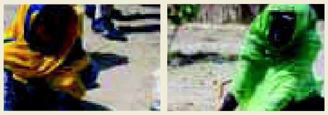

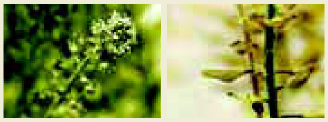

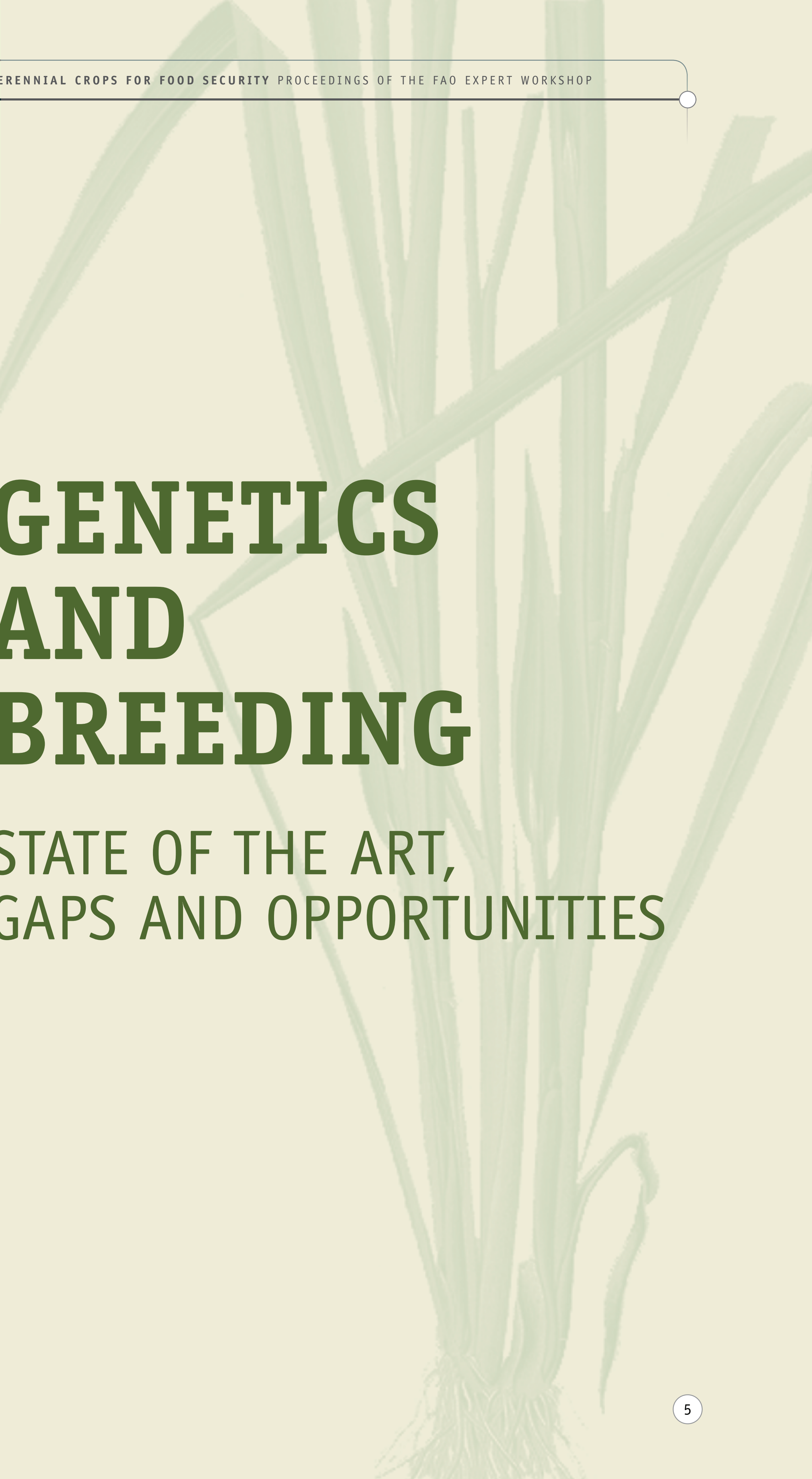

GENETICS AND BREEDING

STATE OF THE ART, GAPS AND OPPORTUNITIES

01

PERENNIAL CROPS: NEEDS, PERCEPTIONS, ESSENTIALS

Len J. Wade
Charles Sturt University, Graham Centre for Agricultural Innovation
Locked Bag 588, Wagga Wagga NSW 2678, Australia

ABSTRACT

The objective of this paper is to provide an overview of the essential issues for consideration in developing perennial crops. It is not intended to be a comprehensive review, rather it seeks to highlight topics that must be addressed in order to secure a strong future for perennial crops. To do so, the paper addresses a series of questions: Why do we need to develop perennial crops. What are the challenges and opportunities they provide. How do perceptions differ in the priority to develop perennial crops. What can be done to address and change these perceptions. How advanced is development of perennial crops. Two cases are then examined: perennial wheat in Australia as a case study for a developed country, and perennial rice in Asia as a case study for a developing country. Policy implications which may arise from development of perennial crops in developed and developing countries are then considered. The paper concludes by recognizing perennial crops may serve different purposes in different situations, so their roles must be carefully articulated. The issues raised are intended to be positive, meaning the perennial crops community should react positively to address them. The publication of hard evidence in scientific journals is considered essential, along with consideration and discussion of alternative views, in order to build credibility and confidence in the case for development and adoption of perennial crops in sustainable farming systems.

Keywords: perception, policy, publication, perennial rice, perennial wheat

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INTRODUCTION

This paper is the first in the FAO Expert Workshop on Perennial Crops for Food Security. The intent of this viewpoint paper is to outline the essential issues for consideration in any decision to proceed to develop perennial crops. Essentially, this paper provides a road map of key considerations: Why do we need to develop perennial crops; What are the challenges and opportunities; What are the perceptions of perennial grains; What do we need to do to change those perceptions; Where are we up to in developing perennial crops; Perennial wheat in Australia as a case study for a developed country; Perennial rice in Asia as a case study for a developing country; What are the policy implications which may arise; Where will perennial crops be grown and what are the consequences; What do we need to do next; and some overall conclusions. Consequently, the paper is intended to flag the major issues for discussion, and to draw attention to a number of contributions later in the proceedings which deal with these topics in detail. This viewpoint is not intended to be a comprehensive review; rather it seeks to highlight topics that need to be addressed in order to secure a strong future for perennial grains. The issues raised are intended to be positive, meaning the perennial grains community should seek to address them.

WHY DO WE NEED PERENNIAL GRAINS?

Global population and demand for food are increasing, while arable land is limited and faces increasing risk of degradation. To ensure food and ecosystem security, development of perennial crops could provide more options under diverse and generally more marginal conditions (Glover *et al*. 2010a,b). Perennial crops should offer more stable surface cover against soil erosion, and improved nutrient balance against soil acidification, rising water tables and salinity, thereby improving ecosystem services. Systems which include perennial crops should also offer farmers greater flexibility and diversity of enterprise, including livestock, and greater stability of income. At different scales, the result should be improved farmer livelihood, improved ecosystem services, and improved food security (see Snapp *et al*. 2014; Runck, 2014; Snapp *et al*. 2014; Van den Putten, 2014; Reganold, 2014; Leakey, 2014, this volume).

WHAT ARE THE CHALLENGES AND OPPORTUNITIES THEY PROVIDE?

To be successful, perennial crops would need to be able to regrow after normal harvest, and able to retain floret fertility and set grain, despite wide hybridization with perennial species. The progeny would require selection for agronomic type, including plant height, flowering time, seed size, and non-shattering. Appropriate resistances would be important against disease, submergence, drought and soil constraints, depending on the characteristics of the target environment. Finally, the successful perennial crop would need to be compatible with its farming system (see Hayes *et al.* 2014; Bell, 2014; Dost, 2014, this volume).

HOW DO PERCEPTIONS DIFFER ON THE PRIORITY TO DEVELOP PERENNIAL GRAINS?

While we can see the benefits, many see problems, at least initially. The genetic challenges in wide crosses are complex, with reports of low seed set in amphiploids capable of regrowth after harvest (Cox *et al.* 2002). Consequently, some have dismissed prospects for the development of perennial crops as just too difficult.

Developed countries have expressed several concerns about potential threats to their established annual crops from perennial relatives. Given perennial species often have robust rhizomes, concern has been expressed that perennial crops may possess potential to become serious weeds, via hard-to-kill rhizomes. Many have been concerned with the possibility of the perennial providing a “green bridge” for disease, via the availability of living tissue able to propagate additional generations of disease, thereby increasing inoculum availability early in the season, encouraging earlier infection and increased risk of epidemics. These extra cycles of disease could enhance probabilities for mutations to bypass current plant resistance, thereby reducing the longevity of resistant cultivars. Finally, wide crosses are likely to result in shattering, small grains and reduced grain quality, necessitating separation of perennial grains from others, perhaps consigning them to feed grain only.

A different set of concerns may apply in developing countries, where the priority is food security, especially the availability of sufficient food for the family. The Consultative Group for International Agricultural Research (CGIAR) sees a need to prioritise increase in yield potential and closing the yield gap in high-yielding annual crops, especially under irrigation, to meet projected food demand. They discourage investment in perennial crops, as this may dilute yield gains. Such an analysis only considers grain supply, however, and not ecosystem services, such as minimising soil erosion, maintaining soil fertility, and providing diversified and stabilised systems contributing livestock as well for balanced nutrition.

At issue is a perceived trade-off with perennial crops (Connor *et al.,* 2011), which have to invest in perennial structures, at the expense of potentially directing that investment to further grain yield in the annual crop (see Cattani *et al.* 2014, this volume). Nevertheless, the additional investment in the perennial crop could result in increased acquisition of resources, which could compensate for their redeployment to perennial structures, or even result in yield gains overall (Glover, 2010; Glover *et al.* 2010a,b). There is a dearth of hard data on these issues, however, and it is essential that the perennial crops community address this.

There is also a wider imperative for developing perennial crops, in order not to cater only to those living and farming in productive irrigated areas. The green revolution neglected those remote from favourable ecosystems, and such input-dependent solutions had many pest and ecological concerns. Impact is needed in all farming systems, including the oft-neglected mixed farming systems in the diversified remote uplands of Asia, Africa and Latin America.

WHAT CAN BE DONE TO ADDRESS AND CHANGE THOSE PERCEPTIONS?

We need to show what these materials can do: ground cover, regrowth, floret fertility, forage dry matter (DM), resource capture, soil health, grain yield, disease, quality, biodiversity, ecosystem benefits, runoff, percolation, leaching. To do this well, we need to use a systems approach, and explore where the perennial crops would fit into the current and future farming systems.

Would the perennial displace the annual crop, or more likely, would it be preferentially suited to particular soils or situations, thereby contributing to the diversification and stability of the whole farming system and landscape. What is the plant requirement?? What traits should they possess?? What materials are available, and what are they capable of at this stage of development?? How do we go about improving them?? What are the next steps??

WHERE ARE WE UP TO IN DEVELOPING PERENNIAL CROPS?

As the papers in this volume attest, development of perennial crops ranges from its infancy (e.g. perennial maize and others, Murray and Jessop, 2014; Van Tassel *et al., 2014;* Geleta *et al.,* 2014; Gross and Miller, 2014, this volume), to intermediate (e.g. perennial wheat, Jones *et al.,* 2014, this volume), to approaching reality (e.g. perennial sorghum, Paterson *et al., 2014;* Cox *et al., 2014;* Bozzini, 2014; perennial rice, Sacks *et al., 2014;* Hu *et al.,* 2014*;* Hill, 2014; and perennial legumes, Snapp *et al.,* 2014, this volume). The level of progress has depended in part on the duration of effort, and the complexity of genetic barriers encountered between domesticated and related wild species (Cox *et al.,* 2002). The latter issue has prompted the alternative approach of domesticating the wild species instead (e.g. *Thinopyrum intermedium*, Dehaan *et al.,* 2014, this volume). The diversity of species, approaches, and ecosystems targeted is positive, as we sort out what opportunities may arise. Nevertheless, as researchers and donors consider their strategies for the future, some targeting of investment by farmer demand, ecological need, breeding progress, and policy implication is likely (see below, and also Dixon and Garrity, 2014, this volume).

Further, sustained progress and the development of perennial crops targeted to farmer needs is likely to require systems approaches, in order to assist compatibility with farmer practice and the likelihood of adoption. To explore this, and to set up a basis for examining policy implications, two cases are examined: perennial wheat in Australia as a case study for a developed country, and perennial rice in Asia as a case study for a developing country.

PERENNIAL WHEAT IN AUSTRALIA AS A CASE STUDY FOR A DEVELOPED COUNTRY

An example of the use of the systems approach is provided by research in perennial wheat in Australia, supported by related efforts in the United States of America. Bell *et al.* (2008) used MIDAS, a bioeconomic model of a mixed crop-livestock farming system to explore what role perennial wheat may play in the farming system. Perennial wheat used solely for grain production was not selected as part of an optimal farm plan under the standard assumptions. In contrast, dual-purpose perennial wheat that produces grain and additional forage during summer and autumn could increase farm profitability substantially (AU$20/ha over the whole farm) and 20 percent of farm area was selected for perennial wheat production on the optimal farm plan under the standard assumptions. As little as 800 kg/ha of forage from perennial wheat could reduce demands on stubble over summer and grain supplement at break of season and increase farm stock numbers. The additional value of this timely grazing reduced the relative yield required for perennial wheat to be profitable to just 40 percent of that of the annual wheat crop. This analysis suggested that dual-purpose perennial wheat would be a profitable option for mixed crop/livestock farmers.

The challenges in developing perennial wheat for Australia were reviewed by Bell *et al.* (2010), following ground-breaking work in the United States to generate amphiploids between annual *Triticum* and perennial *Thinopyrum* species (Cox *et al.* 2010; Murphy *et al.* 2010). Experimental efforts in Australia commenced by evaluating a diverse array of putative perennial wheat derivatives including germplasm imported from the United States perennial wheat programs, Russian and Chinese wide-cross germplasm and assorted lines from the Australian Wheat Collection. This initial evaluation of over 150 wheat x wheatgrass derivatives assessed capacity to regrow post-harvest, and yield grain over successive years, thereby identifying characteristics common to surviving breeding lines (Hayes *et al.* 2012; Jaikumar *et al.* 2012). Several entries persisted to produce grain over three successive years. Regrowth was associated with the presence of at least one whole genome equivalent (14 chromosomes) from the perennial donor species. This research established that developing a perennial wheat may be feasible, even though existing germplasm was not intended for Australian conditions, nor was it yet sufficiently developed to be deployed commercially.

The research continues with further field evaluations of perennial wheat derivatives, including previously untested germplasm, and an additional fourth year of those entries surviving from the initial report. In addition, three more-detailed experiments were examined, which used a common set of six genotypes to evaluate forage biomass production under serial defoliation, changes in root-shoot partitioning of DM over successive regrowth cycles, and dehydration tolerance and plant survival under severe water deficit and re-watering. Based on this evidence, a breeding approach for developing adapted perennial wheat for Australian farmers has been proposed (Larkin *et al.* 2014; Larkin and Newell, 2014, this volume).

Further, this research establishes a need to pursue mechanistic understanding in order to make sustained progress. Some of the United States material is reported to be perennial in the glasshouse, but fails to survive in the field. When grown in Australia, however, some of this material has survived and regrown in the field for three seasons. Is this due to the severity of abiotic stresses encountered, such as cold and snow cover in winter, and severe drought and high temperature in summer. Is survival due to better agronomy such as rotation with Brassicas, a different soil or its key attributes such as pH or drainage, or is it related to tolerance to particular diseases such as root and crown rots. By understanding why materials fail in different situations, sustained breeding and agronomic progress is more likely to result.

PERENNIAL RICE IN ASIA AS A CASE STUDY FOR A DEVELOPING COUNTRY

A second case study is drawn from perennial rice in Asia. Perennial rice was originally proposed as a plant type to improve soil stability on sloping uplands, while contributing forage for livestock and grain for the farmer. Initially, crosses were made between *Oryza sativa* and *Oryza longistaminata*, and between *Oryza sativa* and *Oryza rufipogon* (Sacks *et al.* 2014, this volume), which provided sources of nematode and drought resistance from the wild species. The materials were passed to Yunnan Academy of Agricultural Sciences in Kunming China, where Professor Fengyi Hu and his team continued the breeding effort with great success. By selection and repeated backcrossing, they were able to increase spikelet fertility while retaining perennial traits allowing regrowth. Suitable plant types for favourable lowland environments have resulted, with one entry, PR23, now in pre-release testing in Yunnan province. In addition, two QTL for rhizome development have been identified and sequenced (Hu *et al.* 2014, this volume). Breeding and genetic progress in perennial rice has been impressive, but the associated understanding of target environments, and how the materials generated perform in them, is at its early stages, though is now being addressed in field experiments in Yunnan Province in China, and in Savanakhet and Champassak Provinces in neighbouring Lao PDR (Wade and Sengxua, 2014). There is a need to build on this collaboration to further strengthen the characterisation, agronomy, physiology and field testing in association with the breeding program, to ensure sustained progress in the future. In particular, success in addressing the original target, perennial rice for the drought-prone rainfed lowland and upland environments, will require additional investment and collaboration, especially for perennial survival and regrowth in harsh dry conditions.

WHAT ARE THE POLICY IMPLICATIONS FOR THE DEVELOPED COUNTRY?

In the developed country, high priority was allocated to protection of the established annual crop, its production and marketing system. At issue is concern with disease, weediness or poor grain quality. Any proposed breeding effort must ensure levels of disease resistance at least equal to currently released cultivars, for the most common diseases such as stem rust, leaf rust, stripe rust, and septorias. The intent is to ensure the perennial crop could not act as an out-of-season stepping stone to development of spore epidemics, nor to encourage mutation and breakdown of resistance of useful genes. To an extent, the risk here may be less than envisaged, as there is unlikely to be a large green canopy on the perennial crop when soil water is limiting after harvest. The perennial nature, however, may render certain diseases even more important than in the annual crop. Examples include insect-transmitted viruses such as wheat streak mosaic virus and barley yellow dwarf virus. Nevertheless, the wild progenitors possess strong resistance to these viruses, which the evidence suggests is passed to the progeny. For Australia, the root and crown rots may be a special threat for perennials, with the intention to grow the perennial crop for several seasons. Here, crop rotation with brassicas and soil health will be important to ensure levels of infection are initially low, as resistance is not strong against many root and crown rots. Nevertheless, it will be important to include the best resistance available for these critical diseases. To address these concerns, the developed country may require specified levels of resistance to particular diseases, or require the perennial only be grown for a specified number of seasons before rotating to another crop.

The perennial parent of many of the perennial wheats, *Thinopyrum intermedium*, is a prohibited species in Australia, requiring stringent quarantine procedures before the perennial wheats could be grown in the field in Australia. The concern was robust or long rhizomes, which may be hard to kill, and which could pose a significant weediness threat. Fortunately, the introduced lines did not possess rhizomes, and their regrowth was in the form of tillers in the next cycle from the crown of the plant. Hence the risk of weediness was greatly reduced, but it is still worthwhile checking progeny to make sure. To address this, the developed country may require that released perennial wheats only regrow as tillers not rhizomes.

Grain quality is a critical element of successful marketing of Australian wheat to meet quality requirements for a number of carefully defined markets. Grain is delivered to separate silos, and handled and marketed separately, to ensure consistent quality. Were a lower grain quality perennial wheat to be released, its grain would need to be segregated, perhaps as feed wheat only. Such arrangements are already in place, so should be acceptable, though no doubt there would be concern if larger quantities of low quality wheat were delivered, relative to the more desirable high-quality, higher-priced grain. To address this concern, grain must be segregated by quality and sufficient facilities must be available to cope, until grains of comparable quality become available. The importance of grain quality, and the associated benefits to human health, is considered in several papers in this proceeding (Pogna *et al.* 2014; Sands *et al.* 2014, this volume).

WHAT ARE THE POLICY IMPLICATIONS FOR THE DEVELOPING COUNTRY?

To an extent, the issues for the developing country may include aspects of those for the developed country above, but the contrast may relate to grain for export versus feed for the family. In the developing country, food security and livelihood for the farmer and the family are paramount, especially assurance of food supplies until the next harvest. Governments in developing countries may be more willing to explore opportunities, to see what benefits could accrue for subsistence farmers, and those governments are very concerned with sustained production. Hence, developing countries may more readily recognize the potential opportunities with perennial crops and allow farmers to explore them.

WHERE WILL PERENNIAL CROPS BE GROWN AND WHAT WILL BE THE CONSEQUENCES?

The discussion above is designed to draw attention to the reality that perennial crops are unlikely to soon replace high-yielding annual crops as the mainstay of grain production. More likely, they will fulfil niche roles across the landscape, perhaps being selected to stabilise land intermediate between prime cropping and grazing only, especially if some soil remediation is needed. As such, a perennial crop is most likely to be dual purpose, including provision of timely feed to livestock. The analyses of Bell *et al.* (2008) and Pimentel *et al.* (2012) provide examples of the potential roles of perennial wheat. Likewise, mountain uplands may provide a scenario ideal for inclusion of perennial rice. The lowland paddy at altitude would still support high-yielding annual rice and other crops, but the surrounding sloping uplands, where upland rice is normally grown, may be ideal for perennial rice for grain and grazing, perhaps in conjunction with either agroforestry or self-regenerating annual legumes for further diversification and system stability. Another example is intercropping of annual peanut and perennial pigeon pea in Malawi (Snapp *et al.* 2014 this volume), to provide not only feed for farmer and livestock, but also nitrogen to a following maize crop. In each of these examples, the landscape is improved, with a more productive and stable multi-purpose farming system.

WHAT WE NEED TO DO NEXT

The most critical issue facing the perennial crops community is to build credibility and confidence in the broader scientific population, and especially in the donor community. To do this, we need to collect and publish hard data on the performance of perennial crops, and especially on tradeoffs in performance, benefits to resource capture and timeliness, and alternative systems benefits such as via livestock or ecosystem services. Previously, publications from the perennial crops community tended to be longer on exciting concepts, but shorter on evidence to demonstrate what they could actually deliver. Some publication streams are now in progress to address this, but further effort is needed. I believe donor success is related not only to concept development in exciting proposals, but also to hard evidence and strong track records of delivery. Given these are questions about the validity of developing perennial grains, e.g. yield tradeoffs, grain security, we must assemble and publish evidence to demonstrate the viability of our visions, and also, a likely time frame to their achievement. This workshop is an ideal foundation for a coordinated effort to develop perennial crops, based on sound scientific evidence, relevance to farmer needs, and in a manner compatible with sustainable and profitable farming systems (see also Snapp *et al.* 2014 and final chapter, this volume).

CONCLUSIONS

Prospects for developing perennial crops are bright, especially if efforts are targeted to appropriate environmental niches with appropriate species. For example, the Grains Research and Development Corporation has recently listed perennial wheat as a technology on the horizon, which has potential to contribute to the next substantial advance in Australia. Perennial rice line PR23 is now in pre-release testing in the Yunnan Province of China, raising prospects of commercial release to farmers shortly. Annual peanut-perennial pigeon pea-maize is already being used by farmers in Malawi. Hence, opportunities to include perennial crops in productive, stable and sustainable dual-purpose systems are appearing already. We need to continue our efforts and document our progress as we proceed in order to ensure support of scientific and donor communities towards a perennial cropping systems future.

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02

PERENNIAL RICE: CHALLENGES AND OPPORTUNITIES

Erik J. Sacks
University of Illinois, 1206 W. Gregory Dr., Urbana, IL 61801, USA.
Email: esacks@illinois.edu

ABSTRACT

As the human population continues to grow, and governments increasingly incentivize the migration of people from rural areas to cities, there is pressure to produce more food with fewer resources, including water, fertilizer, and especially labour. The development of high-yielding, perennial cultivars of rice could help meet the need for more food while reducing the inputs required. Domesticated Asian rice (*Oryza sativa*) was derived from perennial ancestors. Moreover, the traditional practice of ratoon cropping demonstrates that domesticated Asian rice retains some of its original perennial character, though this varies greatly by cultivar. However, with currently available cultivars, the yields of first ratoon crops are typically 40 percent or less of main crop yields and subsequent decreases make further cycles of ratooning uneconomical. Thus, the key research and development challenges for perennial rice are to improve ratoon yields, increase longevity, and improve drought tolerance with the goal of facilitating perennial rice regrowth and production beyond irrigated systems and into seasonally dry upland and rainfed lowland environments. Opportunities (in order of increasing difficulty and potential gain) for developing high-yielding cultivars of perennial rice include: 1) breeding within domesticated Asian rice germplasm for improved ratooning ability and yield, 2) introgressing genes from the perennial, rhizomatous A-genome species *O. longistaminata*, 3) domesticating the rhizomatous species *O. longistaminata* and *O. australiensis*.

Keywords: perennial rice, *Oryza sativa*, *O. longistaminata*, ratoon, introgression

INTRODUCTION

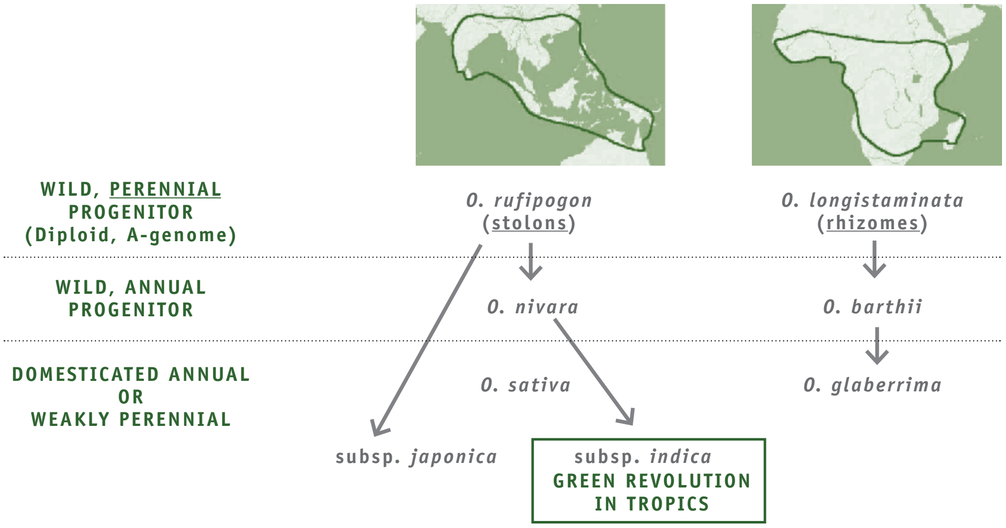
Many rice cultivars grown today are perennials that are cultivated as annuals (Figure 1). Genotypes of *Oryza sativa* vary greatly in their propensity to ratoon (regrow) after an initial harvest of grain (Chauhan *et al.* 1985; Krishnamurthy, 1988). Ratoon cropping of rice was a traditional practice, especially in East Asia (Hill, 2010), that became less common during the second half of the twentieth century. However, a notable modern exception has been an emphasis on ratoon crop production in the United States Gulf Coast to increase overall seasonal yields (Bollich and Turner, 1988), which is an economically viable strategy where warm weather persists long enough for a ratoon crop but is insufficient in duration for a new planting from seed to mature.

FIGURE 1. A RICE FIELD IN JAPAN DURING AUTUMN SHOWS VIGOROUS REGROWTH AFTER HARVEST OF THE FIRST CROP

Cool weather prevents development and harvest of a ratoon grain crop at this location.

Both species of domesticated rice, *O. sativa* from Asia and *O. glaberrima* from Africa, are derived from perennial ancestors, either directly or via an annual intermediary (Figure 2). Cheng *et al.* (2003) found that the progenitor species of *O. sativa*, *O. rufipogon*, consisted of four major clades, three of which were perennial and one of which was annual (the annual clade is sometimes referred to as *O. nivara*). Moreover, Cheng *et al.* (2003) found that the *indica* subspecies of *O. sativa* was derived from the annual *O. rufipogon* clade and that the *japonica* subspecies was derived from one of the perennial *O. rufipogon* clades. Though there is currently much debate about whether *O. sativa* was derived from a single or multiple domestications, the results of Cheng *et al.* (2003) are consistent with observations of some workers that *japonica* cultivars are typically better than *indica* cultivars for ratooning (Shahi and Raharinirian, 1988) and as parents for breeding strongly perennial rice (Sacks *et al.* 2007). In the Southern United States, which is the primary example of successful ratoon cropping of rice on a large commercial scale, production is based on tropical *japonica* cultivars (Lu *et al.* 2004). Recently, New Rice for Africa (NERICA) cultivars and their japonica parent were found to have higher ratoon yields under upland conditions than their *O. glaberrima* parent (Sanni *et al.* 2009). However, some of the green revolution *indica* cultivars, such as IR8 and IR64, have also been observed to ratoon well (Chauhan *et al.* 1985; Munda *et al.* 2009). In any case, it is clear that there is considerable genetic diversity within *O. sativa* for perennial growth, and additional diversity is present in its wild relatives, *O. rufipogon* and *O. longistaminata*. Perennation in *O. sativa* is by growth of axillary buds on older tillers (i.e. tillering), whereas *O. rufipogon* can additionally propagate from stolons, and *O. longistaminata* produces many long rhizomes that are the primary source of new shoots.

FIGURE 2. ORIGINS OF DOMESTICATED RICE IN RELATION TO PERENNIAL GROWTH



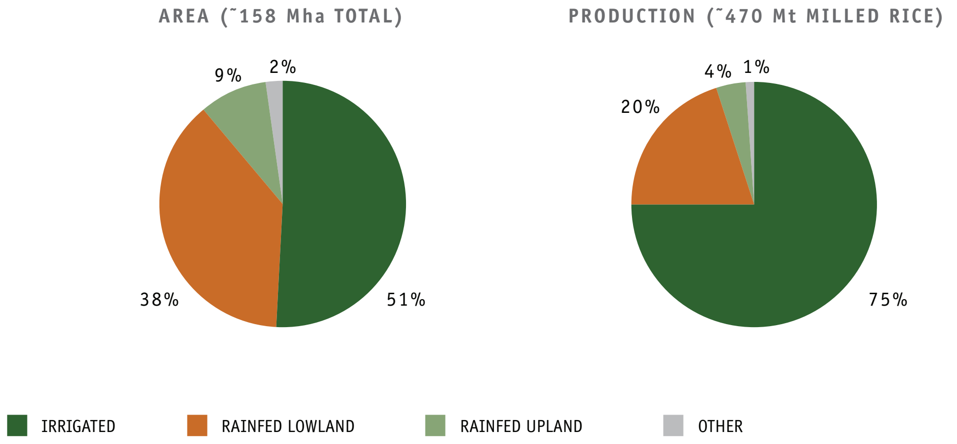
Source: http://www.knowledgebank.irri.org/extension/wild-rice-taxonomy.html

PROBLEMS THAT PERENNIAL RICE COULD HELP SOLVE

Rice is a critically important staple food and the demand for more production is expected to continue to increase, especially in developing countries. Deployment of perennial rice could meet important needs, such as increased production per growing season, reduced risks for farmers, lower labour requirements, less water needed, and protecting soil from erosion.

Irrigated rice accounts for 51 percent of the area planted to rice but 75 percent of rice production (Figure 3). In contrast, rainfed lowland rice accounts for 38 percent of the area but just 20 percent of production, and rainfed upland rice accounts for 9 percent of rice lands but only 4 percent of production (Figure 3). Thus, a major challenge for improving availability of rice is to increase production in rainfed systems. Where rainfed fields in tropical monsoonal environments cannot be converted to irrigated, a potentially valuable strategy for increasing production is to maximize use of rainfall via a ratoon crop. In contrast to annual rice cultivation, ratoon rice could take advantage of end-of-season moisture in a typical year, and also be positioned for even greater yields in wet years. Such a use of season-limiting moisture would be analogous to the use of ratooning in the Southern United States of America to take advantage of end-of-season warmth. Cultivars with tolerance to drought, in addition to high ratooning ability would be especially useful for rainfed production of a ratoon crop. By modifying crop duration from a single long-season crop to a medium duration main crop plus a short-medium duration ratoon crop, it may also be possible to reduce production risks associated with variable abiotic and biotic stress pressures. Adoption of early maturing rice cultivars was critical to the establishment of ratooning in the Southern United States of America (Bollich and Turner, 1988).

FIGURE 3. RICE AREA AND PRODUCTION BY CULTIVATION SYSTEM



Source: http://irri.org/index.php?option=com_k2&view=item&layout=item&id=9151&lang=en

Field preparation and planting of paddy rice, typically via initial establishment of seedbeds followed by transplanting of seedlings, is labour intensive. For upland rice, planting and especially weeding can require a major investment of labour. For example, in Central and West Africa, upland rice accounts for ~40 percent of the rice area, but employs ~70 percent of the region's rice farmers (http://irri.org/). As governments increasingly incentivize urbanization, the availability of labour for farm work will continue to be reduced. Thus, there is a great and increasing need to maximize production of rice per unit of labour. Perennial rice enables multiple harvests from a single planting, thereby improving labour efficiency. Though improvements in labour efficiency may be expected via mechanization, such gains come with capital costs that are often too great for many farmers in developing countries, whereas seed of improved perennial cultivars can be made available for little or no additional cost to farmers.

Hybrid rice has brought yield benefits to farmers who can afford to invest in this technology. However, hybrid rice seed is expensive to produce and thus is sold at a premium. If additional production in farmers' fields can be obtained from hybrid rice crops via ratooning, then the return on investment for a farmer would be greater (Bollich and Turner, 1988; de la Rosa, 2004) and this advantage could make it economically feasible for more farmers to adopt.

Water is a major input for rice production. Global climate change is expected to negatively impact availability of water for rice production by reducing winter snow-packs on mountains and by the melting of glaciers that feed rivers used to supply irrigation water for rice fields. It is well established that ratoon crops of rice require less water than main crops, thereby improving the overall efficiency of water use for rice production. Thus, increasing production of rice via cultivating more ratoon crops and increasing ratoon yields would further improve efficiency of water use.

Upland rice cultivation is source of soil erosion. This is especially true for hilly lands, such as those in Southeast Asia, where upland rice is grown in a traditional swidden system or under new plantings of tree crops such as rubber (Figure 4). Erosion of soil in the uplands fills downstream reservoirs with silt, thereby reducing the amount of water available for irrigated rice in the lowlands. Thus soil erosion has negative consequences on a regional level. The cultivation of perennial crops, such as perennial upland rice, would be expected to reduce soil erosion relative to current practices. This goal was the driving force behind the International Rice Research Institute (IRRI) Perennial Upland Rice Project during the 1990s. However, a successful cultivar of perennial upland rice would need exceptional drought tolerance to survive the long dry season that is typical in mainland SE Asia. Development of adapted perennial rice cultivars would be considerably less challenging for areas without a dry season or only a short dry season, than for long dry season environments. For example, in Mato Grosso, Brazil, some modern and high yielding cultivars of upland rice produce good ratoon crops under favourable environmental conditions (Santos *et al.* 2003).

FIGURE 4. UPLAND RICE PRODUCTION ON HILLY LANDS IN SOUTHEAST ASIA

Top: note upland rice on left side of photo among tree stumps from recently cleared land, and also note the large area of soil erosion on the steep area in the centre of the photo. Bottom left: upland rice growing under a new rubber plantation. Bottom right: a new rubber plantation with bare, eroding soil associated with a gap between the cycles of harvesting and planting of annual understory crops.

IMPROVING RATOONING ABILITY AND YIELD WITHIN *O. SATIVA*

Though ratoon cropping is practiced commercially in the southern United States of America and parts of southern China (Bollich and Turner, 1988; Xue-Bin *et al.* 1988), substantial improvements in regrowth ability and yield potential of ratoon crops would be needed for ratoon cropping to compete economically with new plantings from seed/seedlings in areas where conducive environmental conditions do not limit the time available for growing a subsequent rice crop. For those cultivars that can regrow well after harvest of an initial crop, first ratoon yields are typically 40 percent or less of main crop yields and subsequent decreases in yield make further cycles of ratooning currently uneconomical (Chauhan *et al.* 1985; Krishnamurthy, 1988). Thus, a key challenge is to breed cultivars that have high overall yield potential yet have high ratoon yields (at least on a per growing day basis) as well as high main crop yields. Though most ratoon crops have lower yields than main crops, there have been documented cases where both main crop and ratoon crop yields were both high and similar to each other, indicating that a possible path forward is to identify combinations of genotype and environment that result in multiple high yields. Another key challenge is to select genotypes that maintain high yields for more than one ratoon crop. Such a development would be a step-change for rice that would potentially make ratooning an economically attractive option for irrigated production environments that are not limited in duration by cold weather or other season-limiting conditions. High ratooning ability and low rates of missing hills are necessary but not sufficient for achieving high yields for one or more ratoon crops. Other desirable traits for improving ratoon cropping are drought tolerance, cold tolerance (especially at flowering stage for subtropical and temperate environments), duration adapted to the target environment, and highly resistant to pests and diseases.

Little information is available on the genetics of ratooning ability in rice. However, choice of parents can have a large effect, and heritabilities may be moderate to low (Shifen and Tingwen, 1988). Late stage selection is likely to result in slow and modest improvements. For greater and more rapid gains, early generation selection coupled with replication of genotypes would be desirable. Replicated early generation selection could be accomplished with doubled-haploid populations or by vegetatively propagating F_2 individuals.

INTROGRESSING GENES FOR PERENNIAL GROWTH FROM *O. RUFIPOGON* AND *O. LONGISTAMINATA*

Both *O. rufipogon* and *O. longistaminata* are sources of genes for improving perennation in *O. sativa*. Previous studies at IRRI indicated that choice of genotype within both the domesticated and undomesticated parent species has a large effect on the progenies' ability to grow perennially (Sacks *et al.* 2006, 2007). The main advantage of using *O. rufipogon* as a donor of genes for perennial growth to domesticated Asian rice is its high cross compatibility with *O. sativa*. However, stolons, which are the key perennating structure of *O. rufipogon*, are not well-suited to surviving drought because they may be exposed to sun and dry air on the surface of the soil if grown under upland conditions, or under rainfed production during the dry season. In contrast, the rhizomes of *O. longistaminata* are protected from desiccation by insulating soil. Additionally, *O. longistaminata*, which can form large monocultures in the wild, is more vigorous than *O. rufipogon*. Breeding barriers have typically limited the production of F_1 *O. sativa/O. longistaminata* progeny severely, and embryo rescue has usually been required to obtain early generation backcross progeny in large numbers (Chu and Oka, 1970; Hu *et al.* 2003; Ishikawa *et al.* 2011; Tao and Sripichitt, 2000). Moreover, associations between infertility and rhizomatous growth in early generations have required considerable breeding work to break (Chen *et al.* 2009). Recently however, Kanya *et al.* (2012) reported that crosses between *O. sativa* 'Basmati 370' and a Kenyan accession of *O. longistaminata* resulted in the production of over 500 hybrid seed (6 percent success) and that the F_1 hybrids, which germinated without embryo rescue, were remarkably fertile, producing more seed per plant than either parent. It is likely that *O. longistaminata* is the best species source of genes for improving regrowth potential of domesticated rice via introgression, given that it has vigorous perennial growth, great genetic diversity associated with broad geographic distribution and self-incompatibility, and the same A-genome as *O. sativa* (Kiambi *et al.* 2008; Melaku *et al.* 2013). The recent development of a whole genome fosmid library for *O. longistaminata* should facilitate identification and introgression of key genes from this species into domesticated rice (Li *et al.* 2012). Additionally, Shim (2012) has described several breeding strategies for introgressing genes from *O. longistaminata*. Though *O. longistaminata* is a promising source of genes for improving many traits in domesticated rice, few interspecific hybrids between *O. sativa* and *O. longistaminata* have yet been produced. A substantial public collection of *O. longistaminata* germplasm is available but it remains largely untapped.

In a hybrid population of *O. sativa* and *O. longistaminata*, rhizomatous growth was conferred by two dominant complementary genes for rhizome presence and many QTL of modest effect for degree of rhizome expression (Hu *et al.* 2003). Subsequently, candidate genes for rhizome expression were identified by gene expression analyses (Hu *et al.* 2011), which should further facilitate introgression efforts. Diligent breeding work over many years at the Yunnan Academy of Agricultural Sciences has resulted in the production of interspecifc progenies that have both long rhizomes and high fertility. Moreover, non-rhizomatous backcross progenies of *O. sativa/O. longistaminata* have been selected for their ability, under irrigated conditions, to produce three sequential harvests (one main crop and two ratoon crops) from a single planting, yet have high yields for all three harvests. Thus, introgression from *O. longistaminata* is also a useful strategy for improving ratooning in domesticated rice. Further use of *O. longistaminata* accessions and genes is expected to be valuable for rice improvement.

DOMESTICATING THE PERENNIAL RHIZOMATOUS SPECIES *O. LONGISTAMINATA* AND *O. AUSTRALIENSIS*

The main advantage of domestication as a breeding strategy over introgression is that breeding barriers associated with wide crosses are avoided. However, the main obstacle to domesticating a wild species is that desirable alleles for domestication and agronomic traits are in low frequency in the founder population. Thus, domestication can be expected to take considerable time and effort to produce acceptable cultivars for use by farmers. Nevertheless, domestication may be a viable option if breeding barriers will significantly limit introgression, and if many genes are needed from a wild species to achieve a particular breeding goal. For example, adaptation of rice to perennial growth while surviving seasonal drought may be a case in which domestication is the best option. Both *O. longistaminata* and *O. australiensis* have rhizomes which may enable the plants to survive in a dormant state during drought (Henry *et al.* 2010). Though this dormancy response has not been thoroughly explored, we expect that the trait is most strongly expressed in *O. australiensis* because its native habitat is more drought prone than that of *O. longistaminata*. Introgression may be a competitive option for developing drought-resistant perennial upland rice from *O. longistaminata*, but for the E-genome *O. australiensis*, severe breeding barriers to crossing with *O. sativa* would make domestication the only practical option. With their long, vigorous rhizomes and shattering seed, both *O. longistaminata* and *O. australiensis* are potentially invasive, which would be of particular concern outside of their native range. Thus, any domestication efforts would need to select for a moderate rhizome length, which balances the need for survival during drought with the need to minimize potential invasiveness. For initial breeding work to domesticate *O. longistaminata* or *O. australiensis*, a location where the species are native would be most desirable, so as to avoid introducing potentially invasive early generation plants to areas where they are non-native. In addition to reduced rhizome length, domesticated versions of these species would need to be non-shattering, semi-dwarf, and high-yielding. Fortunately, the sequence of many key genes for domestication of *O. sativa*, such as the non-shattering genes *sh4* and *qSH1*, and the semi-dwarf gene *sd1* are known (Konishi *et al.* 2006; Li *et al.* 2006; Monna *et al.* 2002), which should allow for relatively rapid domestication of wild rice species via targeted screening of germplasm and selection.

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03

THE PROGRESSION OF PERENNIAL RICE BREEDING AND GENETICS RESEARCH IN CHINA

Shila Zhang[1], *Wensheng Wang*[2], *Jing Zhang*[1], *Zhang Ting*[2], *Wangqi Huang*[1], *Peng Xu*[1], *Dayun Tao*[1], *Binyin Fu*[2], *Fengyi Hu*[1,3]

1 Food Crops Research Institute, Yunnan Academy of Agricultural Sciences, Kunming, 650205 China
2 Crops Sciences Research Institute, China Academy of Agricultural Sciences, Beijing, 100018 China
3 Correspondence should be addressed to Fengyi Hu: hfengyiyaas@aliyun.com

ABSTRACT

Soil erosion is a worldwide problem of increasing concern, and perennial grain crops could be an important part of the solution. In Southeast Asia, upland rice (*O. sativa*) contributes to regional soil erosion problems because it is an annual crop grown on hilly lands. The perennial cultivars of upland rice could reduce soil erosion and meet the needs of subsistence farmers. From the viewpoint of breeding, *O. longistaminata*, with same genome, AA, similar to *O. sativa*, would be the most logical donor of genes for rhizome expression for perennial rice cultivar development, several donor traits, such as rhizome and stolon have been employed for perenniality. Up to now, there are some results as following: 1) based on the fine mapping of the rhizome genes (*Rhz*), via genomic library (BAC, Fosmid, rhizome cDNA library) construction and analysis, confirming the genetic regularity that the rhizome was controlled by two pairs of dominant complementary genes, *Rhz2*、*Rhz3*, and obtaining 15 rhizome locus and candidate functional genes; 2) the perennial rice breeding is on the way and some breeding lines that hold the rhizome genes were made. There are five perennial rice (PR) lines, namely PR23, PR57, PR129, PR137 and PR139, that have been bred; 3) the potential perennial materials were screened at different sites, such as Lao, Africa and China for perennial ability investigation. Thus development of perennial rice cultivars from *O. longistaminata* faces two large challenges: 1) the need to pyramid in an *O. sativa* background multiple rhizome QTL in order to get strong rhizome expression, and 2) the need to get rid of QTLs for low pollen fertility without losing linked QTLs for rhizomes.

Keywords: perennial rice, breeding, genetics research, *Oryza longistaminata*

INTRODUCTION

Soil erosion is a serious problem in the uplands of Southeast Asia (Figure 1). Once forest is cleared on sloping uplands and replaced with annual crops such as upland rice, nutrients are rapidly leached and soils are eroded, so lands need to return to forest for some years before being suitable again for crop production. Population pressure is reducing the duration of fallow, so performance of upland rice, associated crops and livestock are declining, as soil quality and fertility progressively deteriorate. A way is needed to stabilise these fragile soils (IRRI, 1998).

FIGURE 1. SOIL EROSION IN UPLAND RICE FIELD IN YUNNAN, CHINA

Soil erosion in uplands of southeast Asia has been a serious problem that led to the project of developing perennial upland rice at IRRI (IRRI 1989)

TABLE 1. *ORYZA* SPECIES, THE SPECIES COMPLEX, CHROM., GENOME GROUP AND DISTRIBUTION

SECTION	COMPLEX	SPECIES	CHROMOSOME NUMBER	GENOME GROUP	DISTRIBUTION
ORYZA	*O. sativa* complex	*O. sativa* L.	24	AA	worldwide
		O. nivara Sharma et Shastry	24	AA	Tropical and Sub. Asia
		O. rufipogon Griff	24	AA	Tropical and Sub. Asia
		O. meridionalis Ng	24	A^mA^m	Tropical and Australia
		O. glumaepatula Steud.	24	$A^{gl}A^{gl}$	South America
		O. glaberrima Steud	24	A^gA^g	Africa (mainly West)
		O. barthii A. Chev.	24	A^gA^g	Africa
		O. longistaminata Chev. et Roher	24	A^lA^l	Africa
	O. officinalis complex	*O. officinalis* Wall ex Watt	24	CC	Tropical and Sub. Asia
		O. minuta Presl. et Presl.	48	BBCC	Philippines
		O. eichingeri Peter	24	CC	Sri Lanka, Africa
		O. rhizomatis Vaughan	24	CC	Sri Lanka
		O. punctata Kotschy ex Steud.	24, 48	BB, BBCC	Africa
		O. latifolia Desv.	48	CCDD	Latin America
		O. alta Swallen	48	CCDD	Latin America
		O. grandiglumis (Doell) Prod.	48	CCDD	South America
		O. australiensis Domin	24	EE	Australia
RIDLEYANAE TATEOKA		*O. brachyantha* Chev. et Roehr.	24	FF	Africa
		O. schlechteri Pilger	48	HHKK	Papua New Guinea
	O. ridleyi complex	*O. ridleyi* Hook. f.	48	HHJJ	SE Asia
		O. longiglumis Jansen	48	HHJJ	Irian Jaya, Indonesia
GRANULATA ROSCHEV.	O. meyeriana complex	*O. meyeriana* Baill	24	GG	SE Asia
		O. granulata Nees et Arn. ex Watt	24	GG	S and SE Asia

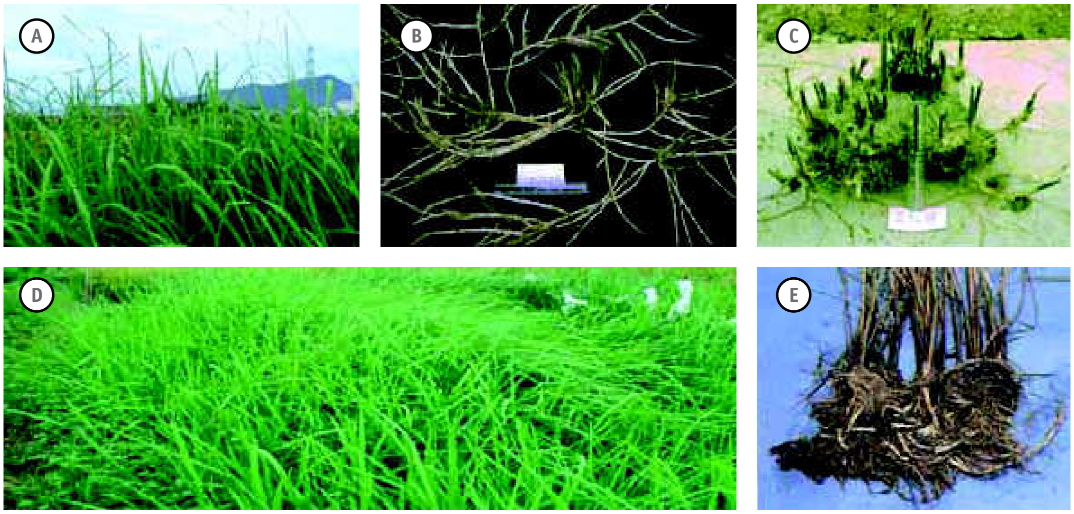
Following reports of a successful cross between *Oryza sativa* and *O. longistaminata* at the Yunnan Academy of Agricultural Sciences (Tao, 2000), development of perennial rice was proposed as one way to maintain surface cover after clearing, with potential benefits to nutrient and soil retention, and rice and livestock performance (IRRI, 1998). With donor support from Europe, research commenced at IRRI, with several reports explaining the concept, outlining the approach and reporting some initial results (Xiu, 1995; Schmit, 1996; Tao, 2000; Sacks, 2003).

METHODS

The *Oryza* species are quite diverse, as indicated in Table 1, which lists the species with their chromosome number, genome group and distribution by *Oryza* complex. The species arrowed express perenniality, including *O. longistaminata* in the *O. sativa* complex, and *O. rhizomatis* and *O. australiensis* in the *O. officianalis* complex.

Initially, *O. sativa* was crossed with *O. longistaminata* and *O. rufipogon*, but the crosses were more successful with *O. longistaminata*, as it is in the same complex as *O. sativa*. Consequently, it was decided to use *O. longistaminata* predominantly as the source of perenniality. *O. longistaminata* is AA genome like *O. sativa*, and develops extensive rhizomes in its native wetland habitat (Figure 2).

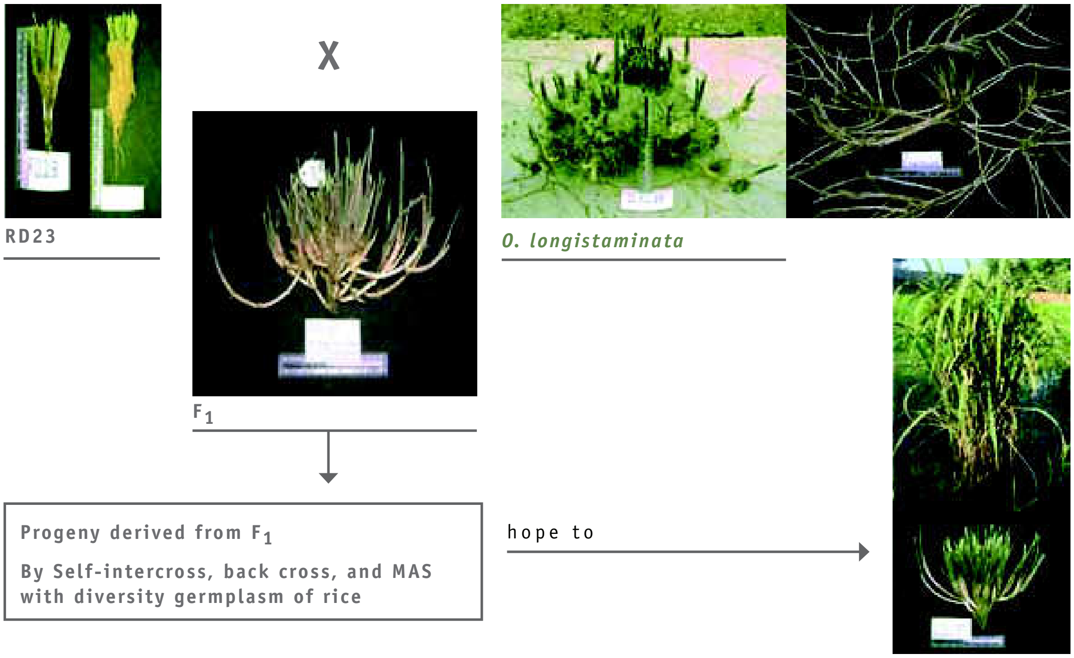
FIGURE 2. THE *O. LONGISTAMINATA* (AA GENOME)



The *Oryza longistaminata*
A. The panicle of the *O. longistaminata*
B,C,E. The strong rhizomes of *O. longistaminata*
D. The performance of *O. longistaminata* in field

The breeding strategy adopted to develop perennial rice at Yunnan Academy of Agricultural Sciences was as follows. RD23 was chosen as the *O. sativa* parent, as this cultivar was widely grown in lowland or upland, high yielding, good grain quality glutenous, and with disease resistance to rust etc. It was crossed with *O. longistaminata*, and the F_1 was intermediate in characteristics between the parents (Figure 3).

FIGURE 3. THE STRATEGY FOR PERENNIAL RICE BREEDING



From a combination of intercrossing among the F_1 progeny, backcrossing to RD23, and selection for desired traits, progress was made in developing perennial rice (Figure 3). This process was repeated a number of times using a wide range of successful cultivars as the *O. sativa* parent, but to date, the most successful has been with RD23. Selection, including marker-aided selection (MAS), was used to assist in transferring the perenniality traits from *O. longistaminata* into perennial rice. This followed successful research to identify QTL associated with rhizome development in *O. longistaminata*.

RESULTS

1. Genetic analysis of rhizome production

Genetics of rhizome expression was explored with Simple-Sequence Repeats (SSR) markers on the F_2 population from RD23/ *O. longistaminata*, using field and marker data. The PCR-based molecular genetic map (Figure 4) revealed three regions on chromosomes 3 and chromosome 4 of rice that indicated the two dominant complementary genes for rhizome expression, which were designated as *Rhz2* and *Rhz3*, respectively (Hu, 2001; Hu, 2003).

FIGURE 4. MOLECULAR GENETIC MAP OF RD23_LONGI BY SSR MARKERS

PCR-BASE MOLECULAR GENETIC MAP

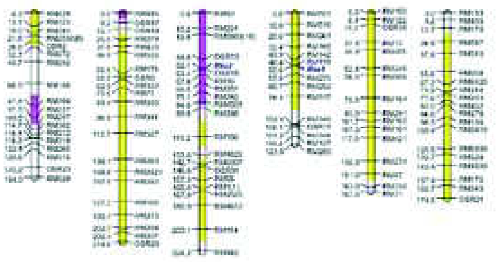
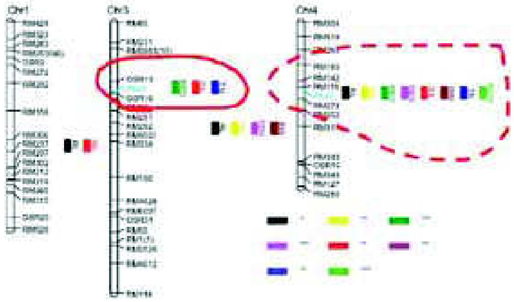


FIGURE 5. QTLs ANALYSIS OF RHIZOME RELATED TRAITS

THE QTLS OF RHIZOME TRAITS MAPPING ON CHROMOSOME



Rhz3 on chromosome 4 co-located with many related root traits, including root length, root number, root branching density, root branching number, root internode length, root internode number, tiller number and root dry weight (Figure 5). Likewise, *Rhz2* on Chromosome 3 was co-located with root branching density, root internode length and tiller number, with the other traits closely located on the same arm of chromosome 3. The strong association between rhizome QTLs and related root QTLs lends confidence, as rhizome formation should lead to changes in the other parameters (Hu, 2003).

Subsequent fine mapping for *Rhz3* identified flanking markers RM14603 and OSR16 about 35 kb apart for *Rhz2* in 2008, and in 2012, a functional 5 scaffold was determined (Figure 6). Likewise, for Rhz3, the flanking markers were RM119 and RM17000 at 9.528 kb apart, with a 6 scaffold determined (Figure 7).

FIGURE 6. THE *RHZ2* FINE MAPPING

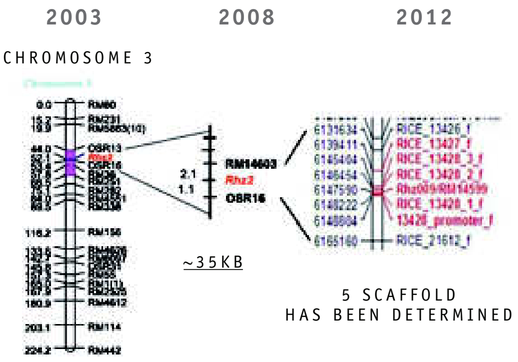
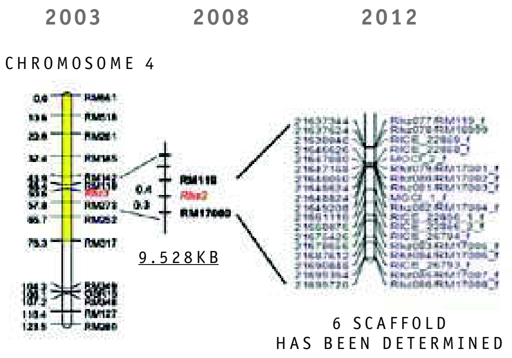


FIGURE 7. THE *RHZ3* FINE MAPPING



2. Breeding of perennial rice

Materials were initially evaluated and selected in the greenhouse, before being evaluated and selected in several field environments. Sanya on Hainan Island in the south of China was used from 2007 to 2010 to allow rapid generation advance under tropical lowland paddy conditions, as two crops per year were possible there. From 2011, however, the breeding station was moved from Sanya to Jing Hong in southern Yunnan Province, with additional field sites established on different soils at Puer and Menglien, which were perceived to be more appropriate environments for perennial rice.

Field evaluation demonstrated that plants were able to regrow successfully in the field for at least three seasons under these conditions, demonstrating not only regrowth and survival, but also reproductive success and grain set in the field.

The outstanding line was PR23 derived from RD23/ *O. longistaminata*, although PR57, PR129 and PR137 were also promising. Field performance of PR23 is after grain harvest in Simao, during regrowth in Simao and Jing Hong, and as a mature crop close to harvest in its third year in Jing Hong (Figure 8). A close-up of PR23 regrowth relative to senesced stems cut in the previous year is shown (Figure 9), along with a close-up of dry season survival at Puer (Figure 10).

FIGURE 8. THE PR LINES: PR23

The phenotype of PR23 in Jinghong, 2012/5 during Len Wade visiting there

FIGURE 9. THE REPRODUCTION ABILITY OF PR23


RE-GROWTH FROM UNDERGROUND

THE OLD STEMS

The growth situation of PR23 after cutting the stub back to 10-15 cm 2 months in Jinghong (The third growth season)

FIGURE 10. THE REPRODUCTION ABILITY OF PR23


PR23 leave in field for next season growth 2011/12, Simao, Yunnan

TABLE 2. YIELD VARIATION OF PR23 BETWEEN DIFFERENT GROWTH SEASONS AND SITES

CROP SEASON	PHENOLOGICAL STAGE	YIELD OF PER UNIT AREA (KG/HA)	DEATH RATE (OVER YEAR)	SITE
First growth season	2011/1/10-2011/6/10 150d	5619.83		Jinghong, Yunnan, China
Second growth season	2011/6/13-2011/10/22 131d	3905.05		
Third growth season	2012/2/10-2012/6/5 116d	4027.57[a]	3.5%	
First growth season	2011/3/7-2011/8/10 157d	7350.00		Simao, Yunnan, China
Second growth season	2011/8/30-	The temperature was too low that the grain setting rate was not very good		
Third growth season	2012/3/28-2012/8/15 137d	6720.00	5.4%	

The yield performance of PR23 perennial rice over three seasons in the field at Jing Hong and Simao is shown in Table 2. Yields from the first wet season were impressive at 5.6 and 7.4 tonnes/ha. Not surprisingly, yields were lower in the drier second season when temperatures were lower in winter. Survival in the second wet season was impressive, with about 5 percent loss in plant stand over the first year. Nevertheless, wet season yields in the second year declined to about 70 and 90 percent of yields in the first wet season, respectively, which may reflect different seasonal conditions, some decline in plant vigour, or both. What factors may contribute to such changes needs further investigation, even though yields were still impressive in season three at 4.0 tonnes/ha (with some rat damage) and 6.7 tonnes/ha.

As a result of the strong performance of PR23 in the field, and farmer and district interest at the field sites, PR23 has entered pre-release testing for release as a perennial rice cultivar in Yunnan Province. At this stage, an individual farmer can try a small area, but a further three years of field evaluation data at several sites in Yunnan is needed before formal release of the cultivar, and any approval to increase and release seed to farmers. Nevertheless, the entry of PR23 to pre-release testing in Yunnan is a first for the perennial crops community. While some perennial species have been improved, and some fortuitous discoveries of unknown origin have been identified and grown by a few farmers, this is the first report of the intentional selection of a perennial cultivar from a cross with a related wild perennial species. This represents a milestone in combining a capacity to regrow with a capacity to set grain in subsequent generations. Hopefully, this heralds the advent of further breeding success in the perennial grains community.

3. Collaborations in perennial rice

In 2011, collaboration was established in neighbouring Lao, PDR via the ACIAR project on developing improved farming and marketing systems for rainfed regions of southern Lao PDR (Wade and Sengxua, 2014). A set of 13 recombinant inbred lines from the cross RD23/ *O. longistaminata* was planted at Xepon in Savannakhet Province and Phone Ngam in Champassak province in southern Lao PDR (Figure 11). The lines performed well in the first wet season (Table 3), but there was some stand loss in the harsher conditions encountered on light-textured soils in southern Lao, PDR, despite life-saving applications of water during the dry season. Some plants of most entries did regrow at both sites in the second wet season, but at one site, the farmer allowed livestock to graze at break of wet season, and heavy rain and flooding shortly thereafter resulted in total crop loss at Xepon. At the other site, the crop survived the typhoon, but plant vigour was affected and greater weed competition resulted, so yields at Phone Ngam were much lower in the second wet season (Table 3). A second set of 22 recombinant lines has now been sent to Lao, PDR for evaluation, and with the experience provided by this initial attempt, should be conducted with better management next time. Nevertheless, the capacity to regrow and produce forage and grain in the second year was recognised by the collaborators, who wish to continue this research in perennial rice. The plant breeders in particular were keen to collaborate in this research.

FIGURE 11. PERENNIAL RICE LINES GROWN IN XEPON, SAVANNAKHET AND PHONENGAM, PAKXE, CHAMPASAK, FOR ONE (2011) AND TWO (2011-2012) YEARS, RESPECTIVELY

TABLE 3. ANALYSIS OF VARIANCE OF GRAIN YIELD (g/m^2) OF 13 PERENNIAL RICE LINES GROWN IN XEPON, SAVANNAKHET AND PHONENGAM, PAKXE, CHAMPASAK, FOR ONE (2011) AND TWO (2011-2012) YEARS, RESPECTIVELY

SOURCE	df	TYPE II SS	MS	F	P
MAIN EFFECTS					
Year	1	1172206.515	1172206.5	1138.8705	.0000***
Site	1	413.1565707	413.15657	0.4014069	.5283 ns
Entry	12	140679.6121	11723.301	11.389905	.0000***
INTERACTION					
Year x Entry	11	39082.49769	3552.9543	3.4519128	.0007***
Site x Entry	11	30768.72232	2797.1566	2.717609	.0053**
Error	74	76166.06598	1029.2712<-		
Total	110	1893016.645			
Model	26	1816850.579	50468.072	49.032824	.0000***

R^2 = SSmodel/SStotal = 0.95976471407

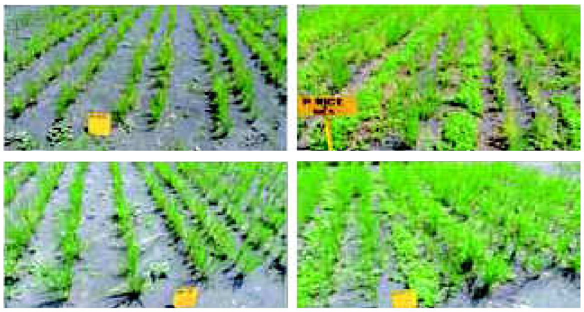
Root MSerror = sqrt (MSerror) = 32.0822561844

Mean Y = 188.996693703

Coefficient of Variation = (Root MSerror)/ abs (Mean Y) * 100% = 16.975036%

A related evaluation conducted in Nairobi, Kenya has confirmed the initial set of materials could not cope well with dry periods and their associated water deficits (Figure 12). Further research is needed to develop perennial rices able to cope with the severity of abiotic stresses under rainfed conditions, including drought in rainfed lowland and especially rainfed uplands. An optimistic note was provided by plant survival after three seasons on rainfall alone at Puer. The soil there is heavier with greater water-holding capacity, and temperatures in the dry season are cooler at higher elevation, so water loss is reduced, and plants survived. This observation lends confidence to the prospect for further improvements in dry season survival.

FIGURE 12. THE PERENNIAL ABILITY TEST OF PR23 IN AFRICA



DES: DESMODIUM
did not respond well to extreme drought conditions

CONCLUSIONS

A successful perennial rice breeding program has been established at Yunnan Academy of Agricultural Sciences, with the line PR23 now in pre-release testing in Yunnan Province. This success is based on a strategy of intercrossing F_1 lines, backcrossing to the cultivated rice parent, and rigorous selection for survival and seed set in the field. Development of perennial rice is consequently at the forefront of perennial grain development, and will hopefully act as an incentive to success in other species. The time is ripe to build on this success by establishing a consortium of perennial crop researchers, supported by a suite of donors to ensure the continuity of efforts needed for success in this challenging but important endeavour. Despite some success in developing a perennial rice phenotype which may be suitable for more favourable lowland conditions in which abiotic stresses are minimal, significant challenges remain in developing a robust perennial rice for the harsher rainfed lowland and especially upland ecosystem, where perennial rice is really needed.

ACKNOWLEDGEMENTS

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04

PERENNIAL WHEAT BREEDING: CURRENT GERMPLASM AND A WAY FORWARD FOR BREEDING AND GLOBAL COOPERATION

Philip J. Larkin[1, 2], *Matthew T. Newell* [2, 3]

1 CSIRO Plant Industry, GPO Box 1600, Canberra, ACT 2601, Australia. Email: Philip.larkin@csiro.au
Corresponding author, phone (+61) 2 6246 5060
2 Future Farm Industries Cooperative Research Centre, 35 Stirling Highway, Crawley WA 6009, Australia
3 New South Wales (NSW) Department of Primary Industries, Cowra Agricultural Research and Advisory Station, PO Box 129, Cowra, NSW 2794, Australia. Email: matt.newell@dpi.nsw.gov.au

ABSTRACT

The Australian perennial wheat team has collected and assessed a wide range of global germplasm derived from wheat x perennial-relative crosses (Hayes *et al.* 2012 and unpublished). Some lines were able to perenniate in one or both field environments where they were grown and a few were able to regrow through four seasons. Without exception the only wheat-derived lines that could perenniate contained seven or more pairs of chromosomes from the perennial parent. The donor perennial parents included *Thinopyrum ponticum* (2n = 70, decaploid), *Th. intermedium* (2n = 42, hexaploid) and *Th. elongatum* (2n = 14, diploid). This has led us to conclude that the best near-term prospect for a perennial wheat-like grain crop is a full or partial amphiploid, containing the full set of tetraploid (AABB) or hexaploid (AABBDD) wheat chromosomes plus one genome (XX) from the donor. When the perennial donor is a polyploidy, the extra genome is usually a synthetic genome, consisting of a mixture of chromosomes from the polyploid parental genomes. This creates a significant difficulty in that each time a primary partial amphiploid is produced, the synthetic genome may consist of a different mix of donor chromosomes, and therefore interbreeding of these primary partial amphiploids may result in poor fertility and loss of the perennial donor chromosomes. That, in turn, would be problematic in trying to establish a breeding program. Consequently we would advocate a breeding program based on a diploid perennial donor, such as *Th. elongatum* (EE). This is analogous to triticale breeding. The amphiploids produced could be AABBEE (analogous to hexaploid triticale, AABBRR) or AABBDDEE (analogous to octoploid triticale, AABBDDRR). We would further advocate a multinational effort to produce many primary amphiploids using locally adapted wheat parents and diverse accessions of *Th. elongatum*; the primary amphiploids would be shared among participating groups, and intercrossed to permit subsequent selection of robust, productive, locally adapted perennials. The analogy with triticale is instructive also in suggesting that many generations of selection may be required before full fertility, high yielding types can be recovered.

Keywords: amphiploidy, genomic changes, perennial wheat, rye, *Thinopyrum*, triticale

INTRODUCTION

This paper explores the question of the preferred breeding route to a successful wheat-like and wheat-derived perennial cereal. Other papers in the Workshop will explore related aspects such as the physiology and field performance of available perennial wheat germplasm and how perennial wheat might usefully fit into a mixed farming situation. Some of that information is assumed as a starting point for this consideration of the best genetic configuration for a perennial wheat and how to instigate a breeding program to move us from basic proof of principle to genotypes that can be successfully and usefully deployed into real farming systems.

LIKELIHOOD OF A PERENNIAL TRUE WHEAT

Our studies of the available germplasm have established the essential feasibility of the concept of a perennial wheat (Hayes *et al.* 2012 and unpublished). Lines were found that could regrow after grain harvest for up to four seasons; simulated grazing of several lines showed an ability to produce both valuable forage and grain; and in addition we could demonstrate their ability to accumulate much greater root biomass after the first season as an indicator of the environmental benefit that could accrue (Larkin *et al.* 2014). The grain quality of these lines even had potential for bread making, and we along with others have also demonstrated the disease resistances that had been contributed from the donors of perenniality (Cox *et al.* 2002; 2005; Murphy *et al.* 2009; Hayes *et al.* 2012). Despite this promise, few of the lines were developed from adapted wheat species and their grain yields were generally low and declining in subsequent years. The available germplasm does not meet the standard required for robustness of the perennial habit or the consistency of grain yield from one season to the next. What we concluded for the Australian environment, Murphy *et al.*(2010), Cox *et al.*(2010) and Jaikumar *et al.*(2012) concluded for North America, namely that better adapted and more productive germplasm is required. For decades perennial wheat breeding attempts in the United States and the former USSR held some hope that it might be possible to introgress the controlling genes for the perennial habit into the wheat genomes through recombination or chromosomal translocation. Indeed Lammer *et al.* (2004) gave impetus to this hope when they showed some capacity to regrow in the wheat cv. Chinese Spring, carrying an extra pair of 4E chromosomes from *Thinopyrum elongatum*, a perennial *Triticeae* species. However, the ability of this chromosome addition line to regrow and set seed a second time was not as strong as the perennial amphiploid progenitor, which itself was not very robust. Perenniality appears to be a polygenic trait (Zhao *et al.* 2012), and it will not be readily conferred by simple introgression from a perennial to an annual species. Indeed the likely genetic complexity of the perennial habit suggests the possibility that it might be easier to transfer domestication traits to an existing perennial *Triticeae* species. These traits would include non-shattering heads, free-threshing grain, more determinate flowering and larger seed size. The genetic controls for some domestication traits are relatively simple (Faris *et al.* 2003; Sang *et al.* 2009; Gegas *et al.* 2010; Takahashi and Shimamoto, 2011; Peng *et al.* 2011). Others such as threshability may be multigenic (Peleg *et al.* 2011).

PERENNIAL GRAIN BY DOMESTICATION

In Australia some herbaceous native legumes have been investigated as having potential as perennial grain crops through a process of domestication (Bell *et al.* 2012). Attempts have been made to commercialize grasses such as *Microlaena stipodies* and *Distichlis palmeri* as perennial grain crops (O'Neill, 2007; Kasema *et al.* 2010; Pearlstein *et al.* 2012). However, this has met with limited success. For the Australian native rice-related perennial grass *M. stipoides,* the major missing traits included synchronous maturity and resistance to shattering (Davies *et al.* 2005). The seeds are structurally similar to rice and some ecotypes have seeds almost as large as domesticated rice. Malory *et al.* (2011) characterized 18 genes from *Microlaena* which are homologues of rice genes known to be important in domestication. If successful, domestication of wild perennial grains will produce crops, which are only likely to have value for niche markets in the medium term. Lower flour yields and higher bran and fibre content per kernel are often an impediment to acceptance in standard milling markets for new domesticated grains (Bell *et al.* 2010). We will hear more from others at this meeting on the prospects for domesticating perennial species.

The Land Institute is also making good progress in direct domestication of a number of perennial species including the wheat relative *Thinopyrum intermedium* (kernza) (DeHaan *et al.* 2005; Van Tassel *et al.* 2010; Culman *et al.* 2013). What might be needed to advance success more rapidly down this domestication option is to utilize the expanding understanding of domestication genes in a more targeted way. Transgenic technology offers a powerful way to test the utility of specific genes in achieving domestication traits. Thereafter, if the course to commercial release is deemed too difficult for the transgenic plants, the same traits might be pursued through targeted mutations using technology such as TILLING or one of the emerging methods for site-directed genome modifications (Curtin *et al.* 2012). Genome editing technology employs sequence-specific nucleases to generate site-specific double-stranded Deoxyribonucleic Acid (DNA) breaks. Nuclease systems in development include: zinc finger nucleases; transcription activator-like effector nucleases (TALENs); and LAGLIDADG homing endonucleases (Curtin *et al.* 2012). The site-specific double strand DNA breaks are followed by informed and designed mutations, sequence insertions or replacements. Rapidly evolving genomic and molecular genetic technology may make rapid domestication of existing well-adapted perennials feasible, but not without substantial investment in the various species of interest to this Workshop and for the goal of a perennial grain crop.

PERENNIAL CEREAL GRAIN THROUGH HYBRIDISATION AND ALLOPOLYPLOID FORMATION

Hybridisation between annual grain crops and perennial relatives offers an avenue to combine the traits of perenniality and grain quality in a new crop species. We believe this would reduce the time of development, compared with domestication, and offer a product that might directly replace annual grains in the market place. There have been attempts to achieve this historically in Russia and the United States (Tsitsin and Lubimova, 1959; Suneson *et al.* 1964). Experimental breeding lines of hybrid perennial cereals have shown higher mineral and protein content than annual cultivars (Murphy *et al.* 2009) and some have demonstrated useful flour, dough and baking properties (Hayes *et al.* 2012). Perennial grains will need to be profitable if they are to be adopted widely in agriculture (Bell *et al.* 2008; Reeling *et al.* 2012) and direct access to already established markets will be important for the acceptance of perennial grains.

The rest of this paper focuses on wide hybridisation to combine perenniality with already domesticated wheat. Our experience thus far consistently indicates that reasonable rates of post-harvest regrowth (PHR) are only observed when many chromosomes are added to wheat from the perennial donor species (Hayes *et al.* 2012 and unpublished). The germplasm examined was derived from wide crosses between wheat and perennial species such as *Th. intermedium, Th. elongatum,* and *Th. ponticum*. Figure 1 is a plot of many of the hybrid derivatives, partial amphiploids and amphiploids that we have examined in the field, showing the relationship between chromosome counts and ability to regrow after the first grain harvest. This figure is based on a similar figure in Hayes *et al.* (2012), but with some new lines and field data added. Some lines were able to perenniate in one or both field environments where they were grown and a few were able to regrow through four seasons (Table 1). The wheat-derived lines that were capable of perennial regrowth contained seven or more pairs of chromosomes from the perennial parent. In the case of the wheat x *Th. elongatum* derivatives, the exception seems to be CPI147232, which has 42 chromosomes but is likely to have substituted the E genome for one of the wheat genomes, just as in the durum wheat x *Th. elongatum* amphiploid (Figure 1A). The situation is even clearer for wheat x *Th. intermedium*, wheat x *Th. ponticum* and wheat x unknown *Agropyron sp.* derivatives, where only 56 chromosomes lines show significant perenniality (Figure 1B). Individual *Triticeae* genomes have seven pairs of chromosomes, and therefore, the chromosome constitutions of stable allopolyploids, both naturally occurring and synthetic amphiploids, stabilise at multiples of 14. Many fertile and stable wheat derived amphiploids have been formed at the octoploid level (2n = 56; Mujeeb-Kazi and Hettel, 1995; Jauhar, 1995; Sepsi *et al.* 2008), and it seems this is a benchmark of stability as well as a general requirement for retaining perenniality. Figure 1 also illustrates that although the wheat hybrid derivatives do not regrow with the same consistency as perennial benchmarks such as *Th. intermedium* or *S. montanum*, some do well.

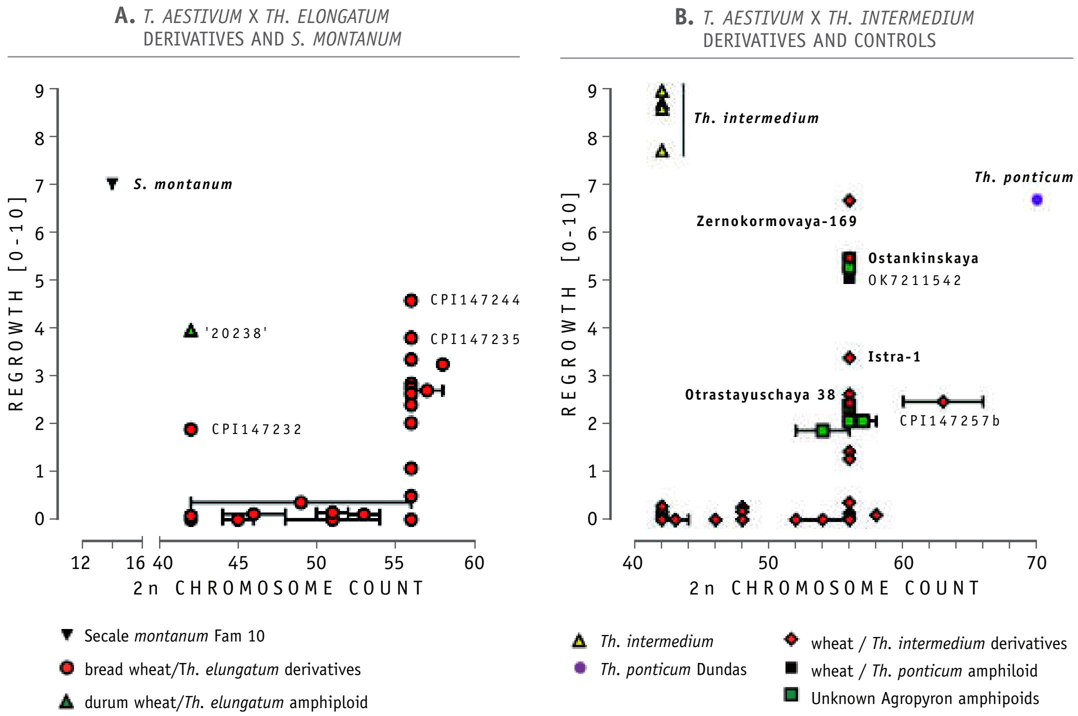
TABLE 1. SUCCESSIVE GRAIN YIELDS OF HYBRID WHEAT DERIVATIVES FROM WHEAT X *TH. ELONGATUM* OR WHEAT X *TH. INTERMEDIUM*, AND THE PERENNIAL GRASS *TH. PONTICUM*, GROWN IN AUSTRALIA

The weights are means of three replicate 1 m rows. Decline in yield, of the hybrids, over time was due to plant mortality within populations.

	GRAIN wt (g) 2008	GRAIN wt (g) 2009	GRAIN wt (g) 2010	GRAIN wt (g) 2011
CPI 147235a	73.5	25.9	0.0	0.0
CPI 147236a	81.7	45.9	11.2	0.0
CPI 147236b	65.9	8.9	0.0	0.0
CPI 147251b	70.9	9.4	0.5	0.4
CPI 147257b	7.8	12.6	4.7	0.7
CPI 147258a	4.7	0.3	0.3	0.0
CPI 147286a	36.3	0.4	0.0	0.0
Th. ponticum	0.0	9.4	207.7	268.8
lsd	**45.7**			

FIGURE 1. CHROMOSOME COUNTS (2) AND POST-HARVEST REGROWTH (PHR) SCORE IN THE FIELD FOR A RANGE OF CONTROL PERENNIAL SPECIES AND WHEAT HYBRID DERIVATIVES

The bars show the range where variable chromosome counts were observed. Some of the data plotted are from Hayes *et al.* (2012).
A. shows mainly wheat x *Th. elongatum* derivatives.
B. shows mainly wheat x *Th. intermedium* derivatives.



A. *T. AESTIVUM* X *TH. ELONGATUM* DERIVATIVES AND *S. MONTANUM*

B. *T. AESTIVUM* X *TH. INTERMEDIUM* DERIVATIVES AND CONTROLS

Perennial amphiploids or partial amphipoids have been produced between wheat and *Th. ponticum* (2n = 70, decaploid) (Berezhnoi, 1987; Zhang *et al.* 1996; Chen *et al.* 1998), *Leymus arenarius* (2n = 56, octoploid) (Anamthawat-Jonsson, 1995), *Th. intermedium* (2n = 42, hexaploid) (Tsitsin and Lubimova, 1959; Cauderon, 1966; Sun, 1981; Jones *et al.* 1999; Cox *et al.* 2002) and *Th. elongatum* (2n = 14, diploid) (Jenkins and Mochizuki, 1957; Rommel and Jenkins, 1959; Cai *et al.* 2001; Murphy *et al.* 2007; Mujeeb-Kazi *et al.* 2008). Taken together, this experience suggests that the best near-term prospect for a perennial wheat-like grain crop is a full or partial amphiploid, containing the full set of tetraploid (AABB) or hexaploid (AABBDD) wheat chromosomes plus one genome equivalent (XX) from the donor. If the perennial donor is a polyploidy, the extra genome in the allopolyploid (partial amphiploid) is usually a synthetic genome. In this context, "synthetic genome" means one consisting of a mixture of chromosomes from the multiple perennial parent genomes, but where each of the seven homologous chromosome groups are represented in the synthetic genome. If each of the homologous groups are not represented the synthetic genome is unstable. So, while a partial amphiploid with a synthetic genome may be stable, a significant difficulty results for any ongoing breeding program. Each time a primary partial amphiploid is produced, the synthetic genome may consist of a different mix of donor chromosomes (Jones *et al.* 1999; Han *et al.* 2004; Liu *et al.* 2005), and therefore interbreeding of primary partial amphiploids often will result in a loss of the donor chromosomes, genetic instability and consequent likely loss of the perennial trait. Banks *et al.* (1993) amply demonstrated this problem through experiments in which a set of independent primary wheat-*Th. intermedium* partial amphiploids (2n = 56) were inter-crossed. We would argue that the ability to inter-breed from independent primary amphiploids is essential to enable a practical rate of breeding progress with hybrid perennial wheats. Robust perennials might be achievable with partial amphiploids using a polyploidy perennial donor; some of the best performing lines in our experiments have derived from polyploid donors such as wheat x *Th. ponticum* (10x) derivatives or wheat x *Th. intermedium* (6x) (Figure1B). However, substantial progress will be made best, if ongoing breeding programs can be established through developing genetically diverse primary amphiploids, inter-crossing them to generate large scale genetic segregation on which to impose heavy selection.

Consequently, although perennial allopolyploid *Triticeae* might make good donors for primary partial amphiploids, we believe the best prospects for a productive breeding program in the medium term should focus on a diploid perennial donor such as *Th. elongatum* (Host) D. R. Dewey (EE, 2n = 2x = 14, synonyms *Lophopyrum elongatum, Elytrigia elongata, Elymus elongatus, Agropyron elongatum*). The Washington State University (WSU) program used this donor species introduced as the Chinese Spring wheat x *Th. elongatum* amphiploid they called AgCs (Jenkins, 1957; Cai *et al.* 2001). It is helpful to consider the analogy with the breeding of the human-made cereal triticale. If a tetraploid wheat is used, the amphiploid would be AABBEE (analogous to hexaploid triticale, AABBRR); if a hexaploid wheat is used, the amphiploid would be AABBDDEE (analogous to octoploid triticale, AABBDDRR). Lelley (2006) gives a useful summary of the history and breeding of triticale. The first fertile triticale, an octoploid, was produced by Rimpau in 1888. It was only in the 1930s, that induced chromosome doubling techniques using colchicine enabled routine production of fertile primary triticales. Research switched to hexaploid triticales in the 1950s, and progress was made by extracting secondary hexaploids from crosses between hexaploid and octoploid triticales. The International Maize and Wheat Improvement Center (CIMMYT) achieved another step forward by producing hexaploid cv. Armadillo carrying a 2D (2R) chromosomal substitution, though subsequent developments have shown that the full potential of triticale is best served with a full rye genome and without substitutions. Decades of breeding and selection have been required to produce high yielding triticale cultivars. There is evidence that during this time genomic sequence modifications were important (Ma and Gustafson, 2008; Tang *et al.* 2008; Ozkan and Feldman, 2009; Feldman and Levy, 2012). The observed changes were mainly losses of Amplified fragment length polymorphism (AFLP) and Restriction fragment length polymorphism (RFLP) bands, predominantly from the rye genome and largely involving repetitive DNA sequences. A positive relationship was found between increased chromosome bivalent pairing in meiosis, improved fertility and elimination of low-copy non-coding DNA sequences (Ozkan and Feldman, 2009). These chromosomal rearrangements may have facilitated the wheat and rye genomes coordinating the timing of their division processes during meiotic division. Both genetic and epigenetic changes have been demonstrated to occur in newly synthesized allotetraploid wheat lines with diverse genome compositions (Qi *et al.* 2010). Some of the changes seemed to be required, which they called *directed*, while others were highly variable, which they called *stochastic*. Feldman and Levy (2012) prefer the word *revolutionary* to describe rapid changes required in new allopolyploids. The changes observed included non-random loss of non-coding, low- and high-copy sequences, resulting in a DNA loss of 2-10 percent compared with the sum of the parental DNA contents. In the case of triticale, the loss was about 9 percent in octoploid triticale and 28-30 percent in hexaploid triticale, compared with the wheat and rye parental DNA. Intergenomic invasion by sequences such as transposons, and reduction in repeats of Ribosomal ribonucleic acid (rRNA) genes are also noted as revolutionary genetic changes occurring rapidly after allopolyploidisation. Epigenetic (DNA methylation) changes were also observed. More recently Hu *et al.* (2012) looked specifically at newly formed wheat x *Th. elongatum* amphiploids using genome specific molecular markers and found chromosome rearrangements and sequence duplications occurring. Interestingly, the whole genome shot-gun sequencing of wheat has revealed the apparent dynamism of hexaploid wheat genomes, in which there has been significant loss of members of multigene families during polyploidization and domestication (Brenchley *et al.* 2012).

Most of the wheat x *Th. elongatum* amphiploids we have examined have been from WSU and are octoploid (2n = 56, presumably AABBDDEE). However, one of the perennial WSU lines, CPI147232, is hexaploid (2n = 42). Its genomic composition needs to be confirmed.

Another hexaploid amphiploid is derived from cv. Stewart (*T. turgidum*) x *Th. elongatum* line (AABBEE) (Jenkins and Mochizuki, 1957). This hexaploid is too tall but should be crossable to CPI147232, provided the latter is also AABBEE, in which case the progeny should segregate to form populations at the hexaploid level useful for selections. Almouslem and Amleh (1999) also report a durum wheat x *Th. elongatum* hybrid. The greater genomic proportion of the perennial genome to annual genomes may confer a more dominant perennial habit; however this remains to be tested.

More recently we have obtained new wheat x *Th. elongatum* amphiploids at the octoploid level from CIMMYT, in particular two lines using wheat cv. Goshawk (Mujeeb-Kazi *et al.* 2008). These had good semi-dwarf stature, strong straw and reasonable PHR in the greenhouse. However, fertility was reduced and the heads were very susceptible to shattering (Larkin, unpublished). Shattering was not a problem with the perennial wheat *Th. elongatum* amphiploids from WSU. Crosses between the two types have been successful. And hopefully will allow the recovery of progeny with non-shattering heads, but with semi-dwarf stature and improved fertility. *Th. elongatum* accessions are available from dry and hot locations such as Israel, North Africa and Mediterranean France. Although this diploid species is not endemic in Australia, some accessions may exhibit the type of summer survival and persistent perenniality required. These should be exploited to make new primary amphiploids with adapted annual wheats.

Th. elongatum is not the only perennial diploid that might serve as the donor of the perennial habit to wheat. It is noteworthy that most species of the *Triticeae* are perennial (Barkworth *et al.* 2009) with about ten basic genome types represented, including *Australopyrum retrofractum* (W), *Agropyron cristatum* (P), *Thinopyrum bessarabicum* (Eb), *Dasypyrum villosum* (V), *Psathyrostachys hushanica* (Ns), *Pseudoroegneria spicata* (St); and wheat can be hybridised to some of these species. So there should be many other possibilities for generating perennial amphiploids. The diploid *Australopyrum* species (2n = 2x = 14, WW) might appear to be an attractive donor of perenniality in an Australian context. However, there are no reports of hybrids with wheat. Furthermore, *Australopyrum* species lack the broader adaptation to various climatic regions of southern Australia associated with the other native perennial *Triticeae* species, *Elymus scaber*.

Some attention has been given to producing a perennial triticale derived from perennial rye, *Secale montanum,* instead of cereal rye to combine with wheat. Schlegel (1980) produced F1 hybrids and amphiploids from wheat x *S. montanum* crosses; the amphiploids at meiosis had an average of 26.55 bivalents compared with 27.30 in an established octoploid triticale, suggesting a reasonable genetic stability early in the breeding cycle. There was a correlation between the amount of telomeric rye heterochromatin and the frequency of univalents in meiosis, suggesting the heterochromatin difference between the wheat and rye chromosomes was an impediment to full fertility. Delayed DNA replication at the heterochromatic telomeres may be a problem for coordinating the timing of wheat and rye chromosome division processes during meiotic division. The reduced amount of telomeric heterochromatin in *S. montanum* relative to *S. cereale* is postulated to explain why wheat *S. montanum* amphiploids were more regular in pairing than primary wheat *S. cereale* amphiploids (Thomas and Kaltsikes, 1974; Schlegel, 1980). Established fully fertile triticales have lost much of the telomeric heterochromatin on the rye chromosomes. This is another example of a genetic change required to enable newly formed allopolyploids to be fully fertile.

There has been some success in breeding perennial rye itself as a grain crop through intercrossing *S.cereale* and *S. montanum* and the release of tetraploid cvs. Permontra and Sopertra, diploid cv. Benmonta in Germany (Reimann-Philipp, 1995), and diploid cvs. Perenne and Kriszta in Hungary (Kotvics *et al.* 2001; Füle *et al.* 2005; Sipos and Halasz, 2007). In Canada, cv ACE-1 was developed by selection from German germplasm (Acharya *et al.* 2004). Initial attempts had difficulties with fertility and retention of perenniality. Success came only after decades of selection. In Australia, a perennial rye cv. Black Mountain was bred from a *S. cereale x S. montanum* cross with two backcrosses to the perennial parent; selection for this release was based mainly on perenniality and forage yield, rather than grain yield. Following cell culture and six generations of selection for fertility, non-shattering heads and grain yield, a higher grain yield perennial selection was recovered called Family 10 (Oram, 1996; personal communication, 2010).

CONCLUSIONS

With the increasing power and reach of genomic analyses, greater genetic insight will come eventually into the genes controlling the perennial habit. Transcriptomic comparisons between annual and perennial *Brachypodium* species, being arguably the closest model species to wheat, might prove particularly informative. Other potentially informative comparisons include annual and perennial rice (*Oryza rufipogon*) (Zhao *et al.* 2012), *Sorghum bicolor* and *S. halepense*, *Hordeum vulgare* and *H. bulbosum, Panicum miliaceum* and *P. turgidum*. Eventually such studies could lead to the ability to engineer perenniality into wheat through transgenics or genome editing. In the meantime we would contend that the best near term prospect of a productive breeding program for a perennial wheat-derived cereal will involve the following steps:

1. The generation of many primary amphiploids between wheat and a perennial diploid such as *Th. elongatum*. Importantly, this should involve a diversity of *Th. elongatum* accessions and a diversity of annual wheat cultivars adapted to various target zones globally.
2. Intercrossing primary amphiploids and advancing segregating populations with selection.
3. Early generation selection at F2 to F4 would emphasise traits such as semi-dwarf plant height, non-shattering heads, stable amphiploid chromosome count, large seed size, good self-fertility, and regrowth in pots.
4. Later generation selections would emphasise traits such as maturity, robust post-harvest growth, grain yield, forage yield, stability of grain yield across seasons, and disease resistance.

In addition to a number of rounds of such breeding and trait selection cycles, progress might be further accelerated through specific attention and selection for the *revolutionary* genetic changes so frequently observed in newly forming allopolyploids (Feldman and Levy, 2012), that signal the accommodation between genomes and the rise in fertility and stability. While triticale took over 40 years to begin to deliver on its potential, the time required to deliver a successful perennial wheat might be greatly compressed through a coordinated international effort and application of our greater understanding of the genomic changes required before a newly formed allopolyploid becomes stable and productive. International cooperation could be encouraged through the formation of a *community-of-practice* with mutually agreed *open-source* style terms of engagement. We already have the beginnings of such a community. High priority should be given to collecting diverse accessions of diploid *Th. elongatum*, crossing to wheat and forming amphiploids, sharing diverse primary amphiploids, intercrossing to form secondary amphiploids, and coordinated multi-environment testing of derived secondary amphiploid populations.

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05

EVALUATION OF NINE PERENNIAL WHEAT DERIVATIVES GROWN IN ITALY

Norberto E. Pogna, Elena Galassi, Roberto Ciccoritti, Ester De Stefanis, Daniela Sgrulletta, Pierino Cacciatori, Laura Gazza, Alessandro Bozzini

Consiglio per la Ricerca e la sperimentazione in Agricoltura, Unità di Ricerca per la Valorizzazione Qualitativa dei Cereali (CRA-QCE), Rome, Italy

ABSTRACT

As part of an international network coordinated by the Australian NSW Department of Primary Industries, nine lines of perennial wheat obtained from crosses between *Triticum aestivum* and *Thinopyrum* spp. were grown at Montelibretti (Rome) in randomized blocks with three replications during two years of testing, and compared for their agronomical, nutritional and technological properties with common wheat cultivars (cvs) Wedgetail and Enesco. All perennial genotypes were characterized by post-harvest regrowth (PHR), lateness of ear emergence, small kernels, loose spikes, variable number of seeds/spikes and high number of tillers. In addition, perennial lines had medium test weight, low percentages of hull-less kernels, high protein content, reduced sodium dodecyl sulphate (SDS) sedimentation volume and kernel texture typical of soft or medium-hard wheat. The hard-textured lines showed novel genes coding for puroindolines A and B inherited from wheatgrass (*Thinopyrum* spp.). Analysis of single seeds revealed a marked inter- and intra-line variation for gliadins and HMW-glutenin subunits (HMW-GS). The total content in bioactive compounds 5-n-alkylresorcinols and soluble polyphenols (SP) was high in perennial lines compared with their annual counterparts. Furthermore, perennial lines exhibited high yellow pigment content and resistant-starch percentage. The poor gluten quality of some perennial lines was associated with the presence of prolamins inherited from the wheatgrass parent and the absence of high-quality, HMW-GS from the wheat parent. Evidence was obtained that chromosome substitution or allosyndetic recombination between E-genome and ABD-genome chromosomes likely occurred in some perennial lines with *Th. elongatum* in their pedigree. The perennial genotypes were found to be valuable for their PHR potential and nutritional value. However, they deserve closer attention for some negative agronomical and quality traits.

Keywords: bioactive compounds, gluten quality, perennial wheat, puroindolines, storage proteins

INTRODUCTION

In the last few decades, one third of Earth's arable land has been lost due to erosion (Pimentel *et al.* 1995) and the production systems based on annual grain crops such as wheat, maize, rice and soybean have been considered among the primary causes of this soil erosion (Glover, 2005). By contrast, the high productive potential and the efficient use of natural resources such as light, water, CO_2, nitrogen compounds and minerals by perennial plant communities (Crews, 2005) suggest that perennial grain crops could meet the increasing demands of food while reducing soil erosion. Moreover, perennial grain crops are seen as an opportunity to improve water, minerals and fertilizers management, while increasing biodiversity, underground biomass and carbon sequestration in the soil (DeHaan *et al.* 2005; Jordan *et al.* 2007). Perenniality seems to be under the control of multiple genes responsible for specific biological structures such as bulbs, rhizomes and meristems, as well as for physiological traits such as resistance to cold, drought and biotic stresses. Therefore, transformation of annual crops into perennial crops with high grain yield, coupled with superior technological and nutritional quality could turn out to be a very demanding and challenging goal. In addition, annual species supply much of the photosynthetic energy for seed development, whereas perennial species allocate a proportion of the photosynthate to their roots and green tissues late in the growing season, after the annuals have senesced. This "energy tradeoff" between grain and perennating structures would result in decreased grain production per hectare of perennial species as compared with their annual counterparts (Wagoner, 1990). However, the biological superiority of annual species in producing a high amount of seed could be the result of their evolutionary history and selection, both natural and human-oriented, rather than an integrant consequence of their annual habit (DeWet, 1981; DeHaan *et al.* 2005). On the other hand, in certain conditions, the decreased input costs of a perennial grain crop can make up the difference in profit and provide additional ecosystem services (Bell *et al.* 2008).

Common wheat (*Triticum aestivum*) is the most widespread annual grain crop grown on more than 220 million hectares. Cultivation of this cereal species is claimed to be one of the primary causes of soil erosion, with annual losses of soil as high as 31.5 tonnes/ha in the case of wheat monocultures (Reganold *et al*. 1987).

The earliest forms of perennial wheat were produced in Russia in the second decade of the last century (Tsitsin and Lubinova, 1959), whereas more recent material has been developed by The Land Institute and Washington State University in the United States (Cox *et al*. 2010; Murphy *et al*. 2010). These perennial genotypes derive from crosses between wheat and one of three species of *Thinopyrum*, namely (i) *Th. elongatum* (synonymous *Agropyrum elongatum*), a diploid species ($2n = 2x = 14$) with the E genome, (ii) *Th. intermedium* (synonyms *Ag. intermedium*, *Elymus hispidus* or *Elytrigia intermedia*), an hexaploid species ($2n = 6x = 42$) with the JJ^SS genome, and (iii) *Th. ponticum* (synonyms *Ag. ponticum*, *Elymus elongatus* spp. *ponticus* or *Elytrigia pontica*), a decaploid species ($2n = 10x = 70$) with three copies of the J genome and two copies of the J^s genome. In some cases, tetraploid AB-genome wheat *T. turgidum* ssp *carthlicum* was used as a bridge species with *Th. intermedium*, and the hybrid progeny was crossed with common wheat.

Among 176 wheat genotypes with wheatgrass (*Thinopyrum* spp.) in the pedigree, 107 lines demonstrated some ability to regrow after the sexual cycle PHR over three consecutive years of cultivation in two Australian locations (Hayes *et al*. 2012). As part of an international network of field trials coordinated by the Australian NSW Department of Primary Industries, nine of the wheat x wheatgrass derivatives with a relatively high PHR capacity mentioned above were seeded in Italy in 2011. The present study aimed to compare these lines with two commercial common wheat cultivars for their agronomical, technological and nutritional traits during two years of testing.

MATERIALS AND METHODS

Plant material

Nine perennial wheat breeding lines kindly provided by Richard Hayes (Graham Centre for Agricultural Innovation, NSW, Wagga Wagga, Australia) were grown at Montelibretti (Rome) in the Tiber valley on sandy soil, with an average annual rainfall of 954 mm. Plants were sown in December 2011 in 1 m rows, 0.5 m apart, with 30 kernels/row in a randomized block experimental design with three replications. Two annual common wheat cultivars, the Australian cv. Wedgetail and the Italian cv. Enesco, were used as controls. Perennial accessions of *Secale montanum*, *Hordeum bulbosum*, *Thinopyrum ponticum* and *Th. intermedium* were included in the experiment as well. The full list of entries is given in Table 1. At sowing, 31 Kg/ha nitrogen and 20 Kg/ha of phosphorus were applied in the form of urea and diammonium phosphate. The date of anthesis was recorded and maturity of each genotype was calculated as time between sowing and anthesis. At harvest, length of the main stem and total number of tillers of each plant were recorded and plants were cut at approximately 10 cm from the soil surface. In addition, spikes from the main stems were threshed in a bench micro-thresher to determine percentage of hull-less kernels, number of kernels per spike, 1 000-kernel weight and test weight. Rows were visually monitored for signs of regrowth every four weeks for three months from harvest and each genotype received a PHR score based on the average percentage of the original plant population that was regrowing in the three replicates.

TABLE 1. PEDIGREE, CHROMOSOME NUMBER AND POST-HARVEST REGROWTH (PHR) OF NINE WHEAT X WHEATGRASS DERIVATIVES

WHEAT DERIVATIVES	PEDIGREE AND ORIGIN	NO. OF CHROMOSOMES[c]
235A	*Th. elongatum / T. aestivum*[a] *// T. aestivum*[b] (WSU)	42, 44, 56
236A	*Th. elongatum / T. aestivum*[a] *// T. aestivum*[b] (WSU)	56, 58
244B	*Th. elongatum / T. aestivum*[a] *// T. aestivum*[b] (WSU)	56
251B	*Th. elongatum / T. aestivum*[a] *// T. aestivum*[b] (WSU)	56
280B	*Th. intermedium / T. carthlicum // T. aestivum* (TLI)	56
281B	*Th. intermedium / T. carthlicum // T. aestivum* (TLI)	56
11955	*Triticum* spp./ *Thinopyrum* spp.(USA)	56
OK72	*T. aestivum/Th. ponticum* (USA)	56
OT38	*T. aestivum/Th. intermedium* (ex USSR)	56

a Cv. Chinese Spring
b Cv. Madsen
c According to Hayes *et al.* (2012)
WSU = Washington State University
TLI = The Land Institute

Technological analyses

Analyses were performed on wholemeal from mature kernels of each replication ground with a laboratory mill (Cyclotec, mod. 1093-Tecator/Hoganas, Sweden) equipped with a 1.0 mm sieve. Protein content was determined by micro-Kjeldhal nitrogen analysis (N x 5.7), whereas gluten quality was evaluated by the SDS sedimentation test using a solution of 2 percent sodium dodecyl sulfate as described by the standard method 56-70 (AACC, 1995), and the sedimentation volumes were expressed in milliliters. The sequential extraction of protein in the wholemeal was carried out according to Wang *et al.* (2007). Kernel hardness was evaluated on 50 hull-less kernels by the Perten Single Kernel Characterization System (SKCS) 4100 (Springfield, IL, USA) following the manufacturer's operating procedure. The instrument was set in a range of hardness between -40 and +120.

Puroindoline and storage protein analysis

DNA was extracted from leaves by the cetyltrimethyl ammonium bromide (CTAB) method and puroindoline genes were amplified by PCR as described by Gautier *et al.* (1994). Puroindolines were extracted with 50 mM NaCl and 50 percent (v/v) propan-2-ol from 50 mg of air-dried starch granules as described previously (Corona *et al.* 2001). Fractionation of puroindolines by acidic poly-acrylamide gel electrophoresis (A-PAGE) at pH 3.1 was carried out as described by Corona *et al.* (2001). Gliadins and total proteins were extracted and fractionated by A-PAGE and SDS-PAGE, respectively, as described by Pogna *et al.* (1990).

Extraction of phenolic compounds and alkylresorcinols

Immediately after harvest, grain samples from two replicates were milled with a laboratory cyclone mill (Cyclotec 1093, Foss, Italy) to pass through a 0.5 mm sieve and kept at 4°C until extraction and analysis. All determinations were carried out in triplicate on two independent aliquots of each composite sample. Moisture content was determined at 120°C with a thermobalance (Sartorius MA 40, Gottingen, Germany).

Samples (1 g) of wholemeal were extracted to determine SP compounds and 5-n-alkylresorcinol (AR) content. Samples were placed in 50 mL tubes and extracted with 40 mL acetone for 24 hours by continuous mechanical shaking at room temperature. The extracts were then filtered through a Whatman n.42 filter paper and evaporated to dryness at 60°C in a rotary evaporator (Buchi R-114, Switzerland). The dry residues were then dissolved in pure methanol (1 mL) and immediately analysed. All reagents were of analytical spectrophotometric grade (Carlo Erba, Rome, Italy).

Determination of total soluble phenolic compounds (TSPCs)

SP of wholemeal extracts were determined using the Folin–Ciocalteu (F-C) method as reported by Moore and Yu (2008). SP content was calculated from a calibration curve, using (+) catechin as standard. Results were expressed as micrograms of catechin equivalents per gram of wholemeal sample.

Gas chromatography-mass spectrometry (GC-MS) analysis of alkylresorcinols

Total AR content in wholemeal extracts was determined by GC-MS analysis according to Landberg *et al.* (2009) using methyl behenate as internal standard. The AR extract (10 mL) of each sample was dried under nitrogen and a mixture (400 µL) of pyridine and TMCS (9:1, v/v) was added. The mixture was then shaken and heated at 70°C for 60 min. GC-MS analysis was performed with a Perkin Elmer gas chromatograph GC Clarus 600 series coupled to the mass spectrometer Clarus 580D (Perkin Elmer, Milan, Italy) equipped with a split/splitless injector, a RTX-5MS column (0.25 mm 30 m, 0.25 mm film thickness, Restek, Milan, Italy) and a quadrupole mass spectrometer (Clarus 580D, Perkin Elmer, Milan, Italy) operating in electronic impact (EI) ionisation mode (70 eV). The chromatographic conditions employed were reported by Bellato *et al.* (2013). AR content was determined by comparing the relative retention times with those obtained for a mix of the AR homologue standards C15:0, C17:0, C19:0, C21:0, C23:0 and C25:0. Chromatographic peak areas of the AR homologues in each sample were summed to yield total AR content.

Total dietary fibre, resistant starch and yellow pigments

Total dietary fibre (DF) content was determined using an enzymatic-gravimetric method (AOAC, 1995) and an automatic filtration of the hydrolysed products (Fibertec system, FossItalia, Italy). Total and resistant starch (RS) contents were evaluated by enzymatic methods using Megazyme kits, K-TSTA and K-RSTAR (Mc Cleary *et al.* 1997; Mc Cleary and Monaghan, 2002; Mc Cleary *et al.* 2002). Total yellow pigment (YP) content was determined following the AOAC 14045 (1975) method and expressed as ppm of β-carotene.

Statistical analysis

As reported by Li *et al.* (2009), two independent aliquots of composite wholemeal sample were considered as statistical replicates of each genotype. Analysis of variance was performed with the Microcomputer Program for the Design, Management, and Analysis of Agronomic Research Experiments (MSTATC) program (Michigan State University, East Lansing, MI). Simple correlation coefficients were calculated as well.

RESULTS

Post-Harvest Regrowth

Three months after the first harvest in 2012, the PHR of the nine wheat x wheatgrass derivatives varied between 5.6 and 42.1 percent, without any evident association between PHR score and genetic origin (Table 2). In some genotypes, new plants arose at the level of the ground surface or immediately below, while in others they emerged at the level of the first or the second internode. The accessions of *Thinopyrum intermedium*, *Th. ponticum* and *Hordeum bulbosum* showed PHR scores between 60.0 and 87.8 percent, approximately double that of the best perennial wheat derivatives. *Secale cereale* had a moderate (23.8 percent) PHR score, whereas annual common wheat cvs Wedgetail and Enesco did not exhibit any sign of regrowth. In 2013, the perennial wheat derivatives in the three months following the second harvest revealed a modest decline in their PHR scores with respect to the those recorded in 2012 (Table 2).

Morphological and physiological traits

The perennial wheat derivatives proved to be significantly different for all the morpho-phisiological traits analysed with respect to cvs Wedgetail and Enesco (Table 2). On average, the nine lines were characterized by lateness of ear emergence (20-30 days later compared with annual varieties), high number of tillers (13.4 *vs* 7.3, except line 236A), tall plants (88.6 *vs* 70.2 cm), loose spikes (1.13 *vs* 1.79 spikelets/cm), reduced number of kernels per spike (39.8 *vs*

63.0) and small kernels (21-33 mg *vs* 40-41 mg). However, lines 280B and 281B, which have in their pedigree the bridge tetraploid species *Triticum turgidum* ssp. *carthlicum*, were comparable with cvs Wedgetail and Enesco for plant height and ear length. In addition, the perennial wheat derivatives revealed mean test weights intermediate between those of annual controls Wedgetail (66.6 kg/hL) and Enesco 74.2(kg/hL), with the only exception of lines 244B and 280B, which showed test weights lower than 58.5 Kg/hL. Upon a single passage through a micro-thresher, spikes of perennial wheat genotypes released a low percentage of hull-less kernels (20.7 to 63.0 percent) compared with control cultivars (84.9 and 91.8 percent). Leaves and stems of perennial wheat derivatives showed no damage due to powdery mildew and rusts (*Puccinia* spp.), with the only exception being lines 251B and 236A, which revealed slight symptoms of stem rust (*Puccinia graminis tritici*). By contrast, line 244B was heavily attacked by *Helminthosporium* spp.

TABLE 2. AGRONOMIC TRAITS AND POST-HARVEST REGROWTH (PHR) OF NINE PERENNIAL WHEAT DERIVATIVES, TWO COMMON WHEAT CULTIVARS AND FOUR PERENNIAL CEREAL SPECIES[a]

GENOTYPE	HEADING TIME*	NO. OF TILLERS	PLANT HEIGHT (CM)		SPIKE LENGTH (CM)	NO. OF SPIKELETS/ SPIKE	NO. OF SPIKELETS/ (CM)	NO. OF KERNELS/ SPIKE	KERNEL WT (MG)	TEST WT (KG/ HL)	HULL-LESS KERNELS (%)	PHR (%)	
			2012	2013								2012	2013
235A	155.0c	10.6d	94.6b	139.3a	15.7a	18.5b	1.18b	59.8b	24.5d	71.2bc	46.0ef	28.1d	25.0d
236A	150.0d	7.1e	85.7c	138.5a	15.3ab	16.5c	1.08b	43.9e	24.8d	68.9c	50.3de	11.1f	9.2f
244B	157.7b	11.2d	80.5d	90.0f	14.0b	15.7c	1.13b	13.1g	23.2de	58.3e	38.8g	5.6g	5.6g
251B	150.0d	11.4d	95.8b	133.0b	16.7a	16.5c	0.98b	58.4b	25.2d	70.2c	41.7fg	36.1b	33.3c
280B	147.0e	12.1cd	64.5f	114.3e	9.8d	11.7e	1.19b	18.5f	21.4e	58.2e	25.0h	20.6e	17.6e
281B	142.3f	18.7a	79.1d	119.3d	10.3cd	13.3de	1.29b	9.2h	32.9b	70.0c	20.7h	40.5a	37.8b
11955	146.3e	15.6b	102.3a	130.5b	15.7a	15.6c	0.99b	53.5c	30.1c	68.7c	48.3de	33.3c	33.3c
OK72	141.3f	19.6a	98.4b	126.2c	15.2ab	14.8cd	0.98b	53.2c	28.3c	74.0a	51.7d	42.1a	42.1a
OT38	160.0a	14.4bc	96.8b	127.7c	15.9a	20.8a	1.31b	48.7d	23.5de	72.0b	63.0c	35.0b	30.0c
Mean	150.0	13.4	88.6	124.3	14.3	15.9	1.13	39.8	26.0	68.0	42.8	27.8	25.9
Wedgetail	129.3g	7.5e	70.3e	-	11.5c	20.7a	1.79a	63.2a	39.9a	66.6d	84.9b	0.0	0.0
Enesco	120.1h	7.0e	70.0e	-	11.1c	21.0a	1.80a	62.8a	41.0a	74.2a	91.8a	0.0	0.0
Th. intermedium	171.2	20.3	129.8	167.7	33.1	26.1	0.79	nd	nd	nd	nd	66.7	60.0
Th. ponticum	193.4	22.1	166.7	194.0	29.7	22.8	0.77	nd	nd	nd	nd	80.0	80.0
S. montanum	133.4	7.7	130.1	142.3	12.0	18.8	1.57	nd	nd	nd	nd	23.8	19.0
H. bulbosum	120.1	14.3	160.8	170.7	11.3	17.6	1.56	nd	nd	nd	nd	87.8	87.8

[a] Agronomic traits of plants harvested in 2012 (first harvest); plant height and PHR were recorded in 2013 (second harvest) as well.
*Number of days from sowing.

nd, not determined. In each column, means followed by the same letter do not differ significantly from one another (Duncan test at P<0.05).

Quality traits

Protein contents as high as 19.7 to 23.7 percent were observed in the perennial wheat derivatives, with an average value of 20.6 percent, 3.3 percentage units higher than those of their annual counterparts (Table 3). On average, the perennial wheat derivatives revealed a high proportion of gliadins (37.2 percent of total proteins *vs* 33.0 percent in cv. Wedgetail) coupled with a significantly low proportion of HMW-GS (on average 9.3 percent of total protein *vs* 10.7 percent in cv. Wedgetail). This was particularly evident in lines 235A, 236A, 244B and 251B developed at the Washington State University, and was associated with poor gluten quality as determined by the SDS sedimentation test, line 235A being unique in showing an SDS sedimentation volume as high as 58 ml. The contrasting behaviour of these lines was likely due to their HMW-GS, which are known to play an important role in the visco-elastic properties of dough.

TABLE 3. PROTEIN CONTENT, PROPORTION OF FOUR PROTEIN FRACTIONS AND SDS SEDIMENTATION VOLUME OF NINE PERENNIAL WHEAT DERIVATIVES AND TWO COMMON WHEAT CULTIVARS

GENOTYPE	PROTEIN CONTENT %	ALBUMIN & GLOBULIN %	GLIADIN %	HMW-GS %	LMW-GS %	SEDIMENTATION VOLUME (ml)
235A	19.7	11.1	39.8	9.7	19.4	58c
236A	19.4	15.1	41.9	7.4	15.4	45f
244B	23.7	15.1	42.7	8.0	13.7	30i
251B	19.9	9.3	41.8	9.7	18.8	43g
280B	20.8	14.3	24.8	12.0	27.1	50d
281B	21.5	10.4	35.1	11.2	22.4	41h
11955	19.7	24.3	31.5	7.9	18.5	50d
OK72	20.6	10.9	38.1	10.1	20.0	50d
OT38	20.7	17.3	38.2	8.1	16.3	47e
Mean	20.6	14.2	37.2	9.3	19.1	46
Wedgetail	17.3	14.2	33.0	10.7	18.8	67b
Enesco	17.4	nd	nd	nd	nd	79a
F value	**	ns	***	**	ns	

**, ***Significant at $P<0.05$ and $P<0.01$ respectively; ns, not significant. In the last column, means followed by the same letter do not differ significantly from one another ($P<0.05$).

The HMW-GS of the perennial material were fractionated by SDS-PAGE and classified according to the nomenclature described by Payne and Lawrence (1983) and Pogna *et al.* (1989) (Figure 1). Six perennial lines (11955, OK72, OT38, 235A, 280B and 281B) showed no trace of HMW-GS inherited from the wheatgrass parent and exhibited the commonly occurring subunits 1 or 2* encoded by the *Glu-A1* locus on the long arm of chromosome 1A together with subunits 20, 7*+ 8 or 7+9 encoded by the *Glu-B1* locus (chromosome 1BL) and subunit pairs 2+12 or 5+10 encoded by the *Glu-D1* locus (chromosome 1DL) (Table 4). By contrast, lines 236A, 244B and

251B exhibited unusual HMW-GS, likely inherited from the wheatgrass parent (Figure 1, arrows) and lacked HMW-GS encoded by the *Glu-D1* locus on chromosome 1DL. In addition, line 244B did not show any subunit encoded by the *Glu-B1* locus. SDS-PAGE fractionation of 10 single kernels from each genotype revealed that the perennial wheat derivatives were homogeneous for their HWM-GS patterns, with the only exception being line 281B, which turned out to be a mixture of three different genotypes (biotypes) with contrasting HMW-GS compositions at *Glu-A1* (subunit 1 or Null) and *Glu-D1* (subunit pair 2+12 or 5+10) (Figure 2 and Table 4).

FIGURE 1. SDS-PAGE FRACTIONATION OF TOTAL PROTEINS FROM NINE PERENNIAL WHEAT DERIVATIVES

(1) line 235A, (2) line 236A, (3) line 244B, (4) line 251B, (5) line 280B, (6) line 281B, (7) line 11955, (8) line OK72 and (9) line OT38. HMW-GS are numbered. Arrowheads indicate HMW-GS inherited from wheatgrass (*Thinopyrum* ssp).

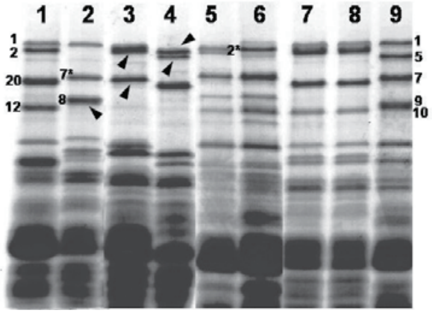


FIGURE 2. SDS-PAGE PATTERN OF TOTAL PROTEINS FROM THREE SINGLE SEEDS OF PERENNIAL WHEAT LINE 281B

HMW-GS are numbered.

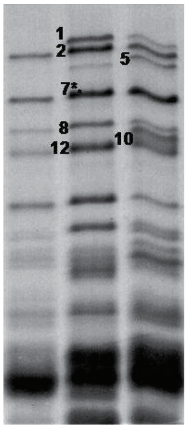


TABLE 4. HMW-GS COMPOSITION OF NINE PERENNIAL WHEAT DERIVATIVES

WHEAT GENOTYPE	WHEAT LOCUS			WHEATGRASS LOCUS
	GLU-A1	*GLU-B1*	*GLU-D1*	
11955	1	20	2+12	absent
OK72	1	20	2+12	absent
OT38	1	7+9	5+10	absent
235A	1	20	2+12	absent
236A	1	7+8	absent	1 subunit
244B	1	absent	absent	2 subunits
251B	Null	20	absent	2 subunits
280B	2*	7+8	2+12	absent
281B-1*	1	7+8	2+12	absent
281B-2	Null	7+8	2+12	absent
281B-3	1	7+8	5+10	absent

*Line 281B contains 3 biotypes with contrasting HMW-GS compositions

Gliadin patterns of the perennial wheat derivatives fractionated by A-PAGE were comparable with those of annual wheat cvs. Bolero and Chinese Spring. However, lines 236A, 244B, 251B and OT38 revealed some ω- or γ-gliadins inherited from the wheatgrass parent (Figure 3, arrowhead). Upon A-PAGE fractionation of gliadins from single seeds, line 236A (Figure 4), 235A and 281B turned out to be a mixture of two or more biotypes with contrasting gliadin bands encoded by homoeologous group 1 chromosomes of common wheat.

FIGURE 3. A-PAGE FRACTIONATION OF GLIADINS FROM NINE PERENNIAL WHEAT DERIVATIVES

(1) Line 235A, (2) line 236A, (3) common wheat cv. Enesco,(4) line 244B, (5) line 251B, (6) line 280B, (7) line 281B, (8) line 11955, (9) line OK72 and (10) line OT38. Arrowheads indicated gliadin inherited from wheatgrass (*Thinopyrum* spp.).

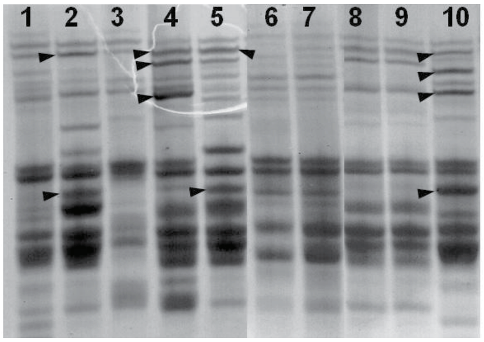
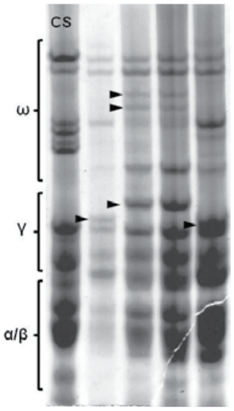


FIGURE 4. A-PAGE FRACTIONATION OF GLIADINS FROM COMMON WHEAT CV. CHINESE SPRING (CS) AND FOUR SINGLE SEEDS OF PERENNIAL WHEAT LINE 236A

Arrowheads indicate differential gliadin bands.



Kernel hardness was determined by the SKCS method using 50 grains for each line and found to be typical of soft-textured (mean SKCS index=30) or medium-hard common wheat (mean SKCS index =68) (Table 5).

TABLE 5. MEAN SKCS VALUE AND ALLELE COMPOSITION AT THE PUROINDOLINE LOCI IN NINE PERENNIAL WHEAT DERIVATIVES

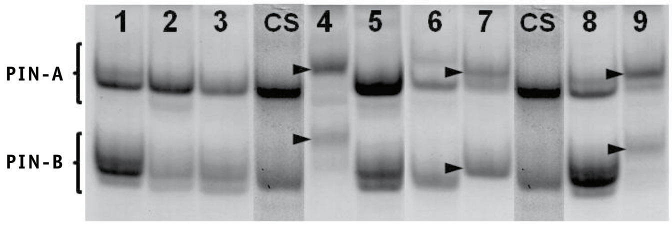
PERENNIAL WHEAT LINE	WHEAT LOCUS		WHEATGRASS LOCUS		SKCS
	PIN-A	*PIN-B*	*PIN-A*	*PIN-B*	
235A			Novel allele	Novel allele	55.4b
236A	*Pina-D1a*	*Pinb-D1a*			34.5c
244B	*Pina-D1a*	*Pinb-D1a*			46.7bc
251B	*Pina-D1a*	*Pinb-D1a*			35.4c
280B			FJ898232.1*	nd	69.1b
281B			FJ898232.1*	nd	60.0b
11955	*Pina-D1a*	*Pinb-D1a*			29.5c
OK72	*Pina-D1a*	*Pinb-D1a*			32.5c
OT38	*Pina-D1a*	nd			37.7c
Wedgetail	*Pina-D1a*	*Pinb-D1b*			61.5b
Enesco	*Pina-D1b*	*Pinb-D1a*			79.8a

nd, not determined ; * GenBank number. In the last column, means followed by the same letter do not differ significantly from one another (P<0.05).

Upon A-PAGE fractionation, the perennial wheat derivatives exhibited puroindoline-A (PIN-A) and puroindoline-B (PIN-B) inherited from either wheatgrass or common wheat. Novel, slow-moving PIN-A and PIN-B likely inherited from wheatgrass (*Thinopyrum spp.*) occurred in the medium-hard kernels produced by lines 235A, 280B and 281B (Figure 5, arrowheads). By contrast soft-textured perennial lines 236A, 244B, 251B, 11955, OK72 and OT38 exhibited wild-type PIN-A and PIN-B. When submitted to PCR amplification and sequencing, these latter soft lines revealed alleles *Pina-D1a* (coding for PIN-A) and *Pinb-D1a* (PIN-B) inherited from common wheat, whereas the medium-hard lines showed three unusual alleles (Table 5). In particular, the PIN-A allele in lines 280B and 281B was found to be identical to that amplified in *Aegilops tauschii* isolate TT52 (GenBank reference no. FJ898232.1), whereas the PIN-A and PIN-B alleles in line 235A were novel sequences never described before.

FIGURE 5. A-PAGE FRACTIONATION OF PUROINDOLINES A (PIN-A) AND B (PIN-B) IN WHEAT CV. CHINESE SPRING (CS) AND PERENNIAL WHEAT LINES (1) OK72, (2) 244B, (3) 251B, (4) 280B, (5) 236A, (6) OT38, (7) 235A, (8) 11955 AND (9) 281B

Arrowheads indicate puroindolines inherited from wheatgrass (*Thinopyrum* spp.).



Phytochemical profile of perennial wheat derivatives

Wholemeals from perennial wheat derivatives and cv. Wedgetail harvested in 2012 (first harvest) were compared for their content in resistant starch (RS), AR, soluble polyphenols (SP), and DF. The YP content of the representatives of perennial wheat varied in the ranges 5.12 to 11.37 ppm, their average content being 43 percent higher than that of cv. Wedgetail (Table 6).

TABLE 6. YELLOW PIGMENT (YP), DIETARY FIBRE (DF), 5-N-ALKYLRESORCINOLS (AR), SOLUBLE POLYPHENOLS (SP), TOTAL STARCH (TS), RESISTANT STARCH (RS) AND RS/TS RATIO IN 9 PERENNIAL WHEAT DERIVATIVES AND ANNUAL WHEAT CV. WEDGETAIL HARVESTED IN 2012 (FIRST HARVEST)

GENOTYPE	YP (PPM)	DF (%)	AR (µg/g)	SP (mg/g)	TS (%)	RS (%)	RS/TS (X100)
235A	5.1 e	15.9 bc	384 d	206 h	57.8 b	0.71 ab	1.2
236A	6.8 c	15.1 d	266 h	287 e	46.4 f	0.47ab	1.0
244B	6.0 d	16.9 a	329 g	231 g	49.9 e	0.74 a	1.5
251B	7.0 c	13.9 e	391 c	255 f	57.9 b	0.59 ab	1.0
280B	11.4 a	15.3 cd	500 b	640 a	57.4 bc	0.49 ab	0.9
281B	9.4 b	13.6 e	372 e	309 d	55.4 c	0.46 b	0.8
11955	6.9 c	16.3 ab	522 a	406 b	51.4 de	0.63 ab	1.2
OK72	6.2 d	16.3 ab	346 f	193 i	68.3 a	0.58 ab	0.8
OT38	7.1 c	12.8 f	182 i	340 c	53.3 d	0.45 b	0.9
MEAN	7.3±1.9	15.1±1.4	366±105	319±280	55.3±6.2	0.6±0.1	1.0±0.22
cv. Wedgetail	5.1	13.4	308	280	73.7	0.4	0.5

In each column, means followed by the same letter do not differ significantly from one another ($P<0.05$).

On average, the total content in 5-alkylresorcinol (AR), soluble polyphenols (SP) and DF was high in the perennial wheat derivatives compared with cv. Wedgetail. However, there was a considerable variability for these bioactive compounds among the germplasm in this study. For instance, the lowest and highest SP values of 193 mg and 600 mg were determined in lines OK72 and 280B, the range of variation among these lines being as high as 407 mg. Another example of variability includes lines OT38 and 11955, which showed 5-n-alkylresorcinol contents of 182 mg/g and 522 mg/g, respectively. As expected, the high protein content of the perennial wheat lines was associated with a reduced amount of total starch (TS) compared with the annual wheat control. Interestingly, all perennial wheat lines exhibited a high concentration of RS, which resulted in a high RS/TS ratio (Table 6). No significant correlation was found between seed weight and the amount of phytochemicals AR, SP, DF and RS.

GC-MS analysis was used to determine the alkylresorcinol homologue composition of grain harvested in 2012. Compared with common wheat cv. Wedgetail, perennial wheat lines 236A, OK72 and OT38 revealed an unusual AR pattern, with a prevalence (> 50 percent) of C19:0 homologue and a relatively high percentage (11-16 percent) of C17: 0 (Table 7). In addition, the nine perennial wheat representatives were found to belong to two groups based on the C17/C21 ratio, which is peculiar of the different *Triticum* species. The first group includes six lines with a C17/C21 ratio of 0.09 to 0.22, comparable to that of the control cv. Wedgetail (0.11), while the second group includes three lines (236A, OK72 and OT38) with a C17/C21 ratio of 0.34 to 0.76.

TABLE 7. HOMOLOGUE PROFILES (%) OF 5-N-ALKYLRESORCINOLS IN NINE PERENNIAL WHEAT DERIVATIVES

GENOTYPE	HOMOLOGUE						C17/C21 RATIO
	C15:0	C17:0	C19:0	C21:0	C23:0	C25:0	
235A	0.54±0.03	5.00±0.23	29.80±1.25	43.21±1.90	13.49±0.86	7.97±0.28	0.12
236A	0.21±0.13	12.61±1.63	50.89±0.50	31.77±1.69	3.98±0.22	0.54±0.24	0.40
244B	0.77±0.12	7.19±0.13	43.05±1.66	36.85±0.89	8.73±0.83	3.41±0.54	0.20
251B	0.46±0.19	6.52±0.63	39.62±1.65	40.66±2.13	8.72±0.19	4.02±0.24	0.16
280B	0.64±0.15	8.06±0.21	38.04±1.18	36.87±0.34	10.22±0.70	6.16±0.65	0.22
281B	0.51±0.19	4.21±0.22	30.72±1.09	47.80±1.27	11.67±0.32	5.09±0.85	0.09
11955	0.44±0.09	6.70±0.24	43.75±0.75	39.78±0.63	6.82±0.19	2.52±0.12	0.17
OK72	0.43±0.11	11.20±0.89	51.02±1.45	32.91±1.60	3.71±0.67	0.73±0.17	0.34
OT38	0.24±0.16	16.33±2.39	60.73±3.17	21.57±0.95	0.99±0.12	0.14±0.11	0.76
MEAN	0.47±0.16	8.65±0.99	43.07±1.35	36.82±1.11	7.59±0.23	3.40±0.25	
LSD (0.05)	0.38	1.20	1.40	1.25	0.78	0.57	
Cv. Wedgetail	0.68±0.31	4.84±0.26	36.72±0.33	45.23±0.27	9.22±0.19	3.30±0.04	0.11

DISCUSSION

Agronomic traits and PHR

The significant differences in the mean PHR value and the wide variation of this trait (5.6 to 42.0 percent) in the nine perennial wheat derivatives developed by the Land Institute and Washington State University indicate that several genes interacting with environment and climatic conditions play an important role in modulating regrowth after harvest. Survival of regrowing plants through the 2012 winter following the first harvest was very high, approaching 100 percent in most lines and leading to a small decline of PHR in 2013. This was likely due to the mild temperatures registered during autumn 2012-winter 2013 in the Tiber valley. The mean PHR scores of 27.8 percent in 2012 and 25.9 percent in 2013 and the performance of lines OK72, 251B, 281B and 11955 which showed regrowth scores greater than 33 percent indicates that the present germplasm is valuable for its regrowth potential. These results confirm the recent conclusions by Hayes *et al.* (2012), who pointed out the strong relationship between PHR and the presence of at least one whole genome equivalent of 14 chromosomes from the wheatgrass parent. According to these authors, lines with $2n = 56$ chromosomes in Table 1 likely contain 42 wheat chromosomes plus 14 wheatgrass chromosomes. However, wheat derivatives with a relatively high regrowth score may contain a reduced number of chromosomes due to substitution of one or more wheat chromosomes by wheatgrass homoeologous (Hayes *et al.* 2012). Here, evidence has been obtained that substitution or recombination involving E-genome and ABD-genome chromosomes likely occurred in lines 236A, 244B and 251B, which have $2n=56$ and *Th. elongatum* in their pedigree. In addition, all perennial wheat derivatives showed endosperm proteins inherited from either wheat or wheatgrass parents. These aspects will be discussed later. The current study identified some negative agronomic attributes, mainly tenacious glumes and reduced kernel weight, together with many desirable traits such as reduced plant height, high tiller number and disease resistance, which can contribute to increase the agronomic potential of perennial wheat.

Quality traits

The HMW-GS account for only about 1 percent of the dry weight of wheat kernel (Payne *et al.* 1987) Nevertheless the results presented in this paper are consistent with accumulated evidence that they are the principal subunits that impart elasticity to gluten. Variation in composition of HMW-GS among the perennial material was found to make a large contribution to the gluten quality of these genotypes. In particular, the absence of HMW-GS encoded by the *Glu-D1* locus on the long arm of chromosome 1D in lines 236A, 244B and 251B (Figure 1, lanes 2-4) proved to be deleterious to gluten quality as determined by the SDS sedimentation volume (Table 3). In these lines, the presence of HMW-GS inherited from wheatgrass (Figure 1, arrowheads) could additionally affect gluten quality. Furthermore, lines 236A, 244B and 251B contain 1-3 w-gliadins plus one prominent g-gliadin arrowed in Figure 3, which have been likely inherited from their wheatgrass parent. In common wheat, all ω-gliadins and most γ-gliadins are encoded by genes on the short arms the homoeologous group 1 chromosomes (Payne *et al.* 1984). Moreover, a comparative study showed that all species in the genera *Triticum*, *Aegilops*, *Secale* and *Hordeum* contain genes coding for prolamins HMW-GS, gliadins or gliadin-type proteins such as secalins and hordeins) on homoeologous chromosome 1, suggesting that wheatgrass prolamins in lines 236A, 244B and 251B are likely encoded by chromosome 1E from *Thinopyrum elongatum*. As introgression of wheatgrass prolamin genes into perennial wheat lines 236A, 244B and 251B has been accompanied by concomitant removal of the *Glu-D1* locus, it is not clear whether it is the presence of wheatgrass prolamins or the absence of wheat HMW-GS that negatively impact the bread-making quality. Line OT30 could offer an opportunity to elucidate this aspect. According to the quality score assigned to each HMW subunit or subunit pair based on its effect on gluten quality (Payne *et al.* 1987), the HMW-GS composition of line OT38 (subunit 1 of chromosome 1A, subunits 7+9 of 1B and subunits 5+10 of 1D, Table 4) has the high *Glu-1* quality score of nine, the maximum score being ten and the minimum three. The finding that the presence of wheatgrass gliadins in line OT38 (Figure 10, arrowheads) is associated with a SDS sedimentation volume as low as 47 ml (Table 3) suggests a direct negative effect of these proteins on gluten quality. In this context it is worth noting that some European-grown wheat cultivars contain the short arm of chromosome 1R from rye combined with the long arm of chromosome 1B (1BL/1RS). This translocated chromosome causes a decrease in gluten quality due to the presence of 1RS-encoded secalins, which increases dough stickiness (Zeller *et al.* 1982). The negative effects of rye prolamins on bread making quality of hexaploid AABBRR triticale are documented as well, and substitution of chromosome 1D for chromosome 1R dramatically improved such parameters of bread-making quality of triticale as SDS-sedimentation, mixing time, mixing tolerance, and loaf volume (Kazman and Lelley, 1996).

Kernel texture, a major determinant of flour quality and end-use quality of wheat, is mainly modulated by allele variation at the *Pina-D1* and *Pinb-D1* loci on chromosome 5DS coding for PIN-A PIN-B, respectively. In the present work, the molecular analysis of puroindoline genes from perennial wheat lines of different genetic origins identified novel alleles coding for PIN-A and PIN-B in line 235A with *Th. elongatum* in the pedigree. When compared with wild-type *Pinb-D1a* allele, the gene coding for PIN-B in this line shows 19 SNPs, whereas the encoded PIN-B protein contains nine amino acid substitutions in its mature form. The novel *Pina-E1a* and *Pinb-E1a* alleles in the homoeologous chromosome 5ES inherited from of *Th. elongatum* conferred a medium hard texture to line 235A (SKCS value = 55.4, Table 5). Interestingly, the absence of *Pina-D1* and *Pinb-D1* sequences from chromosome 5DS suggests that 5E (5D) chromosome substitution or homoeologous recombination between wheatgrass chromosome 5E and 5D of wheat occurred in line 235A.

Medium-hard kernel texture in lines 280B and 281B was associated with the presence of two unusual puroindolines with reduced mobility with respect to wild-tipe PIN-A and PIN-B (Figure 5, lanes 4 and 9). As *T. carthlicum* does not contain *Pina* and *Pinb* genes, puroindolines in lines 280B and 281B have been likely inherited from *Th. intermedium*. The 100 percent similarity between the DNA sequence coding for PIN-A in lines 280B and 281B and the *Pina-D1* allele in *Aegilops tauschii* isolate TT52 (GenBank reference no. FJ898232.1) suggests a strong phylogenetic relationship between D genome and J, J^S or S genome of *Th. intermedium*. As observed in line 235A, the absence of *Pina-D1* and *Pinb-D1* sequences from chromosome 5DS as determined by PCR amplification suggests chromosome substitution or allosyndetic recombination involving chromosome 5D of wheat and a homoeologous chromosome of *Th. intermedium*.

Nutritional traits

Based on the genotypes studied here, there is a great variation in the phytochemical composition among wheat cv. Wedgetail and the nine representatives of perennial wheat. In particular, there is an increased amount of yellow pigments, dietary fibre and RS in most of the perennial wheat derivatives analysed. In addition, unique bioactive phytochemical patterns with high levels of both 5-alkylresorcinol (AR) and soluble polyphenols (SP) were detected in lines 280B and 11955. Among the nine perennial wheat genotypes, line 11955 is characterized by relatively high values for kernel weight (30.1 mg), number of kernels/spike and PHR (33.3 percent). In addition, line 11955 showed the *Glu-1* quality score of six coupled with an above-average SDS sedimentation volume of 50 ml, suggesting its use as a component of a range of traditional and specialty products naturally enriched with health-promoting compounds.

The C17:0/C21:0 ratio of AR homologues has been used to distinguish between different cereal species. This ratio ranges between 0.01 in *Triticum monococcum*, 0.02 in *Triticum turgidum* ssp *dicoccum*, 0.05 in T. *turgidum* ssp *durum*, 0.06 in *T. timopheevii*, 0.11 to 0.18 in common wheat and *T. turgidum* ssp *turanicum,* and 0.25 in *T. zhukovskyi* (Ross *et al.* 2003; Ciccoritti *et al.* 2013). On average, the nine perennial wheats showed a high proportion of C17:0 and a low proportion of C21:0 homologues compared with cv. Wedgetail. This was mainly due to lines OT38, 236A and OK72, which were unique in having C17/C21 ratios as high as 0.34 to 0.76 (Table 7). By contrast, the remaining six perennial lines exhibited C17/C21 ratios of 0.09 to 0.22 comparable to those observed in the different species of the genus *Triticum*. Interestingly, lines 235A and 281 showed unusual AR homologue compositions with a high proportion of C21:0, C23:0 and C25:0 homologues (complessively about 64.5 percent as compared with 55.8 percent in cv. Wedgetail). As long-chain resorcinolic lipids affect protein structure and activity (Stasiuk *et al.* 2008), these lines may have some potential as a source of cereal foods for prevention of cardiovascular diseases and cancer.

The successful development of perennial wheat cultivars and their widespread adoption by millers, bakers and consumers will be facilitated by improvement of kernel threshability, milling and bread making quality, and nutritional characteristics including gluten digestibility (tolerance). Therefore, in addition to addressing the major agronomic traits (grain yield, PHR and disease resistance), good milling and baking quality and superior nutritional quality seem to be key traits to target for genetic improvement. The wide variation in storage protein composition and bioactive compounds detected in the germplasm analysed here can be easily exploited by breeders in the development of new perennial wheat genotypes with improved end-use qualities.

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06

CURRENT EFFORTS TO DEVELOP PERENNIAL WHEAT AND DOMESTICATE *THINOPYRUM INTERMEDIUM* AS A PERENNIAL GRAIN

Lee R. DeHaan[1], Shuwen Wang[1], Steven R. Larson[2], Douglas J. Cattani[3], Xiaofei Zhang[4], Traci Kantarski[5]*

1 The Land Institute, 2440 E. Water Well Rd., Salina, KS, 67401
2 USDA-ARS Forage and Range Research Laboratory, Utah State University, Logan, UT 84322-6300.
3 Department of Plant Science, University of Manitoba
4 Department of Agronomy & Plant Genetics, University of Minnesota,
411 Borlaug Hall, 1991 Buford Circle, St. Paul, MN 55108
5 University of Chicago, Chicago, IL 60642
* Corresponding author. Email: dehaan@landinstitute.org (+1); Phone: 785 823 5376

ABSTRACT

We are developing a new perennial grain by domesticating the perennial grass *Thinopyrum intermedium* (intermediate wheatgrass). In 1983, intermediate wheatgrass was selected for domestication by the Rodale Institute (Kutztown, Penn., USA). Nearly 100 species of perennial grasses were evaluated for promise as a perennial grain before choosing intermediate wheatgrass to domesticate. The Rodale Institute performed two cycles of selection, beginning in 1988. Using selections made by Rodale, breeding work began at The Land Institute (Salina, Kan., USA) in 2002.

Selection has been for yield per head, increased seed mass, free threshing ability, reduced height, and early maturity. Two cycles of selection at The Land Institute have increased seed yield by about 77 percent and seed mass by about 23 percent, when grown in a solid stand. Selected materials have been found to possess a higher harvest index and reduced plant spread.

Molecular tools are being developed for intermediate wheatgrass. A combination of expressed sequence tag (EST), SSRs and AFLP markers will be used to genotype an experimental mapping population comprises 268 full-sib progeny derived from a reciprocal cross of two experimental genotypes. Genotyping by sequencing is also being used to identify ~3 000 high quality single-nucleotide polymorphisms (SNPs) in a population derived from one selfed individual. Phenotype data has been collected and will be used to identify QTL associated with SNPs. Using gene cloning, protein separation and identification, and sequence alignments; we were able to identify five HMW-GS genes and their allelic variants in intermediate wheatgrass plants.

Since 2001, we have been working to develop perennial wheat by crossing wheat (*Triticum* spp.) with perennial *Thinopyrum* species. We have obtained a few stable lines with one set (12-14) *Thinopyrum* chromosomes and 42 wheat chromosomes. These have better agronomic performance in Kansas than other materials, but lack perenniality. Crosses between winter durum wheat and *Thinopyrum intermedium* have been recently developed, and they are promising in terms of perenniality, seed weight, winter hardiness, and vigor. To study the impact of annual/perennial genome dosage on perenniality and agronomic performance, we have crossed diploid and tetraploid wheat lines with tetraploid and hexaploid *Thinopyrum* species. All F_1 plants are perennial, and many have been doubled with colchicine. Wheat chromosome-specific markers have been used to characterize 94 plants that were perennial in the field. Plants with more wheat chromosomes eliminated tended to be more perennial, but specific chromosomes were not associated with perenniality or annuality.

INTRODUCTION

Two major approaches are being used to develop perennial small grains: wide hybridization and domestication. The two approaches present unique strengths and challenges. Wide hybridization involves crossing an annual grain such as wheat with related perennial species. Wide crosses will in theory make available genes controlling traits such as yield, seed size, free threshing ability, and quality, which have been accumulated in current grain crops. With wheat the challenge has been to obtain cytogenetic stability in wide hybrids while preserving perenniality and domestication traits. Direct domestication of wild perennials has the potential benefit of working with populations that are vigorous perennials. However, the necessary genetic variation for domestication may be lacking in perennial species, or substantial time may be required for selection to achieve adequate seed size, yield, or other domestic traits.

The first sustained effort to directly domesticate a perennial grass for grain, of which we are aware, was initiated by researchers at The Rodale Institute in Pennsylvania, United States of America (Wagoner, 1990). The researchers leading this domestication effort cite Jackson (1980) as the inspiration for developing perennial grasses for grain. In 1983, researchers began to evaluate nearly 100 perennial grasses to determine their suitability for grain production. Based on numerous criteria, intermediate wheatgrass (*Thinopyrum intermedium*) was selected as a perennial grain candidate. Beginning in 1988, two cycles of selection for improved fertility, seed size, and other traits were performed in Pennsylvania.

Selection for grain production in intermediate wheatgrass was continued by workers at The Land Institute in Kansas, United States of America, beginning in 2003 (Cox *et al.* 2010). Four cycles of selection using an index based primarily on seed yield per head, seed mass, and free threshing ability have been performed at The Land Institute. Improved populations resulting from this selection program are currently being evaluated and further selected by collaborators in diverse environments.

We began a perennial wheat (*Agrotriticum*) breeding program in 2001. Perennial wheat has potential in reducing soil erosion and promoting the sustainability of agriculture (Cox *et al.* 2002; Cox *et al.* 2006). Through perennial wheat, or wheat-*Thinopyrum* hybrids, more than 18 agronomically important traits other than perenniality have been transferred from *Thinopyrum* species into wheat (Chen *et al.* 2005). At the beginning of our breeding program, we introduced perennial wheat lines from other institutions. All of them died at a time point after grain harvest during the hot summer of Kansas (Cox *et al.* 2006). Therefore, we have sought fresh approaches to developing truly perennial wheat. Among these new approaches are management techniques. Because we have yet to identify agronomic strategies to induce perenniality in Kansas, here we will focus on genetic approaches.

RECENT PROGRESS

Breeding Perennial Wheat

In our search for an approach to perennial wheat with good grain yield and perenniality, we have crossed wheat with *Th. ponticum* (10x = 70), *Th. intermedium* (6x = 42), and *Th. elongatum* (2x = 14). We have also attempted crosses with other *Thinopyrum* species. Crosses involving *Th. junceiforme* (4x = 28), *Th. bessarabicum* (2x = 14), and a *Th. bessarabicum*-*Th. elongatum* hybrid (4x = 28) have produced hybrid F_1 plants successfully. The crosses with *Th. junceum* (6x = 42) and *Th. scerpium* (4x = 28) could form well-developed embryos. But the plantlets died shortly after germination on nutrient medium. One *Thinopyrum* accession (6x = 42; PI531731) is unique in that it is strictly self-pollinated. We have attempted to cross 6x and 4x wheat with this accession. However, no hybrid embryos have been obtained as the caryopses aborted at early stage. We have successfully obtained F_1 hybrids between *Thinopyrum* species and both rye and triticale. So far, no crosses involving rye have resulted in lines with perenniality and seed fertility.

In recent years, we have created a couple of genetically stable lines which were derived from the crosses between common wheat (*T. aestivum*) and *Th. intermedium* wheatgrass. Similar to a great number of perennial wheat lines developed by other institutions, these lines had chromosome numbers from 54 to 56 with 12 to14 chromosomes from wheatgrass. Minor translocations involving wheatgrass chromosomes were observed on some wheat chromosomes. In our plot experiments, four breeding lines named B373, B1126#1, B1126#2 and B1321 did much better than the others. They looked similar to annual wheat cultivars, ripened early, and had excellent seed fertility and better-filled grain. Interestingly, these four lines shared a common parent, B373. They were similar to B373 in many aspects morphologically, but had improved regrowth ability. The line B373 was just a few days later in maturity than local common wheat cultivars. The wheat cultivar Jagger, a leading wheat cultivar in Kansas from the 1990s through the 2000s, was the donor of wheat chromosomes to B373. 'Jagger' might have contributed to the improved adaptability of these lines. By contrast, perennial wheat lines from the former Soviet Union and Washington State did not present similar adaptability. They flowered extremely late, produced fewer heads, and filled grain poorly.

In a root-tube experiment, we compared the three "perennial" wheat lines B373, B1126#2, and OK7211542 with wheat cultivar Jagger and a *Th. intermedium* selection C3-2627. The total biomass, root biomass, shoot biomass, and grain weight of the three perennial wheat lines are intermediate to the wheat and wheatgrass controls, respectively, except that OK7211542 had larger total biomass and shoot biomass than both controls. Like perennial wheatgrass, perennial wheat lines showed larger root mass than the wheat cultivar, especially at depth. At maturity, part of their leaves and stems were still green. These differences appear to be associated with maturity time and the strength of perenniality.

The regrowth ability of our breeding lines varies widely in different years or environments, which was also observed in investigations by other researchers (Tsitsin, 1978; Murphy *et al.* 2009; Hayes *et al.* 2011; Jaikumar *et al.* 2012). Extremely hot, cold, and dry weather conditions all can suppress the regrowth or cause the death of new tillers. We noticed that irrigation or rain after anthesis could promote regrowth. We have kept a number of lines in the greenhouse for continuous observation. The plants were watered every day, so that they were not stressed by moisture. However, the plants died with no more than three grain harvests. The number of tillers declined over life cycles. Unlike wheatgrass plants, the position in the crown where new tillers originated moved upward over time, which could make buds and new tillers vulnerable to environmental stresses. Furthermore, the new tillers entered the reproductive stage quickly without the need of vernalization. We have not found a line among our materials showing a typical winter-type regrowth profile in which the new tillers stay dormant like wheatgrass. Tsitsin (1978) suggested that winter-type regrowth was desirable for stronger perenniality. Common to these tested lines is that they carried about one set of 14 wheatgrass chromosomes.

An exception is MT-2, which was selected for forage production by Montana State University. In the Kansas environment, this hybrid line among all those we have obtained from other institutions is the most perennial. This line has lived in our field for two years up to present. It roughly contains two wheat genomes (mean = 26.2 wheat chromosomes) and two wheatgrass genomes (mean = 29.2 wheatgrass chromosomes) and is genetically unstable (Jones *et al.* 1999). The instability of this durum wheat (*T. turgidum* ssp. *durum*) hybrid and similar durum derivatives has led some to question the feasibility of durum as a parent of perennial wheat.

The problem of genetic instability may possibly be overcome by taking advantage of genetic variability within tetraploid species. Among the progenies derived from a cross of durum wheat with *Th. junceiforme*, we have found lines likely to be stable, predicted on the basis of seed fertility. Tetraploid wheat species may also possess genes that could promote genetic stability. It seems unnecessary to backcross with common wheat as advised by Ellneskog-Staam and Merker (2002).

By means of backcrossing to wheatgrass or intermating different perennial hybrid progenies at their early generations, we obtained 843 truly perennial plants that survived the hot Kansas summer in 2010 for the first time. These plants varied greatly in perenniality, seed fertility, vigour, regrowth habit, and other traits. Using the genomic *in situ* hybridization (GISH) technique, we examined the genomic constitutions of 11 plants. They possessed chromosome numbers ranging from 53 to 70, with some wheat chromosomes lost. In order to identify which wheat chromosomes might be associated with the elevated perenniality, we screened 154 wheat chromosome-specific markers (http://probes.pw.usda.gov:8080/snpworld/Search) on a panel of wheat and wheatgrass parents. We chose these markers because they had been assigned to 21 wheat chromosomes individually and had the capacity to distinguish homologous chromosomes. The presence of a marker clearly indicated the presence of a wheat chromosome. Fifty-six markers that amplified only in wheat were selected eventually to genotype a subset of 94 perennial plants (at F_2 to F_5 generations). The results showed that the patterns of wheat chromosome elimination differed by plants. All 94 plants had lost more than one wheat chromosome. With more wheat chromosomes eliminated, a plant tended to be more perennial. However, we did not see a specific wheat chromosome that was missing consistently across all plants, which would have indicated that a single wheat chromosome had a deciding impact on perenniality. It might be concluded that decreased abundance of wheat chromosomes is necessary for perennial wheat to live longer in the Kansas environment. Alternatively, the ratio of annual to perennial chromosomes may be critical to perenniality.

Cox *et al.* (2006) proposed that more than one wheatgrass genome would be required for perennial wheat to live in the field for many years. With the increased abundance of wheatgrass chromosomes and decrease in wheat chromosomes, some new issues need to be addressed. For instance, at present we do not know what percentage of wheatgrass chromosomes is appropriate for stronger perenniality that will ensure survival for multiple years. We also do not know if the decrease in wheat chromosomes will lead to more severe genetic instability. When polyploid wheatgrass species are used in crosses, wheatgrass chromosomes tend to be eliminated gradually in subsequent generations until a stable genomic constitution is reached (Tsitsin, 1978). It is impossible to assess the effects of complete genomes adequately using advanced generation materials due to chromosome elimination. To investigate these issues, we are establishing a panel of full amphiploids with different genomic constitutions that are composed of wheat: wheatgrass chromosome ratios of 3:3, 3:2, 2:3, 2:2, 1:3, and 1:2, respectively. Diploid, tetraploid, and hexaploid wheat species have been crossed with tetraploid and hexaploid wheatgrass species successfully. A fraction of such full amphiploids have been chromosome doubled, including the F_1 hybrid (2n = 84) between common wheat and *Th. intermedium* wheatgrass. Comparisons among them will help us understand how many wheat and wheatgrass genomes will enable perenniality across diverse environments.

The role of cytoplasm in the crosses of wheat with wheatgrass species may be worth exploring. Wide hybridizations in wheat and oilseeds have indicated that sequence deletion was different in reciprocal crosses, reflecting the interaction between nucleus and cytoplasm (Gill, 1991; Song *et al.* 1995; Ma and Gustafson, 2008; Ozkan and Feldman, 2009). To the best of our knowledge, all the existing perennial wheat lines bear wheat cytoplasm. We speculate that due to incompatibility with wheat cytoplasm, some wheatgrass chromosomes or genes important to perenniality are preferentially eliminated or silenced. This may be why all existing lines are not as persistent as the F_1 hybrids or perennial parents. Viable embryos are very difficult to obtain when wheatgrass is used as the female parent. Out of about 120 heads pollinated, we obtained a single plant with cytoplasm from *Th. intermedium*. We are investigating whether wheatgrass cytoplasm will aid the retention of wheatgrass chromosomes important to perenniality.

The easy crossability of wheat with *Thinopyrum* species provides opportunities for the exchange of genetic information between annual and perennial grass species. Both genera have their own pros and cons. We regard perennial wheat a hybrid species that bridges two genera. Putting more wheatgrass chromosomes/genes into wheat or vice versa appears to be the approach to 'truly' perennial wheat. As indicated by MT-2 and our recent investigations, the cross of durum wheat and *Th. intermedium* might be the best approach to explore in the coming years.

Perennial wheat breeding has been conducted for over 80 years. Hundreds of perennial wheat lines have been developed by different institutions. Certainly survival is dependent upon environmental variables, and agronomic practices developed specifically for perennial wheat may be critical to the persistence of perennial wheat. But declining stands in even the most moderate environments leads us to conclude that genetic improvement will be essential to persistence. The lack of sufficient persistence remains the biggest problem in the development of perennial wheat. The existing lines (with one set of wheatgrass chromosomes) have partly inherited the traits (e.g. large roots, tolerance to abiotic and biotic stresses) that make perennial wheatgrass persistent across varied environments. Before we have 'truly' perennial wheat, the use of these traits could yield some agronomic and ecological benefits for the time being. For instance, recent study of hybrid lines revealed novel disease resistance (Turner *et al*. 2013). Although current lines lack perenniality, their grain traits are similar to wheat. Therefore, these types could be economically viable in the near term while reliably perennial wheat varieties are developed.

Intermediate Wheatgrass Breeding in Kansas

Selection at the Land Institute has been based on individual plants spaced 0.91 metres apart. The first selection cycle comprises 1 000 clones replicated three times. The second selection cycle used about 4 000 individual plants. After two cycles of selection primarily for seed yield per head and seed mass, the resulting populations were planted in a replicated solid-seeded trial at two locations. For controls, the base population (consisting of seed obtained from the Rodale Institute) and several forage varieties were used. For simplicity we are presenting data from the irrigated location in the second year of production.

Two cycles of selection resulted in a 77 percent increase in seed yield (Table 1). If progress were to continue in a linear manner, an additional 12 cycles of selection would result in yields of approximately 2 500 kg ha^{-1}, similar to annual wheat in Kansas. Since this evaluation was initiated, an additional two cycles of selection have been performed, requiring two years per cycle. Therefore, we expect that with sustained efforts yields in Kansas may be similar to wheat within 20 years.

Seed size has responded weakly to index selection in space plants, when evaluated in a solid stand (Table 1). If trends from the first two cycles continue, about 110 years of sustained effort would be necessary to achieve a seed size of 30 mg seed^{-1}, similar to annual wheat. Therefore, large seed size may only be attained by using other approaches. We are currently beginning efforts to introgress genes controlling seed size from wheat. Also, genomic approaches may be useful in screening diverse collections for alleles conferring increased seed size.

TABLE 1. PERFORMANCE OF INTERMEDIATE WHEATGRASS POPULATIONS BEFORE (CYCLE 0) AND FOLLOWING ONE (CYCLE 1) AND TWO (CYCLE 2) CYCLES OF SELECTION

Three intermediate wheatgrass cultivars (Luna, Manska, and Rush) and one tall wheatgrass cultivar (Jose) are included as controls.

	SEED YIELD	BIOMASS YIELD	SEED MASS	HARVEST INDEX	CP[a]	NDF[a]	ADF[a]
	$g\ m^{-2}$		$mg\ seed^{-1}$	%	$g\ Kg^{-1}$		
Cycle 0	39.2	1 770	3.92	2.21	37.4	687	465.3
Cycle 1	56.5	1 920	4.48	2.94	30.6	680	461.1
Cycle 2	69.3	1 740	4.84	3.93	33.5	662	444.5
Luna	17.8	1 480	4.10	1.22	45.0	630	447.5
Manska	21.6	1 610	3.41	1.35	37.0	653	435.7
Rush	26.3	1 730	3.61	1.55	34.5	655	440.6
Jose	6.6	1 500	4.06	0.46	44.7	683	478.7
SEM[b]	7.4	120	0.15	0.39	3.0	11	8.2

a Crude protein (CP), neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined for the non-seed biomass collected at seed maturity.
b Standard error of the mean.

Biomass production has remained mostly unchanged during selection for seed traits (Table 1). Therefore, rising grain yields are apparently occurring due to increased harvest index. In fact, across the populations evaluated the correlation between grain yield and harvest index was 0.996.

Forage quality indicators of the biomass residue were measured to determine whether selection for seed production was reducing forage quality (Table 1). So far the effects of selection have been minor relative to forage varieties, at least when measured at the late stage when seed is ripe. However, we expect that increased allocation of nitrogen to seed will ultimately reduce protein content of the residue. For systems involving both grain and forage production, selection for forage quality at seed maturity may be necessary.

During the third cycle of selection in space-planted nurseries, several individuals with outstanding yield per head were identified. A second set of plants with large seed size were also selected. These two groups of plants were cloned by dividing their crowns and used to establish bordered plots 0.91 metres square at two locations: Kansas and Minnesota. The Kansas location has higher summer temperatures, but was irrigated. The Minnesota location was not irrigated. Although the plants were selected in the Kansas environment, difference in seed yield between selected and unselected types was larger at the more moderate Minnesota location (Table 2). Total aboveground biomass was similar among genotypes, and higher than potential perennial biofuel crops for this region (Parrish and Fike, 2005). In Minnesota, the largest-seed genotypes had seed yield more than 200 g/m^2 (Table 2). These yields were obtained from the best genotypes in small hand-harvested plots, but they indicate that there is potential to obtain substantial seed yields from this perennial grass.

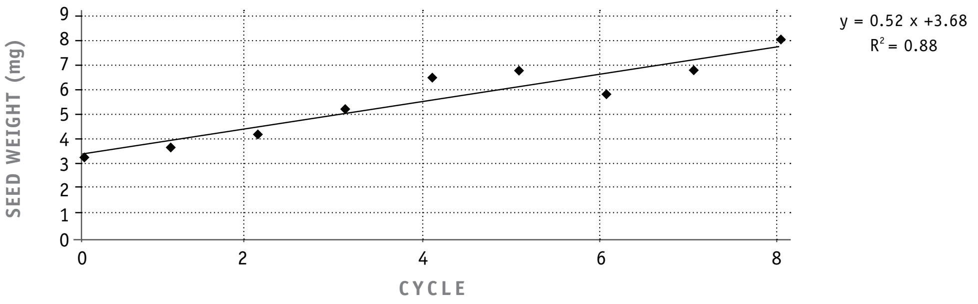
TABLE 2. PERFORMANCE OF INTERMEDIATE WHEATGRASS AT TWO LOCATIONS IN THE SECOND YEAR

Sets of clones (seed weight and seed yield) were compared with the starting population (Cycle 0) and a forage cultivar (Clarke). Predicted means from a mixed model, ± standard errors, are presented.

POPULATION	BIOMASS				SEED YIELD				SEED WEIGHT			
	MINNESOTA		KANSAS		MINNESOTA		KANSAS		MINNESOTA		KANSAS	
	g m^{-2}								mg seed^{-1}			
Cycle 0	1 690	±160	1 650	±160	84	±14	68	±14	5.73	±0.39	4.10	±0.39
Clarke	2 000	±160	2 170	±160	117	±14	49	±14	5.10	±0.39	3.73	±0.39
Seed Weight	2 380	±220	1 660	±110	212	±19	88	±10	9.26	±0.87	7.57	±0.66
Seed Yield	1 830	±140	1 810	±100	192	±12	100	±9	7.60	±0.68	6.07	±0.62

Since 2003 we have been performing mass selection for seed size. Initially, we began by harvesting a set of plants and obtaining the naked seed by floating off seed remaining in hulls. The naked seed was then passed through a set of sieves to obtain the largest seeds. These were planted, and then the process was repeated in the following year. After three cycles of selection, we began using a machine to automatically weigh each naked seed and sort out the largest seeds. We became concerned that this selection method might cause increased see sterility, since seed number and size are often inversely correlated (Sadras, 2007). So beginning with the fifth selection cycle we threshed each plant separately to obtain a seed yield per plant. Seed from the lowest-yield plants was discarded prior to sorting out the largest naked seed for selection. The selection intensity has varied from year to year, depending on seed yield. But in the later selection cycles about 120 plants have been established each cycle, selected out of about 40 000 naked seeds harvested. Seed size has fluctuated over the years depending on growing conditions and selection protocol. But the trend of increasing seed size has been strong (Figure 1). Eight cycles of selection have more than doubled seed weight when grown in a spaced plant selection nursery.

FIGURE 1. RESPONSE OF INTERMEDIATE WHEATGRASS TO MASS SELECTION BASED ON INDIVIDUAL SEED WEIGHT



Intermediate wheatgrass breeding in Manitoba

In the spring of 2011, a 4 500 plant nursery was established at the Carman, Manitoba research farm of the Department of Plant Science at the University of Manitoba. Accessions from the Germplasm Resources Information Network (GRIN) system of the United States Department of Agriculture (USDA) plus advanced generation materials from the Land Institute were planted.

The winter of 2011 to 2012 was relatively short with snowfall arriving in early December 2011 and snowmelt completed by 13 March 2012 on our Carman, Manitoba plots. For six days beginning on 19 March 2012, the daytime high was at least 19.9°C on four days with low temperatures above 0°C for the six days. This was followed by a four day period beginning 9 April 2012 where low temperatures were -5.5°C or lower. Approximately 50 percent of the 4 500 plants in the field sustained severe die-back. An additional 15 percent lodged prior to flowering, mostly due to reduced crown density caused by low temperature damage. The winter of 2012 to2013 was by comparison long and cold. Overnight lows below 0°C began on 31 October 2012 and lasted until 25 April 2013. Snow cover remained until 6 May 2013. An additional 15 percent of the remaining plants were lost due to these winter conditions. We have now experienced two sets of vastly divergent winter conditions and we now feel that we have excellent materials from which to base our selections. Yield stability will be evaluated utilizing the yields from the first two harvests (2012 and 2013). In 2013, some plants were removed from the nursery due to visually high levels of ergot.

Selection has taken place on the materials received from The Land Institute. A subsample of 100 plants was chosen at random to encompass the range of plant morphology within the nursery to investigate yield and its components. A comparison of the two sources indicates that the increased grain production has resulted in a higher harvest index (Figure 2). This has in part been achieved by a reduction for reduced spread (Figure 3). A major result of selection appears to be the increase in the seed yield per unit area of the plant (Figure 4). Initial selection will be based upon consistency of yield between years with an emphasis towards high yield per unit area. This would appear to lead towards a reduced plant size (at least plant area) and potentially higher yields in solid-seeded plantings.

Plant establishment greatly impacts competitiveness. The ability to rapidly emerge from the soil and compete with other species will impact productivity. Six half-sibling families were selected to study the effect of seed size on germination and emergence.

FIGURE 2. RELATIONSHIP BETWEEN HARVEST INDEX AND SEED YIELD PLANT^{-1} IN IMPROVED AND NON-IMPROVED INTERMEDIATE WHEATGRASS

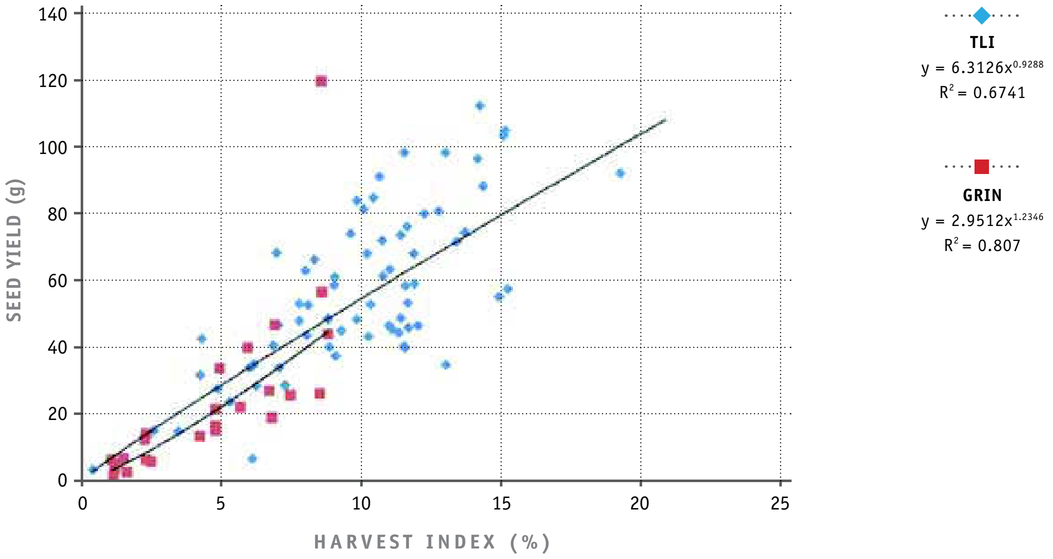


FIGURE 3. RELATIONSHIP BETWEEN PLANT AREA AND SEED YIELD PLANT^{-1} IN IMPROVED AND NON-IMPROVED INTERMEDIATE WHEATGRASS

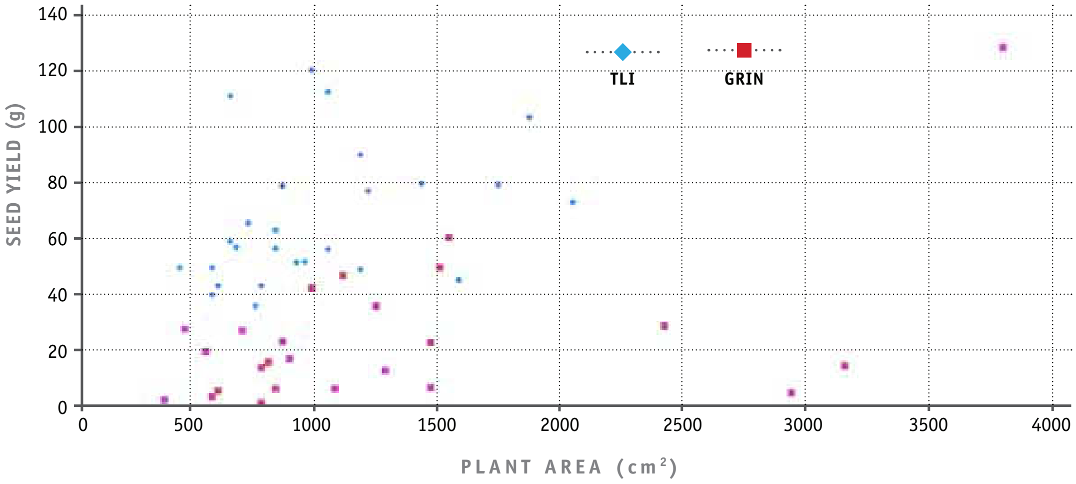


FIGURE 4. RELATIONSHIP BETWEEN PLANT AREA AND SEED YIELD CM^{-2} OF PLANTS IN IMPROVED AND NON-IMPROVED INTERMEDIATE WHEATGRASS

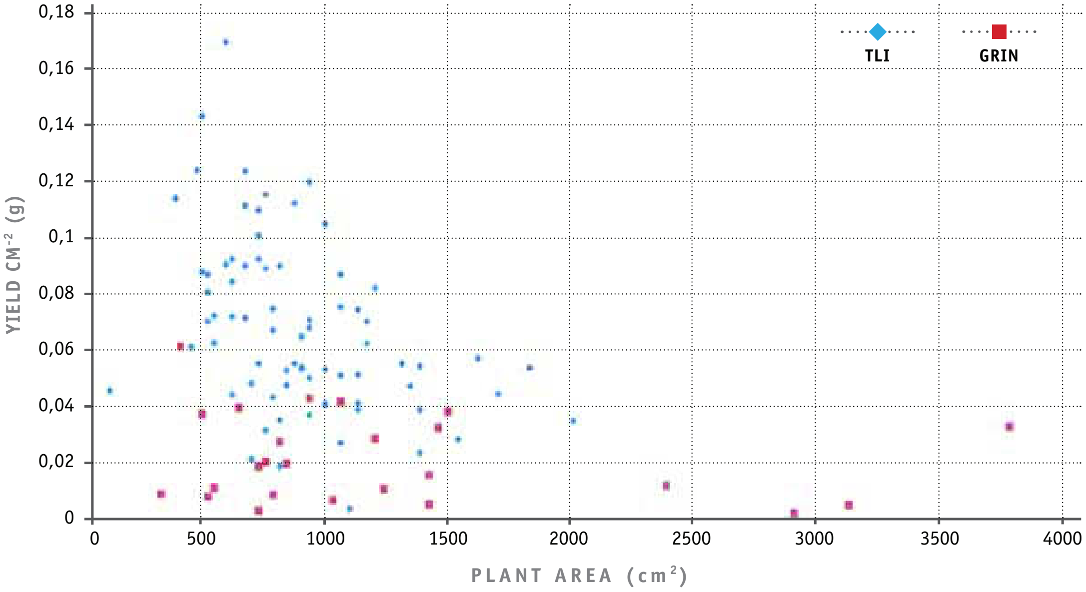
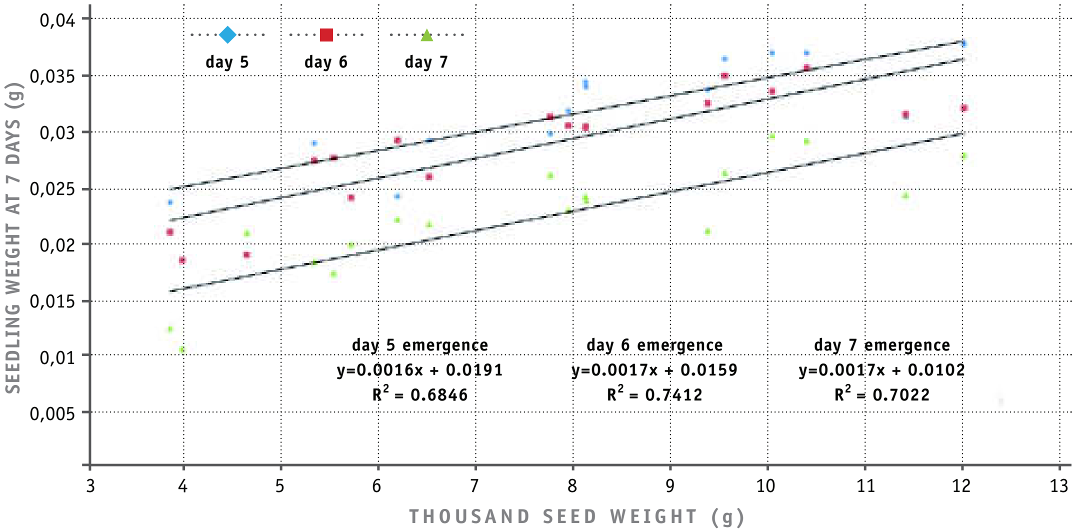


FIGURE 5. RELATIONSHIP BETWEEN THOUSAND SEED WEIGHT AND INITIAL BIOMASS ACCUMULATION IN INTERMEDIATE WHEATGRASS IN THE SEVEN DAYS AFTER EMERGENCE



Germination tests were conducted to ascertain seed viability, and emergence tests from a 2 cm depth (both field and greenhouse) were used to study relative ability to establish. Correlation between the germination tests and greenhouse emergence was high (r = 0.901), while emergence in the greenhouse and field was r = 0.781. Field emergence and germination tests showed the lowest correlation (r = 0.623). Emergence was not necessarily related to seed weight and the ability of plants to amass dry weight was relatively uniform for the first three days of emergence (Figure 5). The earlier the emergence, the greater the seedling weight after seven days of growth (Figure 5). This may be attributed to less energy being expended during emergence, thus resulting in a higher intercept value. Alternatively, early-emerging seedlings may possess genes contributing to rapid growth both before and after emergence.

Improving intermediate wheatgrass quality

Intermediate wheatgrass grain is currently inferior to wheat for most potential uses. Many of the limitations are due to a small grain size and the resulting high fibre and protein of whole grain flour. Small seed produces a low milling yield of white flour. Small grain size is being addressed through breeding, but an additional limitation is the low gluten quality that limits utility in raised breads. We have found that the low gluten quality of intermediate wheatgrass may be due to a very low quantity of HMW-GS.

In fact, HMW-GS genes have been transferred from intermediate wheatgrass to wheat to improve quality (Li *et al.* 2013; Niu *et al.* 2011). Therefore, we investigated the diversity and potential function of HMW-GS alleles in intermediate wheatgrass. Using gene cloning, protein separation and identification, and sequence alignments, we were able to identify five HMW-GS genes and their allelic variants in intermediate wheatgrass plants. Because the species is diverse and heterozygous, one or two allelic variants of each gene were detected in each plant, and an average of five HMW-GS proteins were found in individual plants.

Protein chemistry has demonstrated that all the identified HMW-GS were involved in protein polymers. Sequence alignments showed that two genes share high identities (>96 percent) with the HMW-GS genes from the D-genome of common wheat. We predict that with selection for high expression of superior allelic variants, HMW-GS might contribute to the bread-making quality of intermediate wheatgrass.

Intermediate wheatgrass molecular work

EST markers were developed for allohexaploid intermediate wheatgrass (*Thinopyrum intermedium*) and three closely related diploid species *Pseudoroegneria spicata, Thinopyrum bessarabicum*, and *Thinopyrum elongatum* (Table 3).

TABLE 3. DESCRIPTION OF ESTS AND EST MARKER RESOURCES FOR INTERMEDIATE WHEATGRASS

	P. spicata	*Th. intermedium*	*Th. elongatum*	*Th. bessarabicum*
Platform	Sanger	Roche 454	Roche 454	Roche 454
Reads (avg. bp)	16 128	566 681 (432)	447 936 (421)	364 193 (426)
Contigs (avg. bp)	6 450 (1 017)	29 851 (637)	30 858 (650)	15 700 (701)
Singletons (avg. bp)	2 330 (670)	45 760 (374)	38 216 (365)	22 709 (348)
EST isotigs (avg. bp)	8 780 (924)	75 611 (478)	69 074 (492)	38 409 (492)
Total bp	8 120 750	36 153 671	34 024 034	18 928 290
EST-SSR markers	1 375	672	596	384
Hv1[a]	152	98	70	68
Hv2	185	109	87	45
Hv3	172	106	90	54
Hv4	176	100	87	55
Hv5	204	98	95	77
Hv6	158	70	76	41
Hv7	187	91	71	44

a Correspondence of EST-SSR markers to the seven barley chromosomes (Hv1 to Hv7).

It is thought that the three subgenomes of allohexaploid intermediate wheatgrass may include one *Pseudoroegneria* (**St**) genome and two *Thinopyrum* (**E**) genomes similar to *Th. elongatum* ($\mathbf{E}^{e}$) and/or *Th. bessarabicium* ($\mathbf{E}^{b}$) genomes (Liu and Wang, 1993; Zhang *et al.* 1996; Zhang *et al.* 1997). A total of 1 375 Pspi EST-SSR primer pairs were previously developed from *P. spicata* (Table 3) (Bushman *et al.* 2008). Another 1 652 EST-SSR markers were recently designed from Roche 454 ESTs from intermediate wheatgrass, *Th. elongatum*, and *Th. bessarabicium* (Table 3). All of the *Thinopyrum* EST-SSR markers were designed from ESTs corresponding to one of the seven barley (Hv) chromosomes (Table 3). Likewise, most of the previously designed *P. spicata* EST-SSR markers also correspond to one of the seven barley (Hv) chromosomes (Table 3) based on alignments to the barley genome sequence (The International Barley Genome Sequencing Consortium, 2012).

The *P. spicata* and intermediate wheatgrass EST-SSR primers have been tested for amplification and/or polymorphism in intermediate wheatgrass and related species. Approximately 79 percent (1 083) of the *P. spicata* EST-SSR primers amplified products from *P. spicata* (Bushman *et al.* 2008). More recent experiments also showed that about 80 percent (1086) of the *P. spicata* EST-SSR primers amplified products from both *P. spicata* and intermediate wheatgrass. Similarly, about 84 percent (564) of the intermediate wheatgrass EST-SSR primers amplified products from intermediate wheatgrass. Moreover, about 68 percent of the intermediate wheatgrass EST-SSR primers amplified products from Chinese Spring wheat.

A combination of EST-SSR and AFLP markers will be used to genotype an experimental mapping population comprises 268 full-sib progeny derived from reciprocal crosses of two experimental genotypes, M35 × M26 (137) or M26 × M35 (131). The M26 genotype derives from a cross of C3-3471 × C3-3941. The C3-3941 genotype was also the female parent of M35. So far, six AFLP primers have also been genotyped on M26, M35, C3-3471, C3-3941 and 268 full-sib mapping progeny. These six AFLP primers detected 133 AFLP markers that were polymorphic between M35 and M26 with an average marker frequency of 0.52 among the 268 progeny. These six AFLP primers also detected 75 AFLP markers that were present in both M35 and M26 with an average marker frequency of 0.76 among the 268 progeny. Thus, a total of 208 polymorphic markers showing expected 1:1 or 3:1 segregation ratios were detected using the first six AFLP primer pairs. Another 13 AFLP markers that were present in both M35 and M26 showed marker frequencies greater than 0.9, which could be the result of segregation distortion or non-disomic inheritance. However, initial results were largely consistent with disomic inheritance. Although the mapping population has not yet been genotyped with EST-SSR markers, the M26, M35, C3-3471, and C3-3941 parents and grandparents have been screened using the 672 intermediate wheatgrass EST-SSR markers. Nearly 17 percent of the 564 amplified markers showed clear polymorphism between the M26 and M35 parents of this intermediate wheatgrass mapping population. Thus, we estimate that approximately 440 EST-SSR markers will be informative if this rate of polymorphism applies to *P. spicata*, *Th. elongatum*, and *Th. elongatum* EST-SSR markers.

Genotyping-by-sequencing (GBS) combines molecular marker discovery and genotyping and has been successfully used in species with large, complex genomes, such as barley and wheat. Intermediate wheatgrass is an allohexaploid with an estimated 14 GB genome and no reference genome. The combination of a large and understudied genome makes genetic studies and genomic assisted breeding for intermediate wheatgrass challenging. To address these constraints, we are utilizing the GBS approach to discover SNPs, and then using them to construct a genetic map and map important agronomic traits.

GBS involves simultaneously sequencing DNA from many individuals, but only specific sites in the organism's genome. The GBS approach increases the sequence coverage per site and dramatically reduces the sequencing cost per individual. These specific sites are targeted by restriction enzymes; for intermediate wheatgrass, we are using the restriction enzyme *PstI*, which identifies all sites in the genome with the DNA sequence 'CTGCAG' and cuts the DNA between the A and G nucleotides. A DNA barcode (unique 5-10 bp sequence) and barcode adapter are attached to the DNA fragments from each individual. DNA from many individuals is then pooled and sequenced as if it were from one individual (we sequence 96 individuals at a time). DNA is currently sequenced using Illumina's HiSeq platform and SNPs are called using the Universal Network Enabled Analysis Kit (UNEAK) bioinformatics pipeline.

The genetic map analysis began with ~3000 high quality SNPs from 285 selfs, part of a larger population of ~750 selfs from one individual. Intermediate wheatgrass is primarily outcrossing, but a large effort was concentrated on selfing an individual with an important set of agronomic traits: free-threshing, large seed and non-shattering. Preliminary phenotypic analyses suggest that these traits are correlated, dominant, and segregating in the selfed population. The framework map has been constructed with MapMaker and includes 859 SNPs from 271 individuals, divided into 22 linkage groups (there are 21 chromosomes). One small linkage group shows linkage with two larger linkage groups, but cannot be precisely placed. Preliminary analyses suggest that GBS can be utilized in intermediate wheatgrass for *de novo* genotyping to develop high-density genetic maps and genomic selection models for crop improvement.

Genetic studies of intermediate wheatgrass are complicated by the plant's high degree of heterozygosity, hexaploid nature, and self-incompatibility. Future work would be simplified by access to completely inbred individuals. We have been attempting to obtain fully homozygous individuals by producing doubled haploids through another culture. So far approximately 20 000 anthers have been cultured. Hundreds of calluses have formed. Dozens of calluses have initiated shoots, but most have been albino. We have obtained 5 plantlets that are green. The haploid nature of three young plants has been confirmed, and we are attempting to double their chromosome numbers with colchicine.

CONCLUSIONS

"Perennial wheat" hybrids and intermediate wheatgrass are currently at far ends of a spectrum. While the hybrid types have good grain yield, large seed, and other favourable traits, they remain functionally annual in most environments. On the other hand, intermediate wheatgrass is a vigorous perennial that is easily grown in diverse environments. But it lacks adequate seed yield, seed size, and grain quality to be commercially successful. Working from both directions, we expect that these programs will meet somewhere in the middle. Along the way, much of what is learned and developed in one approach will be useful to the other. For instance, genetic maps developed for intermediate wheatgrass will be critical for perennial wheat development. Understanding the physiology, morphology, phenology, and genomic functioning of perennial wheatgrass will be useful to those developing perennial wheat. Similarly, much that is known about the quality, pathology, yield, and genomics of wheat is being readily applied to intermediate wheatgrass.

We should not choose between the strategies of wide hybridization or domestication in the development of perennial small grains. The most rapid progress will be made by simultaneously working from both ends of the spectrum in order to produce perennial grain crops. Whether the perennial grain of the future will be wheat with grass-like traits added or a perennial grass with the addition of wheat-like traits is a question that we need not dwell on. What is clear is that progress is being made at an accelerating pace toward the day when useful, high-yield, long-lived small grains are a reality.

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07

VIEWPOINT: MULTIPLE-HARVEST SORGHUMS TOWARD IMPROVED FOOD SECURITY

Andrew H. Paterson[1], *T. Stan Cox*[2], *Wenqian Kong*[1], *Maria Navarro*[3]

1 Plant Genome Mapping Laboratory, University of Georgia, Athens GA, USA
2 The Land Institute, Salina KS, USA
3 Department of Agricultural Leadership, Education, and Communication, University of Georgia, Athens GA, USA
Correspondence to: Paterson@plantbio.uga.edu

ABSTRACT

Perennial crops offer an avenue by which marginal lands might be brought into sustainable production while arresting or even reversing losses of ecological capital. The natural adaptation of sorghum to its native Sahelian region makes it essential to some of the poorest and most food-insecure human populations, dependent upon some of the world's most degraded soils. Increased demand for limited fresh water, together with rising global temperature and aridity, suggest that the inherent drought tolerance of sorghum will be of growing importance. With some genotypes already known to be suitable for ratoon crop production, and with two sources of perenniality, the *Sorghum* genus has become a model for dissecting the molecular control of ratooning and perenniality. We are pursuing two broad approaches to investigate the efficacy of ratoon/perennial sorghum production and to develop genotypes suitable for these production systems. First, the identification of diploid progeny from crosses between *S. bicolor* and *S. propinquum* offers a starting point for investigation of these traits in conventional sorghums, by crossing to elite sorghums and evaluation of resulting progeny (potentially also employing DNA-marker aided selection to increase the frequency of genotypes that contain known Quantitative trait loci (QTLs) for ratooning and/or perenniality-related traits). Second, crosses between tetraploid forms of *S. bicolor* and their naturally-tetraploid relative *S. halepense*, may offer the possibility to benefit from many traits that differentiate this widespread weedy/invasive plant from its progenitors. While initial efforts have focused on grain production, substantial forage/biomass is also produced by ratoon/perennial sorghums and in principle there appears to be no insurmountable obstacle to also developing perennial 'sweet sorghums' suitable for sugar production. Perennial cropping systems may be especially attractive to smallholder agriculture (particularly benefitting women farmers), in which poor seedling emergence and stand establishment can lead to crop failure and costs of seed, energy, and equipment are limiting factors. However, the benefits of perennials may also apply to intensive agriculture, especially in situations where erosion, nutrient leaching, and declining organic matter levels occur. A fringe benefit of increased knowledge of perenniality may be new tools and approaches by which to control widespread agricultural weeds. Comparative data from sorghum and rice suggest that findings with regard to perenniality may extrapolate broadly to a wide range of taxa, accelerating progress in implementing new production systems (or weed control systems) in other genera.

Keywords: ratoon crop, perennial, *Sorghum propinquum, Sorghum halepense*

INTRODUCTION TO SORGHUM

About 20 million years ago , a population of 20-chromosome grassy plants had a singularly bright future. An early branch diverged from this population ~10-15 million years ago, experiencing genome-wide duplication and genome size expansion accompanied by chromosome number reduction leading to the modern maize genome (Gaut, Clark, *et al.* 1997; Swigonova, Lai, *et al.* 2004). About 8-9 million years ago (Jannoo *et al.* 2007; Sobral *et al.*

1994) another branch diverged, also experiencing genome-wide duplication and leading to the modern genomes of Saccharum. It was perhaps the most important biomass/biofuels crop worldwide, and its close relative *Miscanthus*, which is among the highest-yielding herbaceous temperate biomass crops known (Heaton, Dohleman, et al. 2008). A third branch spawned lineages with a wide range of fates (Spangler *et al.* 1999). One being domesticated into cultivated sorghum, and another experiencing polyploidy to form one of the world's most aggressive weeds (*Sorghum halepense*).

Among these important grasses, all members of the Andropogoneae clade, the genome of *Sorghum bicolor* L. Moench (sorghum) is of central importance both as a botanical model and as a major crop. Sorghum is the most drought-resistant of the world's top five cereal crops, and an important dual-purpose (grain + straw) crop. Its drought resistance is essential in the United States Southern Plains that often receive too little rain for other grains. And in arid countries of northeast and West Africa, sorghum contributes as much as 26-39 percent of calories in the human diet (www.fao.org). Nonetheless, sorghum improvement lags that of maize, wheat and rice, each of which have more than doubled in worldwide average yield in the last 38 years. Sorghum yields only gained 51 percent (www.fao.org). Increased demand for limited fresh water, together with rising global temperature and aridity, suggest that sorghum will be of growing importance.

While grain is of special importance, the value of other sorghum products should not be underestimated. Sweet sorghum for stock feed (indirectly sustaining protein production) is a large and growing market, and the possibility of production systems based on perenniality combined with low/no-till farming are very attractive. Further, bioenergy sorghums are a promising source of cellulosic ethanol (Rooney *et al.* 2007). They are even better suited to tropical regions (such as much of Africa) than temperate regions, because a more continuous supply of feedstock is possible in the tropics.

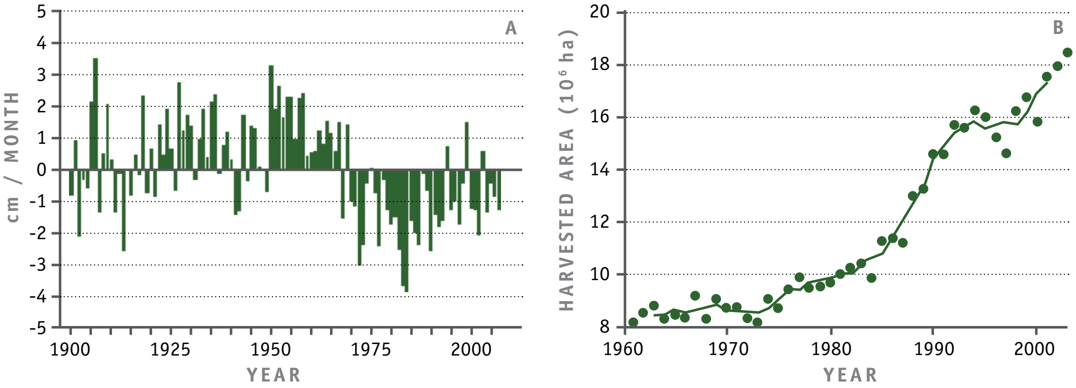
The sequenced ~740 Mb sorghum genome (Paterson *et al.* 2009) is a logical complement to that of *Oryza* (rice) for grass functional genomics. It is a representative of tropical grasses in which biochemical and morphological specializations ('C4' photosynthesis) improve carbon assimilation at high temperatures. The sorghum karyotype is suggested by parsimony to have been shared by the Andropogoneae common ancestor, with only ~3 percent differential sorghum-rice gene loss and less sorghum-rice structural rearrangement in 50 million years than sorghum-maize rearrangement in about 15 million years (Bowers *et al.* 2003). With 70 million years of 'abstinence' from genome duplication, functions of *S. bicolor* genes also may still resemble those of the common cereal ancestor.

Sorghum evolution and improvement have yielded a remarkable diversity of morphologies. Differences in inflorescence and seed morphology are used to classify *S. bicolor* races. Also they have been embellished by divergent selection applied to forms cultivated for purposes ranging from 'broomcorn' to the compact inflorescences and large seed of grain types. Divergent selection has also affected plant stature, with stalks of up to 6 metres being a key building material in Africa, but less than 1 metre favoured for mechanized harvest. Selection for carbohydrate rich seed/grain has increased harvest index (carbon partition to grain) in grain types, while selection for sugar-rich stalks and/or total biomass has reduced seed size and output in others to an extreme exemplified by *S. propinquum* (Figure 1). Human selection has been superimposed on rich natural variation in plant architecture and carbon allocation, for example ranging from one to 100+ tillers per crown, and branching along a tiller ranging from none to secondary, tertiary, and even quaternary. Its morphological and biochemical diversity makes sorghum the only crop that is compatible with fuel production from each of three routes, including starch (seed), cellulose (stalks), and sugar ('sweet' sorghums have sugar yields comparable to those of sugarcane).

FIGURE 1. COMPARISON OF ANNUAL *SORGHUM BICOLOR* AND PERENNIAL *S. PROPINQUUM*

(A) Crowns – including extensive rhizomes of *S. propinquum*.
(B) Seeds – those of *S. bicolor* grain types are large and carbohydrate rich, while those of *S. propinquum* are very small.

FIGURE 2. (A) SAHEL PRECIPITATION, 1900-2007; (B) SAHEL CROPPING AREA, 1960-2010



A: modified from Haywood *et al.*, 2013
B: *source*: Kandji *et al.*, 2006

Rich resources are in place to link sorghum phenotypic diversity to its molecular basis. More than 30 sorghum genomes and 40 transcriptomes, sampling all botanical races of cultivated sorghum, wild *S. bicolor*, and appropriate outgroups, have now been sequenced by the United States Department of Energy Joint Genome Institute. GBS has been performed on several reference collections (Morris *et al.* 2013), including two developed by the International Crops Research Institute for the Semi-Arid-Tropics (ICRISAT) that have been focal points of phenotyping for morphological variation, yield components, and drought responses (Upadhyaya *et al.* 2009).

OVERVIEW OF RESEARCH ON PERENNIALITY IN SORGHUM

The natural adaptation of sorghum to its native Sahelian region makes it essential to some of the poorest and most food-insecure human populations and dependent upon some of the world's most degraded soils. Years of above average rainfall were followed by drought in the Sahel starting in the late 1960s (Figure 2A). A dramatic increase in the Sahelian area devoted to crops began at that time, with falling yields necessitating greater effort to cultivate ever-more fragile lands to feed growing populations (Figure 2B). These considerations are thought to contribute greatly to the fact that the Sahel now includes some of the most degraded lands on the planet.

Recently, the potential benefits of perennial grain crops have gained wider recognition (Glover *et al.* 2010). Perennial crops are essential to bringing marginal lands into sustainable production (Cox *et al.* 2002; Scheinost, 2001; Wagoner, 1990), maximizing ecosystem productivity (Field, 2001) and minimizing losses of topsoil (Pimentel *et al.* 1995), water, and nutrients. Perennial cover is >50 times more effective than annual crops in maintaining topsoil (Gantzer *et al.* 1990), and 30-50 times more effective than annuals at preventing nitrogen losses (Randall and Mulla, 2001). Perennial cropping systems (Cox *et al.* 2006; Jackson and Jackson, 1999; Piper, 1998) may be especially attractive to smallholder agriculture, in which poor seedling emergence and stand establishment can lead to crop failure, and costs of seed, energy, and equipment are limiting factors. It also may benefit mechanized agriculture in situations where erosion, nitrate (and other nutrient) leaching, and declining organic matter levels occur.

The *Sorghum* genus has become a model for dissecting the molecular control of ratooning and perenniality largely due to the availability of the diploid perennial *S. propinquum* which is abundantly tillering and has rhizomes that contribute to perenniality (Hu *et al.* 2003; Jang *et al.* 2006; Jang *et al.* 2008; Paterson *et al.* 1995). Tillers and rhizomes both develop from axillary buds at the lowermost nodes of the erect leafy shoot of the plant, with acropetally increasing tendency to develop into tillers (Gizmawy *et al.* 1985). There is appreciable correspondence in the locations of QTLs determining tillering and rhizomatousness (Paterson *et al.* 1995), thought to be related to the number of buds available. The commitment of a bud to rhizome development, tiller development, or quiescence is genetically-determined (Paterson *et al.* 1995), and is associated with marked differences in gene expression (Jang *et al.* 2006). Finally, a high level of correspondence of rhizome QTLs between sorghum and rice (Hu *et al.* 2003), thought to have diverged from common ancestry about 50 million years ago, suggests that key genes conferring perenniality-related traits may be shared by many Poaceae cereals. In other words, fundamental information about these traits in one cereal may extrapolate broadly to many other cereals.

While no members of the cultivated species, *Sorghum bicolor,* are rhizomatous, there is variation among cultivars for ratoon crop productivity (Duncan *et al.* 1980; Duncan and Moss, 1987), and close relatives sugarcane and *Miscanthus* are usually grown as ratoon crops. Ratooning (regrowth) is a complex trait, showing quantitative inheritance (Duncan *et al.* 1980). Likewise, QTL mapping in an F2 population of a cross between an elite grain sorghum inbred BTx623 (later used for the reference sequence) and *S. propinquum*, showed regrowth to be closely related to both rhizomatousness and tillering, but was only able to account for 14-30 percent of phenotypic variance in these traits (Paterson *et al.* 1995). A recombinant inbred line population for this cross (Kong *et al.* 2013), now provides for replicated genetic analysis of productivity, and includes genotypes with a range of 'degrees' of ratoon cropping from near-zero to fully perennial. The recombinant inbred line population has been genetically mapped with both the population itself and map publicly available (Kong *et al.* 2013), and with three years of phenotyping for tillering, rhizomatousness, and basic metrics related to grain production (yield components) presently being analysed for peer-reviewed publication.

A relative of sorghum, *S. halepense*, is a wild perennial polyploid resulting from natural hybridization between *S. bicolor* (2n = 20) and *S. propinquum* (2n = 20), a wild perennial diploid native to Southeast Asia and estimated to have diverged from *S. bicolor* ~1-2 million years ago. *S. halepense* finds occasional use as forage and even food (seed/flour), but is most noted as one of the world's most noxious weeds, having spread from its west Asian centre of diversity across much of Asia, Africa, Europe, North and South America, and Australia. Its establishment in the United States of America is probably typical of its spread to other continents, including intentional introduction as a prospective forage and/or unintentional introduction as a contaminant of sorghum seedlots (McWhorter, 1971). However, while *S. bicolor* has remained largely confined to cultivation, *S. halepense* has readily naturalized – revealing genetic potential for adaptation that goes well beyond that of sorghum. *S. halepense* crosses readily with *S. bicolor* (Arriola and Ellstrand, 1996) –more than 90 percent of plants in naturalized *S. halepense* populations growing adjacent to long-term sorghum production fields contain alleles introgressed from sorghum (Morrell *et al.* 2005).

RESEARCH AGENDA

We are pursuing two broad approaches to investigate the efficacy of ratoon/perennial sorghum production, and to develop genotypes suitable for these production systems.

First, the identification of diploid progeny from crosses between *S. bicolor* and *S. propinquum* (Kong *et al.* 2013) offers a starting point for investigation of these traits in conventional sorghums, by crossing to elite sorghums and evaluation of resulting progeny (potentially also employing DNA-marker aided selection to increase the frequency of genotypes that contain known QTLs for ratooning and/or perenniality-related traits).

Second, crosses between tetraploid forms of *S. bicolor* and their naturally-tetraploid relative *S. halepense*, may offer the possibility to benefit from many traits that differentiate this widespread plant from its progenitors. While *S. halepense* is generally under selection for weediness-related attributes that are undesirable in annual row-crop production, some selection pressures may favour *S. halepense* alleles that are of potential value in sorghum, for example conferring rapid vegetative development and early flowering. An attractive but unproven hypothesis, which the ability of *S. halepense* to naturalize in a much wider range of environments than sorghum is cultivated may have been accompanied by selection for alleles associated with valuable attributes such as cold tolerance (for example in the northern United States of America and Russia), drought tolerance (Africa, Arabia, United States of America in the southwest), disease resistance (many) or other traits. Moreover, a host of data now show that unpredictable, presumably epistatic, effects of allele introgressions from seemingly undesirable sources can confer valuable traits to crops (Bernacchi *et al.* 1998a; Bernacchi *et al.* 1998b; Chee *et al.* 2005; Eshed and Zamir, 1995; Fridman *et al.* 2004; Fulton *et al.* 1997; Gur and Zamir, 2004; Schauer *et al.* 2006; Tanksley *et al.* 1996; Xiao *et al.* 1996; Zamir, 2001).

At present, we are phenotyping and analysing F2 populations, their F3 progeny and the progeny of backcrosses to *S. bicolor* for perenniality, plant architecture, yield components and domestication traits. While we find some of these tetraploids to have fecundity comparable to diploid sorghums and are potentially directly usable, another attractive route for their utilization would be to extract diploids with selected properties of *S. halepense* either by backcrossing or by the development of haploids.

IMPLICATIONS

The benefits of perenniality as a cropping system are receiving renewed interest. Perennial plants have four advantages over annuals: a longer growing season; better access to water and nutrients; more conservative use of nutrients and better adaptation to marginal lands (Cox *et al.* 2006). The first three of those characteristics are expressed on all landscapes, from prime farmland to poor or sloping soils unsuitable for annual cropping. The expansion of agriculture to provide plant biomass for production of fuels or chemical feedstocks with little or no carbon release (Fargione *et al.* 2008) will require greater utilization of marginal land (Tilman *et al.* 2009), maximizing ecosystem productivity (Field, 2001) and minimizing losses of topsoil (Pimentel *et al.* 1995), water, and nutrients.

The benefits of perenniality apply to food, as well as biomass, crops. Annual food crops have dominated the agricultural landscape since the time of the earliest farmers. In the last few decades alone, one-third of the planet's arable land has been lost to soil erosion (Pimentel *et al.* 1995). No-till production of annual crops, designed to control soil loss, has so far required increased use of herbicides. Direct-seeded annual cropping systems produce as high or higher nitrate emissions as tillage systems (Mummey *et al.* 1998). 'Natural systems agriculture', based on developing a "domestic prairie" with herbaceous, perennial grain-producing crops grown in polyculture (Jackson and Jackson, 1999; Piper, 1998), has been proposed as a means to produce food on otherwise marginal lands while arresting or even reversing losses of ecological capital.

In smallholder production systems that are widespread in regions where sorghum is a staple in the human diet, conservation-agriculture approaches may particularly benefit women farmers, by reducing dependence on duties generally performed by men (ploughing) and mitigating the need for duties performed by women and children (uprooting grass, de-stumping) (Milder *et al.* 2011). Moreover, women frequently are given marginal fields that should be left fallow (Kevane, 2011), for example those which will be the greatest beneficiaries of multi-cropping approaches. Male farmers, with access to generally higher levels of land, labour, and cash, have a somewhat greater tendency to grow cash crops (Gilbert *et al.* 2005) – further indicating that early adopters of reduced-cost, reduced-labour multi-cropping sorghum production systems may tend to be female. A reduced requirement for seed selection in multi-cropping production systems (which propagate vegetatively), may also reduce male investment as seed selection is largely male-dominated (Freeman and Boateng, 2012).

Perenniality plays two contrasting roles in agriculture, being: (i) a valuable asset in establishment and persistence of dense, productive stands of plants suitable for multiple harvests, and (ii) an essential component of "weediness" of many of our most noxious weeds. Better understanding of the biology of *S. halepense* itself, one of the most noxious weeds affecting world agriculture (Holm *et al.* 1977), may also be of high value. *S. halepense* is a major contaminant in sorghum seed production, an alternate host and means of over-wintering for pests and pathogens of both monocot and dicot crops. It is also a highly-effective competitor for sunlight and other resources, causing reductions in economic yield of 45 percent or greater in crops as diverse as monocots such as sugarcane (Millhollen, 1970) and dicots such as soybean (McWhorter and Hartwig, 1972). The first United States federal appropriation for weed control research targeted Johnsongrass (House Bill #121, 1900), yet it remains largely unchecked today.

The *S. halepense* problem constrains improvement of sorghum through biotechnology. *S. halepense* is a particular problem in and around sorghum fields because no known herbicide can selectively eliminate it without damaging the sorghum. Transformation of sorghum was first accomplished in 1993 (Casas *et al.* 1993), and introduction of transgenes for resistance to insects, diseases, or herbicides could have a major impact on sorghum productivity. However, the risk of transgene escape constrains the ability of regulatory agencies to approve the release of sorghum cultivars with transgenes that could enhance agricultural productivity.

Better understanding of the biology of *S. halepense* and associated new functional genomics tools might aid greatly in identification of genes, regulatory elements, and biochemical functions that are important to its weediness, particularly its rhizomes, but dispensable to other plant processes (Jang *et al.* 2006). Identification of such genes would provide the foundation to search for plant growth regulators that specifically target rhizomes, perhaps providing for control of rhizomatous weeds even in closely related crops such as *S. halepense* in sorghum. Moreover, better understanding of rhizome development may also benefit development of plant genotypes optimal for biomass/biofeedstock production on marginal lands, as well as improvement of a host of forage and turf grasses.

Our demonstration that most QTLs responsible for rhizomatousness in *Sorghum* and *Oryza* respectively, map to corresponding locations (Hu *et al.* 2003), suggests that *information about rhizomatousness from a few models (that are also major crops) may extrapolate broadly to a wide range of taxa*. Successes in clarifying the genetics of perenniality in this genus, may accelerate progress in implementing new production systems in other genera, and also to controlling a wide range of related agricultural weeds.

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08

BREEDING AND GENETICS OF PERENNIAL MAIZE: PROGRESS, OPPORTUNITIES AND CHALLENGES

Seth C. Murray[1,2], *Russell W. Jessup*[1,3]

1 Department of Soil and Crop Sciences, 370 Olsen Blvd, MS2474
Texas A&M University, College Station, TX 77845-2474 USA

2 Email: sethmurray@tamu.edu - Phone: (+1) 979 845 3469

3 Email: rjessup@neo.tamu.edu - Phone: (+1) 979 315 4242

ABSTRACT

Perennial crops have many hypothesized benefits (i.e. intercepted sunlight and nutrients over longer periods, increased yields, decreased soil erosion, improve stability of grain production, etc.) making them attractive for sustainable production. Annual maize (*Zea mays* L.), domesticated ~9 000 years ago, is one of the most important world crops and billions of dollars have been spent towards its improvement. However, there has been minimal effort to breed perennial maize and benefits hypothesized remain untested. Through crosses between annual maize and its two interfertile perennial maize relatives: tetraploid *Z. perennis* and diploid *Z. diploperennis*, maize germplasm and genomics knowledge can be leveraged to develop perennial maize. A few crosses and populations have been made by other investigators, notably Brewbaker, Carlson, Doebley, Holland, and Shaver but no adequate-yielding perennial maize has been developed. Compared with breeding perennial sorghum (a close maize relative) maize appears to have additional recessive quantitative traits that must be pyramided (senescence, tillering, perennial rhizomes/crowns, regrowth, heat and cold tolerance). I have now integrated the aforementioned perennial derived germplasm for nine breeding seasons with additional crosses to *Z. diploperennis* but very few to annual maize. My methodology has involved a cycle of 1) selfing crosses to purge deleterious recessive alleles; 2) selecting at the end of each season for non-senescence, regrowth/tillering and large ear size and 3) crossing between germplasm derived from different sources to pyramid complementary advantageous traits. Dramatic progress in adaptation, ear size, non-senescence and stability has been obtained, but this is confounded with evaluation environments. As of yet, we have not found a derived plant that oversummers, overwinters and produces rhizomes in Texas as the wild species do. A number of novel phenotypes have been observed including "ear forest", clumping grass types versus large tillered stalks, and prolificacy. A "one-best" ideotype for perennial maize still remains elusive.

Keywords: perennial, *Zea*, maize, sorghum, breeding, *Z. diploperennis*

INTRODUCTION

Nearly all domesticated grain crops are annuals that senesce and die after reproduction within the growing season in which they were planted, or they are managed as annuals, as in the case of sorghum, which is weakly perennial in warm climates. Perennials unlike our annual crop plants have an ability to overwinter and generally survive three or more successive years. Despite great successes in production, annual grain systems suffer from negative externalities including high input costs, soil erosion, and a limited growing season. Specific perceived economic and environmental benefits of perennial crops include increased vegetative cover over longer growing periods leading to increased photosynthate assimilation, decreased planting costs and reduction of energy-intensive inputs. Expanded root architecture of perennial crops could increase soil sequestration of carbon, further decrease off-season soil erosion, increase temporal access to water and nutrients among many other benefits. Perennial crops are one likely approach to improving sustainable food, feed, and fuel production with decreased land availability and access to inputs under a changing climate (Pimentel *et al.* 2012). Pastures and grasslands, which are generally managed as perennial systems, demonstrate improvements in critical ecosystem services; however, they do not produce grain needed for food, feed, and fuel uses. Despite these benefits the successful development of perennial grain crops has been sparse and largely limited to a few C3 grasses such as rice (Sacks *et al.* 2003, 2006), wheat (Scheinost *et al.* 2001; Murphy *et al.* 2009), and intermediate wheatgrass 'Kernza' (Cox *et al.* 2002; Strand 2010).

Among the productive and widely grown grain crops that humans have domesticated is maize (*Zea mays* L.). Genetic/breeding and agronomic improvement of maize production in the United States of America and the rest of the developed world has been incredibly successful, increasing production as much as 800 percent from a century ago; meaning eight times less land is needed to produce the same amount of grain (Brummer *et al.* 2011). While yields continue to increase at 1-2 percent in areas of the Midwestern United States of America with the heaviest investment, they have remained stable over the last 10 years in lower yielding and lower investment areas such as Texas (Barerro *et al.* 2013). In the developing world, yields have also not increased at the same rate, which is likely due to both lack of agronomic inputs (fertilizer and irrigation) and limited genetic improvement of the crop. Maize could provide the most immediate impact from the development of perennial cultivars if these cultivars would maintain or increase productivity while providing improved ecosystem services and/or decreased costs of production and allow mitigation and improved adaptation to climate change.

PERENNIALISM ACROSS THE GRASSES

From a comparative breeding, physiology and developmental perspective, it is important to note that all C4 grass crops have close perennial relatives within their genus or are perennial themselves. Sorghum (*Sorghum bicolor* L. Moenech) is an important grain, forage, sugar and bioenergy crop grown as an annual that is also closely related to maize. *Sorghum propinquum* and *Sorghum halepense* (also known as Johnsongrass) are both perennial. Close relative Sugarcane (*Saccharum officinarum* L.), is already grown and managed as a perennial. Close relative pearl millet (*Pennisetum glaucum*) has perennial relative Napier grass (*Pennisetum purpureum)*. Napier grass, miscanthus (*Miscanthus giganteus)*, switchgrass (*Panicum virgatum*) and giant reed (*Arundo donax* L.) are undergoing domestication and improvement for forage, restoration, and bioenergy systems specifically because they are perennial (Lewandowski *et al.* 2003; Jessup 2009). Generating and synthesizing information from across all of these species will likely yield the most fruitful understanding of improving perennial phenotypes. Among these species, *Zea* and *Sorghum* provide the most complementary applied and research targets for perennialism with their wild relatives. Maize and sorghum are the 1st and 5th most important cereal crops worldwide, respectively (FAOSTAT, 2013). These crops also have active breeding and genetics programs throughout the world and have extensive genomic information available. Genome annotations have been completed for *Z. mays* (Schnabel *et al.* 2009; Rokhsar *et al.* 2009; Vielle-Calzada *et al.* 2009) and *S. bicolor* (Paterson *et al.* 2009). Because little is known of the molecular mechanisms conditioning the annual/perennial divide across grass species and given the complexity of the trait, molecular dissection would likely be fruitful.

WHAT IS PERENNIAL MAIZE?

Maize has two perennial relatives in its genus; *Zea perennis* (tetraploid – Mexico), *Z. diploperennis* (diploid –Mexico). One of the first reports of crosses within a *Zea* perennial species was that of Emerson and Beadle (1929) who crossed maize to what we now know as *Z. perennis*. Mangelsdorf and Reeves (1939) went further in the investigations of multiple maize crosses with *Z. perennis* and eastern gamagrass, a temperately-adapted, perennial native grass in the genus *Tripsacum* that resides in the tertiary gene pool of maize. Shaver (1964) was one of the first to seriously investigate the possibility of creating perennial domesticated maize. Shaver continued Emerson and Beadles populations and also crossed *Z. perennis* with colchicine doubled maize to investigate the genetic inheritance. From these crosses, Shaver derived perennial 4N (tetraploid) and 2N (diploid through pollen sieving) populations. The 2N, however, appeared to nearly lose the perennial phenotype, likely because of the complex suite of genes/alleles needed. The 4N and 2N populations have now been selected for over 20 generations each and have been shared with many other investigators (Shaver, pers. comm.). While many investigators have given up on these populations, a few have gone on to report interesting traits such as nodal propagation (Ting and Tran, 1999). Nodal propagation is a trait clearly possible with perennial sugarcane, which is how cultivars of sugarcane are propagated, but not possible in annual maize.

In 1979, Iltis *et al.* reported a new diploid perennial *Z. diploperennis* had been discovered and since that time it has been crossed by only a few other investigators and generally not for perennialism (Srinivasan and Brewbaker, 1999). J. Holland successfully crossed *Z. diploperennis* to B73 (the maize reference line with the sequenced genome) and NC300 (an elite inbred line important in the southern United States). F_2 progeny of these crosses were investigated both in North Carolina by Holland and by Murray in College Station and Weslaco, Texas. Both Holland and Murray found that combining with B73 was quite poor compared with NC300 in hybrid combination. In Weslaco, the F_1 and F_2 were crossed to late flowering elite Texas maize lines and were also allowed to open pollinate among themselves. From this, 250 progeny were planted in College Station, TX in 2009 and those that demonstrated at least one perennial type trait, (indeterminacy/staygreen, tillering, etc.; described later) were self-pollinated. Shaver's 2N (diploid) and 4N perennial populations (tetraploid) were also late planted in College Station in 2009 and demonstrated a range of perennial phenotypes but due to extreme summer and winter conditions none were found to overwinter. From Shavers populations out of ~5000 2N plants and ~5000 4N plants, 99 desirable plants from 2N were pollinated, and 34 from 4N were pollinated and planted ear to row in 2010. Plants in this population have been primarily sib-mated but also self-pollinated, crossed with the Holland derived material and crossed with perennial wild relatives by Murray now for a total of nine additional seasons. Germplasm generated and selected has greatly improved for ear size and grain yield which is critical for perennial seed and grain production. A range of other phenotypes has been observed to improve including tillering, totipotency of stem tissue and delayed or eliminated senescence. However, no rhizomes have been observed and plants left in the field have generally died from drought, heat or freezing. Meanwhile, multiple wild *Z. diploperennis* plants have survived for over four years. This continues to suggest that perennial maize could be developed through incorporation of key genes but more work is needed.

GENETICS OF PERENNIALISM

One QTL mapping study of 425 F_2 plants derived from *Z. diploperennis* crossed to the annual teosinte *Z. mays* ssp. *parviglumis* has been reported that used 95 RFLPs (Westerbergh and Doebley, 2004). Very few of the plants had rhizomes and only two QTL for rhizomes were identified but explained less than 12 percent of the variation. They attributed this low amount of variation to low trait heritability as we might expect in an F_2 study of a threshold trait where the phenotype of interest needs a suite of genes and a favourable environment for screening. However, these findings compare poorly to those in sorghum. Paterson *et al.* (1995) reported a mapping study of 370 F_2 and 378 BC_1 plants in a cross between *S. bicolor* × *S. propinquum* with 78 RFLPs. In this study many more plants had rhizomes and 10 QTL were detected explaining 48 percent of the variance. These findings between perennial maize and perennial sorghum dissection are very consistent with my personal observations having worked with both species. Both the rhizomes and the perennialism observed in *Sorghum* are much more robust than that in *Zea*.

What do we need to make maize perennial?

For a maize plant to act as a perennial a number of conditions must be met: the plant must not senesce at the end of the season; the plant must accumulate energy into structures that can overwinter; the plant must be able to prevent its overwintering structures from both freezing and degradation; and finally the plant must remobilize energy from the overwintering structures into new regrowth in the spring. For perennial maize to actually be grown by farmers this must all be accomplished in a plant that can produce adequate grain yields and can switch back and forth between reproductive and vegetative growth – in addition to the other suites of traits that farmers desire such as disease resistance, nutrient and water use efficiency. The breeding progress for selection on these many different quantitative traits is certain to be slow, but I feel all must be selected simultaneously to avoid breeding into a corner.

There are many quantitative tendencies referred to as perennial-like that do not necessarily result in overwintering. First, stay green, or delayed senescence; from evidence in grain sorghum and maize this is an important trait to maximize grain filling under stress (Thomas and Howarth, 2000; Campos *et al.* 2004). Progeny of perennial by domesticated crosses often show much greater stay green. However, even the most staygreen types of commercial maize senesce completely at the end of grain filling and senescence will be the most important trait to select against in perennial x domesticated *Zea* crosses. Some progeny have had a deceiving staygreen appearance that seems to be due to delayed flowering time and maturity. Domesticated annual sorghum does not naturally senesce and does not appear to be a limiting trait for a perennial phenotype, as many researchers already consider sorghum a weak non-overwintering perennial. This may be the cause of the more heritable perennialism in sorghum. Second, the ability to tiller, ratoon or produce additional stalks from the crown is likely an important component to perennialism. However, basal tillering is also found in many landrace maize cultivars, sweet corn, and many grasses including most annual sorghum; therefore Shaver (1964) cautions against using tillering as a proxy selection criteria alone for perennialism in maize. It has been my observation that often tillering is an underground branching not specifically related to active meristem tissue. Third, ratooning or the ability to regrow tillers when the main stalk is cut is a phenotype expected from perennial plants. However, annual maize and many types of sorghum harvested green for silage can also demonstrate ratooning ability under the rare proper environmental conditions (Coors *et al.* 2007; Livingston and Coffman, 1997). These annual ratooning plants, however, are not able to overwinter because they often senesce and also do not produce rhizomes. Fourth, the ability to produce rhizomes, modified underground storage stems that allow vegetative reproduction, is an important and heritable phenotype that is found in many perennial plants but no annual ones. Rhizomes are totipotent, can overwinter underground and produce new plantlets (rammets) in the same seasons or in following years from nodes that grow tillers. This totipotency of rhizomes makes them important in perennial growth habit. Perennialism in *S. halepense* (Johnsongrass) has been found to require rhizomes and to be primarily, but not solely, a function of rhizome depth (Warwick *et al.* 1987; Washburn 2012). While the goal is to produce a high-yielding perennial crop and not a noxious weed difficulty killing vigorous regrowth alone is not a desired criterion. As an alternative to rhizomes, many temperate plants (switchgrass, cordgrass, bahiagrass, etc.) regrow from the crowns via carbohydrate reserves in short, knotty proaxes occasionally mistaken for rhizomes (Haferkamp and Copeland, 1984; Boe *et al.* 2009). This is the overwintering mechanism in close *Zea* relative eastern gamagrass (*Tripsacum dactyloides* L.) (Jackson and Dewald, 1994), its interspecific hybrids with maize (Jatimliansky *et al.* 2004). This may also be the case for *Z. diploperennis*, in which rhizomes are clearly but only occasionally produced. Regrowth from the crown proaxes in combination with rhizomes would provide an avenue for developing robust perennial maize with multiple overwintering mechanisms. Finally, overwintering, which is the most important component of true perennials will be conditioned by all of these traits and their interaction with the environment. Because of this complexity perennialism is a threshold trait, or one that if even one component or gene/allele is missing, or if the material is screened in a poor environment, we will not be able to observe any plants as perennial. Selection on a complex quantitative trait like perennialism will not be as efficient as selection on a simple additive trait. In both maize and in sorghum we have already developed and/or evaluated genetic resources that improve the chances of rescuing a perennial plant.

CHALLENGES AND INTERESTING TRAITS

While perennialism is the primary goal of this research, a number of other traits of interest have arisen in the germplasm that could be useful for farmers, environment or society. *Later canopy cover* – At the end of an annual maize growing season, not only the nutrients but also the sunlight is typically striking bare ground and not being turned into useful biomass for humans or agro-ecosystems. We observe that the perennial derived material has much greater green leaf tissue at the end of the season, whether through branching, tillering, and/or delayed senescence. *Delayed/non-senescence* – the wild species and many of the crosses do exhibit staygreen or delayed senescense, this is believed to be associated with increased stress tolerance, grain filling period and increased yield. Many breeders look for increased staygreen, especially in hot, arid, tropical climates. *Prolificacy* – the ability to produce many ears on the same plant has been a long selected trait by maize breeders. These plants tend to produce ears under even the most stressful condition. Many plants derived in the perennial maize crossing program have multiple ears on each stalk or multiple stalks with at least one ear. *Indeterminant regrowth* – the ability to produce biomass after initial flowering and seed set would be very advantageous for making the most of environmental resources. This biomass would be useful for preventing soil erosion, capturing carbon, and providing additional silage or cellulosic biofuel feedstocks. *Ear forest* – some of the germplasm continues to produce a succession of ears that flower at the base of the plant until drought, heat or freeze kills the plant. A potential application for this trait is to graze livestock that can digest starch. *Deeper rooting* – We observe the root systems of perennial *Zea* to typically be much larger and deeper than annual maize. This suggests that they can access additional water and nutrients and may contribute additional biomass to the soil. *Increased sinks* – In the case of sorghum and sugarcane it has been clearly shown that increased diversity of sink tissue results in an increase of potential harvestable energy and that "tradeoffs" are unlikely to be a problem if cultivars are selected for multiple sinks (Murray *et al.* 2012). This is in large part because photosynthesis has been shown to be sink as opposed to source limited.

CONCLUSIONS

The domestication of crops was a long-term proposition and it has taken over 100 years and billions of research dollars to get maize to reach its current productivity. Developing high-yielding perennial maize is likely to take an additional 10 to 40 years, at which point hypotheses of yield and eco-system service comparisons can be formally tested. This long-term high risk research is most appropriate for the public sector. This is concerning given the erosion of public research funds, especially in agriculture. Substantial commitments and investments will be needed to make perennial maize a reality.

FIGURE 1. *ZEA DIPLOPERENNIS*

Fig1A-B: Division of part of a *Z. diploperennis* rhizome from a pot.
Fig1C: Appearance of *Z. diploperennis* in late summer / fall (College Station, 2010). This has not started flowering while everything else is long dead.

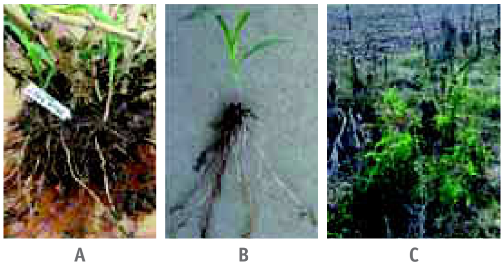
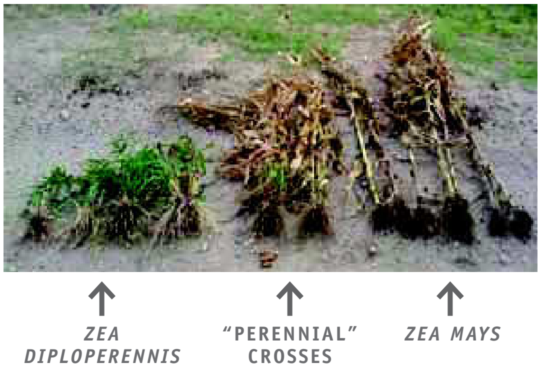


FIGURE 2. DIFFERENCES IN ROOTS

Weslaco, December 2013



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09

EVALUATING PERENNIAL CANDIDATES FOR DOMESTICATION: LESSONS FROM WILD SUNFLOWER RELATIVES

David L. Van Tassel[1*], *Sean R. Asselin*[2], *Sheila A. Cox*[1], *Gina Sideli*[3], *Douglas J. Cattani*[2]

1 The Land Institute, Salina, Kansas, USA
2 University of Manitoba, Department of Plant Science, Winnipeg, Manitoba, Canada.
3 California State University, Biological Sciences Department, Chico, California, USA
* Corresponding Author: The Land Institute, 2440 E. Water Well Rd., Salina, Kansas, 67401, USA.
Phone: (+1) 785 823 5376; Email: vantassel@landinstitute.org

ABSTRACT

Recurrent selection for increased seed production has been successful in two wild perennial species related to sunflower, *Helianthus maximiliani* and *Silphium integrifolium*. A third species, *Helianthus pauciflorus* has highly heritable variation for seed yield and should respond to selection. Each wild species has a different set of traits that limit its usefulness as a grain crop and each has unique adaptations beyond the perennial habit that could enhance its value or accelerate its domestication. For example, *S. integrifolium* is deeply rooted and relatively unaffected by short term droughts, while *H. pauciflorus* is easily asexually propagated by tubers making clonal cultivars feasible.

More generally, we ask which traits make a wild perennial more genetically and agronomically tractable and which complex but ecologically important traits might only be brought to the agro-ecosystem through domestication? Any perennial grain reduces the need for tillage, but perennials are not equally drought tolerant, competitive with weeds or carbon sequestering. We suggest, from both experience and deduction, that seed yield and the classic domestication traits such as shattering may often be less important in selecting perennial species for domestication than genetically complex traits such as growth rate, phenology or branching pattern.

Keywords: perennial grains, oil crops, oilseeds, domestication, landscape genetics, crop wild relatives, functional traits, *Silphium, Helianthus, Linum*

INTRODUCTION

Perennial grains, including perennial cereals, pulses and oilseeds, are being investigated or developed at several institutions as a long-term solution to the ongoing problem of soil erosion and degradation associated with annual grain cropping systems. The rationale for increasing the proportion of agricultural land planted to perennial crops has been more fully articulated by Glover *et al*. 2010; while the potential physiological and genetic obstacles to combining high grain yield and multi-year lifespan—and suggestions for how these obstacles can be overcome-have also been explored elsewhere (DeHaan *et al*. 2007; Van Tassel *et al*. 2010). In this volume given this rationale we assume that high-yielding perennial oilseeds are both beneficial and possible to develop. We provide a rationale for investing in new oilseed crops in addition to the more obvious cereals and for choosing domestication in these cases instead of using wide hybridization to “perennialize” an existing crop. Next we survey the native North American species being domesticated as perennial oilseeds. We report progress and observations with these species, and describe our plans for continued domestication. Finally, we compare the candidates and look for principles that could help future domesticators identify candidates likely to respond rapidly to domesticating selection from among the many seed-bearing wild species.

I. Rationale

Perennial oilseed grains will contribute to long-term food security and renewable energy. Oil crops contribute to food security in every region because they are less expensive than animal fats and dietary lipids supply both calories and the essential fatty acids alpha-linolenic acid (an omega-3 fatty acid) and linoleic acid (an omega-6 fatty acid) that humans cannot synthesize. Furthermore, some fat in the diet greatly improves the bioavailability of at least one vitamin precursor, B-carotene (van het Hof *et al.* 2000), and could increase the uptake of other fat-soluble nutrients such as lycopene, lutein, Vitamins K and D (Brown *et al.* 2004).

Oilseed crops play an important role in the diversity of cropping systems, providing functional diversity to compliment grain and legume rotations. Many oilseeds are duel or multi-purpose crops, filling multiple end uses (Vollamann and Rajcan, 2010). For instance crops such as sunflower, soybean and canola provide human nutrients, protein-rich meal for animal consumption, and the raw feedstock for biofuels (Agarwal, 2007) and bioplastics and other industrial products (Xia and Larock, 2010) which can aid in reducing the dependency of petroleum based fossil fuels.

Perhaps the most compelling argument in favour of perennial oilseed research is simply that oil crops currently occupy 281 million hectares (FAOstat, 2012 data). This is about 26 percent of the world's acreage devoted to staple crops (cereals, pulses, oilcrops and roots/tubers) and about 18 percent of all land classified as arable or under permanent crops (FAOstat, 2011 data). For whatever reasons, humanity has chosen to devote a large proportion of the planet's land area to oil crops and providing more perennial oil crop options could reduce the ecological disservices associated with annual oil crops. Encouragingly, 41 percent of all vegetable oil already comes from perennials such as olive, oil palm, and coconut (FAOstat, 2012 data).

However, these perennial oil crops are all tropical or subtropical tree-like species and virtually all the oil crops in temperate regions are annuals such as soybean, sunflower, flaxseed and canola. Furthermore, tree-like crops are slow to establish and expensive to harvest and immobilize moisture and nutrients from the environment for extended periods. Perennial oilseed forbs could provide additional options even in the tropics, supplying some of the ecosystem services of the tree crops, as well as new services, such as fodder, and new harvesting and management (mowing, grazing and burning) options.

In both tropical and temperate regions, the development of perennial oilseeds, particularly those with diverse end-uses and capable of producing on marginal lands where annual cropping is at a high risk of failure, has the potential to increase the productivity of global agricultural systems.

Domestication of perennial oilseeds, perennialization of domestic oilseeds and intermediate approaches. There are currently four main routes to combining the yield of an annual crop with the longevity of a wild perennial. The first three require wide hybridization between an annual species and a perennial species. Under the "triticale option", a full or partial amphiploids with whole chromosomes of both species is created. If the chromosome constitution remains stable in successive generations, a new species has been created. Option 2 utilizes introgression breeding to move "domestication genes" from the cultigen into the wild perennial background. Option 3 is similar, but aims to move "perennialism genes" from the wild species into the genetic background of the cultivated species. The fourth option—domestication—attempts to find rare alleles in the primary gene pool of a wild perennial that confer favourable "domestication syndrome" traits such as reduced shattering, increased threshability, etc.

All of these routes are being attempted with members of the sunflower family. Researchers at The University of Minnesota (Hulke and Wyse, 2008; Kantar *et al.* 2010; Kantar *et al.* 2014) made wide hybrids between *Helianthus tuberosus* and the crop sunflower (*Helianthus annuus*) followed by using the annual (diploid or tetraploid lines) as the recurrent parent. Two of us (Van Tassel and Cox) have attempted to select for stable amphiploids from among the progeny of intermated wide hybrids between either *Helianthus tuberosus* or *H. pauciflorus* (perennials) and the crop sunflower. While the details of these programs are beyond the scope of this report, the summary is that stable amphiploids are very rare and exhibit low fertility. Even if found, these lines are unlikely to contain the allelic diversity needed for long-term crop improvement and cultivar development. Additional amphiploids of the same karyotype will have to be developed for that. Similarly, crossing hybrids with either the annual or perennial parent often appears to result in the elimination of many or all of the chromosomes of one species or the other.

The situation appears to be very similar to that encountered when trying to transfer genes from *Tripsacum* to *Zea*. Harlan and DeWet (1977) wrote:

The cytogenetic interactions in maize x *Tripsacum* hybrid derivatives can be remarkably complex. The number of viable, somewhat female fertile chromosome combinations is astonishing. Fifty-four different combinations are listed in this paper, and others are certainly possible. In successive backcrosses to maize, chromosome numbers scale both up and down with ease, sometimes stabilizing for a few generations via apomixis, but in most cases finally generate balanced maize genomes and emerge as recovered maize. The maize, however, can produce strange and unusual phenotypes as a result of genetic transfers from *Tripsacum*. Highly tripsacoid maize lines with 2n = 20 chromosomes were recovered in pathways where the maize genome had first contaminated the *Tripsacum* genome in early backcross generations. In other pathways interaction of the two genomes is rare.

Cox is currently attempting to develop "annuus-oid" *H. pauciflorus* by making numerous F1 hybrids between *H. pauciflorus* and *H. annuus*, using the perennial as the female and then "backcrossing" the F1s to the perennial until a fertile, balanced *H. pauciflorus* genome is recovered. Her aim is to introgress a few "domestication genes" into *H. pauciflorus*, and her hypothesis is that even a few alien alleles increasing seed or head size could greatly accelerate the domestication of *H. pauciflorus*. It seems to these authors that introgressing the perennial habit into the annual background is a more daunting task because several to many alien genes are likely to be required simultaneously in order to produce a perennial and winter-hardy phenotype.

Domestication: slow but steady. In contrast with wide hybridization, fertile parents in a domestication population are intermated and fertile offspring are produced which can be easily intermated, crossed with new, wild individuals, or backcrossed to their parents. The "astonishing" number of cytotypes and dramatic "strange and unusual" phenotypes that Harlan and DeWet (1977) saw with the derivatives of interspecific hybrids and that Van Tassel and Cox have seen with interspecific Helianthus are not generated by this approach. There is no possibility of making a single cross that fortuitously combines all the desirable traits of the domestic species and the longevity and hardiness of the perennial. But, on the other hand, serious problems with sterility, chromosome number fluctuation and development of small populations with strong barriers to gene flow with other sources of genetic variation are unlikely to be encountered. Domestication is a slow and steady strategy unlikely to produce dramatic "early wins" but also unlikely to fail if given enough time. In experimental populations, recurrent phenotypic selection on individuals has repeatedly resulted in the evolution of novel phenotypes (e.g. Ratcliff *et al.* 2012; reviewed by Travisano and Shaw, 2013).

Recombination load is the reduction of fitness caused by breaking favourable genetic associations that have been generated by selection (Otto and Lenormand, 2002). Presumably the compliment of chromosomes in a genome represents one of the strongest forms of genetic association. The addition of entire or large pieces of alien chromosomes is almost certain to disrupt the previous genetic associations. Beyond disrupting the process of meiosis itself, wide crossing recombines whole developmental programs sometimes leading to misshapen, fragile or non-functional organs (Siikamaki, 1999). Any adaptations involving whole plant architecture and/or finely tuned phenology are likely to be lost following wide hybridization. On the other hand, the crop field may represent such a novel environment that reductions in fitness in the wild environment are irrelevant.

Domestication as a source of new, complex and functional traits for the farm. The wild relatives of crops can be little more than genetic resources to be mined for novel alleles when only a few genes are likely to be successfully transferred to the domestic species by either introgression or transgenics. However, through domestication, entire biochemical pathways, plant organs or life history strategies are available.

Conceptually treating all domesticated grain species as a single ecological guild we can draw on the emerging field of landscape genetics (Manel *et al.* 2010) to suggest the value of dramatically increasing the genetic and phenotypic variation within grain crops by adding new species with new complex traits. Landscape variables play a vital role in conventional plant breeding as genotype x environment interactions determine the suitability of a particular crop or cultivar to a given region. While the aim of population genetics, a cornerstone of plant breeding, is the study of allele frequencies under various adaptive and neutral processes (Falconer and MacKay, 1996), landscape ecology aims to characterize the relationship between various environmental ecological processes and ecosystems (Ewers *et al.* 2013). The discipline of landscape genetics aims to examine how specific geographic and environmental factors shape population structure and can be useful in identifying traits of ecological significance. By extension ecological genetics is the specific study of these traits of ecological significance (Lesica and Allendorf, 1999) some of which are of interest to plant breeders.

Grain crop traits related to adaptation to novel habitats and abiotic stresses such as flooding and drought stress are increasingly sought after by plant breeders for the maintenance of yield in marginal or unpredictable environments. An ecological genetics approach may force breeders to look outside of conventional selection criteria because the domestication and later crop improvement of our current crops occurred under a particular set of “idealized” conditions; including growing regions with favourable rainfall or irrigation, fertile valley soils, monoculture cropping, and weed control. This process is inherently selective for genotypes—and species--that do well under these conditions and not necessarily marginal landscapes. Many wild species on the other hand have undergone selection under extreme environmental conditions that do not normally present themselves in ideal agricultural systems and provide novel materials for crop development (Vear, 2010).

Domestication can now be accelerated. Advances in sequencing technology and statistical modelling are starting to eliminate barriers to the genetic dissection of complex quantitative and plastic ecological traits even in species which lack genomic resources or known pedigree (Sillanpää, 2011; Davey *et al.* 2011; Baxter *et al.* 2011; Narum *et al.* 2013). While important ecological traits such as nitrogen or water use efficiency may be the result of a combination of many genes each contributing small portions to the total variance in the trait throughout the genome, high molecular marker saturation is allowing for the development of whole genome breeding values and GS for complex traits using whole genome marker saturation. Whole genome GS using markers development techniques such as genotype-by-sequencing and RAD-seq have been effective in predicting selection candidates for complex traits such as grain yield in wheat (Poland *et al.* 2012) and are becoming more common in both plant breeding and ecological genomics (Narum *et al.* 2013).

II. Candidates for Domestication

Perennial sunflowers (*Helianthus* ssp., Asteraceae)

Annual sunflower (*Helianthus annuus* L.) is the only major grain crop domesticated in North America. The domestication of annual sunflower is believed to have been rapid as most of the traits which distinguish wild and domesticated plants such as achene and disc size are polymorphic or quantitative traits that show continuous distributions (Burke *et al.* 2002; Chapman *et al.* 2008; Blackman *et al.* 2011; Chapman and Burke, 2012).

Similar to annual sunflower (*Helianthus annuus* L.), the perennial North American species Maximilian sunflower (*Helianthus maximiliani* Schard.) and stiff sunflower (*Helianthus pauciflorus* Nutt.) have a range that encompasses most of North America, stretching from southern Texas to southern Manitoba. Both *H. maximiliani* and *H. pauciflorus* die to the ground each autumn surviving the winter through tuberous rhizomes. Similar to other wild sunflower species (including wild *H. annuus*) they are self-incompatible outcrossers and the seed requires an extended (4-6 weeks) cool, moist stratification period for full germination.

H. maximiliani and wild *H. annuus* show clinal variation for a number of morphological and life history traits influenced by genetic drift and natural selection (Blackman, Michaels, and Rieseberg 2011; Kawakami *et al.* 2011). For example, Kawakami *et al.* (2011) found that Maximilian sunflower traits such as flowering time, disc size, plant height and growth rate exhibit a clinal distribution across a latitudinal gradient of North America. Discs ranged between 9.8-26.2 mm in populations collected from the southern range of the species in Texas to its Northern range in Manitoba.

The range of habitats in which these widely distributed perennial species persist leads us to predict that, like the wild annual sunflower, they harbour the genetic variation necessary for domestication. New mutations may not be required. Consistent with this prediction, we have found a great deal of variation for traits of interest within and between accessions collected from our locations in Kansas and Manitoba. Families collected across Manitoba grown under growth chamber conditions show considerable variation in plant height, days to flowering, disc size (3.8-18.3 mm), and branching architecture.

Helianthus maximiliani: Maximilian sunflower grows vigorously as a seedling, flowering in the first year. It has good seed fertility and well documented disease resistance including rust and *sclerotinia* (Rashid and Seiler, 2004; Muellenborn *et al.* 2011) and seed oil content equal or surpassing that of wild annual sunflower (Seiler and Brothers, 1999). The oil is rich in the essential fatty acid linolenic acid and higher in tocopherols (compounds with vitamin E activity) than any other wild Helianthus species evaluated (Velasco *et al.* 2004). As with many species of Helianthus interspecific gene transfer is possible though the viability and fertility of F1 interspecific hybrids is low (Atlagic *et al.* 1995). Chromosome doubling through colchicine has been proven to be effective in developing fertile *H. annuus* X *H. maximiliani* amphidiploids (Feng and Jan, 2008) and *H. maximiliani* has served as a source of novel restorer genes and male-sterile cytoplasms in *H. annuus* for hybrid breeding.

Maximilian sunflower is being domesticated simultaneously in Kansas and Manitoba. While genetic materials have been exchanged between these programs, the day length and climate differences make it probable that two distinct domesticated races are likely to emerge. In both locations the basic approach has been to collect seeds from a number of wild populations in the region of the breeding station, stratify seeds and transplant the seedlings to the field for evaluation. The program in Manitoba is still in the evaluation phase, although crosses have been made between wild Manitoba and improved Kansas individuals for the purpose of developing mapping populations. Initial results suggest that wild Manitoba populations are capable of persisting and will readily set seed in nutient deficient soils. Phophorus and nitrogen levels ranging between 2-16 ppm and 4-36 lbs/acre. respectively are capable of sustaining large stands though the total number of seed bearing heads per plant are reduced under these conditions. Substantial variation in floral disk dimaeter, plant height and days to flowering have been observed between sub-populations in Manitoba suggesting that there is enough genetic variance to make genetic advancement for these traits within the population base.

In Kansas, selections from the initial evaluation nursery were intermated in 2003 to produce Cycle 1 plants. Selections from Cycles 1, 2 and 3 were intermated in 2006, 2009 and 20012 respectively. The general method has been to plant spaced-plant evaluation nurseries, select 20-50 individual plants using information about the performance of each plant's half siblings in the second year, dig the selected plants early in the third year and polycross them in a crossing nursery. For polycrossing, controlled pollinations were made using a bulk of pollen obtained by pooling pollen from the selected plants. Although many traits have been measured over the years, the selection indices were based primarily on seed per stalk, seed per head and average seed mass.

A side-by-side, multiyear comparison of the yield of selected Cycle 3 plants, random unselected Cycle 3 plants, and wild plants is currently in the field, so we cannot definitively estimate the gains made to date. However, heritabilities of the traits under selection (Table 1) and the data from each population (Figure 1), though grown in different years, suggest that yield has increased.

FIGURE 1. AVERAGE YIELD COMPONENTS OVER SEVERAL YEARS IN A *HELIANTHUS MAXIMILIANI* POPULATION UNDERGOING ARTIFICIAL SELECTION FOR INCREASED SEED YIELD

Each point on the graph shows traits from plants sampled in their second year (after transplanting). Experimental designs are summarized in Table 2. All experiments were established in Salina Kansas by transplanting clones or seedlings and were managed without fertilizer addition or irrigation beyond what was required to establish transplants. Weeds were controlled by cultivation except in the case of Experiment 2 (2005) where sunflowers were intercropped with alfalfa (*Medicago sativa*). The alfalfa and weeds were controlled by mowing between rows and within rows as necessary.

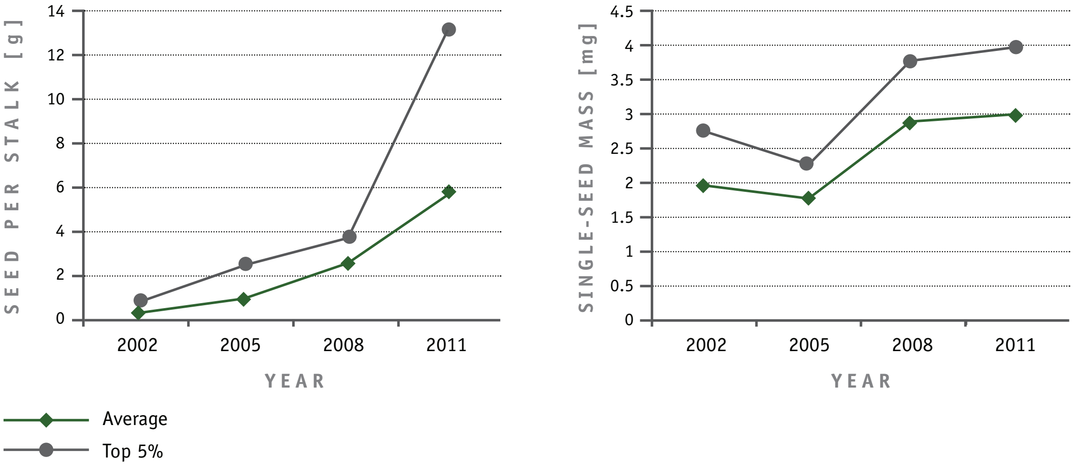

TABLE 1. MEANS, STANDARD DEVIATIONS AND RANGES, AND HERITABILITIES FOR SEVERAL TRAITS MEASURED IN SEVERAL DIFFERENT BREEDING POPULATIONS

Experiments were performed in different years and traits were not always measured the same ways, so only approximate comparisons are possible. Seedlings were germinated in controlled conditions and transplanted to the field in May. All experiments were performed in Salina, Kansas without fertilizer application or irrigation beyond the minimum required for transplant establishment. Only data from 2-year old plants is shown here unless otherwise indicated. Heritabilities were calculated from individual genotypes in known half-sib families using REML processes (Genstat, London) that included spatial components and blocking terms in the model only if they improved the model for a particular trait.

SPECIES, YEAR	NUM. PLANTS PHENOTYPED [NO. HALF-SIB FAMILIES]	SPACE BETWEEN ROWS (AND WITHIN ROWS) (m)	SINGLE-SEED WEIGHT (mg)			SEED YIELD PER STALK (g)		
			Her	Mean (stdev)	Range	Her	Mean (stdev)	Range
S. integrifolium 2008	2246 [224]	0.9 (0.9)	27	23.7(3.9)	12.0 - 42.5		10.9 (9.9)	0 - 83.1
S. integrifolium 2012	2720 [109]	0.9 (0.45)	61	15.6 (3.3)	5.6 - 29.5	27	3.46 (2.3)	0 - 19.9
H. pauciflorus 2012	2555 [100]	0.9 (0.45)	69	8.2 (1.7)	2.0 - 14.9			
H. maximiliani 2008	2240 [20]	0.9 (0.9)	27	2.86 (0.05)	0.42 - 7.68	14	1.33 (1.07)	0.01 - 12.9
H. maximiliani 2011	710 [50]	1.8 (1.8)	34	2.98 (0.80)	0.407 - 15.7	42	5.74 (3.72)	0.01 - 36.9
H. maximiliani 2012*	1925 [113]	0.9 (0.9)	49	4.88 (0.75)	3.33 - 7.65			

* Data taken in first season on a subset of plants, selected visually for reduced branching

SEED YIELD PER HEAD (mg)			SEED YIELD PER HEAD AFTER SHATTERING (mg)			PERCENT NOT SHATTERED			OTHER TRAIT OR NOTES			
Her	Mean (stdev)	Range	Her	Mean (stdev)	Range	Her	Mean (stdev)	Range	Trait	Her	Mean (stdev)	Range
13	423 (142)	0 - 992							Stalk diameter		9.78 (3.6)	2 - 20
									Num ray florets per head	78	51.8 (13.6)	8 - 165
71	291 (197)	0 - 135	67	107 (82.2)	0 - 641	42	43.3 (22.0)	0 - 100	Seed not shattered (%)	42	43.3 (22.0)	0 - 100
42	415 (122)	14 - 844		35.0 (27.1)	0 - 824	16	10.74 (7.46)	0 - 100	Number of stalks per plant	16	19.4 (8.97)	2 - 80
	206 (112)	3.17 - 554		12.2 (13.1)	0 - 153.5	51	9.56 (12.9)	0 - 96.8	Number of stalks per plant	48	24.3(11.0)	3 - 75
31	640 (362)	0 - 2240	33	296 (199)	0 - 1470		48.9 (20.3	0 - 100				

In 2007, a single unusual individual was observed in the Cycle 2 evaluation nursery. This plant had strongly reduced branching and a single, larger than average head on each main branch. Open pollinated progeny from this plant were intermated in 2008 to produce a segregating population in 2009. Plants with strongly reduced branching in their first season were intermated that same year and this process was repeated in 2010 and 2011. Data from the initial reduced branching segregants (2009) show reduced seed mass per head thereby confirming our observation of malformed heads and florets (Figure 2). By 2011 the average yield per head was approximately equal to that of the population from which the first reduced branching plant was taken in 2007, and some individuals had yields per head exceeding anything in the normally branching population, again, confirming our observation that reduced branching is associated with a wider range of disk diameters. We attribute the restored seed fertility to natural selection in which more fertile individuals contributed more pollen and more seeds to the subsequent generation than less fertile individuals. Reduced branching materials are currently being crossed with wild-type materials from Manitoba to develop mapping populations and to introduce this trait into a northern adapted genetic background to expand the diversity of the uniheaded lines and develop divergent breeding pools.

FIGURE 2. QUANTITY OF SEED PER HEAD HISTOGRAMS SHOW CHANGES IN A *HELIANTHUS MAXIMILIANI* POPULATION UNDERGOING ARTIFICIAL SELECTION FOR INCREASED APICAL DOMINANCE

Heads were sampled in the same year as seedling establishment. The 2007 sub population is the same as the population shown in Figure 1. Open pollinated progeny of a single unusually large-headed, nearly unbranched plant in that experiment were intermated to produce the sub-population evaluated in 2009.

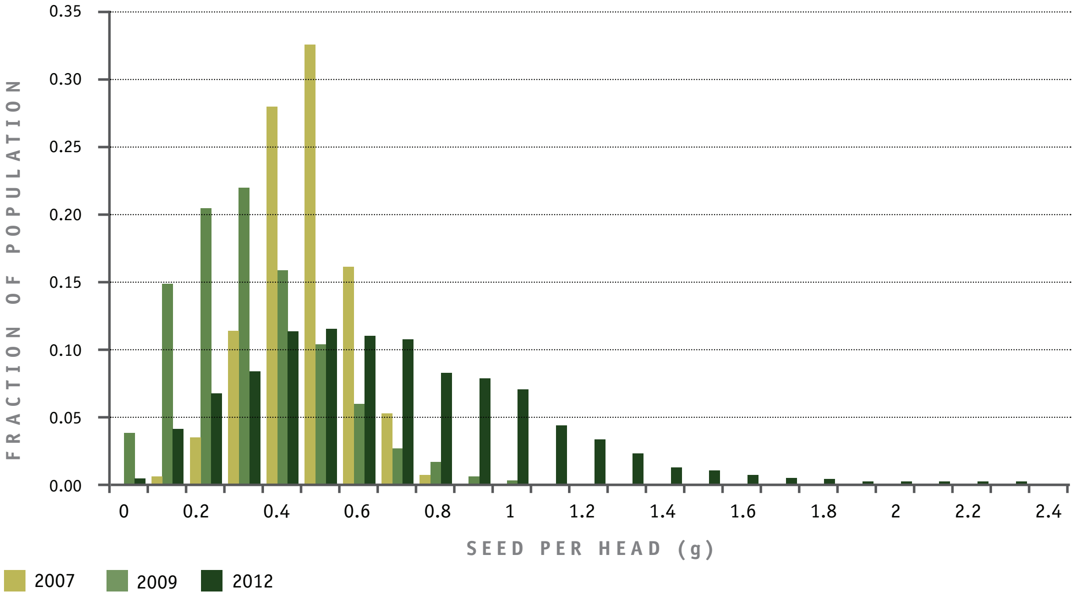

TABLE 2. EXPERIMENTAL DESIGNS

EXPERIMENT	YEAR PLANTED	YEAR DATA TAKEN	EXPERIMENTAL FACTORS			SELECTION		NOTES
			Plots	Number of families	Number of genotypes	Num. of genotypes selected to advance	Percent of genotypes selected to advance	
1	2001	2002	960	96	960	150	15.6	Families are batches of seeds collected from different wild populations
2	2004	2005	900	15	150	20	13.3	Genotypes in this experiment are a subset of the genotypes in the first experiment. Selected individuals were dug and their rhizomes divided and transplanted to the new nursery with clonal replication
3	2007	2008	2240	20	2240	50	2.2	Selected plants from Experiment 2 were intermated to produce seed for Experiment 3
4	2010	2011	710	50	710	50	7.0	Selected plants from Experiment 3 were intermated to produce seed for Experiment 4

***Helianthus pauciflorus* (= *H. rigidus*) and *H. tuberosus*:** these are tuberous, hexaploid perennial sunflower species (n = 51). We consider them together here because they are known to hybridize naturally forming fertile populations (Brooks and Schofield, 1986) that have previously been given species status and are now considered subspecies or hybrids. Clevenger and Heiser (1963) commented that "In their extreme forms, the [hexaploid *Helianthus*] taxa considered here are quite distinct, but intergradations are frequent in nature." In Kansas, *H. pauciflorus* and *H. tuberosus* are readily distinguished when collecting seeds or plants from wild populations. However, open-pollinated progeny of wild-collected individuals grown in a common garden are much more difficult to categorize than their parents, presumably due to interpollination.

Both tuberous species were used as parents in interspecific crosses with *H. annuus,* mainly between 2004 and 2009. *H. pauciflorus* gained attention as a candidate for direct domestication because some individuals had larger seeds than *H. maximiliani* and large heads bourn on long peduncles. The branching pattern and peduncle length place the infructescences largely above the leafy portion of the plant, making mechanical harvesting easier. Although The Land Institue population under consideration for perennial oilseed development is referred to as *H. pauciflorus* it could more accurately be considered a hybrid swarm with individuals positioned all along the intergradation between the two wild hexaploid species.

Both tuberous species are sources of resistance to rust (*Puccinia helianthi*) (Zimmer and Rehder 1976), and both are crossable with *H. annuus*, though, as described above, the hybrids are only weakly fertile (Georgieva-Todorova, 1964; Jan, 1997). Despite the barriers to gene flow, *H. tuberosus* has been the source of important disease resistance genes in *H. annuus* (Rogers *et al.* 1982), and *H. pauciflorus* has provided cytoplasmic male sterility genes (Jan *et al.* 2002). The cytoplasm of many commercial sunflower cultivars may actually be from *H. tuberosus* (Jan and Ruso, 2000). *H. tuberosus* was domesticated as a tuber crop and both tubers and vegetation could be used for bioethanol production or livestock fodder (Kays and Nottingham, 2007). *H. pauciflorus*, though never domesticated, is also strongly tuberous.

The yield potential of this species remains unknown, though initial field trials were established at the Land Institute in 2011, the University of Manitoba in 2012, and Organic Valley Co-op (Cashton, Wisconsin) in 2013 to evaluate this species. Perhaps because of the large phenotypic variation resulting from the intergradation between the two species and their respective hexaploid genomes, the heritabilities for traits of interest measured in a large Land Institute evaluation nursery in 2012 were high (Table 1), suggesting that a breeding program could be successful. However, the extreme sensitivity of sexual reproduction in this population to drought led the Land Institute to phase out its evaluation of this species in Kansas, focusing efforts on the collaborative breeding effort in Wisconsin where this species may be better adapted to the shorter growing season and lower potential evapotranspiration.

Helianthus cusickii: Cusick's sunflower is a diploid perennial (2*n*=34) adapted to dry, rocky soils of high mountain desert, surviving with annual precipitation of only 25-60 cm (Seiler, 1992) by means of a robust taproot. It flowers earlier in the year than the other perennial sunflowers discussed here and is noted for morphologically being the most extreme species in the genus (Heiser, 1965). Counterintuitively, it is more closely related to *H. annuus* than the other perennial species that have been used as sources of genes for the improvement or perennialization of *H. annuus* (Timme *et al. 2007)*. However, both pre and post zygotic barriers to gene flow are expected (Sideli *et al.* 2013). We have made preliminary attempts to produce interspecific hybrids between *H. cusickii* and *H. annuus*. Putative hybrids are being observed and additional attempts at hybridization will be made and it remains to be seen if genes for perenniality, fleshy taproots, or other drought tolerance traits can be introgressed to the annual crop sunflower.

Its early flowering, small pubescent leaves, modest height and deep roots make this species a possible candidate for domestication as a drought tolerant oilseed, especially if domestication related genes could be introgressed from *H. annuus*. However, after planting out hundreds of individuals representing a large portion of the species' native range (seeds collected in California, Nevada, Washington and Oregon) in typical agricultural soils in Kansas, we found poor survival and low vigor compared to several other annual and perennial *Helianthus* species. Possibly this species has not been exposed to soil pathogens typical to agricultural soils but uncommon in desert soils. However, a few individuals did survive into a second year and, if intermated, could be used to develop a population with greater adaptation to agricultural conditions.

CANDIDATES FROM OTHER GENERA

While the genus Helianthus is an obvious source of perennials that could be domesticated as oilseeds because it includes numerous perennial species as well as two domesticated species (*Helianthus annuus* and *Helianthus tuberosus*), the North American grasslands contain many other species with oil rich seeds. Some of these are likely to have chemical or anatomical adaptations not available in *Helianthus*.

***Silphium* (Asteraceae):** The species of the genus *Silphium* have much larger seeds (e.g. 21 mg reported by Kowalski and Wiercinski, 2004) than most other native North American forbs and grasses. The flavor and composition of major nutrients is similar to that of the domesticated sunflower (Kowalski and Wiercinski, 2004). The heads resemble that of a sunflower, but whereas the disk florets of *Helianthus* are perfect and the ray florets are sterile, in *Silphium* the ray florets are pistilate and the disk florets are staminate (Brooks and Schofield, 1986). Several observations of wild stands of *Silphium integrifolium*, a fairly common prairie species in Kansas, led us to consider this species for domestication. In addition to the size of its seeds, we observed variable seed shattering. Mature seeds remain on some heads well into the autumn or even the winter. This is easy to see because the large seeds remain attached to the heads after other floral structures and sterile florets have fallen off. The other key observation was the performance of this species during dry intervals or even serious droughts. In 2012, the drought in Kansas was severe enough that test plots of commercial hybrid sunflowers administered by Kansas State University in nearby Hutchinson, Kansas, were terminated due to crop failure (Stewart Duncan, personal communication). The average seed yield of S. integrifiolium in The Land Institute breeding nursery, where plants had been transplanted the year before in 0.9 m rows, 0.45 m within rows, with no fertilization, was 312 kg/ha and some exceptional plant were estimated to have yielded up to 1182 kg/ha.

In 2001-2002 we evaluated both *Silphium integrifolium* and *S. laciniatum*, another drought tolerant, perennial species, for their adaptation to agricultural conditions. We found that neither species flowers in the first year. They produce large basal leaves and no stems. In the second year *S. integrifolium* reliably produces stout stalks and flowers. *S. laciniatum* is much slower to develop and does not always flower in the second year. We judged its establishment phase to be too slow for agriculture and focused on *S. integrifolium*.

The most obvious barrier to high seed yield in all *Silphium* species is the production of seeds by only the ray florets which number from 15-32 per head in *Silphium integrifolium*. To test whether genetic variation existed for this trait, and whether the number of fertile florets could be increased to at least 100 per head, we practiced recurrent selection for that trait alone. Seed from 21 Kansas populations were allowed to interpollinate in 2004. Approximately 100 000 progeny were planted in 2005 and screened in 2006. Because scoring the number of ray florets is very time consuming, we never measured the population average thoroughly, but estimated it to be about 28-30. The ray florets on plants visually identified as having heads with high numbers of ligules (the long, strap-like corollas of ray florets) were carefully counted. Eighty three plants with 38 or more ligules were found and intermated that year. The progeny of this polycross were planted in 2007 and similar recurrent selection and intermating was done in 2008 and the process repeated in 2009-2010. In 2012, finding that the average number of ray florets in a population of 2 720 was 52, that several plants had over 100 ligules and that one had over 150, instead of simply intermating the plants with the most ray florets, we decided to make selections using an index based on seed yield per stalk and seed size (mass). We reasoned that we had good evidence that the population contained adequate genetic variation for ray floret number and that pushing the population mean higher prematurely might result in loss of variation for seed size or other traits of interest. Furthermore, some of the plants with very high ray floret numbers are now close to being male sterile.

***Linum* (Linaceae):** Lewis flax (*Linum lewisii* Pursh.) and stiffstem flax (*Linum rigidum* Pursh.) are both perennial North American species in the same genus as domesticated annual flax (*Linum usitatissimum*). Antinutritional saponins are reported in *L. rigidum*, limiting its usefulness to agriculture. Cultivars of *L. lewisii* with improved longevity, drought resistance and disease resistance have been developed as ornamentals and for use in landscape reclamation. This species also has good forage value.

L. lewisii has fewer chromosomes (n=9) than annual domesticated flax *Linum usitatissimum* L. (n=15) which could pose a barrier to interspecific hybridization. Fortunately unlike the large complex genome of polyploid candidate perennial grain species (perennial wheat ~17 Gb) or those with highly repetitive genomes (*Helianthus* spp. ~3.5 Gb) the flax genome is small (~370 Mb) and whole genome resequencing is emerging as a technique for improving domesticated flax (Fu, 2012). The small genome size in the genus *Linum* in theory should facilitate whole genome association studies into the genetics of domestication and for screening germplasm for favorable traits.

III. Discussion

Assuming that we have successfully argued the case for domesticating perennial oilseeds, we (authors, scientific community and society) are still faced with difficult decisions about which candidate species are most promising.

The candidates from the Asteraceae, described above, provide an excellent study in contrasts between "perennials." Whenever we have done the experiment, we have found that the traits of interest are heritable. It is difficult—maybe impossible—to judge which species has the highest yield potential, given unlimited cycles of selection for yield. We argued above that it might be simpler to domesticate species by directional selection on quantitative traits like yield and (in sunflowers) shattering than to attempt to introgress complex traits such as lifespan. The same logic may apply within the subset of perennial species. Root structure or vegetative spread by tubers may involve complex developmental pathways. Other traits may simply be very expensive to measure due to the instrumentation required or need for controlled/multiple environments. It would be well to consider the following traits carefully when selecting a perennial species for further investment.

ABOVE-GROUND PLASTICITY

To our surprise, we discovered that *H. pauciflorus* and *H. maximiliani* plants when dug from a mature prairie and transplanted to an experimental plot were likely to triple or quadruple in height. Leaf size, stem diameter and number of inflorescences are also altered in this environment. In short, the plants look like a different species. One implication of this phenomenon is that it is impossible to judge the agricultural phenotype of a wild species without growing it in rows or as spaced plants in an experimental plot where they have a different nutrient status and are released from competition with other plants. A more subtle implication is that we have found that plants with this level of plasticity are highly sensitive to both drought and self-crowding and may require nutrient limitations to modulate overabundance of growth. A positive implication may be that these species are capable of producing seed under resource poor conditions.

Domesticated sunflower has a high rate of evapotranspiration, a trait often exploited as an ecosystem service to lower the water table in areas dealing with salinity. Under drier conditions this can result in drought stress later in the growing season greatly impacting yield potential because the reproductive growth stages of sunflower are more sensitive to moisture stress than the vegetative stages (Göksoy *et al.* 2004). In the southern part of its range (e.g. Kansas), Maximilian and stiff sunflower undergo an extended vegetative period beginning with emergence in May, expend soil moisture and accumulate biomass throughout the season. If moisture or fertility is available early in the season, plants can grow quite tall and branch freely. Frequently as temperatures rise throughout the season these species often endure mid-season drought resulting in a loss of accumulated biomass and potential volatization of nutrients prior to reaching its necessary short day photoperiod requirement for flowering in September.

Thus the plasticity of *H. maximiliani* and *H. pauciflorus* cause—from the perspective of maximizing seed production per unit area—excessive vegetative growth early in the season and excessive vegetative growth in high rainfall years. Both problems result in reduced harvest index (if abscised leaves are included in the calculation) and premature depletion of soil moisture and potential for nutrient volatization. A second issue is that many of the phenotypes of interest to breeders are likely to respond to the environment in ways that are difficult to quantify, impeding breeding progress. For example, small scale patchiness in soil texture or drainage within a breeding nursery can be difficult to model using conventional blocking or even spatial statistics. Neighboring plants also modify each other's environments in ways that are difficult to control or model especially early in the domestication process when the phenotypic variance of the population is great, when gaps are produced irregularly in the field due to plant mortality, or when transplanting or cultivating errors create slight differences in the spacing between or within rows.

In contrast, *H. cusickii* and *S. laciniatum* showed only modest, if any, increase in growth or stature when grown with reduced competition. Unfortunately, this makes them very uncompetitive with weeds, at least in their first few years, and therefore difficult to work with. *S. integrifolium* has an intermediate response growing about twice as tall in cultivation than in the wild. This species also appears to have much greater seed yield and height stability from year to year. One reason for its reduced plasticity is that like the other species it ceases to grow in height at the time of flowering but unlike the Helianthus species, in Kansas it flowers beginning in early July whereas *Helianthus pauciflorus* begins flowering in late August and *H. maximiliani* in early September.

This gives us a clue about a strategy for reducing the plasticity of the *Helianthus* species in the southern part of its range: introgress flowering time genes from points further north. Manitoba *H. maximiliani* populations flower in July.

RHIZOMATOUS SPREADING

S. integrifolium is less plastic than *H. maximiliani* and *H. pauciflorus* in another way: it forms a crown that does not increase in diameter much over time. This means that the number of stems per unit area increases very slowly, if at all (after the establishment year). At the other extreme, *H. pauciflorus* produces rhizomes that radiate 30-50 cm from each stem in all directions. The number of stems per unit area increases geometrically from year to year, leading to severe crowding and stunting of the stems. Without thinning, by the third season, the plants are as short and produce as few heads as when this species is found growing in a mature prairie.

Aggressive asexual spreading impedes plant breeding progress because adjacent genotypes in a nursery are usually impossible to separate in the second season when they are regrowing from large overlapping rings of rhizomes/tubers. This makes it difficult and risky to make selections of individual plants based on their reproductive traits from the year before. Instead, a subset of individuals in a segregating population would be visually selected and their tubers dug before the rhizomes connecting them to the original seed-grown plant rot. Tubers would be planted in small plots and genotypes selected for intermating on the basis of plot yield. Although this digging of tubers and establishment of plots of clones would be labourious and eliminate the possibility of making selections the first year after intermating, some traits could be measured this way that cannot be measured on individual spaced plants (see the Tubers section below).

A more serious problem is the phenomenon of stand decline. *H. pauciflorus* stands decline in seed yield rapidly after the first year. As described above, as stalk density becomes greater, the stalks become shorter and more slender. Many completely fail to flower and those that flower produce much smaller heads. We have not studied the effect of ramet density on root traits, but we hypothesize that roots also become shorter and more slender. The “luxury uptake” of water by such dense stands early in the season may be another factor in stand decline. A second-year population in Kansas, 2012—a dry year, declined so strongly that by midsummer most stalks were extremely stunted and had only brown leaves. In contrast, *S. integrifolium*, planted in the same field at the same time grew and flowered normally.

TUBERS

The tubers of *H. pauciflorus* and *H. tuberosus* are a curse when trying to achieve and maintain an optimal plant density, as described above, but also a potential blessing. Although asexual reproduction of this kind would require farmers to diligently cultivate between and possibly within rows to maintain stalk density within target range, it would also provide an easy method for propagating genetically uniform cultivars, bypassing the need for years of inbred line or synthetic cultivar development. The tubers are easy to dig, store and transport, fairly uniform in size, and could be planted with standard potato planting machinery. Possible applications of tuber propagation:

1. Fixed specific combining ability: Fortuitous crosses creating even a single outstanding F1 individual could be all that is required to instantly create a superior genetically fixed cultivar despite chromosome compliment. Emasculations and careful pollination would not be required to generate propagules each year. And unlike potato, where micro-tubers are sometimes produced in vitro to reduce the costs of shipping whole field-grown tubers or cutting them into pieces, the tuberus rhizomes of *H. pauciflorus* and *H. maximiliani* are already small.
2. Locally adapted cultivars: Ecovars $^{(TM)}$—improved ecotypes—have been proposed as a way of exploiting the adaptations that have evolved in local populations of a species. Attempting to produce numerous locally adapted cultivars of an outcrossing species, while retaining hard-won domestication traits and population uniformity in phenology and plant height, is a daunting prospect. Paradoxically, the possibility of eliminating genetic variation at the field

scale through asexual propagation may make it more feasible to release numerous genetically distinct locally adapted cultivars.

3. Ecological genomics research: Ecological genomics aims to 'find the genes that matter', that is those who influence adaptive variation in the context of their natural environments (Edwards 2013). Being able to propagate individual genotypes asexually (and inexpensively) would allow new phenotypes to be mapped in segregating populations or diverse association mapping panels. The phenotypes of most interest to agronomists, yield, water use, nutrient uptake, stalk density over time, are so easily confounded by inter-genotype competition and plant spacing, that they can not be meaningfully measured on individual seedling-grown plants. These traits can be measured using inbred families derived from segregating populations, but this is much more difficult to accomplish with outcrossing species and likely to be confounded by inbreeding depression therefore association as opposed to pedigree based approaches may be better suited for these species.

ROOTS

Belowground traits are difficult to observe and, therefore, difficult and expensive to change directly using conventional phenotypic selection. The range of root traits among seed-producing perennials is very great. In selecting between candidates for domestication as new crops, it would be wise to carefully consider which kind of root system is needed to provide the desired ecosystem and economic services in the target environment. We have speculated that with a few exceptions (wheat, sorghum) it may often prove to be easier to domesticate species in order to capture their life history and cold tolerance traits (perenniation) than to try to breed or engineer such complex traits in an annual crop using de novo variation or variation brought in from wild relatives. If this logic applies to the easily scored trait of winter survival, it is applies more strongly for the possibly equally complex rooting traits which are far more expensive to "phenotype" under realistic field conditions.

In contrasting the response of experimental populations of *Silphium integrifolium*, *Helianthus maximiliani* and *H. pauciflorus* to drought it is impossible to separate the effects of root depth and increasing stem density. Adjacent, same-age plots of *S. integrifolium* appeared unwilted throughout the 2012 growing season while in midsummer, *H. pauciflorus* appeared scorched and green material was only seen after autumn rain and lower temperatures. The response of *H. maximiliani* was intermediate. The plants were shorter than normal, many leaves dropped, and the remaining leaves were permanently wilted during July and August. A counterintuitive aspect of these observations is that *H. pauciflorus* is often found in dry, rocky, shallow-soil locations, while *S. integrifolium* is often seen at lower landscape positions where soil and moisture are more abundant.

Weaver and Stoddart (1935) noted similar contrasts between *H. pauciflorus* and *S. integrifolium* during the drought of 1934 (the "dust bowl" years), writing, "A few species such as … *Silphium integrifolium*, grew normally and stood unwilted above the dried grasses." They attributed the drought avoidance of these forbs to their deep roots: "Among the forbs, resistance to drought was closely correlated with root extent. Species with root systems penetrating 8 to 20 feet into the moist subsoil were little affected."

These authors also noted that, "*Helianthus rigidus* [= *H. pauciflorus*] was often completely wilted and the leaves dried [in the prairie] while a foot or two distant in cultivated land the same species was 3 feet or more tall, abundantly supplied with flower heads and completely turgid." This statement is consistent with our observations of great differences in height, number of heads and drought stress between seedlings ("cultivated land") and second or third year stands in which the plant density approaches that of the prairie and presumably results in soil water depletion. The authors clearly characterize this species as drought tolerant, stating that "the tenacity with which [*Helianthus rigidus*] persisted after weeks of semi-wilting was impressive."

In climates experiencing variable rainfall within and between growing seasons, the advantages of drought avoidance through persistent deep root systems over mere drought survival through osmotic adjustment, wilting, and stunting seem obvious. For subsistence agriculture, yield stability is more important than yield potential, and crops that yield well during dry years would increase food security.

Increasing the rooting depth of crops has been proposed as a method for sequestering meaningful amounts of carbon (Kell, 2011). If deep roots persist from one year to the next (low turnover) they may not require much more photosynthate for maintenance than shorter root systems. However, in the first few weeks or months of life, the construction of this root system will almost certainly require greater allocation of photosynthate belowground than for other types of root systems. The cost, then, is likely to be reduced aboveground growth in the first year. This translates to poor weed competition (*Silphium* spp. and *H. cusickii*) and/or no flowering in in the first year (*Silphium* spp.). A less obvious side effect of deep persistent roots is reduced seedling nutrient and water acquisition. The surface area of a few large-diameter seedling roots heading almost vertically downwards must be lower than a finer more branching seedling root system. We have seen that following transplanting *S. integrifolium* seedlings are more vulnerable to water stress, experiencing higher mortality in unirrigated plots, than perennial *Helianthus* seedlings. Thus long-term drought avoidance comes at the cost of poor drought tolerance, especially in the first few weeks.

We do not know the costs or benefits of the various rooting strategies in other circumstances. In locations with poor drainage or frequent floods, are fast-growing "disposable" root systems a better investment than costly, deep, permanent ones? Which root systems tolerate root predation better? Do root systems with large root systems and low root turnover tend to immobilize soil nitrogen leading to nitrogen deficiencies?

APICAL DOMINANCE

Dramatic changes in aboveground branching have happened during the domestication of many crops including annual maize and sunflower. So on the one hand, the branching pattern of a wild plant need not eliminate it from consideration for domestication. On the other hand, branching in perennials is much more complex than in annuals. Herbaceous perennials regulate their architecture by controlling branching and internode length at several points including the below-ground branches (rhizomes, tillers), main lateral branches on stalks, and short flowering branches.

As described above, *Helianthus maximiliani* families with a single head per stalk are now available, as are multiheaded types and intermediate lines that produce only a few short flowering branches just below the terminal inflorescence. Increased apical dominance resulting in fewer, larger infructescences and resulted in the characteristic large head of domesticated sunflower. Depending on the context of the habitat in question apical dominance is observed to both positively and negatively impact relative fitness (in the form of intact harvestable seed) in annual sunflower (Reagon and Snow, 2005). The development of a single determinant head increases seed size but can leave the plant vulnerable to seed predation or head loss by the native head clipping weevil. Reduced branching and number of sub-terminal inflorescences on the main stem increases both the synchrony of head maturation and the uniformity of head position on the plant. Both facilitate harvest and reduction in losses to seed predators and shattering. Synchrony of anthesis and maturation means that mature heads do not need to be left in the field—where they are exposed to shattering and bird predation--while immature heads continue to develop. Positioning heads near the top of the plant means that less leafy material must be processed by mechanical harvesters. Excessive foliage near the heads can slow the harvesting process, require suboptimal winnowing settings, or result in contamination of the harvested seed by wet material.

In general, apical dominance combined with early flowering may present an opportunity for increasing the harvest index of perennial oilseeds while reducing the phenotypic plasticity. The longer growing season of perennials compared with annuals allows them to produce more biomass, but in opportunistic, highly indeterminate species, even if stems were harvested as a co-product with the seeds, much of this productivity may be unharvestable (dropped leaves, withered, headless stems, aborted heads) in a dry year or present harvesting difficulties (thick woody stalks, excessive wet, green material) in a good year. Where oil production is the main goal it is clear that either the plants must stop growing vegetatively and enter the sexual phase early in the growing season or that the plants must be low-growing early in the season, accumulating carbohydrate reserves belowground and then bolt late in the season, producing heads on largely leafless stems.

While in theory breeding *H. maximiliani* to flower earlier in the season in the southern part of its range could accomplish these goals, it remains to be seen whether this will succeed. One possibility is that after flowering, apical dominance will be released and new primary stalks may emerge from the rhizomes or the existing stalks may continue to branch and produce leaves without a means of accumulating reserves for the following year. Another possibility is that the plant may simply die back. This would accomplish the goal of conserving soil moisture for the next season, but would reduce the productivity of the cropping system in good and average years. Both of these phenomenons have been observed under growth chamber conditions suggesting possible genetic variance for the regulation of rhizome dormancy following flowering and could potentially be manipulated as well. In the case of tuberous perennials, early flowering may help reduce the harvest index, but the opportunity for storing carbohydrates after seed filling is limited. This is because the main sink at this point is the new tubers, but only a single replacement tuber is required to maintain the stand density and excess tubers may need to be removed to prevent overcrowding. For regions with short growing seasons, these points may be irrelevant. Water stress may be a rare problem and winter imposes a limit to excessive vegetative growth. This may be the only suitable environment for tuberous perennials.

SENESCENCE

One of the most useful features of the annual habit is the programmed death of the stems and leaves bearing the heads to be harvested. Senescence allows the grain and the stem to dry prior to harvest. While the individual heads of the native oilseeds surveyed here senesce when the seeds are mature, the stalks and leaves generally do not until killed by frost. At that point, the weather is cool and the plant material dries slowly. Opportunities for remobilizing biochemical contained in the leaves and stems are also missed. Conversely under growth chamber conditions following flowering senescence has been observed in plants despite optimal temperature, moisture and nutrient conditions suggesting a killing frost may not be necessary to trigger senescence.

If candidates with autumn foliar senescence are not found, chemical defoliants--already used to facilitate dry down and harvest of some annual grain and perennial hay crops-- could be used with perennial oil seeds. An alternative would be to look for species that produce most of their leaves close to the ground and send up a specialized stem with fewer or smaller leaves to display the inflorescences above the canopy. Even if this stem did not senesce after seed maturation, it might be less succulent and leafy than the stems of other species and thus present fewer problems for harvest. Some prairie plants produce such structures, including *Tripsacum dactyloides*, *Silphium laciniatum* and *S. terebinthaceum*. Perhaps these *Silphium* species should be re-evaluated as potential oilseeds. It would be necessary to demonstrate that such a species also had genetic variation for increasing the diameter and rigidity of such a stem in order to bear the weight of larger, heavier seed heads.

Plant senescence in annual crops also gives the farmer a high level of control over the plant density each year. We alluded above to the potential problem of ramet proliferation through rhizomatous and tuberous asexual propagation. Even when the fine roots or leaves of perennials senesce, shoot and root apical meristems may not. With woody crops, pruning to control the number of aboveground meristems (buds) is crucial for maintaining fruit size and plant productivity from year to year (Marcel-les-Valence, 2006). In the absence of programmed meristem population control, agronomists may need to develop methods to “prune” back the above and below-ground meristems of perennial herbs.

The predictable senescence of all the plants in a field of an annual crop provides an opportunity for tillage or pesticide application to kill any invading vegetation and to disrupt the lifecycles and habitats of many kinds of agricultural pests. Of course, the downside of this is that the soil is exposed to erosion and the habitat of beneficial or threatened animal species is compromised. How can perennial grain farmers achieve the necessary function resetting secondary ecological succession annually without the options available to annual grain farmers? Controlled burning and grazing have been used for centuries to arrest succession at a point that provides abundant palatable herbaceous species for grazing livestock. Both of these interventions can prevent the invasion of woody plants that lose their competitive advantage when their aboveground meristems are killed each year. However, many kinds of perennial or biennial weeds are herbs and tolerate these stresses necessitating a diversified integrated approach. We have found that improved populations of perennial oilseed candidates, once established are quite competitive with other herbs and prevent their invasion. Breeding to increase determinacy, advance flowering in the season, reduce asexual reproduction, and increase harvest index is likely to make these species less competitive with other herbs. Perhaps this is an ecological tradeoff. An agroecosystem in an un-invadable state is also in a state in which asexual structures and processes dominate. The *H. pauciflorus* population at The Land Institute in 2013 illustrates this. The density of ramets has increased each year since seedlings were transplanted in 2011. The rows have completely disappeared but no cultivation has been practiced for two years and yet it is a single species monoculture. On the other hand, the plants are nitrogen and moisture stressed and very few, tiny heads were produced.

Maintaining perennial forbs in rows and lightly cultivating between the rows early in the growing season may be one way that farmers can prevent overcrowding yet control invading perennials. Alternatively integrating animals which exhibit root scavenging behavior such as pigs in between rows may be one way of reducing unwanted spread while transporting nutrients across the landscape. If it is necessary to thin (and then weed) the stand within the row also, specialized cultivators or a companion cover crops may need to be developed.

CONCLUSION

Before investing heavily in new perennial crop domestication for a particular commodity and target environment, it would be worth thinking very strategically about what traits other than multi-year survival need to be captured. The breeders should begin discussions with agricultural engineers and agronomists to develop new kinds of equipment and procedures to enable farmers to fulfill the functions that the candidate species cannot do itself. The integration of companion species, equipment capable of root/rhizome "pruning", strip tillage and possibly mowing or cultivation within rows may prove to be necessary in many cases. At a more philosophical level, we wonder if it is possible to predict *a priori* what histories of natural selection are likely to have produced species with complex traits compatible with perennial agriculture.

Hypothesis 1: The life forms that dominate the native ecosystem in the regions and landscape positions targeted for grain production serve as useful models but may themselves not be useful for domestication as grains. Once a particular kind of life form (e.g. warm-season, sod-forming grasses in Kansas) outcompetes other life forms the major source of competition is intraspecific or at least intra-guild. This kind of competition leads to "arms races" which lead to the evolution of highly competitive morphologies such as trees with long trunks and prairie grasses with massive root systems (Craine, 2006). Undoing the effects of these arms races to produce a more efficient canopy or root community could be very difficult.

Non-dominant species have had to evolve strategies for escaping direct competition with the dominant life forms. Thus, in Kansas prairies, deep rooted forbs in the absence of disturbance may never be able to outcompete sod-forming grasses and become dominant, but they can survive as dormant tubers or seeds until a serious drought kills some grasses opening up niche spaces for new growth. The deep-root lifeform may be more useful in the context of agriculture in Kansas than the warm-season sod forming life form. On some landscape positions in this biome, *H. pauciflorus* and *H. maximiliani* can form large stands that are co-dominant with some grasses. *H. maximiliani* and *H. tuberosus* have been described as having allelopathic properties, this coupled with abundant adventitious growth in the presence of available resources tells us these species are highly competitive through a series of diverse mechanisms. However, in different environmental contexts, these species may never be able to form dense stands and thus may not be as competitive with other species…or itself.

Hypothesis 2: Climates or landscape positions where neither perennial nor annual agriculture is possible might seem to be sources of species well adapted to the kinds of stresses perennial crops in moderate-to-low input systems are likely to face. However, we suggest that species found mainly in these extreme locations may have experienced selection for stress tolerance rather than stress avoidance. Reduced growth rate is one way of surviving in a stressful environment, but this strategy is likely to be inimical to any form of agriculture. Evidence for this comes from switchgrass (*Panicum virgatum*) where cultivars derived from the wild upland ecotype showed less physiological stress under water and nitrogen limitations than lowland cultivars, but nevertheless were out-yielded by the lowland types in both stressed and unstressed treatments (Stroup *et al.* 2003).

Another strategy that approaches stress escape (fully realized in the annual habit) is highly plastic development. Such plants may be diminutive and asexual most years but capable of opportunistic growth and profuse flowering following disturbance or in favorable seasons when limitations have been temporarily relaxed (c.f. description above of *H. pauciflorus* in sod versus tilled ground). Stress tolerance/escape adaptations enable a species to survive but may not be useful in an artificial ecosystem designed to both take full advantage of available resources and to minimize seed yield instability from year to year and over the course of centuries.

The ideal perennial grain has adaptations for avoiding water and nutrient limitations, for example by exploiting deep soil moisture unavailable to other species, or by interacting with mutualists to obtain nitrogen or phosphorus in forms usable by plants. We suggest, therefore, that the best candidates for a region may be those capable of persisting and fruiting annually (though perhaps not as the dominant life form, according to Hypothesis 1) in the absence of agricultural inputs or nutrient-releasing disturbance but with the benefit of the soils and landscape positions where perennial agriculture is possible to be practiced.

These hypotheses are suggested as criteria for quickly identifying initial candidates and their adaptations to their environmental constraints; there is still no substitute for rigorous evaluation of all candidates for adaptation to the target range of stresses and input levels.

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10

DOMESTICATION OF *LEPIDIUM CAMPESTRE* AS PART OF MISTRA BIOTECH, A RESEARCH PROGRAMME FOCUSED ON AGRO-BIOTECHNOLOGY FOR SUSTAINABLE FOOD

Mulatu Geleta[1], Li-Hua Zhu[1], Sten Stymne[1], Anna Lehrman[2], Sven Ove Hansson[3]

1 Department of Plant Breeding, Swedish University of Agricultural Sciences, P.O. Box 101, 230 53 Alnarp, Sweden
2 Department of Crop Production Ecology, Swedish University of Agricultural Sciences, P.O. Box 7043, 750 07 Uppsala, Sweden
3 Division of Philosophy, Royal Institute of Technology, Teknikringen 78, 100 44 Stockholm, Sweden

ABSTRACT

Several important challenges are facing agriculture. In the stride towards lowering the negative environmental impact of food production while maintaining and increasing the production, both basic farming practices and novel technologies are important tools. The use of biotechnology in breeding, however, is not uncontroversial. The criticism has many angles and often relates to the applications brought forward by large, multinational companies, and farmers growing dependence on these companies when it comes to seeds. Questions are being raised about ethical acceptability,

and about the health and environmental impacts. A general aversion to what is often referred to as the "industrialization" of agriculture, and to "unnaturalness", also emerges in the debate.

In the research programme referred to as *Mistra Biotech*, we include both philosophy and natural and social sciences. The overall goal is to facilitate production systems that are sustainable from ecological, social and economic perspectives.

The objectives of the programme include developing:

* new elite plant lines that have benefits for consumers, farmers, the food industry and the environment;
* agribiotechnology tools that are important for achieving new product qualities, healthier crops and livestock, and for solving environmental problems in agriculture;
* basis for sustainable production systems that contribute to increased competitiveness in Swedish agriculture and food production;
* tools for ethical scrutiny of agricultural biotechnology that combine high demands on safety with encouragement of innovations;
* basis for improved regulatory approaches and private-public relationships.

The programme includes six component projects, in which domestication of a new biennial oilseed crop *Lepidium campestre* (field cress) is a major research focus. Questions we will try to answer within this programme include: Can biotechnology be used to improve crops which mitigate climate change or benefit the environment? What potential is there to commercialize such a crop? How would the consumers react to products made from it? Can breeding technology be improved further? Why does the market for genetically improved plant and animal materials look the way it does? What ethical concerns does the use of biotechnology raise? And how do all these issues feed into future agricultural systems? The results are integrated in the synthesis project called the Centre for Agriculture and Food Systems Analysis and Synthesis (AgriSA).

Here we emphasise the research within Component Project1, with a focus on the domestication of field cress.

PLANT BIOTECHNOLOGY FOR INNOVATIVE PRODUCTS (Component Project 1)

Domestication of *Lepidium campestre* through GM and non-GM approach

The demand of plant oils for food and biodiesel is expected to increase steadily in the coming 20 years. However, the potential of increasing production from the existing oilseed crops is limited. For example, in Sweden, the only economically viable oilseed crop is winter rapeseed, but it can only be grown in the southern part of the country.

We are pursuing a fast-track domestication of *Lepidium campestre* (field cress), a biennial *Brassicaceae* species, into a combined novel oil- and catch crop. Late professor Arnulf Merker at the Swedish University of Agricultural Sciences (SLU) identified *L. campestre* as a promising species for domestication - a high seed yielding plant (5-6 tonnes/ha, which is higher than the average yield of winter oilseed rape) with an upright stature and synchronous flowering. Moreover, it is biennial with a potential to be a perennial crop. As a cover crop, it is sown under cereal crops during spring, and seeds are harvested the following year; thus reducing nutrient leaching and tillage - a major factor that causes groundwater contamination, especially under intensive use of nitrogen based fertilizers and tillage. Planting biennial or perennial crops and use of cover crops could alleviate this problem. Field cress could be a promising crop species that can fit into such cropping system: a system that is very limited in Sweden. Field cress is also cold-hardy and can be successfully grown in the northern parts of Sweden. However, some of its properties must be altered in order for the plant to be an economically viable oil crop.

In the new cereal-oilseed cropping system being developed, we are using field cress and barley (*Hordeum vulgare L.*) as a model. For genetic improvement of field cress we are using both conventional methods as well as genetic engineering for speeding up the breeding process and for comparing the breeding efficiency of the two methods. The main targeted traits are oil content, oil quality, seed yield and pod shatter. Pod shatter, which is the dropping of seeds before harvest, causes huge losses in seed yield. A number of transgenic lines with genes for improving the aforementioned traits have been recovered and are currently under screening to identify homozygous lines. The preliminary results have shown increased oil content in some transgenic lines, and an increased level of the healthy oleic acid (omega-9), a monosaturated fatty acid in others. The transgenic lines with improved shatter-resistance genes are under evaluation.

To provide more populations for conventional domestication and breeding, we collected a large number of field cress populations from several locations in Sweden in addition to a large number of accessions obtained from various gene banks and botanical gardens around the world. The screening in the greenhouse and outdoors has been focused on the identification of genotypes with one or more of the following desirable traits: low pod-shattering, resistant to major diseases and pests, high seed yield, high oil content, large seed size, synchronised maturity, well-developed root systems and perenniality. Seed oil content and composition analyses have shown a wide variation in oil content, but small variations in fatty acid composition among different accessions with linoleic acid (polyunsaturated) as the highest of all fatty acids. Among the mono-unsaturated fatty acids, erucic acid was dominant, followed by oleic acid. The seed also contains tocopherols and cholesterol. Promising genotypes with various combinations of these traits have now been selected for further evaluation. Intra-and inter-specific hybridizations have also been made. In the case of intra-specific hybridization, crosses have been made between genotypes with elevated levels of oil content, between high seed yielding genotypes, as well as between relatively low pod-shattering genotypes in an attempt to create superior genotypes. Hybrids that are superior to their parents are under further evaluation. Inter-specific hybridizations were made between field cress and other species in the genus *Lepidium* to transfer desirable traits from the latter to the former. The most successful inter-specific hybridization was between field cress and *Lepidium heterophyllum*, a perennial close relative of field cress. The F_1 hybrids produced from these species were perennial and showed very strong hybrid vigour with a significantly higher seed yield and a root system superior to those of both parents. Interesting lines have been selected from the F_2 populations for further breeding. Interspecific hybridizations were also made between field cress and *Lepidium draba* to transfer shatter-proof genes from *L. draba* to field cress; and between field cress and *Lepidium graminifolium* to increase the oil content in field cress. Overall, a significant increase in oil content and seed yield, a significant decrease in pod shattering, and improvement in root systems are all highly promising developments, as is the progress in developing perennial field cress.

At Component Project 1 we are also investigating and improving other crops and traits:

Improve barley and potato: Here we focus on improving nitrogen uptake and pathogen resistance using various genetic technologies. One of the technologies used is site directed mutagenesis, which consists of a number of techniques including TALEN (transcription activator-like effector nuclease) to improve disease resistance in barley and potato. So far, we have synthesised four nucleases for potato and transfection of potato with these nucleases is under evaluation. For improving nitrogen uptake in barley, the transgenic barley lines with introduced genes responsible for root uptake of amino acids were field tested, mainly for producing seeds for further evaluation. The seeds from the field trial are currently under evaluation in controlled growing conditions in a greenhouse for their nitrogen uptake ability.

Starch quality analysis: We have investigated the fine molecular structure of the amylopectin isolated from various genotypes of barley and have found that a mutation linked to starch biosynthesis results in a modified amylopectin structure. We are now investigating how different molecular units in amylopectin are interconnected in these barley samples. A certain category of glucose chains, building up the amylopectin molecule, is thought to play a role in cluster interconnection, and thereby in the compiling of domains. Other categories of shorter chain-lengths are believed to interconnect smaller amylopectin building blocks. More information on the fine structure of amylopectin will improve our understanding of the relationship between starch structure and several functional properties. These relationships will be studied during the coming year.

High amylose potato: Potato starch is usually made up of 25 percent amylose and 75 percent amylopectin. By turning off two genes controlling amylopectin biosynthesis, we could increase the amylose level. The field trial on the transgenic lines shows that high amylose potato had reduced starch content and an increased tuber yield. The preliminary analysis shows that the starch content is about 35–70 percent of the non-transgenic control. Further studies on circumventing the starch yield drag through crossing are underway. Transcriptome analyses will be carried out to elucidate molecular mechanisms underlying the starch yield drag.

OTHER PROJECTS WITHIN MISTRA BIOTECH

Novel molecular breeding tools (Component Project 2)

The majority of economically important traits in crops and livestock, such as product yield, product quality and disease resistance are complex traits governed by many genes and environmental factors. Traditional breeding approaches have used pedigree information and statistical tools to estimate the proportion of variation that is due to heritable factors, but treated the genome as a "black box". Today new technologies facilitate the sequencing at a fraction of the original costs. We will be providing methods and tools for the use of whole genome sequence data in breeding – that is, selecting plants and animals using information about their entire DNA sequence instead of looking at specific genes. Additionally, we will be investigating the potential to use information about proteins, the genetic product, in breeding. The gain here is the ability to screen for and select suitable plants and animals at an early stage in the breeding process.

Ethics (Component Project 3)

The debate about ethical issues in biotechnology and its applications is very polarized. Some people are against, some in favour, and these views are often firmly held. Despite the large literature on ethics of technology in general, there is a shortage of studies carried out in close collaboration with the scientists developing actual technologies. Therefore, much of the debate is insufficiently informed by recent developments and rather sweeping in character. Also, few applications of ethical technology assessment involve new biotechnologies, and even fewer take into account the potentially positive environmental and health impacts of agricultural applications of biotechnology in a systematic way. We hope to provide a structured method of making this debate less polarized, allowing everyone to better understand each other's arguments. We will also investigate what a "precautionary" approach might involve in the context of agricultural biotechnology And we will study some concepts that are common in the public debate, but which are sometimes cursorily treated in the scholarly discourse, such as naturalness and sustainability.

Consumer attitudes and behaviour (Component Project 4)

What are the driving forces behind attitudes and behaviours when it comes to food produced using agricultural biotechnology? What is our perception of risks and trust? We hope to reach a better understanding of the underlying consumer-related issues that will play an essential role in the uptake and use of any application of agricultural biotechnology in Sweden. Our first results come from a meta-study combining the results from over 1 600 questions in 241 different studies in 58 regions. The study shows that previous conclusions on the Europeans' negative attitude towards GM food might be the result of slightly different questions having been asked in Europe compared with other countries.

Driving forces behind applications of biotechnology (Component Project 5)

The economic and regulatory environment in which firms operate has a direct effect on their ability to produce and to adopt new technologies. Firms will produce innovations when they have the ability to commercialize, to sell a product or service at a profit. The profitability of an innovation depends on the degree to which they are able to capture the economic rents generated by their innovations. Farmers will adopt innovations for similar reasons. However, the ability of agrifood value chain to distribute the benefits from consumers, to farmers, from retailers to processors and to biotech firms is the challenge. The competition along the agrifood value chain, the governance of transactions and the regulatory framework are the determining factors and the subject of research in this team.

We analyse the structure and governance of the Swedish agrifood system and the national and international regulatory environments. We also explore Sweden's capacity to produce and distribute innovative products and processes, constraints on this capacity, and the impact of all this on the Swedish economy. We plan to provide a synthesis which will be part of the basis for discussion of policy recommendations. The results will be relevant to actors in the primary agriculture sector, the biotech industry, and other stakeholders in the processing and distribution agrifood industry.

Centre for agriculture and food systems analysis and synthesis (Component Project 6, AgriSA)

The work in AgriSA focuses on whole production systems and stretches across disciplines within the human, agricultural, natural, and social sciences. The aim of this work is to understand and facilitate the implementation of sustainable food production using biotechnology as a tool. AgriSA is the hub where the information and results from all Mistra Biotech projects are processed and where overall syntheses are made and communicated to stakeholder groups. The work will include, among other things, scenario development, lifecycle assessment and cost-benefit analysis. Scientists from all the Mistra Biotech projects are working together in AgriSA. The work will also involve stakeholders and experts on food production systems and methods of system analysis.

Issues currently in focus in AgriSA:

1. What is included in the concept of sustainability in relation to green biotechnology?
2. Ecological consequences of biotechnology in plant and animal breeding.
3. Field trials, communication and relations with producers and consumers.
4. Sustainability assessments of different production systems.
5. Goal conflicts – can biotechnology help to solve conflicts between different environmental goals and between environmental goals and other goals of the society?
6. Ability to improve the nutritional value of food with plant and animal breeding

Mistra Biotech

Mistra Biotech involves over 50 researchers. Most of them work at SLU, while some work at the Royal Institute of Technology, Lund University, and other academic institutions. Mistra Biotech is funded by the Swedish Foundation for Strategic Environmental Research (Mistra) and SLU. Many companies, agencies and organizations also support the programme with their knowledge, experience and valuable feedback.

11

AGRICULTURE REDESIGN THROUGH PERENNIAL GRAINS: CASE STUDIES

Sieglinde Snapp

Department of Plant, Soil and Microbial Sciences, W.K. Kellogg Biological Station,
Michigan State University, East Lansing, MI, USA

ABSTRACT

Multiple purpose perennial crops are potentially the most adoptable by farmers and of most value to society, as means to provide grain, forage for livestock systems, recycling of nutrients to protect water quality, and build soils. This paper examines the services provided by two perennial grain crops as case studies: perennial wheat and semi-perennial pigeon pea. In Michigan, intermediate wheatgrass provided significant benefit to environmental services when investigated in field experimentation, especially through reducing leaching of nitrogen to almost nil. The results were consistent with perennial wheat as providing valuable environmental services, particularly in places with steep, marginal lands, such as Nepal. In Africa, diversification with legumes has a long history, and is the basis of the natural regeneration of fertility in extended bush fallows which dominated agricultural production until recent decades. A new type of perenniation is urgently needed, one where semi-perennial legume food crops are integrated with cereals and tuber crops. Improved varieties of legumes have tended to emphasize short-duration growth types with large grain to shoot ratios (high harvest index); this has come at the cost of extended vegetative cover which can double or triple photosynthesis and nitrogen fixation potential. Judicious combinations of short-duration food legumes (soybean, cowpea, groundnut and bean) and shrubby or viney grain legumes (pigeon pea, mucuna and climbing bean) with cereal crops is a promising way forward, to sustainably intensify production while gaining desirable perennial traits. A 'doubled up legume' system of pigeon pea grown in mixtures with short-statured food legumes, where pigeon pea is ratooned to grow a second year as an intercrop with maize has doubled fertilizer efficiency compared to sole maize, based on hundreds of participatory research actions on-farm in Malawi. Over 10 000 farmers are now pursuing innovations with various combinations of improved pigeon pea germplasm and integrated crop, soil and residue management. Participating villages have demonstrated, measurable improvements in yield stability and child nutrition. This is some of the emerging evidence that perennial grains can help communities overcome degraded soils and build family health for a resilient future.

Keywords: participatory research, agricultural systems, pigeon pea, perennial wheat, doubled up legumes

INTRODUCTION

The purpose of this paper is to illustrate the potential of two perennial grain crops — (i) perennial wheat (*Triticum aestivum x Thinopyrum elongatum*) and (ii) a semi-perennial legume crop, pigeon pea (*Cajanus cajan*) — to address critical production issues. The contribution of perennial wheat is explored using intermediate wheatgrass in Michigan. A doubled up legume system with pigeon pea is explored in Malawi. The results are used to illustrate how perennial grains can improve ecosystem sustainability and family health.

PERENNIAL WHEAT

A comprehensive, 4-year field study conducted in southwest Michigan is quantifying environmental services associated with perennial cereals, including high nitrogen retention, pure water quality, and soil building (Culman *et al.* 2013). Perennial wheat is a novel cereal crop under development through two different pathways. One pathway is the domestication of a perennial relative of annual wheat (*Triticum aestivum*), a forage grass called intermediate wheatgrass (*Thinopyrum intermedium)*. Another pathway that plant breeders are pursuing is to cross perennial grasses with annual wheat to develop a perennial form of wheat. Both pathways have the potential to produce multi-purpose crops; that is, crops that can be grown for grain or fodder and provide environmental benefits (Glover *et al.* 2010).

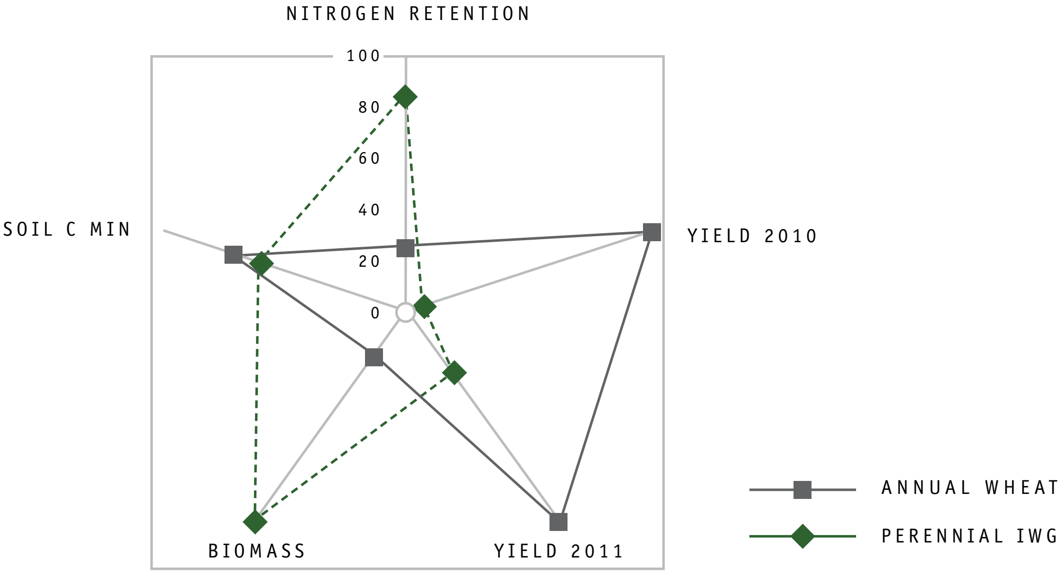
Dozens of lines of perennial wheat have been developed by plant breeders through repeated crossing of annual wheat with perennial grasses including intermediate wheatgrass and *Thinopyrum elongatum*. The new crop is under selection, as breeders choose lines that have a perennial growth habit (with post-sexual reproduction regrowth), while exhibiting grain characteristics similar to that of the annual wheat parent. The majority of the genetic makeup of this new crop is that of annual wheat, at least among the lines being developed at Washington State University by Drs. Stephen Jones and Kevin Murphy (Murphy *et al.* 2009).

Since 2007, field studies have been underway in Michigan at the W.K. Kellogg Biological Station, evaluating intermediate wheat grass (IWG) and perennial wheat lines for production potential and other ecosystem services such as soil building properties. Grain yields remain low to moderate, from about 0.3 to 1.8 mg per ha grain yield of perennial cereals relative to 2 to 4 mg per ha from annual wheat (Jaikumar *et al.* 2012). From an initial, 2 year assessment, IWG produces modest to almost zero grain yield and is a vigorous, true perennial grass that can be grown as a forage crop as it is highly productive and of good quality fodder (Figure 1), and it has substantial environmental benefits (such as reducing nitrogen leaching to almost undetectable amounts). (See Culman *et al.* 2013 for the first report on this novel cropping system). The root growth and biomass belowground of IWG is consistently five-fold higher, relative to annual wheat (C. Sprunger, unpublished data).

Perennial wheat has the advantage that it produces a crop that is recognizable as wheat, so the grain can be sold in an already-existing market, and there is the potential for growers to use it as a dual purpose crop whereby farmers who have livestock graze it during the spring, then plants are allowed to grow back and grain harvest occurs later that same year. Farmers reap an immediate return and have the added benefit of gaining from its long-term impact: large root systems that we predict will improve soil-organic matter, carbon sequestration, and water quality. There is considerable evidence that water quality is improved markedly by growing a crop of IWG, with 80 percent or higher retention of nitrogen in the soil-crop system, reducing nitrate leaching to almost zero through water loss pathways, as indicated by field experiments in the Snapp lab at Michigan State University (Figure 1) (Culman *et al.* 2013).

Somewhat surprisingly, there is limited evidence from these field experiments that IWG or perennial wheat lines have been effective at supporting rapid gains in soil carbon. The active soil carbon pool and nitrogen recycling is indeed markedly improved by IWG, but total soil carbon has been slow to respond in the initial years of these long-term perennial grain experiments.

FIGURE 1. BASED ON DATA PRESENTED IN CULMAN *ET AL.* (2013) COMPARING THE ECOSYSTEM SERVICES SUPPORTED BY PERENNIAL GRAIN IWG WITH THOSE PRODUCED BY ANNUAL WHEAT IN A FIELD TRIAL CONDUCTED AT KELLOGG BIOLOGICAL STATION, MSU, IN SOUTHWEST MICHIGAN



Soil C respiration measurements are consistent with active soil C pools being modestly enhanced in the presence of IWG, by about 15 percent relative to annual wheat (Figure 1). But no effect of a perennial grain (relative to annual wheat) has been observed for soil carbon sequestration pathways, as indicated by soil permaginate oxidizable carbon measurements (Culman *et al.* 2013). The almost complete lack of overlap between the prototype perennial grain IWG and annual wheat shows that the benefits of annual wheat are primarily grain yield as yet, whereas IWG has considerable fodder and environmental services and is not yet a significant producer of grain.

Taken together, the results are consistent with IWG as fitting well into a dairy or cattle farm. In contrast, perennial wheat shows potential as a cover crop to grow in marginal areas of the farm to protect fragile areas such as along riverbeds, but the unique property of perennial wheat is that, in addition to soil conservation, these new genotypes can also produce yields of grain and fodder. These results have implications for other regions of the world, such as Western Nepal, where wheat is the dominant crop and where its production as an annual crop requires excessive amounts of labour, provides too few additional benefits (e.g. to livestock), and is highly variable due to variable weather. Perennial wheat offers a range of opportunities for overcoming the challenges of annual wheat production (Figure 2).

FIGURE 2. DR. DHRUBA THAPA, A WHEAT BREEDER WITH THE NEPAL AGRICULTURAL RESEARCH COUNCIL, HIGHLIGHTS THE STRONG REGROWTH OF SOME OF HIS PERENNIAL WHEAT HYBRIDS

He believes perennial wheat will improve the lives of women farmers, increase wheat yields and quality, and meet multiple farming system needs.

FIGURE 3. TALLER, SLOWER GROWING PIGEON PEAS COMPLEMENT LOWER- AND FASTER-GROWING GROUNDNUTS, WHICH ARE READY FOR HARVEST SEVERAL WEEKS BEFORE PIGEON PEAS MATURE

SEMI-PERENNIAL PIGEON PEA

Another case study, highly relevant to tropical farming systems, is that of the perennial legume species, pigeon pea (*Cajanus Cajan*) (Snapp *et al.* 2003). It is often grown as an annual but traditional cropping systems still involve production of pigeon pea as a short-lived perennial (two to three years). It is a tropical legume that grows as a shrubby semi-perennial crop, and demonstrates a unique set of multi-functional properties, which is being documented in Malawi, southern Africa (Glover *et al.* 2012; Snapp *et al.* 2010). Farmers have long relied on pigeon pea to provide flexible options for livestock feeding and crop production, enhancing flexibility and productivity of the entire farm system. Recent genetic improvements in pigeon pea include new varieties that are broadly adapted to diverse environments. Farmer research groups are testing agronomic innovations through participatory research approaches that promote farmer innovation (Bezner-Kerr *et al.* 2007).

Over 9 000 farm families have newly adopted pigeon pea in the northern Ekwendeni region of Malawi (Snapp *et al.* 2010). Farmer education through participatory action research has promoted experimentation, which includes planting arrangements, crop residue management, and improved fodder for dairy cattle. Because parts of Africa's climate are tropical, farmers can incorporate pigeon pea into cash crops as they can grow for one to three years. After it is harvested, growers can cut it back and use the vegetative material as fodder for livestock or to improve soil fertility, and can sell the peas for profit or use them as a protein-rich food.

Agro-ecology training of farmers has supported local testing of a range of perennial-diversified options, and older, drought tolerant crops such as land races of sorghum are being grown as intercrops with pigeon pea and with other grain legumes (e.g. doubled up legume mixtures of pigeon pea and groundnut or pigeon pea and soybean), and in maize mixed systems (Figures 3 and 4). Growing pigeon pea and doubled up legume systems (with pigeon pea intercropped with soybean or groundnut) has been tested in Kenya and Malawi, with growing evidence that they can substantially enhance production of nutrient-enriched grain compared to sole cropped maize (Snapp and Silim, 2002; Snapp *et al.* 2010; Figure 5). Pigeon pea has a deep root system and leafy residues that enhances soil fertility for consistent yield gains in rotated crops such as maize, as well as supporting production of grain directly.

FIGURE 4. PIGEON PEAS PROVIDE INTERCROPPING OPPORTUNITIES FOR FARMERS

Because of their slow growth rates in the first year, they do not compete aggressively with faster growing legumes such as groundnuts. As they regrow in the second season, they can compete with more aggressive crops such as maize.

DOUBLED-UP LEGUME SYSTEM

- increases plants' efficiency of fertilizer use
- improves yield of protein-rich grains
- decreseas labour requirements
- improves families' diets

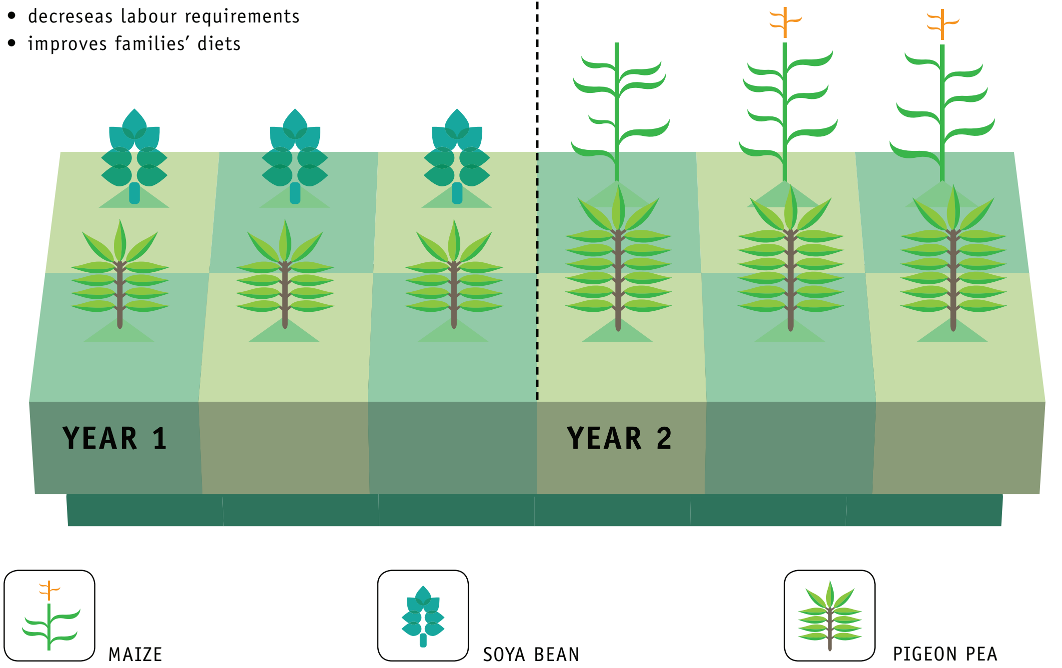
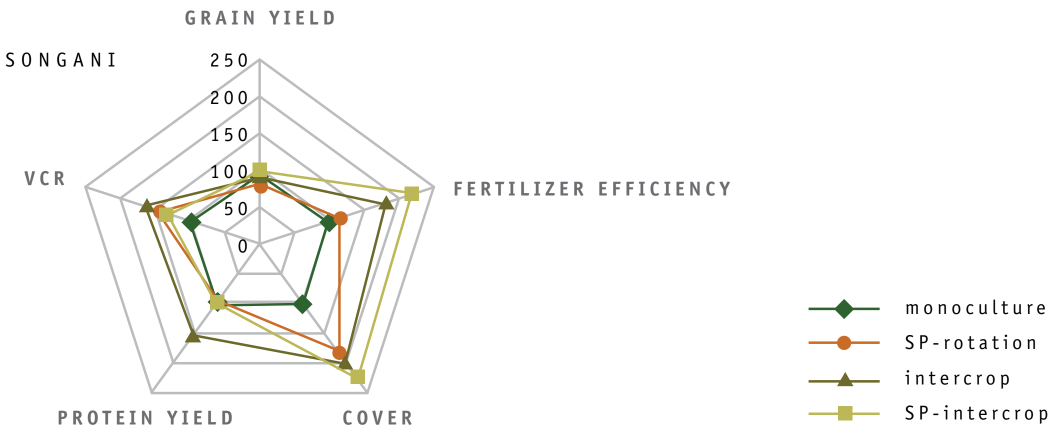


FIGURE 5. SHRUBBY PIGEON PEA INTERCROPS (SP-INTERCROP) AND SHRUBBY PIGEON PEA ROTATIONS (SP-ROTATIONS) DECREASE FERTILIZER REQUIREMENTS; IMPROVE THE VALUE COST RATIO (VCR), FERTILIZER USE EFFICIENCY, AND PROTEIN YIELDS; INCREASE CARBON AND NITROGEN ASSIMILATION AND PHOSPHORUS AVAILABILITY; AND PROVIDE GREATER COVER THAN MONOCULTURE MAIZE



Source: Snapp *et al.* 2010

Long-term field experiments and on-farm monitoring in Malawi have documented the unique ability of these polyculture perennial systems to support gains in soil organic carbon, nitrogen, and water use efficiency and adaptation of crops to climate variability. These are some of the lines of evidence suggesting that perennial crops provide farmers with important new options as a foundation for sustainable intensification of smallholder production in Africa. Further, we predict that the environmental benefits that are associated with perennial grains will prove vital to smallholder farmers' ability to buffer crop production and cope with the on-going variability that is associated with weather patterns in sub-Saharan Africa. Global warming is predicted to induce enhanced variability in the near future, so coping with droughts, rising temperatures, and variable weather will be even more important in the coming decades. The examples presented here illustrate how perennial grains can be used in combination with judicious inputs to provide a sound foundation for wise resource use, to protect the environment, and to support sustainable production in a rapidly changing world.

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