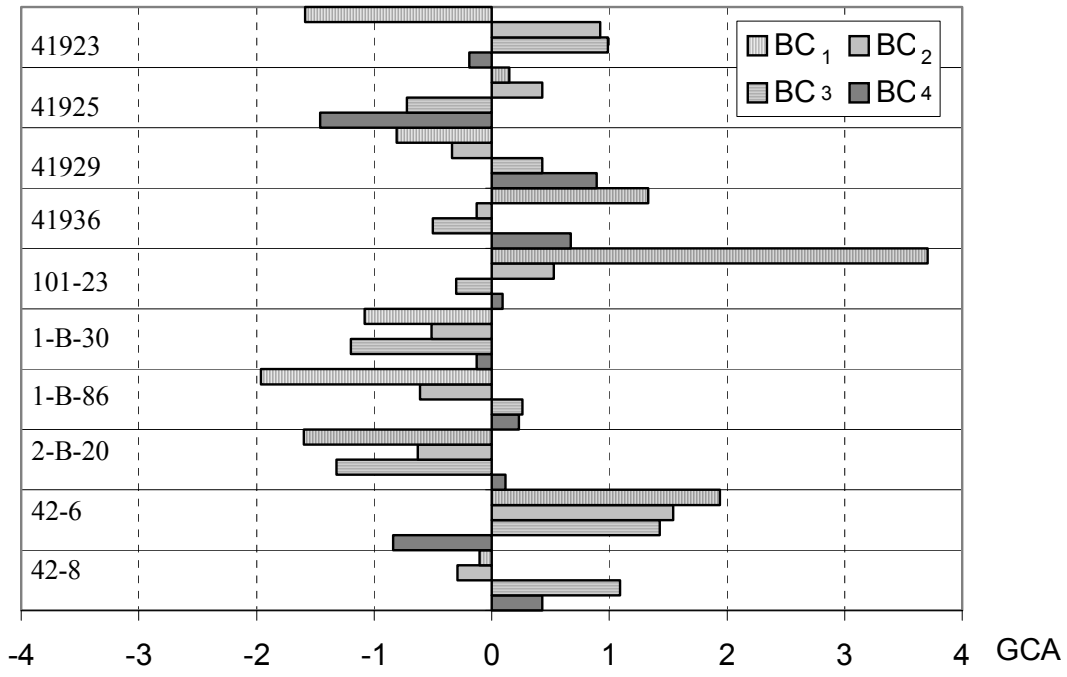

Figure 3: Effects of the general combining ability of the H. spontaneum accessions in four backcross generations for the trait seed weight.

The general combining ability of the seed weight is shown in Figure 3. Even for this trait, with a high heritability, the effects specific parents achieve in different backcross generations vary widely. In most cases the effects are not even either positive or negative in all generations.

Transgression

The ability of the *H. spontaneum* accessions to improve the performance of the modern varieties used as parents is an important aspect of this study. To asses the possibilities *H. spontaneum* offers, the number of transgressive lines in crosses with specific wild parents was calculated. Transgressive lines were defined as lines with grain yields higher than those of their respective *H. vulgare* parents by at least two standard deviations.

Transgressive lines were found for all wild parents. The number of transgressive lines varies widely from generation to generation for individual parents even though there are more in later than in early generations. The wild accessions 42-8 and 41923 produced a higher number of transgressive lines in all backcross generations than any other parent, in some generations their results were not as good as those of other parents. Correlations between the percentage of transgressive in the individual generations were low and seldom significant in some cases they were even negative. Predicting transgression by the performance in earlier generations is not possible.

Tab. 2: Percentage of transgressive lines for the trait grain yield in four backcross generations.

	Transgressive lines in percent				
Crosses with ***H. spontaneum***	BC_1	BC_2	BC_3	BC_4	BC_1-BC_4
101-23 * Apex, Dorett, Harry	0,00	1,85	3,06	1,32	1,43
1-B-30 * Apex, Aura, Dorett	2,22	0,65	0,00	**6,25**	2,43
1-B-86 *Beate, Golf, Klaxon	0,00	2,13	**5,31**	0,65	1,83
2-B-20 *Aura, Beate, Dorett	1,32	1,61	**4,32**	**4,11**	2,85
42-6 *Berolina, Harry, Lerche	0,00	1,90	2,63	**5,65**	2,36
42-8 *Arena, Golf, Klaxon	**4,17**	2,00	**7,55**	3,85	**4,14**
41923 *Arena, Berolina, Golf	1,31	3,23	**7,86**	**11,11**	**5,71**
41925 *Arena, Berolina, Lerche	**4,49**	1,94	1,54	2,60	2,69
41929 *Aura, Beate, Klaxon	1,40	2,88	**4,20**	4,08	3,15
41936 *Apex, Harry, Lerche	0,70	0,67	2,00	**5,26**	2,19
Factoriell Ø	1,59	1,85	3,89	**4,33**	2,89
Control group (*H. vulgare*) Ø			4,08		

Choosing suitable parents from exotic material remains problematic. Neither the theoretical model developed by COX (1984) nor the general combining ability of the *H. spontaneum* parents seem to allow reliable predictions about the performance of progeny for quantitative traits. Exotic material definitely has the potential for improving even traits like grain yield, that have been subjected to intensive breeding. The decision which *H. spontaneum* accession to use in a breeding programme should be made under consideration of other criteria e.g. disease resistance.

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Allelic Diversity and Genetic Gain in Midwestern Six-Rowed Barley Germplasm

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Abstract
United States Midwestern six-rowed malting barley varieties constitute a narrow germplasm tracing back to approximately 40 ancestors. Varietal candidates developed at the University of Minnesota have shown genetic improvement for many traits despite the fact that most of the crosses leading to these lines in the past 50 years have been among elite lines from within the breeding program. We initiated a study to estimate genetic gain for traits that have been under selection in Minnesota breeding germplasm. We evaluated 62 varieties and variety candidates developed from 1958-1998 in five locations in 2002 and 2003. Genetic gains for yield, plump yield, and malt extract are estimated to be 9.5 kg ha^{-1} $year^{-1}$, 20.5 kg ha^{-1} $year^{-1}$, and 0.065 % $year^{-1}$, respectively. Parallel to this genetic improvement of selected traits, we have observed a decrease in genetic diversity within this same germplasm. Allelic diversity, based on 65 SSR markers distributed throughout the genome, has decreased from a mean of 6.0 alleles per locus for lines developed from the first decade to 2.3 alleles per locus for lines developed during the last decade. These phenotype and marker data will be the basis for an association mapping study to identify loci for important agronomic, disease resistance, and malting quality traits that have been under selection during breeding.

Introduction

Plant breeding in six rowed malting barley (*Hordeum vulgare* L.) in the North American Midwest has been successful in developing varieties with combinations of desirable agronomic traits (yield, lodging resistance and disease resistance) and malt quality parameters (malt extract, alpha amylase, distatic power, etc,). This has led to highly accepted varieties by both the malting and brewing industry and farmers.

Breeding for such a complex target has led to the development of a regional narrow germplasm pool, characterized by close pedigree relationships. This phenomenon was assessed by MARTIN *et al*, (1991) using a co ancestry index. They concluded that for the main cultivars grown in the area, "*52% of the germplasm was contributed by the 5 ancestors with the highest mean (Trebi, Coast, Lion, Manchuria and Mensuri*)". RASMUSSON & PHILLIPS (1997) suggested that the main reasons for the narrowness or isolation of this germplasm was germplasm exchange between the three main breeding programs in the region and the need to maintain gene complexes for general environmental adaptation and acceptable malting quality. The fact that the dominant varieties in the region were highly related to each other was due, in part, to industry quality standards that dictated that new cultivars be similar in malting quality to those currently in use. This high level of relatedness has also been reflected in molecular marker studies that detected limited polymorphism in SSR and RFLP markers (DAHLEEN 1997; CONDON *et al.* 2002). The high level of relatedness among lines is reflected in the pedigrees of the variety candidates used in this study. When the germplasm was grouped by the decade it was developed, the average number of SSR alleles per locus decreased from 6.5 to 2.3 between the first and last decade

(CONDON *et al.* 2002). Fifteen of the 65 loci studied were fixed over time. This reduction in allelic diversity was significant overall, but not constant across the genome.

Another factor that has contributed to the genetic narrowness of the Midwest six-rowed germplasm is the successful deployment of major disease resistance genes for spot blotch (*Cochiobulus sativus*) and stem rust (*Puccinia graminis f.* sp. *tritici*). The resistance to spot blotch pathotype ND85F race 1 was derived from NDB112 and/or Dickson (STEFFENSON *et al.* 1996). Stem rust resitance was introduced via the gene *Rpg1*, located on chromosome 1P (STEFFENSON 1992; KILIAN *et al.* 1997).

Despite the narrowness of the germplasm used for breeding Midwest six-rowed barley, genetic improvement has been reported. Studying a set of six cultivars (Manchuria, Kindred, Trail, Larker and Morex) WYCH and RASMUSSON (1983) reported significant genetic gain for yield, yield components, lodging resistance, plant height, and quality traits (malt extract, alpha amylase and diastatic power). They estimated yield gains of 0.98 % or 22.1 kg ha^{-1} yr^{-1} for all the cultivars evaluated. For the period between the release of Kindred (1942) and Morex (1978), yield rates doubled to 2% or 45.7 Kg ha^{-1} yr^{-1}. HORSLEY *et al.* (1995) also found a considerable diversity for several malting quality traits available within Midwestern barley cultivars. They concluded, that although these varieties were closely related, crosses made from within a single breeding program would produce sufficient segregation for several important malting quality to make progress in breeding.

In general, evaluation of achieved genetic gains in breeding has been done at a regional level, between varieties released over a long timeframe, and across different breeding programs. The goal of this research is to assess the genetic gain within a single breeding program by examining variety candidates developed over a 40 year period. Has genetic gain been achieved in a gradual manner, or by steps determined by the major released varieties? Has progress reached a plateau within this period as a result of limited genetic diversity?

Objectives

The overall objective is to assess the rate of genetic gain within the Minnesota Barley breeding program between 1958 and 1998, using the following traits: yield, plump yield, plant height, days to heading and quality traits like plumpness, kernel weight, malt extract, alpha amylase, diastatic power, and the ratio between soluble and total protein.

Material and Methods

Germplasm

Sixty two varieties and variety candidates developed in the Minnesota breeding program between 1958 and 1998 were selected to assess genetic gains made through that period. This set included 50% of the of the variety candidates designated during that period. Lines included in the study were selected on the basis of pedigree to include lines that made a significant contribution as parents in the breeding program. The names, pedigrees and years in which the crosses were made are described in Table 1.

Table 1. Name, pedigree and year in which the cross was made for the germplasm included in the study

Genotype	Year	Pedigree	Genotype	Year	Pedigree
M1	1960	TRAILL*3/BR. 5750-2	M46	1977	NORDIC/MANKER//RBST
M2	1958	PARKLAND*2/ND B112	M48	1977	MRX/MANKER//M72-25/BR-YGT-4
M6	1960	LARKER*2/II-51-43	M49	1978	MANKER*2/STEPTOE//M34
M7	1961	SWAN/WISC. 691-1	M50	1980	ROBUST/BUMPER
M8	1962	M1/M59-8 II-51-43*2/B112)	EXCEL	1981	ROBUST*2/3/CREE/BONANZA//MANKER
M9	1962	M3/LARKER	M55	1981	M46/M44
M10	1963	LARKER*4/B112	M57	1981	RB//M28/MANKER/3/BOWERS/M34//MX
CREE	1963	M1/DICKSON	M59	1982	M47/M44R
M15	1964	TROPHY*2/CI47802/M2/3/NDB130	STANDER	1985	EXCEL//RBST/BUMPER
MANKER	1965	CREE/4/M2/3/VANTAGE/2/KINDRED/JOTUN	M66	1987	M44/EXCEL
M17	1966	TROPHY*7/BR5720-2/2/NDB130	M69	1982	M44/EXCEL
M18	1966	LARKER*7/BR. 5750-2//CREE	M71	1988	M34/EXCEL
M21	1964	JTN/KIND/2/VNTG/3/TROPHY/4/DKN/5/M60-105	M72	1988	M55/EXCEL
M22	1965	JTN/KIND/2/VANT/3/TROPHY/4/DCKSN/5/M59-38/6/BEACON	M73	1988	M57/STANDER
M23	1968	M21/CREE	ROYAL	1990	M62/AZURE
M24	1967	CREE/PARAGON	M75	1989	M66/EXCEL
MOREX	1969	CREE/BONANZA	M76	1989	STANDER SIB//ROBUST/M79-20
M26	1969	M2/M59-24/2/CREE/3/M62-144/2/TRPHY 4*/B112	M81	1992	M66/STANDER
M27	1969	M66-83/M66-124	M82	1992	M86-151/STANDER
M29	1969	CREE/M66-123	M83	1992	M73/EXCEL
M30	1970	M18/M14	M84	1992	CHERI / M66 // EX /3/ ST (GD-C2)
M31	1970	M21/ND B133//CREE	MNBRITE	1992	M90-89/M69
M32	1972	MANKER//M21/ND B134/3/M21/CREE	MNS93	1994	CHEVRON / M69
M33	1972	M28/MANKER	M96	1993	STANDER / EXCEL
M35	1974	M70-43/M69-77//MOREX	LACEY	1996	M78 / M79
ROBUST	1973	MOREX/MANKER	M100	1995	M78 / EXCEL
M37	1974	M18/63AB2987-32//MANKER	M101	1995	M81 / M89-358
M38	1976	MOREX/BONANZA//M32	M104, M105	1993	M92-211 / M83
M39	1976	MOREX/MANKER//M30	M106	1993	M92-334 / M81
M44	1976	NORDIC/MNKR/3/MOREX//MNKR/63AB2987-32	M108	1997	M92-395 / M83

Field Evaluation

The genotypes were evaluated during 2002 and 2003 seasons in five different environments (Saint Paul and Crookston, in 2002 and Saint Paul, Crookston and Morris in 2003) using a randomized complete block design with two replicates. Seed was sown in 2 row plots 3.0 m in length 30 cm apart. Yield (YLD) was determined by harvesting 2.0 meters in the center of the plot. Plant height (PHT) was determined by taking the average of 3 different points in each plot. Days to heading (HD) was recorded as the number of days after planting when the heads of 90% of the plants in the plot had emerged from the boot. Plump grain percentage (PG%) was measured as the proportion (by weight) of grains larger than a 2.25 mm mesh . Plump yield (PYD) was calculated by multiplying yield by the plump grain percentage. The data was analyzed using the SAS PROC GLM (SAS Institute, Cary, NC) in a combined analysis for all environments, considering each year-location combination as a fixed effect. Regression was performed using the least squared means estimated in the previous analysis against the year in which the crosses were made to estimate genetic gain. For quality variables only Crookston and Saint Paul 2002 are included in the analysis. The grain harvested was pooled across reps within locations, cleaned and sent to the USDA-ARS Cereal Crops Research Unit in Madison, WI for evaluation of the following malting quality

parameters: malt extract (ME), diastatic power (DP), kernel weight (KW), barley protein (BP), soluble over total protein (S/T), alpha amylase (AA) and beta-glucans (BG).

Results and Discussion

Significant effects of genotypes and genotype-by-environment interaction were observed for all the agronomic traits evaluated (Table 2). Significant genotype-by-environment interaction was detected for ME and S/T among the quality traits. Calculations of genetic gain were done using the data pooled across environments despite the GxE interaction, since the breeding target for this program is represented by the locations used in the study.

Table 2. Germplasm statistics (mean, minimum, maximum) and analysis of variance results, mean square (MS) and F statistic for 12 agronomic and malt quality variables

	Germplasm				Genotype		Genotype x Environment	
Variable[1]	Mean	Min	Max	CV (%)	MS	F	MS	F
YLD (Kg ha$^{-1)}$	4420	3453	5187	14.9	2058543	4.74**	629069	1.45**
PG (%)	84.6	54.5	93.5	9.7	568	8.36**	97	1.43**
PYD (Kg ha^{-1})	3799	2290	4714	18.2	4023695	8.40**	735778	1.54**
HT (cm)	78.8	66.1	89.6	6.2	395	16.4**	39	1.64**
HD (d)	53.1	51.2	55.8	2.4	8.33	4.99**	2.49	1.49**
KW (mg)	31.2	27.5	34.5	2.3	5.67	10.6**	1.59	2.97**
ME (%)	77.1	73.6	79.8	1.0	3.89	6.76**	0.91	1.59**
BP (%)	15.4	14.2	17.6	5.5	1.06	6.76**	0.94	0.1
S/T	51.2	42.2	60.8	3.8	42.7	11.0**	7.8	2.01*
DP	153.6	109.0	209.3	12.5	1359	3.67**	568	1.83
AA	68.9	47.2	85.6	11.5	125	1.98**	69.7	1.1
BG	205.4	71.2	517.9	0.9	16464	4.47**	4496	1.22

[1] YLD, yield in Kg ha-1; PG%, percentage of plump grain; PYD, plump grain yield; ME%, percentage of malt extract; HT, height in cm; HD, days to heading in days after June 1st; KWT, kernel weight; S/T, soluble over total solids ratio; DP, diastatic power; AA, alpha amylase activity; BG, beta-glucan content.

** significant at P<0.01, * significant at P<0.05,

The estimated YLD gain per year was 20 kg ha^{-1} year^{-1} (Table 3), representing a 0.49% increase per year, with an observed significant $r^2 = 0.20$. This corresponds to about half the increase reported by WYCH & RASMUSSON (1983). This estimate is similar to other estimates of genetic gain made for wheat (DOMNEZ *et al.* 2001) and soybeans (USTUN *et al.* 2001). Percent plum grain increased 0.51% per year (r^2 =0.36) and PYD increased 34.4 kg ha^{-1} year^{-1} (1.09% per year), indicating a combined selection for both increased yield and grain plumpness (Figure 1).

There was no change in heading date and a slight reduction in plant height (Table 3). This suggests that the reduction in plant height in the University of Minnesota released varieties has not been achieved through major genes. Significant gains were detected for KWT, ME (Figure 1), AA, and S/T. We can conclude that even within a very narrow germplasm, significant genetic gains for several important traits has been achieved. This progress can be modeled adequately with simple linear regression with no apparent evidence of a plateau for the traits measured. There does also not appear to be signs of depletion of phenotypic variability for the traits studied. Future research will address the relationship between genetic gain and the changes in allelic frequency detected by SSR marker analysis.

Table 3. Estimates of linear regression parameters for the different variables studied

Variable [1]	R^2	Year (b)	Pr > |t|
YLD	0.20	19.9	0.0003
PG%	0.36	0.511	<0.0001
PYD	0.39	34.4	<0.0001
ME(%)	0.41	0.074	<0.0001
HT	0.06	- 0.12	0.05
HD	0.04	0.01	ns [2]
KWT	0.25	0.07	<0.0001
S/T	0.40	0.27	<0.0001
DP	0.005	Ns	Ns
AA	0.14	0.25	0.001

[1] YLD, yield in Kg ha-1; PG%, percentage of plump grain; PYD, plump grain yield; ME%, percentage of malt extract; HT, height in cm; HD, days to heading in days after June 1st; KWT, kernel weight; S/T, soluble over total solids ratio; DP, diastatic power; AA, alpha amylase activity.
[2] ns, not significant.

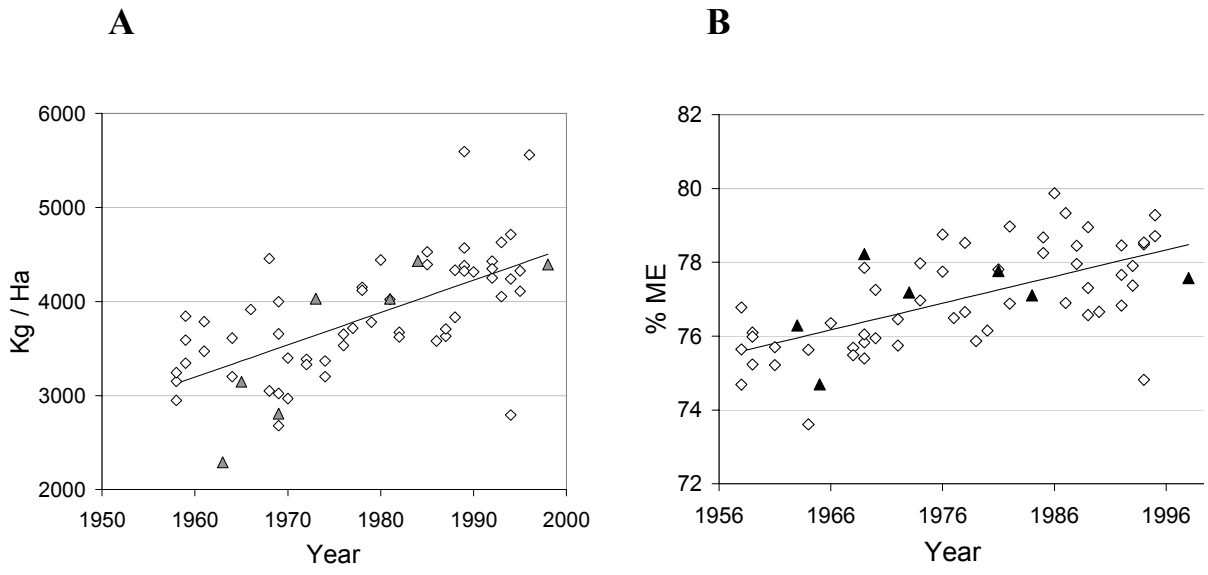

Figure 1. **A** Genetic gain for Plump yield. **B** Genetic gain for percent malt extract (% ME). Diamonds represent variety candidates. Triangles represent released varieties: Cree (1963), Manker (1965), Morex (1969), Robust (1973), Excel (1981), Stander (1984), Royal (1989), MNBrite (1992), Lacey (1998)

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The Oregon Wolfe Barley Population: An International Resource for Barley Genetics Research and Instruction

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Abstract
The Oregon Wolfe Barley (OWB) Population - a set of doubled haploids (DH) developed at Oregon State University (OSU) from the F1 of the cross between Dr. R. Wolfe's Dominant and Recessive marker stocks - is a publicly available resource for plant genomics research and serves as a vehicle for stimulating public awareness and interest in genetic diversity and agricultural biotechnology. The OWB population, which displays a stunning level of morphological trait diversity and a high level of DNA-level polymorphism, is available in both real and virtual formats. Seed of the population can be obtained from the Oregon State University Barley Project (www.barleyworld.org) and this site also serves as a gateway to a spectrum of information and tools for research and instruction involving the OWBs. A subset of the OWBs will be featured on the 9th International Barley Genetics Symposium field tour, and the poster will describe some of the many research and teaching activities around the world that involve this unique germplasm resource.

Introduction
The OWB population is visually interesting and stimulating due to segregation, linkage, independent assortment, and epistatic interactions of alleles at morphological trait loci. The population is also useful and interesting as a research tool, since in addition to the high level of polymorphism at morphological trait loci, there is a remarkable level of DNA sequence polymorphism. The OWB population is, accordingly, a useful tool for integrating all types of markers (e.g. morphological, RFLP, SSR, STS, and EST-based) in a single reference linkage map. This has led to the development of an OWB-based BIN map, which should, in the future, also be useful for aligning linkage and physical maps. Since the OWB population consists of doubled haploid (DH) lines, it is an "immortal" resource. Data are available on the Web at *http:// www.barleyworld.org /owbs.html* and at GrainGenes *http://wheat.pw.usda.gov/OWB*. The power and interest of the OWBs lies in their simultaneous utility for genetics outreach and research. Seed is available upon request.

Material and Methods
There are so many ongoing OWB-related projects that it is not possible to describe all of them in this report, or to present them in the poster. Accordingly, a standard Material and Methods section is omitted from this report. In cases where results have been published or posted on the Web, citations or URLs are provided. In the case of work in progress, please contact the authors for details. Progress reports will be periodically updated on the Oregon Wolfe Barley homepage (*http:// www.barleyworld.org /owbs.html)*.

Results and Discussion

The OWBs as a Tool for Genetics Outreach

In order to facilitate outreach activities, we have developed the Informative and Spectacular Subset (ISS) of 18 OWB DH lines (plus the two parents). With these 20 plants, one can capture most of the morphological diversity as well as demonstrate key genetic principles, including allelic variation at the phenotype and genotype levels, segregation, independent assortment, linkage, and epistasis (Table 1). Thanks to V. Carollo (WRRC, USDA/ARS)[1] an ISS image gallery is posted at GrainGenes, as are additional resources including (i) animations demonstrating the meiotic events giving rise to specific ISS DH lines and (ii) illustrative examples of the RFLP, SSR, and EST markers used for the animations. If you are interested in using the OWBs as an outreach component, please join the OWB "nation": contact the authors for details.

The OWBs as a Tool for Genetics Research

The OWB population has proven to be a useful tool for genetic analyses. Brief profiles are presented for several of these projects. Readers are encouraged to become involved! Please contact the authors with your research plan.

- *The OWB BIN Map:* Because the OWB map includes a range of marker types, it is useful as a standard reference for integrating marker and Quantitative Trait Locus (QTL) information from multiple mapping populations. L. Marquez-Cedillo (OSU[2]), A. Kleinhofs (WSU[3]), P. Szucs (MRI[4]), and MI. Vales (OSU) collaborated on mapping the SCRI SSRs and North American Barley Genome Project (NABGP) RFLPs in the OWBs and L. Marquez-Cedillo aligned this map with the BINs defined by Kleinhofs and Graner (2001). The OWB-based BIN map will evolve as new markers are added. The current version, as shown in Figure 1, features 14 morphological markers, 49 SSRs, 122 RFLPs, one mildew (*Blumeria graminis*) resistance gene, four STS markers and 15 *DsT* insertions.
- *Towards a barley transcript map:* More than 360,000 barley ESTs are reported, most of which still have no assigned function or map location. As a first step in this direction, and a proof of concept, J. Russell, W. Powell (SCRI[4]), and L. Marquez-Cedillo are collaborating on mapping selected ESTs in the OWBs.
- *Mapping Ds insertion sites in transposon tagging stocks:* L. Cooper (OSU) and cooperators have used the OWB population as the principle vehicle for characterizing Ds insertion sites in transposon tagging stocks developed in "Golden Promise". Please see the paper in these Proceedings by Cooper et al. for additional detail.
- *Gene discovery: characterizing the partners in an epistatic interaction:* L. Cooper, S. Bell, R. Riera-Lizarazu, and I. Vales (OSU) are characterizing the intriguing epistatic interaction that results in the hooded phenotype. In individuals that are homozygous for the dominant allele *Kap* and the recessive allele *lks2,* the hooded phenotype is masked, resulting in a non-hooded, short-awned inflorescence. Non-radioactive differential

display in combination with bulked segregant analysis is being used to isolate cDNAs involved in the epistatic interaction of hooded (*Kap)* and short awn (*lks2)* The OWB population was divided into four groups, based upon the genetic makeup at the *Kap* and *lks2* loci. Bulked cDNAs were generated from messenger RNA, extracted from floral tissue at the terminal period of the elongating lemma primordia stage from individuals of each group. A total of 240 primer combinations were used to screen the OWB cDNA bulks by differential display. The differentially displayed fragments were grouped into five expression patterns based upon the allelic composition of the OWB bulks. Two cDNAs are currently being characterized and mapped in the OWB population that were up-regulated, based on quantitative real-time RT-PCR analysis, in bulks that display the epistatic interaction (*KapKap lks2lks2*). BLAST search results indicate that these are unidentified barley gene transcripts with strong sequence similarity to published ESTs of unknown function. This group is currently characterizing the expression patterns of two cDNAs identified as being specific to the long awn phenotype (*kapkap Lks2Lks2*). This work should contribute to the understanding of the epistatic interaction of *Kap* and *lks2*.

- *QTL mapping:* The OWB DH population offers a unique opportunity to relate QTLs with major genes determining morphological characters. The population segregates for ~13 morphological traits, and some of these traits can be mapped genetically based on qualitative evaluations. Other traits, including awn length, spike length, heading date, and number of hairs on leaves, can be evaluated quantitatively. As an example, MI Vales (OSU) has measured the awn length trait in the OWB DH mapping population and the parental lines. Subsequently, composite interval mapping (CIM) implemented in QTL Cartographer was used to identify QTL that affected this trait. Significant QTL on chromosomes 2H, 4HS 4HL, 5H and 7H were detected. A major QTL was located on 4HS that explained up to 59.1% of the phenotypic variance. Tests for epistasis between QTL were evaluated using the MIM (Multiple Interval Mapping) method of QTL Cartographer. Significant epistatic interactions were detected between the 4HS and 7H QTL, that coincided with the positions of *HvKnox3* (*Kap* or *Hooded*) and *lks2,* respectively. The epistatic interaction explained 39.9% of the phenotypic variance. These analyses confirmed the epistatic interaction phenomenon previously described in this population. When the OWB DH lines with hooded spikes were eliminated from the data set for QTL analysis, a major QTL was identified on chromosome 7H; this QTL explained 61.3% of the phenotypic variance and coincided with the location of *lks2*. In addition, two other QTL were found on 2H and 5H; these QTL explained 1.8 and 1.1% of the phenotypic variance, respectively. The QTL on 2H coincided with the position of *Zeo1*.

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Table 1. Morphological loci and chromosome locations segregating in the Informative and Spectacular Subset (ISS) of Oregon Wolfe Barley DH lines. "A" = dominant parent allele; "B" = recessive parent allele. See www/*barleyworld.org* for locus definitions.

Chromosome	**1H**	**2H**			**3H**		**4H**		**5H**		**6H**	**7H**	
Locus	***Blp***	***vrs1***	***Zeo1***	***wst7***	***alm***	***Pub***	***Kap***	***Hsh***	***Srh***	***raw1***	***rob***	***nud***	***lks2***
DH#													
1	A	B	B	B	B	B	A	A	B	-	A	A	A
2	B	B	A	A	A	B	B	A	B	B	B	B	B
5	A	B	B	B	A	B	B	B	A	B	A	B	A
6	B	A	A	A	B	A	B	B	A	A	B	B	B
9	B	B	B	A	B	A	A	B	B	A	A	B	A
10	B	A	A	B	A	A	A	B	B	A	A	A	A
11	B	A	A	B	B	B	B	A	A	B	B	B	A
13	B	A	B	B	B	A	B	A	A	B	A	A	A
14	B	B	A	A	B	B	A	B	A	-	A	B	A
16	A	A	B	B	A	B	A	A	B	B	A	A	B
22	B	B	B	B	A	B	B	B	A	B	B	B	B
39	B	A	A	A	A	B	B	A	A	B	A	A	A
44	B	A	B	B	B	B	A	A	B	A	A	B	B
46	B	B	B	B	A	B	B	A	A	B	B	A	A
49	B	B	A	A	A	A	B	A	B	A	A	A	A
55	A	A	B	B	B	B	A	A	A	B	B	A	A
57	A	B	A	A	A	B	B	A	B	B	A	B	B
90	B	A	B	B	A	B	A	B	A	B	A	A	A
A	**5**	**9**	**8**	**7**	**10**	**5**	**8**	**11**	**10**	**5**	**12**	**9**	**12**
B	**13**	**9**	**10**	**11**	**8**	**13**	**10**	**7**	**8**	**10**	**6**	**9**	**6**

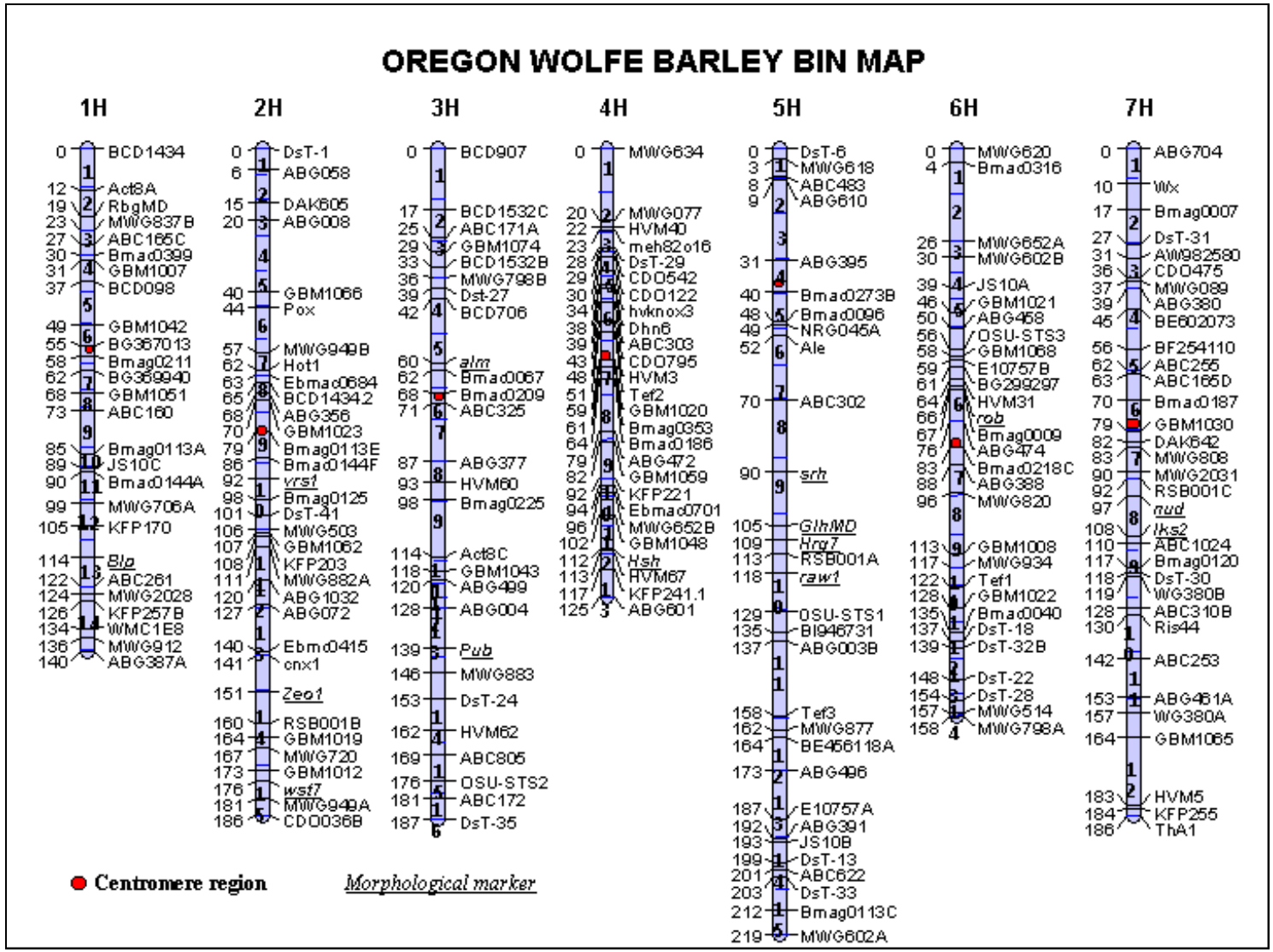

Figure 1. A BIN map based on the Oregon Wolfe Barley population consisting of 205 markers

Tunisian Insular Barley Landraces: A Significant Hordeins Drift

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Abstract
Barley genetic resources play a key role in dry farming and poverty alleviation in dry areas in Tunisia. In semi-arid regions, barley is mostly cultivated by sheep owners and grazed one or two times. It's also grown for grain production in intermediate zones and for hay making. Barley is used mainly for feed (85%) and occasionally for food (10-15%). In southern zones, barley landraces are submitted to various constraints, essentially their substitution by improved cultivars, and are threatened of disappearance and extinction. To safeguard these resources, 423 barley accessions were collected from diverse regions of Tunisia and specifically from the islands of Djerba and Kerkenna. Sixty-one accessions, collected from Djerba [Jorf 2 & 8, Houmet Essouk , Houmet Beni Dighet and El Khmara], were evaluated using SDS-PAGE. Results showed high B and C hordeins segregation. 33 chemotypes were characterized. Beni Dighet accessions showed a fatal drift for A and D hordeins. It seems that certain important quality traits have been loosed and dramatically influence may occur on nutritional value. Presence or absence of some hordein groups or components also could indicate the quality of technological traits of the barley grain.
Keywords: insular barley landraces; hordeins; SDS-PAGE; genetic drift

Introduction

Barley is cultivated on about 450.000 hectares in Tunisia. During centuries, early domestication and local knowledge have generated diverse local barley landraces used mainly for feed and lowly for food (VAVILOV 1951). In semi-arid regions (Central and Southern zones), barley is mostly cultivated by sheep owners, and grazed one or two times as early winter crop when forage and pasture are not available. It's also grown for grain and for hay making. The mostly grown six-rowed improved barley varieties are Martin, Rihane and Manel. Rihane is grown on more than 50% of the barley cultivated areas. Manel, a new six-rowed variety was officially registered in 1996 and released for sub-humid areas. Momtaz, a drought tolerant genotype, was proposed officially for release in 1999. Local barley cultivars are still grown essentially for double use purpose. The most grown local barley landraces are Souihli, Ardhaoui, Frigui, Beldi and Djebali. Sfira and Djerbi are cultivated in limited niches in the South and essentially used for food to prepare special recipes for restricted rural communities in the island of Djerba (EL FELAH 1998).

The conservation and use of plant genetic resources are essential to the continued maintenance and improvement of agricultural production, sustainable development and poverty alleviation. Plant genetic resources include, reproductive or vegetatively propagated material of wild and weedy relatives of cultivated species, cultivars in current use and newly developed varieties, obsolete cultivars, special genetic stocks including elite and current breeders lines, aneuploids and mutants, and farmer' traditional cultivars and landraces (KARP *et al*. 1997). The objective of plant genetic resources is to preserve and to use as broad a sample of the extent genetic diversity, and conduct a participatory plant breeding program only possible way for crops grown in limited agroecological niches. The major storage proteins in the barley endosperm are the alcohol-soluble prolamins, the hordeins which have

been classified into four groups, namely, B-, C-, D-, and gamma hordein (HIROTA *et al*. 2000). In another hand, barley storage proteins can be divided into 4 groups A, B, C and D according to their molecular weight and to their hydrophobicity (SHEWRY *et al*. 1994). Polyacrylamide gel electrophoresis of cereals storage proteins is a vluable tool for assessing variation in germplasm samples (DAMANIA *et al*. 1983).
Indigenous barley accessions were collected from diverse ecogeographic regions of Tunisia and specially from the island of Djerba. This work describes an extraction technique using a modified SDS-PAGE that is capable of resolving barley endosperm proteins into clear and repeatable band patterns.

Material and Methods
Nine barley landraces were collected from farmers' fields in Houmet Beni Dighet in the island of Djerba in 1983. These accessions were grown in unreplicated small plots of 2.5 m row length with a row spacing of 25 cm. These head-rows allow us to obtain by mass selection pure line accessions. 10 kernels in total of each entry were analysed to provide some indication of sample purity.
Single half-kernel extracts were prepared, in a single electrophoretic analysis for the majority of Dighet accessions. Electrophoretic analysis of these 9 accessions was achieved using SDS-PAGE method according to SHEWRY *et al*. (1978) and COOKE *et al.* (1983). For each endosperm analysed, 50 µl of extracting solution is required instead of 15µl.
Endosperm color frequencies were evaluated in Technology Lab. of INRAT using visual method.

Results and Discussion
A total of 9 single seed anlysis was made to identify variation in this material. Each population was represented by several chemotypes, and these are indicated in figure 1. Each seed having a distinct electrophoretic profile with respect to position of bands and their intensities are recognised as a different chemotype. Landraces collected in Houmet Ben Dighet were highly variable with many chemotypes in each population.
Morphological analysis of these populations showed that all barlys from the island of Djerba are 6-rowed cultivars. SDS-PAGE analysis of seed proteins from 9 local barley landraces (112, 114, 115, 116, 117, 118, 119, 120 et 121) collected from Houmet Beni Dighet (Djerba island-South-Tunisia) have segregated into five patterns: Pat. 1, Pat. 2, Pat.3, Pat.4 and Pat. 5 (Figure 1).
Results showed a clear and significant drift of D-hordeins bands in the pattern 1 (accession 112) and in the pattern 4 (accession 119). In another hand, a gradual drift of D-hordeins bands was observed in the patterns 2, 3 and 5 regarding to the band intensities of the accessions 114, 115, 117, 118, 120 and 121 (Figure 1). Regeneration will be needed and has to be carried out to ensure that genetic drift, or change in genetic structure of the population, is reduced to a minimum (KARP *et al*. 1997).
The local barley evolution is based on a gradient of endosperm color, shape and size. The food barley survey revealed that 91% of Tunisians preferred consume the white to yellow barley grain endosperm, whereas 9% of them choose the grey barley (Figure 2). Traditional and industrial processing preferred elongated to round than the oblong shape, and the big size to medium barley grain. Presence or absence of some hordein groups or components could indicate a drift of the quality of technological traits (endosperm color) of the barley grain (PEROVIC *et al*. 2000). Food barley genetic resources should be managed following socio-economic and agronomic modeling strategies with a participatory/decentralized breeding program aiming to *in situ* conservation, farmer's property rights and poverty alleviation (CECCARELLI *et al*. 2001). Changing world (global warming, globalization, consumer

behavior, biosafety, quality and sustainability) needs new strategies toward new priorities. Such information is useful to initiate a breeding program aiming to a genetic gain in barley quality improvement in advanced material in the coming years.

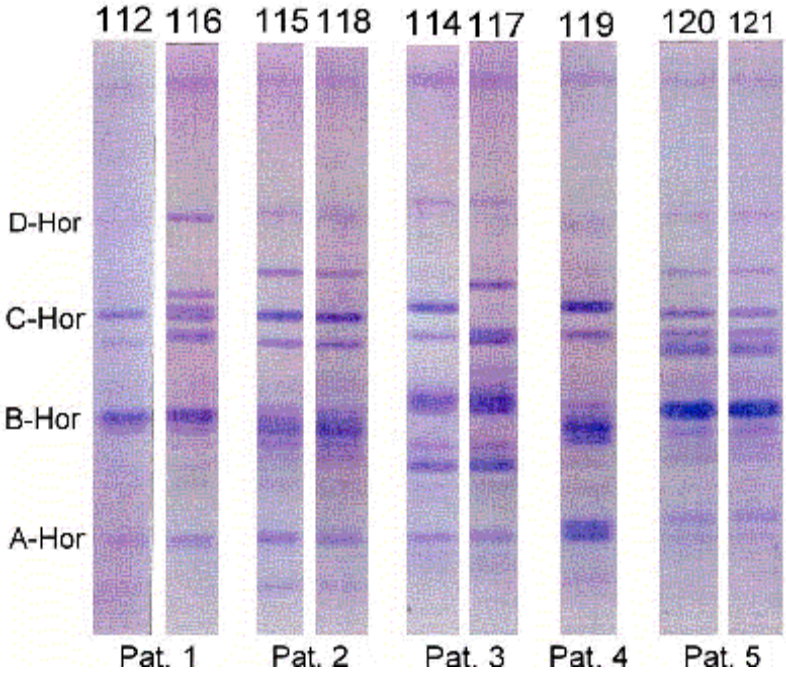

Figure 1. SDS-PAGE analysis of seed proteins from 9 local barley landraces (112, 114, 115, 116, 117, 118, 119, 120 et 121) collected from Houmet Beni Dighet (Djerba island-South-Tunisia) segregating 5 patterns (Pat. 1 to Pat. 5).
Protein markers (PM) were Phosphorylase (94 kd), Bovine Serum Albumine (67 Kd), Ovalbumine (43 kd), Anydrase Carbonique (30 kd), Trypsine I de soja (20 kd) and α-Lactoglobuline (14 kd) (BETTAIEB-BEN KAAB & EL FELAH 2003).

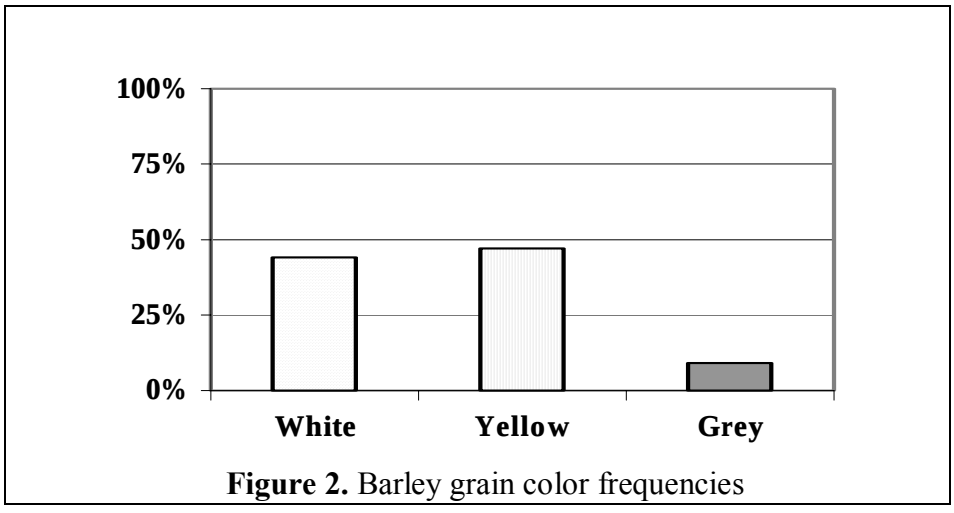

Figure 2. Barley grain color frequencies

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Bridging the Widening Chasm between Exotic Germplasm and Elite Breeding Populations Using Recurrent Introgressive Population Enrichment (RIPE)

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Abstract
Recurrent Introgressive Population Enrichment (RIPE) is a method of utilizing basic evolutionary principles in breeding. The RIPE system is focused on accumulating desirable alleles and allele combinations for specific traits in populations from which well-adapted, high-performing lines can be selected. The limitations of the number of crosses needed for population development and recycling, and the length of the breeding cycle have largely been overcome in the RIPE system through the use of genetic male sterility (linked to a xenia-expressing phenotypic marker for pre-sowing selection) for crossing. The use of controlled environments for rapid generation advance, offseason nurseries for seed increase, and effective evaluation of derived lines in the target environment complete the efficient system. Moderate levels of heritability, moderate selection intensity, reasonable breeding population size, and reduced cycle times have resulted in a very efficient, effective, and therefore economical, breeding system. The RIPE system is useful for incorporating new genetic diversity into elite breeding populations, while maintaining performance and adaptation. There has been more than 50% increase in yield over the foundation parents in ten years with several superior cultivars in commercial production.
Keywords: yield; genetic male sterility; barley

Introduction

Plant breeding is a short-term, accelerated form of artificial evolution used to improve specific traits in specific populations from which desirable genotypes will be extracted, evaluated, and may eventually be commercialized. As such, the more knowledgeable a breeder is of the theory and mechanics of evolution, the more effectively and efficiently they can practice the art of plant breeding. Charles DARWIN (1859) developed the concept of evolution around small, random variations upon which natural selection acted to accumulate those factors associated with increased 'fitness'. Sewall Wright developed much of the modern theory of the relationships among the forces influencing evolution and has explained it in terms that can be used in a breeding context. WRIGHT (1963) wrote that “exploitation of the enormously amplified field of variability provided by recombination speeds up evolutionary change enormously, if it can be coupled with an adequate process of selection”. McPROUD (1979) analyzed three major international barley breeding programs and described them all as various forms of recurrent selection. He used pedigree information to show that they all generally created variability through crossing, isolated inbred lines, evaluated them to identify the superior lines, then recombined the best lines for the next cycle of breeding. McPROUD (1979) goes on to point out some of the shortcomings of the programs he studied as being based on low numbers of founding parents, introduction of few new sources of germplasm in recent cycles, and long recombination cycle times. FOUILLOUX (1980) demonstrated that several cycles of recurrent selection were much more effective in accumulating desirable alleles than even large increases in single cycle population size. The amazing progress of 20-30 standard deviation units of improvement over the original population mean in the Illinois

long-term high oil and protein selection populations (DUDLEY & LAMBERT 2004) provides additional inspiration for attempting to apply recurrent selection methods to cereal breeding. Recurrent selection, in a general context, could be used to describe the vast majority of established cereal breeding programs.

Many breeders do not use unadapted germplasm because of fear of diluting their elite populations with inferior alleles and breaking up desirable linkage blocks. 'Exotic' germplasm can be characterized as an unadapted background with [potentially] a few new, desirable alleles (a weak chain with few strong links). 'Elite' germplasm is generally an adapted background with only a few undesirable alleles (a strong chain with a few weak links). The process of producing and selecting the best possible lines in the existing population through recurrent selection, then bringing in new, desirable alleles to further enhance the population, and producing the best possible new combinations is the essence of an 'open-concept' population breeding approach. The RIPE system described for barley (see KANNENBERG & FALK 1995) details the integration of a recurrent selection system with a structured introgression mechanism to continually upgrade the potential of the core breeding population.

Material and Methods

The RIPE system used at the University of Guelph has undergone considerable evolution in structure and function over the decade since it was first implemented (KANNENBERG & FALK 1995). The coupling of the recessive male sterile gene *msg6,* on barley chromosome 6H, with the very closely linked (less than 0.1% recombination) recessive, xenia-expressing shrunken endosperm *sex1* gene gives a mechanism that allows seeds which will produce male sterile plants to be identified prior to planting. This is based on the phenotype of the endosperm (*sex1msg6/sex1msg6* are shrunken seeds giving male sterile plants; *Sex1Msg6/___*are normal, plump seeds giving fertile plants; see FALK *et al.* 1981). The recessive orange lemma gene (*o*) located between the Sex1 and Msg6 loci is now being used as a repulsion marker to identify seeds from plants that are homozygous for the fertile and plump alleles (*Sex1oMsg6/Sex1oMsg6*) in the F2 population without progeny tests nor a detailed examination of the seed of individual plant progenies (Figure 1). [NB the orange lemma was coupled with the shrunken endosperm and the male sterile alleles in the original RIPE system] Heterozygous plants produce two phenotypes of seeds, based on the genotype at the Sex1 locus, in a ratio of three plump to one shrunken seed. The plants grown from shrunken seeds will be male sterile and are used for further crossing. The plump seeds will give two heterozygotes to one homozygote with orange seed (due to *oo*).

Controlled environments (growth rooms) are used for crossing the male sterile plants with selected male parents (September-December) and for growing the resulting F1 plants (January-April) for selfing resulting in two generations in the offseason. The F2 seed is then available for the normal field season (May-August) (Figure 2). The F2 populations are grown in the field environment as bulk populations where some natural selection likely occurs. Very little breeder selection is practiced in these populations because heritability for quantitative traits in such highly heterozygous populations is generally low. F2 populations are chosen for further selection primarily based on performance of the male parents in concurrent yield trials. After harvest, well-filled orange F3 seeds (*Sex1oMsg6/Sex1oMsg6*) are selected from the chosen F2 populations and sent to an offseason nursery (Southern California) where they are grown as spaced plants. Selection in the F3 is practiced for tillering, height, maturity, BYDV tolerance, spike and grain size. Only plants that produce enough seed (about 60 gr) for unreplicated yield trials back in Canada are selected. The F3:4 generation is grown in yield plots where basic agronomic and disease traits, and yield, relative to the checks, are

determined. Principle component biplots (YAN & KANG, 2003) based on all the available data, are used to select the best lines for further evaluation in multiple location, replicated trials the following years. Superior selected lines (F3:5 seed from the F3:4 plots) are used as male parents in the following round of crossing onto male sterile plants. Shrunken seeds (= male sterile plants) from remnant F2 or F3 seed of the most recent crosses (the same populations selected for winter increase) are used as females in the crossing. This completes one full breeding cycle of the Elite population with five generations being grown in two years and culminating with a yield trial. The cycle is actually one year on the female side and two years on the male side (see Figure 2).

The Elite population was founded by backcrossing the *sex1msg6* gene block into four well-adapted, successful but somewhat diverse cultivars (Bruce, Leger, OAC Kippen, Chapais) and then intermating the resulting lines in all possible combinations in two cycles so that all F1 plants making up the initial population had all four cultivars as parents. This launched the first cycle of recombination among the founders. Adaptation was considered to be the most difficult 'trait' to breed for, and thus, it was important to include only well-adapted material in the initial population. The focus was on recombination to eliminate defects and ultimately, to enhance the traits related to yield, disease resistance/tolerance and agronomic type. New germplasm is introgressed into the Elite population by crossing with Elite male sterile plants (Figure 3). The resulting F1 plants are crossed again with Elite male steriles during the winter grow-out. The F1 seed from this second cross should be 50% *sex1sex1* (= male sterile) and 50% *Sex1sex1* (= fertile). The male sterile F1 plants grown from the shrunken seed are then crossed with the selected Elite males (*oo*) in the next crossing cycle to give F1 plants that contain approximately 87.5% Elite germplasm (these populations are designated as the 'High' level). The F1 plants from this last cross are selfed and the F2, F3, and F4 populations are grown out in parallel with the corresponding generations of the Elite population. The best High lines are selected from the F4 yield trial, based on the same index as the Elite population. Those High lines with additional desired traits which may be missing or deficient in the Elite population are selected for use as male parents in the next round of crossing (these are Norman Borlaug's 'toros nuevos' = 'new bulls'). It takes eight generations, but only three years, to introgress new material into the Elite population in this way. After the selected High lines are crossed with the Elite male steriles, they should contain 93.75% Elite germplasm and their progeny will be considered to be part of the Elite population in the next round of evaluation. They should also contain about 6% 'new' alleles which complement the Elite genetic background. Thus, some new genetic ‘information’ can be introduced into the Elite gene pool without disrupting (nor polluting) the existing adapted, high-performing Elite gene configuration. Because the ‘new’ lines are selected on performance in the mostly Elite background and in the target environment, they should compliment the existing epistatic combinations that have been developed in the Elite population.

Results and Discussion

The shrunken endosperm marker system is highly efficient and very economical to use because male sterile plants can be selected prior to planting the crossing block (FALK *et al.* 1981). Crosses with male steriles typically set about 95% crossed seed and 99% of the crosses produce at least one seed. The repulsion linkage of the orange lemma marker allows elimination of both the male sterility and the shrunken endosperm in bulk plots prior to winter increase and without requiring progeny testing. Initially, crosses were made using the NC Design 2 (see KANNENBERG & FALK 1995), however, in recent cycles, this has been relaxed to where an average of three crosses are made with each male onto a random choice of females. The F1 plants are grown separately, but the F2 seed is composited by common

male parents across females to give a *de facto* NC Design 1. Generally 15-25 male parents are used in the Elite population with another 10-20 at the High level. Up to 25 new 'exotic' lines are introduced into the introgression system each year.

Since its inception, a number of high-yielding, agronomically desirable cultivars have been produced. The cultivars OAC Baxter, OAC Staffa, Celebrity, and Prosper are currently in commercial production. In 2002 and 2003, in 17 official Performance trials across Ontario, three of the top four cultivars for yield were from the RIPE system. These cultivars include the top two lines for test weight and the top line for lodging resistance. They also have good powdery mildew resistance, desirable plant height, and appropriate maturity. In the Provincial Performance trials in 2003, nine of the 23 entries were developed through the RIPE system. The top five for yield were all from the RIPE system, and eight of the top ten were from the system. They had the top test weight, kernel weight, lodging resistance, powdery mildew resistance, and leaf rust resistance. (Figure 4). All of them have combinations of desirable agronomic traits coupled with high yield. The RIPE system is producing more than just germplasm; the lines derived directly from the Elite population are fully competitive and commercially desirable. Sixteen lines from the RIPE program have been supported for registration in the past three years. Once most of the undesirable alleles have been eliminated from the Elite population, many of the best lines produced each cycle will be an improvement on the previous cycle. The lines being developed are equal, or superior, to lines coming out of conventional and doubled haploid breeding programs being used by other breeders for the same target environments.

In 2003, a trial which included the four parents used to create the original foundation population was grown at the same site as the official yield trials with the five most recently developed lines; all trials included the common check variety Brucefield so comparisons could be made across trials (Figure 5). The original parents averaged 24% lower yield than Brucefield while the derived lines were 18% higher, giving more than a 50% increase in yield over the original parents. The parents averaged 9% lower than Brucefield in kernel weight while the lines were 25% higher than Brucefield; an improvement of about 35% in kernel weight. The derived RIPE lines were also better than any of the parents in powdery mildew and leaf rust resistance. Thus, significant improvements in yield were achieved concurrently with significant increases in grain quality in the RIPE system.

The RIPE system comes very close to meeting WRIGHT's (1963) conditions for maximizing the 'enormous evolutionary potential' of a population in the short time frame of a breeding program. This system also addresses McPROUD's (1979) concerns about low numbers of founding parents, few new introductions and long cycle times. The RIPE system, as it has evolved, is highly efficient and effective in developing new cultivars which maintain the suite of genes necessary for adaptation and high yield, and incorporate improved agronomic performance and disease resistance. Using exotic germplasm in an applied breeding program does not necessarily mean taking a step backwards in adaptation nor performance. Combining recombination and introgression in a recurrent selection population is effective in bridging the widening chasm between high-performing Elite lines and the potential genetic contributions of unadapted exotic germplasm currently languishing in the gene banks.

Acknowledgements
This paper is the result of numerous discussions, some in person over a glass of beer and some through the literature, with biologists, plant breeders, geneticists, and farmers who knew there must be a better way. Many of them have passed away over the years but hopefully the

gist of their ideas has been preserved and their legacy can inspire the next generation of breeders to develop even more efficient and effective ways of harnessing the tremendous genetic potential in the plant and animal kingdoms to improve the lot of human kind.

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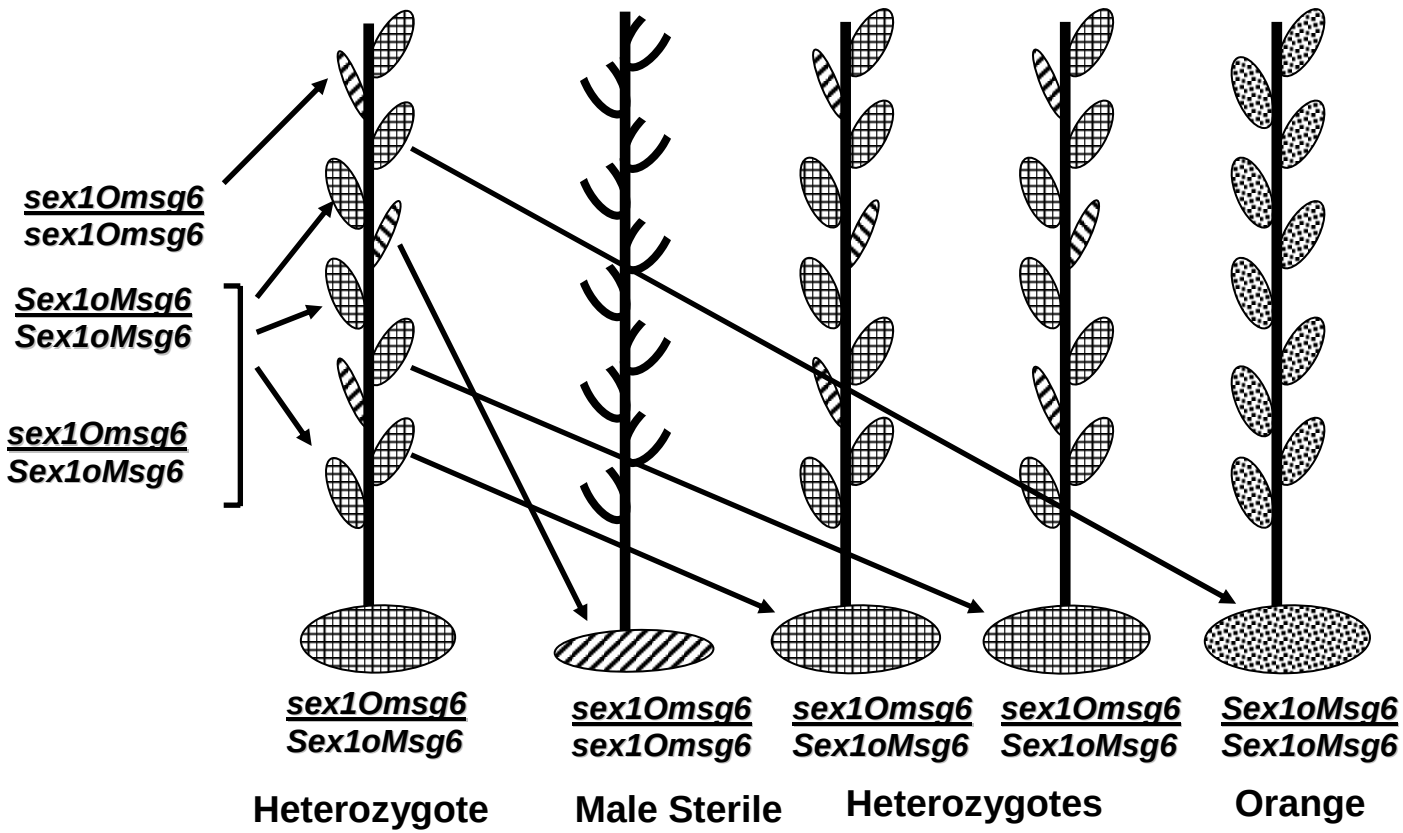

Figure 1. Genotypes and phenotypes of seeds and plants from a heterozygote.

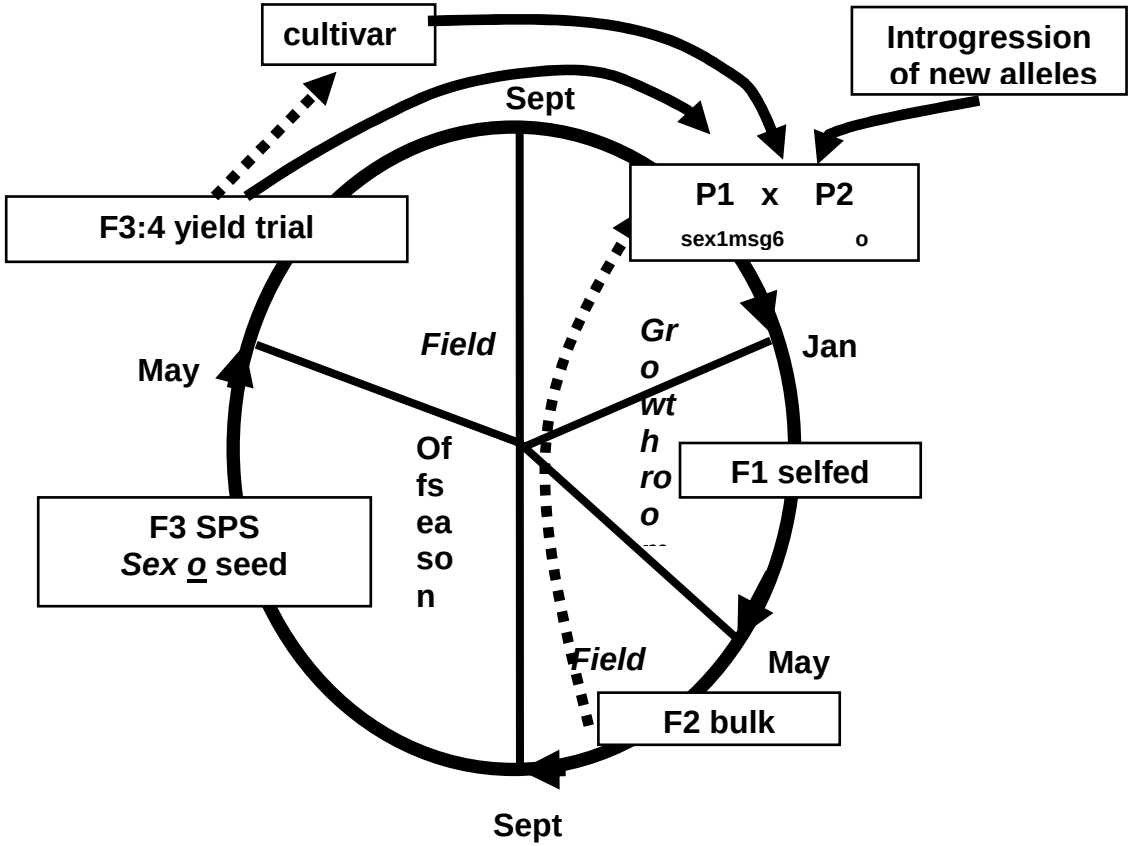

Figure 2. One complete cycle of recurrent selection of the Elite population in the RIPE system has five generations and takes two years.

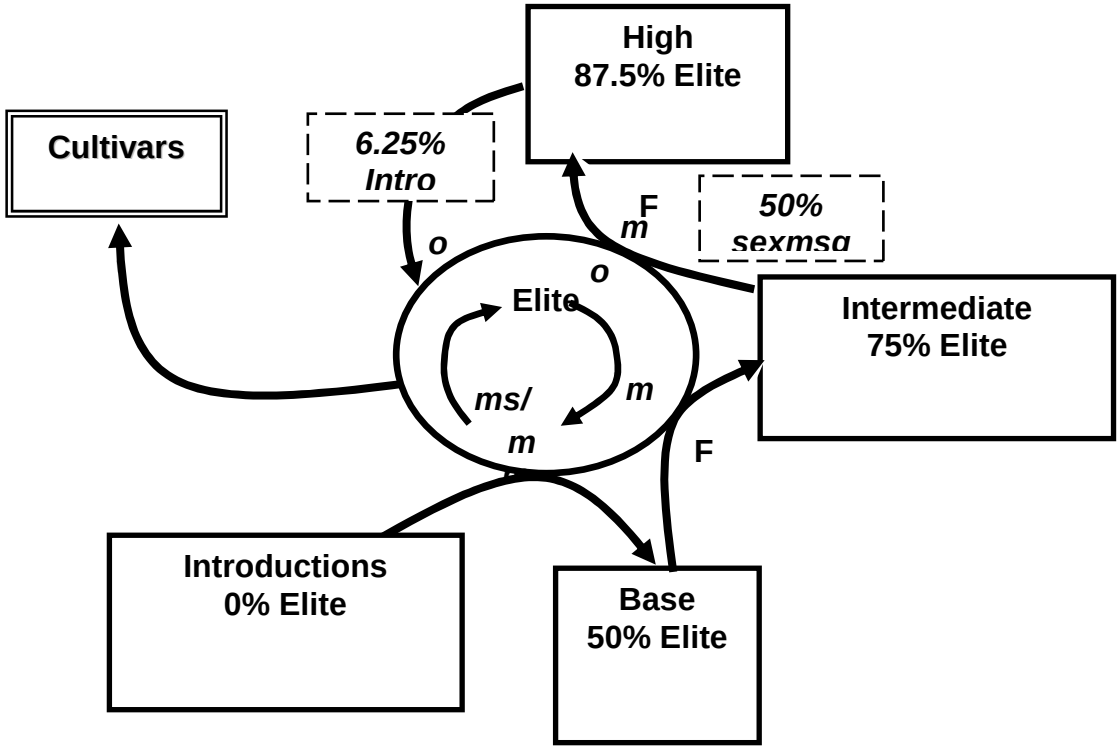

Figure 3. The accelerated introgression of exotic material into the Elite level of the RIPE system has eight generations and takes three years.

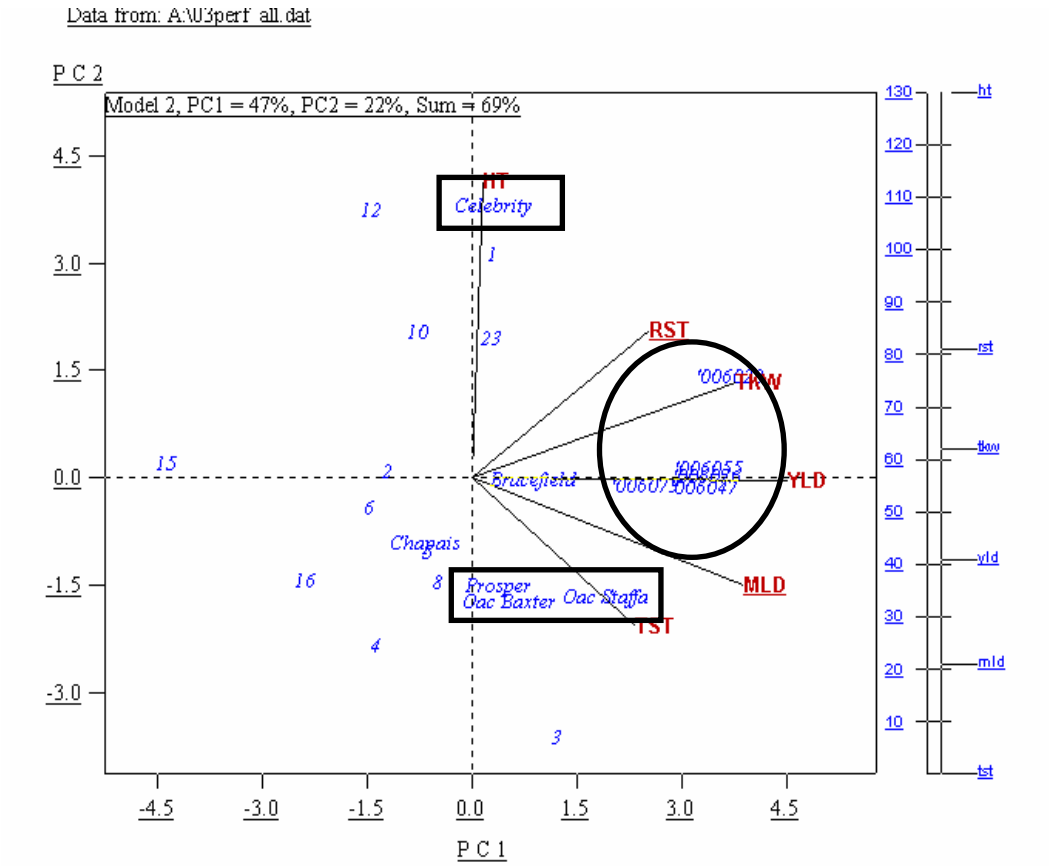

Figure 4. The top five lines and eight of the top ten lines for yield in the Provincial Performance trials in 2003 were from the RIPE system.

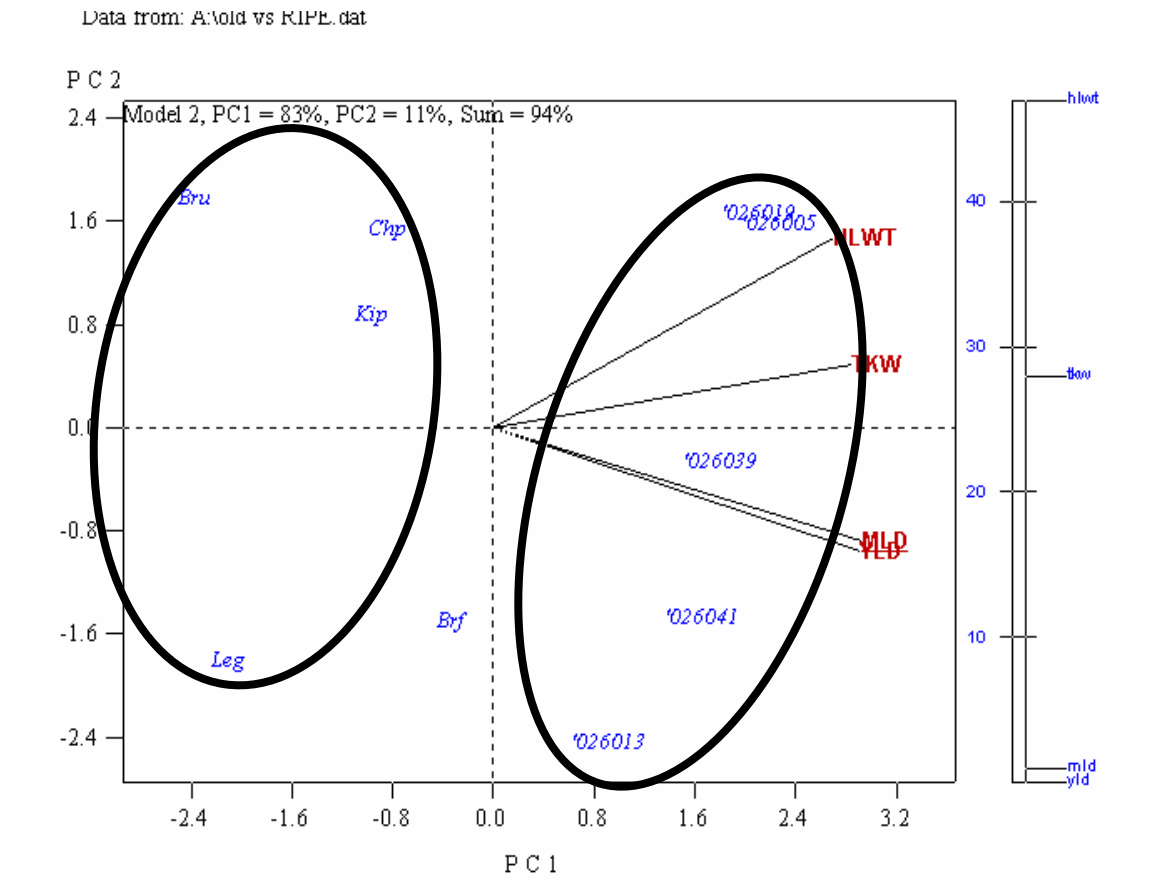

Figure 5. The most recent lines from the RIPE system are significant advances in yield, test weight, kernel weight, and disease resistance compared to the founding cultivars.

QTL for Seedling Root Traits in Barley

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Abstract
A 2-dimensional observation chamber was used to measure seedling root number, root length and root spread in a barley genetic mapping population. Tests were conducted in the dark at 12°C and images scanned and analysed over a 10-day period from germination. The test was used to screen the Derkado x B83-12/21/5 doubled haploid population and QTLs were found on four of the seven chromosomes. QTLs for root length were located on chromosomes 5H and 7H; six root spread QTLs were found on 3H (2), 4H (1), 5H (2) and 7H (1) and two QTLs for root number were located on 4H 5H. The mapping population segregates for two dwarfing genes, *sdw*1 (3H) and *ari-e*.GP (5H), as expected both were associated with reduced seedling shoot length, but only *ari-e*.GP was associated with short roots. Seedling shoot length QTLs were also located on 4H and 7H. Over 60 traits have been mapped in this population and it provided an opportunity to look for associations between root traits and other characters.
Keywords: barley; QTL; seedling root traits; mapping

Introduction

Root systems have until recently been a neglected area of crop research. It is now recognised that root systems vary according to genotype and environment and their effects on plant performance are the subject of much interest. Root traits known to vary with genotype include many vital for crop performance, e.g. rooting depth, density, penetration, length, branching, mass, root hairs, number of seminal roots, exudate composition, nutrient uptake and rhizosphere community. Limited studies have revealed genetic differences for some root characteristics in barley. For example longer and more prolific root hairs have been identified for phosphate deficient soils (GAHOONIA *et al.* 2001). In addition, a recent study has highlighted that whilst there are differences amongst some elite barley cultivars, the differences are relatively small and did not appear to relate to simulated drought conditions. Some differences were apparent in the stability of a yield character with a short-rooted cultivar being more sensitive (BINGHAM & MCCABE 2003). Thus, whilst differences in root characteristics may be small, they may well interact with physiological mechanisms to have a profound effect upon the phenotype. However, for most root traits little is known about the number of genes involved, allelic variation and the magnitude of individual gene effects. Environmental conditions that affect root growth and function include; extremes of weather, waterlogging, drought, mineral deficiencies and toxicities, soil compaction and soil type.

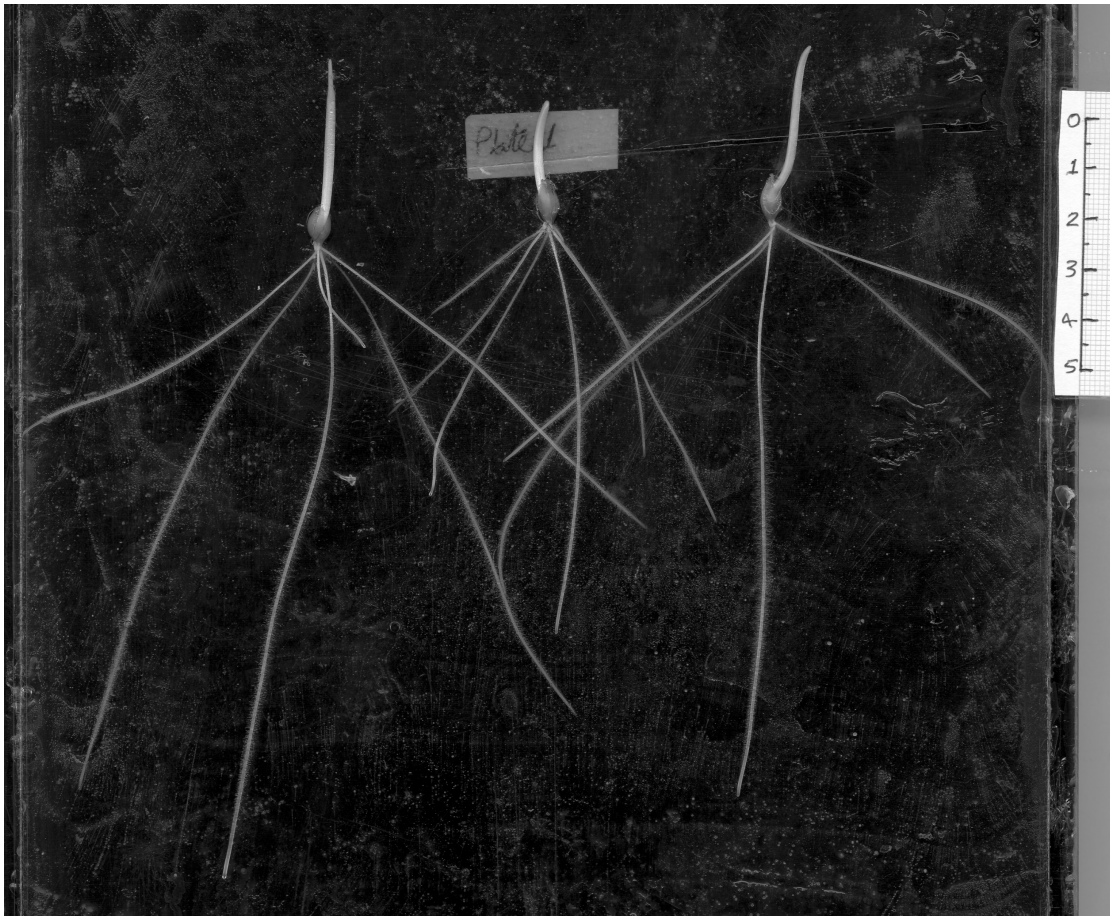
As roots grow in soil, the expression of their various characteristics is difficult to observe and can be modified considerably by differences within and between soil samples. We have therefore utilised a gel observation chamber that has recently been developed in which root traits of cereal seedlings can express their full potential, be easily observed and measured and therefore screened efficiently (BENGOUGH *et al*. 2004). The chamber is formed from two gel-coated plates, one being transparent in which chitted seedlings are placed and allowed to grow. The test is simple, non-invasive, non-destructive, transportable and can been applied to large populations for genetic analysis. Seedling traits that can be scored include shoot length, root length, root number and root spread. We have used this two-dimensional observation

chamber to gather and analyse data on seedling root traits in a barley genetic mapping population.

Material and Methods

Initial tests showed genotypic differences in seedling root traits among parents of genetic mapping populations and the influence of seed size on some traits (BENGOUGH *et al*. 2004). The test population here involved 156 random doubled haploids (DHs) from the F_1 of the cross Derkado x B83-12/21/5. Seed size of the DH lines along with the two parental controls was standardised by sieving (<2.8 mm and >2.5 mm). Seed germination procedures and the seedling root observation chambers were set up as described by BENGOUGH *et al*. (2004). The experiment consisted of four randomised blocks, each comprising of 53 plates with three genotypes per plate (Fig. 1). The experiment was conducted in the dark at 12°C and images of seedling development taken at Day 2 and Day 9 after the transfer of chitted seed to the observation chambers. ScionImage was used to analyse the images and collect data on shoot length, individual and total root length, root number and angular spread.

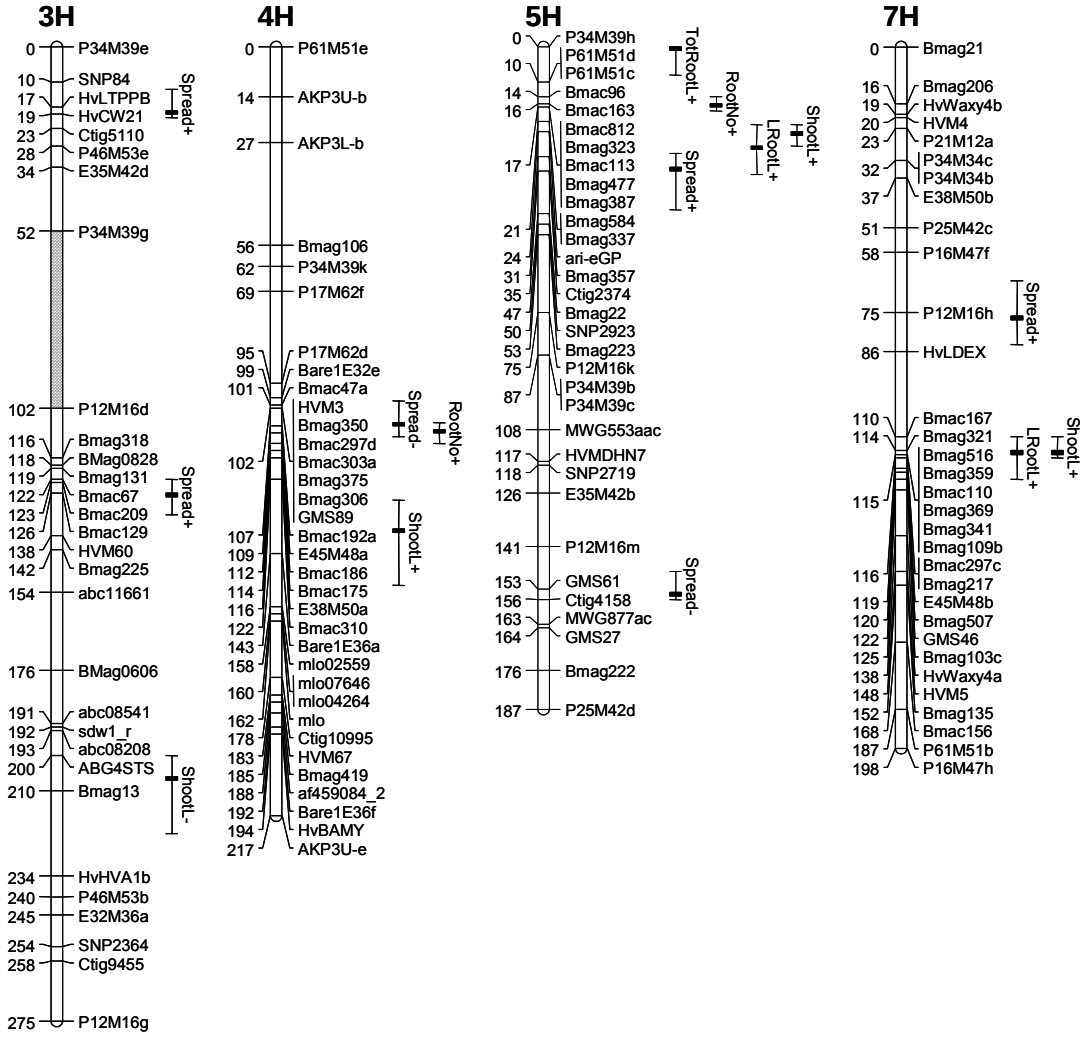
Figure 1. An example of seedlings growing in the observation chamber



The ANOVA directive in GENSTAT was used to analyse the data and produce genotypic means. The updated marker data and genetic map of Derkado x B83-12/21/5 (NEWTON *et al* 2004) was combined with the phenotypic means to search for QTLs using PLABQTL (UTZ & MELCHINGER1996). The default values of PLABQTL were used to identify cofactors for Composite Interval Mapping and this set was selectively refined as described by (NEWTON *et al.*, 2004). MAPCHART (VOORRIPS 2002) was used to display the results (Fig. 2).

Results and Discussion

Figure 2. Location of QTLs for seedling traits shoot length (ShootL), longest root length (LRootL), total root length (TotRootL), angular spread of roots (Spread) and number of roots (RootNo) in the Derkado x B83-12/21/5 population. Thick bars are the QTL peaks and the whiskers their 1 LOD confidence intervals. Sign indicates effects of Derkado allele.



Between two and 8 roots had developed over the whole experiment but some seeds failed to germinate properly and were not included. Analysis of variance revealed significant differences for each of the characters measured, indicating that there was genetic variation for them. For brevity, we shall present results for shoot length, angular spread, root number, total root length and length of the longest root. The highest correlation observed was between length of the longest root and total root length, which is not surprising. Shoot length was also highly correlated with length of the longest root and total root length, suggesting a general correlated growth response in seedlings. The only other moderately high correlation was between root number and total root length. Angular spread was not highly correlated with any of the other characters.

Fifteen QTLs were detected by PLABQTL for seedling root traits on four chromosomes, with the most being found for angular spread (6) and the fewest for total root length (1). After cross validation, the QTLs were found to account for between 37 (shoot length) and 1% (total root length) of the phenotypic variation for the characters. The locations of the QTLs and their confidence intervals are given in Figure 2. Note that no significant QTLs for the characters were detected on chromosomes 1H, 2H and 6H. The only association with a visible phenotype is that of decreased root and shoot length with the semi-dwarfing gene, *ari-e*.GP on chromosome 5H. This association has also found in the soil rhizo-trunking experiments of AL-MENAIE (2003). In contrast, the *sdw1* dwarfing gene on chromosome 3H showed no direct association with any seedling root trait, although it appears to be linked to a QTL decreasing shoot length (Figure 2). These are interesting findings as the dwarfing gene effects on height are manifest right at the seedling stage but only one appears to have an effect on early root development. These two dwarfing genes are known to have effects on a number of traits, although it is not clear whether these are due to linkage or pleiotropy. We have used the updated map to re-analyse the data from hydroponic experiments on shoot weight, root weight, $\delta^{13}C$ in the shoot, and $\delta^{15}N$ in the root presented by ELLIS *et al*. (2002). Our results are consistent with the results from hydroponics experiments as both dwarfing genes were found to have similar effects upon shoot weight as we detected for shoot length. In addition, the *ari-e*GP dwarfing gene was associated with a reduced root weight in the hydroponics experiments, which is consistent with its effects on reduced root number and length detected in the current study.

It is noteworthy that the Derkado QTL allele for increased root number on 4H is associated with another QTL allele from Derkado for reduced root spread. In fact, this is the only clear instance of an association between root spread and any other character measured in the present study or the hydroponics study of ELLIS *et al.* (2002). These are surprising findings as one would have thought that the conditions of our seedling root test may well have forced an apparent association of increased root number with increased spread. It does, therefore, appear that there is potential to manipulate the spread of roots within the elite gene-pool but the implications for rooting depth would require further study. Four of the QTLs for root spread are associated with functional markers. HvCW21 on chromosome 3H and HVM3 on chromosome 4H are genomic SSR markers for a non-specific lipid transfer protein and rubisco activase respectively and Ctig2374 and Ctig4158 on chromosome 5H are EST derived SSR markers for a nucleoporin and a receptor like protein kinase respectively. The significance of these associations is far from clear and requires validation from further, more extensive studies.

In general, the heritability of the seedling root characters was far less than that of the shoot characters, reducing the likelihood of QTL detection for the former. Thus, whilst there are regions such as those on 3H and 4H where shoot length appears independent of root length, there are no instances where QTLs for root number and/or root length are not associated with shoot length. This suggests similar genes and/or mechanisms operate in the growth of these tissues. The major QTLs for plot yield detected in the same population are associated with the two dwarfing gene loci (data not shown). With the exception of the QTL for root spread in the region of Ctig4158 on chromosome 5H, none of the other QTLs detected for the seedling root traits are associated with QTLs for plot yield. This suggests that it might be possible to alter root structure by targeting these QTL and not affect plot yield. Seedling root QTLs were also found to be independent of the developmental QTL for leaf and tiller emergence (data not shown). The lack of associations between seedling root traits and subsequent plant characteristics may be a reflection on the development of an adequate adventitious root

system in the experiments and/or population analysed. Seminal roots may be more important in stressed conditions, e.g. drought where barley plants can survive solely on a seminal root system (BRIGGS 1978). We are therefore studying these characters in another mapping population known to segregate for a range of physiological characteristics related to drought tolerance to test this hypothesis.

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A New Barley Phenotype for North Africa

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Abstract
Barley is a traditional crop of low rainfall areas of North Africa where a common phenotype is non-dwarf plant with six-row ears. In a recent EU INCO-DC programme semi-dwarf and two-row barleys were tested with national checks in rain-fed and irrigated field trials in Egypt, Tunisian and Morocco. Two row and semi-dwarf genotypes performed well and often better than traditional types in certain North African environments. In addition to height and ear-type, flowering time was also found to have a major influence on plot performance. Major genes controlling these traits had strong pleiotropic effects on important agronomic and physiological traits throughout the crop cycle, from germination to maturity date and ultimately yield. Two-row types performed well in Egypt, Morocco and Tunisia and selected lines have now entered breeding programmes. Semi-dwarf types were also competitive in Egypt and Tunisia, but data from Morocco were less convincing. Since major genes have large effects on adaptation to low input conditions of North Africa there is a need to select the most appropriate alleles in matching plant development with environment. Our data challenge the dogma that tall, six-row barleys are the best ideotype for North Africa.
Keywords: barley; North Africa; phenotype; phenology

Introduction

Drought is a major constraint to crop production in North Africa. Barley is drought tolerant and constitutes a reliable risk crop in low input, dry land agriculture. In North Africa barley production is dominated by tall genotypes with six-rowed ears. The crop is used primarily as a multipurpose animal feed for which green and dry shoots are grazed and later grain is fed. Decreasing amounts of rain over the last 40 years has seriously affected barley production in North Africa. In Morocco for example drought years have increased from 1 in 10 to 1 in 2 years and a lack of rain can result in crop failure. Climate change in Europe has also resulted in drought, which now seriously threatens traditional cereal production areas throughout Europe.

In 1998 the EU funded a European/African collaborative project "Stable yields in Mediterranean barley: application of molecular technologies in improving drought tolerance and mildew resistance". The research involved three contrasting approaches; backcross conversion using modern cultivars, physiological analysis of adapted lines developed from landraces and advanced backcross quantitative trait locus (QTL) analysis. All three approaches involved mapping populations that aimed to identify regions of the genome influencing performance in drought-stressed conditions. A key component of the work was the application of molecular genetics to develop genetic maps and detect associations with physiological and agronomic traits. Populations were specifically developed using a wide range of material including cultivars, landrace derived lines and wild genotypes. Over 600 lines were tested in two or more seasons in non-irrigated (rain-fed) and irrigated field trials in

Morocco, Tunisia and Egypt. Comparisons with local standards allowed the identification of superior lines for use in breeding.

QTL Analysis

Populations

Three populations were investigated for QTL analysis:-

1) recombinant inbred lines (RILs) from the Tadmor x ER/Apm cross
2) doubled haploids (DHs) from the cross Derkado x B83-12/21/5
3) advanced backcross QTL analysis (ABQA) population developed from the cross Barke x Hor11508.

The RIL population (167 individuals) was initiated and developed jointly by CIMMYT and ICARDA to investigate the genetics of drought tolerance. The parental lines, Tadmor and ER/Apm are selections from Mediterranean landraces, both have two-row ears, but contrast for physiological responses to water stress (TEULAT-MERAH *et al*. 2000). The population has been studied extensively by QTL analysis of physiological traits in controlled environment experiments. Physiological traits studied included: plant water status, relative water content, water-soluble carbohydrate, osmotic adjustment, carbon isotope discrimination and chlorophyll content (TEULAT *et al*. 1997a; 1997b 2001c; TEULAT *et al*. 1998; THIS *et al*. 2000; TEULAT *et al*. 2001a). The RIL genetic map was developed further during the INCO project and now contains over 150 markers (RAPDs, AFLPs, SSRs and candidate genes: see the GrainGenes data-base for updates). The marker map was used to detect QTL for agronomic as well as physiological traits detected in data from field trials conducted in Morocco, Tunisia and Egypt (1999/2000 and 2000/2001 seasons). Agronomic traits included: plant height, heading date, aerial biomass, grains per spike, thousand-corn weight and grain yield (TEULAT *et al*. 2002; TEULAT-MERAH *et al*. 2000, 2003). A comparison of physiological and agronomic QTL maps is given in FORSTER *et al*. (2004).

The Derkado x B83-12/21/5 population consisted of 156 DH lines. Both parental lines have two row and semi-dwarf phenotypes (Derkado carries the *sdw1* dwarfing gene and B83-12/21/5 the *ari-e*.GP dwarfing gene). The population has been studied intensively for over 60 traits, including quality, yield, disease resistance and abiotic stress tolerance (THOMAS 2003; ELLIS *et al.* 2002). This population segregates for plant stature (tall, semi-dwarfs and doubled-dwarf types in a ratio of 1:1:1:1) and provided an opportunity to compare semi-dwarfs, that have received little attention in North Africa, with tall types in a similar genetic background. In contrast, the semi-prostrate semi-dwarf habit has been highly developed in European spring barley and shows greater yield potential than tall genotypes. In addition co-location with other QTLs can be investigated. The DH population was grown in irrigated and non-irrigated sites in Egypt and non-irrigated sites in Morocco and Tunisia. Agronomic data were collected throughout the season, from seedling emergence to yield and subjected to QTL analysis (FORSTER *et al*. 2004).

Hor11508 is a wild barley (*Hordeum spontaneum*) line that was used in a backrossing programme with the modern European semi-dwarf (*sdw1*) cultivar Barke to create a population for advanced backcross QTL analysis, ABQA (TANKSLEY & NELSON 1996). The method aimed to introgress positive alleles for drought tolerance into elite material from the wild, and to identify linked markers. A DH population was developed from the BC_1F_2 generation. DH lines with negative, wild traits (dormancy, inappropriate flowering time, shattering, low grain weight, etc) were discarded. The remaining 123 selected DHs were evaluated in the field under varying conditions of water stress in Italy, Morocco and Tunisia (TALAMÈ *et al.* 2004).

Effects of Genes for Flowering Time and Height

The biggest QTL effects were found to be associated with major developmental genes for height and flowering time and these are the results of pleiotropy. In the Derkado x B83-12/21/5 DHs over 10 QTLs (seedling establishment, vigour, height and height components, heading date, yield and yield components) were associated with both dwarfing genes, *sdw1* on chromosome 3H and *ari-e*.GP on 5H (FORSTER *et al*. 2004). The Tadmor x ER/Apm RILs segregate for vernalisation response and QTL analysis revealed a cluster of 4 QTLs (yield and yield components, and osmotic adjustment) on the long arm of chromosome 4H in the region of the spring growth habit gene, *sgh1* (FORSTER *et al*. 2004). Large QTL were also found to be associated with flowering time loci (*Ppd* on 2H and 5H) and height (*sdw1*, 3H) in the ABQA (TALAMÈ *et al*. 2004) indicating segregation for photoperiod response. Although the effects of these genes can be masked in certain environments there is a need to check segregation for such genes as they have large effects.

Although it was possible to detect associations with major genes for flowering time and height in these populations none segregated for two-row/six-row ear type (all were two row). However, comparisons of ear type were part of a phenotype survey (see below).

Other Effects

Over 20 agronomic and over 30 physiological QTLs were detected in the RIL population with all seven chromosomes being involved (TEULAT *et al*. 1998, 2001a, 2002b, 2001c; TEULAT-MERAH *et al*. 2002, 2003). In addition to the QTL clustering at the *sgh1* locus, three other hot spots were detected in the RILs in which agronomic and physiological traits were coincident. A centromeric region on 2H was associated with QTLs for thousand grain weight (TGW) and osmotic adjustment, relative water content (RWC), osmotic potential (OP) and water soluble carbohydrate. Another QTL for TGW was associated with carbon isotope discrimination on the long arm of 6H, and a QTL for plant height co-located with QTLs for RWC and OP on 7H (centromeric). Although *sdw1* and *ari-e*.GP loci accounted for most (28) QTLs another 20 were identified over 6 of the 7 chromosomes in the Derkado x B83-12/21/5 DHs (1H proved to be inert, data to be published elsewhere). Interestingly, some commonality was found between the DH and RIL QTL data, e.g. grain yield/TGW QTL on 6H and plant height on 7H.

Phenotype Survey

All three QTL populations were tested at multiple sites in replicated field experiments in Morocco, Tunisia and Egypt. Over 600 genotypes were tested including a standard check, Rihane common to all three countries, and the best local standards. All the standards were tall six row types against which semi-dwarf and two row types could be compared, Table 1 gives some examples for the main yield traits, aerial biomass and grain yield. From Table 1 it can be seen that the top lines included a mixture of ear and height types, except in Noubaria trials where the best lines were all two row. Several lines from the RIL population were selected in Tunisia, eight of which out yielded Rihane and the local check Souihli. Clearly two row genotypes are competitive with traditional six row barleys in North African. Semi-dwarf genotypes also performed well in Tunisia and Egypt, increased tiller production was found to compensate for height reduction in biomass and grain yield. This was somewhat surprising as all semi-dwarf genotypes were of European origin and had been bred for yield potential under high inputs. The results suggest that breeding with two row barleys should be encouraged and that the incorporation of dwarfing genes into North African breeding programmes can provide further additional benefits.

Table 1. Top lines in various test sites

Test site	Condition	Trait	Top lines	Phenotype	
				Ear	Height
Meknès, Morocco	Irrigated	Biomass	Local*	6 row	Tall
			Rihane	6 row	Tall
		Grain yield	Three RILs	2 row	Tall
Le Kef, Tunisia	Non-irrigated	Biomass	Eight RILs	2 row	Tall
Noubaria, Egypt	Non-irrigated	Biomass	Prisma	2 row	Semi-dwarf
			Atem	2 row	Tall
			DxB DH line	2 row	Semi-dwarf
			ER/Apm	2 row	Tall
Giza, Egypt	Irrigated	Biomass	Giza 126*	6 row	Tall
			B83-12/21/5	2 row	Semi-dwarf
			RIL81	2 row	Tall
		Grain yield	ER/Apm	2 row	Tall

* Local standards

Conclusions

The results of QTL analysis can be broken down into two component parts; 1) effects of major genes, and 2) other effects. The strong pleiotropic effects of developmental genes cannot be ignored. Positive effects of ear type and plant stature genes have been noted and bring into question the dominance of six-row, tall barleys in North Africa. Two-row types were among the top performing lines in all three countries and should be given greater consideration in future breeding efforts. A case for semi-dwarf genotypes can also be made for Tunisia and Egypt. Flowering time is also critical in matching phenotype to the environment but this can be achieved by the manipulation of a number of genes/alleles. Since flowering time genes can have strong pleiotropic effects it is necessary to trawl among the available diversity to select the most beneficial source. In addition to developmental genes the work has highlighted other regions of the barley genome that can be targeted in improving drought tolerance in barley. Some of these are associated with physiological characteristics that may themselves be used as selection criteria for practical plant breeding, or alternatively used to study drought tolerance mechanisms. The primary genepool of barley has proved to be a rich genetic resource with cultivated, landrace and wild lines possessing valuable traits (ELLIS *et al*. 2000). The genetics and physiological dynamics of drought tolerance in relation to the Mediterranean environment is now the subject of a new EU INCO-DC project: "Mapping adaptation of barley to drought environments" (ICA3-2002-100073).

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Genetic Variation of Barley Seed Lipoxygenase-1: Thermostability

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Abstract
Barley lipoxygenase-1 (LOX-1) is believed to affect the flavor- and foam-stability of beer. We have screened more than 1000 barley landraces worldwide for the LOX-1 relative thermostability (LOX-RTS). The frequency of the LOX-RTS value was distributed in a bimodal manner. Based on these values, the barley lines were categorized into 2 groups: H-type with relatively thermostable LOX-1, and L-type with relatively thermolabile LOX-1. The qualitative trait locus (QTL) analysis with Steptoe/Morex doubled haploid lines revealed a major QTL of the LOX-RTS value located at around the *LoxA* locus on the barley chromosome 4H (LOD score = 46.97, Variance explained = 81.9 %). Geographic distribution of these H- and L-type barley lines implied how these LOX-1 genes spread.

Introduction

Lipoxygenase (EC.1.13.11.12) (LOX) is a non-heme ferrus protein, which catalyzes hydroperoxidation of polyunsaturated fatty acids with the 1.4-*cis*-*cis*-pentadiene structure. In barley (*Hordeum vulgare* L.) seed, two isozymes have been characterized, namely LOX-1 and LOX-2 (BAXTER 1982; YANG & SCHWARZ 1995). VAN MECHELEN *et al*. (1995, 1999) characterized the cDNA structures for LOX-1 and LOX-2, and mapped them to *LoxA* locus on chromosome 4H and *LoxC* locus on chromosome 5H, respectively.
In the brewing industry, LOX-1 is regard as a negative enzyme that deteriorates the flavor- and foam-stabilities of beer (DROST *et al*. 1990; KURODA *et al*. 2002). WU *et al*. (1997) found the genetic variation of LOX-1 activity using several barley cultivars, suggesting that the LOX-1 activity might be reduced through breeding. An alternative way to reduce the LOX-1 activity in the brewing process through breeding is to create a variety with heat-labile LOX-1. YANG and SCHWARZ (1995) suggested that kiln condition and genotypic variation may have an influence on the LOX isoenzyme survival in finished malt. However, intensive research on the thermostability of LOX-1 has never been reported.
The purpose of this study is to investigate the genetic variation of the LOX-1 thermostability, and to analyze the mode of inheritance of this trait.

Material and Methods

Plant Material
Barley cultivars (Collection of Sapproro Breweries Ltd.) and landrace lines (Collection of Okayama University) were used to evaluate the thermostability of LOX. Steptoe/Morex doubled haploid lines (DHLs) were used for the QTL analysis of the LOX-1 thermostability. The plant materials and genotype data of the Steptoe/Morex DHLs were supplied by the North American Barley Genome Mapping Project (NABGMP). The DHLs were grown in the Kurashiki field, Okayama, Japan.

LOX Thermostability Assay
Crude enzyme was extracted from a single silent seed with cold extraction buffer (0.1 M sodium acetate, pH 5.5). After centrifugation, the supernatant was incubated at 24°C for 90 min in a cocktail (2 mM linoleic acid, 0.05 % tween 20, 0.1 M sodium acetate, pH5.5). The

reaction was stopped by adding an equal volume of BHT solution (0.8 mM 2-6-*t*-butyl-cresol in methanol). After centrifugation, the supernatant was subjected to a hydroperoxide measurement according to JIANG *et al*. (1991) using cumene hydroperoxide as the standard. The heat treatment condition to evaluate the thermostability is described in Results and Discussion. A negative control of the individual samples was prepared by inactivating the crude enzyme through heat treatment at 100°C for 5 min. All heat treatments were carried out using PTC-200 Peltier Thermocycler (MJ Research, Inc.).

QTL Analysis

The QTL analysis was performed using genotype data of RFLP markers for the 150 lines in the Steptoe/Morex DHLs, referred to as NABGMP. The QTL analysis was performed using a computer program, Mapmaker QTL (Lincoln *et al*. 1993) in the back cross population analysis mode. The threshold of the log-likelihood (LOD) score was set at 2.0 for detecting the QTLs.

Results and Discussion

Heat Treatment-Dependent Decline of LOX Activity

In a preliminary experiment, the crude extract from the barley landrace lines, OUC619, OUN647 and OUU025, showed relatively high, medium and low thermostabilities of the silent seed LOX, respectively. Crude enzymes from these landraces were incubated from 46 to 70°C for 30 min, and the decline of the LOX activity was traced. As shown in Figure 1, the LOX activities declined depending on the treatment temperature. The heat treatment at 60°C for 30 min was suitable to evaluate the thermostability of LOX in these three lines. In a subsequent experiment, we adopted this condition to assess the thermostability of the barley seed LOX. For an index to represent the thermostability of the seed LOX, the relative remaining activity was defined as the activity with heat treatment over the activity without heat treatment: LOX-RTS (%).

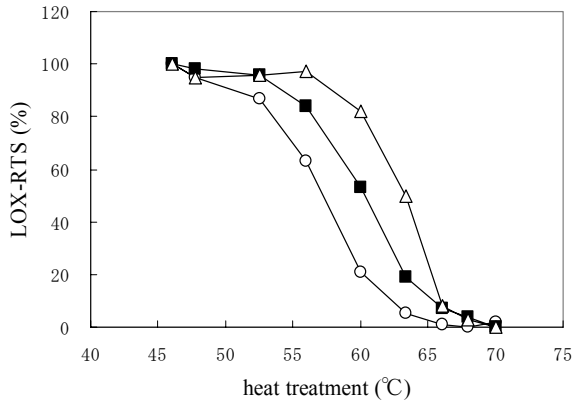

Figure 1. Heat treatment-dependent decline of LOX activity
Open triangle: OUC619, closed square: OUN647, open circle: OUU025

Variation of LOX Thermostability in Barley

The LOX-RTSs of 153 barley cultivars were surveyed. The frequency distributions of the LOX-RTSs were broad, but two major peaks were observed (Figure 2). Based on this result, seed LOX in the silent seed of barley can be mainly categorized into two groups in terms of its thermostability: high thermostability type (H-type) and low thermostability type (L-type). Further analysis showed that the thermostability types could be discriminated based on a LOX-RTS value of 52.5 % (data not shown). In the barley silent seed, it was reported that LOX-1 showed predominant LOX activity, whereas the LOX-2 activity was not detected

(YANG *et al*. 1993). In addition, we confirmed the predominance of LOX-1 in the protein level using the LOX-antibody (data not shown). Therefore, the thermostability of LOX analyzed in this study was regarded to be that of LOX-1.

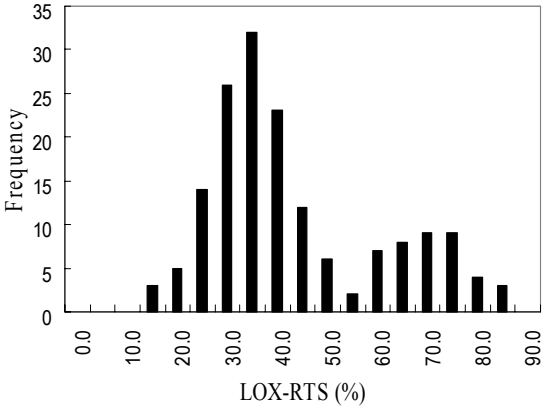

Figure 2. Frequency distributions of LOX-RTS in barley cultivars

Barley landrace lines worldwide (1040 lines) were surveyed for the thermostability of LOX-1. We observed bimodal frequency distributions of LOX-RTSs in the lines, as well as in the cultivars (Figure 3). The geographical distributions of the thermostability types are shown in Figure 4. The frequencies of the H- and L-type were almost equal to each other in western Asia. The occurrence of the H-type predominated in eastern Asia (Japan, Korea and China) and Africa (Ethiopia and north African countries), whereas in Europe and Turkey, the L-type did. This result is consistent with the idea that various phenotypes that exist in the Middle East area differentiated into the Western type and the Eastern type (TAKAHASHI 1955; TAKEDA 1996; TAKEDA & CHANG 1996; COOKE 1996; KANEKO *et al*. 2001).

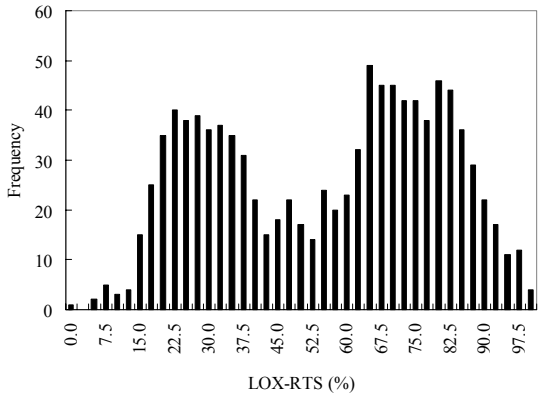

Figure 3. Frequency distributions of LOX-RTS in landrace lines

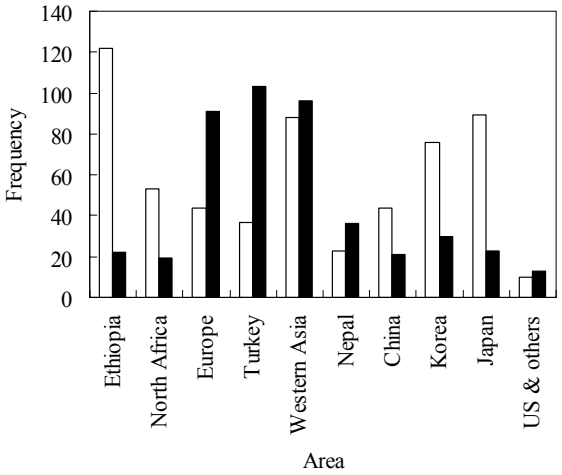

Figure 4. Geographical distributions of LOX-1 thermostability types
Open bar: H-type, closed bar: L-type

QTL and Segregation Analysis

In order to investigate the factor controlling the thermostability, a QTL analysis was carried out with the Steptoe/Morex DHLs. The LOX-RTS values of the parents were 71.3 % in Steptoe (H-type) and 35.0 % in Morex (L-type) (n = 4). The frequency of the LOX-RTSs for

the DHLs showed a bimodal distribution around the LOX-RTSs of each parent (data not shown). The QTL analysis revealed two QTLs detected on the short arm of chromosome 4H (LOD score = 46.97, Variance explained = 81.9 %) and on the short arm of chromosome 7H (LOD score = 3.12, Variance explained = 9.1 %) (Table 1). The major QTL on chromosome 4H is regarded to determine the thermostability of the seed LOX predominantly.

Table 1. Location and effect of QTLs on LOX-1 thermostability

Chromosome	Marker interval	LOD of peak	Variance explained (%)	Weight
4H	CDO669-B32E	46.97	81.9	-0.407 (S)
7H	iEst5-His3A	3.12	9.1	-0.136 (S)

The individual LOX-RTS values of the Steptoe/Morex DHLs were translated into the phenotype of the LOX thermostability (H-type and L-type) based on the borderline of the LOX-RTS value (52.5 %). The segregation ratio of the H-type to L-type was 80:70. The ratio fit to the 1:1 ratio (χ^2 = 0.67, p = 0.41), indicating that LOX-RTS was governed by a single allele. The factor controlling the LOX-RTS in this population was mapped on chromosome 4H at a distance of 13.4 and 4.2 cM from CDO669 and B32E, respectively (Figure 5). The QTL was near the *LoxA* locus, which corresponds to the LOX-1 structural gene (VAN MECHELEN *et al*. 1999). Furthermore, in enzyme expression experiments in *E. coli,* the LOX-1 cDNAs derived from Steptoe and Morex were translated into the H-type and L-type LOX-1, respectively (data not shown). Thus, we concluded that the thermostability of LOX-1 was attributed to the gene structure. We are analysing the cDNA sequences which directly affect the thermostability of LOX-1.

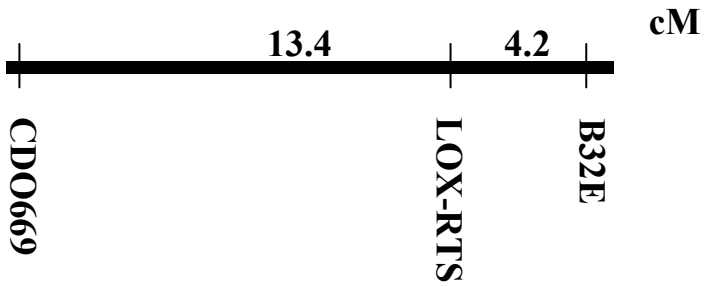

Figure 5. A chromosome 4H segment showing molecular markers linked to the LOX-1 thermostability

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Genetic Variation of Barley Seed Lipoxygenase-1: Null Mutants

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Abstract

Barley lipoxygenase-1 (LOX-1) is regarded to be involved in the formation of beer-deteriorating substances. We have screened barley germplasms worldwide (Collection of Okayama University, Japan) for LOX-1 activity, and discovered six barley lines without significant LOX-1 activity. An immunological analysis showed that these germplasms lacked the authentic LOX-1 protein. The segregation analysis revealed that the LOX-1-null phenotype was governed by a single recessive gene that was located at the *LoxA* locus on chromosome 4H. The six LOX-1 null mutants shared similar features, implying that these mutants might be derived from the same ancestral origin.

Introduction

Lipoxygenase (EC.1.13.11.12) (LOX) is a non-heme ferrus protein, which catalyzes hydroperoxidation of polyunsaturated fatty acids with the 1.4-*cis*-*cis*-pentadiene structure. Two isozymes of LOX in barley (*Hordeum vulgare* L.), LOX-1 and LOX-2, have been identified (BAXTER 1982; YANG & SCHWARZ 1995). The genes of LOX-1 and LOX-2 were cloned and mapped to the *LoxA* locus on chromosome 4H and to the *LoxC* locus on chromosome 5H, respectively (VAN MECHELEN *et al*. 1995; VAN MECHELEN *et al*. 1999). LOX-1 produces predominant LOX activity in malt, and has a relatively low pI compared to LOX-2 (YANG *et al*. 1993; YANG & SCHWARZ 1995).

Brewing scientists have paid attention to LOX-1 because this enzyme is involved in the formation of beer-deteriorating substances in the brewing process (KURODA *et al*. 2002; KURODA *et al*. 2003). In contrast to these functional characterizations of LOX-1 in the brewing process, little is known about its genetic variation in barley. If available, barley germplasms without LOX-1 activity may make it possible to breed barley varieties with the potential to improve beer quality. The purpose of this study is to search for the LOX-1 null mutant barley, and to analyze the mode of inheritance of this trait.

Material and Methods

Plant Material

Barley germplasms from the Research Institute for Bioresources, Okayama University, Japan, were used to screen for the LOX-1 activity. Segregation analysis of a LOX-1-null trait was performed using the F_2 seeds (n = 136) derived from a cross between cultivar CDC Kendall and LOX-1-null barley landrace SBOU2. The mapping of the LOX-1 null gene was performed using DNAs of the F_2 plants (n = 144) derived from the same cross.

LOX Enzyme Assay

Crude enzyme was extracted from a single silent seed with cold extraction buffer (0.1 M sodium acetate, pH 5.5). After centrifugation, the supernatant was incubated at 24°C for 5 min in a cocktail (2 mM linoleic acid, 0.05 % tween 20, 0.1 M sodium acetate, pH5.5). The reaction was stopped by adding an equal volume of BHT solution (0.8 mM 2-6-*t*-butyl-cresol in methanol). After centrifugation, the supernatant was subjected to measurement of

hydroperoxide according to JIANG et al. (1991) using cumene hydroperoxide as the standard. Negative controls of the samples were prepared by inactivating individual crude enzymes through heat treatment at 100°C for 5 min.

Western Blot Analysis

Total protein was extracted from a single silent seed with 20 mM Tris-HCl (pH 7.5) containing Complete Mini (Roche diagnostic, GmbH). The extracted protein (3 µg) was separated by 10 % (w/v) SDS polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to an Immobilon-P membrane (Millipore) by electroblotting. The LOX protein was detected with the LOX-antibody (KURODA *et al*. 2003). This antibody recognized both LOX-1 and LOX-2. Isoelectric focusing (IEF) were performed using PhastGel pI 3-9 (Pharmacia Biotech AB).

RFLP and CAPS Analysis

Isolation of genomic DNA, Southern blotting, and restriction fragment length polymorphism (RFLP) analysis were carried out as previously described (KANEKO *et al*. 1999). JBC970, which was previously mapped on the short arm of chromosome 4H (MIYAZAKI *et al*. 2000), was used as the RFLP probe. For the cleaved amplified polymorphic sequence (CAPS) analysis of the LOX-1 structural gene, two primers (5'-CCATCACGCAGGGCATCCTG-3', 5'-GCGTTGATGAGCGTCTGCCG-3') were designed based on a known LOX-1 cDNA sequence (VAN MECHELEN *et al*. 1995). The polymorphism was detected by digesting the amplified DNA with a restriction enzyme, *Afa*I. Linkage relationships were calculated using the MAPMAKER/Version 3.0b program with a LOD threshold of 3.00 (LANDER *et al*. 1987).

Results and Discussion

Screening of Landrace Lines for LOX Activity

LOX activity in the silent seed was investigated for 1,152 landrace lines stocked at the Research Institute for Bioresources, Okayama University. Six lines, SBOU1, SBOU2, SBOU3, SBOU4, SBPOU5 and SBOU6 (SBOU2 type lines), did not exhibit significant LOX activity compared to each negative control (at the 5 % probability level, n = 8)(Table1). All of the lines shared common features regardless of their geographic origin: covered kernel, 6-kernel rows, blue kernel, occidental type of rachis-brittleness (TAKAHASHI 1955), 'A type' of β-amylase thermostability (KANEKO *et al*. 2001), and so on (Table1). Moreover, the CAPS analysis for the LOX-1 structural gene revealed that the six lines exhibited the same unique pattern, which has not been observed for the other analyzed lines (Figure 1).

These facts imply that these lines are derived from the same ancestral origin. This mutant probably originated in the Indo-Nepal region, and was introduced into Taiwan. We will continue a further investigation on the relationship among these lines.

Table 1. Characteristics of LOX-1 null mutant lines

Characteristics	SBOU1	SBOU2	SBOU3	SBOU4	SBOU5	SBOU6
Seed LOX-1 activity	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]
Covered or naked kernel	Covered	Covered	Covered	Covered	Covered	Covered
Kernel rows	6	6	6	6	6	6
Ear-awn type	Lax-long	Lax-long	Lax-long	Lax-long	Lax-long	Lax-long
Leaf-sheath hair	Absence	Absence	Absence	Absence	Absence	Absence
Heading time[b]	Mid-Apr.	Mid-Apr.	Mid-Apr.	Mid-Apr.	Late-Apr.	Mid-Apr.
Kernel color	Blue	Blue	Blue	Blue	Blue	Blue
Rachilla hair	Long	Long	Long	Long	Long	Long
Vernalization requirement	Highly spring	Highly spring	Highly spring	Highly spring	Spring	Spring
Brittleness of rachis	Occidental	Occidental	Occidental	Occidental	Occidental	Occidental
β-amylase thermostability	Type A	Type A	Type A	Type A	Type A	Type A
Origin	India	India	Taiwan	Taiwan	Nepal	Nepal

[a] Not detectable.

[b] Data in Okayama, Japan.

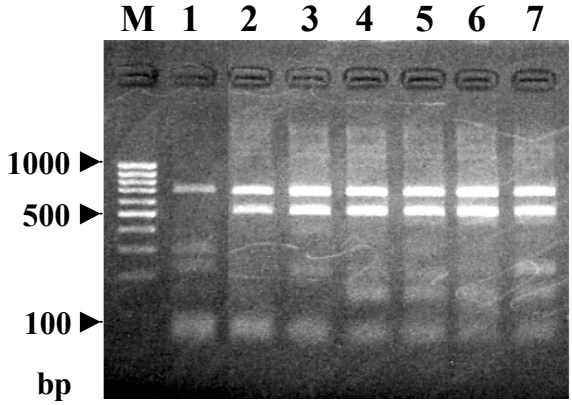

Figure 1. CAPS patterns of the LOX-1 structural genes
M: molecular marker, 1: wild type, 2: SBOU1, 3: SBOU2, 4: SBOU3, 5: SBOU4, 6: SBOU5, 7: SBOU6

Immunological Analysis of LOX Protein from Silent Seed

Western analysis of the silent seed protein after SDS-PAGE revealed two faint bands about 95 kDa and 57 kDa for the SBOU2 type lines, while one major band about 95 kDa for CDC Kendall (Figure 2a). Western analysis after IEF separation did not reveal a lower pI band around pI 4.8, but high pI band around pI 6.5 in the SBOU2 extract. This indicates that the 95 kDa band of the SBOU2 type lines in SDS-PAGE/Western analysis is derived from the LOX-2 protein based on its pI (YANG *et al*. 1993) (Figure 2b). YANG *et al*. (1993) also showed that the LOX-2 activity was detected only in germinating barley. Therefore, it can be concluded that the SBOU2 type lines are LOX-1 null mutants. LOX null mutants have been isolated and well characterized in soybean (KITAMURA *et al*. 1983). However, such a LOX null mutant has never been reported in barley. This is the first report of the LOX-1 null mutant lines in barley.

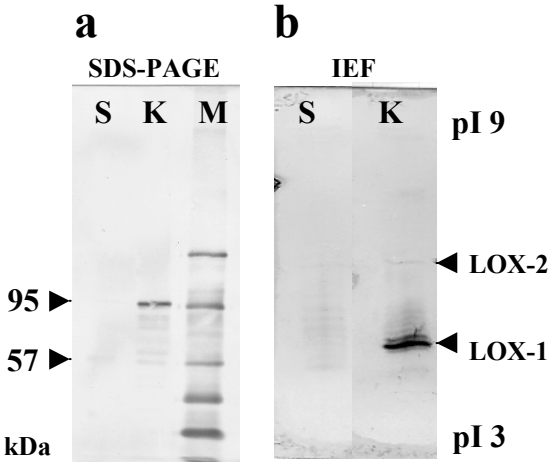

Figure 2. Western blot analysis of LOX-1 protein extracted from SBOU2 and CDC Kendall. a: Western blot analysis after SDS-PAGE separation. b: Western blot analysis after IEF separation. Lane M: molecular marker, lane S: SBOU2, lane K: CDC Kendall

The Segregation and Linkage Analysis

Segregation of the LOX-1 null phenotype was investigated using F_2 generation seeds (n = 136) derived from the cross between CDC Kendall and SBOU2. The segregation ratio of the wild type to the null phenotype was 104:32. The ratio well fit to the 3:1 ratio ($\chi^2 = 0.157$, p = 0.69), indicating that the LOX-1 null trait is governed by a single recessive gene in Mendelian inheritance.

For linkage analysis, the LOX-1 phenotype of the F_2 plants was estimated based on the LOX assay data for F_3 seeds (n = 5) derived from individual F_2 plants (n = 144). The CAPS analysis for the LOX-1 structural gene was carried out for their F_2 DNAs. All the plants giving the homozygous SBOU2 type CAPS pattern exhibited the LOX-1 null phenotype, and vice versa. The location on chromosome 4H was confirmed using the previously mapped RFLP probe (JBC970). Linkage analysis with MAPMAKER revealed that the LOX-1 null trait was assigned to a locus at a distance of 0 and 4.1 cM from the LOX-1 structural gene and JBC970, respectively. (Figure 3). The location is consistent with a previous report describing that the barley LOX-1 gene was located at the *LoxA* locus on the short arm of chromosome 4H (VAN MECHELEN *et al*. 1999). Therefore, these results suggested that the deficiency of LOX-1 was due to a homozygous allele, *loxA*, which was recessive to the one (*LoxA*) required for the presence of the LOX-1 activity and the protein (Figure 3). This information will be effectively used in breeding programs to create advanced LOX-1 null malting barley varieties.
We have already confirmed the effectiveness of the LOX-1 null barley in a trial brewing, indicating that this trait will become one of the powerful tools to improve beer quality.

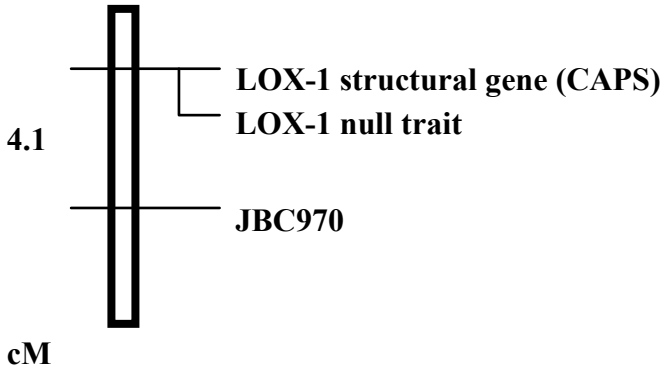

Figure 3. A chromosome 4H segment showing molecular markers linked to the LOX-1 null trait

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Characterization of Genetic Diversity in ICARDA Core Collection of Cultivated Barley (*Hordeum vulgare* L.)

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Abstract
A core collection consisting of 153 accessions originating from different countries of The CWANA was established out of the entire collection of cultivated barley (*Hordeum vulgare* subsp. *vulgare* L.) held at ICARDA. Genetic diversity of the CWANA core collection was studied using AFLP markers. The accessions were grouped into different geographic sub-regions and the total genetic variation was estimated using Popgene software. Genetic distance matrix was computed and hierarchical unrooted tree was performed using Phylip software package. Our results show that the AFLP markers were highly informative and were useful in generating a meaningful classification of the cultivated barley that we determined as subset of core collection.

Introduction
Cultivated barley, *Hordeum vulgare* subsp. *vulgare* L., is widely distributed over the eastern Mediterranean region and western Asian countries. The cultivated core collection investigated in this study representing the geographical distribution of *H. vulgare* was analyzed using Amplified Fragment Length Polymorphism (AFLP) to assess the geographical pattern of genetic diversity.

Plant Material
The core collection, a total of 153 accessions was collected from thirteen countries. The ICARDA core collection was selected from the entire *H. vulgare* collection held at the center to represent the species diversity across its geographical distribution area. Three plants were analyzed per accession.

AFLP Analysis
Total DNA was extracted from 50 mg of fresh leaves of each individual plant with the modified CTAB method. Approximately 500 ng of total DNA per sample were used for AFLP reaction. Three primer combinations were used for all the 450 samples. Bands were scored manually and by eye within a range from 100 bp to 300 bp.

Statistical Analysis
To evaluate the genetic diversity within and among populations, Phylip and Popgene software's were used. The total gene diversity of cultivated barley originated from different countries was estimated, which is partitioned into the mean of allelic variation within the *Hordeum vulgare* subsp. *vulgare* L. from diverse countries.

Results
The three primer combinations gave highly informative patterns that were polymorphic among individuals within and between the cultivated barley accessions. A total of 165 AFLP's markers

were detected for all three-primer pair combinations and the percentage of polymorphic loci was equal to 92 %.
To assess the relationship of the accessions listed in different regions, consensus tree was calculated. The barley landraces from Morocco were the most diverse followed by accessions from Oman and Iran. Landraces from Turkey, Egypt, Syria, Yemen and Iran showed an intermediate genetic diversity. The third group is represented by accessions from Algeria, Tunisia, Yemen and Jordan. The lowest genetic variation was for barley landraces from Cyprus and Uzbekistan.

Discussion

Landraces of barley are known to offer rich sources of genetic variation for crop improvement. Accessions from Morocco were found to be more diverse than others. This result corroborates those revealed by agro-morphological characterization where a great genetic variability between and within landraces for different characters has been found. The same observation is valid for populations from Oman. Barley landraces from Uzbekistan and Cyprus were found to be genetically very distinct from the rest of populations. The results of this study suggest that the genetic variation in the ICARDA *H. vulgare* subsp. *vulgare* subset of core collection is structured geographically, but still a major part of the genetic diversity can be found within the geographical subregions.

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Isozyme, ISSR and SSR Analysis of Genetic Diversity in Nordic and Baltic Spring Barley

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Abstract

Variation of isozymes, inter-simple sequence repeats (ISSRs) and simple sequence repeats (SSRs) were surveyed in 197 spring barley accessions from the Nordic and Baltic countries. The investigation included landraces, cultivars from the end of the 19th century, cultivars from the 20th century and breeding lines. The objective of this study was to assay putative genetic erosion probably due to the intensive breeding during the last century. The results indicated that there are differences in changes of genetic diversity over time in two and six row barleys. When evaluating each country's material separately, differences in diversity changes over time were found. In some cases trends of diversity differed depending on the markers used.

Introduction

There is a concern throughout the world about the genetic narrowing of elite barley germplasm (GRANER *et al.* 2003). The question is how reasonable this concern is and whether it would be an issue in all the regions of barley growing where the commercial plant breeding is practised. In the Nordic and Baltic countries this began at the end of 19th century (GAIKE 1992; PERSSON 1997). The common objectives for barley breeders are to develop new cultivars with high, stable yield and outstanding quality. However, there are differences in breeding strategies, based on differences in climate, soil, pests and diseases. The Northern European eco-geographic zone, for example, represents a marginal agro-ecological area for barley growing, which brings specific demands for adaptation breeding. In this study we evaluate the changes of genetic diversity that might have occurred over more than a century of barley breeding. To follow the changes of diversity and identify differences between markers, we choose three marker systems, namely isozymes, ISSRs and SSRs.

Material and Methods

Plant Material

The material used in this study comprised 197 spring barley accessions from Nordic and Baltic countries including landraces, cultivars from the end of 19th century until modern cultivars and breeding lines. The material was obtained from gene banks in the Baltic countries, the Nordic Gene Bank, and plant breeding companies (Boreal Plant Breeding Ltd. in Finland, Kvithamar Research Centre in Norway, Svalöf Weibull AB in Sweden, The Abed Foundation and Sejet Plant Breeding in Denmark, Priekuli and Stende Plant Breeding Stations in Latvia, Jõgeva Plant Breeding Institute in Estonia, Lithuanian Institute of Agriculture, Dotnuva-Akademia in Lithuania). The material consisted of 129 two row barley accessions and 68 six row barley accessions (Table 1).

Table 1. Number of accessions from different breeding periods and origins included in the study

	period	Landraces and cultivars before 1930		Cultivars 1931 - 1970		Cultivars after 1971 and breeding lines		Total
	type	six-rowed	two-rowed	six-rowed	two-rowed	six-rowed	two-rowed	
Origin	Denmark	5	6	0	8	0	11	30
	Estonia	0	0	1	1	2	16	20
	Finland	5	4	5	4	11	3	32
	Latvia	1	1	1	1	1	19	24
	Lithuania	1	1	0	2	0	16	20
	Norway	8	0	6	3	13	0	30
	Sweden	5	7	3	9	0	17	41
	Nordic and Baltic	25	19	16	28	27	82	197

Isozymes

Ten days old seedlings grown in a greenhouse were used for electrophoresis. The methods for electrophoresis and staining procedures for aconitate hydrotase (*Aco*, E.C.4.2.1.3), phosphogluconate dehydrogenase (Pgd, E.C. 1.1.1.43) and esterase (Est, E.C.3.1.1.2) were previously described in detail by LIU *et al.* (1999, 2000). The enzyme names and their abbreviations followed those recommended by the Nomenclature Committee of the International Union of Biochemists (IUBNC 1984). The designations of alleles at the Aco-1, and Pgd-2 loci follow NIELSEN and JOHANSEN (1986), at Est-1, Est-2, Est-4 and Est-5 loci – Hvid and Nielsen (1977), at the Aco-2 locus LIU *et al.* (1999) and at the Pgd-1 locus – KONISHI and YOSHIMI (1993).

ISSRs and SSRs

DNA Extraction

DNA was extracted from leaf material from glasshouse-grown plants as described by Cheung *et al.* (1993) with minor modifications: for tissue breakage Crush Express (Resistenz Labor GmbH) was used and for pellet re-suspension only TE without RNAse was added.

ISSRs

Each PCR reaction for ISSR was carried out in a total volume of 25 µl, containing 20.87 µl deionised water, 2.5 µl PCR buffer (10mM Tris-HCl (pH 8.0), 50 mM KCL, 2mM $MgCl_2$ and 0.02% gelatin), 0.33 µl of primer (15 µM), 0.2 µl d'NTP (100mM), 0.1 µl Taq polymerase (SIGMA 5U/ µl)and 10-20 ng of genomic DNA (1 µl volume).

Initial denaturation was carried out for 1 min at 94 ℃, followed by 30 cycles of 1min at 94°C 0.5°C/s to 55℃, 2 min at 55℃, 1.3℃ /s to 72℃, 0.5 min at 72℃, 1.3°C /s to 94℃, and a fina 5 min extension at 72℃. Four ISSR primers (#888, #889, #890, #891) were obtained from th University of British Colombia Biotechnology Laboratory. The polymerase chain reactio (PCR) was performed in a PTC-200 Peltier Thermal Cycler (MJ Research). Products wer analysed on polyacrylamide gel (CleanGel 48S, Amersham Pharmacia Biotech AB) and ban size was estimated from a 100 bp ladder. Loci were named based on the primer and observe band size.

SSRs

Each PCR reaction for SSR was carried out in total volume of 5µl, containing. 2.39 µl deionised water, PCR buffer 0.5 µl (SIGMA), 0.4 µl $MgCl_2$(SIGMA), 0.1 µl of Forward primer (0.15 pM), 0.1 µl of fluorescent labelled Reverse primer (0.15 pM), 0.5 µl d'NTP

(2mM)0.01 µl Taq polymerase (SIGMA 5U/ µl) and 10-20 ng of genomic DNA (1 µl volume). A total of 21 primer pairs (Bmac0399, Bmac0032, WMC1E8, HVM36, Bmag0125, Ebmac0415, HvLTPBB, Bmac0067, Bmac0013, Bmac0384, Ebmac0701, HVM67, EBmac0970, Bmac0223, AF043094, Bmag0173, EBmac0806, Bmac0040, Bmac0007, Bmac0273, Bmag0135) from the Scottish Crop Research Institute Barley (SCRI) Genomics were used. The PCR was performed in a PTC-200 Peltier Thermal Cycler (MJ Research), according to the programmes from SCRI for each primer pair. The PCR products were analysed on a 96 capillaries electrophoresis sequencer (SPECTRUMEDIX, LLC), the sequencer data images were analysed using the DNA Fragment analysis software GENOSPECTRUM^{TM} VERSION 2.06.

Statistical Analysis

Gene diversity (h) at each locus was calculated using the gene diversity index of NEI (1973): $h = 1-\sum p_i^2$, where p_i is the frequency of the *i*-th allele of the locus. The average value of gene diversity (h) was calculated over all loci (H) for all entries and groups of cultivars. To analyse electrophoretic data, the POPGENE statistical package was used (YEH *et al.* 1999).

Results and Discussion

From eight isozyme loci (Aco-1, Aco-2, Est-1, Est-2, Est-4, Est-5, Pgd-1, and Pgd-2) analysed, all except Pgd-1 were polymorph. For ISSR 48 polymorph loci and for SSR 22 polymorph loci were analysed. Overall in Nordic and Baltic material the gene diversity for isozyme loci ranged from 0 to 0.494, for ISSR loci from 0.023 to 0.450 and for SSR loci from 0.033 to 0.8913. The average gene diversity for isozymes was 0.193, for ISSRs 0.395 and for SSRs 0.623 (Table 2).

For isozymes and SSRs the highest average gene diversity value and mean number of alleles per locus was found in landraces and old cultivars released before 1930. ISSR data showed the highest gene diversity value in cultivars released from 1931 to 1970. Cultivars released after 1971 and breeding lines had the lowest gene diversity value for isozyme and ISSR data, whereas for SSR the lowest gene diversity value was in cultivars from 1931 to 1970.

Table 2. Levels of genetic variation detected using isozymes, ISSRs and SSRs in Nordic and Baltic spring barley overall and in different breeding periods,
A=mean number of alleles per locus, N_p=number of polymorphic loci and H=average gene diversity (Nei, 1973)

	A			N_p			H		
	isozymes	ISSRs	SSRs	isozymes	ISSRs	SSRs	isozymes	ISSRs	SSRs
Landraces and cultivars before 1930	2.625	2.000	7.454	7	48	21	0.214	0.373	0.604
Cultivars 1931 - 1971	2.125	1.958	5.727	7	46	20	0.189	0.388	0.577
Cultivars after 1971 and breeding lines	2.125	2.000	7.182	6	48	22	0.174	0.342	0.602
Nordic and Baltic (overall)	2.750	2.000	8.636	7	48	22	0.193	0.395	0.623

Differences were found in gene diversity changes over time for two rowed and six row barley cultivars (Figure 1). Modern two row cultivars had low average gene diversity value compared to the old ones according isozyme and ISSR data, whereas for six row cultivars it was not observed. SSRs data indicated a drop of gene diversity for two row cultivars in the

middle of 20th century, but diversity of cultivars bred after 1971 and breeding lines was similar to the landraces and cultivars released before 1930.

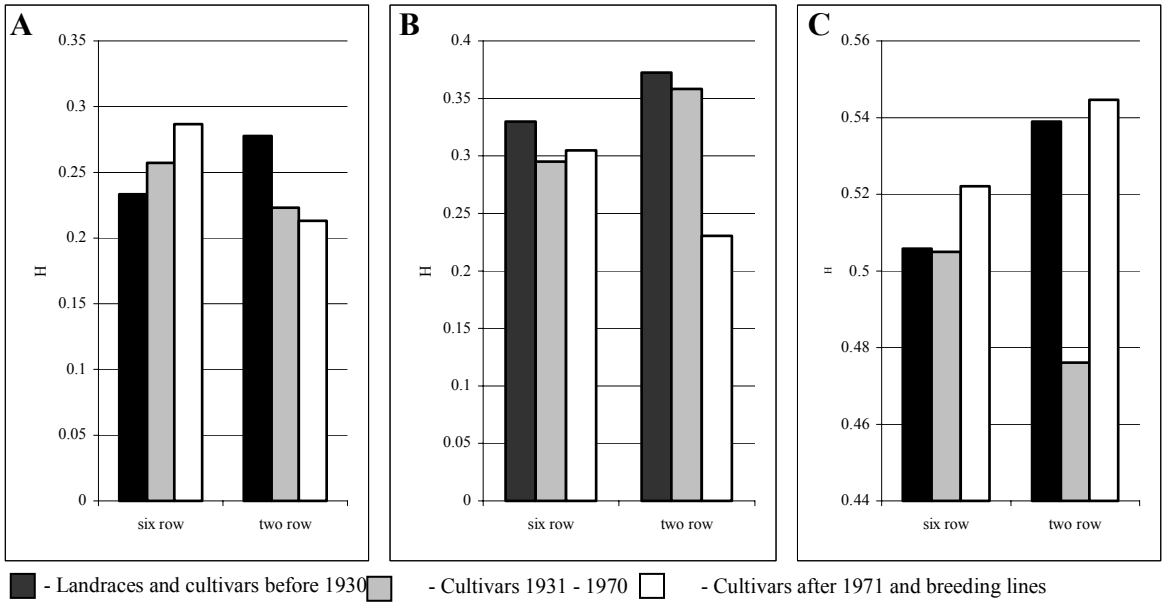

Figure 1. Average gene diversity value H (Nei, 1973) for six row and two row cultivars from different breeding periods, A-isozyme; B – ISSR; C - SSR.

Since the Nordic and Baltic regions comprise a large variation in climate and soils there are also differences in breeding programmes. When comparing the cultivars from different breeding periods within the countries we have found differences (Figure 2) (Baltic accessions from different periods were not compared among the countries due to the low number of accessions representing some of the breeding periods.). For example, data from all markers showed that in Norway the average gene diversity in landraces and cultivars before 1930 is lower compared to modern cultivars. Norwegian landraces and old cultivars also had the lowest average gene diversity value compared to landraces and old cultivars from other Nordic countries. On the other hand for Swedish material different markers showed different trends of diversity changes. Isozymes showed a decrease in genetic diversity over time, whereas SSR and ISSR indicated rather high values of gene diversity in modern material. The highest average gene diversity within Swedish material according to ISSR data was in cultivars bred in the middle of the century.

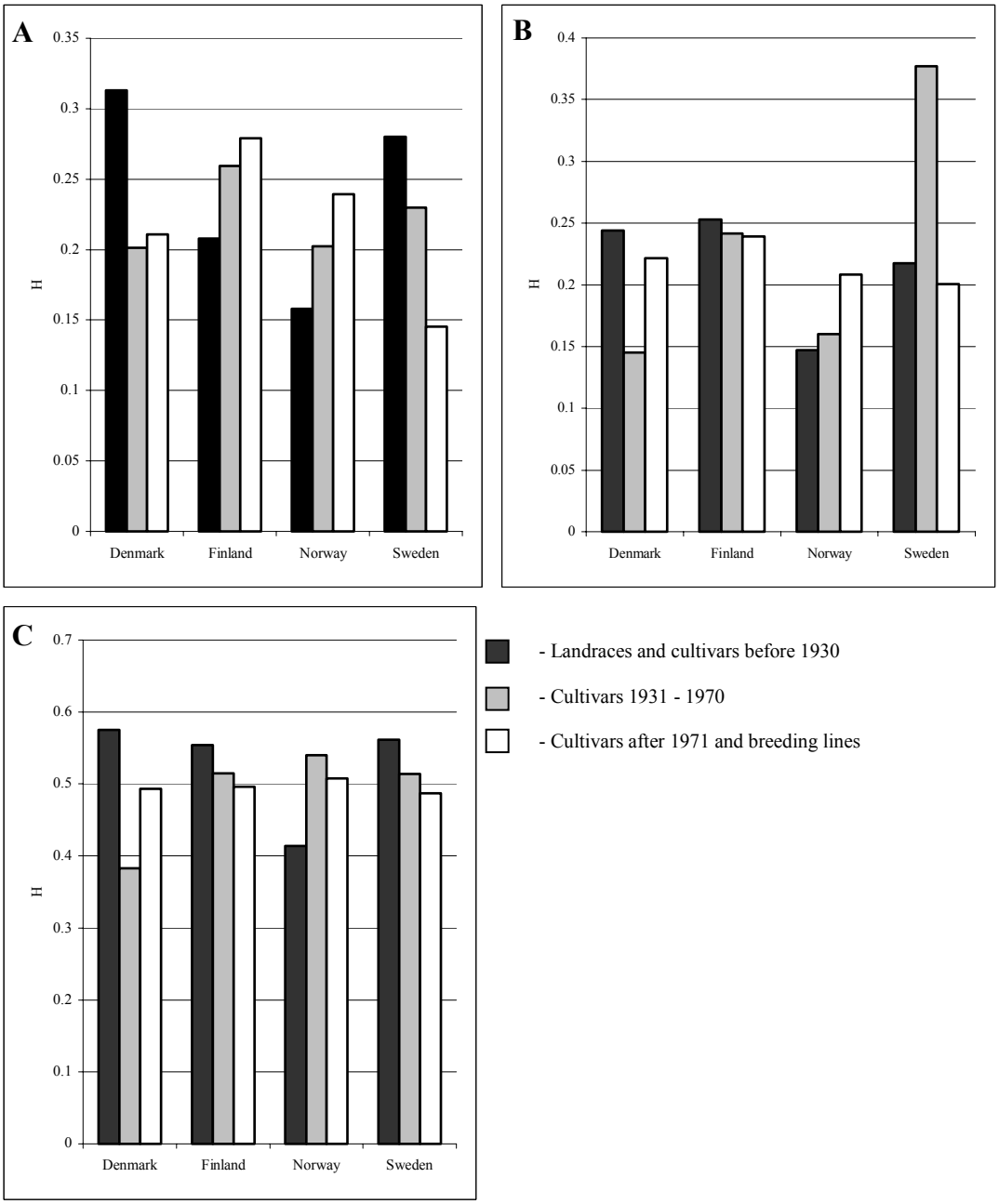

Figure 2. Average gene diversity value H (Nei, 1973) for cultivars from different Nordic countries and breeding periods, A-isozyme; B – ISSR; C - SSR.

The differences of trends in changes of genetic diversity, depending on marker should be taken in consideration when conclusions are drawn. Commercial breeding has affected the diversity of spring barley in Nordic and Baltic countries, but this effect might differ depending what part of the genome is studied and how the breeding practice has been carried out. However in total for the region we could not find definite decrease of diversity.

Acknowledgements
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High-Density Map of Non-Brittle Rachis Genes and Domestication Pattern of Barley

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Abstract

Wild barley disperse their seeds at maturity by means of their brittle rachis. Brittleness of rachis was lost in cultivated barley during domestication. Non-brittle rachis of occidental barley lines is controlled by a single gene (*btr1*) on chromosome 3H. However, non-brittle rachis of oriental barley lines is controlled by a major gene (*btr2*) on chromosome 3H and two quantitative trait loci (QTLs) on chromosomes 5HL and 7H. This result suggests multiple mutations of the genes involved in the formation of brittle rachis in oriental lines. A high-density amplified fragment-length polymorphism (AFLP) map of the *btr1*/*btr2* region was constructed. A phylogenetic tree based on the AFLPs showed clear separation of occidental and oriental barley lines indicating at least two lineages of cultivated barley.

Keywords: seed dispersal; shattering; disarticulation; quantitative trait loci (QTLs); domestication

Introduction

Wild barley has complementary genes, *Btr1* and *Btr2*, for the formation of brittle rachis, and cultivated barley carries recessive alleles at either of the loci, resulting in non-brittle rachis (UBISCH 1915; SCHIEMANN 1921; JOHNSON & ÅBERG 1943; TAKAHASHI & HAYASHI 1959). The *btr1* and *btr2* loci have been mapped to chromosome 3HS, and the two loci are tightly linked (TAKAHASHI & HAYASHI 1964). Most occidental cultivars carry the *btr1* allele, and most oriental cultivars carry the *btr2* allele, indicating a clear differentiation pattern of the two groups (TAKAHASHI 1955). We previously identified AFLP loci flanking the *btr1* locus (KOMATSUDA & MANO 2002), but the position of the *btr2* locus was ambiguous. We detected a new gene factor, '***d***', independently inherited on chromosome 7H, which affected the mapping of the *btr2* locus. In this study, composite interval mapping of QTLs was performed in order 1) to test the hypothesis that the *btr1* and *btr2* loci are tightly linked (TAKAHASHI & HAYASHI 1964); 2) to identify the modifier gene '***d***' previously detected in chromosome 7H (KOMATSUDA & MANO 2002); and 3) to detect any other QTLs that affect the rachis brittleness of barley. High-density AFLP of the *btr1* and *btr2* were constructed. Phylogenetic patterns of cultivated and wild barley were inferred by using AFLP markers. In this study, we assumed that possession of both the *Btr1* and *Btr2* alleles is a necessary condition for producing brittle rachis in barley (TAKAHASHI & HAYASHI 1964).

Material and Methods

Hordeum vulgare ssp. *vulgare* cvv. 'Azumamugi', 'Kanto Nakate Gold' were obtained from the Barley Breeding Laboratory, National Institute of Crop Science, Tsukuba, Japan. *H.*

vulgare ssp. *spontaneum*, lines were obtained from the Research Institute for Bioresources, OkayamaUniversity.

Azumamugi is a non-brittle (*Btr1Btr1btr2btr2*) six-rowed cultivar; Kanto Nakate Gold is a non-brittle (*btr1btr1Btr2Btr2*) two-rowed cultivar (KOMATSUDA & MANO 2002). Ninety-nine RILs (F_{11}) have been developed from the Azumamugi × Kanto Nakate Gold cross by single-seed descent. A single plant of each of 87 RILs was pollinated with Natsudaikon Mugi (*btr1btr1Btr2Btr2*), and a single plant of each of these and of five other RILs was pollinated with Hayakiso 2 (*Btr1Btr1btr2btr2*). Natsudaikon Mugi is a spring barley (*sgh1*/*Sgh2*/*sgh3*) and Hayakiso 2 is a winter barley (*Sgh1*/*sgh2*/*sgh3*). These 2 testers are used to test rachis brittleness and spring habit of growth at the Research Institute for Bioresources, Okayama University. Rachis brittleness was evaluated as 100% × (number of rachis nodes disarticulated) / (number of rachis nodes in a spike).

MANO and KOMATSUDA (2002) constructed a base map eliminating the clustering markers and using the remaining 100 markers to achieve a density of 5 to 10 cM/locus. On the basis of the map, we performed composite interval mapping of QTLs by using the computer program QTL Cartographer version 1.14 (BASTEN *et al.* 2000) on arcsin √p-transformed brittleness data (%). The mapping was run with the default setting for model 6 (five background markers and a window size of 10 cM). The inclusion of background markers makes the analysis more precise and permits efficient mapping of QTLs. A log-likelihood (LOD) score threshold of 3.0 was used to identify regions containing putative loci associated with the trait.

We carried out DNA restriction by *Eco*RI and *Mse*I, ligation of adapters, non-selective preamplification, selective amplification using primers having three selective nucleotides, and polyacrylamide gel electrophoresis, as described by MANO *et al.* (2001). Bulked-segregant analysis was carried out to identify AFLP markers tightly linked to the *btr1* and *btr2* loci. Two bulked DNAs were made by combining preamplified DNAs of eight RILs of *btr1btr1* genotype and eight RILs of *Btr1Btr1* genotype selected from 99 RILs of Azumamugi × Kanto Nakate Gold on the basis of F_9 generation data (KOMATSUDA & MANO 2002).

Fourteen cultivars of *H. vulgare* ssp. *vulgare*, two lines of var. *agriocrithon*, and 19 lines of ssp. *spontaneum* were analyzed by using primer combinations that generated AFLP loci linked to the *btr1* and *btr2* loci. As a comparison, we scored another 46 AFLP loci that were independently inherited with the *btr1* and *btr2* loci, and used them to construct another phylogenetic tree. Phylogenetic trees were constructed by the minimum evolution method (NEI & KUMAR 2000).

Results

QTL Mapping of Brittle Rachis Genes

In the RILs × Natsudaikon Mugi population, only one major QTL was detected, flanked by *e14m27-4-1* and *e15m19-7* on chromosome 3H. The QTL is identical to the *btr1* locus. This QTL explained 72.4% of phenotypic. The result indicates that the *Btr1* allele from Azumamugi was almost sufficient to complement the genotype of Natsudaikon Mugi to produce brittle rachis. In the RILs × Hayakiso 2 population, three QTLs were detected. A major QTL (LOD = 27.9) was located at the same position as the *btr1* locus, between *e14m27-4-1* and *e15m19-7* on chromosome 3H. The result indicates that this QTL is the *btr2* locus (TAKAHASHI & HAYASHI 1964). This QTL explained 70.3% of phenotypic

variance. A secondary QTL (LOD = 6.9) was located between *cMWG704* and *e11m17-10-2* on chromosome 7H. The region overlapped the '*d*' (modifier) locus located between *cMWG704* and *e12m22-10-2* (KOMATSUDA & MANO 2002), indicating that the QTL and '*d*' are identical. This QTL explained 10.8% of phenotypic variance, and the allele of Kanto Nakate Gold had a positive effect. The third QTL (LOD = 3.7) was located between *e07m25-03* and *e12m19-09-1* on chromosome 5HL. This QTL explained 5.2% of phenotypic variance, and the allele of Kanto Nakate Gold had a positive effect. Thus, Kanto Nakate Gold alleles at all three loci were necessary to complement the genotype of Hayakiso 2 to produce brittle rachis.

High-Density AFLP Map of the btr1 *and* btr2 *Loci*

A total of 4096 combinations of 64 *Eco*RI+3 and 64 *Mse*I+3 primers were subjected to the analysis between two bulked DNAs, and 148 AFLP markers were developed. These 148 markers were tested against 13 RILs of Azumamugi × Kanto Nakate Gold that revealed a recombination between *e14m27-4-1* and *e15m19-7* (MANO *et al.* 2001). As a check, another 22 RILs without recombination between the two AFLP loci were also included. The analysis placed 84 markers on or between the two AFLP loci. Ten AFLP loci co-segregated with the *btr1* or *btr2* loci. Four AFLP loci were located 0.5 cM distal and *e37m44-13* was located 2.7 cM proximal to the *btr1*/*btr2* complex.

Phylogenetic Analysis Based on the btr1/btr2 *Region*

Unrooted phylogenetic tree of wild and cultivated barley was constructed by using 74 AFLP loci assigned in the 9.9-cM region covering the *btr1* and *btr2* loci. The tree shows a clear separation of cultivated barley into two groups. One group consists of "W" (Western)-type barley lines (*btr1btr1Btr2Btr2*). Modern Japanese two-rowed cultivars are included in this group, because they have the W-type genes inherited from European barley lines. A Bulgarian cultivar, 'Caveda', and two Moroccan weedy barley lines formed a small clade highly supported by bootstrap analysis. A clade of 'Golden Promise', 'Bonus', and 'Kristina' was also highly supported by bootstrap analysis, and was more or less separated from the other cultivars in the W-type group. A wild barley line from Jordan (OUH638) was included in the W-type group, and two wild barley lines from Iraq (OUH742) and Tibet (OUH825, var. *spontaneum* type) were close neighbors of the W-group. The second group representing "E" (Eastern)-type barley lines (*Btr1Btr1btr2btr2*) included 'Azumamugi', 'Hayakiso 2', and 'Soren Oomugi 19329'. The clade was highly supported by the bootstrap value of 98. Including a wild barley line from Libya (OUH783) and a Tibetan line of var. *agriocrithon* (OUH786) gave the clade the bootstrap value of 100.

Discussion

Identification of QTLs for Non-Brittle Rachis

Although non-allelic modifier genes have been suggested for the non-brittle rachis (SCHIEMANN 1921; TAKAHASHI & HAYASHI 1959; KOMATSUDA & MANO 2002), this study is the first in which modifier genes were precisely identified in the linkage maps. Oriental barley cultivars may represent additional steps of mutation from ancestral barley to landraces or local cultivars. Probably some Oriental cultivars have these modifier genes. The QTL on chromosome 7H is probably identical to the dense spike 1 (*dsp1*, formerly *l*) locus, because a major QTL controlling spike internode length (or spike density) was located at the same position in work using the same RILs (SAMERI & KOMATSUDA, accompanying paper). The *dsp1* gene occurs naturally in local cultivars from Korea and Japan, and both

Azumamugi and Hayakiso 2 have the *dsp1* allele (TAKAHASHI 1951, 1955; TAKAHASHI *et al.* 1979, 1983). Dense spikes tended to show a lower degree of rachis brittleness than those with normal (or lax) spikes (TAKAHASHI & YAMAMOTO 1949, 1951).Some barley cultivars show disarticulation at only one or a few rachis nodes in a spike, resulting in loss of a spike segment. The phenomenon is called 'rachis break' or 'head shattering' and causes loss of yield. Head shattering QTLs were detected on chromosomes 2H, 3H, and 5HL (KANDEMIR *et al.* 2000). Head shattering can be regarded as a special case of brittle rachis, so it is natural that some of the genes or QTLs controlling the two traits are common. The head shattering QTL detected on chromosome 5HL and the brittle rachis QTL that we detected on chromosome 5HL could be identical, but their relative positions remain to be confirmed by the use of common markers. The *btr1*/*btr2* complex is not allelic to the head shattering QTL on chromosome 3H (KANDEMIR *et al.* 2000).

Organization of the btr1 *and* btr2 *Loci*

Our high-density map includes 84 AFLP loci in the interval of 6.7 cM harboring the *btr1* and *btr2* loci, providing an average density of 0.08 cM/locus. The map shows a fairly even distribution of AFLP loci, except for the clustering detected at one of the flanking markers. TAKAHASHI (1963) reported on the basis of the analysis of 1267 cultivars from different regions of the world that all cultivars were either E-type or W-type, and none was *btr1btr1btr2btr2* type. Why have double recessive types not been identified? The most adequate explanation is that the two loci are physically too close to allow the breeding of recombinant lines (TAKAHASHI & HAYASHI 1964). However, it is not clear why a double recessive *btr1btr1btr2btr2* does not occur by spontaneous mutation in cultivated barley. Does it exist in other cultivars but has escaped detection? One explanation is that double recessive plants are lethal, although this explanation does not account for the fact that double dominant *Btr1Btr1Btr2Btr2* was not bred in segregating F_2 or RILs (TAKAHASHI & HAYASHI 1964, and this study). We maintain the hypothesis of tight-linkage between the *btr1* and *btr2* loci. The hypothesis may be tested by simply increasing the size of segregating populations followed by test crosses. Hayakiso 2 may not be the best tester, because it has a *dsp1* gene. It would be favorable to use barley lines with lax spike density in further studies.

Domestication Patterns Indicated by Molecular Loci in the btr1/btr2 *Region*

Wild and cultivated barley are positioned at subspecies level because the two taxa are interfertile (BOTHMER *et al.* 1995). Gene flow between them caused by natural pollinators is possible, so it is difficult to define what is wild barley and what is cultivated barley. Non-brittle rachis therefore could be used to discriminate cultivated barley from wild barley. The phylogenetic tree based on the AFLP loci linked to the *btr1/btr2* loci may represent the gene genealogy for the brittle rachis genes. The tree clearly shows the differentiation of cultivated barley into two clades, E-type and W-type, in good agreement with the differentiation pattern of cultivated barley (TAKAHASHI 1955).

It seems surprising that a Libyan wild barley line (OUH783) has a strong connection with the E-type cultivars. However, more than 90% of North African barley cultivars carry E-type gene sequences for brittle rachis (TAKAHASHI *et al.* 1983), in clear contrast to the fact that more than 95% of Abyssinian barley cultivars carry W-type gene sequences (TAKAHASHI 1963; TAKAHASHI *et al.* 1983). Our phylogenetic analysis suggests that the E-type barley lines of Asia and North Africa have the same lineage. What is the origin of var. *agriocrithon* inferred from the analysis? In the present study, the Tibetan line of var. *agriocrithon* (OUH786) was distinctly separate from the ssp. *spontaneum* group, being much more

distantly separated from the Tibetan line of ssp. *spontaneum* (OUH825). Therefore, the result was not consistent with the theory of hybrid origin or reverse mutations from non-brittle to brittle forms. Other than Tibet, var. *agriocrithon* has been found frequently in Israel, Cyprus, and Libya (for review see BOTHMER & JACOBSEN 1985). In this case our results agree with the hypothesis of hybrid origin between ssp. *spontaneum* and six-rowed cultivated barley (Bothmer et al. 1995), because the var. *agriocrithon* from Israel (OUH802) was included in the group of ssp. *spontaneum*. Probably OUH802 has a *btr1*/*btr2* gene region obtained from sympatric populations of ssp. *spontaneum*. The var. *agriocrithon* lines from Tibet (or China) and Israel may have different origins.

Acknowledgements
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Relationship between Phenotypic and Genetic Distances in a Set of Barley Breeding Lines

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Abstract

The aim of the present study was to estimate a relationship between phenotypic (PD) and genetic (GD) distances in a set of barley genotypes. Material for the study covered 18 spring barley cultivars and breeding lines different in their morphological characters: Apex, Grit, Havila, Krystal, Maresi, Roland, A35, C54, RK22, RK58, RK63/1, B33, B61 (2-rowed, hulled), Klimek, Pomo (6-rowed, hulled), IN86, POA0325, A39 (2-rowed, naked). Cultivars and lines were examined with regard to yield-related traits in a field experiment carried out in a randomised block design with 3 replications. Grain yield per plot, 1000-grain weight, grain weight per ear, plant hight and spike length were observed. The data were processed using multivariate analysis of variance. Mahalanobis distance (i.e., distance between genotypes evaluated for all the studied traits treated simultaneously) was used as a measure of phenotypic distances between studied genotypes. Besides, RAPD (random amplified polymorphic DNA) polymorphism was examined in the studied genotypes. Fifty 10-mer primers were tested in each cultivar or line, giving altogether 389 amplification products, 55% of which were polymorphic. Genetic distance for all pairs of compared genotypes was estimated and a dendrogram was constructed using unweighted pair group method. Correlation between PD and GD distances appeared to be a weak but statistically significant.

Keywords: barley; genetic distance; phenotypic distance; RAPD

Introduction

The simplest method to perform in order to promptly detect changes in the DNA sequence throughout the whole genome is a method using random amplified polymorphic DNA (RAPD). Due to a very large number of various sequences of 10-mer primers (4^{10}), the RAPD method has become especially useful in the search for markers of agronomically important traits. In cereals the RAPD markers are used, among other, to localise quantitative trait loci (QTLs), to construct genetic maps, in taxonomic studies and in the assessment of genetic diversity (BARUA *et al.* 1993; BUSTOS *et al.* 1998; HANG *et al.* 2000; KUCZYŃSKA *et al.* 2001). The aim of the study was to detect genetic and phenotypic differences among cultivars of selected spring barley genotypes.

Material and Methods

The material for investigations included 18 spring barley (*Hordeum vulgare* L.) cultivars and breeding lines different in their morphological characters: Apex, Grit, Havila, Krystal, Maresi, Roland, A35, C54, RK22, RK58, RK63/1, B33, B61 (2-rowed, hulled), Klimek, Pomo (6-rowed, hulled), 1N86, POA0325, A39 (2-rowed, naked). The DNA of the analysed cultivars was extracted from the leaves of 3-week old seedlings (THOMSON & HENRY 1995).

The RAPD-PCR reactions were performed for 50 10-nucleotide primers, selected previously from 300 with a random sequence. The reaction was performed according to the following protocol: 5 min. at 95°C , 45 cycles of: 94°C for 1min., 36°C for 2 min., 72°C for 2 min. and final extension at 72°C for 10 min. (KUCZYŃSKA *et al.* 2001).

The electrophoresis of DNA fragments was conducted for 2.5 h at the voltage of 100 V in 1.5% agar gel containing ethidium bromide.
The coefficients of genetic similarity (GS) of the investigated genotypes were calculated using the following formula (NEI & LI 1979):

$$GS_{ij} = \frac{2N_{ij}}{N_i + N_j},$$

where N_{ij} is the number of alleles present at *i*-th and *j*-th objects, N_i - the number of alleles present at the *i*-th object, N_j - the number of alleles present at the *j*-th object; $i, j = 1, 2, \ldots, 18$.
Genetic distance (GD) were calculated by transformation of GS as follows: GD = 1 – GS. The coefficients were used to group the cultivars hierarchically using the unweighted pair group method of arithmetic means. The results of the performed grouping are presented in the form of a dendrogram.
Grain yield per plot, 1000-grain weight, grain weight per ear, plant height and spike length were observed. The data were processed using multivariate analysis of variance. Mahalanobis distance (i.e., distances between genotypes evaluated for all the studied traits treated simultaneously) was used as a measure of phenotypic distances between studied genotypes. Correlation coefficient was estimated between phenotypic and genetic distances.

Results and Discussion
As a result of the PCR-RAPD reactions performed for the investigated barley cultivars and lines with the use of 50 primers, a total of 389 amplified DNA fragments were obtained, out of which 214 were polymorphic. The number of polymorphic bands revealed by one primer ranged from 1 to 6, with an average of 4.3, and the size of amplification products ranged from 100 to 2000 bp (Fig. 1).
Investigated genotypes appeared to be different on the phenotypic and molecular levels. Values of genetic distance ranged from 0.107 to 0.798. Dendrogram constructed on the basis of genetic distances is presented in Figure 2. The lowest genetic distance was found for lines A39 and B33, whereas the highest GD was revealed between cultivars Apex and Pomo. Mahalanobis distance between pairs of genotypes ranged from 1.076 to 36.411. The most similar genotypes on phenotypical level were cultivar Grit and line A35, and the most different were cultivar Pomo and line C54.
Values of GD and PD are presented in Table 1. Correlation coefficient between PD and GD distances appeared to be a weak (-0.200) but statistically significant (at level $\alpha = 0.05$).

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Figure 1. Results of RAPD analysis for studied 18 lines and cultivars of barley with primer Genset1 (GTGCCTAACG)
M – Gene Ruler^{TM} 100 bp DNA Ladder Plus
1 – Apex, 2 – Roland, 3 – Krystal, 4 – Maresi, 5 – Pomo, 6 – Klimek, 7 – Havila, 8 – Grit, 9 – RK 63/1, 10 – RK 22, 11 – RK 58, 12 – 1N86, 13 – C 54, 14 – B 61, 15 – B 33, 16 – A 39, 17 – A 35, 18 – POA 0325

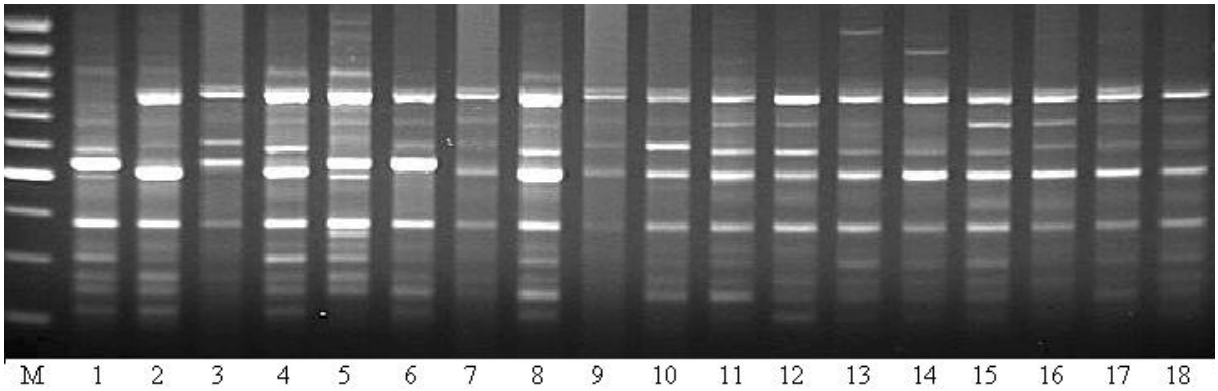

Figure 2. Genetic distance presented on the dendrogram

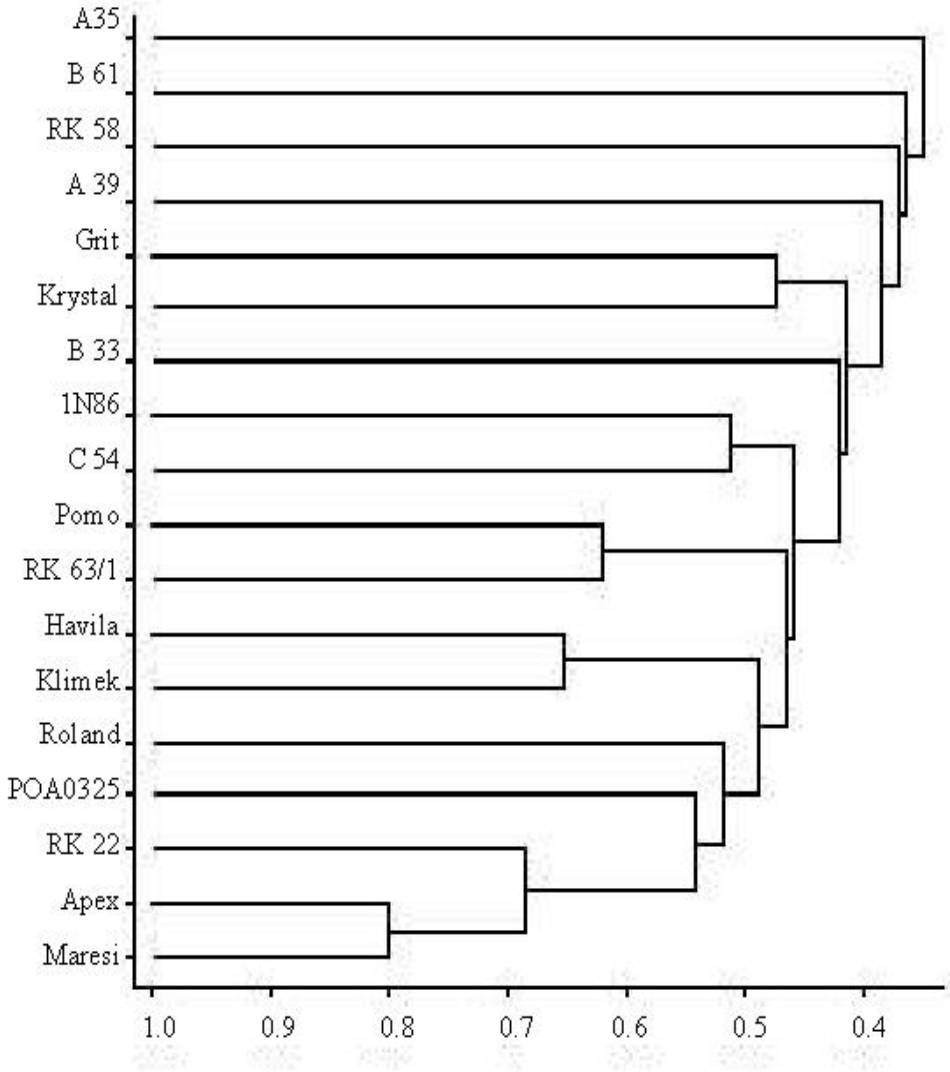

Table 1. Genetic and phenotypic distances between investigated barley cultivars and lines

		Phenotypic distance																	
Genetic distance	1N86	-	2.979	4.355	6.036	6.396	6.498	7.902	2.856	4.355	28.39	5.941	2.617	3.638	29.57	4.209	3.670	6.191	3.683
	A 35	0.301	-	5.191	4.239	5.168	6.215	8.121	1.076	2.748	27.20	4.479	1.989	3.001	28.72	2.020	1.325	4.439	1.775
	A 39	0.407	0.217	-	8.067	7.485	4.565	7.522	5.207	7.079	28.89	6.865	5.533	4.070	29.91	5.843	5.336	8.590	6.342
	Apex	0.718	0.714	0.735	-	5.421	8.953	7.088	4.171	3.398	29.53	4.135	4.030	6.016	31.53	2.507	4.466	2.670	2.884
	B 33	0.458	0.217	0.107	0.735	-	5.593	6.347	6.040	4.116	31.03	1.872	5.872	6.699	32.64	4.718	4.118	3.587	5.532
	B 61	0.488	0.277	0.188	0.721	0.128	-	8.141	6.957	7.355	29.52	5.960	7.369	5.820	30.62	6.821	5.408	8.276	7.627
	C 54	0.513	0.266	0.267	0.691	0.267	0.239	-	8.273	7.494	34.96	5.413	7.567	8.765	36.41	7.010	7.811	7.311	8.007
	Grit	0.471	0.376	0.413	0.721	0.474	0.382	0.448	-	3.269	27.05	5.172	1.650	2.916	28.59	2.147	2.363	4.922	1.538
	Havila	0.407	0.362	0.339	0.735	0.385	0.353	0.267	0.399	-	28.91	3.352	2.584	5.580	30.43	2.750	2.491	2.502	2.667
	Klimek	0.431	0.442	0.587	0.729	0.537	0.484	0.252	0.307	0.653	-	30.85	28.21	26.68	5.713	28.32	27.67	30.02	27.68
	Krystal	0.295	0.310	0.284	0.701	0.284	0.314	0.393	0.475	0.343	0.440	-	4.865	6.236	32.47	3.568	3.586	3.030	4.657
	Maresi	0.513	0.289	0.417	0.800	0.417	0.323	0.375	0.308	0.433	0.389	0.542	-	4.266	29.59	2.602	2.806	4.603	2.069
	POA0325	0.423	0.389	0.349	0.789	0.349	0.365	0.419	0.365	0.301	0.439	0.354	0.383	-	28.31	3.748	3.669	6.677	3.746
	Pomo	0.333	0.328	0.365	0.798	0.413	0.318	0.480	0.318	0.444	0.333	0.354	0.268	0.328	-	30.09	29.15	31.90	29.43
	RK 22	0.358	0.395	0.306	0.729	0.355	0.387	0.475	0.452	0.355	0.518	0.248	0.644	0.456	0.539	-	2.331	3.431	1.527
	RK 58	0.312	0.283	0.372	0.689	0.372	0.343	0.420	0.329	0.255	0.453	0.135	0.493	0.385	0.308	0.219	-	4.059	2.671
	RK 63/1	0.456	0.529	0.496	0.706	0.496	0.475	0.500	0.458	0.478	0.415	0.395	0.482	0.444	0.622	0.396	0.409	-	3.703
	Roland	0.504	0.270	0.333	0.756	0.225	0.349	0.385	0.485	0.550	0.433	0.396	0.476	0.475	0.536	0.367	0.427	0.368	-

A New Web Site for Barley Genetic Resources: the Spanish Barley Core Collection

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Abstract
The Spanish Barley Core Collection was created to represent the genetic variability of the over 2000 accessions present in the Spanish National barley collection. It consists of 159 inbred lines derived from local landraces, as well as 16 cultivars with an extended history of cultivation in the country. Data of several sorts have been gathered, including passport, morphological traits, plant pictures at several growth stages, agronomic traits, have been compiled and will be presented, at the end of the year, in a new website, http://www.eead.csic.es/barleybreeding/SBCC, accessible to researchers. Currently ongoing studies on new agronomic traits and genetic characterization will be incorporated in the future. This collection represents a pool of largely underused genetic variability for barley breeding, as revealed by previous studies, and also a potential tool to study barley population genetics and adaptation on landrace materials.
Keywords: germplasm; genetic resources; landraces; variability

Introduction

Genetic narrowness of elite germplasm in barley breeding is a cause of concern for breeders (MELCHINGER *et al*. 1994). The use of landraces as source of new variability has not been fully exploited, especially in the displaced landraces from peripheral European countries (AHOKAS & POUKKULA 1999). These materials are usually kept in large germplasm collections. From a practical point of view, the use of plant materials coming from large collections, necessarily with a low level of characterization, seems to be quite hopeless. To get around this problem, and to properly evaluate representative samples of germplasm, big efforts for generating core collections were recently undertaken in Europe (KNUPFFER & VAN HINTUM 1995) and in USDA-ARS collection (BOCKELMAN 1996).
In Spain, the Spanish Barley Core collection was assembled (IGARTUA *et al.* 1998), incorporating 159 inbred lines derived from landraces, and 16 old varieties. This Core Collection is being characterized for the last three years; some preliminary results were published in LASA *et al*. (2001).

Material and Methods

Three Spanish groups collaborate in the agronomic evaluation and characterizations of this collection, under different climatic conditions in the Spanish provinces of Zaragoza, Lleida and Valladolid.
Molecular markers analysis is being carried out with microsatellites, to establish membership to genetic groups, results that will be published elsewhere. The results produced are being compiled in data bases, that will be presented in a Web site that is described in this paper. The

initial contents of the web site will include passport data (Table 1), and twenty-six morphological and agronomic evaluation traits (Table 2).

Table 1. Passport data available for the Spanish Barley Core Collection

PASSPORT DATA	
1.1	Accession number
1.1.1	BNG number
1.2	Donor name
1.3	Donor number
1.7	Cultivar name
1.9	Acquisition date
2.1	Collecting Institute
2.2	Collecting number
2.4	Collecting date
2.5	Country of collecting
2.6	Region
2.7	Province
2.8	Department
2.9	Latitude
2.10	Longitude
2.11	Elevation
2.14	Status of sample
2.22	Local name

Characterization was developed following the IPGRI Descriptors for Barley (1994), on the characters presented in Table 2.

Table 2. List and key for the description of morphological, and agronomic evaluation traits, to be included in the web page of the Spanish Barley Core Collection

IPGRI Code	Character/Class	1	2	3	4	5
7.1.1	Growth Class	Winter	Facultative	Spring		
7.1.2	Growth Habit	3-Prostrate - 5-Intermediate - 7-Erect				
7.1.3	Plant height (cm)	<60.2	60.2-67.3	67.3-74.3	74.4-81.5	>81.6
7.1.4	Stem pigmentation	Green	Purple			
7.1.5	Auricle pigmentation	Green	Purple			
7.2.1	Photoperiod sensitivity	1-very low - 3-low - 5-Intermediate - 7-High				
7.2.2	Heading (days to)	<96	96-102	103-111	112-118	>118
7.2.2.1	Grain filling period (days)	<31	31-34	35-38	39-42	>42
7.2.3	Row number	2-two row - 6-six row				
7.2.4	Spike density	3-lax - 5-intermediate - 7-dense				
7.2.5	Spikelets/spike	<13.5	13.5-16.5	16.5-19.5	19.5-22.5	>22.5
7.2.6	Lemma awn/hood	Awnless	Awnleted	Awned	Sessile hoods	Elevat. Hoods
7.2.7	Lemma awn barbs	3-smooth – 5-intermediate – 7-rough				
7.2.8	Glume and glume awn	Shorter th kern	As kernel	Longer th ker..		
7.2.9	Glume colour	White	Yellow	Brown	Black	
7.2.10	Lemma type	No teeth	Teeth	Hair		
7.2.11	Awn colour	White	Yellow	Brown	Reddish	Black
7.2.12	Length of rachilla hairs	Short	Long			
7.2.13	Length of spike (cm)	<4.90	4.90-6.46	6.46-8.02	8.02-9.58	>9.58
7.3.1	Kernel covering	Naked	Semi-cover.	Covered		
7.3.6	1000-kernel weight (g)	<29	29-33.9	34-39	39.1-44	>44
8.1.3	Test weight (kg/Hl)	<51.7	51.7-55.5	55.6-59.3	59.4-63.2	>63.2
9.3	Yield under drought	1-resistant - 9-susceptible				
9.8	Lodging	1-resistant - 9-susceptible				
10.2.3	Reaction to Puccinia hordei	1-resistant - 9-susceptible				
10.2.4	Reaction to Blumeria graminis	1-resistant - 9-susceptible				

Results

The Web site will present passport and characterization data for each entry. As a summary, mean, standard deviation and distribution of the different classes is presented in Table 3.Spike

and grain photographs for each entry will be presented. Other types of data will be incorporated as they become available. The initial version is expected to be launched by the end of 2004.

Table 3. Summary of distribution of morphological and agronomic evaluation traits

IPGRI Code	Character	Mean	Sd	1	2	3	4	5	6	7	8	9	Total
7.1.1	Growth Class	2.64	0.71	23	16	133							172
7.1.2	Growth Habit	4.68	1.05			40		122		12			174
7.1.3	Plant height (cm)	3.92	0.94	3	13	25	84	47					172
7.1.4	Stem pigmentation	1.63	0.48	64	111								175
7.1.5	Auricle pigmentation	1.63	0.48	65	110								175
7.2.1	Photoperiod sensitivity	3.33	1.84	40		85		26		21			172
7.2.2	Heading (days to)	3.33	1.84	8	43	80	25	15					171
7.2.2.1	Grain filling period (days)	36.25	3.72	16	35	68	47	6					172
7.2.3	Row number	5.57	1.25		19				156				175
7.2.4	Spike density	4.37	1.35			76		78		21			175
7.2.5	Spikelets/spike	2.99	0.90	5	44	84	29	12					174
7.2.6	Lemma awn/hood	3.00	0.00	0	0	175	0	0					175
7.2.7	Lemma awn barbs	6.58	0.92			4		29		142			175
7.2.8	Glume and glume awn	1.78	0.59	54	106	15							175
7.2.9	Glume colour	2.73	0.69	24	0	151	0						175
7.2.10	Lemma type	2.73	0.59	13	21	141							175
7.2.11	Awn colour	2.73	0.69	24	0	151	0	0					175
7.2.12	Length of rachilla hairs	1.47	0.50	93	82								175
7.2.13	Length of spike (cm)	7.24	1.56	8	41	90	23	13					175
7.3.1	Kernel covering	2.99	0.15	1	0	174							175
7.3.6	1000-kernel weight (g)	2.98	1.02	11	46	61	43	11					172
8.1.3	Test weight (kg/Hl)	2.97	1.02	9	42	62	30	13					156
9.3	Yield under drought	5.02	1.49	0	11	28	47	43	25	8	7	3	172
9.8	Lodging	3.95	1.87	25	15	31	29	34	22	14	2	0	172
10.2.3	Reaction to Puccinia hordei	7.07	2.05	4	3	6	11	4	12	29	47	40	156
10.2.4	Reaction to Erysiphe graminis	5.83	1.61	3	4	8	19	27	42	48	21	0	172

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The International Database for Barley Genes and Barley Genetic Stocks

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Abstract
The request of an International Database for Barley Genes and Barley Genetic Stocks was already discussed in 1989. In 1991, Sigfus Bjarnasson demonstrated a proposal of a constructed database for Barley Genes and Genetic Stocks. In 1993, Dave Matthews and coworkers introduced the Triticeae Genome Database "GrainGenes" where compiled data include all information on genetics, cytogenetics, maps, phenotypes, molecular markers, genetic stocks and other germplasms of most small grain grass species. The selection of AceDB as a database server system was natural as it has several important advantages over conventional rational db-systems in handling biological information. The usable data model already existed for wheat genes at "GrainGenes". Compiling data for barley genes and genetic stocks resulted in several publications in Barley Genetics Newsletter and thanks to well formatted descriptions, it was easy to convert the enormous amount of information into this database system. Descriptions of more than 600 genes, over 3700 alleles and over 1700 references are included. Most of the described genes are illustrated with images, both overviews and detailed close-up pictures. The database is easy to handle starting with Basic and Simple Search, and it is possible to select the object you are looking for. It is available at http://www.untamo.net/bgs

Historical Development and Review

The request and necessity to establish an International Database for Barley Genes and Genetic Stocks was already discussed in 1989 at a FAO working group on Plant Genetic Resources in Lund, Sweden. One of the above authors as the International Overall Chairman for the barley linkage groups and barley collections since 1986 has been asked to help in establishing an "International Barley Genetic Resources Database", including induced mutants and Barley Genetic Stocks. In the following years inventories of existing accessions by regional databases world-wide have been started. During 1991 together with Sigfus Bjarnasson, the head of the Nordic Gene Bank at that time, a call has been sent to the barley coordinators for the seven chromosomes and the different collections to investigate weather the yearly coordinator reports for Barley Genetics Newsletter were based on own research work, literature studies or other information, and if the reported materials are available in regional Gene Banks or in the Main Stock Center for barley.

At the IBPGR (The International Board for Plant Genetic Resources), to-day IPGRI, workshop in Helsingborg, Sweden, in 1991, Sigfus Bjarnasson (BJARNASSON 1991) demonstrated a proposal of his constructed possible database for Barley Genes and Barley Genetic Stocks. In his proposal he stressed the importance of gene, allele, synonym, stock and reference tables. The participants of this workshop urgently called upon the Scandinavian barley geneticists in corporation with the Nordic Gene Bank to proceed in developing and constructing this detailed database. During the following years more inquiries were performed

in order to collect information of different barley genes and mutants from all over the world. This was a rather difficult task, especially as many barley researchers have turned into new molecular biotechnology research.

At ”The First Meeting of the Coordinating Committee of the International Barley Genetic Resources Network” arranged by IPGRI in Guelph, Canada, 1993, the members again recommended and stressed strongly the importance of the continuation of this project. Also in Guelph, 1993, at the ”15th North American Barley Researchers workshop” Dave Matthews and coworkers (ALTENBACH *et al.* 1993) introduced the Triticeae Genome Database ”GrainGenes” developed under the Plant Genome Research Program of the U.S. Department of Agriculture at Cornell University, Ithaca, USA, with additional support from the International Triticeae Mapping Initiative, USA. All data compiled in this database is including all information on genetics, cytogenetics, physical and molecular maps, information on phenotypes, quantitative traits and QTLs as well as genotypes and pedigrees of cultivars, genetic stocks and other germplasms of most small grain grass species. Additionally, efforts are made to incorporate digitized images of mapping and protein data as well as images depicting plant morphology and pathology. Also at these meetings barley researchers called upon the International Overall Chairman for barley linkage groups and barley collections to collect images of barley morphological characters. GrainGenes also relies upon corporation of the international research community for suggestions and data input.

”GrainGenes” and its software, AceDB, was later to become the model for the ’International Database for Barley Genes and Barley Genetic Stocks’. Since AceDB has several important advantages over other conventional relational db-systems in handling biological information and because a usable data model already existed for wheat mutants at ’GrainGenes’, the decision was natural. Dave Matthews at Cornell University, Ithaca, USA, kindly worked as an advisor and support person throughout the project, e.g. in adapting the data model to the barley’s and providing the GrainGenes model for linking data to references.

The Barley Genetics Stock Descriptions

During several years hundreds of genetic stocks containing morphological, physiological and resistance characters and genes were brought together world-wide from different regions, different genetic backgrounds and different cultivation environments. By Jerome D. Franckowiak’s, Fargo, North Dakota, USA, tremendous and skilful crossing and selection work, it was possible to transfer most of these genes into a common genetic background. The two-rowed cultivar ”Bowman”, a common high malting cultivar from North Dakota, USA, was used. These isogenic back-crossed derived lines are extremely useful for linkage studies, assessment of specific marker genes, determination of linkage drag and marker assisted gene transfer. All detailed studies and information resulted in detailed descriptions of more than 600 morphological, physiological and resistance barley genes published in Barley Genetics Newsletter, mainly in Volume 26 (DAVIS *et al*. 1997) and later issues (LUNDQVIST & FRANCKOWIAK 1997; JENDE-STRID *et al.* 1999; FRANCKOWIAK & LUNDQVIST 2002), both electronically and by hardcopies.

These descriptions include information about gene expression, its locus name and its symbol with the use of a three letter code. These recommended locus symbols are based on the utilization of a three letter code for barley genes as approved at the business meeting of the ’Seventh International Barley Genetics Symposium’ at Saskatoon, Saskatchewan, Canada, on August 5, 1996. Also, the previous nomenclature and synonyms are covered. Every locus or

germplasm is associated with a BGS stock number and corresponds to an accession GSHO or NGB number held at the Barley Genetics Stock Collection in Aberdeen, Idaho, USA and at The Nordic Gene Bank, Alnarp, Sweden, respectively. One allele of each description is kept at the Main Stock Center. Further, information of the chromosomal position, if known, of the critical locus and other mutants at that locus is given. The chromosome numbers and arm designations are based on the Triticeae system. The utilization of this system for the barley chromosomes was recommended (LINDE-LAURSEN 1997) and adopted at the business meeting of the 'Seventh International Barley Genetics Symposium' at Saskatoon, Saskatchewan, Canada, on August 5, 1996. Furthermore, the descriptions include information of the origin of the first described mutant at that locus, all mutational events and all references of published literature. All the many listed Swedish mutational events are available in the Nordic Gene Bank.

The Barley Genetics Stocks AceDB Database

The procedure followed in order to populate the AceDB system with data can be described in separate steps: designing of the data model, conversion of the source data into a format usable by the AceDB import software, installation of AceDB server and the user interfaces, import of the data, addition of additional information and performing corrections and updates. The last step, especially keeping the database up-to-date, remains a continuous process (DURBIN & MIEG 1991 -).

The source data was extracted from the barley mutant descriptions published in the Barley Genetics Newsletter, mainly volume 26, and later ones. Thanks to the authors and editors who agreed to provide in a uniform format, it was possible to convert the enormous amount of information into a database system that allows field-based searches, e.g. gene or allele names. The extraction was performed by first converting the original documents (usually in WordPerfect or MS-Word format) into plain text files. A script (written in the Perl programming language) then extracted and converted the source text to AceDB's import format. Because of this semi-automatic procedure, the conversion script could be run again on new descriptions in later Barley Genetics Newsletter publications, or repeated any time to allow corrections or adjustments.

The Barley Genetics Stocks Database now comprises more than six hundred genes with descriptions, more than 3700 alleles and more than 1700 references. Almost 4000 germplasm objects are referenced (mutant and parents). Many of the germplasm accessions are hyperlinks to webpages of the holding genebanks (ARS/GRIN/NSCG or NGB) in order to facilitate easy ordering of material containing interesting alleles. Many of the genes are illustrated with images, both overviews and detailed close-up character photographs. Most of them are taken in the Bowman back-crossed derived lines and compared with the normal cultivar. More than 900 digitized images are incorporated in the database. In addition, chromosome maps with its known positions as described in the inheritance and chromosome part are shown. Finally, 736 induced Swedish Translocation and Swedish Chromosome Segment Duplication lines, derived from Translocations, with their genetic background are incorporated in the database as an extension project, also associated with NGB stock numbers. Interactive karyotype maps are available for part of the material where breakpoint positions are sufficiently well known.

The database is easy to handle, it starts with Basic and Simple Search, and it is possible to select the object you are looking for. When using anything it searches for the entered text across the whole database. During the following years this International Barley Database will

get incorporated into the Triticeae Database ”GrainGenes” after some more proofreading and when some remaining problems especially in the Germplasm part are solved and revised. When only one gene locus is detected for a special character with several alleles, all Germplasm should be designed with an ”1”. Today, this is not done consequently.

The present database is available at http://www.untmo.net/bgs

The aim of this International Database for Barley Genes and Barley Genetic Stocks is to maintain the knowledge, retain the competence and transmit its distribution to the barley community. The use of existing morphological and resistance gene markers will always form a major input for future gene mapping and is immensely valuable for all molecular genetic analyses of cloned mutant genes. In any case, it is our intention to try to maintain the database for future achievements in both mutation and informatics research and it will be updated continuously. Any suggestions, information, mistakes and questions are welcome to the authors.

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Non-Random Distribution of Nuclear and Chloroplast Variation in Wild Barley from "Evolution Canyon"

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Abstract
Nuclear and chloroplast SSR diversity were studied in a population of wild barley from "Evolution Canyon" in Israel. Over 200 alleles for 19 mapped nuclear SSRs and 12 chloroplast haplotypes were detected among 276 individuals. This level of diversity was detected in populations separated by a distance of 400 m and is comparable to studies that sampled material from a much wider eco-geographic range. A phylogenetic tree of nuclear SSR variation showed clear differences between individuals collected from opposing slopes of the canyon. Inter-slope contrasts for chloroplast haplotype frequency were also observed. Most genetic diversity was observed on the North Facing Slope rather than the highly irradiated, hotter and drier South Facing Slope. Evidence for strong and structured cyto-nuclear interactions (disequilibria) were also detected. The SSR data are discussed with reference to adaptation and divergence to contrasting environments colonized by wild barley in "Evolution Canyon".
Keywords: nuclear; chloroplast; SSR; diversity; wild barley; "Evolution Canyon"

Introduction

Hordeum spontaneum, the wild ancestor of cultivated barley occurs in a wide range of ecologically diverse habitats from primary habitats of the Fertile Crescent (from Israel and Jordan to south Turkey, Iraq and south-western Iran) to secondary habitats in the Aegean region as well as central Asia. Previous diversity studies using SSRs have focused on examining variation in wild barley populations across a wide range of environments (BAEK *et al*. 2003; HUANG *et al*. 2002). These authors observed non-random SSR allele distributions associated with temperature and water stresses. In order to address the question of non-random associations at a micro site population level, we have chosen to assess SSR variation in wild barley, collected at "Evolution Canyon", Israel. "Evolution Canyon" has contrasting north (characterized by a 'European' type environment) and south facing slopes, the latter receives more sunlight (and as a consequence is characterized by 'African' flora and fauna; NEVO 1995). Most recent studies have focused on genetic variation within the nucleus (NEVO *et al*. 1998; OWUOR *et al*. 1999; TURPEINEN *et al*. 2003; HUANG *et al*. 2002). However, SAGHAI-MAROOF *et al*. (1992) highlight the importance of interactions between chloroplast and nuclear genes for a number of physiological and biochemical processes. This raises the question whether the variation encoded in the nuclear and chloroplast genomes are related and how associations are distributed eco-geographically. The current study was conducted to investigate both nuclear and chloroplast SSR diversity within a single population with and *intra-* and *interslope* at "Evolution Canyon" in Mount Carmel, Israel.

Material and Methods

Lower Nahal Oren, Mt. Carmel, Israel (32°43'N; 34°58'E), known as "Evolution Canyon", is a seasonally dry valley draining Mt. Carmel from east to west into the Mediterranean Sea. The opposing south-facing (SFS) or "African", and north-facing (NFS) or "European" slopes are 100 m apart at bottom and 400 m apart at top. The slopes share an identical Plio-Pleistocene evolutionary history (dating back 3-5 million years), geology (Upper Cenomanian Limestone), pedology and regional Mediterranean climate. The slopes diverge in topology (the SFS has a gradient of 35° and the NFS 25°) and in geographic orientation, resulting in differential reception of solar radiation, interslope micro-climatic and biotic differences.

H. spontaneum was sampled in "Evolution Canyon" at seven micro-sites, at three elevations 30m apart (60, 90 and 120m above sea level) on each slope and one at the floor of the canyon: "African", xeric, south facing slope: micro-sites 1 (high), 2 (middle) and 3 (low); canyon bottom (CB): micro-site 4, and "European", mesic, north facing slope: micro-sites 5 (low), 6 (middle), and 7 (high). Seeds were collected from 276 plants 1-2 metres apart at seven microsites. These sub-populations consisted of 19 to 32 genotypes and were analyzed for nuclear SSR loci, and 31 to 51 genotypes were analyzed for chloroplast SSR loci. One seed of each genotype was grown and bulked in glasshouse conditions at the Scottish Crop Research Institute, Dundee, Scotland and DNA extracted using the Nucleon Phytopure DNA Extraction Kit (Nucleon Biosciences), following the manufacturer's instructions.

The 19 nuclear and 4 chloroplast SSR loci used are listed in Tables 1 and 2, respectively. PCR conditions for nuclear and chloroplast SSRs are described in RUSSELL et al. (1997) and PROVAN et al (1999) respectively. PCR products were sized according to the M13 sequencing marker, and scored as allele sizes in base-pairs. PCR products were sized according to the M13 sequencing marker, and scored as allele sizes in base-pairs (bps). Diversity indices were calculated for individual primer pairs as $\hat{H} = (n/n\text{-}1)1\text{-}\Sigma p_i^2$ where p_i is the frequency of the ith allele or haplotype.

Results

Nineteen previously mapped SSRs successfully amplified 226 alleles in 276 genotypes (Table 1). The number of alleles ranged from 1 (HVLEU and Bmac0216) to 23 (HVM3) with corresponding diversity values of 0.000 to 0.932 respectively. Three of the four chloroplast SSRs were polymorphic, and a total of ten alleles were detected. Since the four chloroplast loci are linked the data can be expressed as haplotypes: a total of 12 haplotypes were found with an overall diversity value of 0.692 (Table 2). The NFS had significantly more alleles (178) than the SFS (138 alleles, $p<0.05$) with the mean number of alleles decreasing from low to high sub-populations on both slopes, the highest being CB 4 and NFS 5 (Table 1). Similarly the highest diversity values were observed in the bottom and North Low sites (0.715 and 0.718) compared to the South Middle and South High (0.556,0.583)(Table 1). The highest number of private alleles (16) were found in the NFS with 11 on the SFS and 12 in the CB sub-population. Similarly a significant ($p<0.01$) inter-slope difference was found in the frequencies of 106 (47.3%) alleles, from all polymorphic loci. The level of diversity does not differ significantly between slopes, but allelic composition and distribution varies across several loci (Table 1).

Table 1. Statistical analysis of nuclear SSR variation in the seven sub-populations

SSR (chromosome)	NFS7	NFS6	NFS5	CB4	SFS3	SFS2	SFS1	Mean diversity
	Diversity (no. alleles)	Diversity (no. alleles)	Diversity (no. alleles)	Diversity (no. alleles	Diversity (no. alleles)	Diversity (no. alleles)	Diversity (no. alleles)	
HVM20 (1H)	0.706 (8)	0.683 (5)	0.699 (6)	0.819 (10)	0.692 (6)	0.567 (6)	0.681 (5)	0.692
HvHVA1 (1H)	0.390 (3)	0.490 (2)	0.170 (2)	0.580 (2)	0.300 (2)	0.230 (2)	0.040 (2)	0.314
Ebmac0403 (2H)	0.819 (7)	0.579 (8)	0.808 (10)	0.739 (6)	0.799 (6)	0.606 (5)	0.590 (5)	0.706
Bmac0216 (2H)	0.000 (1)	0.000 (1)	0.000 (1)	0.000 (1)	0.000 (1)	0.000 (1)	0.000 (1)	0.000
HVM54 (2H)	0.602 (6)	0.504 (3)	0.802 (8)	0.840 (8)	0.745 (6)	0.606 (4)	0.648 (3)	0.678
HvLTPPB (3H)	0.369 (3)	0.571 (3)	0.530 (3)	0.766 (6)	0.611 (3)	0.527 (3)	0.492 (2)	0.552
Bmac0029 (3H)	0.646 (4)	0.506 (5)	0.740 (5)	0.792 (8)	0.612 (5)	0.653 (3)	0.527 (3)	0.639
HvOLE (4H)	0.790 (7)	0.597 (6)	0.849 (8)	0.738 (8)	0.787 (6)	0.748 (6)	0.735 (7)	0.749
HVM3 (4H)	0.829 (10)	0.834 (8)	0.711 (10)	0.843 (11)	0.774 (5)	0.583 (7)	0.723 (7)	0.757
Bmac0030 (4H)	0.843 (11)	0.605 (4)	0.820 (11)	0.795 (9)	0.819 (7)	0.508 (5)	0.699 (5)	0.727
Bmac0186 (4H)	0.706 (4)	0.512 (3)	0.715 (4)	0.353 (3)	0.632 (3)	0.646 (3)	0.547 (4)	0.587
Bmac0096 (5H)	0.709 (6)	0.694 (5)	0.716 (8)	0.601 (5)	0.768 (5)	0.715 (4)	0.549 (4)	0.679
Bmac0113 (5H)	0.813 (8)	0.719 (8)	0.893 (13)	0.845 (10)	0.870 (10)	0.754 (10)	0.761 (8)	0.808
HvLOX (5H)	0.501 (2)	0.295 (3)	0.678 (4)	0.467 (4)	0.566 (3)	0.402 (2)	0.248 (2)	0.451
HVLEU (5H)	0.000 (1)	0.000 (1)	0.000 (1)	0.000 (1)	0.000 (1)	0.000 (1)	0.000 (1)	0.000
Bmac0316 (6H)	0.811 (6)	0.865 (10)	0.800 (9)	0.824 (9)	0.660 (6)	0.452 (4)	0.720 (6)	0.733
Bmac0218 (6H)	0.837 (9)	0.784 (7)	0.887 (12)	0.881 (12)	0.781 (5)	0.682 (6)	0.809 (9)	0.809
HVCMA (7H)	0.580 (3)	0.532 (3)	0.488 (3)	0.366 (2)	0.506 (2)	0.000 (1)	0.455 (2)	0.418
Bmag0135 (7H)	0.879 (11)	0.823 (11)	0.907 (13)	0.899 (14)	0.806 (6)	0.770 (8)	0.692 (6)	0.825
sum	(11)	(96)	(131)	(129)	(88)	(81)	(82)	
mean (poly)	0.696 (6.35)	0.623 (5.53)	0.718 (7.59)	0.715 (7.47)	0.690 (5.06)	0.556 (4.65)	0.583 (4.71)	
stdev	0.155 (2.81)	0.147 (2.64)	0.177 (3.53)	0.171 (3.40)	0.138 (1.95)	0.194 (2.27)	0.191 (2.16)	
st.err	0.036 (0.64)	0.034 (0.61)	0.041 (0.81)	0.039 (0.78)	0.032 (0,45)	0.044 (0.52)	0.044 (0.45)	

Table 2. Chloroplast haplotype frequency and diversity values for the sub-populations

Haplotype (*)	NFS7	NFS6	NFS5	CB4	SFS3	SFS2	SFS1
A (113, 101, 140)	0.205	0.267	0.333	0.19	0.387	0.658	0.523
B (113, 100, 141)							0.045
C (114, 100, 140)	0.718	0.667	0.412	0.262	0.419	0.237	0.205
D (113, 100, 140)		0.033	0.137	0.381			
E (115, 100, 140)				0.119			
F (113, 101, 141)	0.026	0.033	0.02	0.024	0.194	0.079	0.045
G(114, 100, 141)	0.051		0.02	0.024			
H (113, 102, 140)			0.02				
I(112, 100, 141)			0.02				
J(112, 100, 140)			0.039				
K(114, 101, 140)							
N(114, 101, 140)							
Diversity	0.451	0.499	0.711	0.753	0.658	0.518	0.677
No. Haplotypes	4	4	8	6	3	4	7

* allele sizes at HvctmS2, Hvcptmlf and OSCP6, respectively

Further evidence for differentiation between slopes was observed by comparing the number and distribution of chloroplast haplotypes. Although similar numbers of haplotypes were observed between the two slopes (NFS (8) and SFS (7) the distribution of the two most frequent (Haplotype A=0.367 and Haplotype C=0.404) haplotypes showed significant ($p<0.01$) inter-slope differences in their frequencies Table 1).

Discussion

Genetic Diversity in "Evolution Canyon"

The overall diversity (mean diversity 0.788) and allelic composition (mean number of alleles, 13.1) found here was comparable to previous studies (BAEK *et al*. 2003) in which the

populations sampled were from a much wider eco-geographical range (mean diversity, 0.512; mean number of alleles, 13.8). The maximum distance between populations in this study was only 400 m. Interestingly, the genetic divergence between sampling sites is as great as that found between the Judean mountain and Negev desert populations, separated by 100 km (TURPEINEN *et al*. 2001). Furthermore, the chloroplast diversity was remarkably high and exhibited a haplotype cline from one side of the canyon to the other. Twelve haplotypes were observed with a corresponding diversity value of 0.692, mirroring the nuclear diversity. PROVAN *et al*. (1999), examined chloroplast variation across the natural distribution of wild barleys (51 accessions), and observed a similar number of haplotypes and variation. Comparisons between slopes revealed greater nuclear SSR variation in the "European" slope (NFS), compared to the "African" slope (SFS). This is in agreement with the study in the Neve Yaar region, with a lower level of variation observed in individuals from sunny and rocky sites compared with those from milder, shady and deep-soil micro-sites (HUANG *et al*. 2002). The trend of greater diversity in low stress compared to high stress is also evident within the slopes of "Evolution Canyon" with both the NFS and SFS sub-populations increasing in genetic diversity with decreasing altitude (decreasing irradiance, temperature and water stress). These results however, contrast with most other studies (NEVO *et al*. 1979; OWUOR *et al*. 1997) using other marker systems such as isozymes and RAPDS, which showed great variation in more stressed environments.

Early studies (GUTTERMAN & NEVO 1994; LAVIE *et al*. 1993) have suggested that the sharp contrast between adjacent populations may be of adaptive significance, but that random genetic drift cannot be excluded as probable cause for population divergence (SAGHAI-MAROOF *et al*. 1992). Seed dispersal of wild barley by animal and other vectors would tend to homogenize the population, but clearly this is not the case in the canyon and diversity is non-random. The high levels of diversity at the base of the canyon and at the low north facing site may however be indicative of seed dispersal by wind and/or water, which would result in low areas acting as repositories for genetic variation. Maternally inherited markers such as chloroplast SSR haplotypes can monitor seed dispersal. Twelve chloroplast haplotypes were identified in the population indicating twelve maternal lineages. Again these are not randomly distributed in the population. Six of the twelve haplotypes were private to sub-populations, generally found within the bottom site of the canyon, five of which were present at very low frequencies suggesting that these lineages are in decline or else newly introduced. Haplotype E was only observed in the bottom sub-population (CB) at a relatively high frequency (0.119) and may represent a more established lineage.

The inter-slope genetic pattern observed for SSRs and other markers suggest adaptive divergence by natural selection. This conclusion is supported by the dramatic inter-slope differences in germination patterns (GUTTERMAN & NEVO 1994) and by transplant experiments (LAVIE *et al*. 1993) in wild barley. The distribution of SSR allele data described here are however in contrast to other marker data sets of wild barley at Evolution Canyon and elsewhere. In this study the lesser stressed slope (NFS) showed greater diversity than the highly irradiated, hotter and drier slope (SFS). In most other studies the reverse is true, e.g. greater diversity of RAPDs (BAUM *et al*. 1997; NEVO *et al*. 1998) and AFLPs (TURPEINEN *et al*. 2003) is found in the most stressed environments. Since SSRs are associated with non-repetitive DNA (MORGANTE *et al*. 2003) they sample a different portion of the genome and may reveal new information on genetic diversity.

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In situ Conservation of Genetic Diversity at Small-Scale Farmer in Eritrea

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Abstract
In this study, the genetic diversity of barley accessions *Hordeum vulgare* ssp. *vulgare* collected from Eritrean farmer fields' was studied within and among field populations. A total of 240 single spikes were sampled from 24 farmers' field *i.e.* 10 spikes per field. Forty five microsatellite covering the whole barley genome were employed and analysed on ABI PRISM 377 DNA sequencer and Mega Base 1000 sequencer. Analysis of Molecular Variance (AMOVA) based on genetic distances was conducted. The level of molecular variance among fields and regions was 5.43% and 7.08%, respectively. Interestingly, high level of variance was observed within the field that reached up to 87.5 %. Average Gene Diversity (AGD) values in fields ranged from 0.35 to 0.55.
Keywords: Eritrea; genetic diversity; SSRs; *in situ* conservation

Introduction

Barley (*Hordeum volgare* L.) is considered as one of the most important cereals crop worldwide. It is grown in West Asia and North Africa as a landraces in marginal, low-input, drought-stressed environments for both grain and straw (CECCARELLI *et al.* 1987). Barley is an important crop in Ethiopia, Eritrea and Sudan (RASMUNSSON 1985). In Eritrea barley is one of the most important crops for poor resource farmers. It is grown in the Central Highland zone on about 40,000 hectares with an average yield of about 500 kg/ha. It's production represents 20% of the total agricultural production. Barley is one of the staple crops in the highlands of Eritrea where is widely used as human food. In addition, straw is an important source of animal feed, as well as it is grown not only as a single crop but also in a mixture with wheat to control disease during we t years and to ensure a production during drought years.

In the search for diversity in cereal crops, farmer varieties or landraces (locally adapted population) are usually an important source of genetics variation (GHEBRU *et al.*2002). Using barley landraces in breeding programs offers other advantages including the incorporation of desirable agronomic traits such as drought tolerance (CECCARELLI *et al*., 1987; YAHYAOUI *et al*., 1996).

The microsatellites (SSRs) have proven to be very useful in the study of genetic diversity, the variability of microsatellites loci is due to the difference in the number of repeat units, and it is easier to detect the variation in length of DNA fragments obtained through PCR amplification. SSRs have several advantages over other DNA markers such as RFLPs,

RAPDs, or AFLPs. The advantages include uniform distribution over the whole genome; high levels of polymorphism, and co-dominant inheritance (PEJIC *et al.*1998).

Material and Methods

A total of 240 Eritrean single spikes were collected from 24 farmers' field located in different agro-climate zones (Fig. 1). DNA was extracted according to the protocol described by SAGHI-MAROOF *et al*. (1984). PCR for genomic microsatellite was performed in Thermo-Fast 96 plate from ABgene in a final reaction volume of 20 µl containing 100 ng genomic DNA, 1x Mg free PCR buffer, X $MgCl_2$, 0.1 units *Taq* polymerase (last three from Promega), 250 µM dNTPs, and 1 µM of forward and reverse primers. PCR program for each primer was performed according to (RAMSY *et al.,* 2000; LIU *et al.,* 1996) and (BECKER & HEUN 1995). All samples were tested with forty-five microsatellite markers covering the whole barley genome. Forward primers were labelled with one of the following fluorescent dye: HEX, VIC, TET and FAM for C filter, NED, HEX or VIC and FAM for D filter. The PCR products were run and analysed on ABI PRISM 377 DNA sequencer from Applied Biosystem and MegaBace 1000 sequencer from Molecular Dynamics, using GeneScan® and Genotyper® version 2.1 programs in ABI and Instrument Control Manager and Genetic profiler version 1.1 programs in MegaBace.

Results and Discussion

Land races from Eritrea showed high level of polymorphism. A total of 315 polymorphic alleles were detected gained from applying 45 microsatellites primers on 239 lines with an average of 8 polymorphic alleles per microsatellite. The highest polymorphism was detected with the primer Bmac0156 with 23 alleles, and the lowest polymorphisms were found with the primers HvLOX and bmag0218 giving only three alleles. Monomorphic bands were obtained with the primers HvLEU and WMC1E8.

Analysis of Molecular Variance (AMOVA) was conducted based on genetic distances. Highest level of variance was observed within the field that reached up to 87.5 %, While the level of molecular variance among fields was 5.43% and among regions was 7.08%. High percentage of variation within the field could be referring to the absence of the breeding activities in Eritrea that saved high variation in the fields.

Average Gene Diversity (AGD) at field's level was ranged from 0.35 to 0.55. The highest AGD were found in Adi hizbay field that is located in Dubarwa Halhale region, while the lowest AGD was in Abi adi in Medenfera Adi Quala region.

Cluster analysis based on Unweighted Pair Group Method Average (UPMGA) for 239 lines with 38 SSRs showed high level of variation among the lines. Only two lines were identical even though collected from different locations, all the other accessions differed from each other; this result shows the high percentage of AGD found within a field.

The study revealed the complexity of genetic variability found in Eritrea that should be further studied and exploited in breeding programs.

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Picture 1. The sites of Eritrean barley collection

Assessment of Genetic Diversity of Hull-Less Barley (*Hordeum vulgare* L.) Germplasm in the High Altitude Himalayas of Nepal

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Abstract
The highlands of Nepal representing almost one-third of the Himalayan-range are known to harbour valuable barley genetic resources. Previous studies have shown considerable variation in morphology, maturity and disease reaction among Nepalese hull-less barley (*Hordeum vulgare* L.) landraces. However, precise and detailed information on genetic diversity is lacking. Therefore, a set of 107 naked barley genotypes from Nepal including 8 selected German and Canadian cultivars were analysed using 27 SSRs covering each barley chromosome. Based on these analyses 165 alleles were detected with an average of 6.1 allele per SSR. Taking into account only Nepalese barley on average 4.8 alleles were found. UPGMA clustering based on DICE similarity index ranging from 0.07 to 1.0 clearly separates European and Canadian from Nepalese hull-less barley. However, also within the Nepalese collection a large degree of genetic variability was detected. The Nepalese collection is grouped in two distinct clusters and several sub-clusters which in some cases are in accordance with the collection site.
Keywords: *Hordeum vulgare* L.; hull-less barley; Nepal; genetic relatedness; SSRs

Introduction

The hull-less or naked caryopsis character of barley seed is controlled by the single recessive gene '*nud'* located on the long arm of chromosome 7H (KIKUCHI *et al*. 2003). Hull-less barley is less common than the hulled type and grown in limited parts of the world, mainly in East Asia. In this region, the grains are often consumed as human food, particularly in the high mountains. There is an increasing concern on this crop in recent days due to it's high feed value and also for human consumption (ATANASSOV *et al*. 2001).

The Himalayan region is considered rich in barley diversity (BADR *et al*. 2000). In Nepal barley is cultivated mainly in the Northern highlands and naked barley is grown predominantly above 2,800 m (BANIYA *et al*. 1997) from east to the west extending along almost one-third of the massive Himalayan chain. Due to high cold tolerance and performance under poor management condition, hull-less barley is a successful cereal in the highlands (DAHAL & SHARMA 1994). Naked barley is a high-value crop in the highlands of the Himalayas and is tightly associated with livelihood of the people. It is mainly consumed as staple food and besides this, in ethnic communities as animal feed, for alcohol preparation and for medicinal as well as religious purposes.

Due to a large geographic variation, multi-ethnic society and primitive farming practices, a large number of hull-less barley landraces has been preserved in farmers fields since centuries. Previous studies have shown considerable variation in morphology, maturity and disease reaction among landraces (SHARMA *et al*. 1994; BANIYA *et al*. 1997; GUPTA *et al*. 2000). However, there is a lack of precise and detailed information on genetic diversity. Such information is vital

for crop improvement and conservation of genetic resources for future use. In this study, a large number of naked barley accessions originally collected from Nepal were analysed using SSR markers.

Material and Methods

A total of 115 barley genotypes were analysed using 27 SSR primers located at each of the barley chromosomes (Table1). The plant material consisted of 107 six-rowed Nepalese hull-less barley genotypes, five Canadian naked cultivars and three German hulled cultivars. The seed material of the Nepalese landraces was obtained from the Barley Germplasm Center, Okayama University, Kurashiki, Japan. Genomic DNA was extracted from the leaf tissues of two weeks-old plants grown in the green-house following the CTAB extraction protocol (DOYLE & DOYLE 1990). The SSR assay was carried out according to RAMSAY *et al*. (2000) with modifications in a Geneamp 9700 thermal cycler (Perkin Elmer).

Respective SSR profiles were detected on an automatic DNA-sequencer (LiCor 4200-S2). The presence or absence of each band was coded as 1 or 0, respectively, using the software RFLP-scan 2.1. The resulting 1/0 data matrix was employed to compute DICE similarity index (DICE 1945). Based on these data UPGMA-clustering using the SAHN option of the software NTSYS-pc (ROHLF 2000) was carried out. To measure the informativeness of each marker, polymorphic information content (PIC, ANDERSON *et al*. 1993) was calculated: $PIC = 1- \Sigma (p_i)^2$, where (p_i) is the frequency of the i^{th} allele of the SSR locus across the 115 genotypes studied. The mean diversity index (DI) over all the loci in 115 genotypes studied and with in the Nepalese collection was estimated according to NEI (1973):

$DI = \frac{1}{n} \sum_j (1- \sum_i x_{ij}^2)$, where x_{ij} is the frequency of the i^{th} allele of locus j and n the number of loci.

Results and Discussion

All the 27 SSRs used in the study were polymorphic on the 115 genotypes analysed and a total of 165 alleles were detected. The number of alleles per locus ranged from 2 (HvMLO3, Ebmac0970, HVLEU) to15 (Bmag0007) with an average of 6.1 alleles per SSR (Table1). The polymorphic information content (PIC) varied largely among the 27 SSRs used. The smallest PIC value was estimated for HVLUE and Bmac0316 (0.07) and the maximum for Bmac0273 (0.89). The total genetic diversity (DI) was estimated at 0.53 for all the 115 genotypes analysed. The DICE similarity estimates varied from 0.07 to 1.00. The minimum value was observed between the Nepalese landraces (collected from Annapurna-South, upper basins of river KaliGandaki and Marshyangdi) Canadian (Candle, Freedom, Silly), and German (Alexis, Ludmilla) cultivars. The maximum similarity estimate was found within the genotypes from Bimtakothi, Philem, Nepal, Sipche, Sikha locality and between Jomson1 and Kagbeni3. The 115 genotypes were clustered in two well distinct groups, clearly differentiating German and Canadian cultivars from Nepalese germplasm (Fig.1). The 27 SSRs used for the analysis were able to discriminate the genotypes of different origin.

Genetic Diversity within Nepalese Germplasm

Out of 27 SSRs applied to 107 Nepalese genotypes, except HVLUE, 26 were polymorphic and in total 130 alleles were scored resulting in 4.8 alleles per SSR (Table 1). The maximum number of

alleles scored for Bmag0007 (14) and minimum for HVLUE (1). The genetic diversity index (DI) based on 26 polymorphic SSRs was 0.48. The genetic similarity estimates (DICE) ranged from 0.22 to 1.00. The minimum genetic similarity within the Nepalese germplasm was estimated for Solu-Uwa vs Pisang7, Pisang6 vs Ghara2 and Pisang7 vs Ghara1. Solu-Uwa is a cultivar developed by selection of a local landrace from the Mt. Everest region of East Nepal, whereas Pisang7 and Pisang6 were collected from upper Annapurna; Ghara1 and Ghara2 from the lower Annapurna in Central Nepal.

Table 1. SSRs used for the analysis, repeat motifs, chromosomal locations (MACAULAY *et al*. 2001); number of alleles and PIC values for Nepalese genotypes and over all 115 genotypes

SSR	Repeat	Chromosomal location	No.of alleles		PIC	
			a	b	a	b
Bmac0399	(AC)21	1H	9	7	0.72	0.69
WMC1E8	(AC)24	1H	3	2	0.10	0.07
Bmac0093	(AC)24	2H	5	5	0.67	0.66
Bmag0378	(AG)14	2H	6	3	0.50	0.46
EBmac0415	(AC)17	2H	3	3	0.66	0.65
HVM54	(GA)14	2H	4	4	0.65	0.60
Bmac0067	(AC)18	3H	9	7	0.77	0.76
Bmac0209	(AC)13	3H	8	4	0.50	0.43
Bmag0013	(CT)21	3H	11	8	0.36	0.26
Bmag0136	(AG)6-(AG)10-(AG)6	3H	3	3	0.16	0.04
Bmag0353	(AG)21	4H	7	6	0.68	0.66
EBmac0701	(AC)23	4H	9	4	0.56	0.49
HVM40	(GA)6(GT)4(GA)7	4H	6	4	0.55	0.49
HVM67	(GA)11	4H	7	5	0.43	0.34
HvMLO3	(CTT)6	4H	2	2	0.43	0.42
Bmag0223	(AG)16	5H	12	11	0.86	0.85
EBmac0684	(TA)7(TG)11(TG)11 (TTTG)5	5H	6	6	0.63	0.60
Ebmac0970	(AC)8	5H	2	2	0.11	0.07
HVLEU	(ATTT)4	5H	2	1	0.07	0.00
Bmac0018	(AC)11	6H	3	3	0.56	0.51
Bmac0316	(AC)19	6H	3	2	0.07	0.02
Bmag0009	(AG)13	6H	6	5	0.60	0.54
Bmag0218	(AG)6(AG)6	6H	3	2	0.35	0.25
Bmac0273	(AC)20(AG)20	7H	11	11	0.89	0.88
Bmag0007	(AG)16(AC)16	7H	15	14	0.88	0.87
Bmag0120	(AG)15	7H	7	4	0.59	0.53
HVCMA	(AT)9	7H	3	2	0.43	0.35
Average			6.1	4.8		

a, all genotypes analysed (115)
b, Nepalese genotypes

Fig.1. Dendrogram of UPGMA-cluster analysis of 115 barley genotypes based on DICE similarity index (German cvs: Alexis, Ludmilla, Verena; Canadian cvs: Alama, Candle, Freedom, McGwire, Silley)

In contrast to this, landraces: Bimtakothi-3, -11; Philem-2, -3; Jomson1, Kagbeni3; Nepal-3, -4, -5, -6; Sipche-3,-4,-6,-7,-9; Sikha-1,-2; Sikha-5,-6 and Nepal-1,-2 are not differentiated from each other by the 27 SSRs tested up to now. Respective genotypes clustering together have been collected from the same location, possibly being duplicated.
The clustering of genotypes in two broad groups did not follow defined agro-ecological origin, however, some of the genotypes from Bimtakothi and Pisang areas are grouped in distinct sub-clustures. The naked barley germplasm analysed in this study were mainly collected from the hills and mountains of Annapurna, Manaslu and Lantang Himalaya-range in Central Nepal which represents about one-third of the Nepalese Himalayan highlands (Catalogue of Barley Germplasm, Okayama Univ.,1983). Like in other studies, eg. based on morphological traits (WITCOMBE & MURPHY 1986, a,b), or isozymes (KONOSHI & MATSUUARA 1991; LIU *et al*. 1999) results of the present study indicate considerable genetic variability in these naked barley germplasm. By analysing additional SSRs a more detailed insight in the genetic relatedness of hull-less barley from Nepal will be gained. Together with phenotypic data well characterized germplasm for hulles barley breeding will be available in future.

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The Conservation of Genetic Diversity in Crops: Establishment of a Local Barley (*Hordeum vulgare* L.) Core Collection for North Rhine-Westphalia (NRW, Germany)

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Abstract
Since centuries, barley (*Hordeum vulgare* L.) is one of most important crops for mankind. In the course of industrialization of farming, old "barley landraces" were replaced by modern high yielding cultivars. A limitation of elite cultivars results in a leakage of diversity. This leakage cannot even be replaced by advanced genetic methods. If exotic and wild forms extinguish, this will result in an irreversible loss of genetic diversity.
Therefore the federal state of North Rhine-Westphalia (NRW), Germany, endeavour in increasing the genetic diversity of cultivated crop species. Hereto a barley core collection should be established by the project at hand. The chosen accessions should be grown *in situ*, so they could participate, unlike *ex situ* accessions of gene banks, in evolution and changing environmental conditions. *In situ* conservation is a proper alternative for *ex situ* conservation by gene banks. In distinct cases a loss of genetic diversity in gene bank accessions is observed by "seed rejuvenation cycles".
In the present project 150 spring barley and 144 winter barley accessions were chosen to be analysed for their genetic diversity by different methods of examination. The accessions were analysed for morphological, taxonomical, historical, geographical, qualitative and genetic differences. Field tests where conducted in 3 blocks for 2 years and SSR-markers were used to analyse the relationship of the different accessions.
Keywords: barley (*Hordeum vulgare* L.); genetic diversity; core collection; SSR markers; genetic distance; genetic similarity; UPGMA; DICE; cluster analysis; dendrogram

Introduction

Barley (*Hordeum vulgare* L.) is one of the oldest cultivated crops of mankind and still has a major economic impact. As a result of intensive breeding efforts, barley is not only very good analysed, but also sufficient characterised. More than 320.000 accessions stored worldwide in gene banks (FAO, 1993) and numbers are still rising.
The great number of accessions brings up several problems. On the one hand, conservation is labour-intensive, high- priced and time-consuming, on the other hand utilisation of accessions is very complex.
The information currently available is not sufficient for users (scientists and plant breeders) and curators. To alleviate the management difficulties, the identification and use of core collections has been suggested. A core collection is a subset of an existing germplasm collection, which represents with a minimum of size the widest range of the genetic diversity.
The question is: what material to choose for plant breeding purposes from a germplasm collection, what material to include in the collection and what material to remove from the collection?
Different sampling strategies of the concept of core collections have been suggested since Frankel (BROWN, 1989a). BROWN (1989b) makes the following assumption: `70% of the

alleles present in the whole collection would be retained in 10% of the total collection´s size based in the theory of selectively neutral alleles.` For several plant species, core collections have been established and compared for their effectiveness to maintain the diversity of the base collection (SPAGNOLETTI ZEULI and QUALSET, 1993; BATAILLON, 1994; CORDEIRO *et al.* 1995; DIWAN *et al.*, 1995; GALWEY, 1995; HINTUM VAN *et al.*, 1995; IGARTUA, 1998).
At present, the collection of cultivated barley (*Hordeum vulgare* ssp. *vulgare*) maintained at the IPK Gatersleben contains more than 90.000 accessions. The objective of this study was to identify the accessions with regional connection to NRW and then to identify a ´local´ subset or core collection of these regional accessions that retained the widest range of genetic diversity with a minimum of repetitiveness.

Material and Methods

Plant Material

The material consisted of 150 spring barley and 144 winter barley accessions obtained from the Institute of Plant Genetics and Crop Plant Research (IPK, Gatersleben), Germany and from the former Federal Centre for Breeding Research of Cultivated Plants (BAZ, Braunschweig), Germany. They were chosen in relevance to crop growing in North Rhine-Westphalia based on the "Beschreibende Bundessortenliste" of the Bundessortenamt (Hannover), Germany.

SSR Analysis

Leaf tissue of 10 days old plants was harvested, freeze dried, and ground into powder. The DNA was extracted using a slightly modified protocol of the CTAB method by SAGHAI-MAROOF (1984). PCR fragments were amplified by a total of 23 SSR markers. The amplified fragments were electrophoresed on 6% polyacrylamid gels and were visualised by silverstainig with $AgNO_3$.
All SSR markers amplify single, distinguishable bands for each accession and are mapped on the seven chromosomes of barley. The distribution and the number of SSR marker per chromosome is shown in Table 1.

Table 1. Position and distribution of the 23 SSR marker. [1)] PILLEN *et al.*, 2000; [2)] LIU *et al.*, 1996 ; [3)] RAMSEY *et al.*, 2000 ; [4)]THIEL *et al.*, 2003.

Chromosome	Number	Name
1H	2	HVALAAT[1)], Bmag0579[3)],
2H	5	HVM36[2)], HVM54[2)], EBmac0415[3)], GBM1016[4)], GBM1047[4)]
3H	6	HVLTPPB[1)], Bmac0209[3)], Bamg0225[3)], Bmag0013[3)], EBmac0541[3)], EBmac0705[3)]
4H	5	EBmac0701[3)], EBmac0788[3)], HVOLE[3)], EBmac0635[3)], GBM1048[4)]
5H	2	Bmag0337[3)], GBM1026[4)]
6H	2	Bmac0316[3)], Bmac0040[3)]
7H	1	Bmac0156[3)],

Statistical Analysis

Bands were scored as present (1) or absent (0). Genetic similarity (GS_{ij}) was estimated with the DICE algorithm described by NEI and LI (1979)

$$GS_{ij} = \frac{2N_{ij}}{T_{ij}}$$

where N_{ij} was the number of bands common in i and j, and T_{ij} was the sum of the number of

bands in *i* and the number of bands in *j*. A similarity matrix was further on calculated by using an UPGMA (unweighted pair group method with arithmetic average) cluster analysis to generate a dendrogram illustrating the relationship between the accessions. The analyses were performed by using the NTSYS-pc vers.2.2i computer software (ROHLF, 2000)

Results

Allelic Distribution

23 SSR markers were analysed, showing clear banding patterns at 150 allele loci. Number of amplified alleles varied from three alleles with the SSR marker EBmac0415[3)] up to ten distinct alleles at EBmac0701[3)]. The average number of alleles per SSR marker was at 6.84 alleles.

Genetic Diversity among Spring Barley and Winter Barley Accessions

The percentage of number of alleles in spring barley accessions (135 in total; 90,0%) is higher, than the number of alleles in the winter barley accessions (109 in total; 72,7%). So one can say that the genetic diversity is lower in the evaluated winter barley accessions than in the spring barley accessions shown by the number of alleles per locus.

Table 2. Number of alleles at each SSR locus in spring and winter barley accessions.

SSR locus	Number of alleles in:			SSR locus	Number of alleles in:		
	total	Spring barley	Winter barley		total	Spring barley	Winter barley
EBmac0705	5	4	4	Bmac0316	8	8	2
EBmac0701	10	6	9	HvOLE	5	5	4
HVALAAT	5	5	4	EBmac0788	8	7	7
HVM36	6	5	5	Bmag0579	5	4	5
Bmac0156	9	8	6	EBmac0635	9	6	7
EBmac0415	3	3	3	Bmac0337	5	5	4
Bmac0209	5	5	3	Bmac0040	6	5	4
HVM54	5	5	3	GBM1016	7	6	5
HVLTPPB	4	4	2	GBM1026	8	8	4
Bmag0225	8	7	8	GBM1047	6	6	6
Bmac0013	9	9	7	GBM1048	6	6	4
EBmac0541	8	8	3				
				total	150	135	109
				%		90,0	72,7

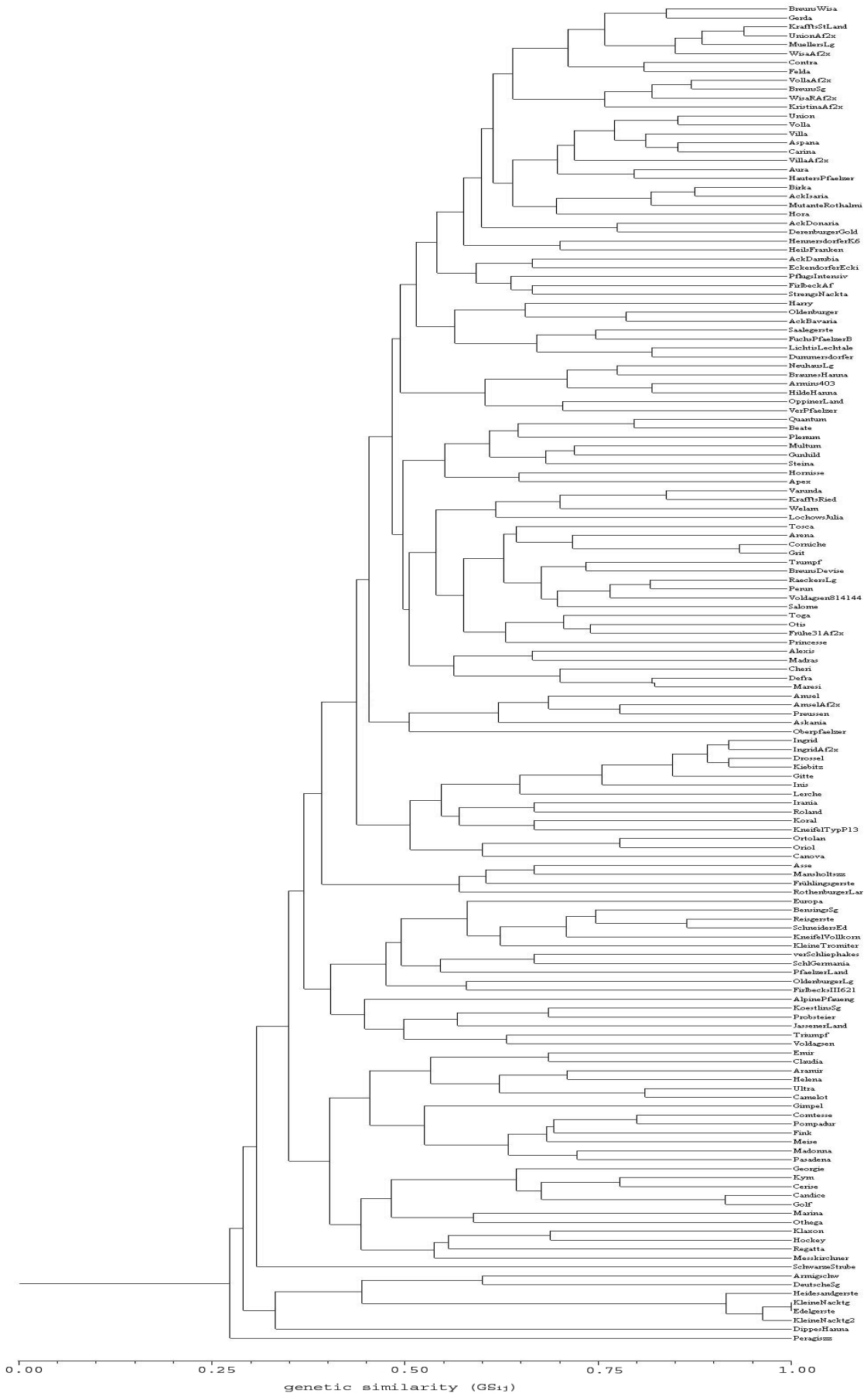
BreunsWisa
Gerda
KrafftsStLand
UnionAf2x
MuellersLg
WisaAf2x
Contra
Felda
VollaAf2x
BreunsSg
WisaRAf2x
KristinaAf2x
Union
Volla
Villa
Aspana
Carina
VillaAf2x
Aura
HautersPfaelzer
Birka
AckIsaria
MutanteRothalmi
Hora
AckDonaria
DerenburgerGold
HennersdorferK6
HeilsFranken
AckDanubia
EckendorferEcki
PflugsIntensiv
FirBeckAf
StrengsNackta
Harry
Oldenburger
AckBavaria
Saalegerste
FuchsPfaelzerB
LichtisLechtale
Dummersdorfer
NeuhausLg
BraunesHanna
Arminu403
HildeHanna
OppinerLand
VerPfaelzer
Quantum
Beate
Plenum
Multum
Gunhild
Steina
Hornisse
Apex
Varunda
KrafftsRied
Welam
LochowsJulia
Tosca
Arena
Corniche
Grit
Trumpf
BreunsDevise
RaeckersLg
Perun
Voldagsen814144
Salome
Toga
Otis
Fruhe31Af2x
Princesse
Alexis
Madras
Cheri
Defra
Maresi
Amsel
AmselAf2x
Preussen
Askania
Oberpfaelzer
Ingrid
IngridAf2x
Drossel
Kiebitz
Gitte
Iris
Lerche
Irania
Roland
Koral
KneifelTypP13
Ortolan
Oriol
Canova
Asse
Manholtszm
Frühlingsgerste
RothenburgerLar
Europa
BensingSg
Reisgerste
SchneidersEd
KneifelVollkorn
KleineTromiter
verSchliephakes
SchlGermania
PfaelzerLand
OldenburgerLg
FirBecksIII621
AlpinePfaueng
KoestlinsSg
Probsteier
JaseneerLand
Triumpf
Voldagsen
Emir
Claudia
Aramir
Helena
Ultra
Camelot
Gimpel
Comtesse
Pompadur
Fink
Meise
Madonna
Pasadena
Georgie
Eyra
Cerise
Candice
Golf
Marina
Othega
Klaxon
Hockey
Regatta
Messkirchner
SchwarzeStrube
Armigschw
DeutscheSg
Heidesandgerste
KleineNacktg
Edelgerste
KleineNacktg2
DippesHanna
Peragrizm
0.00
0.25
0.50
0.75
1.00
genetic similarity (GSij)

Figure 1. Groupings of 150 spring barley accessions on the basis of genetic similarity (GS_{ij}) by DICE at 23 SSR marker loci.

For example the locus Bmac0316 has a total number of eight alleles over the spring and winter accessions. But while 8 alleles were detected over the spring barley accessions, only 2 alleles were detected in the winter barley accessions.

Grouping of Spring Barley Accessions
Figure 1 shows the grouping of the 150 spring barley accessions based on their SSR data. For illustrating the formed cluster, a tree plot (dendrogram) was chosen. Several distinct groups (clusters) were formed. One accession separates from all accessions. Examination has to be done, whether this accession is not right described by literature or these accessions has a special trait, which separates it from all the other.

Discussion
The molecular data presented here is not sufficient for establishing a core collection for North Rhine-Westphalia. A clustering of distinct groups could be shown. Additional data has to be compared in combination with the dendrogram to explain the clustering and make further sub-assembly groups. The additional data already exists but has to be examined. It is up to show, whether this morphological data goes along with the molecular data attained by the SSR markers.
Phenotypic data will be examined by "step by step" backward elimination. This procedure assures, that only accessions remains, which represent the maximum variance of all accessions.

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Differentiation of UK Barley Varieties Using Microsatellite Markers

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Abstract
The accurate identification of barley cultivars is necessary to assure seed quality, protect intellectual property rights and support plant breeder's rights. Traditional techniques for identification that involve morphological and physiological characters are increasingly being replaced with genotypic methods, with simple sequence repeats (SSRs) being the current marker of choice. More than 600 SSR barley markers are available but a manageable set of quality markers has not been identified that will differentiate large groups of UK barley varieties. This study set out to identify a robust set of SSR primers and establish a database that would differentiate barley varieties on the UK National list. Twenty-nine SSR markers were screened using capillary gel electrophoresis. A set of 7 primers was chosen that amplified 34 alleles and differentiated 116 of the 134 varieties on the National List. Individual seed tests on 8 varieties at 2 SSR loci revealed heterogeneity at a level of 0.67% unexpected alleles. Peaks from unexpected alleles were also detected in bulk samples of identical varieties.
Keywords: barley; microsatellite; SSR; uniformity; variety identification

Introduction

The accurate identification of barley is necessary to address issues of commercial interest. For example, assurance of seed quality, protection of intellectual property rights and the support of plant breeders' rights and patents. Traditionally, variety identification has been determined using a combination of morphological and physiological markers.

Identification methods based on plant phenotype require time consuming assessments of adult plant morphology, microscopic examination of seed and can be influenced by environmental conditions. Physiological markers have been developed for barley differentiation that includes the use of isoenzymes (ANDERSON 1982) and gel electrophoresis of hordein proteins (GEBRE *et al.* 1986; HEISEL *et al.* 1986; NIELSON & JOHANSEN 1986). However, physiological markers are not ideal. Profiles can be difficult to interpret, poor resolution of bands can occur and there exists no one method that can differentiate a large set of barley varieties. These problems have led to the need for more accurate means of identifying barley varieties.

Much work has centered on DNA based methods including the use of Restriction Fragment Length Polymorphisms (RFLP) (BOTSTEIN *et al*. 1980), Random Amplified Polymorphic DNA (RAPD) (WILLIAMS *et al*. 1991), Amplified Fragment Length Polymorphism (AFLP) (QI & LINDHOUT 1997), Simple Sequence Repeats (SSR) (RUSSELL *et a.*, 1997) and Single Nucleotide Polymorphisms (SNPs). An ideal DNA marker method for variety identification would be simple and quick to use, be robust, have low development and running costs, have high inter-variety differences yet have no intra-variety differences (be uniform and stable). Studies comparing methods (POWELL *et al.* 1996; RUSSELL *et al.* 1997;

KRAIC *et al.* 1998) have highlighted the benefits of SSRs. This, coupled with their amenability to high throughput and in the development of large databases (DONINI *et al.* 1998), has led SSRs to be the current marker of choice. Databases for varietal identification have been constructed and assessed for several plant species including wheat (RODER *et al.* 2002) and tomato (BREDEMEIJER *et al.* 2002). The use of SNPs has also much potential but the current cost and time required for the development of SNP genotyping means that there is a present need for a marker system before SNPs are applied.

SSRs have been developed and mapped in barley (SAGHAI-MAROOF *et al.* 1994; BECKER and HEUN, 1995; Liu *et al.*, 1996; STRUSS & PLIESKE 1998; PETERSEN & SEBERG, 1998; RAMSAY *et al.* 2000). However, at present there does not exist a published database of markers that have been tested for reliability, robustness and uniformity using a capillary electrophoresis platform, that will distinguish the varieties on the UK National List.

Material and Methods

Plant Material

Seed of the 134 barley varieties on the UK National List (February 2003) was obtained from the definitive collection held by the Cereals Section of the Scottish Agricultural Science Agency (SASA) and was stored at 4°C. Plant material (leaf, seed and root), for tests on reproducibility of allele scores between plant parts, was obtained from farm grown plots at SASA. Plants were identified as being 'true to type' whilst growing and were stored at -70°C.

DNA Extraction

DNA from the 134 National List varieties was isolated using the Nucleon Phytopure Kit (Tepnel, Manchester). Approximately 3 seeds (0.1g) were ground in liquid nitrogen and the kit protocol followed with minor modifications. Five spring barley varieties (Decanter, Spire, Optic, Cellar and Riviera) were used to determine whether allele size calls were consistently obtained from different plant parts of the same plant. Three samples were taken from each of the 3 plant parts: root, leaf and seed to give a total of 9 DNA extracts from each variety using the GeneScan (Adgen) protocol.

DNA from Individual seeds for tests on intra-variety uniformity was extracted using GeneScan. For tests on uniformity in seed 6 separate bulks of 100 seed samples from the varieties Muscat and Antonia were used. The bulks were ground to a flour using a Moulinex (Birmingham) Super Junior's Coffee Mill. Approximately 15ml of the sample was used for DNA extraction following the GeneScan protocol. To analyse the sensitivity of the method for allele detection, seed from varieties Angela and Prisma were bulked in 8 ratios (Angela: Prisma; 1:99; 3:97; 5:95; 10:90; 20:80; 30:70; 40:60; 50:50). The 100 seed mixtures were ground to a flour and DNA extracted using the GeneScan protocol.

SSR Markers and Protocol

29 microsatellite primers were screened from over 600 available in the public domain (RAMSAY *et al.* 2000). Forward primers were fluorescently labeled at the 5' with one of PET™, FAM™, NED™, VIC™ (Applied Biosystems), to allow for detection in capillary electrophoresis. Reverse primers (MWG) were "PIG-tailed"(Brownstein *et al.*, 1996) to facilitate accurate genotyping. Amplification reactions were carried out in 10µl volumes, each containing 10ng DNA, 1mM dNTP mix, 2.5mM $MgCl_2$ 2pmol forward primer, 50pmol reverse primer, 0.5 units AmpliTaq Gold® (Applied Biosystems), 1 x reaction buffer. Initially, each

primer pair was screened using a temperature gradient PCR reaction using a standard set of PCR conditions: 10 minute hotStart at 94°C; 30 cycles of 30 seconds at 94°C, 30 seconds at 47-70°C, 30 seconds at 72°C; and; a final elongation step of 10 minutes at 72°C. PCR profiles for Primers chosen to screen the whole National List either followed the standard program using the respective optomised temperature or one of 2 'touchdown' programs (Touchdown 1[Bmag0211 and Bmag0120]: 10 minutes at 95°C; 7 cycles of 30 seconds at 94°C, 30 seconds at 65°C - 58°C [1°C reduction each cycle], 30 seconds at 72°C; 30 cycles of 30 seconds at 94°C, 30 seconds at 58°C; 5 minute hold at 72°C. Touchdown 2 [HvM62 and Bmag0093]: 10 minutes at 95°C; 10 cycles of 1 minute at 94°C, 1 minute at 64°C - 55°C [1°C reduction each cycle], 1 minute at 72°C; 30 cycles of 1 minute at 94°C, 1 minute at 55°C, 1 minute at 72°C; 5 minute hold at 72°C). Amplifications were performed in either an MJ Research (Massachusetts, USA) PTC-200 Peltier Thermal Cycler or a GeneAmp®PCR system 9700 (Applied Biosystems, Warrington, UK) thermal cycler.

Fragment Analysis

To 1µl PCR reaction was added 0.25µl GeneScan™ -500 LIZ® size standard and 8.75µl Hi-Di™ Formamide 1 (Applied Biosystems). The samples were denatured by incubation at 95°C for 5 minutes and cooling on ice for 5 minutes prior to loading. Samples were loaded onto the ABI Prism® 3100*Avant* Genetic Analyzer (Applied Biosystems), utilising the 36cm capillary array and POP–4™ Performance Optimised Polymer. Data was collected using Data Collection software (Applied Biosystems) and analysed using GeneScan® Analysis Software version 3.7 (Applied Biosystems).

Data Analysis

Alleles were recorded manually as present (1) or absent (0) for each primer and variety and were entered into a database constructed using Microsoft Excel. Discrimination of the varieties by the primer sets was determined using BioNumerics™ version 3.0 (Applied Maths).

Number of alleles observed for each marker was recorded. Rare alleles were detected and classified as such if their frequency within the data set for that marker was less than 2% (RODER *et al.* 2002). In practice this corresponded to an allele frequency of less than 21.44 in a data set of 268 (134 diploid varieties). PIC values were calculated using Microsoft Excel software using:

$$\text{Gene diversity} = 1 - \sum P^2 ij$$

(Struss and Plieske, 1998)

Where *i* and *j* are the frequencies of the *i* and *j* alleles at that locus. Differentiation of varieties was calculated in Bionumerics™ using the Dice coefficient for binary data that measures similarity based upon common and different alleles. A Dendrogram was produced for the composite marker set using the Unweighted Pair group Method using Arithmetic averages (UPGMA).

Results and Discussion

Differentiation of Varieties Using SSRs

Primers were chosen initially based on 2 criteria; those that had similar annealing temperatures, to facilitate future multiplexing and those with a high PIC-value. To be useful in a system that differentiates barley varieties primers need to be reproducible and reliable. Of the 29 primers

Table I. SSRs chosen to screen UK National List of Barley varieties including number of alleles, PIC-values and degree of heterozygosity displayed

Primer	Allele size range (bp)	Number of alleles ( ) – rare alleles	PIC value	% Heterozygosity ( ) – number of varieties displaying heterozygosity
Bmag0211	185-191	4 (1)	0.69	0.75 (1)
Bmag0120	229-263	8 (4)	0.78	3.73 (5)
HvM62	null,235-265	6 (3)	0.68	1.49 (2)
Bmac0093	157-166	5 (1)	0.72	0.75 (1)
Bmag0009	174-180	4 (0)	0.63	2.24(3)
HvLEU	171-175	2 (0)	0.40	0
HvM36	111-141	5 (2)	0.72	3.0(4)

screened 2 produced no product (Bmag0378; Bmag0384), 6 displayed weak signal (Bmac0134; Bmac0156; Bmac0378; Bmag0223; Bmag0021; HvM03), 3 amplified inconsistently (Bmac0067; Ebmac0701; Bmag0206), 5 were either monomorphic or gave only 1 or 2 deviants when screened on the Scottish Agricultural College (SAC) list, and 2 displayed profiles that were difficult to interpret (Bmag0353; HvCMA). Of the set, 7 proved to be useful and were used to screen the whole National list (Table 1). Of the 134 varieties 14 pairs and 1 group of 4 were undifferentiated using the 7 primer set (Fig.1) Further screening of the unresolved varieties with HvM54 and Bmag0125 (data not shown) leaves 7 barley pairs undifferentiated. 34 alleles were detected over the 7 loci with a null allele being observed in 1 variety at the HvM62 locus, which was verified by repeat DNA extraction and amplifications. The occurrence of null alleles in barley corresponds with previous findings (RUSSELL *et al.* 1997; DONINI *et al.* 1998; WILLIAM *et al.* 1997; KRAIC *et al.* 2002)

PIC values, which included rare and null alleles ranged from 0.32–0.75 with an average of 0.62. Heterozygotes were detected at 6 of the 7 loci and were verified by repeat amplifications. A level of 1.7% heterozygosity over the entire data set was observed with Bmag0120 displaying the largest number, in addition to revealing the greatest number of alleles.

As more than 1 seed was used for DNA extraction the observed heterozygosity could be due to admixture from contamination of the seed batch. However, seed was obtained from the SASA definitive collection and no variety displayed heterozygosity across the range of markers suggesting that such variability is not due to contamination with another variety. Hetereozygosity could therefore be due to fixed heterogeneity within a variety or due to outcrossing that has occurred in the batch used for sampling. Previous study on heterogeneity in wheat varieties, also a self-fertilising plant, revealed a higher level of heterozygosity (RODER *et al.* 2002).

Reproducibility between Plant Parts

Alleles called from different plant parts of the same plant were consistent (data not shown). These results support the findings of WILLIAMS *et al.* (1997) whom analysed leaf and seed SSR profiles using Polyacrylamide Gel Electrophoresis and silver staining. However, profiles obtained from most of the root samples were poor which was attributed to the DNA quality obtained from root extraction.

Intra-Variety Uniformity

Assessment of varietal uniformity at 2 SSR loci in 8 varieties revealed heterogeneity (Table). Of the 748 data points scored there were 5 unexpected alleles observed which equates to 0.67%

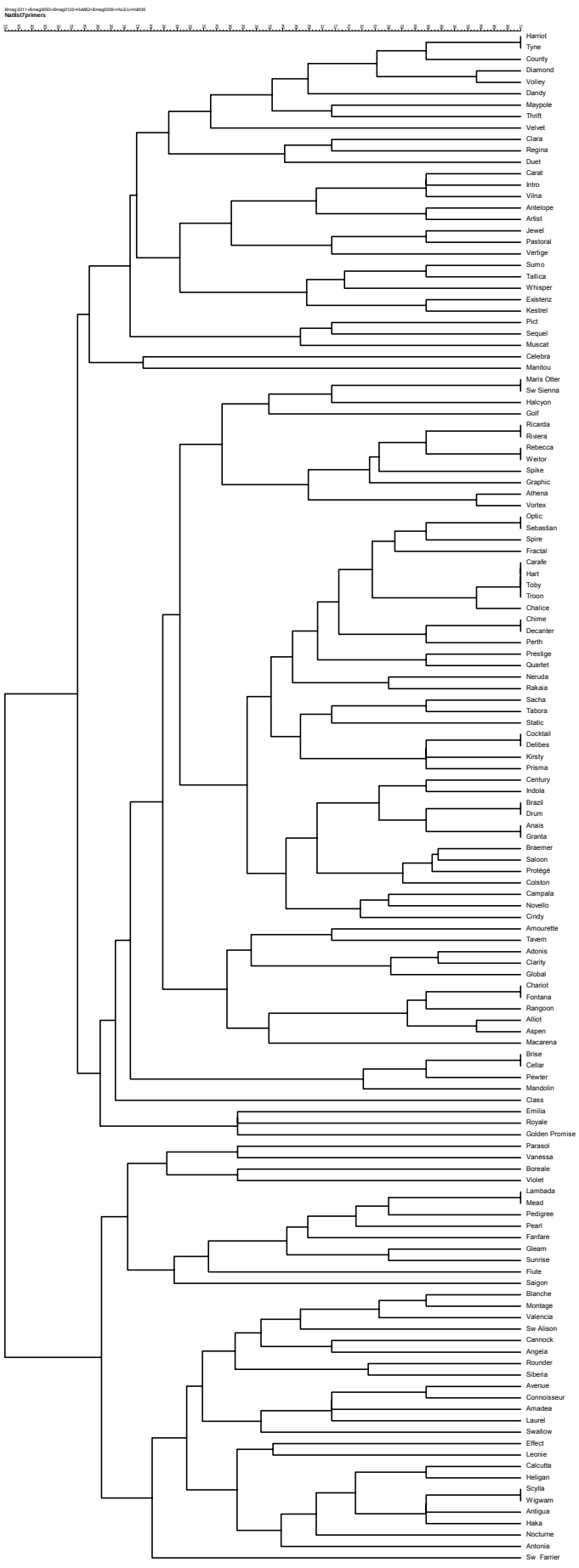

Figure 1. Dendrogram showing differentiation of the 134 barley varieties on the UK |National List using 7 SSR primers

variation. Two individuals from different varieties displayed homozygous heterogeneity, whilst 1 individual of the variety Regina was heterozygous at the Bmag0009 locus. The occurrence of non-uniform homozygous individuals could be due to admixture or outcrossing. Heterozygous non-uniformity, however, suggests outcrossing. It is possible that such heterozygous heterogeneity is fixed within a variety. Interestingly, the variety Regina that displayed heterozygosity in 1 individual at the Bmag0009 locus did not show heterozygosity in the National List screening. This suggests that within varieties homozygosity and heterozygosity at SSR loci exists. Therefore, to establish database accuracy it would be necessary to assess individual seed profiles for varieties that initially display heterozygosity at an SSR locus.

Table II. Uniformity data for 8 barley varieties at 2 microsatellite loci. Non-uniform individuals are italicised

Primer	Antonia	Angela	Muscat	Regina	Optic	Carat	Prisma	Static
Bmag0009	22[a] x180[b] *1x 174*	23 x176	23 x176	23 x174 *1 x 174/180*	22 x174	24 x174	24 x178	23 x174
HvLEU	24 x176	21 x176	22 x172 *1 x176*	24 x172	24 x172	24 x172	24 x172	24 x172

[a] number of individual seed profiles
[b] length of fragment scored (bp)

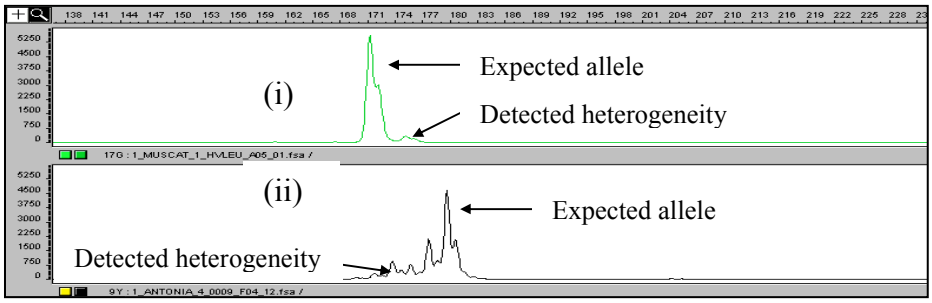

Figure 2. Example of Genescan profiles obtained from bulk seed samples of (i) Muscat using HvLEU primers and (ii) Antonia using Bmag0009 primers. Expected allele peaks for the 2 varieties and unexpected allele peaks, that demonstrate heterogeneity in the seed bulk, are highlighted.

To ascertain whether varieties in the individual seed tests, that displayed heterogeneity, were representitive of the seed batch profiles from bulks of 100 seed for varieties Antonia (Bmag0009 locus) and Muscat (HvLEU locus) were analysed (Fig. 2). Low levels of the unexpected allele that had been identified in the individual seed tests were clearly visible. Thus, verifying the initial observations made in the individual seed tests. Further study would be required using bulk samples obtained from different sources to determine whether such heterogeneity was fixed within that variety.

Summary

This study set out to establish a data set that could reliably differentiate the barley varieties on the UK National List. Primer screening has highlighted the difficulties in establishing a robust and informative set of SSR markers to meet this aim. Heterogeneity has been detected within varieties and will need to be assessed in a larger data set before the construction of the database.

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S 2 – BREEDING METHODOLOGIES I – MAPPING

Differences between South American H Haplome Diploids and I Haplome Diploids, from the Perspective of the 5S rDNA Gene in the Genus *Hordeum*

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Abstract
Twelve South American diploid *Hordeum* species belonging to the H genome and three diploid species belonging to the I genome (including cultivated barley), were investigated for their 5S rDNA sequence diversity. The 374 sequenced clones were assigned to classes, called "unit classes" which were further assigned to haplomes. Two unit classes were found to be present in each haplome. These were labelled to reflect the haplomes, *viz*. the long H1 and short I1 unit classes for the I haplome diploids, and the long H2 and long Y2 unit classes for the South American **H** genome diploids. The aligned sequences were subjected to a series of Maximum Likelihood analyses and various tests, including molecular clock, which are presented and discussed. The divergence among the unit classes suggest that the genus *Hordeum* might be of paleopolyploid origin.
Keywords: 5S DNA gene; molecular clock; paleopolyploid

Introduction
5S rRNA genes in the Triticeae are organized into tandem repeats with the highly conserved genes separated by the more variable, non-transcribed spacer region (henceforth NTS). In several publications (e.g., BAUM & BAILEY 1997; 2000; 2001; BAUM & JOHNSON 1994; 1996; 1998; 1999; 2000; 2002; BAUM *et al*. 2001; 2003), we have described the molecular diversity of 5S rDNA sequences in species within *Elymus*, *Hordeum*, *Kengyilia* and *Triticum*. We were able to classify them into putative orthologous groups, which we call unit classes, and moreover to assign the different unit classes to haplomes. Our objectives were: 1) to examine the diversity of the 5S rRNA gene sequences among the South American diploid species belonging to the **H**-genome group (BOTHMER & JACOBSEN 1991); 2) to classify them into unit classes; and 3) to compare the unit classes of these South American diploids with the unit classes previously found in the **I**-genome group of which cultivated barley (*H. vulgare* L.) is a member. The present study describes the results of these analyses with respect to the evolution and time of divergence of these genes.

Material and Methods.
Plant Material and 5S rDNA Cloning
Material from the following 11 taxa, represented by 38 accessions of seeds collected in their natural habitats in Argentina and Chile, was investigated: *H. chilense* Roem. & Schult., *H. comosum* Presl, *H. cordobense* Bothmer, N. Jacobsen & Nicora, *H. erectifolium* Bothmer, N. Jacobsen & Jørgensen, *H. muticum* Presl, *H. patagonicum* (Hauman) Covas ssp. *magellanicum* (Parodi & Nicora) Bothmer, Giles & N. Jacobsen, *H. patagonicum* ssp. *mustersii* (Nicora) Bothmer, Giles & N. Jacobsen, *H. patagonicum* ssp. *patagonicum*, *H. patagonicum* ssp. *santacrusense* (Parodi & Nicora) Bothmer, Giles & N. Jacobsen, *H. patagonicum* ssp. *setifolium* (Parodi & Nicora) Bothmer, Giles & N. Jacobsen, *H. pubiflorum*

Hook. f., and *H. stenostachys* Godr. The five subspecies according to the taxonomy of BOTHMER *et al*. (1991) are also recognized as species especially by South American authors. Several sequences from species containing the **I** haplome published earlier (BAUM & JOHNSON 1994, 1996) were retrieved from GenBank® and used for comparisons. The isolation of genomic DNA, PCR amplification of the 5S rRNA genes, cloning of PCR products and sequencing of plasmid DNA have been described, e.g. BAUM and JOHNSON (1994, 1996, 1998, 1999, 2000, 2002). The PCR primers used target the coding regions in tandem repeats and amplify a sequence starting from 5' from the BamH1 site within the transcribed region, through the NTS, to a site 3' of the BamH1 site within the adjacent unit in the array. Amplimers were either digested with BamH1, cloned into the BamH1 site of pUC19 (YANISCH-PERRON *et al*. 1985), and transformed into *Escherichia coli* strain DH5α or latterly ligated directed into pGEM-T (Promerga Biotech) and transformed into DH5α. A total of 374 clones were isolated and for each clone, both strands were sequenced.

Sequence Analysis

Sequences were routinely checked to ensure removal of vector sequences using the VecScreen program at NCBI (National Center for Biotechnology Information, USA). For each sequence a search for direct and inverted repeats was carried out using DNAMAN (Lynnon Biosoft®). The sequences were then submitted to NCBI and an accession number for each was obtained.

The 374 sequences were subsequently aligned using CLUSTALW (THOMPSON *et al*. 1994). The alignment was further improved by visual examination and editing using GeneDoc© Version 2.6.002 (NICHOLAS & NICHOLAS 1997). GeneDoc© was used to assign similar sequences to sequence groups, i.e., unit classes, based on the refined alignments for each putative orthologous group. At this stage, the alignment would reveal any sequences that would appear to have been assigned to the wrong unit class. Several sequences representative of each unit class were then subjected to similarity searches of the GenBank® and EMBL (European Molecular Biology Laboratory) databases using the NCBI Web-based BLAST service (ALTSCHUL *et al*. 1990) to identify the unit class with an already established, i.e. published unit class. The sequence identified as being the most similar, i.e., having the highest scoring segment pairs and the lowest P(N) value (as defined in ALTSCHUL *et al*. 1990), was subsequently aligned *in toto* with the representative unit class, and also with other sequences of interest among previously defined unit class sequences mentioned above. All the 374 sequences were then re-assembled for a final step of alignment and manual refinement. This process and the method of unit class determination and recognition has been discussed in more detail in BAUM *et al*. (2001).

The data were subjected to the program WinModeltest© 4.b (POSADA & CRANDALL 1998) to test the fit of various maximum likelihood (ML) models and to choose the model that best fits the data using the hierarchical likelihood ratio test. To conduct the following ML analyses, a reduced set of sequences, representative of each unit class within each taxon, was used after first subjecting them to WinModeltest in order to verify that the model with the best fit for the reduced data set was identical to the model derived for the total data set. Phylogenetic analyses were then conducted with the following ML methods and bootstrapping with neighbour-joining (NJ) search: ML clock not enforced, ML clock enforced, using both PAUP*© (SWOFFORD 1998) and PHYLIP© (FELSENSTEIN 1993), tree calibration by nonparametric rate smoothing (NPRS) and NJ using TREEFINDER© (JOBB 2003). The time calibration in NPRS was based on the estimated time of divergence between barley and wheat of 13 million years (MY) (GAUT 2002). All the trees were imported to PAUP* and subjected to the KH (KISHINO & HASGAWA 1989) and the SH

(SHIMODAIRA & HASEGAWA 1999) tests for the best tree. The latter was then subjected to tree analysis under the maximum parsimony and minimum evolution criteria using PAUP*.

Results and Discussion

All of the South American diploid taxa in this study were found to possess the same two 5S rDNA unit classes, i.e., we found 159 sequences that were assigned to the long H2 and 215 sequences to the long Y2. This is consistent with our previous findings in *H. cordobense* (BAUM & JOHNSON 2002) and *H. muticum* (BAUM & JOHNSON 2003).

An analysis of these sequences was carried out with the addition of exemplars of two unit classes from **I** genome species, i.e., long H1 and short I1. Modeltest selected the following settings as best, namely the HKY+G model with transition/transversion ratio = 1.0799, the assumed nucleotide frequencies of A=0.269, C=0.1743, G=0.2492 and T=0.3075, the shape parameter = 2.9192 and the rates=gamma.

These analyses yielded eight trees - one ML tree, one molecular clock tree (PAUP and PHYLIP), one NPRS tree and four NJ trees (TREEFINDER), and one NJ tree from PAUP.

The KH and SH tests selected the molecular clock tree as best. However, all the eight trees have identical topologies in the sense that the major branches are identical and the four unit classes (Fig. 1) are clearly identified and similar. The differences relate to branch size and specific details that depend upon the optimality criteria used in the tree analyses. Some specifics apply to a particular optimality criterion, e.g. with the parsimony criterion branch lengths are expressed in number of nucleotide step changes that clearly do not apply to the other criteria. The Phylogram (Fig. 1) combines the results obtained from all the three criteria (parsimony, likelihood and distance) superimposed on the (distance) minimum evolution tree.

The estimated time of divergence of 13 MY between *Hordeum* and *Triticum* (GAUT 2002), likely based on comparisons of cultivated species (*H. vulgare* and *Triticum aestivum* L.), is close to the previous estimate of WOLFE *et al*. (1989) based on chloroplast DNA variation. But the genus *Hordeum*, with 31 species (BOTHMER *et al*. (1991), or more depending on the taxonomy, is probably much older judged from its distribution with a center of diversity in South America, one species in South Africa, a center in the Near East and a diffuse distribution across Eurasia. Although phylogenetic estimates of organisms form the basis for genomic and evolutionary studies, in this paper we have examined genome evolution through the 5S rDNA. Clearly different unit classes might have arisen at different times (Fig. 1). What is puzzling is that the long H1 and short I1 unit class, both present in the **I**-genome species and characteristic to them, are the most divergent ones. Furthermore, the South American diploid species belonging to the **H**-genome species group (BOTHMER *et al*. 1991) contain both the long H2 and long Y2 unit classes which are nearly as divergent as the former two unit classes. Does this indicate that *Hordeum* as we know it today is a diploidized paleopolyploid? (Paleopolyploids have a disomic inheritance and their progenitors cannot be detected by cytology or DNA markers). Sequence analysis of full genomes in the classical diploids *Arabidopsis thaliana* and *Oryza sativa*, has revealed that they are apparently paleopolyploids. Alternatively, could two different unit classes have evolved so rapidly in each group during roughly six MY? Both case scenarios point to an earlier origin of the genus *Hordeum*. In this study the 5S rDNA has shown itself to be a valuable tool for the detection of paleopolyploidy status, at least in the Triticeae. The non-transcribed spacer varies considerably more than the coding region of the 5S DNA which is consistent with the greater than 10-fold synonymous substitution rate variation among homeologous loci in *Arabidopsis* (ZHANG *et al*. 2002). In addition it appears that paralogs do not evolve at different rates and that the unit classes appear to have evolved by the molecular clock fashion. Based upon these conclusions, it can be postulated that the 5S rDNA units featuring two different unit classes

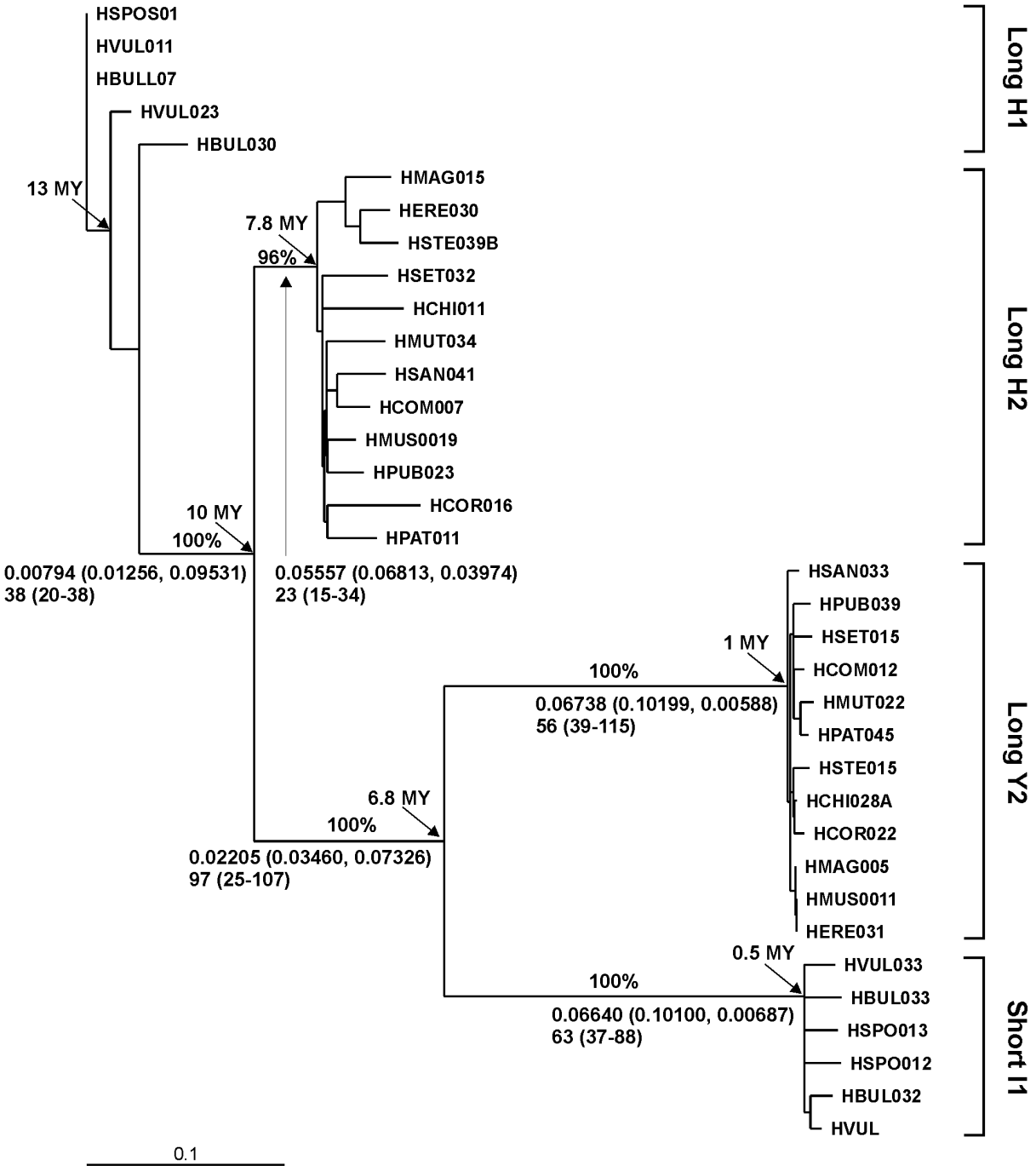

Figure 1. Phylogram of 5S rDNA sequences representative of the four unit classes, based on minimum evolution tree. The Phylogram combines information of the best maximum likelihood tree, the molecular clock, parsimony and NPRS trees. MY: Million Years since divergence; above major brances: bootstrap support (%); below major branches: branches lengths (distance from root, distance from tip) and further below: assigned branch length under the parsimony criterion (Min. possible length – Max. possible length); scale bar: distance from minimum evolution distances; on the right the four unit classes.

were present in two unidentified diploid progenitors. In the past, they contributed to the formation of a paleopolyploid through horizontal transfer of chromosomal segments or genes (LEVY & FELDMAN 2002) giving rise to the present day diploid species.

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Use of the 'Functional Map' to Identify QTLs and Explore the Genetics of Biometric Agronomic Traits in the Oregon Wolfe Barleys

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Abstract

The Oregon Wolfe Barleys (OWBs) are a well-characterised, phenotypically polymorphic mapping population that has been used for a whole range of mapping and QTL studies. The population was grown in the greenhouse twice in 1999/2000 and 2003, and biometric (organ lengths) and phenological (flowering time) traits were established in a systematic fashion. Additionally, the mapping population was used to prepare a 'functional map' by employing ESTs in different marker assays like RFLPs, SSRs and SNPs. The 'functional map' (or 'transcript map') based on the OWB population contains >500 genes. This map has been used for QTL analysis for the above traits. The combination of the functional map with information on biometric morphological traits promises to give new insights into the nature and quantitative inheritance of these traits and is hoped to provide a decisive progress in the field of expression mapping.

Introduction

Genetic studies in plants have revealed that a selection of traits is inherited by major genes. A qualitative inheritance has been described for several genes determining plant morphology or phenology. Major genes for dwarfness are located on all seven barley chromosomes. Another group of genes determining flowering time via vernalisation or photoperiod response are located on six out of the seven linkage groups (FRANCKOWIAK 1997; LUNDQVIST *et al.* 1997).

Beside the major genes many loci inherited quantitatively are present in the genome. They are, however, difficult to detect. With the development of various DNA marker based linkage maps and quantitative trait mapping methods the discovery of such quantitative trait loci (QTLs) became possible in many plant species, including cereals. Starting in the early Nineties of the last century (e.g. HAYES *et al.* 1993; BACKES *et al.* 1995) QTLs for many characters were detected and put in relation to RFLP, AFLP or SSR markers of 'unknown function'. Recently, EST based markers have been developed and successfully mapped (KOTA *et al.* 2001; THIEL *et al.* 2003; VARSHNEY *et al.* unpubl. data). These 'functional maps' are useful tools for mapping any trait of interest. In the present paper we used the 'Oregon Wolfe Barley' (OWB) mapping population, saturated with EST based markers to study a range of morphological (plant organ and grain characters) and phenological (flowering time) traits.

Material and Methods

Preparation of Functional Map of Barley

In context of the barley genomics programme so far more than 110,981 ESTs from 22

different cDNA libraries have been generated (MICHALEK *et al.* 2001; http://pgrc.ipk-gatersleben.de/b-est/). Cluster analysis yielded a tentative unigene set comprising 25,224 genes. To facilitate the integration of EST data with mapped traits, the non-redundant set of ESTs was exploited for construction of a high density 'transcript map' by using 3 different mapping populations, i.e. OWB_{Dom} x OWB_{Rec}, Steptoe x Morex and Igri x Franka. In the OWB_{Dom} x OWB_{Rec} mapping population, 293 ESTs were mapped as RFLP probes while 157 ESTs were mapped as SNP markers (KOTA *et al.* 2001) and 144 ESTs as SSR markers (Thiel et al. 2003; Varshney et al. unpubl. data). In addition to these ESTs, the OWB map has another set of 137 markers including 16 morphological markers. Thus the OWB map consisting of a total of 731 markers (including a total of 594 ESTs/genes) was used for QTL analysis.

Plant Culture

Ninety-three lines of the Oregon Wolfe Barley (OWB) mapping population plus parents (REC and DOM) were grown in a greenhouse at IPK, Gatersleben, Germany (51N82, 11E28) from October 20, 1999 to April 2000 (experiment 1), and from February to June 2003 (experiment 2) under normal light and temperature conditions, with automatic shading applied to avoid temperature extremes. Watering as well as application of fertilizers and herbicides followed common horticultural practise. Details for experiment 1 are described in BÖRNER *et al.* (2002). In experiment 2, sowing was carried out on February 13, 2003, at a density of one seed per pot, into 14 cm plastic pots using as substrate a standard soil mixture previously established for cereals. Seven replications of each genotype were set up in a total of three equally sized and lit chambers of the same greenhouse (i.e. three replicates per chamber, plus an additional replicate as a backup in a separate chamber, as not all seeds germinated). Pots in experiment 2 were arranged according to genotype number, with ascending or descending order alternating according to the replication. However, the distribution of the replicates over the three chambers had no influence on the data set obtained (as concluded from a One-Way Analysis of Variance; data not shown) so that the replicated data obtained for one genotype were pooled subsequently.

Measurements and Statistical Analysis

Phenological and morphometrical data were obtained on living plants during development using a ruler (accuracy 1 mm). If not mentioned otherwise, only the main shoot was considered. Parameters obtained during development were the final lengths of blades of ranks 1 to 3, as well as the percentage of germinated seedlings and the time of anthesis (i.e. flowering, here defined as the time of dehiscence of anthers, which usually occurs while the ear is still in the sheath of the flag leaf and which was regulary checked using a forceps and magnification lens, s.a. PRYSTUPA *et al.* (2003)). All plants were harvested at full maturity (DC 90-92). Due to considerable variation in time to maturity, the harvest period stretched over several weeks though the bulk was harvested during the first week of June, 2003. The ears were separated from the culm at the height of the collar and the remainder of the plant was removed from the pot, the soil removed and roots clipped to a minimum length. Plants as well as ears were stored separately in paper bags in a dry store until further use. Up to 16 viable kernels of one ear per genotype were scanned using a HP flatbed scanner (HP scanjet 5400c) and the resulting bmp-format images analysed using QWin software (Leica, Wetzlar, Germany) and the own software Korn (Brandenburg Technical University, Cottbus, Germany). Parameters thus automatically obtained were the mean values of kernel length, width, circumference, area and roundness (i.e. deviation of shape from perfect roundness, which latter is one). The main shoot was detached and the following measurements carried out using a ruler: length of the flag leaf blade and the lengths of the first three internodes

measured in experiment 2. Raw data were stored in MS Excel worksheets and mean values calculated for all measured parameters therein. For the purpose of QTL analysis, the mean values were transferred to the software QGene v. 2.30 (NELSON 1997) where QTL analysis was carried out as a simple regression analysis. The two main significantly independent markers were identified (Table 1). If more than these two markers were found for a trait, these are indicated as QTLs in the map (Figure 1).

Results and Discussion

Table 1 summarizes the results of main QTLs for the 14 traits considered, whereas Figure 1 indicates the position of all QTLs on the chromosomes. Overall, 68 QTLs (of which 44 with LOD scores $\geq$ 3) were detected, which were associated with 49 markers. The distribution of the involved markers over the genome was not homogenous but concentrated mainly on chromosomes 2H (24 QTLs, 14 markers) and 7H (18 QTLs, 12 markers), with the other five chromosomes harbouring 4 (6H), 5 (4H, 5H), and 6 (1H, 3H) markers correlated with measured traits. Most markers (40) contributed to only a single QTL each, 7 markers to 2 QTLs, 2 to 3 QTLs, and one each to 4 and 6 QTLs. Among the latter, the morphological marker *Zeo* (*Zeocriton*), a biallelic dominant Mendelian gene inducing an overall dwarfing phenotype (culm length reduction, dense ears), was the marker involved in most QTLs, followed by two further morphological markers, *Vrs1* (number of kernel rows, i.e. fertility of lateral rows) and *Nud* (hulled or nude grain). The average number of QTLs per trait was 2.14 (range: 2 to 3) in experiment 1, and 2.86 (range: 1 to 7) in experiment 2, with seven QTLs found for flowering.

Internode lengths (Fig. 1a) were – as was expected – strongly associated with *Zeo*. This effect was most pronounced in the peduncle (= internode 1) with a LOD score of 24.28 for *Zeo*, and decreased in internodes 2 and 3. Long peduncles (mean 26.86 cm, Table 1) were characteristic for the absence of *Zeo*, thus it is a recessive trait (coming from the parent REC). As mentioned above the three internode traits were measured only once (in exp. 2), however, comparison with internode length data of the same population (Patrick HAYES, pers. comm.) showed that similar markers were involved in both cases, thus confirming our findings.

For the length of the flag leaf blade (Fig. 1b) three respective four QTLs were found, surprisingly with completely different markers and on different chromosomes for the two experiments (exp. 1: chr. 7H, 5H, 4H; exp. 2: chr. 3H, 1H, 6H). This could not be explained, since the flag leaf length in a winter barley population grown under four different conditions exhibited a very uniform QTL pattern (BUCK-SORLIN, unpubl. data). The results for the first three leaves exhibited a similar pattern as the flag leaves: In experiment 1, the main QTL for leaf 1 was found on 6H, whilst in experiment 2, one significant QTL was found on 2H and none on 6H. For blade length of leaf 2, no significant QTL (LOD $\geq$ 3) could be found in either experiment. In leaf 3, two QTLs (of which one significant) were found on 4H and 7H. Overall, leaf blade lengths did not exhibit a reproducible pattern of QTLs. It remains the subject of further investigation whether this result means that the trait blade length is not genetically inherited in the OWB population. The first three or four leaves on the main stem are usually preformed – along with the coleoptile – in the embryo (KIRBY & APPLEYARD 1981) and their lengths should thus be predetermined at some stage of embryo development while the grain is still attached to the parental ear.

QTLs for the grain-associated traits were mainly clustered around the morphological markers *Nud* and *Vrs1* or with molecular markers in their vicinity on chromosomes 7H (near *Nud*) or 2H (near *Vrs1*) (Table 1, Fig. 1 c). Grain area yielded two QTLs each in both experiments which co-located on *Vrs1* and *Nud*. A similar situation held for grain width, with reproducible QTLs at *Vrs1*, plus one further QTL on 1H in experiment 1 and 2 QTLs on 1H and 2H (near *Zeo*) in experiment 2. Four QTLs (of which 3 significant) were found for grain length, with

Table 1. Summary of main QTLs identified for measured traits in two experiments

Trait	Experiment	Marker	Chr.	LOD	F	Parent	Mean AA	Mean aa
Length of 1st internode	**2**	*Zeo*	2H	15.63	106.82	REC	14.89	26.86
		GBM1405	3H	1.76	8.32	DOM	25.20	20.44
Length of 2nd internode	**2**	*Zeo*	2H	7.59	41.59	REC	13.96	16.86
		KFP203	2H	4.71	23.93	REC	14.21	16.65
Length of 3rd internode	**2**	*Zeo*	2H	15.39	104.44	REC	9.12	12.30
		GBS0092	2H	7.59	41.59	REC	9.61	12.04
Length of flag leaf blade	**1**	GBM1030	7H	4.80	24.45	REC	24.10	28.70
		GBM1054	5H	3.47	17.08	REC	24.15	28.10
	2	GBR016b	3H	5.77	30.56	REC	7.62	9.65
		GBM1204	1H	3.84	19.12	REC	7.48	9.06
Grain area	**1**	*Vrs1*	2H	9.34	53.51	DOM	114.07	91.60
		Nud	7H	3.59	17.73	DOM	108.48	93.58
	2	*Vrs1*	2H	6.87	37.32	DOM	21.45	17.32
		GBR1478	7H	6.85	36.73	DOM	21.26	16.98
Grain length	**1**	*Nud*	7H	8.41	47.21	DOM	9.21	8.26
		GBR095	2H	2.95	14.37	DOM	9.04	8.42
	2	GBR1478	7H	16.07	114.6	DOM	9.32	7.87
		GBS0081	4H	3.61	17.82	DOM	8.97	8.19
Grain width	**1**	*Vrs1*	2H	13.14	83.43	DOM	3.51	3.00
		GBS0125	1H	3.61	17.82	REC	3.06	3.36
	2	*Vrs1*	2H	9.92	57,72	DOM	3.42	2.96
		Zeo	2H	6.76	36.24	DOM	3.40	2.99
Grain circumference	**1**	*Nud*	7H	9.33	53.77	DOM	23.31	20.53
		GBS0705	2H	3.26	15.94	DOM	22.88	21.13
	2	GBR1478	7H	14.51	98.69	DOM	22.28	18.91
		GBR1012	7H	8.72	49.86	DOM	21.96	19.19
Grain roundness	**1**	GBS0378	7H	4.85	24.73	DOM	1.91	1.69
		Bmac399	1H	3.24	15.86	DOM	1.95	1.75
	2	GBS0250	7H	11.51	69.9	DOM	1.78	1.59
		GBR1012	7H	8.56	48.72	DOM	1.77	1.60
Germination	**1**	GBM1498	2H	3.60	17.78	REC	78.18	92.07
		GBS0265	3H	2.39	11.43	REC	81.36	92.24
	2	*Zeo*	2H	3.80	18.85	REC	86.11	97.96
		GBR1319	5H	1.73	8.15	DOM	97.56	89.28
Length of 1st leaf	**1**	GBR504	6H	3.11	15.22	DOM	21.49	19.22
		GBR586	2H	2.21	10.56	REC	19.46	21.38
	2	GBM1498	2H	4.29	21.58	REC	10.33	11.50
Length of 2nd leaf	**1**	MWG652b	4H	2.05	9.73	DOM	19.33	17.26
		Bmac047a	6H	1.80	8.50	DOM	19.11	16.98
	2	GBM1385	2H	2.6	12.56	DOM	15.07	16.66
Length of 3rd leaf	**1**	KFP221	4H	4.21	21.11	DOM	25.77	23.53
		GBR1610	7H	2.28	10.92	REC	23.78	25.55
	2	GBM1218	2H	4.30	21.72	DOM	21.37	19.26
		GBM1385	2H	3.08	15.05	REC	19.24	21.12
Flowering (LD)	**1**	GBM1204	1H	6.87	37.17	REC	52.83	66.19
		GBR283	7H	4.38	22.21	REC	54.11	65.28
	2	GBM1030	7H	5.08	26.09	REC	63.81	70.94
		GBS0379	2H	4.63	23.45	DOM	71.43	64.51

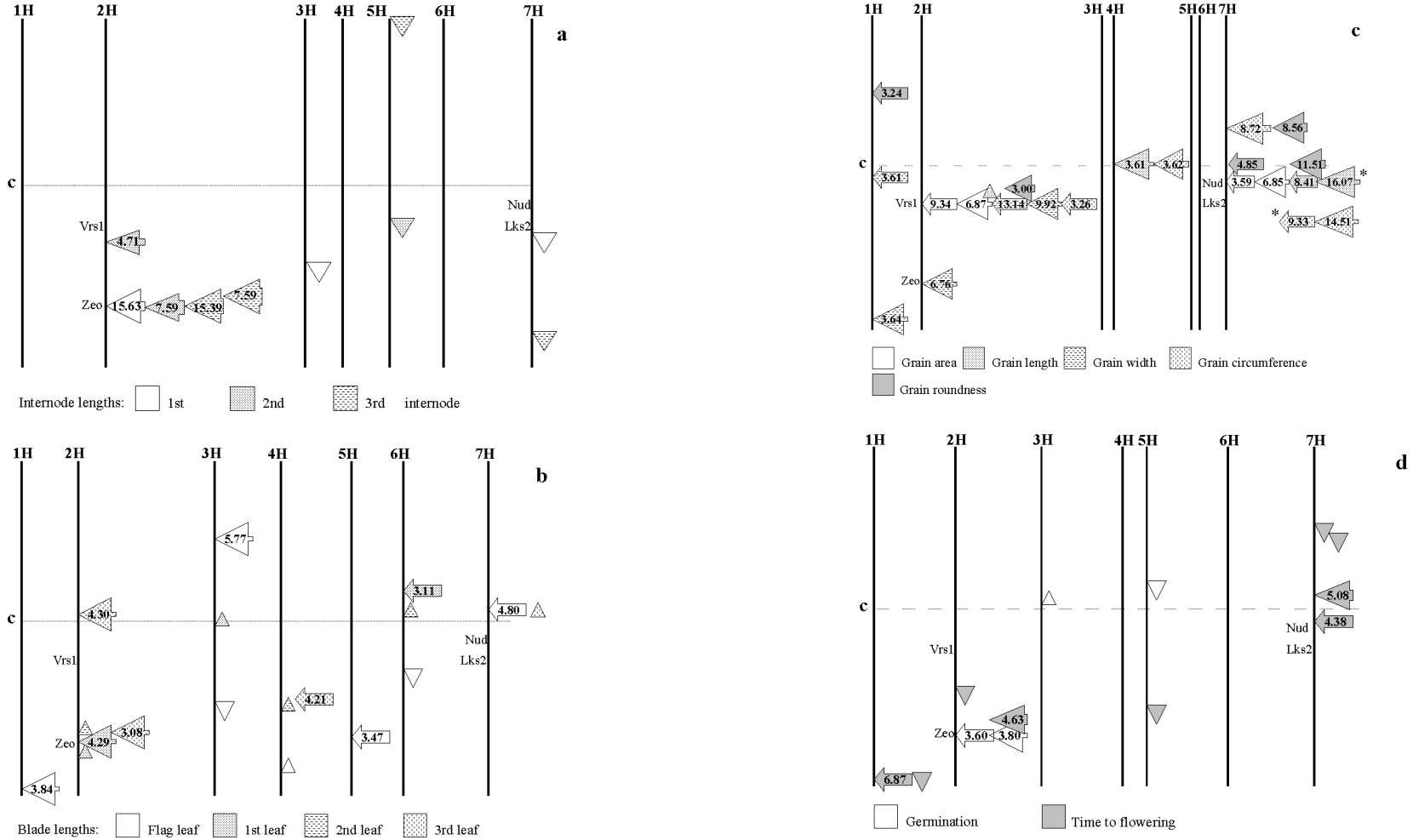

Figure 1 a-d. QTLs found for 14 different traits. Significant (LOD > 3) QTLs are represented by arrows, subsignificant (LOD 1.5 – 3) by triangles. Data from experiment 1 is designated by thin-headed arrows and up-pointing triangles, from experiment 2, by thick-headed arrows and downward-pointing triangles. Numbers inside arrows indicate LOD scores. Different shadings apply to the different parameters (see legend on each figure)

one interesting one near the centromere of 4H, co-locating with a QTL for the derived trait grain circumference. The latter trait exhibited – as expected - a very similar QTL pattern to that of grain length and width, with an interesting exception, a highly significant QTL on 7HS, co-locating for a QTL for the trait grain roundness. This trait, a classical shape factor, apart from being influenced again by grain size and, accordingly, *Vrs1* and *Nud*, yielded in experiment 1 an additional QTL on 1HS, which deserves further attention. Overall, reproducibility of QTLs in the grain-associated traits was moderate to good yet might have been masked by the strong effects exerted by *Vrs1* and *Nud*, i.e. in a mapping population lacking variability of these traits, QTLs might crop up at different locations on the genome.
The trait germination (measured as percentage of established seedlings) yielded two significant QTLs, near *Nud* in experiment 1, and near *Zeo* in experiment 2, with one subsignificant QTL on 3H (exp. 1) and 5H (exp. 2, Fig. 1d). The QTLs obtained were thus not reproducible and indicate in this case that environmental factors at the time of germination might exert a more significant effect than heredity.
Time to flowering yielded two significant QTLs in experiment 1 (on 1HL and 7HL, near *Nud*), and seven QTLs (of which 2 significant) in experiment 2, which partly co-locate with those from experiment 1, indicating moderate reproducibility of these QTLs and thus quantitative genetic control of this trait.
Use of the functional map for QTL analysis is useful as the putative gene/enzyme involved in a particular trait can be identified by deducing the function of the EST (corresponding to an identified marker). For instance, the marker GBM1498 is associated with a significant QTL (LOD 3.6) for germination and has been derived from the EST encoding for 'dehydration responsive element binding protein', generated from a seed germination cDNA library (embryo plus scutellum, 0-16 hrs after imbibition).

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A Consensus Molecular Genetic Map of Barley

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Abstract
An extensive collection of barley maps has allowed us to compile a consensus map of barley. The consensus map was formed using four barley maps produced in our laboratory as well as a number of other Australian and international published genetic maps and includes over 2000 markers. CMap software was used to view the consensus map and validate marker order by comparison of the consensus map to the individual maps that contributed to the consensus map. The options in CMap such as the matrix were found useful for quickly assessing the occurrence of duplicate loci. The alignment of different genetic maps was generally unambiguous with respect to the order of loci and examples of alignments will be presented together with an estimate of the error inherent in producing the consensus map. QTLs were also inserted into map in a searchable format within the software and this has enhanced the value of the map considerably.
Keywords: consensus map; CMap; molecular markers

Introduction

Consensus maps are essential tools for the selection of markers for a corresponding QTL region for marker assisted selection in a breeding program. These maps are build based on the common markers between two or more populations. If a marker is mapped to a defined region of a chromosome in two populations then the markers surrounding the common markers in both maps are put together in one linkage group conserving their orders in respective maps.

Several consensus maps were constructed for barley (LANDRIDGE *et al.* 1995; QI *et al.* 1996; KARAKOUSIS *et al.* 2003a). These maps serve good for the research community to identify/select markers for further mapping activities. The power of the consensus map in this study is that it allows users to do 'live' search at the CMap platform. CMap allows user to navigate around different maps and combination of maps of choice.

Material and Methods

The availability of saturated barley genetic maps constructed predominantly with RFLP and SSR markers provided the basis for the comparison and construction of consensus maps (LANDRIDGE *et al.* 1995; QI *et al.* 1996; KARAKOUSIS *et al.* 2003a). Mostly JOINMAP software was used in the construction of these maps. This software essentially uses the common markers, regularly distributed along barley chromosomes, across several populations to generate new linkage groups.

The consensus map in the study was constructed "by hand" with the method developed by APPELS (2003). For each chromosome a map with the most common markers (bridging markers) with other maps was used as the starting point. In most cases, RFLPs and SSRs were used as bridging markers. Lina x H. *Spontaneum* Canada Park (RAMSAY *at el.* 2000) and Igri x Franka (GRANER *et al.* 1991) maps were the most utilised bridging maps since they included majority of the SSR and RFLP markers, respectively, used across the maps studied (Table 1). Most distantly located bridging markers between the pairs of maps for each chromosome were identified and then the genetic distance between these markers was normalised in a new map to match the respective distance found in the reference map. Bridging markers within a chromosome region analysed provided an estimate of the error in generating the consensus map. For example, a particular marker may be at position 15 cM (normalised) in the new map and at 21 cM in the reference map and this difference in the position was not considered to be significant. If the difference exceeded 20 cM, the marker in new map was given a new designation (a or b etc.) to indicate that marker may be duplicated. These decision points were derived from several iterations of examining the maps available and were intended to minimise the loss of information from the consensus map. The process has essentially utilised floating bins of 20 cM units to develop the final map.

Table 1. List of the crosses that were used for the barley maps and references

Name of Cross	Reference
Alexis x Sloop	Barr *et al.* (2003a)
Amagi Nijo x W12585	Pallotta *et al.* (2003)
Chebec x Harrington	Barr *et al.* (2003b)
Clipper x Sahara	Karakousis *et al.* (2003b)
Dicktoo x Morex	Hayes *et al.* (1993)
Galleon x Haruna Nijo	Karakousis *et al.* (2003c)
Igra x Franka	Graner *et al.* (1991)
Patty x Tallon	Cakir *et al.* (unpublished)
Lina x H.s. Canada Park	Ramsay *et al.* (2000)
Steptoe x Morex	Kleinhofs *et al.* (1993)
Tallon x Kaputar	Cakir *et al.* (2003)
VB9104 x Dash	Cakir *et al.* (unpublished)
Baudin x AC Metcalf	Cakir *et al.* (unpublished)

Results and Discussion

Thirteen published and unpublished maps with a total of more that 2000 markers were used to construct a comprehensive consensus map of barley. One of the main challenges faced during the construction process was the inconsistent use of marker names across different maps. Especially the names of RFLP and SSR markers that were used as bridging markers were carefully edited. For example names such as Bmag010 was changed to Bmag10. CMap software was also modified to tackle the problem due to upper or lower case letters used in the names.

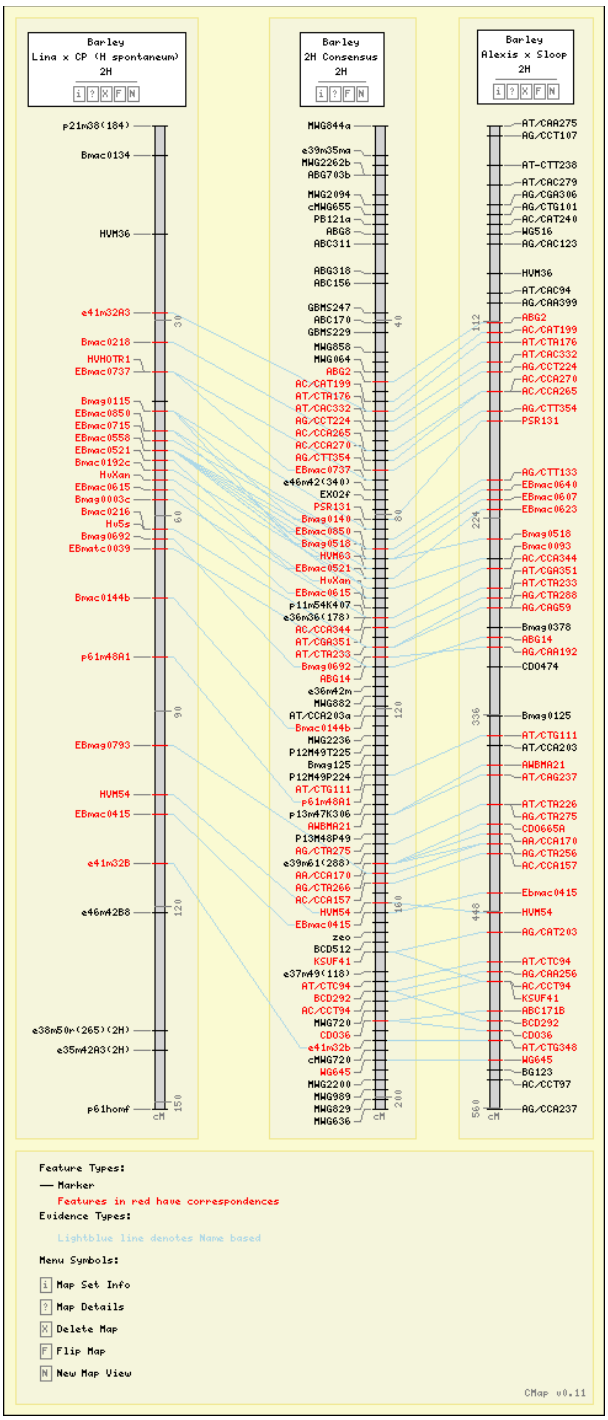

Figure 1. Alignment of 2H consensus map with the 2H chromosomes of Lina x *H. spontaneum* Canada Park and Alexis x Sloop maps

The alignment of consensus map back to the original maps indicated a high level of consistency in the marker order in most cases (Fig 1). The more bridging markers individual maps had the greater accuracy in the order of bridging markers in the consensus map was achieved. This was generally within 5-10 cM.

For illustration purpose we have used the chromosome 2H. The consensus map for this chromosome included 401 markers (Fig 1). Due to space limitation CMap only shows 78 of those markers in Fig 1. Consensus map in this study indicated a good alignment with the most recently published barley consensus map derived from 5 individual Australian barley maps (KARAKOUSIS *et al.* 2003).

QTLs, where available, were also recorded for each map, and included in the consensus map as a reference source. Since CMap is a very interactive software it allows researchers to search for the location of the QTLs for certain traits then choose a variety of markers from the QTL region to be used in barley breeding programs.

The consensus map constructed in this study serves the purpose of summarising extensive datasets in the area of molecular genetic mapping as well as in the application of molecular markers in plant breeding programs.

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Molecular Marker Validation and Physiological Determinants of QTL Effect on Grain Protein Concentration of Two-Rowed Barley

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Abstract

In previous studies, QTLs that influenced variations in barley grain protein concentration (GPC) were identified on chromosome 5H and 7H. The objectives of the present studies were verify QTL effects through realised heritability and to observe whether low GPC in these lines was associated with (1) differences in tiller capacity and biomass accumulation, or (2) differences in nitrogen uptake, translocation and deposition in the grains.

In the first study, QTL effects were confirmed using three doubled haploid populations with different combinations of low and high GPC parental lines. In the second study, doubled haploid lines with allelic variation at these QTL were grown at Horsham, Victoria, in 2001 and 2002, at 0 and 80 Kg ha^{-1} of nitrogen application, and assessed for differences in the contribution made by pre- and post-anthesis assimilation to GPC. For most traits, there were no significant differences among lines with different allelic combinations at the genetic loci. However, highly significant ($P \leq 0.01$) differences were observed in traits associated with spike morphology (spike weight, spike length and kernels per spike). At anthesis, there was no significant difference in straw carbon and nitrogen content among the DH lines. However, a highly significant difference ($P < 0.001$) was observed for nitrogen content of spikes at anthesis, and also for spike C/N ratio ($P = 0.001$). At maturity, these differences were not significant. The implications on germplasm utilisation were discussed.

Keywords: barley; grain protein; molecular marker; QTL; validation

Introduction

Grain protein concentration (GPC) in barley is influenced to a high degree by the environment and this may increase or decrease the protein level above the limit for malting barley. Breeding for low and consistent GPC, however, is difficult due to low trait heritability and marked genotype-environment interaction. With the advent of DNA markers, it has become possible to locate quantitative trait loci (QTL) for numerous phenotypes in plants. Molecular markers associated with such regions can allow breeders to efficiently select for such traits without interference of environmental effects.

Beyond QTL identification, a number of issues still need to be addressed in order to integrate quantitative genetic information into genome-based breeding programs. Among these include validation of QTL effects and also a determination of the possible physiological basis of gene action. In a previous study, QTLs that influenced variations in barley GPC were identified on chromosome 5H and 7H (EMEBIRI *et al*. 2003). In subsequent studies, the QTL effects were found to be consistent across diverse environments in Australia and the USA (EMEBIRI *et al*. 2004), making them ideal candidates for marker-assisted selection. Our objectives in the present study were to verify QTL effects through realised heritability and to observe whether low GPC in these lines was associated with (1) differences in tiller capacity and biomass accumulation, and/or (2) differences in nitrogen uptake, translocation and deposition in the grains.

Material and Methods

QTL Verification

Two approaches were used for verification of QTL effects in this study. The first approach relied on realised heritability for GPC in DH lines selected from within the original mapping population. For this purpose, twenty DH lines representing the four possible marker genotypic classes at the 5H and 7H loci were grown in field trials carried out at Horsham, Victoria, Australia (Lat. 36.4^{o} S, Long. 142.^{o} E) during the 2001 and 2002 cropping seasons. The lines were sown in a randomised complete block design with four replications and two levels of nitrogen application (0 and 80 kg ha^{-1}). Other lines included in the trials were the parents (VB9524 and ND11231*12) and 5 to 7 check varieties.

In the second approach, DH lines were randomly selected from surplus materials not used for the original QTL mapping. These lines were included in the stage 4 trials of the Barley breeding program at DPI, Horsham. A line (VB0229) with consistently low GPC was identified from trials carried out at 18 environments. Six individuals were randomly selected from within this line and fingerprinted using markers closest to identified QTLs on chromosome 5H and 7H.

Physiological Studies

Physiological determinants of GPC were investigated using the DH lines selected from within the original mapping population. Plants were sampled at awn emergence and at maturity. At each stage, 5 plants were sampled per plot, oven-dried at 70 ^{o}C for 48 hrs and measured for total aboveground dry weight and tiller numbers. Other traits measured relate to spike morphology (spike number, weight, length), 1000-grain weight, grain yield, protein (%), and grain plumpness. Duplicate samples of spike and straw were also processed for nitrogen and carbon concentrations at the Sate Chemistry Laboratory, Werribee, and expressed on a dry matter basis.

Statistical Analysis

Analysis of variance of the data were performed using IRRISTAT 4.4 for Windows and GENSTAT 6 software packages, while graphical representation of mean values were drawn with QUATTRO PRO and SIGMAPLOT.

Results and Discussion

QTL Verification

Realised heritability estimates are widely used in plant breeding to reflect the progress made from divergent selection (eg. GUTHRIE *et al*. 1984). In verification of QTL effects, the estimates can be used as a measure of marker-trait association after divergently selecting on the basis of marker genotypes. Estimates of realised heritability in the present study varied from 0.92 under conditions of 0 kg applied nitrogen to 1.16 under 80 kg of applied N, indicating a strong association between GPC variation and loci previously identified by EMEBIRI *et al*. (2003, 2004) on chromosome 5H and 7H.

Further evidence of the QTL effect on GPC was obtained from DH lines selected from outside the original mapping population. These lines were evaluated as part of stage 4 trial of breeding lines carried out between 2000 and 2003 at 13 to 18 locations in South Australia. From these trials, VB0229 was found to be consistently lower in GPC than any of the check varieties, which included standard malting barley varieties (Franklin and Schooner) and newly

released varieties (Dhow and Baudin) (Fig. 1). Six individuals were randomly selected from within this line and fingerprinted using SSR markers, XBmag323 and XEBmac603, linked to low GPC at 5H and 7H. The assays showed that five of these lines had fragment sizes characteristic of the parents, ND11231*12 and VB9524, at these loci.

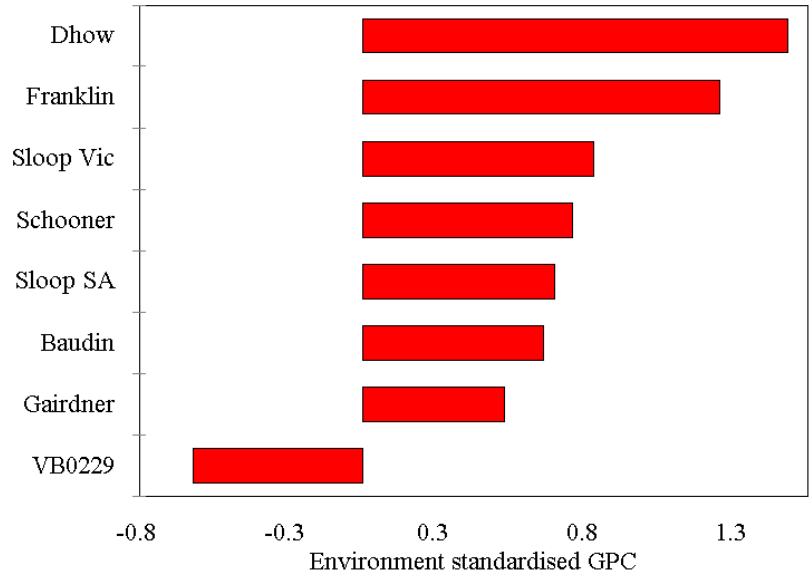

Figure 1. GPC of VB0229 and checks from stage 4 trials at 18 site-year environments in Southern Australia

QTL Effects and Associated Morphological Traits

There was a no difference among genotype classes for tillering or dry matter accumulation in the straw (data not shown). There was also no difference in the number of fertile tillers, but highly significant differences were observed in spike morphology (weight, length and kernels per spike) (Table 1). The differences were particularly noticeable amongst genotypes with low-protein (NDVB) versus high-protein (VBND) alleles.

QTL Effects and Associated Physiological Basis

There was a highly significant response to nitrogen application, as measured by N content of straw and spike (Table 2). Genotype classes had similar levels of N in the straw at anthesis and maturity, indicating that the differences in GPC were not related to N uptake. A highly significant difference was observed for N content of the spike at anthesis, but this was not present at maturity (Table 2). The highly significant difference in GPC (Table 1) would therefore suggest that the lower GPC conferred by favourable alleles at the QTLs might be due to reduced efficiency of N translocation to the grains during the phase of grain filling.

Acknowledgements

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Table 1. Mean squares from analysis of variance of dry matter accumulation and kernel morphology measured in 2001 and 2002 trials

Source of variation	DF	Spike DM at anthesis	Spike DM at maturity	Spike length	Kernels per spike	Grain protein	Screenings (< 2.5)
Replication	3	0.6	15.2	0.7	3.4	9.1**	65.2
Year (Y)	1	23.2**	456.8**	25.0**	91.8**	136.2**	17018.3**
Genotype class (G)	3	2.1*	128.1**	26.3**	136.8**	24.6**	861.3**
NDVB vs VBND	1	0.1	230.3**	29.6**	247.4**	42.7**	818.3**
LF Residual	2	3.2	76.9	24.7	81.5	15.6	882.9
N applied (N)	1	0.7	1.2	0.6	6.1	3.6**	326.5
G x Y	3	2.2*	131.7**	0.8	29.1**	1.7*	1060.2**
G x N	3	0.3	19.4	0.4	15.7	0.5	10.1
G x Y x N	4	1.1	31.0	1.2	14.7	1.1	15.5
Residual	231	0.7	29.6	1.0	7.8	0.5	94.4

*, ** Significant mean squares at P < 0.05 and 0.01, respectively

Table 2. Mean squares from analysis of variance of tissue nitrogen and carbon content measured in the 2001 trial

Trait	Source of variation	df	Tissue Nitrogen content (% w/w)	Tissue carbon content (% w/w)	Carbon-Nitrogen ratio
Straw at anthesis					
	N applied (N)	1	2.16**	2.13*	1039.45**
	Genotype class (G)	3	0.28	0.08	143.46
	G x N	3	0.10	0.09	73.70
	Residual	22	0.21	0.37	79.74
	v.a.f (%)		22.10	0.30	32.90
Spike at anthesis					
	N applied (N)	1	0.05	0.30	13.66*
	Genotype class (G)	3	0.12**	0.30	22.52**
	G x N	3	0.00	0.25	0.38
	Residual	22	0.01	0.43	3.28
	v.a.f (%)		42.00	0.00	38.50
Spike at maturity					
	N applied (N)	1	0.79**	1.02	247.58**
	Genotype class (G)	3	0.04	0.06	13.07
	G x N	3	0.08	0.17	18.89
	Residual	22	0.05	0.25	15.84
	v.a.f (%)		37.80	0.00	32.90

*, ** Significant mean squares at P < 0.05 and 0.01, respectively

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Development of Functional Genetic Markers by Combination of cDNA-AFLP Based Expression Profiling and Marker Assisted Genotype Pooling

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Abstract
Malting quality of barley represents the manifestation of the well-adjusted interaction of several different genes. Due to this complex genetic basis, quantitatively inherited traits and in particular malting quality, require sophisticated methods to be tagged by genetic markers. Induction of gene expression by a standardised micromalting process was used to identify differentially expressed genes both in varieties and in segregating populations. Based on a DH-population which was previously used to construct a QTL map for malting quality, phenotypic pools were assembled utilizing the information about markers which flank significant QTL intervals for malting quality traits as well as the observations of these malting quality traits. cDNA-AFLP analysis was performed with a selected subpopulation of the segregating progeny and differential TDFs were integrated into the linkage map. Several polymorphic fragments could be assigned to QTL intervals of the reference map and their correlation to malting quality traits was calculated. The favourite alleles of differential TDFs increased the means of malting quality parameters significantly compared to the means of the entire subpopulation. Differential TDFs which are located within significant QTL intervals represent candidates for functional genetic markers for malting quality and are therefore well suitable for efficient selection in early stages of plant breeding.
Keywords: malting quality; cDNA-AFLP; expression profiling; functional markers; marker assisted selection

Introduction

Estimation and selection for malting quality in barley require a comprehensive knowledge of the genetic background of this complex trait. Since the inheritance of malting quality is based on the multiple interaction of a number of genes which are unknown to a major part, it is very complicated to identify single genes which contribute to this important trait. However the information about the sequence of such functional genes is a prerequisite for the development of specific molecular markers which can be used to identify favourable genotypes providing the basis to generate the desired quality of raw material.
The development of QTL maps for malting quality is an important and necessary basic step to narrow down the genetic background to particular genome regions. Nevertheless markers linked to QTLs are difficult to use for marker assisted selection (MAS) because they may be specific only in the genetic background which was used to construct the QTL map.
The use of gene expression analysis for the detection of genes in context with seed germination has been demonstrated by POTOKINA *et al*. (2002) who compared the expression of germinating seeds from barley varieties with contrasting malting quality by the use of a macroarray composed of selected barley ESTs.
The introduction of sensitive molecular methods to screen for differentially expressed genes like Differential Display (DD) (LIANG & PARDEE 1998) and cDNA-AFLP (BACHEM *et al*. 1996) paved the way to target specifically induced genes even in complex plant genomes.

In contrast to the array hybridisation experiments which are a very efficient method to examine the expression of known genes the expression profiling technologies have the capability to detect yet unknown genes which contribute to the focussed trait (APPEL *et al*. 1999).
The assignment of transcript derived fragments (TDF) obtained by expression profiling to a genetic linkage map was introduced for potato by BRUGMANS *et al*. (2002). This method allows to include differentially expressed genes into an existing linkage map in order to assign functional markers to genomic regions which contribute to interesting traits.
Since most genes which are responsible for malting quality are expected to be induced during the malting process the analysis of samples taken directly out of a micromalting facility represents the best type of material to analyse the expression of genes correlated to malting quality (HERZ *et al*. 2003). The combination of a well founded QTL map and a method for specific analysis of gene expression promises to identify yet unknown genes with strong influence on malting quality.
In the presented study an approach is described, integrating expression profiling and the analysis of chromosome specific marker based phenotypic individual pools derived from a QTL mapping population. The assignment of differential cDNA-AFLP fragments to a QTL interval is introduced as an initial point for the development of functional genetic markers.

Material and Methods

Plant Material and Generation of Phenotypic Pools

From the DH-Population used for QTL mapping (HARTL *et al*. 2000) 50 individual lines selected for their phenotypic and genotypic uniqueness were grown together with the parental lines as a standard in 10m^2 field plots in two replications at the location Pettenbrunn in the years 2000 and 2001. The field trial was designed as a randomised block. For micromalting the replications of each line were combined. The 50 lines were used to assign differential cDNA-fragments to the QTL interval on chromosome 6H.
Differential pools have been constructed by bulking ten selected lines from the segregating population described above, carrying the desired marker interval for the QTL region on chromosome 6H and displaying the best performance of malting quality traits. Likewise, pools for the opposite alleles of the QTL have been designed. The composition of the pools and their phenotypic characteristics are shown in table 1. For selection up to four AFLP markers bordering each QTL interval have been used. To facilitate marker based selection of the differential pools the software "Graphical Genotypes" (VAN BERLOO *et al*. 1999) was employed.

Micromalting and Sampling

From each sample 250.0 g kernels of the harvested sieve fraction above 2.5 mm were malted in a micromalting machine (Automated Malting System, Phoenix-Biosystems, Australia). The micromalting process and the analysis of malting quality were performed according to MEBAK (1997). During the malting process samples were taken during 18 particular points of time ranging from the unmalted kernel to 76h after starting the micromalting process. Collected grains were immediately frozen in liquid nitrogen and stored at –80°C until processed. For the present study only the material from the samples taken at time point 24h during malting was utilized.

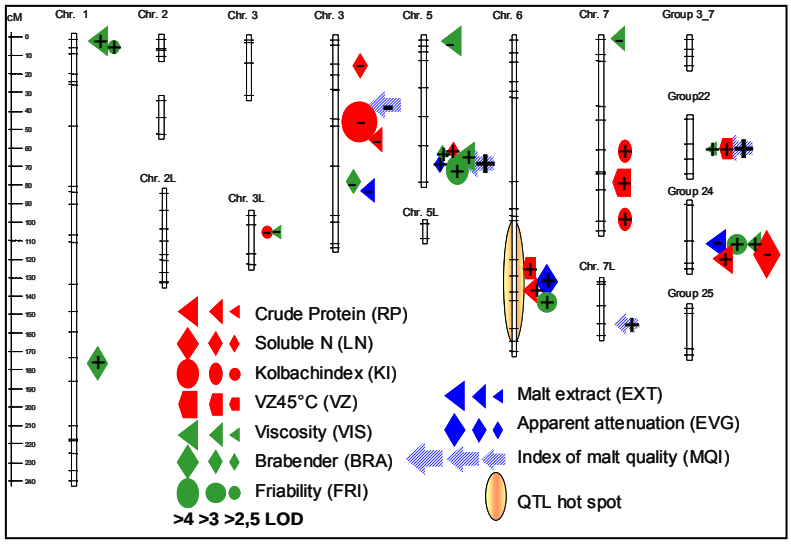

Figure 1: Schematic QTL map for malting quality traits of the Alexis x Steina cross. The analysed QTL hot spot for chromosome 6 is highlighted (HARTL *et al*., 2000).

RNA Extraction and Pool Formation

Total RNA was isolated from 400 mg of malted material, ground under liquid N_2 with pestle and mortar using the PURESCRIPT® RNA Isolation Kit as recommended by the manufacturer (Gentra Systems, USA). Remaining DNA was removed by DNAse I digestion. Final clean up of the total RNA was performed using the RNeasy Kit (Qiagen). RNA of the lines selected for the differential pools was bulked to a total amount of 1.0 µg for each pool. Each single DH-line was represented in the pool with equal RNA concentration. Alexis and Steina as parental lines of the cross were included into the analysis as a control using the equal amount of RNA.

cDNA Synthesis and cDNA-AFLP Analysis

Double-stranded cDNA was synthesised from 1.0 µg total RNA using the SMART^{TM} PCR cDNA Synthesis Kit (Clontech Laboratories AG, Switzerland) according to the manufacturers protocol. The cDNA-AFLP analysis was performed as described by BACHEM *et al*. (1996) with following modifications: 300 ng ds cDNA was digested by 5.0 U *Pst*I and 2.5 U *Mse*I, and ligated to 2.5 pmol *Pst*I (*Sse*I) adapter and 25 pmol *Mse*I adapter by 0.96 Wise-U T4 DNA ligase (New England Biolabs Inc.). The reaction was performed in a total volume of 12.5 µl containing 1.0 mM ATP and 0.1 mg/ml BSA in 1 x NEB buffer 2 (New England Biolabs Inc.). The reactions were incubated for 3 h at 37°C and afterwards at 4°C over night in an MJ PTC-200 Thermal Cycler (Biozym). Ligated fragments were diluted 1:4 in $TE_{0.1}$ (20 mM Tris/HCl, 0.1 mM EDTA, pH 8.0) and used as template for further cDNA-AFLP reactions, performed and analysed as described for genomic AFLPs by HARTL & SEEFELDER (1998). As *Mse*I specific primer for the preselective amplification M00, without selective nucleotides was used.

Fragment Analysis and Linkage Mapping

The cDNA-AFLP patterns were analysed with the computer software Cross Checker (BUNTJER 2000). The data matrix generated by this program was used as input data file for the software JoinMap 3.0 (Plant Research international BV, Netherlands). Linkage mapping was performed using JoinMap 3.0 with a LOD threshold of 6.0 for the calculation of groups. Calculation of the linkage map was carried out using the default settings of the software.

Results and Discussion

The screening of the differential pools for chromosome 6H with 25 cDNA-AFLP primer combinations resulted in a total of 63 fragments which distinguished between the differential pools comprising good and poor quality respectively and both parental cultivars.

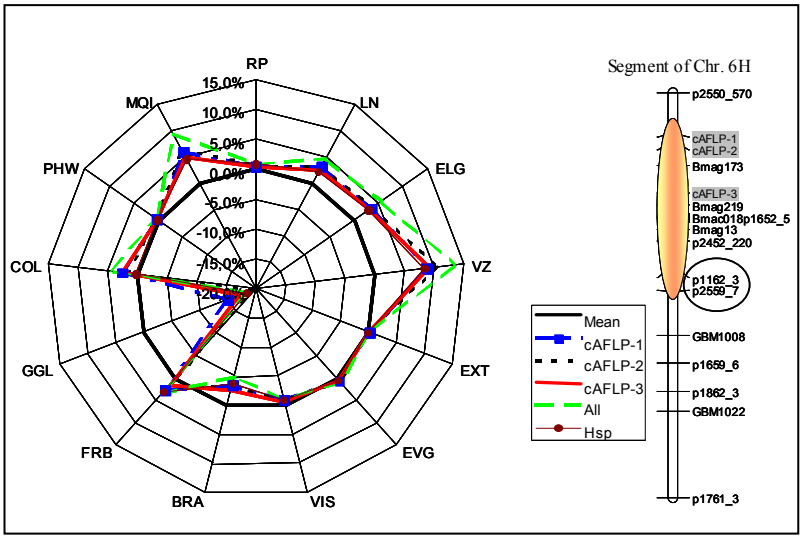

Figure 2. Segment of the linkage map of chromosome 6H in the Alexis x Steina population containing the QTL interval of interest. cDNA-AFLP markers identified by pool screening are highlighted. Encircled markers have been used for selection of pools. The web diagraph shows the influence of the favourable marker alleles on malting quality parameters in comparison to the mean of a subpopulation consisting of 50 DH lines from the mapping population. Labels for traits on the web diagraph are as described in fig. 1.

Detailed sequence analysis of these fragments is in progress.

To exclude artefacts, linkage analysis was performed which resulted in 17 markers which could be assigned to chromosome 6H. Three of the differential TDFs detected by the pool screening could be located close to the genomic AFLP markers which were used to select for the pools (Fig. 2). One particular differential TDF of which sequence information is already available and which is homologous to the sequence of a gene from the family of the heat shock proteins, *Hsp*70 could not be included into the linkage map until now. This suggests that this gene is located in a chromosomal region which is not covered by the existing linkage map. A further explanation for the low ratio of accurately mapped to actually detected differential fragments is that only the negative pools which do not show a cDNA-AFLP fragment are indubitably pure. If only a small number of individuals out of the ten forming a pool displays a fragment, the pool will be scored as "positive" for this fragment. When subsequently the single individuals of the pool are analysed separately, it might be observed that only a part of the pool individuals show the fragment. This was observed for the TDF with homology to the *Hsp*70 gene and explains why this fragment was detected by the pool screening but could not be integrated into the linkage map at the QTL interval on chromosome 6H (Fig. 3). A third explanation for the observed discrepancies between detected and mapped TDFs is, that the lines of one single pool contribute to more than one QTL interval.

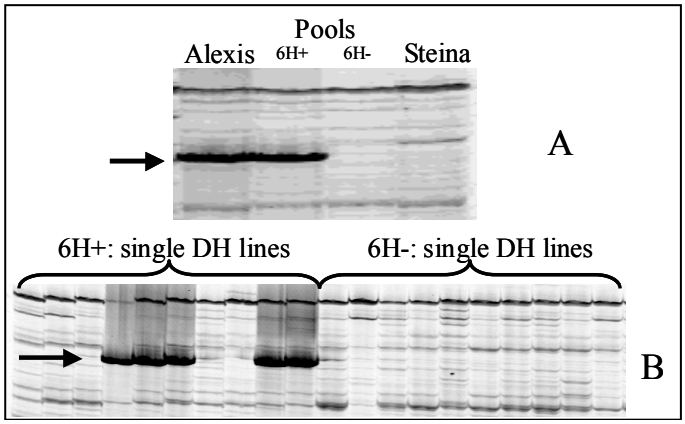

Figure 3. cDNA-AFLP expression profile showing **A**: the comparison between the two differential pools for the QTL interval of chromosome 6H with the parents and **B**, the single DH lines forming the differential pools. Arrows mark the differential TDF with sequence homology to the *Hsp*70 gene.

The latter is the most probable reason for the unlinkable fragments detected by the pool screening. The analysis of the remaining differential pools for the three QTL hot spots on chromosomes 1H, 4H and 5H will uncover such interrelations. Because the fragment with the sequence homology to *Hsp*70 was also detected by a previous study which assessed varietal differences in gene expression during malting (MIKOLAJEWSKI *et al.* 2001) the influence of this cDNA-AFLP marker on malting quality traits in the mapping population has also been calculated and is depicted in figure 2.

Performing a linkage analysis with the differential TDFs offers the opportunity to identify systematically differential TDFs which are located within a particular genomic region. As illustrated in Fig 1. three TDFs could be mapped adjacent to genomic AFLP markers which have been used to select for the individuals to compose the pool for the chromosome 6H QTL-hot spot.

The mean trait values of the individuals carrying the favourable allele of the cDNA-AFLP markers were compared to the mean of the entire subpopulation. The results show that the DH lines carrying the allele from Alexis show a better average performance of all malting quality traits than the mean of the entire population (Fig. 2). The means of all lines which possess all three positive cDNA-AFLP marker alleles is significant higher than the mean of lines with only one cDNA-AFLP marker. If this effect is caused due to the existence of different genes in the QTL interval or just by effects of the QTL has to be revealed by sequence analysis of the respective fragments and detailed QTL analysis.

Particularly the trait VZ 45°C is increased to 13.3% over the population mean by selection for the positive alleles of the cDNA-AFLP markers. This confirms the selection of the parental varieties of the mapping population which had been chosen due to their contrasting performance in the cytolytic parameters.

When sequence information of the presented three differential TDFs will be available, useful genetic markers can be developed to select specifically and effectively for malting quality traits.

Table 1. Means of the malting quality parameters in the marker assisted selected phenotypic pools, the entire population and the parental lines of the cross. **RP:** Crude protein**, LN:** Soluble N, **ELG:** Kolbachindex, **VZ:** VZ 45°C, **EX:** Extract, **EV:** Apparent attenuation, **VIS:** Viscosity, **BRA:** Brabender, **FRI:** Friability, **PH:** pH, **MQI:** Index of malt quality.

	RP	LN	ELG	VZ	EX	EV	VIS	BRA	FRI	PH	MQI
Population	10.13	689.40	42.63	42.31	82.25	82.39	1.50	111.40	80.22	5.79	7.80
Pool 6H+	9.92	702.69	44.34	47.73	82.54	83.22	1.48	103.49	84.32	5.78	8.71
Pool 6H-	10.36	641.28	38.79	35.45	81.81	81.58	1.52	121.69	75.48	5.80	6.78
Alexis	9.96	685.79	43.09	45.72	81.94	83.60	1.49	102.81	83.32	5.80	8.30
Steina	10.62	664.20	39.33	36.23	81.79	81.25	1.52	126.82	73.93	5.79	6.62

The presented results show that differential expression profiling using marker based phenotypic pools and the cDNA-AFLP technology is an efficient conception to generate functional genetic markers in the context of quantitative characters particularly with regard to malting quality.

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Characterization and Mapping of a Wild Barley *eibi1* Mutation Identifying a Gene Essential for Leaf Water Conservation

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Abstract
A spontaneous wilty mutant (*eibi1*) hypersensitive to drought was identified in wild barley, *Hordeum spontaneum* Koch. *Eibi1* had a highest relative water loss rate among the known wilty mutants, indicating that is one of the most drought sensitive mutants. When compared with wild type, *eibi1* had the same ABA level, the same ability to accumulate stress -induced ABA, and the same stomatal movement in response to light, dark, and drought, revealing that *eibi1* was neither an ABA -deficient nor an ABA-insensitive mutant. *eibi1* leaves had a larger chlorophyll efflux rate in 80% ethanol than the wild-type leaves; demonstrating that *eibi1* was defective in an actual barrier layer in cuticle that limits water loss of the plant. An approximately 3:1 segregation ratio of wild type to mutant among 158 F_2 plants derived from a cross of *eibi1* by Morex revealed that the *eibi1* high water loss rate was caused by a single recessive nuclear mutation. This mutation was mapped on chromosome 3H short arm between simple sequence repeat markers (SSR) Bmag0603 and Bmac0067, and cosegregating with Bmac0828.
Keywords: abscisic acid; cuticle; *eibi1* mutant; mapping; stomata; wild barley

Introduction

Well-developed cuticles and stomata exhibited in fossil specimens of the very earliest terrestrial plants known (Edwards et al., 1998) indicate the critical importance of the regulation of plant water status. Defects in stomata closure and cuticular layer cause wilty phenotype due to excess water loss under drought conditions. Wilty phenotype of ABA-deficient mutants (SCHWARTZ *et al.* 1997) and of some ABA-insensitive mutants (LI *et al.* 2000) reveals the fundamental role of ABA in higher plant drought resistance. Mutants defected in cuticular wax such as *eceriferum* mutants in barley and *Arabidopsis* have been identified (LUNQVIST & LUNQVIST 1988; JENKS *et al*. 2002).

While testing drought resistance of wild barley genotypes from Wadi Qilt population (longitude 35.38 Decimal, latitude 31.83 Decimal, altitude 50 m, annual termperature 24.8 ^{o}C, annual rainfall 144 mm, annual evaporation 3300 mm (NEVO *et al*. 1979)) in Israel, we discovered a spontaneous mutant genotype hypersensitive to drought, readily exhibiting a wilty phenotype under water deficit. This spontaneous wilty mutant was investigated and its genetic mapping was conducted in the present study.

Material and Methods

Plant Materials

A wilty mutant, discovered from a zeric wild barley ecotype 23-19, was found to be hypersensitive to water stress. Its second and third generation offsprings showed the same wilty phenotype. This wilty mutant and its near-isogenic line, wild type 23-19, were used in the present study. For comparison of detached leaf water loss rate, tomato flacca and its near-isogenic line RR were also used in the present study.

Physiological Analysis

For leaf drying test, fragments of the basal part of the leaf blades were dried abaxial side up on a lab bench; leaf water loss rate was expressed as percent of initial weight. Drought stress was applied as water withholding on seedlings at four-leaf stage. Shoot samples were extracted and assayed for ABA according to the protocol detailed in the ABA enzyme immunoassay test kit for the quantitative determination of abscisic acid (IDEXX Laboratories, Inc., Westbrook, ME 04092) (MERTENS *et al.* 1985). For transpiration rate in response to transition from dark to light and from light to dark, The youngest fully expanded leaves were cut 1 cm above sheath and immediately inserted the cut ends into distilled water. Detached leaf transpiration was expressed as weight (g) of water lost per m^2 per h – g water m^{-2} h^{-1}. Cuticle permeability was measured using chlorophyll efflux. Four replicates of mutant and wild type leaves were immersed in 80% ethanol solution in 12 ml tubes with covers. The amount of chlorophyll extracted into the solution was quantified using a UV-160A spectrophotometer (Shimadzu, Japan) and calculated from UV light absorption at 647 and 664 nm as described by LOLLE *et al.* (1998). Total chlorophyl was expressed for each time point as a percentage of total chlorophyll extracted after 24 h.

Genetic Analysis

Two crosses were made to investigate the segregation of the mutant in F2: (i) Morex (a cultivar) × mutant, and (ii) 23-19 (a near-isogenic line wild type *Hordeum spontaneum*) × mutant. F_2 seedlings were allowed to grow in Petri dishes in a transparent box to retain high humidity for additional three days. The expanded first leaves were cut for leaf-drying test as described later. A genetic map was constructed with DNA extracted from F_2 populations. Methods of genotyping with SSR were employed as described by PENG *et al.* (2000).

Results and Discussion

Identification of a Wild Barley Mutant

One out of ten of ecotype 23-19 seedlings was found to be hypersensitive to water stress while drought resistance experiment was conducted with Israel xeric wild barley. Two seeds were harvested from this drought sensitive plant (M1). Two M2 plants and all the M3 plants exhibited the same phenotype as the M1 plant. This spontaneous mutant was named *eibi1* following the name Eibi (Professor Eviatar Nevo), the supervisor of the first author of this paper. The *eibi1* detached leaves lost significantly more water than the tomato *flacca* (a typical wilty mutant) detached leaves within one-hour dehydration (Fig. 1). To our knowledge *eibi1* has the highest relative water loss rate among known wilty mutants. This tremendous relative water loss rate of *eibi1* implies that there may be a novel mechanism underlying the water conservation in plant leaves and consequently drought resistance.

Segregation analysis of the F2 progenies of two crosses: Morex (a cultivar) × eibi1 and 23-19 (a near-isogenic line of eibi1) × eibi1 showed an approximately 3:1 segregation ratio of wild type to mutant, indicating that the eibi1 mutation was caused by a single recessive nuclear mutation.

ABA Contents of eibi1 and Wild Type

Wild-type and *eibi1* shoots had the same ABA contents under control conditions, whereas drought stress increased ABA contents in both *eibi1* and wild type to the same level (Fig. 2), indicating that *eibi1* had the normal ABA content and the ability to accumulate ABA under drought stress.

Transpiration of Detached Leaves Responding to Light and Dark

The fluctuation of transpiration of *eibi1* and wild type in response to light and dark revealed that *eibi1* had the normal stomatal movement as wild type (Fig. 3). Detached *eibi1* leaves had more than 10 times larger transpiration rate than wild type leaves in dark implying that *eibi1* had a defected waxy cuticle.

Chlorophyll Efflux Rate

Chlorophyll efflux rate of leaves in 80% ethanol was much greater in *eibi1* than in wild type (Fig. 4), suggesting that *eibi1* is defective in cuticle.

Genetic Mapping of the eibi1 Mutation

The *eibi1* was mapped on chromosome 3H short arm (Fig. 5). The high collinearity of barley chromosome 3H with rice chromosome 1 will guide fine mapping of eibi1 using the small rice genome as a vehicle.

Conclusion

The *eibi1* mutation identified a novel gene that functions in the transport-limited layer in cuticle that is essential for plant drought resistance. This *eibi1* mutation was located on chromosome 3HS.

Acknowledgements

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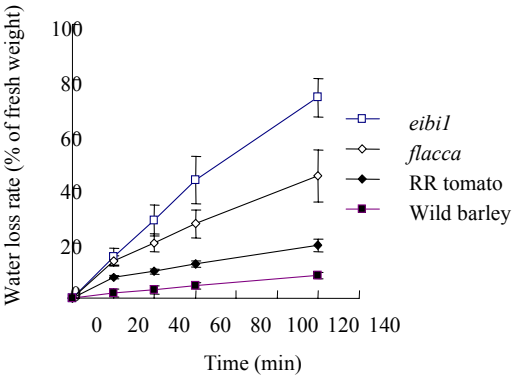

Fig. 1 Water loss rates of detached leaves from *eibi1, flacca*, wild barley, and RR

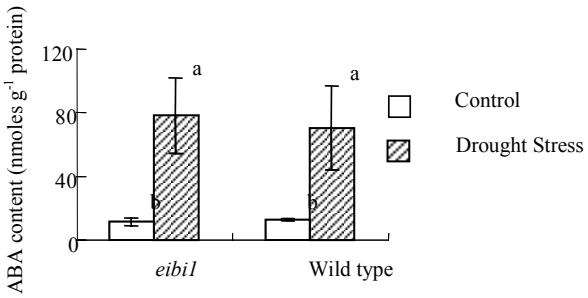

Fig. 2 ABA contents of *eibi1* and wild type shoot with or without drought stress

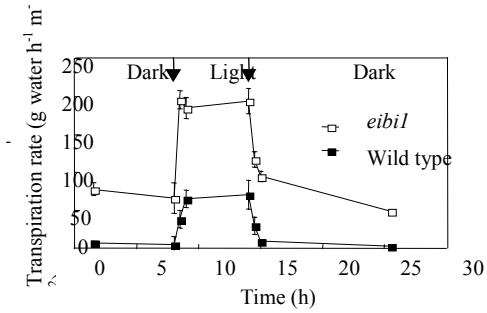

Fig. 3 Transpirations rate of *eibi1* and wild type in response to light and dark

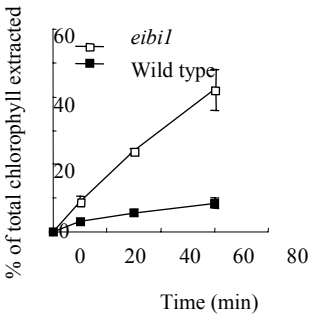

Fig. 4 Leaf permeability to chlorophyll of *eibi1* and wild type

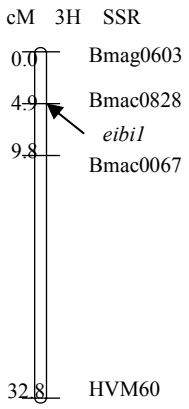

Fig. 5 Genetic map of *eibi1*

Flowering Time Markers for Barley Breeding

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Abstract

Adjustment of crop phenology to the resources of the production environment is among the most important traits for barley adaptation. Three genes, *Sgh*$_1$, *Sgh*$_2$ and *Sgh*$_3$, are responsible for winter/spring growth habit and vernalization response. Two more genes, *Ppd-H1* and *Ppd-H2*, control photoperiod response. In most studies, the main photoperiod and vernalization QTL coincide with the putative location of the corresponding major genes. There are also a number of QTLs with lesser effects on flowering, known as *earliness per se* loci, more abundant, but showing less consistency across studies. All these loci have been detected in a number of studies involving biparental progenies. But their use in MAS requires knowledge of their effects and interactions at the germplasm-pool level. For this reason, seventeen populations of up to 20 doubled haploids each, made of combinations of 14 parents routinely used in a Spanish breeding program, are being phenotyped for heading time, and genotyped with markers located in the vicinity of major photoperiod and vernalization response loci. The objective is to identify a set of markers that can be used to make predictions of flowering time of genotypes and candidate crosses. These predictions would also be useful to perform MAS towards favorable loci combinations. A progress report on association between markers and field heading time for two genomic regions (*Ppd-H2* and *Eam6*) is presented.

Introduction

The adjustment of crop growth to available resources is one of the main factors of crop adaptation. For grain crops, flowering time is a crucial event in the life cycle, and the main trait affecting adaptation to cultivation under water stress conditions (LUDLOW and MUCHOW, 1988).

Heading date in barley and wheat is determined by the concurrence of three phenomena: responses to cold temperatures (vernalization), responses to photoperiod, and intrinsic earliness, also related to temperature (ROBERTS *et al.* 1988; ELLIS *et al.* 1988, 1989; SLAFER 1996).
Genetic studies of heading date (a surrogate measure of flowering time in winter cereals) reveal its complex nature. TAKAHASHI and YASUDA (1970) proposed the existence of three genes controlling winter-spring growth patterns in barley, now known as *Sgh1*, *Sgh2* and *Sgh3*, in chromosomes 4H, 5H and 1H, respectively. Only the first two are known to segregate in Western materials. Molecular marker studies confirmed the presence of these loci: *Sgh1* on the long arm of chromosome 4H, closely linked to the β-amylase locus (HACKETT *et al.* 1992); *Sgh2* on the long arm of chromosome 5H, close to RFLPs CDO504 and WG644 (LAURIE *et al.* 1995), syntenic to similar loci in rye and wheat (PLASCHKE *et al.* 1993; GALIBA *et al.* 1995).

Recently, vernalization locus VRN1 in wheat has been found by comparative mapping (YAN *et al.* 2003), which also shows homeology with barley loci (DUBCOVSKY *et al.* 2001). LAURIE *et al.* (1994) identified a photoperiod response locus on the short arm of chromosome 2H, close to RFLP MWG858. This finding was confirmed in other studies in barley (HAYES *et al.* 1993; PAN *et al.*, 1994). This locus, known as *Ppd-H1*, is detected under long photoperiod. Another major photoperiod response locus, *Ppd-H2*, whose effect is evident under short photoperiods, is located on the long arm of chromosome 1H (LAURIE *et al.* 1995). Any other loci have been found to affect intrinsic earliness in mapping population studies, usually with lower effects compared with photoperiod and vernalization response loci. Among them, the most important is *Eam6*, located near the centromere on chromosome 2H (BOYD *et al.* 2003). These major vernalization and photoperiod response loci are not totally independent. Some studies revealed interactions among them (LAURIE *et al.* 1995; KARSAI *et al.* 1997).

The combination of all detected flowering time QTLs in a single map is not straightforward, as different markers were used in each mapping population experiment, and QTL polymorphisms vary from cross to cross. Nevertheless, this is necessary task to render the information on QTL location useful to plant breeders. To this end, studies on degree of polymorphism over a wider range of germplasm, number of alleles and their effect for each QTL are needed.

This study is a first attempt to extrapolate the information on major heading time loci, found in a number of barley mapping population studies to a wider range of plant materials.

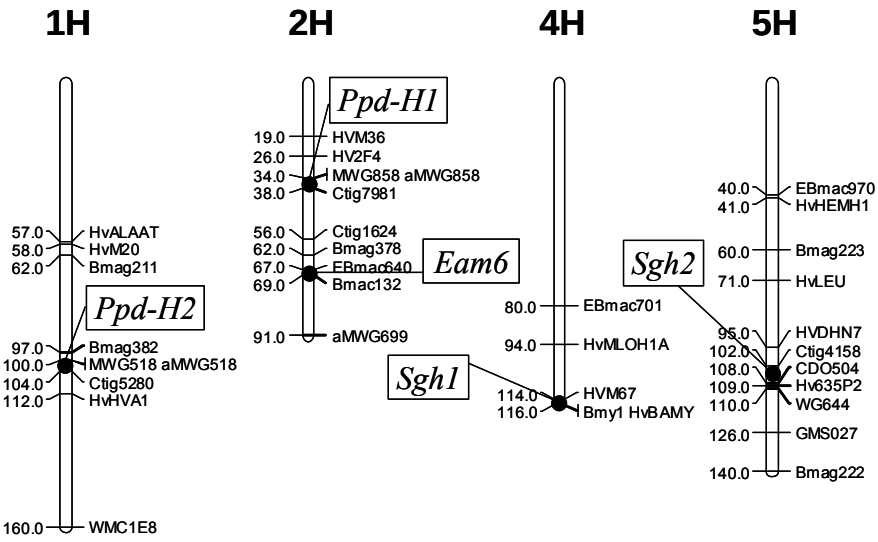

Figure 1. Molecular markers analyzed in this project, and approximate position of major heading time loci.

Material and Methods

Plant Material: Seventeen small populations of doubled haploid (DH) lines, from representative crosses of the Spanish public barley breeding program were used (Table 1). A maximum of twenty lines per population were selected. Parents were cultivars from Spain and other European countries. Winter, Spring and Facultative growth types were represented. There were fourteen different parents, thus each parent was present at least in two crosses.

Field Trials: Heading dates of the collection of the small DH populations and parents were recorded at two field trials planted in November 2002 at two locations in the provinces of Huesca and Zaragoza (North-Eastern Spain). The field trials consisted of three replications of two-row

plots, 1m long, following a randomised complete block design. Heading date was recorded as the moment when 50% of the tillers exhibited 2 cm of protruding awns.

Marker Analysis: Markers were selected at five different regions of the barley genome, carrying the major vernalization (*Shg1*, *Sgh2*), photoperiod (*Ppd-H1*, *Ppd-H2*), and earliness per se loci (Eam6), as shown in Fig 1. Markers aMWG858, and aMGW518 are STS derived from RFLPs (Casas, unpublished), which showed linkage to *Ppd-H1* (LAURIE *et al.* 1994) and *Ppd-H2* (IGARTUA *et al.* 1999), respectively. These STS produce the same result as the RFLPs in a mapping population, and in a collection of cultivars (data not shown). A total of 32 markers are being evaluated, over a total of 285 DH lines and 14 parents. Results are presented on markers in the region of *Ppd-H2* and *Eam6*.

Table 1. Description of the seventeen small populations used in this study

Parents		Growth habit[1]	Row type	DH number[2]
Albacete	Monlon	F-F	6-6	7
Albacete	Plaisant	F-W	6-6	20
Alexis	Pane	S-W	2-6	20
Angora	Clarine	W-W	2-2	20
Barberousse	Albacete	W-F	6-6	10
Barberousse	Monlon	W-F	6-6	12
Barberousse	Plaisant	W-W	6-6	20
Barberousse	Tipper	W-W	2-6	8
Beka	Monlon	S-F	2-6	20
Clarine	Plaisant	W-W	2-6	20
Gaelic	Tipper	W-F	2-2	8
Nevada	Beka	S-S	2-2	20
Pané	Plaisant	W-W	6-6	20
Plaisant	Orria	F-W	6-6	20
Seira	Alexis	S-S	2-2	20
Seira	Tipper	S-W	2-2	20
Seira	Orria	S-F	2-6	20

[1] W: winter ; S: spring; F: facultative
[2] Number of doubled haploid lines per population

Results and Discussion

Heading dates at the two locations were very similar (r=0.86). Thus, the mean heading date for the two trials was calculated, and all further results are based on this mean. Field heading dates of the entire DH collection presented a wide range of variation, for the mean of the two autumn sown trials (Table 2). There was a difference of over 19 days between the earliest and latest heading dates. The range of variation was 6.4 for the parents, and it varied between 6.0 and 16.4 days for the 17 populations, with an average of 9.7 days. Transgressive segregation was evident for all crosses: the average difference between heading dates for the parents was 2.3 days, whereas it was 9.5 for the DH lines.

Marker analysis is complete only for the regions of *Ppd-H2* (chromosome 1H) and *Eam6* (chromosome 2H, near the centromere). Thus, only the results for these two regions are presented. A rough estimation of the overall influence of these regions on heading time is revealed by the fact that the range of heading dates for populations polymorphic at these regions was 11.3 days, whereas this value was only 8.1 days for populations not polymorphic at both loci.

As the markers are not part of the flowering time loci themselves, we chose to use the haplotype defined by the two flanking markers as an approximation to characterize QTL polymorphism, which may be good enough to track it in the doubled haploid populations.

There were five different haplotypes defined by the combination of flanking markers: microsatellites Bmac132 and Ebmac640 presented 3 and 2 alleles, respectively, combined in five different ways (denominated a through e). Twelve populations presented polymorphism at this region. There were three different haplotypes in the *Ppd-H2* region, defined by the combination of flanking markers aMWG518 (STS), and the microsatellite Ctig5280, each with two alleles.

Overall effect of the two regions considered in this study was detected by analyses of variance (Table 2) using the haplotype as classifying factor. This approach assumed that haplotypes defined by bands of similar size had similar phylogenetic origin. Though the overall effect of each region was significant, the amount of heading time variance explained was 8% for each region.

There was not complete consistency of the results for the *Eam6* region. There seems to be at least two alleles, one represented by haplotype V, and possibly haplotype IV, conferring earliness (Table 2). Another allele, conferring lateness seems to be shared by haplotypes I and II. The effect of haplotype III cannot be ascertained clearly. Only one parent (Pané, a primitive Spanish cultivar) presented haplotype III. It was present in two crosses, and its effect was intermediate between the other two alleles, but there were not enough crosses, nor with differences significant enough, to conclude about its effect.

Only six populations were polymorphic at the *Ppd-H2* region. Three haplotypes (I, II, III) were identified using flanking markers, and the general analysis of variance for all populations detected three different effects for them. The effect of this region, however, was only evident in two crosses when considering single population analyses.

There was not exact equivalence of haplotypes with QTL alleles. Haplotype V for the *Eam6* region is characteristic of earliness in most cases. Monlon and Plaisant both have this allele. They were crossed to Albacete, and should present a similar effect, but only Monlon's allele confers earliness, whereas Plaisant's allele has no effect on heading date crossed to Albacete. For the *Ppd-H2* region, crosses Beka x Monlon and Alexis x Pané showed similar polymorphism. However, a significant effect on heading date was evident only in the first cross. This study has to be completed with a yet more thorough analysis of all five regions considered. Also, the study of plant responses under controlled conditions, currently underway, will shed more light on the effects of these major heading time loci.

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Table 2. Population by population analysis of field heading date. Range of variation, and effect of two genomic regions at chromosomes 1H, and 2H on deviation of heading date on population mean.

Parents		Heading date range (days) Parents[1]	Heading date range (days) DH[2]	Haplotypes, *Eam6* region[3]						Haplotypes, *Ppd-H2* region[3]			
				I	II	III	IV	V	P(T<=t)[4]	I	II	III	P(T<=t)[4]
Albacete	Monlon	1.0	7.5		1.7			-1.8	0.09			0.0	
Albacete	Plaisant	0.3	6.0		0.3			-0.3	0.49			0.0	
Alexis	Pané	1.9	10.2	1.4		-0.9			0.14	-0.1		0.2	0.84
Angora	Clarine	1.0	7.2					0.0				0.0	
Barberousse	Albacet	1.5	9.0		2.6			-3.2	0.01			0.0	
Barberousse	Monlon	5.2	8.7					0.0				0.0	
Barberousse	Plaisant	1.8	6.8					0.0				0.0	
Barberousse	Tipper	0.3	11.2		5.0			-3.9	0.25	0.0			
Beka	Monlon	6.4	16.4		2.9			-1.4	0.04	-2.2		3.3	0.00
Clarine	Plaisant	0.2	9.8					0.0				0.0	
Gaelic	Tipper	2.8	10.8		5.8		-1.9		0.01			0.0	
Nevada	Beka	3.0	9.7	-0.4	0.7				0.52	-0.3	0.2		0.72
Pané	Plaisant	1.3	10.5			1.6		-0.7	0.09			0.0	
Plaisant	Orria	2.6	9.0				-0.5	0.5	0.41			0.0	
Seira	Alexis	1.3	9.2	0.0					0.41	-0.7	0.2		0.02
Seira	Tipper	4.5	13.2	-0.3			0.2		0.84		3.0	-0.8	0.49
Seira	Orria	4.0	9.2	0.2	-0.3				0.67	-0.2	0.5		0.72
All populations				0.2 b[5]	1.5 c	-0.1	-0.1	-1.1	0.003[6]	-0.7	1.4 c	0.7 b	0.001[6]

[1] Heading date difference (in days) between the parents of each population
[2] Heading date range (maximum-minimum, in days), for each population of doubled haploid lines
[3] Average effect of each haplotype for each population, measured as the overall deviation (in days) from population mean
[4] Probability of finding a statistic equal or larger than the observed, using a two-tailed t comparison for samples with equal variances
[5] Values followed by the same letter are not significantly different according to a F-protected LSD, P<0.05.
[6] F test probability of an analysis of variance including polymorphic populations for each QTL region

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The Distribution of Retrotransposon-Based (S-SAP) Markers in European Barley Cultivars

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Abstract

The retrotransposon based S-SAP technique has been shown to be a highly informative method for genetic analysis. We have evaluated primers designed from the LTRs of six retrotransposon families in conjunction with a large number of selective *Mse*I primers to evaluate the most informative primer combinations that generate high quality profiles. Following extensive screening, we selected six of these primer combinations from four of the retrotransposon families for wider application. This subset of primer combinations has been used to profile a set of over 500 barley cultivars using the S-SAP technique. The barley cultivars were chosen to represent the barley germplasm cultivated across Europe during the past 50 years. This has allowed us to determine the relationships among and between a range of genotype/ germplasm classes at these loci. Thus, our research gives insight into trends and events that have shaped barley breeding programmes over the past 50 years.

Introduction

The 'Gediflux' project is an EU collaborative project that aims to determine changes in the genetic diversity of four crops; barley, maize, potato and wheat over the past 50 years. To achieve this, a set of more than 500 barley varieties has been created from the varieties in commercial use in European countries that evenly represents the material grown in each decade. This material encompasses the winter / spring and 2 / 6 row phenotypes, as well as including selections that represent the introduction of disease resistances and key donor germplasm based on pedigree analysis. The genetic diversity of these barley varieties has been assessed using a range of molecular markers including the retrotransposon based marker system S-SAP (sequence – specific amplification polymorphism). Preliminary analysis of this data has allowed easy classification of the varieties into groups that reflect 2 phenotypic characteristics.

Material and Methods

DNA was extracted from the flour of 30 seeds of each variety using the Qiagen Dneasy kit. DNA was genotyped using the S-SAP protocol of WAUGH *et al.* (1997) was followed with minor modifications, detailed in LEIGH *et al.* (2003).

Results and Discussion

A set of 101 alleles was identified from 4 retrotransposon primer combinations. The map location of 23 of these alleles was known whilst the remaining 78 alleles were unmapped. The mapped alleles were evenly distributed through the barley genome.

Material was classified into spring and winter varieties, and 2- and 6-row varieties. Jaccards analysis was performed followed by principle co-ordinate analysis. Scatterplots revealed that two distinct clusters were generated; these corresponded to the 2-row spring barley and the 6-row winter barely varieties. The 6-row spring and 2-row winter barley varieties formed two clusters

between these extremes. The clustering was sufficiently strong to allow the unknown habit of a variety to be predicted from the position of the variety within the plot. Indeed, the efficiency of this prediction was 97.5% in 2-row spring barley's and 89.8% efficient overall.
To understand the power of the mapped markers, the efficiency of this subset of 23 mapped markers at predicting the habit of a variety was calculated in the same way. The mapped markers were only 11.3% less efficient than the complete set of 101 markers (of which they were a subset) whilst the set of 78 'anonymous' markers was 2.1% less efficient than the complete marker set. Therefore, the 23 mapped markers are 88.7% as efficient as the complete set of 101 markers, require only a quarter of the effort and provide information across the whole genome.
Future work will investigate the key alleles that are responsible for the classification of the germplasm into four groups. Additionally, changes in the genetic diversity of the set of varieties will be calculated and related to events that have shaped barley breeding where possible.

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Identification and Mapping of Disease Resistance-Related DNA Sequences in Barley

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Abstract
Over 150 barley RGA clones were generated by using two pairs of degenerate primers designed from nucleotide binding domain (NBD) of several previously cloned disease resistance genes. The resultant RGAs were characterized based on both DNA sequence data and RFLP patterns after Southern hybridization. Representative clones were mapped using NABGMP mapping populations and disease resistance near-isogenic lines. Thirteen different barley RGA classes were identified. The encoding amino acids indicated that all these 13 clones, except one, contained continuous open reading frames and had characteristic regions of NBD. RGA-related clones were mapped to all barley chromosomes except chromosome 7. One RGA mapped to chromosome 4 and at least 2 markers mapped to each of the remaining barley chromosomes. Almost all of the RGAs mapped to barley chromosomal regions which previously had been reported to contain known disease resistance factors. DNA sequence comparisons showed that a diverse group of RGAs has been identified.

Introduction

Most of the cloned plant resistance genes encode common sequence motifs, including nucleotide-binding site (NBS) and a series of leucine-rich repeats (LRR). The NBS/LRR domains are speculated to be involved in recognition or signal transduction during the interaction of resistance gene and pathogen molecules (ELLIS *et al*. 2000). PCR amplification of these motifs using degenerate primers is a rapid approach for the identification, isolation and PCR cloning of RGAs (KANAZIN *et al.* 1996; YU *et al.* 1996; LEISTER *et al.* 1998; MAGO *et al*. 1999). RGAs have been isolated from many plant species including grain crops rice, wheat, barley and maize (COLLINS *et al*. 2001; LEISTER *et al*. 1998). Genetic mapping of RGAs demonstrated their clustering in the vicinity of previously mapped disease resistance (R) genes (GRAHAM *et al*. 2000; COLLINS *et al*. 2001). This makes RGAs very useful for the discovery of R-genes. The objectives of this study were (1) to identify RGAs from barley, (2) to localize them in the barley genome, and (3) to examine any possible associations with known barley resistance genes.

Material and Methods

Plants Materials

Genomic DNA of barley cultivars 'Steptoe', 'Morex', 'Harrington', 'TR306', and 'LUGC' were used for PCR amplification of the RGA sequences. Three barley double haploid (DH) NABGMP populations of the parents Steptoe × Morex (SM) (150 DH lines) Harrington × TR306 (HT) (150 DH lines) and LUGC × Bowman BC (LB) (106 DH lines) were used for the genetic mapping of the RGA sequences. To search for possible association of the RGA clones with specific resistance genes, a number of barley near isogenic lines (NILs) were screened with the above-mentioned RGAs.

Amplification and Cloning of the PCR Products

Two pairs of degenerate primers were used in the study. They were designed from the

conserved motifs of the NBS in tobacco *N* and *Arabidopsis RPS*2 genes. For the first pair of the primers, the forward primer was designed based on kinase-1a region, and the reverse primer was designed near the kinase-3a region (YU *et al*. 1996). This primer pair is hereafter referred to as BN primers and clones derived from the sequences amplified with this primer pair are referred to as BN-class RGAs. For the second pair of primers, the forward primer (5'-GGIGGIGTIGGIAAIACIAC-3') was slightly different from that of the BN primer, and the reverse primer (5'-ARIGCIARIGGIARICC-3') was designed from the downstream region (GLPLAL). The second primer pair is hereafter referred to as GL primers and clones as GL class RGAs. For PCR with BN primers, 2.5mM of $MgCl_2$, 200µM of dNTPs, 1.0µM of each primer and 2.5u of Taq DNA polymerase (Invitrogen) and 50ng of DNA template. PCR profile: 94°C for 3 min, 18 cycles of 1 min at 94°C and 1 min at 72°C; annealing began at 69°C and was reduced by 1°C per cycle to 61°C followed by 25 cycles of 1 min at 94°C, 1.25 min at 60°C and 1 min at 72°C. The PCR conditions with GL primers consisted of a hot start (3 min at 94°C), and 42 cycles of 1 min at 94°C, 1 min at 45°C, and 1 min at 72°C. The PCR products amplified with the GL and BN primers were purified from agarose gel slices using Qiaex II gel extraction kit (Qiagen, Valencia, Calif.). The purified products were subcloned into pCNTR vector (5prime-3prime, Boulder, Co.) following the manufacturer's instructions.

RFLP Analysis

DNA extraction and RFLP analysis were essentially as previously described (SAGHAI MAROOF *et al*. 1984) using six restriction enzymes, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III, *Sst*I, and *Xba*I. Mapmaker/exp 3.0 at LOD = 3.0 with maximum Haldane distance of 50 cM was used for genetic linkage and distance determination (LANDER *et al*. 1987).

DNA Sequencing

DNA was sequenced using an ABI377 DNA sequencer. Plasmid template was prepared using standard alkaline-lysis method followed by purification with QiaexII (Qiagen Inc., Valencia, CA). Dye-terminator cycle sequencing was done based on the manufacturer's protocols (Perkin Elmer, Foster City, CA).

Results and Discussion

Cloning, Characterization and Mapping of Barley RGAs

PCR-amplification of barley genomic DNA using BN- and GL-class primers resulted in major products of the expected sizes of 340 bp and 540 bp, respectively. These PCR products were gel-purified and cloned. A total of 109 BN-class and 81 GL-class RGAs were analyzed by RFLP using a set of diagnostic blots containing six barley parental lines digested with six restriction enzymes. Clones were grouped based on RFLP patterns. High copy number clones were discarded. Remaining single or low copy BN and GL RGAs were grouped into six and ten classes, respectively. 70% of the BN and 53% of GL clones belonged to one class. At least one representative was sequenced from each class. DNA sequences from three BN and five GL clones contained open reading frames (ORF). These clones, representing eight different classes, are BN1-C7, BN1-C8, BN1-D5, GL2-B1, GL2-B4, GL2-B11, GL2-B5 and GL3-H11. Also, sequence analysis showed that three BN classes represented by BN1-A10, BN1-B10 and BN2-D3 and four GL classes represented by GL1-A2, GL2-D5, GL2-E4, and GL4-A2 were interrupted by stop codons.

RFLPs, identified in the hybridization studies, were used to map the above-mentioned RGAs in the barley genome using three DH populations. A total of 15 sequences were placed on 6 chromosome (except 5H). Because RGA are potentially derived from disease resistance genes, experimental RGAs were expected to be located in the vicinity of the previously mapped disease resistance genes. The comparison of the location of the RGAs with the

position of R-genes was based on the position of common markers linked to RGAs in our maps as well as R-genes in other maps. Three RGAs, GL2-E4, GL2-D5 and GL4-E1 were mapped onto the chromosome 7H (Fig. 1). GL2-E4 was mapped onto the centromeric region in the SM population. This region harbors several QTLs conferring resistance to fusarium head blight (FHB) and kernel discoloration (KD) (DE la PENA *et al*. 1999), and *Rpt4* conferring resistance to the spot form of the net blotch (NB) (WILLIAMS *et al*. 1999). Two other RGAs, GL2-D5 and GL4-E1, were clustered in the long arm of the chromosome 7H, where three single leaf rust resistance genes *Rphq9, Rph3, Rph19* were mapped and QTL for barley yellow dwarf virus (BYDV) was identified (TOOJINDA *et al*. 2000) (Fig. 1). Four RGAs were positioned onto the long arm of the chromosome 2H covering the genetic distance of about 23 cM (Fig. 1). These RGAs may have associations with *Ha2* – cereal cyst nematode resistance gene (KRETSCHER *et al*. 1997), QTLs for FHB (DE la PENA *et al*. 1999) or BYDV (SCHEURER *et al*. 2001) (Fig. 1). GL2-B1 and GL2-B4 were assigned to the short and long arms of the chromosome 3H, respectively in the SM population (Fig. 1). Position of GL2-B1 clone is of great interest, since several QTLs such as FHB (DE la PENA *et al*. 1999), BYDV (SCHEURER *et al*. 2001) and NB (RICHTER *et al*. 1998) as well as single BYDV resistance gene *Yd2* were previously mapped within this region. With respect to GL2-B4, this clone was mapped near the QTL for stripe rust (SR) (TOOJINDA *et al*. 2000) and KD (DE la PENA *et al*. 1999). GL1-A2 appears to be closely linked to the *Mlg* gene on the chromosome 4H (Fig. 1). The close linkage between GL1-A2 and *Mlg* was also confirmed by RFLP analysis of the NILs. GL4-A2 and BN1-A10 were mapped near *Mla12* on the chromosome 1H (Fig. 1).

The discovery of conserved sequence motifs between plant disease resistance genes has resulted in their use to design candidate gene approaches for identifying resistance genes and analyzing their distribution in plant genome. In this study, 13 different classes of barley RGAs were isolated from barley using two pairs of degenerate primers. The results of our genome-wide mapping of the RGAs identified a number of new barley RGA loci: on chromosome 1H: BN1-A10 and GL4-A2; on chromosome 2H: BN1-C7; on chromosome 4H: GL1-A2; on chromosome 6H: BN2-D3 and on chromosome 7H GL2-D5, GL4-E1 and GL2-E4. All the above-mentioned RGAs did not find any significant matches during BLAST search and can be considered as unique RGA loci. However, some RGAs such as GL2-B11, GL2-B1 and BN1-D5 exhibited high similarity with the previously reported ones. Many authors have reported close associations between RGAs and disease resistance loci and QTLs (WANG *et al*. 2001, GRAHAM *et al*. 2000). In some cases genetic linkage of RGAs with already mapped R-genes manifests the physical proximity (~100 Kb) (WEI *et al*. 2002). This fact makes them extremely attractive for map based cloning of disease resistance genes. In addition, such RGAs can be useful markers in marker-assisted selection of resistant genotypes. In this study, many experimental RGAs were mapped in the vicinity of known R-genes.

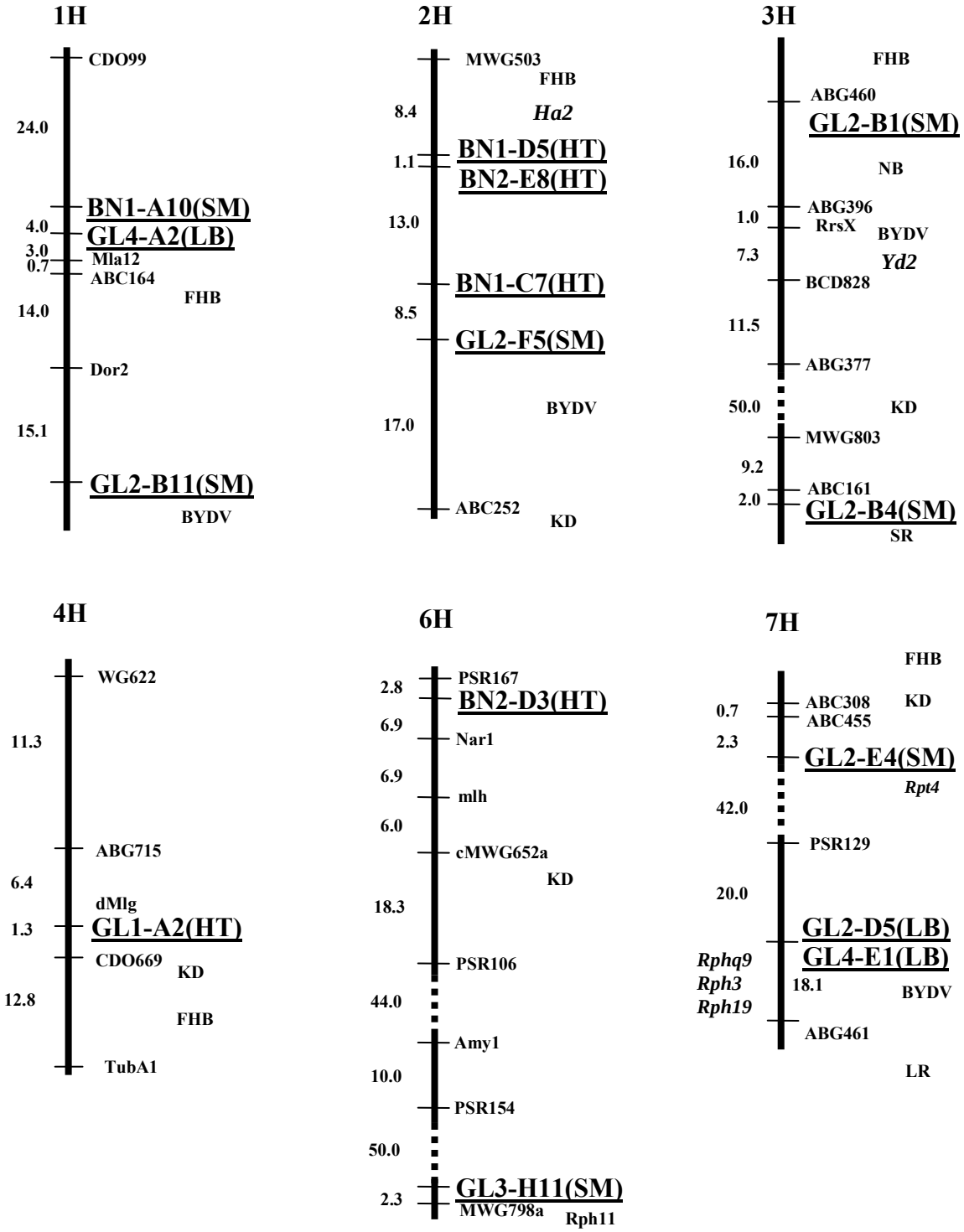

Figure 1. Genetic map of barley RGAs

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Identification of Marker Trait Associations in a Barley Four Way Cross

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Abstract
This paper presents the preliminary results from the genetic analysis of a barley four way cross. The population has been extensively phenotyped for malt quality and adaptation characteristics, through evalution as a mainstream breeding population within the South Australian Barley Improvement Program (SABIP). Of the 841 Doubled Haploid (DH) lines 837 lines were evaluated in double row trials, with 350 individuals promoted to stage one, 70 individuals to stage two and ten individuals to stage three. One line (WI3408) has subsequently progressed to commercial scale evaluation, with likely commercial release. Due to the large size of this population, high throughput technology was adopted for the genotyping. There were 290 microsatellite markers (SSRs), 90 of which were EST derived that were selected for the initial parental screening, based on their association with the traits of interest for this population. Of these combined markers, approximately 60% were polymorphic between one or more parents. An initial skeletal map is currently under construction using a minimum of 48 microsatellites from the parental screens. The 350 stage one lines will be screened more comprehensively as will the 70 stage two lines and the ten stage three lines respectively.
Keywords: barley; whole genome analysis; microsatellite markers

Introduction

Genetic mapping in cereals has been almost exclusively applied to populations derived from simple crosses. Genetic studies are now beginning to target broader population structures to take advantage of association mapping and whole genome analysis techniques. The germplasm used in this experiment is a significant departure from typical mapping population structures. This population will assess the ability of feed varieties to improve the agronomic performance of elite, malting barley varieties for Australian growing conditions, and the possibilities of breeding for a molecular ideotype.

There have been significant developments in marker technology allowing for marker saturation of key regions in the genome. This development has been made possible by the availability of markers closely linked to traits of interest within the breeding program.

Material and Methods

Germplasm

Initial crosses were made between Chieftan and Barque and similarly between Manley and VB9104, resulting in 'single cross' F_1 plants. These F_1 plants were then intercrossed, and 841 DH lines were derived from nine different intercross F_1 plants. The number of these DH lines generated per plant ranged from four to 332 with 206 lines and 332 lines being the two largest sets.

Phenotyping
Of the 841 DH lines, 837 were evaluated in double row trials in 1999 and are under evaluation again in 2004. All lines from 1999 trials were harvested, and data collected on screenings and quality traits were assessed by near infra-red spectroscopy (NIR). There were 350 selected individuals promoted to stage one yield trials in 1999, grown as one replicate at three sites, with seven cultivars as grid checks. Agronomic observations were recorded, yield measured and IOB wet-chemistry quality data obtained. 70 individuals were advanced to stage two yield trials in 2000 and evaluated in unreplicated trials at eight sites. Data was collected as in stage one but with more detailed quality analyses. Ten individuals were advanced to stage three yield trials in 2001, which were evaluated in replicated trials at eight sites.

Genotyping
DNA extractions were performed as described in ROGOWSKY *et al.* (1991) with some minor adjustments to facilitate the high throughput grinding of leaf material. An SSR based marker system was chosen to genotype this population using a combination of fluorescently labelled and unlabelled primers. These primers were amplified using a touchdown PCR reaction with an annealing temperature of either 50^{o} C or 55^{o} C. Each 12.5µL reaction contained 2µL DNA, reaction buffer (Qiagen), Q solution (Qiagen), 1.50mM $MgCl_2$, 10µM of each forward and reverse primer, 0.24mM dNTPs, 0.25 unit of Qiagen Taq polymerase. Three fluorescent labels FAM, HEX and NED were used to facilitate multipooling, or simultaneous analysis of these markers. Labelled samples were electrophoresed on the ABI 3100 and analysed with Genotyper software. Unlabelled SSRs were analysed on 8% acrylamide gels, stained with ethidium bromide and visualised using a gel documentation system. Due to the size of the population a 384 well plate format was used in conjunction with the Corbett CAS-3800 pipetting robot.

Results
The minimum density of SSR markers for linkage map construction was selected based on their map positions around known QTL and specific traits of interest (Table 1).

The parental germplasm was screened with 290 SSR markers. From this screening there were 174 polymorphic markers detected (142 fluorescent labelled and 32 unlabelled). Of these 174 markers, 20 could distinguish between each parental barley genotype in this study i.e. produce four alleles (Figure 1).

Up to eight PCR products, which were amplified using fluorescently labelled primers, were resolved simultaneously on the ABI 3100 (Figure 2). Markers were then assigned to groups also more commonly referred to as kits based on the expected size of the PCR product. Each group comprised markers with all three of the fluorescent labels. To date, 22 kits have been formulated for the labelled primers of which six kits have been completed and analysed for the initial parental screen.

For the purpose of the initial skeletal map, approximately four markers per chromosome arm (48 SSRs, 40 fluorescently labelled, eight unlabelled) were selected ensuring even coverage across the genome and relevance to the traits of interest. The molecular marker work for the skeletal map has been completed and the map construction is in progress.

Discussion

The full set of DH lines of the Barque/Chieftan//Manley/VB9104 cross is similar to a very large conventional mapping population. However, the population is derived from intercrossed F_1 plants and from four parents that are not all necessarily homogeneous and homozygous. Some markers appear to have more than four alleles present, suggesting that one or more of these parents could be heterogeneous and/or heterozygous. Preliminary analysis suggests that the additional alleles were derived from Barque. However, further investigation would also support that VB9104 is not a fixed line and is heterozygous for some loci. Using additional SSR markers, a high density map will be generated on a sub group of the initial 841 DH lines, comprising the 350 stage one lines. This will facilitate measurement of frequency changes to alleles in response to pragmatic selection, and the identification of conserved linkage blocks associated with superior agronomic or quality phenotypes.

This analysis will determine the genetic basis of the elite line WI3408, which is a member of the largest family of 332 lines. WI3408 is currently being considered for commercial release, targeting medium-low rainfall production environments and export markets requiring high diastase and highly fermentable malt.

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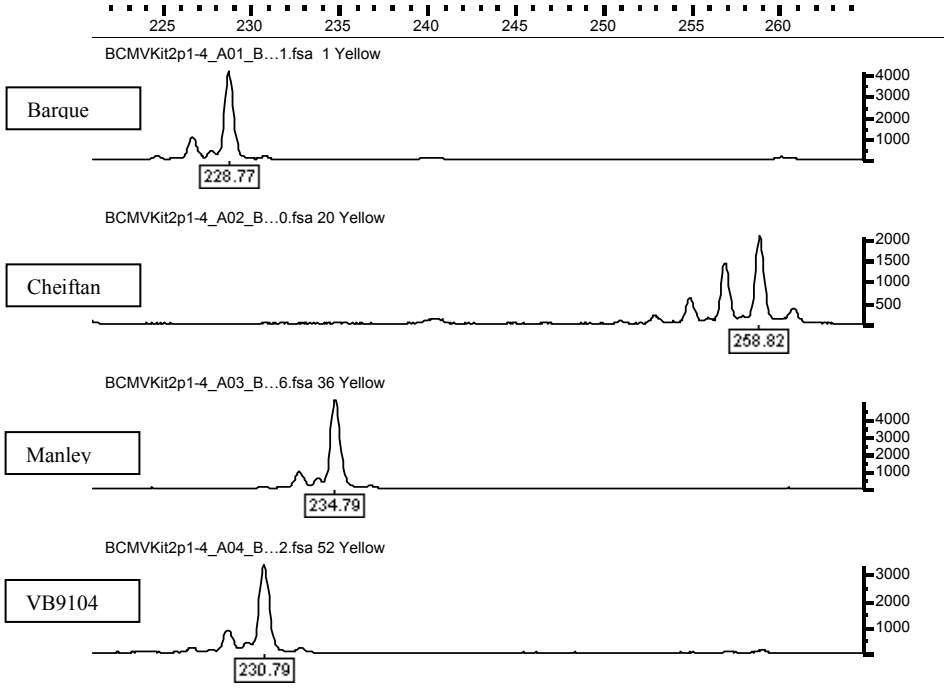

Figure 1. Results of parental screen using a fluorescent labelled SSR marker which distinguishes between the four barley genotypes

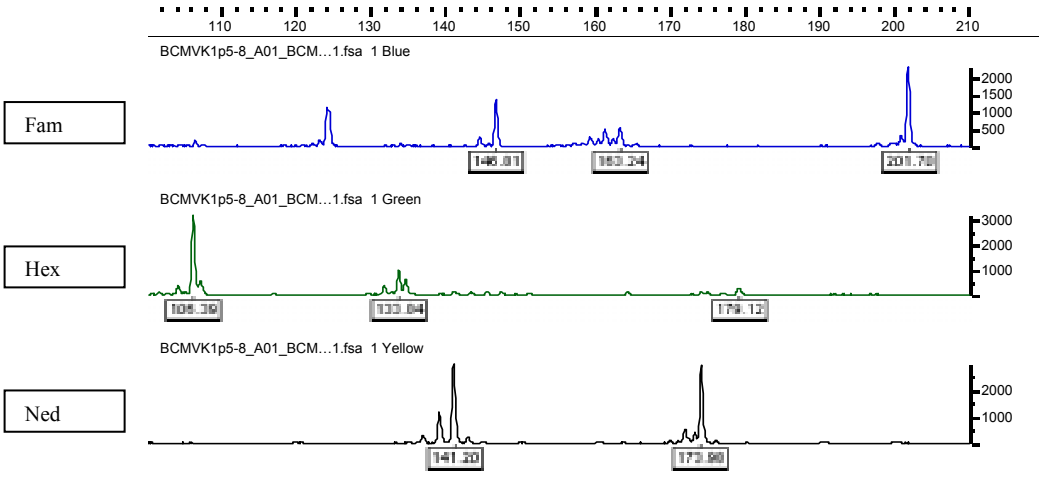

Figure 2. Multiplexing results with eight SSR markers on parental genotype Barque

Table 1. Characteristics of the four parental lines, Chieftan, Barque, Manley and VB9104. The key genetic locations presumed to influence the traits are also shown.

	Chieftan	Barque	Manley	VB9104	Chromosome
Scald	R	S	S	MR	3H
SFNB	S	R	S	MR	7H
Mildew	R	MR	S	S	2H,4H
Height	SD	T	T	T	3H
Maturity	L	E	L	E	2H
Leaf rust	R	S	S	S	5H,7H
Lodging	R	MS		MS	2H
Extract	H	L	H	M	1H,2H,5H
DP	M	L	VH	M	1H,4H,5H
Viscosity	L	H	L	MH	1H
Fermentability	M	L	VH	M	4H,6H
CCN(Ha4)	S	R	S	S	5H
Yield	M	H	L	H	2H,3H,4H,7H
Grain size	SM	ML	M	VL	2H
Ppd		sens		sens	2H
Eps		E		E	2H

SFNB – spot form net bloch; DP - diastatic power; CCN – cereal cyst nematode; *ppd* – photo period response; *eps* – earliness *per se*; R – resistant; S – susceptible; MR – moderately resistant; MS – moderately susceptible SD – semi-dwarf; T – tall; L – late; E – early; H – high; L – low; M – moderate; VH – very high; MH – moderately high; SM – small to medium; ML – medium to large; M – medium; VL – very large; sens - sensitivity

The Multivariate Data Analysis Revolution in Genetics, Plant Breeding and Biotechnology

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Abstract
The main focus in genetics since the rediscovering of the laws of Mendel 100 years ago has been centered around gene analyses. As the developmental geneticist Waddington in 1969 pointed out: "They say nothing what so ever about the actual phenotypes concerned". Classic statistics is set up for studying gene populations and has difficulties in characterising biological individuals and the imprint of genes on the phenotype. This paper presents supplementary experiments to our earlier publications and aims at proving that NIR spectroscopy and multivariate data analysis will be able to revolutionise the view of the mutual connections between the genome and phenome aspects of data.
Near infrared reflection spectroscopy (NIR) technology, now widespread in quality control, makes it possible with high precision to obtain a total multivariate physical chemical fingerprint of the barley endosperm. Whole spectroscopic fingerprints of the physics and chemistry of barley seeds can be interpreted by multivariate analyses such as Principal Component Analysis (PCA) for classification and Partial Least Squares Regression (PLSR) for correlation. PCA classification of NIR spectra can differentiate between mutants and alleles in the *lys3* and *lys5* loci and can as well be used as specific multivariate selection criterion for improving a multigene quality complex in barley as a whole increasing starch and reducing fibre content. Based on classification of NIR spectra two alleles in the *lys5* locus were characterised as a new class of (1→3,1→4)-β-glucan compensating starch mutants.

Material and Methods

Two barley materials are used: Material I: 49 samples of *lys5f* and *lys5g* low starch mutants (DOLL 1983; JACOBSEN *et al*. 2004)) with moderate increase in lysine (appr.10%) and recombinant crosses as well as normal barley lines were grown in greenhouse (n=13), outdoor pots (n=7) and field (n=6) in 2000. Material II: 14 samples of the very high mutants *lys3a* and *lys3m* and seven segregants (0502, 0505, 0531, 0538, 0556, Lysimax, Lysiba) as well as six normal barley lines (two samples of Minerva, Bomi and Triumph) were grown in the field in 1991. Multiplicative signal corrected (MSC) NIR measurements were obtained on flour from all samples (MUNCK *et al*. 2001). Protein, Amide, Starch and (1→3,1→4)-ß-glucan were determined according to MUNCK *et al*. (2001). The "Unscrambler" software (Camo A/S, Trondheim, Norway) was used.

Results and Discussion

Differentiating between Gene Specific and Environmental Effects on NIR Spectroscopy Patterns from Barley Endosperms

Figure 1A shows the MSC-corrected NIR spectra from 49 samples of barley grown in greenhouses, outdoor pots and in the field. The barley material consists of normal barley varieties (N) and two low starch mutants (*lys5f* and *lys5g*) selected at Risø (MUNCK *et al*. 2004) as moderate high-lysine lines with the dye-binding method. The *lys5f* mutant is more

drastic than *lys5g*. The mutants are in the same locus and are identical to Risø mutant 13 (background Bomi) and mutant 29 respectively (background Carlsberg II). In this material there are additional homozygotic mutant recombinants (doubled monoploids) from crosses with normal barley.

By using the unsupervised classification algorithm PCA in evaluating the whole spectra in Figure 1A, six clusters according to genetic parameters as well as for environment can be interpreted by consulting the field book (Figure 1B). Normal samples are located diagonally in the top left corner, whereas *lys5* mutants and recombinants are spread diagonally in the bottom right corner. There is a tendency that the more drastic mutant *lys5f* is placed in the periphery of the *lys5* clusters (Figure 1B). Furthermore, it is seen that samples in the two clusters grown in greenhouse are located diagonally in the bottom left corner below the division line, samples from field (bold) are located in the middle and samples grown in outdoor pots are placed diagonally in the top right corner.

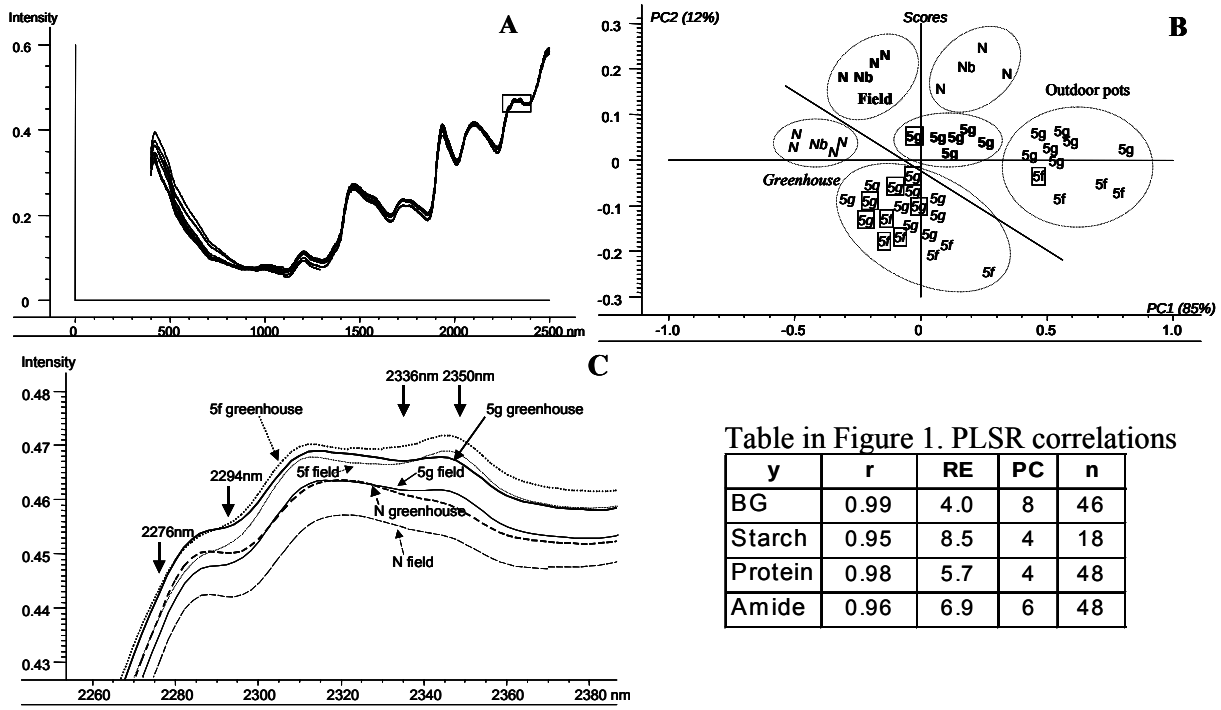

Table in Figure 1. PLSR correlations

y	r	RE	PC	n
BG	0.99	4.0	8	46
Starch	0.95	8.5	4	18
Protein	0.98	5.7	4	48
Amide	0.96	6.9	6	48

Figure 1A. Average NIR spectra (MSC) from normal barley grown in greenhouse (n=5) and field (n=8), *lys5f* mutants and crosses grown in greenhouse (n=6) and outdoor pots (n=4) and *lys5g* mutants and mutant recombinants crosses grown in greenhouse (n=13), outdoor pots (n=7) and field (n=6). **B.** PCA (PC1:2) score plot of NIR spectra (MSC) for 49 samples of normal barley (N, Nb=Bomi) and mutants and mutant recombinants crossings of *lys5f* (*5f*) and *lys5g* (*5g*) grown in three environments: *greenhouse*, **field** and in pots outdoors. Squared samples are original mutants. **C.** Average spectra of normal barley (N) and mutants of *lys5f* (*5f*) and *lys5g* (*5g*) grown in greenhouse and field. **Table.** PLSR predictions of chemical properties (BG=1→3,1→4)-β-glucan) from NIR spectra (MSC). RE = relative error in percentage, PC = number of Principal Components used in the model.

Because samples located near each other in a PCA plot indicate similar pattern of variables, here NIR wavelengths, mean spectra from the six different clusters in Figure 1B can be characterised by visual inspection. In Figure 1C an interesting small area from 2260-2380nm marked with a square in Figure 1A is enlarged for the average spectra of the six classes in the PCA in Figure 1B. It is easily recognised that the average curve form in Figure 1C of the normal barley (N, stippled lines) is quite different from those of the mutants (punctuated line

lys5f, whole line *lys5g*). While the normal barley´s have a decided plateau at 2285-2295nm the slope is here much steeper with the two *lys5* mutants. Likewise the mutants have a characteristic bulb at approximately 2350nm which are almost non existent in normal barley. This bulb is larger in the spectra of the *lys5f* mutant than for that of *lys5g*.

When comparing growing locations in Figure 1C it is seen that the curve form for each mutant genotype is approximately the same while an offset base line indicates growing location where the greenhouse has a higher intensity than the field. As indicated from the PCA in Figure 1B, the original *lys5f* and *lys5g* mutations (marked with a square) are drastic enough to obtain full penetrance in the recombinants. Thus the recombinants are classified together with the original mutants in the PCA. As demonstrated by us (JACOBSEN *et al*. 2004; MUNCK *et al*. 2004) it is also possible to differentiate between several mutants and alleles from the *lys3*, *lys5* loci and other loci in the same data set with PCA.

Verifying the Chemical Nature of the lys5 PCA Cluster as (1→3,1→4)-β-Glucan Compensated Starch Mutants

In an unsupervised PCA, where one does not know the origin of the samples, the spectra may be inspected using prior knowledge regarding critical wavelengths indicative for certain chemical bonds in order to define analyses for chemical validation. In comparing the wavelength areas indicating different chemical compounds and bonds (Figure 1C), it is seen that the mean spectra from the mutants and normal barleys are different from each other at the wavelength about 2276nm correlating to starch, at 2294nm describing amino acids, at 2336 and 2352nm characteristic for cellulose content and unsaturated fat at 2347nm. From here it is possible to make hypotheses about differences in chemical composition and to evaluate these by choosing proper reference analyses for final validation.

Several authors like DOLL (1983) and GREBER *et al*. (2000) have suggested that the high lysine mutants of barley could relate to starch synthesis which is more or less reduced in these mutants. It was therefore surprising when we found (JACOBSEN *et al*. 2004; MUNCK *et al*. 2004) that *lys5f* and *lys5g*, which all were classified by NIR spectroscopy and PCA in the same cluster, had a strongly increased content of (1→3,1→4)-β-glucan which to a large extent compensated the low starch content. In Table 1 the chemical composition of the six clusters and the individual genotypes classified in the experiment in Figure 1 are shown. The drastic increase in (1→3,1→4)-β-glucan especially for the *lys5f* genotype - up to 20% in dry matter - is clearly seen. The *lys5g* genotype seems to fully compensate the loss in starch with (1→3,1→4)-β-glucan as seen from the total figure of (1→3,1→4)-β-glucan plus starch (BG +S, Table 1) which is comparable to the normal controls.

Table 1. Chemical properties (BG=(1→3,1→4)-β-glucan) of the six groups in Figure 1 as well as for *lys5f* and *lys5g* compared to Bomi.

		n	BG	Starch (S)	BG + S	Protein	Amide
N	Greenhouse	5	7.4±1.7	50.1±3.9[a]	57.5±2.5[a]	15.0±1.4	0.41±0.05
5	Greenhouse	19	14.6±3.0	33.5±7.5[a]	51.6±4.5[a]	17.5±1.3	0.44±0.04
N	Field	4	5.0±0.9	55.9±3.5	60.9±2.8	10.5±0.8	0.26±0.03
5	Field	6	8.5±0.9	43.2±4.0[a]	55.1±4.4[a]	12.4±0.7	0.28±0.02
N	Outdoor pots	4	7.4±0.6[b]	44.6±1.3[c]	52.0±0.6[c]	16.7±0.4[b]	0.44±0.02[b]
5	Outdoor pots	11	13.3±2.6	-	-	18.1±1.1	0.44±0.04
Bomi compared to *lys5f* and *lys5g* mutants							
Bomi	Greenhouse	1	6.8	48.8	55.6	14.6	0.4
lys5f	Greenhouse	3	19.6±0.4	29.8±0.6	49.4±1.0	17.0±1.4	0.42±0.06
lys5g	Greenhouse	6	13.1±0.8	44.7[d]	58.2d	17.4±1.0	0.43±0.04

[a]n=4, [b]n=3, [c]n=2, [d]n=1

Greenhouse conditions increases both protein and (1→3,1→4)-β-glucan while keeping the relative differences between the different genotypes. The function of the highly reproducable NIR spectrograph as a "multimeter" is clearly pointed out in Figure 1 (Table) displaying fully cross validated PLSR prediction values for whole spectra in correlations to the different chemical analyses including (1→3,1→4)-β-glucan. This explains also the high discriminating power of the PCA in Figure 1C using the same NIR spectral data.
The question is now if NIR spectroscopy interpreted by chemometrics is restricted to detecting mutants with major effects on chemical composition? Is it possible to use this technology in regular plant breeding e.g. to improve starch and reduce fibre content in the very high lysine genotype *lys3a* - Risø mutant 1508 by crossbreeding and selection?

The Feasibility of Selecting for an Improved Gene Background for High Starch, Low Fiber to the lys3 Gene for Starch by NIR Spectroscopy and Chemometrics

As demonstrated by MUNCK (1992) it is possible to breed for an improved kernel quality and yield with the *lys3a* mutant to reach the level of normal barley's. This results in an improved starch content from about 48 to 53% which is equal to normal feed barley's. A library of the original mutants, normal barley lines and yield-improved crosses was evaluated by NIR. A section of this data bank from material grown in the field in 1991 is demonstrated in a PCA (Figure 2A) which shows the pattern of the 14 NIR spectra 400-2500nm characteristic for six normal barleys, two mutants and seven crosses in F5-10.

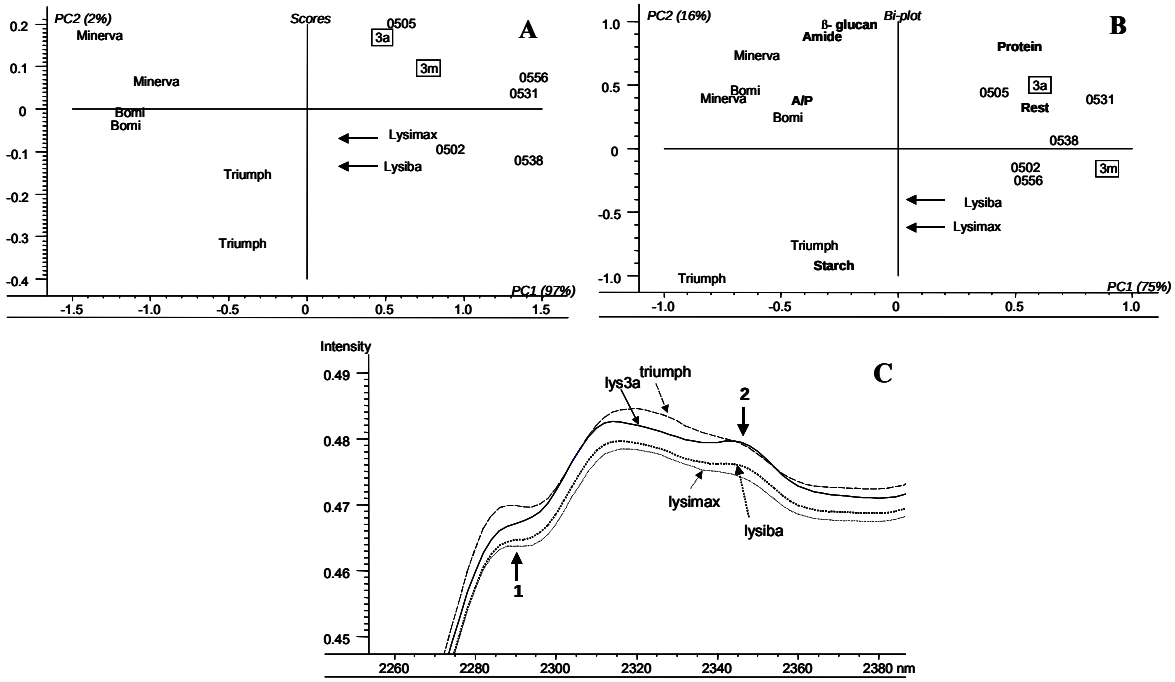

Figure 2A. PCA score plot of NIR (MSC) spectra from normal barley (Bomi, Minerva, Triumph), mutants (*lys3a*, *lys3m*) as well as high lysine recombinant lines (0502, 0505, 0531, 0538, 0556, Lysiba, Lysimax). **B.** PCA biplot of chemical data (protein, ß-glucan, amide, A/P, starch) at the same material. **C.** Comparison of the MSC NIR area 2260-2380nm for samples of Triumph, mutant *lys3a* and high lysine recombinant lines Lysiba and Lysimax. Numbers are referred to in text.

It is clearly seen that the samples divide into two clusters: one with normal barleys (Bomi, Minerva and Triumph) to the left, and the original *lys3* mutants (squared) *lys3a* and *lys3m* and their high-lysine segregates to the right. Lysiba and Lysimax are semi-commercial high-lysine varieties with verified improved yield (about 10-15% better than the original mutant). The lines Lysiba and Lysimax are thus *a priori* defined as positive selections. They are in the NIR

PCA situated closer to the normal samples than for example lines 502, 531 and 538, which are negative selections confirmed by their locations above and to the far right in the PCA (Figure 2A). If an unsupervised PCA is performed on chemical data (protein, amide, A/P, starch, ß-glucan and rest = (100-(protein + starch +ß-glucan)), almost the same pattern (Figure 2B) as with NIR spectra (Figure 2A) is seen, indicating the physical-chemical basis of the NIR measurements.

The PCA bi-plot of chemical data in Figure 2B shows that samples of Triumph, which are known to have a very high starch content, are located close to the starch variable, indicating that these samples are highly influenced by this variable. Opposite, still to the left, are Minerva and Bomi samples highly influenced by amide, A/P and ß-glucan. To the very right the mutants *lys3a* and *lys3m* are placed with the high-lysine segregates and together with the variables protein and rest. The samples of Lysiba and Lysimax (Group 1) are located closer to the normal varieties than the others, indicating a positive selection (with regard to starch) as in the PCA with the NIR spectra. Samples 502 and 556 (Group 2) are located close together, whereas the negatively selected samples 505, 531 and 538 (Group 3) are placed in between the mutants.

When comparing the chemical data of these four groups one can easily see (Table 2) that Group 1 with Lysiba and Lysimax has protein and starch content closer to normal varieties. The amide content and A/P index is, however, more closely related to the mutants. This means that they still are likely to have a content of essential amino acids as high as the original high-lysine mutants combined with an increase in starch (mean) from 48.7 to 52.6 %. Group 2 is intermediate in starch content with reduced protein content, while Group 3 has as high protein content as high as the original mutants with only a slight increase in starch. The wavelength area used in earlier investigations (2260-2380nm) is compared for spectra from samples of the normal barley Triumph, *lys3a* and from the positive selected lines Lysiba and Lysimax. From Figure 2C it is seen that the curve forms for Triumph and *lys3a* are very different, and that those of Lysiba and Lysimax are intermediate between *lys3a* and normal barley.

Table 2. Average and standard deviation of chemical data for the five groups from the *lys3* breeding experiment.

	Normal (n=6)	Group 1	Group 2	Group 3	Group 4
Protein (P)	11.3±0.4	11.7±0.1	11.7±0.1	12.6±0.2	12.5±0.2
Amide (A)	0.28±0.03	0.21±0.007	0.21±0.007	0.22±0.02	0.23
A/P	15.5±0.9	11.0±0.3	10.9±0.4	10.7±0.8	11.4
Starch	54.6±2.5	52.6±0.5	50.0±0.1	49.4±1.5	48.7±0.2
ß-glucan	4.7±1.1	3.1±0.1	3.1±0.2	3.1±0.3	2.8±0.5
Rest (100-P+S+BG)	29.5±1.8	32.7±0.5	35.3±0.3	34.9±1.8	36.1±0.5

Group 1= Lysiba, Lysimax Group 2= 502, 556 Group 3= 505,531,538 Group 4= *lys3a*, *lys3m*

The improved high-lysine lines have adopted some characteristics from the normal curve form. In the wavelength area 2285 to 2295nm (arrow marked 1) the Triumph curve form is horizontal and *lys3a* is diagonal, while Lysiba and especially Lysimax are approaching the normal horizontal condition.

It is also seen that the bulb in *lys3a* at the arrow marked 2 (Figure 2C) is much reduced in Lysiba and especially Lysimax indicating a more normal barley state. This area correlates to unsaturated fat (2347nm) and cellulose (2352nm). The content of these analytes should be tested further to detect the reasons why the samples separate from each other. A lower content of unsaturated fat and cellulose (fibre) in Lysiba and Lysimax should be expected.

Data Breeding - NIR Spectroscopy and Chemometrics in Breeding for Improved Physical-Chemical Composition as Whole Integrated Genetic Complex

We first introduced the concept of "data breeding" at the IBGS-VIII meeting in Adelaide in 2000 (MUNCK *et al*. 2000). As seen in the PCA of the whole NIR spectra in Figure 2A it is possible by crossbreeding and selection to move the *lys3a* segregants Lysimax and Lysiba in the direction of the arrow towards normal barley (Triumph), signifying an improved starch content verified by the PCA for the chemical analyses in Figure 2B and Table 2. Near Infrared Transmission (NIT) spectroscopy is today used as a "blackbox" technology in plant breeding by analysing for specific chemical parameters such as protein and water by a closed chemometric prediction model given by the instrument manufacturer. By introducing chemometrics to be used directly by the plant breeders the closed box can be opened so that they themselves can make their own NIT barley seed classifications and correlation models tailored to their own materials and needs. This is demonstrated for malting barley in these proceedings by MØLLER and MUNCK (2004).

The need for a paradigm shift to obtain a more balanced view between the genotype and phenotype by classifying the (barley seed/endosperm) phenotype as a whole physical-chemical pattern to monitor gene expression was forecasted already in 1969 by the great geneticist C.F. Waddington (1969). First now such a much needed paradigm shift is possible by combining spectroscopy (NIR) with chemometrics (PCA) as visualised in this paper. **We conclude that different mathematical models should be used for the gamete and zygot levels of biological organization (MUNCK 2003) where classic probability statistics effective on the gene recombination level should be complemented by pattern recognition data analysis (chemometrics) for analysing gene expression at the phenotype level.**

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Identification of RAPD Markers Linked to Salt Tolerance in Cultivated and Wild Barleys

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Abstract
Randomly amplified polymorphic DNAs (RAPDs) were used to search for markers associated with salt tolerance in barley. Initial screens involved growing 63 cultivated and wild barley genotypes in saline conditions and testing for shoot sodium content along with other physiological traits. From these tests 5 tolerant and 5 non-tolerant genotypes were selected. DNA from the tolerant and non-tolerant genotypes were formed into two contrasting bulks and interrogated using 30 different 10-mer RAPD primers. One primer (P15) produced a band found only in tolerant lines, and additionally produced a smaller product found only in the non-tolerant group. Primer P10 produced a band specific to the tolerant bulk and P22 produced a band specific to the non-tolerant group.
Keywords: RAPD markers; salt tolerance; cultivated and wild barley

Introduction

Soil salinity is a major concern in arid and semi-arid areas of the world, including Iran. The exploitation of tolerant crops is one approach to the problem and barley is considered to be the most salt tolerant cereal crop (MASS et al. 1977; GILL & DUTT 1987). Wide genetic variation exists for salt tolerance in both cultivated (*Hordeum vulgare*) and wild (*H. spontaneum*) barley germplasm (PAKNIYAT *et al*. 1997). Common tests for salt tolerance in barley have involved measuring tissue sodium content, carbon isotope composition and proline content which have been correlated with dry matter content in salinity tests (FORSTER *et al*. 1994; PAKNIYAT *et al*. 1997, 2003).

A wide range of genetic markers is available for study in barley. Here we use RAPDs as a quick and easy method to search for DNA markers associated with salt tolerance in selected lines contrasting for tolerance. In a previous study PAKNIYAT *et al*. (2003) used shoot sodium and proline content to screen for salt tolerance in 63 Iranian genotypes of cultivated and wild barley. From this study we selected the most tolerant and most non-tolerant genotypes. RAPD fingerprinting of the two contrasting bulks was used to identify markers associated with tolerance and non-tolerance and tested further in other material where tolerance had been measured.

Material and Methods

Plant Materials and Genomic Isolation

A total of 14 wild and cultivated Iranian genotypes were selected; 5 most tolerant, 5 most non-tolerant, and an additional group of 4 tolerant genotypes (Table 1).

DNA was extracted from two week old seedlings of the 14 genotypes using the CTAB method (DOYLE & DOYLE 1987). DNA was extracted separately from each individual genotype. Two bulks were formed, a tolerant and a non-tolerant bulk (Table 1) and used in a bulked segregant analysis (BSA, MICHELMORE *et al*. 1991). DNA bulks consisted of 3µl (15ng μl^{-1} DNA) from each contributing genotype. The additional tolerant genotypes were used to test further any selected marker from the BSA.

Table 1. Fourteen selected barley lines formed into tolerant and non-tolerant groups using shoot Na^+ content data from PAKNIYAT *et al.* (2003)

Genotypic identification	Na^+ content mg/g dry weight
Tolerant bulk genotype	
Vineyard (Hs)	10.77
Afzal (Hv)	12.17
Victoria (Hv)	14.83
Plot. No. 21 (Hs)	15.50
Na-cc-4000-123/Walfajre (Hv)	15.75
Non-tolerant bulk genotypes	
Vineyard (Hs)	24.33
Asse/Karoon (Hv)	24.67
Reihan (Hv)	25.17
Star/Jenusa/em//Rihan-03(Hv)	27.00
Additional tolerant genotypes	
Plot. No. 34 (Hs)	16.20
80-5010/Mona (Hv)	17.77
Valfajre (Hv)	16.90
Black grain (Hv)	17.67

Table 2. Primer nucleotide sequence used to amplify DNA

Primer designation	Sequence 5′-3′	Primer designation	Sequence 5′-3′
P1	ACACAGAGGG	P16	CCTGGGCTTC
P2	CCTCTCGACA	P17	CCTGGGCTTG
P3	TCTCAGCTGG	P18	CCTGGGCCTA
P4	GTGTGCCCCA	P19	CCTGGGCCTC
P5	CCACGGGAAG	P20	TGCCCCGAGC
P6	TCGGCGGTTC	P21	TTCCCCGACC
P7	CTGCATCGTG	P22	GAGGGCGGGA
P8	TGAGCCTCAC	P23	AGGGGCGGGA
P9	TCGGCACGCA	P24	GAGGTCCAGA
P10	CTGCGCTGGA	P25	GGGGGTTAGG
P11	CCATTCCCCA	P26	ATCGGGTCCG
P12	GGTGAACGCT	P27	CCGTGCAGTA
P13	CTCCCTGAGC	P28	TAGCCGTGGC
P14	TTCCGGGTGC	P29	GGCTAGGGGG
P15	GAGCTCGCGA	P30	TACGTGCCCG

Polymerase Chain Reaction Conditions

PCR was carried out essentially as described by WILLIAMS *et al*. (1990). Thirty 10-mer oligonucleotide primers were used for DNA amplification (Table 2). Amplifications were performed in a Technogene Co. thermocycler with one cycle of 30 mins at 94°C followed by

40 cycles of: 1 min at 94°C, 1 min at 38°C and 1 min at 72°C. After the final cycle samples were incubated at 72°C for 4 minutes and then held at 4°C prior to analysis. Amplification products were separated by electrophoresis in 0.7% agarose gels and detected using ethidium bromide and UV light. Each amplification was performed using a single primer and gels scored for the presence and absence of products.

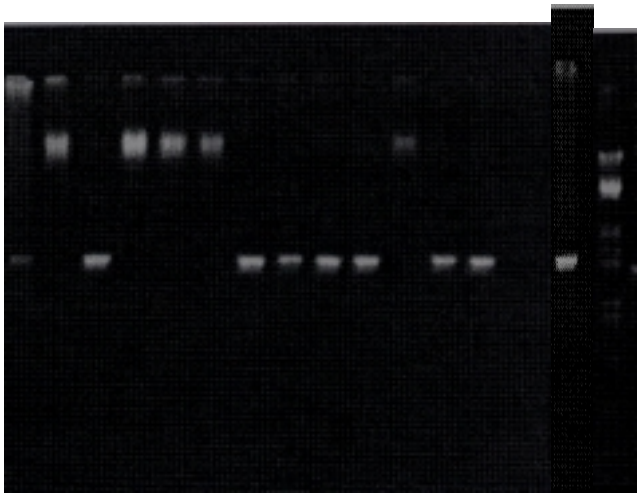
Results and Discussion

Four primers (P3, P26, P27 and P30) did not produce amplification products, two (P7 and P28) produced vague un-scorable bands and P17 and P20 did not produce any polymorphic bands. The remaining 22 primers produced polymorphic bands suitable for BSA, of these 20 produced bands that were specific to one or other of the two contrasting bulks. P10, for instance, produced a band that was present only in the non-tolerant bulk and P22 generated a band that was present only in the salt tolerant bulk.

Once discriminating primers were identified they were tested further and applied to each individual making up the bulks. P15 was particularly interesting as it produced two polymorphic bands: a large band product that was present only in non-tolerant genotypes and a smaller band that was present only in salt tolerant genotypes including the additional salt tolerant genotypes (Figure 1). These bands are therefore useful in screening for salt tolerance in both cultivated and wild genotypes and may be the first case where two RAPD markers from a single primer alternate between extremes for a trait.

The results indicate that RAPD technology is a powerful tool in quickly identifying markers in BSA, in this case for salt tolerance. It is hoped that the discovery of markers associated with salt tolerance with aid the identification of the genes involved. One strategy for the P15 products is to sequence the smaller band and search against stress-related ESTs in public databases to identify the gene involved. T strategy can then be repeated for the larger band to see if the products are allelic and the result of an indel. Sequence data can also be used to develop more robust PCR primers as diagnostics for salt tolerance. Another strategy is to map the two P15 products to confirm or otherwise their genetic co-location.

Figure 1. P15 RAPD banding profile of the 14 barley genotypes screened. The larger band (upper) is present only in non-tolerant genotypes and the smaller (lower) band is present only in tolerant genotypes.



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Small Mapping Crosses and Their Use To Establish a Broad Based QTL Map for Barley

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Abstract
Quantitative traits (QTLs) are identified by selecting and hybridising parental lines that differ in one or more QTL. Thus to examine numerous agronomic traits many different crosses are required: This cross specific nature limits the adoption of marker assisted breeding (MAS) in barley. We propose to maximise the number of cross specific populations thereby enabling the production of one robust, broad based QTL map: We have used 11 small mapping populations of a target size of 20 individuals. These F_1 doubled haploid (DH) populations were derived from crosses made from UK national list trial entries in 1999. The populations were grown at two locations (SCRI and NIAB) to account for genotype and environment interactions. Microsatellite markers from the SCRI barley framework map have been used to genotyped the 200+ DH lines, facilitating the construction of one combined linkage map. We have then used this map to search for malting quality components QTLs using single market analysis. A number of component characters appear to co-segregate, limiting the opportunities for individual manipulation. Nevertheless, the results have identified markers for QTLs that are relevant to elite breeding populations, demonstrating that there is still usable genetic variation within an apparently restricted gene pool.
Keywords: barley; mapping; QTLs; malt quality; elite cultivars; small cross mapping

Introduction

For the successful implementation of Marker Assisted Selection (MAS) the genetic architecture of traits needs de-convoluting and the components associated to reliable markers. Numerous efficient molecular marker systems have been developed providing hundreds of publicly available markers. The dissection of complex traits, as quantitative trait loci (QTLs), is now underway in earnest. And, whilst model plant species are useful for revealing generalised biological functions, they cannot provide information on crop specific traits, i.e. malting quality of barley.

Nevertheless, barley has been described as a model crop for the Triticae species (HORI *et al*. 2003). It is the fourth most important cereal crop worldwide; second most within the UK being grown over 1.25 Mha and producing 6.7 Mt of grain in 2001/2 (THOMAS 2003). When meeting quality criteria barley grain has long been recognised as a good source of free sugars by malting. To these ends, in the EU over 20 breeders are yearly submitting lines for trial (THOMAS 2003).

The malting process involves the controlled germination of barley grain, where proteolytic enzymes digest cell walls allowing amyolytic diastatic enzymes to degrade starch granules. Polymeric carbohydrates and proteins are then broken down to component sugars, amino acids and peptides, providing the substrate sugars and nitrogenous compounds for fermentation by yeast. Current cultivars are defined by phenotypic measurements of yield and malting quality, the later being primarily measured by the amount of material that is solublised by hot water extraction following micro-malting under controlled conditions.

To discern components of these complex traits barley has been the subject of considerable genetic mapping and QTL studies. The construction of a meiotic linkage map with molecular markers has been described as the key step in the dissection of biological or agronomically important traits (HORI *et al*. 2003). However it is clear that no one molecular map of barley satisfies the demands of both breeders and geneticists alike (STAUB *et al.* 1996). Indeed over 40 maps of barley have been produced since 1991 (THOMAS 2003). In order to maximise the polymorphism-recombination levels and locate the most markers, then Elite x Exotic crosses are prescribed (STAUB *et al.* 1996). However, the genomes from such crosses are unreflective of current breeding practice and so results of QTL studies have had little impact on plant breeding up to now. Another approach would be to create Elite x Elite maps with information relevant to current cultivars. Concern has been raised however that the genetic diversity of barley is diminishing due to breeding and selection. KOEBNER *et al*. (2003) have investigated the genetic diversity of current cultivars, with those from the 1970's founding, however, little reduction. Further, 34 loci from the SCRI core Simple Sequence Repeat markers (SSRs) revealed 70 different alleles from within candidates for the 2002 UK recommended list (providing $6x10^9$ different allele combinations) (THOMAS 2003). Whilst no one elite pair cross adequately samples this diversity, the formation of a composite population from a number of elite pair crosses, each of relatively few individuals provides a good sample. We term this approach Small Cross Mapping (SCM) and it has the added advantage of being able to identify QTLs that are robust over several different genetic backgrounds. This paper describes the application of SCM to some elite UK germplasm to identify some QTL from component characteristics of malt quality.

Material and Methods

From pair crosses between 14 elite parents, 11 Doubled Haploid (DH) populations were derived by anther culture, and multiplied in the glasshouse. In 2001, the trials were grown at the SCRI site near Dundee, Scotland. There was only sufficient need to sew a single replicate and a Modified Augmented Design (MAD) 2 (LIN & POUSHINSKY 1985) was used with Optic as the main control and Decanter and Static as the sub-controls. In 2002, there was sufficient need to grow a replicated trial in a row and column design at the SCRI site and another single replicate MAD2 design at the NIAB site in Cambridge, England. A standard malting fertilizer regime was applied to each trial at each site with fungicides applied to prevent the development of foliar pathogens. At maturity, the plots were harvested with a small plot combine and the seed dried to a constant moisture content of 12% and then weighed. The samples were then cleaned and sieved and the fraction passing over a 2.5mm sieve retained and micro-malted by NIAB. Malt samples were then passed to Heriot Watt for extraction and analysis of the wort for concentrations of β-glucan, glucose, fructose, sucrose, maltose and activities of α-amylase, β-amylase, β-glucanase and limit dextrinase. In addition the Free Amino Nitrogen content (FAN) was measured. Grain samples were retained by SCRI and the MARVIN digital seed analyser (GTA Sensorik Gmbh, Germany) used to measure average grain length and width (from which the width to length ratio was calculated) and thousand kernel weight (TKW). Finally, milling energy was estimated by the Comparomill (ALLISON *et al.* 1979).

DNA was extracted from young leaf material from these individuals using a Qiagen Dneasy96 Plant DNA extraction system. Each DH individual was genotyped using simple sequence repeat (SSR) markers designated *BMAC, BMAG, EBMAC and HVM,* plus an arbitrary number (http://ukcrop.net/barley.html). Amplification condition were described RAMSAY *et al*. (2000); briefly, though forward primers were end-labelled with 0.3μCi [γ-^{32}P]ATP and the temperatures used for annealing were either 58^{o}C or 55^{o}C for 1 min, or alternatively a touchdown annealing cycle of -0.5^{o}C from an initial temperature of 64^{o}C and

then additional rounds at 55°C for 1min. The marker alleles were scored from X-ray film exposures according to size (scored A…G).

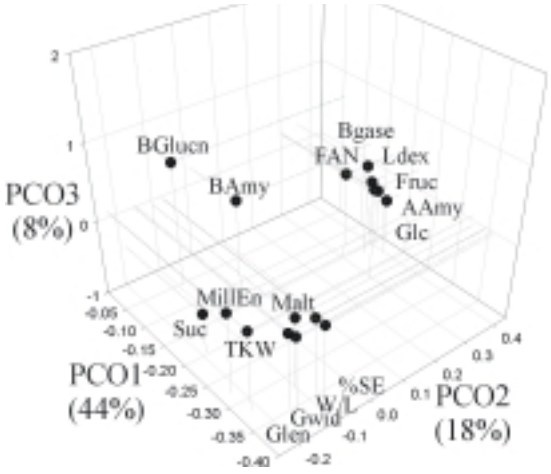
The means from each trial for each variate were used to calculate the overall mean for each individual. Principal Component Analysis (GENSTAT) was then used to show the multi-variate relationships between the variables using the correlation matrix. The means were then combined with genotypic data and ANOVA (GENSTAT) used to identify SSR loci that were associated with significant differences (P<0.005) between allelic classes for each character. MAPCHART (VOORRIPS 2002) was used to display the SSR map locations and QTLs using the map information presented by RAMSAY *et al*. (2000) with some modifications.

Results and Discussion

Discluding five monomorphic markers, on average 43% of the SMC populations segregated for each marker. Given an average of nine markers per linkage group then, at this level of population polymorphism, three markers in one population would be expected to also segregate in another: These would provide the basis for map joining and the creation of one combined map using JOINMAP (VAN OOIJEN & VOORRIPS 2001).

An increasing number of quality characters are used in evaluating malting barley but these analyses may not necessarily be independent (NIELSEN & MUNCH 2003). Principal Component Analysis (PCO) of the correlations between the malt quality traits showed that there were two groups with broadly similar partitions on the first three axes (Figure I).

Figure I. Partitioning of malt quality traits



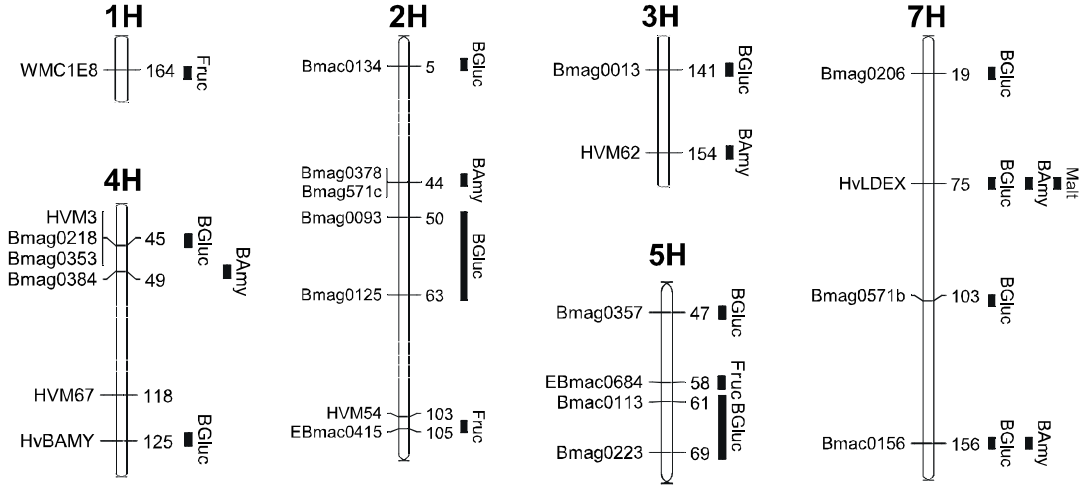
Notes: Plots showing covariance of malt quality QTLs. QTL Notations are detail in text

Thus, the grain shape parameters (width (Gwth), length (Glen) and width to length ratio (W/L)), % soluble extract (%SE), maltose (Malt), sucrose (Suc), thousand kernel weight (TKW) and milling energy (MillEn) were clearly differentiated by loadings on PCO2 from the enzyme activities (α-amylase (AAmy), β-glucanase (BGase) and L-dextrinase (LDex)), the monosacharide (glucose (Gluc) and fructose (Frut) and free amino nitrogen (FAN). Finally, β-glucan and β-amylase appeared relatively distinct from the other two groupings and from each other. For ease of presentation, we have chosen one 'functional' character to represent each of the groupings, namely fructose (PCO group1) and maltose (PCO group2). With the addition of β-glucan and β-amylase, we included four characters in the QTL analysis.

At this stage, single locus QTL association analysis is a valuable tool to test the association between trait values and genotypes at marker loci. It considers each marker separately and so does not require that the marker loci be mapped relative to one another (MANLY & OLSON 1999). The markers used in this study were positioned with their

associated QTLs using the SCRI SSR map (Figure II) (RAMSAY *et al.* 2000). On average the interval between markers was 18 cM. Any gaps (defined here as >50 cM) found were consistent with RAMSAY *et al*. (2000) with the exception of 3H where the gap was largely due to the availability of markers.

Figure II. Single locus QTL associations on the barley Lina x *H. spontaneum* map



Minimal map (Ramsay *et al*., 2000) detailing only markers associated with malt quality QTLs. QTL Notations are β-amylase (BAmy), β-glucan (BGluc), Fructose (Fruc) and Maltose (Malt).

β-glucan has significant associations with all linkage groups except for 6H where none of the selected traits seemingly have any associations. β-amylase has significant associations on linkage groups 2H, 3H, 4H and 7H. Fructose (PCO-group1) was significantly associated with markers on 1H, 2H and 5H and maltose (PCO-group2) with just one marker on 7H. Without knowing the genetic and environmental correlations between the characters, we cannot infer that fructose and maltose are representative of all the traits in their respective PCO groupings. They should, therefore, be best described as individual characters.

Comparisons of trait locations between studies of barley have been hampered by the numerous methodologies for QTL analyses (regression, SIM, CIM etc…), which together with the many different populations that have be used (e.g. 'Steptoe x Morex' (SM), 'Harrington x Morex' (HM), 'Harrington x TR306' (HT), and 'Derkado x B83 12/21/5' (DB)) can produce different results (AYOUB & MATHER 2002). Comparisons of QTLs between studies have therefore been made at times very generally in terms of only the mapping population versus chromosome number. More precisely, work to link physical maps (or BAC contigs), linkage maps and QTL maps have led to an approach called 'BIN-mapping'. In BIN-mapping all the markers or traits falling into a chromosome region (of between 10-20cM) are treated together allowing the fragmentation of a linkage group into between 12 to 16 sections thereby allowing finer, more consistent comparisons between studies (http://barleygenomics.wsu.edu) (THOMAS 2003). If we use this approach to compare our results with those from the aforementioned populations (AYOUB & MATHER 2002; CLANCY *et al*. 2003), we have detected QTLs that have previously been reported for: β-glucan at 2H.2 (2H, BIN No.2) and 4H.5, β-amylase at 2H.8 and 7H.5. As maltose and fructose are rarely analysed individually but are components within the complex trait 'malt-extract' then comparisons can be made to this trait but should be interpreted with caution. QTLs that have been reported for malt extract at 2H.10, 4H.6, 5H.6 and 7H.5are relevant to the current study and we detected QTLs for fructose and maltose in Bins 5H.6 and 7H.5 respectively. Maltose, amongst other traits, has however been specifically studied within the DB population by (MEYER *et al.* 2001) but none of the locations found there correspond to

the BIN 7H.5. With DB the location of the most significant maltose trait was in 4H.6, which does not correspond to previously reported BINs for malt extract, indicating that maltose alone may not give a true picture of malt extract. In this study, however, some QTL loci are consistent with those detected in other studies. Additionally, numerous other loci for β-glucan, β-amylase, fructose and maltose have been identified. Thus clearly there is not only sufficient molecular diversity within these populations but also sufficient phenotypic variation between the malt quality traits of the SMC parents to reveal 'novel' QTLs in their populations.

It is interesting that at BIN 7H.5 there are associations of maltose with β-amylase (and β-glucan). The association of these traits together in part is reflective of some of the biochemistry taking place during malting, i.e. the degradation of starch to simple sugars (maltose, glucose and L-dextrins) by the concerted actions of α-amylase, β-amylase, limit dextrinase and α-glucosidase (LI *et al*. 1999). The limit dextrinase gene, denoted by loci marker HvLDex, is in BIN 7H.5.

As yet we have not discerned whether co-location of traits, or QTL "hot-spots" are due to linkage or pleiotropy. Considering the associations detected with Bmac0156 in Bin 7H.11, we might expect some correlated responses as high β-glucan is associated with inadequate modification of the kernels during malting and β-amylase activity is a major component of diastatic power. Hence, the association of allele 'B' from of Bmac0156 with increased β-glucan but decreased β-amylase and fructose is functionally consistent.

Therefore, although we have used elite cultivars as parents we have found they were sufficiently diverse genetically that only 8% of a universal core set of SSR markers were found to be monomorphic. There is more than enough polymorphism to enable the production of a composite map from combining the data from several small populations, demonstrating that the SMC approach is viable. By means of PCO analysis it is interesting that the phenotypic characters broadly fell into two groupings. So far, only one of our original 16 traits, only %SE had no marker genotype associated with it, all the other phenotypic traits had numerous highly significant (P<0.005%) associations. Some of the QTLs were in common with other published locations, despite the fact that many of these other maps were not wholely relevant to current malting barley cultivars. We have detected some QTLs that appear to be novel and this is likely to be due to the inherent differences between mapping populations, where the SMC populations revealed trait variations between only elite parents and so would be most likely to identify different QTL associations than the typical malt x feed populations. Nevertheless, it should be the 'novel' QTLs that are of most interest to molecular breeders for it these are regions which are currently being manipulated in breeding programmes through phenotypic selection. These 'novel' QTLs therefore represent more relevant targets for MAS. Our current QTL analysis is relatively crude, however, and does not identify the true multi-locus picture of genetic control of the traits that we have studied. Neither are the map locations precise, which may partly explain why we do detect few or no associations for some characters. We plan to use the composite map from the SMC populations in an interval mapping approach that we expect will provide the most credible, consistent QTL map for European malting barley qualities. This composite multi-trait map will be yet another tool assisting MAS breeding.

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Genetic Control of Grain Damage in a Spring Barley Mapping Population

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Abstract
DNA-based markers were used to construct a genetic map of the spring barley cross Tankard x Livet, which was developed to study the genetic control of grain damage and agronomic traits in a malting barley background. Quantitative trait loci (QTL) that accounted for considerable portions of the phenotypic variation were detected for split grains, skinned grains, gape between the lemma and palea and measures of grain shape and size. Considerable clustering of the traits studied was observed on chromosomes 1H, 4H, 5H, 6H and 7H suggesting that changes in grain shape and size parameters has a consequent affect upon grain damage parameters. Transgressive segregation was also observed for the grain damage parameters, indicating that lines that are more prone to splitting than Tankard could, unless actively selected against, be released and therefore affect the supply of malting quality barley. The markers that we have identified could therefore be valuable tools in marker-assisted selection schemes to eliminate lines with high expression of grain damage traits.
Keywords: barley; grain damage; splitting; skinning; mapping

Introduction

Damage to harvested grain in the UK is manifested in two major forms – splitting and skinning. Splitting is a visible rupture of the grain and can often be seen by the naked eye due to exposure of the white starchy endosperm. Under unfavourable environmental conditions, degradation of the exposed starch can take place and maltsters therefore reject loads of barley with noticeable levels of splitting. The phenomenon can also be seen in other parts of the world, e.g. Australia where it is called cleaving and resulted in the down-grading of some of the 2003 crop (http://e-malt.com/mcommon/newsletter08a_2004.htm#O0). In the UK, the recommended cv Tankard suffered extensive splitting in the 1997 Scottish harvest, which led to it being rejected as a malting cultivar. This was an expensive waste not only for the growers but also for all involved in the breeding and recommendation of the cultivar. In some grain samples, the lemma and palea do not wholly cover the grain, a condition known as gape, and this exposure of the pericarp is thought to pre-dispose genotypes to splitting and/or skinning. Skinning, which could affect filtration and lead to uneven malting due to varying permeability, is the physical removal of part of the barley husk to expose part of the testa. It can be caused during threshing of the grain due to a tough awn combined with poor adherence of the lemma and palea, or due to abrasion of individual grains in handling. Cultivars vary in their levels of splitting and skinning (HOAD *et al.* 2003) and thus both characters must be under some genetic control as well as being influenced by environment.

Phenotypic assessment of splitting, gape and skinning requires a suitable environment for genetic potential to be expressed and so cannot be relied upon. The use of molecular markers would enable Marker-Assisted Selection (MAS) and early elimination from breeding and/or testing programmes of genotypes pre-disposed to high levels of grain damage. We therefore carried out phenotypic and genotypic assessments of a population of lines from a cross between two spring barley genotypes (Tankard and Livet), which showed contrasting levels of splitting. The results that we present in identifying lines with low expression of these damage traits build upon the preliminary findings of RAJASEKARAN *et al.* (2000).

Material and Methods

A population of 184 RILs was developed by multiplying seed of random F_3 plants from a cross between the spring barleys Tankard and Livet cross in the field in 1998. The population was grown with its parents and controls in plot trials at Dundee in 1999 (F_5 generation) and 2000 (F_6 generation), using a row and column design in two replicates. The 1999 trial was grown in 3 m^2 plots and the 2000 trial in 7.625 m^2 plots at seed rates of 180 kg ha^{-1} and 425 seeds m^{-2} respectively. The trials were grown under a typical fertiliser regime for malting barley and kept free of foliar pathogens by a prophylactic fungicide regime. The plots were scored for heading date (Head), i.e. days from June 1st when 50% of the plot was at GS 53 and Height, measured in m^{-2} from the ground to the average position of the collar. The plots were harvested with a small plot combine at maturity, the seed dried to approx 12% moisture content and then weighed to estimate yield in t ha^{-1} (Yield). Sub-samples of cleaned grain were then sieved, the proportion retained by a 2.5mm sieve calculated (GT25Sv). This fraction was itself sub-sampled to determine milling energy (MillEn (J)), using the Comparamill (ALLISON *et al.* 1979), and average grain length and width (GLength & GWidth (m^{-3}) respectively), using the MARVIN Digital Seed Analyser (GTA Sensorik GmbH, Germany). The grain width to length ratio was derived from GWidth and GLength. The thousand kernel weight of the sample (TKW (g)) was also estimated by MARVIN. Splitting, Gape and Skinning were estimated from observations made under a 10x binocular microscope of a sub-sample of 100 seeds from the fraction passing over a 2.5mm sieve. Grain that had a visible rupture in the pericarp/testa were classified as being split, those that had lost more than 25% of the lemma and/or palea as being skinned and those that had a gap between the lemma and palea of 1mm or more as gaping. For each sample, these data were expressed to derive percentages of grain in each sample that were split (Split), showed less than 25% skinning (LT25Sk) and were gaping (Gape1). Data from the 1999 and 2000 trials were combined as Randomised Complete Block designs for analysis by GENSTAT (Lawes Agricultural Trust, Rothamsted Experimental Station, UK) over years because a different design was used each year. Many of the percentages in the data for GT25Sv, Split, LT25Sk and Gape1 were either <30% or >60% so an angular transformation was applied in an attempt to normalise the data.

DNA extraction was carried out upon plants grown from seed harvested from the 1999 trial to minimise the possibility of handling errors. At this stage, each RIL was represented by F_6 seed that had been bulked from a single F_3 plant, which was likely to be heterozygous at a number of loci, so that each RIL could be a product of subsequent segregation. We therefore sampled leaves from 10 plants of each RIL and isolated DNA from this bulk to give a good chance of identifying loci that had been heterozygous. The RILs were then genotyped with a range of molecular markers, including Simple Sequence Repeats (SSRs), Amplified Fragment Length Polymorphisms (AFLPs), Sequence-Specific Amplified Polymorphisms (S-SAPs), Retrotransposon-Microsatellite Amplified Polymorphism (REMAPs), Inter-Retrotransposon Amplified Polymorphism (IRAPs) and Sequence Tagged Sites (STSs). This enabled the population to be genotyped with 158 polymorphic markers which were used to construct a genetic map with JOINMAP 2.0 (STAM & VAN OOIJEN 1995). This resulted in 17 linkage groups of three or more markers, 14 of which contained at least one previously mapped marker and could therefore be assigned to specific barley chromosomes. The maps and the genotypic data were then combined with the overall phenotypic means for each trait and scanned for QTLs using PLABQTL (UTZ & MELCHINGER 1996). The program default values were used to select significant co-factors followed by a stepwise elimination of those that were not associated with QTLs that exceeded the default LOD threshold of 2.5. Permutation was then applied to establish the threshold LOD for a genome-wide error rate of 5% and co-factors associated with QTL that did not exceed this LOD were also eliminated.

The significance of any dominance or epistatic effects at these loci was then tested, assuming an F_3 segregation ratio.

Results

Tankard had a higher level of Split and Gape1 than Livet and a lower GLength and a higher GWidth leading to a higher Wid/Len, which resulted in a higher TKW and GT25Sv. Livet had harder grain than Tankard and was later and shorter (Table 1). Non-additive effects appeared to be involved in the genetic control of Gape1, GT25Sv, TKW and GLength as the RIL mean differed significantly from the mid-parent (Table 1). This could be due to dominance or epistasis as each RIL was derived from a single F_3 plant.

Table 1. Population and QTL statistics for eight grain and four agronomic characters measured on Tankard, Livet and their RILs over trials grown in 1999 and 2000. Bold figures indicate significant differences between parents or the RIL mean and mid-parental value ($P<0.05$).

Character	Tankard	Livet	RI Min	RI Mean	RI Max	SED	QTLs	Other Effects	% Variation	
									Phenotypic	Genotypic
Split	8.72	**2.93**	0.00	5.27	21.73	1.88	5	E[1]	28.9	62.7
Gape1	40.0	**17.5**	4.5	**24.1**	57.5	3.0	7	D	35.0	51.9
LT25Sk	65.6	69.9	57.3	67.3	77.6	2.4	5		13.1	57.1
GT25Sv	78.0	**66.6**	57.1	**71.0**	78.8	1.2	5		15.4	24.3
TKW	50.6	**45.6**	40.6	**46.1**	55.5	1.1	5		45.2	64.0
GLength	7.88	**8.11**	7.35	**7.87**	8.43	0.09	6		50.6	62.4
GWidth	4.05	**3.85**	3.73	3.91	4.25	0.05	5		36.5	66.3
Wid/Len	0.514	**0.475**	0.461	0.498	0.534	0.005	7		39.0	67.5
Head	20.9	**27.3**	18.3	24.7	29.8	0.8	3		21.2	40.8
Height	62.5	**55.3**	45.8	57.4	69.8	2.0	5	D	25.4	51.3
Yield	6.96	7.41	6.02	7.06	8.13	0.32	3		4.5	30.8
MillEn	582	**711**	550	648	742	19	8	D	36.5	71.2

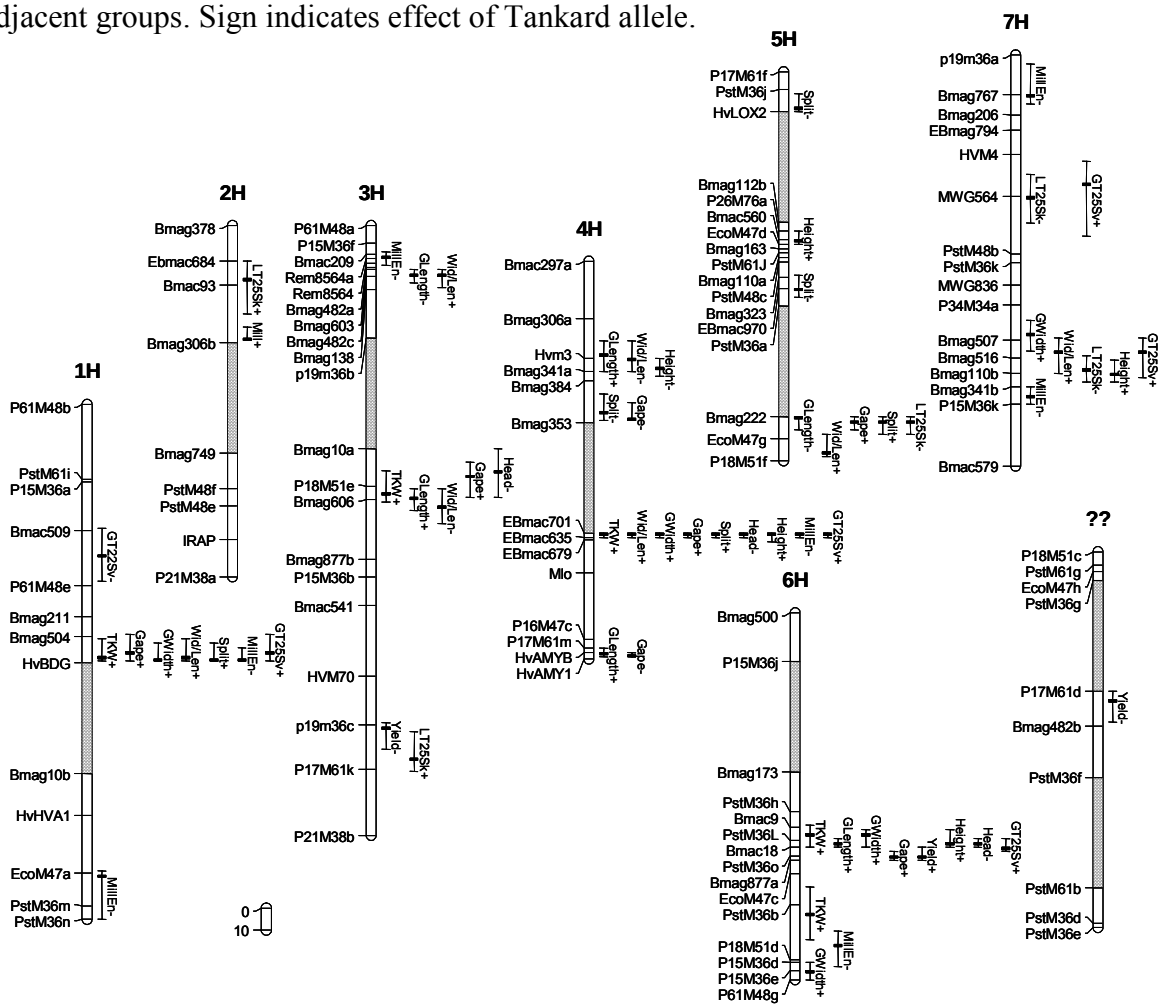
[1] Other effects are Epistasis (E) and Dominance (D).

Between 5 (GT25Sv) and 7 (Wid/Len) QTLs were detected for every grain trait which, after cross-validation, accounted for an average of over 10% and 20% of the phenotypic and genotypic variation respectively. It was particularly noticeable that the QTLs detected accounted for over 60% of the genetic variation in Split and nearly 60% in LT25Sk and so there is a good chance that we can identify molecular markers of value in MAS for low expression of these two traits. The numbers of QTLs that we detected for the agronomic traits ranged from 3 (Head and Yield) to 8 (MillEn). Generally, the amount of phenotypic and genetic variation accounted for was much less than for the grain traits. For example, three QTLs were detected for Yield but together only accounted for 4.5% of the phenotypic and 30.8% of the genetic variation (Table 1).

We found a high degree of co-location of the QTLs (Figure 1), particularly for the grain traits, which probably reflects high correlations between them (data not shown). There are three QTL clusters on chromosomes 1H, 4H and 5H where at least three traits from Wid/Len, Gape1, TKW and Split are co-located with the Tankard allele increasing expression of each. The increases in Wid/Len are either due to an increased GWidth (1H and 4H) or a decreased GLength (5H), suggesting compensatory growth mechanisms are operating. In contrast, another QTL cluster on chromosome 6H may be due to a general growth factor as Tankard alleles increase both GLength and GWidth, resulting in an association of Gape1 and TKW

without an increase in Wid/Len but this did not appear to have any effect upon Split. There are, however, three other QTL for Split that are not co-located with QTLs for any other character from the present study. These are located on chromosomes 4H and 5H and the Tankard allele at two of these loci was associated with reduced expression. Given that Tankard was found to have a high degree of Split, it was surprising to find that there were some loci acting to reduce expression. These QTL were, however, less significant than the others and may also be in part due to relatively low levels of splitting observed over the two years of trials.

Figure 1. QTL Maps for Tankard x Livet population. Thick lines indicate QTL peaks and whiskers 1 LOD confidence intervals. Hatched portions indicate no linkage between adjacent groups. Sign indicates effect of Tankard allele.



There was less evidence of consistent co-location of QTLs for LT25Sk. On chromosomes 5H and 7H, QTLs for LT25Sk were co-located in repulsion with QTLs for Wid/Len suggesting that rounder, well-filled grain might exhibit more skinning. Some support for this can be seen in another region of 7H where a QTL for LT25Sk is co-located in repulsion with a QTL for GT25Sv. One of the other two QTLs for LT25Sk is, however, co-located in repulsion with a QTL for Yield on chromosome 3H but there is no evidence that this is due to grain size effects. The remaining QTL for LT25Sk on chromosome 2H is not co-located with any other trait in the present study.

Epistasis was detected for just one character (Split) and all the interactions involved the QTL on 5H in the region of Bmag222. Dominance was detected for three characters (Gape1, Height and MillEn). For the last two, dominance was always in the direction of the Livet

phenotype, i.e. shorter plants and harder grain but the effects were mixed for Gape1. Taken over the whole experiment, these effects are negligible and, whilst they may represent genuine effects, could be due to under-parameterisation of characters with a partial genome map and, with the exception of Gape1, do not reflect the findings of the biometrical analysis (Table 1).

Discussion

As much of the phenotypic variation for Split is accounted for by the QTLs detected in the current study, there would appear to be potential for developing an effective MAS scheme to reduce expression of the character. Given that some QTL alleles for high expression of Split are co-located with QTLs for high expression of TKW, selection of lines with low Split might limit expression of Yield. There is slight evidence for this problem in the current study as the QTL increasing Yield on chromosome 6H in the Tankard x Livet population is co-located with a QTL increasing TKW although, whilst this region is associated with a QTL for Gape1, the region is not associated with QTLs for Split.

Transgressive segregation for both Split and LT25Sk was observed in the Tankard x Livet RILs, which means that lines that show higher expressions of both characters could be produced. This finding is re-inforced by the discovery of Tankard QTL alleles in the region of Bmag353 on 4H and Bmag323 and HvLOX2 on 5H that reduce Split. None of these three QTLs are associated with any yield related QTLs so might be attractive targets for selection for reduced Split whilst maintaining grain size. Such a strategy could still result in the development of cultivars with levels of Split comparable to Tankard so would need to be combined with MAS at other loci. Selection against the Tankard allele increasing Split located in the region of Bmag222 on 5H is an attractive target as it accounts for the largest amount of variation in a multi-locus model (data not shown) and is not associated with any TKW or Yield QTLs. A QTL for LT25Sk is, however, located in the region and there would be a potential increase in skinning coupled with a reduction in splitting.

Only two other mapping studies of grain damage parameters have been published. KANATANI *et al.* (1998) studied "hull-cracked grain" in the North American two-row spring barley population Harrington x TR306 and COLLINS *et al.* (2000) studied skinning in a cross between the Australian spring barley Galleon and the Japanese spring barley Haruna Nijo. "Hull-cracked grain was defined by KANATANI *et al.* (1998) as the exposure of the caryopsis through the lemma and palea, which could be similar to Gape1 in the present study, and three QTL were detected in the regions of ABG609B, MWG502 and MWG511 on chromosomes 3H, 5H and 7H respectively, although the QTL on 5H was not significant in a multi-locus model. Using the barley bin map (KLEINHOFS *et al.* 1998), the Oregon Wolfe map (COSTA *et al.* 2001) and the Tadmor x ER/APM map (TEULAT *et al.* 2001), we can compare the approximate locations of these QTLs with those from the present study. The QTL for "hull-cracked grain" were located in the region of Bins 15 and 16 on 3H, Bin 1 on 5H, and Bin 7 on 7H, regions in which we did not detect any QTL for Gape1 but did detect a QTL for Wid/Len, a possible determinant of Gape1, in Bin 7 of 7H. It is also possible that HvLOX2 is located in Bin 1 of 5H because Lox1a is also located in that region but we do not have any supplementary information to support this hypothesis. If true, however, it would mean that the QTL in the region of HvLOX2 that we detected for Split in the Tankard x Livet is in the same region as the QTL for "hull-cracked grain" detected by KANATANI *et al.* (1998). The study of skinning by COLLINS *et al.* (2000) was limited to chromosome 2H and whilst a QTL for skinning was detected in the Bin4 region, we did not detect any polymorphism for this region of the genome in the present study.

Resources did not permit the genotyping and phenotyping of an independent sample of lines to validate the associations of markers with Split that we detected. Some validation is provided from the results of a trial of 11 lines with high and 11 lines with low mean expression of Split over the 1999 and 2000 trials that was grown in 2001, a year with higher levels of expression than either 1999 or 2000. We used the genotype at the marker closest to each of the five QTLs detected in the Tankard x Livet population to predict the expression of Split in each of the 22 lines and compared it to that observed in the phenotypes of the 2001 trial. This approach correctly identified eight of the 11 lines with the highest expression of Split and could therefore be used to eliminate lines with confidence. It is, however, not an independent test and genotyping and phenotyping of an independent sample is required to fully assess the value of the markers detected in the present study in MAS. In addition, the identification of more closely linked, or even direct, gene markers would vastly improve the potential to deploy MAS to select against grain damage parameters.

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Mapping of Resistance Genes to Powdery Mildew in Barley

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Abstract

The introduction of resistance genes to powdery mildew (*Blumeria graminis* f. sp. *hordei*) present in two accessions (PI354949 and PI466495) of wild barley (*Hordeum vulgare* subsp. *spontaneum*) into *H. vulgare* was performed by the cross with the variety Tiffany. Powdery mildew resistance tests on plants of F_2 generation revealed the segregation ratio 15 : 1 in Tiffany x PI354949 and 3 : 1 in Tiffany x PI466495, which indicated presence of two and one dominant resistance genes, respectively. To identify individual *R* genes, technology of microsatellite DNA markers and linkage recombination analysis was applied. One of resistance genes in PI354949 was determined to be located in *Mla* locus linked with Bmac0213 (25.0 cM in distal position) on chromosome 1H and the other on chromosome 7H linked with Bmag0507 (14.0 cM in proximal position). In PI466495, *R* gene was linked with Bmac0213 (8.4 cM in distal position) and it is also located in *Mla* locus. The perspective aim of this work is fine-mapping of the promising gene located on 7H chromosome and identification of tightly linked DNA markers for marker assisted selection.

Keywords: *Hordeum vulgare*; *Blumeria graminis* f. sp. *hordei*; DNA markers; microsatellites; gene mapping

Introduction

The present knowledge of genetic determination of resistance to powdery mildew (*Blumeria graminis* f. sp. *hordei*) involves a multitude of genes (JØRGENSEN 1994) and the study of their presumptive functions is under the progress (PANSTRUGA & SCHULZE-LEFERT 2002). Top-priority has been devoted to race-specific loci and to the resistance controlled by them. In the absence of matching *R* genes, *Avr* gene products presumably enhance pathogen virulence as a consequence of gene-for-gene interactions between the two corresponding genes. Concerning the breeding efficiency, plant breeders must continuously implement new *R* genes from wild relatives (NOËL *et al.* 1999). Technology such as DNA markers proved to be very efficient and promising on the way how individual *R* genes and their alleles could be not only identified but even combined into new resistant varieties of barley. Following hybridization-based RFLP markers, profound interest has been devoted to PCR-based markers, in particular those based on simple sequence repeats (SSR). A considerable number of microsatellites have been located into genetic maps of barley (RAMSAY *et al.* 2000; COSTA *et al.* 2001; THIEL *et al.* 2003) which made them a meaningful tool for a gene mapping.

The starting point of our work was selection of altogether 15 resources of wild barley (*H. vulgare* subsp. *spontaneum*) with newly detected resistance to powdery mildew fungus (DREISEITL & BOCKELMAN 2003). The introduction of fully effective resistance genes from the wild barley (PI354949 and PI466495) into *H. vulgare* was performed by the cross with the variety Tiffany as a female parent. Recently, plants of F_2 generation were subjected to mildew resistance tests revealing the segregation ratio 15 : 1 (resistant : susceptible) in PI354949 and 3 : 1 in PI466495, which indicated presence of 2 and 1 dominant resistance

genes, respectively. In addition, tests of allelism for *Mla* locus were carried out by the inoculation of individual F_2 plants with *Aa7* pathotype of powdery mildew, which assigned at least one gene in this locus (DREISEITL *et al.* 2003). This investigation was aimed at identification of linked PCR-based markers for the two crosses with perspective fine mapping of region around the genes of interest.

Material and Methods

Plant Material

Two F_2 populations developed from the cross *H. vulgare* variety Tiffany as a female parent with *H. vulgare* subsp. *spontaneum* bearing newly detected resistance to powdery mildew fungus (PI354949 and PI466495) were used. Mildew resistance tests with virulent pathotype performed on the F_2 individuals enabled the phenotypic typing of plants from the viewpoint of their resistance into nine categories, from 0 to 4, corresponding the infectious types.

Molecular Analysis

DNA extractions from leaves of parental and F_2 individual plants were performed by GeneElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich Co.) and quantified by the spectrophotometer. Altogether 59 microsatellite DNA markers (RAMSAY *et al.* 2000) were used for polymorphism evaluation between the two parental plants of each cross. Polymorphic DNA markers were used for identification of markers linked with the gene of interest and the location of genes on a genetic map with simplifying assumption that one is in *Mla* locus was performed. The genes in *Mla* locus were mapped by means of *Bmac0213*, *Bmac0063* and *Bmac0032* markers. The other *R* gene in PI354949 was traced by means of bulked segregant analysis (BSA) when each of resistant (infection type of 0) and susceptible bulk (infection type 4) consisted of 10 plants. The PCR amplified fragments were separated by electrophoresis either in 3% agarose gel or in 10% polyacrylamide gel. The gels were visualised by ethidiumbromide or by silver-staining method.

To estimate the recombination rate (*r*, %) between the *R* gene of interest and a DNA marker, we restrict the molecular typing efforts to only recessive segregants *rr* (RONIN *et al.* 1996). In accordance with genetic analyses and conclusions that resistance is dominant over susceptibility in all the target genes, this category represents the susceptible plants. The recombination rate was determined as a ratio of the number of recombination events between the two alleles under study (i.e. nonparental combinations) and a total number of chromosomes tested. The estimate of map distance (*D*, cM) was given by the Kosambi´s mapping function (KOSAMBI 1944).

Results and Discussion

Genetic analysis of Tiffany x PI354949 cross revealed 2 dominant genes coding the powdery mildew resistance, one confering the infectious type 0 and the other for infectious type 1 (Fig. 1). Presence of the only infectious type of 0 in resistant parent and both in F_1 indicated epistatic effect of one gene which overrides effect of another non allelic gene. Genetic analysis of Tiffany x PI466495 cross confirmed one gene in *Mla* locus but the way of phenotypic distribution of the trait in F_2 population indicated rather two genes in this locus. Infectious type of 1 in parent and 1-2 and 2 in F_1 generation indicates incomplete dominant type of heritability (Fig. 2).

Out of 51 microsatellite markers used for polymorphism testing 44 (86%) proved to be polymorphic between Tiffany and PI354949. Between Tiffany and PI466495 polymorphism was confirmed for 41 (69%) microsatellites out of 59 ones. For Tiffany x PI354949 cross, linkage with Bmac0213 (r = 23.1%, D = 25 cM) confirmed the presence of an allele in *Mla* locus. Total 35 microsatellite primer pairs were screened using the BSA and a marker on

chromosome 7H(1) appeared to be linked. For Bmag0507 linkage with a dominant gene was indicated. Therefore, Bmag0507 (111 cM) and neighbouring Bmag0120 (118 cM) were used for linkage analyses on F_2 plants with infectious type 4. Linkage was confirmed with Bmag0507, r = 13.6 %. Bmac0120 confirmed very weak linkage (r = 38.5%), which indicated location of the *R* gene proximal to Bmag0507. Known *Mlf* locus is presented somewhat in distal position. SCHÖNFELD *et al.* (1996) identified relatively tight linkage between *Mlf* gene and MWG539 RFLP marker on 7H(1) chromosome and the gene was located on genetic map in position 135 cM (BACKES et al. 2003). In the further research it will be verified if the gene identified in this study is a new original locus for powdery mildew resistance or it agrees with *Mlf*. Recombinant analysis of Tiffany x PI466495 cross confirmed linkage with Bmac0213, r =8.3 % and D = 8.4 cM. Independent assortment with Bmac0063 indicates distal position to Bmac0213.
The perspective aim and the next step of this work will be the identification of DNA markers tightly linked to promising resistance gene and the development of markers for marker assisted selection.

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Figure 1. Distribution of barley resistance to powdery mildew *Va7* pathotype in F2 generation after Tiffany x PI354949 cross and comparison with parental and F1 generation

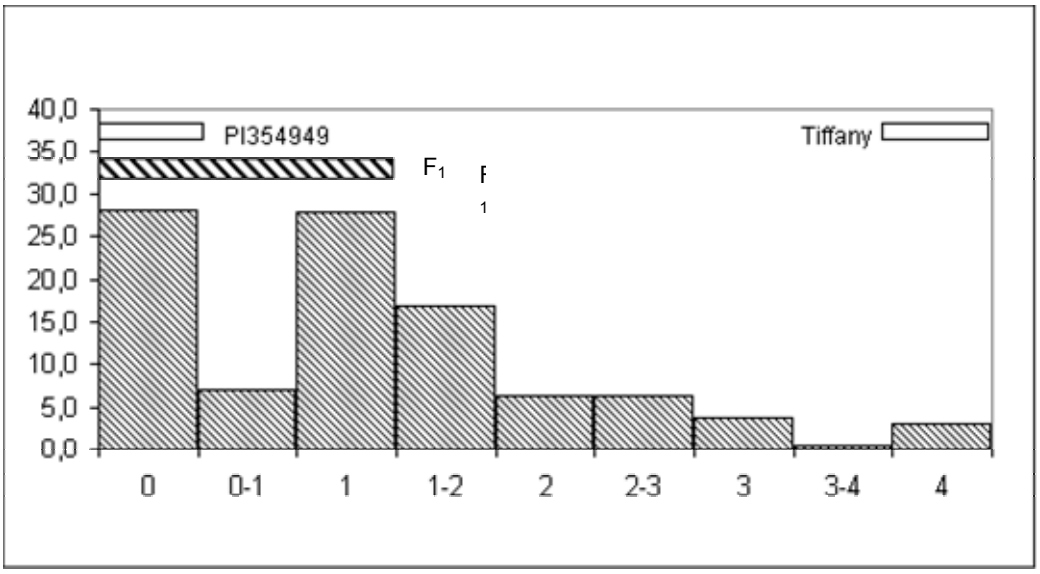
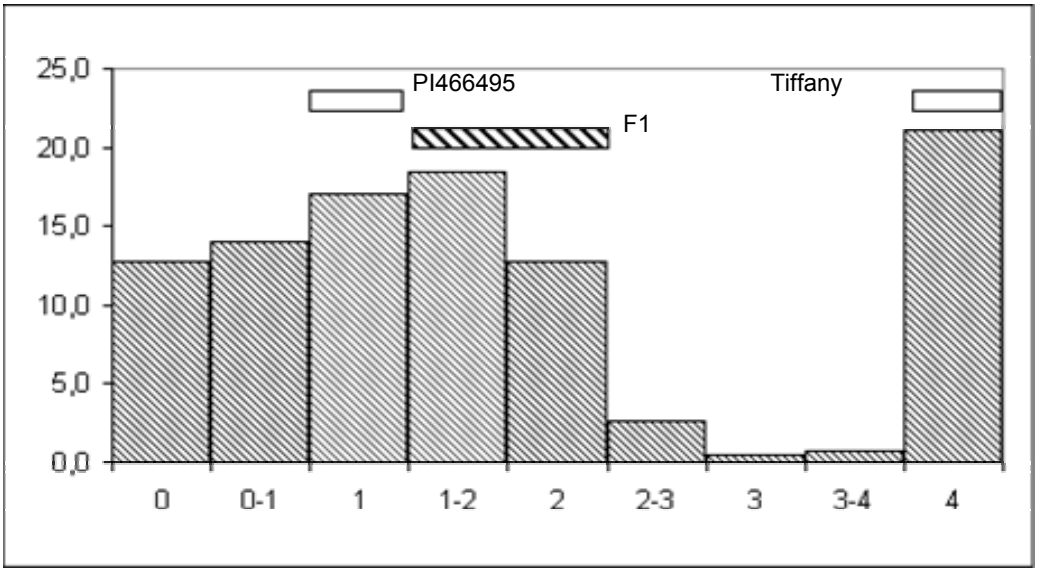


Figure 2. Distribution of barley resistance to powdery mildew *Va7* pathotype in F_2 generation after Tiffany x PI466495 cross and comparison with parental and F_1 generation



x – single phenotypic categories (0 resistant to 4 susceptible)
y – frequencies of plants in single phenotypic categories

Single Nucleotide Polymorphism Mapping of the Barley Genes Involved in Abiotic Stresses

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Abstract

Changes in environmental conditions and need for increased agricultural production and quality require plant varieties tolerant to various abiotic stresses. EST sequencing projects have provided information about significant proportion of expressed plant genes and allow to develop sequence-based polymorphic markers amenable to automation and high-throughput. Recently, various microarray platforms have become available in model plants and barley allowing to carry out genome-wide studies of genes responding to abiotic stresses. Genetic mapping of these genes would facilitate associations with known plant traits. Our goal is to identify and characterize Single Nucleotide Polymorphisms (SNPs) in 1000 barley genes associated with responses to abiotic stress in barley or homologous to such genes in other plant species and to employ SNP markers for genetic mapping. A non-redundant ca. 3000 HarvEST EST contig list has been compiled primarily based on microarray studies using Barley1 GeneChip, but also including barley homologues of stress-associated genes from other plant species. Currently we are analyzing genomic PCR products corresponding to 1370 contigs from 8 barley varieties for the presence of SNP markers. Our preliminary results indicated that up to 90% PCR and sequencing success could be achieved and that 70-80% of sequenced contigs contained at least one mutation. Thus, direct sequencing is a robust solution for SNP discovery in barley. An average mutation frequency was 1 per 113 bp, however, frequencies of up to 1 mutation every 17 bp were observed. To further validate our SNP discovery approach, 32 genes were mapped genetically.

Keywords: abiotic stress; barley; microarray; single nucleotide polymorphism; SNP; transcription profiling

Introduction

Ability of a crop plant to tolerate (or adapt to) stresses encountered in natural environments is a crucial factor determining the yield and quality. Barley is a crop plant of great economic value grown throughout the world in extremely varying environments. Since its domestication in the Fertile Crescent (BADR *et al.* 2000), it has been adapted to fundamentally different growth temperatures, humidity, day length and seasonal changes. The genetic diversity was unknowingly employed by breeders throughout the ages to create varieties adapted to diverse environments. Identification of genetic factors underlying the adaptability could potentially expedite development of new varieties.

Large-scale EST sequencing projects have provided information on significant portion of plant genes, while availability of different platforms of microarrays enables whole genome expression profiling both in model and in crop plants. A number of such studies identified genes responsive to different abiotic stresses, e.g., low temperature (FOWLER *et al.* 2002; KREPS *et al.* 2002; SEKI *et al.* 2001; SEKI *et al.* 2002; PROVART *et al.* 2003), high light (KIMURA *et al.* 2003), salt (KREPS *et al.* 2002; OZTURK *et al.* 2002; SEKI *et al.* 2002),

drought (OZTURK *et al.* 2002; SEKI *et al.* 2001; SEKI *et al.* 2002) and nitrate (WANG *et al.* 2003). Studies by FOWLER and THOMASHOW (2002), KREPS *et al.* (2002), PROVART *et al.* (2003) and WANG *et al.* (2003) used *Arabidopsis* Affymetrix GeneChip, while other studies employed cDNA microarrays and only one of them (OZTURK *et al.* 2002) investigated stress responses in barley.

The goal of our project was to identify barley genes that are associated with different abiotic stresses and then select a subset of these genes for single nucleotide polymorphism (SNP) discovery and genetic mapping in a set of North American, European and *Hordeum vulgare* ssp. *spontaneum* mapping populations. The majority of the list consists of genes responsive to drought, salt, cold, low nitrogen and waterlog stresses which were identified using the new Barley1 Affymetrix GeneChip (CLOSE *et al.* 2004), as well as homologues of model plant genes. Here we report our SNP discovery platform based on robust, high-throughput *de novo* sequencing, preliminary SNP characteristics and genetic mapping of 32 barley genes using SNPs.

Material and Methods

Plant Material and DNA Extractions

Leaf material was collected from one – two weeks old OwbD, OwbR, Steptoe, Morex, Lina, HS92, Optic and Golden Promise plants and DH lines from Steptoe x Morex minimapping and OwbD x OwbR populations grown in the greenhouse. Genomic DNA was extracted by conventional procedures.

Stress Conditions, RNA Extractions and Microarray Hybridizations

1. Microarray experiments conducted at UC Riverside.

Salt stress was imposed on cv. Morex plants in a hydroponic system by gradually increasing NaCl concentration from day 14 after transplanting until day 18. Plants were harvested at 3, 8 and 27 hours after reaching the final concentration of 100 mM NaCl. For drought stress analysis, plants (cv. Morex) were grown in soil at 23^{o}C day, 20^{o}C night temperature, 12 h photoperiod. Water was withheld ten days after planting and samples were obtained at specific values of soil water content. Seven day old barley seedlings (cvs. Dicktoo and Morex) were subjected to a low temperature treatment in three stages and samples were collected at each stage, cold acclimation (4°C), freeze/thaw cycling (4°C day/-10°C night) and de-acclimation (20°C). Three independent experiments were done for the salt, drought and low temperature experiments and triplicate samples were analyzed on Barley1 GeneChip.

2. Microarray experiments conducted at SCRI.

Barley cv. Optic root material for microarray analysis was previously produced at the SCRI to generate cDNA libraries used for EST sequencing. Libraries and stress conditions are described in HarvEST database (http://harvest.ucr.edu). Three RNA extractions from root tissues for each condition (control and salt, low nitrogen, drought and waterlog stresses) were made. Equal amounts of RNA from replicate preparations were combined and the pooled RNAs for control and each stress condition were hybridized each to a single Barley1 GeneChip.

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA was treated with DNaseI (Ambion, Austin, TX, USA) and cleaned with Qiagen RNeasy Midi kit (Qiagen, Hilden, Germany).

Microarray hybridizations were as described by CLOSE *et al.* (2004). Analysis of the microarray data was performed using Silicon Genetics GeneSpring software.

Primer Design and PCR

Oligonucleotide primers were designed in a batch process using locally installed Primer3 program (ROZEN & SKALETSKY 2000) using HarvEST assembly #21 cDNA contigs as templates. In order to maximize chances of finding SNPs, 400-700 bp region from 3' ends of contigs was targeted. The quality of resulting primers was controlled by Blastn search against HarvEST #21 contigs to identify unique primer pairs. Primers were supplied by Illumina (San Diego, CA, USA).

PCR was done in a 25 µl reaction volume consisting of 0.6 units of Qiagen HotStar *Taq* polymerase and reaction buffer supplemented with 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 0.4 µM primers. Thermocycling consisted of 15 min at 95°C followed by 40 cycles of 30 s at 95°C, 45 s at 60°C and 2 min at 72°C, followed by 10 min at 72°C.

Sequencing and SNP Discovery

PCR products were treated with ExoSAP-IT (USB, Cleveland, OH, USA) and sequenced from both ends with the same primers as for amplification using BigDye fluorescent terminator kit 3.1 (Applied Biosystems, Foster City, CA, USA). Trace files were quality-trimmed by phred (EWING *et al.* 1998; EWING & GREEN 1998) at a threshold of 40 and standard chromatogram files (SCF) were generated. SCFs were processed using Mutation Surveyor software (SoftGenetics, State College, PA, USA) which performed base calling, assembly and polymorphism detection.

Genetic Mapping

Steptoe x Morex minimapper (KLEINHOFS & GRANER 2001) and OwbD x OwbR (COSTA *et al.* 2001) populations were used for genetic mapping. Primers and PCR were the same as for SNP discovery. SNP detection in PCR fragments generated from DH lines was done by one of the following methods: sequencing, denaturing HPLC, restriction enzyme digestion or agarose gel-based detection of large indel.

Polymorphism Database

Mutations called by Mutation Surveyor software were confirmed by manual inspection of chromatogram alignments and submitted to a relational database. The Polymorphism Database is built using mySQL RDBMS on Solaris. The database is accessed and amended through a web browser interface using a set of Perl 5 CGI scripts on an Apache web server.

Results and Discussion

Transcription Profiling of Barley Abiotic Stress Response Genes Using Affymetrix Barley1 GeneChip

Table 1. Number of differentially expressed genes in barley (cv. Optic) roots under salt, low nitrogen, waterlog and drought stresses

Stress	**Salt**	**Low nitrogen**	**Waterlog**	**Drought**
Up-regulated	401	389	331	632
Down-regulated	67	130	98	47
Total	468	519	429	679
Up-regulated in only one stress	13	42	8	101

Compilation of the Barley Abiotic Stress Response Gene List

The lists of genes from microarray experiment done at UC Riverside, the lists of barley genes homologous to published stress-related genes and the lists of barley genes homologous to SwissProt database entries associated with abiotic stress were merged in a single master list consisting of 5932 members. All the genes were referenced to the HarvEST #21 contigs. Redundant entries were removed resulting in a 2883 member short list (Table 2).

The SCRI root microarray experiment resulted in 862 genes expressed differentially in at least one stress. This list overlapped with the previous short list in only 108 members based on identical HarvEST #21 contig numbers.

Table 2. Composition of the barley abiotic stress gene short list

Number of contigs	**Stress**	**Organism**	**Reference**
24	Salt	Rice	SAHI *et al.* (2003)
40	High light	*Arabidopsis*	KIMURA *et al.* (2003)
46	Salt	Barley	UC Riverside
88	Drought, salt	Barley	OZTURK *et al.* (2002)
122	Low temperature	*Arabidopsis*	FOWLER & THOMASHOW (2002)
137	Drought, cold, high-salinity	*Arabidopsis*	SEKI *et al.* (2002)
174	Salt, osmotic stress, cold	*Arabidopsis*	KREPS *et al.* (2002)
584	Various stresses	*Viridiplantae*	SwissProt
658	Drought	Barley	UC Riverside
1010	Low temperature	Barley	UC Riverside

SNP Characteristics in Barley

In an attempt to evaluate different SNP discovery and mapping approaches, a set of HarvEST #21 contigs was selected containing differentially expressed barley ESTs from the study by OZTURK *et al.* (2002). Primers were designed from 67 EST contigs and PCR products from OwbD, OwbR, Steptoe, Morex, Lina and HS92 were sequenced and analyzed for presence of SNPs. 73% PCR amplification and sequencing success was achieved. More than 90% of successfully sequenced contigs contained at least 1 SNP in at least 1 genotype. 32 contigs polymorphic between Steptoe and Morex and OwbD and OwbR were positioned on barley linkage map using different SNP detection platforms (data not shown).

A more systematic study was carried out to determine SNP discovery success rate and to obtain information on SNP frequency in barley. 189 contigs from the barley abiotic stress response gene list were analyzed from OwbD, OwbR, Steptoe, Morex, Lina, HS92, Golden Promise and Optic. 2262 sequencing reads (810684 bp total length) with phred quality score >40 were obtained for 171 contigs (90% combined PCR and sequencing success rate). The total length of consensus sequences was 68848 bp. 123 of 171 contigs (72%) showed at least one mutation. In total, 607 distinct mutations were detected corresponding to 1 mutation per 113 bp of consensus sequence, which is somewhat higher than 1 per 189 bp found by KANAZIN *et al.* (2002), and comparable to mutation frequency in barley P450 genes (1 per 131 bp) (BUNDOCK *et al.* 2003). However, the mutation frequencies varied widely and frequency as high as 1 mutation per 17 bp was observed.

Distribution of mutations allowed to distinguish between haplotypes. The number of haplotypes varied from 1 (no SNPs) to 7 in different contigs (Fig. 1), however the majority (90%) of contigs was composed of only 1 – 4 haplotypes.

Thus we have developed a high-throughput SNP discovery platform based on *de novo* sequencing of selected barley germplasm. It is similar to the study by KANAZIN *et al.*

(2002), although our platform is different in targeted analysis of a specific group of genes. Alternative SNP discovery approach has been reported by (KOTA *et al.* 2003) using barley EST collections to pre-select SNP-containing contigs electronically. While in principle it increases the chance of finding SNPs, validation of electronic SNPs is still required. Application of electronic SNPs for genetic mapping necessarily involves sequencing of at least one parent of a mapping population, because the barley EST collections do not have sequences from both parents of any available mapping populations. Our approach of direct sequencing of PCR products derived from gDNA allows us to detect SNPs present in intron sequences which can not be detected electronically in ESTs. Even if no SNPs are detected in any particular contig, such information is useful for an unbiased assessment of genetic diversity. As the costs of sequencing decrease, direct sequencing of 3' ends of genes is becoming a robust and straightforward approach for SNP discovery and marker development in barley.

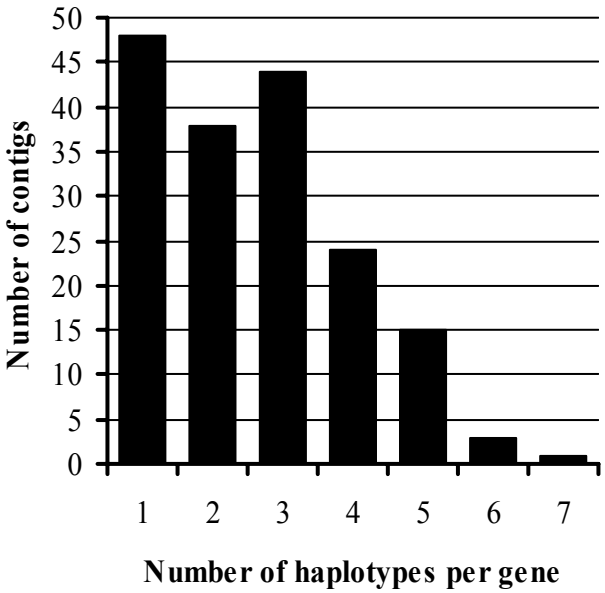

Figure 1. Distribution of haplotype number in barley contigs

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Identification of Barley Semi-Dwarf Gene '*uzu*'

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Abstract

Semi-dwarf varieties of cereal crops have contributed to high yield and resistance to lodging. The adaptation of these varieties is well known as the 'Green Revolution' in rice and wheat since 1960s. In barley, there is a single recessive gene, called *uzu*, which shows a plant type similar to semi-dwarf varieties of rice and wheat. The 'uzu' lines are distributed only in east Asia involving southern part of Japan and Korean peninsula. In 1930s, the majority of cultivars in these regions possessed *uzu* gene. On the basis of comprehensive morphological and physiological studies, we found that 'uzu' line was insensitive mutant to exogenous brassinolide. The insensitivity was ascribed to single substitution of amino acid residue in the *Brassinosteroid insensitive 1* gene homologue (*HvBRI1*), which completely co-segregated with *uzu* locus in F_2 segregating population. Furthermore, the mutation was commonly observed in more than 260 'uzu' accessions. We propose that *uzu* is responsible to mutation in *HvBRI1*.

Introduction

Semi-dwarf genes for short stature are widely used for breeding of the cereals to increase grain yield by reducing lodging and improving canopy structure. In rice and wheat, semi-dwarf varieties originated from east Asia contributed to achieve major yield increases since 1960s and the widespread adoptions was known as 'Green Revolution'. As for barley, although there is no semi-dwarf variety expanding in the world such as rice and wheat, several semi-dwarf genes were contributed to the breeding programs for developing the short-stature cultivars. Among them, the semi-dwarf varieties possessing *uzu* gene have been distributed in central and southern part of Japan and southern coastal region of Korea. In Japan, 'uzu' lines have been cultivated for over a century (MIYAKE & IMAI 1922).

The semi-dwarf plant type of 'uzu' barley is conditioned by a single recessive gene *uzu* located on the 3H chromosome (TAKAHASHI & YAMAMOTO 1951). Although the 'Green Revolution' genes in rice and wheat, *sd1* and *Rht*s, respectively, were caused by the mutations in giberellin (GA)-related genes, it had been known that the *uzu* gene was independent to GA

(SUGE 1972). In order to reveal the genetic functions of *uzu* gene, we comprehensively analyzed the *uzu* gene from both morphological and physiological aspects.

Material and Methods

Plant Materials

Two hundred sixty three 'uzu' lines and 55 normal lines of barley (*Hordeum vulgare* L.) derived from Japan, Korean peninsula and China were used in this study. A number of 'uzu' and normal isogenic lines were also used.

Analysis of Exogenous Brassinolide (BL) Response

In order to investigate the response to exogeneous BL, several isogenic lines of 'uzu' and normal were grown on the distilled agar plates with or without 1μM BL in continuous light, 25 °C, for 9 days after imbibition.

dCAPS Analysis

To detect the SNP in *HvBRI1* gene, dCAPS method was performed as described by MICHAELS & AMASINO (1998). Genomic DNAs from 263 'uzu' and 55 normal barley lines were used as templates for mismatch-polymerase chain reaction (PCR). Restriction enzyme *Hha* I was used for the detection of the SNP in *HvBRI1*.

Results and Discussion

Morphological and Physiological Characteristics of uzu Gene

The *uzu* gene represents pleiotropic effects on the elongation of various part of plant including leaf, culm, rachis internode, awn and glume. (Fig. 1a). Furthermore, in 'uzu' line, grain size is also reduced in contrast to normal line (Fig. 1b). Grain size of 'uzu' line is almost same with that of rice. The reduced grain size of 'uzu' line seemed to be highly desirable and accepted by the people for mixing the rice and the barley grains because the polished barley was cooked with rice to supply the shortage of rice production before 1950s.

We found that, in contrast to normal lines, the 'uzu' line showed the unique elongation pattern of second internode with temperature dependent manner. Fig. 1c shows the elongation pattern of second internode from the top in 'uzu' line. The elongation of second internode in 'uzu' lines is specifically inhibited under 25˚C growth condition. This is the same phenotype with one of the internode elongation classes 'dm' in rice (TAKEDA 1977).

In this study, we also clearly detected the insensitivity of 'uzu' lines in germination test on agar plates with or without 1μM BL. On coleoptiles, leaves and root elongation, 'uzu' lines were not affected by exogenous BL. In contrast, normal lines showed screwed shapes of coleoptiles and severe inhibition of root elongation by BL treatment (data not shown).

Allelism of the Mutation in Barley HvBRI1 and uzu Gene

Recently, it has been revealed that one of the dm-type dwarfing rice mutants, *d61* showed the brassinosteroid (BR)-insensitive phenotype and that *D61* encoded the putative BR receptor gene *OsBRI1*, which located on rice chromosome 1 (YAMAMURO *et al*. 2000). It has been well known that the synteneus conservation was highly maintained between rice chromosome 1 and barley chromosome 3H where *uzu* was located (SMILDE *et al*. 2001). In order to confirm the morphological and physiological similarities between *d61* and *uzu*, we isolated the barley homologue of *OsBRI1* from normal ('Haruna Nijo' and 'H602'; ssp. *spontaneum*) and 'uzu' ('Akashinriki') lines and compared the DNA sequences followed by conventional method (The sequences have been deposited in DDBJ, accession number; AB109213, AB109214 and AB109215, respectively).

We found the single amino acid substitution responsible for single nucleotide polymorphism (SNP) in the barley homologous gene of *OsBRI1*, *HvBRI1*, between normal and 'uzu' lines. The 'uzu' line specific SNP generates arginine-missense at 857 residue mutation substitute for histidine in putative kinase domain IV (Fig. 2a). Furthermore, 'uzu' specific SNP found in *HvBRI1* was completely co-segregated with *uzu* gene in F_2 population, which were derived from 'H602' x 'Akashinriki' (Fig. 2b & 2c). These results strongly indicate that the SNP with amino acid substitution of *HvBRI1* is *uzu* mutation itself.

Phylogeny of BR Insensitive Barley Mutants in East Asia

Despite the limited geographic distribution, it has been well known that 'uzu' lines represent a very wide range of phenotypes including ear-awn type and degree of vernalization requirement (see for detail: http://www.rib.okayama-u.ac.jp/barley). In order to reveal whether these various 'uzu' lines had the SNP in common with the 'Akashinriki' *uzu* locus or not, we performed the dCAPS analysis. Two hundred sixty three 'uzu' lines were selected from Japan, Korean peninsula and China. Fifty five normal type accessions from the same region were also selected for the comparison.

Results of the dCAPS analysis showed that all of the investigated 'uzu' lines had the same mutation contributing to one amino acid residue substitution in the kinase domain of HvBRI1 (His_{857} to Arg_{857}) and that dCAPS marker developed in this study based on the SNP in *HvBRI1* gene could completely distinguish between 'uzu' and normal lines without exception (Table 1). These evidences suggested that various 'uzu' lines derived from spontaneous out crossing after a single mutation event of *uzu* gene.

In this study, we presented the morphological and physiological evidence that the *uzu* gene was the counterpart of rice *d61*. Empirically many of the gene mutations are repeatable, but *uzu* may be a sole mutant in the world and we have not obtained artificial mutant on this locus (LUNDQVIST *et al*. 1996). As well known 'Green Revolution' in rice *sd1* and wheat *Rht*s, the plant type with short, thick and erect leaves is an ideal semi-dwarf type of cereal crops,

especially adapted to heavy fertilizer application in intensive farming (TAKAHASHI 1964) and, in both of the semi-dwarf genes, several allelic mutations were reported (ASHIKARI *et al*. 2002; GALE & YOUSSEFIAN 1985). The unique mutant of *HvBRI1* gene in barley, *uzu*, shows the similar plant type to rice *sd1* and wheat *Rht*s in the view of intensive farming and has been also introduced into commercial varieties of barley to increase lodging resistance at least for a century. Our results suggest the single mutation in *HvBRI1* might be widely distributed in east Asia as a high-yielding semi dwarf gene of barley. Furthermore, although "Green Revolution" varieties in rice and wheat have defects in GA-biosynthesis and/or GA-signaling pathway, respectively (SASAKI *et al*. 2002; PENG *et al*. 1999), a practical dwarf gene concerning with the BR-signal transduction pathway or BR-biosynthesis has not been reported. This is the first report on the BR-related dwarfism in commercial cereal crops.

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Fig. 1. Morphology of normal and 'uzu' isogenic lines of 'Akashinriki'. **a**, Plant type: left; normal, right; 'uzu'. **b**, Grain shape: left; normal, center; 'uzu', right; rice. **c**, Second internode elongation under low (15 °C; left) and high (25 °C; right) temperature. White arrow heads show the position of nodes.

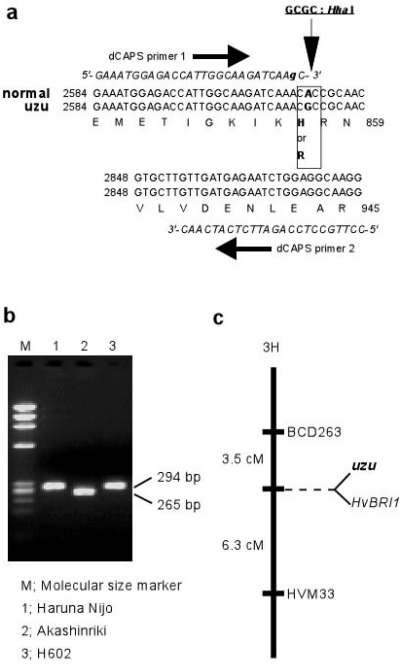

Fig. 2. Detection of the SNP between normal and 'uzu' lines and mapping. **a**, The method of dCAPS for detecting the SNP in *HvBRI*. Partial alignment of the deduced amino acid sequences of normal (upper) and 'uzu' (lower) lines around subdomain VI in *HvBRI1* is shown. The position of single nucleotide substitution is represented by bold. The primers for PCR are shown in the upper (dCAPS primer 1) and lower (dCAPS primer 2) parts of alignment in italics. The base written in lower-case in dCAPS primer 1 represents the mismatch-base to generate the restriction enzyme *Hha* I recognition site (GCGC) in the PCR product derived from 'uzu' line(s). **b**, dCAPS products from 'Haruna Nijo' (normal line; lane 1), 'Akashinriki' ('uzu' line; lane 2) and 'H602' (ssp. *spontaneum*, normal line; lane 3) on agarose gel. When the PCR product includes the unique *Hha* I recognition site in *HvBRI1* such as 'Akashinriki' (lane 2), the size becomes 265 bp after *Hha* I digestion because of the separation of part of the dCAPS primer 1 (28 bp) by enzymatic digestion. M; molecular size marker. **c**, Partial linkage map of barley chromosome 3H in F_2 population of 'H602' x 'Akashinriki'. The location of *uzu* gene is shown in bold, and the SNP detected in *HvBRI1* in italic.

Table 1. dCAPS patterns of *HvBRI1* in 'uzu' and normal accessions.

Plant type	Region	*HvBRI1*	
		Cleaved	Not cleaved
'uzu' (263)	Japan	225	0
	Korean peninsula	34	0
	China	4	0
normal (55)	Japan	0	30
	Korean peninsula	0	23
	China	0	2

QTL Analysis in *Hordeum bulbosum* L. for Interspecific Crossability and Hybrid Formation with Barley

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Abstract
H. bulbosum is important in barley breeding for haploid production and for the introgression of useful genes. An RFLP map of *H. bulbosum* was created which covers about 90% of the *H. bulbosum* genome (SALVO *et al*. 2001). The two parents of this mapping population were also chosen on differences when used as pollinators onto barley. PB1 had high crossability and gave high rates of haploid production; in contrast, PB11 had a lower crossability and gave a high frequency of hybrids. Experiments were carried out to map QTL involved in these different responses. To phenotype the 75 recombinant *H. bulbosum* F_1 clones, all were pollinated onto a barley cultivar, Triumph, under controlled environmental conditions. QTLs for crossability were mapped on chromosomes $2H^b$, $3H^b$, $6H^b$ and $7H^b$. A novel QTL promoting hybrid formation, heterozygous in the PB11 parent, was mapped on chromosome 6H*b*, and explained about 40% of the phenotypic variation for this trait. This QTL would enable more efficient selection of *H. bulbosum* clones for use in introgressing traits from *H. bulbosum* into barley. The QTLs for crossability identified will enable the selection of more efficient clones for haploid production by MAS.

Introduction

H. bulbosum is the closest relative of barley and is important for haploid production and for the introgression of useful genes into barley. However, unlike barley, *H. bulbosum* is a perennial, out-crossing, self-incompatible species, and therefore, each separate accession is a unique heterozygous individual which can be maintained by clonal propagation. To study genome relationships between *H. bulbosum* and barley SALVO *et al*. (2000) developed a recombinant F_1 population between two clones, PB1 and PB 11 maintained at the John Innes Centre. A composite RFLP map was developed which covered about 90% of the *H. bulbosum* genome by combining separate maps developed for the two parents. This map showed that the barley and *H. bulbosum* genomes have complete co-linearity, although there were some differences in recombination frequencies between syntenic loci, indicating differences in chiasma distribution between the species.

The availability of this map also allows a study of trait differences between the parents of the cross by QTL analysis, in particular with respect to crossability and haploid production differences, since the two parents of this mapping population showed very different results when used as pollinators onto barley. PB1 had high crossability and gave high rates of haploid production; in contrast, PB11 had a lower crossability and gave a high frequency of hybrids. Thus, this cross is ideal for dissecting the genetic control of these crossability and haploid production characteristics.

Material and Methods

Experimental Design and Trait Evaluation

The *H. bulbosum* crossing, embryo rescue and *in vitro* culture techniques were carried out as described by SIMPSON and SNAPE (1981) and PICKERING (1991). A completely randomized design was used with four replications (spikes) for each clone. Crosses were

randomized over plant and days, and the experiment was carried out over two years with half of the population crossed each year. In each year 12 replications of the parents, PB1 and PB11 were carried out as controls. The crossing programme was carried out during the same months of the corresponding years.
With the female parent, Triumph, each replication was represented by one spike with 20 flowers. For each recombinant clone, a mixture of fresh pollen from about 5-10 spikes per individual was used for pollination, with the aim of having a homogeneous sample of pollen. The following crossability traits were evaluated as a percentage:

- Seeds per florets pollinated = S/F (%) →(number of seeds set / number of floret pollinated)*100
- Embyos per seeds = E/S (%) → (number of embryos obtained / number of seeds fertilized)*100
- Plants per embryos = P/E (%) →(number of plants obtained / number of embryos excised)*100
- Callus per embryos = C/E (%) →(number of calli obtained / number of embryos obtained)*100
- Hybrids per embryos = Hy/E (%) →(number of hybrid plants produced / number of embryos obtained)*100

Mapping Population, Design and QTL Mapping Methods
For analysis, 75 recombinant clones were phenotyped. For QTL mapping, MapQTL, version-3 was used, which allows QTL analysis in full sib-families. Single marker analysis, non-parametric mapping, interval mapping and multiple-QTL models (MQM) were used to detect significant QTL for different crossability traits.

Results

Phenotypic Performance of Parents and F_1 Full-Sibs
Differences between parent and recombinant clones were highly significant for all traits under study. The frequency distribution for almost all traits was approximately normally distributed, indicating polygenic inheritance. However, the frequency of hybrid plants per embryos was non-normally distributed, where few recombinant clones produced hybrid plants. This might indicate that this trait is under the control of either one or few recessive genes. Transgressive segregation was present for almost all traits, which is significant with respect to crop improvement, since it represents a potential source of genetic variation, where both parents can contribute useful alleles to enhance a particular trait.

QTLs for Pre-Fertilization Interspecific Crossability Barriers
Pollen tube-stylar incompatibility is related to seed set per total number of florets pollinated (S/F), which has been attributed to a pre-fertilization interspecific crossability barrier. QTLs controlling this trait were mapped on chromosomes $3H^b$ (near *Xbcd134*), $6H^b$ (near *Xmwg652*), and $7H^b$ (near *Xpsr466b*). The three independent QTLs explained about 65% of the total phenotypic variance for the trait. The most crossable parent, PB1, had an additive effect of 7.4% and the less crossable parent also possessed some positive alleles with an additive effect of 4%, giving an overall additive effect of 12%. These loci are not associated with the S and Z self-incompatibility loci which are located on $1H^b$ and $2H^b$, respectively. Homoeologous chromosome 3H, 7H, wheat group 3 and 6D have been reported to have significant QTLs for crossability (SNAPE *et al*. 1979; MILLER *et al*. 1983; ROMERO and CUADRADO 1992; TAKETA *et al*. 1998). Since anchor markers appeared in similar confidence intervals, it is possible that these QTLs are indicating the presence of homoeoallelic loci in *H. bulbosum*.

QTLs for Post-Fertilization Interspecific Barriers

Endosperm degeneration and embryo aberrations are more related to post-fertilization interspecific barriers. In this study, no significant correlation was found between S/F and P/E (plants obtained per embryos excised); E/S (embryos obtained per seed fertilized) and P/E. This indicates that the pre- and post-fertilization interspecific barriers are under different genetic control. Two QTLs for P/E were mapped in the distal region of the short and long arms of chromosome $7H^b$ (near *Xcdo545* and *Xwg240b*) in the combined map. The two independent QTLs explained about 36% of the total phenotypic variation for the trait. This confirms the presence of transgressive segregation, where favourable alleles from PB11 had an additive effect of 6%.

The same situation was observed for a QTL for E/S mapped on chromosome $2H^b$ (near *Xpsb24*) where both parents contributed positive alleles for crossability. Since the QTL in the individual parental maps had fully informative markers, the QTLs were mapped in the same genomic region of the combined map. The QTLs explained about 15% of the phenotypic variation for the trait.

QTLs for Hybrid Production Frequency and Callus Formation

The production of hybrid plants represent a detrimental effect on the efficiency of haploid production, but it is a positive factor when the aim is to produce hybrids for the introgression of genes from *H. bulbosum* into barley. A QTL for hybrid formation was mapped on the PB11 map on chromosome $6H^b$. Differences in the alleles from this parent explained 15% of the phenotypic variation for the trait. The non-parametric mapping method (Kruskal-Wallis) and single marker analyses indicated that the marker *Xpsb84* was co-segregating with variation for the trait ($P<0.0005$), Figure 1, where the "*c*" allele of the PB11 parent for this fully informative locus was highly associated with hybrid formation in the segregating population. These data suggest that the trait might be under the genetic control of a single partially recessive gene because of the low frequency of hybrid formation and only one significant QTL. In barley, this locus is on 6HL, near the centromere. It is interesting to note that the greatest number of introgressions and chromosome substitutions has been achieved with $6H^b$ for/onto chromosome 6H (PICKERING *et al*. 2000). This might be indicating that there is a gene associated with chromosome elimination and homoeologous pairing between these two closely related species.

Callus formation is a negative factor in haploid production. A QTL for embryos producing callus rather than germinating (C/E) was mapped on the proximal region of chromosome $5H^b$ (near *Xpsb134*).

The QTL for callus formation was highly associated with the QTL alleles for lower crossability from the PB11 parent. Previous studies have pointed out that in wheat, the short arm of 5B carries alleles suppressing crossability (TIXIER *et al*. 1998). This might be indicating that the alleles for callus formation and crossability are pleiotropic, but most probably different but linked, but nevertheless combine to make PB11 a less desirable parent for haploid production.

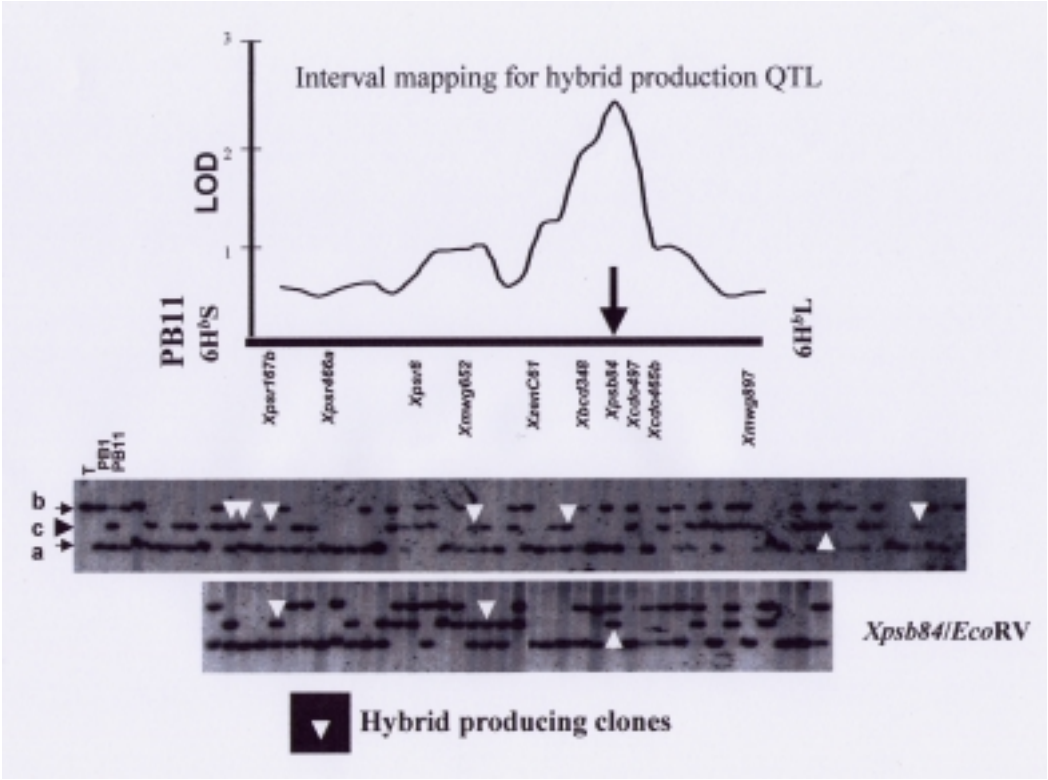

Figure 1. QTL analysis on chromosome $6H^b$ for hybrid formation frequency in the PB1 x PB11 F_1 population

Conclusions

The QTLs located in this study are indicating that the genetic control of interspecific crossability barriers and hybrid formation can be elucidated at the genetic and, possibly, molecular levels. These traits can be now manipulated by marker-assisted-selection to improve haploid production efficiency and the introgression of valuable traits from this *H. bulbosum* into barley.

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Identification of Quantitative Trait Loci Controlling Morphological and Physiological Traits, which are Characteristic between Oriental and Occidental Barley Cultivars (*Hordeum vulgare* L.)

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Abstract

A total of 99 recombinant inbred lines (RILs) derived from a cross of Oriental six-rowed × Occidental two-rowed cultivars were grown during the two seasons 2001 and 2002 to identify quantitative trait loci (QTL) controlling agronomic traits such as spike characters (length, density, grain number, triplet number, awn length), plant height, tiller number and days to heading. The RILs showed wide variations for each trait, showing the wide range of genetic diversification between Oriental and Occidental varieties and segregation in the progenies. The composite interval mapping identified three QTLs affecting plant height, of which one QTL on chromosome 7HL was newly identified in addition to *uzu* gene and *dsp1* gene. For spike length and density one new QTL was identified on chromosome 2HL, which is closely linked to the cleistogamy gene and Fusarium head blight resistance QTL. For heading date one new QTL was identified on chromosome 7H. This study showed the Oriental × Occidental barley RILs are good resources for discovering of novel genes with agronomic importance.

Keywords: *Hordeum vulgare*; agronomic traits; *uzu* gene; *dsp1* gene; quantitative trait loci (QTL)

Introduction

In contrast to qualitative trait in nature, the variability exhibited by many agriculturally important traits fails to fit into separate phenotypic classes. Economically important traits are quantitative with continuous variability. The inheritance of these characters is complex and may be governed by many genes that interact with environmental component; therefore their individual effects cannot be detected by Mendelian methods (STANSFIELD 1991).

Six-rowed winter cultivar 'Azumamugi' (AZ) and two-rowed spring cultivar 'Kanto Nakate Gold' (KNG) are highly polymorphic for morphological, physiological, isozyme and molecular markers (KOMATSUDA *et al.* 1993, MANO *et al.* 2001) probably due the two cultivars belong to Oriental and Occidental type, respectively (TAKAHASHI *et al.* 1983). We developed a linkage map of recombinant inbred lines (RILs) of a cross between AZ × KNG (MANO *et al.* 2001), which is suitable to detect genes controlling agronomic traits. The aim of the present study is to identify QTLs for morphological and agronomical traits in a population of recombinant inbred lines from the cross between cultivars.

Material and Methods

The experimental plots of RILs were grown with their parents in two replications for 2002 and without replication for 2001. Within blocks each RIL was represented by a row of ten seeds and space between two seeds was 20cm. Rows spaced 80cm apart. During the growing season, all measurements for the following characters at National Institute of Agrobiological Sciences (NIAS), Tsukuba, Japan were scored: Days to heading (DHe) number of days from sowing to heading when the base of 50% of the ears has emerged from

the flag leaf sheath; Spike Length (SPL $_{cm}$) measured from the base of spike to the top of the terminal spikelet (exclude awn); Spike-Internode Length or spike density (SIL $_{mm}$) calculated by formula 10(SPL / TPN - 1 $_{mm}$); Awn Length (AWL $_{cm}$) length of the awn from tip of the seed measured in central spikelet of third triplet from base of spike; Plant Height (PLH $_{cm}$) from the ground to the top of the terminal spikelet (exclude awn); Tiller Number (TN), number of tillers which contain fertile spike; Triplet Number (TPN) in two-rowed only central spikelet is fertile but in six-rowed all three spikelets are fertile, in this case Grain Number (GN) per ear in two-rowed is equal of triplets number and Thousand Kernel Weight (KWT $_{gr}$) was calculated from the weight of 250 kernels.

For QTL analysis we used the base map with 100 markers (MANO & KOMATSUDA 2002) to provide a density of 5 to 10 cM intervals without the clustering markers. Map distances were estimated by using kosambi's map function (KOSAMBI 1944). Composite interval mapping (CIM) was performed using the computer program QTL Cartographer version 1.14 (BASTEN *et al.* 2000). A log-likelihood (LOD) score threshold of 3.0 was used to identify genomic regions containing putative loci associated with the trait. Comparisons between the results of the present study and those already reported were obtained with the help of and IMM map (Integrating Molecular and Morphological/Physiological Marker Maps (KLEINHOFS 1994)).

Results and Discussion

'uzu' Gene

Numerous studies have been conducted for the identification of QTLs for agronomic traits in the previous studies but none of the parents carried the *uzu* gene. The *uzu* gene is on chromosome 3HL and coming from the Japanese cultivar such as Azumamugi (TSUCHIYA 1984). However this investigation revealed that, this gene pleiotropically affected important characters such as PLH, SPL, SIL, and AWL, which were expected (Table 1). Also expression of the QTLs for these traits was associated with the *uzu* locus and the effects were highly consistent over environments and the allele of Azumamugi (*uzu* type) decreased the traits. For comparison of this locus with the other map, we could place the *uzu* locus in the *BCD828-MWG571B* interval (in IMM map). In addition *uzu* locus was located 30 cM distance to *ABG471*, which is located on short arm of chromosome 3H.

'*dsp1*' *Gene*

The QTL was located on chromosome 7HS close the marker *cMWG704* probably identical to the dense spike 1 (*dsp1,* formerly *l)* locus, because the QTL controlling the traits SPL, SIL, AWL and PLH was located at the same position. The *dsp1* gene occurs naturally in local cultivars from Korea and Japan, and Azumamugi have the *dsp1* allele at this locus (TAKAHASHI 1951). The *dsp1* gene has pleiotropic effects with reduction effect on these traits and the QTL on chromosome 7HS has the same effect as *dsp1* gene. Also *dsp1* gene in many cultivars of Oriental origin often associated with the short awn gene (TAKAHASHI 1951).

Days to Heading

Three QTLs were detected for DHe on chromosomes 1HL, 2HS and 5HL for two years and one on chromosome 7H only for 2002. The QTL on chromosome 1HL can be identical with that identified by TOHNO-OKA *et al.* (2000) between *ABC322B* and *ABC261* using Steptoe × Morex doubled haploid lines in autumn sowing condition. The *ABC261* was common marker for the two QTLs and two QTLs are closely linked. Also MARQUEZ-CEDILLO *et al.* (2001) identified a QTL for heading date in spring sown on chromosome

1HL using Harrington × Morex doubled haploid lines. The QTL detected in our study was overlapped with this QTL. Also this QTL can be identical with *eam8* (early maturity 8, TAKAHASHI & YASUDA 1970), because the *eam8* was around 14 cM distal to *ABC261* in chromosome 1HL. The *eam8* naturally occurred in Japanese two–rowed barley (ex. Kinai 5 and Kagoshima Gold), so probably Kanto Nakate Gold (two-rowed) has a homologous gene (QTL). The QTL on chromosome 2HS closely linked with *ABG602* can be identical to *eps2S* (earliness per se) gene. LAURIE *et al.* (1995) detected *eps2S* gene on chromosome 2HS and it was located 36.4 cM proximal to the marker *MWG865* at the same position as QTL in present study.

The *Sgh2* locus presented in many spring barley cultivars such as Kanto Nakate Gold and under the non-vernalized condition made these cultivars earlier flowering (TAKAHASHI & YASUDA 1970). The QTL on chromosome 5HL was mapped at the same interval covering *Sgh2* locus (SAMERI & KOMATSUDA, unpublished data), but lines with the Kanto Nakate Gold (spring cultivar) allele were "later" flowering. This interval probably contains both *Sgh2* and an earliness gene (QTL), or the *Sgh2* responds in two different directions under the different environment. We detected another QTL on chromosome 7H at 18 cM to the marker *ABG701*. The QTL did not cover *eps7L* (LAURIE *et al.* 1995). The 2002 early spring had higher temprature than normal years, so that this QTL was highly affected by environment.

Spike Length and Spike Inter-Node Length (Spike Density)

QTLs for the traits SPL and SIL were found on chromosomes 2HL, 3HL and 7HS in present study. In addition to *uzu* gene and *dsp1* gene, one QTL was identified on chromosome 2HL at 8cM proximal to marker *ABG613*. Azumamugi allele made the SPL and SIL larger and it was opposite to the effects by *uzu* and *dsp1* gene. This QTL is probably identical to the zeocriton1 (*zeo1*) locus, although *zeo1* is an individual mutant (FRANCKOWIAK & R.I. WOLFE 1997). With the help of IMM map the *zeo1* gene was located on chromosome 2HL at *ABC157-ABC165* interval. The marker *ABG613* (present study) was located at 10 cM distal to the marker *ABC165*; therefore our QTL is 2 cM distal to the *zeo1* region. Plants with the *zeo1* gene have short culms and compact (dense) spikes (FRANCKOWIAK & R.I. WOLFE 1997). In addition this QTL is closely linked to the cleistogamy gene, which was mapped at the same interval marker (TURUSPEKOV *et al.* in press) and also Fusarium head blight resistance QTL (MESFIN *et al.* 2003). The QTL of SPL on chromosome 2HS close to *ABG602* might correspond to earliness gene (*eps2S*).

Awn Length

In addition to *uzu* and *dsp1* gene one QTL was located on chromosome 2HL close the *vrs1* locus. The complex *vrs1* locus may include awnless and reduced awn length mutant (TAKAHASHI *et al.* 1982). Therefore, the QTL on 2HL might be identical with the *vrs1* locus.

Plant Height

In addition to *uzu* gene and *dsp1* gene, one major QTL was found on chromosome 7HL for two years. This QTL was located at 1 cM proximal to the marker *ABG608* on chromosome 7HL and Azumamugi allele increased the trait. No QTL was reported at this region for PLH and this QTL might be a new one controlling PLH. Our QTL at centromere region on chromosome 7HS corresponded closely with plant height QTL detected by HAYES *et al.* 1993 at *ABG701 – ABG119* interval, TINKER *et al.* 1996 at *Brz – MWG003* interval and BEZANT *et al.* 1996 at *Xpsb23 – Xpsr56* interval. The QTL on chromosome 4HL was mapped at the same interval covering *sgh1* (spring growth habit) locus (SAMERI &

unpublished data) for plant height in 2001 and lines with the Azumamugi (winter cultivar) allele increased plant height. The QTL detected in this study on chromosome 4HL is presumably the pleiotropic effect of spring growth habit gene. The QTL on chromosome 5HL was mapped at the same interval covering *Sgh2* locus (SAMERI & KOMATSUDA, unpublished data), and lines with the Azumamugi allele made shorter plant height. Our QTL on chromosome 5HL at *Sgh2* locus corresponded closely with plant height QTL detected by THOMAS *et al.* 1995 between the marker *OPA19-H1000* and – *CDO504*. The marker *CDO504* is located at 1.5 cM proximal to the marker *WG644,* which is very close to the Sgh2 locus (LAURIE *et al.* 1995). However the location of two QTLs on chromosomes 4HL and 5HL was unstable across the environment but we should not negligent spring habit gene effect on plant height.

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Table 1. QTLs detected by composite interval mapping with QTL Cartographer for several agronomic traits in two years (2001and 2002).

	2001						2002				
Trait	Chr	Flanking marker	Pos cM	LOD	AE	R^2	Chr	Flanking marker	Pos cM	LOD	AE	R^2
	1HL	*ABC261 - ABG055*	142.97	5.38	2.00	0.115	1HL	*ABC261 - ABG055*	144.97	5.66	3.31	0.127
DHe	2HS	*e13m23.6 - e13m31.7.1*	49.82	11.20	-2.86	0.235	2HS	*e13m23.6 - e13m31.7.1*	49.82	4.58	-2.82	0.092
	5HL	*e07m25.3 - e12m19.9.1*	106.13	9.60	-2.80	0.226	5HL	*e07m25.3 - e12m19.9.1*	106.13	15.90	-6.60	0.430
							7H	*e11m17.10.2 - e12m19.10.3*	93.5	3.3	2.6	0.07
	2HS	*ABG602 - e13m23.6*	47.29	3.12	-0.40	0.036						
	2HL	*e11m19.3 - ABG613*	122.2	13.17	0.93	0.176	2HL	*e15m31.1 - e11m19.3*	121.80	14.10	0.95	0.187
SPL	3HL	*uzu - e06m30.10.1*	65.53	18.51	-1.16	0.280	3HL	*uzu - e06m30.10.1*	65.53	18.52	-1.11	0.251
	7HS	*cMWG704 - e11m17.10.2*	84.02	20.70	-1.23	0.340	7HS	*cMWG704 - e11m17.10.2*	82.02	24.83	-1.29	0.361
	2HL	*e11m19.3 - ABG613*	122.2	10.78	0.29	0.136	2HL	*e15m31.1 - e11m19.3*	121.80	13.35	0.30	0.160
SIL	3HL	*uzu - e06m30.10.1*	65.53	18.81	-0.42	0.296	3HL	*uzu - e06m30.10.1*	65.53	22.14	-0.42	0.324
	7HS	*cMWG704 - e11m17.10.2*	84.02	20.51	-0.44	0.347	7HS	*cMWG704 - e11m17.10.2*	84.02	23.85	-0.44	0.367
	2HL	*vrs1 - MWG503*	79.56	6.37	-0.69	0.026	2HL	*vrs1 - MWG503*	79.56	5.90	-0.67	0.026
AWL	3HL	*uzu - e06m30.10.1*	65.53	51.39	-3.69	0.764	3HS	*uzu - e06m30.10.1*	65.53	51.13	-3.79	0.773
	7HS	*cMWG704 - e11m17.10.2*	84.02	4.63	-0.60	0.022	7HS	*cMWG704 - e11m17.10.2*	82.02	7.24	-0.77	0.037
	3HL	*uzu - e06m30.10.1*	65.53	31.71	-12.13	0.564	3HL	*uzu - e06m30.10.1*	65.53	25.87	-12.03	0.478
	4HL	*e04m32.8 - e12m19.8*	73.38	3.61	2.86	0.033						
PLH							5HL	*e07m25.3 - e12m19.9.1*	104.13	3.70	-3.36	0.040
	7HS	*cMWG704 - e11m17.10.2*	84.02	8.63	-5.08	0.107	7HS	*cMWG704 - e11m17.10.2*	80.02	7.48	-4.96	0.088
	7HL	*e12m22.10.2 - ABG608*	142.29	11.41	5.86	0.139	7HL	*e12m22.10.2 - ABG608*	139.76	14.42	7.70	0.212
TN	3HL	*uzu - e06m30.10.1*	67.53	3.98	-2.77	0.140						
	5HL	*e07m25.3 - e12m19.9.1*	112.13	3.56	-2.23	0.120	5HL	*e07m25.3 - e12m19.9.1*	112.13	3.63	-2.10	0.128
TPN	2HS	*ABG602 - e13m23.6*	47.29	12.16	-2.28	0.375	2HS	*ABG602 - e13m23.6*	47.29	7.67	-1.53	0.256
	5HL	*e14m27.4.4 - MWG2230*	88.37	3.80	-1.04	0.083	5HL	*e07m25.3 - e12m19.9.1*	106.13	4.07	-1.09	0.129
GN	2HS	*ABG602 - e13m23.6*	47.29	8.25	-4.88	0.028	2HS	*ABG602 - e13m23.6*	47.29	4.01	-3.57	0.013
	2HL	*vrs1 - MWG503*	79.56	66.86	28.01	0.910	2HL	*vrs1 - MWG503*	79.56	73.94	30.05	0.860
	2HS	*e13m31.7.1 - e15m19.8.1*	57.49	4.09	1.59	0.043						
KWT	2HL	*vrs1 - MWG503*	79.56	32.88	-6.57	0.715	2HL	*vrs1 - MWG503*	79.56	25.52	-5.95	0.629
	5HL	*e07m25.3 - e12m19.9.1*	112.13	4.42	2.02	0.049						

AE: Additive Effect of "Azumamugi"allele. R^2 :Proportion of the phenotypic variance explained.

Molecular Markers and Marker Assisted Selection in Winter Barley Breeding

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Abstract

Molecular markers linked to *rym4* and *rym11* resistance genes and marker assisted selection principle have been used for creation of BaMMV, BaYMV, and BaYMV-2 winter barley resistant lines. Seventy-five individuals carrying *rym4*-linked DNA marker, 78 carrying *rym11*-linked marker, and 6 individuals possessing markers for both resistance genes have been identified. Both codominant DNA markers fulfil the conditions for advanced markers for molecular breeding.

Introduction

Barley (*Hordeum vulgare* L.) is one of the oldest crops used by man, especially for animal feed and brewing. Both, winter and spring barleys are grown in our agro-ecological conditions. The main reasons of high yield losses in barley are fungal and virus diseases, which are spreading with increasing of barley cultivation area. The production of East-Asian and European winter barley is seriously damaged by viruses. Disease is caused by complex of at least three viruses – barley mild mosaic virus - BaMMV, barley yellow mosaic virus – BaYMV, and BaYMV-2. All of these viruses are transmitted by soil-borne fungus *Polymyxa garaminis* (TOYAMA, KUSABA, 1970). Chemical control against these viruses is inefficient and unacceptable from ecological or economical reasons (ORDON *et al*., 1999). The most common approach for prevention of infection by these viruses is the introgression of resistance genes into breeding lines and cultivars (OKADA *et al*., 2003). The resistance of European barley cultivars against BaMMV and BaYMV has been exclusively based on the recessive resistance gene *rym4* (GRANER *et al*., 1999). But this gene does not confers resistance against strain BaYMV-2 (HUTH, 1989; FRIEDT *et al*., 1990). Just recently cultivars Tokyo and Kamoto carrying *rym5* have been released being also resistant to BaYMV-2 (HEMKER, pers. com. ex WERNER *et al*., 2003). Another resistance genes have been identified (WERNER *et al*., 2000 and ORDON *et al*., 2003), the last one is *rym13* gene present in cultivar Taihoku A from Taiwan (WERNER *et al*., 2003). New resistance genes are predominantly carried by exotic cultivars which usually show very poor agronomic performance under European environmental conditions. So classical breeding for resistant cultivars using these genotypes is long and difficult (GOUIS *et al*., 2000). Marker assisted selection as well as using of better adapted gene donors and searching for resistant cultivars among old native ones and local landraces can be helpful.

The RFLP and different PCR-based markers (i.e. RAPDs, AFLPs, SSRs, STSs, CAPS) have been developed for *rym1*, *rym4*, *rym5*, *rym9*, and *rym11* genes (ORDON *et al*., 1999, WERNER *et al*., 2003, OKADA *et al*., 2003). Today these markers enable an efficient marker-assisted selection for these genes avoiding the need for mechanical inoculations, ELISA or tissue-blotting techniques, and time-consuming field tests. They transfer selection from the phenotype to the genotype level. Codominant markers also enable to enhance backcrossing procedures by detecting of heterozygous carriers of the resistant alelle directly in F_1, thereby saving one year for each backcrossing cycle. These markers also offer opportunity for gene pyramiding, i.e. combination of different resistance genes in line or

cultivar for longer lasting resistance. In this process markers are needed also due to lack of differentiating pathogen strains (ORDON *et al*., 1999).
The BaYMV disease is not present at this time in Slovakia and Czech Republic, but according to studies of ŠPUNAR *et al*. (1999) any cultivar or line possesses resistance against BaYMV-2. With increasing of the acreage of winter barley, obtaining of resistance to the BaYMV complex belongs to breeding goals (ŠPUNAR *et al*., 1999). In our study we decided to create resistant lines by introducing of resistance genes *rym4* (resistance against BaYMV and BaMMV) and *rym11* (resistance against BaYMV-2, BAUER, 1997) into winter barley varieties by combination of classical hybridization and MAS techniques.

Material and Methods
The acceptors of both resistance genes were cultivars Copia and Tiffany. Cultivar Romanze (obtained from the Genebank, VÚRV Piešťany) has been used as a donor of *rym4* gene and landrace Russia 57 (obtained from the Genenank, IPK Gatersleben) as gene *rym11* donor. We used classical hybridization among these materials and MAS as indirect selection. Codominant STS marker derived from RFLP marker MWG838 was used as marker linked to *rym*4 gene (TUVESSON *et al*., 1998), while codominant SSR marker HVM3 was used as marker linked to *rym11* gene (BAUER *et al*., 1997). Genomic DNA was isolated from segments of young leaves by Plant DNAzol (Invitrogen). Amplification using the SSR primer pair HVM3 was carried out in a 25-µl reaction volume containing 50 ng of DNA, 1.5 mM $MgCl_2$, 100 µM of each dNTP, 200 nM of each primer, and 1 U of Taq-DNA polymerase. PCR reactions were performed using the following conditions: denaturation (94ºC for 3 min) was followed by 35 cycles of amplification (94ºC for 30 s, 55ºC for 30 s, 72ºC for 1 min) and final extension step (72ºC for 5 min). Amplification products were separated in 1.8% agarose gels. Amplification using the STS primer pair HWG838 was carried out in a 25 µl reactions containing 50 ng of DNA, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 200 nM of each primer, and 0.5 U of Taq-DNA polymerase. PCR reactions were: denaturation (96ºC for 5 min) followed by 35 cycles of amplification (95ºC for 1 min, 59ºC for 1 min, 72ºC for 30 s, the polymerization step was extended 3 s per each cycle) and final extension step (72ºC for 10 min). Amplification product was digested by *Rsa*I and DNA fragments were separated in 2% agarose gels.

Results and Discussion
The cultivar Tiffany was selected due to its ability to better agronomical parameters and comparable malting quality to cultivars Akcent and Tolar (ŠPUNAR *et al*., 2001, 2002). The Russia 57 was also used for classical hybridization with progeny of Copia x Romanze carrying the marker linked to *rym4* gene in order to obtain plants with combination of both genes. After hybridization, F_1 progenies were self-pollinated and DNA analyses were performed from young plants of F_2 progenies. As both these genes are inherited recessively, linked codominant molecular markers (STS marker MWG838 for gene *rym4* and SSR marker HVM3 for gene *rym11*) were used for their detection in both resistant parents as well as in F_2 progenies of all crosses. Total number of analyzed individuals of F_2 progenies was 150. Both codominant markers linked to desired resistance genes showed a clear differentiation between individuals possessing resistant (recessive homozygote) and susceptible (dominant homozygote and heterozygote) linked markers. Homozygous and heterozygous individuals were excluded from further analyses. Expected segregation in F_2 generation was 1:2:1 for both genes. This was in good coincidence with experimental ratios for all combinations, differences were not statistically significant ($\alpha = 0.05$). Out of 150 analyzed plants of combination Copia x Romanze only 37 plants had genotype *rym4rym4* detected by linked marker, while from combination Tiffany x Romanze 38 plants carrying marker were detected.

Segregation in F_2 progeny suggests that resistance is controlled by single gene (ORDON, FRIEDT, 1993; KONISHI *et al*., 1997; PELIO *et al*., 2000). F_2 plants identified by molecular markers as probable carriers of *rym4* gene were self-pollinated and re-tested. We did not find dominant homozygous or heterozygous genotypes. Out of 150 analyzed plants from the combination Tiffany x Russia 57, 48 plants had genotype *rym11rym11*, while from combination (Copia x Romanze) x Russia 57, 30 plants were detected. Out of these 30 plants carrying marker linked to gene *rym11* only 6 plants were carrying also marker linked to gene *rym4*. Unfortunately all these plants produced only seeds which were not able to germinate, what probably means that this combination of cultivars is not suitable for pyramiding of these genes. After process of backcrossing (5 backcross generations) with the help of MAS, new created winter barley lines carrying marker linked to gene *rym4* and lines carrying marker linked to gene *rym11* will be undergo resistance tests on infested fields and will be tested by agronomic tests.

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Genetic and Physical Mapping of Genic Microsatellites in Barley (*Hordeum vulgare* L.)

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Abstract
Due to the availability of sequence data from large scale EST (expressed sequence tag) projects, it has become feasible to develop microsatellite or simple sequence repeat (SSR) markers from genes. A set of 111,090 barley ESTs (corresponding to 55.9 Mb of sequence) was employed for the identification of microsatellites with the help of a PERL5 script called *MISA*. As a result, a total of 9,564 microsatellites were identified in 8,766 ESTs (SSR-ESTs). Cluster-analysis revealed the presence of 2,823 non-redundant SSR-ESTs in this set. From these 754 primer pairs were designed and analysed in a set of seven genotypes including the parents of three mapping populations. Finally, 185 microsatellite (EST-SSRs) loci were placed onto the barley genetic map. These markers show a uniform distribution on all the linkage groups ranging from 21 markers (on 7H) to 35 markers (3H). The polymorphism information content (PIC) for the markers developed ranges from 0.24 to 0.78 with an average of 0.48. For the asignment of these markers to BAC clones, a PCR-based strategy was established to screen the "Morex"-BAC library. By using this strategy BAC addresses were obtained for a total of 127 mapped EST-SSRs, which may provide at least two markers located on a single BAC. This observation is indicative of an uneven distribution of genes and may lead to the identification of gene-rich regions in barley genome.
Keywords: EST-SSRs; genic-microsatellites; genetic mapping; physical mapping

Introduction

The analysis of DNA sequence variation is of major importance in genetic studies. In this context, molecular markers represent a major tool for genome mapping and have revolutionised the genetic analysis of crop plants. In the past, a variety of molecular markers including RFLPs (*R*estriction *F*ragment *L*ength *P*olymorphism*s*), RAPDs (*R*apid *A*mplification of *P*olymorphic *D*NA*s*), AFLP*s* (*A*mplified *F*ragment *L*ength *P*olymorphsim*s*) and microsatellites or SSRs (*S*imple *S*equence *R*epeats) have been developed in barley (see VARSHNEY *et al.* 2004). Among different classes of molecular markers, SSR markers have proven as markers of choice for a variety of applications in plant genetics and breeding because of their multiallelic nature, codominant inheritance, relative abundance and extensive genome coverage (reviewed by GUPTA & VARSHNEY 2000). In barley, about ~400 SSR loci have been mapped (RAMSAY *et al.* 2000; PILLEN *et al.* 2000; LI *et al.* 2003).

With the establishment of EST sequencing projects, a wealth of sequence information is being generated allowing the possibility for identification and development of SSR markers from ESTs

(KANTETY *et al.* 2002; VARSHNEY *et al.* 2002). EST-SSR markers are superior to genomic SSRs in terms of transferability and comparative mapping in related species as they are derived from conserved portions of the genome. To further increase the resource of barley microsatellite markers, we utilized a set of barley ESTs for the computer-assisted identification of SSRs and the development of corresponding markers (THIEL *et al.* 2003).

In this paper, we report on identification of SSRs in 111,090 barley ESTs generated at IPK and the development and genetic mapping of a non-redundant set comprising 185 genic microsatellite markers. In addition, a PCR-based strategy was employed to screen the "Morex"-BAC library with SSR (PCR-based) markers and BAC-addresses were obtained for 127 SSR-ESTs.

Material and Methods

Plant Materials

For detection of SSR polymorphism, a set of 7 barley (*Hordeum vulgare* L.) cultivars was used. This set included 'Barke', 'Igri', 'Franka', 'Steptoe', 'Morex', Oregon Wolfe Barley 'OWB_{Dom}' and OWB_{Rec}'. 'Barke' was used as a standard because this cultivar was used for the construction of most EST libraries, while the other six genotypes represent the parents of three doubled haploid (DH) mapping populations. Genomic DNA isolation was carried out as described in THIEL *et al.* (2003).

Database Mining

A total of 111,090 barley EST sequences, generated from 22 cDNA libraries (MICHALEK *et al.* 2002; http://pgrc.ipk-gatersleben.de/b-est/) were screened for microsatellites by using the *MISA* software module described by THIEL *et al.* (2003) and available under http://pgrc.ipk-gatersleben.de/misa/. To improve the efficiency of the identification of polymorphic SSRs, we included another set of 207,449 barley ESTs present in GenBank (NCBI, USA), which were developed from cultivars other than Barke (see Table 1).

Redundancy Analysis, Primer Designing and Genetic Mapping

Cluster analysis and primer designing for development of non-redundant set of markers was performed stackPACK2.1 and PRIMER3 programmes, respectively as described earlier (VARSHNEY *et al.* 2002; THIEL et al. 2003). PCR amplification of microsatellite loci, their separation, visualization and linkage mapping were performed as given in THIEL *et al.* (2003).

PCR-Based Screening of the Barley BAC Library

To identify gene-containing clones in an ordered BAC library of barley ("Morex"- cultivar) with more than 300,000 clones (YU et al. 2000) a four step, PCR-based screening protocol was established. The same primer pairs as were used for the mapping of SSR markers were applied for the screening of the BAC library using a touchdown PCR protocol (95°C, 3 min / 10 cycles: 95°C, 1 min; 65°C-0.5°C/cycle, 1 min; 72°C, 1 min / 25 cycles: 95°C, 1 min; 60°C, 1 min; 72°C, 1 min / 72°C, 3 min / 15°C) in a total volume of 20 µl (buffer: 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100, 2 mM $MgCl_2$), 0.2 mM each of dATP, dCTP, dGTP and dTTP, 100 pmol of each primer and approximately 1 U of Taq polymerase). The first round of screening was performed on 90 super pools comprising BAC-DNA 3456 clones from 9 consecutive 384-well microtiter plates (see Table 3). PCR products with a typical size of 100 - 500 bp were analysed on 3% agarose gels. For those super pools which yielded a fragment of the same length as

genomic DNA from *H. vulgare* cv. Morex all 9 individual plate pools of BAC DNA were examined during the second round of screening. The third round of screening was performed on 16 row and 24 column pools running through a positive plate. These row and column pools were derived from rows and columns running through a rectangular arrangement of 24 by 34 microtiter plates of the BAC library to minimize the number of DNA preparations.

Results and Discussion

Occurrence of SSRs in ESTs

A set of 111,090 barley ESTs, corresponding to 55.9 Mb, was employed for searching of microsatellites as a source for marker development. With the help of the *MISA* software tool, 9,564 microsatellites (EST-SSRs) were identified in a total of 8,766 ESTs (SSR-ESTs). Cluster-analysis revealed 2,823 non-redundant SSR-ESTs that contain 3,122 (2.8%) non-redundant SSRs. As expected, trimeric SSRs constituted the major portion as 52.6% and 63.4% of the total SSRs identified in non-redundant and redundant SSR-EST sets, respectively. Pentameric and hexameric microsatellites were present at less than 1% of total SSRs searched. These results are in accordance with earlier studies on database mining of SSRs in ESTs in cereal genomes (KANTETY *et al.* 2002; VARSHNEY *et al.* 2002; THIEL *et al.* 2003)

Genetic Mapping of EST-SSRs

A set of seven genotypes was used to screen for polymorphism with the primer pairs for EST-SSRs. For designing and analysing the primer pairs, SSR-ESTs were selected by using two approaches. In the first approach, a set 656 SSR-ESTs selected from the non-redundant SSR-ESTs from IPK-dataset. Of the 455 primer pairs (69.6%), which yielded an amplicon in the genotypes analysed, 156 primer pairs (34.3%) displayed polymorphism between the parents of at least one mapping population (Table 1).

Table 1. An overview on polymorphism and development of EST-SSR markers in barley

	First approach (database mining of IPK- ESTs)	**Second approach** (clustering of IPK SSR-ESTs with public SSR-ESTs*)	**Total**
Identified potential SSR-ESTs	3,122	197	
Primer pairs designed	656	98	754
Functional primer pairs	455 (69.4%)	70 (71.4%)	525 (69.6%)
Polymorphic primer pairs in Parents of at least one mapping population	156 (34.3%)	29 (41.5%)	185 (35.2%)

* In this approach, 8,766 SSR-ESTs from IPK and 18,041 SSR-ESTs from public domain were clustered and mixed clusters were analysed to select the IPK-ESTs that had variation in SSR length than that of public domain

To enhance the level of polymorphism in the genotypes of our interest, we adopted a second strategy based on additional barley ESTs from the public domain. While the IPK-ESTs were generally derived from the 'Barke' genotype, ESTs from other sources were developed from a series of different cultivars (KOTA *et al.* 2003). Hence, a comparison of Barke to non-Barke ESTs allows a preselection of polymorphic SSRs. After clustering the 18,041 SSR-ESTs containing 24, 623 SSRs (out of 207,449 barley ESTs, the then available in public domain) with 8,766 SSR-ESTs of the IPK set, we identified a total of 197 mixed clusters containing SSR-ESTs that had variation in SSR length between ESTs of IPK and public domain. Of this set, we selected 98 IPK SSR-ESTs, for amplifcation of the microsatellite locus in the set of 7 genotypes (Table 2). Amplicons were obtained with 70 (71.4%) primer pairs. Of these, 29 (41.5%) detected polymorphisms that could be mapped in the populations used in this study. However, this increment in level of polymorphism statistically (χ^2- test) was not significant. Therefore, the presence of a polymorphic SSR in the EST databases was not a good predictor for the presence of a polymorphism in any of the three mapping populations used in this study. This observation is in contrast to the results obtained from database mining for SNPs, where the presence of a SNP in the EST-database greatly enhanced the likelihood to also detect polymorphism in our mapping populations (KOTA *et al.* 2003).

Using both approaches a total of 754 primer pairs were analysed with the set of 7 genotypes. 525 (69.6%) primer pairs yielded amplicons of which 185 (35.2%) primer pairs detected polymorphism in parents of at least one mapping population. Of the 185 polymorphic markers 129 were mapped in the OWB_{Rec} x OWB_{Dom} (R/D), 47 in the Steptoe x Morex (S/M) and 23 in the Igri x Franka (I/F) population. Twelve SSRs were mapped in both Igri/Franka and OWB while two SSRs were mapped in Steptoe/ Morex and OWB. In addition to these common SSR markers a set of anchor markers was used to construct a consensus map of all three mapping populations. On this map, the EST-SSR markers were fairly even distributed with numbers ranging from 21 (7H) to 35 (3H) with an average of 27 per chromosome (Table 2).

Table 2. Summary of genetic mapping of barley EST-SSRs in different mapping populations

Chromosome / **Population**	**1H**	**2H**	**3H**	**4H**	**5H**	**6H**	**7H**	**Total**
I/F	5 (3)*	1	3 (2)	1 (1)	2 (1)	7 (4)	4 (1)	23 (12)
S/M	5	8	12 (1)	7 (1)	4	5	6	47 (2)
R/D	18 (3)	22	23 (3)	20 (2)	17 (1)	17 (4)	12 (1)	129 (14)
Total	25	31	35	26	22	25	21	185

*number in parentheses represent the number of common markers mapped in two mapping populations.

For all mapped markers, the polymorphism information content (PIC) was calculated on the basis of observed alleles in 6 (with 76 markers) to 7 genotypes (with remaining 109 markers). In the studied set of genotypes, the mapped markers detected 2 to 5 alleles with an average of 2.7 alleles per locus. The PIC value ranges from 0.24 to 0.78 with an average of 0.48 (Fig. 1), which is lower than that of genomic DNA-derived microsatellites (RAMSAY *et al.* 2000; LI *et al.* 2003) and is comparable to that of EST-derived SSRs (PILLEN *et al.* 2000; THIEL *et al.* 2003).

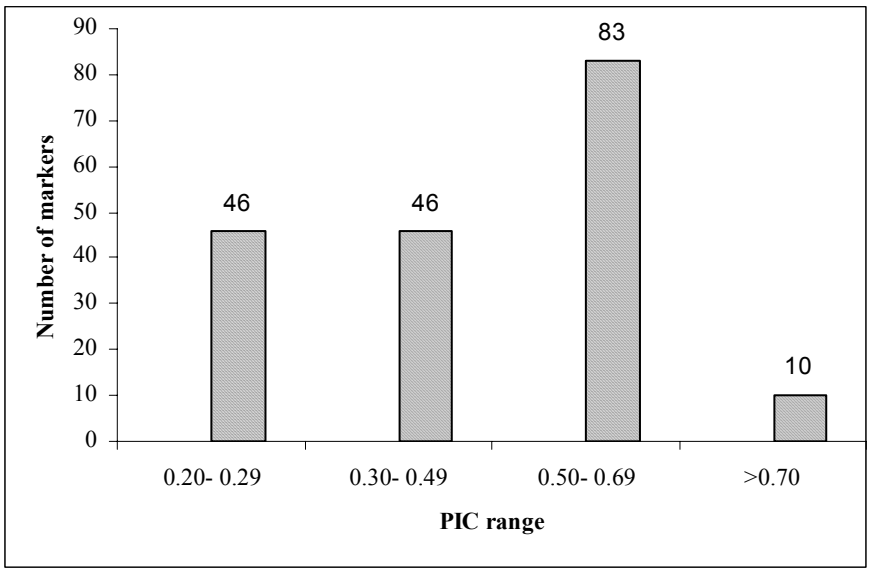

Figure 1. PIC of developed EST- SSR markers

Physical Anchoring of EST-SSRs

For 130 EST-based SSR markers an attempt was made to identify BAC clones in a genomic library of barley (YU *et al.* 2000). A four-step, PCR-based screening strategy was developed, employing DNA of BAC pools and a confirmation step at the level of individual clones (see Table 3).

Table 3. PCR-based strategy to screen the BAC library

Pool type	Numbers	Pool size	Assembly of	PCR reactions
Super	90	3,456	9 MTPs	90
Plate	810	384	1 MTP	6x9=54
Row	544	576	row through 24 MTPs	6x16=96
Column	576	544	column through 34 MTPs	6x24=144
Clone	311,040	1	Clone	6

This strategy requires as initial step the screening of 90 super-pools, each containing the DNA of 3,456 BAC clones. During the following steps, positive pools are deconvoluted to platepools and to BAC addresses using special row- and columnpools. These row- and columnpools run through rows of 24 plates and columns of 34 plates of the library, respectively. Finally, BAC addresses for genes are confirmed by PCR at the level of individual clones. In theory, this four step screening procedure should require on average 390 reactions plus controls and their analysis on agarose gels for the identification of all BAC clones in the library containing a single-copy

sequence. Using that strategy one or more BAC clones were obtained for 127 (98%) of the SSR markers analysed.

BAC library screening and genetic mapping revealed closely linked groups of EST-SSR markers (maximum distance of 5 cM on genetic map) of which more than one hit the same BAC clone. A closer inspection of the clustering data of these markers revealed two distinct groups.

The first group comprised 14 markers that were derived from 6 consensi of overlapping EST sequences (five contained 2 while one contained 4 different SSR motifs, respectively). Thus, the 14 markers represent 6 pairs, which were derived from one gene each, respectively. Evidently, these marker pairs are expected to co-segregate on the genetic map (within the limited resolution of the consensus map distances of up to 0.5 cM were observed) and to co-locate on a single BAC.

The second group of 28 markers comprised EST-SSR that were not part of the same cluster/contig/consensus sequence and the corresponding ESTs yielded different BLASTX hits. Therefore, these EST-SSRs very likely originated from different genes. PCR screening of the library with these 28 markers revealed 11 groups of BACs harbouring at least 2 genes. Resequencing of the BAC derived amplicons confirmed that independent and physically separated sequences were amplified by the respective primer pairs. In some cases contigs were formed from the selected BACs, with the largest contig comprising 5 physically linked markers (on 4 overlapping BAC clones) and another 3 contigs comprising 3 markers, each. The remaining 7 groups of BACs comprised 2 markers, respectively. The regions on genetic map, where the 'gene-rich' BAC clones were observed in the present study could be assigned to recombination hot-spots on the translocation-breakpoints based physical map of KÜNZEL *et al.* (2000). Therefore this dataset, although being small is in agreement with earlier studies, where gene-rich regions in Triticeae genomes were identified in regions high in recombination (for references see GILL 2004).

In summary, the present study contributes a set of 185 gene-derived microsatellite markers. These can be amplified using a standardised single PCR profile and are uniformly distributed on all seven barley linkage groups without any obvious centromeric clustering. BAC addresses identified for 127 SSR-ESTs will provide useful anchoring points to correlate the genetic map with a "future" physical map of the barley genome. Furthermore, at least eleven regions identified on six chromosomes offer the possibility for preparation of contigs leading to preparation of sub-genome physical maps gene-dense regions of the barley genome.

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Multi-QTL Mapping of Caryopsis Dormancy and Seedling Desiccation Tolerance of Barley

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Abstract

The genomic regions controlling caryopsis dormancy and seedling desiccation tolerance were identified with 152 F_4 lines derived from a cross between Mona, a Swedish cultivar, and an Israeli xeric wild barley *Hordeum spontaneum* genotype collected at Wadi Qilt. Dormancy, the inability of a viable seed to germinate, and desiccation tolerance, the ability of the desiccated seedlings to revive after rehydration were characterized by fitting the germination and revival data with growth curves, using three parameters (instead of initial seven scores obtained at 3-day intervals): minimum, maximum, and slope of germination or revival rate derived by least square method. The genetic map (2058 cM in length) was constructed with 85 genetic markers (SSRs, AFLPs, STSs and Dhn) using MultiPoint-mapping algorithm. QTL mapping was conducted with MultiQTL package (http://esti.haifa.ac.il/~poptheor/MultiQtl/MultiQtl.htm). Fourteen genomic regions were detected affecting the target traits, and six out of these regions affected both dormancy and desiccation tolerance traits. No QTL was identified for germination speed, while five QTLs were for revival speed. More QTLs were for minimum germination than maximum germination, whereas more QTLs were for maximum revival rate than minimum revival rate. Most of the QTLs with strong effects were located on chromosome 4H. Both xeric wild barley and cultivar Mona contributed favorite alleles for caryopsis dormancy and seedling desiccation tolerance. The results revealed that the QTL effects on minimum germination rate underlay the genetic control of caryopsis dormancy, and that the seedling desiccation tolerance was based on the QTL effects on the maximum revival rate.

Keywords: barley; caryopsis dormancy; desiccation tolerance; germination; QTL mapping; revival rate

Introduction

The plant's life circle begins with seed germination, followed by seedling emergence (TAKAHASHI *et al.*, 2001). This is the transition period of the plant life from the most tolerant stage, seeds, to the most sensitive stage, seedlings (EVENARI & GUTTERMAN, 1976). The characteristic of primary dormancy of barley in nature is an important survival strategy (EVENARI & GUTTERMAN, 1976). The dormancy allows plants to escape drought and high temperatures in the warmer summer months (SNAPE *et al.*, 2001). Understanding the genetics of dormancy of caryopses and drought tolerance of young seedlings of barley in one context, is important for establishing an efficient breeding system of drought-tolerant barley. It has been

proved that the expression of seed dormancy of barley has strong genetic and environmental components (OBERTHUR *et al.*, 1995). During past decades, many efforts have been made to identify genes controlling responses of barley at the dormancy or seedling stages to abiotic stresses (BURASS & SKINNES, 1984; HAYES *et al.*, 1996; HACKETT *et al.*, 2001; KATO *et al.*, 2001). The objective of the current study was to identify chromosome locations of genes involved in caryopsis dormancy and seedlings drought tolerance of barley by employing the powerful multiple trait QTL analysis (KOROL *et al.*, 2001) combined with Multiple Interval Mapping (MIM) (KAO *et al.*, 1999).

Material and Methods

Plant Materials: An F_4 mapping population (152 lines) was constructed by a cross of a wild barley (*Hordeum spontaneum* C. Koch) xeric genotype 23-39, originated from Wadi Qilt (140 mm annual rainfall), Israel, by a cultivar *H. vulgare* cv. Mona (Swedish). The caryopses (F_4) used in the present study were produced by F_3 lines which were grown under two water regimes, control (400 mm) and drought (200mm).

Test of Dormancy: 6 X 100 caryopses of each F_4 line were wetted with 4 ml distilled water in 90-mm Petri dishes on a Whatman No. 1 filter paper at 20°C (the first and the last week) and 5ºC (the second week) in darkness for three weeks. Germination was checked 3, 5, 7, 10, 14, 17, and 21 days after wetting. The seedlings (germinated caryopses) were removed and dried on filter paper in open Petri dishes under laboratory conditions for 4 weeks.

Test of Revival Ability of Seedlings: After 4 weeks of drying, the seedlings derived from the test of dormancy were rewetted and moved to a dark chamber at 15°C for 5 days. On the fifth day, the revival rate was evaluated by checking re-growth and the appearance of adventitious roots (ASHBY & MAY, 1941).

Traits for Mapping: The dynamic process of the germination rate and revival rate at the designated times was fitted by growth curve, characterized by a genotype-specific minimum value (X^0_g), maximum value (X^m_g), and slope parameter (S_g),

$$X_g(t) = X^m_g/\{1+(X^m_g/X^0_g-1)\exp[-S_g(t-t_0)]\},$$

where t_0 and t are the initial and current days of measurements. Twelve traits, GCmin, GCmax, GCs, GDmin, GDmax, GDs, RCmin, RCmax, RCs, RDmin, RDmax, and RDs, representing minimum (min), maximum (max) and slope parameter (S) of germination rate (G) and revival rate (R) of caryopsis derived from control (C) and drought stressed (D) F3 lines, were mapped.

***Genetic Linkage Map*:** DNA markers include 48 SSRs, 4 STSs, 2 Dhns and 31 AFLPs were used to construct genetic linkage map being 2058 cM in length, based on Evolutionary Strategy algorithm implemented in MultiPoint mapping software (MESTER *et al.*, 2003).

***QTL Mapping*:** The software used in the analysis was MultiQTL package. Each chromosome was dissected into two or three segments due to big gaps (intervals longer than about 30 cM). These segments were used separately as linkage groups for QTL mapping which employed four approaches, (1) single-trait analysis, (2) MIM-single-trait analysis, (3) multiple-trait analysis, and (4) MIM-multiple-trait analysis.

Results and Discussion

Trait Distributions and Correlations. A large variation was observed in the progeny for all traits with coefficient of variation (CV) ranging from 34.1 to 99.6% (Table 1). The distributions of all the traits showed strong transgressive segregations, suggesting that the Mona genotype also contains positive alleles for the traits. The traits correlated to one another and all the significant correlations were positive. The significant positive correlations presented in germination rate vs revival rate, control vs drought stressed, and maximum vs minimum. The four slope parameters (GCs, GDs, RCs and RDs) significantly correlated to each other, but not to other traits.

Detected QTLs. Out of 31 QTL effects, 12 were detected by single-trait analysis, 20 by MIM-single-trait analysis, 6 by multiple-trait analysis, 4 by MIM-multiple-trait analysis (Fig.1). These QTLs were located at 14 genomic regions out of which 6 regions affected both dormancy and desiccation tolerance traits, whereas 3 and 5 regions affected germination rate and revival rate, respectively. No region was identified for germination slope parameter, while 5 regions were for revival slope parameter. More QTLs were for minimum germination than maximum germination, whereas more QTLs were for maximum revival rate than minimum revival rate. Most of the QTLs with strong effects were located on chromosome 4H. Grain dormancy of barley is genetically controlled by QTLs and these loci were located earlier in four regions of barley genome on chromosome 4, 5, and 7H, which were associated with most of the differential genotypic expression for dormancy in Steptoe/Morex mapping populations (OBERTHUR *et al.*, 1995; HAN *et al.*, 1996). Both xeric wild barley and cultivar Mona contributed favorite alleles for caryopsis dormancy and seedling desiccation tolerance. The results revealed that the QTL effects on minimum germination rate underlay the genetic control of caryopsis dormancy, and that the seedling desiccation tolerance was based on the QTL effects on the maximum revival rate.

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Table 1. Trait values of two parents and 152 F_4 lines. GCmin, GCmax, GCs, GDmin, GDmax, GDs, RCmin, RCmax, RCr, RDmin, RDmax, and RDr, representing minimum (min), maximum (max) and slope (s) of germination rate (G) and revival rate (R) of caryopsis derived from control (C) and drought stressed (D) F3 lines.

Trait	**F_4 lines**				**23-39**	**Mona**
	Mean	**Minimum**	**Maximum**	**Std.Dev.**	**Mean**	**Mean**
GCmax	62.5	10.4	98.1	21.3	36.2±15.6	96.1±4.4
GCmin	28.8	0.1	94.3	23.8	1.4±2.1	89.3±16.7
GCs	0.5	0.1	3.5	0.5	0.4±0.2	0.6±0.4
GDmax	59.9	5.0	100.0	23.2	30.8±15.9	98.9±2.8
GDmin	29.5	0.1	95.7	24.9	1.0±1.8	96.6±6.4
GDs	0.6	0.1	2.3	0.4	0.5±0.2	0.6±0.5
RCmax	33.1	2.7	78.3	17.8	15.7±8.9	66.8±9.5
RCmin	15.9	0.0	62.7	15.0	0.8±1.4	64.8±11.4
RCs	0.7	0.1	4.4	0.6	0.5±0.2	0.1±0.1
Dmax	32.0	0.7	82.3	18.6	14.9±9.9	57.8±22.2
RDmin	16.5	0.0	76.0	16.4	0.6±1.0	57.6±22.1
RDs	0.7	0.1	3.4	0.6	0.5±0.2	0.2±0.3

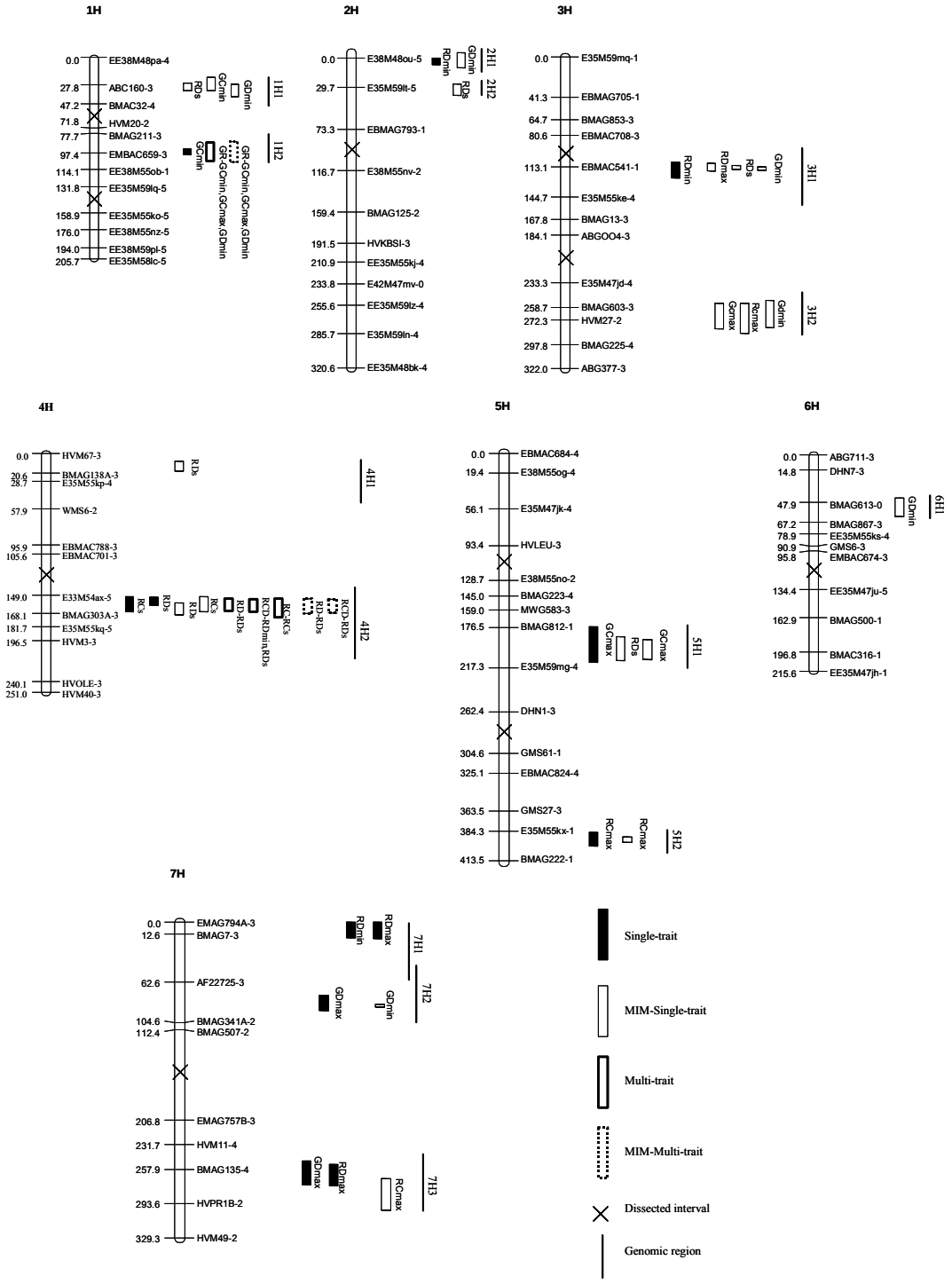

Fig. 1. The genomic regions controlling caryopsis dormancy and seedling desiccation tolerance. QTLs were identified with 152 F_4 lines derived from a cross between Mona, a Swedish cultivar, and 23-19, an Israeli xeric wild barley *Hordeum spontaneum* genotype collected at Wadi Qilt. Each chromosome was dissected into 2-3 linkage groups due to >30 cM gaps. These linkage groups were used separately for QTL mapping which employed four approaches, (1) single-trait analysis, (2) MIM-single-trait analysis, (3) multiple-trait analysis, (4) MIM-multiple-trait analysis.

S 3 – BREEDING METHODOLOGIES II – GENOMICS

Ac/*Ds* Transposon-Mediated Gene Tagging in Barley

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Abstract
A transposon tagging system, based upon maize *Ac/Ds* elements, was developed in barley (*Hordeum vulgare subsp. vulgare*). The long-term objective of this project is to identify a set of lines with *Ds* insertions dispersed throughout the genome as a comprehensive tool for gene discovery and reverse genetics. *Ac*TPase and *Ds-bar* elements were introduced into immature embryos of Golden Promise by biolistic transformation. Subsequent transposition and segregation of *Ds* away from *Ac*TPase and the original site of integration resulted in new lines, each containing a stabilized *Ds* element in a new location. The sequence of the genomic DNA flanking the *Ds* elements was obtained by inverse PCR and TAIL-PCR. Using a sequence-based mapping strategy, we determined the genome locations of the *Ds* insertions in 17 independent lines using primarily restriction digest-based assays of PCR-amplified single nucleotide polymorphisms and PCR-based assays of insertions or deletions. The principal strategy was to identify and map sequence polymorphisms in the region corresponding to the flanking DNA using the Oregon Wolfe Barley mapping population. The mapping results obtained by the sequence-based approach were confirmed by RFLP analyses in four of the lines. In addition, cloned DNA sequences corresponding to the flanking DNA were used to assign map locations to Morex genomic BAC library inserts, thus integrating barley genetic and physical maps. BLAST search results indicate that the majority of the transposed *Ds* elements are found within predicted or known coding sequences. Transposon tagging in barley using *Ac/Ds* promises to provide a useful tool for Triticeae functional genomics.
Keywords: transposon tagging; barley; reverse genetics; *Ac/Ds*; mapping

Introduction
Maize *Activator* (*Ac)* and *Dissociation (Ds)* elements have been utilized as mutagens in heterologous systems since the cloning of the transposons themselves (FEDOROFF *et al.* 1983) and the demonstration that these elements retain the ability to transpose in heterologous transgenic plants (BAKER *et al.* 1986). Based upon the ability of *Ds* to transpose to linked sites (PARINOV *et al.* 1999; ITO *et al.* 2002), targeted mutagenesis

of loci genetically linked to one of the mapped *Ds* elements can be accomplished by reactivating the *Ds* element in crosses with transposase expressing lines.

The set of *Ds* insertions described are in the variety Golden Promise, in which the successful transformation of barley was first shown (WAN & LEMAUX 1994). In order to map the *Ds* insertions we are utilizing the Oregon Wolfe Barleys (OWBs). By integrating the virtual bin map Restriction Fragment Length Polymorphism (RFLP) markers of KLEINHOFS and GRANER (2001) into the OWB map, the OWBs will connect the mapped *Ds* elements to the array of available genomic resources in barley. For more information on the OWBs and the bin map, please see: "The Oregon Wolfe Barley Population: An International Resource for Barley Genetics Research and Instruction" by Corey et al, in these Proceedings.

We utilized a sequence-based strategy for assigning linkage map coordinates on the OWB map to each *Ds* insertion in Golden Promise. The principal strategy involved sequencing the genomic DNA flanking the *Ds* insertions in each Golden Promise TNP line, followed by cloning and sequencing the corresponding alleles from OWB-D and OWB-R (or in other barley varieties, as necessary). By locating the *Ds* insertions on the OWB map, and in reference to the bin map, researchers interested in a specific region of the genome could choose the appropriate Golden Promise *Ds* insertion line for reactivation experiments

Our long-term goal is to develop and make available to the research community a set of *Ds* insertion lines in barley, each containing a single, mapped, *Ds* element assigned to a linkage map bin. Our principal objective in this phase of the project was to determine the location of the *Ds* insertions in a number of Golden Promise TNP lines, utilizing the OWB population, and the preliminary characterization of those insertion sites, using BLAST searches and by addressing the mapped flanking sequences to BAC clones.

Material and Methods

Stable, single copy *Ds* insertion lines in Golden Promise were previously developed as described by KOPREK *et al.* (2000; 2001). Genomic DNA flanking the *Ds* insertions was isolated by Inverse PCR (iPCR) (OCHMAN *et al.* 1988; 1993; LI *et al.* 2001) or by TAIL PCR (LIU *et al.* 1995; KOPREK *et al.* 2000). Primers for PCR, based on the flanking sequences in the TNP lines, were designed to identify Single Nucleotide Polymorphisms (SNPs) or insertion/deletion (indels) polymorphisms in the sequences of the corresponding alleles from OWB-D and OWB-R. Where a SNP was identified, a Cleaved Amplified Polymorphic Sequence (CAPS) assay was designed to differentiate between the parental alleles. The corresponding sequence was amplified from each member of the OWB DH mapping population and digested with the appropriate restriction enzyme. Indels were assayed after amplifying the corresponding sequences from each member of the mapping population. Depending upon the size of the amplified DNA fragments, the polymorphisms were assayed and scored on 2 or 3% agarose gels stained with ethidium bromide.

Four of the Ds insertions were also mapped by RFLP analysis, following procedures as in KLEINHOFS *et al.* (1993). The mapped mapped *DsT* loci were addressed to barley BAC clones by hybridizing cloned flanking DNA to a subset of the 17 filters comprising the Morex genomic BAC library (YU *et al.* 2000). BLASTN and TBLASTX

searches were performed using genomic DNA sequences from wild type Golden Promise corresponding to the DNA flanking each *Ds* insertion event.

Results and Discussion

We assigned linkage map positions to *Ds* insertions in 17 Golden Promise TNP lines by mapping polymorphisms in the genomic DNA flanking each *Ds* insertion. Fifteen *DsT* loci polymorphisms were scored in the OWB DH population and directly placed on the OWB linkage map (Table. 1). The remaining two *DsT* loci have inferred positions based on bin assignments. In these cases, we did not identify any polymorphisms in the OWB-D and OWB-R genomic DNA corresponding to the flanking sequence, so we utilized other barley mapping populations. *DsT-34* was mapped in the Steptoe x Morex mini population while *DsT-11* was mapped in the Dicktoo x Morex population. The inferred linkage map positions of these loci were assigned based on the presence of markers common to the OWB population in the bin map.

Mapping the *Ds* insertions in the OWB population, together with consensus RFLP markers, places each *Ds* element into a specific bin. This will facilitate integration of functional and structural genomics resources in barley. By assigning the *DsT* loci to bins on the OWB map, researchers can identify the closest *Ds* insertions to mapped morphological loci, genes of known function or QTL regions identified in other cultivars. These *Ds* insertions will be targets for reactivation in order to tag linked genes. The sequences generated from for mapping the *Ds* insertions have been deposited in GenBank. (Accession #AY505351-AY505353, AY505357-AY505363, AY505366-AY505371, AY505375-AY505409, AY505413-AY505415, AY520812 and AY594683-AY5946850). To date, we have *DsT* loci in 15 of the 99 bins, on six of the seven chromosomes, (Table 1), while the long term goal is to have at least one *DsT* insertion in each bin.

Twelve *DsT* loci were mapped (Table 1) using a CAPS marker strategy (KONIECZNY & AUSUBEL, 1993) to assay SNPs (*DsT 13 , 22, 24, 27, 28, 29, 30, 31, 32B, 34, 35, 41*) using the appropriate restriction enzymes. SNPs are the most common type of sequence difference between alleles (RAFALSKI 2002) and have been used in maize (BATLEY *et al.* 2003) and barley (KOTA *et al.* 2003) for mapping ESTs. Five of the *DsT* loci were mapped (Table 1) by assaying insertions or deletions in the sequences flanking the *Ds* elements using agarose gel electrophoresis.

DsT-11, *30, 32B* and *41* were mapped by RFLP analysis to confirm the results of the sequence-based mapping approach. All four of the RFLP probes detected single or low-copy number sequences in the DNA hybridization analyses. In addition to providing map locations, the results from the RFLP analysis indicated that all four of the *DsT* loci studied were in non-repetitive, low-copy number DNA sequences, which was verified by the results of the BLAST searches of the sequence flanking the insertion site and by hybridization of the flanking sequence to BAC clones (Table 1). These results support the hypothesis that in barley, as observed in other heterologous plant systems such as *Arabidopsis* (PARINOV *et al.* 1999; TISSIER *et al.* 1999; ITO *et al.* 2002) and rice (ENOKI *et al.* 1999; GRECO *et al.* 2001; KOLESNIK *et al.* 2004) the *Ds* element transposes preferentially into predicted coding regions. The development of this transposon tagging resource in barley promises to provide a useful tool for Triticeae functional genomics.

Table 1. Summary of *Ds* insertion site mapping in barley based on chromosome and bin location, number of positive BAC clones identified, cereal EST matches and E values

DsT-	Chrom.-bin[a]	Positive BAC Clones	Cereal EST matches[b]	E Value[c]
1	2H-1	16	Maize EST CB885436	2.3e-7
6	5H-1	5	Wheat EST BE443030	6.0e-16
11	5H-10	32	Barley EST AL505601, Putative wall-associated protein kinase	2.9e-141
13	5H-14	4	Barley EST BF266272.2	6.5e-5
18	6H-12	2	Wheat EST Contig TC94268	4.8e-28
22	6H-13	6	Barley Array Contig #10453, similar to -putative aristolochene synthase (*Oryza sativa*)	1e-111
24	3H-14	4	Rice EST Contig TC128765	6.1e-89
27	3H-4	1	Unknown	3.3e-2*
28	6H-14	1	Unknown	2e-3*
29	4H-3	1	Barley Array Contig #2257, similar to putative EREBP-type transcription factor (*Oryza sativa*)	4e-8
30	7H-9	6	Unknown	0.13*
31	7H-3	3	Unknown	0.35*
32B	6H-12	3	Wheat EST AL818129	2.5e-10
33	5H-14	11	Unknown	2.0e-3*
34	5H-2	1	Unknown	9.2e-2*
35	3H-16	2	Barley Array Contig #39284	5e-4
41	2H-10	2	Rice TC196496, probable ubiquitin-conjugating enzyme	2.1e-42

* Below significance threshold

a. See text for details of the mapping strategies.
b. BLASTN and TBLASTX searches were performed using genomic DNA sequences from wild type Golden Promise corresponding to the DNA flanking each *Ds* insertion event.
c. For the purposes of this table, the best match from all the searches is shown, although any match with an E value greater than $1e\text{-}^{4}$ is not considered to be significant, so the putative identities of those are not shown.

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A Simple and Effective Procedure for Molecular Marker-Assisted Screening

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Abstract

Molecular markers are tools for the indirect selection of traits in breeding populations. To be relevant, markers must demonstrate benefits in accuracy or cost over conventional screening methods. We present a simple, accurate, and cost effective MMAS approach that can be adapted to breeding programs of various sizes and capabilities. DNA is isolated from seedlings germinated in an 8x12, 96-well format, that is conserved throughout the process. Template preparations are carried out in 96-well low-profile microplates sealed with a pierceable foil to allow for multichannel pipetting. The preparations involve the addition of sodium hydroxide, and Tris buffer solutions with two brief incubations. The released DNA is of sufficient quality for repeatable analysis with SCAR, RAPD, or microsatellite markers, and can be stored for several weeks for re-analysis or analysis with other markers. PCR reactions are carried out in 96-well, heated lid thermal cyclers, and PCR products are visualized on agarose gels with 96 wells spaced suitably for multi-channel pipetting. Cost per sample is estimated at less than $2.00 (CDN) including all costs for materials (including thermostable polymerase), and labour. These costs are further reduced when screening the same DNA with additional markers either in separate or multiplexed PCR reactions. The procedure allows for the screening of 1000 breeding lines per technician week.

Introduction

Molecular marker-assisted selection (MMAS) is the use of DNA tags or fingerprints associated (linked) with particular traits of interest to select for these traits based on genotype rather than phenotype. MMAS has been promoted as a promising new tool to assist plant breeders in the selection of favourable lines from thousands of progeny created every breeding cycle. For this tool to be relevant to breeding programs, it must provide a benefit in terms of accuracy, ease of screening, time, cost, or a combination thereof.

For certain traits, an increase in accuracy alone may justify the use of MMAS. Instances such as this include screening for disease resistance in which multiple genes may confer resistance thereby masking the effect of the particular gene of interest. Back-cross breeding, where a trait is continually being selected in heterogeneous material, is another example where accuracy gained from the use of markers justifies their use.

Traits that cannot be selected for locally due to lack of disease pressure, favourable environmental conditions, equipment, or expertise, may also be candidates. In such instances, costs for MMAS may well be less than those involved in preparing samples and arranging for phenotyping services by another party.

Traits that require repeated phenotyping, especially over several seasons or multiple generations, also benefit from MMAS. Nevertheless, for most traits, plant selections in breeding programs are carried out in the field where numerous traits are evaluated at the same time. This “whole plant” approach to selection reduces the cost per data point to minimal levels, even with inefficiencies such as environmental influences factored in. In most cases then, if MMAS is to confer an economic benefit, it must be simple, reliable, and inexpensive.

MMAS related costs include material costs (equipment and consumables) and labour. The fixed costs for specialized equipment are often difficult to justify, especially in smaller

breeding programs where the use of MMAS may be beneficial, but the volume of plant material to be evaluated does not warrant robotic systems and automation. Costs for consumables are reasonably stable and may in fact be decreasing as supplies involved in the procedures become more common. Labour is the major component in cost calculations for MMAS. Therefore, technically challenging, inconsistent, and time consuming protocols, dramatically increase the costs associated with MMAS.

Our MMAS procedure has been developed to address the issues of simplicity (minimal need for specialized equipment) and reliability (minimal handling to decrease mistakes, robust protocols). In addition, the procedure is based on the premise that successful MMAS does not require large amounts of clean DNA nor does the DNA need to be stable for long term storage. The procedure includes a template preparation method modified from KLIMYUK *et al.* (1993), and is used routinely for three markers in barley (Un8, Rpg1) and oat (Pc68), and on occasion, others traits (leaf scald, covered smut, barley yellow dwarf, barley *beta*-amylase, oat smut). All markers are based on PCR (SCAR or ASA) and agarose gel electrophoresis.

Material and Methods

All procedure steps are performed in an 8 row x 12 column format to suit commercially available 96-well microplates, and 96-well thermal cycler blocks. The conservation of this format eliminates the need for labelling of individuals at every step of the process. An identification template is created at the beginning of the process which specifies the individual line at any given coordinate within the 8 x 12 format.

Preparation of Plant Tissue

DNA template is obtained from seeds germinated in "plug trays" (example; Landmark Plastics, Akron, Ohio, USA) trimmed to an 8 x 12, 96-well format (Figure 1). Each plug contains a large absorbent (cotton) ball moistened with water, several seeds per line in each cell, and a clear cover to retain moisture. Seed of the parents of the cross (controls) are placed in the first two cells, with an additional positive control at random within the rest of the tray. For barley and oat, 4 to 6 days are required for the seed to germinate.

Preparation of Alkali Template

A small coleoptile piece (2 mm) is harvested from as close to the seed as possible by pinching with tweezers. The tissue is transferred, and lightly macerated with the tweezers into the bottom of a good quality, thin-wall, V-bottom, low profile microplate (ABgene LP962108-10/N, Surrey, UK). The microplate is placed on ice throughout the harvesting process. V-bottom wells ensure good contact between tissue and chemical solutions in the subsequent steps, and the low profile simplifies the placement of the tissue at the bottom of the well. The tweezers are placed in 95% ethanol between samples. The amount of plant tissue is not critical, but it should be noted that more plant tissue is not necessarily better, and may in fact inhibit the preparation.

Forty microlitres of 0.25 M sodium hydroxide is added to each sample well. The uncovered microplate is placed in a temperature cycler block (or other well fitted heating block), pre-set for 94°C, for exactly 35 seconds. The plate is removed and kept at room temperature while sixty microlitres of a solution of 0.5 M Tris-HCl pH 8.0, 0.25% Nonidet P-40 is added to each sample well. The microplate is sealed using a pierceable foil (3M Aluminum Microplate Sealing Tape). The microplate is returned to the heating block, pre-set to 94°C, for 120 seconds after which the plate is removed and placed on ice. The microplates containing the prepared templates may be stored at 4°C.

PCR Preparation

Reactions are prepared as per standardized conditions, in 96 well microplates, strip tubes, or individual reaction tubes as suited for the available temperature cycler. Two to three microlitres of the template preparation are added to the polymerase chain reactions (25 µL total volume) in place of purified DNA template. The template is transferred from the microplate to the PCR tubes using a multichannel (8 or 12) pipet and pipet tips that pierce the aluminum foil.

Agarose Gel Analysis

PCR samples are analysed on agarose gels using equipment that allows for all 96 samples to be electrophoresed and viewed simultaneously (Sunrise™ 96, Whatman Biometra®). Well spacings should be suitable for multichannel pipettors.

Results and Discussion

Figures 1 to 3 illustrate steps of the MMAS procedure. Results have indicated few failed reactions (Figure 3) and demonstrate the reliability of the method. Although the data has not been subjected to rigourous analysis, we average less than one failed reaction per plate of 96, which is acceptable for our purposes especially when taken in the context of the inherent error rates associated with linked markers.

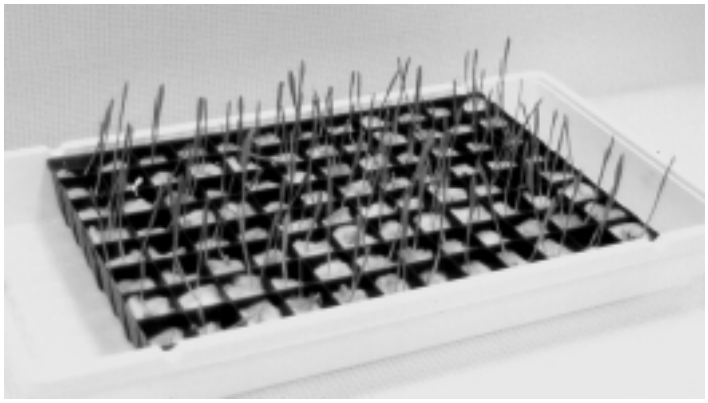


Figure 1. Germination of seed in 96-well plug trays

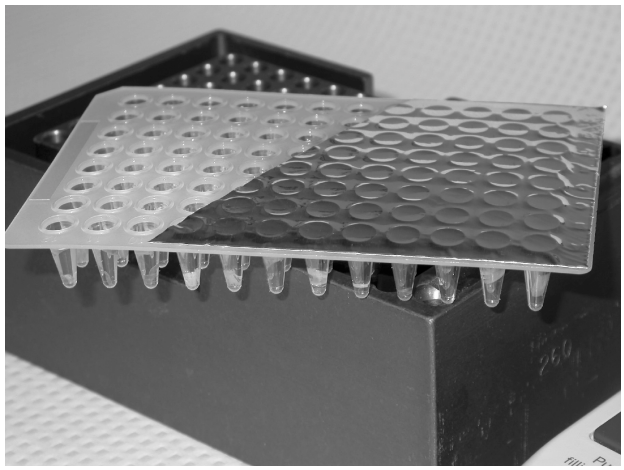
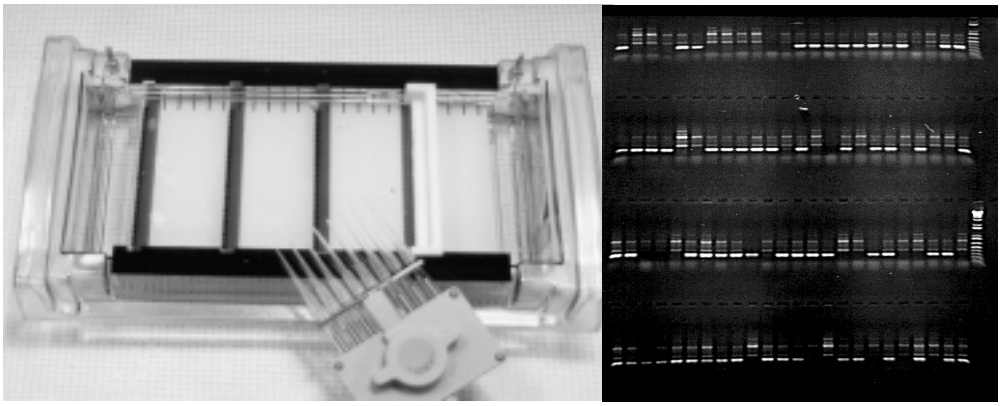


Figure 2. 96-well microplate for template preparations with partial aluminum pierceable foil cover

Figure 3. Agarose gel electrophoresis apparatus that allow 96 samples to be analysed in 4 rows, wells spacings are compatible with multichannel pipets



As described, 1000 samples can be comfortably analysed by a single person per 40 hour week. At an annual salary of $40,000 (CDN), labour costs are therefore less than $1.00 per reaction. The estimated cost, including labour and consumables (calculated on the basis of 100 reactions, not including DNA polymerase), is approximately $1.35 per sample. Thermostable polymerase may presently be obtained at $0.25 per Unit, to total $1.60 (US $1.25) per sample.

It should be noted that efficiencies in the procedure are gained by several adaptations. Addition of solutions, and liquids transfer, is accelerated through the use of repeater pipets or multichannel pipets as available. Use of a pierceable foil cover allows for pipetting of solutions without removal of the seal and the risk of cross-contamination.

In general, PCR and cycling conditions do not need to be modified from those optimized for the particular primers in use. The template amounts per reaction are a guideline only and do not appear to be critical. For some primers, the optimum annealing temperature may need to be adjusted. The template is also compatible with microsatellite (SSR) markers.

Experiences with field collected samples were awkward, slow, and prone to collection error, so we have elected to prepare template from lab germinated seed. Tissue can be collected and combined from multiple coleoptiles (if heterogeneity is a concern).

Some of the most successful examples of the implementation of molecular markers have been for highly valuable traits under simple genetic control, but which are difficult to phenotype (YOUNG 1999). In barley, true loose smut resistance is conferred by the single gene (*Un*8), but disease assessments need to be done over at least two cycles, and need to be repeated for accuracy. Stem rust resistance is also conferred by a single gene (*Rpg*1), but we lack the pathogen locally, and the marker is based within the gene itself. In oat, *Pc*68 provides durable resistance to crown rust, but we lack local disease pressure and the resistance may be masked by other genes. Markers for these three traits form the backbone of the MMAS program, allowing the use of other markers on the same template at lower incremental cost.

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Allele-Specific Markers and Molecular Diversity at the *Bmy1* Locus Determining Enzyme Thermostability

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Abstract

The *Bmy*1 locus on chromosome 4H determines enzyme activity, thermostability, isoenzyme form, and free/bound enzyme ratio. The enzyme can be classified into types Sd1 and Sd2, with a sub-group (Sd2-H) being more thermostable. Based on DNA sequence comparisons of the *beta*-amylase gene from the Canadian Sd1 cultivar Harrington and sequences from several other Sd1 and Sd2 cultivars, allele-specific PCR based markers were constructed for each of six amino acid substitutions. PCR markers that identify variation in a microsatellite region, a 126 bp palindromic insertion/deletion, and a 21 bp insertion/deletion, all located in intron III of the gene, were also developed. Analysis of 110 cultivars from Australia, Canada, China, Europe, Mexico (CIMMYT), and the USA, revealed that the Sd1 and Sd2 isoenzyme types are determined by 4 of 6 amino acid substitution markers which co-segregate with the 21 bp insertion/deletion. Only one amino acid substitution marker and the microsatellite marker show variability within Sd1 types. Divergence within Sd2 types was greater with four of nine markers showing variability. All known Sd2-H cultivars could be clustered according to the amino acid present at position M527 and the absence of the 126 bp in intron III, suggesting that isoenzyme type and thermostability are influenced by regions within the intron. In addition, we found that several Canadian breeding lines have the same *Bmy*1 DNA sequence as thermostable cultivars.

Keywords: barley; *beta*-amylase; diversity; isoenzyme; molecular marker; thermostability

Introduction

Beta-amylase is the most important of four enzymes that combine to hydrolyse starch into fermentable sugars necessary for the brewing process. In contrast to the other enzymes (*alpha*-amylase, limit dextrinase, *alpha*-glucosidase) which are synthesized *de novo* during grain germination, *beta*-amylase accumulates during grain development (HARDIE 1975). *Beta*-amylase in barley grain is highly heterogeneous with at least eight different isoenzyme bands being detected by chromatofocussing (LABERGE & MARCHYLO 1983) though most of these are thought to be the result of post-translational modification. Two distinct isoenzyme forms are evident in cultivated barley (NIELSEN & JOHANSEN 1986; FORSTER *et al*. 1991; EVANS *et al*. 1997) and malt (ALLISON 1973; ALLISON & SWANSTON 1974) referred to as Sd1 (starch degrading enzyme) and Sd2. A third form (Sd3) has been identified in a *Hordeum spontaneum* accession (EGLINTON *et al*. 1998). The *Bmy*1 gene is located on the long arm of chromosome 4H and encodes the *beta*-amylase stored in barley grain (KREIS *et al*. 1987; LI *et al*. 2002). *Bmy*1 determines *beta*-amylase activity (ERKKILA *et al*. 1998), isoenzyme form (LI *et al*. 2002), and enzyme thermostability (EGLINTON *et al*. 1998). Thermostability is important since *beta*-amylase activity decreases rapidly as temperature increases above 55°C, but temperatures >60°C are required for rapid and complete enzymatic starch degradation. The isoenzyme forms Sd2-L (low), Sd1, Sd2-H (high), and Sd3 show enzyme thermostabilities that differ by approximately 5°C in $T_{50,}$ from lowest to highest respectively (EGLINTON *et al*. 1998). Using PCR-based markers designed to take advantage

of nucleotide sequence variation in five cloned Sd1 and Sd2 barley cDNAs, we investigated genetic diversity at the *Bmy*1 locus of 110 barley cultivars/lines to determine the molecular basis for isoenzyme form and thermostability.

Material and Methods

Plant Materials

One hundred and ten barley lines originating from Australia, Canada, China, Europe, Mexico (CIMMYT), Japan, and the USA were evaluated. DNA from each line was extracted from young leaves using a CTAB based method (LI *et al*. 2002), modified for small scale extractions.

Alignment of the Bmy1 cDNA Sequences

DNA sequences of *Bmy*1 from cultivars Hiproly (accession # X52321) (KREIS *et al*. 1987), Haruna Nijo (accession #s D21349, D49999) (YOSHIGI *et al*. 1994, 1995), Adorra (accession # AF061203) (ERKKILA *et al*. 1998), and Harrington and Galleon (LI *et al*. 2002) were aligned using DNAMAN software (Lynnon Biosoft, Vaudreuil, Canada).

PCR Primers for Bmy1 Markers

PCR primer pairs were designed based on: six nucleotide (amino acid) substitutions within the coding regions of *Bmy*1 (Table 1); a 126 bp palindromic insertion/deletion (in/del) in intron III (ERKILLA *et al*. 1998); a 21 bp in/del in intron III; a microsatellite region in intron III (ERKILLA *et al*. 1998; YOSHIGI *et al*. 1995). Primer sequences, PCR product sizes, and primer annealing temperatures for all nine markers are listed in Table 2.

Table 1. Nucleotide changes that result in amino acid substitutions at six loci in *Bmy*1. Substitutions are shown Haruna Nijo↔alternate cultivar. The numeral indicates the position in the coding region of *Bmy*1.

Nucleotides	C343 ⇔ T	C495 ⇔ G	C698 ⇔ T	C1040 ⇔ T	T1289 ⇔ C	G1581 ⇔ A
Amino acids	R115 ⇔ C	D165 ⇔ E	A233 ⇔ V	S347 ⇔ L	V430 ⇔ A	M527 ⇔ I
Location	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6	Exon 7

*PCR Reaction Conditions for Bmy*1 *Markers*

PCR reactions (25 µL) were as follows: 20 mM Tris-HCl, 50 mM KCl, 2.0 mM $MgCl_2$, 400 µM dNTP, 5 pmol primer, 100 ng template DNA, and 1 Unit *Taq* DNA polymerase. Template DNA was initially denatured for 4 minutes, followed by 35 cycles of 94°C for 45 seconds, anneal for 45 seconds, and 72°C for 1 minute. The PCR products were separated on a 1.0-1.5% agarose gel, or a 6% denaturing polyacrylamide gel (microsatellite marker).

Cluster Analysis

NTSYSpc (Version 2.0) was used to calculate genetic similarity (Jaccard's coefficient), and cluster analysis (Complete method).

Table 2. PCR primer sequences, annealing temperatures (Ta), and DNA fragment sizes of molecular markers used to study genetic diversity in *Bmy*1.

Marker Name	Primer sequence	Fragment length (bp)	Ta (°C)
BmyR115	5'GCC GTG AGC GTG AAC AAC AG 5'GTA GAA AAT GTC GGG ATC ACG	306	69
BmyE165	5'AAG GCT GGG CTG AAG CTA 5'CGA TAA CAC CAG CAT CCA AGA AC	654	68
BmyA233B	5'CAG ACA GTT CAC CCA TAA GCT 5'GTG TCA TTG TAC TGT CCG G	482	61
BmyS347B	5'CAG ACA GTT CAC CCA TAA GCT 5'CCT GCG AGC TTT GCT CCG	1023	62
BmyA430	5'GGT GGA GGG ACA AAA CTA TGC 5'GGT TTC TCT GTC ACA CTC ACA	508	69
BmyM527	5'AGC TGG TGG AGG GAC AAA 5'CAG GGA GCT CCC CAC CC	451	67
BmyIntron3	5'CAG GCA TCG GAG AAT TCA 5'AGA GAC TAC AAT GTG CCG AA	471/597	58
BmyMicros	5'TTA GCA CAA ACC TTG ATG CAA G 5'TGC TGC TGC TTT GAA GTC TGC	352 (in H.Nijo)	56.5
Bmy21I/D	5'CAG ACA GTT CAC CCA TAA GCT 5'TGC TGC TGC TTT GAA GTC TGC	400/421	56.5

Results

The six allele-specific markers (BmyR115, BmyE165, BmyA233B, BmyS347B, BmyA430 and BmyM527) amplified fragments from 60, 52, 60, 47, 52 and 88 of the 110 lines, respectively. BmyIntron3 amplified either of the two possible alleles with the longer fragment being produced from 41 lines, and both fragments from the DNA of SH98585. The Bmy21I/D marker amplified the long or short fragments from 58 and 51 lines respectively, and both fragments from DNA of SH98585. The line SH98585 (heterozygous) was omitted from further analysis. Four alleles were detected at the microsatellite locus.

Genetic similarity at the *Bmy*1 locus was determined based on data from eight markers, excluding the microsatellite locus. Cluster analysis (Figure 1) divided the 109 barley lines into two large groups of 58 (Sd2) and 51 (Sd1) lines, based on the nucleotide (amino acid) present at five co-segregating loci. With the exception of the cultivar Lindwall, which showed variation at amino acid (aa) positions 115 and 233, the Sd1 group can be characterized by the conserved amino acids Glutamic acid 165, Alanine 430, and Methionine 527, as well as the 21 bp and 126 bp deletions in intron III. Only the microsatellite region and the Ser347/Leu substitution varied among lines in the Sd1 group with leucine being a rare allele. The Sd2 group showed more divergence than the Sd1 group. Beyond the five conserved alleles that define the Sd2 group, polymorphisms were evident with markers BmyS347B, BmyM527, and BmyIntron3, all of which contribute to the determination of distinct Sd2 subgroups. The evaluated Japanese cultivars, all known to carry the thermostable form of *beta*–amylase, grouped together (Sd2-H) with the thermostable Australian cultivar Arapiles, a Chinese line CI4196B, and several related breeding lines from the University of Saskatchewan. In addition, a subgroup (Sd2-L) that includes lines known to be poorly thermostable was formed. The microsatellite marker was polymorphic within all of the groups and subgroups.

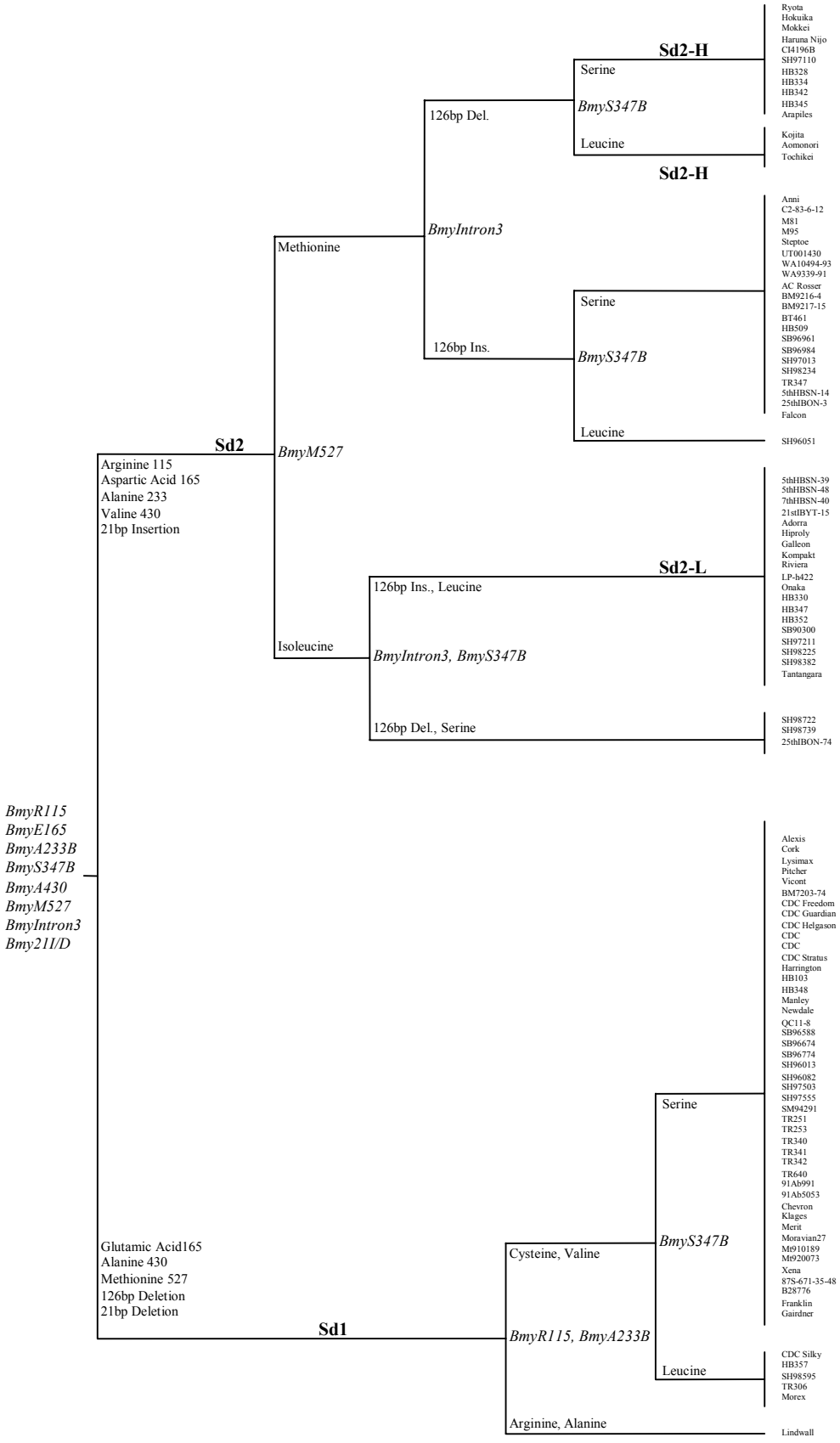

Figure 1. Phylogenetic relationship of 109 barley lines based on genetic variation at *Bmy*1

Discussion

In this study, 109 barley lines could be separated into distinct groups based on variation in the DNA of *Bmy*1. The groups correlate well with *beta*-amylase isoenzyme type and thermostability. Figure 1 has been annotated for the aa (or other DNA variations) that are involved in the determinations of the groupings. Two major groups are evident that correspond to the Sd1 and Sd2 isoenzyme forms based on the placement of lines that have been characterized for *Bmy*1 isoenzyme type. Analysis of the groups revealed that the division of Sd1 and Sd2 was consistent with the Cys115/Arg substitution identified by LI *et al*. (2002). In addition, several other aa and in/del are conserved and as a group separate Sd1 from Sd2. It should be noted that only one cultivar had a genotype intermediate to Sd1/Sd2.

Greater variation was evident at the *Bmy*1 locus among lines with the Sd2 isoenzyme than those carrying Sd1. This is consistent with results for *beta*-amylase thermostability, for which only one Sd1 *beta*-amylase allele but two Sd2 alleles (Sd2-L and Sd2-H) were found (EGLINTON *et al*. 1998). The Sd2 isoenzyme group showed variation at Met527/Ile, Ser347/Leu, and the 126 bp in/del. The Sd1-H subgroup is characterized by Met527 and the 126 bp deletion, while the opposite combination gives rise to Sd2-L. Previous attempts to relate enzyme activity and thermostability to variations at the molecular level have suggested an involvement of the aa at positions 233 and 347 (EGLINTON *et al*. 1998). While Val233/Ala contributes to the determination of Sd1 from Sd2, our analysis indicates that variation at Ser347/Leu does not correlate with enzyme thermostability. ERKKILA *et al*. (1998) proposed that the 126 bp palindromic in/del in intron III of the *Bmy*1 gene determines *beta*-amylase thermostability. Our results support this hypothesis and clearly implicate a role for the 126 bp in/del in the determination of the Sd2-H subgroup. Hypotheses for the molecular mechanisms by which intron DNA is involved in the determination of enzyme form have been reviewed by ERKILLA *et al*. (1998), and a similar role has been suggested by CLOSE *et al*. (2000) for an intron in the *Dhn*4 gene of barley.

Evidence from our study further suggests a role for Met527/Ile substitution. The Sd2-L enzyme type seems to be defined by the presence of Ile527. Met527 with the 126 bp deletion gives rise to the Sd2-H enzyme type while Met527 with the 126 bp insertion results in an enzyme with normal thermostability, suggesting an interaction between the two loci.

Recent Japanese malting barley cultivars exhibit high thermostability while most current North American, European, and Australian cultivars predominantly exhibit intermediate or low thermostability (KIHARA *et al*. 1998, 1999). In our results, the Sd1 intermediate thermostable group includes mostly North American genotypes with a few from Europe and Australia. Other genotypes from Europe, Australia, and North America (including all CIMMYT lines) group as Sd2 with intermediate thermostability. All East Asian genotypes tested grouped as Sd2-H, while in contrast, several of the European and Australian genotypes grouped as Sd2-L.

The seven Japanese cultivars, one Chinese line (CI4196B), the Australian cultivar Arapiles, and five CDC breeding lines (HB328, HB334, HB342, HB345, SH97110), formed the Sd2-H subgroup. This was as expected for the Japanese cultivars, is not unreasonable for CI4196B, and would also be expected for Arapiles since it is one of the few Australian cultivars known to carry thermostable *beta*-amylase (EGLINTON *et al*. 1998). The presence of the five CDC lines was unexpected but is not surprising since the lines are feed-barley types and have not been evaluated for *beta*-amylase thermostability. All five lines share a common line in their recent pedigree. The DNA of HB334 and HB345 was subsequently sequenced and found to be identical to that of Haruna Nijo.

The present study has identified PCR-based markers that, when used in combination, can be used to select for *Bmy*1 isoenzyme type and thermostability in a marker-assisted breeding program.

Acknowledgements
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Changes in SSR Alleles Frequency and Diversity Index in a Decentralized-Participatory Barley Breeding Program

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Abstract
A total of 181 barley entries, 105 fixed genotypes and 76 heterogeneous populations, were grown in seven locations in trials planted in farmers' fields in Jordan. The changes in allele type, allele frequency and genetic diversity due to selection by individual farmers and breeders were assessed using Simple Sequence Repeats (SSRs) during one cycle of selection in a program of decentralized participatory barley breeding (DPBB). The average number of alleles retained per locus after the one cycle of selection at almost all of the locations was significantly lower than the number of alleles in the original population of entries. The reduction in diversity indices was not as high as the reduction in the number of alleles. The allelic composition and the diversity level of the populations were maintained after one cycle of decentralized-participatory selection regardless whether breeders or farmers conducted the selection. This study demonstrates the importance of decentralized participatory plant breeding in maintaining genetic diversity.

Introduction

The emphases of conventional plant breeding (CPB) programs on genetic uniformity and wide adaptation have lead to a continuous reduction in the genetic diversity of cultivated barley (*Hordeum vulgare* L.), 2n = 2x =14, gene pool (CECCARELLI *et al*. 1997) which itself is a sub-sample of the total variation in the species. In a highly centralized non-participatory breeding program in Europe, the number of SSR alleles in post-1985 barley cultivars has been found to be 40% less than the number of alleles in 19 landraces (RUSSELL *et al*. 2000). Farmers in environmentally and economically marginal production environments tend to maintain genetic diversity to maximize production stability and reduce risk of total crop failure (BINSWANGER & BARAH 1980). Decentralized-participatory plant breeding (DPPB) in general and in barley in particular, has been proposed not only as a strategy to reach those areas and farmers by-passed by CPB programs but also as a means of maintaining or enhancing genetic diversity in harsh environments where production stability over time is one of the most important farmers' objectives (CECCARELLI *et al*. 1996). The increase in the number of genotypes retained after participatory variety selection (PVS) has been reported in rice (STHAPIT *et al*. 1996), in common bean (KORNEGAY *et al*. 1996), and in decentralized-participatory barley breeding (DPBB) (CECCARELLI *et al*. 2000). However, no studies have been conducted to quantify the level of genetic diversity in both PVS and participatory plant breeding (PPB) in terms of allele type, frequency and diversity index.
The present study was conducted with the objective of determining changes in SSR allele types, frequency and genetic diversity due to farmers' and breeders' selection in DPBB.

Material and Methods

Genetic Material: A total of 181 barley entries grown in farmers' fields in trials called Farmer Initial Trials (FIT) at seven locations (Ghweer on-farm and on-station, Rabba, Mohay, Khanasri, Ramtha East, Ramtha West) were included in the study. The genetic materials consisted of 104 fixed homogeneous genotypes, and 77 heterogeneous populations. A total of 379 individual plants were assayed from the heterogeneous populations (five plants per population). Each of the 104 fixed genotypes was represented by one sample.

SSR Analysis: DNA extraction was conducted following the cationic hexadcetyl trimethyl ammonium bromide (CTAB) mini-preparation procedure. A total of 12 SSR primer pairs were used in assaying the 104 fixed genotypes and a total of 8 in assaying the 379 individual plants obtained from the heterogeneous populations according to standard PCR procedures on Perkin Elmer 9600 thermocycler. The following primers were used according to the protocols of RAMSEY *et al.* (2000). SSR analysis was conducted using polyacrylamide gel electrophoresis and visualized using silver staining method.

SSR Data Score: Scoring of the SSR data was carried out manually using binary code, present (1) and absent (0). The size (bp) of each allele per locus was determined in reference to the pGEM DNA size markers loaded from the two sides of each gel.

SSR Data Analysis: Every allele of each locus is designated by its size (bp) connected to the locus as a subscript by one dash (-). For example, the allele with a size of 178bp at the HVM68 locus on chromosome 4H is designated as HVM68-$_{178}$ and is identified by this designation in this paper unless and otherwise specified.

Diversity Index: Genetic diversity index (H) was obtained at each of the 12 SSR loci for the fixed genotypes and at the eight loci for the heterogeneous populations using Nei's unbiased statistic (NEI 1987) given as: $H = \underline{n[1 - \Sigma(P_i^2)]/(n-1)}$

where n is the number of individuals analyzed and P_i is the frequency of the i^{th} allele at a given SSR locus. Changes in genetic diversity due to farmers' selection preferences were determined separately in the heterogeneous populations and fixed genotypes in the FIT. Likewise, changes due to breeders' selection were determined in both heterogeneous populations and fixed genotypes.

Results

Heterogeneous Populations

Change in Number of Alleles and Frequency and Change in Genetic Diversity Index: Both farmers' and breeders' selection pressures resulted in reduction of the number of alleles at each locus in almost all locations included in the study (Table 1). Averaged over eight SSR loci, the number of alleles in both farmers' and breeders' selections at all locations, except Rabba and Ramtha West in farmers' selections was significantly lower than the number of alleles in the original populations in the FIT. The highest number of alleles retained was at Ramtha West in farmers' selections. The lowest number per locus per location, three alleles (about 38% of the number in the original population), was retained at the BMS90 locus (1H) at Khanasri in farmers' selections. At Rabba, Mohay and Ramtha East, farmers retained significantly higher number of alleles than breeders (Table 1). Added overall loci per location, the highest loss was in breeders' selection at Mohay (39 alleles out of 87 detected in the starting populations; Fig 1). Only three alleles

were lost one at each of HVM68 locus (4H), Bmag0011 (7H) and HVM67 (4H) due to farmers' selections at Rabba while the corresponding loss due to breeders' selections was 27 alleles.

There was a tendency towards a reduction in diversity index at most of the loci. For example, at HVM68 locus the index changed from 0.90 in the original populations to 0.78 in farmers' selections at Khanasri. At Bmag0125 locus, the diversity index decreased from 0.82 to 0.62 at Khanasri in farmers' selections, and to 0.68 and 0.69 in breeders' selections at Khanasri and Mohay, respectively. At Bmag0011 locus, the diversity value was 0.62 at Ghweer on-farm in breeders' selections with the value in the starting populations 0.78. However, although there was a very high reduction in the number of alleles at each SSR locus and location due to both breeders' and farmers' selection pressures, the change in the level of genetic diversity index was not equally high.

Table 1. Number of alleles and diversity index (H) at SSR loci in heterogeneous populations in trials grown at seven locations (GW-Ghweer on-farm, OS- Ghweer on-station, RB-Rabba, MO-Mohay, KN-Khanasri, RE-Ramtha East, RW-Ramtha West), after one cycle of farmers' (FS) and breeders' (BS) selections.

		GW		OS		RB		MO		KN		RE		RW
SSR Locus	FIT[y] (379)	BS (90)	FS (79)	BS (89)	FS (78)	BS (44)	FS (146)	BS (25)	FS (60)	BS (53)	FS (44)	BS (40)	FS (64)	FS (183)
HVM68	14	14	13	13	12	10	13	8	11	10	9	12	13	14
H	0.902	0.907	0.907	0.895	0.901	0.895	0.887	0.883	0.814	0.877	0.783	0.919	0.91	0.886
Bmag0013	10	9	9	8	7	7	10	5	6	7	7	6	6	10
H	0.848	0.859	0.826	0.802	0.824	0.829	0.860	0.807	0.841	0.836	0.810	0.823	0.817	0.826
Bmag0125	9	8	7	8	8	7	9	5	6	7	7	7	7	9
H	0.817	0.800	0.769	0.795	0.841	0.812	0.812	0.690	0.781	0.676	0.624	0.774	0.813	0.796
Bmag0135	9	8	7	6	7	5	9	5	8	8	7	7	7	9
H	0.788	0.823	0.798	0.721	0.745	0.747	0.825	0.768	0.811	0.834	0.810	0.867	0.840	0.782
Bmag0011	10	8	8	8	8	8	9	5	7	8	7	7	8	10
H	0.776	0.619	0.754	0.839	0.768	0.855	0.754	0.740	0.703	0.770	0.805	0.820	0.785	0.825
BMS90	8	7	6	6	5	5	8	4	6	5	3	4	5	8
H	0.772	0.738	0.780	0.767	0.743	0.735	0.757	0.737	0.755	0.747	0.629	0.735	0.737	0.776
Bmac0040	14	10	13	13	12	10	14	7	10	10	9	12	13	14
H	0.912	0.883	0.898	0.914	0.900	0.892	0.902	0.830	0.857	0.832	0.820	0.914	0.923	0.906
HVM67	13	11	9	9	9	8	12	9	11	9	9	9	9	12
H	0.859	0.871	0.823	0.822	0.833	0.821	0.887	0.890	0.887	0.865	0.848	0.851	0.841	0.829
Average[y]	10.9	9.4a	9.0a	8.9a	8.5a	7.5b	10.5a	6.0b	8.1a	8.0a	7.3b	8.0b	8.5a	10.8
^		**	**	**	**	**	NS	**	**	**	**	**	**	NS

[y], means followed by the same letter at each location are not significantly different at the 5% level in paired sample t-test; ^,**, NS significantly different ($P \leq 0.05$) and not significant different (P=0.05) from the average number of alleles (10.9) in the FIT using paired sample t-test; [y], numbers in brackets indicate number of lines assayed from heterogeneous populations in the FIT or number of lines in populations retained in FS or BS at each location.

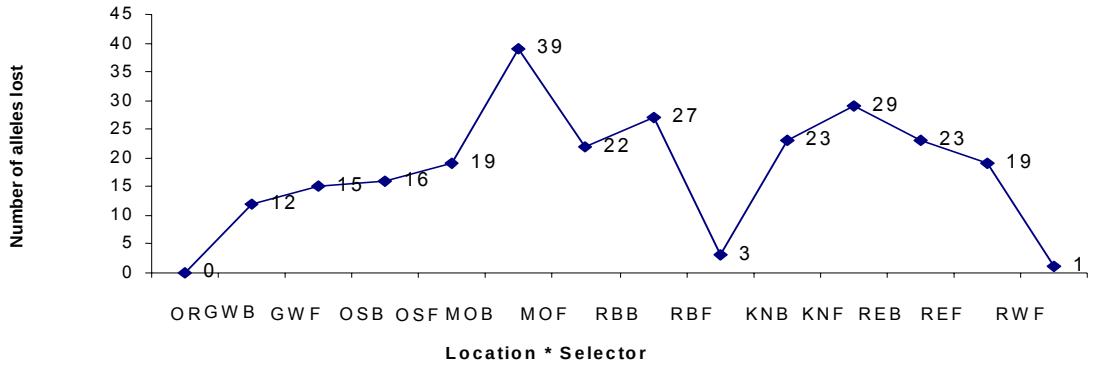

Figure 1. Number of allele lost from the original 87 alleles in heterogeneous populations, added over eight SSR loci, due to farmers' and breeders' selection pressures at various locations. OR-loss in original population, GW-Ghweer On-farm, OS- Ghweer On-station, RB-Rabba, MO-Mohay, KN-Khanasri, RE-Ramtha East, RW-Ramtha West, B-Breeders' Selections, and F-Farmers' Selections; B-Breeders' Selections, and F-Farmers' Selections.

Fixed Genotypes

Change in Number of Alleles and Frequency and Change in Genetic Diversity Index:

The number of alleles retained in farmers' selections and breeders' selections, averaged over eight SSR loci at each location, was significantly lower than the number of alleles in the original fixed breeding materials in the FIT (Table 2). Like in the case of heterogeneous populations, a number of alleles were lost at each locus and location due to breeders' and farmers' selection pressures. Although there was a significant reduction in the number of alleles due to selection in all locations, there were alleles whose frequency increased after selection. The allele HVM68-$_{185}$ increased from 0.14 to as high as 0.30 at Ghweer on-farm in farmers' selections. Most of the frequent alleles in the original population maintained their proportion (increased in most locations) after selection. In most locations, rare alleles in the original population became either lost or reduced though the frequency of some increased.

The level of genetic diversity was reduced at some loci and locations (Table 2). At the Bmag0125 locus the diversity index was reduced from 0.80 in the starting population to 0.67 at Ghweer on-farm in breeders' selection. The index at BMS90 locus was reduced from 0.76 to 0.67 and at Bmac0040 from 0.84 to 0.72 both at Mohay in breeders' selection.

Table 2. Number of alleles and diversity index (H) at SSR loci in in the fixed genotypes in trials grown at seven locations (GW-Ghweer on-farm, OS- Ghweer on-station, RB-Rabba, MO-Mohay, KN-Khanasri, RE-Ramtha East, RW-Ramtha West), after one cycle of farmers' (FS) and breeders' (BS) selections

SSR Locus	FITy (104)	GW		OS		RB		MO		KN		RE		RW
		BS (14)	FS (23)	BS (46)	FS (31)	BS (30)	FS (31)	BS (30)	FS (34)	BS (45)	FS (44)	BS (27)	FS (38)	FS (44)
HVM68	13	8	9	12	11	12	12	11	12	12	13	11	11	13
H	0.884	0.742	0.852	0.843	0.863	0.902	0.873	0.852	0.896	0.821	0.822	0.832	0.834	0.894
Bmag0013	9	8	8	8	8	7	7	8	9	8	8	8	8	9
H	0.844	0.912	0.885	0.819	0.817	0.802	0.852	0.830	0.850	0.832	0.844	0.823	0.801	0.840
Bmag0125	7	5	7	7	7	7	7	7	7	6	7	6	7	6
H	0.803	0.670	0.779	0.790	0.776	0.809	0.824	0.756	0.776	0.749	0.739	0.758	0.784	0.756
Bmag0135	8	6	7	7	7	7	6	8	8	7	7	7	7	7
H	0.811	0.747	0.840	0.800	0.802	0.770	0.790	0.839	0.840	0.801	0.834	0.863	0.844	0.780
Bmag0011	9	5	7	8	7	8	5	7	7	8	9	8	9	8
H	0.775	0.731	0.814	0.750	0.800	0.793	0.713	0.720	0.715	0.751	0.759	0.772	0.733	0.808
BMS90	7	4	5	5	4	5	7	4	5	4	4	4	4	6
H	0.759	0.703	0.751	0.700	0.712	0.747	0.770	0.669	0.738	0.699	0.747	0.701	0.741	0.736
Bmac0040	14	9	8	10	9	8	11	9	12	11	10	9	10	13
H	0.841	0.835	0.877	0.793	0.828	0.803	0.871	0.718	1.000	0.753	0.770	0.724	0.788	0.862
HVM67	12	8	7	11	9	10	11	9	10	11	12	11	11	12
H	0.908	0.912	0.881	0.888	0.869	0.871	0.918	0.876	0.913	0.875	0.887	0.866	0.869	0.915
Bmac0067	12	8	9	12	10	12	9	12	11	12	12	12	12	12
H	0.911	0.912	0.909	0.926	0.892	0.920	0.860	0.931	0.906	0.920	0.918	0.917	0.920	0.913
Bmag0120	9	6	7	6	7	5	6	7	8	8	8	6	7	8
H	0.747	0.791	0.830	0.694	0.737	0.736	0.807	0.657	0.734	0.697	0.706	0.707	0.679	0.769
HVM03	8	5	7	7	7	6	6	8	8	8	8	6	7	7
H	0.658	0.670	0.625	0.535	0.497	0.586	0.776	0.632	0.699	0.574	0.635	0.500	0.584	0.654
Bmac0316	9	5	7	8	8	7	6	7	8	8	8	7	7	8
H	0.817	0.835	0.733	0.836	0.809	0.848	0.768	0.837	0.810	0.814	0.831	0.849	0.814	0.820
Average[y]	9.8	6.4a	7.3a	8.4a	7.8a	7.8a	7.8a	8.1a	8.8a	8.6a	8.8a	7.9a	8.3a	9.1
^		**	**	**	**	**	**	**	**	**	**	**	**	**

[y], means followed by the same letter at each location are not significantly different at the 5% level in paired sample t-test; ^,** significantly different ($P \leq 0.05$) from the average number of alleles (9.8) in the FIT using paired sample t-test; [y], numbers in brackets indicate number of genotypes in the FIT or number retained in FS or BS at each location.

Discussion

In the heterogeneous populations, the largest number of allelic loss (39 alleles out of 87 in the original populations) averaged overall loci was in breeders' selection at Mohay (Fig 1). At Raba, only three alleles were lost due to farmers' selection, while the corresponding loss due to breeders' selection was 27 alleles (Fig 1). This indicates that more allelic variation was retained in farmers' selections. In a highly centralized non-participatory breeding program in Europe, the number of SSR alleles in post-1985 barley cultivars has been found to be 40% less than the number of alleles in 19 key progenitors ('termed foundation genotypes') (RUSSELL *et al*. 2000).

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QTLs for Straw Traits Identified in Recombinant Inbred Lines of the Cross 'Arta' x *Hordeum spontaneum* 41-1

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Abstract
A genetic linkage map has been developed for recombinant inbred lines (RILs) derived from the cross 'Arta' x *Hordeum spontaneum* 41-1.194 RILs randomly chosen from a population of 494 RILs were mapped with 189 markers including one morphological trait (*btr* = brittle rachis locus). The linkage map extended to 890 cM. Straw samples from RILs grown at Tel Hadya and Breda ICARDA's research stations in the years 1996/97 and 1997/98 were analysed by NIRS for acid detergent fiber, neutral detergent fiber, lignin, dry organic matter digestibility, voluntary intake, and crude protein. QTL analysis was performed using QTL cartographer software package and QTLs were localised. For acid detergent fiber, none of the identified QTLs was common between the environments. However, for neutral detergent fiber, lignin, dry organic matter digestibility, and crude protein one of the identified QTLs each was common between the two environments. There were four main locations within the barley map where a number of QTLs were clustered. The 1H-5-8 interval had 7 QTLs, the 2H-3-6 interval 6 QTLs, the 7H 5-8 interval 5 QTLs, and the 5H-5 interval 6 QTLs. The identified QTL locations could be used to initiate marker-assisted selection for straw quality traits.

Introduction
Barley is the main rainfed crop in the semi-arid areas of West Asia and North Africa (WANA) and is largely grown as feed for animals, particularly small ruminants (sheep and goats). The crop is normally harvested by combine, but when is too short because of drought, it may either be grazed at maturity or hand-harvested by uprooting. In general both grain and straw are used for feed. *Hordeum spontaneum* is expected to have a potential in contributing useful genes in barley breeding as donor of adaptive traits to extreme stress conditions, as suggested by its distribution in the driest areas of the West Asia. Most of the current genetic improvement programs for grain crops do not include direct selection for the quality of their residues as ruminant feed, and genetic markers on which to base selection have rarely been identified. The objective of this study was to identify trait-marker linkages in a population of recombinant inbred lines (RILs) of a cross between the *H. vulgare* cv 'Arta' and *H. spontaneum* 41-1 using the QTL approach. Of particular interest was to analyze straw characteristics and to determine the locations of the genes involved in their control.

Material and Methods
Plant Material and Growth Conditions
494 F_7 RILs, derived by single seed descent from the cross between the *H. vulgare* cv 'Arta' and *H. spontaneum* 41-1, were planted with the parents in the cropping seasons 1996/97 and 1997/98, at ICARDA's research stations located near Tel Hadya (36°01' N; 37°20' E, elevation 300 m asl.) and near Breda (35°56' N; 37°10' E, elevation 354 m asl.) in Syria. In

both years and locations, the trials were grown under rainfed conditions (see BAUM *et al.* 2003 for details).

Straw-Related Traits
Complete plants for each plot were hand-harvested. Roots were cut off by scissors, and samples weighed in the field and collected in plastic bags for further processing. Straw samples were milled by Wiley mill No.4 fitted with a 6-mm screen, and analyzed by NIRSystems Model 5000 scanning monochromator, wave length range 1100-2500 mm, using the coarse sample cell (half volume). The traits measured were: acid detergent fiber (ADF), neutral detergent fiber (NDF), lignin (LIG), dry organic matter digestibility (DOM), voluntary intake (DMI), crude protein (CP), and ash percentage (ASH) (Table 1).

Table 1. Units and means of the traits analyzed

Trait	Unit	Br97	Br98	Th97	Th98
ADF	%	30.54	37.92	37.64	49.40
NDF	%	71.17	76.84	75.43	78.68
LIG	%	3.747	4.202	6.820	6.122
DOM	%	58.50	53.75	53.27	52.80
DMI	g/kg LW/d	20.52	18.01	14.25	12.60
CP	‰	4.09	2.44	2.44	1.26
ASH	%	–	3.25	–	–

DNA Extraction and Analysis of SSRs and AFLPs
One hundred and ninety-four RILs were used to construct a genetic linkage map. Total genomic DNA was extracted and genetic mapping was carried out using Amplified Fragment Length Polymorphic (AFLP) markers and microsatellite-based markers (see BAUM *et al.* 2003).

Results
The linkage map based on the 'Arta' x *H. spontaneum* 41-1 population originally contained 189 marker loci, including 1 morphological marker locus (*btr* = brittle rachis) (BAUM *et al.* 2003). For the purpose of the QTL analysis, a reduced map was constructed (Fig.1), containing 129 marker loci. The Join Map v. 2.0 software package was employed for map construction and recombination fractions were converted to centiMorgans (cM) according to the Kosambi's mapping function. The QTL analysis was performed using Windows QTL Cartographer v. 2.0.

In Table 2, the QTLs are ordered by chromosomal position. The numbers on the right side of the chromosomes in Fig. 1 correspond to the number of the chromosomal position in Table 2. The LR-value of the locus, originates from either from Multi Trait Analysis – or from composite interval analysis. The effect and the explained phenotypic variance were estimated by Multi Interval Mapping.

Figure 1. Linkage map of the cross ‘Arta’ x H. spontaneum 41-1 with positions of QTLs on the right side of the chromosomes.

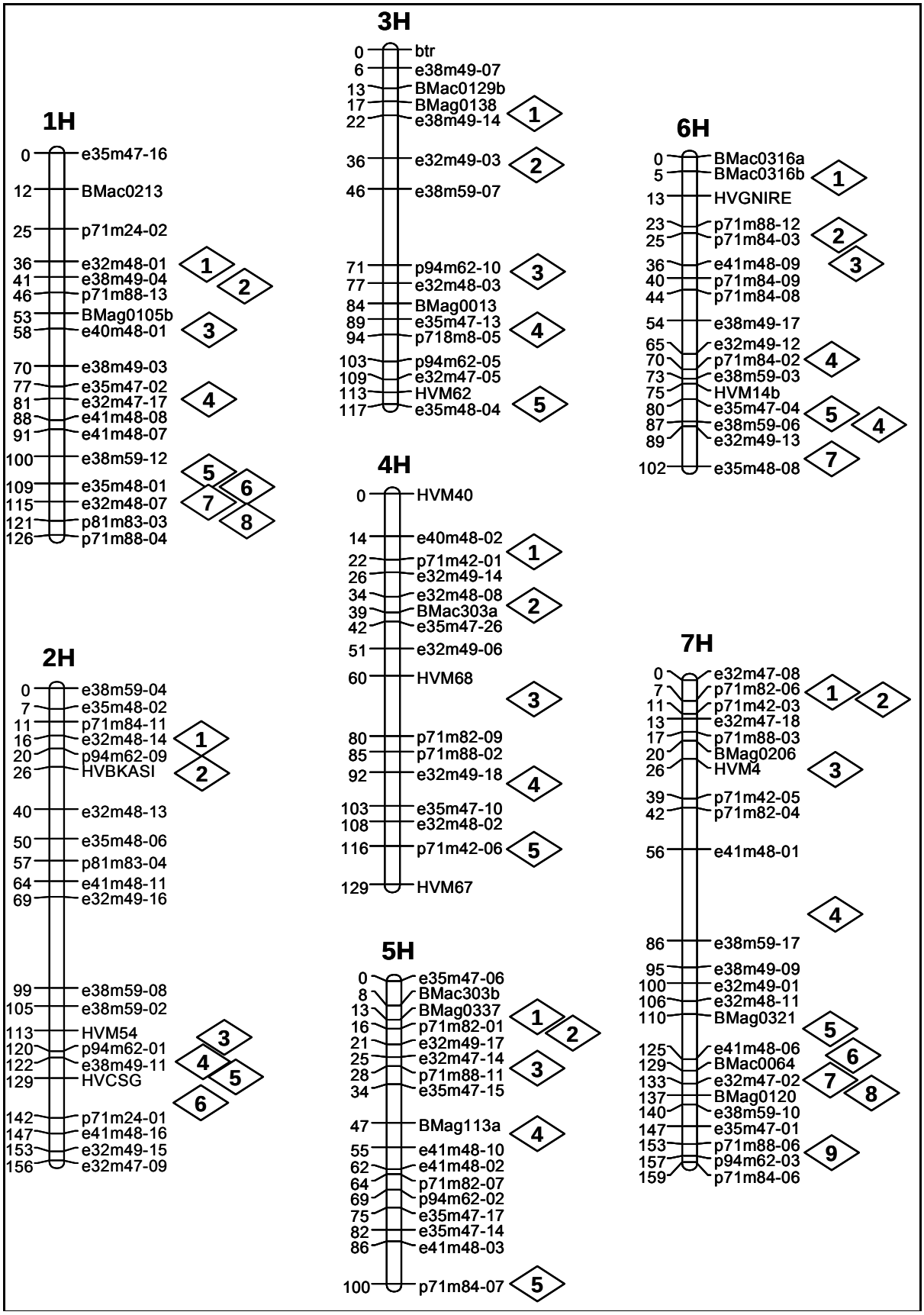

Table 2. Explained variance of the QTLs for trait-environment combinations and the sum of explained variance

Trait/Env.	**QTLs**					**Sum**
ADF-Br97	1: 1H-2	2: 3H-3	3: 6H-1	4: 7H-6	5: 7H-8	
	4.1%	2.5%	4.1%	5.9%	2.0%	18.6%
ADF-Br98	1: 6H-6					
	3.2%					
ADF-Th97	1: 4H-2	2: 7H-1	3: 7H-2			
	2.3%	0.7%	16.2%			19.2%
ADF-Th98	1: 2H-1					
	6.6%					6.6%
ASH-Br98	1: 1H-8	2: 2H-4	3: 3H-1	4: 5H-1	5: 5H-2	
	4.6%	2.5%	8.3%	1.1%	9.3%	25.8%
DMI-Br97	1: 1H-4	2: 1H-8	3: 1H-9	4: 2H-6	5: 6H-4	
	7.2%	2.2%	8.4%	10.1%	6.0%	33.9%
DMI-Br98	1: 1H-1	2: 3H-4	3: 5H-5	4: 6H-7	5: 7H-4	
	7.4%	5.2%	4.0%	5.2%	4.8%	30.3%
DMI-Th98	1: 1H-6	2: 1H-8	3: 4H-2	4: 5H-5		
	13.7%	0.5%	11.6%	5.6%		31.4%
LIG-Br97	1: 6H-7					
	4.2%					4.2%
LIG-Br98	1: 2H-4	2: 3H-4	3: 5H-5	4: 6H-7	5: 7H-7	
	5.8%	4.4%	4.3%	7.5%	5.2%	30.0%
LIG-Th97	1: 1H-5	2: 3H-5	3: 7H-5			
	7.6%	5.4%	7.1%			20.1%
LIG-Th98	1: 3H-2	2: 4H-4	3: 6H-5			
	8.9%	3.7%	7.1%			19.7%
NDF-Br97	1: 1H-5	2: 2H-2	3: 2H-4	4: 6H-3		
	3.8%	3.1%	14.7%	3.8%		30.1%
CP-Th97	1: 1H-7	2: 5H-5	3: 7H-5			
	6.0%	3.3%	5.3%			14.6%
CP-Th98	1: 5H-5					
	4.2%					4.2%
DOM-Br97	1: 1H-9	2: 2H-5	3: 5H-3	4: 6H-4		
	4.8%	12.0%	4.2%	7.8%		28.8%
DOM-Br98	1: 2H-3	2: 3H-3	3: 4H-3	4: 5H-4		
	5.9%	4.1%	6.3%	3.6%		19.9%
DOM-Th97	1: 5H-5	2: 6H-5				
	6.1%	7.3%				13.4%
DOM-Th98	1: 4H-1	2: 4H-4	3: 5H-4			
	4.4%	6.2%	4.5%			15.1%

For ADF, ten QTLs were detected; none of them was common between the environments. The QTLs detected in Br97 explained in total 18.6 % of the phenotypic variation and the ones found in Th97 explained 19.2 %. For NDF, one QTL was detected being common to two

environments (1H-1: Br98 and Th98). Eleven QTLs were localized for LIG (Tab.4). One of them was common to two environments (6H-7 in Br97 and Br98). For DOM, twelve QTLs were localized; one of them was common to Br98 and Th98 (on 5H-4). Twelve QTLs were localized for DMI, one of which common to Br97 and Th98 (on 1H-8) and one common to Br98 and Th98 (on 5H-5). Only for these two QTLs, the *H. spontaneum* line contributed the allele with the higher value, while for all other QTLs detected for this trait, the higher allele was found in 'Arta'. Specific QTLs for DMI were detected in Br97, in Br98, and in Th98. Epistatic effects were found in Br98 between the QTL on 5H-5 and the one on 6H-7. No QTLs were found in Th97. The explained phenotypic variance was about 30 %. For CP, no QTLs were found in Br97, while one QTL was common to Th97 and Th98 (on 5H-5) and three additional specific QTLs were localized. Like for the case of LIG, DOM and DMI, the origin of the allele with the higher value for the locus on 5H-5 was opposite to the rest of the QTLs: In this case, it was the only QTL where the *H. spontaneum* line contributed with the allele leading to the higher value of the trait. QTLs for ASH were found only in Br98. Five QTLs were identified, explaining together 25.8 % of the phenotypic variance.

Discussion

The "plant height under drought" QTLs in the 'Arta' x *H. spontaneum* 41-1 population, especially the one on 3H, showed pleiotropic effects on traits such as days to heading, grain yield and biological yield (Baum et al. 2003). However, there was no co-localization of straw quality QTLs at the same position. Only one QTL for LIG and one for DMI, both detected in Breda 1998, were identified in this interval (at 3H-4).

The feeding value of barley straw is of greater importance in years with favorable rainfall when not only is feeding value generally poor, but high straw yields permit the straw to be stored for future years (GOODCHILD 1997). Conditions likely to decrease grain yield tend to increase the feeding value of straw, such as low winter minimum temperature, low rainfall during vegetative growth, and high temperature during grain maturation. Precipitation before January affects the yield of grain and straw but had little effect on the feeding value of straw (GOODCHILD 1997). Two negative correlations between feeding value and agronomic traits are expected: with plant height under drought, and with lodging resistance. These factors would compromise yield and harvestability in dry and in wet conditions, respectively (CECCARELLI *et al*. 1991).

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Segregation Analysis of SSRs Markers and Two Morphological Traits in a Doubled-Haploid Population of Barley

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Abstract
Fifty nine doubled-haploid barley (*Hordeum vulgare* L) population obtained from a cross between the Tunisian cultivar 'Roho' and the local line '90' was analysed using eighteen polymorphic simple sequence repeats (SSRs) markers localised specially on chromosomes 3, 4 and 6 (RAMSAY *et al.* 2000). Thirteen SSR loci indicated a good fit to a 1:1 segregation ration. Among five distorted segregation three were localised on chromosome 3. However, this distortion may be due to small size of the population screened. The morphological traits, row number and stem colour were observed. Two-genes (3:1) segregation ratios were observed for the row number and stem colour. A significant linkage was observed between Bmag 571 and stem colour using correlation analysis. Sinteny was observed between SSRs linkage group and the mapping data obtained by RAMSAY *et al.* (2000). However, the multi-loci marker Bmac 144 showed 1:1 segregation without linkage with the SRR markers mapped on chromosome 6. This is a preliminary genetic map result for QTL identification of resistance and agronomic traits for which the parents differentiated.
Keywords: *Hordeum vulgare*; SSRs markers; segregation distortion; linkage analysis

Introduction

Various DNA markers and molecular linkage maps have been developed in a large number of plants and make effective tools for phylogenetic analysis, marker-assisted selection (MAS) in breeding programs, genetic analysis of quantitative traits, cloning of the genes responsible for various phenotypes (map-based cloning), and construction of a physical map.

Microsatellite markers, also known as SSRs (Simple Sequence Repeats) use primers designed from sequences flanking short repeat blocks made di-, tri- or tetra-nucleotides. These sequences initially described by HAMADA *et al.* (1982) are widely dispersed and have been shown to make up to 44% of genomes in some plant species (LEVINSON & GUTMAN 1997). They have been recognised as the marker of choice for molecular mapping and marker assisted selection because of their ubiquitous distribution in the genome, easy assay and scoring, robust and reliable genotype information, co-dominant Mendelian inheritance and higher heterozygosity index (POWELL *et al.* 1996).

Genetic maps of barley have been constructed using morphological, isozyme and molecular markers. Several kinds of markers have been developed These include RAPD markers (GIESE *et al.* 1994), SSRs (LIU *et al.* 1996), AFLPs (QI *et al.* 1998) STSs (MANO *et al.* 1999) and randomly amplified SSRs (DAVILA *et al.* 1999). Many other segregating progenies have also been used to construct partial maps and to determine locations of interesting genes. The aims of the present investigation are: (i) to study segregation analysis of morphological traits and polymorphic SSRs loci in a doubled-haploid barley population and (ii) to identify linkage group between the used markers.

Material and Methods

Plant Material

A total of fifty nine doubled-haploid barley (*Hordeum vulgare* L.) population were obtained from F1 plants of the cross between the Tunisian cultivar 'Roho' and the local line '90'. These DH lines were produced by anther culture (KAO *et al.* 1991) as well as by the *Hordeum bulbosum* method (KASHA & KAO 1970). The parents were selected because they exhibited different responses to foliar disease and contrasting morphological and physiological characters.

DNA Extraction

Total genomic DNA was extracted from fresh leaves of the parents, F1 and the fifty nine DH population using a standard CTAB method from SAGHAI-MAROOF *et al.* (1984). The DNA was RNAase-treated and quantified by measuring the OD_{260} on a spectrophotometer.

Microsatellite Markers

The fifty three (53) SSR markers used in this study were obtained from published sequences (RAMSAY *et al.* 2000) and were specially localised on chromosomes 3, 4 and 6. The primers were synthesised by GENSET OLIGO. PCR amplification was performed with 20 ng DNA in 15 µl volume reaction according to the protocol developed by LIU *et al.* (1996). The amplification products were resolved on 6% polyacrylamide gels (PAGE) followed by silver-staining according to the protocol described by PILLEN *et al.* (2000).

Morphological Markers

The DH population was grown in the field with the parents and the F1 at ESA-Mograne research station during 2002-2003 and were evaluated for the morphological characters row number (Six-row and two-row) and stem colour (green and purple).

Data Analysis

The segregation of SSRs markers in the doubled haploid progeny was tested against an expected 1:1 ratio using X^2 analysis. For morphological traits, a one-gene model 1:1 and a two-gene model 3:1 were tested by the X^2 to determine the number of genes controlling these traits. To identify linkage groups, pairwise comparisons and grouping of markers was carried out using simple correlation analysis.

Results and Discussion

Fifty three SSRs markers were screened for polymorphism between 'Roho' and '90', 30 (56.6%) were found to be polymorphic, 9 (16.9%) monomorphic and 14 (26.4%) exhibited any or smeary amplification. The polymorphic SSRs markers detected one to two polymorphic alleles segregated co-dominantly and showed no biased segregation in the HD population.

A X^2 analysis of 30 SSRs loci revealed that 12 (40%) deviated from the expected 1:1 Mendelian segregation ratio at $P<0.05$ and 5 (16.6%) at $P<0.01$ (Table 1). Skewed segregation ratios have been reported in many species and for all types of markers (morphological, isozyme, RFLP, SSR and AFLP) (KY *et al.* 2000) with a percentage of loci showing segregation distortions varying from 1% to 80%. For barley, the percentage of loci showing segregation distortions was highly variable: 4% with RAPD markers (MANNINEN 2000), 6.66% with SSRs markers (MARTIN *et al.* 2000), 10% with co-dominant RFLP and AFLP markers (RICHTER *et al.* 1998), and 29% with BARE-1 retrotransposon markers (MANNINEN *et al.* 2000). In our study, the higher level of distorted loci calculated at

P<0.05 may be due to the small size of the population screened, so we can consider that only 16,6% of the SSRs markers exhibited distorted segregation (P< 0.01) .

The majority of distorted loci were located on chromosomes 3H and 2H. This result is in agreement with those observed by MANNINEN *et al.* (2000) using BARE-1 retrotransposon markers. GRANER *et al.* (1996) indicated that all the linked RFLP markers to the gene for resistance to *P. teres* (Pta) located on the long arm of chromosome 3, are characterised by heavily distorted segregation ratios.

The morphological traits, row number and stem colour were observed. For row number, the DH lines segregated 3:1 for two rows: six rows (41:18) indicating that two genes were involved in the control of row number (Table 1). For stem colour, the DH lines segregated 38 (purple): 21 (green). The best fit for this segregation pattern was with a 3:1 ratio although, the X^2 value (4.89) was still significant at P = 0.027 for 1:1 ratio.

Segregation data were used for genetic linkage analysis using correlation test with SPSS program. The analysis revealed six linkage groups comprising 2 to 9 SSRs markers (Table 2). Sinteny was observed between SSRs linkage group and mapping data obtained by RAMSAY *et al.* (2000). However, the SSRs markers HVM31, and Ebmac806 known to be localised on chromosome 3 (table1) were genetically linked to four markers localised on chromosome 6. Also, the markers HVM11 and HVM49 with different map localisation (Table1) belong to same group in our study. In the other hand, the multi-loci marker Bmac 144 (Table 1), showed an independent segregation.

Significant correlation was observed between Bmag 571, and stem colour which is governed by two genes. Because Bmag 571 is multi-loci marker (Table1) stem colour was then assigned to chromosomes 2, 6 or 7.

Clustering of distorted loci at P< 0.05 was observed for markers in linkage group 2 and 4. A clustering of distorted loci have often been reported within the linkage groups constructed in several species (GRANER *et al.* 1991) and might be explained by linkage to incompatibility (WRICKE and WEHLING 1985) or else linkage to a lethal allele in gametes (PILLEN *et al.* 1992).

This is a preliminary genetic map result for QTL identification for resistance and agronomic traits for which the parents differentiated .

Table 1. Segregation analysis of SSR markers and two morphological traits in a barley doubled haploid lines derived from a cross: Roho x 90

SSRs Marker and Morphological traits	Location (RAMSAY et al.) 2000	N° of DH lines P1 alleles	P2 alleles	Expected ratio[a]	X^2	Probability
HVM 11	6	30	29	1:1	0.02	0.896
HVM 27	3	38	21	1:1	4.90	0.027
HVM 31	3	33	26	1:1	0.89	0.362
HVM 33	3	37	22	1:1	3.81	0.051
HVM 36	2	27	32	1:1	0.42	0.515
HVM 49	1	27	32	1:1	0.42	0.515
HVM 54	2	41	18	1:1	8.96	0.003
HVM 62	3	40	19	1:1	7.47	0.006
HVM 67	4	34	25	1:1	1.37	0.241
HVM 68	4	36	23	1:1	2.86	0.091
HVM 70	3	41	18	1:1	8.96	0.003
HVM 74	6	33	26	1:1	0.83	0.362
Bmac 67	3	38	21	1:1	4.90	0.027
Bmac 144	1/2/4/5/6	34	25	1:1	1.37	0.241
Bmac 209	3	21	38	1:1	4.90	0.027
Bmac 316	6	31	28	1:1	0.15	0.696
Bmag 136	3	21	38	1:1	4.90	0.027
Bmag 138	3/4	38	21	1:1	4.90	0.027
Bmag 225	3	37	22	1:1	3.81	0.051
Bmag 496	6	26	33	1:1	0.83	0.362
Bmag 500	6	21	34	1:1	3.07	0.080
Bmag 571	2/6/7	16	43	1:1	12.35	0.000
Bmag 603	3	21	38	1:1	4.90	0.027
EBmac 701	?	30	29	1:1	0.02	0.896
EBmac 708	3	17	41	1:1	9.93	0.002
EBmac 775	4	23	36	1:1	2.86	0.091
EBmac 788	4	30	29	1:1	0.02	0.896
EBmac 806	6	21	38	1:1	4.90	0.027
WMS6	4	28	31	1:1	0.15	0.696
HvLTPPB	3	35	24	1:1	2.05	0.152
Row number	?	41	18	1:1	8.97	0.003
				3:1	0.95	0.400
Stem color	?	21	38	1:1	4.89	0.027
				3:1	3.53	0.063

[a]: The 1:1 ratio was derived from a one-gene model, and the 3:1 ratio from a two-gene model

Table 2. Linkage groups based on SSR markers and morphological characters

Linkage groups	markers_
Group1:	HVM 67 - HVM 68 - Ebmac 701 - Ebmac 775 - Ebmac 788 - WMS6
Group 2:	HVM 62 - HVM 70 - Ebmac708
Group 3:	HVM 31 - HVM 74 - Bmac 316 - Bmag 496 - - Bmag 500 - Ebmac806
Group 4:	HVM 27 - HVM 33 - Bmac 67 - Bmac 209 - Bmag 136 - Bmag 138 - Bmag 225 – Bmag 603 - HVlTPPB
Group 5:	HVM11 - HVM 49
Group 6:	Bmag 571 - Stem color

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Effect of *Vrn-H2* Vernalization Response Locus (4H) on Plant Development in a Winter Barley Mapping Population

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Abstract

Large QTL peaks of heading date in controlled environment tests were identified on the long arm of chromosome 4H in the Dicktoo (D) x Kompolti korai (KK) winter barley mapping population. It was proven to be the effect of the vernalization response gene *Vrn-H2* itself through mapping the tightly linked *HvSnf2* transcriptional regulator gene in the population. The segregation of this gene explained more than 90 % of the phenotypic variance in first node appearance and heading date in unvernalized and 80 % of the variance in vernalized treatments under long photoperiod regimes. Lines carrying the KK allele headed significantly later both in unvernalized and vernalized treatments. In this population neither *Ppd-H1* (2H) nor *Vrn-H1* (5H) had significant effect on heading date.

Introduction

Low temperature and the length of photoperiod are the two most important environmental factors, which influence both flowering and winterhardiness. Vernalization requirement is the sensitivity of the plant to cold treatment. In genotypes with true winter growth habit the transition from the vegetative to the generative growth phase does not occur without vernalization treatment, or it is extremely delayed. Parallel to the inhibition of the regulating genes of flowering, vernalization requirement is also a key component of winterhardiness as genes controlling frost tolerance stay up regulated until the low temperature requirement of a genotype is not saturated (DANYLUK *et al.* 2003).

Vernalization in barley is determined by three loci, *Sh* (*Vrn-H2*; based on the common nomenclature within the cereal species) on chromosome 4H, *Sh2* (*Vrn-H1*) on chromosome 5H, and *Sh3* (*Vrn-H3*) on chromosome 1H (TAKAHASHI & YASUDA 1970; TRANQUILLI *et al.* 2000); the more exact positions of the first two loci have been determined through marker map based analyses (HACKETT *et al.* 1992; HAYES *et al.* 1993; LAURIE *et al.* 1995). Barley requires winter alleles at these loci in order to show a distinct vernalization response, the winter alleles being recessive at *Vrn-H1* and *Vrn-H3*, while dominant at *Vrn-H2* locus, with epistatic interactions between them (TAKAHASHI & YASUDA 1970; PAN *et al.* 1994; TRANQUILLI *et al.* 2000). The candidate genes and their mode of actions have been recently published both for *Vrn-A1* (YAN *et al.* 2003; DANYLUK *et al.* 2003) and for *Vrn-A2* (YAN *et al.* 2004).

To analyse the complex controlling mechanism of plant development we started a series of experiments applying comparative mapping using two barley populations with one common parent. In the Dicktoo x Morex winter x spring barley population both the photoperiod response locus *Ppd-H1* (2H) and *Vrn-H1* locus significantly determined heading date under controlled environment tests and field-sown experiments, and their epistatic interactions resulted in a number of transgressive segregants in the population (PAN *et al.* 1994; KARSAI *et al.* 1997). In the Dicktoo x Kompolti korai winter barley population both parents represented an unusual combination of adaptation traits, making them unique within the group of winter barleys (KARSAI *et al.* 2001). In this paper we described the effect of *Vrn-H2* gene (4H) on plant development in the winter barley genetic background of Dicktoo x Kompolti korai cross.

Material and Methods

Plant Materials

Doubled haploid population of 98 lines was developed using anther culture technique from the cross of Dicktoo x Kompolti korai winter barley varieties. Of these, the US variety Dicktoo possesses a good frost tolerance, has no vernalization response, and photoperiod sensitive. The old Hungarian variety, Kompolti korai (Ager/Ledeci beta) has a moderate frost tolerance, strong vernalization requirement but this variety is photoperiod insensitive.

Phenotypic Characterization

The 98 DH lines were phenotyped in a series of controlled environment tests involving combinations of vernalization and photoperiod (based on the methodology of KARSAI *et al.* 1997; KARSAI *et al.* 2001). The photoperiod treatments consisted of 8, 16 and 24 h light regimes per 24 h period and were carried out in the phytotron facilities of the Agricultural Research Institute of HAS (Martonvásár, Hungary). The temperature was kept constant at 18°C day and night, in all the treatments. The days to heading were recorded for each experimental unit. Vernalization response of each DH line was measured as the difference in heading date of unvernalized and vernalized plants for each photoperiod regime. All 98 DH lines were sown in field in head-rows replicated twice in the autumn of 1995. Their heading date characteristics were measured on the basis of the days to heading from January 1.

Genotypic Characterization

Molecular marker linkage map was created in the 98 DH lines of the cross between Dicktoo x Kompolti korai using sets of different marker types. The RFLP probes originated from the NABGM project, the STS primers developed from RFLP probes were kindly provided by Dr. Blake, Montana State Univ., Bozeman. The RAPD primers were produced by OPERON, while the SSR primer information was obtained from the Internet site of www.genetics.org (RAMSAY *et al.* 2000). The *HvSnf2* gene sequences of the two varieties were determined based on the results of YAN *et al.* (2002) and a 167 bp deletion/insertion difference was utilised in mapping this gene in the DH population. The linkage map was constructed using the MAPMAKER 3.0 software. The QTL analyses of phenotypic traits were carried out using the MAPMAKER/QTL software.

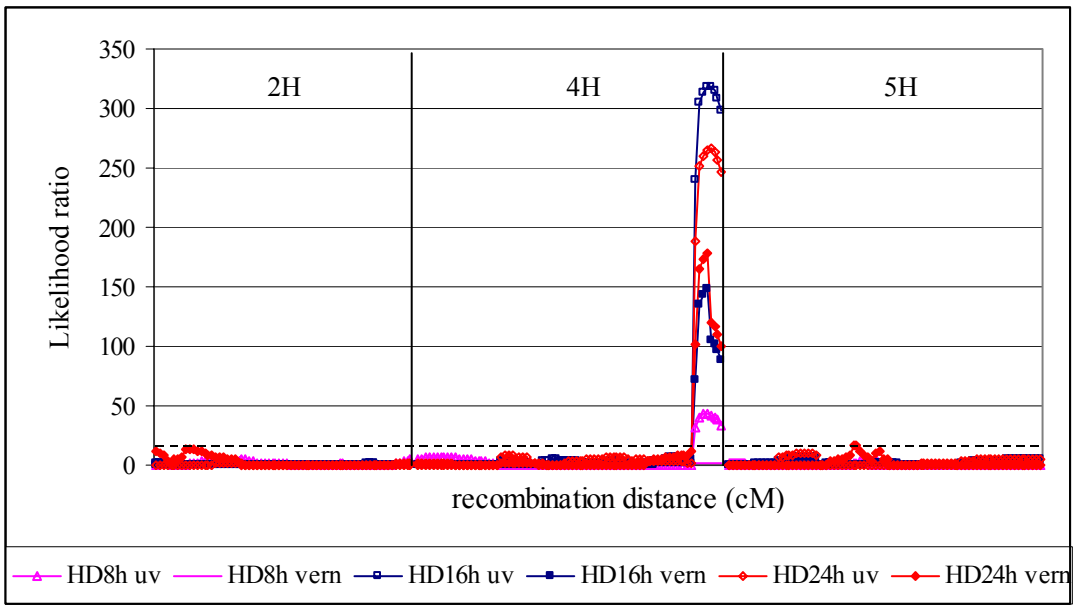
Results and Discussions

Under controlled environments there was a large difference in heading date in the DH population in all treatments. The range of heading was between 34-166 days for HD16uv, 33.5-94 days for HD16v, 24-134.5 for HD24uv and 22-90 days for HD24v. Under long photoperiod regimes the distribution was not normal, irrespective of vernalization treatment; there were two distinct groups around the respective parent separated from each other with more than 50 days under 16 h photoperiod regime in unvernalized treatments. This underlined the significance of one locus determining heading date in this population.

The marker linkage groups of the three chromosomes 2H, 4H and 5H in Dicktoo x Kompolti korai DH population, contain several markers, which were mapped in Steptoe x Morex and in Dicktoo x Morex barley populations or in both to similar locations. These three chromosomes are known to carry major genes of plant development (TAKAHASHI & YASUDA 1970; PAN *et al.* 1994; LAURIE *et al.* 1995). Chromosome 2H spans to 173.1 cM with three already identified markers around *Ppd-H1* locus on the short arms; *ABG358* and *ABG459* RFLP probes and *Bmac222a* SSR marker. The length of the linkage group on chromosome 4H is 155.4 cM. At the distal part of the chromosome the *HvM67* marker is linked proximal to the *Hdamyb* (*Bmy1*) marker with a recombination value of 16.8 cM, which is comparable with the published results (barley BIN map http://www.barley.genomics.wsu.edu/arnis/linkage_maps, RAMSAY *et al.* 2000). HACKETT *et al.* (1992) and LAURIE *et al.* (1995) established the position of *Vrn-H2* vernalization response locus, proximal to the *Bmy1* with a recombination distance of 4.3 and 2.6 cM, respectively. The

chromosome 5H linkage group spans to a distance of 113.6 cM with the *mR* morphological marker and *BCD265b* RFLP marker located on the long arm in close vicinity of the *Vrn-H1* locus (TAKAHASHI & YASUDA 1970; PAN *et al.* 1994).

Figure 1. QTL analysis of heading date measured in controlled environments in the full DH population of Dicktoo x Kompolti korai barley population. Dashed line represents the significance threshold



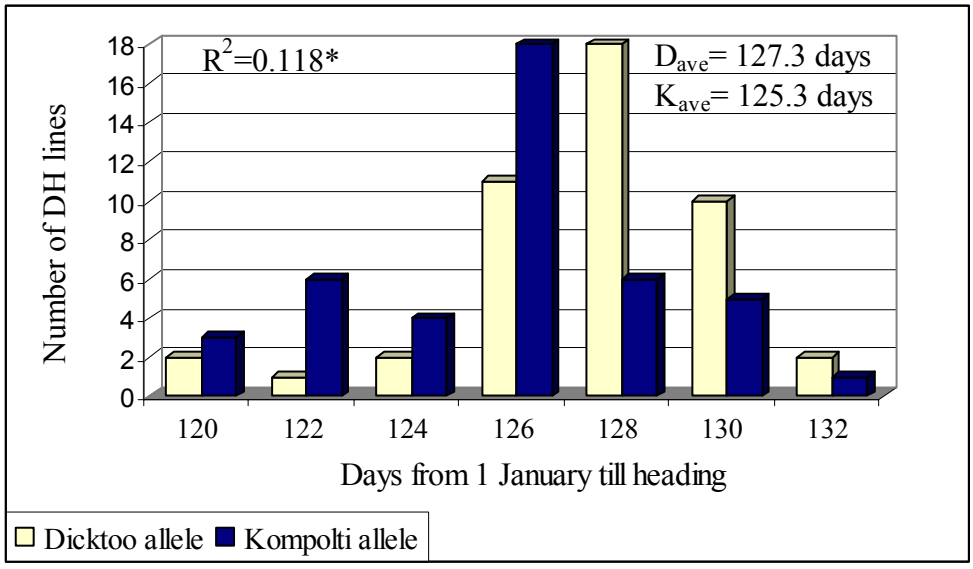
The QTL analysis showed large peaks of heading date under long photoperiod regimes at the most distal part of the chromosome 4HL, in the marker interval of *OPS31* and *Hdamyb* and no effects at the *Ppd-H1* locus on chromosome 2H and at the *Vrn-H1* locus on chromosome 5H (Figure 1). In unvernalized treatments the segregation of this region explained 67.5 and 66.5 % of the phenotypic variation in 16 and 24 hour photoperiod regimes, respectively. In vernalized treatments the effect of this region was smaller, but still highly significant, explaining 51.5 and 42.3 % of the phenotypic variation, respectively. In all the four cases it was the Kompolti korai allele, which delayed heading. Based on the comparative marker order, we hypothesised that the segregation in the *Vrn-H2* gene results in the huge QTL effects in this winter barley population (HACKETT *et al.* 1992; LAURIE *et al.* 1995). YAN *et al.* (2002) identified that the *Snf2* gene that has important role in transcriptional regulation is physically tightly linked to the *Vrn-A2* gene; there was no segregation found between *Snf2* and vernalization response in a population consisting of more than three thousand F_2 plants of *Triticum monocccum*. We found an INDEL between the *HvSnf2* sequences of Dicktoo and Kompolti korai. The Dicktoo sequence contained a 167 bp fragment, which was missing from Kompolti korai, making it possible to use as a co-dominant marker in the DH population. The *HvSnf2* was mapped on the distal part of the long arm of chromosome 4H between the marker interval of *OPS31* and *Hdamyb*, 8.0 cM proximal to *Hdamyb*. The *HvSnf2* marker type distribution explained more than 90 % of the phenotypic variance in heading date in unvernalized treatments at long photoperiod regimes and about 80 % of the variation in vernalized treatments (Table 1). Thus it was proven that the *Vrn-H2* gene is the major determinant of heading date in the Dicktoo x Kompolti korai winter barley population under long photoperiod regimes in controlled environment tests.

Table 1. Effect of *Vrn-H2* gene on heading date in controlled environment in the Dicktoo x Kompolti korai DH barley population as represented by the physically closely linked *HvSnf2* marker

Trait	***HvSnf2 marker***		
	R^2 value	***Average values of lines with Dicktoo allele***	***Average values of lines with Kompolti korai allele***
Heading at 8 h unvernalized (days)	0.318***	198.0	238.3
Heading at 8 h vernalized (days)	0.013^{ns}	167.2	176.5
Heading at 16 h unvernalized (days)	0.961***	40.4	140.2
Heading at 16 h vernalized (days)	0.798***	39.0	66.5
Heading at 24 h unvernalized (days)	0.942***	27.7	111.0
Heading at 24 h vernalized (days)	0.837***	29.0	55.7

YAN *et al.* (2004) have recently identified *ZCCT1*, the candidate gene of *Vrn-A2* in *Triticum monococcum* with positional cloning. The gene contains a putative zinc finger motif and a CCT domain, and is in close relationship with the *CO*, and *CO*-like genes of Arabidopsis, which have a major regulatory role in the photoperiod pathways. However the proteins from grasses formed a separate group within the *CO*-like protein family and their activity was regulated by vernalization and not photoperiod (YAN *et al.* 2004). This gene is a dominant repressor of flowering, down regulated by vernalization. This down-regulation of *ZCCT1* during vernalization was found to be concomitant with an increase in wheat *Vrn-A1* transcription (TRANQUILLI & DUBCOVSKY 2000; YAN *et al.* 2003). In spring growth type genotypes the *Vrn-A2* gene is not functional due to a point mutation in the CCT domain or the complete lack of the gene. While these two possibilities occur approximately at a similar rate in *T. monococcum*, in barley the lack of the gene was characteristic to all of the spring types studied by YAN *et al.* (2004).

Figure 2. Distribution of heading date of DH lines with Dicktoo or Kompolti korai marker allele at the *HvSnf2* marker locus, respectively in field-sown experiment



Under field-sown condition the effect of this region was much smaller but still significant, it explained 11.8 % of the phenotypic variation in heading date. There was a shift in the higher value adding allele. While in the controlled environment tests the Kompolti korai allele always delayed

heading, under field conditions it resulted in significantly earlier heading (Figure 2). It is interesting that the effect of *Vrn-H2* is almost impossible to detect under field-grown conditions, as under controlled environments its effect was highly significant even in vernalized treatments. The reason for this result together with the shift in the higher adding allele may be that not only the cold temperature regulate the activity of the *Vrn-H2* gene but the complex effects of changes in photoperiod and/or in temperature during the early growing season. It is also possible that this chromosome region contain other regulating genes in addition to *HvSnf2* and *Vrn-H2*.

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Molecular Analysis of T-DNA Integration into the Barley Genome

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Abstract

Several studies have shown that barley can be transformed routinely with *Agrobacterium*. However, the technique still has a number of shortcomings. The transformation frequencies are moderate, often more than one T-DNA copy is inserted and vector backbone is integrated into the barley genome on a regular basis.

In our experiments barley immature embryos were transformed with pVec8-GFP, using hygromycin as selectable marker and the gene for green fluorescent protein (GFP) as reporter. The transformation frequency ranged from 2-22%. It was apparent that the growth conditions of the donor plants were major determinants for the transformation frequencies.

Integration of T-DNA and non-T-DNA parts into the barley genome was investigated. Southern blot analysis of GFP expressing lines showed that 66% had single copy integrations while two to five copies were found in the remaining 34% of the lines. We also found examples of that one embryo could give rise to several transformants with different integration patterns, indicating multiple transformation events in a single embryo.

PCR screening for the presence of the spectinomycin gene, localised on the vector backbone was performed on 191 hygromycin-resistent independent transgenic plants. Twenty-six percent of the lines were positive in this screen, but preliminary Southern blot analyses indicate a lower frequency.

Introduction

Over the last half century several attempts have been made to improve the amino acid composition of the cereal grain using spontaneous variations and mutation breeding. However, these mutations in general have pleitropic effects on a number of grain characteristics and have a negative effect on yield (SHEWRY *et al.* 1994). There is accordingly a great demand for further investigations into these basic mechanisms in order to devise suitable strategies for manipulating amino acid composition in the cereal grain. Our strategy was to alter storage protein synthesis using gene-silencing technology.

The most widely used method to introduce genes into barley has until recently been biolistic transformation. In the initial study by WAN and LEMAUX (1994) a 7.9% frequency of transformation (percentage of immature embryos giving rise to a transgenic lines) was reported and multicopy integration pattern was observed (WAN & LEMAUX. 1994). Several factors such as quality of the plant material, the bombardment procedure (gold coating, inefficient shooting) or the tissue culture regime can influence the frequency obtained. In 1997 the first successful *Agrobacterium* mediated barley transformation was reported (TINGAY *et al.* 1997). *Agrobacterium* mediated barley transformation promises many advantages compared to the alternative gene transfer methods. For example the number of transformation events resulting in a single insert is significantly higher with *Agrobacterium* mediated transformation compared to the biolistic approach, when using a similar construct, the same cultivar, regeneration and selection protocol (CHENG *et al.* 1997). To establish and to optimise the *Agrobacterium* mediated transformation system in our laboratory a procedure involving pVec8-GFP vector (MURRAY *et al.* 2004) carrying a constitutive expressed *GFP* gene was implemented.

Material and Methods

Plant Material and Growth Conditions

Barley plants (*Hordeum vulgare cv. Golden Promise*) were grown either in soil or pots in the green house or in growth cabinets at 12°C or 15°C. In soil they were grown under a cycle of 16 h illumination and 8 h darkness at 23°C and 18°C respectively or they were grown in 50:50 peat perlite mix in pots at 12°C, 18/6 day/night regime. In growth cabinets they were grown in pure Sm-soil with 20 g of Osmocote super added at sowing and an additional 10 g added after 7 weeks. The day/night regime was 16/8 hours and the temperature regimes 15°C and 10°C, respectively. For transformation experiments were used immature zygotic embryos approximately 14 DAF with an embryo length of 1-2 mm.

Barley Transformation

Agrobacterium mediated transformation of barley was carried out as described by TINGAY *et al.* (1997) modified as described in MATTHEWS *et al.* (2001) using the hygromycin resistance gene as selectable marker.

Reporter Gene Construct

The pVec8-GFP binary vector (MURRAY *et al.* 2004) was used. pVec8-GFP is carrying the sGFP(S65T) gene (CHIU *et al.* 1996) between the maize *Ubi1* promoter (CHRISTENSEN *et al.* 1992) and *A. tumefasciens* nopaline synthase (nos) terminator (BEVAN *et al.* 1983). The hygromycin gene was driven by the cauliflower mosaic virus (CAMV) 35S promoter.

Microscopy Analysis for GFP Expression

The GFP expression in barley embryos, shoot and roots was visualised under a stereomicroscope (WILD M3Z, Heerbrugg, CH) with fluorescence GFP Plus filter module (Leica Microsystems), which contains a 480/40 nm excitation filter, a 505 nm LP dichromatic beam-splitting mirror and a 510 nm LP barrier filter. The embryos were analysed three days after infection, the calli were analysed three weeks after infection and the shoots and roots six weeks after infection.

Genomic DNA Gel Blot Analysis

Genomic DNA extraction was essentially carried out as described by GUIDET *et al.* (1991). DNA (5-8 µg/lane) was digested with the restriction endonuclease *EcoRV* and electrophoresed through 0.7% agarose gels and blotted onto Hybond N^+ membrane (Amersham Pharmacia Biotech Inc) according to the manufactures instructions. The radioactive probe was made by random priming of a 3kb T-DNA fragment of the pVec8-GFP containing the maize ubiquitin promoter and the GFP gene using the Ready-To-Go™ DNA labeling beads according to the manufactures instruction (Amersham Biosciences).

Filters were prehybridised 4h and hybridised for 16h at 65°C in hybridisation solution (0.25M sodium phosphate, 7% SDS, pH 7.2). They were then washed 2 times 1h at 65°C in 1x SSPE and 1% SDS (SAMBROOK *et al.* 1989).

PCR Analysis

The barley plants transformed by *Agrobacterium* were screened for the presence of the GFP gene, hygromycin gene and the spectinomycin gene.

A 671 bp fragment from the GFP gene was amplified by PCR with the following primer set: LDS 40 (5'- AGT TCA TCT GCA CCA CCG GC-3') and LDS29 (5'- TAA TCA TCG CAA GAC CGG CAA-3'). For the spectinomycin gene a 485 bp fragment was amplified by PCR by SPEC F2 (5'- GTT GTG CAC GAC GAC ATC-3') and SPEC R2 (5'- CCT TGG TGA

TCT CGC CTT TC- 3'). For the hygromycin gene a 917 bp fragment (MATTHEWS *et al.* 2001) was amplified by PCR by HYG F1 (5'- ACT CAC CGC GAC GTC TGT C-3') and HYG R1 (5'- GCG CGT CTG CTG CTC CAT-3').

Results and Discussion

Transient and Stable GFP Expression in Barley

Three days after infection the embryos were analysed for transient expression under the fluorescence microscope (Fig. 1 A).

Transient expression was seen in 90% to 98% of the embryos. After three weeks of callus induction the calli were analysed for GFP expression indicating stable integration (Fig. 1 B). After 8 weeks GFP expressing roots and shoots could be identified (Fig. 1 C, D).

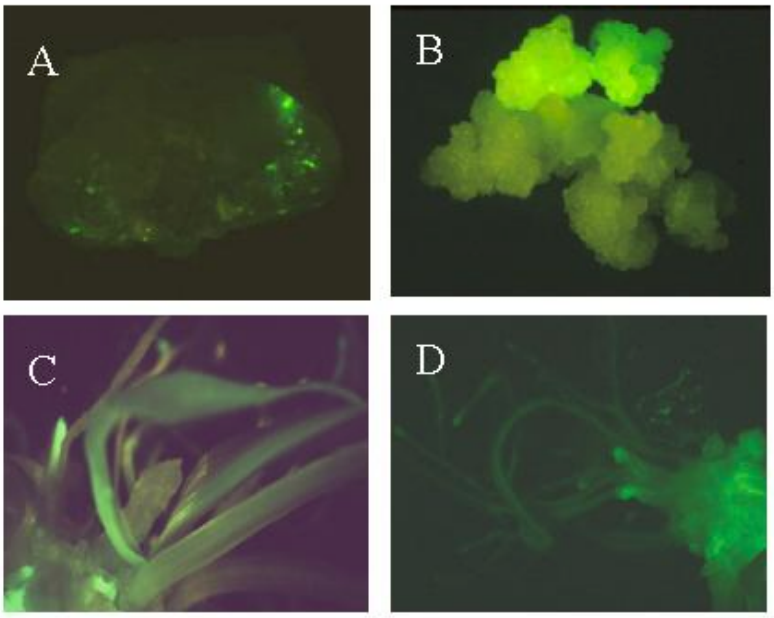

Figure 1. GFP expression in barley tissues. A: An embryo showing transient GFP expression three days after infection B: GFP expressing calli, three weeks after infection C: GFP expressing shoots D: GFP expressing roots

The Quality of the Plant Material Is Influencing the Transformation Frequency

Nearly 1000 immature embryos were isolated and inoculated with *Agrobacterium* strain AGL0, carrying the pWBVecGFP vector. The regenerated plantlets were analysed for the presence of the hygromycin gene by PCR. Results from six experiments can be seen in table 1. The transformation frequency differed within experiments with an average of 10.8%.

Transformation experiments carried out with material from the growth cabinets gave the highest frequency (25%) while the material grown in pots in the greenhouse at 22°C gave the lowest transformation frequency (3.7%).

Table 1. The results of the experiments with different donor materials. Four different materials were used: Material from growth cabinet 12°C/15°C, material grown in soil or in pots in greenhouse at 22°C.

Experiment	Growth cabinet (12/15°C)	Soil	Pots (22°C)	Pots (12°C)	Total
A	52 (27%)	5 (6.8%)	-	-	57 (22.2%)
B	2 (7.7%)	6 (7.8%)	1 (2%)	1 (1.8%)	10 (4.7%)
C	-	9 (18%)	2 (6.3%)	3 (8.6%)	14 (12%)
D	-	1 (4%)	-	0	1 (2%)
E	-	-	-	6 (12%)	6 (12%)
F	-	9 (9.4%)	-	8 (8%)	17 (7.3%)
Total	54 (25%)	30 (9.3%)	3 (3.7%)	18 (7.5%)	105 (10.8%)

Integration of Non-T-DNA and Multiple Copies of T-DNA

The putative transgenic plants (in total 191 plants) were tested by PCR to check for integration of the three genes: GFP, hygromycin (both localized on the T-DNA), and spectinomycin (localized on the vector backbone) individually and in combination. Fig. 2A shows a typical result of the PCR reactions. In the PCR analysis plant number 1, 3, 4, 5, 6 has integrated the GFP and hygromycin gene and plant one and five showed integration of the spectinomycin resistance gene. The majority of the plants (63.8%) contained the hygromycin and the GFP genes. In some cases (9.5%) the *Agrobacteria* transferred only the hygromycin gene into the plant genome. In other cases (26.6%) the transformation resulted in integration of the spectinomycin gene, although the spectinomycin resistance gene is outside the T-DNA on the backbone of pWBVec8GFP.

To further confirm the integration pattern of T-DNA, we performed a Southern blot analysis of lines shown by PCR to possess the GFP gene. Of the 36 plants analysed, 66% had single copy integrations while two to five copies were found in the remaining 34% of the lines. There is a single *EcoRV* site downstream of the GFP gene in the plasmid pVec8-GFP.

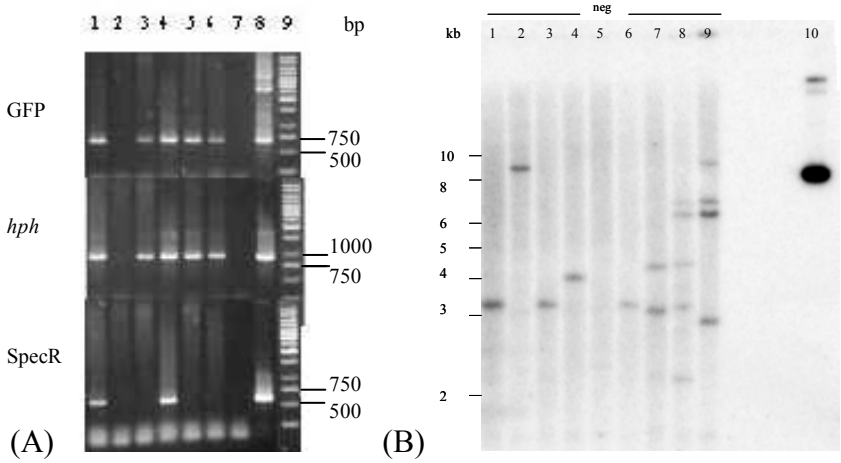

Figure 2. (A). The figure shows three different PCR experiments. GFP: PCR with primers within the GFP gene, hph: PCR with primers within the hygromycin resistance gene, Spec R: PCR with primers within the Spectinomycin gene on the non-T-DNA. Lane 1-6: DNA extractions from putative transgenic plants. Lane 8: negative control, lane 8: pVec8GFP plasmid, lane 9: 1kb DNA marker.

(B). Southern blot of DNA extracted from transformed barley plants. DNA was digested *EcoRV* and then hybridised with a radioactive probe of the T-DNA. All hybridising bands should be at least 2.6kb (representing 2.0kb of the maize Ubi1 promoter and 0.6kb of the GFP gene). Lane 1-4, 6-9: transformed plants, lane 5: negative control, lane 10: pVec8GFP.

One Single Embryo Could Give Rise to Different Transgenic Lines

In total, 86 transformed embryos generated lines of transgenic plants and 47 embryos gave rise to more than one transgenic plant. By PCR we analysed the integration pattern of the GFP, the hygromycin and the spectinomycin genes in transgenic lines originating from the same embryo. We observed three possible integration patterns. Six plants showed integration of only the hygromycin gene, other six plants showed integration of the hygromycin and the GFP gene and one plant showed integration of all three genes, hygromycin, spectinomycin and GFP (data not shown). These results could indicate that the plants had developed from three independent transformation events.

In our experiments a total of 978 embryos were infected and 68 gave rise to transgenic plants. However, some plants originating from the same embryo showed different integration patterns and were therefore considered to be independent transformation events, increasing the numbers of the transgenic lines to 105. A similar observation was reported by TINGAY *et al.* (1997) and TRIFONOVA *et al.* (2001). In our individual experiments the average transformation frequency was 10.8% ranging from 1.8% to 27%. Although a transformation frequency as high as 27% has not previously been reported, our average transformation efficiency (10.8%) correlates with the results reported in the literature: TINGAY *et al.* (1997) reported a transformation frequency of 4.2%, TRIFONOVA *et al.* (2001) frequencies from 1.7% to 6.3% and MATTHEWS *et al.* (2001) frequencies from 2-12%. The marked differences in transformation frequency observed among our six experiments could be caused by several factors such as the quality of the plant material or the strength of the *Agrobacteria* inoculum. The results strongly indicate that the quality of the plant material, as evidenced by optimal growth in a growth cabinet, is a major determinant of the transformation frequency.

In this study we found that the spectinomycin gene was present in 26% of the regenerated barley transformants, although the spectinomycin gene was encoded by the backbone of the pVec8-GFP vector. From the PCR analysis that have been carried out so far we could not conclude if these transgenic plants contained the entire vector or part of the backbone only.

The frequency of non-T- DNA transfer in barley (26%) found in this study is considerably lower than that reported for rice (33.2%, Yin and Wang, 1999), tobacco (75%, KONONOV *et al*. 1997) and Arabidopsis (62%) (WENCK *et al.* 1997). However, the plants in this report were only screened for the presence of the spectinomycin resistance gene and preliminary Southern blot analysis indicate a lower frequency. Further studies of the backbone must be performed in order to elucidate whether the obtained 26% is a representative frequency of non-T-DNA integration. It is a variance though with the recent report by FANG *et al.* (2002) that *Agrobacterium* mediated transformation of barley results in insertion of defined T-DNA's with exclusion of vector DNA. We have no explanation for this discrepancy but are currently analysing in more detail the vector backbone integration in more detail in our materials.

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Use of SSR Marker Data to Study Linkage Disequilibrium and Population Structure in *Hordeum vulgare*: Prospects for Association Mapping in Barley

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Abstract

Random samples of lines developed for genetic mapping are in extreme linkage disequilibrium and have no population structure. In contrast, non-random sets of existing cultivars, breeding lines or accessions have unknown linkage disequilibrium and may have complex structure. We examined simple-sequence repeat (SSR) marker information for several such germplasm sets. Disequilibrium was high among cultivars and lines of cultivated barley (*H. vulgare* ssp. *vulgare*) and low among accessions of *H. vulgare* ssp. *spontaneum*. Within diverse sets of barley germplasm, there was frequent disequilibrium among non-linked loci, suggesting that association mapping without consideration of population structure would have a high rate of Type-I error. Among subsets representing known germplasm groups, disequilibrium between non-linked loci was greatly reduced while disequilibrium among closely linked loci was largely maintained. Similar effects were obtained using subsets identified by model-based analysis of population structure. With sufficient marker density and control of population structure, patterns of disequilibrium among loci in barley may be appropriate for association mapping of trait loci.

Introduction

In barley (*Hordeum vulgare* L.), genetic mapping often involves the development, genotyping and phenotyping of a random sample of doubled haploid lines derived from the F_1 generation of a cross between two highly homozygous lines. This type of mapping population shows extreme disequilibrium between linked loci (with only one opportunity for recombination during population development). It should not have any population structure (due to the random sampling) and there should be no disequilibrium between non-linked loci. This combination of features favours the detection and approximate mapping of quantitative trait loci (QTLs).

In contrast, in human genetics, genetic mapping relies upon analysis ('association mapping') of genotypic and phenotypic data sampled in a non-random fashion from existing populations of complex but often unknown structure. In such populations, linkage disequilibrium may decline within relatively short distances in the genome, making fine-mapping possible, provided that adequate controls for population structure are included in the experimental design and/or the statistical analysis.

Plant geneticists and breeders are now showing interest in using sets of germplasm (potentially including cultivars, breeding lines, ancestral materials, and/or germplasm

as association mapping populations, with sequencing within candidate genes and/or genotyping of random markers across the genome (e.g., REMINGTON *et al.* 2001; THORNSBERRY *et al.* 2001; IVANDIC *et al.* 2003). These approaches could provide opportunities for fine mapping and for discovery of new alleles in diverse sources. Furthermore, they could allow mapping to be carried out using materials of direct interest to breeders without requiring the development and phenotyping of dedicated mapping populations.

Association mapping on a whole-genome basis requires either an extremely high density of markers (as currently being developed for the human genome) or the maintenance of linkage disequilibrium over quite long distances in the genome. Using current technologies, it would be prohibitively expensive to develop and use extremely large numbers of markers in crop plants. However, at least for self-pollinated crop plants such as barley, it seems worth investigating whether linkage disequilibrium is maintained over adequate distances to permit genome-wide association mapping with an affordable density of markers.

In this study, we used genotypic data on simple-sequence repeat (SSR) markers that had been assayed in several germplasm sets of barley to investigate population structure and linkage disequilibrium. We assessed whether linkage disequilibrium was present among marker loci, whether it was more prevalent for pairs of linked loci than for pairs of non-linked loci, and whether disequilibrium between non-linked markers could be reduced by accounting for population structure.

Material and Methods

Germplasm Sets and SSR Markers

Four germplasm sets were used:

1. A set of 32 cultivars and breeding lines of barley (*H. vulgare* ssp. *vulgare*) (including spring-habit, winter-habit and facultative types) and 22 accessions of wild barley (*H. vulgare* ssp. *spontaneum*). This set has previously been described by MATUS & HAYES (2002), who reported the results of a diversity analysis.
2. A set of 9 two-rowed and 87 six-rowed cultivars and breeding lines of spring-habit barley. This set has previously been described by MATUS & HAYES (2002). Many of the accessions in this set are elite lines from the Busch Agricultural Resources Inc. (BARI) malting barley breeding program.
3. A set of 95 two-rowed cultivars and breeding lines of barley including Australian cultivars, elite lines and parental materials of interest to Australian barley breeding programs.
4. A set of 113 two-rowed and 71 six-rowed cultivars and breeding lines of barley, including both spring-habit and winter-habit types. Many of the two-rowed accessions in this set are elite lines from the BARI malting barley breeding program.

For each of these germplasm sets, mapped SSR markers were assayed with fluorescent primers. Map positions for the marker loci were estimated based on previously constructed maps, principally the one published by RAMSAY *et al*. (2000). Markers for which genotypes had not been scored for 15% or more of the accessions within a set were excluded from the analysis for that set. This left 54 marker loci for set 1 (between 4 and 11 per chromosome), 42 marker loci for set 2 (between 4 and 9 per chromosome), 76 marker loci for set 3 (between 7

and 17 per chromosome) and 46 markers for set 4 (between 5 and 7 per chromosome) Analyses of linkage disequilibrium used data for all of these markers. For analyses of population structure, further markers were removed to obtain at spacing of at least 1 cM between adjacent markers. No attempt was made to combine the four datasets, as the genotyping had been done at different times in different laboratories, using different equipment.

Analysis of Population Structure

Markov-chain Monte Carlo analysis of population structure was conducted, using the linkage model of the software Structure 2.0 (pritch.bsd.uchicago.edu) for values of K (assumed number of ancestral populations) from 1 to 5. Four replicates were conducted at each value of K, with each replicate involving a 'burnin' of at least 10,000 runs followed by data collection on at least 100,000 runs. Results were examined to identify subsets of lines that consistently clustered together, without substantial admixture (i.e., each member of subset n having a Q_n value of at least 0.8.).

Analysis of Linkage Disequilibrium

The software TASSEL (www.maizegenetics.net) was used to assess linkage disequilibrium for pairwise combinations of markers for each germplasm set and for subsets of accessions defined according to their known biological characteristics (two-rowed *vs*. six-rowed barley; *vulgare vs. spontaneum*) or according to the results of the analyses of population structure. For marker pairs with two alleles at each locus, significance of linkage disequilibrium was tested with Fisher's exact test. For marker pairs with more than two alleles at one or both loci, significance of linkage disequilibrium was tested using 1000 permutations. The results of the TASSEL analyses were then manipulated with SAS® (www.sas.com) to calculate frequencies of significant ($P \leq 0.01$) linkage disequilibrium for closely linked markers (no more than 5 cM apart), for linked markers (over 5 cM apart) and for non-linked markers (on different chromosomes).

Results and Discussion

Population Structure

For germplasm sets 1, 2 and 3, a burnin of 10,000 runs followed by data collection on 100,000 runs appeared to be sufficient, giving quite consistent values of *ln prob data (X|K)* across replicates. The same was true for set 4, but only for K values of 1, 2 and 3. At K=4, the analysis program repeatedly failed to report any results for set 4, and we were unable to determine the reason for this. At K=5, the results for set 4 were inconsistent, with the *ln prob data (X|K)* value varying considerably among replicates. The analysis for set 4 was repeated using a burnin of 30,000 runs followed by data collection on 300,000 runs. More consistent results were obtained at K=5, but the K=4 analysis continued to fail. For this dataset, a longer burnin and/or more data collection runs may be needed to obtain consistent results.

For all four germplasm sets, the value of *ln prob data (X|K)* changed gradually as K was increased from 1 to 5, giving no clear indication of a 'true' number of ancestral populations. This is not surprising, considering that the cultivated accessions within these sets cannot reasonably be said to be derived from several distinct ancestral populations. Rather, such

accessions would have had a complex breeding history involving intercrossing and introgression between germplasm groups, overlaid with strong selection pressure for agronomic and quality characteristics.

For germplasm sets 1 and 2, analysis at *K*=2 yielded biologically meaningful subsets of accessions, corresponding closely, but not exactly, to their classifications into subspecies (set 1) or spike morphology (set 2).

Germplasm set 3 consists entirely of two-rowed lines with good adaptation to Australian growing conditions. Analysis at *K*=2 yielded subsets of 21 lines (subset 3a) and 33 lines (subset 3b). The remaining 41 lines showed too much admixture to be classified into either of these subsets. Most members of subset 3a were developed by from breeding programs in South Australia, Victoria or New South Wales. Most members of subset 3b were developed by breeding programs in Queensland or Western Australia.

For germplasm set 4, analysis at *K*=2 yielded a subset (4a) consisting of 87 two-rowed spring lines and a subset (4b) of 98 lines that included two-rowed, six-rowed, spring and winter materials,. One line (a selection from a two-rowed by six-rowed cross) was associated with subset 4a in some replicates and with subset 4b in others. With analysis at *K*=3, subset 4a remained intact but subset 4b split into a subset (4b-1) of 34 six-rowed spring lines (subset 4b-1) and a diverse subset (4b-2) of 50 lines (two-rowed and six-rowed; winter and spring). At *K*=5, the clustering of lines was not consistent across replicates.

Linkage Disequilibrium

In each of germplasm sets 1, 2 and 3, the proportion of marker pairs exhibiting significant linkage disequilibrium was highest for the closely linked markers, intermediate for the linked markers more than 5 cM apart and lowest for markers on different chromosomes (Table 1). Among these three germplasm sets, set 2 (the two-rowed and six-rowed barley lines) exhibited the most disequilibrium and set 3 (the Australian barley lines) exhibited the least disequilibrium. Germplasm set 4, for which there were no closely linked marker pairs assayed, had high proportions of disequilibrium for both linked and non-linked markers (Table 2).

When the 22 *spontaneum* accessions were excluded from the analysis for germplasm set 1 and when the 9 two-rowed accessions were excluded from the analysis for germplasm set 2, most of the associations between non-linked markers were eliminated (Table 1). Thus, for each of these two germplasm sets, the two biologically obvious subsets seem to be the main sources of population structure. For germplasm set 3, there was no obvious basis on which to classify the lines. In germplasm set 4, separation into two-rowed and six-rowed subsets reduced the proportion of non-linked loci exhibiting linkage disequilibrium, but only to 0.55 (Table 2). This set may have a more complex structure that can not be explained simply on the basis of subgroups with contrasting spike morphology.

For germplasm sets 1 and 2, the subsets obtained by analysis of population structure were almost identical to those defined by subspecies or spike morphology, so the linkage disequilibrium results (not shown) were also very close to those reported for the biologically-defined subsets.

Table 1. Proportions of marker pairs for which linkage disequilibrium was significant ($P \leq 0.01$) for germplasm sets 1, 2 and 3 and for subsets of those germplasm sets

		Proportion of marker pairs with significant disequilibrium		
		Markers on the same chromosome		Markers on different chromosomes
	N[1]	≤ 5 cM apart	> 5 cM apart	
Germplasm set 1				
Entire set	54	0.43	0.26	0.19
vulgare subset	32	0.40	0.12	0.07
spontaneum subset	22	0.07	0.05	0.03
Germplasm set 2				
Entire set	96	0.89	0.64	0.66
Six-rowed subset	87	0.58	0.13	0.06
Germplasm set 3				
Entire set	95	0.31	0.25	0.09
Subset 3a	21	0.27	0.08	0.02
Subset 3b	33	0.24	0.11	0.05

[1] Number of accessions within the germplasm set or subset.

Table 2. Proportions of marker pairs for which linkage disequilibrium was significant ($P \leq 0.01$) for germplasm set 4 and for six subsets of germplasm set 4

		Proportion of marker pairs with significant disequilibrium	
	N[1]	Markers on the same chromosome	Markers on different chromosomes
Entire set	186	0.67	0.97
Two-rowed subset	113	0.64	0.55
Six-rowed subset	73	0.59	0.55
Subset 4a	87	0.18	0.09
Subset 4b	98	065	0.64
Subset 4b-1	34	0.18	0.06
Subset 4b-2	50	0.44	0.39

[1] Number of accessions within the germplasm set or subset.

For germplasm set 3, separate analyses of the two moderate-sized subsets obtained by population structure analysis reduced the frequency of significant associations between markers that are on the same chromosome but not closely linked, and reduced the already low frequency of associations between non-linked loci (Table 1).

For germplasm set 4, the use of subsets obtained by population structure analysis had marked effects on the linkage disequilibrium results. The proportion of significant associations between non-linked loci dropped from 0.55 for all 113 two-rowed lines to 0.09 for subset 4a , which consisted of 34 of these lines. Similarly, this proportion dropped from 0.55 for all 73 six-rowed lines to 0.06 for subset 4b-1, which consisted of 34 of these lines. However, this proportion remained quite high (0.39) for subset 4b-2 (Table 2), indicating that this subset may still have quite complex population structure. There were no closely linked pairs of markers assayed in germplasm set 4, so it is not possible to see whether the linkage disequilibrium between closely linked markers was retained as population structure was accounted for.

Implications for Association Mapping

In the sets of cultivated barley examined here, linkage disequilibrium is present on a scale that could be useful for association mapping. Genome-scale association mapping should be possible, provided adequate methods are implemented to control for the effects of population structure, Without controls for population structure, spurious associations between non-linked loci could lead to high rates of Type-I error (false positives). Model-based analyses of population structure, such as those conducted here, may be helpful for characterizing patterns of population structure, providing information that could be incorporated into association mapping analyses.

Acknowledgements
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Genetic Study of Adaptational Traits in a Set of Winter Barley Varieties (Hordeum vulgare L.) Using Molecular Markers

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Abstract
For studying adaptation traits 38 barley varieties were involved in a series of experiments, which combined different photoperiod and vernalization treatment, and in freezing tests. The varieties were parallel genotyped using 31 RAPD, 33 STS and 21 SSR markers. To examine the association between the markers and the phenotypic traits of the varieties marker regression was applied. The marker difference matrix and the phenotypic data matrices were obtained by calculating the real difference in phenotypic and genotypic value for each pair-wise comparison. Fifteen marker loci showed significant correlation with 11 adaptational traits, some of them being in similar positions with previously described loci affecting adaptation. The *Vrn-H2* vernalization response locus on chromosome 4HL influenced almost all of the adaptation traits. We determined the marker allele compositions of a set of barley varieties for HvM67, OPS31, and Hdamyb, which were closely linked to the *Vrn-H2* locus. The 38 varieties were placed in six groups. The winter barley varieties carrying OPS31 fragments (430bp), which was typical for the two-rowed spring barley, has a special adaptation type.
Keywords: barley varieties; adaptational traits; molecular markers; association analysis

Introduction

Adaptation of cultivated barley is determined by earliness *per se* (*eps*), photoperiod sensitivity vernalization response and frost tolerance through influencing winter hardiness and heading date. The development of varieties with photoperiod sensitivity are slowing down as the day length decreases in autumn, thence their winter hardiness are increasing. In springtime these varieties do not begin to grow until the daylenght pass a critical duration, thus avoiding early spring frost damage. The increasing of day length accelerates the development and heading of the photoperiod sensitive types, and helps to accumulate the maximum biomass before dry summer period (LAURIE *et al.* 1995). The vernalization requirement is one of the characters determining cold tolerance. The vernalization treatment accelerates frost tolerance of varieties, which have vernalization requirement. On the other hand the vernalization treatment allows the transmission from the vegetative phase to the reproductive phase. In the case of winter wheat it is essential for the flowering, but winter barley varieties can head without vernalization, though their development and heading date become delayed (KLAIMI *et al.*1973). These traits are determined by many genes inherited independently, and also influenced by environmental factors (TAKAHASHI & YASUDA 1970; HAYES *et al.* 1993). It makes difficult the selection for favorable alleles.

In cereals, adaptational traits have been studied extensively and several major loci have been identified. These experiments were carried out mostly on doubled haploid population or other segregating population. In order to verify the general roles of these loci we carried out a series of controlled environment tests combining vernalization and photoperiod treatments in a set of winter barley varieties of different origin, and we examined the connection between molecular markers and adaptational traits.

Material and Methods

Thirty-eight barley varieties of different origin were included in the experiment. All the varieties were phenotyped in a series of controlled environment tests involving combinations of vernalization and photoperiod. The photoperiod treatments consisted of 10, 14 and 18 h light regimes per 24 h period and were carried out in the phytotron facilities of the Agricultural Research Institute (Martonvásár, Hungary) as described by KARSAI *et al*. (1997). The days to heading were recorded for each experimental unit. The photoperiod sensitivity of the varieties was estimated by the difference in heading date under 10 hv and 18 hv treatments, while the vernalization response was estimated from the difference in heading date under 18 huv and 18 hv treatments. The frost tolerance of the varieties was evaluated in artificial freezing tests based on the methodology of TISCHNER *et al.* (1997), applying two freezing temperatures of -10 and -13°C. After freezing the plant survival percentages were determined. Based on the phenotypic data the adaptation type of the varieties was determined by discriminant analysis (KARSAI *et al.* 2001).

The 38 barley varieties were characterised with 93 molecular markers, which include 31 RAPD, 33 STS and 21 SSR markers. There are average13 markers on each chromosome. The chromosomal position of RAPD and STS markers were determined in mapping them in Dictoo x Kompolti korai and/or in Dictoo x Morex population. The chromosomal localization of SSR markers has been published by RAMSAY *et al*. (2000). To examine the association between the markers and the phenotypic traits marker regression was used. The marker difference matrix and the phenotypic data matrices were obtained by calculating the real difference in phenotypic value for each pair-wise comparison. Correlation coefficients were obtained on the phenotypic and the genotypic matrices.

On the 4HL chromosome the 38 cultivated barley varieties were characterized with the three markers (HvM67, OPS31, Hdamyb), which showed close linkage with the *Vrn-H2* (*Sh*) locus on chromosome 4H. To establish the three-locus haplotype of these varieties cluster analysis was carried out on the three-marker matrix of the varieties using the average linkage method of the SPSS for Windows program package after Z-score standardization. The distances are rescaled in a scale of 0-25 units by the SPSS program, where 0 unit represents the smallest distance and 25 unit represents the largest distance in the range of genotypes examined.

Results and Discussion

Fifteen markers of the 93 molecular markers showed significant correlation with some of the 11 adaptational traits (Table 1). Heading date under different photoperiod regimes correlated with 10 markers, which were located on all chromosomes except to 6H. The highest correlation was between heading date and *HdAMYB1* marker on chromosome 4HL, which explained 22% of variance in unvernalized treatment. Some of these markers are located in chromosomal regions where *eps* loci have been described (LAURIE *et al.* 1995). The *eps* loci influence heading date independent from vernalization and daylength.

Photoperiod sensitivity correlated with four loci on chromosome 4H, Bmag353 on the short arm, and the three markers linked to the *Vrn-H2* locus, and two additional markers on chromosome 7HS. HvM4 and Bmag369 are placed above and under *eps7*. The winter allele in *eps7* resulted in late flowering under short photoperiod (LAURIE *et al.* 1995).

Vernalization requirement associated with six markers on chromosomes 2H, 3H, 4H (HvM67, OPS31, HdAMYB1) and 7H. These markers are located near to eps2S, eps3L, Vrn-H2, and eps7L respectively. The vernalization treatment includes also a short daylength treatment, which may influence the development of the varieties. There are some varieties where the cold treatment is replaceable with short day treatment (ROBERTS *et al.* 1988). LAURIE *et al.* (1995) found that in the field condition there was no correlation between photoperiod sensitivity and vernalization requirement, but it was between vernalization requirement and *eps* loci.

Table 1. Marker loci associated with adaptational traits

	1H	*2H*	*3H*	*4H*	*5H*	*6H*	*7H*
Heading date	OPH82	WG5472	OPK111	HvM67,OPS31, HvAMYB1	OPL31,GMS001		OPL12, HvID
Photoperiod sensitivity				BMAG 353, HvM67,OPS31, HvAMYB1			HvM4 BMAG369
Vernalization requirement		OPK112	HvM70	HvM67,OPS31, HvAMYB1			HvID
Frost tolerance			HvM70	HvM67,OPS31, HvAMYB1			HvID

Frost tolerance associated with five markers. The strongest correlation was between frost tolerance and Hvm70; it explained 32 % of the phenotypic variance. Three additional markers showed significant correlation with frost tolerance on chromosome 4HL linked with *Vrn-H2*, and HvID on 7HL. All these markers also have effect on vernalization requirement. In this group of barley varieties correlation between vernalization requirement and frost tolerance at – 10˚C was found to be significant (r^2=0.62) (KARSAI *et al*. 2001).

All of the traits affecting adaptation have significant correlation with markers HvM67, OPS31 and HdAMYB1 on chromosome 4HL linked to the Vrn-H2 locus. We determined the marker allele compositions of this set of barley varieties for these markers and the 38 varieties were placed in six groups (Figure 1). The common character of Group 1, 2, and 6 was the lack of the OPS31 band. In addition, Group 1 and 2 had the same HvM67 fragment length, while they differed in the Hdamyb. The varieties of Group 6 were similar to Group 1 in their marker allele composition of Hdamyb, but they carried a very short fragment unique to this group for HvM67. The other common characteristic of these three groups was that the majority of the varieties were six-rowed (18 out of 20), the two exceptions were both in Group 1, the spring barley Gobernadora, and the winter barley Montana having two-rowed ear type. Group 1 and 2 contained both winter and spring barleys approximately in the same portion, while the three varieties in Group 6 were all winter growth habit.

The common character of Group 3, 4 and 5 is the presence of the 430 bp. polymorphic band of the OPS3 marker. Group 3 contained only spring barley varieties, the majority of which being two-rowed type. They were of both American and European origins. The only exception was the CIMMYT breeding line LBIRAN with 6-rowed ear type. In addition to the OPS31 band, they carried a 112 bp long fragment of HvM67 and they had the shortest fragment length of Hdamyb1. The Hdamyb1 fragment length of all the European varieties was 195 bp., while the American varieties carried a fragment of 200 or 202 bp. Thus the marker haplotype of this group may represent the typical marker composition of spring two-rowed types around the region of *Vrn-H1* locus. Group 4 contained two winter barley varieties with six-rowed head type. With the exception of OPS31 their haplotypes were the same as characteristic to the six-rowed varieties in Group 1. The six varieties of Group 5 were of European origin and all carried the two-rowed marker types for both Hvm67 and OPS31. In Hdamyb1 Rex, Rodnik and Kompolti 4 carried the fragment length more characteristic to six-rowed varieties, while the other three varieties possessed a unique band. This Group was also a mixture of both head-type and growth type; Baronesse is the only spring barley variety in this group, and half of the varieties were six-rowed (Kompolti korai, Manas, Kompolti 4).

As half of the cluster groups contained both winter and spring-growth habit barley varieties the association studies with the heading date characteristics, frost tolerance, vernalization response and photoperiod sensitivity the single marker locus analyses gave significant associations (Table 2).

Table 2. Correlation between marker compositions (HvM67, OPS31 and Hdamyb1) linked to *Vrn-H2* locus on chromosome 4H and adaptational traits in a group of cultivated barley varieties

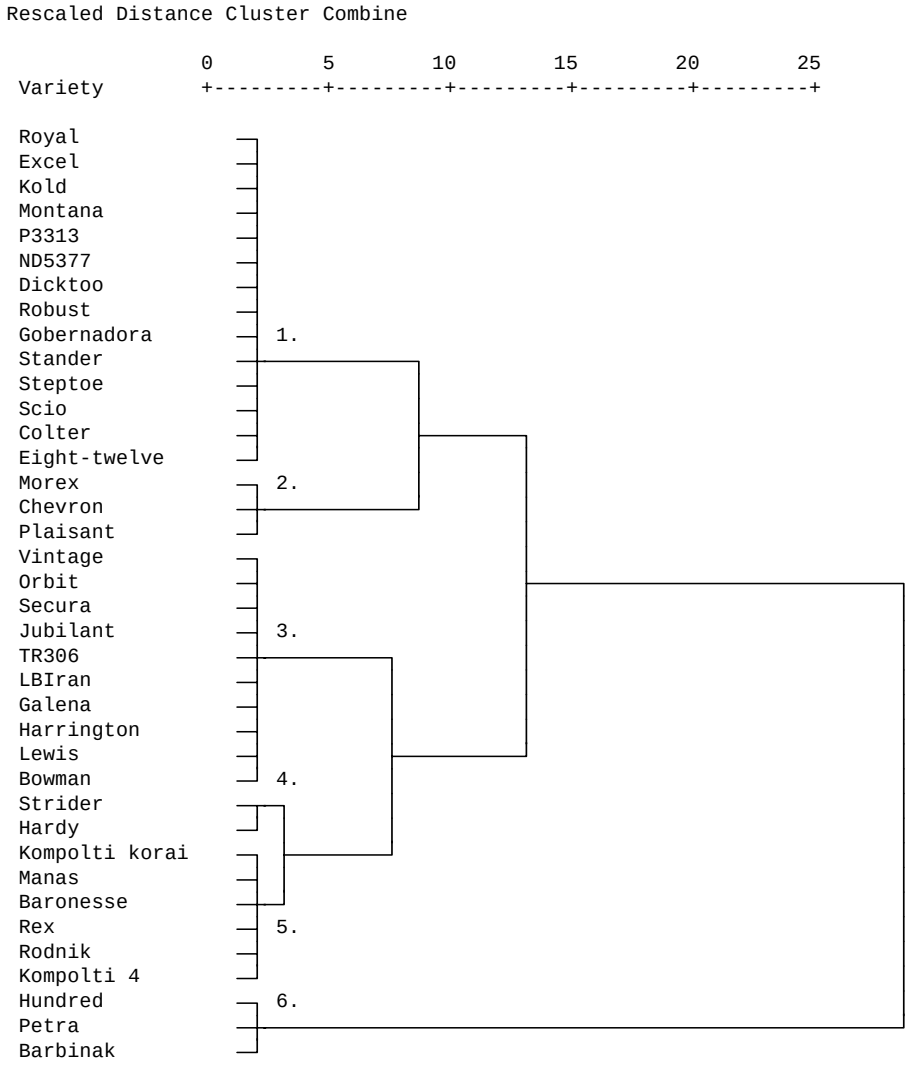
Trait	*38 barley varieties*			*20 spring barley varieties*			*17 winter barley varieties*		
	HvM67	*OPS31*	*Hdamyb1*	*HvM67*	**OPS31**	**Hdamyb1**	*HvM67*	*OPS31*	*Hdamyb1*
HvM67	-			-			-		
OPS31	0.54***	-		0.96***	-		0.50*	-	
Hdamyb1	-0.44**	-0.48**	-	-0.65**	-0.75***	-	-0.32	-0.06	-
Growth type	0.35*	0.14	-0.53**	-	-	-	-	-	-
Ear type	-0.42*	-0.70***	0.51**	-0.77***	0.80***	0.60**	-0.29	-0.45+	-0.05
HD18uv	-0.27	0.07	0.43*	0.62**	0.63**	-0.48*	0.04	0.44+	0.06
HD14uv	-0.35*	0.04	0.41*	0.65**	0.59**	-0.60**	-0.16	0.32	0.04
HD10uv	-0.42*	-0.11	0.46**	0.48*	0.44*	-0.03	-0.34	-0.19	-0.05
HD18v	-0.39*	-0.01	0.34*	0.55*	0.57**	-0.36	-0.28	-0.03	0.17
HD14v	-0.39*	0.17	0.17	0.84***	0.77***	-0.61**	-0.50*	-0.08	0.29
HD10v	-0.35*	-0.28	0.39*	0.42+	0.40+	-0.27	-0.30	-0.58*	0.36
Sur10	-0.40*	-0.24	0.57***	-0.36	-0.31	0.30	-0.41	-0.47+	0.12
Sur13	-0.46**	-0.27	0.53***	-0.22	-0.25	0.24	-0.34	-0.53*	0.48+
Daydiff1018	-0.23	-0.29	0.28	0.36	0.33	-0.23	-0.17	-0.50*	0.25
Vern. response	-0.22	0.09	0.43*	0.37	0.36	-0.32	0.16	0.56*	0.00

When all the barley varieties were examined the effect of Hdamyb1 and HvM67 were the greatest, significantly associating with almost all traits. This was due to their correlation with the growth type. It is especially interesting as IGARTUA *et al.* (1999) proved that the allele type of Bmy1 strongly correlated with the growth habit in a set of barley varieties as it was expected from the previous results (HACKETT *et al.* 1972). In our group of varieties there was a much stronger correlation between the markers and head type, showing that the genotypes of the two germ plasm pools may carry different sequences near the vicinity of the major loci of adaptation as a consequence of their divergent origin (TANNO *et al*. 2002).

In the spring barley group all the three markers significantly associated with the heading date parameters, but the effects of HvM67 and OPS31 was the strongest. The two-rowed varieties (with longer fragment of HvM67 and with the presence of the polymorph band of OPS31 which were characteristic to the vernalization responsive winter barley parent in the mapping population) headed significantly later, irrespective to vernalization and photoperiod regime, than the six-rowed group. In the group of winter barley varieties the association of OPS31 was more pronounced with several traits. This marker significantly correlated with heading date in 10 hour vernalized treatment, frost tolerance, photoperiod sensitivity and vernalization response of the winter barleys. Varieties with the presence of OPS31 band headed earlier under short photoperiod regime when vernalized, were less sensitive to photoperiod, had a higher vernalization response, but a lower frost tolerance than those with the lack of this band.

Figure 1. Haplotypes of 38 barley varieties on chromosome 4HL based on the cluster analysis of their compositions in the three markers (HvM67, OPS31 and Hdamyb) linked to the *Vrn-H2* vernalization response locus

HvM67	*OPS31*	*Hdamyb1*
107	0	216
107	0	216
107	0	216
107	0	216
107	0	216
107	0	216
107	0	214
107	0	214
107	0	214
107	0	214
107	0	214
107	0	214
107	0	220
107	0	220
105	0	200
107	0	200
107	0	195
112	1	195
112	1	195
112	1	195
112	1	195
112	1	202
112	1	202
112	1	200
112	1	200
112	1	202
114	1	200
107	1	220
107	1	216
112	1	210
112	1	210
112	1	210
112	1	214
112	1	214
112	1	216
85	0	218
85	0	216
80	0	220



Introgressions of the two head types may result in specific combinations of sequences around the *Vrn-H2* locus, of which then may arise genotypes representing special adaptation types. We found that the few winter barleys with OPS31 fragment at the *Vrn-H2* locus, was mostly those, which represented special adaptation types. Like the six-rowed Kompolti korai, Manas and Strider with the two-rowed marker alleles were carrying special component traits, not characteristic to winter barleys. Thus Kompolti korai was photoperiod insensitive, while Manas had as low earliness *per se* as the spring barley varieties and Strider also belonged to the earliest winter wheat varieties under long photoperiod (KARSAI *et al*. 2001). When the varieties were separeted by adaptational traits these varieties formed separate groups.

Based on our results several markers were identified among barley varieties, which are correlated with adaptational traits. Some of them could be connected with previously described chromosomal regions affecting adaptation. The *Vrn-H2* locus on chromosome 4H has a significant effect on development of barley varieties, and the winter barley varieties carrying OPS31 fragments (430bp) represented special adaptation types.

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AB-QTL Analysis in Spring Barley and Three Strategies of QTL Verification

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Introduction

This report illustrates how the genetic variation in wild barley can be exploited by means of DNA marker technologies in order to improve quantitative traits in elite barley. The report is sub-divided into three chapters: (1) The AB-QTL project in barley, (2) Verification of QTLs, (3) Construction of introgression lines (ILs).

(1) The AB-QTL Project in Barley

In 1996 TANKSLEY and NELSON (1996) integrated the mapping of favorable quantitative trait loci (QTL) and the introgression of these QTLs into a single process. In order to achieve this goal, they proposed to utilize wild species germplasm as a genetic resource for improvement of quantitative agronomic traits and to begin with marker and phenotype analysis in advanced backcross generations like BC_2 or BC_3. The strategy was coined advanced backcross (AB)-QTL analysis.

So far, several reports on the application of the AB-QTL strategy are available for rice, tomato, wheat and barley. In all cases favorable wild species QTL alleles for important agronomic traits have been identified. For instance, two wild species alleles could be localized in rice which were associated with an increase of yield by 18 and 17 %, respectively (XIAO *et al.* 1996). In tomato, besides other traits, fruit yield could be improved through introgression of wild species alleles by 17 and 34 %, respectively (TANKSLEY *et al.* 1996, FULTON *et al.* 1997). A further AB-QTL study in tomato revealed 25 favorable wild species QTL alleles out of 121 detected QTLs (20 %, BERNACCHI *et al.* 1998a). Again the authors found wild species alleles which increased yield by 15 %.

The favorable wild species QTL alleles are useful as a breeding resource after they have been fixed in near isogenic lines (QTL-NILs) and after the superior performance of a QTL-NIL has been confirmed in comparison to the recurrent elite line. BERNACCHI *et al.* (1998b) have already demonstrated the stability of identified exotic QTL alleles in QTL-NILs. In field evaluations at five locations worldwide, 22 QTL-NILs out of 25 tested (88 %) exhibited the expected phenotypic improvement over the recurrent parent. For instance, the QTL-NIL possessing the exotic QTL allele for a 15 % yield increase did, in deed, outperform the control line by 12 %. These reports clearly illustrate that the AB-QTL strategy is a powerful tool for improvement of quantitative agronomic characters.

Our own lab has already finished the first barley AB-QTL project using spring barley as a model (PILLEN *et al.* 2003). An advanced backcross population comprising 136 BC_2F_2 individuals from the cross Apex *(Hv)* x ISR101-23 *(Hsp),* abbreviated A101, was genotyped with 45 SSR markers and, after two rounds of seed propagation, subjected to field trials at three locations during two seasons. Altogether, 13 quantitative traits were evaluated. Subsequently, a two-way ANOVA with marker genotype *(Hv* vs. *Hsp),* environment (6x) and marker x environment (MxE) interaction as effects was applied in order to locate QTLs (see Fig. 1).

In total, 86 significant effects were located as environment-stable marker main effects (64) or as environment-dependent MxE interactions with P<0.01. Fig. 2 shows map locations of 34 QTLs in A101 restricted to the traits heading time, plant height and yield. The significant effects of adjacent SSR markers with less than 20 cM distance are considered as a single QTL as indicated by vertical bars in Fig. 2. Favorable *Hsp* alleles, which improve a trait relative to the *Hv* allele, are specified with a "+".

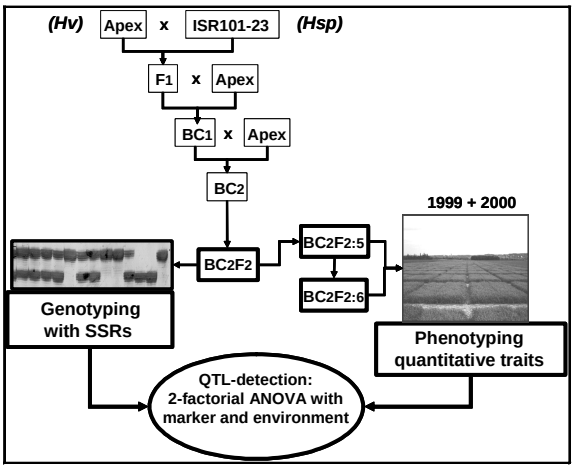

Fig. 1. Strategy of AB-QTL analysis in barley (PILLEN *et al.* 2003)

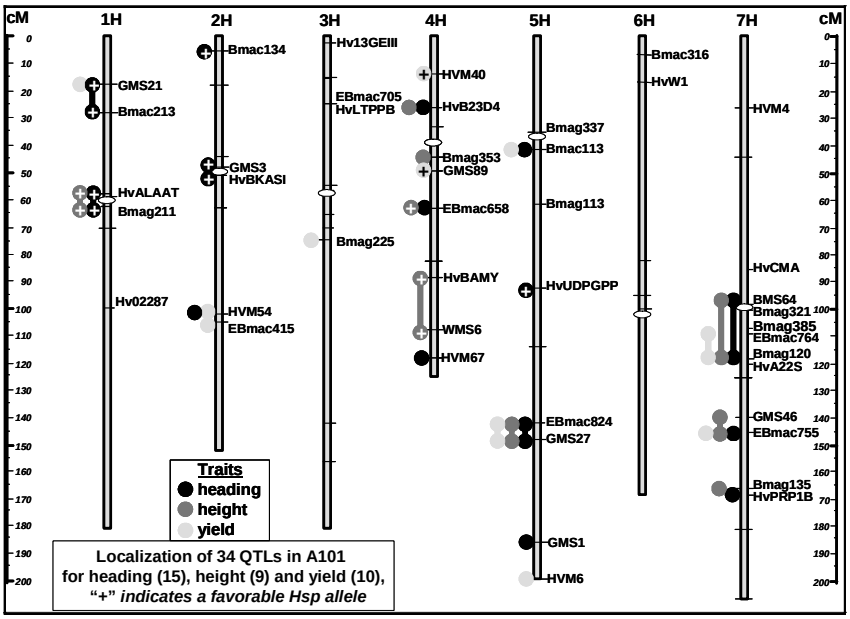

Fig. 2. Thirty-four QTLs located for heading time, plant height and yield in population A101

Favorable *Hsp* alleles improve a trait relative to the *Hv* allele. The dots and bars indicate the approximate position of QTL effects associated with a single marker or adjacent markers.

In total, 10 favorable QTL alleles from *Hsp* (29%) were identified among 34 located QTLs (see PILLEN *et al.* 2003). For example, the exotic *Hsp* QTL allele at $GMS3_{[2H]}$ was associated with an average heading time reduction of 6.0 days (-9.6%). Likewise, the *Hsp* allele at $HvBAMY_{[4H]}$ was associated with an average plant height reduction of 8.6 cm (-10.4%). And the *Hsp* allele at $GMS89_{[4H]}$ was associated with an average yield increase of 0.5

t/ha (+ 7.7%). These QTLs are excellent candidates for introgression into elite cultivars after the effects have been reconfirmed.

(2) Verification of QTLs

The verification of a QTL effect is extremely important in regard to its exploitation. In the following, three independent lines of verification of the A101 QTL data are presented. These are: (1) a cross validation of the QTL effects using a second backcross population, (2) the search for supporting map locations of candidate genes and (3) the test of QTL effects in derived nearly isogenic lines harboring a single QTL segment from *Hsp.*

Cross Validation

For cross validation, we analyzed 164 BC_2F_2 individuals of the cross Harry x ISR101-23 (H101). The same traits as in A101 were measured in H101 and the population was genotyped with 50 SSR markers (PILLEN *et al.* 2004). Since both populations share the same donor accession, the trait effect of the *Hsp* allele at a given SSR locus can be compared between the two recipient cultivars Apex and Harry. In total, the location of 17 QTL effects (50%) could be verified in H101 (Fig. 3). These are 10 QTLs for heading time on chromosomes 1H, 2H, 4H, 5H and 7H, 3 QTLs for plant height on chromosomes 4H and 5H and 4 QTLs for yield on chromosomes 3H, 4H and 5H. At locus $GMS27_{[5H]}$, all three QTL effects were verified.

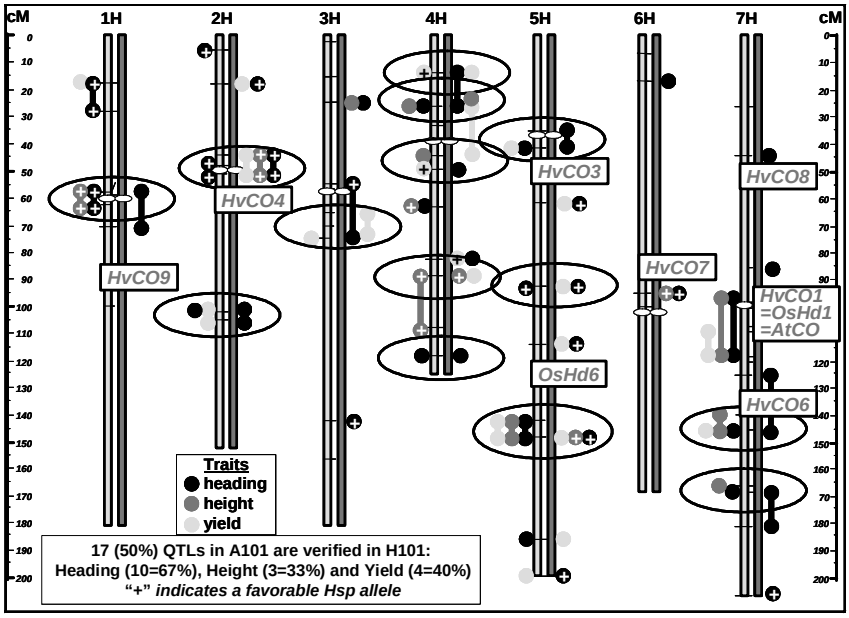

Fig. 3. Verification of A101 QTLs for heading time, plant height and yield

QTLs in A101 are labeled to the left and QTLs in H101 to the right of each chromosome. Seventeen QTLs in common between A101 and H101 are encircled. Eight QTLs for heading correspond to map positions of cloned genes (boxed names) involved or possibly involved in regulation of heading time in *Arabidopsis (At),* rice *(Os)* and barley *(Hv).*

Although a considerable portion of the QTL effects are in common between the two populations, four *Hsp* alleles revealed opposite QTL effects in both populations. These are the effects of $HvALAAT_{[1H]}$ and $GMS27_{[5H]}$ on heading date, the effect of $GMS27_{[5H]}$ on plant height and the effect of $GMS40_{[5H]}$ on yield (see PILLEN *et al.* 2004). The latter findings indicate that even if a favorable QTL effect of an exotic allele has been found, its reaction in a new genetic background can not be predicted with certainty. In this regard, epistatic

interactions of an exotic QTL allele with the recipients remaining genome complement might cause the contrasting QTL effects.

QTL-Supporting Map Locations of Candidate Genes

As an example that the existence of QTLs can be supported by corresponding map locations of candidate genes, we compared the genetic map location of published genes involved in heading time control in *Arabidopsis* (*CONSTANS*, PUTTERILL *et al.* 1995), rice (*Hd,* YANO *et al.* 2000, TAKAHASHI *et al.* 2001) and *CONSTANS*-like genes from barley (GRIFFITHS *et al.* 2003). In total, the approximate map location of eight heading time genes corresponds to QTLs for heading time in A101 and/or H101 (see Fig. 3). Two of these genes are the rice heading time QTLs *OsHd6* and *OsHd1* which are already cloned. Presumably, the latter is the ortholog of the *Arabidopsis CONSTANS* gene *(AtCO)* which promotes flowering under long day conditions. In order to further test if these genes are true candidate genes for heading time QTLs in barley, we will map them in the A101 and H101 populations. In case a candidate gene reveals the QTL effect on heading time, a knock out experiment by transposon tagging (KOPREK *et al.* 2000) or TILLING (MCCALLUM *et al.* 2000) could be launched in order to clone the underlying QTL factor.

Verification of QTL Effects by QTL-NILs

The final proof of a QTL effect would be to construct a QTL-NIL through backcrossing and selection with informative markers and then evaluate the QTL-NIL performance relative to the recurrent parent (see Fig. 4). The exotic donor genome in the QTL-NIL would be restricted to a small segment harboring the original *Hsp* allele which was originally associated with the QTL.

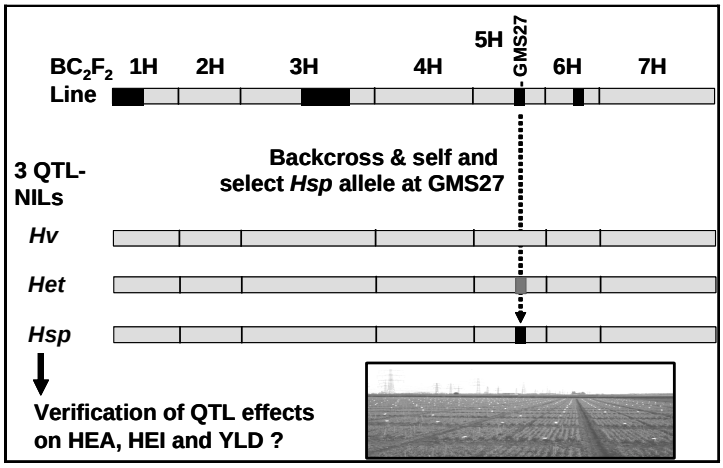

Fig. 4. Construction of QTL-NILs for locus GMS27 on chromosome 5H
Grey and black squares represent *Hv* and *Hsp* genome portions, respectively.

We have applied the QTL-NIL concept in order to verify the QTL effects of the *Hsp* allele at locus $GMS27_{[5H]}$. Based on limited results from the first year of evaluation, 2 out of 6 QTL effects in A101 and H101 could be verified in derived QTL-NILs in $BC_3S_{1:3}$ These are the yield reduction effect in A101 and the heading time decreasing effect of the *Hsp* allele in H101. Fig. 5 presents results for the latter case.

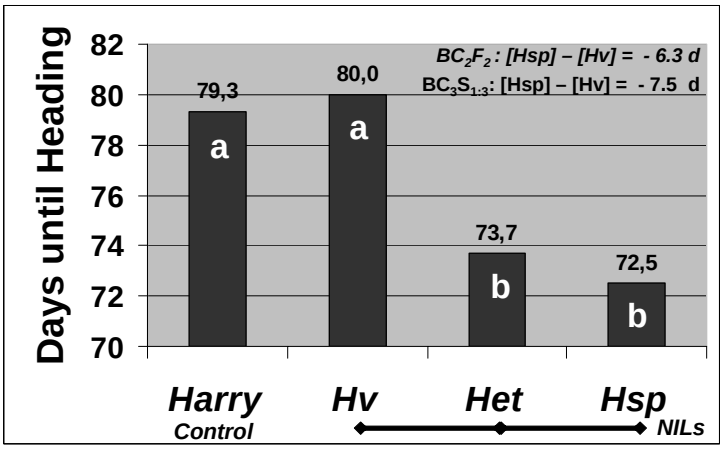

Fig. 5. Verification of a QTL effect on heading time at locus GMS27 with QTL-NILs
QTL NILs in $BC_3S_{1:3}$ containing the *Hsp* allele homozygous *(Hsp)* or heterozygous *(Het)* at locus $GMS27_{[5H]}$ reveal a significant reduced heading time (-7.5 d) compared to the control and to the homozygous *Hv* QTL-NILs. Different letters in columns indicate significant differences ($P<0.05$) between the classes based on a Tukey test (N=44). The original QTL effect of the *Hsp* allele at $GMS27_{[5H]}$, measured in the BC_2F_2 H101 population, disclosed a reduced heading time of -6.3 days.

In summary, we could support our A101 QTL data through three independent lines of verification. Based on the cross validation with H101, 50% of the A101 QTLs could be verified. The comparison of the map location of candidate genes supported the identification of 8 QTLs for heading time. Finally, QTL-NILs, which are derived from backcrossing AB lines and selecting for the presence of the *Hsp* allele at locus $GMS27_{[5H]}$, verified 2 out of 6 original QTL effects (33%) on heading time, plant height and yield in A101 and H101.

(3) Construction of Introgression Lines (ILs)

Based on the existing AB lines, a complete set of overlapping introgression lines can be selected straightforwardly. For this, appropriate AB lines have to be backcrossed and selfed and, thereafter, selected with SSR markers for the presence/absence of *Hsp* alleles at each locus (Fig. 6). The average size of *Hsp* donor segments should be between 20 and 40 cM with an overlap of 10-50%. Thus, the entire exotic barley genome of ca. 1,200 cM could be represented by a set of 30-80 ILs.

The ILs are fixed genotypes which can be multiplied and tested in replicated field trials. Significant QTL effects can be pin-pointed to a narrow chromosomal segment. In addition, the genes responsible for a QTL effect of a particular IL can be studied on the transcript, protein and metabolite level by comparing the IL with the recurrent parent (Apex). Furthermore, the IL which bears a QTL effect is an ideal launching pad for a map-based cloning project. Starting from the original IL, the size of the exotic QTL segment can be further reduced by backcrossing and checking for recombination events with flanking markers until the QTL introgression fits to the dimensions of a bacterial artificial chromosome (BAC). After the QTL is limited to a BAC, there is a good chance that the gene can be identified by means of a complementation experiment. Alternative QTL cloning strategies like transposon tagging and TILLING were already introduced in the chapter about candidate genes. The map-based cloning of a QTL starting from ILs was already demonstrated by FRIDMAN *et al.* (2000). The authors could proof that the tomato sugar content QTL *Brix9-2-5* was identical with the apoplastic invertase gene *Lin5*, which hydrolyses sucrose into glucose and fructose.

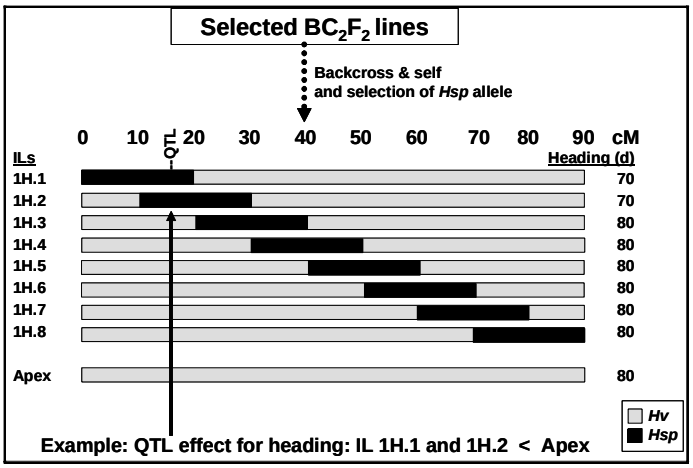

Fig. 6. Development of introgression lines (ILs) and hypothetical localization of a QTL
A complete set of ILs can be selected after backcrossing and selfing of appropriate BC_2F_2 lines. The ILs should harbor overlapping segments from the exotic *Hsp* donor. Testing the complete set of ILs would allow to confine a QTL to a narrow interval shared by two adjacent ILs (in this example: 10 cM between IL-1H.1 and IL-1H.2).

In future, we anticipate that a wealth of exotic barley genes will be identified by means of the AB-QTL strategy or related procedures. A considerable fraction of these exotic genes may be beneficial to both applied breeding and molecular genetics. Starting from verified QTL-NILs or ILs, the purified exotic QTLs can be readily incorporated into a breeders elite germplasm. The same resources can also be utilized by plant molecular geneticists to clone the QTL effect-causing genes and study their action and regulation in detail.

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The Semi-Dwarfing Gene *sdw*1 in European Spring Barley

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Abstract
The semi dwarf mutation *sdw*1 is present in almost all spring barley cultivars grown in North-West Europe, e.g. in all but one of the spring varieties on the current UK recommended list. Most current varieties carry the gene from one particular source, the mutation in the Czech variety Valticky that produced the variety Diamant.
The map position of *sdw*1 on the long arm of 3H(3) allows the genomic sequence of the broadly homologous rice chromosome 1 to be used to provide molecular markers that delineate the *sdw*1 region in barley and also give more detailed data on the syntenic relationship between these two species in this genomic region. These markers can also be used to determine the extent of the Valticky derived linkage block that has survived into the many varieties that can be traced backed to Diamant and its derivative Trumpf (Triumph). Work will be presented that utilises molecular markers to characterise this region in a range of germplasm and also reports on the progress made in the more detailed characterisation of the *sdw*1 gene.
Keywords: barley; semi-dwarf; *denso*; *sdw*; synteny; Single Nucleotide Polymorphism

Introduction

The mutation *sdw*1in barley confers a semidwarf phenotype that is sensitive to exogenous GA unlike the dominant GA insensitive dwarfing *Rht* genes found in bread wheat. *sdw*1 is utilised in the majority of spring barley cultivars grown in North-West Europe. It is present, for example in all but one of the spring varieties on the current UK recommended list.
Although multiple sources of the mutation are known (HAAHR & VON WETTSTEIN 1976) most current varieties carry the gene from one particular source, the mutation in the Czech variety Valticky that produced the variety Diamant in 1965. Its prevalence is due in part to the great success of the high malting quality variety Trumpf (Triumph) bred in Germany in 1973 that included Diamant in its parentage (Figure 1). The success of Trumpf, due to its combination of high yield potential with different good malting attributes, meant that it was subsequently used widely as a parent (FISCHBECK 1992) which has ensured the penetrance of the Diamant source of *sdw*1 in North-Western European spring barleys.

In order to investigate the effect of this gene on elite European barley germplasm a study was undertaken to determine the extent of the Valticky derived linkage block that has survived around *sdw*1 in such material. This entailed the generation of new molecular markers in order to saturate a relatively sparsely covered region of the genetic map. The map position of *sdw*1 on the long arm of 3H(3) (BARUA *et al.* 1993; LAURIE *et al*. 1993) allowed the genomic sequence of the broadly homologous rice chromosome 1 to be used to provide markers that delineate the *sdw*1 region in barley and also give more detailed data on the syntenic relationship between these two species in this genomic region. The initial results of the study are presented here along with discussion of future work.

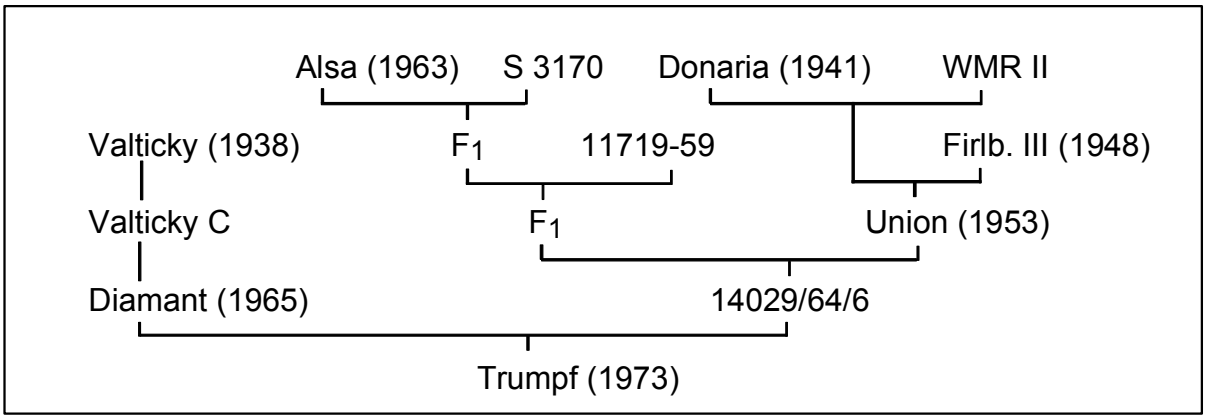


Figure 1. Pedigree of Trumpf (adapted from FISCHBECK 1992)

Material and Methods

Plant Material

Leaf material was collected from glasshouse grown plants of the parents and doubled haploid populations of Lina x *Hordeum spontaneum* (Canada Park) (RAMSAY *et al*. 2000), Steptoe x Morex (KLEINHOFS *et al*. 1993) and Derkado x B83-12/21/5 (THOMAS *et al*. 1998) and also from a range of current and recent elite UK spring barleys and varieties that figures in their pedigrees.

Molecular Marker Development

The genomic sequence of rice PACs from the *sd*1 genomic region was used to find putative homologous barley sequences from the assembly of public barley ESTs in HarvEST Barley v1.07 (WANAMAKER & CLOSE 2003). Primer pairs were then designed using Primer3 (ROZEN & SKALETSKY 2000) from these barley sequences targeting either polymorphisms shown in the HarvEST assemblies or to regions likely to contain polymorphisms, (3'UTRs and introns). Markers (SNPs, indels and SSRs) were verified/discovered through the sequencing of PCR products from several mapping parents and the polymorphisms mapped in the relevant populations.

Results and Discussion

Mapping

The presence of the recently cloned *sd*1 gene (coding for gibberellin 20-oxidase) on the long arm of rice chromosome 1 (SPIELMEYER *et al*. 2002) gave the focus of a candidate gene for this study. Mutations at the rice gene *sd*1 have been widely used in the 'Green Revolution' and give a dwarf phenotype that is also sensitive to exogenous GA as is *sdw*1 in barley. The use of the rice chromosome 1 sequence to generate barley markers was successful in populating the marker-poor region of *sdw*1 on the barley genetic maps and indicates that *sdw*1 maps to Bin 12 on 3HL rather than Bin 13. From the genetic map order it appears that the co-linearity in rice may be interrupted by two inversions in this region in barley. In addition the comparison of the barley genetic maps shows some variation in recombination frequency as well as a reduction in polymorphism in the narrower crosses.

The mapping work also supported the working hypothesis that a *sd*1 homologue is the candidate for *sdw*1 as two markers that showed homology to genes on the same rice PAC as *sd*1 were shown to map within a recombination unit of *sdw*1 mapped from the phenotypic scores on the Derkado x B83-12/21/5 population (Figure 2).

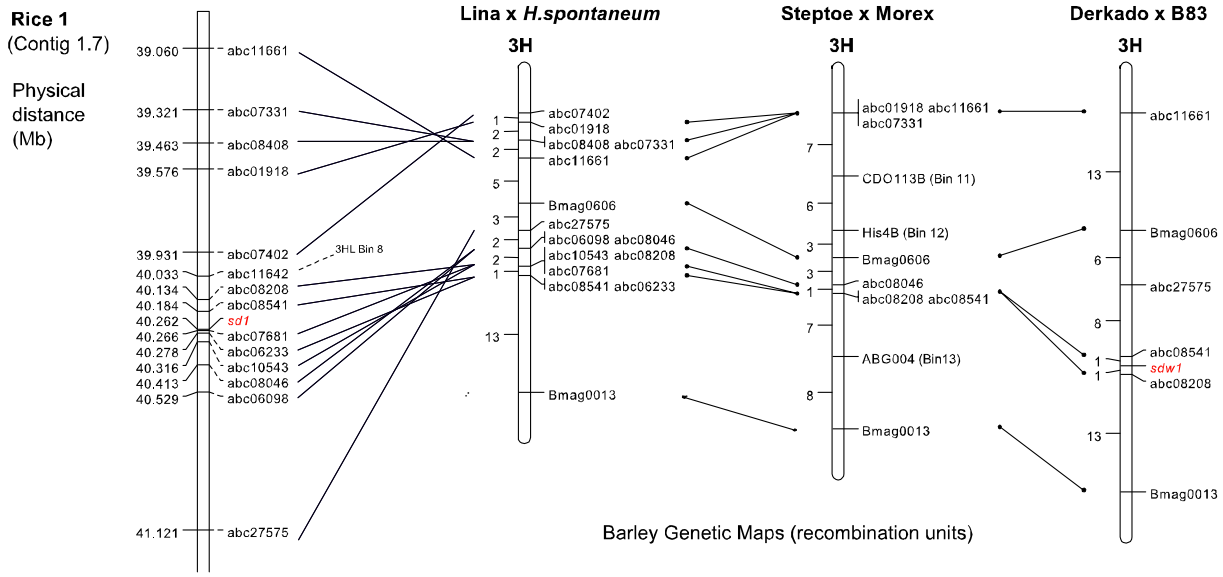
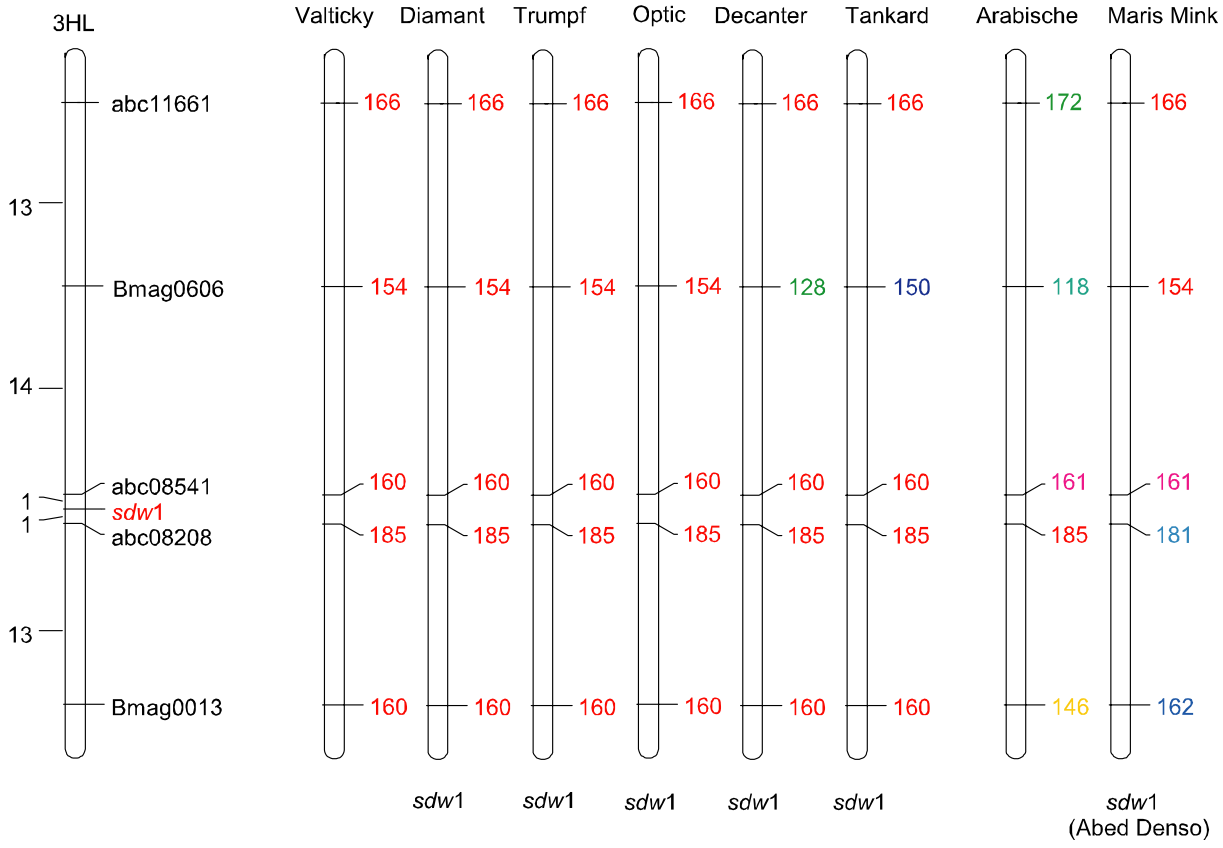


Figure 2. Use of barley-rice synteny for the mapping of *sdw*1 region

Introgression

The mapping of polymorphic markers in the *sdw*1 region allowed us to genotype a range of elite spring barley varieties. These studies indicated that a region between the genomic SSRs, Bmag0606 and Bmag0013, almost 30 cM, is conserved from Diamant in some modern varieties such as Optic (Figure 3). Significant linkage disequilibrium was shown between these makers in the elite spring material screened.



Although some recombination can be observed the haplotype across this region is indicative of the presence of the *sdw*1 gene from Diamant. The maintenance of such a large linkage block is intriguing and may relate to the good quality of the genetic background of the Diamant *sdw*1 mutant. QTL studies have highlighted a "hot-spot" associated with *sdw1*, particularly for quality characters (MEYER *et al.* 2000; ELLIS *et al*. 2002). This could be due to pleiotropy or close linkage but, whatever the cause, it does indicate a reason why LD might have persisted in the breeding of North-West European spring barley. It is interesting that alternative haplotypes are observed in non-*sdw*1 lines or in varieties with *sdw*1 from a different source e.g. Maris Mink, the latter category not providing such good malting quality as found in Triumph and its derivatives.

Future Work

We are presently engaged in cloning *sdw*1 in order to fully characterise the mutant alleles from the various sources available. To this end we have screened a 3x genome coverage subset of the Morex BAC library with a probe derived from abc07681 which is homologous to a gene which is less than 1.5 kb from the gibberellin 20-oxidase *sd*1 gene in rice. This hybidisation highlighted four BAC clones that are presently being fingerprinted and subcloned. Ultimately, this will enable the associations of *sdw1* with other characters to be ascribed to either pleiotropy and/or close linkage and appropriate breeding strategies formulated.

Other work will include a further elucidation of the rice/barley synteny in this region with particular emphasis on the region distal to *sdw*1. The putative translocations relative to rice are also of interest and will continue to be investigated. In particular confirmation of breakpoints indicated on the barley genetic maps will be sought using physical mapping approaches. In addition we will be utilising the markers that this study provides within an ongoing genotyping survey of elite barley germplasm in order to elucidate the patterns of linkage disequilibrium within modern barley varieties.

Acknowledgements

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Regions of the Genome Affecting Hull Peeling in Two-Row Barley and Malt

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Abstract
Hull peeling resistance in barley and malt is desired for malting and brewing. To investigate the inheritance of these traits in Canadian two-row malting barley, a molecular map consisting of 60 microsatellites and 120 AFLP's was constructed for the Harrington (susceptible)/ Manley (resistant) cross using 160 F_2-derived F_9 recombinant inbred lines (RIL's). Barley samples from standard yield test plots grown at two sites in each of Manitoba and Saskatchewan during 1999 and 2000 were evaluated for % hull peeling on a weight basis direct from the plot combine (RPWB) and after inducing peeling with an air-blast de-huller (APWB). Micromalted samples were evaluated for % hull peeling of the malt "as is" (RPWM) and after air-blast de-hulling (APWM). Two QTL were identified for RPWB, three for APWB, two for RPWM, and two for APWM. Both RPWB QTL were coincident with two for APWB. The two QTL for APWM and RPWM were coincident, and one of these may be coincident with a QTL for RPWB and APWB. The QTL were consistent over locations and years, indicating they could be candidate targets for molecular breeding.
Keywords: hull peeling; hull adherence; skinning; barley; malt; molecular markers; QTL

Introduction

Resistance to hull peeling (hull adherence or skinning) in barley and malt is highly desired by the malting and brewing industry. This is partly due to the increasing importance of the export market which increases the amount of handling. There have been customer concerns about Harrington's peeling susceptibility so this deficiency must be improved in new malting cultivars. Hulls protect the developing embryo and acrospire from mechanical damage. Hull peeling increases the rate of water uptake and results in uneven germination during malting. It may also promote mould growth which results in off-flavours and gives the malt an unattractive appearance. The recent development of two-row malting barley lines, such as TR244 and TR251, with promising malting quality but high susceptibility to hull peeling, has also been a concern.

Little research has been done on the genetics of hull peeling. AIDUN *et al.* (1990) crossed Harrington to two lines (Ellice and WM797-83) that were more resistant to hull peeling. Heritability was relatively low to moderate and peeling was largely an environmentally influenced trait. A study to determine the genetic control of resistance to hull peeling through the use of molecular genetic techniques will increase our understanding of this trait. Knowing the location of hull peeling resistance genes in the genome in relation to other traits such as disease resistance, quality and agronomic performance is useful in developing a breeding strategy. The development of molecular markers for resistance to hull peeling would allow breeders to use molecular marker assisted selection (MMAS) to increase the precision of selection and more rapidly develop new cultivars with improved resistance to hull peeling. The objectives of this project were to determine the inheritance of resistance to hull peeling of barley and malt in two-row Canadian malting barley, and identify molecular markers for these traits that could be used for MMAS.

Material and Methods

160 F_2-derived F_9 recombinant inbred lines (RIL's) from the Harrington (susceptible)/Manley (resistant) cross were developed at Agriculture and Agri-Food Canada (AAFC) Brandon. The RIL's were grown in a single replication of standard yield test plots at two sites near Brandon, Manitoba and Saskatoon, Saskatchewan during 1999 and 2000. Harrington and Manley were replicated 10 times each and alternated at regular intervals within each test.

Ten gram barley samples from each plot were evaluated for % hull peeling on a weight basis direct from the plot combine (RPWB). Since RPWB values were low and differences between the two parents small, % hull peeling of barley was also determined on a weight basis after inducing peeling with an air-blast de-huller (APWB). The air-blast de-huller was originally used for de-hulling oats at the Grain Research Laboratory, Canadian Grain Commission, Winnipeg, and a protocol for inducing peeling in barley was developed by Dr. N.T. Kendall (personal communication) formerly of the Brewing and Malting Barley Research Institute (BMBRI) with further modifications at AAFC Brandon. The device consists of a brass sample chamber 61 mm in diameter x 64 mm deep (interior measurements) mounted horizontally to a brass upright with a wire screen the same diameter as the sample chamber and adjacent to a plastic cyclone chamber of similar diameter. The cyclone chamber is about 36 mm wide, the side nearest the sample chamber is open while the opposite side is closed, and there is an open tube about 31 mm in diameter x 63 mm long extending perpendicular from the cyclone. Air at a specific pressure is directed through a metal tube through the screen to the sample chamber where it swirls the seed inducing peeling. Excess air escapes back through the screen and out the cyclone chamber. There is a valve to turn the air on or off, and a pressure gauge that can be used to regulate and monitor the pressure of the air going into the sample chamber. Degree of peeling is controlled by the air pressure level and the length of time that the air is in the sample chamber. This is determined empirically prior to inducing peeling for each test by using samples of Harrington and Manley from that particular test. The combination of air pressure and time that maximized the difference between Harrington and Manley while minimizing peeling of Manley was used for each test. All tests for barley used 69 KPa pressure with the length of time ranging from 10 to 40 sec.

Forty gram samples from the above tests were micromalted at the Cereal Research Centre, Winnipeg, using procedures consistent with preliminary malt analyses. Roots were gently removed manually by rubbing the malted grain over a sieve, so as not to loosen or affect the hull. A 10-gram subsample was evaluated for % hull peeling of the malt "as is" on a weight basis (RPWM). The % hull peeling of malt was also determined on a weight basis after air-blast de-hulling (APWM) as described above for barley. Since malt was more prone to breakage, a less aggressive regime was used with a pressure of 35 KPa for 5 to 25 sec.

For all peeling measurements, % peeled was assessed according to the Canadian Grain Commission (2003) grading standard which states that peeled kernels have at least one of the following: one-third or more of the hull is removed, germ is fully exposed, hull is badly frayed or ruptured over the germ without evidence of germination or the hull is removed along both edges. Since this definition is subjective, most of the peeling assessments were done by the same person at AAFC Brandon to reduce error. The 10-gram sample from each plot for each peeling treatment was rated visually and hand sorted into peeled and non-peeled portions, each portion was weighed, and the % peeled was calculated as the weight of the peeled portion divided by the total weight of peeled and non-peeled portions multiplied by 100. Broken kernels were removed and not included in the calculation. Preliminary research

indicated a near perfect correlation between % peeled based on weight vs. actual seed counts so all subsequent peeling measurements were conducted on a weight basis.

Peeling measurements were combined over locations and years with each site treated as one replication. Within each site, the 10 measurements for each parent were averaged and entered as a single value. Analysis of variance was conducted and entry means calculated for each year and over years using AGROBASE'98 (Agronomix Software Inc., Winnipeg, MB).

A molecular map consisting of 60 microsatellites (Scottish Crop Research Institute, Dundee, Scotland) and 120 AFLP's was constructed using MapMaker V.3.0 (LANDER *et al.* 1987) at the Cereal Research Centre, Winnipeg, for the Harrington/Manley cross by genotyping a subset of 93 RIL's from the population. The AFLP protocol of VOS *et al.* (1995) was followed and genomic DNA digested with PstI and Mse prior to pre-amplification and selective amplification with PCR. A quantitative trait locus (QTL) analysis was conducted for RPWB, APWB, RPWM and APWM using QTL Cartographer (http://statgen.ncsu.edu).

Results and Discussion

The air-blast de-huller was effective in inducing peeling in barley samples and differentiated susceptible from more resistant barley lines. The unit can handle small lots of seed which is important to breeding programs where large samples may not be available. The small size of this device and the way seed is swirled in the sample chamber may not be a true simulation of the commercial grain handling system, but it does peel or remove the hull, reflecting potential problems. It was less effective in malt because malt was more prone to breakage, so lower pressure for a shorter time was used to induce peeling. As a result, the differential in % hull peeling of malt after air-blasting (weight basis) between the parents Harrington and Manley was lower than for barley. Inducing peeling in barley with the air-blast de-huller would appear to be more effective than measuring it directly from the plot combine. For malt, measuring peeling "as is" would be more efficient than inducing peeling with the air-blast de-huller. Which ever method is used, determination of % peeled is subjective, time-consuming and tedious.

A QTL analysis was conducted for RPWB and APWB since the frequency data for these two traits suggested that they were quantitatively inherited. The means over 4 locations in each of two years for both traits were significantly lower for Manley (RPWB = 1.0%, APWB = 19.0%) than Harrington (RPWB = 2.3%, APWB = 35.5%) as expected. Population means were slightly skewed toward Manley for both traits (RPWB = 1.4%, APWB = 26.2%). Three QTL were identified for APWB: the QTL with the largest effect was found on chromosome 6H near marker P1515e, the second QTL was near marker P1311a on chromosome 1H, and the third QTL was near marker P0906d which could not be assigned to a specific chromosome location and was designated as linkage group 8 (Table 1, Figure 1). Two QTL were identified for RPWB and appeared to be in the same region as the QTL for APWB near markers P1311a and P0906d. The individual QTL for RPWB and APWB explained from 9 to 21% of the variation only, and appeared to be additive. Combining the first 2 QTL above for APWB explained 30% of the variation. All resistant alleles were contributed by Manley.

For malt, similar trends were observed in the frequency data with Manley (RPWM = 2.4%, APWM = 9.8%) having significantly less peeling than Harrington (RPWM = 7.3%, APWM = 22.0%) for both traits with the population mean (RPWM = 4.2%, APWM = 15.2%) skewed toward Manley. Two QTL affected RPWM with one QTL located near marker P1403a on chromosome 3H and the other near marker P0908a on linkage group 8 (Table 1, Figure 1).

Two QTL identified for APWM were coincident with the QTL on chromosome 3H and linkage group 8 associated with RPWM. Thus, measuring RPWM would be more effective since it is determined by the same two QTL affecting APWM, and it is less time consuming to determine RPWM as noted above.

It is not known why more QTL were not found, but environment and genotype by environment interactions appear to strongly influence peeling. Also, only one cross was used for this project, and may not have been the optimal choice since there are barley lines, such as TR244 and TR251, that are significantly more susceptible to peeling than Harrington.

For the most part, % malt peeling was under different genetic control than % barley hull peeling with only the QTL associated with linkage group 8 being potentially coincident with RPWB and APWB. These findings suggest that breeders must select for hull peeling resistance of both barley and malt. The QTL detected in this project were generally consistent over locations and years, suggesting they could be candidate targets for MMAS to improve the efficiency of selection for hull peeling resistance of barley and malt. This is thought to be the first report in which QTL affecting % hull peeling of malt and barley have been identified.

Acknowledgements
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Table 1. Contribution of QTL determining % hull peeling in barley and malt

Trait[a]	Chromo-some	Marker	% Hull Peeling			ANOVA r^2
			Harrington allele(s)	Manley allele(s)	Trait mean	
RPWB grand	1H	P1311a	1.5	1.2	1.4	0.18
RPWB 1999	1H	P1311a	2.5	1.9	2.2	0.21
RPWB grand	8[c]	P0906d	1.6	1.2	1.4	0.17
RPWB 1999	8	P0906d	2.6	1.9	2.2	0.18
RPWB grand[b]	1H+8	P1311a + P0906d	1.8	1.1	1.4	0.34
APWB grand	1H	P1311a	28.3	24.6	26.7	0.11
APWB 1999	1H	P1311a	29.1	24.5	27.0	0.13
APWB grand	6H	P1515e	29.1	24.1	26.7	0.19
APWB 1999	6H	P1515e	29.5	24.3	27.0	0.17
APWB 2000	6H	P1515e	28.5	23.9	26.3	0.16
APWB 1999	8	P0906d	29.1	25.3	27.0	0.09
APWB grand[b]	1H+6H	P1311a + P1515e	31.3	23.0	26.7	0.30
RPWM grand	3H	P1403a	4.7	3.8	4.2	0.12
RPWM 1999	3H	P1403a	6.1	4.7	5.3	0.12
RPWM grand	8	P0908a	4.7	3.8	4.3	0.09
RPWM 1999	8	P0908a	5.9	4.8	5.3	0.08
RPWM 2000	8	P0908a	3.4	2.7	3.1	0.07
RPWM grand[b]	3H+8	P1403a + P0908a	4.9	3.4	4.3	0.19
APWM grand	3H	P1403a	16.5	14.2	15.2	0.09
APWM 1999	3H	P1403a	18.4	15.5	16.7	0.09
APWM 2000	3H	P1403a	14.9	12,6	13.6	0.07
APWM grand	8	P0908a	16.6	14.2	16.9	0.07
APWM 1999	8	P0908a	18.7	15.3	15.4	0.12
APWM grand[b]	3H+8	P1403a + P0908a	16.8	13.0	15.5	0.16

[a] RPWB = % hull peeling of barley on a weight basis direct from the plot combine; APWB = % hull peeling of barley on a weight basis after inducing peeling with an air-blast de-huller; RPWM = % hull peeling of the malt "as is" on a weight basis; APWM = % hull peeling of the malt on a weight basis after inducing peeling with an air-blast de-huller; grand = grand mean over 4 locations and 2 years (8 tests); 1999 = mean over 4 locations in 1999; 2000 = mean over 4 locations in 2000.

[b] Combined effect of alleles from two different chromosomal locations on the grand mean.

[c] Linkage group 8 could not be located to a particular chromosome.

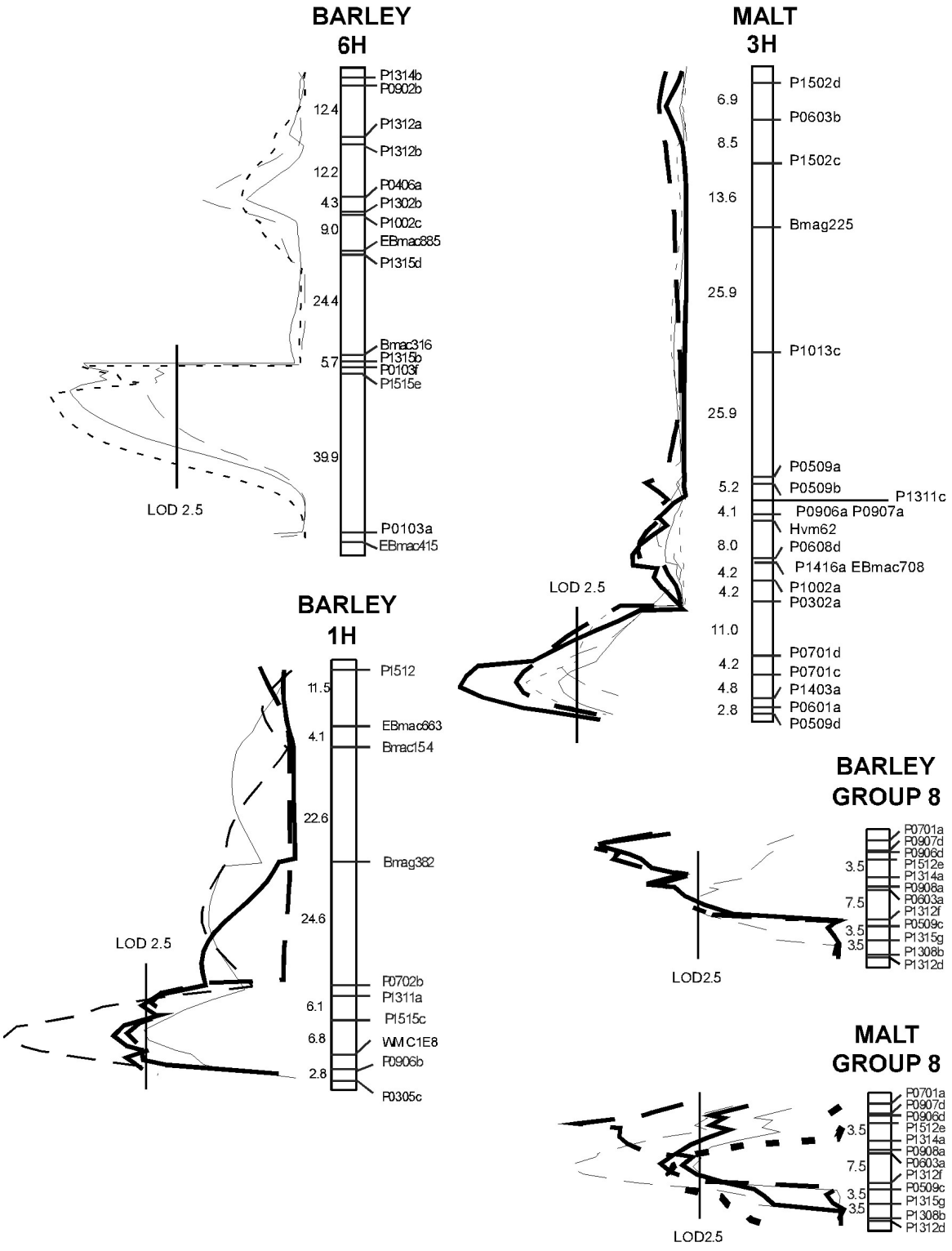

Figure 1. Genetic map of four linkage groups controlling hull adherence in barley. Hull adherence was determined on either barley or on malt as indicated above each linkage group map. Further, hull adherence was determined with (thin lines) or without (thick lines) an air-blast-dehuller treatment. QTL analysis was performed on data averaged from four locations in 1999 (long dash), 2000 (short dash) or as a grand mean (solid).

EST-Derived Markers and Transcript Map of Barley: A Resource for Interspecific Transferability and Comparative Mapping in Cereals

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Abstract
The availability of sequence data from large-scale EST (expressed sequence tag) projects has made it possible to develop markers directly from the genes. In order to develop functional markers and preparation of 'transcript map' (integrating of genes/transcripts to genetic map) of barley, more than 1,000 ESTs/cDNAs including 589 RFLP (*R*estriction *F*ragment *L*ength *P*olymorphism), 255 SNP (*S*ingle *N*ucleotide *P*olymorphism) and 185 SSR (*S*imple *S*equence *R*epeat or microsatellite) markers have been developed. These markers provide a good resource for a variety of purposes like interspecific transferability and comparative mapping in other cereals, pedigree analysis, marker-trait association etc. A computational study suggests a theoretical transferability of barley markers to wheat (95.2%), rice (70.3%), maize (69.3%), sorghum (65.9%), rye (38.1%) and even to dicot species (~16%). Furthermore, comparative mapping of barley ESTs in rice showed that a total of 311 markers exhibit collinearity between barley and rice with an average of 40 syntenic barley ESTs for each rice chromosome. The transcript map enables for a rapid identification of target regions in rice, for efficient marker saturation of defined regions of the barley genome and also serves as a resource of mapped candidate genes for agronomic traits.
Keywords: gene-derived markers; functional markers; ESTs; transcript map

Introduction

DNA-based molecular markers are the most powerful diagnostic tools to detect DNA polymorphism both at the level of specific loci and at the whole genome level. In the past, these DNA-based molecular markers were developed either from genomic DNA libraries (RFLPs and microsatellites) or from random PCR amplification of genomic DNA (RAPDs- *R*apid *A*mplification of *P*olymorphic *D*NA*s*) or both (AFLPs - *A*mplified *F*ragment *L*ength *P*olymorphsim*s*). These DNA markers can be generated in large numbers and can prove to be very useful for a variety of purposes relevant to crop improvement. For instance, these markers have been utilized extensively for the preparation of saturated molecular maps (genetical and physical). Their association with genes/QTLs controlling the traits of economic importance has also been utilized in some cases for indirect marker assisted selection (MAS). Other uses of molecular markers include gene introgression through backcrossing, germplasm characterization, genetic diagnostics, characterization of transformants, study of genome organization and phylogenetic analysis (see JAIN *et al.* 2002)

The availability of genomic DNA and cDNA sequences (ESTs) in public databases (e.g. http://www.ncbi.nlm.nih.gov; http://www.ebi.ac.uk) has made marker development more direct and sometimes also cost effective. The majority of the markers, developed and used in the past,

belonged to genomic DNA, and therefore could belong to either the transcribed region or the non-transcribed region of the genome without any information available on their functions. In contrast, markers developed from coding sequences like ESTs or fully characterized genes in recent times represent the class of markers with known functions and therefore are known as functional markers (ANDERSEN & LÜBBERSTEDT 2003). Functional or transcriptome-based or gene-derived molecular markers could detect both length and sequence polymorphism in the expressed region of the genome. These markers are superior to random DNA markers (derived from genomic DNA) owing to complete linkage with potential trait locus alleles.

Keeping in view the importance of functional markers, we have exploited the barley ESTs generated at IPK in a systematic manner for genetic mapping. In this article, we describe the development of gene-derived markers and the preparation of a transcript map of barley and discuss the use of the developed resource for interspecific transferability and comparative mapping in cereals.

Material and Methods

Plant Material and DNA Isolation

To screen for markers, parental genotypes and DH (doubled haploid) lines of three mapping populations 'Igri', 'Franka', 'Steptoe', 'Morex' and Oregon Wolfe Barley 'OWB_{Dom}' x 'OWB_{Rec}' along with 'Barke' (reference genotype, since the majority of the IPK barley ESTs were generated from Barke) were employed. Total genomic DNA was extracted from leaf material as described in GRANER *et al.* (1991).

RFLP Analysis

A set of six restriction enzymes (*Bam*HI, *Hind*III, *Eco*RI, *Eco*RV, *Xba*I and *Dra*I) was used to digest genomic DNA. Southern blotting and probe labelling was carried out according to GRANER *et al.* (1991). Autoradiography was performed by exposure of hybridised blots to imaging plates (Fuji Photo Film, Japan) and subsequent signal detection on a phosphoimager (Fuji, Japan).

SSR Analysis

For development of EST derived SSR markers, 111,090 ESTs from the IPK barley EST database (B-EST) were searched for all possible repeats with the help of a PERL5 script (see VARSHNEY *et al.* 2004). The method for PCR and separation of PCR products are described in THIEL *et al.* 2003.

SNP Analysis

To perform the PCR, primers were designed to amplify ESTs derived from cDNA libraries constructed using *H. vulgare* cv. Barke. All primers were designed using the Primer Express software (Perkin-Elmer, USA). The method for PCR and denaturing high-performance liquid chromatography was carried out on automated HPLC instrumentation equipped with a DNASep column as described in KOTA *et al.* (2001).

To identify SNPs, PCR products amplified among the parental genotypes were sequenced in both directions on an ABI 377 automated sequencer using big dye-terminator chemistry (Perkin-Elmer, USA). DNA sequence data was checked for sequencing errors using the "Sequencher"

software (Gene Codes Corporation, USA). Sequences were aligned using the GCG program pileup and polymorphisms between the parents were identified manually.

Preparation of Consensus Map
Polymorphic RFLP, SSR and SNP markers were mapped in respective mapping population. Finally, the consensus map was prepared by using JoinMap using a LOD score of 3.0 (STAM 1993).

In silico Transferability and Comparative Mapping
To examine theoretical transferability of barley EST-derived markers, BLASTN analysis (homology filter of expectation value ≤ 1.00E-10) of mapped barley SSR-ESTs was conducted against ESTs of 6 cereal (wheat, rye, rice, maize, sorghum and oats) and 3 dicot species (Arabidopsis, Medicago and Lotus), respectively. For this purpose, publicly available EST sequences for wheat, rye, rice, maize, sorghum, oats, Arabidopsis, Medicago and Lotus were acquired by the Sequence Retrieval System (SRS6, http://srs.ebi.ac.uk/) from the EMBL database [release 77 dt. 20 November 2003; query for: ORGANISM ('*Triticum aestivum*'/ '*Secale cereale*'/ '*Oryza sativa*' / '*Zea mays*'/ '*Sorghum*'/ '*Avena*'/ '*Arabidopsis*'/ '*Medicago*'/ *Lotus*) and DIVISION ('*EST*')]. The obtained 524,720 wheat, 9,194 rye, 266,169 rice, 384,391 maize, 184,708 sorghum, 501 oats, 191,302 Arabidopsis, 188,642 Medicago and 36,311 Lotus ESTs were transferred into 'BLASTable' databases by using the NCBI BLAST package ver. 2.26.

In order to associate the genetic location of experimentally mapped barley ESTs with the genetic map of rice, a BLASTN search was conducted against publicly available rice BAC/PAC sequences that were obtained from GenBank via NCBI's Entrez system (http://www.ncbi.nlm.nih.gov/Entrez/). We used the mapping position of rice BAC/PAC sequences provided by the IRGSP consortium (http://rgp.dna.affrc.go.jp/IRGSP/) and TIGR (http://www.tigr.org/tdb/e2k1/osa1/), where the rice BAC/PAC clones were associated to genetic linkage groups (YUAN *et al.* 2000;WU *et al.* 2002).

Results and Discussion

Development of Gene-Derived Molecular Markers
A flow chart for systematic development of gene-derived molecular markers is presented in Fig.1. In the context of a barley genomics programme, more than 111,090 ESTs from 22 different cDNA libraries representing different tissues at various stages were generated at IPK (MICHALEK *et al.* 2002; http://pgrc.ipk-gatersleben.de/b-est/; ZHANG *et al.* submitted). Cluster analysis yielded a tentative unigene set comprising of 25,000 genes. This unigene set was targeted for development of functional markers and preparation of the transcript map of barley by using following approaches:

*Direct Mapping of ESTs***.** The cDNA clones (corresponding to ESTs) can be mapped directly by using RFLP, STS (*S*equence *T*agged *S*ite), CAPS (*C*leaved *A*mplified *P*olymorphic *S*equence) or SNP assays. Using an RFLP assay, 543 cDNA clones leading to a total number of 558 loci were integrated into the genetic map of barley. 46 cMWG markers detecting 53 cMWG loci were also included in the dataset (GRANER *et al.* 1991). In total 589 cDNAs were mapped as RFLP to a total number of 611 loci on the genetic map.

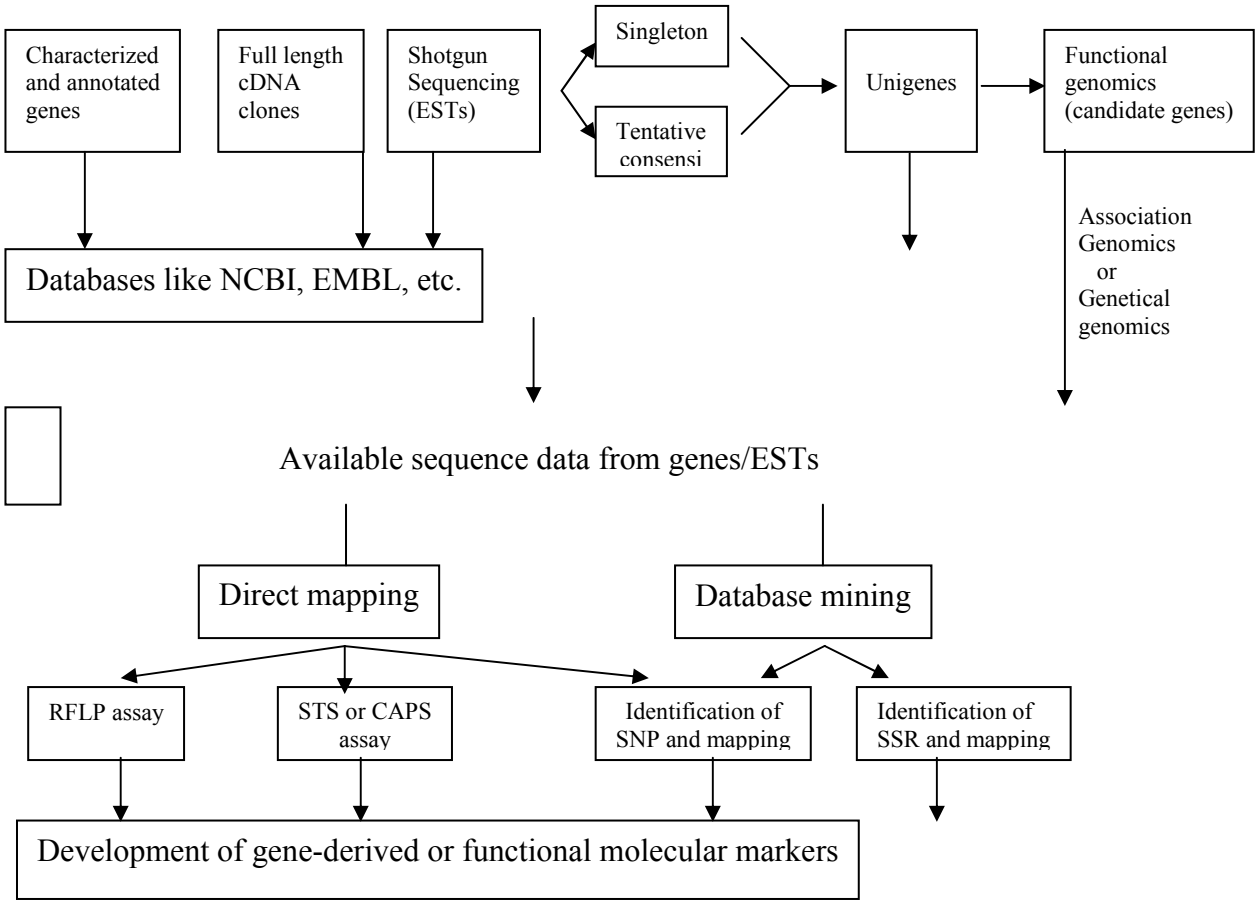

Figure 1. A flow chart showing different approaches for development of gene-derived markers

Many of those ESTs, which could not be mapped as RFLPs, were further analyzed for the presence of SNPs by allele-specific sequencing of EST-based amplicons and subsequently mapped by DHPLC (KOTA *et al.* 2001). Using this approach, a total of 255 non-redundant SNP markers were developed and placed on a consensus map (Table 1).

Database Mining: Identification of SSRs or SNPs in ESTs. Several computer programmes or scripts are available in the public domain for recognition of sequence pattern in EST sequences leading to identification of microsatellites/SSRs in a particular EST or SNP in two ESTs (of the same gene) derived from two genotypes. These programmes can be used to exploit the potential of ESTs for the development of EST-SSR or EST-SNP markers by using database mining approaches. For instance, the *MISA* (*MI*cro*SA*tellites, http://pgrc.ipk-gatersleben.de/misa) is a PERL scripts-based search module for the identification of different types of SSRs in ESTs (THIEL *et al.* 2003). Screening of the 111,090 barley ESTs with *MISA* yielded 9,564 redundant and 3,122 non-redundant microsatellites (EST-SSRs). After analysing a total of 755 primer pairs in a set of 7 genotypes, 185 EST-SSR markers were developed (THIEL *et al.* 2003; VARSHNEY *et al.* 2004).

Similarly, the SNiPpER alogrithm was developed and used to identiy 3,069 candidate inter-varietal SNPs by using 271,630 ESTs from different cDNA libraries, representing 23 barley

varieties (KOTA *et al.* 2003). By using the database mining approach, 28 EST-based SNP marker were developed. The polymorphic information content (PIC) and nucleotide diversity (π) values of the SNPs identified by the SniPpER algorithm are significantly higher than those that were obtained by random sequencing.

Transcript Map of Barley

A consensus map was produced from three individual maps by using JOINMAP V3.0. The highest number of markers was mapped in the OWB population followed by 'SxM' and 'IxF'. Altogether the transcript map contains a total of 1,039 loci including 611 RFLP, 255 SNP and 185 SSR loci (Table 1). The map covers 1,131.5 cM, with an average marker interval distance of 1.04 cM.

Table 1. Distribution of different type of markers on the transcript map of barley

Markers	**Barley-chromosomes**							**Total**
	1H	**2H**	**3H**	**4H**	**5H**	**6H**	**7H**	
RFLP	84	111	95	51	98	65	107	611
SNP	30	41	45	26	44	28	41	255
SSR	25	31	35	26	22	25	21	185
Total	139	185	176	103	164	118	170	**1051-12* (1039)**

* number of redundant ESTs mapped either as RFLPs, SSRs or SNPs

EST-Derived Markers for Interspecific Transferability

One of the most important features of gene-derived markers is their use in related species as gene sequences are known to remain highly conserved during evolution. BLASTN analysis of 974 mapped ESTs (from the transcript map) with the available 1,369,683 ESTs of six cereal species and 286,255 ESTs of three dicot species showed the presence of barley homologues in all the species, examined (Table 2). Among cereals, barley EST-derived markers showed *in silico* transferability as 95.2% in wheat, 70.3% in rice, 69.3% in maize and 65.9% in sorghum. A lower transferability of only 38.1%, which was observed for rye and 3.1% for oats has to be attributed to the small datasets (9,194 ESTs in rye and 501 ESTs in oats), which were available for analysis and may be biased regarding the content of conserved sequences. Significant homology of barley ESTs with an average of 16% ESTs of even dicot species suggest that a 'conserved ortholog set' (COS) of markers could be developed as demonstrated earlier by FULTON *et al.* (2002). These markers will prove useful in comparative mapping among fairly divergent genomes, and therefore, may also prove useful for taxonomic studies and in deducing phylogenetic relationships between different genera and species.

Transcript Map for Comparative Mapping between Barley and Rice

Comparative mapping within the grass family (Poaceae), involving major cereal species like

Table 2. Homology of mapped barley ESTs with ESTs of other plant species

Marker type	*ESTs BLAST(N)ed	**Wheat** *Trticum aesitvum* (524,720 ESTs)	**Rye** *Secale cereale* (9,194 ESTs)	**Maize** *Zea mays* (384,391 ESTs)	**Sorghum** *Sorghum bicolor* (184,708 ESTs)	**Rice** *Oryza sativa* (266,169 ESTs)	**Oats** *Avena sativa* (501 ESTs)	**Arabidopsis** *Arabidopsis thaliana* (191,302 ESTs)	**Medicago** *Medicago sativa* (188,642 ESTs)	**Lotus** *Lotus japonicus* (36,311 ESTs)
RFLP	536	511	210	388	372	393	25	107	111	87
SNP	253	243	99	191	174	186	6	47	41	36
SSR	185	173	62	96	96	106	3	18	16	12
Total	974	927 (95.2%)	371 (38.1%)	675 (69.3%)	642 (65.9%)	685 (70.3%)	34 (3.1%)	172 (17.7%)	168 (17.2%)	135 (13.9%)

*Sequences of EST corresponding to mapped markers were BLAST(N)ed against the available ESTs of different plant species. Significant homology was considered if barley EST gives a hit with the EST of examined species at expectation value ≤ 1E-10.

barley, maize, rice and wheat, demonstrated conservation of gene content and gene orders in many studies (see DEVOS and GALE 2000). The availability of a transcript map of barley faciliates the study of marker synteny and colinearity among related cereal genomes. Taking a criterion of E < 1E-05 and an identity more than 80%, 769 homologous markers were found in barley and rice (PRASAD *et al.* submitted). By correlating their mapping positions, a total of 505 (65%) ESTs displayed a syntenic relationship at the linkage group level including 311 (40%) that exhibited the collinearity. On average more than 40 syntenic barley ESTs were identified for each rice chromosome. The study mainly confirmed the established known conserved genetic linkage blocks between both species but also provided new starting points for further investigations about previously unknown linkage blocks or ancient duplications within the target genomes.

Conclusions

A comprehensive resource for gene-derived markers including SSRs and SNPs has become available in barley. The widespread application of these functional markers in a variety of studies will have far reaching implications relevant to crop improvement. The transcript map enables barley geneticists to rapidly identify target regions in rice, for an efficient marker saturation of defined regions in the barley genome. In addition to application of gene-derived markers for crop improvement in barley, developed computer tools like *MISA* and SniPpER are useful for the development of additional markers in barley or any other plant or animal system.

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Genetic Conversion of Feed Barley Varieties to Malting Types

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Abstract
Currently there is a gap of at least 10% in yield between malt and feed varieties, as well as significantly better disease resistance profiles in the feed types. Recent advances in the genetics of malt quality and in marker assisted selection for malt quality have opened the possibility of the "conversion" of feed varieties to malting varieties.
Keel was used as the recurrent parent to produce backcross lines containing the key quality genes from international malting lines, Alexis, Haruna nijo and AC Metcalfe. The BC_1 and BC_2 generations for each introgression stream were screened using molecular markers for a range of malt quality loci. These populations were evaluated in double row trials in the 2002 and 2003 season. Agronomic selection was applied to identify individuals exhibiting the Keel phenotype. The subsequent grain samples were evaluated for grain size and Near Infra Red (NIR) predicted malt quality. Preliminary results for the BC_1 generations suggest that the strategy has successfully introgressed a range of malt quality loci and has largely retained the characteristics of the recurrent parent. Populations derived from crosses between the introgression streams have also been developed, to pyramid the malt quality genes from the three international varieties into a Keel genetic background.
Preliminary agronomic and malting quality profiles of this germplasm will be presented, and the prospects for developing malting quality barley using this novel breeding strategy will be discussed.
Keywords: malting quality; breeding; SSR; STS

Introduction

Malting quality in barley is considered to be a complex genetic trait. As such, breeding programs have typically applied conservative strategies for the introgression of traits from non-malting germplasm. However, these strategies intrinsically limit the rate of genetic gain for adaptation. The drought conditions in Australia in 2002 have again highlighted the superior adaptation of Australian feed varieties, particularly in low rainfall environments.

This paper presents preliminary results from a novel strategy that tests a new paradigm for breeding malting barley. The project aims to introduce key malting quality genes from a range of elite international malting varieties into the well adapted feed variety Keel, while maintaining the superior adaptation and agronomic profile of the feed barley. This strategy suggests that the genetics of malting quality is now better understood than the genetics of adaptation.

Keel is a high yielding, well adapted Australian feed variety (released by SABIP 1999) and was used as the recurrent parent to produce backcross lines derived from Alexis, WI3284 and WI3405. These Waite breeding lines were derived from Haruna nijo and AC Metcalfe respectively. Individuals were screened with molecular markers and selected for key malt quality loci including malt extract on chromosomes 1H, 2HS, 2HL and 5H, (COLLINS *et al*. 2003) and the ß-amylase gene on 4H which influences diastatic power and fermentability

(EGLINTON *et al*. 1998). In the case of the Alexis cross, the opportunity was also taken to select the Alexis genes for resistance to powdery mildew and leaf rust (PARK *et al*. 2003), to further improve the agronomic profile of Keel.

The subsequent populations were evaluated in double row trials in the 2002 and 2003 season. Agronomic selection was applied to identify individuals exhibiting the Keel phenotype, the subsequent grain samples were evaluated for grain size and NIR predicted malt quality. Elite lines identified in the double row trials advanced to yield trials the following year, where further agronomic selection and micromalting will be performed.

In conjunction with the assessment of the individual streams genetically and the progressive agronomic and malt quality performance, elite lines from the three introgression streams were intercrossed. This strategy aims to pyramid the malt quality genes from the international malting varieties into a Keel genetic background.

Material and Methods

BC_1 and BC_2 generations of the three malt donor parents using Keel as the recurrent parent, were crossed at different times, hence some are more advanced than others. The BC_1F_1 generations were screened using molecular markers for a range of malt quality loci. Selected individuals were screened as BC_1F_2 single plants to identify individuals homozygous for the target loci. The subsequent populations were evaluated in a double row trial at Strathalbyn in the 2002 season. Agronomic selection was applied to identify individuals exhibiting the Keel plant type, the subsequent grain samples were evaluated for grain size and NIR predicted malt quality. Elite lines were identified and promoted for further agronomic evaluation in yield plots in the 2003 season. Selected BC_1F_2 individuals were used to develop BC_2 generations for each of the introgression streams. This germplasm is currently being analysed from a double row trial at Strathalbyn 2003, for agronomic and predicted malt quality evaluation.

DNA was extracted using a modified phenol/chloroform extraction method described in ROGOWSKY *et al.* (1991) from plant material approximately three weeks old. Two types of PCR-based DNA markers were employed to marker screen, predominantly simple sequence repeats (SSRs), however sequence tagged sites (STSs) were utilized to select for ß-amylase gene on 4H. SSR samples were loaded on 8% polyacrylamide gels and the STS markers were loaded on 1% TAE gels.

A total of 3783 marker assays were performed to select elite lines from the BC_2 generations for the three malt donor genotypes. Table 1 shows the breakdown of the development of the individual backcross lines. Marker screening was carried out a second time on two F_2 populations derived from one elite Alexis BC_1 and one elite WI3284 BC_1, to identify homozygous alleles. Four Alexis BC_1F_2's and five WI3284 BC_1F_2's were selected and intercrossed, combining the malt quality alleles of Alexis and WI3284. The intercrossed F_1's (Alexis/KeelBC_1//WI3284/KeelBC_1) were marker screened, to select for the Alexis and WI3284 quality alleles and to select against Keel alleles, when markers were not polymorphic between Alexis and WI3284. Eight lines were selected and crossed with eight elite WI3405/KeelBC_2's, to pyramid the malt quality alleles for all three malt donor parents. Approximately 1000 pyramid F_1's were produced and harvested from summer nursery plots 2002-03. The F_2's were culled based on their disease resistance to scald and leaf rust at Turretfield disease nursery in July 2003. Currently the F_3's are growing in an observation summer nursery 2003-2004, where they will be assessed for photoperiod sensitivity. The development of the pyramid lines is also summarised in Table 1.

Table 1. Summary of the development of individual backcross and pyramided lines

Donor Parent	Level of cross	No. F1's	No. marker assays	No. lines selected
Alexis	BC_1F_1	45	315	5
	BC_1F_2	100	400	4
	BC_2F_1	70	420	3
WI3284 (Haruna nijo derivative)	BC_1F_1	32	128	5
	BC_1F_2	100	300	5
	BC_2F_1	118	472	61
WI3405 (AC Metcalfe derivative)	BC_1F_1	65	260	13
	BC_2F_1	496	1488	28
Alexis and WI3284	Alexis/KeelBC_1// WI3284/KeelBC_1	88	704	8
Alexis and WI3284 and WI3405	Alexis/KeelBC_1// WI3284/KeelBC_1 /3/ WI3405/KeelBC_2	1012	-	1000

After MAS, selected lines were multiplied and entered D/Row trials where agronomic and malt quality performance was assessed. Post harvest lines were assessed for grain size and predicted malt quality based on whole grain Near Infra Red (NIR) analysis. Disease assessment was not possible due to the low incidence of diseases caused by the drought of 2002. Following D/Row trials promising lines will enter yield trials, where further agronomic selection and micromalting will be performed. The most advanced feed conversion lines have entered yield trials in 2003. The progress and projected progress for the different feed conversion lines is outlined in Table 2.

Table 2. The projected progress for the different feed conversion lines. Where 2 years have been stated, more MAS was carried out on the next generation.

	Development / Evaluation Stage (Year)				
Germplasm	Crossing	MAS	D/Row trials	Yield trials	MBQIP Stage 2
Alexis/Keel BC_1		2001 / 2002	2002	2003	2004
WI3284/Keel BC_1		2001 / 2002	2002	2004	2005
WI3405/Keel BC_1		2002	2003	2004	2005
Alexis/Keel BC_2	2001	2002	2003	2004	2005
WI3284/Keel BC_2	2002	2002	2003	2004	2005
WI3405/Keel BC_2	2001	2002	2003	2004	2005
Pyramided Lines Alexis/KeelBC_1//WI3284/ KeelBC_1/3/WI3405/KeelBC_2	2002	2002 / 2003	2004	2005	2006

Results

Agronomic and Malt Quality Analysis

3.1 Alexis/Keel BC_1 & BC_2

Nine elite lines were identified from 89 Alexis/KeelBC_1F_3's, derived from one elite Alexis/KeelBC_1F_1. Unfortunately 29 lines from this population were unable to be analysed by NIR, because of their low yield due to drought effects. Approximately 60% of the population exhibited early or moderately early maturity similar Keel. 18% had screenings of or lower than Keel, 76% had a malt extract value of or higher than Schooner (a commercial malting variety) and 23% exhibited diastatic power (DP) higher than Schooner. The promising malt

extract values from this population are significantly greater than Mendelian expectation, demonstrating the effect of marker assisted selection.

Table 3 displays the agronomic and malt quality values for the nine elite lines, including Keel, Schooner, Sloop and Gairdner as controls. One of these lines (BX00;~35) with the key locus for malt extract on chromosome 1H from Alexis, has exhibited a maturity close to that of Keel, low screenings, and extract and DP values higher than Schooner. This line also has powdery mildew resistance and the leaf rust resistance (5H) gene.

Table 3. NIR predicted malt quality and relative maturity for selected Alexis/KeelBC_1F_3 lines grown at Strathalbyn 2002 compared to four control varieties.

Cross	Maturity score	Screenings (%)	Grain Protein (%)	Extract (EBC) (%)	DP (*)	Alleles
BX00;~92	4	14.23	14.22	76.93	502	Bmy, mlo, 5HLR
BX00;~51	4	16.67	14.54	76.80	506	1Hext, 1Hext, 5HLR
BX00;~82	4	8.48	13.94	77.13	459	1Hext, mlo, 5HLR
BX00;~38	4	16.02	14.29	77.04	464	Bmy,1Hext, 5Hext, mlo, 5HLR
BX00;~35	4	16.46	15.20	76.70	571	1Hext, mlo, 5HLR
BX00;~91	3	11.4	12.97	77.74	387	Bmy, 5Hext, mlo, 5HLR
BX00;~84	3	9.69	12.95	77.80	402	1Hext, mlo, 5HLR
BX00;~33	3	19.24	14.27	77.33	491	1Hext, 5Hext, mlo, 5HLR
BX00;~86	3	13.01	13.34	78.39	441	5Hext, mlo, 5HLR
Keel	3	11.96	14.23	74.77	451	
Schooner	5	9.21	15.51	75.70	535	
Sloop	5	11.36	16.21	76.74	668	
Gairdner	7	20.78	15.46	77.70	565	

Pedigree for selected lines - Keel/Alexis//Keel

* μmoles of maltose equivalents released per minute per gram dry weight

The three Alexis/KeelBC_2F_2 lines, included in 2002 double row trials, exhibited significant variation within plots. However one potential line (Table 4) has the desired Alexis quality alleles, the maturity of Keel, low screenings and malt extract higher than Sloop.

Table 4. NIR predicted malt quality and relative maturity for the selected Alexis/KeelBC_2F_2 line grown at Strathalbyn 2002 compared to four control varieties

Cross	Maturity score	Screenings (%)	Grain Protein (%)	Extract (EBC) (%)	DP (*)	Alleles
BX01A;025DNA180	3	9.34	12.72	78.03	379	Bmy,1Hext,2HLext,mlo
Keel	3	8.64	13.16	75.64	382	
Schooner	5	7.99	15.04	76.95	506	
Sloop	5	13.22	14.46	76.75	528	
Gairdner	7	22.11	14.73	78.13	553	

Pedigree for selected line - Keel/Alexis//Keel-4821/3/Keel

* μmoles of maltose equivalents released per minute per gram dry weight

3.2 WI3284/Keel BC_1

Over half of the 91 WI3284/KeelBC_1F_3 s derived from an elite WI3284/KeelBC_1F_1, have the same maturity as Keel and almost a quarter have screening percentages similar to Keel or

lower. An impressive 90% of this population have extract values greater than Schooner, with 56% having values greater than Sloop, possibly reflecting the impact of the thin husk trait from Haruna nijo on NIR predicted malt extract. The DP values however are less impressive, with 10% having a DP greater than Sloop.

Eleven out of 91 lines have been selected for promotion to yield trials, all have some of the desired WI3284 quality alleles and an extract higher than Sloop, shown in Table 5. BX00;~150 is one of these lines, it has the key WI3284 malt quality alleles, the maturity of Keel, excellent screenings, and an extract value greater than that of Gairdner.

Table 5. NIR predicted malt quality and maturity scores relative maturity for the selected WI3284/KeelBC_1F_3 lines grown at Strathalbyn 2002 compared to four control varieties

Cross	Maturity Score	Screenings (%)	Grain Protein (%)	Extract (EBC) (%)	DP (*)	Alleles
BX00;~156	3	9.47	13.37	77.49	422	Bmy,1Hext
BX00;~183	3	10.07	12.87	77.75	385	Bmy,1Hext
BX00;~152	3	5.64	12.83	77.81	389	Bmy,2HSext, 2HLext
BX00;~159	3	9.58	13.33	77.79	427	Bmy,2HLext
BX00;~155	3	6.73	14.33	77.50	486	Bmy,1Hext, 2HLext
BX00;~142	3	7.52	12.82	78.23	401	Bmy,1Hext,2HSext,2HLext
BX00;~144	3	7.69	11.95	78.67	327	Bmy,1Hext, 2HLext
BX00;~173	3	9.97	12.86	78.35	382	1Hext,2HLext
BX00;~150	3	9.26	13.06	78.34	425	Bmy,1Hext,2HSext
BX00;~184	3	9.42	13.63	78.18	442	Bmy,2HLext
BX00;~145	3	8.31	11.20	79.32	314	Bmy,1Hext,2HLext
Keel mean	3	10.01	13.66	75.33	417	
Schooner mean	5	14.58	16.49	75.06	574	
Sloop mean	5	13.63	14.95	76.53	522	
Gairdner mean	7	20.78	15.46	77.70	565	

Pedigree for selected line - Keel/WI3284//Keel

* μmoles of maltose equivalents released per minute per gram dry weight

3.3 WI3405/Keel BC_1

Table 6. NIR predicted malt quality and relative maturity for selected WI3405/KeelBC_1F_2 lines grown at Strathalbyn 2002 compared to four control varieties.

Cross	Maturity score	Screenings (%)	Grain Protein (%)	Extract (EBC) (%)	DP (*)	Alleles
BX01A;033DNA20	4	14.38	13.97	76.17	456	Bmy,1Hext,2HLext,5Hext
BX01A;033DNA62	4	15.29	12.89	76.75	375	Bmy,1Hext, 5Hext
BX01A;033DNA58	4	12.75	13.42	76.58	414	Bmy,1Hext, 2HLext
Keel	3	7.51	13.60	75.64	366	
Schooner	5	7.99	15.04	76.95	506	
Sloop	5	13.22	14.46	76.75	528	
Gairdner	7	22.11	14.73	78.13	553	

Pedigree for selected line - Keel//WI3405/Keel

∗ μmoles of maltose equivalents released per minute per gram dry weight

Three potential lines carrying WI3405 quality alleles were identified out of 13 WI3405/KeelBC_1F_2 lines (Table 6). The DP and malt extract values are higher than Keel, but lower than Schooner. These BC_1 lines are one generation behind the Alexis and WI3284 BC_1F_3 populations, hence there would be greater genetic variation within each line. The three promising lines will be evaluated in yield plots and subjected to single plant selection, to generate more homogeneous lines for subsequent evaluation.

Discussion

Conventional barley breeding programs have traditionally attempted to introgress improved adaptation into elite malting quality genetic backgrounds. Despite considerable progress, there is still a significant gap between feed and malting varieties in terms of general adaptation, disease resistance profiles and yield potential. Progress in QTL mapping across a range of germplasm has improved our understanding of the complex genetics of malting quality and delivered molecular markers for key genomic regions. These tools have had a significant impact on breeding within traditional breeding methodologies. In addition, these tools provide an opportunity to attempt more aggressive plant breeding strategies.

The level of polymorphism between the donor and the recurrent parent was a limiting factor in this strategy, sometimes preventing the use of more than one molecular marker and the use of flanking markers to select desirable malt quality QTL. This was an especially limiting factor in the pyramid populations, when markers were used to retain desired malt quality QTL. This could increase linkage drag of unwanted donor genotype and the donor genotype could have a deleterious effect the overall adaptation of the variety.

The approach outlined in the current paper aims to perform a complete quality conversion, from feed to malting quality, while retaining the agronomic advantage of the feed variety. The preliminary results suggest this strategy has successfully introgressed a range of malt quality QTL and largely retained the characteristics of the recurrent parent. However 2002 was a season of severe drought in Australia and there is need for more typical seasonal data, sites and replication. Hence more detailed characterisation of the malt quality, yield potential and disease resistance profiles of the germplasm, will be required to fully assess the merits of the alternative breeding strategy.

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Construction of a Barley Recombinant Chromosome Substitution Library

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Abstract

Four barley genotypes cvs. Haruna Nijo, Morex, a land race from Western Syria and a *Hordeum spontaneum* were used in a marker assisted backcrossing programme with cv. Chime to produce a series of isolines possessing different segments of donor genome in an otherwise uniform genetic background. Analysis of SSR data over the whole genome for the BC1F1 generations showed that the Chime contribution was approximately 75% but there were regions where the donor contribution was either less or greater than expected. Using GENEFLOW to analyse the SSR data, we were generally able to detect BC1F1 individuals that were heterozygous at a marker locus but carried entirely recipient alleles at the remaining loci on the chromosome and these were used in BC2 construction. Where such lines were not detected, lines that carried donor alleles in regions remote from the marker in question were chosen for BC2 construction. Because we do not know exactly where cross-overs have occurred, we also used lines that carried donor alleles for two adjacent markers in the BC2 construction. This strategy minimised the chances of missing donor segments of the genome. On average, prior genotyping of the population reduced the amount of crossing by 67%.

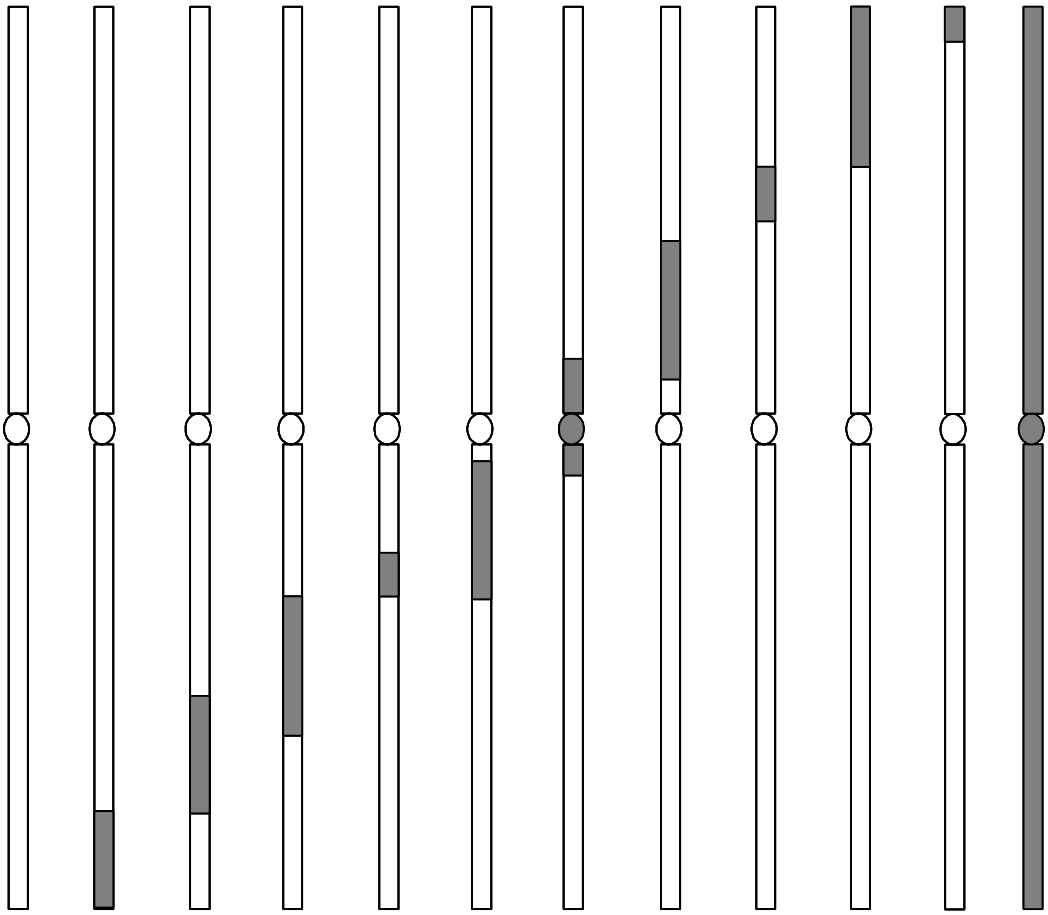
Keywords: barley; introgression; marker-assisted selection; mapping; isolines

Introduction

Whilst molecular markers have been developed and used successfully for some major-gene traits, the results from barley QTL mapping studies have, so far, not been widely taken up by plant breeders, particularly those in the commercial sector (THOMAS 2003). There are a number of reasons for this lack of uptake but it is clear that, for complex characters such as yield and quality, there are either no obvious targets or the studies so far have merely identified regions that are approaching fixation in elite germplasm. Multi-variate QTL studies have also highlighted the fact that there are often QTL "hot-spots", where a region is associated with effects upon a number of characters. More often than not, a particular allele in this region may produce the desired effect upon one character but an undesired effect upon another. Current QTL mapping methodology cannot identify whether these effects are due to close linkage or to the pleiotropic effects of one locus. Another problem is that current QTL mapping procedures do not reliably estimate effects at individual QTL loci, although methods have been developed to restrict the amount of bias (UTZ *et al.* 2000).

One way to solve this problem is to design alternative populations in which to estimate QTL effects. PATERSON *et al.* (1990) proposed the development of a series of isolines in which small segments of a donor genome are introgressed into a common recipient to give a library of lines which covers the whole donor genome. Such isolines are called Recombinant Chromosome Substitution Lines (RCSLs), which are depicted graphically in Figure 1. Marker-Assisted Selection (MAS) can be combined with backcrossing to select individuals that represent a tiled series of introgressed segments from a donor. Positive selection can be practised to maintain the donor segment at the target locus with negative selection to progressively eliminate donor segments at other loci. RCSLs enable fine mapping of the genome and will solve many of the problems identified above. In *Brassica oleracea*, phenotypic and genotypic analysis of RCSLs has not only confirmed the location of QTLs revealed by mapping studies but has also identified extra loci (RAE *et al.* 1999). A similar approach has been developed for *Arabidopsis thaliana* in which markers are used to first

Figure 1. Diagrammatic representation of Recombinant Chromosome substitution lines for one chromosome. Shaded portions represent overlapping introgressions of varying length that provide a tiled representation of complete introgression.



identify single chromosome introgressions, followed by further MAS to progressively eliminate greater segments of the introgression starting from one telomere. This approach is called Stepped Aligned Inbred Recombinant Strains (STAIRS) (KOUMPROGLOU *et al.* 2002). We describe the development of a series of barley RCSLs designed to distinguish between linkage and pleiotropy in the genetic control of performance characters in key regions of the barley genome. We believe that RCSLs will be valuable tool in the identification of those QTLs that are sub-significant in QTL mapping studies but can distinguish the very best genotypes from a pool of elite germplasm. We will also be able to investigate the economic value of novel alleles contained by some of the donor genotypes.

Our initial goal is to develop 'coarse focus' sets of RCSLs with large portions of genome introgressed from each donor, using our library of SSR markers to monitor the process. We then propose to construct 'fine focus' sets of lines to closely study a target area of the barley genome. We will make these sets of 'coarse' and 'fine focus' lines available to the wider barley research community for further study and development.

Material and Methods

The two-row spring barley cv. Chime was chosen as the recipient genotype for the RCSL crossing programme. Chime was bred by Nickersons Seeds from the cross 92.25 x Heron and is known to have the *sdw1*, *mlo11* and *eph* genes for semi-dwarf stature, mildew resistance and non-production of epiheterodendrin respectively. Chime was placed on the HGCA/CEL recommended list between 2000 and 2002 inclusive and, at the time of its first

recommendation, had the third highest level of hot water extract corrected to 1.5% N (HWEc) of all the recommended cultivars. Despite this, Chime failed to gain commercial acceptance and was removed from the recommended list. We chose the four barley genotypes listed in Table 1 as donors. With the exception of Morex, all were two row genotypes.

Table 1. Donor genotypes used in production of Recombinant Chromosome Substitution Lines together with their origin and characteristics

Donor	Origin	Characteristics
Haruna Nijo	Japan	Photo period insensitive, Thermostable β-amylase
Morex	USA	6-row, High DP malting cultivar
WS241	Syria	Genotypically divergent landrace
Mehola	Israel	Genotypically divergent wild barley

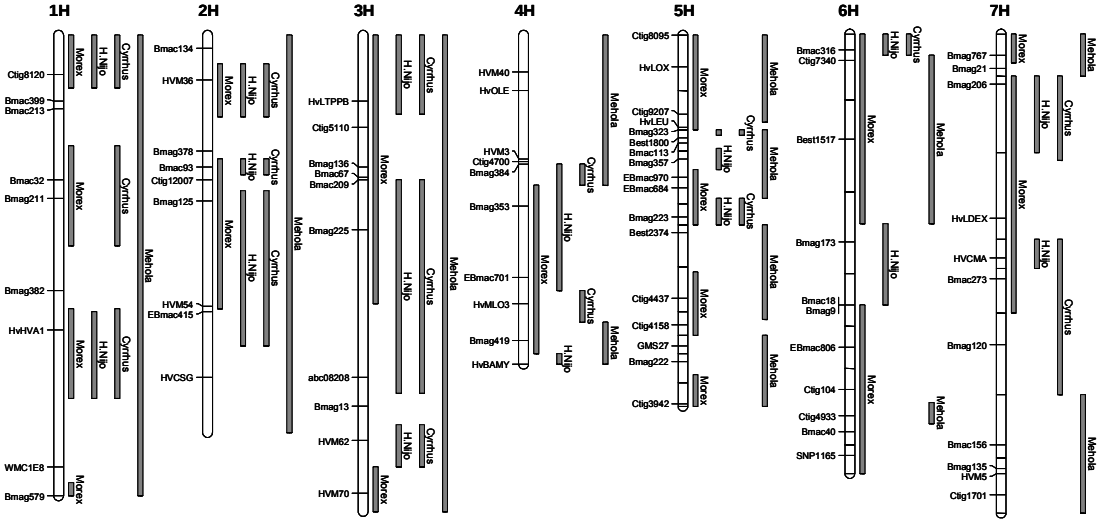
Each donor was crossed to Chime to produce the F1, which was then backcrossed to Chime to give the BC1 generation. BC1 individuals were then genotyped with a range of SSR markers from SCRI's library of genomic SSRs, together with some markers derived from ESTs that are listed as Best or Ctig SNP (Figure 2). GENEFLOW (Geneflow inc, USA) was then used to identify individual BC1 genotypes that carried the fewest donor alleles but were heterozygous at each SSR locus in turn and therefore carried the donor allele but, where possible, only had recipient alleles at neighbouring loci. These individuals were then re-crossed to Chime to produce the BC2 generation. As resources proved limiting, BC2 seed of the Cyrrhus and Haruna Nijo series was shelved to concentrate resources upon the other two donors. Up to eight BC2 individuals from each selected BC1 were re-genotyped with SSR markers from Figure 2 selected according to polymorphism in the relevant BC1.

To facilitate map construction, the BC1 generation of the Morex and Mehola series were also genotyped with a series of AFLP primers that had already been used to genotype the Steptoe x Morex population and had established map locations. Twenty primer combinations were used to survey the parents and 13 and 10 were chosen to run over the Mehola and Morex BC1 populations respectively.

Results

The expected differences between the donor and recipient parents according to SSR polymorphisms are depicted in Figure 2, which shows that the wild barley from Mehola differs completely from Chime for the whole of chromosomes 1H, 2H and 3H. It is also quite different over much of the remaining four chromosomes with the least differences on 6H and 7H. There are also considerable differences between the Morex donor and Chime, notably on 5H with fewest differences on 4H. The differences between the Cyrrhus and Haruna Nijo donors from Chime are apparently less from Figure 2 but the BC1 of these was less comprehensively genotyped compared to the other two donors. The AFLP primer combinations generated 80 and 68 polymorphic bands in the Mehola and Morex BC1 populations respectively. Five of the primer combinations were common to both populations but there were very few instances of bands of the same size being generated by the same combination from the Morex and Mehola series. This limited the value of the AFLPs as markers for generating a composite map. Even fewer bands were of the same size as those detected as polymorphic between Steptoe and Morex, which means that there was little opportunity to use known AFLP map locations to guide formation of linkage groups and aid RCSL development. The inclusion of the AFLPs did facilitate the bridging of linkage groups within each population and the map positions of the SSR markers were largely as expected from existing maps (COSTA *et al.* 2001; MEYER *et al.* 2001; RAMSAY *et al.* 2000).

Figure 1. Location of genomic SSR and EST derived markers used in development of Recombinant Substitution Lines. Shaded bars indicate regions where each of the four donors is polymorphic with Chime.



Over all the SSR markers, the percentage recipient genome in the BC1 generations varied from 75 to 77% i.e. in accordance with genetical expectations. There was a slight trend of more donor genome persisting in the landrace and wild barley but the difference in amounts between these and the two cultivars was very slight. Within these figures, there was considerable fluctuation with up to 90% recipient genome recovery in Morex detected by HvLEU but just 58% recovered in Cyrrhus as detected by Bmac93. Considering the individual genotypes within each series, the maximum recipient genome was over 91% for all series apart from the wild barley Mehola, which was much lower at 87%. The minimum recipient genome varied from 59 (Morex) to 68% (Haruna Nijo). Thus, within a relatively small sample of BC1 individuals, we were able to detect genotypes equivalent to the BC3 generation in terms of their donor genome content.

The initial genotyping of the BC2 generation was targeted to markers on chromosome 5H as we wished to develop a 'fine-focus' library for this chromosome. The genotyping therefore enabled us to select suitable individuals for re-crossing to Chime to produce BC3s. Genotyping of the BC2 for the remaining markers is still in progress but preliminary results reveal that the average number of heterozygous loci per BC2 line was just under five with up to 15 heterozygous loci in one BC2 line. Just under 1% of the lines are heterozygous at one marker locus only and just over 2% are heterozygous at two marker loci. These lines are therefore ideal candidates for production of individual RCSLs, although a number of these are multiple samplings of the same region. The lines selected for RCSL production will be selfed and genotyped at the target heterozygous locus to identify individuals that are homozygous for the donor segment. These lines will then be multiplied as bulks up to the F4 generation and single plants selected from each bulk and genotyped to confirm that they are indeed individual RCSLs. Aberrant single plants will be discarded and the remainder re-bulked to form a nuclear stock for further multiplication to produce sufficient seed for a replicated trial series.

Discussion

We have made significant progress in the development of two RCSL libraries of barley, one with a wild barley donor and one with a contrasting cultivated barley donor. We have identified a number of single segment introgressions and will commence an inbreeding programme to fix these and will continue development until we have a complete library of

single segment introgressions that cover the genomic SSR and EST polymorphic markers that we have sampled. As recombination could have taken place anywhere in the interval between adjacent marker loci, we are also developing introgressions that include both loci to ensure that the library has as full a genome coverage as possible. As more EST derived markers become available, we will endeavour to locate these on individual RCSLs and, if necessary, conduct further backcrossing so that our libraries are isogenic for various functional genes and can therefore be used to evaluate the effect of allelic differences at such loci upon phenotype. RCSLs do appear to have value in identifying QTLs for economically important traits as another set has been developed using the Canadian 2 row spring malting barley Harrington as the recipient and a *Hordeum spontaneum* accession from Caesarea as the donor. QTLs for characters such as yield and malting quality were detected in the Harrington / Caesarea set, some of which corresponded with the locations of previously reported QTLs but others that were in apparently novel locations (MATUS *et al.* 2003). In such cases, the desirable allele came from the cultivated parent so the novel locations may represent alleles that have been progressively eliminated from the cultivated gene-pool but more extensive studies are required before any firm conclusions can be reached. The lack of any beneficial alleles for yield from the wild barley contrasts with results from an Advanced Backcross QTL analysis in barley where the wild barley was found to contain desirable alleles for yield (PILLEN *et al.* 2003). What will be of more value for QTL deployment will be to demonstrate that RCSLs have the potential to identify the smaller effect loci that are beyond the power of detection of conventional QTL mapping studies but are being manipulated through phenotypic selection in elite breeding programmes.

There are some interesting features in the polymorphic loci in the Chime / Morex RCSL set. Chime and Morex carry the same allele at abc08028 on chromosome 3H but only Chime carries the *sdw1* dwarfing gene that is located within 1cM of the marker (RAMSAY *et al.* 2004). This suggests that Morex may share some of its ancestry with the Moravian barley Valticky but further evidence may be gleaned from additional genotyping, particularly proximal to *sdw1*. Chime and Morex are clearly polymorphic in the region of *vrs1* on chromosome 2H but, surprisingly, appear to be monomorphic for the short arm of chromosome 4H where *int-c* is located. It is also curious that Chime and Morex appear to share alleles on the long arm of 7H, although the cause of this is unknown. In addition, Morex is a producer of epiheterodendrin (data not shown) but carries the same Bmac399 allele as Chime. Clearly the genomic SSRs that we have used are not an adequate representation of the functional diversity that exists between barley cultivars, which has motivated our switch to including EST derived markers in the RCSL development programme.

The use of markers to select appropriate BC1 individuals for further crossing has greatly streamlined the RCSL development programme, reducing the amount of crossing and subsequent genotyping by approximately 67%. The use of MAS at the BC1 stage probably also contributed to finding some 9% of the BC2 individuals heterozygous at just one donor locus. Like the development of the Harrington / Caesarea set (MATUS *et al.* 2003), we found some BC2 individuals (4%) with no donor alleles but we conclude that this is most likely the result of segregation within BC2's derived from a BC1 individual with minimal donor genome. In conclusion, we are nearing the end of the development of a tiled isogenic series of RCSLs that we believe will be of great value to the worldwide barley research community in relating genotype to phenotype as well as providing genetically defined isolines for physiologists and biochemists to unravel the complex pathways and processes that lead to performance characters such as yield and malting quality.

Acknowledgements
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S 4 – BIOCHEMISTRY, CYTOGENETICS, TRANSGENIC BARLEY

Production of Recombinant Gelatin in Barley

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Abstract
Gelatin, derived from collagen, is an attractive mammalian protein to be used as model for the commercial production of complex heterologous proteins in plants. Collagen requires post-translational modifications and triple helical assembly. The availability of a plant-derived product will provide a safer, more homogeneous product than the current animal-derived material. The aim of the project is to develop a production system for the accumulation of recombinant gelatin and of recombinant collagen for conversion to gelatin using barley seed. Two strong transcriptional promoters will be used for production of recombinant collagen and gelatin in transgenic barley during germination and maturation of barley seeds. The 5'-end of the Cocksfoot mottle virus (CfMV; genus Sobemovirus) genomic RNA sequence, called CfMV ε-element, has been shown to enhance recombinant protein synthesis in barley. The ε-element will be used to study whether accumulation levels of complex mammalian proteins can be further increased, using collagen and gelatin as models that will serve as basis for exploring the expression of other complex proteins and the commercial production of recombinant gelatin from plants. In order to achieve proper post-translational modification, mammalian prolyl-4-hydroxylase will be co-expressed with collagen in barley seeds.
Keywords: transgenic barley; recombinant gelatin; *Hordeum vulgare*

Gelatin

Recombinant gelatin is currently being developed for use in plasma expander applications and in capsule/tablet manufacturing. The production of recombinant gelatin and of recombinant collagen for conversion to gelatin has been demonstrated in several recombinant eukaryotic expression systems (mammalian, plant, insect cell culture, yeast). The availability of recombinant gelatin produced in barley could enhance the introduction of plant-derived recombinant gelatin in various medical and pharmaceutical applications.

Constructs

The collagen gene to be used for expression of recombinant gelatin in barley contains the complete collagen I alpha 1 chain or sections of the helical region of the collagen I alpha 1 chain, the N- and C-terminal telopeptides, and a C-terminal propeptide. This gene, optimized for monocot expression, is fused to the Cocksfoot mottle virus (CfMV) epsilon-sequence (WO 01/55298 published 2 August 2001), known to be a translational enhancer, a signal sequence, and an ER retention signal. The human prolyl-4-hydroxylase required for increased stability of the expressed collagen contains two subunits. Accumulation of both subunits of prolyl-4-hydroxylases using genes optimized for monocot expression is also targeted to the ER. The aim is to co-express the collagen gene together with the prolyl-4-hydroxylase genes

to produce and accumulate hydroxylated, processed and assembled collagen for conversion to gelatin.

Transformation of barley will be carried out with two types of constructs:
1. Collagen/gelatin gene linked to a promoter, which is active during germination
2. Collagen/gelatin gene linked to a promoter, which is active during seed maturation.

Translational Enhancer Sequence

Plant viruses provide control elements having useful activities to enhance the production of heterologous proteins. The 5'untranslated regions of several plant viruses are able to enhance mRNA translation efficiency. The genome of *Cocksfoot mottle virus* (CfMV; genus *Sobemovirus*) is a positive stranded RNA molecule of 4082 bases (MÄKINEN *et al.* 1995). High amounts of CfMV viral particles accumulate in the cereal hosts suggesting that the CfMV genome contains elements for efficient gene expression in cereal cells. We have shown that the CfMVε, the untranslated leader sequence of CfMV RNA, enhances reporter gene expression in tobacco and in barley (WO 01/55298 published 2 August 2001).

Expression in Barley Seeds

Barley (*Hordeum vulgare* L.) is one of the most important cereal crops worldwide and especially relevant to northern European regions. It is the major cereal crop in Finland. Generally the main use of barley is for animal feed but about fifteen percent of the world annual barley crop is used for malting and brewing. Barley stores large amounts of protein and starch in an endosperm that undergoes programmed cell death. Barley seeds can be stored for 5 to 10 years. The first gene transfers to barley aiming at commercial applications, *e.g.* improvement of malting and feed quality, have been published recently (e.g. NUUTILA *et al.* 1999). These studies have clearly demonstrated the concept that active, heterologous enzymes can be produced in transgenic barley plants and barley grains, thus indicating that barley seed is a suitable host for recombinant protein production. We have also developed a transient expression system for barley cells that allows us to evaluate different constructs and to optimize expression.

Biosafety

In developing our technology we will aim at clean transformation technologies, antibiotic-free transgenic plants, and tissue specific and inducible expression. Barley is a self-pollinating, diploid plant that will not cross with any known wild plant. It has been shown in field trials that gene flow through pollen dispersal is extremely low in barley cultivation, and that the cultivation of special varieties of barley can be sufficiently controlled in Nordic conditions. Thus, barley can be considered as a suitable host for production of novel recombinant proteins (RITALA *et al.* 2002). Malting barley has been traditionally bred for high enzyme production capabilities during germination. Taking advantage of this property, heterologous proteins could be produced in large scale with germination specific promoters. In addition, the fact that the recombinant protein product is not present in the transgenic seed prior to germination (i.e. in the field and in the storage) is an important biosafety issue.

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Differential Expression Proteomics in Barley and Its Wild Relatives

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Abstract

Proteome approaches prove important in studying differential expression of genes. For differential analysis of protein expression in barley and its wild relatives, we used and compared two methods, two-dimensional gel electrophoresis (2-DE) and isotope-coded affinity tagging (ICAT) analysis. In the 2-DE analysis searching for self-incompatibility (*S*) allele-specific proteins from the pistil of *Hordeum bulbosum*, we detected 4 protein spots that were specific to individual *S* genotypes and identified those proteins by LC-MS/MS. In contrast, we found much more peptide fragments that could be specific to each genotype by using the ICAT analysis coupled with MALDI-TOF MS. The result demonstrates that ICAT is a powerful tool in investigating differential protein expression and suggests that it will be efficiently applied to profiling protein expression during seed development, malting processes etc. in barley.

Keywords: protein expression; isotope-coded affinity tagging; mass spectrometry; *Hordeum*

Introduction

Large amounts of EST (Expressed Sequence Tag) sequences have been available in barley. A cDNA microarray technique using ESTs is a powerful tool for the large-scale comparative analysis of gene expression. However, gene expression profiles at the mRNA level are often different from those at the protein level. Post-transcriptional modifications and degradations of proteins also influence the expression of genes. These indicate the importance of proteome approaches to study differential expression of genes. Two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) techniques are most commonly used for differential expression proteomic analysis. Recently an alternative technique, isotope-coded affinity tagging (ICAT) has been developed (GYGI *et al.* 1999). In this technique, the proteins from two samples are labeled separately with light and heavy ICAT reagents, combined and digested. Then the ICAT reagent-labeled cysteine-containing peptides are selectively isolated and analyzed quantitatively by MS. This approach enables the identification and quantification of proteins expressed differently between, e.g., genotypes, tissues, developmental stages, etc. As a model study for differential expression proteomics in barley and its wild relatives, we describe here the application of the ICAT method to identify the self-incompatibility (*S*) allele-specific proteins from the pistil of *H.bulbosum*, and compare its feasibility with the conventional 2-DE method.

Material and Methods

Plant Materials and Sample Preparation

Diploid *Hordeum bulbosum* has self-incompatibility controlled gametophytically by two unlinked loci, *S* and *Z* (KAKEDA *et al.* 2000). Progeny plants homozygous for either S_2 or S_3 allele were obtained from the cross S_2S_3 Z_2Z_2 x S_2S_3 Z_1Z_2. Pistils at the flowering stage were collected from 10 plants for each *S* homozygote. Proteins were extracted by homogenizing pistils in Reagent 2 (ReadyPrep Sequential Extraction kit, Bio-Rad) with the addition of DeStreak Reagent (12 µl/ml, Amersham Biosciences), purified using 2-D Clean-Up Kit (Amersham Biosciences) and resuspended in the same solution. Protein concentration of each sample was measured by the Lowry method using DC Protein Assay Kit (Bio-Rad).

Two-Dimensional Gel Electrophoresis (2-DE) and LC-MS/MS Analysis

For each genotype (S_2S_2 or S_3S_3), an equal amount of pistil protein was mixed from 10 plants and a total of 50-100 µg of proteins were used for 2-DE. 2-DE was performed with Multiphor II system (Amersham Biosciences) using pH 4-7 IPG strips and 12.5% SDS gels (ExcelGel 2-D Homogeneous 12.5, Amersham Biosciences). Gels were stained with Silver Staining Kit, Protein (Amersham Biosciences) and genotype-specific protein spots were detected. To identify the proteins, the spots were excised from the 2-D gels after staining with Silver Stain MS Kit (Wako), destained and digested with trypsin (35 C, 20h). The resultant peptides were subjected to LC-ESI MS/MS analysis using a Q-Tof2 (Micromass) mass spectrometer equipped with a MAGIC 2002 HPLC system (Magic C18 column, Michrom BioResources). The spectra were searched against NCBInr and BaEST databases using the Mascot (Matrix Science).

ICAT and MALDI-TOF MS Analysis

The ICAT method using the Cleavable ICAT Reagents (Applied Biosystems) is outlined in Fig. 1. Pistil proteins (100 µg; mixture from 10 plant samples for each genotype) were precipitated with 2-D Clean-Up Kit (Amersham Biosciences), redissolved in 80 µl of the Denaturing Buffer (50 mM Tris-HCl, pH8.5, 0.1% SDS), and denatured by boiling for 10 min after adding 2 µl of the Reducing Reagent (50 mM TCEP). Proteins from S_2S_2 and S_3S_3 genotypes were labeled with cleavable ICAT reagents Light (cICAT Light) and Heavy (cICAT Heavy), respectively, for 2h at 37 C, then combined and digested with trypsin for 16h at 37 C.

Tryptic digest samples were diluted with 10 mM KH_2PO_4/ 20% acetonitrile (ACN), pH 3.0 and separated by cation exchange chromatography using a Polysulfoethyl A column (Poly LC). Peptides were eluted by a gradient from 0-0.5M KCl (Fig. 3a). Fractionated

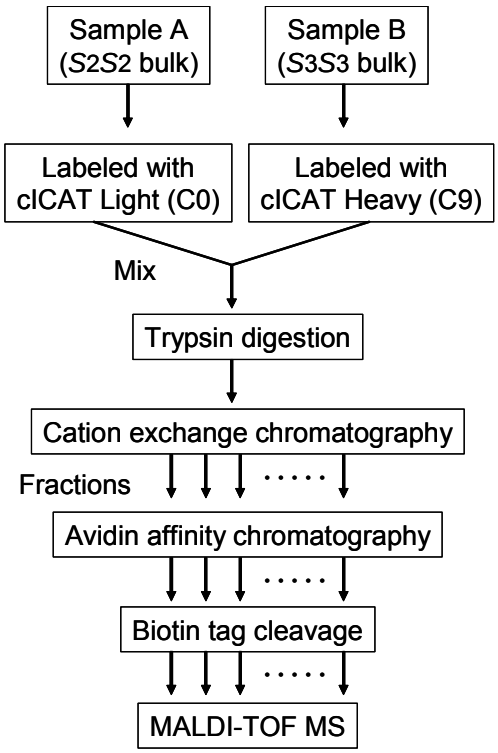

Figure 1. Outline of ICAT analysis used in this study.

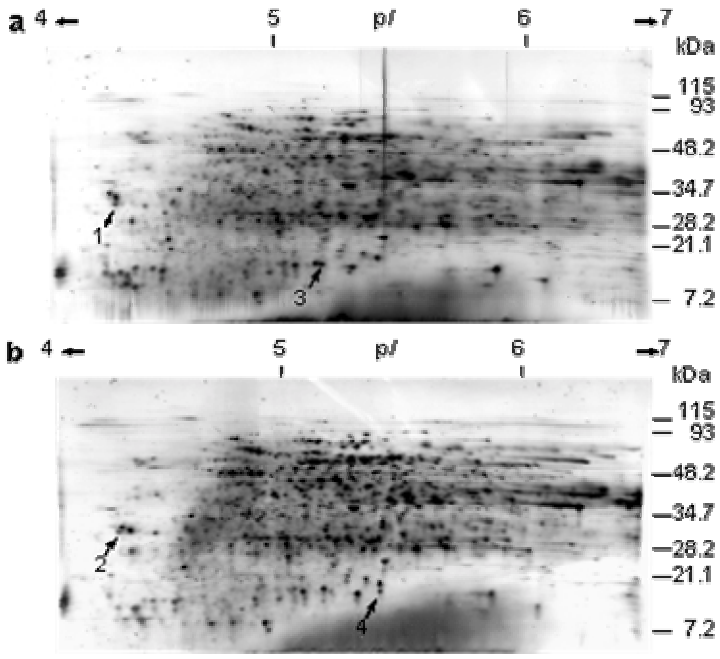

Figure 2. 2-DE profiles of pistil proteins from *H. bulbosum* genotypes (a) S_2S_2 and (b) S_3S_3. Arrows indicate genotype-specific spots (nos. 1-4, see Table 1).

peptides chosen for further analysis were applied to the Avidin Cartridge (supplied by Cleavable ICAT Reagent Kit) to purify labeled-peptides and the affinity tags (biotin) were cleaved according to the manufacture's instruction. The cleaved samples were vacuum-dried, dissolved in 10% ACN/0.1% TFA, and subjected to analysis by MALDI-TOF MS (Voyager-DE STR, Applied Biosystems).

Results and Discussion

The self-incompatibility (*S*) gene in the grasses including *H. bulbosum* has not yet been identified. However, it is predicted that the gene product (S protein) is expressed in mature pistils and anthers, and highly polymorphic among different alleles. To identify the S protein, we decided to search for pistil proteins that are expressed specifically in either the S_2S_2 or S_3S_3 genotype. Fig. 2 shows 2-DE profiles (p*I* 4-7) of pistil proteins from two genotypes. Two profiles were very similar and there were no prominent spots. We could detect only four reproducible spots that were specific to S_2S_2 (nos. 1 and 3) or S_3S_3 (nos. 2 and 4) as indicated by arrows in Figs. 2a and 2b. We also performed 2-DE using a pH 6-11 gel strip for the first dimension but could not observe any genotype-specific spots reproducibly (data not shown). The four specific spots were excised, digested with trypsin and subjected to LC-MS/MS analysis. Table 1 shows the result of protein identification. Spot nos. 1 (S_2–specific) and 2 (S_3–specific) were identified as a single protein homologous to p23 co-chaperone in the

Mascot search against BaEST database, indicating that they were variants of the same protein. For spot nos. 3 and 4, although each MS/MS spectrum matched two different proteins (data not shown). However, because one of them (universal stress protein) was common to both the spots, which showed the same molecular weight on the 2-D gel, we identified them as the same protein. A slight difference in p*I* between them was later confirmed by sequencing the corresponding PCR products amplified from the two genotypes and comparing the p*I*s predicted from their amino acid sequences (data not shown).

Table 1. Protein identification of 2-DE spots by LC-MS/MS analysis

Spot no.	Peptide matched	EST no.	Mr (kDa)	p*I*	Protein homology
1, 2	VYITVQLPDAK LDLNDKVNVEASK SIFCILEK	BaEST102422	20.0	4.81	p23 co-chaperone
3	WVGLAVDFSEGSR AALQWAADNLLR YGVKPDAETLDMLNTIAK LCQAIHDMPISCLVIGSR	BaEST102015	19.2	5.25	Universal stress protein (USP)
4	WVGLAVDFSEGSR SGDNLLLLHVLK YGVKPDAETLDMLNTIAK LCQAIHDMPISCLVIGSR	BaEST102015	19.2	5.25	Universal stress protein (USP)

Molecular weight (Mr) and p*I* value are calculated from the BaEST database entry without any processing. Protein homology is based on BLASTP search.

We conducted ICAT analysis with the same samples as used in 2-DE analysis. Pistil proteins from S_2S_2 and S_3S_3 genotypes were labeled with cICAT reagents Light and Heavy, respectively (Fig. 1). The Heavy reagent is 9 Daltons heavier than the Light reagent, because nine ^{13}C atoms are incorporated into the linker chain of Heavy reagent. The labeling is selective to reduced cysteine residues of proteins, and a biotin affinity tag on the reagent can reduce the complexity by selecting the labeled cysteine-containing peptides. In this study, we fractionated tryptic peptides by cation exchange HPLC and analyzed individual fractions to reduce sample complexity and obtain enhanced sensitivity in MS analysis. Fig. 3 illustrates an example of the ICAT analysis. We obtained 50 fractions of tryptic peptides by cation exchange HPLC (Fig. 3a). It is known empirically that a high peak in fraction 12 is ascribed to ICAT reagents and labeled peptides are eluted from this fraction. Thus, we first selected 3 fractions (nos. 12, 16 and 21) for further avidin affinity chromatography and MALDI-TOF MS analysis. Fig. 3b exemplifies the monoisotopic mass spectrum of peptides from fraction 16. An expanded view (m/z 2060-2260, Fig. 3c) shows a pair of signals that differ by a mass of 9 (m/z 2156.1289 and 2165.1547), which indicate the same peptides labeled with Light and Heavy reagents. As signals observed in pairs like this example, a total of nine pairs were detected and the ratios of Heavy to Light (H/L) in peak intensity were calculated (Table 2a). Five and three pairs of such signals were also detected in fractions 12 and 21, respectively

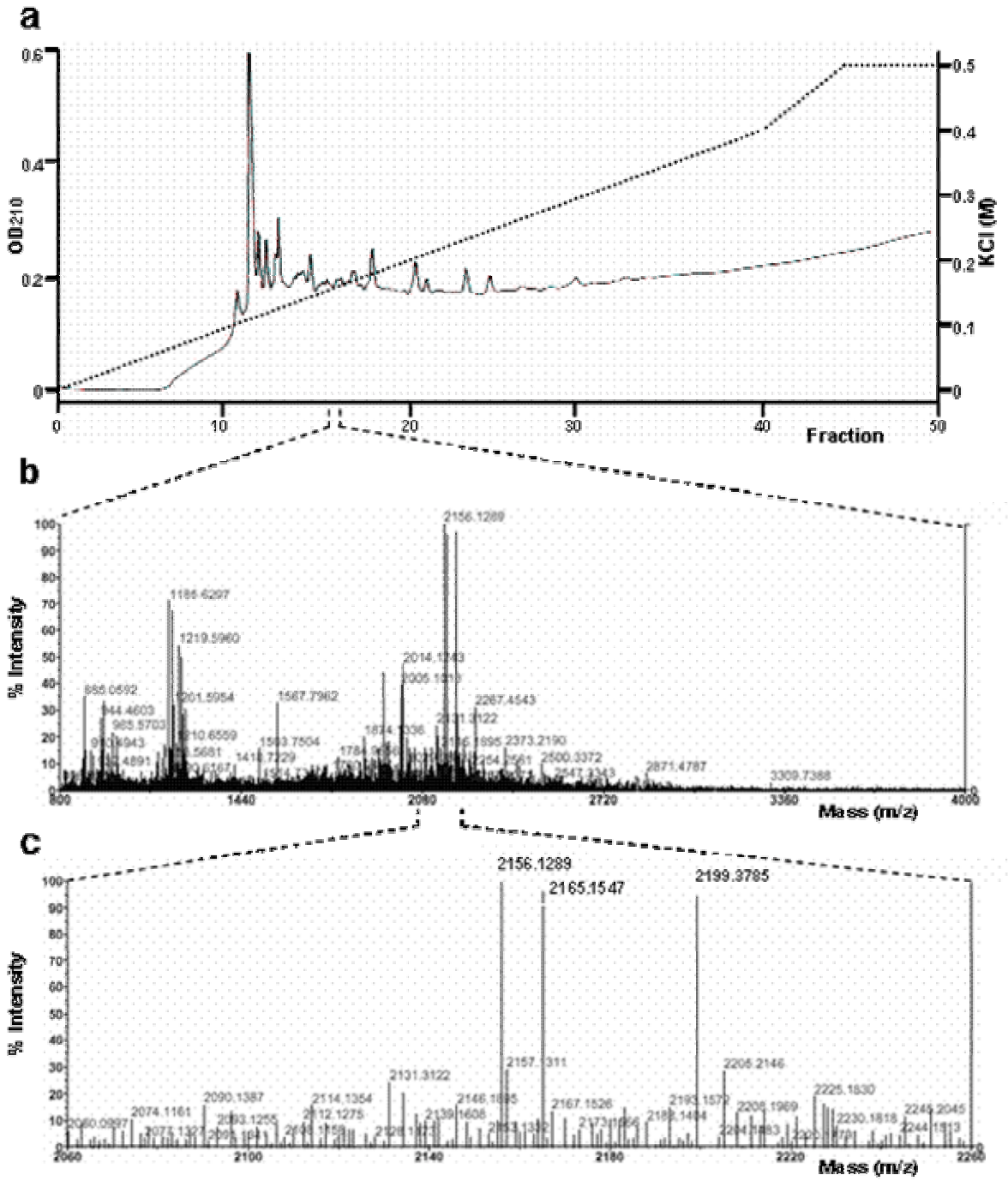

Figure 3. ICAT analysis of pistil proteins in *H. bulbosum*. Light- and Heavy-labeled samples were combined and digested with trypsin. (a) Cation exchange HPLC chromatogram of digested peptides. (b) MALDI-TOF MS spectrum (monoisotopic mass, m/z) of peptides from fraction 16 after avidin affinity chromatography and cleavage of biotin tag. (c) An expanded view of the spectrum from m/z 2060-2260 in b.

Table 2. cICAT-labeled peptide signals analyzed by MALDI-TOF MS

a. Signals labeled with Light and Heavy reagents in pairs

Pair no.	Monoisotopic mass (m/z)		Peak intensity		Ratio*
	Light	Heavy	Light	Heavy	(H/L)
1	1139.6369	1148.6616	13703	18405	1.41
2	1185.6297	1194.6595	91370	86422	1.00
3	1219.5960	1228.6274	69872	64314	0.97
4	1235.5918	1244.6204	36704	38887	1.12
5	1909.0105	1927.0007	14702	19680	1.41
6	1935.0303	1953.1218	13736	22503	1.73
7	2001.0970	2010.0780	30385	48216	1.67
8	2005.1013	2014.1243	50682	60673	1.26
9	2156.1289	2165.1547	128667	123122	1.01

b. Signals with a single peak

Signal no.	Monoisotopic mass (m/z)	Peak intensity
1	951.5995	19595
2	985.5703	27750
3	1001.5643	25718
4	1503.7504	20225
5	1567.7962	41893
6	1942.2265	56217
7	2131.3122	31033
8	2199.3785	124531
9	2267.4543	39600

*In calculation of average intensity ratio of Heavy to Light (H/L), a value of H/0.95 was used for the intensity value of Heavy because ^{13}C isotope content in the Heavy reagent used in this study was reported to be 95%.

(data not shown). The second class of signals checked in this study were signals with a single peak (e.g., a prominent signal (m/z 2199.3785) shown in Fig. 3c), which should be peptides present in either of two samples. Nine such signals were detected in fraction 16 (Table 2b), and 2 and 11 signals detected also in fractions 12 and 21, respectively (data not shown). These signals are rather important than signals observed in L/H pairs, in order to identify proteins highly polymorphic between two genotypes. Although MALDI-TOF MS analysis for the other fractions separated by cation exchange HPLC is in progress, the next step of the study is selecting signals expressed differently between two samples and identifying the peptides and proteins by ESI-MS/MS analysis. Although the ICAT analysis shown in this study is preliminary, it appears to be more powerful than 2-DE analysis in investigating differential expression of proteins. It will also be applied to profiling protein expression during seed development, malting processes, etc. in barley.

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Exclusive Generation of True-Breeding Transgenic Plants via *Agrobacterium*-Mediated Transformation of Barley Pollen Cultures

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Abstract

Methods used to identify and clone genes and promoters which are of scientific interest or have putative relevant function for plant breeding are becoming increasingly powerful. Therefore, reliable and efficient systems for genetic transformation of important plant species are needed for comprehensive functional gene analyses as well as for crop improvement approaches. *Agrobacterium*-mediated transformation of pollen cultures has been used for efficient barley transformation in our lab, yet only a few plants which are true-breeding with regard to the transgene can be directly generated by this method, and tedious as well as time-consuming segregation analyses are necessary to phenotypically distinguish these individuals from hemizygous plants. Pursuing a novel concept, haploid primary transgenic regenerants which appear to be useless in the first instance were routinely subjected to induced genome doubling. As a result, almost all of these plants showed partial or full seed set. Although the inflorescences from many colchicine-treated plants appear to be ploidy-chimaeric, the caryopses obtained are always homozygous for the transgene, since fertile flowers can only be formed from diploid (doubled haploid) cellular origines. Thanks to stable hereditary transmission and expression of the transgene, exclusive generation of true-breeding transgenic plants will substantially contribute to increased efficiency, reproducibility and reliability of overexpression, knock-out and mutant complementation experiments.

Introduction

Immature barley pollen at the microspore or early bicellular stage can deviate from the normal process of pollen formation to undergo embryogenesis. Different kinds of stress application, e.g. cold, heat and/or starvation, turned out to be effective in inducing this developmental pathway. Immature pollen shows by far the highest potential for multiple plant regeneration per barley donor plant: from a single spike, up to 10,000 plants can be generated. In conventional barley pollen cultures, about 80 percent of the regenerants have spontaneously doubled their genome during the first androgenic cell divisions.

Pollen cultures have been used previously for biolistic transformation, but the mechanical impact on the isolated pollen appeared to be too severe, and thus this method turned out to be very inefficient. Compared with direct gene transfer methods, *Agrobacterium*-mediated transformation has the advantage, that the transfer of the foreign DNA is efficiently guided by an evolutionarily developed system, i.e. the piece of DNA to be tranferred is precisely excised out of the bacterial vector, actively transferred into the plant cell, prevented from being fragmented within the plant cell, transported into the plant nucleus, and eventually integrated into actively transcribed regions of the plant chromosomes by a set of bacterial gene products. In many cases, only a single copy of the transgene is integrated per target cell, which ensures expression stability for subsequent generations. On the other hand, agrobacteria have evolved

their system to genetically transform other plants than grasses, which is reflected by the difficulties to employ their potential for these species. There are some reasons to regard *Agrobacterium*-mediated transformation of androgenic barley pollen as a particular challenge: the pollen exine is likely to be a barrier for the agrobacteria, a relatively high population density of agrobacteria is expected to be indispensible to obtain a reasonable frequency of transformation events, and the delicate target cells thus may be overgrown and damaged too heavily. On the other hand, barley pollen cultures have an incomparable regeneration potential, and transformation of haploid cells along with subsequent genome doubling would result in immediate formation of transgenic plants homozygous for the gene integrated.

Material and Methods

Donor plants of the barley cultivar Igri were grown and pollen cultures started according to MORDHORST & LOERZ (1993). *Agrobacterium*-mediated transformation of pollen cultures was performed as was described by KUMLEHN *et al.* (2004). The hypervirulent strain LBA4404 (KOMARI *et al.* 1996) carrying a binary vector with a green fluorescence protein gene under control of the maize ubiquitin promoter was used for transformation. Flow cytometry was conducted by use of a Ploidy Analyser 1 (Partec, Germany) following the instructions of the manufacturer. Standard molecular techniques were performed largely following to SAMBROOK *et al.* (1983).

Results and Discussion

A method for *Agrobacterium*-mediated transformation of pollen cultures was established and transgenic barley has been obtained routinely. As preculture of the isolated pollen for about one week turned out to be necessary for efficient transformation, and spontaneous genome doubling typically happens very early in culture, only a few plants which are true-breeding with regard to the transgene can be directly generated by this method. Moreover, tedious as well as time-consuming segregation analyses have to be conducted to phenotypically distinguish these individuals from hemizygous plants. Furthermore, about 50 percent of the transgenic plants obtained were haploids which seem to be useless in the first instance because they are generally sterile. The advantage of androgenic pollen as transformation target compared with immature embryos (TINGAY *et al.* 1997) thus appeared to be questionable.

The novel concept presented here includes routine identification of the haploid primary transgenic regenerants by flow cytometry at an early stage of development and subsequent artificial induction of genome doubling. We expected exclusive formation of homozygous T_1 plants because fertile flowers can only be formed from diploid (doubled haploid) cellular origines. To proof this approach experimentally, we transformed androgenic pollen cultures of the barley cultivar Igri by use of Agrobacteria carrying a green fluorescence protein gene transcriptionally controlled by the constitutive maize ubiquitin promoter within the T-DNA of the binary vector. Upon flow cytometric analysis and assessment of the transgene copy numbers by Southern blot, we selected single-copy primary transformants which were haploid. As a result of induced genome doubling by use of colchicine, almost all of these originally haploid T_0 plants showed partial or full seed set. Although the inflorescences from many colchicine-treated T_0 plants appeared to be ploidy-chimaeric, all T_1 plants obtained were fertile and all of their progeny (T_2) plants investigated carried the transgene as was

shown by PCR analyses. Hemizygous T_1 plants carrying a single copy of the transgene would result in a three-to-one segregation whereas the homozygous T_1 plants exclusively obtained here lead to uniform expression of the reporter gene in the T_2 generation. Furthermore, sets of T_2 sister plants were analysed by Southern blot and all of them showed a uniform integration site of the transgene. As additional proof of the exclusive formation of homozygous transgenic T_1 plants, we assessed expression of the reporter gene in pollen populations of T_2 plants. Again, this analysis revealed uniform expression of *gfp* in all viable pollen grains.

The method presented here will substantially contribute to increased efficiency, reproducibility and reliability of overexpression, knock-out and mutant complementation experiments in fundamental as well as applied research.

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Mainstem Leaf Development during Tillering in Spring Barley

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Abstract
Time interval between appearances of successive leaves is designated as phyllochron interval (PI). The objective of this study was to evaluate PI of mainstem leaf (MSL) development during tillering of spring barley. Six spring barley varieties were grown in Novi Sad, Serbia and Montenegro (45° 20' N, 15° 51' E, 86m asl) from 1999 to 2001. Phyllochron was estimated using Haun scale where growing-degree-days (GDD) served as time scale. PI ranged from 60.5 GDD in the fifth to 75.8 GDD in the third leaf. All three factors, i.e., year, variety, and their interaction, were included in phyllochron determination of the first leaf. The highest percentage of variance component for first leaf phyllochron belonged to year. Variation in phyllochron of the second leaf was controlled by variety and interaction year x variety, variations in phyllochron of the third, fourth, and fifth leaf by year and interaction, and variation in the sixth leaf by interaction only. The linearity suggests that MSL stage can be used as a predictive measure of plant development and can retroactively show the quality of the preemergent seedbed environment.
Keywords: barley (*Hordeum vulgare* L.); Haun scale; phyllochron

Introduction

Morphological changes of barley plant can be used for the evaluation of both, plant development and growth environment (KLEPPER *et al*. 1982; RICKMAN *et al*. 1983). Mainstem leaf (MSL) appearance and proportion of tiller formation are parameters useful for the evaluation of germination and vegetative development. HAUN (1973) developed a scale which quantitatively describes the vegetative developmental stages in spring wheat. The time spent for leaf elongation, i.e., the time from leaf appearance to the appearance of the next leaf, measured in growing-degree-days (GDD), is designated as phyllochron interval (PI). Fast emergence is positively related with MSL (KRENZER & NIPP 1991). Smaller PI, i.e., lower GDD, represents faster development and leaf appearance. PI is influenced by temperature and daylength (CAO and MOSS 1989a, 1989b). BAKER *et al*. (1986) found a decrease and KENZER *et al*. (1991) found an increase in PI in wheat under water deficit conditions, while BAUER *et al*. (1984) did not establish a dependence of MSL appearance on soil moisture content.
Investigations of DOFING (1995) and SHARRATT (1999) referred to the subarctic environment, while little is known about MSL growth of spring barley in semiarid conditions of growing. The objective of this study was to evaluate MSL development during tillering of spring barleys, which differed in origin, earliness, spike type and some other traits, grown in a semiarid environment.

Material and Methods

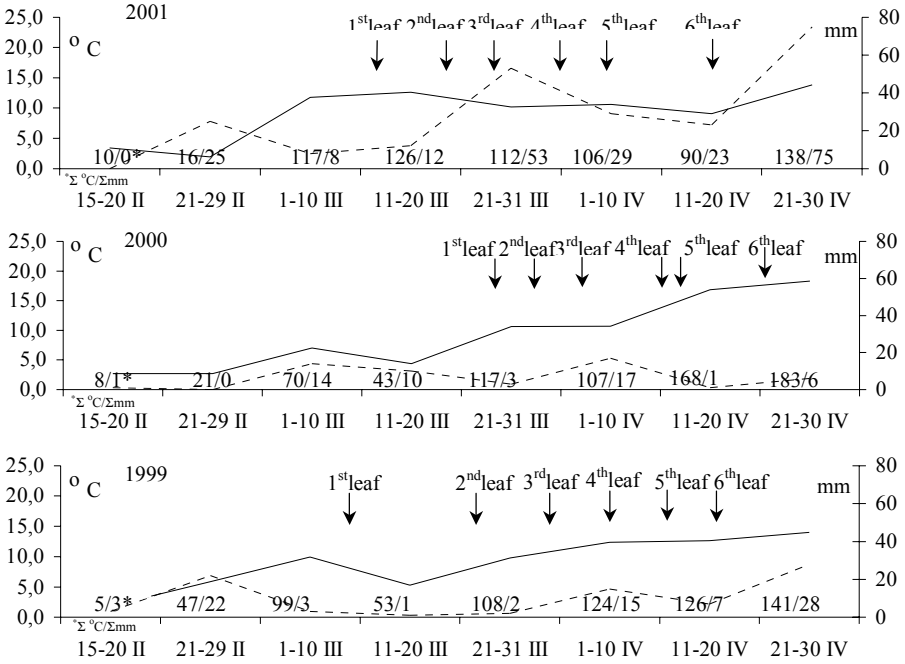
Field trials. Six spring barley varieties were grown in Novi Sad, Serbia and Montenegro (45° 20' N, 15° 51' E, 86m asl) from 1999 to 2001. The experiments were arranged in a randomized complete block design with three replications with plots 2m long and 1m wide, containing 6 rows

20 cm apart. The planting rate was 200 viable seeds per m^{-2}. Three random plants in each plot were labeled for monitoring during growing. Phyllochron was estimated every 2-3 days using Haun scale (HAUN 1973). Growing-degree-days (GDD) were used as time scale with base temperature of 0°C.

PI, measured as GDD per leaf, was the reciprocal of the slope determined by regression of MSL against GDD using data from each plant within a cultivar (BAKER *et al*. 1986). Only MSL measurements greater than or equal to 1.0 were used in the regression because it is difficult to estimate partial emergence of the first leaf since there is no antecedent leaf for comparison.

Statistical analysis. A mixed model was used with varieties considered as fixed and years as random effects (ZAR, 1996). Heritability for a single year was estimated as the ratio $\sigma^2_G/\sigma^2_{G+}\sigma^2_E$, (SINGH *et al*. 1993).

*Weather***.** At the time of planting in 1999, the mean daily temperature was increased and this year had the highest sum (Σ=52ºC) in pre-emergence period (Figure 1). The amount of available water during that period was appropriate. In the period following first leaf appearance, there occurred a temperature decrease and a water deficit. In the last third of March, the temperatures were moderate, with a slow increasing trend, and a water deficit was recorded. In the year 2000, low temperatures persisted till the last third of March, with the lowest temperature sums for 10-day periods among the three years (Figure 1).



*∑ ºC for the period /∑mm of precipitation for the period

Figure 1. Ten-day average air temperature (——) and total ten-day precipitation (-------) in 1999, 2000, and 2001 growing seasons of spring barley in Novi Sad

From the middle of the last third of March, the temperature continually increased. Water deficit was again recorded in this year. In 2001, low temperatures were recorded till the end of February and after that they continually increased till the end of tillering. The periods of emergence and tillering in this year were well supplied with water.

Results and Discussion

ANOVA showed that all three factors, i.e., year, variety, and their interaction, were included in the phyllochron determination of the first leaf (Table 1).

The phyllochron of the first leaf was not precisely defined, since GDD from sowing to first leaf appearance was supposed to be the phyllochron. Year had a lower range of variation than variety (Table 2), although its component of variance was higher. Across cultivars, the lowest phyllochron was in 1999 and the highest in 2001 (Table 3). The Russian six-rowed variety Avans had the longest phylochron interval for first leaf appearance. Interaction GxY occurred in all cultivars; Jelen, Nora, Alexis, and NS-135 had the shortest PI in 1999 while differences in the other two years could not be established. Avans significantly differed in all three years, while Gustoe had longer PI in 2001 than in the previous two years (data not shown). Significance of variety in first leaf appearance was also confirmed by a high value of heritability (Table 1).

Table 1. ANOVA and percentage of components of variance for phyllochron during tillering of six spring barley varieties in 1999-2001 growing seasons

Source of ariation	Df	1st leaf	2nd leaf	3rd leaf	4th leaf	5th leaf	6th leaf
		Square mean					
Year (Y)	2	**	ns	**	**	**	ns
Variety (G)	5	*	**	Ns	ns	ns	ns
G x Y	10	**	**	**	**	**	**
		% of components of variance					
Year (Y)		38	0	22	30	5	0
Variety (G)		31	75	0	0	0	23
G x Y		27	20	72	53	75	49
Error		4	5	6	17	20	28
Heritability		96.7	94.1	89.5	64.4	64.0	80.6

*,** Significant at 0.05 and 0.01 level, respectively; ns - not significant

Table 2. Ranges of cultivar and year mean values for phyllochron during tillering in six spring barley varieties in 1999-2001 growing seasons

Leaf	Variety (n=6)	Year (n=3)	Mean
1st leaf	127.5-202.8	142.4-167.8	155,2
2nd leaf	53.3-125.3	72.1-77.8	74,1
3rd leaf	40.3-114.8	62.8-83.3	75,8
4th leaf	49.2-94.1	66.7-81.6	71,8
5th leaf	41.8-79.0	54.5-63.9	60,5
6th leaf	61.3-86.8	73.8-75.0	74,3

In 1999, high temperatures during emergence caused a faster second leaf development so that it appeared 214.5 GDD after sowing (from Table 4). Low mean daily temperatures and water deficit after first leaf development in that year caused relatively more days for the second leaf appearance in relation to the other two years. Year did not effect second leaf appearance (Table 1) and PI across cultivars ranged from 72.1 in 1999 to 77.8 GDD in 2001 (Table 3). Total sum of GDD from sowing to second leaf appearance was shortest in 1999 (214.5) and longest in 2001 (245.6). Of all six leaves studied, the second leaf was most strongly influenced by genetic constitution of cultivars and 75% of total variation belonged to this component of variation (Table 1). Range of variation for cultivars was from 53.3 (Nora) to 125.2 GDD (Gustoe) (Table 2).

The two-rowed cultivars had shorter phyllochron of the second leaf than the six-rowed cultivars. Thermal requirements for the second leaf were consistent among years in the two-rowed cultivars, while significant differences occurred in all three six-rowed cultivars. High heritability values (Table 1) showed that the established variability for second leaf appearance was under strong genetic control.

Variation of phyllochron of the next three leaves, third, fourth and fifth, was mainly determined by interaction GxY and year (Table 1). In the components of variance, year participated from 5 to 30% and interaction from 53 to 75% of total variation (Table 1). Range for variation for these leaves, similar to the previous two, was larger among years than among cultivars (Table 2). The shortest PI for these three leaves was registered in 2000.

Table 3. Means of phyllochron across cultivars and years in GDD during tillering of six spring barley varieties in 1999-2001 growing seasons

Year	1st leaf	2nd leaf	3rd leaf	4th leaf	5th leaf	6th leaf
	GDD					
1999	142.4	72.1	83.3	81.6	63.9	75.0
2000	155.5	72.4	62.8	66.7	54.5	74.0
2001	167.8	77.8	81.5	67.2	63.1	73.8
$LSD_{0.05}$	*2.63*		*3.30*	*3.84*	*3.67*	
$LSD_{0.01}$	*3.53*		*4.43*	*5.15*	*4.92*	
Jelen	146.9^{cd}	61.2^{d*}	87.4	70.4	53.5	80.1
Nora	145.3^{d}	57.3^{d}	78.8	66.4	61.6	69.1
Alexis	148.7^{cd}	61.9^{d}	77.9	72.1	58.2	76.9
NS-135	150.6^{c}	67.8^{c}	76.7	72.6	62.1	76.7
Avans	176.8a	78.2^{b}	62.0	80.5	60.5	77.4
Gustoe	163.1^{b}	118.0^{a}	73.2	68.9	67.2	65.5
$LSD_{0.05}$	*3.72*	*5.05*				
$LSD_{0.01}$		*6.78*				

* at 0.05 level of significance

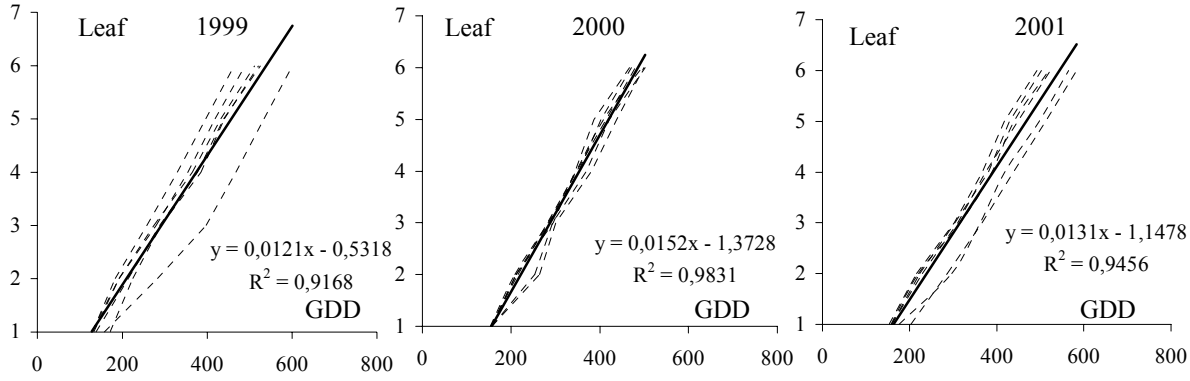


Figure 2. Heat units (GDD) required for the leaf appearance in six spring barley cultivars (—— illustrates regression of leaf appearance against GDD for each investigated variety, ------- illustrates regression of average values of the six cultivars against GDD)

The third and fifth leaf had close phyllochrons in 1999 and 2000, while the fourth leaf had the longest PI in 1999 and similar lengths of PI in the other two years. GxY interaction for these

three leaves occurred in almost all cultivars. Variation in third leaf appearance was mainly controlled by cultivar, while about one third of variation in fourth and fifth leaf appearance was due to non-genetic factors. GxY interaction had the highest responsibility for PI of the sixth leaf.

Variation across cultivars, i.e., among years, was minor, while across years it varied from 61.3 to 86.6 (Table 2). The cultivars Jelen and Gustoe did not differ in the phyllochron of the sixth leaf, while the other cultivars showed significant differences among the three years (data not shown). There was no consistency in the differences in PI among the cultivars; Alexis had the longer PI in 2000 NS-135 in 1999 and Avans the shortest in 2001.

The differences in the heat units for first leaf appearance suggest that the cultivars responded to accumulated GDD at this phase which agrees with observations of BAKER *et al*. (1986) and BAUER *et al.* (1984). Avans and Gustoe originated from different ecological conditions in relation to the location of this experiment and it could be partially the reason for longer PI of the first leaf. Fast and uniform seedling emergence is especially desirable in short-season areas (semiartic, semiarid, arid), which provides adequate plant stand and establishment of effective plant canopy structure and yield. Early emerging seedlings have a longer time for growth in relation to late emerging ones. The results of early emergence is higher tillering, i.e., higher grain yield (GAN *et al*. 1992).

In all three years, first leaf demanded more days for appearance due to lower temperatures and shorter daylength. CAO and MOSS (1989a), found that decrease in phyllochron interval and increase in the rate of leaf appearance (leaves day^{-1}) was associated with increase in daylength. It seems that in our investigation temperatures had a strong effect on leaf appearance. Indeed, in 1999, temperatures during sowing and germination were rather high and the first leaf appeared soon after sowing; after that, the temperatures dropped and more days were required for second leaf appearance (Figure 1). Water deficit that occurred at the time of sowing in 1999 could also be responsible for the increased rate of first leaf appearance. BAKER *et al.* (1986) found lower PI values in wheat under non-irrigated field conditions, i.e., a higher rate of mainstem leaf. High air temperature at the time of sowing and emergence is expected to cause longer PI. That was confirmed in this study where high temperatures in the first 10 days of March 2001 (Figure 2, sum of mean daily temperature was 117˚C) caused the longest PI of the first leaf in the three years. Appearance of the other five leaves in 1999 and all leaves in 2001 was in close symmetrical intervals, since temperatures were steady and without large fluctuations. In 2000, temperatures were lowest among the three years and the appearance of the first leaf was latest.

The relation between leaf development and GDD as illustrated in Figure 2 shows the close dependence of leaf development on accumulated GDD. The coefficients of determination (R^2) were 0.92, 0.98, and 0.95 for 1999, 2000, and 2001, respectively, indicating that leaf development was mainly influenced by thermal conditions. SHARRATT (1999) also reported high values of coefficients of regression for two spring barleys grown in interior Alaska (R^2=0.98 and R^2=0.95). These results are consistent with KLEPER *et al*. (1982), whose results were the basis for suggesting a linear relationship between GDD and leaf emergence. The high dependence of spring barley leaf appearance on temperature found in this investigation confirmed the previously determined thermal requirements for leaf development in small grains.

Leaf appearance is a highly heritable trait and there is a strong relationship with accumulated thermal units. The linearity suggests that MSL stage can be used as a predictive measure of plant development. Also it can retroactively show the quality of the preemergent seedbed environment.

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Barley Population Structure and Adaptability with Small Radiation Doses Effects

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Abstract
In result of perennial observation of radiation hormesis effects in spring barley populations it was estimated that low doses of radiation induce a diversity of reactions on different biological organization levels (cellular, organism, population) among consistent postradiational generations. Radiation hormesis reactions induced by low doses have system character and pronounced adaptive direction. It was discovered that irradiation of barley in low doses (0.5-1.0 Gy) causes increase of heterogeneity of populations structural organization among generations (M_1-M_3). Use of many-dimensional analysis methods (cluster, factor, discriminant) allowed to reveal individual plant groups in structure of experimental populations of barley, with different type of epigenetic organization of morphogenesis. Literary and own data analysis allows starting a proposal about epigenetic nature of low doses effects in barley populations. It was determined that to low doses radiation action in contrast to high doses modification of structural organization of population adaptability take place in increase's direction. It supposes that low and high radiation doses produce fundamentally different genetic processes in barley populations. Possibility of line selection from experimental populations M_2 with low irradiation doses which are characterize by heightened total adaptability and modified complex of quantitative trait is showed.

Introduction

The problem of genetic effects of small dozes of radiation is one of the great significant ones in the theory of radiation mutagenesis. Up to present the paradigm of common knowledge states that small dose influence causes similar effects as big doses but only with less frequency. However, within the framework of classical paradigm many of the effects caused by small radiation zones (radioadaptive response, growth and development stimulation, raising of viability and adaptivity, etc.) have no explanation (SAGAN 1989). During recent years the hypothesis of "radiation hormesis" has been widely spread according to which small radiation dosage is of positive influence (LUCKEY 1999, 2000).

It is believed that genetic effects of small radiation doses are related to epigenetic mechanisms of heritage and variability (ZAYNULLYN 1997). The responses of biological systems to small radiation dosage are considered to be adaptive ones. As a result of our many-years system researches dealing with radiation hormesis effects in populations in spring barley it has been found out that the small doses induce a number of responses on different levels of biological organization in a row of subsequent generations. The responses induced by small radiation doses are of systems character and have a distinct adaptive feature (PROSKURNIN & KRYVORUCHENKO 1999).

Up to the present time the problem of influence of small radiation doses on structural organization of agricultural crops populations has remained not investigated.

Material and Methods

The object of research were control and experimental (M_1-M_3) populations of spring barley varieties having various ecological and geographic origin: Zvershennya, Spomyn, Fillippe.

Gamma radiation of dry seeds was carried out in doses 0.5; 1.0; 2.5 and 5.0 Gy source of gamma-rays was Co^{60}. The plants were analyzed on base of 18 quantitative traits which mainly reflect peculiarities of development at all stages of ontogenesis. The populations M_2-M_3 were sown in families. Estimation of the family was done visually, the degree of traits development was done on a 5-grade scale. General adaptive capability was defined based on the level of field plant surviving within each family. In the third generation family selection was based on adaptivity rate, singling out 3 groups of selection – M^0 –average adaptive level (3 grades of surviving), M^+ – high level (4-5 grades) and M^- – low level (1-2 grades). Multivariate statistic analyses of experimental data (principal factor and discriminate methods) were carried out by means of standard of applied statistic programmes.

Result and Discussion

Representing populations as massives of dots in the space of two principal factors, one can get visual presentation of structural population organisation. In this case the point is for separate plant and its location in the factor space of complicated traits corresponds to peculiarities of correlation between the processes of development traits included in these factors. On the other hand, peculiarities of point aggregate locations in the space of main factors correspond to specific interrelations between the population elements in the process of its development, i.e. they reflect peculiarities of structural organisation variability under definite agroecological conditions. Interpreting the experimental results obtained we proceeded from the fact that the more densely were the dots located in the massive, the more homogeneous structure the population possessed.

In has been found out that radiation of all the examined doses resulted in raising heterogenness of interpopulation structure of the experimental (M_1-M_3) populations (Fig 1-3). Small (0.5-1.0 Gy) and big (2.5-5.0 Gy) doses of radiation, however, caused different changes in the barley population structures.

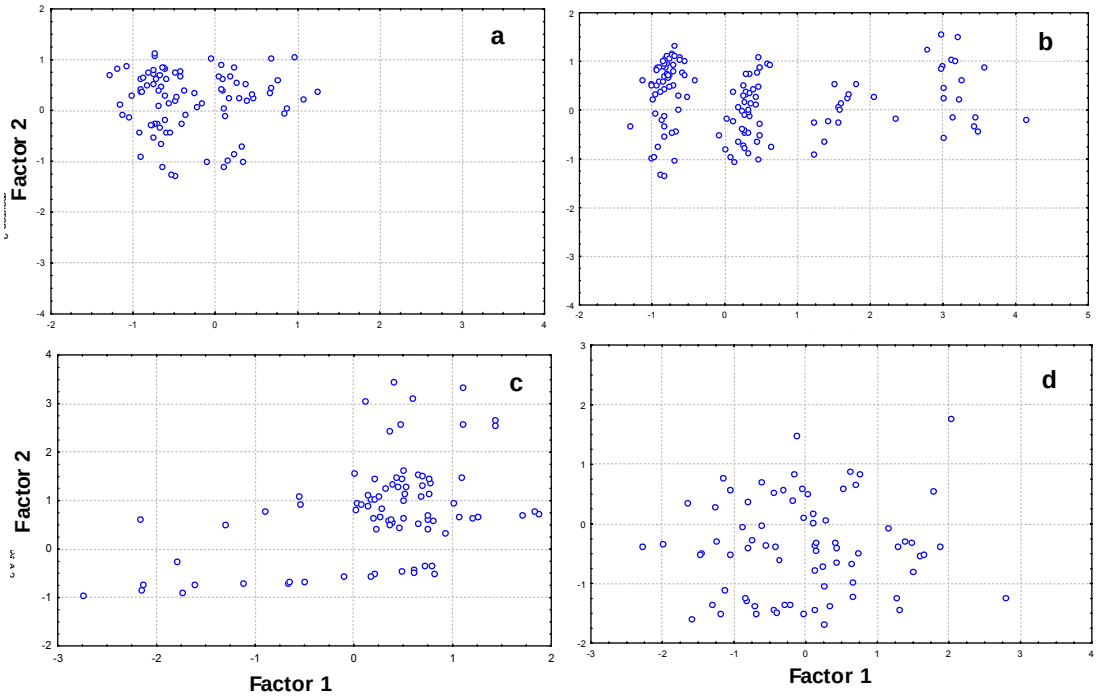

Fig 1. Structure of control and experimental M_1 barley populations variety – Spomyn (a – control, b – 0.5 Gy, c – 1.0 Gy, d – 5.0 Gy)

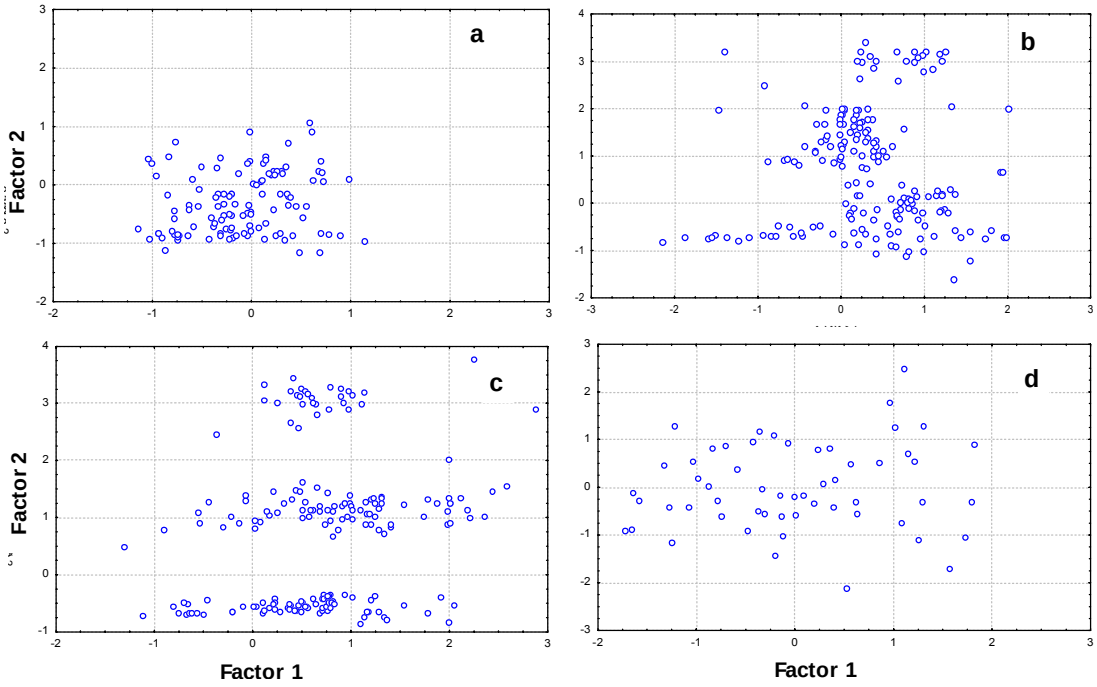

Fig 2. Structure of control and experimental M_2 barley populations variety – Spomyn (a – control, b – 0.5 Gy, c – 1.0 Gy, d – 5.0 Gy)

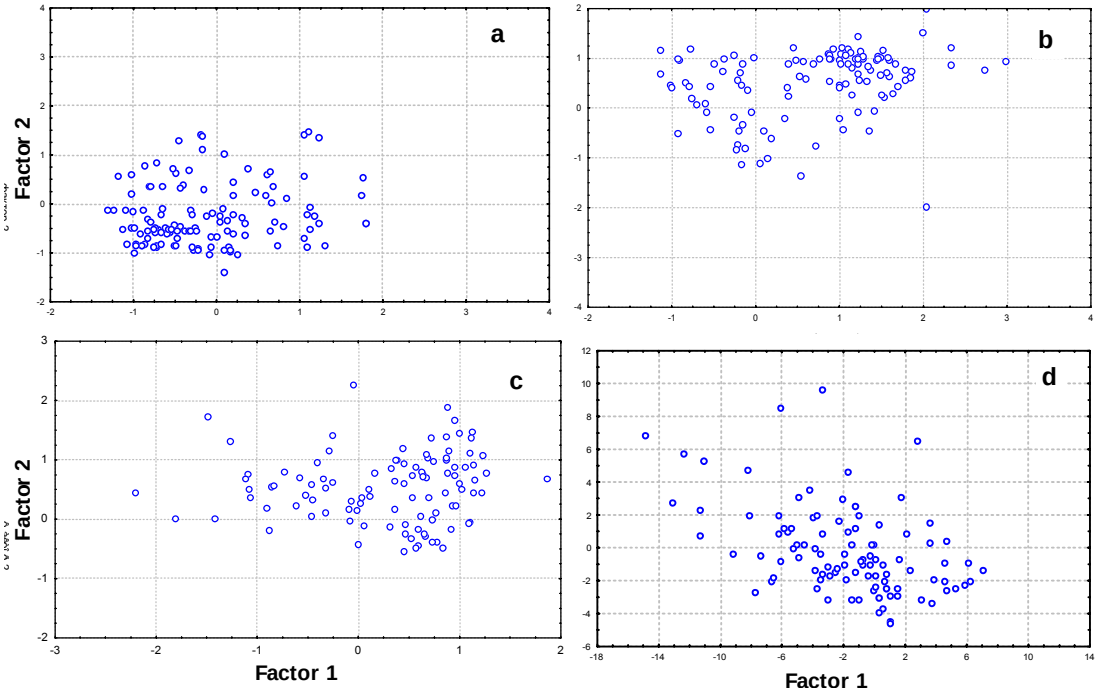

Fig. 3. Structure of control and experimental M_3 barley populations variety – Spomyn (a – control, b – 0.5 Gy, c – 1.0 Gy, d – 5.0 Gy)

The effect of big doses showed themselves in a highly increased population heterogenness, which was followed with destruction of "the median phenotype" or population "nucleus" (Fig 1.d-3.d), i.e. destruction of integral of structural population organisation in phylogenesis. Such restructuring changes in the experimental populations in a number of generations are, obviously related to displaining intensive mutation variability in M_2-M_3, as well as the effects of depression in M_1 barley plant development.

Variability of structural population organization induced by small radiation doses is connected with singling out some definite plant groups within their structure ("phenoelements")

(Fig. 1.b,c-3.b,c). Such plant groups possess different types of epigenetic organisation in their development processes. Some separate "phenoelements" are characterised with specific interrelations between traits of the first and second principal factors in the morphogenesis processes. It's obvious that these plant groups are different not only morphogenetically but also have different adaptivity level.

It had been established before, that barley populations are characterised with multifactorness in structure-and-functional development (PROSKURNIN & LITUN 1996) what indicates to existence of various epigenetic development programmes. The given research includes the traits of plant productivity (number productive steam, number and mass of grains produced by a plant) in the first factor, while the second factor includes features of the main steam productivity (the length ear, the number earlet in ear, the number and mass of grains ear). Thus peculiarities of interrelations between epigenetic development traits programmes of the whole plant and the main ear define the peculiarities of the singled out "phenoelements".

Maximum effect of changeability in experimental populations structure under small radiation doses was observed in M_2. With the lack of selection pressing there was observed a gradual homogenization of barley population structure and in the later (M_5-M_6) generations the structure of experimental populations was stabilized and was close to the control populations of barley varieties. That's why it was of great interest to research the effect of selection on changeability of structural organisation in the experimental barley populations.

In the second generation there has been found a significant differentiation between control and experimental families of barley populations in the index of general adaptive capability (Table 1). The populations under small radiation doses were characterised with significant increase of the number of families having a high (4 grades) and very high (5 grades) level of adaptivity while the effect of the big doses resulted in quite opposite results. With this such differentiation was followed by different level of traits development, what was confirmed by results of discriminant analysis on complex of traits. The results of classification of linear functions discrimination testify to significance of differences between the families.

Table 1. Structure of control and experimental population of M_2 variety Spomyn on common family adaptivity

Population	Relative frequency of families belonging to different adaptivity groups,%					Validity of classification, %
	1	2	3	4	5	
control	34.88	32.56	24.80	7.75	-	69.7
0.5 Gy	18.05	22.95	40.16	12.30	6.56	77.1
1.0 Gy	17.28	20.73	34.41	20.43	7.15	76.7
2.5 Gy	28.80	26.40	34.40	10.40	-	68.8
5.0 Gy	36.43	35.71	22.86	5.0	-	66.7

The purposeful selection based on the general adaptivity level led to higher differentiation on structural organisation of experimental population adaptivity (Table 2). The maximum effect of selection aimed at raising general adaptivity (selection group M^+) was noticed in the populations exposed to small doses. Effectiveness of selection based on eliminating the groups with low adaptivity in M^+ groups with small doses ranged within the limits 91.7-100%. The most significant selection effect directed to decreased family adaptivity (selection group M^-) was observed in the population under big doses.

Table 2. Influence of selection of general adaptivity structure in control and experimental populations M_3, variety - Spomyn

Population	Group of selection	Relative frequency of adaptivity group,%					Validity of classification, %
		1	2	3	4	5	
control	M^-	50.00	26.32	23.68	-	-	74.2
	M^0	10.34	37.93	37.93	13.79	-	
	M^+	-	40.00	30.00	20.00	10.00	
0.5 Gy	M^-	27.50	40.00	25.00	7.50	-	87.1
	M^0	5.13	25.64	48.71	15.38	5.13	
	M^+	4.55	-	44.54	40.91	10.00	
1.0 Gy	M^-	44.12	26.47	29.41	-	-	89.7
	M^0	37.04	14.81	40.74	7.41	-	
	M^+	-	8.33	33.33	45.33	19.00	
2.5 Gy	M^-	47.37	28.95	18.42	5.26	-	71.8
	M^0	15.79	26.32	42.11	15.79	-	
	M^+	-	22.22	33.33	38.88	5.55	
5.0 Gy	M^-	37.50	27.50	32.50	2.50	-	65.6
	M^0	23.08	11.54	53.85	4.54	-	
	M^+	-	33.33	33.33	33.33	-	

Carrying out selection based on adaptivity level resulted in higher differentiation between families on the level of traits development as well what was stated after a discriminant analysis had been made. Validity of differences between the selected groups is confirmed with the meanings of discrimination correctness.

Effectiveness of different forms of selection in barley population with different irradiation dosage may testify to the fact that the processes going on in them are of different genetic nature and require adequate approaches in their selection on adaptivity.

Conclusions

On the base of carried out investigations we may state that small irradiation dosage cause significant reconstruction in structural and adaptive organization of barley field populations. The obtained experimental data allow to assume rather an epigenetic nature of reaction induced with small doses. Possibility of small radiation dosage use for raising general adaptive capability in barley field population is shown

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In silico Expression Analysis of Barley ESTs: Tissue-Specificity of Starch Accumulation and Mobilization

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Abstract

To provide resources for functional genomics studies of the development of barley caryopses, approximately 40,000 ESTs were produced from four cDNA libraries covering pre-storage (HZ, HA: maternal and filial tissues, respectively, 0-7 Days After Flowering, DAF), middle storage (HB: whole barley seeds, 8-15 DAF) and late storage phase (HF: whole barley seeds, 16-25 DAF). About 36% of the ESTs have no match to sequences in Arabidopsis functional gene catalogues, suggesting that a reasonable number of transcripts are specific for monocots and/or barley. StackPack clustering of the 40,000 barley seed ESTs resulted in the identification of 4,782 tentative unigene consensi (TUCs) and 7,005 singletons, altogether a set of 11,787 unigenes. By computing abundantly expressed ESTs, correlated expression patterns of genes belonging to specific pathways were inferred for specific developmental stages during seed development. Further data mining results indicate that, while metabolism of starch synthesis and storage proteins dominates during middle storage phase (8-15 DAF), lipid metabolism genes are highly expressed during late storage phase (16-25 DAF). Multiple alignments of TUCs allowed us to estimate the number of genes in gene families responsible for starch metabolism. To identify key regulators of starch-, storage protein- and lipid metabolism, we surveyed the abundantly expressed putative transcription factors as well as kinases during storage and maturation phase.

Introduction

Barley is an important diploid cereal crop predominantly used in malting industry for beer production (SWANSTON *et al.* 1999). Seed contents accumulated during development decide over the usefulness of barley grains not only for the brewing industry but also for nutrition and feeding of animals. Developmental processes of complex organs like seeds are especially difficult to analyse due to the dramatic changes occurring in time and space and the multiple interactions between the tissues. During early seed development, cellularization processes dominate the filial tissues whereas the maternal pericarp functions in transient storage (starch accumulation) and represents, therefore, the main sink of the young grain. As development proceeds, the filial tissues (endosperm/embryo) enter the storage phase and accumulate starch, storage proteins and lipids. While the endosperm starts accumulation, the pericarp is drastically reduced. The analysis of gene expression networks controlling seed traits is a central step in understanding seed biology and supporting cause-related engineering. In the past, knowledge on seed development has been accumulated mainly from investigation of a few genes or enzymes (cf. BEWLEY & BLACK 1994; KIGEL & GALILI 1995). With the recent development of high-throughput genomic technologies allowing the concomitant analysis of thousands of genes, proteins and/or metabolites (see AHARONI &VORST 2001; FIEHN *et al.* 2001; SREENIVASULU *et al.* 2002a) the analysis of complex networks governing developmental and metabolic processes has become possible (RUUSKA *et al.* 2002, SREENIVASULU *et al.* 2004). For barley, an initial transcript profiling study was performed in maternal (pericarp) and filial (endosperm/embryo) tissues during early seed development by using a 700 and a 1400 cDNA macroarray, respectively (SREENIVASULU *et al.* 2002b; SREENIVASULU *et al.* 2004). To study seed development and the associated

metabolism, especially during mid and late storage phase, high throughput genomic technology has been employed. For gene discovery and elucidation of metabolic pathways more than 40,000 Expressed Sequence Tags (ESTs) were generated from four different cDNA libraries covering various stages of barley grain development. Further, by employing *in silico* expression analysis cellular and metabolic events of seed filling were revealed.

Material and Methods

To construct cDNA libraries from different tissues (male inflorescence, female inflorescence, maternal and filial tissues of the developing caryopsis 0-7 DAF, whole caryopses 8-15 and 16-25 DAF), 250 µg of total RNA were used to isolate polyA$^+$-RNA. The pBluescript II XR cDNA Library Construction Kit (Stratagene) was used for cDNA library construction following the manufacturer's protocol. Bacterial colonies encoding cloned cDNA were re-arrayed into 384 well plates and plasmid DNA was isolated using the Qiagen Plasmid kit. Sequencing of cDNA clones was performed by the companies QIAGEN (Hilden, Germany) and AGOWA (Berlin, Germany). Base calling and sequence quality were evaluated using Phred (Ewing and Green 1998). Sequences with a Phred score less than 20 were rejected. After vector trimming and removal of polyA or polyT stretches, sequences shorter than 100 bases were discarded. Long sequences were restricted to 700 bases by cutting extra sequences from the 3'-end. The software StackPACK v2.1.1 (available at http://www.sanbi.ac.za/CODES) was applied with default parameters for EST clustering and generation of tentative unigene consensi (TUCs). Annotation of individual ESTs and TUCs were performed by BLASTX2 search against NRPEP protein database using HUSAR (Heidelberg Unix Sequence Analysis Resources; http://genius.embnet.dkfz.heidelberg.de) software package. Functional classification of ESTs and TUCs was performed according to the functional catalogues of MIPS (Munich Information Center for Protein sequences, http://mips.gsf.de). To analyse the gene expression computationally, we considered abundant TUCs (at least 5 ESTs) from cDNA libraries of male and female inflorescences and developing seeds. *In silico* expression data were obtained by estimating the relative numbers of sequences within specific clusters distributed over the different cDNA libraries representing specific tissues or developmental stages. This digital *in silico* expression quantification method has been implemented to assess genes predominantly expressed in seeds.

Results and Discussion

A functional genomics approach was employed to study the transcriptome involved in storage product synthesis in barley seeds. More than 40,000 ESTs were derived from four seed-specific cDNA libraries (HZ, HA, HB, HF) representing the maternal and the filial seed part (HZ and HA, respectively) during the pre-storage phase (0 – 7 DAF), and whole caryopses during the early to mid storage (8-15 DAF; HB) and the late storage phase (16-25 DAF; HF). In addition, 4,940 and 5,038 ESTs were produced from the female- and the male-inflorescence cDNA library, respectively. StackPack clustering of 40,752 ESTs from developing barley seeds resulted in the identification of 4,782 tentative unigene consensi (TUCs) and 7,005 singletons. Altogether, a set of 11,787 unigenes was derived and high-density macroarrays were constructed. In Fig. 1, we have outlined the functional genomic tools used to identify developmental stage-specific genes.

Barley ESTs were functionally classified by using integrated databases such as KEGG, SWISSPROT, MIPS, TRANSFAC, PlantsP and PlantsT. Only 14.0% of the ESTs belong to primary metabolism, 19.7% to translation and cellular organization, 32.6% of the ESTs have no match to sequences in the Arabidopsis functional gene catalogues. 3.1% of the ESTs

belong to the category transcription factor, 2.8% to kinases and 1.3% to transport-associated proteins (Fig. 2). Sequences belonging to the latter three categories were predicted based on their functional domains.

Fig. 1 Schematic representation of the functional genomic approach based on ESTs

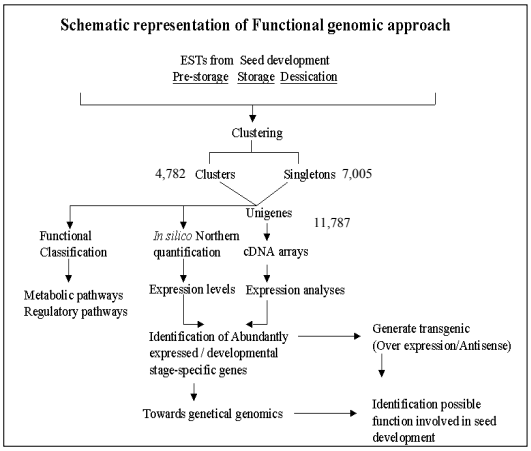
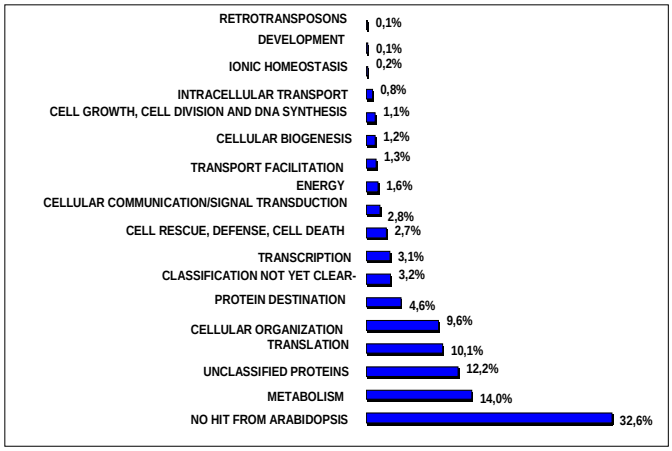


Fig. 2 Functional annotation of a large collection of ESTs from developing barley seeds



Because of the non-normalized character of the four libraries, the number of ESTs representing a specific transcribed gene can be used as a measure of this gene's activity. Data mining of the four different libraries resulted in the identification of high numbers of genes associated with cellular organization expressed during the pre-storage phase (0-7 DAF) of maternal and filial tissues. High numbers of starch metabolism and storage protein genes are expressed during the storage phase of seed development (8-15 DAF). The *in silico* data mining results compare nicely with expression profiling results (based on a macro array containing 1,400 cDNA fragments; SREENIVASULU *et al.* 2004) related to the accumulation of starch and storage proteins in the filial tissue during 8 - 12 DAF. Interestingly, relatively high expression of genes associated with lipid metabolism was detectable by data mining during the late storage phase of seed development (16-25 DAF). Two possibilities (identification of stage specific isoforms and relative quantification of mRNA abundance by *in silico* expression analysis) were used to analyse tissue- and development specificity as well as transcriptional activity mainly of genes involved in starch metabolism.

TUCs result from multiple EST alignments, and the examination of TUC sub-structures identificaties gene family members (qualitative information). Furthermore, because TUCS are derived from homologous sequences out of different non-normalized libraries, they contain quantitative information regarding the number of ESTs transcribed from each iso-gene active in a specific tissue and/or developmental stage. Hence, TUCs open the possibility to estimate digital expression levels of individual members of gene families characteristic for different tissues/developmental stages. Here we employed this method to depict gene expression patterns of different members of gene families related to starch metabolism. In cDNA libraries of developing seeds, differences in relative mRNA expression patterns among gene family members were observed not only for genes associated with starch biosynthesis, but also for starch catabolism genes. The developing barley seed is composed mainly of pericarp, endosperm and embryo. Early during development, the seed accumulates starch transiently in the maternal pericarp to supply the developing filial tissues, but later on starch is stored in the filial starchy endosperm to feed the future seedling. Comparable processes (starch

accumulation and subsequent mobilization) are also known from female and especially male inflorescences. What we do not know is whether distinct isoforms of starch synthesizing and degrading enzymes are expressed in the different tissues. Digital expression analysis based on the examination of TUC composition can provide such information.

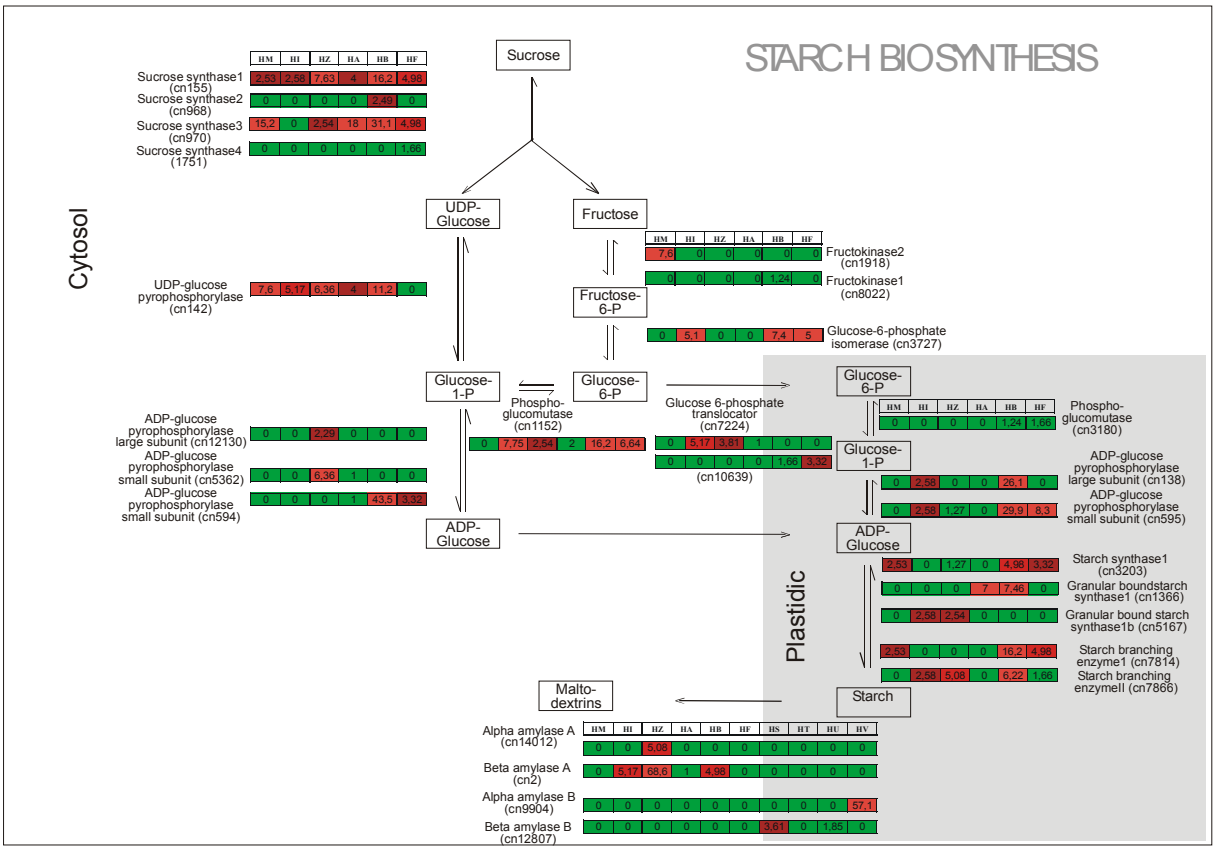
The starch synthesis pathway involves three key steps (i) sucrose cleavage, (ii) generation of ADP-glucose and (iii) synthesis of amylose and amylopectin. The sucrose cleavage gene sucrose synthase (SUS) is encoded by a small multigene family. Out of our EST collection, 4 putative SUS genes were predicted [SUS1 (cn155), SUS2 (cn968) SUS3 (970) and SUS4 (cn1751), see Fig. 3], which show considerable differences in their expression patterns. While high transcript levels of SUS1 were found in the pericarp during the pre-storage phase (0-7 DAF, HZ) and in the starchy endosperm during the middle storage phase (8-15 DAF, HB), SUS3 mRNA is expressed at higher levels in the male inflorescence (HM), in filial tissue during the pre-storage phase (HA) and between 8 and 15 DAF in the starchy endosperm (HB). SUS2 and SUS4 show confined expression in the middle and late storage phase, respectively. SUS catalyzes the reversible cleavage of sucrose into fructose and UDP-glucose, but different SUS isoforms could serve different functions. Thus, expression of SUS3 in young dividing cells of filial tissue seems to be correlated with cellulose biosynthesis (SREENIVASULU *et al.* 2004). Of specific interest is the finding that the SUS2 and SUS4 gene products can be correlated with the ongoing storage product accumulation, i. e. with starch biosynthesis, but also with the accumulation of storage proteins and lipids. Early in grain development, SUS1 transcripts were localized by *in situ* hybridization in different regions of the pericarp, and during storage phase in the starchy endosperm (WOBUS *et al.* 2004). These previous findings are in agreement with our *in silico* data mining results and underline an expected function of SUS1 in transfer processes (*in situ* localization in companien cells of the main vascular bundle within the early pericarp) as well as starch accumulation in both, parenchyma cells of the pericarp and the starchy endosperm. There, SUS activity produces UDP-glucose, which serves as precursor for starch biosynthesis. UDP-glucose pyrophosphorylase (UGP) metabolizes UDP-glucose to glucose-1-phosphate, which is utilized by ADP-glucose pyrophosphorylase (AGP) for the synthesis of ADP-glucose, the direct precursor for starch biosynthesis. However, high relative mRNA levels of the cytosolic isoform of phosphoglucomutase found in library HB (see Fig. 3) suggest cytosolic production of glucose-6-phosphate from glucose-1-phosphate during the middle storage phase and support the hypothesis (KAMMERER *et al.* 1998) that glucose-6-phosphate is taken up by the plastids of dicot storage organs but also monocot seeds. The hypothesis is further supported by the expression of glucose 6-phosphate translocator mRNA in all tissues responsible for starch accumulation as the maternal and the filial part of the developing seed (HZ and HF, respectively) and the female inflorescence (HI). Interestingly, two different isoforms of the transporter (cn10639 and cn7224) serve both tissues (see Fig. 3). However, we have detected only small mRNA amounts of the plastidic isoform of phosphoglucomutase in the accumulating seed tissues (HB, HF) and no respective EST in cDNA libraries produced from the maternal seed part (HZ) and female inflorescences (HI). Hence, cytosolic production and transport of glucose-6-phosphate into barley plastids seems to be likely, but the plastid-localized conversion of glucose-6-phosphate into glucose-1-phosphate necessary for starch biosynthesis cannot be illustrated by our *in silico* expression analysis.

Production of ADP-glucose is catalyzed by AGP. The enzyme is a heterotrimer made of small (AGP-S) and large subunits (AGP-L). In endosperm of monocots, the enzyme is found in both, cytosol and amyloplasts (NEUHAUS & EMES 2000). We have found mRNA of cytosolic as well as the plastidic AGP isoforms of both, the small and the large subunit (see

Fig. 3). While the maternal tissues are obviously served by cytosolic isoforms (AGP-L, cn12130; AGP-S, cn5362), the starchy endosperm as well as female inflorescence encodes plastidic (AGP-L, cn138; AGP-S, cn595) and a different set of cytosolic isoform (AGP-S, cn594). These reported data provide a hint that ADP-glucose formation takes place both in plastids and in the cytosol in female inflorescence and in endosperm cells of caryopses. In contrast, BURTON *et al.* (2002) reported recently biochemical data supporting a purely cytosolic site of AGP synthesis in wheat.

Expression of granule-bound starch synthase (GBSS) as well as starch branching enzyme (SBE) is generally co-regulated with AGP. GBSS and SBE constitute the final step of starch biosynthesis, i. e. transfer of the glucose moiety of ADP-glucose to the non-reducing end of the starch molecule. We found transcripts of 3 different starch synthase isoforms, one coding for soluble starch synthase (cn3203) and two others for granule-bound starch synthase (cn1366 and cn5167) (see Fig. 3). In the middle storage phase, soluble (cn3203) as well as granule-bound starch synthase genes (cn1366) (known to contribute to amylose synthesis; SMITH & MARTIN 1993) are highly expressed. The second GBSS isoform 1b (cn5167) is expressed only in the female inflorescence and in the maternal part of the seed. The same seems to be true for the starch branching enzyme. One specific isoform serves female inflorescence and maternal seed tissues (cn7866), another one (cn7814) the male inflorescence and the accumulating endosperm. These observations suggest that distinct isoforms of starch synthase and starch branching enzyme are specifically expressed in different tissues, creating possibly different amylose to amylopectine ratios, which may lead to tissue-specific structures of the starch granule.

Fig.3 *In silico* expression profiles of starch biosynthetic pathway genes



Library specific abbreviations: HM-male inflorescence; HI- female inflorescence; HA-filial tissues of developing seeds (0-7 DAF); HZ-maternal tissues of developing seeds (0-7 DAF); HB- whole developing seeds (8-15 DAF; storage phase I); HF-whole developing seed (16-25 DAF; storage phase II); HT- aleuron/endosperm of germinating seeds 2HAI (hours after imbibition); HS-embryo+scutellum of germinating seeds 2 HAI; HU and HV- germinating seeds 16-48 and 48-96 HAI, respectively.

To our knowledge up to now unknown details and interesting results were noticed in the current study regarding the expression of the starch degrading amylases. Generally, two sub-types, α- and β-amylases, exist, each represented by an A and a B isoform. The maternal pericarp, which typically accumulates transient starch, expresses α- as well as β-amylase, but in both cases exclusively the A-isoform. Isoform A of α-amylase is present only in the pericarp. Isoform A of the β-amylase was found to a lower extent in female inflorescence and in accumulating starchy endosperm 8-15 DAF. The B isoforms of α- as well as β-amylase are characteristic for germinating seeds. β-amylase B is expressed in embryo/scutellum 2 hours after imbibition (HAI) and, to a lower extent, in germinating seeds 16-48 HAI, whereas α-amylase B shows high expression 48-96 HAI. The processes of starch degradation during germination are well analyzed and described (LARKIN *et al.* 1993; GUBLER *et al.* 1995). However, not so much is known about the transcriptional regulation of the different isoforms and specially the expression of amylases in the female inflorescence and in the maternal tissues of the barley grain.

Recently, a high-density array containing 11,787 tentative unigenes from developing seeds was developed in our group. The 11,787 cDNA array is employed for more sophisticated experiments, mainly to conduct high-throughput expression profiling during complete seed development. It opens the possibility to analyze regulatory phenomena connected, besides of others, to the expression of isoforms of α- and β-amylases in tissues of the barley seed.

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Advances in Flow Cytogenetics of Barley

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Abstract

Flow cytogenetics is an attractive tool that facilitates genome mapping. Flow cytometric analysis permits classification of isolated chromosomes according to their DNA content. As thousands of chromosomes are analysed in each sample, quantitative detection of structural and numerical chromosome changes is possible. Any chromosome that can be discriminated can also be sorted in large quantities and high purities for subsequent molecular analysis. Unfortunately, only the smallest chromosome 5(1H) can be discriminated and sorted in barley. This is due to similarity in relative DNA content among the chromosomes 1 – 4, 6 and 7 (2H – 7H). Cytogenetic stocks facilitate sorting of those translocation chromosomes that differ in DNA content. Even if the sorted translocation chromosomes were found useful for mapping DNA sequences to subchromosomal regions, their use remained limited. Here we describe a novel approach that facilitates sorting of any of the 14 barley chromosome arms. The strategy is based on using wheat-barley addition lines carrying barley chromosome arms (telocentric chromosomes). Furthermore, we will be able to sort subarm chromosomal segments that are generated in the wheat-barley chromosome addition lines by the gametocidal system. These advances make flow cytogenetics an attractive tool that can simplify barley genome mapping. A possibility to purify large quantities of individual chromosome arms and their segments opens avenues for targeted isolation of low-copy "genic" sequences, preparation of specific probes for screening EST arrays, HAPPY mapping, and construction of chromosome arm-specific BAC libraries.

Introduction

The nuclear genome of barley (*Hordeum vulgare*, $2n = 2x = 14$) is complex (~ 5100 Mb / 1C), consisting mainly of various classes of repetitive DNA sequences. These features hamper its physical mapping and map-based gene cloning. Various strategies have been considered to overcome the difficulties. One of them is to fractionate the genome into small and defined parts. This could be achieved by chromosome sorting using flow cytometry.

Protocols for chromosome analysis and sorting using flow cytometry (flow cytogenetics) were developed for human and some animal species during 1980s. Subsequently, flow cytogenetics has been developed for 17 plant species, including major legume and cereal crops (DOLEŽEL *et al.* 2004a). Flow cytometry analyses suspensions of intact chromosomes stained with a DNA-specific fluorochrome. The chromosomes are classified according to relative fluorescence intensity at rates of $10^2 – 10^3$ chromosomes / sec, and therefore large populations can be measured in a short time. The result of analysis is displayed as histogram of relative fluorescence intensity (flow karyotype). Ideally, each chromosome is represented by a single peak on a flow karyotype. A chromosome that can be discriminated on a flow karyotype can be sorted at large quantities (DOLEŽEL *et al.* 2004b).

Flow karyotyping is suitable for quantitative detection of structural and numerical chromosome changes (KUBALÁKOVÁ *et al.* 2002, 2003). The use of flow-sorted chromosomes encompasses a broad range of applications, including physical mapping of DNA sequences by PCR with sequence specific primers and fluorescence *in situ* hybridization on super-stretched chromosomes (NEUMANN *et al.* 1993; VALÁRIK *et al.* 2004). Sorted chromosomes were used for targeted isolation of molecular markers (POŽÁRKOVÁ *et al.* 2002) and construction of chromosome-specific DNA libraries (MACAS *et al.* 1996). Methods for preparation of high molecular weight DNA from sorted chromosomes were developed (ŠIMKOVÁ *et al.* 2003) and facilitated production of subgenomic, chromosome-specific and chromosome-arm specific BAC libraries (JANDA *et al.* in preparation; ŠAFÁŘ *et al.* 2004).

The application of flow cytogenetics for barley genome mapping has been hampered by difficulties in sorting individual chromosome types. Due to small differences in relative DNA content among the chromosomes, only chromosome 5 could be sorted. This paper evaluates various strategies for chromosome sorting in barley and describes a novel approach that facilitates sorting each of the 14 barley chromosome arms.

Material and Methods

Seeds of barley cultivars 'Akcent' and 'Morex' were obtained from a commercial supplier. Six chromosome translocation lines ('Elgina', 'Triumph', 'Bonus', 'St.13559', 'Frigga' and 'Mut.4841') were obtained from the germplasm collection of the Institute of Plant Genetics and Crop Plant Research (Gatersleben, Germany). Seeds of wheat-barley addition lines carrying containing barley chromosome arms (telocentric chromosomes) were obtained from The National Bioresource Project/Wheat (Japan). The lines were originally developed by ISLAM *et al.* (1981) and ISLAM (1983). In order to maintain a compatibility with the nomenclature of chromosome translocations by KÜNZEL *et al.* (2000), the older chromosome nomenclature (BURNHAM & HAGBERG 1956) is used in this work instead of the new one (LINDE-LAURSEN *et al.* 1997), which is based on homoeologous relationship with the chromosomes of the wheat genomes (a chromosome number followed by a letter H).

Chromosome suspensions were prepared from synchronized root tips of barley (*Hordeum vulgare* L., $2n = 2x = 14$) cultivars and translocation lines according to LYSÁK *et al.* (1999), and from hexaploid wheat (*Triticum aestivum* L. $2n = 6x = 42$) and wheat-barley addition lines according to VRÁNA *et al.* (2000) and KUBALÁKOVÁ *et al.* (2002). Suspensions of isolated chromosomes were stained with a DNA-specific fluorochrome DAPI, and their fluorescence was analysed at rates of 200 – 500/sec using the Becton Dickinson FACSVantage SE flow cytometer and sorter. In each sample, at least ten thousand chromosomes were analysed and the results were displayed as histograms of relative fluorescence intensity (flow karyotypes). Chromosomes were sorted at rates of 5 – 20/sec as described by LYSÁK *et al.* (1999) and VRÁNA *et al.* (2000). PCR on flow-sorted chromosomes with chromosome-specific markers was performed according to LYSÁK *et al.* (1999). For each of the 14 barley chromosome arms, two STS primer pairs (KÜNZEL *et al.* 2000) were used. Fluorescence *in situ* hybridization (FISH) with probes for GAA microsatellite and a telomeric repeat was done on chromosomes sorted onto microscope slides according to KUBALÁKOVÁ *et al.* (2002).

Flow cytometric analysis of chromosome suspensions prepared from various barley cultivars resulted in flow karyotypes consisting of a single dominant composite peak representing chromosomes 1 – 4, 6 and 7, and a well-separated peak representing chromosome 5. In addition, minor peaks representing chromatids, chromosome fragments and debris were observed (Figure 1a) (LYSÁK *et al.* 1999). The identity of sorted barley chromosomes was determined by PCR with primers for STS markers. However, quantification of contaminating particles was done best by microscopic observation of sorted chromosomes after fluorescent labelling GAA microsatellites using FISH (Figure 1a, insert). The results showed that only chromosome 5(1H) could be sorted from barley cultivars with a standard karyotype.

Analysis of Barley Chromosome Translocation Lines

When compared to barley cultivars with a standard karyotype, flow karyotypes of barley translocation lines differed in the number of peaks representing single chromosome types (LYSÁK *et al.* 1999). The numbers ranged from one to three, depending on the translocation type. In 'Elgina' (T1-7an), only chromosome 7^1 could be discriminated and sorted as the peak of chromosome 1^7 overlapped partially with the peak of 1H. Two chromosome-specific peaks were resolved in 'Mut.4841' (T3-5af) possessing a translocation involving chromosome 5. In most of the lines, both translocation chromosomes could be resolved in addition to chromosome 5 (Figure 1b). These results clearly demonstrated a possibility that translocation chromosomes of barley could be sorted. Flow-sorted translocations were found suitable for PCR for physical sequence mapping at the subchromosomal level (LYSÁK *et al.* 1999).

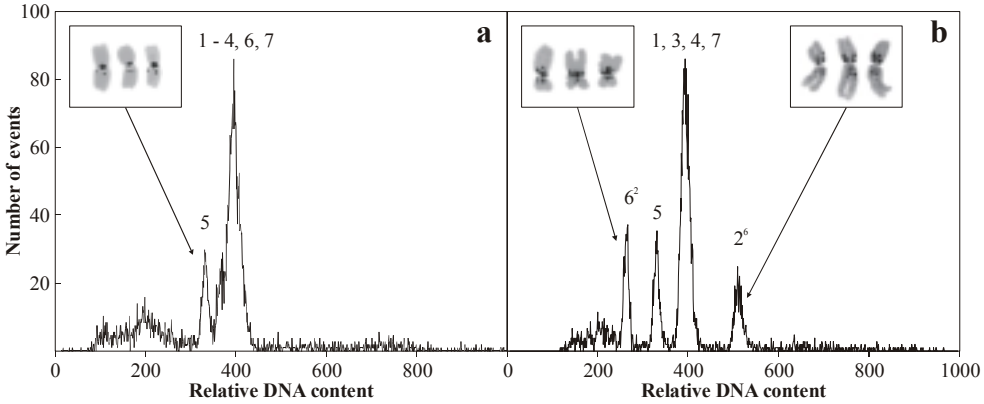

Figure 1. Flow karyotypes obtained after the analysis of DAPI-stained chromosome suspensions prepared from two barley lines. (**a**) Flow karyotype of 'Morex' consists of a composite peak representing chromosomes 1 – 4, 6 and 7 and a minor peak corresponding to chromosome 5 (insert: chromosome 5 after GAA banding); (**b**) Flow karyotype of barley translocation line 'St. 13559' in which the three chromosomes 2^6, 5 and 6^2 could be sorted (inserts: chromosomes 2^6 and 6^2 after GAA banding).

Flow Karyotyping in Wheat-Barley Telosome Addition Lines

Inability to sort other wildtype barley chromosomes than 5 stimulated a search for other approaches to overcome this problem. The success of sorting single chromosome arms from telosomic lines of hexaploid wheat (KUBALÁKOVÁ *et al.* 2002) prompted us to test wheat-barley telosome addition lines. This approach turned out to be extremely successful. Flow cytometric analysis resulted in flow karyotypes, on which the peaks corresponding to barley arms were clearly resolved (Figure 2).

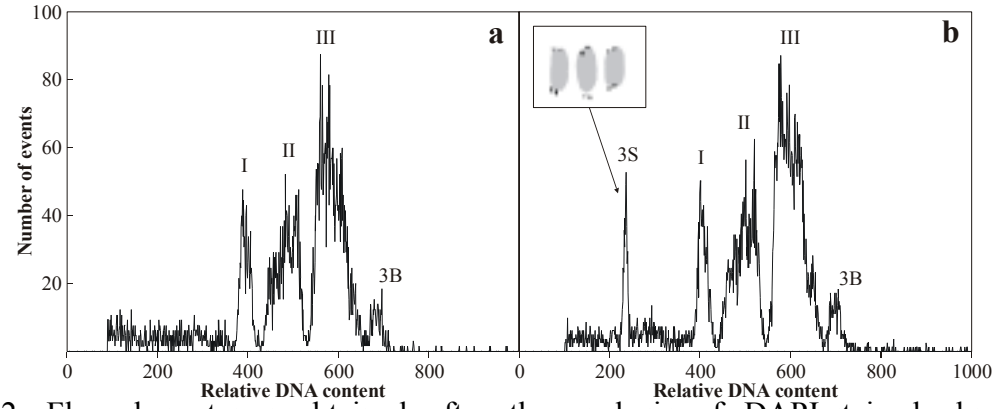

Figure 2. Flow karyotypes obtained after the analysis of DAPI-stained chromosome suspensions prepared from wheat and wheat-barley telosome addition line. (**a**) Flow karyotype of hexaploid wheat 'Chinese Spring' consists of three composite peaks (I – III) representing groups of chromosomes and peak representing chromosome 3B; (**b**) Flow karyotype of wheat-barley telosome addition line with peaks containing wheat chromosomes (I – III, 3B) and a peak representing barley chromosome arm 3S (insert: chromosome arm 3S after FISH with a probe for telomeric repeat).

Since no barley telosome is larger than the smallest wheat chromosome, any barley chromosome arm could be sorted using this system (Figure 3). The identity and purity in sorted fractions was determined after double FISH on sorted arms with probes for GAA microsatellite and telomeric repeat. A probe for telomeric repeat was included in order to discriminate broken chromosome arms occurring at low frequencies from intact telocentric chromosomes (Figure 2b insert). The analysis showed that the arms could be sorted at purities approaching 95%. In addition to microscopic analysis of sorted particles, the identity of sorted barley arms was confirmed using PCR with specific primers (data not shown).

Conclusions

Until recently, a potential of flow cytogenetics for barley genome analysis has been greatly limited by difficulties in sorting individual chromosomes. From the seven barley chromosomes, only the chromosome 5 could be sorted (LYSÁK *et al.* 1999). The strategy for sorting single chromosome arms from wheat-barley telosome additions described here represents a novel and powerful approach that brings barley flow cytogenetics back to the spotlight. It is now possible to dissect the large genome of barley into small and defined fractions representing only 5% – 9% of the whole genome. Furthermore, we expect that we will be able to sort subarm chromosomal segments that are generated in the wheat-barley chromosome addition lines by the gametocidal system (SHI & ENDO 2000).

A possibility to sort any chromosome arm and segments of chromosome arms opens new horizons for the application of barley flow cytogenetics in physical mapping and gene cloning. The arms can be sorted at purities of up to 95% and at rates of 5 – 20 / sec. Thus, even microgram quantities of chromosome arm-specific DNA can be obtained to produce specific BAC libraries. We expect that the use of sorted arm fractions will include targeted isolation of low-copy "genic" sequences, preparation of specific probes for screening EST arrays, HAPPY mapping, and construction of chromosome arm-specific BAC libraries.

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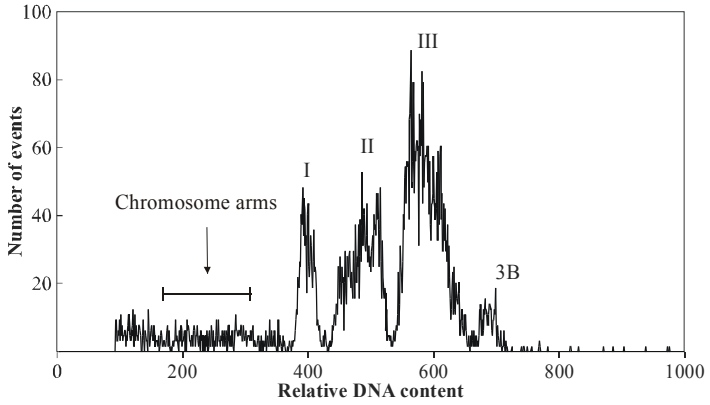

Figure 3. Flow karyotype of hexaploid wheat "Chinese Spring' showing the range of peak positions for barley chromosome arms. As can be seen, none of the arms is expected to overlap with peaks that represent chromosomes of wheat (labelled I - III and 3B).

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Variability of Oil Content in By-Products of Australian Barley

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Abstract
Processing, such as pearling and malting of barley, results in significant quantities of by-product that is either sold for minimal return or is discarded at cost to the producer. Barley oil has been shown to significantly lower total serum cholesterol and LDL-C without lowering HDL-C in humans with hypercholesterolemia. High concentrations of barley oil have been found in flour pearled from barley and in brewers grain, therefore extraction of oil from these by-products may be a viable, value-adding process. In this study we measured the oil content of various genotypes of barley to determine if differences could be found among barley destined for the pearling and malting markets.
The total lipid content of whole barley has previously been estimated at 3% and occurs mainly in the aleurone and germ layers, mostly as free lipids in the form of triglycerides. There have been few reports of significant variation of lipid content between genotypes of barley including the cultivar Riso 1508 (4.1% total lipid), and some hulless waxy genotypes compared to normal starch covered types. In this paper we discuss the lipid content of whole barley and pearled flour from Australian hulless waxy genotypes and other Australian cultivars and also from brewers grain of "Torrens" hulless barley. Significant variation was found between the samples and higher concentrations of free lipids were found in pearled flour of hulless waxy genotypes.
Keywords: barley; waxy; hulless; oil; malting; pearling; value-adding

Introduction
The health benefits of barley oil, in particular its content of Vitamin E, are still under investigation. Vitamin E research in general, has focussed mainly on its antioxidant properties particularly in relation to cancer and heart disease prevention, however many non-antioxidant functions are starting to emerge, such as anticoagulant and neuroprotective properties, anti-inflammatory actions, effects on gene regulation and immune function. Vitamin E exists as eight different compounds: four tocopherols and four tocotrienols (designated as α, β, γ, and δ). For structures see Figure 1.

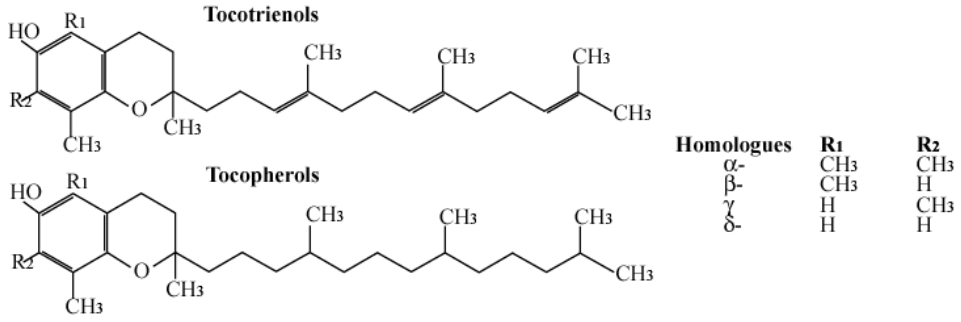

Figure 1. 2D structure of tocols

In the past, only one, α-tocopherol, was thought to be important for nutrition and health. However, the other tocols have important roles too. Tocotrienols are most abundant in cereal grains including barley, rice, rye, and wheat, and the fruit of palm. Unfortunately, the common commercial sources of natural vitamin E (soy, corn, cottonseed, canola, and

sunflower oil distillates) contain little or no tocotrienols. There is reasonable evidence to suggest that barley oil (which contains all of the tocol isomers) can lower cholesterol in hypercholesterolemic humans (LUPTON *et al.* 1994).
Evidence, from avian research, suggests that a higher ratio of tocotrienols to tocopherols in the diet is more beneficial for the metabolic regulation of cholesterol (QURESHI *et al.* 1989) and tocotrienols are a more potent form of vitamin E (antioxidant) activity (DUTHIE *et al.* 1992). Barley is one of the best sources of tocols, containing a high concentration of total tocols and a favourable distribution of the most biologically active isomers (PETERSON & QURESHI 1993). The most predominant tocol isomers in barley are α-tocotrienol and α-tocopherol. β-Tocotrienol and γ-tocotrienol are also present in significant amounts. KOOYENGA *et al.* (1997) showed that in humans, a mixture of tocopherols and tocotrienols could slow down the narrowing of carotid arteries and in forty percent of the patients tested appeared to reverse the condition. For high-risk individuals, the recommended daily intake of tocols is estimated to be 400IU of natural α-tocopherol plus 400mg of mixed tocols. To obtain these amounts of tocols, it would be necessary to consume large amounts of natural foods, e.g. 1 cup of palm oil, 3.0kg Barley or 4.0kg Oats. Therefore, it is desirable to ingest the tocols in the form of supplements, fortified foods or enriched food products. Due to the possible synergy of health benefits provided by the individual tocols, it is highly desirable to maintain the most natural form. This would also lower processing costs. PETERSON & QURESHI (1993) have reported variability of oil content in products from different genotypes of barley. JADHAV *et al.* (1998) reported Wang's thesis findings, of a high percentage of oil in the pearlings of hulless, waxy barley containing 6-12% oil. PETERSON (1994) found that barley tocols were enriched in brewers spent grains (BG) 152.9mg/kg. WANG *et al* (1993a) found that the pearling flour of waxy hulless barley had 2.7-4.4 times more α-tocotrienol, α-tocopherol, total vitamin E, and oil concentration than did the barley grain. This suggests that the Pearled Flour (PF) by-product from waxy hulless barley would provide a nutrient-rich, health promoting food ingredient. The extraction of oil and Vitamin E isomers from barley by-products may add value to pearling and brewing processes. Such products may be used in human nutrition and cosmetics. In this paper we discuss the preliminary findings of oil content and tocol analysis of whole and malted barley, spent grain, and the pearling flour from pearled barley using a number of Australian covered, hulless and hulless, waxy barley genotypes.

Material and Methods

Material

Waxy hulless barley (CDC Alamo, Merlin, CDC Candle, Sumire mochi, WI3693 and WI3874), hulless barley (Torrens, Namoi, WI3152, WI3044 and 12 breeders lines) and the covered variety Schooner were evaluated. Samples were grown at Strathalbyn, South Australia during 2002 and 2003. Hulless and waxy barleys were chosen for their potential use for human food. The covered malt variety, Gairdner, from the 2002 season, was supplied by AusMalt Ltd, Cavan, South Australia, and was used for commercial malting.

Pearling

100g of barley were pearled on a "Satake" test mill (model TM05, Satake, Tokyo, Japan) to varying degrees for 5-8mins each. The flour (PF), approx. 20-35% wet weight of grain, and grains (PG), approx. 65-80% wet weight, were collected and ground in a UDY mill fitted with a 0.5mm screen prior to oil extraction.

Malting
12 hulless barley breeders' lines were malted using a Phoenix Micromalter and standard micro-malting procedure (7hrs steep, 8hrs air, 9hrs steep, 94.5hrs germination, 20hrs kiln to a maximum temperature of 80°C). The malt was analysed for oil content and compared with covered, control malt (Gairdner). AusMalt Ltd performed large scale malting of Torrens (Australian hulless) and Gairdner (Australian covered malt), using a procedure described by STEWART *et al.* (2004). Gairdner (Control) malt and Torrens mixed malt (50% Torrens: 50% Gairdner) were brewed at Coopers Brewery, Regency Park, South Australia. The Brewers Grain (BG) of these samples, along with a commercially produced BG, was obtained from the mash-filtration of the malt after milling, mashing and wort separation. These samples were freeze-dried and ground as described earlier, prior to oil extraction and tocol analysis.

Oil Extraction and Tocol Analysis
AusBulk Ltd performed the oil extraction using an ISCO Fast Fat Procedure based on a modified AOCS method Am 3-96 (1997) that extracts all lipids using CO_2. The oil content is expressed as % of whole sample. The resulting oil was protected from light and heat. Australian Government Analytical Laboratories (AGAL) performed the Tocol analysis. Tocols were separated by Normal phase HPLC on a 5u Silica Novapak column using an iso-propanol in tri-methyl pentane mobile phase. Detection was made using fluorescence with excitation at 292nm and emission at 350nm. Quantitation was made against tocopherol and tocotrienol isomer standards (Merck), whose concentration was determined by absorbance measurements. Results are expressed as ug/100g.

Results and Discussion
Whole grain (WG) oil content of barley samples ranged from 2.1%-3.0%, with the hulless, waxy barleys exhibiting the highest oil content (Table 1). The WG oil content of CDC Alamo, Merlin and a breeders line derived from Azhul (WI3693) were significantly different from the samples mean.

Table 1. Oil content of whole grain genotypes

Sample	Type	WG oil content%
Namoi	Hulless	2.2[a]
Torrens	Hulless	2.2[a]
WI3152	Hulless	2.4[a]
WI3044	Hulless	2.1[a]
CDC Alamo	Hulless, waxy	2.9[b]
Merlin	Hulless, waxy	3.0[b]
WI3693	Hulless, waxy	2.9[b]
WI3874	Hulless, waxy	2.5[a]
Schooner	Covered	2.4[a]
Gairdner	Covered	2.0[c]

[a] results followed by the same letter are not significantly different ($P<0.05$)

There were reasonably large differences between oil content of normal hulless barley PF, waxy barley PF and covered barley PF when similar pearling yields were considered (Table 2). Statistics were not performed on oil content of pearled PF because samples were not pearled to an identical yield and time constraints did not allow further analysis. The range in oil content for normal hulless PF was 6.2-7.7%, waxy hulless PF was 7.7-8.1%, and Schooner

covered barley was lowest at 6.1% (Table 2). When the amount of flour pearled from the waxy barleys was increased by approximately 10%, a 1% decrease in oil content was obtained in the waxy PF i.e. CDC Alamo PF at 24% yield, produced 8.9% oil compared to 8% oil from 34% PF yield. Similar results were found for Merlin PF (Table 2). When waxy samples were pearled to approximately 25% PF yield (range 23-27%), the highest oil content was obtained from Sumire mochi, a Japanese waxy variety. Oil content of barley pearled to lesser degrees was not investigated at this stage. YEUNG & VASANTHAN (2001) reported that a 10-12% flour yield was optimal for oil yield (6.2%±0.2 was obtained from a CDC Candle waxy barley). Interestingly, we found that at 27% flour yield CDC Candle produced 9.5% oil. Further pearling and oil analysis is required to clarify this result, however, methods of oil extraction differ and may influence yield. WANG *et al* (1993a) reported PF oil contents for two waxy barleys (Azhul and Waxbar) to be 8.9±0.9% (average) at 20% flour yield. This result compares favourably with results obtained from our analysis. Azhul was not available for our analysis, however a cross, derived from Azhul (WI3693) produced 8.9% oil from flour pearled to 28% yield. This cross also provided excellent pearling characteristics (Data not shown.)

Table 2. Oil content of pearled flour

Sample	Degree of pearling %	Pearling rep	Yield of pearled grain %	Yield of pearled flour %	Oil content pearled flour %
Namoi	28-35		71	29	7.2
Torrens	28-35		69	31	6.7
WI3152	28-35		72	28	7.7
WI3044	28-35		70	30	6.2
CDC Alamo	**22-28**	Rep1	76	24	8.9
CDC Alamo	28-35	Rep2	66	34	8
Merlin	**22-28**	Rep1	75	25	9.3
Merlin	28-35	Rep2	65	35	8.1
CDC Candle	**22-28**		73	27	9.5
Sumire mochi	**22-28**		77	23	10.2
WI3693	28-35		72	28	8.9
WI3874	28-35		70	30	7.7
Schooner	28-35		67	33	6.1

Oil content of commercially produced brewers spent grain (BG) ranged from 9.5-11.7% (Table 3). This is in agreement with KANAUCHI & AGATA (1997) who reported the lipid content of BG as 10.2%. BG from hulless barley malt did not appear to offer any significant increase in oil content over BG from covered barley malt. As predicted, oil was enriched in BG compared to malt and whole barley (Table 3). Rootlets were also analysed but were not a significant source of oil. BONNELY *et al.* (2000) obtained 1.65% oil from malt rootlets. They found only low quantities of tocopherols. This result did not justify the specific extraction of Vitamin E from rootlet oil. Waxy barley was not analysed due to its inappropriateness for malting and brewing. WEBER *et al.* (1991) and LUPTON *et al.* (1994) reported 3g/day of barley oil extracted from BG or 30g/day of bran from BG given as supplements to hypercholesterolemic patients was sufficient to reduce total cholesterol, and LDL-cholesterol, and increased blood tocotrienol without affecting HDL-cholesterol. γ- Tocotrienol and δ-tocotrienol were demonstrated to have the most significant effect. The same effect was observed with oat bran diets, however approximately twice the amount of oat bran was

required for a similar effect. WANG *et al.* (1993b) reported that α-tocotrienol and γ-tocotrienol concentrations of barley oil were 24 and 17 times greater than in corn oil.

Table 3. Oil content of whole grain and malt products

Sample	Whole grain	Spent grain	Malt	Rootlets
Torrens 100%	2.2	nd	2.5	1.85
Gairdner 100% (2 reps)	2	10.3, 9.5	2.6	nd
50%Torrens + 50%Gairdner (2 reps)	2.2	11.7, 9.8	2.2	nd
Mixed micro-malts	nd	nd	nd	1.4
Hulless micro-malts (average 12 lines)	nd	nd	2.3	nd

PETERSON (1994) reported that BG is a rich source of tocols (Table 4).

Table 4. Tocol concentrations of Morex Whole Barley and Brewers' Grains (mg/kg)

Sample	α-T	β-T	δ-T	γ-T	α-T3	β-T3	δ-T3	γ-T3	Total	T3 (%)
WG	10.96	0.78	0.39	0.62	32.52	6.09	0.72	4.66	56.7	77.5
BG	20.24	3.05	1.21	0.9	92.06	16.00	1.75	17.64	152.9	83.4

T=tocopherols, T3=tocotrienols. From PETERSON (1994)

Preliminary investigation of tocol content in PF (Table 5) indicated that similar or higher tocol concentrations were obtained from PF from Australian waxy genotypes compared to results reported by WANG (1993). It is not known whether genotype or method of sample preparation is responsible for the higher results. BG contained low amounts of total tocols (results not shown). PETERSON (1994) reported lower amounts of tocols in BG (see Table 4) compared to PF (Table 5). Interestingly, the Japanese variety, Sumire mochi, contained significantly higher amounts of all tocols tested in the PF fraction. This may be associated with its purple aleurone and potentially high phenol levels. Further investigation is required.

Table 5. Tocol content of BG and PF compared to results from WANG *et al.* 1993. (mg/kg)

Pearled Flour (PF) Sample	α-T	β-T	δ-T	γ-T	α-T3	β-T3	δ-T3	γ-T3	Total
Sumire mochi	230	20	67	34	nd	nd	nd	nd	nd
WI3693	38	2	4	2	155	7	<1	61	269
WI3874	30	1	3	3	143	5	<1	52	237
Two waxy, hulless varieties (mean±*SD*)	35.4	2.0	0.8	21.3	115.8		2.1	28.1	202.5
(WANG *et al*, 1993)	*±9.1*	*±0.3*	*±0.2*	*± 5.1*	*±2.5*		*±1.0*	*±10.4*	

T=tocopherols, T3=tocotrienols.

In summary, preliminary results show that a significant quantity of barley oil can be extracted from brewing and pearling by-products of Australian barley genotypes. A waxy hulless breeders line derived from Azhul, WI3693, exhibited high oil content and high concentrations of tocols in its pearled flour. The extraction of oil from this particular barley may provide a value-added process to a genotype with high pearling quality characteristics. Approximately 300gm of PF or 3gm of oil extracted from PF, would supply useful quantities of tocols for a wide variety of health benefits. Not only are waxy cultivars more suitable for pearling for human food (EDNEY *et al.* 2002; WASHINGTON *et al.* 1999), Waxy PF would provide approximately 1-2% higher oil yield than PF from normal cultivars, which gives them an economic and health advantage over normal cultivars. Based on the toco l analysis, PF from

waxy genotypes would provide a better source of tocols than BG. Further work will focus on large-scale quality evaluation.

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Expression of Heterologous *Vitreoscilla* Hemoglobin in Barley

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Abstract
Anaerobiosis occurs in seeds of many higher plants during imbibition. After successful germination followed by penetration of the enclosing structures by the radicle, the embryo switches from fermentative to aerobic energy metabolism. The microbial population, which is naturally present on the covering layers of barley grains, may lead to oxygen deficiency by competing for oxygen with the grain tissues. Although imbibed barley grains can survive several days under anoxia, they require oxygen to complete germination. Therefore, novel methods are useful to improve oxygen transfer and enhance germination of seeds. The advances in genetic engineering have opened new possibilities to study oxygen limitation and other critical mechanisms of germination.
Previously, heterologous expression of *Vitreoscilla* hemoglobin (VHb) has been shown to improve either growth properties or productivity of various microbes and plants. Therefore, our metabolic engineering approach was to express the *Vitreoscilla* hemoglobin gene (*vhb*) in germinating barley to alleviate adverse effects of inadequate oxygen availability. However, our results show that VHb expression did not improve germination or growth of barley.
Keywords: barley; germination; anaerobiosis; *Vitreoscilla*; hemoglobin; transgenic plant

Introduction

Oxygen Deficiency in Germinating Seeds

Germination of seeds is a chain of events, which commences when a viable dry seed imbibes water and terminates with the elongation of the embryonic axis. Anaerobiosis occurs in seeds of many higher plants during imbibition (BEWLEY & BLACK 1994). After penetration of the enclosing structures by the radicle the embryo switches from anaerobic to respiratory metabolism. Barley aleurone cells react to oxygen deficiency by expressing lactate and alcohol dehydrogenase enzymes (HANSON & JACOBSEN 1984; HANSON *et al*. 1984) and indeed ethanol and acetaldehyde have been detected in the gaseous atmosphere prevailing in industrial germination of non-dormant barley (WILHELMSON *et al*. 2003). These results indicate that barley embryos and/or aleurone cells are suffering from anoxia. These metabolites disappeared when germination was successfully completed and chitting of the grains was observed. Studies also suggest that the aerobic microbial populations normally colonizing the barley kernel may compete with the grain tissues for dissolved oxygen during the steeping phase in industrial scale malting (DORAN & BRIGGS 1993; KELLY & BRIGGS 1992a).

Freshly harvested seeds are often dormant to some extent. Seed coat imposed dormancy is typical for cereal grains. Dormant cereal grains usually germinate well at relatively low temperatures (10-12°C) but germination becomes increasingly difficult at higher temperatures (20-25°C) (CÔME *et al.* 1984). The tissues enclosing the embryo (testa, pericarp and husk) can interfere with water uptake and gas exchange. In cereal grains the tissues enclosing the embryo probably interfere with gas exchange because dormant grains germinate better in conditions of high partial pressure of oxygen and facilitated access to air (BEWLEY &

BLACK 1994). Therefore, oxygen deficiency has been suggested to play an important role both in germination and coat imposed dormancy.

Heterologous Expression of Vitreoscilla Hemoglobin
The obligate aerobe, gram-negative bacterium *Vitreoscilla* synthesizes large amounts of hemoglobin (VHb) when growing in oxygen-limited conditions. Heterologous expression of VHb has been shown to improve growth properties and productivity of various micro-organisms in conditions where oxygen availability has been considered to be a growth limiting factor (FREY & KALLIO 2003). While the mechanisms of the effects of VHb in heterologous hosts are not yet fully characterized, it has been suggested that the expression of *Vitreoscilla* VHb in *E. coli* increases the intracellular effective oxygen concentration under microaerobic environment (KALLIO *et al.* 1994). Thus, VHb-expressing cells may experience a more oxygenated cytoplasmic environment than the external milieu.

Recently, the *vhb* gene has also been expressed in plants. Transgenic tobacco plants expressing VHb have been shown to experience a faster germination and growth rate, to exhibit increased chlorophyll content and enhanced nicotine production (HOLMBERG *et al.* 1997). Recently, FARRÉS & KALLIO (2002) have demonstrated improved cell growth in tobacco suspension cultures expressing *Vitreoscilla* hemoglobin. VHb-expressing cultures showed no lag phase and achieved higher final cell densities compared to the control cultures. However, the physiological effects of VHb expression are still unknown in plants.

In order to alleviate the adverse affects of oxygen deficiency in germinating barley kernels and to study the role of oxygen availability in germination, the *Vitreoscilla vhb* gene was transferred to barley.

Material and Methods
Cloning of the Hemoglobin Gene into the Plant Expression Vector pAHC25
The *vhb* carrying plasmid pUVHb has been described in FARRÉS & KALLIO (2002). The *vhb* gene was PCR amplified (SAIKI *et al.* 1988) and cloned into *Bam*HI–*Sac*I digested pUC19 (YANISCH-PERRON *et al*. 1985). The DNA fragment carrying the *vhb* gene was DNA sequenced, isolated and subcloned into the plasmid pAHC25 (CHRISTENSEN & QUAIL 1996), which was kindly provided by Peter Quaile (USDA, Plant Gene Expression Center, Albany). The recombinant plasmid was named pALI13. All DNA techniques were performed according to the standard protocols (SAMBROOK *et al.* 1989).

Barley Transformations
Barley was transformed using particle bombardment with the procedure of WAN & LEMAUX (1994) as described in NUUTILA *et al*. (1999). Excised immature embryos of barley cv. Golden Promise were used as target material. After bombardment, the embryos were cultured on callus induction medium in the presence of a selective agent. After four subcultures, the resistant callus cultures were transferred to a regeneration medium containing the selective agent.

Analysis of Transformants
Putative transgenic regenerants were initially screened for the presence of the transgene by PCR. The stable integration of the *vhb* gene was confirmed by Southern blot hybridization (SOUTHERN 1975). Probe hybridization and detection of *vhb* were performed according to the DIG High Prime DNA Labeling and detection Starter Kit II (Roche Diagnostics GmbH, Mannheim, Germany).

Western Blot Analysis
The expression of VHb was determined using Western blot analysis as described by FARRÉS & KALLIO (2002) with the following modifications. Complete proteinase inhibitor cocktail tablets (Roche Diagnostics GmbH, Mannheim, Germany) were used instead of PMSF for inhibition of proteolytic activity in the barley extracts. Extracts were concentrated using centrifugal filter devices (Microcon YM-3, Millipore).

Analysis of Germination and Growth
Kernels from transgenic barley plants were germinated in conditions mimicking industrial malting practice. Kernels were immersed in water, followed by germination on moist filter paper (20°C, darkness). When roots and shoots had developed, the plantlets were planted in soil and transferred to the greenhouse. The time span from immersion to completion of germination was recorded and the length of the main stem and the total amount of stems were monitored. The inheritance of the *vhb* gene and expression of VHb was confirmed by PCR and Western Blot analysis, respectively. The negative segregants of each line were used as controls.

Results and Discussion
Bombardments of 1545 immature Golden Promise embryos with the plasmid pALI13 resulted in 157 transgenic barley plants representing at least seven separate lines (Table 1). Line 28c did not produce detectable amounts of VHb protein although the *vhb* gene was detected by PCR. In addition, no VHb protein was detected in the T1 generation of line 30ij although the gene was detected by PCR in 74% of the T1 plants. Therefore, these lines were excluded from further studies. Two VHb-producing lines (29a and 32i) did not produce any seeds, leaving three fertile, VHb-producing lines (3b, 16a, and 32h) that were suitable for further studies.

Table 1. Regeneration of barley plants expressing VHb protein

Line	Number of regenerated plants	Number of fertile, VHb-producing plants
3b	85	16
16a	29	1
28c	1	0
29a	1	0
30ij	8	2
32h	23	15
32i	10	0

Therefore, germination and other experiments were conducted with three transgenic barley lines: 3b, 16a and 32h. Line 16a was heavily contaminated with fungi, and had to be excluded from the study because of extremely poor germination. The data from positive and negative segregants of the lines 3b and 32h were compared to evaluate the effect of VHb expression on germination and growth.

VHb expression did not lead to faster germination rate of barely kernels and no differences in the elongation growth or in the formation of stems were detected. These results differ from those of HOLMBERG *et al.* (1997) who observed much faster germination and growth of VHb-expressing tobacco plants than of controls. However, HÄGGMAN *et al.* (2003) did not observe improved growth of VHb-expressing hybrid aspen in optimal greenhouse experiments, but enhanced growth differences could be detected under suboptimal conditions.

Therefore, they suggested that VHb expression is able to improve growth of long-living forest trees only under suboptimal growth conditions. The greenhouse conditions used in the present study can be considered optimal for germination and growth of barley. Therefore, our results seem to indicate that VHb expression does not affect barley growth in optimal conditions.

It has been hypothesized that cereal grains suffer from anoxia even under optimal germination conditions (CÔME *et al.* 1984). If so, why did not VHb expression improve barely germination in our study? The germination conditions used in the present study were chosen to mimic industrial malting practice, where the kernels are immersed in water before germination. It has been shown that germinating kernels in general quickly consume the dissolved oxygen available (KELLY & BRIGGS 1992b). Therefore, our results suggest that oxygen availability may not be the rate limiting factor in the experimental conditions we have used. It is also possible that the dead, covering layers (husk, testa and pericarp) are efficiently able to reduce the diffusion of oxygen to the VHb-expressing embryo and aleurone layers. Our future studies will analyze the role of more severe oxygen limitations and the physiological effects of VHb expression during barley germination.

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Variation of Starch Pasting Properties among Lines and Pearled Fractions of Waxy Barley Determined by the Rapid-Visco Analyzer (RVA)

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Abstract

Starch pasting properties of waxy barley lines (a *waxy* gene originating from indigenous and introgressed lines and artificial mutants) were measured by the Rapid-Visco Analyzer (RVA). RVA patterns in high-amylose lines (8-10%) showed more stable hot paste viscosity than those of low-amylose lines. Amylose content and hot paste viscosity stability are highly correlated. In starch from different pearled fractions of indigenous and introgressed waxy lines, amylose content in outer parts of the endosperm is higher than that in inner parts. We measure and discuss the amount of waxy protein and enzyme activity of GBSS I in uneven distributions of amylose in indigenous waxy barley. We also discuss the variation in pasting properties dependent on different amylose contents of lines and the waxy protein expression responsible for amylose synthesis.

Keywords: waxy; starch pasting property; RVA; amylose content; NIL

Introduction

Cereal grain endosperm starch generally consists of amylose and amylopectin. Starch composed of amylopectin alone is called waxy. The indigenous waxy barley lines originating in East Asia are mostly hull-less and have been used mainly for specialized food, e.g., in Japan and Korea, hull-less barley was used for making miso and boiled pearled barley as a rice extender. The waxy phenotype produces stickiness, a quality preferred by Japanese, so the uses of waxy barley will be expanded. Intensive breeding efforts have gone into developing modern and agriculturally improved waxy barley cultivars such as “daishimochi,” whose flour and wheat flour mixtures are used for making bread, biscuits, cake, and noodles. In contrast to other cereals, indigenous waxy barley lines have storage starch containing 2-10% amylose. Amylose-free waxy mutant barley was recently bred by chemical mutagen (ISHIKAWA *et al*. 1994) to expand the applications of waxy barley. Amylose content plays an important role in the quality of barley because it affects starch properties. The highly variable amylose content of starch granules from the waxy phenotype of barley may thus be an important consideration in assessing the physicochemical properties of starch. Waxy protein,

the *waxy* (*Wx*) gene product controlling amylose synthesis, is granule-bound starch synthase I (GBSSI) (NELSON and RINES 1962; ECHT & SCHWARZ 1981). We measured the amount of waxy protein and enzyme activity of GBSSI of near-isogenic lines for the *waxy* gene to clarify the starch pasting properties of waxy barley flour by using a Rapid-Visco Analyzer (RVA). We then discuss the variation in starch pasting properties dependent on different amylose content in lines and the expression of waxy protein responsible for amylose synthesis.

Material and Methods

Waxy Lines

We studied 20 waxy barley lines, of which the near-isogenic lines (NILs) were Yon-kei 9548 (B_5F_3; the waxy donor parent is the introgressed line Shikoku-hadaka 96) and Yon-kei 9550 (B_2F_6; the waxy donor parent is the artificial mutant line Shikoku-hadaka 97). The ecurrent parent is Shikoku-hadaka 84, which is the original line of Shikoku-hadaka 97.

Starch Extraction and Amylose Content

After grains were pearled to 60% (hull-less) or 55% (hulled), they were ground using a cyclotec mill (Cyclotec 1093). Barley flour was homogenized with 0.08% NaOH solution and centrifuged at 3000 rpm for 10 min. The starch residue was washed three times with 0.08% NaOH, neutralized, and washed with distilled water and dried. Amylose content of starch was determined based on colorimetric measurement of the iodine-starch complex (absorbance at 620 nm). A potato amylose (Sigma Type III) (100% amylose) and artificial waxy barley (total fraction of starch in Shikoku-hadaka 97: 0% amylose) starch mixture was used as the standard. Experiments were repeated at least five times.

Starch Paste Viscosity

Starch paste viscosity was measured by an RVA. Starch (2.5 g) was mixed with 25 ml of distilled water. The suspension was heated at 35ºC to 95ºC at 3ºC/min and held at 95ºC for 5 min, then cooled to 35ºC at 3ºC/min. The peak viscosity temperature and time maintained above 80% of PV (KIRIBUCHI-OTOBE *et al.* 2001b) was selected to determine the stability of hot paste viscosity.

SDS-PAGE

SDS-PAGE was conducted according to YANAGISAWA *et al*. (2004). To determine the amount of Wx protein, densitometric analysis was conducted using a public-domain Scion image Beta 4.02 program for Windows (available from an anonymous Internet FTP at http://www.scioncorp.com).

Starch synthase activity of granule-bound proteins

Immature seeds (Shikoku-hadaka 84, Yon-kei 9548, and Yon-kei 9550) were collected 15 days after anthesis by removing grains from panicles, immediately frozen, and stored at -80ºC until processed. Starch synthase activity was measured according to NAKAMURA *et al*. (1989). Experiments were repeated 5 times.

Results and Discussion

RVA profiles of some lines (Shikoku-hadaka 96, Daishimochi, and Shikoku-hadaka 97) are shown in Fig. 1. Compared to starch from Shikoku-hadaka 96, time maintained above 80% of peak viscosity was shorter than that of starch from Shikoku-hadaka 97, and final viscosity and total setback were also higher than that of Shikoku-hadaka 97. RVA parameters for starch pasting properties were analyzed by an RVA. Peak time and time maintained above 80% of peak viscosity correlated significantly with amylose content (Fig. 2). Correlation efficiency was 0.78. RVA profiles of near-isogenic lines for *waxy* gene (Yon-kei 9548 and Yon-kei 9550) suggest that amylose affected the stability of hot paste viscosity. Amylose may have inhibited starch swelling. Amylose synthesis is

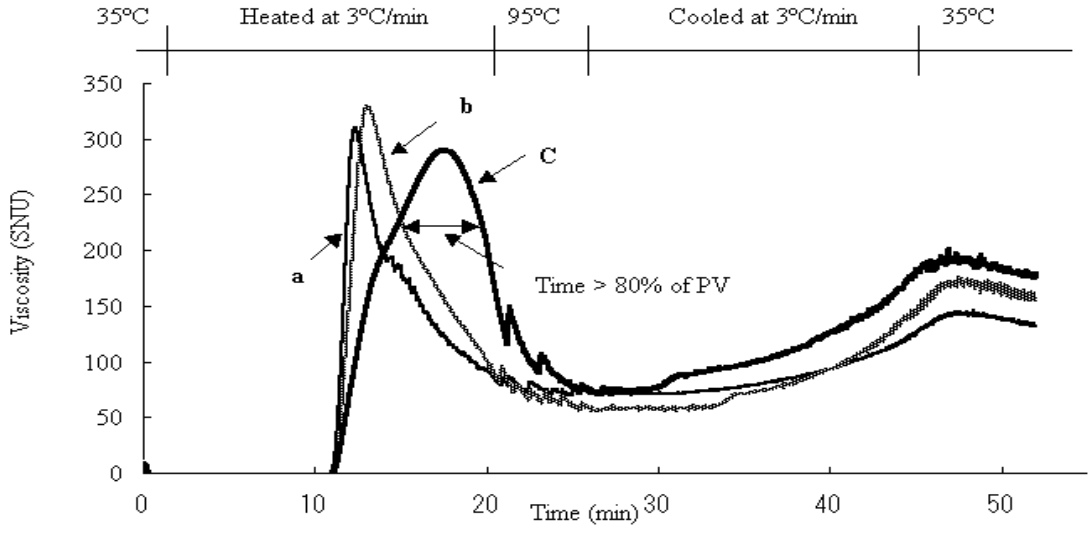

Fig. 1. Starch pasting curves in RVA measurement of waxy barley
a: Shikoku hadaka 97 b: Daishimochi, c: Shikoku hadaka 96

controlled by Wx proteins, and the amount of Wx protein correlates with amylose content. Indigenous and introgressed waxy lines have little Wx protein. Amylose content in outer parts of grain is higher than that in inner parst in these lines (Table 1). Densitometric analysis of the Wx protein from the starch of different pearled fraction in introgressed waxy lines showed that the amount of Wx protein in outer parts exceeded that in inner parts (Table 1). The indigenous *waxy* gene has deletion of the 5'-untranslated region (PATRON *et al.* 2002; DOMON *et al.*

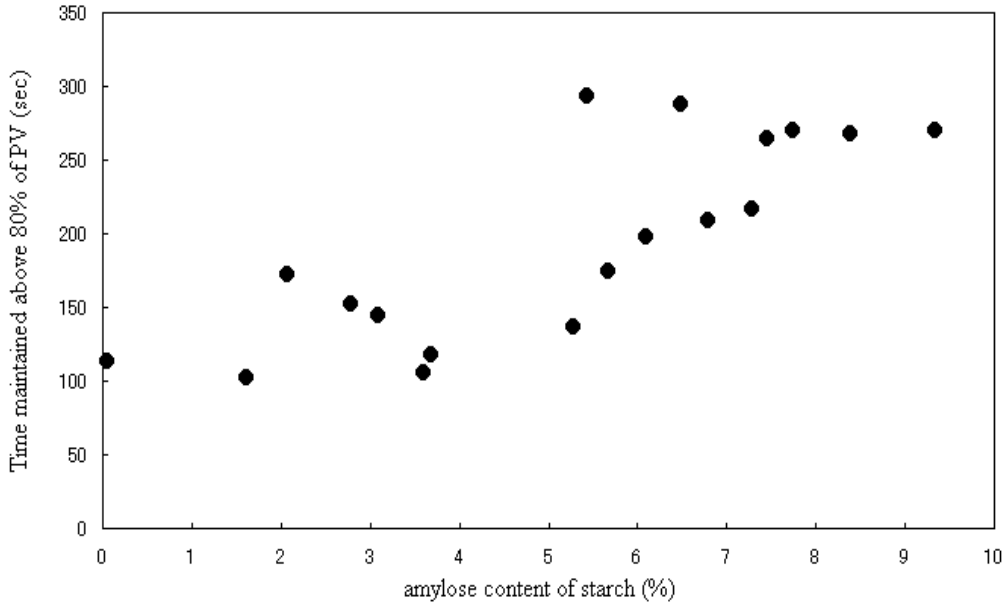

Fig. 2 Relationship between amylose content and stabilty of hot paste viscosity

2002). They invariably found 418 bp in all indigenous Japanese and Korean waxy barley lines (DOMON *et al.*, 2002) and state that alleles in all waxy lines are derived from a single origin. If this hypothesis is true, it remains to be clarified

Table 1 Amylose content and the amount of Wx protein in pearled fraction in introgressed waxy barley lines.

line name	pearled fraction	amylose content (%)	The relative amount of Wx protein (The value of 0%-20% is 100)
Shikoku-hadaka 96	0%-20% (inner part)	4.2	100.0
	20%-40%	6.4	ND
	40%-60%(outer part)	7.6	160.0
Daishimochi	0%-20% (inner part)	3.1	100.0
	20%-40%	4.3	ND
	40%-60%(outer part)	5.2	156.9
Yon kei 9548	0%-20% (inner part)	5.4	100.0
	20%-40%	8.2	ND
	40%-60%(outer part)	9.5	147.5

why amylose content in indigenous and introgressed waxy lines varies.Food products made using waxy barley flour are more difficult to harden after production than those made using nonwaxy flour because waxy starch is more difficult to retrograde than nonwaxy starch. The RVA parameters of final viscosity and total setback are important as indicators of retrogradation. Waxy starch is widely used because of its unique starch pasting property. Waxy starches are not usually used unmodified in the food industry, where they are cross-linked and often substituted. Cross-linked starch is more resistant to acid, heat, and shearing than native starch, and is therefore suitable for applications such as canning. We found that native waxy barley starches from some indigenous and introgressed lines have hot

paste stability, suggesting that these starches are more widely used because most consumers prefer them to natural ingredients. Such findings are particularly useful to barley breeders and food chemists.

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S 5 – MALTING AND BREWING – TECHNOLOGICAL PARAMETERS

An Investigation of the Relative Rates of Protein and Carbohydrate Modification of a Number of International Malting Varieties, Grown in Three Countries

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Abstract
In this study, eleven international malting varieties and one feed variety were grown in Scotland, Canada and Australia in 2002. They were malted in a Phoenix® micomalter using a typical micromalting regime from each country. These regimes varied greatly, with large differences in both the number and length of steeps and the length of time for germination. In an attempt to gain a greater understanding of the influences of both the growing environment and the malting regime, a number of traits influenced by the modification process were compared with the activity of a number of the proteolytic and hydrolytic enzymes present in the malt and the level of the fermentable sugars in the wort. Both the environment and micromalter regime influenced the degree of modification of the grain. The moisture content of the grain at the end of steep had a greater influence on both the level of modification of the cell walls and proteins than the length of the germination process. The strongest relationships were found between both cell wall and protein modification, and limit dextrinase activity and wort glucose levels.
Keywords: barley; modification; protein; carbohydrate; malting

Introduction

During malting, barley is steeped in water and allowed to germinate. During this process some of the complex carbohydrates and proteins are degraded and important enzymes are produced. This process is generically termed modification. The overall degree and relative proportion of starch, protein and cell wall modification during malting are critical in determining the quality of the final product. The modification process is greatly influenced by both the variety investigated and the environment in which the variety is grown. Currently, there are a large number of high malting quality varieties being grown worldwide that differ dramatically in the way in which they modify. These varieties are adapted to vastly different growing environments and often perform poorly when removed from their ideal environment. Additionally, the malting regime used to produce the malt has a large influence on the final product, reflecting brewer or distiller specification.

Traditionally, a number of different methods have been used to describe malt modification (MOLL 1996a, 1996b, 1996c, 1997). In this study, we used hot water extract (HWE) as a best measure of overall malting quality, kollbach index (KI), the ratio between soluble and malt protein, as an indicator of protein modification, malt beta glucan (MBG) as an indicator of cell wall modification and the total fermentable sugar content (TFS) in the wort as an indicator of starch modification. In an attempt to gain a greater understanding of the influence

of both the growing environment and the malting regime, these have been compared with the activity of a number of the proteolytic and hydrolytic enzymes present in the malt, and the level of individual sugars in the wort.

Material and Methods

Twelve varieties were grown in 2002, in duplicate, at Dundee in Scotland and Charlick in South Australia, and in single replicate trials at the University of Saskatchewen Kernan and Campus Crop Research Farms at Saskatoon, Canada. The varieties chosen were Cooper, Alexis and Optic from Europe, CDC Copeland, Harrington, CDC Dolly, AC Metcalfe and Newdale from Canada, and Arapiles, Franklin, Sloop and Stirling from Australia. The feed variety CDC Dolly was included as a poor quality baseline. Each sample was malted in a Phoenix Automated Micromalter, using three micromalter regimes (Table 1). These were chosen to represent a typical laboratory regime from each of the regions in the study. The moisture content of the grain was calculated at the end of steep from the change of weight in grain, of known moisture content at the outset. For the following discussion the two Canadian sites were combined in order to compare the differences between the countries.

Table 1. Micromalter regimes

	Steep		**Germination**		**Kiln**		
	Time (hours)	**Temp (°C)**	**Time (hours)**	**Temp (°C)**	**Time (hours)**	**Temp (°C)**	**Total Time (hours)**
South Australian (LOGUE, 1997)	7 8 (AR)[a] 9	15 15 15	94.5	15	9 4 2 4.5 0.5	30-40 40-60 60-70 70-80 80-25	138.5
Canadian (LANGRELL AND EDNEY, 2001)	10 18 (AR) 8	13 13 13	12 108	13 15	12 6 2 4 0.5	30-55 55-65 65-75 75-85 85-25	180.5
Scottish	8 10 (AR) 12 6 (AR) 4	16 16 16 16 16	104	16	24 0.5	60 25	168.5

[a]AR: Air rest

Malt protein (MP) was measured using near infrared reflectance and SP was measured using the spectrophotometric method according to the ASBC method (ASBC, wort17, 1987). HWE was measured using a small-scale version of the EBC method (EBC ANALYTICA, 1998). SP was measured on the wort produced by the HWE method. KI was measured by dividing the soluble protein by the malt protein and was expressed as a percent. Limit dextrinase activity (LD), β-Glucanase activity, MBG and α-amylase activity were measured using appropriate Megazyme assay kits according to the manufacturers instructions. Endoproteinase activity was measured using azogelatin as a substrate following the method of JONES *et al.* (1998). The activity was assessed at pH 4.8 in both the presence (proteinase-c) and absence (proteinase) of cysteine (4mM) as a reducing agent (JONES *et al.* 2000).

Fermentable sugar analysis was performed using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using a Dionex GP40 gradient pump, a Dionex ED40 electrochemical detector and a Shimadzu SIL-10AD autoinjector. It was fitted with a Dionex CarboPac PA-1 column and PA-1 precolumn. The wort was eluted using a linear gradient of sodium acetate (16-120mM) in sodium hydroxide (100mM, made from low CO_2, 50% w/w liquid sodium hydroxide) over 34 minutes with a flow rate of 1ml/min. TFS was calculated by combining the levels of glucose, fructose, sucrose and maltose in the wort.

Statistical analysis was performed using the program JMP IN® (version 4.0.3, SAS Institute Inc.) and Genstat® (release 6.1, version 6).

Results and Discussion

Micromalter Regimes

The three micromalter regimes were very different (Table 1). The amount of time the grain was under water varied greatly from 16 hours in the South Australian regime to 24 hours for the Scottish regime. Consequently, the steep out moistures were significantly different ($P<0.05$) (Table 2). This difference was reflected in the Scottish regime producing the highest levels of modification of the cell walls and proteins and the Australian regime producing the lowest levels. The Canadian regime produced the highest levels of starch modification and the Australian regime the lowest. However, HWE levels were similar across all regimes. The length of germination varied from 94.5 hours in the South Australian regime to 120 hours in the Canadian regime. This appeared to play a minor role in influencing protein and cell wall modification despite the differences in steep-out moisture.

Table 2. The mean and least significant difference (lsd) of a number of traits, malted using three micromalter regimes

	Micromalter Regime			
Trait	**Canadian**	**Sth Australian**	**Scottish**	**lsd[a]**
Steep out moistures (%)	44.4	42.6	47.2	0.5
HWE (%db)	77.5	77.3	77.6	0.9
MBG (%db)	0.9	1.6	0.6	0.1
KI (%)	39.6	35.2	43.1	2.4
TFS (mmols/L)	200	187	195	5.4
LD (U/kg)	403	252	499	43
Protease-c(units)	0.40	0.26	0.26	0.03
Protease (units)	0.18	0.19	0.18	0.02
ß-Glucanase (U/kg)	598	564	319	37
α-amylase (μm/min/g)	267	183	259	20
MP (%db)	13.6	12.8	12.8	0.7
Glucose (mmols/L)	55.5	45.8	56.0	3.2
Fructose (mmols/L)	9.3	8.5	9.2	0.9
Sucrose (mmols/L)	8.0	7.2	7.9	1.0
Maltose (mmols/L)	128	129	123	5
Maltotriose (mmols/L)	15.9	15.7	14.6	0.6

[a]least significant difference ($P<0.05$)

The only enzyme that reflected a similar trend to that of cell wall and protein modification was limit dextrinase (Table 2). The Scottish regime produced significantly lower levels of β-glucanase activity ($P<0.05$) than the other regimes. This may have been due to differences in kilning. While the South Australian and the Canadian regimes reached 80 and 85°C, the rise was gradual and the moisture content of the grain would have been low before the highest temperatures were achieved. In the Scottish regime the kilning involved a single temperature of 60°C. β-glucanases are heat labile and their activity is reduced during kilning (LOI *et al.* 1987; BARBER *et al.* 1994). This reduction would have been greatest if the temperature was high while the grain was still very wet, as in the case of the Scottish regime. By contrast, levels of α-amylase were highest in the Canadian regime, indicating that the three regimes produce different products, reflecting end-use. Slightly under-modified, low diastase malts are suited to Australian brewing, with sugar-based adjuncts, while starch-based adjuncts, used in North America require higher amylase activity. In Scotland, malt is used primarily in distilling, where initial extraction is at 65°C, so β-glucanase activity must occur during malting to give a well modified product. Differing levels of starch breakdown in the three regimes were indicated as the South Australian regime produced significantly lower ($P<0.05$) levels of wort glucose and fructose. The Canadian regime produced significantly lower ($P<0.05$) levels of maltose and the Scottish regime significantly lower ($P<0.05$) levels of maltotriose in the wort.

Country Differences

All the growing environments experienced atypical conditions in 2002. The Australian and Canadian environments experienced drought conditions, while the Scottish site produced poor grain filling, due to colder, wetter conditions than usual. Proteins at all sites were thus higher than would normally be expected for malting samples (Table 3). Consequently HWE levels were lower at all three sites than generally reported for malting varieties. Differences across seasons will be determined following completed analysis on samples grown in 2003.

Large differences were found between the levels of HWE and KI produced from the three countries. This can partially be attributed to the significantly different ($P<0.05$) levels of protein found between the three sites. However, Canada produced the highest levels of protein, while Australia produced the lowest levels of HWE.

Table 3. The country mean and least significant difference (lsd) of malt protein and the four indicators of modification of twelve varieties, malted using three micromalting regimes

	Country Grown			
Trait	**Australia**	**Canada**	**Europe**	**lsd[a]**
HWE (%db)	75.0	77.4	78.7	0.7
MBG (%db)	1.0	1.2	1.1	0.2
KI (%)	34.4	37.7	45.1	2.2
TFS (mmols/L)	194	191	197	5.7
MP (%db)	13.7	14.7	11.8	0.6

[a]least significant difference ($P<0.05$)

To gain a greater understanding of the influence of the growing environment on the modification profile the indicators of modification have been compared with the activity of a number of the proteolytic and hydrolytic enzymes present in the malt, and the level of individual sugars in the wort for each country (Table 5). Due to the large number of samples in the trial significant correlations were found between numerous traits. However, this discussion will be limited to the traits with the strongest.

LD formed strong correlations with KI and MBG from all countries. As LD degrades branched dextrins present in starch it is unlikely that this is a direct relationship. The relationship between LD and HWE or TFS was significant at most of the sites but was not as strong.

Glucose formed strong correlations with KI, MBG and TFS for all countries. As glucose contributes approximately 25% of the total fermentable sugars in the wort it is not surprising that a significant relationship was found between TFS and Glucose. The negative relationship between MBG and glucose would be directly attributable to the hydrolysis of the MBG to glucose during both malting and mashing. Strong correlations were also found between α-amylase and MBG for all countries, and α-amylase and KI at the Australian site.

Table 4. The correlation (r) between four indicators of modification and a number of other traits, for each country, of twelve varieties, malted using three micromalting regimes

	HWE			KI			MBG			TFS		
	Aust	Can	Scot	Aust	Can	Scot	Aust	Can	Scot	Aust	Can	Scot
Protease	0.17	0.07	0.07	0.31	0.19	0.00	-0.06	-0.05	0.17	0.10	-0.05	-0.18
Protease-c	0.32	0.12	0.04	0.46	0.19	0.10	-0.36	-0.24	-0.23	0.37	0.13	0.33
ß-Glucanase	0.14	0.23	0.07	0.09	0.05	-0.21	0.11	0.09	0.23	0.21	0.21	0.05
α-amylase	0.20	-0.04	0.06	**0.70**	0.48	0.42	**-0.56**	**-0.58**	**-0.50**	0.35	0.32	0.40
LD	0.36	0.30	0.41	**0.76**	**0.72**	**0.70**	**-0.79**	**-0.70**	**-0.71**	0.19	0.24	0.39
Glucose	0.38	0.27	0.46	**0.80**	**0.76**	**0.73**	**-0.72**	**-0.75**	**-0.73**	**0.66**	**0.67**	**0.75**
Fructose	0.05	-0.01	.24	**0.51**	0.46	**0.65**	-0.48	**-0.52**	**-0.55**	0.45	0.42	**0.49**
Sucrose	0.41	0.17	0.11	-0.05	0.05	0.21	-0.09	-0.02	-0.27	0.48	0.21	0.41
Maltose	0.35	0.40	0.15	-0.12	-0.25	-0.07	-0.13	0.21	-0.09	**0.76**	**0.67**	**0.84**
Maltotriose	0.42	0.24	**0.53**	-0.15	-0.34	0.32	-0.07	0.25	-0.41	0.49	0.43	**0.79**

$|r|>0.22$ significant $P<0.001$, **bold**: $|r|>0.5$

Conclusion

This trial assessed the influence of both environment and micromalter regime on the modification of high malting quality varieties. By growing the trial in three countries large environmental differences were experienced. Additionally, three very different micromalter regimes were used. Modification of the cell walls and proteins was influenced by the moisture content of the grain at the end of steep. Limit dextrinase activity and wort glucose showed the strongest relationships with both cell wall and protein modification.

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Understanding the Genetic Bases of Modification

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Abstract
The level of modification of barley grain during malting is one of the factors that determines the quality of the final malt and beer product. It is important that maltsters achieve a suitable level of protein, starch and cell wall modification**.** It is important to not only achieve high levels of major hydrolytic and proteolytic enzymes by the end of the malting process, but also during the malting process. High cell wall and starch modification are likely to occur if high levels of β-glucanase, endoproteinases and limit dextrinase are present in the malt within 48 to 72 hours of the start of germination. Endoproteinase activity was found to be associated with the telomere region on the long arm of chromosome 5H in the Sloop/ Alexis mapping population. The fermentable sugars glucose and fructose were found to be associated with regions on Chromosomes 1H and 5H.
Keywords: barley; malt; modification; protein; carbohydrate; QTL

Introduction

The proportion and degree of starch, protein and cell wall modification during malting are critical in determining the quality of the final product. While high levels of cell wall modification are desirable to prevent filtration problems, only partial degradation of protein is required, with too much being detrimental. The relationship between cell wall and protein modification is complex with numerous genes likely to be involved. A number of commonly measured traits give an indication of the level of modification. Free amino nitrogen (FAN), kolbach index (KI) and soluble protein (SP) are used as indicators of the level of modification of the proteins, while malt β-glucan (MBG), wort beta glucan (WBG) and wort viscosity (WV) indicate the level of modification of the cell walls. The Australian Winter Cereals Molecular Marker Program (AWCMMP) has investigated these traits using a number of Australian mapping populations. Due to the complex relationship between protein and carbohydrate modification this information will not be useful to barley breeders unless an understanding of how and why these regions are influencing modification is gained. The aims of this study were to gain a greater understanding of the processes involved in modification in two ways. The first is by assessing the changes that occur in the grain during the malting (modification) process. The second is by assessing regions of the barley genome found to be associated with modification related traits.

Material and Methods

Hot water extract (HWE) was measured using a small-scale version of the EBC method (EBC Analytica, 1998). Extractable (soluble) protein was measured by heating 250mg of ground green malt with 2ml of water, at 65ºC for 1 hour. Limit dextrinase activity (LD), β-Glucanase (BGase) activity, α-amylase activity (AA) and MBG levels were measured using the appropriate Megazyme assay kit, according to the manufacturers instructions. Endoproteinase activity was measured following the method of JONES *et al.* (1998), using azogelatin as a substrate, at a pH of 4.8, in both the presence (protease-c) and absence (protease) of cysteine (4mM) as a reducing agent (JONES *et al.* 2000).

The Assessment of the Modification Changes that Occur during Modification (Malting)
Four varieties (Alexis, Harrington, Sloop and Arapiles) were grown at Charlick, South Australia, in 2000. These were malted, in triplicate using the malting schedule: 22 hour steep (7 wet, 10 dry, 5 wet) and followed by germination for 94 hours, at 15˚C. Samples were taken at the end of steep and every 24 hours during germination and frozen before being freeze dried. After 72 and 94 hours of germination the samples were kilned for 20 hours, from 30˚C to 80˚C.

Mapping
The Sloop/Alexis and Sloop-sib/Alexis mapping populations were grown in duplicate by the South Australian Barley Improvement Program at Charlick in 2000. The Sloop/Alexis population consists of 109 F_1 derived doubled haploid lines. The linkage map was constructed from 304 markers (BARR *et al.* 2003). The Sloop-sib/Alexis population consists of 152 F_4 derived recombinant inbred lines (RIL). The linkage map was constructed from 282 markers BARR *et al.,* 2003). The combined linkage map consisted of 261 markers (BARR *et al.,* 2003). Simple regression and interval analyses were performed using the computer software program "Qgene" (NELSON 1997).

Protease activity was measured on freeze dried, green malt, after 72 hours of germination. The level of fermentable sugars in the wort was measured by high performance anion exchange chromatography using a pulsed amperometric detector, as described in COLLINS *et al.* (2004). Statistical analysis was performed using the program JMP IN® (version 4.0.3, SAS Institute Inc.).

Results and Discussion
Cell Wall Modification
The cell walls within the endosperm of the barley grain consist of approximately 75% (1→3)(1→4)-β-glucans and 20% arabinoxylans (FINCHER 1975). The breakdown of the cell walls during malting is one of the most important processes involved in the modification of the grain. The presence of the cell wall material within the endosperm can physically hinder the access of amylolytic enzymes to starch granules. Therefore the partial or complete removal of these during malting is important. Common indicators of cell wall modification are WBG, MBG and WV. Levels of both β-glucans and arabinoxylans within the wort can influence WV. Cell wall modification was found to be associated with BGase, LD, wort glucose and wort fructose levels (COLLINS *et al.* 2003b; COLLINS *et al*. 2004).

It was found that by 48 hours into germination Sloop had significantly higher levels of BGase ($P<0.05$) than Alexis and Harrington (Figure 2b). This continued throughout the germination process and during kilning. As a consequence of this, by 48 hours Sloop had produced significantly lower levels of MBG ($P<0.05$) than all other varieties (Figure 2b), and significantly lower levels of WBG in the wort ($P<0.05$, data not shown) than the other varieties.

Wort glucose and fructose were found to be associated with regions on chromosomes 1H and 5H in the combined Sloop/Alexis and Sloop-sib/Alexis mapping population (Table 1). The region on chromosome 1H was also found to be associated with WBG and WV in this population (Figure 2, PANOZZO *et al.,* in preparation) with Sloop donating the alleles for higher levels of WV and WBG and lower levels of wort glucose.

Protein Modification
During the malting process approximately 50% of the protein is degraded (BARRRETT & KIRSOP 1971), however this is dependant on the malting conditions. Most importantly the proteins adhering to the starch granules need to be degraded to allow the starch degrading enzymes access to the starch granules. Common indicators of protein modification are FAN, KI and SP, although levels of these are also dependant on the amount of protein present in the grain before malting. FAN and SP were found to be associated with BGase, (COLLINS *et al.* 2003b). KI was found to be associated with LD, protease-c, wort glucose and wort fructose levels (COLLINS *et al.* 2003b; COLLINS *et al*. 2004).

In this study it was found that after 72 hours of germination there was no significant difference between the levels of the protease when cysteine was added into the reaction (data not shown). However when no cysteine was added Sloop and Harrington produced significantly higher levels of protease than Alexis ($P<0.05$) (Figure 2a). After kilning of the 94 hour malt Sloop and Harrington both had statistically higher levels of protease than Alexis and Arapiles. As a consequence of this by 72 hours into germination Sloop had produced significantly higher ($P<0.05$) levels of soluble protein than the other varieties (Figure 2a). Additionally Sloop produced significantly higher levels of FAN, SP and KI ($P<0.05$, data not shown) than the other varieties and Harrington produced higher SP and KI than Alexis ($P<0.05$, data not shown).

Protease-c was found to be associated with a region on the telomere of chromosomes 5H in the combined Sloop/Alexis mapping population (Table 1). This region was also found to be associated with FAN in this population (Figure 2, PANOZZO *et al.,* in preparation). It has also been found to be associated with numerous other traits in other populations (Figure 2) including SP and KI in the Harrington/TR306 population (OZIEL *et al*. 1996; MATHER *et al.* 1997). No significant region was found to be associated with protease.

Starch Modification
During malting, starch granules undergo a number of changes. These include the partial or complete removal of protective proteins and cell wall material to allow amylolytic enzymes to attack. The assessment of the modification of starch is quite difficult. Other studies have used HWE and total fermentable sugars (COLLINS *et al.* 2003b; COLLINS *et al*. 2004). In one of these studies HWE was found to be associated with LD (COLLINS *et al.* 2003b).

After 48 hours of germination Harrington was found to have significantly higher levels of LD than the other varieties ($P<0.05$, Figure 2d). After kilning of the 94 hour malt Harrington had significantly higher levels of both LD and HWE than the other three varieties ($P<0.05$). Harrington was also found to have significantly higher ($P<0.05$) levels of AA than all other varieties after kilning of the 72 hour malt (Figure 2c).

LD has not been investigated in the Sloop/Alexis population. However a region was found to be associated with LD on chromosome 2H in the Galleon/Haruna nijo mapping population (LANGRIDGE *et al.* 1996). This region was also found to be associated with HWE in the same mapping population (COLLINS *et al.* 2003a)

Conclusion
High levels of carbohydrate and cell wall modification are likely to occur if β-glucanase, endoproteinases and LD are present in the malt within 48 to 72 hours of the start of germination. Endoproteinase activity was found to be associated with the telomere region on

the long arm of chromosome 5H in the Sloop/ Alexis mapping population The fermentable sugars glucose and fructose were found to be associated with regions on chromosomes 1H and 5H.

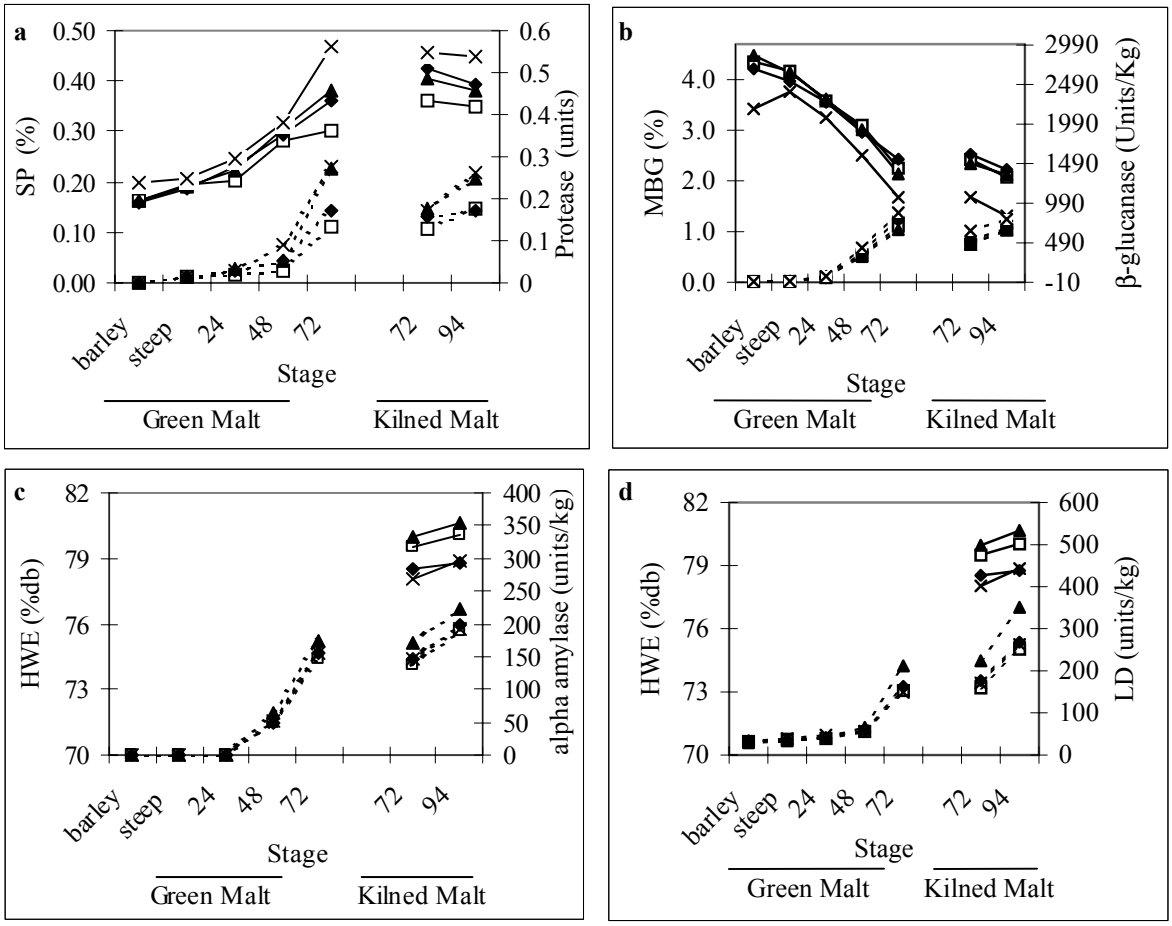

Figure 1. The change in activity of four enzymes, during the malting process (dashed line), compared to the indicators of modification (solid line). a: soluble (extractable) protein and protease without cysteine, b: MBG and ß-glucanase, c: HWE and α-amylase, d: HWE and LD. × Sloop, □ Alexis, ▲ Harrington, ◆ Arapiles

Table 1. Regions of the barley genome found to be associated with protease-c and wort sugars in the Sloop/Alexis and Sloop-sib/Alexis mapping populations

Trait	Population	Chromosome	Most Significant Marker	Source	R^2	LOD	Bin
Protease-c	Sloop/Alexis	5H	*XP11/M51-193*	Alexis	0.20	4.6	14-15
Glucose	Combined Sloop/Alexis and Sloop-sib/Alexis	1H	*XEBmac0501*	Alexis	0.07	3.2	6-7
		5H	*XBmag0222*	Alexis	0.08	3.9	13-14
		7H	*XP14/M60-177*	Alexis	0.10	4.4	9-10
Fructose	Combined Sloop/Alexis and Sloop-sib/Alexis	1H	*XEBmac0501*	Alexis	0.07	3.2	6-7
		3H	*Xabg4*	Sloop	0.13	6.3	12-13
		5H	*XBmag0222*	Alexis	0.09	4.4	13-14
Sucrose	Combined Sloop/Alexis and Sloop-sib/Alexis	1H	*XEBmac0501*	Sloop	0.07	3.6	6-7
		2H	*XP13/M62-133*	Alexis	0.13	5.4	6-8
		3H	*Xabg4*	Alexis	0.08	3.9	12-13

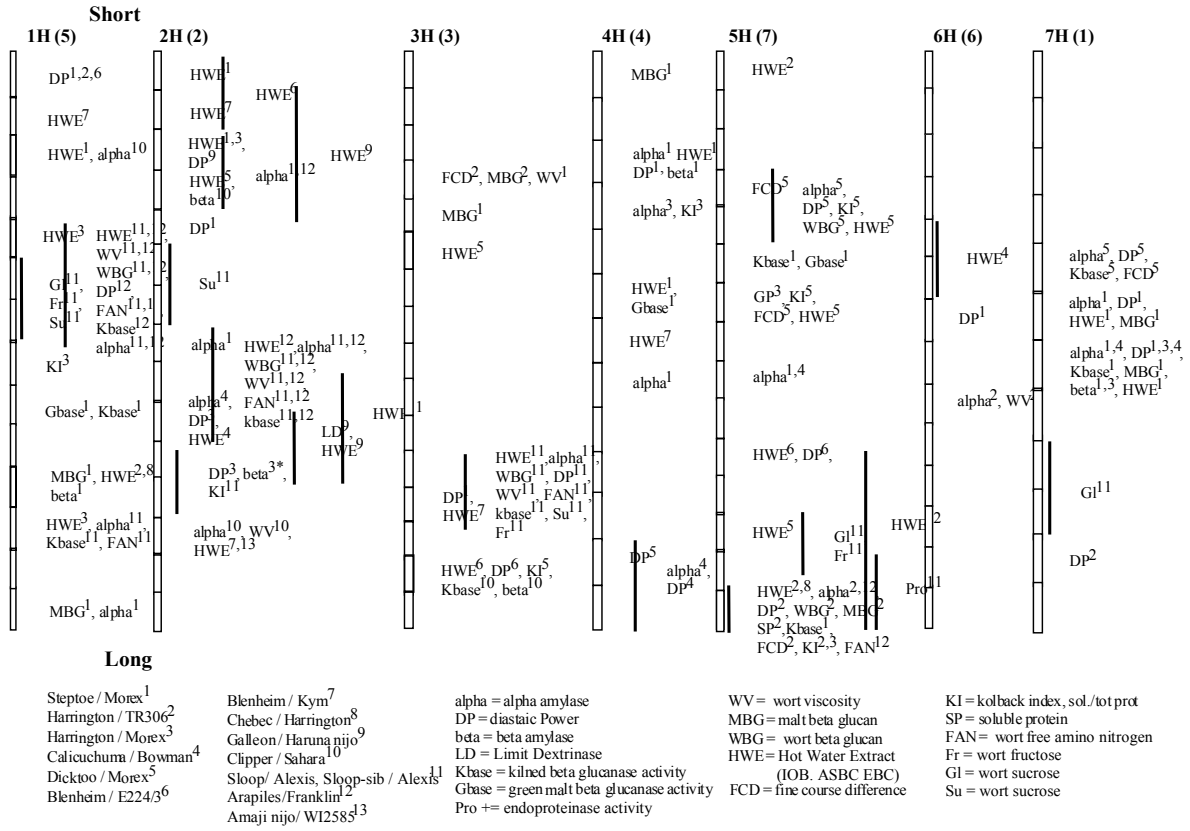

Figure 2: A schematic diagram of a number of regions found to be associated with modification and enzyme traits. Each chromosome is separated into a number of regions called Bins as described by KLEINHOFS AND HAN (2002).

(HAYES *et al.* 1993; HAN *et al.* 1995; THOMAS *et al.* 1995; HAYES *et al.* 1996; LANGRIDGE *et al*., 1996; LARSON *et al.* 1996; LI *et al.* 1996; OZIEL *et al.* 1996; THOMAS *et al.* 1996; ZWICKERT-MENTEUR *et al.* 1996; HAYES *et al.* 1997; MATHER *et al.* 1997; POWELL *et al.* 1997; ULLRICH *et al.* 1997; BEZANT *et al.* 1997; MARQUEZ-CEDILLO *et al.* 2000; ZALE *et al.* 2000; HAYES *et al.* 2001; COLLINS *et al.* 2003a; PALLOTTA *et al.* 2003; BARR *et al.* 2003a; KARAKOUSIS *et al.* 2003a; KARAKOUSIS *et al.* 2003b; BARR *et al.* 2003b)

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Malting Quality of a Hulless/Covered Doubled Haploid Barley Population

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Abstract

The potential to improve malting quality of hulless barley was investigated in a two-row doubled haploid population (TR251/HB345). TR251 is a covered, breeding line with good malting potential while HB345 is a hulless, breeding line with good agronomic traits and the allele for heat stable beta-amylase (sd2H). Barley grown in standard yield test plots at Brandon, Manitoba during 2002 was micromalted and analysed using standard conditions. The 54 hulless lines, on average, had significantly higher levels of malt extract than the 54 covered lines. A significantly lower percentage of the hulless extract was fermentable but the hulless lines still fermented a greater amount of total extract. Levels of diastatic power were similar among hulless and covered malts but alpha-amylase was significantly lower in the hulless lines. Heat stable beta-amylase improved fermentability but not significantly. Samples showed an acceptable degree of malt modification but beta-glucan levels still had a negative effect on fermentability. Levels of total free amino acids were similar among hulless and covered worts; however, levels of several individual amino acids were significantly lower in the hulless worts which had a negative effect on fermentability. In summary, hulless malt had some superior attributes compared to covered malt but increased levels of some specific enzymes would further enhance its appeal.

Keywords: hulless barley; malt; fermentability; alpha-amylase; ß-glucan; doubled haploid population

Introduction

The malting and brewing industries continue to express interest in the commercial use of hulless barley malt. They are intrigued by potential economic returns such as increased levels of malt extract, less production of spent grains and reduced shipping costs for both barley and malt. Current hulless varieties have been shown to produce significantly higher levels of malt extract than covered barley varieties. The present varieties are also capable, during malting, of producing an adequately modified endosperm given an appropriate malting regime (EDNEY & ROSSNAGEL 2000), and levels of malt diastatic power have been shown to be similar between the two types (EVANS *et al.* 1998). However, α-Amylase levels have tended to be lower (EDNEY & LANGRELL 2004) and fermentability of hulless wort has received limited attention.

The acceptable malt quality of the current hulless varieties has been achieved with only limited attention to breeding for malting quality. Breeders are showing increased interest in breeding hulless barley for malt quality but are uncertain of breeding targets and have tended to aim for the quality targets of covered barley. Most research on hulless barley malt has concentrated on comparisons with established varieties of covered malting barley. The relevancy of such comparison could be questioned given possible effects of removing the hull on processing. Hulless barley has been shown to be more susceptible to the severe conditions of kilning, which resulted in poorer friability values (EDNEY & LANGRELL 2004).

The present study investigated differences in malt quality between covered and hulless barley using a doubled haploid population from a covered/hulless barley cross (TR251/HB345). The doubled haploid nature of the population, which randomizes genetic traits across lines in the population, allowed the hulless trait to be isolated from other genetically-controlled traits. The result was an excellent opportunity to study the effect of hull removal on malt quality.

Material and Methods

The doubled haploid population (54 covered and 54 hulless lines) was produced by anther culture techniques at Agriculture and Agri-Food Canada (AAFC) Brandon from the cross, TR251/HB345. TR251 is a covered breeding line with good malting potential while HB345 is a hulless breeding line with good agronomic traits and the allele for heat stable beta-amylase (sd2H). The 109 lines along with two replicates of each parent, were grown at Hamiota, Manitoba in 2002. Samples of the lines and parents (500 grams) were micromalted in a Phoenix Automated Micromalting machine (Adelaide, SA, Australia) according to the following schedule: Wet steep 6 hours, Air rest 2 hours, Wet steep 4 hours, Air rest 12 hours, Wet steep 4 hours, Air rest 4 hours, Wet steep 4 hours, Air rest 4 hours, Wet steep 4 hours (steeping at 13°C); Germination 100 hours (15°C), Kiln 12 hours @ 55°C, 6 hours @ 65°C, 2 hours @ 75°C, 4 hours @ 85°.

Standard methods of the ASBC (AMERICAN SOCIETY OF BREWING CHEMISTS 1992) were used to prepare fine grind Congress malt extracts and to analyse for wort ß-glucan (Calcofluor), soluble protein, free amino nitrogen, diastatic power, alpha-amylase activity and alcohol (GC method). Analysis of free amino acids was based on the method of GARZA-ULLOA *et al* (1986) and was performed on a Beckman 7300 High Performance Amino Acid Analyzer (Beckman Coulter, Inc., Fullerton, CA 92834-3100).

A small scale method for measuring apparent attenuation limit (AAL) was used to determine the fermentation properties of the samples. The method incubated 40 ml of EBC wort (EUROPEAN BREWERY CONVENTION 1998) with 160 mg dried yeast (Mauribrew lager yeast, Toowoomba, Australia) at 25°C for 24 hours (LOGUE 1997). Levels of fermentable sugars in worts were determined using a DX-500 HPLC system (Dionex).

Results and Discussion

Hulless barley lines produced significantly higher levels of Congress malt extract than the covered lines (Figure 1). Higher extract levels are routinely observed in hulless barley malt and are a major reason for the brewing industry's interest in hulless malt. Higher extracts are direct result of absence of hull which contributes nothing to malt extract and can represent 10% or more of the weight of a barley kernel.

Extracts from the hulless lines had significantly lower apparent attenuation limits than from the covered lines (Figure 2). However, despite poorer apparent attenuation limits, malts from the hulless lines were still able to produce significantly higher levels of alcohol (Figure 3), the result of higher initial extract levels. Improvements to hulless malt fermentability could further extend its advantage of producing greater amounts of alcohol (beer) per unit weight of malt.

The fermentability of a wort is dependent on a number of malt parameters including levels of starch degrading enzymes. Hulless lines had significantly lower alpha-amylase levels compared with covered lines (Figure 4) but levels of diastatic power were not significantly

different (Figure 5). MACGREGOR *et al.* (1999) have shown that alpha-amylase can play a significant role in producing fermentable sugars, an indicator of fermentability. Therefore, lower levels of alpha-amylase in hulless lines may have contributed to reduced fermentation potential of the hulless malts. However, diastatic power, consisting mainly of beta-amylase, is usually considered to be of greatest importance in determining fermentability (EVANS *et al.* 2003).

The reduced levels of alpha-amylase in hulless lines was likely connected with the physical loss of hull. Unless linked to the covered/hulless locus, any variability of alpha-amylase under genetic control would have been randomized across the hulless and covered lines, due the doubled haploid nature of the samples tested. Absence of hull does result in the outer layers of hulless malt being more affected by the extreme temperatures of kilning (EDNEY & LANGRELL 2004). Therefore, it is possible that some alpha-amylase activity, which is synthesised in the outer aleurone layers of the malt, could have been inactivated during kilning. In contrast, beta-amylase, as indicated by diastatic power in this study and which did not differ between the hulless and covered malts, is located in the endosperm of malt and would have been more protected during kilning.

Fermentability of a malt can also be reduced by poor endosperm modifcation (BATHGATE *et al.* 1978). The present study did indicate that malt from hulless lines had significantly higher levels of ß-glucan than that from covered lines (Figure 6). ß-Glucan levels were also significantly, negatively correlated with apparent attenuation limit (r=0.51***). Specialized malting conditions may be required to achieve adequate endosperm modification in hulless barley compared with covered barley (EDNEY & ROSSNAGEL 2000). However, the present study used an extended regime that resulted in adequately modified malts as indicated by acceptable levels of ß-glucan in malt from both barley types (Figure 6). The higher levels of ß-glucan in hulless malts were most likely the result of poorer germination (Figure 7). Poor germination has often been cited as a potential problem with hulless barley where embryos can easily be dislodged or damaged at harvest or during malting.

An adequate supply of free amino acids is also required for optimum fermentation (O'CONNOR-COX & INGLEDEW 1989). The present study found no significant difference in overall levels of free amino nitrogen (data not shown) in worts from the two barley types. However, several individual amino acids including; alanine, aspartic acid, cysteine, glutamic acid, proline, serine and tryptophan, did differ significantly in worts from hulless versus covered barley lines. Serine differences (Figure 8) were the most interesting as they were also found to have a significant, positive effect on apparent attenuation limit (r=0.577***). The doubled haploid nature of the population, once again assuming no linkage between the free serine levels and the covered/hulless locus, indicated that significant differences in free serine levels between hulless and covered malts were the result of physical not genetic differences. A large number of protease enzymes, with varying degrees of thermostability, are involved in production of free amino acids. They are predominately synthesised during malting in the outer aleurone layers of the kernel and could have been inactivated during the kilning of hulless lines because of the greater exposure to high temperatures in these kernels lacking the insulating effect of the hull.

Conclusions

The potential advantage of hulless barley as malt was confirmed by the production of significantly higher levels of malt extract in hulless versus covered malts. The hulless malts also produced significantly higher levels of alcohol in the fermented wort. However, the

hulless malts did tend to have lower apparent attenuation limits suggesting room for further improvement in the quality of future varieties of hulless barley for malt. In particular, heat stability of enzymes located in the outer aleurone layers of the kernels could be addressed. Higher levels of ß-glucan in hulless worts could also have reduced the fermentation potential of the hulless worts. The high ß-glucan levels were likely the result of lower germination levels in hulless barley, the cause of which warrants further investigation.

A doubled haploid population was found to be a good tool for investigating non-genetic factors that could affect malt quality of covered versus hulless barley. Doubled haploids by nature should have had a random distribution of genetically defined malt quality across all lines in the population. Therefore, assuming no linkage between the traits investigated and the covered/hulless locus, any significant differences found between hulless and covered lines should be related to the absence versus presence of hull.

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Figure 1. Levels of Congress malt extract were significantly lower ($p<0.001$***) in malts from covered versus hulless barley lines

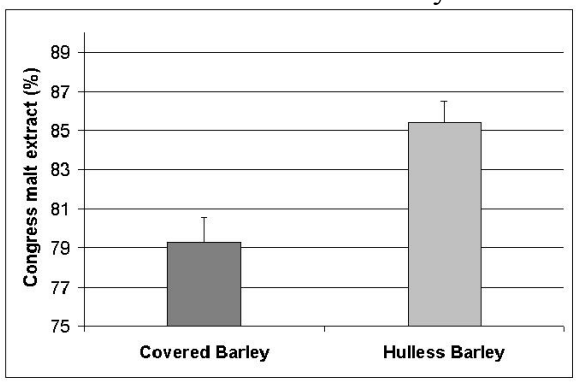


Figure 2. Apparent attenuation limits were significantly higher ($p<0.001$***) in malts from covered versus hulless barley lines

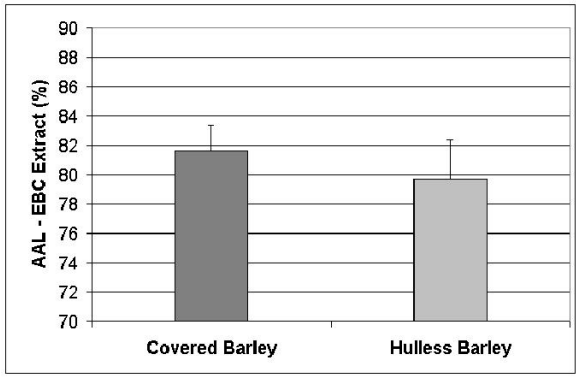


Figure 3. Alcohol levels were significantly lower ($p<0.001$***) in fermented worts from covered versus hulless barley lines

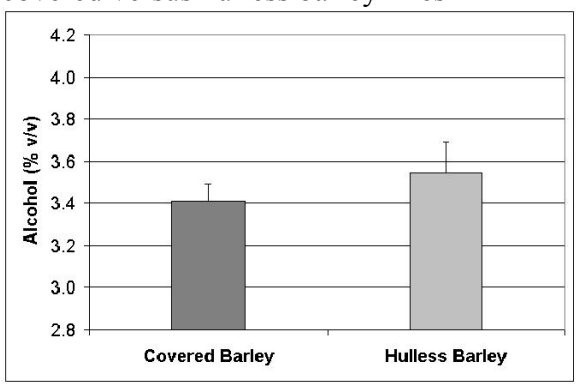


Figure 4. Levels of alpha-amylase were significantly higher ($p<0.001$***) in malts from covered versus hulless barley lines

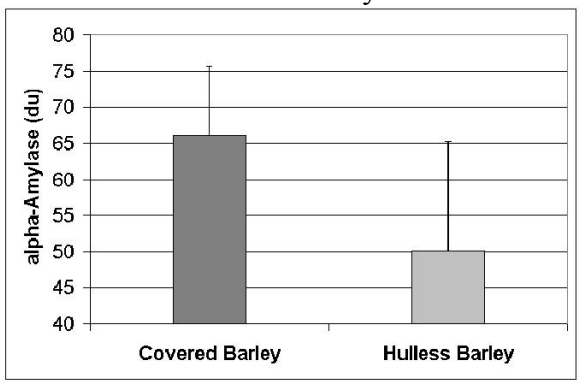


Figure 5. Levels of diastatic power were not significantly different ($p>.48$) in malts from covered versus hulless barley lines

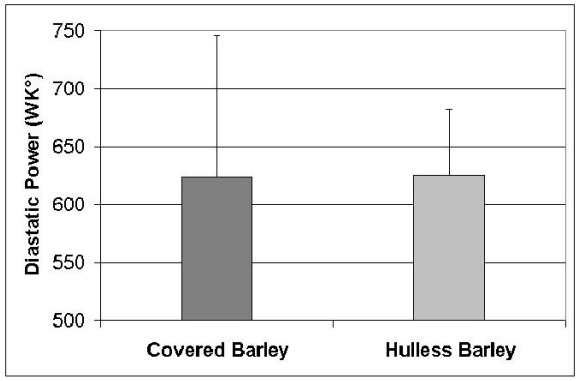


Figure 6. ß-glucan levels were significantly lower ($p<0.001$***) in worts made from covered versus hulless barley lines

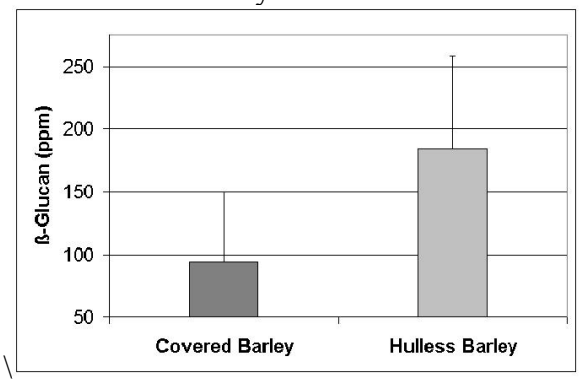


Figure 7. Germination levels (4 ml test) were significantly higher ($p<0.001$***) in covered versus hulless barley lines

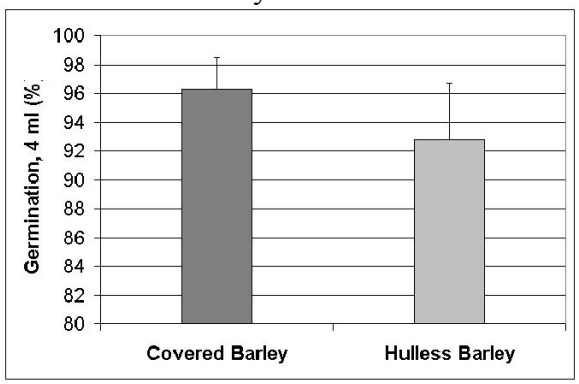
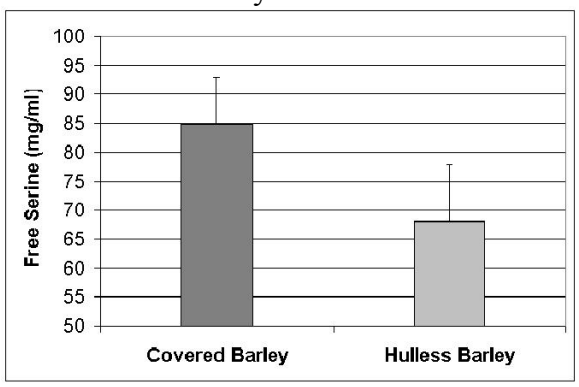


Figure 8. Levels of free serine were significantly higher in worts made from malts of covered versus hulless barley lines



Biochemical and Genetic Basis of Wort Fermentability

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Abstract

Factors that affect wort fermentability were investigated with 176 doubled haploid lines from an Arapiles/Franklin mapping population. Barley, grown in South Australia during 2001, was micromalted and analysed using standard methods. Samples were found to have a wide range in malting quality with lines both superior and inferior to the parents for all traits analysed. Quantitative trait loci analysis and statistical analysis of data indicated, for this population, that poor malt modification, beta-glucan in particular, had the greatest negative effect on fermentability. Fermentability was also negatively correlated with the maltotetrose content of wort. A significant quantitative trait loci for this sugar was coincident with the chromosome 4H locus for the heat-stable beta-amylase gene (sd2H). Lines with the heat stable enzyme, inherited from Arapiles, had improved fermentabilities of European Brewery Convention extracts but not of Institute of Brewing extracts. Increased levels of wort glucose, possibly coming from beta-glucan hydrolysis, had a positive effect on fermentability. A significant quantitative trait loci for wort fructose was found in the area of the *sdw* semi-dwarfing gene on chromosome 3H, highlighting the potential impact of agronomic traits on the genetic analysis of malt quality.

Keywords: barley; malt; fermentation; quantitative trait loci; beta-amylase thermostability; ß-glucan

Introduction

The fermentability of a malt is of great importance in determining the malt's value in the brewery. Fermentability is important because it determines the amount of alcohol that can be produced from a given weight of malt, an economic concern of any brewer. Fermentability also affects the sugar profile in a finished beer and, thus, knowledge of a malt's fermentation potential is essential if the sugar profiles required for specific beer types or brands are to be achieved. Breeders of malting barley, therefore, should be aware of the potential fermentability of their breeding lines. However, the malt properties that affect fermentability, as well as their interactions, are not well understood making it difficult to breed malting barley with a specific fermentation potential.

Fermentability will obviously be dependent on the level of fermentable sugars offered to the yeast at fermentation. However, research has often found poor correlations between fermentability and levels of fermentable sugars (FOX *et al.* 2001) suggesting the complexity of the process and the importance of other factors and their interactions. Levels of fermentable sugars will themselves be affected by a number of factors. The amount of malt solubilized at mashing, usually referred to as malt extract, will directly affect potential levels of fermentable sugars. The level of malt extract, in turn, is restricted by the degree to which the endosperm is modified during malting. The factor often deemed to be of greatest importance in determining levels of fermentable sugars is the production or activation, during malting, of starch degrading enzymes. Recent research has even demonstrated the importance, in fermentability,

of the heat stability of the starch degrading enzyme, beta-amylase (EGLINTON *et al.* 1998), further evidence of the complexity of fermentability and the factors that affect it.

Several quantitative trait loci (QTL) have been shown to be associated with fermentability (SWANSTON *et al.* 1999; FOX *et al.* 2001). QTL analysis of doubled haploid populations is particularly good at isolating individual factors that affect a complicated factor (ULLRICH *et al.* 1997) such as fermentability.

The present research investigated fermentability using a large doubled haploid population (Arapiles/Franklin) with a known range in malt quality (MOODY *et al.* – personal communication). The population was good for investigating the importance of beta-amylase thermostability in determining fermentability as the population segregated for this factor (MOODY *et al.* – personal communication). The effect on fermentability of other malt quality parameters, such as levels of starch degrading enzymes, fermentable sugars and indices of endosperm modification, were also investigated. QTL analysis was used as an additional tool for unravelling the complexity of fermentation.

Material and Methods

A doubled haploid population (176 lines) from an Arapiles/Franklin cross (MOODY *et al.* – personal communication), along with three replicates of the two parents, were grown at Charlick, South Australia in 2001. Samples of the lines and parents (60 grams) were micromalted in a Phoenix Automated Micromalting machine (Adelaide, SA, Australia) according to the following schedule: Wet steep 7.5 hours, Air rest 8 hours, Wet steep 9 hours, Germination 95 hours, Kiln 9 hours à 40°C, 4 hours à 60°C, 2 hours à 70°C, 4.5 hours à 80°, 0.5 hours à 25°C. Steeping and germination were carried out at 15°C.

Malt extracts (worts) were prepared using small-scale versions of the EBC (EUROPEAN BREWING CONVENTION 1998) or the IOB extract methods (INSTITUTE OF BREWING 1997). All other malt parameters; wort ß-glucan (enzymatic), soluble protein and free amino nitrogen, were measured using standard EBC methods (EUROPEAN BREWING CONVENTION 1998). Diastatic power, β-amylase and α-amylase activity were measured spectrophotometrically using PAHBAH (LOGUE 1997). Levels of fermentable sugars in worts were determined using a DX-500 HPLC system (Dionex).

A small scale method for measuring apparent attenuation limit (AAL) was used to determine the fermentation properties of the samples. The method incubated 40 ml of EBC wort or 40 ml of IOB wort with 160 mg dried yeast (Mauribrew lager yeast, Toowoomba, Australia) at 25°C for 24 hours (LOGUE 1997).

Detection and mapping of QTLs were performed by simple regression and interval analysis with the software package "Qgene" (NELSON 1997), using an Arapiles/Franklin map with 240 markers, most of which were AFLPs (MOODY *et al.* – personal communication).

Results and Discussion

The doubled haploid population (176 lines) from the Arapiles/Franklin cross showed a wide range in malting quality. Apparent attenuation limit, an indicator of fermentability, was found to range from 77% to over 84% (Figure 1) with a significant number of lines having values lower than the poorer check, Arapiles. However, a large number of lines also showed superior fermentability to Franklin, the best check (Figure 1). This set of samples, therefore, offered an excellent opportunity to investigate factors that affect fermentability.

Levels of ß-glucan in malt extract were found to have the greatest negative effect on fermentability of lines in our population. Statistical analysis indicated a significant negative effect of wort ß-glucan levels on fermentability of the wort (Figure 2). A significant QTL for levels of wort ß-glucan was observed on chromosome 1H (Figure 3) with high ß-glucan associated with Arapiles alleles. Coincident to this general location, significant QTLs were observed for EBC (Figure 3) and IOB fermentabilities (Figure 4) with high values associated with Franklin alleles. The poor modification of lines with Arapiles alleles at this location, therefore, appeared to be limiting the fermentability of the extracts.

Previous research has shown that under modified malts produce less fermentable extract (BATHGATE *et al.* 1978). It was suggested that the poor modification restricted the exposure of starch to enzyme degradation, thus reducing fermentable extract. However, in the present study the only fermentable sugar found to have a significant QTL in the same general area of the genome associated with endosperm modification, was glucose. This QTL was detected on chromosome 1H (Figure 4) coincidental to QTLs for both IOB fermentability and wort ß-glucan levels. Statistical analysis also showed that glucose levels in IOB wort had a significant positive correlation with IOB fermentability (r=0.43***). Therefore, the effect of under modifcation on fermentability in this population appeared to be related to limiting levels of glucose which were alleviated in better modified lines by complete hydrolysis of ß-glucan to glucose.

The chromosome 4H beta-amylase gene, Bmyl, also appeared to have a significant effect on fermentability in the Arapiles/Franklin population. The Sd2H allele from Arapiles was found to have a significant association with the fermentability of EBC wort (Figure 5). A significant QTL for levels of maltotetrose in EBC wort was also found on chromosome 4H but high levels for this trait were associated with the Franklin allele. Both of these QTLs were coincidental with the gene for beta-amylase. Statistical analysis of the malt data also showed a significant negative correlation between maltotetrose levels in EBC wort and fermentability of the EBC wort (Figure 6). The heat stable beta-amylase was apparently able, at least with an EBC extract method, to degrade a significant portion of the non-fermentable sugar, maltotetrose, to fermentable sugars. It was interesting that no significant QTL for IOB fermentability was observed on 4H indicating the heat stable beta-amylase was unable to increase fermentability of a hot water IOB extract, all other parameters being equal.

Several research reports have found a significant QTL for fermentability on chromosome 3H coincidental to the sdw semi-dwarfing gene (SWANSTON *et al.* 1999; FOX *et al.* 2001). In the present study no significant QTLs were found on chromosome 3H for fermentability, although, a significant QTL was found coincidental to the sdw gene for the fermentable sugar, fructose, suggesting a possible connection, even in the Arapiles/Franklin population, of this sdw gene and fermentability.

Conclusions

Fermentability was found to be significantly reduced by poor endosperm modification in the Arapiles/Franklin population. Future research could show that the effect was related to the production of the fermentable sugar glucose from ß-glucan hydrolysis. The heat stable beta-amylase gene (sd2H) significantly increased the fermentability of EBC extracts but not IOB extracts indicating the importance for fermentability of a thermostable beta-amylase. The results also indicated the significance of different testing methods (IOB versus EBC) and how they may affect the determination of a variety's malt quality.

Acknowledgements
The authors acknowledge the support of Australian farmers and the Australian Commonwealth Government through the Grains Research and Development Corporation.

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Figure 1. Distribution, among the Arapiles/Franklin lines, including parents, of fermentability (AAL) of EBC extract

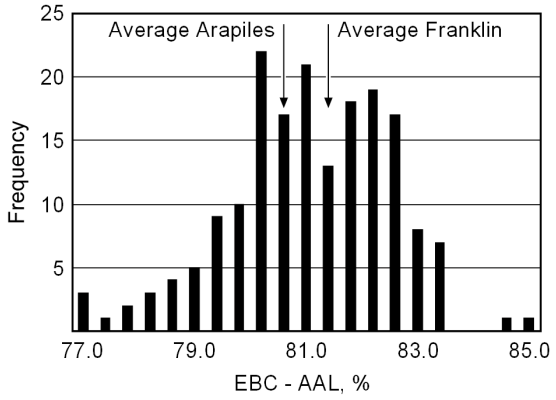


Figure 2. Relationship between levels of ß-glucan in EBC extract and fermentability (AAL) of the extract (r^2=0.52***)

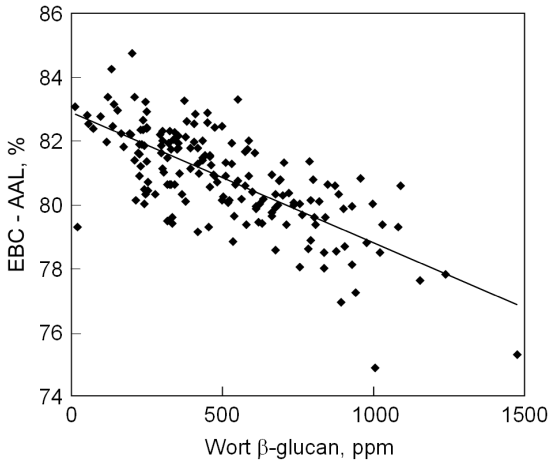


Figure 3. Single marker regression analysis map of fermentability of EBC extract (dark line) and ß-glucan content of EBC extract (light line) in the Arapiles/Franklin mapping population. Franklin alleles were associated with the increased fermentability on chromosome 1H while Arapiles alleles were associated with the increased levels of ß-glucan (poorer modification) on 1H.

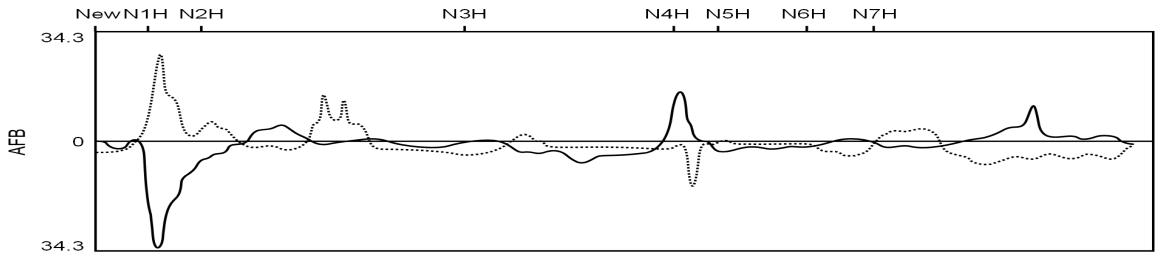


Figure 4. Single marker regression analysis map of fermentability of IOB extract (dark line) and glucose levels in IOB extract (light line) for the Arapiles/Franklin mapping population. Franklin alleles were associated with the increased fermentability and the increased glucose on chromosome 1H.

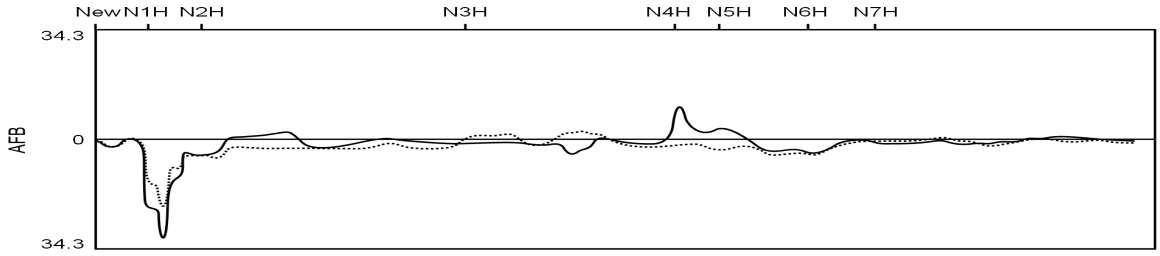


Figure 5. Single marker regression analysis map of fermentability of EBC extract (dark line) and maltotetrose levels in EBC wort (light line) for the Arapiles/Franklin mapping population. Arapiles alleles were associated with increased fermentability AAL on chromosome 4H while Franklin alleles were associated with the increased levels of maltotetrose on 4H.

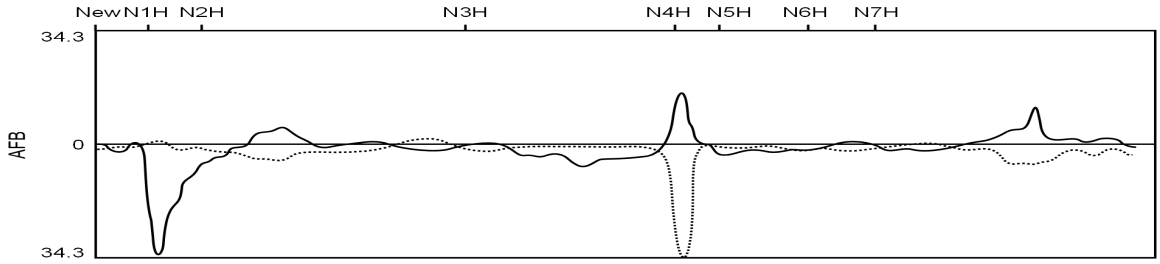
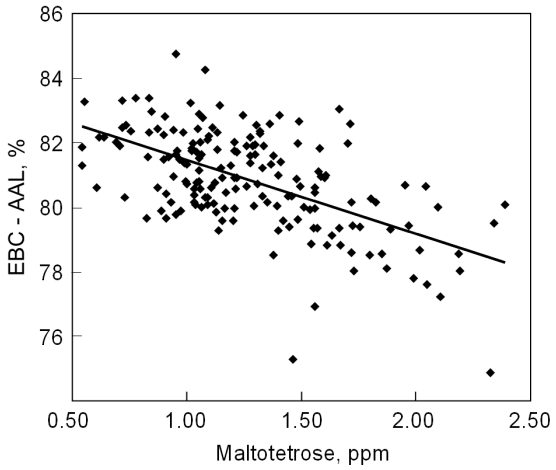


Figure 6. Relationship between levels of maltotetrose in EBC extract and fermentability (AAL) of the extract (r^2=0.33***)



Proteome Analysis of Barley Seeds and Malt

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Abstract

Barley proteomics, using 2D-gel electrophoresis and mass spectrometry for protein identification, provides biologically relevant information even without genome sequence. Proteins in over 260 spots in developing and germinating seeds and dissected tissues have been identified, including multiple forms of α-amylase, β-amylase, α-amylase/subtilisin inhibitor, serpins, CM-proteins, peroxidases, enzymes involved in defence: PR-proteins, chitinase, thaumatin-like proteins, and oxidative or desiccation stress: ascorbate peroxidase, peroxiredoxins, sHSP, LEA-proteins. Previously uncharacterised proteins are identified by searching EST databases with peptide masses and post-translational modifications are identified by advanced mass spectrometry.

The project aims to identify proteins important for malting. Sixteen cultivars differing in malting quality were grouped using independent methods: (a) clustering analysis of spot pattern differences, (b) malt quality scoring from micromalting. The best malting cultivars were grouped identically by both methods, thus malting quality is reflected by the proteome. Analysis of additional spot differences and cultivars will explore the potential for predicting malting quality. Most spot differences are genetically determined, illustrated by spots containing allelic β-amylase forms. Spot differences segregated in a doubled haploid population (Scarlett x Meltan) and thus were located on the genetic map. Micromalt analysis of this population enabled correlation of proteome, genetic information and malting quality.

Introduction

Determination and prediction of the quality of barley seed is of particular importance for the malting industry. The proteins contained in barley seeds are major determinants of malting quality and have as such been studied for decades. Several of the proteins with major roles in germination and malting are well studied at the molecular level, for example α-amylase, β-amylase and β-glucanase. More recently, developments in high-resolution two-dimensional gel electrophoresis for separation of proteins in complex mixtures, and mass spectrometry for protein identification, have enabled new approaches to study cereal seed proteins. We have undertaken a study of the soluble proteome of barley seeds and analysed changes in protein appearance during grain-filling through the mature seeds and the germination process, in intact seeds and dissected seed tissues to identify proteins that influence malting quality. This has resulted in identification of proteins in over 260 spots by mass spectrometry (FINNIE *et al*. 2002; ØSTERGAARD *et al.* 2002; FINNIE & SVENSSON 2003; BAK-JENSEN *et al.* 2004; ØSTERGAARD *et al.* in press).

Different cultivars vary in their suitability for malting. These differences might be expected to be reflected in the proteomes of the seeds. To investigate this, a number of barley

cultivars have been compared and differing proteins identified. It has been shown that the seed proteomes of barley seeds are highly similar, although specific differences in protein spots can be observed that might be related to cultivar properties (FINNIE *et* al. in press). Differences in the protein patterns can result either from genetic differences or from environmental effects. Doubled haploid technology is commonly used in plant breeding for rapid production of homozygous lines, and also for genetic mapping purposes in research. We have used a doubled haploid population derived from a malting barley (Scarlett) and a feed barley (Meltan) in order to investigate the genetic basis for spot pattern differences and relate these differences to malting quality.

Material and Methods

Barley cultivars were field grown in the 2000 season in Fyn, Denmark. Doubled haploid lines were generated and kindly supplied by Sejet Plantbreeding and Risø National Laboratory. Micromalt analysis was carried out according to standard methods. Proteins were extracted from developing, mature and micromalted seeds in 5 mM TrisHCL, 1 mM $CaCl_2$, pH 7.5, as previously described (FINNIE *et al*. 2002; ØSTERGAARD *et al.* 2002). Protein extracts were separated by two dimensional gel electrophoresis as previously described in the pI intervals 4-7 (FINNIE *et al*. 2002; ØSTERGAARD *et al.* 2002) and 6-11 (BAK-JENSEN *et al.* 2004). Gels were stained with silver nitrate according to (HEUKESHOVEN & DERNICK 1988) or colloidal Coomassie brilliant blue (RABILLOUD & CHARMONT 2000). Protein spots were excised from 2D-gels and digested with trypsin according to (SCHEVCHENKO *et al.* 1996). Tryptic peptides were micropurified according to (GOBOM *et al*. 1999) and analysed on a Bruker Reflex III MALDI-TOF mass spectrometer as previously described (FINNIE *et al*. 2002; ØSTERGAARD *et al.* 2002). Proteins were identified by using peptide mass data to search the NCBI sequence databases. In cases where no identification could be made, EST databases were used. Positive matches were then cross checked against the Institute of Genome Research (TIGR) barley gene data bank.

Results and Discussion

Identification of Protein Spots in the Barley Seed Proteome

Cultivar Barke, a widely used malting cultivar, was chosen as a reference cultivar for the project, in order to provide a basis for comparison of cultivar properties. Although an important aim of this project was to identify proteins that differ among cultivars, it was also considered important to obtain a general overview of the proteins present that were common to many cultivars. For this reason, a comprehensive effort was made to identify the proteins in the major spots on the 2D-gels from cultivar Barke. Proteins in mature seed extracts were separated on 2D-gels in the pH intervals 4-7 and 6-11.

Currently, relatively few barley protein sequences are available in databases. This hinders protein identification using peptide mass mapping since this technique relies on highly identical sequence to the protein under analysis. However, there is a large and growing amount of EST sequence information available for barley, which has proved invaluable for identification of previously uncharacterized proteins from 2D-gels. Thus, about one third of protein identifications in this project have been based on EST sequence information. This makes it possible to identify proteins that have not previously been detected or characterized, since they are known only as EST sequences. Since so little is known about their function some of the proteins identified in this manner are among those most interesting for further study.

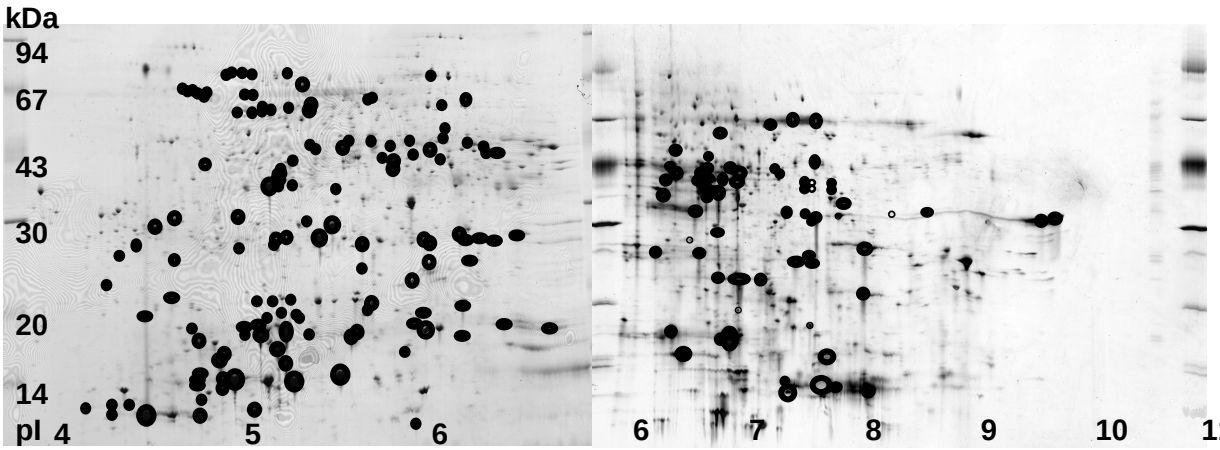

Figure 1. Protein spots identified in mature seeds of cultivar Barke in the overlapping pH intervals 4-7 and 6-11

Comparison of the Seed Proteomes from Different Barley Cultivars

The overall spot patterns from sixteen different cultivars were highly identical. A study of the variation in spot pattern when cultivars are grown in different locations and over different harvest years showed variation in only a few spots (JENSEN *et al*. unpublished). A comparison of Golden Promise and Barke seeds (FINNIE *et al.* in press) showed over 16 spots differing between the cultivars while in parallel, four spots were found to increase in intensity in relation to increased protein content of the seeds as a result of added nitrogen. Thus, it is possible to distinguish between environmental and genetic effects in proteomes, and differences in spot pattern between barley varieties are significant. Sixteen cultivars, representing different properties with respect to malting, were analysed. These varieties have almost identical seed proteomes but with characteristic variations. Spots differing between the cultivars were pinpointed. Malt quality is a complex property that is the result of interactions between the various components of the seed. To simplify the analysis, an initial comparison was made of nine simple spot patterns from local areas of the 2D-gels, instead of analysing every spot difference for each variety. Nine subpattern variations were chosen that were present in a unique combination in the sixteen cultivars. A clustering analysis of the subpattern variations was used to group the cultivars such that the groups were determined by the proteome.

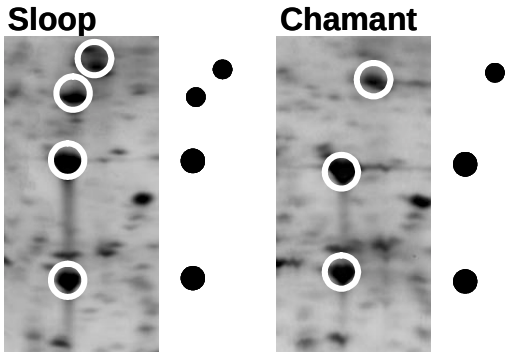

Figure 2. Example of subpattern variation between barley cultivars Sloop and Chamant. An additional spot is present in the cultivar Sloop pattern. This spot is present in several other cultivars and has been found to contain serpins (FINNIE *et al.,* in press).

Segregation of Spot Pattern Differences among a Population of Doubled Haploid Lines

Although relatively little is yet known about the relationship between the proteome and malting quality, a large amount of data at the genetic level is available. Currently this data cannot be applied directly for interpretation of the barley proteome. In order to link the barley seed proteome to the genetic map as well as malting quality, a doubled haploid population was used that was derived from a malt barley (Scarlett) and a feed barley (Meltan). The 2D-gel patterns from these cultivars were examined and 20 spot pattern differences, involving 43 protein spots, were pinpointed. The segregation of these spot patterns was followed in 30 randomly selected doubled haploid lines. The patterns segregated giving rise to 14 linkage groups. Co-segregation of protein spots from different areas of the 2D-gels allows relationships between these proteins to be observed even in the absence of an identification. The segregation data was used to place the spot pattern differences on the genetic map available for this cross, allowing the proteome and genome to be directly linked.

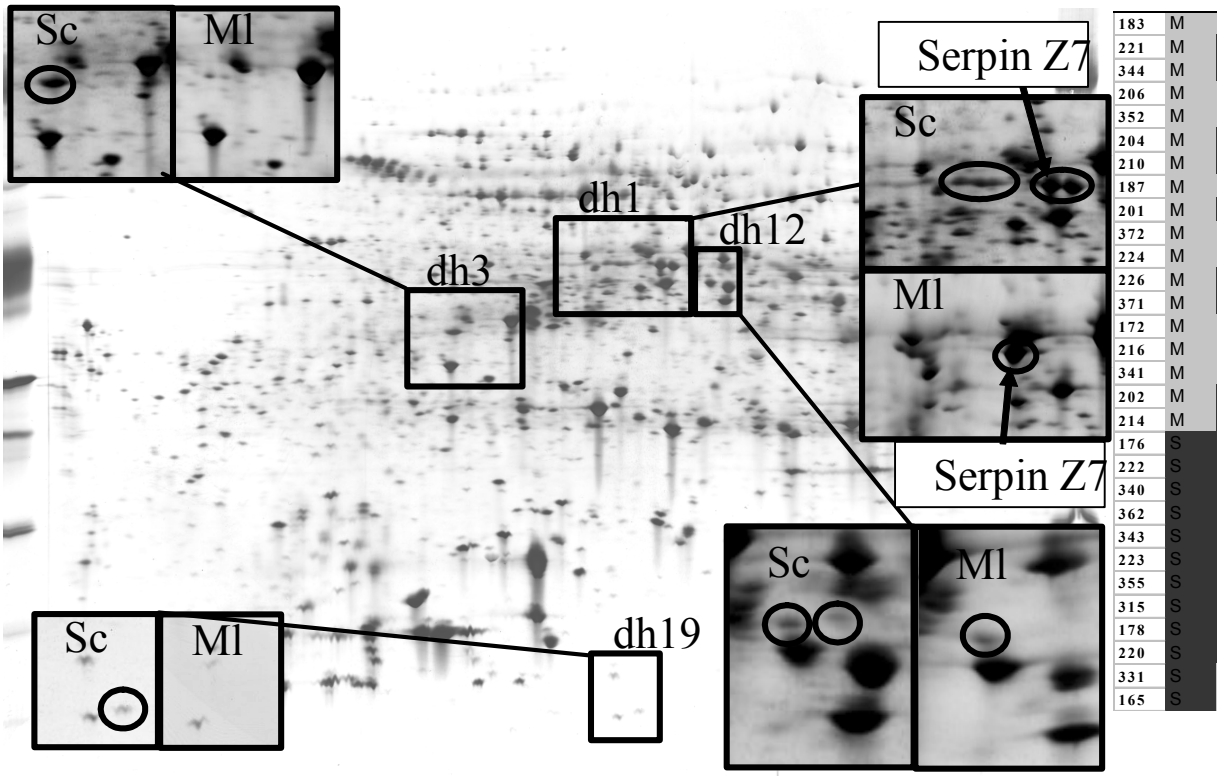

Figure 3. Co-segregation of four spot pattern differences in a doubled haploid population derived from Scarlett (Sc) and Meltan (Ml). Segregation data for the 30 lines is shown (right; S, Scarlett pattern, M, Meltan pattern). Several of the segregating spots have been identified as serpin Z7. The segregation data enabled mapping to chromosome 5H(7). This is the known location of a gene encoding serpin Z7 (HEJGAARD, 1984).

Correlation of the Barley Seed Proteome with Malting Quality

Standard micromalt analysis was carried out for each of the sixteen cultivars and the thirty doubled haploid lines. The cultivars were grouped based on a weighted scoring of the micromalt parameters. The group evaluated on this basis to contain the best five malting cultivars was identical with one of the groups determined by clustering analysis based on the nine 2D gel subpatterns.

A similar clustering analysis of the sixteen cultivars was carried out using the larger number of spot pattern differences identified in the doubled haploid study, that could also be seen to vary among the other cultivars. Again, one of the groups that resulted from the analysis contained four out of five of the best malting cultivars. This group was characterized by a combination of three spot patterns that were not found in any of the other cultivars. These spot patterns will be mapped onto the chromosomes as part of the doubled haploid analysis. The proteins in the spots are targets for identification by mass spectrometry.

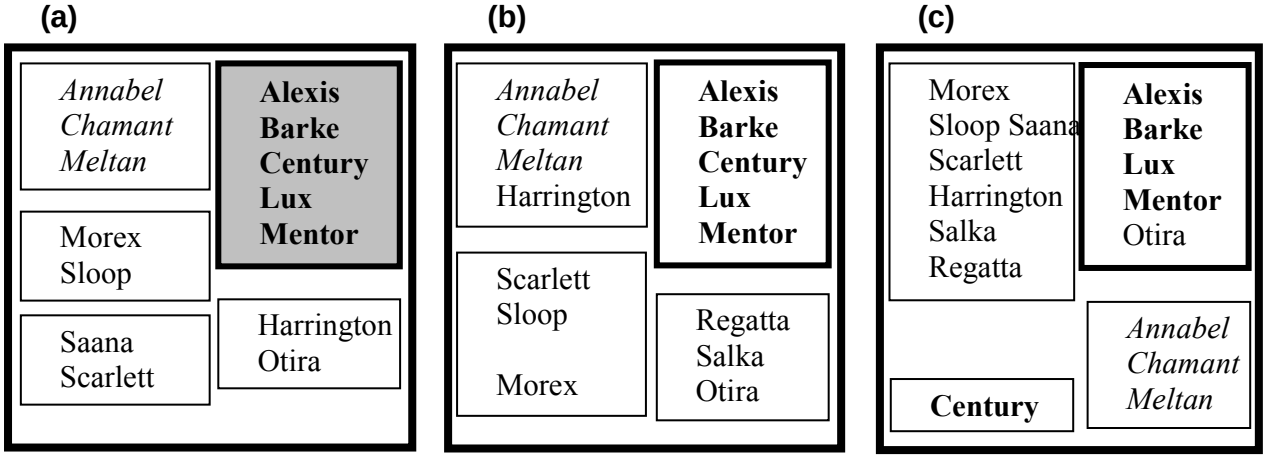

Figure 4. Grouping of cultivars based on (a) micromalt analysis (b) nine spot subpatterns (c) double haploid spot differences. The box containing the best malting cultivars according to the analysis in (a) is shaded, and these cultivars are in **boldface** in (b) and (c). Cultivars Annabel, Chamant and Meltan (indicated in *italics*) also grouped together in each analysis.

Conclusion

Information contained in the barley seed proteome can be combined with genetic mapping and micromalt analysis to provide new insight into the problem of prediction of malting quality.

Acknowledgements

Mette Hersom Bien and Ella Meiling are acknowledged for excellent technical assistance. Kurt Hjortsholm and Birger Eriksen are thanked for providing plant material and micromalt analysis and Torben Steenholdt for mass spectrometry. This work was funded by the Danish Research Agency's SUE programme grant no. 9901194 and a Ph.D. fellowship to OØ (#EF803) from the Danish Academy of Technical Sciences.

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Hull-Cracked Grain and Grain with Ventral Swelling as Damaged Grains in Malting Barley

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Abstract

Hull-cracked grain (HCG) and grain with ventral swelling (GVS) are damaged grains that engender deteriorated grain qualities in malting barley. This study was undertaken to solve this problem by developing evaluation methods for occurrence of these damaged grains. It was possible to select HCG-tolerant varieties by early seeding and cultivation under insufficient sunshine during the internode elongation stage. When water was sprinkled on the barley materials in the early-ripening stage, the middle-ripening stage and the late-ripening stage, sprinkling in the late-ripening stage most notably reproduced the GVS occurrence. We established a method to evaluate occurrence of GVS by intermittent, artificial water sprinkling in the late-ripening stage. We screened DNA markers to detect polymorphisms between Kinuyutaka (low occurrence of HCG and high of GVS) and Yoshikei 15 (high occurrence of HCG and low of GVS) to establish a Marker Assisted Selection (MAS) system for lines with a low occurrence of HCG and GVS. Thereafter, we constructed a genetic map for Kinuyutaka × Yoshikei 15 using 150 doubled haploid lines. The QTL analyses revealed three QTLs for HCG on 2H, 3H, and 6H and one for GVS on 1H of barley chromosomes, respectively.

Keywords: DNA marker; grain with ventral swelling; hull-cracked grain; malting barley; QTL analysis

Introduction

Malting barley is a useful winter crop in northern Kyushu, Japan because it matures early and is well adapted to the double cropping system. Recently, however, the occurrence of damaged grains, including hull-cracked grain (HCG) and grain with ventral swelling (GVS), poses a serious problem for malting barley production. These damaged grains decrease the inspection grade and malting quality of grain. HAMACHI *et al.* (1989) suggested that poor hull-vessel growth causes HCG instead of grain bursting by increasing of contents. Low-temperature exposure, lack of sunshine, and water injury during hull development retard hull growth and accelerate HCG. On the other hand, YAMAGUCHI *et al.* (2000) reported environmental factors surrounding GVS' occurrence: it is mainly caused by precipitation over 25 mm during the 15 days preceding maturity.

Malting barley production demands the breeding of a high quality variety with tolerance to these types of grain damage. Toward that goal, this study developed an evaluation method of occurrence of these types of grain damage and a method to select a tolerant variety.

In addition, marker assisted selection (MAS) is useful because it does not require a vast

test field; moreover, this method is unaffected by the environment. Genetic and quantitative trait loci (QTL) analyses for many agronomic traits have been conducted using these markers. In QTL analysis for HCG, KANATANI *et al.* (1998) reported some QTLs using doubled haploid lines (DHLs) produced from a cross between Harrington and TR306, but no other reports have addressed GVS in Japanese malting barley.

Material and Methods

Selecting Method for HCG and GVS in Malting Barley

Twenty-six Japanese two-rowed malting barley varieties were seeded around November 10 about 15–20 days earlier than ordinal seeding. They were 50% shaded with cheesecloth for 30 days before heading. Thereby, we evaluated the ease of occurrence of HCG by early seeding and shading treatment during the stem shooting stage using these varieties.

Artificial precipitation tests were done to develop the selection method of GVS tolerance lines using 13 Japanese two-rowed malting barley varieties because GVS occurrence is caused mainly by precipitation over 25 mm during the 15 days preceding maturity. The artificial test was conducted by sprinkling 50 mm water/day five times each day for two contiguous days at two-day intervals during the late-ripening stage. The percentages of occurrence of GVS for artificial treatment and natural conditions were then compared to evaluate the treatment efficiency.

The newly developed malting barley line Kyushu Nijo 16 was tested for tolerance of HCG and GVS with six Japanese malting barley varieties.

The QTL Analysis of HCG and GVS in Japanese Malting Barley

We produced 150 F_1-derived doubled haploid lines (DHLs) from a cross between Kinuyutaka and Yoshikei 15 using the *bulbosum* technique (FURUSHO, 1993). Kinuyutaka is a two-rowed malting barley with low occurrence of HCG and high of GVS. Yoshikei 15 is a two-rowed malting barley with high occurrence of HCG and low of GVS. About 15 green leaves were harvested from each line at the heading date and used to extract genomic DNA. Evaluation of HCG occurrence was conducted in 1999 and 2000, and GVS in 2000 and 2002 by the method developed in this study.

We used DNA markers that were previously mapped and some that have not been utilized for mapping. Initially, 1,269 DNA markers – 255 RFLP, 422 SSR, 325 RAPD and 267 CAPS – were screened using Kinuyutaka and Yoshikei 15. We selected the DNA markers that detected polymorphisms between the parents. The segregation ratios of the DHLs' genotypes at all markers were checked using the Chi-Square Test using computer software MAPL (UKAI *et al*., 1995). QTL analysis of HCG and GVS was done using MAPL with a log likelihood (LOD) of 2.0 for the DH model.

Results and Discussion

Selecting Method for HCG and GVS in Malting Barley

Figure 1 shows a frequency distribution of occurrence of HCG in 26 Japanese two-rowed malting barley varieties comparing early seeding with shading treatment during stem shooting

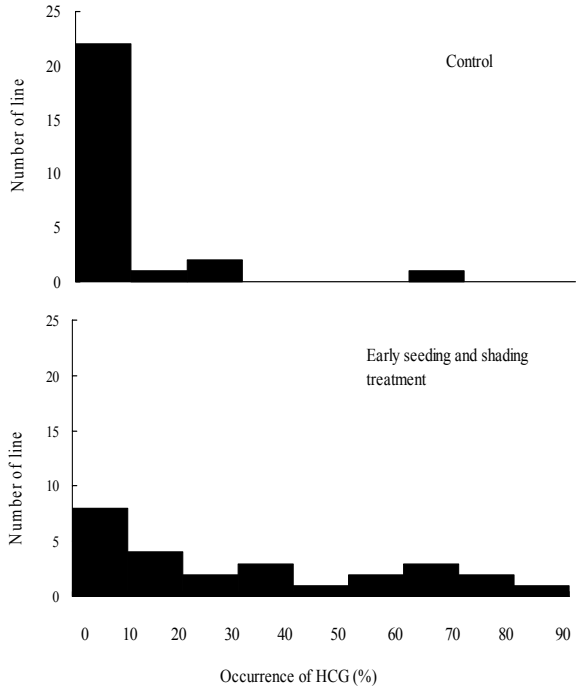

Fig. 1. Frequency distribution of occurrence of HCG in 26 lines by early seeding and shading treatment(bottom) and control(top).

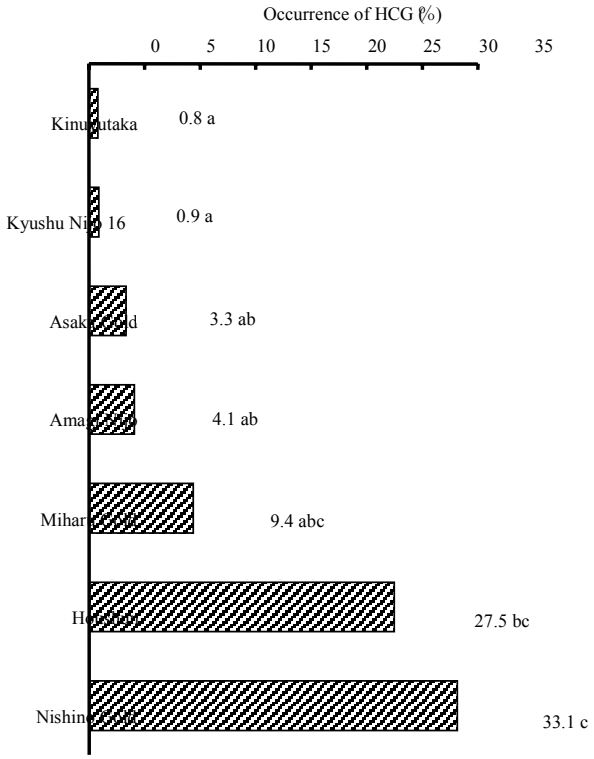

Fig. 2. Varietal difference in occurrence of HCG by early seeding and shading treatment during the stem shooting stage.

stage and control. Occurrence of HCG was higher in the group with combined early seeding and shading treatment than in control. This result shows that the treatment is efficient for evaluation of occurrence of HCG. HAMACHI *et al.* (1989) suggested that low-temperature exposure and lack of sunshine during hull development retarded hull growth and accelerated HCG. Early seeding and shading treatment produced an environment that engendered and accelerated HCG. Figure 2 shows the varietal differences in HCG occurrence by early seeding and shading treatment during the stem shooting stage. Kyushu Nijo 16 is a newly developing breeding line with good malting quality and high agronomic performance. This line showed low occurrence of HCG and was selected as an HCG tolerant line.

Figure 3 shows the relationship between GVS occurrence by artificial sprinkling water treatment and average data of GVS obtained at natural growth conditions in 1996 and 1997. A significant correlation existed in the occurrence of GVS between these two conditions, indicating that the treatment could be used as an evaluation method of GVS occurrence. Figure 4 shows the varietal difference in GVS occurrence by the artificial sprinkling water treatment. Kyushu Nijo 16 is also significantly tolerant to GVS.

In HCG and GVS, both evaluation methods were established and the tolerant line was selected in this study.

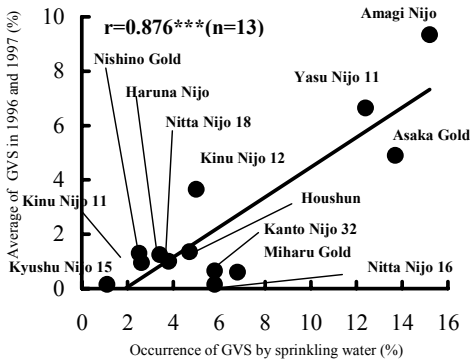

Fig. 3. Relationship between occurrence of GVS by sprinkling water and average of GVS at natural condition in 1996 and 1997.

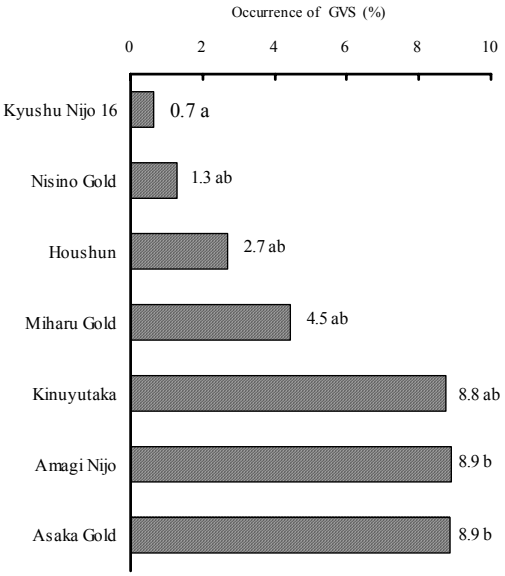

Fig. 4. Varietal difference in occurrence of GVS by sprinkling water during the late-ripening stage.

Detection of Polymorphism, Map Construction and QTL Analysis

The rate of total polymorphism was 5.1% of total 1,269 DNA markers. Three RFLP markers, 8 SSR markers, 17 RAPD markers, and 37 CAPS markers fit a 1:1 segregation ratio and tested for map construction (Fig. 5). Six linkage groups with 65 markers were assigned to barley chromosomes. This linkage map covered 699.2 cM. We detected three QTLs for HCG on 2H, 3H and 6H, and for GVS on 1H in barley chromosomes. The major QTL for HCG on the nearest RAPD marker RA55 (2H) had LOD scores of 5.09 and 5.52, explaining 15.1% and 11.4% of the total variance in 2000 and 1999, respectively. The QTL for GVS on the nearest RAPD marker OPBD5 (1H) had LOD scores of 4.03 and 3.10, explaining 12.9% and 9.8% of

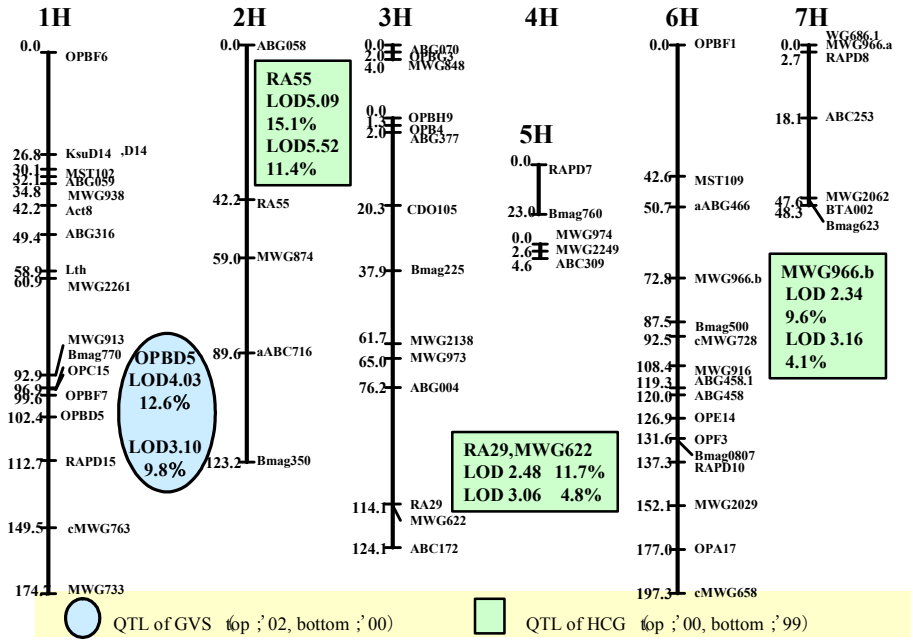

Fig.5. The Kinuyutaka × Yoshikei 15 linkage map constructed by MAPL (UKAI *et al*. 1995) using 65 markers.

the total variance in 2002 and 2000, respectively. These markers on QTLs for HCG and GVS might be useful to select tolerant lines for the damaged grains.

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Beta-Glucan Content of Old and Modern Spring Barleys

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Abstract
The interest in barley as food source is currently increasing. Numerous studies have verified the cholesterol-lowering benefits of barley foods due to barley β-glucan, the major soluble fiber component. The health benefit of barley, therefore, depends on the direct relationship between blood cholesterol and the risk of heart attacks. On the other hand high β-glucan contents can cause serious problems both in the brewing process – if the enzymatic degradation during malting is too low – and in poultry feeding. The β-glucan content of spring barley landraces and cultivated varieties from a time period of more than 160 years was studied over three years under the low-rainfall conditions of eastern Austria. Hypothesis was that probably old barleys exhibit higher β-glucan contents because of a less intensive selection for malting quality. Moreover, a few hull-less varieties from the 2003 harvest were analysed in order to compare husked and hull-less types of barley. A significant variation in β-glucan content was estimated both for genotypes, as well as for years and the interaction, however, the genotypic effect was much greater than the other effects. Generally, no clear trend in the β-glucan content over time of registration/cultivation of the varieties was observed, except that the most recently registered malting barleys exhibited significantly lower contents than the rest. The highest contents were observed for some hull-less barleys.
Keywords: dietary fiber; health benefit; *Hordeum vulgare*; hull-less barley

Introduction

Mixed-linkage (1→3),(1→4)-β-D-glucans (termed barley β-glucan) are the major components (≈70%) of barley endosperm cell walls. During malting β-glucan is partially degraded by enzymes synthesized *de novo*. Incomplete cell wall degradation leads to higher values of malt β-glucan and lower values of malt extract: on the one hand the non-starch polysaccharide β-glucan can act as a physical barrier to starch and protein degrading enzymes, on the other hand undegraded β-glucan does not contribute itself to fermentable sugars. Moreover, high molecular weight, viscous material can be released into solution and cause filtration problems at various stages in the brewing process, as well as beer hazes (McCLEARY 1986; HENRY 1988; EDNEY 1996). In animal nutrition β-glucan reduces the availability of energy and protein in poultry fed diets (EDNEY 1996).

Besides the detrimental effects of barley β-glucan in brewing and animal nutrition cell wall polysaccharides contribute to dietary fiber in human diets and are now considered to be nutritionally beneficial. The hypocholesterolemic effect of barley β-glucan was demonstrated in both experimental animals and humans (NEWMAN *et al.* 1989; KAHLON *et al.* 1993; HECKER *et al.* 1998; HALLFRISCH *et al.* 2003). Due to the healthful advantages of β-glucan barley has the potential for a much wider food use than at present. As a cereal component or ingredient in chemically leavened baked products, barley functions satisfactorily. Extracted β-glucan or milling fractions with high dietary fiber content have

promise as a fiber supplement in baked and/or pasta products (KNUCKLES *et al.* 1997; YOKOYAMA *et al.* 1997; NEWMAN *et al.* 1998).

The level of β-glucan in barley may vary considerably, depending on barley type and genotype, method of determination, and growing conditions. A range of 2% to more than 8% has been published in the literature. Husked normal-starch barleys contain comparable concentrations of β-glucan to oats, twice the contents of rye and the six- to tenfold amount of wheats (HENRY 1987; WAGNER & KUHN 1996). The effect of barley type is somewhat unclear. LEHTONEN & AIKASALO (1987) report that two-rowed barleys contain somewhat more β-glucans than six-rowed barleys. PÉREZ-VENDRELL *et al.* (1996) found that winter barleys have higher contents than spring barleys, while NARZISS *et al.* (1989) report higher contents in spring than in winter barleys. In the latter study spring barleys, however, showed a better β-glucan breakdown during malting resulting in lower malt β-glucan values than in winter barleys. While XUE *et al.* (1997) found higher contents in hull-less (naked) barleys, WOOD *et al.* (2003) showed that the increase in β-glucan from husked to naked barleys would be expected from the absence of ≈11% husk, since no β-glucan is in this part of the grain. Significant higher contents, however, are found in waxy barleys.

The development of commercial barley varieties with best food and health characteristics is a new task for the barley breeder. Heretofore, emphasis has been placed on developing barley varieties suited to malting for beer. Hence, selection was concentrated on low contents of β-glucan. In the presented study old and modern barley landraces and/or cultivated varieties were investigated for their total β-glucan content over three years. The aim of the work was to search for genetic resources with high genotypic β-glucan levels and to study whether older barley material contains higher levels because of a less intensive selection for malting quality during their breeding process. For that question Austrian-grown landraces and/or cultivars from a period of more than 160 years were investigated.

Material and Methods

Plant Material and Field Trials

The investigated nursery comprised 20 spring barleys covering the period from 1832 to 1996 (Table 1). The field trials were carried out in 2000 and 2003 at Raasdorf (16°35′ E, 48°14′ N) and in 2002 at Groß-Enzersdorf (16°33′ E, 48°11′ N). The trials in 2000 and 2002 were fertilised with 32 N and 8 S (kg ha^{-1}), and 67 N, respectively. The 2003 trial was organically grown with 2 years alfalfa as precrop. In 2003 also five naked barleys (Figure 1) grown at Vienna and 10 cultivars from a conventional trial at Groß-Enzersdorf, fertilised with 49 kg N ha^{-1} were analysed. Grain samples were sifted with a Sortimat laboratory machine (Pfeuffer GmbH, Kitzingen, Germany). Only plump grains >2.5 mm were used for analysis.

β-*Glucan Analysis*

Total β-glucan content was determined enzymatically using Megazyme kits (Megazyme Int. Ireland Ltd., Bray, Wicklow, Ireland) according to ICC Standard Method No. 168 using a mixed linkage β-glucan assay procedure (McCleary method). Milled barley samples were suspended and hydrated in a sodium phosphate buffer solution of pH 6.5 and incubated with purified lichenase enzyme (specific, endo-(1→3),(1→4)-β-D-glucan 4-glucanohydrolase, EC 3.2.1.73). An aliquot of the filtrate was then reacted to completion with purified β-glucosidase enzyme (EC 3.2.1.21). The glucose produced was assayed using a glucose/peroxidase (GOPOD) reagent. The β-glucan contents are reported as % on dry basis. Statistical analyses were carried out with SAS 8.2 software. The values of the naked barleys were adjusted by multiplying with 0.89 assuming 11% husk. The husk is known to contain no β-glucan.

Results and Discussion

Total β-glucan content ranged from 3.4 to 5.9% (Table 1, Figure 1). The analysis of variance revealed significant genotype, year and genotype by year interaction effects. The main effects remained significant if tested against the interaction. The genotypic effect was much more important (61% of total sum of squares) than the year (11.3%) and interaction effect (13.5%). Genotypic differences as well as effects of environment and genotype by environment interaction have been well established in numerous other studies, all revealing the genetic factor being more important than environmental factors. Generally, β-glucan content was significantly higher in 2000 (4.77%) and 2002 (4.67%) than in 2003 (4.35%). Significantly lower values than for the rest were observed for 'Viva 1', 'Penelope' and 'Prosa', all malting barleys released in the 1990s. The genotype by year interaction is demonstrated in Figure 1. It is obvious that some genotypes show stable low, i.e. 'Penelope', 'Viva 1' and/or high, i.e. 'Ebra', '1877/27', β-glucan contents over the years, while other genotypes exhibit significant cross-over interaction, i.e. 'Liechtenstein', 'Adora'. Comparing the ten cultivars grown in 2003 both under conventional and organic farming system revealed significant lower β-glucan contents for the organically grown barleys (4.21 vs. 4.54%). Contents of two cultivars remained constant in both farming systems. Supposing that less nitrogen was available during the grain filling period for the organic grown plants this result is consistent and similar in values to the investigations of OSCARSSON *et al.* (1998). PETR *et al.* (2000) reported significantly lower wort β-glucan levels for organically grown malting barleys.

Table 1. Mean β-glucan content of the investigated husked barleys in the respective years

Barley	Origin[1]	Growing period	Use[2]	β-Glucan (% db)		
				2000	2002	2003
Nürnberg I	D	Before 1900	?	4.64	4.84	4.31
Nürnberg II	D	"	?	4.86	4.59	4.84
1877/27	A, H	"	?	4.96	4.93	5.23
1877/31	A, H	"	?	5.03	4.57	4.86
Tschermaks Hanna-Kargyn	A	1900-1930	M	5.23	4.79	4.53
Kneifel	A, CZ	"	F	4.46	4.47	4.16
Vollkorn	A	1930-1960	F	4.70	4.67	4.23
Angerner früh	A	"	F	4.62	4.78	3.80
Fisser Imperial	A	"	F	5.37	5.08	4.71
Heines Haisa II	D	"	F	5.89	5.23	4.69
Liechtenstein	A	1960-1980	M	4.94	5.19	4.23
Firlbecks Union	D	"	M/F	4.77	4.67	4.46
Eura II	A	"	M	4.70	4.74	4.27
Adora	A	"	M	4.88	5.08	4.28
Berta	A	1980-2004	F/M	4.64	4.66	3.83
Ebra	A	"	M/F	5.05	5.02	5.08
Viva 1	A	"	M	3.97	4.08	3.91
Elisa	A	"	M	4.83	4.32	4.16
Prosa	A	"	M	3.80	3.69	3.44
Penelope	D	"	M	4.03	3.97	3.96

[1] A, Austria; CZ, Czech Republic; D, Germany; H, Hungary
[2] F, feed barley; M, malting barley; ? main use unknown

Considering climatic conditions during the six weeks following anthesis reveals that the highest β-glucan contents were realized in the driest and hottest year (2000: 34.4 mm precipitation, 98 hours >30°C; 2002: 94.8 mm, 70 hrs; 2003: 45.2 mm, 55 hrs). FASTNAUGHT *et al.* (1996) also reported higher contents in low-moisture, hot environmental conditions. β-Glucans are synthesised early during grain filling. This period appears to be the critical time in determining the concentration that will be present in the mature grain. A levelling off or small decrease in concentration was noted at later stages of development, especially in more humid and cooler environments (ÅMAN *et al.* 1989; SWANSTON *et al.* 1997). The effect of the interaction of climatic factors is still somewhat unclear. Drought is suggested to enhance, rainfall to reduce β-glucan contents (MORGAN & RIGGS 1981; ÅMAN & GRAHAM 1987; PÉREZ-VENDRELL *et al.* 1996; EHRENBERGEROVÁ *et al.* 2003) either because final grain-filling is adversely affected by drought through the impairment of starch and protein deposition or because β-glucan synthesis *per se* is enhanced in dry conditions. On the other hand high temperatures *per se* are reported to have diminishing or no apparent effect (SAVIN *et al.* 1997; WALLWORK *et al.* 1998). However, it must be considered that the latter studies were carried out in growth chambers and not under field conditions where a precise and independent timing of a single stress factor is impossible.

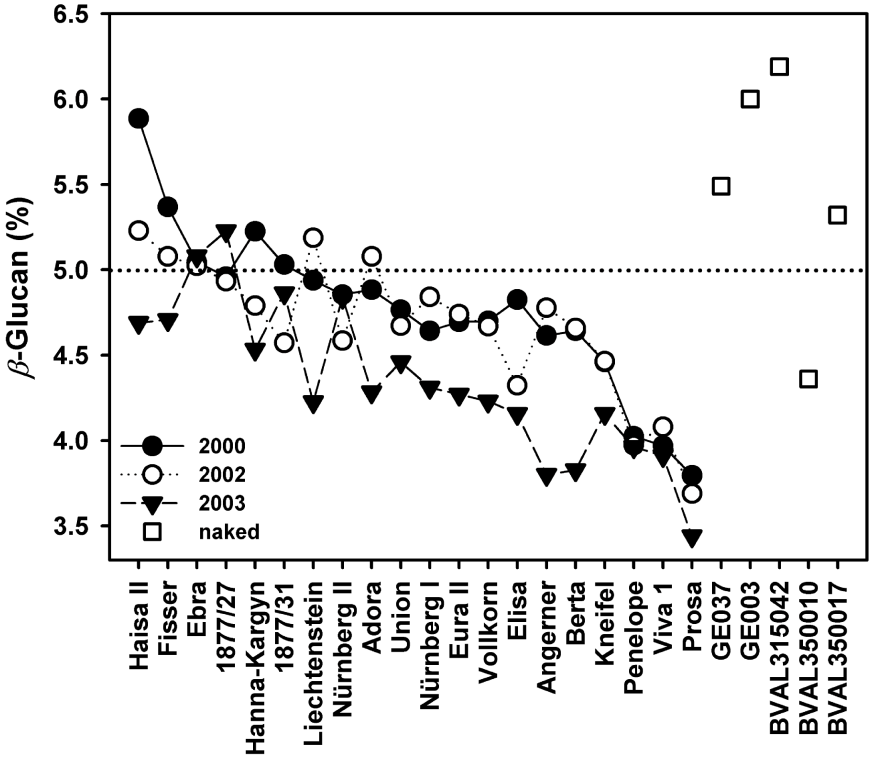

Figure 1. Genotype by environment interaction in total β-glucan content (% db) of the investigated barley genotypes. The dotted line represents the maximum desirable content for malting barleys (FOX *et al.* 2003).

With the exception of BVAL350010 naked barleys showed significant higher β-glucan contents than husked barleys (Figure 1). This result is in agreement with those of XUE *et al.* (1997). The significant lower value of BVAL350010 shows that variation within naked barleys seems to be of similar order than in husked barley. Nevertheless the results

demonstrate that naked barleys probably have a genetic potential to synthesise more β-glucan during grain filling than husked barleys. Moreover, naked barleys would have several advantages in processing, e.g. milling, pearling and, therefore, represent an interesting raw material for the production of barley-based, β-glucan rich food products.

Conclusions

The results of the presented study revealed a considerable genetic variability in total β-glucan content of spring barleys. The genetic factor was found to be significant more important than environmental, interaction and/or cultivation factors. Although the recently released malting barleys exhibited the lowest contents no trend over time similar to that for yield, disease resistance, protein content, malt extract etc. (GRAUSGRUBER *et al.* 2002) was apparent for β-glucan. Hence, for the production of food barley rich in β-glucan the use of more modern barleys with a significant better agronomic performance, e.g. higher yields, better disease and lodging resistance is not only possible, but advisable. For the production of barley-based, β-glucan rich food products naked barleys are probably most promising since they contain somewhat higher levels of β-glucan and could have advantages at various stages of processing through the absence of the husk. Hull-less barleys, however, will be only of increasing interest if the significant lower yields are diminished and/or overcome by the barley breeder.

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Investigation of the Molecular Characteristics of Barley Hordeins by HPLC and MALDI-TOF MS Techniques

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Abstract

The effect of barley proteins on malt and beer quality characteristics is complex because of the large number of proteins present in barley grain, their diverse characteristics, and their extensive modification during the malting and mashing processes. A better understanding of the protein-related phenomena associated with beer quality will assist in developing improved malting barley. In this study, ground barley samples were extracted sequentially with 0.5M NaCl, 50% (v/v) propanol, and 50% (v/v) propanol containing 1% dithiothreitol. The monomeric and polymeric proteins were separated by selective precipitation. The extracts were subjected to HPSEC combined with RI, UV, and multiangle light scattering (MALS) detectors. The average molecular weight of intact monomeric and polymeric fractions was determined. The reduced polymeric fraction was separated by RP-HPLC and the collected peaks were subjected to MALDI-TOF MS to obtain masses of polypeptides in the B, C, and D hordeins. Tryptic peptide maps of the major polypeptides in the C hordeins were also obtained.

Introduction

The influence of barley protein on malt and beer quality is complex and not fully elucidated because of the large number of proteins present in the barley grain and the diversity of functional roles they play during the malting and brewing processes as well as in the final product (SHEWRY & DARLINGTON 2002). Proteins account for about 8-15% of the dry weight of the mature barley grain. Albumins and globulins – the water- and salt-soluble proteins, respectively – function as enzymes, metabolic regulators, or inhibitors of particular enzymes. Hordeins, the major storage proteins, are insoluble in salt solution but dissolve in warm aqueous alcohol. The residual insoluble material – glutelins – constitutes the fourth group of barley proteins, which contain storage and structural proteins.

Recent advances in mass spectrometry, particularly the development of matrix assisted laser desorption/ionization time of flight mass spectrometry, have allowed precise determination of the molecular masses of purified proteins and other biopolymers at extremely low concentrations over a wide mass range. The technique has also proven useful for rapid analysis of crude or partially purified complex protein and/or peptide mixtures, due to its tolerance to impurities and the 'soft' nature of the ionization process, which allows the injection of intact molecular ions. Following extraction, results can normally be obtained in less than 2 min and multiple samples can be analyzed through automated sampling. Peptide patterns and MS/MS peptide sequencing can be used to identify the proteins types they are derived from.

The objective of this study was to evaluate the potential of matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) as a complementary technique to reverse phase (RP-HPLC) and high performance size exclusion chromatography (HPSEC) for the purpose of identifying and characterizing proteins from various barley cultivars.

Material and Methods

Several different barley cultivars, including malting, feed, and hull-less varieties were used in this study. Hordeins were isolated by direct extraction with 50% propanol/ 1% dithiothreitol (DTT). Alternatively, barley proteins were sequentially extracted with NaCl (0.5M) followed by 50% propanol, and finally 50% propanol/DTT. The sequential extraction accomplished separation of barley water and salt soluble proteins (albumins and globulins) from alcohol-soluble hordeins. Following extraction with NaCl, the fractions extracted with 50% propanol and propanol/DTT were designated hordeins I and hordeins II, respectively. Proteins in the residual material constituted the glutelin fraction. Protein content was determined by combustion nitrogen analysis (CNA) using a LECO Model FP-428 CNA analyzer (Leco Corp. St. Joseph, MI) calibrated against ethylenediaminetetraacetic acid (EDTA).

The polypeptides of hordein I (before and after reduction with 1% DTT) and hordein II groups were separated by reverse phase chromatography (RP-HLPC) using a Waters chromatography system (Alliance 2695, 2487 Waters UV detector) controlled by Millenium software. Separation was performed on a Zorbax SB300-C18 column (15 cm x 4.6 mm i.d.) at 60^{o}C using a linear gradient of aqueous acetonitrile (containing 0.1% trifluoroacetic acid) at a flow rate 1 ml/min..

Intact and reduced hordein I fractions were subjected to high performance size exclusion chromatography (HPSEC) on three columns (G5000 PW TSC, Tosohaas Corp. and two S-4000 SW, Phenomenex, Torrance, CA). Column eluent was monitored by absorbance at 210 nm, refractive index detector (Waters 410, Waters, Mississauga, ON, Canada) and by MALLS detector using a DAWN DSP Laser Photometer (Wyatt Technology, Santa Barbara, CA). The columns were kept at room temperature. The flow rate of mobile phase (50% acetonitrile), which was filtered through 0.2 Φm and then 0.1 Φm of cellulose acetate membranes, was 0.4 ml/min. Weight average molecular weight (M_w) was determined using ASTRA software (V4.72, Wyatt Technology) from the equation $R_\theta/K^*c = MP(\theta)\text{-}2A_2cM^2P^2(\theta)$ from ZIMM (1948) where the second virial coefficient (A_2) was assumed to be zero.

Hordein extracts and specific fractions obtained from the RP-HPLC were mixed with sinapic acid and deposited on metal targets for analysis by linear MALDI-TOF MS (Ciphergen PBS-II) to obtain intact polypeptide masses. Some of the fractions were digested with trypsin; tryptic peptide maps and MS/MS on selected peptides were obtained using a MALDI QqTOF mass spectrometer and 2,5-dihydroxybenzoic acid (DHB) as matrix. Fractions obtained by collection of individual peaks of C hordeins were subjected to reduction, alkylation, trypsin digestion, and C18 RP-HPLC separation. The eluting fragments were mixed on-line with DHB and deposited on a MALDI target with a custom built fraction collector for analysis by MALDI Qq TOF.

Results and Conclusions

Composition of Proteins in Feed, Malting, and Hull-Less Barley

Substantial differences in the distribution of various protein groups were found in different barley varieties (Table I). In general, the malting varieties contained higher amounts of albumins and globulins than some feed and hull-less varieties. The total amount of hordeins I and II was significantly lower in malting varieties than in the feed and hull-less varieties. These differences could be associated with the higher protein content of feed and hull-less varieties. We have observed a significant decrease of albumins and globulins, but an increase in hordeins with increasing protein content in malting barley varieties (LEACH *et al*. 2002). The feed and hull-less varieties contained significantly higher amounts of hordeins I compared to the 2-row malting varieties (Table 1). Hordeins I are readily soluble in 50% propanol; whereas hordeins II are extracted only when a reducing agent is added to 50% propanol. As a consequence of the higher amount of hordeins I, all feed and hulless barleys tested in this study had a very high ratio of hordeins I/hordeins II. The 6-row malting varieties (B1602 and Robust) examined in this study also had high ratios of hordein I/II.

Table 1. Protein content and distribution of hordeins I and II in different barley varieties

Variety	Protein Content (%) dwb	Albumin & Globulins (% of total proteins)	Hordein I (% of total proteins)	Hordein II (% of total proteins)	Hordein I & II	Glutelins (% of total proteins)	Ratio Hordein I/Hordein II
Feed Barley							
TR306	15.9	13.0±3.9	39.6±1.5	10.5±0.0	50.1	36.9±5.4	3.8
CDC Dolly	13.0	19.5±2.4	38.2±2.6	8.0±0.2	46.2	34.3±4.8	4.8
Malting Barley							
B1602	11.9	24.4±1.0	29.8±0.3	8.1±1.5	37.9	37.7±0.8	**3.7**
Robust	12.3	19.9±1.0	38.3±0.6	9.7±1.1	48.0	32.1±0.7	**4.0**
Harrington	13.8	18.1±6.7	27.6±1.0	14.4±1.0	42.0	40.4±6.8	**1.9**
CDC Kendall	13.7	19.6±2.1	28.7±2.2	13.6±0.8	42.3	38.2±5.1	**2.1**
AC Metcalfe	13.2	21.5±2.9	31.4±2.9	11.1±1.4	42.5	36.0±4.4	**2.8**
CDC Copeland	13.5	25.3±2.8	27.7±2.6	11.7±0.7	39.4	35.3±4.7	**2.4**
CDC Stratus	12.0	25.0±0.6	29.9±0.1	9.4±0.4	39.3	35.7±0.9	**3.2**
Hulless barley							
CDC Dawn	15.2	23.9±1.3	35.2±0.5	7.5±1.1	42.7	33.4±0.4	4.7
CDC Alamo	18.1	12.3±0.5	40.8±2.1	6.2±1.1	47.0	40.7±0.5	6.6
HA	16.9	16.6±0.1	37.7±1.9	8.8±0.7	46.5	36.9±1.1	4.3

Size Exclusion Chromatography

Extractions with 50% propanol, preceded by extraction of albumins and globulins with NaCl solution, afforded isolation of free and intact monomeric (C) and polymeric (B) hordeins. The first small peak eluting with retention time (RT) 52 - 56 min had a very high average molecular weight (M_w) (11-15x10^6 Da); the average M_w of the second, broad peak with RT 56-86 min was ~1x10^6 Da. Proteins eluting in this region were polydispersed as indicated by a high value of M_w/M_n (~1.9). The low M_w protein populations eluted at RT 88-99 min. The M_w/M_n values of the low M_w protein populations were small (1.01-1.14) compared with the high M_w populations. A greater proportion of high molecular weight populations in feed and hull-less barley varieties distinguished these samples from the malting varieties.

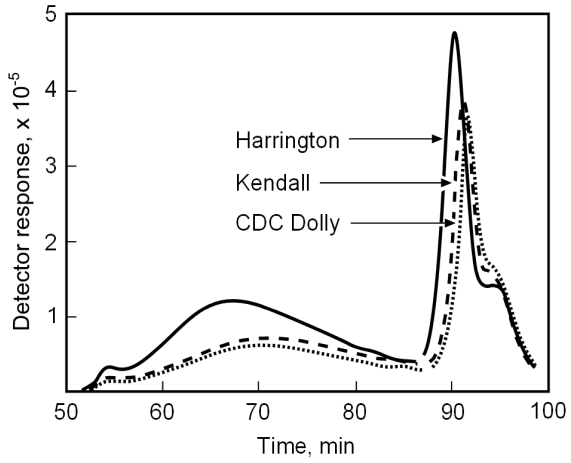

Figure 1. HPSEC profiles of unreduced hordeins I.

Reverse-Phase Chromatography

The RP-HPLC patterns of reduced hordeins I of different barley varieties are shown in Fig. 2 (left chromatogram). The hordeins II extracted from barley with 50% propanol and DTT, preceded by extraction of albumins, globulins, and hordeins I are shown in Fig 2 (right chromatogram).

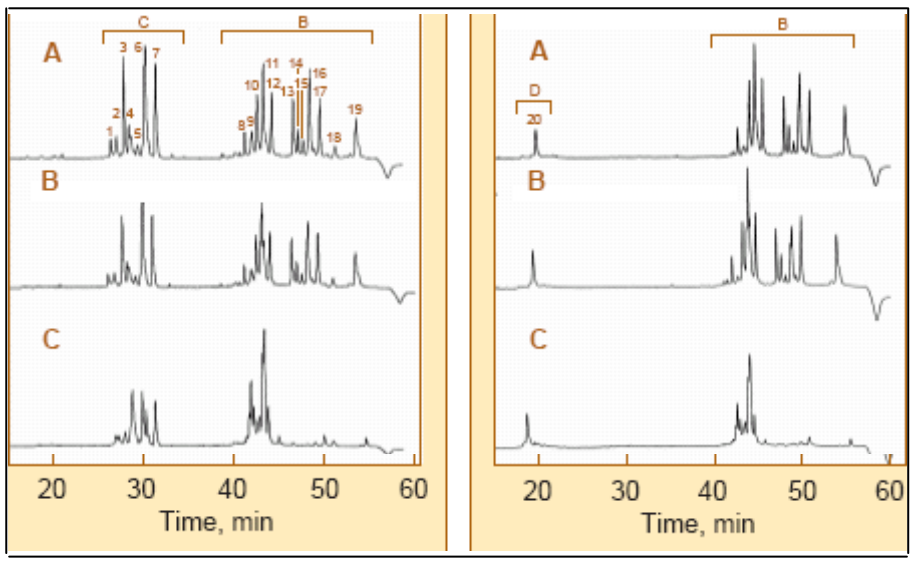

Figure 2. RP-HPLC profiles of reduced hordeins I (left) and hordeins II (right). A: Harrington; B: CDC Kendall; C; CDC Dolly. Polypeptides constituting the C, B, and D hordeins are indicated above the peaks.

The RP-HPLC revealed substantial differences in the amount and composition of C, B, and D polypeptides in hordeins I and II in different barley types (Table 2). In contrast to the malting varieties, the feed and hull-less barley contained a high proportion of B1 polypeptides (peaks 8-12) and very little B2 (peaks 13-17), B3 (peak 18), and B4 (peak 19) polypeptides. Some differences in the composition of B hordeins were also observed between the 2- and 6-row varieties.

Table 2. Distribution of proteins and polypeptides in hordein I fraction. Results from RP-HPLC

Variety	C	B	B1	B2	B3	B4
	(% of hordein I)		(% of B in hordein I)			
CDC Dolly	40.0	59.99	**90.0**	3.8	4.3	1.8
B1602	40	60.2	79.3	18.3	2.4	-
Robust	41.6	58.4	80.3	18.0	1.7	-
Harrington	42.4	57.6	47.0	**41.7**	2.5	**8.7**
AC Metcalfe	35.7	64.3	49.0	**39.3**	1.6	**10.1**
CDC Copeland	41.2	58.8	46.3	**42.1**	2.1	**9.4**
CDC Stratus	32.8	67.2	75.0	23.3	1.7	-
CDC Alamo	42.3	57.7	**81.92**	18.06	-	-
HA	43.3	52.2	**82.61**	11.54	5.85	-

MALDI-TOF MS

In order to obtain detailed mass patterns of polypeptides constituting the C, B, and D groups, individual peaks of reduced hordeins I and II were collected and analysed by linear MALDI-TOF MS. The intact polypeptide mass patterns were also obtained directly from the hordeins extracts (Fig. 3). These experiments allowed to obtain a map of polypeptides constituting the C, B, and D hordeins for each barley variety. The molar masses of polypeptides in the B group (peaks # 8-19 in Fig. 2) ranged from 30 to 38 kDa; the number of polypeptides with unique molar masses differed for different varieties. Interestingly, the number of polypeptides found in the RP-HPLC chromatograms, for the B hordeins, did not correspond to the number of polypeptides with unique molar masses indicating that some polypeptides may have the same molar mass, but differ in properties (hydrophobicity). The majority of polypeptides in the C group (peaks # 1-7 in Fig. 2) had a very narrow range of molar masses 43 to 44 kDa. Polypeptides with molar mass 48-51 kDa constituted a very small portion of the C hordeins. The D hordeins contained mainly one polypeptide (peak # 20) with m/z ~ 71 kDa; other polypeptides with molar masses ranging from 64-87 kDa constituted only a very minor portion of the D hordeins.

The seven polypeptides of the C hordeins in cv Harrington (peaks # 1-7) were further characterized by digestion of individual fractions with trypsin and analysis of the peptide maps. Between 35 to 67 tryptic peptides were obtained from the digested fractions. Fourteen peptides appeared to be common to all the fractions according to their peptide masses and

MS/MS peptide sequences. About 30% of the peptides in each fraction were unique. When the peptide maps of the C group of CDC Dolly were compared with those of Harrington, about 60% of the peptides were common to both varieties, indicating a highly conservative nature of the C hordeins in barley. Further work is needed to characterize the composition of polypeptides present in B hordeins.

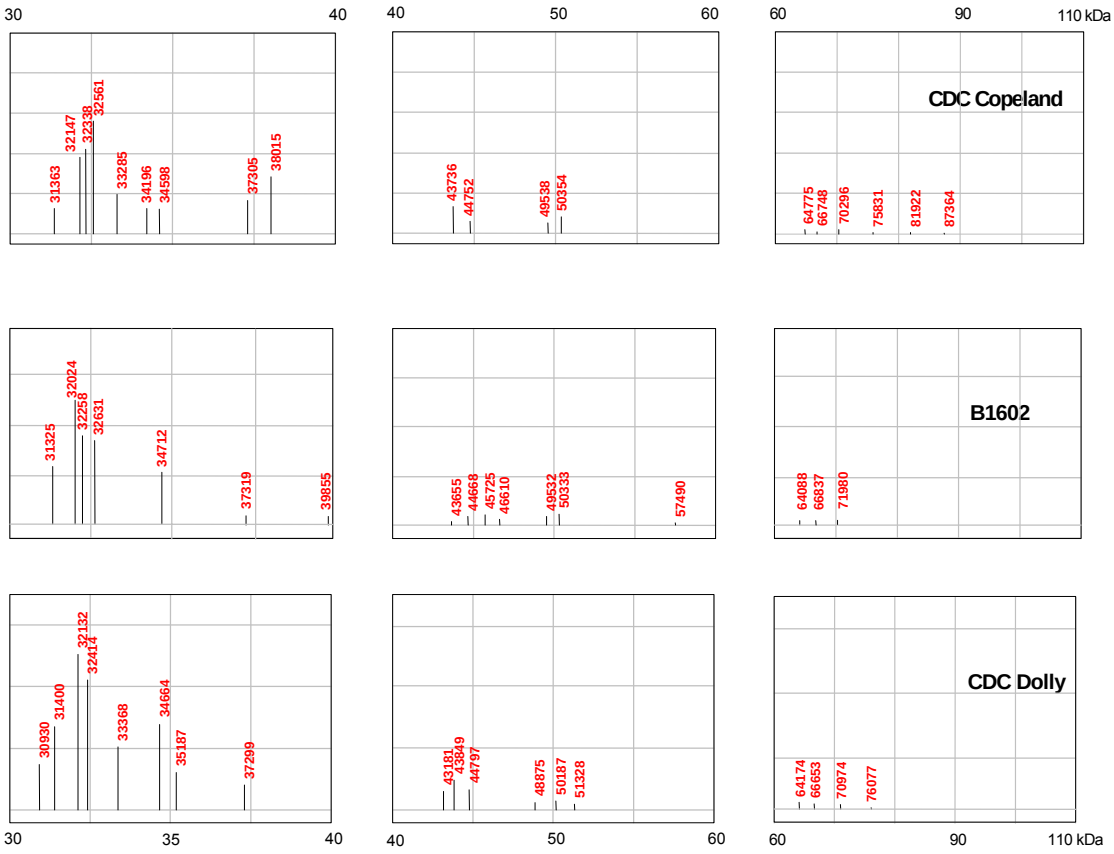

Figure 3. Mass spectra of polypeptides constituting the B (left column), C (middle column), and D (right column) hordeins in selected barley varieties

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Impact of Chromosome 5HL QTL on the Malting Quality and Proteinase Activity in Malt

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Abstract

We investigated the QTLs for the malting quality and proteinase activity in the doubled-haploid lines derived from a cross between 'Mikamo Golden', a Japanese malting barley, and 'Harrington', a North American malting barley. Our results clearly indicated that one QTL located on chromosome 5HL had a large influence on the Kolbach index and proteinase activity in malt. In order to clarify the effects of chromosome 5HL QTL, we analyzed the malting quality and proteinase activity of 27 malting barley cultivars and breeding lines with various origins, and also examined RFLP pattern of the QTL region. Kolbach index and proteinase activity of the malt varied among the lines tested. The RFLP pattern showed polymorphism of the QTL region and was classified into several groups. One of the groups including 'Klages', a North American malting barley, showed a higher Kolbach index and proteinase activity than the other groups, and the RFLP pattern was closely related with these characteristics. These results suggest that the QTL located on chromosome 5HL has a considerable effect on the malting quality.

Keywords: malting barley; QTL analysis; chromosome 5HL; proteinase activity; Kolbach index

Introduction

The degradation process of seed storage protein is of vital importance to the commercial malting process as well as the germination process. The content of soluble nitrogen on malt (SN) or the percentage of soluble nitrogen among the total nitrogen on malt (Kolbach index; KI), which indicate the degradation efficiency of storage protein in germinating barley, have a large influence on the beer quality (ENARI 1986; MACGREGOR 1996). The proteinases of germinating barley are responsible for the breakdown of large, generally insoluble, storage proteins into soluble proteins, peptide and amino acids (SIMPSON 2001). Endoproteinase plays a major role in the degradation of storage proteins in barley endosperm, and among them, cysteine proteinase is considered the most important group (ENARI & SOPANEN 1986; KIHARA *et al*. 2002) as well as in other cereal grains

(SIMPSON 2001). We applied QTL analysis for proteinase activity to the doubled-haploid lines (DHLs) of two crosses between Japanese and North American malting barley lines with different levels of malting quality (OKADA *et al*. submitted), and discussed the relation between QTLs for proteinase activity and malting quality (KIHARA *et al*. submitted). Our results indicated that the QTL region of chromosome 5HL had a significant influence on the proteinase activities, soluble nitrogen on malt and the Kolbach index.

In the present report, we investigated the RFLP using three DNA markers that were located on the chromosome 5HL QTL, and clarified the effect of this QTL on the malting quality and enzyme activity in various barley lines.

Material and Methods

Plant Material

Barley samples (Material 1 and Material 2) were used for micromalting. Material 1 consisted of 6 barley lines from the 'Hokuiku 27'pedigree. 'Hokuiku 27' had a high soluble nitrogen content and high Kolbach index in the previous malting quality analysis. Material 2 consisted of 27 barley lines that contained 10 Japanese, 9 North American, 4 Australian and 4 European lines. Both materials were grown in our experimental field (Gumma, Japan), and the mean $\pm$ standard deviation for total nitrogen level was 1.563 $\pm$ 0.044 and 1.575 $\pm$ 0.109 in Material 1 and 2, respectively.

Micromalting and Malting Quality Analysis

Grain (250 g) (>2.5 mm screen) was micromalted in an Automatic Micromalting System (Phoenix Biosystems, Australia). Prior to germination, the steeping moisture was set to approximately 43.0 – 43.5% by adjusting the steep time. The stepping time varied among samples, and its mean $\pm$ standard deviation was 52.3 $\pm$ 4.2 h and 45.2 $\pm$ 3.0 h in Material 1 and 2. Germination lasted 6 days at 15°C and the kilning scheme was; 10 h at 45°C, 8 h at 55°C, 3.5 h at 65°C, 3.5 h at 75°C and 4 h at 83.5°C. These malt samples were stored at 5°C until crude enzyme preparation.

Malting quality analysis for the content of soluble nitrogen in the malt and the Kolbach index followed the EBC methods.

Assay of Proteinase Activity

The assay method for proteinase activity was the same as previously reported (KIHARA *et al*. 2002). Proteinase activity was measured using a resorufin-labeled casein (Universal Protease Substrate) (Roche, Germany), and were calculated on a malt basis (pmol/min/mg malt).

Southern Blot Analysis

Genomic DNA was extracted from the mature leaves of the plants as described in the standard protocol of the CTAB method (MURRAY & THOMPSON 1980). Three micrograms of each DNA sample was individually digested with restriction enzymes. These digested DNAs were electrophoresed on a 0.8% agarose gel and transferred to nylon membranes by the capillary method (SAMBROOK *et al.* 1989). The prehybridization, hybridization, detection, and probe labeling procedures were performed according to the *Gene Images*^{TM} system (Amersham Biosciences, UK) manual.

RFLP Probes

We applied ABG463, ABG314, and ABC622 as DNA markers. Prof. A. KLEINHOFS (Washington State Univ., Pullman, WA) donated these markers, which were used for North American Barley Genome Mapping Project (NABGMP). These markers were located close to chromosome 5HL QTLs for malting quality and proteinase activity (OKADA *et al.* submitted, KIHARA *et al.* submitted).

Results and Discussion

We investigated the RFLP pattern of 'Hokuiku 27' pedigree using three DNA markers (ABG463, ABG314 and ABC622). RFLP pattern varied among six pedigree lines (Fig. 1), and 'Hokuiku 27' and 'Klages,' which showed high Kolbach index and high proteinase activity (KIHARA *et al*. 2002), had the same RFLP pattern (type A) with all DNA markers, although other lines had different RFLP types (Table 1).

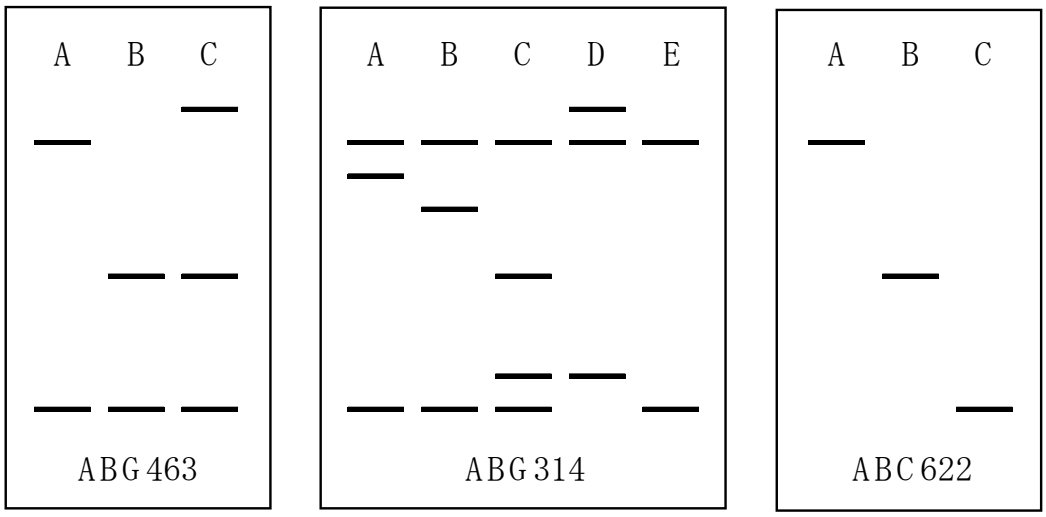

Figure 1. RFLP patterns with three DNA markers

Table 1. Characteristics of 'Hokuiku 27' pedigree lines

Lines	Kolbach index	Proteinase activity (pmol/min/mg malt)				Each RFLP types using three DNA markers		
		Total	Cysteine	Aspartic	Other	ABG463	ABG314	ABC622
Hokuiku 15	39.5	6.83	4.29	1.34	1.19	B	B	C
Hokuiku 17	38.5	8.65	2.85	3.53	2.27	B	C	B
Hokuiku 19	45.1	10.54	4.41	4.13	2.00	B	B	B
Hokuiku 27	59.2	13.10	8.32	2.38	2.40	A	A	A
Haruna Nijo	43.3	10.19	4.80	3.41	1.98	B	B	B
Klages	53.3	12.55	6.69	3.24	2.61	A	A	A

In order to clarify the effects of chromosome 5HL QTLs in more detail, we analyzed the malting quality and proteinase activity of 27 barley lines, and examined the RFLP pattern with three DNA markers. The Kolbach index and proteinase activity varied among 27 barley lines tested, and the mean $\pm$ standard deviation was 44.8 $\pm$ 5.7 (31.2 min – 56.5 max) and 9.82 $\pm$ 2.37 (4.32 min -14.02 max) pmol/min/mg malt, respectively. RFLP patterns with ABG463, ABG314 and ABC622 were divided into 3, 5 and 3 types, respectively (Fig. 1). Therefore, we analyzed the relationship among these characteristics. The barley lines, which had higher Kolbach indexes and higher proteinase activities, showed the same RFLP pattern (type A) as 'Hokuiku 27' and 'Klages' with three DNA markers (Fig. 2).

We have already clarified that the QTL region of chromosome 5HL had a significant influence on the malting quality (soluble nitrogen content, Kolbach index, extract yield and wort β-glucan content) and enzyme activity (α-amylase and proteinase) in the doubled-haploid progeny of standard Japanese malting barley, 'Mikamo Golden', and North American malting barley 'Harrington' (OKADA *et al*. submitted; KIHARA *et al*. submitted). In the previous report, the QTLs for several malting qualities were also located on the same region of chromosome 5HL in Harrington/TR306 (MATHER *et al*. 1997) and Harrington/Morex (MARQUEZ-CEDILLO *et al*. 2000). In the present report, we confirm that the QTL region of chromosome 5HL has a large effect on the degradation of storage protein and proteinase activity among various barley lines. MANO and TAKEDA (1997) reported that, in the DHLs of Harrington/TR306, the QTL for the most effective ABA response at germination were located close to our QTL region. These facts suggest the importance of 5HL QTL during the germination process in barley grain. Further experiments will be needed to clarify the role of this region at the germination stage.

The control of the degradation level of seed storage protein has a significant influence on the production of high quality malt and beer. Marker assisted selection using a close-linked marker with this QTL could accelerate the improvement of both malt and beer quality by malting barley breeding.

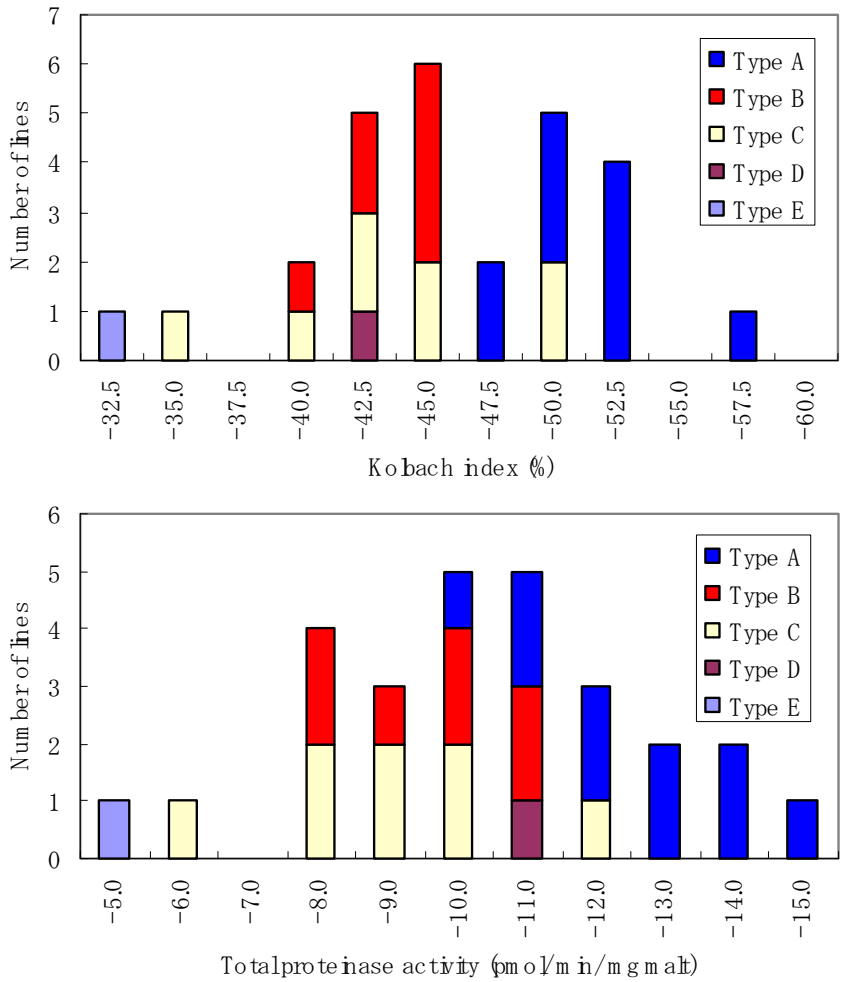

Figure 2. Relationship between RFLP patterns (ABG314) and Kolbach index or proteinase activity

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Can Anonymous QTLs Be Introgressed Successfully into Another Genetic Background? Results from a Barley Malting Quality Parameter

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Abstract
Spirit yield, the product of hot water extract and its fermentability, is an important production parameter for Scotch Whisky. We utilised a barley mapping population to identify a QTL associated with an increase in fermentability and used a backcrossing programme coupled with doubled haploidy to transfer it into cv. Landlord, in an attempt to improve its predicted spirit yield (PSY). We genotyped and phenotyped the resultant backcross population to establish whether or not the QTL in the introgressed region was detectable in a different genetic background. The introgressed region was also associated with a decrease in hot water extract. Whilst the effect of the introgressed region on hot water extract was still detectable in the Landlord background, we found evidence of a cross-over QTL x environment interaction for fermentability. Effects of the introgression upon grain nitrogen content and soluble nitrogen ratio suggest that QTLs detected in a relatively poor malting quality background, such as the introgression, may not be expressed to the same extent in a better background. Fine mapping in the region is required to establish whether or not the observed effects are due to closely linked loci or the pleiotropic effects of a single locus.
Keywords: barley; QTLs; mapping, quality; marker-assisted selection

Introduction

The Scotch Whisky industry consumes between 400,000 and 500,000 tonnes of barley annually, which accounts for an average 30-40% of spring barley production in Scotland. The vast majority of this requirement is used in the production of malt whisky where the distiller's aim is to balance the maximum production of spirit (Spirit Yield) with the minimum of processing problems. Spirit Yield is the product of the hot water extract (HWE) following malting and the fermentability of that extract. HWE is the major measure of malting quality because it is a measure of the amount of material that can be solubilised following malting. Not all of the solubilised material is, however, fermentable by yeast for conversion into alcohol and the proportion that is fermentable varies both with variety and environment. Thus, whilst fermentability depends upon HWE, there is some potential to manipulate both characters independently. Measurement of fermentability requires controlled fermentation of the extract of a malted sample (SWANSTON *et al.* 2000) and is therefore not suitable as a high throughput assay or for early generation selection. The use of molecular markers in a Marker-Assisted Selection (MAS) scheme is therefore an attractive alternative. In an earlier report, we described the use a barley mapping population to identify QTLs for fermentability that might be suitable targets for MAS (MEYER *et al.* 2000). Here we update the findings of the earlier report to include some EST derived markers that have been scored on the mapping population and also describe an attempt to validate the QTL by transferring the donor segment into another genetic background.

Material and Methods

We previously reported the use of the Derkado x B83-12/21/5 mapping population to identify QTLs for fermentability and hot water extract (HWE) in the region of the *ari-e*GP dwarfing gene on barley chromosome 5H (MEYER *et al.* 2000). Since then, we have added some extra markers to the cross, notably some EST derived markers, either as Single Nucleotide Polymorphisms (SNPs) or Simple Sequence Repeats (SSRs). We used JOINMAP 3.0 (VAN OOIJEN & VOORRIPS 2001) to produce an updated genetic map of the region and PLABQTL (UTZ & MELCHINGER 1996) to detect QTLs from an analyses of the overall means of fermentability and HWE data obtained from four replicated trials grown between 1995 and 1996.

We produced a BC1DH population to validate the effects of the QTL mapped on chromosome 5H in an alternative genetic background. We selected high fermentability lines that also possessed the appropriate genotype in the region and crossed those to Landlord as it then represented a potential new malting quality cultivar. We crossed the F1's back to Landlord to produce BC1F1 seed from which we derived doubled haploid (DH) lines. The BC1DHs were a random sample of the BC1F1 and therefore had not undergone any selection beyond any selection that the DH protocol might have exerted.

DNA was extracted from the BC1DH plants and used to genotype the population with 44 previously mapped SSR markers, including four closely linked markers on 5H to monitor segregation in the region of the hot water extract and fermentability QTLs. The lines were first multiplied in the field in 1999 and then a large scale multiplication of 200 lines was carried out by Advanta Seeds UK in New Zealand in 1999/2000. Seed from 125 BC1DHs from a common donor were returned for growing in three randomised plot trials in the UK in 2000. Two trials, grown nr Dundee, Tayside and nr Docking, Norfolk, were replicated and received a full prophylactic fungicide programme. The third trial was also grown nr Dundee but in a single replicate MAD-2 design (LIN & POUSHINSKY 1985) without any fungicide treatment. Landlord was the control main plot and the mapping population parents, Derkado and B83-12/21/5, were the control sub-plots. All trials were sown in 7.5m^2 plots, including gaps, and received a typical spring malting barley fertiliser application. During the growing season, the plots were scored for juvenile growth habit, which also enabled the lines to be classified for alleleic differences at the *sdw1* and *ari-e*GP loci. At maturity, the plots were harvested with a small plot combine and the grain from each dried to a constant 13% moisture content. Samples were cleaned and sieved and the fraction passing over a 2.5mm sieve retained for grain quality analysis. The cleaned and sieved grain samples were micro-malted and malt samples used to measure hot water extract. The fermentabilities of the extracts were then measured according to the protocol described by (SWANSTON *et al.* 2000).

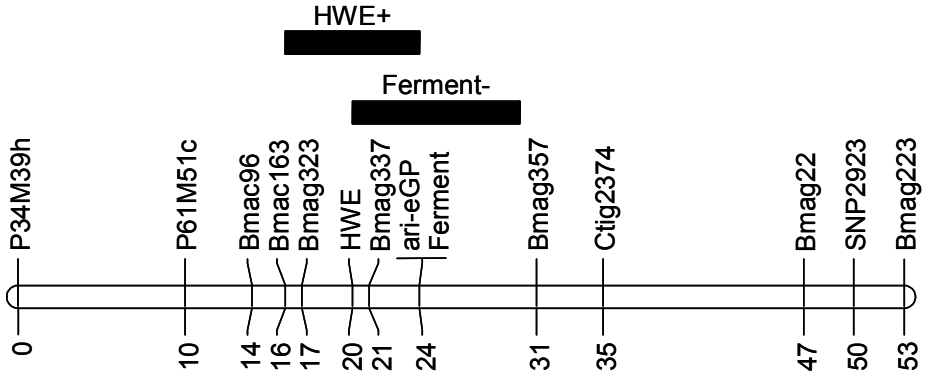
We used the markers in the region of *ari-e*GP to classify the BC1DH lines according to whether or not they possessed donor alleles at the location of the QTL peaks for hot water extract and fermentability. We then carried out an ANOVA of the phenotypic data using GENSTAT (Lawes Agricultural Trust, Rothamsted Experimental Station, UK) to determine whether or not there were significant differences in the allelic classes for each trial separately. In addition, we derived means over all three trials for the phenotypic data and analysed those in the same manner. Finally, we used all the genotypic data and the overall phenotypic means to conduct a genome wide search for QTLs affecting HWE and fermentability using GENSTAT to conduct a multiple regression analysis.

Results

Twenty-four EST derived markers were located on the Derkado x B83-12/21/5 map with a Single Nucleotide Polymorphism (SNP3923) and an EST-SSR (Ctig2374) mapping in the

region of *ari-e*GP. Neither was within the confidence intervals of either the HWE or fermentability QTLs located in the region (Figure 1). The fact that, whilst the QTL peaks in the region for the characters are separated by 4cM, the confidence intervals overlap raises the question of whether there are two separate QTLs in repulsion or that fermentability is the opposite of HWE.

Figure 1. Partial genetic map of chromosome 5H of Derkado x B83-12/21/5. Shaded bars indicate 1 LOD confidence intervals associated with QTL peak location on the map.



The overall phenotypic means from the three trials of the BC1DH lines for selected characters are shown in Table 1. The donor parent from the Derkado x B83-12/21/5 mapping population possessed the *ari-e*GP dwarfing gene whereas Landlord had the *sdw1* dwarfing gene. This meant that both were segregating in the BC1DH population and there was a slight excess of double dwarves, resulting in a significant difference of the BC1DH mean from the recurrent parent. Double dwarves are small-grained and hence have a high grain nitrogen content, which is the most likely explanation for the significant difference between the BC1DH mean and Landlord. In malting the trial, it was apparent that there was considerable water sensitivity in the BC1DH population and Landlord, which persisted 10 months after harvest. These problems were not apparent in the established cultivars, Derkado and Optic, nor the breeding line B83-12/21/5 and the reasons for it are not clear. There was considerable development of "Ramularia-Like Spots" in SCRI trials in 2000 and scores were obtained from the untreated trial, which were significantly negatively correlated with germination in 8mls of water. Susceptibility to Ramularia was one of the reasons why Landlord failed to become an established cultivar and it could be that its genetic background adversely affected the malting performance of some of the BC1DH lines in 2000.

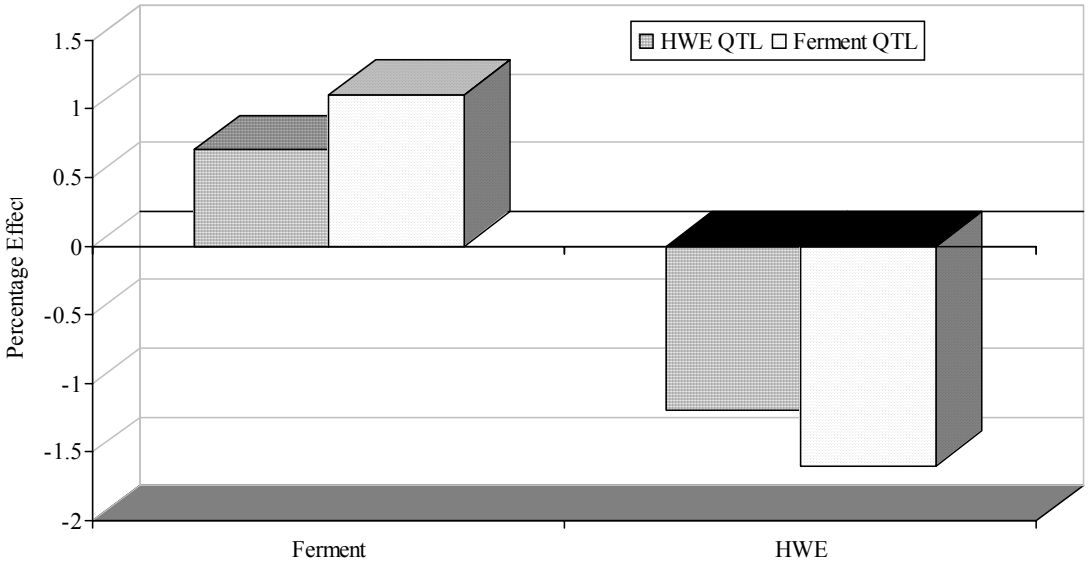
Table 1. Population statistics from the overall means of three trials of Landlord BC1DH lines. Figures in bold are significantly different from Landlord (P<0.05).

	Yield (t/ha)	Head (days)	Height (cm)	Grain Nitrogen (%)	HWE (L°/kg)	Ferment (%)	PSY (l/t)
Optic	5.31	20.1	61.5	1.15	319	80.6	407
Landlord	5.02	19.4	61.8	1.25	261	82.4	333
Derkado	4.76	23.4	64.3	1.34	337	78.0	412
B83-12/21/5	5.16	21.6	60.5	1.38	325	80.9	410
BC1DHMin	**4.07**	**15.0**	**38.3**	1.27	**210**	**77.9**	**267**
BC1DHMean	4.84	18.7	**56.9**	**1.40**	268	80.9	341
BC1DHMax	**5.96**	**28.5**	**75.3**	**1.66**	**317**	83.7	**406**
SED(pair)	0.25	1.5	2.8	0.08	21	1.7	29

The poor malting performance of lines in the 2000 SCRI trials was not randomly distributed across the different allelic classes in the regions of the HWE and fermentability QTLs on 5H,

thus confounding a thorough evaluation of their effects in a different genetic background. From more typical malting results obtained from the Norfolk trial site, we found donor alleles in the region of the fermentability QTL peak significantly increased fermentability and decreased HWE. The same pattern was observed at the HWE QTL but the effect of the donor alleles was slightly less than that of donor alleles at the fermentability locus for both characters (Figure 2). This was slightly surprising as we would have predicted that, if the two QTLs were separate, the HWE locus should have a greater effect upon HWE phenotype than the fermentability locus. Given that the fermentability locus co-segregates with the *ari-e*GP dwarfing gene, it may well be that the effects that we have detected are merely due to pleiotropy. Fine mapping in the region is, however, required to resolve this question.

Figure 1. Percentage effect of donor alleles at mapped QTL loci for fermentability and hot water extract that have been transferred to a Landlord genetic background (Norfolk trial only).

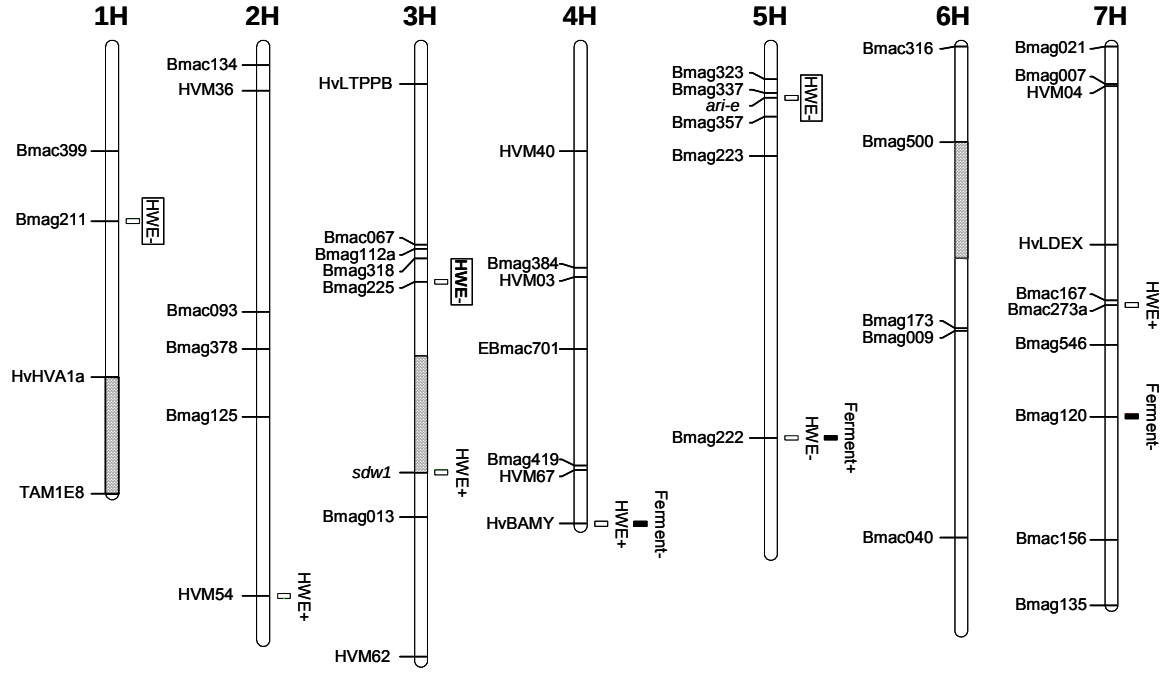


Considering the overall means from the three sites, multiple regression of phenotype against the allelic classes at the 46 marker loci identified 8 and 3 significant associations with HWE and fermentability respectively (Figure 3.) Three of the HWE QTLs were in similar regions to those detected in the original mapping study (data not shown) and 5 were not detected in the earlier study. None of the three fermentability QTLs were detected in the earlier mapping study. We would have expected the multiple regression analysis to detect a significant fermentability QTL in the region of *ari*-eGP if the introgressed segment was effective in another genetic background. The QTLs detected by the multiple regression analysis were all significant and accounted for 69 and 20% of the phenotypic variation in HWE and fermentability respectively.

Despite the fact that the malting quality analyses of the BC1DH population were compromised by excessive water-sensitivity, donor alleles in the region of *ari-e*GP on chromosome 5H did have the predicted negative effect in another genetic background but the predicted effect on the overall fermentability phenotype was not significant. Looking at the environments individually, donor alleles at the fermentability QTL resulted in a significant decrease in fermentability in the SCRI fungicide treated trial whereas they increased fermentability in the Norfolk trial and did not have a significant effect in the SCRI untreated trial. Clearly the locus is subject to QTL x environment interactions that render it an unsuitable target for MAS as the interactions are of the cross-over type. Inspection of the data reveals that donor alleles at *ari-e*GP resulted in 21 and 17% decreases in the Soluble Nitrogen

Ratio means in the SCRI treated and untreated trials respectively whereas there was no significant effect upon the character in the Norfolk trial. Donor alleles at the *ari*-eGP locus also resulted in slight but highly significant increases in grain nitrogen content in both SCRI trials whereas there was no significant effect in the Norfolk trial. Non-random distribution of under-modified malts therefore appears to have resulted in the QTL x environment interactions for fermentability. BATHGATE *et al.* (1978) found that fermentability peaks earlier in modification than HWE. Together with increased water sensitivity, this might indicate that the performance of the SCRI malt samples that possessed the *ari-e*GP allele resulted from under-modification.

Figure 1. Map locations of markers used to genotype Landlord BC1DH population and their significant associations with hot water extract (HWE) and fermentability. Sign is the effect of the donor allele and characters in a box denote locations detected in Derkado x B83-12/21/5.



Discussion

The significant effects of the donor allele at *ari*-eGP on grain nitrogen content were both detected in trials where the overall mean for the character was low whereas there was no significant effect in a trial with a more normal mean value for malting barley. Whilst the results are derived from just three trials, there was a trend of increasing effect of the donor allele with reducing N. There does, however, appear to be a complex interaction between the QTL, the environment and the genetic background that limits the value of this particular locus as a target for MAS. It is possible that past selection has resulted in a QTL that is more effective in scavenging N in a low N environment but releases less during malting, resulting in less N in extract. Landlord was being developed as a malting cultivar, until the Ramularia problem became apparent, and is a different genetic background from the feed type B83-12/21/5, which was the origin of the donor. There is evidence from winter barley that successful varieties possess different combinations of alleles at seven key loci (MACAULAY *et al.* 2004), suggesting that good malting quality is derived from the summation of a number of different attributes. This is confirmed by breeding experience, which shows that crosses between good malting quality cultivars often result in populations with a HWE mean that is less than the poorer parent. This suggests that there are alternative balances of these characteristics that result in the same final product and, unless one has a clear understanding of the underlying genes and their interactions, the success of MAS schemes for anonymous

QTLs will de due to chance. Detailed functional marker maps and expression analyses provide two tools that, when combined with biochemistry, may help in deriving a more predictive approach for malting quality that will lead to suitable targets for MAS.

The value of studying crosses between good and poor malting quality cultivars clearly maximises the chances of detecting significant QTLs but does not reflect routine barley breeding. The QTLs that are detected in such analyses have most likely already been fixed in the elite gene-pool that barley breeders use and therefore do not offer realistic targets for MAS. The challenge is to identify QTLs for the relatively minor differences that distinguish between good malting quality and the very highest levels because these are the differences that determine the commercial success of a variety. At SCRI, we are currently exploring the use of a composite population made up from several small populations from crosses between elite genotypes to identify such QTLs (RAE *et al.* 2004).

The confidence intervals of the fermentability and HWE QTLs that we detected in the original population overlap and also cover the *ari-e*GP dwarfing gene locus and results from the BC1DH study found that the dwarfing gene has the strongest effect upon both characters. All the effects that we detected could therefore be due to pleiotropy of *ari-e*GP but fine mapping and associated phenotyping is required to establish whether or not recombinants in the region can be identified. Studies of Recombinant Chromosome Substitution Lines (THOMAS *et al.* 2000; YOUNG *et al.* 2004) might help determine whether or not the effects are due to linkage or pleiotropy.

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A New Two Dimensional Germinative Classification for Malting Barley Quality Based on Separate Estimates for Vigour and Viability

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Abstract
It is surprising that germination data not even today seem fully integrated with malting data in barley quality evaluation. In order to implement such an integration, pattern recognition multivariate data analysis (chemometrics) is essential. Inspired by the results from chemometric analyses of whole germination curves we have tested a two-dimensional classification plot of barley samples based on separate estimates for "vigour" (g%1) germination energy (GE) as abscissa with limits at 70% and 30% and "viability" (g%3) as ordinate with limits at 98% and 92%. The seven barley classes obtained visualises the quality differences in a consistent and instructive way clearly differencing and ordering malting barley's with falling extract% and increasing wort ß-glucan mg/L according to a subsequent validation analysis. "Vigour" g%1 could surprisingly by predicted by Partial Least Squares Regression (PLSR) correlation by Near Infrared Transmission (NIT) and by a separate set of ten physical-chemical analyses. In both correlations, samples with "viability" g%3 lower than 92% were outliers. It was concluded that germination speed is connected to the structure of the seed in barley of malting quality detected by the two methods, which regulates the availability of substrate for germ growth near connected to the speed of malt modification. It is suggested that a NIT PLSR prediction model for "vigour" can be used directly "on-line" for quality control in the grain industry and by plant breeders. A fast germinative classification plot can be established with NIT spectroscopy for "vigour" and the Tetrazolium germ-staining test for "viability" within two hours.

Introduction

Optimal germination performance is with no doubt the most important quality criterion for malting barley. The industry and trade are dependent on reproducible and representive analyses as expressed in European Brewery Convention (EBC) Analytica (3.5-3.7) (ANONYMOUS 1998) regarding germinative energy (GE) % (at 3 and 5 days) and capacity (GC) % as well as germination index (GI) and homogeneity (GH). Quality classification indices based on elaborate pilot malting analyses and expert evaluation without the germinative analyses has been developed by MOLINA-CANO (1987) for EBC and recently by our brewing research group (NIELSEN *et al*. 2002) using fuzzy logic analyses. The latter index could be predicted (NIELSEN *et al*. 2002) by NIT spectroscopy in a PLSR chemometric model. MONNEZ (1987) found it difficult to embrace the quality complex in one figure and suggested a more complex classification obtaining two hierarchical indices based on malting and brewing parameters by multivariate analysis still not including germinative data in the analysis. We therefore (MUNCK & MØLLER 2004A, 2004B; MØLLER 2004) decided to combine germinative, chemical, malting and brewing data in a multivariate data analysis starting with investigating the pattern of germination response curves by Principal Component Analysis (PCA).

Material and Methods

Two barley materials are used

I. 17 samples of the malting barley Alexis grown all over Europe in 1994 collected and analysed for malting quality at Centre UdL-IRTA, Spain by Dr. José Luis Molina-Cano (MØLLER *et al*. 2002).

II. 42 barley samples of the varieties Alexis, Blenheim and Meltan grown in Southern Scandinavia collected in 1993-1996 were analysed for germination energy (GE), seed physical-chemical analyses and malt quality after cold storage (7°C, 13.5% water) in 1999. Percentage of germination day1-day3 was determined according to EBC Analytica 3:6 (ANONYMOUS, 1998).

The "Unscrambler" chemometric software (Camo A/S, Norway) was used.

Results and Discussion

Germinative Classification

The germination curves (percentage of germinated grains for days 1-8 (g%1-g%8), Figure 1A) were used in an unsupervised PCA calculation for the 17 samples of the variety Alexis grown at different locations all over Europe (Figure 1B). The PCA biplot shows that three samples grown in Spain (E) are located to the left in the plot, whereas all the samples from Finland (SU) are located in bottom right corner. In the top right corner are samples from Germany (D), Czech Republic (CZ), The Netherlands (NE) and two of the samples grown in Denmark (DK). Variables germination% from days 1 to 8 are shown in the plot as loadings (**1**,**2**,**3**,**4**,**5**,**6**,7,**8**). It is seen that day 1 percentages are located in the top, day 2 closer to the rest of the loadings, and days 3-8 are located near each other in a group. This can also be seen from the germination curves in Figure 1A, while the curve form after 3-8 days is more levelled for the 17 samples.

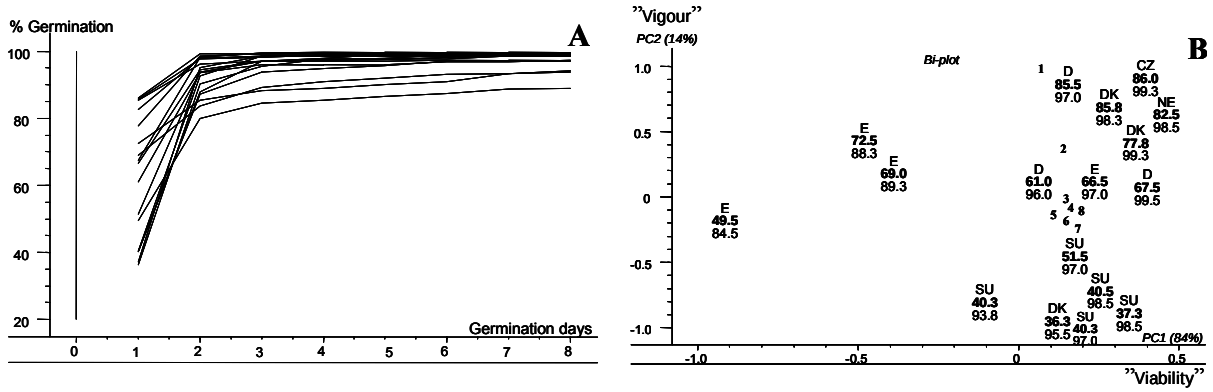

Figure 1. Multivariate evaluation of germination profiles g%1-g%8 for 17 untreated Alexis barley seed samples grown in EBC trials in Europe in 1994. **A.** Germination profiles for the 17 samples. **B.** PCA biplot (PC1:2) of the germination profiles for the 17 samples with identification of each sample position by figures for g%1 in bold and g%3 in normal font. Letters denote country symbols. Figures in large and bold are loadings g%1(**1**) – g%8 (**8**).

To investigate in more detail why the samples are located as they are in the PCA plot in Figure 1B, the notation refers to germination percentage after one day (bold) and after three days. The 3-day germination percentage is taken as the most conveniently measured representative for the close loading cluster 3-8. From this it is seen that there is a clear gradient in germination percentage after three days along the abscissa from left to right, and for germination percentage after one day along the ordinate from below to above. Now it is possible to ascertain the meaning of the "hidden" principal components PC1 and PC2 in the plot. PC1 mainly describes the variation due to germination percentage day 3. This axis can

approximately be described to represent "viability" or germination energy (GE). The germination percentage after three days does not increase very much to day 8 (Figure 1A), and this factor can therefore give an estimate of living grains. PC2 mainly describes the variation due to germination percentage day 1. Concluded from earlier investigations where GI and g%1 correlates well, PC2 can be described as an expression of germination speed "vigour".

From the PCA in Figure 1 a hypothesis can be generated that g%1 and g%3 could be used in a simple two-dimensional germinative abscissa-ordinate plot for malting quality classification.

This hypothesis was further confirmed in studying another data set where 42 micro malted barley samples with a large variation in "vigour" (abscissa) and "viability" (ordinate) are plotted as seen in Figure 2. Two quality levels of both "viability" (92 and 98%) and "vigour" (30 and 70%) are introduced in the plot. This leads to a division of the barley samples into 7 classes: 1.1, 1.2, 1.3, 2.1, 2.2, 2.3 and 3.0.

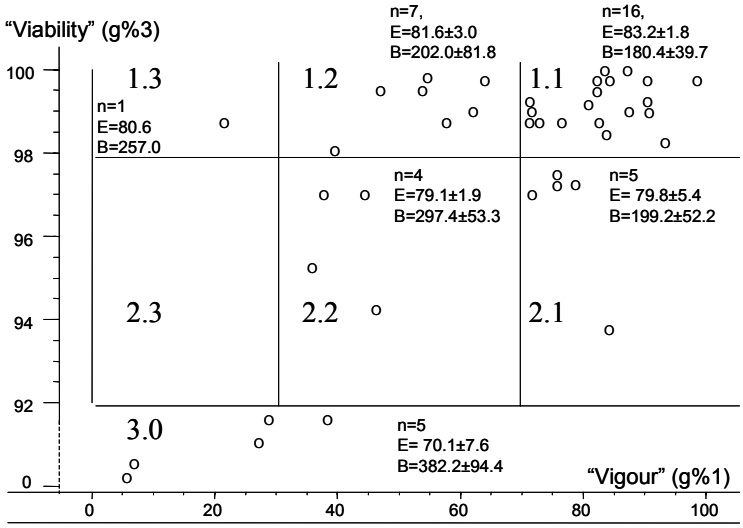

Figure 2. Germinative energy classification for a malting barley material (n=42). "Vigour" g%1 (abscissa) and "viability" g%3 (ordinate). Enlargement of classification plot with "viability" ≥ 92%. See text for discussion. E=Extract %, B= mg/L (1→3,1→4)-ß-glucan in wort.

It is clearly seen that the "vigour" component is complementary to the germinative energy component "viability" g%3 in differentiating the whole material with regard to extract % and to an even greater degree with regard to the critical quality criteria (ß-glucan in wort), revealing the dependence of cytolytic activity in the malt on a swift and complete germination. The mean values of the malting barley classes in Figure 2 reveal clear gradients in these important quality criteria. The feed barley Class 3 is clearly unsatisfactory for malting with mean figures of 70.1% for extract and 382.2 mg/L for ß-glucan in wort. The germination index has a high correlation of r=0.99 in this material with g%1 and can thus be used as an indication for vigour. g%1 should however, be prefered because it is faster to measure and is much more responsive.

It is concluded that the proposed two dimensional classification system with the barley material tested here is highly sensitive for predicting and discriminating the levels of extract (%) and ß-glucan in wort (mg/L) which are central parameters in the barley malt quality complex. It is suggested that further malting barley quality research should be directed to utilise vigour and viability information from germination curves for quality classification.

Prediction by ten physical-chemical parameters

It would be advantageous if it were possible to predict the indirect parameters such as germination properties from manifest physical-chemical parameters from ungerminated barley. Here we use a set of ten physical-chemical parameters: Protein (P), β-glucan (BG), single seed hardness (HI, Perten SKCS 4100) and six morphological imaging barley data (Graincheck Foss A/S) as well as thousand kernel weight (TKW). The supervised chemometric algorithm PLSR can test this. The prediction of the germination properties are shown in Table 1.

Table 1. PLSR Jack-knife correlations between the ten manifest parameters (TKW, HI, P, BG, width, length, area, volume, round, intensity) as (**X**) and hidden germination and malting variables (**y**). Samples with low viability (<92%) = underlined, medium viability (92-98%) = **bold**, high viability (>98%) = normal.

y (GE)	No.	Step*	r	RE	PC**	n	Total outlier samples removed	Significant variables***
g%1	a01	0	0.73	18.1	1	42		P, Round, Length, Width, Volume, Intensity
	a02	I	0.84	14.5	1	35	~~M07~~, A08, A09, ~~A12~~, **B21**, A37, B41	P, Round, Length, Width, Volume
	a03	I	0.94	9.1	4	35	~~M07~~, A08, A09, ~~A12~~, **B21**, A37, B41	P,Width, Round, Length, Volume
g%3	a04	0	0.39	16.5	1	42		P, TKW
	a05	I	0.56	14.5	1	40	~~M07,A12~~	P, HI, TKW
	a06	II	0.73	14.5	2	39	~~M07,A12, M16~~	P, INT, TKW
GH	a07	0	0.70	14.9	2	42		Length, Round, P, Area

* Step of outlier selection from influence plot
** Minimum value of residual validation variance
*** Variables ordered after degree of importance

With respect to GE determined after three to six years of storage, the PLSR correlation to the set of the ten physical-chemical variables is r=0.73 (one PC, RE=18.1) with a characteristic sequence of significant variables (Protein, Round, Length, Width, Volume, Intensity). Seven outliers are detected (removed in two steps, Table 1), two of which have extremely low "viability" g%3 (M07 and A12) and B21 with reduced "viability" g%3.
When the seven outliers are removed, the correlation improves to r=0.84 (one PC, RE=14.5) with r=0.94 for four PC's (RE=9.1). The pattern of importance is unchanged. There is a clear tendency for low "viability" outliers in the other correlation models given in Table 1. This is especially apparent in the "viability" g%3 GE prediction in Table 1 where the correlation coefficient is improved from r=0.39 (one PC, RE=16.5) to r=0.73 (two PC, RE=14.5) when removing three outliers which all have low "viability" (g%3). It is also concluded that the prediction of "viability" g%3 from the ten parameters has a significantly less correlation coefficient than that of "vigour" g%1.

We can thus conclude that "vigour" g%1 to a surprisingly great extent can be predicted by the set of the ten physical-chemical parameters. From this information a new hypothesis can be generated stating the physical-chemical nature of "vigour"(MUNCK & MØLLER 2004A,B).

Prediction by Near Infrared Transmission spectroscopy

It is seen that the ten physical-chemical barley parameters were able to roughly predict germination properties of a sample. Using NIT spectroscopy a physical-chemical fingerprint is likewise obtained. The ten physical-chemical manifest parameters are here expanded to 100 variables. Therefore, it is expected that NIT also will be able to predict germination.

In Table 2 a relatively high correlation of r=0.80 for "vigour" g%1 and for "viability" g%3 GC (three PC's) are seen using the first derivate of NIT spectra. As with the prediction of indirect germination variables using the ten manifest parameters in Table 1 there is a clear tendency that g%3 gives lower predictions with NIT than g%1 and that outliers have a low viability. The low vigour outliers in **y** in the NIT correlations can obviously not contribute to the prediction of germination speed or "vigour" g%1 on the basis of analysing not-germinated kernels.

Table 2. NIT (1. derivate) prediction of germination, malting data and chemical-physical data for samples of Alexis, Blenheim and Meltan. Samples with low viability (<92%) = underlined, medium viability (92-98%) = bold, high viability >98% = normal.

y (GE)	No.	Step*	r	RE	PC**	n	Outliers***
g%1 (GE)	b01	0	0.74	17.8	4	42	
	b02	I	0.77	15.7	4	41	A12
	b03	II	0.80	14.3	3	38	A12,M16,**A20,A27**
g%3 (GE)	b04	0	0.31	17.0	1	42	
	b05	I	0.68	15.7	1	39	M07,A12,M16
	b06	II	0.80	3.4	3	37	B04,M07,A10,A12,M16
GH (GE)	b07	0	0.59	24.4	4	42	
	b08	I	0.75	17.1	4	37	**B21**,A31, A37, A39, M44

* Step of outlier selection from influence plot

** Minimum value of residual validation variance

*** Total outlier samples removed from correlation

The most important variable in the prediction of "vigour" in Table 1 is Protein, followed by Round, Length, Width and Volume. Most of these parameters are obtainable with NIT with e.g. correlations of r=0.97 for protein, r=0.77 for Round and r=0.94 for HI. The rather good prediction for "vigour" from NIT measurements is therefore expected to stem from the physical-chemical properties manifest in the grains, which are essential for access of nutrients to the embryo influencing germination speed (g%1).

With the strategy of focusing on the structural factor by PLSR and identifying the physiological (viability) nature of the outliers in **y** the surprising conclusion is reached that germination speed "vigour" in this investigation has a much more pronounced structural component than physiological within the range of viability which is characteristic for malting barley (above g%3, 98%). The g%3 variable also reflects seed structure to some degree but with a much lower correlation to the structural parameters than g%1. It is therefore concluded that the structural physical-chemical factor is the main determinator for "vigour" g%1, defined as the early growth rate of the emerging plantlet in barley of malting grade. As in Table 1 with the ten physical-chemical barley parameters, NIT predictions of GE g%1 and GE g%3 are improved by removing the outliers which in NIT spectroscopy all were found to be low in "viability" g%3.

These preliminary results can be interpreted as follows (MUNCK & MØLLER 2004): Substrate availability for the germ is of importance for fast sprouting and is related to the function of how to "unlock" the complex physical and chemical structure of the food store – the endosperm. This function should also be identical with the aims of the maltster to obtain a fast malt modification (a low malt modification resistance) in dissolving cell walls and in enzyme spreading in the endosperm. Fast germination, i.e. high "vigour", should therefore be operative for the malsters as an indicator of an efficient malt modification representing the structural functional factor related to physics and chemistry. Thus, by securing a high

"viability" above g%3: 92% the structural functional factor becomes limiting in malting and brewing performance (MUNCK & MØLLER 2004). Thus "vigour" g%1 can be estimated directly "on-line" by a NIT calibration.

The outliers with low "viability" (g%3) that have been found in the models in Tables 1 and 2, are deviates in **y** (g%3) and not in **X** (NIT or the ten physical-chemical parameters). When removal of outliers determined in **X** no improvements in correlation coefficients are found. The detected outliers in **X** do not show low "viability". This indicates that neither NIT nor the ten physical-chemical parameters can be used for predictions of "viability" in unknown samples (MØLLER 2004). This is in accordance with the initial hypothesis that physical-chemical analyses should not be able to trace the physiological properties (low viability) dependent on an ungerminated embryo only contributing less than 5% of the intact barley seed. A separate method for "viability" is thus needed as a supplement to "vigour" (g%1) to remove low "viability" outliers. The germination percentage after 3 days (or more correctly 8 days germination) or the Tetrazolium embryo staining test could be used for that purpose. A complete germinative classification could thus be done within one-two hours.

Alternatively to NIT measurements the maltsters could germinate samples in 24 hours as well as determine the percentage of living grains with the fast Tetrazolium test (approximately one hour analysis time), and from here obtain a 24 hours germinative classification plot where samples will cluster according to malt quality. The g%1-g%3 germinative classification as such or based on data derived from the Tetrazolium test and/or NIT calibrations should be able to be developed to a convenient tool in classification of barley for malt quality (MUNCK & MØLLER 2004; MØLLER 2004) .

Conclusion

The advantages and possibilities for plant breeders and industry of using multivariate data analyses such as PCA for early prediction of malting barley quality (extract % and wort β-glucan mg/L) in a germinative classification plot with separate estimates for "vigour"(g%1) as abscissa and "viability" (g%3) as ordinate has thus been shown. We have in another contribution to IBGS-IX further examplified the great advantage of using multivariate analysis in plant breeding, genetics and biotechnology (MUNCK & MØLLER 2004B). The physical-chemical basis of germination speed "vigour" g%1 revealed in this paper and by MUNCK & MØLLER (2004A); MØLLER (2004) can be used to develop PLSR multivariate predictions of "vigour" by fast non destructive instrumental methods (grain image analysis /hardness or NIT spectroscopy) to be used "at-line" or "on-line".

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Genotypic and Environmental Variation of Some Chemical Compounds Related to Quality of Malting Barley

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Abstract

The improvement of malt barley quality is imperative in China for its weak competition on the markets mainly due to inferior quality, which results in a substantial decline in barley production accompanied by a rapid increase in input of both barley grain and malt. The main parameters involved in malt barley quality include malt extract, Kolbach index, diastatic power and viscosity, which are in turn dependent on the carbohydrate and protein content and their components as well as activities of the hydrolysis enzymes associated with these chemicals. It has been well documented that protein and β-glucan content, β-glucanase and β-amylase activities are the chemical compounds related closely to quality of malt barley. In the present review on the genotypic and environmental effect of these compounds and their relationships with malt quality, genetic variation of Chinese barley germplasm in these compounds and the characterization of main malt barleys planted on the different ecological locations are comprehensively illustrated. The influence of climatic and agronomic factors on these chemical compounds is discussed. Moreover, the future approaches of improving malt barley quality are proposed in reduction of β-glucan content and increase of diastatic power through enhancing the activities of both β-glucanase and β-amylase.

Keywords: barley (*Hordeum vulgare* L); environment; genotype; malt; quality

Barley is one of the major cereal grains, grown all over the world. One reason for this is its ability to grow in a wide diversity of environment. There are three distinct end-uses for barley: malting or brewing, human foods and feed for livestock. It is evident that more and more barley have been used as the materials for malting and brewing worldwide. The certain quality is required for malt barley, as indicated by malt quality, including extract, diastatic power, viscosity and Kolbach index. On the whole malt quality of present barleys is far away from the requirement by malting or brewing industries, although great effort has been done in the improvement of malt barley both in breeding and agronomy.

There are many traits affecting the quality of malt and brewing. It has been well documented that some chemical compounds in grains and their changes during malting are closely associated with malting or brewing quality. Of those, contents of protein and β-glucan, activity of β-glucanase and β-amylase is particular important. In China, there is the longest history of barley planting in the world, and its wide distribution and rich germplasm gives rise to well-known genetic diversity. Correspondingly, there are remarkable differences in ecological conditions and managements in barley production from location to location. Therefore, it is great significant to determine the situation and genetic potentials of malt barley cultivars in quality and illustrate the mechanisms by which genotypic and environmental difference of malt quality is caused. In

this paper, the genotypic and environmental variation of several chemical compounds and their relationships to malt quality were reviewed based on many relevant reports as well as results of our previous research.

1. Protein Content

The protein content in barley grains has been considered as an important trait closely associated with malt quality. According to European Brewing Association, the barley used for malting should have protein content below 11.5%. Generally, malt extract is reduced with the increase of protein content in grains, and higher protein content is also a major factor attributed to deterioration of flavor stability and formation of turbidity in beer. It is well documented that protein content in barley grains is genetically controlled, but easily affected by the environmental conditions (SMITH 1990; KACZMAREK *et al*. 1999). By planting 10 two-rowed barley cultivars at 7 locations with a wide difference in climatic conditions for two successive years, ZHANG *et al.* (2002a) found a highly significant difference among cultivars and locations, and between years in grain protein content, meanwhile the significant interaction between cultivar and environment (location or year) was detected. The largest variation was caused by environment, accounting for more than 50% of total variation, followed by the interactive effect between region and year. The variation attributed to cultivar was relatively smaller. It indicates that protein content in barley grains is mainly dependent on the environment, and thus shows a large difference for a given cultivar when it is grown under quite diverse environments (ZHANG *et al*. 2001). The analysis of the multiple regression among protein content and meteorological factors showed that protein content in barley grains was closely related to the temperature and radiation during grain-filling stage, high temperature and low humidity being favorable to the synthesis and accumulation of protein in grain (WANG *et al*. 2001).

The soil fertility and fertilization, in particular N fertilizer, have the dramatic impact on protein content in barley grains (BIRCH *et al*. 1997; EAGLES *et al*. 1995). According to an experiment conducted in Hangzhou, China, the protein content of barley cultivar Xiumai 3 was 9.86% when N fertilizer was applied at a rate of 90 kg/ha, and increased to 11.5 % when the rate of N fertilizer was doubled (180 kg/ha) (CHEN *et al*. 2001). On the other hand, with the same total amount of N fertilizer, the protein content in barley grains increased when a larger proportion of N fertilizer was top-dressed at later growth stage. In a field experiment, the total amount of N fertilizer was 140 kg/ha, and half of which was applied as basal fertilizer and the other half was top-dressed with the following application timing: (1) all applied at tillering stage; (2) all applied at booting stage; (3) each 50% applied at tillering and booting stages, respectively. The barley cultivars used in the experiment were Xiumai 3 and 92-11. The results showed that the protein contents in barley grains were 9.86%, 11.71% and 11.07% for above three treatments of N application timing, respectively. Meanwhile it was found that two cultivars had the different response to N fertilizer in terms of protein content. Hence, Xiumai3 had the protein content consistently below 11.5% in 3 N treatments, while 92-11 exceeded 11.5% in the two treatments with N fertilizer application at booting stage (ZHANG *et al*. 1998). It could be concluded that the heavy application of N fertilizer at late growth stage should be avoided in the production of malting barley, or there is a risk that that protein content in grains would be enhanced, resulting in lower malting quality. Moreover, it is also suggested that the selection and utilization of the cultivars with weak sensitivity to N fertilizer application might be an effective approach for overcoming the contradiction between high yield and quality, in light of the fact that high N

nutrition at late stage, such as booting and heading, is generally favorable to the formation of more florets and larger grain weight (BERTHOLDSSON 1998).

The uniformity in the germination rate of same lot of grains is a basic trait for malt barley with high quality. There are many inherent factors affecting germination rate. Among them, protein content is mainly involved. The difference in protein content among individual seeds could result in inconsistent germination (YIN *et al*. 2001). By examining the grains located on different positions (top, middle and bottom) of spikes, sampled from the plants with different amount of spikes in the same cultivar and 5 barley cultivars, it was showed that protein content in grains varied greatly in the their positions on spikes, and the position on which grains had the highest protein content was dependent on spike number per plant. The plants with only one spike had the highest protein content in the grains located on the top, while the plants with two and three spikes happened to on those on the middle and bottom, respectively. For all cultivars examined, the grains on the top of spikes from main shoots had the highest protein content. Protein content in grains of different positions of spikes was also affected by agronomic factors. Application of much more N fertilizer at later growth stage would caused an increased protein content in grains, being more affected in the grains on the bottom and middle than those on the top of spikes. By contrast, grain weight in the middle and bottom decreased. In addition, grain protein content, in general, increased with the increase of spike number per unit area. However, the response of protein content to spike number per unit area differed among cultivars. For instance, the largest changes in the protein content happened in the grains on the bottom of spike for 92-11, but on the top for Xiumai3. The treatments leading to decrease in the capacity of source and sink increased grain protein content [11].

There have been many reports on the influence of grain protein content on malt quality. Protein content was negatively correlated with malt extract and Kolbach index and positively correlated with diastatic power (YIN *et al*. 2003; XU *et al*. 1987; MOLINA-CANO *et al*. 1995). It could be seen that protein content has dual effects on malt quality, i.e. negative aspect by deceasing malt extract and Kolbach index and positive one by increasing diastatic power. Diastatic power is closely related to β-amylase activity. For a given cultivar, protein content is positively correlated with β-amylase activity (CHEN in press). Thus it is imperative to control protein content in grains by genetic improvement so as to improve malt quality.

2. β-Glucan Content

β-glucan, referred to (1-3, 1-4)-β-D-glucan, is one of polysaccharides, mainly present in cell wall of cereals. Barley and oat have the higher β-glucan content, compared with other cereals (NEWMAN *et al*. 1992). β-glucan is one of the important quality traits whether barley is used for malting and fodder or for human food. When barley is used for malting and brewing, β-glucan is unfavorable, because it would enhance viscosity in wort, resulting in difficulty of filtration, and consequently turbidity or deposition of beer (MORGAN *et al*. 1977; PRENTICE *et al*. 1980). Similarly, low β-glucan content is also expected when barley is used as animal feed, as it will cause the viscous state in the intestine of non-ruminant animals, leading to decreased nutrient digestibility an absorption. Therefore, β-glucan is commonly considered as an anti-nutritional factor in animals (WANG *et al*. 1992). On the contrary, β-glucan is a chemical component beneficial to human health when barley is used as food, as it shows the effective function in the decline of cholesterol and low-density blood lipid levels (BENGTSSON *et al*. 1990).

Genotypic and environmental variation of β-glucan content in barley has been extensively studied. The study of 50 six-rowed and 68 two-rowed cultivars in Finland showed that β-glucan content in two-rowed cultivars ranged 3.5%-5.3% in the south areas and 4.0%-5.2% in the middle areas. Correspondingly, β-glucan content in six-rowed cultivars ranged 2.8%-4.3%, 3.3%-5.6% and 3.6%-4.0% in the south, middle and north areas, respectively (LEHTONEN *et al*. 1987) NARASIMHALU *et al.* (1995) studied β-glucan variation of 75 barley cultivars from the eastern and western regions of Canada under 6 environmental conditions. The results showed that environment had the dramatic effect, and β-glucan content from the eastern region was significantly higher than that from the western region. The great difference also existed among cultivars. β-glucan content in 32 hull-less feed cultivars from the eastern region varied from 3.71% to 4.41% and that in 43 feed and malt cultivars from the western region varied from 3.37 to 4.42%. PEREZ-VEDRELL *et al.* (1996) determined β-glucan content of 10 two-rowed and six-rowed cultivars grown at 7 locations for 3 successive years in Spain. Highly significant differences were detected among cultivars, locations and years, with mean β-glucan content of 3.5% and the range of 1.9%-5.5%. Winter barleys had higher β-glucan content than spring barleys (PEREZ-VENDRELL *et al*. 1996). KATO (1995) reported a mean β-glucan content of 4.26% and the range of 2.86%-6.35% for 268 barley cultivars in Japan (KATO *et al*. 1995). In an earlier study, we found the large difference in β-glucan content among 85 barley genotypes (WANG *et al* 1998). After that we examined 185 barley genotypes with wide ecological difference and the results showed that β-glucan content ranged from 2.98% to 8.62%. It was found that the genotypes from Tibet and Xinjiang had remarkably high β-glucan content, and all of the genotypes with β-glucan content over 8% were limited to the accession from Tibet. On an average β-glucan content of all genotypes from winter barley region in China was 4.24%, being slightly higher than that (4.02%) of malt barleys from both Australia and Canada. β-glucan content, averaged over all Chinese genotypes planted in Hangzhou, was only 3.69%. It indicates that inferior quality of malt barley produced in Hangzhou, compared to those from Australia and Canada, could be not attributed to higher β-glucan content (ZHANG *et al*. 2002b). The multi-site experiments were carried out to make clear of the cultivar and environmental effects on β-glucan content. It was showed that there were significant differences in β-glucan content among cultivars and locations, and between years. On an average of two 2 years, Xiumai 3 and Gangpi 1 had the highest and lowest β-glucan content, respectively; and Taian and Hangzhou were the locations with the highest and lowest β-glucan content, respectively. Analysis of AMMI model showed that the effect of interaction between cultivars and environments (locations) was highly significant in two-year's experiments; however the extent of the interaction differed, depending on the cultivars. Thus, it is demonstrated that the utilization of the cultivars suitable for the local environments is also of significant in controlling of β-glucan content in barley grains (ZHANG *et al*. 2002c).

The difference in β-glucan content of a given cultivar among locations and years is definitely caused by different environmental conditions. The effect of climatic conditions on β-glucan content has been extensively studied, but no consistent results are reached up to date (NARASIMHALU *et al*. 1994; PEREZ-VENDRELL *et al*. 1996; FASTNAUGHT *et al*. 1996; WALLWORK *et al*. 1998). Analysis of multi-variant regression between climatic factors and β-glucan content demonstrated the major climatic factors affecting β-glucan content. They included total, ≥25 °C and ≥30 °C cumulative temperatures, total precipitation and raining days

during flowering to mature. High temperature and drought during grain filling stage enhanced synthesis and accumulation of β-glucan content. On the contrary, high moisture could be beneficial for the reduction of β-glucan content (ZHANG *et al*. 2001). So far, little research has been done on the effect of agronomic practices on β-glucan content. In a study, we found that β-glucan content was higher when plants were supplied with a moderate rate of N fertilizer, and less or more N supply lead to lower β-glucan content. On the other hand, heavy application of N fertilizer at booting stage caused dramatic increase of β-glucan content in grains (CHEN *et al*. 2001). β-glucan content is also affected by sowing date. In Hangzhou, The plants sown in normal season, as indicated by yield formation, had lower β-glucan content than those sown earlier or later.

There was significant difference in accumulation β-glucan during grain-filling stage among cultivars and years. However, the interaction between cultivar and year was not significant. Accumulating dynamics of β-glucan content during grain-filling stage was basically similar for different cultivars in the same year, but varied in experimental years. The accumulating pattern was, to great extent determined by temperature during grain-filling stage. With the increase of temperature, the maximum rate of accumulation appeared earlier and the duration was shorten, as a result, final β-glucan content was reduced (CHEN *et al*. 2002). Remarkable difference was found in β-glucan contents among grains in the different positions of the spike, with grains in the middle being the highest. In addition, the plants with only one spike had significantly higher β-glucan content and larger difference among grains within a spike than those with more spikes. Although there was no significant difference between the spikes derived from main shoots and tillers, but the difference among grains within a spike was much larger in main-shoot spikes than in tiller ones (ZHANG *et al*. 2002d).

Advance in study of genetics, breeding, physiology and ecology on β-glucan is closely related to the improvement of methodology in the measurement of β-glucan content. Before the middle of 80's in last century, the slow advance in these fields was completely due to the deficiency in accurate and repeatable measurements. After that, chromatographic, fluorometric and enzymatic methods come to birth successively and soon are widely accepted worldwide. These methods have proved plausible in determination of β-glucan content, but obvious disadvantage exists still for each of them. Both chromatographic fluorometric methods are characterized by the complex procedures, and cost much time and money. Comparatively, enzymatic method, which is now being most commonly used, is relatively simple and faster. However, the price of assay kit appears to be a bit high if it is used in routine breeding program, in particular for the laboratories in developing countries. Therefore, rapid, economic and accurate method has been to be developed. We analyzed more than 500 samples with a near infrared reflectance analyzer and by comparing the results with those obtained in enzymatic method (assay kit), established a working curve for further measurement of barley samples. The highly significant correlation between the values of β-glucan content determined by these two methods indicates that near infrared reflectance could be used in the determination of barley β-glucan content, at least for initial screening in early generations of breeding.

3. β-Glucanase Activity

Prior to brewing, barley should be malted. During malting, decomposition and release of starch, a major component in barley grains is involved in a series of hydrolytic enzymes. Starch is mainly present in endosperm cells, so cell wall becomes the barrier to entrance of hydrolytic

enzymes. As endosperm cell wall is principally composed of β-glucan, rapid and complete degradation of β-glucan is quite important in malting (JENSEN *et al*. 1996). The degradation is mediated by β-glucanase (EC3.2.1.73).

β-glucanase, referred as to (1-3, 1-4)-β-D-glucan 4-β-glucanase is concentrated in endosperm and aleurone layer, and also present in scutellum and embryo with very little amount. The enzyme mainly reacts to β-1,4-bond and its substrate is non-reducing end of β-1, 3 chains and reducing end of β-1, 4 chains. The reactive product is 3(O)-β-cellobiose-D-glucan and 3(O)-β-cellotriose-D-glucan. At present, two kinds of β-glucanases have been found in barley, i.e. isozyme EI and EII. The both isozymes consist of 306 amino acids, and have 92% homology in nucleotide and amino acid levels. DOAN *et al.* (1988) reported that isoelectric point and molecular weight were 8.5 and 30 KD for isozyme EI, and 10.6 and 32 KD for isozyme EII, respectively DOAN *et al*. 1988). Several methods in determination of β-glucanase activity have been developed, including fluorometric, high efficiency liquid chromatography and enzymatic methods. Among them, enzymatic method is being most widely used at present.

There have been some reports about the genotypic and environmental variation of β-glucanase activity. Henry (1989) reported that β-glucanase activity in 41 barley cultivars of Australia varied from 148 U/Kg to 766 U/Kg during germination (HENRY *et al*. 1989). HAN (1995) analyzed 150 DH populations and found that malt β-glucanase activity ranged from 541 U/Kg to 1116 U/Kg, with a mean of 842 U/Kg (HAN *et al*. 1995). ELLIS (1997) studied variation of β-glucanase activity of two cultivars planted in Scotland and Spain for two successive years. The result showed that the grains from Spain had substantially higher β-glucanase activity than those from Scotland during germination and the effect of genotype and environment was highly significant (ELLIS *et al*. 1997). KNUCKLES *et al* (1999) studied the variation of β-glucanase activity in grains and malt of 10 cultivars in America and found that the range in grains was 52-65 U/Kg, being significantly lower than that in the malt (390 U/Kg) (KNUCKLES *et al*. 1999). MACGREGOR *et al.* (1994) studied the change of β-glucanase activity under controlled environment and showed that drought stress during grain filling period increased β-glucanase activity (MACGREGO *et al*. 1994). Analysis of β-glucanase activity in grains and malt of 8 cultivars planted at 7 different sites of China (Zhengzhou, Yancheng, Taian, Jingzhou, Nanchong, Putian and Hangzhou) showed that β-glucanase activity in mature grains was very low, although significant difference was found among sites, ranging from 38.74 U/kg (Zhengzhou) to 57.24 U/kg (Hangzhou). In contrast, there was no significant difference among cultivars. After malting, β-glucanase activity increased considerably and there was significant difference among cultivars and sites. In addition, it was demonstrated that β-glucan content in malt was positively correlated to that in grains, but more dependent on β-glucanase activity in malt. During malting, about 80% of β-glucan was degraded. It may be suggested that enhancement of β-glucanase activity in malt is great significant for improving malt quality (WANG *et al*. 2003). β-glucanase activity in malt was significantly and positively correlated to that in grains, so it seems possible to predict β-glucanase activity in malt by measuring that in grains in early breeding generation, so that the difficult in malting due to few grains or high cost in malting could be, to the great extent overcome.

Timing of N fertilizer application has remarkable effect on β-glucanase activity. It was showed that β-glucanase activity in malt was significantly higher in the treatment (N3), in which all top-dressing N was applied at booting stage, than in the treatment (N2), in which top-dressing

N was applied in the equal amount at two-leaf and booting stages respectively, and in the treatment (N1), in which that all top-dressing N was applied at two-leaf stage. Timing of N fertilizer application also affected malt quality. Malt extract and Kolbach index was significantly lower in the N3 treatment than in the N1 and N2 treatments, but diastatic power showed the opposite trend. Correlation analysis showed that β-glucanase activity in malt was positively correlated with malt extract, Kolbach index and diastatic power, and negatively correlated with wort viscosity and β-glucan content. The variation of β-glucanase activity in malt attributed to the environment was much larger than that caused by genotype (CHEN in press). Highly significant differences were noted among cultivars and environments (locations) for all malt quality traits (diastatic power, wort viscosity, Kolbach index and malt extract), except diastatic power. In terms of variation coefficient, the difference among locations sites was greater than that among cultivars for each quality trait mentioned above. Of these, diastatic power and Kolbach was most pronounced. It could be seen that environment (locations) is predominant factor affecting malt quality.

β-glucanase activity was generally increased with increasing N nutrition and showed a positive correlation with protein content in grains for a given cultivar. However, no significant correlation was found between β-glucanase activity and protein content when different cultivars was used in the study. So the genotypic and environmental variation in protein components should be studied for comprehensively understanding of the mechanisms in the cultivar and environmental variation of malt quality and identifying the relevant protein components or molecular markers closely related to malt quality for the practical use in malting barley breeding.

4. β-Amylase Activity

In beer industry, diastatic power is an important trait associated with malt quality. It is generally considered as the capacity of all hydrolases in starch degradation and its level is mainly dominated by β-amylase (ARENDS *et al*. 1995; GIBSON *et al*. 1995; EVANS *et al*. 1995). β-amylase (EC 3.2.1.2), refereed as to α-1, 4-glucan malt hydrolase, is a exo-hydrolase which releases β-maltose from the non-reducing ends of α-1, 4-linked poly- and oligoglucans. The enzyme is synthesized and accumulated during seed maturation and no longer synthesized during germination. In the mature barley seed, β-amylase exists in the two forms: free and bound, with the latter being predominant (DUNN. 1974). The total amount of β-amylase in barley grain measures up to 1% of total grain protein content (LAURIERE *et al*. 1986). Although many methods of measuring β-amylase are available (HEJGAARD *et al*. 1980; HARA-NISHIMURA *et al*. 1986; SANTOS *et al*. 1996), the most commonly used is that developed by McCLEARY *et al.* (1989), and now the special assay kit has been produced by Megazyme International Company (Ireland).

There are considerable differences among cultivars and environments in β-amylase activity (SWANSTON 1980; AHOKAS *et al*. 1990). Arends *et al* (1995) reported that the variation among 11 cultivars ranged from 501 U/g to 1100 U/g and among 4 sites from 389 U/g-1290 U/g, with environmental effect being larger than genotypic effect (AREND *et al*. 1995). GEORG-KRAEMER (2001) examined β-amylase activity of 10 Brazilian barley cultivars and found that there was significant difference among cultivars, ranging from 716.72 U/g to 1470.55 U/g (GEORG-KRAEMER *et al*. 2001). MCNICOL *et al.* (1993) studied the changes of β-amylase activity under the controlled environments and found that water stress in the middle of the grain

filling stage enhanced accumulation of β-amylase, while heat stress had little effect (MACNICOL *et al*. 1993).

In a study, we measured β-amylase activity of 56 malting barley cultivars collected from China, Australia and Canada and planted in Hangzhou. The results showed that the range of β-amylase activity for all cultivars was 458 U/g-1024 U/g, with mean of is 738 U/g. Moreover, as a whole, the cultivars from Australia and Canada had apparently higher β-amylase activity than those from China. There was no significant correlation between β-amylase activity and protein content. In fact, some cultivars, such as Ganda, Harrington and Stein were found containing high β-amylase activity but low protein content (WANG *et al*. 2003). In the experiment of 8 cultivars planted at 4 locations in Zhejiang, China, the variation β-amylase activity ranged from 608 U/g (ZAU 6) to 843 U/g (92-11) among cultivars, and from 710 U/g (Jiaxing) to 813 U/g (Yuyao) among locations. The variation of β-amylase activity over the locations, as indicated by coefficient of variation, differed considerably among cultivars. For instance, Xiumai3 has much less variation than ZAU 6. β-amylase activity also varies in the positions of grains in spike and the difference among various positions of spike is dependent on cultivar. For an example, 92-11 showed β-amylase activity in the order: top>middle>bottom and the differences between them were significant, while Xiumai 3 was top>bottom>middle and the difference between bottom and top was relatively small. Some agronomic factors have substantial effect on β-amylase activity. In Hangzhou, the plants sown at suitable dates in terms of yield formation had higher β-amylase activity than those sown earlier or later. Timing of N fertilizer application had considerable influence on β-amylase activity, the activity being enhanced with the increase of N fertilizer applied at booting stage. The enhancement was more pronounced for grains in the middle and bottom of the spike than those in the top, thus leading to the reduced difference between positions within a spike (YIN *et al*. 2002).

Because diastatic power is mainly associated with β-amylase activity, improvement of β-amylase activity should cause substantial increase of diastatic power. However, it is commonly considered that β-amylase activity was significantly and positively correlated with N or protein content in grains (AREND *et al*. 1995; SANTOS *et al*. 1996). In an experiment, we also found that N fertilizer increased protein content and β-amylase activity simultaneously. It is implied that the method of N fertilizer application has a considerable influence on quality of malt barley. On the other hand, as stated previously, no significant correlation was detected between β-amylase activity and protein content among different cultivars planted in the same environment. Therefore it seems possible to develop a cultivar with high β-amylase activity and low protein content through breeding. Similar to the study on β-glucanase activity, further research should be focused on the protein components closely associated with β-amylase to make clear of genotypic environmental variation of these components and their impact on expression of β-amylase activity and to find biochemical or molecular markers for use in malt barley breeding.

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Transgenic Barley in Brewing - Detection of Transgene and Heterologous Protein Levels

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Abstract

Genetically modified (GM) raw materials and products have appeared on the market. The aim was to find out through a model case whether the threshold of 1 % of GM contamination in foods is detectable using the present analytical methods. Transgenic barley lines were homozygotized through microspore culture for production of doubled haploid plants. The transgenic and non-transgenic barley samples were malted, mashed and fermented. The produced malts, worts and beers were analyzed in respect to the transgene and the corresponding heterologous protein. The heterologous protein was not detectable from protein extracts of malted grains. However, presence of the heterologous enzyme in the aleurone layer and scutellum of the transgenic malt was verified by immunomicroscopy. This suggested that the heterologous protein was expressed in germinating transgenic seeds. Real-time PCR proved to be repeatable and extremely sensitive for the detection of the transgene. The threshold of 1 part in 1000 (0.1%), nicely in the range of the legal requirements, was obtainable with high confidence. Detectable foreign DNA was also obtainable from wort, but repeated trials of DNA extraction from beer were not successful.

Keywords: genetically modified (GM); transgenic; doubled haploid (DH); barley; malting; brewing; real-time PCR; immunomicroscopy

Introduction

Genetically modified (GM) raw materials and products have appeared on the market. The global area for GM crop plants was approximately 40 million hectares in 2000 (Braun, 2001) and 68 million hectares in 2003 (JAMES 2003). In Europe, the GM foods are governed by the Novel Foods Regulations (EC No. 258/97, 1139/98, 49/2000, 1829/2003, 1830/2003). In addition GM additives and GM flavouring have to be labelled according to EC Regulation No. 50/2000. In the beginning of this research, in 2001, the Novel Foods Regulation included the need for labelling if the modified food differed from the conventional product. Basically, this was construed that if a product contained GM material it needed to be stated in labelling. Thus, standardized methods were required to guarantee an efficient surveillance. At that time, the labelling threshold for presence of GM material adopted by the European Commission was 1 %. In the end of 2002 a new draft law and in 2003 the EC regulation 1830/2003 concerning the tracability and labelling of genetically modified organisms were approved. The draft law will add to the current rules the labelling of all food produced from GM organisms irrespective of whether there is DNA or protein of GM origin in the final product. In addition, all genetically modified feed need to be labelled. For example, glucose syrup produced from starch of genetically modified maize needs to be labelled according to the draft law. The threshold was agreed to be no higher than 0.9 %. Under current legislation, there is no tolerance threshold for the adventitous presence of GM material in food or feed. It has

become a reality, that minute traces of GM organisms in conventional food and feed can arise during cultivation, harvest, transport and processing. The tolerance threshold was decided to be set at 0.5 %. (For details see: www.europa.eu.int/, DN: PRES/02/359, IP/02/1770, IP/03/1056).
In general, marker genes are utilized for the production of transgenic plants. Often markers are followed only through the few first generations, the real interest being in the application genes. This restricts the use of marker genes in surveillance of foods and food raw-materials. The objective of the research reported here was through a model case to find out whether the threshold of detection was obtainable with the present analytical methods. Transgenic barley carrying a herbicide resistance gene (*bar*) as a transformation marker and a fungal enzyme encoding gene (*egl1*) as an application gene was used as experimental material. Our aim was to determine if the heterologous DNA, protein or other modifications caused in the plant material by the transferred genes could be used for detection of GM contamination in barley grain, malt, wort or beer.

Material and Methods
Genetically modified barley carrying the *egl1* gene coding for the fungal thermotolerant endo-1,4-β-glucanase under control of a high-pI α-amylase promoter was used as research material (NUUTILA *et al*. 1999). Under control of this promoter, the *egl1* gene should be expressed during germination mainly in the aleurone layer of the seed and the heterologous protein should be present in the malt. The transgenic barley material was homozygotized through microspore culture according to Ritala and coworkers (2001) and propagated in greenhouse conditions. Efficient extraction methods for DNA and proteins from malt, wort and beer were developed. The PCR parameters were optimized and the sensitivity of Western blot hybridization was checked.

Transgenic and non-transgenic barley were malted, mashed and the resulting worts were fermented to beers. Transgenic and non-transgenic malts, worts and beers were mixed in ratios of 100:0, 50:50, 10:90, 1:99 and 0:100. Each malt, wort, and beer mixture was analyzed in respect to the presence of the transgene and the heterologous protein. The presence of transgene was analyzed by PCR, both modifications "End point" and "Real time" were utilized. The heterologous protein was detected by Western blot analysis and by combined IEF / activity assay and its activity was evaluated (NUUTILA *et al.* 1999). Furthermore, the heterologous protein was localized *in situ* in the malt samples by immunomicroscopy (Marttila et al. 1996). The modification of malt by the transgenic enzyme was evaluated by scanning electron microscopy (SEM; WALLWORK *et al.* 1998). A sensory analysis of the produced beers was performed by a trained panel of 10 assessors. A schematic presentation of the experimental plan is given in Figure 1.

Results and Discussion
Production and Screening of Transgenic Doubled Haploid (DH) Plants
Transgenic barley carrying the *egl1* gene coding for the fungal thermotolerant endo-1,4-β-glucanase under control of the high-pI α-amylase promoter was used as research material (NUUTILA *et al*. 1999). Under control of this promoter, the *egl1* gene was expressed during germination. Transgenic barley lines (43, 46 and 417) were propagated in greenhouse conditions. From the transgenic plants, spikes were collected for homozygotization through microspore culture. The microspore culture and regeneration of plants were performed according to Ritala *et al.* (2001). After rooting the plantlets were treated with colchicine in order to double the genome and to produce doubled haploid (DH) plants. The presence of transgene was verified by PCR according to NUUTILA *et al.* (1999) (Table1, Figure 2).

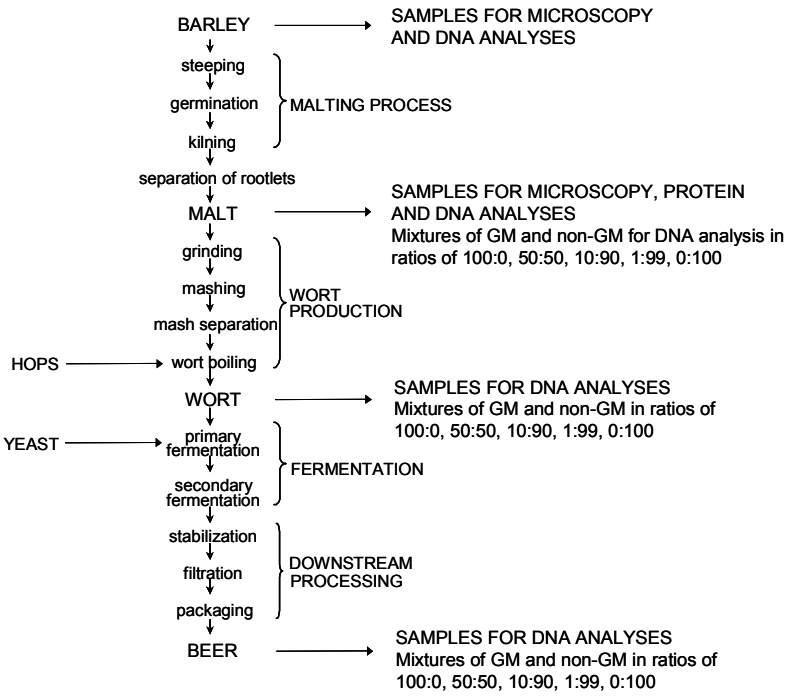

Figure 1. A schematic presentation of the experimental set-up

The microspore culture resulted in 927 green regenerants of which 476 were transferred to soil mix after rooting and colchicine treatment. Of these 476 plants, 380 were transgenic, 358 reached maturity and 350 were fertile with normal seed set. The transgenic DH plants were grown to maturity in greenhouse and the seeds were harvested. A yield of 6.9 kg for the transgenic seed was obtained. These transgenic DH seeds were used in malting, mashing and brewing experiments. Non-transgenic barley (c.v. Golden Promise) was also propagated in greenhouse conditions and used as control.

Malting, Mashing and Brewing of Transgenic Materials

The behaviour of non-transgenic- and transgenic barley samples in malting, mashing and brewing were similar. No major differences were seen in the structure of transgenic and non-transgenic material by SEM. However, dry transgenic malt was more friable than non-transgenic malt which may refer to more efficient modification of the endosperm. In addition, no clear differences in β-glucanase activities or β-glucan contents were observed. The overall rating in sensory analysis of beers produced from transgenic and non-transgenic materials ranged from fairly good to poor, which is typical for beer produced in small scale. The quality of beers produced from greenhouse grown barleys suffered mainly from a strong buttery aroma. This was however, caused by the insufficient secondary fermentation, which could not be controlled due to the small batch size.

Table 1. Summary of produced transgenic doubled haploid barley plants

Line	Starting material spikes / Mother plant	Greens	Albinos	Greens in regenerants (%)	Greens in soil	PCR +	PCR + (%)	Mature plants in GH	Fertile plants in GH	Fertility (%)	Yield (kg)
43	30	17	18	49	7	7	100	7	7	100	
	22	2	3	40	1	1	100	1	1	100	
	12	7	11	39	8	5	63	5	5	100	
	31,5	101	67	60	65	35	54	34	32	94	
	42,5	48	28	63	25	23	92	20	18	90	
Total	**138**	**175**	**127**	**58**	**106**	**71**	**67**	**67**	**63**	**94**	**1.2**
46	36	29	13	69	14	14	100	13	13	100	
	37,5	3	15	17	0	0	-	0	0	-	
	19	4	7	36	3	2	67	2	2	100	
	9,5	0	0	0	0	0	-	0	0	-	
	11,5	0	0	0	0	0	-	0	0	-	
Total	**113,5**	**36**	**35**	**51**	**17**	**16**	**94**	**15**	**15**	**100**	**0.3**
417	44	21	11	66	6	6	100	5	4	80	
	7	0	0	0	0	0	-	0	0	-	
	31,5	263	128	67	142	111	78	111	111	100	
	44	118	26	82	91	62	68	57	56	98	
	22	2	0	100	1	1	100	1	1	100	
	33,5	312	31	91	113	113	100	102	100	98	
Total	**182**	**716**	**196**	**79**	**353**	**293**	**83**	**276**	**272**	**99**	**5.4**
ALL	**433,5**	**927**	**358**	**72**	**476**	**380**	**80**	**358**	**350**	**98**	**6.9**

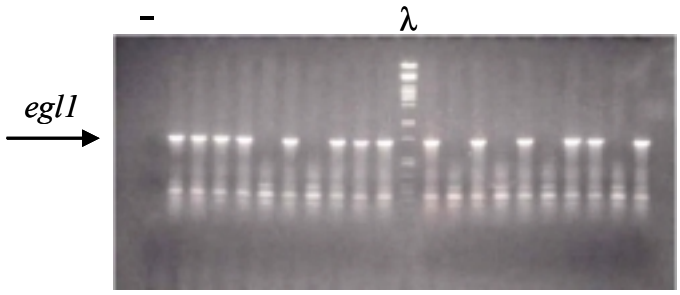

Figure 2. The doubled haploid plants were screened by PCR (according to NUUTILA *et al*. 1999) in order to confirm the presence of transgene (pointed by arrow in the figure). The lane (-) represents negative non-transgenic control and λ a molecular weight marker.

Detection of Heterologous Protein Levels

The detection of the heterologous protein from the malted transgenic grains by Western blot analysis and by combined IEF / activity (according to NUUTILA *et al.* 1999) was not successful. Thus, the homozygous transgenic seeds were germinated *in vitro* at +10°C for 7, 12, 14 days, but the heterologous protein was still not detectable at any of the time points. This meant that either the expression was below the detection level or that the homozygotization of the transgenic lines had resulted in gene silencing. The possibility of gene silencing was not studied further.

Immunomicroscopy tests were performed on paraffin sections of transgenic grains and malt. A polyclonal β-glucanase antibody was used, specificity of which had been tested by immunoblotting. High concentration of this antibody raised labelling in the non-transgenic seeds as well. This meant that the antibody was able to recognize endogenous glucanases. Probably this was due to different conformations of the target proteins in the immunoblotting and *in situ* detection. However, with a properly diluted antibody the labelling of endogenous

glucanases could be reduced so that the true labelling for the heterologous glucanase could be seen. Sections treated with a non-immune serum instead of the glucanase antibody gave no signal. In transgenic malt, the signal for the heterologous glucanase was mainly localized in the aleurone layer (Figure 3) with a minor signal in the scutellum, indicating synthesis of the enzyme in these tissues. On longitudinal grain sections, a gradual expression was seen in the aleurone layer, the label being weak or absent in the distal part of the malted grain. No signal was observed in the starchy endosperm. These results gave evidence that the heterologous enzyme was indeed expressed in transgenic seeds and was not silenced by the plant. Furthermore, the expression pattern was according to the known specificity of the promoter used to control the transgene (JACOBSEN *et al.* 1986).

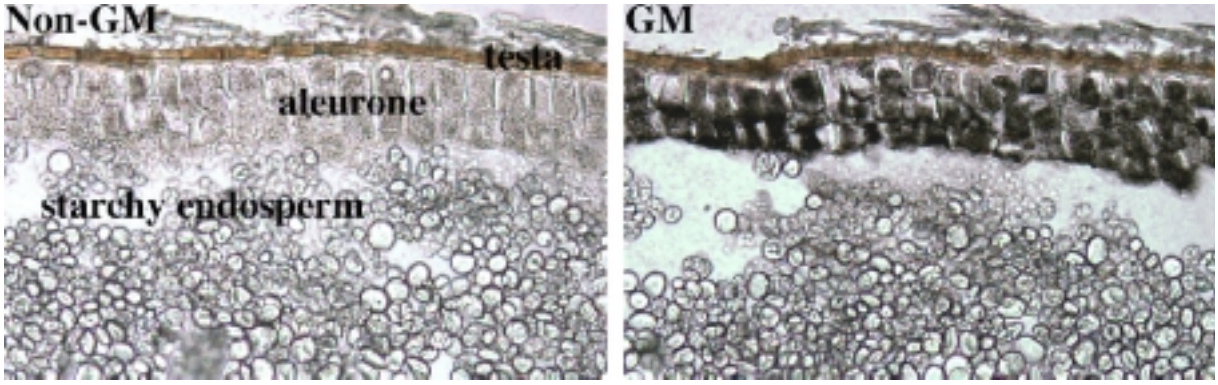

Figure 3. Localization of the heterologous endo-β-glucanase in the aleurone layer of non-transgenic malt (non-GM) and transgenic malt (GM). The dark brown colour in the aleurone layer of the transgenic malt revealed the location of the heterologous protein. Starchy endosperm appeared similar in both samples. Testa shows a natural golden colour due to phenolic compounds.

Tracking the Transgene

DNA analysis by real-time PCR proved to be repeatable and extremely sensitive for detection of transgenic material. A threshold of 1 part in 1000 (0.1%) which was in the range of the legal requirements in EU was obtainable with high confidence (Figure 4). Sample preparation requirements for real-time PCR were robust, since the DNA did not need to be of high molecular weight. Our experience showed that detectable DNA was obtainable from grains, malt and wort, but repeated trials of DNA extraction from beer were not successful. Quantitative PCR was shown to be the most reliable method for tracking GM-material.

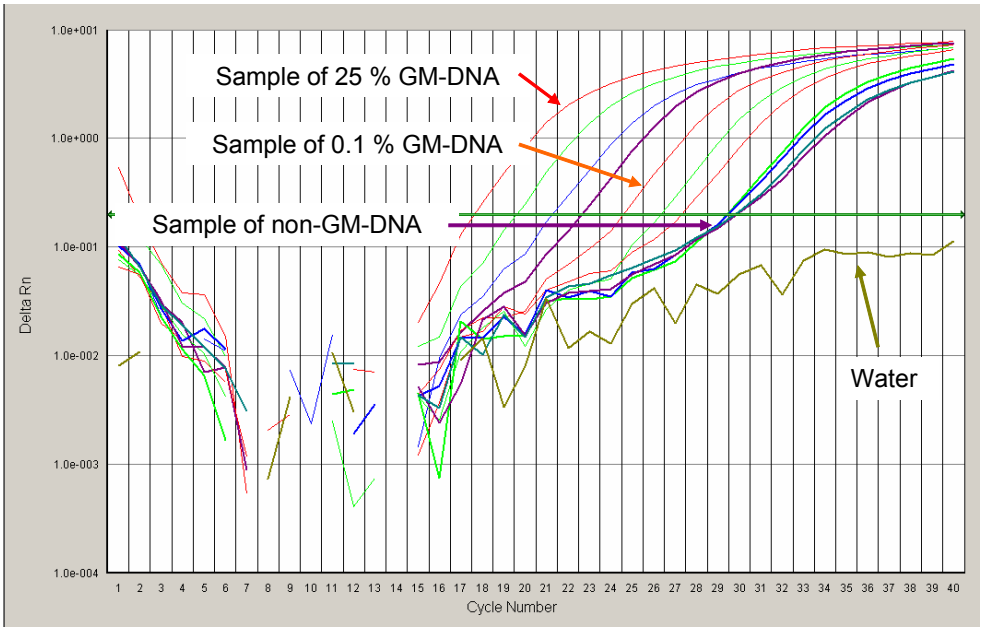

Figure 4. DNA analysis carried out by real-time PCR. GM-samples were diluted with non-GM DNA in order to determine at which level of dilution detection was still possible.

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http://europa.eu.int/comm/food/food/biotechnology/index_en.htm

The Genetic Basis of Improved Beer Haze Stability

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Abstract
The formation of permanent haze in beer is a serious quality problem, which limits the storage life of the product. From silica gel, used for beer colloidal stabilisation, a silica eluent (SE) protein fraction was isolated and an antibody raised against this fraction. SDS-PAGE immunoblot analysis using the SE antiserum detected a range of protein bands in barley, malt, beer and haze. Interestingly, a polymorphism was observed in some barley varieties which contained a ~12 kDa band (SE +ve) while in other varieties this band was absent (SE -ve). The genetic basis for the presence/absence of the SE protein was determined by QTL analysis which found that the ~12 kDa band mapped to the short arm of chromosome 3H. Two-dimensional gel electrophoresis and sequence analysis was used to identify the SE protein as the barley trypsin inhibitor CMe precursor (BTI-CMe). This protein has a molecular weight of 13.3 kDa and the functional gene is located on chromosome 3H, consistent with information available on the SE protein. Pilot brewing trials found that beer brewed from varieties that did not contain the ~12kDa SE band, "SE -ve varieties", developed less haze in haze force testing trials than beer produced from SE +ve varieties. These results demonstrate that the selection of SE -ve varieties will provide an opportunity to improve the colloidal stability of beer.
Keywords: malt; barley trypsin inhibitor; brewing; haze

Introduction

In bright beers, the formation of haze is a serious quality problem, which places limitations on the products storage life. Beer contains a number of barley proteins that are modified both chemically and proteolytically during the malting and brewing processes, which can influence haze stability. Haze active proteins isolated from beer have been found to be derived primarily from barley (*Hordeum vulgare* L.) storage proteins (hordeins). These proteins are comprised of fragments of several different molecular weights, and are relatively rich in the amino acid proline (ASANO *et al.* 1982).

During the malting and brewing processes, the proteinases of barley and malt partially hydrolyze the hordeins (JONES & MARINAC 2002). In particular, protein degradation in barley begins with the insoluble storage proteins being hydrolysed by the endopeptidases (proteases) that then generate 'soluble protein' and peptides, upon which the exopeptidases (carboxypeptidases) act to release amino acids (BAMFORTH & BARCLAY 1993). The solubilization of these proteins in the malt, can affect many aspects of beer made from these worts, including their haziness. Forty-two proteases have been isolated from malted barley (ZHANG & JONES 1995) and they are representative of all four protease classes (aspartic, cysteine, metallo- and serine). Serine proteinase inhibitors present in barley include; the chymotrypsin/subtilisin inhibitors 1 and 2 (CI – 1 and 2), a bifunctional barley α-amylase/subtilisin inhibitor (BASI), a barley Bowman-Birk type trypsin inhibitor (BBBI), trypsin α-amylase inhibitors (Chloroform/methanol – soluble proteins (CM proteins)) and

serpins (protein z) (SHEWRY 1999; CARBONERO & GARCÍA-OLMEDO 1999). The CM proteins belong to the trypsin/a-amylase inhibitor family. Within the barley endosperm, one of the most abundant members of this family is the 13-kDa monomer CMe. A barley trypsin inhibitor (BTI), which was initially sequenced by ODANI *et al.* (1983) has been subsequently shown to be identical to protein CMe (SALCEDO *et al.* 1984; LAZARO *et al.* 1985), and the corresponding gene for BTI-CMe has been shown to be located on chromosome 3H (HEJGAARD *et al.* 1984; SALCEDO *et al.* 1984).

In this paper, we report the identification, characterisation, mapping and function of a protein in barley that can influence the haze stability of beer.

Material and Methods

Barley and Malt Samples

Barley (*Hordeum vulgare* L. cv.) seed was obtained from the 2000 Australian Winter Cereals Collection, Tamworth (Australia); the 2000 University of Adelaide growing seasons breeding experiments conducted at Charlick, Port Wakefield, Tuckey; the 1998 season experiments at Yeelanna and the 1997 season experiments at Brinkworth. Samples were also obtained from Dr. Silja Home (VTT Biotechnology, Finland) and Dr. Berne Jones (WI, U.S.A.). Commercial malt samples were obtained from Joe White Maltings Ltd, Kirin Australia, Barrett Burston Malting Company, International Malting Company, Coors Brewing Company and Carlsberg Breweries.

Protein Extraction, SDS-PAGE and Immunoblotting

Immunoblotting and SDS-PAGE were carried out essentially as described by EVANS *et al.* (2003). Briefly, ground barley and malt samples (20 mg) were extracted or diluted into SDS-PAGE sample buffer (5M Urea, 4% SDS, Tris buffer pH 8.0) and 1% (v/v) 2-mercapto-ethanol. The extracted protein was separated by SDS-PAGE (15% gels), using a Mini-Protean II electrophoresis unit (Bio-Rad, Richmond, CA, U.S.A.). The separated proteins were transferred to nitrocellulose (100V, 1h) (0.2 mm pore size; Schleicher and Schuell, NH, U.S.A.) and incubated overnight with the polyclonal anti-SE antibody (EVANS *et al.* 2003). (1/1000 dilution). Antibody binding was detected using a goat anti-rabbit antibody, horse radish peroxidase conjugate (1/5000 dilution) (Bio-Rad) and the immunoblots were developed using 4-chloro-1-naphthol (Bio-Rad) as the substrate.

Mapping of SE Trait

The SE polymorphism was screened by immunoblot in 92 lines from the Chebec x Harrington (KRETSCHMER *et al.* 1997) mapping population and 84 lines from the Galleon (SE +ve) x Haruna nijo (SE -ve) (KARAKOUSIS *et al.* 2003) mapping populations.

Pilot Brewing Trials

Pilot brewing trials were conducted using SE +ve and SE –ve malts at the Lion Nathan brewery (Sydney) (50 L) scale (EVANS *et al.* 2003) and at VTT Biotechnology (Finland) (10 L and 100 L) scale (ROBINSON *et al.* 2004). The colloidal stability of beer produced was analysed in triplicate using a (5 day 55°C, 1 day 0°C) force test procedure. Haze measurements were recorded (EBC FU) using a Hach 2100N Turbidimeter (Hach Loveland, CO, U.S.A) (50 L) or using a HZ-013 Lg – automatic aps (Denmark) (10 L and 100 L).

Characterisation of the SE Protein

Samples for 2-D electrophoresis were extracted in 1M Urea/1% (v/v) 2-mecapto-ethanol. First-dimension separation was achieved using IPG strips, Immobiline DryStrip gels

(Amersham Biosciences) (3-10 non-linear 18 cm). The second dimension was resolved by SDS-PAGE (15% gels) (LAEMMLI 1970). For in-gel digestion, gels were stained overnight with a 0.05% G-250 colloidal coomassie stain, modified from (NEUHOFF 1988). The appropriate spots were excised from the gel, washed, digested with trypsin and eluted according to HELLMAN *et al.* (1995). The eluted peptides were separated by reversed phase HPLC (Hewlett-Packard (1090 LC), Rockville, IL, USA). Separations were achieved using a 2.1 x 250 mm Vydac C18 protein column (Vydac Separations Group, Hesperia, CA). Eluent A was 0.05% TFA, eluent B was 0.04% TFA in acetonitrile, flow rate of 0.2 mL min-1. Fractions were collected manually for sequencing. N-terminal sequencing using automated Edman chemistry was performed using a Hewlett-Packard G1000A protein sequencer. (Hewlett-Packard, Rockville, IL, USA). Sequence analysis and comparison of results was completed using the NCBI (http://www.ncbi.nlm.nih.gov) and SWISSPROT/TrEMBL protein databases (http://www.au.expasy.org/sprot). Sequences were aligned using the ClustalW program (http://www.ebi.ac.uk/clustalw/).

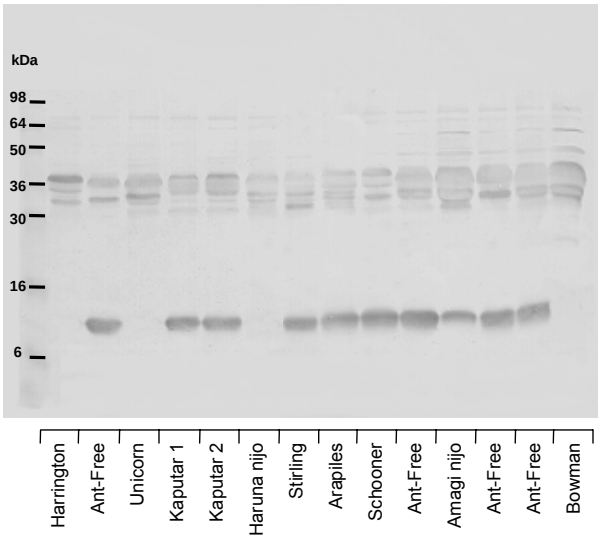
Results and Discussion

Immunodetection of SE Polymorphism

In bright beers, the formation of haze is a serious quality problem, which places limitations on the storage life of the product. The hordein polypeptides which are present in both barley and malt have been well characterised (SHEWRY 1993), but to date the identities and roles of these hordein-derived polypeptides found in beer have not been characterised. This study investigated a polymorphism which was observed in some barley varieties which contained a MW ~12000 band (SE +ve), while in other varieties this band was absent (SE -ve).

From silica gel, used for the colloidal stabilisation of beer, a silica eluent (SE) protein fraction was isolated and an antibody raised against this fraction (EVANS *et al.* 2003). One-dimensional SDS-PAGE immunoblot analysis using the SE antiserum detected a range of protein bands in barley, malt, beer and haze samples. A polymorphism was observed in some barley varieties which contained a MW ~12000 band (SE +ve), while in other varieties this band was absent (SE -ve) (Figure 1).

Figure 1. Immunoblot analysis of the SE trait.



One hundred and eighty eight Australian and international barley varieties, including a comprehensive selection of current and past malting varieties were screened. One hundred and sixty two varieties were identified as SE +ve, twenty-six were identified as SE –ve (data not shown).

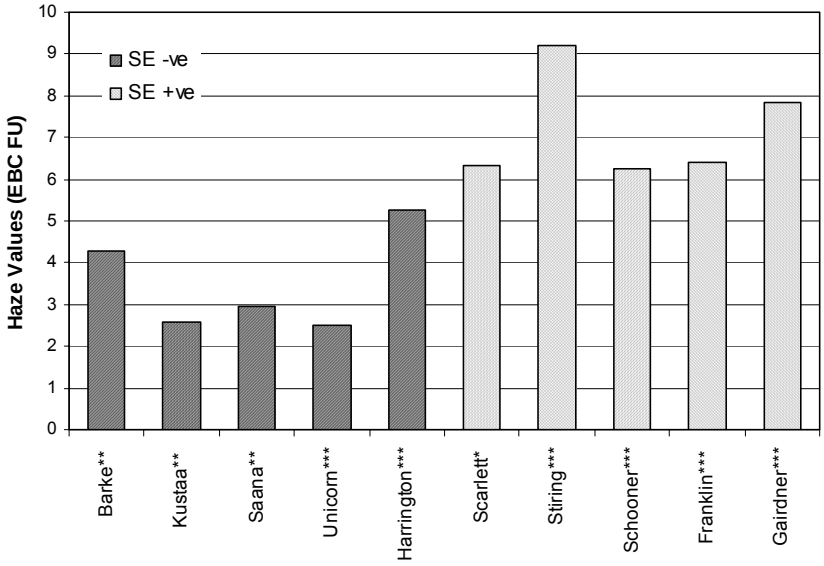
Mapping of the SE Trait

The SE polymorphism was screened by immunoblot in 92 lines from the Chebec (SE +ve) x Harrington (SE -ve) (KRETSCHMER *et al.* 1997) and 84 lines from the Galleon (SE +ve) x Haruna nijo (SE -ve) (KARAKOUSIS *et al.* 2003) mapping populations. The genetic basis for the presence/absence of the SE protein was determined by QTL analysis which found that the ~12 kDa band mapped to the short arm of chromosome 3H (data not shown).

Brewing Trials

Pilot brewing trials found that beer which was brewed from SE –ve malt varieties, formed less haze in haze force testing trials (5 days at 55˚C, 1 day at 1˚C) than beer which was brewed using SE +ve malt varieties (Figure 2) (ROBINSON *et al.* 2004 and EVANS *et al.* 2003).

Figure 2. The colloidal stability of beer produced from pilot brewing trials (10 L*, 100 L** and 50 L***) using a 5 day 55°C, 1 day at 0°C chill haze force protocol.



Characterisation of the SE Protein

The SE protein was characterised using comparative two-dimensional (2-D) gel electrophoresis immunoblots of barley extracts from SE +ve and SE -ve varieties. The protein spot identified by this investigation was excised and its partial sequence determined, after in-gel cleavage of the protein spot using trypsin and the separation of the resulting fragments by reversed-phase HPLC (ROBINSON *et al*. 2003). Tryptic digestions of the SE –ve/SE +ve preparations produced at least 12 peptide fractions, which were resolved by reversed-phase HPLC. Three of these fractions were selected for N-terminal sequencing. A SE +ve fraction was sequenced for 25 cycles and the following sequence was obtained; QTSYAANLVTPQECNLGTIHGSAY, a second SE +ve fraction was also obtained; FGDSCAPGDALPH, along with the following SE –ve fraction; QTSYAANLVT-QE-NL--. Results of non-redundant BLAST searches of these three sequences revealed 100% sequence identity to the *Hordeum vulgare* L. trypsin inhibitor CMe precursor (Chloroform/methanol-soluble protein CMe). The mature protein is 13.3 kDa and the gene which codes for trypsin inhibitor BTI-CMe in barley, *Itr1* is located on chromosome 3H. This confirms the identification of the SE trait on the short arm of chromosome 3H in the Chebec (SE +ve) x Harrington (SE -ve) and Galleon (SE +ve) x Haruna nijo (SE –ve) mapping populations. The gene for trypsin inhibitor CMe is regulated in trans by the *lys 3a* locus in the endosperm of barley (RODRIGUEZ-PALENZUELA *et al.* 1989). The *lys 3a* locus which is located on chromosome 5H regulates in *trans* the expression of the *Itr1* gene, which is located on chromosome 3H. The barley *lys 3a* gene is thought to also control the expression of several genes in the barley endosperm, such as those that encode for B and C hordeins, β-amylase and protein Z (BRANDT *el al.* 1990; ENTWISTLE 1988; HOPP *et a.* 1983; KRIES *et al.* 1984; KRIES *et al.* 1987).

Conclusions

In this study a polymorphism for haze active proteins was identified and surveyed by immunoblot throughout the brewing process, in barley, malt, beer and in haze. The SE protein has been characterised as the barley trypsin inhibitor CMe (BTI-CMe). These results suggest that the selection of SE -ve varieties may provide an opportunity to genetically improve the colloidal stability of beer, thus providing an alternative option to the use of traditional colloidal stabilisation treatments in the brewery.

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Comparison of Malting Quality Parameters of Spring and Winter Barley Genotypes in the Czech Republic

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Abstract

The Czech Republic is a country in Central Europe where cultivation and particularly breeding of top-quality malting spring barley has a long tradition. Czech local varieties have been extensively used in the breeding programmes of many countries in Europe and overseas since the beginning of the 20th century. At the present time, spring barley is cultivated on the area of about 400,000 ha, which represents 30% of the total area of cereal crops. Two-row barley with the top malting quality strongly dominates among varieties. On the contrary, six-row barley dominates in winter barley. The total area of winter barley represents about 180,000 ha, i.e. about 10% of the total area of cereals. In the frame of winter barley, 80% of six-row barley and 20% of two-row barley are cultivated. In 1999, the first variety of two-row winter barley Tiffany (Germany) for malting purposes was registered, but malting industry strongly prefers spring barley. The demands of malting industry are characterised by parameters as follows: protein content, extract content, relative extract, final degree of attenuation, β-glucan content, Kolbach number and friability. There was recorded a significant increase in malting parameters, particularly extract content of both Czech (Malz, Respekt) and foreign varieties (Jersey, Prestige, Biathlon) registered in the period 2000-2003. As for malting winter barley, there is a relevant problem of winterhardiness.

Keywords: spring barley; winter barley; malting quality

Introduction

Barley is in the Czech Republic the second crop as for the area of cultivation in 2002 (Fig. 1). From the total area of roughly 600,000 ha, spring barley is cultivated on 350-450,000 ha and winter barley on 150-200,000 ha. It represents the total area of cereal cultivation in the relative comparison 30% and 10%, respectively. Table 1 demonstrates more precisely the comparison and differences in the cultivation area and yield in the period of 1974-1998. Spring and winter barley have the same spectrum of parasites but the occurrence and damages are different. There are also differences in yield potential, yield and malting quality.

Material and Methods

The broad assortment of varieties of spring (with both hulled and hulless grain) and winter barley was evaluated. Particularly the results of the Official State Trials were analysed. Analyses of malting quality were carried out by the Research Institute of Brewing and Malting Brno. In micro-malting analyses, samples were analysed by European Brewing Convention (EBC 1998) and Mitteleuropäischen Brautechnische Analysenkommission (Mebak 1997) recommended methods for a range of parameters: protein content of barley (Pb), extract of malt, congress mash (E), mash method according to Hartong and Kretschmer VZ 45 °C (VZ45), Kolbach index (K), diastatic power (DP), final attenuation of laboratory wort from malt (FA), friability (F), high molecular weight β-glucan content of malt (BGw), viscosity of laboratory wort from malt (Vw). For carrying out analyses on the national scale the official statistic data are used.

Figure 1. The cultivation area of individual cereals in the Czech Republic, 2002

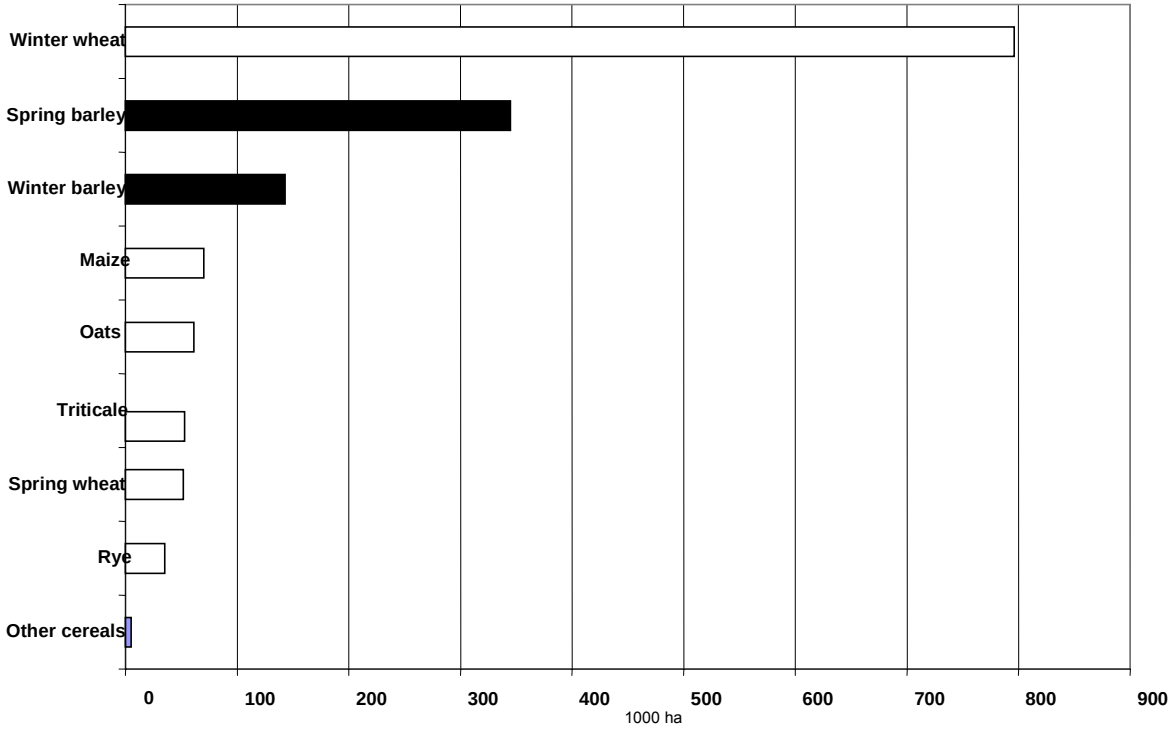

Table 1. Development of cultivation area and yields of winter and spring barley in the Czech Republic

Year	Winter barley		Spring barley		Difference	
	1000 ha	t/ha-1	1000 ha	t/ha-1	t/ha-1	%
1974	4	3.5	649	3.9	-0.36	-11
1984	123	5.1	469	4.5	0.53	13
1990	243	6.1	339	5.4	0.62	12
1994	185	4.2	495	3.7	0.47	13
1995	195	4.4	370	3.8	0.62	14
1998	187	4.1	393	3.8	0.31	8
2000	142	4.0	354	3.0	1.00	25
2001	157	4.4	338	3.7	0.70	18
2002	141	3.7	345	4.0	-0.30	-6
2003	98	3.1	450	3.9	-0.80	-22

Results - Malting quality

The parameters of malting quality required by the malting industry according to PSOTA and KOSAR (2002) are presented in Table 2. The particular importance of extract content and relative extract at 45 °C representing 50% of the total malting quality weight is quite clearly manifested.

Table 2. Limit values and weights of quality parameters required by the Czech malting industry

	Unacceptable limit 1	Optimal limit 9	Regression equation coefficients a	b	Weight W
Pb	**9,5**	**10,2**	**-107,57**	**11,43**	**0,01**
P̄b	**11,7**	**11,0**	**134,71**	**-11,43**	
E	**81,5**	**83,0**	**-433,67**	**5,33**	**0,30**
VZ45	**35,0**	**40,0**	**-55,00**	**1,60**	**0,20**
VZ45	**53,0**	**48,0**	**85,80**	**-1,60**	
K	**40,0**	**42,0**	**-159,00**	**4,00**	**0,10**
K	**53,0**	**48,0**	**85,80**	**-1,60**	
DP	**220**	**300**	**-21,00**	**0,10**	**0,10**
FA	**79,0**	**82,0**	**-209,67**	**2,67**	**0,10**
FI	**79,0**	**86,0**	**-89,29**	**1,14**	**0,10**
BGw	**250**	**100**	**14,33**	**-0,05**	**0,10**
			Sum of weights		**1,01**

Abbreviations:
Pb - grain protein content
E - extract yield in d.m.
VZ45 - relative extract at 45 °C
K - Kolbach index
DP - diastatic power
FA - apparent final attenuation
F - friability
BGw - glucan in wort

Table 3 demonstrates the increase in malting quality parameters by systematic breeding in the period 1950-2003. The particular progress was reached in extract content as the new Czech varieties like Olbram and Malz reach more than 83%. As well as other parameters were improved. The particular attention has been paid to β-glucan content since 1990 and newly registered varieties since 2003 reach the content even less than 100 mg/l in wort.

Table 3. Development of spring barley malting quality parameters in the period 1950-2003

Variety	Country of origin	Period	Pb %	E %	VZ45 %	K u.WK	DP %	FA %	F %	BGw mg/l
Valticky	CZ	1967-69	11.7	81.2	37.9	39.4	317	77.1		
Diamant	CZ	1973-75	10.9	81.6	38.1	41.7	305	78.4		
Favorit	CZ	1978-80	11.4	80.7	39.6	39.8	301	79.2		
Rubin	CZ	1988-90	11.4	81.4	45.6	42.3	282	80.8		
Akcent	CZ	1994-97	11.1	81.5	45.9	46.9	280	81.3	82.2	221
Olbram	CZ	1994-97	11.0	83.0	43.2	47.8	256	82.1	86.8	149
Maridol	CZ	1996-98	11.1	82.2	40.1	43.7	250	80.1	83.3	203
Malz	CZ	2002	10.9	83.1	40.2	44.5	317	81.7	86	171
Respekt	CZ	2003	11.2	82.5	44.1	45.9	379	81.6	83	192
Jersey	NL	2000	10.7	82.2	45.2	47.6	385	82.8	89	120
Prestige	GB	2001	10.9	82.2	48.3	44.9	424	83.1	85	119
Biathlon	GB	2003	10.5	83.4	47.8	50.5	303	81.1	90	90

The MQI values of individual parameters are calculated according to the method described by PSOTA *et al.* (1995) and KOSAR (2002) to determine the malting quality index (MQI). If the variety reaches the MQI lower than 4, the variety is not considered as a malting one. On the contrary, the varieties having a value of the MQI higher than 6, are considered as varieties of high malting quality. The maximum value of the MQI is 9. Table 5 demonstrates that the variety Jersey (NL) reached the MQI 8, among the winter barley varieties, Tiffany and

Vanessa (D) reached the MQI 2-3 only. As well as the variety Esterel did not reach the minimal level acceptable for malting industry.

Table 4. Comparison of malting quality parameters in spring and winter barley varieties cultivated in the Czech Republic, 2002

Varieties	Country	Pb	E	VZ45	K	DP	FA	F	BGw	MQI
		%	%	%		W.K.	%	%	mg/l	9-1
Spring										
Jersey	NL	10,4	82,5	45,6	49,0	370	83	91	115	8
Winter										
Tiffany	D	11,1	80,2	37,8	45,0	380	82	75	208,875	3
Vanessa	D	11,2	80,4	35,2	42,7	396	82	77	255,25	2
Esterel	F	10,0	80,7	32,4	40,1	406	81	79	339,75	2

Abbreviations

Pb - grain protein

E - extract yield d.m.

VZ45 - relative extract at 45

K - Kolbach index

DP - diastatic

FA - apparent final

F - friability

BGw - glucan in wort

MQI - malting quality

The specific item was to study the malting performance in hulless barley. As the first results show (VACULOVA & PSOTA 2003), these genotypes are able to reach very high values of extract content. This character could be a very interesting alternative for the malting industry, particularly in processing technologies where highly effective mash filters are used. The influence of different malting regimes (times) and the mutual correlations between the malting quality parameters and important agronomic traits are presented in Table 5.

Table 5. Spearman´s rank correlations between selected agronomic characters and malting parameters of hulless spring barley lines

Character/ Parameter[1)]	E	VZ45	K	DP	FA	F	Pb	BGw	Vw
				144 h					
Grain yield (t/ha)	-0.54*	-0.12	0.04	0.45	0.70**	0.64**	-0.08	-0.58	-0.26
Screening[2)] (%)	-0.29	-0.43	-0.47	-0.03	0.21	0.23	0.56*	-0.09	0.15
TGW (g)	-0.33	0.14	0.16	0.05	0.29	0.37	-0.03	-0.32	-0.39
Lodging (9-1) [3)]	0.27	0.74**	0.68**	-0.19	-0.09	0.31	-0.51*	-0.06	-0.67
Height (cm)	-0.34	-0.82**	-0.73**	0.22	0.30	-0.03	0.61*	-0.10	0.55*
				168 h					
Grain yield (t/ha)	-0.52*	0.52*	0.23	0.35	0.45	0.72**	-0.05	-0.63*	-0.55*
Screening[2)] (%)	-0.16	-0.07	-0.37	0.10	0.31	0.24	0.57*	-0.19	-0.17
TGW (g)	-0.33	0.11	0.19	-0.09	0.30	0.36	-0.02	-0.27	-0.30
Lodging (9-1) [3)]	0.21	0.23	0.54	-0.22	-0.09	0.12	-0.54*	0.03	-0.18
Height (cm)	-0.36	-0.16	-0.58*	0.27	0.31	0.09	0.64*	-0.18	0.05

[1)] - see Material and Methods

[2)] - samples were sreened on a 2.2 mm sieve

[3)] - scale 9-1, where 9= max. and 1= min. ; *, ** - significant at P≤0.05 and 0.01

As the table indicates, different time of malting did not influence existing relations, caused particularly by the genotype of the lines used.

Discussion

The malting quality parameters of spring barley varieties reached great progress particularly in the period 1995-2003. In the year 2000, there was lack of malting barley in the Czech Republic due to very low yields of spring barley accompanied by the unfavourable climatic conditions in the period of harvest. In that time, the variety of winter barley Tiffany was required by the malting industry (SPUNAR *et al*. 1996, 2002). This variety suffered strongly from high susceptibility to BYDV in 2002 and medium winterhardiness in the year 2003. In the period 2002-2003, there were registered in the Czech Republic varieties of spring barley manifesting very high yield potential and malting quality (Prestige, Biathlon, Malz, Respekt) and both malting industry and farmers have strongly oriented on the cultivation of malting barley. In spite of the progress in barley breeding in Germany (FRIEDT 2000), new genotypes and varieties tested in Germany for economically important traits (BAUMER *et al*. 2000, 2001, 2002, 2003) and for malting and brewing quality (RATH *et al*., 2000, 2001, 2002, 2003), it seems that winter barley can be considered as a reserve raw material for the malting industry. Though the winter barley variety Diamond (GB) has very high parameters of malting, it seems to be impossible for cultivation in Central Europe due to low winterhardiness. In this respect, the situation can be different in France (BERNICOT *et al*. 2000). Nevertheless, in the year 2003, the damages of winter barley due to winterkill were reported (GATE & VIGNIER 2003).

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Do Components of Barley Variety Mixtures Converge for Malting Quality Attributes?

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Abstract
Barley plants grown in a variety mixture may be subject to competitive and/or complementary interactions, that alter how the plants respond to their environment. In some situations, mixture components appear to converge in their expression of certain agronomic characters. Whether quality characters are similarly affected has not been investigated, although convergence could reduce heterogeneity, perceived as a major obstacle to the commercial utilisation of mixtures. Three diverse spring barley varieties were, therefore, grown in replicated trial as pure stands, as equal proportion, three-component mixtures and as alternate row mixtures. Manual harvesting was carried out to preserve the identity of individual plants, upon which mean values for height and grain size were assessed. For both characters, two of the varieties performed differently when grown as random mixture components compared to monoculture. Bulk samples of each genotype, from the three different plot arrangements, were assessed for grain nitrogen and milling energy, then malted for measurement of extract and fermentability. For all characters, one or more of the mixture components gave values different to those of the same variety grown in monoculture.
Keywords: variety mixtures; malting; extract; fermentability

Introduction

Varietal mixtures have been used to control disease in a diversity of crops such as wheat, coffee and rice (FINCKH 1997; WOLFE 2000), over a wide range of environments. In addition to reducing the spread of pathogens, mixtures may confer advantages such as higher yield, even in the absence of disease (NEWTON *et al*. 1997) and greater stability of yield between sites and seasons (FINCKH *et al*. 2002). Laboratory data have challenged the view that barley mixtures are too heterogeneous for malting (NEWTON *et al.* 1998) and mixtures have been used for malting and brewing, commercially, in the former German Democratic Republic (WOLFE 1992). For yield, while many mixtures may give similar values to the mean of their components, some combinations may equal or exceed the best of the components (CZEMBOR *et al.* 1991). This has also been noted for quality characters in both winter and spring barley (NEWTON *et al*. 1998; SWANSTON *et al*. 2000). The basis of the yield advantage is considered to be a better exploitation of resources both above and below ground (FINCKH *et al*. 2002) and this could also have positive implications for quality in barley. Better grain filling could lead to lower nitrogen content, more starch and, consequently, higher extract.

While mixture components may have complementary effects upon each other, it is also possible to observe compensation and inter-plant competition within mixtures. The former may explain the greater stability of performance ascribed to mixtures, since one or more components may thrive in any given environment. The less favoured components will thus not exhibit as large a deleterious effect as they would if grown in monoculture, but will comprise a lower proportion of the mixture after harvest than they did at sowing. The composition of a mixture may also be

changed by competition between mixture components, e.g. VALENTINE (1982) suggested that, in barley, a more erect growth habit conferred a competitive advantage over a prostrate habit.

Growing within a mixture may also influence the expression of certain traits by the individual components. Data from Poland showed a cold-sensitive winter barley to suffer less winter kill when grown in a mixture with a cold-tolerant type (FINCKH *et al*. 2002). Farmer observation of mixtures of two-row and six-row barley, grown in Scotland to optimise yield and specific weight, indicated the six-row component to be slightly shorter than when grown as a monoculture (SWALLOW, 2002). Phenotypic expression of a trait like height has an environmental and a genotypic component. Growing within a mixture may thus alter the environmental aspect, so that the difference between mixture components is reduced. If such convergence can occur for agronomic traits, it may also be observed in those concerned with quality. This could reduce heterogenity within mixtures and would strengthen the argument for their use in malting, brewing and distilling. In the preliminary experiment described here, three barley varieties, phenotypically contrasting, to permit ready identification, were grown as monocultures, in alternate row mixtures and as equal component mixtures, with the grain of each component being randomly distributed within the plot. Grain and malt quality results were compared, within and between varieties to determine whether there was convergence within the mixtures for any quality traits.

Material and Methods

The three malting varieties Chalice, Harrington and Morex, were included in a trial of two replications grown at the Scottish Crop Research Institute near Dundee in 2003. Chalice is a short-strawed two-row variety that ripens slightly earlier than other spring barleys recommended for the UK, but was the latest variety here. Harrington, from Canada is also two-rowed, while Morex is a 6-row type from Minnesota. Neither contains a dwarfing gene. In addition to being grown as monocultures, the three varieties were also grown as alternate row mixtures and as 'random' mixtures i.e. grain mixed in equal proportions by number. Equal numbers of grain were sown in each case through adjustment of seed quantity, based on the thousand corn weights of the varieties. Plots, 1.5m wide and comprising six rows 2m long, were given an appropriate fungicide regime to prevent infection by pathogens that might have introduced variability in disease levels.

Individual plants were harvested by hand, avoiding those at ends or edges of plots, and, in the case of the random mixtures, allocated to the appropriate variety by length of straw and ear-type. Six samples were thus obtained from each variety, from monoculture, from alternate row mixture and from random mixture, all replicated twice. Ten plants were taken at random from each sample and, for each, the length of the main tiller to the base of the ear was taken as a measure of plant height. After threshing by hand, thousand corn weight (TCW) was determined by counting a given weight of grain using a MARVIN digital seed analyser (GTA Sensorik GmbH, Neubrandenburg, Germany). Mean values for grain length and width were also recorded. The remaining plants within each sample were threshed as a bulk and the seed retained by a 2.5mm sieve was used for further testing. Germinative energy was measured two weeks after harvest with 100 grains placed on filter paper in 9cm petri dishes, to each of which 4mls of water was added. The number of grains germinating after 3 days was counted. Grain nitrogen was measured by near infra-red transmission and grain milling energy by use of a Comparamill (ALLISON *et al*. 1979). Grain was malted as described by SWANSTON (1997) and extract and fermentability measured by the method of SWANSTON & THOMAS (1996) except that, for hot water extract, 5g of grist was made up to a final volume of 51.5mls. Of this, 40mls were used for fermentability

determination, with 0.2g of yeast. Mean values from the two replicates were used to compare varieties and between monocultures and the two mixture configurations.

Results and Discussion

Height, Thousand Corn Weight and Grain Dimensions

Analysis of variance (data not shown) was carried out on all the single plant measurements. For height there was a highly significant difference between varieties, but not between treatments (i.e. monoculture v the two mixture configurations). There was, however a significant variety x treatment interaction, resulting from Chalice being slightly taller and Morex slightly shorter when grown in the random mixture (Table 1). For TCW, Morex produced larger grain in both mixture configurations compared to monoculture and Chalice also showed an increase, particularly when grown in random mixture. Harrington, which gave the highest TCW of the monocultures (Table 1) showed no increase in either mixture configuration. These results were reflected in an analysis of variance that showed significant genotype by treatment interactions as well as highly significant effects of genotype and treatment. A significant interaction and effect of treatment was also observed for grain width, but not length, while significant genotype effects were observed for both characters. The data presented in Table 1, therefore, suggests some evidence of convergence, particularly in the random mixtures.

Table 1. Heights, Thousand Corn Weights and Mean Grain Widths of three barley varieties grown in monoculture and in two mixture configurations.

	Chalice	Harrington	Morex	Chal.	Harr.	Morex	Chal.	Harr.	Morex
	Height (cm)			Thousand Corn Weight (g)			Grain Width (mm)		
Monoculture	72.4	91.4	94.3	52.4	54.0	46.7	4.03	4.08	3.87
Alt. Row Mixture	71.8	90.3	94.7	53.7	53.1	49.9	4.08	4.05	3.96
Random Mixture	74.9	91.7	92.8	55.0	54.4	49.6	4.08	4.08	3.96

Grain Quality Tests

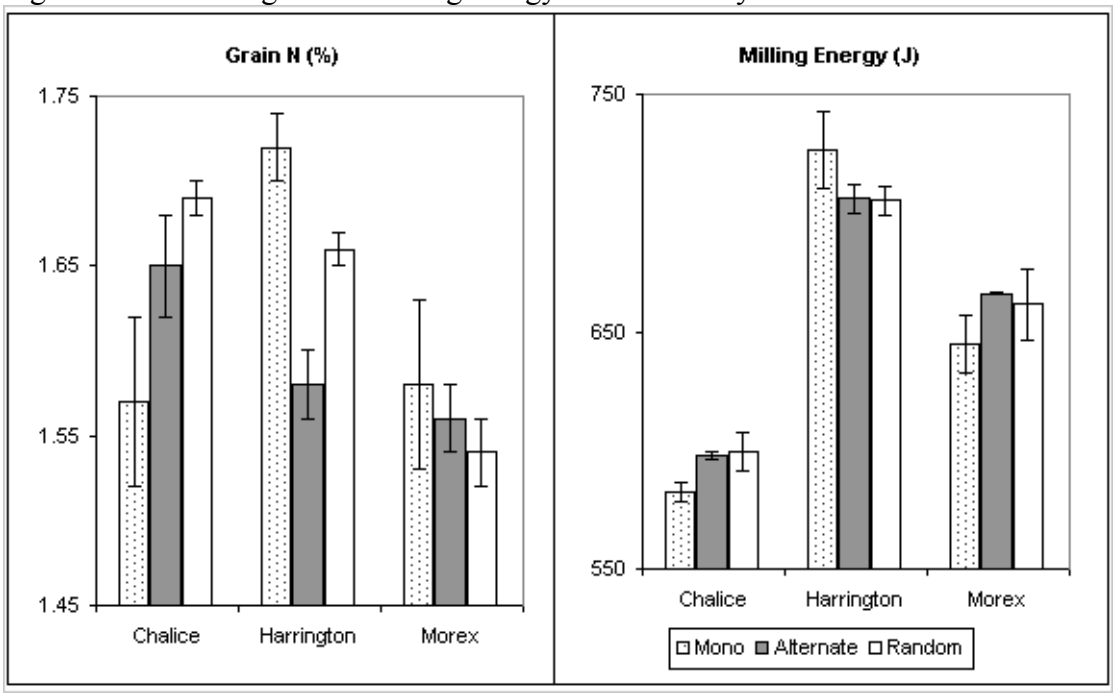
For grain nitrogen, there were fairly large differences between replicates in some cases (Fig. 1), but it was still clear that Harrington had a lower nitrogen content when grown in either mixture configuration, whereas Chalice had significantly higher, particularly when grown in the random mixture. These results were mirrored to some extent by the milling energy data, with Chalice having higher levels when grown in mixtures compared to monoculture. Although Harrington appeared to give slightly lower values in mixtures, differences were not significant. This is in accordance with the findings of COWE *et al*. (1989) who noted a negative correlation between milling energy and grain nitrogen content that varied between varieties.

Germination and Malting Tests

Harrington showed a very rapid recovery from dormancy, with 95% of grain germinating in 3 days (GE3), when measured two weeks after harvest (Fig. 2). Morex showed significantly lower germination that Harrington, but was very much higher than Chalice. Although this ranking order remained the same in both mixture configurations and differences between varieties remained significant, Harrington showed a slightly, but significantly, lower and Chalice a slightly higher rate of germination when grown in the random mixture.

For malting characters, there were some fairly large differences between replicates, but there were also clear differences between varieties and between monocultures and mixtures (Fig. 2). Chalice, the variety best adapted to Scottish growing conditions gave higher extracts than either Harrington or Morex in monoculture. The latter two gave significantly higher extracts in alternate row mixture compared to monoculture and Harrington also showed an improvement in extract when grown in random mixture. These results for Harrington are likely to reflect the lower nitrogen content in the grain, while better grain filling by Morex when grown in mixtures may explain the higher extract in the alternate row mixture compared to the monoculture.

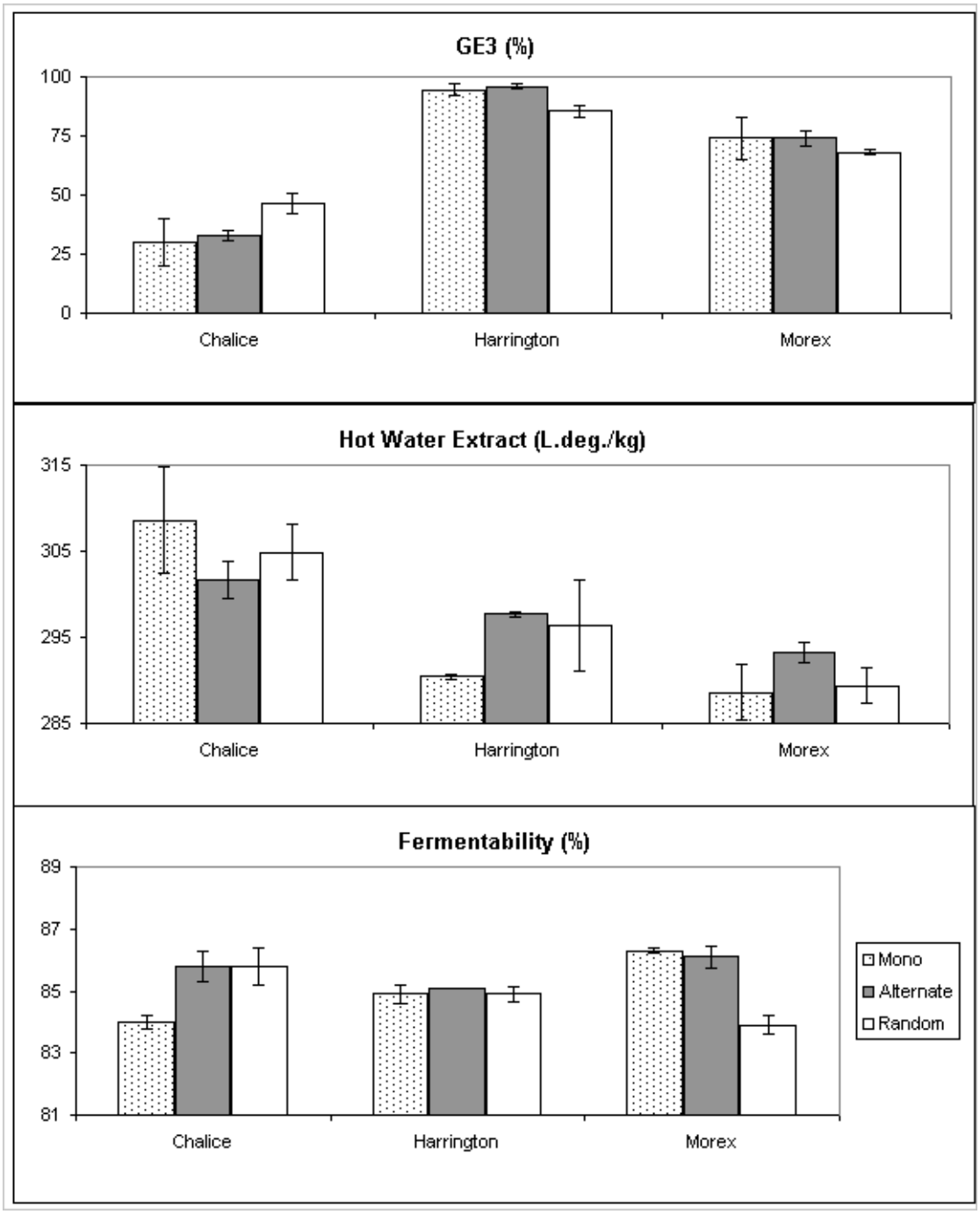
Figure1. Grain nitrogen and milling energy of three barley varieties in monoculture and mixtures



While Chalice showed slightly, but not significantly lower extract, when grown in mixtures, this was compensated for by higher fermentability compared to that in monoculture. The yield of alcohol from a given quantity of malt, which is derived from the product of extract and fermentability would therefore be similar for all the Chalice samples. Grown in mixtures, Chalice had slightly higher milling energy and this could be linked to slower breakdown of the grain structure (modification) during malting. BATHGATE *et al*. (1978) noted fermentability to peak at a slightly earlier stage of modification than did extract, so the results, here, for Chalice are consistent with that observation. Fermentability values for Harrington were similar in both mixtures and monocultures, but Morex showed a significant reduction in fermentability when grown in random mixture. The three varieties were all significantly different when grown in monoculture, but, in the alternate row mixtures, Chalice and Morex gave similar values. These results showed that, when grown in mixtures, barley varieties could give a different malting performance to that observed when they were grown in monoculture and, for some characters, there was evidence of convergence between at least two of the components. Some of these effects could be predicted from the tests on unmalted grain. The three varieties here were distinct phenotypically, so further research will be aimed at determining whether similar results are observed in other mixtures, particularly those comprising closely related varieties. It will also be

necessary to determine effects on the performance of the mixture as well as those of the individual components.

Figure 2. Germination and malting data from three barley varieties in monoculture and mixtures



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Genotypic Differences of Translocated Dry Matter at Spring Barley

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Abstract
In the years 2000 and 2001 plot field experiments with spring barley were established (in the region west Slovakia, lowland climatic region, maize-barley productive region). Five parental varieties and 135 $F_{2.3}$ lines from 9 crossings in F_3 and 5 parental varieties and 42 $F_{2.4}$ lines from 5 crossings in F_4 were evaluated. In the year 2000, with shorter period from sowing to heading in 14 days in comparison to the year 2001, lower biomass yield were produced before heading from total dry matter yield at maturity (for varieties 42.9 % and for lines 52.4 %) than in the favourable year 2001 (50.8 %, 56.9 %, respectively). Dry matter translocation was higher in the favourable year 2001, than in the year 2000, but in both years high differences were among varieties and lines in portion of translocated dry matter in grain. In the year 2000 portion of translocated dry matter in grain varied among varieties from –32.3 % to 14.7 % and among crossings from –14.0 % to 24.8 % and in the year 2001 in the same order from –13.3 % to 33.1 % and 6.2 % to 23.8 %. Genotypes with higher portion of translocated dry matter in grain had lower grain yield, in comparison to genotypes producing from heading to maturity higher amount of assimilates exceeding needs for grain formation. Selection for higher biomass yield can be indirect selection on grain yield.
Keywords: spring barley; varieties; F_3 lines; F_4 lines; accumulated dry matter; translocation

Introduction

Increasing of grain yield of modern cereal varieties with respect to old ones was due to increasing harvest index (AUSTIN *et al.* 1989), however biomass yield was not changed substantially. Additional increasing of grain yield can be achieved by increasing of biomass yield without modification of harvest index (SIDDIQUE *et al.* 1989b), or by increasing of harvest index without modification of biomass yield (ABDIN *et al.* 1996), or by combination of both traits. Assimilates inevitable for grain formation are from continuous assimilates produced from anthesis to maturity and from retranslocated assimilates produced before anthesis and stored up generative organs (SIDDIQUE *et al.* 1989a; SLAFER *et al.* 1990; BLUM 1998). Portion of retranslocated assimilates depends on year condition, on nutrition mainly nitrogen (PRZULJ & MOMCILOVIC 2001), on genotype and locality (FLOOD *et al.* 1995).
The aim of research was to find out effect of retranslocated dry matter on grain yield at local spring barley varieties and their lines in generations F_3 and F_4.

Material and Methods

Experiment in F_3 generation consisted of 135 $F_{2.3}$ lines from 9 crossings and 7 parental varieties (Table 2). In F_3 generation field experiment was established on April, 7, 2000 in Piešťany, in three replications, on three levels of nitrogen nutrition (N_1=0, N_2=60, N_3=120 kg $N.ha^{-1}$) by split plot method, where plot = crossing, subplot = lines and parental varieties (1 row length 2 m, space between rows 0,15 m). The first replication was cut at heading time (from June, 6 to Jun, 8). Biomass yield was estimated and samples were taken for dry matter. At maturity (from July 25 to July, 26) in the second replication on each subplot plant height,

number of ears, grain yield were determined and in the third replication only grain yield was determined.
Into F_4 generation was randomly selected 42 $F_{2.4}$ lines from 5 crossings and 5 parental varieties (Table 4). Field experiment was established on March 29, 2001 in Borovce (near Piešťany) in four replications and on two levels of nitrogen nutrition (N_1= 0, N_2 = 120 kg $N.ha^{-1}$) at randomised lines and varieties (space between rows 0.125 m, harvest plot 5 m^2). At heading on each plot plant height was measured. From two replications, cut at heading (June, 13) samples for dry matter were taken.
From primary data following parameters were calculated: -translocated dry matter ($g.m^{-2}$) as the difference in dry matter yield at heading and dry matter yield at maturity, -portion of translocated dry matter in grain, -effectivity of translocation as the portion of translocated dry matter from dry matter yield at heading.
Results were elaborated by analysis of variance according to model and from component of variance expected variance among family means was estimated:

$$X_{ijk} = \mu + N_j + C_i + NC_{ij} + L_{k(i)} + e_{ijk}$$

$$\sigma^2_{PC} = \sigma^2_C + \frac{\sigma^2_{LC}}{n} + \frac{\sigma^2_{NC}}{l} + \frac{\sigma^2_e}{nl}$$

where: i – crossings, i = 1,..., 9 for F_3, respectively 5 for F_4, j – N-nutrition, j = 1, 2, 3 for F_3, respectively 1, 2 for F_4, k – lines, k = 1, ..., 135 for F_3, respectively 1, ..., 42 for F_4, μ_i – total average, N_j – effect of N-nutrition, C_i – effect of crossings, NC_{ij} – effect of interaction N-nutrition x crossings, $L_{k(i)}$ – effect of lines within crossing, e_{ijk} – error.

Results and Discussion
F_3 Generation (Table 1, 2)
From parental varieties, high *dry matter yield at heading* had varieties Progres and Amos and low one Galan and SK 13-9. Among crossings differences were not significant, what could be due to significant interaction N-nutrition x crossings. Between average *dry matter yield at maturity* of parents (1087 $g.m^{-2}$) and crossings (1006 $g.m^{-2}$) were not significant differences. Varieties Stabil and Galan were below average in dry matter yield, which was manifested in crossings 1, 2, 6 and 7, where at least one of them was parent, but on the contrary from the crossings of above average parental varieties in dry matter yield Kosan, Amos, Progres, only crossing 3 (Amos x Kosan) was above average. For portion of ear weight to total dry matter yield at heading (*ear index*), all sources of variability were significant at crossings and lines. The low ear index had varieties with low biomass yield at heading. Among crossings ear index ranged from 0.180 to 0.273. Low ear index had crossings 1 and 8 and high one crossing 7, originated from crossings, where at least one parent had low ear index (Galan), or high one (Progres). Among varieties in *grain yield* were nonsignificant differences in spite of variation which ranged from 469.9 $g.m^{-2}$ (Galan) to 624 $g.m^{-2}$ (Kosan). Grain yield at crossings, although varied from 435.1 $g.m^{-2}$ to 587 $g.m^{-2}$, unexpectedly was significantly lower (506.3 $g.m^{-2}$) than at parental varieties (558 $g.m^{-2}$). Below average, or only average grain yield had crossings originated from low yielding variety Galan (1-Vladan x Galan, 6- Progres x Galan, 8-SK 13-9 x Galan) and on the contrary high grain yield had crossing 3 (Amos x Kosan) originated from variety Kosan with above average grain yield. *Translocated dry matter* as difference between dry matter yield at heading and vegetative dry matter yield at maturity varied at parents from -202.1 $g.m^{-2}$ (Kosan) to +66.1 $g.m^{-2}$ (Stabil). All varieties, except for variety Stabil from heading to maturity, produced besides amount of assimilates equal to grain yield, another one allocated in vegetative biomass. Almost opposite situation was at crossings. At the most crossings from accumulated dry matter was translocated to grain from 25.2 $g.m^{-2}$

(crossing 1) to 101.1 g.m^{-2} (crossing 2), what was from 8.3 % (crossing 1) to 24.8 % (crossing 2) from total grain yield. Among crossings were significant differences. Amount of translocated dry matter per area or portion in grain was not possible to explain by their origin. Dry matter translocation of variety Stabil was expressed in crossings 2 (Vladan x Stabil) and 7 (Progres x Stabil), however opposite high ability to produce dry matter from heading to maturity, besides assimilates inevitable for grain as well assimilates accumulated into vegetative biomass, at variety Kosan was only partially expressed at crossing 3 and at variety SK 13-9 at crossings 8 and 9.

F_4 Generation (Table 3, 4)
In F_4 generation *at heading*, *dry matter yield* (645 g.m^{-2}) was higher than in F_3 generation (497.3 g.m^{-2}) (Table 2, 4) conditioned by 14 days longer vegetative period from sowing to heading and a smaller row width in F_4 generation. Unexpected dry matter yield had varieties Galan and SK 13-9, significantly higher than the others (Table 4), although in the previous year both varieties had below average dry matter yield. It could be caused by slower initial growth of mentioned varieties till heading, what was in shorter period from emergence till heading in F_3 generation (in the year 2000) manifested by their under average biomass yield. Average dry matter yield of parents (649.2 g.m^{-2}) corresponded with average dry matter yield of crossings (641.4 g.m^{-2}). Among crossings dry matter yield varied from 611.7 g.m^{-2} 7 (Progres x Stabil) to 669.1 g.m^{-2} 6 (Progres x Galan), however the last mentioned crossing with the highest dry matter yield in F_4 had below average dry matter yield in F_3, what is in agreement with reaction of parental variety Galan (Table 2, 4). *Ear index* in F_4 had higher value than in F_3 (Table 4, 2), however below average values for varieties Galan and SK 13-9 and above average one at variety Progres corresponded with the past year (Table 2). Among crossings, ear index varied from 0.251 6 (Progres x Galan) to 0.296 7 (Progres x Stabil), corresponded with value of parental varieties. Above average *grain yield* had varieties Progres and SK 13-9, similarly as in F_3 generation, however variety Kosan had unexpected relatively low grain yield in comparison to F_3 generation (Table 2) . Among crossings grain yield varied from 532.2 g.m^{-2} 1 (Amos x Kosan) to 604.3 g.m^{-2} 9 (SK 13-9 x Stabil). Above average grain yield at crossings 6 (Progres x Galan) and 9 (SK 13-9 x Stabil) corresponded with above average grain yield of varieties Progres and SK 13-9 and on the contrary, low grain yield at crossings 3 and 5 corresponded with low grain yield of variety Kosan (Table 4). In opposite to F_3 generation low grain yield had crossing 3 (Amos x Kosan) similarly as variety Kosan, what could be caused by similar reaction of parental variety and their offspring.
Essential difference among the years 2000 and 2001 was in the amount of *dry matter* produced from emergence till heading. Different dynamism in dry matter yield between parents and crossings and among lines within crossing explains different amount of translocated dry matter yield between years and between parents and lines, too. In the year 2000, for the reason of short period from emergence to heading lower dry matter yield (497.3 g.m^{-2}), was developed than in the year 2001 (645.3 g.m^{-2}) and therefore even if nearly the same amount of dry matter yield produced from heading to maturity in both years, in the year 2000 assimilates produced from heading to maturity exceed grain capacity and part of them was left in vegetative organs. In the year 2001, amount of produced assimilates from heading to maturity was not sufficient for sink capacity which caused translocation of assimilates. These results, on one side, were confirmed by research of PRZULJ and MOMCILOVIC (2001), translocation occurs only in favourable years, on the other side, results are not comparable with the research of the other authors (FLOOD *et al.* 1995). According to their results in unfavourable years translocation is higher.
For selection is important portion of variance conditioned by crossings and lines within crossing. At all evaluated traits except for biomass yield at heading (in F_3), significant portion

of variance was among crossings (Table 5). At ear index and translocated dry matter higher portion of variance was for crossings than for lines within crossing and therefore selection on these traits should be based on average value of crossings, while at grain yield and dry matter yield at maturity significant portion was for both sources of variability and therefore at selection average value of crossings and lines could be considered. Although from evaluated traits in F_3 generation nearly at all interaction crossing x N-nutrition was significant (Table 1), their portion in expected variance among crossing means was low and ranged from 0.0 (ear index) to 14.4 % (grain yield) (Table 5). In F_4 generation in comparison to F_3, number of crossings was reduced from 9 to 5 and number of lines from 135 to 42, what resulted in turn for portion of variability for crossings and lines among crossings (Table 5). For the most traits at F_4 generation, there were no portion of variability among lines within crossing and therefore next effectivity of selection is only conditioned by selection among crossings.

Table 5. Components of expected variance among average of crossings (portion $\sigma^2_{Pc}=1$) in F_3 and F_4 generation

Components	Yield of dry matter ($g.m^{-2}$) at time		Ear index	Grain yield ($g.m^{-2}$)	Translocated dry matter	
	heading	maturity			($g.m^{-2}$)	in grain
F_3 generation						
σ^2_{Pc} abs	570	6542	1025	1384	3023	0.0131
σ^2_{e}	0.450	0.104	0.00307	0.167	0.187	0.221
σ^2_{NC}	0.115	0.079	0.000896	0.144	0.0307	0.0310
$\sigma^2_{L(C)}$	0.433	0.232	0.0174	0.364	0.0069	0.114
σ^2_{C}	0	0.59	0.978	0.323	0.774	0.603
F_4 generation						
σ^2_{Pc} abs	765.6	7751	0.648	1259	1723	6119
σ^2_{e}	0.813	0.137	0.073	0.270	0.572	0.558
σ^2_{NC}	0.020	0.068	0.00	0.056	0.020	0.158
$\sigma^2_{L(C)}$	0.00	0.082	0.096	0.00	0.00	0.014
σ^2_{C}	0.167	0.713	0.833	0.672	0.407	0.268

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Table 1. Mean squares from analysis of variance of traits evaluated at heading and in technological maturity of spring barley varieties and F_3 lines

Source of Variability	Df	Yield of dry matter ($g.m^{-2}$) at time		Ear index (x1000)	Grain yield ($g.m^{-2}$)	Translocated dry matter		
		heading	maturity			($g.m^{-2}$)	portion (x 1000)	
							in grain	from heading
Parental varieties								
N–nutrition	2	4270	15232	14.06^{+}	6040	11219	35.9	49.9
Variety	6	4094	44577	5.38	9037	19188	62.2	96.7
Error	12	6894	16227	3.34	9654	8190	28.0	47.2
Total	20							
Crossings and lines								
N–nutrition	2	72394^{++}	111058^{+}	39.21^{++}	25588	203170^{++}	1106.1^{++}	687.0^{++}
Crossings	8	12175	322053^{++}	45.52^{++}	75502^{++}	151869^{++}	690.6^{++}	629.5^{++}
Lines within crossings	126	13821	44482^{++}	3.03^{++}	15263^{++}	25724	148.1	119.3
N–nutrition x crossings	16	26585^{++}	148180^{++}	3.49^{++}	55336^{++}	46450^{+}	332.0^{++}	174.6
Error	252	11590	30778	1.41	10451	25534	134.2	107.7
Total	404							

Table 2. Average value of traits of spring barley varieties and F_3 lines evaluated at heading and technological maturity

Factor	Yield of dry matter ($g.m^{-2}$) at time		Ear Index	Grain yield ($g.m^{-2}$)	Translocated dry matter		
					($g.m^{-2}$)	portion	
	heading	maturity				in grain	from heading
Parental varieties							
Vladan	454.5	1070.0	0.279	553.6	-61.00	-0.119	-0.181
Stabil	469.3	903.6	0.244	500.4	66.17	0.147	0.126
Kosan	457.4	1284.0	0.240	624.0	-202.18	-0.323	-0.460
Amos	499.9	1122.0	0.261	596.8	-25.18	-0.012	-0.065
Progres	528.5	1158.3	0.265	578.4	-51.31	-0.079	-0.097
Galan	447.5	986.3	0.171	469.9	-68.85	-0.133	-0.173
SK 13 - 9	415.2	1088.0	0.180	584.5	-88.22	-0.158	-0.245
$\bar{x}_P$	467.5	1087.4	0.234	558.2	-61.67	-0.097	-0.156
$LSD_{(0.05)}$	147.3	226.62	0.067	174.7	161.00	0.297	0.386
Crossings and lines							
N_1	544.6	983.9	0.238	509.5	70.21	0.192	0.080
N_2	535.6	1038.7	0.240	518.2	15.20	0.045	-0.039
N_3	500.7	996.7	0.210	491.3	-4.67	0.027	-0.045
SE	9.6	16.1	0.003	9.4	13.40	0.032	0.028
1-Vladan x Galan	520.0	968.6	0.195	473.7	25.23	0.083	0.009
2-Vladan x Stabil	259.5	863.5	0.256	435.1	101.12	0.248	0.144
3-Amos x Kosan	539.5	1159.1	0.221	587.3	-32.31	-0.034	-0.130
4-Amos x Galan	543.6	977.3	0.256	508.0	74.27	0.204	0.091
5-Progres x Kosan	534.7	985.6	0.241	510.6	59.75	0.149	0.057
6-Progres x Galan	521.9	977.2	0.202	490.8	35.64	0.136	0.004
7-Progres x Stabil	526.2	978.7	0.273	521.5	69.13	0.144	0.101
8-SK 13-9 x Galan	488.8	1076.9	0.180	508.3	-79.73	-0.140	-0.212
9-SK 13-9 x Stabil	538.7	1071.2	0.240	521.6	-10.87	0.006	-0.081
$\bar{x}_L$	527.0	1006.5	0.229	506.3	26.91	0.088	-0.001
$LSD_{(0.05)}$	46.3	77.93	0.018	45.5	64.57	0.154	0.138
$\bar{x}_T$	497.3	1046.9	0.232	523.3	-17.37	-0.040	-0.079

Table 3. Mean squares from analysis of variance of traits evaluated at heading and in technological maturity of spring barley varieties and F_4 lines

Source of variability	Df	Yield of dry matter ($g.m^{-2}$) at time		Ear index (x1000)	Grain yield ($g.m^{-2}$)	Translocated dry matter		
		heading	maturity			($g.m^{-2}$)	portion (x 1000)	
							in grain	from heading
Parental varieties								
N–nutrition	1	19706	1742	2.19	327	13625	45.30	29.70
Variety	4	18020	40993	2.94^{+}	16656	21682	67.80	48.10
Error	4	1385	6622	0.486	2818	5315	11.20	13.20
Total	9							
Crossings and lines								
N–nutrition	1	79763^{++}	254606^{++}	1.347	82603^{++}	4257	1.99	10.09
Crossings	4	7898	79113^{++}	5.879^{++}	13649^{++}	17763	74.94	4.04
Lines within crossings	37	6179	13156	0.716	3248	9467	35.07	2.07
N–nutrition x crossings	4	6619	23794	0.425	5182	18674	58.52	37.43
Error	37	6217	10635	0.472	3410	9858	34.21	22.54
Total	83							

Table 4. Average value of traits evaluated at heading and technological maturity of spring barley varieties and F_4 lines

Factor	Yield of matter ($g.m^{-2}$) at time		Ear Index	Grain yield ($g.m^{-2}$)	Translocated dry matter		
	heading	maturity			($g.m^{-2}$)	portion	
						in grain*	from heading
Parental varieties							
Stabil	597.5	1234.0	0.284	657.6	21.16	0.033	0.032
Kosan	576.5	1192.0	0.248	560.4	-55.01	-0.102	-0.107
Progres	566.9	1388.0	0.296	722.4	-98.67	-0.133	-0.171
Galan	750.2	1108.0	0.198	531.8	174.09	0.331	0.231
SK 13-9	754.6	1456.0	0.248	730.4	29.02	0.035	0.025
$\bar{x}_P$	649.2	1275.6	0.255	640.5	14.12	0.032	0.002
$LSD_{(0.05)}$	103.3	225.9	0.058	147.4	202.4	0.294	0.319
Crossings and lines							
N_1	610.4	1070.9	0.277	535.6	74.5	0.149	0.103
N_2	672.5	1181.8	0.269	598.4	88.8	0.159	0.125
SE	12.2	16.0	0.003	9.0	15.4	0.028	0.022
3-Amos x Kosan	652.5	1066.1	0.263	532.2	118.6	0.238	0.160
5-Progres x Kosan	630.3	1091.7	0.289	559.1	97.7	0.178	0.145
6-Progres x Galan	669.1	1184.3	0.251	582.7	67.5	0.131	0.096
7-Progres x Stabil	611.7	1077.9	0.296	554.7	88.6	0.162	0.127
9-SK 13-9 x Stabil	643.6	1212.0	0.266	604.3	35.9	0.062	0.041
$\bar{x}_L$	641.4	1126.4	0.273	566.6	81.7	0.154	0.114
$LSD_{(0.05)}$	57.46	81.47	0.017	46.63	71.40	0.135	0.107
$\bar{x}_T$	645.3	1190.4	0.267	597.6	52.47	0.100	0.068

S 6 – BARLEY PROTEIN, FEED AND FOOD

The Characteristics of Barley Germplasm with Increased Crude Protein Content

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Abstract
Evaluation of barley germplasm received from USDA-ARS Small Grain Collection with increased protein content was carried out in Latvia University of Agriculture. There was wide variability in yield, crude protein and starch content. The yield of hulled two-row barley (126 genotypes) was in average 4.12 t ha^{-1} (1.7 - 5.75 t ha^{-1}), crude protein content in average 15.5 % (12.9 - 18.6 %). There were 63.8 % samples with protein content higher than 15 %. The starch content was in average 51.7 % (49.7 - 54.4 %). The yield of hulled six–row barley (56 genotypes) was lower: in average 3.19 t ha^{-1} (1.7 - 5.07 t ha^{-1}) with mean crude protein content 17.0 % (13.3 - 20.1 %), and starch content 51.3 % (49.2 - 52.6 %). The highest content of crude protein was observed in hulless barley (30 genotypes) - 17.2 % (15.2 - 22.4 %), but they are characterized with lower starch content 49.7 % (40.6-51,9 %) and the lowest yield in average 2.49 t ha^{-1} (1.09-3.83 t ha^{-1}). With increasing the yield, the crude protein content decreased and starch content increased. Our experiments showed that barley germplasm collection comprises genotypes with increased crude protein content and good agronomic traits. It is possible to select the initial material for crossing, and to include it in barley breeding programme for feed.
Keywords: hulled and hulless barley; crude protein; starch

Introduction
Barley is an excellent source of concentrated nutrients for all classes of livestock. It is not practical to define single ideal barley for feed when considering all of the various animals and fowl, their ages and production requirements (NEWMAN & NEWMAN 1992). Most barley improvement research and breeding activities have been devoted toward malting barley, but relatively little efforts has gone into feed or food improvement, little support has come from those industries (MOLINO-CANO *et al.* 2000). Starch is the primary nutrient in barley and is principal source of available energy. Barley containing higher levels of this nutrient has higher digestible energy values. Barley kernel starch is predominantly amylopectin (branched chain, average 75 %) and amylose (straight chain, average 25 %) (NEWMAN & McGUIRE 1985; NEWMAN & NEWMAN 1989; WASHINGTON *et al.* 2000). The amylose content of barley starch can affect the quality of barley for malt, food and animal feed. Three main classes of barley varieties have been characterized with respect to amylose content: low or waxy (0-12 %), normal (25 %) and high (>35 %). Waxy barley has been tested for use as an animal feed and observed its readily digestibility. Identification of agronomically viable, hulless, waxy barley with reduced beta glucan content may prove to be useful for animal feed (WASHINGTON *et al.* 2000).
Compared with hulled barley cultivars, hulless cultivars have reduced fibre content and increased starch content due to absence of the hull (ANDERSON *et al.* 1999; BOWMAN *et al.* 2000) and lower acid detergent fibre (ADF) content than hulled types (BELICKA 2000). Due to reduced fibre content and increased starch content, hulless barley would have better feed quality for beef cattle compared to hulled cultivars (BOWMAN *et al.* 2000).

Protein is the second most important nutrient in barley. In areas of the world where protein is deficient, high protein barley should be considered (NEWMAN & NEWMAN 1989). Breeders strive for high grain protein if the barley is to be used for livestock feed or human food. Analysing the 6770 barley samples of USDA-ARS National Small Grain collection, protein ranged from 6.9 - 25.0 %, with mean 14.8 % (PETERSON *et al.* 1996). There are possibilities to select donors of high protein content for including them in breeding program. Like other cereal protein, protein in barley is nutritive unbalanced due to deficiency of the essential amino acids (NEWMAN & McGUIRE 1985). Hulless barley is characterized with higher protein and increased essential amino acid content (BELICKA 1999, 2000; BELICKA & LEGZDINA 2000, 2002; ROSSNAGEL 2000) and that is why they are interesting as animal feed. Therefore, high starch and moderate protein levels in barley grains are consistent with high quality malting, brewing and feed end uses. Barley germplasm with increased protein content was in focus of our investigation.

Material and Methods

In 1997 Latvia University of Agriculture (LUA) received the barley germplasm collection (450 samples) with increased crude protein content (15.0 –23.5 %) from USDA-ARS National Small Grain Collection. During 1997-1999 this material was sown in small plots, multiplied and evaluated. One part of genotypes was very susceptible to different diseases and lodging; some were with very long vegetation period and other undesirable characteristics. From this collection more suitable accessions were selected.

In 2001 the hulled and hulless varieties/lines with different origin were evaluated. There were hulled and hulless, both 2-row and 6-row genotypes from different continents and states.

Hulled 2-row** type*: ***Europe (n = 83) from Austria, England, Armenia, Bulgaria, Belgium, Bosnia and Herzegovina, the Czech Republic, Denmark, the Netherlands, Croatia, Italy, Israel, Russia, Latvia, Lithuania, Norway, Portugal, Finland, Slovakia, Spain, Germany, Poland, Sweden, Turkey, Ukraine, France, Hungary, Macedonia; ***America:*** (n = 24) from Argentina, Brazil, Chile, Venezuela, Canada, USA; ***Australia and New Zealand*** (n = 9), ***Africa:*** (n = 7) from Ethiopia, South Africa.

Hulled 6-row** type*: ***Europe (n = 29) from Norway, Denmark, Estonia, England, Finland, Sweden, Spain, Greece; ***America:*** (n = 15) from Canada, Colombia, Mexico; ***Asia:*** (n = 11) from China, Iran, India, Yemen, Nepal, Uzbekistan.

Hulless 2-row: (n = 12) from the Czech Republic, the Netherlands, Italy, Romania, Turkestan, Guatemala, Ethiopia; ***6-row*:** (n = 10) from Finland, Romania, Italy, Jordan, Nepal, China, Mongolia, Tajikistan, Pakistan, Bhutan.

The trials were conducted at the Research and Study Farm "Vecauce" of LUA. Soil conditions: sod-podzolic sandy loam, high content of available K_2O and P_2O_5, pH_{KCl} - 6.9, content of organic matter 18.0 g kg^{-1}. Before sowing the fertilizer 70 kg N ha^{-1}, 90 kg P_2O_5 ha^{-1} and 90 kg K_2O ha^{-1} were used. Previous crop was maize. The plot's size was 5.4 m^2 without replication, sown with Hege-80 sowing machine. The crop was harvested with harvester Hege-140. In 2001, the vegetation period was characterized with drought in May and with abundant rainfall in June (200 % from the norm) and July (205 % from the norm). Precipitation in August was in norm.

Protein and starch content was determined in grain by express method using Infratec 1275 Analyser Foss Tecator. The results were statistically evaluated with t-Test (Two Sample Assuming Unequal Variances). Coefficient of variation (%) of traits, coefficient of correlation between yield and different traits were calculated.

Results and Discussion

Evaluation of barley collection with increased protein content received from USDA-ARS (USA) was carried out in Latvia University of Agriculture prebreeding programme. Investigated barley collection material was divided in tree groups: I - hulled 2-row; II - hulled 6-row and III - hulless barley**.**

Hulled 2-Row Barley

In Table 1 are presented summarized data of yield, crude protein and starch content and 1000 grain weight (TGW) of hulled 2-row barley genotypes of European, American, Australian and New Zealand and African origin. There are not significant differences in mean **yield** among the genotypes presented Europe (4.22 t ha^{-1}), America (4.17 t ha^{-1}), Australia and New Zealand (3.97 t ha^{-1}) (p>0,05). Only the yield of genotypes from Africa differed significantly (p<0.05) from other groups. The yield variation of barley was in average wide in all sample groups, coefficient of variation values ranged from 14.50 % to 18.30 %.

Table 1. The characteristics of hulled 2-row barley of different origin

Indices		Europe (n = 83)	America (n = 24)	Australia, New Zealand (n = 9)	Africa (n = 7)
Yield, t ha^{-1}	Min - max	1.73 - 5.75	2.30 - 5.38	3.61 - 5.48	2.43 - 4.20
	Mean ± standard error	4.22 ± 0.08	4.17 ± 0.16	4.46 ± 0.21	3.45 ± 0.20
	Confidence level (95.0 %)	4.07 - 4.37	3.85 - 4.49	3.97 - 4.95	2.97 - 3.93
	Coefficient of variation	16.81	18.30	14.50	16.63
Crude protein, %	Min - max	12.9 - 18.5	13.2 - 18.3	14.1 - 17.2	14.8 - 18.6
	Mean ± standard error	15.40 ± 0.12	15.78 ± 0.25	15.10 ± 0.31	16.40 ± 0.53
	Confidence level (95.0 %)	15.16 - 15.64	15.26 - 16.30	14.40 - 15.80	15.10 - 17.80
	Coefficient of variation	7.24	7.87	6.07	8.62
Starch, %	Min - max	49.7 - 54.4	50.1 - 53.5	51.3 - 53.5	51.2 - 52.0
	Mean ± standard error	51.64 ± 0.09	51.77 ± 0.19	52.06 ± 0.22	51.70 ± 0.12
	Confidence level (95.0 %)	51.45 - 51.83	51.38 - 52.16	51.54 - 52.57	51.41 - 51.99
	Coefficient of variation	1.7	1.77	1.29	0.6
TGW, g	Min - max	29.26 - 51.76	25.32 - 50.13	38.39 - 44.29	37.05 - 47.61
	Mean ± standard error	40.22 ± 0.45	40.12 ± 1.13	40.52 ± 0.87	42.82 ± 1.25
	Confidence level (95.0 %)	39.33 - 41.11	37.77 - 42.47	38.39 - 42.66	39.86 - 45.78
	Coefficient of variation	10.00	13.53	5.71	8.27

The genotypes of Africa are characterized with the highest average **crude protein** content (16.4 %), but only those of Australia and New Zealand (15.1 %) differed significantly (p<0.05). There were not observed significant differences (p>0.05) in average data of crude protein content (15.1-15.7 %) among other investigated groups. The genetic variability of this parameter was low, coefficient of variation - 6.07 to 8.62 %. **Starch** content of barley genotypes with high protein content was the less variable. This parameter was noted with the lowest coefficient of variation – 0.60 % to 1.77 % and there were no significant differences (p>0.05) among investigated accessions. Investigated barley genotypes characterized with comparatively low **1000 grain weight** (in average 40.12 to 42.82 g), there were no significant differences (p>0.05) among accessions from different continents, the coefficient of variation of this trait in groups was from low to medium (5.71 to 13.53 %).

Hulled 6-Row Barley

The results of investigated indices: yield, crude protein, starch and TGW of hulled 6-row barley are presented in Table 2. The yield of genotypes from Europe (3.29 t ha^{-1}) and America

(3.63 t ha^{-1}) were significantly higher ($p<0.05$) if compared to genotypes from Asia (2.48 t ha^{-1}), but differences in mean yield between genotypes of Europe and America were not verified as significant ($p>0.05$). The coefficient of variation was high (25.46 to 33.87 %).

Table 2. The characteristics of hulled 6-row barley of different origin

Indices		Europe (n = 29)	America (n = 15)	Asia (n = 11)
Yield, t ha^{-1}	Min - max	1.17 - 4.95	2.12 - 5.07	1.36 - 3.87
	Mean ± standard error	3.29 ± 0.19	3.63 ± 0.24	2.48 ± 0.25
	Confidence Level (95.0 %)	2.90 - 3.68	3.12 - 4.14	1.92 - 3.04
	Coefficient of variation	31.06	25.46	33.87
Crude protein, %	Min - max	13.3 - 18.5	14.2 - 19.0	15.9 - 20.1
	Mean ± standard error	15.83 ± 0.22	16.99 ± 0.32	17.61 ± 0.43
	Confidence Level (95.0 %)	15.37 - 16.29	16.31 - 17.66	16.66 - 18.56
	Coefficient of variation	7.65	7.29	8.11
Starch, %	Min - max	50.3 - 52.6	51.0 - 52.5	49.2 - 51.4
	Mean ± standard error	51.45 ± 0.11	51.60 ± 0.13	50.39 ± 0.22
	Confidence Level (95.0 %)	51.22 - 51.68	51.33 - 51.87	49.91 - 50.87
	Coefficient of variation	1.17	0.95	1.43
TGW, g	Min - max	33.78 - 48.57	31.38 - 48.25	34.74 - 51.96
	Mean ± standard error	39.56 ± 0.85	38.53 ± 1.26	39.05 ± 1.41
	Confidence Level (95.0 %)	37.83 - 41.29	35.12 - 41.24	35.91 - 42.19
	Coefficient of variation	11.53	12.71	11.97

Accessions of 6-row barley are characterized with **crude protein** content 15.83 % for European genotypes, 16.99 % for American, 17.61 % for Asian. Significantly higher ($p<0.05$) this indices were for Asian genotypes, if compared to genotypes from Europe and America. Genetic variability was low: 7.29 % to 8.11 %. The content of **starch** ranges from 49.2 % to 52.6 %, with low coefficient of variation: from 0.95 % to 1.43 % depending on the group. The

Table 3. The characteristics of hulless 2-row and 6-row barley

Indices		2 - row (n = 12)	6 - row (n = 10)
Yield, t ha^{-1}	Min - max	1.89 - 3.83	1.09 - 3.95
	Mean ± standard error	2.72 ± 0.15	2.00 ± 0.27
	Confidence level (95.0 %)	2.39 - 3.05	1.38 - 2.62
	Coefficient of variation	21.12	40.44
Crude protein, %	Min - max	15.9 - 18.4	16.6 - 22.4
	Mean ± standard error	17.6 ± 0.26	18.2 ± 0.51
	Confidence level (95.0 %)	17.34 - 17.86	17.69 - 18.71
	Coefficient of variation	5.14	8.84
Starch, %	Min - max	49.1 - 51.9	46.6 - 51.2
	Mean ± standard error	50.3 ± 0.24	49.67 ± 0.40
	Confidence level (95.0 %)	49.77 - 50.83	48.76 - 50.58
	Coefficient of variation	1.67	2.56
TKW, g	Min - max	34.65 - 47.65	29.15 - 49.03
	Mean ± standard error	43.69 ± 0.86	37.28 ± 1.94
	Confidence level (95.0 %)	41.83 - 45.54	32.9 - 41.64
	Coefficient of variation	7.37	16.42

starch content of Asian genotypes was lower ($p<0.05$), if compared that from America and Europe. Regarding to TGW, there were not established significantly determined differences ($p>0.05$) in mean values (38.53-39.56 g) among genotypes of investigated continents. The variability of this trait was medium (s % = 11.97 % to 12.71 %).

Hulless Barley

In our collection 12 two-row and 10 six-row type accessions of hulless barley of different origin were included (Table 3). The yield and TGW of hulless barley 2-row types exceeded ($p<0.05$) these of 6-row types and were characterized with high variability (s % = 21.12 % and 40.44 % respectively). There were no differences ($p>0.05$) in crude protein and starch content between 2-row (accordingly crude protein 17.34 %; starch 50.35 %) and 6-row genotypes (accordingly 18.2 % and 49.67 %). The variability of these indices was low.

Frequency of Traits

The yield of hulled 2-row barley was 1.73 – 5.75 t ha^{-1}, average 4.12 t ha^{-1}. Accessions with yield level 3.5 - 4.5 t ha^{-1} were dominant (51.56 %) in the hulled 2-row barley group. The yield of hulled 6-row type was lower (1.17 –5.07 t ha^{-1}), average 3.19 t ha^{-1}.

Yield 2.5 - 3.5 t ha^{-1} was the most frequent group (33.9 %) in hulled 6-row barley. The 2-row hulless barley mainly corresponded to the group with the yield level less than 2.5 t ha^{-1}, but it has also 14.3 % of genotypes with the yield level higher than 3. 5 t ha^{-1} (Fig. 1).

Most of the 2-row hulled barley samples (56.8 %) had crude protein content 13.5 - 14.5 %, but 21.6 % of them were higher in crude protein 14.5 – 15.5 %. In 6-row barley group 33.9 % of accessions were characterized with crude protein content 15.5-16.5 %, and 25.0 % of samples with 16.5-17.5 %. If compared with hulled barley, more than 50 % of hulless both type barley had the highest protein content (>17.5 %) (Fig. 2).

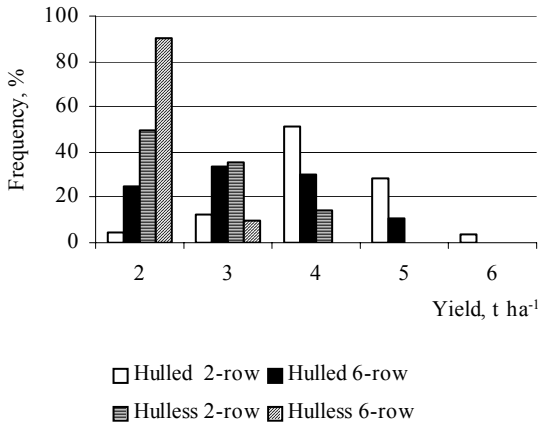

Figure 1. Grain yield, t ha^{-1}

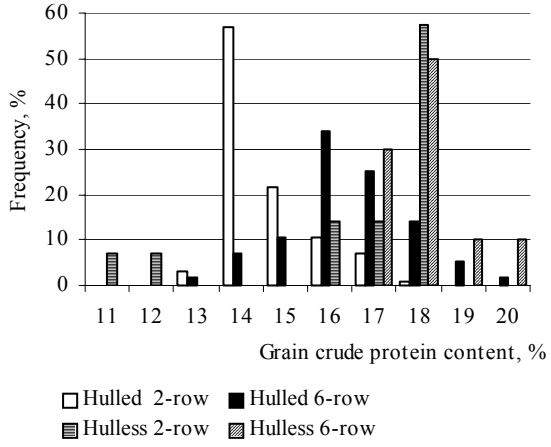

Figure 2. Crude protein, %

Fig. 3 presents the division of genotypes by starch content. This parameter was higher for hulled 2-row barley, but there were no remarkable differences among genotypes.

TGW of included hulless 2-row barley group in our trials was higher than that of other group, having 78 % samples with TGW > 45.0 g. In other groups were dominant barley genotypes with average TGW 35.0 g and 40.0 g (Fig. 4).

Our experiments showed that barley germplasm comprised genotypes with increased crude protein content and good agronomic traits. It is possible to select the initial material for crossing, and to include it in barley breeding programme for feed. The crude protein and

starch content of investigated barley depending on the yield is given in Table 4. With increased yield, the crude protein content decreased ($r_{2\text{-row}}$ = -0,307 $r_{6\text{-row}}$ = -0,541, $r_{0,05}$ = 0,253) and starch content increased. This tendency was noted in all the barley groups, but in every group we could find the genotypes with different relations of these indices. There are possible to select genotypes where these traits are in balance. Considering it, using as initial material the forms with desirable indices for breeding work it is possible to develop varieties with good agronomic traits and improved end use quality.

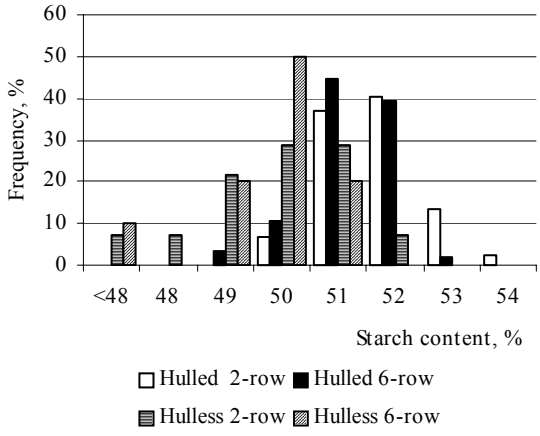

Figure 3. Starch, %

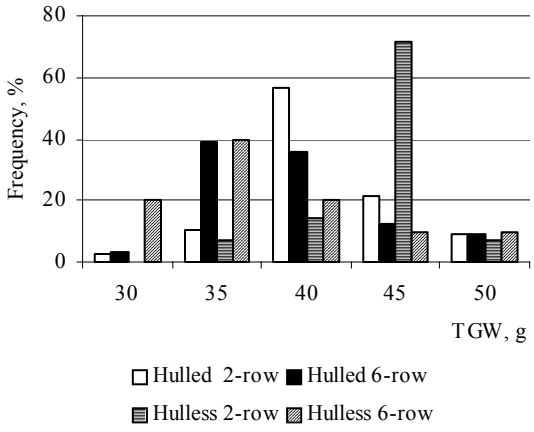

Figure 4. TGW, %

Table 4. The crude protein and starch content of hulled 2-row barley depending on yield

Barley type	Yield, t ha^{-1}	n	Crude protein, %		Starch, %	
			Min - max	Mean	Min - max	Mean
Hulled 2-row	≤ 2.50	5	16.4 - 18.5	17.6	50.5 - 51.9	50.96
	2.51 - 3.50	16	14.1 - 18.6	16.4	50.3 - 51.8	51.16
	3.51 - 4.50	65	14.0 - 18.2	15.4	50.3 - 54.4	51.77
	4.51 - 5.50	36	13.3 - 17.4	14.7	49.9 - 54.2	51.72
	>5.50	4	13.4 - 14.5	14.0	52.4 - 53.8	52.87
Hulled 6-row	≤ 2.50	14	15.8 - 20.1	17.4	49.2 - 52.1	50.95
	2.51 - 3.50	19	14.9 - 19.0	17.0	50.3 - 52.0	51.20
	3.51 - 4.50	17	13.8 - 18.2	15.6	50.8 - 52.6	51.58
	> 4.50	6	13.3 - 16.8	15.3	50.7 - 51.7	51.35
Hulless 2-row	≤ 2.50	5	18.3 - 18.4	18.3	49.1 - 51.9	49.84
	> 2.5	7	15.9 - 18.4	17.1	49.6 - 51.9	50.65
Hulless 6-row	≤ 2.50	9	17.0 - 22.4	18.4	46.6 - 51.2	49.60
	> 2.5	1	16.6	16.6	50.6	50.60

Summary

Hulled and hulless 2-and 6-row barley with high protein content has a wide diversity in yield, crude protein and starch content. By the yield level the genotypes are arranged into following way: hulled 2-row barley, hulled 6-row, then hulless 2-row and hulless 6-row. The hulled 2-row barley was characterized with lower crude protein, this parameter is higher for hulled 6-row barley, but the highest crude protein content had hulless barley. With increased yield, the crude protein content decreased and starch content increased. Barley germplasm collection comprises genotypes with increased crude protein and good agronomic traits. It is possible to

select the initial material for crossing, and to include it in barley breeding programme for feed.

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Protein Fractions and the Assessment of Their Genotype Variability

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Abstract

Proteins represent an important part of barley grain, mainly from the point of view of the food industry application. In this presentation, 20 barley genotypes (cultivars and new breeding lines) of world-wide range were evaluated for their total protein content and relative percentage of protein solubility fraction. The modified method of extraction of protein fractions (Albumins + Globulins, Prolamins, Glutelins) based on the Osborne's principle was used. Total protein content and protein fraction contents were determined as total nitrogen content in dry matter (N x 6.25) by modified Dumas method on elementar analyser Flash EA 1112 (ThermoQuest, Italy). Genotype variability in protein fraction composition was evaluated by electrophoresis (SDS-PAGE). In total protein content, the statistically significant differences among analyzed genotypes were found. The values of total protein content were in range 11 to 18.5 % of dry matter. The significant differences were also found in individual protein fraction - mean proportions of albumins + globulins, prolamins and, glutelins represent 16.1 %, 22.4 %, and 38.2 % of the crude protein, respectively. SDS-PAGE profiles of prolamin fraction may be considered as a suitable tool for barley genotype characterization.

Introduction

Barley (*Hordeum vulgare* L.) is the fourth most spread cereal in the world after wheat, rice a maize. Barley seeds are indispensable material for malting industry (production of beer and spirits) and animal feeding, and also for food industry (hulled barley and barley flour). The crude protein represents an important part of nutrients in barley grain – it has the controlling role in genotype evaluation from the point of view of the feed quality and industry processing (EDNEY 1997). Total content of crude protein in barley grain fluctuates from 8.1 to 18.1 %, in growing conditions of the Czech Republic only 10.5 – 12 %. It is generally known that naked barley genotypes have higher content of crude protein, about 1-2 % more than husked genotypes, but their amino-acid composition is not significantly different (OVESNÁ & POLÁKOVÁ, non-published).

From the point of view of feeding, easy soluble protein fractions – albumins and globulins - are important, possibly also glutelins (hordeinins), for their more positive amino-acid composition in comparison with basic storage proteins – prolamins (hordeins) (ČERNÝ & ŠAŠEK 1998). According to VELÍŠEK *et al.* (1999), barley proteins include 12.1 % albumins, 8.4 % globulins, 25 % prolamins, and 54.5 glutelins.

The main aim of this presentation is the evaluation of genotype variability in crude protein content and their solubility fractions.

Material and Methods

Plant Material

Plant material for analyses - barley seeds of 20 genotypes from growing year 2001 - was obtained from the Agricultural Research Institute Kromeriz, Ltd. The list of cultivars used in this study as well as their basic characterisation (variety, grain type, spike type, country of

origin) are given in table 1. Whole grain barley meal (absolutely dry matter) was used for all performed analyses.

Analysis of Crude Protein Content

Crude (total) protein content was determined as total nitrogen content in dry matter of barley meal multiplied by factor 6.25. Total nitrogen content was analysed by modified Dumas method on nitrogen/protein analyser Flash EA 1112, ThermoQuest, Italy (THERMOQUEST 1999). Two samples, in amount 100 mg, were analysed parallel.

Analysis of Protein Solubility Fractions

In this study, the modified method of extraction of protein solubility fractions by KRÁLOVÁ *et al.* (1991) was used (based on the Osborne´s principle). The barley meal samples (500 mg) were successively extracted using following solvents: 0.1 M sodium phosphate buffer, pH 7.0 with 1 M NaCl (for extraction of albumins + globulins); 70 % ethanol (for extraction prolamins); 0.2 % NaOH (for extraction glutelins). Procedure was based on negative principle. Each analysed variant was composed from three particular samples which were successively extracted (first only with sodium phosphate buffer, second with sodium phosphate buffer and with ethanol, third with sodium phosphate buffer, ethanol and with NaOH; washing system among successive extraction was used). Residual nitrogen content in particular meal samples (after freeze-drying and homogenization) was analysed as above reported crude protein content. Contents of protein fractions were determined by deduction of nitrogen content values in particular samples (negative principle) and using multiplying factor 6.25. Two independent replicates of each variant were analysed.

SDS-PAGE of Prolamins

For SDS-PAGE analysis, "waste" ethanol extracts of prolamins were used. Protein extracts were denatured by a special buffer (0.0625 M Tris-HCl, pH 6.8, 5 % 2-mercaptoethanol, 2 % SDS) in proportion 1:1, for 4 hours at 4°C. After centrifugation (10,000 rpm, 3 min), 10 µl of supernatant + 2.5 µl of loading buffer (5 ml 1.25 M Tris-HCl, pH 6.8, 2.3 g SDS, 10 ml glycerol, 5 mg Bromophenol Blue; to 500 µl of this buffer was added 170 µl 2-mercaptoethanol) was used as electrophoretic run sample. SDS-PAGE of denatured proteins was performed by standard cooled dual vertical slab units SE 600 (Hoefer Scientific Instruments, San Francisco, USA) under conditions of 0.025 M Tris, 0.192 M glycine, 0.1 % SDS (pH 8.3) buffer system. The discontinuous gel system (HAMES & RICKWOOD 1987) was used – 4 % stacking gel (0.125 M Tris-HCl, pH 6.8) and 10 % separating gel (0.375 M Tris-HCl, pH 8.8). Proteins were detected by staining the gels overnight in a staining solution (1 g Coomassie Brilliant Blue R-250 was dissolved in 500 ml methanol + 100 ml acetic acid + 400 ml distilled water), gel processing after protein detection was performed according to HAMES & RICKWOOD (1987). Electrophoretic data were processed by computerised image analysis. Scanned gel record was used as input information for special software BIOPROFIL 1D++ (VILBER LOURMAT 1999).

Results and Discussion

Although presented values are only one-year results, found differences among studied genotypes are significant. On the other hand, the contribution of year and site effects is also important, so that genotype potential may be modified by other factors.

Performed analysis of crude protein content in barley meal showed a wide range of values within the framework of evaluated barley genotypes from 11.04 % (Nordus) to 18.45 % (Kavkazský golozernyj) of dry matter (table 2). Genotype effect on the content of crude protein in barley grain is very important in world-wide scale. Results also confirm a

presumption (OVESNÁ & POLÁKOVÁ, non-published) that husked genotypes contain lower content of crude proteins in comparison with naked genotypes. All seven analysed husked genotypes had the content of crude protein lower than found average 14.30 % (table 2).

Nutritive and feed values of barley proteins are linked with easy soluble protein fractions - albumins and globulins - with respect to their more positive amino-acid composition. Results presented on figure 1 documented that the genotype is a source of marked variability in soluble protein fractions. It was found the range from 5.8 % (KM 2283) to 25.5% (Wapana). It is possible to include Annabel, Jersey, Nordus and Wapana to the barley genotypes with higher content of albumins and globulins. All these genotypes are husked, which is interesting for evaluation of both grain types. Contrary, the lowest content of albumins and globulins (10 % and less of crude protein) showed genotypes KM 2283, Lyallpur 3647, and Miloňov nudum. Glutelin fraction fluctuated between 31.2 % (Washonubet) and 50.3 % (Lyallpur 3647).

Frequently discussed prolamin fraction (hordeins) ranged from 8.7 to 31.4 % - two genotypes (Annabel and Jersey) had the prolamin content below 10 %, and two genotypes (KM 2283, Shimabara) over 30 %. Hordein proteins were studied in detail by SDS-PAGE (Figure 2). Prolamin subunits were found in the region from 30 to 66 kDa, which is in agreement with two main groups of prolamins - group B (28 - 49 kDa) and group C (50 - 58 kDa), as reported KOIBE *et al.* (1976). The prolamin group A (14 - 22 kDa), that represents the form of atypical prolamins with differences in molecular weight, isoelectric point, amino-acid composition (higher content of lysine), can be identified as very weak bands.

Presumptive high level of prolamin polymorphism in groups B and C (SHEWRY 1981) documented Figure 3. It was found genotype differences up to 35 %.

Acknowledgements

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Table 1. List of analysed barley genotypes

Code	genotype	variety	country of origin	grain type	spike type
1	Shimabara	Var. brevisetum R. Reg.	PKR	naked	six-rowed
2	Washonubet	var. nudum L.	USA	naked, waxy	two-rowed
3	Annabell	var. nutans Schubl.	Germany	husked	two-rowed
4	KM2062	var. nudum L.	Czech Republic	naked	two-rowed
5	Miloňov nudum	var.nudum L.	Czechoslovakia	naked	two-rowed
6	Wapana	var. nutans Schubl.	USA	husked, waxy	two-rowed
7	Tolar	var. nutans Schubl.	Czechoslovakia	husked	two-rowed
8	Kavkazkyj golozernyj	var. nudum L.	Soviet Union	naked	two-rowed
9	KM2087	var. nudum L.	Czech Republic	naked	two-rowed
10	Jersey	var. nutans Schubl.	Netherlands	husked	two-rowed
11	Ebsdorfer nackt	var.coeleste L.	Germany	naked	six-rowed
12	KM1057	var. nudum L.	Czech Republic	naked	two-rowed
13	Wanubet	var. nudum L.	USA	naked, waxy	two-rowed
14	Pax	var. nutans Schubl.	Slovakia	husked	two-rowed
15	Nordus	var. nutans Schubl.	Germany	husked	two-rowed
16	KM2283	var. nudum L.	Czech Republic	naked	two-rowed
17	KM1910	var. nudum L.	Czech Republic	naked	two-rowed
18	Wabet	var. nutans Schubl.	USA	husked, waxy	two-rowed
19	KM2001	var. nudum L.	Czech Republic	naked	two-rowed
20	Lyallpur 3647	var.nudidubium Koern.	India	naked	two-rowed

Note: PRK - Democratic People's Republic of Korea

Table 2. Crude protein content in barley grain (% of dry matter)

Genotype	n	mean	SD	diff.*
Shimabara	2	16,19	0,07	c
Washonubet	2	13,96	0,02	f
Annabel	2	12,50	0,23	hi
KM 2062	2	14,56	0,06	f
Miloňov nudum	2	16,19	0,03	c
Wapana	2	11,84	0,10	i
Tolar	2	13,05	0,07	gh
Kavkazský golozernyj	2	18,45	0,03	a
KM 2087	2	15,31	0,08	ed
Jersey	2	12,71	0,03	gh
Ebsdorfer nackt	2	17,42	0,46	b
KM 1057	2	14,55	0,08	f
Wanubet	2	14,20	0,28	f
Pax	2	12,00	0,11	i
Nordus	2	11,04	0,18	j
KM 2283	2	15,39	0,15	d
KM 1910	2	13,13	0,08	gh
Wabet	2	13,24	0,13	g
KM 2001	2	14,65	0,34	ef
Lyallpur 3647	2	15,63	0,07	cd
Total	40	14,30	1,90	

* Different letters mean significant difference at $P < 0.05$ (Tukey HSD). The letters are given in alphabetical order with decreasing level of a parameter.

Figure 1. Proportion of protein solubility fractions (% of crude protein content)

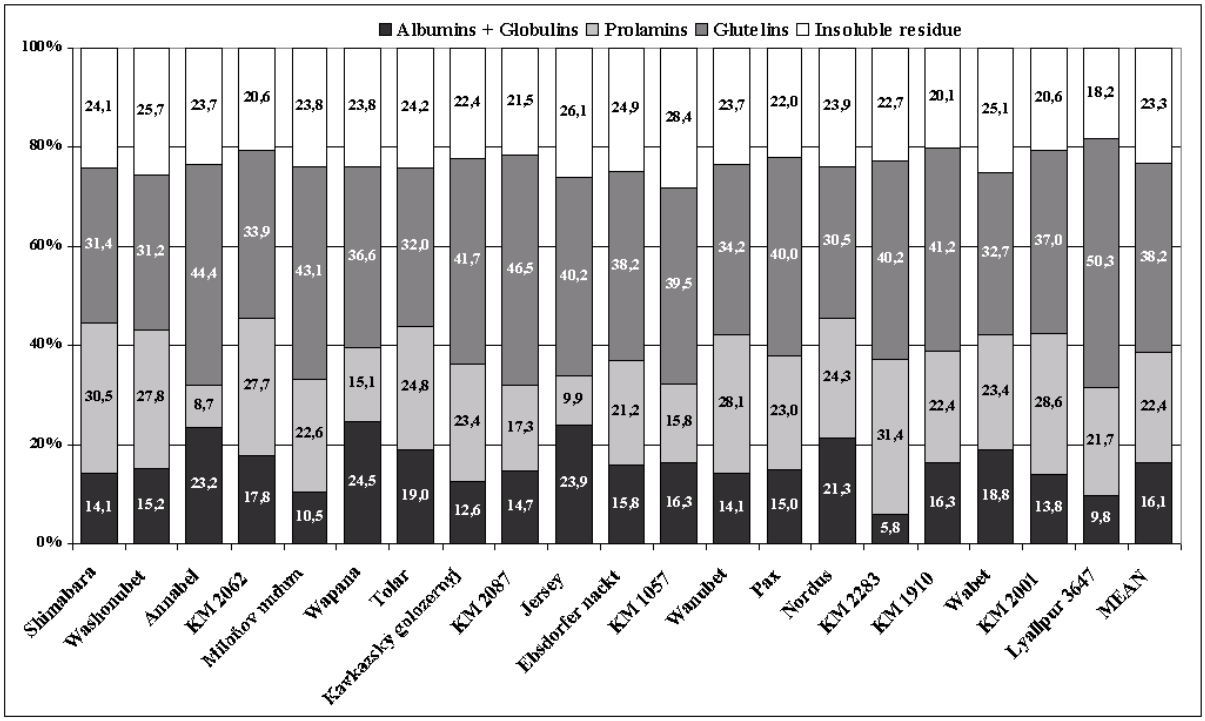
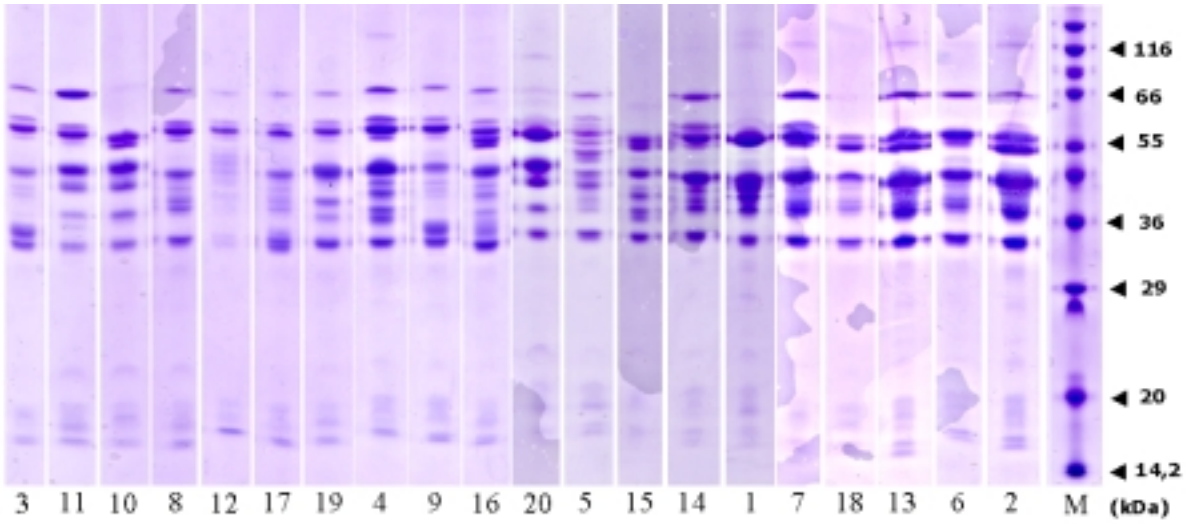
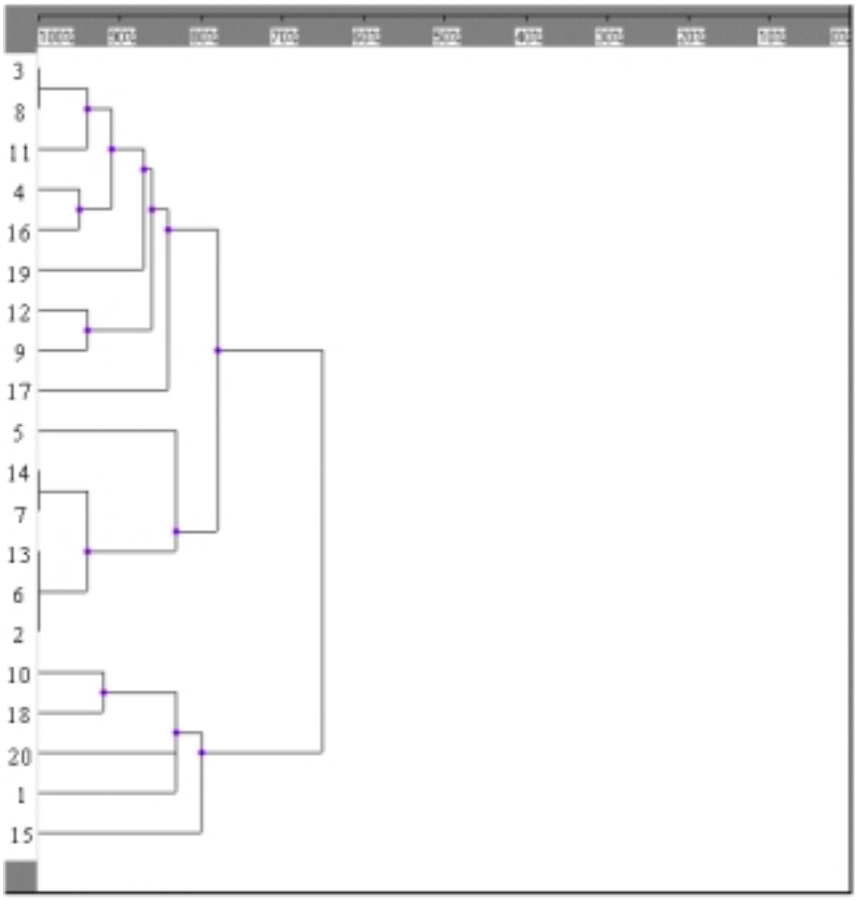


Figure 2. SDS-PAGE profiles of prolamin protein fraction



1 Shimabara, 2 Washonubet, 3 Annabell, 4 KM2062, 5 Miloňov nudum, 6 Wapana, 7 Tolar, 8 Kavkazkyj golozernyj, 9 KM2087, 10 Jersey, 11 Ebsdorfer nackt, 12 KM1057, 13 Wanubet, 14 Pax, 15 Nordus, 16 KM2283, 17 KM1910, 18 Wabet, 19 KM2001, 20 Lyallpur 3647, M weight marker (Sigma co. USA)

Figure 3. Dendrogram based on SDS-PAGE profiles of prolamin protein fraction (Nei & Li similarity coefficient, confiden. int. 5 %; BioProfil 1D++)



Hulled and Hull-Less Barley Genotypes for the Development of Functional Foods

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Abstract

Among cereals, barley is the best grain for the development of functional foods, as it contains two classes of compounds of strong nutritional interest: tocols and β-glucans.

Barley contains a high concentration of β–glucans, which have beneficial effects on blood cholesterol level and on glycemic response. They are a complex class of polysaccharides and are the major constituent of barley endosperm cell walls and their viscosity-enhancing property contributes to the beneficial physiological effects reported in animal and human trials.

Tocols are well recognised for their biological effects, including antioxidant activity and hypocholesterolemic action. Barley is a good source of this class of compounds, containing both a high concentration of total tocols and a favourable distribution of the most biologically active isomers.

Introduction

In these last years, many authors have shown that there is a strong relation between the quality of consumed foods and human health, and a beneficial correlation between the function of various food components, treatment and prevention of specific illness (KOIDE *et al*. 1996; ALDOORI *et al*. 1998; ANDERSOON & HANNA 1999; McBURNEY 2001). The abundant evidence regarding the vital role that nutritional factors play in maintaining health and the role of diet in the occurrence of the major causes of death, including heart disease, cancer, stroke, diabetes, arteriosclerosis and liver diseases, have contribute to increase the interest in functional foods. Functional foods may be defined as any food, in a natural or processed form, which contains, in addition to its nutritional components, substances which favour the good health, physical capacity and the mental health of an individual.

Among cereals, barley is the main cereal grain for the development of functional foods, as it contains two classes of compounds of strong nutritional interest: tocols (vitamin E) and β-glucan (soluble fibre).

Barley contains a high concentration, as well as a large range, of the non-starch polysaccharide (1→3),(1→4) mixed linked β–glucan (β–glucan). This polysaccharide is the major constituent of barley endosperm cell walls and its viscosity enhancing property contributes to the beneficial effects reported on blood cholesterol level, in animal and human trials, and on glycemic response (LILJEBERG *et al*. 1996; YOKOYAMA *et al*. 1997; HECKER *et al*. 1998; BOURDON *et al*. 1999).

For these reasons, researchers' interest has focused on the use of high-fibre cereals, such as barley and oats, which are rich in β–glucan, as potential ingredients for lowering glycemic index (GI) of most cereal based foods (BJIÖRCK *et al*. 2000).

A number of techniques have been developed to enrich the β–glucan content of barley flour (SUNDBERG & ÅMAN 1994; KLAMCZYNSKI & CZUCHAJOWSKA 1999). This has provided the opportunity to significantly enhance the β–glucan content of foods prepared from flour blends using barley fractions as a minor ingredient without affecting the sensory

characteristics of the traditional wheat-based products (KNUCKLES *et al*. 1997; NEWMAN *et al*. 1998; MARCONI *et al*. 2000).
Tocols (tocopherols and tocotrienols) are well recognised for their biological effects, including antioxidant activity (KAMAL-ELDIN & APPELQVIST 1996) and reduction of serum LDL-cholesterol (QURESHI *et al*. 1991; WANG *et al*. 1993). While tocopherols, and mainly α-tocopherol, are considered to have the greater biologically activity (KAMAL-ELDIN & APPELQVIST 1996), tocotrienols have been the focus of growing research interest as unique nutritional compounds for their hypocholesterolemic action.
Barley grains are a good source of this class of compounds, containing both a high concentration of total tocols and a favourable distribution of the most biologically active isomers. The development of high-tocols barley cultivars would help food industries, but the lack of knowledge of the genotype potentials, in term of tocols productivity, has limited the possible application of this cereal as a primary tocols source. This is particularly true for naked barley, which has been largely investigated for its β-glucan content and that seem to be the most promising barley genotypes for food industries (BHATTY 1999), but need to be compared with hulled variety for tocols productivity (CAVALLERO *et al*. 2004).
Since the important qualitative characteristics of these barley components, we carried out two experiments, one with the aim to test barley whole-grain flour and β-glucan enriched flour fractions to evaluate bread- and pasta-making quality and to evaluate the postprandial glucose response after consumption of different bread meals.
In the other experiment we evaluated naked and hulled barley genotypes, grown in different environments, for tocols content with the object to investigate the effects of genotype and environmental conditions, the presence of a location-genotype interaction and to verify the relationships between all the isomers and naked/hulled trait.

Material and Methods
The hull-less barley variety Zacinto was used for all the enrichment and baking trials. To evaluate the bread-making quality, barley whole-grain flour and two β–glucan enriched flour fractions, obtained from a naked barley variety, were used. The three barley fractions were: barley flour (BF), obtained by grinding whole grain, the sieved fraction (SF), produced processing the BF on an automatic siever, and a water-extracted fraction (WF), extracted from the SF. Different blends were baked in a pilot plant and each bread was evaluated for sensory attributes and chemical composition. Commercial bread wheat flour (BW) was used as base flour.
For tocols analysis, the extraction was performed according to PETERSON and QURESHI (1993). Tocols were then separated by HPLC and quantified by a fluorimetric detector according to AOCS method (1989).

Results and Discussion
The enrichment in β-glucan concentration in the different barley fractions was successfully achieved. The total β-glucan concentration of SF was 1.8 times higher than BF. This increase is due to the elimination of starch with the finer flour fraction (Table 1).
To keep an acceptable rheological quality with the highest sustainable β-glucan content, one level of BW substitution was selected for each barley fraction: 50% for SF and 20% for WF. This selection was based on a preliminary farinograph screening and on previous results (CAVALLERO *et al*. 2000). Farinograph water absorption and degree of softness were generally higher in blends compared to BW. The increase in water absorption was as high as 29% for 50% SF, 14% for 20% WF and 11% for 50% BF. Farinograph stability, a property related to protein content, decreased in all bends, confirming the negative effects of β-glucan (CAVALLERO *et al*. 2002).

Table 1. Chemical composition of bread wheat flour and barley flour fractions (g/100g db) (CAVALLERO *et al*. 2002)

Fraction	Protein (N x 6.25)	Fat	Ash	Total β-glucan (%)	Soluble β-glucan(%)
Bread wheat flour	16.8	2.1	0.6	0.1	0.1
Barley whole grain Flour (BF)	15.5	2.6	2.6	4.6	3.2
Sieved Fraction (SF)	17.8	3.2	3.1	8.5	3.7
Water Extracted Fraction (WF)	17.9	0.9	11.6	33.2	31.9

The three blends and one bread wheat tester were both baked according to AACC method 10-10B (1995) (with minor modifications to simulate the traditional procedure used in Italy) and baked in a pilot plant. In the baking test, dilution of the wheat flour with the barley fractions increased mixing times; in the case of the 20% WF, it was not possible to achieve an acceptable dough consistency with the mechanical mixer and manual mixing was necessary. As compared to 100% BW, bread volumes of 50% BF and 50% SF were lower by 46% and 51% in the case of AACC trials and 51% and 45%, respectively, in the pilot plant baking trials. Bread volume for 20% WF was 49% lower than BW when baked according to AACC procedure, probably as consequence of the low efficiency of manual mixing, but was only 13% lower when baked in the pilot plant. Bread with high β-glucan content and an acceptable volume was obtained only in this last case, thus confirming the need to reduce the barley flour fraction, which acts by depressing loaf volume, in the mixture to reach a loaf volume comparable to plain wheat bread (CAVALLERO *et al*. 2002).

Moreover, all products were subjected to sensory quality analyses and were judged acceptable, but the 20% WF and 100% BW reached an acceptability score significantly higher than the other barley breads. So the addition of a high concentrated β-glucan fraction, WF at 20% of the total flour, allowed the production of bread comparable in sensory quality to plain wheat bread, with a lower caloric content and the added value of a high, soluble β-glucan content (CAVALLERO *et al*. 2002).

It has been also identified the best condition for preparation, by traditional methods, of pasta using mixture of barley flour and semolina, to evaluate the acceptability of the final products by sensory analysis, and to define the barley contribution in terms of β-glucan.

Commercial samples of durum wheat semolina were mixed with barley flour (85:15, 75:30 respectively) and spaghetti (classified as n°5) were produced on a laboratory extruder and dried in a pilot plant. Spaghetti were cooked for 8 minutes in boiled water (ratio: spaghetti/water=1:10).

The cooking value of spaghetti with 15% barley flour was of the same magnitude as the standard spaghetti. This value was poorer when 30% barley flour was included in the semolina. Also the sensory evaluation of firmness and stickiness significantly decreased with 30% of barley flour in the mixture (CAVALLERO *et al.* 2000).

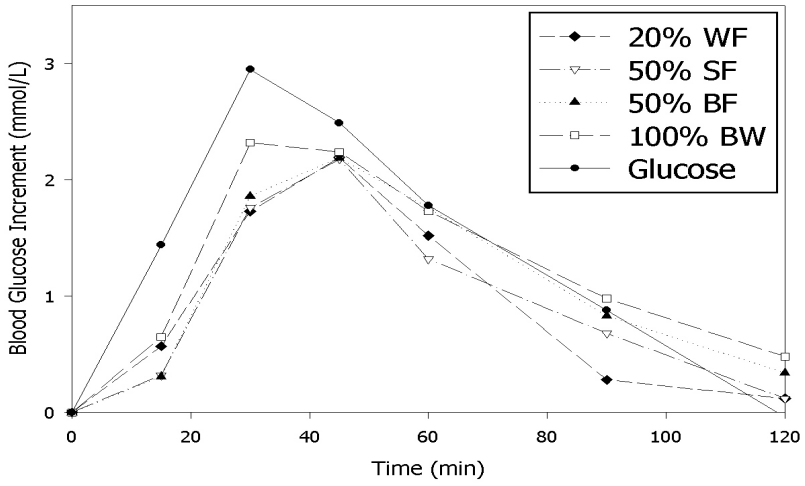
β-Glucan content of pasta was not affected by the processing and cooking (Table 2), and is close to predicted values reaching nutritionally interesting levels with the highest barley flour/wheat flour ratio (CAVALLERO *et al*. 2000).

Table 2. β-glucan content and cooking value of spaghetti made with durum wheat semolina and a mixture of durum semolina-barley flour (85:15, 70:30) (CAVALLERO *et al*. 2000)

	β-glucan content	Cooking value (%)
Spaghetti barley 15%	1.3%	90-92
Spaghetti barley 30%	2.5%	75-80
Spaghetti semolina	0.5%	100

The postprandial glucose response was determined after consumption of the different bread meals and related to β-glucan content (Figure 1). The mean peak value of blood glucose was found at 30 min for BW bread, whereas for the other experimental breads the peak was reached at 45 min. However, 30 min glucose values resulted in significant differences only for 50% SF and 20% WF bread compared to 100% BW bread. Similarly, by indexing the incremental area under the curve of bread, compared to pure glucose or to 100% BW bread (to calculate GI), only WF and BW gave a significant difference (CAVALLERO *et al*. 2002). Nevertheless, a clear negative trend can be discerned for GI indicating that barley β-glucan is dose-effective in reducing the blood glucose response to bread.
Regarding all these aspects, barley represent an excellent starting point for the development of functional foods.

Figure 1. blood glucose concentration increment in healthy volunteers following ingestion of glucose and different bread meals. Values are means, *n*=8 (except for 20% WF: *n*=7). WF= water-extracted fraction; SF= sieved fraction; BF= barley flour; BW= bread wheat flour (CAVALLERO *et al*. 2002).



In the second experiment, the results achieved evidenced that genotype and environment influence tocol content of barley (Cavallero *et al*. 2004) and it has been possible to identify varieties and locations with a significant higher tocol production. While genotype fingerprint is visible in the tocol isomer relative ratio, the environment impact on tocol concentration is not clearly understood.

The naked genotypes examined, in spite of their lower total tocol content, present significant amount of the most interesting isomers (γT3 and δT3) with the perspective to develop high-value genotypes for human nutrition by starting a breeding program from naked genotypes with these characteristics.
For this purpose more naked barley genotypes will be evaluated in the near future to define the genetic heritability qualities in barley and to adopt a selection method to assemble alleles for these traits and for grain yield.

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A Functional Genomics Approach for Improving Amino Acid Composition in the Developing Barley Grain

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Abstract

In Denmark barley grain is a major feed source for pigs and chickens despite the fact that the content of the essential amino acids lysine, methionine and threonine are at sub optimal level. This deficiency results primarily from the shortage of these amino acids in the major group of storage proteins, the hordeins. The second problem is that hordeins contain large quantities glutamine and proline that are not fully utilized but are degraded and the nitrogen excreted in the urine into the environment.

Our objective is to get more insight into amino acid metabolism in the barley grain at the molecular level and thereby provide a foundation for breeding of barley with a better nutritional quality. The strategy is to generate "pathway specific microarrays" for developing barley grains where a comprehensive set of genes involved in nitrogen mobilization; transport and amino acid metabolism are assembled. Genes of major significance for carbon and lipid metabolism are also included in order to assess the interconnections between the basic metabolic pathways in the grain. The collected target materials used for microarrays are grains of barley cultivars and a high lysine mutant, *lys3a* from the field grown under different nitrogen regimes.

Introduction

Lysine, methionine and threonine are only present in low amounts in the barley storage proteins, the hordeins. This is a major problem since barley primarily is used as the feed source for monogastric animals. In order to obtain the optimal amino acid compositions in the feed large-scale supplementation is required with soybean protein, microbial derived lysine and threonine and industrial synthesized methionine. The prolamins termed B and C hordeins are the two major groups that account for 40 – 50% of the total seed protein. The B and C hordeins constitute 95% of the total hordein fractions while the D and γ hordeins comprise the remaining 5%. The second problem is that hordeins possess a high amount of the amino acids glutamine and proline. These cannot be fully utilized but are degraded and the nitrogen excreted in the urine making a significant contribution the environmental nitrogen load in regions with intense livestock production. Excess nitrogen fertilisation attenuates this problem since it results in a preferential synthesis of the hordein fraction rather than the lysine-rich endosperm proteins, known as glutelins and albumins (SHEWRY 1980).

The three essential amino acids are synthesized in plants from aspartate via different branches of the aspartate family pathway. Numerous transformation studies have been performed in order to increase the levels of the nutritional limiting amino acids in seeds. The strategy has typically been expression of microbial genes encoding the two key regulatory enzymes in the lysine biosynthetic pathway, aspartate kinase (AK) and dihydrodipicolinate synthase (DHPS) that are not like their endogenous counterparts feedback regulated by threonine and lysine. However, although significant increases of the free pool of these amino acids were observed in developing seeds most studies have only found marginal increases in the total content in the mature seed (SHAUL & GALILI 1992; KARCHI *et al*. 1994; BRINCH-PEDERSEN *et*

al. 1994; BRINCH-PEDERSEN *et al.* 1996). Using a *Corynebacterium* feedback insensitive DHPS FALCO *et al.* (1995) obtained major increases in total lysine but very high levels were accompanied by seed germination problems. These studies further revealed that elevated lysine content triggers extensive lysine catabolism by lysine-ketoglutarate reductase/saccharopine dehydrogenase (LKR/SDH). In agreement with this slight increase in lysine were observed in a T-DNA knockout mutant of this gene in *Arabidopsis thaliana* (ZHU *et al*. 2001). The latest approach has been the manipulation of both the biosynthesis and catabolism of lysine in *Arabidopsis thaliana*. The expression of a bacterial insensitive feedback DHPS showed an 80-fold increase in seed free lysine level, when coupled to the knockout of the LKR/SDH enzyme (ZHU & GALILI 2003). The significant increase of methionine and lysine had some unexpected consequences like germination retardation effects. The described examples are only a few of several attempts to understand the complex metabolic regulatory mechanisms of the three essential amino acids lysine, methionine and threonine.

Over the last 40-50 years many attempts have been made to obtain higher lysine levels in seeds by using mutagenesis, selection in tissue culture or searching for spontaneous variants. The first successful approach led to the identification of the high lysine maize mutant *opaque2* (MERTZ *et al*. 1964). The *Opaque2* gene is a basic leucine zipper class (bZIP) transcriptional factor expressed in maize endosperm. Research has demonstrated that the transcriptional factor regulates both the LKR/SDH gene of lysine catabolism and the activation of the maize prolamine biosynthesis. Mutation in the *Opaque2* gene results in the synthesis of lysine poor storage proteins and reduced catabolism of lysine. Similar approaches have been directed in barley to obtain identical improvements of the seeds. Among many mutants a high lysine mutant has been identified in barley, *lys3a* (from Risø 1508) with a reduction of 50 – 60% of lysine-poor hordeins and no less than 44% increase of lysine. The N-load into the environment was reduced 20% when feeding pigs with the high lysine mutant (MUNCK 1992). However, the barley mutant possesses 10 – 20% lower yield compared to the best conventional cultivars in spite of comprehensive breeding efforts.

The nature of the *lys3a* gene and the mutations is still elusive. It is apparent that the mutations confer pleiotropic effects. Hordein biosynthesis is regulated by a number of transcription factors like BPBF, BLZ and GAMYB. Besides, also methylation of the hordein gene promoters is affecting transcription (SØRENSEN 1992).

The complexity of amino acid and storage protein metabolism and the pleiotropic effects conferred in mutants warrants the implementation of more global techniques for elucidating the key regulatory mechanism. In the present project we would like to increase the knowledge base on genes involved in amino acid transport, mobilization and metabolism in the barley grain by microarray analysis. We have also included major genes of interconnected pathways as carbon and lipid metabolism to gain more insight into the basic metabolic pathways in the grain. The strategy is to generate "pathway specific microarrays" for amino acid metabolism in developing barley grains. In our initial approach we will analyse the effect of different nitrogen supply to conventional grown barley as well as the effect of the *lys3a* mutations on the gene expression profile. Through microarray analysis the understanding of the complex regulation of amino acid metabolisms could provide the knowledge required for increasing the level of essential amino acids and at the same time reduce the amounts of proline and glutamine.

Material and Methods

Plant Material

We collected barley spikes 20 days after pollination (DAP) from winter barley (Clara) grown on normal nitrogen condition (60 kg/ha) and on high nitrogen (120 kg/ha) and from the field where no extra nitrogen was added to the soil. Similar materials were collected from two

spring barleys (Barke and Prestige) and a high lysine mutant barley *lys3a*. The material was collected from field trials at Sejet Plant Breeding, Horsens, Denmark.
From another location in Denmark, Flakkebjerg, barley spikes (20 DAP) were collected from two spring barleys (Barke and Cork) grown under different levels of nitrogen (no added nitrogen, 60 kg/ha and 120 ha/kg).

RNA Extraction and Cy3/Cy5 - Labelling
RNA was extracted from developing barley grains using TRIZOL REAGENT™ and Dynabeads according to manufacturer's instructions from SIGMA and DYNAL® Biotech, respectively. The extracted mRNA of the control and sample were Cy3/Cy5 (Cy™Dye Post-labelling Reactive Dye Pack, Amersham Biosciences) labelled according to the manufactures manual from DYNAL® Biotech.

Barley cDNA Library
Two tissue specific cDNA libraries of *Hordeum vulgare* cv. Morex were obtained from CUGI (Clemson University Genomics Institute), namely the HVSMEi (20 DAP spike) and HVSMEk (Testa/Pericarp) barley EST libraries in pBluescript SK(-).

Growth of cDNA Library EST Clones
The growth of EST clones was modified according to Holloway *et al*. (2003). A 96-well plate was prepared containing 150 µl sterile LB Ampicillin (100µg/ml). Each well was inoculated with a pipette tip from the frozen EST clone cultures. The plate was incubated 16-17 hours (h) in 30^{o}C shaking with 220 r.p.m.

Polymerase Chain Reaction
The PCR reactions were carried out from 1.5 µl overnight culture as template in a final volume of 50 µl in the 96-well plates. The inserts from the pBluescript SK(-) were amplified using forward primer, 5'-CGCGCGTAATACGACTCACT-3' and reverse primer, 5'-CGCGCAATTAACCCTCACTA-3' and the BD Advantage™ 2 PCR kit (BD Biosciences). The PCR program was 35 cycles of 95 ^{o}C for 1 min, 55 ^{o}C for 1 min and 72 ^{o}C for 3 min using the PTC-225, Peltier Thermal Cycler from MJ Research. 1.5 µl PCR product is mixed in running buffer and run at 100 V (agarose gel 1%) and stained in Ethidium bromide for 20 min.

Spotting and Hybridisation
PCR products were vacuum dried and dissolved in 50% DMSO to the final volume of 10 µl. The prepared DNA fragments were spotted on Poly-L-Lysine- (Menzel-Gläser®) and Aminosilane slides A+ (Schott, Nexterion). The spots were immobilized by cross-linking with 60 mJ/cm^2 (UV Stratalinker 1800). Slides were incubated for 20 min. in succinic anhydride (SA) pre-hybridisation buffer containing 12.5 mL sodium borate and 5 g SA in 280 mL 1-methyl-2-pyrrolidinone. The blocking was followed by a short wash in 0.1% SDS and diH_2O and quenched in boiling diH_2O followed by spin-drying. A second pre-hybridisation solution (50% formamide, 0.1%SDS, 0.1% BSA) was also performed incubating slides in a coplin jar for 1 h at 42 ^{o}C followed by three times wash in diH_2O and spin-drying.
The blocked slides were hybridised in a 30 µl hybridisation solution (deionised 50% formamide, 3 X SSC, 5 X Denhardt's reagent, 0.1% SDS, 100 µg/mL salmon sperm DNA and 20 µg/mL polyA DNA). The hybridisation was allowed to proceed for 16 h at 42 ^{o}C in a hybridisation chamber (MWG). After hybridisation, the arrays were sequentially washed

with medium stringency buffer (1 X SSC, 0.2% SDS), high stringency buffer (0.2 X SSC, 0.1% SDS) and post-wash buffer (0.1 X SSC) followed by spin-dry. Each microarray was scanned using an AppliedPrecision ArrayWoRX microarray scanner.

Results and Discussion

The first major task was to assemble the comprehensive set of unigenes for "pathway specific microarrays" among ~5000 attenuated genes from the two cDNA libraries. The careful assembly of unigenes had different criteria such as to choose similar genes present in both cDNA libraries to cover the possible tissue specificity. Furthermore, unigenes were collected that could be identified as an isoform (below 35% homology of non barley genes) or had a hypothetical function. The outcome of the selection resulted in ~1100 unigenes involved in different functions as e.g. transport, signal transduction, transcription, photosynthesis, amino acid metabolism. In Figure 1 the metabolic pathway of proline and arginine is exemplified to indicate the high coverage of important unigenes provided from the two cDNA libraries. A circle around the enzyme number indicates unigenes present in our "pathway specific microarrays".

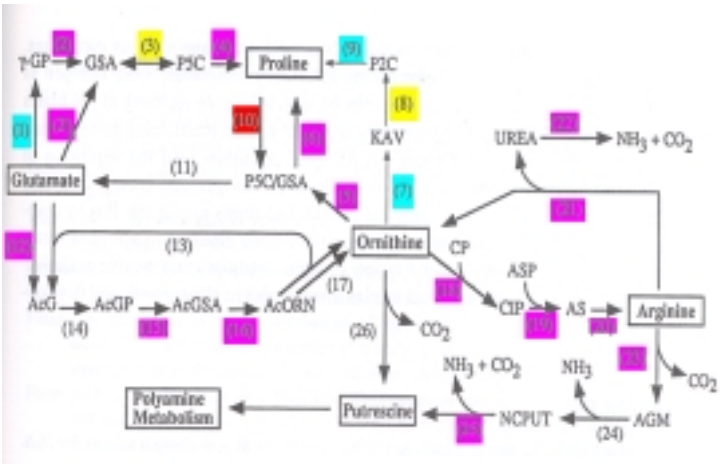

Figure 1. Biosynthetic pathways of proline and arginine (VERMA & ZHANG 1999). The dark box around the enzyme number indicates that the coding gene is present in our "pathway specific microarrays". The brighter box indicates a bacterial gene and the brightest is a spontaneous reaction where no enzymes are present.

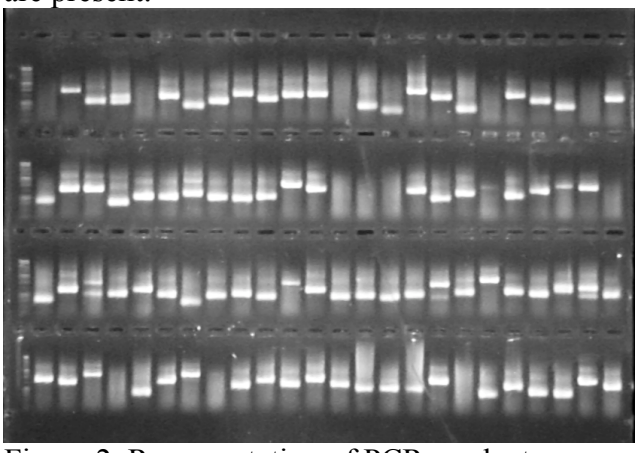

Figure 2. Representation of PCR products

The figure represents a typically first round agarose gel loaded with 96 PCR products with a 90% success rate.

The chosen unigenes were amplified by colony PCR (figure 2). The concentration of the PCR products ranged from 250 – 500 ng/µl. In the first round of the PCR amplification of the 1100 clones 10% of the amplification products were missing. The PCR reactions from those clones were repeated after we verified that the DNA was present. To obtain DNA amplification we modified the growth condition (37 ^{o}C instead of 30 ^{o}C) of the culture or/and used different Taq DNA polymerases (Advantage 2 from BD BioSciences, Herculase from Stratagene and the Eppendorf HotMaster) for PCR amplification. As a final result we obtained PCR products from 97% of the clones, i.e. a total of 1067 clones to be spotted. The non-amplifiable templates could be due to e.g. a high GC-ratio in the sequence or sequence repeats.
In the next stage amplified DNA fragments were spotted on slides coated with either the substrate Poly-L-lysine or Aminosilane. The procedure of blocking and hybridisation to determine the optimised conditions for the microarray slides are in progress.
The aim is to identify candidate genes that are significantly up or down regulated in response to different nitrogen regimes and the *lys3a* mutation involved in e.g. the amino acid metabolism. As a next step we will modulate the candidate gene expression using double stranded RNA antisense technology in both barley and the model plant *Arabidopsis*. Various mutant libraries of barley and *Arabidopsis* will also be screened. The elucidation of specific key candidate genes essential for regulation of amino acid and prolamine metabolism could provide a powerful resource for future breeding initiatives for barley with improved nutritional composition and lower environmental impact.

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Inheritance of (1-3) (1-4)-Beta-D-Glucan Content in Barley (Hordeum vulgare L.)

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Abstract

β-glucan is the soluble dietary fiber component and occurs in highest amounts in the endosperm of barley. Our objectives were to study the inheritance of β-glucan content in barley grains and mapping quantitative trait loci (QTL) associated with this trait. F_5-derived 107 lines from the cross of the 6-rowed waxy hulless barley cultivar originating from Japan 'Yonezawa Mochi' and the 6-rowed non-waxy hulless barley cultivar originating from Korea 'Neulssalbori' were measured for the agronomic traits and β-glucan level at four environments. Eighty-eight lines of them were analyzed for 90 genetic markers to map QTLs. The β-glucan content of recombinant lines was normally distributed with a range of 43.6-62.1g Kg^{-1} across environments. There was significant ($P < 0.01$) genotypic variation for β-glucan among the lines in the population at each environment and across environments. The broad-sense heritability estimates for β-glucan content ranged from 0.42 to 0.82 for individual environments. By one-factor analysis, 7 markers were found to be significantly associated with β-glucan content in each and across environments. Major QTL could be mapped on chromosome 1(7H) with genetic markers explaining 14.1 – 52.7% of the variability for the mean of β-glucan content across environments. In particular, the SSR marker HVM4 on linkage group 1 assigned to barley chromosome 1 (7H) had the greatest association with β-glucan content, explaining 52.7% of the variation in the population.

Keywords: barley; β-glucan; DNA marker; mapping; QTL

Introduction

Barley has been one of the major food crops as the source of starch. Recently, barley cereal food has become human diet of great interest because of its nutritional function. This trend is quite prominent in the world where it has been mainly used as either animal feed or brewery. Barley cereals are especially good source of water-soluble dietary fibers. β-glucan is the major component of these fibers. It is present in the cell walls surrounding the starch granules in barley endosperm. Structurally, β-glucan is polymers of beta-D-glucose with about 70% of the glucose residues 1,4-linked, and about 30% 1,3-linked. Overall size of the molecule and the relative order of 1,4 and 1,3 linkages can vary (ROUBROEKS *et al.* 2001). As a healthy diet for humans, these fibers are known to have hypocholesterolemic effects with

decreased serum and low-density lipoprotein cholesterol (KLOPFENSTEIN & HOSENEY 1987). High β-glucan content is not always good for end-use quality of barley. As contrasted with human diet, barley β-glucan is undesirable component for the brewery and animal feed. They contribute considerable viscosity to the mash and may cause slow wort filtration and haze formation in the beer. When fed with barley grains, β-glucan may also cause sticky droppings and decreased growth rates in poultry (GOHL *et al.* 1978). Accordingly, β-glucan content is an important factor for barley grain quality with respect to its end-use. As compared to other cereals, barley has relatively high content of β-glucan between 2 and 10% (BAMFORTH 1982).

Genetic enhancement for β-glucan content is one of the major concerns in Korean barley breeding to improve the end-use quality. With development of various molecular markers and extensive studies, quantitative trait loci were mapped in the barley genome and a few closely flanking markers associated with traits of interest have been identified in the world-wide. Practically, the breeders can select the genotypes with these markers to develop the enriched germplasms without field and laboratory evaluations. As for β-glucan content of barley grains a few regions of the genome were reported depending on different genetic background. JEUNG (2000) found six QTL for β-glucan content in the cross between Mtaz90-123 (waxy hulless and high fiber content) and MTH3860224 (high yielding feed barley line). HAN *et al.* (1995) also mapped 3 QTL for barley β-glucan on chromosomes 2 and 5 on the 6-row Steptoe/Morex mapping population. In the 2-row barley cross 'Harrington'/'TR306, major QTL affecting extract β-glucan content were mapped on chromosomes 3, 6 and 7 (MATHER *et al.* 1997).

Although its functional property is quite prominent, there is little knowledge of the β-glucan content of Korean barley cultivars under various genetic and environmental background. The objectives of our studies are to elucidate genomic regions for β-glucan content of barley grains and to determine genetic and environmental effects on this trait.

Material and Methods

A total of 100 barley accessions including Korean barley varieties and foreign introduction lines were primarily evaluated for β-glucan content (data not shown). A population of F_5-derived lines derived from the cross between a Japanese barley cultivar with low β-glucan content, 'Neulssalbori' was developed and used for map construction and QTL analysis. Both parental genotypes had 6-rowed head with naked kernel types.

One hundred seven lines and the parents were grown in four environments: Suwon (2001 2002, and 2003) and Jinju (2002). Experimental plots were arranged in a randomized complete block design with two replications. The plants of individual lines were sown in the plots with 3-m row length and 40-cm row spacing. Cultivation of plant materials including

the quantity of sowed seeds and fertilization followed the standard cultivation method for winter cereal crops developed by Rural Development Administration (R.D.A.) in Korea. In general, seeds were sown at a rate of 13-14kg/10a. Individual genotypes were evaluated for agronomic traits such as heading date and plant height in the field. Bulked seeds harvested from individual genotypes were measured for β-glucan content. Measurement of β-glucan content was followed by McCleary method which was dependent on a streamlined enzymatic reactions and absolutely specific for mixed-linkage β-glucan (McCLEARY & MUGFORD 1997).

For DNA marker analysis, DNA was isolated from a bulk of fresh leaf tissues of 2-week-old plants. RAPD, STS, SSR, and AFLP analysis were performed according to the protocols described by JEUNG (2000), respectively. Segregation data of 88 DNA markers and two morphological markers were analyzed and linkage map was partially constructed using MAPMAKER/EXP3.0. Linkage groups were assigned to the barley chromosomes on the basis of anchor markers of the barley frame map. For single-marker approaches to detect QTL associations between β-glucan content and markers on linkage groups were tested with one-factor analysis of variance using PROC GLM of SAS. Data from each environment were analyzed separately, and also a combined analysis of variance for all environments were conducted.

Results and Discussion

The parent, 'Yonezawa Mochi' always exhibited significantly higher β-glucan content ($P < 0.05$) than the other parent, 'Neulssalbori' at each environment and across environments (Table 1). The mean β-glucan content across all lines in the population was not significantly different from the mean of the parents for each environment and across environments. The β-glucan values of the lines in the population were normally distributed for each and across environments (data not shown). There was significant transgressive segregation with lines from the population having higher or lower β-glucan content than 'Yonezawa Mochi' and 'Neulssalbori', respectively.

There was significant ($P < 0.01$) genotypic variation for β-glucan among the lines in the population at each environment and across environments. The genotype × environments interactons, i.e., genotype × locations for 2002 or genotype × years for 2001-03, for β-glucan content were also significant. The correlations for β-glucan content of lines were highly significant between the means of individual environments and the range of its values were 0.6-0.73. Some lines exhibited constantly higher or lower β-glucan content than the others in spite of different locations and years.

The broad-sense heritability estimates for β-glucan content ranged from 0.42 to 0.82 for individual environments (Table 1). Even if the trait of β-glucan content is quantitatively

inherited, the observed heritability estimates in this study suggest that the selection for this trait on the single plant basis can be quite effective for the next generation. In oat, similar range of heritability estimates in the broad-sense was observed in grain β-glucan content

Table 1. Range and means of β-glucan content (unit: g Kg^{-1}), broad-sense heritability estimates for the parents and a population for individual environments and across four environments

	01 Suwon	02 Suwon	02 Jinju	03 Suwon	Across Environments
Parents					
Yonezawa Mochi	61.0	60.9	56.8	65.6	61.1
Neulssalbori	40.9	39.6	42.7	39.7	40.7
Population					
Range	38.1-66.5	41.9-61.6	38.3-61.9	44.0-67.8	43.6-62.1
Mean	52.8	50.8	49.9	54.1	51.9
LSD‡	9.3	6.5	8.9	6.7	7.8
h^2 (broad sense)	0.42	0.76	0.71	0.82	0.86

‡ Least significant difference for comparing the means of individual lines at $P = 0.05$

(KIANIAN *et al.* 2000; HOLTHAUS *et al.* 1996). Based on intermediate-high level of genetic factors in the expressed phenotypic variance and the existing genetic variability of barley and oat germplasms, Long-term goal of genetic gain by selection will be achieved for the improvement of β-glucan content.

Linkage analysis of the 90 marker loci resulted in the formation of 13 linkage groups with 17 markers remaining unlinked. Some of these linkage groups were assigned to barley chromosomes by using STS and SSR markers as anchors. More anchor markers with known genomic regions were required to accurately place marker loci on the linkage groups. Single-factor analysis of variance was conducted for individual markers mapped to the linkage groups to identify their significant association with β-glucan content. Seven markers consisting of one morphological trait (waxy gene), 2 SSR , 1 RAPD, and 3 AFLP markers were found to be significantly ($P < 0.01$) associated with β-glucan content in each and across environments (Table 2). Some of these markers could be assigned to individual barley chromosomal regions by anchor positions. In particular, the SSR marker HVM4 on linkage group 1 assigned to barley chromosome 1 (7H) had the greatest association with β-glucan content, explaining 52.7% of the variation in the population. The mean β-glucan content of individuals homozygous for HVM4 marker allele from 'Yonezawa Mochi' was 55g Kg^{-1},

which was higher than that from 'Neulssalbori' (48g Kg^{-1}) across all environments. Some AFLP markers such as AFYN37-1, AFYN60-1, AFYN61-1, and AFYN64-2 were significant only at Jinju environment in 2002.

When considering small population size in this study, the magnitude of the QTL identified in the chromosome 1 (7H) might be over-estimated (BEAVIS 1998). Though, waxy gene has been previously implicated as having a significant influence on the β-glucan content (WOOD et al. 2001; JEUNG, 2000). Our results also revealed that the genomic regions around waxy locus under 'Yonezawa Mochi'-originated genetic background significantly contributed β-glucan content. Genetic markers mapped in these genomic segments may be available for selection of QTL associated with barley β-glucan content.

Table 2. Markers significantly associated with β-glucan content of barley grains at the 0.01 probability level in each and across environments based on one-factor analysis. The estimates shown in the table were calculated with the average β-glucan content values for individual lines across environments 2001-2003.

Marker	LG	Chr.	Pr	R^2 (%)	Genotypic means‡ (g Kg^{-1})		
					YY	YN	NN
Waxy	1	7H	***	45.9	55.2	53.7	47.9
HVM4	1	7H	***	52.7	55.1	53.2	47.8
HVM49	2	7H	**	15.2	54.2	50.8	50.5
					YY	Y_ / _N	NN
OpU01	1	7H	***	24.5		54.1	49.4
AFYN47_1	1	7H	***	14.1		53.9	50.4
AFYN29_3	7	?	***	14.8	54.6	51.0	
AFYN33_1	8	?	**	10.3		53.7	50.7

** ,*** significant at 0.01 and 0.001 probability levels, respectively

‡ YY designates homozygous 'Yonezawa Mochi' class; YN designates segregating class; NN designates homozygous 'Neulssalbori' class; Y_ designates segregating and homozygous 'Yonezawa Mochi' class; _N designates segregating and homozygous 'Neulssalbori' class.

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Development of New Waxy Barley Cultivars for Healthy Diet Purpose in Korea

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Abstract

Improvement of cooking quality and specific nutrition components of food barley is facing a new challenge with the growing awareness of the beneficial effects of a healthy diet. In our studies, various isogenic lines were developed with four genetic combinations of waxy/hulled, waxy/hulless, non-waxy/hulled, and non-waxy/hulless forms by backcrossing a waxy hulless Korean landrace, 'Masangwamaek' and a waxy hulless Japanese cultivar, 'Yonezawa Mochi' to the non-waxy hulled Korean cultivars, 'Kangbori' and 'Olbori', respectively. The effects of these genes were analyzed on the agronomic traits, cooking and texture properties. No typical differences were observed among isogenic lines for agronomic traits and yield except for winter-hardiness. The amylase content of waxy lines (2.1 - 2.9%) was lower than that of non-waxy lines (18.9 – 19.7%) regardless of hulled/hulless forms. The addition of waxy gene could promote gelatinization of grain starch at lower temperature and in a shorter time than non-waxy starch grains by 13 ^{o}C and 11 minutes, respectively. There was no significant difference between hulled and hulless lines for amylase content and cooking qualities. With respect to cooking quality and palatability, some superior varieties including a waxy and hulless barley genotype with two-row heads, 'Duwonchapssalbori' have been developed in an effort to introduce waxy gene into hulless barley as well as hulled barley in Korea. Hulless barley breeding programs would be reinforced for the production of functional human diets.

Keywords: barley; isogenic line; hulled/hulless; waxy/non-waxy; quality; functional food

Introduction

In Korea, barley has been used as one of the major food crops with a form of whole grain diets usually cooked with rice. Generally, the polishing barley grains are mixed and boiled with rice and eaten as a human diet. Most breeding efforts for Korean barley were made aiming at early maturity and high yield until 1970s. Since 1980s, various attempts have been made to improve quality for raising consumption through development of value-added barley products. One of them was to introduce waxy gene into the cultivated barley germplasm to improve palatability and cooking quality. The wax locus is known to be responsible for the starch component and content in seed endosperm (ONO & SUZUKI 1957) and located on the short arm of chromosome 1 (7H) (KLEINHOFS 1997). The barley endosperm with

recessively homozygous waxy type (wx/wx) at this locus has normally 2-10% amylase and 90-98% amylopection (ISHIKAWA *et al.* 1995).

Since the first waxy hulled barley variety, 'Chalbori' was developed via introducing waxy gene from a Korean landrace, 'Masangwamaek', development of waxy barley varieties has been more extensively conducted for hulless type rather than hulled type in Korea. Hulless barley grains easily released during trashing and lacking of glumelets, can be very useful for cooking and food processing without any special postharvest management. In our studies, the effects of waxy/non-waxy and hulled/hulless genes with different genetic background were evaluated on the agronomic traits and yield, chemical composition of grain endosperm and cooking quality of barley.

Material and Methods

Four different genetic combinations for waxy/non-waxy and hulled/hulless types were made: wxwxNN (waxy and hulled), wxwxnn (waxy and hulless), WxWxnn (non-waxy and hulless), and WxWxNN (non-waxy and hulled). In order to develop isogenic lines for these genotypes, a Korean barley variety with non-waxy and hulled type, 'Kangbori' and a Korean landrace with waxy and hulless type, 'Masangwamaek' were crossed and then recurrently backcrossed and selected for individual traits. For development of the other isogenic line population with different genetic background, a Korean barley variety with non-waxy and hulled type, 'Olbori' and a Japanese barley variety with waxy and hulless type, 'Yonezawa Mochi' were used. With respect to four genetic combinations, these isogenic population lines were evaluated for agronomic and yield-related traits in the field at Suwon, Korea. The harvested grain materials of individual isogenic lines were also measured for chemical composition and cooking quality in the laboratory.

Results and Discussion

The mean values of isogenic line groups for some agronomic characteristics are shown in Table 1. There were no significant differences among groups without regard to waxy/non-waxy and hulled/hulless genotypes except for winter-hardiness. 'Masangwamaek' was matured later than 'Kangbori', but no significant difference was observed between their isogenic progeny groups. This suggested maturity was not affected by waxy/non-waxy and hulled/hulless grain types. As expected, hulless genotypes showing higher winter-killed withering rates were weaker than hulled genotypes in winter-hardiness, but this trait was not related with the kernel waxiness. Similar result was observed for Olbori/Yonezawa Mochi combination. For grain yield, waxy and hulled genotype (wxwxNN) was highest, but was not significantly different from the other three genotypes including wxwxnn, WxWxnn, and WxWxNN. No specific combination of genes of isogenic lines for waxy/non-waxy and hulled/hulless types was apparent for high yield potential (Table 2).

Table 1. Agronomic characteristics of recurrent and donor parents and their isogenic progeny lines for waxy/non-waxy and hulled/hulless genotypes

Genotype	Days For heading	Days for maturity	Plant height	Head length	Winter killed rate	Earli-ness ¶	Short day response	Vernal-ization
	(days)	(days)	(cm)	(cm)	(%)	(days)	(days)	
Kangbori (R)‡	30.6	60.4	85.7	4.7	61.5	25	38	I
Masangwamaek (D)	37.1	75.2	85.6	7.7	100.0	33	64	II
wxwxNN	29.0	68.2	81.9	4.1	60.0	22	32	I
wxwxnn	29.0	68.0	82.1	4.4	28.5	25	45	I
WxWxnn	29.3	68.6	81.1	4.4	41.0	23	36	I
WxWxNN	28.3	68.0	80.8	4.1	34.5	22	32	I
Olbori (R)	30.2	69.7	91.3	4.1	24.0	27	37	IV
Yonezawa Mochi (D)	37.3	80.3	70.2	6.0	100.0	32	77	II
wxwxNN	30.7	70.2	85.3	3.9	16.6	31	49	III
wxwxnn	30.5	70.2	83.3	4.2	24.6	32	48	III
WxWxnn	30.7	70.3	90.5	4.6	20.4	31	48	III
WxWxNN	30.8	70.0	87.0	3.8	28.6	32	50	III

‡ R – recurrent parent, D – donor parent

¶ Earliness in narrow sense

Table 2. Effects of waxy/non-waxy and hulled/hulless genes of barley on yield and its related traits

	Isogenic genotype groups			
	wxwxNN	wxwxnn	WxWxnn	WxWxNN
1000-seed wt. (g)	28.5	26.5	26.9	29.4
1 liter wt. (g)	658.1	691.0	706.9	675.7
Yield (kg/10a)	331.0	317.6	313.6	307.7

The endosperm quality of barley for cooking property was evaluated with respect to waxy/non-waxy starch component and hulled/hulless kernel types in this study (Table 3). The rating values of hulled and hulless types for quality traits were almost similar each other within either waxy or non-waxy types. On the other hand, the waxy genotype gave superior physico-chemical and nutritional values for food as compared to non-waxy genotypes. As

expected, the average amylase content of the waxy genotypes was 2.1-2.9% and significantly lower than the non-waxy genotypes (18.9-19.7%). The presence of the waxy gene was associated with the elevated level of β-glucan content, a soluble fiber with functional property of lowering serum cholesterol in humans. About 2-3 times of more β-glucan was contained in the waxy barley endosperm than the non-waxy ones. Similar result was reported in XUE *et al*. (1997). Changes of physical property in the endosperm starch fractions were also evident with the presence of waxy gene. Water absorption and volume-expansion rates of the waxy grain endosperm were greater than those of the non-waxy grain endosperm. Temperatures required for gelatinization and the highest viscosity of grain starch were lower in the waxy type than those in the non-waxy type by 13 °C and 15 °C, respectively. These results indicated that cooking and food processing quality of waxy barley type was better than non-waxy type without regard to kernel hullessness and its interaction.

With respect to cooking quality and palatability, some superior varieties have been developed in an effort to introduce waxy gene into hulless barley genotypes as well as hulled barley genotypes (Table 4). In particular, a waxy and hulless barley genotype with two-row heads, 'Duwonchapssalbori' was first developed for healthy diet purpose in Korea. This variety has large grain size with 37.3g of 1000-seed weight and nutritionally high level of β-glucan (6.1%). With continuous efforts of incorporating winter-hardiness into hulless barley cultivars, it was possible to proceed the boundary line of hulless barley cultivation northward in Korea. It is expected that hulless barley breeding programs will be more reinforced for the production of functional human diets.

Table 3. Effects of waxy/non-waxy and hulled/hulless genes of barley on grain endosperm and cooking/processing quality

Traits	Isogenic genotype groups			
	wxwxNN	wxwxnn	WxWxnn	WxWxNN
Amylose content (%)	2.9	2.1	18.9	19.7
β-glucan content (cSt)	19.3	23.6	7.7	7.8
Water absorption rate (%)	258	257	238	225
Volume expansion rate (%)	434	438	412	395
Temperature for gelatinization (°C)	60.3	61.0	73.0	74.3
Time elapsed for gelatinization (min)	35.7	34.8	46.4	45.8
Temperature at highest viscosity (°C)	78.2	77.2	92.7	92.5
Solidification (Bu)	0	90	530	558

Table 4. Development of Korean barley cultivars by improving cooking quality and palatability

Factors improved	Six-rowed Non-waxy	Waxy	Hulless Waxy	Two-rowed Waxy Hulless
Variety	Kangbori Olbori	Chalbori Seodunchalbori	Chalssalbori Saechalssalbori Jinmichapssalbori	Duwonchapssal -bori
Temperature for gelatinization (°C)	74.0	62.5	61.0	61.0
Water absorption rate (%)	220	254	268	282
Cooking with rice	Pre-treat.	Simultaneous	Simultaneous	Simultaneous

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Genotypic Variations in Phytate-Phosphorus of Barley and Other Cereals

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Abstract

Phytate is a salt of phytic acid (myo-inositol-1,2,3,4,5,6-hexakiphosphate) and it is the most abundant form of phosporus (P) in seeds where it is used as a phosphorus reservoir during seed germination. This study describes the range of phytate-P in cereal genotypes of barley, wheat, triticale, rye and oat. Phytate is indigestible by humans and non-ruminant livestock. Phytic acid in feed grain seeds can bind with micronutrients (e.g., iron, manganese and zinc) and macronutrients (e.g., calcium and magnesium). Excessive non-digested phytate from feed could end up in animal manure. This could pollute the environment and cause eutrophication of waterways. Samples of 423 barley, 114 wheat, 38 triticale, 24 oat, and 8 rye genotypes samples were analyzed for phytate-P and total P on a wet and dry basis. The phytate-P percent of total P, on a dry matter basis, ranged between 2.6 and 70.2 for barley, 39.3 and 84.1 for wheat, 44.6 and 84.8 for triticale, 43.5 and 64.3 for oats, 52.1 and 63.5 for rye. The widest phytate-P variability was determined in barley compared to other cereal species. Results of the current study show that there is variability in phytate-P content in the genotypes of cereals analyzed. This variability could be exploited for the benefit of livestock feed and human food.

Introduction

Barley and other cereal genotypes may contain varying amounts of phytate-P, but the magnitude of this variability within specific Canadian barley and other cereal varieties is not fully known. Characterizing different Canadian barley and other cereal varieties in terms of their phytate-P levels could lead to selection and development of varieties for use in the livestock feed industry leading to more efficient in livestock production. There is inadequate information on phytate-P in various Canadian cereal varieties. The phytate-P levels could affect the livestock feed industry especially in Alberta where livestock industry is a major contributor to both domestic and export markets.

There are growing public concerns regarding the potential of environmental pollution due to increasing intensive livestock production. Environmental pollution represents a major constraint to the development of the hog industry regarding manure management. Nitrogen (N) and phosphorus (P) are the two major nutrients often associated with environmental contamination and therefore their levels in manure should be controlled (JONGBLOED & LENIS 1998). Dietary manipulations could offer the most cost-effective approach of minimizing the amounts of these nutrients excreted in hog manure (LENEMAN *et al.* 1993; SUTTON *et al.* 1999; GRANDHI 2000).

Supplementing non-ruminant diets with sources of extrinsic phytase has been proposed as a means of improving P, energy and amino acid retention in pigs and poultry (JONGBLOED & LENIS 1998). However, these benefits are not always achieved mainly because of the differences in the efficacy of phytase in different commercial sources (de LANGE *et al.* 1999). An alternative approach is to use ingredients with low phytate-P, such as low phytate-P barley genotypes, that will contribute to improved nutrient availability (de LANGE 1997; JONGBLOED & LENIS 1998). Low phytate cereal grains (such as barley, wheat, triticale, oat) have the potential of benefiting the primary producers, industry and consumers. The benefits could be through reduced phosphorus (P) pollution resulting from increased P availability in low phytate grain feeds fed to monogastrics, poultry and dairy animals. Low phytate grains also have the potential of enhancing the availability of elements such as zinc, manganese, iron, magnesium and cobalt. This could be of nutritional value to humans and livestock. The objective of this study was to determine and compare the range of phytate-P and total P content in Canadian cereals (barley, wheat, triticale, oat and rye) genotypes.

Material and Methods

Selection of Cereal Genotypes

Some of the barley genotypes in the present study were selected randomly from the germplasm pool to represent genetic diversity, selection history, pedigree and morphological differences. Other genotypes were included in the study based on prior knowledge or the perceived phytate levels. Known low phytate-P lines and mutants were included in the study as check lines. The other cereal genotypes of wheat, triticale, oat and rye were included in the study mainly for comparing with barley. These other cereal genotypes, within each group, were selected to represent genetic variability based on the pedigrees.

Analysis of Total P and Phytate-P

This experiment was conducted to determine the genotype and species influence on total P and phytate-P content in 630 samples comprising 423 barley, 117 wheat, 38 triticale, 24 oat, and 8 rye genotypes. For each genotype, 25 g were dried overnight at 60 ^{o}C, then ground to pass through 1-mm sieve using a Brinkman ZM grinder. The ground samples were dried in the oven at 104 ^{o}C for 3 hr before analysis. The moisture content of each sample was measured using the Moisture in Malt Gravimetric Method No. 935.29A.29C described in the Official Analytical Chemists – Association of Official Analytical Chemists (OAC 15th Edition 1990). Total phosphorus, as a percentage of wet weight and dry-matter weight, was measured using protocol No. 985.01 of Metals in Plants (OAC 15th Edition 1990). Phytate-P based on wet weight and dry-matter weight was determined using the sensitive method for the rapid determination of phytate described by HANG and LANTZSCH (1983).

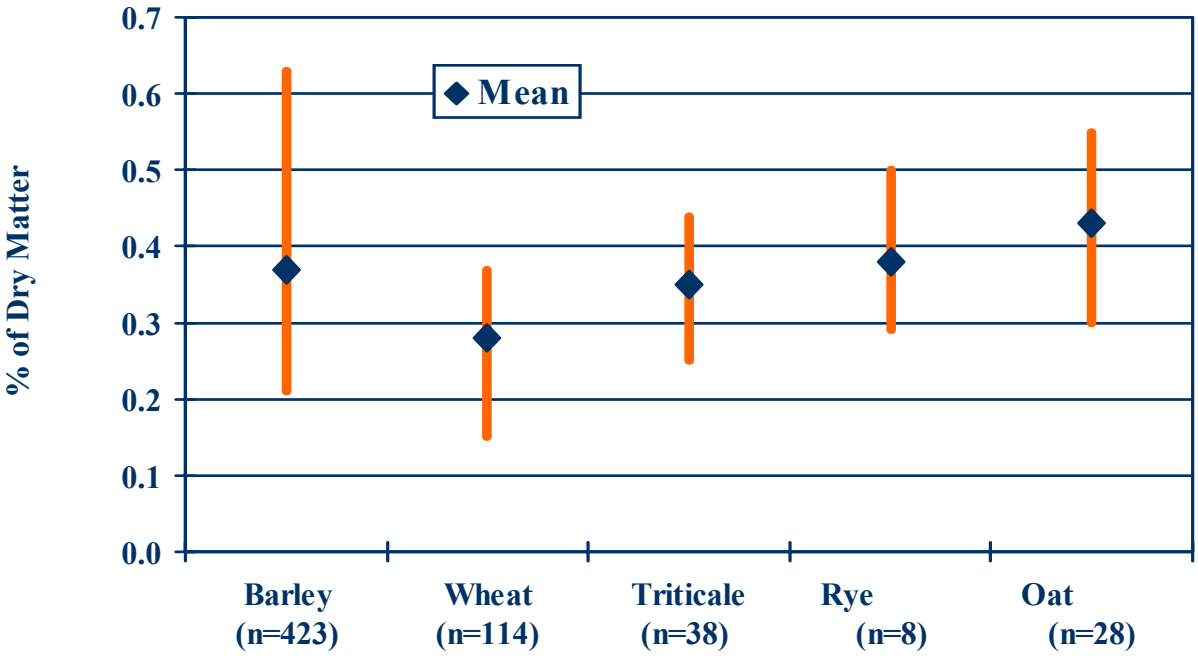
Results and Discussion

Percentage of Total P and Phytate-P Content on Dry Weight Basis

A wide range in total P content, on dry matter basis, was observed in genotypes within different cereal species (Figure 1). Wheat showed the lowest total percentage P in the grain. Triticale and

rye were close to barley in percentage total P, whereas oat was slightly higher in percentage P content compared to barley.

Fig. 1. Range of Total Phosphorus for Cereal Grain



Barley

The total P and percentage phytate-P on a dry-weight basis was examined using the mean value, and the range (minimum and maximum) values (Fig. 1 and Fig. 2). The results showed genotypes had more phytate-P variability (Fig. 2) compared with total P (Fig. 1). The high variability in phytate-P suggests the existence of genetic diversity in phytate-P among the barley genotypes tested in this study. As expected, the range of phytate-P values was lower compared to the range of total P (Fig. 2). This test did not account for the separation of genetic and environmental effects.

Wheat

The mean total P in wheat was lower by 25% compared to the mean percentage total P in barley (Fig. 1). The range of total P in wheat was different compared to the range in barley, as was the range in phytate-P (Fig. 2). Generally, wheat showed less variability in phytate-P compared with barley. Most of this was to one sample that was very low in phytate-P. This could be due to a combination of genetic or environmental effects.

Triticale

Triticale showed a mean total P value close to barley (Fig. 1) except the maximum range value (0.63) of barley was higher compared to the value (0.44) of triticale. The mean percentage phytate-P in triticale was slightly more than the mean in barley. There was significantly less phytate-P variability in triticale compared to barley (Fig. 2).

Rye

The mean total P value in rye was similar to the value in barley (Fig. 1). The percentage of phytate-P in rye was a minimum of 0.15 in rye compared to 0.01 in barley. The results indicate there was less variability in rye phytate-P compared with barley (Fig. 2). This low variability could indicate that the rye samples in this study are not different in phytate-P content but this does not rule out other rye genotypes that were not included in this study.

Oat

The results show the mean total P value of oat was higher by 16% compared to the value of barley (Fig. 1). The mean percentage phytate-P value in oat was higher by 35% compared to the value in barley (Fig. 2). The maximum percentage phytate-P range of 0.28 in oat was similar to barley although barley showed a lower minimum range of 0.01 compared to 0.17 in oat.

More research is required to elucidate the impact of low phytate-P barley in the livestock industry. There is need for further studies of phytate-P in cereals, on a large scale, involving animal feeding trials to compare cereal genotypes with low phytate-P to those with high phytate-P. This study has pointed out that there is variability in phytate-P among cereal species in the current study. This variability could be exploited to benefit the industry and advancement of agricultural knowledge.

Conclusions

This study has demonstrated that there are substantial differences in the percentage of total P and phytate-P of the current samples analyzed. Barley appears to have more variability in phytate-P content compared to wheat, triticale, rye, oat or ground feed samples in the current study and the low phytate lines are significantly lower in total phytate-P. Based on the results of this study, there are possibilities of making relatively large improvements in breeding and selecting for phytate-P content in barley.

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Improving the Plumpness and Yield of Barley in Western Australia

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Abstract
In order to identify quantitative trait loci (QTLs) controlling grain yield, average grain weight and grain plumpness, the progeny of the mapping population VB9104 x Dash and their parents were grown at three locations (Esperance 2001, Kendenup 2001 and Kendenup 2002) in single replicate trials. QTLs for grain yield, average grain weight and grain plumpness were generally located on chromosome 5H and to a lesser extent on chromosome 2H. Numerous QTLs were common to several locations. The marker *Bmag0337* on chromosome 5H was highly associated with grain yield and average grain weight. The positive alleles for grain weight and grain plumpness were from VB9104 and Dash, respectively.

Introduction

The Western Australian cereal belt (28°S to 35°S, 114°E to 122°E) is often described as exhibiting a Mediterranean environment. Crop growth is reliant on rainfall with 60 to 70 percent of the annual rainfall received in the growing season between May and October. Crops emerge or initiate their first leaves just before the winter solstice. Flag leaf appearance and subsequent grain fill occurs in spring as daily radiation receipt and air temperature is increasing and rainfall is declining. Low moisture supply and/or high temperatures in isolation or in combination can have significant effects on the yield, grain size and grain quality of barley (*Hordeum vulgare* L.) (SAVIN 1996; PAYNTER & YOUNG 2004).

Over 80 percent of the area currently sown to barley in Western Australia are with cultivars suitable for the malting market (PAYNTER *et al*. 2004). The reason that more feed barley is not grown is related to the price differential between feed and malting, typically $20 to $40/t. In order to make the same return from feed barley as from malting barley, feed barley needs to yield at least 25% more (pool feed price $160/t, malting premium $40/t). Even if only 50 percent of a grower's malt crop is received as malting, feed barley needs to yield at least 12% more. Very few feed barley cultivars out yield malt cultivars by over 12% (LITTLEWOOD 2003) to make growing a feed cultivar an economic alternative in Western Australia.

Producing grain to meet industry receival standards is a challenge. The target is hectolitre weight above 65 kg/hl, protein range between 10% to 12% (db), more than 70% of grains above a 2.5mm slotted sieve and a brightness above 58 Minolta L units (FOX *et al*. 2003). Whilst choosing the appropriate cultivar for the environment is important, so too is sowing at the correct date for the maturity of that cultivar in combination with the right rotation and fertiliser, appropriate foliar, seed and in-furrow disease control, insect management and weed management (YOUNG 1995). Even getting all things right does not guarantee success due to our reliance on rainfall during the grain filling period to finish the crop off.

The main cultivar grown in Western Australia over the last twenty years has been Stirling barley (PAYNTER *et al*. 2004). As barley breeders seek to breed and release cultivars (ie. Franklin, Gairdner and Baudin) with improvements in grain yield and malting quality over Stirling for this environment, they have also reduced grain plumpness.

The question for the breeders is how do they achieve genetic improvements in yield, disease resistance and malting quality concurrent with grain size. This is the subject of a paper by COVENTRY *et al.* (2003) that examines prospects of marker assisted selection to breed for improved grain shape. Such breeding studies are being initiated in Western Australia.

This paper describes some preliminary work to map the QTLs for grain yield and grain quality using the VB9104 x Dash mapping population as a precursor to developing a marker assisted selection-breeding strategy in Western Australia.

Material and Methods

Background

The population used for this study, VB9104 x Dash (182 lines), was developed for the Grains Research and Development Corporation's National Barley Molecular Marker Program by D.B. MOODY *et al*. (unpublished data). VB9104 (Europa/IBON7.148) is a high yielding, plump grained crossbred from Victoria and Dash (Chad/Joline//Cask) is a narrow grained, high yielding variety from the United Kingdom.

The VB9104/Dash lines were planted at two locations (Esperance [ES01] and Kendenup [KD01]) in 2001 and two locations (Katanning and Kendenup [KD02]) in 2002. The site at Katanning was not included in this analysis due to poor growing conditions. They were sown in single replicate, randomised designs with parental cultivars as duplicate random controls. An additional nine duplicate controls were also sown at each site. Each line was planted in a 5-m long by 5 row wide plot with a precision seeder. The middle 3-m of each plot were harvested and weighed and the whole plot sample retained for subsequent analysis.

Grain Quality Analysis

Grain was de-awned and cleaned over a 1.5-mm sieve in a Rationel Kornservice Model SLN3 cleaner. Physical grain analysis was then performed on each sample: hectolitre weight (kg/hl), screenings percentage (Pfeuffer Sortimat with a 2.2-mm, 2.5-mm and 2.8-mm sieve stack) and average grain weight (mg, db) from a 1000 grain count.

QTL Analysis

A molecular linkage map of the Dash x VB9104 population was constructed according to CAKIR *et al*. (2003), which consisted of 164 markers spread over the seven chromosomes. The detail map location of molecular markers were listed in the barley consensus maps (CAKIR *et al.* 2004). QTL analysis was performed using the method described in ECKERMANN *et al*. (2001). The model fitted was a linear mixed model in which QTL effects, residual genetic effects, and environmental effects were included together in a one-stage approach. QTL effects were modelled using regression interval mapping, where each interval along the genome was scanned for putative QTLs. Intervals containing significant QTL effects were added to the model as cofactors, the genome was rescanned, then a final model was fitted to determine the significance of each QTL in the presence of cofactors. Residual genetic effects were included as random effects and environmental effects such as row and column trends were included as fixed or random effects. A logarithm of odds ratio (LOD) > 3 was used to claim existence of a QTL for the trait.

Results

Grain Yield

Across the three sites, the difference in grain yield between parental lines was less than 400 kg/ha. Dash was generally lower yielding than VB9104 (Table 1). There was a large range in grain yields of the DH lines at each location. QTLs were identified on chromosomes 1H, 5H, 6H and 7H for grain yield.

QTLs on chromosome 5H associated with the marker *Bmag0337* (positive allele from Dash) were detected at two locations (LOD > 6, Table 2), which accounted for approximately a third of the genotypic variation. QTLs on 6H associated with marker *P11/M50.13* (positive allele from VB9104) were found at two locations (LOD ~ 4) and accounted for 10% to 20% of the genotypic variation.

Table 1. Distribution of grain yield and grain quality traits across three locations

Location	VB9104	Dash	Population mean	Range
a) Grain yield (kg/ha)				
Esperance 2001	1658	1290	1534	504-2233
Kendenup 2001	1832	1683	1506	981-1930
Kendenup 2002	1642	1505	1515	966-1985
b) Average grain weight (mg, db)				
Esperance 2001	52.0	34.5	42.7	33.6-52.6
Kendenup 2001	48.4	35.1	39.7	30.3-47.4
Kendenup 2002	43.4	29.3	36.2	25.7-44.8
c) Screenings (% < 2.8mm)				
Esperance 2001	7.4	60.6	29.4	8.4-74.8
Kendenup 2001	8.2	44.3	34.0	11.1-89.8
Kendenup 2002	40.3	89.0	75.8	38.2-97.4
d) Screenings (% < 2.5mm)				
Esperance 2001	1.0	23.3	6.4	1.2-42.4
Kendenup 2001	1.4	9.7	7.0	1.9-36.4
Kendenup 2002	8.8	42.0	27.3	6.6-73.0
e) Screenings (% < 2.2mm)				
Esperance 2001	0.2	8.0	1.4	0.1-32.1
Kendenup 2001	0.5	2.1	1.4	0.6-6.3
Kendenup 2002	1.2	10.4	5.4	0.6-31.1

Grain Weight and Plumpness

The variation in average grain weight (mg, db) and grain plumpness (% < 2.8 mm or 2.5 mm or 2.2 mm) amongst lines in the VB9104 x Dash population and between parents was large (Table 1). Grains of the parent, Dash were lighter than those of VB9104 and produced grain with a higher screenings.

QTLs on chromosome 5H accounted for approximately half of the genotypic variation in average grain weight. QTLs detected at ES01 on chromosomes 1H and 2H each accounted for less than 5% of the genotypic variation. The marker associated on chromosome 5H with grain yield, *Bmag0337*, was also found to be associated with average grain weight at all thee locations (LOD > 12, Table 2). The grain weight allele was contributed from VB9104 rather than Dash as found for grain yield. This marker accounted for approximately a third of the genotypic variation. Another marker, *P12/M55.2* on chromosome 5H was detected at two locations an accounted for less than one tenth of the genotypic variation.

QTLs controlling grain plumpness were found on 1H, 2H, 3H, 5H and 7H (Table 2). QTLs contributing most to grain plumpness were found on chromosome 5H, with a lesser effect of

QTLs on 1H, 2H, 3H and 7H. QTLs on chromosome 5H accounted for just under a half of the genotypic variation when measured on a 2.8-mm sieve at all three locations. When measured on a 2.5-mm sieve, they accounted for between a quarter to a half of the variation and on a 2.2-mm sieve, less than an eighth. The contribution of QTLs detected on the 2H chromosome to genotypic variation were variable depending on location. The contribution ranged from less than 10% (KD02) to nearly 90% (KD01).

Table 2. Presence of QTLs controlling grain yield and grain quality traits across three locations

Chromosome	Marker	Interval	Logarithm of odds ratio (LOD) Esperance 2001	Kendenup 2001	Kendenup 2002	Positive allele
a) Grain yield						
1H	P13/M49.16	13			3.6	Dash
1H	P12/M47.3	19			3.2	VB9104
5H	P12/M50.8	88	3.2			Dash
5H	Bmag0337	90	13.5		6.8	Dash
6H	P11/M50.13	121	3.9		3.8	VB9104
7H	P11/M47.17	142	4.6			Dash
b) Average grain weight						
1H	P13/M50.3	3	3.3			VB9104
2H	P13/M55.2	49	3.3			VB9104
5H	P12/M47.4	87			3.0	VB9104
5H	Bmag0337	91	15.0	15.0	12.6	VB9104
5H	P12/M55.2	100		5.2	3.6	VB9104
5H	P13/M55.3	102		7.8		VB9104
5H	GMS061	112		3.1		VB9104
5H	P11/M47.12	113	3.7			VB9104
c) Screenings (% < 2.8mm)						
1H	P12/M62.4	14	4.5			Dash
2H	P12/M47.5	34			5.7	VB9104
5H	P12/M50.8	88		4.9	3.1	Dash
5H	P12/M47.2	89	3.6			Dash
5H	P11/M62.9	92			3.6	Dash
5H	P12/M55.2	99	5.2	4.5	3.7	Dash
5H	P13/M55.3	101	6.9	5.5		Dash
5H	P11/M55.2	102			3.9	Dash
5H	GMS061	111	3.6	9.0	3.0	Dash
d) Screenings (% < 2.5mm)						
2H	P12/M47.5	34			4.6	VB9104
2H	P13/M55.2	49			4.5	Dash
5H	P12/M47.4	87		4.8	4.2	Dash
5H	Bmag0337	90			3.1	Dash
5H	P13/M55.3	101	4.3		3.5	Dash
5H	GMS061	111		3.7		Dash
e) Screenings (% < 2.2mm)						
2H	EBmac0415	39		3.5		VB9104
2H	HVM54	41		4.7		Dash
2H	P13/M55.2	49			3.7	Dash
2H	P13/M50.15	52	6.3			Dash
3H	P12/M55.4	70		3.1		Dash
5H	P12/M47.4	87		5.9	4.3	Dash
5H	GMS061	111	3.6			Dash
7H	P13/M62.2	144	3.5			Dash

For grain plumpness when measured through a 2.8-mm sieve, the following markers on chromosome 5H were detected at two locations, *P12/M50.8* and *P13/M55.3* or three locations, *P12/M55.2* and *GMS061*. When measured through a 2.5-mm sieve, the following markers were detected at two locations, *P12/M47.4* and *P13/M55.3*. When measured through a 2.2-mm sieve, the marker *P12/M47.4* was detected at two locations. All these markers were from the positive allele Dash indicating that they were associated with a narrow grain shape. Contributions to grain shape from the positive allele VB9014 were found to occur only on chromosome 2H. No markers were detected at multiple locations on any other chromosome.

Discussion

The development of cultivars with a grain shape and plumpness that assists growers to deliver a reliable supply of malting barley to the market is very important to the viability of the industry. The development of quantitative trait loci (QTLs) for a marker assisted approach to improving grain weight and plumpness concurrent with grain yield are important goals of breeding programs for Mediterranean type environments. The review of COVENTRY *et al*. (2003) concludes that most of the grain weight and plumpness QTLs mapped are coincident with genetic loci determining flowering date, plant stature and plant morphology. Often this causes pleiotrophic effects.

In this study, QTLs for average grain weight (mg, db) and grain plumpness (%) were found predominantly on chromosome 5H (Table 2). There are two developmental loci found on this chromosome associated with spring habit (*sgh2*) and earliness per se (*eps5Hl*) (COVENTRY *et al*. 2003). There may be an association between developmental loci on 5H and average grain weight in the VB9104 x Dash population as the grain yield and grain weight marker *Bmag0337* was also associated with flowering date when measured at one site (KD02).

The SSR marker *Bmag0337* has been detected in two other Australian mapping populations, VB9524 x ND11231-12 (EMEBIRI *et al*. 2003) and Alexis x Sloop (BARR *et al*. 2003). It is found on the Scottish Crop Research Institute SSR map (RAMSAY *et al*. 2000) and the Australian consensus maps of ABLETT *et al*. (2003) and KARAKOUSIS *et al*. (2003). It has also been associated with grain brightness (Minolta L) and grain yellowness (Minolta b) in the VB9104 x Dash population (LI *et al*. 2003; PAYNTER *et al*. unpublished).

The SSR marker *GMS061* was associated with average grain weight and grain plumpness in this study (Table 2). It has also been detected in the following Australian mapping populations: Alexis x Sloop (BARR *et al*. 2003), Franklin x Arapiles (ABLETT *et al*. 2003) and Sloop x Halcyon (READ *et al*. 2003). The *GMS061* marker is found on the consensus linkage maps constructed by RAMSAY *et al*. (2000), ABLETT *et al*. (2003) and KARAKOUSIS *et al*. (2003).

A number of markers were detected at numerous locations and associated with average grain weight and grain plumpness, *P12/M47.4*, *P12/M50.8*, *P12/M55.2* and *P13/M55.3* (Table 2).

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Selection of Barley Lines with Waxy Endosperm and Hulless Grains: Genotyping and Phenotyping

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Abstract

Traditional barley breeding has taken many years of selection based on phenotypic expression to develop lines for a commercial distribution. It is necessary to bridge the gap between plant molecular biology and conventional plant breeding. DNA-marker-assisted selection (DNA-MAS) accelerates the breeding process.

Here we present DNA-MAS of F2 lines with waxy starch and hulless grains. We used three types of co-dominant molecular markers. A high throughput DNA extraction system was used in this study. Genomic DNA was extracted from leaf segments of seedling plants, which enabled direct characterization of individual plants with molecular markers immediately followed by application of the DNA-MAS. Therefore, selected plants could be used for further breeding purposes in the same vegetative season.

Keywords: barley; hulless; waxy; marker assisted selection

Introduction

Hulless barley development is now more emphasised because of its great potential for feed, food, and industrial purposes. Waxy starch is often required together with hulless grain. The combination of hullless and waxy characteristics produces barleys high in soluble dietary fiber, especially β-glucans (ULLRICH *et al.* 1986).

Barley starch is composed of two glucose polymers - amylose and amylopectin differing in the way of branching of α-D-glucopyranosyl units (WHISTLER & DANIEL 1984). The amylose:amylopectin ratio affects the swelling power and gel characteristics, solubility, water absorption, cooking ability etc., which influence the main technological properties of the barley starch and therefore, also the grain quality for malting, food and animal feed production. Starch low in amylose (<10%) and high in amylopectin is known as waxy starch (WASHINGTON et al. 2000).

Amylose is synthesized by the granule-bound starch synthase I (GBSSI). The GBSSI is encoded in the *waxy* gene in cereals. The *waxy* gene is responsible for the amylose content in endosperm and pollen grain (NAKAO 1950; ROSICHAN *et al.* 1979). In most cereal species, *waxy* mutants lack any detectable amylose in the starch of endosperm. The insertion/deletion marker (DOMON *et al.* 2002) and the SSR marker which we have named Waxy06 $(AG)_6$ (unpublished data) can be both used for waxy character determination. Both markers lie in the promotor region of the *waxy* gene. The insertion/deletion marker specifically detects recessive *waxy*, dominant *Waxy*, and novel *Waxy* alleles. The Waxy06

Table 1. Waxy06 microssatellite marker detecting waxy alleles and distinguishing between cultivars carrying *Wax* or novel *Wax* alleles

Allele	ins/del	Waxy06
wax	600 bp	154bp
Wax	800bp	151bp; 160bp
novel Wax	1000bp	154bp; 156bp

marker detects all three types of alleles. Moreover, it distinguishes between genotypes carrying one type of alleles (Tab.1). However, Waxy06 does not specifically detect the *waxy* allele. Waxy06 generates a 154bp PCR product in cultivars bearing the *waxy* allele and in some hulless cultivars carrying the novel *Waxy* allele (unpublished data). In this study, we used the newly developed SSR marker to verify its possible use in DNA-MAS.
In our recent study (POLAKOVA *et al.* 2003) we have characterised a molecular cSNP (coding Single Nucleotide Polymorphism) (G/A) marker located in the fourth exon of the alpha-amylase gene named *Amy32b* (WHITTIER *et al.* 1987). We have found that the G allele is present in waxy hulless cultivars, whereas the A allele is present in covered and non-waxy hulless cultivars.

The progress in biotechnology holds a great potential for plant breeding as the time to produce new crop varieties with desirable characters is shortened. The main goal of this study was to select lines with low amylose content and hulless grain. To accomplish our goals, we used three types of co-dominant markers which detect superior alleles.

Material and Methods

Plant Material

Two spring barley hybrid combinations (HC) were selected for our trial. The line No94609D7 was crossed with the cultivar CDC Candle (1nd HC, designed No411) and the cultivar Nordus also with the cultivar CDC Candle (2nd HC, designed No412). CDC Candle is a waxy hulless (or naked, n) cultivar, bred at the CDC Saskatoon, Canada, registered in 1995 (labelling No HB 313 waxy). Nordus is a covered (cov) malting cultivar from Germany (NORDSAAT Saatzuchtgesellschaft mbH), registered for cultivation in the Czech Republic in 1998. The line No94609D7 is a covered breeding line of the spring barley with normal type of endosperm, bred at the same firm as Nordus. The parental materials were crossed under the glasshouse conditions and the F1 generation was grown in the field. The grain was harvested manually and sowed in the field in 2003. All barley trials were established in the breeding nursery (3.75 x 7.5 cm, hand sowing) after winter rape as a preceding crop.

Young leaves (vegetation phase 13-21 according to the DC scale by Zadoks) were sampled from 800 seedlings of both F2 populations. Samples were taken directly in the field without causing any damage to the plants. Leaves were frozen and stored at -80 ºC. 192 samples of each cross were randomly selected for the DNA analysis.

Important agronomic data (such as seedling emergence, stalk shooting, heading time, and leaf diseases attack) were evaluated for both crosses during the vegetation period. All plants were harvested during the full ripening. Randomly selected plants (123 from the cross No411 and 142 from the cross No412) were taken for assessment of the following yield parameters: number of tillers (NAT), number of spikes per plant (NSP), number of kernel per plant (NKP), grain weight per plant (GWP), 1000 kernel weight (TKW), number of kernel per spike (NKS), and grain weight per spike (GWS).

DNA Extraction

DNA from young leaves was extracted using a kit for rapid extraction (DNeasy® 96 Kit, Qiagen). The extraction protocol followed the manufacturer's instructions apart from the first step, which was slightly modified. 50mg of frozen leaves were directly transferred into the tubes containing carbide beads and mixture of the AP1 Buffer, RnaseA, and DX Reagent (Qiagen). All the other steps followed the Qiagen Handbook. DNA concentration and quality were evaluated after electrophoretic separation in 0.8% agarose gel and visualisation by means of ethidium bromide staining under the UV light. All DNA samples were diluted to the final concentration of approximately 100ng/µl.

Molecular Markers Analyses

Amy32b SNP analysis:

A 205bp PCR fragment containing the cSNP was generated using primer pair 5'AGCTCAGCCTCGGTCTCAGT 3' (forward) and 5'ACGTAGGCGTCTCTTCGTG3' (reverse) (Invitrogene). PCR amplification was performed in 20-µl reaction volume containing 1x Buffer with 15mM $MgCl_2$ (Qiagen), each dNTP at 200µM (Invitrogene), each primer at 0.1 µM, 1 unit of Taq DNA Polymerase (Qiagen), and 100ng of template DNA. PCR amplification was carried out for 2 min at 94 ºC, followed by 35 cycles of 30s 94ºC, 30s 58ºC, 30s 72ºC, with a final 5 min incubation at 72ºC. PCR products were checked by means of electrophoresis in 2% agarose gel after staining with ethidium bromide.

Afterwards, the CAPS analysis (Cleaved Amplified Polymorphism Sequence) was used to detect cSNP. The cSNP lies in the recognition sequence of the restriction endonuclease XhoI. 10µl of PCR product was incubated with enzyme mixture containing 1x Buffer (containing BSA, Fermentas) and 1U of XhoI (Fermentas) at 37°C for 60 min. The products of digestion were separated in 2% of agarose gel, stained with ethidium bromide and visualised under the UV light. Non-digested and digested PCR products corresponded to A or G genotypes, respectively.

SSR analysis:

PCR products of the Waxy06 marker were amplified using primer pair 5'CCAAAAAGCGAAGAGGAAGGA3' (forward) and 5'GTTTCCGGTGGGTGTACGTA3' (reverse) (Applied Biosystems). Forward primer was flourescently labelled with fluorochrom FAM6. PCR reaction was performed in 20µl reaction volume containing 1x Buffer with 15mM $MgCl_2$ (Qiagen), each dNTP at 200µM (Invitrogene), each primer at 0.1 µM, 1 unit of Taq DNA Polymerase (Qiagen), and 100ng of template DNA. PCR amplification was carried out for 2 min at 94ºC, followed by 35 cycles of 30 s 94ºC, 55ºC 30 s, 72ºC 30 s with final 5 min incubation at 72ºC.

The length polymorphism of PCR products was evaluated after capillary electrophoretic separation using genetic analyser ABI PRISM 310 (PerkinElmer).

Ins/del marker analysis:

PCR products were generated using primer pair 5'CAAACAGACGACAAGCGGAGAA3' (forward) and (5'TAGAAAAAGAAAACATCAAGCA3' (reverse) (DOMON *et al.* 2002). PCR reaction was performed as described by authors. The length polymorphism of PCR products was evaluated after electrophoretic separation in 2% agarose gel and visualisation with ethidium bromide under the UV light.

Results and Discussion

265 lines from crosses between No94609D7 x CDC Candle (No411) and Nordus x CDC Candle (No412) were randomly selected and genotyped. CDC Candle is a waxy hulless cultivar containing approximately 3% of amylose and 97% of amylopectin, whereas covered cultivar Nordus has about 20% of amylose and 80% of amylopectin. The other parental material No94609D7 is a covered line, which has 16% of amylose and 84% of amylopectin (unpublished data). We used three types of co-dominant markers. The polymorphism of PCR products within the parental cultivars is summarised in Table 2. We used the *Amy32b* cSNP (G/A) marker distinguishing among waxy hulless and other hulless and covered cultivars as it was found in the previous study (POLAKOVA *et al.* 2003). We used the Waxy06 SSR marker, which lied in the promoter region of the barley *waxy* gene (EMBL: X07931, ROHDE *et al.* 1988), and generated 4 PCR products differing in length. The insertion/deletion marker specifically detected three types of *waxy* alleles (DOMON *et al*. 2002). Using both waxy

Table 2. The PCR product lengths of parental cultivars using the ins/del, Waxy06 markers, and SNP genotypes using cSNP marker

	cSNP *Amy32b*	ins/del	Waxy06
CDC Candle	G	600bp	154bp
Nordus	A	800bp	160bp
No94609D7	A	1000bp	156bp

markers, we obtained identical data. In case of all applied molecular markers among randomly selected F2 lines, we obtained the expected segregation ratio 1:2:1 (P=0,5-0,77).

It was reported that hulless barley had grater nutritional value compared to covered barley in all studies on feeding monogastric animals (BHATTY *et al.* 1986; BHATTY 1993). Although waxy barley has no nutritional advantages as feed for swine and ruminants over other barleys, it was found that waxy hulless lines with low milling energy had much lower β-glucan levels than those with high milling energy. The waxy hulless lines with low milling energy also demonstrated much more extensive cell wall modification during malting (XUE *et al*. 1997). However, waxy types of barley remain to be a valuable source of soluble fibre for human diets (NEWMAN *et al*. 1989b). A recent clinical trial and other studies involving diabetic subjects have revealed several health benefits of waxy barleys, including blood cholesterol decrease and improvement of glycemic level and blood lipid profile (McINTOSH *et al*. 1991; BOURDON *et al.* 1999; BEHALL *et al*. 2004; NEWMAN *et al*. 1989a). Washonubet, a waxy type of barley, was found to be a great source of tocotrienol (VACULOVA *et al.* 2001). Tocotrienol is an isoform of the vitamin E and a very effective antioxidant. Even though the waxy character is not associated with the total elimination of amylose, the produced starch has implications for the food industry as it has unique processing and cooking characteristics. Although most studies had found high β-glucan content in waxy background, others assayed a different β-glucan gene expression (SWANSTON 1997).

To reach the main aim of our study, the development of a high yield waxy hulless cultivar adapted to Czech agro climate conditions, we performed a hybridization between selected parental cultivars differing in their genotypes. CDC Candle is a Canadian waxy hulless cultivar, with very good proportional yield performance in the Czech field conditions, nevertheless the yield is lower in comparison to the best Czech hulless barleys (VACULOVA *et al.* in the press). The covered cultivar Nordus and the breeding line No94609D7, which were originated in Germany, belong to the high productive types of malting barleys.

Each molecular marker used in this study lies within a gene. Such molecular markers are highly valuable for breeding because the recombination between the marker and the gene is eliminated. We used two co-dominant markers, which specifically flank polymorphic region in the promotor of the waxy gene. DOMON *et al.* (2002) found a 403 bp deletion in the promotor region in some Japanesse cultivars. The deletion includes the TATA box and exon I. Therefore, the deletion is responsible for the low amylose content by reducing *waxy* gene transcription. Authors also revealed a 193bp insertion in the intron I of the *Waxy* allele. The *Waxy* allele leads to about 27% of amylose and 73% of amylopectin in the endosperm, whereas homozygous *waxy* endosperm contains 2-10% of amylose and 98-90% of amylopectin (WASHINGTON *et al.* 2000). DOMON *et al*. (2002) observed three PCR products differing in length using a PCR-based ins/del marker among studied cultivars. The PCR products of 600bp, 800bp, and 1000bp in length corresponded to the recessive *waxy*, dominant *Waxy*, and novel *Waxy* alleles. The novel *Waxy* allele probably evolved from the *Waxy* allele. We developed Waxy06, a microsatellite marker, which lied in the promotor region of the *waxy* gene (EMBL: X07931). It contained a $(AG)_6$ repetitive motive. We observed 4 PCR products differing in length in a collection of 70 genetic resources

Table 3. Means of yield components of hybrids divided according *Amy32b* allele cSNP marker

Allele/ character*	No411*		No412		Allele/ character*	No411*		No412		Allele/ character*	No411*		No412	
	n	cov	n	cov		n	cov	n	cov		n	cov	n	cov
A					**G**					**H**				
NAT	5,3	5,4	5,5	6,1	NAT	8,1	6,3	5,9	4,5	NAT	6,0	6,7	4,3	5,2
NSP	5,0	4,8	5,5	5,3	NSP	6,8	6,0	5,1	3,5	NSP	5,0	5,7	3,8	4,7
NKP	125,0	113,0	134,5	130,3	NKP	148,8	140,8	125,8	94,0	NKP	107,0	137,1	73,5	115,7
GWP	5,0	5,4	5,3	6,5	GWP	6,3	6,2	5,5	5,1	GWP	4,8	6,6	3,1	5,5
TKW	39,5	46,0	39,2	48,8	TKW	41,1	43,3	41,2	41,4	TKW	44,9	46,5	42,1	46,7
NKS	25,0	22,6	24,4	24,3	NKS	22,1	23,2	23,8	21,9	NKS	21,3	23,5	19,2	24,1
GWS	0,93	0,94	0,96	1,19	GWS	0,77	0,97	1,00	1,01	GWS	0,79	0,94	0,81	1,14

*- see Material and Methods

(unpublished data). The Waxy06 marker detects all three types of alleles. Moreover, it distinguishes among genotypes carrying one type of alleles (Tab.1).

The *Amy32b* cSNP marker (POLAKOVA *et al.* 2003) distinguishes among waxy hulless cultivars (G genotypes) and other hulless non-waxy and covered cultivars (A genotypes). The cSNP lies in the exon 4 and results in an amino acid substitution (glutamic acid→lysin; GAG→AAG). This substitution may play an important role in the final protein structure, which may affect enzyme activity and bound interaction to starch granules. It was previously reported that the hulless waxy barley had increased level of maltose, suggesting that it was caused by a higher level of alpha-amylase activity (XUE *et al.* 1997; GOERING & ESLICK 1976). GOERING and ESLICK (1976) found that alpha-amylase was associated with isolated starch in waxy hulless cultivars.

To ensure a routine marker-assisted selection that is cost effective, it is necessary to use protocols that allow a rapid DNA isolation from leaf tissue. We used a rapid kit (DNeasy® 96 Kit, Qiagen), which was a high-throughput isolation kit for plant DNA. DNAs from 2x96 samples were extracted within 2 hours. Tube labelling and manipulation with frozen leaves turned out to be the most time consuming steps. The techniques used for DNA marker analyses were based on PCR. The polymorphism was evaluated in 2% agarose gel after visualisation by means of ethidium bromide under the UV light. In case of the Waxy06 marker, we used a capillary electrophoresis, which required one fluorescently labelled primer. Finally, the DNA-MAS of 265 samples, including the DNA extraction, was not expensive, as the time of the F2 lines selection and further work were shortened.

The selected lines were characterised on phenotype level. JEUNG *et al.* (1998) reported that barleys with the waxy allele did not have decreased yield characters. The authors showed that whereas the *Nud* locus (formerly *N*) had the second-greatest contributing factor for grain yield, the *waxy* gene contributed positively to β-glucan content without any measurable impact on grain yield. We observed similar data. Hybrids with the G genotype (carrying waxy, Waxy (novel Waxy) or both alleles) did not have a decreased mean of yield characters compared to hybrids with the A genotype (carrying waxy, Waxy (novel Waxy) or both alleles). In our experiment, we also did not detect any significant differences among the covered and hulless hybrids, based on the number of tillers, spikes and kernels per plant, especially among the hybrids with the G genotypes (Tab. 3). The variation of the yield components in the hulless and covered plant groups showed importance of the parental selection, which affected the result of the hybridization (Fig. 1 and 2; see also poster). Based on the DNA analyses, we selected hybrids with the G genotype (the *waxy* allele together with the hulless grain). It is likely to obtain other nud- and waxy- hybrids from the F3 segregating population of the F2 plants, which were G/A heterozygots and waxy homozygots. There is also a real chance to select required genotypes with better yield characters as shown in Fig. 1 and Fig.2.

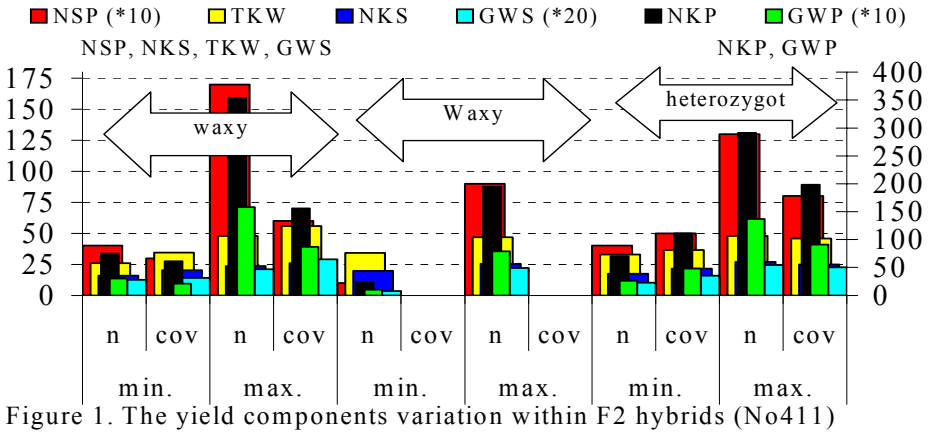

Figure 1. The yield components variation within F2 hybrids (No411) carrying G-allele (*Amy 32b* cSNP marker) with waxy, Waxy or both alleles.

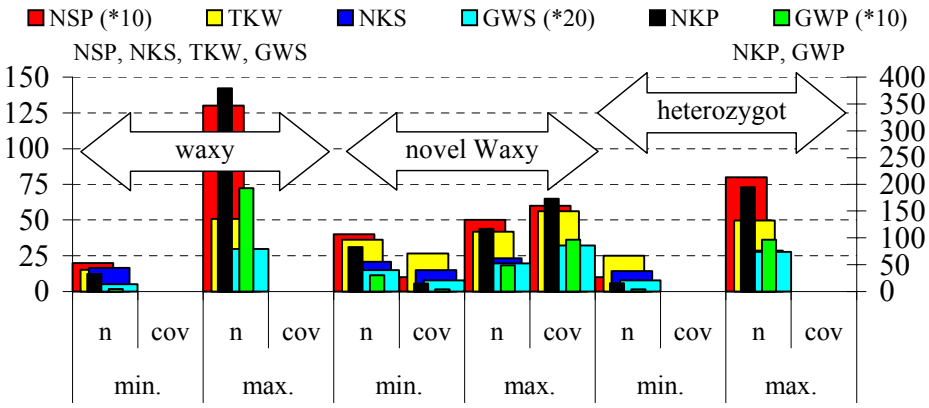

Figure 2. The yield components variation within F2 hybrids (No412) carrying G-allele (*Amy 32b* cSNP marker) with waxy, Waxy or both alleles.

We demonstrated an effective DNA-MAS of F2 lines from two crosses carrying desirable alleles. Further, we detected new hybrid lines with the low amylose content and hulless grain. During harvesting, we selected mature plants with required alleles based on the DNA data and we made phenotype analyses. The selected plants will be used for further breeding and experiments, which will be carried out under strict glasshouse conditions to find possible associations between cSNP in the *Amy32b* gene and in alpha-amylase activity.

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Verification of Nutritive Value and Hypocholesterolemic Effect of Spring Barley Lines

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Abstract

The aim of a growth model experiment in laboratory rats was to check the nutritive value of the set of newly bred spring barley lines with different grain characteristics. The followed set contained hulless lines bred in the ARI Kroměříž: feed type - KM 1771 (1) and KM 1057 (2) , food type - KM 2082 (3), KM 2092 (4), KM 2062 (5) and lines formed on the basis of crossing with food donors of a *waxy* endosperm type – (hulless line Wabet x Washonubet (6), hulled line Wabet x Krona (7) and Wabet x Kompakt (8)). The line Kompakt x Krona (9) and the malting variety Nordus (10) were used as a control.

The best results of an average daily gain and feed conversion (9.19g; 2.29g/g) were achieved by feeding the line KM 1057 (2), conversely, the worst by feeding food barley of the *waxy* type (6 - average daily gain in animals was only 8.01g and feed conversion 2.54g/g). This material also had the highest β-glucan (7.1%) out of the followed set. The lowest β-glucan content was detected in the sample 9 (3.88%). Polynomial dependence between β-glucan content (x) and cholesterol content ($y = 0.0061x^2 - 0.419x + 8.9265$, $R^2 = 0.5387$, $P<0.01$) was calculated from the statistical data.

The highest values of digestibility coefficients of nitrogen substances - NS (75.96%) and fibre (49.63%) were measured in animals in a group 2, on the contrary the lowest digestibility of fibre (31.10%), fat (56.96%) and ash (45.65%) was exhibited by a control group 10. Differences in digestibility of all followed nutrients after the 14th day and at the end (28th day) of the experiment were observed (NS digestibility was lower by 5.65%, ash digestibility by 11.47%, fibre by 10.85% and fat by 3.75%).

It was confirmed that the tested *waxy* lines exhibited hypocholesterolemic effect; we-however, do not recommend them for feeding of monogastric animals due to high β-glucan content, reduced intake of mixture and impaired feed conversion.

Keywords: barley nutritive value; growth; organic nutrients; hypocholesterolemic effect; β-glucans

Introduction

Barley is our significant feeding cereal and annually about 70 % of the total grain production is used for feeding purposes. Barley grain is an important source of energy and protein for pigs, it supports production of eggs in laying hens and is a source of feeding fibre for ruminants and horses.

Considerable renaissance of barley use also in human nutrition has been registered all over the world. This trend is a result of numerous scientific and clinical research studies accentuating a health – preventive role of fibre consumption.

A group of structural non-starch polysaccharides (NSP) participates in the construction of a barley grain cell wall together with lignin. The barley grain contains 15-20% of dietetically beneficial fibre, of which even 86 % falls on NSP, of this ca 23% are arabinoxylans and 56%

of β-glucans (β -G). These NSP belong to the most significant in nutrition of young livestock from the view of antinutritive barley effects. They swell, thicken chime, increase its viscosity and slow down its shifting in animals‘ bowels and stomach (PETR 2003). Thus mobility of nutrients and digestive enzymes is deteriorated and absorption of nutrients, mainly fats are limited (KALAČ & MÍKA 1997).

It means that biological effects of NSP from the point of view of human nutrition, nutrition of poultry and piglets are diametrically different. While in human nutrition food fibre is appreciated favourably, first of all as one of the factors decreasing cholesterol level, in poultry and to a lesser degree in growing pigs, it reduces intake and utilizability of nutrients and utilization of barley in feeding rations of poultry or piglets without enzymatic preparations would be considerably problematic (JEROCH & DÄNICKE 1995).

Obviously, barley breeding must consider the given facts and breed new varieties of various utility types with respect to the final utilization of grain.

Various forms and types of barley are used as feed barley – six-row, two-row, winter and spring, hulled and hulless forms. But the high protein content and the lowest possible β-G level is always required. The current feed varieties contain few essential amino acids - lysin (Lys), threonin, valin and izoleucin (BHATTY & WHITAKER 1987). Pursuant to VELÍŠEK (1999), Lys and leucin are the limiting amino acids. An ideotype of feed barley should therefore contain minimally 11.5% of nitrogen substances (NS) but at the least 5g of Lys/16g of N, maximally 4.5% of fibre and minimal content of β-G (PETR & HÚSKA 1997). Considerable increase of digestibility of an energetic grain component can be expected after elimination or reduction of a husk portion (VACULOVÁ 1995), therefore the question of creation of hulless barley varieties for feeding specific groups of monogasters is often discussed.

The spring form is grown predominantly for malting purposes in this country. NS content is important here too – barley workability to malt is limited by minimal (9.5%) and maximal content of NS (11.7%) and β-G content - max. to 4 % (PELIKÁN *et al.* 1996).

Barley grain utilization for direct food consumption is relatively low in the Czech Republic. According to the statistical data less than 30 000 tonnes, i.e. 5% of the total consumption is used for these purposes annually (VACULOVÁ 1999). On the worldwide scale food barley is considered a very suitable material for the production of dietary products. Hypocholesterolemic and further health-preventive effect of β-G, α-tokotrienol and active antioxidants contained in grain is verified. Varieties with increased β-G content (above 5%) and higher content of digestible fibre are bred; special attention is devoted to hulless barleys and especially to barleys of the type *waxy* with the increased NSP content.

Number of studies on the favourable effect of the food fibre, especially its soluble fraction, on the cholesterol content in blood serum (mainly its undesirable LDL fractions) have been published, new data with health significance, however, have been supplemented all the time (LIFSCHITZ *et al.* 2002,).

The object of the submitted study was to verify the nutritive value and hypocholesterolemic effect of feeding the grain of new spring barley hulled and hulless lines in a model experiment on laboratory rats.

Methods

The model experiment was carried out in the experimental facility of the Department of Animal Nutrition of AF MUAF in Brno. Conditions of the facility conform to the methodology pursuant to ”Law on Protection of the Maltreated Animals” no. 246/1992 Coll.

Laboratory rat males, strain Wistar, were used as an experimental model for the growth experiment. Animals 29 days old were used in the experiment. Their average initial weight

ranged within 82.1 – 87.1g, which complies with the standard requiring maximal differences of 5g in weight between the test groups (KACEROVSKÝ 1990).

The animals were divided into 10 groups (8 experimental groups and 2 controls), and different barley material was tested in each group (assignment of materials to a number of groups is presented in Table 1). Experimental feed mixtures were prepared from the followed line (80%), soybean extracted meal (16%) and mineral and vitamin concentrate for laboratory rats (4%).

Rats were stalled in plastic cages with a slotted floor at temperature of 23 ± 1°C, air humidity 60% and light regime 12 hours night and 12 hours day. Duration of the experiment was 28 days. The animals were weighed every day, body weight gains, intake feed conversion organic substance digestibility and health were followed. Residues of feeds and excrements of individual groups were separated, weighed, dried and analyzed for the assessment of net feed intake and digestibility coefficients (%).

Effect of β-G content on changes of cholesterol levels was studied. At the beginning and at he end of the experiment, total cholesterol in blood (mmol/l) was followed in animals of the control group 9 (with the lowest β-G content in caryopses) and groups fed with food barley lines of the type *waxy* (groups 6, 7, 8) spectrophotometrically using Bio-La-Test.

Analyses of nutrient content of grain samples of experimental barley lines and experimental feed mixtures made from them were conducted based on the methods described inthenoticeno. 451/2000 to the law no. 244/2000 Coll. Determination of β-G in barley caryopses was conducted in the Research Institute of Brewing and Malting in Brno by the method FIA. Content of gross energy in barley samples and in complete trial mixtures was assessed on the equipment PARR 1281 Bomb Calorimeter.

The obtained results were evaluated using the standard statistical methods (SNEDECOR & COCHRAN 1967).

Results and Discussion

The amount of dry matter in samples varied from 88.9 to 89.4%. The highest NS content was determined in the sample of feed barley 2 (151g/kg of d. m.), conversely the lowest content (118.3g/kg of d. m.) was exhibited by a control group 10. The average content of NS was higher in hulless barleys (135g/kg of d. m.) as compared with hulled barleys (127g/kg of d. m.), and similarly it was higher in barley of a type *waxy* (130 g/kg of d. m.) compared with the standard lines and variety (133g/kg of d. m.). Content of organic nutrients and gross energy in grain of the followed lines and feed mixtures are given in Table 2.

The highest average daily gain and the best conversion of feed (9.70g and 2.29g/g, respectively) were achieved by feeding the line KM 1057 (group 2). Conversely, the worst results (gain 8.01g, conversion 2.54g/g) were achieved by feeding food barley of the type *waxy* (group 6) – see Figure II and I.

The results of digestibility coefficients of NS, fibres, fats, ash and NNES of the followed utility groups in the second and the fourth week of the experiment are presented in Table 3. Differences in digestibility of all followed nutrients were observed between evaluation after the 14th day and at the end of the experiment (28th day). In comparison with the second week, digestibility of all followed nutrients at the end of the experiment was significantly lower. The average values of coefficients of the actual digestibility of the whole set indicate that the biggest drop occurred in ash digestibility (11.47%), then in fibre (10.85%), digestibility of NS was lower by 5.65% and fat only by 3.75%. Concerning the course of digestibility of single utility barley types, the lowest drops were observed in groups 1 and 2, i.e. in the original feed types of barley (digestibility coefficient of NS was reduced only by 3.63%, fibre by 4.74%, fat by 2.2% and ash by 9.66% in comparison with the other

week). Conversely, in the control groups, i.e. 9 and 10, NS digestibility coefficient was reduced by 10.40%, coefficient of fibre even by 19.71%, fat by 7.09% and ash by 21.51%.

The highest content of β-G in absolute dry matter of grain (Table 2) was measured in the hulless food line Wabet x Washonubet - group 6 (7.1%) and the whole group of *waxy* types showed significantly higher average content of β-G (5.8%) versus classical lines (4.2%). The average content of the total cholesterol in blood of animals was 1.99 mmol/l at the beginning of the growth experiment. At the end of the experiment this value was reduced in all lines of the type *waxy* (in groups 6, 7 and 8 these values were 1,837, 1,981 and 1,927 mmol/l, respectively), conversely in the control group 9 (line Kompakt x Krona) the level of the total cholesterol compared to the initial value was increased to 2.83 mmol/l. Polynomial dependence between the intake of β-G (x) and cholesterol content (y) was calculated based on the statistical data: $y = 0{,}0061x^2 - 0{,}419x + 8{,}9265$, $R^2 = 0{,}5387$ (Figure III). The found dependence was statistically highly significant ($P<0{,}01$).

Conclusion

The results suggest that the usually fed malting varieties are not the best for intensive fattening of livestock and it is necessary to look for feed barleys, preferably with hulless grain and increased content of desirable nutrients and reduced content of substances with antinutritive effect.

It has been confirmed that the tested *waxy* lines show hypocholesterolemic effect; but we do not recommend them for feeding monogastric animals due to the high β-glucan content, reduced intake of mixture and deteriorated feed conversion.

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Table 1. Experimental lines and barley varieties

IDENTIFICATION		CHARACTERISTICS
1_B 2_B	KM 1771* KM 1057	Lines with hulless grain (of an extensive type). KM 1771 has a higher β-G content and KM 1057 lower starch content and a higher share of essential amino acid lysine than the malting barley varieties. They were bred for **feed** utilization, line KM 1771, however, appears to be more suitable for food use.
3_B 4_B 5_B	KM 2082 KM 2092 KM 2062	Lines with hulless grain (of an intensive type). With a higher β-G content than the registered varieties of the malting type and they are determined for **food** purposes.
6_B 7_P 8_P	Wabet x Washonubet Wanubet x Krona Wabet x Kompakt	Lines of an extensive type (created in the framework of the project GAČR-521/97/1202, solved in collaboration with DCSPB of MUAF in Brno, ARI in Kroměříž and RIBM in Brno), **food type**. Grains are of the type ***waxy*** with the increased content of proteins, β-glucans, vitamin E and its isomers. They have an increased share of amylopectin.
9_P 10_P	Kompakt x Krona Nordus	Line Kompakt x Krona comes from crossing the **varieties of a malting type**, with intensive yield. Malting variety with a low content of proteins and β-glucans, with intensive yield.

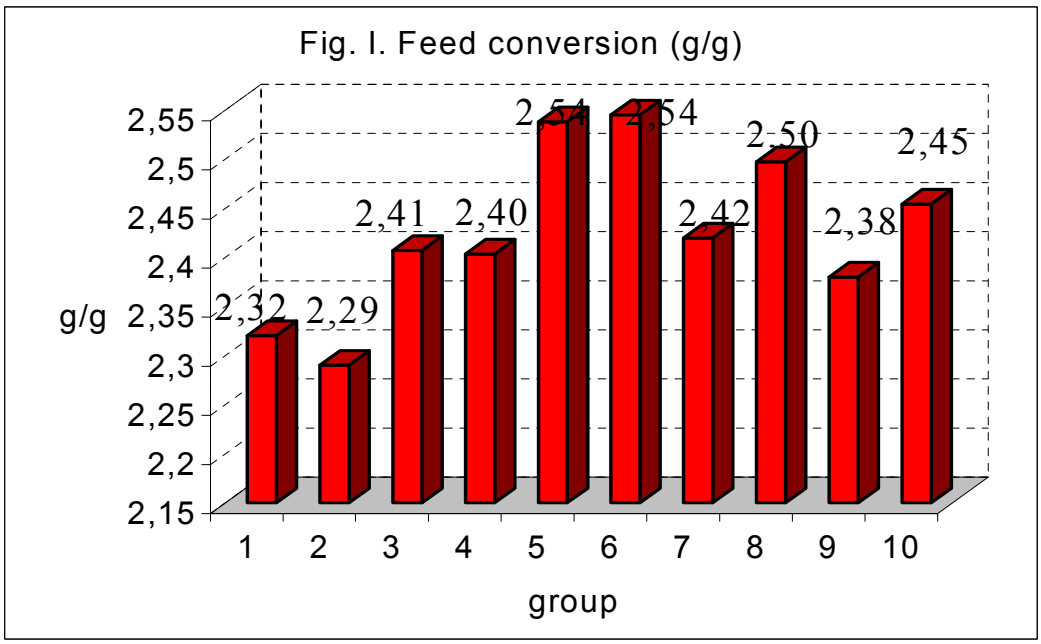
Notes: mark $_P$ - hulled, $_B$ – hulless barley, * - Lines marked KM – bred in ARI Kroměříž, Ltd.

Table 2. Content of organic nutrients, gross energy (GE), β-glucans (β-G)

Group	protein (%)		crude fibre (%)		fat (%)		ash (%)		nitrogen free extract (%)		BE (kJ/kg)		β-G (%)
	Grain	Feed mixture	Grain	Feed mixture	Grain	Feed mixture	Grain	Feed mixture	Grain	Feed mixture	Grain	Feed mixture	Grain
1	13.16	19.10	1.82	1.82	2.20	3.40	1.79	4.48	81.02	71.20	18.07	17.87	4.35
2	15.10	21.48	2.40	2.09	2.62	3.72	2.06	4.71	77.82	68.00	18.46	18.14	3.52
3	13.03	20.05	1.61	1.72	2.42	3.14	2.03	4.41	80.92	70.68	18.03	17.95	4.34
4	13.18	19.47	1.65	1.65	2.51	3.02	1.91	4.42	80.75	71.44	18.0	17.85	4.34
5	14.58	19.41	1.77	1.76	2.51	3.15	1.77	4.50	79.37	71.18	18.12	16.25	4.34
6	12.30	19.07	2.08	1.41	4.47	4.80	2.29	5.02	78.86	69.70	18.15	16.41	7.10
7	13.12	18.73	1.42	1.71	4.37	4.74	2.18	4.75	78.91	70.07	18.23	16.53	5.60
8	13.85	18.79	1.30	1.54	4.17	4.43	2.34	4.85	78.35	70.39	18.23	16.28	4.90
9	12.27	18.07	1.69	1.58	3.83	4.56	2.30	4.62	79.91	71.17	18.23	16.32	3.88
10	11.83	18.23	1.41	1.29	3.81	4.13	2.18	4.60	80.77	71.76	17.98	16.32	4.15

Table 3. Apparent digestibility coefficient (%)

BARLEYS	protein		crude fibre		fat		ash		nitrogen free extract	
	2nd week	4th week	2nd week	4th week	2nd week	4th week	2nd week	4th week	2nd week	4th week
Hulless feed	74.00	70.37	48.16	43.42	64.03	61.83	49.48	39.83	90.97	89.62
Hulless food	73.91	70.02	43.73	35.74	65.35	61.66	49.26	41.91	91.66	90.25
Food *waxy*	73.85	69.17	40.09	29.12	65.09	63.03	49.55	42.17	88.91	87.92
Standards	72.79	62.39	36.33	16.62	60.62	53.53	46.12	24.61	88.97	86,49



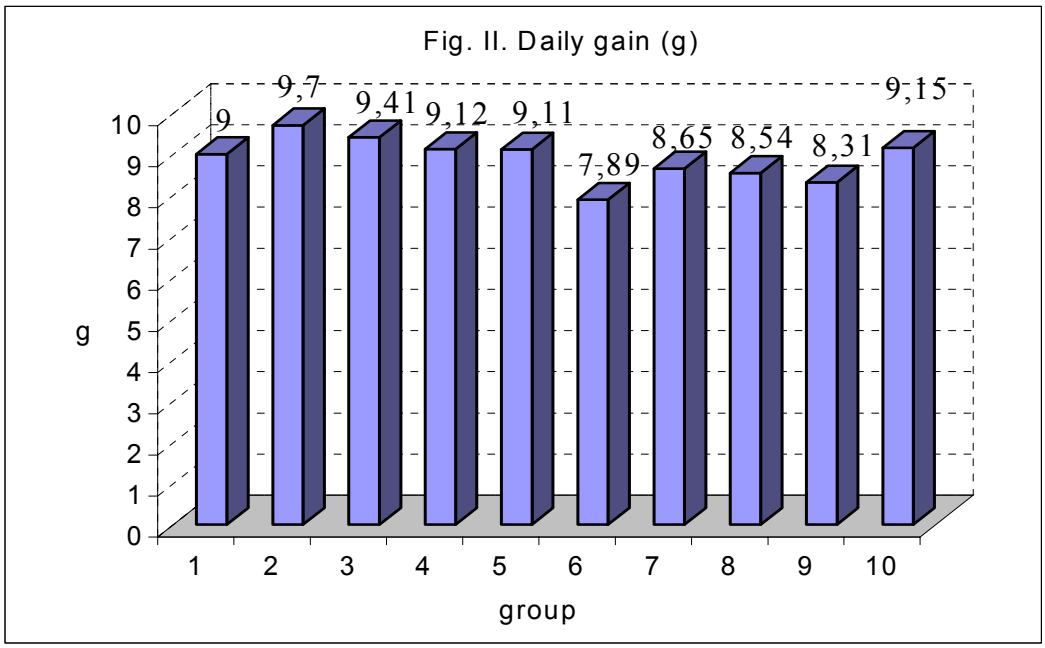
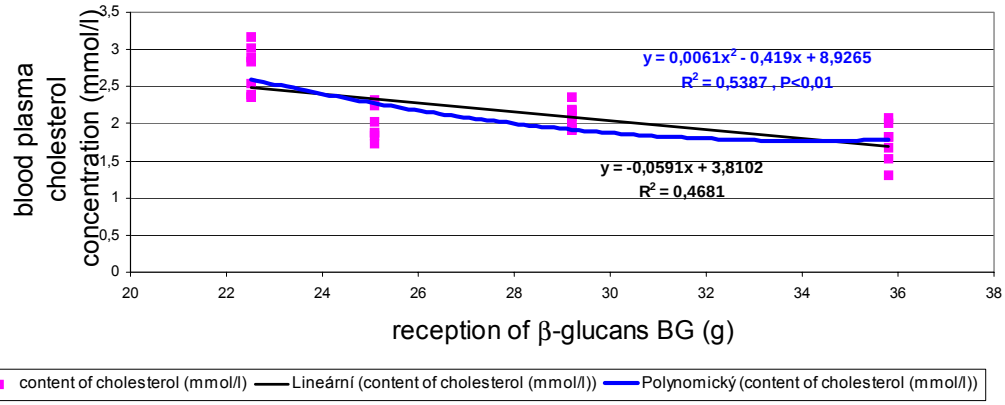


Fig III. The linear and polynomial dependence of reception β- glucans on blood plasma cholesterol concentration



Molecular Marker Development for Low Phytate Barley (Hordeum vulgare)

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Abstract

Barley grain contains significant levels of phosphorous, however most is bound in the form of phytic acid (phytate) and is unavailable to monogastrics. USDA researchers have recently developed several low phytate barley mutants with significantly less phytate. Of the three low phytate mutants investigated, the *lpa1-1* locus shows a 50% decrease in phytic acid and has been mapped to the long arm of chromosome 2H. A RFLP marker (ABC153) located in this region has been converted to a SCAR marker. Segregation analysis in the doubled haploid population from the cross CDC McGwire by low phytate mutant Hvlpa1-1 has confirmed linkage between the SCAR marker and *lpa1-1* with 15% recombination. The second low phytate mutant, M635, (75% phytate decrease) has been mapped to the long arm of chromosome 1H through linkage with an ISSR marker (LP75). This locus has been designated *lpa3-1*. The mutation in M955 (95% decrease) and *lpa3-1* are in the same region on chromosome 1H, and may be allelic based on an analysis of recombination between the mutations and molecular markers.

Keywords: barley; phytic acid; low phytate; mutation; molecular marker

Introduction

Barley (*Hordeum vulgare* L.) is one of the principal components of animal feed in Western Canada. Unfortunately much of the phosphorous present in the grain is in the form of phytate, a complex of phytic acid (*myo*-inositol 1,2,3,4,5,6 hexakisphosphate) and other minerals (RABOY 1997). This phytate phosphorous is unavailable to monogastric animals as they do not produce phytase, an enzyme capable of degrading phytate, and unlike ruminants do not possess phytase-producing micro-flora (ERTL *et al*. 1998). Consequently the diet must be supplemented with either inorganic phosphorous or a microbial form of phytase to ensure adequate phosphorous availability (KETAREN *et al*. 1993). Phytate generates high levels of phosphorous in effluent leading to potential environmental problems (DANIEL *et al*. 1998). Phytate also decreases the availability of some essential elements, in particular iron, zinc, calcium, potassium and magnesium, due to bonding between the negatively charged phytic acid and the positively charged elements (RABOY 1997). Thus diets consisting primarily of cereal grains can lead to additional specific nutrient deficiencies (SPENCER *et al*. 2000; POULSEN *et al*. 2001).

USDA researchers have recently identified low phytic acid mutants in grains. These lines have substantial reductions in phytate and equivalent increases in inorganic phosphorous. Low phytate corn (RABOY *et al*. 2000), rice (LARSON *et al*. 2000), soybean (WILCOX *et al*. 2000) and barley (LARSON *et al*. 1998; RASMUSSEN & HATZACK 1998) have been developed. Preliminary feeding trials with the low phytate grains have demonstrated increased phosphorous availability and a decrease in the amount of phosphorous excreted (ERTL *et al*. 1998; SPENCER *et al*. 2000; THACKER *et al*. 2003).

Four distinct low phytic acid mutants have been developed in the barley cultivar Harrington. The mutant line Hvlpa1-1 has 50% less phytic acid with a comparable increase in inorganic phosphorous. This locus has been mapped to the long arm of chromosome 2H (LARSON *et al*. 1998). Two other mutants (M635 and M955) with 75% and 95% less phytate respectively, again have concomitant increases in inorganic phosphorous (RABOY & COOK 1999). Mutant Hvlpa2-1 exhibits a 70% decrease in phytic acid but is associated with an increase in inorganic phosphorous as well as increases in inositol pentakisphosphate, an intermediate from the phytate biochemical pathway. The Hvlpa2-1 locus has been mapped to chromosome 7H (LARSON *et al*. 1998). Here we report on the characterization of the Hvlpa1-1, M635, and M955 mutations through the discovery and development of molecular markers linked to these loci and suitable for molecular marker-assisted selection (MMAS).

Material and Methods

Plant Material

The molecular work was carried out on the following populations segregating for the individual mutations; CDC McGwire x Hvlpa1-1 doubled haploid lines (DH) (41 lines), HB358 x M955 recombinant inbred lines (RIL) (91 lines), CDC Freedom x M635 RILs (20 lines). All populations were created at the Crop Development Centre, University of Saskatchewan, Saskatoon, Canada. Markers, where possible, were mapped using the mapping populations Harrington x Morex and Harrington x TR306, developed by the North American Barley Genome Mapping Project (NABGMP).

Phytate Quantification

Phytic acid levels in mature seeds were determined for all lines from the low phytate crosses utilizing the technique of RABOY *et al*. (2000).

ABC153 SCAR marker

A copy of the genomic DNA probe ABC153 was kindly provided by Dr. A. Kleinhofs, Washington State University, USA. Sequencing was performed by standard dideoxy chain termination protocols. Primer sequences for the SCAR marker were 5'-TTCATCATCATCGTCATCGTG-3' (ABC153F) and 5'-CCTCTGCCGCTGGAACTA-3' (ABC153R). The PCR reactions (25 μL) were composed of 20 mM Tris-HCl, 50 mM KCL, 2 mM $MgCl_2$, 400 μM dNTP, 200 pM each primer, 1 Unit of *Taq* DNA polymerase (Invitrogen), and 100 ng of genomic DNA. The PCR cycling conditions consisted of an initial denaturation at 94°C for 5 minutes, followed by 37 cycles of 94°C (1 min), 62.5°C (dominant) or 55°C (co-dominant) (1 min), 72°C (1 min), and terminated with a 10 minute dwell at 72°C. All PCRs were performed in a Thermolyne Amplitron II temperature cycler (Barnstead/Thermolyne).

ISSR and LP75 Analysis

Inter-simple sequence repeat (ISSR) primers were acquired from the Biotechnology Laboratory, University of British Columbia, Vancouver, Canada. Two bulk DNA samples were produced from the CDC Freedom x M635 cross. DNA from six low phytate lines was pooled to generate the low phytate bulk and six normal lines were utilized to produce the normal phytate bulk. The protocol of HUANG & SUN (2000) was followed with slight modifications. ISSR reactions (20 μL) consisted of 20 mM Tris-HCl, 50 mM KCL, 1.5 mM $MgCl_2$, 800 μM dNTPs, 2% formamide, 200 pM primer, 1 Unit *Taq* DNA polymerase, and 60 ng genomic DNA. Amplification consisted of an initial denaturation at 94°C for 5 minutes, and 40 cycles of 94°C (45 sec), 49°C-59°C (45 sec), 72°C (1 min), and terminated with a 10 minute dwell at 72°C. All reactions were analyzed on 1.5% agarose gels.

Primers sequences for the LP75 marker were 5'-CACACACACACACACAA-3' (UBC817) and 5 '- CAAGTCAAGTCTAGTAGG-3' (LP75F). Reaction and cycling

conditions were similar to ISSR conditions except for the elimination of formamide, and a decrease in primer concentration to 125 pM. The optimum annealing temperature for these primers was 57°C. PCR products were visualized on 1% agarose gels.

LP75 post-amplification restrictions using *Cla*1 involved the addition of 8.5 mM $MgCl_2$ and 4 Units of restriction enzyme to the cycled PCR. Samples were incubated at 37ºC. DNA fragments were resolved on 1.5% agarose gels.

Un8-700R Analysis

Un8-700R reaction and amplification conditions are described in ECKSTEIN *et al*. (2002).

Results

Segregation of ABC153

The ABC153 RFLP marker was converted to a SCAR marker based on DNA sequence of the locus from the cultivar CDC McGwire. The PCR primers were designed to be complementary to regions near the ends of the fragment, and the marker could be used in two forms depending on annealing temperature. The primers, when annealed at 55ºC, produced a co-dominant banding pattern that included a 350 bp fragment from normal phytate lines and fragments of approximately 340 bp plus 310 bp from low phytate lines (Figure 1A). An annealing temperature of 62.5°C produced a dominant banding pattern, with the positive allele (350 bp fragment) being amplified in repulsion to the low phytate allele (Figure 1B). To be effective, the co-dominant marker requires high percentage agarose gels and long run times to descriminate between the two fragments of similar size, but offers the advantage of eliminating false negative results and the ability to identify heterozygotes. The map location of the SCAR marker was confirmed to be the same as the original RFLP marker on 50 DH lines of the Harrington x TR306 mapping population.

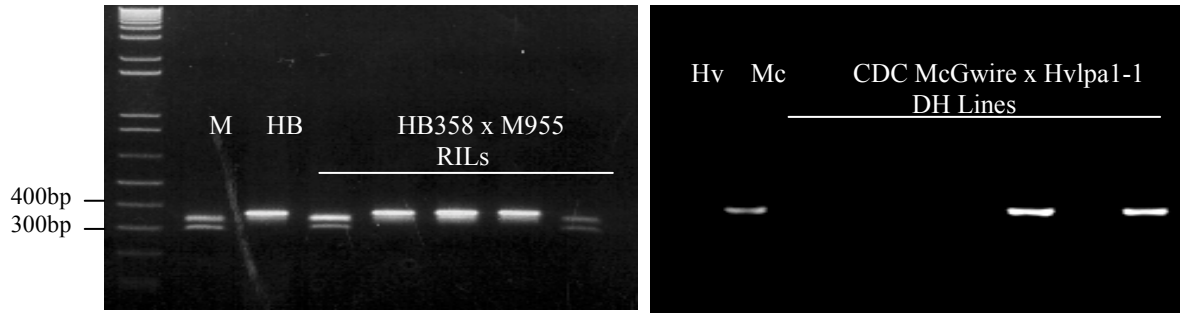

Figure 1. A. ABC153 co-dominant SCAR marker banding pattern on M955 (M), HB358 (HB), and five segregating RILs. B. ABC153 dominant SCAR marker banding pattern on Hvlpa1-1 (Hv), CDC McGwire (Mc), and seven segregating DH lines.

The ABC153 SCAR marker was polymorphic in all three crosses involving the individual phytate mutants (CDC McGwire x Hvlpa1-1, CDC Freedom x M635, HB358 x M955). ABC153 co-segregated with the *lpa1-1* locus in 35 of the 41 doubled haploid lines examined for an estimated genetic distance of 15 cM (Fig. 2). This related well with published map locations and distances for several markers in this area.

The SCAR marker segregated independently from the M635 locus. Of the 20 lines tested, the phenotype/marker-indicated genotype agreed in 12 lines, and disagreed in seven lines, with one line still segregating. The SCAR marker also segregated independently from the M955 locus. Of the 91 lines tested only 46 were concordant. The *lpa1-1* mutation was

the M955 locus. Of the 91 lines tested only 46 were concordant. The *lpa1-1* mutation was therefore not allelic to the M635 and M955 mutations and the ABC153 marker could not be used to select for the 75% and 95% reductions in phytate.

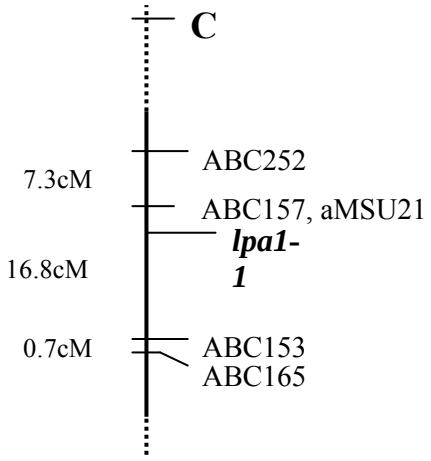

Figure 2. Linkage group of the long arm of barley chromosome 2H. The locus *lpa1-1* has been placed in relation to ABC153, and other markers on the Steptoe/Morex map (NABGMP).

CDC Freedom x M635

ISSR analysis was performed to detect a marker linked to the mutation in M635. Two DNA bulks (low phytate and normal phytate) were screened with ISSR primers. Primer UBC817 amplified an approximately 800 bp fragment from the low phytate DNA bulk which was absent from the bulked DNA of normal phytate lines. Analysis of two seeds of each of 20 individual lines determined that the marker banding pattern agreed with the phenotype in 16 cases and that two lines were segregating at the locus, suggesting a genetic distance of approximately 11 cM between the marker and the M635 locus. The banding pattern and polymorphism generated by UBC817 was weak, unreliable, and not suited for use in MMAS. To develop a robust marker, the 800 bp polymorphic fragment from an individual low phytate line was cloned and sequenced. A primer, complementary to sequence near the 3' end of the fragment, was designed to work with the original UBC817 primer to amplify an 800 bp fragment in coupling to the low phytate characteristic (Figure 3). The primer pair (marker), termed LP75, generated results identical to the original ISSR primer amplification in the 20 segregating lines.

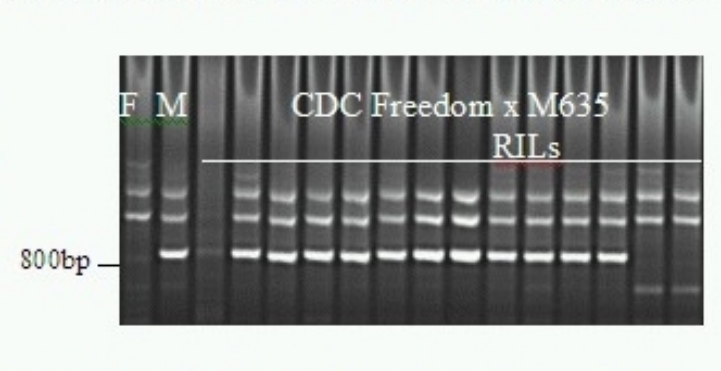

Figure 3. LP75 banding pattern on RILs of CDC Freedom x M635. The polymorphic band at 800 bp is linked in coupling with low phytate in M635 and M955.

The fragment generated by LP75 contains an internal *Cla*1 restriction enzyme site present in the cultivar Harrington and absent in Morex. Analysis of this site in individual lines of the Harrington x Morex mapping population maps this locus to the long arm of chromosome 1H (Figure 4). The map location of M635 was further confirmed through analysis with marker Un8-700R, a SCAR marker for true loose smut resistance located on the

long arm of chromosome 1H (Figure 4). Because the low phytate mutation in M635 mapped to a locus different from *lpa1-1*, it is suggested that the locus in M635 be designated *lpa3-1*.

HB358 x M955

The LP75 marker demonstrated an identical polymorphism in the HB358 x M955 cross. The 800 bp fragment was amplified in M955 and absent in HB358 (not shown). The phenotype/marker indicated genotype agreed in 79 of 90 lines, suggesting an approximate 12 cM distance between the LP75 marker and the M955 locus. Linkage analysis between the M955 locus and Un8-700R was not possible due to a lack of polymorphism.

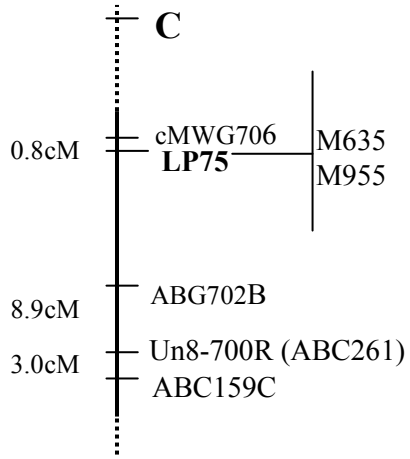

Figure 4. Linkage group of the long arm of barley chromosome 1H. The marker LP75 has been placed in relation to other markers on the Harrington/Morex map (NABGMP). The low phytate mutations M635 and M955 are placed in relation to LP75.

Discussion

LARSON *et al.* (1998) and SHIN *et al*. (1990) located the *lpa1-1* low phytate locus on barley chromosome 2H through linkage to marker aMSU21. While this marker was tightly linked, it was polymorphic largely only between 2-row and 6-row barley types, and therefore not applicable for selection in most of the crosses in the Crop Development Centre barley breeding program. Further examination of the aMSU21 locus DNA sequence indicated a lack of sequence variation, thus no possibility of marker conversion. The location of aMSU21 was used to identify other map-based neighboring markers. Of several candidate markers, ABC153 was successfully converted to a PCR-based marker suitable for MMAS.

As expected the ABC153 SCAR marker co-segregated with the *lpa1-1* low phytate mutation, confirming the previously identified map location of the locus (LARSON *et al*. 1998). While the SCAR marker is not as tightly linked as aMSU21, it is robust (especially when used as a co-dominant marker), and is polymorphic between barleys of the same head type.

The allelic relationship between the three mutants was unknown. Our analysis indicates that the Hvlpa1-1 line contains a unique mutation versus that in M635 and M955.

The M635 and M955 mutations have both been located to the long arm of chromosome 1H through linkage with marker LP75. The mutation in M635 has been named *lpa3-1*. The low phytate mutation in M955 is located near, or may even be allelic with *lpa3-1,* although this requires further clarification. Additional RIL populations from the crosses M635 x Morex and M955 x Morex, in which both LP75 and Un8-700R are polymorphic, are being tested to elucidate the allelic relationship between the two mutations. Screening of a large population (553 lines) is in progress to determine a more accurate estimate of genetic distance between *lpa3-1* and LP75.

The SCAR markers ABC153 and LP75 may be used for MMAS, and will be especially useful if the different mutations are to be combined into a single line.

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High-Resolution Mapping of the Naked-Caryopsis Gene (*nud*) and the Origin of Naked Barley

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Abstract

The hulled or naked caryopsis character of barley is an important trait for utility and to follow its domestication process. It is well established that a single recessive gene, *nud*, located on the long arm of chromosome 7H, controls the naked caryopsis character. With an ultimate goal of map-based cloning of this economically important gene, we initiated marker development by bulked segregant analysis combined with the amplified fragment length polymorphism (AFLP) technique. From analyses of 2,742 primer combinations, 17 AFLP fragments were selected as linked markers, and 8 of them were converted into easy-to-use PCR based markers. A fine map surrounding the *nud* locus was developed in an F_2 population of 460 individuals derived from the cross between Kobinkatagi (naked) and Triumph (hulled). This map has one order higher resolution than those reported before. One dominant PCR-based marker sKT7, which has been cosegregated with the *nud* gene in coupling phase, was used to trace the origin of naked barley. A total of 259 barley accessions including 47 wild barleys, were examined for molecular variation at this locus. Our data indicate that naked domesticated barley accessions today were originated from a single, mutation event.

Introduction

Domesticated barley (*Hordeum vulgare* subsp. *vulgare*) includes two contrasting grain types, namely, hulled barley and naked barley. Hulled barley has caryopses with the husk adhering to the grain, while naked barley grows with easily separable husks upon threshing. Naked caryopsis is controlled by a single recessive gene *nud*, located on the long arm of chromosome 7H. Most barley varieties grown worldwide are of the hulled form, which is suitable for brewing malt and animal feed, while naked barley is produced on a small scale and used mainly as human food for its ease in processing and edibility. Recently, in North America, naked barley is attracting attention as feed and as a healthy food because of the high feed-value and abundance of dietary fiber, respectively (LIU *et al.* 1996). According to a survey by TAKAHASHI (1955), naked barley is widely distributed, but it is predominant in East Asia, where naked barley is consumed as an important staple food.

The hulled or naked caryopsis character is one of the key traits to follow the origin and domestication process of barley, along with row-type and non-brittle rachis characters (TAKAHASHI 1955; HARLAN 1995; SALAMINI *et al.* 2002; TANNO *et al.* 2002). It is generally accepted that barley was domesticated from the wild ancestor *H. vulgare* subsp. *spontaneum*, which has brittle rachises, two-rowed spikes and hulled caryopses. According to the archeological evidence, the earliest domesticated barley grown in the Near East about 8000 B.C. was of the hulled form, and the naked form appeared by about 6500 B.C. (ZOHARY & HOPF 2000). However, it has not yet been clarified where naked barley originated and how it migrated.

We have been attempting positional cloning of the *nud* gene to clarify its function in the control of grain type in barley. Molecular markers closely linked to the *nud* gene can be effectively used to trace the origin of naked barley. Previously, we (KIKUCHI *et al.* 2003) developed amplified fragment length polymorphism (AFLP) (VOS *et al.* 1995) markers closely linked to the *nud* gene, using bulked segregant analysis (MICHELMORE *et al.* 1991) and the High Efficiency Genome Scanning (HEGS) electrophoresis system (KAWASAKI & MURAKAMI 2000). Some of them were used to construct a linkage map near the *nud* locus in a F_2 population of 151 plants. In this paper, we summarize our recent progress in fine mapping and phylogenetic analysis of the naked caryopsis character.

Material and Methods

Plant Materials

For mapping, 460 F_2 plants from the cross between Kobinkatagi and Triumph were used. Kobinkatagi is a Japanese six-rowed naked cultivar, and Triumph is a European two-rowed hulled cultivar. Genotypes of hulled F_2 plants were determined by the progeny test. For phylogenetic analysis, a total of 259 barley accessions, consisting of 53 wild (all hulled) and 206 domesticated (106 hulled and 100 naked) accessions, were selected on the basis of collection sites and morphological characters (see TAKETA *et al.* in press).

Linked AFLP Marker Screening and Conversion into PCR-Based Markers

AFLP analysis was performed as previously reported (KIKUCHI *et al.* 2003). For reliable detection of linked AFLP markers, screening was carried out in three steps. Linked AFLP bands were excised from the gel, cloned and sequenced. Primers for sequence-characterized amplified region (SCAR) markers (PARAN and MICHELMORE 1993) were designed as previously reported (KIKUCHI *et al.* 2003).

Molecular Variation at the sKT7 Locus

sKT7 is a dominant SCAR marker converted from an AFLP marker KT7, and it is completely linked in coupling phase with the *nud* allele in the mapping population of 460 F_2 plants. database search of the sKT7 sequence of Kobinkatagi using the BLAST program revealed no

significant homology to any sequences in the DNA Data Bank of Japan (DDBJ). sKT7-PCR products of barley germplasms were examined. PCR amplification, restriction digestion with *Nsp*I and electrophoresis were performed as reported elsewhere (TAKETA *et al.* in press). Selected accessions were also examined for variation at the DNA sequence level.

Results and Discussion

Marker Screening and High Resolution Mapping

From analysis of 2,742 primer combinations, 17 potentially linked AFLP markers were selected. Of nine markers characterized, 8 were successfully converted into polymorphic SCAR markers (data not shown). Five SCAR markers, named sKT2, sKT3, sKT7, sKT9 and sKT14, were used in mapping. sKT2, sKT3 and sKT9 are codominant markers, and the rest are dominant ones. Primer information for sKT2, sKT3 and sKT7 is available in our previous publication (KIKUCHI *et al.* 2003). The *nud* gene was flanked by two markers, sKT3 (proximal) and sKT14 (distal), with the genetic distances of 0.2cM and 3.1cM, respectively. Further mapping work is under the way, in order to resolve the order of sKT7 and sKT9 relative to the *nud* gene. Analysis of marker-hitting bacterial artificial chromosome (BAC) clones is also in progress toward the physical map construction.

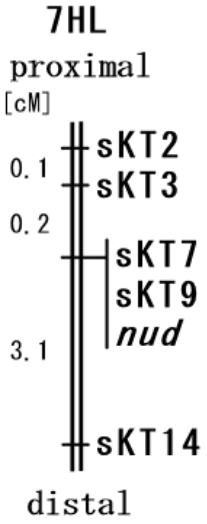

Fig.1. A high resolution map surrounding the *nud* locus in 460 F_2 plants derived from the cross between Kobinkatagi (naked) and Triumph (hulled).

Molecular Analysis of the sKT7 PCR-Amplified Product

Of the 259 accessions examined, 133 (51.4%) amplified a single PCR fragment of about 470 bp. The remaining 126 accessions (48.6%) produced no amplification products, carrying a null allele (named allele I). Allele I was found only in hulled accessions of both wild and domesticated barley, and totally absent in naked barley accessions. On the other hand, the ca. 470-bp band was amplified in 23 accessions (43.4%) of wild barley, ten accessions (9.4%) of hulled domesticated accessions and all naked domesticated accessions. Restriction analysis of the sKT7 PCR-amplified products with *Nsp*I revealed three distinct alleles, named II, III and IV. The amplified fragment size was all 467 bp long.

In wild barley, all four alleles (I to IV) were detected, but allele IV was rare (1.9%) and found only in one accession collected in southwestern Iran. In hulled domesticated barley, three alleles (I to III) were found, but allele I was predominant (90.6 %). All the naked domesticated barley examined had allele IV. Detailed information on the geographical distribution of the four sKT7 alleles is available in TAKETA *et al.* (in press).

Sequence analysis of the sKT7-PCR products was carried out in 41 accessions (11 hulled and 30 naked) representing the alleles II, III and IV. Altogether, 12 haplotypes were detected. Allele IV included 5 haplotypes, which shared 7 nucleotide substitutions unique to this allele (Table 1) and were clearly distinguished from the 7 haplotypes found in the alleles II and III. Although haplotype IVa found in wild barley showed a rather different sequence, four haplotypes found in naked domesticated barley (IVb to IVe) were very homogeneous. Compared to the most predominant haplotype IVb (66.7%), haplotypes IVc, IVd and IVe each had only one single nucleotide polymorphism (SNP). The second most frequent haplotype IVc (33.3 %) was distributed in Central Asia (Russia and Afghanistan) and eastward from China to Japan. Haplotype IVd was localized in eastern Europe. Thus, haplotype IVb was considered as the prototype, and haplotypes IVc, IVd, and IVe are considered as derivatives (Fig. 2). Haplotype IVb included both two- and six-rowed spikes, while haplotype IVc was all six-rowed, and haplotype IVd included only two-rowed spikes. Haplotype IVe was found in a Spanish six-rowed naked barley.

Table 1 Nucleotide polymorphism in 5 haplotypes of allele IV at the sKT7 locus

Haplo-	No. of	Polymorphic site[a]														
type	access.	40	108	141	149	181	193	211	226	239	244	272	287	298	366	44
IVa	1	A	G	C	T	T	G	T	C	A	G	A	T	A	T	C
IVb	20	*	A	C	*	T	*	C	*	G	G	G	*	*	*	T
IVc	7	*	A	C	*	T	*	C	*	G	**A**	G	*	*	*	T
IVd	2	*	A	C	*	**G**	*	C	*	G	G	G	*	*	*	T
IVe	1	*	A	**T**	*	T	*	C	*	G	G	G	*	*	*	T

[a] : Undelined numbers indicate the positions of 7 nucleotides unique to the allele IV.
Bold letters denote SNPs relative to haplotype IVb. * denote the same sequence as that of IVa.

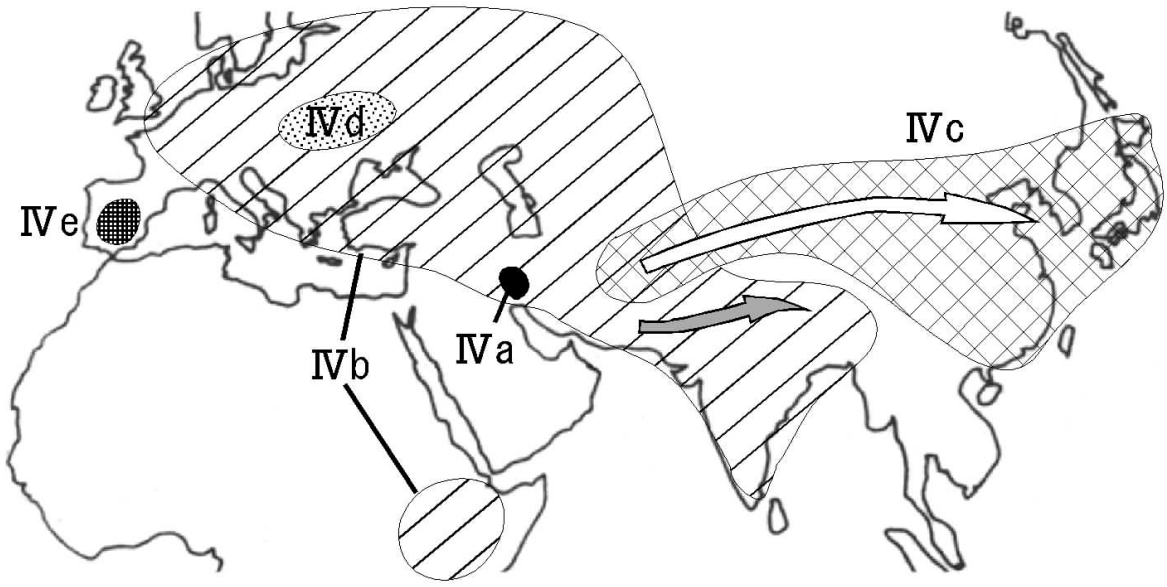

Fig.2. Geographical distribution of five haplotypes of allele IV at the sKT7 locus. Haplotype IVa was found only in a single wild barley accession, and the rest were found in naked domesticated barley. Arrows indicate the two migratory routes of naked domesticated barley to east Asia, which are proposed in this study.

Monophyletic Origin of Naked Barley

The localized distribution of the allele IV in wild barley and its fixation in naked domesticated accessions indicate that naked barley has a monophyletic origin, probably in southwestern Iran. This conclusion is further corroborated by the DNA sequence data (Table 1). The present conclusion coincides with the archeological evidence that the oldest carbonized naked domesticated barley (before 6000 B.C.) was found in the remains within the Fertile Crescent (ZOHARY & HOPF 2000). However, the available results on the sKT7 locus can not unequivocally decide whether naked barley derived directly from wild barley or from hulled domesticated barley. The sKT7 haplotype difference between naked accessions in China, Korea and Japan (fixed with IVc) and those in the Himalayan regions (Nepal, Bhutan and Tibet, fixed with IVb) indicate two independent migration routes of naked domesticated barley to east Asia, as shown in Fig. 2. The SNPs at the sKT7 locus are useful, to some extent, for discrimination among naked barley accessions. Closely linked SCAR markers developed in this study can be effectively used for marker assisted selection in crosses between naked barley and hulled barley, because they allow screening of plants with the *nud* gene at early growth stages in a wide range of materials.

Acknowledgements

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Relationship between Grain Hardness and Endosperm Cell Wall Polysaccharides in Barley

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Abstract

Grain hardness is one of important quality in pearling process of barley. To clarify factors contributing to hardness of barley grain, relationship between hardness of endosperm and major endosperm components (starch, protein, beta-glucan, arabinoxylan) were analyzed. Beta-glucan and arabinoxylan which were major components of cell walls in barley endosperm had a high correlation with hardness of that. On the other hand the effect of protein content on hardness was low; however protein content had a significant correlation with vitreosity which influenced endosperm hardness. These results showed that endosperm cell wall polysaccharides such as beta-glucan and arabinoxylan were principal factors contributing to hardness. Beta-glucan contained in barley endosperm is useful as dietary fiber, but its content influences grain hardness.

Introduction

Barley has been revaluated for food and source of dietary fiber. Beta-glucan richly contained in barley endosperm is especially very useful. Pearling process is needed to use barley grain as food, so pearling quality, such as grain hardness and whiteness of endosperm is one of very important objectives in breeding. Grain hardness of barley is evaluated as the time to grind grain to certain pearl yield *e.g.* 55% in pearling test mill, and endosperm hardness is an important factor in that. Vitreosity (or endosperm texture) contributes to hardness (KAWADA & TSURU 1984) and protein content has a significant correlation with degree of vitreosity (SOHTOME *et al*. 1991). In our previous study (TOHNO-OKA *et al*. 2002) it was suggested that content of not only protein but also cell wall polysaccharides was related to grain hardness. Therefore we analyzed relationship between endosperm hardness and cell wall polysaccharides (beta-glucan and arabinoxylan) in consideration of protein level and degree of vitreosity.

Material and Methods

22 varieties (containing six-, two-rowed, naked, hulled, waxy and non-waxy varieties) which reveal various grain hardness were experimented. To obtain grain having different level of protein content and vitreosity, they were cultivated in the two fields which consisted of

different soil. The one consisted of volcanic ash soil and was used as an upland field all the year around, but the other consisted of alluvial soil and was used as a paddy field where rice was cultivated during the summer. They were planted late in October, 2001 and harvested late in May to early June, 2002. Grain was sifted to adjust to 2.4-2.8mm (for six-rowed varieties), >2.6mm (for two-rowed with large grain) and >2.4mm (for two-rowed with small grain) grain size distribution. Endosperm hardness was evaluated as the time to grind grain from 55% to 40% (for hulled varieties) or 60% to 45% (for naked) pearl yield in pearling test mill (TM-05, Satake Corp., Japan) to avoid influence of husk and seed coat. Before pearling moisture of grain was adjusted to 11-12% level. Content of protein, starch, beta-glucan and arabinoxylan in the bran generated during pearling were analyzed. And also protein content of whole grain was measured. Protein was determined by nitrogen content (Nx5.83) with nitrogen analyzer (rapid N, Elementar Corp., Germany). Starch and beta-(1-3,1-4)-glucan were determined by glucose content with total starch assay kit and mixed-linkage beta-glucan assay kit (Megazyme Corp., Ireland), respectively. Arabinoxylan content was determined by arabinan assay procedure published by Megazyme Corp. (www.megazyme.com/booklets/KARAB.pdf) after hydrolysis with sulfuric acid. Vitreosity (endosperm texture) was evaluated as per cent of vitreous grain by observation of horizontal section of 100 kernels.

Results and Discussion

Barley grain revealed low level of protein content and vitreosity in the alluvial but high in the volcanic ash soil field (Fig. 1). Varieties carrying *wax* (waxy) or *fra* (fractured starch granule, CHUNG 1982) gene showed almost complete floury texture in spite of protein content both in the two soil conditions. In the varieties which did not have those genes vitreosity had a significant correlation with protein content and hardness (Table 1) as previously reported (KAWADA & TSURU 1984; SOHTOME *et al*. 1991). Protein content of whole grain was reported to have effect on grain hardness (ALLISON *et al*. 1979; TOHNO-OKA *et al*. 2002). Nevertheless, significant correlation was not detected between protein content in the bran and endosperm hardness both in mealy (in the alluvial) and vitreous (in the volcanic ash soil field) grain condition (Fig. 2). This meant that the effect of total content of protein on endosperm hardness was low; however protein content had a significant correlation with vitreosity which affected endosperm hardness. In wheat *Ha* gene and puroindoline located on the short arm of chromosome 5D were reported to affect endosperm texture and grain hardness (SYMES 1965; BAKER 1977; GREENWELL & SCHOFIELD 1986; GIROUX & MORRIS 1998). Recently the homologue of it, hordoindoline locus, was found on the short arm of barley chromosome 5H (ROUVES *et al*. 1996) and indicated to affect hardness also in barley (CHALMERS *et al*. 1993; BEECHER *et al*. 2002). But the relationship with endosperm texture was not clear (DARLINGTON *et al*. 2001). In our study hordoindoline might have effect on hardness, but it was not analyzed.

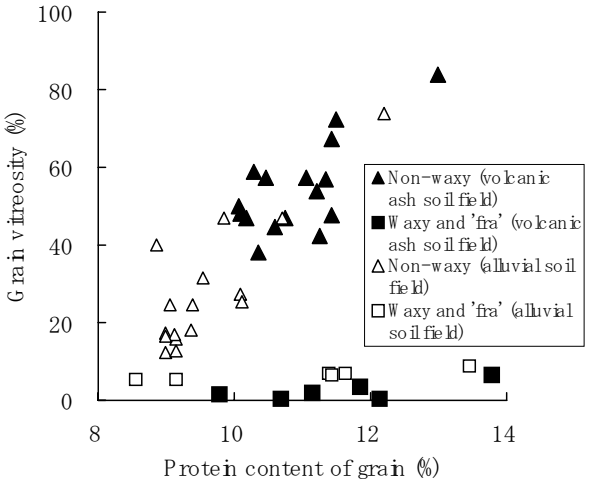

Fig. 1. Relationship between protein content of grain and vitreosity

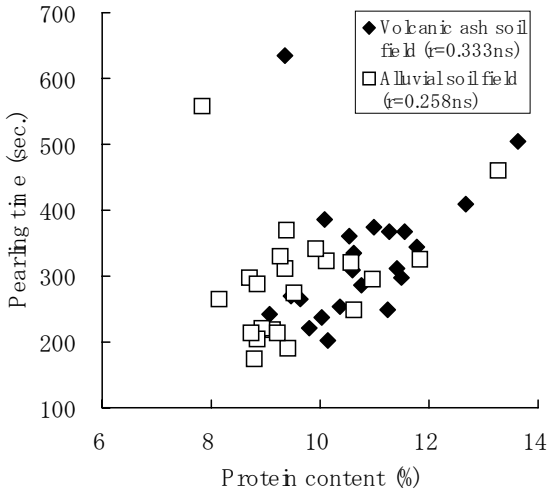

Fig. 2. Relationship between protein content and endosperm hardness

Table 1. Correlation coefficient between grain protein content, endosperm hardness and vitreosity (n=16)

	Volcanic ash soil field	Alluvial soil field
Grain protein content	0.712**	0.837**
Endosperm hardness	0.618**	0.578**

**: Significant at the 1% level

Beta-glucan and arabinoxylan, by contrast with protein, had a high correlation with endosperm hardness in spite of soil conditions (Fig. 3, 4). The effect of beta-glucan was higher than that of arabinoxylan. They are major polysaccharides in endosperm cell wall of barley, and it consists of 75% beta-glucan and 20% arabinoxylan (FINCHER 1975, 1976; BALANCE & MANNERS 1978). It was concluded that endosperm cell wall polysaccharides such as beta-glucan and arabinoxylan have higher effect on endosperm hardness than protein content or endosperm texture (vitreosity). These polysaccharides had a correlation with protein content (Table 2), but the relationship was unknown. Also in wheat polysaccharides in endosperm cell wall was reported to have an effect on its hardness (KATO *et al*. 1997). High correlation was detected between beta-glucan and arabinoxylan content (Table 2), but correlation of endosperm hardness and total content of beta-glucan and arabinoxylan was the same level as sole content of beta-glucan (Table 3).

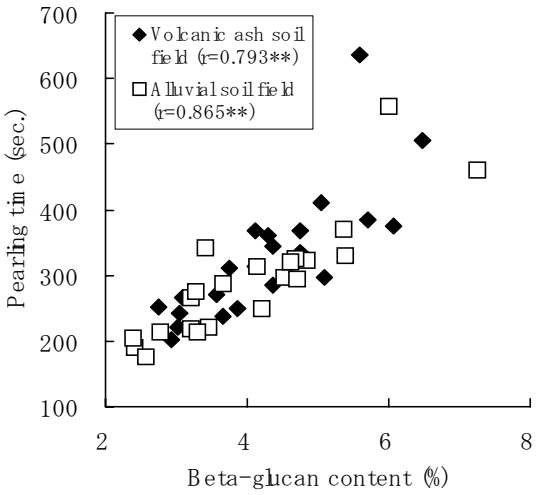

Fig. 3. Relationship between beta-glucan content and endosperm hardness

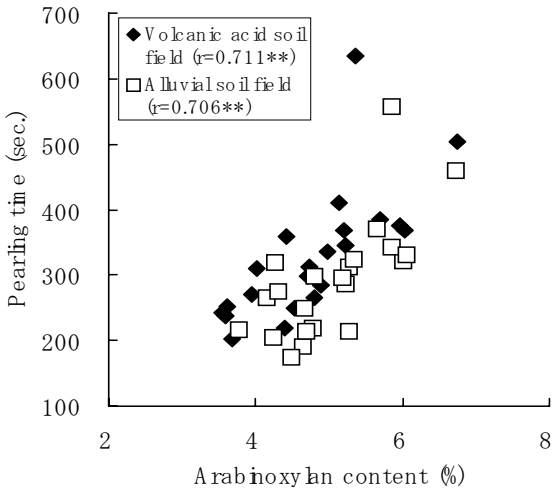

Fig. 4. Relationship between arabinoxylan content and endosperm hardness

Table 2. Correlation table among content of endosperm components (n=22)

	Starch	Beta-glucan (BG)	Arabinoxylan (AX)	BG+AX
Protein[1)]	−0.790**	0.544**	0.618**	0.603**
	−0.780**	0.513*	0.410*	0.505*
Starch[2)]	−	−0.885**	−0.868**	−0.917**
	−	−0.839**	−0.759**	−0.861**
Beta-glucan[3)]	−	−	0.831**	−
	−	−	0.749**	−

**, *: Significant at the 1%, 5% level respectively
1),2),3) Upper: in volcanic ash soil field
Lower: in alluvial soil field

Table 3. Correlation table of content of endosperm components with hardness (n=22)

	Volcanic ash soil field	Alluvial soil field
Protein	0.333	0.258
Starch	−0.738**	−0.636**
Beta-glucan (BG)	0.793**	0.865**
Arabinoxylan (AX)	0.711**	0.706**
BG+AX	0.790**	0.857**

**: Significant at the 1% level

There was a negative correlation between starch content and hardness. It was thought to be derived from negative relationship of starch with beta-glucan and arabinoxylan content (Table 2). In our experiment arabinoxylan level in the bran was the same as that of beta-glucan. That means much aleurone layer remained on the surface of even pearled grain of 55% or 60% pearl yield, since arabinoxylan is a main component of aleurone cell walls (BASIC & STONE

1981a, 1981b). Beta-glucan and arabinoxylan forms matrix and cellulose microfibrils are embedded in it (FINCHER & STONE 1986); therefore, their contribution to endosperm hardness indicates that strength, thickness or combination of cell walls is related to its hardness. Beta-glucan contained in barley grain is useful dietary fiber and high content of it is desirable for food, but its content has a negative effect on pearling process.

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Ways of Hulless Barley Grain Processing and Nutritional Quality

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Abstract

Different technological processing methods (separated milling, malting, puffing) were applied in two our own hulless breeding lines of spring barley, KM 1910 and KM 2283, with different genetic backgrounds of grain chemical composition. Based on nutritionally differentiated products (barley and malt flour, barley and barley malt flakes, scratch bran, malt honey, and others), recipes for preparation of salty and sweet bakery and pastry products (bread, pasta, bulgur, griddle-cakes, pancakes, fancy bread, gingerbread, etc.), products with beneficial health effects (Granola, a basis for milk fermented products, malt drink), children nutrition and products of "Chalva" and "Nugeta" types have been developed. Both sensory evaluations and chemical analyses of traditional and new types of products showed that hulless barley is a suitable raw material to enlarge the foodstuff assortment in the Czech Republic for both wholesome preventive human nutrition of the population and consumers of specific dietary requirements.

Keywords: spring barley; hulless grain; processing; milling; malting; puffing; food products; food ingredients; product recipes; preventive human nutrition; specific dietary requirements

Introduction

Direct use of barley grain has been stressed in its recent history for more than 10 years, however no considerable progress in actual consumption has been recorded in the Czech Republic (CR). Such a tendency is also reported from other countries in Central and East Europe, where, on the contrary, the supplement per capita has been decreasing since 1995 (the first available data of FAO). The trends in consumption are also different in economically developed countries. Whereas in some countries with traditionally high barley supplement (Japan and Korea) remarkable reduction has been recorded, the direct food barley consumption has been steadily increasing in countries of North Europe (Norway and Finland) since 1965 (Fig. 1).

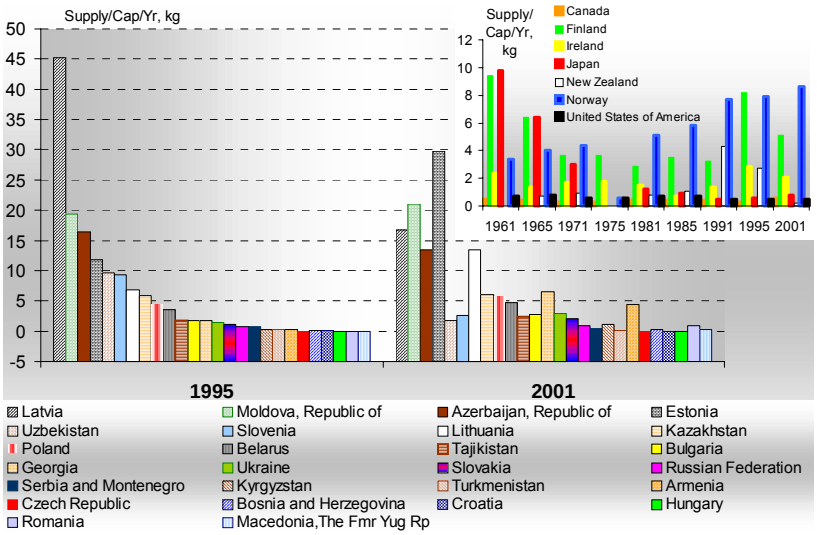

Figure 1. Supplement of the barley grain for direct food per capita in the East and Central Europe countries and selected developed countries of the World (FAOSTAT data, 2004)

There is no doubt that barley ranks among the most valuable cereal crops, which is confirmed by a large number of both foreign and home studies and clinical tests, and new findings about beneficial effects of barley grain in prevention of numerous chronic diseases (NEWMAN *et al.* 1989; HECKER *et al.* 1998; KEOGH *et al.* 2003; KERCKHOFFS *et al.* 2002, etc.). What is the cause that barley is rather less accepted as a nutritionally important food? There are more reasons, but the principal one is apparently its grain chemical composition which is characterized by fewer friendly properties necessary for processing in comparison with widely used wheat. According to foreign data, many of new or non-traditional processing technologies allow to improve sensory characteristics of barley cereal products (INGLETT *et al.* 2004). Their introduction in the CR is difficult not only due to conservative attitude of home consumers and the processing industry as it could seem at first sight, but as well as high costs of such investments.

If the state in the year of holding the 7th IBGS (VACULOVÁ *et al.* 1996) is considered, some progress has been recorded, particularly in the study and food use of hulless barley grain. Novel materials comparable to foreign varieties are tested in interstational trials at more locations and are supposed to be included in the State Variety Trials of the CR from 2004.

Even though the grain of hulless barley is more suitable for food use than hulled grain (BHATTY 1986), only people with naturalistic life attitude and not common consumers are ready to direct consumption. Therefore, further increase in the consumption necessitates to develop acceptable processing technologies that would emphasise our national boarding habits and aim at products comparable to well-known food kinds and/or better ones in sensory characteristics. One of possibilities how to enrich our market with cereal products is preparation of "half-finished products" that can be used by both small-scale producers and common consumers, even within groups with specific diet requirements.

In this paper, some processing methods and use of hulless barley grain, which are studied within a similar research project, are discussed.

Material and Methods

Plant Material

Grain of two own lines of spring hulless barley (*Hordeum vulgare* L., subsp. *distichon*, var. *nudum*) with different chemical composition developed at the Agricultural Research Institute Kroměříž (the pedigrees in VACULOVÁ & PSOTA 2003) were used for all processing methods. The line KM 2283-80/93 (hereafter KM 2283) has higher yield, medium to higher starch content and increased beta-glucan content in grain.

The line KM 1910-6/91 (hereafter KM 1910) ranks among standard hulless barleys. It is characteristic of high starch content, significantly higher extractability in comparison with hulled varieties, higher grain weight and light colour. It produced the highest content of methionine amino acid among the examined accessions.

Processing Technology

Separate Milling

Two methods for grain processing were employed. Designations of the obtained products are given in notes to Fig. 2. Milling fractions break bran, scratch bran and barley flour were obtained by standard processing in the wheat mill Buehler. Debranning was performed using PTMT device (Prokop Firm, Pardubice), exploiting Tkac preprocessing procedure (TKAC 1992).

Puffing

This technological method was carried out in the device for expansive drying developed and manufactured at the Food Research Institute Prague. During the processing, optimum conditions for puffing were examined, such as a gun temperature, operating pressure and raw

material moisture. The following parameters were used after preliminary tests: barley moisture range from 11.03 to 20.65 %, batch weight 6-9 kg, operating temperature 450-550 °C, operating pressure 0.8-1.2 MPa. Measures of a final product quality were a puffing index (raw material repose weigh/product ratio) and sensory quality (particularly off-flavour and smell of burn, puffed grain hardness).

Malting

The technological process was based on the following methodologies: a) preparation of standard malt – 2 days steeping, the 1st day 4 h under water, 2nd day 6 h under water, water content of 45 % at germination, a total malting period 6 days, standard kilning at kiln temperature of 80 °C for 4 h, b) malting at water content of 38 % at germination, a total malting period 4 days, standard kilning at kiln temperature of 80 °C for 4 h, c) malting at water content of 38 % at germination, 6 days germination (a total malting period), 12 h kilning at 55 °C, then the pre-dried malt was brought to the temperature of 105, 125 and 150 °C in 3 h and roasted for 4 h.

Chemical Analyses

The chemical analyses (dry matter, ash, standard parameters of malt and wort, contents of sugars, starch, N-substances, beta-glucans-BG, fat, total dietary fibre-TDF and non-dietary fibre-NDF in grain and barley products, etc.) were carried out according to standard methodologies (EBC, AOAC, etc.).

Development of Recipes for Preparation and Sensory Evaluation of Final Products

Recipes for preparation of salty and sweet bakery and pastry products, products for healthy and children nutrition were based on traditional Czech recipes. The final products were evaluated for sensory characteristics using a 1-7 scoring scale (1=excellent sample, 4=average sample, 7=unacceptable sample). Appearance, aroma, texture, flavour and total sensory acceptability by a consumer were evaluated.

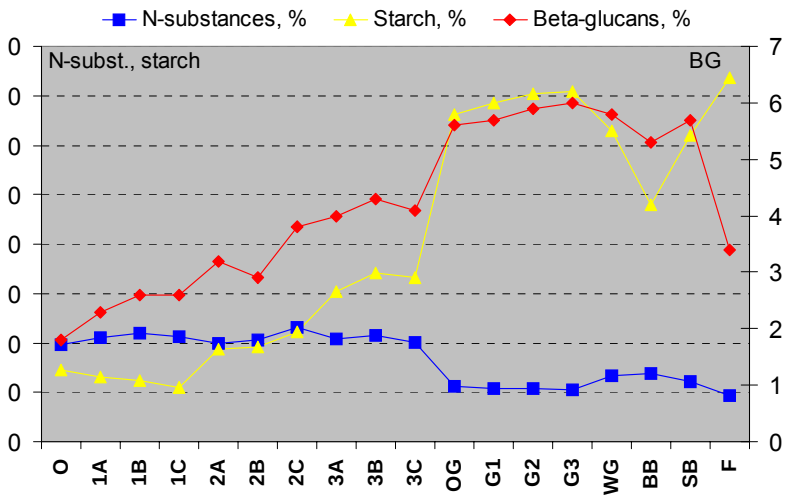

Figure 2. Composition of grain and by-products after debranning and separate milling (BS,SB,BF) (Kroměříž, 2002)

Products: 0 - Passage after friction, 1A - 1st by-product from abrasion, 1B - 2nd by-product from abrasion, 1C - 3rd by-product from abrasion, WG- Whole grain, 0G - Grain after friction, G1 - grain after 1st abrasion, 2A, 2B, 2C, 2G - i.d. for 2nd round of abrasion, 3A, 3B, 3C, 3G - i.d. for 3rd round of abrasion, BB - Break bran, SB - Scratch bran, F - Barley flour

Results and Discussion

A large number of technological processes are referred to in the literature that can be used for barley grain processing or improvement of its partial components (IZYDORCZYK *et al.* 2003)

We used processes from the methodology for milling fractions with added partial nutritionally important components, particularly beta-glucans.

The technology of **debranning** uses modified rice polishers aiming at the same primary goal as in conventional milling – that means separating bran from the

endosperm, however it considers physico-chemical characteristics of various bran layers. Figure 2 shows that if the standard process is adjusted, debranning allows to obtain very interesting by-products. Especially passages after the 3rd abrasion were significantly different from the original ones. Along with gradual abrasion of individual grain layers, beta-glucan and starch contents increase, whereas the original higher N-substance content remains practically identical. In contrast to conventional milling, it allows to obtain two types of food raw materials from hulless barleys during one technological process. Bran layers from internal parts of grain and finely abraded grain of oval shape is more suitable for various food applications than classical pearled barley produced in a pearling device. The following milling process is simpler and shorter than in conventional milling. BHATTY (1996) reports that barley requires longer germination due to hard grain. In the case of debranning, this character becomes advantageous because it improves results of abrasion. According to KLAMCZYNSKI *et al.* (1998), the changes in composition and microstructure of the kernels due to abrasion affect the rate of water imbibition, hardness of cooked kernels, etc. However, such a process is problematic if barley grain structure is damaged by internal sprouting and the grain becomes friable (brittle).

Puffing or explosion drying is a quite well-known and widely used process (SULLIVAN & CRAIG 1984). It is applied, for example, in production of expanded (puffed) rice and thermal treatment of present starch components (amylose and amylopectin). For hulless barley, there was no knowledge, particularly in relation to heating-up period, batch moisture and various operating conditions, and therefore it was necessary to conduct comprehensive investigations. It was found that for lower temperatures the heating-up period was longer and puffing indices were higher. The shorter heating-up period positively affected sensory quality of the product (lower percentage of burned surface of puffed grains). For a set of tests at the operating temperature of 550 °C for various conditions (batch weight and moisture, pressure), the regression equation to calculate the puffing index depending on modified operating conditions is as follows:

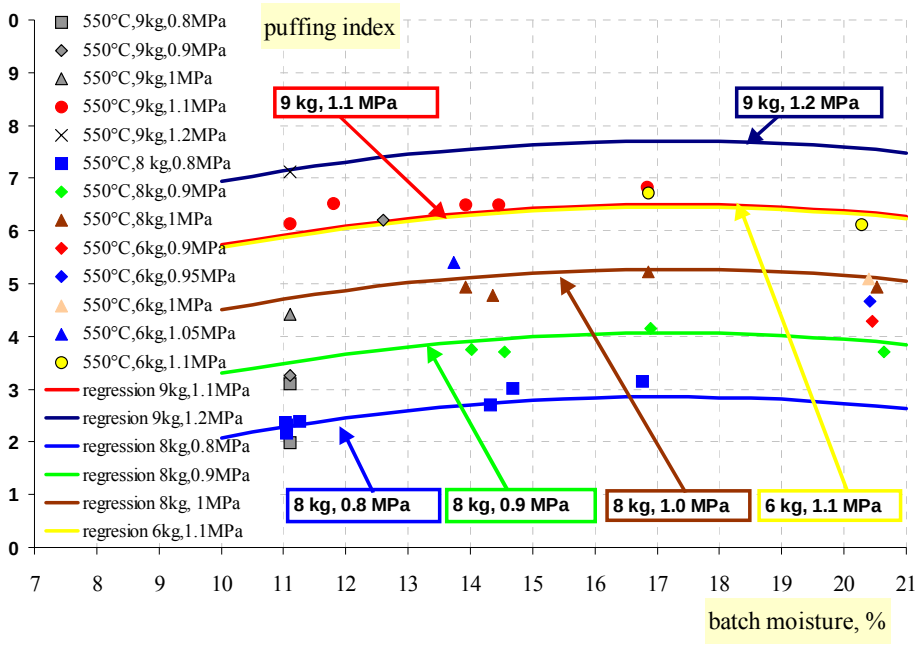

Figure 3. Comparison of measured and calculated puffing index depending on batch moisture and working conditions (T= 550 °C)

$$PI = a.x_1 + b.x_2^2 + c.x_2 + d.x_3 + e$$

where PI – puffing index
a, b, c, d, e – regression constants
x1 – regression variable – batch weight [kg]

x2 – regression variable – batch moisture [%]
x3 – regression variable – pressure [MPa]
The regression equation calculated values of regression constants (a = 0.0149, b = -0.0149, c = 0.5152, d = 12.0895, e = -11.3569). The standard error of the puffing index calculation is 0.29; 97 % variability is explained; correlation coefficient is 0.986.
The results illustrated in Fig. 3 and calculated values confirm the increase in the puffing index along with the increasing pressure; negligible differences in puffing indices were measured for various batch weight. The maximum puffing index can be achieved, under given operating conditions, at the batch moisture of approximately 17.2 %. Nevertheless, in accordance with conclusions on barley flour extrusion reported by VASANTHAN *et al.* (2002), further research on the functionality of the beta-glucan (and also other nutrients) in puffed barley grain is needed.
Malting of hulless barley induces enzyme activity and basic nutrients hydrolysis, which increases a potential of biologically active substances in the kernel and its nutritional value. Therefore, malts and malt flours are considered as beneficial food supplements. Especially in malts from hulless grain there is no need of removing undesirable hulls or nutrient extraction, so they can be directly used in food production (BHATTY 1996). Similarly to findings abroad (BOX 1999; EDNEY & ROSSNAGELL 2000), our results proved that some hulless barley have extremely high extractability (VACULOVÁ & PSOTA 2003). The highest mean extract content (86.95%) was found just in the line KM 1910, which was also confirmed using various malting regimes applied during this study.
Malting of hulless barley, however, requires to modify the technology applied in hulled barleys due to different grain structure. Therefore, we also investigated various malting technologies and their modifications (combinations of steeping, germination, kilning, roasting and extracting) aiming at the development of novel products either for direct use or further processing. Chemical analyses of sugar forms confirmed that the line KM 1910 showed the

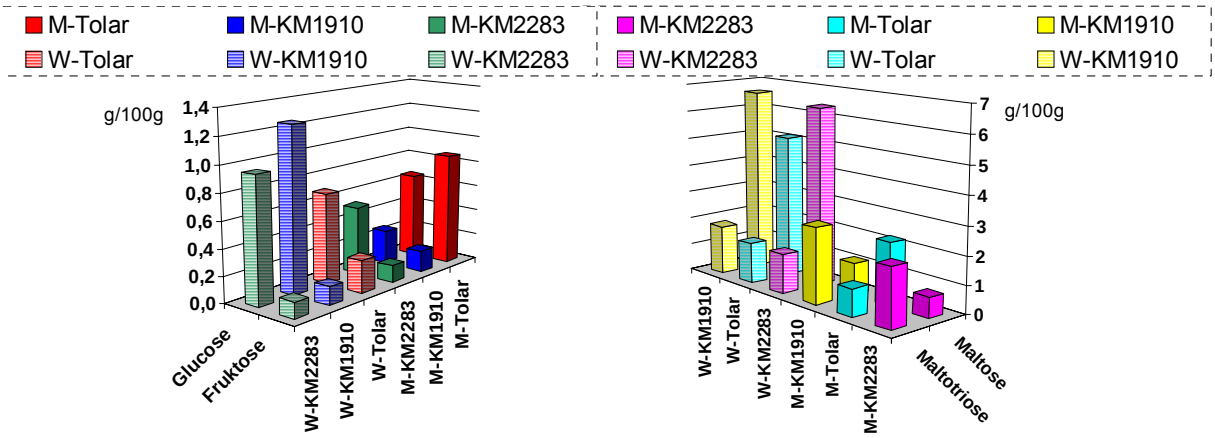

Figure 4. Content of sugars in malt (M) and wort (W) of hulless barley lines and standard hulled variety Tolar

highest contents of glucose and maltose in malt, which proves its high enzyme activity in comparison with the hulled variety Tolar and other line of hulless barley KM 2283 (Fig. 4). On the contrary, KM 2283 with high beta-glucan content is more suitable for other food applications (data see poster).
To produce malt flours for preparation of bakery products, the most suitable variant was the malt with water content of 38 % at germination, a total malting period of 6 days and final heating-up at 105 °C. This malt fulfilled requirements related to sensory characteristics and was used for the development of recipes of bakery and pastry products.

Vacuum thickened water extracts from malts with enzymatic activity were used for "cereal honeys" that can be used as natural sweetening agents, in pastry or drink production. Nutritional values for selected parameters of barley malt honey are given in Table 1.

Table 1. Contents of selected nutritional parameters of barley malt honey

Water content (%)	36.0	Maltotriose	6.29
Dry weight (%)	64.0	DP-4	4.71
pH	5.8	DP-5	1.05
Beta-glucans (mg/l)	60	DP-6	1.01
Soluble N (mg/100 ml)	1350	DP-7	0.91
Sugars (g/100 g)		DP-8	0.65
Fructose	3.67	DP-9	0.90
Glucose	12.05	DP-10	0.78
Maltose	29.34	Total	61.34

Based on nutritionally differentiated products (barley and malt flour, barley and barley malt flakes, scratch bran, and others), recipes for preparation of salty and sweet bakery and pastry products have been developed. Data on basic composition of the products, bread and oil fancy bread, that exhibit the best sensory characteristics, are presented in Tables 2 and 3. The sensory evaluation shows that in some recipes wheat flour can be completely replaced by barley flour without decreasing both sensory and nutritional characteristics of the certain product. In other products, more suitable barley flour:wheat flour ratio is 1:1 or lower. Nevertheless, BHATTY (1986) suggested following mixogram data to add from 5-10 % of barley flour only without seriously affecting loaf volume and bread appearance. Considering sensory evaluation, malt flour was better in drinks and children nutrition, by contrast, barley flour was more acceptable in bakery products.

Table 2. Chemical composition of various bread kinds with barley and barley malt flour

Parameter/ Product	Control Vita bread with wheat flour	Vita bread with barley flour	Vita bread with malt flour	Vita bread with barley flour and carob	Vita bread with malt flower	Šumava bread
Dry matter	69.3	65.1	75.1	66.1	67.2	69.0
N-subst.	7.10	7.90	10.0	8.20	9.40	8.30
Fat	7.20	7.00	6.00	8.20	7.70	6.40
Ash	1.93	1.72	1.90	1.94	2.17	2.28
TDF	8.20	5.40	8.60	9.18		5.60

Table 3. Basic composition of oil fancy bread

Parameter/ Product	Fancy bread (control-wheat flour)	Fancy bread (barley flour)
Dry weight	75.73	77.34
Protein	6.57	5.09
Fat	21.8	21.85
Ash	1.05	1.21
Total dietary fibre	5.7	6.99

There were no distinct differences in the nutritional value in Vita breads depending on flour used, however the Vita bread with barley flour was preferred in all parameters in sensory evaluation. Likewise, evaluation of fancy bread with barley flour was better than the average in all parameters, except of the texture, because of crumbliness. A higher TDF

content vs. a conventional variant was confirmed in both products. Similar values were obtained for cookies from barley flour, except for taste, that was worse. Gingerbread with barley flour was scored better than the average, but malt flour showed to be unacceptable.
MARCONI *et al.* (2000) used pearling by-products enriched with beta-glucan for pasta production in blending with 50% of standard durum wheat semolina. In our products, low sensory values were found for pasta, where namely appearance and colour received negative evaluation. Pure barley flour was not suitable for production of griddle-cakes. Better values for these products were obtained if a mixture of wheat and barley flour was used. Chemical composition and nutritional evaluation of other products with expected health preventive benefits (Granola, a basis for milk fermented products, children nutrition, products of "Chalva" and "Nugeta" types, etc.) were influenced by a proportion of the barley component. A group of health beneficial products can also include other evaluated recipes for barley and malt drinks. However, they exhibited lower values than bakery products (Table 4).

Table 4. Sensory evaluation of the barley and malt drinks

Feature/sample	Barley	Malt
Appearance	4.8	3.4
Aroma	3.7	2.9
Flavour	3.7	3
Acceptability	4	3

Conclusions

This study shows that even current technological equipment of processing plants in the CR provides suitable methods for processing the raw material and development of barley products acceptable by common consumers. Both sensory evaluations and chemical analyses of the products developed and tested until now showed that hulless barley is a suitable raw material to enlarge the foodstuffs assortment in the CR.
The above-mentioned recipes can be used in both small- and large-scale bakeries and manufactories of dough mixtures, pastry production, products of healthy nutrition, etc. In the future, the research will focus on enrichment of the barley foodstuff assortment with fermented milk products, improvement of both sensory and nutritive characteristics of products, and thus their wide use for both wholesome preventive human nutrition of the population and consumers of specific dietary requirements.

Acknowledgements

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S 7 – DISEASE AND PEST RESISTANCE I – GENERALLY, LEAF DISEASES

Evolutionary Potential of *Rhynchosporium secalis* Populations and Resistance Breeding Strategies

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Abstract
Scald (*Rhynchosporium secalis*) is a damaging barley disease. Breeding programs emphasize the incorporation of major resistance genes to control barley scald but rapid evolution of pathogen virulence leads to loss of resistance following widespread deployment. While knowledge of genetic structure may provide useful insights into the evolutionary processes that affect pathogen population genetics, experimental approaches ultimately are needed to provide a sound basis for the prediction of pathogen evolutionary potential. This paper presents preliminary results from a replicated mark-release-recapture field experiment designed to quantify the relative impacts of sexual reproduction, asexual propagation, immigration and selection on the genetic structure of an experimental population of *R. secalis*. Four barley varieties with differential scald resistance and a 1:1 mixture were planted in randomized complete block design and inoculated with an equiproportional mixture of 10 *R. secalis* isolates (inoculants). A total of 288 isolates were collected and assayed for 8 individual microsatellite loci. Two inoculants, BT6 and BT9, were particularly successful in infecting, competing, and reproducing on the susceptible hosts, Arabi Abiad and Rihane-03. Significant differences were observed in the frequencies of BT6 and BT9 on the two hosts over time. These early findings have validated the principle of the mark-release-recapture strategy in the barley/*R. secalis* pathosystem and showed the possible effects of selection due to specific host-pathogen interactions. If sexual recombinants constitute a significant source of inoculum for scald epidemics, then plant breeders should adjust their breeding strategies to focus on quantitative resistance, as well as increasing diversity in host populations.

Keywords: barley scald; evolutionary potential; mark-release-recapture; microsatellites; resistance breeding; *R. secalis*

Introduction

Scald, caused by the fungal pathogen *Rhynchosporium secalis* (Oudem.) J. J. Davis, is a damaging barley disease worldwide. Breeding efforts have focused on incorporating major resistance genes to control this disease but rapid evolution of pathogen virulence leads to loss of host resistance following widespread deployment.

Hierarchical sampling combined with DNA markers has substantially increased our knowledge of the amount and distribution of genetic variation within and among populations of *R. secalis* (McDONALD *et al*. 1999; SALAMATI *et al*. 2000; von KORFF *et al*. 2004). The source of the rapid evolution in *R. secalis*, however, remains unclear. SALAMATI *et al*.

(2000) examined the available evidence for recombination in *R. secalis* populations and concluded that regular sexual recombination is the most likely source of the high genetic diversity found in field populations of *R. secalis*. It was postulated that the teleomorph of *R. secalis*, though still not recognized, could play a significant role in the epidemiology and population biology of barley scald. If the ascospores of the putative teleomorph are dispersed by air, this could account for gene flow on a regional scale of tens or hundreds of kilometers. While knowledge of genetic structure may offer significant insight into the evolutionary processes that affect the population genetics of pathogens, experimental approaches ultimately are needed to test hypotheses and validate evolutionary concepts that emerge from population surveys.

Few field experiments have been conducted to test specific inferences (or hypotheses) regarding pathogen evolution under controllable and repeatable environments. The Phytopathology Group at ETH, Zurich is at the forefront of this field of experimental evolution with plant pathogens, and has already developed and applied many of the tools needed for experimental evolution studies in the model wheat pathogen *Mycosphaerella graminicola* (anamorph *Septoria tritici*) (CHEN & McDONALD 1996; ZHAN *et al.* 1998, 2001, 2002). In *M. graminicola*, ZHAN *et al.* (1998, 2000) showed that immigration and sexual recombination play significant roles in the epidemiology of the disease, and quantified their contributions to an epidemic. They also measured how quickly pathogen populations adapt to different host genotypes and showed results consistent with disruptive selection operating in mixed host populations (ZHAN *et al.* 2002). If sexual recombinants constitute a significant source of inoculum for scald epidemics, then plant breeders should adjust their breeding strategies to focus on quantitative resistance, as well as increasing diversity in host populations.

In this paper, we present preliminary data obtained in replicated field experiments designed to measure the relative impacts of mating system, immigration and host selection on the genetic structure of an experimental population of *R. secalis*.

Material and Methods

The barley varieties Rihane-03 (susceptible) and Line 9-26 F27 (resistant), a 1:1 mixture of Rihane 03 and Line 9-26 F27, and barley landraces Arabi Abiad (susceptible) and Arabi Aswad (moderately resistant) were planted in November 2002 in an RCBD with four replications at Tel Hadya-Aleppo, Syria (36° 01′N, 36° 56′E, 284 masl). Three replications were inoculated artificially at the seedling stage with an equiproportional mixture of 10 isolates of *R. secalis*. The ten inoculants (BT1-BT10) originated from various locations in Syria and were collected in previous years. The fourth replication served as negative control and was inoculated naturally by wind-blown conidia or ascospores that originated from outside of the experimental field. The inoculated plots were sampled two times, early (1 April 2003) and late (6 May 2003), during the growing season. It was not possible to conduct a planned third sampling because of the late planting and the sudden, extremely high temperatures (over 30°C) experienced with the onset of summer in April/May (Fig. 1). Infected leaves were collected from each plot at each sampling date and one fungal isolation was made from each leaf sampled from a different plant. A total of 288 isolates were collected and assayed for 8 individual microsatellite loci.

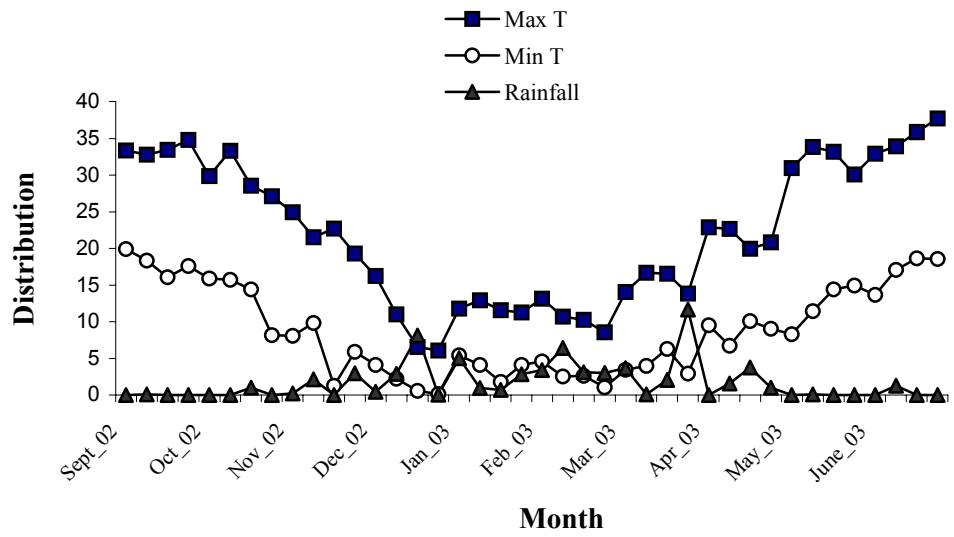

Fig. 1 Mean weekly minimum and maximum air temperature (°C), and rainfall (mm) at Tel Hadya for the period from September 1 2002 to June 30 2003

Total genomic DNA was extracted according to von KORFF *et al.* (2004) with minor modifications. Fungal biomass was either obtained by filtration of cultures grown in flasks containing potato dextrose broth (Difco, Detroit, USA) or directly from mycelium/conidia scraped off petri dishes containing yeast malt sucrose agar (4 g sucrose, 4 g malt extract, 4 g yeast extract, 4 g lima bean extract, 10 agar, 0.01 g kanamycin in 1 l distilled water). Eight *R. secalis* microsatellite loci were analysed for all 10 inoculants and the 288 *R. secalis* isolates. Forward and reverse primers of the *R. secalis* microsatellites TAC SSR1, TAC SSR6, CA SSR1, and GA SSR4 were multiplexed in one PCR (group I PCR) while those of GA SSR7, GA SSR1, GA SSR3 and R2 were multiplexed in another (group II PCR).

Microsatellite DNA amplification reactions consisted of 5-20 ng DNA, 1.5 µl of 10X PCR buffer, 0.1 mM dNTPs, ~10 pmol of each forward and reverse primer (slightly less primer was used for loci that gave a particularly strong product), and 0.5 U Taq DNA polymerase (Applied Biosystems, Foster City, California) in a total volume of 15 µl. The forward primers were 5′ modified with TAMRA, TET, 6-FAM, or HEX fluorescent labels (Applied Biosystems). Amplification conditions were as follows: initial denaturing at 96°C for 2.5 min; 35 cycles of 96°C denaturing for 30 s, 56°C annealing for 30 s, 72°C extension for 1 min; and a final extension at 72°C for 20 min using a T-gradient Thermoblock thermal cycler (Biometra GmbH, Gottingen, Germany).

For each sample, 4 µl and 3 µl of group I and group II amplified products, respectively, were diluted with 8 µl deionized water, mixed thoroughly and ran through sephadex into a sequencer plate. Four microliters of this dilution was added to 0.45 µl of the GENESCAN-500 LIZ internal size standard (Applied Biosystems) and 9 µl of Hi-Di formamide. The mixture was heat-denatured at 95°C for 2 min, placed immediately on ice for 2 min, and then subjected to capillary electrophoresis on an ABI PRISM 310 Genetic Analyzer at a run time of 3500 seconds run time and 12 seconds injection time. Fragment size data were obtained

using Genescan. Microsatellite allele sizes were scored by comparing the length of the PCR fragments to the standard 35-500 bp range size standard.

Proportions of novel genotypes in inoculated plots due to either recombination or immigration were obtained by determining the frequency of asexual progeny with multilocus haplotypes identical to those of the inoculants. The frequency of the inoculants on the different host treatments over time was also determined.

Results and Discussion

We present preliminary results on the use of replicated field experiments and microsatellite markers to investigate the evolutionary potential of R. secalis. Asexual progeny retrieved from inoculated field plots were found to have identical multilocus haplotypes to those of the inoculants - a crucial proof of the principle of the mark-release-recapture strategy in the barley/R. secalis pathosystem. This demonstrated the efficiency of the microsatellite markers used and validated the methodology chosen to address the research questions.

Microsatellite analysis revealed identical chromatograms with DNA obtained from cultures grown in liquid medium compared to cultures scraped directly from agar plates, indicating that the use of DNA extracted directly from cultures grown on yeast malt sucrose agar had no effect on the PCR amplification of the R. secalis microsatellites. This saves the cost of conical flasks and shakers, and eliminates the time (up to three weeks) required the shake the cultures. These cost- and time-saving measures are especially relevant in experiments requiring the analysis of thousands of fungal strains.

Significant differences were observed in the frequencies of the inoculants. Fifty-seven novel genotypes (represented by 113 isolates or 39 %) were observed among the 288 isolates analysed. Given the available data, however, it is premature to say exactly what proportions of novel genotypes in inoculated plots were due to recombination and/or immigration.

Two inoculants, BT6 and BT9, were particularly successful in infecting, competing, and reproducing on the susceptible hosts, Arabi Abiad and Rihane-03. Preliminary results indicate that host selection played an important role in influencing the frequency of R. secalis genotypes over the course of a growing season (Fig. 2). More BT9 than BT6 representatives

were sampled from Arabi Abiad plots (67% and 61% BT9 compared to 10% and 4% BT6, in April and May respectively), while more BT6 than BT9 representatives were sampled from Rihane-03 plots at the two sampling dates (41% and 42% BT6 compared to 28% and 17% BT9, in April and May respectively). It is interesting to note that between April and May, the frequency of BT6 decreased from 10% to 4% on Arabi Abiad but remained the same on Rihane-03 (41% to 42%). The frequency of BT9 decreased slightly from 67% to 61% on Arabi Abiad but fell sharply on Rihane-03 (28% to 17%).

This preliminary data has provided valuable information on the possible effects of selection due to specific host-pathogen interactions. ZHAN et al. (2002) reported intense competition among different M. graminicola genotypes in the field and significant differences in the frequencies of specific pathogen genotypes during the growing season. Some strains showed evidence for adaptation to particular hosts while others provided good evidence of host nonspecificity. In addition to gene flow and mating system, interactions among other biological, ecological and evolutionary processes might lead pathogens to increase their performance in some host populations, be unchanged in others and decrease in others, depending on the hosts compared and the pathogens used (KALTZ & SHYKOFF 1998).

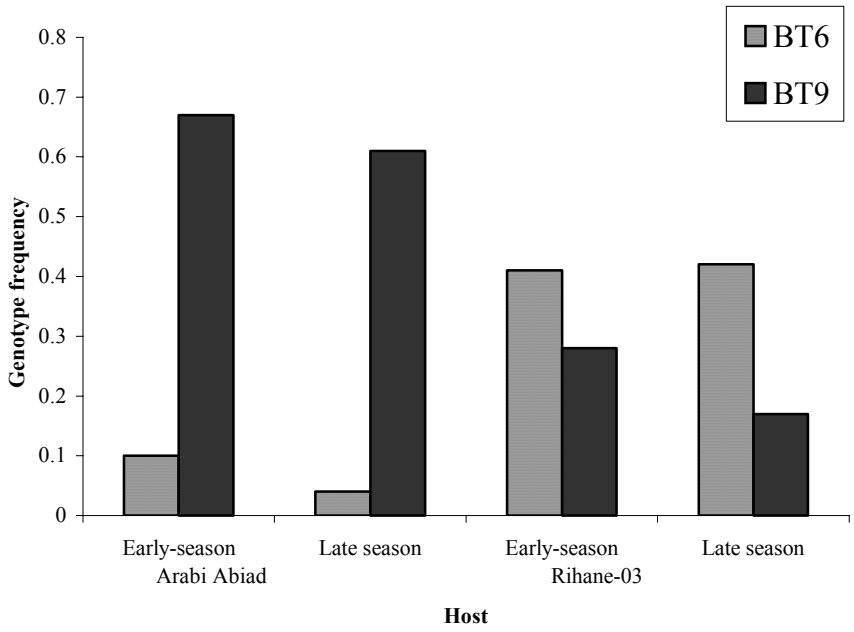

Fig. 2 Frequencies of *Rhynchosporium secalis* strains BT6 and BT9 on different host treatments during the growing season

The 288 isolates obtained from the field experiment at Tel Hadya fell short of the sample size expected to provide a complete dataset. More data will be needed before reliable inferences can be made on the significance of selection and host adaptation on the frequency of *R. secalis* genotypes over the course of a growing season. Similarly, the significance of recombination in field populations of *R. secalis* during and between growing seasons cannot be established with certainty at this stage. By placing the field experiment at Tel Hadya and Lattakia (35° 30′N, 35° 47′E, 7 masl), two locations conducive to scald but with sharply contrasting climatic conditions, there is a good chance of obtaining a complete dataset (microsatellite data on 2700 isolates) from at least one of the two locations.

McDONALD and LINDE (2002) recently formulated a risk assessment framework which predicts that pathogens with the highest evolutionary potential are most likely to become resistant to fungicides or to evolve virulence against resistance genes. Pathogens with the lowest evolutionary potential are least likely to rapidly adapt to control measures, and hence pose a lower risk in agroecosystems. Based on the predictions of the risk assessment framework, they also proposed a decision-diagram that can be used to offer advice to plant breeders on what type of resistance to use and how to deploy the resistance according to the evolutionary potential of the pathogen. Conducting replicated field experiments to understand the evolution of plant pathogens and differentiate among the different evolutionary forces is in its infancy. Field experiments to test the predictions of the risk assessment model concerning the barley/*R. secalis* pathosytem are now possible with robust and precise microsatellite markers coupled with advanced experimental designs and novel analytical methods. Their application in this project will contribute immensely to our knowledge of the

evolution of *R. secalis*, and thus to the development of sustainable breeding strategies for scald resistance in barley.

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Genetic Resources of Barley Resistance to Net Blotch

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Abstract

Resistance of more than 6000 barley accessions were investigated in field and laboratory conditions. Most of resistant barley genotypes were found among the landraces from centres of barley evolution. Effectiveness of known and novel sources and donors of barley resistance to different *Pyrenophora teres* populations was studied by inoculation with 200 isolates from Russia and Finland. Virulent isolates were found to any investigated barley genotype but resistance of new sources are more effective than of known donors CI 9819 and CI 5791. The segregation on resistance in hybrid population from crosses of resistant barley accessions c 15811 and c 23874 with susceptible cultivar *Pirkka* was depended on isolate genotype. Data of parallel analysis of segregation on resistance in barley and on virulence in fungus crosses are supported the model of gene-for gene interaction in pathosystem *P. teres/Hordeum vulgare*.

Keywords: *Pyrenophora teres*; barley; sources of resistance; genetic of resistance; genetic of virulence

Introduction

Problems of barley blights are in great importance all over the world (SMEDEGARD-PETERSEN 1974; STEFFENSON 1988; WALLWORK 1993). One of the most widely distributed and harmful diseases of barley in Russia are net blotch caused by *Pyrenophora teres* Drechsler f. sp. *teres* (anamorph *Drechslera teres (Sacc) Shoem*, f. sp. *teres*). In some areas of Russia yield losses on the susceptible cultivars can reach nearly 40% under epidemic conditions (SERENKO 1994; KASHEMIROVA 1995).

The development of resistant barley cultivars is the most cost effective and environmentally sound means of controlling this disease. The purpose of our research was to study the genetic resources of barley resistance to net blotch.

Material and Methods

Fungus Cultivation

Single conidial isolates of P. teres were obtained from infected barley leaves on the media (g/l): 0.5 KH_2PO_4, 0.5 $MgSO_4$, 0.5 KCL, 1.2 urea, 20 lactose, and 20 agars (BENKEN *et a.* 1969). Dishes were incubated during 5-7 days at 20-22^{o}C under constant illumination with a daylight lamps (5000 lx). Single conidia were transferred on the above mentioned medium and incubated in same conditions under constant illumination for propagation.

Virulence and Resistance Tests

For virulence test attached first barley seedling leaves and benzimidazole (0.004%) technique were used (AFANASENKO *et al*. 1995). Four or five days after inoculation (5000 conidia per ml), the types of seedling reactions to different single conidial isolates were estimated on a scale of 1 to 4 : (1) dots of necrotic lesions, no chlorosis (high resistance, avirulence); (2) necrotic brown spots, no or slight chlorosis, necrotic lesions are not spread

along the leaf segment, but restricted to the size of an infectious drop (resistance, avirulence); (1,2) point necrotic lesions and chlorosis (intermediate reaction type, resistance, avirulence); (3) necrosis develops all over the leaf surface surrounded by chlorotic regions (susceptibility, virulence); (2,3) necrosis occupies the entire leaf surface, but develops more slowly than in (3) (intermediate reaction type, for typing virulence phenotypes conditionally put down to the avirulence) ; (4) necrotic lessons occupy the entire surface of the leaf segment (high susceptibility, virulence).

Genetic Control of Resistance

Four novel sources of resistance were involved in genetic analysis of resistance (table 1).The F_2 of barley hybrid populations from crosses between resistant and susceptible genotypes were investigated. Four fungus single ascospore isolates were used in this work: 8-3, 8- 4, 9- 1 and 9-4 (progeny from cross 181-6 x A80) and single conidia isolate from Far East of Russia 181- 6. Isolates 8-3 and 8-4 were originated from one octade, also as 9-1 and 9-4.

In order to evaluate the response of the same F_1 or F_2 plants to different isolates, in each hybrid combination, the first leaf of F_1 or F_2 seedlings was cut into four 1,5-2 cm segments and placed in a vertical column on filter paper soaked in benzimidazole in plastic trays. There for a vertical column of four leaf segments represented one plant, while four horizontal rows consisted of different plants. On each leaf segment in a horizontal row, 0,005 ml of conidial suspension (5000-10000 conidia/ml) of one isolate was applied with a micropipette; the next row was inoculated with the second isolate, etc. Segregation for resistance to each isolate and compare the reaction of the same hybrid plant to different isolates was estimated.

Genetic Control of Virulence

Crosses of *P. teres* isolates were conducted by using W. MC DONALD's (1963) method: the mixture of mycelium of two isolates with different mating types, which we determined early, was spread on the autoclaving lemon leaves. Petri dishes with lemon leaves maintain in refrigerator with +15^{o}C and 16 hours photoperiod. Mature pseudothecae were formed after 2-4 months. Single ascospore isolates were obtained by injecting of ascospores from asci to the cover with lay of water agar.

Data Analysis

The χ^2 test was used for comparison of observed and expected segregation for resistance and virulence.

Results and Discussion

Sources of Resistance

In All Russia Research Institute of Plant Protection in collaboration with Plant Industry Institute screening of sources of resistance to *P. teres* have been conducting during more than 20 years (AFANASENKO 1977; TROFIMOVSKAIA *et al.* 1983; AFANASENKO 1995; AFANASENKO 1996). Resistance to *P. teres* more than 6000 barley accessions were investigated in field and laboratory conditions, using the benzimidazole technique. Most of resistant barley genotypes were found among the accessions from centres of barley evolution: from Ethiopia - 10,3%, Manchuria - 8,7%, Mediterranean region - 5,0%, East Asia - 8,7%, Middle Asia - 3,5%, South America – 1,6%.

Effectiveness of known and novel sources and donors of barley resistance to different *P. teres* populations was studied in 2001-2003 by inoculation by isolates from Russia and Finland. Characteristic of effectiveness of resistance of new sources in comparing with known

most resistant donors CI 9819 and CI 5791 are present in table 2. High heterogeneity of *P. teres* populations on virulence and significant differences between geographical populations are the reason of different effectiveness of sources of resistance in different geographical regions (AFANASENKO 2001). Absence of virulent isolates in different *P. teres* populations is the indirect index of durable barley resistance. The data of table 2 demonstrated that virulent isolates could be found to any investigated barley genotype but resistance of new sources are more effective than of known donors CI 9819 and CI 5791.

Genetics of Resistance and Virulence

Data of segregation in hybrid populations from 2 barley crosses to different *P. teres* isolates are presented in table 3. The resistance to isolate 181-6 in barley accessions c 15811 and c 23874 was determined by 1 dominant and 1 recessive gene. 181-6 is the parental isolate of ascospore isolates 8-3, 8-4, 9-1, 9-4. In combination 23874 x Pirkka segregation to isolates 9-1 and 9-4 is corresponded to the existence of one (3:1) or two (13:3) genes of resistance (table 3). We can propose that these isolates have the same virulence genotype as 181-6, but segregation on virulence in ascospore population from cross 9-1 x 9-4 to 23874 (21 avirulent: 6 virulent) is corresponded to the existence of two different avirulence genes in these isolates. These data supported the model of gene-for gene interaction in pathosystem *P. teres/Hordeum vulgare*. Presence of segregation in fungus cross combination of 181-6 x d 8-3, also testifies that the avirulence genes to 15811 and 23874 in these isolates are different.

Data of table 4 demonstrated the part of virulence test of ascospore progenies to barley accessions 15811 and 23874. Only discrepancy of reaction types to some ascospore isolates of barley accessions are showed in the table 4. Other ascospore isolates have the same virulence or avirulence to investigated barley accessions. This test is corresponded different resistance genes in barley accessions 15811 and 23874 which can be used as a source of resistance to different *P. teres* populations.

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Table 1. Barley genotypes - sources of resistance to *Pyrenophora teres f. teres*

Catalog VIR	Origination	Name	Variety
19979	Ethiopia	AHOR-40/65	brunneinudum
23874	Ethiopia	WGA-148-3	parallelum
15811	Chine	landrace	pallidum
15812	Chine	landrace	pallidum
25274	Ethiopia	CI 9819	Deficiens
C 25273	Ethiopia	CI 5791	Nutans
Susceptible control			
18530	Finland	Pirkka	pallidum

Table 2. Effectiveness of sources of resistance to different *P. teres* populations

P. teres populations	Number of virulent isolates in different *P. teres* populations to barley accessions, %					
	c-23874	c-19979	c-15811	c-15812	CI 9819	CI 5791
Russia, 2001						
Central part Kaluga	0	0	38,5	0	7,6	30,7
North West Rozhdestveno	0	7,1	3,6	0	10,7	17,8
Ural Falenki	0	17,6	5,9	0	15,4	5,9
Perm	0	0	0	0	10,0	25,0
Russia, 2002						
Luga	4,0	8,0	0	17,4	16,0	25,0
Rozgdestveno	1,75	2,17	0	19,5	10,0	6,1
Finland, 2001 Jokioinen	0	2,1	27,1	8,3	8,3	4,1
Finland, 2002						
Jokioinen	0	0	0	6,6	9,3	0
Ylistaro 1	0	4,7	0	5,2	19,0	25,0
Ylistaro 2	0	5,2	0	0	7,1	15,7
Total investigated isolates to each barley accession is 332						

Table 3. Segregation in hybrid populations from 2 barley crosses to different P. teres isolates

Crosses	Isolates	Segregation ratio		χ^2
		in experiment	expected ratio	
	8-3	169 : 64	3:1	0,757
15811x Pirkka	8- 4	145 : 88	9:7	3,388
	9- 1	183 : 50	3:1 13:3	1,558 1,123
	9-4	178 : 55	3:1 13:3	0,242 3,605
	181- 6	200 : 33	13:3	3,218
23874 x Pirkka	8-3	61 : 29	3:1	2,504
	8- 4	57 : 33	9:7	1,835
	9- 1	60 : 30	3:1	3,333
	9-4	73 : 17	3:1 13:3	1,793 0,001
	181- 6	77 : 13	13:3	1,095

Table 4. Discrepancy of reaction types to ascospore isolates in two *P. teres* cross combinations to barley genotypes

Ascospore isolates	Reaction type to barley genotypes (A – avirulence, V – virulence)	
	C 23874	C 15811
Cross combination 9-1 x 9-4		
2- 5	A	V
2- 7	V	A
2- 11	A	V
2- 17	V	A
2 -18	V	A.
2- 23	A	V
2- 29	V	A
2- 34	V	A
Total investigated isolates	27	27
Cross combination 181-6 x 8-3		
7- 3	A	V
7- 7	V	A
7- 8	V	À
7- 11	A	V
7- 13	A	V
7- 16	V	A
Total investigated isolates	25	25

Identification of Molecular Markers Linked to a *Pyrenophora teres* Avirulence Gene

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Abstract
This study investigated the genetic control of avirulence in *Pyrenophora teres*, causal agent of barley net blotch. Eighty progeny isolates were derived from a cross between the isolates WRS 1906 and WRS 1607. WRS 1906 is avirulent on the barley variety 'Heartland' while WRS 1607 is highly virulent. The population was evaluated for virulence on Heartland and segregated 38 avirulent: 42 virulent (χ^2 = 0.2, P = 0.70). This suggested a single gene controlled the avirulent phenotype. AFLP fingerprinting identified seven markers linked to the avirulence gene. These markers will be used in future efforts to positionally clone this gene.
Keywords: *Pyrenophora teres*; net blotch; avirulence; AFLP

Introduction

Many plant pathogens are highly specialized parasites capable of infecting a limited number of hosts. Co-evolution between a pathogen and its host is reflected by the presence of pathogen races, each capable of infecting only certain host genotypes. To defend themselves, plants have developed an "immune" system capable of recognizing and destroying these pathogens. To avoid the plant's defense system, pathogens are constantly changing the cues plants use to recognize them. The study of these specialized interactions by FLOR (1946, 1947) led to the gene-for-gene hypothesis. This stated that plant-pathogen interactions were conditioned by pairs of genes, one from the plant (resistance genes) and one from the pathogen (avirulence genes) (FLOR 1955).

The presence of avirulence (*avr*) genes in pathogens compromises their ability to colonize hosts and has led to questions regarding their purpose. Approximately 50 *avr* genes have been cloned, predominantly from bacterial pathogens, but little can be inferred about their function from their sequence data since they show little homology to others genes found in databases. However, studies with bacterial *avr* genes indicate that many function as pathogenicity factors on susceptible hosts (GABRIEL 1999). For example, strains of *Xanthomonas oryzae* carrying mutated copies of several *avr* genes were less aggressive and showed reduced growth rates on susceptible rice cultivars (BAI *et al.* 2000). The dynamic process of pathogens evolving new pathogenicity genes which are eventually recognized by newly evolved resistance (*R*) genes would explain the presence of *avr* genes, as well as, the large number of *avr* genes.

There is a limited understanding of how *avr* genes interact with *R* genes. Of the many cloned *avr*-*R* gene pairs, only two pairs show a physical interaction (TANG *et al.* 1996; JIA *et al.* 2000). This has led to the hypothesis that *avr* proteins interact with their host protein to promote disease (consistent with the idea that they are pathogenicity factors) and that *R* genes "guard" these host proteins to initiate an *avr*-dependent plant hypersensitive response (DIXON *et al.* 2000).

The ability to dispense with *avr* genes through mutation is thought to be the mechanism by which pathogens avoid detection by plant *R* genes (LEACH *et al.* 2001). However, it has been demonstrated that *avr* genes contribute in quantitatively different ways to pathogen fitness on susceptible hosts and that the loss of certain *avr* genes is more detrimental to pathogen survival and found less often in nature (VERA CRUZ *et al.* 2000). Therefore, *R* genes directed towards such *avr* genes would be expected to be more durable, which would have profound effects on the deployment of *R* genes in plant breeding programs. There is still much to be learned about *avr* genes, particularly fungal *avr* genes, which may lead to new methods of disease control.

The goal of this research project is to clone and characterize an *avr* gene from the net blotch pathogen *Pyrenophora teres* (*P. teres*). The initial steps towards this objective are described here, specifically, the creation of a *P. teres* mapping population, phenotyping of the population on a barley differential line and the identification of AFLP markers linked to an *avr* gene using a bulked segregant approach.

Material and Methods

Isolates

Fifteen *P. teres* isolates were evaluated for mating compatibility and virulence on five barley differential lines. These isolates were kindly provided by Dr. A. Tekauz (Cereal Research Centre, Agriculture and Agri-Food Canada, Winnipeg, Manitoba).

Mating

Mating was accomplished as described by WEILAND *et al.* (1999) except after incubating the crossing plates for 12 weeks at 15ºC in the dark, the plates were transferred to a 9 h photoperiod with an increase of 3 min/d for 3 weeks. Mature pseudothecia were produced at this point. Mature asci were released from the pseudothecia by crushing with a sterile needle, ascospores were released from the asci by crushing with a finely drawn glass pipet tip. Individual ascospores were transferred to 5% water agar plates to germinate overnight at room temperature. Germinated ascospores were transferred to V8A plates to produce conidia that were stored as glycerol and silica gel stocks until needed.

Virulence Assays

Plant inoculations were carried out as described by HO *et al.* (1996) with three replications for each isolate. Disease was rated on a 1-10 scale according to TEKAUZ (1985).

DNA Extractions

Each isolate was grown on V8A plates for 10 d at 21ºC with a 12 h photoperiod. Conidia ($2x10^5$) were scraped from the plates, inoculated into 100 mL of Liquid Medium (0.25% $MgSO_4$, 0.27% KH_2PO_4, 0.1% Bacto-Peptone, 0.1% Yeast Extract, 1.0% Sucrose) and shaken at 130 rpm for 48 h. Mycelia was collected on four layers of cheesecloth and washed with 100 mL of sterile water. The mycelia was blotted dry between two pieces of Whatman #3 paper before DNA was extracted using a method modified from PROCUNIER *et al.* (1990). The modifications involved adding Proteinase K to the 2X CTAB buffer at a concentration of 0.35 mg/mL and resuspending DNA pellets in 500 µL water with 50 µg/mL RNase A. Further purification of the DNA was required using three phenol/chloroform/isoamyl alcohol (25:24:1) extractions before finally resuspending the DNA in 100 µL water.

Bulked Segregant Analysis and AFLP Fingerprinting

Three bulks were created for both the avirulent and virulent phenotypes. Each bulk contained DNA from five isolates. AFLP analysis was carried out as described by VOS *et al.* (1995)

except only 250 ng of template DNA was used. Preamplification reactions used primers with one selective base (E-A, E-C, E-G, M-A, M-C, M-T) while selective amplification primers contained two selective bases. Bands were separated on 6% polyacrylamide gels and visualized by silver staining according to the manufacturer's instructions (Promega, Madison, WI). AFLP markers were named using four letters and a number. The first two letters are the selective bases from the *EcoR* I selective primer, the second two are the selective bases from the *Mse* I selective primer and the number represents the band size.

Linkage Analysis

Chi-square analysis was performed on each marker to test for deviations from the expected 1:1 Mendelian segregation ratio using a 0.05 significance level. Mapmaker/Exp version 3.0 (LANDER *et al.* 1987) was used to group and order the markers. Initial linkage groups were defined using the "group" command (LOD score 4.0, maximum distance 15 cM). Markers within linkage groups were ordered using the "compare" command. Linkages between ordered loci were calculated using recombination fractions and the Kosambi mapping function (KOSAMBI 1944).

Results and Discussion

Isolates WRS 1906 and WRS 1607 were chosen as parents for the mapping population based on their ability to mate with one another and their contrasting virulence on the six row variety 'Heartland' (Table 1). Both were highly virulent on the two row variety 'Harrington' which subsequently served as a susceptible check during disease evaluations.

Table 1. Description of parental *P. teres* isolates used to create the mapping population

		Collection		Disease Reaction[a]		Mating
Isolate	Form	Date	Location	Harrington	Heartland	Type
WRS 1906	net	1994	Fredricton, NB	9	1	+
WRS 1607	net	1985	Prince Albert, SK	9	9	-

[a] 1 is a resistant line (i.e., avirulent isolate), 9 is a susceptible line (i.e., virulent isolate).

A total of 80 single ascospore progeny were isolated from the WRS 1906 X WRS 1607 cross. The isolates segregated 38 avirulent: 42 virulent (χ^2 = 0.2, P = 0.70) on Heartland (Figure 1). Additionally, a complete octet of ascospores isolated from one ascus segregated in a ratio of four avirulent to four virulent on Heartland. This strongly suggested a single gene (*avr* gene) controlled the inability of WRS 1906 to cause disease on Heartland. All isolates were virulent on the susceptible control Harrington.

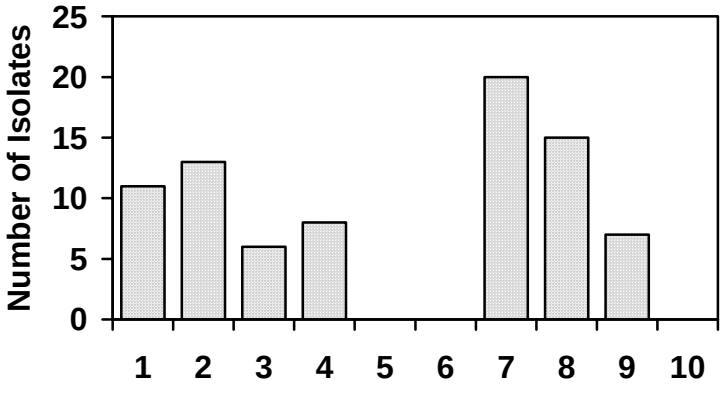

Figure 1. Disease reactions of Heartland to the 80 isolates derived from the WRS 1906 X WRS 1607 cross. An isolate which produces scores of 5 or lower is considered avirulent, while scores of 6 or higher are considered virulent reactions.

The six bulks and both parents were screened with 68 AFLP primer pairs. There were an average of 40 scorable bands per reaction with 3.6 polymorphic loci detected between the parents. Seven of the polymorphic markers were found to be linked to the avirulence gene (Figure 2). Two originated from the avirulent parent (WRS 1906) and five

originated from the virulent parent (WRS 1607). These markers segregated in a 1:1 ratio within the mapping population and formed a linkage group with the *avr* gene that covered 21.3 cM (Figure 3).

Identification of a single gene that controls the incompatibility between *P. teres* isolate WRS 1906 and Heartland supports the finding of WEILAND *et al.* (1999) who also concluded that one gene was responsible for the inability of isolate 0-1 to cause disease on the variety 'Harbin.' It appears that *P. teres* joins a growing number of fungal-host interactions governed by a gene-for-gene model, others include *Magnaporthe grisea*-rice (rice blast), *Cochliobolus sativus*-barley (spot blotch), *Rhynchosporium secalis*-barley (scald) and *Phytophthora infestans*-potato (late blight).

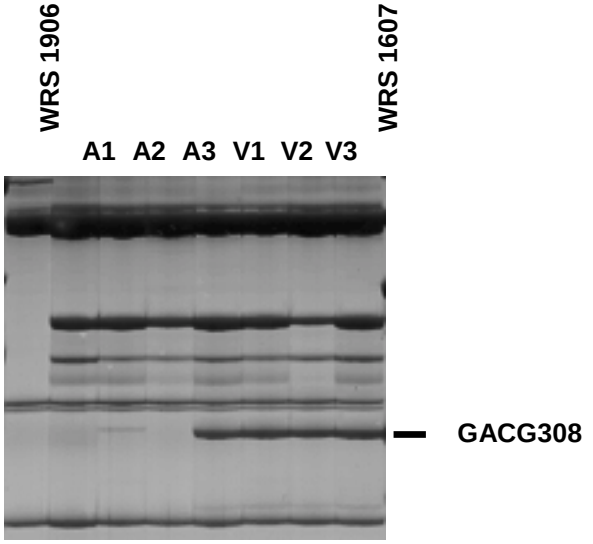

Figure 2. Example of an AFLP marker segregating (in repulsion) with the avirulence gene. A1-A3 are the avirulent bulks, V1-V3 are the virulent bulks.

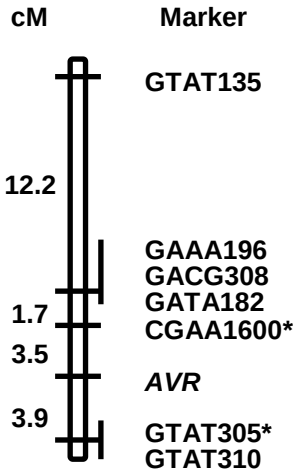

Figure 3. Linkage map containing the *avr* gene. * indicates the marker originated from the avirulent parent.

At present, the *avr* gene tagged in this study is being placed onto a larger map of the *P. teres* genome which will be linked to specific chromosomes. Additionally, a BAC library is being created as part of our effort to clone and characterize this gene. The markers identified here will be used to initiate screening of this library to positionally clone this *avr* gene.

Acknowledgements
This work was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) postgraduate scholarship and the Robert P. Knowles scholarship awarded to A.D. Beattie.

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Inheritance of Resistance to *Pyrenophora graminea* in Barley

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Abstract

An inheritance study of resistance to *Pyrenophora graminea* was undertaken in a diallel cross of 4 barley genotypes (two suceptible local genotypes (Saida & Tichedrett) and two resistant varieties (Alpha & M23).

A large variabilty in the disease incidence was found among parents and their progenies. In the crosses between a susceptible and a resistant parent, an intermediate reaction of the plant to barley stripe was obserdved, the general tendancy was however towards the resistant parent; a 1:2:1 ratio was predominant showing thus that the resistance of M23 and Alpha was contolled by a single dominant major gene.

In the cross between the suceptible parents, all the progenies showed suceptible reactions to *P.graminea*, a cumulatif effect of two genes was observed (9:6:1 ratio). The resistant by resistant parent gave in general resistant progenies and two dominant genes with no cumulatif effect (15:1 ratio).

Introduction and Literature Review

More than ninety percent of the worlds population diet is mainly based on cereals. Barley is one of nine essential cereals (food and feed) that are differently and insufficiently produced around the wold.

The lack of production is mainly due to low yields obtained every where due to abiotic and biotic stresses. The diseases are among the major constraints that limits seriously yields. Yield losses due to the diseases are estimated to 135 million tons per year.

The stripe disease in barley (Hordeum vulgare L.) caused by *Pyrenophora graminea* Ito & Kuribayashi is widespread over the world (2, 11, 14, 15, 6). It is also a prevalent disease in barley fields of Algeria (4). Resistance to the disease appears to be the most practical way to control it. Fungicides are usefull but expensive and can cause damages to the soil and unexperienced users. Sources of resistance to barley stripe are known (3, 8, 9, 13, 16, 17) and can be introduced in breeding programs for disease resistance. Many authors indicates that resistance is a heritable trait and the control is difficult or complex. Isenbeck (7) concluded that resistance was controlled by several dominant genes. Suneson reported that resistance was completely dominant in the cultivar Trebi and partially dominant in male sterile, whereas susceptibility was dominant in Club Mariout. He suggested that resistance was due to six genes. Arny (3) concluded that resistance was dominant and conditionned by three genetic factors in some crosses and incompletely dominant and conditionned by many factors in other crosses. Konak (10) studied resistance in F2 populations of eight crosses between American and Turkish barley varieties and found that resistance was dominant and conditionned by one or two genes. In a similar study Boulif (5) using American and Morroccan parents and their progenies (F1, F2, F3, BC1, BC2) concluded that the resistance of M23 was controlled by a single dominant gene. In other crosses, resistance was found to be conditionned by two genes one of which appeared to have a recessive epistatic effect on the other or by indeterminated number of recessive genes.

The relation of the reaction of the barley stripe disease and several marker genes was studied by Arny (3) that found the resistant genes in six out of seven linkage groups suggesting that these resistant genes have the same effect in the control of the resistance.

The objective of this work is to study the inheritance and genetics of resistance to barley stripe using local adapted genotypes, resistant American varieties and their progenies.

Material and Methods

Four barley genotypes were studied because their reactions to stripe had been observed in previous studies (1, 5).

Saïda and Tichedrett are Algerian local landraces developped in the 1950's from indegenous barley populations (1) are very sucseptible to stripe disease. Minnesota 23 and Alpha, of American origin showed resistance to Moroccan isolates (5).

The susceptible genotypes, Saïda & Tichedrett, were crossed to the resistant genotypes M23 & Alpha. Crosses between the susceptible parents (Saida x Tichedrett) or the resistant genotypes (M23 & Alpha) were also done.

F1 plants grown in the field at El Khroub (Algeria) and backcrosses were made to parental cultivars. F2 populations were generated and the F3 populations were harvested from individual F2 plants. Progenies of the F1, F2, F3, and backcross generations (BC1 & BC2) were tested along with parental genotypes in 1996 and 1997 at INRA Meknès (Morocco).

The virulent isolate obtained from El Khroub (Algeria) was used to test resistance. Inoculum was prepared from stock cultures of the isolates of *P.graminea* stored in infected dried barley leaves. Each culture was derived from a single conidium isolate of *P.graminea*. Inoculation was with vigorously growing cultures that were 10-12 days old, using sandwich method of Houston & Oswald and Mohammed & Mahmood (12).

Data analysis:

Because the data were expressed as percentages of plants diseased, they were transformed to arcsin x½, where x was the percentage of diseased plants. Comparisons among the parents, the F1, BC1, BC2 and F2 generations were made on transformed data. In the F3 generation, the frequency distributions of the F3 families were used to help interpret F2 segregation ratios. F3 families were classified as resistant, susceptible, or segregating on the basis of observed ranges of parental reactions.The number of F3 families in each class was compared with the number of expected in those classes. The chi-square test was used to check the goodness of fit of the proposed hypothesis to the observed distributions.

Results and Discussion

In this study, parental, F1, F2, BC1, BC2 and F3 generations were tested under similar experimental conditions. The data obtained showed great variability between the parents and the different progenies. The two local parents (Saïda & Tichedrett) showed a large susceptibility to *P.graminea* (49.9% -60%) and the resistant parents (M23 & Alpha) exhibited low levels of disease incidence (4.8% - 14.4%) to the El khroub isolate used (Table.1).

Table 1. Incidence of Elkhroub isolate of *P. graminea* on the different parents

Genotypes	Saïda	Tichedrett	Minnesota 23	Alpha
Incidence %	49.9 – 60	46.5 – 56.8	5 – 14.4	4.8 – 8.2
Reaction	S	S	R	R

S susceptible, R resistant.

In the crosses between a resistant (M23 or Alpha) and a susceptible (Saïda or Tichedrett), the mean of the BC1 generation was similar to that of the resistant parents, indicating complete dominance of resistance of M23 and Alpha over susceptibility of Saïda or Tichedrett. The BC2 generation had an intermediate reaction close to the midpoint of the two parents, approximating a 1:1 segregation ratio expected if resistance was due to a single dominant gene. In the F2 generation, many plants were stripe-free, mean incidence of stripe was about 24.5% (Table.2). This mean stripe incidence

which approaches the incidence of the BC1 and BC2 generations suggest that resistance in Minnesota23 and Alpha may be governed by a single dominant gene.

Table 2. Réaction des différents produits obtenus à partir des croisements diallèles réalisés, à la maladie striée de l'orge

Generations	**F1**	**BC1**	**BC2**	**F2**	**F3**
Resistant x Susceptible :					
Range %	18.8 – 20.2	18.9 – 23.3	17.5 – 30.8	21.8 – 25.7	20.9 – 27.7
Mean %	19.5	20.5	23.3	24.5	23.7
Variance	0.98	1.9	6.7	1.8	2.9
Susceptible x Susceptible :					
Mean %	-	57.07	63.9	56.4	54.8
Variance	-	27.9	31.3	29.6	25.7
Resistant X Resistant :					
Mean %	-	14.8	12.5	12.9	14.03
Variance	-	9.8	6.4	8.1	9.7

The hypothesis that resistance was controlled by a dominant gene was tested with 156 F3 families (Fig. 2, 4, 6, 8). The distribution of the families was skewed toward resistance, thereby suggesting that resistance was dominant in Minnesota 23 and Alpha and susceptibility was recessive in Saïda and Tichedrett. Because the F3 distribution did not group families into distinct classes, the families were grouped into resistant, segregating and susceptible classes on the basis of the range of stripe incidence of the parents (Table 3). The resistant, segregating and susceptible F3 families identified a 1:2:1 ratio, indicative of the segregation of a single gene. The test for the goodness of fit of the 1:2:1 ratio to the observed distribution gave high *P* values (0.5 – 0.7 to 0.85 – 0.9) for M23 x Saïda, Alpha x Saïda and Alpha x Tichedrett) and medium P value (0.3 – 0.5) in M23 x Tichedrett cross, confirming that one gene govern stripe reaction in these crosses.

In the cross between susceptible parents, all the progenies in the different generations exhibited susceptible reactions to the isolate used (54.8 – 63.9%) (Table.2). The obtained ratio in the F2, BC2 and F3 generations of 9:6:1 is indicating the presence of two genes with cumulative effect; however the existance of three F3 families in the resistant class (0 – 4.8%) indicate the possible effect of minor genes that govern resistance.

Table 3. Family number of F3 infected by the Elkhroub isolat of P.graminea, incidence range by class of reaction & Chi² in the different crosses

Resistant		***segregating***		***susceptible***		***Hypothesis***			***Total number***
Nbre	***Range(%)***	***Nbre***	***Range(%)***	***Nbre***	***Range(%)***		***X²***	***P***	***of families***
M23 x Saïda									
10	0 – 12.5	21	17.4 - 30	8	33 - 70	1 :2 :1	0.13	0.85-0.9	39

Alpha x Saïda									
9	4 – 14.8	23	16.3 - 28	8	43.3 - 53	1 :2 :1	0.95	0.5-0.7	40
Min23 x Tichedrett									
12	0 - 10	21	16.7 – 28.3	6	40 - 70	1 :2 :1	2.07	0.3–0.5	39
Alpha x Tichdrett									
9	0 – 12.5	22	16.7 - 30	7	35 - 60	1 :2 :1	1.16	0.5-0.7	38
Saïda x Tichedrett									
3	0 – 4.8	12	18.7 - 37.5	26	52.2 - 100	9 :6:1	1.17	0.8-0.9	41
Alpha x M23									
17	0- 13.6	12	16 - 24	4	35 – 47.3	15 :1	1.99	0.3-05	33

In the cross between the resistant parents, the progenies were resistant in all generations (12.5 - 14.8%). Each variety brings its resistance in the different products having thus an action of two dominant genes with no cumultive effect that expresses the same phenotype of resistance to the stripe disease presenting a 15:1 ratio in the BC1, F2 and F3 generations, the BC2 was completely resistant (Fig.12).

Conclusion

Barley stripe disease caused by *Pyrenophora graminea* is indeed an important constraint to grain yield. The inheritance of resistance to the stripe disease seems complex and varies with the existing inoculum. In this study, a large variability between the parents (resistant and susceptible) and their progenies (F1, F2, BC1, BC2 & F3 generations) appeared in the incidence levels. In the crosses including a resistance and a susceptible genotype, resistance was found governed by a single dominant gene in M23 or in Alpha showing a 1:2:1 ratio. In the cross between the two susceptible or the two resistant parents it has been shown a 9:6:1 and a 15:1 ratios respectively indicating the presence of two genes with or without cumulative effects.

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Fig.1: Class distribution in M23 x Saida (BC1, BC2 & parents)

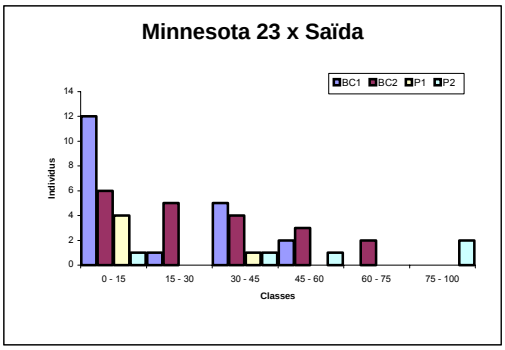


Fig.2: Class distribution in M23 x Saida (F2, F3 & parents)

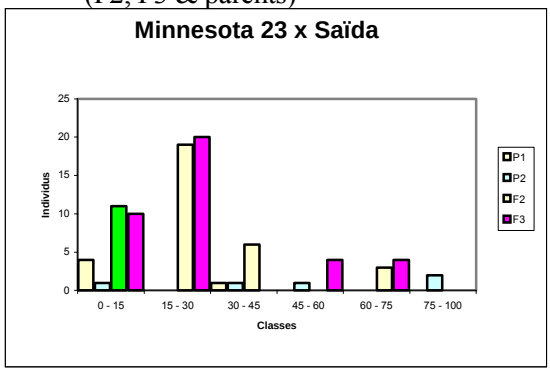


Fig.3: Class distribution in M23 x Tichdrett (BC1, BC2 & parents)

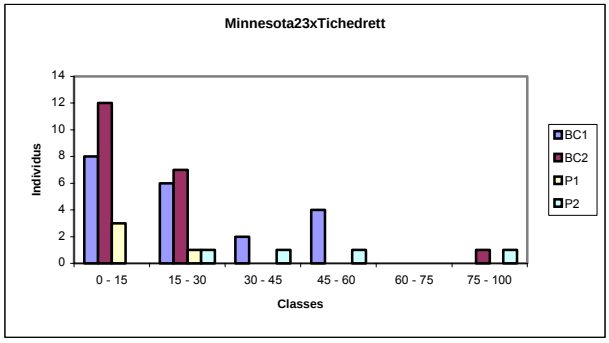


Fig.4: Class distribution in M23 x Tichedrett (F2, F3 & parents)

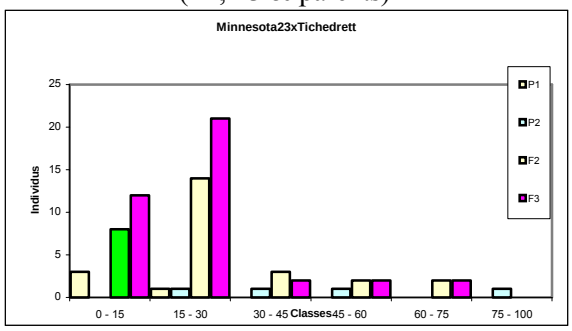


Fig.5: Class distribution in Alpha x Saida (BC1, BC2 & parents)

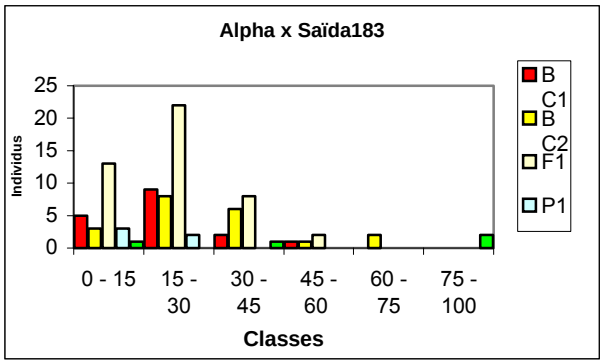


Fig.6: Class distribution in Alpha x Saida (F2, F3 & parents)

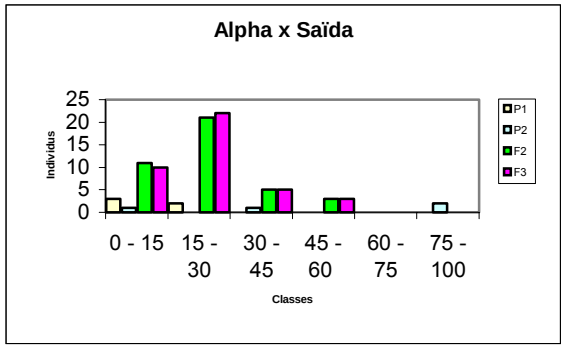


Fig.7: Class distribution in Alpha x Tichedrett (BC1, BC2 & parents)

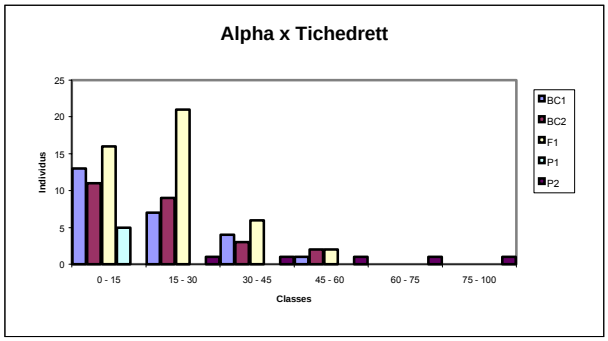


Fig.8: Class distribution in Alpha x Tichedrett (F2, F3 & parents)

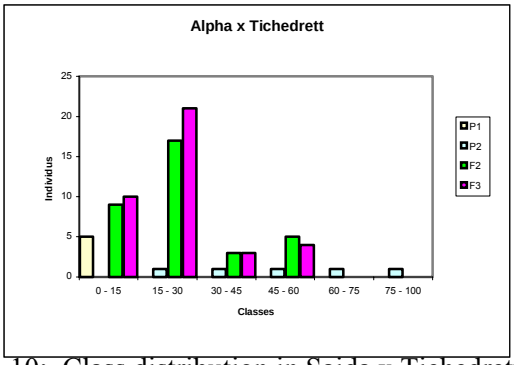
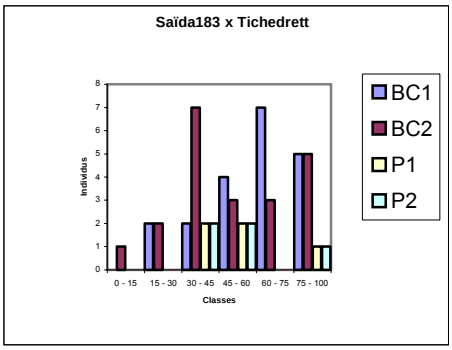


Fig.9: Class distribution in Saida x Tichedrett (BC1, BC2 & parents)

Fig.10: Class distribution in Saida x Tichedrett (F2, F3 & parents)



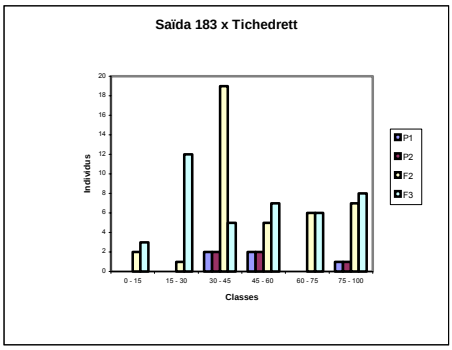
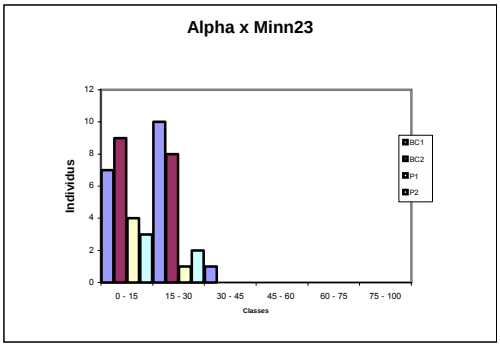
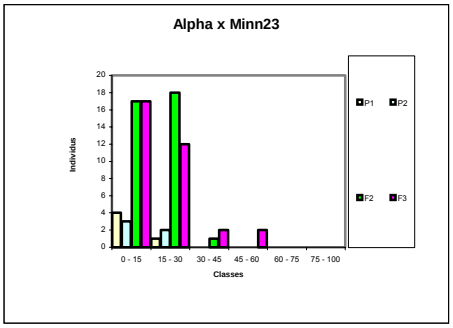


Fig.11: Class distribution in Saida x Tichedrett (BC1, BC2 & parents)

Fig.12: Class distribution in Saida x Tichedrett (F2, F3 & parents)





Profiling of Gene Expression in the Incompatible Interaction between Barley and the Fungus *Pyrenophora teres* (f. *teres* and f. *maculata)*

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Abstract
Net blotch disease of barley (*Hordeum vulgare* L.) is caused by the necrotrophic fungus *Pyrenophora teres*. The two types of leaf symptoms associated with net blotch disease are caused by the two *formae* of *P. teres*, with *P. teres* f. *teres* causing the net form and *P. teres* f. *maculata* responsible for the spot form. In this study, the method of suppression subtractive hybridisation was used to isolate genes differentially expressed in the early stages of both incompatible interactions, with the aim of finding genes involved in the defence response specific to each pathogen or common to both. Two cDNA subtraction libraries were generated resulting in the sequencing of 443 randomly selected clones of low abundance. An overview of the data reveals several genes that have previously been implicated in the defence response and a high proportion of genes that have no known function. Expression profiles during the early stages of infection have been generated for a subset of these genes using a combination of Northern blot analysis and quantitative real time PCR. In this paper, data is presented for a clone up-regulated in the epidermis of the net blotch resistant genotype at 3 and 24 hours after inoculation. There is evidence that this same clone may also play a defence role in the barley - *Rhynchosporium secalis* incompatible interaction.
Keywords: *Pyrenophora teres*; differential gene expression; real time PCR

Introduction
Gene expression analysis in incompatible plant – microbe interactions is a very important area of research because it may provide an insight into the complex network of genes associated with the plant defence response. In economically important cereal crops such as barley, our understanding of the signalling mechanisms that lead to disease resistance is limited. The aim of this project is to identify genes associated with disease resistance when barley (*Hordeum vulgare* L.) is challenged by the necrotrophic fungus *Pyrenophora teres*. The two *formae* of *P. teres* cause two different forms of net blotch disease, with *P. teres* f. *teres* causing the net form (NF), and *P. teres* f. *maculata* responsible for the spot form (SF). In this study, the focus is directed at the very early stages of both plant – microbe interactions with the aim of identifying the molecular basis by which the plant is able to mount a successful defence response. Genes that are differentially expressed in the incompatible interaction may provide targets for manipulation to produce improved resistance against barley net blotch and perhaps to a wider range of fungal diseases.

Material and Methods
Plant Material and Inoculations
The barley varieties CI9214 and B87/14 served as sources of net blotch resistance and susceptibility, respectively. Ten day old seedlings were inoculated with a virulent South Australian NF and SF isolate. After inoculation, the epidermal layers of infected and control leaves were removed and pooled together at various time points - 1, 3, 6, 12, 24, and 48 hours after inoculation (hai).

Suppression Subtractive Hybridisation
The PCR – Select™ cDNA subtraction kit (CLONTECH) was used to isolate differentially expressed genes in the NF and SF incompatible interactions 24 hai. For SSH, RNA extracted from the epidermis of CI9214 and B87/14 was the source of "tester" and "driver" cDNA, respectively. All cDNA fragments generated from SSH were cloned and spotted onto Hybond-N+ nylon membranes to make cDNA dot blots. A selection of clones was sequenced using an ABI 3700 sequencer.

Expression Profiling
Expression profiles for candidate transcripts were generated over 6 time points in the initial 48 hai using a combination of Northern blot analysis and quantitative real time PCR (Q-PCR). For Northern analysis, 10µg of total leaf RNA from infected and control plants was transferred onto Hybond-N+ nylon membranes. For Q-PCR, 0.5µg of epidermal RNA was reverse transcribed using 200 units of Superscript III RT (Invitrogen) to generate cDNA template.

Quantitative Real Time PCR
Q-PCR amplification was performed in a RG 3000 Rotor-Gene Thermal Cycler (Corbett Research) using the method described by Burton et al (2004), with some modifications. Briefly, PCR products amplified using gene specific primers were purified by HPLC and made to a stock solution of 10^9 copies/µL. A dilution series covering 10^7 to 10^1 copies/µL was used to generate a standard curve for each amplicon. This was used to quantify the amount of that amplicon in a cDNA sample of interest. All reactions were performed with the QuantiTect SYBR Green PCR reagent (Qiagen) in a final volume of 12µL using 1µL of a 1 in 10 dilution of cDNA as template.

Normalisation
All gene expression levels quantified by Q-PCR were normalised by applying a normalisation factor generated by the geNorm program (Vandesompele et al., 2002). In this study, the expression levels of four "house keeping" genes (cyclophilin, glyceraldehyde-3-phosphate dehydrogenase, α-tubulin, and a 14-3-3-like protein A) were analysed and the normalisation factor was derived from the three most stably expressed genes as indicated by geNorm.

Results and Discussion
The method of Suppression Subtractive Hybridisation (SSH) was successfully used to generate two cDNA libraries of potential resistance gene mediated defence response genes. Barley interaction with the net and spot form pathogen yielded 335 and 367 cDNA clones respectively following SSH.

To analyse the efficiency of SSH and to look for differential gene expression between the net blotch resistant and susceptible genotype a cDNA dot blot of each library was created. Following a number of hybridisations with "tester", "driver", and subtracted cDNA it was concluded that (a) SSH was successful in enriching for lowly abundant cDNAs and (b) the cDNA dot blots lacked sensitivity and could not be used to identify differentially expressed genes with low expression levels.

When some of the more abundant clones that gave a clear hybridisation signal were sequenced the results showed there to be a high level of redundancy of highly constitutively expressed genes such as ribulose bisphosphate carboxylase. These genes appear to have escaped subtraction and consequently were excluded from further analysis. Attention was shifted towards the cDNA clones of low abundance that gave no or very little signal following cDNA dot blot analysis. This group comprised 72% and 82% of all NF and SF clones respectively. The sequencing of 443 (243 NF and 200 SF) of these clones produced 324 unique sequences of which 249 were represented by 1 copy in the library. These unique sequences were categorised according to probable function based on their alignments with known sequences using BLASTX (Figure 1).

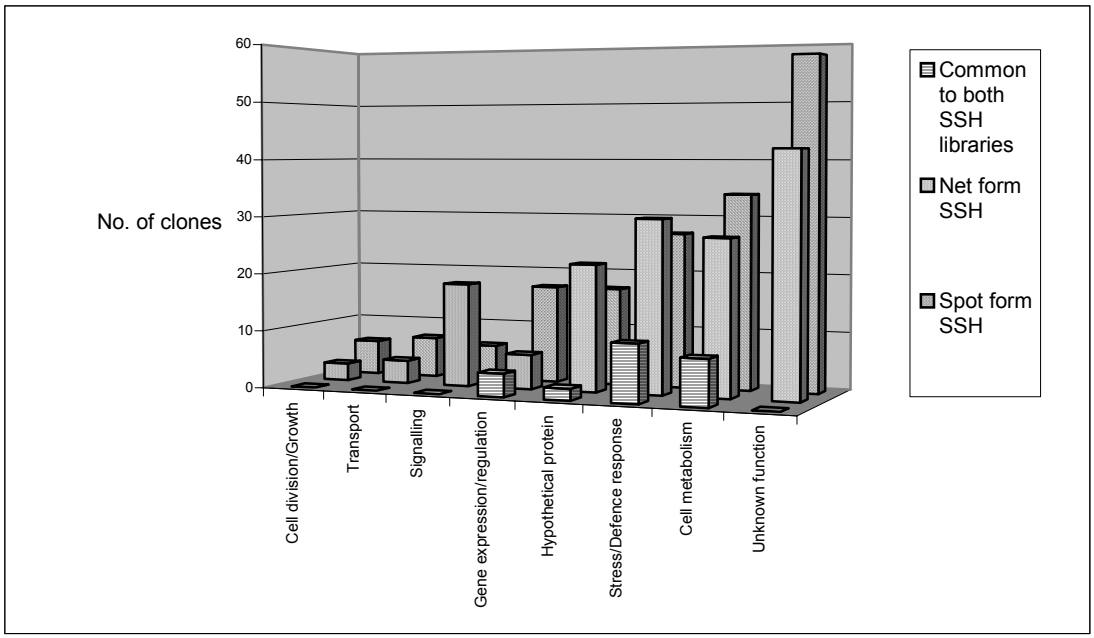

Figure 1. Categorisation of the 324 singletons according to their alignment with known sequences using BLASTX

A mixture of genes exists that have and have not been previously implicated in the defence response of other plant-microbe interactions. The majority of clones (48%) aligned to hypothetical or putative proteins with unknown function or had no known alignments. A small number (7%) of sequences were common to both subtraction libraries and from this group the stress and defence associated clones were most numerous perhaps suggesting a common mode of defence between the net and spot form interactions.

Northern blots were generated from the RNA of total leaf tissue of infected and uninfected plants to determine if the subtracted clones were (a) defence related and (b) differentially expressed between the resistant and susceptible genotypes. Expression profiles were generated over 6 time points in the initial 48 hai for 12 clones. The most interesting expression profile exists for a clone named NF353 (Figure 2). In the initial 6 hours following infection it is expressed at a higher level in the NF/SF resistant genotype. Even at 12 and 24 hai we see elevated expression levels combined with the expression of another perhaps related gene.

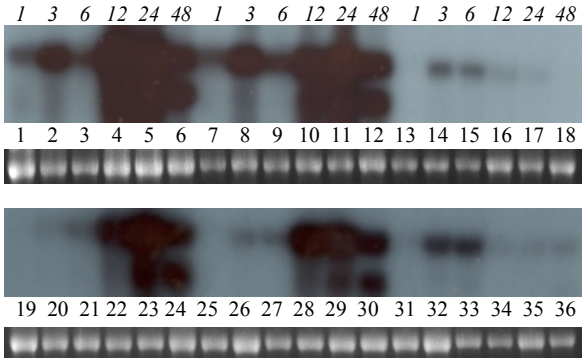

Figure 2. Expression profile for clone NF353 in total leaf tissue following NF and SF inoculation. Lanes 1-6: CI9214 NF inoculation. 7-12: CI9214 SF inoculation. 13-18: CI9214 water inoculation controls. 19-24: B87/14 NF inoculation. 25-30: B87/14 SF inoculation. 31-36: B87/14 water inoculation controls. *Italic numbers indicate hours after inoculation.*

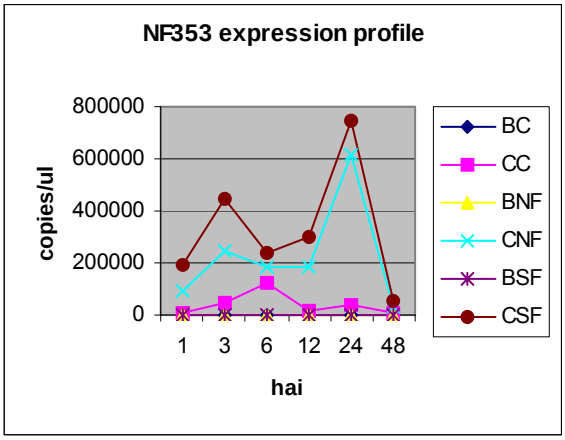

Figure 3. Expression profile generated by Q-PCR for clone NF353 in epidermal tissue following NF and SF inoculation. BC: B87/14 water inoculation controls. CC: CI9214 water inoculation controls. BNF: B87/14 NF inoculation. CNF: CI9214 NF inoculation. BSF: B87/14 SF inoculation. CSF: CI9214 SF inoculation.

Q-PCR analysis was used to generate an expression profile for this clone in epidermal tissue (Figure 3). The results show elevated transcript levels present in the resistant genotype controls compared to the susceptible genotype. At 3 hai this gene appears to be up-regulated slightly above controls followed by a more pronounced up-regulation at 24 hai. It is interesting to note that this clone was also found to be differentially expressed at 24hai in the incompatible interaction between barley and another necrotrophic fungus, *Rhynchosporium secalis* (K. Oldach, personal communication). *R. secalis* causes barley scald disease so it will be interesting to compare gene expression profiles for clone NF353 against net blotch and scald infection to determine a potential role for the corresponding gene in plant defence. Research is under way to optimise individual Q-PCR assays for several genes of interest, including those involved in plant senescence, ubiquination, and molecule recognition.

Acknowledgements

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Leaf Stripe Resistance in Barley: Marker Assisted Selection and Fine Mapping of the Resistance Gene *Rdg2a*

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Abstract

A barley gene conferring resistance to the leaf stripe agent has been mapped on the chromosome arm 7HS. This resistance gene, named as *Rdg2a*, confers resistance towards several isolates having therefore a useful range of activity. To verify the reliability of a PCR-based marker (MWG2018) associated to the resistance gene to assess the leaf stripe resistant phenotype in barley breeding programs, several resistant lines obtained from several crosses were tested for the allelic composition at the MWG2018 locus. The results showed that the resistant phenotype of the lines was always associated with the resistant allele of the molecular marker, evidentiating the reliability of this marker to select for leaf stripe resistance; this marker is routinely used in practical breeding. To saturate the *Rdg2a* chromosomal region with molecular markers, two approaches have substantially been used: 1) several RGAs have been tested for co-segregation with *Rdg2a*; 2) by exploiting the syntenic relationships between the telomeric regions of barley chromosome 7H and rice chromosome 6, rice ESTs informations have been used to generate PCR-based markers then mapped in the *Rdg2a* mapping population. By using these approaches, we have observed tight association of RGAs with *Rdg2a* and conservation of syntenic relationships between the telomeric regions of barley chromosome 7H and rice chromosome 6 for the *Rdg2a* chromosomal region.

Introduction

Leaf stripe, caused by the fungus *Pyrenophora graminea*, is a common disease in barley districts characterized by a cold climate during the sowing season. In susceptible cultivars the disease causes brown stripes on the leaves, stunted growth and severe yield reductions (PORTA-PUGLIA *et al*. 1986).

Both polygenic partial resistance (PECCHIONI *et al.* 1996; ARRU *et al.* 2002, 2003a) and race-specific resistance genes have been identified. *Rdg1a* conferring complete resistance to a subset of *P. graminea* isolates has been mapped on the long arm of barley chromosome 2 (2H) (THOMSEN *et al*. 1997). *Rdg2a* conferring complete resistance to the most virulent Italian isolate of *P. graminea* (Dg2) has been mapped to barley chromosome 1 (7H) (TACCONI *et al.* 2001). Plants containing *Rdg2a* are almost immune to disease caused by avirulent isolates, exhibiting no brown stripes on the leaves. *Rdg2a* containing breeding lines selected using Dg2 also appear to be resistant to the natural field populations of the pathogen, suggesting that *Rdg2a* may have a useful range of activity (ARRU *et al.* 2003b).

In the course of the *Rdg2a* mapping, PCR-based STS and CAPS markers were developed from the sequence of linked RFLP markers. The STS marker MWG2018 was mapped proximal from *Rdg2a* (TACCONI *et al.* 2001). Given this linkage relationship, this marker may represents a useful tool for selecting genotypes with leaf stripe resistance in barley breeding programs. The six-rowed winter cv. Rebelle carries resistance to the Italian field population of *P. graminea* and is used as a source of resistance in conventional breeding programs. It has been verified that the allelic composition of cv. Rebelle at the MWG2018 locus is the same as that of the resistant cv. Thibaut (ARRU *et al.* 2003b). The reliability of the STS marker MWG2018 to assess the leaf stripe resistant phenotype was therefore verified

in several resistant barley lines bred from the crosses between the resistant parent Rebelle, or Rebelle-derived resistant lines, and different susceptible barley cultivars.
In addition, a fine mapping of the *Rdg2a* resistance gene was performed by using a large segregating population. RGA markers mapping in the region, representing potential candidates for *Rdg2a*, were tested for co-segregation with *Rdg2a*. Furthermore, we developed additional markers closely linked to *Rdg2a* using the genomic rice sequence from the related region on rice chromosome 6. In this way, we defined a 115 kbp stretch of sequence as the syntenic interval for *Rdg2a* in rice.

Material and Methods

Plant Materials, Disease Screening and Mapping

Leaf stripe-resistant lines derived from pedigree programs originated from five crosses were tested. 1) F3494 (resistant line derived from Rebelle) X Balkan (susceptible); three F4 resistant lines F6573, F6574 and F6575 were selected for analysis. 2) F3494 (resistant) X Tamaris (susceptible); two resistant F4 lines F6576 and F6577 were selected. 3) F3505 (resistant line derived from Rebelle) X Po609.35 (susceptible); eight resistant F4 lines were chosen. 4) Rebelle (resistant) X Jaidor (susceptible); two F8 resistant lines F3485 and F3486 were used. 5) Rebelle (resistant) X F1341 (susceptible); three F9 sister lines, F3510.A, F3510.B (both susceptible to leaf stripe) and F3510.C (resistant) were selected. In addition, F2 plants derived from two crosses (Figure 2) were genotyped for the MWG2018 allele: Fo4388 (Fior6530, susceptible X Fior3486, resistant) and Fo4389 (Fior6578, resistant X Fior6530, susceptible).
A Thibaut (resistant) x Mirco (susceptible) cross was used to generate F_2 populations for *Rdg2a* fine mapping. The genotype for the *Rdg2a* locus was determined for all F_2 individuals (medium-resolution population) or for selected F_2 recombinants (high-resolution population).
Leaf stripe resistance assay was performed by artificial inoculation with the monoconidial isolate Dg2 using the "sandwich method", as described in TACCONI *et al.* (2001).
Map construction was performed using the MAPMAKER computer program (LANDER *et al.* 1987), using the Kosambi mapping function to convert recombination frequencies into centimorgans (cM).

PCR Marker Development

Genomic DNA to be used as PCR template was isolated from barley leaves according to STEIN *et al.* (2001) or following a standard CTAB (hexadecyltrimethyl-ammonium bromide) protocol (MURRAY & THOMPSON 1980). PCRs from barley genomic DNA template were performed as described by Bulgarelli et al., 2004. Chromosomal positions of rice BAC and PAC clones sequences were obtained from the physical/genetic rice maps (http://rgp.dna.affrc.go.jp/cgi-bin/statusdb/irgsp-status.cgi). EST and rice genomic sequences were downloaded and used in BLAST searches (Altschul et al., 1997) at NCBI (http://www.ncbi.nlm.nih.gov/). Barley or wheat ESTs with a DNA identity of 85% or greater with the rice sequences were considered as potential orthologues. In cases where a barley EST could not be identified, primers based on a wheat EST were used to amplify the corresponding gene from barley DNA. For MWG2018, amplification products were size-fractionated on 5% acrylamide gels, run in 1x TBE (100 mM Tris-HCl, 100 mM boric acid, 2 mM EDTA, pH 8.3) and stained with ethidium bromide. For CAPS markers, 20 µl of PCR mixture was digested overnight in a volume of 25 µl containing 1 x restriction enzyme buffer, 1.5 units of restriction enzyme and 0.5 µg/ul of acetylated BSA. The resulting fragments were size fractionated in 2% agarose gels (or 3% agarose for the marker BV078160).

RFLP Analysis

The ssCH4 RGA clone (AF052396) was kindly provided by Wolfgang Spielmeyer and Evans Lagudah (CSIRO Plant Industry). The RGA probes ABG1019, pic20, b9, BE216309 and S9202 were obtained as described by BULGARELLI *et al.* (2004). Genomic DNA for RFLP analysis and Southern blot analysis were as described in BULGARELLI *et al.* (2004).

Results and Discussion

Validation of MWG2018 as Rdg2a-Linked PCR-Based Marker

MAS effectiveness using the *Rdg2a*-linked marker MWG2018 was investigated in lines derived from five crosses, where the donor parents of leaf stripe resistance were either Rebelle-derived lines, namely F3494 and F3505 (Fig. 1A, B) or the cv. Rebelle itself (Fig. 1C). In Fig. 1A, the two susceptible parents (cvs. Balkan and Tamaris) showed the alternate allele at the MWG2018 locus, whereas all five resistant F4 lines bred from these two crosses (F6573, F6574, F6575 and F6576, F6577) showed the resistant allele of the marker. In Fig. 1B the resistant parent F3505 is heterozygous at the MWG2018 locus; the susceptible parent Po609.35 showed the susceptible marker allele. Four of the resistant F4 lines (F6571, F6579, F6580, F6584) derived from this cross were heterozygous for the marker; nevertheless, all nine selected resistant F4 lines had the resistance allele at the MWG2018 locus. In Fig. 1C the two F8 resistant lines (F3485 and F3486) possessed the allele of the resistant parent. In the last cross, three sister lines were analysed (F3510.A, F3510.B and F3510.C) and two (F3510.A and F3510.B) were susceptible to leaf stripe, while the third line, F3510.C, was found resistant to the disease. The analysis of the allelic composition at the MWG2018 locus with the STS primers revealed that the two susceptible lines have the same band pattern as the susceptible parent F1341, while the resistant sister line F3510.C shows the allelic band pattern of the resistant parent.

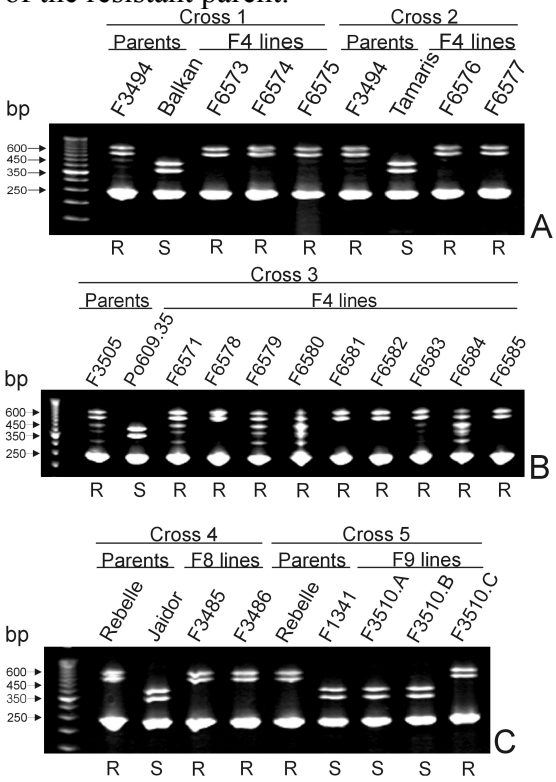

Figure 1. STS analysis of the marker verification lines derived from five crosses

Additional evidences of the suitability of MWG2018 for MAS have been obtained from analysis of the marker allele in F2 plants derived from two crosses (F04388 and Fo4389,

where Rebelle was the donor of the resistance, Figure 2) between resistant and susceptible lines (Table 1). The genotype at the resistance gene locus was verified by artificial inoculation of the F3 progenies, and the results showed that the resistant phenotype (with one exception for one F3 line of the cross Fo4389) was always associated with the resistant allele of the molecular marker.

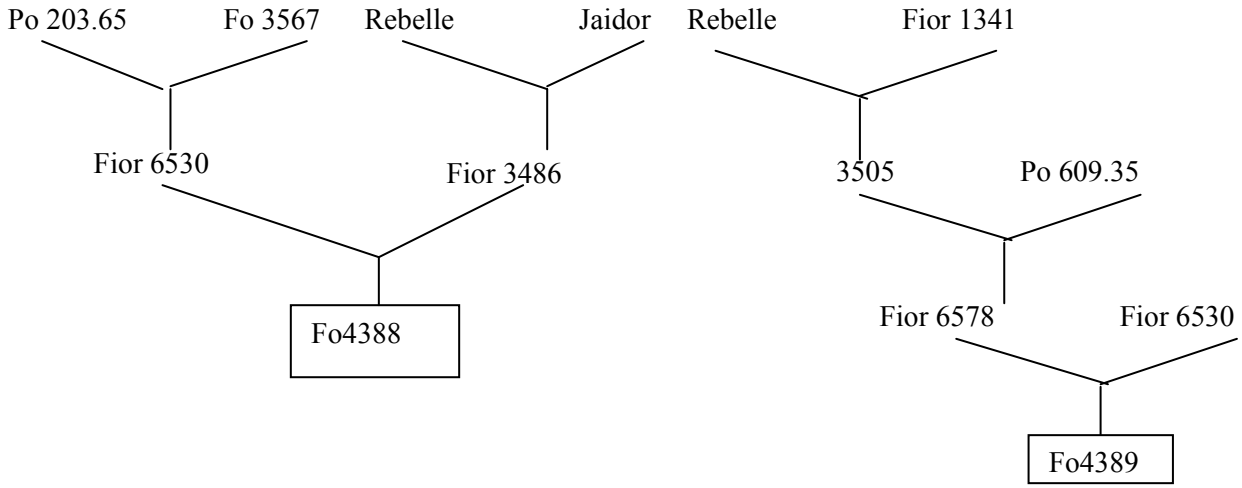

Figure 2. Pedigree of the two crosses in which Rebelle acted as donor of leaf stripe resistance

Table 1. Correspondance between the *Rdg2a* allele, as detected by artificial inoculation of F3 lines, and MWG2018 allele of the F2 plants

Fo4388			Fo4389		
Line	*Rdg2a* allele	MWG2018 allele	line	*Rdg2a* allele	MWG2018 allele
1	A	A	1	A	H
2	H	H	2	H	H
3	A	A	3	H	H
4	H	H	4	A	A
5	B	B	5	H	H
6	H	H	6	A	A
7	H	H	7	H	H
8	H	H	8	A	A
9	H	H	9	H	H
10	B	B	10	H	H

The utilization of the co-dominant marker MWG2018 can therefore allow identification of homozygous resistant plants in which no further segregation of the resistant phenotype will occour.

The reliability of the *Rdg2a*-linked PCR-based marker MWG2018 to detect leaf stripe resistance was therefore followed in a total of seven crosses where cv. Rebelle, or resistant lines derived from it, acted as donors of resistance. All the parents, resistant and susceptible,

proved to possess alternate alleles at the marker locus. For the resistant lines derived from the crosses, the marker MWG2018 correctly predicted the resistant phenotype. This PCR-based marker, which lies within few cMs from the resistance gene, thus proved to be suited to MAS for the resistance of both the cv. Rebelle and of the resistant lines derived from it.
The *Rdg2a P. graminea* resistance gene on barley chromosome arm 7HS was then subjected to high-resolution mapping using an F_2 population representing 2,800 gametes (Figure 3). *Rdg2a* was located to a marker interval defined by ssCH4, located 0.07 cM (2 recombinants) distally, and MWG851, located 0.07 cM proximally (Figure 3B).

Figure 3. Consecutive stages of high-resolution mapping at the *Rdg2a* locus. The first map (**A**), was constructed using a population of 218 F_2 plants. Markers ABG704 and ScOPQ9 were used to screen a population of 1,400 F_2 plants, and the resulting 93 recombinants used to construct the second map (**B**). RGAs in **B** are shown in bold. The three CAPS markers derived from rice ESTs (BV078155, BV078153, BV078160) enabled alignment to a rice physical contig of 453,648 kbp comprising three PAC clones and one BAC clone (**C**). Arrows between **B** and **C** indicate the position of homologues present in the rice sequence. Question marks indicate loci which mapped to positions unlinked to *Rdg2a* in barley.

We used a single high-resolution mapping population to map six classes of RGAs (in bold in Figure 3B; BULGARELLI *et al.* 2004) previously reported to map to the distal region of chromosome arm 7HS (SEAH *et al.,* 1998; LEISTER *et al.* 1999; AYLIFFE *et al.* 2000; ROSTOKS *et al.* 2002; MADSEN *et al.* 2003). Therefore, this region of 7HS contains a high concentration of RGAs of diverse sequence, in addition to the *Rpg1* resistance gene encoding a receptor-kinase like protein, and the presently uncloned *Rdg2a* resistance gene. These studies demonstrate that RGAs can provide a useful source of markers closely linked to resistance genes. High-resolution resistance-segregating mapping populations such as the one in our study are essential for eliminating closely linked RGAs as candidates for functional resistance genes. Rice sequences from the interval spanned by rice genomic PAC clones P0644B06, P0514G12 and P0029D06 (Figure 3C) were used to identify ESTs from barley or

wheat, representing potential orthologues of the rice genes and PCR markers were developed (Figure 3B, 3C; Bulgarelli et al., 2004). The markers BV078155 and BV078153 define the smallest *Rdg2a* syntenic interval in rice (Figure 3C). The current annotations for this 115 kbp sequence interval did not include any predicted protein with assigned similarity to a known resistance protein (BULGARELLI *et al.* 2004). Additionally, our own analysis of this DNA sequence interval did not reveal any potential to encode any resistance protein -like sequence. This indicates that either an orthologue of *Rdg2a* does not occur in this rice interval due to a breakdown in synteny, or that *Rdg2a* encodes a type of resistance protein not yet described. The recombinants and markers identified durin this work will be invaluable resources for the isolation of this gene.

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Identification of a New Adult Plant Resistance Gene for Scald (*Rhynchosporium secalis*) in Barley

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Abstract
The identification and deployment of disease resistance genes are key objectives of Australian barley breeding programs. A doubled haploid (DH) population derived from the cross VB9104 X Dash was used to identify markers for resistance to scald (*Rhynchosporium secalis*). The population was assessed for severity of scald in field trials in South Australia and Western Australia. A genetic map comprised of 205 markers including SSRs and AFLPs was used for gene discovery. Marker analysis was performed using the software packages Mapmanager and Qgene. QTL analysis identified a region on chromosome 1H and 3H, also associated with scald resistance in a number of other studies, and a region on 4H, which has not previously been associated with scald resistance at the adult plant stage. These associations were consistent in both locations. Multiple regression analysis of these three QTLs explained up to 48% of the variation. The markers within these QTLs present an opportunity for marker-assisted selection of lines with resistance to scald in barley breeding programs.
Keywords: *Rhynchosporium secalis*; adult plant resistance; genetic mapping; SSRs; AFLPs

Introduction

The disease scald, or leaf blotch, caused by the pathogen *Rhynchosporium secalis* is a foliar disease that is widespread in the temperate barley growing areas of the world. Yield losses of 15% to 45% have been reported due to this disease in Australia (BROWN 1985; KHAN 1986). *R. secalis* is a highly variable pathogen and this results in selection of new pathotypes that can overcome host plant resistance (GARVIN *et al.* 1997), which is a challenge to breeders.

Inheritance studies have identified many genes from different sources. Genetic mapping of some of these genes, reviewed by GENGER *et al*. (2003) and WILLIAMS (2003), identified the chromosomal locations of these genes. The most common locus is situated on the long arm of chromosome 3H. Additional genes were also mapped on chromosomes 1H, 6H and 7H (GENGER *et al*. 2003). Most of the studies involved genes that are effective at the seedling stage. The objective of this study was to identify genes in a barley population that are effective at the adult plant stage.

Material and Methods

Mapping Population

The barley parent varieties VB9104 and Dash were used to construct a DH population of 180 lines using the anther culture technique.

Phenotyping for Scald in the Field

Turretfield Nursery

180 DH lines and parents of the VB9104/Dash mapping population were sown in 2m long paired rows with 2 replications in a disease nursery at Turretfield in South Australia in 2002. At mid-tillering, barley straw infected with scald and spot form net blotch from the previous trial was spread through the nursery. The trial was assessed for scald and spot form net blotch after all lines had reached flowering and some were at late grain filling. Scores of scald infection were obtained using a 0-9 scale where 0 represented no disease and 9 was very severely infected.

Medina Nursery

The same 180 DH lines and parents were sawn in the field at Medina, Western Australia in 2m row plot length in a randomized block design. The plants at growth stage 15 (Zadoks et al. 1974) were infected with the scald-infested straw with the Western Australian pathotype (no formal pathotype names are given yet in WA) for the disease development. The trial was also inoculated with the spore suspension with the same strain during the season at GS 40. The field assessments were done at GS 60) using the CIMMYT scale 0 - 9, where 0 is no disease and 9 is very susceptible (Saari and Prescott, 1975).

Marker Analysis

The genetic map with 205 DNA markers (M. CAKIR *unpublished data*) was used to identify marker loci associated with the scald resistance. QTL analyses were performed using software packages Mapmanager QTX (MANLY *et al*. 2001) and Qgene (NELSON 1997). A threshold LOD (logarithm of odds ratio) score of 3.0 was chosen for declaring the existence of a QTL. Wherever appropriate, simple regression and interval mapping analyses were used to find the associations. Separate analyses for each site and a joint analysis over two sites were performed.

Results and Discussion

QTL analysis has identified 3 significant regions on chromosome 1H, 3H and 4H using the WA phenotypic data. The data from SA identified the same regions with a difference that the QTL region on Chromosome 1H was less significant than the threshold level of LOD 3. As shown in figure 1, QTL diagrams from both WA and SA were identical in pattern. The region on 3H was the most significant with an R^2 value of 29% and 42% in SA and WA, respectively. A scald gene located on the short arm of chromosome 4H has not been reported before. This gene was identified from both locations (Fig. 1) and explained 22% and 7% of the phenotypic variation in SA and WA locations, respectively (Table 1). JENSEN *et al.* (2002) and GRØNNERØD *et al.* (2002) also reported presence of scald genes with smaller effect on chromosome 4H but

the location of the gene in their study was on the long arm. For the combined effect of three genes multiple regression analysis explained 42% and 48% of the variation in this trait in SA and WA environments, respectively.

Table 1. Markers associated with scald field resistance data collected from South Australia (SA) and Western Australia (WA), their LOD scores and R^2 values.

Chromosome	Location	Most significant marker	LOD score	R^2 (%)
1H	SA	ISCOO4_AO2	1.0	3
	WA	ISCOO4_AO2	3.3	9
3H	SA	Bmag0131	11.6	29
	WA	Bmag0131	18.4	42
4H	SA	M9P1_7	9.4	22
	WA	M9P1_7	3.0	7

The gene on the long arm of chromosome 3H was identified in previous studies as summarised by GENGER *et al.* (2003) and WILLIAMS (2003). Besides genes on chromosomes 1H, 3H and 4H, resistance genes have also been reported on chromosome 6H (ABBOTT *et al.* 1995) and 7HS (SCHWEIZER *et al.* 1995).

The newly identified 4HS gene may be useful to breeding programs for pyramiding in combinations with genes on 3H and other chromosomes. The three genes identified in this study were at least partially effective in both SA and WA.

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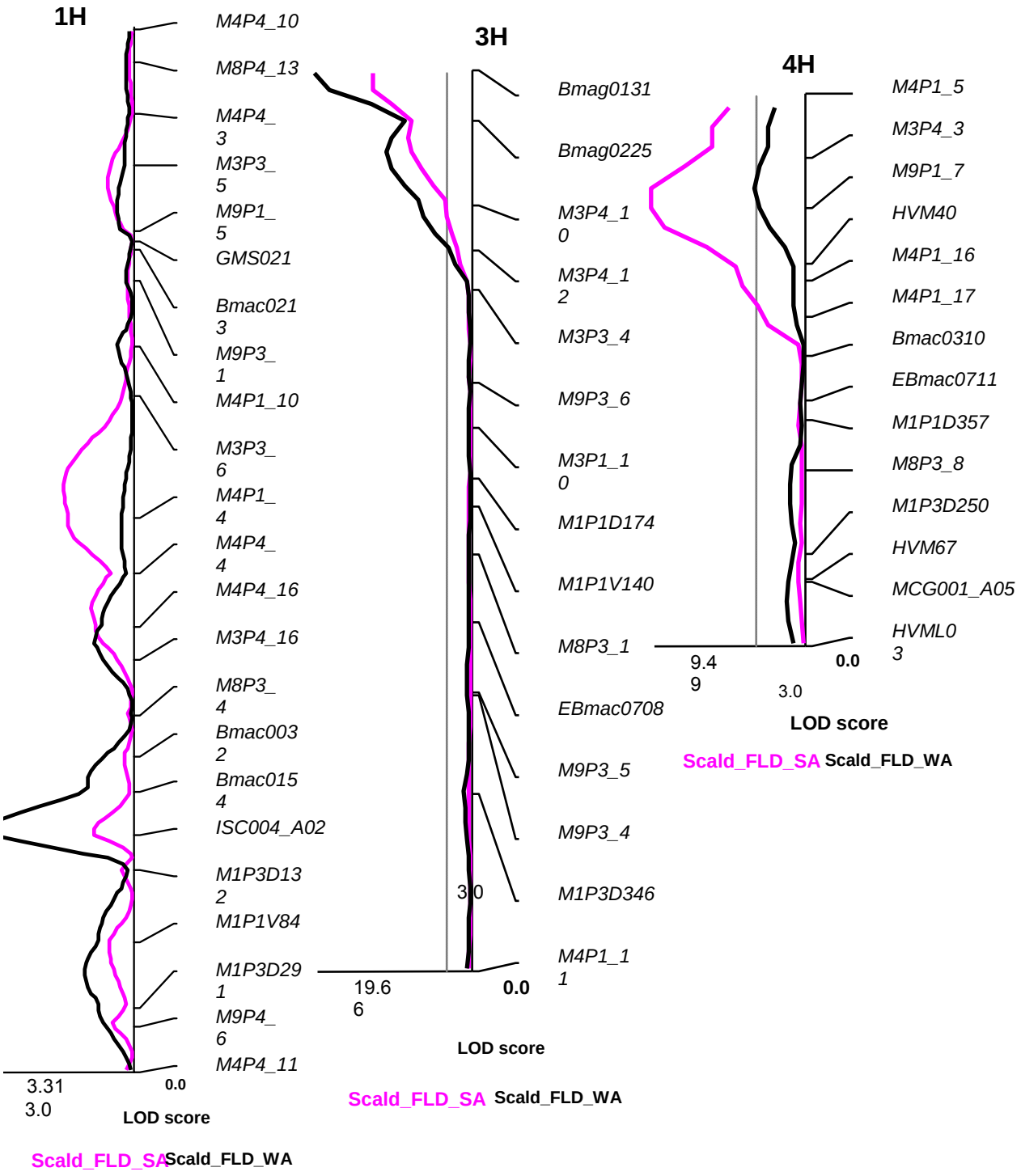

Fig. 1. QTL locations on chromosomes 1H, 3H and 4H.

Pyramiding Quantitative and Qualitative Resistance to Barley Stripe Rust

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Abstract
Barley stripe rust (caused by *Puccinia hordei* fsp. *hordei*) is an important disease of barley. Qualitatively and quantitatively inherited resistance loci are distributed throughout the genome, allowing pyramiding of multiple alleles in single genotypes. For the past ten years we have been detecting, mapping, combining and exploiting different sources of resistance to barley stripe rust. Using three quantitative resistance loci mapped in two ICARDA/CIMMYT accessions - Cali-sib (on chromosomes 4H and 5H) and Shyri (on chromosome 1H)- and an adult plant Mendelian resistance gene mapped in CI10587 (on chromosome 7H), we developed pyramids of both types of resistance. In the first stage we used a complex cross to combine alleles from the the three QTL. Lines with resistance alleles at these QTL had lower disease severities, confirming the additive effect of the QTL, and showing that pyramiding resistance QTL is effective. In the next step we combined the QTL resistance alleles with the qualitative resistance allele. The results show that this combination of alleles is also effective in significantly reducing disease severity, and preliminary studies suggested that the effectiveness is maintained even when challenged with a new pathotype.

Introduction
Barley stripe rust (*Puccinia striiformis* f.sp. *hordei*) (BSR) is an important barley disease first reported in South America in 1975, and in the U.S. in 1991 (MARSHALL & SUTTON 1995). BSR in the Americas was first described in terms of race 24, which was reported in Europe in the early 1960's and first detected in Colombia in 1975 (DUBIN & STUBBS 1985). Genetic resistance is the most cost-effective and environmentally appropriate technique for crop disease management. Host pathogen resistance to biotic stresses can be classified as qualitative or quantitative. The former refers to Mendelian genes of large effect that clearly interact on a gene-for-gene basis with the pathogen, whereas the latter describes resistance that shows continuous variation and is usually incomplete in expression. Qualitative resistance can be measured as the reaction of either seedling or adult plants to inoculation, and its use for the development of new varieties can be straightforward. Its main problem is the lack of durability. Quantitative resistance (QR) is often determined in the field and it is more likely to be durable. Pyramiding multiple QR alleles in single genotypes may be an approach to increasing the level of resistance relative to that conferred by a single QR locus and multigenic QR may also lead to greater durability. The combination of both types of resistance genes (e.g. qualitative and quantitative) offers the possibility of exploiting both the complete effect of the qualitative resistance gene, or genes, with the theoretical durability of the quantitative resistance gene, or genes.
Quantitative trait locus (QTL) analysis procedures have facilitated the dissection of QR. Some plant breeders have embraced QTL mapping tools in order to increase selection efficiency via marker-assisted selection (MAS). However, QTL analysis has been shown to be subject to serious limitations, including bias in QTL estimation, lack of studies validating QTL alleles in

different genetic backgrounds, and few examples of successful MAS (DEKKERS & HOSPITAL 2002).
The lack of durability of qualitative resistance in the BSR system has been particularly problematic (HAYES *et al*. 2001). In a collaborative effort to map and exploit quantitative resistance using molecular tools, we have used barley germplasm developed by ICARDA/CIMMYT with quantitative resistance (SANDOVAL-ISLAS *et al*. 1998), and mapped resistance QTL in several regions of the barley genome (HAYES *et al*. 2001)
In this project we used three quantitative resistance loci mapped in two ICARDA/CIMMYT accessions - Cali-sib (on chromosomes 4H and 5H) (CHEN *et al*. 1994) and Shyri (on chromosome 1H) (TOOJINDA *et a*l. 2000) - and an adult plant Mendelian resistance gene mapped in CI10587 (on chromosome 7H) to develop pyramids of both types of resistance. In the first stage we used a complex cross to combine alleles from the the three QTL. In the next step we combined the QTL resistance alleles with the qualitative resistance allele.

Material and Methods

Development of Resistance Pyramids

A population of 115 doubled haploid (DH) lines (population BCD) was developed from the cross Harrington*2/Orca/2/D1-72. Harrington is a two-rowed malting barley variety developed by the University of Saskatchewan. Orca (Calicuchima-sib/Bowman) is a two-rowed barley variety and has resistance alleles at chromosomes 4H and 5H (QTL4 and QTL5) tracing to Calicuchima-sib (CHEN *et al.* 1994). D1-72 is a line from the Shyri/Galena population that has a resistance allele at chromosome 1H (QTL1) tracing to Shyri (TOOJINDA *et al*. 2000). One cycle of marker-assisted selection was performed for resistance alleles at QTL4 and QTL5 in the BC_1 generation (Harrington*2/Orca). Four BC_1 plants with Orca alleles at marker loci flanking both QTL crossed with D1-72. DH lines were derived from the F_1 plants of these crosses, following the procedures described by CHEN and HAYES (1989).
A line from CI10587 x Galena population (D3-6), carrying the qualitative resistance gene from CI10587 on chromosome 7H (*Rpsx*) was selected and crossed with the variety Baronesse. One hundred doubled-haploid lines were derived from the F_1 of this cross, following the procedures already described. Two DH lines (D3-6/B-23 and D3-6/B-61) from this population were selected based on their BSR resistance and crossed with two DH lines from the BCD population. One of these lines (BCD47) carries stripe rust resistance QTL alleles on QTL4 and QTL5, while the other (BCD12) carries a stripe rust resistance QTL allele on QTL1. Three crosses were made between the quantitative and qualitative resistance sources and three DH populations were obtained from the F_1 of each cross following the procedures described above. Seventy DH lines were derived from the D3-6/B-23 x BCD47 cross (population BU), seventy-seven DH lines were derived from the D3-6/B-61 x BCD47 cross (AJ), and eighty-five DH lines were derived from the D3-6/B-61 x BCD12 cross (OP).

Phenotyping

The BCD population and the three parents were phenotyped at Toluca, Mexico in 1996, 1998 and 1999 for BSR severity, in a total of seven experiments. Epidemics were generated with local bulk isolates. This procedure was used to detect and measure resistance in the original mapping populations. BSR severity ratings made at multiple growth stages allowed calculation of the area under the disease progress curve (AUDPC) in six of the seven experiments. The BU, AJ, and OP populations were assessed for field resistance in four tests over three years at Toluca, Mexico, in 1999-2001 following the procedures described above. Stripe rust was rated as percentage severity on a plot basis.

Genotyping
The BCD population was genotyped in the regions defining the BSR QR QTL on chromosomes 1H, 4H, and 5H using thirteen SSR markers. As the interest is to define the region of chromosomes 4H and 5H that were introgressed from Orca, map positions of markers in these regions was confirmed in the Cali-sib x Bowman mapping population. In chromosome 1H, the interest is in saturating the region tracing to Shyri (via D1-72). The reference population for confirming map position is Shyri x Galena. The AJ, BU, and OP populations were genotyped using SSR markers of known position on chromosomes 1H, 4H, 5H and 7H. After screening all available PCR-based markers mapping to those regions, we we able to map twenty markers in the BU population, twenty-two in the AJ population, and twenty-three in the OP population. SSRs were assayed as described by LIU *et al*. (1996) and RUSSELL *et al*. (1997).

Data Analysis
Individual effects were estimated using an approach analogous to candidate gene analysis, where the genotypes at the QTL region are used as independent variables. Therefore, the independent variables had two levels each, with one level corresponding to the resistance allele and the other level corresponding to the alternative alleles at these QTL. The treatment design was a 2 x n factorial, where n is the number of genome regions considered. The difference between parental marker class means estimates the additive effect of the QTL flanked by the markers. In the BCD population, disease severity analysis was performed using data from seven environments, while AUDPC and infection rate analyses were performed using data from six environments. Infection rate was determined by adjusting a multiple regression model for all of the data. The dependent variable was the infected area transformed with the Gompertz transformation y = -log(-log(infected area)). The independent variables were individual experiments and QTL (dummy variables), QTL x QTL interactions, date of observation (expressed as days after emergence), QTL x date interactions, and experiment x date interactions. The model was selected using backward selection.
In the AJ, BU, and OPS populations, QTL were mapped using MultiQTL version 1.5. Each dataset was analyzed with 1000 permutations in order to establish the significance of the QTL. Genome regions, revealed by the QTL scans, that determine resistance to stripe rust were used in performing a QTL analysis analogous to candidate gene analysis, as described above.
Statistical analyses were performed using the GLM procedure of SAS 7.1 (SAS INST. 2001).

Results and Discussion
In the analysis of the QTL allele pyramid (BCD population) we detected significant main effects of the three QTL under study on the different measures of BSR infection considered (Table 1). In all cases the presence of resistance alleles at the three QTL regions was significantly associated with lower disease levels, providing evidence of these alleles' effect on reducing BSR symptoms in a genetic background different from that in which they were originally discovered, and thus validating the effects of these alleles. For the phenotypes of disease severity and AUDPC, there was only one significant QTL X QTL interaction. The QTL resistance allele on QTL4, contributed by the variety Orca, showed an interaction with the QTL resistance allele contributed by D1-72 on QTL1. The presence of two QTL resistance alleles led to greater resistance than the presence of either resistance allele alone, but the reduction in disease conferred by the two resistance alleles did not reduce disease symptoms as much as would be expected by the sum of the individual effects of the two alleles. For the infection rate, two other QTL x QTL interactions were also detected (QTL4 x QTL5, and the three-way QTL interaction). As in the previous case, they were magnitude

interactions, resulting in smaller that expected reductions of infection rates. The same QTL were associated with disease severity and AUDPC. QTL effects were confirmed in each experiment, regardless of epidemic intensity. No QTL x experiment interaction was detected. At the level of resolution afforded by our disease measurements, resistance alleles at the three QTL showed quantitative differences in the magnitude of their effects.

Table 1. Least squares means of disease severity and area under the disease progress curve (AUDPC), and least squares estimates of the infection rate according to the presence or absence of resistance alleles in the QTL regions under study on chromosomes 1H, 4H and 5H, in the BCD population. Values in the same column followed by the same letter are not significantly different ($p < 0.05$) based on pairwise comparisons (t-test).

QTL location						
4H (from Orca)	1H (from D1-72)	5H (from Orca)	n	Disease Severity[a]	***AUDPC***[b]	Infection rate[c]
-	-	-	25	50.8 a	850 a	0.0402 a
+	-	-	18	25.0 d	438 c	0.0227 d
-	+	-	29	31.7 c	587 b	0.0278 c
-	-	+	9	40.2 b	648 b	0.0317 b
+	+	-	19	15.8 e	241 d	0.0137 f
+	-	+	2	7.7 f	160 d	0.0085 g
-	+	+	11	21.1 d	341 cd	0.0194 e
+	+	+	2	8.9 ef	226 d	0.0130 f
r^2 of the multiple regression model (%)				46.0	44.6	61.1

[a] Measured as maximum % of leaf area infected.
[b] Measured as the integral of the disease progress curve.
[c] Calculated as the coefficient of regression after adjusting multiple regression models on transformed data (using the Gompertz transformation).

Our results on the BCD porpulation validate the effects of resistance alleles at three QTL regions on disease severity and AUDPC, proving that they are significant determinants of adult plant stripe rust resistance. We also showed that, in the population under study, these QTL explain 94% of the genetic variation of the trait expression (data not presented). The effects of the three QTL were quite similar for two measures of the QR phenotype: disease severity and AUDPC. From a breeder's perspective, this finding is of great practical utility: disease severity is based on a single point reading, whereas AUDPC requires multiple observations.

In the three complex pyramid populations (AJ, BU, OP), the QTL analysis detected a significant QTL on chromosome 7H in approximately the same region where *Rpsx* was expected to map to. We considered *Rpsx* as the candidate gene for this QTL, as the resistance alleles corresponded to D3-6/B23 (in BU) and D3-6/B61 (in AJ and OP. No other QTL was detected in the AJ and BU populations in this analysis. In the OP population an additional QTL with a smaller effect was detected on chromosome 1H, located in the expected QTL1 position.

Considering the potentially masking effect of *Rpsx* on the other resistance QTL's effect, we performed a second QTL analysis using the subset of lines with susceptible alleles at *Rpsx* in each population. The small sizes of the subpopulations (32 in AJ, 36 in BU, 37 in OP) limited the power of the QTL análisis. Via this analysis, we located BSR resistance effects in the QTL4 region in the BU and AJ populations, as well as in the QTL1 region in the OP population. Another QTL candidate QTL4B) was mapped on chromosome 4H in the AJ and

BU population, linked to *Bmac310*, more than 40 cM proximal to QTL4. A third QTL candidate was detected in the OP population linked to *Bmag353* (QTL4C), also on chromosome 4H. *Bmac310* and *Bmag353* are tightly linked (RAMSAY *et al.* 2000). The resistance alleles at these QTL candidates traced to the BCD parent.

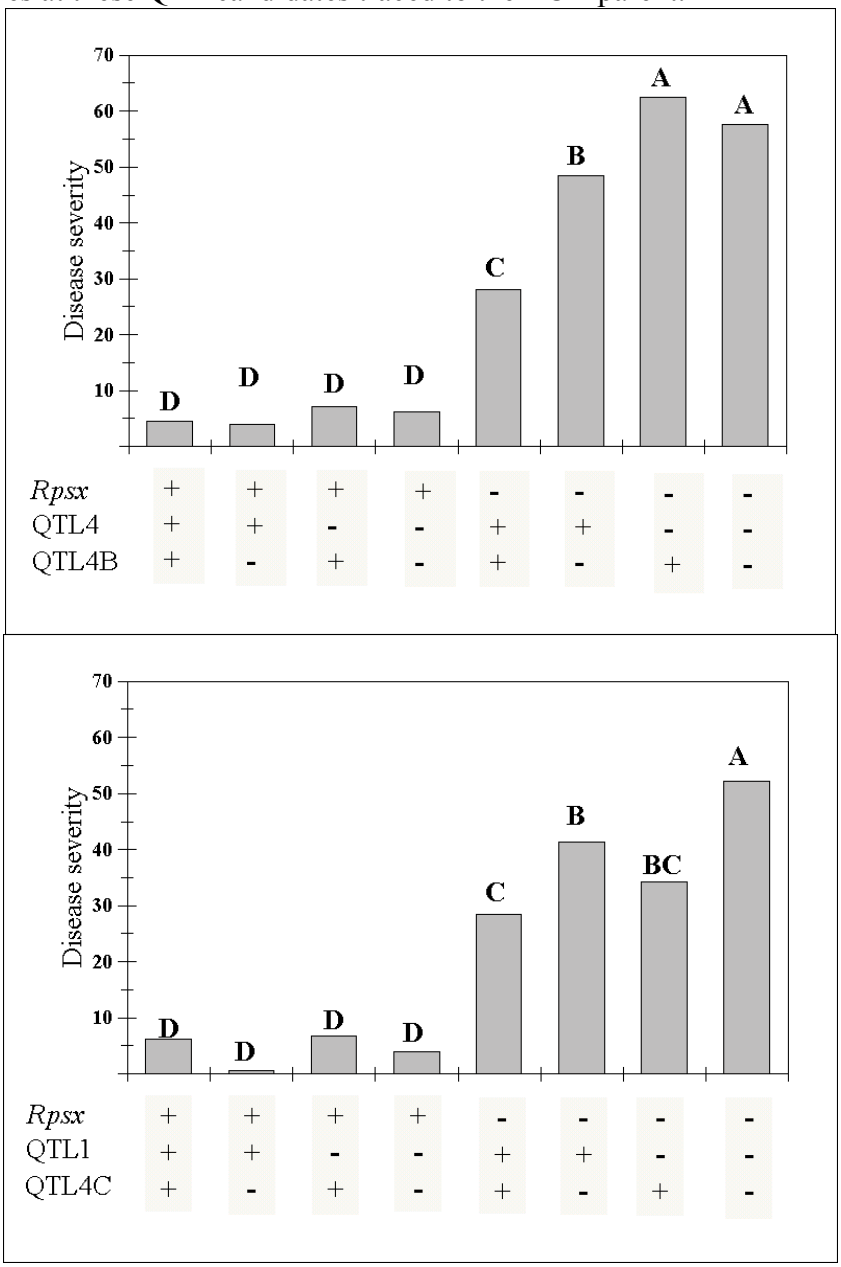

Figure 1. Least squares means of disease severity in DH lines of the AJ and BU (top figure), and OP (bottom figure) populations classified according to the presence or absence of the resistance alleles at *Rpsx*, QTL1, QTL4, QTL4B and QTL4C QTL regions. Bars with the same letter are not significantly different ($p < 0.05$) based on pairwise comparisons.

In the candidate gene analysis of AJ, BU, and OP populations, we pooled the AJ and BU populations, considering that the same QTL were detected in both and the common origin of these QTL alleles is BCD47. The ANOVA results confirmed the effects of *Rpsx*, QTL4, QTL4B, QTL4C, and QTL1, and the lack of significance of the QTL5 main effect. The qualitative nature of the *Rpsx* effect is reflected in the significant interactions with the different QTL. As seen in Figure 1, the different QTL alleles show their effects only in the absence of the resistance allele at *Rpsx*. The disease severity of lines with the resistance allele at *Rpsx* in all the populations was significantly lower. In the absence of the *Rpsx* resistance

allele, the presence of resistance alleles at QTL4 and QTL4B in AJ and BU, and at QTL1 and QTL4C in OP, significantly decreased disease severity.

These results on the AJ, BU and OP populations validate the map location of *Rpsx*. Of particular interest is the lack of significance of QTL5. The original mapping population estimates of QTL4 and QTL5 (CHEN *et al*. 1994) revealed QTL5 as having a much larger effect than QTL4, but we found a change of rank in importance of these QTL based on the estimates of QTL effects in the BCD population. The AJ and BU populations represent a second generation of QTL alleles from the original mapping population and confirmed that these "realized heritability" estimates of QTL effect are indeed more robust estimates of allele value than those obtained in the original mapping population. QTL effect estimates are reported to be biased, raising concerns about their use in understanding and manipulating genetic determinants of quantitative characters (BEAVIS 1998). We have found that QTL effect estimates based on derived populations provide better estimates of QTL effects than source mapping populations, but whether this is due to bias estimation or to genetic background is not known.

Our results support the utility of molecular markers and QTL analysis for understanding and manipulating genes determining qualitative and quantitative resistance to barley stripe rust. We mapped one qualitative resistance gene (*Rpsx*) and validated its importance, as well as the importance of two QTL (QTL4 and QTL1) that determine quantitative resistance to barley stripe rust, in a new genetic background. We also found that, in the populations under study, the effects of QTL estimates based on derived populations were different from those in the original mapping populations.

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Resistance to European Isolates of *Blumeria graminis* f. sp. *hordei* in Selections from Barley Landraces Collected in Israel

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Abstract
Seed samples of 22 barley landraces were used for screening for resistance to powdery mildew. These landraces were collected in Israel and originated from Centre for Genetic Resources the Netherlands (CGN). The infection types were scored according to a 0 - 4 scale and the cultivar Manchuria (CI 2330) was used as a susceptible control. In preliminary study, about 30 plants per landrace were evaluated in greenhouse with isolate 33. Isolate 33 represented the most avirulent isolate available allowing the expression of maximum number of resistance genes. The 14 landraces tested showed powdery mildew resistance reaction and 14 single plant lines were selected. These lines were tested in seedling stage with 21 differential isolates of powdery mildew. The isolates were chosen according to their virulence spectra on the Pallas isolines differential set and 7 additional differential cultivars. These isolates had virulences corresponding to all major resistance genes used in Europe. Twelve tested lines were resistant to all solates used. The results showed that barley landraces collected from Israel are very valuable source of resistance to powdery mildew. Twelve highly resistant lines identified in this study should be used in barley breeding.
Keywords: barley; powdery mildew; resistance; landraces

Introduction

Barley (*Hordeum vulgare* L.) is the fourth most important cereal crop in the world, after wheat, maize and rice. In European Union (EU) barley is the second (after wheat) most important cereal crop with more than 30% of EU total cereals acreage. In West Asia and North Africa (WANA) region barley is often grown in marginal agricultural areas with low annual precipitation (often less than 220 mm). Landraces in this area are important as they are often the only rain-fed crop possible and they are cultivated on mountain slopes at elevations higher than other cereals (CECCARELLI *et al*. 2000; CZEMBOR 2000, 2002). Powdery mildew, caused by the pathogen *Blumeria graminis* f. sp. *hordei*, is one of the most destructive foliar diseases of barley in central and Western Europe, WANA region, Japan and the eastern and southern barley producing areas of North America. In countries where mildew is a problem, yield losses in experimental tests usually exceed 25%, although average losses in barley production are smaller and about 10%. Yield reduction is due to loss of functional green leaf area, reduced root growth, reduced kernel weight, smaller numbers of kernels per spike and tillers per plant. Reduction in quality characteristics is particularly detrimental for malting barley (ZWATZ 1987; WOLFE & McDERMOTT 1994; CZEMBOR 1996; HOVMØLLER *et al.* 2000).

Barley landraces constitutes a rich genetic resource, and many examples of their successful use have been reported (CZEMBOR 1996; JØRGENSEN & JENSEN 1997). However only for less than 2 percent of barley landraces the attempts were made to identify powdery mildew resistance genes using differential lines and isolates. These type of studies were mostly conducted in Germany, Denmark and Sweden and on smaller scale in other countries

such as Czech Republic, The Netherlands, USA and Poland (HONECKER 1938; BRÜCKNER 1964; CZEMBOR 1976, 1996, 2000, 2002; JØRGENSEN & JENSEN 1997; CZEMBOR & FRESE 2003). Based on these reports it may be assumed that barley landraces collected in Israel may possess mildew resistance genes different from those, which already have been introduced into barley cultivars. Such genes would be of value in the further diversification of resistance genes available to breeding programs (FINCKH *et al*. 1999; HOVMØLLER *et al.* 2000).

The objective of this study was to determine the identity of powdery mildew resistance genes in selections from barley landraces collected in Israel.

Material and Methods

Seed samples of 22 barley landraces were used for screening for resistance to powdery mildew. These landraces were collected in Israel and originated from Centre for Genetic Resources the Netherlands (CGN). The infection types were scored according to a 0 - 4 scale and the cultivar Manchuria (CI 2330) was used as a susceptible control. In preliminary study, about 30 plants per landrace were evaluated in greenhouse with isolate 33. Isolate 33 represented the most avirulent isolate available allowing the expression of maximum number of resistance genes. The 14 landraces tested showed powdery mildew resistance reaction and 14 single plant lines were selected. These lines were tested in seedling stage with 21 differential isolates of powdery mildew. These isolates were kindly provided by Dr. H. J. Schaerer (ETH, Zurich, Switzerland) and originated from collections of the Risø National Laboratory, Roskilde, Denmark; Danish Institute for Plant and Soil Science, Lyngby, Denmark and Edigenossische Technische Hochschule – ETH, Zurich, Switzerland. The isolates were chosen according to their virulence spectra on the Pallas isolines differential set and 7 additional differential cultivars.

Hypotheses about the specific resistance genes present were made from the comparison of the reaction spectra of the tested lines with those of differential lines. The lines giving the same reaction spectra with all isolates were classified in the same group. Identification of resistance genes was made by eliminating resistance genes not present in tested lines. The next step was determination of postulated and possible resistance genes present and was done based on the gene for gene hypothesis.

Results and Discussion

Geneticists, plant pathologists and breeders working with barley are constantly looking for gene pools from which new genes can be introduced into existing cultivars in order to improve their resistance to major diseases including powdery mildew. Such gene pool are barley landraces, especially those originated from origin centres of barley such as Mediterranean region, and most of the powdery mildew resistance genes used commercially (e.g. *Mla1*, *Mla3*, *Mla6*, *Mla7*, *Mla9*, *Mla12*, *Mla13*, *Mlat*, *Mlk*, *Mlg*, *Mlh* and *Mlra*) are derived from these landraces (CZEMBOR 1976, 1996; JØRGENSEN 1994). This was confirmed in this study. Twelve tested lines (39338-1-2, 39367-1-4, 39386-1-2, 39387-1-3, 39395-1-2, 39406-2-4, 39407-1-3, 39408-3-5, 39410-1-1, 39413-2-2, 39336-2-1, 39401-2-1) were resistant to all isolates used. Based on the fact that isolates used in this experiment had collectively virulences to all major resistance genes used in the past and currently in Europe, it may be concluded that these lines showed high level of resistance to the powdery mildew virulence genes occurring in Europe. Therefore, this germplasm should be very useful in barley breeding programs as new sources of resistance to powdery mildew. In most selected lines the presence of unknown genes were postulated. Presence of a high number of unknown genes in barley landraces is in agreement with findings from other studies (BRÜCKNER

1964; CZEMBOR 1976, 2002; JØRGENSEN & JENSEN 1997; CZEMBOR & FRESE 2003).

Selected lines were characterised by resistance reaction type 2 (about 88% of observed resistance reactions). Infection type 2 is different from infection types conferred by most of powdery mildew resistance genes used in Europe. These genes confer mostly infection type 0 and 1 (JØRGENSEN 1994, DREISEITL & STEFFENSON 1996, DREISEITL *et al*. 1996, DREISEITL & JØRGENSEN 2000). Barley accessions expressing intermediate resistance to a wide-array of isolates provide a more stable resistance in barley cultivars to powdery mildew (CZEMBOR 1976, 1996; JØRGENSEN 1994). A good example of this is deployment of *Ml(La)* resistance gene conferring 2 or 3 infection types. This resistance gene have been effective for more than 10 years, despite the fact that it has been present in many barley cultivars throughout Europe (JØRGENSEN 1994).

The use fungicides and resistant cultivars are available for the effective control of powdery mildew. During last thirty years fungicide control of *E. graminis* f. sp. *hordei* has been used to reduce the severity of powdery mildew in the field. However, many pathotypes of *E*. *graminis* f. sp. *hordei* that are resistant to commonly used fungicides have been identified. In addition, fungicide cost and environmental concerns regarding pesticide use have led to a gradual reduction in their use for control of powdery mildew (WOLFE & McDERMOTT 1994). Breeding for resistance, as an alternative approach to control of powdery mildew, has been very successful, inexpensive and environmentally safe (FINCKH *et al.* 1996; CZEMBOR & FRESE 2003).

Many strategies for effective use of resistance genes in order to increase their durability were developed. Such strategies are: use of multiline cultivars, combining ('pyramiding') different resistance genes into one variety and deploying many cultivars with different resistance genes over space (e.g. cultivar mixtures) and time (winter versus spring barley) (WOLFE & McDERMOTT 1994; FINCKH *et al.* 1996). However, there are resistance genes which have not been exploited, and new sources of resistance are still being found in barley landraces and wild relatives (JØRGENSEN 1994; DREISEITL & STEFFENSON 1996; CZEMBOR 2000, 2002). Such new sources of resistance to powdery mildew have been described in this study.

Determination of powdery mildew resistance genes based on tests performed on seedlings using isolates with different virulence spectra is effective and sufficient for breeders and pathologist needs (DREISEITL & STEFFENSON 1996; CZEMBOR 2000, 2002; DREISEITL & JØRGENSEN 2000). However, it may not always predict adult plant resistance. Confirmation of putative resistance genes or alleles can only be established through evaluation of progeny from crosses and backcrosses among appropriate genotypes. In addition, different levels of partial resistance in tested lines may influence the postulation of presence of specific resistance genes (JØRGENSEN 1994; CZEMBOR 1996, 2000, 2002).

This investigation identified new sources of resistance to barley powdery mildew in lines selected from barley landraces collected in Israel. These new sources confer resistance to all or a large number of powdery mildew virulence genes prevalent in Europe and may contribute significantly to the diversity of the powdery mildew resistance gene pool available for barley breeders.

Acknowledgements

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Tab. 1. Differential isolates of *Blumeria graminis* f. sp. *hordei* and their infection types on Pallas isolines set and on 7 additional cultivars.

NO.	DIFFERENTIAL SET		ISOLATES																				
	ISOLINES AND CULTIVARS	RESISTANCE GENES	1	2	3	4	8	9	11	13	14	24	27	28	29	31	36	39	40	48	51	57	63
1.	Pallas	*Mla8*	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
2.	P01	*Mla1*	0	0	4	4	4	0	0	0	0	0	0	0	4	0	4	0	0	0	4	0	0
3.	P02	*Mla3*	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	4	0	0	0	0
4.	P03	*Mla6,a14*	0	0	0	0	0	0	4	0	0	0	0	4	0	0	4	0	4	4	4	0	4
5.	P04A	*Mla7Mlk*	4	4	4	0	0	0	1	4	2	0	0	0	0	4	4	4	2	0	2	4	4
6.	P04B	*Mla7+?*	4	4	4	1	0	1	1	4	4	0	0	1	0	4	4	4	2	0	2	4	4
7.	P06	*Mla7,Ml(LG2)*	4	4	4	0	0	0	0	4	4	0	0	0	0	4	2	4	1	0	1	4	4
8.	P07	*Mla7, Mlk*	4	0	4	0	0	0	0	4	0	0	0	0	4	0	0	0	0	0	0	4	0
9.	P08A	*Mla9,Mlk*	4	0	4	0	0	4	0	4	0	0	0	0	4	0	0	0	0	0	0	4	0
10.	P08B	*Mla9*	4	0	4	0	0	4	0	4	0	4	0	0	4	0	0	0	0	0	0	4	0
11.	P09	*Mla10, Ml(Du2)*	4	4	4	0	0	0	0	4	0	0	4	0	4	4	4	4	0	0	4	4	4
12.	P10	*Mla12*	0	0	4	0	0	0	0	0	1	0	0	0	4	4	4	0	4	0	4	0	4
13.	P11	*Mla13, Ml(Ru3)*	4	0	4	0	0	0	0	0	4	0	0	0	0	4	0	0	0	0	4	0	4
14.	P12	*Mla22*	4	4	0	4	4	0	4	0	4	4	0	4	4	4	0	4	4	4	4	0	0
15.	P13	*Mla23*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
16.	P14	*Mlra*	4	4	4	2	4	4	4	4	0	4	4	4	4	4	4	4	4	4	4	4	4
17.	P15	*Ml(Ru2)*	2	3	4	4	2	4	2	2	2	2	4	2	2	4	4	4	4	4	4	4	2
18.	P17	*Mlk*	4	4	4	2	2	2	2	4	2	2	2	2	4	4	4	4	4	2	4	4	4
19.	P18	*Mlnn*	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
20.	P19	*Mlp*	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
21.	P20	*Mlat*	2	2	2	4	2	2	2	4	2	2	2	2	2	2	2	2	4	2	4	2	2
22.	P21	*Mlg*	4	4	4	0	0	0	4	0	4	0	4	4	4	4	4	4	4	0	4	0	4
23.	P22	*mlo5*	0/4	0/4	0/4	0/4	0/4	0/4	3	0/4	0/4	0/4	0	0/4	0/4	0/4	0/4	0/4	0/4	0	0/4	0	0/4
24.	P23	*MlLa*	4	4	4	4	4	2	2	4	2	3	4	4	4	4	4	4	4	2	4	2	2
25.	P24	*Mlh*	4	4	4	0	4	4	4	4	4	4	4	4	4	4	0	4	4	4	4	4	4
26.	Benedicte	*Mla9, Ml(IM9)*	0	0	4	0	0	0	0	0	2	0	0	0	4	4	4	0	4	0	4	0	4
27.	Lenka	*Mla13, Ml(Ab)*	2	0	4	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	4	0	4
28.	Gunar	*Mlaa, Ml(3Tu2)*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Tab. 1. Continued

NO.	DIFFERENTIAL SET		ISOLATES																				
	ISOLINES AND CULTIVARS	RESISTANCE GENES	1	2	3	4	8	9	11	13	14	24	27	28	29	31	36	39	40	48	51	57	63
29.	Steffi	*Ml(St1), Ml(St2)*	1	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4
30.	Kredit	*Ml(Kr)*	4	1	4	1	0	0	0	0	4	1	0	0	2	4	0	0	0	0	4	0	4
31.	Jarek	*Ml(1192) +?*	4	4	4	4	4	2	4	4	4	4	4	4	2	4	4	4	4	4	4	4	2
32.	Trumph	*Mla7, Ml(Ab)*	4	4	4	4	4	4	4	4	4	0	0	0	4	4	4	4	4	4	4	4	4
33.	Borwina	*Ml(Bw)*	2	2	3	2	3	3	2	2	3	3	2	2	2	4	4	3	4	4	4	4	2
34	Manchuria	-	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4

Tab. 2. Resistance alleles and infection types of 14 lines to infection by 21 isolates of *Blumeria graminis* f. sp. *hordei*

No.	LINES	ISOLATES																					POSTULATED RESISTANCE ALLELES
		1	2	3	4	9	11	13	14	24	27	28	29	31	33	36	39	40	48	51	57	63	
1	39338-1-2	1	2	2	2	2	2	2	2	2	1	2	2	2	2	2	2	2	2	2	2	2	?
2	39367-1-4	2	2	2	2	2	2	2	2	2	0	2	2	2	2	2	2	2	2	2	2	2	?
3	39386-1-2	2	2	2	2	2	2	2	2	2	1	2	2	2	2	2	2	2	2	2	2	1	?
4	39387-1-3	2	2	2	2	2	2	2	1	2	2	2	2	2	2	2	2	2	2	2	2	1	?
5	39395-1-2	2	*	2	2	2	2	2	2	2	2	2	2	*	2	2	2	2	2	2	*	1	?
6	39406-2-4	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	?
7	39407-1-3	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	2	2	2	?
8	39408-3-5	2	2	2	2	2	2	2	2	2	1	2	2	2	2	2	2	2	2	2	2	2	?
9	39410-1-1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	?
10	39413-2-2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	?
11	39389-2-3	2	2	2	2	4	2	2	2	2	1	2	2	2	2	2	2	2	1	2	2	1	?
12	39336-2-1	2	2	2	2	2	2	2	2	2	2	2	1	2	2	2	2	2	2	2	2	2	?
13	39401-2-1	2	2	2	2	2	2	2	2	2	1	2	2	2	2	2	2	2	2	2	2	2	?
14	39402-1-1	4	4	4	4	4	0	4	4	4	0	0	4	4	2	4	4	4	0	4	4	4	?

* - no data

QTL for *Drechslera teres*-Resistance in Barley

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Abstract
Quantitative trait loci (QTL) for *Drechslera teres*-resistance were detected in a barley backcross population resulting from a cross between the *H. v. spontaneum* accession BGRC 41923 and the German spring barley cultivar Arena as recurrent parent. BGRC 41923 was resistant against *Drechslera teres* in a previous study, in which Arena was medium to highly susceptible. To verify the results three additionally produced populations were created.
QTL were located on chromosome 1H, 2H, 3H, 4H and 6H. Since not all of QTL markers were polymorphic in verification populations, only the QTL on 4H was verified in all three populations (Berolina, Golf and Pasadena). The QTL on 3H was confirmed in the population Golf and Pasadena and the QTL on 1H in Pasadena x 41923. None of the QTL was able to reduce the infection scores to very low values. It is shown that only a combination of QTL can reveal a high degree of resistance.
Keywords: *Drechslera teres*; net blotch disease; SSR-marker; QTL; resistance; barley

Introduction

The pathogen *Drechslera teres* Sacc. (SHOEMAKER, 1959) of the net blotch disease (perfect state *Pyrenophora teres*) is gaining economic importance in the group of leave diseases in barley and occurs in most barley-producing countries (MATHRE, 1997).
Drechslera teres resistance controlling loci have been described for all seven barley chromosomes. BOCKELMAN *et al.* (1977) found three independently segregating resistance genes on 1H, 2H and 3H. STEFFENSON (1996) detected seven QTL for adult plant resistance on 2H, 3H (two QTL), 4H, 5H, 6H and 7H and two loci for seedling resistance on 4H and on 6H. In a F_2-population Arena x Hor9088 twelve QTL were identified on chromosome 1H, 2H, 3H, 4H and 6H by RICHTER *et al.* (1998).
The objective of this study was to evaluate a putative new source of resistance against *P. teres* which was found in the *H. spontaneum* line BGRC 41923. Backcross populations of this line and the German cultivar Arena as recurrent parent in the BC_2F_2-generation were evaluated in an *in vitro* test with the *D. teres* isolate 67/1 and accordingly genotyped with SSR-markers in order to find QTL for *D. teres* resistance. To verify the results two additional BC_2F_2 and a F_2 population were tested.

Material and Methods

Plant Material
The *H. vulgare spontaneum* line BGRC 41923, which showed resistance against *Drechslera teres* in a previous study, was crossed onto the susceptible German spring barley cultivars Arena, Berolina and Golf. Each F_1 was backcrossed twice to the respective cultivar. For the cross combination we selected the Arena population for detecting of QTL and the other for verification. 636 plants of the Arena BC_2F_2-population were chosen for the QTL detection test whereas ca. 170 BC_2F_2-plants of Berolina- and Golf-population were used for verification. Additionally 167 plants of a F_2-population, Pasadena (German spring barley cultivar) with BGRC 41923, had been included into the project for the verification.

Drechslera teres Isolate
We tested three *Drechslera teres* isolates before we conducted the screening test of the BC_2F_2 progenies. In this primarily test, the monoconidial isolate 67/1 (BBA, Sachs) revealed large differences between the parents, further susceptible elite lines, and the resistant *H. v. spontaneum* line BGRC 41923.

Resistance Test
The second and third leafs of all BC_2F_2-plant were stored in biological plates (15-16 plants per plate) with wet filter paper containing 60 ppm Benzimidazol. They were inoculated with a conidia-suspension of 5000 conidia/ml with one drop Tween20/800 ml water.
Additionally, each plate contained second and third leafs of the parents as a control. On the 5^{th}, 7^{th} and 9^{th} day after inoculation these leafs were scored for grade of infection (on a percent basis).

SSR-Marker Analysis and QTL Analysis
286 SSR-marker were used for a test of polymorphism in the Arena and BGRC 41923 population. In order to reduce the amount of plants, which have to be genotyped, we split the population into a group with low and a group with high infection scores. This strategy can be compared to the *bulked segregant analysis* (MICHELMORE, 1991), expect that we analysed individual plants within groups. Each group contains app. 15 % of the original population. Analysing both groups with a *single-point analysis* 8 highly significant ($\alpha \leq 0{,}001$) SSR-markers were identified, which also showed a trend of change in their allele frequency between the groups in the way of reduced frequency of BGRC 41923 alleles in the susceptible group and increased frequency in the resistant group. These markers were defined as putative QTL markers and were tested together with their flanking markers in the Arena x BGRC 41923 population with all 636 individuals. Scores were transformed using the "arcsin (square root of score value)"-function for statistical analysis. In order for a better interpretation, the tables contain retransformed means.
The verification populations were genotyped with the 8 highly significant SSR-markers out of the *single-point analysis* from the bulks. However not all of these 8 markers were polymorphic in these populations. In this case flanking markers were used instead.

Results and Discussion
Analysing the selected groups with the *single-point analysis* putative QTL were found on chromosome 1H (GMS021), 2H (HVM36), 3H (Bmag0606 and EBmac0708), 4H (EBmac0708 and HVPDIA) and 6H (HVM74 and Bmac0613) (Tab. 1). These and their respective flanking markers were genotyped for all 636 individuals of the Arena population. The result of the statistical analysis is shown in Tab. 2. We applied a *combined analysis*, in which the respective marker with the highest F-value was included first. Each additionally included marker has to explain the remaining variation. Only if a marker is able to explain a significant part of the remaining variance (that means not explained by the already included markers) is recognised as a putative QTL marker. Using this analysis and a significance level of $\alpha \leq 0{,}001$, ten markers were identified as QTL markers. Only a few of these markers result in a decrease in infection score. Regarding HVM36 (2H) the genotypes with homozygosis

Table 1. List of the high significant (α ≤ 0,001 ***) SSR-markers of the *single-point analysis* from the transformed dataset of the Arena-Population bulks

SSR-Marker	chromosome	Position in cM	F-Value	probability of error	R^2
GMS021	1H	17	21,63	<,0001 ***	0,142
HVM36	2H	17	11,28	0,0008 ***	0,133
Bmag0606	3H	125	14,90	0,0001 ***	0,131
EBmac0708	3H	156	11,22	0,0008 ***	0,131
EBmac0906	4H	37	18,11	<,0001 ***	0,141
HVPDIA	4H	?	21,15	<,0000 ***	0,141
HVM74	6H	66	42,10	<,0001 ***	0,157
Bmag0613	6H	112	46,86	<,0001 ***	0,159

of BGRC 41923 alleles showed a reduction in infection scores of 77,4% compared to the Arena alleles. The same was true for following markers Bmag0606 (3H), EBmac0906 (4H) and EBmac0775 (4H) with a reduction of 49,4 %, 50,3 and 47,8 %, respectively. The lowest mean infection score (3,7) was observed on locus HVM36 with homozygosity of the BGRC 41923 alleles.

Table 2. Mean infection scores and significance level of the *combined analysis* analysing 636 individuals of the Arena x BGRC 41923 backcross population (α ≤ 0,001 ***, α ≤ 0,01 **, α ≤ 0,05 *, n.s.: not significant), only the grey marked loci show an infection score under 10 %

SSR-marker	chromosome	position in cM	homozygote elite parental genotype	Heterozygote genotype	Homozygote wild parental genotype	significance level
GMS021	1H	17	16,4	14,0	14,8	**
Bmac0213		28	15,8	15,8	14,7	*
HVALAAT		57	14,9	17,8	15,7	***
Bmag0347		70	14,8	16,2	18,9	***
HVM36	2H	17	15,9	10,7	3,7	***
Bmag0378		43	15,7	9,4	19,8	***
Bmag0841	3H	99	15,7	16,0	14,6	n.s.
Bmag0606		125	16,2	17,7	8,2	***
HVM62		154	16,0	15,7	13,1	*
EBmac0708		156	15,5	15,9	17,2	n.s.
Bmac0029		166	15,6	15,7	16,1	n.s.
HVOLE	4H	21	15,3	19,4	21,3	***
EBmac0906		37	15,9	15,3	7,9	***
HVPDIA		?	16,0	14,5	7,9	*
EBmac0775		42	15,9	12,3	8,3	***
EBmac0635		82	15,1	15,2	23,3	***
EBmac0679		83	15,3	14,0	24,2	**
HVM74	6H	66	15,2	23,2	16,6	***
Bmag0613		112	15,1	20,6	16,4	n.s.

None of the QTL was able to reduce the scores to very low values. Table 3 shows that only a combination of QTL can reveal a high degree of resistance. To achieve resistant lines a

putative QTL located at 1H has to be combined with a QTL located at 4H. Another interesting combination appears on 3H (Bmag0606 and HVM62). It has to be mentioned that the analysis of the selected groups were not able to detect all of these markers as QTL relevant marker.
A high resistance seems to be depended on epitasis as has been shown in Table 3. Therefore it is necessary to combine the relevant marker alleles in a selection procedure.

Table 3. List of marker-combinations in the Arena-population, which result in a mean infection score below 5 %

marker-combination						genotype		significance of interaction
SSR-marker 1	chromosome	position	SSR-marker 2	chromosome	position	homozygote elite	homozygote wild parent	*Epitasis*
Bmac0213	1H	28	Bmag0378	2H	43	15,7	1,8	***
GMS021	1H	17	Bmag0606	3H	125	16,8	3,0	***
HVALAAT	1H	57	EBmac0708	3H	156	15,1	4,0	***
HVALAAT	1H	57	EBmac0906	4H	37	15,0	0,6	***
HVALAAT	1H	57	HVPDIA	4H	?	14,9	0,6	***
HVALAAT	1H	57	EBmac0775	4H	42	15,2	0,7	***
Bmag0347	1H	70	EBmac0906	4H	37	14,9	0,6	***
Bmag0347	1H	70	HVPDIA	4H	?	14,9	0,6	***
Bmag0347	1H	70	EBmac0775	4H	42	15,2	0,7	***
GMS021	1H	17	Bmag0613	6H	112	15,6	4,2	***
Bmac0213	1H	28	Bmag0613	6H	112	14,8	4,2	***
HVALAAT	1H	57	Bmag0613	6H	112	13,6	4,1	***
Bmag0347	1H	70	Bmag0613	6H	112	13,6	4,2	***
HVM36	2H	17	Bmag0606	3H	125	16,5	3,7	***
Bmag0606	3H	125	Bmag0841	3H	99	15,8	2,4	***
Bmag0606	3H	125	HVM62	3H	154	16,4	0,3	***
Bmac0029	3H	166	Bmag0841	3H	99	15,3	0,8	***
Bmag0606	3H	125	EBmac0906	4H	37	16,3	1,8	***
Bmag0606	3H	125	HVPDIA	4H	?	16,3	1,8	***
Bmag0606	3H	125	EBmac0635	4H	82	15,9	2,4	***
Bmag0606	3H	125	EBmac0679	4H	83	16,0	2,2	***
EBmac0708	3H	156	EBmac0906	4H	37	15,6	0,7	**
EBmac0708	3H	156	EBmac0775	4H	42	15,7	0,7	***
EBmac0708	3H	156	HVPDIA	4H	?	15,5	0,7	**
Bmag0841	3H	99	EBmac0906	4H	37	15,9	2,4	**
Bmag0841	3H	99	HVPDIA	4H	?	15,9	2,4	*
Bmag0841	3H	99	HVM74	6H	66	15,2	4,4	***
Bmag0841	3H	99	Bmag0613	6H	112	15,1	4,4	***
EBmac0906	4H	37	EBmac0775	4H	42	16,1	4,3	**
HVPDIA	4H	?	Bmac0775	4H	42	16,0	4,3	*
EBmac0906	4H	37	EBmac0635	4H	82	15,2	2,4	***
HVPDIA	4H	?	EBmac0635	4H	82	15,3	2,4	***

In figure 1 putative QTL and loci combinations are marked, which indicated a high effect on infection score. E.g. on loci EBmac0906 (4H, Pt BIVa) the homozygote elite genotypes possessed an average infection score of 15,9 % whereas the homozygote wild form genotype had a score of 7,9 % (compare table 2). It is remarkable that in combination with locus Bmac0347 (1H, Pt BIb) the same locus showed an average infection score of only 0,6 %

(compare table 3). This reduction is not due to additive effects of the loci. We detected significant interaction between these loci, which means that epistatic effects are present.
This striking infection reduction of two resistance loci suggested the importance of epistatic effects in the resistance against *Drechslera teres*.

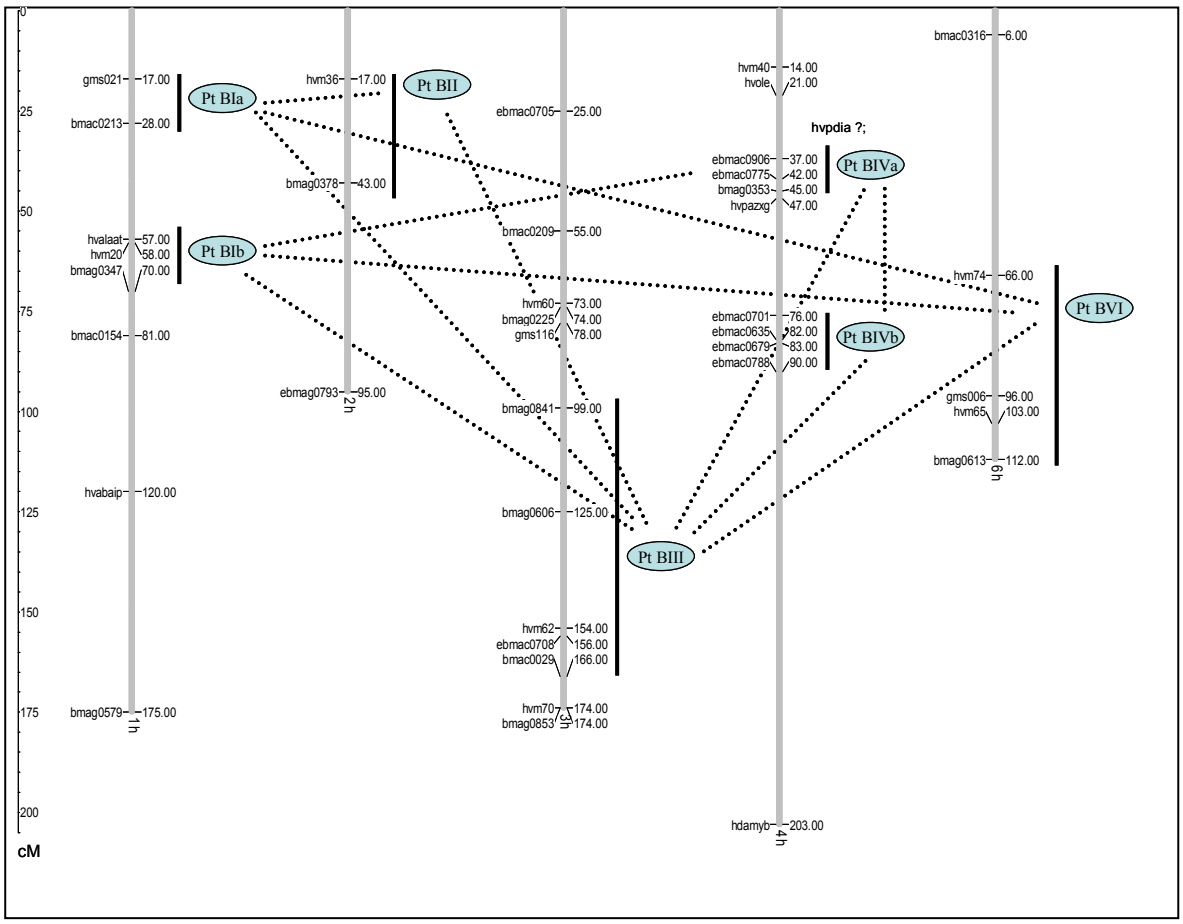

Figure 1. Putative QTL regions (Pt BI-VI) involved in resistance against *Drechslera teres* and significant epistatic effects between two resistance loci regions (**…..**)

The marker effect of EBmac0906 (4H) was verified in the Berolina-population with an average infection score of 0,7 % for the homozygous genotype with BGRC 41923 alleles. The *single-point analysis* of the Golf-population showed one highly significant ($\alpha \leq 0{,}001$ ***) marker on 1H (Bmac0213) and significant markers ($\alpha \leq 0{,}01$ **) on 3H (HVM62) and 4H (EBmac0775). In the Pasadena-population the markers Bmag0606 and HVM62 (both 3H) and HVPDIA (4H) were verified as QTL. Due to the fact, that not all markers were polymorphic in the verification populations, we were not able to verify all results of the Arena backcross population.
Some of these marker loci have already been described in literature. KOPAHNKE and SAKER (2002) identified one SSR-Marker on 1H (Bmac0213) which distinguishes between resistant and susceptible plants. Using a trisomic analysis BOCKELMAN *et al.* (1977) located two resistance genes from the wild form C.I. 9819 on chromosome 1H and 3H. Furthermore one resistance gene was found to be linked with spike type and lemma colour, which were localised on 2H (HO *et al.*, 1996). In a RFLP-analysis of the population Igri x Franka one dominant resistance gene *Pt,,a* was located on chromosome 3H (GRANER *et al.* 1996). The marker HVM74 (6H) and loci on 2H and 3H were associated with the resistance

to *Drechslera teres* (CAKIR *et al.*, 2003). EBmac0906 (4H) and one loci on 2H and 3H were related to resistance against *Drechslera teres* in the study of RAMAN *et al.* (2003).
As a conclusion of all these studies it seems clear that several loci are related to the resistance against *Drechslera teres*. A result of our study is to show the relevance of epistatic effects between these QTL regions.

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Gene Distribution and SSR Markers Linked with Net type Net Blotch Resistance in Barley

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Abstract
Four doubled haploid and F_2 populations generated from resistant net type net blotch lines, Pompadour, WPG8412, WA4794 and CI9214 crossed with susceptible widely adapted variety Stirling. Each line was found to have one to four resistance genes effective against different Australian pathotypes. These genes were mapped on chromosomes 2H, 3H, 4H and 6H using simple sequence repeat (SSR) markers. 6H, 4H and 3H were found to be major genes depending upon the pathotype. The closest SSR markers provide R^2 values up to 92% for 6H, 56% for 3H and 53% for 4H. For 2H, the maximum R^2 value was up to 7%. These genes can be used in combinations and the markers closely linked to these genes provide an efficient tool for marker assisted selection for breeding.
Keywords: *Pyrenophora teres* f. *teres*; resistance genes; SSR markers

Introduction

Net type net blotch of barley caused by *Pyrenophora teres* f. *teres* occurs widely throughout the barley cropping regions of Australia and overseas. It reduces grain yield by up to 33% (KHAN 1987) mainly through reduced grain size. One of the major objectives of Australian breeding programmes is to develop barley varieties with sufficient resistance to this disease. Sources of resistances in barley were identified against Australian pathotypes (GUPTA *et al*. 2003). Inheritance studies conducted worldwide in different barley lines indicated that the resistance is governed by one to three genes against the pathotypes representing different geographical regions (WILCOXSON *et al*. 1992; AFANASENKO *et al*. 1999; JONSSON *et al*. 1999). Identification of molecular markers linked with net type net blotch resistance genes helps to identify the breeding material using marker assisted selection procedures.

In this paper we report the gene distribution in four resistant barley lines against Australian pathotypes, chromosome locations and closely linked microsatellite markers which can be used in marker assisted selection.

Material and Methods

Four resistant lines, Pompadour (FDO192/Patty), WPG8412 (Bowman//Ellice/TR451), WA4794 (103IBON91 = Arupo'S'*2/3/PI2325/Maf 102//Cossack) and CI9214 (Collected from South Korea) were crossed with Stirling (Dampier//Prior/Ymer/3/Piroline), a susceptible but well adapted cultivar in Western Australia. Doubled haploid (DH) and F_2 populations were generated through anther culture and by selfing F_1s respectively. All the material was

screened as seedlings in duplicate against two Western Australian net type net blotch pathotypes. Seedling inoculations were undertaken at the two leaf stage with a suspension of ~2 x 10^4 spores/ml. Plants were incubated at 19-20^{0}C with complete leaf wetness for the first 24 hr and symptom severity was assessed on the ninth day using a 1 - 10 scale developed by TEKAUZ (1985).

Subsets of 70 - 80 DHs from each population were planted in replicated 1m rows in disease nurseries in randomised block designs at Wongan Hills, Western Australia. The lines were infested with net type net blotch straw at the seedling stage and subsequent disease development occurred naturally. Disease was assessed at the start of flowering.

DNA extractions were done according to ROGOWSKI *et al*. (1991). Bulk segregant analysis was carried out by grouping lines in resistant and susceptible categories for the initial identification of microsatellite markers linked with the resistance. Partial linkage maps were developed for the chromosomes wherever the genes were located. QTL analyses were performed using MapManager QTX (MANLY *et al*. 2001) software.

Results and Discussion

The infection types for resistant parents were between 1 to 2 for all resistant lines and 7 to 9 for the susceptible parent Stirling. Adult field reactions went up to 3 for resistant lines and were around 7 for Stirling. A summary of net type net blotch resistant gene distribution against Australian pathotypes in four doubled haploid populations indicates that resistance is governed by one to four genes (Table 1) (GUPTA *et al*. 2000, 2002).

In Pompadour, genes were mapped on chromosomes 6 (6H) and 3 (3H) near the centromere. The R^2 value for the SSR markers with greatest effect ranged from 18% - 84% for 6H and 56% for 3H. The gene on 6H was effective against all the pathotypes and 3H against NB50 and NB52B (Table 2). HVM74 was found to be the closest SSR marker against pathotypes 97NB1, 95NB100, NB81 and Beecher virulent (adult), whereas Bmag0496 against NB50 and NB52B. Linkage analysis indicated that these two SSRs were 1.1cM apart on the maps. The closest SSR for 3H was Bmag0603.

In WPG8412, only one gene on 6H was mapped. HVM74 was found to be closest marker giving R^2 values from 61% - 92% depending upon the pathotype test. For field evaluation as adult plants, Bmag0173 was found to be closest along with HVM74. Linkage analysis indicated that these two SSRs were 2.7cM apart.

In WA4794, three resistance genes were mapped on 6H, 4H and 2H. The gene present on 6H was found to be effective against 97NB1, 97NB100, NB81 and Beecher virulent (adult) pathotypes with R^2 values from 30% - 56%. The closest linked SSR markers Bmag0496 and Ebmac0874 were 6.2 cM distant apart from each other based on the linkage analysis conducted on this population. HVM74 was not found to be polymorphic between WA4794 and Stirling. The gene on 4H was found to be effective against 97NB1, NB50, NB81 and NB52B with R^2 values from 13% - 53%. The closest markers GMS089 and GBM1422 were co-segregated in this population and were 2.2 cM distant away from the other nearby marker Ebmac0906. The gene present on 2H had a minor effect as the closest markers, GBM1214 for 95NB100, Bmag0813 for Beecher virulent (adult), and GBM1214, Bmag0692 for NB50 explain percent variation (R^2) of only 5 - 7%. The distance between GBM1214 and Bmag0692 is 13.7 whereas between Bmag0692 and Bmag0813 is 48.8cM. There is a possibility that the seedling and adult plant resistances were controlled by two nearby 2H

regions as the distance between the closest marker Bmag0692 (seedling) and Bmag0813 (adult) is comparatively large.

Table 1. Gene distribution for net type net blotch resistance in barley populations against different Australian pathotypes (GUPTA *et al.* 2000; 2002)

DH Population	Pathotype	Putative Gene	Total Number of Different Genes
Pompadour x Stirling	97NB1	1	
	95NB100	1	
	Beecher virulent (adult)	1	2
	NB50	2	
	NB81	1	
	NB52B	2	
WPG8412 x Stirling	97NB1	1	
	Beecher avirulent (adult)	1	
	95NB100	1	1 - 2
	NB50	1 - 2	
	NB81	1	
	NB52B	1 - 2	
WA4794 x Stirling	97NB1	2	
	95NB100	2	
	Beecher virulent (adult)	2	3 - 4
	NB50	1	
	NB81	2	
	NB52B	2	
CI9214 x Stirling	97NB1	2	
	Beecher avirulent (adult)	2	
	95NB100	2	Complex gene distribution
	NB50	3	
	NB81	2	
	NB52B	3	

In CI9214, four genes were mapped on 6H, 4H, 3H and 2H. 6H is a major gene effective against 97NB1, Beecher avirulent (adult), 95NB100 and NB81 with a maximum R^2 value of 54%. Similarly 3H is also a major gene with a R^2 value of 40% against NB50 but the same gene acts as a minor gene against Beecher avirulent (adult), 95NB100 and NB52B. 4H and 2H genes have maximum R^2 value of 7% against different pathotypes and thus can be considered as minor genes in this parent. The distance between Ebmac0853 and HVM74 – the closest SSR markers to the gene on 6H is 3 cM. As found in WA4794, the seedling and adult plant resistance might be controlled by two nearby 2H regions in CI9214 as the distance between the closest marker Ebmag0793 (seedling) and Bmac0134 (adult) was nearly 150 cM.

Table 2. SSR markers linked with the resistance against different net type net blotch pathotypes in four doubled haploid populations

Pathotype	Closest SSR Marker	Chromosome	R^2	P
Population 1: Pompadour x Stirling				
97NB1	HVM74	6	0.83	0.00000
95NB100	HVM74	6	0.84	0.00000
Beecher vir (adult)	HVM74	6	0.51	0.00000
NB50	Bmag0496	6	0.18	0.00000
"	Bmag0603	3	0.56	0.00000
NB81	HVM74	6	0.75	0.00000
NB52B	Bmag0496	6	0.19	0.00000
"	Bmag0603	3	0.57	0.00000
Population 2: WPG8412 x Stirling				
97NB1	HVM74	6	0.88	0.00000
Beecher avir (adult)	HVM74 & Bmag0173	6	0.74	0.00000
95NB100	HVM74	6	0.92	0.00000
NB50	HVM74	6	0.61	0.00000
NB81	HVM74	6	0.86	0.00000
NB52B	HVM74	6	0.75	0.00000
Population 3: WA4794 x Stirling				
97NB1	Bmag0496	6	0.25	0.00000
"	GMS089	4	0.4	0.00000
95NB100	EBmac0874	6	0.3	0.00000
"	GBM1214	2	0.05	0.00110
Beecher vir (adult)	EBmac0874	6	0.35	0.00000
"	Bmag0813	2	0.06	0.04306
NB50	GBM1422 & EBmac0906	4	0.53	0.00000
"	GBM1214 & Bmag0692	2	0.07	0.00166 & 0.00172
NB81	EBmac0874	6	0.56	0.00000
"	EBmac0906	4	0.13	0.00001
NB52B	GMS089	4	0.41	0.00000
Population 4: CI9214 x Stirling				
97NB1	EBmac0853	6	0.54	0.00000
"	EBmac0701	4	0.06	0.00031
"	EBmag0793	2	0.05	0.00190
Beecher avir (adult)	EBmac0853	6	0.11	0.00206
"	EBmac0871	3	0.07	0.00878
"	Bmac0134	2	0.07	0.01279
95NB100	EBmac0853	6	0.48	0.00000
"	EBmac0701	4	0.03	0.01045
"	EBmac0871	3	0.05	0.00160
"	EBmag0793	2	0.05	0.00201
NB50	EBmac0871	3	0.4	0.00000
NB81	HVM74	6	0.45	0.00000
"	EBmac0701	4	0.07	0.00024
NB52B	Ebmac0871	3	0.06	0.00032

All the four resistant parents contain 6H gene (Table 3). Genes on 2H and 4H are present in WA4794 and CI9214, whereas 3H is present in Pompadour and CI9214.

Table 3. Loci distribution among the parents for net-type net blotch resistance

Locus	Resistant donor contributing favourable allele
2H	WA4794 and CI9214
3H	Pompadour and CI9214
4H	WA 4794 and CI9214
6H	Pompadour, WPG8412, WA4794, and CI9214

The genes on 2H, 3H and 6H were also mapped in another population developed from adapted varieties Tallon and Kaputar which was screened against pathotypes NB50, NB52B and NB81 (CAKIR *et al*. 2003). The 4H locus was also reported to be effective in Halcyon in the Australian germplasm (RAMAN *et al*. 2003). These loci except 2H have also been previously reported overseas (GRANER *et al*.1996; STEFFENSON *et al.* 1996; RICHTER *et al*. 1998; SPANER *et al*. 1998; MANNINEN *et al*. 2000). It has been found that 6H is the most common locus contributing resistance to net type net blotch in barley. Closely linked SSR markers would facilitate for efficient selection for net type net blotch resistance genes combinations.

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Mapping of Quantitative Genes in Barley that Determine the Resistance to the Heterologous Wheat Leaf Rust Fungus (Puccinia triticina)

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Abstract
Very little is known on the genetic basis of nonhost resistance of crops to specialised pathogens. In order to investigate the inheritance of this resistance, we chose barley, since in this species some accessions are in the seedling stage still somewhat susceptible to heterologous rust species like the wheat leaf rust, *Puccinia triticina*, implying that barley is nearly a nonhost to this rust species. By accumulation of genes for susceptibility we developed a barley research line, SusPtrit, that is fully susceptible to wheat leaf rust. This line was crossed with the regular fully resistant cv Vada to produce 102 Recombinant Inbred Lines (RILs in F_8). By using 24 primer combinations, 363 segregating AFLP markers were scored and used to construct a molecular marker map. The susceptibility of the RILs to *P. triticina* was quantified by Infection Frequency (IF) and Frequency of Visible Infection Sites (FVIS) in the seedling stage. The RILs were also evaluated for Latency Period (LP) of *P. hordei* in the seedling stage. The RIL population showed a quantitative segregation for both VIS and IF of *P. triticina* and LP of *P. hordei*, without transgression. Five QTLs were identified for resistance to *P. triticina*. LOD profiles for both traits were similar except that LOD scores for FVIS were always higher than for IF. Two QTLs were on chromosome 1, one on chromosome 6, and two QTLs on chromosome 2. One of the QTLs on chromosome 1 was close to the locus for naked caryopses, explaining the relatively high susceptibility of naked seed barley germplasm to *P. triticina*. Two QTLs for LP of *P. hordei*, the most important component of partial resistance, also were revealed in this research: one on chromosome 2 and the other on chromosome 1. The QTLs for partial resistance to *P. hordei* were close to, but probably not coinciding with those for resistance to *P. triticina*. The data show that the complete resistance of Vada to the wheat leaf rust *P. triticina* has a polygenic basis.
Keywords: barley; *Hordeum vulgare*; wheat leaf rust; non-host resistance; partial resistance; *Puccinia triticina*; *Puccinia hordei*; Quantitative Trait Loci (QTL)

Introduction

Many plant pathogenic organisms can infect only a limited number of species, which are called hosts. It is one of the intriguing questions which characters determine whether a species is a host or a non-host to a given pathogenic organism and which genes are involved. There is some evidence that plants often respond in similar ways to host and non-host pathogens as reviewed by THORDAL-CHRISTENSEN (2003) but it is not clear whether these two types of resistance are under similar genetic control. Detecting the genetic basis of reaction of plants to non-host pathogens is not straightforward because of lack of variation for this trait within plant species. Barley exhibits a complete resistance to the many heterologous rust pathogens viz. rye leaf rust fungus (*Puccinia recondita*). However, some barley lines are in the seedling stage somewhat susceptible to heterologous leaf rust fungi such as the wheat leaf rust and the leaf rust fungus of *Hordeum murinum* (NIKS *et al*. 1996; HOOGKAMP *et al*. 1998). It is likely that in such "near-non-host" plant-pathosystems similar types of resistance occur as in full non-hosts, and they can therefore serve as model for the genetic basis of this type of resistance. In addition, barley as a diploid crop with rather simple genome and chromosome

homology to common wheat (MOORE *et al*. 1995) seems to be an ideal model to study the genetics of nonhost resistance that can be extrapolated to the other cereals with more complex genomes. The main obstacle to develop this kind of study could be the low level of susceptibility of barley to heterologous rust species that is insufficient to allow a study of the underlying genetics of this resistance (ATIENZA *et al*. in press). Recently a research line of barley, named SusPtrit has been developed that is fully susceptible to wheat leaf rust *P. triticina* and some other heterologous rust fungi (ATIENZA *et al*. in press). In order to map QTLs involved in nonhost resistance of barley to heterologous rust fungi, a cross between SusPtrit (as susceptible parent) and Vada was used to develop a mapping population. The latter accession represents "normal" barley i.e. it is fully resistant to heterologous rusts (NIKS *et al*. 1996) and has several QTLs for partial resistance to *P. hordei* (QI *et al.* 1998b). Determination of the level of resistance in this mapping population to both *P. triticina* and *P. hordei* allowed mapping of genes for host and non-host resistance in barley. Therefore the results can be used to compare the number, location and effect of genes determining these two types of resistance in barley.

Material and Methods

Plant Material

The fully susceptible research line to *P. triticina* (SusPtrit) was crossed with the W. European cultivar Vada. The latter accession is fully resistant to *P. triticina* and partially resistant to *P. hordei* (QI *et al.* 1998b). After seven generations of Single Seed Decent, a population of 102 Recombinant Inbred Lines (RILs) was obtained. Within the population two morphological markers were scored: naked caryopses and number of rows (two or six rows). More than 50 additional RILs will be added to the current mapping population soon.

Disease Test with P. triticina

Seedlings were grown in boxes (37x 39 cm). Urediospores of wheat leaf rust (isolate Flamingo) were produced on susceptible wheat seedlings (Little Club). The inoculations were carried out with about 10 mg freshly collected spores per box resulting in a deposition of about 600 urediospores per cm^2. About ten times (w/w) the volume of *Lycopodium* spores was added to the Urediospores to homogenise the distribution of the rust spores. The inoculum was applied in a settling tower (HOOGKAMP *et al*. 1998). The plants were incubated overnight in a dew chamber during 9.5 hours (17-18 ºC) at 100% relative humidity and then transferred to a greenhouse compartment at about 22/18ºC (day/night). Twelve days after inoculation the level of infection was quantified by estimating the Frequency of Visible Infection Sites per cm^2 (FVIS). Visible infection sites included flecks as well as sporulating pustules. An additional parameter used in QTL mapping was the number of pustules per cm^2 (Infection Frequency, IF). Three seedlings of each RIL were tested in three separate replications and the average of three replications was used as phenotypic value for QTL mapping.

Disease Test with P. hordei

The RILs were grown as described above and tested for *P. hordei* (isolate 1.2.1), as described by QI *et al.* (1998b). The latency period (LP) of each plant was evaluated by estimating the period (in hours) at which 50% of the ultimate number of pustules became visible. The relative latency period of seedlings (RLP) was then calculated relative to the LP of L94 plants, where L94 was set at 100, as described by PARLEVLIET (1975). Two plants per RIL were tested in two replications and the average over two seedlings was considered to reflect the level of partial resistance of each RIL.

Construction a Molecular Marker Map

Genomic DNA of the 102 RILs and the parents (SusPtrit and Vada) was isolated according to CTAB based protocol of STEWARD and VIA (1993) adjusted for 96-well format. Isolated DNA was washed with 76 % ethanol for 15 minutes, dried and diluted to approximately 100 ng/ml in $T_{0.1}E$ buffer for next experiments. The AFLP procedure was performed according to the two step amplification as described by VOS *et al*. (1995) with some minor modifications. In this study 20 *Eco*RI/*Mse*I primer combinations each with three selective nucleotides which had already been evaluated as excellent and informative primer combinations to create the molecular marker map in L94 × Vada (QI *et al.* 1998a), were employed to generate the AFLP markers. Four *Pst*I primers with two selective nucleotides also were used in combination with *Mse*I primers. The AFLP amplification products were designated with the name of two primer combinations used (e.g.E32M61) followed by the molecular size as estimated from the mobility in the gel compared to a 10 bp size standard. The scoring of the AFLP products was based on absence or presence of amplification products. Segregating markers showing polymorphism between two parents were scored in mapping population by using Quantar-Pro software developed by Keygene.

Linkage Analysis and QTL Mapping

Linkage analysis was performed with JoinMap® 3.0 (VAN OOIJEN & VOORRIPS 2001). Linkage groups were assigned to the corresponding barley chromosomes by using the morphological markers and common AFLP markers that had already been mapped by QI *et al*. (1998a) in L94 × Vada. The software Map QTL 4.0 (VAN OOIJEN *et al*. 2002) was used to perform QTL mapping. The Interval Mapping method and MQM Mapping method were used on the data of the disease tests with *P. triticina* and *P. hordei.* After interval mapping in the region of putative QTLs the peak marker with highest LOD value was selected as co-factor for running MQM mapping (JANSEN and STAM 1994).

Results and Discussion

Disease Test for P. triticina *on RIL Population*

The results obtained from the disease test showed a wide range in susceptibility to *P. triticina* isolate Flamingo among 102 RILs for both IF and FVIS. There was no RIL as susceptible as SusPtrit while four lines were immune as Vada. Different levels of susceptibility occurred from immune (no pustules and less than 5 flecks per cm^2) to fully susceptible (maximium 87 sproulating pustules per cm^2) among RILs. There was a very high correlation between IF and FVIS and between the three separate replications as well (data not shown). The continuous segregation among the RILs suggests contribution of several minor genes, each with a quantitative effect. The FVIS and IF for RILs with naked seeds were approximately three and two times more than RILs with covered seed respectively. This association between the gene for naked seeds and relative susceptibility to heterologous rusts had already been reported in the L94 (naked) × Vada (covered) mapping population (NIKS *et al*. 2000**)** and in a germplasm collection by ATIENZA *et al*. (In press).

Disease Test for P. hordei *on RIL Population*

The RIL population also showed a quantitative segregation for susceptibility to *P. hordei* isolate 1.2.1. The values of RILs for RLP in two replications showed no transgressive segregation in this population. SusPtrit was as susceptible as L94 and in some boxes even more susceptible. For RLP there was strong correlation between the two replications. There was a significant negative correlation (r = -0.292) between FVIS (*P. triticina*) and RLP (*P. hordei*) suggesting an association between host and non-host resistance in this RIL population.

AFLP Analysis and Map Construction

The 20 *Eco*RI/*Mse*I primer combinations resulted in 322 easily distinguishable markers. The numbers of segregating markers were from 6 (E33M58) to 28 (E39M61) per primer pair with an average 16 markers per primer combination. From four *Pst*I/*Mse*I primer combinations an additional 44 segregating markers were scored. The *Pst*I primers had two selective nucleotides, and consequently the AFLP fingerprints were frequently full of amplification products. Therefore, only few clear and sharp markers for any primer combination were selected to avoid mistakes in scoring. The 366 markers were split into 18 linkage groups at a LOD threshold of 6.0. Two morphological markers were used as anchor markers. By comparing our mapping markers with those which had been mapped in Vada × L94 (QI *et al*. (1998a) 59 markers were identified as common markers. The morphological markers and those AFLP markers in common were used to assign the linkage groups to barley chromosomes. Chromosome 2 (2H) had the highest number of markers (60 markers) in a total genetic length of 106 cM. In contrast, chromosome 5 (1H) had the lowest number of markers (31) in a total genetic length of 123cM. The full linkage map comprised a linkage distance of 757 cM in total. The majority of markers (76%) showed 1:1 segregation ratio for two parental alleles ($P< 0.05$) but a severe skewness in linkage groups corresponding to chromosome 3 (3H) and chromosome7 (1H) was observed towards SusPtrit and Vada with 16 and 22 markers, respectively. In addition, the morphological marker *vrs* (six- rowed spike) located on chromosome 2 (2H) deviated towards Vada alleles. The rather high skewness of segregation of AFLP markers is in contrast with results of QI *et al*. (1998a). They found for only 8 % of the AFLP markers skewness in the L94 × Vada RIL population (F_9) while in the Oregon Wolf barley doubled-haploid population 26 % of the AFLP markers deviated from expected 1:1 ratio (COSTA *et al*. 2001).

Clustering of AFLP markers was clearly observed on all chromosomes especially around centromeric regions as reported by QI *et al*. (1998a) and COSTA *et al*. (2001) in barley and JEUKEN *et al*. (2001) in lettuce. The order of the markers was mostly consistent with the previously published map (QI *et al*. 1998a). We will improve the map by developing some SSR and additional AFLP markers and adding about 50 more RILs.

QTLs for Non-Host Resistance

The QTL mapping was performed on two data sets of evaluated traits: FVIS and IF each in three replications and the average separately. By using interval mapping five QTLs were identified and the results were qualified by MQM to improve the accuracy of QTL mapping. LOD profiles of QTLs on barley chromosomes for three replications was almost identical. For both traits the same QTLs were detected. LOD scores of QTLs found for FVIS were always higher than LOD scores for IF. Two QTLs on chromosome 1 for FVIS and IF, one QTL on chromosome 6 for both traits with high LOD score especially for FVIS, and two QTLs on chromosome 2 were identified. One of the QTLs for non-host resistance was located on chromosome 1, close to gene for naked seed as described in L94 × Vada mapping population (NIKS *et al*., 2000). It may explain the relative high susceptibility of naked seed barley lines to *P. triticina* (ATIENZA *et al.* in press).

QTLs for Partial Resistance

In the same way as described above, QTL mapping was performed for RLP of *P. hordei*. One major QTL was observed on chromosome 2 and one other was revealed on chromosome 1 with LOD score 2.85. On chromosome 6 a suspected QTL was observed with a LOD score just below the threshold value. This putative QTL will be subjected to further analysis when the 50 additional RILs have been added. The identified QTLs for RLP in this research partly

agree with those mapped by QI *et al*. (1998b) in mapping population L94 × Vada. They found three QTLs for partial resistance in the seedling stage. The QTL with the largest effect in the seedling stage was on chromosome 2 as we found here. They found also one other major QTL with a great effect on chromosome 6, which did not appear in the present mapping population. In addition, one minor-effect QTL on chromosome 1 seems to be located at identical position in both mapping populations.
Our preliminary QTL mapping data suggested that the QTLs for resistance to *P. triticina* and *P. hordei* are located in each other's vicinity, but not at identical positions. Since SusPtrit is also susceptible to several other heterologous rust species to which Vada is fully resistant, we will soon map also the genes responsible for that resistance and compare them to the QTLs identified in the present work.

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Evaluation and Mapping of a Leaf Rust Resistance Gene Derived from *Hordeum vulgare* spp. *spontaneum*

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Abstract

The use of resistant cultivars is an efficient way of controlling leaf rust in barley. Because all resistance genes identified in *H. vulgare* ssp. *vulgare* have been overcome by the fungus meanwhile, 500 *H. vulgare* ssp. *spontaneum* accessions were screened for resistance. Out of these, 38 lines with complete resistance to a set of known leaf rust races including *Rph*7 virulence were identified.

Studies for marker development were performed on a doubled haploid population derived from the cross of a highly resistant line *H. spontaneum* 677 x Krona (susceptible). Previous segregation studies on F_2 and F_3 populations revealed that the resistance of *H. spontaneum* 677 is most likely due to a single dominant gene.

Bulked segregant analysis using AFLPs and SSRs was conducted to identify markers linked to this leaf rust resistance gene. By this approach the resistance gene was located on barley chromosome 2H with the closest markers linked at 6.1 cM (E35M54b) and 13.6 cM (Bmac0218) based on the analysis of 83 DH-lines. In order to get first hints whether this gene may be allelic to *rph16* located on chromosome 2H STS marker MWG 2133 cosegregating with *rph*16 was tested but turned out to be monomorphic. However, in a resistance test with a set of four different isolates of *Puccinia hordei, H. spontaneum* 677 showed a different reaction pattern than *H. spontaneum* 680, the source of *rph*16. Tests for allelism to confirm these results are in progress.

Introduction

Leaf rust caused by *Puccinia hordei* Otth is an important disease of barley in Central Europe. New virulent races as well as combinations of virulent genes have overcome most of the resistance genes known so far and only the leaf rust resistance gene *Rph*7 is still effective in Europe. For this reason it is necessary to identify new sources of resistance. Resistance in *Hordeum vulgare* ssp. *vulgare* was shown to be very limited but a large variability was found in the wild progenitor *Hordeum vulgare* ssp. *spontaneum* (JIN *et al.* 1996; WEIBULL *et al.* 2003), which is a valuable source for broadening the genetic base of resistance to *Puccinia hordei*, therefore. In this respect *rph16* derived from *Hordeum spontaneum* 680 has already been mapped on chromosome 2H (IVANDIC *et al*. 1998). The present study aims at mapping of leaf rust resistance of *H. spontaneum* 677, which is most likely due to a dominant gene (WALTHER *et al.* 1999).

Respective markers will be useful tools for marker-assisted selection and gene pyramiding in breeding programs for leaf rust resistance.

Material and Methods

Plant Material

Genetic mapping was performed in a population of 83 doubled haploid lines (DH) which was produced by anther culture from F_1 plants derived from a cross between *Hordeum spontaneum* 677 (resistant*)* x Krona (susceptible).

Resistance Tests

The standard leaf rust isolate I-80 virulent for *Rph*1, 2, 3, 4, 8, 9, 10, 11 and 12, but avirulent for the resistance gene in *H. spontaneum* 677 was used for phenotyping the *H. spontaneum* 677 x Krona mapping population.

The determination of the qualitative resistance to *P. hordei* is carried out by means of a seedling test in the greenhouse. Seedlings were incubated with uredospores for 24 h at 18°C and 100% humidity in a growth chamber. Plants were scored 8-10 days after inoculation according to LEVINE and CHEREWICK (1952). Infection types 0, 1 and 2 indicate resistance, 3 and 4 susceptibility. The chi^2 test was used to assess segregation ratios.

All races found in Europe are avirulent for *Rph*7. The standard isolate I-80 is the isolate with the highest virulence in Germany (Tab. 1). For further differentiation of the resistant *H. spontaneum* accessions resistance tests were carried out in the U. S. Isolates (90-3, 92-7, 90-5) with virulence/avirulence patterns that have not been observed in the *P. hordei* population present in Europe were used (Tab. 1).

DNA Isolation and Linkage Analysis

DNA samples were prepared from fresh leaf tissue of green-house-grown barley plants. Standard procedures like CTAB-based DNA isolation was carried out as described by SAGHAI MAROOF *et al.* (1984). Besides this, a fast small-scale DNA isolation according to DOROKHOV *et al.* (1997) was applied. DNA concentration was measured on the fluorometer DyNA Quant 200 (Hoefer/Amersham Biosciences).

For marker identification bulked segregant analysis (BSA) was carried out using equal amounts of DNA from 10 resistant and 10 susceptible DH lines (MICHELMORE *et al.* (1991). For marker development SSRs (RAMSAY *et al.* 2000) and AFLPs were used (VOS *et al.* 1995). In order to get information about the chromosomal location of the gene, 5 SSRs per chromosome were analysed in a first step.

The PCR reactions for SSRs were performed in a total volume of 20 µl) in a thermal cycler PTC 200 (Biozym Diagnostics GmbH) and consisted of 50 ng template DNA, 1 x PCR buffer, 1,5 mM $MgCl_2$, 0,3 µM of forward and reverse primer, 200 µM dNTPs, 1 unit Taq polymerase (Roche Diagnostics GmbH. The amplification products were separated in a denaturing polyacrylamide gel in a Sequi-Gen Cell (BioRad Laboratories Inc.) The DNA fragments were detected using the silver-staining method.

For AFLP analysis, template DNA (300 ng) from the parents and bulks was digested with 5 units of the restriction enzymes *Eco*RI and *Mse*I. For template preparation the selection of biotinylated DNA restriction fragments was omitted. The adapter ligation, pre-amplification and selective amplification with Cy5-labelled *Eco*RI +3 primers was carried out according to the AFLP protocol suggested by GibcoBRL. The detection of the amplified DNA fragments was performed on an automatic laser fluorescence sequencing machine (ALFexpress, Amersham Biosciences).

Linkage analysis was performed with the MAPMAKER software program, version 3.0 (LANDER *et al.* 1987). The Kosambi function was used to convert recombination frequencies to map distances in centimorgan (KOSAMBI 1944).

Results and Discussion

38 completely resistant *H. vulgare* ssp. *spontaneum* lines were selected out of 500 accessions tested. Analysing these lines with 4 isolate including *Rph*7 virulence, some were found to possess leaf rust resistance genes different from recently known ones (PROCHNOW 1998).

Reactions of the host differential lines and of the *H. spontaneum* accessions to four isolates differentiating resistant lines are presented in Table 1. *H. spontaneum* 677 turned out to be resistant to isolates 92-7, 90-3 and I-80 but susceptible to isolate 90-5. In contrast to this, *H spontaneum* 680 (*rph16*) is exclusively susceptible to isolate 90-3 and shows the same reaction like l line 195-282-2 (*Rph*15).

By analysing the progeny of the cross L 94 x *H. spontaneum* 677 in F_2 and F_3 a good fit to a segregation ratio of 3r : 1s was observed, giving hint to a single dominant gene encoding resistance to *P. hordei* in this line (WALTHER *et al.* 1999).

Table 1. Reactions of a set of barley differentials and of *H. spontaneum* accessions to inoculation with four isolates of *Puccinia hordei*

Differential lines	**Isolate 90-3**	**Isolate 92-7**	**Isolate 90-5**	**Isolate I-80**
Sudan (*Rph*1)	S	S	S	S
Peruvian (*Rph*2)	S	S	S	S
Estata (*Rph*3)	R	S	R	S
Gold (*Rph*4)	S	S	S	S
Magnificent (*Rph*5)	S	S	R	R
Bowman/Bol (*Rph*6)	S	S	S	R
Cebada capa (*Rph*7)	R	S	R	R
Egypt 4 (*Rph*8)	S	S	S	S
Hor 2596 (*Rph*9)	S	S	S	S
Clipper BC8 (*Rph*10)	S	S	S	S
Clipper BC67 (*Rph*11)	S	S	S	R
Triumph (*Rph*12)	S	S	S	S
PI 531849 (*Rph*13)	R	R	S	R
PI 584760 (*Rph*14)	S	S	S	R
I95-282-2 (*Rph*15)	S	R	R	R
Resistant accessions				
H. sp. 680 (r*ph*16)	S	R	R	R
H. sp. 677	R	R	S	R

The results of the disease scoring of DH-lines of the *H. spontaneum* 677 x Krona cross using rust isolate I-80 is shown in Figure 1.

A segregation ratio of 50r : 33s was observed. Chi^2 value for a 1r : 1s segregation is 3.48 and for 3r : 1s segregation indicative for the presence of two resistance genes 9.60 suggesting that one gene is involved in resistance to isolate I-80 in *H. spontaneum* 677. However, it has to be noticed that *H. spontaneum* 677 is scored 0 while Krona is scored 4. In the DH population also lines showing a reduced level of resistance or susceptibility, respectively were observed. Therefore, additional genes influencing the level of resistance my be involved.

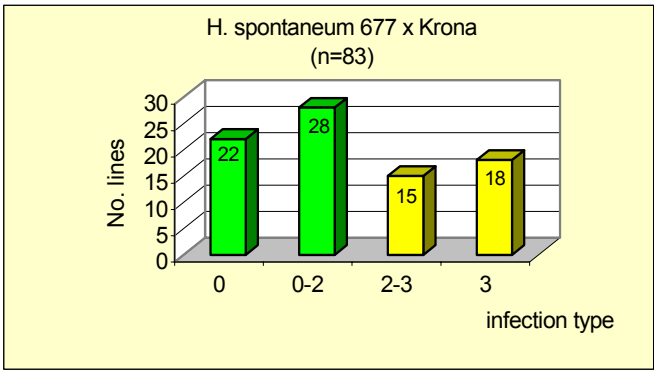

Figure 1. Reaction to leaf rust isolate I-80 of 83 doubled-haploid lines derived from the cross *H. spontaneum* 677 x Krona

A set of 35 previously described SSR markers were screened for detection of polymorphic bands between the resistant and susceptible bulks (RAMSEY *et al.* 2000). As the result of the bulked segregant analysis polymorphisms were detected on chromosome 2H. In a next step additional SSR markers for chromosome 2H were analysed and mapped.Linkage was detected between the resistance locus and the SSR markers Bmac0218, Bmag0518, EBmac0521, EBmac0558, Bmac0093 and EBmac0715 (Fig.2).SSRs EBmac 0415, Bmac 0134, HVM26, HVM63, EBmac0557 and Bmag0003 were monomorphic. The closest linked SSR markers are Bmac0218 and Bmag0518, which are flanking the gene at a distance of 13.6 cM and 17.8 cM, respectively.

Because SSRs are quite distantly linked to the resistance derived from *H. spontaneum* 677, AFLP marker saturation was conducted using about 200 AFLP primer combinations.

Polymorphic DNA fragments between the parents and the bulks were amplified by using the AFLP primer combinations E39M58, E42M48, E37M33 and E36M54. Out of these the AFLP marker E36M54b was mapped at a distance of 6.1 cM to the resistance gene. The total map including six SSR markers and one AFLP marker constitutes 51.4 cM. The map position of the leaf rust resistance gene is shown in Figure 2.

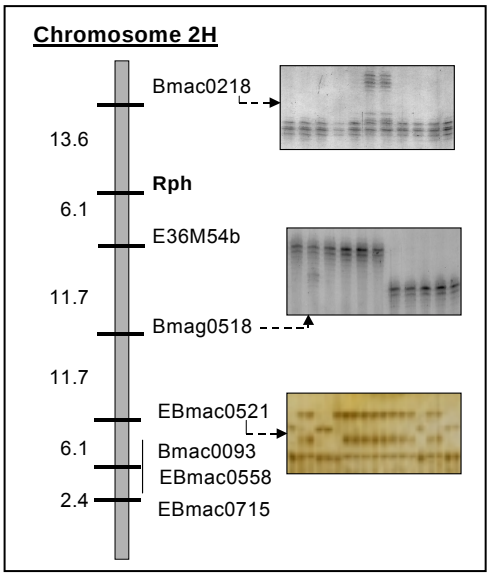

Figure 2 Partial Map of barley chromosome 2H using the mapping population *H. spontaneum* 677 x Krona. Genetic distance is given in centimorgans (cM)

Unfortunately, linkage detected up to now is quite loose. Therefore, additional AFLPs will be screened and besides this phenotypic analysis will be repeated on those genotypes showing no unequivocal reactions, i.e. 2-3 scores. Analysing 42 DH lines of this population which were e scored resistant (infection type 0) or susceptible (infection type 3) closer linkage is observed, i.e. 4.8 cM for Bmac0218 and 9.7 cM for Bmag0518.

As the gene of *H. spontaneum* 677 like *rph16* is located on chromosome 2H the STS marker MWG 2133 developed by IVANDIC *et al.* (1998) and co-segregating with *rph*16 was analysed but turned out to be monomorphic on our DH-population. Therefore, no information could be gained whether resistance of *H. spontaneum* 677 is located at exactly the same chromosomal region. As it just recently turned out that *Rph15* and *rph16* are allelic (WEERASENA *et al.* 2004), extensive tests for allelism will be carried out in the future.
Independent from chromosomal localisation resistance to *Puccinia hordei* derived from *H. spontaneum* 677 will be interesting in future breeding programmes as it shows a different resistance spectrum in comparison to the *Rph15/rph16* locus.

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Detection and Localisation of Resistance Genes against Powdery Mildew and Leaf Rust Introgressed from Wild Barley (*H. vulgare* ssp. *spontaneum*)

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Abstract
The objective of this study is to map new resistance genes against powdery mildew (*Blumeria graminis f.* sp. *hordei* L.) and leaf rust (*Puccinia hordei* L.) in a BC2DH population derived from a cross between the spring barley cultivar Scarlett and the wild barley accession ISR42-8 (*H. v*. ssp. *spontaneum*). Using field scored data of disease severity under natural infestation, we detected eight QTL for powdery mildew and seven QTL for leaf rust resistance. The QTL for powdery mildew resistance were distributed over all chromosomes with the exception of 5H. The QTL for leaf rust resistance were located on 2H, 3H, 4H and 7H and the exotic allele reduced disease severity in all cases. Some of the detected QTL may correspond to previously identified qualitative (i.e. *Mla*) and to quantitative resistance genes, others may be newly identified resistance genes against powdery mildew and leaf rust. For the majority of resistance QTL the wild barley contributed the favourable allele demonstrating the usefulness of wild barley in the quest for resistant cultivars.
Keywords: powdery mildew; leaf rust; *H. v.* ssp. *spontaneum*; quantitative disease resistance

Introduction

Powdery mildew caused by *Blumeria graminis* f. sp. *horde*i and leaf rust caused by *Puccinia hordei* are two of the economically most important foliar diseases on barley in the temperate climate zone, as these can reduce yield dramatically. The use of resistant barley varieties has proved an efficient tool to control disease and to prevent yield losses in susceptible cultivars. The wide use of qualitative resistance genes, however, has led to a rapid adaptation of pathogens and thus to a devaluation of these resistance genes. Quantitative or partial resistance, which is primarily non-specific, is assumed to be more durable, because the selection pressure is low and resistance is difficult for the fungus to overcome. The development of molecular markers and of statistical procedures for the detection of quantitative trait loci, has led to a highly effective tool for studying quantitative resistance.

The restricted availability of new resistance genes in the gene pool of cultivated barley has directed the focus of research on wild barley as potential donor of new resistance genes. Qualitative and quantitative resistance genes derived from exotic barley have been identified in different elite genetic backgrounds (BACKES *et al.* 2003, IVANDIC *et al.* 1998, QI *et al.* 1998). In order to isolate individual QTL from exotic barley and to gain knowledge about gene function, it has been proposed to develop advanced backcross populations. Repeated backcrossing with the elite parent reduces the number and size of exotic introgressions. The resolution and accuracy of QTL detection is thus increased and favourable exotic alleles may be rapidly isolated and transferred into elite varieties.

Disease resistance tests are conventionally based on data from primary detached leaves infected with a single defined isolate or from artificial inoculation in controlled environment conditions (IVANDIC *et al.* 1998, SAYED *et al.* 2004). Few people have studied disease severity and infection type of naturally occurring symptoms in the field (FALAK *et al.* 1999, SPANER *et al.* 1998). Although quantitative, field resistance of adult plants is assumed to be

more durable than race-specific resistance. BACKES *et al.* (2003) found different QTL for powdery mildew resistance scored on detached leaves and in the field. The authors concluded that results obtained on primary detached leaves might not be representative for adult plant resistance and that factors effective in the field might not be detectable on detached leaves.
In the present study we attempt to identify favourable exotic alleles, which improve disease resistance in a BC2DH population derived from a cross between the spring barley cultivar Scarlett and the wild barley accession, ISR42-8 from Israel. Here we present preliminary data for naturally occurring leave symptoms of powdery mildew and leaf rust from field tests at three locations in the season 2003.

Material and Methods

Plant Material

The development of a BC2DH population was conducted according to the advanced backcross strategy of TANKSLEY and NELSON (1996). An exotic accession of *H. vulgare* ssp. *spontaneum* from Israel (ISR42-8) was crossed with a spring barley cultivar (*Hordeum vulgare* ssp. v*ulgare*). The German spring barley cultivar Scarlett was obtained from the breeder Saatzucht Josef Breun GdbR. The wild barley accession, ISR42-8, from Eastern Lower Galilee, Israel, was provided by Prof. G. Fischbeck, Weihenstephan. After two cycles of backcrossing with the recurrent parent Scarlett, the BC2DH population with 301 DH lines was developed by anther culture (in the lab of the Saaten-Union Resistenzlabor, Leopoldshöhe, Germany).

Molecular Characterisation

The BC2DH population was genotyped with 96 SSR markers, which were fairly evenly distributed over the chromosomes. The following prefixes of SSR names indicate the published sources from which the primer sequence information was primarily taken: *HVM,* LIU *et al.* (1996), *Bmag, Bmac, Ebmag, Ebmac*, RAMSAY *et al.* (2000), *Hv*, BECKER and HEUN (1995) and PILLEN *et al.* (2000), *GBM*, THIEL *et al.* (2003), and *MGB*, VON KORFF *et al.* (in prep). The construction of the SSR consensus map is reported elsewhere (VON KORFF *et al.*, submitted). The genotyped markers were assigned to bins according to information by KLEINHOFS and GRANER (2001) and by the OWB population (http://barleyworld.org).

Evaluation of Powdery Mildew and Rust Leaf Symptoms

Phenotypic evaluation of the BC2DH plants was carried out under field conditions at three different locations in the season 2003. The test locations were the experimental station of the University of Bonn (D03, West Germany), and the breeders' experimental stations in Gudow (G03, Nordsaat Saatzucht, North Germany), and Morgenrot (M03, Saatzucht Josef Breun GdbR, East Germany). The field experiment was designed as randomised plots without replications. Plot size was 1.5 m x 1.8 m (D03) or two rows per BC2DH line (G03, M03). As a control, the recurrent parent Scarlett and the two barley cultivars Alexis and Pasadena (as susceptible checks) were tested with eight replications per block. Disease severity was recorded for naturally occurring powdery mildew and rust leaf symptoms. Disease severity was screened at the maximum stage of disease development in a scale from 1 (resistant) to 9 (susceptible).

QTL Analysis

The QTL detection from the genetic data and field data from three (powdery mildew) and two (leaf rust) environments was conducted using the procedure GLM (SAS INSTITUTE 1999). The GLM model included the marker (M) as fixed effect, and the environment (E) and the

MxE interactions as random effects (mixed model). Here we report on significant marker main effects (M) and marker environment interactions (MxE) based on a 0.01 probability threshold (PILLEN *et al*. 2003). Only the most significant single locus from each group of linked loci is recorded. The relative performance (RP[exotic]) of the homozygous exotic genotype was calculated as follows:

$$\text{RP [exotic]} = \frac{aa - AA}{AA} * 100$$

Where for each trait aa and AA are the least square means of the homozygous exotic and the homozygous elite genotypes, respectively, calculated over all environments. LSMEANS of disease severity for the BC2DH population and the recurrent parent Scarlett were calculated for each environment separately (SAS INSTITUTE 1999).

Results and Discussion

Powdery Mildew

Powdery mildew symptoms were recorded in three environments and the mean disease severity in the BC2DH population ranged from 2.7 in D03 to 7.4 in M03. The mean disease severity recorded for the recurrent parent Scarlett was in all three locations above that of the BC2DH population mean (Table 1). The phenotypic distribution of powdery mildew severity in the BC2DH lines averaged over the three environments did not exhibit concrete classes.

Table 1. LSMEANS of disease severity for the recurrent parent Scarlett, LSMEANS and distribution for the BC2DH lines presented separately by environment. (See text for abbreviations of locations.)

	BC2DH				Scarlett
Environments	**LSMEANS**	**Std Dev**	**Min**	**Max**	**LSMEANS**
		Powdery mildew severity			
D03	2.7	1.2	1	7	2.8
G03	3.6	1.5	1	7	4.0
M03	7.4	2.7	1	9	8.4
		Leaf rust severity			
D03	2.8	1.1	1	6	3.9
M03	1.7	1.6	1	9	2.0

The QTL analysis detected five marker main effects, and three loci exhibited a significant MxE interaction (Table 2). These were distributed over all chromosomes with the exception of 5H (Figure 1). For six QTL the exotic parent contributed the favourable allele. The strongest effect was measured at locus $MGB402_{1H}$ where the exotic allele reduced disease severity by 54 %. This locus corresponds to the *Mla* locus, which carries a cluster of race-specific powdery mildew resistance genes (JORGENSEN 1994). The recurrent parent Scarlett is known to carry the *Mlg* powdery mildew resistance locus. The only QTL on 4H with the positive effect from Scarlett, however, did not coincide with the *Mlg* locus mapped in bin 6 by KURTH *et al.* (2001). SAYED *et al.* (2003) reported accordingly that most European mildew populations carry close to 100 % virulence against the *Mlg* resistance gene. SCHÖNFELD *et al.* (1996) described a resistance gene *Mlf* derived from *H. v.* ssp. *spontaneum* on chromosome 7HL, which could be the same as the QTL localised at $HVCHI26A_{7H}$. The two QTL on 2HS and 3HL for which the MxE effect was significant, map to the same location as the *Ppd-H1* gene for early heading and *denso* gene for reduced plant height, respectively. The colocalisation of QTL for disease resistance with candidate genes for early heading and plant height indicate that the latter may influence disease escape. Three of the detected loci

coincide with quantitative resistance loci on 2H, 3H, and 7H detected in an RI population derived from a cross between a cultivar and a wild barley accession (BACKES *et al.* 2003).

Leaf Rust

Leaf rust symptoms were only recorded in two environments and disease severity was low to moderate (Table 1). The mean disease severity of the recurrent parent was above that of the BC2DH population in both environments. The QTL analysis detected seven significant marker main effects (Table 2). The QTL for leaf rust resistance were located on 2H, 3H, 4H and 7H, while two and three QTL mapped to chromosomes 4H and 7H, respectively (Figure 1). The exotic allele reduced disease severity in all cases and the strongest effect was measured for the QTL on the long arm of 4H. Here the exotic allele reduced disease severity by 28 %.

Table 2. Presentation of eight QTL for powdery mildew and seven QTL for leaf rust resistance in the BC2DH population

Symptom	*Marker*	*Chr*	*Pos (cM)*	*Bin*	*Effect*	*RP[exotic]*
Powdery mildew	MGB402	1H	0-20	1	M	- 54.5
	GBM1035	2H	17-27	3	MxE	- 10.5
	EBmac415	2H	146	13	M	- 22.1
	MGB410	3H	49-70	5	M	- 22.4
	HVM62	3H	152-154	14	MxE	- 9.6
	GBM1015	4H	170	12	MxE	+ 6.8
	GBM1008	6H	135-155	10	M	+ 9.5
	HVCHI26A	7H	166-181	12	M	- 28.0
Leaf rust	MGB334	2H	159	14	M	- 15.2
	MGB410	3H	65	5	M	- 16.7
	MGB396	4H	95	8	M	- 16.9
	Ebmac788	4H	150	11	M	- 29.0
	Bmag11	7H	93	6	M	- 11.1
	GMS56	7H	133	8	M	- 18.0
	Ebmac755	7H	166	11	M	- 16.5

The chromosome assignments and positions in cM are given for the significant markers. For each group of linked markers with significant effect only one representative marker is listed. Then the cM position of the QTL are indicated from the position of the first significant to the last significant marker in the linkage group. Bin classification follows Kleinhofs and Graner (2001). M = marker main effect, MxE = marker environment interaction; RP[exotic] = relative performance (aa-AA)*100/AA).

In barley, about 16 race-specific resistance genes to leaf rust (designated as *Rph* loci) have been reported (FRANCKOWIAK *et al.* 1997). Four of these genes have been identified in *H. v*. ssp. *spontaneum*. FEUERSTEIN *et al.* (1990) detected two resistance genes, *Rph10* and *Rph11*, on chromosomes 3HL and 6H, and IVANDIC *et al.* (1998) mapped *Rph16* at the centromeric region of 2H. JIN *et al.* (1996) identified *Rph15* on chromosome 2HL distal to *Rph16*. None of these major disease resistance genes previously identified in wild barley mapped close to the QTL detected in this study. On the other hand, QI *et al.* (1998) found the quantitative resistance loci *Rphq1*, *Rphq2* and *Rphq5*, which confer partial resistance to leaf rust by prolonging the latent period. The QTL detected in this study may confirm these loci localised on 7H, 2H and 4H, respectively.

Three loci on 3HS, 4HL and 7HL were significant for both, powdery mildew and leaf rust resistance (Figure 1). However, on the basis of the present results it is difficult to conclude

whether the same genes regulate partial resistance against both diseases or whether tightly linked genes could not be resolved by the current QTL mapping.

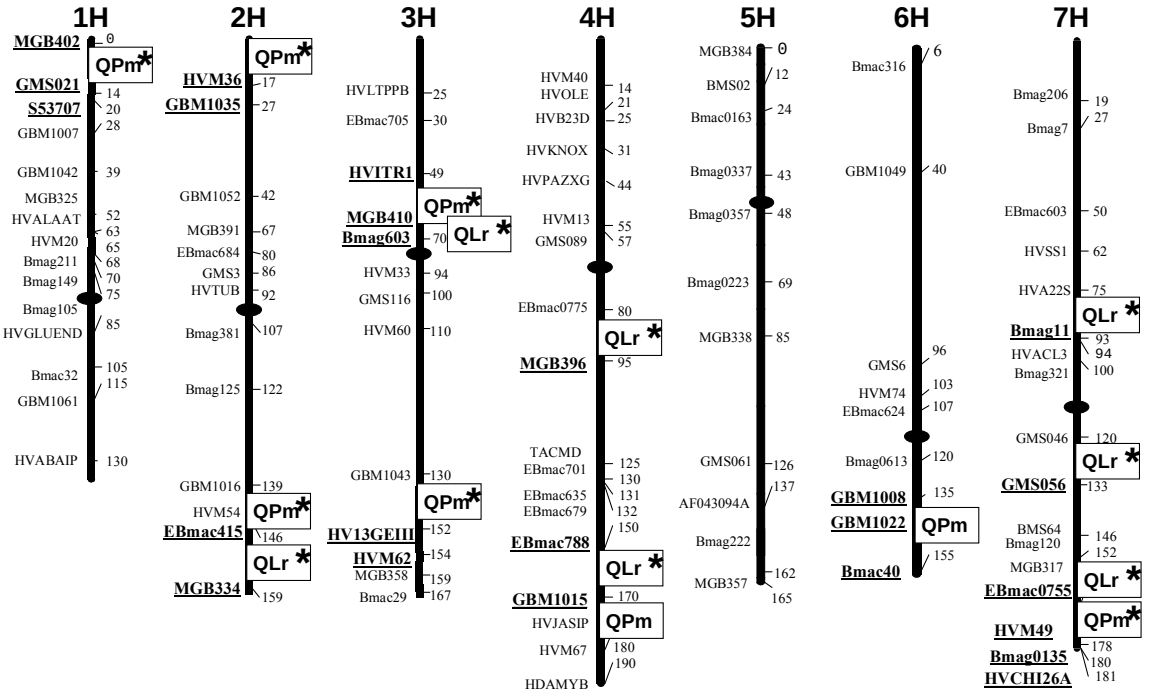

Figure 1. Locations of QTL for resistance to powdery mildew (QPm) and leaf rust (QLr) on a consensus map with 96 SSR markers. The QTL are indicated to the right of the significant markers. These are typed in bold and underlined. The asterisks next to the QTL indicate that the wild barley contributes the resistant allele.

Conclusion

We were able to demonstrate that QTL for powdery mildew and leaf rust resistance could be detected based on field score data. We observed no simple monogenic control of field resistance for any of the naturally occurring diseases studied in three environments. Disease resistance varied quantitatively because of the presence of multiple resistance loci and substantial environmental variance. We found QTL with major and minor effects. Some of them may correspond to previously identified qualitative (i.e. *Mla*, *Mlf*) and to quantitative resistance genes, others may be newly identified resistance genes against powdery mildew and leaf rust. For the majority of disease QTL the wild barley contributed the favourable allele demonstrating the usefulness of wild barley in the quest for resistant cultivars. The population structure employed in this study will allow us to rapidly isolate the resistance QTL and use them for the study of gene function or for breeding new cultivars.

In the future, we plan to finish a thorough AB-QTL study for naturally occurring leaf symptoms recorded in four environments and two years. These QTL will be verified in introgression lines and will be used to initiate new projects on high resolution mapping and finally on map-based cloning of verified QTL from the *H. v.* ssp. *spontaneum* accession ISR42-8.

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German Network for the Evaluation of Cereals for Disease Resistance (EVA II)

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Abstract
EVA II aims at a more efficient use of genetic resources of barley and wheat in resistance breeding. The goal of this project is to evaluate genetic resources for resistance and to transfer and spread new resistances into breeding programs to support sustainability and genetic diversity. To achieve this, a network of private German plant breeders and research institutes will jointly evaluate germplasm (gene bank material, domestic and foreign varieties, and actual breeding material) of barley and wheat, that is pre-selected according to interesting resistances. The nurseries are screened in multi-site field trials for resistance to most important fungal and viral pathogens. Besides race specific resistance (qualitative resistance), quantitative resistance is evaluated by determining the area under disease progress curve by repeated scoring. To characterise the level of infection at different sites resistant and susceptible standards are included. Commonly used evaluation methods are refined to be simultaneously applicable to several cereal diseases. Based on field observations respective germplasms are analysed for known resistance genes by PCR-based markers. An information system for an effective data management is developed. In the growing season 2003 out of 38 spring barley lines, which were described as leaf rust resistant 9 turned out to be better than the resistant standard. Out of 7 barley genotypes selected for powdery mildew resistance all turned out to be equal to the resistant standard. In the growing season 2002-2003 out of 87 winter barley lines 13 genotypes were scored for net blotch resistance better than the resistant standard and also for powdery mildew 13 genotypes turned out to be better than the resistant standard.
Keywords: barley; genetic resources; evaluation; information system

Introduction

To improve crops for resistance to diseases, plant breeders have relied on the genetic diversity within their breeding populations, on collections of genetic resources stored in gene banks, and on those varieties maintained and selected by farmers.
Public concern has focused on the potential for plant disease epidemics due to the uniformity of the genetic base of resistance (SMALE & SINGH 1998). Many virulence genes in pathogens correlate to the corresponding resistance genes in host plants. Specific resistances to such diseases e.g. to the cereal rusts contribute to a 'boom-bust' cycle of resistance and vulnerability (VANDERPLANK 1963). The average duration of a resistance based on major resistance genes appeared to be only a few years (BEEK 1988). One can distinguish between vertical (qualitative), i.e. race-specific, often only temporary resistance, which is mostly inherited in a monogenic manner, and horizontal or quantitative resistance, which is race-non-specific, (more) durable, and normally involves several genes. Recently, efforts have been concentrated to enhance the horizontal resistance in crops (WALTHER *et al*. 1996).

In the past, huge amounts of evaluation data have been collected for various species, but often data collected under different conditions using different scoring schemes cannot be summarized in data collections. EVA II aims to provide plant breeders with accelerated access to resistant genotypes, thereby supporting the sustainability of agriculture by increasing the genetic diversity present in cultivars. The overall goal is a better transfer and dissemination of new resistance genes into (commercial) breeding programs. For this purpose, secondary evaluations of wheat and barley are carried out and a network-information system is developed. EVA II also pursues the refinement of standardized systems for resistance evaluation.

Material and Methods

For barley (both spring and winter) sets of a maximum of 100 genotypes are chosen for testing. All tested genotypes are expected to carry new or unknown resistance genes or combinations. At least two standards (susceptible and resistant) for each fungal pathogen are included to characterise the infestation conditions of all sites. Resistance to most important fungal pathogens in barley (Table 1) is evaluated. Additionally, virus resistance (BaMMV, BaYMV, BaYMV-2) is screened at several sites.

Table 1. Fungal pathogens screened on spring and winter barley and standards for evaluation

Pathogen:		Standards:	
Common name	Latin name	Resistant	susceptible
Spring barley			
Powdery mildew	*Blumeria graminis* f.sp. *hordei*	Alexis	Prisma,
Net blotch	*Drechslera teres*	Annabell	Barke, Pasadena,
Leaf rust	*Puccinia hordei*	Barke, Hanka	Alexis, Prisma,
Scald	*Rhynchosporium secalis*	Sissy	Pasadena
Winter barley			
Powdery mildew	*Blumeria graminis* f.sp. *hordei*	Verena	Regina, HJ171
Net blotch	*Drechslera teres*	Camera	Krimhild
Leaf rust	*Puccinia hordei*	Carola	Cornelia, Vogelsanger Gold
Scald	*Rhynchosporium secalis*	Leonie	Intro, MS Scald

The sets are evaluated for field resistance in multi-site field trials with 17 locations for winter barley and 10 locations for spring barley. Usually, micro-plots of 1 m^2 are used; the trials are set up in one or three replications. Each partner, i.e. 13 breeding companies, 3 research institutes and the BAZ Aschersleben for winter barley and 8 breeding companies, 1 research institute and the BAZ Aschersleben for spring barley, screens the whole set of genotypes for all appearing diseases.

Evaluation scoring is be made by % infected leaf area. Scores (1-9) are transformed by logarithmic scale to % (WALTHER *et al.* 1996). In contrast to vertical resistance, for a rating of quantitative resistance, the extent and the development of the infection has to be quantified. For this purpose, the area under the disease progress curve and the mean disease severity is determined based on several scoring dates (WALTHER *et al.* 1996), i.e. three scorings for every occurring disease; starting, when first symptoms are visible followed by scoring every seven to 14 days.

Comparison is made between the scores of the tested lines and the standards. Besides this, respective genotypes are evaluated for the presence of known resistance genes mainly to viral diseases (BaMMV/BaYMV, BYDV) using molecular markers. For this purpose DNA extraction is carried out according to Dolye and Dolye (1990) and analysis is carried out using Bmac29 for the *Rym4/Rym5* locus (GRANER *et al.* 1999) and YLpPCRM for the *Ryd2* locus (FORD *et al.* 1998).

The results are summarized and soon after harvest shared with the project partners for direct use in their own breeding programs. The network-information system for data acquisition, overview, and retrieval was developed by the German Centre for Documentation and Information in Agriculture (ZADI/IBV, Bonn).

Results and Discussion

The structure of the project is shown in figure 1. The group of experts consists of members of the different breeding companies and of research facilities. This group meets twice a year and decides on the genotypes to be tested and on the future focus of the evaluation sets.

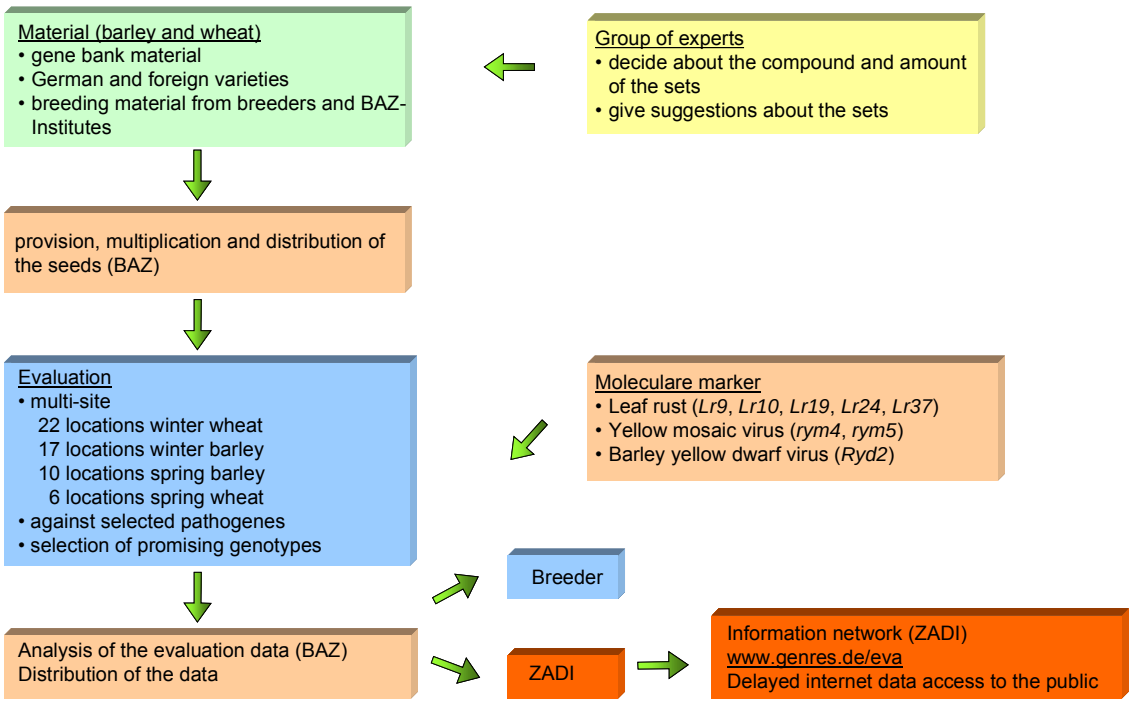

Figure 1. Structure of the EVA II project

With respect to barley, accessions of different sources have been tested in the period 2001 to 2003 (Table 2).

Table 2. Overview of evaluated genotypes

	BAZ-lines	gene bank material	foreign varieties	German varieties	Breeding lines	Standards (res., sus.)	Differential* lines	total
WB 2001/02	**18**	**52**	**12**	**-**	**5**	**12**	**-**	**99**
WB 2002/03	**45**	**9**	**9**	**-**	**3**	**12**	**7**	**87**
WB 2003/04	**7**	**20**	**50**	**5**	**4**	**10**	**4**	**100**
SB 2001	**20**	**13**	**9**	**-**	**3**	**7**	**27**	**79**
SB 2002	**26**	**30**	**33**	**-**	**-**	**11**	**-**	**100**
SB 2003	**29**	**11**	**33**	**-**	**4**	**11**	**-**	**88**
SB 2004	**2**	**42**	**22**	**-**	**8**	**11**	**7**	**92**

WB=Winter barley, SB=Spring barley, BAZ-lines=lines derived from the Federal Centre of Breeding Research, *lines to differentiate specific races of pathogens.

The material from the Institutes of the Federal Centre for Breeding Research was pre-selected for rust and BYDV tolerance. The gene bank material was selected for resistance to powdery mildew, BYDV, net blotch and/or rust resistance. In all evaluated sets genotypes with a better

resistance than the resistant standard could be identified. The disease occurence at the different locations in growing seasons 2001-2003 in spring barley is shown in table 3.

Table 3. Disease occurrence (% mean infected leaf area) in spring barley at the different locations throughout Germany

	powdery mildew			net blotch			leaf rust			scald		
Year	2001	2002	2003	2001	2002	2003	2001	2002	2003	2001	2002	2003
Irlbach	-	0.4	-	-	0.2	26.6	17.5	0.4	-	-	7.9	-
Herzogenaurach	46.1	-	-	18.1	-	-	26.3	36.4	-	-	-	-
Rieste	8.0	5.9	-	-	-	-	-	-	-	-	-	-
Hadmersleben	-	13.0	2.3	-	18.0	2.2	28.9	7.7	7.0	-	-	-
Weihenstephan	14.5	35.0	-	-	-	5.5	-	-	-	-	-	-
Silstedt	16.7	6.0	5.7	-	6.2	-	-	3.3	-	-	3.5	-
Lemgo	20.0	25.2	12.5	-	-	1.5	-	-	-	-	6.8	3.8
Uffenheim	11.9	7.0	11.4	4.8	-	1.8	16.6	-	8.5	-	-	-
Aschersleben	12.1	14.5	1.8	2.0	1.1	-	3.9	9.7	-	-	-	-
Braunschweig	-	20.5	6.1	-	15.9	11.0	-	22.6	3.9	-	30.9	4.4
Neu Darchau	-	14.4	23.3	-	-	-	-	-	-	-	-	-

- no disease appearance

Summarized results over all genotypes and locations of the growing periodes 2001 to 2003 are presented in figure 2. For powdery mildew (*Blumeria graminis*) resistance scores ranged from 1 to 7. For resistance to leaf rust (*Puccinia hordei*) the scores ranged between 2 and 7. For *Rhynchosporium secalis* the scores ranged from 2 to 7. Net blotch (*Drechslera teres*) was scored over all locations from 1 to 6.
Figure 2: Frequencies for the disease scores for the evaluated diseases of spring barley in the years 2001-2003.

Looking in detail at the results of spring barley concerning powdery mildew out of 7 lines described as resistant to powdery mildew all are equal to the resistant standard ‘Alexis’. The variation in the scores are smaller than for the resistant standard (Figure 3).

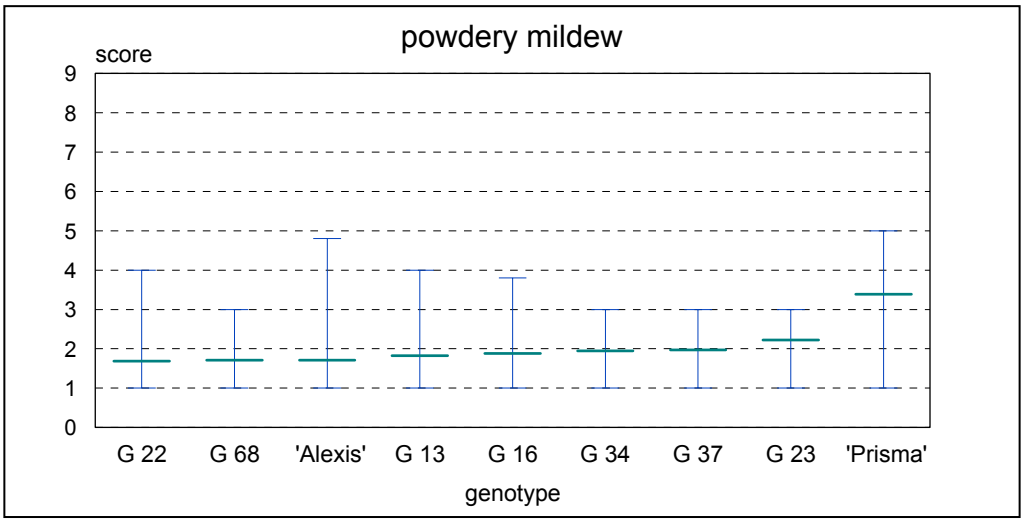

Figure 3. Powdery mildew disease scores (minimum, average, maximum score) in the field 2003 for the accessions described as resistant to powdery mildew (resistant cultivar: ‘Alexis’, susceptible cultivar ‘Prisma’)

For 38 spring barley genotypes selected for leaf rust resistance, 9 genotypes turned out to be more resistant than the resistant standard and the rest of the genotypes tested were scored intermediate between the resistant and susceptible standard (Figure 4).

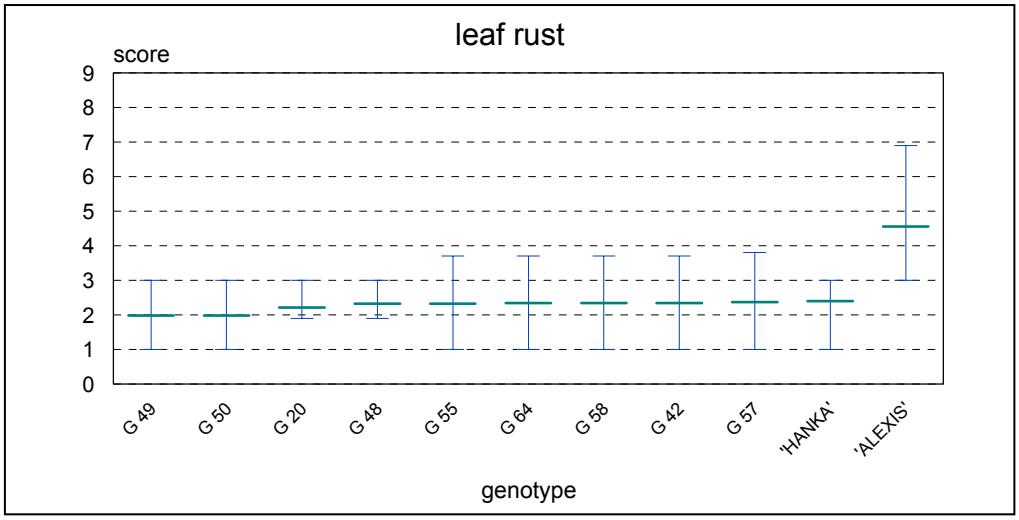

Figure 4. Leaf rust (*Puccinia hordei*) disease scores (minimum, average, maximum score) in the field 2003 for the accessions described as resistant to leaf rust (resistant cultivar: 'Hanka', susceptible cultivar 'Alexis')

In the growing season 2002-2003 out of 87 winter barley lines 13 genotypes were scored for net blotch resistance better than the resistant standard 'Camera'. 'Camera' was scored with 2.1 and the best genotype with 1.3. For powdery mildew resistance 13 genotypes turned out to be better than the resistant standard 'Verena'. For leaf rust resistance no genotype with a better disease score than the resistant cultivar 'Carola' was found. 'Carola' was scored with 1.0 and 10 genotypes were equal to this.

To investigate the presence of known resistance genes against viral diseases PCR markers closely linked to these genes were analysed on a set of genotypes. Using Bmac29 up to now 78 genotypes showing the allele indicative for *rym4* and 20 genotypes showing the fragment linked to *rym5* were detected out of 414 accessions tested. Besides this, using YLpPCRM 27 genotypes revealed the banding pattern linked to the resistance encoding allele *Ryd2* (Table 4).

Table 4. Result of the moleculare charakterisation of known resistance genes

Resistance gene	Banding pattern indicative for the resistance encoding allele	Number of genotypes analysed
rym4	78	414
rym5	20	414
Ryd2	27	414

The results of the EVA II project are represented in the world wide web:
http://www.genres.de/eva/eva_2/index.htm
Partners of EVA II have full access to the data and can search the results by year, crop species and disease or by location. Also a combined search is possible. The first right of access and use of the results belongs to the participants of EVA II. After a three years period the data are publicly available.

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Variability of *Rhynchosporium secalis* (Oud.) J.J. Davis Populations in Morphological Characteristics, Isozymes and Fungicide Resistance Markers in the Czech Republic

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Abstract

The aim of our research was to study the diversity of the *Rhynchosporium secalis* population in the Czech Republic using morphological (colony colors), biochemical (α–esterase, β-esterase, superoxidedismutase, aspartataminotransferase) and fungicide resistance markers. A total of 128 isolates (one isolate from one scald lesion) from five sampling sites of the Czech Republic were studied. Single-spore cultures were divided by color into five groups (from black to beige). The majority of the single-spore cultures (89.8%) kept their colors during the whole period of growth. Most single-spore cultures were genetically homogeneous in the studied trait. Four enzymes of the fungus were studied - α–esterase, β-esterase, superoxidedismutase *(SOD)*, aspartataminotransferase *(AAT)* by using vertical slab polyacrylamide gel electrophoresis. A minimum of three replicates were examined for each isolate. All investigated enzymes of *R. secalis* were polymorphic. A total of nine patterns of *SOD*, fifteen - α–esterase, three - β-esterase and two - *AAT* were obtained. Almost every studied site had the certain major type of polymorphism in esterases and *SOD*. Unique patterns were identified in certain regions of the Czech Republic. The *in vitro* reaction to some widely used fungicides was assessed. There were found significant differences between particular isolates and their LD_{50} levels.

Keywords: barley scald; genetic diversity; population genetics; resistance to fungicides; strobilurins

Introduction

Barley scald is caused by the haploid imperfect fungus *Rhynchosporium secalis* (Oud.) J.J. Davis. It is an important disease of barley (*Hordeum vulgare* L.) in the cool, humid areas of the world. Yield losses ranging from 10 to 40 % have been reported (McDONALD *et al.* 1999). In the last few years, severe epidemics have occurred especially in the northern and central areas of Europe after a more intensive cultivation of barley and the widespread usage of foreign cultivars that are not adapted to the local scald population (BROWN 1985; McDERMOTT *et al.* 1989; JØRGENSEN & SMEDEGAARD-PETERSEN 1995). The main difficulty in the genetic control of scald is variability of pathogen populations. The *R. secalis* population from each of the barley producing areas in the world comprises several unique races that differ in their ability to attack different barley cultivars. Furthermore, virulence structure of populations of *R. secalis* may change significantly over a relatively short period. For example, the resistant cultivar Atlas 46, released in California in 1947, was rated as extremely susceptible throughout the state 9 years later. In contrast, Pennrad barley remained resistant to scald for over 20 years in Pennsylvania (GOODWIN *et al.* 1992). However, besides markers of the virulence, other markers, such as morphological and isozymes, can be used for the studying of variability of pathogen populations.

On the other hand, other important approach to control barley diseases is the use of fungicides. Cereals are protected against fungal diseases by frequent spraying with DMI

(inhibitors of C-14 demethylation of lanosterol or 24-methylendihydrolanosterol) fungicides. DMIs clearly belong to the group of site-specific fungicides, which are in general more prone to resistance than conventional multisite inhibitors (DEKKER 1985). The strobilurins have started to be an important class of agricultural fungicides during the last decade. The huge impact of the strobilurin fungicides on agriculture is well reflected by the current status of azoxystrobin, which is now registered for use on 84 different crops in 72 countries (BARTLETT *et al.* 2002).

The aim of our research was to study the diversity of *R. secalis* population in the Czech Republic, represented by 5 local groups of isolates, using morphological (colony colors), biochemical (variability in enzymes: α–esterase, β-esterase, superoxidedismutase, aspartataminotransferase) and fungicides resistance markers. Furthermore, the aim of this work was to assess possible development of resistance to fungicides from the both higher mentioned chemical groups. The obtained collection of *R. secalis* strains was subjected to the assessment of *in vitro* sensitivity.

Material and Methods

Collection and Isolation of the Fungus

Leaves of barley plants with scald symptoms were collected from five sampling sites of the Moravia region of the Czech Republic during the summer of 2003 (Figure 1). Leaf pieces with scald lesions were sterilized with 70% ethanol and 1% sodium hypochlorite for 30 s, rinsed in pure water and placed on potato-saccharose agar (PSA) with 1% yeastrel. Leaf sections were incubated at 18°C in darkness for two weeks, until the sporulating colonies were visible. A total of 127 isolates (one isolate from one scald lesion) were obtained (Table 1). Single-spore cultures were selected from each isolate and incubated at18°C for 6 weeks.

Sample Preparation

Isozyme analyses were performed on enzyme extracts prepared from single-spore cultures grown in PSA medium. Mycelium of the fungus was ground with a little quantity of glass (Polyclar AT) and added extraction buffer (sucrose, ascorbic acid, cysteine hydrochloride in tris-*HCL* buffer, pH 8.0, based on the recipe of Sako, Stachmann, 1972. These homogenates were transferred to tubes and centrifuged at 12 000 g at 0°C for 15 min. The resulting supernanant was used for electrophoresis.

Electrophoresis

Division of isozymes has been carried out in the device for electrophoresis with vertical slab polyacrylamide gel with tris-glyzyn buffer (pH 8.3). 7.5% polyacrylamide gel was used for *AAT* and *SOD,* 6.0% one for esterases. A constant current from 50 to 100 mA was maintained throughout electrophoresis for 2.5 h.

Gel slices were stained for enzyme activity following published protocols: *AAT* by BROWN *et al.* (1978), *SOD* by BEAUCHAUMP & FRIDOVICH (1971), α-esterase and β-esterase by WEHLING (1986) where α and β-naphtylacetates were utilized as substrates.

A minimum of three replicates were examined for each isolate.

Data Analysis

A relative mobility value (R_f) was assigned to each band of enzyme activity detected. For each isolate, presence or absence of a band of enzyme activity for each enzyme used in this study was defined as an electrophoretic phenotype. Electrophoretic phenotype similarities were inferred from a cluster analysis. The dendrogram was constructed by using UPGMA (unweighted pair-group method using arithmetic averages) procedure in Neighboor-Joining program of PHYLIP v.3.6 package. Standard and molecular diversity indices and genetic

differentiation between and within isolate groups, collected from different sites were calculated by Arlequin software.

Fungicidal Treatment

The methodological approaches are derived from the FRAC Methods for Monitoring Fungicide Resistance (1991). Ninety-two *R. secalis* isolates originating from the Czech Republic were tested.

Description of the Sensitivity Assay

Petri dishes containing PDA-agar with or without fungicides were prepared. The four strobilurin fungicides used were azoxystrobin, trifloxistrobin, kresoxym-methyl and picoxystrobin. One DMI-fungicide (propiconazole) and one MBC fungicide (thiophanate-methyl) were assessed too. The following rates were tested: 0.0, 0.25, 0.5 and 1.0 $g.ml^{-1}$ of fungicides. The test included three replications of each concentration/isolate/fungicide combination as well as three replications of the fungicide-free controls.

The colony diameter was measured after 15 days incubation in the dark at 21°C. The percentage of growth reductions caused by fungicides in comparison with the fungicide-free control was subjected to probit transformation. ED_{50} (effective dose inhibiting 50 % of mycelial growth) values were then calculated using regression analysis of transformed growth reductions caused by fungicides and "ln" of fungicide concentrations. ED_{50} values thus obtained were compared with ANOVA and correlation analysis.

Results

The colonies of *R. secalis* become visible usually only after 14 days growing on PSA with 1% yeast extract. Small, light, yeast-like, growing upward colonies were formed on media. After 6 weeks of growth on PSA, the greater part of isolates with light color darkened and changed color into black, brown and black with a white bloom. The isolates became more dense and developed mycelium. Only 1.2 % of all isolates kept beige color and yeast-like structure. Single-spore cultures were divided by color into five groups (from black to beige color). The majority of the single-spore cultures (89.8 %) kept their color during the whole period of growth (Figure 2).

All investigated enzymes of *R. secalis* were polymorphic. A total of nine patterns of *SOD*, fifteen - α–esterase, three - β-esterase and two *AAT* were obtained. Six *SOD*, two *AAT*, six α–esterase and two β-esterase bands were identified.

Cluster analysis with UPCMA resulted in splitting of all isolates of *R.secalis* into two separate clusters (Figure 3). The first (upper) one was composed of two subclusters: small, including 22 (17.19 %) isolates and large – 93 (72.66 %) ones. Within the small cluster, all isolates were obtained from one field at Kroměříž, but from different cultivars of barley – Kompakt, Malz and Nelly. The large cluster is formed by isolates collected from different sites of the Czech Republic, but the majority of isolates of this cluster was collected from two sites: Rýmařov and Lysice.

The second (down) cluster comprises of 13 (10.15 %) isolates. 69.23 % of all isolates located in this cluster were collected in the Czech-Moravian Highlands (CMH region). It demonstrated a similarity in isozymes among *R. secalis* isolates found in the CMH region.

Genetic diversity of the Czech population is represented by 67 different electrophoretic phenotypes (Figure 3). In other words, almost 50 % of *R. secalis* isolates had unique genotypes.

The pairwise comparison of genetic variations between isolates collected at different sites of the Czech Republic and within each site was tested statistically. Values of F_{ST}

(population differentiation) greater than 0.25 indicate significant genetic differentiation (HARTL & CLARKE 1997). The level of differentiation among five sites ranged from 0.09 to 0.38 (Table 2).

According to these data, groups 1 and 3 can be considered as one subpopulation, because of low F_{ST} between them and the same for sites 2, 4 and 5.

Significant levels of gene and genotype diversity were found at the five sites of the Czech Republic (Table 3). Gene diversity ranged from 1.00 to 0.93 within sites. The collection of isolates from Medlov (site 2) had the highest genotype diversity, which can be explained by very small numbers of isolates (5).

Different isolates of *R. secalis* highly significantly influenced ED_{50} levels. There were also highly significant differences between mean levels of ED_{50} among the fungicides tested (Table 4).

ED_{50} values were significantly lower for picoxystrobin, azoxystrobin, thiophanate-methyl and kresoxym-methyl than for trifloxistrobin and propiconazole (Table 5).

The highest variability was found in the reaction of particular isolates to propiconazole characterized by s_x level. There were no correlations in the reaction of particular isolates among all fungicides of different chemical groups.

Discussion

The results of this experiment show that populations of *R. secalis*, collected from five sampling sites of the Czech Republic, are significantly variable phenotypically for morphology (color of colony), biochemically by isozymes structure and agronomically by susceptibility to fungicides. The great majority of genetic variation in *R. secalis* was distributed within as well as among five places of isolates originating from the Czech Republic. Sixty-seven different electrophoretic phenotypes were identified: 36 from 128 isolates were unique. Similar genetic variability for pathogenicity, isozyme, ribosomal DNA and colony color variants in populations of *R. secalis* have been reported by other investigators (McDONALD *et al.* 1989; GOODWIN *et al.* 1994).

The highest level of genetic diversity in *R. secalis* in our study may be explained by several possible sources: spontaneous mutation and migration or parasexual recombination (JACKSON & WEBSTER 1976; NEWMAN & OWEN 1985). Although the sexual stage has not been reported for *R. secalis* till now, the study of geographically diverse collections of isolates from Australia, California, Finland and Norway using isozyme (BURDON *et al.* 1994), RFLP markers and DNA fingerprints (McDONALD *et al.* 1999, SALAMATI *et al.* 2000) provided additional evidence for regular recombination in studying populations.

The presence of the isolates with different genotypes at one large subcluster confirms higher gene flow between these sites of the Moravian region of the Czech Republic. This gene flow can be considered as a result of longer-distance dispersal of conidia by windswirling during storms or as a result of infected seed transmission or windblowing of small particles of infected straw during harvest. It is also possible that the observed genetic distances are due to differences in the historical movement of barley seeds between these sites. It is known, the Czech Republic is agrarian country with movement of farm products (barley seeds and straw used for animal feed).

There were high similarities in isozymes among *R. secalis* isolates found in the Czech-Moravian Highlands. It is possible that the influence of gene flow at this site was not so significant. It is the mountainous area with the average above-sea level around 500 meters, dividing the Czech Republic from south to north into two parts. The mountains are a barrier in transfer of pathogen conidia to the barley-host.

We can believe that low pathogen genotype diversity at this site was mainly due to the higher plasticity of the pathogen, quicker adaptation to changes in the environment, including introduction of new resistance genes. Therefore, the traditional method of introducing single major resistance genes into productive cultivars may not provide long-term control of barley scald disease.

Strobilurin fungicides have become an integral part of disease-management programmes on a wide range of crops (BARTLETT *et al.* 2002). The major reasons for the success of strobilurins have varied among individual active ingredients, but have consisted of one or more of the following: broad-spectrum activity and control of fungal isolates resistant to other fungicide modes of action.

A relatively important problem of the management of resistance to fungicides is the existence of the so-called "cross-resistance". We found any correlation in reaction of strobilurins assessed neither between each other nor with reaction to fungicides from the other chemical groups.

Within integrated plant protection, leaf diseases of cereals are often suppressed with fungicides. Search for changes in reaction to the broad-spectrum fungicides can help to detect the development of possible shift of resistance and to correct the advisory recommendations to farmers as early as possible.

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Possibilities of *Pyrenophora teres* Detection in Barley Leaf Tissue

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Abstract

Specific polymerase chain reaction (PCR) primers were developed from AFLP bands of DNA of *Pyrenophora teres,* the causal agent of net blotch on barley leaves. The primers were designed to specifically amplify DNA from *P. teres* f. sp. *teres* and allow its differentiation from *P. teres* f.sp. *maculata*, which is morphologically very similar to *P. teres* f.sp. *teres* in culture. The PCR amplification was carried out successfully from DNA extracted from fungi mycelium. The PCR assay was validated with 60 samples from 10 barley hosts originating from several regions across the Czech Republic. No cross reaction was observed with DNA of several other species like *P. tritici repentis*, *P. graminea* and *Helminthosporium sativum*. This method is prepared to be used to detect the pathogen from environmental samples for survey and management purposes.

Introduction

Net blotch caused by *Pyrenophora teres* Drechs. (anamorph: *Drechslera teres* [Sacc.] Shoem.) is an important disease of barley (*Hordeum vulgare* L.). It is widespread and causes considerable yield losses. In the Czech Republic they range from 10 to 40% (MINARIKOVA & POLISENSKA 1999).

There are two forms of the pathogen: *Pyrenophora teres* f. *teres* Drechs. (anamorph. *Drechslera teres* f. *teres* [Sacc.] Shoem.) and *Pyrenophora teres* f. *maculata* Drechs. (anamorph. *Drechslera teres* f. *maculata* [Sacc.] Shoem.) which differ in the symptoms induced on barley leaves (SMEDEGARD-PETERSEN 1977).

In spite of the symptoms, it is difficult to distinguish the two forms morphologically in culture. As resistance to the two forms of net blotch is inherited independently (Ho et al., 1996), it is important that the pathogen is correctly identified. Traditionally, this pathogen has been identified by plating seeds on a culture medium, and after an incubation period, by examination under microscope. These methods are time consuming and unreliable. In recent years DNA technology has become a major tool for the identification and detection of fungal plant pathogens (MUGNIER 1995).

Amplified Fragment Length Polymorphism (AFLP) is a reliable, PCR based, marker system for obtaining quantitative estimates of genetic relationships (VOS *et al.* 1995). Recently, AFLPs have been used to evaluate *P. teres* and *P. graminea* isolates' variability in Australia (WILLIAMS *et al.* 2001).

This paper describes the use of specific primers, derived from AFLP products, that differentiate spot form and net form isolates of the net blotch fungus *Pyrenophora teres*.

Material and Methods

30 isolates of *Pyrenophora teres* f. *teres*, 36 isolates of *Pyrenophora teres* f. *maculata*, 5 isolates of *Pyrenophora graminea*, 3 isolates of *Pyrenophora tritici repentis,* 1 isolate of *Pyrenophora flavispora* and 4 isolates of *Helminthosporium sativum* were evaluated. These

isolates were collected from different barley growing regions of the Czech and Slovak Republics; five isolates that originated from Norway, Germany and Syria. All isolates were derived from single conidia taken from leaf tissue.

DNA was extracted from mycelia cultured in potato-dextrose broth, using CTAB detergent according to the optimised protocol. The quality and the concentration of the extracted DNA were verified electrophoretically in 0.8% agarose gel. DNA was visualized by ethidium-bromide and detected under a UV lamp. As to size and concentration standards, λ Hind III was used.

The AFLP analysis was done according to the optimised protocol in the system of MseI and EcoRI restriction endonucleases. The amplification products were separated by the capillary electrophoresis in ABI PRISM 310 (Perkin-Elmer). As to internal size standards, Rox500 was used (Applied Biosystems). Chromatograms were processed by the software GeneScan and Genotyper. For each isolate, a binary matrix, reflecting specific AFLP fragment presence (1) or absence (0), was generated. Pair-wise distances between the isolates, based on Hamman similarity metrics (Armstrong et al., 1994), were calculated using the Microsoft® Excel VBA (Visual Basic for Applications) macros. Cluster analysis was performed by STATISTICA for Windows (StatSoft, Inc.) by means of an unweighted pair-group method that used an arithmetic averaging algorithm (UPGMA).

Form specific AFLP fragments were extracted from 3% agarose gel using a QIAquick Gel Extraction Kit (QIAGEN) and cleaned using a StrataPrep PCR purification kit (Stratagene). The blunt-ended fragments were cloned in a pPCR-Script™ Amp SK(+) vector. The recombined plasmids were transformed into XL 10-Gold Ultracompetent cells *E. coli* (Stratagene).

The sequencing was done using Big Dye Terminator 3.1 Sequencing kit (Applied Biosystems). Products of the sequencing reaction were separated on an ABI PRISM 310 sequencer (Perkin-Elmer). Electrophoretic data were analysed using Sequence Analysis for Windows NT software (Applied Biosystems). Sequence alignments were assembled using T-COFFEE software (Notredame et al., 2000). Specific primers were designed using the Primer Express for Windows NT 1.5 software (Applied Biosystems).

Amplifications were performed in total volume of 15µl according to the optimised protocol. Products (10µl of PCR reaction) were visualized and photographed under UV, after electrophoresis, in ethidium bromide containing agarose gels. As primers were fluorescently labelled, the method of capillary electrophoresis in ABI PRIM 310 (Perkin-Elmer) was used as well. The analysis was performed as a multiplex of three reactions (fam, hex, ned). As to internal size standards, Rox500 was used. The data were analysed using GeneScan and Genotyper software.

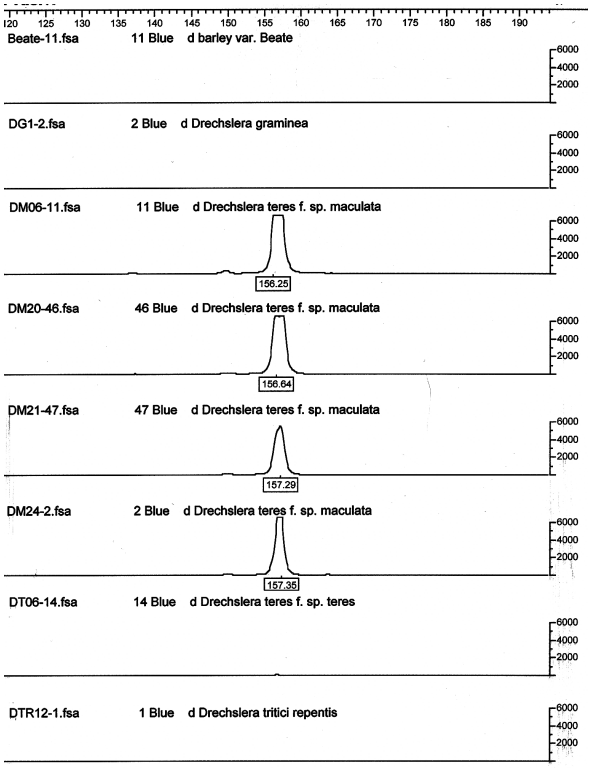
Results and Discussion

AFLP analysis was conducted on DNA prepared, from 35 isolates of *Pyrenophora teres*, *P. graminea*, *P. tritici repentis* and *Helminthosporium sativum*, using 11 primer combinations. For each primer pair, 27 to 50 polymorphic DNA fragments of 50 to 500 bp in size were detected. With the 33 primer pair combinations, a total of 1247 AFLP polymorphic bands were recorded across all studied isolates. When subjected to a UPGMA clustering analysis based on genetic distances, 5 groups were found in accordance with studying 4 species, including *P. teres* which had two forms (*P. teres* f. *teres* and *P. teres* f. *maculata*).

Using a two coloured representation of AFLP data (presence/ absence of bands) markers specific to one of P. *teres* forms, were found. Sequences of 4 original AFLP amplicons were used to design *Pyrenophora teres* form specific primers.

Three primer pairs: DTT429g, DTT471h, and DT340i were designed to detect P. *teres* f. *teres*. The amplification products' sizes were: 93, 87 and 81bp. Three other primer pairs:

DTM494d, DTM379d7, and DT350j were designed to detect P. *teres* f. *maculata*. The amplification products sizes were: 157, 379 and 66bp. Each of them was validated on a panel of isolates of both forms of P. *teres* and other related species. The PCR products' analysis showed that only DTT471h and DT340i were specific to P. *teres* f. *teres*. As for P. *teres* f. *maculata*, only DTM494d was specific to it:



Amplification with DT350j provided two different products; the *P. teres* f. *teres* amplification product had a size of 66 bp, unlike the *P. teres* f. *maculata* amplification product that had a size of 60 bp. Primer pairs DTT429g and DTM379d7 amplified both forms of *P. teres,* even *P. graminea* isolates.

The assays were validated, during a survey of isolates of P. *teres,* from major barley producing areas of the Czech Republic. The results confirmed the initial AFLP data and morphological and physiological evaluation (ONDREJ, personal communication). The four isolates collected as *P. teres* f. *teres* were genotyped as *P. teres* f. *maculata*. The three isolates collected as *P. teres* f. *maculata* were genotyped as *P. teres* f. *teres*. The isolate number H306 that was collected from a barley leaf from Syria, as *P. teres* f. *maculata,* was genotyped as an unknown species. It was subsequently determined to be *Pyrenophora flavispora* (ONDREJ, personal communication).

The AFLP was found as a suitable method to generate specific PCR-based assays. The preliminary results of using these assays to identify the pathogen directly in infected leaves (data not shown) indicated that they are suitable for routine diagnosis everywhere correct identification of the pathogen plays a key role.

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Molecular Dissection of a QTL Region for Partial Resistance to Barley Leaf Rust

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Abstract
Partial resistance to leaf rust (*Puccinia hordei*) in barley is a quantitative resistance that is not based on hypersensitivity. This resistance is prehaustorial and characterized by a long latent period in greenhouse tests. Six quantitative trait loci (QTLs) have been mapped on a population of 103 recombinant inbred lines obtained from the cross L94 x 'Vada', respectively susceptible and partially resistant lines. The most consistent QTLs *Rphq-2*, *Rphq-3* and *Rphq-4* have been introgressed into L94 background to obtain near isogenic lines (NILs). Seedling disease tests revealed a clear effect of *Rphq-2* NIL while *Rphq-3* NIL had a moderate effect and *Rphq-4* NIL had no effect at this development stage. The development of flanking PCR markers, based on mapped RFLP sequences, allowed locating *Rphq-2*, *Rphq-3* and *Rphq-4* in physical regions of high (1.1 Mb/cM), suppressed (>4.4 Mb/cM) and very high (0.2 Mb/cM) recombination respectively. Based on those results *Rphq-2* seems to be the best candidate gene for cloning. Fine-mapping the *Rphq-2* region should open the way towards cloning the first gene for partial resistance to a pathogen.
Keywords: barley; *Hordeum vulgare*; leaf rust; *Puccinia hordei*; partial resistance; Quantitative Trait Loci (QTL), Cleaved Amplified Polymorphic Sequence (CAPS), Allele-Specific PCR (ASPCR)

Introduction

At Wageningen University the partial resistance of barley to leaf rust is studied since 1973. To evaluate partial resistance through greenhouse tests, the measurement of latent period (LP) has been shown to be the most reliable and effective method (NEERVOORT & PARLEVLIET 1978). The two cultivars with the most extreme values for LP identified, L94 and 'Vada' (PARLEVLIET & VAN OMMEREN 1975), were crossed and a population of 103 F_9 recombinant inbred lines (RILs) was obtained. The AFLP technique was then applied to create a high-density linkage map of L94 x 'Vada' (QI *et al.* 1998a) on which six QTLs for partial resistance were identified (QI *et al.* 1998b). The most consistent QTLs, *Rphq-2*, *Rphq-3* and *Rphq-4*, were introgressed by marker assisted backcrossing into L94 background to obtain near isogenic lines (NILs) (VAN BERLOO *et al.* 2001). Those NILs allow the evaluation of QTLs in a nearly uniform genetic background, overcoming the special difficulties of identifying QTL phenotypes, and they are the starting material for the fine-mapping and cloning of the QTL. The reliability of the phenotypic measures and the unambiguous assignment of genotypes by progeny testing is an important requirement for successful map-based cloning (REMINGTON *et al.* 2001; BOREVITZ & CHORY 2004).

The accuracy with which a QTL can be identified and located increases with the heritability of the QTL and the size of the population but the confidence interval on the genetic map is seldom less than 5 centimorgans (cM) and frequently up to 30 cM (POONI & KEARSEY 2002). And even if greater precision can be obtained from NILs, in map-based cloning the marker interval containing the gene should be refined by genetic mapping to a region small enough to allow physical mapping (REMINGTON *et al.* 2001). Because the AFLP markers that flank *Rphq-2*, *Rphq- 3* and *Rphq-4* in the L94 x 'Vada' population are of

dominant nature and technically rather cumbersome to work with, new molecular markers technically easy to apply and co-dominant in expression are required. In this study web-available sequences of RFLP probes positioned on the physical barley map of KÜNZEL *et al.* (2000) were used to develop allele-specific PCR (ASPCR) (WU *et al.* 1989), and cleaved amplified polymorphic sequence (CAPS) (KONIECZNY & AUSUBEL 1993) markers flanking the three QTLs. Information on recombination frequencies in the three QTL regions and on the size of the effect of each QTL in its NIL-background are the basis on which to select the best candidate to clone a gene for partial resistance to a pathogen.

Material and Methods

Evaluation of the Near Isogenic Lines in Greenhouse

To estimate the individual effects of *Rphq-2*, *Rphq-3* and *Rphq-4*, NILs in their BC_3S_{3+n} generation were evaluated in a greenhouse compartment on seedlings. The evaluation of the NILs was performed with isolate 1.2.1, which had also been used to map QTLs in L94 x 'Vada' by QI *et al.* (1998b).

Greenhouse disease tests were performed as described by QI *et al.* (1998b). For seedling evaluation 3 mg of urediospores from a –80°C freezer were dusted over small trays of 37x39 cm. The latent period (LP) of each plant was evaluated by estimating the period (in hours) at which 50% of the ultimate number of pustules became visible. The relative latent period of seedlings (RLP50S) was then calculated relative to the LP of L94 plants, where L94 was set at 100, as described by PARLEVLIET (1975). Between December 2001 and April 2003 the effects of *Rphq-2*, *Rphq-3*, and *Rphq-4* were estimated twelve, ten, and six times respectively. Each experiment contained from 3 to 15 seedlings per genotype tested and all the values obtained were averaged to give an estimation of the QTL effects.

Primer Design and Polymorphism Detection

Twenty-two primer pairs were designed based on DNA sequences of barley genomic clones mapped in the supposed region of *Rphq2*, *Rphq3* and *Rphq4*. The DNA sequences were downloaded from the GrainGenes database (URL: http://www.graingenes.org/), and the optimal annealing temperature (Tm) of each primer pair was determined by gradient-PCR. The generated PCR products of parental lines L94 and 'Vada' were then digested with the 24 restriction enzymes of the CAPS-kit described by BAI *et al.* (in press) in order to detect polymorphism. If after testing those 24 restriction enzymes no polymorphism was detected, PCR products were sent for direct sequencing (BaseClear, Leiden, The Netherlands). The sequences were then analysed for the presence of single nucleotide polymorphism (SNP). Primer design and sequence analyses were done with the Lasergene software (DNASTAR Inc., Madison, WI, USA).

Each PCR reaction contained 100 ng of genomic DNA, 8 pM of each primer, 0.2 mM dNTPs, 1x PCR reaction buffer, and 0.5 U of *Taq* DNA polymerase in a total volume of 25 µl. The PCR conditions were: 1 cycle at 94°C for 5 min, followed by 39 cycles of 30 s at 94°C, 30 s at Tm, 45 s at 72°C, and a final extension of 7 min at 72°C. The PCR products were separated on 1% agarose gel stained with ethidium bromide, and DNA fragments were visualized by UV light. About 3 µl of crude PCR product was digested in a total volume of 15 µl for 3 hours with 1 unit of the appropriate restriction endonuclease. After digestion, DNA fragments were separated on 2 to 3% agarose gel and visualized by UV light.

Linkage Analyses

DNA of the 103 RILs and parents of the L94 x 'Vada' population was isolated essentially following the CTAB based protocol of STEWARD and VIA (1993), adjusted for 96-well format.

The converted RFLP molecular markers were mapped on the high-density AFLP linkage map of the L94 x 'Vada' population (QI *et al.* 1998a) in order to verify their position relative to the confidence interval of the three QTLs. Linkage analyses were done with JoinMap® 3.0 (VAN OOIJEN & VOORRIPS 2001) applying the Kosambi's mapping function. Then, genetic distances between the QTL-flanking markers mapped on L94 x 'Vada' were related to physical distances between the corresponding RFLP markers mapped on the cytologically integrated physical maps of KÜNZEL *et al.* (2000).

Results and Discussion

Phenotypic Evaluation of the Near Isogenic Lines

A successful map-based cloning approach requires that genotypes of single plants can be unambiguously assigned according to their phenotype. Also, a fast and easy-to-apply method for phenotype evaluation is preferred. Compared to adult plants, testing seedlings is much faster. It also requires less space and is much easier and reliable since adult plant leaves often show age differences and various lesions and damages hampering the latent period measurements. Therefore, tests on seedlings are preferred in a map-based cloning procedure.

Table 1. Relative latent period at seedling stage of *Puccinia hordei* isolate 1.2.1 on barley RILs and NILs that differ for *Rphq*-genes

	Rphq-2	*Rphq-3*	*Rphq-4*
Fitted value - RILs[a] (QI *et al.* 1998b)	111.4	108.6	102.5
Observed mean - RILs[b] (QI *et al.* 1998b)	108.0	109.7	98.5
Near Isogenic Line	**110.7**	**103.3**	**99.8**

[a], theoretical value calculated based on the population mean and the allelic effect of each QTL

[b], average value of the RILs carrying the 'Vada'-allele of the QTL considered and the L94-allele of the other QTLs

The effects of *Rphq-2*, *Rphq-3*, and *Rphq-4*, were measured in their NIL-background and compared to their effect assessed in the mapping population (Tab. 1). The effect of each QTL discovered in the mapping population was estimated in two ways by QI *et al.* (1998b). The "fitted value" was based on the average RLP50S of the whole population to which the effect of the alleles from 'Vada', for the QTLs others than the one considered, were substracted. The "observed mean" was based on the average RLP50S of those RILs that carry only the 'Vada'-allele of the considered QTL and the L94-allele of the other QTLs.

In seedlings QI *et al.* repetitively identified *Rphq-2* and *Rphq-3* as being the two QTLs with the highest effect, while *Rphq-4* was never identified at this development stage. Those previous results are confirmed by the partial resistance levels in these isolines (Tab. 1). However, the effect of *Rphq-3* in the NILs was prolonging the LP less than predicted by the mapping population.

Development and Mapping of ASPCR and CAPS Markers

From the 22 primer pairs designed, five did not amplify anything or amplified multiple bands in L94 and in 'Vada'. Fourteen primer pairs amplified clear and single bands in both parents. Three primer pairs gave direct dominant ASPCR markers (Tab. 2): ABG458, MWG618 and MWG835.

The 14 primer pairs which amplified clear and single bands were screened with the CAPS-kit enzymes and revealed four CAPS markers: MWG2068 digested with *Hae III*, cMWG679 digested with *BssK I*, ABG388 digested with *Nla III*, and MWG502 digested

with *Hpa II* (Tab. 2). Sequencing of the amplified DNA fragments of the 10 primer pairs for which CAPS-kit enzymes did not reveal any polymorphism, allowed the design of only one additional co-dominant CAPS marker: MWG2200 digested with *Ava I* (Tab. 2).

Table 2. Primer sequences and polymerase chain reaction (PCR) conditions for the developed CAPS and ASPCR markers

Name	Location	Marker type	Primer sequence (5'-3')	Tm (°C)[a]	Product size	Restriction enzyme
MWG2068	2 (2H)	CAPS	ccgtgagatgtaagttgctgg atgcgttggcccattgg	58	700 bp	*Hae III*
MWG2200	2 (2H)	CAPS	atcctggagtacctcgaagccg agcacggcctccagcaccac	58	358 bp	*Ava I*
cMWG679	6 (6H)	CAPS	gagcagcccgtgacatggaa ctccaccagtgcaacctcgt	61	300 bp	*BssK I*
ABG458	6 (6H)	ASPCR	gagagccgatgacggtatgt cttggacacatgccatatcc	65	248 bp	- [b]
ABG388	6 (6H)	CAPS	gcactggcatagtctcacaa cgatgctggttcggtcatac	58	303 bp	*Nla III*
MWG502	7 (5H)	CAPS	tgtttagctaagctgttgctgagg ctctgaagcatggaaccagatttg	55	500 bp	*Hpa II* *Msp I*
MWG618	7 (5H)	ASPCR	tattttcagtcccgcacac gccgctaaaacacatcaaac	65	231 bp	- [c]
MWG835	7 (5H)	ASPCR	ttccctattcaagccatcagcaaa gtaacccatagatagcatcttgtg	65	565 bp	- [c]

[a], PCR annealing temperature. [b], only amplification product for 'Vada'-DNA. [c], only amplification product for L94-DNA

The developed PCR-based markers were mapped in the 103 RILs of the L94 x 'Vada' population. With the exception of cMWG679, all mapped markers were found to be located close to one of the three QTLs. The CAPS marker cMWG679 mapped at the telomeric end of chromosome 6HS in L94 x 'Vada' (data not shown) while the corresponding RFLP marker was mapped 60 cM proximal on that chromosome arm (GRANER *et al.* 1991). Markers that were closely located to one of the QTLs were also verified in the NILs. If the 'Vada'-allele of the marker is present in the NIL, this marker can be used later to detect recombinants for fine-mapping the corresponding QTL.

The two co-dominant CAPS markers MWG2200 and MWG2068 were linked to *Rphq-2* on chromosome 2HL. The *Rphq-2* NIL had the 'Vada' allele of MWG2200 but the L94 allele of MWG2068. Those two markers were linked at 8 cM interval and flanked *Rphq-2*. They delimit a physical region of high recombination of 1.1 Mega-base per centiMorgan (Mb/cM) on the physical maps of KÜNZEL *et al.* (2000) (Fig. 1a). The two markers ABG458 and ABG388 were linked to *Rphq-3* and mapped at 20 cM interval on both sides of the centromeric region of chromosome 6H. For both markers *Rphq-3* NIL had the 'Vada' allele. On the physical maps of KÜNZEL *et al.* ABG458 and ABG388 delimit a physical region of suppressed recombination of 3.4 to more than 42 Mb/cM (Fig. 1b). The co-dominant CAPS marker MWG502 and the two dominant ASPCR markers MWG618 and MWG835 were linked to *Rphq-4* on chromosome 5HS. For the three markers the *Rphq-4* NIL had the 'Vada'

allele. MWG502 and MWG835 flanked *Rphq-4* at 11 cM interval and fell in a physical region of very high recombination of less than 0.2 Mb/cM on the maps of KÜNZEL *et al.* (Fig. 1c).

Selection of a Candidate to Clone a Gene for Partial Resistance

By applying disease tests on seedlings the evaluation of the developed isolines for the three QTLs showed that *Rphq-2* is the easiest QTL to detect. The increase of about 10% in RLP50S of *Rphq-2* isoline corresponds to an average difference of more than 18 hours with the susceptible line L94. The detection of *Rphq-3* could be hampered by its low effect and the lack of effect of *Rphq-4* in seedlings disqualifies it as candidate gene for cloning.

Another important aspect to look at is the variation of the effect of a particular QTL and its potential overlap in range between L94 and 'Vada' alleles. The standard deviations of RLP50S on the line L94 and on the *Rphq-2* isoline are respectively 2.7% and 4.7%, implying that any individual plant containing the 'Vada'-allele of *Rphq-2* can be distinguished from the susceptible line L94 by the longer LP of leaf rust isolate 1.2.1.

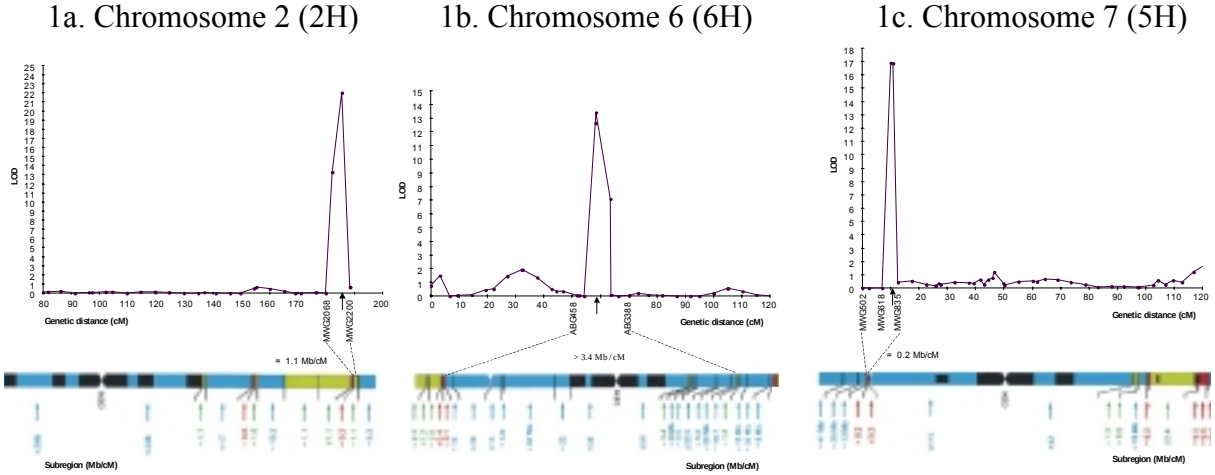

Figure 1. LOD profile of *Rphq-2* (1a), *Rphq-3* (1b), and *Rphq-4* (1c) along the genetic distances of the L94 x 'Vada' AFLP map of QI *et al.* (1998a) obtained by composite interval mapping and their relationship to the physical distances of the map of KÜNZEL *et al.* (2000). Blue indicates suppressed (>4.4 Mb/cM), green increased (1.0 – 4.4 Mb/cM) and red strongly increased (≤1.0 Mb/cM) recombination.

The set of PCR-based markers developed allowed the estimation of the relationship between genetic and physical distances in the regions of the three QTLs (Fig. 1a, 1b, 1c). The QTLs *Rphq-2*, *Rphq-3*, and *Rphq-4*, fall respectively in physical regions of high, suppressed and very high recombination. *Rphq-3*, which mapped above the centromere of chromosome 6 (6H), is likely to be in a region of highly suppressed recombination, making this gene, also for this aspect, less suitable than *Rphq-2* for map-based cloning.

The flanking ASPCR and CAPS markers developed could also be valuable tools for breeding purposes. Those simple PCR-based markers should allow the detection of QTLs for partial resistance to leaf rust in commercial breeding lines and introgression of the 'Vada'-alleles into germplasm with low level of partial resistance should be feasible. The combination of *Rphq-2* and *Rphq-3* should confer a high level of partial resistance at seedling stage and the combination of *Rphq-3* and *Rphq-4* should confer a high level of partial resistance at adult plant stage.

Rphq-2 is the most suitable gene for map-based cloning because its effect can be clearly determined in seedling tests and because its genetic to physical distances ratio

indicates a reasonable frequency of recombinations. A bulked-segregant analysis to saturate the *Rphq-2* region with AFLP and microsatellite markers is currently underway. Then fine-mapping of *Rphq-2* using the near isogenic line should open the way to the first cloning of a gene for partial resistance to a pathogen.

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Genetic Variability in Ethiopian, Jordanian and Syrian Populations of the Scald Pathogen, *Rhynchosporium secalis*

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Abstract
Barley scald, caused by the fungal pathogen *Rhynchosporium secalis*, is an important disease in many barley production areas of North Africa, West Asia, Middle East and Ethiopia. To assess genetic diversity using amplified fragment length polymorphism (AFLP) markers in this pathogen, the isolates were sampled from Ethiopia, Jordan and Syria during the year 2002 - 2003. AFLP analysis could amplify a total of 51 loci and out of which 48 loci (94.12%) were found to be polymorphic among the isolates. The genotypic diversity (GD) measure within individual populations (as measured by percentage of GD) was 82.5, 62.3, 89.2 and 80 respectively for Ethiopia-1, Ethiopia-2, Jordan and Syria while the overall genotypic diversity was 78% of the theoretical maximum (100%), indicating moderate to high genetic diversity in these populations.
Keywords: *Rhynchosporium secalis*; PCR, AFLP; genetic diversity; barley

Introduction

Leaf scald of barley (*Hordeum vulgare* L.), caused by the fungus *Rhynchosporium secalis* (Oud.) Davis, is an economically important disease of barley world wide (SALAMATI *et al.* 2000). Yield losses under favorable conditions can be 40% (KHAN 1986; SALAMATI *et al*. 2000) or greater in highly susceptible cultivars (WILLIAMS *et al*. 2003).

Pathogenicity, isozyme, colony color, ribosomal DNA, genomic restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) DNA markers have been used to study genetic variability in *R. secalis* populations (McDERMOTT *et al*. 1989; GOODWIN *et al.* 1993; BURDON *et al*. 1994; McDONALD *et al*. 1999; NEWTON *et al*. 2001) (SALAMATI *et al*. 2000; WILLIAMS *et al*. 2003). These studies confirmed that the pathogen possesses a high level of pathogenic variation (ALI *et al*. 1976; CEOLONI 1980; FOWLER *et al*. 1971; TEKAUZ 1991; GOODWIN *et al.* 1992) and that most populations of *R. secalis* are highly variable for many genetic markers despite the absence of a known teleomorph. Asexual recombination (NEWMAN & OWEN 1985; NEWTON 1989), spontaneous mutation (GOODWIN *et al*. 1994) and sexual reproduction (McDONALD *et al*. 1999) have been some of the mechanisms suggested as being the sources of the high level of genetic diversity in *R. secalis*.

Knowledge of the amount and distribution of genetic variation within and among populations is an important component of understanding the population biology of pathogenic fungi (McDONALD *et al*. 1999). Genetic structure may be used to infer the relative impact of different forces that influence the evolution of the pathogen populations. In turn, a better understanding of evolutionary forces may allow us to predict the potential for the pathogen populations to evolve in agricultural ecosystems (WATSON 1981; McDONALD *et al*. 1999). Furthermore, information on evolution of the pathogen may help in designing suitable integrated disease management strategies to combat the problem of the disease. However, there is no information available on genetic diversity of *R. secalis* populations in Ethiopia, Jordan and Syria. Here we report the level of genetic variation within and among populations of *R. secalis* sampled from Ethiopia, Jordan and Syria.

Material and Methods

Field Sampling Method, Isolation and Culturing of R. secalis *Isolates*

Barley leaves infected with *R. secalis* were sampled from fields of local cultivated barley cultivars in Ethiopia (2 populations from Haro-Wonchi and Negash, designated as Ethiopia-1, Ethiopia-2), Jordan (from Al-Barra) and Syria (from Khandaria) using a hierarchical sampling method (McDONALD *et al.* 1999) where four sites were sampled along two parallel transects. Transects and collection sites along transects were separated by 10m. Infected leaves (10 total) were sampled from each site within the field (80 leaves per field total), during 2002 and 2003 cropping seasons. Isolation and culturing of the fungus was done using the method described by McDONALD *et al.* (1999) with slight modification. The fungal material was extracted by passing through a filter paper using a suction pump and then lypholized in a freeze drier.

DNA Extraction and AFLP Analysis

Approximately 0.05g of freeze-dried fungal tissue was ground in to fine powder using a mixer-mill (mixer-mill Retsch MM 2000) and grinding balls in a 2ml Eppendorf tubes. DNA extraction and AFLP analysis were performed by adapting the previously published methods (von KORFF *et al*. 2004) with minor modifications. The primer combination M301(TATA) and P24(TC) was selected for selective PCR amplification of AFLP analysis after screening 16 different *Tru*9I - *Pst*I selective primer combinations.

Data Analysis

Scoring of the amplified DNA profile was performed on the basis of the presence or absence of AFLP fragments. Genotype diversity (GD) in a population based on multi-locus haplotypes was calculated using the measure proposed by (STODDART & TAYLOR 1988). Genetic distance analyses were estimated using Nei's unbiased genetic distance, *D* (NEI 1978).

Results and Discussion

The use of morphological and pathogenicity characters in distinguishing variations among different isolates of the pathogen had been less satisfactory than the use of genetic markers (MEYER *et al*. 1992; NEWTON *et al*. 2001). Various genetic markers such as Isozyme, RFLP, RAPD, and AFLP markers have been used to study the genetic variability in various pathogen populations including *R. secalis* (McDERMOTT *et al*. 1989; GOODWIN *et al*. 1993; McDONALD *et al.* 1999; NEWTON *et al*. 2001; FLIER *et al*. 2003). Compared to the widely used RFLP technique, AFLPs are faster, cheaper, less labour intensive, more reliable than RAPDs, it is practical and provide more information (McDONALD 1997; MATTHES *et al*. 1998). Therefore we have selected the AFLP technique to study the population diversity of *R. secalis* populations from Ethiopia, Jordan and Syria.

In the present study, we analysed the genetic variation within the populations using AFLP markers and detected more than 50 distinct bands, out of which 14 to 31 were polymorphic in individual populations (Table 1). The number of polymorphic bands detected was slightly higher than von KORFF *et al*. (2004), with a different primer combination and different sets of isolates representing Ethiopian, Jordanian and Syrian populations.

The present collection described here with 159 isolates was richer in genotype diversity (over all percentage of maximum genotypic diversity = 77.99), with total 124 genotypes detected in all the 3 countries. The pathogen populations in all the 3 countries are also apparently distinct from each other (over all genetic distance ranged from 0.022 to 0.228), which is in line with the observations of von KORFF *et al*. (2004). However, our study also showed high within population genotypic diversity (within population genotypic diversity as indicated by

percentage of GD of 82.5, 62.3, 89.2 and 80 respectively for Ethiopia-1, Ethiopia-2, Jordan and Syria), which supported the observations of SALAMATI *et al*. (2000). The amount of genetic variation detected in our study was slightly lower than the variation reported in previous studies based on RFLPs (SALAMATI *et al.* 2000). Future diversity studies on *R. secalis* will include additional isolates from both barley and its wild relatives, from different agro-climatic regions and characterizing them with DNA markers in order to address issues related to origin and diversity of this fungus.

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Table 1. Number of total- and polymorphic-loci, genetic distance and genotypic diversity

	Population				
	Ethiopia-1	Ethiopia-2	Jordan	Syria	Overall
No. of isolates	40	42	37	40	159
No. of genotypes	33	27	33	32	124
No. of polymorphic loci	24	27	31	17	48
Total number of loci	38	32	37	38	52
Genetic distance (range)	0.0 - 0.0757	0.0035 - 0.1208	0.009- 0.2203	0.0 - 0.12	0.0222-0.228
Genotypic diversity (GD)[a]	28.57	19.6	30.42	22.86	96.13
% of maximum GD	82.5	64.29	89.19	80	77.99

[a] STODDART and TAYLOR (1988)

Disease Resistance Mapping in Spring Barley

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Abstract
We used scores obtained from a barley mapping population grown in scald nurseries to show that the resistance loci detected were co-incident with either known dwarfing gene loci or other QTL detected for height variation in the same cross. We therefore utilised a detached leaf test to measure scald infection on the same mapping population to detect non-morphological and specificity responses, conducting the test with several isolates and with leaves of varying ages from GS13 to 33. The detached leaf data showed that, whilst there was significant genetic variation, there were considerable genotype x isolate effects. We detected several QTLs for the overall mean response to infection in the detached leaf tests, none of which were co-incident with the QTLs detected from the disease nursery scores. We consider that some of these loci could be potential sources of non-specific scald resistance. The same mapping population was also scored for expression of resistance to physiological spotting, a leaf-spotting complex and Rice blast. The *mlo* mildew resistance allele played a major role in increasing physiological spotting and intereacted with another QTL of large effect on chromosome 6H. QTLs for physiological spotting were largely independent of those for the leaf spotting complex, which we presume to be due to infection by *Ramularia collo-cygni*. The *mlo* allele also increased susceptibility to Rice blast but other QTLs detected for resistance to the disease were more significant.
Keywords: barley; mapping; QTLs Ramularia; Rhynchosporium; physiological spotting; Rice blast

Introduction

Scald (caused by *Rhynchosporium secalis*) is a problematic disease of spring barley in the UK as chemical control is difficult and, with the exception of Doyen, there are no cultivars on the UK 2004 recommended list that have a high resistance rating (www.hgca.com). Attempts to deploy major-gene resistance to the disease in spring cultivars in the UK have not been generally supported by the market, possibly because such cultivars fail to achieve the highest levels of malting quality necessary to achieve recommendation by the Institute of Brewing and consequent widespread cultivation. Whether this apparent association of slightly reduced malting quality with major-gene resistance to the disease is due to an adverse linkage or pleiotropy is not clear. Evidence from the UK Cereal Pathogen Virulence Survey also suggests that, with the exception of Digger (BRR-8), there is virulence to most of the major-gene sources of resistance to the disease (www.hgca.com), which indicates that deployment of major-gene resistance may just be a short-term solution. Effective sources of durable resistance to the disease that can be combined with the highest malting quality are therefore required. Previous studies have indicated that apparent QTLs for non-specific resistance to scald are associated with genes increasing plant height (THOMAS *et al.* 2000). In addition, it has been suggested that recommended list cultivars possessing *mlo* mildew resistance alleles show increased susceptibility to scald, although a DH population indicated the opposite (BROWN & JONES 1996).

We therefore studied infection of a mapping population that segregated for the *mlo11* mildew resistance allele by a range of *R. secalis* isolates in detached leaf tests and compared the findings with those obtained from infection in field-grown scald nurseries. As *mlo* alleles have been implicated in a range of biotic and abiotic stresses (PIFFANELLI *et al.* 2002), we also studied infection levels in the same population to the rice blast pathogen (*Magnaporthe grisea)* and naturally occurring symptoms of physiological spotting and the post-anthesis spotting complex thought to be due to *Ramularia collo-cygni*.

Material and Methods

Data on scald infection on the DH lines from the Derkado x B83-12/21/5 population was available from 7 nursery scores gathered from experiments conducted between 1994 and 1997 and used to derive an overall mean score. Similarly, data on post-anthesis spotting of the flag leaf was available from scores gathered on trials conducted between 1999 and 2002. Whether these spots were due to infection by *Ramularia collo-cygni* and/or due to physiological causes was not clear but we will term these scores "Ramularia-Like Spots" (RLS). Prior to anthesis, clear physiological spotting (PSpot) of some of the forms described by SUTHERLAND and LENNARD (1988) were apparent in trials grown at a site near Sleaford (Lincs, UK) and the degree of leaf coverage scored. All these data were scored using a 1-9 scale where 1 = absence of symptoms and 9 = complete coverage. In addition, the Derkado x B83-12/21/5 population was inoculated with isolate 96-1-27 of rice blast at the seedling stage and scored for levels of infection on a 0-4 scale where 0 = resistant and 4 = susceptible. The experiment was replicated and conducted three times.

A detached leaf test (NEWTON *et al.* 2001) was used to study scald infection of the same Derkado x B83-12/21/5 population. The detached leaves were scored on four points of a 1-9 scale where 2 = absence of visible infection and 8 = presence of large scald lesion(s) covering most of the leaf segment. Seven separate experiments were carried out in randomised complete blocks with five replicates and each was inoculated with a different race of *R. secalis*. One experiment was inoculated at two separate growth stages with the same race, making a total of 8 measurements. The leaf segments were taken from the plants between GS13 and GS33.

The data from each of the field grown experiments for each pathogen were analysed with the ANOVA directive in GENSTAT (Lawes Agricultural Trust, Rothamsted Experimental Station, UK) to derive means and an overall mean was derived for each DH line's response to each disease. Similarly, means were derived for the detached leaf data and an overall mean derived. To study the relationships between the scores for infection by the pathogens and the distribution of scores for individual isolates in the detached leaf tests, Principal Components Analysis (GENSTAT) was used to explore the relationships between the correlations of the characters.

Since publication of the latest genetic map of the Derkado x B83-12/21/5 population (ELLIS *et al.* 2002), 24 further markers have been scored on the population. The majority (22) of these were EST derived markers, either Single Nucleotide Polymorphisms (SNP and abc) or Simple Sequence Repeats (Ctig) and the other two were genomic SSRs. The augmented marker set was used to produce a revised map of the population using JOINMAP 3.0 (VAN OOIJEN & VOORRIPS 2001). The phenotypic means for the degree of infection by each pathogen were then combined with the maps and genotypic data to scan for QTLs with PLABQTL (UTZ & MELCHINGER 1996). The program defaults were used to identify significant co-factors for infection response and those that were not associated with any significant QTLs were iteratively removed. Permutation was then used to establish a 5% error threshold for the whole experiment and further iterative co-factor removal carried out if

necessary. The presence of any significant epistatic effects was tested and then cross-validation was used to estimate the overall amount of phenotypic variation accounted for by the QTLs detected.

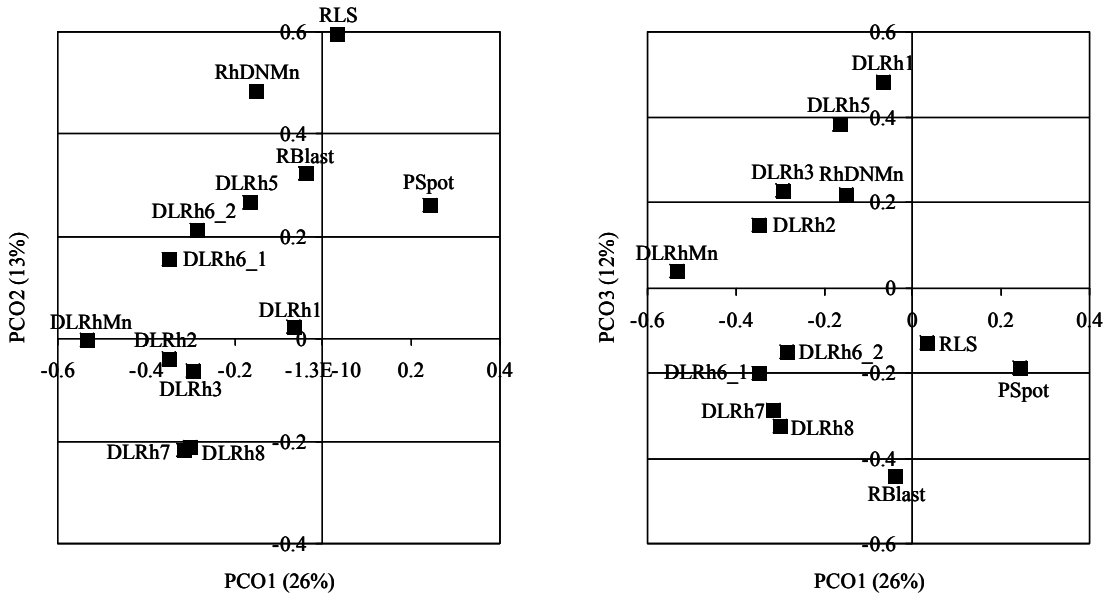
Results

Table 1. Population statistics and summary of results from QTL analysis of infection levels by five separate biotic and abiotic stresses

	Derkado	B83-12/21/5	DH Mean	QTLs	5% LOD Threshold	%Variation
RhDNMn	5.4	**4.4**	4.9	3	2.95	71.2
RhDLMn	5.8	**4.0**	4.0	5	2.96	18.0
RLS	3.6	**2.6**	3.6	6	3.12	17.3
PSpot	4.0	**1.8**	3.1	4	2.84	62.0
RBlast	3.5	**0.5**	1.8	3	2.91	13.2

There was significant genetic variation amongst the DH lines for the overall means of the characters with Derkado showing a significantly higher level of infection than B83-12/21/5 for each one (Table 1). The differences between the two parents were most marked for PSpot and RBlast. For RhDL and RLS, the DH population mean was equivalent to the mean of B83-12/21/5 and Derkado respectively, indicating the possible presence of epistatic effects (SNAPE & SIMPSON 1981).

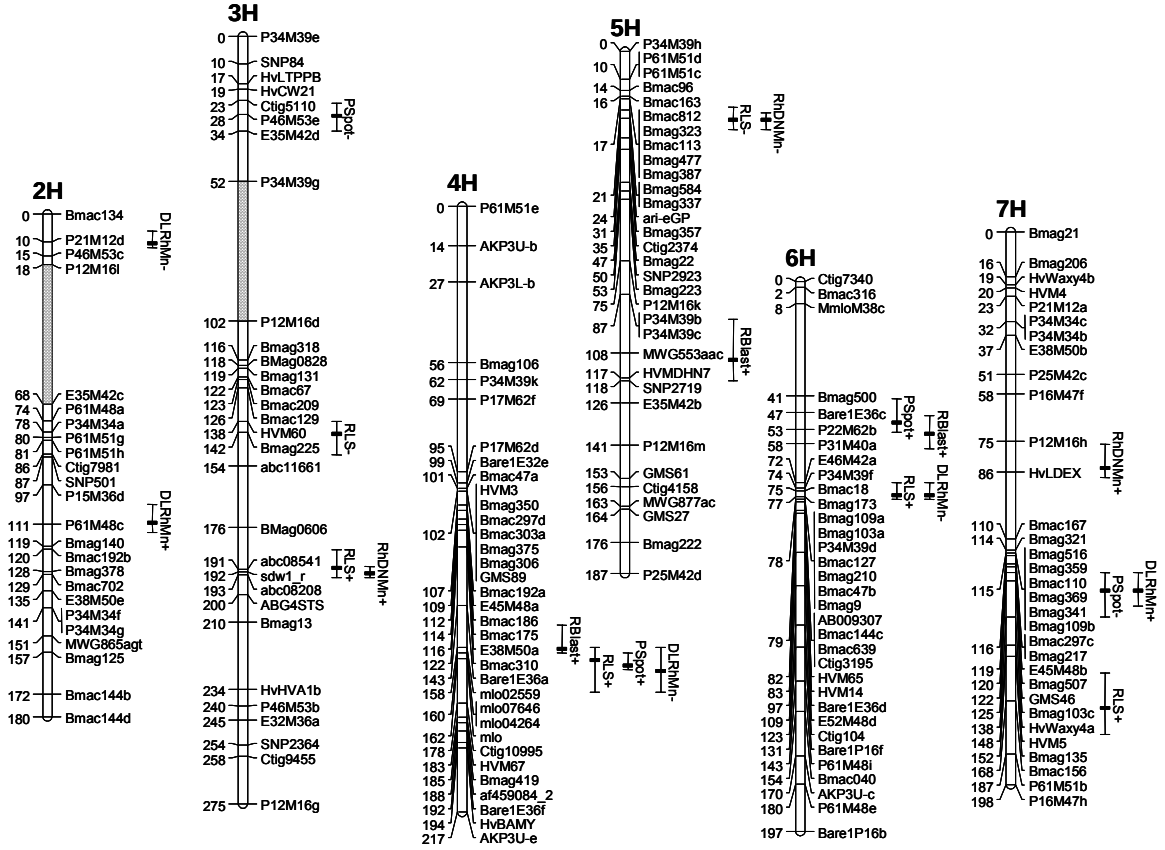
Figure 1. Principal components analysis of reaction to scald infection by the Derkado x B83-12/21/5 mapping population in 8 detached leaf tests (DL1-8), their mean (RhDLMn) and the overall mean from field nurseries (RhDNMn). Also included is the reaction of the population to physiological spotting (PSpot), "Ramularia-Like Spots" (RLS) and rice blast (RBlast).



The first three principal components accounted for over 50% of the variation amongst the 13 variables and the loadings of the variables are shown in Figure 1. From the loading of the variables upon the principal components, it can be seen that Pspot is clearly separated from the rest of the variables by PCO1 and has a markedly opposite loading to RhDNMn, suggesting a negative relationship between the two. The various detached leaf scores show considerable variation amongst themselves, indicating considerable interactions of infection

levels with race and/or physiologic age of the lines. The individual scores have fairly similar loadings on PCO1 but have a range of loadings on both PCO2 and PCO3. Some groupings of two scores can be seen, e.g. RhDL7 and RhDL8 and RhDL2 and RhDL3. RhDNMn is clearly separated from RhDLMn by PCO2 and the two overall measures of scald infection do not appear to be highly related to each other. Similarly, Pspot and RLS are separated by both PCO1 and PCO2 and, although they have similar loadings on PCO3, do not appear to be highly related. Rblast has a similar loading to RLS on PCO1 but is clearly separated by both PCO2 and PCO3 and does not appear to be highly related to any other character. In general, the field scores tend to group together on PCO1 and PCO2 but are more separated by PCO3.

Figure 2. Updated map of Derkado x B83-12/21/5 and location of QTLs for five measures of resistance to biotic and abiotic stresses. Sign indicates effect of Derkado allele on expression of a character. Thick line indicates QTL peak and whiskers its 1 LOD confidence interval. No QTLs were detected on chromosome 1H and it has been omitted for clarity.



Between three and six QTLs were detected for the mean infection levels and accounted for between 72 and 13% of the phenotypic variation (Table 1). QTLs were found on six of the seven chromosomes (Figure 2) with the region around the *mlo* locus affecting each character apart from RhDNMn. Three loci were detected for RhDNMn with two large effects at the two dwarfing gene loci, *sdw1* and *ari-e*GP and another effect in the region of HvLDEX on chromosome 7H, where a height QTL was also found (not shown). As reported previously by THOMAS *et al.* (2000), the associations are such that tall plants have less disease. QTL loci for RhDLMn show no signs of co-location with RhDNMn suggesting independence of the two scores of scald infection. Two of the five QTLs for RhDLMn are clearly co-located with RLS on chromosomes 4H and 7H and, in each case, are in repulsion. Apart from the region on chromosome 4H, QTLs for RLS are not co-located with Pspot, suggesting some independent genetic control for the two characters implicated in the spotting phenomenon. Derkado alleles at the *mlo* region on chromosome 4H increase infection levels of rice blast, RLS and Pspot

but decrease RhDLMn. Epistasis was only detected for PSpot, possibly indicating a more complex inheritance of processes leading to physiological spotting. In general the co-location and effects of the QTLs reflect the picture provided by principal components analysis.

Discussion

Neither Derkado nor B83-12/21/5 possess any known major-gene resistance to scald so any resistance that we detect must be viewed as the action of minor-genes probably effecting non-specific resistance. Each genotype does possess a separate major dwarfing gene and the action of the two in the segregating population clearly influences infection levels on plants grown in disease nurseries as two of the three QTLs are located at the dwarfing gene loci and, with a third, account for over 70% of the phenotypic variation for the character, i.e. major-gene levels. Even the third QTL is located in a region where QTLs for height have been detected in the Derkado x B83-12/21/5 population (unpublished data). These findings therefore reinforce those of an earlier study by THOMAS *et al.* (2000). More telling evidence for the influence of plant height comes from the fact that none of the QTLs for the detached leaf tests co-locate with any from the disease nursery scores for scald. This suggests that QTL studies of non-specific resistance for splash-borne diseases such as scald should be treated with extreme caution unless the effects of height variation are taken into account. Further work is needed to validate the findings of the detached leaf tests and the finding that a QTL for reduced lesion development in detached leaf tests is twice co-located with one for increased physiological spotting is clearly of interest but may represent an artefact of the methodology. There is no evidence from this study to suggest that *mlo* alleles are linked with increased susceptibility to scald as was found in a survey of recommended list cultivars by BROWN & JONES (1996). Results from the detached leaf tests suggest the opposite to be the case, similar to the results from a study of another DH population (loc cit.) and it may be that the mlo cultivars in the recommended list survey had a lower overall height thus reducing their escape from the disease.

The co-location of QTLs for increased infection by rice blast and increased physiological spotting with the mlo locus is in line with the findings that *mlo* alleles enhance susceptibility to rice blast (JAROSCH *et al.* 1999) and abiotic stresses (PIFFANELLI *et al.* 2002). Whilst the QTL for PSpot is clearly the most significant (LOD=30) and indicative of the action of a major-gene, the QTL for Rice blast is only just significant (LOD=3) with the other two being much more significant although none has a LOD greater than 6. The QTLs for PSpot and RBlast on 6H are both highly significant (LOD>5.5) and the Derkado allele at each increase expression of symptoms. There is a significant epistatic interaction between the QTLs for PSpot on 4H and 6H that increases expression of symptoms, suggesting that allelic variation at the locus on 6H can modify expression of the *mlo* QTL.

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Mapping and Dissection of Barley Stripe Rust Resistance

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Abstract

Barley stripe rust (BSR), caused by *Puccinia striiformis* fsp. *hordei*, is an important fungal disease of barley. Genetic resistance to BSR can be qualitatively or quantitatively inherited. Qualitative resistance alone is risky due to evidence that pathogen virulence can evolve faster than breeders can release new resistant varieties. Pyramiding of quantitative trait loci (QTL) and combining quantitative and qualitative sources of resistance are effective strategies for lowering disease severity. The goal of this project is to identify genetic factors that determine BSR resistance and to measure the effects and interactions of each resistance factor. A large doubled haploid population was developed from the cross of BCD47 x Baronesse. We have identified QTL on chromosomes 3H, 4H, 5H and 6H where the resistant parent (BCD47) contributed favorable alleles, and QTL on chromosomes 2H and 7H where the susceptible parent (Baronesse) contributed favorable alleles. Currently, quantitative and qualitative resistance genes are deployed in complex genetic backgrounds involving multiple donors. A set of backcross near-isogenic lines (BISONs) was created to allow measurement of resistance allele effects in the same genetic background and the systematic analysis of resistance allele interactions. The BISON lines represent the quantitative resistance alleles on chromosomes 4H and 5H from BCD47, a quantitative resistance allele on chromosome 1H from BCD12 and a qualitative resistance allele on chromosome 7H from D3-6/B23 introgressed, separately, into a Baronesse background.

Introduction

Genetic resistance is the most desirable disease control method because of its low cost to crop producers and environmental appropriateness. Plant disease resistance can be classified as qualitative or quantitative, based on inheritance of the resistance and degree of symptom expression. Qualitative resistance can be manipulated most easily in breeding programs. It facilitates genetic analysis and selection but it is likely to be non-durable due to the evolution of virulence in the pathogen population that can quickly overcome the host's resistance (BROWNING & FREY 1969). There is also evidence that this virulence evolution happens more quickly than plant breeders can deploy single qualitative resistance genes in new varieties. Germplasm developed by the ICARDA/CIMMYT Barley Program in Mexico can be considered a source of 'durable' resistance to BSR because it allows limited symptom development when exposed to a spectrum of virulences. One of several strategies to extend the longevity of host plant resistance is to select host genotypes with quantitative resistance (MUNDT 1994). Quantitative resistance is more complicated, due to complex inheritance, but it is considered to be more durable. Molecular mapping has been widely used to study QTL-type resistance. BSR resistance QTL were mapped to barley chromosomes 2H, 3H, 4H, 5H, 6H, and 7H. CASTRO *et al.* (2002) showed that three of these QTL give differential

resistance. More precise genetic characterization of quantitative resistance will aid in the development of improved selection methodologies. The goal of this project is to identify genetic factors that determine BSR resistance and to measure their effects and interactions. A large doubled haploid population was developed from the cross of BCD47 x Baronesse. From this mapping population QTL were identified on chromosomes 3H, 4H, 5H and 6H where the resistant parent (BCD47) contributed favorable alleles, and QTL on chromosomes 2H and 7H where the susceptible parent (Baronesse) contributed favorable alleles. Currently, the sources of resistance are in complex genetic backgrounds involving multiple donors. A set of backcross near-isogenic lines (BISONs) was created to allow measurement of resistance allele effects in the same genetic background and the systematic analysis of resistance allele interactions. The BISON lines represent the quantitative resistance alleles on chromosomes 4H and 5H from BCD47, a quantitative resistance allele on chromosome 1H from BCD12 and a qualitative resistance allele on chromosome 7H from D3-6/B23 introgressed, separately, into a Baronesse background. If all, or at least most, of the genes controlling quantitative resistance can be identified and tagged, the corresponding regions of the genome can be tracked and incorporated into new genotypes via marker-assisted selection. Information on markers defining quantitative resistance regions is also essential for pyramiding resistance QTL, since based on phenotype alone, it may not be possible to distinguish intervals with different numbers and combinations of resistance genes. Also, understanding the genetic basis of quantitative resistance is critical in order to predict how pathogen populations may respond to deployment of such resistance.

Material and Methods

Mapping of BSR Resistance QTL

A large doubled haploid population, the ORO population, was developed from the cross of BCD47 x Baronesse. BCD47 is a two-rowed, spring, doubled haploid barley line developed via marker-assisted selection for BSR resistance QTL alleles on chromosomes 4H and 5H. Baronesse is a two-rowed, spring, feed barley variety grown extensively in the Pacific Northwest and is susceptible to BSR in the Toluca Valley (Mexico). The ORO population (n=409) was screened with 58 SSRs and a genetic map was generated using JoinMap software. Phenotypic analysis was performed with two reps and five experiments: three in the Toluca Valley (Mexico) and two in Washington State (USA). QTL analysis was performed by composite interval mapping using QTL cartographer software.

Dissection of BSR Resistance QTL

The "F1" the BISON population (n=237), was developed from the cross of BCD47/Baronesse x BCD12/Baronesse. BCD12 is a two-rowed, spring, doubled haploid barley line developed via marker-assisted selection for the BSR resistance QTL allele on chromosome 1H. The BISON population was screened with SSRs (RAMSAY *et al*. 2000) that flank the QTL on chromosomes 1H, 4H, and 5H. Genescan® and Genotyper® software was used to identify and select plants heterozygous for the resistant parent allele. The selected plants were selfed to give rise to the "F2" BISON population. The "F2" BISON population was screened with the previously used SSRs and a C.B.S. polyacrylamide gel sequencing system (WANG *et al*. 2003) was used to identify and select plants homozygous for the resistant parent allele. The selected lines were advanced to the "F4" generation by single seed descent. Seed from selected "F4" BISON population lines (and the parents as controls) was evaluated for BSR resistance under field and greenhouse conditions in Montana State University and Colegio de Postgraduados, Montecillo in Mexico. Lines that contain the BSR QTL or gene of interest and showed a predictable BSR phenotype were selected. The selected lines are being backcrossed to Baronesse to develop the near-isogenic lines, which

represent the quantitative resistance alleles on chromosomes 4H and 5H from BCD47, a quantitative resistance allele on chromosome 1H from BCD12 and a qualitative resistance allele on chromosome 7H from D3-6/B23 introgressed, separately, into a Baronesse background.

Results and Discussion

Mapping of BSR Resistance QTL

In the ORO population, eight QTL in the Toluca Valley and five in Washington State were identified on chromosomes 3H, 4H, 5H and 6H where the resistant parent (BCD47) contributed favorable alleles, and QTL on chromosomes 2H and 7H where the susceptible parent (Baronesse) contributed favorable alleles. It was determined that Baronesse, the susceptible parent, contains some resistance alleles. No epistatic interactions between QTL were detected. There were significant QTL by environment interactions, but all were a change in magnitude rather than a change in rank.

Dissection of BSR Resistance QTL

The data collected from the Montana and Mexico experimental locations showed similar infection types for the tested "F4" BISON lines, which would be expected for robust QTL. The data for the controls (parents) showed infection types that match data collected in prior experiments. This data confirmed the QTL effects in a 90% homozygous background. The lines from the phenotypic analysis have been backcrossed to Baronesse once. Heterozygosity (successful hybridization) was confirmed by genotypic screening. The selected lines had fixed the resistance allele at the target BSR QTL. The allele state at the other QTL regions was also identified. Several disease resistance parameters (latent period, lesion length, infection efficiency, and sporulation density) are currently being evaluated in the "F5" BISON lines in order to dissect the genetic basis of the components of resistance.

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Introgression and Mapping of Novel Resistance Genes from the Secondary Genepool of Barley, *Hordeum bulbosum*

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Abstract
Enhancement of the genetic diversity in our crops is of a major importance especially in respect to disease resistance. *Hordeum bulbosum* represents the secondary gene pool of barley (*Hordeum vulgare* L.) and is a potential source of desirable agronomic-trait genes. Despite the existence of crossing barriers, recombination events between the two *Hordeum* genomes may occur and lead to introgressions of *H. bulbosum* genomic segments which carry novel major resistance genes. Telomeric introgressions have been identified that confer resistance to the soil-borne virus complex (BaMMV, BaYMV-1, -2) as well as to leaf rust and to powdery mildew, respectively. Based on diploid resistant recombinants mapping population were developed. For each of the resistances, a monogenical, dominant inheritance could be demonstrated. Analysis of molecular anchor markers led to the localization of virus-resistance genes on barley chromosomes 6HS and 2HL, as well as leaf-rust and powdery-mildew resistance genes on 2H and 5HL. PCR markers were developed which are suited for routine use in breeding programmes. A diagnostic marker was obtained from a cDNA-AFLP analysis that cosegregated with the soil-borne virus resistance gene *Rym14*^{Hb}.
Keywords: plant-genetic resources; *Hordeum bulbosum*; resistance genes; mapping

Introduction

Use of plant-genetic resources (PGR) is a prerequisite for maintaining a broad genetic basis in the breeding of our crops. The use of PGR by breeders for unlocking novel genes of resistances to diseases and pests and of tolerances to abiotic stresses constitutes an essential component in concepts towards an environmentally compatible and sustainable agriculture.
The virus complex of BaMMV/BaYMV-1/BaYMV-2 is transmitted by the soil-borne fungus *Polymyxa graminis* and represents one of the most important diseases in barley. Chemical measures to defeat the vector are neither efficient nor acceptable for ecological and economical reasons. Virus resistance of recent European cultivars is based on the resistance genes *rym4* and *rym5*, with the latter being effective to BaMMV, BaYMV-1, and BaYMV-2. To date, at least 14 virus resistance genes with recessive inheritance have been introduced from the primary genepool into barley. Molecular mapping led to the localization of these genes on chromosomes 1H, 3H, 4H, 5H, 6H and 7H, respectively (KONISHI *et al.* 2002; LE GOIS *et al.* 2004).

[*] The authors dedicate this paper to Prof. Dr. H.H. Geiger, University of Hohenheim, on the occasion of his 65th birthday.

Another barley disease is leaf rust, caused by the fungus *Puccinia graminis*. Strategies towards yield stability encompass the search for novel leaf-rust resistance genes as well. To date, barley leaf-rust resistance genes *Rph1* through *Rph19* have been described (LUNDQVIST 2000, http://www.cdl.umn.edu/res_gene). Several of these resistance genes became ineffective after they had extensively been utilised in barley breeding. *Rph3* has been overcome in Europe (CLIFFORD 1985) and *Rph12* in Europe and Australia (CLIFFORD 1985; COTTERILL *et al.* 1992). While most of the *Rph* genes were derived from the primary barley gene pool two of them have been tranferred from *H. bulbosum*. *Rph17* was found to cosegregate with *MWG682*, which is an anchor marker for the short arm of barley chromosome 2H (LUNDQVIST 2000; PICKERING *et al.* 1998), while a second *H. bulbosum* gene, *Rph18*, was mapped on the long arm of this chromosome (LUNDQVIST 2000; PICKERING *et al.* 2000). *H. bulbosum* proved also useful as a source of genes conferring resistance to powdery mildew. Two such genes, which were named *Mlhb1*.a and *Mlhb2.b*, respectively (STEFFENSON 1997), were described in a barley-genetic background and found to be non-allelic to all other known powdery mildew resistance genes in barley (PICKERING *et al.* 1995; XU and KASHA 1992). An additional powdery-mildew resistance from *H. bulbosum* was located on chromosome 2HS and reported to cosegregate with leaf-rust resistance (Pickering et al. 1998). Most of the resistance genes effective against powdery mildew in barley are located on chromosomes 1H (*Mla* locus), 4H (*mlo* locus) and 6H (JØRGENSON 1993). To date, additional resistance genes were located on chromosomes 3H (BAUM *et al.* 1996), 5H (SCHÖNFELD *et al.* 1996; FALAK *et al.* 1999) and 7H (SCHÖNFELD *et al.* 1996).
In the present study, part of our work on the genetic analysis and molecular mapping of seven disease-resistance genes from *H. bulbosum* is summarized.

Material and Methods

Plant Material

The F5 families *BAZ-4006* and *BAZ-4034* were both derived from the same resistant, recombinant (VV^b) F2 individual, which had been obtained from a tetraploid *H. vulgare* cv. 'Borwina' x *H. bulbosum* hybrid (VVBB; SZIGAT and SZIGAT 1991). The F6 family *BAZ-5061* was obtained by selfing the F5 individual *BAZ-4034/80* which was heterozygous for the introgression. The F4 mapping population *BAZ-3026* traced back to the same tetraploid hybrid but was obtained from a different diploid recombinant offspring plant. The F2 families *BAZ-1282* and *BAZ-1283* were developed from a BBVV and a VVBB hybrid, respectively, and were kindly provided by Dr. U. Walther (BAZ, Aschersleben). The winter barley cv. 'Vogelsanger Gold' had been used as the *H. vulgare* parent in these two interspecific hybrids. F3 family *BAZ-2113* represents the progeny-tested (*PT*) offspring of the heterozygous resistant genotype *BAZ-1282/85*.

Resistance Tests

Soil-borne virus

For virus-resistance tests, plants were mechanically inoculated with BaMMV in the greenhouse. For segregation analyses, all plants were assessed by DAS-ELISA according to PROESELER (1993). For progeny testing, plants were selfed and 15-20 offspring individuals per selfed parent were evaluated after inoculation with BaMMV. Field tests in different contamination areas were performed as described by RUGE *et al.* (2003).

Leaf rust

In situ testing for leaf-rust resistance was performed in detached-leaf tests according to a protocol utilised for leaf rust in rye (WEHLING *et al.* 2003). The inoculation was performed using air-pressure driven infection towers with 20 mg uredospores/shot for inoculation of

1200 leaf segments. Three single-pustule isolates, showing different levels of virulence, were used for genotyping every plant of the mapping families. Plants were scored on a scale from 1-6 according to FRAUENSTEIN and REICHEL (1978), with infection types (IT) of 1 through 4 indicating resistance and IT of 5 and 6 denoting susceptibility. For progeny tests, plants with a given IT score were selfed and 15-20 offspring individuals were evaluated in detached-leaf tests for their reaction to leaf rust.

Powdery mildew

Testing and genotyping of mapping populations were performed as described for leaf rust. Five different powdery-mildew isolates (*19*, *75*, *90*, *106*, *108*) which differ in their level of virulence were used. Scoring of genotypes was assessed according to JAHOOR (1986) with ITs of 0 through 2 indicating resistance and ITs 3 and 4 denoting susceptibility. Progeny tests were done as described above.

Marker Analysis

For RFLP genotyping, total plant DNA was isolated as described by WILKIE (1989). Genomic DNA was digested with *Dra*I, *Xba*I, or *Hin*dIII, electrophoresed on 0.8% agarose gels and transferred onto nylon membranes (Roche) by capillary blotting (SAMBROOK *et al.* 1989). Labeling of the probes and non-radioactive Southern hybridizations were carried out according to the protocols of the manufacturer (Roche). Marker analysis with RFLP probes positioned in the barley consensus map (QI et al. 1996) and located on chromosomes 6H, 2H and 5H, respectively, comprised MWG clones from barley (GRANER *et al.* 1991) as well as PSR clones from wheat (GALE *et al.* 1995). The STS marker *Xiac500* was developed from a differential cDNA-AFLP sequence (RUGE *et al.* 2003). *Xiac504* (Ruge, unpublished) was obtained using primers based on leucine-rich repeat regions of the *Xa21* gene in rice (CHEN *et al*. 1998). For PCR of STS markers, 50-100 ng of genomic DNA was used in a solution containing 1 x reaction buffer (Qiagen), 200µM dNTPs, 5 pmol primers and 0.5 U of *Taq* DNA polymerase (Qiagen). PCR products were separated either on 1% agarose gels or 10 % polyacrylamide gels followed by ethidium bromide and silver staining (BUDOWLE *et al.* 1991), respectively.

Mapping

Except of family *BAZ-4034* linkage analysis was performed by using the data obtained by progeny tests, which allowed to identify all the three genotypes at a segregating resistance locus (*PT* segregation data in Table 1). Mapping was accomplished with JoinMap (VAN OOIJEN and VOORRIPS 2001). Where applicable (see above), recombination values were calculated based on the model of codominance and converted into genetic map distances (cM) by use of the Kosambi function.

Results and Discussion

Inheritance of Resistances Caused by H. bulbosum Introgressions on 2HS

Resistance tests in family *BAZ-3026* performed in respect to leaf rust and powdery-mildew revealed segregation of a dominantly inherited resistance factor, but were not consistent with a 3:1 segregation. The *PT* data suggested gametic selection against the *H. bulbosum* introgression as the cause of the deviation (Table 1). PICKERING *et al.* (1998) already described a *H. bulbosum* introgression on 2HS which conferred resistance to both pathogens, but the segregation data obtained with their F2 mapping population did not differ significantly from a 3:1 segregation. On account of the different *H. bulbosum* parent used as resistance donor in *BAZ-3026*, the 2HS introgression in this family may be of different size and/or genomic organisation as compared to that described by PICKERING *et al.* (1998). The occurrence of resistant offspring in *BAZ-3026* demonstrates that despite the strong gametic selection, the resistance trait may be separated from the selecting factor via recombination to produce

homozygous resistant introgression lines for breeding purposes. The resistance genes introgressed into family *BAZ-3026* and effective toward leaf rust and powdery mildew, respectively, were designated *Rph20*^{Hb} and *Ml*^{Hb}, respectively. These genes could be separated in family *BAZ-3026PT* where one recombinant plant was observed between leaf-rust and powdery-mildew resistance (Table 1, Fig. 1).

Inheritance of Resistances Causes by H. bulbosum Introgressions on 2HL

F5 family *BAZ-4034* was mechanically inoculated with BaMMV and revealed a 3:1 segregation of resistant vs susceptible genotypes (Table 1). Besides *Rym14*^{Hb} which had been mapped to chromosome 6HS (RUGE *et al.* 2003), the resistance factor on 2HL represents the second dominantly inherited soil-borne virus resistance gene derived from the secondary gene pool. We designate this gene as *Rym16*^{Hb}. Progeny tests of 46 F6 individuals of family *BAZ-4034* revealed pronounced deviation from the expected 1:2:1 ratio, the type of deviation suggesting that zygotic selection had taken place against homozygosity of the introgressed *H. bulbosum* segment (Table 1). Resistance tests without further progeny testing of additional F5 families that carried the same introgression displayed segregations deviating towards a 2:1 ratio of resistant vs susceptible plants (not shown), which is expected with linkage of the resistance with a recessive letality factor. Although the segregation of mapping population *BAZ-4034* (Table 1) fitted a 3:1 ratio of resistant to susceptible plants, this data is also still consistent with a ratio skewed towards 2:1 which was observed in the F5 families mentioned above. The orginal size of the introgression present in *BAZ-4034* could be reduced via recombination. This reduction in size was traced with molecular markers (not shown) and led to the elimination of the zygotic-selection factor, resulting in a non-disturbed 3:1 segregation ratio in mapping family *BAZ-5061* (Table 1).

Family *BAZ-1282* also carried a 2HL introgression, but was obtained from a different source. This family segregated for leaf-rust resistance in a 3:1 and 1:2:1 ratio, respectively (Table 1). The resistance factor was given the designation *Rph21*^{Hb}. In family *BAZ-2113*, 91 individuals were inoculated both in situ with leaf rust and in planta with BaMMV by mechanical inoculation. Of the 91 plants, 64 and 23 proved to be resistant and susceptible, respectively, to both pathogens. Four individuals were identified to be resistant to leaf rust but susceptible to BaMMV (Table 1).

The appearance of recombination between the two resistance loci suggests a relatively small linkage drag within the *H. bulbosum* introgression. This assumption could be confirmed by molecular markers which flank the resistance loci. The RFLP anchor markers *MWG2076* and *MWG949* span a distance of approximately 5.5 cM in the barley consensus map (QI et al. 1996). In the mapping families *BAZ-4034* and *BAZ-1282* where recombination takes place between barley and *H. bulbosum* chromatin, these markers were found to map 4.2 cM and 7.7 cM, respectively, away from each other (Fig. 1).

At present, identity of the two leaf-rust resistance genes in families *BAZ-3026* and *BAZ-1282* in relation the known genes *Rph17* and *Rph18*, respectively, remains uncertain. *Rph17* was reported to cosegregate with marker *MWG682* among 82 individuals (PICKERING *et al.* 1998.). In contrast, the *Rph* gene in family *BAZ-3026* was found to recombine with this marker among 62 individuals, corresponding to a genetic distance of 0.9 cM (Fig. 1). Concerning the *Rph18* gene on chromosome 2HL, RFLP analysis with individual and pooled samples from the BC2F2 population confirmed cosegregation with *MWG949* (PICKERING *et al.* 2000), while the same marker mapped 6.7 cM away from leaf-rust resistance among the 105 individuals of family *BAZ-1282* (Fig. 1). These differing mapping data may indicate that different resistance genes or gene complexes were introgressed on the same barley chromosomes. They may as well be due, though, to differing recombination activities in the

Table 1. Inheritance and location on barley chromosomes of different disease-resistance genes introgressed from *H. bulbosum*

Family	N	Resistance	Segregation res. vs susc.	$Chi^2_{3:1}$	Segregation (1:2:1)	$Chi^2_{1:2:1}$	Loc.	Resistance gene
BAZ-4034	69	BaMMV	52:17	0.005	-	-	2HL	*Rym16*^{Hb}
4034 PT [a]	46	BaMMV	-	-	3:34:9	12.08	2HL	*Rym16*^{Hb}
BAZ-5061	99	BaMMV	72:27	0.27	-	-	2HL	*Rym16*^{Hb}
BAZ-1282	105	LR[b]	76:29	0.39	-	-	2HL	*Rph21*^{Hb}
1282 PT [a]	85	LR	-	-	16:46:23	1.72		*Rph21*^{Hb}
BAZ-2113	91	BaMMV LR	64:27 68:23	1.07 0.003	- -	- -	2HL	*Rym*^{Hb} *Rph21*^{Hb}
BAZ-3026	78	LR, PM[c]	46:32	10.68 2.51 [d]	-	-	2HS	*Rph20*^{Hb} *Ml*^{Hb}
3026 PT [a]	62	LR PM	- -	- -	3:28:31 2:29:31	25.87 0.00 [d] 26.96 0.00 [d]	2HS	*Rph20*^{Hb} *Ml*^{Hb}
BAZ-1283	105	LR	75:30	0.72	-	-	5HL	*Rph22*^{Hb}
1283 PT [a]	93	LR	-	-	18:47:28	2.16		*Rph22*^{Hb}

[a] *PT*, progeny test
[b] LR, leaf rust
[c] PM, powdery mildew
[d] $Chi^2_{1:1}$

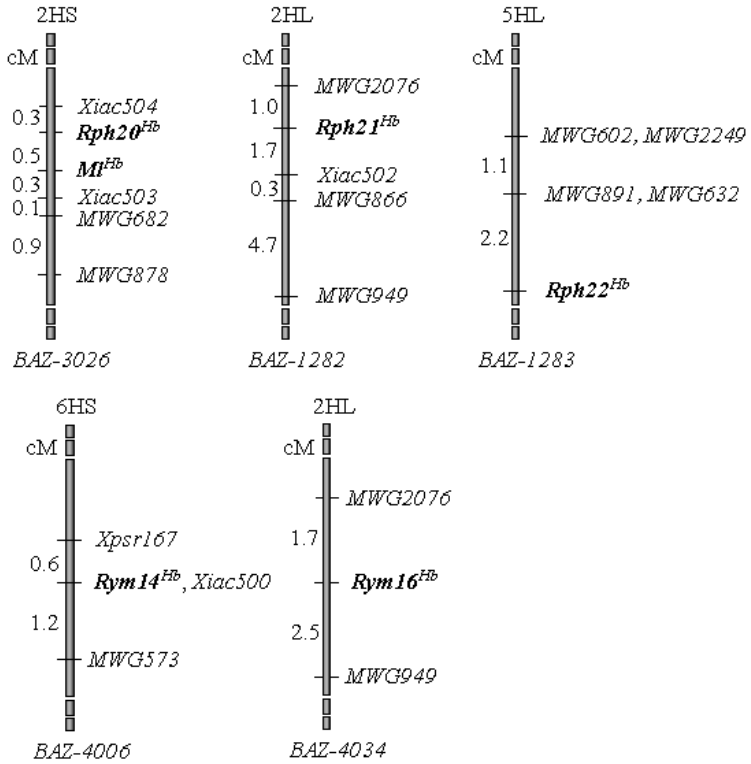

Fig 1. Mapping of different resistances genes in barley derived from the secondary genepool

introgressed genome segments, in dependence on their different origins and sizes. For the reasons noted, the designations *Rph20*^{Hb} and *Rph21*^{Hb} (Fig. 1) of the two leaf-rust resistance genes mapped in families *BAZ-3026* and *BAZ-1282*, respectively, should be taken as preliminary. The relationships of leaf-rust resistance genes *Rph20* and *Rph21* vs *Rph17* and *Rph18*, respectively, may be clarified by determining their effectiveness toward a variety of leaf-rust isolates.

Inheritance of Resistances Caused by H. bulbosum Introgressions on 5HL
Besides the leaf-rust resistances located on the distal regions of chromosomes 2HS and 2HL, a *H. bulbosum* introgression conferring leaf-rust resistance was identified on chromosome 5HL.

The resistance was inherited as a single dominant factor, which was corroborated by the 1:2:1 ratio obtained with the progeny test (Table 1). This resistance factor was designated *Rph22*^{Hb}.

Mapping of Rph20^{Hb} *and Ml*^{Hb} *on Barley Chromosome 2HS*
The introgressed resistance loci *Rph20*^{Hb} and *Ml*^{Hb} proved to be tightly linked to *MWG682*, the latter of which is located on the short arm of chromosome 2H (QI *et al.* 1996). As a second, more closely linked proximal marker *Xiac503* was obtained by use of PCR primers which have been reported for converting the RFLP marker *MWG878* into a STS marker (http://www.graingenes.org). In our hands, though, *Xiac503* displayed recombination with *MWG878* and mapped 1.2 cM away from it (Fig. 1). On the opposite side of the resistance locus, *Xiac504* could be mapped with a genetic distance of 0.6 cM (Fig. 1). This RGA marker was derived from a conserved region (XLLR) of the rice resistance gene *Xa21* (CHEN *et al.* 1998).

Mapping of Rym16^{Hb} *and Rph21*^{Hb} *on Barley Chromosome 2HL*
Resistance conferred by *Rym16*^{Hb} in family *BAZ-4034* was found to be flanked distally by the RFLP marker *MWG949* and proximally by the STS marker *MWG2076* (Fig. 1). Primers of *Xiac502* were designed from a partial sequence of *MWG866* (AJ235601, GrainGenes). With a distance of 0.3 cM to resistance loci in family *BAZ-1282* this marker represents an efficient tool for marker-assisted selection.

Mapping of Rph22^{Hb} *on Barley Chromosome 5HL*
Due to its linkage to anchor markers, *Rph22*^{Hb} was assigned to the long arm of chromosome 5H (Fig. 1). All markers mapped proximal to *Rph22*^{Hb}.

Molecular Markers Located on H. bulbosum Introgression 6HS
Molecular-marker development for *Rym14*^{Hb} yielded a PCR marker (*Xiac500*) that was derived from a cDNA-AFLP analysis and is cosegregating with the resistance (Fig. 1). *Xiac500* has been already integrated in practical breeding programmes.

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Fungicidal Effect of Plant Extracts from Some Medicinal Plants against Powdery Mildew on Barley *(Blumeria graminis* f. sp. *hordei)*

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Abstract

Plant extracts, alone or in mixture with olive oil, from 17 species of medicinal plants, already known for their fungicidal activity in *in vitro* assays were tested for their effect against powdery mildew *(Blumeria graminis f.sp. hordei)* under glasshouse conditions. All treatments created from plant extracts showed statistically significant suppression of powdery mildew development.

Plant extracts of *Abies balsamea* (L) Mill (ABBA) showed the highest fungicidal effect. The treatment was effective under all concentrations tested, especially in mixture with plant oil. Other effective treatments included extract from *Lavandula officinalis Chaix et Kitt*. (LAVAO), *Ptelea trifoliata L.* (PTETRIO) with oil, and *Allium sativum L.* (ALSAT) all in mixture with oil and *Ligusticum changxiong Hort* (LUX).

Thirteen plant extracts from a total of seventeen tested showed increased fungicidal effects when in combination with plant oil.

The highest fungicidal activity was obtained when four plant extracts were combined: *Alium ursinum* (AURS), *Pinus nigra Arnold* (PINIG), *Ligusticum changxiong Hort*. (LUX) and *Schisandra chinensis (Turcz)* – SANDRA. Addition of GREEMAX to this mixture increase the fungicidal effect compared to original extracts as well as when in mixture with plant oil.

Keywords: barley; powdery mildew; plant extracts; fungicidal effect; GREEMAX

Introduction

Protection of plants against fungal diseases can be achieved by different means. Currently, chemical protection is most common. Synthetically produced fungicides are highly effective at relatively low rates, and when correctly used can achieve profitable results. However many of them are toxic for people and animals (SINCLAIR & PRESSINGER 2002).

A possible solution to this problem is the use of biological protection. The most important method is breeding for resistance, which is, however a long term process and the final resistance is not durable in all cases. Another possibility is the use of biopreparates. Fungicidal activity may be found in natural materials like the cultures of microorganisms (fungi and bacteria), extracts of animal origin and plant extracts. Their advantage is usually low toxicity and low prices for their production.

This paper is based on results concerning fungicidal effects of plant extracts which have been assessed in *in vitro* conditions (TVARŮŽKOVÁ 2003). I selected highly effective plant extracts and used them on intact plants.

Fungicidal effects of plant extracts against powdery mildew in barley *(Blumeria graminis* f. sp. *hordei)* were studied under glasshouse conditions. Extracts were tested in different concentrations and combinations with other extracts or with some additional compounds.

Material and Methods

Extraction

50 g of fresh or frozen plant material was cut into small pieces (equal to 25 g of dry plants in powder form) and then homogenized by laboratory blender in 100 ml of 96 % ethanol. The mixture was then placed in Erlenmeyer flasks and placed on a laboratory shaker for 8 hours. The final extract was filtered after 24 hours of extraction process and stored in refrigerator at 5°C.

Glasshouse Experiment

Establishment of Experiment

Spring barley variety Tolar, extremly susceptible to powdery mildew was used. For each treatment there were 4 replications with five plants per container. Barley plants were grown until they reached the two leaf stage.

Plant Treatments

In the first step plant extracts with demonstrated fungicidal activity against *Microdochium nivale /Fr.Samuels, I.C.Halle/t* in *in vitro* cultures were tested (TVARŮŽKOVÁ 2003): balsam fir */Abies balsamea (L.) Mill./*, lesser stitchwort */Stellaria graminea L./*, hoptree */Ptelea trifoliata L./*, garlic */Allium sativum L./*, common walnut /*Juglans regia L./*, lavender */Lavandula officinalis CHAIX et KITT./*, northern white cedar */Thuja occidentalis L./*, tasmanian blue gum */Eucalyptus globulus Labill /*, thyme */Thymus serpyllum L./*, mugwort */Artemisia vulgaris L./*, austrian pine */Pinus nigra Arnold/*, Szechuan lovage root */Ligusticum chanxiong Hort/*, schisandra */Schisandra chinensis(Turcz) Bail/* and chinese sumac */Rhus chinensis Mill/*. Extracts were named based on abbreviations of the latin name of the original plant species (Table 1).

Extracts were tested in the following concentrations: 2.5%, 10%, 20% and mixtures of extract 20% + GREEMAX(G) and extract 20% + olive oil (O). Colloidal concentrate GREEMAX (Stallen Company, Switzerland) was used to improve penetration, translocation and utilization of the extracts at the recommended rate of 40ml/ha. The second part of the experiment was to continue use of those extracts with high fungicidal activity against powdery mildew discovered in part one. In addition, two new plant extracts were prepared from henna *(Lawsonia innermis L.)* originated in North Africa and wild species of *Mentha spp*. collected in Zakynthos Island (Greece).

Wild garlic */Alllium ursinum* L./, balsam fir */Abies balsamea (L.) Mill./*, austrian pine */Pinus nigra Arnold/*, Szechuan lovage root */Ligusticum chanxiong Hort/* and schisandra */Schisandra chinensis(Turcz) Bail/* were tested in monocomponent extracts in concentration 2,5%, 10%, 20%, 20% + GREEMAX and 20% + olive oil, in two-component and in four-component mixtures of extracts in final concentration 20 % (10%+10%, and 5% + 5% + 5% + 5%, respectively). The list of experimental treatments is summarized in Table 2.

10 ml of extract solution was sprayed per treatment and replication with a hand sprayer. After treatment containers were left until plant surfaces were dry and then inoculated with *B. graminis* f.sp. *hordei* by shaking from above severely infected plants with developed conidia of powdery mildew. Temperature was maintained between 15 - 20°C for following 10 days. In both parts of the experiment the plants treated with water were used as controls.

Assessment

A standardized scale was used to estimate infected leaf area: 0%, 1%, 5%, 10%, 25% and 50% (ANONYMOUS 1997). Each treatment was assessed in 4 replications with 5 plants per replication. Results were compared using ANOVA.

Results

Practically all extracts and their combinations showed statistically significant supression of powdery mildew development (Table 3,4). The lowest infection rate was found in the first part of experiment after the treatments with ABBA 20%+O (0.67%) and STEGR 20%+O (1.0%). Between 5 and 10% of infection level were LAVAO 20%+O, ABBA 2,5%, PTETRIO 20%+O, ABBA 10%, ALSAT 20%+O, LUX 20% and ABBA 20%. On the contrary the highest infection over 40 % were found after LAVAO 20%+G, THYM 20%, PINIG 2.5%, AURS 2.5%, PTETRIO 2.5%, EUCA 20% and JUREG 20%+G (50.0%). The results are summarized in Figures 1 – 3.

The lowest powdery mildew infection in the second part of the experiment was found after the following treatments: AURS+PINIG+LUX+SANDRA+G (4.17%), AURS+PINIG +LUX +SANDRA+O (6.08%), AURS+ABBA+LUX+SANDRA (6.33%), AURS+ABBA +PINIG+LUX (6.67%), AUARS+HENNA+MINT+SANDRA (6.92%), LUX 20% (7.50%), AURS+PINIG+LUX+SANDRA (7.58%), AURS+HENNA +MINT+PINIG (9.17%), ABBA 20% (10.00%) (Fig. 4). Infection rate of non-treated checked plants reached 47.92%.

Discussion

The plant extract originating from balsam fir needles showed the highest fungicidal activity against powdery mildew on barley in glass-house experiments. The treatment was highly effective across all concentrations used, but the best result was achieved when the extract was mixed with oil. The combination with GREEMAX was moderately effective only. Extract from balsam fir is known as Canada terpentine which is composed of 23 to 24 % volatile oils, 48 to 50 % alpha and beta canadinolic acids and 11 to 12% resene (Kaufman et al., 1999). The fungicidal active compounds from balsam fir may explain the suppression of *B. graminis* f.sp *hordei* when sprayed on the barley leaf surface. This effect was increased with addition of oil which helped adherence of the fungicidal compounds on the leaf surface. On the contrary GREEMAX which helps to increase penetration of compounds into the leaf tissue may have removed them from the place of maximal utilization.

Thirteen plant extracts from total number of seventeen tested showed an increase of fungicidal effectivity against powdery mildew in combination with plant oil. The effect of oil as an additive to fungicidal products is commonly known and currently used in production of chemical preparates (EC formulation). QUARLES (2000) describes enhanced fungicidal effects of treatments containing different kinds of oils. KREUTER (2000) described bioporeparates effective against fungal diseases of plants, which are based on combinations with natural oils: „Mehltau-Kombipack“ used against mildew or „Neudo-Vital“ which increases resistance of strawbery against *Botrytis cynerea* and rose plants against mildew, rust and black spot disease.

The effectivness of treatment was significantly better in combination with GREEMAX in 6 experimental treatments. The most significant difference between GREEMAX and GREEMAX free treatment was found for extract from *Eucalyptus globulus* Labill., (EUCA). A similar high reduction of powdery mildew was also found in mixture of EUCA with oil.

Tasmanian blue gum leaves are a traditional Aboriginal herbal remedy. The essential oil found in the leaves is a powerful antiseptic. The most important constituent is eucalyptol, present in up to 70 per cent of its volume. It consists chiefly of a terpene and a cymene (LEYEL 1931). Eucalyptus oil contains also, after exposure to the air, a crystallizable resin, derived from eucalyptol. Some active compounds have a strong penetration effect and therefore it can be concluded that EUCA extract showed both contact fungicidal influence on the leaf surface and internal fungicidal activity inside the leaf tissue.

A mixture of 4 extracts - AURS+PINIG+LUX+SANDRA was the most effective experimental treatment. *Schisandra chinensis (Turcz)* (SANDRA) powder prepared from dry

fruits contains lignins and essential oils (LI 1991). The fungicidal effect of extract from *Alium ursinum L*. (AURS) is based on sulphur essence. The active compounds of *Ligusticum chanxiong Hort.* (LUX) are alkaloids, tetramethylpyrazine and phenols (SHAO *et al.* 1994). The composition of *Pinus nigra* (PINIG) extract is based on fixed or volatile oils, and solution of potassium or sodium hydrate. There are many compounds in this mixture which have various biochemical and physiological properties. Classic Chinese herbal prescriptions also include between five and ten herbs per formula and contain hundreds of potentially active ingredients (BENSKY & GAMBLE 1993). They become difficult to evaluate using the Western pharmacological model of analyzing a solitary agent for a specific effect. It is therefore necessary to perform wide screening of possible extract combinations and to increase the probability of creating a combination with optimal influence on plant diseases.

The use of natural compounds for treatment of plant diseases protects the environment and is safe for human health. Every year new findings concerning the succesfull utilization of new plant extracts are published.

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Table 1. List of plant species common and scientific names and the names of plant extracts

common name	scientific name	extract name
balsam fir	*Abies balsamea (L.) Mill.*	ABBA
stitchwort	*Stellaria graminea L.*	STEGR
hoptree	*Ptelea trifoliata L.*	PTETRIO
garlic	*Allium sativum L.*	ALSAT
common walnut	*Juglans regia L.*	JUREG
lavender	*Lavandula officinalis CHAIX et KITT.*	LAVAO
Northern white cedar	*Thuja ocidentalis L.*	TOCIDENT
tasmanian blue gum	*Eucalyptus globulus Labill.*	EUCA
thym	*Thymus serpyllum L.*	THYM
mugwort	*Artemisia vulgaris L.*	MISIA
Austrian pine	*Pinus nigra Arnold*	PINIG
Szechuan lovage root	*Ligusticum chanxiong Hort.*	LUX
schisandra	*Schisandra chinensis (Turcz)*	SANDRA
Chinese sumac	*Rhus chinensis Mill.*	RUS
wild garlic	*Allium ursinum L.*	AURS
henna	*Lawsonia innermis L.*	HENNA
mentha	*Mentha spp.*	ZAKYNTH

Chemical Induced Resistance (CIR) and Detection of QTL for Resistance against *Rhynchosporium secalis* in Barley (*Hordeum vulgare* L.)

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Abstract

Scald, caused be the fungus *Rhynchosporium secalis* (Oud.) J.J. Davis, is an important disease of barley (*Hordeum vulgare* L.) world wide. Various major resistance genes against scald are known but have been overcome, already. Another approach to reduce yield losses caused by *R. secalis* may be the use of chemical Induced Resistance (cIR) in combination with R-genes. Therefore, studies were carried out in order to identify QTL for the effectiveness of cIR against *R. secalis*. Sixty doubled haploid (DH) lines derived from the cross ´Igri´ (*rh*; 2-row) x ´Triton´ (*Rh*; 6-row) were analysed in pot and field experiments for quantitative scald resistance after artificial infection with a mixture of pathotypes virulent on *Rh* in three variants, i.e. healthy control, artificially infected, dichloroisonicotinic acid (DCINA), jasmonic acid (JA) and acetyl-salicyl-methylesther (ASM) treated and artificially infected, too. Regarding the effects of treatment with the chemical inducers DCINA, JA and ASM a genotype specific response was observed but could not be reproduced in different experiments due to environmental factors. Consequently, no QTL for this trait could be identified based on these phenotypic data and a skeleton map of 163 RAPD, AFLP and SSR markers comprising 1,344 cM. However, for *R. secalis* resistance 6 QTL could be detected on chromosomes 1H, 3H and 7H. Further studies now aim at confirming these QTL by enlargement of the mapping population to about 120 DH-lines. Based on these data cDNA-AFLP using RNA isolated during early stages of infection, i.e. forming of papillae and development of subcuticular hyphae, will be carried out on lines with positive and negative alleles at respective QTL in order to identify differentiating fragments which will be sequenced and remapped. By this approach ESTs involved in *R. secalis* resistance will identified and mapped.

Introduction

Scald or leaf blotch, caused by *Rhynchosporium secalis*, is an important disease of barley throughout the world. One approach to control scald is the use of resistance genes. In previous studies of different sources of resistance fourteen major genes conferring race-specific resistance to *R. secalis* have been identified (PENNER *et al.* 1996; JENSEN *et al.* 2002), and some of them were incorporated into barley cultivars.

Unfortunately, different resistance genes, initially found to be effective under field conditions, have been overcome due to the highly variable nature of *R. secalis*. The loss of sources for effective qualitative resistance against scald increases the importance of quantitative resistance in breeding programmes and the development of molecular markers for quantitative trait loci (QTL, THOMAS *et al*. 1995). QTL for scald resistance were reported by BACKES *et al.* (1995) on barley chromosomes 1 (7H), 3 (3H), 6 (6H) and 7 (5H), by Thomas *et al*. (1995) on chromosome 3 (3H), by SPANER *et al*. (1998) and JENSEN *et al*. (2002) on chromosomes 3 (3H), 4 (4H) and 6 (6H). However, not much is known about the genes involved in this quantitative resistance.

Another approach to control *R. secalis* is the use of chemical Induced Resistance (cIR), i.e. to strengthen the resistance potential of plants by the application of chemical inducers prior to

disease infestation. Our objective was to map QTL contributing to scald resistance and to the effectiveness of cIR against scald in barley. For this purpose, simple-sequence repeats (SSRs) were used as framework markers, and randomly amplified polymorphic DNAs (RAPDs) and amplified fragment length polymorphisms (AFLPs) to construct a linkage map as a prerequisite for the detection and localization of QTL. Furthermore, the detection and mapping of expressed sequence tags (ESTs) related to resistance of barley against scald should facilitate more efficient marker assisted selection (MAS) procedures by providing markers more closely linked to respective QTL.

Inducable Resistance and the Variability of Scald Resistance

The general effect of the chemical inducers DCINA, JA and ASM on scald infestation was tested in greenhouse studies with the highly susceptible cv. ´Intro´ using PTA-ELISA. Effects on scald infestation were found for each chemical inducer but observed to be highly variable between replications (Fig.1).

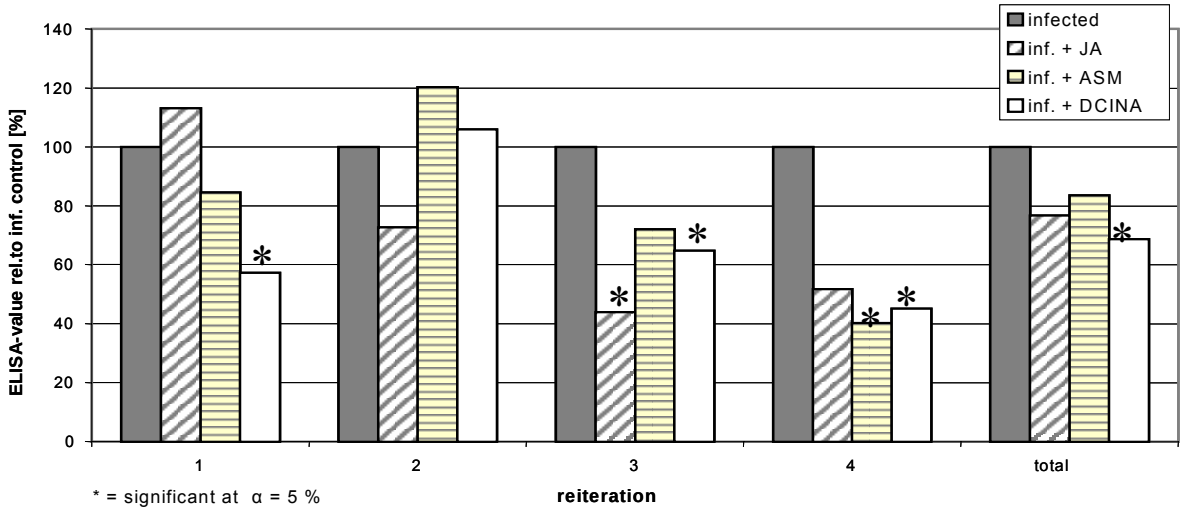

Figure l. ELISA-values relatively to infected control after treatment with ASM, DCINA and JA, each, 3 weeks after inoculation of cv. ´Intro´with *R. secalis*

In order to obtain a first hint on genotypic differences after treatment with one chemical resistance inducer a set of 60 DH lines derived from the F_1 of the cross ´Igri´ (2-row) x ´Triton´ (6-row) segregating regarding the resistance gene *Rh* were investigated. In greenhouse studies genotypic differences regarding the effect of DCINA were measured by PTA-ELISA showing a reduction of the ELISA-value for 70% of the genotypes whereas for 30% of them the ELISA values were increased by DCINA treatment (Fig. 2).

In order to check the general inducibility and any coherences between resistance gene and inducibility all DH lines were proven to be inducible in Northern analysis and RT-PCR with the cIR specific markers *Bci4* and *LOX:2Hv1*, whereas *Bci4* was shown to be more suitable

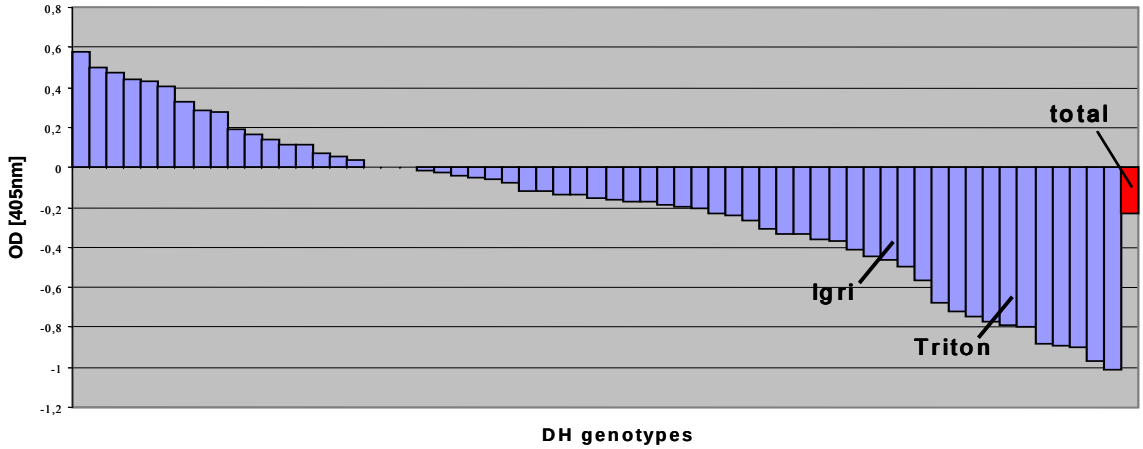

Figure 2. Differences regarding ELISA-values between DCINA-treated and untreated plants of the same genotypes two weeks after artifical inoculation

for inducibility because RT-PCR with *LOX:2Hv1* revealed a fragment in the control too, indicating constitutive expression of *LOX:2Hv1* (Fig. 3, 4). Furtheron, there were not obvious relations between inducibility and presence of the resistance gene.

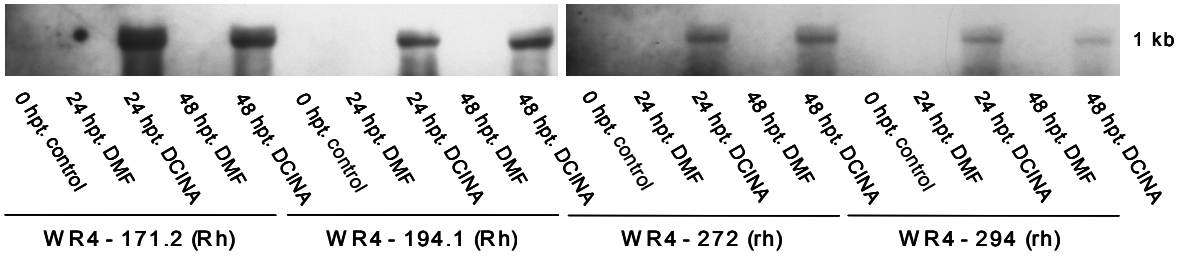

Figure 3. Differences in the expression level of *Bci*-4 proven by Northern-analyis directly, 24-and 48 h after DCINA and DMF application, respectively

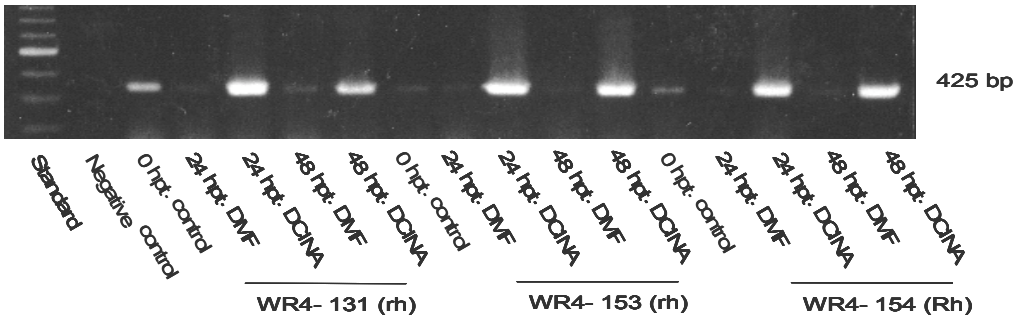

Figure 4. Gene expression evidence of *LOX*:2*Hv*1 after DCINA-application of selected DH lines

In 2000 and 2001 the DH-population was assessed for scald resistance and the effect of treatment with the chemical inducer ASM in Mitscherlich pot experiments as well as in the field (only 2001). The percentage leaf area diseased (PLAD, CSELENYI *et al*. 1997) was

estimated at two stages (EC31, EC51) to determine the level of resistance of parents and DH lines assessed on infected, infected plus ASM-treated and non-infected controls. In pot experiments as well as in the field a quantitative distribution of scald resistance was observed. Regarding the effect of ASM on *R. secalis* the genotypes responded highly variable to the treatment within the experiments so that a positive effect of treatment on scald infestation was found, but it seems to be dependent on unknown environmental factors. Moreover, phytotoxic effects of ASM resulting in yield reduction were often observed to be stronger than the positive effect of reducing scald infestation.

QTL Mapping of Scald Resistance and the Influence of Chemical Inducers

The lines were genotyped with a total of 163 markers including 28 SSRs (LIU *et al.* 1996; RAMSAY *et al*. 2000; PILLEN *et al*. 2000), 30 RAPDs and 95 AFLPs. A sequence tagged site marker (cMWG680, GRANER & TEKAUTZ 1993) regarding *Rhynchosporium* resistance (*Rh*) and one morphological marker (spike rows) were also scored. The genetic map was constructed using MAPMAKER (LANDER *et al.* 1987) employing a procedure described by SCHÄFER-PREGL *et al.* (1999) using Kosambi mapping function. Two-point analysis LOD and multipoint analysis LOD was preset 3.0. SSRs with known chromosomal location were used as anchor markers to assign linkage groups to chromosomes. All mapped markers were tested for the expected 1:1 segregation ratio using χ^2 goodness of fit test. The resulting skeleton map comprises a length of 1,344 cM with an average marker distance of 8.6 cM. QTL analysis of scald resistance was performed as a composite interval analysis (CIM) using the software package PLABQTL 1.0. For detection of putative QTL a LOD threshold of 3.0 was chosen for declaring significance corresponding to a significance of 0.05 assuming a χ^2 distribution for the test statistic.

Due to the highly variable reactions of the genotypes towards a treatment with chemical inducers no QTL-analysis could be performed, because the phenotypic data did not show unique effect over the three experiments for each DH line. At both stages (EC31 and EC51) of estimating the PLAD genotypes were found reacting with an increase or decrease of disease, respectively. These results emphasize the complexiticity of this trait and its highly variable nature.

In a QTL analysis for quantitative resistance against *R. secalis* (Tab. 1) in the seedling stage (EC31) of the two pot experiments two QTL were identified both having its positive allele from the resistant cv. ´Triton´ explaining 20% of the total phenotypic variance. One QTL localized on chromosome 3H in the region of the *Rh*-locus showed the strongest additive effect (7.5%) explaining about 18% of the phenotypic variance. This result confirms the findings of other studies where QTL for resistances to *R. secalis* were localized in regions of qualitative resistance genes already being overcome (BACKES *et al.* 1995). The other QTL was localized on chromosome 7H explaining 8.7% of the phenotypic variance. QTL analysis for all three environments revealed a third QTL expressed in the seedling stage (EC31) on the long arm of chromosome 3H explaining 32.7% of the phenotypic variance and having its positive allele from the resistant cv. ´Triton´. The QTL analysis for resistance against scald in the adult plant stage (EC51) revealed three QTL explaining 49.5% of the total phenotypic variance. One was localized on chromosome 1H having its positive effect from ´Igri´ explaining 29.8% of the phenotypic variance. The other QTL were localized on chromosome 3H, one explaining 37.6% of the phenotypic variance having its positive allele from ´Triton´ and the other one explaining 21.0% of the phenotypic variance having its positive allele from ´Igri´. Verification of these QTL by enlargement of the mapping population to about 120 DH lines is still in progress.

Table 1. QTL of infested leave surface in EC31 and EC51 respectively after *Rhynchosporium* infection

Chromosome	Marker-Intervall	Position [cM]	LOD-value	$\sigma^2_{part\ P}$	Additive-Effect [%]	Positive Allel from
QTL of 2 environments regarding infested leave surface EC31						
3HL	E3347350 - E3148160	78	3.57	17.9	7.5	´Triton´
7HL	E3847350 - R16_1290	216	3.09	8.7	7.0	´Triton´
Total				20.2		
QTL of 3 environments regarding infested leave surface EC31						
3HL	HvITR1 - E3149260	5	4.98	32.7	6.9	´Triton´
QTL of 3 environments regarding infested leave surface EC51						
1HS	E3451320 - E3449255	52	6.30	29.8	4.0	´Igri´
3HL	E3151210 - E3347500	139	5.35	37.6	6.6	´Triton´
3HL	Q141280 - E4349565	57	4.98	21.0	6.6	´Igri´
Total				49.5		

Detection and Mapping of *R. secalis* Resistance Related Allelspecific ESTs

Resistance associated allele specific ESTs (Expressed Sequence Tags) should be detected by two different strategies. One is to identify differentially expressed genes by cDNA-AFLP analysis based on RNA isolation of artificially infected DH selected due to possessing the *Rh* gene and different QTL. The second strategy comprises the use of known resistance-related ESTs mainly published in public databases (IPK-Gatersleben, NCBI, EMBL) detected from cDNA libraries after artifically infection with *Blumeria graminis*. Up to now, the cDNA-AFLP method is established for detection of ESTs, specifically related to resistance reaction after infestation with *R. secalis*. Furthermore, around 35 ESTs from databases as well as around 60 ESTs from colaboraters are available and will be screened for polymorphismes and allele specific differences for a later ongoing mapping into the enlarged QTL-map.

Conclusions

The QTL identified for resistance against *R. secalis* should facilitate the strengthening of the resistance potential of barley against this pathogen. Especially the one identified for seedling stage resistance (EC31) localized at the *Rh*-resistance locus derived from the resistant cultivar ´Triton´ is of special importance because in other populations this region is reported also to be involved in resistance reactions against scald (BACKES *et al*. 1995; THOMAS *et al.* 1995). Markers flanking this region might be useful for marker-assisted pre-selection for scald resistance in the future. Especially with respect to scald resistance, marker-based selection is of importance, because selection in the field turns out to be difficult due to a lack of infection in years without favourable conditions for the pathogen. Furthermore, artificial infection is difficult to be handled in practical breeding programmes. Therefore, a combination of different positive alleles for scald resistance using molecular markers may be useful for strengthening the resistance potential of barley against this pathogen. Work being undertaken with more genotypes as well as the detection and mapping of resistance related ESTs will be performed to verify the resistance QTL localized in this study and to identify more specific markers.

Acknowledgements
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Resistance to Scald Identified Using Differential Isolates

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Abstract
The presence of specific scald resistance genes is being identified through the use of single spore derived differential isolates of *Rhynchosporium secalis*. Using controlled growth conditions and stable isolates, seedling tests have been found to be more reliable than previous reports have suggested. Specific isolates are being repeatably used to detect the presence of two of the most widely distributed genes, *Rrs1* and *Rrs2,* either separately or in combination. Adult plant resistance genes cannot be detected in our tests where these genes occur singly, but evidence is being collected which suggests that using certain isolates and in combination with other resistance genes they can be identified in seedling plants.

Introduction
The genetic control of resistance to scald in barley has always been problematic. The published literature has provided some contradictory evidence for the presence of specific resistance genes in a range of varieties that have been used as international differentials (GOODWIN *et al*. 1990). Considerable discrepancies have also arisen between tests conducted by individual research groups. The reasons for these differences may include the use of different isolates, mixed isolates, different or impure seed lots and the use of different environmental conditions. In addition the fungus is known to be highly variable and the virulence of some isolates may change significantly over time. In this paper we describe a system for the identification of specific resistance genes through the use of a reliable set of differential scald isolates and controlled environment conditions. As an example we investigated the genetic basis of resistance to scald in the variety Gairdner which has been widely used by barley breeders around Australia as a source of good malting quality, high yield and resistance to scald.

Used in conjunction with DNA marker technology, these isolates also offer a powerful tool for the identification of novel resistances and gene locations. The value of these differential isolates in plant breeding is discussed.

Material and Methods
Seedling Inoculation
Single spore cultures were grown on LBA containing 0.01g/l streptomycin sulphate at 16^0C with a 12 hour photoperiod until the plates were covered in spores (~ 7 days). A spore solution for inoculation was obtained after dilution to 1 x 10^6 spores/ml with sterile RO water. 100ml of this inoculum, with 2 droplets of Tween 20 added, was sprayed on to 60 x 8cm pots each containing 2 seedlings at the 3 leaf stage. 3 replicates of each variety for each isolate were used. These were placed in the dark with 100% humidity in a controlled atmosphere growth room for 24 hours at 16^0 C. The temperature was then maintained at 16^0 C for a 12 hour photoperiod with 100% humidity. First signs of infection were observed after 7 to 10 days and the first scores taken after 11 days. Final scores were obtained 21 days after inoculation.

Scoring Scale
Assessment of resistance is carried out using a four-point scale where S represents leaves where many large lesions are observed. MS leaves show only a few large lesions. MR leaves have only few, small lesions, typically along the leaf margins or on the leaf sheaths. R reactions are where no lesions are observed.

Barley Varieties and Breeding Lines
The internationally used differential varieties Turk, cited to have the gene *Rh3* (DYCK & SCHALLER 1961; ROHE 1995), Atlas, cited to carry *Rh2* (DYCK & SCHALLER 1961; SCHWEIZER *et al*. 1995) and Atlas 46 with both *Rh2* and *Rh3* (DYCK & SCHALLER 1961), have been used to identify specific scald isolates with virulence on the genes *Rh2* and Rh3. The Australian varieties, Forrest, Onslow and Gairdner, which have these differential varieties in their pedigree, were tested to identify which of these two genes they carried. Subsequently Franklin, Keel and breeding lines derived from Keel and Gairdner, have been tested to deduce the presence of *Rh2* in those lines. A collection of 26 breeding lines used by ICARDA (YAHYAOUI *pers. comm*.) as a source of scald resistance were also used to investigate the genetic base of resistance in those lines.

Scald Isolates
Following the isolation and culturing of large number of isolates and testing on a range of international differential and Australian varieties, four isolates have been identified which are stable in culture and have been used to selectively identify the presence of the resistance genes *Rh*2 and *Rh*3. Isolate 102f is virulent on varieties with just *Rh2* such as Atlas. Isolate 8 is virulent on varieties with just *Rh3* such as Turk. Isolate 332a is virulent on varieties with both *Rh*2 and *Rh*3 such as Atlas 46. These interactions are illustrated in Table 1, which includes isolate 6, which is avirulent on both *Rh*2 and *Rh*3.

Results and Discussion
Resistance in Gairdner
Based on an observation of its pedigree it was postulated that Gairdner may have inherited resistance to scald from Atlas 46 via Atlas 57, Forrest and Onslow. The results of screening these parents with four differential isolates (Table 1), has indicated that Forrest, which is derived from a cross between Atlas 57 and a susceptible line, inherited both *Rh*2 and *Rh*3 from Atlas 57 and thereby from Atlas 46. Onslow, which is derived from a cross between Forrest and a susceptible line Aapo only inherited the *Rh*2 gene from Forrest. Gairdner is derived from a cross between a Franklin sib and Onslow and Table I suggests that it inherited *Rh*2 from Onslow. Franklin is a variety that shows good resistance as adult plants (APR) in the field but is almost universally susceptible at the seedling stage.
In these tests, Gairdner gave a resistant reaction to isolate 102f. This could be explained by additive or complementary action between *Rh*2 and the APR gene in Franklin. Further evidence for this suggestion was observed when the reactions of Onslow, Gairdner and Franklin were compared using 31 different isolates (Data not shown). Using these isolates Gairdner was observed to have a higher level of resistance than Onslow to 20 isolates, to have a similar reaction to 10 isolates and in only one case, isolate 20, did Gairdner show reduced resistance compared to Onslow.

Resistance in Breeding Lines Derived from Gairdner and Keel
The variety Keel, like Franklin, has APR when observed in the field despite being almost uniformly susceptible to a wide range of isolates as seedlings under the above defined controlled conditions. Using isolates 8, 6 and 332a on a set of breeding lines derived from a

Table 1. Reaction of three international differential and four Australian barley varieties to four differential isolates of *R. secalis*. The genes in Forrest, Onslow and Gairdner are deduced from the results.

		Isolate			
Variety	Genes	102f (Vr *Rh2*)	6	8 (Vr *Rh3*)	332a (Vr *Rh2, Rh3*)
Atlas	*Rh2*	MS	R	MR	S
Turk	*Rh3*	R	R	S	S
Atlas 46	*Rh2, Rh3*	R	R	MR	S
Forrest	*Rh2, Rh3*	R	R	MR	S
Onslow	*Rh2*	S	R	R	S
Gairdner	*Rh2,* APR_F	R	R	R	S
Franklin	APR_F	S	S	S	S

cross Gairdner/Keel//Gairdner, it was observed that transgressive segregation for resistance was occurring (Table 2). In this test Gairdner reacted with an MS to isolate 332a, as did three breeding lines. It is likely that this reaction is the result of the combined action both of *Rh*2 from Onslow and the APR gene in Franklin. The last four lines in Table 2 all show a resistant reaction. It is our suggestion that this enhanced resistance is due to the combined action of both genes in Gairdner as well as an APR gene from Keel. Similarly it is our suggestion that the resistant reactions of lines WI3584, WI3585 and WI3587 to isolate 8 may be due to the combined actions of the APR genes from Keel and Franklin. A proper test of this hypothesis will be conducted with new doubled populations being prepared from crosses between these varieties.

Table 2. Reaction of ten breeding lines from the cross Gairdner/Keel//Gairdner, to three differential isolates of *R. secalis*.

	Isolate		
Line	8 (Vr *Rh3*)	6	332a (Vr *Rh2, Rh3*)
Gairdner	R	R	MS
Keel	MS	S	S
Onslow	R	R	S
Franklin	S	S	S
WI3584	R	S	S
WI3585	MR	S	S
WI3586	R	R	S
WI3587	R	S	MS
WI3588	R	R	MS
WI3599	R	R	MS
WI3600	R	R	R
WI3601	R	R	R
WI3602	R	R	R
WI3603	R	R	R

ICARDA Resistance Sources

Four differential isolates were used in an investigation into the resistance genes present in 26 lines identified by ICARDA as useful sources of resistance to scald (Table 3).

Table 3. Reaction of 26 resistance sources in the ICARDA program to 4 scald isolates.

	Isolate			
ICARDA Line	6	102F (Vr *Rh2*)	8 (Vr *Rh3*)	332a (Vr *Rh2, Rh3*)
1	R	R	MR	S
2	R	R	S	S
3	R	R	MS	S
4	R	R	R	R
5	R	R	MR	MS
6	R	R	R	R
7	R	R	S	S
8	R	R	R	MS
9	R	R	R	R
10	R	R	MR	MS
11	R	R	S	S
12	R	R	MS	S
13	R	R	S	S
14	R	R	MR	MS
15	R	R	S	S
16	R	R	S	S
17	R	R	S	S
18	R	R	MR	S
19	R	R	MS	MS
20	R	R	MS	S
21	R	R	R	S
22	R	R	R	S
23	R	R	R	S
24	R	R	MS	MS
25	R	R	R	S
26	R	R	MS	S

The resistant responses of the ICARDA lines to isolate 102f suggest that none of these lines has resistance solely from *Rh*2. However, a number of lines that are susceptible to isolate 8 are likely to carry resistance due to *Rh3*. A majority of the lines that are susceptible to isolate 332a but not to isolate 8, most likely possess both the *Rh*2 and *Rh*3 genes. In addition there are likely to be some APR genes present that can be seen to modify the reaction types of some lines to a degree. These will need further investigation.

From Table 3 it can be seen that three lines, ICARDA 4, 6 (pedigree: Arar/Lignee27), and 9 (CI07117-9/Deir Alla 106//Badia/3/Arar) showed a resistant reaction to all four isolates. This indicated that these lines possess one or two genes different to *Rh2* and *Rh3*. However subsequent repeat testing of these lines have given some conflicting results with some tests showing a susceptible reaction to isolates 8 and 332a. Using doubled haploid populations and bulked segregent analysis, the resistance in lines 2, 4 and 9 have been located near to the *Rh3* locus on chromosome 3 (GENGER *et al.* 2003). The result from line 2 supports the hypothesis that this resistance is *Rh3*. The results from lines 4 and 9 suggests that these lines and line 6 either also have *Rh3* or else have genes closely linked to or are alleles of *Rh3*. Testing these lines with an isolate, 385, which has been virulent on all varieties except for

those known to contain *Rh3* has indicated that each of the lines does contain *Rh3* and that the initial resistant reactions recorded may have been brought about by subtle changes in the testing conditions. This is being investigated in further studies.

The results discussed above, whilst incomplete and requiring further confirmation, show the value that can be gained from the systematic use of differential isolates to investigate the genetic basis of resistance to scald. The use made of differential fungal isolates is not new and is very advanced in studying diseases such as the cereal rusts. That it has not been established in scald to date is because of the difficulty in obtaining reliable and repeatable results and because of variability in both the fungus and in its response to environmental variation. The apparent success reported here is most likely due to the use of single spore cultures, the extensive search for stable differential isolates and the availability of growth room conditions that ensure a very repeatable environment for each test.

Preliminary results have indicated that seedling tests can be used to detect APR genes when present in combinations such that together they provide higher levels of resistance. Whether this can be termed additive or complementary gene action is a moot point. Either way it is likely that at least some of the published records of complementary gene action (HABGOOD & HAYES 1971; BAKER & LARTER 1963) may be interpreted as illustrations of adult plant resistance genes being combined. This will need testing.

Evaluation of 26 resistance sources from the ICARDA program suggest that the origin of resistance amongst these lines is very narrow and dependent on the *Rh*3 gene in combination with *Rh*2 and possibly some other minor adult plant resistance genes. It is possible that this reflects a widespread stability of the *Rh*2 and *Rh*3 resistance gene combination and that there is a price in fitness lost by isolates virulent to this gene combination.

Further isolates, virulent on Osiris, La Mesita and other varieties or germplasm with different alleles or genes, are being used to resolve the genetic control of resistance in these lines and to identify the diversity of resistance currently available in barley collections.

The differential isolates described here as well as others are being used to provide a resistance gene identification service to barley breeders around Australia. The service can identify varieties with resistance derived from *Rh2*, *Rh3* and also from genes that are present in La Mesita, Osiris, some *Hordeum spontaneum* sources (GARVIN *et al.* 1997) and some other Australian varieties. The system is particularly useful for identifying gene pyramids, confirming the use of molecular marker screening, and in identifying the level of resistance diversity in breeding programs. With improvements in testing protocols and new screening populations we aim to improve the detection of adult plant resistance and increase the number of genes which can be readily identified in combination with other genes.

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Determining the Genetics of Leaf Scald and Spot Form of Net Blotch Resistance Using Molecular Markers

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Abstract

Leaf scald (caused by *Rhynchosporium secalis*) and spot form of net blotch (SFNB) (caused by *Pyrenophora teres* f. *maculata*) are economically damaging foliar diseases of barley. Our objective has been to identify, and tag with molecular markers, loci providing resistance to these diseases. Genetic mapping and bulked-segregant analysis of doubled-haploid populations were used to identify loci for seedling and adult plant resistance to these pathogens in twelve genotypes. A high level of co-location of resistance genes was observed. Loci for seedling resistance to leaf scald in five lines mapped to the *Rrs1* locus previously identified on chromosome 3H. One line has a quantitative trait locus (QTL) for adult plant resistance on chromosome 6H. A limited number of loci have also been observed for resistance to SFNB, with five lines having a seedling resistance gene at the *Rpt4* locus on chromosome 7H. Markers linked to *Rpt4* explained a large part of the seedling variation for SFNB, but little of the adult plant resistance (APR). In two mapped populations, major QTLs for APR were identified near *Rpt4* on chromosome 7H. QTL contributing to APR on chromosomes 4H or 5H were also identified in each population.

Introduction

Barley (*Hordeum vulgare*) leaf scald disease, which annually reduces yield by 10-15% in southern Australia, is caused by the fungal pathogen *Rhynchosporium secalis*. The fungus is highly variable and is able to quickly overcome new resistance genes as they are deployed in new cultivars. New sources of resistance are constantly being sought for use in gene-pyramiding strategies. Numerous sources of resistance to scald have been identified, with several loci mapped to barley chromosomes (reviewed in WILLIAMS 2003). The spot form of net blotch (SFNB), caused by the fungus *Pyrenophora teres* forma *maculata* is a foliar disease of barley found on crops grown in Scandinavia, Canada, South Africa and Australia. SFNB has been responsible for severe disease outbreaks in Western Australia, and has become a serious problem in southern Australia. In this paper we describe the identification of scald and SFNB resistance loci in cultivated barley varieties.

Material and Methods

Plant Material and Bioassay

The line B87/14 was obtained from the South African Small Grains Research Institute and is a selection from Pye (S) from CIMMYT. Galleon, Keel, Chebec, Sultan and O'Connor were obtained from Prof. A. Barr, University of Adelaide. Tilga was obtained from Dr B. Read, NSW Agriculture. ICARDA lines 2 (Apm/11012-2//NP CI 00593/3/IFB 974), 4(Arar/Lignee 527), and 9(CI 07117-9/Deir Alla 106//Badia/3/Arar) were obtained from Dr A. Yahyaoui, ICARDA. Phenotyping of *H. vulgare* reaction to scald was conducted as described in Williams et al. (2001). For phenotyping of reaction to SFNB, *P. teres* f *maculata* inoculum was prepared according to TEKAUZ (1990). Seedling resistance tests were performed as described in WILLIAMS *et al*. (1998). Scoring for seedling SFNB phenotype was based on the numerical lesion type scale developed by TEKAUZ (1985). Adult plant resistance

screening: The Galleon/Haruna Nijo population was sown as paired rows in an irrigated disease nursery at Turretfield in South Australia in 2002. Two incompletely duplicated replicates were assessed after flowering using a 1-9 scale reflecting disease severity. The VB9104/Dash population was sown as a single replicate in the Turretfield nursery in 2001.

Bulked Segregant Analysis
Resistant and susceptible "bulks" were formed by pooling 1µg of DNA from eight very resistant and eight susceptible lines as determined by the bioassay. Amplified Fragment Length Polymorphism (AFLP) analysis was performed essentially using the technique of VOS et al. (1995) with some modifications (WILLIAMS *et al.* 2001).

Mapping and Marker Validation
The position of putative AFLP markers was determined on an "Alexis x Sloop" map using Map Manager QT. Simple sequence repeat (SSR) markers developed and mapped by RAMSAY et al. (2000) were amplified using a 'touchdown' profile (WILLIAMS et al. 2001) in 10µl reaction mixes containing 20mM Tris-HCl pH 8.4, 50mM KCl, 1.0mM dNTP 1.875mM $MgCl_2$, 15ng of each primer, 0.5 U of *Taq* DNA polymerase (Life Technologies) 16ng template DNA.

Results and Discussion
Leaf Scald
Bulked-segregant analysis (BSA) was used to map a scald resistance locus in the *H. vulgare* genotype 'B87/14' (WILLIAMS et al. 2001) on chromosome 3H, possibly within the complex *Rrs1* scald locus. Microsatellite markers adjacent to the locus were identified and validated for their linkage to scald resistance in a second segregating population. These markers were used to screen other populations segregating for scald resistance. It was found that populations based on Sultan and ICARDA lines 2, 4 and 9 all had genes for scald resistance at or near the complex *Rrs1* locus on chromosome 3H, as evidenced by their linkage with microsatellite markers at this locus (Figure 1).

A population consisting of 116 double haploid (DH) lines produced from the F1 of a cross between the barley variety 'Schooner' (susceptible) and 'O'Connor'(resistant) was tested for field resistance to barley leaf scald disease. BSA was used to identify amplified fragment length polymorphism (AFLP) markers linked to a scald resistance locus in the barley cultivar 'O'Connor'. One of the AFLPs linked (12% recombination) to the scald resistance was mapped in a 'Chebec'/'Harrington' DH mapping population to the distal end of the short arm of chromosome 6H. Microsatellite markers in this region were selected from several maps and tested for polymorphism on the 'Schooner'/'O'Connor' population. Together with the BSA-derived AFLPs, the SSR markers explained up to 51% of the total phenotypic variation (LOD $\leq$ 15.6), confirming the chromosome 6H assignment of an 'O'Connor' scald resistance gene (Figure 1). This gene is near the *Hordeum vulgare* ssp. *spontaneum*-derived scald seedling resistance gene, *Rrs13* (ABBOT *et al.* 1995). Differential reactions to eighteen *R. secalis* isolates comprising five pathotypes indicates that although these loci are close together, they encode different specificities to *R. secalis* pathotypes. We have designated the scald resistance gene on chromosome 6H of O'Connor as *Rrs15*.

Spot Form of Net Blotch

Resistance to SFNB was located on chromosome 7H of the feed variety Galleon using QTL mapping. This locus, designated *Rpt4*, was the first definitively mapped SFNB resistance locus (WILLIAMS *et al*. 1998). One of the *Rpt4*-linked markers identified, PSR117(D)/EcoR1, was shown to be about 92% reliable in predicting resistance in an advanced cross, verifying the original mapping results. New sources of resistance to SFNB were used to develop populations. These were phenotyped and when SSR markers linked to *Rpt4* were used to genotype the populations, it was found that lines Keel, Chebec, Tilga and CI9214 all had seedling resistance to SFNB at the *Rpt4* locus (Figure 1).

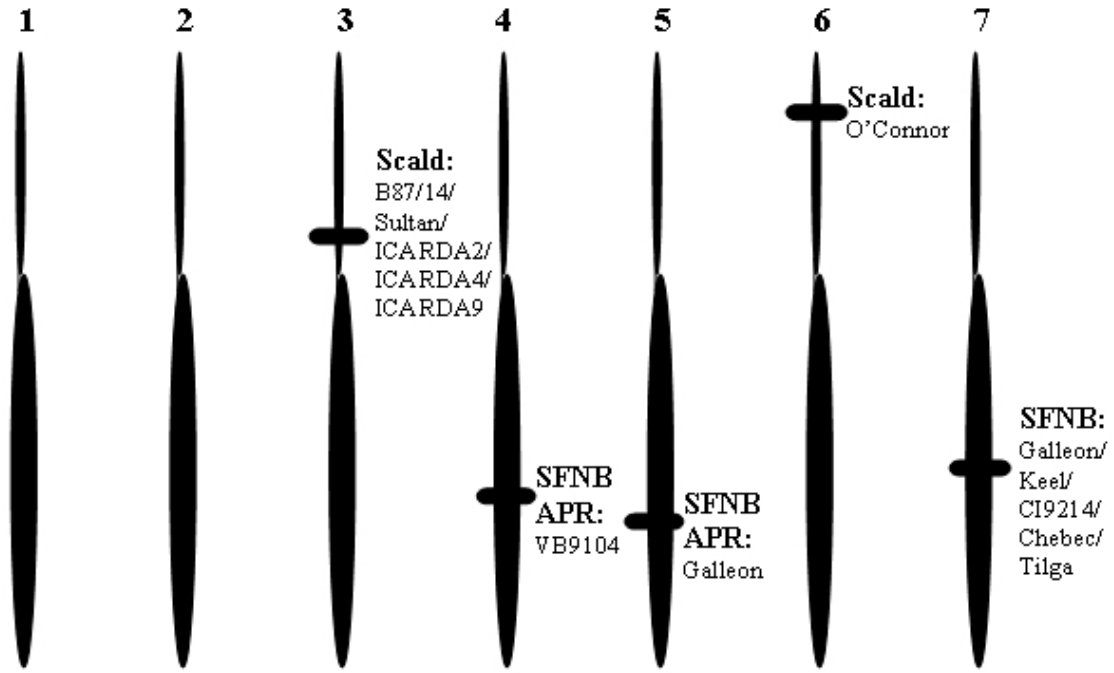

Figure 1. Location of genes for resistance to scald and spot form of net blotch, with sources of each gene

The Galleon/Haruna Nijo cross was used to identify quantitative trait loci contributing to adult plant resistance to SFNB. QTL were identified on chromosomes 5H and 7H by simple interval mapping (Figure 1). Bootstrap re-sampling showed that while the 5H QTL had a clearly defined confidence interval within 12 cM, the significant 7H QTL were distributed along the long arm and many marker loci the short arm of this chromosome also had a suggested (P=0.63) contribution to SFNB APR. The 5H and 7H QTLs provided greatest resistance when combined, suggesting that the two QTLs are additive in effect. Composite interval mapping (CIM) confirmed the significance of the 5H and 7H QTL, as well as revealing a QTL of minor effect on chromosome 4H.

Separate QTL analysis of the Turretfield and Hermitage field data for the VB9104/Dash population revealed a QTL with significant effect on APR on chromosome 7H of VB9104. This QTL was 20 cM distal to *Rpt4*, but was at approximately the same location as the APR QTL from Galleon. Another significant QTL for expression of APR at both sites was detected on chromosome 4H of VB9104. CIM confirmed the significance of the 4H and 7H QTL.

Additionally, CIM of the Turretfield data revealed a QTL of minor effect on chromosome 5H, close to the APR QTL identified in Galleon (WILLIAMS *et al*. 2003).

Summary
Our search for new loci for resistance to scald and SFNB has uncovered considerable redundancy of loci in cultivated barley lines, even when they were not believed to be closely related. This has implications for the ability of breeders to "pyramid" resistance genes, a strategy designed to slow the development of virulent pathogen isolates. Mapping of seedling resistance to SFNB only identified one locus necessary for APR, with another two loci revealed by mapping data collected from field screening. Searches of the wild relatives of barley should be conducted to identify new genes for resistance to both scald and net blotch. Markers for known resistance genes may be useful in rapidly identifying whether populations segregating for 'new' sources of resistance carry resistance genes at previously identified locations.

Acknowledgements
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Molecular Marker Development for Scald Resistance in 'Seebe' Barley

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Scald (*Rhynchosporium secalis*) of barley is prevalent in central Alberta, Canada and causes considerable yield and quality losses. Scald has the ability of rapidly changing in pathotype composition and frequency. This makes it difficult to develop durable scald resistance in barley. Previous studies have shown that the cultivar 'Seebe' carries durable genetic resistance, however, barley breeders have found this trait difficult to transfer into new barley lines. Therefore, we are trying to develop molecular markers for scald resistance from Seebe. Recombinant inbred lines were created from the genetic cross of 'Harrington' (scald susceptible) and Seebe (scald resistant). Progeny of about 175 individual F_2 seedlings were advanced by single-seed descent to the F_8 generation. Disease resistance to a major scald race was phenotyped at the seedling stage in a green house. By utilizing bulked segregant analysis, resistant and susceptible pooled populations were compared by AFLP analysis. A total of 255 AFLP primer combinations were used to analyze the genetic population and several *Eco*RI-*Mse*I and *Pst*I-*Mse*I fragments were found linked to scald disease resistance. These AFLP fragments identified are currently being verified, sequenced and transformed into a site-specific marker. As well AFLP and SSRs markers are being used to map the putative scald resistance genomic location.

S 8 – DISEASE AND PEST RESISTANCE II – EAR DISEASES, FUSARIUM HEAD BLIGHT, VIROSES

Cloning of AFLP Fragments Linked to Resistance Genes for Fusarium Head Blight in Barley

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Abstract
Genetic mapping of resistance genes to Fusarium head blight (FHB) in barley, caused by *Fusarium graminearum*, revealed multiple locus inheritance. A segregating population of seventy-five double haploid lines, developed from the three-way cross Zhedar 2/ND9712//Foster, was used for genome mapping and FHB evaluation. Four amplified fragment length polymorphism (AFLP) markers were identified and mapped to chromosomes 2H and 5H at regions closely associated with FHB resistance and low deoxynivalenol (DON) concentration. An additional AFLP marker was associated with disease susceptibility. These five AFLP fragments were purified, cloned and sequenced. Sequence-characterized amplified regions (SCARs) will be produced and used for assessing the presence of FHB resistance genes for comparisons in other barley genotypes. These markers may therefore be useful in marker-assisted selection of resistance genes in barley breeding programs and will facilitate map-based cloning.

Introduction
The development and use of molecular marker technologies has facilitated the subsequent cloning and characterization of disease, insect, and pest resistance genes in several plant species. Sequence-characterized amplified region (SCAR) markers have been converted from RAPD and AFLP markers and have proven useful in identifying resistance genes (ARDIEL *et al*. 2002; WEERASENA *et al*. 2004). The conversion of these markers into SCARs by the development of more specific primers significantly improves the reproducibility and reliability of PCR assays, and their utility for marker-assisted selection.
Genetic mapping of resistance genes to Fusarium head blight (FHB) in barley has revealed multiple locus inheritance. A segregating population of 75 double haploid lines, developed from the three-way cross Zhedar 2/ND9712//Foster, was used for genome mapping and FHB evaluation (AGRAMA *et al*. 2003; DAHLEEN *et al*. 2004). Fourteen amplified fragment length polymorphism (AFLP) markers were identified and mapped to chromosomes 2H and 5H at regions closely associated with FHB resistance and low deoxynivalenol (DON) concentration. An additional AFLP marker was associated with disease susceptibility. Our objective in this study was to develop SCAR markers linked to the genes for resistance to *Fusarium graminearum* on chromosome 2H.

Material and Methods

Three barley genotypes were used in this study: Foster, ND9712 and Zhedar 2. Foster and ND9712 are US six-rowed genotypes susceptible to FHB infection caused by *F. graminearum*. Zhedar 2 is a two-rowed accession from China that has shown some resistance to FHB (PROM *et al*. 1997) with a different origin than the other sources of FHB resistance previously mapped. The initial crosses were made between ND9712 and Zhedar 2 in Fargo, ND. The resulting F_1 plants were crossed to Foster to increase adaptation. The F_1's were crossed with *H. bulbosum* (CHEN & HAYES 1989) to develop a Zhedar 2/ND9712//Foster doubled haploid (DH) population consisting of 75 lines. The DH lines, parents, and the resistant check cultivar 'Chevron' were evaluated for FHB severity and DON level in the US and China in five environments from 1996-1997.

AFLP analysis (VOS *et al*. 1995) was carried out using AFLP Analysis System I (Gibco-BRL Gaithersburg, MD, USA). AFLP markers of interest that flanked one of the QTLs in chromosome 2H were eluted from the polyacrylamide gels, re-amplified, separated in agarose gels, excised and purified using the Qiaex gel-extraction kit (Qiagen). The amplified fragments were cloned into the plasmid vector TOPO-TA (Invitrogen, Carlsbad, Calif) following the manufacturer's instructions. Cloned fragments were completely sequenced by Northwoods DNA (Becida, MN). For each isolated AFLP marker, sequences of approximately five to ten different clones were checked to ensure that the correct AFLP band had been cloned. Linkage maps were developed using Mapmaker 3.0 (LANDER *et al*. 1987).

Results and Discussion

All 64 AFLP primer combinations were applied to the 75 DH lines. One-hundred and twenty five AFLP markers were mapped (DAHLEEN *et al*. 2003). Of these, 14 fragments were identified within the QTL for FHB resistance and low DON. A 300bp AFLP fragment (M8E8-3) was located in the main QTL (QFhb.Dah-2H.1, QDon.Dah-2H.1; http://wheat.pw.usda.gov/ggpages/FHBmapathon.htm) on chromosome 2H that was significantly associated with FHB resistance and low DON in the five environments. The fragment was cloned and sequenced to develop forward and reverse primers (5′-TAGTTGGTCTCTACTTCAGTTGGT-3′, 5′-TGCGTACCAATTCAGGGAGTAT-3′). The segregation of the SCAR marker, M8E8-3, in a set of resistant and susceptible DH is shown in Figure 1. SCAR M8E8-3 was placed on the previous Zhedar 2/ND9712//Foster map (DAHLEEN *et al*. 2003). The linkage showed that the SCAR marker mapped in the QTL region on chromosome 2H (Figure 2).

This marker suggests that the resistance may be conferred by either recessive allele due to deletion or other mutations of a genomic region in barley that might contain some host factor(s) required for the pathogen. NOGUCHI *et al*. (1999) have reported that resistance to potato virus Y in tobacco was due to deletions of a large genomic segment. Also, some recessive genes that confer resistance have been reported in mutant lines of *Arabidopsis* (OHSHIMA *et al*. 1998; YOSHII *et al*. 1998). A recessive barley gene that confers disease resistance, *ml-o*, is associated with broad-spectrum resistance to powdery mildew and a leaf lesion phenotype (BÜSCHGES 1997). It is not clear why these deletions were associated with resistance (NOGUCHI *et al*. 1999). The use of DNA SCAR markers is a very effective way of obtaining essential information about the genomic region around a given gene (Young *et al*., 1988; MESSEGUER *et al*. 1991), selecting resistant lines in breeding programs, and ultimately isolating the gene of interest (Van DOMMELEN *et al*. 2002).

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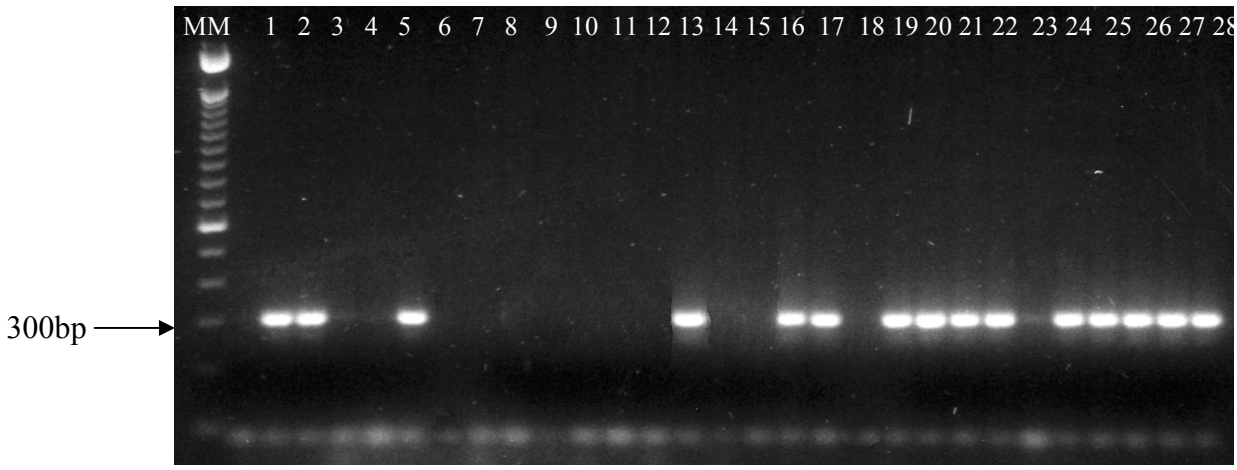

Figure 1. The AFLP marker M8E8-3 was converted to a SCAR marker. MM is 100bp standard marker, lanes 1, Gobernadora; 2, Foster; 3, Zhedar 2; 4, Zhedar 1; 5, ND9712; 6-15 resistance DH lines; 16-28 susceptible DH lines.

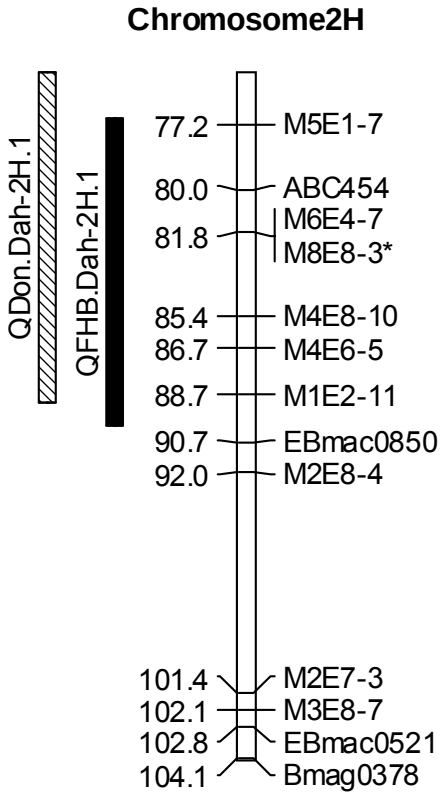

Figure 2. Partial map of barley chromosome 2H in the area surrounding the QTL (Dahleen *et al*. 2003; http://wheat.pw.usda.gov/ggpages/FHBmapathon.htm). Linked AFLP SCAR marker M8E8-3 was identified from the screen of the 75 DH lines. Genetic distance is given in centiMorgans (cM).

Mapping QTL for Resistance to Pathotypes of *Cochliobolus sativus* in Barley

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Abstract
Spot blotch, caused by *Cochliobolus sativus*, is an important disease of barley in many production areas. The parents of the doubled haploid mapping population Calicuchima-sib/Bowman-BC (C/B) exhibit differential reactions to pathotypes 1 and 2 of *C. sativus.* To elucidate the genetics of resistance, C/B progeny were evaluated to both pathotypes at the seedling stage in the greenhouse and at the adult plant stage in the field. Seedling resistance to pathotype 1 was conferred by a single QTL on chromosome 4(4H), and adult plant resistance was conferred by QTL on chromosome 2(2H) and chromosome 3(3H). Bowman-BC contributed both resistance alleles. A single gene on the short arm of chromosome 5(1H) conferred both seedling and adult plant resistance to pathotype 2 and was contributed by Calicuchima-sib. Progeny with resistance to both pathotypes were identified and may be useful in programs breeding for spot blotch resistance.
Keywords: QTL; barley; *Hordeum vulgare*; spot blotch; *Cochliobolus sativus*; disease resistance; Calicuchima-sib/Bowman-BC

Introduction

One of the most important diseases of barley in the Upper Midwest region of the United States is spot blotch caused by the fungus *Cochliobolus sativus*. Yield losses as high as 35% have been reported for susceptible barley cultivars under epidemic conditions (CLARK 1979). Six-rowed malting cultivars possess durable spot blotch resistance controlled primarily by a major effect QTL on chromosome 5(1H) (STEFFENSON *et al*., 1996). In contrast, the resistance of two-rowed feed cultivars like Bowman has been short-lived due to the appearance of virulent *C. sativus* pathotypes (FETCH & STEFFENSON 1994). Calicuchima-sib (a six-rowed line from the ICARDA/CIMMYT barley program also known as LUGC) and Bowman-BC (a BYDV-resistant backcross line of two-rowed cultivar Bowman) exhibit a differential response to pathotypes 1 and 2 of *C. sativus* (Table 1). Calicuchima-sib is susceptible to pathotype 1 and resistant to pathotype 2, whereas Bowman-BC is resistant to pathotype 1 and susceptible to pathotype 2. A doubled haploid population was developed from these parents by HAYES *et al*. (1996). To increase our understanding of the genetic basis of spot blotch resistance in both two- and six-rowed types, we evaluated the C/B doubled haploid mapping population to pathotypes 1 and 2 of *C. sativus* at both the seedling and adult plant stages.

Material and Methods

The C/B population (110 progeny) was evaluated to pathotypes 1 and 2 of *C. sativus* at the seedling stage in the greenhouse and at the adult plant stage in the field according to the methods of FETCH and STEFFENSON (1999) and STEFFENSON *et al*. (1996), respectively. Two *C. sativus* isolates from the United States representing pathotypes 1 (isolate ND85F) and 2 (isolate ND90Pr) were used. For QTL analyses at the seedling stage, the most common infection response observed on the second leaves of plants across all replications was used. For QTL analyses at the adult stage, the average disease severity (in percent) on the top two leaves across all replications was used. Data analyses were performed

using MapMaker (Version 2.0) and QTLCartographer (Version 2.0) for linkage and QTL analyses, respectively. Composite interval mapping (CIM) was applied with 1000 permutations at a significance level α=0.05. To fill in gaps in the previously published molecular map of the C/B population, simple sequence repeat (SSR) markers developed by RAMSAY *et al*. (2000), LIU *et al*. (1996) and M. S. RODER (IPK, Gatersleben Germany) were screened for polymorphism and mapped.

Table 1. Seedling and adult plant reactions of Calicuchima-sib and Bowman-BC to *Cochliobolus sativus* pathotypes 1 and 2

		Seedling		Adult	
		Mean Infection Response[1]	General Reaction Class	Mean Severity %[2]	Most Common Infection Response[3]
Pathotype 1	Cali-sib	5.8	Susceptible	38.8	MS-S
	Bow-BC	2.8	Resistant	18.2	MR
Pathotype 2	Cali-sib	2.0	Resistant	6.4	MR-R
	Bow-BC	7.0	Susceptible	64.4	S

[1]Based on a 0-9 scale where 0 is most resistant and 9 is most susceptible.
[2]Disease severity is the percentage of leaf area affected by disease on a 0-100 scale.
[3]Adult plant infection responses were based on the size and type (presence of chlorosis) of lesions observed where R=Resistant; MR=Moderately Resistant; MS=Moderately Susceptible; and S=Susceptible (FETCH and STEFFENSON 1999).

Results and Discussion

Seedling resistance to pathotype 1 was conferred by a single QTL on chromosome 4(4H) that explained only 14% of the total variation. Adult plant resistance was conferred by two QTL: one on chromosome 3(3H) explaining 35% of the total variation and a second on chromosome 2(2H) explaining 17% of the variation. The C/B population segregated 56:54 (X^2=0.04 with P=0.85) for resistance:susceptibility to pathotype 2 at both the seedling and adult plant stages. Progeny resistant at the seedling stage were also resistant at the adult stage; thus, the same single gene confers resistance at both growth stages. This gene was mapped to the *cer-yy* to *Hor2* interval of chromosome 5(1H) (short arm) and was designated *Rcs6*. This result was corroborated from quantitative data analyses as seedling and adult plant QTL mapped to the same region of chromosome 5(1H) and explained 93% and 87% of the variation, respectively (Table 2 and 3).

The previously published map of C/B contained 63 markers (CASTRO *et al.* 2002). To define more precisely the position of the resistance loci, we added two new morphological markers: *cer-yy* (controlling waxy-glossy spike) and *srh* (controlling rachilla hair length). Additionally, twelve SSR markers were added around the target regions containing spot blotch resistance QTL. Six of these SSR markers were mapped on chromosome 3(3H): EBmac705, GBM1073, Bmac225, KAB05-66, GBM1034, and Bmag905. The six SSR markers mapped onto chromosome 2(2H) were HVCSG, Bmag518, EBmac623, EBmac715, KAB05-45, and GBM1024. With the exception of HVCSG, all SSR markers were linked to each other on either chromosome 3(3H) or 2(2H) and helped to define the spot blotch resistance QTL more precisely.

Table 2. Summary of QTL (chromosomal location and phenotypic variance explained) conferring resistance to pathotypes 1 and 2 of *Cochliobolus sativus* at the seedling stage in the C/B double haploid population

Seedling Stage		
Pathotype	Chromosome 4 (4H) WG114 to HvAmy1	Chromosome 5 (1H) *cer-yy* to*Hor2*
Pathotype 1	14%	--[1]
Pathotype 2	--[1]	93%

[1]No QTL detected

Table 3. Summary of QTL (chromosomal location and phenotypic variance explained) conferring resistance to pathotypes 1 and 2 of *Cochliobolus sativus* at the adult stage in the C/B double haploid population

Adult Stage			
Pathotype	Chromosome 2 (2H) EBmac623 to BCD265D	Chromosome 3 (3H) GBM1073 to KAB05-66	Chromosome 5 (1H) *cer-yy* to *Hor2*
Pathotype 1	17%	35%	--[1]
Pathotype 2	--[1]	--[1]	87%

[1]No QTL detected

Previously published studies reported five different loci for resistance to *C. sativus* in barley. GRIFFEE (1925) postulated the position of three spot blotch resistance genes on chromosome 2(2H), chromosome 5(1H), and chromosome 7(5H) by their general association with morphological markers. GONZALEZ CENICEROS (1990) identified two genes for spot blotch resistance: one on chromosome 3(3H) and second on chromosome 5(1H). STEFFENSON *et al*. (1996) mapped two QTL for spot blotch resistance on chromosome 1(7H) and chromosome 5(1H). In the Steptoe/Morex population, STEFFENSON *et al*. (1996) identified a single gene (*Rcs5*) on chromosome 5 (1H) conferring seedling resistance and a major effect QTL on chromosome 5 conferring adult plant resistance. The spot blotch resistance loci identified in this study were located proximal to previously reported resistance loci on chromosome 2(2H) and chromosome 3(3H) and may therefore be unique. The major resistance gene identified on chromosome 5(1H) was positioned near the telomeric region and is unique. It was therefore designated as a new resistance locus, *Rcs6*. The position of the chromosome 4(4H) QTL conferring seedling resistance to pathotype 1 is also unique for spot blotch resistance loci (Table 2). This QTL maps to the same general region where a stripe rust (*Puccinia striiformis* f. sp. *hordei*) resistance QTL was reported in C/B population (CASTRO *et al.* 2002).

This study revealed the genetic basis of spot blotch resistance against two distinct pathotypes of *C. sativus*. The information obtained from this study will have practical implications for barley breeding programs in the Upper Midwest region and beyond. Moreover, our results may help to answer the question as to why two-rowed feed cultivars like Bowman do not possess the durable spot blotch resistance found in six-rowed malting cultivars (FETCH and STEFFENSON 1994).

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Population Structure of *Bipolaris sorokiniana* in Western Canada

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Abstract
Foliar fungal diseases are a significant limiting factor to barley production in Canada and elsewhere in the world. Spot blotch, caused by *Bipolaris sorokiniana*, has recently become one of the most damaging diseases of barley in western Canada especially in the eastern prairie region. Spot blotch can cause yield reductions of up to 20%, and additionally, reduce grain quality. Determination of a pathogen's virulence can provide useful information regarding pathogenic variability and the usefulness of resistance genes available for deployment in the host. Three pathotypes of *B. sorokiniana* were identified in North Dakota, the main spring barley growing state of the USA, based on infection responses on three barley differential lines. In this study, the infection responses of 87 isolates of *B. sorokiniana* from western Canada were evaluated on 12 barley differentials to determine the pathogen's variability in this region. The results indicate more pathogenic variation than that reported previously for North Dakota. Based on infection responses induced on the differentials, the isolates were classified into 5 main groups. Isolates of groups 1, 2 and 4 were similar to those previously designated as pathotypes 0, 1, 2 in ND, respectively. A new pathotype of *B. sorokiniana* was identified that is highly virulent on most of the differential lines, including six-row barley ND B112 that has been used as a source of resistance.
Keywords: *Bipolaris sorokiniana*; infection response; pathotype; virulence

Introduction

Spot blotch, caused by *Bipolaris sorokiniana* (Sacc.) Shoemaker [(telomorph *Cochliobolus sativus,* (Ito and Kuribayshi) Drechs. ex Dastur. syn. *Helminthosporium sativum* Pamm. King and Bakke.] is considered to be one the major diseases of barley in Canada. The disease can cause annual yield losses up to 20% (CLARK 1979). High humidity and warm temperatures during the growing season enhance the damage caused by spot blotch (BAILEY *et al.* 2003).

To breed for effective spot blotch resistance, it is necessary to know the pathogenic variability (virulence) in the causal organism. Virulence assessments can provide useful information regarding physiologic variation in plant pathogenic fungi and the likely effectiveness of host resistance genes. Physiological specialization in B. *sorokiniana* was first described by CHRISTENSON (1926), who showed that isolates of the fungus varied considerably in their virulence to wheat and barley. In North Dakota, FETCH and STEFFENSON (1994) identified two pathotypes of *B. sorokiniana* that exhibited a differential virulence pattern on several barley genotypes. Subsequently, VALJAVEC-GRATIAN and STEFFENSON (1997) evaluated thirty-three isolate from North Dakota, and on the basis of infection phenotypes with three barley differentials, identified three pathotypes of *B. sorokiniana* (pathotypes 0, 1 and 2). In Australia, MELDRUM *et al.* (2000) identified six pathotypes of *B. sorokiniana* among 34 isolates tested on 20 differential lines.

Recently, the prevalence of spot blotch has increased dramatically in western Canada and in 2002 it was the predominant leaf spot pathogen of barley and occurred in every field sampled from Manitoba (TEKAUZ 2003). Although fungicides can be used to reduce disease severity, the most economical and environmentally sound means of control is the use of resistant cultivars. Considerable interaction between different pathotypes of *B. sorokiniana* and its relevant hosts indicates the importance of evaluating the virulence present in the pathogen population. The objectives of this study were to characterize the population of *B. sorokiniana* in western Canada, and to identify and compare the pathotypes found with those reported from ND.

Material and Methods

Collection of Isolates

Eighty-seven isolates of *B. sorokiniana* collected from different regions of western Canada including Manitoba, Saskatchewan and Alberta, were evaluated for their virulence in this study. Seventy-four of the isolates were from Manitoba, and among these 8, 4, 19 and 43 isolates were collected in 1999, 2000, 2001 and 2002, respectively. The 8 isolates from Saskatchewan and 5 isolates from Alberta were collected in 1999 and 2001, respectively. Most of the isolates were obtained from infected barley leaf samples; some were either from seed of barley (all samples from Saskatchewan) or from infected leaves of wheat or oat.

Differential Lines

A set of 12 barley lines/varieties (the differential set) including ND B112, ND 5883 and 'Bowman' used by VALJAVEC-GRATIAN and STEFFENSON (1997), 'Conlon', TR 251, TR 261, 'Newdale', 'AC Metcalfe', 'CDC Stratus', 'CDC Bold', 'Robust' and 'Stander' were employed to differentiate among various isolates. Two weeks prior to inoculation, each differential was sown to produce a clamp of eight plants, with 4 clamps being sown per 30 cm clay pot. Plants were grown in a controlled environment at 20 ^{o}C and an 18 hour photoperiod.

Preparation of Inoculum

Leaf sections containing putative spot blotch lesions were surface-sterilized and rinsed in sterile distilled water. They were then placed on dry filter paper in petri dishes with another piece of filter paper, moistened with distilled water, placed in the lids, and incubated at 20 ^{o} C and a 12 hours photoperiod to promote pathogen sporulation in the lesions. After 3- 4 days single conidia were transferred to a 9 cm diameter plastic petri plate containing 10% V-8 juice agar. The plates were incubated for 10-12 days and then were used to prepare the final inoculum. For preparation of the final inoculum, the plates were flooded with sterile distilled water and the colony surface was rubbed with a sterile wire loop. The resulting suspension was homogenized for 1 min in a Waring blender and filtered through two layers of cheesecloth. The inoculum concentration was adjusted to 3,000 conidia per ml. A drop of 'Tween 20' was added per 50 ml of suspension as a spreader and sticker.

Inoculation and Scoring

Inoculum was applied to plants with an atomizer nozzle connected to an electric air pump. Sufficient inoculum was used to cause run-off on seedlings, approximately 10 ml per clump. Inoculated plants were incubated in darkness for 18 h at 20-22^{o} C and 100% relative humidity. They were then grown under an 18-h photoperiod at 20 ^{o} C. The second leaves of seedlings were scored three times for their infection responses, 8, 10 and 12 days after inoculation using the 0-9

infection response scale for spot blotch developed by FETCH and STEFFENSON (1999). Infection responses 1-4 and 5-9 were considered as 1ow and high virulence, respectively. Most of the isolates were tested twice and the data presented is the average of replications and the three scores taken for each. For further analysis, three second leaves of each differential line with typical symptoms were scanned using an HP Scanjet 5300C.

Results and Discussion

Based on infection responses induced by the 87 isolates of *B. sorokiniana* on the 12 differential lines, the isolates could be classified into 5 main groups each with some similar symptoms on differentials (Table 1 and Figure 1). Group 1 isolates were similar to 'pathotype 0' described for ND, i.e., the avirulent pathotype. Isolates of this group induced minute to small necrotic foliar lesions without any marginal chlorosis on all differential lines; most were scored as infection responses of 2-3 (Fig. 1). A few isolates in this group produce moderately-sized lesions (4-5) on some of the differentials. In general, however, the virulence of those isolates was considered low, and they were included in group 1.

Isolates of group 2 and group 3 had a similar virulence pattern on most of the differentials, except for ND B112 and 'Bowman'. The high infection response of line ND 5883 to the isolates of these two groups is a unique characteristic that can readily separate these isolates from those of other classes. Groups 2 and 3 contain isolates that are virulent on ND 5883, 'Conlon', 'AC Metcalfe', 'CDC Stratus' and 'CDC Bold'. 'Robust' barley was considered as susceptible when inoculated by isolates of these groups, but its infection responses were lower (near 5) than those of the preceding genotypes. Group 2 isolates appear to be similar to 'pathotype 1' described for ND. The low virulence of isolates of group 2 on ND B112 and 'Bowman' is a characteristic that can distinguish them from isolates of group 3. Isolates of group 3 are somewhat similar to isolates of group 2 except for their moderate virulence on line ND B112 and cv. 'Bowman'. Induction of medium- sized necrotic lesions on ND B112 and 'Bowman' is specific characteristic of isolates of group 3.

Table 1. Infection response groups differentiated among 87 isolates of B. sorokiniana from western Canada.

	Group 1	**Group 2**	**Group 3**	**Group 4**	**Group 5**
Number of Isolates	20	23	29	2	13
(Percent of total)	23%	27%	33%	2%	15%
Differential Lines	**Infection Responses**				
CDC Bold, CDC Stratus	R†	S††	S	S	S
Conlon,Robust	R	S	S	R	S
Bowman	R	R	S	S	S
AC Metcalfe, ND 5883	R	S	S	R	R
ND B112	R	R	S	R	S
Stander	R	R	R	R	S
TR 251, TR 261, Newdale	R	R	R	R	R

† R represents infection responses of 1-4 (low virulence)

†† S represents infection responses of 5-9 (high virulence)

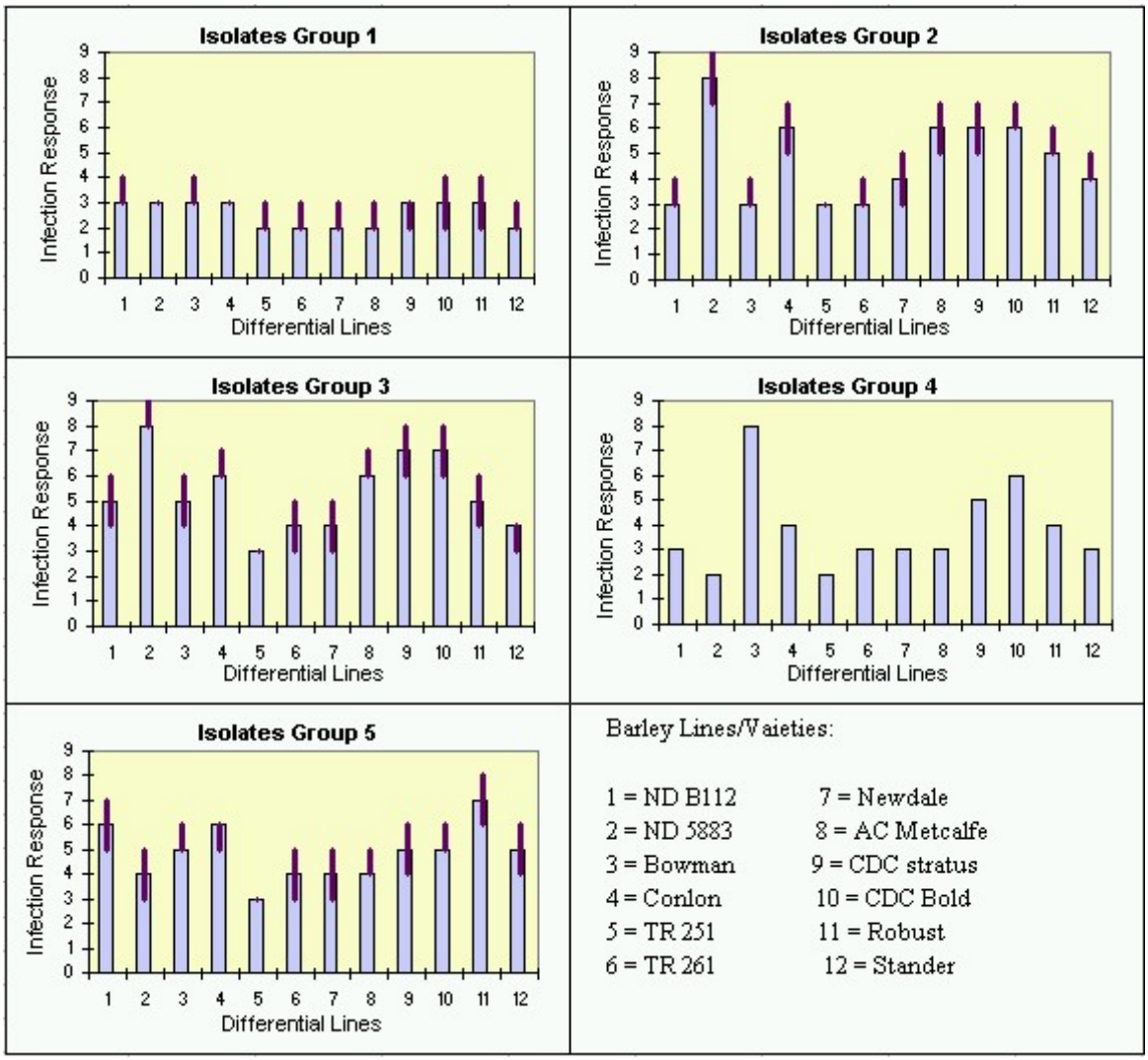

Figure 1. Infection responses observed on 12 barley differential lines inoculated with 87 isolates of *Bipolaris sorokiniana* from western Canada using the 0-9 rating scale developed by FETCH and STEFFENSON (1999). Bars indicate the variability in infection response among isolates of each group.

Group 4 isolates were composed of isolates inducing infection responses similar to those of ND pathotype 2. Isolates of this group were highly virulent on cvs. 'Bowman' and 'CDC Bold' and moderately virulent on cv. 'CDC Stratus'. Group 5 includes isolates with moderate to high virulence on most of the differential lines. Isolates of this group were virulent on line ND B112 and cvs. 'Bowman', 'Conlon', 'CDC Stratus', 'CDC Bold', 'Robust' and 'Stander'. In general, the lines ND 5883 and TR 261 as well as the cvs. 'Newdale' and 'AC Metcalfe' had moderately-resistant infection responses to isolates of this group, with line TR 251 being the only one showing a clear low infection response to isolates of this group.

These results indicate a greater level of pathogenic variability among western Canadian isolates of *B. sorokiniana* than that reported for ND. Isolates in groups 1 to 5 comprised 23%, 27%, 33%, 2% and 15% of total the number tested. Group 4 (ND pathotype 2), with only 2 isolates, had the

lowest isolates frequency among the groups. Our results also demonstrates a lower frequency of group1 (pathotype 0) and group 4 (pathotype 2) isolates compared to that reported for ND. In contrast, the frequency of group 2 isolates (pathotype 1) was higher than reported from ND. Isolates of Group 3 with highest frequency were found among all isolates collected from 1999 to 2002. This virulence group was detected only in isolates from Manitoba and Saskatchewan. However, the low number of isolates (5) tested from Alberta does not provide strong evidence for absence of group 3 isolates in Alberta regions. The presence of this type of isolates indicates broader variability of pathogen in western prairie of Canada than in ND. Group 5 isolates may represent a new pathotype in this region because they were found only from collections in Manitoba in 2002. None of the isolates collected prior to 2002 showed the same virulence pattern. This suggests that this pathotype either has emerged recently, or that the frequency of the pathotype previously had been too low to be identified in the population. Although the frequency of this newly-emerged group of isolates was only 15% of the total, it was 32% of the 43 isolates of *B. sorokiniana* collected in 2002. Three AFLP markers unique to isolates of group 5 have been identified, supporting the hypothesis of a more recent origin of this pathotype (H. Ghazvini, D. J. Somers and A. Tekauz, *unpublished data*). Based on the isolates analysed, this pathotype was found primarily in central Manitoba (Figure 2). However, as there were only a few isolates collected from western Manitoba during the 2002 annual barley leaf spot disease survey, additional more collections from this and other parts of province should be made to determine the extent of its occurrence.

Our data demonstrate that isolates of group 5 can overcome the resistance present in the North American six-rowed barley germplasm. Susceptible infection responses on seedlings of ND B112 (Figure 3) and 'Robust' barleys caused by isolates of this group are indicative of this. ND B112 is resistant to all pathotypes of *B. sorokiniana* from North Dakota and has remained resistant for more than 30 years (VALJAVEC-GRATIAN & STEFFENSON 1997). Most of the resistance present in American and Canadian 6-rowed barley, and in some 2-rowed cultivars, is derived from ND B112. The outbreaks of spot blotch in western Canada, especially in Manitoba, in 2001 and 2002 may be related to the emergence of this new pathotype of *B. sorokiniana.* TR 251 barley was the only differential line exhibiting great resistance to all isolates/pathotypes tested, and can be used as a source of effective resistance to spot blotch in barley breeding programs. Although, in our classification, line TR 261 and cv. 'Newdale', were categorized as resistant varieties (Table 1), the infection response values of these lines to most isolates were higher than observed in TR 251 (Fig. 1). 'Stander' can also be considered a good source of resistance to *B. sorokiniana*; except for its moderately susceptible response to isolates of group 5 it was resistant to isolates of other groups. Genes conferring resistance in TR 261, 'Newdale' and 'Stander' could also be employed to incorporate into barley germplasm.

Although most isolates could be placed in the 5 distinct groups, a few did not fit into any of these. Additionally, some minor differences occurred among isolates in groups 1-5 that may indicate an even greater level of diversity among this pathogen population. These minor differences could not be clearly differentiated using the set of 12 barley lines used here.

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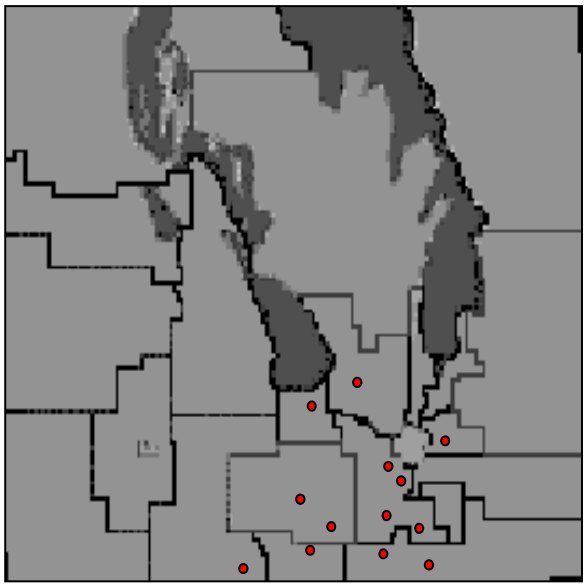

Figure 2 (left). Distribution of group 5 isolates of *B. sorokiniana* in Manitoba in 2002.
Figure 3 (right). Infection response of barley line ND B112 inoculated with isolate WRS 1983 representative of isolates of group 5.

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Yield Loss in Barley Inoculated with High and Low Virulence Isolates of *Bipolaris sorokiniana*

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Abstract
Recently, *Bipolaris sorokiniana*, causal agent of spot blotch has become a serious problem to barley cultivation in the western Canada. In 2001, spot blotch was the predominant leaf spot of barley in Manitoba and occurred in every field surveyed. Isolates were found to vary in their virulence on barley seedlings and could be classified into two main groups, high virulence (HV) and low virulence (LV). The recent emergence of a new pathotype of *B. sorokiniana* that is highly virulent on most of commercial six-row barleys has been reported in Manitoba. To determine if this new pathotype causes higher levels of damage and grain yield loss compared to LV isolates, 6 barley lines were inoculated with a single HV (WRS 1986) or LV isolate (WRS 1949) in field trials in 2003. Average grain yield losses caused by the HV and LV isolates were 11% and 6 %, respectively. Resistant barley line TR 251 had lower reductions in yield (5% and 3%) than the susceptible cultivars 'Conlon' (6% and 23%) and 'CDC Bold' (13% and 11%) when inoculated with HV and LV isolates, respectively. However, 'Stander' barley had the lowest overall yield loss, suggesting it may possess a superior and usable level of adult plant resistance. This suggests that adult plant resistance should be examined as components of an effective strategy in breeding for resistance to *B. sorokiniana*.
Keywords: *Bipolaris sorokiniana*; barley; virulence; pathotype; yield loss

Introduction

Barley (*Hordeum vulgare* L.) is the second most important cereal grain in Canada, occupying a land base of about 4.3 million hectares. Annual barley production in Canada, based on the five-year average, is about 12.7 million tonnes (Statistics Canada, 2002). Spot blotch, caused by *Bipolaris sorokiniana* (Sacc.) Shoemaker (telomorph *Cochliobolus sativus,* [Ito and Kuribayshi] Drechs. ex Dastur.) is considered to be one the major diseases of barley in Canada. In a study done at Ottawa, Ontario, spot blotch caused average yield losses of 26% and 16% in 1976 and 1977, respectively, and 10% reduction in kernel weight (CLARK 1979). In North Dakota, NUTTER *et al*. (1985) found that yield losses in six-rowed barleys inoculated at specific growth stages by *B. sorokiniana* ranged from 4% to 20%. An annual grain yield loss of 5-10% was estimated for barley production in Manitoba when plants were damaged by the leaf spot complex of net blotch (*Pyrenophora teres*) and spot blotch (TEKAUZ 2003a). In wheat in South Asia, where spot blotch and tan spot form a disease complex referred to as "Helminthosporium leaf blight" yield losses have amounted to 16% in India, 20% in Nepal, and 23% in Bangladesh (DUBIN & GINKEL 1991; SAARI 1998).

Recently, the importance of spot blotch as a component of the leaf spot complex on barley in southern Manitoba has increased markedly. Spot blotch has become the predominant foliar disease in the region and is responsible for much of the damage and yield loss observed (TEKAUZ *et al*. 2003). Previous studies have shown that infection phenotypes (lesion types) in adult plants are more resistant than in seedling, and within adult plants, more resistant in 6-rowed

than 2-rowed lines (TEKAUZ 2003b). The identification of a newly-emerged pathotype of *B. sorokiniana* in western Canada that is virulent on seedling of previously resistant six-rowed barley cultivars in Manitoba has been reported recently (GHAZVINI & TEKAUZ 2003).
The objective of the study was to determine if high virulence (HV) and low virulence (LV) pathotypes cause differential levels of damage in adult plants.

Material and Methods

The experiment was arranged in a randomized, split plot design with treatments as main plots and cultivars as sub-plots. Plots were 1.5 x 5 m and contained six rows 30 cm spaces. The three treatments consisted of inoculation with HV and LV pathotypes of *B. sorokiniana*, plus a non-inoculated control. The four replicates and the treatments in each replication were separated by 2-meter wide paths to minimize interplot interference. Treatments consisting of *B. sorokiniana* conidia were applied 3 times to coincide with specific barley growth stages: GS 36 (just prior to flag leaf expansion), GS 57 (three-forth of inflorescence emerged), or GS 73 (early milk) (ZADOKS *et al.* 1974).
Isolate WRS 1986 identified as a newly-emerged pathotype of *B. sorokiniana* in Manitoba (GHAZVINI & TEKAUZ 2003) was used as a high virulent isolate in this study to allow for better recognition of the pathogenicity of this pathotype on adult plants. Single conidia of isolates WRS 1986 (HV pathotype) and WRS 1949 (LV pathotype) were first transferred to 9 cm diameter plastic plates containing 10% V-8 juice agar. Plates were incubated for 10-12 days at 20 ^{o} C and a 12 hour photoperiod. Subsequently, plates were flooded with sterile distilled water and the colony surface rubbed with a sterile wire loop. The resulting suspension was homogenized for 1 min in a Waring blender and filtered through two layers of cheesecloth. The inoculum concentration was adjusted to 5×10^3 conidia per ml and a drop of 'Tween 20' added per 50 ml of suspension as a spreader and sticker. Two litres of conidial suspension, containing a total of 10×10^6 spores of *B. sorokiniana,* were applied per subplot using a CO_2-pressurized single nozzle spray boom. Inoculations were done on calm, clear evenings when there was little or no wind, minimizing drift and the likelihood of dew formation to facilitate conidial germination and host infection.
The barley genotypes used included 'Newdale', 'Stander' and TR 251 (resistant) and 'CDC Bold', 'Conlon' and 'Robust' (susceptible). These resistance ratings were based on previous inoculation studies using *B. sorokiniana* isolate WRS 1903, a prevalent type in Manitoba (TEKAUZ, *unpublished*).
Infection phenotypes were recorded at 7-day intervals for 21 days, beginning 5 days after the third inoculation, using the adult plant infection response scale (R, MR, MS, S) developed by FETCH and STEFFENSON (1999). At this time, plants were at the late milk through late dough stages of plant development. Five randomly selected tillers from each plot were assessed for their infection responses at each date.
A few days prior to harvest, a half meter row length from each end of the plots was removed to reduce interplot interference. At maturity, a 6 m^2 portion of each plot was harvested with a Wintersteiger combine and the grain air-dried, cleaned, and weighed. Kernel weight was determined from a sample of grain from each subplot by weighing 250 kernels and multiplying by 4 to determine the thousand kernel weight (TKW). The Test weight of half a liter volume of grain was determined for each subplot using an electric balance and data were converted to hectoliter weight (HW or kg m^{-3}). Data were analyzed as a Split-Plot design with PROC GLM (SAS Inst. Inc., 1998). Based on estimators of variance components, appropriate F-tests were done.

Results and Discussion

The mean infection responses of the barley genotypes inoculated by the two isolates of *B. sorokiniana* (Table 1), indicated some differences compared to those of the seedling stage (GHAZVINI & TEKAUZ 2003). 'Robust' barley had a somewhat lower infection response to the HV pathotype than is normally found at the seedling stage. In contrast, 'Conlon' was highly infected by the HV pathotype; normally it exhibits a moderately susceptible infection response to isolates of this group. 'Newdale' also had higher infection response to the HV pathotype. Moreover, 'CDC Bold' and 'Colon' had higher infection responses to the LV pathotype than observed at the seedling stage. Analysis of variance indicated significant differences for yield, TKW and HW among genotypes at the $p \geq 1\%$ level (Table 2). Treatments, the indicator of different types of pathotypes, had a significant influence on TKW and HW at the 1% and 5% levels, respectively. However, treatments did not have a significant effect on yield, although considerable yield differences could be observed (Table 3 and Figs. 1.A & 1.D). The lack of an effect on yield may be due to the split plot design used, in which the precision in estimating the average effects of treatments assigned to main plots would usually be sacrificed to provide higher precision for estimation of subplots. In addition, no significant interactions between genotypes and treatments were observed for yield, TKW and HW (Table 2). This indicates constant changes in yield, TKW and HW for all barley genotypes when inoculated with different treatments (Fig. 1.A, 1.B, 1.C). Average yields, TKWs and HWs of the genotypes inoculated with the HV pathotype were lower than those for the LV pathotype. Among these, 'Stander' and 'Conlon' with 2238 and 1648 g/plot had the highest and lowest average yields, respectively. However, 'Conlon' had the highest TKW and HW.

Table 1. Average adult plant infection response (AP-IR) and seedling infection response (S-IR observed on barley genotypes infected with HV and LV pathotypes of *B. sorokiniana*

	Varieties					
Pathotypes	Robust	Stander	Conlon	CDC Bold	TR 251	Newdale
HV (AP-IR)	MS	MR-MS	S	MS	R	MS-MR
HV (S-IR)	7	5,6	5,6	5,6	3	4
LV (AP-IR)	MR-MS	MR	MS	MS	R-MR	MR
LV (S-IR)	3,4	2,3	3	3,4	2,3	2,3

Table 2. Analysis of variance (split-plot design) for yield, TKW and HW

		Yield		TKW		TW	
Class variable	DF	MS	F	MS	F	MS	F
Treatment[a]	3	335995	0.79 [ns]	21.12	12.96 **	845.32	8.04 *
Rep (treatment)	2	425807		1.63		105.12	
Variety[b]	6	501285	10.38 **	265.88	265.88 **	1682.36	48.08 *
Treatment x Variety[b]	5	28746	0.60 [ns]	0.45	0.45 [ns]	42.23	1.21 n
Rep x Variety (treatment)	10	48305		1.00		34.99	

[a] Error term for treatment is Rep (treatment).

[b] Error term is Rep x Variety (treatment).

[ns] Not significant.

* & ** Significant at levels of the 5% and 1% probability, respectively.

Table 3. Average yield (g/plot), thousand kernel weight (g) and hectolitre weight (kg m-^{3}) for varieties and treatments.

Varieties	Yield	TKW	HW
Robust	1976	38	603
Stander	2238	39	598
Conlon	1648	51	625
CDC Bold	2001	43	595
TR 251	2017	46	593
Newdale	2168	44	595
Treatments	**Yield**	**TKW**	**HW**
Control	2129	45	608
HV pathotype	1892	43	597
LV pathotype	2003	43	599

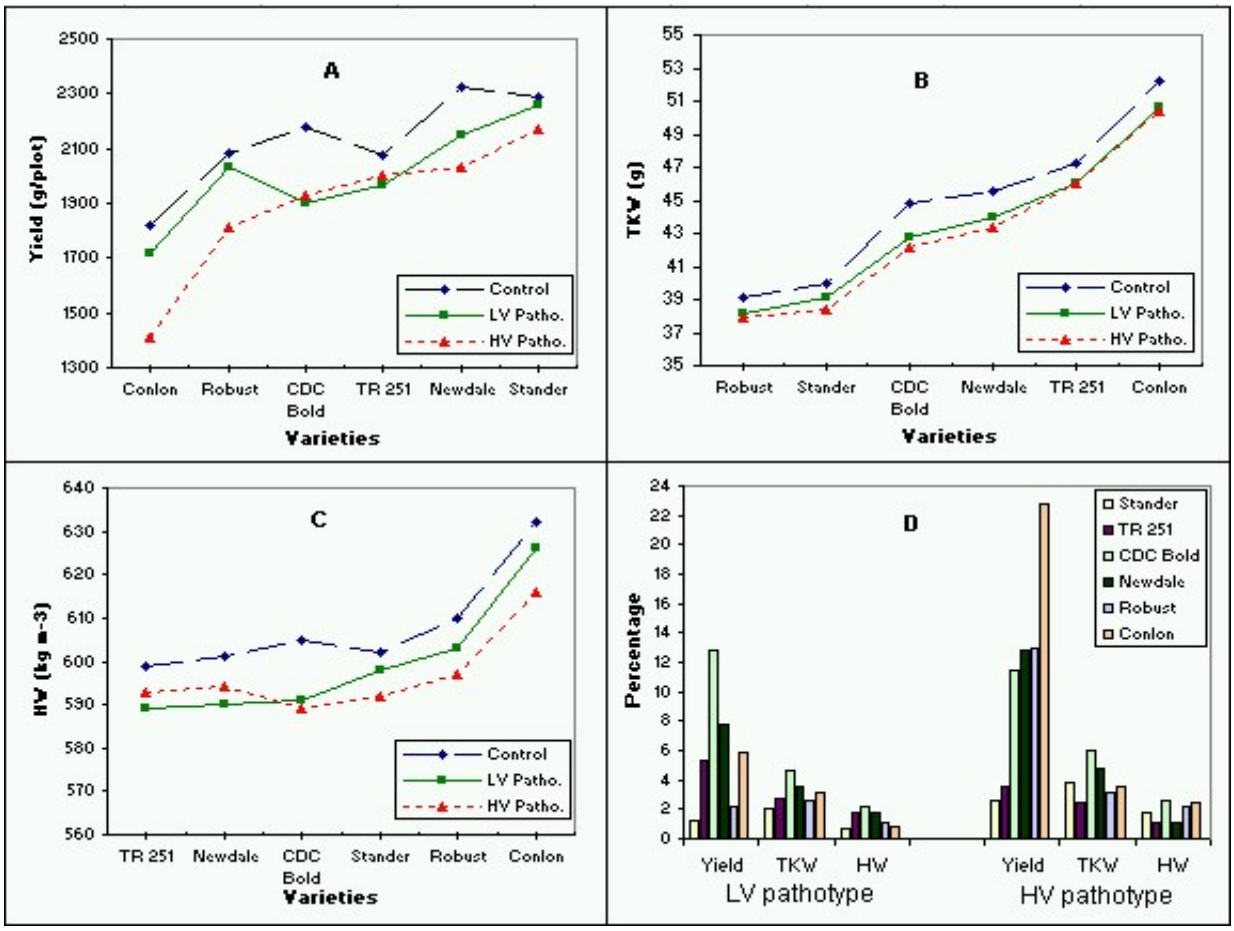

Figure 1. Interaction of barley genotypes with HV and LV pathotypes of *Bipolaris sorokiniana* and the control: (**A, B and C**) Effect of different treatments on yield, TKW and HW, respectively. (**D**) Effect of HV and LV pathotypes on yield components (% reduction vs. control). Genotypes 'Stander', TR 251, 'CDC Bold', 'Newdale', 'Robust' and 'Conlon' are illustrated from left to right, respectively.

Total grain yield losses over all genotypes following inoculation with HV and LV isolates were 11% and 6 %, respectively (Table 4). 'Stander' with 1% and 3%, and TR 251 with 5% and 3% yield reductions, when inoculated with LV and HV pathotypes, respectively, demonstrated significant adult plant resistance. 'Colon' with a 23% yield reduction possessed the weakest adult plant response when inoculated with HV pathotype. 'Robust' and 'Newdale', each with 13%, and 'CDC Bold' with an 11% yield loss, indicated moderate to high susceptibility to the HV pathotype at adult plant stage. Excluding 'CDC Bold' and 'Newdale' with 13% and 8% yield reductions, the other genotypes had lower levels of yield loss (between 1% to 6%), when inoculated by the LV pathotype. Surprisingly, the 13% yield reduction observed in 'CDC Bold' when inoculated with the LV pathotype was higher than that (11%) caused by the HV pathotype. TR 251 with 5% and 3% yield loss when inoculated with the LV and HV pathotypes, respectively, also showed more damage from the LV pathotype versus the HV pathotype. Compared to the average yield loss of 6% caused by the LV pathotype, the lower level of yield loss of 5% in TR 251 can be considered as a deviation likely caused by experimental error. However, in the case of 'CDC Bold', the significant yield loss induced by LV pathotype can not be justified by such errors. This may be related to the higher infection response of 'CDC Bold' to LV isolates of *B. sorokiniana* at the adult plant stage.

In comparison to their corresponding control plots, an average reduction of 3% and 4% in TKW, and 1% and 2% in HW were observed following inoculation with LV and HV pathotypes, respectively. Reductions were no greater than 6% for TKW, and 3% for HW, in any genotype. As such, only a portion of the yield reductions can be attributed to lower TKW and HW. The appearance of spot blotch at later stages of a plant's life cycle may have little effect on the numbers of tillers, but can cause a small reduction in the number of kernels per spike. CALLAGHER *et al.* (1976) found that the number of kernels per unit ground area was largely determined prior to anthesis, while the mean kernel weight was mainly determined during the growth period following anthesis. NUTTER *et al.* (1985) found that the timing of inoculation did not significantly reduce the number of kernels per spike in 'Larker' and 'Dickson' barley compared to the non-inoculated controls. Their results were in agreement with those of CALLAGHER *et al.* (1976). In a separate study, NUTTER (1983) found that inoculation with increasing spore concentrations of *B. sorokiniana* at GS 36 reduced the number of kernels per spike of cv. 'Larker' by as much as 20%, while 'Dickson' was unaffected. In our study, much of the yield loss can be attributed to thin and shrunken kernels that were blown out the back of the combine used to harvest the plots, or that were lost during cleaning.

Table 4. Yield, thousand kernel weight and hectolitre weight reductions in barley genotypes inoculated with HV and LV isolates of *B. sorokiniana* compared to their corresponding controls

	LV pathotype			HV pathotype		
Varieties	Yield	TKW	HW	Yield	TKW	HW
Robust	0.02	0.03	0.01	0.13	0.03	0.02
Stander	0.01	0.02	0.01	0.03	0.04	0.02
Conlon	0.06	0.03	0.01	0.23	0.04	0.02
CDC Bold	0.13	0.05	0.02	0.11	0.06	0.03
TR 251	0.05	0.03	0.02	0.03	0.02	0.01
Newdale	0.08	0.04	0.02	0.13	0.05	0.01
Average loss	0.06	0.03	0.01	0.11	0.04	0.02

An accurate estimation of yield loss caused by spot blotch is of interest to barley-growers in Manitoba. The results of this study show that epidemics of spot blotch caused by *B. sorokiniana* can reduce yields, although the amount of damage caused would vary based on pathogen pathotype and barley genotype. In general, HV pathotypes appear to be more aggressive and result in more damage to the crop. The average yield loss of 11%, caused by HV pathotype, increased to 13% and 23% for 'Robust' and 'Conlon'. 'Robust' and 'Conlon' were planted on 29% and 11% (40% in total) of the area under barley cultivation in Manitoba in 2003 (Yield Manitoba, 2004). This indicates that the newly-emerged HV pathotype of *B. sorokiniana* has become a potential limiting factor to barley production in Manitoba. Although the LV pathotype of *B. sorokiniana* caused average losses of only 6%, its pathogenicity is still substantial and it should not be considered as 'avirulent'. The effect of environmental conditions, an important component of host-pathogen-environment epidemic triangle, was not assessed in our study. However, it has been shown that high humidity and warm temperature increase the damage caused by spot blotch (BAILEY *et al.* 2003).

The identification of a newly-emerged more virulent pathotype of *B. sorokiniana,* indicates that a greater effort must be made to suppress epidemics of this pathogen. Based on our results, TR 251 and 'Stander' barley possess usable levels of resistance at the adult plant stage, and are good sources of resistance to spot blotch for use in two-rowed and six-rowed barley breeding program, respectively.

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Greenhouse Screening and NIRS for Fusarium Head Blight in Barley

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Abstract
Fusarium head blight (FHB) is a significant disease of barley in Canada. Grain quality reduction due to deoxynivalenol (DON) makes barley unsuitable for malting or feed. Effective, efficient indoor screening techniques are critical for breeding resistant cultivars as field screening is not efficient due to environmental effects where plant architecture, heading date and other diseases can confound FHB development. An indoor screening technique using spray inoculation and a humidity chamber has been evaluated and a screening protocol developed. FHB severity increased with the duration of humidity chamber incubation. A 48 h incubation period was the best. When inoculated at spike emergence, early heading lines showed less disease than mid or late heading lines. All lines had high disease severity when inoculated 14 days after spike emergence. Testing 14 cultivars with six isolates of *F. graminearum* demonstrated significant cultivar × isolate interaction. Single isolates are being used to screen segregating populations against FHB with inoculation at ear emergence followed by 48 h of humidity chamber incubation. Near infrared spectroscopy (NIRS) shows promise for simplified estimation of barley DON levels and has the potential to be used as a screening tool to eliminate samples with high DON levels.

Introduction

Fusarium head blight (FHB), caused by *Fusarium graminearum* Schwabe, is a significant disease of barley in Canada and many areas of the world (TEKAUZ *et al*. 2000; STEFFENSON 2003). Effective, efficient indoor screening techniques are critical for breeding resistant cultivars especially for barley breeding programs where the disease is not endemic. Field screening methods are not efficient due to environmental effects and confounding effects of plant architecture, heading date and other diseases.

DON can be measured by several methods. The most commonly used method (ELISA) is slow, labour intensive and expensive. Near infrared spectroscopic (NIRS) methods have the advantage of short analysis time, limited sample preparation and relatively low cost (RUAN *et al*. 2002). The present studies were conducted to standardize screening techniques under greenhouse conditions and develop a NIRS prediction equation to estimate DON levels in ground barley samples.

Material and Methods

Preliminary experiments were conducted to evaluate three inoculation methods viz. injection, spray and infected canaryseed and humidity chamber conditions. Three *F. graminearum* isolates (Fg 02-1, Fg 02-2, Fg 02-3) were obtained from J. Tucker, Brandon Research Centre, AAFC, Brandon, Manitoba (originally from Dr. A. Tekauz, AAFC, Cereal Research Centre, Winnipeg,

Manitoba). A mixture of these isolates with a final concentration of 5 X 10^4 macroconidia/mL was used for inoculation. For spray inoculation, 0.5 mL of a 5 x 10^4 macroconidia/mL was applied to the entire spike, using a Duray® 2 oz. Pump Sprayer (Goody Products, Inc., Peachtree City, GA). For the injection method, 10 µL of 5 x 10^4 macroconidia/mL suspension was micropipette injected into a single spikelet in the middle of each spike. For the canaryseed method, a single infested canaryseed was placed inside a single spikelet in the middle of the spike. For controls, sterilized ddH_2O was sprayed or injected instead of the spore suspension and in the canaryseed method, a sterilized seed was placed in the middle spikelet. Following inoculation, plants were placed in a humidity chamber.

Twenty-one lines were screened with the spray inoculation method using standardized humidity chamber conditions and 10 replications. These lines included the parents of potential segregating populations and susceptible and resistant checks. Each replication consisted of one pot with 3 plants. Spray inoculations were conducted when spikes were fully emerged (in some, spikes did not emerge from the boot early enough and it was necessary to open the boot to expose the spike). Inoculated plants were incubated in the humidity chamber for 72 h. FHB severity was recorded on a 0-100 scale (Stack and McMullen 1998) two weeks after inoculation. These lines were screened using different humidity chamber incubation periods (24 h, 48 h, 72 h) with four reps/line. Checks and seven early heading lines were tested at three growth stages viz. spike emergence (SE), 7 (7 DASE) and 14 days after spike emergence (14 DASE) again with four reps. Fourteen lines were tested with six isolates of *F. graminearum* (Fg 02-1, Fg 02-2, Fg 02-3, a mixture of these three isolates, FG99 and MN42) with four reps/line - isolate combination.

DON analyses were performed at either the Eastern Cereal and Oilseeds Research Centre (ECORC), AAFC, Ottawa, Ontario or Norwest Labs, Winnipeg, Manitoba using the ELISA method. Statistical analysis was performed using SAS (SAS Institute Inc. 1999).

A FOSS NIR Systems 6500 Near Infrared Spectrometer was used to collect the spectra from 577 samples harvested from FHB field screening nurseries at Brandon, Manitoba and Ottawa, Ontario in 2001 with ELISA DON levels ranging from 0 to 161 ppm. Computer programs CENTER and SELECT were used to define population boundaries and to select samples to be used for calibration. Population boundaries were established with a maximum standardized H distance from the average spectrum of 3. Calibration samples were selected with a minimum standardized H distance between samples of 0.6. Calibrations were developed using different mathematical treatments with multiplicative scatter correction (de-trend). This equation was used to predict the DON levels in sample sets harvested from the AAFC Brandon Research Centre FHB screening nursery in 2002 and 2003.

Results and Discussion

Standardization of Greenhouse Screening Technique

Preliminary results showed high disease severity with the spray method whereas symptoms were restricted to inoculated spikelets in case of the injection and canaryseed methods. These results confirm the earlier reports (STEFFENSON 2003) that barley has 'acceptable' Type II FHB resistance (resistance to spread). Consequently, only the spray method of inoculation was used in further experiments.

Humidity chamber conditions were standardized to obtain high disease incidence. Optimum levels of disease were obtained with misting for one minute every 3 h from 7 AM to 10 PM; two humidifiers running continuously; the humidity tent sealed and greenhouse shade curtains in place on the greenhouse roof and south side wall.

Screening of 21 barley lines using the above conditions resulted in high disease severity and DON levels. Many lines viz. AC Metcalfe, CI4196, Chevron and CDC Sisler which have moderate field resistance showed high disease severity in the greenhouse (data not shown). This may be due to the overwhelming disease pressure under humidity chamber conditions. Alternatively field resistance may be due to plant architecture or late heading which are non-

Table 1. Effect of humidity chamber incubation periods in the on FHB % disease severity and DON levels

		Incubation periods			
Cultivar/line	Barley Type	24 h	48 h	72 h	Mean
2ND16092	2R h+	0 (**0**)*	26 (**23**)	41 (**142**)	23
Robust	6R h+	0 (<1)	13 (10)	40 (39)	18
CDC Freedom	2R h-	2 (<1)	4 (7)	33 (22)	13
TR340	2R h+	0 (<1)	22 (22)	65 (38)	25
Falcon	6R h-	8 (2)	66 (35)	84 (47)	53
AC Metcalfe	2R h+	9 (4)	74 (28)	74 (44)	52
HB353	2R h-	14 (5)	91 (31)	70 (42)	58
CDC Sisler	6R h+	12 (3)	81 (62)	77 (54)	57
BM9203-74	2R h+	22 (2)	79 (26)	86 (45)	62
CDC McGwire	2R h-	47 (5)	86 (31)	89 (48)	71
CDC Silky	6R h-	38 (12)	88 (47)	93 (54)	73
CDC Kendall	2R h+	54 (18)	96 (42)	94 (54)	81
MC9813-08	2R h+	23 (11)	79 (36)	99 (46)	71
Stander	6R h+	50 (16)	79 (30)	93 (41)	74
TR360	2R h+	24 (10)	94 (40)	92 (49)	70
CDC Bold	2R h+	45 (**12**)	96 (**76**)	97 (**195**)	79
Harrington	2R h+	80 (15)	99 (32)	99 (43)	93
CI4196	2R h+	13 (**7**)	46 (**67**)	76 (**222**)	45
CDC Helgason	2R h+	47 (**15**)	88 (**108**)	96 (**262**)	77
Chevron	6R h+	48 (14)	92 (36)	90 (46)	77
HDE84194	2R h+	23 (4)	48 (26)	71 (44)	47
Mean		28	70	79	
$LSD_{0.05}$ for cultivars		- 11			
$LSD_{0.05}$ for treatments		- 4			
$LSD_{0.05}$ for cultivars * treatments		- 19			

* Data in brackets are DON (ppm) values, estimated by **ECORC Lab, Ottawa, Ontario** and Norwest Labs, Winnipeg, Manitoba.

effective under controlled conditions. DON levels correlated (r = 0.74) with the FHB severity with some exceptions: CDC Kendall, Harrington and Chevron exhibited low DON but high disease severity.

Disease severity increased with increasing duration in the humidity chamber (Table 1). There were some escapes with a 24 h incubation period and the 72 h incubation led to very high disease and DON levels. In many lines, disease severity at 48 h and 72 h was the same but DON levels increased with incubation period (DON values from Norwest labs are lower because a water extract was used instead of an alcohol extract, however the trend is similar). Therefore, the 48 h incubation period was deemed best to differentiate resistant and susceptible lines.

Early-heading lines consistently showed less disease when inoculated at spike emergence. An experiment was conducted to determine how long resistance was maintained. All lines showed high disease incidence when inoculated 14 DASE (Table 2). This is in contrast to wheat where spikes become more resistant after anthesis. However, inoculating barley 14 DASE is not appropriate as in some lines, spikes are too mature. Thus spike emergence stage has been used as the inoculation stage in subsequent experiments. However, in early heading lines, there is need to wait until 3-4 days after spike emergence for optimum disease development.

Table 2. Effect of plant growth stage on FHB severity

Cultivar/Line	Growth stage at inoculation			
	Spike Emergence	7 DASE*	14 DASE	Mean
HDE84194	11	74	95	60
2ND16092	18	62	94	58
TR340	47	91	100	79
CDC Freedom	42	85	100	75
HB353	65	97	100	87
Stander	44	88	98	77
BM9203-74	29	80	100	70
CDC Bold	56	89	100	82
CDC Helgason	63	92	100	85
CI4196	8	66	96	56
Mean	38	82	98	
$LSD_{0.05}$ for cultivars		- 9		
$LSD_{0.05}$ for treatments			- 5	
$LSD_{0.05}$ for cultivars * treatments		- 16		

* Days after spike emergence.

Testing 14 barley lines with six *F. graminearum* isolates demonstrated significant cultivar × isolate interaction. The parents of potential segregating populations showed differential reactions to many isolates (Table 3).

Table 3. Screening of barley lines with single *F. graminearum* isolates

Line	*F. graminearum* isolates					
	Fg 02-1*	Fg 02-2	Fg 02-3	Mixture	FG99	MN42
	FHB severity					
HDE84194	20 (34)**	11 (14)	2 (2)	11 (17)	19 (25)	1(<1)
2ND16092	6 (6)	1 (<1)	0 (<1)	2 (3)	8 (18)	5 (5)
HB353	15 (22)	5 (2)	17 (2)	8 (6)	37 (39)	30 (21)
BM9203-74	3 (2)	1 (<1)	4 (<1)	3 (4)	23 (30)	23 (31)
CDC Bold	54 (47)	11 (7)	17 (13)	45 (33)	78 (53)	58 (56)
CDC Freedom	59 (22)	33 (8)	26 (6)	38 (18)	55 (42)	45 (35)
Chevron	50 (2)	15 (<1)	23 (2)	35 (4)	57 (25)	66 (18)
TR360	7 (4)	5 (2)	25 (17)	46 (22)	54 (54)	41 (49)
MC9813-08	55 (21)	43 (8)	17 (5)	33 (16)	52 (37)	61 (42)
CDC McGwire	57 (12)	25 (2)	13 (3)	27 (10)	41 (42)	74 (59)
TR306	18 (15)	13 (8)	17 (3)	16 (4)	29 (36)	46 (47)
Harrington	35 (15)	38 (8)	31 (7)	32 (21)	63 (62)	73 (66)
CDC Helgason	19 (10)	41 (13)	12 (8)	51 (6)	51 (48)	48 (40)
CI4196	11 (2)	6 (<1)	20 (6)	12 (15)	16 (23)	43 (42)

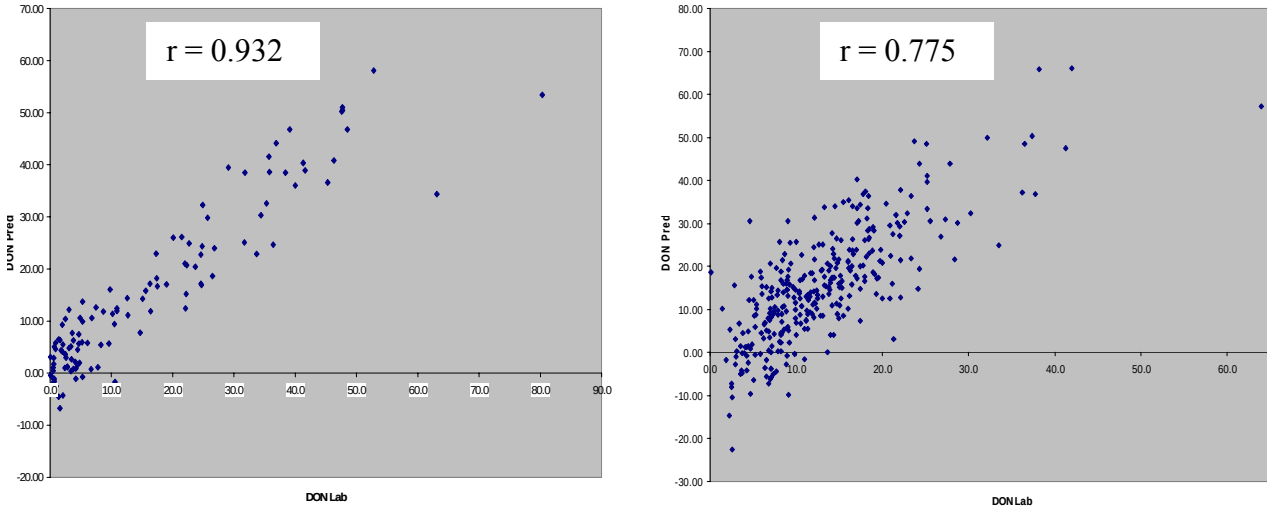
$LSD_{0.05}$ for cultivars * isolates - 23.4

*Fg 02-1, Fg 02-2 and Fg 02-3 are from J. Tucker, AAFC Brandon, MB, Canada.
The mixture contains the three isolates Fg 02-1, Fg 02-2 and Fg 02-3.
FG99 - received from Dr. Linnea Skoglund, BARI Fort Collins, CO, USA
MN42 - received from Dr. Ruth Dill-Macky, University of Minnesota, St. Paul, MN, USA
** Data in brackets are DON (ppm) values, estimated by Norwest Labs, Winnipeg, MB.

DON Estimation Using NIRS

The math treatment giving the lowest standard error of cross validation (SECV), the highest R-squared (RSQ) and 1-VR (1 minus the variation ratio) was used as the final equation. This equation was used to predict the DON values of the validation set (Fig. 1a). The correlation between the laboratory DON values and the predicted values was 0.93. Laboratory DON values ranged from 0.1 to 80.3 ppm whereas the predicted values ranged from -6.8 to 58.1 ppm.

This equation was also used to predict DON values for samples from six experiments grown in the AAFC Brandon FHB nursery in 2002 and 2003. Correlations between the laboratory and predicted DON values were 0.690 and 0.775 for 2002 and 2003 (Fig. 1b), respectively. These correlations compare favourably with that of DOWELL *et al*. (1999) who developed an equation to estimate DON concentration in single wheat kernels with a correlation of 0.64. An equation based on whole seed also shows promise, although more samples need to be analysed. This would eliminate grinding making screening prior to ELISA significantly more efficient.



A B

Figure 1. Laboratory vs predicted DON values for the validation set (a) and 2003 FHB samples from Brandon FHB nursery (b).

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Hordeum bulbosum - a Source for BYDV Resistance

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Abstract
In a pre-screening of 15 *Hordeum bulbosum* genotypes maintained for several years under natural *Barley yellow dwarf virus* infection in the field and in the greenhouse one accession free of virus was identified by ELISA. In several inoculation tests with high infection pressure using *Barley yellow dwarf virus*-PAV, -MAV and *Cereal yellow dwarf virus* the accession remained symptomless and no virus could be detected by DAS-ELISA. First results of penetration behaviour of *Rhopalosiphum padi* females, studied with the Electrical Penetration Graph method, show that the duration of phloem penetration is much shorter on *H. bulbosum* clone A 17 than on virus susceptible clone A 21. Additional studies on this subject and electronmicroscopic investigations of leaf morphology are in progress.
Keywords: *Barley yellow dwarf virus*; BYDV; resistance; *Hordeum bulbosum*; probing behaviour of aphids; electrical penetration graph; EPG; scanning electron microscope

Introduction

Yield losses caused by the aphid-transmitted *Barley yellow dwarf virus* (BYDV) and *Cereal yellow dwarf virus* (CYDV) are of economic importance all over the world (LISTER & RANIERI 1995). Besides agronomic practices and insecticide treatment against the virus vectors the cultivation of resistant or tolerant cultivars is the most effective and environment-protecting method to control these viruses. However, up to now only two genes, namely *ryd1* (SUNESON 1955) and *Ryd2* (SCHALLER *et al.* 1963), were identified. Out of these the more effective *Ryd2* gene found in Ethiopian barleys was used to develop numerous resistant or tolerant cultivars (BURNETT *et al.* 1995). But this gene does not confer complete resistance or immunity and its effectiveness is depending on the genetic background, the virus and the environmental conditions (JONES & CATHERALL 1970; SCHALLER 1984; SKARIA *et al.* 1985; BALTENBERGER *et al.* 1987). Therefore, in several countries continuous efforts are undertaken to identify resources of resistance (e.g. HABEKUSS *et al.* 2000; OVESNA *et al.* 2002).

Different *H. bulbosum* clones were found to be resistant to the soilborne mosaic virus complex (BaMMV, BaYMV and BaYMV-2), powdery mildew, leaf rust and typhula blight (MICHEL 1996). Furthermore, one clone also stayed free of virus after numerous inoculation tests with different viruses of barley yellow dwarf. This paper reports about the results of these resistance tests and the investigations to find out the reason for the observed resistance reaction.

Material and Methods

BYDV Resistance Tests

Vegetatively multiplied plants of the *H. bulbosum* clones A 17 and A 21 and seedlings of the virus susceptible *H. vulgare* cultivar 'Rubina' as control were inoculated by viruliferous aphids (15-20 *Rhopalosiphum padi* / plant, BYDV-PAV1 Aschersleben and CYDV-RPV Dittersbach or 15-20 *Sitobion avenae* / plant, BYDV-MAV1 Aschersleben of the virus collection of the Institute of Epidemiology and Resistance – see http://www.bafz.de/) for 5 days. After this time aphids were killed by insecticide spraying. Plants were cultivated in a greenhouse for 6 weeks before the virus content was estimated by DAS-ELISA using selfproduced polyclonal antisera (BYDV-PAV) and bought reagents of BIOREBA (BYDV-MAV, CYDV-RPV).

Investigation of the Probing Behaviour of Aphids

The Electrical Penetration Graph (EPG) technique (TJALLINGII and HOGEN ESCH 1993) was used to study the feeding behaviour of *Rhopalosiphum padi* on plants of the *H. bulbosum* clones A 17 (resistant) and A 21 (susceptible), and of *H. vulgare* cv. 'Rubina' or 'Erfa' (susceptible). Aphids were connected by a fine gold wire and water soluble conductive silver glue to a 'Giga4'-amplifier and 4 plants were tested simultaneously. The digitalized signals were recorded for 8 h and stored on the PC harddisk. The different graphical signal patterns of stylet pathway (C, F, pd), xylem sucking (G) and phloem penetration (E1, E2) were analysed by the Stylet 3.0 Software according to TJALLINGII (1978) and PRADO and TJALLINGII (1994). 47 parameters, combining the presents and duration of the different waveforms, was used for the statistical analysis. Analysis of variance was conducted by the procedure GLM of the SAS Software (Version 6.12).

Investigation of Leaf Structure

For characterisation of the leaf surface, cross sections of fresh material of the *H. bulbosum* clones A 17 and A 21, and of *H. vulgare* cv. 'Rubina' were investigated with a Philips XL 30 ESEM scanning electron microscope. The amounts of selected chemical elements like oxygen, silicon, potassium and calcium were determined by x-ray microanalysis using a DX4-system attached to the microscope.

Results and Discussion

H. bulbosum is known to be a valuable source to improve resistance of *H. vulgare* to different fungi and to the barley mosaic virus complex (WALTHER *et al.* 2000). In a pre-screening of 15 *H. bulbosum* genotypes maintained for several years under natural *Barley yellow dwarf virus* (BYDV) infection pressure in the field and in the greenhouse in the Institute of Agricultural Crops at Groß Lüsewitz one accession free of BYDV was identified by ELISA. In numerous inoculation tests with viruliferous aphids of BYDV-PAV (14 tests), BYDV-MAV (2 tests) and CYDV-RPV (1 test), respectively and a long inoculation time (5 d) no virus infection was detected by ELISA, although infection rates of the virus susceptible cv. 'Rubina' amount to 98, 74 and 46 % for the different viruses (Table 1).

Table 1. Number of inoculation tests carried out to infect *H. bulbosum* clone A 17, comparison of infection rates with that of virus susceptible 'Rubina'

Virus	Number of Tests	Number of Inoculated Plants [1]		Infection Rate (%) [2]	
		A 17	Rubina	A 17	Rubina
BYDV-PAV	14	26	10	0	98
BYDV-MAV	2	26	10	0	74
CYDV-RPV	1	28	10	0	46

[1] per test; [2] mean of all tests

An inhibited sucking behaviour of the aphids on the phloem could be a possible explanation for the lack of successful infections of clone A 17. By using the EPG-technique this character was investigated for apterous *Rhopalosiphum padi* probing on the resistant A 17 in comparison to the susceptible A 21 and *H. vulgare*. Table 2 shows the mean values of some of the most important penetration periods influencing infection.

Table 2. Number of probes and duration (s) of characteristic waveforms during the electrical registration of the probing behaviour of single *Rhopalosiphum padi* on *H. bulbosum* clones A 17, A 21 and *H. vulgare*

Character	A 17	A 21	*H. vulgare*
Time to the 1st probe	444.7	379.4	52.5
Duration of the 1st probe	621.3	1377.5	4154.5
Total time of phloem penetration (E1 + E2)	703.8	773.2	10060.0
Total time of phloem sucking (E2)	401.0	735.5	9921.3
Duration of early phloem sucking period (E1)	302.8	37.8	138.8
Duration of xylem sucking (G)	410.8	1030.0	0.0
Duration of stylet work (F)	6568.5	6820.5	718.8
Number of probes	14.3	11.9	9.3
Number of short probes until the first phloem contact	3.9	3.9	1.3

The time until the first probe is quite similar on both clones but significantly longer as on *H. vulgare*. The duration of the first probe is shorter on A 17 than on A 21 and *H. vulgare*, resulting in a higher number of probes on A 17. On *H. bulbosum* aphids spend longer times with stylet work (F-pattern) than on *H. vulgare*. Furthermore, significant differences were observed for phloem penetration and sucking. Aphids suck considerably shorter in *H. bulbosum* phloem than in that of *H. vulgare*, and shorter in A 17 than in A 21. These results support the assumption that virus resistance of A 17 is at least partly caused by a reduced phloem sucking of the aphids.

First investigations of the morphological structure of cross sections of leaf surface by scanning electron microscope (Fig. 1) do not show any obvious differences neither between the clones nor to the susceptible cv. 'Rubina' that could explain the various aphid behaviour on the clones.

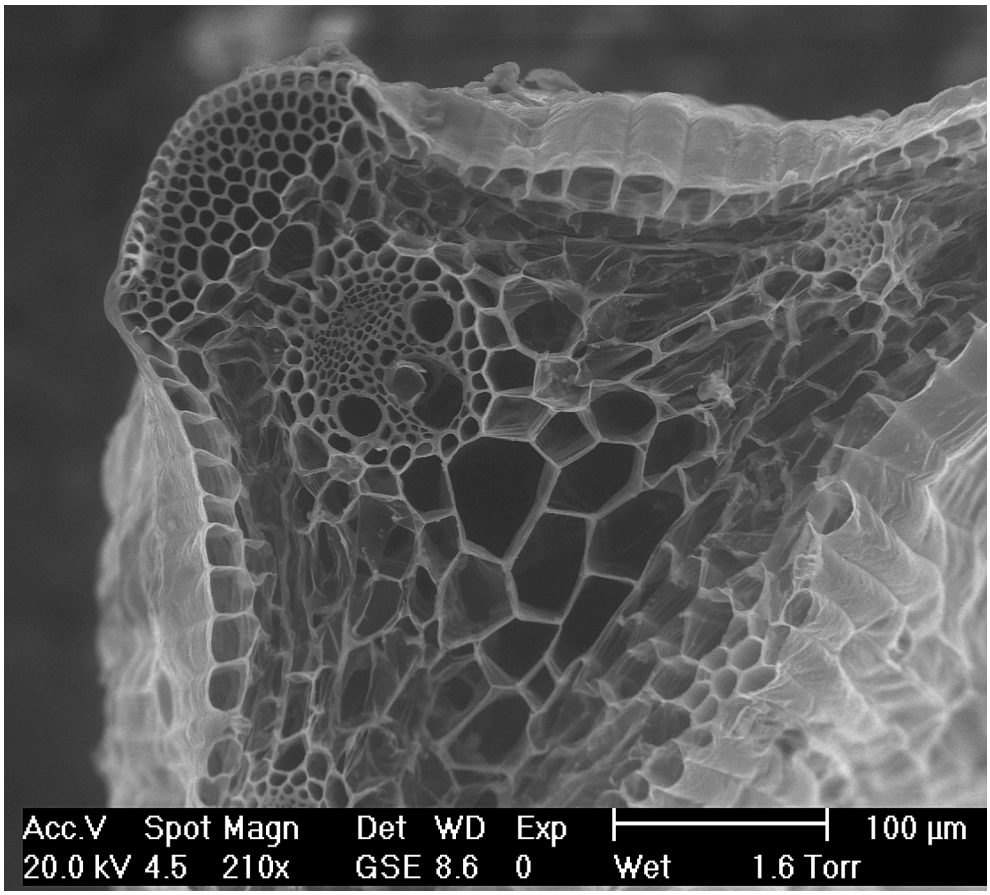

Figure 1. Cross section of A 21 leaf

The most frequent chemical elements of the leaves of all three genotypes are oxygen, silicon, potassium and calcium (Table 3). But in A 17 the content of calcium in both investigated leaf areas, the middle leaf vein and between the leaf veins, is higher in the sum than in A 21 and in 'Rubina'. Calcium is an important element contributing to the strength of the cell membrane. Its higher amount in A 17 could be one of the factors for the resistance reaction of this genotype.

Table 3. Chemical composition of leaves (weight %) of *H. bulbosum* clones A 17 and A 21 in comparison to cv. 'Rubina'

Chemical element	**Rubina**		**A 17**		**A 21**	
	MLV[1]	**BLV[2]**	**MLV**	**BLV**	**MLV**	**BLV**
Oxygen	93.1	93.2	91.0	92.6	75.3	81.8
Silicon	1.7	0.8	3.6	1.2	10.4	4.4
Chlor	1.7	2.1	1.9	2.4	5.6	5.8
Potassium	2.8	3.2	2.0	1.9	6.9	7.1
Calcium	0.7	0.7	**1.6**	**1.9**	1.9	0.9

[1] middle leaf vein
[2] between leaf veins

These studies as well as the EPG-tests are still in progress. Furthermore, recently interspecific hybrids have been developed and tested for their BYDV-reaction (SCHOLZ *et al.*, this volume).

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QTL Analysis of Resistance to Fusarium Head Blight in Barley RI Populations

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Abstract

We evaluated Fusarium head blight (FHB) resistance using 'cut-spike test' in two recombinant inbred populations derived from Russia 6 × H.E.S. 4 (RI1) and Harbin 2-row × Turkey 6 (RI2). We constructed high-density linkage maps including AFLP and SSR markers on two RI populations. At the same time, resistant gene analog (RGA) markers developed from degenerated primers based on highly conserved domain of R-genes, or sequence specific primers based barley EST markers that showed high homology to R-gene sequences were mapped on the high-density linkage maps. In the RI1 population, the results of simple interval mapping (SIM) and composite interval mapping (CIM) for FHB resistance detected two QTLs on the long arm of chromosome 2H and one QTL located on the short arm of chromosome 5H. One of the QTLs located on chromosome 2H was coincident with *vrs1* locus that determines inflorescence row type. In the RI2 population, CIM detected one QTL on chromosome 2H. This QTL might be identical with the one detected in RI1 since the cleistogamy locus and one RGA marker were mapped on the vicinity of these QTLs in both populations. The closely linked markers to QTLs for FHB resistance are useful for the marker-assisted selection or the identification of candidate resistance genes.

Introduction

Fusarium head blight (FHB) or scab is one of the most serious diseases that cause significant reductions of yield and quality in wheat and barley. A considerable number of genetic studies to get over FHB epidemics have been conducted to improve resistance in barley. Breeding for FHB resistance has been difficult due to the multiple components to control the resistance, genotype × environment interaction for resistant expression, and high-cost of phenotyping the resistance (MESFIN *et al*. 2003). Moreover, there were also strong associations between FHB resistance and certain morphological or physiological traits. Two-rowed varieties generally showed higher FHB resistance than six-rowed varieties in several studies (TAKEDA & HETA 1989; STEFFENSON *et al*. 1996). Heading date may also strongly influence the expression

of FHB resistance in barley (MA *et al*. 2000).

Resistant gene analog (RGA) makers were generated from conserved motifs of disease resistant genes isolated from a wide range of plant species (SPIELMEYER *et al*. 1998), and were successfully used to localize on the linkage maps in various species (LEISTER *et al*. 1998; SPIELMEYER *et al*. 1998). Some QTL analyses for barley disease resistance were reported that the positions of detected QTLs were coincident with RGA markers. QTL analysis based on a linkage map including RGA markers is important to confirm the association between FHB resistance and resistant gene motifs.

The objectives of the present research were (1) to determine and compare locations and effects of QTLs for FHB resistance in barley RI populations, and (2) to dissect the relations between FHB resistance and agronomic traits or RGA markers.

Material and Methods

Plant Material

Two RI populations derived from the cross between Russia 6 and H.E.S. 4 (RI1), and Harbin 2-row and Turkey 6 (RI2) were developed by single seed descent. Russia 6 and Harbin 2-row are two-rowed spring barleys, which are selected as the highly resistant cultivars (TAKEDA & HETA 1989). Contrary, H.E.S. 4 (six-rowed) and Turkey 6 (two-rowed) were susceptible cultivars by the same authors. RI1 population was grown on the field in 2000-2001 and 2002-2003 seasons. RI2 population was grown in only 2000-2001 season.

Phenotypic Evaluation

Phenotypes of two RI populations were scored for FHB resistance and 14 agronomic traits including row type, heading date, flowering type (cleistogamy). The FHB resistance of RI lines was scored using ‘cut-spike test’ developed by TAKEDA and HETA (1989).

Linkage Map Construction

Five morphological traits including row type and flowering type were scored in the field and were integrated as morphological markers on the linkage maps. AFLP, SSR and RFLP procedures were described by HORI *et al*. (2003). AFLP markers located on the vicinity of detected QTLs were converted into sequence tagged site (STS) markers. RGA markers were amplified with degenerate primers followed by CHEN *et al*. (1998). EST sequences that showed high similarity for R-genes were collected from barley EST database (HarvEST: barley; http://harvest.ucr.edu/) by tBLASTn search, and were integrated into the linkage maps.

Data Analysis

The segregation data of morphological, AFLP, SSR, RFLP, STS and RGA markers were used for linkage map construction by software package MAPMAKER/EXP. QTL analysis was carried out by the simple interval mapping (SIM) and by the composite interval mapping (CIM), using software packages MAPMAKER/QTL and QTL Cartographer, respectively. A LOD threshold 2.0 was used as the presence of putative QTLs for each trait. LOD peak of each significant QTL interval was considered as the QTL location on the linkage map.

Results and Discussion

Linkage Map Construction

A high-density linkage map including 1,172 loci (1,595.7 cM) was constructed in RI1 population as reported in HORI *et al*. (2003). Forty RGA and 38 EST markers were additionally localized on the linkage map in RI1 population to characterize detected QTLs for FHB resistance.

In RI2 population, a linkage map including 382 loci (1,377.1 cM) was constructed by morphological, AFLP, SSR, RFLP, STS and RGA markers. Most of the locations of morphological, SSR, RFLP and STS markers were not different from the previous reports.

QTL Analysis in RI1 Population

QTL analyses by SIM and CIM were carried out for FHB resistance and nine agronomic traits in two RI populations. Most of the marker intervals including QTLs existed in coincident or very close region on the linkage map between SIM and CIM analyses. Several QTLs for agronomic traits were localized at similar chromosomal regions as reported in other studies.

By both SIM and CIM analyses on the RI1 population, three and two QTLs for FHB resistance were detected in 2001 and 2003 seasons, respectively (Fig. 1). Alleles originated from the resistant parent Russia 6 contributed to FHB resistance for all the QTLs. One QTL on chromosome 2H was coincident with the *vrs1* locus, while no significant QTL was observed near the *vrs1* locus in 2003. This QTL explained the phenotypic variance of 13.0% (SIM) and 10.1% (CIM), respectively. Another QTL on chromosome 2H was detected in both years with LOD=3.5 by SIM (16.7%) and LOD=3.2 by CIM (13.5%) in 2001, LOD=6.0 by SIM (26.1%) and LOD=6.0 by CIM (25.9%) in 2003. Cleistogamy locus and one RGA marker (FXLRRfor_XLRRrev170) were localized on the vicinity of this QTL (Fig. 1). The

QTL on chromosome 5H was also detected in both years with LOD=2.6 by SIM (18.7%) and LOD=3.4, by CIM (15.5%) in 2001 and LOD=2.3 by SIM (11.2%) and LOD=3.4 by CIM (15.9%) in 2003.

The results of QTL analyses for heading date detected three QTLs on chromosome 2H, 6H and 7H both in 2001 and in 2003. Correlation coefficient between heading date and FHB resistance was significant in 2001 ($r=0.22$, $P<0.01$), while FHB resistance was not influenced by heading date in 2003 season ($r=-0.06$, $P>0.90$). Therefore, significant correlation with FHB resistance in 2001 might be due to the linkage between the QTL for heading date and the *vrs1* locus on chromosome 2H.

QTL Analysis in RI2 Population

One QTL for FHB resistance was detected by CIM analysis on chromosomes 2H in 2001 season (Fig. 1). The QTL for FHB resistance by CIM showed LOD score 2.6 and explained the phenotypic variance by 12.3%. The QTL allele originated from the resistant parent Harbin 2-row contributed higher FHB resistance. Cleistogamy locus and one RGA marker (FXLRRfor_XLRRrev119) were localized on the vicinity of this QTL (Fig. 1).

In this study, RI2 population derived from the cross between two-rowed varieties was used to evaluate association between *vrs1* locus and FHB resistance. The result of QTL analysis didn't detect any QTL on the vicinity of the *vrs1* locus (cMWG699) in RI2 population. However, ZHU *et al*. (1999) reported QTLs for FHB resistance on the vicinity of the *vrs1* locus on chromosome 2H in a population derived from the cross between two-rowed parents. Further analyses should be necessary to conclude about the *vrs1* effect on FHB resistance. FHB resistance of RI2 population was not significantly correlated with heading date in 2001 season ($r=-0.06$, $P>0.90$), and no QTL for heading date was detected on the vicinity of the QTL for FHB resistance.

Comparison of QTL Position between Two RI Populations

LUBBERSTEDT *et al*. (1998) and CHEN *et al*. (2003) were described that comparison of QTLs among multiple populations was necessary to collect more QTLs and to reveal conservation of the QTL regions among varieties or species. In this study, three markers (HVM54, FXLRRfor_XLRRrev119 (170) and STS_FEgtaMacg677) and cleistogamy locus were commonly localized on the same order on the long arm of chromosome 2H between two linkage maps of RI1 and RI2 populations, and were linked with the significant QTL for FHB resistance in both populations (Fig. 1). Thus, the QTL region for FHB resistance detected

commonly between RI1 and RI2 populations in this study might be consistent resistance factor for FHB in barley, and these markers could be useful for MAS in barley breeding strategies.

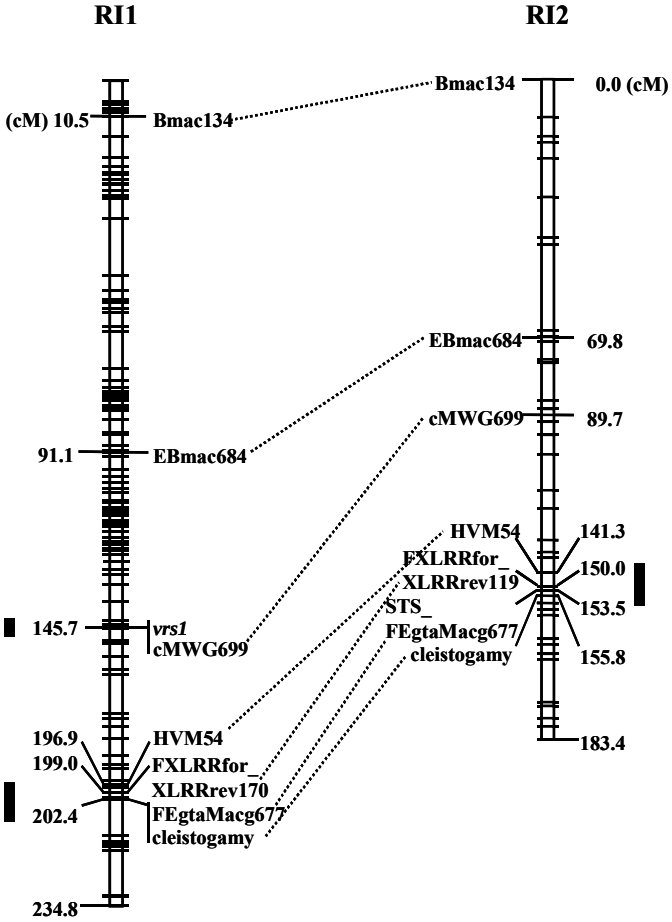

Figure 1. Comparison of morphological, AFLP, SSR, RFLP, STS and RGA markers on linkage maps on the RI populations derived from Russia 6 × H.E.S. 4 and Harbin 2-row × Turkey 6. *Broken lines* connect common marker between two linkage maps. QTL positions for FHB resistance were shown by *black boxes* on the left side of Russia 6 × H.E.S. 4 linkage map and on the right side of Harbin 2-row × Turley 6 linkage map, respectively.

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Identification and Mapping of Resistance Genes against the Barley Yellow Mosaic Virus Complex in Barley

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Abstract
Breeding for resistant cultivars is the only way to prevent high yield losses in barley caused by a complex of soil-borne viruses (BaMMV, BaYMV, BaYMV-2). In order to identify donors of resistance carrying genes different from *rym4* and *rym5* which are widely used in European barley breeding 120 gene bank accessions, resistant against BaYMV in Japan, were analysed by the SSR marker Bmac29 being to some extent diagnostic for this locus. Out of the 120 accessions analysed, 43 genotypes were detected carrying *rym5* and 12 carrying *rym4*. Those which are not carrying *rym5* or *rym4* are potential candidates for detecting new resistance genes after running extensive tests for allelism. Besides this, DH populations derived from crosses to different resistant exotic germplasms are analysed by bulked segregant analysis (BSA) using genome covering SSRs. In this respect the BaMMV resistance of 'Chikurin Ibaraki 1' was assigned to chromosome 6H by the analysis of 163 F_1-derived doubled haploid (DH) lines of a cross to the susceptible cv. ´Igri´.
Keywords: barley (*Hordeum vulgare*); barley yellow mosaic virus disease (BaMMV, BaYMV, BaYMV-2); molecular markers; SSRs

Introduction

Barley yellow mosaic virus disease, caused by barley mild mosaic virus (BaMMV), barley yellow mosaic virus (BaYMV), and BaYMV-2 (HUTH 1990), is one of the most important diseases of winter barley in most European barley growing regions. Because of transmission of virus particles into the root cells of barley plants by the soil-borne fungus *Polymyxa graminis* (TOYAMA & KUSABA 1970) chemical measures against the disease are neither efficient nor economic. Therefore, breeding for resistance against yellow mosaic inducing viruses is of special interest in order to ensure winter barley production in the increasing area of infested fields. Resistance to BaMMV and BaYMV in Europe is based on the recessive resistance genes *rym4* and *rym5*. *rym5* derived from the Chinese landrace 'Mokusseko 3', additionally confers resistance to BaYMV-2 and has been incorporated into adapted cultivars such as ´Kyoto´ or ´Kamoto´ (ANONYMOUS 2003). Both genes are located on chromosome 3 HL and are allelic with regard to BaMMV (GRANER *et al.* 1999). Besides these two genes, several resistance genes against the different yellow mosaic inducing viruses have been identified (for overview cf. WERNER *et al.* 2003). These genes are well suited for broadening the genetic base of resistance in European barley breeding. Regarding the present situation in Japan and Europe the potential risk of the selection of new virus strains, which are able to overcome resistance genes is obvious. In France a new strain of BaMMV has been already reported infecting carriers of *rym5* (HARIRI *et al.* 2003). Therefore, it is still an important task to identify donors of resistance against BaYMV disease carrying genes

different from *rym4* and *rym5*. Respective genes and markers will be valuable sources for broadening the genetic base of resistance.

Material and Methods

Genetic analysis was carried out on 163 DH-lines of the cross of 'Chikurin Ibaraki 1' to the susceptible cultivar 'Igri'. 'Chikurin Ibaraki 1' is resistant against European BaMMV, BaYMV and BaYMV-2 (GÖTZ & FRIEDT 1993).

Reaction to BaMMV was estimated on 10 plants of each DH-line by mechanical inoculation in the greenhouse according to Friedt (1983) followed by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using a BaMMV-specific antiserum (kindly provided by Dr. Frank Rabenstein, Federal Centre for Breeding Research, Aschersleben). Optical density was estimated photometrically at a wavelength of 405 nm and a reference wavelength of 620 nm.

DNA of each DH line was extracted according to DOYLE and DOYLE (1990). DNA concentration was determinate using a Fluorometer (Hofer Scientific Instruments) and diluted to a final concentration of 25 ng/µl. PCR was carried out in a GeneAmp System 9700 (Perkin Elmer, Almeda USA). According to the phenotypic data bulks comprising equal amounts of DNA of 15 DH-lines each were constructed for bulked segregant analysis (MICHELMORE *et al.* 1991). In order to assign resistance genes to chromosomes four SSR's per chromosome were analysed in a first step. SSRs were amplified according to LIU *et al.* (1996) and RAMSAY et al. (2000). PCR products were separated on a 8% Long Ranger Gel and detected fluorescence labelled on a LI-COR DNA sequencer Genreader 4200.

Determination of linkage was carried out using the Mapmaker software (LANDER *et al.* 1987). Crossover units were converted into map distances (cM) by appling the Kosambi functions (KOSAMBI 1949).

On the 120 exotic germplasm derived from the Barley Germplasm Center, Okayama University, Kurashiki, Japan molecular analysis was carried out as described above using Bmac29 closely linked to the *Rym4/Rym5* locus (GRANER *et al.* 1999).

Results and Discussion

120 gene bank accesions derived from Okayama classified as resistant against BaYMV in Japan were analysed by the SSR marker Bmac29 (GRANER *et al.* 1999) in order to identify new donors of resistance carrying genes different from *rym4* and *rym5*. It turned out, that out of these exotic germplasm 43 show the allele indicative for *rym5* and 12 the allel for *rym4*. The remaining genotypes not carrying *rym4* and *rym5* are potential candidates for detecting new resistance genes. Field tests for BaMMV, BaYMV and BaYMV-2 resistance in Europe are presently carried out and will be followed by extensive tests for allelism.

	rym4	*rym5*	different alleles
Number of Genotypes	12	43	65

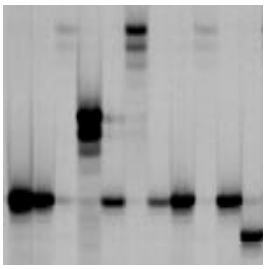

Fig. 1. Results of screening 120 exotic barley accessions for the presence of *rym4/rym5* by the SSR Bmac 29 (banding pattern of selected varieties)

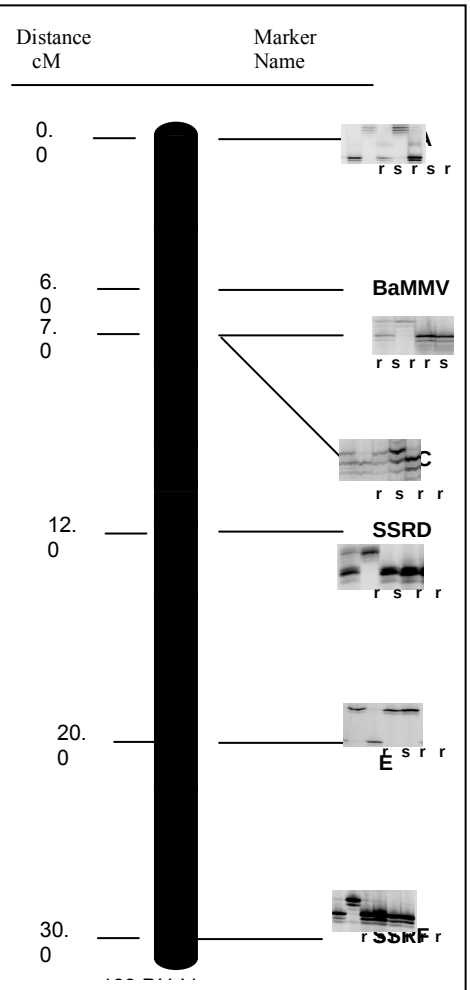

Fig.2. Partial map of barley chromosome 6H including the BaMMV resistance locus of 'Chikurin Ibaraki 1'

In order to develop markers for new resistance genes attempts were carried out to map the BaMMV resistance of the cultivar 'Chikurin Ibaraki 1'in a first step. The BaYMV/BaYMV-2 resistance of this cultivar has already been assigned to chromosome 5H (WERNER *et al.* 2003a).

Based on the ELISA-results a segregation ratio of 78r:85s (Chi^2=0.301) was observed giving hint to a single recessive gene effective against BaMMV. Using bulked segregant analysis employing 26 SSRs (RAMSAY *et al.* 2000) the BaMMV resistance of 'Chikurin Ibaraki 1' was mapped on chromosome 6H. Polymorphisms between the bulks containing 15 completly susceptible lines and 15 lines being resistant to BaMMV were observed with SSR B and SSR E. Additional SSRs located in the same chromosomal region were used for marker saturation. Well defined polymorphisms between the bulks were detected for SSR A, SSR C, SSR D and SSR F. Consequently, linkage analysis was carried out on the whole mapping population of 163 DH- lines with these SSR markers. The actual linkage map shown in Fig. 2 comprises six microsatellites with the closest linked at a distance of 1.0 cM.

Similar results concerning the BaMMV resistance of 'Chikurin Ibaraki 1' were observed by LE GOUIS et al. (2004). The knowledge of the chromosomal localization and especially the availability of molecular markers closely linked to the genes enables an efficient integration of these resistance genes into modern barley cultivars. With respect of mapping new resistance genes against BaMMV and BaYMV/BaYMV-2 additional DH-line populations are currently analysed.

Regarding the present situation in Japan and Europe, the potential risk of the selection of new virus strains of soil-borne mosaic inducing viruses, which are able to overcome resistance genes is obvious. In Japan seven strains of BaYMV and two of BaMMV have been described (NOMURA *et al.* 1996) and in France a new strain of BaMMV has been already reported infecting carriers of *rym5* (HARIRI *et al.* 2003). In this respect enlarging the resistance spectrum in modern barley cultivars is an important task for barley breeding. Therefore, exotic *Hordeum vulgare* germplasm are of special interest as donors of resistance genes. Many of these exotic cultivars possess resistance against all agents of the barley yellow mosaic virus complex with a BaMMV resistance not allelic to *rym4* (ORDON & FRIEDT 1993). This applies for example to the genes *rym11* from 'Russia 57' (BAUER *et al.* 1997) and to *rym13* from the cultivar 'Taihoku A' (WERNER *et al.* 2003b). Concerning 'Chikurin Ibaraki 1' the BaMMV resistance has been located in the present study on chromosome 6HS. These results are in accordance with those of LE GOUIS *et al.* (2004), who mapped the BaMMV resistance of this cultivar also on chromosome 6H.

The availability of easy to handle markers e.g. the SSR markers developed in this study is a prerequisite for an efficient integration of new resistance genes into modern barley cultivars as well as for combining these resistance genes (WERNER *et al.* 2000).

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Spike Morphology and Flowering Behavior Affecting the Resistance to Fusarium Head Blight in Barley

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Abstract

Barley varieties show a wide range of resistance to Fusarium Head Blight (FHB) and two-rowed type has been recognized more resistant than six-rowed one. And also, from our test for the resistance, two-rowed and closed-flowering, cleistogamous, barley cultivars form Japan belonged to the highest resistant group. In order to define the effect of spike morphology and flowering behavior to the resistance, we investigated the degree of the resistance for near-isogenic lines (NILs) of barley differing for these traits, using two testing methods, "pot-plant" and "cut-spike", in which spikes exactly at anthesis were inoculated with macroconidia suspension of *F. graminearum*. The largest difference of FHB severity was observed between chasmogamy and cleistogamy NIL pair and obviously greater than that of two-rowed and six-rowed spike NIL pair. Significant differences were not observed between lax/dense spike, normal/uzu-type and wax/wax-less spike NIL pair. The results indicate that cleistogamy and genetic background of Japanese two-rowed varieties make a great contribution to the FHB resistance and are useful germplasm for the resistance breeding.

Introduction

Fusarium head blight (FHB), caused by several *Fusarium* species, is a wide spread and destructive disease of barley. FHB reduces yield and quality, and also produces mycotoxins which are toxic to human and animal. Barley varieties show a wide range of resistance to FHB and the resistant cultivars is most cost effective for controlling the disease. The resistant breeding have been carried on for several decades, and two-rowed cultivars which show resistant ware released and cultivated in southern reaches of Japan. But the breeding for highly resistance to FHB has been difficult, because genetic resources of immunity to FHB is unsuspected, and also inheritance of the resistance is complex and have been found to associate with various spike characters such as row type (HETA & HIURA 1963; TAKEDA & HETA 1989), kernel density (CHEN *et al*. 1991) and anther exsertion (HETA & HIURA 1963) in barley.

The effect of row type have been noted, because two-rowed types generally are more resistant than six-rowed types and the most resistant varieties recognized so far are all two-rowed types (HETA & HIURA 1963; TAKEDA & HETA 1989). The association between *Vrs1* locus and FHB resistance was demonstrate by genetic study (TAKEDA 1990) and QTL analysis with the Frederickson (two-rowed, moderately resistant)/Stander (six-rowed, susceptible) population (MESFIN *et al*. 2003). However, it has not been clear whether the resistance comes from pleiotropic effect of *Vrs1* locus or tightly linkage.

Flowering behavior, which is directly related to initial infection of the pathogen into florets and anther exsertion, attracted our notice. Almost all of Japanese two-rowed resistant cultivars are cleistogamous (closed-flowering type) while six-rowed cultivars are mostly chasmogamous (opened-flowering type) and are moderate or susceptible to FHB. HETA & HIURA (1963) found that the resistant varieties have more florets without anther exsertion

than susceptible varieties, suggesting cleistogamous varieties are more resistant than chasmogamous ones. Therefore, it seemed likely that flowering behavior may affect FHB resistance and contribute to the difference of FHB resistance level between two- and six-rowed cultivars.

In order to develop the breeding strategy, we need to know the effect of such spike characters to FHB resistance. The best way available to assess contribution of such traits to the resistance would be by comparing the response to FHB of near-isogenic lines (NILs) for the target traits. Previously, STEFFENSON *et al*. (1996) assessed contribution of the traits to FHB development using NILs in the field. They found six-rowed NILs and dense spike NILs had more FHB infection than two-rowed NILs and lax spike NILs, respectively, however, no significant difference was detected in the NIL pairs. To know the effects of the traits well, it is desirable to reassess them in well-controlled testing environment and/or in different genetic background. In this study, we investigated the effect of several spike characters, such as, row type, flowering behavior, wax-coating on the spike, kernel density, uzu or semi-brachtic type and lateral floret size, to FHB resistance, using NILs for the characters.

Material and Methods

NILs differing for the target traits with genetic background of Japanese varieties, 'Misato Golden', 'Kanto Nijo 29' and 'New Golden', were developed and tested for FHB resistance (Table 1). The parent varieties are all two-rowed, cleistogamy, hulled, and classified as 'resistant' to FHB. The NILs introduced with chasmogamy have more lax spike than the parent. All the materials were planted in plastic pots of 18cm diameter (four plants in each pot) and grown in a greenhouse until heading.

A strain of *F. graminearum* H-3 originally isolated from a wheat spike in Japan was used in this study. Macroconidia of the strain were produced by growing the fungus in mungbean liquid medium (20g of mungbean is boiled in 1L of water). The macroconidia suspension was filtered to remove hyphae, centrifuged, and the pellet was suspended in water. The macroconidia concentration was finally adjusted to 5×10^5/mL.

The reaction to FHB was performed using 'pot-plant' method (YOSHIDA *et al*. 2003) and 'cut-spike' method (TAKEDA & HETA 1989) in the early spring. On the 'pot-plant' method, the spikes of potted plants exactly at anthesis were spray-inoculated, then nursed in a greenhouse at 20±5°C with 100% humidity overnight with humidifiers. The inoculation procedure was performed 2 times for each pot in 2 consecutive days in order to avoid escapes. The plants after inoculation were placed in the greenhouse (20±5°C) equipped with sprinkler system to keep the inoculated spikes wet during the test. The 'cut-spike' test was performed according to the procedure developed by Takeda and Heta (1989) with some modifications. Three spikes were cut off at third internodes exactly at anthesis and spray-inoculated together. The inoculated spike sets were cultured in tap water and infection was promoted in a dew chamber at 100% humidity and 25°C for a day. After that, the spike sets were cultured on running water in a growth chamber at 18-25°C with 80-100% humidity.

In both of the testing methods, the FHB severity was observed at a week after inoculation and scored from 0 to 9 as follows: 0= no disease; 1= a floret/spike is diseased; 2= two florets/spike – up to 10% of the florets are diseased; 3, 4, 5, 6, 7, 8= up to approximately 30%, 40%, 50%, 60%, 80%, 90% of the florets are diseased respectively; 9= almost or all florets are diseased. The tests were performed using both of the two methods from 2001 to 2003 with more than three replications. Differences of the means of FHB severity between NILs and the parents were tested for statistical significance using t-test.

Table 1. Near isogenic lines used in the present study

No.	Line [1]	Introduced character [2]	Pedigree [3]	Generation [4]		
				2001	2002	2003
1	Misato Golden	-	-	P	P	P
2	iso vrs1 of MG-1	six-rowed	Kashimamugi/8*MG	B_4F_3	B_6F_3	B_7F_3
3	iso vrs1 of MG-2	six-rowed	Kashimamugi/8*MG	-	B_6F_3	B_7F_3
4	iso vrs1 of MG-3	six-rowed	MG Mut. /3*MG	$M_2B_1F_3$	$M_2B_2F_3$	$M_2B_2F_4$
5	iso cly of MG-1	chasmogamy	Satsuki Nijo/6*MG	-	M_5F_3	B_5F_4
6	iso cly of MG-2	chasmogamy	Satsuki Nijo/6*MG	-	M_5F_3	B_5F_4
7	iso cly of MG-3	chasmogamy	Satsuki Nijo/8*MG	-	-	B_7F_2
8	iso cly of MG-4	chasmogamy	Satsuki Nijo/8*MG	-	-	B_7F_2
9	iso cly of MG-5	chasmogamy	Bozu/6*MG	-	B_4F_3	M_5F_3
10	iso cly of MG-6	chasmogamy	MG Mut.	-	-	M_3
11	iso cer(-) of MG	eceriferum(-)	MG Mut. /MG	M_6	M_5F_3	-
12	iso uzu of MG	uzu, semi-brachtic	Kashimamugi/8*MG	B_5F_3	B_6F_3	B_7F_3
13	Kanto Nijo 29	-	-	P	P	P
14	iso Vrs1.t of KN29	deficiens	KN29 Mut. /2*KN29	M_6	M_6	$M_5B_1F_3$
15	iso cer(-) of KN29	eceriferum(-)	KN29 Mut. /KN29	M_6	M_5F_3	-
16	iso cer(+) of N29	eceriferum(+)	KN29 Mut.	M_6	M_7	-
17	iso dsp of KN29-1	dense spike	KN29 Mut. /KN29	-	M_7	M_5F_3
18	iso dsp of KN29-2	dense spike	KN29 Mut. /KN29	-	M_7	M_5F_3
19	New Golden	-	-	P	P	P
20	iso vrs1 of NG	six-rowed	NG /NG Mut.	M_7	M_6F_3	M_6F_4
21	iso cer(-) of NG	eceriferum(-)	NG Mut.	M_4	M_4	-

1) The parent varieties are shown with under line. MG, KN29 and NG are abbreviations of the parents, Misato Golden, Kanto Nijo 29 and New Golden, respectively.
2) eceriferum: surface wax coating on the spike appears absent(-) or reduced(+).
3) Mut.: induced mutant lines.
4) Generation of each line in the tested year is shown. -: Not tested.

Results and Discussion

The resistance to FHB of the NILs and those parents are shown in Table 2. All the parents of the NILs presented with resistance scored from 0.5 to 2.3 as compared to the susceptible check 'Minorimugi' throughout the tests. Significant difference of FHB severity between NILs and its parents were found in some cases. The largest difference was observed between chasmogamous NILs and the parent. All the chasmogamous NILs were more diseased than the parent throughout the tests, and the differences were significant ($P<0.01$ or $P<0.001$) with one exception (No. 7, in the 'pot-plant' test). Six-rowed NILs also showed significantly higher FHB severity than the parents ($P<0.05$ or $P<0.01$) in some cases of the both tests, however, in the other cases no difference was detected in the two-/six-rowed pairs. The non-glaucous (wax-less in spike) NILs and the deficiens NIL exhibited significantly greater FHB severity than the parents in only one of more than three cases for each line ($P<0.05$). The less-glaucous (wax-reduced in spike) NIL , uzu type NIL and dense spike NILs didn't show significant difference in FHB severity with the parent through the tests.

Previous reports and our tests showed that two-rowed types generally are more resistant than six-rowed types, and also, the highest resistant group were cleistogamous two-rowed varieties and chasmogamous six-rowed varieties were mostly susceptible (HETA & HIURA 1963; TAKEDA & HETA 1989). Row type and flowering behavior may have a potential for the difference of FHB resistance in barley, however, we can't assess the effect of such traits to

the resistance only by comparing varieties differing in the traits, since such varieties have different genetic background and would have other factors contributing to FHB resistance. In order to evaluate the possible effect of such characters to the resistance, we developed NILs for several spike traits including row type and flowering type, and evaluated the resistance of them.

In the present tests, cleistogamous/chasmogamous NIL pairs showed the greatest difference in some traits of spike (Table 2). It demonstrates that chasmogamy contributes to FHB infection in accord with the previous studies by evaluation of varieties or breeding lines (HETA & HIURA 1963; VIVAR *et al*. 1997). This trait open a floret at anthesis and may allow the pathogen to enter easily into inside of the floret for infection, and/or, an anther shoot out of florets and may promote the pathogen colonization on the florets. Previous studies on wheat have shown that the initial infection with FHB occurs on anthers that are either extruded or caught in the mouth of florets (PUGH *et al*. 1933). Barley commonly undergo anthesis after heading, however, in some other barley growing environments these events occur while the spike is still enclosed within the flag leaf sheath (STEFFENSON 2003). Contribution of chasmogamy to FHB infection is important trait for the resistance when anthesis occurs after heading.

Six-rowed NILs were more diseased than the two-rowed parent, and the difference was not always found and not so great (Table 2). It suggests that row type affects FHB resistance, however, the effect is little and unstable, and obviously smaller than that of flowering behavior. In a previous study testing NILs in the field, six-rowed NILs were more infected than two-rowed NILs, but without significant difference (STEFFENSON *et al*. 1996). It seems to also suggest that the effect of row type is not so great and the effect of *Vrs1* locus on FHB resistance is just due to the effects of the spike morphology itself. The difference of resistance among the two- and six-rowed varieties is greater than difference estimated from the row type effect, indicating that other factors than row type is involved in the difference between two- and six-rowed varieties, however, it is unclear from our present results.

With regard to other spike traits, no or little differences were observed in the NIL pairs (Table 2). There have not been any reports describing association between wax-coating on spike and FHB resistance. In our NILs test, non-glaucous NILs were slightly more infected than the parent in only a few cases and the less-glaucous spike NIL didn't show significant difference through the tests, suggesting that wax-coating may have a potential to reduce FHB infection, though the effect would be very small. Kernel density and lateral floret size have been reported to associate with FHB resistance, however, the relationship has not been clear. Recent molecular mapping studies (ZHU *et al*. 1999; MA *et al*. 2000) suggested the quantity traits loci for the resistance coincided with kernel density and lateral floret size in barley populations. However, weak negative correlation between FHB severity and spike density was reported (URREA *et al*. 2002). The present study using NILs indicated that lateral floret size (deficiens) and dense spike including uzu-type would have practically no effect to FHB resistance.

The present study indicated that flowering behavior would have the greatest effect on FHB resistance and row type would only have small effect, however, a great difference of the resistance among two- and six-rowed Japanese varieties couldn't be explained only by the row type effect estimated by the NILs test, suggesting flowering behavior and/or other factors would be affecting the resistance. All the materials used in our study have genetic background of Japanese two-rowed barley which possesses good FHB resistance. Cleistogamy and the genetic background of the resistant two-rowed varieties contribute to the resistance more than row type. Therefore, it would be possible to develop six-rowed varieties with good FHB resistance nearly as the resistant two-rowed varieties of Japan. The resistant germplasms generally have undesirable agronomic traits such as plant height and late heading,

moreover, in many cases such traits are linked or associated with FHB resistance (URREA *et al*. 2002; STEFFENSON 2003). Such situations have made FHB resistance breeding more difficult. However, most of the resistant two-rowed germplasms we showed in this paper are commercial malting barley in Japan, which don't possess such undesirable agronomic traits. Therefore, development of FHB resistant six-rowed cultivar by transferring resistance from such two-rowed germplasms can be expected to work successfully.

Table 2. Comparison of the reaction to FHB between the NIL pairs and the parent

No.	Line	Susceptible score [1)]					
		'pot-plant' test			'cut-spike' test		
		2001	2002	2003	2001	2002	2003
1	Misato Golden	0.5	0.5	0.7	0.4	0.4	0.0
2	iso vrs1 of MG-1	0.2	1.3 *	-	0.6	0.1	-
3	iso vrs1 of MG-2	-	0.7	-	-	0.1	-
4	iso vrs1 of MG-3	0.8	2.0 **	-	1.0 *	0.1	-
5	iso cly of MG-1	-	1.5 **	-	-	2.1 ***	-
6	iso cly of MG-2	-	1.5 **	-	-	2.1 ***	2.1 ***
7	iso cly of MG-3	-	-	0.9	-	-	1.8 **
8	iso cly of MG-4	-	-	-	-	-	2.5 ***
9	iso cly of MG-5	-	2.0 **	-	-	3.0 ***	-
10	iso cly of MG-6	-	-	1.8 **	-	-	-
11	iso cer(-) of MG	0.7	0.5	-	1.2 *	0.1	-
12	iso uzu of MG	1.0	1.2	0.5	0.2	0.2	-
13	Kanto Nijo 29	0.5	1.0	2.3	1.8	0.0	0.4
14	iso Vrs1.t of KN29	1.5 *	1.2	1.3	0.3	0.0	-
15	iso cer(-) of KN29	0.8	0.8	-	1.6	0.3 *	-
16	iso cer(+) of N29	0.5	0.5	-	0.3	0.2	-
17	iso dsp of KN29-1	-	1.5	-	-	0.1	-
18	iso dsp of KN29-2	-	0.5	-	-	0.1	-
19	New Golden	1.3	0.9	0.3	0.4	0.0	0.1
20	iso vrs1 of NG	2.7 **	1.7	-	0.7	0.0	-
21	iso cer(-) of NG	2.0 *	1.2	-	-	0.2	-
	Minorimugi [2)]	6.0	5.0	4.3	6.5	3.2	3.5

1) *, **, *** indicates statistical significance (P<0.05, 0.01, 0.001, respectively) between the NILs and the parent (shown with under lines) according to t-test. -: Not tested.

2) The data of a chasmogamous six-rowed variety 'Minorimugi', as a susceptible check, were shown.

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Mapping of Resistance to BaYMV/BaYMV-2 in the Japanese Winter Barley Accession HHOR 4224

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Abstract
The inheritance of resistance to *Barley mild mosaic virus* (BaMMV) and *Barley yellow mosaic virus* (BaYMV, BaYMV-2) of the Japanese winter barley accession HHOR 4224 was analysed in a doubled haploid population (DH) derived from a cross to the susceptible variety HHOR 10714. Reactions to the viruses were assessed in infested fields (BaMMV, BaYMV, BaYMV-2) and by mechanical inoculation in a growth chamber (BaMMV). Reaction to the different viruses was determined by DAS-ELISA. Results of these genetic analyses suggest the presence of two independently inherited genes effective against BaMMV and against BaYMV/BaYMV-2, respectively. Using bulked segregant analysis (BSA) employing SSRs and AFLPs the gene determining resistance to BaYMV-/BaYMV-2 was mapped on chromosome 5H with the two closest markers Bmac303 and Bmac096 flanking it in a distance of 1 cM.
Keywords: *Hordeum vulgare*; Barley mild mosaic virus; Barley yellow mosaic virus; resistance; SSRs; AFLPs; genetic mapping

Introduction

The viruses of the barley yellow mosaic virus complex (*Barley mild mosaic virus*, BaMMV, *Barley yellow mosaic virus*, BaYMV and BaYMV-2) cause one of the most important diseases of winter barley in Europe. Due to transmission by the soil-borne fungus *Polymyxa graminis* (TOYAMA & KUSABA 1970) growing of resistant cultivars is the only possibility to control the disease. This fact is reflected in the list of registered German cultivars: out of the 81 cultivars registered in Germany 45 cultivars are resistant to BaMMV and BaYMV (ANONYMOUS 2003). With the exception of the six-rowed cultivars 'Anastasia', 'Structura' and the two-rowed cultivars 'Kamoto' and 'Kyoto' which are in addition resistant to BaYMV-2 due to *rym5,* the resistance of these cultivars is based on *rym4*. Due to the fact that the genetic base of resistance in German cultivars is quite narrow large screening programmes were carried out mainly on the barley germplasm collection of the Institute of Plant Genetics and Crop Plant Research, Gatersleben to identify winter barley accessions with complete resistance to the soil-borne mosaic viruses.
In order to find accessions being resistant to all agents of the barley yellow mosaic virus complex known in Germany but not carrying *rym5* respective germplasms were analysed using the SSR marker Bmac29 closely linked to *rym5* (HABEKUSS *et al.* 2000). Selected genotypes were crossed to susceptible cultivars and F_1 derived doubled haploid populations were produced. In the frame of these studies the inheritance of complete mosaic resistance in the barley accession HHOR 4224 was investigated on a doubled haploid progeny of a cross to the susceptible genotype HHOR 10714.

Material and Methods

Phenotypic Evaluation

The DH population including 107 lines was produced using the '*bulbosum*' technique (KASHA & KAO 1970; PICKERING & DEVAUX 1992). Virus resistance tests were carried out on naturally infested fields, artificially layed out plots and by mechanical inoculation in a growth chamber (Fig. 1). In early spring all lines were serologically analysed by DAS-ELISA to determine the presence of the different viruses in leaf tissue.

Figure 1. Resistance tests in the field; Evaluation of the DH progeny to BaMMV, BaYMV and BaYMV-2

DNA Preparation

Leaf tissue for DNA extraction was harvested, freeze-dried and grinded into powder. DNA was isolated from each sample according to GRANER *et al.* (1990) and quantified by fluorometric analysis (DyNA Quant 200, Hoefer).

DNA Marker Screening

According to the phenotypic results bulks were created by combining equal amounts of DNA from ten DH lines for bulked segregant analysis (MICHELMORE *et al.* 1991). Seven microsatellites per chromosome were investigated in order to assign resistance genes to chromosomes. PCR amplifications were performed in a GeneAmpSystem 9700 (Perkin Elmer). Reactions and cycler conditions for microsatellites followed BECKER and HEUN (1995), LIU *et al.* (1996) and RAMSAY *et al.* (2000). Furthermore, the bulks were screened using 144 AFLP primer combinations. The restriction enzymes used in preparation of AFLP templates were *Eco*RI and *Mse*I. A first pre-amplification was carried out with *Eco*RI + 0 and *Mse*I + 0 primers. Primers with one additional nucleotide (*Eco*RI primer + A and *Mse*I primer + C or + A) were used for the second pre-amplification, whereas primers with three additional nucleotides were used for selective amplifications. AFLP analyses were conducted using the GIBCO BRL AFLP Core Reagent Kit following respective protocols. Amplified fragments were separated on automated laser fluorescence sequencer (ALF Express, Amersham Biosciences) on ReproGel High Resolution (Amersham Biosciences).

Linkage Analysis

Linkage analyses were performed using the JoinMap program, version 3.0 (VAN OOIJEN & VOORRIPS 2001). The segregation of 29 markers was studied and a LOD score of 5.0 was used to estimate the position of these markers on the map. The Kosambi mapping function was used to convert recombination frequencies to map distances (cM).

Results and Discussion

Results of resistance tests including BaMMV, BaYMV and BaYMV-2 are shown in Table 1. In genetic analyses of the DH population consisting of 107 DH lines a segregation in four different phenotypic classes was observed: 21 DH lines were exclusively resistant to BaMMV, 22 DH lines were resistant to BaYMV and BaYMV-2, 28 DH lines completely susceptible and 36 DH lines completely resistant. The $\chi 2$ test determined that the ratio 21 : 22 : 28 : 36 fitted well to the expected segregation ratio 1 : 1 : 1 : 1, indicating that two genes control the resistance to the mosaic viruses. The results give hint that BaMMV in HHOR 4224 is inherited independently from the resistance to BaYMV and BaYMV-2.

Table 1. Segregation of DH lines of the cross HHOR 4224 x HHOR 10714

Virus	Phenotypic classes				X^2 (FG 3)
	1	2	3	4	
BaMMV	s	r	s	r	
BaYMV/BaYMV-2	s	s	r	r	
Number DH lines	28	21	22	36	5.336

s = susceptible; r = resistant

Consequently bulked segregant analysis using microsatellites was carried out in order to localise respective resistance genes on the barley chromosomes. Out of the 49 SSR markers investigated three microsatellites (Bmag337, Bmac096 and HVM30) located on chromosome 5H were polymorphic between the bulk being susceptible to BaYMV/BaYMV-2 (class 1 and 2, Table 1) and the bulk containing DH-lines resistant to BaYMV/BaYMV-2 (class 3 and 4, Table 1). Consequently, additional SSRs located on chromosome 5H were analysed and linkage was detected for Bmac303, Bmag387, Bmac113, Bmag005 and HVACL1 (Fig. 2). By this approach resistance to BaYMV/BaYMV-2 was assigned to chromosome 5H. In addition to SSRs, AFLPs were applied in order to develop closely linked markers. Altogether eight microsatellites and 21 AFLP markers could be detected with linkage to the resistance locus. Subsequently, the whole DH progeny was analysed with the selected markers to map the BaYMV/BaYMV-2 resistance. The genetic map of chromosome 5H including the BaYMV/BaYMV-2 resistance is shown in Fig. 2. The resistance locus is flanked by the SSRs Bmac096 and Bmac303 at a distance of 1 cM. Current experiments are focused on mapping the BaMMV resistance of HHOR 4224.

In the same region of chromosome 5HS the gene conferring resistance to BaYMV/BaYMV-2 in the Japanese cultivar ‘Chikurin Ibaraki 1’ has been mapped (WERNER *et al.* 2003) as well as the resistance gene *rym3* of ‘Ea 52’ (SAEKI *et al.* 1999) which is a gamma ray induced mutant of ‘Chikurin Ibaraki 1’ (UKAI 1984). In this respect it is interesting to notice that ‘Chikurin Ibaraki 1’ is susceptible in Japan but completely resistant in Germany while ‘Ea 52‘ is resistant to BaYMV and BaYMV-2, only (GÖTZ and FRIEDT 1993). The BaMMV resistance of ‘Chikurin Ibaraki 1’ has recently been mapped on chromosome 6H (LE GOUIS *et al.* 2004). Therefore, attempts to locate the BaMMV resistance of HHOR 4244 will focus on this chromosome. Closely linked markers, which are available for many resistance genes against the barley yellow mosaic virus complex (cf. WERNER *et al.* 2003) meanwhile, on the one hand facilitate efficient marker based selection procedures and on the other hand pyramiding of resistance genes (WERNER *et al.* 2000) may be leading to longer lasting resistances.

This is of special importance because recently a new strain of BaMMV has been detected in France (HARIRI *et al.* 2003).

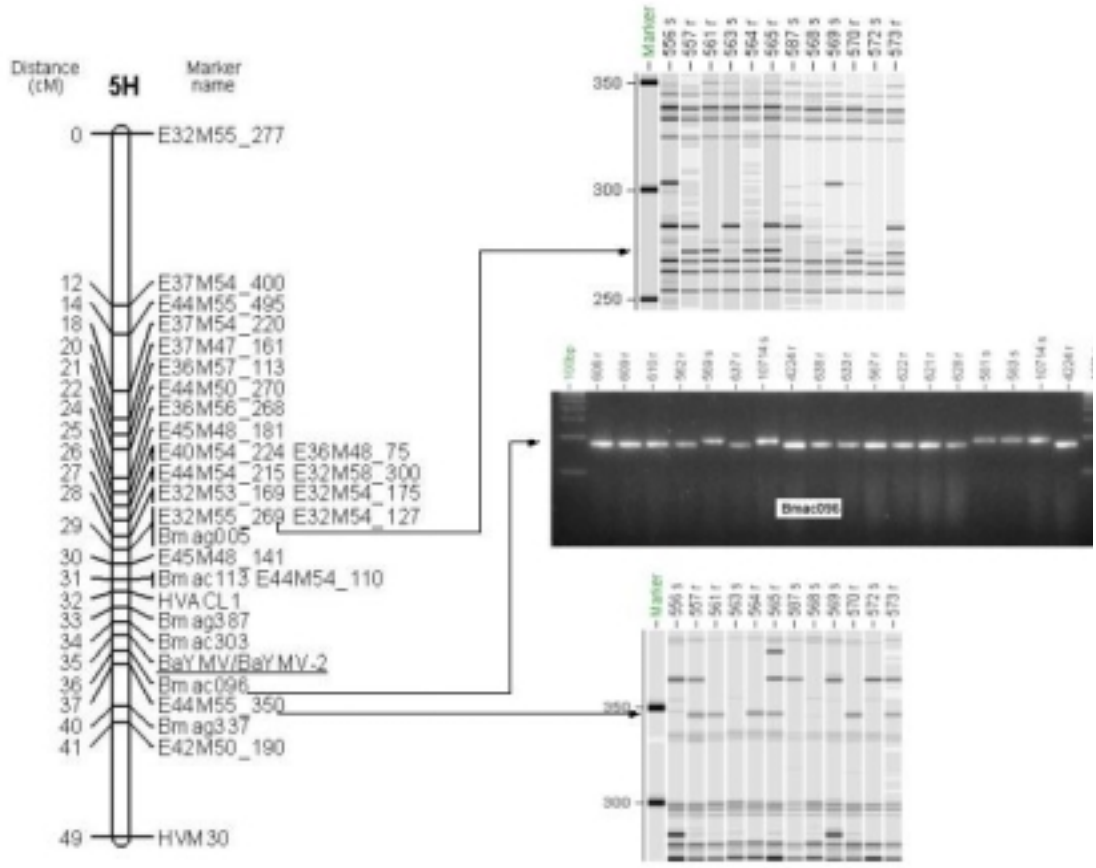

Figure 2. Genetic linkage map of BaYMV/ BaYMV-2 resistance locus with SSR and AFLP markers on chromosome 5H
(s = susceptible; r = resistant)

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Genetic Associations of FHB Reaction and Morphological Traits in Barley

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Abstract

Partial resistance of barley (*Hordeum vulgare* L.) accessions to Fusarium head blight (FHB), incited by *Fusarium graminearum* Schwabe, is often associated with morphological traits such as spike type, plant height, and maturity. Disease occurrence and severity is often lower in tall, late, two-rowed lines, which are agronomically undesirable. Also, expression of traits such as late heading, tall culms, and two-rowed spikes can interfere with accurate measurement of FHB resistance and may increase escape from infection. Several quantitative trait loci (QTLs) for FHB resistance have been mapped in chromosome 2H, which contains genes for the six-rowed spike type (*vrs1*), two long-day sensitive maturity factors (*Eam1* and *Eam6*), and at least two short culm traits (*hcm1* and *lin1*). Near-isogenic lines and 'Harrington'/'Morex' (HM) mapping population were evaluated to study the linkage vs. pleiotropy hypothesis for morphological traits in chromosome 2H. The results suggest that QTLs for FHB reaction and several morphological traits are tightly linked. A significant effort will be necessary to break several unfavorable linkages or to replace the maturity and plant height genes currently present in barley cultivars adapted to the Upper Midwest of the USA.

Introduction

Fusarium head blight of barley is a serious problem worldwide causing devastating economic losses. Besides the reduction of yield and quality, FHB results in the accumulation of the mycotoxin deoxynivalenol (DON). DON is of concern in food safety and can make barley unacceptable for malting and brewing. Development of spring barley cultivars with resistance to FHB is difficult because the inheritance of FHB resistance and its interactions with morphological traits are complex (MARQUEZ-CEDILLO *et al*. 2001). FHB severity is often lower in two-rowed genotypes than in six-rowed genotypes. Spike morphology in barley is controlled primarily by the six-rowed spike 1 (*vrs1*) locus located in chromosome 2HL. The *vrs1.a* allele is present in most six-rowed cultivars and produces well-developed lateral spikelets. The *Vrs1.b* allele reduces lateral spikelets to sterile bracts with a rounded tip in most two-rowed cultivars. The *Vrs1.t* (deficiens) allele causes an extreme reduction in the size of lateral spikelets. Alleles at the intermedium spike-c locus (*int-c*) in chromosome 4HS alter the size of lateral spikelets in the presence of *vrs1.a, Vrs1.b,* and *Vrs1.d,* but not when *Vrs1.t* is present. Development of large lateral kernels in six-rowed barley is enhanced by the *Int-c.a* allele at the *int-c* locus (DAVIS *et al*. 1997). FHB occurrence and severity were thought to be determined in part by the spike morphology, because six-rowed spikes may alter air-movement around the spikelets and produce conditions favorable for FHB onset. QTLs for FHB resistance were mapped in 2H close to the *vrs1* locus suggesting that a genetic association between spike morphology and FHB reaction might exist (ZHU *et al*. 1999). The expression of traits such as heading date and plant height might interfere with the accurate measurement of resistance because late and tall plants may partially escape infection. QTLs for heading date and FHB resistance were found to be coincident (de la PENA *et al.* 1999), and a QTL for plant height appeared to be in the same region of 2H (MA *et al*. 2000). Coincident QTLs may be due to

linkage or pleiotropy (MESFIN *et al*. 2003). If this association is caused by pleiotropy, breeding FHB resistant barley cultivars will be very difficult.
The objectives of this study were: 1) to test the hypothesis that FHB severity is determined by the spike morphology in barley; and 2) to evaluate the Harrington/Morex doubled-haploid population for FHB reaction, heading date, and plant height in both long- and short-day environments. Harrington, a Canadian two-rowed cultivar, and Morex, a Midwest six-rowed cultivar, are recommended for malting in the US. More information about genetic control of maturity and plant height in barley can aid in development of breeding strategies.

Material and Methods
For the first experiment, 36 cultivars and backcross-derived lines containing spike morphology variants were inoculated and screened for FHB resistance. The backcross-derived lines were produced by crossing marker stocks five to seven times to the cultivar Bowman. A partially balanced lattice design with three replicates was applied. Entries were planted in 2001 and 2002 at three locations, one in Hangzhou (China), and two in North Dakota (ND), Langdon and Osnabrock, in hill plots. For the second experiment 140 HM lines, their parents (Harrington and Morex), and the checks (CIho 4196, Bowman, and 'Conlon') were arranged in a randomized complete block design with two replicates, and grown in two environments in 2002: Langdon, ND (LA02) and Osnabrock, ND (OS02); and three environments in 2003: Hangzhou, China (CN03), Langdon, ND (LA03), and Osnabrock, ND (OS03). Plant height was measured in the CN03, LA03, and OS03 nurseries as centimeters from the soil surface to the tip of inflorescence excluding awns. Heading date was estimated in the CN03 and OS03 nurseries as the number of days from January 1 to when approximately 50% of the heads were half emerged from the boot.
Fusarium inoculum was prepared according to PROM *et al*. (1996). Disease readings were taken at the soft dough stage. The percent severity of FHB was determined by counting the number of infected kernels and dividing that quantity by the total number of kernels in that spike, multiplied by 100. Assessments were made on 10 randomly selected spikes per plot. Grain samples were harvested from the LA03 and CN03 nurseries for DON determinations. Tests of DON accumulation in grain were performed on the harvested and threshed grain samples in the Barley Quality Laboratory of the Plant Sciences Department at NDSU. Acetonitrile method with water extraction, silylation, and DON quantitation by gas chromatography with electron capture detection was applied (TACKE & CASPER, 1996).
Analyses of variance for FHB severity, heading date, and plant height were conducted for each environment by means of GLM procedures of SAS (1990). Error mean squares across all the environments were not homogeneous as determined by Bartlett's chi-square test; thus, a combined ANOVA across environments was not conducted. Phenotypic data sets on HM lines from the five environments, and the published 107-marker linkage map for the HM population (MARQUEZ-CEDILLO *et al*. 2001) were used to perform QTL analyses with the software package NQTL. Both simple interval mapping (SIM) and simplified composite interval mapping (sCIM) techniques were used for QTL detection (TINKER & MATHER, 1995). Each data set was analyzed with 1000 permutations, a 5-cM walking speed, and a Type-I error rate of 5%. Coincident peaks with both SIM and sCIM analysis above the significance threshold were used to declare the presence of QTL.

Results and Discussion
The analysis of FHB severity data for lines with different spike morphologies shows that most of the genotypes responded similarly across environments, with the exception of Chinese nursery

(Table 1). A significant association was observed between FHB severity and spike type. The presence of *vrs1.a* and *Int-c.a* alleles that promote the development of lateral spikelets seemed to enhance the disease susceptibility. This appeared due to tight linkage between the spike morphology and FHB susceptibility genes in 2HL and 4HS. In the case of the elongated outer glume mutants (*eog1.a* and *eog1.c*), which add extra tissue to the spike, the disease readings were not significantly different from those of Bowman. Reduced size of lateral spikelets determined by the *sls1.a* allele or the absence of lateral spikelets (*Vrs1.t*) did not reduce FHB severity. Entries with *Int-c.a*, *int-c.5*, and *vrs5.n* alleles at the *int-c* locus or the *vrs1.a* and *vrs1.c* alleles at the *vrs1* locus tended to have higher FHB severity readings.

Table 1. Mean FHB severity value (%) of selected barley cultivars and Bowman backcross-derived lines.

Cultivar	Alleles			2001		2002		
				North Dakota		China	North Dakota	
	vrs1	***int-c***	**other**	**Langdon**	**Osnabrock**		**Langdon**	**Osnabrock**
LACEY	*vrs1.a*	*Int-c.a*		45.2	37.1	55.8	39.3	28.5
ALELI	*Vrs1.t*	*int-c.b*		34.3	26.2	48.5	29.2	43.1
MOREX DHP	*vrs1.a*	*Int-c.a*		45.1	36.4	45.9	21.2	25.9
CANELA	*Vrs1.t*	*int-c.b*		33.5	22.4	64.9	21.5	28.6
CONLON	*Vrs1.b*	*int-c.b*		28.8	18.6	48.6	20.2	22.5
BOWMAN	*Vrs1.b*	*int-c.b*		26.4	16.7	46.7	19.3	23.9
Six-rowed spike 1 locus								
I94-403	***vrs1.c***	*int-c.b*		40.7	31.0	74.1	42.9	40.6
I93-520	***vrs1.a***	*int-c.b*		46.6	42.0	54.8	41.9	31.7
I90-137	***Vrs1.t***	*int-c.b*		30.0	21.5	49.9	14.9	28.4
Intermedium-c locus								
I98-504	*Vrs1.b*	***int-c.5***		54.3	41.6	47.6	51.8	57.9
I97-404	*Vrs1.b*	***Int-c.a***	*Vbn1.a*	26.9	24.4	48.5	26.3	38.3
Elongated outer glume								
I93-515	*Vrs1.b*	*int-c.b*	***eog1.a***	30.3	22.9	33.3	13.6	19.9
I92-512	*Vrs1.b*	*int-c.b*	***eog1.c***	27.4	18.6	44.8	18.9	27.9
Small lateral spikes								
I91-499	*Vrs1.b*	*int-c.b*	***sls1.a***	25.8	18.3	52.8	25.3	28.5
LSD (0.05)				7.5	6.8	13.5	13.4	12.9
CV				19.4	22.1	16.5	32.4	25.0

Harrington/Morex population - The frequency distributions in the HM population were continuous for all traits, except spike type at each of the test locations.

FHB and DON Results. Significant differences among HM lines were observed for all traits that were evaluated. The differentiation among lines for FHB reactions appeared best for the OS02, CN03, and LA03 nurseries (Table 2). Phenotypic correlation coefficient values for FHB severity among all environments were significant with a range of 0.40 to 0.69. The moderately resistant parent (Harrington) exhibited lower FHB severity than the moderately susceptible parent (Morex). Few HM lines had lower FHB scores than Harrington, but several had higher FHB

scores than Morex. With the exception of Chinese nursery, most lines were within the bounds of LSD (P=0.05) and were not transgressive segregates for increased FHB resistance. There was a notable trend for two-rowed lines to be more FHB resistant. Some HM lines showed similar levels of resistance as of the resistant check CIho 4196.

Tests showed unacceptable levels of DON accumulation in all HM lines, their parents and checks. Mean values ranged from 1.5 to 47.5 ppm at LA03 and from 12.9 to 120.6 ppm at CN03. Correlations between FHB severity and DON accumulation were significant (P<.001) and positive: 0.41 at LA03 and 0.51 at CN03. These results are consistent with the previous findings that FHB susceptibility and DON accumulation have low correlation values, and might be controlled partially by independent genetic factors (de la PENA *et al.* 1999; MA *et al*. 2000).

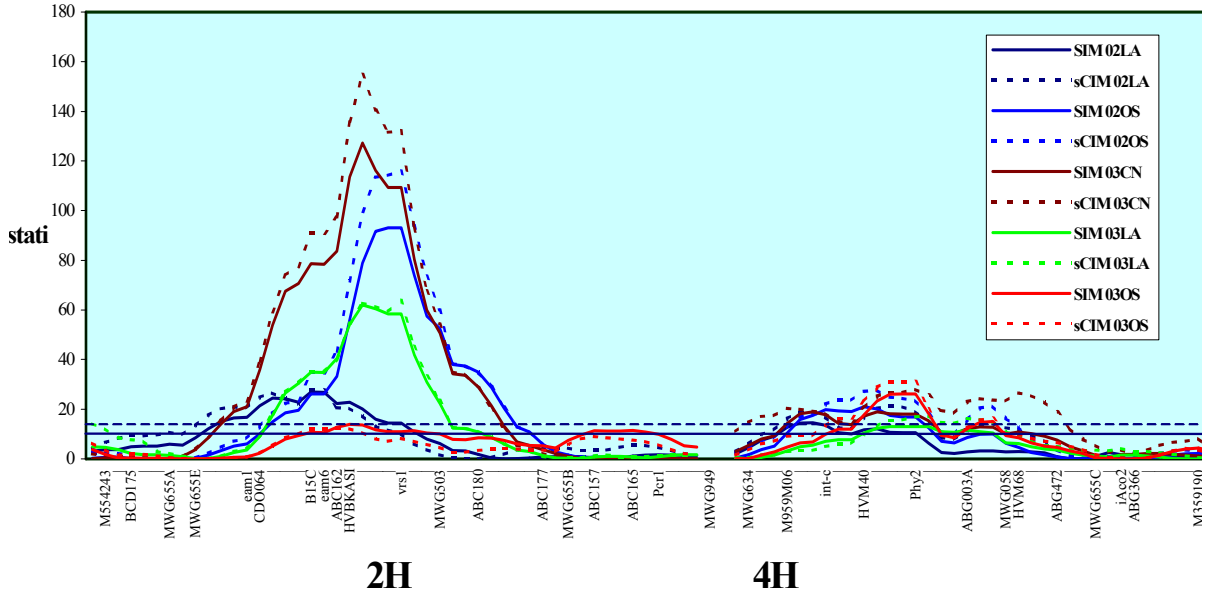
Table 2. Mean values for FHB severity, heading date, and plant height for HM lines, parents, and checks.

Cultivar	Spike Type	FHB %			Heading date days from Jan 1		Plant height cm	
		2002	2003		2003		2003	
		Osnabrock	China	Langdon	China	Osnabrock	China	Langdon
Checks								
CIho 4196	2	8.7	0.1	22.1	115.4	201.5	109.1	114.0
Harrington	2	**10.3**	**20.3**	**30.5**	**108.0**	**206.5**	**93.1**	**92.5**
Bowman	2	11.0	32.5	32.9	102.2	198.0	91.8	87.0
Conlon	2		29.8	37.9	101.4	193.0	93.2	88.5
Morex	6	**25.2**	**37.1**	**40.9**	**97.2**	**195.0**	**110.2**	**96.0**
HM mean		21.1	26.4	39.7	105.3	200.2	104.2	96.7
Standard deviation		3.9	4.5	6.6	1.2	1.8	6.7	2.9
Less susceptible								
HM 124	2	6.6	8.8	26.0	107.3	206.0	116.7	110.5
HM 1	2	12.1	2.0	19.1	111.8	204.5	115.2	125.0
HM 244	2	4.4	6.6	22.5	107.1	199.5	114.7	108.0
More susceptible								
HM 72	6	41.1	69.3	45.6	101.1	201.0	91.7	76.5
HM 33	6	35.2	66.3	60.1	103.9	196.0	88.4	81.5
HM 145	6	35.9	50.4	53.8	100.9	198.0	93.8	74.5
LSD 0.05		10.9	12.5	18.5	3.2	5.1	18.5	8.0
CV		26.1	23.9	23.6	1.5	1.3	9.1	4.2

<u>*Heading Date and Plant Height Results*</u>. Harrington was later and shorter than Morex in all tests (Table 2). A few transgressive segregates for plant height were noted, but none for heading date. Maturity in both long- and short-day conditions was significantly and positively correlated. Plant height was significantly and negatively correlated with FHB severity. Maturity was significantly and negatively correlated with FHB severity in the two environments where days to heading were recorded. These data support previous findings about the general tendency of tall, late plants to be more FHB resistant.

Identification of QTLs -The largest QTL for FHB resistance was present in chromosome 2H and was detected in all five environments (Fig. 1). A second, but smaller, QTL for FHB was found in 4H in all tests. The largest QTL for heading date at both locations was detected in 2H. At the short-day site, heading date peaks in 4H and 7H were significant and peaks in 1H and 5H approached significance. QTLs for plant height were detected in 2H and 4H according to the coincident peaks of SIM and sCIM analyses. An additional plant height QTL in 7H was found in only two environments. MARQUEZ-CEDILLO *et al.* (2001) previously reported plant height and maturity QTLs in 2H in the HM population. The largest QTL for DON accumulation was detected in 2H in all environments, while smaller ones were found in 4H at LA03, and in 7H at CN03.

Figure 1. Scans of test statistics (Y-axis) for simple interval mapping (SIM, solid lines), and simple composite interval mapping (sCIM, broken lines) for Harrington/Morex DH lines. Chromosome numbers are shown on the X-axis. Horizontal lines indicate approximate thresholds for testing SIM and sCIM.



Trait relationships - Harrington contributed QTLs for FHB resistance, increased plant height, and late heading. The HM lines with the lowest FHB readings were tall and two-rowed, while the lines with highest FHB readings were short and six-rowed (Table 2). These associations are likely caused by linkage. Other studies placed at least two plant height genes (*hcm1* and *lin1*) and one heading date gene (*Eam6*) near the *vrs1* locus in 2H (FRANCKOWIAK and LUNDQVIST, 2002). These genes are present in Morex and can partially explain the trait associations observed in 2H. Previous studies suggested that the *Eam6* gene is expressed in both long and short-day environments. TOHNO-OKA *et al*. (2000) reported that the QTL for short-day response in the Steptoe/Morex doubled-haploid population is located in 2H with minor factors in 1H, 4H, and 7H. Testing of the HM lines under short-day conditions revealed the presence of several QTLs that contribute to earliness in Morex. MESFIN *et al*. (2003) found that QTL for FHB resistance was associated with the heading date locus (presumably *Eam6*) in a greenhouse experiment.

In conclusion, QTLs for resistance to initial FHB infection, or type I resistance, were located primarily in 2H near major genes that control spike type, plant height, and maturity. Both adverse linkages and a number of minor genes for early heading have made development of early, FHB resistant barley cultivars very difficult.

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Yield Reduction in Naked and Hulled Barley Doubled Haploids Inoculated with *Fusarium culmorum* (W.G.Sm.) Sac.

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Abstract
The influence of inoculation with *Fusarium culmorum* on performance of yield-related traits in naked and hulled barley doubled haploids was studied in experiment carried out in 6 environments. Doubled haploids were derived by "bulbosum" method from F_1 hybrids between naked (1N86) and hulled (RK63/1) breeding lines. In each year and locality the experiment was established in a randomised complete block design with three replications. In each replication 50 ears of each line were inoculated with a single isolate of *Fusarium culmorum*. In inoculated and control lines kernel yield per ear, 1000-kernel weight and percentage of plump kernels (> 2.5 mm) were analysed and then percentage of reduction in particular traits was estimated. Genotype-environment (GE) interactions and their structure were analysed using the computer program SERGEN. It was found that reduction in yield-related traits was higher in naked than hulled lines, especially in the case of plump kernels, for which the mean reduction was equal to 31.10% in hulled, and 53.85% in naked genotypes.
Keywords: barley; doubled haploids; *Fusarium culmorum*; yield reduction

Introduction

Fusarium head blight caused by *Fusarium culmorum* reduces yield and its quality in small grain cereals (PROM 1997; SALAS *et al*., 1999). In barley, it was found that reduction in yield components after infection with *F. culmorum* is dependent on genotypes and environmental conditions: 6-rowed genotypes appeared to be more susceptible than 2-rowed ones. The aim of the present studies was to examine the influence of *F. culmorum* on reduction of yield-related traits in naked and hulled barley lines.

Material and Methods

Material for the studies covered 15 hulled and 15 naked barley doubled haploid (DH) lines derived from F1 hybrids between naked breeding line IN86 and hulled DH line RK63/1. DHs were produced using the *Hordeum bulbosum* method (KASHA, KAO 1970). The lines, their parents and hybrids (RN63F2, RN63F3) were examined in an experiment conducted in 6 environments. The experiment was carried out in a randomised block design with 3 replications. At the full anthesis in each replication 50 ears of each line were inoculated with a single isolate of *Fusarium culmorum* (IPO 348-01). In inoculated and control lines kernel yield per ear, 1000-kernel weight and percentage of plump kernels (> 2.5 mm) were analysed and then percentage of reduction in particular traits was estimated. Genotype-environment (GE) interactions and their structure were analysed using the computer program SERGEN (CALIŃSKI *et al*., 1996). The main effect for each genotype over environments was evaluated and tested by F statistic. Information about sensitivity of genotypes to environments was determined by the regression analysis. Genotypes were regarded as stable when their GE interaction was non-significant and as unstable when their GE interaction was significant at P = 0.05.

Results and Discussion

Mean values of the studied traits for inoculated and control lines are presented in Table 1. Generally, inoculation with *F. culmorum* resulted in a reduction in the studied yield-related traits. It was found that the reduction was stronger in naked than hulled DH lines, especially in the case of plump kernels, for which the mean reduction was equal to 31.10% in hulled, and 53.85% in naked genotypes.

Estimates of main effects for reduction in particular traits and results of testing their significance are given in Tables 2-4. The most of studied genotypes was characterised by non-significant main effects. From the breeding point of view the lines with negative and significant main effect are desirable, because it indicates on a weak susceptibility to infection. Among the examined genotypes only one such genotype was found - hulled line RN63/9 that was characterised by significant negative main effects for all the traits.

F-statistics for GE interaction, given in Tables 2-4, showed that studied genotypes were the most stable in the case of kernel weight per ear – only 6 lines among 34 genotypes (17.6%) had significant GE interaction. In contrast, for reduction in 1000-kernel weight 73.5% of all genotypes appeared to be unstable (F-statistics for GE interaction were higher than $F_{0.05}$). Generally, hulled DH lines were more stable than naked ones. It may be noticed that only in few cases the GE interaction could be explained by regression on environments (3 lines regarding 1000-kernel weight and 5 lines for plump kernels). That is not unexpected because parental genotypes of the studied DH population, RK63/1 and 1N86, were observed to be of undetermined tendency in their reaction on environments that is visible in non-significant regression coefficients and low values of determination coefficients.

The present results indicated that reduction in yield-related traits in barley lines after inoculation with *F. culmorum* depends on environmental conditions and ear morphology. Similar results were obtained for DH population of two- and six-rowed barley DHs (ADAMSKI *et al.*, 1999; CHEŁKOWSKI *et al*., 2000). In those studies was shown that six-rowed barley genotypes were more susceptible to infection than two-rowed.

A significant environment-genotype interaction for reduction in yield-related traits indicates that selection of barley genotypes less susceptible to *F. culmorum* should be based on series of experiments performed in different environments.

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Table 1. Mean values for yield-related traits in barley DH lines inoculated with *Fusarium culmorum*

Line	Kernel weight per ear (g)		1000-kernel weight (g)		Plump kernels (%)	
	control	inoculated	control	inoculated	control	inoculated.
Hulled genotypes						
R63N/1	1.14	0.59	51.21	39.99	86.56	62.31
R63N/3	0.99	0.39	47.43	34.40	74.96	49.39
R63N/4	0.86	0.43	43.12	33.58	77.10	56.02
R63N/9	0.99	0.58	43.17	34.33	76.42	53.94
R63N/18	1.08	0.59	47.35	36.94	82.61	57.76
R63N/21	1.05	0.51	51.60	38.28	84.21	62.93
R63N/22	1.04	0.41	48.67	33.55	82.55	51.50
R63N/27	0.85	0.39	44.16	32.52	77.55	49.73
R63N/28	1.01	0.48	45.29	33.97	79.41	57.32
R63N/34	0.95	0.45	47.05	34.43	84.67	62.21
R63N/35	0.84	0.44	39.88	31.64	62.59	38.80
R63N/61	1.07	0.57	51.12	38.44	88.24	70.26
R63N/63	0.98	0.52	45.01	34.65	68.85	42.70
R63N/67	0.87	0.40	42.93	33.86	72.62	45.46
R63N/74	1.13	0.47	47.58	34.06	80.88	53.00
Naked genotypes						
R63N/16	0.86	0.44	39.20	28.75	60.94	23.26
R63N/19	0.81	0.34	39.42	26.67	47.38	16.09
R63N/24	0.71	0.37	35.11	27.40	40.42	11.94
R63N/29	0.73	0.33	39.92	28.93	57.20	15.27
R63N/31	0.91	0.43	43.80	33.76	70.12	39.65
R63N/39	0.92	0.39	43.67	34.80	60.10	26.31
R63N/42	0.83	0.36	42.81	29.90	56.92	19.53
R63N/43	0.75	0.41	38.04	30.50	52.71	27.06
R63N/44	0.91	0.45	43.68	31.43	71.34	31.74
R63N/46	0.72	0.39	35.69	28.51	53.54	29.05
R63N/47	0.85	0.42	45.61	34.48	66.63	29.81
R63N/55	0.93	0.40	47.76	33.41	67.80	31.17
R63N/70	0.82	0.46	47.08	36.21	74.91	49.37
R63N/71	0.86	0.45	44.90	34.32	75.54	43.38
R63N/75	0.67	0.35	36.31	27.28	58.49	24.81
1N86 – P1	0.98	0.45	41.04	29.30	69.64	35.50
RK63/1– P2	0.96	0.48	46.35	34.57	77.51	52.60
RK63NF2	0.94	0.46	46.65	34.24	78.26	53.48
RK63NF3	0.95	0.43	45.41	32.12	77.41	52.48

Table 2. Main effects of barley DH lines and testing their interaction with environments for reduction in kernel weight per ear after inoculation with *F. culmorum*

Line	Main effect	F-statistic for		Coefficient of		F-statistic for	
		main effect	GE interac-tion	regres-sion	determina-tion	regres-sion	deviation from regress-ion
Hulled genotypes							
R63N/1	-1.901	0.13	1.20	0.025	0.34	0.01	1.50
R63N/3	10.370	13.18	1.14	-0.063	2.29	0.09	1.39
R63N/4	-1.211	0.54	0.25	-0.080	17.01	0.82	0.25
R63N/9	-11.096	268.88	1.93	-0.255	21.89	1.12	1.88
R63N/18	-4.916	1.22	1.18	0.061	2.02	0.08	1.45
R63N/21	-0.626	0.04	1.53	0.362	55.34	4.96	0.86
R63N/22	8.308	8.24	0.59	0.055	3.32	0.14	0.72
R63N/27	0.469	0.01	1.46	-0.010	0.05	0.00	1.83
R63N/28	0.740	0.02	1.87	0.034	0.41	0.02	2.32
R63N/34	1.896	0.95	1.48	-0.041	0.74	0.03	1.83
R63N/35	-4.332	5.57	0.87	0.038	1.08	0.04	1.08
R63N/61	-4.006	5.49	0.58	-0.202	45.35	3.32	0.40
R63N/63	-3.778	8.86	0.14	-0.021	2.03	0.08	0.18
R63N/67	0.137	0.00	3.25	-0.142	4.01	0.17	3.90
R63N/74	7.875	76.94	0.52	0.038	1.83	0.07	0.64
Naked genotypes							
R63N/16	-0.942	0.03	1.57	0.154	9.80	0.43	1.77
R63N/19	8.767	2.90	2.35	0.027	0.20	0.01	2.93
R63N/24	-3.291	0.22	5.80	-0.367	15.02	0.71	6.16
R63N/29	4.199	0.53	1.38	-0.260	31.61	1.85	1.18
R63N/31	1.251	0.07	1.89	-0.157	8.49	0.37	2.16
R63N/39	5.946	0.84	1.64	-0.096	3.63	0.15	1.98
R63N/42	4.802	2.65	0.54	0.182	39.61	2.62	0.41
R63N/43	-6.715	3.10	0.84	0.137	14.41	0.67	0.90
R63N/44	-1.205	0.66	2.77	0.287	19.31	0.96	2.79
R63N/46	-2.502	0.95	2.93	0.438	42.53	2.96	2.10
R63N/47	-1.190	0.31	0.29	-0.112	28.35	1.58	0.26
R63N/55	7.251	2.04	1.66	-0.019	0.14	0.01	2.07
R63N/70	-7.051	1.96	1.72	-0.042	0.66	0.03	2.13
R63N/71	-5.225	0.57	2.07	-0.066	1.35	0.05	2.55
R63N/75	-3.519	0.27	2.50	-0.253	16.51	0.79	2.61
1N86	3.190	0.61	0.74	-0.019	0.33	0.01	0.92
RK63/1	-1.276	0.05	1.22	-0.015	0.11	0.00	1.52
R63NF2	1.394	0.23	1.09	0.203	24.46	1.30	1.03
R63NF3	-1.809	0.23	1.57	0.178	13.12	0.60	1.71
F-statistic at the level							
$\propto$=0.05		6.61	2.24			7.71	2.39
$\propto$=0.01		16.26	3.06			21.20	3.37

Table 3. Main effects of barley DH lines and testing their interaction with environments for reduction in1000-karnel weight after inoculation with *F. culmorum*

Line	Main effect	F-statistic for		Coefficient		F-statistic for	
		main effect	GE intera-ction	regres-sion	determina-tion	regres-sion	deviation from regressio b
Hulled genotypes							
R63N/1	-0.832	0.16	1.28	-0.076	3.18	0.13	1.55
R63N/3	1.807	0.10	6.46	0.309	10.41	0.46	7.23
R63N/4	-3.707	0.67	7.45	-0.640	38.61	2.52	5.72
R63N/9	-5.605	8.43	4.00	-0.306	16.47	0.79	4.18
R63N/18	-2.912	0.87	2.85	-0.500	61.77	6.46	1.36
R63N/21	1.52	0.21	3.55	0.289	16.53	0.79	3.71
R63N/22	6.465	2.88	8.92	0.921	66.86	8.07	3.69
R63N/27	0.579	0.02	3.07	-0.279	17.90	0.87	3.15
R63N/28	-0.391	0.04	5.87	-0.656	51.59	4.26	3.55
R63N/34	1.657	0.20	6.40	0.369	14.95	0.70	6.80
R63N/35	-4.557	2.16	2.24	-0.383	46.03	3.41	1.51
R63N/61	-0.395	0.03	1.28	0.040	0.89	0.04	1.59
R63N/63	-2.030	0.61	2.05	-0.074	1.89	0.08	2.51
R63N/67	-4.314	0.54	5.13	-0.182	4.57	0.19	6.12
R63N/74	4.189	4.57	1.88	0.253	23.94	1.26	1.79
Naked genotypes							
R63N/16	0.520	0.01	6.57	-0.413	18.24	0.89	6.72
R63N/19	7.738	27.88	2.63	0.123	4.02	0.17	3.16
R63N/24	-3.089	1.31	4.88	-0.457	30.13	1.73	4.27
R63N/29	1.917	1.06	1.13	-0.104	6.70	0.29	1.32
R63N/31	-1.989	0.11	10.39	0.116	0.91	0.04	12.87
R63N/39	-4.510	0.50	5.58	-0.370	17.25	0.83	5.77
R63N/42	4.676	36.01	1.91	0.395	57.38	5.39	1.02
R63N/43	5.070	1.08	4.85	-0.053	0.40	0.02	6.04
R63N/44	3.029	1.32	2.91	0.459	50.80	4.13	1.79
R63N/46	-4.699	6.51	3.18	-0.045	0.45	0.02	3.95
R63N/47	-0.071	0.01	0.48	-0.074	7.91	0.34	0.56
R63N/55	4.759	7.34	2.28	0.354	38.61	2.52	1.75
R63N/70	-1.120	0.29	2.20	0.360	41.25	2.81	1.62
R63N/71	-1.498	1.86	0.93	0.335	84.81	22.34	0.18
R63N/75	-0.272	0.01	3.16	-0.344	26.32	1.43	2.91
1N86	3.825	0.75	4.63	-0.492	36.79	2.33	3.66
RK63/1	-0.283	0.00	4.14	0.076	0.99	0.04	5.13
R63NF2	1.696	0.40	4.91	0.497	35.45	2.20	3.96
R63NF3	4.334	3.21	3.83	0.554	56.28	5.15	2.09
F-statistic at the level::							
$\propto$=0.05		6.61	2.24			7.71	2.39
$\propto$=0.01		16.26	3.06			21.20	3.37

Table 4. Main effects of barley DH lines and testing their interaction with environments for reduction in plump kernels after inoculation with *F. culmorum*

Line	Main effect	F-statistic for		Coefficient of		F-statistic for	
		main effect	GE interac-tion	regres-sion	determina-tion	regres-sion	deviation from regress-ion
Hulled genotypes							
R63N/1	-14.319	71.52	0.90	-0.290	28.54	1.60	0.80
R63N/3	-9.319	1.97	2.01	0.098	1.46	0.06	2.48
R63N/4	-15.454	4.91	1.65	-0.171	5.41	0.23	1.95
R63N/9	-13.244	8.52	1.34	-0.438	43.85	3.12	0.94
R63N/18	-13.335	8.83	1.08	-0.029	0.23	0.01	1.35
R63N/21	-18.132	42.35	0.81	0.015	0.09	0.00	0.89
R63N/22	-5.226	1.38	3.67	1.005	84.23	21.37	0.72
R63N/27	-7.344	2.24	0.70	0.061	1.62	0.07	0.86
R63N/28	-15.558	6.68	3.21	-0.722	49.71	3.95	2.02
R63N/34	-16.889	12.73	1.79	-0.399	27.24	1.50	1.62
R63N/35	-2.494	0.06	3.70	-0.074	0.45	0.02	4.61
R63N/61	-22.360	62.06	1.59	-0.589	66.81	8.05	0.66
R63N/63	-5.892	0.71	2.95	-0.404	16.92	0.81	3.06
R63N/67	-4.664	0.73	1.92	-0.260	10.73	0.48	2.14
R63N/74	-6.616	12.23	0.95	0.531	90.43	37.78	0.11
Naked genotypes							
R63N/16	22.850	14.25	2.18	-0.245	8.39	0.37	2.50
R63N/19	21.095	37.62	3.00	-0.471	22.55	1.16	2.91
R63N/24	24.116	19.00	2.74	0.117	1.52	0.06	3.37
R63N/29	29.084	714.39	1.44	0.122	3.13	0.13	1.75
R63N/31	0.514	0.00	5.13	-0.008	0.00	0.00	6.42
R63N/39	11.398	1.06	4.88	-0.267	4.48	0.19	5.83
R63N/42	22.009	70.69	0.59	-0.035	0.62	0.02	0.74
R63N/43	13.021	1.86	2.82	0.290	4.40	0.18	6.96
R63N/44	13.536	65.41	1.01	0.461	64.49	7.26	0.45
R63N/46	1.092	0.08	1.40	-0.102	2.28	0.09	1.72
R63N/47	14.567	11.13	2.08	0.465	31.88	1.87	1.77
R63N/55	12.334	12.74	1.21	0.168	7.15	0.31	1.41
R63N/70	-5.783	3.37	2.48	0.748	69.00	8.90	0.96
R63N/71	1.462	0.06	2.41	0.738	69.21	8.99	0.93
R63N/75	12.925	9.61	2.57	0.077	0.71	0.03	3.18
1N86	7.822	3.99	0.90	-0.276	25.82	1.39	0.84
RK63/1-	-11.018	4.52	1.37	-0.371	30.81	1.78	1.18
R63NF2	-9.617	4.23	1.22	0.080	1.60	0.07	1.50
R63NF3	-10.561	8.44	1.09	0.173	8.38	0.37	1.25
F-statistic at the level							
$\propto$=0.05		6.61	2.24			7.71	2.39
$\propto$=0.01		16.26	3.06			21.20	3.37

Comparison of Hulless and Covered Barley for Infection with *Fusarium* Head Blight and Accumulation of B-Trichothecene Mycotoxins in Grain

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Abstract
We have evaluated 145 covered and 29 hulless barley genotypes under artificial infection with *Fusarium graminearum* Schw. in a field experiment. Significant genetic variation for *Fusarium* head blight severity measured by the area under the disease progress curve and for mycotoxin contamination was found among covered and hulless barley lines. The overall mean area under the disease progress curve of covered and hulless barley did not differ significantly. From the evaluated genotypes we chose 29 hulless-covered pairs with approximately the same disease severity, based on visual symptoms on the heads, for analysis of mycotoxins (B-trichothecenes) in the harvested grain. The average content of deoxynivalenol of the 29 covered and 29 hulless barley samples was 15520 μg kg^{-1} and 12964 μg kg^{-1}, respectively. The results showed that the mean deoxynivalenol, 3-acetyl-deoxynivalenol and 15-acetyl-deoxynivalenol content of covered barley was significantly higher than that of hulless barley (p<0.01), whereas for nivalenol the difference between the mean values of covered and hulless barley was not significant. The results support the hypothesis that hulless barley appears to be less prone to accumulation of mycotoxins in the harvested crop than covered barley. Explanation of this finding may be that a considerable proportion of the mycotoxin resides in the barley hulls.
Keywords: hulless barley; covered barley; *Fusarium graminearum*; B-trichothecenes

Introduction

Fusarium head blight (FHB) is a fungal disease, which can not only significantly reduce the yield of barley (BOTTALICO 1998; MCMULLEN *et al*. 1997), but also lower grain quality. Grain contaminated with mycotoxins produced by *Fusarium* spp. may be completely unusable for feed, food and malting purposes. This problem has actualised with the spreading of FHB in the world (HYSEK *et al*. 2002; SPUNAROVA *et al*. 2002).

Fusarium graminearum Schw. is one of the most frequently found *Fusarium* species on the cereals in Europe. It is more usual in the wet and warm climate of Central and Southern Europe. Common species in cooler and maritime regions are *F. culmorum* and *F. avenaceum*. The most common *Fusarium* mycotoxins in European barley grain are deoxynivalenol (DON) and zearalenone. Nivalenol (NIV) and other trichothecenes are usually found in cereals together with DON, but the occurrence of DON is more frequent and the concentration higher and therefore considered a major food safety issue (BOTTALICO 1998). ROSSNAGEL (2000) has reported about lower accumulation of mycotoxins as one of the possible advantages of hulless barley (HB) over conventionally grown covered barley. Investigations of CLEAR *et al*. (1997) showed that a major portion of mycotoxins accumulated in the hulls of barley. The grain infection level of HB grain with hulls detached from the grain during harvesting was 95% lower compared to grain with hulls, and the content of DON was 59% lower. Hulless barley grain threshes free from hulls and the hulls are left on the field after harvesting. In consequence, hulless barley is supposed to contain a lower

content of mycotoxins than covered barley assuming a similar *Fusarium* head blight severity in the field.

T.M. Choo (personal communication) informed that HB variety AC Alberte was less contaminated with DON among 49 barley genotypes under natural infection conditions and the contamination of this variety was comparatively low also under artificial infection. We did not find any published data, which would show differences in mycotoxin content of various hulless and covered barley genotypes. This experiment was carried out with the purpose to compare the infection level with FHB and accumulation of *Fusarium* mycotoxins in the grain of hulless and covered barley grown under artificial inoculation with *F. graminearum*.

Material and Methods

Experiments were carried out at the Institute for Agrobiotechnology (IFA-Tulln, Austria) in 2002. A field experiment (1 m long 2-row plots, 3 replications, randomised complete block design) with 145 covered and 29 hulless barley accessions with diverse origin was established (Table 1). For inoculation the aggressive single-spore *F. graminearum* isolate IFA-65 was used, which was originally isolated from a naturally infected *Ttritcum durum* grain and is maintained at IFA-Tulln's isolate collection. This isolate is a producer of trichothecenes and zearalenone. Plots were individually inoculated at 50% anthesis by spraying 100 ml of a macroconidia inoculum (5×10^4 spores ml^{-1} in water) on the heads and the inoculation was repeated 2 days later to ensure a uniform inoculation of all heads in each plot. An automated mist-irrigation system maintained humidity and kept plants wet for 20 h after inoculation. This inoculation procedure is well established and proofed to yield repeatable FHB infections on wheat and barley (BUERSTMAYR *et al*. 2000, 2004). The percentage of visually infected spikelets was estimated on days 10, 14, 18, 22 and 26 after inoculation on a whole plot basis. The area under the disease progress curve (AUDPC), as described by BUERSTMAYR *et al*. (2000) was calculated for each plot and used as the measure of disease severity. Plant height was measured for each plot shortly before harvesting time. Plots were threshed with a plot combine (Wintersteiger nursery master) at low wind speed. Harvested samples were cleaned manually, ground on a sample mill and 10 grams of each sample from three replications were pooled and used for toxin analysis. 29 pairs of hulless – covered genotypes with similar AUDPC scores were selected for further analysis of mycotoxins (B-trichothecenes) in grain.

The content of the B-trichothecenes deoxynivalenol (DON), 3-acetyl-deoxynivalenol (3-Ac-DON), 15-acetyl-deoxynivalenol (15-Ac-DON) and nivalenol (NIV) were determined in pooled grain samples by gas chromatography and electron capture detection (GC-ECD) after clean-up with Mycosep 225 columns (KRSKA *et al*. 2001). Each sample was analysed once. The number of visually infected grains in 100-grain samples from three replicates was counted.

The analysis of variance, t-test of paired two sample for means and correlation analysis were applied.

Results and Discussion

FHB Severity

Large genetic variation was found among both the covered and the hulless barley genotypes for FHB severity. The mean infection level (AUDPC) with *F. graminearum* of covered and hulless barley (261.5 and 300.5, $LSD_{0.05}$ = 196.3) did not differ significantly. AUDPC of hulless barley genotypes ranged from 46.7 to 891.3 (Table 1). The lowest infection was found for line CIMMYT 106 and AUDPC did not significantly differ from 0 for genotypes L-302, HOR-1867 (a Czech line, obtained from genebank at IPK Gatersleben), CIMMYT 75, CDC Buck, CDC Richard, KM 2083, and KM 2062 as well.

Table 1. FHB severity measured by AUDPC and content of DON in the harvested grains of hulless and covered barley genotypes evaluated in Tulln in 2002

	Hulless barley				Covered barley			
Nr.	Genotype	Origin[a)]	AUDPC	Content of DON, µg kg^{-1}	Genotype	Origin[a)]	AUDPC	Content of DON, µg kg^{-1}
1	CIMMYT 106	MEX	47	7121	Hellana	A	53	4531
2	L-302	LV	114	10669	Bios	RU	112	8728
3	HOR-1867	CZ	117	1556	Chevron	CH	92	8474
4	CIMMYT 75	MEX	123	8092	Thuringia	D	120	7804
5	CDC Buck	CDN	142	6790	Stander	USA	125	5896
6	KM 2083	CZ	154	9211	Peggy	D	161	9217
7	CDC Richard	CDN	170	5448	Abava	LV	169	7444
8	KM 2062	CZ	177	7778	Adagio	F	181	11794
9	C-43.1	LV	202	9944	Elisa	A	198	15212
10	Taiga	D	207	10906	Brenda	D	205	10062
11	KM 2092	CZ	211	8266	Krona	D	212	11192
12	SW 1291	S	214	12926	Prosa	A	214	13909
13	KM 2037	CZ	247	8557	Madras	D	245	15520
14	CDC McGwire	CDN	256	6798	HOR-10986	TN	256	14559
15	CDC Freedom	CDN	258	8782	Maresi	D	276	16203
16	KM 1910	CZ	285	10899	Viva1	A	285	12673
17	CDC Dawn	CDN	292	6857	Annabell	D	294	8864
18	KM 2283	CZ	323	16631	Madeira	D	323	15139
19	KM 2084	CZ	336	16677	Druvis	LV	336	10384
20	SW 1290	S	342	10841	Tolar	CZ	342	21126
21	L-238	LV	356	11772	Madonna	D	363	17015
22	KM 2045	CZ	364	12230	Pasadena	D	373	20827
23	CDC Gainer	CDN	366	9369	Linga	LV	378	18401
24	KM 2001	CZ	428	21555	Ricarda	D	450	21719
25	CDC Silky	CDN	473	19761	HOR-9726	LY	470	26677
26	Falcon	CDN	492	25105	HOR-7447	EC	491	23991
27	CIMMYT 39	MEX	612	28737	Bartok	UK	474	35205
28	Cebada Petunia	MEX	701	32746	HOR-2940	IND	649	28729
29	Merlin	USA	891	29921	HOR-214	J	752	28801
Mean			**307**	**12964**	**Mean**		**297**	**15520**
LSD $_{0.05}$			196.3		LSD $_{0.05}$		196.3	

[a)] Country of origin, abbreviated by the international car plate identification code

High infection levels (AUDPC > 400) were recorded for the following hulless barley varieties and lines: Merlin, Cebada Petunia, CIMMYT 39, Falcon, CDC Silky and KM 2001. The majority of genotypes with high FHB infection were 6-row barleys, e.g. several accessions from the genebank at IPK Gatersleben originating from diverse collection sites (HOR-lines), and the hulless genotypes CDC Silky, Falcon, CIMMYT 39 and Cebada Petunia. Among the

covered varieties, low FHB severity was observed e.g. on the 2-row varieties Hellana, Bios and Thuringia and the 6-row variety Chevron, which has been used as a source of FHB resistance in breeding (RUDD *et al*. 2001). Other well-known European 2-row malting barley varieties like Annabell and Pasadena showed comparatively high infection levels. More detailed information on FHB resistance of the covered barley genotypes will be published (BUERSTMAYR *et al*. 2004).

Comparison of Mycotoxin Content in Hulless and Covered Barley

From the 174 genotypes, 29 hulless-covered barley pairs with similar AUDPC values were selected for further analysis of mycotoxins (B-trichothecenes). Among the analysed samples, DON was the most prevalent mycotoxin (1556 – 35205 μg kg^{-1}), followed by 15-Ac-DON (103 – 1625 μg kg^{-1}), NIV (0 – 859 μg kg^{-1}) and 3-Ac-DON (0 – 510 μg kg^{-1}).

The content of DON was higher in covered than in hulless barley in 66% of hulless-covered barley pairs (Table 1). On average, the covered barleys contained 2557 μg kg^{-1} more DON than the hulless barleys and this difference was statistically significant (p-value = 0.004).

3-Ac-DON content was higher for covered genotypes in 86% of the pair-wise comparisons, and the difference between mean content in covered and hulless barley (103 μg kg^{-1}) was significant (p-value < 0.001).

Similarly, the content of 15-Ac-DON was higher in covered barley in most of pairs (83%), and the difference between mean values of covered and hulless genotypes (225 μg kg^{-1}) was significant as well (p-value < 0.001).

On the contrary, the content of NIV was higher in hulless barley for the majority of pairs (63%), and the difference between mean values of covered and hulless barley (10 μg kg^{-1}) was not significant (p-value = 0.85). LEE *et al*. (1992) found that NIV is accumulated not only in the external part of the barley grain like other mycotoxins, but also in the inner part, and grain polishing does not significantly reduce NIV content. This may explain why the results obtained from NIV analysis were different from the other trichothecenes analysed.

Relationships between Mycotoxin Content and Other Traits

The correlation of AUDPC with the content of mycotoxins DON, 3-Ac-DON and 15-Ac-DON in grain, as well as with the percentage of infected kernels was significant for both hulless and covered barley genotypes, whereas the correlation with NIV content was not significant (Table 2). Consequently the infection severity on the spikes is a useful indicator for grain contamination with DON, 3-Ac-DON and 15-Ac-DON, but not for NIV.

The percentage of visibly infected grain in harvested samples ranged 6.0-27.3% for hulless barley and 2.3-38.7% for covered barley (data not shown). The correlation between AUDPC and the amount of infected grain was larger for covered barley than for hulless barley, which shows that it may be more reliable to estimate disease severity from infected grain for covered barley than for hulless barley.

AUDPC showed a larger correlation with the content of DON, 3-Ac-DON and 15-Ac-DON than the percentage of infected kernels in harvested samples, which means that FHB severity evaluation on the field is better suited for indirect approximation of mycotoxin content than scoring FHB infection on harvested grain samples. Opposite results were reported for wheat by MESTERHAZY *et al*. (1999), who found that the correlation between DON content and amount of infected wheat grain was higher than correlation between DON content and spike infection level with FHB. Possibly the visual identification of FHB infected grains is more difficult for barley than for wheat.

Table 2. Pearson correlation coefficients between mycotoxin content in grain and other plant traits

Traits	Grain type	AUDPC	Content of mycotoxins, μg kg^{-1}			
			DON	3-Ac-DON	15-Ac-DON	NIV
AUDPC	hulless	-	0.90**	0.90**	0.92**	0.08
	covered	-	0.88**	0.73**	0.69**	-0.27
Infected grain (%)	hulless	0.70**	0.65**	0.68**	0.61**	-0.14
	covered	0.87**	0.73**	0.59**	0.59**	-0.38*
Plant height (cm)	hulless	-0.77**	-0.83**	-0.82**	-0.87**	-0.18
	covered	-0.47*	-0.43*	-0.31	-0.31	0.00

* $p<0.05$; ** $p<0.01$

Plant height of hulless genotypes showed a significant negative correlation with AUDPC and content of DON, 3-Ac-DON and 15-Ac-DON in grain (Table 2), which is in agreement with other findings (DE LA PENA *et al*. 1999; FRANCKOWIAK 2000; MA *et al*. 2000) reporting a significant association of plant height and resistance to *Fusarium* head blight: taller genotypes tend to be less infected and their grain accumulates smaller amounts of mycotoxins. For the covered genotypes, plant height was significantly correlated with AUDPC and DON content, but not with 3-Ac-DON, 15-Ac-DON and NIV (Table 2). Results indicate, that some exceptions are possible and high FHB severity and accumulation of mycotoxins is not necessarily associated with short plant types. For example, covered variety Adagio with short stems had a comparatively weak infection, but some of the hulless genotypes (CDC Gainer, CDC Dawn) with taller stems were among the more severely infected genotypes.

To summarise, hulless barley varieties, with similar levels of *Fusarium* head blight infection, accumulated significantly lower amounts of DON, 3-Ac-DON and 15-Ac-DON than covered barley varieties, but the difference in NIV content was not significant. On the whole, the results support the hypothesis that hulless barley tends to be less prone to mycotoxin contamination than covered barley. Further investigations using segregating populations or near isogenic lines should allow study the differences in mycotoxin accumulation in more detail.

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Barley Seed Health as Affected by Seed-Borne *Bipolaris sorokiniana*

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Abstract
Spot blotch, caused by *Bipolaris sorokiniana* (teleomorph: *Cochliobolus sativus*), in barley (*Hordeum vulgare*) has increased significantly in Manitoba, Canada. Based on isolations from infected leaf tissue, spot blotch was the most prevalent disease in barley fields surveyed in the province in 2001 and 2002. *Bipolaris sorokiniana* can also infect developing kernels to cause discoloration and contribute to shrivelling of the grain. Sampling of barley kernels from 2-rowed barley fields severely affected by spot blotch has indicated a 90-100% incidence of *B. sorokiniana*. The effects of high levels of *B. sorokiniana* seed-borne infection on subsequent germination and plant emergence have not been studied in barley. Also, the effects of management options (new seed treatment fungicides, seed placement) to enhance germination and emergence need to be determined. Initial results indicated that germination and emergence of highly infected, untreated barley seed were significantly reduced, but that these negative effects could be mitigated by certain seed treatments. Seed treatment also significantly increased yields, indicating that this could be an effective management tool. However, yields were highest with seed lots having low levels of *B. sorokiniana* infection, suggesting that prevention of infection should be a component of a holistic management strategy.
Keywords: *Bipolaris sorokiniana*; barley; seed health; fungicides

Introduction

The fungus *Bipolaris sorokiniana* (Sacc. in Sorok.) Shoemaker, Syn. *Helminthosporium sativum* Pamm. King and Bakke, (teleomorph: *Cochliobolus sativus* (ITO & KURIBAYASHI) Drechsler ex. Dastur) is a significant pathogen of barley (*Hordeum vulgare*) in Canada and is the causal agent of seedling blight, common root rot, foliar spot blotch, and kernel black point (BAILEY *et al.* 2003). Inoculum of *B. sorokiniana* may be seed or soil borne and can over-winter on crop residues with dispersal of conidia by wind and rain (STEFFENSON 1997). CLARK and WALLEN (1969) demonstrated that barley seedlings can readily become diseased from infected seed when grown under high humidity. Under warm, moist conditions, the pathogen may sporulate on affected seedling tissue producing conidia that can infect developing leaves and cause spot blotch (BAILEY *et al*. 2003; RICHARDSON 1972). Simultaneous with the spread of foliar infection, developing kernels can also be infected by airborne conidia resulting in discoloration and shrivelling of the grain. STEVENSON (1981) found the percentage of barley seed internally-infected with *B. sorokiniana* paralleled the incidence of airborne spores and the progression of spot blotch. The same study found that the stage of kernel development during which infection takes place does not appear to be restricted; infection events were observed from the end of flowering through to maturity.

High levels of kernel infection have frequently been found on barley produced in Eastern Canada, including Ontario, Quebec, and the Maritime Provinces, due to conditions of high relative humidity that favour the development of spot blotch (STERLING *et al*. 1977; COUTURE &

SUTTON 1978). Recently, spot blotch in barley has increased significantly in the prairie province of Manitoba, Canada. Based on isolations from infected leaf tissue, spot blotch caused by *B. sorokiniana* was the most prevalent disease in barley fields surveyed in the province of Manitoba in 2001 and 2002 (TEKAUZ *et al*. 2002; TEKAUZ *et al*. 2003). In addition, sampling in Manitoba of barley kernels from 2-rowed barley fields severely affected by spot blotch has indicated a 90-100% incidence of *B. sorokiniana*. The intensity of internal kernel infection raises concern because these infection levels were previously not observed in Manitoba.

Variety surveys by the Canadian Wheat Board (2003) indicated that in Manitoba in 2003, 47.2% of the hectares seeded to malting barley were two-row cultivars. In general, 2-rowed barley cultivars possess a lower level of resistance to spot blotch compared to six-row cultivars (FETCH & STEFFENSON 1994). As such, more conidial inoculum is likely to be produced in 2-rowed crops making their spikes and kernels more vulnerable to infection.

Foliar fungicides used to control spot blotch in barley have had some degree of success in preventing subsequent kernel infection by reducing the availability of inoculum. Couture and Sutton (1978) found the internal colonization of kernels by *B. sorokiniana* to be significantly reduced in plots sprayed with a select group of foliar fungicides, compared to untreated plots. However, even frequent fungicide applications applied up to 12 days before harvest were not able to eliminate kernel infection entirely (COUTURE & SUTTON 1978). The most desirable means to control kernel black point would be the use of resistant cultivars; however, this has not been exploited in barley breeding programs. Incorporation of this resistance has been challenging because available sources of resistance are inferior in both agronomic and quality performance (GEBHARDT *et al*. 1992). Thus, cultivar resistance is currently not being used as a control measure for kernel infection.

The effects of high levels of *B. sorokiniana* seed-borne infection on subsequent germination and plant emergence have not been studied in contemporary 2-row barley cultivars. Likewise, the effects of management options (new seed treatment fungicides, seed placement) to enhance germination and emergence need to be determined. The objectives of this study were to evaluate the effects of depth of seeding and fungicide seed treatment on barley seed and seedling health, in two seed lots with different levels of seed-borne *B. sorokiniana* infection.

Material and Methods

Kernel Infection, Germination, and Seed Treatments

The cultivar used, 'AC Metcalfe', is currently the most widely grown 2-row malting barley in Manitoba (Manitoba Agriculture, 2003). Two seed lots (designated 'A' and 'B') of 'AC Metcalfe', both produced in 2002 and originating from the same region of Manitoba, were selected on the basis of their different levels of internal kernel infection by *B. sorokiniana* to represent 'low' and 'high' infections. Kernel infection levels were determined simultaneously with germination procedures which were based on Association of Official Seed Analysists (1981) protocols. Briefly, 200 kernels from each seed lot were surface sterilized in 0.3% NaOCl solution for 3 minutes, dried, and placed in 10-cm diameter glass Petri plate moisture chambers for incubation. Each moisture chamber contained 25 kernels with seeds placed in a circular pattern on 90mm diameter filter paper (Whatman International Ltd., Maidstone, England) moistened with 3 ml of distilled/autoclaved water. Kernels were incubated at 20°C and a 16 h photoperiod under cool white fluorescent light, and 15°C during the 8 h of darkness. After 7 days' incubation,

the level of kernel infection was determined by examining each kernel microscopically and recording the number from which *B. sorokiniana* conidia sporulated; conidial production was considered the visual manifestation of viable mycelium within the kernel. At the same time, germination (plumule and at least one root growing from the radicle) was recorded.

Liquid systemic seed treatments were applied to each seed lot according to rates and procedures recommended by the manufacturer; each treatment was applied using a Hege 11 (Wintersteiger, Reid, Austria) liquid seed dresser. The treatments included: Charter (25 g/L triticonazole à 25 mL concentrate per 25 kg seed), Dividend® XL RTA (3.21% difenoconazole, 0.27% metalaxyl-M à 325 and 650 mL/100 kg seed), and Raxil® 250 FL (6 g/L tebuconazole à 83 mL/25 kg seed) manufactured by BASF Canada, Syngenta Crop Protection, and Gustafson respectively.

Controlled Environment Experiments
Greenhouse experiments were carried out using a four replicate, randomized complete block design with plastic flats arranged as blocks on greenhouse benches. Flat dimensions were 50 x 38 x 15 cm; each flat contained 4 rows of seeds spaced 8 cm apart. Treatment rows were randomized within flats with treatments consisting of untreated seed (control), Dividend XL RTA à 325 mL/100 kg seed, Dividend XL RTA à 650 mL/100kg seed, and Charter treated seed. Kernels were placed on a 50 mm thick soil bed at 1 cm intervals generating 50 seeds per row. Experiments were conducted for each seed lot at seeding depths of 25 mm and 50 mm. Soil, a mix of four parts clay loam, 1 part Turface MPV (Profile Products, Buffalo Grove, Illinois, USA), was initially watered to capacity, allowed to drain, and subsequently watered at regular intervals to maintain moisture. Temperature was recorded and observed to range from 20-23°C; light was artificially supplemented with sodium halide lamps to provide a 16 hour photoperiod. Seedling emergence was recorded after 7 and 14 days. Plants were maintained in flats until day 35 when all flats were harvested. As a measure of seedling vigour, all shoots were cut off at soil level prior to the removal of roots from soil; shoots were collected, dried at 40°C for 48 hours, cooled to room temperature and weighted. Roots were exhumed and were washed with water to loosen and remove soil and were dried and weighed. Prior to washing, roots were assessed for 'seedling blight' symptoms by examining the crowns and sub-crown internodes. Disease incidence was recorded for each plant if brown colored lesions were present. Seedling blight was rated using a severity scale of 0-4 on the crown and sub-crown internode of each plant where 0 = healthy (no lesions), 1 = slightly lesioned (1-25%), 2 = moderately lesioned (26-50%), 3 = severely lesioned (51-75%), and 4 = blighted (76-100%). Percent severity was calculated according to the formula: disease rating = (sum of ratings/4)x(100/total number of plants), (MEAD 1942).

Field Experiments
Field trials were conducted in 2003 at two sites located near Portage la Prairie and Glenlea, Manitoba. Trials for each seed lot were established as a four replicate, randomized complete block design containing five treatments each. Trials at Portage la Prairie were seeded using a Hege 80 (Wintersteiger, Reid, Austria) single cone seeder. Individual plot dimensions were 1.5 x 6 m and contained 7 seed rows with 25 cm seed row spacing and a seeding rate of 76 g/plot (10.9 g/row). Trials at Glenlea were seeded using a custom built plot drill with individual cones for each of the 6 seed rows. Plot dimensions were 1.5 x 6 m and contained 6 seed rows with 30 cm row spacing; seeding rate was 76 g/plot (12.7 g/row). Treatment plots were randomized within each block. Treatments consisted of untreated seed (control), Dividend XL RTA à 325 mL/100

kg seed, Dividend XL RTA à 650 mL/100kg seed, Charter treated seed, and Raxil treated seed. A single plot of untreated seed was sown as a border on each side of a block. Seeding depth was approximately 30mm at both sites. Seedling emergence was determined at the 1 to 2-leaf stage by counting the number of seedlings in the center 2 rows of each plot. At maturity, plots were harvested with a plot combine to determine yield.

Statistical Analysis

Analysis of variance was carried out for all observations using SAS PROC GLM (SAS Institute Inc., 2000). SAS PROC t-test was used to test equality of means and variance among sites. Data were pooled across sites where the variance between sites was equal. Where appropriate, Duncan's New Multiple Range Test was used for comparison of means.

Results and Discussion

Kernel Infection, Germination, and Seed Treatments

The incidence of internal kernel infection by *B. sorokiniana* in seed lots A and B was 95% and 29.5% respectively (Table 1). In each lot, fungicide seed treatment significantly reduced the incidence of pathogen sporulation (as evidenced by the presence of mature conidia of *B. sorokiniana*) during kernel germination. The ability of the seed treatment fungicides to suppress pathogen sporulation indicated these curtailed pathogen development. In seed lot A, the higher rate of Dividend enhanced the level of control. All treatments reduced conidia development in seed lot A; the Dividend seed treatments had the greatest effect. In lot B, all fungicide seed treatments significantly reduced conidial development, but treatments did not differ from each other. The level of control was greatest in seed lot A where kernel infection incidence was the highest.

Table 1. Incidence of internal kernel infection by *Bipolaris sorokiniana* and germination in untreated and fungicide-treated seed of 'AC Metcalfe' barley

			least squares means (%)	
Seed lot	treatment	rate	incidence	germination
A	untreated control		95 *a*	64 *a*
	Dividend XL RTA	325 ml/ 100kg	70.5 *c*	66 *a*
	Dividend XL RTA	650 ml/ 100kg	40 *d*	72.5 *a*
	Charter	25 ml/25 kg	87 *b*	51.5 *b*
	Raxil 250 FL	83 ml/25 kg	88 *b*	64.5 *a*
B	untreated control		29.5 *a*	90.5 *a*
	Dividend XL RTA	325 ml/ 100kg	11.5 *b*	90 *a*
	Dividend XL RTA	650 ml/ 100kg	11 *b*	89.5 *a*
	Charter	25 ml/25 kg	20 *b*	88 *a*
	Raxil 250 FL	83 ml/25 kg	20 *b*	86.5 *a*

a-c Means in a column within each seed lot, followed by a common letter, are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test.

Germination appeared to be inversely related with the level of *B. sorokiniana* kernel infection. In the untreated control, seed lot A kernel infection was 95% and germination 64%; by contrast, lot B with 29.5% kernel infection had a germination of 90.5%. Seed treatments did not significantly improve germination in either seed lot, although the high rate of Dividend provided a numerical 8.5% improvement. Overall, germination in lot A was significantly lower than seed lot B (data not shown). Despite lowering the incidence of kernels subtending conidia, no treatment was able to completely eradicate the pathogen or improve germination of infected seed in either seed lot. STEVENSON (1981) attributed the failure of fungicide seed treatments (those available in 1981) to control *B. sorokiniana* to the presence of seed coats (silicified and lignified hull, tightly

compressed floral glumes - lemma and palea) of mature barley and their ability to protect fungal propagules, located either within or between these structures. When examining internal kernel infection, MEAD (1942) discovered that while the embryo was not infected, tissues and organs surrounding the developing embryo contained mycelium. During germination, the emerging coleoptile forces its way through infected tissue and is exposed to living hyphae; such mycelial invasion can rapidly halt the development and growth of the plumule. It appears that the fungicides used in this study may only control surface-borne *B. sorokiniana*; systemic activity of the fungicide does not appear to penetrate barley seed coats, at least not to the extent of completely eradicating the pathogen within.

Controlled Environment Experiments

Initial results at the two seeding depths indicated that the highly infected seed (lot A) had significantly lower emergence relative to seed with low kernel infection. However, these preliminary results also suggested that seed treatment can mitigate these effects and improve emergence of highly infected seed. Fungicide seed treatment appeared to have little to no effect on emergence in seed lots with a low level of kernel infection. As such, emergence was relatively high for all treatments in seed lot B. This suggests that emergence in fungicide-treated, highly infected seed lots cannot reach levels comparable to seed lots with an initial low level of kernel infection. Plant vigour, assessed by comparing the dry mass per plant, did not shown any differences among treatments within seed lots seeded at the same depth; differences only existed within lots seeded at different depths. Preliminary results also suggested that fungicide seed treatment can prevent or reduce levels of seedling blight. Some seed treatments appeared to significantly reduce both incidence and severity of subcrown internode infection by *B. sorokiniana* relative to untreated controls, in both seed lots and at both seed depths. Thus, while fungicide seed treatment may not always improve emergence of barley seedlings, they may provide an additional benefit by mitigating post-emergence development of seedling blight.

Field Experiments

Emergence and yield data for the two locations were pooled because variances between sites were considered equal (data not shown). A significant response to fungicide seed treatment was observed only in seed lot A (high infection level) (Table 2). Emergence in seed lot A was low and only the high rate of Dividend resulted in an improved plant count; however, this improvement was marginal relative to the much higher emergence in seed lot B.

Table 2. Emergence and yield of 'AC Metcalfe' barley seed lots differing in their level of internal kernel infection by *Bipolaris sorokiniana*

			least squares mean	
Seed lot	treatment	rate	Emergence (plants/m of row)	Yield (kg/ha)
A	untreated control		7.3 *b*	2712.6 *c*
	Dividend XL RTA	325 ml/ 100kg	7.9 *b*	2898.2 *b*
	Dividend XL RTA	650 ml/ 100kg	8.9 *a*	3073.4 *a*
	Charter	25 ml/25 kg	7.6 *b*	2860.7 *bc*
	Raxil 250 FL	83 ml/25 kg	7.8 *b*	2933.0 *ab*
B	untreated control		28.2 *a*	3703.0 *a*
	Dividend XL RTA	325 ml/ 100kg	27.7 *a*	3760.8 *a*
	Dividend XL RTA	650 ml/ 100kg	28.0 *a*	3699.4 *a*
	Charter	25 ml/25 kg	27.0 *a*	3661.9 *a*
	Raxil 250 FL	83 ml/25 kg	26.9 *a*	3722.7 *a*

a-c Means in a column within each seed lot, followed by a common letter, are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test.

Both rates of Dividend, and the Raxil seed treatment increased grain yields in seed lot A relative to the untreated control. Similar to studies by STERLING *et al*. (1977), yield response to seed treatment was highest in the most highly contaminated seed. The high rate of Dividend provided the highest yield increase, 360.8 kg/ha over the control. No significant differences were observed between treatments in seed lot B for either emergence or yield. However, both emergence and yield were significantly higher ($P \leq 0.01$, data not shown) across treatments in seed lot B compared to seed lot A. This is contrary to the findings of MACHACEK *et al*. (1954) and CLARK and WALLEN (1969) who found that the level of seed infection had little effect on final yields. CLARK and WALLEN (1969) concluded there was little practical value in treating highly infected barley seed because no yield benefit accrued. However, our results suggest that the application of newly-developed seed treatment fungicides, and the selection of seed with a low level of kernel infection, can be effective components of a holistic strategy to manage barley seed infected with *B. sorokiniana*.

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Proteinases Secreted by *Fusarium* and Their Inhibitors in Barley Grains

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Abstract
Fusarium head blight (FHB) has become a common problem for cultivated barley around the world. Infected grains have poor quality: they are small, shrivelled, discoloured, and often contain mycotoxins. The problem is especially difficult for malting barley. More knowledge about the defense mechanisms that barley uses against *Fusarium* infection is needed in order to breed for more tolerant malting barley cultivars. When grown on barley grains, *F. culmorum* secretes trypsin-like (TL) and subtilisin-like (SL) alkaline proteinases which can hydrolyze barley storage proteins. Barley grains contain proteinase inhibitor proteins, such as the Bowman-Birk trypsin inhibitor, BASI and CI-2A, that can decrease the activities of these fungal proteinases. We have studied the spatial and temporal localization of the two fungal proteinases and their barley inhibitors in infected and non-infected barley grains using immunomicroscopy. In this report, we show the results obtained with the trypsin inhibitor BBBI and the fungal trypin-like proteinase. BBBI was present in both the non-infected and infected grains. The fungal TL proteinase was detected only in the infected grains. It ocurred in the outer layers of the grain, in collapsed starchy endosperm cells and especially in the deformed aleurone cells. BBBI was localized in the same tissues. The intensity of the inhibitor labelling decreased at the later stage of infection, possibly because it was bound into a proteinase/inhibitor-complex.
Keywords: Fusarium; barley; proteinases; inhibitors

Introduction

Fusarium head blight (FHB, scab) is a serious fungal disease that reduces the quality and yield of cultivated barleys. The present malting barley cultivars are at best only moderately resistant to FHB. The use of improved tillage and crop rotation methods, applying less fertilizer and using fungicides have all been used to reduce the severity of *Fusarium* infections (reviewed by STEFFENSON 2003). However, more research on the molecular interactions between barley and various *Fusarium* spp is needed to better understand the natural defense mechanisms that barley uses to protect itself from the fungi.

FHB is a soil-borne disease that usually becomes a problem on rainy summers or in humid areas, because moisture favors the growth of the fungus (PARRY *et al*. 1995). In order to penetrate the plant tissues, the fungus needs to access either the inner side of the glumes or palea/lemma, or the hairs of the pistil (KANG & BUCHENAUER 2000). For this reason, barley and wheat are are most susceptible to *Fusarium* attack when the flower opens for pollination. The hyphae can quickly spread into the other plant tissues after the initial penetration.

Heavily infected grains are small, shrivelled, miscoloured and often contain mycotoxins (e.g. SCHWARZ *et al*. 2001). The *Fusarium* also alters the composition of grain by secreting hydrolytic enzymes and toxic compounds into the plant tissues. Two alkaline serine proteinases, a subtilisin-like proteinase (SL) and a trypsin-like proteinase (TL), are secreted

by *F. culmorum* when it is grown in media that contain cereal proteins. These proteinases have recently been purified and characterized and shown to hydrolyze C- and D-hordeins *in vitro* (PEKKARINEN *et al*. 2002; PEKKARINEN & JONES 2002; PEKKARINEN *et al*. 2003).

The barley grain can possibly counteract these fungal proteinases, because it contains endogenous proteins that inhibit their activities (PEKKARINEN & JONES 2003). The SL proteinase is inhibited by chymotrypsin/subtilisin class inhibitors (CI-1, CI-2) and a barley amylase/subtilisin inhibitor (BASI), whereas the TL proteinase is inhibited by a barley Bowman-Birk type trypsin inhibitor (BBBI).

The aim of the present work was to study whether the *Fusarium* SL and TL proteinases and their endogenous inhibitors exist together in the same tissues and cell types of infected barley grains. In other words, if the inhibition can take place *in vivo*. The results of our studies of the TL proteinase and its inhibitor BBBI are reported.

Material and Methods

Plant Material and Infection

Seeds of the Swedish malting barley cultivar 'Mentor' were treated with a fungicide and grown in a greenhouse. The plants were infected with *F. culmorum* (strain VTT D-80148) by spraying the axes with a conidia suspension as soon as the the awns could be seen. Control barleys were sprayed with sterile water. The samples were collected immediately after spraying, and then weekly until the grain matured.

Immunoblotting

The awns of the whole spikes were removed and the rest was frozen in liquid nitrogen and held at –80°C until powdered and extracted with 50 mM Na-acetate, pH 5.0 in ratio 0.4 g/ml. The proteins of the extracts were separated by SDS-PAGE on a 15% gel and electroblotted to a membrane, which was then treated with polyclonal antibodies raised against either the TL proteinase or BBBI.

Immunohistochemistry

Single grains were cut in half either longitudinally or transversely, and fixed with 4 % paraformaldehyde in PBS (10 mM Na-phosphate, pH 7.2, 150 mM NaCl) overnight at +4°C, dehydrated and infiltrated with paraffin (MARTTILA *et al*. 1995). Ten μm sections were cut and placed on glass slides, and counterstained with 0.1% Light Green (protein) and 50% Lugol solution (starch). Alternatively, the sections were treated with antibodies against the TL proteinase or BBBI as in MARTTILA *et al*. (1995).

Results and Discussion

The Structure of the Infected Barley Grains

The heavily infected grains were small and malformed, and their color varied from reddish to later brownish with black spots. The dry infected grains were very hard. When comparing the structure of non-infected and infected nearly mature barley grain sections, the malformation is very obvious (Fig. 1). The outer layers of the infected grain are thin and consist mostly of palea/lemma (husk), with almost no pericarp. The cells of the aleurone layer were badly misshapen. In the healthy grain, the Light Green staining showed that the protein contents of the aleurone and subaleurone cells were normal. The Lugol solution stained starch heavily in the starchy endosperm. In the infected grain, the cell walls of the aleurone layer were very green, indicating that they had a high cell wall protein contents. The subaleurone and nearby

starchy endosperm cells contained little starch, but a high protein content. The collapsed structure in the outermost starchy endosperm cells is likely to be due to the activities of the fungal hydrolytic enzymes.

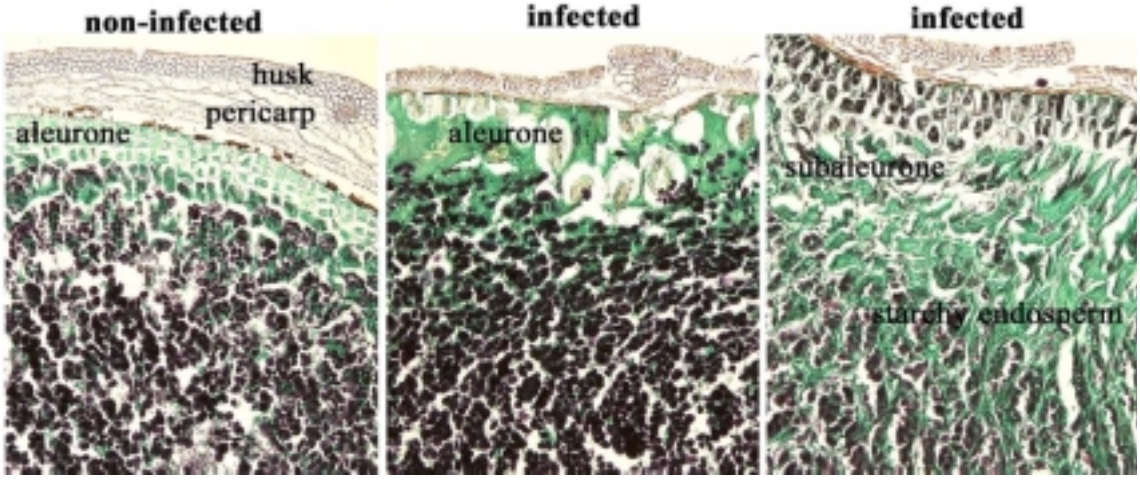

Figure 1. Structure of the infected grains. Non-infected nearly-mature grain had a normal appearance. In the infected grains, the aleurone layer could be badly misshapen. The starchy endosperm cells had partially collapsed. Green color shows protein distribution. Starch is stained black. All three pictures have the same magnification.

Detection of Trypsin Inhibitor and Trypsin-Like Proteinase in Barley Extracts

Immunoblotting showed that BBBI trypsin inhibitor was present both in control seeds and in the infected seeds (Fig. 2). The antibody recognized only one lane of approximately 15 kD. The fungal proteinase TL was not detected in the control seed extract, although naturally contaminated seeds might contain fungal proteins. TL antibody detected two close lanes of approximately 25 kD in the infected barley grains. These samples were taken four weeks after the infection date.

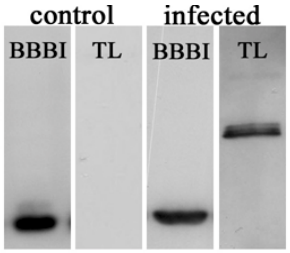

Figure 2. Immunoblotting of trypsin inhibitor (BBBI) and a fungal trypsin-like proteinase (TL) in the extracts of non-infected control barley and *Fusarium*-infected barley. The molecular weights were approximately 15 kD for BBBI and 25 kD for TL. The samples were taken four weeks after infection date.

The Localization of the Trypsin Inhibitor in Barley Grain

We used a specific antibody to localize BBBI, a putative inhibitor of the fungal TL proteinase, in healthy and infected barley grains. Previous studies have reported that BBBI is found in barley embryos and aleurone layers, and in the rootlets and coleoptiles of barley plants (BOISEN & DJURTOFT 1982; NAGASUE *et al*. 1988; KIRSI & MIKOLA 1971). In a near-mature barley grain, BBBI was indeed found in the aleurone layer and even in the

pericarp and husk (Fig. 3). However, the most intensive labelling was seen in the subaleurone layer of the non-infected grain. The protein in the embryo was clearly concentrated in the coleoptile and coleorhiza, the tissues that protect the embryonic shoot and root (data not shown). In the infected grain, the BBBI was located in the aleurone layer; especially in the misshapen aleurone cells (Fig. 4). The layers outside the aleurone layer contained BBBI as well. Surprisingly, BBBI was hardly detected in the subaleurone of the infected grain. This might be due to the formation of an inhibitor/proteinase complex in which the antibody may not be able to recognize the BBBI.

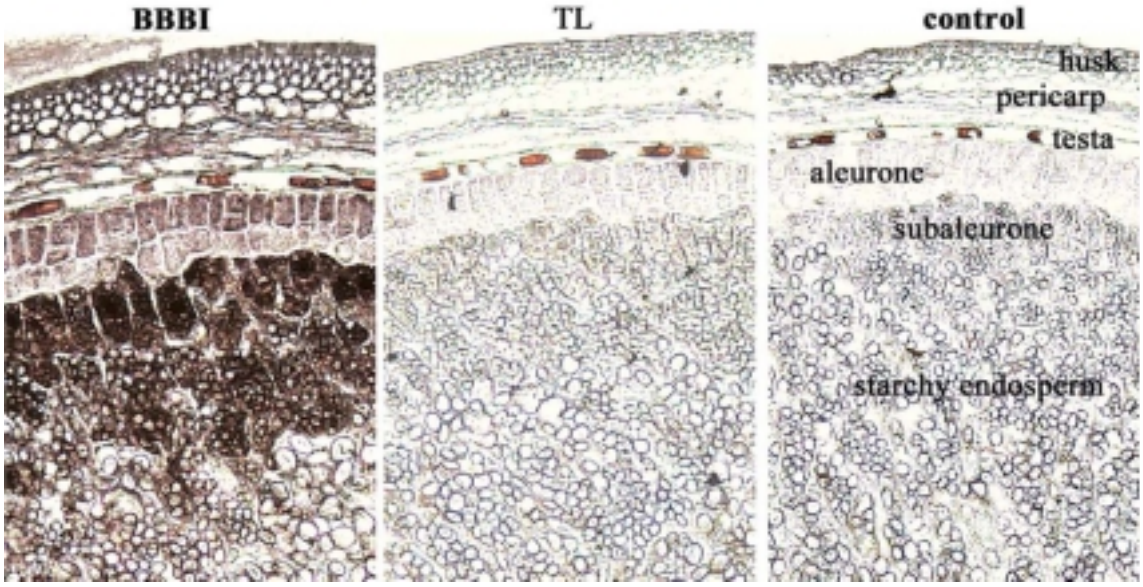

Figure 3. Immunolocalization of trypsin inhibitor (BBBI) and a fungal trypsin-like proteinase (TL) in non-infected barley grains. Positive signal is seen as a dark color. BBBI was strongly detected in the subaleurone layer, whereas TL was not detected in the healthy grain.

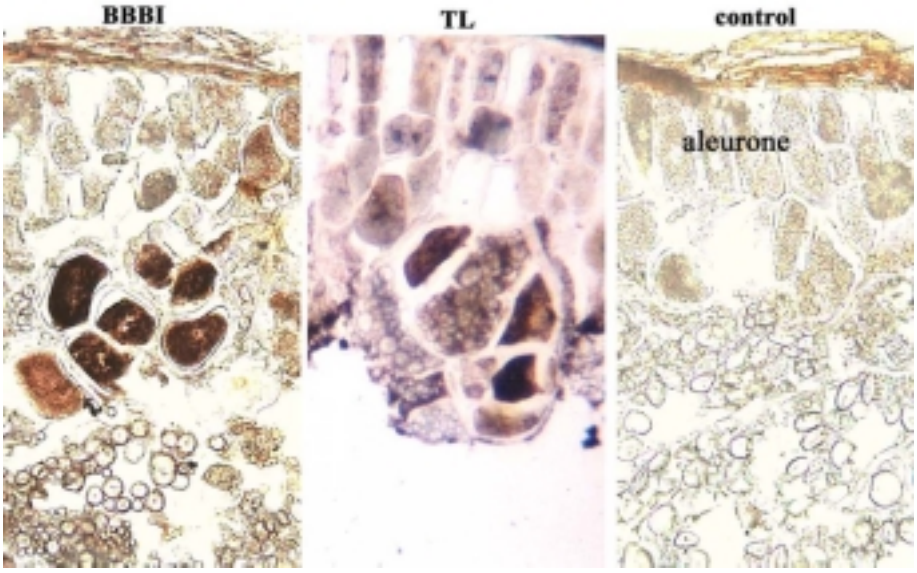

Figure 4. Immunolocalization of trypsin inhibitor (BBBI) and a trypsin-like proteinase (TL) in infected barley grains. BBBI and TL were detected in the misshapen aleurone cells (dark color).

The Detection of the Trypsin-Like Fungal Proteinase in Barley Grain

The TL proteinase was not detected in healthy barley grains, confirming that the antibody did not react with any barley proteins (Fig. 3). In the infected grain, a strong labelling was seen in the malformed aleurone cells (Fig. 4). The TL proteinase was also present in some collapsed starchy endosperm cells near the aleurone layer. In comparison to the endogenous proteins, the fungal proteinase was not evenly distributed in certain types of cells. It was only present occasionally; usually in the most misshapen cells.

Conclusions

These results show that the BBBI trypsin inhibitor and a fungal TL proteinase occur in the same type of cells within barley grains indicating that the fungal proteinase may well be inhibited *in vivo*. However, more detailed studies on the subcellular distribution of these proteins are still needed.

Acknowledgements

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Improvement of Malting Barley Resistance to Fusarium Head Blight Using Molecular Markers

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Abstract
The worldwide-distributed fungal disease called Fusarium Head Blight (FHB) and successive grain contamination by mycotoxins cause a considerable decrease in yield, quality and economic use of cereal crops. The infection of cereals by *Fusarium* fungi affects not only hygienic safety, but as well as grain technological quality. For instance, deoxynivalenol passes from malting barley grain infected by FHB up to a final product, beer, and is one of factors inducing beer gushing. Therefore, a great attention has been paid to diseases caused by fusaria in the Czech Republic over the recent years. A set of spring and winter barley varieties artificially infected with head fusaria were subjected to a test analysis. Based on visual and laboratory assessments and determination of the deoxynivalenol concentration, initial types displaying different resistance/susceptibility to FHB were selected. Hybridization of resistant and susceptible resources was carried out. Doubled haploid lines were derived from the hybrid progenies using *in vitro* induced androgenesis and used for further testing. RAPD markers and AFLP analyses were employed to detect differences in FHB resistance of barley lines.
Keywords: malting barley; Fusarium Head Blight; molecular markers; deoxynivalenol

Introduction

Fusarium Head Blight (FHB) is one of the most damaging diseases of barley. Though FHB ranked among minor and sporadic diseases of barley for a long period, it has become a significant factor limiting the yield and quality of cereal crops in a number of production areas worldwide over the last decade (STEFFENSON 2003; WINDELS 2000; PSOTA 2000).
The disease is caused by several species of the *Fusarium* genus which is known for production of a number of mycotoxins that are hazardous to both human and animal health. Even though FHB does not necessarily reduce yields, a low level of infection and grain contamination by mycotoxins can induce serious implications to grain processing and consumers – malthouses and brewhouses, and other food and feed use (SCHWARZ 2003).
Primary factors that are favourable for FHB outbreaks are changes in cropping practices, growing susceptible varieties and wet weather conditions during the grain filling stage. The best control of FHB is integrated cropping practices, fungicide application and breeding and releasing new resistant varieties (STEFFENSON 2003). Besides conventional methods, breeding programmes more often comprise molecular markers for identification of FHB resistance and development of new, more resistant varieties during the last years (QI *et al.* 1997; CASTIGLIONI *et al.* 1998; ZHU *et al.* 2001; ARMSTRONG *et al.* 2001; MUELLER & WOLFENBARGER 1999).

Material and Methods

Plant Material

The varieties and lines of spring barley that were used to evaluate and prepare markers for FHB resistance are characterized in Table 1. Doubled haploid lines examined were produced according to VAGERA and OHNOUTKOVÁ (1993).

Table 1. Pedigrees of original parental materials

Variety/line	Pedigree	Country	FHB reaction	Row no.
Chevron	CIho 1111 (PI 38061) = Landrace from Luzerne	CHE	Resistant	2
PEC 210	Embrapa 128	BRA	Resistant	2
CI 4196	PI 64275 (hang wang ta mai) = Landrace from Beijing	USA	resistant	2
Zao Zhou 3	Cultivar in East China, Zhejiang University, Hangzhou	ČHN	med. resistant	2
6NDRFG-1	PI 615583; North Dakota Agric. Experiment Station, USA	USA	med. resistant	6
Foster	Robust/3/Hazen//Glenn/Karl	USA	very susceptible	6
PI 383933	Kanto Nijo 2 = Ko. 1018/Kyoto Nakate from Japan	USA	very susceptible	6

Evaluation of Resistance and DON Content

The experiments with artificial inoculation of plots with conidium suspension of FHB were established and evaluated according to the scale by Horsfall-Barrett (STACK & Mc MULLEN 1995). The content of deoxynivalenol (DON) in ppm was determined by comparison to the standard curve using HPLC method (Liquid Chromatograph PU 4100M, Philips) according to LANGSETH *et al.* (1998) and PFOHL-LESZKOWICZ *et al.* (1999). Every measurement was twice replicated.

RAPD Assays

Genomic DNAs were extracted from leaves of seedlings (1st true leaf stage) using DNAesy Plant Mini Kit (Qiagen Firm). Thirty-five RAPD primers were used for RAPD assays. The reaction mix consisted of a total volume for reaction 25 µl, 1x buffer for *Taq* polymerase, 0.4 U *Taq* polymerase (Finnzymes), 0.25 mM mixture dNTP, 30 ng primer and 30 ng tDNA. Reaction conditions: 1 min. initial denaturation at 94 ^{o}C; 45 cycles – 1 min. denaturation at 94 ^{o}C, 2 min. annealing at 35 ^{o}C, 1 min. elongation at 72 ^{o}C; followed by final synthesis at 72 ^{o}C for 10 min. Electrophoretic separation was carried out on 1.5% agarose gel and visualization using ethidium bromide.

AFLP Assays

Genomic DNAs were extracted from leaves of 14 days old plants by a method based on selective precipitation in CTAB (SAGHAAI-MAROOF *et al.* 1984). Quality and quantity were estimated using electrophoresis and spectrophotometry. The DNA concentration was determined by comparison with the weight standard lambda/*Hind*III. Furthermore, 0.5µg of DNA at the volume of 5.5 µl were used. Restriction segregation using enzymes *Eco* RI a *Mse* I, ligation adapters and pre-selective amplifications were performed according to the Manual of Perkin Elmer Firm (Rev. Manual 1995, Part No. 569933) except for *Taq* polymerase. *Taq* polymerase of the Qiagen Firm (1U reaction, relevant buffer, 5mM dNTP, 10 µM *Mse*I selective primer and 1 µM *Eco*RI labelled primer) was used for selective amplification. The thermocycler Perkin-Elmer 9600 at the profile recommended in the AFLP manual was used. Amplification products were separated by capillary electrophoresis using Perkin-Elmer Genetic Analyser ABI 310. The results were evaluated by GeneScan and Genotyper software.

Data Analysis
Based on product presence (1) and absence (0) for AFLP and RAPD reactions, matrices were generated and graphically assessed using the Freetree software and TreeView, respectively.

Results and Discussion
Seven varieties (lines) of spring barley were tested in field experiments (Table 1). Results of these experiments showed that the level of the foreign materials corresponded with declared resistance or susceptibility also under climatic conditions of the Czech Republic (PROM *et al.* 1997; STEFFENSON 1999). This finding was confirmed by both laboratory assays and DON content measurement. The average infection of the lines PEC 210 and CI 4196 and variety Chevron with declared FHB resistance in 2 years field tests was 2 %, which is an upper limit for spring barley according to the European Brewery Convention (EBC 2000). The DON content in these resistant genotypes ranged from 0.2 to 0.3 ppm, whereas the acceptable hygienic limit is 2 ppm in the Czech Republic (PAPOUŠKOVÁ & SÝKOROVÁ 2001). Based on the results obtained, the highly positive correlation (r=0.92) between field evaluation and DON content as well as positive correlation (r=0.72) between values of laboratory assay and DON content were calculated. This was also confirmed by other authors, for example by LEMMENS *et al.* (1997) who deal with these problems. In contrast, ŠÍP (2001) refers to high variability in the DON content in his experiments, even in materials with medium resistance.

Table 2. Evaluation of original barley resources for reaction to FHB (2000-2001)

Genotype	Field eval. (%)	Labor.eval. (%)	DON content(ppm)
	Mean of 2000-2001		
Chevron	2	9.3	0.2
PEC 210	2.3	12	0.2
CI 4196	2	9	0.3
Zao Zhou 3	2	12.3	0.2
6NDRFG-1 *	3	15	1.8
Foster	6.5	27.7	1.1
PI 383933	9	34	1.3

* one year data from 2001

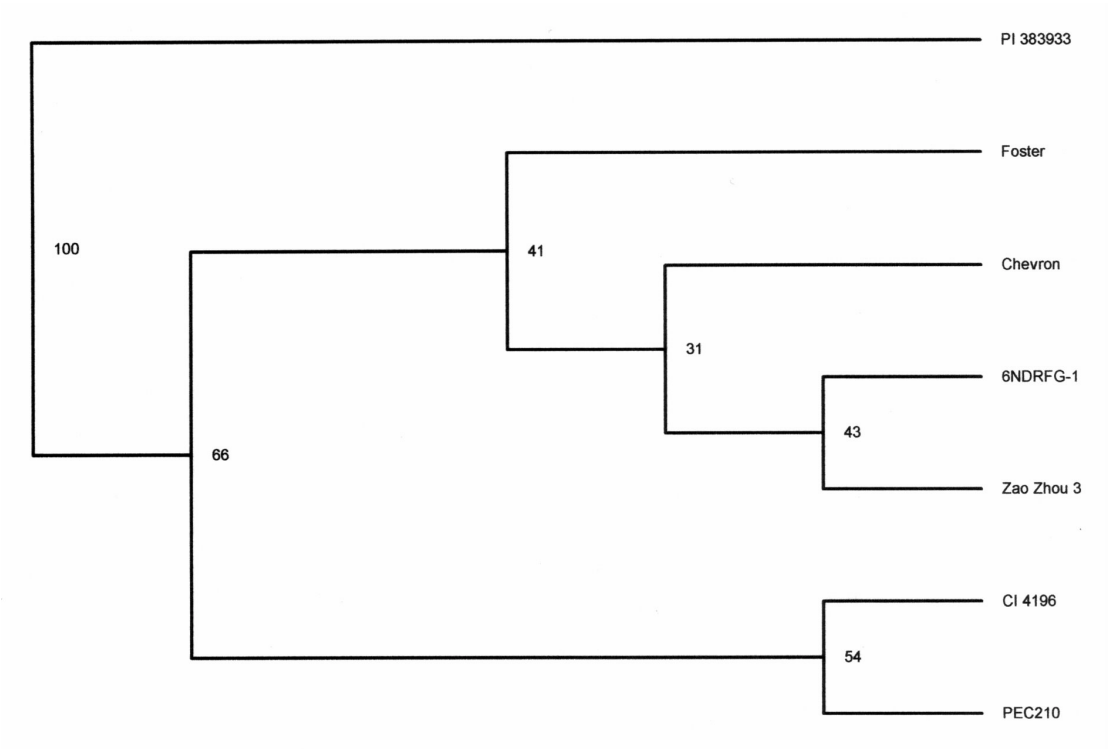
Based on preliminary screening, the original FHB resistance and susceptibility resources were involved in hybridization followed by doubled haploid (DH) line production. A method of *in vitro* androgenesis was used to derive 11 DH lines from the cross PEC 210 x PI 383933 and 6 DH lines from Chevron x PI 383933, which were further investigated. In laboratory tests, the average value of resistance in DH lines (Chevron x PI 383933) was 21.3±8.1 %, which seemed to be an acceptable value in comparison with parental materials. This value was 93.5 % for the susceptible genotype PI 383933 and 19 % for the resistant genotype Chevron.
Larger differences in the resistance level among DH lines were found for DH which originated from PEC 210 x PI 383933, where some lines overcome their resistant parent (Table 3). The highest resistance to FHB infection in laboratory tests (6 % in both years) was recorded in the line DH55.
AFLP and RAPD markers were employed to assess genetic diversity among examined barley resources with declared resistance/susceptibility to FHB. Based on 35 RAPD primers used, the studied spring barley genotypes were divided into three groups (Fig. 1). The first group consists of the genotypes CI 4196 and PEC 210 that are reported in literature to be resistant. The second group includes four genotypes: Chevron, which is considered as a donor of resistance to FHB, Zao Zhou 3, 6NDRFG-1 – the genotypes moderately susceptible and the

Table 3. Laboratory tests of DH lines in comparison with controls (2002-2003)

Variety/line	Row no.	% fusaria (2002)	% fusaria (2003)	Mean
PEC 210 x PI 383933 (DH40)	2	27	0	13
PEC 210 x PI 383933 (DH41)	6	29	26	28
PEC 210 x PI 383933 (DH42)	2	24	0	12
PEC 210 x PI 383933 (DH43)	6	17	5	11
PEC 210 x PI 383933 (DH47)	2	21	9	15
PEC 210 x PI 383933 (DH48)	2	13	11	12
PEC 210 x PI 383933 (DH53)	6	83	27	55
PEC 210 x PI 383933 (DH55)	6	6	6	6
PEC 210 x PI 383933 (DH58)	2	19	13	16
PEC 210 x PI 383933 (DH60)	6	34	11	23
PEC 210 x PI 383933 (DH61)	2	27	24	26
PEC 210 (control)	2	11	5	8
PI 383933 (control)	6	93	72	83

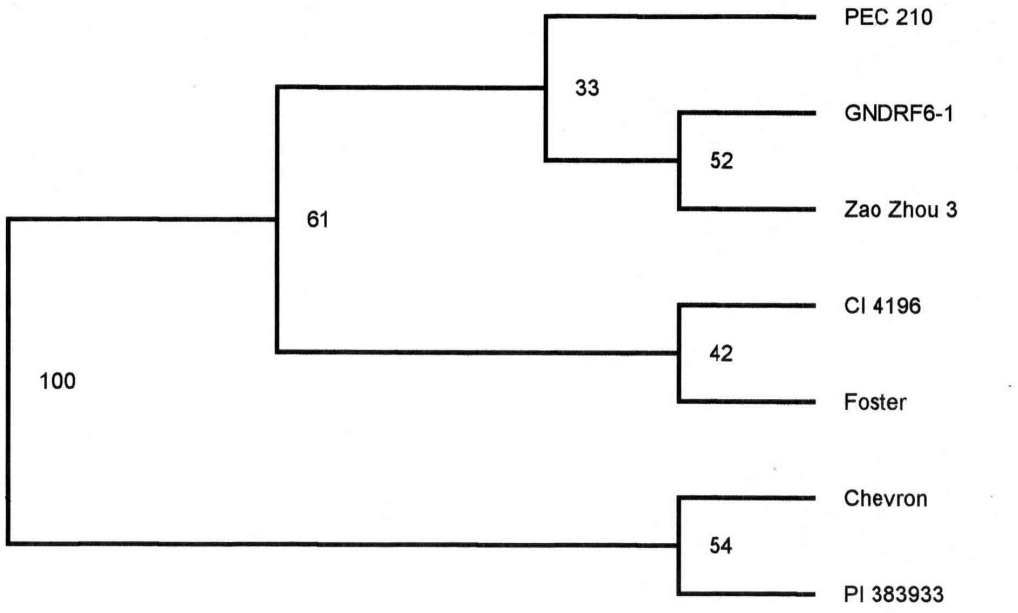
susceptible variety Foster. The third group comprises the susceptible genotype PI 383933, which is quite different from the other examined spring barley genotypes. The identical spring barley genotypes were subjected to AFLP assays with markedly different results. After evaluation of 84 polymorphic AFLP markers and their statistical analysis, three groups could be distinguished, however proportions of susceptible and resistant genotypes were different (Fig. 2). The first group, which considerably differed from the other two ones, included the

Figure 1. Dendrogram generated by UPGMA analysis calculated from RAPD markers using Nei and Li metrics showing relative genetic distance among analyzed genotypes of spring barley



resistant genotype Chevron and the susceptible spring barley PI 383933. The second group comprised the susceptible genotype Foster together with the resistant CI 4196. This group was genetically more similar to the third one consisting of the three genotypes: PEC 210 (resistant) and two moderately susceptible genotypes (6NDRFG-1 and Zao Zhou 3). Different results obtained with applications of RAPD and AFLP protocols could be due to higher sensitivity of the AFLP assay in comparison with RAPD. Differentiation of the developed DH lines to susceptible and resistant ones using molecular markers will be further investigated.

Figure 2. Dendrogram generated by UPGMA analysis calculated from AFLP markers using Nei and Li metrics showing relative genetic distance among analyzed genotypes of spring barley



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Characterization of Barley Yellow Mosaic Disease Resistant Gene *rym1* and Breeding of a Novel BaYMV and BaMMV Resistant Malting Barley

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Abstract

Mokusekko 3, a Chinese barley landrace is completely resistant to all strains of the *Barley yellow mosaic virus* (BaYMV) and *Barley mild mosaic virus* (BaMMV). It is also well known that Mokusekko 3 has at least two resistant genes *rym1* and *rym5*, with only *rym5* having been utilized for BaYMV resistant barley breeding in Japan. In order to clarify the effect of *rym1* on BaYMV and BaMMV, and to utilize the gene for resistant barley breeding, the susceptibilities of lines carrying only *rym1* against BaYMV and BaMMV were investigated. Our results showed that *rym1* was completely resistant to BaYMV-I, -II, BaMMV-Ka1 and -Na1, and had an acceptable level of resistance to BaYMV-III. Therefore, to introduce *rym1*, a novel BaYMV resistant gene, to malting barley, the Mokkei 01530 was selected from a cross between Mokusekko 3, a donor for *rym1*, and Haruna Nijo, an excellent malting variety. Mokkei 01530 was completely resistant to BaYMV-I and had an acceptable level of resistance to BaYMV-III. In comparison with its high quality parent Haruna Nijo, malting quality of Mokkei 01530 was almost identical with Haruna Nijo. Our results clearly indicated that *rym1* is another promising gene for the breeding of BaYMV and BaMMV resistance malting barley.

Keywords: virus resistance; *rym1*; marker assisted selection; malting barley; BaYMV; BaMMV

Introduction

East Asian and European winter barley cultivars are seriously damaged by BaYMV and BaMMV (HUTH & LESEMANN 1978). The most common approach for the prevention of infection with BaYMV and BaMMV is the introgression of the resistance genes identified in barley germplasm accessions into modern barley cultivars. A Chinese six-rowed barley landrace, Mokusekko 3, is unique in being completely resistant to all the strains of BaYMV and BaMMV in Japan (KASHIWAZAKI *et al.* 1989; IIDA *et al.* 1992; NOMURA *et al.* 1996). KONISHI *et al.* (1997) have indicated that at least two resistance genes, *rym1* and *rym5*, confer resistance to BaYMV in Mokusekko 3. In Japan, many BaYMV-resistant malting barley cultivars have been developed using Mokusekko 3 as a cross parent. Although Mokusekko 3 harbors at least two BaYMV resistance genes, only *rym5* was studied for barley breeding against BaYMV. However, the breakdown of its resistance was detected in the field by the appearance of a new virus strain, BaYMV-III (OGAWA et al. 1987). Another gene resistant to all strains of BaYMV is *rym3*. The *rym3* gene was found in Haganemugi and Ea 52 (UKAI & YAMASHITA 1980) and displays resistance to all strains of BaYMV in Japan. However, *rym3* is not

effective against BaMMV. Consequently, the effects of the *rym5* and *rym3* gene on infection by strains of BaYMV and BaMMV in Japan have been reported, but the effect of the *rym1* gene is unknown. In addition, an RFLP marker linked to *rym5* (GRANER *et al.* 1999) and *rym3* (SAEKI *et al.* 1999) has already been reported. The location of the *rym1* locus and *rym5* locus in Mokusekko 3, RFLP linkage maps has recently been reported (MIYAZAKI *et al.* 2001). This study first raises the issue of only *rym1* introgressed to barley cultivar using DNA markers, and investigates the effect of the *rym1* gene on infection by strains of BaYMV and BaMMV. The effect of *rym1* gene to the malting quality and agronomic performance including virus resistance will be shown. A discussion of the reason why the important resistance gene *rym1* was eliminated along with resistant cultivars during breeding for resistance to BaYMV will be discussed.

Material and Methods

DNA Extraction and Screening by Southern Hybridization

DNA was extracted from the mature leaves of the plants and their parents as described in the standard protocol of the CTAB method (MURRAY & THOMPSON 1980). Then, 3 μg of each DNA sample was individually digested with *Bam*HI or *Dra*I. These digested DNAs were electrophoresed on a 0.8% agarose gel and transferred to nylon membranes by the capillary method (SAMBROOK *et al.* 1989). The prehybridization, hybridization, detection, and probe labeling procedures were performed according to the Gene Images^{TM} system (Amersham Pharmacia Biotech) manual.

Plant Materials and Resistance Assessment

A BaYMV resistant F_4 progeny derived from Ko A×Mokusekko 3 carrying only *rym1* was screened by DNA markers linked to *rym1* (MWG2134, MWG2159 and MWG58) and linked to *rym5* (ABC172). Its progeny, Y4, carrying only *rym1* isolated from Mokusekko 3, proved homozygous for resistance. 341 F_2 populations were derived from a cross between the resistant line Y4 and the susceptible cv. Haruna Nijo. These were grown in a field infected with only BaYMV-I, and segregation of the reaction was investigated in the 1999-2000 season. Furthermore, nine resistant F_2-derived F_3 lines (40 seedlings / line) were grown in the same field and the reaction was investigated in the 2000-2001 season. The disease reaction was evaluated individually based on the mosaic symptoms on the leaves. If at least one leaf with mosaic symptoms was detected, the plant was scored as susceptible. In the assessment of resistance to BaYMV-III, the same nine F_3 lines were derived from F_2 plants that are resistant to BaYMV-I and selected homozygotes using DNA markers linked to *rym1*. In the 2000-2001 season, 40 seedlings per F_3 line were grown in a field infected with only BaYMV-III and investigated for their reaction to BaYMV-III. The assessment of resistance to BaYMV-III was based on the mosaic symptoms and yellowing on the leaves. The scoring of symptoms and assessment used the observed values of 0.0 to 6.0. All assessment plants were individually harvested and their agronomic characters were examined. Reaction to BaYMV-II, BaMMV-Ka1, and BaYMV-Na1 were performed by sap inoculation. The infection rate of sap inoculation is lower than field infection. In addition, infection rate of sap inoculation is more effective to genotypes (KASHIWAZAKI *et al.* 1989). On the other hand, we have various information of sap inoculation on Haruna Nijo. Therefore, plant materials were screened by DNA markers linked to *rym1*, derived from a Y4 × Haruna Nijo, and a 2 times

backcrossed with Haruna Nijo and selfed BC_2F_3 lines. DNA markers selected fifteen BC2F2 lines, and these seeds were bulked and used for plant materials. The sap inoculation was performed according to the procedure of KASHIWAZAKI *et al.* (1989), and the disease reaction was evaluated in each individual plant, as a substrate according to the method of KASHIWAZAKI *et al.* (1989).

Parent and Breeding

A cross was made between 'Y4' as the female parent and 'Haruna Nijo' as the male parent at the Plant Bioengineering Research Laboratories (PBRL), Sapporo Breweries Ltd., Gunma, Japan in 1999. 'Haruna Nijo', bred by Sapporo Breweries Ltd., is a Japanese malting variety with high malting and brewing quality. However, it is susceptible to BaYMV and BaMMV. F_1 plants were grown in a summer nursery and then backcrossed with 'Haruna Nijo'. Furthermore, the selection was made from 321 BC_1F_1 plants using DNA markers. After marker selection of BC_1F_1, hetero types were selected and selfed, and a second marker selection of progeny homozygous for *rym1* was made from BC_1F_2. These selections were carried out from the F_1 to BC_1F_4 generations at PBRL up to 2001. Cultivation for malting quality analysis and investigation of agronomic characters were carried out at Kizaki, Gunma, Japan in the 2001-2002 season. For the assessment of resistance to BaYMV-I and -III, barley samples were grown in a field infected with only BaYMV-I and -III, and the consequent reaction was investigated in the 2001-2002 season.

Micromalting and Malting Quality Analysis

Two hundred and fifty grams of grain (> 2.5 mm screen) were micromalted in an Automatic Micromalting System (Phoenix Biosystems, Torrens Park, Australia). Before germination, the steeping moisture was set to approximately 43.0-43.5% by adjusting the steep time of each sample (steeping utilized a 5-hour air rest after every 7 hours steep in water, 15°C). Germination lasted 6 days at 15°C, and the kilning scheme was 10 hours at 45°C, 8 hours at 55°C, 3.5 hours at 65°C, 3.5 hours at 75°C and 4 hours at 83.5°C. Malt samples were evaluated by the EBC analytical methods (Analytica-EBC 4.5.).

Results and Discussion

Effect of rym1's Resistance to Strains of BaYMV and BaMMV

In the assessment of resistance to BaYMV-I, 341 F_2 population derived from a cross between the resistant line Y4 with only *rym1* and the susceptible cv. Haruna Nijo shows that the segregation loosely fits a 1R : 3S ratio ($0.05 > P > 0.01$), suggesting that the resistance is controlled by a single recessive gene, *rym1*. Furthermore, none of the F_3 lines derived from the nine resistant F_2 plants showed any disease symptoms in the field infected by BaYMV-I. The same nine F_3 lines showed almost the same agronomic characters in the field infected by BaYMV-III as those in the uninfected field, apart from the symptom of showing numerous mosaics (Table 1). All assessment plants were individually harvested and the culm lengths were examined. The culm lengths were especially highly significant, the susceptible Haruna Nijo being dwarfed (Fig. 1). The infected plants developed systemic symptoms such as faint mosaic, mild mosaic, mosaic, necrosis, yellowing and severe mosaic in sap inoculation tests of BaYMV-II, BaMMV-Ka1 and -Na1. The infection rates of the susceptible control Ko A were

100%, 94% and 71%, and in the other susceptible control, Haruna Nijo, were 63%, 87% and 72%, respectively. On the other hand, the resistant control Mokusekko 3 did not show any disease symptoms. The *rym1* gene carrying lines also did not show any disease symptoms, which was the same as the level of resistance shown by Mokusekko 3. Moreover, the absence of the *rym1* gene in the BC_2F_3 lines showed the same reaction as Haruna Nijo, and the infection rates were 58%, 90% and 72%, respectively.

Table 1. Reaction of cultivars and Mokkei lines to BaYMV-I and -III. These were grown in a field infected with only BaYMV-I and -III segregation of the reaction was investigated in the 1999-2001 season.

Cultivars and Lines	Resistant gene	Reaction to BaYMV-I R / S*	Reaction to BaYMV-III		
			Score of Mosaic	Score of yellowing	R / S*
Amagi Nijo	-	S	6.0	5.5	S
Haruna Nijo	-	S	6.0	4.0	S
Mikamo Golden	*rym5*	R	5.0	4.0	S
Mokusekko 3	*rym1* , *rym5*	R	0.0	0.0	R
Kanto Nijo 34	*rym3* , *rym5*	R	0.0	1.0	R
Kanto Nijo 29	*rym3*	R	0.0	0.5	R
SA 22	*rym1*	R	5.0	2.0	AR
SA 52	*rym1*	R	4.5	2.5	AR
SA 58	*rym1*	R	4.5	1.5	AR
SA 75	*rym1*	R	4.5	1.0	AR
SA 141	*rym1*	R	4.5	1.5	AR
SA 144	*rym1*	R	4.5	1.5	AR
SA 154	*rym1*	R	4.5	2.0	AR
SA 184	*rym1*	R	4.5	2.0	AR
SA 201	*rym1*	R	4.5	1.5	AR

* R, resistant; S, susceptible; AR, acceptable resistance

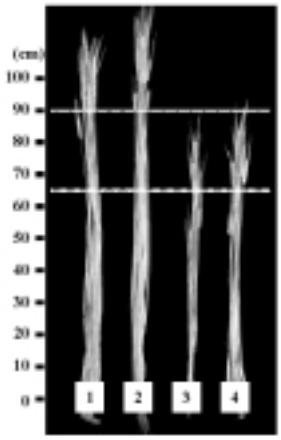

Fig. 1 These were grown in an infected field and harvested. There were significant differences between the *rym1*-carrying line and the susceptible Haruna Nijo grown in a field infected with BaYMV-III. The susceptible cultivar Haruna Nijo and *rym5*-carrying lines were dwarfed in infected field.
1;, grown in uninfested field Haruna Nijo,
2; *rym1*-carrying line Mokkei 01530,
3; susceptible control, Haruna Nijo,
4; *rym5*-carrying cv. Mikamo Golden

In this study, only *rym1* was introgressed into a modern barley cultivar using RFLP markers, with the effect of the gene being investigated on the infection by BaYMV and BaMMV strains. The above results indicated that *rym1* was completely resistant to BaYMV-I, -II, BaMMV-Ka1 and -Na1, and had an acceptable level of resistance to BaYMV-III. Mokusekko 3 is completely resistant to all the strains of BaYMV and BaMMV in Japan (KASHIWAZAKI *et al.* 1989; IIDA *et al.* 1992; NOMURA *et al.* 1996) and it was carrying at least two resistance genes, *rym1* and *rym5* (KONISHI *et al.* 1997). There are two interpretations of *rym1*'s reaction to BaYMV-III. One interpretation of the reaction to BaYMV-III of Mokusekko 3 is the incidence of multiplicative gene action. On the other hand, MIYAZAKI *et al.* (2001) recently reported that three QTLs were detected from Mokusekko 3 for BaYMV resistance. These QTLs were located on chromosome 4H for *rym1*, located on chromosome 3HL *rym5* and a new QTL was identified in the terminal region of chromosome 7HS. Therefore, another interpretation is the possibility that the new QTL had an effect on BaYMV-III. However, our result of this QTL region, revealed segregation in the assessment F_3 lines (data not shown). Therefore, it seems reasonable to conclude that Mokusekko 3 acquired a complete resistance to BaYMV-III by the multiplicative gene action of *rym1* and *rym5*. However, further investigation is now required.

Effect of rym1 to the Agronomic Performance and Malting Quality

This study introduces only *rym1* to the modern malting barley cultivar using DNA markers and investigates virus resistance and malting quality. The above results indicate that Mokkei 01530 was completely resistant to BaYMV-I and had an acceptable level of resistance to BaYMV-III and field resistance (Table 2). The important agronomic traits data of Mokkei 01530 are summarized in Table 3 comparing it with the Japanese malting variety, Haruna Nijo. Mokkei 01530 shows similar agronomic potential to Haruna Nijo in all aspects. As shown in Table 3 all the malting quality character values of Mokkei 01530 were in the desirable range and compared favorably with its high quality parent, Haruna

Nijo. The agronomic performance and malting quality of the introgression to the *rym1* line, Mokkei 01530, were comparable to the standard and acceptable level of the normal malting barley variety. The *rym1* gene has not been commonly introgressed into the established cultivars in previously breeding programs. However, in this program, the application of DNA marker-assisted selection to malting barley lead to successful breeding of novel virus resistance in a high malting quality barley line in only 3 years. Molecular marker assisted selection may further contribute to the introduction of a resistance gene, as well as combining resistance genes into a cultivar to stabilize resistance.

Many BaYMV-resistant malting barley cultivars have been developed using Mokusekko 3 as a cross parent in Japan. Though Mokusekko 3 harbors at least two BaYMV resistance genes, only *rym5* was used for barley breeding against BaYMV. As described above, in the breeding programs for BaYMV resistance, *rym1* was not commonly introgressed into the established cultivars. The question arises why the important resistance gene *rym1,* was eliminated in common with resistant cultivars during the breeding for resistance to BaYMV. Assuming a close linkage between *rym1* and an unfavorable QTL for malting barley, *rym1* could be easily dropped by eliminating the QTL. The malting quality was also investigated using BC_1F_4 carrying a *rym1* gene derived from Y4×Haruna Nijo, backcrossed with Haruna Nijo and selected using DNA markers. As a result, there were no significant differences between BC_1F_4 lines and Haruna Nijo (Table 3). On the other hand, the elimination of *rym1* might be due to the linkage drag between *rym1* and QTLs with the unfavorable agronomic characteristics being derived from Mokusekko 3. In our investigation, the *rym1* locus was found to be located on chromosome 4H and most tightly linked to the RFLP marker MWG2134 with a recombinant value of 4.17%, and MWG2134 was linked to the morphological marker *Bl* with a recombinant value of 8.42%. These results suggested that the *rym1* locus and the *Bl* locus were tightly linked on chromosome 4H. Given that these results are accurate, the elimination of *rym1* might be due to the linkage drag between *rym1* and the unfavorable agronomic character *Bl* derived from Mokusekko 3. In Japan, the blue aleurone tends to be regarded as an unfavorable character and it was eliminated in the malting barley breeding program. In consequence, it might be inferred from these results that in past breeding programs for BaYMV resistance, *rym1* was not introgressed into the established cultivars. This study has already broken the tightly linked *rym1* and *Bl*, and breeding a new line of malting barley carrying only the *rym1* gene resistant to all Japanese strains of BaYMV and BaMMV. In addition, a combination of *rym1*, *rym3,* and *rym5* is now being attempted using DNA markers and field infection.

Table 2. Reaction of Mokkei 01530 and cultivars to BaYMV-I and -III. In the assessment of resistar to BaYMV-I and -III barley samples were grown in a field infected with only BaYMV-I and -III, the reaction was investigated in the 2001-2002 season.

Cultivars and Lines	Resistant gene	Reaction to BaYMV-I R / S*	Reaction to BaYMV-III		
			Score of Mosaic	Score of yellowing	R / S*
Amagi Nijo	-	S	5.5	4.0	S
Haruna Nijo	-	S	5.5	4.0	S
Misato Golden	*rym5*	R	5.5	3.5	S
Mikamo Golden	*rym5*	R	5.5	4.0	S
Mokusekko 3	*rym1* , *rym5*	R	0.0	0.0	R
Mokkei 01512	-	S	5.0	3.0	S
Mokkei 01523	-	S	5.5	3.0	S
Mokkei 01530	*rym1*	R	4.5	2.0	AR

* R, resistant; S, susceptible; AR, acceptable resistance

Table 3. Important agronomic character and malting quality of Mokkei 01530 in comparison with the control variety Haruna Nijo

Character	Mokkei 01530	Haruna Nijo
Heading date	2002/4/6	2002/4/7
Culm length (cm)	91.7±3.0	84.6±6.1
Panicle length (cm)	5.3±0.07	5.1±0.3
Panicle number /m^2	812.5±73.2	839.7±56.1
Number of grains per panicle	22.8±0.09	22.1±2.1
Grain yield (kg/a)	47.3±2.0	50.2±5.6
Yield of plump (kg/a)	38.1±2.1	40.1±3.7
Plumpness (> 2.5 mm) (%)	80.6±0.9	80.1±1.6
1000 kernel weight (g)	33.0±0.9	31.9±0.5
Plump kernel weight (g)	36.1±0.09	35.4±1.2
Hot water extract (%, db)	83.7	84.2
Total nitrogen on malt (%, db)	1.57	1.67
Soluble nitrogen on malt (%, db)	0.66	0.68
Kolbach index	42.2	40.7
Diastatic power (°WK)	218	227
Apparent attenuation limit (%)	84.5	84.3
Hartong index (VZ45)	37.6	37.2
Wort β-glucan (mg/l)	66	79
Friability (%)	88.6	86.4

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Molecular Mapping of Virus Resistance in Barley (*H. vulgare* L.)

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Abstract

Virus diseases, i.e. soil-borne barley yellow mosaic virus disease (BaMMV, BaYMV BaYMV-2) and the aphid transmitted barley yellow dwarf virus (BYDV) have gained evident importance in European barley breeding. Resistance to barley yellow mosaic virus disease has been identified in exotic germplasms and several recessive resistance genes, e.g. *rym4* (3H), *rym5* (3H), *rym9* (4H), *rym11* (4H), *rym13* (4H) have been mapped. Recently the BaYMV/BaYMV-2 resistance of ´Chikurin Ibaraki 1´ has been assigned to chromosome 5H in the region of *rym3* while the BaMMV resistance (*rym15*) of this accession has been located on chromosome 6H. Genetic analyses of DH-populations to map additional genes is in progress. For the above mentioned genes easy to handle PCR-based markers have been developed (RAPDs, AFLPs, SSRs, STSs, SNPs). In contrast to barley yellow mosaic virus disease, no complete resistance to BYDV is known in the barley gene pool, but tolerant accessions have been identified and QTL for BYDV-tolerance have been detected on chromosomes 2HL and 3HL in different crosses and different trials explaining about 50 % of the phenotypic variance. Respective markers will facilitate a more efficient use of genetic diversity with regard to virus resistance in barley and are suited to combine tolerance to BYDV with resistance to soil-borne viruses.

Introduction

Barley mild mosaic virus (BaMMV) and barley yellow mosaic virus (BaYMV) belonging to the bymoviruses which are transmitted by the soil-borne fungus *Polymyxa graminis* (TOYAMA & KUSABA 1970) are the agents of barley yellow mosaic disease. These viruses either separately or in mixed infections (HUTH & ADAMS 1990) cause severe yield losses and have gained evident importance in European barley breeding. With respect to pathogenicity, seven strains of BaYMV and two of BaMMV have been distinguished in Japan (NOMURA *et al*. 1996). In Europe, up to now, only two strains of BaYMV are known (HUTH 1989) but a second strain of BaMMV has been recently detected in France (HARIRI *et al*. 2003). Due to transmission by the fungal vector *Polymyxa graminis* which has been detected viruliferous up to a soil depth of about 60 cm, chemical measures against the disease are neither efficient nor economic. Therefore, breeding for resistance has to be considered as the only possibility to ensure winter barley cultivation in the expanding area of fields infested by these viruses.

Barley yellow dwarf caused by *Barley yellow dwarf luteoviruses* BYDV-PAV and BYDV-MAV, the BYDV-RMV, which has not been classified up to now, and *Cereal yellow dwarf*

polerovirus CYDV-RPV, respectively (VAN REGENMORTEL *et al*. 2000) is widely distributed and causes one of the economically most important diseases of cereals world-wide. In barley, yield losses up to 40 % have been reported. The virus is transmitted by aphids, of which *Rhopalosiphum padi* and *Macrosiphum* (*Sitobion*) *avenae* are the prevalent vectors in Northern Europe. Symptoms caused by the virus are dwarfing of shoots and roots and leaf yellowing. Besides this, the number of spikes per plant and grain weight are reduced, the heading-date is delayed and the plants are more susceptible to abiotic stress and fungal diseases compared to healthy plants.

Breeding for resistance to these viruses can be enhanced today by biotechnology like the use of anther and microspore techniques giving rise to homozygous doubled haploid plants in A_1 (F_2) already, and especially the development of molecular marker systems transferring selection to some extend from the phenotyic to the genotypic level (cf. Friedt and Ordon, this volume).

Mapping of Resistance Genes against the Barley Yellow Mosaic Virus Complex

In extensive screening programmes resistance against barley yellow mosaic virus disease has been identified within the primary barley gene pool and genotypic differences concerning reaction to the different members of the barley yellow mosaic virus complex have been observed (ORDON *et al*. 1993). Besides this, resistances have been detected in *Hordeum spontaneum* (ERDOGAN *et al*. 1994) and *Hordeum bulbosum* (RUGE *et al*. 2003).

By segregation analyses it turned out that these germplasms carry genes different from *rym4,* which has for a long time been the only source of resistance to BaMMV and BaYMV in European barley breeding, and different from each other (GÖTZ & FRIEDT 1993, ORDON & FRIEDT 1993). Respective resistance genes have been subsequently assigned to barley chromosomes by morphological, isozyme and especially molecular markers (GRANER *et al*. 2000). An overview on mapped resistance genes against barley yellow mosaic virus disease, the resistance of the donor in Germany and the virus or virus strains used for mapping is given in Table 1 (WERNER *et al*. 2003a).

Out of the genes mentioned in Table 1 closely linked PCR-based markers are available for *rym4*, *rym5*, *rym9*, *rym11*, *rym13*, $Rym14^{Hb}$, *rym15,* and the BaYMV/BaYMV-2 resistance of ´Chikurin Ibaraki 1´ which may be allelic to *rym3*. Besides this, just recently the resistance of the accession ´PI 1963´ has been assigned to chromosome 4HL by RFLP and SSR analysis and it turned out by F_1-tests for allelism using mechanical inoculation with BaMMV that ´PI 1963´ carries a gene allelic to *rym11*. Using a targeted AFLP approach based on the analysis of 191 and 161 DH-lines, respectively, co-segregating markers have been developed for *rym11* (NISSAN-AZZOUZ *et al*. 2004). Besides this, in order to develop more or less diagnostic markers STS closely linked to the resistance locus *Rym4/Rym5* are recently screened for SNPs (NEUHAUS *et al*. 2003).

Based on easy to handle PCR-based markers the use of these resistances in barley breeding can be considerably improved, as these markers facilitate (i) a reliable selection on a single plant level independent from symptom expression in the field (ii) the acceleration of back crossing procedures, (iii) the pyramiding of resistance genes (cf. Friedt and Ordon, this volume).

Mapping of QTL for Tolerance against BYDV

In contrast to barley yellow mosaic virus disease no complete resistance to barley yellow dwarf virus (BYDV) is known in barley. However, genes conferring tolerance, i.e. ryd1 derived from the cultivar ‘Rojo’ (Suneson 1955) and Ryd2 identified in Ethiopian landraces (Rasmusson & Schaller 1959), have been identified. But, due to its low efficiency, *ryd1* was

Table 1. Mapped resistance genes against barley yellow mosaic virus disease, their source, resistance of the donor in Germany, and virus used for mapping (from WERNER *et al.* 2003a, mod.)

Resistance Gene	Chromosome	Source	Resistance of donor in Germany	Virus used for mapping	Reference
Rym1	4HL	Mokusekko 3	BaMMV, BaYMV, BaYMV-2	BaYMV*	1, 2, 4, 10
Rym2	7HL	Mihori Hadaka 3	BaMMV, BaYMV, BaYMV-2	BaYMV*	1, 2, 4
Rym3	5HS	Ea 52 Ishuku Shirazu	BaYMV, BaYMV-2	BaYMV*	2, 4, 14
Rym4	3HL	Ragusa, Franka	BaMMV, BaYMV	BaMMV BaYMV	2, 3, 4, 7
Rym5	3HL	Mokusekko 3, Resistant Ym No.1, W122/37.1	BaMMV, BaYMV, BaYMV-2	BaMMV BaYMV BaYMV-2 BaYMV*	2, 4, 6, 10, 11, 20
Rym6	3HL	Prior, Amagi Nijo	Susceptible	BaYMV*	5, 13, 15
Rym7	1HS	HHor 3365	BaMMV	BaMMV	12
Rym8	4HL	10247	BaMMV, BaYMV	BaMMV	2, 4, 9, 12
Rym9	4HL	Bulgarian 347	BaMMV	BaMMV	2, 4, 9
Rym10	3HL	Hiberna	BaYMV, BaYMV-2	BaYMV BaYMV-2	6, 11
Rym11	4HL	Russia 57	BaMMV, BaYMV, BaYMV-2	BaMMV	2, 4, 9
Rym12	4HL	Muju covered 2	BaMMV, BaYMV, BaYMV-2	BaMMV	2, 4, 8
Rym13	4HL	Taihoku A	BaMMV, BaYMV, BaYMV-2	BaMMV	2, 4, 16
Rym14^{Hb}	6HS	*Hordeum bulbosum*	BaMMV, BaYMV, BaYMV-2	BaMMV BaYMV BaYMV-2	17
Rym15	6HS	Chikurin Ibaraki 1	BaMMV, BAYMV, BaYMV-2	BaMMV	18
	5HS	Chikurin Ibaraki 1	BaMMV, BaYMV, BaYMV-2	BaYMV BaYMV-2	19

*Japanese strain of BaYMV

[1]TAKAHASHI *et al.* (1973), [2]GÖTZ and FRIEDT 1993, [3]GRANER and BAUER (1993), [4]Ordon *et al.* (1993), [5]IIDA and KONISHI (1994), [6]GRANER *et al.* (1995), [7]ORDON *et al.* (1995), [8]GRANER *et al.* (1996), [9]BAUER *et al.* (1997), [10]KONISHI *et al.* (1997), [11]GRANER *et al.* (1999a), [12]GRANER *et al.* (1999b), [13]IIDA *et al.* (1999), [14]SAEKI *et al.* (1999), [15]KONISHI *et al.* (2002), [16]WERNER *et al.* (2003b), [17]RUGE *et al.* (2003), [18]LE GOUIS *et al.* (2004), [19]WERNER *et al.* (2003a), [20]PELLIO *et al.* (2004)

only rarely used in barley breeding. In contrast to this, *Ryd2* has been incorporated into several barley cultivars tolerant to BYDV, e.g. 'Vixen' (PARRY & HABGOOD 1986) and other modern cultivars. This gene which has been mapped to the centromeric region of the long arm of chromosome 3H (COLLINS *et al*. 1996) and for which different PCR-based markers are available (PALTRIDGE *et al*. 1998, FORD *et al*. 1998) confers tolerance to BYDV which is additionally influenced to some extend by the genetic background, the virus isolate and the environmental conditions. Besides *Ryd2*, different sources of tolerance such as that found in ´Post´ have been identified. By analysing DH-lines of the crosses ´Post´ x ´Vixen´ and ´Post´ x ´Nixe´ after artificial inoculation with BYDV-PAV bearing aphids in three years field and pot trials a quantitative mode of inheritance of tolerance to a German

isolate of BYDV-PAV was demonstrated (SCHEURER *et al*. 2001). Tolerance to BYDV was estimated relative to healthy control plants of the same DH-line. QTL analysis for 'Post' x 'Vixen' revealed two major QTL for the relative grain yield after BYDV-infection. The positive allele for one was derived from cv. 'Vixen' on chromosome 3HL in the *Ryd2* region (LOD 7.7). The second QTL with the positive allele derived from 'Post' was detected on the long arm of chromosome 2H (LOD 3.3). These two QTL together explain 46.8 % of the phenotypic and 73.7 % of the genotypic variance (SCHEURER *et al*. 2001). In order to verify these results the original mapping population of ´Post´ x ´Vixen´ was enlarged to 117 DH-lines and again tested in two years trials. Respective QTL were again found on chromosomes 2HL and 3HL explaining 41.5 % of the phenotypic variance (WEISKORN 2003). Besides this, additional tests in an independent DH-population of the cross ´Post´ x ´Vixen´ carried out at the Institute of Epidemiology and Resistance at Aschersleben again confirmed the importance of these chromosomal regions for tolerance to BYDV, as those genotypes carrying the positive alleles showed a significantly higher relative grain yield after BYDV-PAV infection. Additional QTL analysis for BYDV-tolerance on the population ´Post´ x ´Nixe´ also revealed the importance of the QTL on chromosome 2HL derived from ´Post´ which was detected at exactly the same marker interval as in ´Post´ x ´Vixen´, i.e. HVCSG-W20H480 (SCHEURER *et al*. 2001). In addition to these analyses, QTL analysis was also carried out on 61 DH-lines of the cross ´MOB3561´ x ´Asorbia´ (FRIEDT et al. 2003). For this reason a skeleton map comprising 1289 cM with an average marker distance of 13.2 cM was constructed. Based on this map and phenotypic data obtained in three years trials at Aschersleben and Braunschweig, i.e. six environments (SCHEURER 2001) QTL for all traits analysed were detected in the marker interval YLpPCRM-AB04H500 in the vicinity of *Ryd2* (Table 2, WEISKORN 2003).

Table 2. Putative QTL in the marker interval YLpPCRM-AB04H500 averaged over six environments after BYDV-PAV infection estimated on 61 DHLs of the cross ´MOB3561´ x ´Asorbia´ (WEISKORN 2003)

Trait	Position [cM]	Marker interval	LOD	σ^2_p	additive effects [%]
Kernel/Yield (%)	3H, 124	YLpPCRM-AB04H500	14.4	74.3	28.8
Ears/Plant (%)	3H, 124	YLpPCRM-AB04H500	21.7	76.3	26.4
TKW (%)	3H, 124	YLpPCRM-AB04H500	3.9	37.7	3.5
Kernel/ear (%)	3H, 124	YLpPCRM-AB04H500	4.4	19.6	8.8
Plant Height (%)	3H, 124	YLpPCRM-AB04H500	10.6	50.9	17.2

σ^2_p = phenotypic variance

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Unlocking the Secondary Gene Pool of Barley as a Genetic Resource for Resistance to Barley Yellow Dwarf Virus (BYDV)

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Abstract
Barley yellow dwarf virus (BYDV) causes high yield losses in most of the major cereal crops worldwide. In contrast to the widely used resistance sources to BYDV based on the *Ryd2* gene and conferring tolerance to BYDV, a new type of resistance has been identified within the tetraploid wild species *Hordeum bulbosum* (2n=4x=28), (HABEKUSS *et al.,* this volume). Interspecific reciprocal crosses between the tetraploid *H. bulbosum* accession and diploid (2n=2x=14) and tetraploid (2n=4x=28) cultivars, respectively, of *H. vulgare* were used to transfer resistance to BYDV into cultivated barley. Triploid (2n=3x=21) and tetraploid (2n=4x=28) hybrids were obtained with the aid of embryo culture. The number of embryos produced per seed and regeneration rate of plants was strongly influenced by the genotype of the *H. vulgare* parents and the direction of the cross. Barley anchor markers were used to verify the hybrid character of the interspecific F1 offspring. Fifty hybrids were tested for their resistance to BYDV via inoculation with viruliferous aphids (BYDV-PAV, *Rhopalosiphum padi*). Thirteen of these 50 F1 plants did not develop symptoms and stayed virusfree according to ELISA. Twenty-five diploid plants have been produced by backcrossing one triploid BYDV resistant hybrid with partial fertility to *H. vulgare*. Their resistance to BYDV remains to be tested.
Keywords: barley yellow dwarf virus (BYDV); resistance; *Hordeum bulbosum*; interspecific crosses

Introduction

Barley yellow dwarf (BYD) is the most widely distributed viral disease of cereals in the world. The range of economically important crops affected by BYD luteoviruses (BYDVs) is very large. More than 20 species of aphids may act as vectors. The symptoms depend on the age of the plants at the time of infection, the strain of the virus, and environmental conditions. The virus causes the vascular degradation of the phloem sieve tubes. This results in a limited transport of assimilates and reduced photosynthesis, leaf yellowing and stunting, reduced root and shoot growth, leaf stiffness, delayed heading, reduced tillering and the occurrence of numerous sterile florets. HEWINGS and EASTMAN (1995) calculated that 5 % losses caused by BYDVs in the United States in 1989 would have resulted in crop losses corresponding to $48.5 million for barley. Infected plants are more susceptible to fungal diseases, water deficiency and frost injury (D'ARCY 1995). The development of resistant or tolerant cultivars is an efficient strategy to control this disease. Various levels of tolerance have been found in a number of Ethiopian barleys. The *ryd1* and *Ryd2* genes in barley confer subtotal protection against BYDV. *Ryd2* identified in Ethiopian barleys, provides resistance only to subgroup I BYDVs (BALTENBERGER *et al.* 1987). *Ryd2* does not prevent the virus spreading after infection, it rather appears to act by reducing the rate of replication of the virus in the phloem (SKARIA *et al.* 1985). OVESNA *et al.* (2002) tested barley cultivars, breeding lines, and resistance sources from world collections for more than ten years. Most genotypes

were found to be susceptible or moderately susceptible to BYDV. Resistance reactions were due to the presence of the *Ryd2* gene. They also found genes that were non–allelic to *Ryd2* and conferred a moderate resistance in barley cultivars. QTL analysis suggested a quantitative mode of inheritance for tolerance against BYDV-PAV (SCHEURER *et al.* 2001). QTLs responsible for effects of BYDV infection on yield components, plant height, and heading date were mapped on chromosomes 2HL and 3HL (SCHEURER *et al.* 2001). To date, no barley source for immunity to BYDV is known.

MICHEL (1996) identified an accession of *H. bulbosum* that confers complete resistance (immunity) to BYDV. This accession additionally contains resistances to powdery mildew, leaf rust, the soilborne mosaic virus complex (BaMMV, BaYMV-1 and -2) and typhula blight (MICHEL 1996). It is a potential genetic source of novel genes for barley improvement by introgression. The wild species *H. bulbosum* L. represents the secondary gene pool of barley. It has been scarcely used to improve cultivated barley. Despite the crossing barriers successful introgressions of agronomically useful genes, e.g. resistance to powdery mildew (POHLER & SZIGAT 1982; XU & KASHA 1992; PICKERING *et al.* 1995), leaf rust (SZIGAT *et al.* 1997; PICKERING *et al.* 1998) and soilborne virus complex (RUGE *et al*. 2003) have been achieved. Using molecular markers these dominantly inherited resistances were mapped on *H. bulbosum* introgressions located on barley chromosomes 2HL, 2HS and 6HS, respectively (PICKERING *et al.* 1995, 1998, 2000; KASHA *et al.* 1996; RUGE *et al.* 2003).

To further unlock the secondary gene pool as a plant-genetic resource for barely breeding, a crossing programme has been initiated to develop interspecific *Hordeum vulgare* × *H. bulbosum* hybrids which serve a starting point for the transfer of resistance to BYDV into cultivated barley. The present paper describes the hybrids obtained so far.

Material and Methods

Plant Material

Interspecific hybridisation was carried out using the three diploid (2n=2x=14) *H. vulgare* cvs. 'Igri' (VV-1), 'Nikel' (VV-2), and 'Borwina' (VV-3), one tetraploid (2n=4x=28) cultivar 'Borwina' (VVVV) and one tetraploid (2n=4x=28) *H. bulbosum* accession with resistance to BYDV (BBBB). VV-1, VV-2 and VV-3 were used as female parents. Reciprocal crosses were also made between VV-1 and BBBB as well as VVVV and BBBB. VV-1 was used to produce BC1 offspring, with the triploid (2n=3x=21), partially fertile F_1 hybrid used as pollinator. Plants were vernalized at 2-4 °C under short-day conditions (8h light, 2000-4000 lux/16 h dark) for ten weeks. Finally, the vernalized plants were transferred to a climate chamber under following conditions: 18 h artificial light (Philips SON-T-Agro and Philips HPI-T-Plus, about 17000 lux) per day and average temperature of 15 °C (day/night temperatures: max. 22 °C/min 11 °C). Plants were cultivated in 13 x 13 cm pots filled with a peat dust to which fertilizers had been added (2 g/l: at time of vegetative growth Hakaphos Blue [15N+10P +15K+2Mg], at time of tillering Hakaphos Red [8N+12P+24K+4Mg]) every two weeks.

Embryo-Rescue Technique

Spikes were cut 14-20 days after pollination, surface-sterilized in 70 % ethanol for 30 sec., followed by 3 % sodium hypochlorite for 20 min and three times washed in sterile water. The embryos were aseptically excised under 10 x magnification on a laminar flow bench and placed on the culture medium (macro- and microelements L3, vitamins L2 [JÄHNE *et al.* 1991], 750 mg/l glutamine, 100 mg/l asparagine, 17.1 g/l sucrose, 0.04 mg/l BAP, pH 5.8, solidified with 6 g/l phytagel). Embryos were kept in a culture cabinet with low light intensity (1000-2000 lux, 16 h) and 16 °C temperature. Plants were potted about four weeks later.

Cytological Analysis
Chromosome counts were performed on root tips using the Feulgen technique. The nuclear DNA content was analysed using a method described by De LAAT *et al.* (1987).

Molecular-Marker Analysis
Total plant DNA was isolated as described by WILKIE (1989). For PCR of STS markers, 50-100 ng of genomic DNA was used in a solution containing 1 x reaction buffer (Qiagen), 200 µM dNTPs, 5 pmol primers and 0.5 U of *Taq* DNA polymerase (Qiagen). PCR products were separated either on 1 % agarose gels or 10 % polyacrylamide gels followed by ethidium bromide and silver staining (BUDOWLE *et al.* 1991), respectively.

BYDV-Resistance Tests
Interspecific hybrids were inoculated via viruliferous aphids (15-20 *Rhopalosiphum padi* per plant, BYDV-PAV1 Aschersleben) for 5 days. As a control, seedlings of *H. vulgare* cv. 'Rubina' were also inoculated. Subsequently, aphids were killed by insecticide spraying. Plants were cultivated in a greenhouse for 6 weeks before virus content was estimated by DAS-ELISA using a self-produced polyclonal antisera. Non-infected plants were vegetatively cloned by dividing them into ten plants, and tested again for virus resistance as described above.

Leaf-Rust Resistance Tests
Leaf-rust resistance was tested according to RUGE *et al.* (this volume).

Results and Discussion
The seed set per flower and the yield of embryos and plantles from the caryopses and embryos, respectively, in *Hordeum vulgare* × *H. bulbosum* crosses highly depended on the *H. vulgare* cultivar that was used as a female parent. Cv. 'Borwina' (2x, 4x) led to the highest frequency of embryos (VV-3: 67.9 %/VVVV: 44.3 %) compared to cv. 'Igri' (VV-1: 5.9 %, Table 1). When cv. 'Borwina' (4x) was used as pollinator, however, only 7 % of the embryos differentiated compared to 44.3 % achieved with the opposite direction. The regeneration rate of embryos into plants was also influenced by the genotype and the cross direction. When cv. 'Nikel' (VV-2) was used as female parent only 6 plants out of 69 embryos could be regenerated compared to 17 plants out of 20 embryos from 'Igri' × *H. bulbosum* crosses. The regeneration rates of embryos into plants were much lower when *H. bulbosum* was used as female parent (36.4 %: BBBB×VV-1; 22.1 %: BBBB×VVVV; not shown in Table 1). MICHEL (1996) also reported a slower seed set in this cross direction. Apparently there is an effect of the cytoplasma of *H. bulbosum* on the vigour of the embryos. The influence of the genotype on the hybridisation rate observed in the present study was in accordance with that reported by LANGE (1969) and PICKERING (1984).
All hybrids from VV/VVVV×BBBB crosses that were analysed proved to be triploid (2n=3x=21). Hybrids from BBBB×VV crosses were triploid or tetraploid and all hybrids from BBBB×VVVV crosses were tetraploid (2n=4x=28). In contrast to other reports (LANGE 1969; KASHA & KAO 1970; SUBRAHMANYAM & KASHA 1973), elimination of chromosomes was never observed cyologically. SUBRAHMANYAM (1982) found differences between the species in their ability to retain their genome after fertilisation. Chromosome stability is probably dependent on the genotype of *H. bulbosum* that had been used in these interspecific crosses.
As reported by LANGE (1969) and SZIGAT (1984), tetraploid hybrids may result from reciprocal VVVV×BBBB crosses. SZIGAT (1984) and RUGE *et al.* (2003) demonstrated that fertile tetraploid hybrids are useful for gene transfer into *H. vulgare*. It remains to be analysed

whether the new tetraploid and triploid interspecific hybrids of the present study are suited for resistance-gene transfer into barley.

Table 1. Yields of hybrid plants obtained from reciprocal crosses between *H. vulgare* (2n=14: VV-1, -2, -3; 2n=28: VVVV) and *H. bulbosum* (2n=28, BBBB)

Combinations		Florets pollinated	Embryos cultured Number	(%)	Hybrid Plants Number	(%)
VV-1	BBBB	339	20	5.9	17	5.0
VV-2	BBBB	401	69	17.2	6	1.5
VV-3	BBBB	78	53	67.9	50	64.0
VVVV	BBBB	463	205	44.3	200	43.2
BBBB	VV-1	1108	74	6.7	36	3.2
BBBB	VVVV	752	53	7.0	40	5.3

A differentiation of molecular-marker alleles from *H. bulbosum* (*b* alleles) and *H. vulgare* (*v* alleles) in the hybrids and their parents was attempted by using STS anchor markers located on the seven chromosomes of barley. The hybrid character of three interspecific F1 offspring plants from BBBB×VV (H1, H3) and VV×BBBB crosses (H2), respectively, could be demonstrated (Table 2).

Table 2. Identification of interspecific hybrids (H1-H3) using barley STS markers derived from RFLP ancor markers

	STS Marker	**Expected size (bp)**	**Hybrid H1 BBBB×VV**	**Hybrid H2 VV×BBBB**	**Hybrid H3 BBBB×VV**
Expected Genome constitution			BBV	VBB	BBV
Chromosome **1H**	*ABG373*	444 (900)*	*bv*	*vb*	*v.*
Chromosome **2H**	*MWG2133*	380	*bv*	*vb*	*bv*
	MWG2146	324	*bv*	*vb*	*bv*
	MWG520	800	*bv*	*vb*	*bv*
Chromosome **3H**	*MWG549*	392	*bv*	*vb*	*bv*
Chromosome **4H**	*WG622*	161	*bv*	*vb*	*bv*
Chromosome **5H**	*MWG877*	1150	*bv*	*vb*	*bv*
	MWG583	350	*bv*	*vb*	*bv*
Chromosome **6H**	*MWG2218*	200	*bv*	*vb*	*bv*
	MWG934	314	*bv*	*vb*	*bv*
Chromosome **7H**	*MWG530*	178 (600)*	*bv*	*v.*	*bv*
	MWG2031	1200	*bv*	*vb*	*v.*

*Size of *v* alleles in cv. 'Igri'

In three instances, anchor markers failed to verify the presence of the respective orthologous *H. bulbosum* chromosome or chromosome region (Table 2, shaded entries). In hybrid H3, *H. bulbosum* chromosome 1 could not be detected since marker *STS-ABG373* displayed the *v* allele only. *ABG373* is located distally on the long arm of barley chromosome 1H (QI *et al.* 1996). STS primers for additional 1H anchor markers gave no polymorphismen between the

two genomes (not shown). Absence of the *b* alleles of markers was also observed in hybrids H2 and H3 for chromosomes 7HS and 7HL, respectively, as demonstrated by markers *MWG530* and *MWG2031*, respectively (Table 2, Fig. 1). Cytological analysis displayed 21 chromosomes for all three hybrids. However, the absence of *b*-marker alleles for the distally located markers on 1HL (hybrid H3) and 7HS (hybrid H2), respectively, as well as the proximally located marker *MWG2031* (~85 cM) on 7HL (hybrid H3) may be indicative for partial elimination of *H. bulbosum* chromatin.

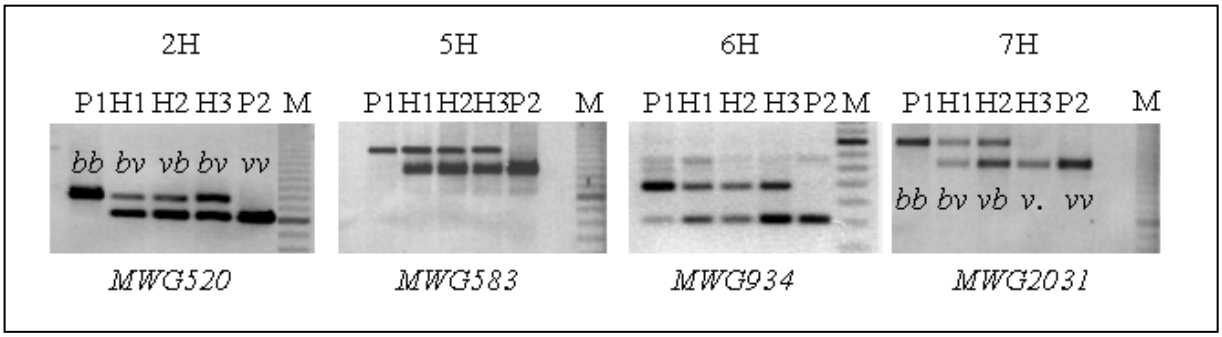

Figure 1. Identification of interspecific hybrids by means of STS markers. P1, *H. bulbosum* parent; P2, *H. vulgare* parent; H1, H2, and H3, interspecific hybrids

Elimination of marker alleles of the *H. bulbosum* parent in the two hybrids appears to be independent on whether this parent was used as male or female (Table 2). The loss of *H. bulbosum* chromatin in the two interspecific hybrids will have to be analyzed in its extension in more detail.

Upon inoculation with the strain BYDV-PAV1 Aschersleben, thirteen hybrids did not develop any symptoms and stayed virusfree according to ELISA results (Table 3). Of the three hybrids verified with molecular markers above, all proved non-infectable by BYDV-PAV. In addition, hybrids H1 and H2 also displayed resistance to leaf rust (not shown).

Table 3. Reaction of different hybrids to BYDV-PAV (ELISA)

Combination	Number of F1 plants tested	Number of infected plants	Number of non-infected plants
VV × BBBB	27	25	2^{H2}
BBBB × VV	23	12	$11^{H1, H3}$

After backcrossing the partially fertile hybrid H2 to 'Igri', 25 offspring plants (6.4% of pollinated florets) were obtained. Of these, 18 plants displayed 14 chromosomes and were fertile. All plants resembled *H. vulgare* morphologically except of the leaves which were intermediate in width. Furthermore, the plants differed in their reaction to leaf rust as compared to cv. 'Igri'. To clarify whether *H. bulbosum* chromatin conferring BYDV resistance has been introgressed into this offspring, BYDV-PAV resistance tests will be conducted together with molecular-marker analysis.

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Pathogenic Variability of Fusarium Head Blight Pathogens on Spring Barley

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Abstract

Fusarium Head Blight (FHB) threatens the barley (*Hordeum vulgare*) production in Austria under humid and warm weather conditions and has the potential of reduced food and feed safety for barley products. Sources of resistance to *Fusarium graminearum* have been identified in spring barley collections. However, very little is known about the reaction of barley to other *Fusarium* species to effectively manage FHB resistance. Two *F. graminearum* susceptible and two resistant six- and two-rowed spring barley cultivars were investigated for their reaction towards Austrian isolates of *F. graminearum*, *F. culmorum*, *F. poae*, *F. avenaceum* and *F. sporotrichioides* in pot and field experiments under moderate and severe disease pressure, respectively. In both experiments, the six-row spring barley variety Chevron was resistant against all *Fusarium* species, while both susceptible lines, the two-rowed barley line ICB 111809 and six-rowed barley variety Stander were highly susceptible for all Austrian *Fusarium* species. Overall, *F. graminearum* presented high aggressiveness at moderate and high disease pressure, while *F. poae* exhibited higher aggressiveness at lower humidity and *F. sporotrichioides* and *F. culmorum* were more adapted to more humid screening conditions. The ranking of *Fusarium* species severity on Stander and Chevron 14 and 21 days after inoculation was very similar between moderate and severe disease pressure. Based on observations for both two-rowed barley lines, a potential species × genotype interaction requires further investigation.

Introduction

Fusarium Head Blight (FHB) threatens the barley (*Hordeum vulgare*) production in Austria under humid and warm weather conditions and has the potential of reduced food and feed safety for barley products. Sources of resistance to *F. graminearum* have been profoundly identified in North American spring barley collections (SCHOLZ & STEFFENSON 2002; McCALLUM *et al.* 2004). However, very little is known about the reaction of resistance sources to other *Fusarium* species (*F. poae*, *F. avenaceum*, *F. sporotrichioides* and *F. culmorum*) causing FHB in Austria and beyond. To effectively manage FHB using host resistance, it is important that cultivars are developed with a broad-based resistance against all potential *Fusarium* pathogens.

Material and Methods

Two *F. graminearum* susceptible (Stander, six-rowed; ICB 111809; two-rowed) and resistant (Chevron, six-rowed; CIho 4196, two-rowed) six- and two-rowed spring barley cultivars were investigated for their susceptibility towards Austrian isolates of *F. graminearum* (isolate number 66), *F. culmorum* (104), *F. poae* (36), *F. avenaceum* (60) and *F. sporotrichioides* (197). For each variety a non-inoculated control check (sprayed with double distilled water) was included. The experiments were carried out in plastic pots and field plots under moderate and severe disease pressure, respectively. All experiments were arranged in a randomized complete block design with four replicates. At the late-milk to early-dough stage, pot grown plants were spray-inoculated at dusk, applying 10000 cfu/ml (macroconidia based), and immediately covered with plastic bags to maintain high humidity. Bags were removed the

following morning to avoid excessive heat development. For the field experiments, varieties were grown in double rows. In every second row, spikes at the late-milk to early-dough stage were spray-inoculated at dusk. Each treatment was inoculated twice (interval 2 days) with a macroconidial suspension of *Fusarium* spp.with an air-pressured backpack sprayer at 10 psi, applying 10000 cfu/ml (macroconidia based). After inoculation, the nursery was mist-irrigated for 18h. The irrigation system was operated by a leaf wetness sensor. For disease evaluation, the average percentage of infected kernels (on 5-10 randomly chosen spikes, counting the total number of kernels and the number of infected kernels/spike) was assessed on each accession at the mid-dough stage (14 days after inoculation) and then one week later at late-dough stage (21 days after inoculation) to record possible changes in the infection level.

Results and Discussion

In both experiments, the six-rowed spring barley variety Chevron was resistant against all *Fusarium* species, while both susceptible lines, the two-rowed barley line ICB 111809 and six-rowed barley variety Stander were highly susceptible to all Austrian *Fusarium* species. Chevron exhibited similar resistance, when inoculated with the *Fusarium* species from North Dakota, USA (SCHOLZ *et al.* 2002). The two-rowed line CIho 4196 showed moderate resistance under severe disease pressure, but was similarly susceptible as the six-rowed variety Stander under moderate disease pressure (Table 1). The susceptible non-inoculated control check (ICB 111809) had a 38% infection under field conditions. This might be attributed to the long-term inoculum build up in the soil, insufficient rotation system, and/or warm and humid weather conditions in 2003.

Overall, *F. graminearum* presented higher aggressiveness at moderate and high disease pressure, while *F. poae* exhibited higher aggressiveness at lower humidity and *F. sporotrichioides* and *F. culmorum* were more adapted to more humid screening conditions (Table 2). A low pathogenicity of *F. poae* was observed by SALAS *et al.* (1999). This discrepancy might be due to the fact, that in the present investigation, only macroconidia were considered for inoculum calibration. SCHOLZ *et al.* (2002) reported higher pathogenicity for *F. graminearum* and *F. culmorum* compared to other *Fusarium* species in 2001 under field conditions in North Dakota.

In the field experiment, the most aggressive *Fusarium* species was *F. graminearum*, while the other four species tested (*F. avenaceum*, *F. culmorum*, *F. poae* and *F. sporotrichioides* demonstrated an overall similar aggressiveness. In the same experiment, 14 days after inoculation, the ranking for *F. culmorum* and *F. avenaceum* is similar for both susceptible varieties and differences between some *Fusarium* species are significant, while the severity caused by *F.graminearum*, *F. poae* and *F. sporotrichioides* differs between both lines. On the other hand, 21 days after inoculation, the ranking for the *Fusarium* species (except *F. graminearum*, which is highest in Stander) is similar for both susceptible lines, but no significant differences between the *Fusarium* species were found. Remarkably, the ranking of *Fusarium* species severity on the susceptible Stander as well as on the resistant Chevron 14 and 21 days after inoculation was very similar between the moderate and severe disease pressure, except for the *F. avenaceum*, which was weakest in Stander, and *F. sporotrichioides*, which was weakest in Chevron after 21 days. On ICB 111809, *F. graminearum*, *F. culmorum* and *F. avenaceum* reacted relatively similar in both experiments, while *F. poae* was the most pathogenic species under moderate disease pressure and *F. sporotrichioides* more pathogenic under severe disease pressure at both reading dates. Interestingly, *F. sporotrichioides* showed the highest increase in severity from mid-dough to

Table 1. FHB severities under moderate and severe disease pressure

	Stander		Chevron		ICB 111809		CIho 4196	
Moderate disease pressure								
Reading at 14 days after inoculation								
Control	2.3	C	0.2	D	missing	-	9.4	C
F. avenaceum	14.8	AB	2.4	AB	23.3	B	17.4	AB
F. culmorum	18.4	A	2.6	AB	27.1	B	10.5	C
F. graminearum	20.7	A	3.6	A	28.1	B	13.3	BC
F. poae	12.8	AB	1.7	BC	40.5	A	21.5	A
F. sporotrichioides	9.1	BC	1.0	CD	25.4	B	13.3	BC
Reading at 21 days after inoculation								
Control	3.8	B	0.2	D	missing	-	12.9	C
F. avenaceum	17.5	AB	2.4	AB	37.2	B	27.3	A
F. culmorum	28.8	A	2.6	AB	36.2	B	20.8	B
F. graminearum	29.4	A	3.6	A	36.0	B	24.8	AB
F. poae	20.6	A	1.7	BC	45.5	A	25.0	AB
F. sporotrichioides	26.8	A	1.0	CD	31.9	B	23.0	AB
Severe disease pressure								
Reading at 14 days after inoculation								
Control	7.3	C*	1.3	C	38.2	B	6.4	B
F. avenaceum	34.4	B	3.2	ABC	45.0	AB	13.0	AB
F. culmorum	65.4	A	6.3	A	57.7	A	16.1	AB
F. graminearum	60.6	A	5.8	AB	41.1	AB	21.1	A
F. poae	29.3	B	2.3	BC	37.5	B	17.6	AB
F. sporotrichioides	24.7	CB	3.1	ABC	48.9	AB	12.5	AB
Reading at 21 days after inoculation								
Control	39.7	B	1.3	A	23.1	B	7.8	C
F. avenaceum	61.0	A	2.0	A	38.8	AB	14.5	BC
F. culmorum	62.8	A	2.8	A	41.6	AB	22.6	AB
F. graminearum	68.2	A	2.9	A	40.6	AB	26.5	AB
F. poae	61.6	A	1.6	A	40.4	AB	17.4	ABC
F. sporotrichioides	66.2	A	1.3	A	43.9	A	18.0	AB

Adjusted means of treatments followed by the same letter are not significantly different at $P<0.05$.

* original values were transformed by log10 and analyzed

late-dough stage compared to the remaining *Fusarium* species. The *F. sporotrichioides* isolate, tested in this study, exhibited lower pathogenicity (~20%) than *F. graminearum* (90%) using seedling pathogenicity tests (M. Lemmens, pers. comm.). These data represent additional hints about the involvement of unidentified factors for *Fusarium* species pathogenicity at adult-plant and seedling development stages. Based on relative high FHB severity readings under moderate disease pressure in CIho 4196, all *Fusarium* species reacted differently, but as in ICB 111809, *F. poae* was more pathogenic under moderate disease pressure than under severe disease pressure, which indicates its better adaptation to less humid and warm conditions than other *Fusarium* species. Recent report by TEKAUZ (2002) showed higher infection rates for *F. poae* compared to other Fusarium species in Canada.

Table 2. Overall FHB severities caused by different Fusarium species at two reading dates under moderate and severe disease pressure

	Moderate disease pressure		Severe disease pressure	
Reading at 14 days after inoculation				
Control	4.1	D	9.9	C
F. avenaceum	15.3	BC	23.9	B
F. culmorum	13.3	C	32.2	A
F. graminearum	16.8	AB	32.9	A
F. poae	18.4	A	21.7	B
F. sporotrichioides	12.8	C	20.5	B
Reading at 21 days after inoculation				
Control	5.8	B	13.5	B
F. avenaceum	22.8	A	29.1	A
F. culmorum	20.8	A	30.4	A
F. graminearum	24.6	A	35.1	A
F. poae	22.3	A	30.3	A
F. sporotrichioides	21.2	A	31.6	A

Adjusted means of treatments followed by the same letter are not significantly different at $P<0.05$.

Table 3. Influence and interaction of variety and Fusarium species on FHB severity under moderate and severe disease pressure 14 and 21 days after inoculation

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Moderate disease pressure					
Severity 14 days after inoculation					
Variety	3	10680.9	3560.3	46.8	<.0001
Fusarium species	5	1283.2	256.6	3.4	0.0053
Variety × *Fusarium* species	14	6968.3	497.7	6.6	<.0001
Severity 21 days after inoculation					
Variety	3	17043.4	5681.1	27.4	<.0001
Fusarium species	5	856.0	171.2	0.8	0.5323
Variety × *Fusarium* species	14	6179.5	441.4	2.1	0.0098
Severe disease pressure					
Severity 14 days after inoculation					
Variety	3	4451.2	1483.7	13.5	<.0001
Fusarium species	5	624.7	124.9	1.1	0.3700
Variety × *Fusarium* species	13	3421.7	263.2	2.4	0.0341
Severity 21 days after inoculation					
Variety	3	15097.2	5032.4	51.2	<.0001
Fusarium species	5	559.4	111.9	1.1	0.3686
Variety × *Fusarium* species	13	457.3	35.2	0.4	0.9708

Based on observations for the two two-rowed barley lines and on the reaction of *F. avenaceum* and *F. sporotrichioides* on Stander and Chevron after 21 days, a potential species × genotype interaction requires further investigation. The two-factorial analysis (Table 3)

yielded the significant influence of the variety (Chevron < CIho 4196 < Stander < ICB 111809) and the nonsignificant influence of the factor *Fusarium* species (except for the first reading under moderate disease pressure). The variety × *Fusarium* species interaction was significant except for the second reading under severe disease pressure. TAKEDA *et al.* (1995) reported significant isolate × host interaction, where 89% of the overall variation was accounted to the general pathogenicity.

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Development of Tissue-Specific Gene Promoters for Targeting Anti-Fusarium Gene Expression in Barley

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Abstract
We identified lemma and pericarp epithelium tissues as those most rapidly infected by *Fusarium graminearum*. Genes specifically expressed in these tissues were cloned so that their promoters could eventually be used to express antifungal protein genes. The tissue-specific genes included a lipid transfer protein homologue ("*Ltp6*") highly expressed in the pericarp epithelium but not in vegetative leaves and a jacaline-like gene, *Lem2*, preferentially expressed in the lemma/palea, compared with the flag leaf. *Ltp6* is also expressed in coleoptiles and embryos and responds to salt, cold, abscisic acid and salicylic acid in a pattern distinct from other barley *Ltp*s. Transient expression analysis of the promoter showed that an upstream sequence confers tissue-specific expression and retains most promoter activity. All 4 *Lem2* genes are located in a single BAC and map to chromosome 5(1H). *Lem2* is specifically expressed in the lemma/palea and coleoptile. The two LEM2 jacalin-like domains may be involved in pathogen recognition. Salicilic acid induces *Lem2* within 4 h, suggesting that it is a defensive gene. Particle bombardment with the cloned *Lem2* promoter showed strong expression of a green fluorescent protein gene (*gfp*) in the lemma/palea and very weak expression in leaves.
Keywords: Fusarium graminearum; tissue-specific promoters; Ltp; jacalin; epicarp; lemma

Introduction

Since 1993, high moisture and no-till cultural practices have sustained high levels *F. graminearum* infections of barley and wheat in the U.S. and Canada. Significant infection levels also occur in China, Europe, Argentina and other countries. Inoculum on crop residues infects spike tissues and causes a disease known and Fusarium head blight (FHB) or scab (www.scabusa.org/). Mycotoxins (e.g., DON) produced by Fusarium make the harvest unsuitable for food, feed or malting. The cumulative primary and secondary U.S. economic loss for the 1998-2000 barley and wheat crop years totaled $2.7 billion (NGANJE *et al*., 2001). In addition, as the risk of consuming mycotoxins becomes better defined, presumably leading to lowered acceptable ppm limits, the negative affects on these crops will be more widely appreciated. The growing popularity of no-till cultivation and the growing acreage in maize ensure that a source of inoculum will be present in the future. There are no known barley cultivars with biochemical resistance to Fusarium. We are developing tissue-specific gene promoters in order to target expression of antifungal genes to the lemma/palea and the pericarp epithelium. A strain of *F. graminearum* transformed with the green fluorescent protein gene (*gfp*) was used to determine that these organs are the main paths of infection (SKADSEN & HOHN 2004).

A great deal of effort is going into mapping the barley genome, developing EST sequence databanks and examining gene expression through microarrays. Ultimately, it will be necessary to learn how these genes are controlled by their various promoter elements. Several labs are producing stable transformants of barley in attempts to constitutively express

various antifungal genes. Ultimately, tissue-specific promoters will be needed to restrict expression to specific spike tissues in order to reduce selection for resistant strains of Fusarium and to reduce plant stress. In order to identify the most appropriate tissues to target, and from which to obtain tissue-specific genes, we conducted infection studies with a strain of Fusarium transformed with *gfp* (strain GZT501; SKADSEN & HOHN 2004). These showed that Fusarium can directly infect the lemma/palea and proliferates rapidly when it reaches the pericarp. Infections occur much more rapidly at the exposed tip of the seed, particularly in the ovary epithelial hairs, and rapidly spread downward along the pericarp epithelium, interior to the lemma and palea. It is therefore essential to express antifungal genes in both the lemma/palea and pericarp epithelium.

We previously used the differential display technique to detect tissue-specific genes. This resulted in the cloning of the lemma-specific *Lem1* gene (S. Puthigae) and the pericarp epithelium-specific *Ltp6* gene (M. L. Federico) and their corresponding promoters. *Lem1* is a novel single copy intronless gene that encodes a 102 amino acid product. The promoter was ligated in place of the constitutive ubiquitin (*Ubi*) promoter in our *Ubi*/*gfp* vector (pAHCSGFP; KAEPPLER *et al.*, 1999) and bombarded into barley and wheat spikes and leaves. Proving the promoter's specificity, g*fp* was expressed in young lemma, palea and awn tissues, but not in leaves (SKADSEN *et al*., 2002). Deletion analysis showed that the strength and specificity of the promoter were dictated by a short 77 bp sequence located immediately upstream from the start of transcription. The *Ltp6* gene encodes a protein related to lipid transfer proteins and is expressed mainly in the pericarp epithelium, although there is also some expression in the epithelium of the developing embryonic axis and the coleoptile (Federico *et al*., manuscript submitted).

More recently, the suppression subtractive hybridization (SSH) technique (DIATCHENKO *et al*., 1998) was employed to develop a library of lemma-specific cDNA clones (ABEBE *et al*. 2004). In this procedure, lemmas and paleas were harvested from florets in which the endosperms were in the elongating (ca. 2-3 DAP) through the mid-dough stages of development. Flag leaves (controls) were also harvested at the same times. This technique identifies transcripts that are unique to the target tissue or expressed in great abundance over control tissue (Fig. 1).

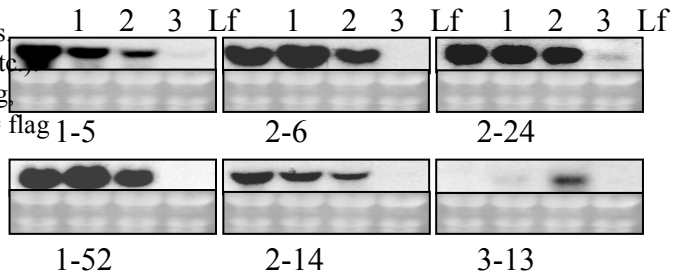

Fig. 1. Northern analysis of selected SSH clones. Blots were probed with insert DNA from 1-5 (etc.). Lanes 1, 2, 3 - RNA from lemmas of elongating, gelatinous and dough development stages. Lf = flag leaf. Lower panels are RNA stained with EtBr.

Lemmas expressed a high proportion of genes for reactive oxygen species (ROS) scavenging, structure, defense, photosynthesis, and biosynthesis of translocated amino acids. Preferential expression of genes for photosynthesis and amino acid biosynthesis supports the suggestion that lemma and palea are major sources of carbon and nitrogen for the growing kernel. Expression of genes for fibrous structures provides physical barriers against pathogen invasion. Expression of ROS scavenging genes is necessary for protection from oxidative damage, which is common in highly photosynthetically active tissues. (To view sequences in GenBank, conduct nucleotide search with "Abebe + Skadsen" query.)

One clone, 1-5 (now named *Lem2*; see Fig. 1), was chosen from the SSH library for further development. This was used as a probe by Andy Kleinhofs to locate several BAC clones containing this gene. One clone with an 80,000 bp insert was found to have several repeated *Lem2* genes. The full transcripts and about 1500 bp of upstream sequences were determined for two repeats (designated *Lem2* and *Lem2b*). These proved to be novel genes, each with two domains that have moderate sequence homology to jacalin lectins. *Lem2b* differs from the *Lem2* gene in only one site, a 25 bp addition in the (*Lem2b*) promoter. *Lem2* is expressed in the lemma and not in leaves.

Material and Methods

Barley plants (*Hordeum vulgare* L. cvs. Morex and Golden Promise) were grown in a greenhouse maintained at 16-21°C. Plants received supplemental lighting from sodium arc vapor lights for 16 h per day. Total RNA was extracted from lemmas, paleas, flag leaves, and coleoptiles with guanidinium thiocyanate (CHIRGWIN *et al*. 1979). Genomic DNA was extracted from leaves using the CTAB procedure (MURRAY & THOMPSON 1980). For northern analysis, 5 µg of total RNA from each organ was fractionated on 1.2% (w/v) agarose-formaldehyde gels and transferred to Nytran N membranes using 10X SSC. Southern analysis was performed using 10 µg of genomic DNA digested to completion with *EcoR* V, *BamH* I, and *Hind* III, fractionated through 0.8% agarose and transferred to Nytran N membranes. Blots were hybridized with [α-^{32}P]dCTP-labeled *Lem2* or *Ltp6* probes. Probe labeling and hybridization were performed as described previously (SKADSEN *et al*. 1995). For northern analysis, equal loading and transfer of RNA was verified by reprobing blots with a [α-^{32}P]dCTP-labeled barley 18S rRNA clone (GenBank Accession AY552749). Leaf DNA from transformed plants was extracted with CTAB and tested for presence of the transgene by PCR using a *Lem2* or *Ltp6* upstream primer and a NOS downstream primer.

Deletion analysis of promoters and transformation - Core promoter sequences were identified through analyzing a deletion series of the region upstream from the start of transcription. A series of 5' and 3' deletions of the *Lem2* and *Ltp6* promoters was synthesized by PCRing with nested primers and *Pfx* polymerase. These were ligated to the 5' end of *gfp* in the blue-SGFP-TYG-nos SK vector of Jen Sheen (sGFPSK). The full promoters and deletions were cloned into the pAHC25 *Ubi*/*GUS* vector (CHRISTENSON & QUAIL 1996), in place of *Ubi*. The constructs were tested for transient promoter activity in lemmas, epicarps and leaves by particle bombardment, conducted similarly to WAN & LEMAUX (1994). Promoter effectiveness in transient assays was assessed by particle bombardment of excised lemmas and leaves using the PDS-1000/He Biolistic gene gun (BioRad).

Quantification of promoter strength in deletion studies was done through real time PCR. Tissues were collected and frozen. Total RNA was used to produce cDNA. Fluorescent probes were synthesized for *gfp*, *BAR* and *GUS*, along with their flanking PCR primers. *BAR* and *GUS* were used as internal standards. Internal standards and *gfp* were PCRd separately in an ABI 7000 real time PCR machine. The ratio of *gfp* to internal control transcripts was used as a measure of promoter strength. This approach avoids the potential problems of GFP and GUS protein activity measurements, which adds additional layers of regulation to interpret.

Stress treatments - Preferential expression of *Lem2* or *Ltp6* in protective organs and epidermal tissues suggest that they are defense or stress related genes. For *Lem2* analysis, spikes at the gelatinous stage of kernel development were subjected to wounding, elicitor (SA and MeJA), and water stress treatments. Spikes were sprayed with each elicitor to runoff and covered with clear plastic bags to avoid drying. Lemmas were collected 4 to 24 h after

treatment for RNA extraction. For *Ltp6* analysis, various seedling tissues were analyzed following stress treatments.

Results and Discussion

Lem2 promoter - We have characterized a novel lemma-, palea-, and coleoptile-specific gene, *Lem2*. Induction of *Lem2* by SA occurred within 4 h of treatment. However, *Lem2* was not responsive to methyl jasmonate (MeJA) or wounding. Therefore, it is not involved in the MeJA-dependent defense response. Taken together, these results suggest that *Lem2* is involved in systemic acquired resistance (SAR). Moreover, *Lem2* was down-regulated by drought, dehydration, and ABA; thus, it does not play a role in mitigating water stress.
Southern analysis indicated that Morex barley has at least three copies of the *Lem2* gene arranged in tandem on chromosome 5 (1H) Bin 02, near the short arm telomere. *Lem2* is not present in the barley cultivars Steptoe, Harrington, Golden Promise, and Q21861.
Following particle bombardment of the lemma, GFP was produced under all promoter deletions down to within 100 bp of the start of transcription. Real time PCR has identified the promoter sequence responsible for most of the promoter activity.

Ltp6 promoter - We have cloned a novel barley *Ltp* gene (*Ltp6*) that is expressed mainly in the pericarp epithelium, and to a degree in the coleoptile and embryonic seedling axis. The ability of the *Ltp6* upstream region to serve as a promoter was demonstrated by transient particle bombardment experiments. The full *Ltp6* promoter drove *gfp* expression in the pericarp epidermis (epicarp), coleoptiles and embryos, but expression was negligible in leaves. The expression of *uidA* from the co-bombarded pAHC25 internal control vector demonstrated that the leaves were competent to express transgenes. *Ltp6*-driven expression of *gfp* in embryos was very strong in the embryonic axis but very weak in the scutellum. In contrast, *Ubiquitin*-driven expression of *uidA* was present in all embryo tissues when the co-bombarded pAHC25 vector was tested. A series of 5' and 3' *Ltp6* promoter deletions was constructed to locate regions conferring tissue-specificity and promoter strength. All deletion constructs containing ca. 300 bp of upstream sequence expressed *gfp* with an intensity equal to that of the full *Ltp6* promoter. Quantitative real time PCR was used to assess the level of transcription conferred by these constructs in transient expression assays. Constructs with roughly 200 bp of upstream sequence retained full promoter activity. A large loss in expression was observed when a small fragment was deleted. This decline suggests that important *cis*-acting DNA elements must be located in this region, which contains ABA- and stress-related motifs.

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QTL Mapping Fusarium Head Blight Resistance in Barley

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Abstract

Enhancing genetic resistance to Fusarium head blight (FHB) is complex because resistance is quantitative, resistance QTLs are coincident with plant morphology QTLs that can influence disease severity, and allelic effects are inconsistent across environments. We have investigated four sources of FHB resistance to identify molecular markers that will enable pyramiding resistance genes through marker assisted selection. Two of eleven QTLs identified in a mapping study with the cultivar Chevron were validated on chromosomes 2 and 6 and are associated heading date (HD), which is conditioned by the *Eam6* locus, and grain protein, respectively. Two of three QTLs identified in a mapping study with the cultivar Frederickson have been validated. One of these is coincident with the QTL on chromosome 2 identified in the Chevron study. The other is associated with the *Vrs1* locus. We have created fine mapping populations to determine whether these plant morphology genes (*Eam6* and *Vrs1*) condition resistance to FHB or are tightly linked to resistance loci. We have developed mapping populations using new FHB resistant cultivars Atahualpa and Hor211. Preliminary data indicate that these sources contain new genes that could complement genes discovered in Frederickson and Chevron. We will present current data on the positions and effects of QTLs for FHB resistance derived from these four sources of resistance.

Introduction

Fusarium head blight (FHB), caused primarily by *Fusarium graminearum* Schwabe in North America, is a devastating disease of wheat and barley. The primary concern is the mycotoxin deoxynivalenol (DON), produced by the pathogen, which accumulates in the grain and can render it useless for malting and brewing as well as for some animal feed uses. Understanding genetic resistance to FHB in barley offers hope of exploiting resistance genes through traditional or marker assisted breeding to produce resistant cultivars. Quantifying levels of disease is challenging because disease severity is strongly influenced by the environment and assessment must be done on adult plants using labor intensive field experiments (,STEFFENSON 2002). Studies, to date, have shown that resistance to FHB is complex, controlled by multiple loci, with relatively small effects, and expressed inconsistently across environments (KOLB *et al.* 2001). Furthermore, resistance is often linked to other traits that can confound disease assessment, are undesirable from a breeding perspective, or both (de la PEÑA *et al*. 1999; MA *et al.* 2000; ZHU *et al.* 1999; MESFIN *et al.* 2003; DAHLEEN *et al.* 2003).

The inherent statistical limitations associated with quantitative trait locus (QTL) mapping studies and the complexity of this disease necessitate validation of QTLs prior to implementing marker assisted selection (MAS). In the Chevron source of resistance (de la PEÑA *et al.* 1999), two important QTLs for resistance have been validated (CANCI *et al.* 2004). Likewise, two regions have been validated in the Frederickson source of resistance, one of which was identified in the Chevron study (MESFIN *et al.* 2003). The three regions that have been identified and validated in these studies are on chromosome 2H in BIN 8 (2H-08), chromosome 2H in BIN 10 (2H-10), and chromosome 6H in BIN 5 (6H-05). The 2H-08 QTL, which was identified and validated in both studies, is also associated with heading date.

The heading date QTL in this region is likely the *eams6* gene (FRANCKOWIAK & LUNDQVIST 1997). The 2H-10 region includes the *vrs1* locus and resistance to FHB is linked to two-rowed spike type. The 6H-05 region is associated with FHB severity, grain protein concentration, and the disease complex kernel discoloration (CANCI *et al.* 2003). In this region, FHB resistance is linked to kernel discoloration resistance and high grain protein concentration.

We evaluated MAS for the Chevron allele in the 2H-08 and 6H-05 regions using simple sequence repeat (SSR) markers and breeding lines from a cross between resistant breeding lines that trace back to Chevron and our most recent malting variety release, Lacey (RASMUSSON *et al.* 2001). Selection for the Chevron allele at 2H-08 resulted in a 43% reduction in FHB severity and increased heading date by 2 days (GUSTUS *et al.* 2001). Selection for the Chevron allele at 6H-05 resulted in a 22% reduction in FHB severity and increased grain protein concentration by 14g kg^{-1}. We are currently fine mapping these regions to attempt to separate these linked effects and develop more effective MAS strategies.

Given the challenge of QTL mapping studies for FHB and the fact that several studies have identified QTL in similar regions of the genome, it is necessary to carefully select new sources of resistance for mapping. Genetic diversity analysis using a set of 59 SSR markers distributed across the genome identified Hor211 and Atahualpa as two sources of resistance that were different from other mapped sources of resistance (BELINA *et al.* 2002). We conducted a selective genotyping study to determine whether resistance segregating in breeding populations was due to allelic variation at previously reported QTLs for FHB resistance (WINGBERMUEHLE *et al.* 2002). This study indicated that the sources of resistance Hor211 and Atahualpa likely carried resistance alleles at loci not previously described.

The purpose of this report is to compare QTL results for FHB that have been reported for the sources Chevron and Frederickson with preliminary results for the sources Hor211 and Atahualpa. In particular, we focus on chromosomes 2H and 6H.

Material and Methods

Atahualpa/M81 (AM) Mapping Population

Atahualpa is a two-rowed hulless barley cultivar from Ecuador that is moderately resistant to FHB. Atahualpa was crossed with the susceptible breeding line, M81, from our six-rowed malting barley breeding program. The population (101 lines) was advanced by single seed decent to the F4 generation and bulked in subsequent generations for use in field evaluations. The preliminary linkage maps of chromosomes 2H and 6H consists of 19 SSR markers and were constructed using JOINMAP (Van OOIJEN & VOORRIPS 2001).

Hor211/Lacey (HL) Mapping Population

Hor211 is a six-rowed hulless cultivar from the Ukraine that is moderately resistant to FHB. The population (101 lines) was advanced by single seed decent to the F6 generation and bulked in subsequent generations for use in field studies. The preliminary linkage maps of chromosome 2H and 6H consists of 16 SSR markers and were constructed using JOINMAP.

Trait Evaluation

The parents and lines for the populations were evaluated in field nurseries supplied with mist irrigation and inoculated with *F. graminearum* as described by MESFIN *et al.* (2003). Seed was sown in 2.5 m single row plots in a randomized complete block design with three replications. Data were collected on heading date, plant height, percent FHB disease severity,

and DON concentration in harvested grain. The AM population was evaluated in Crookston, MN in 2002 and 2003, and in Hangzhou China in 2003. The HL population was evaluated in Hangzhou, China in 2001, and in St. Paul, MN in 2002 and 2003. We used grain spawn inoculum at the Hangzhou and Crookston locations and macroconidia spray inoculum at the St. Paul location as described by MESFIN *et al.* (2003).

Data Analysis
Analysis of variance was conducted on the trait data with lines, environments, and blocks in the model using SAS Proc GLM (SAS Institute, Cary, NC). Simple interval mapping analysis was conducted using the software PLABQTL (UTZ & MELCHINGER 1996).

Results and Discussion
We detected significant variation among lines in both the AM and HL populations for FHB severity in all three environments and for heading date for two environments (data not shown). We did not collect heading date data at the Hangzhou location. For the AM population we found significant correlations among the three locations for FHB severity. The correlation for heading date between the Crookston 2002 and 2003 evaluations was 0.53. For the HL population we found significant correlations among the three environments for FHB severity ranging from 0.19 to 0.37. The correlation for heading date between the St. Paul 2002 and 2003 evaluations was 0.88.

Chromosome 2H has at least three QTLs for FHB based on previous studies and every study published, to date, has detected a QTL on chromosome 2H. In the AM population, we detected one of these previously detected QTLs in the 2H-08 region (Figure 1). This QTL was detected in two of the three environments tested (Table 1). Interestingly, at this locus the M81 allele was associated with lower disease severity. In previous studies with the resistant sources Chevron (CM) and Frederickson (FS) Chevron, the resistant parent allele was associated with lower disease severity (de la PEÑA *et al.* 1999, MESFIN *et al.* 2003). In the AM population, this region was not associated with HD as it was in both the CM and FS population. Quantitative trait loci for HD have often been coincident with FHB severity begging the question as to whether this is due to linkage or pleiotrophy. We have initiated fine mapping in the 2H-08 region with Chevron derived material to attempt to answer that question. Apparently, the resistance allele in this region from Atahaulpa is not linked to heading date. The 2H-10 region containing the *vrs1* locus was not associated with FHB severity in the three environments that the AM population was tested. In the Hor211 population, we detected no QTL for FHB on the mapped portion of chromosome 2H.

Chromosome 6H has one region that has been associated with FHB severity in multiple studies. In the AM population, we detected a QTL in this region in two of the three environments tested (Table 1). No FHB QTL in this region was detected using the HL population. Resistance from Chevron conditioned by this locus is associated with kernel discoloration resistance and high protein concentration. We are currently evaluating these traits in the AM population to determine if similar linkages exist.

Thus far, it appears that the genetics of resistance to FHB in Atahualpa and Hor211 is somewhat different from the other sources of resistance that have been investigated. Continued mapping and analyses of these populations will hopefully lead to new QTLs that can be utilized in MAS for FHB resistance.

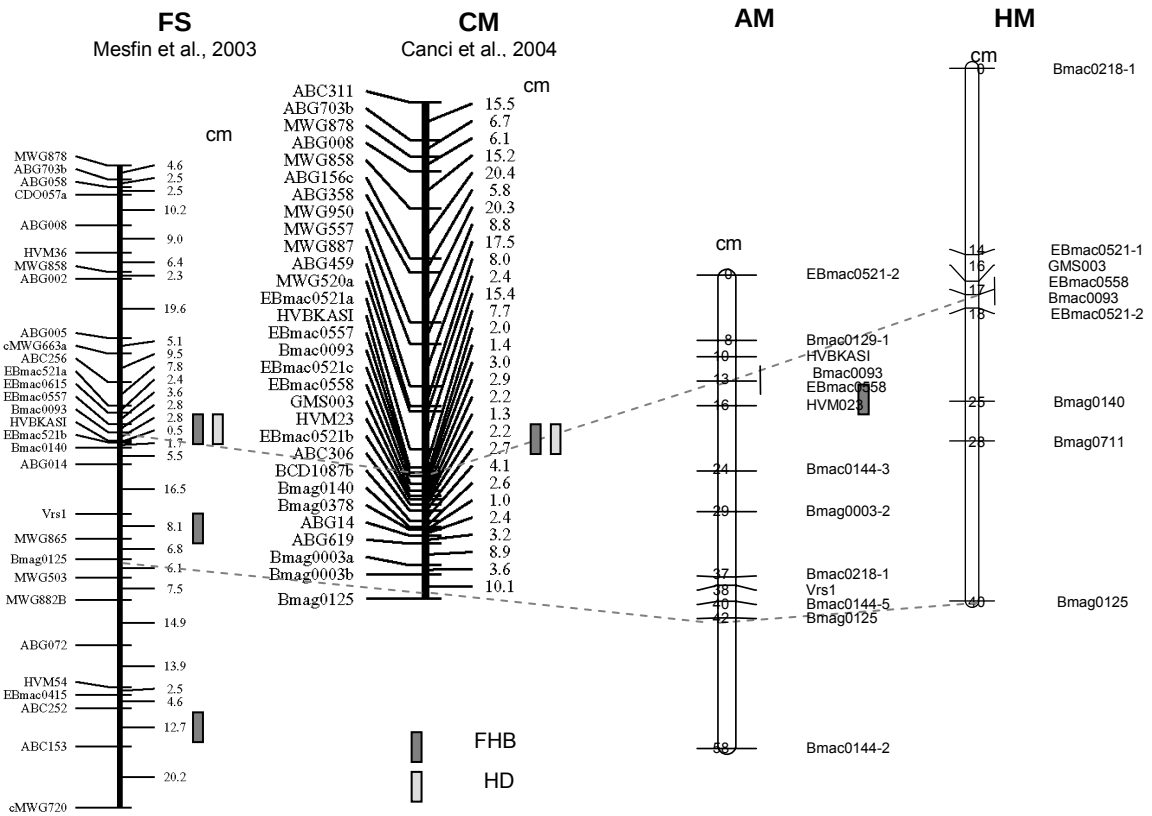

Figure 1. Detection of quantitative trait loci (QTLs) for Fusarium head blight (FHB) severity and heading date (HD) on chromosome 2H using four mapping populations. FS, Frederickson/Stander; CM, Chevron/M69; AM, Atahualpa/M81; HL, Hor211/Lacey. Shaded bars to the right of the map indicate the position of a QTL.

Table 1. Quantitative trait loci (QTLs) detected for Fusarium head blight (FHB) in the Atahualpa/M81 (AM) mapping population.

Population	Trait	Environment[1]	LOD Peak [2]	LOD	R^2	Alpha[3]
AM	FHB	CR03	2H 14 cM	2.2	8.3	1.4
AM	FHB	HZ03	2H 14 cM	5.0	22.5	4.9
AM	FHB	CR02	6H 4 cM	2.1	8.0	-4.4
AM	FHB	HZ03	6H 4 cM	3.4	8.4	-1.8

[1] CR03 = Crookston, 2003; CR02 = Crookston, 2002; HZ03 = Hangzhou, China, 2003.

[2] Linkage group, centimorgan position based on maps in Figures 1 and 2.

[3] Effect of the Atahualpa allele

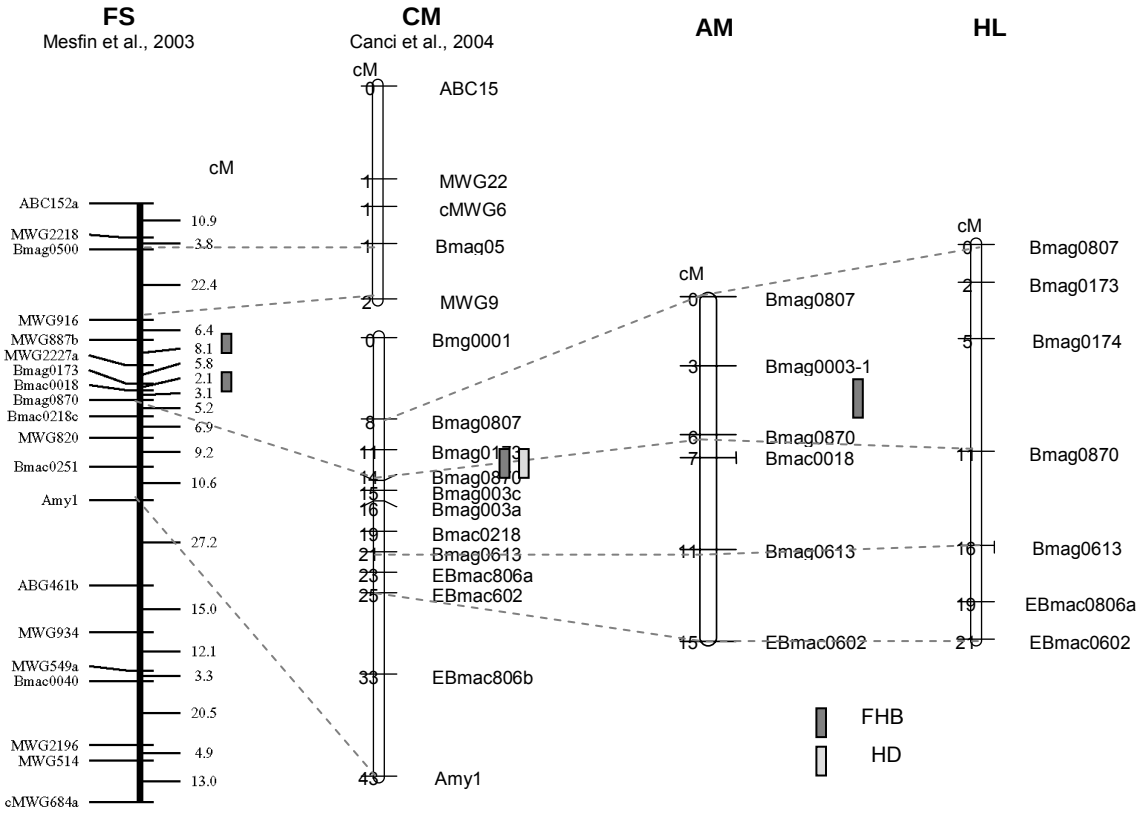

Figure 2. Detection of quantitative trait loci (QTLs) for Fusarium head blight (FHB) severity and heading date (HD) on chromosome 6H using four mapping populations. FS, Frederickson/Stander; CM, Chevron/M69; AM, Atahualpa/M81; HL, Hor211/Lacey. Shaded bars to the right of the map indicate the position of a QTL.

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Correlations among Components of Fusarium Head Blight in Barley

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Abstract
The reactions to fusarium head blight (FHB) of a group of feed barley cultivars from western Canada were assessed at four field locations in southern Manitoba in 2000. Disease components measured included the FHB Index, total *Fusarium* spp., *F. graminearum*, *F. poae*, Fusarium damaged kernels (FDK) and deoxynivalenol (DON). Significant (P=<0.05) positive correlations were found among all components, except for *F. poae*; correlations were highest (0.86, P<0.001) between *F. graminearum* and DON. Several of the cultivars tested were as susceptible (S) to FHB as the S check AC Lacombe, while others were moderately resistant (MR), similar to the MR check AC Metcalfe. Six-rowed barleys alone were in the S group, but the MR group included both 2-and 6-rowed types. Both groups included hulled and hulless cultivars. The MR level of FHB resistance identified in the feed cvs. AC Hawkeye (6-rowed, hulless,) and Tukwa (6-rowed, hulled) is noteworthy, as this level is rarely found in 6-rowed genotypes. Together with the 2-rowed cvs. Phoenix, Seebe and Tercel, these genotypes may provide alternative resistance(s) to that detected previously in 2-rowed malting cultivars such as AC Metcalfe, CDC Kendall and CDC Stratus.
Key words: Fusarium head blight; *Fusarium graminearum*; barley; mycotoxins

Introduction

Fusarium head blight (FHB) has been the most important disease of barley (*Hordeum vulgare* L.) in the eastern Canadian prairies (Manitoba and eastern Saskatchewan) for the last 10 years. Since 1994, when first reported as widespread in Manitoba (TEKAUZ *et al.* 1995), FHB has affected barley crops annually resulting in reduced yields, and lower grain quality due to kernel discolouration and contamination with mycotoxins; this has limited the grain's potential end-uses (TEKAUZ *et al*. 2000). To protect the health and safety of both humans and animals, and ensure livestock productivity, particularly that of hogs, barley must be tested for mycotoxin (deoxynivalenol or DON) content prior to its use as feed or food. The nil to low tolerances for DON in barley destined for feed or food, including the malting and brewing industries, have resulted in less barley being selected from the region, in favour of regions further west (western Saskatchewan and Alberta) where FHB is less severe and (or) relatively rare (PEARSE *et al.* 2003; TURKINGTON *et al.* 2003). As a result, the disease likely is responsible, at least in part, for the 27% reduction in barley acreage in Manitoba from 1.1 to 0.8 million acres (0.45 to 0.32 million ha) between 1992 and 2003.

Fusarium head blight in western Canada is caused primarily by *Fusarium graminearum* Schwabe, (teleomorph: *Gibberella zeae* (Schwein.) Petch), a species that produces DON during the infection process. Other *Fusarium* spp. can be recovered from diseased spikes and kernels, indicating that these also may be involved in the disease syndrome (CLEAR *et al.* 1996; TEKAUZ *et al.* 2000). The ability of four *Fusarium* spp. to promote FHB in barley was studied

by McCALLUM and TEKAUZ (2003) who found that all could cause FHB, although one or another predominated, depending on environmental conditions. Of these causal species, *F. avenaceum* (Fr.) Sacc. and *F. sporotrichioides* Scherb. are capable of producing and contaminating grain with mycotoxins other than DON, i.e., with moniliformin, and HT-2 and T-2, respectively, during the infection process (ABRAMSON *et al.* 2002; 2004).

Barley accessions with partial resistance to FHB has been identified and used as parents in crosses to improve resistance in adapted genotypes (McCALLUM *et al.* 2004, PROM *et al.* 1996). Differences in the reactions of registered Canadian barleys to FHB have been reported previously (TEKAUZ *et al.* 2000). In general, 2-rowed cultivars appeared to be more resistant to FHB than 6-rowed ones; many of the more resistant (MR-MS) 2-rowed cultivars were of malting quality, and included 'AC Metcalfe', currently one of the most widely planted cultivars in western Canada. Subsequently, the relative resistance status to FHB (based primarily on DON accumulation) of most western Canadian barley cultivars was determined, based on multi-year data from the inoculated FHB nursery established at the Agriculture and Agri-Food Canada, Brandon Research Centre, in 2000. The resistance ratings, ranging from 'fair' to 'very poor' (on a 5-category scale of: v. good, good, fair, poor, v. poor) have been published annually for the information of the industry in provincial production guides (ANONYMOUS 2004). The FHB nursery also has been used to evaluate putative sources of resistance, and the resistance status of advanced breeding lines adapted for production in the region (TUCKER *et al.* 2003).

Symptoms of FHB in a growing barley crop are not always apparent, partly because the awns can mask the spikes, and because the opportunity for best visualization appears to be limited. Additionally, it is difficult to diagnose the disease symptoms, in the field or in the harvested grain, unequivocally, because similar discoloration(s) of the spike and kernels can be produced by other fungal pathogens, saprophytes, or by abiotic factors (TEKAUZ *et al.* 2000). In wheat, measurable components of FHB all are correlated, and each can provide a valid determination of FHB (TEKAUZ *et al.* 1997). In barley, these relationships are less clear, and no, or very weak significant correlations have been found between mycotoxin levels and visual symptoms, whether FHB-I or FDK (JONES & MIROCHA 1999; SALAS *et al.* 1999; TEKAUZ & McCALLUM 1999). Because of this, the levels of DON, an expensive test, has been used by industry and researchers to evaluate FHB in barley grain.

The possibility in future that FHB may become more prevalent in western prairie regions is of concern, as much of the barley produced in western Canada originates from Alberta (~50%) and northwestern Saskatchewan. Feed barley is an important segment of the crop in Alberta, where the extensive livestock (mainly beef cattle) industry in the province uses this grain as the principal feed to 'finish off' animals prior to utilization. Feed quality barleys, including semi-dwarf stature hulled and hulless types suited for production in high nutrient situations (livestock manure spread on farm fields), adapted to this region have not been tested comprehensively for their reactions to FHB. This information is vital to evaluating the potential impact of FHB on agriculture here, should the disease become better established. The information also is needed to develop an effective multi-faceted strategy to manage the disease. The objectives of this study were to assess the reactions to FHB of a group of Canadian feed quality barleys, and to use the

data generated to evaluate the correlations among the various components by which FHB can be assessed.

Material and Methods

Fourteen feed barley cultivars, including hulled and hulless, 2- and 6-rowed types (Table 1), plus two check cultivars, 'AC Metcalfe', a popular 2-rowed malting barley, moderately resistant to moderately susceptible (MR-MS) to FHB, and 'AC Lacombe', a 6-rowed feed barley, susceptible (S) to FHB, were seeded in field trials at four locations in southern Manitoba in 2000. Seeding dates were: East Selkirk, May 5; Rosebank, May 15; Grosse Isle and St. Adolphe, May 17. Experimental plots were 1 x 5 m and composed of four rows with 0.3 m spacing, with two replications. To promote infection, natural *Fusarium* levels were supplemented by spreading *F. graminearum*-infested corn kernel inoculum on the soil surface at a rate of 40 g per m^2, about 3 weeks prior to crop heading. No misting or irrigation water were supplied.

Disease components measured, included: 1) visual in-crop severity, or the FHB-Index (FHB-I), calculated as the % disease incidence x % disease severity / 100 (range 0-100%) on a sample of 10 randomly selected spikes from each plot row (40 in total) at GS 78 (ZADOKS *et al.* 1974). Incidence was computed as the percentage of spikes with symptoms of FHB; severity as the average percentage of diseased spikelets in affected heads; and 2) at maturity on harvested grain, levels of Fusarium damaged kernels (FDK), *Fusarium* spp. and DON on sub-samples of seed (10g, 100 kernels, 20g ground seed, respectively) from one central row hand-harvested from each plot.

Fusarium fungi were isolated from surface-sterilized (immersion in 0.3% NaOCl for 3 min. and subsequently air-dried) kernels plated on potato dextrose agar, and incubated at 20C under continuous fluorescent light for 4-5 days. The *Fusarium* and other species present were identified by colony and conidial morphology, using standard keys. Fusarium damaged kernels were quantified by counting the number of discoloured, and at times smaller or thinner, kernels in the total sample. Discoloration most often was a brown darkening of the seed coat involving part or much of the kernel. Occasionally, glume edges of affected kernels were coated with the salmon-coloured sporodochia typical of *Fusarium* fungi. Deoxynivalenol was quantified using ELISA, utilizing 1.0 g of the 20 g ground seed sample. Levels of *Cochliobolus sativus* (Ito & Kuribayashi) Drechsler ex Dastur also were recorded, as this is a common component of barley seed microflora in Manitoba, and is sometimes found in high proportions. *Cochliobolus sativus* is a major barley pathogen in Canada and the sole or main cause of several diseases, including seedling blight, spot blotch, common root rot and black point (BAILEY *et al.* 2003).

Statistical analyses were done on data from each individual site (not presented) and on the combined data for the four trial sites (presented here) using SAS PROC GLM (1999-2001, SAS Institute Inc, Cary, NC, USA). Means were compared using the Ryan-Einot-Gabriel-Welsch Multiple Range Test at $P=<0.05$. Correlations among FHB components were tested using the Pearson Correlation Coefficient. The analyses were done on arc-sine transformed data.

Results and Discussion

Fusarium head blight developed at all sites, but varied in intensity among these. Date of seeding, local environmental conditions, and individual cultivar maturity likely influenced FHB development. The severity of FHB across sites, as measured by various components in the 16 barley cultivars tested, is shown in Tables 1a and 1b. The cultivars are listed according to the level of all, or the sum total, of the *Fusarium* species isolated from seed, from highest incidence to the lowest. Significant differences were found among the cultivars for all *Fusarium*, *F. graminearum*, *F. poae* (Peck) Wollenw., *F. avenaceum*, FDK, DON, FHB-I and *C. sativus*. Levels of *F. equiseti* (Corda) Sacc., a species not associated with FHB, and those of *F. sporotrichioides* did not differ.

Fusarium graminearum was the most prevalent *Fusarium* species isolated, and was about 3x as numerous as all of the other *Fusarium* species combined. This could be expected, as supplementary inoculum of *F. graminearum* was added to the experimental plots, and likely augmented the natural levels present. Under commercial production, and dependent on environmental conditions during crop development and at harvest, the relative levels of other *Fusarium* species, particularly *F. poae* and *F. sporotrichioides*, can be much higher (Tekauz et al. 2003).

Based on *F. graminearum* and DON, and to a lesser extent also on FDK and visual disease severity (FHB-I), FHB generally was more severe in 6-rowed compared to the 2-rowed cultivars tested. Two exceptions were the 6-rowed cultivars 'AC Hawkeye' (hulless) and 'Tukwa'. These, and the 2-rowed feed cultivars 'Phoenix' (hulless), 'Seebe', 'Tercel' (hulless), and possibly 'CDC Dolly', had resistance (MR-MS) to FHB comparable to that of the 2-rowed resistant check 'AC Metcalfe'. The level of *F. graminearum* on seed of 'CDC Dolly' was relatively high in relation to its low level of DON, and if validated, may represent a resistance mechanism influencing DON accumulation, and (or), its subsequent breakdown. The FHB resistance in the 6-rowed cvs. 'AC Hawkeye' and 'Tukwa' is noteworthy, as resistance to FHB in adapted Canadian 6-rowed genotypes is rare. Conversely, some cultivars, all 6-rowed, were as susceptible to FHB as the susceptible check 'AC Lacombe'. The widespread production of susceptible cultivars (those with 'very poor resistance') in regions where FHB currently is not common, could lead to a build-up of inoculum and an increase in disease prevalence and severity, in future.

While levels of FDK were significantly correlated with DON, *F. graminearum*, and FHB-I, the separation between 'discoloured' (FDK) and 'clean' (non-FDK) kernels during their quantification was arbitrary, as discoloration varied both in extent and intensity. As such, the choice of a different 'cut-off' point could influence results. In addition, as other agents are known to discolour kernels in barley, FDK ultimately may not be a reliable criterion to gage levels of FHB.

Correlations among the various components used to assess FHB are presented in Table 2. Data for *F. sporotrichioides* and *F. equiseti* are not included as correlation coefficients were mostly very low and non-significant. Significant correlations were found among several components.

Table 1a. Levels of *Fusarium* species on kernels of barley affected by fusarium head blight

Barley cv.	Description	All *Fus.*	*F. gram.*	*F. sporo.*	*F. poae*	*F. aven.*	*F. equi.*
(n=16)		%	%	%	%	%	%
Trochu	6r	56.9 a	42.8 a	4.0 ns	2.8 a	5.3 a	2.1 ns
AC Lacombe	6r (S check)	55.9 ab	41.4 a	7.1	1.8 ab	3.0 ab	2.6
Vivar	6r	49.6 abc	40.1 a	4.6	1.6 ab	1.8 abc	1.5
Mahigan	6r	48.5 abc	40.8 a	2.1	0.1 b	2.5 ab	3.0
AC Harper	6r	48.0 abc	36.4 a	5.6	2.1 ab	2.1 ab	1.8
Kasota	6r	45.3 abc	38.1 a	2.9	0.8 ab	2.8 ab	0.8
Jaeger	6r hulless	40.4 abc	32.0 a	3.8	1.6 ab	1.1 abc	1.8
CDC Dolly	2r	34.5 abcd	23.9 ab	4.4	2.1 a	1.5 abc	2.6
Tukwa	6r	32.8 bcd	24.0 ab	3.3	2.1 ab	0.9 abc	2.5
Peregrine	6r hulless	32.3 cde	23.4 ab	2.3	2.4 a	2.8 ab	1.5
Bronco	6r	32.1 de	24.6 ab	3.5	2.0 a	1.1 abc	0.9
Seebe	2r	19.1 de	7.8 c	4.9	4.1 a	0.5 bc	1.9
AC Metcalfe	2r ('R' check)	17.6 de	9.9 bc	3.3	2.9 a	0.3 bc	1.1
AC Hawkeye	6r hulless	17.3 de	10.6 bc	3.0	1.5 ab	1.3 abc	0.9
Phoenix	2r hulless	14.3 de	8.8 bc	2.1	2.0 abc	0.8 bc	0.6
Tercel	2r hulless	12.9 e	6.9 c	2.3	2.9 a	0.0 c	0.8
Average		**34.8**	**25.7**	**3.7**	**2.1**	**1.7**	**1.7**

Means in a column followed by the same letter are not different at $P=<0.05$
Fusarium spp. = *F. graminearum, F. sporotrichioides, F. poeae, F. avenaceum, F. equiseti*

Table 1b. Levels of Fusarium damaged kernels, deoxynivalenol, disease severity, and *Cochliobolus sativus* in barley affected by fusarium head blight

Barley cv.	Description	FDK	DON	FHB-I	*C. sativus*
(n=16)		%	Ppm	%	%
Trochu	6r	10.8 abc	2.6 abcd	25.4 ab	9.9 b
AC Lacombe	6r (S check)	10.8 abc	3.4 ab	29.5 a	10.0 b
Vivar	6r	13.2 ab	3.6 abc	20.7 abcd	13.6 ab
Mahigan	6r	9.8 abc	5.3 a	18.7 abcde	21.5 ab
AC Harper	6r	8.1 abc	2.6 abcde	23.3 abc	19.8 ab
Kasota	6r	7.3 abc	4.3 a	13.6 abcdef	20.8 ab
Jaeger	6r hulless	15.8 a	3.4 ab	25.9 ab	27.4 ab
CDC Dolly	2r	11.0 abc	0.9 bcdef	8.8 cdef	28.0 ab
Tukwa	6r	4.8 bc	0.9 def	10.3 bcdef	14.5 ab
Peregrine	6r hulless	12.1 ab	2.5 abc	13.0 abcdef	15.4 ab
Bronco	6r	10.8 abc	3.1 abcdef	9.9 bcdef	10.8 b
Seebe	2r	5.8 bc	0.4 f	6.4 ef	37.4 a
AC Metcalfe	2r ('R' check)	3.4 c	0.3 ef	7.5 def	30.9 ab
AC Hawkeye	6r hulless	6.2 bc	0.9 cdef	19.4 abcde	9.5 b
Phoenix	2r hulless	5.4 bc	0.3 f	4.6 f	22.3 ab
Tercel	2r hulless	9.2 abc	0.7 def	21.5 abcd	31.5 ab
Average		**9.0**	**2.2**	**16.2**	**20.2**

Means in a column followed by the same letter are not different at $P=<0.05$
FDK = Fusarium damaged kernels; incidence. DON = deoxynivalenol; parts per million. FHB-I = Fusarium head blight index = (%incidence x %severity / 100)

The strongest correlations, considering mycotoxin levels in the grain, were found between *F. graminearum* and DON (0.86**), and total *Fusarium* spp. and DON (0.80**). Deoxynivalenol also was correlated, although more weakly, with FDK and the FHB-I. Levels of *F. graminearum*, and *F. avenaceum* were negatively and significantly correlated with *C. sativus*; the correlation for all *Fusarium* was nearly significant (*P* value of 0.051). By contrast, levels of *F. poae*, were positively correlated with *C. sativus*, although not significantly. The positive

Table 2. Pearson correlation coefficients among disease components used to assess fusarium head blight in 16 Canadian barley cultivars

	***F.* spp.**	***F. gram.***	***F. poae***	***F. ave.***	**FDK**	**DON**	**FHB-I**
F. gram.	0.99**						
F. poae	-0.45	-0.56*					
F. ave.	0.80**	0.77**	-0.28				
FDK	0.56*	0.57*	-0.26	0.38			
DON	0.80**	0.86**	-0.72**	0.58*	0.60*		
FHB-I	0.62**	0.61*	-0.28	0.50*	0.59*	0.52*	
C. sativus	-0.50	-0.51*	0.42	-0.58*	-0.22	-0.39	-0.38

F. = *Fusarium*; *gram.* = *gramineaurm*; *ave.* = *avenaceum*
FDK = Fusarium damaged kernels; FHB-I = Fusarium head blight index;
DON = deoxynivalenol
*, ** significant at *P*=<0.05, *P*=<0.01

correlations between DON and visual symptoms, both FHB-I and FDK, are in contrast to results published previously (JONES & MIROCHA 1999; SALAS *et al.* 1999; TEKAUZ *et al.* 1999). This may be due to the different group of barley genotypes sampled here, the 'definition' of FDK, and (or), the comprehensive determination of disease severity (FHB-I) on spikes and mature kernels (FDK) achieved in the laboratory.

Based on the results of this study, several barley genotypes can be added to the list of Canadian cultivars with partial resistance to FHB. These cultivars may possess resistance that is different from that identified previously in Canadian 2-rowed malting cultivars, as, being of feed quality, they likely do not share the similar germplasm. If true, this would be valuable for broadening the basis of resistance to FHB, and (or) pyramiding resistances to enhance their effectiveness. The 'resistance' identified in several of the hulless genotypes tested may not be genetic, as reductions in *Fusarium* and DON have been documented when the hull is removed artificially, or is lost during harvesting (CLEAR *et al.* 1997). However, the higher levels of DON and *Fusarium* found in other hulless cultivars such as 'Jaeger' and 'Peregrine', suggest that the resistance in 'AC Hawkeye', 'Phoenix' and 'Tercel' has, at least in part, a genetic basis. The utilization of partially resistant barley cultivars in regions of Canada where FHB is not yet endemic, should help delay its further spread or intensification.

The negative correlation between *F. graminearum* and *C. sativus* is interesting, and suggest that these fungi may compete for infection sites on barley spikes. Alternatively, they may be unilaterally or mutually antagonistic towards each other. The somewhat higher levels of *C.*

sativus, and generally better resistance to FHB in the 2-rowed, versus the 6-rowed barleys tested, indicate that *C. sativus* may affect *Fusarium* levels, and as such contribute to the differential 'resistance' observed.

The relationship between visual symptoms of FHB in barley and other assessment components requires additional clarification and study. At present, quantification of DON or *F. graminearum* appears to offer the most reliable means to assess the severity of FHB in the crop, and the grain's suitability for value-added uses. Unfortunately, both assessments require specialized equipment and expertise, are labour- and time intensive, and thus are costly. Less onerous means to assess FHB and DON would be welcomed by industry, as part of the protocols needed to deal and manage effectively with this disease.

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Field Reaction of Selected Spring Barley World Collection Accessions to Fusarium Head Blight Infection

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Abstract
Three hundred and sixty spring barley genotypes were tested in field trials under artificial infection with conidium suspension of *Fusarium culmorum*. Of them, 97 were subjected to mycotoxin DON content analysis and following statistical evaluation. Inoculation was made at flowering. Twenty-three entries showed highly significant low accumulation of DON mycotoxin (mg.kg^{-1}) in harvested grains. It positively correlated with low FHB symptoms development on spikes. The mean level of DON in the trials was 9.2 mg.kg^{-1}, minimum concentration was 0.36 mg.kg^{-1} and maximum reached 35.4 mg.kg^{-1}. The lowest DON accumulation combined with zero incidence of powdery mildew (*Blumeria graminis* f. sp. *hordei*) and low and medium net blotch (*Drechslera teres*) infection was found in the varieties Princesse and Union Firlbecks. Plant height was 80 cm for both of these varieties at full heading. Other varieties with low DON grain content (less than 4.0 mg.kg^{-1}) were: Selecta Hanak 1, Spartan, Jersey, KM 1192, Digger, Diplom, Domen, Hodonínský Kvas, Opal, Diamant, Branišovický C, Nolč-Dreg.-Veleraný, Viva, Detenický Kargyn, Ratbořský, Aramir, Krajová St. Hrozenkov, Grosso, Ackermann Donaria, Kompakt and Ingrid.
Keywords: barley; scab; *Fusarium culmorum*; resistance; DON

Introduction

Fusarium head blight (FHB) in barley is a serious problem of current growing this crop for its use in the malting, brewing and feed industries. The disease is most common in humid regions with favourable temperature. In our latitude, the infection considerably increases in crop rotations where maize is a preceding crop.

FHB has been a major disease problem of this crop in the U.S. Midwest since 1993 (CAPETTINI *et al.* 2003). It can make a potentially profitable barley crop unusable for malting, and substantially reduce its value as a feed grain. The main cause of economic loss in malting barley is the presence of deoxynivalenol (DON) or vomitoxin, a mycotoxin produced by the fungus. Studies in Germany revealed that DON has the highest frequency of occurrence in wheat, oats and barley, with a contamination rate of 30-90% (DROCHNER & LAUBER 2001).

In some years, malting barley harvested in Finland, Sweden, Denmark and Scotland has a very heavy infection by Fusarium, and thus not all of the harvest will be accepted by the brewing industry (LARSEN 2000). In Europe, Fusarium infection is responsible for beer gushing, but in North America the presence of mycotoxins discriminates the barley for use in the brewing industry.

Breeding firms develop extensive phytopathological programmes aiming at the detection of donors with higher resistance to FHB. Fruitful international cooperation among gene banks and research centres is one of preconditions of success in this field.

Barley growing and breeding have a long-term tradition in the Czech Republic. The collection of genetic resources housed at the Agricultural Research Institute Kroměříž, Ltd., comprises a large number of foreign genotypes but as well as local varieties and landraces. These

accessions are assumed to be well adapted to the conditions where they were developed. Therefore, it is useful to recognize their reactions to current biotic stresses that also include infection by the most frequent diseases.
The objective of the programme is to detect varieties and genotypes within the world barley collection that are less infected by FHB and offer some other interesting characteristics.

Material and Methods
The trials were conducted in fields of the Agricultural Research Institute Kroměříž, Ltd., in the season 2003 (average annual temperature 8.7 °C, average annual precipitation 599 mm).
Ninety-eight spring barley genotypes were sown in two replications using a small-plot drill. Each replication was 1 m^2 in size.
The heading date and plant height were assessed in all genotypes.
The trials were artificially inoculated with spores of *Fusarium culmorum*. The concentration of the inoculum was adjusted to 6 million conidia per ml. Inoculation was carried out at full anthesis (DC 65) in five terms depending on genotypic differences. The assessment was carried out in 2 - 5 weeks after inoculation in the field as the necrotic area of spikes. We assessed twenty spikes for each genotype.
The DON-toxin content in harvested grains of the barley genotypes was detected using an ELISA method. Data on *Fusarium* infection were completed with field reactions to other leaf diseases. Powdery mildew (*Blumeria graminis* f. sp. *hordei*), net blotch (*Pyrenophora teres*) and leaf stripe disease (*Helminthosporium gramineum*) were assessed during the whole growing season. The results were statistically compared.

Results
ANOVA confirmed highly significant differences in grain contamination by DON mycotoxin (Table 1). The mean value of DON was 9.24 $mg.kg^{-1}$. The highest level was found in the variety Philadelphia (25.4 $mg.kg^{-1}$). Another varieties exhibiting significantly high DON concentrations were: Early Chevalier, Ceres, Morgenrot, Ymer, Isaria Nova and Provost.
The lowest DON content was determined in the variety Princesse being 0.36 $mg.kg^{-1}$ in grain. The values lower than 2 $mg.kg^{-1}$ (the Czech standard of DON content in cereal grain) were found in the following varieties: Selecta Hanak 1, Union Firlbecks, Spartan, Jersey, KM 1192, Diplom, Domen, Hodonínský Kvas and Opál. All tested varieties with DON contents are listed in Table 2.
Among 11 varieties with the lowest DON content, there were seven varieties whose percentage of visual infection by FHB assessed in the field was low too. Two genotypes displayed medium and two genotypes high infection. The important traits of genotypes with low DON in grains are summarized in Table 3.
However, the non-significant correlation was found between grain infection and DON contamination after harvest for all tested varieties (Table 4). Another analyses indicate that the heading date did not affect final fusarium infection, but plant height at the heading stage was in highly significant negative correlation with an infection level. Higher susceptibility to FHB was accompanied by increased susceptibility to net blotch at high significance. No relationship was found between the susceptibility to FHB and that to powdery mildew and leaf stripe disease.

Discussion
Integrated management strategies for control of FHB in barley include fungicide application and sowing of varieties differing in days to maturity (SIMPFENDORFER *et al.* 2003). The role of higher genotype resistance (tolerance) to disease is fundamental.

We assessed the field reaction to FHB in a large collection of genebank accessions which are variable in many agronomic and growth parameters. The main attention was given to resistance to fungal diseases.
There are two varieties with low DON accumulation which were registered in former Czechoslovakia before the Second World War. **Selecta Hanak 1**, registered in 1926, is a midlate variety (111 days), susceptible to powdery mildew, medium susceptible to net blotch and rust. The spike has a high grain number (26) with relatively high TKW. **Hodonínský Kvas**, registered in 1937, is also a midlate variety (105-115 days), susceptible to powdery mildew, medium susceptible to rust, but highly resistant to net blotch.
There are two varieties with low DON grain content which have been registered for growing in the Czech Republic after 2000 and which are now planted on a large area of malting barley fields. The first of them is **Jersey** (Cebeco Zaden, B.V. Vlijmen, The Netherlands). The midlate genotype with medium height of stem (76 cm), high resistance to powdery mildew (Mlo gene), medium susceptibility to scald and high susceptibility to net blotch, and a high level of malting quality. The second one is **Diplom** (Nordsaat Saatzucht, Germany). Medium resistance to powdery mildew, susceptibility to net blotch and rust is combined with high malting quality.
The genotypes with low DON accumulation include **KM 1162** which was bred by the former Cereal Research Institute Kroměříž. The midlate variety has medium height (71-80 cm), medium resistance to resistance to powdery mildew, and it could be used as a resistance source to net blotch and rust.
This short review of some accessions of our experiment with low DON accumulation evidences that the higher resistance to FHB can be found on variable genetic background and can be influenced by different factors. We did not find any significant correlation between traits of FHB resistance and agronomic parameters: height of the plant or heading date. The influence of both these factors, which can significantly change the disease rate in natural epidemic conditions, is completely eliminated under conditions of artificial infection with conidia suspension, which was made regularly when particular genotypes coming to the flowering stage. On the other hand, the significant correlation was found between DON content and net blotch resistance. This finding could probably stand for the set of genotypes tested in this project only.

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Table 1. ANOVA of DON content ($mg.kg^{-1}$) in infected grains of the genotypes assessed

Source of variation	d.f.	Mean square	Significance
genotype	97	113.973	Hs
residual	196	0.0186	

note: hs = significant at α 0.01

Table 2. Reaction to *Fusarium culmorum* infection in spring barley accessions

	DON (mg.kg^{-1})		DON (mg.kg^{-1})
PRINCESSE	0.357	MALTERIA HEDA	8.300
SELECTA HANAK. 1	0.766	MADONNA	8.400
UNION FIRLBECKS	1.058	PRESTIGE	8.700
SPARTAN	1.226	NOMAD	8.860
JERSEY	1.300	THAYA LOOSDORFERS	9.020
KM 1192	1.326	TEPELSKÝ 421	9.060
DIGGER	1.385	AMULET	9.200
DIPLOM	1.500	TELLUS	9.900
DOMEN	1.538	ORTHEGA	9.900
HODONÍNSKÝ KVAS	1.771	RTG VALTICKÝ	9.990
OPÁL	1.959	APEX	10.200
DIAMANT	2.136	SCARLETT	10.400
BRANIŠOVICKÝ C	2.345	CHLUMECKÝ	10.550
NOLČ-DREG.-VELERANÝ	2.459	NORDUS	10.600
VIVA	2.65	DVORAN	10.920
DETENICKÝ KARGYN	2.739	MERKUR	11.050
RATBOŘSKÝ	2.913	JANTAR	11.060
ARAMIR	3.088	TOLAR	11.100
KRAJOVÁ ST. HROZENKOV	3.334	HADOSTRENG	11.260
GROSSO	3.695	BAVARIA ACKERMANNS	11.330
ACKERMANN DONARIA	3.817	SLADAR	11.440
KOMPAKT	3.900	WEIHENSTEPHANER	12.340
INGRID	3.982	STELLA SVALOFS	12.350
BIATLON	4.100	FAUSTINA	12.700
SABEL	4.100	VADA	12.820
DENSO	4.457	SLADKO	13.130
EMIR	4.800	CALGARI	13.700
STEFFI	4.800	TSCHERMAKS	13.890
PLENA	4.850	AMALIA	15.160
CARUSO	4.875	HANÁCKÝ JUBILEJNÍ	15.250
CERESIA ACKERMANNS	5.137	CAMBRINUS	15.290
LUD	5.157	BINDER	15.550
MARIS BADGER	5.17	SALOON	15.700
ATHOS	5.502	VALTICKÝ	16.380
DREGERŮV	5.982	MALZ	16.400
KREDIT	6.021	MAJA	16.590
TRUMPF	6.177	CARLSBERG	16.670
RUBÍN	6.285	EUROPA	16.730
HAISA I HEINES	6.288	RESEARCH	16.860
KORÁL	6.290	QUANTUM	17.610
KAŠTICKÝ	6.292	CARLSBERG II.	19.140
ČELECHOVICKÝ HANÁC.	6.556	PRESTO	19.300
ANNABELL	6.600	PROVOST	20.410
AMETYST	6.784	ISARIA NOVA	21.930
STUPICKÝ PLNORZNNÝ	6.958	YMER	22.450
RESPEKT	7.400	MORGENROT	22.830
ISARIA ACKERMANS	7.499	CERES	22.880
HERIS	7.600	EARLY CHEVALIER	23.360
GERDA	8.300	PHILADELPHIA	25.400

Table 3. Agronomically important traits of barley genotypes with low DON accumulation in grains

genotype	DON ($mg.kg^{-1}$)	Fusarium head blight	Heading date	Height (cm)	*B. graminis*	*P. teres*	*H. gramineum*
PRINCESSE	0.357	LS	06.06.	80	R	LS	HS
SELECTA HANAK. 1	0.766	MS	10.06.	105	LS	LS	LS
UNION FIRLBECKS	1.058	LS	10.06.	80	R	MS	R
SPARTAN	1.226	LS	10.06.	85	MS	LS	R
JERSEY	1.300	HS	06.06.	80	R	HS	R
KM 1192	1.326	LS	10.06.	75	R	LS	R
DIGGER	1.385	LS	10.06.	75	LS	LS	LS
DIPLOM	1.500	HS	06.06.	87	LS	HS	HS
DOMEN	1.538	LS	10.06.	90	LS	LS	LS
HODONÍNSKÝ KVAS	1.771	MS	06.06.	105	MS	LS	LS
OPÁL	1.959	LS	06.06.	95	R	LS	LS

Note: field disease assessment key: R – resistant, LS – low susceptible, MS – medium susceptible, HS – high susceptible

Table 4. Correlation coefficients between *Fusarium* infection traits (spike infection and DON content) and other characteristics

	Spike infection	Significance	DON ($mg.kg^{-1}$)	Significance
Spike infection		ns	0.07	ns
DON ($mg.kg^{-1}$)	0.07	ns		ns
Heading date	0.00	ns	-0.18	ns
Height (cm)	0.02	ns	-0.27	ns
B. graminis	0.00	ns	-0.17	ns
P. teres	-0.13	ns	0.39	hs
H. gramineaum	0.08	ns	-0.07	ns

note: ns = non-significant, hs = significant at α 0.01

S 9 – ABIOTIC STRESS

Improving High Yielding Malting Barley Cultivars for Supplementary Irrigated Areas in Turkish Highlands

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Abstract
Some of the supplementary irrigated areas devoted for barley cultivation after sugar beet production were mainly dominated by six rowed feed barley cultivars in Turkish Highlands. In order to meet good malting quality cultivars demand of malting industry located in the highlands, a collaborative breeding program supported by the private company was commenced in 1994. The short term aim of the project was to improve new malting cultivars having high yield level and desirable quality when compared to six rowed feed cultivars. Breeding efforts in collaboration with cereal quality, plant pathology and agronomy departments during the last ten years have resulted in new winter and facultative two rowed malting cultivars namely Aydanhanim and Zeynelaga that exceeded six rowed feed cultivars in terms of both yield and grain quality over locations in target areas.
Keywords: malting barley; cultivar improvement; supplementary irrigated areas; selection

Introduction
Barley is an important cereal crop in Turkish Agriculture with 3,5 million ha acreage, 8 million tons production and 2,2 yield. Majority of the production (90 %) is directly used for animal feeding and as row material in feed preparation industry (Anonymous, 2002). However, high quality malting barley have been imported by private Turkish malt companies in the last decades. % 60 of barley acreage is rainfed and in the highlands of Turkey. Therefore, production of high quality malting barley is mainly depend on amount and distribution of rainfall during heading and grain filling stages. The second factor is lacking of malting barley cultivars suitable for irrigated conditions of the region. In order to meet increasing demand of new malting barley cultivars of malting industry for supplementary irrigated conditions of the region, a new collaborative project was commenced in 1996 to develop quality malting barleys for irrigated areas.

Material and Methods
Totally 7000 winter facultative barley genetic stocks developed during the last two decades by Central Research Institute for field Crops (CRIFC) Barley Breeding Program and 1200 lines from previous preliminary yield trials, yield and regional yield trials were used as project basic material. In addition to these, nearly 250 lines visually selected from preliminary yield trials were also included in the basic material during the last seven years. Considering the criteria of grain plumpness, thin grain layer, white aleuron colour and soft and milly endosperm (Burger and La Berge, 1985), all germplasm were screened in 1996 and then 250 of them were sowed in preliminary yield trial in experimental station while 64 of them were sowed in yield trials in two locations. In addition to these, sieve analysis and protein content criteria were also included as another selection criteria in the successive years. Some candidate lines with superior yield level and desirable malting criteria were selected and submitted to variety registration trials in 1998 and 2000.

Results and Discussion

According to variety registration trials results conducted in two locations during 2000-2001 seasons the first candidate line, Aydanhanim, outyielded all checks in the trials (Table 1).

Table 1. Yield Performance of Aydanhanım Registration Trials (kg/ha)

Cultivars	Locations				Grand mean
	Konya		Eskişehir		
	1999	2000	1999	2000	
Cumhuriyet (check)	3400	6210	5238	4450	4825
Erginel (check)	3510	7855	5167	4083	5154
Yıldırım (check)	3516	7740	5422	3601	5069
Aydanhanım (candidate line)	5004	7163	4764	4245	5294
LSD	465	1058	506	545	

Considering some physical quality criteria, it can be clearly understood that Aydanhanım has remarkably higher test weight, 1000 kernel weight and kernel size over 2.5 mm sieve than two-rowed check Cumhuriyet, and all six-rowed cultivars, Erginel and Yıldırım (Table 2). As a result of these convincing evidences including micro malt results (Table 5), Aydanhanım was registered as a new malting cultivar in 2000 for supplementary irrigated areas of Turkish highlands.

Table 2. Some Quality Parameters of the First Candidate Line, Aydanhanım, in Registration Trials

Cultivars	Test weight (kg/hl)		1000 kernel weight (g)		Protein content (%)		% of kernel size (over 2,5 mm)	
	Konya	Eskişehir	Konya	Eskişehir	Konya	Eskişehir	Konya	Eskişehir
Cumhuriyet (ch)	68,1	69,8	42,0	37,8	12,6	9,9	67,9	41,1
Erginel (ch)	68,2	71,0	40,8	38,1	11,8	9,4	67,2	43,9
Yıldırım (ch)	69,4	69,0	38,8	40,7	12,7	9,5	63,7	43,7
Aydanhanım (candidate line)	71,6	72,5	49,3	46,7	11,2	10,2	94,5	88,4

The second candidate line selected from the basic material namely Zeynelaga was also tested over two locations during 2001 and 2002 seasons. As was in Aydanhanım, the candidate line with its outstanding yield performance over locations during the successive seasons had higher average yield than all checks including cv. Aydanhanım (Table 3)

Regarding some physical quality criteria, Zeynelaga was also superior to the checks. and It was slightly higher than Aydanhanım, new malting barley cultivar under registration process (Table 4). When two years yield and grain quality traits including micro malt analysis results were taken in to account, Zeynelaga was also registered as the second malting barley cultivar for the irrigated conditions.

Table 3. Yield Performance of the Second Candidate Line, Zeynelaga, in Registration Trials (kg/ha)

Cultivars	Locations				Grand mean
	Konya		Eskişehir		
	2001	2002	2001	2002	
Cumhuriyet (check)	6210	4880	7416	5470	5994
Erginel (check)	7850	7020	6805	6500	7043
Yıldırım (check)	7740	6190	6002	6760	6673
Aydanhanım (check)	7160	7090	7075	6210	6883
Zeynelaga (candidate line)	6530	8080	7250	6900	7190
LSD	1173	1368	1230	1260	

Table 4. Some Quality Parameters of The Second Candidate Line, Zeynelaga, in Registration Trials

Cultivars	Test weight (kg/hl)		1000 kernel weight (g)		Protein content (%)		Kernel size (over 2,5 mm)	
	Konya	Eskişehir	Konya	Eskişehir	Konya	Eskişehir	Konya	Eskişehir
Cumhuriyet (ch)	70,0	69,1	46,2	42,4	11,5	11,9	82,8	85,7
Erginel (ch)	71,0	69,5	38,3	36,9	11,5	10,2	70,2	66,8
Yıldırım (ch)	71,6	69,3	37,3	39,6	9,9	10,8	76,7	77,1
Aydanhanım (ch)	74,0	71,0	44,6	41,2	11,3	11,1	96,0	87,8
Zeynelaga (Candidate line)	72,4	71,2	42,3	42,5	11,7	11,4	96,8	94,3

Malt extract, Kolbach index and friability scores of new cultivars were highly different from those of standard cultivars (Table 5). Enhanced yield and micro malt analysis results of new cultivars have revealed that they can be adopted by farmers in a short time and contribute much to high quality malting barley production in the target areas urgently required by the private industry.

Table 5. Micro Malt Analysis Results of New Malting Barley Cultivars

Cultivars	**Malt Extract (%)**	**Kolbach Index**	**Viscosity**	**Friability (%)**
Cumhuriyet (ch)	76,2	28,3	1,76	54,6
Erginel (ch)	74,2	27,7	1,83	43,4
Yıldırım (ch)	72,1	26,5	1,74	35,6
Aydanhanım (new cultivar)	78,1	34,2	1,52	56,0
Zeynel aga (new cultivar)	78,5	35,1	1,55	58,1

Conclusion

The first period of the project has resulted in developing two new two-rowed malting barley cultivars suitable for supplementary irrigated condition with desirable malting quality and yield level over six- rowed checks, Erginel and Yıldırım. This indicates that Turkish barley germplasm has great genetic variability in terms of yield potential and feed and malting quality traits alike. In order to broaden malting quality background of the germplasm and improve more superior new malting barley cultivars, a malting barley crossing program was commenced three years ago.

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Sequencing, Annotation and Transcriptional Analysis of Molybdenum Cofactor Sulfurase Gene from Wild Barley, *Hordeum spontaneum*

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Abstract
Plant aldehyde oxidases form a multigene family whose members catalyze the final step in the biosynthesis of the phytohormone abscisic acid. Molybdenum cofactor sulfurase catalyzes the generation of the sulfurylated form of molybdenum cofactor, a cofactor required by aldehyde oxidase functioning in the last step of abscisic acid biosynthesis in plants. This function is important in plant development and adaptation to environmental stresses such as drought. The coding region of molybdenum cofactor sulfurase gene has been cloned and sequenced in the genotypes of wild barley, *Hordeum spontaneum* from a northern mesic Mediterranean area, semi-xeric steppes, and a southern xeric desert in Israel. Southern blot analysis of wild barley DNAs revealed evidence that molybdenum cofactor sulfurase is a single copy gene in the genome of wild barley. After hybridization of the molybdenum cofactor sulfurase gene probe with Morex barley BAC filter set, positive BAC clones containing flanking regions, promoters of the corresponding gene were identified by DNA Walking Speed Up Strategy. Chromosomal mapping using Chinese Spring–Betzes addition lines revealed that the molybdenum cofactor sulfurase gene is located on the barley chromosome 1 (7H). Using "reverse transcriptase polymerase chain reaction" and "real time polymerase chain reaction" to study the gene expression analysis of this gene under water stress is currently under investigation.

Introduction
Developing genetically drought and salt resistant cereal crops is a major way of alleviating future threats to food security in a demographically exploding and increasingly starving world (PLUCKNETT *et al*. 1987). Increased resistance to water deficit is considered among the most important abiotic parameter that can contribute to increased grain production. Due to global aridization and salinization trends and the greenhouse effect (increasing temperature), there is an urgent need to provide genomic answers to these challenges. The loss of genetic diversity of some of world's crops has accelerated in recent decades with many crops becoming increasingly susceptible to biotic and abiotic stresses. Genetic diversity, the basis of evolution by natural selection, is gravely threatened in the progenitors of cultivated plants and its exploration, evaluation, conservation in situ and ex situ is imperative to guarantee effective utilization and sustainable development. Wild barley, *Hordeum spontaneum* is one of the principal grain plants on which Neolithic food production in the Near East was founded (ZOHARY & HOPF 1993). Wild barley is the progenitor of cultivated barley indicated by its cross-compatibility, full fertility and sporadic spontaneous hybridization with the cultivars. It is an annual, brittle, two-rowed diploid (2n=14), and is predominantly self-pollinated. Wild barley is a widespread generalist species, found over the eastern Mediterranean Basin and western Asiatic countries. Its highest genetic diversity is displayed in Israel and Jordan, climaxing in the grasslands of the Golan height. The progenitor of cultivated barley, *H. spontaneum*, developed over a long evolutionary history, a wide

range of adaptive diversity to biotic and abiotic stresses including extreme variations in water availability (NEVO 1992).

Abscisic acid (ABA) is a plant hormone that plays an important role in plant responses to various abiotic stresses such as drought. Increased ABA levels alleviate growth inhibition by water deficits via reducing stomatal aperture and limiting water loss through transpiration (LEUNG & GIRAUDAT 1998). In higher plants, ABA is derived from an epoxy-carotenoid precursor that is oxidatively cleaved to produce xanthoxin (PARRY *et al*. 1988). Xanthoxin is converted to ABA by a series of ring modifications to yield abscisic aldehyde, which is oxidized to ABA by the molybdenum cofactor (MoCo) containing aldehyde oxidase (AO) (LEYDECKER *et al.* 1995; WALKER–SIMMONS *et al*. 1989). In plants, xanthine dehydrogenase (XDR) and AO require that the MoCo be modified in the last step of biosynthesis with the insertion of a sulfur atom to replace one of the two terminal oxygen atoms and this sulfuration reaction is catalyzed by MoCo sulfurase.

The objective of the present study is to identify in natural populations of wild barley from across their ecogeographic range, including deserts, the physiological and genomic organization of drought resistance genes in terms of cloning and characterization of ABA biosynthesis genes. The part of coding region of ABA biosynthesis genes; zeaxanthin epoxidasegene (ZEP), 9-cis-epoxycarotenoid dioxygenasegene (NCED), short chain dehydrogenase, reductase gene (SDR), aldehyde oxidase gene (AOO3) and MoCo sulfurase gene have been cloned in wild barley recently (ALTINKUT *et al*. 2004). We describe here sequencing, annotation and transcriptional analysis of molybdenum cofactor sulfurase gene in wild barley.

Material and Methods

Plant Material

3 different wild barley (*Hordeum spontaneum*) genotypes and one barley cultivar *(Hordeum vulgare* L. cv. Morex*)* as a control were used for PCR analysis to amplify the part of coding regions of MoCo sulfurase gene.

Cloning and Sequencing

In order to clone MoCo sulfurase gene, genomic DNA`s of wild barley from mesic (Maalot) (800 mm rainfall), semi-xeric (Mehola) (290 mm rainfall), xeric (Sede Boqer) (91 mm rainfall) regions and Morex cultivar were amplified with the primers based on C-terminal portion of MoCo sulfurase homolog from cultivated barley (*Hordeum vulgare*). Amplified fragments were cloned into the pGEM-T Easy vector system and sequenced. Sequences were identified and analyzed by Bioedit Sequence Alignment Editor Version 5.0.9 and aligned by Gene Tool Lite1.0 Programs.

Southern Blot Analysis

The southern blot hybridization has been used to examine the genomic organization of MoCo sulfurase gene in wild barley. Genomic DNA was digested with 5 restriction enzymes (HindIII, EcoRI, BamHI, HaeIII and AluI) and the resulting restriction fragments are fractionated by molecular weight via agarose gel electrophoresis. Digested genomic DNA of wild barley was hybridized with MoCo sulfurase gene specific probe is denatured and the single strand molecules are labeled with ^{32}P-Dctp. After filter hybridization, the unbound probe is washed off the filter with a series of stringency washes and detection of the DNA:DNA hybrids is done by exposing the membrane directly to an X-ray film.

BAC Library Screening
A 6.64x barley (*Hordeum vulgare*) BAC library 17-filter set was obtained from Clemson University SC, USA and hybridized with the MoCo sulfurase gene probe labeled by ^{32}P-dCTP.

Chromosomal Mapping
Chromosomal mapping of MoCo sulfurase gene has been performed using wheat-barley (Chinese Spring-Beztes) addition lines. Genomic DNA of parents (Chinese Spring and Betzes), their addition lines for each chromosome and wild barley from mesic region were amplified with MoCo sulfurase primers.

Drought Treatment for Expression Analysis
Seedlings were grown in a growth chamber at 22^{o}C, with a photoperiod of 16h light/8h dark, in Murashige Skoog (0.5X) solution circulated by air pumps. Drought stress was applied at three-week old seedlings by draining the solution from the container for defined dehydration periods. Leaf tissues were harvested from control plants (time 0), and after 1, 3 6, and 12 hours of dehydration. Leaves were frozen in liquid nitrogen, and stored at –80^{o}C for RNA extraction.

Results and Discussion
Cloning and Sequencing of MoCo Sulfurase Gene
MoCo sulfurase gene sequences based on conserved regions from cultivated barley have been used to clone and characterize this gene in wild barley. The size of the amplification product in the part of coding region is 946 bp (Figure 1) and the genomic DNA sequence polymorphism in this region between mesic, xeric and semi-xeric genotypes has been observed as single nucleotide polymorphism.

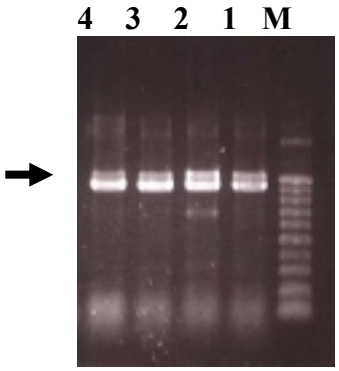

Figure 1. PCR product of wild barley genomic DNAs with MoCo sulfurase gene primers. M: 100 bp Ladder), 1. Maalot (from mesic region), 2.Mehola (from semi-xeric region), 3. Sede Boqer (from xeric region), 4. Morex cv.

Southern Blot Analysis
Southern blot analysis of DNA from wild barleys using the MoCo sulfurase gene probe revealed the presence of only one hybridizing band, providing evidence that MoCo sulfurase is a single copy gene in wild barley (Figure 2).

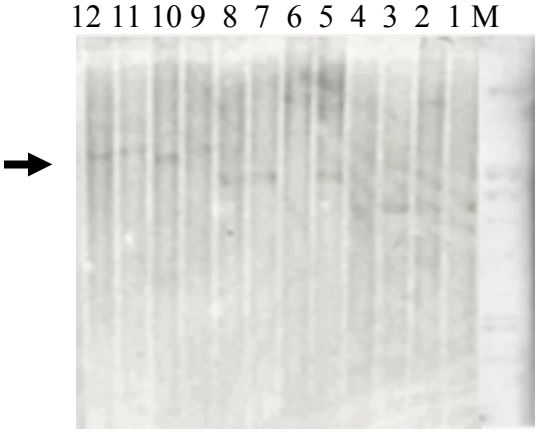

Figure 2. Southern blot analysis of MoCo sulfurase gene in wild barley. M: λ DNA digested with EcoRI. Wild barley genomic DNAs digested with Hind III; 1. Maalot, 2.Mehola, 3. Sede Boqer, 4. Morex. Wild barley genomic DNAs digested with EcoRI; 5. Maalot, 6.Mehola, 7. Sede Boqer, 8. Morex. Wild barley genomic DNAs digested with BamHI; 9. Maalot, 10.Mehola, 11. Sede Boqer, 12. Morex.

BAC Library Screening

After hybridization of MoCo sulfurase gene probe with barley BAC library set, hybridizing clones were observed. DNA from positive BAC clones containing flanking regions, promoters of the corresponding gene (MoCo sulfurase) was be identified by DNA walking speed up strategy.

Chromosomal Mapping

Chromosomal mapping using Chinese Spring-Beztes addition lines revealed that the MoCo sulfurase gene is located on the barley chromosome 7Hα. Only 7Hα additional line's DNA produced 946 bp amplification product of MoCo sulfurase gene (Figure 3). It was previously reported that this chromosome is associated with osmotic adjustment variation in barley (TEULAT *et al*. 1998).

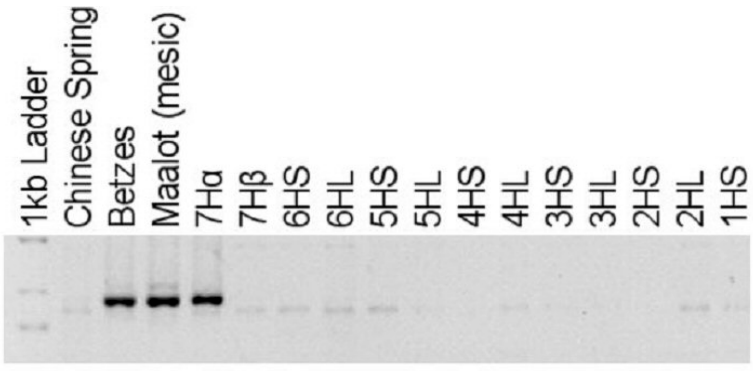

Figure 3. PCR amplification of MoCo sulfurase gene in wheat-barley addition lines

Expression Analysis

To determine the expression of MoCo sulfurase gene, RT-PCR analysis with gene-specific 5`-end and 3`end primers was performed using the same RNA isolated from control (well-watered) and drought stressed plants (after 1h, 3h, 6h and 12h of water stress). Using the RT-PCR with gene specific primers, different size of fragments and intensities could be obtained for comparison of expression of a given gene. The correlation between haplotypes, gene expression patterns and geographic locations such as mesic, semi-xeric and xeric habitats is currently under investigation. It has been reported that MoCo sulfurase gene is expressed ubiquitously in different plant parts, and the expression level increases in response to drought, salt, or ABA treatment in Arabidopsis (XIONG *et al*. 2001).

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QTLs for Drought Tolerance in Barley Grown in a Mediterranean Environment

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Abstract
Breeding for drought tolerance is a challenging task for many researchers. The ability to identify and select genotypes with desirable traits is influenced by the environment. In Mediterranean type environments, yield is limited largely by drought, which is becoming more of a constraint in the last decades. This study aims at contributing to the improvement of the knowledge of drought tolerance determinism, with the main objective of identifying QTLs related to drought performance based on molecular markers and their association with agronomic traits evaluated under field drought conditions. Plant material consists of a population of 167 barley Recombinant Inbred Lines and the two parents Tadmor and ER/Apm. Grain yield, yield components, phenology, plant height, leaf area, relative water content, spike characteristics, and carbon isotope discrimination rate were evaluated during two drought contrasting seasons. Composite interval mapping using QTL cartographer allowed the identification of QTLs associated with agronomic performance. An array of QTLs associated with performance under varying drought conditions were identified for the agronomic traits considered, and favorable allele contributions from the parental lines were determined. The results show colocalization of QTLs for some agronomic traits. These findings, in conjunction with the established association among traits allowed to identify traits, such as earliness, thousand kernel weight and kernel number, presenting a potential value in being used as selection criteria or targeted via marker assisted selection for improving barley performance under the prevailing drought of the Mediterranean basin, while further research is needed to ascertain the value of other traits.
Keywords: drought stress; Mediterranean environment; barley; QTL

Introduction

Morocco has a Mediterranean climate characterized by contrasting seasons and a wide spatial and temporal variability of precipitation. The wide range of climate allows for a wide range of crops. Cereals are predominant and occupy about 5.2 million hectares annually, representing about 70% of the total arable land. As climatic conditions become less favorable, barley becomes the most dominant cereal specie, covering about 2.3 million hectares.

Over the past 40 years, Morocco has known very recurring droughts periods, and drought has become a structural abiotic stress affecting yield and yield stability of cereals. This reinforces the need for identifying barley genotypes with good drought resistance. The present study aims at contributing to the improvement of the knowledge of drought tolerance determinism, with the main objective of identifying QTLs related to drought performance based on molecular markers and their association with agronomic traits evaluated under field drought conditions.

Material and Methods

The barley population studied consisted of 167 recombinant inbred lines (RILs) and the two parents Tadmor (a two-row line selected by ICARDA from Arabi aswad, a Syrian landrace) and ER/Apm (a two-row selected line released in Tunisia). Tadmor and ER/APM have

expressed contrasting physiological traits with respect to drought (FOSTER *et al.* in press), with Tadmor being more resistant to drought than ER/APM. The field evaluation was conducted in the region of Meknès during 1999-2000 (Season 1) and 2000-2001 (Season 2). The RILs population was evaluated in a randomized complete block design with three replicates. The traits measured are total number of tillers (TT), fertile number of tillers (FT), days to heading (DH), days to maturity (DM), fresh leaf area (FLA), turgescent leaf area (TLA), relative water content at heading (RWC) (BARRS &WATHERLEY 1968), spike length (SpL), awns length (AwL), peduncle length (PL), mildew resistance (Mdw), plant height (PH), kernel number per spike (KN), thousand kernel weight (TKW), grain yield (GY), biological yield (BY), and carbon isotope discrimination rate (Ciso) (FARQUHAR *et al*., 1989).

The genetic map used to identify QTL has been generated from 165 genetic markers including RFLP, RAPD, AFLP, SSR, STS, and one morphological marker. This map includes a fairly large number of candidate genes loci, some of them representing differentially expressed sequence tags (AYMAN *et al.*, unpublished). The linkage map construction has been performed by Map Manager QTXb19 (MANLY *et al*. 2001) using the Kosambi function. An analysis of variance (General Linear Model) was performed on the agronomic data; adjusted entry means were generated by fixing the block effect and used for QTL analysis. Composite interval mapping has been performed using QTL cartographer v2.0 (WANG *et al*. 2003) in order to detect QTLs corresponding to associations between agronomic data and molecular markers. During the regression procedure, background markers are selected by the program using a forward and backward method in the standard model, with the probability for into or out being 0.01. The window size used was 10 cM. A threshold of 2.5 has been chosen to declare a QTL significant.

Results and Discussion

Characterization of Climatic Conditions and Drought Severity for the Two Seasons

Total rainfall during the growing cycle of the crop (*Nov. 10 to May 20*) amounted to 175 mm during 99-00 and to 267 mm during 00-01. Both years were below the long term average rainfall of the region, which is about 455 mm. In addition to the low rainfall of both seasons, periods of drought occurred at different periods of the crop growing cycle. Early, mid and late season drought occurred in 99-00, while mid season and late season drought occurred in 00-01. In addition, evapotranspiration of the crop cycle was higher in 99-00 (386 mm) compared to 00-01 (314 mm). The drought severity of the two seasons was characterized using several indices (AGHRAB 2003), the Index of Percent to Normal (HAYES 2003), the Standardized Precipitation Index (HAYES 2003) and the Deciles Index (GIBBS & MAHER 1967). According to these three indices, the 99-00 and 00-01 were classified as being respectively **severe** and **moderate** drought seasons**.**

Characterization of the RIL Barley Population in Terms of Agronomic Performance

The performance of the 167 RILs, as well as the effects of genotype, season and genotype-season interaction are presented in Table 1. In general, the RILs performed better under moderate drought (00-01) compared to the severe drought (99-00) conditions for most of the traits, to the exception of thousand kernel weight, peduncle length and awns length. Although 99-00 is classified as severely dry, the little rainfall that occurred during grain filling (April and May) allowed for appreciable grain plumpness. In the case of peduncle length and awns length, it seems that these two traits are under the genetic control of developmental genes that are affected by drought conditions; therefore, under severe drought, they grow longer to allow for remobilization of carbohydrates to the grain. The severe drought conditions of 99-00 season induced substantial reductions for some traits in comparison to the moderate drought

conditions of 00-01 (71% for leaf area, 50% for plant height, and 33% for kernel number). Despite these important reductions, barley remains a preferred cereal crop by farmers in arid and marginal areas of morocco as it enables to ensure a minimum production for subsistence under the unpredictable prevailing drought conditions. In the case of mildew, the small variability and the lack of significance of genotype-season interaction indicate that drought affected similarly the behavior of the RILs in terms of their resistance to this disease. Carbon isotope discrimination presented values commonly observed for C_3 species with a small variability indicating the repeatability of this trait.

The environment effect was highly significant for all the traits studied; highly significant differences were obtained among genotypes for all the traits, except for grain yield and biological yield. Genotype-season interaction showed highly significant differences for days to heading, days to maturity, leaf area, spike length, peduncle length and thousand kernel weight.

Table 1**.** Mean performance, standard deviation, minimum and maximum, values of the agronomic traits studied for 99-00 and 00-01 seasons, and Genotype, Season and Genotype-season interaction effects from the combined season analysis of variance for the 167 RILs.

Trait (unit)	**99-00 season**				**00-01 season**				**Level of significance**		
	Mean	**STD**	**Min**	**Max**	**Mean**	**STD**	**Min**	**Max**	**G**	**S**	**GxS**
TT	680.4	115.3	415.7	1060.3	1048.6	137.8	717.3	1629.3	***	***	***
FT	426.4	121.0	158.5	787.6	543.6	113.7	286.7	946.7	**	***	*
DH	92.9	4.1	83.7	103.3	104.8	2.8	99.0	111.0	***	***	***
DM	122.1	2.7	116.3	131.7	145.0	1.3	143.0	149.0	***	***	***
FLA	4.0	1.0	2.2	7.2	14.3	2.4	9.2	22.6	***	***	**
TLA	4.5	1.1	2.2	8.1	15.5	2.5	9.8	24.4	***	***	**
RWC	64.2	5.4	49.2	78.1	74.3	6.3	56.2	87.1	**	***	ns
SpL	7.0	0.6	5.7	8.8	9.2	0.80	7.3	11.7	***	***	***
AwL	10.4	1.2	8.3	16.9	8.2	0.96	6.1	11.2	***	***	ns
PL	2.7	0.4	2.1	4.1	0.3	0.08	0.19	0.57	***	***	***
Mdw	6.1	0.8	4.7	9.0	5.3	1.0	1.7	7.3	***	***	ns
PH	52.4	5.0	36.1	66.1	102.5	5.3	86.7	118.3	***	***	ns
KN	19.6	1.6	15.5	22.7	29.8	1.5	26.3	34.0	***	***	ns
TKW	40.8	3.4	30.1	49.8	31.8	4.0	22.1	42.0	***	***	***
GY	224.1	75.5	25.6	429.0	275.8	58.9	122.1	454.3	ns	***	ns
BY	653.6	151.9	349.3	1071.0	1704.4	251.6	933.3	2333.3	ns	***	ns
Ciso	15.1	0.5	13.6	17.0	16.9	0.4	15.9	18.2	-	-	-

G: genotype effect, **S:** season effect, **GxS**: genotypexseason interaction

TT: Total number of tillers; **FT**: Fertile number of tillers, **DH**: Days to heading; **DM**: Days to maturity **FLA**: Frech leaf area **(cm^2), TLA**: Turgescent leaf area **(cm^2), RWC**: Relative water content, **SpL:** Spike length **(cm)**; **AwL:** Awns length **(cm)**; **PL**: Peduncle length **(cm)**, **Mdw:** Mildew resistance, **PH:** Plant height **(cm)**, **KN:** Kernel number per spike; **TKW**: Thousand kernel weight **(g), GY:** Grain yield **(g/m^2)**; **BY:** Biological yield **(g/m^2)**, **Ciso**: Carbon isotope discrimination rate **(‰)**.

*, **, ***: significant at the probability levels of 0.05, 0.01 and 0.001 respectively

Identification of QTLs Associated with the Agronomic Traits

Table 2 and figure 1 present the QTLs identified for the agronomic traits evaluated for the two seasons. A total of 30 QTLs were identified for the severe drought season (99-00) and 35 QTLs for the moderate drought season (00-01) for the agronomic traits evaluated, with many QTL colocalizations. Some QTLs were conserved across the two seasons, while others were season specific.

Phenology seems to be under the genetic control of genomic regions on chromosomes 2H, 3H and 5H. QTLs for days to heading and days to maturity colocalized on the dhn1 and dhn9 on chromosome 5H, known as dehydrin genes, as well as on chromosome 2H. Days to heading has been found negatively associated with grain yield (r=-0.36**) and yield components under severe stress conditions, indicating that early heading genotypes present a drought escape mechanism, giving them an advantage over later heading genotypes in regard to their response to the commonly occurring late season drought. This corroborates the common use of earliness as a selection criterion by local cereal breeding programs.

Tillering capacity, especially the number of fertile tillers, presented strong associations with grain yield and biological yield across environments. However, only the moderate stress environment allowed for the detection of 2 QTLs on chrom 3H and one QTL on chromosome 6H for this trait. Chromosome 5H seems to play a key role in the genetic control of yield and yield components, with kernel number and thousand kernel weight being also associated with conserved genomic portions on chromosomes 4H and 6H. These 2 QTLs have been reported as main effect and main effect and QTLxE effect respectively (Teulat et al. 2001). Thousand kernel weight was consistently correlated with yield (r=0.27** and 0.31**) across environments, and kernel number was associated with yield under severe drought (r=0.31**). These two traits could be considered as potential selection criteria to improve yield under such drought conditions, and QTLs identified for this traits could also be considered as potential candidates for marker assisted selection.

Concerning plant height, a strong and consistent QTL on chromosome 6H, and 3 season specific QTLs on chromosomes 2H and 3H were identified. The QTLs on chromosomes 6H and 2H have been reported as main effects (Teulat et al. 2001). Plant height presented a highly significant association with yield (r=0.51***) and yield components and was negatively associated with plant phenology, indicating that tall early heading genotypes presented good yielding capacity under severe drought conditions.

In the case of carbon isotope discrimination rate, 3 season specific QTLs were identified on chromosomes 7H, 4H, and 1H. No consistent QTL was obtained across environments. The trait presented a positive association with grain yield (r= 0.23**) under severe drought, and a negative association (r=-0.25**) under moderate drought. The lack of consistency of these associations may indicate that the use of Ciso as a selection criterion to improve grain yield under drought should be considered with care, and that further research is needed to determine its reliability.

Mildew showed 2 consistent QTLs both located on chromosome 5H (wg889 and Dhn1), and one season specific QTL on chromosome 3H. Fresh and Turgescent leaf area are under the genetic control of genomic regions located on chromosomes 2H, 3H, 4H and 6H. Spike length showed 2 consistent QTLs on chromosomes 3H and 6H, and 5 season specific QTLs on chromosomes 1H, 2H, 4H and 5H. Peduncle length was controlled by a consistent QTL on chromosome 2H and 2 season specific QTLs on chromosomes 1H and 5H. Awns length showed 2 season specific QTLs on chromosomes 7H and 5H. Relative water content was also controlled by 3 season specific QTLs located on chromosomes 6H and 5H, detailed information on QTLs for RWC has been published (TEULAT *et al.* 2003).

Table 2**.** QTLs identified for 99-00 season for the various agronomic traits evaluated

	Season 99-00						Season 00-01					
Trait	**Chr**	**Left marker**	**Peak position (cM)**	**LOD score**	**R^2 (%)**	**Add**	**Chr**	**Left marker**	**Peak position (cM)**	**LOD score**	**R^2 (%)**	**Add**
TT	2 7	Bmac684 Dhn1	128.1 271.4	2.94 3.11	8.7 11.7	34.09 39.70	3	C1B1b	137.9	3.28	8.7	-42.1
DH	2 3 7	Bmac684 PSR170 Dhn1	116.1 280.8 275.4	2.68 4.85 2.80	11.2 19.6 12.0	1.47 -1.82 1.49	2 3 7	Bnl16_06 mwg595 C1F9a	83.2 68.5 252.1	2.97 3.15 9.49	10.4 13.1 30.3	0.93 1.06 1.59
DM	2 3 7	Bmac684 Bmag013 Dhn1	120.1 287.4 269.4	3.08 5.81 7.49	10.1 11.9 24.8	0.87 -0.97 1.40	*2* 5	*Bmag378* pHva1	134.4 206.5	2.14 4.49	*4.8* 10.4	*0.28* -0.42
FLA	4	wg114	158.0	2.77	8.8	0.30	2 3 6	cdo1417 bcd1127 Bmag173	197.8 205.3 87.8	2.95 2.65 2.96	11.5 6.5 9.7	-0.81 0.62 0.74
TLA	2 4	Bmag378 wg114	156.4 158.0	2.68 3.52	9.0 10.4	-0.34 0.35	2 6	cdo1417 Bmag173	189.8 87.8	3.76 4.37	11.0 15.2	-0.82 0.97
RWC	6 6 7	bcd348B bcd1 C1F9a	68.8 212.5 250.1	3.68 3.04 3.91	11.1 10.7 16.1	-1.82 1.78 2.17	-	-	-	-	-	-
SpL	3 6 7	mwg595 C1F12 mwg502	62.5 103.8 0.0	3.03 3.02 2.86	17.4 11.7 7.2	0.25 0.21 -0.16	2 *3* 4 5 6 7	C1D4 *C1F4b* C1F8a C1B11a cdo497 wg564	317.1 *88.8* 222.2 150.5 113.4 173.2	2.95 *2.44* 2.97 3.02 4.31 2.91	7.5 *9.5* 6.7 8.2 10.6 8.8	-0.23 *0.25* 0.21 -0.23 0.26 -0.24
AwL	- -	- -	- -	- -	- -	- -	1 7	caaaagB C1A4	223.3 118.7	3.59 3.78	8.3 8.9	0.28 -0.30
PL	*2* 5	*C1D4* Bmag211	313.1 76.0	2.48 2.52	*10.2* 6.3	*-0.12* -0.09	2 7	bcd266 Tapk4	317.6 363.7	3.13 2.81	8.1 8.4	-0.02 -0.02
Mdw	7 7	wg889 Dhn1	135.8 271.4	6.86 3.38	20.6 11.7	0.36 -0.27	3 *7* *7*	ABG495 *wg889* *Dhn1*	320.5 129.8 273.4	3.09 2.41 2.47	7.1 *5.6* *8.8*	0.28 *0.25* *-0.30*
PH	2 6	Bmac684 C1F12	114.1 97.8	3.25 3.36	11.1 12.1	-1.68 1.75	2 3 6	bcd266 bcd263 C1F12	317.6 212.5 103.8	4.37 3.58 8.07	10.1 6.8 21.4	-1.75 1.41 2.46
KN	2 4 7	cdo64 C1F9b caaaccS	53.2 171.5 19.3	3.91 4.92 2.62	9.3 18.0 10.9	0.51 -0.71 -0.56	3 4 7	C1F4b wg114 wg889	98.8 150.0 131.8	3.85 3.70 3.45	10.3 7.9 8.8	0.50 -0.43 -0.47
TKW	6 7 7	cdo497 mwg502 C1F9a	109.4 0.0 256.1	6.49 3.09 3.69	16.9 7.7 12.9	1.45 -0.97 1.25	6	cdo497	113.4	2.69	7.9	1.14
GY							5 7	bcd442 C1G8b	210.9 65.3	3.65 2.81	9.5 8.2	-18.4 -19.3
Ciso	*1*	*CDO475*	*24.1*	*2.31*	*8.4*	*-0.16*	*4* 5	*ctgaccF* wg983	*192.0* *201.8*	*2.27* *3.88*	*6.1* 11.9	*1.13* 0.16

Chr: Chromosome; **R^2**:percent of the phenotypic variation explained; **Add:** Additivity (+: from Tadmor; -: from ER/Apm); **GY:** Grain yield; **BY:** Biological yield; **KN:** Kernel number per spike; **TKW**: Thousand kernel weight; **DH**: Days to heading; **DM**: Days to maturity; **TT**: Total number of tillers; **FT**: Fertile number of tillers; **PH:** Plant height; **SpL:** Spike length; **AwL:** Awns length; **PL**: Peduncle length; **Mdw:** Mildew resistance; **RWC**: Relative water content; **Ciso**: Carbon isotope discrimination rate.

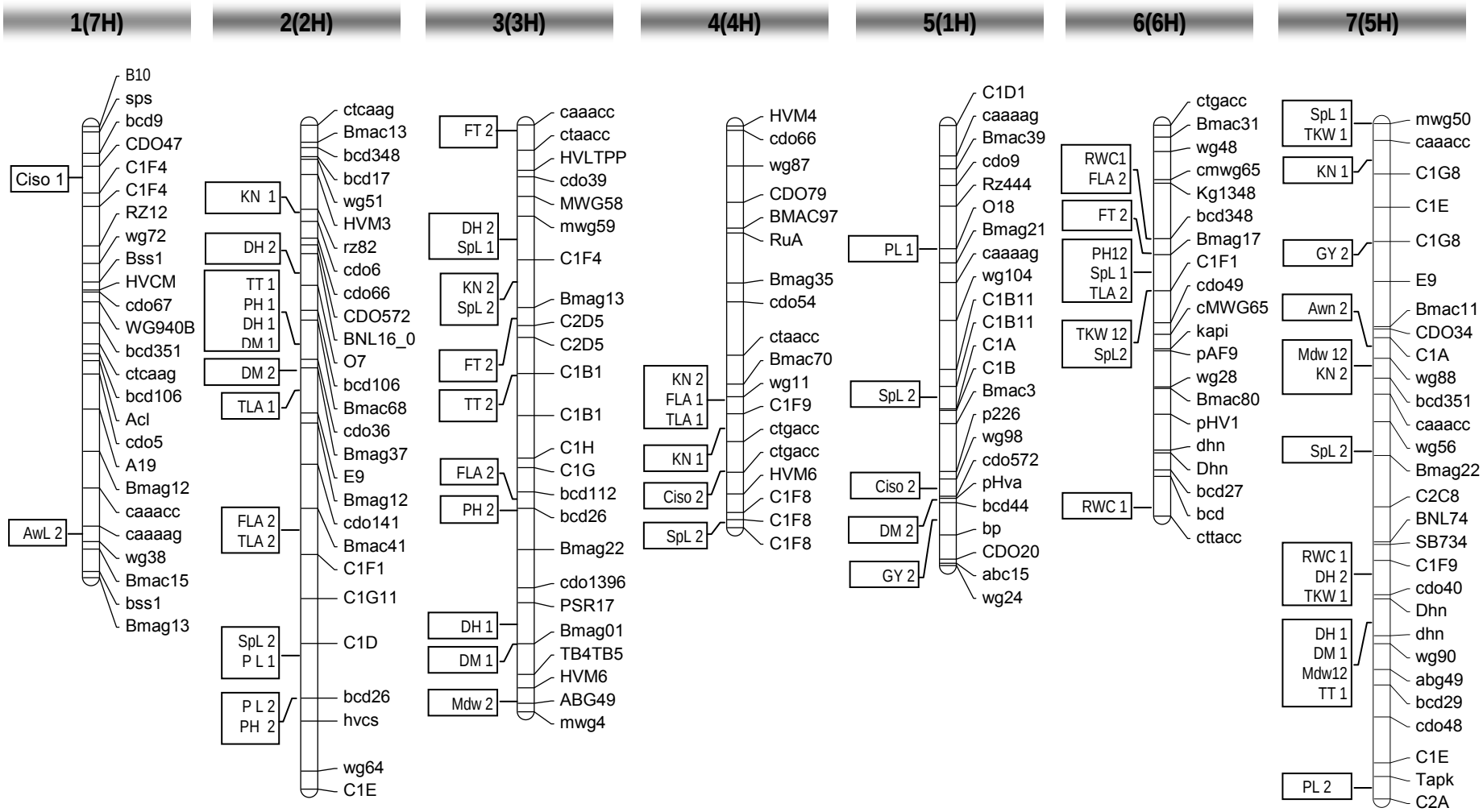

Figure 1 QTLs for the agronomic traits identified for the RIL progeny of Tadmor x ER/Apm; (1) 99-00 season and (2) 00-01 season

Conclusion

The QTLs obtained represent a data base for an array of agronomic traits associated with the performance under varying drought conditions of the barley RILs evaluated. Favorable alleles contributed by each of the two parents were identified for the set of agronomic traits tested. These findings could be valuable for breeding purposes in terms of targeting important traits related to drought resistance via marker assisted selection. Some traits, such us earliness, thousand kernel weight, and kernel number, have been identified as presenting a potential value in being used as selection criteria or targeted via marker assisted selection for improving barley yields under the prevailing drought conditions of the Mediterranean basin. While other traits, such as carbon isotope discrimination rate, contrasting results have been found, and further research is needed to ascertain their value under natural drought conditions.

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The Genetic Basis of Adaptation to Low Rainfall Environments in Australia

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Abstract
Improving the adaptation of barley to low rainfall environments is a challenge, since there are many constraints to productivity in these environments. Specific mapping populations have been grown in Australian low rainfall environments to characterise the genetic basis of adaptation. The mapping populations include the Australian advanced backcross QTL (*H. spontaneum x Barque*73*) mapping population, and ICARDA mapping populations Arta x Harmal-02/Esp//1808-4L and Tadmor x Er/Apm. QTL mapping of yield and grain quality traits has revealed common loci from trials conducted in Australia and Mediterranean low rainfall environments internationally. These genome locations are different to those identified previously in Australian mapping populations that were not specifically designed to investigate the genetics of adaptation to low rainfall environments.
Keywords: drought; adaptation; mapping population; yield

Introduction

Drought stress is a common constraint to the productivity of barley in the low rainfall Mediterranean type environments of Australia. These environments are characterised by winter-dominant rainfall and low temperatures during early crop development, increasing photoperiod, temperature, evaporative demand, and reducing moisture availability as crop development proceeds (NIX 1975). The frequency of drought stress is influenced by the sub-environment, including the variability in winter rainfall events, soil moisture holding capacity, and the incidence of subsoil constraints, which reduce the ability of the plant to obtain available moisture. High temperatures are commonly coincident during late crop development to exacerbate the effects of drought stress.

These combinations of stresses in the low rainfall environments of Australia act to reduce yield, with drought stress constituting a major yield-limiting factor. Low yields of less than 1.5 t/ha are common in the low rainfall environments of Australia (ABARE 1999), which comprise a major component of the cereal producing area. Therefore, improving adaptation to these low rainfall environments is important, however the factors involved are still poorly understood. A better understanding of the direct and indirect tolerance mechanisms, particularly related to drought stress, will make the breeding efforts targeting these areas more successful.

Introgression of alleles from barley originating in the Mediterranean basin has historically led to major advances in the adaptation of Australian barley. Hence, there is an active collaborative breeding effort between ICARDA and the South Australian program, involving germplasm exchange and evaluation in the low rainfall environments. The evaluation of mapping populations specifically developed for low rainfall environments has been important

in investigating the genetic basis of adaptation in Australia. An understanding of the genetics of adaptation of ICARDA germplasm in Australian environments is beginning to emerge, and is compared to populations developed and evaluated in Australia.

Material and Methods

The Low Rainfall Trials

The Arta x Harmal-02/Esp//1808-4L and Advanced Backcross QTL (AB-QTL) population *H. spontaneum* x Barque*73 were evaluated at Port Wakefield (32°50', 135°07') in South Australia in 2002 and 2003. The AB-QTL population was also evaluated at Ouyen in the Victorian Mallee in 2003, and the Tadmor x Er/Apm population at Port Wakefield in 2003 only. There was drought stress (growing season rainfall <200mm; mean site yield <1.5t/ha) at Port Wakefield in 2002, and Ouyen in 2003.

The AB-QTL and Tadmor x Er/Apm trials were unreplicated, with a grided check of Barque or Tadmor and Er/Apm for the respective populations. CPI 71284-48 could not be grown in the field because of quarantine regulations. The Arta x Harmal-02/Esp//1808-4L population was conducted as a randomised complete block design with two replications. Spatial analysis of all trials was conducted using the REML directive in Genstat and the methodology of SINGH *et al.* (in press). QTL-trait associations were detected by simple marker regression for the AB-QTL population, SIM for Tadmor x Er/Apm, and sCIM for the Arta x Harmal-02/Esp//1808-4L population data mapped by ICARDA.

Mapping Populations

A summary of the populations is shown in Table 1. The Arta x Harmal-02/Esp//1808-4L, Tadmor x Er/Apm, and AB-QTL mapping populations, are designed to investigate adaptation to low rainfall environments. Arta and Tadmor are derived from the Syrian landraces Arabi Abiad and Arabi Aswad respectively. Harmal-02/Esp//1808-4L is an improved ICARDA breeding line, and Er/Apm a selected line released in Tunisia. All these parents have adaptation to the low rainfall environments, with Arta conferring high tillering, and reduced height under drought (BAUM *et al.* 2003), Tadmor conferring yield stability, osmotic adjustment under drought, and pale green leaf pigmentation putatively associated with adaptation (TEULAT *et al.* 1998). Barque is an early maturing, semi-dwarf feed variety with good yield stability, released in South Australia (BARR 1998). CPI1284-48 is a *Hordeum spontaneum* genotype from a high rainfall area in Israel, and has large seed and high levels of osmotic adjustment under drought stress (EGLINTON *et al.* 2000).

Table 1. Details of the mapping populations evaluated under low rainfall environments

Population	Population Structure	Environment	Marker No.	Reference
Arta x Harmal-02/Esp//1808-4L	94 RIL	PTW 2002-03	76	(EGLINTON *et al.* 2001)
Tadmor x Er/Apm	167 RIL	PTW 2003	180	(TEULAT *et al.* 1998)
CPI 71284-48 x Barque*73	325 BC_2F_1	PTW 2002-03 OUY 2003	122	(EGLINTON *et al* 2004; this issue)

PTW, Port Wakefield (South Australia); OUY, Ouyen (Victoria)

Trait Assessment

Yield was assessed from the weight of plot samples, expressed as t/ha. Grain plumpness (%>2.5mm) and screenings (%<2.2mm) were determined from plot samples after size fractionation though fixed width slotted sieves, and 1000-grain weight was determined from the weight of 1000 grains, and expressed as mg/grain. Hector-litre weight was assessed using

a test weight apparatus. Plant height was assessed from the base of the plant to the collar, measured to the nearest 0.5cm. Plant growth staging was assessed using the Zadoks scale.

Results and Discussion

A summary of the spatially analysed yield and 1000-grain weight data is presented in Table 2. Other traits were also assessed but not summarised here. Yield under drought stress conditions was low, especially in 2002 at Port Wakefield, where the growing season rainfall was only 143mm. The mapping results are summarised schematically on Figure 1, with QTL positions as approximations only.

Table 2. Summary of spatially adjusted trait values (BLUPs) for yield and 1000-grain weight measured in the Arta x Harmal-02/Esp//1808-4L, Tadmor x ER/Apm, and AB-QTL (CPI 71284-48 x Barque*73) mapping populations, grown at the low rainfall sites of Port Wakefield and Ouyen in 2002 and 2003 under drought stress and more favourable conditions

Port Wakefield 2002 (Severe drought stress)

	Parent Mean		Population						
Arta x Harmal	**Arta**	**Harmal**	**Mean**	**Min**	**Max**	**SED**	**%CV**	**Model**	**h^2**
Yield (t/ha)	0.36	0.39	0.55	0.24	1.01	0.14	31.5	RcAr	0.41
1000-grain weight	44.68	45.2	44.79	29.76	51.43	1.18	2.5	Rcb	0.89
AB-QTL	***H.Spont***	**Barque**							
Yield (t/ha)	N/A	0.59	0.50	0.20	0.88	0.18	34.7	CrdArAr	0.37
1000-grain weight	N/A	42.82	41.82	29.85	48.10	1.87	3.4	Crd	0.83

Port Wakefield 2003 (More favourable)

	Parent Mean		Population						
Arta x Harmal	**Arta**	**Harmal**	**Mean**	**Min**	**Max**	**SED**	**%CV**	**Model**	**h^2**
Yield (t/ha)	1.60	1.69	2.25	1.17	3.00	0.27	19.3	RcArAr	0.46
1000-grain weight	37.5	42.35	38.45	32.64	44.25	1.81	6.2	RcArAr	0.61
AB-QTL	***H.Spont***	**Barque**							
Yield (t/ha)	N/A	3.29	2.73	0.79	3.79	0.20	8.19	CrdLAr	0.82
1000-grain weight	N/A	39.11	41.10	32.21	51.65	2.73	5.9	CrdArAr	0.60
Tadmor x Er/Apm	**Tadmor**	**Er/Apm**							
Yield (t/ha)	2.18	3.21	2.74	2.11	3.63	0.22	8.8	CrdCSAr	0.55
1000-grain weight	31.90	33.43	32.04	27.65	39.11	3.42	7.4	Crd	0.50

Ouyen 2003 (Moderate drought stress)

	Parent Mean		Population						
AB-QTL	***H. spont***	**Barque**	**Mean**	**Min**	**Max**	**SED**	**%CV**	**Model**	**h^2**
Yield (t/ha)	N/A	1.55	1.33	0.79	1.82	0.24	18.4	CrdArAr	0.39
1000-grain weight	N/A	41.07	42.25	34.51	48.31	2.47	4.00	Crd	0.40

Nb. SED, Standard Error of Deviation (significant differences are determined by 2*SED)

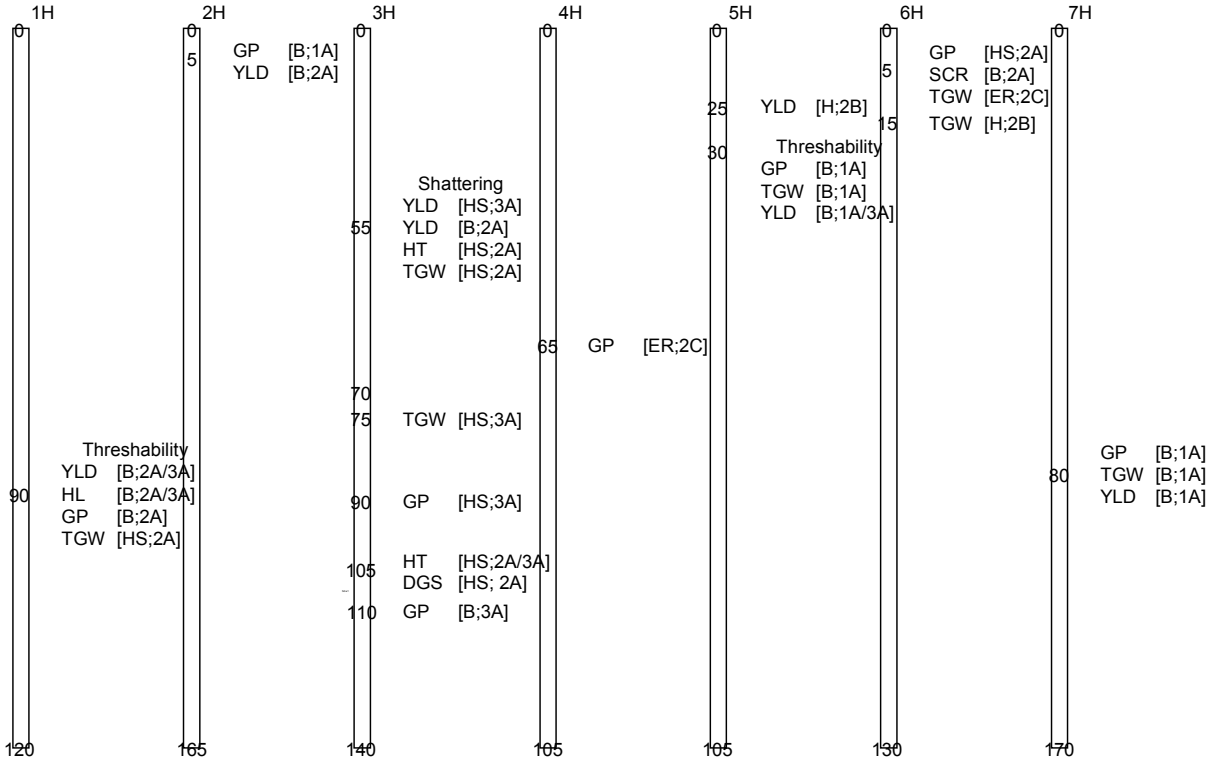
AB-QTL Population

The average yields under drought stress at Port Wakefield in 2002 and Ouyen in 2003 were 0.5 t/ha and 1.33 t/ha respectively. Under the more favourable environment there was wide transgressive variation for yield, and high heritability (0.82). Parental differences could not be determined since only Barque could be grown in the field. The range in 1000-grain weight was similar for all AB-QTL trials, with heritability highest under drought stress at Port Wakefield in 2002. There was clear segregation for height, but segregation for maturity was restricted, especially for heading date, which was relatively uniform.

CPI 71284-48 alleles (hereby referred to as HS alleles), at a chromosome 1H and 5H locus confer poor threshability, and a group of traits map coincidently to the positions 90cM and

30cM respectively on the consensus map. Poor threshability was correlated with low yield, 1000-grain weight, and grain plumpness in all environments, and these coincidences indicate pleiotropic interactions. However a HS allele on the chromosome 1H locus conferred higher 1000-grain weight at Port Wakefield in 2003, an association that could be through extra dry matter attached to the grain (ie. awn stubs).

Figure 1. Summary of QTL mapped under drought stress and favourable conditions in the mapping populations evaluated in low rainfall environments in Australia



Legend

Chromosomes are represented by vertical rectangles (chromosomes 1H-7H) with the chromosomal position of individual QTL referenced by its position in cM based on the relative position of consensus markers published by (KARAKOUSIS *et al.* 2003), linked to the QTL of interest. All QTL have LOD >3, except those of the Arta x Harmal-02/Esp//1808-4L population, where only putative QTL with LOD between 2.5-3.0 have been found. The traits are coded as below, with the allele of largest effect, followed by site and population codes in parenthesis. Trait Codes: YLD, yield; HL, hector-litre weight; GP, grain plumpness; TGW, 1000-grain weight; SCR, screenings %; HT, height; DGS, Zadoks decimal growth stage. Poor threshability is conferred by *Hordeum spontaneum* alleles. Allele Codes: B, Barque allele; HS, *Hordeum spontaneum* allele; ER, Er/Apm allele; H, Harmal-02/Esp//1808-4L allele. Site Codes: 1, Port Wakefield 2002; 2, Port Wakefield 2003; 3, Ouyen 2003. Note that drought stressed environments were sites 1 and 3. Population Codes: A, CPI 71284-48 x Barque*73; B, Arta x Harmal-02/Esp//1808-4L; C, Tadmor x ER/Apm.

There were many marker-trait associations on chromosome 3H in the AB-QTL population. At the consensus position of 55cM, there were interesting QTL x environment interactions. The direction of allele effects for yield QTL mapped to this locus from Port Wakefield and Ouyen in 2003 changed, with HS alleles conferring higher yield at the latter site. A QTL for shattering maps to this region, but is not pleiotropically associated, since all field grown lines are non-shattering. HS alleles at this locus also conferred higher 1000-grain weight and plant height at Port Wakefield in 2003, which were positively correlated in all environments. Height and early plant development mapped to the chromosome 3H *sdw1* locus with HS alleles conferring tallness and faster early plant development. Interestingly, no other traits mapped to this locus, which has been shown to be pleiotropically associated with many traits

mapped to this locus, which has been shown to be pleiotropically associated with many traits in other Australian mapping populations (COVENTRY *et al.* 2003).
Grain plumpness and screenings assessed at Port Wakefield in 2003 mapped to chromosome 6H, with HS alleles conferring plump grain and low screenings in this environment. Since there was an increased site mean screenings percentage across all populations grown, this may represent a locus for adaptation that needs further characterisation. Chromosome 2H and 7H loci were associated with yield, 1000-grain weight, and grain plumpness, with Barque alleles conferring higher trait values. Chromosome 2H has important plant developmental genes which confer phenological adaptation to Australian environments (COVENTRY *et al.* 2003). The 2H locus in the AB-QTL population is not linked with these developmental loci, nor did the developmental traits assessed map to this chromosome. The narrow maturity range indicates that developmental loci with major effects are not segregating in this population, which is important to reduce the confounding effects of development on the traits of interest.

Arta x Harmal-02/Esp//1808-4L Population
The mean yield of the Arta x Harmal-02/Esp//1808-4L population under severe drought stress at Port Wakefield in 2002 was low (0.55 t/ha), and there was no significant difference between the parents. However, this population exhibited wide transgressive segregation and higher maximum yield than the AB-QTL population in this environment. The highest yielding Arta x Harmal-02/Esp//1808-4L line was equivalent to Keel, the highest yielding Australian cultivar in these environment types. This is a significant achievement considering the parents of this population are unrelated to Australian germplasm. Although there was no significant difference between the parents for 1000-grain weight under drought stress, there was a large range and high trait heritability. Mean 1000-grain weight in the more favourable environment was lower, since screenings % was high at Port Wakefield in 2003.

There were few QTL mapped in the Arta x Harmal-02/Esp//1808-4L population, and those reported here are putative, with LOD scores of between 2.5 and 3. At Port Wakefield in 2003, a yield QTL was detected on chromosome 5H, with positive effects from Harmal-02/Esp//1808-4L (referred to as the H allele). Although this had a low LOD (2.6), it explained 12.4% of the variance for yield at this site. Yield QTL at this locus have been previously reported in the Arta x Harmal-02/Esp//1808-4L population grown in drought stressed environments in Syria (Breda) and Jordan (Ramtha and Gweer) in 2000 (EGLINTON *et al.* 2001). The importance of this locus in Australian low rainfall environments needs validation. A QTL for 1000-grain weight at Port Wakefield in 2003 was detected on chromosome 6H, with H alleles of increased effect, but explaining little of the variance for this trait. This is coincident with maturity and yield QTL detected in Syria and Jordan in 2000 (EGLINTON *et al.* 2001).

Tadmor x Er/Apm Population
The Tadmor x Er/Apm population was only assessed at Port Wakefield in 2003. There were significant differences between the parents for yield, but not 1000-grain weight. Tadmor was the lower yielding parent, and suffered from lodging in this environment. Only QTL for grain plumpness and 1000-grain weight were detected in this environment, on chromosomes 4H and 6H respectively. Both QTL had Er/Apm alleles conferring increased effect. The chromosome 6H 1000-grain weight locus, was at a coincidental consensus location to the grain plumpness and 1000-grain weight QTL detected in the AB-QTL population in this environment. This 1000-grain weight QTL was also found by TEULAT *et al.* (2001) for the population grown in Mediterranean environments internationally. This may be an important locus conferring improved 1000-grain weight in Australian low rainfall environments, but this

population needs evaluation under drought stress to validate the effects of this QTL.

Conclusion

The QTL identified conferring grain yield and grain weight/size map to different loci than those found in conventional mapping populations that have been evaluated in Australia. Unlike conventional mapping populations they are not confounded by segregation for key developmental traits, and offer an opportunity to investigate the genetic basis of adaptation relevant to mainstream breeding for the low rainfall environments. These populations offer information on alternative genomic locations that Australian breeders can target for improving the adaptation of Australian germplasm. More evaluation of these populations and alternative populations is needed to decipher the genetics of adaptation. It is already apparent that common chromosomal regions influence traits assessed in Mediterranean environments. The chromosome 5H and 6H yield and 1000-grain weight loci in the Arta x Harmal-02/Esp//1808-4L and Tadmor x Er/Apm populations are common to Australian and Mediterranean environments internationally. The discovery of HS alleles that influence traits in Australian environments shows promise, but needs further evaluation.

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QTLs and Candidate Genes in the 'Nure' x 'Tremois' Mapping Population

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Abstract
In order to study in an unique genetic system cold and drought stress-related traits, together with malting quality traits, a new barley genetic map based on the 'Nure' (winter and feeding) x 'Tremois' (spring and malting) doubled-haploid population has been developed. Two low temperature tolerance QTLs were mapped on chromosome 5H. A first, distal QTL for low temperature tolerance and vernalization requirement was identified at the *VrnH1/FrH1* region. A second proximal QTL of cold tolerance was coincident with two QTLs regulating the accumulation of two different COR (COld Regulated) proteins (COR14b and TMC-Ap3); the peak position of this QTL, named *FrH2*, is coincident with the genetic locus of the *HvCBF4* barley gene, that is in turn the candidate gene underlying this QTL. The 'Nure' x 'Tremois' population is also being tested for malting quality related traits. For this purpose, two separate replicated yield trials have been set up, the first fall-sown in one location (2001/2002), and the second one for validation in different locations in Italy (2002/2003). Moreover, a multilocational irrigated *vs.* non-irrigated field trial in Mediterranean Europe, North Africa and West Asia is being carried out in 2004 for mapping QTLs and candidate genes for drought tolerance.
Keywords: cold tolerance; drought tolerance; malting quality; CBF

Introduction

Barley (*Hordeum vulgare*) is an excellent model system for genetic analysis of the molecular basis of abiotic-stresses tolerance in fall-sown cereals. There is indeed abundant genetic variation for this trait within the primary gene pool and an ever-expanding set of tools for genetic analysis, ranging from mapping populations to arrays. Resistance to low temperature is necessary for winter-habit genotypes grown in areas with subzero winter temperatures. Maximum low-temperature tolerance is achieved after induction—"hardening", i.e. exposure to moderately low temperatures, and is achieved at vegetative growth stages (HAYES *et al*. 1997). Vernalization, low-temperature tolerance, and photoperiod sensitivity are inter-related (LIMIN & FOWLER 2002). The same conditions, essential for vernalization of winter genotypes, promote the hardening process in both spring and winter types, nevertheless, the three phenotypes for vernalization, low-temperature tolerance and photoperiod sensitivity were observed to occur in all possible combinations (KARSAI *et al*. 2001). The *Triticeae* form a homogeneous genetic system and comparative genetics studies confirm that the genetic determinants of winter hardiness are conserved, and therefore results from one species are applicable to other members of the tribe. Quantitative trait locus (QTL) analysis tools, when applied to the *Triticeae*, converge on a limited number of conserved genome regions as being responsible for the components of abiotic-stress tolerance (CATTIVELLI *et al*. 2002). Chromosome group 5 has the highest concentration of QTLs and major loci controlling plant adaptation to the environment, particularly those controlling heading date, frost and salt tolerance, whereas a region with a crucial role in drought tolerance is located on chromosome

group 7. Although the molecular responses to cold and drought stresses share a common set of genes (CATTIVELLI *et al*. 2002), the loci describing the genetic bases of cold and drought tolerance are different. Nevertheless, multiple-stress QTL sand linked markers have also been detected, suggesting the existence of common mechanisms for different stresses, or of clusters of genes controlling different stress tolerance processes. Most consistent is a region on the long arm of chromosome 5H where there are QTL/loci for vernalization response, low-temperature tolerance and photoperiod sensitivity (PAN *et al.* 1994). In barley three *Vrn-H* loci determine the vernalization requirement: *Vrn-H1* on chromosome 5H, *Vrn-H2* on 4H and *Vrn-H3* on 1H. Alleles at these loci interact epistatically, such that a vernalization requirement occurs in plants homozygous recessive at *Vrn-H1* and *Vrn-H3*, and with at least one dominant allele at *Vrn-H2*. The interrelationships of vernalization, photoperiod and low-temperature are most likely attributable to linkage rather than pleiotropy. Moreover, in wheat GALIBA *et al*. (1995) and SUTKA *et al*. (1999) demonstrated that the *Fr-A1* and *Vrn-A1* determinants on chromosome 5A, were distinct loci via linkage mapping as well as physical mapping, respectively.

During the physiological processes of cold acclimation, a number of stress-related genes are up-regulated [see CATTIVELLI *et al*. (2002) for a recent review]. In both wheat and rye, FOWLER *et al*. (1996) hypothesized that a gene, or genes, in the *Vrn-A1/Fr-A1* region were responsible for regulating the *Wcs120* gene-family. VAGUJFALVI *et al.* (2000) hypothesized that two loci (provisionally designated as *Rcg1* and *Rcg2*) on chromosome 5A, regulate the expression of the *cor14b* gene. The first locus, tightly linked to the marker psr911, is more proximal on the chromosome, while the second one is slightly distal to the marker psr2021(ABA2), belonging to the region of *Vrn-A1/Fr-A1*.

A notable advance in plant cold-tolerance research was the discovery of the *CBF* (C-repeat Binding Factor) family of genes. In Arabidopsis, these transcription factors have been shown to be key determinants of both low-temperature and drought-stress tolerance (SHINOZAKI *et al*. 2003). Genes with CBF signature sequences have been reported in the *Triticeae* and have been characterized in terms of their map location, coding sequence, and expression in barley (CHOI *et al*. 2002; Von ZITZEWITZ *et al.* 2003). The mapping of candidate genes, such as transcription factors, can reveal their genetic relationships with previously detected QTLs; our principal interest is thus to determine the role of the *CBFs* in the cold and drought tolerance of barley and the *Triticeae*, and accordingly a first step is to relate their map locations with QTLs for winter hardiness traits.

Material and Methods

Plant Material and Map Construction

One hundred and thirty six doubled-haploid (DH) lines were derived by anther culture from the F1 of the cross of 'Nure' × 'Tremois' at Saaten-Union Resistenzlabor GmbH, Germany. The winter parent—'Nure' [(Fior 40 × Alpha2) × Baraka]—is a modern, frost tolerant, high yielding two-rowed feed-barley cultivar released by the Istituto Sperimentale per la Cerealicoltura Section of Fiorenzuola, Italy. It belongs to the RLT (Recommended-List Trials) of Italy and it is adapted to South European environments, showing high yield stability in irrigated as well as in moderately drought-stressed (400mm rainfall) conditions (Fig. 1);. The spring parent—'Tremois' [(Dram × Aramir) × Berar]—is a modern, high yielding French two-rowed malting variety, adapted to fertile environments, frost and drought sensitive and also included in the RLT. Genomic DNA extraction and linkage map building were performed as described in FRANCIA *et al*. (2004) with morphological, biochemical and molecular markers. The 'Nure' x 'Tremois' molecular map is principally based on AFLP and SSR markers. AFLP primer combinations were chosen in order to facilitate map alignment, based on their previous use in the construction of barley linkage maps (BECKER *et al*. 1995;

QI *et al*. 1998). SSR primers were kindly provided by the Scottish Crop Research Institute, Scotland, and their map position has been previously reported by RAMSAY *et al*. (2000). Four abiotic stress-related genes were mapped as RFLPs (*cor18*, *aba2*, *af93* and *acx1*) and four using PCR [*aba3*(psr2020), *dhn1*, *dhn3* and *dhn6*]. Two CBF loci—*HvCBF4* and *HvCBF8*—were mapped using gene-specific primers (FRANCIA *et al*. 2004). The linkage map was constructed using MAPMAKER/EXP 3.0 (LANDER & BOTSTEIN 1989).

Figure 1. Phenotypes of parental cultivars Nure and Tremois after drought stress and recovery.

Phenotyping

Three measures of low-temperature tolerance were used: winter survival of the population was rated under field conditions at Fiorenzuola d'Arda (Italy) during the 2001/2002 growing season; freezing tolerance data were recorded in a controlled environment (phytotron) at the Agricultural Institute of MTA (Martonvásár - Hungary); and, to evaluate the effect of freezing on the functionality of the Photosystem II (PSII) reaction centers, the maximum quantum yield of the PSII photochemistry was measured by the ratio of variable (Fv) to maximal (Fm) fluorescence in a dark-adapted state, Fv/Fm (FRANCIA *et al*. 2004). As a measure of the vernalization requirement trait the arithmetical difference in days to heading between a late spring and a fall-planting was used, and this value was termed "heading date delay" (FRANCIA *et al*. 2004).

COR Protein Accumulation

The accumulation of two COR proteins—COR14b and TMC-Ap3—was assayed in field-grown plant material grown at Fiorenzuola d'Arda, Italy. Harvest and Western blotting analysis were performed as described by FRANCIA *et al*. (2004) using COR14b and TMC-Ap3 polyclonal antibodies with the ECL Western kit (Amersham).

Statistical and QTL Analyses

For QTL analysis, the winter survival in the field, frost tolerance in the controlled freeze test, frost tolerance measured using the Fv/Fm value and COR protein accumulation data for each DH line, were converted to the corresponding percentage of the 'Nure' parent for QTL mapping. The primary tool for QTL analysis was PLABQTL (UTZ & MELCHINGER 1996) using a LOD threshold of 3.0 and a 2.0 cM scan interval. After a preliminary analysis using Simple Interval Mapping (SIM), the markers with the highest LOD value were used as cofactors for Composite Interval Mapping (CIM). As a validation step, the chromosome 5H QTL data were also analyzed using MapQTL version 4.0 (Van OOIJEN *et al*. 2002) using a LOD threshold of 3.0. After a preliminary analysis using SIM, the most statistically significant markers were selected using the backward elimination procedure as cofactors for CIM.

Results and Discussion

One hundred and twenty eight loci were placed on the NT linkage map spanning 1,182 Kosambi cM with an average density of one non-co-segregating marker every 10 cM, ranging from 16.1 cM for chromosome 4H to 7.4 cM for chromosome 5H (FRANCIA *et al*. 2004). In general, locus orders and distances are in agreement with previous reports. In order to construct a functional map for the study of winter hardiness-related traits, 19 barley *COR* and abiotic stress-induced genes were placed. Based on the presence of markers in common to the NT map and published maps, we were able to infer the positions of genes related to abiotic stresses in other populations.

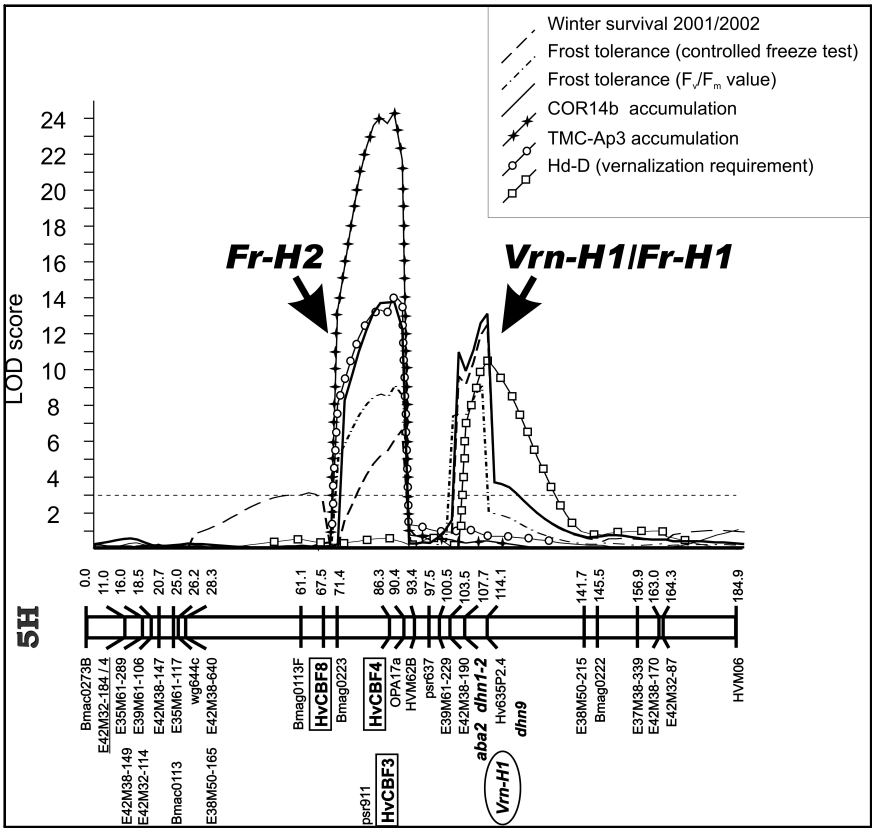

Figure 2. QTL LOD plots of chromosome 5H obtained with CIM analysis of the indicated traits. The 3.0 LOD score threshold is indicated by a hatched line. Chromosome 5H is orientated with short arm at the left; distances are in Kosambi cM; abiotic stress-related genes are in bold italic while *CBF* transcription factor genes are in bold type highlighted by boxes. An ellipse indicate the inferred position of the major gene affecting vernalization.

The linkage map of the NT mapping population underscores the advantages of having openly available, abundant, and informative markers for linkage map construction. Marker order and distance are conserved relative to published maps, facilitating comparative analysis. We have established the linkage map positions of two *CBF* loci: *HvCBF4* and *HvCBF8*. 'Nure' and 'Tremois' are of Italian and French origin, respectively, represent different end uses ('feed' vs 'malting'), display different growth habit ('winter' vs 'spring') and have no known ancestors in common. Chromosome 5H is well-resolved in this population, with 29/128 markers mapped to this chromosome. Of particular interest is the mapping of the *CBF* transcription factor genes to this chromosome. In Arabidopsis, the role of these genes in various abiotic-stress regulons has been an area of intensive research (GILMOUR *et al.* 1998; SHINOZAKY *et al.* 2003): their role in the *Triticeae* is an area of active investigation (VAGUJFALVI *et al.* 2003). We have also directly mapped the positions of eight stress-related genes, and inferred the map positions of 11 others. It is of interest that, out of 19 mapped and/or inferred, the only genes known to be cold responsive, or to be hypothesized to be related to low-temperature

tolerance, map to the same region as the low-temperature tolerance QTLs in this population, i.e. *dhn1*, *dhn2* and *aba2*. These loci form a cluster on 5H that has been consistently implicated in low-temperature tolerance genes and/or QTLs in multiple reports (CATTIVELLI *et al*. 2002). QTLs for three measures of low-temperature tolerance all map to the long arm of chromosome 5H and are coincident (Fig. 2). We report for the first time in the 'Nure' x 'Tremois' population the segregation of two separated low-temperature tolerance loci QTLs on a group-5 chromosome of the *Triticeae*. Moreover, we have preliminary data supporting a functional role for *CBF* genes in determining low-temperature tolerance in barley: *HvCBF4* is a potential positional and functional candidate gene for the QTL. In Arabidposis, the small family of *CBF* transcriptional activators recognize the cold and drought responsive DNA regulatory element designated by CRT (C-repeat)/DRE (dehydration responsive element), and are also associated with a low-temperature tolerance QTL (SALINAS *et al.* 2002). We hypothesize that the *CBF* orthologs in barley have a similar functional role, and preliminary evidence in support of this hypothesis is provided by the coincident QTLs for low-temperature tolerance and COR protein accumulation in conjunction with *HvCBF4*. The accumulation of COR14b and TMC-Ap3 proteins has been implicated in low-temperature tolerance (BALDI *et al*. 1999; VAGUJFALVI *et al*. 2000). The genes encoding these proteins are on chromosomes 2H and 1H (FRANCIA *et al*. 2004). The *cor14b* promoter contains a CRT/DRE recognition site located in a 28-bp fragment (GTCACCCAAAGGTACGTGAGGTCGGCAA) that conferred low-temperature responsiveness (DAL BOSCO *et al.* 2003). This fragment contains a basic Leu zipper protein binding motif, an ABRE (opposite orientation) and a C-repeat/DRE (opposite orientation, underlined), with which a CBF protein could interact. No other C-repeat/DRE motifs are present in the sequenced portion of the promoter. Only the coding region sequence is available for *tmc-ap3*, so additional work is needed before we can hypothesize a regulatory role of *CBF* loci for this *COR* gene.

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The Changes of Aluminum and Mineral Nutrient Contents in Response to Al and Cd Toxicity and Al-Induced Organic Acid Exudation in Two Barley Varieties Differing in Al-Tolerance

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Abstract

A hydroponic experiment was carried out to study varietal difference in effect of Al and Cd on Al and mineral nutrient contents in various plant parts and Al-induced organic acid exudation by using two barley varieties differing in Al tolerance. The results showed that Al sensitive *cv*. Shang70-119 had significantly higher Al content and accumulation in various plant parts than Al tolerant *cv.* Gebeina, especially in root, when subjected to low pH (4.0) and Al treatments (100μM Al and 100μM Al+1.0μM Cd). Without Al addition, 1.0μM Cd treatment caused a slight decrease in Al contents of both cultivars, however at the presence of Al in the solution, Cd addition further increased Al contents in plant parts compared with 100μM Al treatment alone. Both low pH and two Al treatments caused significant reductions in Ca and Mg contents in all plant parts, P and K contents in shoot and leaf, Fe, Zn and Mo contents in leaf, Zn and B contents in shoot, Mn contents both in root and leaf. Moreover, the greater change in nutrient content was detected in the plants exposed to Al and Cd combination treatment than in those exposed to Al treatment alone. A dramatic enhancement of malate, citrate and succinate was found in the plants exposed to 100μM treatment over the whole duration of experiment relative to the control, and Al-tolerant cultivar had a considerable higher exudation of these organic acids than Al-sensitive one, indicating that more Al-induced enhancement of these organic acids is very likely to be associated with Al-tolerance. The exudation of oxalate in response to Al exposure was not consistent during the treatment and showed less difference between the two cultivars, suggesting that oxalate is not likely to act a mechanism of Al tolerance in barley.

Keywords: aluminum; barley *(Hordeum vulgare* L.*)*; cadmium; nutrient content; organic acid

1. Introduction

Aluminum toxicity is a major factor constraining crop performance on acid soils that predominate under tropical climate. The primary toxicity symptom observed in plants is inhibition of root growth (DELHAIZE & RYAN 1995; KOCHIAN 1995), followed by less nutrient and water absorption, resulting in poor growth and production.

Aluminum interferes with uptake, transport and utilization of essential nutrients including Ca, Mg, K, P, Cu, Fe, Mn, and Zn (FOY 1984; FOY 1996; SIMON *et al.* 1994; KIDD *et al.* 2000, GUO *et al.* 2003). The mechanism by which Al inhibits the uptake and utilization of the mineral nutrients is not completely clear to date. It is assumed that Al3+ ions may bind to the phospholipid heads of the plasma membrane, alter the lipid-protein interaction, and modify the activity of the transporters (SUHAYDA & HAUG 1986). Alternatively, Al3+ may reduce the negative charge associated with the plasma membrane phospholipids and proteins by binding to these charged groups or shielding the surface potential (KINRAIDE *et al.* 1992). Another possibility is that Al3+ binds directly to the transport proteins, thereby impairing their function (SCHROEDER 1988).

In fact, soil acidification could bring about many other changes of physical and chemical properties in soil, which in turn affect plant growth and development. On acid soil, some nutrients, such as P, Ca and Mg are usually deficiency, while others, such as Mn and B could attain the very high available concentration toxic to plants. Similarly, the bio-availability of some heavy metals in soil is also strongly affected by pH. It has been demonstrated that Cd availability increases with reduced soil pH (WU & ZHANG 2002). Thus in acid soil, Cd may also produce extra toxicity to plants. However little has been known on combinational effect of Cd and Al on plants, although their individual toxicity is basically clear.

Some plant species and genotypes within a species have developed strategies to avoid or tolerate to aluminum toxicity. Mechanisms of Al-resistance are commonly classified into two categories (PELLET *et al.* 1995): avoidance or exclusion of Al from the roots, and internal or protoplastic tolerance. Many of the current researches point out the central role of certain organic acid in detoxification of Al both externally and internally (MA 2000). Root exudation of organic acid under Al stress has been reported in several Al-tolerant plant species, including wheat (BASU *et al.* 1994; RYAN *et al.* 1995; PELLET *et al.* 1996), maize (PELLET *et al.* 1995; JORGE and ARRUDA 1997), snapbean (MIYASAKA *et al.* 1991), buckwheat (ZHENG *et al.* 1998a,b). Aluminum chelation by organic exudates reduces the activity of free Al ions and, consequently, their binding to the cell wall and /or plasma membrane (KIDD *et al.* 2001). The kinds of organic acids secreted by Al-exposed roots vary depending on the plant species. Malate exudation by wheat is the mechanism that has been investigated most thoroughly (KOCHIAN 1995), while citrate seems to be the most common organic acid anion secreted by Al-tolerant species or varieties (BARCELÒ & POSCHENRIEDER 2002). The exudation of oxalate and succinate in Al-exposed roots has been also investigated, but their the role in detoxification of Al still remains controversy (JORGE and ARRUDA 1997; ZHENG *et al.* 1998a, b; KIDD *et al.* 2001). So far there have been few reports on organic acid exudation in response to Al in barely varieties differing in Al tolerance, while the crop is much sensitive to Al toxicity compared with other cereals, in particular wheat and rye.

The objectives of this study were to investigate (1) cultivar difference in the effect of low pH and Al toxicity on Al and nutrient uptake and accumulation; (2) the effect of toxic Cd concentration on the response of barley to Al toxicity; and (3) the difference in Al-induced enhancement of some organic acid exudation in the barley cultivars differing in Al tolerance.

2. Material and Methods

2.1 Plant Materials and Treatments

The experiment was carried out in 2003 at Huajiachi campus, Zhejiang University, Hangzhou, China. Two barley (*Hordeum vulgare* L.) genotypes differing in acid soil tolerance were used according to a preliminary experiment (GUO *et al.* 2003), Gebeina being relatively tolerant and Shang 70-119 being relatively sensitive. The seeds were surface sterilized in 0.2% $Na(OCl)_2$ for 20 min, rinsed with distilled water 8 times and germinated in moist quartz sand in a greenhouse. When seedlings grew the second leaf (10-day old), they were selected for uniformity and transplanted onto a 4-L container, which was covered with a foamed plastic plate with evenly spaced holes and placed in a greenhouse. The composition of the basic nutrient solution was (mg L^{-1}): $(NH_4)_2SO_4$ 48.2, $MgSO_4$ 65.9, K_2SO_4 15.9, KNO_3 18.5, $Ca(NO_3)_2$ 59.9, KH_2PO_4 24.8, Fe-citrate 5, $MnCl_24H_2O$ 0.9, $ZnSO_4$ $7H_2O$ 0.11, $CuSO_4$ $5H_2O$ 0.04, HBO_3 2.9, H_2MoO_4 0.01. One week after transplanting to the basic solution culture, Al as $AlCl_3.6H_2O$ and Cd as $CdCl_2$ were added to corresponding containers to form 5 treatments, i.e. control (pH6.5), low pH (4.0), 1.0μM Cd (pH6.5), 100μM Al (pH4.0) and 1.0μM Cd+100μM Al (pH4.0), and the solution pH was adjusted with HCl. The solution pH in each container was adjusted every other day with HCl or NaOH as required. The experiment was laid out a completely randomized design with six replicates. The nutrient solution in the growth container was continuously aerated with pumps and renewed every 5 days.

2.2 Measurements and Statistical Analysis

After 7 weeks treatment, plants were separated into roots, shoots and leaves, and roots immersed in distilled water for 2 hours, and then dried at 80 °C for 48h. Mineral elements were determined by ICP-AES (Inductively coupled plasma atomic emission spectroscope) after digesting the samples with HNO_3-$HClO_4$ (2:1,v/v).

At the 12, 17, 22, 27d after beginning of Al treatment, seedlings were gently taken off from the hydroponic system, washed with tap water, distilled water and deioned water 3-5 times in the order respectively, then transferred into 3L pots containing 0.5 mM $CaCl_2$ solution with pH 4.5 for the collection of root exudates. Approximately 1 g thymol was added into the collecting solution to prevent possible growth of microorganisms. The collection was allowed to last for 4 h and then seedlings were removed. The collecting solutions were dried in a rotary evaporator on a hot plate at 65 °C. The residue was filtered and diluted to 25 ml with deionized water for measurement of organic acids by ion chromatography. The exudates were filtered by a 0.45μm filter, and separated with a C18 analytical column (300×3.9 μm). An anion trap column (ATC-1) was installed between the gradient pump and injection valve to remove anionic contaminants from the eluents. The eluents consisted of high purity water, methanol, 1 m*M* NaOH and 100 m*M* NaOH, which were run in a gradient. The system was interfaced with a computer, and samples were injected automatically with a Rheodyne injector equipped with a 50-ml loop. Anionic components in the root exudates were detected using an electrical conductivity cell. Many organic acid peaks were identifiable in chromatograms, but only malate, citrate, succinate and oxalate changed substantially with Al exposure.

All data presented are the mean values. The measurement was done with three replicates. Statistical analysis was carried out by two-way (treatment and cultivar) ANOVA using Student's t-test to test the different significance.

3. Results and Analysis

3.1 Aluminum Content and Accumulation in Plants

There were the significant differences in Al content and accumulation in all plant parts among treatments and between barley cultivars (Table 1). Compared with the control (pH 6.5), lower pH (4.0) resulted in a significant increase in root Al content, but little change in above ground parts for both genotypes, except in leaves of Shang 70-119, which showed the significant difference between two pH treatments. Addition of 100μM Al into the solution dramatically increased Al contents in all plant parts. The effect of Cd treatment on Al content varied in the presence or absence of Al in the solution as well as in plant parts. Thus, without Al, Cd treatment tended to reduce Al content in plant parts, in particular in roots (1.0μM Cd vs pH 4.0), whereas in the presence of Al, Cd treatment further increased root Al content (1.0μM Cd+100μM Al vs 100μM Al), while it caused significant reduction in shoots and less change in leaves. The remarkable difference could be found between the two cultivars in the response of Al content to the treatments, with the relatively tolerant cultivar Gebeina having much lower Al content in root and shoot than the relatively sensitive cultivar Shang70-119 when subjected to Al treatments, i.e. 100μM Al and 1.0μM Cd+100μM Al. On the other hand, there was no obviously detectable difference between cultivars in leaf Al content.

Al accumulation in plant tissue is a function of Al content and biomass (dry matter weight). It may be seen from Table 1 that the effect of the treatments on Al accumulation differed significantly between genotypes and also varied in plant part. Compared with the control, 1.0μM Cd treatment reduced significantly Al accumulation in roots, but had less effect on that in aboveground parts, which was basically consistency with the changes in Al content. On the other hand, low pH treatment resulted in a significant increase in Al accumulation of roots for Gebiena and a reduction in shoots for Shanghai 70-119, mainly due to higher root Al content and more severe inhibition in shoot biomass, respectively. Addition of Al in the solution enhanced dramatically Al accumulation in roots but not in aboveground parts, which may be attributed to severe inhibition of Al toxicity on shoot and leaf growth.

3.2 Effect of the Treatments on Nutrient Content

The effect of both pH and ionic stresses on mineral nutrient content in plant parts of two barley cultivars was shown in Table2, 3 and 4. Compared with the control, except for 1.0μM, other three stress treatments caused significant reductions in Ca and Mg contents in all plant parts, P and K contents in shoot and leaf, Fe, Zn and Mo contents in leaf, Zn and B contents in shoot, Mn content both in root and leaf, and significant increases in Fe content in root and Mo content in shoot. Moreover, the effect of Al and Cd combinational treatment on nutrient contents was more pronounced than that of Al treatment alone. However, 1.0 μM Cd treatment alone caused the significant increase in Mn content and reductions in Cu and Fe contents in root, and significant increases in Ca and Mg contents both in shoot and leaf, and Cu and Fe contents in leaf, compared with the control.

There were the substantial differences between the two cultivars in the mineral nutrient contents in the response to these stress treatments. Under normal nutrient solution (pH 6.5), Al-sensitive cultivar Shang70-119 had significantly higher Ca, Mg and Cu contents in root, Ca, Mg, Fe, Mn and Zn contents in shoot, and Fe, Mn and Zn contents in leaf than Al-tolerant cultivar Gebeina. Although the two cultivars had the similar changed pattern in nutrient content in the response to the stress

treatments, especially for the two Al treatments, the changed extent was mostly much less in Al-tolerant cultivar than in Al-sensitive one.

3.3 Effect of Al Treatments on Organic Acids Exudation

As shown in Fig. 1, there was little difference between the two cultivars in the efflux rate of all detected organic acids when they grew under the normal nutrient solution. A markedly high efflux rate of oxalate was noted at the 12 d after the beginning of Al exposure, but thereafter there was little difference between the control and Al treatment. Moreover there was no substantial difference between the two cultivars in oxalate exudation, suggesting that oxalate is not likely to contribute to Al tolerance. By contrast, Al-induced enhancement in the exudation of other three organic acids was consistent over all sampling dates and differed greatly between the two cultivars, with Al-tolerant cultivar having substantially higher efflux rate than Al sensitive one.

4. Discussion

It was demonstrated that the seedling growth was dramatically inhibited with low pH and both Al treatments (alone and combination with Cd), while 1.0μM Cd showed a slight stimulation of growth. The inhibition was more severe in 1.0μM Cd+100μM Al than in 100μM Al, indicating that the effect of Cd and Al is synergistic. Al-sensitive cultivar Shang 70-119 was more inhibited than Al-tolerant cultivar Gebeina (GUO *et al.* 2004). In the current research, we found low pH and the two Al treatments caused a significant increase in Al content of all plant parts, but the treatment of 1.0 μM Cd had no the effect of Al uptake by roots. Meanwhile, Al content in plants under 1.0μM Cd+100μM Al treatment was much higher than that of 100μM Al treatment alone, showing the stimulation of Al uptake by Cd. The difference between the two cultivars in Al content was distinct, with Gebeina being much lower than Shang 70-119. It could be seen that the inhibition extent of growth caused by Al toxicity is completely consistent with its influence on Al content in plants. Moreover, Al content in root is considerably higher than that in shoot and leaf, which supports the established fact that Al sensitive plants accumulate more Al in the roots than the tolerant ones and aluminum toxicity in plants is frequently associated with increased Al concentrations in roots but not generally in shoots. KIDD *et al*. (2000) reported that the Al concentration of Al-sensitive *betula pendula rothin* race was higher in roots but lower in shoots than that of other races. In this study, root Al concentration and accumulation of Al-sensitive cv. Shang70-119 was much higher than Al-tolerant cv. Gebeina. Therefore it may be concluded that the difference in the tolerance to Al toxicity among barley genotypes is associated with uptake and accumulation of Al by roots, and therefore it is very imperative to make comprehensive clear of the mechanisms leading to the genotypic difference of Al uptake and accumulation in roots.

There have been many reports on the effect of Al toxicity on mineral nutrient uptake and composition in plants. The current study showed that Al exposure inhibited Ca and Mg absorption by roots and restrained K, Fe and Zn from being transferred into shoot and leaf. Without Al addition, the treatment of 1.0μM Cd had no substantial effect on nutrient content in plants compared with the control. However, Cd addition at the presence of Al in the solution caused the greater change in nutrient content relative to Al treatment alone. High concentration of plant-available Mn and Cu has been considered a problem for plant growth in some strongly acid soils (SCOTT *et al*. 1998; FOY

1984). The present results showed that shoot and root Mn contents and root Cu content in Al-sensitive cultivar were significantly higher than those in tolerant one, suggesting the potential association of higher Mn and Cu contents in plants with more Al sensitivity. Moreover, Al and Cd combination treatment enhanced Mn accumulation in roots, which may partially account for the more detrimental effect of Al and Cd combination on the plant growth. Al treatment altered the mineral composition in barley plants, and the changed extent of mineral content differed significantly depending on genotypes. Al-tolerant genotype showed relatively less changes than Al-sensitive one. Hence the disorders in mineral metabolism and ion homeostasis would be more frequently caused under Al stress for Al-sensitive genotypes than for the tolerant ones, while the enhanced disorders in turn result in much more inhibition of growth and biomass synthesis for Al-sensitive genotypes.

Aluminum-induced enhancement of organic acid exudation has been proposed as a means of Al detoxification in Al-resistant varieties of different plant species (MA 2000). Most available evidence comes from investigations on wheat, where malate exudation appears to be an important mechanism for Al detoxfication (DELHAIZE *et al*. 1993). However JORGE and ARRUDA (1997) reported less difference in malate exudation between maize varieties differing in Al resistance. Citrate has been also considered as an important candidate relevant to Al tolerance, with higher exudation in resistant varieties than in sensitive ones (PELLET *et al*. 1995; YANG *et al*. 2000). By contrast, KIDD *et al*. (2001) reported that Al-induced citrate exudation was only observed in Al-sensitive or intermediately sensitive maize varieties, not in Al-tolerant ones. The inconsistent results have been reported on oxalate and succinate exudation in response to Al exposure and their possible role in Al tolerance (JORGE & ARRUDA 1997; ZHENG *et al*. 1998b; KIDD *et al*. 2001). The available results suggests that organic acid exudation in response to Al stress is species-dependent. In our understanding this is the first report on Al-induced organic acid exudation of barley varieties differing in Al-tolerance. The present results tend to exclude the possibility of oxalate exudation as a mechanism in Al tolerance of barley, as Al exposure does not cause consistent and obvious increase in oxalate exudation relative to the control and there is little varietal difference. By contrast, Al exposure resulted in drastic enhancement of malate, citrate and succinate over the whole duration of the experiment, and Al-tolerant cv. Gebeina had consistently much higher exudation of all three organic acids than Al-sensitive cv. Shang 70-119. It may be assumed that increased exudation of these three organic acids may account for the Al resistance in Gebeina.

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Table 1. Al content and accumulation in two barley genotypes under different treatments

Cultivars	Treatments	Al content (μg/g)			Al accumulation (μg/plant)		
		root	Shoot	leaf	root	shoot	leaf
Shang 70-119	CK (pH6.5)	104.87	57.70	8.07	77.60	52.51	7.75
	1μm Cd	55.30	41.45	9.76	46.45	43.11	10.25
	pH4.0	233.53	50.79	14.82	77.07	27.43	9.49
	100μm Al	742.87	114.54	16.32	156.00	48.11	8.16
	100μm Al+1μm Cd	1409.03	82.84	15.82	267.72	40.59	5.85
Gebiena	CK (pH6.5)	79.49	63.08	10.16	60.41	61.82	9.86
	1μm Cd	48.47	56.95	9.74	39.75	58.66	9.93
	pH4.0	208.20	71.89	10.49	108.26	61.83	9.44
	100μm Al	551.10	76.75	15.50	209.42	52.19	11.62
	100μm Al+1μm Cd	782.33	38.77	15.25	250.35	44.81	8.69
LSD 0.05 between cultivars		162.62	23.92	2.52	31.54	14.40	2.79
LSD 0.05 between treatments		122.45	26.26	1.79	28.45	20.24	1.41
Interaction between treatment and cultivar		**#	*	*	ns	ns	*

*, P<0.05; * *, P<0.01; ns: no significant at 95% probability

Table 2. The effect of different treatments on nutrient content in root of two barley genotypes

Cultivars	Treatments	P	K	Ca	Mg	Cu	Fe	Mn	Zn	Mo	B
		mg/g				μg/g					
Shang 70-119	CK (pH6.5)	2.18	2.65	1.21	1.43	23.42	96.56	166.73	237.30	4.44	1.91
	1μM Cd	1.24	2.67	1.20	1.40	7.00	52.32	303.97	193.50	4.12	2.55
	pH4.0	3.13	3.43	0.71	0.53	22.09	225.03	56.38	214.80	11.53	2.05
	100μM Al	1.72	3.47	0.69	0.27	14.93	207.40	26.72	97.48	39.20	1.91
	100μMAl+1μMCd	3.83	3.76	0.59	0.24	15.34	422.48	20.58	79.70	48.69	2.53
Gebeina	CK (pH6.5)	2.21	2.59	1.11	0.76	17.72	100.05	179.73	237.57	5.52	2.27
	1μM Cd	1.66	2.53	1.10	1.07	6.30	57.95	235.32	203.93	4.76	2.38
	pH4.0	2.67	2.82	1.05	0.54	13.37	100.59	49.76	138.60	10.36	2.19
	100μM Al	1.58	3.53	0.86	0.30	10.65	125.16	35.10	79.66	23.22	2.08
	100μMAl+1μMCd	3.01	3.32	0.70	0.28	11.46	227.17	25.87	74.66	30.70	2.27
LSD 0.05 between cultivars		0.83	0.17	0.01	0.04	4.17	142.55	56.06	8.43	2.53	1.37
LSD 0.05 between treatments		1.33	0.26	0.05	0.06	3.32	51.87	48.92	17.19	7.65	1.05
Interaction between treatment and cultivar		ns#	ns	**	**	Ns	**	ns	ns	**	ns

*, P<0.05; * *, P<0.01; ns: no significant at 95% probability

Table 3. The effect of different treatments on nutrient content in shoot of two barley genotypes

Cultivars	Treatments	P	K	Ca	Mg	Cu	Fe	Mn	Zn	Mo	B
		mg/g				μg/g					
Shang 70-119	CK (pH6.5)	5.62	4.01	1.20	1.13	1.87	69.04	16.24	224.27	11.72	3.14
	1μM Cd	5.78	4.00	0.90	0.97	1.63	79.03	13.84	242.50	15.21	3.62
	pH4.0	5.75	3.92	0.60	0.58	2.75	83.71	29.89	190.33	16.40	1.86
	100μM Al	2.98	3.16	0.24	0.16	0.97	36.58	10.89	79.41	5.08	0.93
	100μMAl+1μMCd	2.35	2.85	0.19	0.13	0.49	26.15	8.40	65.29	2.38	0.56
Gebeina	CK (pH6.5)	5.46	4.00	1.04	0.96	1.87	49.40	8.55	158.23	10.89	3.09
	1μM Cd	5.54	3.72	0.98	0.89	1.88	51.25	8.57	168.60	11.07	3.36
	pH4.0	5.01	3.76	0.81	0.48	1.94	51.98	14.17	137.50	12.87	2.72
	100μM Al	3.57	3.53	0.43	0.24	1.35	29.29	6.97	73.14	6.11	1.72
	100μMAl+1μMCd	3.38	3.49	0.39	0.26	1.25	21.61	5.61	65.13	3.95	1.67
LSD 0.05 between cultivars		0.85	0.70	0.13	0.21	0.20	4.01	3.65	13.26	2.40	0.73
LSD 0.05 between treatments		0.41	0.49	0.10	0.10	0.14	13.67	0.84	14.08	1.47	0.84
Interaction between treatment and cultivar		*[#]	ns	**	*	ns	*	**	**	**	ns

*, P<0.05; * *, P<0.01; ns: no significant at 95% probability

Table 4. The effect of different treatments on nutrient content in leaf of two barley genotypes

Cultivars	Treatments	P	K	Ca	Mg	Cu	Fe	Mn	Zn	Mo	B
		mg/g				μg/g					
Shang 70-119	CK (pH6.5)	3.67	3.12	0.89	0.68	1.71	11.18	5.38	44.09	1.23	1.64
	1μM Cd	4.65	2.41	0.51	0.44	0.93	8.18	6.27	50.54	0.83	2.08
	pH4.0	4.12	2.89	0.30	0.20	1.46	5.22	5.55	26.15	0.22	1.24
	100μM Al	2.40	2.36	0.16	0.07	0.41	2.86	3.34	14.06	nd[†]	1.09
	100μMAl+1μMCd	2.00	2.10	0.11	0.05	0.26	1.93	1.96	11.15	nd	2.22
Gebeina	CK (pH6.5)	4.34	2.96	0.82	0.60	1.54	9.27	4.70	38.64	1.50	2.10
	1μM Cd	4.50	2.52	0.50	0.42	1.07	7.83	4.22	36.94	1.08	2.12
	pH4.0	4.23	2.75	0.34	0.24	1.58	6.84	4.97	17.05	0.62	1.37
	100μM Al	3.32	2.46	0.21	0.11	0.62	4.44	2.76	15.48	nd	1.52
	100μMAl+1μMCd	2.93	2.30	0.18	0.09	0.47	3.71	1.89	13.14	nd	2.44
LSD 0.05 between cultivars		0.78	0.35	0.07	0.06	0.15	1.03	0.34	2.15	0.27	0.46
LSD 0.05 between treatments		0.35	0.18	0.12	0.10	0.38	1.07	0.70	5.87	0.14	0.57
Interaction between treatment and cultivar		**[#]	ns	ns	ns	ns	**	ns	ns	*	ns

*, P<0.05; * *, P<0.01; ns: no significant at 95% probability. †, no detected

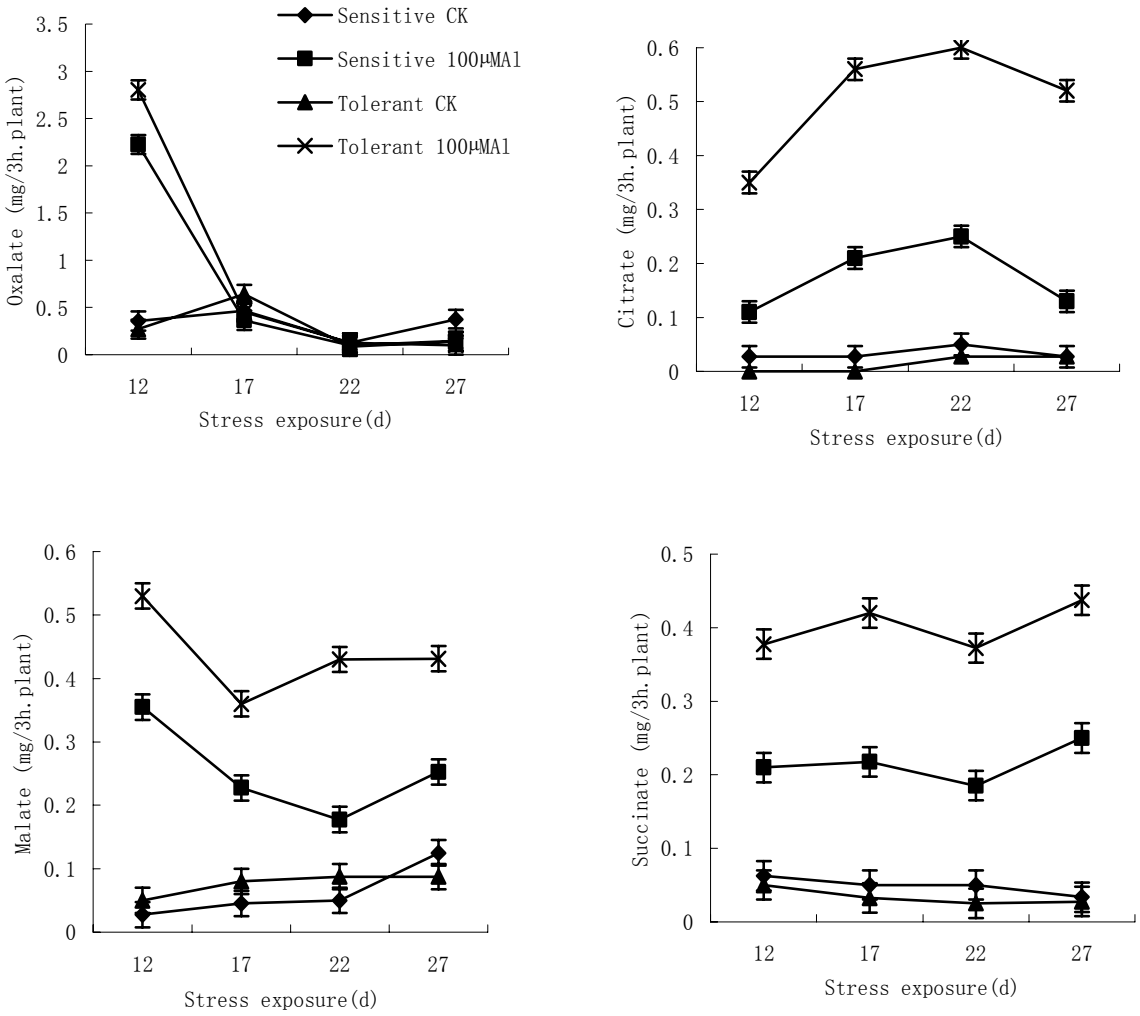

Figure 1. Organic acids exudation from roots of Al-tolerant and -sensitive barley plants exposed to 0 and 100μM Al solutions. The vertical bars represent the standard errors (n=3)

Black Point of Barley: A Possible Enzymatic Browning Reaction

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Abstract

The development of black point in barley has been investigated. Black point discolouration appears to be similar to enzymatic browning characteristic of plant tissues subjected to physical damage, which involves the oxidation of phenolic compounds present in plant tissues by polyphenol oxidase (PPO) or peroxidase (POX) and the transformation of the oxidation products to dark pigments. The presence of high levels of oxidative enzymes such as POX and PPO in barley grain is detrimental as they are implicated to promote haze, off-flavour and staling of the finished beer. In order to determine differences in enzymatic activity of clean and black pointed grains, and hence the suitability of black pointed barley grain for use in malting and brewing, POX and PPO was measured during grain development using enzymatic activity assays and isoelectric focusing.

Introduction

Black point of barley (*Hordeum vulgare* L.) is characterised by a brown-black discolouration of the husk tissue (palea and lemma) covering the embryo end of barley grain. In Australia, the occurrence of moist conditions during grain filling and ripening such as those found in Queensland, northern New South Wales and the warmer northern high rainfall region of Western Australia (WILLIAMSON 2000), appear to increase the intensity and frequency of black point in wheat (REES *et al.* 1984) and barley (SULMAN *et al.* 2001). However, severe black point still occurs in Victoria and South Australia once every three to five years. In this situation approximately 50,000 to 200,000 tonnes of malting barley can be downgraded to feed quality solely due to black point. However, the malting industry has no knowledge of whether black point affected grains behave differently to unaffected grains during the malting process. In Australia, black point in barley is deemed unacceptable by maltsters and brewers and results in downgrading of barley quality at receival if there is greater than ten grains per hundred with black point discolouration. This can result in losses of approximately AUS$50 per tonne to the grower when malting quality barley is downgraded to feed quality.

The cause of black point has been attributed to infection of the grain by several common fungi such as *Alternaria* spp*., Fusarium* spp. *and Helminthosporium* spp. (MALOY & SPECHT 1988; CROMEY & MULHOLLAND 1988). However, study of the infection process in wheat (WILLIAMSON 1997) found no direct link between the presence of such fungi and the development of black point symptoms. Studies into the cause of black point are now focusing on the biochemistry of the discolouration (HADAWAY *et al.* 2003; SULMAN *et al.* 2001). Black point appears to be similar to the enzymatic browning characteristic of plant tissues subjected to physical damage, which involves the oxidation of phenolic compounds by polyphenol oxidase (PPO) and peroxidase (POX) and the transformation of the oxidation products to melanin-like black or brown pigments (DICKO *et al.* 2002). The proposed model for black point formation as a type of enzymatic browning is shown in Figure 1. Oxidative enzymes such as PPO and POX, and phenolic compounds present in barley grain have been proposed to negatively affect the brewing process due to excess colour formation and the

production of stale flavour (CLARKSON & LARGE 1992; ANTROBUS & LARGE 1997; BILLAUD & NICOLAS 2001). Investigating the possible link between black point and enzymatic browning, and the effect of black point affected grain on malting quality is essential.

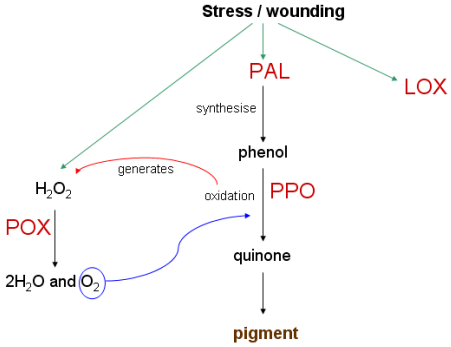

Figure 1. Proposed interaction of phenolic substrates and oxidative enzymes in the process of black point formation. The activities of phenylalanine ammonia lyase (PAL), lipoxygenase (LOX), peroxidase (POX) and polyphenol oxidase (PPO) may be increased in response to stress such as humidity. PAL is important for the formation of phenolic compounds. Phenols can be oxidised by PPO and POX to form highly reactive quinones which continue non-enzymatically to produce dark pigments. PPO is also thought to promote POX activity by generating hydrogen peroxide (H_2O_2) from the oxidation of phenolic compounds.

The current study was performed to determine whether oxidative enzymes participate in an enzymatic browning reaction responsible for the formation of black point in barley. We are also assessing if black point discolouration has a negative impact on grain and malting quality of barley.

Material and Methods

Growing Conditions

Barley varieties were chosen based on their black point score (% BP) from a trial grown in Millicent 2002 of the South Australian Research and Development Institute (SARDI) Stage 4 breeding lines and current commercial varieties. The varieties chosen are shown in Table 1 and cover a wide range of black point susceptibility. The allowable limit of black point discoloured grains in Australian malting specification barley is 10%. Plants were grown in a glasshouse at the Waite Campus of the University of Adelaide in Glen Osmond, South Australia. Both a humid and non-humid environment was used to compare differences in black point occurrence and biochemistry of black point formation. To create a humid environment conducive for the formation of black point discolouration, plants were grown in a glasshouse with overhead misting and surrounded by clear plastic sheeting. Plants were misted for 10 minutes every 6 hours from the maturity stages of anthesis until soft dough. The humidity was measured and this environment provided a relative humidity of 70 to 80%, compared to 40 to 50% relative humidity in the non-enclosed, non-misted environment.

Germination Tests

Grain from both environments was analysed for germinability using the 4mL test for germinative energy and the 8mL test for water sensitivity (Method 3.6.2; EBC, 2000). Two filter papers (Whatman No. 1, 90mm) were placed in the bottom of a plastic petri dish and 100 grains added. For the 8mL test all grains were placed ventral side down to avoid drowning the embryo. 4mL (or 8mL) of reverse osmosis (RO) water was added to the petri dish, and the plates were placed in a resealable bag and incubated at 20°C in the dark. At 24h,

48h and 72h, the number of grains chitted were recorded and removed from the petri dish. The cumulative count at 72h was taken as the germinative energy.

Table 1. Average phenotypic scoring of barley varieties grown at Millicent (2002) for black point susceptibility

Variety	% BP	Variety	% BP
Franklin	3.84	WI-3586	9.0
Alexis	4.28	Sloop	17.83
Torrens	5.12	Schooner	22.33
Dhow	5.84	Barque	23.33
WA2080	6.0	WI-3397	26.5
Gairdner	6.33	Sloop SA	28.5
Mundah	7.83	Keel	37.83
Arapiles	8.00	VB9953	47.67

Enzyme Extraction
Grain was harvested at maturity from both environments and a sample of grain was ground through a 0.5mm screen using a laboratory sample mill. Barley flour (0.2g) was mixed with 1mL 20% sucrose solution for 1 hour then centrifuged at 10,000rpm for 5 minutes and the resulting supernatant used as a source of crude enzyme. The crude enzyme extract was stored at –20^{o}C until used in activity assays and for isoelectric focusing.

Enzyme Activity
Alpha-amylase activity (as a determinant of weather damage) was measured using the Megazyme Amylazyme BG kit as per Megazyme protocol AMZ 7/98. Activity of soluble POX at room temperature (~23^{o}C) was determined by measuring the appearance of pink/brown colour resulting from guaiacol oxidation in the presence of hydrogen peroxide as per Dann and Deverall (2000). Guaiacol (50µL, 0.02M), 0.5mL 0.38M H_2O_2, 2mL 0.2M sodium phosphate buffer pH 5.8 and 50µL of grain extract were added to a 3mL disposable cuvette and the optical density measured at 470nm every 6s for 4 minutes. Results were calculated as ΔOD_{470}mg protein^{-1}min^{-1}. Protein content in crude extracts was calculated using the Bradford protein assay with bovine serum albumin (BSA) as standard (Bradford, 1976).

Isoelectric Focusing
Differences in POX isozyme activity from mature grain and developing grain was observed using isoelectric focusing (IEF) with: cathode (0.5M NaOH), anode (0.25M HEPES). 7.5µL aliquots of grain sample extract were applied on IEF polyacrylamide gels 0.4mm thick with a pH range of 3 to 10.5, and run at a limit of 2200V and 50mA and a constant power of 10 watts for 2 hours. POX isozyme banding patterns were produced by incubating gels in catechol solution (Liu *et al.*, 1990) (1g catechol, 2g Tris, 0.15g boric acid, 0.2g EDTA, and 1.5g calcium chloride dissolved in 100mLs nanopure water) for 10 minutes. Bands appeared immediately after transfer to 0.01M H_2O_2.

Results and Discussion

Germination Tests
Fast and even germination during malting is an essential quality parameter of malting barley. It is important for grain to be evenly modified during malting, and the occurrence of dormant or unviable grain negatively affects the malting process. There was no significant difference in germinative energy between varieties or between environments (humid or non-humid),

with most varieties achieving 95-100% germination in the 4mL test at 72 hours. However, in the 8mL test, varieties with greater susceptibility to black point (as shown in Table 1) and grown in the humid environment, had much reduced germination percentages (data not shown). For example, Schooner grown in the non-humid environment had 99% germination, compared to 73% germination from seed grown in the humid, misted environment. Varieties relatively tolerant to black point, such as Gairdner and Arapiles, had germinations of >95% from both environments. This result suggests that humidity may reduce the viability of grain susceptible to black point, by causing modification of the grain before harvest. A reduction in the level of phenolics, as would occur in the reaction for the formation of black point, may allow grain to germinate more readily. This is because phenols normally impose dormancy on the grain (WEIDNER *et al.* 2000). This fits our model that humidity imposes a stress on susceptible varieties and may lead to enzymatic browning discolouration of black point.

Alpha-Amylase Activity

Alpha-amylase activity was assayed to determine whether the poor germinability of the black point susceptible varieties in the 8mL test was due to secondary dormancy imposed by water, or due to weather damage modification of the grain. Preliminary results show that the black point susceptible varieties grown in the humid environment had increased levels of alpha-amylase indicating that the grain had begun the process of germination while still on the plant (data not shown). MRVA and MARES (2001) found the incidence of black point in wheat grains was also strongly associated with pre-harvest sprouting.

POX Activity in Developing Grain

There were significant differences in the POX isozymes of barley at different stages of grain development. Bands were more intense at advanced stages of grain development, indicating POX activity of the grain increases during growth (Figure 2). The activity of POX as measured using the guaiacol assay supported these results (data not shown). There was also a significant difference in electrophoretic mobility of POX isozymes present in the different barley varieties. Susceptible varieties Schooner and Sloop focus at more alkaline pH than the tolerant varieties Franklin and Alexis, suggesting a correlation between phenotyping of varieties for susceptibility to black point and POX banding patterns.

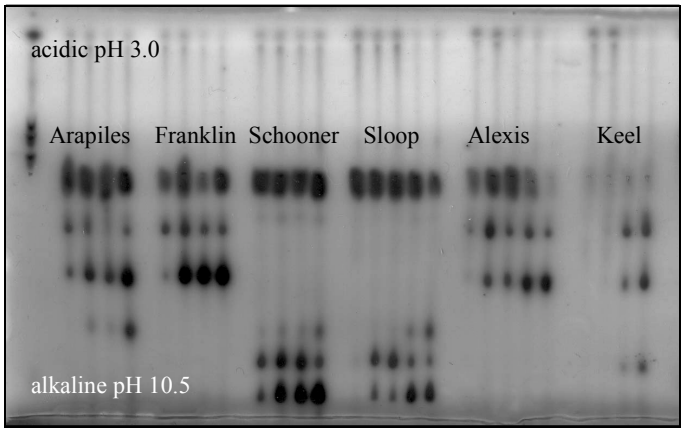

Figure 2. Isoelectric focused (pH 3-10.5) POX isoenzymes of barley varieties (Arapiles, Franklin, Schooner, Sloop, Alexis and Keel) at increasing Zadok's developmental stages (73, 75, 77, 83, and 85).

POX Activity in Mature Grain

There did not appear to be any trend in the levels of POX using the guaiacol assay in black point susceptible barley varieties compared to tolerant varieties (data not shown). There also

did not appear to be any difference in POX activity between environments. However, a significant difference in isozyme banding patterns between barley varieties of differing susceptibility to black point was observed (Figure 3), regardless of environment. There appeared to be four different sets of banding patterns; a set containing one POX isozyme focused at neutral pH (Figure 3a); a set containing two POX isozymes with one at neutral pH and the other at alkaline pH (Figure 3b); a set containing two POX isozymes focused at alkaline pH (Figure 3c), and a set containing two POX isozymes very close together focused at more alkaline pH (Figure 3d). Varieties more susceptible to black point produced banding patterns which focused at increasingly more alkaline pH. There was an exception however, with Keel, a variety highly susceptible to black point (~37.83% black point), with a POX isozyme focusing at neutral pH and the other at alkaline pH (Figure 3b; the banding pattern characteristic of a relatively tolerant variety). The barley variety VB9953 is highly susceptible to black point and this was illustrated by the focusing of two POX isozymes at very alkaline pH (Figure 3d). The difference in POX isozymes between varieties and its correlation with black point susceptibility indicates that POX may be important for the formation of black point in barley.

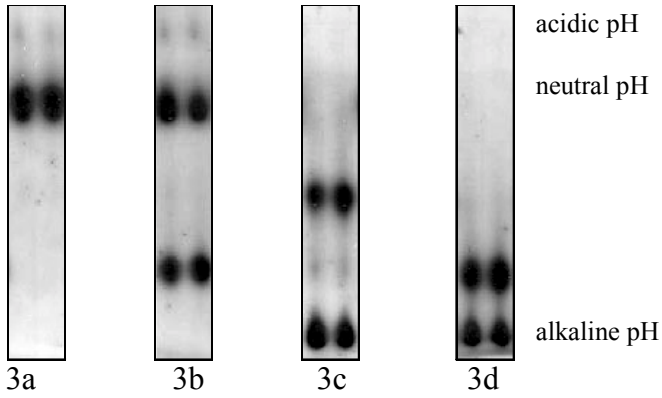

Figure 3. Isoelectric focused (pH 3-10.5) POX isozymes of increasingly black point susceptible barley varieties Figure 3a (Franklin, Gairdner, Torrens), Figure 3b (Namoi, Dhow, Mundah, Keel), Figure 3c (Sloop, Barque, Sloop SA, Schooner) and Figure 3d (VB9953).

Conclusions and Further Work

Differences in peroxidase isozymes and correlation with black point susceptibility suggests that black point is caused by a type of enzymatic browning reaction involving oxidative enzymes such as POX. The possible involvement of POX and other oxidative enzymes in the formation of black point discolouration may suggest that black pointed grain is unsuitable for use in malting as increased levels of oxidative enzymes are unfavourable in brewing. However, malting analysis is required to investigate the impact of using black point discoloured grain for malting and brewing.

The work on this project is continuing and results for the activity of the oxidative enzymes PPO, lipoxygenase (LOX) and the enzyme phenylalanine ammonia lyase (PAL) which is the precursor to phenolic synthesis, and their association with black point, will be available soon. Gene expression during black point formation is also being monitored. The use of these biochemical and molecular techniques in screening for susceptibility to black point may allow for breeding of black point tolerant barley varieties.

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Effects of Cultivar and Provenance on Vigour of Barley Seed

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Abstract

High vigour of barley seed is a precondition for rapid and homogenous field emergence and good malting quality. Seed quality has traditionally been associated with germination and purity. Another important seed quality component is seed vigour. Symptoms of vigour loss include reduction in germination rate and uniformity and consequently inferior seedling emergence and growth.

The study was aimed to determine potential differences in seed quality of spring and winter barley in relation to cultivars and growing conditions. Several tests were performed on each seed lot, i.e. the standard germination test, the low temperature and water stress germination test and germination in a salt solution. Some of the seed samples were also evaluated using the controlled deterioration (CD) test. The results were compared and seed lots with different values were identified. The effects of main factors as cultivar, provenance and year on seed vigour were estimated using analysis of variance. Statistically significant differences were particularly found among cultivars.

Keywords: barley; seed quality; germination; vigour

Introduction

Seed quality has traditionally been associated with germination and purity. Since the high germinating seeds can widely differ in their field emergence or in ability to germinate after storage or transport, seed vigour as another important seed quality component was established. Seed vigour is defined by ISTA (The International Seed Testing Association) as *"an index of the extent of the physiological deterioration and / or mechanical integrity of a high germinating seed lot which governs its ability to perform in a wide range of environments"*. Seed deterioration express in reduction in germination rate and uniformity, reduced tolerance to environmental stress and consequently inferior seedling emergence and growth. Therefore most of vigour tests are based on promoting of seed deterioration or germination under stress conditions (HAMPTON & TEKRONY 1995).

CD test was initially developed for evaluating quality of small seeds of vegetable species as brassicas, carrot and lettuce, but seems to be also suitable for barley. The CD test is based on exposing seeds to high temperature and high seed moisture for a short period of time.

The study was aimed to determine potential differences in seed quality of spring and winter barley in relation to cultivars and growing conditions. The previous research found out that in case of unsuitable climatic conditions during vegetation period seed vigour was affected more by cultivar than by location and vice versa (CHLOUPEK *et al.* 2003).

Material and Methods

The seed samples were obtained from the State Variety Registration Trials, harvested in 2001 and 2002. Four winter barley cultivars and two spring cultivars were chosen from six field trial stations throughout the Czech Republic (Tab. 1). No pesticides were used during growing and only moderate amount of fertilizers was applied. Post-harvest treatment included cleaning with 2.0 mm sieve for winter cultivars and 2.2 mm sieve for spring cultivars. Immediately after harvest the barley seed may exhibit dormancy preventing germination.

Therefore the seed samples were stored for several weeks until they have developed full germination capacity.

Each seed lot was evaluated by two methods for seed vigour estimation, i.e. the low temperature and water stress germination test developed by CHLOUPEK *et al.* (1997) and germination in a salt solution (0.8 % NaCl). According to the results of those tests, seed lots with different performance were selected for the CD test. The procedure is fully described in HAMPTON & TEKRONY (1995). Moisture of weighted samples was adjusted to 22 % and 24 % and the samples sealed in aluminium-coated polyethylene bags were heat-treated at 45 ^{o}C for 24 hours. After this treatment, standard germination test was set up using 100 seed per replication and the results were compared with germination before deterioration.

Results and Discussion

Table 2 and 3 present germination and vigour data on six cultivars of barley from 2001 and 2002 crop years. Although the mean germination was very similar in both years (98.4 % and 98.0 % resp.) significant differences among cultivars were found, particularly in 2002. The germination capacity generally considered acceptable for malting barley is 98 %, but especially some samples of malting cultivar *Nordus* were far below this level. Evaluation of vigour showed even bigger significant differences among cultivars as well as among locations. A genetic variation in seed vigour has been confirmed for several times, e.g. by heredity studies of winter barley and a screening programme for high vigour in spring barley (RIIS *et al.* 1993).

Seed lots with best germination capacity (99 %) and vigour (98 %) were used for the CD test. The samples behaved differently upon the treatment, the seed moisture of 22 % lowered germination approximately to 92.1 %; the moisture of 24 % to 82.4 %. Similar results have been achieved by using accelerated ageing test on barley seeds (HOSNEDL & HONSOVÁ 2002).

Acknowledgements

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Table 1. Main characteristics of 6 locations

Location	Altitude	Sep. 2000 – Aug. 2001		Sep. 2001 – Aug. 2002		30-year means	
	(m)	Sum of rainfall (mm)	Mean temp. (^{0}C)	Sum of rainfall (mm)	Mean temp. (^{0}C)	Sum of rainfall (mm)	Temperature (^{0}C)
Hradec n/Sv.	450	723	7.9	621	8.0	624	6.5
Chrastava	340	808	9.0	953	8.9	798	7.1
Jaroměřice	425	524	9.1	605	8.9	487	7.8
Staňkov	370	525	7.8	754	8.8	511	7.8
Vysoká	610	709	8.4	926	8.6	599	7.4
Žatec	285	472	9.7	637	10.0	451	8.3

Table 2. Analysis of variance for germination, vigour and germination in NaCl solution

2001

Source of variance	d.f.	MS Germination	MS Vigour	MS NaCl germ.
Cultivars	5	66.09 *	229.2 **	70.6
Locations	5	43.81	130.1 **	605.0 **
Interaction	25	55.64 **	85.8 **	94.4 **
Error	108	22.17	38.2	32.6

2002

Source of variance	d.f.	MS Germination	MS Vigour	MS NaCl germ.
Cultivars	5	124.52 **	113.7 *	331.7 **
Locations	5	137.96 **	212.8 **	286.2 **
Interaction	25	104.72 **	107.8 **	137.9 **
Error	108	18.99	46.6	56.6

* Significant at P=0.05; ** Significant at P=0.01.

Table 3. Mean values for germination, vigour and germination in NaCl solution, years 2001 and 2002

Cultivars 2001	Germination (%)		Vigour (%)		Germ. in NaCl sol. (%)	
Akcent	99.2	b	94.9	b	97.8	a
Luran	97.4	a	91.1	a	94.5	a
Luxor	98.5	ab	91.5	ab	94.9	a
Okal	98.9	ab	92.9	ab	94.4	a
Olbram	98.2	ab	95.5	b	95.5	a
Tiffany	98.0	ab	89.0	a	96.9	a
Mean	**98.4**		**92.5**		**95.7**	
Locations 2001						
Hradec	98.2	a	94.3	b	87.1	a
Chrastava	98.1	a	93.3	ab	97.3	bc
Jaroměřice	98.3	a	92.1	ab	98.2	c
Staňkov	98.9	a	94.5	b	97.5	bc
Vysoká	99.1	a	92.2	ab	97.7	bc
Žatec	97.7	a	88.6	a	96.3	b
Mean	**98.4**		**92.5**		**95.7**	
Cultivars 2002						
Luran	98.5	bc	90.5	a	90.4	b
Luxor	99.4	c	95.8	b	82.7	a
Nordus	96.3	a	92.6	ab	91.3	b
Okal	98.6	bc	93.9	ab	87.9	ab
Olbram	97.2	ab	92.4	ab	91.3	b
Tiffany	97.8	ab	94.9	ab	94.9	b
Mean	**98.0**		**93.3**		**89.8**	
Locations 2002						
Hradec	99.8	b	96.8	b	94.9	b
Chrastava	97.7	a	93.5	ab	91.0	ab
Jaroměřice	97.7	a	93.8	ab	89.2	a
Staňkov	97.9	a	90.8	a	89.0	a
Vysoká	96.1	a	90.8	a	91.0	ab
Žatec	98.6	a	94.4	ab	83.5	a
Mean	**98.0**		**93.3**		**89.8**	

Different letters denote statistically significant differences at the 95 % confidence level.

Allelic Variation of Dehydrin Genes in Barley

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Abstract
The aim of our study was to identify the presence and the variation of dehydrin genes in different barley genotypes and to assess the level of dehydrin genes expression induced by drought in fourteen genotypes of barley. . Plants were subjected to drought conditions and leaves were sampled every three to five days. DNA and RNA were extracted from plant subjected to water deficit and from plants used as control (normal watering). Genomic DNA and cDNA reverse transcribed (RT-PCR) was analyzed with specific dehydrin primers. RNA samples were used for northern hybridization with labeled probes of dehydrin genes. Variation in dehydrin gene alleles was detected in the different genotypes of barley at genomic and complementary DNA (cDNA) level. The result of northern hybridization demonstrated that the difference in dehydrin gene expression depended on the genes used for the hybridization.

Introduction

Barley (*Hordeum vulgare* L.) is an important crop grown from sub-arctic to semi-desert regions, showing a wide adaptability to a range of different eco-regions in many barley-growing areas. Where cold is not a problem, drought and heat are probably the most limiting factors to barley production. When barley plants are subjected to dehydration stress, the typical molecular response leads to the accumulation of a class of dehydration-related proteins called dehydrins (CLOSE 1997). Dehydrins are proteins that accumulate during water deficit conditions and are thought to play a role in drought resistance in plants. They are accumulated during the maturing phase of seed development, in seedlings or more mature plants. In barley, it has been estimated that the total number of dehydrin genes should be 12-14, and 12 dehydrin genes have been cloned so far (CHOI et al. 1999, CHOI & CLOSE 2000). They are dispersed over all the barley genome on almost all chromosomes.

Material and Methods

Plant Materials and Growth Conditions

Barley seeds from fourteen genotypes (1.Rihane-03, 2.Aths, 3.W12269/Line 251-11-2, 4.Leb71/CBB37//Leb71/CBB29, 5.*H. spontaneum* 41-1, 6.Arta, 7.Zambaka, 8.Tadmor, 9.Harmal, 10.Sara, 11.Gustoe, 12.M64-76/Bon//Jo/York/3/M5/Galt//As46/4/Hj3480/Astrix /5/NK1272, 13.Harmal-02//Esp/1808-4L, 14.Pitayo/Cam//Avt/RM1508/3/Pon/4/Mona/Ben// Cam,) were used in this study. Seeds were germinated in Petri dishes and seedlings were then transferred to pots in the plastic house. Three plants per pot and three pots of each genotype were used. Two pots of each genotype were subjected to the dehydration treatment (no watering of plants from 10 days after their transfer to pots) and the third pot was watered regularly up to the end of the experiment (control).

Leaf samples were collected after the initiation of water stress; one sampling for each of 5 weeks. Seven-day intervals separated each two collections for the first three samples, while only 3 days separated the last two samplings. Collected samples were frozen in liquid nitrogen and stored at –80°C.

Genomic DNA Isolation and Amplification

Genomic DNA of 14 barley genotypes was extracted from leaves using a modified method of (SAGHAI-MAROOF *et al.* 1984). Polymerase Chain Reaction (PCR) amplification were performed in 50 µl reaction containing 100-150 ng of genomic DNA according to (CHOI *et al*. 1999). Nine *Dhn* gene-specific primers (*Dhn1, Dhn3, Dhn4, Dhn5, Dhn6, Dhn7, Dhn9, Dhn10*, and *Dhn12*) were used for genomic DNA analysis. PCR annealing temperatures were optimized for each *Dhn* gene-specific primer set.

Detection of Polymorphisms

To develop genetic markers of each *Dhn* genotype, we compared 3 *Dhn* gene sequences (*Dhn1*, *Dhn3*, and *Dhn12*) from five barley genotypes (Sara, Zanbaka, Arta, *H. spontaneum* 41-1, and Tadmor). Both strands of DNA fragments were sequenced using ABI PRISM® BigDye™ Terminator Kit on ABI377 automatic sequencer. The nucleotide and deduced amino acid sequences were analyzed with the DNA Sequencing Analysis Software Version 3.2 and SEQUENCHER™ Version 3.2, and compared with sequences in DNA databases using the BLAST server. Amino acid sequence alignments of deduced dehydrin polypeptides were performed using SEQUENCHER™ Version 3.2 program.

Gene-Specific RT-PCR Analysis of Dhn Gene Expression

Total RNA was prepared using LiCl modified method of (LIEVENS AND GOORMACHTIG 1998). Quality of RNA was tested by electrophoresis on 1.8% agarose gel and staining with ethidium bromide. The first-strand cDNA was made from 0.2 µg of total RNA and the gene-specific 3'-end primer in a 20 µl reaction. The resulting single-strand cDNAs were amplified using *Taq* DNA Polymerase and using gene-specific 5'-end and 3'-end primers, (CHOI et al. 1999). PCR products were separated on 1.8% agarose gels and stained with ethidium bromide for photography.

RNA Analysis of Dhn Gene Expression

Total RNAs (10 µg per lane) were denatured in the presence of ethidium bromide (25ng/µl) and fractionated on 1.8% agarose containing formaldehyde (SAMBROOK *et al*. 1989). After electrophoresis, the gels were examined under UV light to ensure RNA integrity and equal sample loading, transferred to nylon membranes, and analyzed for *Dhn* gene expression according to standard DNA:RNA hybridization procedure. The ^{32}P-labeled DNA probes were synthesized from genomic DNA fragments of *H. spontaneum* 41-1 including *Dhn1*, *Dhn3* and *Dhn4*.

Results

Detection of Dhn Gene Polymorphisms

Genomic DNA derived from 14 barley genotypes were amplified with nine *Dhn* gene-specific oligonucleotides primers. The *Dhn1* and *Dhn4* primers produced 4 different amplification fragments in the 14 barley genotypes, Table 1 reflecting the presence of 4 different alleles for those genes in those genotypes. The *Dhn* primers 3, 5, 7, 9, and 12 produced 4 different amplification fragments while the *Dhn6*, and *Dhn10* primers amplified only one fragment each

reflecting the presence of one allele for those two genes in 14 barley genotypes (Figure 1).

Table (1): Polymorphism of fragments amplified by *Dhn*-gene primers

	Barley Genotypes														
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	
Gene	**DNA fragment polymorphisms of PCR products**														**Alleles**
Dhn1	**C**	**C**	**B**	**A**	**C**	**D**	**D**	**D**	**D**	**C**	**A**	**A**	**D**	**D**	**4**
Dhn3	**A**	**A**	**A**	**B**	**A**	**A**	**B**	**A**	**A**	**B**	**A**	**A**	**B**	**B**	**2**
Dhn4	**A**	**A**	**A**	**D**	**A**	**A**	**B**	**C**	**A**	**C**	**A**	**A**	**C**	**D**	**4**
Dhn5	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**B**	**A**	**A**	**A**	**A**	**A**	**A**	**2**
Dhn6	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**1**
Dhn7	**A**	**B**	**A**	**B**	**A**	**A**	**A**	**B**	**A**	**A**	**A**	**A**	**B**	**B**	**2**
Dhn9	**A**	**A**	**A**	**A**	**B**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**2**
Dhn10	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**1**
Dhn12	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**B**	**A**	**A**	**A**	**2**

A, B, C, and D indicate polymorphic DNA fragments for those genes in 14 genotypes
1, 2, 3, … and 14 indicate genotypes numbers (as in material and methods paragraph)

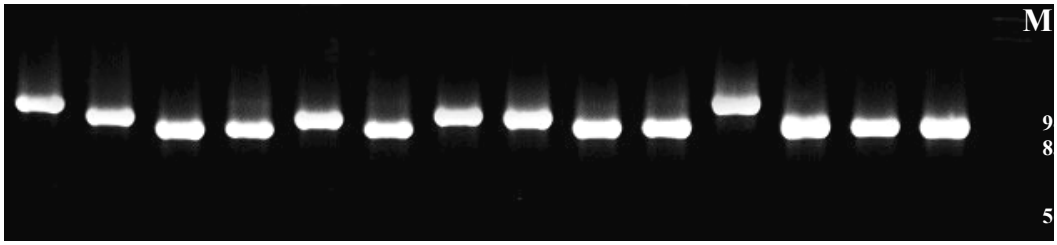

Fig (1): Polymorphism of fragments amplified by *Dhn4*-gene primers on 1.8% agarose gel.
M: molecular marker (base pair)

Sequence Analysis of Dhn Genes

Comparison of the DNA sequences of three *Dhn* genes (*Dhn*1, *Dhn*3, and *Dhn*12) for five barley genotypes identified 3 nucleotide differences for *Dhn1* gene in those genotypes and 11 nucleotide differences for *Dhn3* gene, while 9 differences were determined for the *Dhn1* gene in those genotypes (Fig. 2). These differences reflect in some cases differences in the amino acids sequences (Fig. 3).

```
(Dhn12) Morex      #281          AGCTGTTTATT TTCCGTTACG TGCTTGCAGT CTGCTGAGGA
(Dhn12) Sara       #281          AGCTGCTTAA TTCCGTTACG TGCTTGCAGT CTTCTGAGGA
(Dhn12) Zanbaka    #281          AGCTGCTTAT TTCCGTTACG TGCTTGCAGT CTGCTGAGGA
(Dhn12) Arta(6)    #281          AGCTGCTTAA TTCCGTTACG TGCTTGCAGT CTTCTGAGGA
(Dhn12) H.spont.   #281          AGCTGCTTAA TTCCGTTACG TGCTTGCAGT CTTCTGAGGA
(Dhn12) Tadmor     #281          AGCTGCTTAT TTCCGTTACG TGCTTGCAGT CTTCTGAGGA
                                 ........................................
                   #281          AGCTGCTTAA TTCCGTTACG TGCTTGCAGT CTTCTGAGGA
                                       *   *                          *

(Dhn12) Morex      #321          TGACGGCATG GGCGGGAGGA GGGAGAAGGG CGTGAAGGAG
(Dhn12) Sara       #321          TGACGGCATG GGCGGGAGGA GGAAGAAAGG CGTGAAGGAG
(Dhn12) Zanbaka    #321          TGACGGCATG GGCGGGAGGA GGGAGAAGGG CGTGAAGGAG
(Dhn12) Arta       #321          TGACGGCATG GGCGGGAGGA GGAAGAAAGG CGTGAAGGAG
(Dhn12) H.spont.   #321          TGACGGCATG GGCGGGAGGA GGAAGAAAGG CGTGAAGGAG
(Dhn12) Tadmor     #321          TGACGGCATG GGCGGGAGGA GGGAGAAGGG CGTGAAGGAG
                                 ........................................
                   #321          TGACGGCATG GGCGGGAGGA GGAAGAAAGG CGTGAAGGAG
                                                        *    *
```

```
(Dhn12) Morex      #361          AAGGTCAAGG AGAAGCTCCC CGGTGGGCAG CACATGGCCG
(Dhn12) Sara       #361          AAGGTCAAGG AGAAGCTCCC CGGTGGGCAG CACATGGCCG
(Dhn12) Zanbaka    #361          AAGGTCAAGG AGAAGCTCCC CGGTGGGCAG CACATGGCCG
(Dhn12) Arta       #361          AAGGTCAAGG AGAAGCTCCC CGGTGGGCAG CACATGGCCG
(Dhn12) H.spont.   #361          AAGGTCAAGG AGAAGCTCCC CGGTGGGCAG CACATGGCCG
(Dhn12) Tadmor     #361          AAGGTCAAGG AGAAGCTCCC CGGTGGGCAG CACATGGCCG
                                 ........................................
                   #361          AAGGTCAAGG AGAAGCTCCC CGGTGGGCAG CACATGGCCG

(Dhn12) Morex      #401          CGGGAACTGG AGCTGGCGGG GCTTACGGGC AGCACACGGC
(Dhn12) Sara       #401          CGGGAACTGG AGCTGGCGGG GCTTACGGGC AGCACACGGC
(Dhn12) Zanbaka    #401          CGGGAACTGG AGCTGGCGGG GCTTACGGGC AGCACACGGC
(Dhn12) Arta       #401          CGGGAACTGG AGCTGGCGGG GCTTACGGGC AGCACACGGC
(Dhn12) H.spont.   #401          CGGGAACTGG AGCTGGCGGG GCTTACGGGC AGCACACGGC
(Dhn12) Tadmor     #401          CGGGAACTGG AGCTGGCGGG GCTTACGGGC AGCACACGGC
                                 ........................................
                   #401          CGGGAACTGG AGCTGGCGGG GCTTACGGGC AGCACACGGC
```

Fig. (2): Comparison of *Dhn12* gene nucleotide sequences (281-440) of five barley genotypes and a previously known dehydrin allele (Morex). Asterisks (*) indicate non-identical nucleotides.

Fig. (3): Comparison of *Dhn3* amino acids sequences, (YSK_2 Type) of five barley genotypes and a previously known dehydrin allele from cv. Dicktoo. Asterisks (*) indicate non-identical amino acids.

Gene-Specific RT-PCR Analysis of 9 Dhn Genes in Arta *and H. spontaneum 41-1*

To determine the expression pattern of *Dhn* genes, we amplified mRNAs of two barley genotypes (Arta and *H. spontaneum* 41-1) with 9 *Dhn* gene-specific primers by RT-PCR. For comparison,

Dhn-DNAs from the same samples were amplified with *Dhn* gene-specific primers. The results showed that the expression pattern of the *Dhn* genes was different between those two genotypes, the expression of 5 *Dhn* genes appeared earlier in Arta as compared to *H. spontaneum* 41-1 (Fig. 4).

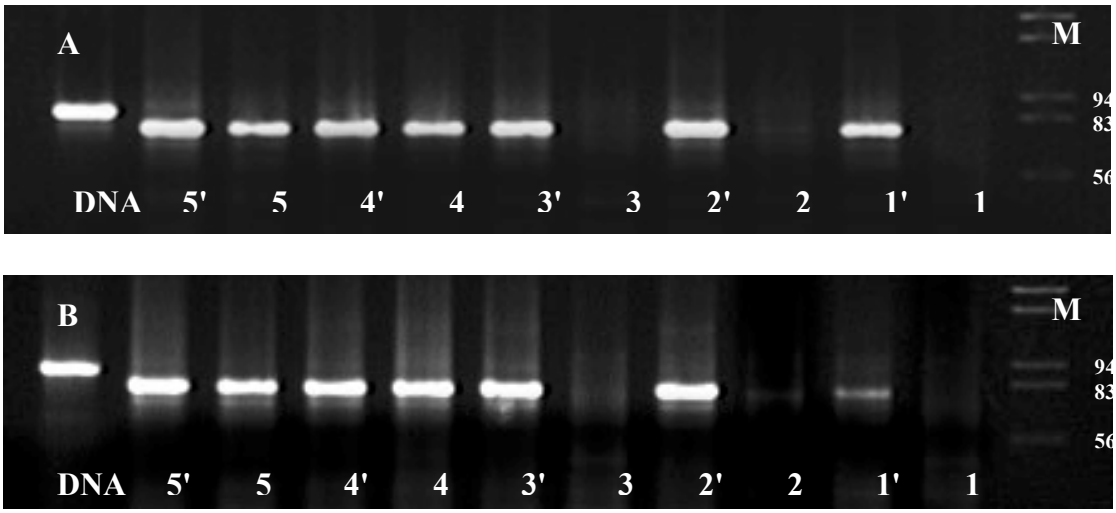

Fig. (4): Gene-specific RT-PCR analysis of *Dhn4* gene expression in two barley genotypes Arta (A) and *H. spontaneum* 41-1(B). 1, 2, 3, ….. indicate the five collections of the control plants, while 1', 2', 3'…. indicate the five collections of the stressed plants. DNA amplification with gene-specific primers where used as control to ensure cDNA amplification.
M: molecular marker (base pair)

Three Dhn Gene Expressions Induced by Drought Stress

To compare the differences of RNA accumulation of *Dhn* gene in 14 barley genotypes, we hybridized total RNA extracted from those genotypes with three *Dhn*-DNA probes (*Dhn1*, *Dhn3*, and *Dhn4*). The results showed different variations in dehydrin-genes expression between barley genotypes, three genotypes (*H. spontaneum*, Tadmor, and *M64-76/Bon//Jo/York/3/M5 /Galt//As46/4/Hj3480/Astrix/5/NK1272*) showed very clear accumulation in late stage of drought stress, Fig. 5.

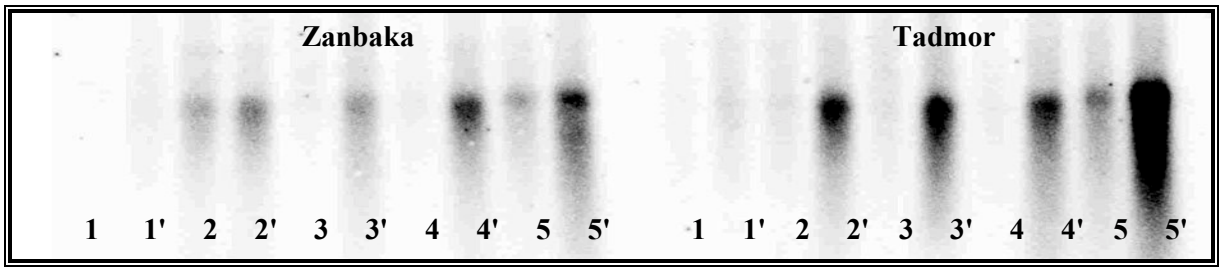

Fig (5): Expression of *Dhn4* gene in two barley genotypes after drought stress treatment. 1, 2, 3, ….. indicate the five collections of the control plants, while 1', 2', 3'…. indicate the five collections of the stressed plants.

Conclusion

1. Analysis of genomic DNA derived from 14 barley genotypes with nine *Dhn* gene-specific primers revealed the presence of different alleles for those specific genes (Loci).
2. Polymorphism in the cDNA of two genotypes of barley was detected by analyzing with specific dehydrin primers.
3. Sequences analysis of three *Dhn* genes (*Dhn*1, *Dhn*3, and *Dhn*12) in five selected genotypes of barley showed variations in the coding and non-coding regions. The variation in the coding sequences caused variations at the amino acid level.
4. Difference in the expression of 3 dehydrin genes was demonstrated by Northern analysis using the 3 *Dhn*-DNA as probes. The level of expression was proportional with the period of drought stress.

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Characterisation of Traits Associated with Improved Growth and Grain Yield of Barley on Sandy Soils of Low Fertility

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Abstract

Superior performance and grain yield on sandy soils was associated with improved establishment, early vigour, phosphorus utilisation efficiency, the reallocation of carbohydrates from the stem to the developing grain post-anthesis, and a deep root system. Besides the genotypic differences, coleoptile length, establishment, early vigour and grain yield could be improved by selecting the large seed size fraction for sowing. Superior varieties also exhibited an erect growth habit and an earlier flowering phenology. The 'over-riding' effect of terminal drought, as highlighted by the yield performance of Keel at drought affected sites, provides evidence to suggest that adaptation response on sand requires drought tolerance to accommodate prevailing environmental conditions that negate the 'sand' effect.

Adaptation response of barley on sandy soils of low fertility is a complex inter-relationship between a number of essential traits. In view of this, it is doubtful whether any one variety will possess the optimum level of expression of all traits, rather a balanced package of traits is likely to be the key. In this context, the variety Mundah exhibited the most effective combination of traits, despite a lack of suitable resistance to economically significant diseases, and a response to drought stress that is inferior to varieties such as Keel.

Introduction

Sandy soils of low fertility constitute approximately 30% of the total area sown to barley in South Australia (SA). With such a significant proportion of the barley sown on sandy soils, the provision of specifically adapted cultivars for this soil type is an integral component of feed barley breeding objectives in the SA Barley Improvement Program (SABIP).

It is generally recognised that barley displays better adaptation on sandy soils than wheat, triticale and oats, but is inferior to cereal rye. Even so, growing barley on sandy textured soils can be unreliable because crop responses can be highly variable (HAMBLIN *et. al.* 1988), and this reflects the inherently poor properties of this soil type. A large part of the lower grain yield potential of crops grown on these soils is believed to be associated with the poor establishment and growth typically observed in these environments. In contrast, heavier textured soils in more favourable environments have a greater yield potential, and this has been attributed largely to improved establishment and early vigour (HAMBLIN *et. al.* 1988; FRENCH & EWING 1989). Barley's greater early vigour than wheat (LOPEZ-CASTANEDA *et al.* 1995), Triticale and Oats may then, in part, confer its better adaptation on sand. It is also possible that drought tolerance related traits (*e.g.* remobilisation of stem reserves for grain filling) provide a mechanism to improve adaptation on sandy soils. A deep root system and a high degree of assimilate transfer to the developing grain, in addition to good early vigour has been suggested as desirable for drought tolerance on light textured soils (TURNER & NICOLAS 1987).

The efficiency and progress in breeding and selecting varieties with superior adaptation on sand is complicated by the low heritability (h^2) of traits important to adaptation in this

environment. The low h^2 is related to low genetic variance, partly related to the germplasm available, and the high environmental and error variance of yield trials conducted on sandy sites. In addition, genetic gain for sand adaptation has been limited by traditional selection methods that tend to discriminate against low yield potential environments.

Some genetic gain for adaptation has been achieved. Yagan, an introduction of unknown pedigree from CIMMYT, was released by the Western Australian (WA) Department of Agriculture barley breeding program in 1988 because of improved yield and superior agronomic features on sandy soils in low rainfall environments (PORTMANN 1989). In 1996, the WA breeding program released Mundah (Yagan/O'Connor). Since being introduced into SA Research and Development Institute field evaluation trials Mundah has consistently shown superior grain yield potential over SA bred varieties on sand, but has ranked lower than SA selections in high yield potential environments (Table 1). Such genotype by environment (g x e) interaction for adaptation response provides evidence of genotypic variability for sand adaptation. While potential genetic variation for sand adaptation has been observed, there has been no concerted effort to identify the physiological, morphological or biochemical characteristics of Mundah (and Yagan) that contribute to superior performance on sand. The objective of this study was to re-address this deficiency in our knowledge of sand adaptation.

Table 1. Long term grain yield (t ha^{-1}) and rankings of selected WA and SA cultivars on sandy and non-sandy soils in South Australia. (Wheeler *et al.*, 1988-2000)

Cultivar	Sandy Soils	Rank	Non-sandy Soils	Rank
Mundah (WA feed)	1.70	1	2.97	3
Forrest (WA feed)	1.64	**3**	2.68	**8**
Galleon (SA feed)	1.47	8	2.85	4
Keel (SA feed)	1.55	**5**	3.02	**1**
Sloop (SA malting)	1.49	7	2.83	5
Schooner (SA malting)	1.52	6	2.77	7
Barque (SA feed)	1.67	2	2.99	2
Chebec (SA feed/malting)	1.56	4	2.80	6

Material and Methods

Variety Comparison Experiment

Barley cultivars were sown in plots, arranged as a randomised complete block design, at three sandy sites in SA, in 1999 and 2000. Traits measured included; establishment, growth habit (upright v prostrate), early vigour (early dry matter (DM) production, leaf area development), relative developmental stage, grain yield, 1000 grain weight, screenings (<2.5) percentage. Non-structural carbohydrate (fructan, glucose, fructose, sucrose) content was measured in main stems at anthesis and physiological maturity (Z92) at one site in 2000.

Seed Size Experiment

Seed of Mundah, sourced from 4 sites, was sorted into four size fractions (<2.2, 2.2-2.5, 2.5-2.8, >2.8mm) and the seeding rate adjusted to sow exactly the same number of germinable seeds per unit area (145 seeds/m^2). Each seed source (site) x size fraction sample was assessed as for the Variety Comparison Experiment.

Controlled Environment Experiment

A subset of the barley varieties used in the field trials was assessed under controlled environmental conditions using soil collected from one field trial site. Sampling occurred at

10, 17, 24 and 31 days after sowing, at which time shoot (leaf area, biomass) and root (maximum root depth, root length density) data was collected.

Statistical Analysis

Field experiment results were analysed by a linear mixed model using residual maximum likelihood (REML) estimates of variance components (PATTERSON & THOMPSON 1971) and multi-environment trial (MET) statistical analysis (CULLIS *et al.* 1998; SMITH *et al.* 2001) using ASREML (GILMOUR *et al.* 1999). The controlled environment experiment was analysed by ANOVA (Genstat® for Windows™, 5th edition).

Results and Discussion

Superior adaptation and grain yield on sandy soils was found to be based largely on the ability to set yield potential through greater early vigour, both in terms of biomass production and leaf area development. This result in general follows several other reports that have shown improved grain yield in low yield potential environments to be associated with superior early vigour (TURNER & NICOLAS 1987; BROWN *et al.* 1987; CECCARELLI 1987; ACEVEDO *et al.* 1991; EL HAFID *et al.* 1998).

At sites demonstrating the typical 'sand' response (*i.e.* not affected by terminal drought) the higher grain yield of Mundah ($\underline{P}$<0.001) was related to superior early vigour ($\underline{P}$<0.001) (Figures 1 & 2a,b). However, at sites with significantly lower than average growing season rainfall (Minnipa, 1999; Figure 3), the prevailing adaptation response appeared to mostly be a function of moisture stress rather than an effect of soil type. Early vigour in this situation was not an important determinant of high yield potential, and varieties with better drought stress tolerance, such as Keel, despite inferior early vigour, out yielded Mundah (Figure 3).

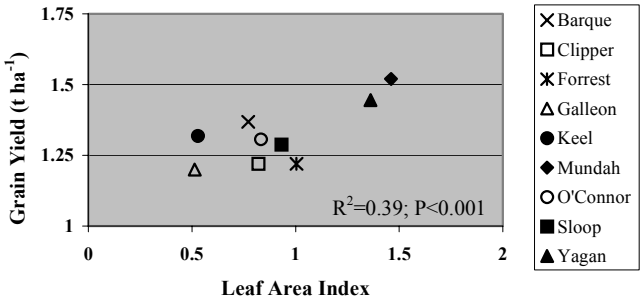
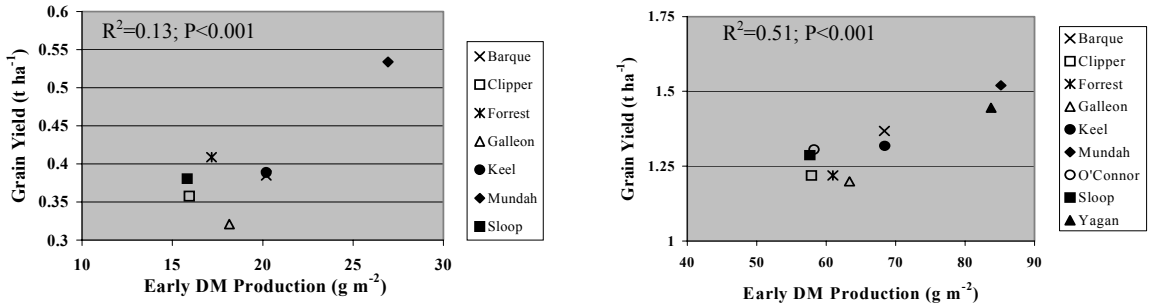


Figure 1. Relationship between grain yield and leaf area index (Lowbank, 2000).



a) b)

Figure 2. Relationship between grain yield and early dry matter production at a) Lowbank 1999, b) Lowbank 2000.

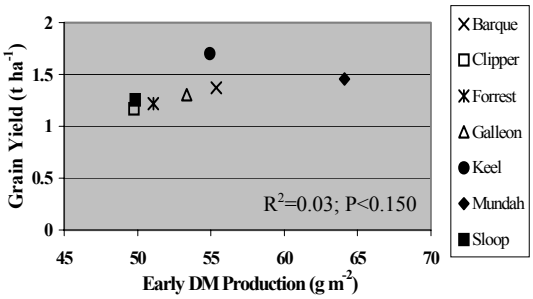
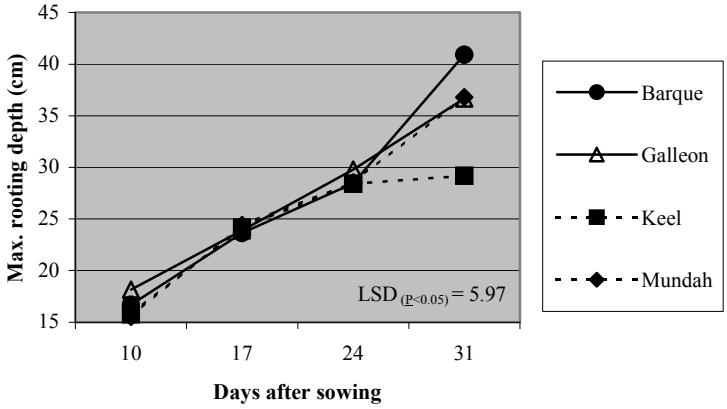


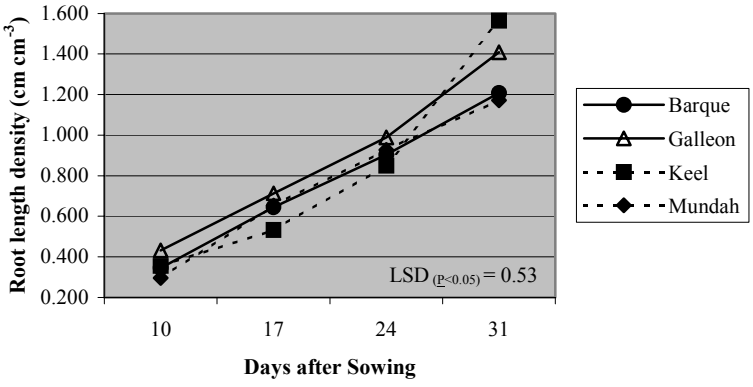
Figure 3. Relationship between grain yield and early dry matter production at Minnipa 1999.

Overall, the yield response of Mundah was relatively stable across environments with a 'sand' effect and those displaying a 'drought' effect. In contrast, the adaptation response of Keel was predominantly determined by prevailing environmental conditions, with a greater grain yield potential than Mundah at sites characterized by terminal drought stress (data not shown). This g x e for grain yield may be explained by Keel possessing traits more important in tolerance to moisture stress at late plant growth stages.

Mundah exhibited a rapidly extending and deeper ($\underline{P}<0.05$), but low density ($\underline{P}<0.01$, square root transformed data), root system in the early stages of growth (Figure 4).



a)



b)

Figure 4. Mean maximum rooting depth and root length density of four barley varieties in the controlled environment experiments. LSD for root length density is for non-transformed data.

The deeper root morphology is likely to be an advantage on sandy soils to counterbalance the characteristically uneven distribution of moisture and the rapid drainage of water down the soil profile. This evidence supports theoretical predictions in recent studies by ASSENG (2002) and DRECCER *et al.* (2002). These authors found, through crop simulation models, that fast early root growth and a greater maximum rooting depth would have a large impact on yield, in their case wheat, on light textured (sandy) soils.

The vagaries of the moisture relations of sandy soils (*i.e.* rapid drainage, uneven distribution through the soil profile because of water repellency) means that plants in the early stages of growth could enter moisture stress very rapidly even after significant rainfall events. In contrast, seedlings growing in heavier soils can maintain early growth in non-moisture stress conditions using very little water, and can set yield potential on smaller rainfall events, because of the greater water holding capacity of these soils. Therefore the superior performance of Mundah on sand is likely to be a function of overall plant growth (*i.e.* root and shoot). The greater early vigour of Mundah protects the soil surface (reduced evaporation) in the early stages of growth, increasing the amount of moisture potentially available to the plants through evapotranspiration, improves crop photosynthesis due to greater light interception, and sets yield potential. Superior early vigour could also improve competitiveness with weeds, reduce erosion, and possibly reduce the damaging effects of sand "blasting". The more rapid and deeper root morphology provides a significant advantage in 'chasing' the depleting moisture through the soil profile. The deep root system of Mundah may also be important in terms of the availability of nitrogen (nitrate form), which is easily lost below the root zone through leaching.

Although increased light interception favours greater evapotranspiration, this does not necessarily translate into better tranpiration efficiency. The erect growth habit of Mundah (data not shown) suggests a compromise, balancing high evapotranspiration with high crop photosynthesis, and moisture conservation. The superior biomass production of Mundah implies better water use efficiency in early growth relative to the other varieties evaluated in this study (Figure 2).

In addition to genotypic differences for adaptation, inferior early vigour and grain yield can be related to the sowing of small seed, and which will also likely impact on tillering ability.

In a seed size field experiment we were able to demonstrate that adjusting seed size modified the response of Mundah, despite an inherent adaptation to sandy soils. Plants grown from large seed were found to produce longer coleoptiles, produced a greater level of emergence and establishment, greater early vigour, and superior grain yield (predominantly through a greater number of grains per m^2, data not shown) than plants grown from small seed (Table 2).

Table 2. Agronomic data for the Mundah seed size trials at Lowbank in 2001

Seed Size (mm)	Average Seed Weight (mg)	Coleoptile length (cm)	Establishment (plants m^{-2})	Early Vigour (g m^{-2})	Grain Yield (t ha^{-1})	1000 grain weight (g)	Screenings (%)
<2.2	26.61	69.84	104.4	17.44	1.08	53.00	4.53
2.2-2.5	37.26	77.85	125.9	24.52	1.14	52.06	4.34
2.5-2.8	47.17	82.84	138.5	27.57	1.17	51.89	4.51
>2.8	54.79	83.98	144.6	30.14	1.18	51.73	4.62
LSD (P<0.05)	*N/A*	*0.86*	*17.55*	*5.86*	*0.14*	*1.01*	*0.87*

N/A=not applicable

Even though early vigour was higher with sowing larger seed, covariate statistical analysis showed, with one exception, that early vigour was not a factor in determining grain yield. This demonstrated that grain yield improvement on sandy soils in these experiments was

determined predominantly by seed size. As discussed above, we showed early vigour to be an essential feature of adaptation on sandy soils in the absence of terminal drought.
These experiments imply that in addition to improving early vigour through variety selection, genetic improvement in grain size could also lead to improved performance on sand. Furthermore, simple, yet effective, management strategies to provide seed of large average size can be employed by growers to improve growth and grain yield on sand (*e.g.* screening seed to improve the average size and weight). However, if it is common practice for growers to sow seed on a weight per hectare basis, the benefits of sowing seed of larger average size will be lost because plant density and early vigour on an area basis will be lower than that for seed of smaller average size. Accordingly, it is advisable to calculate seeding rate based on a recommended plant density for sandy soils, and adjusted for average seed weight.

A significant relationship between coleoptile length, plant establishment, and early vigour could not be directly verified in this study. However several studies have shown a positive relationship between coleoptile length and plant establishment (WHAN 1976; BACALTCHUK & ULRICH 1990; HUGHES & MITCHEL 1987; RADFORD 1987; RADFORD & WILDERMUTH 1987; SHARMA 1990), and coleoptile length and early vigour (GUL & ALLAN 1976; GORNY & PATYNA 1981; REDONA & MACKILL 1996; REBETZKE & RICHARDS 1996; REBETZKE *et al.* 1999). Intuitively, a long coleoptile should be an important component of a variety adapted to sandy soils, precisely because seeding depth is typically highly variable on these soils. Mundah is a large seed variety and has a longer than average coleoptile, however Galleon, which is clearly inferior to Mundah on sandy soils (Table 1) and has smaller seed, produces a significantly longer coleoptile than Mundah (data not shown). This points to genotype as a major source of variation in coleoptile length, and seed size alone does not define variation in coleoptile length. So while a longer coleoptile can improve establishment potential, it is in combination with other adaptation traits that coleoptile length is important to performance on sand. The greater reserves of starch and nutrients in larger seeds also contribute to early seedling development (*e.g.* coleoptile and root growth). However seed nutrient reserves are limited and short term, such that the rapid development of roots into the soil profile is essential for plants to acquire nutrients and moisture to sustain early growth.

Efficient utilisation of phosphorus was found to be a component of the overall package that defines the superior adaptation of Mundah ($15.60g^2$ mg^{-1} P *c.f.* $11.65g^2$ mg^{-1} P for Keel, $\underline{P}<0.001$). Phosphorus nutrition is a key component of successful cropping in South Australia, and many soils are below levels critical to plant health. In our trials the level of phosphorus in the top 10 cm soil fraction was between 9 and 15 mg P kg^{-1} soil, and up to 10 mg P kg^{-1} soil at a depth of 80 cm; well below the critical value for phosphorus (18 mg P kg^{-1} soil, REUTER *et al.* 1995). Phosphorus has a relatively low mobility in the soil, and the effective use and/or an extensive root system to capture the available phosphorus is important. The low root density of Mundah shown in these experiments suggests that the total available phosphorus that can be exploited from the soil may be limited, and that efficiency of use of the available phosphorus arises from high biomass production per unit of phosphorus taken up by the plant.

The superior adaptation of Mundah is also related to an ability to convert the potential into improved grain yield and grain yield stability. This is achieved through a capacity to support the development of larger grain. In general, conditions post-anthesis in SA become increasingly unfavourable for grain development (*e.g.* leaf senescence, moisture and heat stress, increased evaporation) as climatic conditions become hotter and drier. Under these constraints NSCs stored in the stem prior to flowering, and a deep root system, is likely to assist in sustaining grain filling and preventing premature physiological maturity under the

environmental conditions often encountered in SA. The contribution of NSCs to grain filling, and therefore yield and yield stability, has been implied as an important mechanism in drought tolerance (AUSTIN *et al.* 1980; RICHARDS & TOWNLEY-SMITH 1987; BLUM 1998; BLUM *et al.* 1994; GEBBING *et al.* 1999). Mundah achieved a high level of NSC at flowering, and displayed greater utilisation of the carbohydrate resource (Table 3).
Equally important is the early flowering habit of Mundah that maximizes the duration of grain filling. Sloop had a high utilisation of NSC, however it's later flowering phenology, along with an inferior early vigour, makes it poorly adapted to sandy soils. Similarly for Galleon and Clipper, despite equivalent NSC content to Mundah at anthesis. A early flowering habit is essential in combination with increased early vigour in moisture limiting environments (RICHARDS 1991). Further research is required to elucidate completely the contribution of NSC to grain filling on sandy soils. Measuring the rate and duration of grain filling, and changes in NSC in the stem during this period should be components of any future study.

Table 3. Total non-structural carbohydrate content per stem of 9 varieties at anthesis, maturity and utilisation. Site: Lowbank, 2000.

Variety	Total non-structural carbohydrate (mg)		
	Anthesis	Maturity	'Utilisation'†
Yagan	92.29a	5.48bcd	86.07a
Sloop	77.66ab	7.93bcd	73.31ab
Mundah	62.2bc	4.34cd	68.26b
Forrest	65.17bc	11.89a	63.67bc
Clipper	69.73bc	7.91bcd	58.59bc
Galleon	61.59bcd	8.47ab	50.14c
Barque	57.84cd	8.54ab	47.63cd
O'Connor	45.64de	8.27abc	31.12de
Keel	30.37e	4.27d	28.05e
LSD (P<0.05)	*16.34*	*3.94*	*17.80*

†Anthesis, maturity and 'utilisation' data analysed separately, therefore 'utilisation'≠anthesis-maturity

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Two Mutants Affecting Adaptative Responses to Abiotic Stresses in Barley Seedlings

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Abstract
We want to describe here two novel mutants which affect the adaptative responses of barley seedlings to different abiotic stresses. They allow us to explore some aspects of adaptative phenomena which are little known in higher plants. One of those mutants corresponds to a plastid encoded gene which is involved in photosystems I and II stability and, probably indirectly, affects the acclimation of the seedlings to higher temperatures, a fact which seems to occur through the control of unsaturation/saturation levels of the thylakoid membrane fatty acids. The other mutant corresponds to a nuclear gene which, under certain circumstances, induces an additional ethylene production in the seedling roots. This mechanism seems to be involved in eliciting a negative hydrotropic growth of the roots, a phenomenon that we interpret as a waterlogging avoiding response.
Keywords: abiotic stress; barley; root behavior; temperature sensitivity

Introduction

Two barley mutants, each one affecting the response of seedlings to two different abiotic stresses are here presented bearing in mind that they can be useful tools to investigate the highly complex mechanisms by which barley seedlings can respond and adapt to adverse environmental conditions. One of those mutants was identified in hydroponics (MARTINEZ *et al*. 2004) by the sandwich method of MYHILL and KONZAK (1967). In those particular conditions, mutant roots did not show the growth pattern with windings and turnings usually observed on wild type roots before they submerged (MARTINEZ *et al*. 2004). On that paper, it was also demonstrated that the root behavior above mentioned is controlled by a semi-dominant nuclear gene. That differential root behavior was postulated to be associated with ethylene production (see MARTINEZ *et al*. 2004). Indeed, ethylene diffused from mutant roots was significantly lower than that from wild type roots and, in addition, experiments with the ethylene antagonist silver ion supported that hypothesis. Interestingly, no phenotypic differences were noticed between mutant and control root when they grew in humid chambers. The other mutant is a high temperature-sensitive one (PRINA *et al.* 2000), which was previously described as a *viridis* type and has shown maternal inheritance (PRINA 1996).

Material and Methods

Seeds of barley (*Hordeum vulgare*) were employed in all assays. Root studies were carried out on seedlings of the root mutant (MC 169) and of the wild type control (MC 182). Biochemical analyses of the first leaf blade were done on CL3 *viridis* mutant (PRINA 1996) and on wild type control (MC 182) seedlings. In this case, second generation seedlings of the reciprocal crosses were also analyzed.

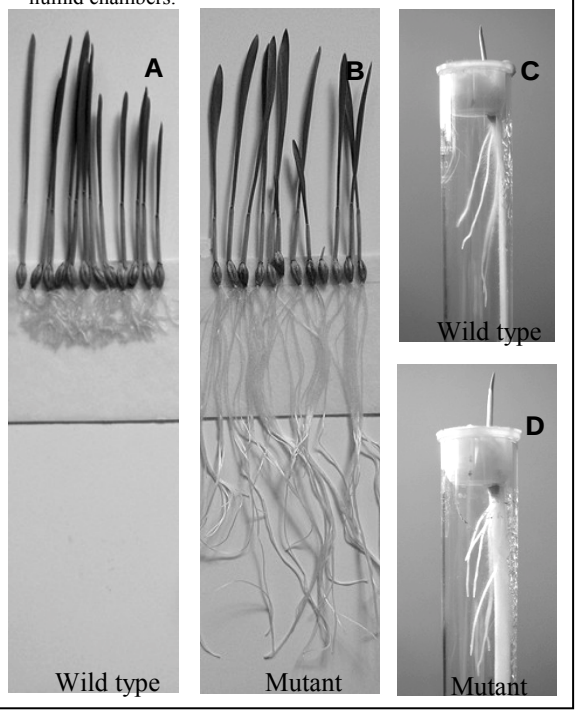
For most of the assays seedlings were grown in hydroponics by the sandwich method (MYHILL & KONZAK 1967). Some assays were carried out in humid chambers made up inside of 15 ml Pyrex test tubes as described in MARTINEZ *et al*. (2004). Root ethylene production was quantified by gas chromatography as described earlier (MARTINEZ *et al*. 2004). The following ethylene related plant growth regulators (PGRs) were added at sowing in the sandwich method assays: silver ions (Ag+, added as silver thiosulfate); aminoethoxyvinil glicine (AVG, Retain®, NUFARM, New Zealand) and aminocyclopropane carboxylic acid (ACC, ICN Biomedicals Inc.). For root analyses, seedlings were grown during seven days in a growth chamber (Conviron EF7 model) with a photoperiod of 14 h at 18°C. The Student's t test for unpaired samples was employed for the estimation of statistically significant changes in response to the different treatments. For biochemical analyses of the first leaf blade environmental conditions were as follows: assays of Figs. 6, 7 and 8 were done in a growth chamber (SANYO Versatile Environmental Test Chamber), with a photoperiod of 18 h of light (100 $\mu E/m^2s$ from white fluorescent tubes) at 18 or 32°C; assay of Fig. 9 was carried out in a growth chamber (Conviron S10H) with a photoperiod of 18 h (100 $\mu E/m^2s$ from white fluorescent tubes and incandescent lamps); for the assay of Fig. 10 two different environments were used: I) at the greenhouse, with natural light and temperatures ranging from 15 to 25°C and II) in a growth chamber (SANYO) at 32°C and 18 h of light (100 $\mu E/m^2s$ from white fluorescent tubes). Pigment analyses were done in a Beckman DB-G spectrophotometer, according to MACLACHLAN and ZALIK (1963) as described in PRINA *et al.* (2003). Thylakoid proteins were analyzed by SDS-PAGE and by Western blotting against D1 or against PSI. Fractionation of thylakoid membranes and SDS-PAGE were made based on CAMM and GREEN (1980). Western blottings were performed according to ECL Western Blotting Analysis System by Amersham. Water content was determined by weight differences between fresh and dry weight after 4½ hours at 105ºC. Lipids were quantified by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). Lipids separation was done according to PEARCY (1978), but using HPLC instead of gas chromatography (GC).

Results and Discussion

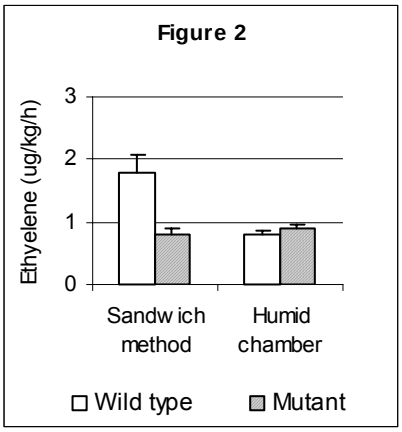
A Mutant Lacking Root Tropic Response to Submergence

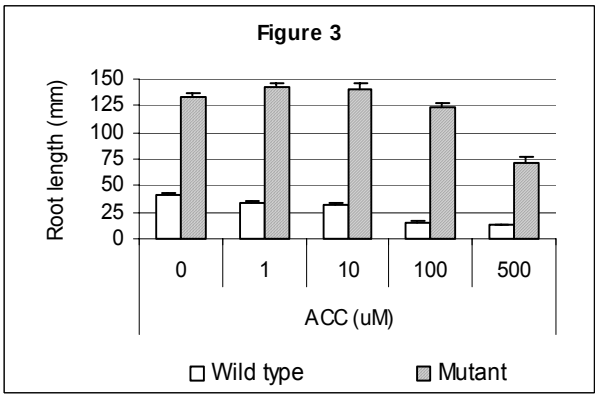
In Fig. 1 (A, B) marked morphological differences can be observed between wild type and mutant roots when seedlings were grown in hydroponics by the sandwich method. Estimations of ethylene released by roots grown in these conditions confirmed results previously found (MARTINEZ *et al*. 2004). Ethylene produced by mutant roots was significantly lower when compared with that of wild type roots (Fig. 2). On the other side, inside humid chambers, where roots grew freely without any physical contact, wild type and mutants roots were morphologically similar (Fig. 1: C, D) and they had also a similar ethylene production (Fig. 2). Moreover, the amount of ethylene produced by both genotypes under these conditions was similar to that produced by mutant roots grown by the sandwich method (Fig. 2). These results suggest that wild type barley roots are able to increase ethylene production in response to the special conditions given by the sandwich method. This response was observed to be associated with a tropic response that results in an erratic growth pattern, with winding and turnings, displayed by wild type roots before they submerged. It was also associated with several morphological changes, like root shortening and others, not informed here, like root thickening and changes in root hair length and distribution. This particular

Figure 1: Barley seedlings grown by sandwich method or in humid chambers.

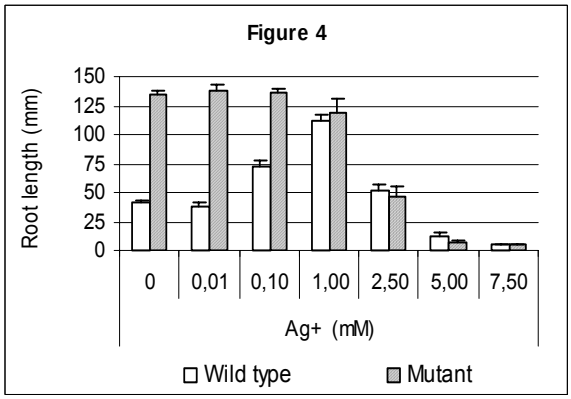


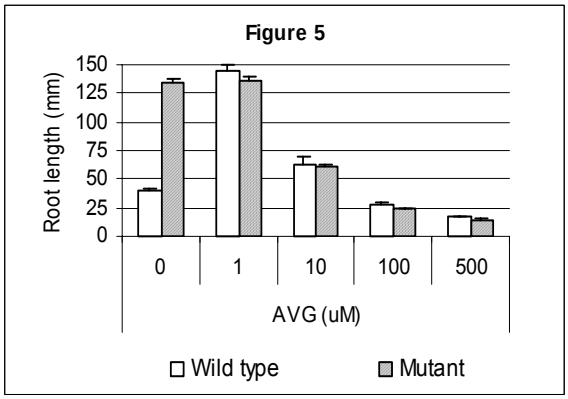
behavior of wild type roots could be interpreted as an adaptative response of barley seedlings to avoid flooding conditions.





In order to further investigate the physiological basis of that root response, we assayed diverse ethylene related PGRs and their effects were quantified by measuring the length of the primary roots after seven days. Addition of the ethylene precursor aminocyclopropane carboxylic acid (ACC, TANIMOTO *et al*. 1995) caused a significant decrease of root length in both genotypes (Fig. 3), indicating that an excess of ethylene had an inhibitory effect on root lengthening in both of them. Previously, silver ions treatments were observed to induce a marked increase in wild type root length, but they did not show any effect on mutant roots (MARTINEZ *et al*. 2004). In the present work a more complete dose-response curve to silver ions was performed (Fig. 4). At the lower doses, the effects were the same as previously observed (MARTINEZ *et al*. 2004), but a marked reduction of the root length was observed

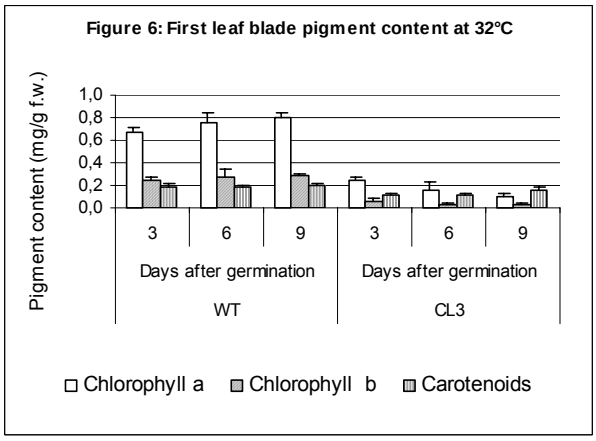


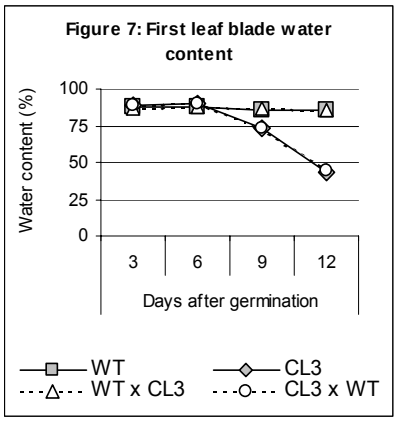


for both genotypes with 2.5 mM Ag+ or higher. Results with the inhibitor of ethylene synthesis aminoethoxyvinil glicine (AVG, HEIDSTRA *et al*. 1997) showed similar effects to those of silver ions (Fig. 5). Both experiments suggest that a certain minimal endogenous ethylene production is necessary for root lengthening, but, this process is inhibited above certain ethylene level. We hypothesized that this happens in wild type roots when an additional ethylene production is induced by the special conditions created by the sandwich method.

A Temperature Sensitive Mutant

Results of pigment content in the first leaf blade of wild type and CL3 seedlings grown at 32°C are presented in Fig. 6. Similar results from reciprocal crosses (data not shown) confirmed the maternal inheritance of these pigment content characteristics (PRINA 1996). In CL3 seedlings the chlorophyll content diminished in time, while carotenoids maintained a





constant level (Fig. 6). In Fig. 7, water content of the same experimental material is represented in relation with time. It can be said that 32°C of constant temperature are markedly lethal for seedlings carrying CL3 cytoplasm, which suffered a dramatic loss of water after 9 days. Fig. 8 shows thylakoid protein SDS-PAGE gels carried out on the same experimental material grown at 18 or 32°C. After 9 days at 32°C the pattern of thylakoid proteins was completely altered in CL3 and the corresponding western blot with D1 antiserum (Fig. 9) showed that D1 protein was no detected by day 9. On the other side, at 18°C (Figs. 8 and 9), slighter differences were observed between CL3 and the control. In Fig. 10 PSI

Figure 8: Thylakoid protein SDS-PAGE gels

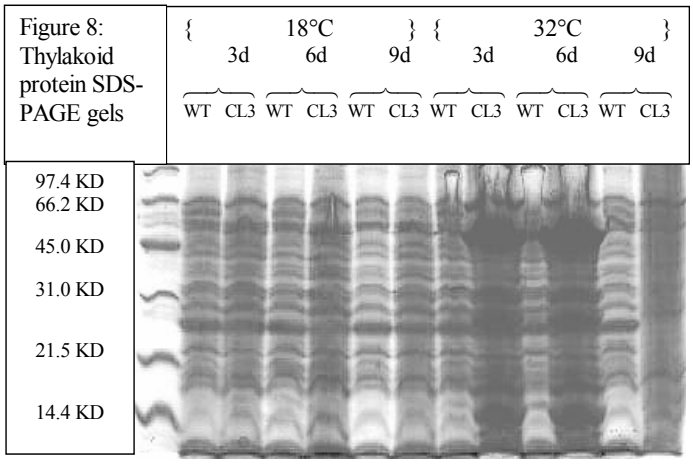
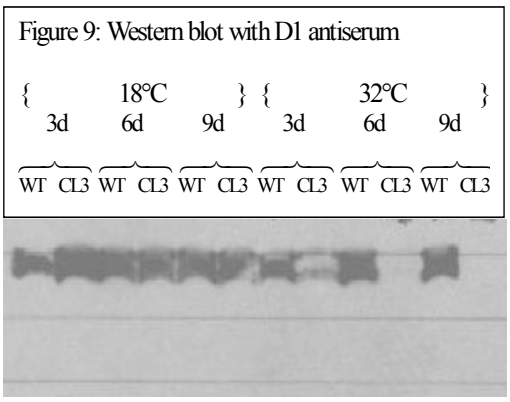
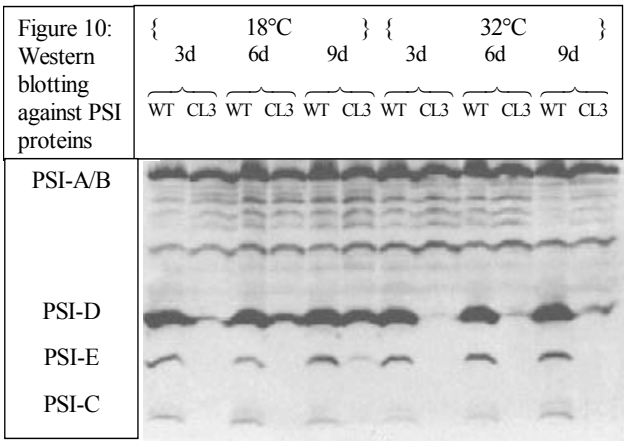


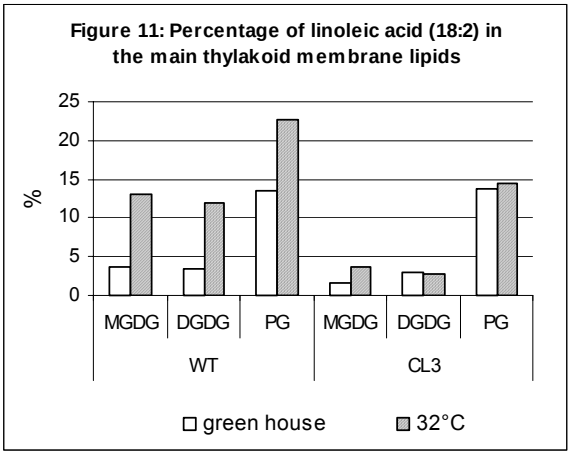
Figure 9: Western blot with D1 antiserum



protein composition of wild type and CL3 seedlings can be observed**.** In the wild type, PSI-A/B, PSI-D, PSI-C and PSI-E were observed as expected. On the other side, in CL3 at 18°C, PSI-D appeared as a weak band at day 3 after germination and it became stronger and with similar intensity to control by day 9. PSI-E and PSI-C were not observed either at day 3 or day 6, but they appeared as very weak bands at day 9. At 32°C, in CL3 PSI-D was observed as a weaker band than at 18°C and PSI-E and C could not be observed either at day 3, 6 or 9.
In relation to adaptative responses the most streaking differences were observed in relation with the fatty acid saturation degree of the main thylakoid-membrane lipids: mono-galactosyl-diacyl-glycerol (MGDG), di-galactosyl-diacyl-glycerol (DGDG) and phosphatidyl-glycerol (PG). In Fig. 11 the proportions of linoleic acid (18:2) are presented. In environment II, at 32°C, the wild type presented a general increase in linoleic acid in relation with environment I

Figure 10: Western blotting against PSI proteins





(Fig. 11), with a concomitant decrease in the more unsaturated linolenic acid (18:3) (data not shown). However, in CL3 seedlings the proportion of linoleic acid did not show marked differences between environment I and II (Fig. 11). Greater lipid saturation might be expected to confer thermal stability to membranes because its higher melting temperatures (PEARCY 1978) and has been observed to play an important role at the chloroplast membrane level in improving thermal tolerance (RAISON *et al*. 1982; MURAKAMI *et al*. 2000; ALFONSO *et al*. 2001; IBA 2002). Even though more detailed investigations need to be done, it can be said that under certain circumstances wild type seedlings seems to display an acclimation response, which was not functional in CL3 seedlings. All results suggest that CL3 has a delayed assembly of PSI components and, as consequence, they could be easily damaged by environmental stresses. On the other side, the observed decrease in PSII probably resulted from photoinhibition caused by a deficient PSI. ALFONSO *et al*. (2001) pointed out that any factor affecting photosynthesis might ultimately influence the activity of fatty acid desaturases and then the capacity to adapt to environmental stresses. It is probably that deficiencies in the photosynthetic machinery would be responsible for the lack of CL3 response to the different environments and suggests that a chloroplast signal would be necessary for such response. TANAKA *et al*. (2000), proposed several models for acclimation mechanisms of the photosynthetic machinery to high temperature in *Chlamydomonas reinhardtii*, which involve different nuclear-chloroplast interactions.

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AB-QTL Analysis for Osmotic Adjustment and Relative Water Content of Drought Tolerance in Spring Barley

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Abstract

The advanced backcross quantitative trait loci (AB-QTL) analysis was used to identify QTLs for osmotic adjustment and relative water content (RWC) in a BC_2DH population derived from a cross between the spring barley variety Scarlett and the wild barley ISR42-8. Ninety-seven markers were employed to genotype 323 BC_2DH lines and phenotypic data were collected for two parameters related to drought tolerance. Two treatments were tested for the drought experiment, 50% field capacity (FC) level for drought stress and 100% FC level as a control. QTLs were detected by means of ANOVA with a significance level of $P < 0.01$ for a marker main effect and a marker × drought treatment (M × D) interaction, respectively. A total of 10 putative QTLs were detected, of which seven were for osmotic adjustment and three for relative water content. Altogether, 5 (50%) favorable effects of exotic alleles were detected for drought tolerance, despite the fact that the exotic barley donor had an overall inferior phenotype with respect to agronomic performance. The present study indicated that favorable QTL alleles for drought tolerance could be transferred from wild barley into an elite barley variety by the advanced backcross QTL strategy and molecular breeding.

Keywords: *Hordeum spontaneum*; drought tolerance; osmotic adjustment; relative leaf water content; AB-QTL analysis

Introduction

All over the world, water is clearly among the most important factors affecting plant survival and function. Water deficit and changes in the environmental conditions may reduce plant growth and yield and impair metabolic processes (HSIAO 1973). The response of plants to stresses depends on their genetic potential to adaptation. Drought resistance in crops could be attributed to either avoiding or tolerating drought. Tolerance to drought could be attained through a mechanism that enhances plant ability to withstand low water potential, (CLARKE *et al.* 1984). Drought avoidance involves rapid morphological development, leaf rolling, leaf shading, reduced leaf area, and increased stomata and cuticular resistance (MORGAN 1984; TURNER 1986). Osmotic adjustment and relative water content are indicators of the plant water status and hence of the plant's genetic potential of adaptation to drought. Osmotic adjustment enables water uptake to continue under increasing drought in many crop species and, in some cases, it was associated with maintenance of growth and stable yield under drought conditions (GUNASEKERA & BERKOWITZ 1992). Evaluation of the relative water content (RWC) provides an idea of the level of the water deficit in the plant at a specific time-point and may closely reflect the balance between water supply to the leaf and transpiration rate.

The genetic heterogeneity of wild barley populations has long been recognized as an important mechanism of adaptation to the environment (IVANDIC *et al.* 2003). Indeed, wild barley has often been considered a valuable resource for increasing stress resistance in modern varieties. In order to isolate single favourable genes from exotic germplasm, TANKSLEY & NELSON (1996) proposed the advanced backcross quantitative trait loci (AB-QTL) strategy. Here, the marker and phenotype analysis is carried out in an advanced backcross generation, thereby integrating the mapping of favorable QTL alleles and the introgression of exotic donor alleles into elite cultivars.
In the present study we analysed osmotic adjustment and relative water content (RWC) as indicators of drought tolerance in a BC_2DH population derived from a cross between a German spring cultivar Scarlett and the wild barley accession ISR42-8 from Middle East.

Material and Methods

Plant Material

The spring barley variety Scarlett was crossed with a wild barley accession ISR42-8. The resulting F1 population was backcrossed twice with Scarlett. The BC2 population was finally subjected to double haploid production (323 BC_2DH lines). These BC2DH lines were examined on their tolerance in relation to drought.

Simple Sequence Repeats Markers (SSR)

The genotypes of 323 BC_2DH lines were determined by means of SSR analysis (Pillen *et al.* 2003). The SSR analysis was performed with a LI-COR detection system (LI-COR, Lincoln, NE, USA).
The following prefixes of SSR names indicate the published sources from which the primer sequences were taken: HVM, LIU *et al.* (1996); Bmac, Bmag, Ebmag and Ebmac, RAMSAY *et al.* (2000); Hv, BECKER and HEUN (1995) and PILLEN *et al.* (2000). Linkage distances between SSR markers were inferred from RAMSAY *et al.* (2000) and PILLEN *et al.* (2000).

Phenotyping the BC2DH Population

The experiments were carried out in the green house during the years 2002 and 2003 at the Poppelsdorf Experimental Station, Department of Crop Science and Plant Breeding, University of Bonn, Germany.
Relative leaf water content (RWC) was measured from two field capacity levels according to (MATIN *et al.* 1989; ALI *et al.* 1999). The relative water content of the leaf tissues was calculated as follows: RWC (%) =(FW- DW) x 100 /(TW-DW), on the last fully expanded leaf according to (BARRS and WEATHERLY 1962), where FW is leaf fresh weight, TW the turgid weight obtained after 24 h floating on distilled water at room temperature under dim light. Dry weight (DW) was measured after the samples had been dried for 24 h at 80 °C.
For osmotic adjustment (OA), the penultimate leaf was cut, wrapped in plastic foil, frozen in liquid nitrogen. Then 500 µl sterile water was added and material was homogenized with an ultrathurax. Then the material was centrifuged at 13,000 rpm for 3 minutes and finally stored at – 20°C until measurement. A sample of 50µl was taken and measured by Osomat 300 (Gonotec, Berlin) with sterile water as a standard. Osmotic adjustment was calculated according to (WILSON *et al.* 1979; LUDLOW *et al.* 1983).

QTL Detection

The QTL detection from BC_2DH genotype and phenotype data was conducted using the GLM procedure (General Linear Model) from the SAS software (SAS Institute *1999*). The model included the effects marker genotype (M), drought treatment (D), and M*D interaction. A mixed model with the marker and the drought treatment was chosen as fixed effects and year as a random effect. Following PILLEN *et al.* (2003), the presence of a stable QTL in the vicinity of a marker locus was accepted, if the marker main effect was significant at $P < 0.01$. Adjacent marker effects (distance <20cM) are considered as one putative QTL. The presence of a drought treatment dependent QTL was accepted, if the M*D interaction was significant at $P < 0.01$.

Results and Discussion

The 97 polymorphic SSRs revealed 10 putative QTLs for drought tolerance (Fig. 1). At these loci, the trait means of the homozygous ISR42-8 (*H. v*. ssp. *spontaneum*, hereafter abbreviated with *Hsp*) genotype and the homozygous (*Hordeum vulgare* L. *distichon*, hereafter abbreviated with *Hvd*) genotype deviated highly significantly (Table 1). Altogether, 5 (50%) favorable QTL effects were detected. At these loci, the homozygous *Hsp* genotype was associated with an improvement of the trait compared to the homozygous *Hvd* genotype (Table1).

Osmotic Adjustment (OA)

A total of 7 putative QTLs, which had effects on osmotic adjustment, were located on chromosomes 1H, 5H, 6H and 7H. Four loci exhibited a significant marker main effect, the other loci showed a significant M*D interaction. Five favorable *Hsp* effects were detected. These loci improved osmotic adjustment by a maximum value of 22.3% at HW01M22T3 [5H]. On the other hand, two *Hsp* alleles decreased OA by 11.2% at Bamc0316 [7H] and HVALAAT [1H]]. The *Hsp* allele of four loci lifted OA in the control treatment by a maximum of 7.2% (HY02J05T3 [5H]), while at three loci, the *Hsp* allele decreased OA in the control treatment by up to 8.6% at HVALAAT [1H]. Two *Hsp* alleles decreased OA in the drought treatment by 13.3% and 18.0% at HVALAAT [1H] and Bmac0316 [6H], respectively. Five *Hsp* alleles increased OA under drought stress of up to 39.5% at HW01M22T3 [5H] (see Table 1).

Relative Leaf Water Content (RWC)

Three putative QTLs for relative leaf water content were located on chromosomes 1H and 4H. All loci exhibited significant M*D interactions. The presence of the *Hsp* allele led to a reduction in relative leaf water content by up to 4.3% at GBM1007 [1H]. All *Hsp* alleles showed positive effects under the control treatment except GMS089 [4H]. The *Hsp* increased relative leaf water content in the control treatment by 4.0% at Ebmac0701 [4H] and TACMD [4H], while the *H*sp allele decreased RWC by 4.8% at GBM1016 [2H]. On the other hand, the *Hsp* allele decreased the RWC under drought stress by a maximum of 12.8% at GBM1007 [1H], while the *Hsp* allele increased the RWC under drought stress by 3.9% at GMS089 [4H] (see Table 1).

Comparison of Results with Other QTL Analyses in Barley

While classical QTL analyses were conducted in early, balanced generations like doubled haploid (DH), our AB-QTL analysis was based on a BC_2DH population. This change was

Table 1. List of 10 putative QTLs detected from the BC_2DH cross Scarlett x ISR42-8

Trait[A]	Marker	Chr[B]	Position[C] (cM)	Effect[D]	RP of *Hsp* genotype (%) [E]	RP of *Hsp* genotype in control treatment (%)[F]	RP of *Hsp* genotype in drought treatment (%)[G]	QTL No. [H]
OA	HVALAAT	1H	63	M	-11.2	-8.6	-13.3	1
	HY02J05T3	5H	0	M	11.5	7.2	15.2	2
	Bmag0223	5H	69	M	8.1	7	9	3
	Bmag0222	5H	162	M*D	19.7	2.6	34	4
	HW01M22T3	5H	165	M*D	22.3	1.6	39.5	4
	Bmac0316	6H	6	M	-11.2	-2.5	-18	5
	HVA22S	7H	75	M*D	8.4	-0.9	16.2	6
	Bmag0011	7H	93	M	8.8	1.2	15.3	6
	GMS056	7H	133	M + M*D	14.8	4.7	23	7
	BMS64	7H	146	M + M*D	14.3	1.4	25	7
	Bmag0120	7H	152	M*D	17.7	3.6	29.8	7
RWC	GBM1007	1H	28	M*D	-4.3	3.4	-12.8	1
	GMS089	4H	57	M*D	-0.8	-4.8	3.9	2
	TACMD	4H	125	M*D	-0.8	4	-6.1	3
	EBmac0701	4H	130	M*D	-0.3	4	-5	3
	EBmac0635	4H	131	M*D	-0.8	3.4	-5.5	3
	EBmac0679	4H	132	M*D	-0.9	3.6	-5.8	3
	EBmac0788	4H	150	M*D	-0.7	3	-4.7	3

[A] OA: osmotic adjustment, RWC: relative water content
[B] Chromosomal assignment of SSRs
[C] Chromosomal position of SSRs deduced from RAMSAY *et al.* (2000) and PILLEN *et al.* (2000)
[D] A QTL was assumed within the vicinity of a marker locus if the marker main effect or the M*D interaction was significant in the three-factorial ANOVA at $P < 0.01$
[E] Relative performance of *Hsp* genotypes across both treatments in % = (Ms-Mv)*100 / Mv
[F] Relative performance of *Hsp* genotypes in control treatment in % = (MsT1-MvT1)*100 / MvT1
[G] Relative performance of *Hsp* genotypes in drought treatment in % = (MsT2-MvT2)*100 / MvT2
Mv = lsmean of *Hvd* genotypes. Ms = lsmean of *Hsp* genotypes.
T1 = control treatment T2 = drought treatment
[H] Consecutive number of QTL for each trait

necessary since we used an exotic cross with the barley progenitor *Hsp* as the donor of potential favorable QTL alleles. The selected SSRs could be associated with 10 putative QTLs in the Scarlett ×ISR42-8 population. The first common QTL is the putative QTL for RWC associated with GMB1007 [1H]. Classical QTLs for RWC were detected in similar regions at AC13 [1H] and MctcEaagM [1H] by TEULAT *et al.* (1998; 2001). The second common QTL was again detected for RWC but associated with GMS089 [4H]. This QTL was also recovered at locus CDO669 [4H] (TEULAT *et al.* 2003). The third common QTL was found for OA associated with Bmag0223 [5H]. This QTL was also detected at McaaEaccS [5H] (TEULAT *et al.* 2001). The fourth common QTL was found for OA associated with Bmac0316 [6H]. In this case, a classical QTL for osmotic adjustment was also detected in the down lapping for marker WG286 [6H] (TEULAT *et al.* 1998).

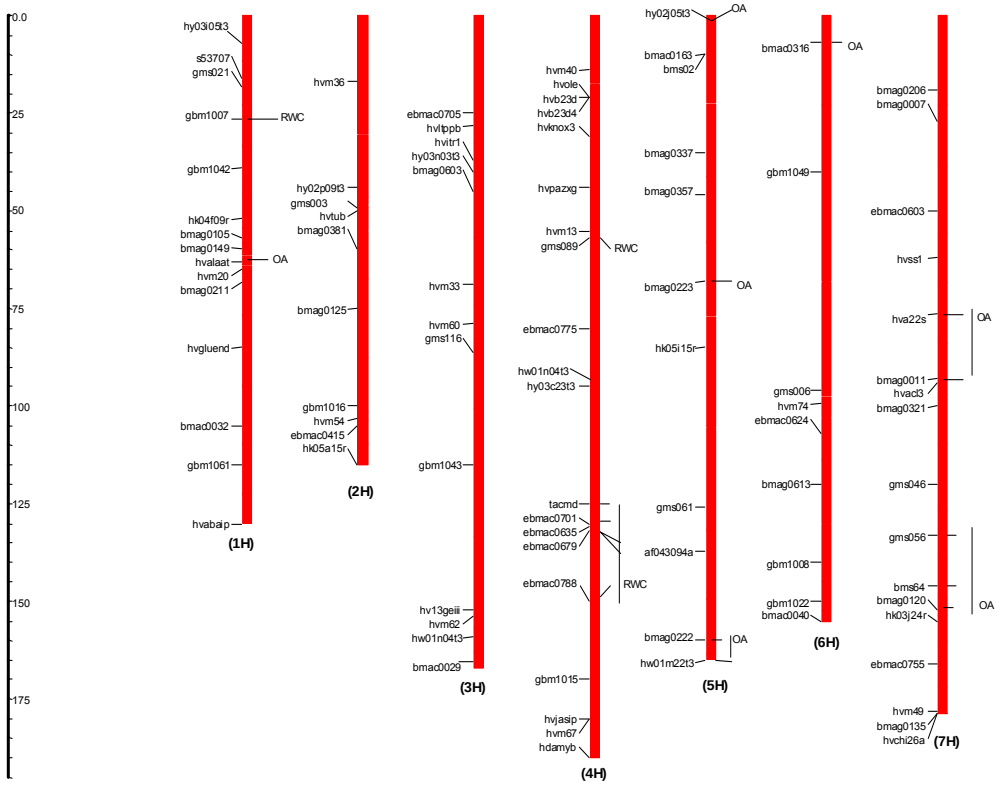

Figure 1: Linkage map containing drought related QTLs in spring barley (Scarlett*ISR42-8). The map contains 10 putative QTLs detected from the BC2DH cross Scarlett x ISR42-8. Putative QTLs which revealed either a significant ($P < 0.01$) marker main effect or M*D interaction are written to the right of the SSR locus. Adjacent marker effects (distance <20cM) are considered as one putative QTL.

Conclusion

We were able to demonstrate that exotic alleles from wild barley can potentially improve osmotic adjustment and relative water content and thus drought tolerance in cultivated barley. The established marker/trait associations allow the asssesment of drought responses at the level of the plant cells and organs. These markers can be used in the selection and transfer of the detected favourable exotic alleles into breeding programs. The population structure chosen for the QTL analysis proved very effective for the detection of exotic QTL. Only few cycles of marker assisted backcrossing will result in QTL-NILs (near isogenic lines) and these can be used for breeding a new drought tolerant variety.

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Growth, Morphological and Anatomical Responses of Two Barley Genotypes to Hypoxia

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Abstract

TX9425 (Chinese variety, waterlogging tolerant) and Naso Nijo (Japanese variety, waterlogging susceptible) were used to investigate the growth, morphological and anatomical responses of barley genotypes to hypoxia, produced experimentally by growing seedlings in de-oxygenated nutrient solution containing 0.1% (w/v) agar, with controls in aerated nutrient solution. Both varieties were subjected to hypoxia for 3 weeks starting from the four-unfolded-leaf stage. After the hypoxia treatment, the growth of seminal roots was reduced in both TX9425 and Naso Nijo, however, TX9425 showed less reduction in dry weight than Naso Nijo. Both varieties had a significant rise in emergence of adventitious roots after hypoxia treatment with TX9425 showing greater increase. Anatomical study of the adventitious root showed that aerenchyma was formed in the cortex in both genotypes after 3 weeks hypoxia treatment, and the percentage of aerenchyma area in the root cross-section area was larger for TX9425 than the corresponding value in Naso Nijo. Hypoxia significantly decreased the percentage of the root cross-section area occupied by the stele in both varieties, especially in TX9425. In plants suffering from hypoxia the ratio of the xylem area to root cross-section area also decreased in both varieties, with a more severe effect for Naso Nijo than for TX9425.

Introduction

Temporary waterlogging, mainly over winter, is widespread in Australia's dry land cropping environments as well as in irrigated areas in the southern regions of Australia (MOORE & McFARLANE 1998). Waterlogged soils are usually characterized by the absence of O_2 and a reduced chemical condition. Under normal conditions, water dissolves about 230 $mmol \cdot m^{-3}$ oxygen, and hypoxia occurs when the oxygen level falls below 50 $mmol \cdot m^{-3}$ within 1 h of flooding, the oxygen partial pressure declines from 20.8 to 7.9 kPa, and further decreases to 1 kPa after 1 d of flooding. Shortly after the onset of flooding, soil micro-organisms consume all of the available oxygen, and different toxin compounds begin to accumulate in the soil (GRICHKO & GLICK 2001).

In drained soils or aerated culture media, inward radial diffusion through the root surface may dominate; in waterlogged soil or stagnant culture solution, cortical gas-phase diffusion from shoot to root may be the only significant means of aeration and there may even be a substantial radial loss of oxygen from the root to the rooting medium (ARMSTRONG *et al.* 1994). Therefore, root growth into waterlogged soils depends upon an internal supply of O_2, which

moves from the atmosphere through aerenchyma in the plant to the root apex.
To improve the efficacy of internal aeration within a root and survival under hypoxic conditions, a series of adaptations may develop, including morphological characteristics, anatomical features, and physiological processes. In this paper, two varieties contrasting in waterlogging tolerance – TX9425 (tolerant) and Naso Nijo (susceptible) were studied to investigate the morphological and anatomical mechanisms involved in waterlogging tolerance.

Material and Methods

Plant Culture

Seeds of Naso Nijo and TX9425 were surface-sterilized by immersion in 1% NaOCl for 10 min and then washed thoroughly in flowing, distilled water for 5 min. The seeds were germinated in a darkened growth cabinet at 21-24 Cº in a petri dish, with the seeds placed between two sheets of filter paper moistened with ¼ strength nutrient solution. The full-strength nutrient solutions contained (mol m^{-3}): $MgSO_4$, 2.0; $Ca(NO_3)_2$, 5.0; KNO_3, 5.0; $NH_4H_2PO_4$, 1.0; together with micronutrients and iron-EDTA. After 3 d the germinated seedlings were transplanted into 9 L pots, each pot received 12 seedlings of one genotype, 10 pots of each genotype were established. Seedlings were grown in aerated ¼ strength nutrient solution during the first week and in the full strength nutrient solution in the following 18 days in a temperature-controlled glasshouse, the day/night temperatures were kept at 20 ±3Cº under natural sunlight conditions.

Oxygen Treatment

Twenty-eight-day-old TX9425 and Naso Nijo seedlings were treated with the de-oxygenated nutrient solution containing 0.1% (w/v) agar which has been shown to prevent convection in the nutrient solution (WIENGWEERA *et al.* 1997), thus simulating the impeded gas movements in waterlogged soils to provide a low O_2 treatment (hypoxia). Before transferring plants, 0.1% (w/v) agar nutrient solution was flushed overnight with N_2 gas to remove dissolved oxygen, with oxygen concentrations in the bulk solution being generally between 0.8 and 1.3 g m^{-3}. An equal number of plants (5 pots for each variety, 10 seedling each pot) were grown as controls in fresh and aerated nutrient solution for 3 weeks. During the growth period, both solutions were renewed weekly. The experiment was a two-factor (O_2 treatment, variety) completely randomized design with 5 replicates.

Root Anatomy

Adventitious roots of one plant of the same genotype from each pot were collected for the measurement, with five replicates of each genotype and treatment sampled. After vacuum-infiltrating the roots with water, freehand cross-sections of roots were cut every 2 cm, starting from 5 mm behind the root tip to the root-shoot junction. Sections mounted on slides were stained with Toluidine blue O (1% w/v in 1% w/v borax solution) for 30 s and photographed under a light microscope for anatomical features. The areas of aerenchyma in the root cortex, stele area, xylem area and total root cross-sectional area were determined with Scion Image analysis software (Scion Image program for windows Beta 4.02, US National Institutes of Health, Bethesda, MD, U.S.A.).

Statistical Analysis

Data on length of the longest seminal root, total length of adventitious roots, and root aerenchyma in waterlogged plants were analyzed using two-way analysis of variance (ANOVA). Data on stele and xylem were analyzed using three-way (genotypes, treatments, and position along roots) ANOVA. Differences between treatments were compared using the least significant differences (LSD) at the 0.05 level of probability.

Results and Discussion

Growth of Plants

After 3 weeks hypoxia treatment, the seminal roots in both TX9425 and Naso Nijo were affected, with the length of the longest seminal root significantly reduced compared with the control plants (Fig. 2A). The dry weight of seminal roots (SRDW) in Naso Nijo and TX9425 was only 34.3% and 48.0% of that in control plants (Fig. 1).

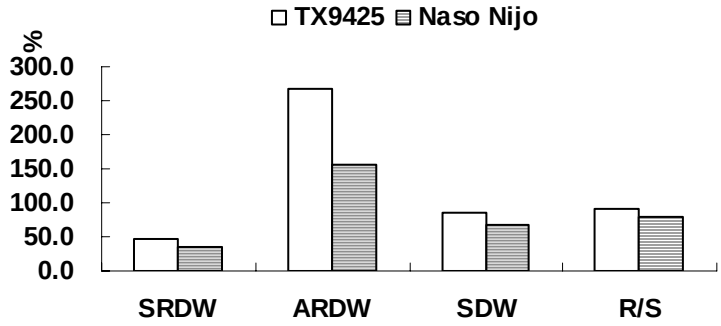

Fig. 1 Percentage of dry weight in hypoxia plants to the control after 3 weeks hypoxia treatment

Accompanying the death of the seminal roots, adventitious roots developed in both varieties. The total length of adventitious roots increased significantly (p=0.05) compared with the control, after 3 weeks hypoxia treatment in both TX9425 and Naso Nijo (Fig. 2B). The dry weight of adventitious roots (ARDW) increased to 154.8% and 267.1% of that for control plants in Naso Nijo and TX9425, respectively (Fig. 1).

Shoot dry weight (SDW) was adversely affected by 3 weeks hypoxia treatment in both varieties However, the adverse effect was more severe for Naso Nijo, which showed 68.3% of the control plants, compared with 86.6% of the control plants in TX9425 variety (Fig. 1).

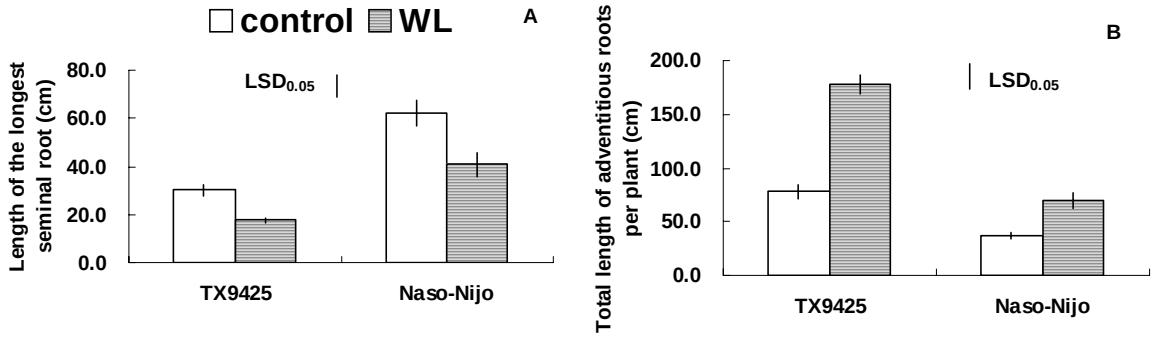

Fig. 2 Change of a) seminal root and b) adventitious root length growth

Compared with the control plants, the ratio of root to shoot (R/S, Fig. 1) weight decreased in hypoxia plants. The value for stressed plants was 79.3% and 90.5% of that for control plants in Naso Nijo and TX9425, respectively, showing that the root of Naso Nijo was more severely damaged compared with TX9425.

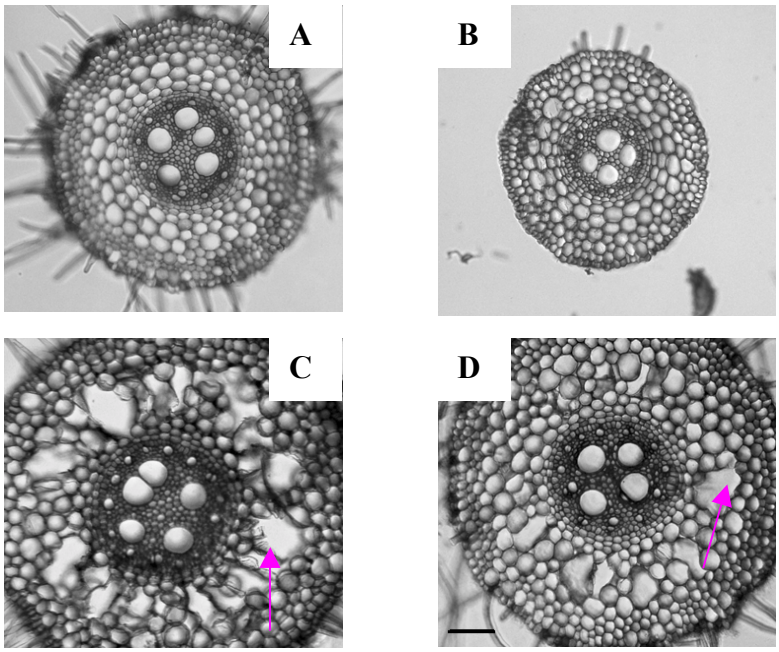

Plate. Light micrographs of transverse sections of adventitious roots, showing aerenchyma at 4.5 cm behind the root tip in (C) TX9425 and (D) Naso Nijo roots after three weeks of waterlogging. No aerenchyma was observed in adventitious roots of (A) TX9425 and (B) Naso Nijo in aerated plants. Bar = 100 μm for all.

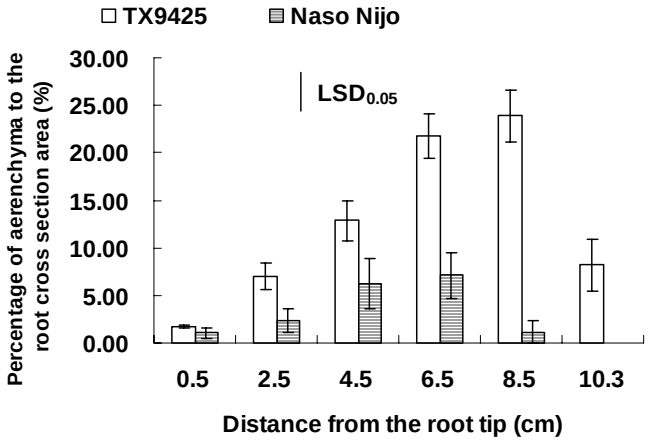

Fig. 3 Aerenchyma along the entire adventitious root

Aerenchyma Formation

Jackson and Armstrong (1999) reported that the key strategy used for long term waterlogging is the development of aerenchyma in roots to facilitate gas diffusion. In order to provide insight into the mechanisms underlying waterlogging tolerance in barley, the root anatomy was investigated in both varieties. For both TX9425 and Naso Nijo, cortical cell breakdown and the formation of air channels (aerenchyma) in adventitious roots were observed from 0.5

behind the root tip and persisted along the entire adventitious root axis in both genotypes after 3 weeks of waterlogging (Figure 3; Plates C and D). No aerenchyma was found in the root of drained plants (Plates A and B).

The percentage of aerenchyma to root cross-section area in TX9425 along the entire adventitious root was obviously much larger than that in Naso Nijo (Fig. 3). Aerenchyma at 0.5 cm behind the adventitious root tip accounted for only 1.7% and 1.0% of the root cross-section area for TX9425 and Naso Nijo, respectively. The proportion increased along the root, reaching a maximum of 23.9% and 7.1% at 8.5 cm and 6.5 cm behind the adventitious root tip for TX9425 and Naso Nijo, with an average root length of 10.3 cm and 8.5 cm, respectively. Both maxima occurred at about 2 cm from the root-shoot junction. At the root base, it reduced to 8.2% and 1.1% in TX9425 and Naso Nijo, respectively.

Stele

After three weeks hypoxia treatment, the percentage of the root cross-section area occupied by the stele along the entire adventitious root was significantly reduced for both genotypes compared with the value in the control plants, especially in TX9425 (Fig. 4). Roots with a small stele volume relative to the whole root would show enhanced longitudinal O_2 diffusion due to (i a proportionally lower O_2 consumption within the root, and (ii) a potentially greater cortex volume, the tissue in which aerenchyma is usually formed (McDONALD *et al.* 2002).

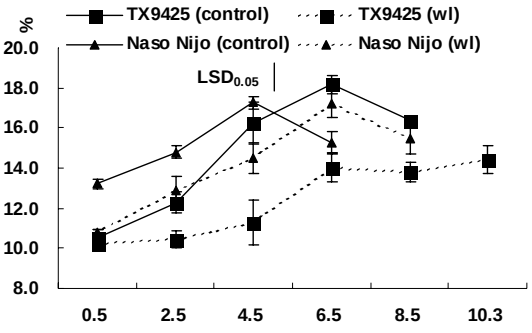

Fig. 4 Percentage of stele to the cross-section area in adventitious root

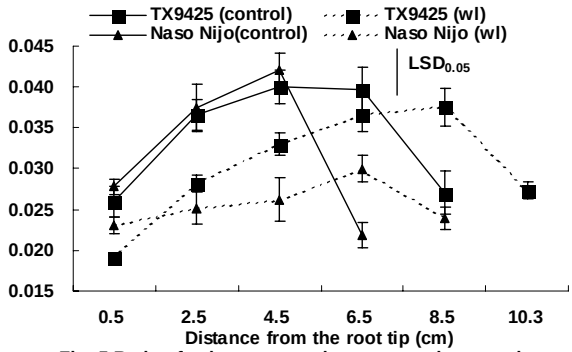

Fig. 5 Ratio of xylem area to the cross-section area in adventitious root

Xylem

The ratio of the root xylem area to the root cross-section area also significantly decreased in adventitious roots of waterlogged plants compared with the control. However, it was more adversely affected in Naso Nijo than TX9425 (Fig. 5). The decrease of the ratio could lead to a relative reduction in root axial conductance for water movement.

Acknowledgements

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QTL Mapping of Reproductive Frost Tolerance in Barley

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Abstract
Spring radiation frost is a major abiotic stress affecting broad acre grain production in Australia. Low temperatures during reproductive stages of cereal development can cause floret and spike abortion as well as damage to developing grain, significantly reducing yield and quality. No previous study has been successful in identifying genetic variation for reproductive frost tolerance in barley.
A survey of diverse germplasm collected from throughout the world was undertaken to identify genetic variation for reproductive frost tolerance in barley. Field based screening nurseries were developed to discriminate between reproductive frost tolerant and susceptible genotypes. Compared to Australian commercial varieties, two genotypes from Japan were identified to have significantly less frost induced sterility and grain damage during a discriminating frost event. Both Japanese lines were used to develop mapping populations from a cross with Australian frost susceptible varieties. Another Australian developed mapping population was found to be segregating for frost tolerance and included in the study. Frost induced sterility mapped to chromosome 2HL in the Japanese populations and 5HL in all three populations. The QTL on chromosome 2HL mapped distal to the 'earliness per se' *Eps2* locus. The QTL on chromosome 5HL mapped close to *Vrn*-H1 and the previously identified vegetative frost tolerance locus. This region is also homoeologous to the *Vrn*-A1, *Fr*-A1 region on wheat chromosome 5AL. The results suggest that this region on chromosome 5H conditions frost tolerance of both reproductive and vegetative plant tissue challenging the traditional view that these traits are independent.

Introduction

While there has been much research carried out into freezing tolerance during vegetative growth of barley, especially in the northern hemisphere, no comprehensive study has been undertaken to determine whether there is genetic variation for spring radiation frost tolerance. This is the most common form of frost damage in barley in Australia and can damage crops from head emergence through to late grain fill.

Plant breeders have made little progress in reproductive frost tolerance due to spatial variation of field trials and overriding maturity effects which favours frost avoidance rather than true tolerance. Since factors such as maturity can confound comparisons between genotypes QTL analysis can be used to separate effects of maturity and plant type from true frost tolerance. Using markers closely linked to QTL, marker-assisted selection may improve the efficiency of frost tolerance screening by reducing these confounding effects.

Material and Methods

Genetic Variation for Spring Radiation Frost Tolerance

A survey of diverse germplasm collected from throughout the world was undertaken to identify genetic variation for reproductive frost tolerance in barley. The genotypes collected included landraces, wild barley introgressions, winter types, Australian varieties and mapping

population parents. Two sites prone to radiation frost at Loxton and Black Rock in South Australia, were selected to locate reproductive frost tolerance screening nurseries. Early seeding times in March/April, compared to the normal of May/June, were used to encourage flowering during the peak frost risk period (July). Irrigation was used to promote early growth and to supplement rainfall events during the growing season. Four seeding times were required to allow for maturity differences between genotypes and to collect data from multiple frost events during the season. Each entry was sown in a single 1.5m row/seeding time. After each frost event, ten tillers/entry within each Decimal Growth Stage (DGS) (ZADOKS *et al.* 1974) maturity class in the range of DGS59-DGS77 were tagged. Ten to twenty days after a frost event, tagged heads were assessed on percentage sterility and grain damage. A discriminating frost event occurred on the 30th of June 2001, where temperatures reached –4.6°C at crop canopy height causing extensive damage to genotypes in the Loxton frost screening nursery. ANOVA was used to analyses genotype effects on Frost Induced Sterility (FIS), Frost Induced Grain Damage (FIGD) and Total Frost Induced Damage (TFID).

QTL Mapping

The Arapiles/Franklin mapping population was grown in a randomised complete block design with two replicates at Horsham, Victoria. Each entry was sown in a 6 meter × 6 row (150mm spacing) plot on the 29th of June 2001. A frost event on the 11th of October 2001, where the minimum temperature was –1.0 °C at Stevenson screen, caused extensive sterility and grain damage. Ten heads were randomly sampled from field plots of both replicates of the Arapiles/Franklin mapping population after all genotypes had reached physiological maturity. This material was then assessed for FIS and FIGD. ANOVA was used to analyse genotype effects on frost damage and means used for QTL analysis.

The Galleon/Haruna Nijo and Amagi Nijo/WI2585 mapping populations were sown at Loxton S.A. in April 2002 at multiple seeding times. On the 2nd of July the minimum canopy temperature of –4.5 °C was recorded. On the 21st of July a minimum canopy temperature of –2.8 °C was recorded. Both events caused damage to reproductive tissue of genotypes post spike emergence. Data was analysed using ANOVA and means analysed using Qgene version 3.04 with a LOD significance threshold of 3.

Results and Discussion

Genetic Variation for Frost Tolerance

A significant genotype effect ($P<0.001$) on FIS was observed at the Loxton frost tolerance screening nursery during the 2001 season. The Japanese varieties Amagi Nijo and Haruna Nijo had significantly lower frost-induced sterility than Australian commercial varieties (Figure 1). The performance of these lines was assessed again in the 2002 season where a subset of the original germplasm was re screened (Figure 2). Mapping populations had been developed from the crosses Amagi Nijo/WI2585 and Haruna Nijo/Galleon with parents differing significantly in frost-induced sterility. This result suggested that genetic variation for frost tolerance existed and that the trait may be segregating in at least two of the Australian developed mapping populations.

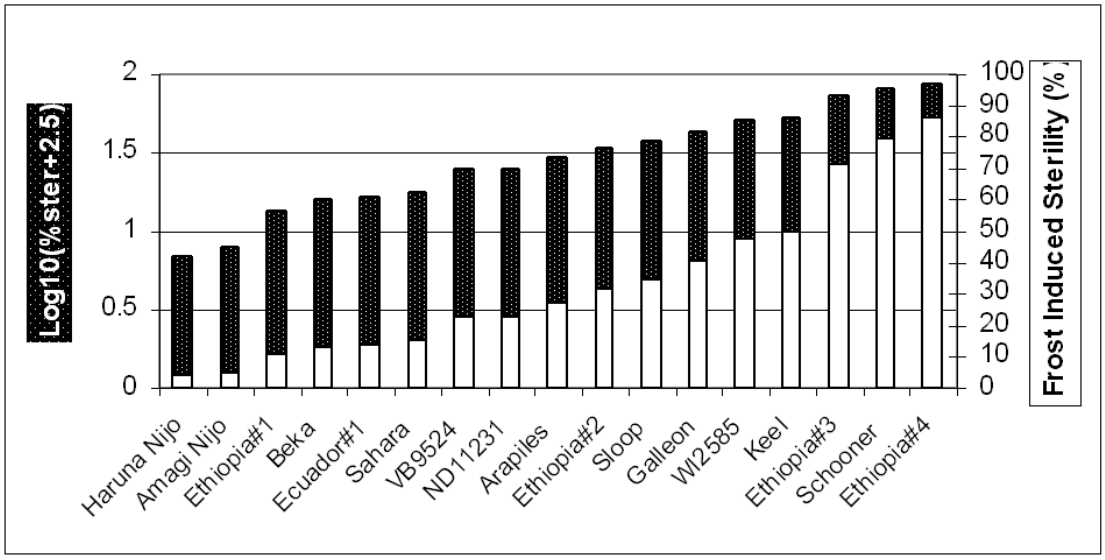

Figure 1. Frost induced sterility observed on barley lines during the 2001 season at Loxton, South Australia. L.S.D.=0.5 and relates to the log converted data (left axis). Raw % frost induced sterility represented by the white bars on right axis.

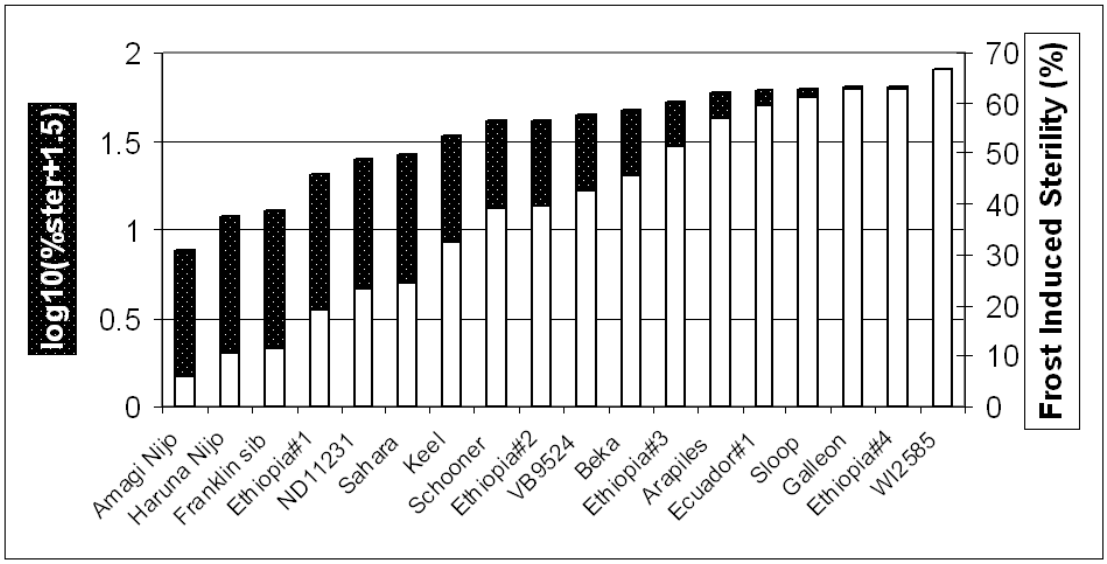

Figure 2. Frost induced sterility observed on barley lines during the 2002 season at Loxton, South Australia. L.S.D.=0.2 and relates to the log converted data (left axis). Raw % frost induced sterility represented by the white bars on right axis.

QTL Mapping Frost Tolerance

Due to maturity effects in the whole Arapiles/Franklin mapping data, a sub set of the population was selected for QTL analysis. Based on maturity data collected from the population, a reduced maturity effect was observed when mapping frost tolerance traits using the sub population. A significant ($P<0.001$) genotype effect on grain damage and total frost damage was observed. FIGD and TFID mapped to chromosome 5HL with the Arapiles allele contributing to the higher damage score indicating that Franklin was the more tolerant parent (Table 1). A significant genotype effect ($P<0.001$) for FIS was observed for both the Galleon/Haruna Nijo and Amagi Nijo/WI2585 mapping populations. In both populations FIS mapped to 5HL and 2HL (Table 1).

Table 1. QTL analysis of Frost Induced Grain Damage (FIGD), Total Frost Induced Damage (TFID) and Frost Induced Sterility (FIS) in the mapping populations Arapiles/Franklin (A/F), Galleon/Haruna Nijo (G/HN) and Amagi Nijo/WI2585 (AN/WI) grown at Horsham, Victoria and Loxton, South Australia in 2001 and 2002. Note: Parent specified is the least tolerant.

Population	Trait	Location	Date	Chromosome	Marker	LOD	R^2	Parent
A/F	FIGD	Horsham	11/10/2001	5H	Hv635P2.4f	3.9	0.19	Arapiles
A/F	TFID	Horsham	11/10/2001	5H	Hv635P2.4f	4.3	0.20	Arapiles
G/HN	FIS	Loxton	21/07/2002	2H	HVM54	5.0	0.42	Galleon
G/HN	FIS	Loxton	21/07/2002	5H	AWBMA13b	3.5	0.44	Galleon
AN/WI	FIS	Loxton	02/07/2002	5H	MWG514	5.9	0.32	WI2585
AN/WI	FIS	Loxton	21/07/2002	2H	HVM54	3.4	0.18	WI2585
AN/WI	FIS	Loxton	21/07/2002	5H	BCD265a	4.5	0.26	WI2585

Mapping data collected from the three populations in this study indicated that two chromosomes, 2H and 5H, were implicated in genetic control of the reproductive frost tolerance traits. Using a bin map system, based on the Steptoe/Morex population (KLEINHOFS and HAN 2002), the relative location of QTL in the three different populations was identified (Figure 3).

QTL identified in the Amagi Nijo/WI2585 and Galleon/Haruna Nijo populations that mapped to chromosome 2HL were located in the same bin location (Figure 3). This locus in both populations was independent of all other traits mapped including developmental and other stress response traits. In other mapping populations, few traits have been identified as significantly associated with this bin location on 2HL. These traits include ear weight, thousand grain weight and hot water extract from the Blenheim/Kym population (BEZANT *et al*. 1997); height and lodging from the Steptoe/Morex population (HAYES *et al*. 1993b); screenings from the Sloop/Alexis population (COVENTRY *et al*. 2003). Although these traits reported in bin 13 can be affected by developmental loci, heading date data collected on the three populations indicated that major developmental loci were in other genomic locations. With no evidence of a major developmental or stress responses in this location, it is possible that this is a stress response locus not previously identified. Chromosome 2HL has been reported as the location of cold regulated genes cor14b and blt14 (FRANCIA *et al*. 2003; CATTIVELLI *et al*. 2002b). Their reported location in bin 9 is proximal to the frost tolerance QTL identified in this study (Figure 3).

The group 5 chromosomes belonging to the tribe *Triticeae* have an important role in abiotic stress tolerance in a number of crops (CATTIVELLI *et al*. 2002a). The region on the long arm of the group 5 chromosomes, where the syntenous *Vrn* genes are located, have been identified as being associated with cold tolerance traits in wheat (SUTKA 2001; TOTH *et al*. 2003) and barley (HAYES *et al*. 1993a; PAN *et al*. 1994; FRANCIA *et al*. 2003). These studies have predominantly focused on leaf and plant survival at the vegetative stage after an extended period of hardening. We have demonstrated that this region is also important in conferring tolerance to frost at the flowering and grain filling stages in barley. The syntenous group 5 chromosomal region may now indicate that an increased tolerance to reproductive frost tolerance in wheat may also reside at this location.

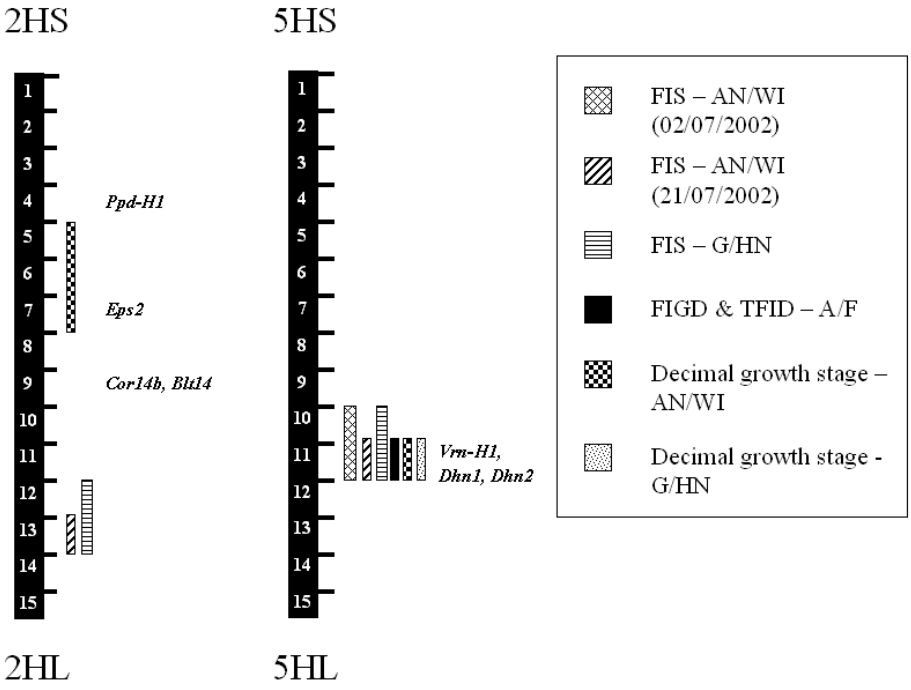

Figure 3. A schematic bin map (KLEINHOFS and HAN 2002) of barley chromosomes 2H and 5H showing locations of QTL for Frost Induced Sterility (FIS), Frost Induced Grain Damage (FIGD), Total Frost Induced Damage (TFID) and decimal growth stage for the Australian mapping populations Amagi Nijo/WI2585 (AN/WI), Galleon/Haruna Nijo (G/HN) and Arapiles/Franklin (A/F). QTL (LOD>3) are represented by vertical bars. Candidate developmental and cold regulated gene loci are in italics, positioned based on reported locations (LAURIE *et al.* 1995; CATTIVELLI *et al.* 2002).

Marker Assisted Selection for Reproductive Frost Tolerance

Current selection methods for reproductive frost tolerance in barley are based on field based phenotypic observations after a frost event. This selection method can be prone to error due to within site variation, seasonal variation, confounding effect of developmental and morphological traits and the sporadic nature of discriminating frost events. Therefore, the use of phenotypic selection in a breeding program, especially on segregating populations, is limited due to the low throughput, high risk and expense. Consequently, very few breeding programs have attempted to improve reproductive frost tolerance. Marker Assisted Selection (MAS) provides a tool for identifying individuals in a segregating population carrying favourable alleles for frost tolerance at the 2HL and 5HL loci with high throughput and relatively low cost. Validation of these QTL will be achieved by integrating them into genetic backgrounds of Australian adapted genotypes. We have now commenced breeding programs where this trait will be actively pursued via genotypic and phenotypic selection.

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Australian farmers and the Commonwealth Government through the Grains Research and Development Corporation.

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Root-Specific *O*-Methyltransferase Gene Expressed in Salt-Tolerant Barley

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Abstract
A cDNA encoding an *O*-methyltransferase (OMT) was isolated from salt-tolerant barley roots by subtraction hybridization. The deduced amino acid sequence showed 41and 21% identity with methyl jasmonate-inducible barley OMT and pathogen-inducible barley OMT, respectively. Southern blot analysis showed that the OMT gene was a single-copy in both salt-tolerant and –sensitive barley genomes and nucleotide sequence of 5'-flanking region of the OMT gene from the tolerant barley was almost same as that from the sensitive barley. Northern blot analysis revealed that the OMT gene was expressed in the roots but not in the leaves and was induced specifically in salt-tolerant barley under the exposure to NaCl.
Keywords: *Hordeum vulgare* L.; *O*-methyltransferase; subtraction hybridization; salt stress; root

Introduction

O-Methylated flavonoids and derivatives play an important role not only in plant growth but also in interactions with environmental factors as regulators of auxin transport, signal molecules in plant-symbiont interactions, precursors of phytoalexins, and precursors of lignin biosynthesis (JACOBS & RUBERY 1988; LONG 1989; DIXON *et al*. 1983; DAKORA & PHILLIPS 1996; LEWIS & YAMAMOTO 1990). Enzymatic *O*-methylation is catalyzed by *O*-methyltranserase [EC 2.1.1.6] (OMT), which transfers the methyl group of *S*-adenosyl-*L*-methionine to the hydroxyl group of an acceptor molecule (POULTON 1981). The OMT gene from barley was induced by fungal pathogens and UV light (GREGERSEN *et al*. 1994) and the gene from ice plant is highly expressed under the exposure to salt stress (NELSON *et al.* 1998). These results expect that the OMT would function in protecting cells from environmental stresses in plants. A variety of genes have been reported to respond to environmental stresses, however, it is important to confirm the type of the stress-inducible genes whether the gene product functions in stress tolerance or the gene is just induced without contributing to stress tolerance. The evaluation of the genes whether the preferential expression in the stress-tolerant plants rather than the stress-sensitive plants is necessary for identify the genes which would possibly play roles in conferring the stress tolerance to plants. In this paper, we present an OMT gene expressed specifically in salt-tolerant barley roots, which is different from those isolated from barley as reported previously (GREGERSEN *et al*. 1994; NELSON *et al*. 1998).

Material and Methods

Plant Materials. Seeds of salt-tolerant barley (*Hordeum vulgare* L.), OUK305, and salt-sensitive barley, OUI743, which were characterized by MANO (1996), were germinated and cultured in a hydroponic solution which consists of 4 mM KNO_3, 1 mM NaH_2PO_4, 1mM $MgSO_4$, 1mM $CaCl_2$, 1 mg/ml Fe-citrate, pH 5.5 (KATSUHARA *et al*. 2001). Plants were

transferred to the culture solution containing 100 mM NaCl and incubated for 12 h. After 12 h incubation, the roots and leaves were harvested, frozen in liquid nitrogen, and stored at –80^{O}C.

DNA, RNA, and cDNA Preparation. Genomic DNA was isolated from leaves of barley by the method of Fang *et al* (1992). Total RNA was isolated by the guanidium isothiocyanate method and poly(A)$^{+}$ RNA was separated from total RNA with Dynabeads Oligo(dT)$_{25}$ (Dynal). cDNA for subtraction hybridization and virtual northern blot (ENDEGE *et al.* 1999) was synthesized and amplified using the SMART PCR cDNA Synthesis Kit (Clontech). Poly(A)$^{+}$ RNA was reverse-transcribed at 42 ^{O}C for 1 h with cDNA synthesis primer and SMART II oligonucleotide. The reaction mixture was amplified with the "PCR primer" supplied in the kit according to the manufacturer's instruction.

Northern Hybridization. One μg of cDNA for virtual northern blot was electrophoresed on 1% agarose gel and transferred onto Hybond-N^{+} nylon membrane (Amersham Biosciences). The membrane was hybridized with a fragment of the OMT cDNA labeled with digoxigenin-11-dUTP using a PCR DIG Labeling Kit (Boehringer Mannheim) with primers 5'-TGAGTGCGCAGACGGGAGCCTGTCC-3' and 5'-GGTGTACTCCATTGCCCAGAAGTT-CGC-3'. Hybridization was performed for 16 h at 42 ^{O}C in 50% formamide, 5 x SSC, 0.25% non-fat milk powder, 0.1% SDS, 5 x Denhardt's reagent. The membrane was washed twice with 1 x SSC, 0.1% SDS at room temperature, and twice with 0.1 x SSC, 0.1% SDS at 68 ^{O}C.

Subtraction Hybridization and Generation of Subtracted Library. Subtraction hybridization (Diatchenko *et at*. 1996; Gurskaya *et al*. 1996) was performed using the PCR-Selected^{TM} cDNA Subtraction Kit (Clontech). Tester and driver cDNAs were prepared from total RNAs isolated from roots of OUK305 and OUI743 exposed to 100mM NaCl for 12 h, respectively. The enriched cDNA fragments were cloned into a pGEM-T vector (Promega) and transformed into *Escherichia. coli* XL1-Blue cells. Colonies were hybridized with the driver cDNA labeled with digoxigenin-11-dUTP as described above and colonies displaying reduced signal were selected as the OUK305 subtracted library.

RACE-PCR. The primers for 3'- and 5'-RACE PCR, 5'-TACAGGCGTCAAGGCCA-GCTACATCT-3' and 5'-GGCTAGCTTCTCGAAGTCCCTGAGGT-3', respectively, were synthesized on the basis of the sequence of the gene fragment isolated from the subtracted library. RACE-PCR was carried out on the cDNA and the PCR products were cloned into a pGEM-T vector (Promega). The nucleotide sequence on both strands was determined by using a BigDye Thermal Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems) with a series of synthetic primers.

Isolation of 5'-Flanking Region of OMT Gene. Genomic DNA was digested separately with *Dra* I, *Eco*R V, *Pvu* II, *Sca* I and *Stu* I, respectively. Each digested DNA was ligated separately with an adaptor provided in Universal GenomeWalker Kit (Clontech). Each adaptor-ligated DNA library was subjected to PCR with a primer 5'-GGTTTCGAAGCTCC-AGTTATGCG-3' and an adaptor primer (AP1) provided in the kit. The PCR product was cloned into a pGEM-T vector and sequenced on both strands as indicated above with a series of synthetic primers.

Results and Discussion

A cDNA fragment preferentially amplified from mRNA of OUK305 roots treated by NaCl was obtained by colony hybridization. The nucleotide sequence of the fragment showed significant identity to plant OMT genes. To obtain the complete nucleotide sequence of the gene, two cDNA fragments were amplified by RACE-PCR with the specific primes and cDNA synthesized from the roots of OUK305 treated by NaCl. The resulting nucleotide sequence, except for the poly(A) sequence, is 1370 bp in length and contains an open reading frame encoding a polypeptide of 352 amino acid residues with a calculated molecular mass of 38.2 kDa (accession number: AB086416). The deduced amino acid sequence of the cDNA showed high identity with those of plant OMTs. OMTs from *Lolium perenne* (accession number: AF033539), *Festuca arundinacea* (accession number: AF153824), *Saccharum officinarum* (accession number: AJ231133), *Zea mays* (accession number: M73235), *Populus tremuloides* (accession number: X62096), *Populus kitakamiensis* (accession number: D49710) *Nicotiana tabacum* (accession number: X74453) and *Clarkia breweri* (accession number: AF006009) had 79, 68, 62, 62, 55, 55, 55 and 54% identity with the deduced protein, respectively. Among barley OMTs, JRG5 protein induced by methyl jasmonate (LEE *et al*. 1997) and HVOMET protein induced by fungal pathogens and UV light (GREGERSEN *et al*. 1994) showed 41 and 21% identity with the deduced protein, respectively. Figure 1 illustrates the alignment of OMTs from monocot plants. On five consensus regions comprising 36 amino acid residues among the majority of plant OMTs, 32 of 36 amino acid residues are conserved in the amino acid sequence of the deduced protein (IBRAHIM 1997; IBRAHIM *et al*. 1998). The gene that encodes the novel OMT was designated as *Sub23*.

The differential expression of *Sub23* gene was analyzed by northern blotting with the cDNA of *Sub23* as a probe (Fig. 2). Our northern blot detected a single 1.4 kb band in the OUK305 roots by both 0 and 12 h NaCl treatment. In OUK305 roots exposed to NaCl for 12 h, densitometry indicated that the OMT mRNA level in total RNA was increased about 1.5 times when compared to OUK305 roots before exposing to the stressful condition. A weak hybridizing band was observed in OUI743 RNA that exposed to NaCl for 12 h. No band was detected in leaves of OUK305 and OUI743 before and after NaCl treatment. It has been reported that *HVOMET* transcript was induced in pathogen- and UV light-treated barley, but not observed in unstressed barley roots (GREGERSEN *et al*. 1994). *JRG5* transcript was found in unstressed barley leaf sheath and induced in methyl jasmomate-treated barley leaves, but not detected after treatment with salicylic acid, sorbitol, NaCl, desiccation, cold stress, or heat stress (LEE *et al*. 1997). These results show that the OMT gene is responsible for barley defense against environmental stress, while organs where the gene is expressed and biological factors by which the gene is induced are different among three barley OMT genes. Furthermore, our result that *Sub23* mRNA is specifically expressed in the roots of salt-tolerant barley is interesting to investigate the molecular mechanisms of salt tolerance because the root is the organ of land plants mostly affected by salt stress, dehydration stress, and drought stress.

Salt, drought, and cold stress-inducible genes at the transcriptional level have been reported and promoter analysis of those genes has identified several promoter regulatory elements and proteins that bind to promoter regulatory elements (SHINOZAKI & YAMAGUCHI-SHINOZAKI 1997). To clarify the induction mechanism of *Sub23* gene, we determined and characterized the nucleotide sequences about 1.2 kb at the 5'-flanking regions of *Sub23* gene from OUK305 and OUI743 (Fig. 3). There are 17 mismatched nucleotides between the 5' regions of OUK305 and OUI743, and 2 deleted nucleotides in the 5' region of OUI743. Computer analysis predicted that the transcription start site is G that is same as the 5'-end of the cDNA. Neither upstream region displayed a consensus CAAT box, however,

both had a TATA box. One MYB.Ph3 motif (Solano *et al*. 1995) (score 94) was found in each 5'-flanking region of OUK305 and OUI743. This result suggests that there is some salt-inducible biosynthesis of protein factors that induce *Sub23* gene expression in salt-tolerant barley, OUK305.

OMT is one of the essential enzymes for lignin biosynthesis (LEWIS *et al*. 1990). The induction of OMT gene by wounding or pathogen invasion suggests the deposition of lignin that provides a barrier for protection of tissues from damage. In the present study we showed the induction of the OMT gene by salt stress in the salt-tolerant barley. The OMT gene is also induced by salt stress in the salt-tolerant plant, the ice plant (NELSON *et al*. 1998). Although it has not been clear what kind of a role the OMT plays to acquire the tolerance against salt stress, these results suggest that the OMT gene would be a good candidate component of anti-environmental stress system in plants.

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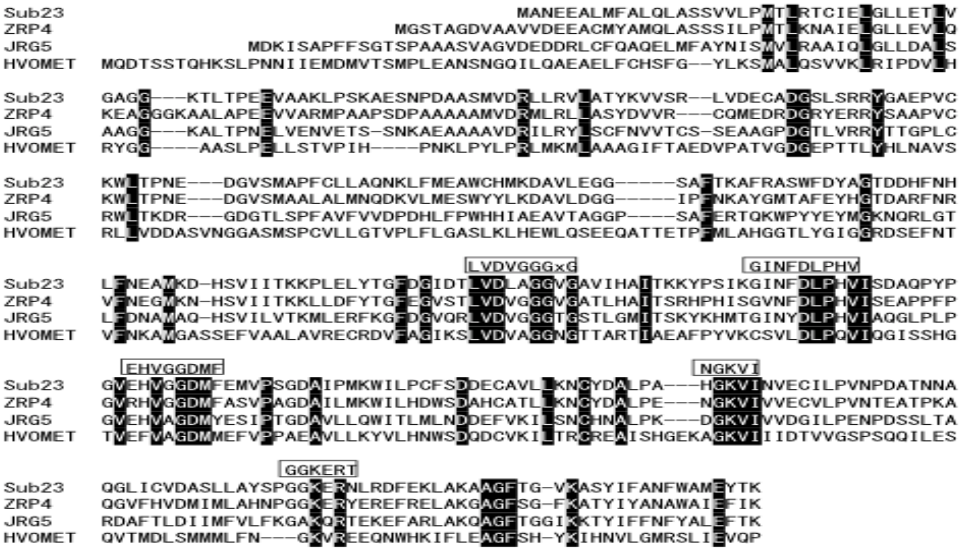

Figure 1. Alignment of the deduced amino acid sequence of Sub23 protein with those of OMTs from monocot plants. The alignments were generated by the CLUSTAL W algorithm (TOMPSON *et al*. 1994). Gaps, indicated by a dash, are introduced in the sequences to maximize the homology. Identical amino acid residues among monocot OMTs are represented by reversal letters. The amino acid sequences of OMT consensus regions are boxed. ZRP4 , maize OMT expressed in roots (Held *et al*. 1993); JRG5, barley methyl jasmonate-inducible OMT; HVOMET, barley fungus-inducible OMT.

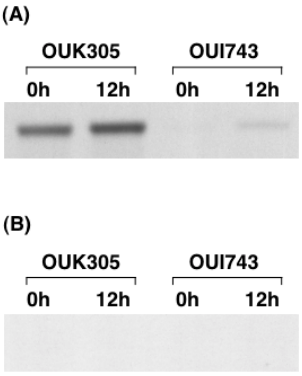

Figure 2. Expression profiles of *Sub23* gene in salt-tolerant barley OUK305 and -sensitive barley OUI743 exposed to NaCl using virtual northern analysis. cDNA samples prepared from roots (A) and leaves (B) of OUK305 and OUI743 exposed to NaCl for 0 and 12 h were electrophoresed on 1% agarose gel and transferred onto a nylon membrane. The membrane was hybridized with a fragment of *Sub23* cDNA.

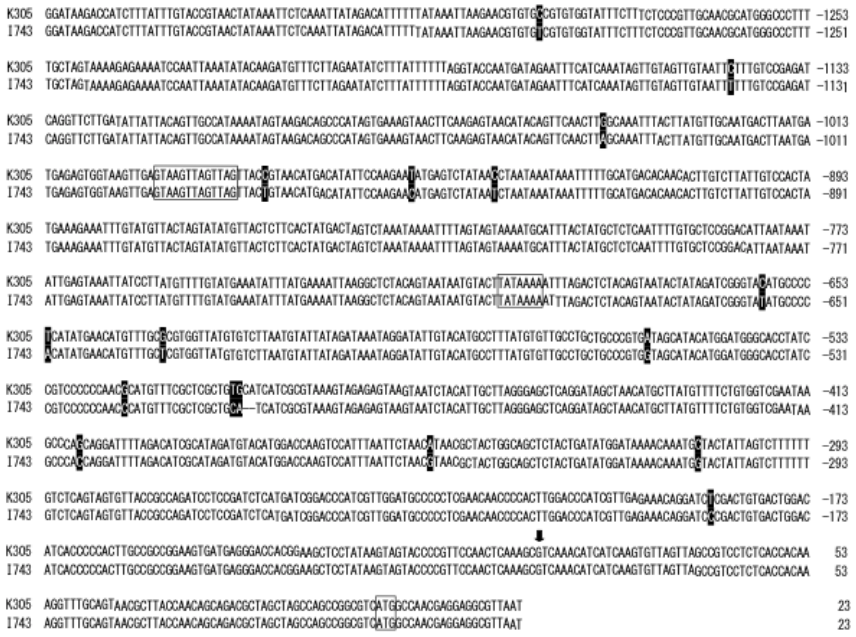

Figure 3. Nucleotide sequences of the 5'-flanking regions of *Sub23* genes from salt-tolerant barley OUK305 (K305) and -sensitive barley OUI743 (I743). Nucleotide residues are numbered relative to translation start site as +1. Deleted and mismatched nucleotides are indicated by a dash and a reversal letter, respectively. Presumptive a TATA box, a motif similar to MYB.Ph3, and a translation start-methionine codon are boxed. A putative transcription start site is indicated by an arrowhead.

Differential Expression of Dehydrin Genes under Water Stress in Wild Barley, *Hordeum spontaneum*

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Abstract

Dehydrin gene (*Dhn*) expression is associated with plant response to dehydration. Our aim was to study the association of *Dhn* gene *(Dhn 1, 3, 5, 6,* and *9*) expression with drought tolerance found in wild barley (*Hordeum spontaneum*). Tolerant and sensitive genotypes were identified from Israeli (Tabigha microsite) and Jordanian (Jarash and Wadi Hassa) populations (based on scoring of water loss rate of 400 genotypes). The expression of the five *Dhn* genes was up-regulated by dehydration in both resistant and sensitive wild barley genotypes. Notably, differences between resistant and sensitive genotypes were detected, mainly in the expression of *Dhn1* and *Dhn6* genes, depending on the duration of dehydration stress. Resistant genotypes showed earlier expression of *Dhn1* (after 3 h) and higher levels of expression (12 h and 24 h) as compared to sensitive genotypes. The level of expression of *Dhn6* was significantly higher in the resistant genotypes in the earlier stages after stress. However, after 12 and 24 h *Dhn6* expression levels were relatively higher in sensitive genotypes. Our results may indicate that these genes have some functional role in the dehydration tolerance of wild barley. We suggest that the observed differences of *Dhn* expression in wild barley, originating from different micro- and macroecogeographic locations, may be the result of edaphic and climatic selective pressures.

Keywords: drought; dehydrin genes; quantitative PCR; local adaptation; edaphic selective pressure; ecogeographic location

Introduction

Water deficit is considered among the most severe environmental stresses that can cause water stress, often referred as to drought, and has an immediate effect on plant growth and yield. Plants respond to water stress through multiple physiological mechanisms at the cellular, tissue, and whole-plant levels (SMITH & GRIFFITHS 1993). Dehydrins (DHNs), peripheral membrane proteins that function in the physical protection of the cell from water deficit or temperature change, are among the most frequently observed proteins in plants under water stress. Most of the *Dhn* genes are up-regulated by environmental stresses as drought, salinity, low temperature, or application of abscisic acid (ABA) (CLOSE 1997). An association between tolerance to drought, freezing and salinity with accumulation of members of the *Dhn* family has been established in some crop species (LABHILILI *et al*. 1995; PELAH *et al.* 1997; CELLIER *et al*. 1998).

Wild barley, *H. spontaneum,* the progenitor of cultivated barley, is one of the major genetic recourses of plant tolerance to stressful environments (HARLAN & ZOHARY 1966; NEVO 1992). The wide ecological range of wild barley differs in water availability, temperature, soil type, altitude, and vegetation generating a high potential of adaptive diversity to abiotic stresses. Genetic diversity and physiology in *H. spontaneum* from Israel and Jordan have been studied previously at micro- and macrogeographic scales (NEVO *et al*. 1979; OWUOR *et al*. 1999; TURPEINEN *et al*., 2003; BAEK *et al.* 2003; IVANDIC *et al.* 2000). The adaptive genetic diversity that was found indicates that wild barley is a potential source for drought resistance alleles for breeding purposes. In this study we focus on the differential expressions

of dehydrin genes in wild barley from the Mediterranean and Irano- Turanian regions, which is associated with resistance to dehydration.

Material and Methods

Plant Material and Drought Treatment

Selection of the most contrasting wild barley genotypes: Two approaches were used: (***i***) large-scale screening of water loss rate (WLR) was conducted using 400 wild barley genotypes from diverse ecogeographic regions of Israel and Jordan. As a result of this screening, two 'putatively sensitive' genotypes from Wadi Hassa (JS1 and JS2) and two 'putatively resistant' genotypes from Jarash (JR1 and JR2) were chosen for gene expression analysis; ***(ii***) two additional genotypes, one resistant from Terra Rossa (TR) soil and one sensitive from Basalt (BA) soil, were chosen based on the evaluation of 15 agronomic, morphological, developmental, and fertility related traits from the Tabigha microsite (IVANDIC *et al*. 2000).

Drought stress: Seedlings were grown in a greenhouse at 22^{o}C, (12h light/12h dark), in Murashige and Skoog basal salt (MS) solution, circulated by air pumps. Drought stress was applied at ten-day-old seedlings by draining the solution from the container. Leaf tissues of two seedlings per genotype were harvested from control plants (time 0); and after 3, 12, and 24 hours of dehydration, frozen in liquid nitrogen, and stored at –80^{o} C for RNA extraction.

Expression Analysis

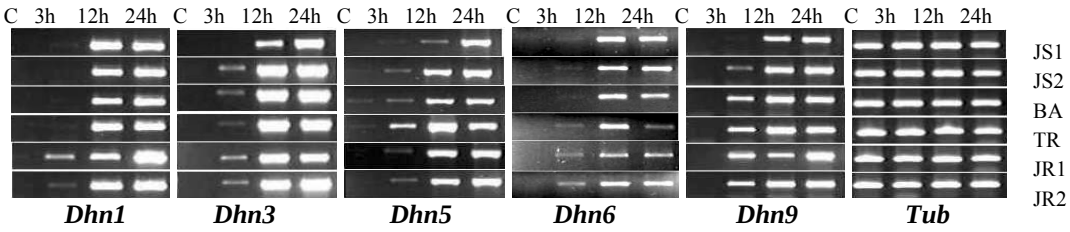
RNA Isolation and RT-PCR: Total RNA was extracted using EZ-RNA Total RNA Isolation Kit (Biological Industries LTD, Israel). RNA was treated by RNase-free DNase I (Ambion) for removal of template DNA. First-strand cDNA was prepared using Oligo(dT)$_{15}$ and SuperScript II reverse transcriptase (GIBCO-BRL). Single-strand cDNA was amplified with gene-specific primers (Choi et al. 1999). Barley α–*tubulin* gene was amplified with specific primers as an internal control for the relative amount of RNA.

Quantitative Real-Time PCR Analysis: Gene quantification was performed using ABI PRISM 7000 Sequence Detection System and SYBR Green PCR Master Mix (Applied Biosystems, USA). Specific primer pairs were designed based on sequences (Access.No AF043087, AF043S091) from NCBI. Calculation is based on the intensity of the reporter dye fluorescence in the threshold cycle (Ct) of each sample interpolated to the standard curve. In order to account for differences in target RNA presented in each sample, both *Dhn1* and *Dhn6* gene quantities were normalized to the barley α–*tubulin* as an internal housekeeping gene. Two independent plant samples for each genotype were examined in triplicate. Data are shown as mean value ± S.E.M

Results and Discussion

The expression characteristics of five *Dhn* genes were studied by RT-PCR in 6 wild barley genotypes that were defined as 'sensitive' and 'resistant' by leaf WLR. Differences in expression patterns were found in each of the *Dhn* genes depending on duration of dehydration stress (Fig. 1). RT-PCR analysis showed that *Dhn1, 3, 6,* and 9 were not expressed in well-watered plants; low-expression levels of *Dhn5* were detected in TR- and BA-genotypes. No differences in expression of *Dhn3* and *Dhn9* were found between the resistant and sensitive genotypes after 3 h, 12 h and 24 h drought stress, with the exception of genotype JS1. *Dhn 1*, *5*, and *6* displayed differences in expression levels between resistant and sensitive genotypes. Resistant genotypes showed earlier expression of *Dhn1* (after 3 h) and higher level of expression (12 h and 24 h) as compared to sensitive genotypes.

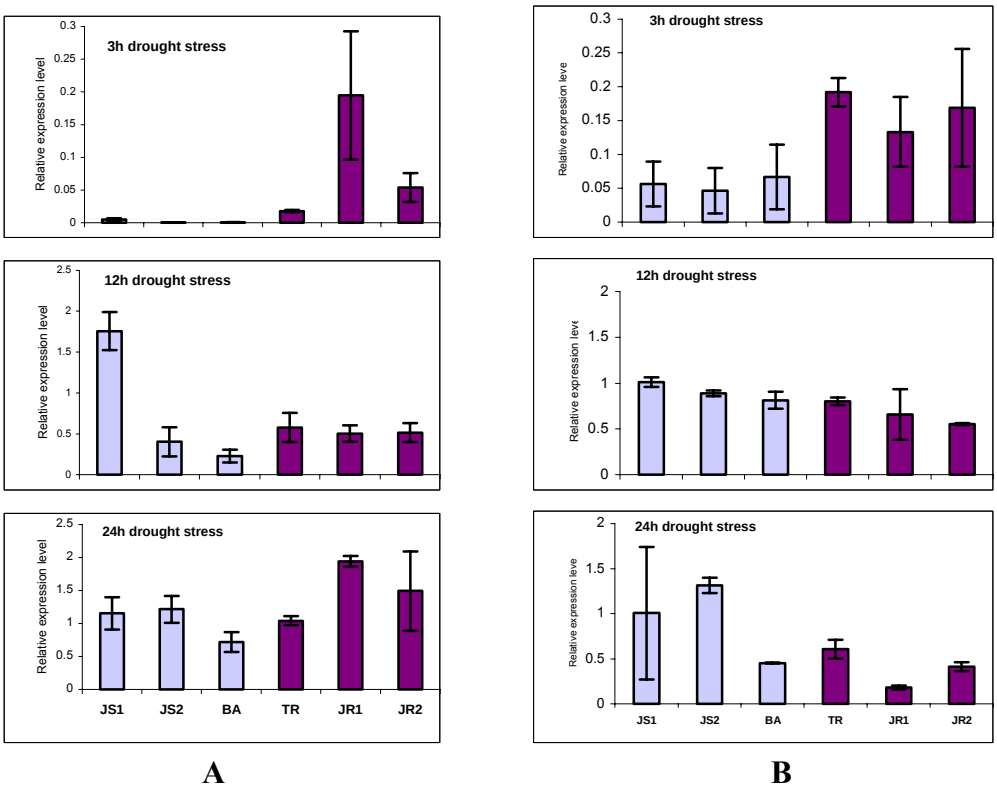
Figure 1. Differential expression patterns of *Dhn1, 3, 5, 6,* and *9* detected by RT- PCR. RT-PCR of six barley genotypes (JS1, JS2, BA, TR, JR1, JR2) after 0 (control -C), 3, 12, and 24 hours of dehydration. *α–tubulin* (*Tub*) was used as a control for relative amount of RNA.



The level of expression of *Dhn6* was significantly higher in the resistant genotypes at the earlier stages after stress. However, after 12 and 24 h *Dhn6* expression level was relatively higher in sensitive genotypes. A slightly higher expression level of *Dhn5* was detected in the resistant genotypes as compared to sensitive ones after 12 h of dehydration (Fig. 1).

Dhn1 and *Dhn6* expression, which showed opposite trends of differential expressions under water stress, was confirmed by Quantitative real time PCR (Fig. 2A&B).

Figure 2. Expression of *Dhn1* (A) and *Dhn6* (B) detected by quantitative real time PCR. Real time PCR using barley genotypes (JS1, JS2, BA, TR, JR1, and JR2) after 3, 12, and 24 hours of dehydration. Quantification is based on Ct values that were normalized using the Ct value corresponding to a barley (housekeeping) α–*tubulin* gene.



Expression of *Dhn1* and *Dhn6* was not detectable in control plants of all genotypes. Earlier induction of *Dhn1* after 3 h was detected in the resistant genotypes. The expression level of JR1 was by far higher (360-fold) than the sensitive plants (Fig 2A). Variation in the

expression of *Dhn1* within the considered resistant group was observed also after 24 h of dehydration. In the sensitive group, the variation was observed after 12 h dehydration. All genotypes reached their maximal level of *Dhn1* expression after 24 h of drought stress with the exception of the sensitive genotype JS1 that after 12 h stress displayed the maximal expression followed by reduced activity of *Dhn1*. The highest level of expression was observed after 24 h in resistant genotypes JR1 and JR2 as compared to JS1 and JS2. In the TR and BA genotypes from Tabigha that were selected by whole plant performance under stress similar trends of expression patterns were found: *Dhn1* expression was higher in resistant plant originating from the TR soil as compared with the genotype from the BA soil (Fig 2A).
Significant differences in the expression level of *Dhn6* were found between sensitive and resistant genotypes (Fig. 2B). After 3 h of dehydration stress, the expression level was higher (2-4-fold) in resistant genotypes (JR1, JR2, and TR) than in sensitive genotypes (JS1, JS2, and BA). The opposite trend of transcript accumulation was observed after 12 h: expression levels detected in the sensitive genotypes (JS1 and JS2) were higher than in the resistant genotypes (JR1 and JR2); the expression levels of BA and TR genotypes were not different. After 24 h of dehydration, expression of *Dhn6* was not changed in the sensitive JS1 and increased slightly in JS2, while reduction was observed in resistant genotypes JR1 and JR2; the TR genotype showed 33% higher expression than the BA genotype.
Our results indicate that each member of the dehydrin gene family have different functions in the process of plant response to drought. The earlier expression of *Dhn1* and *Dhn6* in resistant genotypes may indicate that the resistance is due to one or more of the following mechanisms and/or their combination: (i) earlier perception of the water stress, (ii) more efficient signaling pathways and transcriptional activators, and (iii) higher expression of the *Dhn* gene. Our findings in resistant and sensitive wild barley corroborate well with results obtained by microarray analysis that identified a broad spectrum of transcripts whose expression is modified in response to dehydration in a relatively drought tolerant barley cultivar (OZTRUK *et al*. 2002). Moreover, microarray analysis showed that in the initial response to salt stress, up-regulated transcripts characteristic of salt-tolerant rice were absent in salinity-sensitive rice (KAWASAKI *et al*. 2001). Processes involved in drought tolerance can be dissected by molecular genetic approaches through marker-based detection and mapping of relevant quantitative trait loci (QTL) and/or co-localization of QTLs with candidate genes. Our finding on the role of *Dhn1* in drought tolerance of wild barley is supported by several reports on co-localization of such QTLs with *Dhn* genes in barley overlapping with a cluster of *Dhn* genes on 5H (PAN *et al*., 1994; TEULAT *et al*. 2003).
The drought resistant and sensitive genotypes from Jordan were selected for this study based on their physiological response to dehydration as an objective test, rather than by their site of origin. We found that the main difference between the collection sites of the selected genotypes was soil type. The resistant genotypes from Jordan originated from Terra Rossa soil (Jarash) similar to the resistant genotype from the Tabigha microsite. The sensitive genotypes originated from Wadi Hassa, from Basalt soil type, similar to the sensitive genotypes from Tabigha (IVANDIC *et al*. 2000). Basalt soil possesses higher water-holding capacity as compared to Terra Rossa. Moreover, the soil in Wadi Hassa, formed through weathering of limestone and basalt, is very rich and productive when combined with enough moisture. The populations from the Tabigha microsite share the same annual rainfall (480 mm), and Jarash and Wadi Hassa are similar to each other in their annual rainfall (250-300 mm). However, Wadi Hassa is one of riverbeds which dominate drainage of the Karak plateau. Therefore, plants growing at the bottom of the wadi are exposed to higher water amounts during their life history, despite the macroclimatic xeric surroundings. Water

availability is known to impose strong selective pressure (BOHNERT *et al*. 1995; PÉREZ 1996). The above features support why the sensitive genotypes were found at this collection site. A long history of research provides support for climate as a selective pressure to which populations adapt locally (NAGY 1997; OWUOR *et al.* 1999). We suggest that the observed differences both in physiology and *Dhn* gene expression may be a result of edaphic and climatic selective pressures. Adaptation to environmental changes in populations requires the existence of a large spectrum of genetic diversity and phenotypic plasticity for physiological traits. It characterizes species from highly fluctuating ecosystems (HOFFMANN & PARSONS 1991). Indeed, high genetic diversity was found in the Terra Rossa subpopulation of Tabigha as compared with the Basalt subpopulation (OWUOR *et al.* 1999), and the Jarash population as compared to other populations from Jordan (BAEK *et al*. 2003). Wild barley originating from populations with a high level of genetic diversity and phenotypic plasticity for physiological traits, can serve as a good source for identifying drought-resistant alleles for breeding purposes.

The remarkable finding of this study is in the following two facts: (a) the most contrasting genotypes with respect to drought resistance were found in ecologically divergent microclimatic niches; (b) different patterns in the dynamics of desiccation-induced expression of dehydrin candidate genes (*Dhn1* and *Dhn6*) was displayed by resistant and sensitive genotypes. Our further efforts will be devoted to elucidate whether the observed differential expression relates to allelic variation in the promoter region of these genes or if alternatively, trans-regulatory factors are involved (i.e., alleles of transcription factor genes or other components of the signal transduction pathways).

Acknowledgements

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S 10 – BREEDING SUCCESS STORIES

First Results on SSR Analysis of German Winter Barley Cultivars to Reflect the Breeding Progress over the Last Decades

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Abstract

The substantial increase in winter barley yield in Germany during the last decades is due to an optimisation of plant production systems as well as to the improvement of cultivars achieved by plant breeding. To study the respective breeding progress in more detail a representative set of 65 six-rowed and 52 two-rowed winter barley cultivars was selected. In ongoing multi-site field experiments over two years the performance of these varieties is analysed and compared. At the same time the whole set of 117 cultivars is genotyped by means of SSR (simple sequence repeats) markers. For most of the 20 SSRs analysed up to now we found a high number of alleles per locus and high PIC-values. Interestingly, the distribution of these alleles differs significantly between six-rowed and two-rowed types for 13 of the 20 SSRs. Due to these differences, six-rowed and two-rowed varieties are clearly separated by a principle co-ordinate analysis (PCoA) based on genetic similarities between varieties. The grouping of varieties in PCoAs carried out for the two groups of cultivars separately corresponds in many cases with known pedigree data. Additionally, when taking the year of release into account, differences in the grouping of older and newer cultivars can be found between six-rowed and two-rowed cultivars. While the older six-rowed varieties are more evenly spread, the older two-rowed varieties group relatively close together. How this might reflect the historical differences in breeding of six- and two-row winter barley cultivars in Germany is discussed.

Introduction

During the last five decades winter barley yield in Germany has increased by about 75 kg/ha per annum. Besides enhanced plant production systems this increase is mainly due to strong efforts made in plant breeding during this period.

The first six-rowed winter barley cultivars were derived by selection from a small number of landrace populations, while the next generation of varieties was obtained by cross-breeding of these with other winter barley landraces or with spring barley cultivars. Varieties derived by such crosses are for example 'Vogelsanger Gold' released in 1965 or 'Dura' listed in 1961. These cultivars not only occupied large acreages at the time of release but are still of certain relevance as parents of several recent cultivars (FISCHBECK 1992).

Although two-rowed spring barleys are often found in the pedigree of the early six-rowed winter barley varieties, two-rowed winter barley varieties only gained importance after the release of 'Malta' in 1968 (FISCHBECK 1992). 'Malta' is an ancestor of major two-rowed cultivars like 'Igri', 'Sonja' and 'Trixi', which in turn can be found in the pedigrees of the recently strongly increasing number of two-rowed winter barley varieties (BAUMER & CAIS 2002; FRIEDT *et al.* 2000).

The long-term aim of this study is to investigate how the allelic composition of winter barley cultivars released in Germany during the last decades has changed and whether this is reflected in better agronomic performance. Therefore, 65 six-rowed and 52 two-rowed winter barley cultivars, having been the most important at their time of release and representing important ancestors of modern cultivars, are presently genotyped using a set of SSR (simple sequence repeats) markers covering the whole genome. At the same time these varieties are analysed for agronomic traits in multi-site, biannual field experiments.
Here we present the results of a first marker-analysis based on a set of 20 SSRs.

Material and Methods

Plant Material

The set of experimental material consists of 65 six-rowed winter barley varieties released between 1961 and 2003 and 52 two-rowed winter barley varieties released between 1968 and 2003 in Germany. For DNA extraction 6 to 9 plants of each variety were grown in the greenhouse and pooled leaf samples were taken.

SSR Assay

Genomic DNA was extracted from leaf material using the CTAB method (DOYLE & DOYLE 1987). SSR assays were carried out according to RAMSAY *et al.* (2000).

Statistical Analysis

The polymorphic information content (PIC) of single SSR markers and the mean diversity index (DI) over all loci were calculated according to NEI (1973).
To test for homogeneity of allele distributions between six-rowed and two-rowed varieties χ^2 tests were conducted for all 20 SSRs.
Genetic similarity among varieties was estimated using the Dice coefficient (DICE 1945). The subsequent principle coordinate analysis (PCoA) was carried out with NTSYSpc 2.01 (ROHLF 1997).

Results and Discussion

Varieties Selected for the Study

For selection of the varieties included in this study the year of release, the variety's agronomic importance at that time and its relevance as a parent of later cultivars was taken into account. According to these criteria 65 six- and 52 two-rowed winter barley varieties have been selected. The chosen six-rowed varieties trace back to 1959 - 1961 when 'Mädru' and 'Dura' were released, to cultivars that were registered only recently (Fig. 1). Because of the above mentioned lack of importance of two-rowed winter barley varieties before the release of 'Malta' in 1968, the set of two-rowed varieties spans only 30 years with a special emphasis on lately released new cultivars representing the recent breeding progress (Fig. 1).

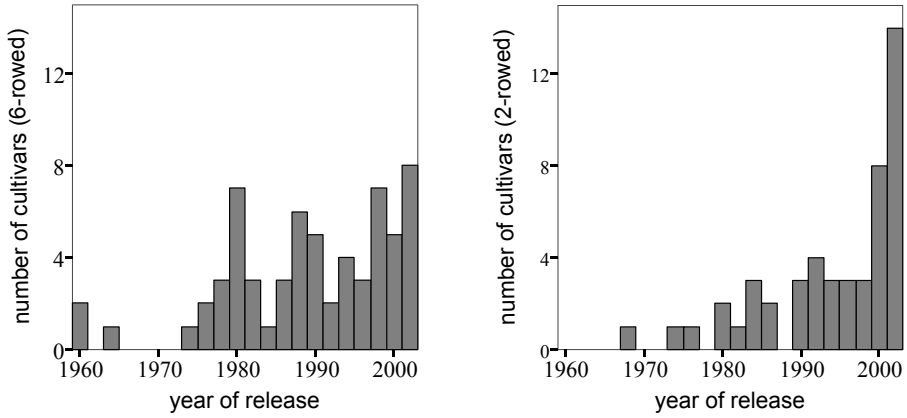

Figure 1. Distributions of six-rowed and two-rowed varieties according to the year of release

Allele Frequencies and PIC-Values

The 117 varieties selected are being genotyped at present with SSRs. The markers were chosen according to their quality score, the chromosomal location and their diversity indices (DI) as denoted in RAMSAY *et al.* (2000).

For the first 20 primers analysed the observed allele sizes, the number of alleles within the six- and two-rowed cultivars and the respective PIC-values are shown in Table 1. Within the six-rowed varieties the number of alleles found at the 20 loci range between 2 to 9 with an average of 4.15 alleles per locus. For the two-rowed cultivars the average number of alleles is smaller (3.80) and ranges between 1 and 7 alleles per locus.

Table 1. SSR primers and their chromosomal location, allele sizes, number of alleles, PIC- and p-values of χ^2-tests for homogeneity of allele distributions between six- and two-rowed varieties

	chrom o-som	allele sizes	total # of alleles	# of alleles (6-rowed)	# of alleles (2-rowed)	p (χ^2-test)	PIC (6-rowed)	PIC (2-rowed)
Bmac032	1H	205-247	8	8	4	<0.001	0.65	0.51
Bmag211	1H	180-190	5	5	4	<0.001	0.65	0.54
Bmag378	2H	139-141	2	2	2	0.931	0.12	0.11
Bmac093	2H	154-160	4	2	4	<0.001	0.46	0.42
HVM054	2H	157-163	3	2	2	0.360	0.03	0.04
EBmac415	2H	236-246	2	2	1	0.203	0.06	0.00
Bmac209	3H	189-193	3	3	2	<0.001	0.50	0.50
Bmag225	3H	143-164	6	6	6	<0.001	0.64	0.97
HVM040	4H	146-162	3	2	3	0.137	0.33	0.44
Bmag353	4H	94-124	7	4	6	<0.001	0.62	0.55
EBmac701	4H	129-151	6	5	5	<0.001	0.29	0.59
HVM067	4H	112-118	3	3	2	<0.001	0.51	0.23
EBmac970	5H	185-189	3	3	1	0.049	0.20	0.00
Bmac113	5H	194-204	3	3	2	0.324	0.14	0.04
Bmac316	6H	129-169	4	4	3	<0.001	0.41	0.62
Bmac018	6H	134-140	4	3	4	<0.001	0.67	0.46
Bmag009	6H	162-176	7	6	6	0.083	0.71	0.69
Bmac040	6H	173-229	12	8	8	<0.001	0.45	0.80
Bmag007	7H	195-219	4	3	4	<0.001	0.67	0.38
Bmac0156	7H	134-216	13	9	7	<0.001	0.69	0.72
Average			5.10	4.15	3.80		0.44	0.43

The PIC-values of the SSRs range from 0 for Ebmac415 and Ebmac790, being monomorphic in the two-rowed material, to 0.97 for Bmag 225. On average the PIC-values, respectively diversity indices (DI), do not differ between six-rowed and two-rowed varieties although for some markers as for example Bmac040 or Bmag007 there is a considerable difference between the two types of barley cultivars. Because of these differences in allele frequencies and due to different allele sizes in the six-rowed and two-rowed types, the χ^2-test for homogeneity of allele distributions at a given locus is highly significant for 13 of the 20 markers.

Genetic Similarity

The results of a principle coordinate analysis (PCoA) based on the genetic similarity of all 117 varieties is displayed in Figure 2. Six-rowed and two-rowed varieties are separated clearly by the first axis explaining 16.7% of the total variance. This precise separation of six- and two-rowed varieties respectively is not unexpected as both populations differ significantly in their allele distributions at most of the 20 loci.

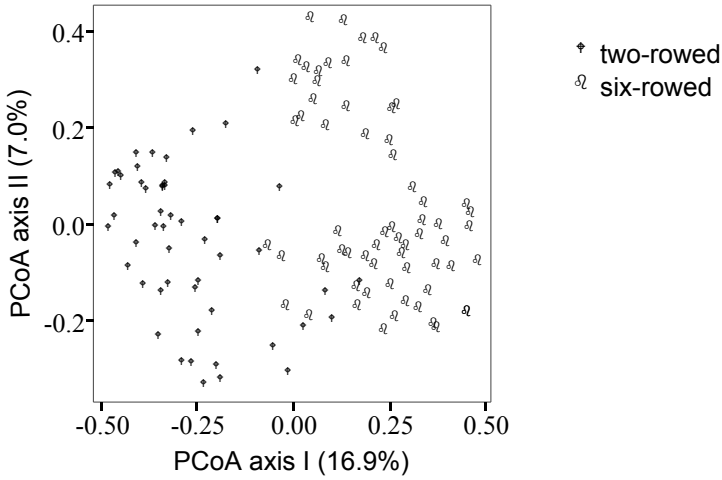

Figure 2. PCoA based on genetic similarities of all varieties studied

For further analysis of the genetic similarity within the two groups PCoA was carried out for six-rowed and two-rowed varieties separately (Fig. 4 and 5). Although these results are preliminary and only based on 20 SSRs it is interesting to notice that the grouping of some cultivars is in accordance with their pedigree.

For example, six-rowed barleys having ‘Banteng’ in their pedigree like ‘Ludmilla’, ‘Alpaca’, ‘Andrea’ and ‘Brunhild’, group in the left part of the chart near ‘Banteng’. On the right site of the diagram ‘Corona’ and derived cultivars (‘Sympax’, ‘Theresa’, ‘Cita’) can be found.

Similarly groups of related varieties can be found in the PCoA-chart of the two-rowed varieties. In the left part of the diagram varieties like ‘Sonja’, ‘Igri’, ‘Trixi’ or ‘Marilyn’ together with their common ancestor ‘Malta’ are found. In the right part of the diagram descendants of crossings with ‘Labea’ like ‘Bistro’, ‘Regina’ and ‘Leonie’ group together.

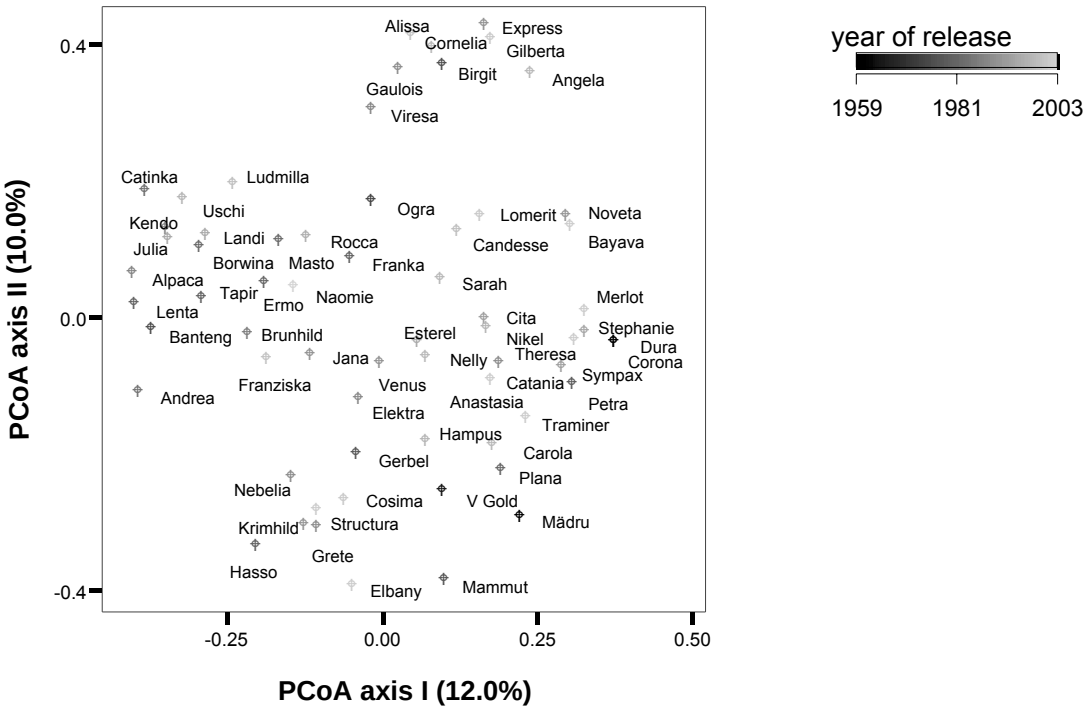

Figure 3. PCoA based on genetic similarities of all six-rowed winter barley varieties

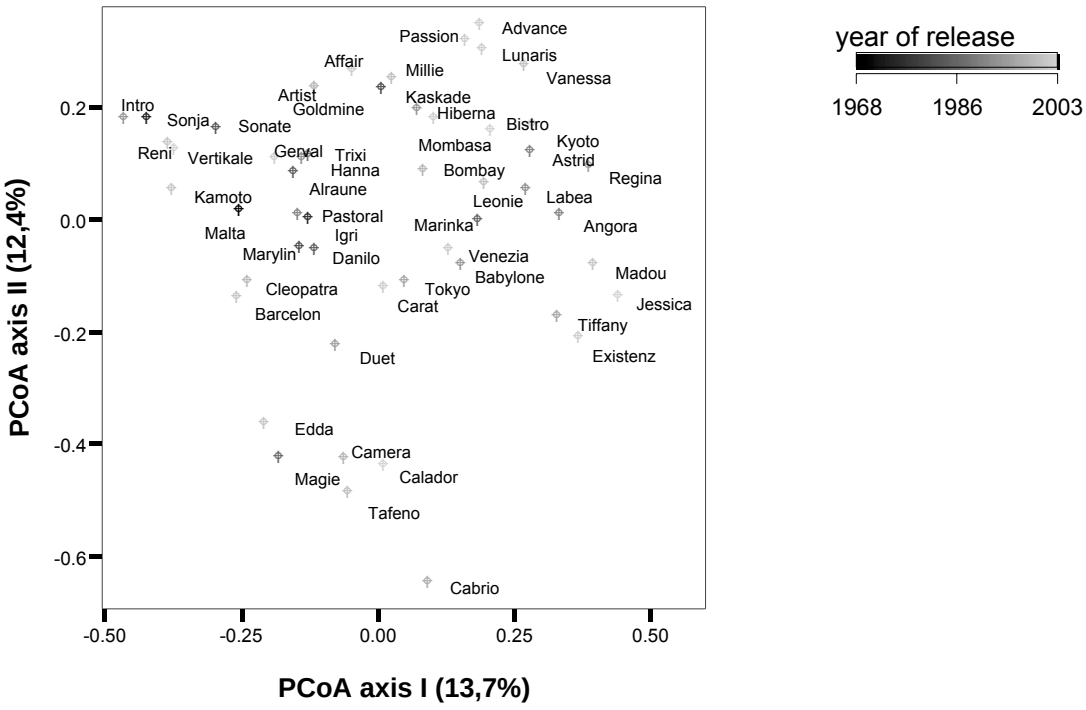

Figure 4. PCoA based on genetic similarities of all two-rowed winter barley varieties

Interestingly, when taking the year of release into account it can be seen that amongst the two-rowed barleys older cultivars like 'Malta', 'Igri', 'Sonja' and 'Kaskade' group relatively close to each other and the newer varieties are found at the other ends of both axes. The opposite is true for the six-rowed varieties, where the older cultivars ('Banteng', 'Birgit', 'Petra', 'Mammut') rather mark the extreme ends of both axes. These differences in the distributions of older and newer cultivars between the two groups of varieties under investigation is not unlikely to reflect the different breeding history of six-rowed and two-rowed winter barley cultivars. While the breeding of six-row varieties in Germany started from the basis of different landrace populations and therefore from material with relatively high genetic diversity, the first two-rowed winter barley cultivars of importance in Germany all have 'Malta' in their pedigree. The genetic basis of the two-rowed varieties was only broadened later.
The planed future enlargement of the SSR marker set will show if these first results regarding the genetic diversity within the two populations are finally confirmed.

Acknowledgements
Ms Ines David is acknowledged for excellent technical assistance. The authors also wish to thank the co-operating breeding companies, represented by the Gemeinschaft zur Förderung der privaten deutschen Pflanzenzüchtung (GFP), for their support. This work is financed by the German Research Foundation (DFG) as part of the Collaborative Research Centre (SFB) 299 at the University of Giessen.

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Evaluation and Selection for Traits Related to Lodging Resistance in Barley

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Abstract

Lodging in barley, due to loss of root anchorage or to stem breakage, causes losses in grain yield, reduction in grain quality, and reduced harvesting efficiency. Lodging resistance is quantitatively inherited, highly influenced by environment, and generally low in heritability. Selection for a combination of easily-measured morphological traits that are related to lodging may lead to greater response than direct selection for lodging itself. A number of culm traits, and their relationships to measured stem breakage and field lodging were examined. Lodging was reduced by shorter peduncles and greater peduncle diameter, peduncle weight/length, peduncle weight/length*diameter and peduncle breaking resistance. Peduncle breaking resistance was positively associated with peduncle weight, diameter, weight/length, and weight/length*diameter. Each of these culm traits was more heritable than lodging itself. There was a significant reduction in lodging in response to indirect selection for plant traits. The use of spaced plants and hill plots was more effective for indirect selection for lodging resistance than head rows and commercial-density yield plots. Selection for culm traits in F2 populations was effective in increasing breaking resistance and in reducing lodging in later generations. Leaf rust resistance was also associated with an improvement in lodging resistance.

Introduction

Lodging is commonly divided into two categories, stem lodging and root lodging. Stem lodging refers to the bending, buckling or breaking of the stem, whereas root lodging involves stems leaning from the crown due to disturbance of the root system and resulting loss of anchorage. In Ontario, stem lodging is the more common lodging type in barley. Breeding for lodging resistance is a challenge due to the various interactions between environmental factors promoting lodging and the plant characters associated with lodging. Because of this it is difficult to identify any reliable selection method for lodging resistance in cereals and the heritability of field lodging is fairly low (CONTI *et al.* 1997). Farmers in Ontario prefer tall cultivars that are more suitable for straw production used for winter bedding, and therefore the use of semi-dwarf cultivars is not an option to reduce the risk of lodging. Studies at the University of Guelph (RODRIGUEZ 1997) indicated a possible relationship between lodging resistance and stem breaking resistance, and a good relationship between breaking resistance and various straw characteristics of barley. The conclusion was that selection for simple culm traits could lead to increased lodging resistance in barley breeding populations.

Barley stems can be considered analogous to a hollow cylindrical rod fastened at one end. The forces of the wind and rain, and the self-weight moment cause bending of the plant; up to a certain limit this bending is reversible and is referred to as elasticity (PINTHUS 1973). The relation between torque (T) and stress (S) for a cylindrical rod is $T = S(2I/d)$ (PINTHUS 1973). I is the inertia and d is the diameter. For a hollow cylinder $I = (\pi(d^4-d_1^4))$, where outer diameter =

d; inner diameter = d_1. JEZOWSKI (1981) studied morphological and physiological characters determining lodging in barley. His elasticity index (E) had a significant linear relationship to lodging (correlation with lodging score = -0.78). The elasticity index (E) showed fairly high broad sense heritability (H_{bs}= 68%). Increased values of stem diameter, stem-wall thickness and elasticity may all increase the straw strength, and lead to improved lodging resistance.

Material and Methods

Culm Parameters

Straw was sampled from the Ontario Barley Performance Trial yield plots from 1999 to 2002, and several different culm traits were measured. The objectives were to examine the relationships among culm traits, the relationships between culm traits and field lodging, and to examine the environmental and genotypic effects on culm traits. Straw was sampled from the innermost four rows of each yield plot. A total of five plants were sampled from each cultivar and one representative culm was selected from each plant. The height and yield of each plot was recorded and the degree of lodging scored on a scale from zero to nine (0= no lodging and 9 = severely lodged). Plants were sampled at maturity, dried for 48 hours, and the peduncle (the uppermost internode of the culm) measured (Figure 1). The spike was removed and weighed, and the weight, length, and diameter of the peduncle were measured and derived parameters calculated. A three-point bending test was performed on the peduncle using a transducer in 1999 and 2000 and an Instron Universal Testing Machine (Instron Corp., Canton, ME, USA; series 4400) in 2001 and 2002. The peduncle was placed on supports 5 cm apart and pressure applied to the center of the peduncle (see Figure1). Breaking resistance (BRK) was recorded as the load at the breaking point of the straw.

Plot Types

In 2001 and 2002, four different plot types of six different cultivars were grown to evaluate the effects on lodging-related characteristics and possible interactions. The plots were space planted (single seeds 10 cm apart in rows of 30 cm); hill plots (25 seeds planted in hills, 50 cm between hills in 50 cm rows); head rows (25 seeds planted in 1 m rows, 50 cm between rows); and yield plots (6 rows of 3 m long with 17.5 cm between rows, density 350 pl/m^2). The cultivars were chosen to represent a range of known lodging types in adapted backgrounds.

Selection Response and Heritability

Two populations were subjected to one or two cycles of selection for BRK on F2 spaced plants and the effects on culm traits in F4 hill and yield plots were determined. One population (Pop A) was produced in 1998 from a random set of adapted 6-row crosses from the breeding program; and the second population (Pop B) was from a more diverse set of crosses made in 2000 which included unadapted lines and 2-row materials. Selection response and heritability for each trait, and the relationship to lodging in hill and yield plots were calculated. The F_2 progenies for C_0 of Pop A were tested as single spaced plants at the Elora Research Station in the summer of 1999 and the F_2-families selected based on their peduncle breaking resistance, as measured by a plunger type transducer. Plants showing low and high breaking resistance were selected from the populations and their progeny grown in a winter nursery in California as single spaced plants. The plants were harvested in California and planted as $F_{3:4}$ yield plots at the Elora Research Station in the summer of 2000. Straw samples were taken from each plot in 2000 and the breaking resistance measured along with the other characters. Plant height and lodging was also recorded for the plots. The $F_{3:4}$ material was used as parents for the next cycle of recurrent

selection based on the breaking resistance test and crosses made in a growth room in the autumn of 2000, and $F_{3:4}$ in 2002 hill and yield plots. The C_1 of Pop A and the C_0 of Pop B were advanced and tested in the same way as C_0 of Pop A.

Results and Discussion

Correlations among Culm Parameters and Their Relationship to Lodging

Of the directly measured straw parameters over the four years of testing, PD is the most closely related to breaking resistance of barley straw (Table 1). SW and PW are also a good indicators of breaking resistance, particularly in six-row cultivars. A stronger correlation existed between breaking resistance and the derived parameters, which included more than one directly measured parameter. Peduncle weight/length*diameter (WLD) is the most consistently correlated to peduncle breaking resistance in both six-row and two-row barley. Very few significant correlations exist among field lodging and any of the measured parameters. In six-row barley, lodging was significantly correlated with PD in 2000 (r=0.48*) and with height in 1999 (r=0.54*). In the case of two-row barley, field lodging was significantly correlated with WL in 1999 (r=0.60*) and BRK in 2000 (r=0.68**). Yield was not significantly associated with measured straw parameters but was negatively associated with lodging (r=-0.19 ns). Plant height was significantly associated with yield (r=0.52*) but not with the lodging score (r=0.39ns). There is a strong linear relationship between peduncle breaking resistance and the directly measured peduncle traits, with the exception of peduncle length. A very strong linear relationship exists between the breaking resistance and WL and WLD over all years and both spike types. SW, PD and, PW in particular, can all be used to predict the breaking resistance of barley culms, but greater accuracy is gained if the combined measurements, WL or WLD are used. This agrees with earlier work of Atkins (1938), who found breaking resistance of winter wheat to be closely related to weight per unit length of culm. The ratio weight/length, can be determined easily, by cutting and weighing a fixed length of a barley culm. This is probably the most efficient way, in terms of time and money, to select for increased breaking resistance without doing an actual breaking test. This should work for barley regardless of spike type.

Plot Types

In general, strong correlations existed among the peduncle parameters, supporting the previous findings. Peduncle breaking resistance was significantly correlated to all parameters for all plot types, except for head rows where the correlations between BRK and WL and WLD were not significant in 2001 and correlations between BRK and PL and PD were not significant in 2002. Plot type effects were significant for all traits except PL and PD. Cultivar effects were significant only for PL and PD. The main effects were greater than the plot type x cultivar interaction effects for all traits. Head rows are the least effective in distinguishing among genotypes (Figure 2) and, therefore, the least effective plot type for selection. In early generations, the seed supply usually precludes the use of yield plots, with space plantings and hill plots as the remaining options. Hill plots take less space and are, therefore, more economical. Additionally, grain yields of hill plots and yield plots are generally correlated (FALK *et al.* 1996). For these reasons spaced plants and hill plots seem to be the most suitable plot types for early evaluation of barley selections for culm traits related to breaking resistance and, ultimately, to field lodging resistance. Selection for a simple parameter such as PW and/or combined parameters such as WL and WLD, within a breeding population planted in spaced planting or hill plots should result in progeny with greater breaking resistance, due to the high correlations detected among these parameters in both

spaced plantings and hill plots. Selection of single spaced plants in early generations with progeny evaluation in hills would be a logical, and efficient, methodology.

Selection Response and Heritability

The correlation coefficients among peduncle characteristics in both populations A and B corresponds with results presented in earlier. A strong relationship existed between peduncle breaking resistance (BRK) and all of the directly measured and derived peduncle parameters with the peduncle length being the lowest (r = 0.31), which is consistent with previous results. The strongest association with peduncle breaking resistance was again peduncle weight/length*diameter, explaining over 50% of the observed variation.

The associations among peduncle parameters in F_4 hill and yield plots were similar to the previous findings. The greatest relative difference due to selection was for peduncle breaking resistance, where the selections for High and Low straw strength differed significantly among all F_2-derived families. There was also residual variation remaining within the families, suggesting that further selection would likely be productive. The differences between High and Low selections within families ranged from 57% to 127%, indicating a very efficient selection for breaking resistance among the space-planted F_2 plants.

Testing of F_4 material in yield plots gave a very good opportunity to test the association of peduncle parameters and field measurements on lodging and diseases. Lodging in the F_4 yield plots was mostly gradual post-maturity lodging and selection for increased straw strength seems to increase lodging resistance. This is a very promising result, as breaking resistance seems to be readily improved, either directly, or through indirect selection for straw traits. Selection for peduncle breaking resistance proved very efficient in both populations. Even though the response to divergent selection was reduced in the second cycle of selection for Pop A compared to the first cycle, the population mean for peduncle breaking resistance increased 47%. Population means for all the peduncle parameters increased significantly from 6% for PL to 40% for PW, and 47% for BRK. There was a highly significant correlation (r=0.82***) between lodging scores in hill plots and lodging scores in yield plots. The error variance was much lower for lodging in the yield plots while the error variance for the straw traits and BRK was higher compared to hill plots. Thus, selecting on the physical traits of the straw is more efficient in hill plots while evaluating lodging is more accurate in yield plots.

Heritability was moderate or high for most peduncle parameters in both populations. The F_2–F_4 heritabilities ranged from 28% for PL to 61% for PD in Pop A and 34% for PL to 37% for PD in Pop B, with WLD having 37% and 43% in Pops A and B, respectively; BRK had 33% to 40% heritability in both populations. The F_2-F_4 heritability was calculated on single plant vs plot basis and the high values suggest a very efficient selection of single F_2 plants for most straw traits. Thus, environmental effects of the different years and plot types are not seriously masking genetic differences.

Peduncle breaking resistance showed significant negative correlations with leaf rust (*Puccinia hordei*) score for C_0 (r = -0.20) and C_1 (r = -0.40) in Pop A and for C_0 Pop B (r = -0.46). Either plants with strong peduncles are giving resistance to leaf rust, or more likely, the rust reduces the breaking resistance of the peduncle. Leaf rust did not affect peduncle length in F_4 hill plots in

2002 but reduced both peduncle weight and peduncle diameter. The peduncle weight/length and, more dramatically, the peduncle weight/length*diameter are, therefore, reduced by the leaf rust and, hence the breaking resistance of the peduncle. There was also a positive relationship between the severity of leaf rust and the degree of lodging in the hill plots. In C_1 of Pop A this correlation is highly significant (r = 0.52**). This indicates that leaf rust reduces lodging resistance through weakening of the stem by reducing both the structural material of the stem and the diameter.

Conclusions

Selection for peduncle parameters related to lodging seems to be very effective in increasing post-maturity stem lodging resistance. Because of the strong associations among peduncle parameters, several traits could be used to select indirectly for increased straw strength and stiffness, ultimately leading to increased lodging resistance. Peduncle weight or spike weight could also be used for indirect selection of breaking resistance but using a combination of parameters, such as the peduncle weight/length or peduncle weight/length*diameter, should give better results. The relationship of spike weight to yield in six-row barley would suggest that including this trait in an index could lead to improved yield along with improved lodging resistance.

This research supports the use of recurrent selection for a simple combination of peduncle parameters, such as peduncle weight/length*diameter, in a barley breeding population for increasing straw strength, ultimately leading to increased stem lodging resistance in the population and the lines derived from it.

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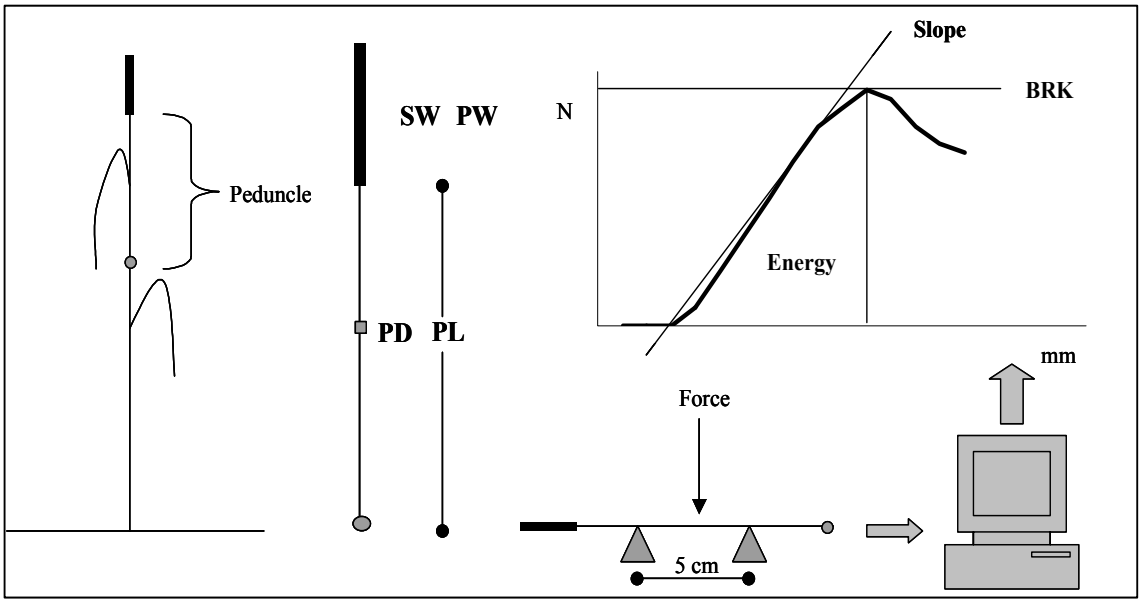

Figure 1. A single culm from each plant was used to determine spike weight (SW), peduncle length (PL), peduncle weight (PW), and peduncle diameter (PD); the same straw segment was broken with the Instron and the force required (BRK) also measured.

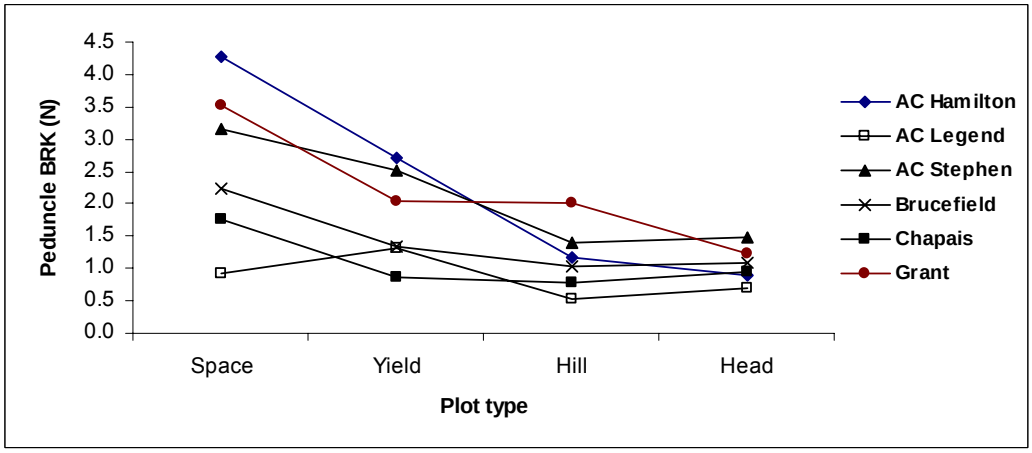

Figure 2. Values for peduncle breaking resistance (BRK) for six cultivars planted in four different plot types

Table 1. Correlations among peduncle parameters, breaking resistance, and lodging in six-row barley cultivars grown at the Elora Research Station 1999-2002 (NB Lodging for 1999-2001 only). (see text for abbreviations).

Trait							
	SW						
PW	0.67**	PW					
PL	0.42*	0.77***	PL				
PD	0.64**	0.77***	0.52*	PD			
WL	0.64**	0.79***	0.25ns	0.70***	WL		
WLD	0.67***	0.85***	0.36ns	0.86***	0.96***	WLD	
BRK	0.71***	0.71***	0.39ns	0.77***	0.74***	0.79***	BRK
LOD	0.11ns	0.07ns	0.04ns	0.17ns	0.06ns	0.11Ns	0.14ns

*** ($p<0.001$), ** ($p<0.01$), * ($p<0.05$), ns ($p>0.05$)

Torrens – a New Hulless Barley Developed for Southern Australia

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Abstract

Torrens is a new hulless barley developed by the South Australian Barley Improvement Program with a national, co-ordinated approach to the development of a hulless barley industry. Torrens was bred from a simple cross between Galleon, a well adapted, CCN resistant; South Australian feed variety, and CIMMYT 42002, a hulless 2-row barley line with bright, white and plump grain selected from CIMMYT, Mexico.

Torrens was developed as a hulless replacement for Namoi averaging 4% higher yields. Torrens has higher yield potential than Namoi in most agro-ecological zones of South Australia. Torrens produces larger grain and has better feed and malt quality than Namoi. Torrens has a similar plant type to Galleon and is shorter and early maturing compared to Namoi. It is less susceptible than Namoi to leaf rust, powdery mildew and spot form of net blotch. It is sensitive to both manganese and zinc deficiencies.

Torrens progressed through early generation selection trials and was promoted to University of Adelaide Stage 3 trials in 1995. Torrens produced grain yields that ranged between 1 and 22 percentage points higher than Namoi in five out of six growing seasons. Subsequently, it was promoted to SARDI (South Australian Research and Development Institute) Stage 3 trials in 1997. Torrens has now completed five years of SARDI Stage 4 trial evaluation in South Australia and will be included in this trial series in 2004. It has also been included in collaborative trials in Victoria, New South Wales, Queensland and Western Australia since 1997. Results from 2002 collaborative trials indicated Torrens yielded up to 23% above Namoi with the best performance at Gibson, Western Australia.

This paper will describe, in detail, results for Torrens compared to Namoi and current Australian covered cultivars for:

- Agronomic performance and adaptation
- Physical grain quality
- Disease resistance
- End use performance:
  - Animal feed evaluation
  - Malt quality evaluation
  - Human food evaluation

Keywords: hulless barley; barley breeding; agronomy; grain quality; disease resistance; end use performance

Breeding

Torrens was bred from a simple cross between Galleon, a well adapted, CCN resistant South Australian feed variety, and CIMMYT 42002, a hulless 2-row barley line with bright, white and plump grain selected from CIMMYT, Mexico.

Selection and Performance in University of Adelaide Trials

Torrens progressed through early generation selection trials and was promoted to University of Adelaide Stage 3 trials in 1995. Table 1 provides a summary of grain yields (expressed as a % of Schooner) from replicated trials grown over eight seasons alongside Namoi and three other major barley varieties as part of the South Australian Barley Improvement Program. Torrens produced grain yields that ranged between 2 and 16 percentage points higher than Namoi in seven out of eight growing seasons. Subsequently, Torrens was promoted to SARDI Stage 3 trials in 1997. Torrens has now completed seven years of SARDI Stage 4 trial evaluation in South Australia and will be included in this trial series in 2004 as a hulless barley control variety. Torrens was released as a variety in 2001.

Table 1. Grain yield of barley varieties (as % of Schooner) in University of Adelaide breeding trials (1996 – 2003)

Genotype	1996 6 Sites	1997 6 Sites	1998 5 Sites	1999 6 sites	2000 6 sites	2001 6 sites	2002 6 sites	2003 6 sites
Torrens	87	101	87	87	95	99	95	89
Namoi	85	86	71	89	81	92	84	85
Barque	-	-	87	112	107	114	-	104
Schooner	100	100	100	100	100	100	100	100

Yield and Adaptation

Figure 1 and Table 2 provide a summary of the South Australian yield of Torrens in SARDI Stage 4 trials between 1998 and 2003. It has also been included in collaborative trials in Victoria, New South Wales, Queensland and Western Australia since 1997. Results from 2002 collaborative trials indicated Torrens yielded up to 23% above Namoi with the best performance at Gibson, Western Australia. Torrens yielded 15% and 17% above Namoi at Formatin, Queensland and Condobolin, New South Wales, respectively. At Wagga Wagga, New South Wales, Torrens yielded 9% lower than Namoi.

Table 3 provides a summary of grain yields for Torrens (expressed as a % of Schooner) from single replicated trials grown at Formatin, Queensland, Condobolin and Wagga Wagga, New South Wales and Gibson, Western Australia in 2002 compared to Namoi, Keel and Schooner.

Figure 1. Grain yield of Torrens in South Australia compared to Namoi in SARDI Stage 4 Trials (expressed as mean of 21 sites from 1998 and 1999 and 20 sites from 2000). Yield expressed as a percentage of Schooner

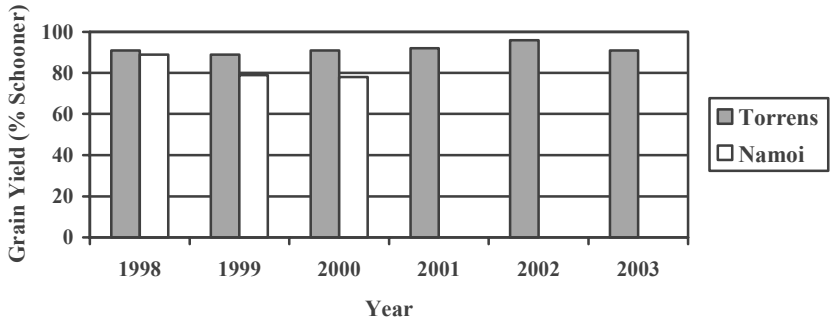


Table 2. Grain yield of barley varieties (as % of Schooner) in different agro-ecological zones of South Australia (Stage 4 Trials, 1998 – 2003)

Genotype	Yorke Peninsula	Mid North	Murray Mallee	South East	Eyre Peninsula
Torrens	95	89	84	92	89
Namoi	90	84	76	83	75
Barque	112	106	111	106	106

Table 3. Grain yield of barley varieties (as % of Schooner) in Collaborative Trials grown in 2002

Genotype	Condobolin	Wagga Wagga	Formatin	Gibson
Torrens	99	69	100	115
Namoi	82	78	85	92
Keel	100	118	116	123

Disease Resistance

Table IV provides a summary of the disease ratings of Torrens and other major barley varieties. These ratings are based on observations made in yield trials and field and laboratory disease screening nurseries by the Field Crops Pathology Unit (SARDI) and University of Adelaide.

Table 4. Disease ratings for Torrens and other major barley varieties

Genotype	Leaf Scald	Powdery Mildew	Leaf Rust	Spot form of net blotch	*P.neglectus*		CCN
					Resistance	Tolerance	
Torrens	MS	MR/MS	MR/S	MS	MR	I	R
Namoi	MR	S	S	MS	MR	I	S
Barque	S	MR/MS	S	MR	MS	MT	R
Schooner	MS/S	S	S	MS/S	MR/MS	MI	S

Disease rating codes: R = resistant, MR = moderately resistant, MS = moderately susceptible, S = susceptible, VS = very susceptible, MT = moderately tolerant, MI = moderately intolerant, I = intolerant

Physical Grain Quality

Figures 2a and 2b provides a summary of grain plumpness and 1000-grain weights of Torrens compared to Namoi and Barque from SARDI Stage 4 trials grown during1998 - 2003 seasons. All results are expressed as a percentage of Schooner. The 2002 season was characterised by a delay in planting dates due to later opening rains and, by anthesis, dry weather conditions were beginning to impact on grain filling, particularly in the low rainfall districts. Consequently, the actual range of grain plumpness for Torrens was between 13% Borrika (Murray Mallee, SA) and 85% at Salter Springs (Mid North, SA). In addition, only 6 sites had grain plumpness greater than 60% of Schooner. By comparison, the range for Schooner was between 50% at Port Clinton (Yorke Peninsula, SA) and 96% at Salter Springs (Mid North, SA). Additionally, Schooner had grain plumpness greater than 60% at 14 out of the 19 sites

tested. Subsequently, the smaller grain size also brought about lower 1000-grain weights for Torrens and Namoi compared to Barque and Schooner for the 2002 season (Figure 2b).

Figure 2a. Grain plumpness (>2.5mm) of Torrens in South Australia compared to Namoi and Barque in SARDI Stage 4 Trials (expressed as mean of 21 sites from 1998, 1999, 2001, 2002 and 2003; and 20 sites from 2000). Yield expressed as a percentage of Schooner.

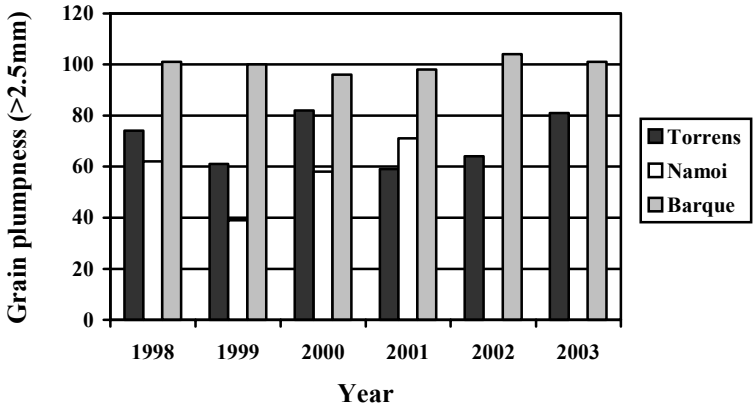
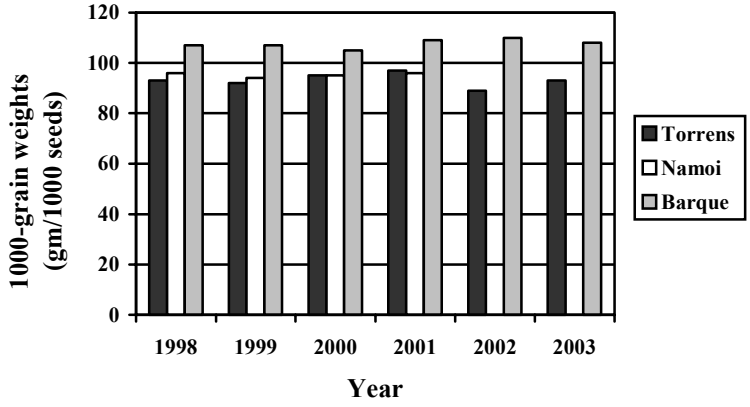


Figure 2b. 1000-grain weights (gm/1000 seeds) of Torrens in South Australia compared to Namoi and Barque in SARDI Stage 4 Trials (expressed as mean of 21 sites from 1998, 1999, 2001, 2002 and 2003; and 20 sites from 2000). Yield expressed as a percentage of Schooner.



Recommended End Use and Grain Quality

Progress with Animal Feed Quality

The primary use of hulless barley in Canada is in pig diets. One of the major objectives of the South Australian program is to develop hulless barleys that have potential in Australia's pig industry. In South Australia, we have evaluated eight hulless barley genotypes in three animal feeding assays - *in vitro* digestible dry matter (DDM), *in vitro* pig digestible energy (DE) and *in vivo* apparent metabolisable energy (AME) in chickens. In all three assays, the hulless genotypes are clearly superior to the covered control Schooner (Table V). BARR and

KNEIPP (1995) estimated that a unit of AME was worth approximately $20 per tonne at 1995 feed prices. On this basis, hulless barley is valued at between $10 (SB85216) and $24 (Namoi) per tonne more than Schooner. This data is encouraging but more research into *in vivo* pig digestible energy and amino acid digestibility is required before hulless barley cultivars could be launched confidently into the Australian pig industry.
Torrens was not included in this preliminary study and was analysed after 2001 and 2002 harvests (Table VI). From all traits assayed and predicted using NIR, both Torrens and Namoi had superior feed quality to Schooner.

Table 5. Least squares means and standard errors for eight hulless barley genotypes compared to Schooner grown in 1995 and 1996 in South Australia tested in three feed quality assays, Digestible dry matter (DDM); *in vivo* poultry Apparent Metabolisable energy (AME) and *in vitro* pig Digestible Energy (DE).

Genotype	DDM (%)	SE	AME (MJ/kg)	SE	DE (MJ/kg)	SE
Schooner	85.4	0.23	13.76	0.28	11.89	0.33
Galleon	84.9	0.23	14.10	0.28	11.90	0.33
Namoi	89.4	0.25	15.16	-	13.02	0.43
Richard (Canada)	89.5	0.37	14.91	0.36	11.92	0.43
SB85216 (Canada)	90.1	0.37	14.24	0.49	13.15	0.43
CIMMYT 42002	90.1	0.37	14.99	0.49	13.33	0.43

Table 6. Animal feed analyses for Torrens and Namoi compared to Schooner grown in 2000 - 2002 in South Australia. Analyses performed by GRDC funded Premium Grains for Livestock Program.

Genotype	% beta-glucan	% Starch (as is)	% DMD*	NIR predicted % DMD* (Hamiliton, Vic. 2001) Ground, Whole	Digestible Energy (MJ/kg)	SARDI NIR Digestible Energy (MJ/kg)
Torrens	4.955	52.50	89.75	88.22, 86.09	14.12	14.10
Namoi	5.430	56.10	-	88.23, 86.36	14.12	-
Schooner	3.5	-	-	83.51, 83.44	13.40	-

**Dry Matter Digestibility*

Progress with Malt Quality Evaluation
The malt quality profile of Torrens is encouraging. In general, Torrens consistently produced malt extracts up to 8% higher and DP up to 5% higher than Schooner (Table7). Wort beta glucan and viscosity levels for Torrens were higher than Schooner. In addition, Torrens produced a friability that was lower than Schooner. This result may be explained by, either, embryo damage during harvesting, resulting in poor grain modification during the malting process, or 'case hardening' during the kilning at completion of the malting process (STEWART *et al*. 2004).

Table 7. Summary of malt quality traits of Torrens compared to Schooner (expressed as mean 6 sites from 2002 SARDI Stage 4 trials). Analyses and results provided by Sophie Roumeliotis and Barley Quality Evaluation Laboratory, School of Agriculture and Wine, University of Adelaide.

Genotype	Friability	DP	Grain Protein	Malt Protein	Soluble Protein	Extract EBC	Visc	Faan	Wort beta-glucan	KI	AAL	Colour L
Torrens	50.01	624	11.52	11.77	3.97	83.73	1.93	127	404	33.79	77.31	62.15
Schooner	62.71	595	11.60	11.90	4.57	77.35	1.64	172	151	38.69	80.40	64.14

Even more encouraging were the results from the commercial malting and brewing evaluation performed by AusMalt and Coopers Brewery, South Australia. This study (STEWART *et al*. 2004) used a modified steeping regime to improve grain modification during the malting process. Subsequently, the malt produced had a higher extract, was well modified and was low in beta-glucan. The hulless malt was then brewed in commercial size batches at 50% hulless grist and 50% control malt. The hulless/control malt blend exhibited an improvement in brewhouse yield over control batches; however, there was a slight reduction in mash filtration efficiency. STEWART *et al*. (2004) concluded that the selection of better quality hulless barley and fine-tuning mash filtration parameters would increase and improve its potential for commercial viability.

Progress with Human Food Evaluation

An alternative prospect for a successful hulless barley industry may include supplying barley to the human food markets in Asian countries, particularly Japan. Presently, over 40,000 tonnes of Australian barley is exported to Japan each year for staple foods and Shochu. Barley for Japanese specialty foods may attract a price equivalent to or greater than malting barley, even for non-premium malting varieties. From a nutritional and processing point of view, barley has enormous potential. In order to utilise barley for food it is necessary to understand primarily, its processing and health characteristics. Japanese food products from barley include Shochu, a fermented and distilled spirit; miso, a soup paste; pearled barley for food, including "rice extender"; and barley tea.

Hulless barley appears to be desirable for these markets since covered barley is pearled to approximately 65% of its original weight to remove the entire husk on the 'crease' for shochu and can be greater for rice extender.

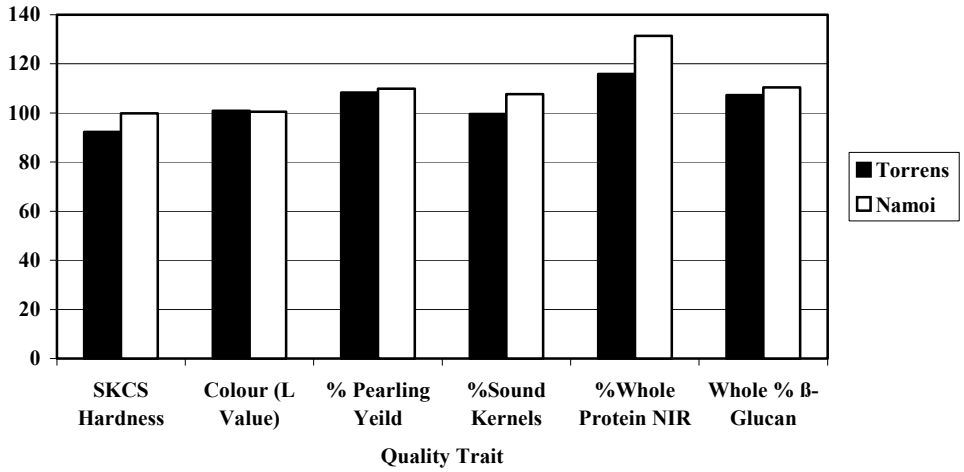
Preliminary investigations into the suitability of Torrens for human food involves measuring the following parameters:

- Grain hardness assessment using 'Single Kernel Characterisation Systems' – Perten Instruments
- % Sound Kernels – Whole pearled kernels greater than 2.0mm in diameter
- Kernel colour assessment – post pearl
- % Pearling Yield – % of kernels remaining after pearling
- Grain protein analysis using NIR wholegrain calibration
- Beta glucan content

Figure 3 provides a summary of results of pearl quality research of Torrens and Namoi compared to Schooner. These results indicate Torrens performing equal to Schooner for colour and % sound kernels, and higher than Schooner for protein and beta-glucan levels. However, Torrens displayed lower grain hardness than the other varieties shown here. Lower grain hardness is not a desirable trait in the pearling process because it may lead to too many

broken kernels in the finish product. However, this was not reflected in the sound kernel measurement when compared to Schooner.
Quality parameters for other food products and hulless barley suitability needs to addressed immediately as it is overall poorly researched. It is important from both an economic and health perspective to understand these quality requirements better.

Figure 3. Summary of preliminary results of pearl quality research of Torrens and Namoi compared to Schooner. Results expressed as a mean of 3 replicates from 2 years and then expressed as a percentage of Schooner. Analyses and results provided by Jennifer Washington, GRDC project UA00030 – 'Alternative End Uses of Barley', School of Agriculture and Wine, University of Adelaide.



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The Results of Barley Breeding in the State Stende Plant Breeding Station on Last 10 Years

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Abstract
Spring barley (*Hordeum vulgare* L.) is the most important cereal crop in Latvia. The aim of our barley breeding program is to produce and introduce for production a new, high yielding, lodging and disease resistant variety suitable for Latvian environmental conditions**.** During 1993 to 2003 three new two-row varieties 'Rasa', 'Sencis' and 'Ansis' and six-row variety 'Druvis' bread at the State Stende Plant Breeding station were included in the Variety Catalogue of Latvia. New variety 'Kristaps' is evaluated in States trials now. The results show the potential of these varieties in competition trials during last five years (1998-2003) characterized with different climatic conditions.
Keywords: spring barley; varieties; breeding programme and characteristics

Introduction

In 2003 the area of barley was 140 thousand ha, it comprises 34,3% of the total area of cereal crops. For a long time (last 20 years) variety 'Abava' is occupied the key position between barley varieties grown in Latvia - more then 50% of barley area. Farmers like this variety due to its plasticity to soil and climatic conditions. Nevertheless there is tendency to increase also area of new varieties bred in Stende after 1993.

Material and Methods

State Stende Plant Breeding Station is located in north – western part of Latvia. Under the influence of the Baltic Sea and the Gulf of Riga the climate is maritime. During the years there are many cloudy days, summers are temperately warm, autumns – long and moist. In spring night frosts may last till June. The climatic conditions from 1998 to 2003 were different and atypical for Latvia.

Soil is well – eroded by carbonates in 1,0 to 1,5 m depths, well – drained. The types of soil are weakly or meanly podsoled loam and sandy loam: humus – 1,6-2,5%, pH_{KCl} 5,4 – 6,6, P_2O_5 – 183-355 mg kg^{-1}, K_2O – 170 – 264 mg kg^{-1}. Given fertilisers: P_2O_5 80-100 kg ha^{-1}, K_2O 80-100 kg ha^{-1}, N 60-70 kg ha^{-1}. Weed control – chemical. Previous crop – potatoes. Sowing time – April 20 to early May, harvesting - early to middle August.

We are used the following steps for producing of the new varieties: the collection testing, breeding work, disease resistance testing on natural and provoked background, grain quality testing, ecological testing and elaboration for agrotechnical recommendations (KALININA *et al*. 1996;)

Results and Discussion

Variety 'Rasa' (Francengold / HE-P-54) is in the Variety Catalogue of Latvia since 1996. Recommended for malt production. Variety has very smooth grains, very high sieving fraction

and low protein content, have resistant to loose smut (*Ustilago nuda* (C.N.Jensen) Rostr.) and powdery mildew *(Blumeria graminis* (DC)E.O. Speer f. sp. *hordei* Em.Marchal*)*. Now in Latvia 'Rasa' is occupied 11,8 % of barley total area.

Table 1. Results and characteristics of barley varieties bred at State Stende PB station, (1998-2003)

Variety	Yield potential t ha^{-1}	Grain quality			Characteristics	
		TKW, g	Volume weight, g l^{-1}	Crude protein content, %	Special	Limit
Rasa	5,0-7,7	44,2-47,5	630-710	10,5-12,6	Very smooth grains, resistance to loose smut and powdery mildew	Sensitive to late harvesting
Sencis	5,0-6,0	46,3-53,1	650-720	10,3-12,4	Early ripening, resistant to disease, middle intensive type	Middle lodging endurance
Ansis	6,0-8,0	47,0-53,4	650-730	10,2-13,3	Short straw, high lodging endurance, intensive type	Susceptible to net blotch, sensitive to frosts and drought
Druvis	5,0-6,5	41,1-48,2	690-704	11,7-13,6	Early ripening	Middle resistance to powdery mildew, loose smut
Kristaps	5,5-7,5	42,3-51,1	670-700	10,5-12,8	Resistance to loose smut and powdery mildew	Middle resistance to *Septoria* spp.

Variety 'Sencis' (Torkel / Stende 7542) was registered in 1998 and is in the Variety Catalogue of Latvia since 2000. 'Sencis' is recommended for malt, the standard for early ripening varieties. 'Sencis' has very good resistance to diseases, has Mla 13, Ml (RU3) and Mlg genes (TUREIAPINA *et al*. 1997). A limited characteristic of variety is middle lodging endurance (KALININA 2000).

Variety 'Ansis' (KM 246-3/78 / Taifun) is included in Variety Catalogue of Latvia since 2000. 'Ansis' is recommended for malt. Variety is middle late ripening, high yielding and lodging endurance; resistant to loose smut and powdery mildew, has u_1u_2 genes (TUREIAPINA *et al*. 1997; KALININA 2001). Variety has short straw, is intensive type therefore is recomended special growing technology (MALECKA 2001). Variety is popular now and seed growing go up

– in 2003 it occupied 17,5 % of barley total area. Since 2002 ‘Ansis’ is a standard for middle late ripening varieties.
Six-row variety ‘Druvis’ (‘Dobryi’ / HVS 115440) characterizes with early-maturity, high yield potential (5.0-6.5 t ha^{-1}) and good resistance to net blotch, good malting quality (KALININA & VIKSNE 2000).
‘Kristaps’ (CF 79502 / Stende 9023) the new two-row variety is tested now in the States trials. ‘Kristaps’ characterizes with high yield potential, high tillering ability. Variety recommended for malt, feed and food production.
Taking into consideration that actually in Latvia barley yield nevertheless is mainly used for feed and food purposes (about 85 %) it is actual task to work out selection criteria of quality also for varieties suitable for those directions of production.

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Variability of Yield of Five Newly Two-Rowed Spring Barley Cultivars in Different Environment

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Abstract
At six newly Yugoslav two rowed spring barley cultivars (Horizont, Ukras, Dragulj, Jadran and Djerdan) were studied morphological and yield related traits. Those barley cultivars tested in different environmental conditions during three seasons. Variation of morphological traits and yield components was observed and evaluated. In this investigation was assessed variability of yield, hectoliter mass, thousand grain mass, grain protein content, stem height, lodging resistance, resistance to diseases. Each cultivar showed variation of analyzed traits in different environmental conditions. It's indicating influence of environment to trait development and expression.
Keywords: spring barley (*Hordeum vulgare* L.); cultivar; grain yield; and environment vegetative period

Introduction

The aim of barley breeding is combine genetically divergent cultivars with wide range of desired traits and genes. Incorporation of desirable genes into new genotypes is very difficult as well estimation of genes effect during selection. Especially it is very difficult for breeders, which are, conducted traditional breeding program in creation new cultivars. In barley breeding programs new cultivars breeders can developed by crossing wild species, land races and commercial cultivars. Utilization of crossing divergent barley genotypes play important role in increasing genetic variability. Also, very important is develop barley genotypes adapted to local conditions as well for various users (CECCARELLI 2000). Breeders may have success by making a series of crosses among parents that possess all the desirable traits. Such developed material requires selection for those types combining desirable traits and further crossing to fix the selected genotypes. Traditional breeding methods have been very successful in improving in improving yield and quality. Evaluation of yield, quality and agronomic characters must occur over sites and seasons prior to cultivar realize. Analysis of barley lines in micro trials allowing reliable testing lines in different environment. On the other hand biotechnology contributing to knowledge of gene structure and function. Recent studies of barley have mapped the genes responsible for many agronomic and some malting quality traits. The ability to select specific genes for traits such as resistance to disease has allowed pyramiding of several different sources of genes to confer improved resistance to multiple strains.

In this paper were analyzed yield and quality traits of spring barley cultivars tested in different environmental conditions. The aim of this study was estimation cultivar variability and their adaptability as well recommend these cultivars for the future breeding programs of development new barley cultivars with increased yield and quality.

Material and Methods

The study of six spring barley cultivars carried out during three successive season at three location in micro trials on 5 m^2 plot in five replications. Rate of seed for sowing was determined on the base of seed size and percentage of germination (400-450 seeds, approximately).

Fertilizers were applied at two split rates (Nitrogen - 40 kg ha^{-1} and Phosphorous 20 kg ha^{-1}). At the sowing time was applied half rte of nutrient and remain rate of fertilizers was applied at the tillering growth stage.

Yield of grain was established by measure and obtained data was computed by using statistical method of variance analysis. Significance of yields differences among cultivars and check cultivars were tested by value of $LSD_{0.05}$ and $LSD_{0.01}$.

Grain protein content was analyzed by using micro Kjeldalh method (NELSON & SUMMERSS 1972).

Heading time, height of plants and lodging resistance were estimated in field conditions.

Disease severity of leaf rust (caused by *Puccinia hordei*), stem rust (caused by *Puccinia graminis* f.sp. *hordei*) and powdery mildew (caused by *Erysiphe graminis* f.sp. *hordei*) was visually estimated as percentage on the uppermost leaves on randomly selected ten main stems of plants in each plot.

Results and Discussion

The results of investigation of grain yield of the five new barley cultivars were approved by Federal commission in 1999, are presented in this paper. According to the botanical classification the five new barley cultivars belongs to *Hordeum sativum*, Jess. *var*. *nutans*. Analyzed barley cultivars showed differences for examined traits per year and per place. All cultivars expressed highly significant higher grain yield than check cultivar NS 294 what were established in average of three year and three different locations. The average grain yield of Djerdan barley cultivar including all places and years of investigation was 5588 kg/ha it mean 1358 kg higher than in check NS 294 barley cultivar. The highest grain yield 6360 kg/ha in first year of investigation and 6276 kg/ha in second year expressed Djerdan cultivar in place Novi Sad, while in third year of investigation Djerdan cultivar expressed the highest yield in Kragujevac place. Analysis of grain yield for all year and location showed that barley cultivar Ukras (5416 kg/ha) expressed higher grain yield in average for 1186 kg/ha tan check NS 294 cultivar, Horizont cultivar (5067 kg/ha) for 837 kg/ha, Jadran cultivar (4996 kg/ha) for 766 kg/ha and Dragulj cultivar (4968 kg/ha) for 738 kg/ha in average higher than check barley cultivar NS 294 (4230 kg/ha) table 1. The great yield response to favorable condition (MAKSIMOVIC *et al.* 1994; KOKHMETOVA & OMAROVA 2000).

Barley cultivars Djerdan, Jadran and Ukras showed in average the highest grain yield in place of Novi Sad, while Horizont and Dragulj as well check cultivar NS 294 expressed the highest grain yield in Kragujevac place in average of three year of examination. Also, value of grain yield of each cultivar varied in each place depends of year of examination. The most differences of grain yield per year were expressed in Zajecar place, mostly for all analyzed barley cultivars, table1.

Table 1. Grain yield in six Yugoslav two row spring barley cultivars

Cultivar	Location	Year of micro trials			Average grain yield /place/year	Average value of grain yield (kg/ha)	Difference of grain yield in relation to check cv. (kg)
		1996	1997	1998			
Horizont	Novi Sad	5740^{++}	5996^{++}	4788^{0}	**5508^{++}**	5067	+837
	Kragujevac	5848^{++}	5456^{++}	5252^{0}	**5519^{++}**		
	Zajecar	2640^{0}	4680^{++}	5200^{0}	**4173^{++}**		
	Average/year/place	**4743^{++}**	**5377^{++}**	**5080^{0}**	**5067^{++}**		
Ukras	Novi Sad	6212^{++}	5940^{++}	5596^{0}	5916^{++}	5416	+1186
	Kragujevac	5800^{++}	5180^{++}	6184^{+}	5721^{++}		
	Zajecar	2880^{0}	5752^{++}	5200^{0}	4611^{++}		
	Average/year/place	**4964^{++}**	**5624^{++}**	**5660^{+}**	**5416^{++}**		
Dragulj	Novi Sad	4968^{++}	5532^{++}	4192^{-}	4897^{++}	4968	+738
	Kragujevac	6112^{++}	5116^{++}	5756^{0}	5661^{++}		
	Zajecar	3040^{0}	5200^{++}	4800^{-}	4347^{++}		
	Average/year/place	**4707^{++}**	**5283^{++}**	**4916^{0}**	**4968^{++}**		
Jadran	Novi Sad	5088^{++}	5852^{++}	5672^{0}	5537^{++}	4996	+766
	Kragujevac	5360^{+}	4684^{0}	5720^{0}	5255^{+}		
	Zajecar	2532^{0}	5380^{++}	4680^{-}	4197^{++}		
	Average/year/place	**4327^{++}**	**5305^{++}**	**5357^{0}**	**4996^{++}**		
Djerdan	Novi Sad	6360^{++}	6276^{++}	5308^{0}	5981^{++}	5588	+1358
	Kragujevac	6144^{++}	5424^{++}	6220^{+}	5929^{++}		
	Zajecar	3200^{++}	5240^{++}	6120^{0}	4853^{++}		
	Average/year/place	**5235^{++}**	**5647^{++}**	**5883^{+}**	**5588^{++}**		
NS 294 check cv.	Novi Sad	3572	3968	5184	4301	4230	000
	Kragujevac	4500	4208	5384	4697		
	Zajecar	2796	3160	5120	3692		
	Average/year/place	**3683**	**3779**	**5229**	**4230**		
LSD 0.05	Novi Sad	611	405	808	402		
	Kragujevac	700	636	692	435		
	Zajecar	291	438	307	206		
	Average/year/place	357	353	393	**214**	214	
LSD 0.01	Novi Sad	812	539	1075	532		
	Kragujevac	931	845	920	576		
	Zajecar	387	583	408	273		
	Average/year/place	473	467	520	**282**	282	

Differences of value of traits in cultivars which growing in different environment found (MAKSIMOVIC *et al.* 1994; PRZULJ *et al.* 1997; KOKHMETOVA & OMAROVA 2000).
Analysis of physical characteristics of grain showed differences among newly barley cultivars and check cultivar NS 294. Jadran cultivar had the highest average value of percentage of seed

first class (92.2%), the highest thousand-grain weight (46.58 g) and very high value of hectoliter mass of grain (73,6 kg) – i.e. on the level of check cultivar. Except that Horizont cultivar the highest value in average of hectoliter mass (77.1 kg) and higher value of thousand grain mass (44.61 g) than check cultivar NS 29 (43.91 g) table 2. Other barley cultivars expressed these analyzed traits on the level of check cultivar or less than in NS 294.
Grain protein content varied from 12.7 % in Djerdan cultivars to 14.8% in check barley cultivar NS 294. Jadran cultivar had lower value grain protein content (13.1%) what is very important for malting barley, table 2. Also, in Jadran cultivar was established higher value of Colbacsh coefficient (74.6%) and Hartongs coefficient- 45 C (77.9) than in check cultivar NS 294 (Colbacsh coefficient - 73.2% and Hartongs coefficient - 77.5%). The values of Colbacsh coefficient and Hartongs coefficient- 45 C were not included in table, but have been consider in determination Jadran cultivar as a malting barley cultivar. Other four barley cultivars were approved as food barley cultivars.

Table 2. Physical characteristics of grain and grain protein contents in six two-row spring barley cultivars

Cultivar	Traits			
	Average percentage of seed First class (over 2.5 mm) (%)	Thousand grain weight of dry matter in %	Hectoliter mass of grain (kg)	Protein contents in grain dry matter (%)
Horizont	68.8	44.61	77.10	13.4
Ukras	78.2	41.84	73.40	13.2
Dragulj	58.0	38.62	72.75	13.2
Jadran	92.2	46.58	73.60	13.1
Djerdan	79.4	40.57	74.52	12.7
NS 294 check cv.	85.3	43.91	74.00	14.8

Height of plants varied for investigated barley cultivars, table 3. In average Horizont and Ukras cultivars had higher height of plants in average 2 cm than check cultivar. Plant height of Djerdan (-0.6 cm), Jadran (0.8 cm) and Dragulj (for 6.5 cm) was lower than in check cultivar NS 294. All newly barley cultivars had later ripening time (in average 5-6 days, except Jadran cultivar which expressed maturity about 2 days later) than NS 294 check cultivar. Days of maturity showed a strong positive correlation with days of heading. Similar results showed in investigation (MAKSIMOVIC *et al.* 1994). Greatest variation was observed for number of effective tillers per plant followed by number of seeds per spike and plant height. The number of tillers per plant varied from 3 to 14. A wide variation was found for number of seeds per spike, which ranged 24 to76.
All barley cultivars investigated for resistance to disease. Each barley cultivars expressed higher resistance to *Puccinia graminis.* Cultivars Dragulj, Jadran had very high resistance and Djerdan good and higher resistance to *Puccinia graminis* than check cultivar. Barley Horizont and Ukras cultivars had the highest resistance to *Puccinia graminis*, table 3. Also, same cultivars showed the highest resistance to *Puccinia hordei*, while Dragulj, Djerdan and Jadran had resistance on the level of check barley cultivar NS 294. Resistance to powdery mildew in Horizont and Ukras

cultivars expressed on the level of check cultivar (30%), while in Dragulj, Jadran and Djerdan cultivars was expressed higher resistance to powdery mildew than in check cultivar NS 294.
The powdery mildew (caused by *Erysiphe graminis* f.sp. *hordei*) in some country is a problem and yield losses my reach in average about 10%. Also in some country powdery mildew is serious disease of barley and yield losses can exceed 25%. This yield reduction is due to loss of green leaf area, reduced of yield components (numbers of kernel per spike, grain weight, tillers per spike) reduced root growth (CZEMBOR & CZEMBOR 2000).

Table 3. Morphological and physiological traits of new Yugoslav spring barley cultivars and check cultivar NS 294

Traits	Cultivars					
	Horizont	Ukras	Dragulj	Jadran	Djerdan	NS 294 check cv.
Plant height (cm)	78.7	78.8	70.2	75.9	76.1	76.7
Heading (days)	+6.2	+5.0	+4.8	+1.9	+4.8	
Resistance to lodging	3.4	3.2	2.2	2.0	3.6	3.6
Resistance to disease						
Puccinia graminis	5.0	5.0	10.0	25.0	35.0	45.0
Puccinia hordei	5.0	5.0	15.0	12.5	15.0	15.0
Erysiphe graminis f.sp. *hordei*	30.0	30.0	20.0	15.0	12.5	27.5

+ later heading than check cv.; - earlier heading than check cv.
0=without loading, 9=100% loaded plants

Conclusion

The newly spring two row barley cultivars included in this investigation expressed differences for grain yield and yield components (plant height, thousand grain weight, hectoliter mass) as well as for other corresponding parameters for yield potential and stability. All of previously quoted traits have been affected by the growing conditions. The variability of those traits was established for each cultivars on the same location in different growing year as well as in different location in the same experimental year. The highest yield in average per year and per location was established for Djerdan (+ 1358 kg/ha) and Horizont (+ 1186 kg/ha than check cultivar). Jadran cultivar expressed the highest percentage of seed first class (92.2%) and better quality parameters of malting barley than check cultivar NS.294. Other investigated cultivars showed traits value better than check cultivar, or at least value on the level of check cultivar NS 294. Analysis of yield and other morphological and physiological traits showed that environment had high influence to expression value of traits. In this investigation were exploited different growing condition and variation of traits indicating adaptability of cultivar.

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Genes for Success – What Can We Learn from Recommended List Trials?

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Abstract
Genotypic data was collected on 120 spring and winter barley lines that were placed on the NIAB and SAC Recommended Lists from 1980 to 2000. In addition, phenotypic data for yield and malting quality parameters was available from UK recommended list trials of both crops conducted since 1993 courtesy of Crop Evaluation Limited. Analysis of the phenotypic data enabled the regression of cultivar means against their year of first recommendation, which demonstrated a significant increase in spring and winter barley yield over the period from 1993 to 2004. In addition, there was a significant increase in hot water extract corrected to 1.5% grain nitrogen over the same period for spring but not for winter barley. Combining the genotypic data with the phenotypic data for winter barley corrected hot water extract identified significant associations of the trait with seven Simple Sequence Repeat loci. Allelic distribution at the seven loci was such that not one established malting quality cultivar carried more than four of the optimal alleles at the seven loci, suggesting several strategies whereby Marker-Assisted Selection could be deployed for malting quality in winter barley.
Keywords: barley; breeding progress; quality; yield; associations; QTLs

Introduction

Barley is the second most important cereal in the EU15 and the UK with production from the 2002 harvest at 49.6 and 6.2 Mt respectively. In Scotland, spring barley is the major combinable crop as the climate is well-suited for the production of malting barley. Consequently, maltsters purchase over 50% of the Scottish spring barley production in many years and the overall usage of malting barley in the UK is approximately 1.9 Mt. UK barley breeding and evaluation therefore focuses upon the development of high yielding cultivars with good malting quality as the main objectives. Barley breeding is very competitive with over 20 European breeders submitting an average of over 35 and 40 new spring and winter barley entries each year respectively to first year National List Trials since 1993. Since 1993, 63 new spring and 62 new winter barley candidates have been evaluated in the HGCA/CEL funded Recommended List Trial system for the UK leading to 27 and 37 new recommendations for growers of spring and winter barley cultivars respectively. This is an overall average of nearly six new recommendations each year, which suggests that real breeding progress is being made as the new recommendations have to show improvements in at least one character over existing cultivars.

Most commercial breeding programmes operate according to variations of the pedigree breeding scheme and rely upon phenotypic selection. Doubled haploids produced from F1's of crosses are used by some breeders to speed up the breeding cycle and/or improve selection efficiency, mainly in the winter crop. Whether or not doubled haploids are used, it is apparent from pedigree records that the breeding cycle has shortened so that new cultivars can be recommended seven years after the cross was made so that a cultivar's progeny may well be entering official trials by the time it is commercially successful. Breeding progress in yield of spring barley in the UK, up to the introduction of Triumph and Atem in 1980, was estimated to be approximately 1% per annum (SILVEY 1986). Analysis of UK Recommended List Trial data from 1992 to 2002 showed that this rate of increase had been maintained in both winter and spring barley (THOMAS 2003).

Moreover, the pedigrees of the recommended cultivars clearly demonstrate that commercial breeders are able to maintain this yield advance by working almost entirely with crosses between elite cultivars or breeding lines. Conventional phenotypic selection is therefore still an efficient means of selection for a complex character such as yield. Within the same time-frame, a number of QTL studies of yield have been published but comparison of the results reveals few genomic regions where genetic control is detected in more than one or two crosses and Marker-Assisted Selection (MAS) methods are, with one or two notable exceptions, not deployed by commercial barley breeders.

Many of the mapping studies that have been published so far are not representative of commercial barley breeding as many of the crosses are either between older cultivars that have long since been superceded and/or between highly divergent parents. The challenge is to integrate genotypic studies with a relevant set of germplasm to begin to identify the important genomic regions that are being manipulated in commercial breeding programmes. The Recommended List Trial data set provides a wealth of high quality phenotypic data that is collected from often over 20 sites each year throughout the UK and is able to identify statistically significant differences in yield of the order of 3% (www.hgca.com). In the following study, we update the breeding progress results of THOMAS (2003) to highlight the advances being made through conventional phenotypic selection as this sets a standard that MAS must either beat or enable to be matched with a greater efficiency. By integrating the phenotypic data with the genotypic data we also demonstrate how the breeding of malting quality winter barley has advanced since the introduction of Maris Otter.

Material and Methods

From harvest 1993 onwards, 72 spring and 82 winter barley genotypes have been grown in fungicide treated Recommended List Trials at sites throughout England, Scotland and Northern Ireland. Yield (t ha-1) data was available from a total of 228 spring and 237 winter trials from harvests 1993 to 2003 inclusive. In addition, a more restricted set of malting quality data was available for 50 spring and 50 winter cultivars from five selected sites each year from 1995 to 2002 inclusive. From each site, both the mean grain nitrogen (GN) content and hot water extract (HWE) were available were available for each genotype under test and were used to derive a hot water extract corrected to 1.5% grain nitrogen content (HWEc) using the formula HWEc=HWE+11(GN-1.5) following BISHOP (1948) as this gives a more accurate representation of varietal performance over contrasting seasons. Some pedigree information was also available for the entries in recommended list trials, either through an annual publications available up to 2000 or from www.stmlf.bayern.de/lbp/forsch/pz/gerstenstamm/.

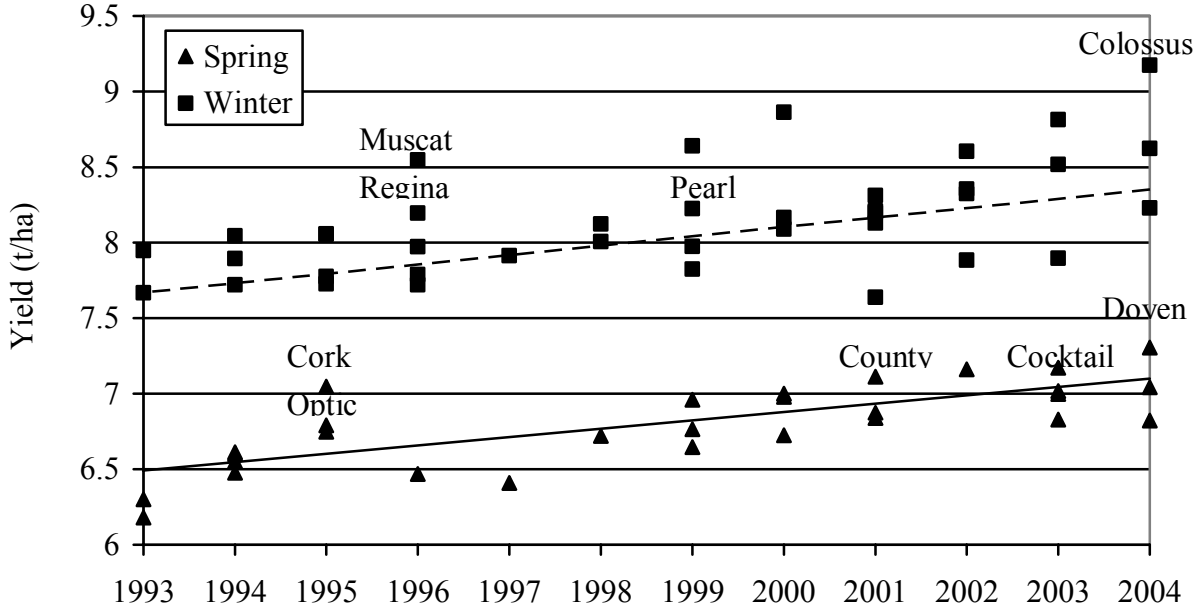
The overall mean for each genotype was estimated using the REML directive in GENSTAT (Lawes Agricultural Trust, Rothamsted Experimental Station, UK) with genotype, site, year and the interaction between the last two as fixed effects. Many of the genotypes were trialled over more than five consecutive years and one (cv Pastoral) and two (cvs Optic and Riviera) were trialled over 11 and 10 years respectively. With the overlaps in the trial periods for each genotype, the analysis is able to smooth out cultural differences over the years that the trials were conducted and generate a reliable estimate of the genetic potential of each line. The year in which each genotype was first recommended can be derived from the UK Recommended Lists or from www.hgca.com and thus establish the state of breeding progress in that year. We were therefore able to estimate the rate of breeding progress between 1993 and 2003 by regressing the mean yield of each recommended cultivar using GENSTAT.

As part of a larger study, we genotyped 62 spring and 58 winter barley cultivars that were recommended to growers over the period 1980 to 2000. The cultivars were scanned for variation at the DNA level with a set of 48 previously mapped SSR markers. The marker fragments from each SSR primer pair were then converted into alleles according to their frequencies and coded accordingly. Malting data was available for 31 of the winter barley cultivars and the ANOVA directive in GENSTAT was used to test for significant differences between the HWEc means of allelic classes at each SSR locus. Pedigree analysis at loci where significant differences were detected was used to follow the inheritance of alleles in the winter barley cultivars Halcyon, Pipkin, Puffin, Fanfare, Angora, Regina and Pearl, all of which were recommended by the Institute of Brewing for use by UK maltsters.

Results

In 1993, the highest yielding spring barley on the recommended list was Nomad at 6.45t ha^{-1} but Doyen, the highest yielding spring barley on the 2004 recommended list offered a 0.86t ha^{-1} increase in performance. Similarly, Colossus, the highest yielding winter barley on the 2004 recommended list has an average yield of 9.17t ha^{-1}, an increase of 0.66t ha^{-1} over Manitou, the highest yielding entry on the 1993 recommended list. Overall, there is a significant regression of treated yield against year of introduction that accounts for 58 and 34% of the variation amongst the cultivar means for spring and winter barley respectively. The average increase in yield is 0.8 and 0.9% for winter and spring barley respectively (Figure 1).

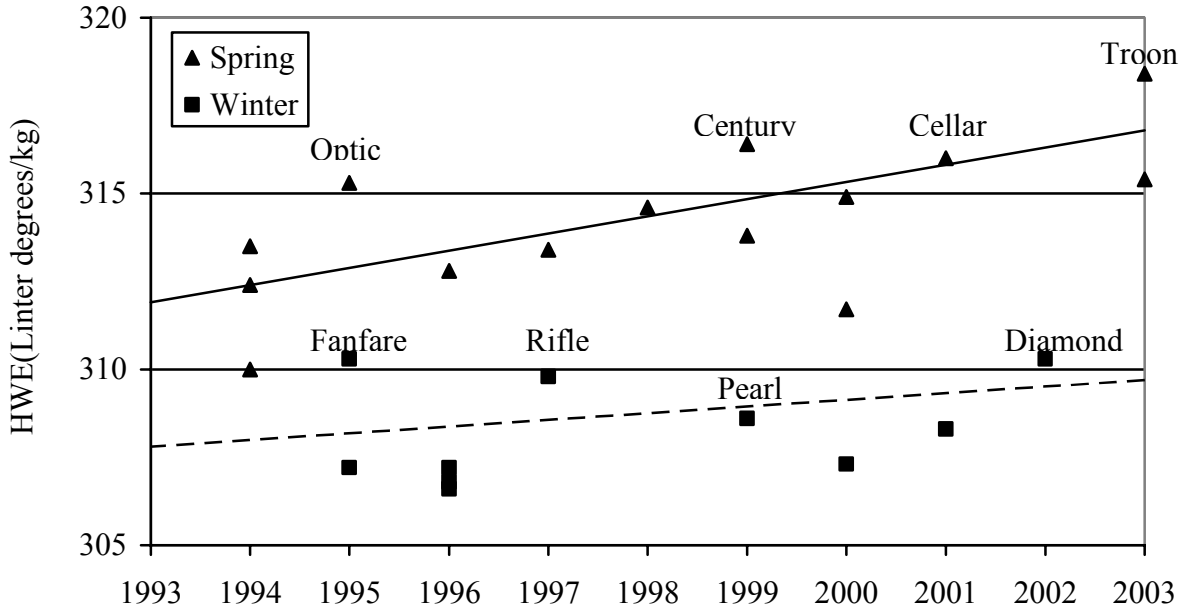
Figure 1. Treated yield plotted against year of introduction of cultivars placed on the UK recommended list 1993-2004



For HWEc content, we restricted the regression to those spring and winter barley cultivars that were recognised as being of malting quality by the Institute of Brewing. Nevertheless, the highest extract of a cultivar on the spring barley recommended list increased from 314.4 (Chariot) to 318.4 Linter° kg^{-1} (Troon) and from 307.4 (Halcyon) to 310.3 Linter° kg^{-1} (Diamond) for winter barley. (Figure 2). Whilst there was a significant regression between corrected hot water extract

and year of introduction for spring barley that accounted for 43% of the variation amongst the 14 cultivar means, the regression for winter barley was non-significant.

Figure 2. Hot water extract plotted against year of introduction of cultivars recommended by the Institute of Brewing and placed on the UK recommended list 1993-2004



Currently, Optic and Pearl dominate the spring and winter barley malting market in the UK respectively. Whilst they both have high levels of HWEc, varieties with still higher levels are, or have been, available. The spring barley Century and the winter barley Diamond appeared to have higher levels of HWEc but neither gained widespread acceptability as a malting variety as processing problems became apparent during their commercial evaluation. This resulted in both being removed from the UK recommended list soon after they were first recommended and represents a waste in development and testing costs. With a number of lines able to satisfy the requirements of a high level of hot water extract, processability will become increasingly important in discriminating between malting types. The spring barley Troon appears, however, to offer a notable improvement in HWEc but is still undergoing commercial evaluation so it is too early to say whether it represents a real alternative to Optic.

Analysis of mean differences in corrected hot water extract for the allelic classes at each of the 48 SSR loci used to genotype a subset of the winter barley recommended list trial genotypes detected significant associations at 10 loci. Three of these were neighbours of other, more significant loci and we considered these associations to be likely due to loose linkage and so discarded them. For the remaining seven loci, we were able to assign values to each allelic class and, in the case of the UK derived varieties, trace their ancestry back to Maris Otter. Both Regina and Angora are derived from the Breun breeding programme in Germany and, from a limited study of their pedigrees, appear to have little in common with Maris Otter. Igri features in the pedigrees of Puffin, Fanfare and Pearl and may represent a partial re-merging of the gene-pools.

Table 1. Allelic distribution and estimated effects on corrected hot water extract at seven SSR loci for selected winter barley cultivars. Patterns and colours indicate alternative alleles at each locus. Net allelic effects are summed and added to the mean to predict hot water extract.

	SSR1	SSR2	SSR3	SSR4	SSR5	SSR6	SSR7	Predicted HWE
M Otter	1.1	1.5	0.7	0.2	0.7	1.4	-0.3	309.0
Halcyon	1.1	1.5	0.7	0.1	0.4	0.4	0.4	309.1
Pipkin	1.1	0.2	0.7	1.3	0.7	0.2	-0.3	307.5
Puffin	1.1	0.2	0.7	1.3	0.7	1.4	0.5	308.2
Fanfare	1.1	1.5	0.7	0.2	0.4	1.4	1.1	309.8
Pearl	1.1	1.5	0.7	1.3	0.7	1.4	-0.3	309.8
Angora	1.0	1.5	0.7	1.5	0.7	0.2	1.1	310.2
Regina	1.0	0.2	0.7	1.3	0.7	1.3	0.4	309.1
Optimal	1.1	1.5	1.3	1.5	0.7	1.4	1.1	312.1
Igri	-1.5	0.2	0.7	0.6	-0.8	0.6	-0.7	302.5
Torrent	-1.5	0.2	-1.3	-2.3	0.4	0.0	0.4	299.4

The UK derived cultivars share considerable similarities in their SSR allele patterns. They all have the second most frequent allele at SSR1 and the most frequent allele at SSR3, both of which appear to have been transmitted from Maris Otter. In addition, the Maris Otter allele is found in three of the UK cultivars for SSR2 and SSR6. The latter is particularly notable, as it is the fourth most frequent allele at that locus. Angora and Regina show notable differences at SSR1 where they possess the third most frequent allele, which appears to be slightly inferior to the second most frequent. By contrast, the allelic distribution at SSR7 appears to be quite diverse over all the cultivars. The two other known parents, Igri and Torrent share few alleles with the malting quality cultivars and each possesses three alleles that appear to decrease malting quality and do not possess the best allele at any one of the seven loci. One notable feature of Table 1 is that, with the exception of Regina, the better cultivars (HWE > 309) possess three or more of the optimal alleles at the seven loci but have slightly different combinations and none possesses all seven optimal alleles. Indeed the best (Maris Otter, Fanfare and Pearl) possess the greatest number with four each. If all seven optimal alleles were combined in one genotype, the data suggest that corrected hot water extract in winter barley could be improved by nearly 2 Lintner° kg^{-1}.

Discussion

Results from conventional mapping studies show very little agreement even in the approximate location of QTLs for hot water extract on the Steptoe x Morex bin map (THOMAS 2003). Results from this study indicate that the best commercially acceptable malting quality winter barley cultivars do not possess more than four out of seven of the QTLs for hot water extract and none possess the best allele at the SSR3 locus. This re-inforces the fact that results from mapping of single cross populations has little practical value for commercial barley breeding. Mapping of a composite population derived from the amalgamation of several populations from a range of crosses, which we term Small Cross Mapping, is a strategy more likely to identify QTLs of value to barley breeders, particularly if applied to elite germplasm (RAE *et al.* 2004). Preliminary

results show that loci that we have identified by the association approach above are also detected by Small Cross Mapping.

The results from this study must, however, be treated with some caution as they assume that the genetic variation controlling corrected hot water extract is additive whereas epistasis is highly likely to also have an effect on such a complex character. Nevertheless, they do indicate some strategies that could be adopted for breeding winter malting barley, either by selecting the appropriate alleles at sub-sets of the significant markers or by trying to derive the optimal genotype. Comparison across the spring gene-pool reveals that the best alleles at some loci (data not shown) may not be even be present in the elite winter gene-pool, offering another strategy.

Our results clearly show that breeding progress is being maintained within the elite gene-pool and there is therefore little incentive for commercial breeders to use un-adapted germplasm in their breeding programmes for the key traits yield and malting quality. Given the lack of diversity amongst elite germplasm, e.g. RUSSELL *et al.* (2000), this might seem a surprising conclusion to reach. Cocktail and Doyen are two recently recommended spring barley cultivars bred by the same breeder (Syngenta Seeds UK) and share an immediate parent. Despite these similarities, genotyping at 35 SSR loci shows that over 8,000 different allelic combinations can be produced from the cross. Indeed, if the survey is extended to include another three recently recommended cultivars (Troon, Kirsty and Rebecca), there are over 200 billion different combinations. Whilst these are results from essentially anonymous markers, they provide strong evidence that there is still much variation that can be exploited in the elite gene-pool. Genotyping with functional markers such as the emerging resources from EST-derived markers will provide a more accurate picture of functional diversity. For use in plant breeding, the challenge is to identify the optimal combinations of alleles. This will, without doubt, require the integrated efforts of a number of different disciplines.

Acknowledgements
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Characterising the New Malting Barley Cultivars – Hamelin and Baudin – from Western Australia

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Abstract

The Western Australian barley industry has recently released two new, two-row spring malting quality barley (*Hordeum vulgare* L.) cultivars – Hamelin and Baudin. These cultivars were bred by the Government of Western Australia's Department of Agriculture. The Western Malting Barley Council oversaw the registration of these two cultivars. Agronomic information on the adaptation of Hamelin and Baudin to Western Australia was compiled from yield performance and agronomic management research trials.

Baudin is a superior malting quality, high yielding barley suited to the high rainfall regions and parts of the medium rainfall regions of Western Australia. Baudin received full registration in 2003 and will compliment sowing of the second most widely sown cultivar, Gairdner. Baudin has a wider regional adaptation than Gairdner because it has rounder grain and more flexible phenology. It has advantages over Gairdner for shortness of its straw, straw strength and head loss, but is more susceptible to the leaf blotch diseases and powdery mildew. Crop management plans released on registration of the cultivar are aimed at maximising adoption, minimising small grain and minimising the impact of leaf diseases.

Hamelin is a high malting quality barley suited to the low and medium rainfall regions of Western Australia. It is seen as a replacement for Stirling with a 4% increase in grain yield and improved grain brightness. In the field Hamelin looks very similar to Stirling, with a slight improvement in straw strength, a similar straw length and the same phenological development pattern. Its weaknesses relative to Stirling are slightly higher levels of screenings and susceptibility to pre-harvest sprouting (which excludes it from being grown in all regions of Western Australia). Hamelin does however offer significant advantages to the malting industry over Stirling due to its superior malting quality attributes.

Introduction

Western Australia is an important supplier of malting barley traded to the international malting barley market. Over the last five years Western Australia has supplied nearly 20% of the malting barley required by the international market, primarily to Japan, China, South America and Korea.

Since 1992, four new malting barley cultivars have been registered in Western Australia. The registration of Franklin (Tasmania), Harrington (Canada) and Unicorn (Japan) has helped improve the supply from Western Australia of high quality malt, but has done little to supplement the large tonnage of Stirling grain that is still exported. The Western Australian bred cultivar Stirling has been the dominant cultivar grown since 1983 (Table 1). However, the release of the Western Australian bred cultivar Gairdner in 1997 is beginning to have an

impact in international markets, in particular in South America and China. Gairdner is now the second most widely grown cultivar with the its area increasing at 5% per annum, primarily at the expense of Stirling.

Table 1. Percentage (%) of the Western Australian barley area sown to different cultivars averaged over a 3 year period since 1983 (source - Cooperative Bulk Handling)

Variety	1983 – 1985	1986 – 1988	1989 – 1991	1992 - 1994	1995 – 1997	1998 - 2000	2001 – 2003
Malting cultivars							
Stirling	35.9	75.4	83.0	76.5	69.5	65.7	54.7
Dampier	8.2	0.4	0.0	-	-	-	-
Clipper	20.0	2.5	0.9	0.5	0.0	-	-
Schooner	0.2	1.0	0.1	0.0	0.1	5.9	6.1
Franklin	-	-	0.0	2.1	6.9	1.6	0.0
Harrington	-	-	-	0.0	1.2	2.1	1.2
Gairdner	-	-	-	-	0.0	8.9	24.2
Unicorn	-	-	-	-	0.0	1.0	1.4
Feed cultivars							
Various	35.7	20.8	15.9	20.9	22.4	14.7	12.4

Franklin, Harrington, Unicorn and Gairdner have all required different management practices to achieve the malting receival specification. The delivery of management guidelines to growers and agronomists at the time of their release has enhanced their adoption and ensured that sufficient quantities of each cultivar has been available for developing markets and supplying malthouses. The impact to the industry from developing robust management guidelines and well targeted extension was demonstrated with the release of Gairdner. Within 2 years of release, Gairdner was sown on 13% of the barley acreage. Gairdner was adopted by growers because it's grain yields were equivalent to the best feed varieties, grain was able to meet export standards for grain size in more situations than Franklin and it had improved levels of disease resistance relative to Stirling.

Despite the successful adoption by growers and the interest being shown in the international market for Gairdner, the Western Australian barley industry is still looking for improved malting barley cultivars. Under the direction of the Western Malting Barley Council, an industry focused research and development group, investment is being made to develop cultivars whose malting quality equals the best European and Canadian varieties, but have adaptation to the Mediterranean environment of Western Australia.

In 2002, two elite breeding lines were considered for commercial release in Western Australia. This paper talks about the process to release WABAR2104 (registered as Hamelin) and WABAR2080 (registered as Baudin), their malting quality, their characteristics, their agronomic evaluation and where they will be adopted in Western Australia.

Commercial Evaluation of Elite Breeding Lines

Decisions about the release and status of malting barley cultivars in Western Australia are undertaken by the Western Malting Barley Council (WMBC). The WMBC comprises different sectors of the barley supply chain – grain growers, research and development organisations, maltsters, brewers, marketers and storage and handling companies. Elite breeding lines being considered for release as cultivars are required to undergo between two to three years of commercial malting and brewing trials before they can be classified as a malting cultivar in Western Australia (Table 2). This involves growing the breeding line at up to 5 locations (50 t production per site) each year. Grain from two of the locations that meet

the malting barley receival specification for Western Australia are then selected and submitted to the Australian malting company Joe White Maltings for commercial malting. Malt is then submitted to a partner brewing company such as the Swan Brewery, Western Australia, Kirin Brewery, Japan or the pilot brewery at the Carlton and United Breweries, Victoria.

On completion of commercial malting and brewing trials, the status of the line is determined. The line can be classified as either General Malting, Domestic-Only Malting, Export-Only Malting or Contract Malting. The explanation of each classification category is detailed in Table 2. If the line fails to meet the expectations of the industry it will not be released or be released as a feed cultivar.

Table 2. Current classification status of released malting barley cultivars as determined by the Western Malting Barley Council in Western Australia

Classification	Explanation	Cultivars
General Malting	Variety successfully undergone commercial evaluation and deemed by the Western Malting Barley Council (WMBC) to be applicable to both domestic and export markets	Stirling Gairdner Baudin
Domestic-Only Malting	Variety successfully undergone commercial evaluation and deemed by the WMBC to be suitable for domestic use in Western Australia.	Unicorn Harrington
Export-Only Malting	Variety successfully undergone commercial evaluation and deemed by the WMBC to be suitable for niche sales on the export market and unsuitable for the domestic market in Western Australia.	Schooner
Contract Malting	Variety subject to a specific contract to be received for malting. For grain not covered under such a contract it will be classified as feed. Variety may not meet all conditions of the commercial protocol.	Franklin
Provisional Malting	Released variety that is deemed to have malting potential and is undergoing commercial evaluation in Western Australia.	Hamelin

Breeding and Release of the Cultivars Baudin and Hamelin

In October 2002, the Government of Western Australia's Department of Agriculture released two barley breeding lines WABAR2104 and WABAR2080. WABAR2104 was named Hamelin (pronounced *Ham-lin*) and WABAR2080 named Baudin (pronounced *Bow-dan*). Both were released with a status of Provisional Malting. Baudin was named after the French explorer, Thomas Nicolas Baudin who reached the Western Australian coastline on 30 May 1801 in the corvette the Geographe. Hamelin was named after the French explorer, Emmanuel Hamelin who was second in charge to Thomas Nicolas Baudin. Hamelin reached the Western Australian coastline on 31 May 1801 in the corvette the Naturaliste.

Both Hamelin and Baudin are two-row spring cultivars bred by the F_2 bulk progeny method at the Department of Agriculture, Western Australia in 1990 (ANON 2002). Hamelin is the result of a controlled pollination cross between seed parent Stirling and pollen parent Harrington. Baudin is from the cross between seed parent Stirling and pollen parent Franklin.

In October 2003, the WMBC upgraded the status of Baudin to the classification of General Malting after completion of two years of successful malting and brewing trials. Baudin represents a significant step forward in malting quality and should be well received by international barley markets. The WMBC expects the release of Baudin will ensure Western Australia continues to be a preferred supplier of high quality malting barley.

Micro-malt tests conducted at the Department of Agriculture compared the malting performance of Baudin to the cultivars, Stirling and Gairdner. Overall the results indicate Baudin is an improvement in malting quality with high malt extract, high diastase and alpha

amylase activity, good protein modification capacity and produces highly fermentable wort with low wort ß-glucan levels (TARR *et al.* 2003). TARR *et al*. (2003) concluded that they considered Baudin to be similar in malting quality to the Canadian cultivar Harrington.

Hamelin has one more year of commercial malting and brewing trials before its final status can be considered by the WMBC. Micro-malt tests have been conducted at the Department of Agriculture comparing the malting performance of Hamelin to the cultivar Stirling. Overall the results indicate that Hamelin malts and modifies very easily giving clear wort with low wort ß-glucan, has a high level of diastase activity and protein modification capacity with high free amino nitrogen levels, is an improvement in malt extract and extract fermentability when compared to Stirling (TARR *et al.* 2003). Hamelin compares favourably to the malting quality achieved by its parent Harrington (TARR *et al*. 2003).

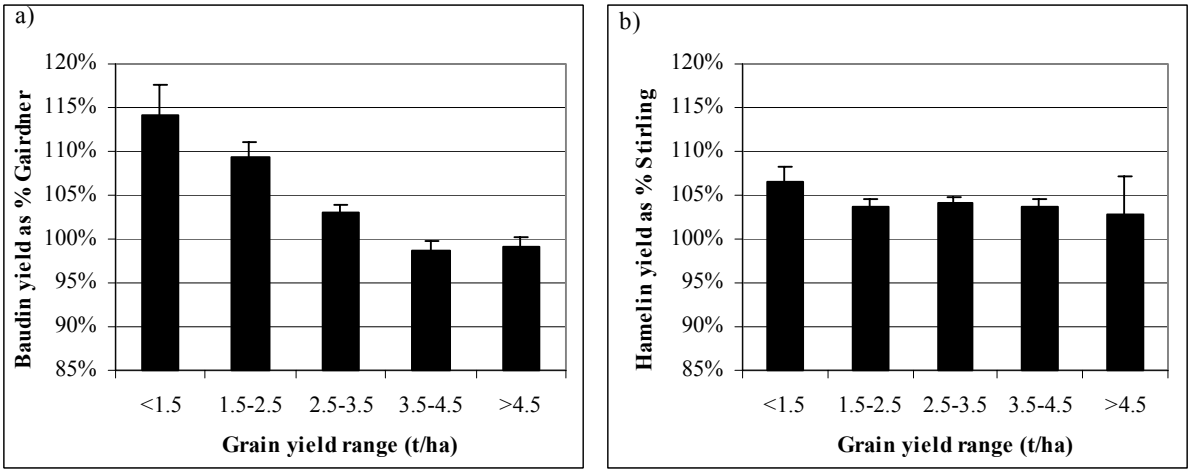

Figure 1. Relative grain yield of a) Baudin versus Gairdner and b) Hamelin versus Stirling over a range of grain yields achieved by the control variety from agronomic management and crop variety testing trials in Western Australia between 1998 to 2003.

Characteristics of Baudin versus Gairdner

Baudin was released for sowing as an alternative to the cultivar Gairdner in the higher rainfall regions of Western Australia. Baudin however has a wider regional adaptation than for Gairdner as its phenological development pattern is based on a very high level of daylength sensitivity and a short basic vegetative period (Table 3). The development pattern of Baudin is considered to be well suited to the higher rainfall environments of Western Australia (YOUNG & ELLIOT 1994).

Baudin has a rounder grain shape and there should be less screenings through a 2.5 mm slotted sieve than for Gairdner (PAYNTER *et al*. 2003). Overall, plumper grain and higher daylength sensitivity means that Baudin grain quality is less sensitive to changes in crop management than Gairdner and has wider adaptation to rainfall zones, sowing dates, rotations and soil types (Table 4).

In summary it has advantages over Gairdner for malting quality, straw length, straw strength and head loss, but is more susceptible to the leaf blotch diseases and powdery mildew (Table 3). Grain yields of Baudin are comparable to Gairdner when the site mean is above 3 t/ha (Figure 1a). As the site mean falls below 3 t/ha however, Baudin shows a grain yield advantage over Gairdner. The grain hardness of Baudin is similar to Stirling and harder than

Gairdner and Schooner, which means it may be suitable as a hard grained cultivar for the Japanese shochu industry.

Table 3. Comparison of Stirling, Hamelin, Gairdner and Baudin for a range of phenological, physical and disease resistance traits

	Stirling	Hamelin	Gairdner	Baudin
(1) Phenology				
Maturity group	Early spring	Early spring	Medium spring	Medium spring
Basic vegetative period	Short	Short	Medium-Long	Short
Daylength sensitivity	High	High	Medium	Very high
(2) Physical traits				
Early growth habit	Erect	Erect	Prostrate	Prostrate
Height	Tall	Tall	Medium	Short
Straw strength	Fair	Fair-Good	Fair-Good	Very good
Head retention	Fair	Fair-Good	Moderately poor	Very good
Grain plumpness	Very good	Good	Fair	Moderately good
Grain protein potential	Moderate	Moderate	Low	Low
Pre-harvest sprouting	Low susceptibility	Very susceptible	Low-mod susc	Low susc
Grain hardness	Hard grained	Hard grained	Soft grained	Hard grained
(3) Disease resistance				
Scald	Susceptible	Susceptible	Intermediate	Intermediate
Net-type net blotch	Susceptible	Susceptible	Intermediate	Susceptible
Spot-type net blotch	Mod susceptible	Mod susceptible	Susceptible	Susceptible
Powdery mildew	Susceptible	Susceptible	Mod susc – Interm	Susceptible
BYDV	Intermediate	-	Resistant	Mod resistant
Barley leaf rust	Susceptible	Susceptible	Susceptible	Susceptible

Characteristics of Hamelin versus Stirling

Hamelin is being considered for release as a cultivar to replace Stirling in the low, medium and parts of the high rainfall regions of Western Australia. Hamelin has a similar regional adaptation as Stirling with its phenological development pattern based on a high level of daylength sensitivity and a short basic vegetative period (Table 3). This development pattern is well adapted to all growing regions in Western Australia (YOUNG & ELLIOT 1994).

Hamelin grains weigh around 0.5 mg (db) lighter than those of Stirling and are slightly narrower in grain shape. The grain shape of Hamelin is equivalent to Schoooner and this translates into slightly higher levels of screenings relative to Stirling. Hamelin has a slight advantage in grain yield over Stirling over a range of yield potentials (Figure 1b). The average yield advantage is around 104% of Stirling.

In summary, Hamelin is adapted to similar rainfall zones, sowing dates, rotations and soil types as Stirling. Like Stirling it is susceptible to high levels of boron and aluminium found in some Western Australia soils. It has a similar appearance in the field as Stirling, but has significant advantages for malting quality with an improvement in grain colour (1 Minolta L unit). Grain hardness is also similar to Stirling, suggesting it may be suitable for use in the shochu industry in Japan where Stirling is already a preferred cultivar. Like Stirling, disease resistance is a weakness of the cultivar (Table 3).

Agronomic Evaluation of Elite Breeding Lines

Elite barley breeding lines entering commercial malting and brewing trials are evaluated in agronomic management trials. Agronomic trials focus on characterising elite breeding lines such as Hamelin and Baudin and determining their adaptation to key environment (Agzones)

and soil types under a range of sowing dates. The Western Australian environment is broken into six key Agzones for cereals (LITTLEWOOD 2003) and sixty major soil groups (SCHOKNECHT 2002). These trials compliment cultivar yield performance trials by providing greater information on the interaction between crop management and adaptation.

The agronomic research group works with key soil groups in each of the Agzones and compares the response of elite breeding lines on one soil group to another soil group. The soil groups chosen differ in textural properties and water holding capacity. The small plot research trials sown on each soil group are often only 100 m apart in the same paddock. Seeding rate for each breeding line is adjusted to establish 150 plants/m^2. This methodology minimises weather differences between comparisons. An example of a soil group comparison might be comparing the performance of breeding lines on a boron toxic sandy duplex (sand overlying clay with boron levels of 5 µg/g) to their performance on an ironstone gravel (sand to 80 cm with 50% ironstone rocks in matrix). Each small plot trial is sown at two dates of seeding on each soil type giving four site-soil-seeding date comparisons at the one location.

Table 4. Comparison between Stirling, Hamelin, Baudin and Gairdner for screenings (% < 2.5mm) when sown at two different dates on either a loamy surfaced soil (loam earth or duplex) or a sandy surfaced soil (sandy duplex) on three sites in the medium rainfall zone of Western Australia

Soil type	Loamy surfaced sites		Sandy surfaced sites	
Variety and trial	TOS1	TOS2	TOS1	TOS2
a) Brookton 2001	15-May	13-June	15-May	13-June
Stirling	2	3	4	2
Hamelin	6	6	10	3
Gairdner	20	30	17	38
Baudin	6	9	4	2
b) Brookton 2002	05-June	27-June	05-June	27-June
Stirling	5	15	9	20
Hamelin	8	17	20	18
Gairdner	12	30	18	36
Baudin	13	13	16	25
c) Calingiri 2003	23-May	17-June	23-May	17-June
Stirling	3	3	2	3
Hamelin	3	2	3	4
Gairdner	7	15	24	54
Baudin	4	3	14	23

This methodology proved to be very successful in developing agronomic management plans for the cultivar Gairdner. Gairdner is a cultivar with a narrow grain shape and tendency to produce higher screenings than the cultivar Stirling (PAYNTER *et al*. 1999b; Table 4). Using the soil type methodology we were able to determine on what soil types and at what sowing dates in different Agzones that Gairdner could successfully be grown for the malting industry. This information formed part of the management guidelines extended to the barley industry in Western Australia (PAYNTER *et al*. 1999a).

Over the last three years, the elite breeding lines Hamelin and Baudin have been compared to the control cultivars Stirling and Gairdner at 26 different locations, giving 104 site-soil-seeding date-year comparisons. Table 4 highlights how Baudin and Hamelin responded to changes in soil type and date of seeding at three locations within Western Australia. Table 4 suggests Baudin is more stable in its grain size than Gairdner and should be more reliable across a range of soil types and sowing dates. There are also situations where the screening levels of Baudin were equivalent to the plump grained cultivar Stirling. Screening levels for Hamelin were slightly above Stirling in most situations.

In addition, the agronomic research group examines relationships between cultural management practices and the grain yield and grain quality of elite breeding lines. This may include seeding rate, nitrogen application, date of harvest and disease management trials. The information generated is used to modify the management plan for the cultivar when it is released and to maximise adoption by the grain growers of Western Australia.

Where Will Baudin and Hamelin Be Grown in Western Australia?

The combination of maturity, responsiveness to daylength, grain shape, adaptation to soil type, disease resistance, yield potential and market potential determine where a new cultivar will be adopted and grown by Western Australian barley growers. The provision of management guidelines from the agronomic research group assists this adoption.

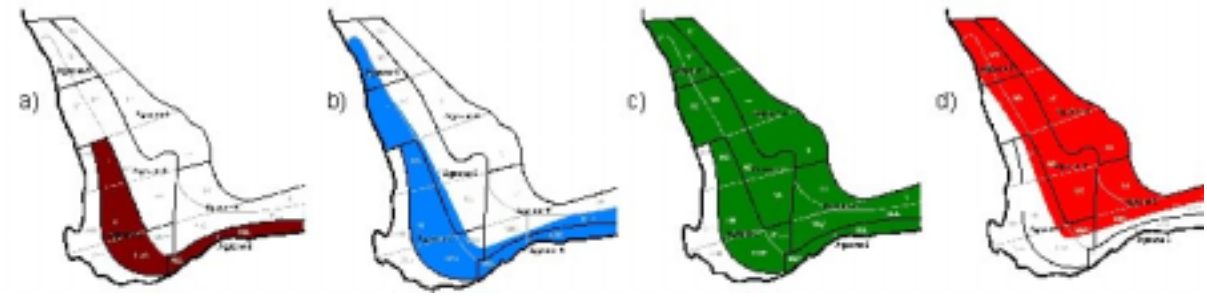

Figure 2. Regional adaptation maps for a) Gairdner and b) Baudin, c) Stirling and d) Hamelin in Western Australia. Shaded areas represent where each cultivar is recommended for sowing with an appropriate management package. Barley is grown in winter (May to October) in the south-western corner of Western Australia between the 29°S and 35°S parallels.

Adoption of Baudin in Western Australia

As Baudin has grain yields equivalent to Gairdner and has a similar flowering date it will be adopted in areas that currently grow Gairdner (Figure 2). In the southern and central high rainfall areas of Western Australia, Gairdner will still be the preferred cultivar where growers are seeking tall crops for swathing and a variety with intermediate resistance to powdery mildew. Baudin will be selected where a short, stiffer strawed cultivar is needed and where powdery mildew can be effectively managed.

In medium rainfall and northern high rainfall areas, Baudin has a greater probability of meeting the malting receival standard (due to rounder grain shape and more flexible phenology) than Gairdner and should become the preferred cultivar. Growers may choose to grow Gairdner because its taller straw offers ease of harvest in uneven terrain and it is less susceptible to net-type net blotch.

Adoption of Hamelin in Western Australia

The WMBC is due to consider the classification Hamelin at the end of the 2004 season. As Hamelin has a similar phenology, disease susceptibility and plant appearance and slightly higher yield potential, Hamelin has the potential to be adopted by growers in all areas of Western Australia where they currently grow Stirling (Figure 1). The exception will be the southern high rainfall areas where there is a risk of weather damage and pre-harvest sprouting. Hamelin is susceptible to pre-harvest sprouting with the genes for pre-harvest sprouting coming from its pollen parent Harrington (C.D. Li *et al*., unpublished data).

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Interrelationships between Phenology and Yield of Winter Barley in Semiarid Environment

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Abstract
Each yield component of barley is determined by developmental events during specific phenological phases. The number of spikes per unit area is established from tillering to jointing. Spikes continue to develop between single ridge and flag leaf elongation and the number of kernels per spike is established from jointing, i.e., it starts from double-ridge of apical meristem development and sets shortly after anthesis. Duration and rate of grain filling determines kernel weight. The objectives of this study were to examine the variation and relationships among phenology and yield components. Twenty-four winter barley cultivars were used in this investigation. The stage of leaf development of the main culm was referenced to the Haun scale. All phenological measurements were assessed using growing degree days (GDD) with a base temperature of 0˚C. The relationship between kernel weight and GDD accumulated from anthesis for each plot was determined by fitting the quadratic polynomial. The duration from planting to flag leaf was 1223 GDD across two-rowed varieties and 1304 GDD across six-rowed varieties. The variety NS 519 had the shortest grain fill period (648 GDD), the variety Marinka the longest (940 GDD). GF rate was mainly determined by genotype (44.3% of total variation) and GxY interaction (31.1% of total variation). GF rate across two-rowed varieties was rather higher (7.251 mg 100 GDD^{-1}) than across six-rowed varieties (6.395 mg 100 GDD^{-1}). The duration of the vegetative and generative phases should be balanced, since neither too early nor too late a flowering will bring maximum yields. Our results show that the pre-heading period varied more than the grain filling period in the tested varieties. Selection for shorter vegetative period and longer grain filling period is recommended in the development of varieties for semiarid conditions of growing.
Keywords: winter barley (*Hordeum vulgare* L); phenology; vegetative period; grain filling; yield components

Introduction

Each yield component of barley is determined by developmental events occurring during specific phenological phases. Number of spikes per unit area is establishing from tillering to jointing. In winter barley, tillering typically starts in the fall and it finishes in the spring. Number of tillers depends on genetic factors, i.e., tillering capacity, ecological factors, which ensure the realization of tillering capacity, and the interaction of these two factors (DAVIDSON & CHEVALIER 1990). Number of kernels per spike, the second most important yield component, sets in the period from double-ridge stage of apical meristem till shortly after anthesis (KIRBY *et al.* 1981). In this period, rate and duration of spikelet development and floret generation as well as the effectiveness of pollination define the final number of kernels. Generally, spikes develop between single ridge and flag leaf elongation. Kernel weight is the third most important yield component. During first two weeks after anthesis, the number of kernel cells is determined (BROCKLEHURS 1977) and, after that, kernel weight is determined by the duration and rate of

grain filling (WIEGAND & CUELLAR 1981). Plant genotype and environment, fist of all temperature and moisture, control the process of kernel filling (SOFIELD *et al.* 1977).
To our knowledge, limited data are available about relationships between phenology and yield components of winter barley in semiarid regions of southeastern Europe. The objectives of this study were to examine (i) the variation of phenology and yield components and (ii) the relationships between growth and yield components.

Material and Methods
Twenty-four winter barley cultivars, twelve two-rowed and twelve six-rowed, differing in origin, duration of vegetative and generative phases and other physiological traits were used in this investigation. The trials consisted of two identical blocks. The experimental design of each block was randomized complete block in three replicates. Each plot consisted of six rows 15 cm apart and 5m long ($5m^2$). Seedling dates were October 4, 1999, October 3, 2000, and October 8, 2001. The sowing rates were 200 seeds m^{-2} for the six-rowed and 250 for the two-rowed cultivars. Shortly after emergence, five random plants in the second row, 30 cm away from plot border, were tagged for observation during the growing season. The stage of leaf development of the main culm was referenced to the Haun scale (HAUN 1973) at intervals of 3-4 days from emergence to heading. The Haun scale is based on the number of leaves and sequence of leaf insertion on the main stem. All phenological measurements were assessed using growing degree days (GDD) with the base temperature of 0˚C.
At anthesis, 60 main spikes from each plot of the first trial that flowered at the same day were tagged. Samples of five tagged spikes/plant were collected from each plot at 3-4 days intervals beginning five days after anthesis till harvest maturity for determination grain filling rate and above ground dry matter accumulation. The relationship between kernel weight and GDD accumulated from anthesis to physiological maturity was determined for each plot by fitting the quadratic polynomial W=a+bt+ct2, where W is kernel dry weight, t is time (GDD) from anthesis and a, b, c are regression coefficients (PRZULJ 2001).
The second trials was used for the determination of yield components in each year and plot, i.e., number of spikes m^{-2}, number of kernels spike-1, kernel weight spike-1, and grain yield. Analysis of variance for each trait was conducted by using MSTAT-C program. Variance components were estimated using expected mean squares to compare the relative magnitude of main effect and interaction variances (COMSTOCK & MOLL 1963). Simple correlation coefficients were calculated for the relationships between vegetative period and yield components and grain filling period and yield components.

Results and Discussion
Durations of periods from planting to flag leaf and from planting to heading were determined mainly by genotype of the tested varieties, although year and GxY interaction also significantly affected these phenological phases (Table 1). Genotype controlled the largest part of the variation, as confirmed by high heritability (0.90). The duration from planting to flag leaf stage was 1223 GDD across the two-rowed varieties and 1304 GDD across the six-rowed varieties (from Table 1). The duration from planting to heading ranged from 1337 GDD, in the two-rowed barley variety NS 331, to 1554 GDD, in the six-rowed variety Gerbel (Table 2). Across years and varieties, the two-rowed type accumulated during this period 87 GDD less than the six-rowed type (from Table 1). A general conclusion can be drawn that the two-rowed varieties from Novi Sad, i.e., from the place where the experiment was conducted, had shorter periods from planting to flag leaf and planting to heading than the two-rowed barley varieties from Germany, France,

England, and Holland which headed 3 to 10 days later. The six-rowed varieties also exhibited significant differences in the duration of vegetative period but these differences amounted to a few days only and they were not consistent in relation to the geographic origin of the varieties.

Table 1. ANOVA, percentages of variance components and heritability for the tested traits

Source of variation	Df	Planting to flag leaf	Planting to heading (GDD)	GF duration	GF rate (mg 100 (GDD^{-1})	Spikes m^{-2}	Kernels $spike^{-1}$	Kernel wt. $spike^{-2}$ (mg)	Grain Yield ($kg\ ha^{-1}$)
Year (Y)	2	**	**	**	**	**	**	**	**
Variety (G)	23	**	**	*	**	**	**	**	*
GY	23	**	**	**	**	**	**	**	**
				Percentage of variance components					
Year (Y)	2	13.7	10.4	26.8	18.6	34.1	8.8	4.0	79.0
Variety (G)	23	62.2	59.6	20.5	44.3	32.3	78.9	67.7	6.3
GY	23	14.8	13.8	50.6	31.1	26.5	7.9	15.0	8.4
Error	142	6.3	16.2	2.1	6.0	7.1	4.4	13.3	6.3
h^2		0.92	0.90	0.55	0.80	0.77	0.96	0.91	0.64

*,** - Significant at P=0.05 and 0.01, respectively

Duration of grain filling stage depended of year, variety and specific behavior of the varieties in the test years ($P<0.01$). The interaction genotype x year determined the main part of variation (50.6%) of this trait. The variety NS 519 had the shortest grain filling period (648 GDD), the variety Marinka the longest (940 GDD). There was no consistency between variety origin and GFD, i.e., among both Serbian and foreign varieties there were genotypes with either short or long GF periods. GF rate was mainly determined by genotype (44.3% of total variation) and by GxY interaction (31.1% of total variation) (Table 1). GF rate across the two-rowed varieties was higher (7.251 mg 100 GDD^{-1}) than across the six-rowed varieties (6.395 mg 100 GDD^{-1}). The negative correlation between the number of kernels per spike and GF rate confirms this finding (Table 3). Sonate and NS 27 had the fastest GF rates within the two-rowed and six-rowed varieties, respectively. The slowest GF rates were found in Marinka among the two-rowed varieties and Kearney among the six-rowed varieties. GF duration was under strong ecological influence ($h^2=0.55$) but GF rate was under strong genetic influence ($h^2=0.80$). Van SANFORD (1985), CAMPBELL *et al.* (1990), HUNT *et al.* (1991) found that genetic factors determine to a larger extent the rate of GF while environmental factors, first of all temperature, determine to a large extent the duration of GF. NASS and REISER (1975), GEBEYEHOU *et al.* (1982), and WONG and BAKER (1986) reported a positive correlation between an effective filling period and grain yield, but van SANFORD (1985) and DARROCH and BAKER (1986) pointed out that high kernel weight is associated with a high rate of GF. In a previous work, PRZULJ (2001) found that in semiarid conditions the environment favors a higher rate and shorter duration of GF, i.e., cultivars with faster rate and shorter duration of GF produce higher yields. The duration of the vegetative and generative phases should be balanced, since neither too early nor too late a flowering will bring maximum yields. Choosing genotypes with a high GF rate, whose developmental dynamics is suitable for particular growing conditions, represents a safer way to develop stable, adaptable and high-yielding cultivars. A negative correlation exists between GF duration and rate which have an opposite effect on grain yield.

Table 2. Effect of cultivar and year on duration of period from planting to flag leaf appearance, planting to heading, flag leaf appearance to heading, grain filling (GF) duration, grain filling rate (mg 100GDD-1), number of spikes per m^2, number of kernels per spike, kernel weight per spike, and grain yield in 24 winter barley cultivars across three years

Cultivar (origin and spike type)	Planting to flag leaf	Planting to heading	GF duration	GF rate	Spikes m^{-2}	Kernels $spike^{-1}$	Kernel wt. $spike^{-1}$	Grain Yield
	GDD							
NS 183 (SMN, 2R)	1190	1351	662	7.379	579	25	1.35	6672
NS 293 (SMN , 2R)	1179	1351	700	7.012	565	25	1.26	6700
NS 331 (SMN, 2R)	1163	1337	660	6.917	578	25	1.19	6539
NS 519 (SMN, 2R)	1075	1343	648	7.385	630	28	1.33	6022
Sonate (DEU, 2R)	1217	1399	687	7.605	702	25	1.45	6817
Hanna (DEU, 2R)	1239	1447	748	7.497	636	26	1.44	6728
Marylin (DEU, 2R)	1178	1365	657	7.469	748	22	1.31	6322
Melusine (FRA, 2R)	1261	1472	758	7.347	564	28	1.35	7151
Belivia (FRA, 2R)	1266	1440	664	7.485	536	24	1.46	6236
Waveney (GBR, 2R)	1286	1447	677	7.231	678	24	1.19	6406
Mallard (GBR, 2R)	1307	1527	753	6.928	683	28	1.40	5639
Marinka (nld, 2R)	1310	1523	940	6.724	457	38	1.76	5464
NS 27 (SMN, 6R)	1304	1492	700	7.264	462	47	2.79	6817
NS 313 (SMN, 6R)	1292	1507	729	6.717	378	54	2.70	6761
Galeb (SMN, 6R)	1305	1502	660	6.861	411	50	2.43	6939
NS 717 (SMN, 6R)	1238	1460	665	6.862	343	50	2.56	6172
Katja (DEU, 6R)	1335	1517	763	6.175	368	51	2.32	6622
Majo (DEU, 6R)	1302	1460	656	6.561	437	55	2.69	5021
Robur (FRA, 6R)	1265	1439	702	6.376	430	51	2.18	5861
Gerbel (FRA, 6R)	1348	1554	826	5.459	350	58	2.63	6277
Kozir (RUS, 6R)	1320	1499	855	5.728	421	55	2.44	8367
Michailo (RUS, 6R)	1328	1498	675	6.501	437	54	2.68	7994
Kearney (USA, 6R)	1326	1523	690	5.874	451	55	2.32	3989
Dundy (RUS, 6R)	1288	1460	710	6.361	385	63	2.84	7539
$LSD_{0.05}$	19	22	16	0.184	42	3.1	0.25	680
$LSD_{0.01}$	25	30	21	0.243	62	4.1	0.33	898
2000	1235	1423	789	6.448	640	45	2.15	9413
2001	1295	1465	683	6.851	452	36	1.86	5404
2002	1260	1476	676	7.166	438	39	1.88	4565
$LSD_{0.05}$	7	8	6	0.065	17	1.1	0.09	241
$LSD_{0.01}$	9	11	7	0.086	22	1.4	0.12	318

Number of spikes m^{-2} was influenced by all three factors approximately to the same measure, while the other two yield components were mainly determined by the genotype, as confirmed by a high heritability value (Table 1). Close to 80% of grain yield variability were due to ecological factors and, as confirmed by a rather low heritability value. The two-rowed varieties had a higher

number of spikes m^{-2}, a lower number of kernels $spike^{-1}$ and a lower kernel weight $spike^{-1}$ than the six-rowed varieties. Number of spikes m^{-2} was negatively correlated with the periods from planting to flag leaf emergence and from planting to heading. This apparently puzzling statement could be explained by the ability of barley to self-regulate stand density. Indeed, varieties with a long period from planting to heading develop a large number of tillers and a large number of kernels per spike in the course of fall and spring. In 2000 and 2002, the droughts that occurred after tillering reduced the number of spikes that had already been formed, which caused a situation that the later-heading varieties had a lower spike number m-2 than the early-heading ones. This explains the negative correlation between the length of vegetative period and grain yield. All three yield components, spikes m^{-2}, kernels $spike^{-1}$, and kernel weight $spike^{-1}$, were in positive correlation with grain yield (Table 3).

Table 3. Simple correlation coefficients between the means of 24 winter barley varieties in the period 2000-2002 (n=216)

	Planting to heading	GF duration	GF rate	Spikes m^{-2}	Kernels $Spike^{-1}$	Kernel wt. $spike^{-1}$	Grain yield
Planting to flag leaf	0.79**	0.15*	-0.39**	-0.51**	0.42**	0.42**	-0.25**
Planting to heading		0.17	-0.29**	-0.55**	0.45**	0.40**	-0.32**
GF duration			-0.61**	-0.03	0.26**	0.14*	0.39**
GF rate				0.25**	-0.63**	-0.47**	-0.28**
Spikes m^{-2}					-0.43**	-0.38**	0.47**
Kernels $spike^{-1}$						0.89**	0.24**
Kernel wt. $spike^{-1}$							0.22**

*,** Significant at P=0.05 and 0.01, respectively

Our results showed that the tested varieties differed more in the duration of the pre-heading period than in the duration of the grain filling period. Selection for reduced vegetative period and increased grain filling period is recommended in the development of varieties for semiarid conditions of growing.

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The Malting Quality of European Malting Barley Varieties in Estonian Conditions

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Abstract

The trials of malting barley varieties were carried out in 1991–2001 at the Jõgeva Plant Breeding Institute in Estonia. The malt analyses were made at the VTT Biotechnology in Finland. The objective of the study was to estimate the malt quality of European malting barley varieties. 70 malting barley varieties of Northen region of European Brewery Convention (EBC) were included in the trial. 'Alexis'was used as a standard in 1991–1995 and 'Scarlett' in 1997–2001. Most of the tested varieties showed excellent or good level of extract yield, β-glucan content, viscosity and diastatic power. 'Maresi', 'Landora' (Germany), 'Caruso', 'Miralix' (Denmark), 'Wikingett' (Sweden) and 'Brise' (Great Britain) showed the highest level of extract yield compared to standard varieties. ß-glucan content of the varieties 'Chariot' (Great Britain), 'Caruso' (Denmark), 'Potter' (Sweden), 'Pasadena' and 'Annabell' (Germany) showed excellent level of this quality trait. The friability of standard varieties was most exceeded by varieties 'Annabell' (Germany), 'Pasadena' (Germany), 'Chariot' (Great Britain), 'Reform' (Denmark) and 'Caruso' (Denmark). The varieties 'Elo' (Estonia), 'Cork' (Great Britain) 'Extract', 'Laura' (Germany) showed the highest level of the diastatic power when compared to the standard varieties.

Keywords: malting barley; malting quality; extract; β-glucan; friability; diastatic power

Introduction

Barley needs to be malted before brewing to enable the synthesis or activate of enzymes. These enzymes soften the grain, rendering it more readily milled; remove polymeric substances that hinder wort separation from spent grains due to their hight viscosity and contribution to "fines" that block the filter bed, convert starch to fermentable sugars during mashing; generate the other nutrients demanded by yeast for efficient fermentation (for example, amino acids and nucleic acid degradation products); eliminate proteins that jeopardize beer handling and product quality (BAMFORTH & BARCLAY 1993).

Extractivity demonstrates a property of malt to form substances that dissolve in wort. The extractivity of the malt is of economic importance to the brewer, therefore it is this property of malt quality that is most important in barley breeding. Many breeders have accomplished remarkable success in the enhancement of malt extractivity (SCHWARZ & HORSLEY 1995; SCHILDBACH 2002). Extractivity depends on the barley variety, the growing conditions, the weather and soil conditions, as well as on the kernel size and the protein content. To reach high extractivity, high strach content in the kernel and a suitable amount starch degrading enzymes synthesized during malting is necessary (HOME 1992). From an economical viewpoint, the most important criterion is extract yield.

ß-glucans are the main constituents of the endosperm cell walls. In the barley grain the β-glucan is located in the endosperm cell walls. It accounts up to two thirds of the walls, the remainder being pentosans and proteins. The thickness of cell wall is different in different varieties and it is

in a negative correletion with the modification of endosperm. There are found that when the cell walls are thick, then there are more β-glucans in the wort and *vice versa* (MUNCK 1987; PALMER 1989). The β-glucan amount is influenced by both genotypic and environmental factors. The major environmental factors that influences β-glycan levels appears to be the availability of water during grain maturation. Dry conditions before harvest result in high β-glucan levels (HOCKETT *et al*. 1987; KASHA & FALK 1993). As a high molecular weight water soluble polymer, several problems in the malting and brewing process have been associated with the presence of high levels of β-glucan (implying thick endosperm cell walls in barley), e.g.: retardation of malting, increased viscosity of wort and beer which increases the process time needed for wort separation and beer filtration (AASTRUP 1987; BAMFORTH & BARCLAY 1993).

The modification process during malting is divided into two: cell wall degradation and the protein matrix degradation. A hard and steely barley endosperm gives an inferior malt compared to soft and mealy one. The steely grains contain less starch but more protein and β-glucans especially the soluble onesthan does the mealy seeds. This principle is valid in comparison between varieties as well as within varieties grown under different conditions where a dry weather causes a more steely endosperm (ENARI 1990; HOME 1991; KUNZE 1996). The hardness is determined with a Friabilimeter.

Diastatic power include both α- and β-amylase activity. These are the main enzymes that degrade the starch to dextrins and sugars. α- amylase breaks down the long starch chains to smaller dextrins, β-amylase splits maltose off from the non-reducing ends of chains, but it also produces glucose and maltotriose (PALMER 1989; LIE 1990). α-amylase is not detectable in ungerminated barley. Most of the α-amylase is produced on the 2nd to 4th days of germination. ß-amylase is already present in large amounts in ungerminated barley and its amount increses considerably on the 2nd and 3rd day of germination. The amylase content is a varietal property which is affected by the climatic conditions. Large kernels of a barley variety form more amylase than small kernels of the same variety (BRIGGS 1992; KUNZE 1996).

Material and Methods

This research project included 70 varieties which belonged to the Northern region of EBC in 1991-2001. 19 varieties from Great Britain, 15 Germany, 15 Denmark, 13 Sweden, 3 France, 2 Netherlands, 2 Estonia and 1 Finland were tested. The malt analyses were made in the same year, when the varieties were included to the list of EBC trials. Standard varieties in evaluation of malting quality were German malting barleys 'Alexis' in 1991-1995 and 'Scarlett' in 1997-2001.

Trials were arranged in 4 replications and the plot size was 10 m². The plots were organised in randomised order according to the NNA method. The plots were sown at 500 seeds per m². The precrop was potato. Fertilizer background was N_{60} P_{13} K_{25}. Chemical weed control was carried out every year at 3–4 leaf growth stage.

The quality analysed at the VTT Biotechnology in Finland according to Analytica-EBC (Analytica-EBC 1987; 1998).

Results and Discussion

Extract Yield

In conlusion, based on the trials carried out at Jõgeva Plant Breeding Institute, it is possible to state, that the extractivity of most of the varieties met the quality requirements, and was either good or very good. The corresponding characteristic of any tested variety was not under 78,0%.

The extractivity level of the standard variety 'Alexis' was exceeded the most (0,6-0,5%) by German varieties 'Maresi' and 'Korinna', the Danish varieties 'Caruso' and 'Miralix', and the Great Britain variety 'Chariot' (Table 1). The Estonian variety 'Elo' was on par with the standard variety. 1,2-3,2% lower extractivity, compared to the standard variety 'Alexis', was shown by Great Britain varieties 'Vintage', 'Forester', 'Cork', 'Teal', and the Danish variety 'Semal', Swedish variety 'Blondie' and the Estonian variety 'Anni'.
The extractivity level of standard variety 'Scarlett' was exceeded 0,8-2,1% by the Swedish variety 'Wikingett', the German variety 'Landora', the Dutch variety 'Jersey' and the Great Britain varieties 'Brise' and 'Extract' (Table 2). 2,7-3,7% lower extractivity than the standard variety was shown by the Finnish variety 'Saana', the German variety 'Optima', the Swedish variety 'Tofta' and the Danish varieties 'Cadeau' and 'Punto'.

ß-glucan
In the years 1991-1995 (excluding 1992) the average β-glucan content of every tested variety was 439 mg/l and in this respect most of the varieties had either good or very good quality characteristics. The varieties analysed in the years 1997-2001 (excluding 1999) showed an average β-glucan content of 147 mg/l, and the corresponding characteristics of all the varieties were either good or very good. The β-glucan content of the newest malting barley varieties was consideraby lower than the β-glucan content of the older varieties.
The trials carried out in Jõgeva during 1991-1995 (excluding 1992) showed, that the Great Britain variety 'Vintage', the French variety 'Nevada' and the Estonian variety 'Anni' have too high β-glucan content. When compared to the standard variety 'Alexis', the lowest β-glucan content was shown by the Great Britain variety 'Chariot' and the Danish variety 'Caruso'. The aforementioned characteristic of the Estonian variety 'Elo' was on par with the standard.
In the trial carried out in 1997-2001 (excluding 1999), the Swedish variety 'Potter' and the German varieties 'Pasadena' and 'Annabell' stood out with a β-glucan content 120-170 mg/l lower than in the case of the standard variety 'Scarlett'. The German varieties 'Landora' and 'Optima' showed a 130-140 mg/l higher β-glucan content than the standard variety 'Scarlett'.

Friability
The average friability of the malting barley varieties in trial during 1991-1995 (excluding 1992) was 80%, reaching 55-97% in case of some varieties. Out of the 35 varieties that were analyzed, 15 had very good and 6 had good friability levels. The average friability of the standard variety 'Alexis' was very high over the trial years – 87%. The Great Britain variety 'Chariot' and the Danish variety 'Caruso' exceeded the standard variety most in this respect. The lowest friablity results compared to the standard were shown by the Danis variety 'Caminant', the French variety 'Nevada' and the Estonian variety 'Anni'.
The average friability of the varieties analyzed from 1997-2001 (excluding 1999) was 84%, varying from 69-98%. The average friability of the standard variety 'Scarlett' was also very high during the trial years – 86%. In the case of most varieties that were analyzed during this period, the friability level was either good or very good. The standard was in this respect most exceeded by German varieties ' Annabell' and 'Pasadena', and the Danish variety 'Reform'. The lowest

Table 1. Malt quality of malting barley varieties compared to the standard variety 'Alexis'

			Extract		ß-glucan		Friability		Diastatic power	
Year	Variety	Origin*	%	+/-Alexis	mg/l	+/-Alexis	%	+/-Alexis	WK	+/-Alexis
1991	**Alexis**	**D**	**82,6**	**0,0**	**453**	**0**	**78**	**0**	**280**	**0**
	Maresi	D	83,2	0,6	636	183	75	-3	270	-10
	Chariot	GB	83,1	0,5	234	-219	87	9	230	-50
	Decor	GB	82,7	0,1	495	42	76	-2	220	-60
	Derkado	D	82,7	0,1	625	172	77	-1	170	-110
	Triumph	D	82,7	0,1	588	135	71	-7	260	-20
	Elo	EST	82,6	0,0	447	-6	70	-8	360	80
	Carula	DK	82,1	-0,5	600	147	71	-7	180	-100
	Loke Abed	DK	81,8	-0,8	529	76	74	-4	210	-70
	Semal	DK	81,2	-1,4	364	-89	74	-4	150	-130
	Blondie	S	81,0	-1,6	504	51	71	-7	170	-110
	Forester	GB	81,0	-1,6	544	91	72	-6	220	-60
1993	**Alexis**	**D**	**80,9**	**0,0**	**574**	**0**	**78**	**0**	**309**	**0**
	Caruso	DK	81,5	0,6	384	-190	85	7	216	-93
	Korinna	D	81,4	0,5	600	26	75	-3	282	-27
	Krona	D	80,9	0,0	438	-136	81	3	264	-45
	Maud	S	80,6	-0,3	410	-164	75	-3	219	-90
	Goldie	S	80,2	-0,7	597	23	73	-5	251	-58
	Jessica	S	80,0	-0,9	778	204	71	-7	173	-136
	Nevada	F	80,0	-0,9	1178	604	55	-23	188	-121
	Senor	DK	80,0	-0,9	588	14	73	-5	205	-104
	Vintage	GB	79,7	-1,2	883	309	65	-13	245	-64
1994	**Alexis**	**D**	**82,9**	**0,0**	**110**	**0**	**97**	**0**	**237**	**0**
	Miralix	DK	83,5	0,6	370	260	86	-11	230	-7
	Brewster	GB	82,8	-0,1	210	100	94	-3	207	-30
	Texane	F	82,6	-0,3	270	160	90	-7	180	-57
	Trebon	S	82,4	-0,5	170	60	89	-8	220	-17
	Caminant	DK	82,2	-0,7	500	390	77	-20	120	-117
	Anni	EST	80,8	-2,1	790	680	65	-32	157	-80
	Teal	GB	79,7	-3,2	280	170	91	-6	250	13
1995	**Alexis**	**D**	**83,1**	**0,0**	**132**	**0**	**95**	**0**	**260**	**0**
	Polygena	D	83,4	0,3	117	-15	96	1	260	0
	Cooper	GB	83,3	0,2	289	157	88	-7	150	-110
	Mie	S	83,3	0,2	211	79	91	-4	200	-60
	Delibes	GB	82,7	-0,4	187	55	89	-6	210	-50
	Mentor	S	82,7	-0,4	108	-24	92	-3	290	30
	Reggae	NL	82,6	-0,5	302	170	83	-12	230	-30
	Cork	GB	81,4	-1,7	202	70	89	-6	300	40

*GB-Great Britain, D-Germany, DK-Denmark, F-France, NL-Netherland, EST-Estonia, FIN-Finland

Table 2. Malt quality of malting barley varieties compared to the standard variety 'Scarlett'

			Extract		ß-glucan		Friability		Diastatic power	
Year	Variety	Origin*	%	+/-Scarlett	mg/l	+/-Scarlett	%	+/-Scarlett	WK	+/-Scarlett
1997	**Scarlett**	**D**	**84,4**	**0,0**	**120**	**0**	**93**	**0**	**270**	**0**
	Barke	D	84,3	-0,1	76	-44	94	1	310	40
	Chalice	GB	83,8	-0,6	45	-75	98	5	180	-90
	Linus	S	83,7	-0,7	100	-20	93	0	240	-30
	Paloma	DK	81,8	-2,6	75	-45	93	0	300	30
	Tofta	S	80,9	-3,5	57	-63	90	-3	180	-90
	Punto	DK	80,7	-3,7	92	-28	86	-7	260	-10
1998	**Scarlett**	**D**	**82,2**	**0,0**	**130**	**0**	**85**	**0**	**300**	**0**
	Extract	GB	83,0	0,8	95	-35	86	1	454	154
	Optic	GB	82,3	0,1	253	123	85	0	287	-13
	Cecilia	S	81,6	-0,6	220	90	76	-9	360	60
	Lux	DK	80,2	-2,0	140	10	79	-6	360	60
	Ferment	GB	79,6	-2,6	190	60	76	-9	220	-80
	Saana	FIN	79,5	-2,7	150	20	80	-5	390	90
	Optima	D	79,4	-2,8	270	140	81	-4	210	-90
	Cadeau	DK	78,9	-3,3	120	-10	80	-5	250	-50
2000	**Scarlett**	D	**82,8**	**0,0**	**250**	**0**	**79**	**0**	**250**	**0**
	Ricarda	GB	83,4	0,6	160	-90	86	7	190	-60
	Annabell	D	82,3	-0,5	90	-160	93	14	190	-60
	Century	GB	82,3	-0,5	280	30	77	-2	300	50
	Prestige	GB	82,3	-0,5	150	-100	81	2	350	100
	Alliot	DK	82,2	-0,6	190	-60	82	3	350	100
	Chamant	DK	81,7	-1,1	150	-100	78	-1	270	20
	Cicero	DK	81,6	-1,2	180	-70	79	0	210	-40
	Potter	S	81,4	-1,4	130	-120	72	-7	350	100
	Saloon	GB	81,3	-1,5	150	-100	79	0	280	30
	Astoria	F	81,2	-1,6	200	-50	77	-2	290	40
	Pasadena	D	81,1	-1,7	80	-170	88	9	340	90
	Laura	D	80,9	-1,9	210	-40	76	-3	400	150
	Pongo	S	80,4	-2,4	190	-60	69	-10	310	60
2001	**Scarlett**	**D**	**82,4**	**0,0**	**120**	**0**	**88**	**0**	**300**	**0**
	Wikingett	S	84,5	2,1	180	60	82	-6	260	-40
	Brise	GB	84,2	1,8	78	-42	92	4	230	-70
	Landora	D	84,0	1,6	250	130	78	-10	310	10
	Jersey	NL	83,3	0,9	220	100	82	-6	310	10
	Neruda	D	82,6	0,2	77	-43	91	3	280	-20
	Reform	DK	82,3	-0,1	20	-100	96	8	260	-40
	County	GB	80,5	-1,9	110	-10	90	2	310	10

*GB-Great Britain, D-Germany, DK-Denmark, F-France, NL-Netherland, EST-Estonia, FIN-Finland

friability levels, compared to the standard, were shown by the Swedish variety 'Pongo' and the German variety 'Landora'.

Diastatic Power

Most of the analyzed varieties had either good or very good diastatic power. The Estonian variety 'Elo', the Great Britain variety 'Cork' and the Swedish variety 'Mentor' stood out as having a higher diastatic power than the standard variety 'Alexis'. The diastatic power of the Estonian variety 'Anni' was satisfactory. The French variety 'Nevada', the Danish variety 'Semal' and the Swedish variety 'Jessica' showed a considerably lower diastatic power than the standard 'Alexis'. The standard variety 'Scarlett' was exceeded by 19 varieties in diastatic power, the highest results were shown by the Great Britain variety 'Extract' and the German variety 'Laura'. The lowest results, compared to the standard 'Scarlett', were shown by the Swedish variety 'Tofta', the Great Britain variety 'Chalise' and the German variety 'Optima'.

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Comparison of Agronomic and Nutritional Parameters Stability in Hulless and Hulled Barley

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Abstract

No cultivars of hulless barley are registered in countries of Central and Eastern Europe even though their development used to be part of breeding programmes aiming particularly at improved feeding grain quality over the past decades. A currently increasing interest in hulless barley grain production results from higher requirements of consumers for a wider assortment of foods and feeds, improved nutritional quality of cereal products and as well as possibilities of effective use of growing areas that are less favourable for production of quality malting barley. A set of selected cultivars, new breeding lines and genetic resources of spring barley with hulless grain was grown at two locations in the Czech Republic, Latvia and Slovakia in 2000-2003. In comparison with hulled barleys, the productive hulless materials exhibited a wider variation in selected agronomic traits and some quality parameters during years (mostly in starch), while interactions associated with the origin, row type and a thoroughbreeding level in individual genotypes are necessary to consider. The obtained results proved that there is a possibility of developing and/or selecting hulless barley materials with a high stability of desired traits and parameters that can simultaneously meet differentiated food requirements for nutritional grain quality. Particular hulless barley genotypes, their responses to different soil and climatic conditions and prospects of their use in practical growing are discussed in detail.

Keywords: spring barley; hulless and hulled grain; agronomic and nutritional parameters; stability; variability; cultivars; breeding lines

Introduction

Growing and breeding spring barley have more than 100-year tradition in the Czech Republic, and particularly in the Moravian region. In contrast to other areas in the world, this tradition is linked exclusively to two-row spring barley with hulled grain and high malting quality, which is acceptable for malt and beer production (LEKEŠ 1997). Change in the attitude to basic directions of breeding, and thus official involvement of barley types with different morphological traits in the conception of barley "ideotype", was reported in a summary publication by a team of breeders from former Czechoslovakia (KOLEKTIV 1976). Hulless grain was required as one of characteristics for the ideotype of fodder barleys. This requirement was based on the aim to improve energetic and nutritional grain quality along with visual morphological distinction from malting barley. However, breeders´ conceptions of nutritional grain quality and yield in fodder barley (Table 1) were not fulfilled during the expected period. Although hulless barley was included in breeding programmes almost at all breeding stations in the 70s and 80s, the development of cultivars with hulless grain receded into the background.

Table 1. Specific requirements of Czechoslovak breeders for the "ideotype" of fodder barley till 2000 (KOLEKTIV 1976)

Author/ parameter	Crude protein, %	Amino acid lysine, %	Other amino acids, %	Grain yield	Other characteristics
Rychtárik	15	3.5-4 in CP	**	> malting barley	**
Brückner	HB*- 15 CB – 13	0.56-0.6 in d.m. 0.6 in d.m.	0.2 meth.	8-10 t/ha	black colour of grain
Haraj	17 (15-20)	0.75 in d.m. 4-4.5 in CP	**	20% higher	percentage of plump grain (97%)
Růžička	CB - 18 HB - 15	0.7 in d.m. (4-4.5 in CP) 0.6 in d.m.	TKW > 45g	**	black colour of grain, morphol. different
Minařík	HB, high technological and nutritional quality				

* - HB=hulless barley, CB=covered (hulled) barley, CP=crude protein, d.m.=dry matter; ** - not special requirements

The development of hulless barley genotypes for alternative use continued within research projects only (VACULOVÁ *et al.* 1996). Interesting materials of spring barley with hulless grain considering technological quality were developed in the last decade of the past century only and were also tested abroad (CANDRAKOVA 1999; LEGZDINA 2003). In recent years, an increasing interest in hulless barley has been recorded resulting from higher demand of consumers on a wider assortment of cereal foods with improved nutritional quality. New barley cultivars with hulless grain can also be interesting to growers as an alternative for growing areas that are less favourable for production of quality malting barleys.

The objective of our paper was to obtain more information on growing potential of selected hulless barleys using evaluation of their agronomic performance, variability in some important agronomic, biological, morphological and quality parameters under different agro-climatic conditions (Czech Republic, Latvia, Slovakia) and as well as to compare a level of stability of individual yield components and agronomic traits in productive hulless breeding lines and cultivars with new European hulled barley cultivars under the identical growing conditions.

Material and Methods

A. In 2000-2003, a set of selected cultivars and new lines of spring barley with hulless grain was evaluated. The set was cultivated at chosen locations in the Czech Republic (CZE - Kroměříž, 2 locations) and Latvia (LVA - Priekuli and Vecauce) in a randomized complete block design, in plots of 2.35-11.7 m^2 with 1-4 replicates. At Kroměříž, a standard crop management practice was applied after a suitable preceding crop (oilseed rape), at reduced chemical inputs (basic fertilization before seeding in the form of a combined fertilizer in the whole dosage at pre-seeding treatment; herbicides after emergence, no fungicide application). In Latvia, a standard crop management practice for given soil and climatic conditions at a total higher rate of mineral fertilizers was applied (LEGZDINA 2003). The seeding rate was 350-400 germinating seeds per m^2. Out of hulled grain controls, spring barley cultivars Tolar (CZE) and Abava (LVA) were used. Hulless barleys were obtained from the collection of genetic resources housed at the Agricultural Research Institute Kroměříž, Ltd. (10 registered cultivars from Canada, USA and Germany) and our own breeding lines from Kroměříž (10 lines designated KM) and 10 materials provided by the CIMMYT (Mexico) thanks to Dr. Legzdina were also included (Fig. 1).

B. In 2001-2003, 28 new cultivars of spring hulled barley developed in neighbouring countries (see the list in a poster) were tested together with 19 hulless barleys at the location Kroměříž using the same crop management practice.

C. Two new lines of spring hulless barley originating from Kroměříž, KM 1910 and KM 2092, with different nutritional and morphological traits were also tested at the location Oponice (Slovak Republic – SVK) in 2001-2003. They were cultivated in exact agronomic experiments in plots of 14 m^2 (3 replicates), after a standard preceding crop and at various levels of mineral fertilization. The fertilization variants were as follows (see figures for identical designations):

1. unfertilized control,
2. nitrogen rate of 20 kg.ha^{-1} (ammonium nitrate with lime) applied at the beginning of tillering,
3. nitrogen rate per hectare calculated for the assumed grain yield of 7 tons (ammonium nitrate with lime) applied at the beginning of tillering based on the agrochemical soil analysis of N_{an} to a soil depth of 0.60 m that was carried out at the beginning of tillering,
4. nitrogen rate per hectare calculated for the assumed grain yield of 7 tons (DAM 390) applied at the end of tillering based on the agrochemical soil analysis of N_{an} to a soil depth of 0.60 m that was carried out at the beginning of tillering.

The nitrogen rates in variants 2, 3 and 4 were based on agrochemical analyses of soil samples taken from the depth of 0.30 and 0.60 m. The nitrogen rate was determined based on the average nutrient requirements for the grain yield of 1 ton and corresponding amount of straw for spring barley being 24 kg. Phosphorus and potassium fertilizers were not applied.

Selected biological, morphological, agronomic and quality traits and parameters of tested barleys were evaluated. In all years and at all locations, data on grain yield in t.ha^{-1} , grain yield in comparison with the hulled control or the mean of the experiment in % (Rel. Yield), 1000-kernel weight in g (TKW) and plant height in cm were obtained. Furthermore, days from seeding to maturity (Veg. Time), starch and crude protein content in % (using NIR device Infratec 1275 and Inframatic 9100), resistance to lodging (both CZE and LVA) and diseases (CZE - *Blumeria graminis*, *Pyrenophora teres*, *Puccinia hordei*) using the scale from 9 (best) to 1 (worst) were evaluated. Resistance to threshability, sprouting and diseases (*Blumeria graminis*, *Ustilago nuda*, leaf spots - *Drechslera teres*, *Helminthosporium sativum*) were measured in LVA according to the scale from 0 (best) to 4 (worst). In experiments LVA and SVK, volume weight in g (VW), in CZE and SVK percentage of plump grain (2.5 mm), plant density (No of plants/m^2), density of tillering (No of productive tillers/m^2) and some of yield components (No of productive tillers per plant-NPT, spike density=no of kernels per spike-NKPS, weight of grain per spike in g-WGS, weight of grain per plant in g-WGP, etc.) were evaluated.

Standard mathematical methods using statistical software S-Plus 4.5 were used to assess the obtained results. Variability was assessed by coefficient of variation (CV, %).

Table 2. Important agronomic and nutritional traits and parameters of hulless barleys grown in different countries and their variability (2000-2003)

Traits, parameters/ type of grain, year		CZE	LVA
		2001	2001
Yield, t ha^{-1}	Min - max	1.68– 10.16	0.97-7.04
	Mean ± st.error	5.39± 0.13	4.14± 0.09
	CV**	30.26	28.82
Yield to hulled std., %	Min - max	23.8–103.95	27.79–121.06
	Mean ± st.error	65.20± 1.34	79.94± 1.35
	CV**	26.48	22.26
TKW, g	Min - max	23.9-48.7	26.7-52.85
	Mean ± st.error	37.9± 0.38	38.69± 0.40
	CV**	12.76	13.69
Crude protein, %	Min-max	11– 19.8	12.1–20.1
	Mean ± st.error	14.66± 0.12	15.01± 0.12
	CV**	10.2	10.83
Starch, %	Min-max	47.6-67.4	48.9-59.6
	Mean ± st.error	61.09±0.21	53.7± 0.17
	CV**	4.35	4.10
Height, cm	Min-max	56-110	45-113
	Mean ± st.error	85.63 ± 0.84	82.62 ± 1.33
	CV**	12.64	21.13
Lodging, 9-1*	Min-max	1-9	3-9
	Mean ± st.error	8.11± 0.13	6.61± 0.13
	CV**	20.59	26.22
Veget. time, days	Min-max	79-115	84-99
	Mean ± st.error	101.1± 0.77	91.64± 0.23
	CV**	9.82	3.35

Results and Discussion

The set of cultivars, lines and genetic resources of hulless barley tested in the Czech Republic and Latvia under different soil and climatic conditions included materials with distinct genetic backgrounds, therefore a high variability in evaluated traits and parameters could be expected. Nevertheless, the analysis of variance proved that significant sources of variation within the set were all factors, i.e. both barley materials and individual years as well as experimental conditions of the given country or location, and namely for all the traits that were common for both countries. The variability in some traits was also markedly influenced by interactions of particular factors, especially by the year-country interaction. Table 2 shows selected traits and parameters, and variability found in both countries. Regardless of two different locations in Latvia and two experimental fields at the location Kroměříž in the Czech Republic, and mean values of individual traits

at a significantly different level, the data on variability in the set of hulless barleys prove that the variability in agronomically important traits was very similar. In general, it was higher in grain yield, average in TKW and protein content and low in starch content. The only exception was plant height, when the growth of some tested materials was depressed in LVA, obviously due to effects of weather conditions. BELICKA (2004) calculated the average coefficient of variation (CV) for grain yield, but similar values of variability for protein and starch content were reported in the study of a large assortment of barley cultivars and genetic resources in the environment of Latvia.

However, the extent of variability determined for parameters of grain nutritional quality of hulless genotypes considerably changed if the set was evaluated at both locations together. Due to a statistically significant lower starch content in LVA, the variability in this trait almost doubled, whereas variability in protein content decreased. Characteristics of individual materials are illustrated in Fig. 1, where also considerable differences in protein content of the materials from the CIMMYT are apparent in comparison particularly with Canadian cultivars CDC Buck (13.12%) and CDC Richard (13.37%) and some lines from CZE.

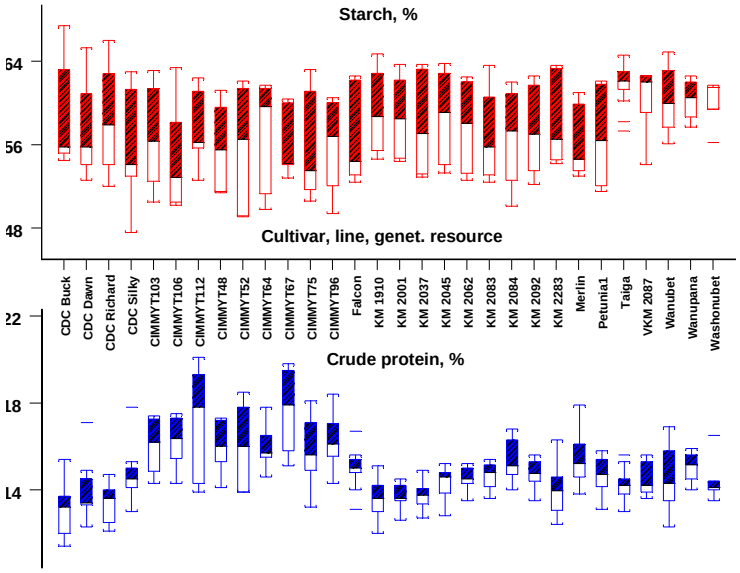

Figure 1. Mean values and variability in nutritional parameters of hulless barley materials in two different environments (CZE, LVA, 2000-2003)

The highest mean protein content was found in the line CIMMYT67 – 17.62% that significantly surpassed all the tested materials, except for the CZE lines KM 1057 (14.75%), KM 1771 (14.80%) and KM 2084 (15.32%), cultivar Merlin (15.34%) and waxy cultivar Wanupana (15.02%) from the USA. The relationship between the grain yield and both nutritionally important parameters corresponded with commonly known rules; the correlations differed in force only. The equation for linear regression for starch in CZE was: y=56.5+0.91x and in LVA there were lower values of intercept and slope (y=51.22+0.62x), whereas, on the contrary, intercept in equation for protein was slightly lower in CZE (y=16.91-0.41x) than in LVA (y=17.40-0.59x).

The materials tested at locations in CZE and LVA provided similar results as those reported by BOWMAN and BLAKE (1996). They found significantly higher effect of the genotype on protein content (heritability 0.9) than on starch content in DH lines of barley cultivated in experiments under various water regimes. Though the both nutritionally important traits were strongly influenced by the environment, a higher starch content and lower protein content were detected in the more humid environment. By contrast, a beta-glucan content and TKW ranked among the traits that were more significantly influenced by the genotype than environment and there were no significant differences in mean values of these parameters of identical materials cultivated in contrast environments.

The highest grain yield was produced by the materials from CZE - KM 2037 and KM 2283, i.e. more than 6 tons per hectare on average of years and locations. With regard to high variability, however, they significantly differed from the CIMMYT materials (designated by numbers 48, 52, 64, 103 and 106), cultivars Falcon and Wanupana only. The grain yield of the

materials tested in different countries also differed in relation to hulled control cultivars. The two-row hulless barleys reached on average 82.47% in LVA and only 69% in CZE and the six-row barleys had a mean yield 69.48% of a hulled cultivar in LVA (≅ the same value as in two-rowed), whereas it was only 56.16% in CZE. These results fully correspond with the findings achieved by JUSKIW and HELM (2003) who found that in the case of late seeding, six-row materials with a shorter or medium vegetation period performed better than two-row and late six-row cultivars. Analogically, a lower level of productivity was manifested when traits and parameters were evaluated in relation to the origin of materials. It is generally valid that cultivars originating from the genepool of a certain geographic area and ecological conditions exhibit both better yield and quality parameters in that environment. However, we confirmed our earlier published partial conclusions that in the less productive environment, hulless barleys provided better performance than hulled cultivars, which was confirmed in LVA in higher values of relative grain yield in comparison with a hulled control. This was valid regardless of the country of origin; the materials cultivated in LVA had also slightly higher protein content in grain and markedly lower starch content. On the contrary, their vegetation period was shorter (Fig. 2).

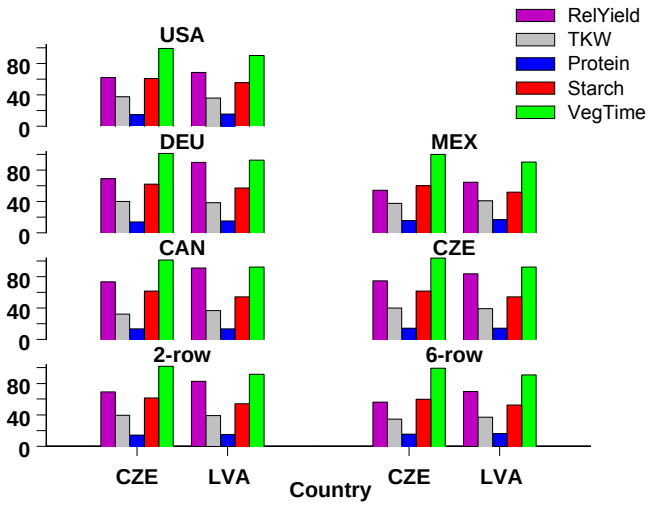

Figure 2. Mean values of selected traits and parameters in different environments (2000-2003)

Separate evaluation of stability of new CZE lines showed (Table 3) that they can be differentiated also from this point of view even though a total level of variability expressed by a coefficient of variation was in accordance with the data on the whole set. Mean values obtained for individual Czech new lines in both environments (CZE and LVA) are presented in Fig. 3 which shows differences in grain yield and another agronomically important traits.

Table 3. Variability in important agronomic and nutritional traits and parameters (CZE, LVA, 2000-2003)

Cultivar, line	Traits, parameters* – Coefficients of variation - CV, %*									
	Yield	TKW	Protein	Starch	Lodging	Height	Blum. gram.**	Pyr. ter.**	Pucc. hord.**	Veg. time
KM 1910	25.62	11.17	6.71	6.70	17.23	10.41	47.48	25.28	20.57	8.81
KM 2001	26.39	8.34	4.19	6.83	28.57	15.83	5.08	16.11	30.84	8.11
KM 2037	32.50	11.59	4.31	8.03	18.40	9.17	31.87	7.04	20.20	8.28
KM 2045	25.07	10.80	5.50	7.03	14.42	15.19	8.58	16.21	14.18	8.11
KM 2062	23.46	9.92	3.90	7.17	17.73	12.49	29.51	26.40	13.23	9.67
KM 2083	29.73	10.73	4.30	7.27	16.62	13.32	26.43	17.11	15.27	8.79
KM 2084	24.46	7.16	5.72	8.02	18.02	17.98	34.57	12.25	14.24	8.60
KM 2092	22.92	9.98	4.78	7.27	23.26	14.06	35.48	25.28	15.80	10.56
KM 2283	29.66	10.33	8.02	7.22	12.12	16.88	37.33	16.52	15.80	9.90
Taiga	22.77	4.83	4.82	2.81	9.85	12.43	41.43	12.25	34.15	13.51

*, ** - see Material and Methods

Likewise, BANATEANU and MOISA (2001), who investigated selected barley cultivars in different years in the area of North-West Romania, found different stability and yielding ability in various cultivars.

The undisputed breeding progress is apparent in new cultivars of malting barley as for a high level of yield potential, grain quality and other stabilizing factors including high resistance to fungal diseases (LANGER 2004). The cultivar, as an important factor of intensification, plays a critical role in economic effectiveness of cereal growing. Considering this aspect, hulless barleys are handicapped because there is no growing tradition under our agro-climatic conditions. Perspectives of their future economic application have to be sought in their morphological, nutritional as well as use distinctions.

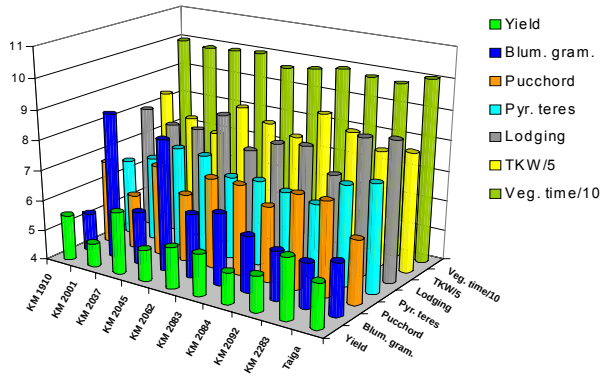

Figure 3. Mean values of selected important agronomic traits of productive hulless barley lines in two environments (CZE, LVA, 2000-2003)

In the Czech Republic, hulless barleys could be used rather for food and/or feed purposes, particularly in the regions that are less favourable for growing malting barley, such as the maize-growing region, where higher protein content in grain dry matter is produced due to drier conditions. Similarly, growing conditions in the grass production region, mostly in submontane area, do not guarantee desired production quality for malting, but for feeding purposes only. In the world, hulless barley is successfully grown in the countries where stable yield and standard grain quality are guaranteed. Under our conditions, individual hulless barley genotypes have not been tested for their responses to cultivation in different production regions yet.

In 2001-2003, a set of registered cultivars and new lines of hulless barley was compared with selected new European cultivars of spring hulled barley in Kroměříž. Data on grain yield, its components and other agronomically important traits and parameters together with parameters of variability of both sets in individual years are presented in Table 4. The obtained results suggest that the range of measured values in hulless materials is of wider amplitude than in the set of hulled barleys. Regardless of the fact that these materials originate from various European countries, a level of their traits and parameters is more balanced than that in hulless barleys. Especially in grain yield, hulled barleys exhibited lower interannual differences that led to the variability by up to 77% lower than in hulless barleys. OVESNÁ *et al.* (2004), based on the study of selected Czech malting cultivars using DNA markers, concluded that a current assortment is characteristic of markedly lower genetic variability. As Table 4 shows, in favourable years hulless barleys were close to the maximum obtained in hulled cultivars (year 2003), which holds up hopes to gradually improve a level of their productivity. CHOO *et al.* (2001) indicate that there is a possibility to develop high-yielding cultivars of hulless barley. The authors found out that grain yield positively correlated with spike density, which corresponds with our earlier (VACULOVÁ *et al.* 2003) as well as current findings. The correlation coefficients calculated from our three-year results showed that in contrast to hulled barleys, where the major yield components were spike density and a number of productive tillers (r=0.76** and r=0.40*, respectively), the relationship between a number of tillers and grain yield is negligible (r=0.08) in hulless barleys, and beside spike density (r=0.76**) the yield is affected by TKW (r=0.42**). In comparison with hulled cultivars, the variability in evaluated yield components (NPT, NKPS and WGS) was also higher. The breeding progress

in hulless barleys is apparent from values of resistance to lodging and leaf diseases, where the obtained parameters are comparable with those in hulled cultivars. The vegetation period in

Table 4. Characterization and variability in important agronomic and nutritional parameters of hulless and hulled barley cultivars and breeding materials (Kroměříž, 2001-2003)

Traits, parameters/ type of grain, year		Hulless			Hulled		
		2001	2002	2003	2001	2002	2003
Grain yield t.ha^{-1}	Min-max	1.9-6.6	4.6-7.9	4.0-10.2	3.7-8.8	5.8-9.4	6.2-10.5
	Mean±st.error	4.2±0.2	6.3±0.2	7.1±0.3	7.3±0.2	8.0±0.2	8.2±0.2
	CV**	26.69	14.65	17.98	15.06	10.77	12.74
TKW g	Min-max	29.8-40.0	33.2-46.7	33.3-48.7	34.7-48.4	34.9- 49.6	34.6-50.8
	Mean±st.error	35.4±0.6	39.1±0.7	42.2±0.8	43.1±0.7	44.9±0.7	44.3±0.7
	CV**	8.08	8.91	9.03	8.90	7.43	7.77
No of product. tillers	Min-max	468-764	300-900	404-776	653-856	542-834	584-887
	Mean±st.error	613.2±18.8	584.6±26.2	559.2±22.2	773.5±9.0	701.8±11.1	792.5±13.0
	CV**	15.03	21.99	19.47	5.95	8.10	8,38
No of kernels per spike	Min-max	10.1-28.9	13.2- 6.1	20.2-47.1	12.8-27.0	18.2- 33.3	18.1- 9.3
	Mean±st.error	19.6±1.1	28.4±1.6	30.8±1.1	22.0±0.6	25.8±0.6	23.7±0.6
	CV**	26.72	27.60	18.00	14.20	11.72	12.08
Weight of grain per spike, g	Min-max	0.30-1.13	0.72-1.81	0.93-1.96	0.56-1.20	0.84 -1.46	0.85-1.28
	Mean±st.error	0.70±0.04	1.12±0.06	1.30±0.05	0.94±0.03	1.15±0.03	1.04±0.02
	CV**	30.24	24.11	18.46	14.94	12.07	9.80
Veget. time, days	Min-max	95-103	105-115	101-110	106-108	103-116	86-107
	Mean±st.error	101.2±0.5	113.7±0.5	106.6±0.6	106.8±0.1	112.3±0.5	101.5±0.9
	CV**	2.30	2.01	2.61	0.63	2.43	3.99
Lodging 9-1*	Min-max	6-9	3-9	6-9	4-9	9-9	5-9
	Mean±st.error	8.8±0.1	8.2±0.3	8.7±0.2	7.0±0.2	9.0±0.0	8.7±0.2
	CV**	7.21	19.34	9.46	17.93	0.00	10.17
Blumeria graminis 9-1*	Min-max	1-9	1-9	4-9	2-9	1-9	6-9
	Mean±st.error	3.3±0.4	4.8±0.4	6.6±0.4	7.7±0.4	7.4±0.4	8.7±0.1
	CV**	64.88	43.01	29.33	29.42	29.62	8.29
Pyreno-phora teres 9-1*	Min-max	6-8	7-9	4-8	6-9	5-9	1-8
	Mean±st.error	7.5±0.1	7.9±0.1	6.6±0.3	7.4±0.1	7.5±0.2	6.0±0.4
	CV**	7.87	8.21	22.21	10.21	15.28	32.61
Puccinia hordei 9-1*	Min-max	1-7	6-8	4-7	5-8	6-9	4-9
	Mean±st.error	4.4±0.3	7.3±0.2	5.9±0.2	6.8±0.2	8.2±0.2	6.4±0.2
	CV**	39.20	11.87	18.37	15.81	10.93	18.31
Crude protein, %	Min-max	13.2-17.9	12-14.9	13-19.8	8.9-14.7	7.8-13.8	18.5-15.1
	Mean±st.error	14.9±0.2	13.3±0.2	14.6±0.3	11.1±0.3	10.8±0.3	11.0±0.3
	CV**	7.81	5.71	8.70	13.90	12.21	15.02

see Material and Methods, **- CV = Coefficient of variation

days exhibited an identical level of variability in both hulled cultivars and hulless materials. Also CHOO *et al.* (2001), studying hybrids of hulless and hulled barleys in the area of Eastern Canada, found that hullessness was associated with 17% lower plant density, 11 to 18% shorter plant height, 15 to 19% lower seed weight, 20 to 21% higher volume weight, and 21 to 36% yield reduction. Hullessness, however, was not associated with heading date, maturity, smut resistance, scald resistance, and spike density.

The only trait that was more stable in our set of hulless barleys in comparison with hulled cultivars was protein content. Hulless barleys had not only higher mean values of this parameter, but the markedly lower negative correlation was between protein and grain yield (r=-0.23 vs. r=-0.63**) and as well as between protein and TKW (r=-0.14 vs. r=-0.72**) in comparison with hulled cultivars. As LANGER (2004) demonstrates, there is a decreasing tendency in grain protein content in new cultivars bred in the Czech Republic and the development of foreign cultivars goes in the same direction.

At three soil locations (CZE, LVA and SVK), two new CZE lines of hulless barley, KM 1910 and KM 2092, were tested in 2001-2003. These materials differ in both morphological traits and nutritional characteristics of grain (VACULOVA *et al.* 2002). The results of analysis of variance for grain yield, TKW and plant height are presented in Table 5. It is interesting that in spite of various plot sizes and differences caused by contrast agro-climatic characteristics at all experimental locations, individual factors were highly significant sources of variation in both grain yield and plant height.

Table 5. ANOVA of selected traits of two hulless lines under different agro-climatic conditions (CZE, SVK, LVA, 2001-2003)

Source of variability	Df	Mean Sq	Pr (F)
Grain yield			
Cultivar	1	2.11	0.01
Year	2	11.71	0.00
Country	2	4.30	0.00
Cultivar:Year	2	1.59	0.01
Cultivar:Country	2	0.34	0.30
Year:Country	4	2.37	0.00
Residuals	18	0.26	
TKW			
Cultivar	1	0.93	0.75
Year	2	292.48	0.00
Country	2	17.13	0.17
Cultivar:Year	2	12.59	0.26
Cultivar:Country	2	3.43	0.68
Year:Country	4	152.17	0.00
Residuals	18	8.80	
Height			
Cultivar	1	902.89	0.00
Year	2	203.63	0.09
Country	2	410.31	0.00
Cultivar:Year	2	29.60	0.20
Cultivar:Country	2	110.68	0.01
Year:Country	4	149.18	0.00
Residuals	18	16.69	

A high variability for TKW induced by unfavourable weather conditions in 2001 resulted in the difference between KM 1910 and KM 2092 on the level of 2.69 g, which was non-significant. Results of multiple comparisons for individual countries and years showed significant differences in all assessed factors that can obviously be ascribed to their interrelationship.

New lines of hulless barley, KM 2062, KM 2082 and KM 2092, were also tested in agronomic experiments of Mendel University of Agriculture and Forestry Brno at Žabčice (EHRENBERGEROVÁ *et al.* 2003). The authors state that these materials are able to accumulate a higher content of nutritionally important substances in grain (starch, protein and beta-glucan) as compared to hulled barley cultivars, while cropping measures, such as chemical treatment and fertilization, affected particularly a protein content; the content of starch and beta-glucan changed mostly due to the year and cultivar.

At the assessment of three-year agronomic experiments with the above-mentioned lines of hulless barley at the location Oponice (SVK), particularly the effect of different variants of mineral fertilization on grain yield and individual yield components was studied. Table 6 shows means and significance of differences between the examined lines KM 1910 and KM 2092 and as well as experimental years and fertilization variants. The year showed to be again a significant source of variation.

The values in Table 6 demonstrate the ability of mutual compensation of yield components in hulless barley, but as well as stress the fact that a considerable yield gain can be achieved even in hulless materials in years with favourable climatic conditions in the period of formation of more basic yield components (in this case the year 2003).

Similarly to ABELEDO *et al.* (2003), who found that the yield of modern cultivars increased along with a higher N rate much more rapidly than that of old cultivars, the experiments conducted in SVK showed that hulless barleys also positively responded to appropriate agronomic measures, in particular to a various level of nutrition. The interaction between a selected material and year with a particular fertilization variant influenced mainly TKW and volume weight (VW). Also, these conclusions complete our earlier findings that higher grain yield in hulless barley can be obtained by increasing NPT and management of TKW. It is apparent from Table 6 that fertilizer application timing and form affected both grain yield and a level of its components in both lines. They tended to higher yields and better grain

Table 6. Means and significance of means differences in selected agronomically important traits (SVK, 2001-2003)

Source of variability	Traits, characters*				
	NPT	Yield	TKW	VW	WGP
KM 1910	1.18 a	6.08 a	39.89 a**	723.7 a	2.15 a
KM2092	1.20 a	5.88 a	40.06 a	751.1 b	1.96 a
2001	1.47 b	5.54 a	22.59 a	712.9 a	1.96 a
2002	1.01 a	5.85 a	46.12 b	782.4 b	1.96 a
2003	1.08 a	6.54 b	51.21 c	716.8 a	2.24 b
Fertilizer1	1.16 a	5.62 a	39.79 a	730.5 a	2.01 a
Fertilizer2	1.23 a	6.23 ab	39.78 a	735.7 ab	2.18 a
Fertilizer3	1.26 a	6.31 ab	40.24 ab	738.7 bc	2.09 a
Fertilizer4	1.11 a	5.75 a	40.08 a	744.7 c	1.94 a
Years : Fertilizers	n.s.	n.s.	$P_{0.05}$***	$P_{0.05}$	n.s.
2001 : Fertilizer1	1.47	5.78	22.30	711.7	1.93
: Fertilizer2	1.54	5.49	22.40	717.30	2.04
: Fertilizer3	1.45	5.56	23.47	710.6	1.95
: Fertilizer4	1.41	5.33	22.19	712.1	1.94
2002 : Fertilizer1	1.10	6.41	47.50	791.8	2.05
: Fertilizer2	1.10	6.25	46.02	781.53	1.93
: Fertilizer3	1.10	6.33	45.97	782.4	2.00
: Fertilizer4	0.92	6.80	47.03	785.8	1.88
2003 : Fertilizer1	1.07	5.76	50.59	699.6	2.06
: Fertilizer2	1.07	6.95	50.93	708.26	2.55
: Fertilizer3	1.22	7.05	51.28	723.08	2.33
: Fertilizer4	1.00	6.41	52.02	736.4	2.00
Line : Fertilizer	n.s.	n.s.	$P_{0.05}$	$P_{0.05}$	n.s.

* see Materials and Methods, ** - different letters for the same trait mean significant difference at $P_{0.05}$, n.s.= non significant***

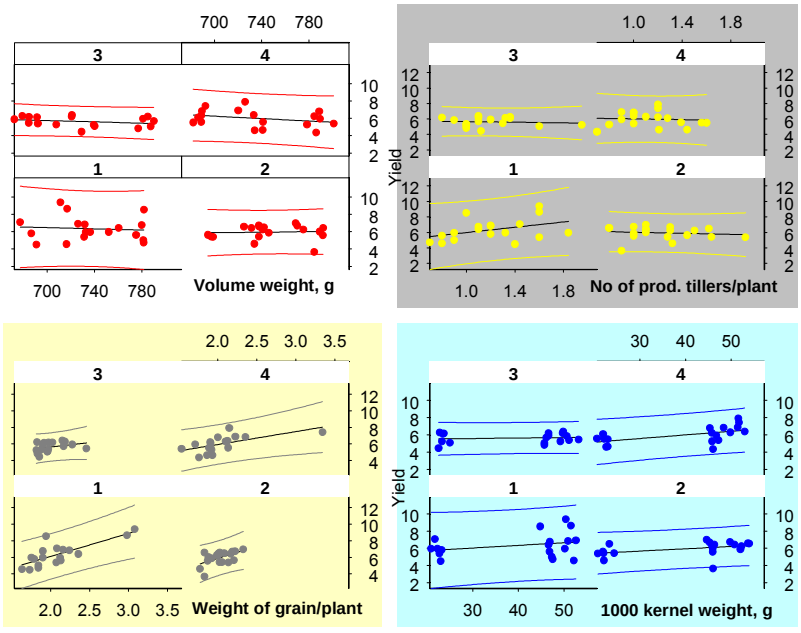

Figure 4. Linear least square of hulless barley lines yield and selected yield components at different application of mineral fertilizers (SVK, 2001-2003 - variants 1-4 see Material and Methods)

characteristics in variant 3 (fertilization with ammonium nitrate calculated according to nitrogen take-off at grain yield of 7 $t.ha^{-1}$), which apparently evinces for positive influence of a slowly affecting nutrition form during grain filling. Variant 3 had also positive effects due to the decrease in variance of measured values of yield components around the regression line of grain yield (Fig. 4). However, it is necessary to notice that such an affirmation is not valid for all years. The last higher nitrogen rate (DAM 390) at the end of tillering showed positive effects in comparison with other variants in increased volume weight and in 2002 and 2003 in higher TKW too. The effect of the year on volume weight and percent of hullessness are stressed also by CHOO *et al.* (2003) who cultivated hulless barley at various seeding rates and found that low yield in hulless barley vs. hulled cultivars might not be associated with poor emergence, and that for some hulless cultivars, increasing seeding rate could increase the yield.

Conclusion

Hulless barleys that were evaluted under identical soil and climatic conditions were characteristic of wider variability in yield traits and parameters and lower variance of grain protein than hulled cultivars. The important yield components in the set of hulless barleys studied were spike density and TKW. Comparing

agronomically important traits and parameters in the set of different hulless barley materials, the significant sources of variation were not only a cultivar, but as well as year, location and interactions of more factors. In multi-year experiments conducted at different soil and climatic locations, only markedly distinct materials of hulless barley can be successful in grain yield. New productive cultivars of hulless barleys differ in response to contrast soil and climatic conditions and can be included in various types according to the reaction to intensification factors.

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