Pollen mRNAs of *Phalaris coerulescens* and their Possible Role in Self-Incompatibility

by

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November 1995

Phalaris coerulescens

Declaration

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November 1995

Acknowledgements

I wish to thank my supervisors, Dr Peter Langridge and Dr David Hayman for their support, advice and constructive criticism during the course of this study. I also thank Ms Margaret Cargill for her assistance with the English language and encouragement during the writing of this manuscript.

Many thanks to all students and staff in the laboratory of Dr. Peter Langridge for their helpful advice, practical assistance and inspiring discussions during the course of this research. Special thanks to Ms Jan Nield for her excellent technical advice, to Ms Carole Smith for assistance with the plants and Mr Angelo Karakousis for help with computer problems.

Finally, I would like to thank my friends André, Jennifer, Fiona and Stephen for their support and understanding.

This work was supported by an Australian Overseas Postgraduate Research Award and the Australian Research Council's Special Research Centre for Basic and Applied Plant Molecular Biology.

List of Abbreviations

aa	amino acid
ATP	adenosine 5'-triphosphate
b	base/s
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BLAST	Basic Local Alignment Research Tool
bp	base pair
BSA	bovine serum albumin
^{o}C	degree centrigrade
cAMP	adenosine 3',5'-cyclic-monophosphate
cDNA	complementary deoxyribonucleic acid
Ci	curie
Da	Dalton
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DEAE	diethylaminoethyl
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine triphosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
ds	double stranded
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetraacetic acid
EMBL	European Molecular Biology Laboratory
g	gram
HEPES	N-2-hydroxyethylpiperazine-N'-2- ethanesulfonic acid
hr	hour
IPTG	isopropyl-1-thio-b-D-galactoside
kb	kilobase
kDa	kiloDalton
mA	milliampere
mg	milligram/s
min	minute/s
µg	microgram/s
ml	milliliter/s
µl	microliter/s

M	molar
MOPS	3-(N-morpholino)propane-sulfonic acid
mRNA	messenger ribonucleic acid
NBT	nitroblue tetrazolium
NTP	nucleoside triphosphate
OD_{260}	optical density at 260 nm
oligo(dT)	oligodeoxythymidylic acid
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
pers. comm.	personal communication
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PIR	Protein Information Resource
PIPES	piperazine-N,N'-bis(2-ethane-sulfonic acid)
poly(A)	polyadenylic acid or polyadenylate
poly(A)+	polyadenylated (mRNA)
RFLP	restriction-fragment-length polymorphisms
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
Sarkosyl	N-lauroylsarcosine
SDS	sodium dodecyl sulfate
ss	single stranded
SSC	sodium chloride/sodium citrate (buffer)
TAE	Tris/acetate (buffer)
Taq	Thermus aquaticus DNA (polymerase)
TBS	Tris-buffered saline
TE	Tris/EDTA (buffer)
Tris-Cl	tris(hydroxymethyl)aminomethane hydrochloride
UV	ultraviolet
v/v	volume/volume
w/v	weight/volume
X-gal	5-bromo-4-chloro-3-indolyl-b-D-galactoside

Summary

Self-incompatibility is a highly specific recognition system between pollen and pistil which acts as a barrier to self-fertilisation. In *Phalaris coerulescens,* gametophytic self-incompatibility is under the control of two unlinked genes, S and Z. An incompatible reaction occurs when both the S and Z alleles of the pollen are present in the genotype of the recipient stigma. In this very rapid reaction either pollen grains fail to germinate or pollen tube growth is arrested shortly after contact with the stigma.

This study examined the contribution of genes specifically expressed in the male gametophyte to the physiological processes during pollen tube germination and pollen-stigma interaction. Among the aims of this study was the isolation of the Z-gene.

To this end, selected cDNA clones from a pollen mRNA library were differentially screened with RNA from non-pollen tissues and pollen RNA from plants of different Z-genotypes. Clones which showed differences in hybridisation intensity with different Z-alleles and were pollen-specific were grouped depending on their behaviour in cross-hybridisation studies. Representative clones of each of the eighteen subgroups were subsequently analysed for linkage to the Z-locus in RFLP studies. However, none of the clones analysed showed linkage to Z.

To investigate the possible role of these 18 cDNA clones in pollen function, their temporal and spatial expression was analysed and their inserts were either partially or fully sequenced. The results of the transcriptional analysis revealed that the majority of the cDNAs were indeed predominantly expressed in pollen, as anticipated from the result of the differential screen. A comparison of their expression in pollen from plants with different self-incompatibility genotypes, including self-fertile mutants, revealed various degrees of variation in expression level but no correlation with the S-Z genotypes of the pollen parents was indicated.

In one case, clone B7b, the transcripts were completely absent in two self-fertile mutants. Further characterisation of B7b showed that the clone represents a gene-family with at least 15 members, which are closely linked since they were inherited as a single locus in a segregation analysis. DNA blot analyses revealed that the lack of B7b-transcripts in the two self-fertile mutants was correlated with the absence of the corresponding DNA sequences. However, examination of a large number of plants identified more, including self-incompatible wildtypes, in which the B7b family was not detectable. It was therefore suggested that the apparent absence of the gene-family and a self-fertile phenotype are independent phenomena. An open reading frame was found in 5 out of 9 cDNAs sequenced. The deduced protein sequences indicated the presence of a putative signal pep tide and demonstrated the presence of a new cysteine-motif. Database searches failed to

show any homology to known sequences. Possible functions for this gene-family are discussed.

Sequence analysis of the other 17 cDNA clones revealed sequence homology of 3 clones to protein kinases, among them a MAP-kinase, a calcium-dependent calmodulin-independent protein kinase and a kinase with homology to the *Pto/Fen* family. In addition, a fructose-1,5-phosphate aldolase, a *LAT52* homolog and a putative glucose-transport protein were identified. The remaining 12 cDNAs did not show any homology to sequences in the databases and are likely to represent new genes.

The involvement of protein kinases in the self-incompatibility reaction of *Phalaris* was addressed by examining phosphorylation of pollen proteins from self-incompatible and self-fertile mutant plants to determine whether the self-compatible phenotype was correlated with failed phosphorylation of any proteins. The results showed that this was not the case with any of the self-fertile mutants of *Phalaris*. Using immunodetection and immuno-precipitation, it was demonstrated that the S-protein is not phosphorylated during pollen germination *in vitro*. Nor was any evidence obtained for the phosphorylation of the S-protein in response to stigma proteins. In addition, an experimental system was developed which allows direct monitoring of protein phosphorylation during pollen germination and tube growth.

Chapter 1 Literature Review

1.1 Introduction

The life cycle of angiosperms is characterised by the alternation between a diploid generation, the sporophyte, and a haploid generation, the gametophyte. Male and female gametophytes are reduced in size and complexity; the female gametophyte usually consists of seven cells whereas the male gametophyte consists of two or three cells.

The female gametophyte is a product of the ovule. The nucellus, which is derived from the apical portion of the ovule, produces the megasporocyte, which undergoes meiosis to form the megaspores. In most plant species only one of the meiotic products becomes a functional megaspore (Reiser and Fischer 1993).This undergoes three mitotic divisions, followed by nuclear migration and cytokinesis, to form the embryo sac, the mature female gametophyte. The female gametophyte stays in the ovary, which together with style and stigma comprises the pistil, the female part of the flower.

Male gametogenesis starts with the division of a diploid sporophytic cell, giving rise to the tapetal initial and the pollen mother cell. The pollen mother cells undergo meiosis, producing tetrads of haploid cells which are subsequently released as free microspores. The microspores undergo an asymmetric division producing two unequal daughter cells, the vegetative cell and the generative cell, which have different structures and developmental fates (McCormick 1993). In the majority of plant families the pollen grain is released from the anther in the bicellular stage and the mitotic division of the generative cell occurs in the germinated pollen grain. In other families, such as the *Gramineae* and the *Cruciferae*, the second mitotic division takes place before the pollen is released.

To complete the cycle the pollen attaches to a suitable stigma, germinates and extrudes the pollen tube which grows down the style and delivers the sperm cells to the embyo sac. During this phase of development, complex interactions occur between the male gametophyte and the cells of the pistil, which ultimately determine whether or not fertilization will take place. A knowledge of the molecular nature of these cell-cell interactions is an essential step towards the understanding of the fertilization process in flowering plants.

The first part of this literature review examines what is possibly the single most important aspect of pollen-stigma interaction, the phenomenon of self-incompatibility (SI). The strategies currently in use for the identification of genes involved in SI and their limitations are then discussed. Finally, current knowledge about genes specifically expressed in pollen is reviewed.

1.2 Self-Incompatibility

Self-incompatibility (SI), defined as " ..the inability of a fertile , hermaphrodite seed-plant to produce zygotes after self-pollination" (Lundqvist 1964) and is a special example of cell-cell communication in plants. SI involves a recognition event between a germinating pollen grain and the pistil, in which the growth of "self" but not "non-self" pollen tubes is stopped, on either the stigmatic surface or the transmitting tract of the style .

The continuing interest in investigating the molecular factors involved in self-incompatibility is due to a number of reasons. Firstly, SI is of agronomical importance, in terms of the establishment or elimination of breeding barriers. Secondly, it is likely that SI was a prerequisite for the emergence of angiosperms in the Cretaceous period and played a key role in their evolutionary success (Whitehouse 1950). Thirdly, SI may function as a model system for the elucidation of the ways plant cells interact during differentiation and morphogenesis, and with pathogens and symbionts (Lewis 1954). Finally, it is critical to our understanding of plant reproduction.

Much of the early work and theories on self-incompatibility have been reviewed by de Nettancourt (1977), Lewis (1979a) and Heslop-Harrison (1978, 1982b). Recent molecular work has been reviewed by Franklin-Tong and Franklin (1993), Hinata et al. (1993), Nasrallah and Nasrallah (1993), Newbigin et al. (1993) and Sims (1993).

Self-incompatibility systems are classified as heteromorphic or homomorphic depending on whether they are associated with differences in flower morphology. Homomorphic systems can be assigned to two major classes which are distinguished by the way the recognition specificity in the pollen is determined. In gametophytic systems the haploid genotype of the individual pollen determines its incompatibility phenotype, whereas in sporophytic systems the incompatibility phenotype is dependent on the diploid genotype of the pollen-producing plant. In the simplest and best studied systems, SI is controlled by a single locus, the "S-locus", which has multiple alleles. In gametophytic systems S-allele specificities are expressed in a co-dominant fashion. A pollen grain is therefore incompatible when its genotype matches either allele in the pistil. Sporophytic systems are characterised by the occurrence of dominance and co-dominance effects within pollen and stigma. A pollen grain is incompatible when the dominant allele(s) of the pollen parent matches the dominant allele(s) in the recipient pistil.

Gametophytic SI is widespread throughout the plant kingdom, whereas sporophytic SI seems to occur only in the *Compositae* and the *Cruciferae* (reviewed by de Nettancourt 1977). In both systems the number of alleles at the S-locus can be very large. For *Brassica oleracea* for example, which has a sporophytic system, over 50 different S-alleles have been

identified in a population (Ockendon 1980, 1982) and in *Papaver rhoeas* more than 30 alleles were found (Campbell and Lawrence 1981).

More complex systems in which several genes are involved have also been identified, such as the two-locus system in grasses (Lundqvist 1954; Hayman 1956) and the four-locus system in *Beta vulgaris* (Larsen 1977).

Since the phenomenon was described by Darwin in 1876 , many aspects of self-incompatibility have been extensively studied but nevertheless most questions raised are yet to be answered. For instance, how did the self-incompatibility systems evolve? Has self-incompatibility evolved only once as suggested by Whitehouse (1950) or several times as argued by Bateman (1952). What is the organisation of the S-locus? Based on mutation studies, Lewis (1949, 1960) suggested that the S-locus had a tripartite structure and consisted of an S-specificity domain, which determined the S-allele specificity of pollen and pistil, a pollen activity domain and a pistil activity domain, which activated the S-allele specificity in pollen and pistil, respectively. Are the S-proteins in pollen and pistil indeed identical or are they entirely different as proposed by van der Donk (1975)? What is the nature of the S-proteins?

In recent years, recombinant DNA techniques have been extensively used in an attempt to understand the molecular mechanisms underlying SI and to answer some of the above questions. Molecular studies of single locus systems have been carried out in *Brassicaceae*, *Solanaceae* and *Rosaceae*, and *Papaveraceae* and are summarised below.

1.2.1 Self-Incompatibility in the *Brassicaceae*

At present the *Brassicaceae* are the only sporophytic system that has been investigated at a molecular level.

Genetic studies have shown that SI in Brassica is controlled by a single complex locus, the S-locus. Molecular analyses of the S-locus region have identified two different genes, the S-locus glycoprotein (*SLG*) and the S-receptor kinase (*SRK*) (Stein et al. 1991), which are separated by less than 220 kb (Boyes and Nasrallah 1993). Together they comprise the so-called "S-haplotype".

Significant evidence indicates that the *SLG* and *SRK* gene represents the S-locus. Firstly, extensive restriction length polymorphism studies have demonstrated perfect co-segregation of the self-incompatibility phenotype with polymorphic fragments detected by hybridisation with DNA sequences specific to *SLG* and/or *SRK* (Chen and Nasrallah 1990; Stein et al. 1991; Kumar and Trick 1994; Delorme et al. 1995). Secondly, both genes are predominantly transcribed in the pistil. *In situ* hybridisation and RNA blot analysis have both shown that *SLG* expression is developmentally regulated, localised in the papillar cells

of the stigma and reach maximum levels with the onset of SI (Nasrallah et al. 1985; Nasrallah et al. 1988). The *SLG* protein can make up 5% of total soluble protein in the stigma (Nasrallah and Nasrallah 1989). *SRK* expression is lower than that of *SLG* by a factor of 140-180 (Stein et al. 1991).

The *SLG* gene comprises a single exon that encodes a secreted protein of about 435 amino acids, including a signal peptide at the N-terminus. The number of potential glycosylation sites varies among alleles. Whether the oligosaccharide moieties play a role in the recognition process is not yet clear (Sims 1993).

The S-receptor kinase gene is predicted to encode a transmembrane protein kinase with intrinsic serine/threonine kinase activity (Stein et al. 1991; Goring and Rothstein 1992; Stein and Nasrallah 1993). The extracellular domain shows high sequence identity to the *SLG* of the same haplotype. Values ranging from 75.9 % to 98.4% have been reported (Stein et al. 1991; Kumar and Trick 1994; Watanabe et al. 1994; Yamakawa et al. 1995; Delorme et al. 1995) suggesting that the genes might have evolved by duplication (Tantikanjana et al. 1993).

The high degree of polymorphism of the S-locus has been demonstrated by overall comparisons of the protein sequences of *SLG-SRK* pairs from different S-haplotypes. Sequence divergence of up to 30% at the protein level have been found between class I haplotypes, which exhibit a strong self-incompatibility reaction, and class II haplotypes, which show a weaker incompatibility phenotype (Stein et al. 1991). Sequence comparisons between different *SLG* alleles display conserved and variable regions in the protein (see Sims 1993 for similarity plot). The highest variability is concentrated at the central part of the protein (Nasrallah et al. 1991; Sims 1993).

The study of mutants added more evidence that the *SLG* and*SRK* genes are required in SI. For instance, the analysis of a self-compatible strain of *B. campestris* showed that, while the expression levels of *SRK* were unchanged, *SLG* expression was significantly reduced in comparison to self-incompatible strains (Nasrallah et al. 1992). Evidence that a functional *SRK* gene is required in SI was provided by Goring et al. (1993). A 1 bp deletion in the *SRK* gene, which resulted in premature termination of translation, conferred self-compatibility. In another study Nasrallah et al. (1994a) investigated self-compatible mutant strains of *B. oleracea* and *B. campestris*. In neither mutant could an *SRK* transcript be detected. In *B. oleracea* this was due to the *SRK* gene being a null allele resulting from a deletion of the promotor region and the extracellular and transmembrane domains. Expression of *SLG* was unaffected.

Recently Shiba et al. (1995) successfully transformed self-incompatible *B. campestris* with an antisense-*SLG* gene construct. The transgenic plant set seeds after self-pollination, and *SLG*- protein was not detectable in immunoblot analysis.

Sporophytic control of SI in *Brassica* implies that the pollen is supplied with the necessary molecules for recognition by the pollen parent. It has been suggested that the tapetum tissue expresses the S-proteins which are then deposited in the pollen wall (Heslop-Harrison 1975).

Transformation experiments indicated that the *SLG* and the *SRK* promotor can drive transcription in both the pistils and anthers (Stein et al. 1991; Sato et al. 1991). However RNA blot analyses revealed expression of the *SRK* gene only in anthers at the binucleate stage (Stein et al. 1991, Delorme et al. 1995). *SLG* expression in young anthers was only detected by using the polymerase chain reaction (Guilluy et al. 1991, Delorme et al. 1995). Watanabe et al. (1991), who approached the question from the protein side, used immunoblotting to identify proteins with homology to *SLG* epitopes in anthers. Their results indicated the presence of an *SLG*-like protein in anther walls, which was first detected in anthers with pollen at the uninucleate stage. The biochemical characteristics of the protein differ from the stigma *SLG* however and its role in SI, if any, has not yet been established.

Since the pollen S-protein has not yet been unambiguously identified, Nasrallah et al. (1994b) in their model for SI in *Brassica* proposed a pollen-borne component, which is possibly a ligand for *SRK*. In an incompatible reaction this pollen component, which would be highly polymorphic and encoded within the S-locus complex, would bind to the *SLG-SRK* complex of the stigma, causing the activation of the S-receptor kinase. The activated signal transduction pathway would lead to a localised change in the papillar cell at the adhesion site of the pollen. As a consequence the adhesion would be weakened and pollen tube growth arrested. This model is supported by physiological studies (reviewed by Dickinson 1995), which suggest self grains as physiologically isolated from the subjacent papilla of the recipient stigma. This leads to an interruption of the hydration of the grain and to inhibition of pollen tube growth.

The study of SI in Brassica is complicated by the fact that the *SLG-SRK* genes belong to a multigene family with at least 15 members (Dwyer et al. 1989). Some members are thought to be pseudogenes while others, the S-Locus-Related genes, *SLR1* and *SLR2,* not only show high homology to *SLG*, but are also expressed in the same spatial and temporal manner (reviewed by Trick and Heizmann 1992, Sims 1993). At present neither the involvement of the *SLRs* in SI nor their function has been determined.

In conclusion, it remains to be proven whether the *SLG-SRK* pair is not only necessary but sufficient for the operation of self-incompatibility in *Brassica*.

Interestingly, the presence of S-locus-glycoproteins and S-receptor kinases is not restricted to self-incompatible plants in the Brassicaceae. A gene encoding a protein similar to *SRK* has been reported in *Zea mays*, called *ZmPK1* (Walker and Zhang, 1990).

Altogether six homologs to the *SLG/SRK* family have been reported in *Arabidopsis*, (Dwyer et al 1992, 1994; Tobias et al. 1992) and an *SLG* homolog, *EP1*, was isolated from carrot (van Engelen et al. 1993). The presence of proteins with an *SLG*-like domain in self-compatible species indicates that the function of these proteins is not restricted to self-incompatibility. It has been suggested that the genes involved in SI were recruited from genes which originally had more generalised roles in fundamental developmental processes both in self-incompatible and self-compatible plants (Nasrallah et al. 1994b).

1.2.2 Self-Incompatibility in the *Solanaceae* and *Rosaceae*

In contrast to the *Brassicaceae*, in which incompatible pollen tubes are arrested on the stigma, in the gametophytically controlled self-incompatibility reaction of the *Solanaceae* and *Rosaceae*, incompatible pollen tubes are arrested in the style transmitting tissue (van der Pluijm and Linskens 1966; de Nettancourt et al. 1973). This observation suggests the possible site of expression of the S-gene. Biochemical work focussing on style proteins of several species led to the identification of glycoproteins which correlated with S-alleles (Kamboj and Jackson 1986; Mau et al. 1986). The subsequent analysis of these proteins and the sequencing of their N-termini was followed by the isolation of the first cDNA clone for the S-gene in *Nicotiana alata* (Anderson et al. 1986). The biochemical work on the S-proteins has been reviewed by Cornish et al. (1988), Hinata et al. (1993) and Sims (1993). At present several S-alleles have been cloned from different species (Table I). Since the features of these genes are very similar they will be considered together.

Table I:

Species	S-allele	Reference
Nicotiana alata	S_2	Anderson et al. (1986)
	S_3, S_6	Anderson et al. (1989)
	S_1, S_z, S_{F11}, S_a	Kheyr-Pour et al. (1990)
Petunia hybrida	S_1, S_2, S_3	Clark et al. (1990)
	S_o, S_x	Ai et al. (1992)
Petunia inflata	S_1,S_2,S_3	Ai et al. (1990)
Solanum chaoense	S_2,S_3	Xu et al. (1990)
	S_{13}, S_{14}	Depres et al. (1994)
	S_{11}	Saba-El-Leil et al. (1994)
Solanum tuberosum	S_1, S_{r1}, S_2	Kaufmann et al. (1991)
Malus x domestica	S_2,S_3	Broothaerts et al. (1995)

The S-gene encodes a basic protein of approximately 220 amino acids, including a signal peptide of 22 amino acids. The mature protein is glycosylated, but as shown by Karunanandaa et al. (1994) the glycosylation is not critical for the recognition process in the self-incompatibility reaction. The S-mRNA is preferentially expressed in the style and

accumulates when the style undergoes transition from bud self-compatible to self-incompatible (Cornish et al. 1987). Low to very low levels of S-mRNA have also been found in ovaries and petals, but not in leaf, mature anthers or pollen. (Cornish et al. 1987; Clark et al. 1990; Sims 1993). DNA blot analysis indicated that the S-gene is a single copy gene (Bernatzky et al. 1988).

The polymorphism at the locus is reflected in the sequences of the S-alleles. Sequence alignment of different alleles revealed a distinct pattern of conserved and variable regions in the proteins. Three of the five conserved regions are quite hydrophobic, the remaining two are hydrophilic (Clark et al. 1990). In a comparative sequence analysis of several S-alleles from *Nicotiana*, *Petunia* and *Solanum*, Ioerger et al. (1990) observed that the similarity between two S-alleles from the same species can be as low as 40%. Furthermore, sequence divergence among alleles within one species can be higher than interspecies sequence divergence. These data suggests that the S-genes arose from an ancestral gene which was recruited for use in SI before speciation.

Work on fungal ribonucleases revealed that there was a partial sequence homology between RNases and the S-protein from *N. alata* (Kawata et al. 1988, 1990). This lead to testing of the S-glycoproteins for RNase activity (McClure et al. 1989). Subsequently, RNase activity of S-glycoproteins from several solanaceous (Broothaerts et al. 1991; Singh and Kao 1991; Ai et al. 1992) and rosaceous (Sassa et al. 1992; Broothaerts et al. 1995) species was shown. Indirect evidence for the involvement of ribonuclease activity in the self-incompatibility reaction was demonstrated by McClure et al. (1990). It was found that pollen rRNA recovered from self-pollinated pistils was degraded, whereas the rRNA remained intact during compatible interactions. Other experiments showed that *S-RNases* are able to enter and inhibit the growth of pollen tubes germinated *in vitro*, but allele specificity could not be demonstrated (Jahnen et al. 1989; Gray et al. 1991).

Clear demonstration that the *S-RNases* are required for SI was provided by transformation experiments. Working with *P. inflata*, Lee et al. (1994) transformed an S_2S_3 plant with an S_3 antisense gene, resulting in several plants which were self-fertile. The breeding behaviour of these plants was in direct correlation with the reduced expression of the *S-RNase* genes, both at the RNA and protein levels. Plants with a significant reduction in S_3 expression failed to reject S_3 pollen, while plants with reduced levels of S_2 and S_3 protein failed to reject both S_2 and S_3 pollen. Furthermore, in a second experiment, when an S_3 transgene was expressed in an S_1S_2 plant, the transgenic plants gained the ability to recognise and reject S_3 pollen.

Using a similar approach, Murfett et al. (1994) introduced S_{A2}-cDNA of *N. alata*, controlled by the promotor of a tomato chitinase gene, into self-compatible hybrids between *N. alata* and *N. langsdorffii*. The resultant transformed plants which expressed high levels of the S_{A2}-protein were able to reject S_{A2} pollen.

As the His 93 codon has been implicated in RNase activity (Ohgi 1992), Huang et al. (1994) transformed a petunia plant of S_1S_2 genotype with a mutated (His93 to Asn) S_3 allele to answer the question of whether the degradation of pollen rRNA is a cause or effect of aborted pollen tube growth in an incompatible reaction. Of the transformants, even those with high levels of mutant S_3 protein could not reject S_3 pollen. Ribonuclease activity of the purified mutant S_3 protein could not be detected. This showed unambiguously that the ribonuclease activity of *S-RNases* is required for the incompatibility reaction.

While the pistil side has been fully elucidated, the pollen component has not yet been unambiguously identified. If one assumes that the pollen and the stylar product of the S-locus are identical (de Nettancourt 1977), then *S-RNase* transcripts should be detected in mature anthers and pollen. However, using PCR Dodds et al. (1993) detected only low levels of *S-RNase* gene expression in immature anthers in *Nicotiana alata*. The expression was restricted to the developing pollen grains, as shown by *in situ* hybridisation. Clark and Sims (1994) reported a similar result for *Petunia hybrida*.

On the other hand, Lee et al. (1994) noted in their transformation experiments that the self-incompatibility behaviour of the pollen of the transgenic plants (mentioned above) was not at all affected, either by the antisense S_3 gene or by the S_3 transgene. This suggests that the SI-phenotype of the pollen is under the control of a separate gene, closely linked to the stigma S-gene. However, the failure of the S3 transgene to modify pollen behaviour might be due to complex allelic interactions, which occur when pollen of species with gametophytic SI carries two S-alleles (Lewis 1979b).

Preliminary data by Okuley and Sims (1991) suggested that a DNA sequence, tightly linked to the S_1-RNase gene in *Petunia* is transcribed in pollen. Whether this transcription unit represents the pollen component of SI in the *Solanaceae* remains to be shown.

At present two working models for SI in the *Solanaceae* are being discussed (Sims 1993). Both models assume that the *S-RNases* are secreted into the intercellular spaces of the transmitting tract of the style, where they are encountered by both compatible and incompatible pollen tubes. In one model, the pollen component mediates the selective uptake of *S-RNases* of the same genotype, which then degrade pollen RNA and lead to the arrest of pollen tube growth. In the second model, *S-RNases* enter pollen tubes regardless of the genotype. A specific inhibitor produced by the pollen then inactivates *S-RNases* of different genotypes, but not *S-RNases* with the same genotype.

As in the *Brassicaceae*, the self-incompatibility gene of the *Solanaceae* and *Rosaceae* might have other functions in other plants. For example, a gene with homology to the *S-RNases* has been identified in *Arabidopsis* (Taylor et al 1993). For an overview on RNases in plants and their function see Green (1994).

1.2.3 Self-incompatibility in *Papaver rhoeas*

Self-incompatibility in *Papaver* is also gametophytically controlled. However, unlike the *Solanaceae* and *Rosaceae*, the site of pollen tube inhibition in an incompatible reaction is at the surface of the stigma, due to flower morphology (Lawrence et al. 1978). This inhibition takes place very rapidly, during or just after germination of the pollen grain.

Franklin-Tong and coworkers developed an *in vitro* bioassay for studying the self-incompatibility reaction (Franklin-Tong et al. 1988). This assay system enabled the researchers to not only isolate the S-protein but also to study the signal transduction mechanism directly (Franklin-Tong et al. 1989).

When pollen tubes growing *in vitro* are challenged with S-protein from incompatible stigmas they respond with a transient increase in internal calcium concentration (Franklin-Tong et al. 1993), followed by protein phosphorylation and changes in gene expression (Franklin-Tong et al. 1992) which ultimately lead to cessation of pollen tube growth. This response, which can be mimicked by injecting calcium into the pollen tube, is absent in a compatible reaction, and it does not occur in response to heat denatured incompatible S-protein (Franklin-Tong et al. 1993).

The S-gene, which has now been cloned, encodes a small, probably secreted, glycoprotein of 22 kDa, which is only expressed in maturing stigmas. The corresponding cDNA hybridises to a single band in a DNA blot analysis, which co-segregates with the S-phenotype. It is a novel gene since database searches did not reveal any significant homology to known sequences (Foote et al. 1994).

When the recombinant protein expressed in *E. coli* was purified and used in the bioassay, the pollen tube response was identical to that of the isolated pistil protein (Foote et al. 1994). This result demonstrates that the cloned gene encodes the S-protein and shows that the carbohydrate moiety is not required for biological activity. Furthermore, the recombinant protein can trigger a transient rise in cytosolic free calcium in incompatible pollen tubes just as the purified native protein does (Franklin-Tong et al. 1995). Therefore post-translational modifications of the S-protein are not required for its biological activity.

The current model of signal transduction in *Papaver* assumes that in an incompatible reaction, the S-glycoprotein binds to a pollen receptor which induces a cellular response, probably via the phophatidylinositol pathway. The expression of genes is subsequently altered, with new proteins being produced specifically in incompatible pollen. Whether these proteins cause the cessation of pollen tube growth or whether they are expressed as the result of the modification of proteins which lead to cell death has not been demonstrated (Franklin-Tong and Franklin 1993). At present the pollen receptor is unidentified.

1.2.4 Self-Incompatibility in the *Poaceae*

The earliest comprehensive studies on self-incompatibility in the *Gramineae* were carried out by several researchers, including Körnicke (1890), Troll (1930, 1931) and Beddows (1931). Connor (1979) summarised and updated the knowledge in this area and his review can be consulted for specific references. SI is widespread among the grasses and present in at least 16 genera (Connor 1979). Self-compatible and self-incompatible species are found in the same genus, the frequency of self-incompatibility being higher among the perennials than the annual species (Körnicke 1890; Beddows 1931).

1.2.4.1 The Genetics of SI in the *Poaceae*

The genetics of SI in the *Poaceae* remained unknown until studies in *Secale cereale* by Lundqvist (1954) and *Phalaris coerulescens* by Hayman (1956). Both researchers proposed that self-incompatibility in grasses was under the gametophytic control of two multiallelic genes, named S and Z, which segregated independently. A pollen grain is incompatible when both its S and Z alleles are matched in the recipient pistil. The S-Z system has been identified in eight species and is likely to be present in another eight (Table II).

Table II: (after Hayman, 1992)

Species	Tribe	Reference
a) Species in which the S-Z system has been identified by studies using parental genotypes of known relationship		
Secale cereale	Triticae	Lundqvist (1954)
Festuca pratensis	Poeae	Lundqvist (1955)
Phalaris coerulescens	Avenae	Hayman (1956)
Hordeum bulbosum	Triticae	Lundqvist (1965)
Dactylis aschersoniana	Poeae	Lundqvist (1965)
Briza media	Poeae	Murray (1974)
Lolium perenne	Poeae	Cornish et al (1979)
Lolium multiflorum	Poeae	Fearon et al. (1983)
b) Species in which a gametophytic system has been identified and there are differences between reciprocal crosses		
Alopecurus myosuroides	Avenae	Leach & Hayman (1987)
Cynosurus cristatus	Poeae	Weimarck (1968)
Holcus lanatus	Avenae	Weimarck (1968)
Alopecurus pratensis	Avenae	Weimarck (1968)
Arrhenatherum elatius	Avenae	Weimarck (1968)
Festuca rubra	Poeae	Weimarck (1968)
Deschampia flexuosa	Avenae	Weimarck (1968)
Phalaris arundinacea	Avenae	Weimarck (1968)

1.2.4.2 Features of the S-Z system

Due to the complementary interaction of two genes, SI in the grasses has features distinct from those of single locus systems. These include differences in reciprocal crosses and the degree of compatibility (i.e. the percentage of compatible pollen) between two plants. The degree of compatibility can be either 0 , 50, 75 or 100%, depending on the genotypes. For instance, a cross between a plant with the genotype S_{11} Z_{12} and S_{12} Z_{13} as

the pollen donor, will show 75% compatible pollen grains, while in the reciprocal cross 50% of the pollen will be compatible.

In addition, the self-incompatibility response in grasses was retained when plants were made tetraploid (Lundqvist 1957, 1962; Fearon et al. 1984a, 1984b), a phenomenon which differs from single locus systems. Also unlike single locus systems (Lewis 1947), there are no dominance or competitive interactions between alleles of the diploid pollen or between the alleles of the tetraploid style. In an autotetraploid grass, a pollen grain will be incompatible if any one of the possible S-Z allele combinations is present in the recipient pistil.

1.2.4.3 Genetic organisation

Genes which are linked to either S or Z will show disturbed segregation in a cross which is only partially compatible. Hence, the analysis of such disturbed segregations should allow the identification of genes linked to the SI loci and might provide clues about their location in the genome. (For a theoretical discussion on this issue see Leach (1988)). Leach and Hayman (1987) used this approach to show that the isozyme phospho-glycoisomerase, PGI-2, which is linked to the S-locus in *Lolium perenne* (Cornish et al. 1980), is also linked to the S-locus in *Alopecurus myusorides*, *Phalaris coerulescens*, *Festuca pratensis, Holcus lanatus* and *Secale cereale.*

Other studies have confirmed this observation and provided further linkage information. The S-gene is now known to be linked to PGI-2 and a leaf peroxidase, Prx-7, and located on chromosome 1R in rye and chromosome 6 in *Lolium* (Gertz and Wricke 1989; Lewis et al. 1980). The Z-gene is linked to beta-glucosidase and Esterases 4/11 and located on chromosome 2R in rye (Wricke and Wehling 1985; Fuong et al. 1993b). In Lolium, linkage was indicated between the Z-locus and the isozyme glutamate oxalacetate-transaminase, GOT-3 (Thorogood and Hayward 1991), which is located on chromosome 2 (Lewis et al. 1980).

All the grasses mentioned above have a haploid genome with n=7 chromosomes. The results of the studies discussed demonstrate that S and Z segregate independently and are located on different chromosomes. They further indicate that there is a conservation of linkage groups in these grasses and a strong likelihood that they share the same SI system.

1.2.4.4 Breakdown of SI in the *Poaceae*

Self-incompatibility in the grasses is rarely absolute; a low number of seeds can be obtained after self-pollination. This partial breakdown is called "pseudocompatibility" and has been studied by Lundqvist (1958, 1964) and others and reviewed by Hayman (1992). Pseudocompatibility can also be caused by environmental factors; for instance high

temperatures have deliberately been used to create homozygous plants of rye (Wricke 1978) and *Lolium* (Wilkins and Thorogood, 1992) for breeding purposes.

The study of the complete breakdown of self-incompatibility has revealed the existence of different kinds of mutants with interesting characteristics. Working on rye, Lundqvist (1958, 1962, 1968) identified mutations at or near the S and Z loci. All mutants were pollen-only (= pollen-part), which means that the pollen has lost its SI specificity whereas SI is retained in the pistil of the same plant.

Thorogood and Hayward (1991) analysed a self-compatible mutant of *Lolium perenne*. Their results indicated that the gene causing the self-fertility was allelic to neither S nor Z suggesting a third locus was involved.

The presence of a third locus was substantiated in an extensive study of self-compatible mutants in *Phalaris coerulescens* by Hayman and Richter (1992). The mutation at the third locus, T, which showed close linkage to a leaf peroxidase isozyme, had an effect only in the pollen but not in the pistil of the mutant plant. Pollen carrying the T-mutation was compatible with any plant irrespectively of its S or Z genotype. Apart from mutations at the T-locus, pollen-only mutants at S and Z and a "complete" mutant at the S locus were identified. Homozygotes for the "complete" mutation are fully fertile (100%) with any plant, as either the pollen or stigma parent.

A third locus conferring self-compatibility was also identified in rye (Fuong et al. 1993a, b). The locus is linked to the Esterase 5-7 complex and located on chromosome 5R. Unfortunately comparative linkage maps are not available, so it is not possible to say whether the additional loci identified are functionally homologous or whether more than three genes influence SI in the grasses.

1.2.4.5 Physiological and biochemical studies in the *Poaceae*

Within minutes after a pollen grain is captured by a stigma several physiological and biochemical events take place. These include hydration, secretion, enzyme activation and tube emergence (Heslop-Harrison 1979a). According to Heslop-Harrison and coworkers, who have studied pollen-pistil interaction in the grasses extensively, no differences were observed between compatible and incompatible grains during the initial events of pollen germination (Shivanna et al. 1982). Only after the pollen tube touches, or in weaker SI reactions, penetrates the stigmatic surface will the self-incompatibility reaction occur, resulting in cessation of pollen tube growth (Heslop-Harrison 1979b; Shivanna et al. 1982; Heslop-Harrison 1982b).

Based on an interpretation of their work, a hypothesis has been formulated for the underlying events in a self-incompatible reaction (Heslop-Harrison 1982a, 1982b). For the pistil side it was proposed that the incompatibility factors were proteins with lectin-like properties present in the stigma surface secretion at the receptive zone and in the intercellular

spaces of the transmitting tract. On the pollen side it was suggested that the S and Z factors were associated with the tube wall. In an incompatible reaction the interaction of the stigma proteins with the pollen tube leads to a disruption of apical growth of the tip by interfering with the re-orientation and extension of polysaccharide microfibrils.

In a recent study, using an entirely different approach, Wehling et al. (1994) proposed that pollen protein phosphorylation was part of the signal transduction mechanism of self-incompatibility in rye. Pollen germinated *in vitro* in the presence of self-stigma extract was found to show a higher phosphorylation activity than pollen germinated in the presence of cross-stigma extract. In addition, significantly reduced basic phosphorylation activity was detected in a self-compatible mutant.

Wehling et al. (1994) also studied the effect of protein kinase inhibitors and Ca^{2+} antagonists on the self-incompatibility response. Isolated pistils were placed on agar medium containing the inhibitor and pollinated with self pollen. Of the inhibitors tested, Lavendustin A, a tyrosine kinase inhibitor, showed the strongest effect and verapamil, a calcium channel blocker, resulted in almost complete suppression of the SI response.

Until the molecules involved are isolated and the signal transduction cascades investigated, it is difficult to judge whether the physiological changes observed are a consequence or cause of the SI reaction.

1.2.4.6 Molecular studies in the *Poaceae*

Molecular investigation of the self-incompatibility system in *Phalaris coerulescens* in our laboratory have resulted in the isolation of a putative S-gene from pollen by Li and coworkers (Li et al. 1994). The gene is about 3 kb long, split by five introns and predominantly expressed in mature pollen. The comparison of deduced amino acid sequences of two completely sequenced alleles, S_1 and S_2, indicated that the overall homology was high at 92% identity. The majority of sequence variations were concentrated in the second exon. Database searches revealed that the carboxy-half of the protein, which was completely conserved among the alleles, showed high homology to thioredoxin h proteins. Indeed, when this region was expressed in *E. coli* and the recombinant protein was used in a functional assay, it catalysed the reduction of the disulfide bonds in insulin and acted as a substrate for *E. coli* thioredoxin reductase (Li et al. 1995).

These results suggest that the S-protein has two functional domains, a conserved C-terminus with a thioredoxin-like catalytic function and a variable N-terminus which determines the allele specificity. Additional support for this concept was provided by PCR amplification of the second exon of the S_3 and S_4 alleles. The second exon of the S_3 allele is about 10 bp longer than that of the S_1 allele and the sequence analysis of S_4 showed 13 differences to S_1, including seven nucleotide deletions (X. Li, pers. comm. 1994).

Apart from the expected allelic variation, further evidence that the S-thioredoxin represents the S-gene was obtained through RFLP studies, which showed perfect co-segregation of a restriction fragment and the S genotype, and by sequence analysis of an S-complete mutant (Li et al. 1994). In the thioredoxin-like domain of the mutant, three amino acid changes have been identified. Although none of the mutations is located at the active site of the thioredoxin domain, it is possible that the mutated gene has lost its catalytic function.

When the S-thioredoxin cDNA clone was used as a probe in an RNA-DNA hybridisation under low stringency conditions, a weak signal was detected in mature stigmas in addition to the expected signal in pollen, but not in leaf. The mRNA in stigmas appeared to be smaller than that in pollen (X. Li, unpublished results). A closer analysis of the possible expression of the S-thioredoxin gene in the stigma is currently underway.

1.2.4.7 The role of Thioredoxin in SI

The presence of a thioredoxin-like domain in the S-protein might provide insight into the possible mechanism of action of the S-gene. Therefore, a brief overview of thioredoxin and thioredoxin-like molecules is presented here.

Thioredoxin itself is a small ubiquitous protein with a conserved active-site sequence, Cys-Gly-Pro-Cys (reviewed by Holmgren 1985, 1989). The protein is thought to play a role in a variety of intracellular reactions: As a protein disulfide oxido-reductase, as a regulatory factor for enzymes and receptors, in cell proliferation and meiosis and in post-translational modification of different proteins (see Buchanan et al. 1994 for specific references).

An intriguing example of thioredoxin function is its regulation of the activity of an inducible transcription factor, NF-κ B, as shown in human T-cells. By reducing a disulfide bond involving the Cys62 of the p50 subunit of NF-κ B, the DNA binding property of the transcription factor increased and gene expression in the cells was altered (Matthews et al 1992; Hayashi et al. 1993).

In plants, three types of thioredoxins have been identified: Thioredoxin m and f, which are located in the chloroplast and reduced by ferredoxin-thioredoxin reductase; and thioredoxin h, which occurs in the cytosol, the endoplasmatic reticulum and mitochondria (Marcus et al. 1991) and is reduced by a NADPH-dependent thioredoxin reductase.

Thioredoxin h has been identified in all plant tissues examined so far (Florencio et al. 1988, Brugidou et al. 1993; Marty et al. 1993). In contrast to the thioredoxins of the chloroplast (Levings and Siedow, 1995), the function of thioredoxin h is largely unknown. Thioredoxin h must however be of considerable importance in many plants species as in rice, for example, it is one of the major proteins of the phloem sap (Ishiwatari et al. 1995).

To date, physiological activity of thioredoxin h has been demonstrated only in seeds. It was found that the NADP/thioredoxin system reduced several soluble low-molecular-weight seed proteins, including thionins, and different alpha-amylase and trypsin inhibitors, thereby regulating their activity (Kobrehel et al. 1991). In addition, reduction of major storage proteins - gliadins, glutenins, albumins and globulins - has been demonstrated (Kobrehel et al. 1992; Wong et al. 1993). It seems that thioredoxin specifically reduces intramolecular disulfides but is ineffective in reducing intermolecular disulfide bonds, as shown by Shin et al. (1993) who investigated the reduction of 2S albumin from castor-seeds. It has been suggested thioredoxin functions as a signal during seed germination through inactivation of protease inhibitors and by reducing the disulfide bonds of seed storage protein and thereby increasing susceptibility to proteolysis.

The S-protein of *Phalaris* is one of a family of proteins with a thioredoxin domain. Examples in the animal kingdom are protein disulphide isomerase (PDI) (Edman et al. 1985), phophoinositol-specific phospholipase C (Bennett et al. 1988) and calcium binding proteins 1 and 2 (CaBP1 and CaBP2 (= ERp72) (Nguyen Van et al. 1989). Interestingly, all these proteins contain two thioredoxin-like domains, with the exception of CaBP2, which has three. It seems that the main functions of these proteins are in the post-translational modification processes. For instance, PDI, CaBP1 and CaBP2 have been shown to be involved in the catalysis of formation, reduction and isomerisation of protein disulphide bonds(Lundström-Ljung et al. 1995). It has also been suggested that CaBP2 and PDI can function as chaperones (Nigam et al. 1994).

Apart from the S-gene, the only other example to date of a plant gene with a putative thioredoxin-like domain is LMP134, which was isolated from mature pollen of *Lilium longiflorum* (Kim et al. 1994). In comparison with the S-thioredoxin it shows relatively low homology to other plant thioredoxins and nothing has yet been published about its function.

1.2.5 Strategies for the Identification of Genes Involved in SI

In three of the four systems described in this review the self-incompatibility gene(s) were isolated from stigmas. In the *Brassicacae*, *Solanaceae* and *Papaveraceae*, the pollen component of SI either has not been identified or its identification is still ambiguous. The question arises as to why the isolation of the SI genes from pistils was successful, but appears to be difficult from pollen.

To approach this question, a brief historical overview is given of strategies employed to identify the SI genes. (For early reviews see Heslop-Harrison 1975, 1978; de Nettancourt 1977; Knox and Clarke 1980.)

In 1929, East postulated that the self-incompatibility reaction might resemble the antigen-antibody reaction. Interestingly, a number of researchers have attempted to identify

the S-gene product using immunobiological methods, an approach which ultimately led to the identification of the S-gene in the *Brassicaceae*. Using immunodiffusion tests, Nasrallah and Wallace (1967a) were able to demonstrate biochemical variability of different incompatibility genotypes in the stigmas, but not in the anthers or pollen . The presence of these stigma-specific antigens increased in developing buds with the change from self-compatible to self-incompatible (Nasrallah and Wallace 1967b). Co-segregation of certain stigma proteins with different S-alleles was subsequently demonstrated using isoelectric focussing (Nishio and Hinata 1977; Hinata and Nishio 1978; Nasrallah and Nasrallah 1984). The detailed characterisation of these S-locus-specific glycoproteins (SLSG) (Nishio and Hinata 1982) facilitated the cloning of the corresponding genes once molecular techniques were available.

A significant step in the isolation of the *S-RNase* genes of the *Solanaceae* was the investigation of the effect of stylar extract and exudates on *in vitro* pollen tube growth (reviewed in Hinata et al. 1993; Sims 1993). Subsequent analysis of style proteins using gel electrophoretic methods identified putative S-associated proteins, of which the N-termini were sequenced. Using oligonucleotides directed towards these N-terminal regions in combination with differential screening researchers isolated the corresponding cDNAs (Anderson et al. 1986, Clark et al. 1990, Kaufmann et al. 1991).

The identification of the S-gene of *Papaver rhoeas* followed a similar path, as already described in Section 1.2.3.

1.2.5.1 Attempts to Isolate the S-Gene from Pollen

The identification of pollen S-antigens has been attempted by several researchers (reviewed in de Nettancourt 1977). For instance Lewis, working with *Oenothera organensis*, a plant with single locus gametophytic self-incompatibility, demonstrated correlation between antigen specificity and S-genotype in pollen by applying the Precipitin ring test (Lewis 1952). A later study substantiated this result, but when pollen proteins were separated electrophoretically no differences were detected between different incompatibility genotypes (Mäkinen and Lewis 1962). Analogous results were obtained by Linskens (1960). Antisera were generated against pollen and style proteins from *Petunia hybrida* of different S-genotypes. From precipitation tests using different combinations of both pollen and style antigens with all antisera, Linskens concluded "that S-alleles produce substances, each of which is antigenically different and highly specific" and that the S-antigens of pollen and style are immunologically closely related if not identical (Linskens 1960).

Kamboj and Jackson (1986) analysed pollen and pistil proteins in *P. hybrida* from different S-genotypes by both one- and two-dimensional gel electrophoresis. While stylar proteins associated with the S-genotype were easily identified, putative pollen S-proteins

were not apparent. Similar results were obtained in an earlier study by Bredemeijer and Blass (1981), where isoelectric focussing (IEF) of pollen and pistil proteins from *Nicotiana alata* revealed the presence of S-specific proteins in pistils but not in pollen. Given the sensitivity of the techniques used, pollen S-proteins would have been missed if they were present in relatively low abundance.

In their analysis of polypeptides present in the pollen coating of *Brassica oleracea*, Doughty et al. (1993) did not identify any major S-specific protein band(s) after gel-electrophoresis. However, when coat polypeptides were mixed with crude stigmatic extracts and subsequently separated by IEF, a protein band corresponding to the *SLSG* had shifted position. Further analysis showed that a 7 kDa protein was responsible for this shift. In a follow-up study it was demonstrated that this 7 kDa protein interacts with *SLSG* and *SLR* proteins (Hiscock et al. 1995). Since sequence analysis and mapping of the 7 kDa protein have not been carried out, it is not clear whether this peptide represents the pollen SI component or simply acts as a non-specific cofactor.

For the sporophytically determined system of the *Brassicaceae*, physiological experiments recently reviewed by Dickinson (1995) indicated that the SI response is likely to take place predominantly in the stigma rather than in the pollen. Three interesting observations should be mentioned. Firstly, compatible and incompatible pollen grains attached to the same stigma papilla are either "accepted" or "rejected", indicating that the stigmatic response is very localised. Secondly, if a stigma papilla is touched briefly with an incompatible grain which is then replaced by a compatible grain, no development of the latter will take place. Lastly, pollen grains placed in between compatible and incompatible stigma papillae produce a tube, suggesting that the contact with the incompatible stigma does not lead to physiological inhibition.

On this basis one could speculate that the low expression of the *SLG* and *SRK* genes detected (see Section 1.1) is sufficient to trigger the localised stigmatic response and hence the S-genes of pollen and stigma would be identical. To explore this possibility, very specific probes and very sensitive methods need to be employed.

An alternative possibility is that a different gene is expressed in pollen, for instance the above mentioned 7 kDa protein.

Although pollen grain inhibition in the *Papaveraceae* takes place at the stigmatic surface as in the *Brassicaceae*, studies suggest that pollen tube growth is arrested due to changes in gene expression in the pollen grain itself following contact with an incompatible stigma (Franklin-Tong et al. 1993).

For gametophytic systems, the pollen gene(s) involved in SI are likely to be expressed after microspore formation since, as already mentioned, the S-genotype of the pollen in these systems is determined by its own haploid genome.

The fact that the stigmatic S-gene in *Papaver* cannot be detected in pollen, argues that the pollen gene is likely to be different.

In gametophytic systems with stylar inhibition two possibilities arise. The first is that the pollen and stigma S-genes are different, as suggested by Lee et al. (1994). In this case the characterisation of genes closely linked to S will eventually lead to the identification of the pollen component. The second possibility is that the pollen S-gene is not expressed until after pollen germination or even later during pollen-tube growth, possibly triggered by the interaction with the stigma. Since it often takes several hours for pollen tube growth to be arrested, for instance more than nine hours after germination in *Petunia* (Singh and Kao 1992), it can be argued that the second possibility has potential. If the pollen S-gene is indeed expressed only after pollen germination, differential screening of cDNA libraries from growing pollen tubes challenged with self- and cross-stigma extract might lead to the identification of the pollen S-gene. The two possibilities are of course not mutually exclusive.

1.2.5.2 Isolation of the Pollen SI Genes in *Phalaris*

Phalaris coerulescens was the first system in which a putative SI gene was isolated from pollen. There were three main reasons why researchers examined the problem from the pollen side, in contrast to the approaches used in other systems. The first was that in the grasses the self-incompatibility reaction takes place very shortly after pollen germination.For instance, the growth of the pollen tube tip was arrested 30 sec after tube emergence in *Gaudinia fragilis* (Heslop-Harrison 1982), which implied that the molecules involved in the reaction were likely to be expressed in mature pollen grains. Secondly, a previous analysis of stigma proteins failed to assign any protein band to either S or Z (Tan and Jackson 1988). Finally, the extensive screen for self-compatible mutants of *Phalaris* carried out by Hayman and Richter (1992) resulted in the isolation of several pollen-only mutants at the S and Z loci, facilitating the molecular identification of putative S- and Z- genes.

The method chosen was differential screening of mature pollen cDNA libraries (Li et al. 1994).

From what has been presented in thc previous sections, it is clear that further characterisation of genes expressed specifically in pollen is required, not only for a full molecular description of the complex phenomenon of SI in plants, but also as an essential step towards understanding the general mechanisms by which pollen and stigmas interact. The next section deals with pollen-expressed genes, since they are the focus of this thesis.

1.3 Gene Expression in the Male Gametophyte

Evidence for gene expression from the haploid genome was obtained as early as the beginning of this century. Several researchers described incidences of pollen dimorphisms which were interpreted as being a consequence of the expression of genes encoded by the haploid genome of the male gametophyte (reviewed by Demerec 1924). One example is the effect of the waxy mutation, which can be easily seen when pollen is stained with iodine (Weatherwax 1922). Furthermore, the mechanisms of gametophytic self-incompatibility as described by East and Mangelsdorf (1925) required that the SI characters be expressed from the haploid genome of the pollen.

More recent studies on dimeric enzymes, such as alcohol-dehydrogenase and glutamate-oxaloacetate transaminase in maize (Schwartz 1971; Frova et al. 1983) and phospho-glycoisomerase in *Clarkia* (Weeden and Gottlieb, 1979), not only provided more evidence for gametophytic gene expression, but also indicated that some genes may be expressed specifically in pollen (reviewed by Ottaviano and Mulcahy, 1989).

In their molecular analysis of gene expression during pollen development, Willing et al. (1988) estimated that there were 24000 different mRNAs present in mature pollen of *Zea mays*, compared to about 31000 different mRNAs in shoots. The pollen mRNAs fell into three abundance classes: 35% of the mRNAs, comprising about 240 different sequences, were very abundant (about 32000 copies per grain), while the low abundance fraction (15%), representing about 17000 different sequences, were present in about 200 copies per grain. The middle abundance class (49% of the mRNAs) consisted of about 6000 different sequences, each present in about 1700 copies per cell.

The majority of these sequences were not pollen specific. Based on colony hybridisation studies with cDNA libraries made from pollen, root and shoot mRNAs, it was estimated that about 10% of the sequences expressed in maize pollen and about 20% of those expressed in *Tradescantia* were pollen-specific (Stinson et al. 1987). The actual number of pollen-specific genes might be even higher, since the method misses pollen-specific expression of genes which have homologous counterparts expressed in other tissues. Examples of such genes are ADP-glucose pyrophosphorylases (Bryce and Nelson 1979), beta-glycosidases (Frova et al. 1987), the actin gene family in tobacco (Thangavelu et al. 1993) and the alpha-tubulin genes (Ludwig et al. 1988).

With regard to their temporal expression, Mascarenhas (1990) divided pollen genes into two groups, the "early" and "late" genes. Early genes, such as represented by the *I3* cDNA clone of *Brassica napus* (Roberts et al. 1991), were first detected after meiosis. Their expression increased during microspore development but was reduced or undetectable in mature pollen. The late expressed genes became active around the time of microspore mitosis and their mRNAs continued to accumulate as the pollen matured. However, not all genes followed this rule (see Scott et al. 1991 for examples).

The temporal pattern of a pollen-expressed gene can give a general indication of its possible function. Early genes are thought to be involved in pollen grain development, whereas the accumulation of the late mRNAs suggests that they play a role during pollen germination or pollen tube growth (Scott, 1993).

Early studies indicated that both protein and RNA synthesis occured during germination and pollen tube growth (Mascarenhas and Bell 1969; Linskens et al. 1970; Tupy 1977). However, studies with transcription and protein synthesis inhibitors indicted that early pollen tube development was independent of newly synthesised proteins and mRNA as shown in (reviewed by Mascarenhas 1975, 1988). In other words, the mature pollen grain was equipped with all the necessary proteins and mRNAs for pollen germination and initial tube growth. It should be noted that there were differences among plants; bicellular pollen is strongly inhibited by cycloheximide, whereas tri cellular pollen is virtually resistant (Hoekstra and Bruinsma 1979).

Several questions arise. For instance, are the mRNAs necessary for pollen function pre-synthesised? Do the genes which were active during the terminal stages of pollen maturation continue to be expressed? Are new genes switched on after contact with the stigma? There are examples of all three expression patterns in the literature.

Working with *Nicotiana* pollen, Storchova et al. (1994) identified pre-synthesised stored mRNA which was translated upon germination. They showed that the mRNA of a 69-kDa protein, which is abundantly synthesised in growing pollen tubes, was present in mature pollen but not the protein itself.

An example of pre- and post-anthesis gene expression has been obtained by Weterings et al. (1992), who found that the pollen-specific gene from tobacco, *NTP303*, was transcribed both during pollen maturation and during pollen germination and tube growth.

De novo transcription of genes in growing pollen tubes was demonstrated by Franklin-Tong et al. (1990). These genes were specifically transcribed in response to self-incompatible stigma extract, and the corresponding mRNAs were not present either in mature ungerminated pollen grains or in pollen germinated in the presence of compatible stigma extract.

1.3.1 Isolation and Characterisation of Pollen-Expressed Genes

In recent years a growing number of anther- and pollen- specific genes have been isolated, either by differential screening of cDNA libraries made from mature pollen or anthers of different stages, or on the basis of the allergenicity of their protein products in susceptible humans.Little is known however about the nature and function of these genes. So far, most information has been obtained through sequence analysis. Recent reviews on pollen- and anther-specific genes are by Mascarenhas (1988, 1990, 1992), McCormick (1991, 1993) and Davies et al. (1992). The present review concentrates on genes which are

late expressed and of indicated or known function (Table III) and their possible involvement in pollen germination, tube growth and pollen-stigma interaction.

Table III: Selected list of pollen-expressed genes

Gene	Species	Sequence homology	Reference
PPE1	*P. inflata*	Pectin esterase	Mu et al. 1994a
LAT56, LAT59	tomato	Pectate lyase	Wing et al. 1989
Amb aI.1,2,3	ragweed	Pectate lyase	Rafnar et al. 1991
Amb a II	ragweed	Pectate lyase	Rogers et al. 1991
TP10	tobacco	Pectate lyase	Rogers et al. 1992
Zm58.1, Zm58.2	maize	Pectate lyase	Turcich et al. 1993
LMP131	lily	Pectate lyase	Kim et al. 1994
P2	*Oenothera*	Polygalacturonase	Brown and Crouch 1990
Pg1	maize	Polygalacturonase	Allen and Lonsdale 1993
G9	cotton	Polygalacturonase	John and Peterson 1994
Npg1	tobacco	Polygalacturonase	Tebutt et al. 1994
Pex1	maize	Extensin-like	Rubinstein et al. 1995
PRK1	*Petunia*	Protein kinase	Mu et al. 1994b
CDPK	maize	Protein kinase	Estruch et al. 1994
*Bet v*II	birch	Profilin	Valenta et al. 1991
*ZmPRO*1,2,3	maize	Profilin	Staiger et al. 1993
(no name)	timothy grass	Profilin	Valenta et al. 1994
"clone 4"	tobacco	Profilin	Mittermann et al. 1995
LMP131A	lily	Actin depolymerization factor	Kim et al. 1993
BMP1	*B. napus*	Actin depolymerization factor	Kim et al. 1993
ZmABP1	maize	Actin depolymerization factor	Rozycka et al. 1995
LAT52	tomato	Kunitz trypsin inhibitor	Twell et al. 1989
Zm13	maize	Kunitz trypsin inhibitor	Hanson et al. 1989
Ole e I	olive	Kunitz trypsin inhibitor	Villalba et al. 1993
PS I	rice	Kunitz trypsin inhibitor	Zou et al. 1994
Msb8	sorghum	Kunitz trypsin inhibitor	Pe et al. 1994
NeIF-4A8	tobacco	Translation initiation factor	Brander &Kuhlemeier 1995
SF3	sunflower	Zinc-finger protein	Baltz et al. 1992
Phl p Vb	timothy grass	RNase	Bufe et al. 1995
LAT51	tomato	Ascorbate oxidase-like	Ursin et al. 1989
NTP303	tobacco	Ascorbate oxidase-like	Weterings et al. 1992
Bp10	*B. napus*	Ascorbate oxidase-like	Albani et al. 1992

Pectinases

Several of the late pollen-expressed genes show sequence homology to pectinases, a family of enzymes involved in the degradation of pectin, a structural component of the primary cell wall. Pectin esterase demethylates pectin to pectate, which is subsequently depolymerised by polygalacturonase and pectate lyase. Bacterial and fungal pectinases play an important role in the host-pathogen interaction, as they are capable of causing the maceration and killing of plant cells (Collmer and Keen 1986).

The presence of pectinases in pollen has already been demonstrated by biochemical methods (Heslop-Harrison 1979a; Pressey and Reger 1989), with these enzymes thought to be necessary for pollen tube emergence and tip growth, as well as for the penetration of the stigma.

The *PPE1* gene from *Petunia inflata* shows sequence homology to fungal, bacterial and other plant pectin esterases: 48 % identity to *Bp19*, an early pollen-expressed gene of *Brassica napus* (Albani et al. 1991) and 47% identity to a tomato pectin esterase (Ray et al. 1988). The gene, which encodes a 374 amino acid long protein, is first expressed in anthers at the uninucleate microspore stage. Its expression increases thereafter and it is present throughout pollen tube growth as shown by Northern blot analysis (Mu et al. 1994a). No hybridisation was detected in roots, leafs, petals or pistils of immature flowers.

Putative pectate lyase encoding genes expressed in pollen have been identified in several plant species. Enzymatic activity has so far only been demonstrated for *Cry j* I, a major allergen of Japanese cedar pollen (Taniguchi et al. 1995). Comparison of the partially determined protein sequence of *Cry j* I revealed significant homology to *Amb a* I and *Amb a* II, major allergens of short ragweed.

In most examples listed in Table III more than one gene has been identified coding for putative pectate lyases. The genes are related to each other to varying extents. For instance in tomato, although the deduced amino acid sequences of the *LAT56* and *LAT59* genes share 54% identity, the cDNA clones do not cross-hybridise (Wing et al. 1989). On the other hand, the three genes of ragweed, *Amb a* I.1, *Amb a* I.2 and *Amb a* I.3, which have been isolated due to their allergenicity, are highly related to each other, with sequence identities ranging from 86% to 91% at the nucleotide level (Rafner et al. 1991). *Amb a* II shows 65% sequence identity to the *Amb a* I gene-family. Sequence analysis of the putative pectate lyase genes has revealed a putative signal peptide sequence at the N-terminal of the proteins, indicating that they are likely to be secreted.

In all studies in which the expression of the genes was analysed, it was found that they were pollen-specific and showed the highest expression in mature pollen.

The *P2* gene of *Oenothera*, which showed sequence homology to a polygalacturonase from tomato fruit, represents a small gene family of 6-8 members which seem to be expressed in the same way during pollen development (Brown and Crouch 1990). Antibodies raised to the β-galactosidase/*P2*-fusion protein were used to demonstrate the presence of the protein in mature pollen grains and pollen tube grown *in vitro* and *in vivo*. In a comparative Northern blot analysis, the *P2* probe hybridised to pollen RNA from monocots and dicots with either bi- or tricellular pollen, including *Brassica napus, Spathophyllum clevandii, Amaryllis vittata* and *Zea mays*. This data suggests that the polygalacturonase genes are highly conserved. The analysis of the polygalacturonase genes in maize, tobacco and cotton confirmed this data, and demonstrated that the spatial and temporal expression of the genes is the same for all these plants.

In summary, the presence in evolutionary distant plants of related pectinase genes specifically expressed in pollen indicates that the pectinase genes play an important, although not yet clearly defined, role in late pollen development and pollen germination and/or pollen tube growth. It remains to be seen, whether this role is limited to stigma penetration and/or pollen tube growth or whether these pectinases function as a signal in pollen-stigma interactions. That pectic wall fragments released by polygalacturonase can function as a signal and are capable of stimulating physiological changes, such as synthesis of proteinase inhibitors and ethylene has been demonstrated in the context of plant-pathogen interactions (Collmer and Keen 1986).

Extensin-like proteins

It has been suggested that the pollen-specific gene *Pex1* has a function in pollen-pistil interactions during compatible pollinations (Cheung 1995). The *Pex1* gene encodes a 1184 aa protein with a two domain structure, an N-terminal possibly globular domain and a C-terminal extensin-like domain (Rubinstein et al. 1995). This structure is unusual for plant extensins and reminiscent of cell wall proteins found in some algae, such as sexual agglutinins of *Chlamydomonas* (Goodenough et al. 1985). The protein is expressed very late during pollen maturation and is likely to be secreted, as indicated by the presence of a signal peptide. Further analysis will show whether *Pex1* mediates recognition between pollen and stigma through interaction with stigma surface molecules and triggering of downstream events in pollination, as proposed by Rubenstein et al. (1995).

Protein kinases

Other studies indicate a possible involvement of protein kinases in mediating the transduction of pollination signals in pollen. The receptor-like protein kinase recently isolated from petunia pollen and the calcium-dependent calmodulin-independent protein kinase from maize pollen might represent such protein kinases.

PRK1 encodes a transmembrane protein with an extracellular domain that contains leucine-rich repeats (Mu et al. 1994b). Its cytoplasmic kinase domain, expressed in *E. coli*, was shown to autophosphorylate at serine, threonine and tyrosine, indicating a dual specificity of the protein. The gene is specifically expressed during late stages of pollen development and immunodetection indicated that the protein is localised in microsomal membranes of pollen and pollen tubes. The presence of leucine-rich repeats suggests that *PRK1* is involved in protein-protein interactions.

The significance of calcium in plant signal transduction and pollen germination and tube growth has been known for many years. For reviews see Roberts and Harmon (1992), Poovaiah and Reedy (1993) and Pierson and Cresti (1992). Germinating pollen grains were shown to take up calcium from the stigma (Bednarsky 1991) and an artificially elevated

concentration of free cytosolic calcium leads to reorientation of pollen tube growth (Malho et al. 1994). Furthermore, in *Papaver rhoeas* signal transduction in the self-incompatibility reaction was demonstrated to be mediated through a transient increase of cytosolic free calcium in pollen tubes (Franklin-Tong et al. 1993).

It may be possible that the pollen-specific calcium-dependent protein kinase (CDPK) from maize acts downstream in the calcium dependent signal transduction cascade. When antisense oligonucleotides directed to CDPK were added to pollen growing *in vitro*, significantly impaired tube growth and disruption of pollen tubes at a high frequency were observed, suggesting that CDPK has a crucial role in pollen germination and pollen tube growth (Estruch et al. 1994). It has been proposed that calcium dependent protein kinases regulate the activity and structure of plant cytoskeletons since CDPKs were co-localised with actin microfilaments (reviewed by Roberts and Harmon 1992). Whether this is the function of the pollen-specific CDPK of maize remains to be demonstrated.

Apart from these two pollen-specific protein kinases, a putative mitogen-activated protein kinase (MAP) was also expressed in pollen from tobacco as well as in all other tissues tested (Wilson et al. 1993). Southern analysis of the MAP-kinase encoding gene, *ntf3*, indicated that there is a family of MAP-kinase genes in tobacco.

Given the data on pollen phosphorylation during pollen tube growth (Polya et al. 1986, Wehling et al. 1994), it is to be expected that more protein kinases will be identified in the future.

Actin-binding proteins

Profilins have also been associated with the re-organisation of the cytoskeleton. Profilin is a small actin- and phosphoinositide-binding protein, which was first discovered in plants as an important pollen allergen (Valenta et al. 1991). To date, plant profilins have been characterised in monocots and dicots (Table III). Sequence comparisons show an average of 75% sequence identity between profilins of monocots and dicots, compared to around 35% to other eukaryotic profilins (Mittermann et al. 1995). The three cDNA clones coding for profilin in maize are highly homologous to each other and show pollen-specific expression in the typical fashion of late-expressed genes. However, when less stringent wash conditions were applied, transcripts could be detected in all tissues tested, indicating the presence of related profilins. In tobacco, the profilin gene was found to be expressed around 50-100 times more strongly in pollen than in other tissues (Mittermann et al. 1995).

One of the roles attributed to profilin is the capacity to promote actin polymerisation through binding to G-actin and thereby accelerating the exchange of adenine nucleotides (Goldschmidt-Clermont et al. 1992). On the other hand, profilin has a strong affinity, 10 times higher than to actin, for phosphoinositol-[4]-phosphate and phosphoinositol-[4,5]-biphosphate (PIP2). It has therefore been suggested that profilin is involved in the transduction of extracellular signals, leading to changes in cytoskeletal architecture (Staiger

et al. 1993). In a recent study, concerned with the role of profilin in the phosphoinositide-signal transducing system in plants, profilin was shown to inhibit the plasma membrane phosphoinositide phospholipase C of bean leaf *in vitro*, by forming complexes with PIP2, which is the substrate for this enzyme (Drobak et al. 1994). The exact function of profilin in pollen has yet to be determined.

In addition to profilin, a second actin binding protein, which shows homology to the actin depolymerization factor (ADF)/cofilin family, has been shown to be preferentially, if not exclusively expressed in mature pollen of at least three plant species (Table III). The corresponding cDNA clones show between 70% and 80% homology to each other and about 40% sequence identity to animal ADF. Vertebrate ADF/cofilin proteins are regulated by pH, phosphorylation and phospholipids and contain a nuclear localisation sequence (see Sun et al. 1995 for review). In contrast to profilin, ADF/cofilins can also bind to actin filaments and can cause their depolymerization. The presence of ADF/cofilin homologues in mature pollen of monocots and dicots suggests that they play an important role in pollen development or pollen tube growth.

Of interest in this context might be a study by Thangavelu et al. (1993), in which the expression of the actin gene family in tobacco was analysed. The researchers found that one member of the family, Tac25, was specifically expressed in mature pollen, while another, Tac9, was expressed in mature pollen and other tissues. Further analysis will show whether the presence of male gametophyte-specific actins reflects a unique function in mature pollen.

Putative proteinase inhibitors

The *LAT52* gene from tomato is one of the few genes which has been found to be necessary for pollen function (Muschietti et al. 1994). The gene codes for a heat stable, glycosylated protein, which is first detectable in anthers at the tetrad stage. The *LAT52* mRNA expression increases thereafter until anthesis. Expression was not restricted to pollen; 20 to 50 times lower levels have been found in petals (Twell et al. 1989). When tomato plants were transformed with an antisense *LAT52* gene driven by the *LAT52* promotor and the pollen phenotype studied *in vitro* and *in vivo,* 50 % of the pollen from those plants showed abnormal behaviour. This was correlated with the reduced expression of the *LAT52* gene. Pollen expressing the antisense gene showed incomplete hydration in pollen germination medium and either did not germinate at all or showed "corkscrew" pollen tubes. Pollen tubes of transformed pollen growing *in vivo* showed abnormal behaviour, with pollen tubes coiling, twisting and bursting in a fashion reminiscent of pollen behaviour in incompatible reactions. In addition, their growth ceased in the style (Muschietti et al. 1994).

LAT52 shows 32% amino acid identity to *Zm13*, a pollen-specific protein of maize and 36% similarity with both the pollen-specific gene *PS1* from rice, and *Ole e I*, a major pollen

allergen from olives. This group of related proteins (Table III) shows some similarity with Kunitz trypsin inhibitors (see Muschietti et al. 1994 for sequence alignment). However, they do not contain the active site domain present in the inhibitors, and proteinase inhibiting activity has not yet been demonstrated. The exact function of the *LAT52* and related proteins is currently unknown.

Potential regulatory proteins

Two pollen-specific genes have been isolated which might play a role in transcriptional and translational regulation of gene expression during pollen development. *SF3*, from sunflower, codes for a 219 amino acid long polypeptide, which contains two potential zinc finger domains alternating with two basic domains. The structural organisation of *SF3* was similar to those of the erythroid-specific transcription factors *Eryf1* and *GF-1*. It has therefore been proposed that *SF3* interacts with a DNA target site in a similar way and might be involved in regulating the expression of a set of pollen-specific genes coding for late pollen function.

A family of related genes has been isolated from tobacco, showing high homology to the eukaryotic translation initiation factor eIF-4A, an RNA helicase belonging to the superfamily of DEAD-box proteins. The expression of one of its members, *NeIF-4A8*, was shown to be restricted to mature pollen and anthers after microspore mitosis, while others, such as *NeIF-4A2* are expressed ubiquitously. In germinating pollen grains from plants which had been transformed with the GUS reporter gene system under the control of the *NeIF-4A8* promotor, GUS activity remained at the same level for more than 30 hrs of *in vitro* germination. This observation led to the suggestion that *NeIF-4A8* might be required to sustain high translational activity during pollen tube growth (Brander and Kuhlemeier, 1995).

Putative RNases

RNase activity of the pollen allergen *Phl p* Vb was recently demonstrated. In comparison with the pancreatic enzymes, the RNase activity of the recombinant protein expressed in *E. coli* was about 1000 times lower (Bufe et al. 1995) . The deduced amino acid sequence of *Phl p* Vb shows high homology to other group V allergens in four different grasses, indicating that the genes have an important physiological function which is currently unknown.

Ascorbate oxidase like proteins

Pollen-specific genes which show homology to ascorbate oxidases have been isolated from several plant species (Table III) and are likely to be ubiquitous as indicated by Northern analysis. The expression pattern of *NTP303* is typical for a late expressed gene,

with highest levels being found in mature pollen (Weterings et al. 1992). Expression analysis of *NTP303* during pollen germination *in vitro* showed that *NTP303* is actively transcribed, especially during the first 2 hours of germination. Sequence analysis of *NTP303* revealed 91% sequence identity to *LAT51* from tomato and 64% to *Bp10* from *Brassica napus*. All three genes show homology to ascorbate oxidases from cucumber and pumpkin. However, the conserved copper binding regions of ascorbate oxidases are not conserved in any of the three genes, indicating that they might have a different function, but share a common ancestral gene. This assumption was supported by an expression study of the recombinant *Bp10* protein in *E. coli* (Albani et al. 1992). When the bacterial cells were growing on minimal medium *Bp10* expression led to inhibition of growth, which could be rescued by high levels of sodium bicarbonate or carbon dioxide, but not by substances known to affect ascorbate oxidase activity. In addition, no ascorbate oxidase activity of the *Bp10* protein was detectable.

It has been suggested, that the *Bp10* proteins have an important metabolic function in the developing pollen grains since they are predominantly present in the binucleate stage of microspore development and less dominant in mature pollen. *NTP303* proteins, on the other hand, are more likely to be important for pollen germination and pollen tube growth.

As is obvious from the discussion above, the analysis of pollen-expressed genes is still in its early stages and further molecular analysis will be necessary to elucidate complex processes such as pollen-stigma interactions. As the number of genes characterised at the molecular level increases, a major goal will be to determine the function of their protein products and the mechanisms underlying their tissue specificity. Although sequence similarities can be helpful in suggesting functional analogies from other organisms, they may be misleading in specific contexts. Similarly it will be necessary to study gene function *in vivo,* since *in vitro* studies do not necessarily reflect what occurs *in vivo* . Some of the tools to approach this problem are mutant analyses and transformation studies with sense and antisense constructs to either overexpress or repress the gene.

1.4 Objective of this thesis

The main objective of this work was to study the contribution of pollen expressed genes to the complex interactions that eventually result in fertilisation or its prevention. To this end, the molecular characterisation of cDNA sequences which are predominantly or specifically expressed in mature pollen was begun, aiming at the identification of potential loci involved in pollen-stigma interactions and specifically in self-incompatibility phenomena.

Chapter 2 Materials and Methods

2.1 Plant Material

Self-incompatible lines and self-compatible mutants of *Phalaris coerulescens* were derived from a collection of plants at the Department of Genetics, University of Adelaide. The self-compatible mutants used in this study were obtained as a result of a mutant screen by Hayman and Richter (1992). All plants were kept under glasshouse conditions.

Seeds used for RNA isolation were germinated on wet Whatman 3MM paper in the dark at 4°C for 2 days, then transferred to room temperature (RT) for a further 2 days. Seedlings were then transferred to the glasshouse and grown for 7 days at RT with natural day-night cycles.

2.2 Transfection of *E. coli*

2.2.1 Plating Bacteria

The *E. coli* strain C_{600} Hfl^- was used for growing recombinant lambda gt10 phages. The bacterial cells were grown to an OD_{600} of 0.6 in LB media (1% (w/v)Bacto-tryptone, 5% (w/v) Bacto-yeast extract, 1% (w/v) NaCl, pH 7.5) containing 0.2% (w/v) maltose and 0.01M $MgSO_4$ and 15 µg/ml tetracyclin. Cells were pelleted by centrifugation and resuspended in 0.5 vol of 0.01M $MgSO_4$ as described by Sambrook et al. (1989).

2.2.2 Production of Masterplates

To generate a bacterial lawn of *E. coli* (C_{600} Hfl^-), 400 µl of the bacteria was mixed with 8 ml Top Agarose and overlayed on a 15 cm diameter LB agar plate. The plates were incubated inverted for 8 hrs at 37°C. 1 µl or 2 µl of phage lysate from purified phages was spotted onto the bacterial lawn and the plates were incubated inverted for 12 hrs at 37°C and 2 hr at 4°C.

2.3 Transfer of Phages onto Membranes

Plaques were directly transferred to 15 cm diameter Hybond-N^+ membranes (Amersham, UK) by overlaying the LB plates for 2 min for the first set of membranes and 7 min for the second set. The DNA was fixed by placing the membranes, DNA face up, on 3MM Whatman paper soaked in denaturing solution (1.5M NaCl, 0.5M NaOH) for 7 min and then on 3MM Whatman paper soaked in neutralising solution (1.5M NaCl, 500 mM Tris-HCl, 10 mM Na_2EDTA, pH 7.2) for 3 min. The membranes were rinsed with 2X SSC and allowed to air dry before being baked at 70°C for 30 min. The DNA was cross linked under shortwave UV light for 7 min. Finally, the membranes were washed (10 min, 65°C) in stripping solution (0.1% (w/v) SDS, 2mM Na_2EDTA, pH 8.0) to remove bacterial debris.

2.4 Isolation of Recombinant Lambda DNA

50 µl of phage lysate was mixed with 400 µl of "plating bacteria" (see 2.2.1) and incubated for 20 min at RT. This mix was used to inoculate 5 ml LB media, containing tetracyclin (15µg/ml) and 5 mM $CaCl_2$. The solution was incubated at 37°C with shaking for 5 to 7 hrs or until lysis occurred. The lysates were centrifuged for 10 min at 6000 rpm (rotor JA20.1, Beckman J2-21M) to remove bacterial debris. The supernatant was transferred to a fresh tube, RNase and DNase were added to a final concentration of 5 µg/ml and the solution was incubated at 37°C for 30 min. Phages were precipitated by adding an equal volumes of precipitation buffer (20% (w/v) PEG, 2.5M NaCl and 10 mM Tris-HCl, pH 7.5) and incubating on ice for at least 4 hrs. The phages were pelleted by centrifugation at 10,000 rpm (rotor JA20.1, Beckman J2-21M) for 20 min at 4°C. The phage pellet was resuspended in 750 µl of LB media and transferred to a 2.0 ml centrifuge tube. To remove residual bacterial nucleic acids, 750 µl of DE52 (Whatman) was mixed by inversion with the resuspended phages. The mix was centrifuged at 12,000 rpm for 5 min at room temperature and the supernatant, which contained the phages, was transferred to a fresh 2.0 ml tube. Phage protein was denatured by addition of 1/100 vol of 10% (w/v) SDS and 1/100 vol of 0.5 M Na_2EDTA and incubation at 68°C for 15 min. This was followed by a phenol/chloroform/iso-amyl alcohol (25:24:1) extraction and a chloroform extraction (according to Sambrook et al. 1989). Phage DNA was precipitated by adding an equal volume of isopropanol and incubating at -80°C for at least 15 min. The solution was centrifuged at 10,000 rpm for 15 min at room temperature and the DNA pellet washed in 70% (v/v) ethanol. The DNA was resuspended in 50 µl of TE (10 mM Tris-HCl pH 8.0, 1mM Na_2EDTA) containing 40 µg/ml of RNase.

2.5 PCR Amplification of Recombinant Phage DNA

For a 50 µl PCR reaction either 1 to 2 µl of phage DNA or 20 µl of boiled phage lysate was used as the template. To the phage DNA or lysate was added 0.15 µg of lambda gt10 F&R primers (see Appendix C), 5 µl of 10X *Taq* reaction buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM each of d(GTP, ATP, CTP, TTP) (Promega) and 1 Unit *Taq* polymerase (Promega). Thermal cycling consisted of an initial 5 min denaturing step at 94°C followed by 35 cycles of 94°C for 1 min, 55°C for 2 min and 72°C for 2 min. Final chain extension was allowed to proceed for 5 min at 72°C. PCR products were separated on a 1% TAE agarose gel.

2.6 Subcloning of Phage Inserts

2.6.1 Dephosphorylation of vectors

Two micrograms of the plasmid vector, pTZ19U or Bluescript KS(-), was digested with the restriction endonuclease *Eco*RI for 2hrs at 37°C. The reaction was extracted once with phenol/chloroform/iso-amyl alcohol. The DNA was precipitated with ethanol and resuspended in 20 µl of TE buffer. An aliquot of 10 µl (10 µg) of digested plasmid was dephosphorylated in a 20 µl reaction volume containing 2 µl of 10X reaction buffer (supplied by the manufacturer), 7 µl water and 1 µl (1 unit) calf intestinal phosphatase (Boehringer Mannheim) and incubated at 37°C for 1 hr. The phosphatase was denatured at 65°C for 10 min and an additional 0.5 ml (0.5 units) of fresh enzyme added for a further incubation at 37°C for 30 min. The reaction was extracted twice with phenol/chloroform/iso-amyl alcohol and the DNA was precipitated with ethanol. The DNA was recovered by centrifugation and resuspended in TE buffer to a final concentration of 25 ng/µl.

2.6.2 Ligation of DNA-Sequences into Plasmid Vectors

Selected cDNAs were digested with the appropriate restriction endonuclease for 2 hrs at 37°C followed by an ethanol precipitation. The DNA was resuspended in TE buffer to a final concentration of 50 ng/µl.

The ligation was performed in a reaction containing 100 ng of digested cDNA clone, 50 ng of dephosphorylated vector, 2 µl of 10X reaction buffer (supplied by the manufacturer), 1 µl of 10 mM ATP (pH 7.0), 1 µl of T4 DNA ligase (1 Weiss unit, Boehringer Mannheim) and water to 20 µl. The reaction was incubated at 15°C overnight after which the DNA was precipitated with ethanol, recovered by centrifugation and resuspended in 10 µl of TE buffer.

2.6.3 Transformation of *Escherichia coli*

2.6.3.1 Preparation of Competent Cells

Competent cells were prepared according to Hanahan (1983). An overnight culture of *E. coli*, strain DH5α was prepared by inoculating cells into LB media and incubating at 37°C. 0.5 ml of these cells was added to 50 ml SOB medium (2% (w/v) Bacto-tryptone, 0.5% (w/v) Bacto-yeast extract, 10 mM NaCl, 2.5 mM KCl) and incubated at 37°C with shaking until the OD_{600} had reached 0.45-0.55. Cells were pelleted by centrifugation at 2,500 rpm for 12 min at 4°C (rotor JA-20, Beckman J2-21M), resuspended in 8.5 ml TFB buffer (10 mM 2-[N-Morpholino]ethanesulfonic acid, 45 mM $MnCl_2$, 100 mM RbCl, 10 mM $CaCl_2$, 3 mM $HACoCl_3$) and chilled on ice for 10 min. The centrifugation step was repeated and the cells resuspended in 2 ml TFB buffer. 70 µl of redistilled DMSO was added and the cells kept on ice for 5 min. 160 µl of 1M DTT was added and the cells were incubated on ice for another 5 min followed by the addition of 75 µl DMSO and a further 10 min incubation on ice.

2.6.3.2 Transformation of Competent Cells

Competent cells (0.2 ml) were transferred to a sterile glass tube and 10 µl of the ligation reaction was added. The solution was gently mixed and placed on ice for 30 min. The cells were heat shocked at 42°C for 2 min and 800 µl SOC medium (SOB media with 10 mM $MgSO_4$, 10 mM $MgCl_2$, 0.35% (w/v) glucose) added prior to incubation at 37°C for 45 min with shaking.
Cells were plated out onto LB plates containing 50 µg/ml ampicillin, 0.004% (w/v) X-gal and 0.1mM IPTG and incubated at 37°C overnight. Recombinant bacteria were identified as white colonies.

2.7 Isolation of Plasmid DNA

2.7.1 Small Scale Isolation of Recombinant Plasmids

For mini-scale plasmid preparations, the alkaline lysis method of Sambrook et al. (1989) was applied .

2.7.2 Large Scale Isolation of Plasmid DNA

The preparation of plasmid DNA was performed following the protocol by Sambrook et al. (1989). Plasmid DNA was purified by equilibrium centrifugation in a CsCl-ethidium bromide gradient according to Sambrook et al. (1989).

2.8 Nested Deletion Library

A nested deletion library was constructed using the Erase-a-Base System (Promega) following the protocol supplied by the manufacturer.

2.9 DNA Sequencing

Sequencing of inserts cloned into pTZ19U or Bluescript KS (-) was performed with the ABI automated DNA sequencer (Applied Biosystems). Template preparations and sequencing reactions were performed as described by the manufacturer.

2.10 DNA Isolation from Plant Material

2.10.1 Small Scale DNA Isolation

Approximately 3-4 young leaves were placed in a 2 ml microcentrifuge tube and frozen in liquid nitrogen. The material was ground to a fine powder and homogenised with 750 µl of DNA extraction buffer (1% (v/v) sarcosyl, 0.1M Tris-HCl, 100 mM NaCl, 10 mM Na_2EDTA, 0.1M Na_2SO_3, pH 8.5). The resulting slurry was extracted with 750 µl of phenol/chloroform/iso-amyl alcohol (25:24:1) and gentle mixing on an orbital rotor for 30 min. The two phases were separated by centrifugation at 10,000 rpm in a table centrifuge for 5 min. The aqueous phase was transferred to a new tube and the phenol/chloroform/iso-amyl alcohol extraction repeated. The DNA was precipitated by the addition of 1.5 ml of ethanol and 60 µl of 3M sodium acetate (pH4.8) and incubated for at least 10 min at room temperature. The DNA was spooled around the tip of a pasteur pipette and transferred to a tube containing 500 µl of 70% (v/v) ethanol. The solution was centrifuged for 10 min at 10,000 rpm and the DNA-pellet was allowed to air dry. The DNA was resuspended in 50 µl of TE buffer (10 mM Tris-HCl pH 7.5, 1 mM Na_2EDTA) containing 40 µg/ml of RNase A.

2.10.2 Large Scale DNA isolation

Leaf material was collected, frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. The powder was resuspended in 100 ml of DNA extraction buffer and further homogenized. The resulting slurry was transferred to a 250 ml centrifuge tube and 100 ml phenol/chloroform/iso-amyl alcohol was added. The solution was mixed for 2 hrs at 4°C using an orbital rotor and then centrifuged at 7000 rpm (rotor JA10, Beckman J2-21M) for 10 mins at 4°C. The supernatant was poured through 3 layers of cheese cloth to remove debris. The DNA was precipitated by adding 2.5 volumes of absolute ethanol and one tenth volume of 3M sodium acetate (pH 4.8) and placed overnight at -20°C. The DNA was washed in 70%

ethanol, allowed to air dry and resuspended in 7 ml of TE buffer by gentle inversion overnight.

The DNA was further purified via CsCl gradient centrifugation. CsCl (7.5 g) was added to 7 ml of DNA solution and dissolved by gentle mixing. The solution was transferred to an ultracentrifuge tube and mixed with 0.5 ml of ethidium bromide (10 mg/ml). The sample was centrifuged at 40,000 rpm for 40 hrs (rotor 70.1 Ti, Beckman L8-70). The DNA band was removed from the gradient by direct piercing of the tube with a 10 ml syringe and 18 gauge needle. Ethidium bromide was removed with water saturated butanol as described in Sambrook et al. (1989). CsCl was removed from the DNA solution by dialysis in TE buffer for 5 hrs at RT.

2.11 Digestion of Genomic DNA with Restriction Endonucleases

Approximately 10 µg of DNA was digested in a reaction volume of 20 µl containing 2 µl of the appropriate 10X restriction buffer (supplied by the manufacturer), 2 µl of 40 mM spermidine, 2 µl of 1 mg/ml acetylated bovine serum albumin (Promega), 1 µl (8-12 units) restriction endonulease (Boehringer/Promega) and water to 20 µl. The digests were incubated at 37°C for 5 hr. An additional 1 µl (8-12 units) of enzyme was added and the digest continued for further 12 hrs. The most commonly used restriction endonucleases were *Bam*HI, *Bgl*II, *Dra*I, *Hind*III, *Eco*RI, *Eco*RV and *Xba*I (Promega/Boehringer).

2.12 Agarose Gel Electrophoresis

Digested DNA was mixed with 1/10 vol of 10X Ficoll dye (100 mM Tris-HCl, 200 mM Na_2EDTA, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 30% (w/v) ficoll type 4000, pH 8.0) and fractionated on a 1% agarose gel in TAE buffer (0.04 M Tris-acetate, 1mM Na_2EDTA, pH 8.0) at 20 mA for 16 hr. After electrophoresis, gels were stained in 10 µg/ml ethidium bromide for 30 min, visualised under UV light and photographed using Polaroid 667 film.

2.13 Transfer of DNA onto Nylon Membranes

Transfer was by capillary blotting as described by Southern (1975). In brief, gels were soaked in denaturing solution (1.5M NaCl, 0.5M NaOH) for 30 minutes and the DNA transferred to a nylon membrane (Hybond-N^+, Amersham, UK) using 20X SSC (3M NaCl, 300 mM Na_3citrate) for 5 to 7 hr. Upon completion of the transfer, the membrane was rinsed briefly in 2X SSC and blot dried using paper towels. DNA was fixed by placing the membrane on 3MM Whatman paper soaked with 0.4 M

NaOH for 30 min. The membrane was rinsed sequentially in neutralising solution (1.5 M NaCl, 500 mM Tris-HCl, 10 mM Na_2EDTA, pH 7.2) and 5X SSC for 5 min. The blot was dried using paper towel.

2.14 Probe Preparation and Labelling

DNA sequences cloned into plasmid vectors were digested with the restriction endonuclease of the cloning site and separated on 1% TAE agarose gels. The insert band was excised under longwave UV light and purified using Geneclean according to the manufacturers instructions (Bio101). DNA sequences cloned into phage vectors were amplified by PCR as described in 2.5, phenol/chloroform extracted and digested with *Eco*RI to remove the priming sites. The DNA was precipitated with 2 volumes of ethanol and ammonium acetate at a final concentration of 2.5 mM.

After purification of inserts, probes were radioactively labelled using the random oligo-priming method. 2 to 4 µl (ca. 50 ng) of purified insert and 3 µl (0.3 µg/µl) of random labelling primers were denatured together by boiling for 7 min, then rapidly cooled in an ice-water slurry for 5 min. To this was added 12.5 µl of 2X random oligolabelling buffer (40 µM d(ATP, GTP, TTP), 100 mM Tris pH 7.6, 100 mM NaCl, 20 mM $MgCl_2$, 200 µg/ml acetylated DNase free bovine serum albumin (Fraction V, Sigma), 4 µl [α-^{32}P]-dCTP (Amersham), 1 µl (2 units, Boehringer Mannheim) of DNA polymerase (Klenow fragment)) and water to a final volume of 25 µl. The reaction was incubated for 1 hr at 37°C and labelled DNA was separated from unincorporated nucleotides on a G-100 Sephadex column (Sambrook et al. 1989).

2.15 Hybridisation and Autoradiography

Prehybridisation of membranes was performed in a hybridisation solution containing 6 ml sterile water, 2 ml 5X HSB (3M NaCl, 100 mM PIPES, 25 mM Na_2EDTA pH 6.8), 1 ml Denhardt's III (2% (w/v) gelatine, 2% (w/v) ficoll, 2% (w/v) polyvinyl pyrollidone (PVP), 10% (w/v) SDS, 5% (w/v) tetra sodium pyrophosphate, filtered through 1MM Whatman paper) and 1 ml denatured salmon sperm DNA (5 mg/ml). The membrane was placed in a hybridisation bottle (Hybaid) and the solution (pre-warmed to 65°C) was added. Membranes were prehybridised at 65°C overnight.

The labelled probe was denatured in boiling water for 10 min and rapidly cooled on ice. The probe was added directly to the hybridisation bottle containing the membrane and hybridization solution (4 ml sterile water, 2 ml 5X HSB, 2 ml Denhardt's III, 1

ml 25% (w/v) dextran sulfate and 1 ml salmon sperm DNA (5 mg/ml)) . Hybridisation was performed at 65°C overnight.
The membranes were then washed in 2X SSC, 0.1% SDS for 30 min at 65°C to remove unbound DNA. Washes were repeated in 1X SSC, 0.1% SDS and 0.5X SSC, 0.1% SDS and 0.1X SSC, 0.1% SDS. The membranes were sealed in plastic and exposed to X-ray film at -80°C for 12 hrs to 5 days, depending on signal intensity.

2.16 Removal of Radioactive Probe from Membranes

Membranes were stripped by pouring 80°C hot stripping solution (0.1% SDS, 2 mM Na_2EDTA, pH 8.0) onto them then incubating at 65°C for 30 min. Membranes were re-exposed to X-ray film for 2 days to be certain abbout the effectiveness of radioactive probe removal.

2.17 RNA Isolation from Plant Material

1-3 g of plant material was frozen in liquid nitrogen and ground to fine powder as described in 2.10.2. Pollen was ground in the presence of sterilised sand. The powder was homogenised in 3 to 4 ml of REB-buffer (100mM Tris-HCl pH 8.0, 4% (v/v) sarcosyl, 10 mM Na_2EDTA) and the slurry was transferred to pre-chilled Corex tubes and immediately centrifuged at 6000 rpm (JA20, Beckman J2-21) at 4°C for 10 min. The supernatant was transferred to a fresh tube, caesium chloride (1g CsCl per ml supernatant) was added. The solution was pipetted onto 3 ml of a CsCl cushion (9.65 g CsCl dissolved in TE to a final volume of 10ml) in ultracentrifuge tubes (Oakridge Polycarbonate for Ti70.1 and Ti65 rotor).The samples were centrifuged at 30,000 rpm for 16 hrs at 10°C (rotor Ti 70.1, Beckman L8-70). The supernatant was removed, the RNA pellet dissolved in 400 ml REB-buffer and phenol/chloroform/isoamyl alcohol (25:24:1) extracted. RNA was precipitated with ethanol at -20°C for 2 hrs to overnight. The pellet was washed with 70% ethanol and resuspended in TE buffer.

2.18 Isolation of Poly-(A^+) RNA

Poly (A+) RNA was isolated using the PolyATract mRNA Isolation System III (Promega) following the protocol supplied by the manufacturer.

2.19 RNA Gel Blots and RNA Blot Hybridization

2.19.1 Formaldehyde Gel Electrophoresis

Approximately 5 µg of total RNA per lane was fractionated on an agarose gel containing 2.2 M formaldehyde (1 % agarose, 0.02 M MOPS pH 7.0, 5 mM sodium acetate, 1 mM Na_2EDTA, 2.2 M formaldehyde). Before loading, the RNA was dried under vacuum and resuspended in 2.0 µl buffer A (0.5 M MOPS, 0.01 M Na_2EDTA pH 7.0) and 13.5 µl formaldehyde/formamide/H_20 (3.5:10:3.5). The solution was incubated at 70°C for 10 min, then chilled on ice. Before loading 1 µl RNA-loading buffer (322 µl buffer A mixed with 178 µl 37% formaldehyde, 500 µl deionized formamide, 5 mg xylene cyanol, 5 mg bromocresol green, 400 mg sucrose) was added. MOPS-electrophoresis buffer (0.02 M MOPS pH 7.0, 5 mM sodium acetate, 1 mM Na_2EDTA) was used for the gel electrophoresis.
After electrophoresis the gel was stained in 2 mg/ml ethidium bromide for 15 min, destained for several hours and photographed using Polaroid 667 film.

2.19.2 Transfer of RNA onto Nylon Membranes

Gels were soaked in 20X SSC for 15 min and the RNA transferred to a nylon membrane (Hybond-N^+, Amersham, UK) by capillary blotting using 20X SSC for 10 to 12 hrs. The membrane was rinsed in 2X SSC, allowed to air dry and baked for 30 min at 70°C. The RNA was crosslinked to the membrane by exposure to shortwave UV light for 7 min.

2.19.3 Radioactive Labelling of cDNA

10 µl of poly (A^+) RNA (about 200 ng) or total RNA (ca. 5 µg) was denatured at 70°C for 10 min in the presence of 2 µl (0.3 µg/µl) of random labelling primers for poly(A^+) RNA or 2 µl of oligo (dT)12-18 (500 ng/µl) for total RNA and then chilled on ice. 5 ml of 5x First Strand Buffer (GIBCO BRL), 2 µl of dNTPs (40 µM d(ATP, GTP, TTP), 4 mM dCTP) 1 µl 0.1 M DTT, 3 µl [α-^{32}P]-dCTP (Amersham) and 2 µl (400 units) reverse transcriptase (SuperScript II, GIBCO BRL) were added and the reaction incubated at 37°C for 2 hrs.

The labelled single stranded (ss) cDNA was separated from unincorporated nucleotides on a G-100 Sephadex column. The RNA was degraded by adding 1M NaOH and the solution was neutralised by adding an equal volume of 1M HCl. The ss cDNA was denatured by boiling for 7 min and chilled on ice for 5 min before it was added to the hybridization bottle.

2.19.4 RNA Blot Hybridization

Membranes were placed in hybridization bottles and prehybridized overnight at 42°C in hybridization solution (50% (v/v) formamide, 5X SSPE, 1X Denhardt's (Sambrook et al. 1989), 1% SDS, 100 µg/ml yeast RNA). The hybridization solution was replaced before the probe was added. Hybridization was at 42°C for 12 to 24 hours.

The membranes were washed in 2X SSC, 0.1% SDS at 65°C, followed by washes at higher stringencies (1X SSC, 0.5X SSC, 0.2X SSC, 0.1X SSC, all with 0.1% SDS). After the washes the membranes were sealed in plastic and exposed to X-ray film at -80°C for varying lengths of time depending on signal intensity.

2.20 Protein Phosphorylation Assay of Pollen Protein

Phosphorylation of whole pollen grains was performed on ice following the protocol by Wehling et al. (1994). In brief, fresh pollen was collected, weighed and 4 µl assay buffer per mg pollen was added. (Assay buffer: 30 mM HEPES (pH 7.4), 1mM DTT, 0.0025% (w/v) BSA, 0.15% (v/v) Triton-X 100, 500 µM $CaCl_2$, 10 mM $MgCl_2$, 3 mM $MnCl_2$, 0.5 mM Pefabloc (Boehringer Mannheim), 5 µg/ml Leupeptin and 30 µM ATP) .

The reaction was started by adding 10 µCi [γ-^{33}P]-ATP (Amersham UK, 2000 Ci/mmol). At different time points, 18 µl aliquots were taken and transferred into reaction tubes containing 18 µl of 8% SDS/40 mM Na_2EDTA, and subsequently frozen in liquid nitrogen.

After the termination of the reaction, samples were boiled for 15 min and 2 µl of 5x PAGE-loading buffer was added. Pollen proteins were separated by SDS polyacrylamide gel electrophoresis (10% T/3% C) as described by Laemmli (1970) using the Mini Protean II Electrophoresis apparatus (Biorad, USA). After electrophoresis the gels were stained with Coomassie blue for 10 min, destained for 1 to 2 hours (after Sambrook et al. 1989) and subsequently treated with Entensify (DuPont) according to the manufacturer's protocol. Gels were dried overnight and exposed to X-ray film for 1 to 5 days at -80°C using intensifier screens (DuPont Cronex Quanta-III).

2.21 Establishment of a Liquid Pollen Germination Medium

In order to determine the optimal osmotic conditions and the optimal $CaCl_2$ concentration for pollen tube growth of *Phalaris* pollen *in vitro*, a series of germination media were prepared and tested. The first series of germination media

contained 2 mM $CaCl_2$ and 2 mM boric acid while the sucrose concentration varied in steps of 5% (w/v) from 10% to 30% . The second series of germination media contained 2 mM boric acid either 20% sucrose or 25% sucrose and increasing concentrations of $CaCl_2$ (2 mM, 5 mM, 7 mM, 10 mM).
Each germination media was tested using freshly collected pollen from at least 4 different plant including self-compatible mutants. Pollen grains were germinated in 100 to 200 µl germination medium in cavity microscope slides and pollen tube growth observed under the microscope for 1 hr at 25°C.

2.22 Protein Phosphorylation Assay of Germinating Pollen Grains

a) without pistil eluate
In order not to disturb the growth of the pollen tubes, separate reaction tubes were used for each time point containing a minimum of 5 mg freshly collected pollen. To each reaction 4 µl of germination medium (25% sucrose, 5 mM $CaCl_2$, 2 mM boric acid) per mg pollen was added and the pollen allowed to germinate at room temperature. At indicated time intervals 15 µCi of [γ-^{33}P]-ATP (Amersham UK, 2000 Ci/mmol) was added and the tubes kept at RT for 1 min before the reaction was terminated by taking an 18 µl aliquot and transferring to a tube containing 18 µl of 8% SDS/40 mM Na_2EDTA and 2 µl of SDS-PAGE-loading buffer. The tubes were kept in liquid nitrogen until all reactions were terminated. Samples were boiled for 10 min, and centrifuged at 6000 rpm for 3 min to pellet cell debris. Pollen proteins were separated on an SDS-PAGE gel as in 2.20. After electrophoresis the gels were treated as described in 2.20.

b) in the presence of pistil eluate
Pistils of either the pollen donor plant itself or plants 100% compatible with the pollen donor plant were incubated in 5 µl germination medium per pistil for 10 to 16 hrs at 4°C. A 1:1 dilution of this stigma eluate with fresh germination medium was used in the phosphorylation assay described above.

2.23 Immunodetection of the S-protein

Polyclonal antibodies against the S-protein were generated by X. Li (unpublished results).
After separation of the pollen proteins by SDS-PAGE, proteins were electrotransferred to nitrocellulose as described by Harlow and Lane (1988). Nitrocellulose membranes were blocked with 5% (w/v) non-fat dry milk in PBS (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO4, 137 mM NaCl, 2.7 mM KCl, pH 7.5) for 1hr at

RT, then washed twice in PBS for 10 min each. Immunodecoration with primary antibody (1:500) was carried out overnight at RT in PBS with 1% (w/v) BSA. Membranes were washed three times in PBS with 0.05% (v/v) Tween 20 for 10 min each, followed by 2 washes in PBS. Membranes were incubated with goat-anti-rabbit alkaline phosphatase conjugates (Biorad, USA) (1:5000) in PBS with 1% BSA for 3 hrs at RT, washed three times in PBS with 0.05% Tween 20 for 10 min each, twice with PBS and then developed with NBT/BCIP according to Knecht and Dimond (1984).

2.24 Immunoprecipitation of S-protein Using Polyclonal Antibodies

The method for the immunoprecipitation was performed using a modification of the protocol of Harlow and Lane (1988).

a) without stigma proteins

After termination of the phosphorylation reaction by transferring a 18 µl aliquot to a fresh tube containing 18 µl of 8% SDS/40 mM Na_2EDTA, the samples were boiled for 5 min. Cell debris was removed by centrifugation (10 min, 10000 rpm, table centrifuge); the supernatant was transferred to a fresh tube and its volume measured. Lysate buffer (50 mM Tris pH 7.5, 150 mM NaCl, 2% Triton X-100) was added to a final volume of 400 µl followed by the addition of 7 µl pre-immune serum. After mixing, the solution was kept on ice for 1 to 2 hrs with occasional inversion of the tube. To precipitate the antibodies present in the pre-immune serum, 20 µl of a 50% (v/v) Protein-A-Sepharose beads (Cl-4B, Sigma) in lysate buffer was added and the solution was kept on ice for further 1 to 2 hours with occasional agitation to mix the components. The beads were pelleted by centrifugation at 3000 rpm for 3 min and kept as a control. To the supernatant 5 µl of Anti-S serum was added, and the solution was incubated on ice for 1 hour. A further 20 µl of 50% Protein A-Sepharose beads were added and the suspension incubated on ice for 1 to 2 hrs. The suspension was centrifuged at 3000 rpm for 3 min, and the Protein A-Sepharose beads were washed once with lysate buffer and twice with wash buffer (0.5% (v/v) Tween 20, 0.15 M NaCl, 10 mM Tris pH 8.0), while the supernatant was kept as a control. After the washes the immuno complexes were resuspended in 5x SDS gel loading buffer (2.17) and boiled for 10 min. Proteins were separated by SDS-PAGE (2.20) and the gels treated as described in 2.20.

b) in the presence of stigma proteins

Mature stigmas were dissected out, immediately frozen in liquid nitrogen and stored at -80°C until used. Stigmas were crushed into a fine powder and homogenized in 4 µl germination medium/stigma. The homogenate was centrifuged at 10,000 rpm for 5

min and the supernatant transferred to a fresh tube. Pollen was germinated in a 1:5 dilution of the stigma proteins in germination medium. The phosphorylation assay was performed as described in 2.22 and immunoprecipitation of the S-protein was performed as described above (2.24 a).

Chapter 3 Isolation and Characterisation of Pollen-Specific Genes

3.1 Introduction

A major objective of the present study was to identify pollen-specific genes that are directly or indirectly involved in self-incompatibility. It was initially decided to take advantage of the detailed genetic analysis of the Z-alleles to identify cDNA sequences potentially belonging to the locus.

The assumptions made to approach this goal were: a) the Z-gene was pollen-specific; b) it was relatively abundant; c) it was a single or low copy gene; and d) the allelic differences between two Z alleles were detectable using differential screening.

All these assumptions were derived from previously published studies on gametophytic self-incompatibility systems and were the basis of the successful attempt to isolate the pollen S gene in *Phalaris* by Dr Xinmin Li and coworkers (1994). These researchers constructed a cDNA library from mature pollen with the genotype $S_{1.2}$ $Z_{1.1}$ in bacteriophage λgt10. The library was subsequently screened with single-stranded radioactively labelled cDNA from pollen of the genotypes $S_{1.2}$ $Z_{2.2}$, $S_{F.F}$ $Z_{1.1}$ (F is used to describe the S-complete mutant) and $S_{1.2}$ $Z_{1.1}$, this last genotype being used as a control for hybridisation signal intensity.

3.2 Results

3.2.1 Screen for Potential Z-Candidates

The starting point for the identification of Z-candidates was a group of 117 plaques originally isolated by Li and coworkers using the procedure outlined in the previous section. These 117 plaques showed either weaker or no hybridisation compared to the control and were therefore selected for further study. However on closer analysis, a relatively high number of the 117 plaques turned out to consist of a heterogeneous population of phages. Therefore, each original plaque was further purified by plating out at low density and randomly selecting 6 individual plaques for later analysis. The resulting 696 cDNA clones (one clone failed to grow) were transferred onto master plates and screened, first for pollen-specificity and then for putative Z-candidates.

3.2.2 Screen for Pollen-Specificity

Since the first assumption of this study was that the Z-gene was pollen-specific, the next step was to identify which of the 696 cDNA clones were expressed only in pollen. The clones were screened with single-stranded radioactively labelled cDNA from each of the following tissues: root, leaf, seed, 7 day old seedlings and florets at the pre-meiotic to meiotic stage.

A complete list of the results can be found in Appendix A. In summary, 193 clones were not pollen specific; 146 clones showed hybridisation signals to cDNA from one or more non-pollen tissues, which could not unambiguously be distinguished from background in the stringency conditions used. These "ambiguous" clones were included in subsequent experiments. A total of 357 clones did not show any hybridisation with cDNA from non-pollen tissues.

3.2.3 Differential Screen for Z-Gene Candidates

Based on assumption (d) above, the Z-gene candidates should show variation in hybridisation signal intensity when hybridised to cDNA from mature pollen of plants carrying different Z-alleles. Therefore, all clones were differentially screened under high stringency conditions with single stranded cDNA from mature pollen of the following genotypes: $S_{1.2}$ $Z_{2.2}$ (wildtype), $S_{1.2}$ $Z_{2°.2°}$ (° refers to pollen-only mutants), $S_{1°.1°}$ $Z_{2.2}$ and $S_{1.2}$ $Z_{1.1}$ (control).

Combining the results of the screen for pollen-specificity and the screen for allelic differences, it was possible to identify a subset of 139 clones out of the 503 putative pollen-specific clones which showed reduction in hybridisation signal intensity with at least one of the Z-alleles tested. The individual result for each of the 139 clones is listed in Table IV. Included in the 139 clones are 31 of the "ambiguous" group (see above). A summary of the result obtained is presented in Table V.

Table V: Clones showing lower expression relative to control genotype

Plant	Genotype	No of clones	%
42-4-8	$S_{1°.1°}$ $Z_{2.2}$	110	21.7
85-2-5	$S_{1.2}$ $Z_{2.2}$	53	10.4
25A-6-2	$S_{1.2}$ $Z_{2°.2°}$	30	5.9

There was little overlap among the clones identified by the differential screening. Only three clones showed a weaker hybridisation signal with all three genotypes. While 54 clones showed weaker hybridisation with both 42-4-8 and 85-2-5, only 7 clones were identified as weaker with both 42-4-8 and 25A-6-2 and 4 clones with both 85-2-5 and 25A-6-2.

3.2.4 Cross-Hybridisation Analysis

Once a number of pollen-specific clones were identified showing differences in hybridisation signal intensity, the question arose of how many of the clones were identical or related.

Table IV: Results of the differential screen

	42-4-8 ($S_{1°.1°}$ $Z_{2.2}$)	85-2-5 ($S_{1.2}$ $Z_{2.2}$)	25A-6-2 ($S_{1.2}$ $Z_{2°.2°}$)
cDNA clone:			
Am2b	w		
A5b	w	w	
A7b	w	w	
A7d	w	w	
A7f	w	w	w
A12a			w
A12b			w
A12c			w
A12d			w
A12e			w
A12f			w
A14a	w		
A14b	w		
A14c	w		
A14d	w		
A14f	w	w	
A15a	w	w	
A15b	w	w	
A16e			w
A28b	w		
A28e	w		
A28f	w		
A29b	w		
A29c	w		
A34a	w	w	
A34b	w		
A34c	w	w	
A34d	w	w	
A34e	w	w	
A34f	w	w	
A36d			w
Bm3a	w	w	
Bm7c		w	
Bm7d		w	w
Bm7e			w
Bm9e		w	w
Bm15b	w		
Bm16a	w		
Bm16b	w	w	
Bm16e	w		
Bm16f	w		
Bm17a	w		
Bm17b	w		
Bm19a	w		
Bm19b	w		

Table IV: continued

	42-4-8 ($S_{1°.1°}\ Z_{2.2}$)	**85-2-5** ($S_{1.2}\ Z_{2.2}$)	**25A-6-2** ($S_{1.2}\ Z_{2°.2°}$)
Bm19c	w	w	
Bm19d	w	w	
Bm19e	w	w	w
Bm19f	w		w
Bm20a	w		
Bm20b	w	w	
Bm20c	w	w	
Bm20d	w	w	
B1e	w		
B2b	w		
B2c	w	w	
B2d	w	w	
B2f	w	w	
B4a	w	w	
B4b	w	w	
B4c	w		
B4d	w		
B4e			w
B4f		w	
B5a		w	
B5c		w	
B5d			w
B5f	w		
B6c	w	w	
B6d	w	w	
B7b	w	w	
Cm5e			w
C2a	w	w	w
C2d	w		w
C6b	w	w	
C6d	w	w	
C6f	w		
C7b	w		w
C7c	w		w
C7d	w	w	
C10b	w		
C10c	w		
C10d	w	w	
C10e	w		
C10f	w	w	
C11c	w	w	
C11e			w
C11f	w	w	
C13a	w		
C13b	w		
C13d	w		
C14d			w

Table IV: continued

	42-4-8 ($S_{1°.1°}$ $Z_{2.2}$)	85-2-5 ($S_{1.2}$ $Z_{2.2}$)	25A-6-2 ($S_{1.2}$ $Z_{2°.2°}$)
C15e	w	w	
C15f	w	w	
C17a			w
C17e	w		
C19a	w		
C19c	w		
C19f	w		
C20a	w	w	
C20b	w	w	
C20d	w		
C20f	w		
C21e	w		
C21f	w		
C22f	w		
C23d	w	w	
C24a			w
C24b	w		
C24c	w		
C24e			w
C25e	w	w	
C26a	w		
C26b	w		
C26c	w		
C26d	w		
C26e	w		
C26f	w		
C27b	w		
C27c	w		
C27d	w		
C27e	w		
C27f	w		
C28a	w		
C28c	w		
C28d	w		
C28f	w		
C29e	w		
C29f	w		
C30f	w		
C37d			w
C37f		w	
C38d	w		w
C39b			w
C40f			w
C41b		w	
C41d		w	

w indicates weaker hybridisation signal in comparison to control

To answer this question the inserts of the phages were amplified using PCR, separated by gel-electrophoresis and transferred to nylon membranes. After purification the amplified DNA-inserts of individual phages were used as probes in DNA blot hybridisations.
The results of the experiments revealed that the 139 clones could be classified into 20 groups. Two phages contained inserts of less than 120 bp (groups 19 and 20, Table VI) and were discarded. For each remaining group the phage carrying the largest insert was chosen for further analysis.

Table VI: Results of Cross-Hybridisation Analyses

Group	Representative clone	Size of insert (bp)	Number of members
1	B7b	552	99
2	A12a	1316	7
3	C41b	678	5
4	C38d	470	5
5	C17a	406	3
6	C11e	ca. 700	3
7	Bm7d	ca. 2200	2
8	C7c	641	2
9	Bm7e	ca. 800	2
10	A12e	ca. 500	1
11	A16e	ca. 900	1
12	A36d	307	1
13	A34b	733	1
14	B4e	ca. 700	1
15	B5c	442	1
16	Cm5e	195	1
17	C7b	633	1
18	C24e	522	1
19	Bm9e	below 120	1
20	Bm17b	below 120	1

The representative clone is the phage which contains the largest insert.

3.2.5 RFLP Analysis

The 18 candidates for the Z-gene were subjected to restriction fragment length polymorphism (RFLP) analyses in order to detect restriction site polymorphisms that could be correlated with specific Z-genotypes. Initially, the clones were hybridised to genomic DNA from plants of the genotypes $S_{1.2}\ Z_{1.1}$, $S_{1.2}\ Z_{1.2}$, and $S_{1.2}\ Z_{2.2}$, which had been digested with different restriction endonucleases. In some cases DNA from a plant with the genotype $S_{1.4}\ Z_{1.3}$ was also used.
The number of bands detected with each clone are summarised in Table VII.
With all clones tested a polymorphic pattern was obtained with at least one of the enzymes used. A typical example of such a polymorphic restriction pattern is presented in Fig. 3.1. C38d was hybridised to DNA from three self-incompatible plants which vary only in their Z-genotype. The DNA had been digested with 6 different restriction endonucleases as

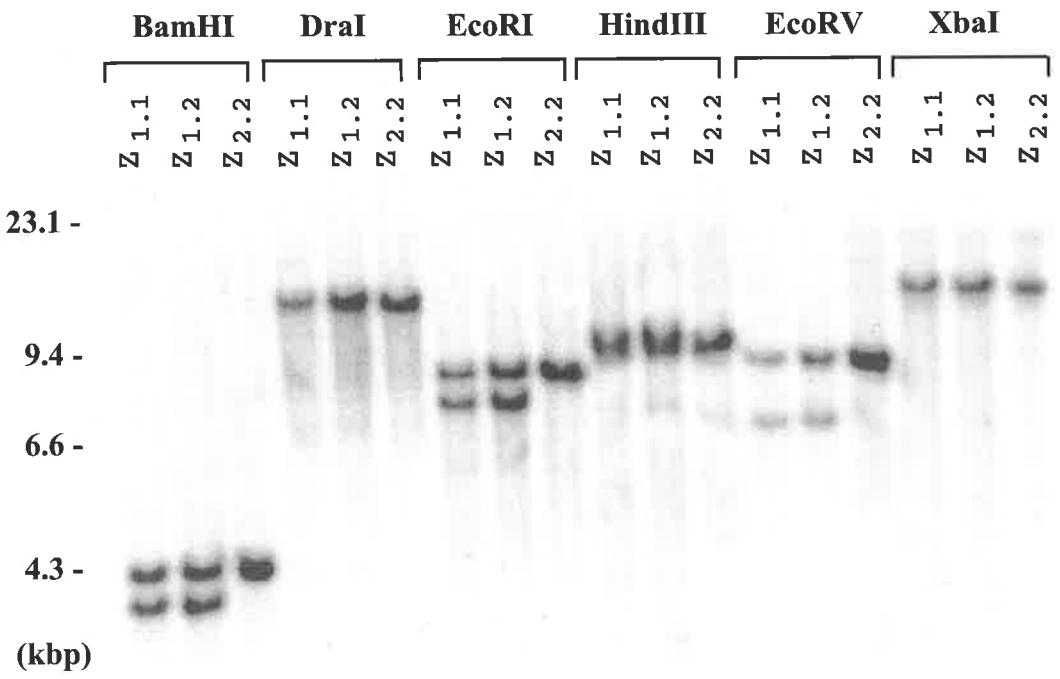


Fig 3.1 Southern blot hybridization of clone C38d to DNA of three self-incompatible plants with different Z-genotypes. The S-genotype is $S_{1.2}$. DNA was digested with the restriction endonucleases indicated. Size markers are in kilobase pairs (kbp).

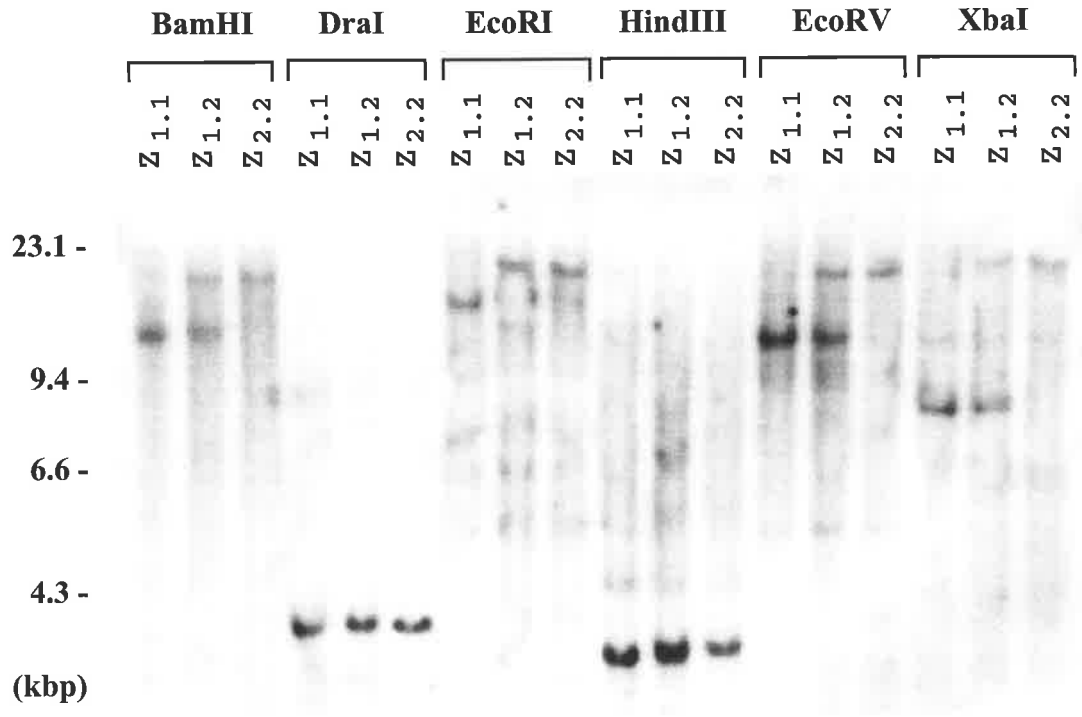


Fig. 3.2 A Southern blot hybridization of clone C7c to DNA of three self-incompatible plants. The S-genotype is $S_{1.2}$. The DNA was digested with the restriction endonucleases indicated. Size markers are in kilobase pairs (kbp).

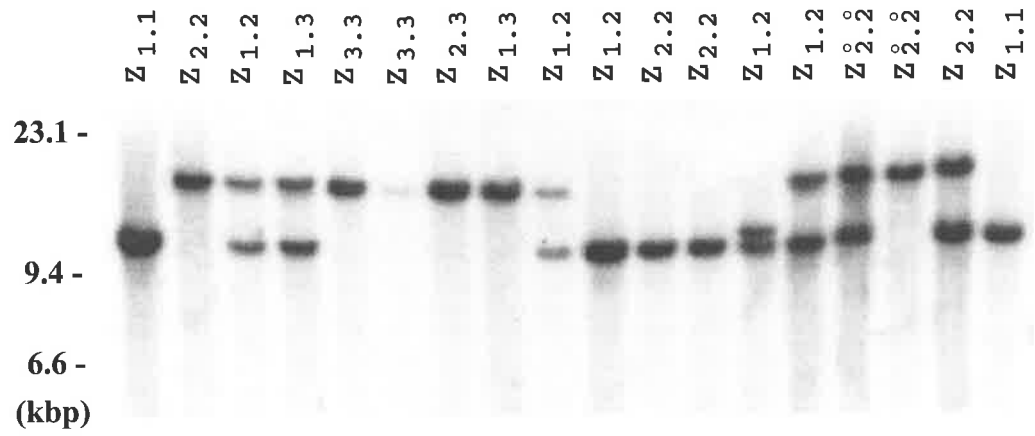


Fig. 3.2 B Southern blot hybridization of clone C7c to DNA of self-incompatible and self-compatible plants. The first three lanes contain the same DNA as Fig.3.2 A. The DNA was digested with the restriction endonuclease *Eco*RV. Size markers are in kilobase pairs (kbp).

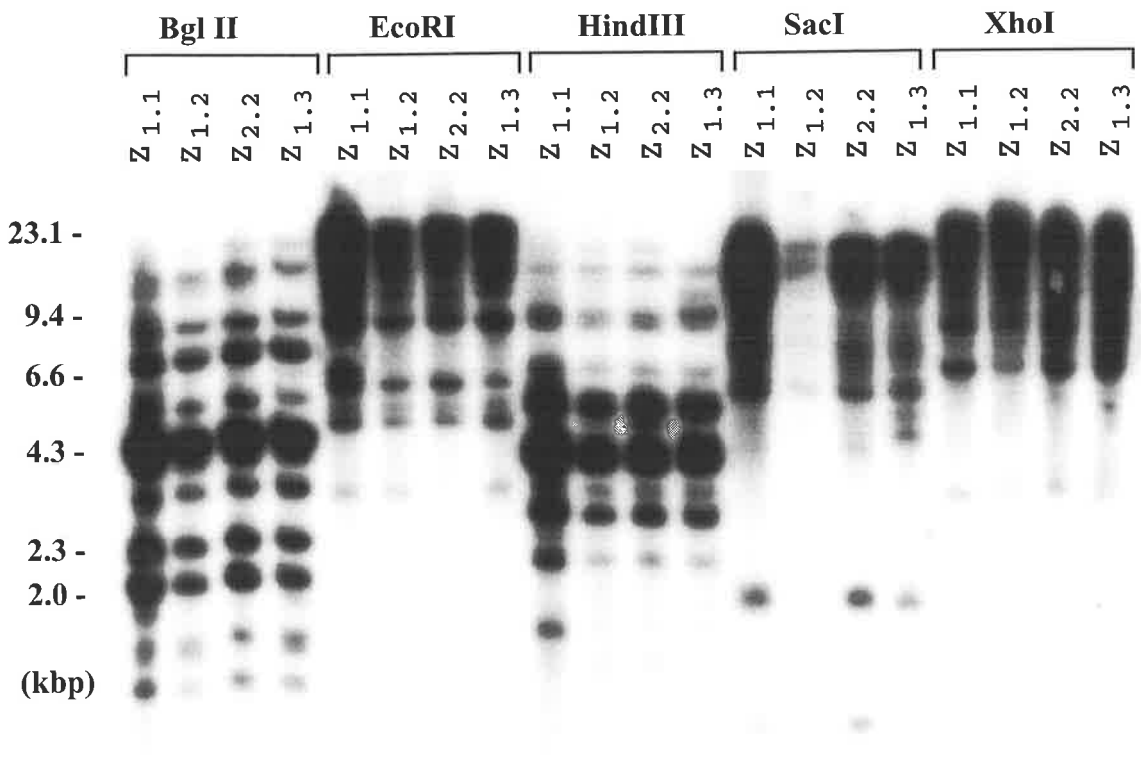


Fig. 3.3 Southern blot hybridization of clone B7b to DNA of four self-incompatible plants. The S-genotype of the plant containing the Z_3 allele is $S_{1.4}$, all others are $S_{1.2}$. The DNA was digested with the restriction endonucleases indicated. Size markers are in kilobase pairs (kbp).

indicated. A polymorphic pattern was obtained with DNA digested with *Bam*HI, *Eco*RI and *Eco*RV .

In the initial series of experiments the cDNA-clones C41b, A34b, C7c and Cm5e showed restriction patterns compatible with linkage to the Z-locus. To find out whether the clones were indeed linked they were used as probes in a second Southern blot hybridisation to genomic DNA from 17 to 20 plants.

Fig. 3.2 A shows the results of the Southern blot hybridisation with C7c as a probe. The restriction pattern obtained with *Bam*HI, *Eco*RI, *Eco*RV and *Xba*I digested DNA indicates potential linkage to the Z-locus, assuming the lower band represents the Z_1 allele and the upper band the Z_2 allele. When C7c was hybridised to *Eco*RV-digested DNA from plants of known SI genotype, the bands potentially representing the Z_1 and Z_2 alleles did not segregate with the Z-genotype in 11 of the 18 plants tested (Fig. 3.2 B) or 7 of the 18 plants tested, assuming that the Z_2 and the Z_3 allele give the same band.

In summary, no clone showed conclusive linkage to the Z-gene.

Table VII: Results of Southern Blot Analysis

cDNA-clone	Number of Bands detected in Southern Blot Analysis
B7b	8 to 12
A12a	1 to 2
C41b	1 to 2
C38d	1 to 2
C17a	1 to 3
C11e	2 to 4
Bm7d	1 to 2
C7c	1 to 2
Bm7e	1 to 2
A12e	3 to 8
A16e	3 to 7
A36d	1 to 3
A34b	3 to 7
B4e	1 to 3
B5c	1 to 2
Cm5e	1 to 2
C7b	1 to 4
C24e	1 to 4

As can been seen from Table VII, the majority of the cDNA clones represent single or low copy genes. One prominent exception was B7b whose restriction pattern indicates that the clone was a member of a gene-family (Fig. 3.3).

3.2.6 Transcriptional Characterisation and Sequence Analysis

Although none of the clones analysed could be unambiguously linked to the Z-gene, the fact that their expression altered in plants of different Z-genotype pointed to their possible involvement in the SI reaction. In addition, their apparent pollen-specific expression

suggests that, irrespectively of their putative roles in SI, they might be essential for late pollen-development and/or pollen-stigma interaction.
For these reasons a systematic transcription analysis was undertaken and the nucleotide sequences of all 18 clones partially determined.

3.2.6.1 Transcriptional Analysis

The spatial and temporal expression of the 18 cDNAs was examined by RNA blot hybridisations. Total RNA was isolated from roots, leaves, 7 day old seedlings, mature pistils and anthers of 4 different developmental stages (Fig. 3.4). In addition, total RNA was isolated from mature pollen of plants with different S and Z genotype as well as from S-, Z- and T-mutants. To be certain of the pollen-specificity of the cDNAs, membranes carrying non-pollen RNA were washed using low stringency conditions and exposed for several days, whereas membranes with pollen RNA were subjected to stringent washes and short exposure times in order to detect potential differences in hybridisation intensity to different SI genotypes. As a control for the RNA amount in each lane, all membranes were hybridised with an rDNA probe. The expression profile of the cDNAs is summarised in Table VIII.

Table VIII: Results of Transcriptional Analyses

	Root	Leaf	Seedling	Pistil	A 1	A 2	A 3	A 4	Pollen
cDNA-clone									
B7b	-	-	-	-	-	-	w	++	+++
A12a	-	-	-	-	-	-	-	w	+++
C41b	-	-	-	-	-	-	-	+	+++
C38d	-	-	-	-	-	-	-	-	++
C17a	-	-	-	-	-	-	-	+	++
C11e	-	(w)	(w)	-	(w)	(w)	(w)	+	++
Bm7d	-	-	-	-	+	+	+	+	+++
C7c	-	-	-	-	-	-	-	w	+++
Bm7e	-	-	-	-	-	-	-	w	++
A12e	-	-	-	-	-	-	-	w	++
A16e	w	w	w	w	w	w	w	w	++
A36d	-	-	-	-	-	-	-	++	+++
A34b	-	-	-	-	-	-	-	-	w
B4e	-	-	-	-	-	-	-	w	+++
B5c	-	-	-	-	-	-	-	-	w
Cm5e	w	w	w	w	-	-	-	-	w
C7b	(+)	-	-	-	-	-	-	+	+++
C24e	-	-	-	-	-	-	-	w	+++

Signal was detectable: +++ after less than 10 hrs exposure
++ overnight exposure
+ 48 hrs exposure
w weak signal, required more than 48 hrs exposure
() transcript size differs from the one expressed in pollen
A1: anther stage 1, etc.

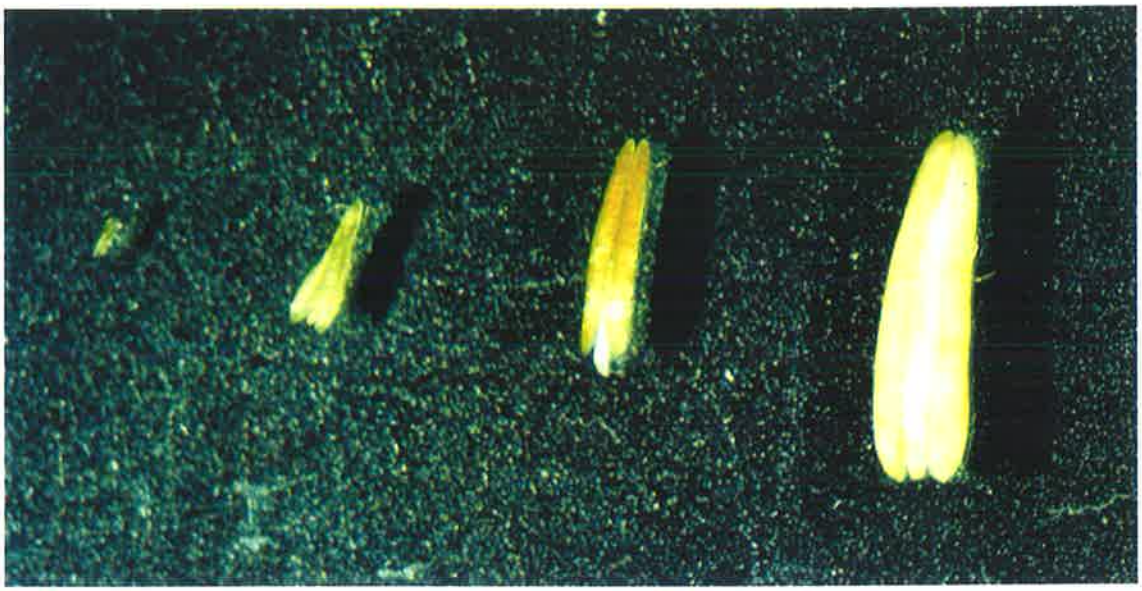
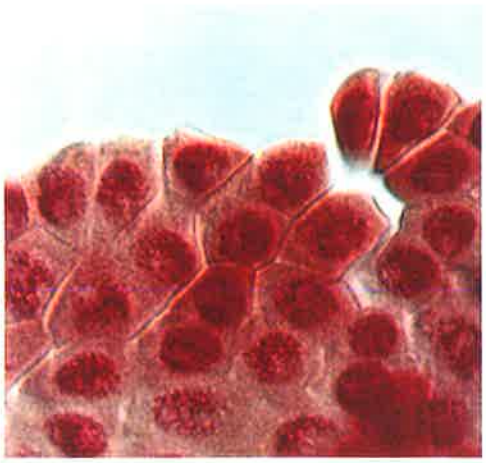
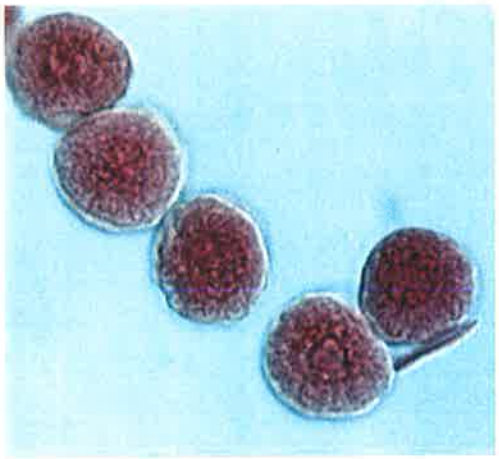


Fig. 3.4 RNA was isolated from the four developmental stages depicted above. The corresponding pollen stages are shown below.



stage 1: pollen mother cells



stage 2: young microspores

stage 3: late microspore

stage 4: pollen

The sizes of the mRNAs detected ranged from approximately 360 bases (b) to 2.7 kilobases (kb). Most of the clones examined seemed to hybridise to only one mRNA species. Exceptions were Bm7d, B7b and A16e.
Bm7d hybridised to 3 mRNAs of approx. 2.7 kb, 1.7 kb and 880 b in size (Fig. 3.5). It was interesting to note that the relative abundance of transcripts varied not only between pollen from plants of different genotype but also within one genotype (see Fig. 3.5 lanes 1 and 2 and 4 and 9). In anthers of stage 1 to 3, the smallest transcript seemed to be absent, whereas the 1.7 kb transcript was only weakly detectable.

The majority of the mRNAs was predominantly, if not exclusively, expressed in pollen, as anticipated from the results of the screen with RNA from non-pollen tissue.
C7b, however, hybridised to several transcripts of root (Fig. 3.6), but the transcripts detected in root were of different sizes from that of the mRNA expressed in pollen.
A16e was one of the "ambiguous" clones putatively expressed in root and seedling (Appendix A). As can be seen in Fig. 3.7, A16e weakly hybridised to a 1.25 kb band in root, seedling, pistil and anther stages 1 to 3. In pollen and anther stage 4, however, A16e hybridised to an additional transcript of ca. 850 b. This transcript was not detectable in any other tissue.
For C11e, a possible expression in leaf was assumed from the screen (Appendix A). However, the two transcripts detected in leaf and seedling, after a 5 day-exposure, were of different sizes (ca. 1.4 kb and 1.1 kb) from the one expressed in pollen (ca. 1.6 kb) and the two present in anther stage 4 (Fig. 3.8). It is likely that the transcripts in non-pollen tissue are related to but not identical with the C11e sequence.
The expression of Cm5e might not be restricted to pollen (Fig. 3.9) Weak hybridisation was also found with leaf, seedling and pistil RNA and, to a lesser extent, with root RNA but not with RNA from anthers. Since Cm5e is of comparatively low abundance, it is not surprising that expression in non-pollen tissue was not detected in the screen.

The most interesting result concerns the relative abundance of the transcripts and their variation among pollens of different SI genotypes. Clear quantitative variation of mRNA expression in pollen from different SI genotypes could be observed with clone C41b (Fig. 3.10) and to a lesser extend with Cm5e (Fig. 3.9). However there seemed to be no obvious correlation with the SI genotype. C41b was hybridised to total RNA from non-pollen tissues, from anthers of 4 developmental stages and from pollen of 10 plants with different SI genotype (Fig.3.10). The detected transcript was only present in pollen and anthers of stage 4. The transcript seemed to be more abundant in pollen from plants with the genotypes $S_{1.2}$ $Z_{2^{\circ}.2^{\circ}}$ (lane 4) and $S_{2.2}$ $Z_{1.2}$ (lane 7) when compared with the control hybridisation using a probe for 18S rRNA. It seems possible that in these two plants the degree of degradation of this particular mRNA is reduced in comparison to the other plants tested.

Fig. 3.5 Northern blot analysis of the expression of clone Bm7d.

Approximately 5 μg of total RNA from the tissues indicated was used in each lane. The upper autoradiograms show the blots hybridised with the Bm7d cDNA. The final wash for the blot carrying pollen RNA was in 0.1x SSC, 1% SDS at 65°C. All other blots were washed in 0.5x SSC. The membrane carrying pollen RNA was exposed for 12 hrs, all others for 24 hrs. After removing the probe the same membranes were hybridised with the 18S rRNA probe and the results are shown in the lower autoradiograms.

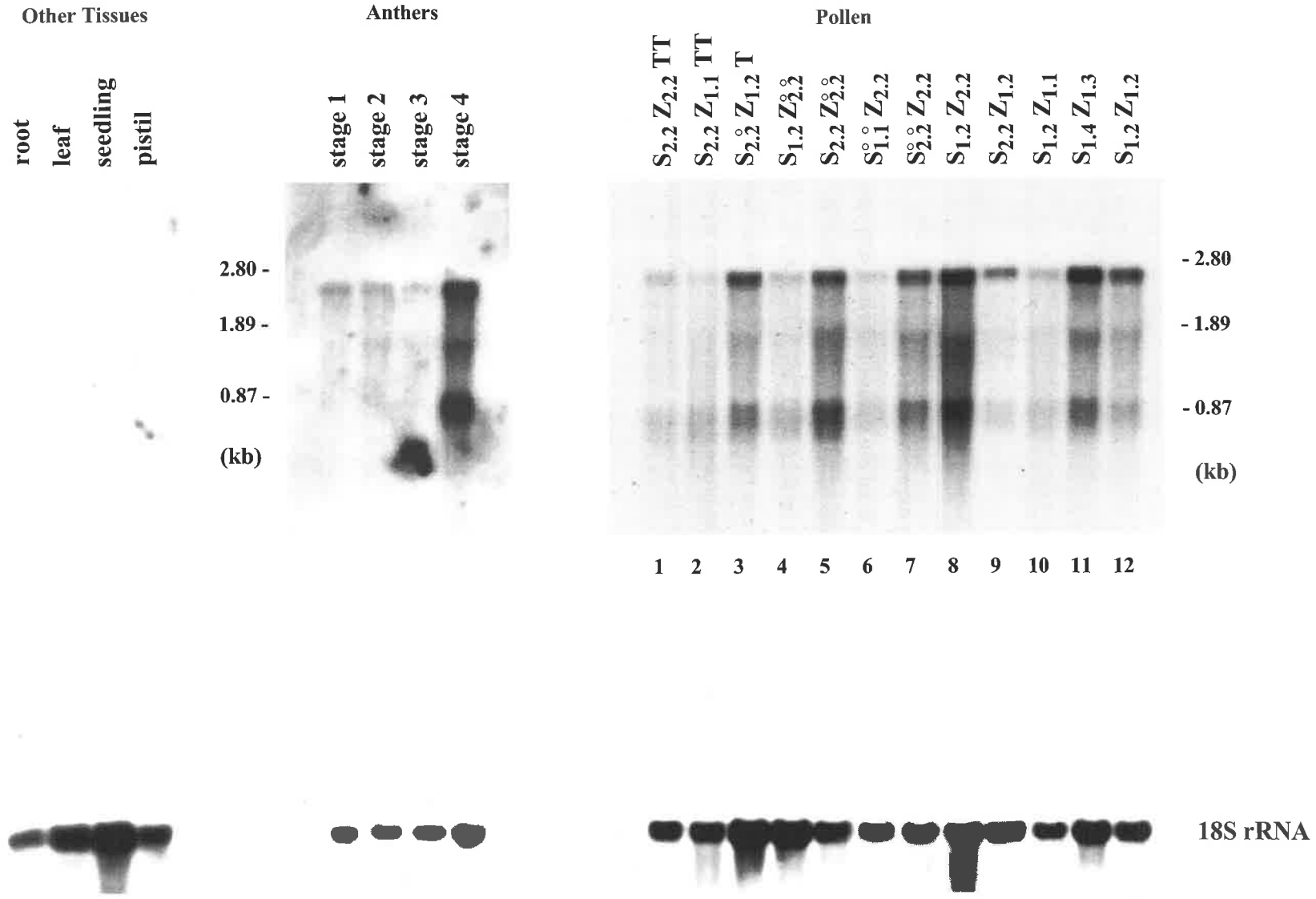

Other Tissues
root
leaf
seedling
pistil
Anthers
stage 1
stage 2
stage 3
stage 4
Pollen
$S_{2.2}$ $Z_{2.2}$ TT
$S_{2.2}$ $Z_{1.1}$ TT
$S_{2.2}^{\circ}$ $Z_{1.2}$ T
$S_{1.2}$ $Z_{2.2}^{\circ}$
$S_{2.2}$ $Z_{2.2}^{\circ}$
$S_{1.1}^{\circ}$ $Z_{2.2}$
$S_{2.2}^{\circ}$ $Z_{2.2}$
$S_{1.2}$ $Z_{2.2}$
$S_{2.2}$ $Z_{1.2}$
$S_{1.2}$ $Z_{1.1}$
$S_{1.4}$ $Z_{1.3}$
$S_{1.2}$ $Z_{1.2}$
2.80 -
1.89 -
0.87 -
(kb)
- 2.80
- 1.89
- 0.87
(kb)
1 2 3 4 5 6 7 8 9 10 11 12
18S rRNA

Fig. 3.6 Northern blot analysis of the expression of clone C7b.

Approximately 5 μg of total RNA from the tissues indicated was used in each lane. The upper autoradiograms show the blots hybridised with the C7b cDNA. The final wash for the blot carrying pollen RNA was in 0.1x SSC, 1% SDS at 65°C. All other blots were washed in 0.5x SSC. The membrane carrying pollen RNA was exposed for 6 hrs, all others for 48 hrs. After removing the probe the same membranes were hybridised with the 18S rRNA probe and the results are shown in the lower autoradiograms.

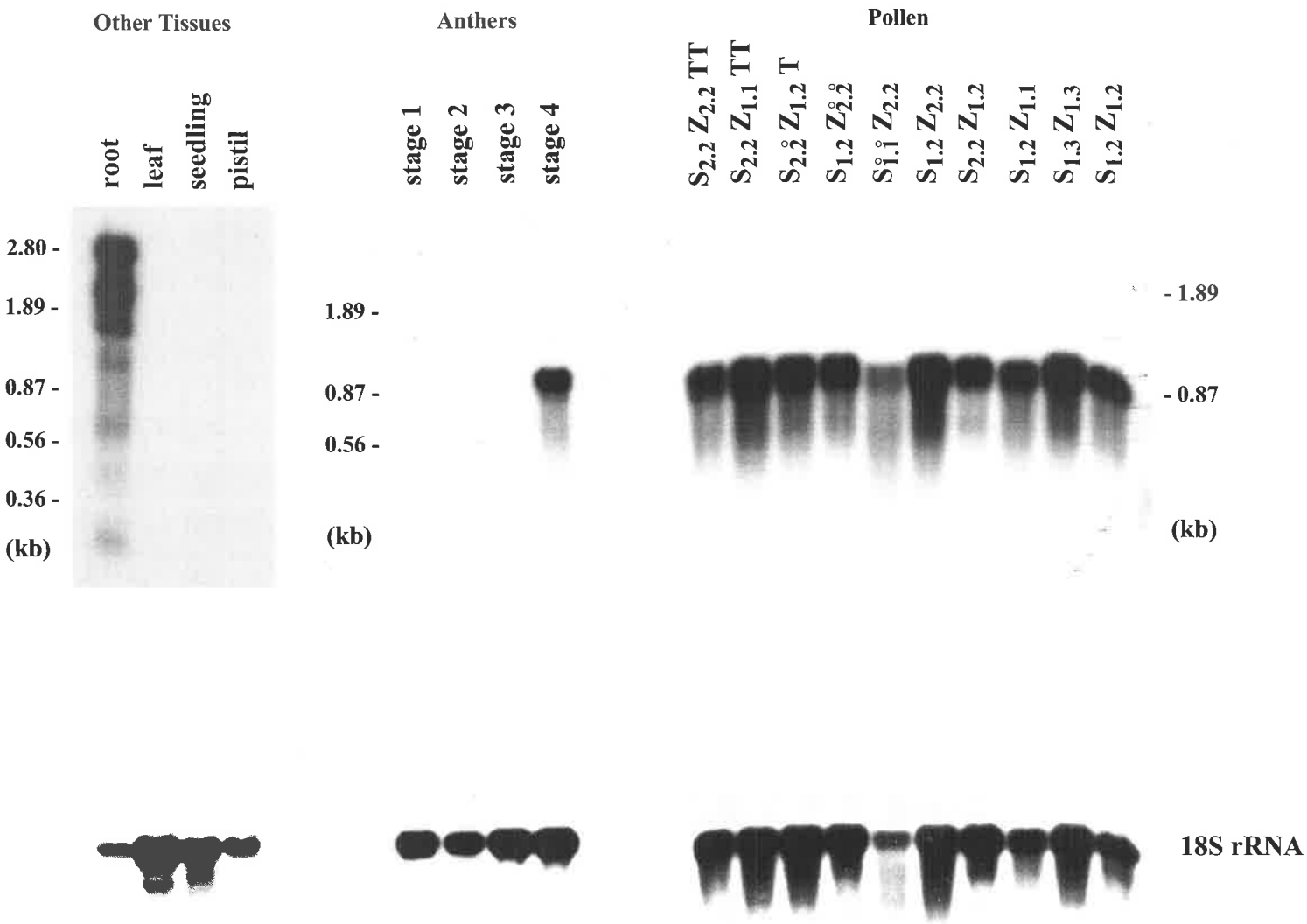

Other Tissues
Anthers
Pollen
root
leaf
seedling
pistil
stage 1
stage 2
stage 3
stage 4
S2.2 Z2.2 TT
S2.2 Z1.1 TT
S2.2 Z1.2 T
S1.2 Z2.2
S1.1 Z2.2
S1.2 Z2.2
S2.2 Z1.2
S1.2 Z1.1
S1.3 Z1.3
S1.2 Z1.2
2.80 -
1.89 -
0.87 -
0.56 -
0.36 -
(kb)
1.89 -
0.87 -
0.56 -
(kb)
- 1.89
- 0.87
(kb)
18S rRNA

Fig. 3.7 Northern blot analysis of the expression of clone A16e.

Approximately 5 μg of total RNA from the tissues indicated was used in each lane. The upper autoradiograms show the blots hybridised with the A16e cDNA. The final wash for the blot carrying pollen RNA was in 0.1x SSC, 1% SDS at 65°C. All other blots were washed in 1x SSC. The membranes carrying pollen and anther RNA were exposed for 2.5 days, the other for 4 days. After removing the probe the same membranes were hybridised with the 18S rRNA probe and the results are shown in the lower autoradiograms.

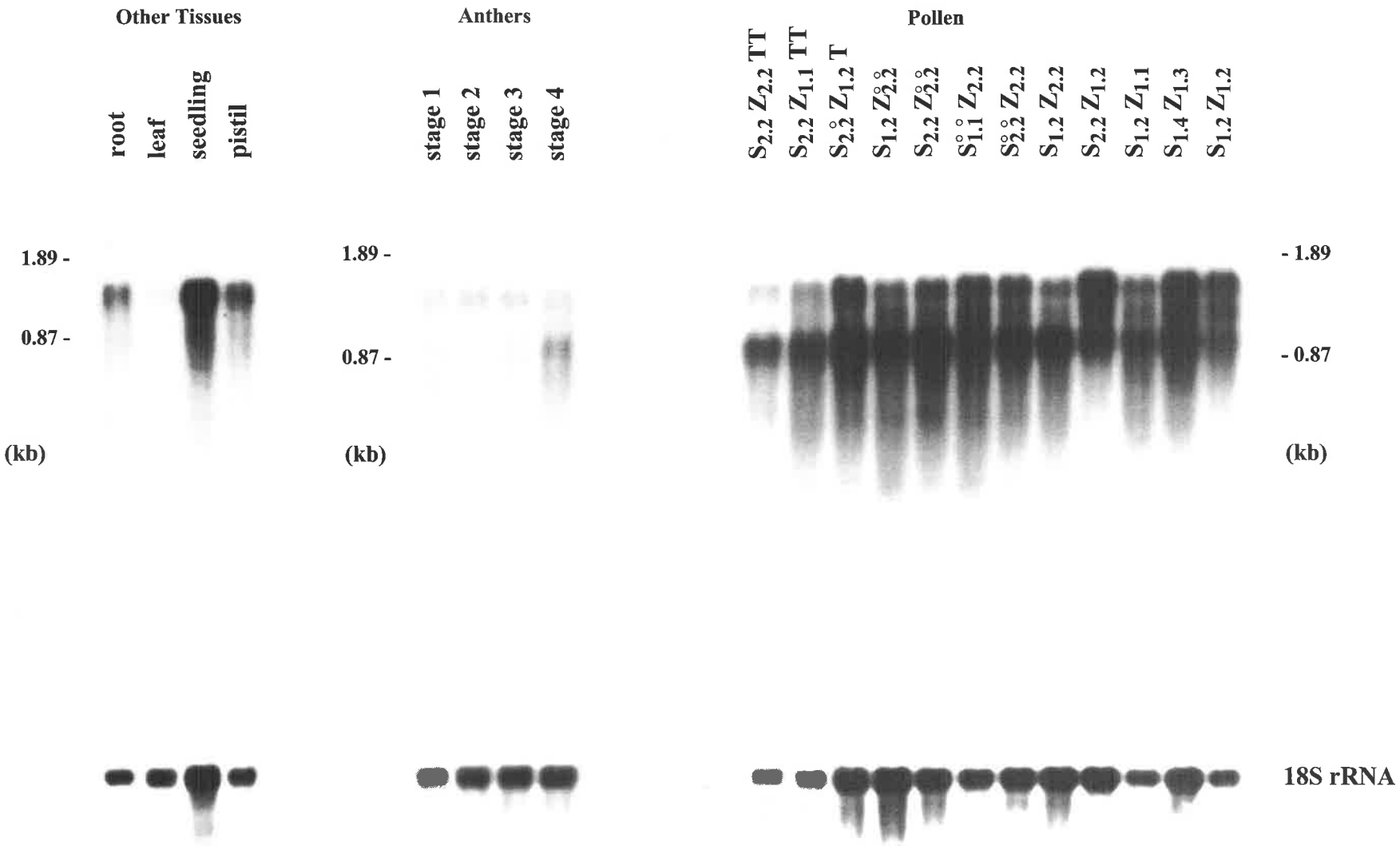

Other Tissues
root
leaf
seedling
pistil
Anthers
stage 1
stage 2
stage 3
stage 4
Pollen
S2.2 Z2.2 TT
S2.2 Z1.1 TT
S2.2 Z1.2 T
S1.2 Z2.2
S2.2 Z2.2
S1.1 Z2.2
S2.2 Z2.2
S1.2 Z2.2
S2.2 Z1.2
S1.2 Z1.1
S1.4 Z1.3
S1.2 Z1.2
1.89 -
0.87 -
(kb)
1.89 -
0.87 -
(kb)
- 1.89
- 0.87
(kb)
18S rRNA

Fig. 3.8 Northern blot analysis of the expression of clone C11e.

Approximately 5 μg of total RNA from the tissues indicated was used in each lane. The upper autoradiograms show the blots hybridised with the C11e cDNA. The final wash for the blot carrying pollen RNA was in 0.1x SSC, 1% SDS at 65°C. All other blots were washed in 1x SSC. The membrane carrying pollen RNA was exposed for 12 hrs, all others for 5 days. After removing the probe the same membranes were hybridised with the 18S rRNA probe and the results are shown in the lower autoradiograms.

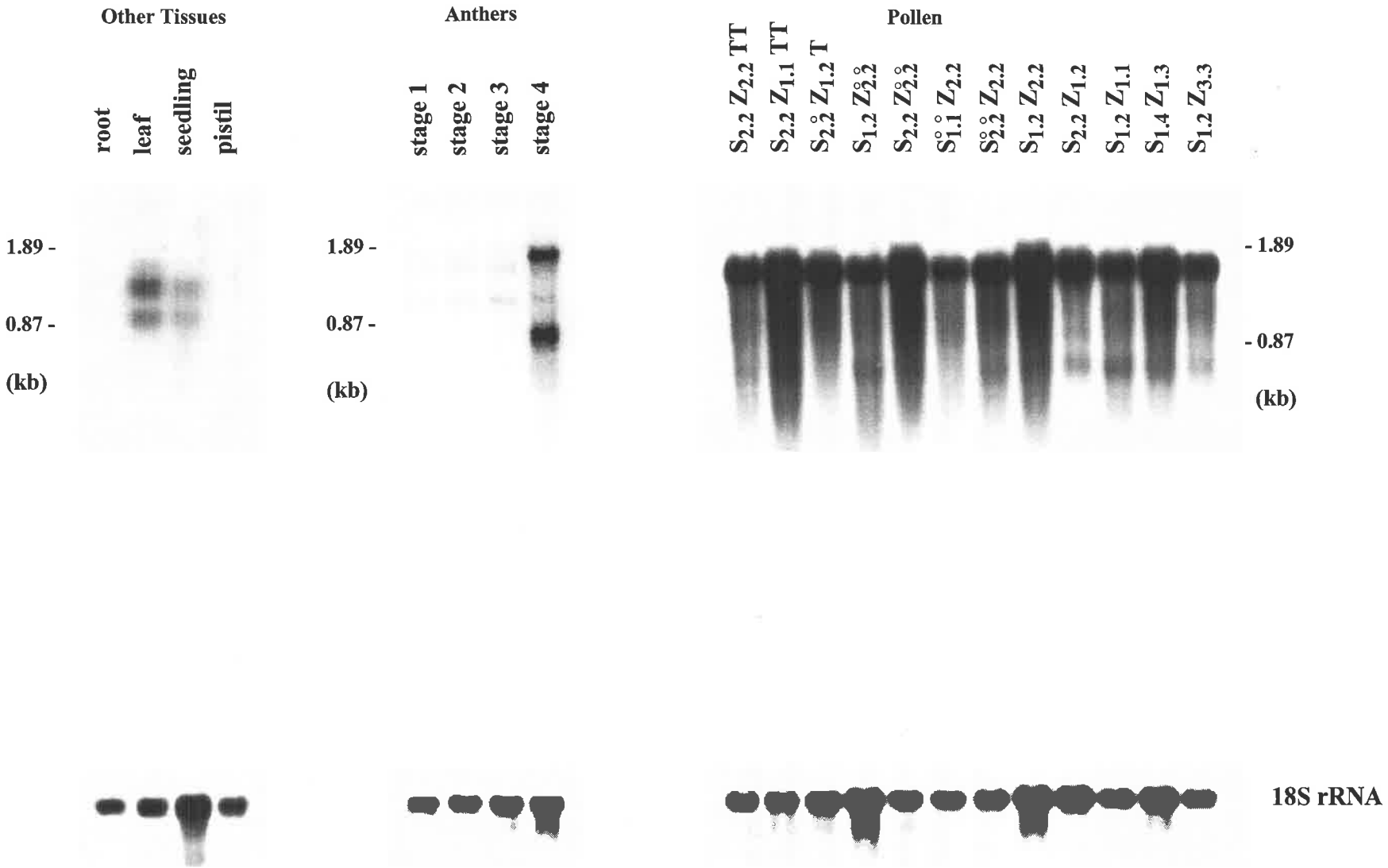

Other Tissues
root
leaf
seedling
pistil
Anthers
stage 1
stage 2
stage 3
stage 4
Pollen
S2.2 Z2.2 TT
S2.2 Z1.1 TT
S2.2 Z1.2 T
S1.2 Z2.2
S2.2 Z2.2
S1.1 Z2.2
S2.2 Z2.2
S1.2 Z2.2
S2.2 Z1.2
S1.2 Z1.1
S1.4 Z1.3
S1.2 Z3.3
1.89 -
0.87 -
(kb)
1.89 -
0.87 -
(kb)
- 1.89
- 0.87
(kb)
18S rRNA

Fig. 3.9 Northern blot analysis of the expression of clone Cm5e.

Approximately 5 μg of total RNA from the tissues indicated was used in each lane. The upper autoradiograms show the blots hybridised with the Cm5e cDNA. The final wash for the blot carrying pollen RNA was in 0.5x SSC, 1% SDS at 65°C. All other blots were washed in 1x SSC. All membranes were exposed for 4 days. After removing the probe the same membranes were hybridised with the 18S rRNA probe and the results are shown in the lower autoradiograms.

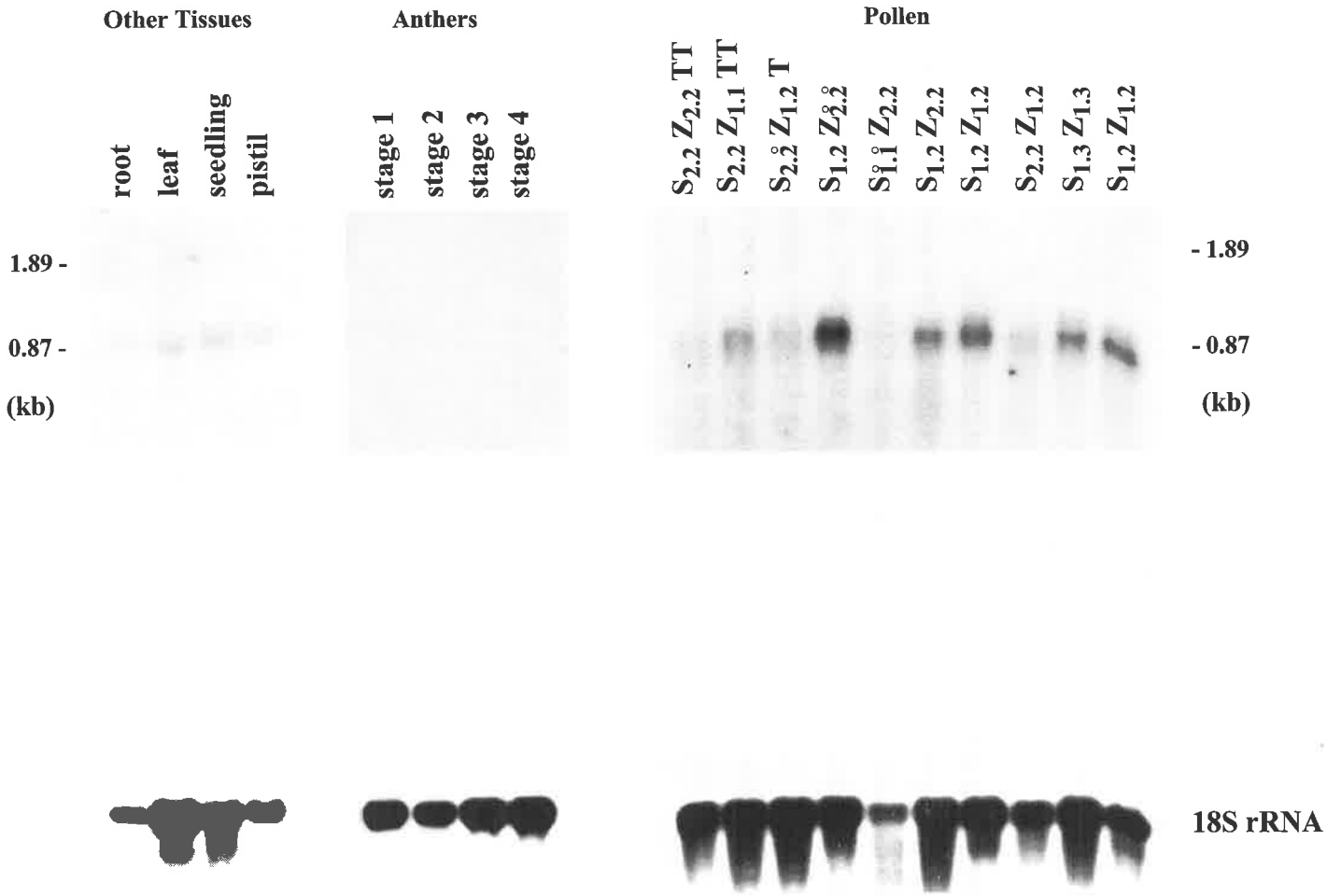

Other Tissues
Anthers
Pollen
root
leaf
seedling
pistil
stage 1
stage 2
stage 3
stage 4
S2.2 Z2.2 TT
S2.2 Z1.1 TT
S2.2♀ Z1.2 T
S1.2 Z2.2♂
S1.1♀ Z2.2
S1.2 Z2.2
S1.2 Z1.2
S2.2 Z1.2
S1.3 Z1.3
S1.2 Z1.2
1.89 -
0.87 -
(kb)
- 1.89
- 0.87
(kb)
18S rRNA

Fig. 3.10 Northern blot analysis of the expression of clone C41b.

Approximately 5 μg of total RNA from the tissues indicated was used in each lane. The upper autoradiograms show the blots hybridised with the C41b cDNA. The final wash for the blot carrying pollen RNA was in 0.1x SSC, 1% SDS at 65°C. All other blots were washed in 1x SSC. The membrane carrying pollen RNA was exposed for 3.5 hrs, all others for 24 hrs. After removing the probe the same membranes were hybridised with the 18S rRNA probe and the results are shown in the lower autoradiograms.

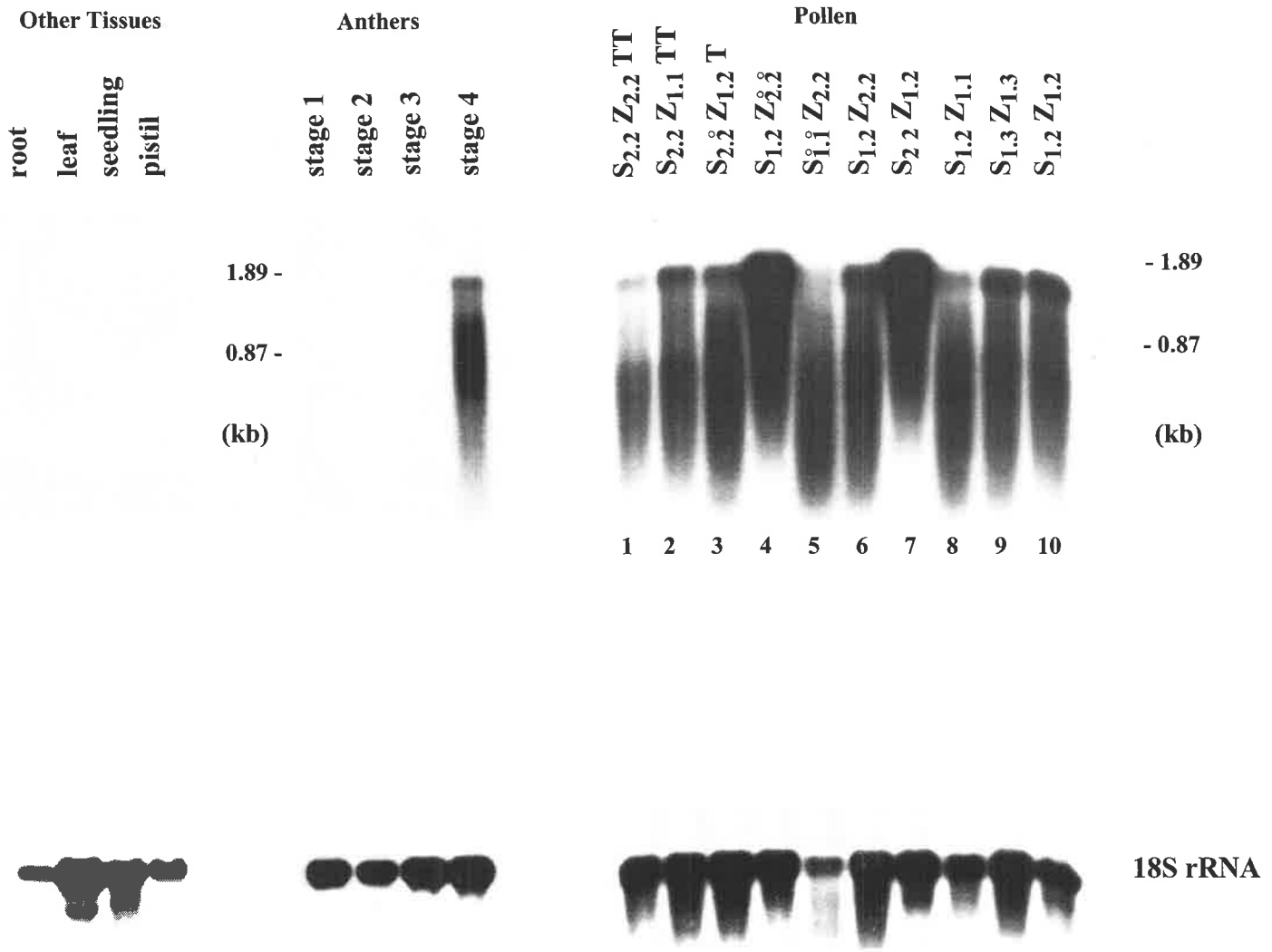
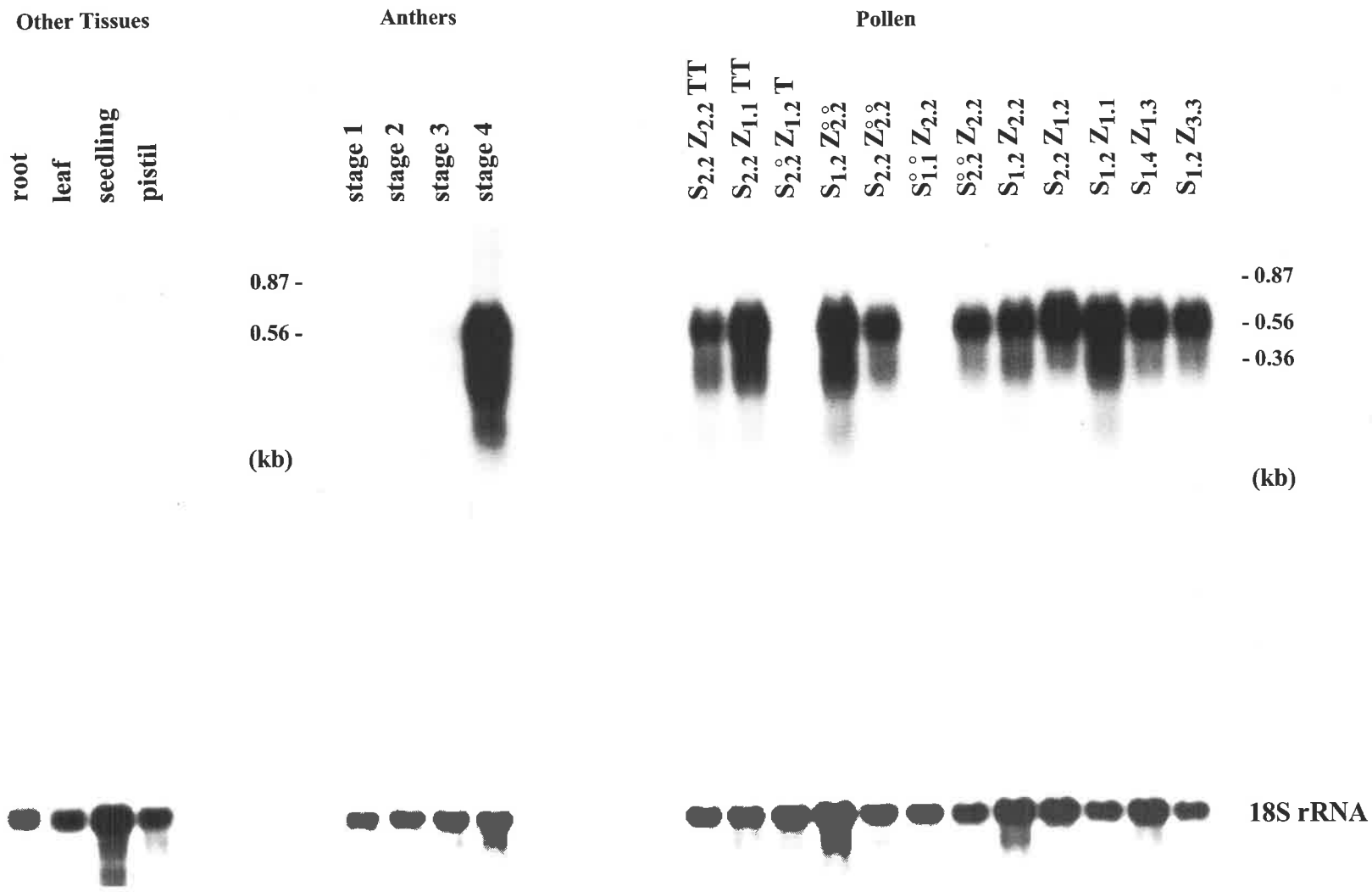

Other Tissues
root
leaf
seedling
pistil
Anthers
stage 1
stage 2
stage 3
stage 4
Pollen
S2.2 Z2.2 TT
S2.2 Z1.1 TT
S2.2 Z1.2 T
S1.2 Z2.2
S1.2 Z2.2
S1.2 Z2.2
S2 2 Z1.2
S1.2 Z1.1
S1.3 Z1.3
S1.2 Z1.2
1.89 -
0.87 -
(kb)
- 1.89
- 0.87
(kb)
1 2 3 4 5 6 7 8 9 10
18S rRNA

Fig. 3.11 Northern blot analysis of the expression of clone B7b.

Approximately 5 μg of total RNA from the tissues indicated was used in each lane. The upper autoradiograms show the blots hybridised with the B7b cDNA. The final wash for the blot carrying pollen RNA was in 0.1x SSC, 1% SDS at 65°C. All other blots were washed in 1x SSC. The membrane carrying pollen RNA was exposed for 4 hrs, all others for 3 days. After removing the probe the same membranes were hybridised with the 18S rRNA probe and the results are shown in the lower autoradiograms.


Other Tissues
Anthers
Pollen
root
leaf
seedling
pistil
stage 1
stage 2
stage 3
stage 4
$S_{2.2}$ $Z_{2.2}$ TT
$S_{2.2}$ $Z_{1.1}$ TT
$S_{2.2}$ $Z_{1.2}$ T
$S_{1.2}$ $Z_{2.2}$
$S_{2.2}$ $Z_{2.2}$
$S_{1.1}$ $Z_{2.2}$
$S_{2.2}$ $Z_{2.2}$
$S_{1.2}$ $Z_{2.2}$
$S_{2.2}$ $Z_{1.2}$
$S_{1.2}$ $Z_{1.1}$
$S_{1.4}$ $Z_{1.3}$
$S_{1.2}$ $Z_{3.3}$
0.87 -
0.56 -
(kb)
- 0.87
- 0.56
- 0.36
(kb)
18S rRNA

Striking was the complete absence of B7b transcripts in pollen from the plants 23A-2x37A-9-6 (genotype: $S_{2.2}{}^{\circ}$ $Z_{1.2}$ T) and 42-4-8 (genotype: $S_{1}{}^{\circ}{}_{.1}{}^{\circ}$ $Z_{2.2}$) (Fig. 3.11), while the transcript (ca. 570 b) was clearly detectable in pollen from other plants tested. B7b also hybridised, although weaker, to a smaller mRNA of approx. 360 b (pollen and anther stage 4).
Further analysis of the clone and its characteristics is presented in Chapter 4.

3.2.6.2. Sequence Analysis

In order to gain insight into the possible function of the cDNAs, the nucleotide sequences of the clones were compared to those of known genes in nucleic acid and protein sequence databases (such as Genbank, EMBL, PIR, Swiss-Prot). Out of the 18 cDNA clones six showed significant similarity to sequences in the databases and will be discussed in more detail below. Sequences which did not reveal any sequence homology to known sequences are listed in Appendix B.

Sequence analysis of the entire cDNA clone A12a revealed a putative although incomplete open reading frame, which translated into an amino acid sequence showing all the properties of a protein kinase (Fig. 3.12). Database searches using the FASTA (Pearson and Lipman 1988) and BLAST (Altschul et al. 1990) programs showed that A12a was homologous to serine/threonine protein kinases as can be seen from the alignment of the protein sequence of A12a with those of other protein kinases (Fig. 3.13). Significant homology was also found at the DNA level.

Sequence analysis of C7c showed a complete open reading frame of 570 bp (Fig 3.14). Database searches indicated that C7c was homologous to pollen-specific genes from rice, maize and tomato (Fig. 3.15) and showed 65% sequence identity with *PS1* from rice and 61% identity to *ZmC13* from maize.

The sequences of C11e, Bm7d, A16e, and B4e were only partially determined by sequencing both ends of the cDNAs, which had been subcloned in pTZ19U, using M13 forward and reverse primers. While the sequences obtained from the M13 forward side did not show any homology to known sequences at both the protein or the DNA level, sequences obtained from the M13 reverse side were highly homologous to known sequences in the databases.
Database searches with the DNA sequence of C11e (Fig. 3.16) indicated that this clone might represent a sugar-transport protein. An alignment of the deduced protein sequence of C11e with glucose transport proteins from is presented in Fig. 3.17. Interestingly, C11e showed the highest degree of homology to glucose-transport proteins of *C.elegans* and yeast with 26% sequence identity in the overlapping region.
Bm7d might represent a Mitogen-Activated Protein (MAP) kinase (Fig.3.18). A comparison of the deduced protein sequence with other MAP-kinases shows that the

clone displays significant homology over a region of 6 protein kinase domains (Fig. 3.19) with conserved amino acids being present.

A16e showed homology to fructose-1,6-biphosphate aldolases identified in plants (Fig. 3.20 and Fig. 3.21). The partial amino acid sequence was 93% identical with fructose-1,6-biphosphate aldolase from maize and 95% with the adolase from rice.

Database searches indicated that B4e was homologous to calcium-dependent protein kinases from rice, maize and *Arabidopsis* (Fig 3.22 and Fig. 3.23). In the overlapping region the amino acid sequence of B4e showed 75% sequence identity with the pollen-specific calcium-dependent calmodulin-independent protein kinase from maize, 65% sequence identity with CDPK6 from *Arabidopsis* and 63% with OSCPK2 from rice.

```
   1 ATCGCCGACCTCGTCAACAACATCTCGGCCAAGTCGGACGTCACCAGGCACGTGTACGCG  60
     I  A  D  L  V  N  N  I  S  A  K  S  D  V  T  R  H  V  Y  A   -

  61 GCGGAGGAGATCCTCCGGATCACCAGCCAGAACATCCCCAGCCGGGTGCTCACGTTCAGG 120
     A  E  E  I  L  R  I  T  S  Q  N  I  P  S  R  V  L  T  F  R   -

                                                        domain I
 121 GAGCTGTCCGACGCCACCGACATGTTCAGCTGCAGCAACCTCGTCGGCGAAGGCGGCTTC 180
     E  L  S  D  A  T  D  M  F  S  C  S  N  L  V  G  E  G  G  F   -

                                                       domain II
 181 GGGATGGTGTACAGGGGGTACCTCAAGAAAACCGAGGAGGCGATCGCCGTCAAGAAGTTC 240
     G  M  V  Y  R  G  Y  L  K  K  T  E  E  A  I  A  V  K  K  F   -

                                                  domain III
 241 GACAAGGAGGGGTTCCAGGGGAACCGCGAGTTCCTGGTGGAGGTGCTGATGCTCAGCCTC 300
     D  K  E  G  F  Q  G  N  R  E  F  L  V  E  V  L  M  L  S  L   -

                                 domain IV
 301 CTGCGCCACCCCAACCTGGTCAAGCTAATCGGGTACAGCACCGACCTCGACCAGCGGATG 360
     L  R  H  P  N  L  V  K  L  I  G  Y  S  T  D  L  D  Q  R  M   -

                                            domain V
 361 CTCGTCTACGAGTACATGCACAACGGCGCGCTGGAGGATCATCTCCTAGATCTCCCGTCG 420
     L  V  Y  E  Y  M  H  N  G  A  L  E  D  H  L  L  D  L  P  S   -

                                       domain VIa
 421 AACGGCAATTGCGCTTCCCTGGCAGACACGGGATTGAAGATCGCGGTGGGCCGCGGCCAA 480
     N  G  N  C  A  S  L  A  D  T  G  L  K  I  A  V  G  R  G  Q   -

                                                      domain VIb
 481 GGGGATCCAGTACCTTGCCACGAGGTGGCCAACCCGCCGGTCATCTACAGGGACCTCAAG 540
     G  D  P  V  P  C  H  E  V  A  N  P  P  V  I  Y  R  D  L  K   -

                                                 domain VII
 541 GCCTCCAACATCCTCCTGGACAAGGACTTCAACTGCAAGCTCTCCGACTTCGGGCTCGCT 600
     A  S  N  I  L  L  D  K  D  F  N  C  K  L  S  D  F  G  L  A   -

 601 AAGCACGGCCCCGTCGGCGACCAGAGCCACGTCAGCACCAGGGTCATGGGAACCTATGGC 660
     K  H  G  P  V  G  D  Q  S  H  V  S  T  R  V  M  G  T  Y  G   -

                                 domain VIII                domain IX
 661 TACTGCGCCCCCGAGTACGCCATGACCGGCAAGCTCACCAAGATGTCCGACATATACAGC 720
     Y  C  A  P  E  Y  A  M  T  G  K  L  T  K  M  S  D  I  Y  S   -

 721 TTCGGCGTCGTGCTCCTGGAGCTCATCACCGGCAGGCGAGCCATCGACACCTCAAGGCCA 780
     F  G  V  V  L  L  E  L  I  T  G  R  R  A  I  D  T  S  R  P   -

                                                 domain IX
 781 TCGGAGGAGCAGGTTCTTATCCACTGGGCGGCGCCTCTTATTCAAAGACAGGAAAAGGTT 840
     S  E  E  Q  V  L  I  H  W  A  A  P  L  I  Q  R  Q  E  K  V   -

 841 CGTGAGGATGGCGGATCCGCTGATGGAGAAGAAGTTCCCGTTGAAGGGGTTGTACCAGGC 900
     R  E  D  G  G  S  A  D  G  E  E  V  P  V  E  G  V  V  P  G   -
```

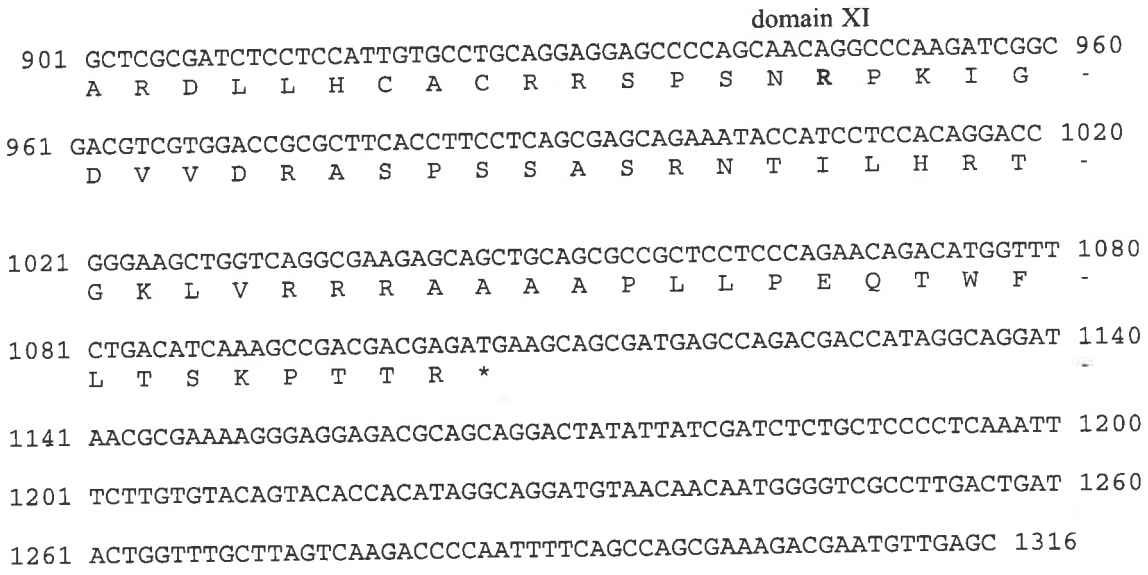


Fig. 3.12 DNA and deduced protein sequence of clone A12a. The 11 protein kinase domains as defined by Hanks et al. (1988) are indicated. Amino acids that are absolutely conserved in protein kinases are shown in boldface letters.

```
      1                                                  50
A12a  .......... .......... ....IADLVN NISAKSDVTR HVYAAEEILR
 Fen  .......... .......... .......MGS KYSKATNSIN DASNLSYGVP
 Pto  .......... .......... .......MGS KYSKATNSIN DALSSSYLVP
APK1  MGICLSAQVK AESSGASTKY DAKDIGSLGS KASS.VSVRP SPRTEGEILQ
 NAK  MGGCFSNRIK TD.IASSTWL SSKFLSRDGS KGSSTASFSY MPRTEGEILQ

      51                                                 100
A12a  ITSQNIPSRV LTFRELSDAT DMFSCSNLVGEGGFGMVYRGYLKK.......
 Fen  FENY....R. VPFVDLEEAT NNFDDNFFIGEGGFGKVYRGVLRD.......
 Pto  FESY....R. VPLVDLEEAT NNFDHKFLIGHGVFGKVYKGVLRD.......
APK1  SPNL....KS FSFAELKSAT RNFRPDSVLGEGGFGCVFKGWIDE KSLTAS
 NAK  NANL....KN FSLSELKSAT RNFRPDSVVGEGGFGCVFKGWIDE SSLAPS
                           ---------- I ----------

      101                                                150
A12a  ...TEEAIAVKKFDKEGF QGNREFLVEVLMLSLL RHPNLVKLIGYSTD LD
 Fen  ....GTKVALKKHKPESS QGIEEFETEIEILSFC SHPHLVSLIGFCDE RN
 Pto  ....GAKVALKRRTPESS QGIEEFETEIETLSFC RHPHLVSLIGFCDE RN
APK1  RPGTGLVIAVKKLNQDGW QGHQEWLAEVNYLGQF SHRHLVKLIGYCLE DE
 NAK  KPGTGIVIAVKRLNQEGF QGHREWLAEINYLGQL DHPNLVKLIGYCLE EE
        ------II------- ------III------- ------IV------ --

      151                                                200
A12a  QRMLVYEYMHNGALEDHLLDLP SNGNCASLADTGLKIAVGRGQGDPVPCH
 Fen  EMILIYDYMENGNLKSHLYGSD LP..SMSW EQRLEICIGAARG..LHYL
 Pto  EMILIYKYMENGNLKRHLYGSD LPTMSMSW EQRLEICIGAARG..LHYL
APK1  HRLLVYEFMPRGSLENHLFRRG LYFQPLSW KLRLKVALGAAKG..LAFL
 NAK  HRLLVYEFMTRGSLENHLFRRG TFYQPLSW NTRVRMALGAARG..LAFL
      ----------V---------- -----------------VIa--------

      201                                                250
A12a  EVANP PVIYRDLKASNILLDKDF NCKLSDFGLAKHGPV GDQSHVSTRVMG
 Fen  HKNA. .VIHRDVKCTNILLDENF VPKITDFGISKTMPE LDQTHLSTVVRG
 Pto  HTRA. .IIHRDVKSINILLDENF VPKITDFGISKKGTE LDQTHLSTVVKG
APK1  HSSET RVIYRDFKTSNILLDSEY NAKLSDFGLAKDGPI GDKSHVSTRVMG
 NAK  HNAQP QVIYRDFKASNILLDSNY NAKLSDFGLARDGPM GDNSHVSTRVMG
      ----- ------VIb--------- ------VII------ ------------

      251                                                300
A12a  TYGYCAPEYA MTGKLTKMSDIYSFGVVLLELITGRRAIDTS RPSEEQVLI
 Fen  NIGYIAPEYA LWGQLTEKSDVYSFGVVLFEVLCARPALDRS ...EIMSLD
 Pto  TLGYIDPEYF IKGRLTEKSDVYSFGVVLFEVLCARSAIVQS LPREMVNLA
APK1  THGYAAPEYL ATGHLTTKSDVYSFGVVLLELLSGRRAVDKN RPSGERNLV
 NAK  TQGYAAPEYL ATGHLSVKSDVYSFGVVLLELLSGRRAIDKN QPVVEHNLV
      -VIII----- --------------IX--------------- ---------

      301                                                350
A12a  HWAAPLIQRQEKVREDGGSADGEEVPVEG VVPGARDLLHCACRRSPSNRP
 Fen  D...E.TQKMGQLEQIVDPTIAAKIRPES LRMFGETAIKCLA.PSSKNRP
 Pto  EWAVE.SHNNGQLEQIVDPNLADKIRPES LRKFGDTAVKCLA.LSSEDRP
APK1  EWAKPYLVNKRKIFRVIDNRLQDQYSMEE ACKVATLSLRCLT.TEIKLRP
 NAK  DWARPYLTNKRRLLRVMDPRLQGQYSLTR ALKIAVLALDCIS.IDAKSRP
      ------------X---------------- --------------XI----
```

```
      351                                                        400
A12a  KIGDVVDRAS PSSASRNTIL HRTGKLVRRR AAAAPLLPEQ TWFLTSKPTT
 Fen  SMGDVLWKLE YALCLQEPTI QDDPE..... .......... ..........
 Pto  SMGDVLWKLE YALRLQESVI .......... .......... ..........
APK1  NMSEVVSHLE HIQSLNAAIG GNMDKTDRRM RRRSDSVVSK KVNAGFARQT
 NAK  TMNEIVKTME ELH.....IQ KEASKEQQNP QISIDNIINK SPQA......
      ----------

      401
A12a  R.....................................................
 Fen  .......... .......... .......... .......... ..........
 Pto  .......... .......... .......... .......... ..........
APK1  AVGSTVVAYP RPSASPLYV. .......... .......... ..........
 NAK  ......VNYP RPSIM..... .......... .......... ..........
```

Fig 3.13 Alignment of the deduced amino acid sequence of A12a with protein kinases. The alignment was made to maximize sequence similarity. The 11 subdomains, I to XI, described by Hanks et al. (1988) and Hanks and Quinn (1991), are indicated. The sources of the protein kinases are Fen of tomato (Martin et al. 1994), Pto of tomato (Martin et al. 1993), APK1 of *Arabidopsis* (Hirayama et al. 1992) and NAK of *Arabidopsis* (Moran et al. 1993). Amino acids conserved in all 5 proteins are in boldface letters.

```
   1 GGAGGTAGGGGAAAACACACACACCGCAGCCATGGCCTCGCTCCGCGCCCTGTCAGTGAT  60
                                     M  A  S  L  R  A  L  S  V  I  -

  61 CGCCGTCGCCGTCGTCCTCTTCGCCCTCGCCGACACAGCCGTCGCCACCAAGGCTCCTGA 120
     A  V  A  V  V  L  F  A  L  A  D  T  A  V  A  T  K  A  P  D  -

 121 CTACGTCGTCCAGGGCCGCGTCTACTGCGACACGTGCCGCGCAGGGTTCGAGACGAACGT 180
     Y  V  V  Q  G  R  V  Y  C  D  T  C  R  A  G  F  E  T  N  V  -

 181 CACCGAGTACATCAAGGGTGCCAAGGTCAGGCTGGAGTGCAGGCACTTCGGCACCAACGT 240
     T  E  Y  I  K  G  A  K  V  R  L  E  C  R  H  F  G  T  N  V  -

 241 CCTTGAGCGCGCGATCGACGGCGTGACCGACGAGACGGGCACCTACAAAATCGAGCTCAG 300
     L  E  R  A  I  D  G  V  T  D  E  T  G  T  Y  K  I  E  L  R  -

 301 GGACAGCCACGTGGAGGACATCTGCGAGGTCGTCCTCGTCAAAAGCCCCTCGCGGACTTG 360
     D  S  H  V  E  D  I  C  E  V  V  L  V  K  S  P  S  R  T  C  -

 361 CCATGAGATCCAGAGCCTCAGGGACCGTGCCCCTGTTCTGCTCACCAGGAACGTCGGCAT 420
     H  E  I  Q  S  L  R  D  R  A  P  V  L  L  T  R  N  V  G  I  -

 421 CAGCGACAACCTGCGCCTAGCCAACCCACTCGGCTACCTCAAGGATGTGCCGCTGCCCGT 480
     S  D  N  L  R  L  A  N  P  L  G  Y  L  K  D  V  P  L  P  V  -

 481 CTGCGGTGACCTGCTAAAGATGTTCAAGTTGGCTGATGATGATGATGATCAGTGGGGGAT 540
     C  G  D  L  L  K  M  F  K  L  A  D  D  D  D  D  Q  W  G  M  -

 541 GGTTGCCATGGCACCACCTGAGAGGCTGCACCGAGCATATATAAGTTACCTGACCTGCAA 600
     V  A  M  A  P  P  E  R  L  H  R  A  Y  I  S  Y  L  T  C  K  -

 601 ATAGCATGGTTGAGTACAGCATCCATGCGTAGCTGTCCCAA 641
     *
```

Fig. 3.14 DNA and deduced protein sequence of C7c.

```
          1                                                  50
  C7c   MASLRALSVI AVAVV..LFA LADTAVATKA ..PDYVVQGR VYCDTCRAGF
  PS1   MASLRTIPVI .FGIL..FYV LASTATATDA ..PDYVVQGR VYCDTCRAEF
ZmC13   MASVPAPATT TAAVILCLCV VLSCAAADDP NLPDYVIQGR VYCDTCRAGF
LAT52   ........MA KAIVLLSALC ILALANFAHC RPEVFDVEGK VYCDTCRVQF

          51                                                 100
  C7c   ETNVTEYIKG AKVRLECRHF GTNVLERAID GVTDETGTYK IELRDSHVED
  PS1   ETNVTEYIKG AKVRLECKHF GTDKVERAID GVTDETGTYK IELKDSHEED
ZmC13   VTNVTEYIAG AKVRLECKHF GTGKLERAID GVTDATGTYT IELKDSHEED
LAT52   ETKLSENLEG ATVKLQCRNI STEAETFSVE GVTDKDGKYK LTVNGDHEND

          101                                                150
  C7c   ICEVVLVKSP SRTCHEIQSL RDRAPVLLTR NVGISDNLRL ANPLGYLKDV
  PS1   ICEVVLVHSP LANCSEIEAE RDRARVLLTR NVGICDNLRL ANPLGYLKDY
ZmC13   ICQVVLVASP RKDCDEVQAL RDRAGVLLTR NVGISDSLRP ANPLGYFKDV
LAT52   ICEVTVVKSP REDCKESVSG YEKARIECSD NVGIHNAVRF ANPLFFMKAE

        151                                              194
  C7c   PLPVCGDLLK MFKLADDDDD QWGMVAMAPP ERLHRAYISY LTCK
  PS1   HCP.SAALLK QFDLADDDNE .......... .......... ....
ZmC13   PLPVCAALLK QLDSDDDDDQ .......... .......... ....
LAT52   SVQGCKEALD ELGLFPLEF. .......... .......... ....
```

Fig. 3.15 Alignment of the deduced protein sequence of C7c with pollen-specific clones from rice, maize and tomato. PS1, pollen-specific clone from rice (Zou et al. 1992); ZmC13, pollen-specific clone from maize (Hanson et al. 1989) and LAT52 from tomato (Twell et al. 1989). Gaps were introduced for better alignment. Amino acids conserved in all four proteins are in boldface letters.

```
  1 ATGCGTGGCGCCATCGCCTCGGTGTTCGCGTTCGTGCCTTCTTCTCCATCGGCTTGGGCC 60
    C  V  A  P  S  P  R  C  S  R  S  C  L  L  L  H  R  L  G  P  -

 61 CATCACAGGTGCCTACAGCTCCGAGGTCATCCCGCTCCGCTGCGCGCGCAGGGCGTCGCG 120
    I  T  G  A  Y  S  S  E  V  I  P  L  R  C  A  R  R  A  S  R  -

121 TCGCGTGGCTTTCAACCGCGTCGCCAATGCCACCGTTGCCCTGACCTTCATTTCCCTGTC 180
    R  V  A  F  N  R  V  A  N  A  T  V  A  L  T  F  I  S  L  S  -

181 CAACGCGATCACCATGGGCGGCGCCTTCTTCCTCTTCGGATTCCTGTCCGTGGGAGCGGC 240
    N  A  I  T  M  G  G  A  F  F  L  F  G  F  L  S  V  G  A  A  -

241 CACATTCTTCTACTTCTTCTGTCCGGAAACTCAGGGCCGGCCGCTGGAGGAGATCGAGGA 300
    T  F  F  Y  F  F  C  P  E  T  Q  G  R  P  L  E  E  I  E  E  -

301 GGTGTTCAGACAGGGGTGGCGCCAGAGACGACACAATGTACACACTCCTTTCACGGCATT 360
    V  F  R  Q  G  W  R  Q  R  R  H  N  V  H  T  P  F  T  A  F  -

361 CCAAATGAGG 370
    Q  M  R
```

Fig 3.16 Partial DNA and deduced protein sequence of clone C11e.

```
          1                                                    50
   C11e   .......... .......... .......... .......... ...CVAPSPR
M01F1.5   SATGICEFSN LTNNGTDFEW EDTYCHTKF. ...TVLPIII MVFYLLSFSA
D9509.7   ...AILLRTI LIMTVGLLLC SVGFGHDQV. ...NLL.LIS VVIYVAAYAS
   STP1   GGTQMLICQA VVAACIGAKF GVDGTPGELP KWYAIVVVTF ICIYVAGFAW

          51                                                   100
   C11e   CSRSCLLLHR LGPITGAYSS EVIPLRCARR A.SRRVAFNR VANATVALTF
M01F1.5   G......... YAPLPWVLNA EFYPLWARST AVSVSTACNW IFNLIVSLTF
D9509.7   A......... MGSVPWT.CV EFLPLNRRSF GASCIACTNW LTNAFVSMTY
   STP1   S......... WGPLGWLVPS EIFPLEIRSA AQSITVSVNM IFTFIIAQIF

          101                                                  150
   C11e   ISLSNAITMG GAFFLFGFLS VGAATFFYFF CPETQGRPLE EIEEVFRQGW
M01F1.5   LSLSQAATKY GTFFIYCGCT MVALVFVFFF VPETKGYSID EVEMLFMTK.
D9509.7   LSTINTIGDE NTMLIFAFFT VCAWFFVYFW YPEVKGLSLE EVGRVFDNG.
   STP1   LTMLCHL.KF GLFLVFAFFV VVMSIFVYIF LPETKGIPIE EMGQVWRSHW

          151                                  185
   C11e   .RQRRHNVHT PFTAFQMR.. .......... .....
M01F1.5   .....EERRK AQKVLDESKE GKHRNSVAMS FDTKF
D9509.7   .....IDVHY VFRTYH.... .......... .....
   STP1   YWSRFVEDGE YGNALEMGKN SNQAGTKHV. .....
```

Fig 3.17 Alignment of the partially determined and deduced protein sequence of C11e with glucose-transport proteins M01F1.5 of *C. elegans* (Wilson et al. 1994), D9509.7 of yeast (Dietrich et al. 1995, unpublished, accession no U32274) and STP1 of *Arabidopsis* (Sauer et al. 1990). Protein sequences have been truncated and gaps introduced for better alignment. Amino acids conserved in at least 3 proteins are given in bold type.

```
  1 GCTACATGATATCTTTGAACACATATCTGATGCGGCACGGATTCTCCGTGAGATCAAGCT 60
    L  H  D  I  F  E  H  I  S  D  A  A  R  I  L  R  E  I  K  L  -

 61 TCTGAGGCTCTTGAGACATCCTGACATTGTTGAGATAAAGCATATCATGCTACCTCCATC 120
    L  R  L  L  R  H  P  D  I  V  E  I  K  H  I  M  L  P  P  S  -

121 GAGAAGGGACTTCAAAGATATTTATGTTGTTTTTGAGCTCATGGAGTCTGATCTCCACCA 180
    R  R  D  F  K  D  I  Y  V  V  F  E  L  M  E  S  D  L  H  Q  -

181 AGTTATAAAGGCTAATGATGACTTGACGAAGGAGCATTACCAGTTCTTTCTTTACCAGTT 240
    V  I  K  A  N  D  D  L  T  K  E  H  Y  Q  F  F  L  Y  Q  L  -

241 AGTCCGGGCTCTCAAGTACATTCACACTGCTAATGTTTATCACCGGGACCTAAAGCCAAA 300
    V  R  A  L  K  Y  I  H  T  A  N  V  Y  H  R  D  L  K  P  K  -

301 GAATATATTGGCAAATTCTAACTGCAAATTGAAAATTTGTGATTTTGGATTGGCACGAGT 360
    N  I  L  A  N  S  N  C  K  L  K  I  C  D  F  G  L  A  R  V  -

361 TGCATTCAACGATACCCCGACGACAATCTTTTGGACGGATTATGTTGCACCAAGATGGTA 240
    A  F  N  D  T  P  T  T  I  F  W  T  D  Y  V  A  P  R  W  Y  -

421 TAGATCTCCAGAGCTCTGTGGGCCTCTTTCAAGGATACACCAGCTATTGTTGTTGGGCAC 480
    R  S  P  E  L  C  G  P  L  S  R  I  H  Q  L  L  L  L  G  T  -

481 GGGTGCACTTGCTGAAGTTTCAGGGGCCTTGTTCCGGAAAA 521
    G  A  L  A  E  V  S  G  A  L  F  R  K
```

Fig. 3.18 Partial DNA and deduced protein sequence of clone Bm7d.

```
                      II                          III                    IV
Bm7d    .......... ..LHDIFEHI SDAARILREI KLLRLLRHPD IVEIKHIMLP 100
ATMPK2  NRESNERVAI KKIHNVFENR IDALRTLREL KLLRHLRHEN VVALKDVMMA
NTF3    NRETNEKVAI KKINNAFENR IDALRTLREL KLLRHLRHEN VIALKDVMMP
ATMPK7  NRETNERVAI KKIHNVFENR VDALRTLREL KLLRHVRHEN VIALKDVMLP

                         V
Bm7d    PSRRDFKDIY VVFELMESDL HQVIKANDDL TKEHYQFFLY QLVRALKYIH 150
ATMPK2  NHKRSFKDVY LVYELMDTDL HQIIKSSQVL SNDHCQYFLF QLLRGLKYIH
NTF3    IHRRSFKDVY LVYELMDTDL HQIIKSSQTL SNDHCQYFLF QLLRGLKYLH
ATMPK7  ANRTSFKDVY LVYELMDTDL HQIIKSSQSL SDDHCKYFLF QLLRGLKYLH

                VI                          VII
Bm7d    TANVYHRDLK PKNILANSNC KLKICDFGLA RVAFNDTPTT IFWTDYVAPRWYR 203
ATMPK2  SANILHRDLK PGNLLVNANC DLKICDFGLA RTS.NTKGQ. .FMTEYVVTRWYR
NTF3    SANILHRDLK PGNLLINANC DLKICDFGLA RTS.SGKDQ. .FMTEYVVTRWYR
ATMPK7  SANILHRDLK PGNLLVNANC DLKICDFGLA RTS.QGNEQ. .FMTEYVVTRWYR

          VIII
Bm7d    SPEL...CGP LSRIHQLLLL GTGALAEVSG ..ALFRK... .......... 253
ATMPK2  APELLLCCDN YGTSIDVWSV G.CIFAELLG RKPVFPGTEC LNQIKLIINI
NTF3    APELLLCCDN YGTSIDVWSV G.CIFAELLG RKPVFPGTEC LNQLKLIINI
ATMPK7  APELLLCCDN YGTSIDVWSV G.CIFAEILG RKPIFPGTEC LNQLKLIINV
```

Fig 3.19 Alignment of the partially determined and deduced protein sequence of Bm7d with MAP-kinases from *Arabidopsis*, ATMPK2 (Mizoguchi et al. 1994) and ATMPK7 Mizoguchi et al. 1993) and tobacco, NTF3 (Wilson et al. 1993) The first 50 and the last 126 amino acids of the kinases have been omitted. Amino acids conserved among all 4 proteins are given in boldtype. Roman numbers indicate protein kinase domains.

```
  1 CAGAACGCTCAGGGTCTTGCTCGCTACGCCATCATCTGCCAGGAGAACGGTCTGGTGCCC 60
    Q  N  A  Q  G  L  A  R  Y  A  I  I  C  Q  E  N  G  L  V  P   -

 61 ATTGTTGAGCCTGAGATCCTTGTTGATGGACCTCATGACATTGACCGCTGCGCTTACGTC 120
    I  V  E  P  E  I  L  V  D  G  P  H  D  I  D  R  C  A  Y  V   -

121 ACCGAGGTGGTCCTTGCTGCCTGCTACAAGGTTCTCAATGACCAGCATGTTCTCCTCGAG 180
    T  E  V  V  L  A  A  C  Y  K  V  L  N  D  Q  H  V  L  L  E   -

181 GGTACCCTCCTGAAGCCCAACATGGTTACCCCTGGTTCTGATGCCAAGAAGGTTGCCCCT 240
    G  T  L  L  K  P  N  M  V  T  P  G  S  D  A  K  K  V  A  P   -

241 GAGGTCATTGCTGAGTACACCGTCCGTACCCTTCAGAGGACTGTTCCACCTGTCGTGCCT 300
    E  V  I  A  E  Y  T  V  R  T  L  Q  R  T  V  P  P  V  V  P   -

301 GCCATTGTCTTCCTCTCTGGTGGACAGAGCGAGGAGGAGTGTC 343
    A  I  V  F  L  S  G  G  Q  S  E  E  E  C    -
```

Fig 3.20 Partial DNA sequence of A16e and its deduced protein sequence

```
          151                                                  200
   A16e   .......... QNAQGLARYA IICQENGLVP IVEPEILVDG PHDIDRCAYV
  maize   PNEPSQLAID LNAQGLARYA IICQENGLVP IVEPEILVDG PHDIDRCAYV
   rice   PNEPSQLAIH LNAQGLARYA IICQENGLVP IVEPEILVDG PHDIDPCAYV
spinach   PTEPSPLAIL ENANGLARYG IICQENGLVP IVEPEILVDG THDIDRCAEV
    pea   PNEPSELSIQ QNAQGLARYA IICQENGLVL FVEPEILTDG SHDIAKCAAV

          201                                                  250
   A16e   TEVVLAACYK VLNDQHVLLE GTLLKPNMVT PGSDAKKVAP EVIAEYTVRT
  maize   TETVLAACYK ALNEHHVLLE GTLLKPNMVT PGSDSKKVTP EVIAEYTVRT
   rice   SEVVLAACYK ALNEHHVLLE GTLLKPNMVT PGSDAKKVSP EVIAEYTVRT
spinach   SERVLAACYK ALNDHHVLLE GTSLKPNMVT PGSESKKVTP EVIAEYTVRT
    pea   TETVLAACYK ALNDQHVLLE GTLLKPNMVT PGSDSPKVSP EVIGEYTVNA

          251                                                  300
   A16e   LQRTVPPVVP AIVFLSGGQS EEEC...... .......... ..........
  maize   LQRTVPAAVP AVLFLSGGQS EEEATRNLNA MNKLSTKKPW SLSFSFGRAL
   rice   LQRTVPAAVP AIVFLSGGQS KEEATRNLNA MNKLSTKKPW SLSFSFGRAL
spinach   LQRTVPQAVP GVMFLSGGQS EEEATLNLNA MNKLETKKPW TLSFSYGRAL
    pea   LRRTVPAAVP GIVFLSGGQS EEQATLNLNA MNKFDVVKPW TLSFSFGRAL
```

Fig 3.21 Alignment of the partially determined and deduced protein sequence of A16e with fructose-1,6-biphophate aldolases from maize (Dennis et al. 1988), rice (Hidaka et al. 1990), spinach (Pelzer-Reith et al. 1993) and pea (Pelzer-Reith et al. unpublished). The first 150 and the last 159 amino acids of the aldolases have been omitted. Amino acids conserved among all 5 proteins are given in boldtype.

```
  1 CCGCTGGACAACGCCGTCATGAACAGGCTGAAGCAGTTCAGGGCTATGAATCAGTTCAAG 60
    P  L  D  N  A  V  M  N  R  L  K  Q  F  R  A  M  N  Q  F  K   -

 61 AAAGCGGCGCTAAGGGTCATCGCCGGATGCCTGTCGGAGGAAGAGATCAGAGGGCTGAAG 120
    K  A  A  L  R  V  I  A  G  C  L  S  E  E  E  I  R  G  L  K   -

121 GAGATGTTCAAGAGCATGGACGCCGACAACAGCGGAACGATCACCGTGGACGAGCTCCGG 180
    E  M  F  K  S  M  D  A  D  N  S  G  T  I  T  V  D  E  L  R   -

181 CGAGGCCTGTCCAAGGAGGGGACCAAGCTGACGGAGGCCGAAGTGGAGCAGCTCATGGAA 240
    R  G  L  S  K  E  G  T  K  L  T  E  A  E  V  E  Q  L  M  E   -

241 GCCGCCGATGCGGACGGGAACGGCACGATCGACTACGAAGAGTTCATCACGGCGACGATG 300
    A  A  D  A  D  G  N  G  T  I  D  Y  E  E  F  I  T  A  T  M   -

301 CACATGAACAGGATGGACAGGGAGGAGCACTTACAACGCTTCAGTACTTTGACAAGGACA 360
    H  M  N  R  M  D  R  E  E  H  L  Q  R  F  S  T  L  T  R  T   -

361 ACAGCGGT 368
    T  A
```

Fig. 3.22 Partial DNA-sequence and putative deduced protein sequence of B4e.

```
          351                                                       400
   B4e   ..PLDNAVMN RLKQFRAMNQ FKKAALRVIA GCLSEEEIRG LKEMFKSMDA
OSCPK2   DKPIDSAVLS RMKQFRAMNK LKKMALKVIA SNLNEEEIKG LKQMFTNMDT
  CDPK   DTPLDNVVLD RLKQFRAMNQ FKKAALRIIA GCLSEEEITG LKEMFKNIDK
 CDPK6   DKPLDNAVLS RMKQFRAMNK LKKMALKVIA ENLSEEEIIG LKEMFKSLDT

          401                                                       450
   B4e   DNSGTITVDE LRRGLSKEGT KLTEAEVEQL MEAADADGNG TIDYEEFITA
OSCPK2   DNSGTITYEE LKAGLAKLGS KLSEAEVKQL MEAADVDGNG SIDYVEFITA
  CDPK   DNSGTITLDE LKHGLAKHGP KLSDSEMEKL MEAADADGNG LIDYDEFVTA
 CDPK6   DNNGIVTLEE LRTGLPKLGS KISEAEIRQL MEAADMDGDG SIDYLEFISA

        451                                                     500
   B4e   TMHMNRMDRE EHL.QRFSTL TRTTA..... .......... ..........
OSCPK2   TMHRHKLERD EHLFKAFQYF DKDNSGFITR DELESALIEH EMGDTSTIKD
  CDPK   TVHMNKLDRE EHLYTAFQYF DKDNSGYITK EELEHALKEQ GLYDADKIKD
 CDPK6   TMHMNRIERE DHLYTAFQFF DNDNSGYITM EELELAMKKY NMGDDKSIKE

          501                                                 542
   B4e   .......... .......... .......... .......... ..
OSCPK2   IISEVDTDND GRINYEEFCA MMRGGGMQQP MRLK...... ..
  CDPK   IISDADSDND GRIDYSEFVA MMRKGTAGAE PMNIKKRRDI VL
 CDPK6   IIAEVDTDRD GKINYEEFVA MMKKGNPELV PNRRRM.... ..
```

Fig. 3.23 Alignment of partial protein sequence of B4e with calcium-dependent protein kinases. The first 350 amino acids of the other protein kinases have been omitted. The sources of the kinases are OSCPK2 of rice (Breviario et al. 1995), CDPK of maize (Estruch et al. 1994) CDPK6 of *Arabidopsis* (Hong et al. 1995, unpublished; accession number: U20623)

Table IX : Summary of the results of the transcriptional and sequence analyses

cDNA clone	predominant expression	No of mRNAs in pollen	Sequence homology
B7b	anther 4, pollen	2	none
A12a	pollen	1	protein kinases
C41b	anther 4, pollen	1	none
C38d	pollen	1	none
C17a	anther 4, pollen	1	none
C11e	anther 4, pollen	1	glucose transport proteins
Bm7d	anthers, pollen	3	MAP-kinases
C7c	pollen	1	ZmC13, LAT52
Bm7e	pollen	1	none
A12e	pollen	1	none
A16e	ubiquitous	2	aldolases
A36d	anther 4, pollen	1	none
A34b	pollen	1	none
B4e	pollen	1	Ca^{2+}-dep. protein kinases
B5c	pollen	1	none
Cm5e	root, leaf, seedling, stigma, pollen	1	none
C7b	root, pollen	1	none
C24e	pollen	1	none

3.3 Discussion

In the study reported in this chapter, the isolation and initial characterisation of pollen-specific cDNAs was undertaken, and their linkage to the Z-locus examined.

The starting point for this analysis was the subset of clones previously isolated from a pollen cDNA library by Li et al. (1994). The differential screen designed by these authors aimed at the identification of putative S- and Z-genes, based on the assumption that different S- and Z alleles can be detected due to variability at the DNA level. From a total of 117 cDNA clones isolated, 42 showed differences in hybridisation signal intensity compared to the control when probed with pollen RNA from the S-complete mutant. 76 clones were isolated as putative Z-candidates (J. Nield, pers. comm. 1995). One clone showed reduced hybridisation with both the S-complete mutant and *85-2-5*, the plants used for the screen for putative Z-candidates. Since nothing was known about how S and Z interact, all 117 clones in this study, as it was possible that a mutant S-gene might influence Z-expression as well as the expression of other genes involved in the SI reaction.

In order to analyse these clones further, it was essential to deal with individual phages. This was achieved by selecting individual, well separated plaques from each original clone for use in subsequent experiments.

The first screen carried out aimed at the identification of pollen-specific clones. "Pollen-specificity" in this context meant lack of expression in vegetative tissue and anthers at the pre-meiotic and meiotic stage. Since the self-incompatibility genes

expressed in the pollen might be identical to those expressed in the pistil, RNA from pistils was deliberately excluded from this screen.

It was observed that more than 50% of the clones were only expressed in pollen. This figure seemed high, when compared to data found in the literature. Stinson et al. (1987) estimated, based on colony hybridisation experiments, that 10% (for maize) and 20% (for *Tradescantia*) of pollen-expressed sequences were pollen-specific. As the clones analysed here represented only a fraction of pollen-expressed sequences and only partial information was available on how many of these clones were unique, the high number of pollen-specific genes might be coincidental.

The second differential screen aimed at the identification of pollen-specific genes which might be putative Z-candidates and genes which might be indirectly involved in the SI reaction.

The 139 clones which resulted from this screen were classified into 20 groups based on their behaviour in the cross-hybridisation experiment. With the exception of group 1 (with 99 members), the number of members per group was small, ranging from 1 to 7. These 20 groups represented the minimum possible number of independent cDNAs present in the original subset of clones selected. Although the possibility remains that related cDNAs derived from different transcription units were grouped together, it is unlikely that the actual number of independent clones is much higher for two reasons. Firstly, several of the grouped clones were derived from the same original phage and contained an insert of the same size. Secondly, random sequencing of cDNAs belonging to the same group failed to show significant differences in the DNA sequences.

The clones containing the longest insert were selected as representatives of each group and used in further analysis. Two groups were not analysed further because their inserts were too short to produce meaningful results.

The search for Z

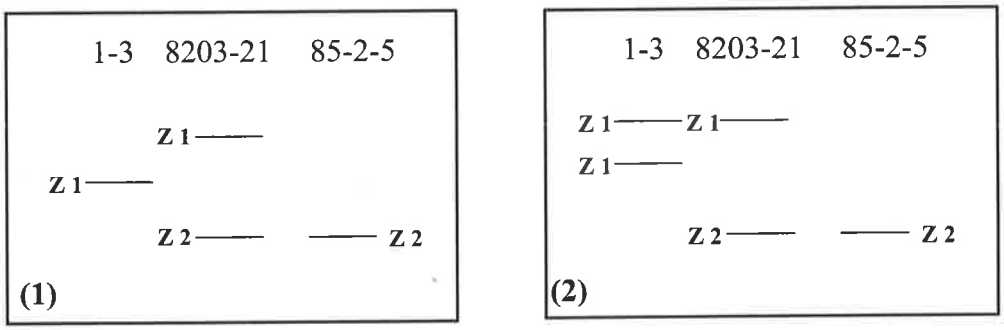
Restriction fragment length polymorphism (RFLP) analysis was chosen as the method for testing whether any of the 18 representative cDNA clones represented the Z-gene. In contrast to "conventional" usage of restriction length polymorphisms as markers to map genes of interest, in this study RFLP analysis was used to target and identify the Z-gene directly. The assumption made was that the sequence variability of different Z-alleles would be reflected by the presence or absence of restriction sites. A cDNA encoding the Z-gene could therefore be identified by a polymorphic pattern which correlated with the Z-genotype of the plants analysed. To this end, advantage was taken of a large population of genetically characterised plants (Hayman and Richter 1992), hereafter called the Adelaide population . The same approach led to the successful isolation of the S-gene in *Phalaris* (Li et al. 1994).

All 18 clones analysed displayed a polymorphic pattern with at least one (and on average 3) of the restriction endonucleases used. This result underlines the efficiency of SI in ensuring a high degree of polymorphism within populations, which is characteristic of strongly outcrossing species. However, the RFLP analyses showed that none of the clones identified co-segregated with the Z-genotype. This implies that Z was not represented in the clones studied.

Several conceivable scenarios could have made it complicated, if not impossible, to identify linkage to the Z-locus. Three of these possibilities will be discussed, since they were taken into consideration when the RFLP results were interpreted. Before describing the scenarios it is necessary to provide more information about the plants used in this study.

Two of the three plants used in the initial round of analysis, plants *1-3* (genotype $S_{1.2}\,Z_{1.1}$) and *8203-21* (genotype $S_{1.2}\,Z_{1.2}$), represented two of the ancestor plants of the Adelaide population (D. Hayman, pers. comm., 1993). The third plant used, *85-2-5* (genotype $S_{1.2}\,Z_{2.2}$), was derived from intercrossing two F1 plants which resulted from crossing *1-3* with *8203-21*. All Z_2 alleles in the Adelaide population were derived from *8203-21*.

Firstly, there may be differences between the Z_1 allele of plant *8203-21* and the Z_1 alleles of plant *1-3* at the sequence level. For instance, a restriction site could be absent or present in an intron or in a non-coding region flanking the gene. In this case one would observe different bands in *8203-21* in comparison to *1-3*, as shown in Diagram (1) below, which depicts the simplest possible pattern.

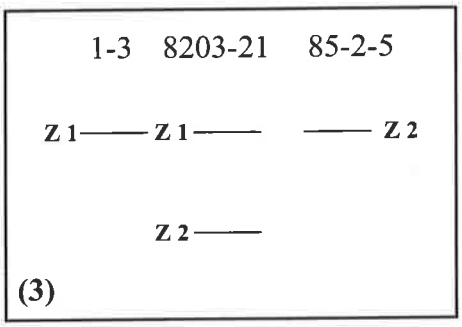


The banding pattern indicated in Diagram (1), which would provide evidence for this hypothesis, was not observed with any of the clones analysed.

Secondly, the two Z_1-alleles present in plant *1-3* may differ at the molecular level. In this case, a banding pattern similar to Diagram (2) would be expected. In the few instances where a pattern consistent with this hypothesis was observed, further analysis of the cDNA clone indicated that the clone was not linked to Z.

Thirdly, a crossing-over event may have occurred when the Adelaide population was generated, which caused the offspring to show a banding pattern inconsistent with their

Z-phenotype. If such an event took place in the gametes of *8203-21,* when the plant was crossed to *1-3* one would expect to see a banding similar to that depicted in Diagram (3).



It should be stated that the likelihood for such an event is very small, but it cannot be excluded *per se* and especially not for the self-fertile mutants in the Adelaide population, since they are derived from a large population of clonal offspring of *8203-21* (Hayman and Richter 1992). On the other hand, one would not expect to find more than one mutant plant showing a banding pattern inconsistent with its SI genotype. The possibility of a cross-over event in mutants was considered when the results of the hybridisation experiments were interpreted.

However, if such a cross-over event took place when the Adelaide population was generated, the identification of the Z-gene by using RFLP would be impossible, and unfortunately such an cross-over could only be traced once the Z-gene has been identified.

In conclusion, even by taking the scenarios discussed above into account, none of the clones analysed showed any linkage with the Z-gene. That 4 clones showed indication of linkage to the Z-gene in the initial RFLP analysis can be explained on the basis of the relatedness of the plants used and the segregation of the different alleles detected.

This prompts the question of whether the failure to isolate the Z-cDNA might be due to one or more of the initial assumptions being incorrect or to the intrinsic lack of sensitivity of the methods employed. The assumptions used in this work postulated that the Z-gene, in order to be detected in the designed screen, must be pollen-specific, be present in relatively high abundance, be a single or low copy gene and that differences in the mRNA-sequence of different alleles could be readily observed.

Molecular analysis of the S-gene in the *Solanaceae* had shown that the gene is of single copy and that sequence identity between alleles ranged from 41% to 80% (Ioerger et al. 1990). Exceptionally high sequence identity of 95% was only found between the S_{11} and S_{13} alleles of *Solanum chacoense* (Saba-el-Leil et al. 1994). The S-gene of *Papaver* was a single copy gene and different S-alleles did not cross-hybridise (V. Franklin-Tong, pers. comm. 1993). In both systems, the expression of the S-gene was

found to be predominantly or exclusively restricted to the pistil of the plants (see Chapter 1).

In contrast, as was discovered during the course of the sequence analysis, the sequences of the S-alleles of *Phalaris* differed only marginally. S_1 and S_2 shared 92 % identical amino acids. The coding sequence of S_2 differed from S_1 by having 4 deletions, 1 insertion, 3 base exchanges which resulted in amino acid changes, and 2 conservative base exchanges. With the exception of the two conservative base exchanges, all differences were located at the 5' end of the sequence (second exon) (Li et al. 1994).

According to Rivers et al. (1993), it has been estimated that sequence divergence of 10 to 40% can be detected by filter hybridisation (hybridisation conditions: 68°C, 0.5 to 0.05 M Na^+), assuming that the melting temperature decreases by 1°C for every 1% mismatch of sequence (Beltz et al. 1983). It is important to take into consideration that the distribution of the mismatched bases within a sequence is critical. Clustered mismatched bases will not reduce the melting temperature dramatically since the other regions of the sequence are identical, whereas dispersed mismatches might prevent hybridisation altogether (Beltz et al. 1983).

As this also applies in the case of the differential screening used for the S-gene, it is obvious that the isolation of the gene was fortuitous and not the result of the screen designed by Li et al (1994). When the sequence divergence at the mRNA level was compared, the two S-alleles differed by only 1.18% and, as mentioned above, the majority of sequence variations were clustered.

If the Z-gene shows similar features to S, i.e. the allelic differences are below 10% and/or clustered, any attempt to isolate the Z-gene by differential screening cannot be successful.

It is conceivable that the Z-cDNA was not represented in the 117 cDNA isolated by Li and coworkers, and consequently was missing from the clones analysed in this study. On the other hand, even if the Z-cDNA was present among the clones examined, it might have been missed due to the limitations of the method. These two possibilities provide the most plausible explanation for the failure to isolate the Z-cDNA. One could argue that Z-gene expression is not restricted to pollen, but there is currently no evidence to support this argument. Alternatively, Z might only be expressed in response to pollen-stigma interaction. However, since cessation of pollen-tube growth in an incompatible reaction takes place within minutes after the pollen attaches to the stigma, this alternative seems unlikely. In addition, it is hard to conceive that the expression of the two SI genes of *Phalaris* is regulated in different ways. Finally, it is possible that the attempt to isolate the Z-gene has failed due to low abundance of the transcript in pollen. For example, if S and Z interacted in a stoichiometric fashion and formed a heterodimer, one would expect that Z-gene expression would be similar to that of S. On the other hand, if S and Z acted independently and it was the sum of their actions which led to pollen tube arrest in an incompatible reaction, Z-expression might be low provided that the signal was amplified

downstream. Currently, we have no knowledge on the interaction of S and Z and therefore no evidence for either hypothesis. Future approaches to identify the Z-gene will have to take account of the possibility that the Z-transcript may be of low abundance.

Characterisation of pollen-expressed genes

Recognition between pollen and stigma is the first step in compatible and incompatible interactions and is followed by a cascade of events which ultimately leads to fertilisation or cessation of pollen tube growth. At present, nothing is known about the genes which act downstream of the recognition event in *Phalaris* pollen. In order to gain insight into the genes expressed in *Phalaris* pollen, their possible function and putative indirect involvement in the SI reaction, the 18 cDNA clones isolated were studied further as follows.

Transcriptional analysis of the cDNAs was carried out to: 1) obtain detailed information of the spatial and temporal expression of the corresponding genes, and 2) analyse whether there was any correlation between the relative expression of the genes in pollen and the SI genotypes of the pollen parents. To this end, pollen RNA from wildtype and self-fertile mutant plants was used in the Northern blot analysis.

The analysis revealed that the majority of the cDNAs represented genes that were expressed only in pollen and anthers of stage 4, which corresponded to the bicellular/tricellular stage of pollen development. This result was consistent with the results from the screen for pollen-specificity. Note that when a comparison was made between the expression levels of the transcript detected in pollen from different plants, all transcripts showed variation in their relative abundances in pollen from different genotypes. The variations observed were in some cases dramatic, as with C41b and B7b, and in other cases less pronounced, as for C11e. Further study is required to determine whether the quantitative differences in abundance are due to allelic variation or a consequence of gene regulation in response to environmental factors. A correlation with the SI-genotype was not observed.

Sequence analysis provided evidence for a possible functional role of six of the 18 cDNA clones. The finding that three of the cDNA clones isolated showed significant sequence homology to protein kinases was unexpected and led to investigation of the involvement of protein kinases in pollen germination and pollen-stigma interaction (Chapter 5).

Bm7d, whose deduced protein sequence is homologous to MAP-kinases, hybridised to 3 transcripts in pollen, the largest one being approx. 2.7 kb in size. The same three transcripts were detected in anther stage 4, whereas in younger anthers only the 2.7 kb transcript was clearly detectable. Southern blot analysis indicated the presence of at least two copies of the gene in *Phalaris*. MAP-kinases isolated from other plants have been shown to be members of gene families. Their protein sequences ranged from 361 to 395

amino acids, and the corresponding transcripts detected varied from 1.5 to 1.8 kb in length (Duerr et al. 1993, Jonak et al. 1993, Wilson et al. 1993, Strafstrom et al. 1993, Mizoguchi et al. 1994, Decroocq-Ferrant et al. 1995). In only one of the studies was an additional transcript of 3.3 kb reported (Strafstrom et al. 1993). This raises the question of the nature of the three transcripts in *Phalaris*. Further analysis is needed to determine whether all three transcripts derive from the same locus and are generated by alternative splicing, or whether they derive from different loci and represent other MAP-kinase like genes. The question of the involvement of protein kinases in pollen function will be discussed in Chapter 5.

C7c is likely to represent a homologue of *Zm13*, *PS* 1, *LAT52* and related genes (Table III, Chapter 1). The *LAT52* gene was shown to be necessary for pollen germination and pollen tube growth in tomato (Muschietti et al. 1994). Similar to *PS*1 of rice (Zou et al. 1994) and *Zm13* of maize (Stinson et al. 1987), the C7c gene of *Phalaris* is single or low copy, as indicated by Southern blot analysis. As expected from the close phylogenetic relatedness, C7c showed higher homology to the genes in the grasses than to *LAT52* of tomato. The deduced protein sequence, however, was 24 amino acids longer at the C-terminus than *PS*1. It is tempting to speculate about an indirect involvement of C7c in the incompatibility reaction, since the phenotypical behaviour of growing pollen tubes of transformed pollen described in the study by Muschietti et al. (1994) was reminiscent of pollen tube behaviour in incompatible reactions. Whether this is the case can only be answered once the molecules involved in the self-incompatibility reaction have been fully identified.

The partial sequence of A16e was highly homologous to cytoplasmic fructose-1,6-biphosphate aldolases of monocots and dicots. Fructose-1,6-biphosphate aldolase (aldolase) is an enzyme of the glycolytic pathway and catalyses the reversible aldol cleavage of fructose-1,6-biphosphate into glyceraldehyde-3-phosphate and dihydroxy-acetone phosphate (Lehninger 1993). Plants contain two forms of aldolases, a cytosolic and a plastidic (Kelley and Tolan 1986; Razdan et al. 1992; Pelzer-Reith et al. 1993). The first cytoplasmic plant aldolase gene was cloned from *Zea mays* (Kelley and Tolan 1986) and was shown to represent one of at least 20 specific polypeptides induced when maize seedlings are grown under anaerobic conditions (Sachs et al. 1980; Hake et al. 1985). The deduced protein sequence was highly conserved with aldolases from *Drosophila,* trypanosomes and mammals (Kelley and Tolan 1986). Further study showed that the promotor of the aldolase gene contained the same regulatory element which was critical for the anaerobic induction of the alcohol dehydrogenase 1 gene of maize (Dennis et al. 1988).

It has long been known that pollen begins to respire at a high rate immediately after hydration, indicating a high rate of oxidative phosphorylation (Dickinson 1965; Hoekstra

and Bruinsma 1975, 1980). However, in a recent study by Bucher et al. (1995) it was demonstrated that ethanolic fermentation may also be a major metabolic pathway during pollen tube development. Measurement of ethanol production of tobacco pollen germinated *in vitro* indicated that about two thirds of the respirated carbon entered the ethanolic fermentation pathway. In addition, it was shown that two enzymes of this pathway, alcohol dehydrogenase (ADH) and pyruvate decarboxylase (PDC), were abundantly expressed in pollen (Bucher et al. 1995). Analysis of PDC expression revealed that two types of PDC are expressed in pollen: PDC1, which was also detected at low levels in all tissues tested; and PDC2, whose abundant expression was restricted to pollen and anthers. The expression of both ADH and PCD2 seemed to be independent of environmental conditions, leading to the conclusion that the regulation of ethanolic fermentation might be different in the sporophyte and the male gametophyte. The abundant expression of A16e in pollen, assuming it represents a fructose-1,6-biphosphate aldolase from *Phalaris* as indicated by the sequence homology, fits well into the picture.

A16e hybridised to two mRNAs in pollen of approx. 1.25 kb and 870 b. The larger transcript was also detected, although weakly, in vegetative tissues, the pistil, and during anther development, whereas the smaller one was only present in pollen and anther stage 4. Southern blot analysis indicated that A16e was a member of a small gene family. For maize (Dennis et al. 1988), the presence of two copies of the aldolase gene has been suggested. In spinach it was assumed to be a single copy gene (Pelzer-Reith et al. 1993), whereas in rice evidence for three genes was presented (Tsutsumi et al. 1994). At present, one can only speculate on the function of the smaller 870 b transcript as it appears too short to encode a pollen-specific full-length aldolase protein and is more likely to represent a related gene or pseudo-gene.

The results of the transcriptional analysis showed that C11e was predominantly expressed in pollen as a transcript of about 1.65 kb. Weak hybridisation indicated the presence of related transcripts in leaves and seedlings, as well as in all anther stages. The sizes of the transcripts detected in these tissues, ca. 1.4 kb and 1.1 kb, differed from that of the one expressed in pollen, indicating that C11e might be a member of a small gene family. This is supported by the result from the genomic Southern blot analysis, which showed the presence of additional weak bands.

Further research is required to determine whether C11e indeed encodes a monosaccharide transport protein, as suggested by sequence comparisons at the DNA and protein level. Sequence identity of C11e with the glucose transport protein STP1 from *Arabidopsis* was low (24%) when compared to sequence homologies amongst different plant monosaccharide transport proteins. For example, STP1 and MST1 from tobacco show 79% sequence identity (Sauer et al. 1993). The 24% value resembles more the similarity between STP1 and SNF3, a glucose transport protein from yeast, which show 28.1% identity (Sauer et al. 1990).

The presence of a pollen-specific monosaccharide transport protein would be an interesting result and might provide insight into the mechanisms by which stigmas provide nutrient support to growing pollen tubes.

The remaining 12 cDNA clones failed to show any similarity to known sequences in the databases and probably represent new genes.

Chapter 4 Detailed Analysis of the B7b-family

4.1 Introduction

The clone B7b represented a sequence that was completely missing in the mRNA population of pollen from 2 lines, but was abundant in other pollen assayed. One of the plants lacking the B7b transcript, named 23A-2x37A-9-6, was heterozygous for pollen-only mutations at the T- and the S-loci, whereas the second plant, 42-4-8, was homozygous for a pollen-only mutation at the S-locus. At present the molecular information about the pollen-only mutation at the S-locus is limited to the sequence analysis of the S_{2° allele from plant 13-15 (genotype $S_{2^\circ.2^\circ}$ $Z_{2.2}$) which does not show any differences to the self-incompatible wildtype (X. Li, pers. comm.). It is therefore conceivable that the mutation which leads to the self-compatible phenotype is not located at the S-locus itself but in a gene tightly linked to S.

Even less is known about the T-locus. Although genetic studies have not provided evidence for distinct mutant T-alleles, it is possible that the T-mutants identified in the population show differences at the molecular level. This possibility cannot be ruled out for the S-mutants either.

On the other hand, the lack of the B7b-transcripts in 23A-2x37A-9-6 argues against the involvement of the transcripts in SI. Their absence cannot be explained on the basis of mutations at the S and T-locus, since the plant is heterozygous at both loci and should therefore produce 25% wildtype pollen.

To clarify whether B7b-transcripts are indeed involved in SI or represent a completely independent phenomenon further analysis was undertaken.

4.2 Results

4.2.1 Southern Blot Analyses

Previous Southern blot analysis of B7b indicated that the clone represented a gene-family (Fig. 3.10). This analysis was performed with DNA from self-incompatible plants.

It was therefore necessary to extend the analysis using more plants including mutants at the SI loci to determine whether the restriction patterns of the two plants which did not express B7b differs from those of the plants which did, and whether there was any indication for linkage to one of the SI-genes.

DNA from 17 plants of the Adelaide population of known SI genotype (Hayman and Richter 1992), was isolated and digested with the restriction endonuclease *Bam*HI.

Among these 17 plants were the two plants which did not express the B7b-family and the wildtype controls, which had also been used in the Northern analysis. B7b was used as the probe for the Southern blot hybridisation.

As can be seen from Fig 4.1, there is little variation in the restriction patterns detected. The patterns of plants 25A-6-2 and 25A-11-2 are very similar to plant 1-3, while all other plants share the same pattern. Most intriguing is the fact that no bands were detectable in plants Z(172) (lane 7), 23A-2x37A-9-6 (lane 8) and 42-4-8 (lane 16), even after prolonged autoradiographic exposure of the membrane.

The apparent absence of the corresponding DNA sequences explains why no B7b transcripts were detected in the Northern blot analysis. However, the results pose even more questions about the nature and function of this gene-family.

Plant Z(172) which turned out to be "negative" for the gene-family is one of the grandchildren of plants 8203-21 and IxIIB16. Its siblings, plants Z(108) (Fig. 4.1 lane 6) and Z(3) (Fig. 4.1 lane 5) as well as the grandparents (lane 3 and 4), do have the gene-family. This observation led to further Southern blot analysis of close relatives of "family-negative" plants. In addition, plants from populations originating in Greece, Portugal and Algeria were tested to investigate whether the absence of the B7b-family is restricted to the Adelaide population, or is of a more general nature. The results are summarised in Table X. In order to make the relationships more understandable, terms commonly applied to human relationships were used.

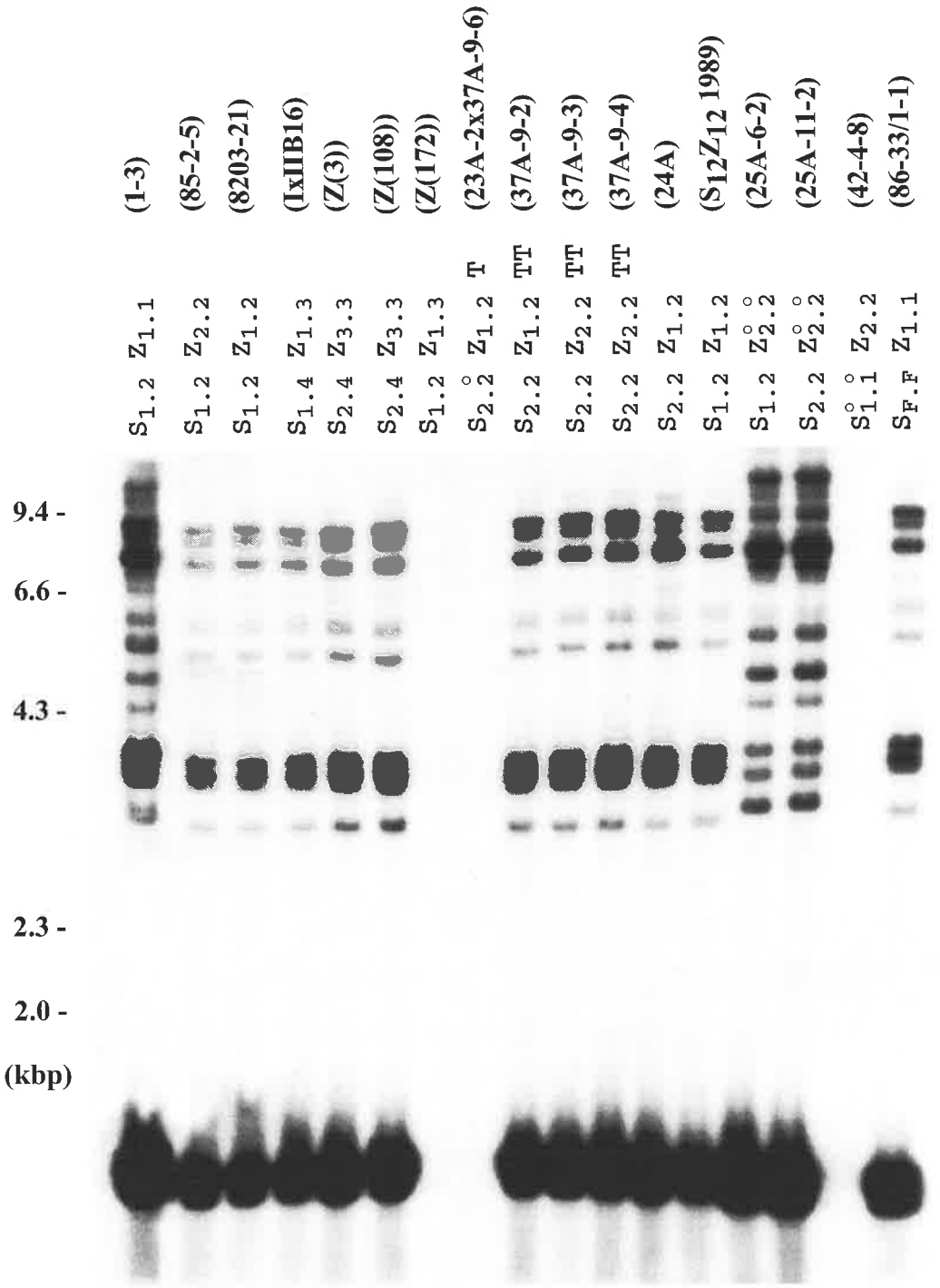


Fig 4.1 Southern blot analysis of the B7b-family. The DNA was digested with *Bam*HI. The names of various plants tested are given in brackets. Size markers are in kilobase pairs (kbp).

Table X: Results of Southern analysis of the B7b-family

Name of Plant	Relationship	SI-genotype	Presence of Gene Family
A) *Relatives of 42-4-8:*			
42-4-5	sibling	$S_{1^\circ.1^\circ}$ $Z_{2.2}$	negative
42-4-11	sibling	$S_{1^\circ.1^\circ}$ $Z_{1.1}$	negative
B) *Relatives of 23A-2x37A-9-6*			
23A-2	parent	$S_{1.2^\circ}$ $Z_{2.2}$	positive
37A-9-6	parent	$S_{2.2}$ $Z_{1.1}$ TT	positive
23A-2-1	"niece"	$S_{2^\circ.2^\circ}$ $Z_{2.2}$	positive
37A-9-2	"aunt"	$S_{2.2}$ $Z_{1.2}$ TT	positive
37A-9-3	"aunt"	$S_{2.2}$ $Z_{2.2}$ TT	positive
37A-9-4	"aunt"	$S_{2.2}$ $Z_{2.2}$ TT	positive
C) *Relatives of Z(172)*			
8203-21	"grandparent"	$S_{1.2}$ $Z_{1.2}$	positive
IxII B16	"grandparent"	$S_{1.4}$ $Z_{1.3}$	positive
(8203-21xIxIIB16)-45	parent	$S_{1.2}$ $Z_{2.3}$	positive
(8203-21xIxIIB16)-23	parent	$S_{1.4}$ $Z_{1.3}$	positive
Z(3)	sibling	$S_{2.4}$ $Z_{3.3}$	positive
Z(42)	sibling	$S_{1.2}$ $Z_{3.3}$	positive
Z(60)	sibling	$S_{1.2}$ $Z_{3.3}$	positive
Z(108)	sibling	S? $Z_{3.3}$	positive
Z(162)	sibling	$S_{2.2}$ $Z_{1.2}$	positive
D) *Plants from other populations*			
Algeria #5		unknown	positive
Greece #2			negative
Greece #4			negative
Greece #6			positive
Greece #10			negative
Portugal #1			positive
Portugal #3			positive

The S-genotype of plant Z(108) was not determined

From this data (Table X) it was possible to draw several conclusions. Firstly, the presence or absence of the B7b-family was not restricted to the Adelaide population and therefore not due to factors such as inbreeding depression. Secondly, the gene-family seemed to be inherited as a "block" in a similar manner to a single gene, since it was either completely absent or completely present. Finally, there was no evidence for linkage of the gene-family with any of the SI-loci, as indicated from Table X (B) and (C).

It was possible that the unusual properties of the B7b-family were due to extrachromosomal inheritance or due to a pollen transmitted virus. This was investigated by analysis of the inheritance of the B7b-family.

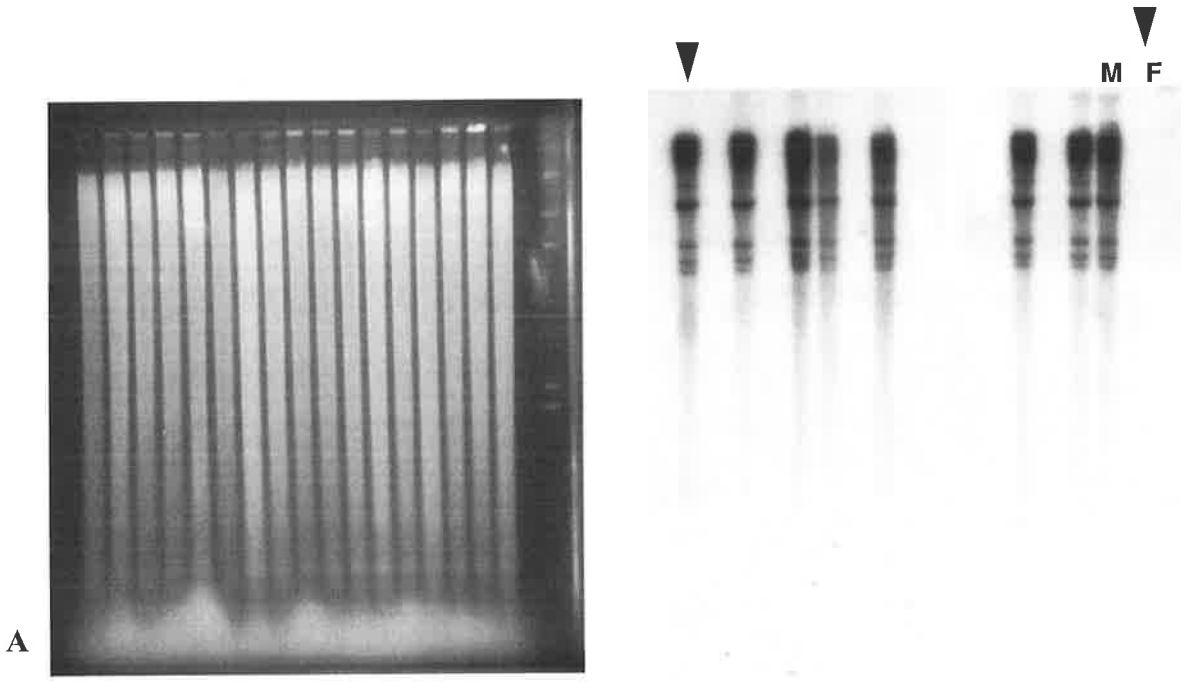
4.2.2 Segregation Analysis of the B7b-family

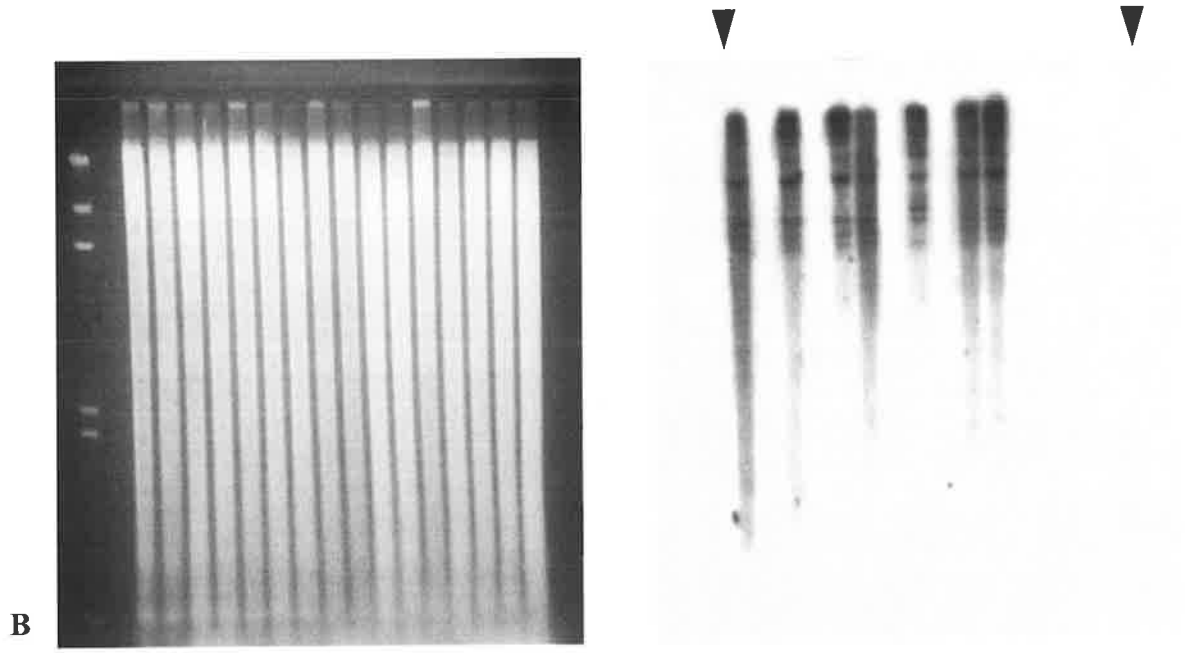
A mapping population was generated by crossing (85-2-5 x Greece6)-5 as the female parent with (85-2-5 x Greece6)-4 as the male parent. These plants had already been tested for the presence of the gene family by genomic Southern analysis (data not shown). The female parent did not contain the B7b-family, while the male parent did. DNA was extracted from 62 F1 plants, digested with *Eco*RV and probed with B7b. The results are presented in Fig. 4.2 A-D. Out of the 62 plants analysed, 29 contained the B7b-family, whereas 33 did not. This result was consistent with normal (1:1) Mendelian segregation.

4.2.3 Sequence Analysis of the B7b-family

Altogether 9 members of the B7b-family were chosen for sequence analysis based on their insert length. B7b contains the longest insert and Bm20-3 the shortest. The inserts of all phages were subcloned in pTZ19U and sequenced from both ends. Fig. 4.3 displays the alignment of the DNA sequences obtained.There is little sequence variation among the clones. Apart from a 15 bp insertion in clones C6b, A15b and C11c, differences are due to one-base deletions (18), transitions (9) and transversions (11). At some positions, a deletion in one of the sequences coincides with a base exchange in another sequence.

The DNA sequences of all clones were analysed for open reading frames (ORF). In five out of the nine clones a putative ORF was identified. Fig. 4.4 shows the DNA and deduced protein sequence of C17e as an example. The DNA sequence contains several putative start codons which are all in frame. The flanking sequences of the first start codon show the highest similarity with the consensus sequence surrounding


M
F
A


B

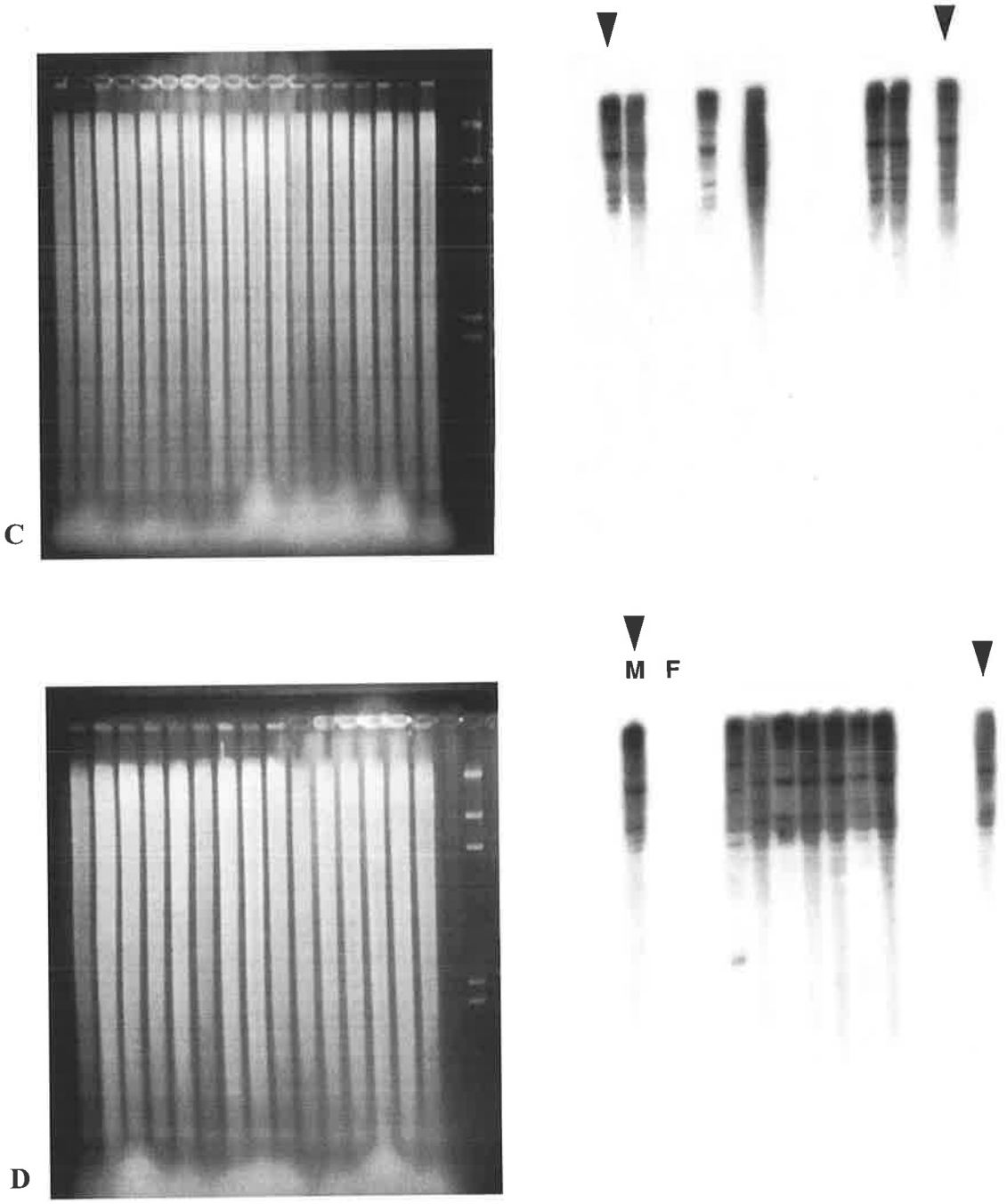


Fig. 4.2 Result of Segregation Analysis
Approximately 10 µg of *Eco*RV digested DNA from each of the 62 F_1-plants was loaded in each lane. DNA from parental plants was loaded on gel A and D and is indicated by M for the male and F for the female parent. Arrows indicate the first and last lane on each autoradiogram. Autoradiograms were exposed for 4 days.
Gel photographs are shown as a control.

```
          1                                                        50
C17e      .......... .......... .......... ....CCGAAG AATGCGACTG
C6b       .......... .......... .......... ....CCGAAG AATGCGACTG
B7b       .......... .......... .......... .......... ..........
Bm20b     .......... .......... .......... .......... ....CGACTG
Bm20d     .......... .......... .......... .......... .....GACTG
B5f       .......... .......... .......... .......... ..........
A15b      .......... .......... .......... .......... ..........
C11c      .......... .......... .......... .......... ..........
Bm20-3    .......... .......... .......... .......... ..........

          51                                                      100
           *                        *
C17e      TGGCGGTACT CCTTATCGCC GTCATGTTGG CTGCTATGTC GGCACCAATG
C6b       TGGCGGTACT CCTTATCGCC GTCATGTTGG CTGCTATGTC GGCACCAATG
B7b       .......... .......... ..CATGTTGG CTGCTATGTC GGCACCAATG
Bm20b     TGGCGGTACT CCTTATCGCC GTCATGTTGG CTGCTATGTC GGCACCAATG
Bm20d     T.GCGGTACT CCTTATCGCC G.CATGTTGG CTGCTATGTC GGCACCAATG
B5f       .......... .......... .......... .......... ..........
A15b      .......... .......... .......... .......... ..........
C11c      .......... .......... .......... .......... ..........
Bm20-3    .......... .......... .......... .......... ..........

          101                                                     150
                  *     *
C17e      GTGACCGCCG CCCAAGGCTG TTGGGAACAA TGCATCCAAA CATGCAAGGG
C6b       GTGACCGCCG CCCAAGGCTG TTGGGAACAA TGCATCCAAA CATGCAAGGG
B7b       GTGACCGCCG CCCAAGGCTG TTGGGAACAA TGCATCCAAA CATGCAAGGG
Bm20b     GTGACCGCCG CCCAAGGCTG TTGGCAACAA TGCATCCAAA CATGCAAGGG
Bm20d     GTGACCG.CG CC.AAGGCTG TTGGCAACAA TGCATCCAAA CATGCAAGGG
B5f       .......... .......... .......... .......... ..........
A15b      .......... .......... .......... .......... ..........
C11c      .......... .......... .......... .......... ..........
Bm20-3    .......... .......... .......... .......... ..........

          151                                                     200
C17e      AGGTAACGAT GTTTGCACCC CAAAGTGCAA TGAATTTTGT AAAAATCAGG
C6b       AGGTAACGAT GTTTGCACCC CAAAGTGCAA TGAATTTTGT AAAAATCAGG
B7b       AGGTAACGAT GTTTGCACCC CAAAGTGCAA TGAATTTTGT AAAAATCAGG
Bm20b     AGGTAACGAT GTTTGCACCC CAAAGTGCAA TGAATTTTGT AAAAATCAGG
Bm20d     AGGTAACGAT GTTTGCACCC CAAAGTGCAA TGAATTTTGT AAAAATCAGG
B5f       .......... .......... .......... .......... ..........
A15b      .......... .......... .......... .......... ..........
C11c      .......... .......... .......... .......... ..........
Bm20-3    .......... .......... .......... .......... ..........

          201                                                     250
                                    * *                 *   *
C17e      CCGCTGCGAT AAGGTATTTT GCCGCTGCCA CTGGTAAATT GAAGGAAGCC
C6b       CCGCTGCGAT AAGGTATTTT GCTGCTGCCA CTGATAAATT GAAGGAAGCC
B7b       CCGCTGCGAT AAGGTATTTT GCCGCTGCCA CTGATAAACT GAAGGAAGCC
Bm20b     CCGCTGCGAT AAGGTATTTT GCCG.TGCCA CTGATAAATT GAAGGAAGCC
Bm20d     CCGCTGCGAT AAGGTATTTT GCCGCTGCCA CTGATAAATT GAAGGAAGCC
B5f       .......... .......... .......... .......... ..........
A15b      .......... .......... ...GCTGCCA CTGATAAA.T G.AGGAAGCC
C11c      .......... ......TTTT GCTGCTGCCA CTGATAAATT GAAGGAAGCC
Bm20-3    .......... .......... .......... .......... ..........
```

```
          251                                                       300
              *                                                      *
C17e      AAAACAGCCT CACCCGAAAA GGCCACCACC CTCAAGAAAG AAGCCGACGA
C6b       AAAACAGCCT CACCCGAAAA GGCCACCACC CTCAAGAAAG AAGCCGACGA
B7b       AAAACAGCCT CACCCGAAAA GGCCACCACC CTCAAGAAAG AAGCCGACGA
Bm20b     AAAACAGCCT CACCCGAAAA GGCCACCACC CTCAAGAAAG AAGCCGACGA
Bm20d     AAAACAGCCT CACCCGAAAA GGCCACCACC CTCAAGAAAG AAGCCGACGA
B5f       .......... .......... .......... .......... ..........
A15b      AAAA.AGCCT CACCCGAAAA GGCCACCACC CTCAAGAAAG AAGCC.ACGA
C11c      AAAACAGCCT CACCCGAAAA GGCCACCACC CTCAAGAAAG AAGCCGACGA
Bm20-3    .......... .......... .......... CTCAAGAAAG AAGCCGACGA

          301                                                       350
                    * * **                      * *   **************
C17e      GTACTTAGCC ACCGCAAAAA AAGTACAACG ACGCTGC... ..........
C6b       GTACTTAGCC ACCGC.AAAA AAGTACAACG ACGCTGCTGG CACCCCGTGA
B7b       GTACTTAGCC ACCGC.AAAA AAGTACAACG ATGCTGC... ..........
Bm20b     GTACTTAGCC ACCGC.AAAA AAGTACAACG ACGCTGC... ..........
Bm20d     GTACTTAGCC ACCGC.AAAA AAGTACAACG ACGCTGC... ..........
B5f       .......... .......... .......... .......... ..........
A15b      GTACTTAGCC ACCG..AAAA AAGTACAACG ACGCTGCTGG CACCCCGTGA
C11c      GTACTTAGCC ACCGC.AAAA AAGTACAACG ACGCTGCTGG CACCCCGTGA
Bm20-3    GTACTTAGCC TCGGCAAAAA AAGTACAACG ACGATGC... ..........

          351                                                       400
          *                              **                  *   *
C17e      .TGGCACCCC GTGAGTGGCA CCCCTTTCAT CCATGGGCGT CATCGATGTG
C6b       GTGGCACCCC GTGAGTGGCA CCCCTTTCAT CCATGGGCGT CATCGATGTG
B7b       .TGGCACCCC GTGAGTGGCA CCCCTTTCAT CCATGGGCGT CATCGATGTG
Bm20b     .TGGCACCCC GTGAGTGGCA CCCCTTTCAT CCATGGGCGT CATCGATGTG
Bm20d     .TGGCACCCC GTGAGTGGCA CCCCTTTCAT CCATGGGCGT CATCGATGTG
B5f       .......... .......... .......... .........C CACCGATGTG
A15b      GTGGCACCCC GTGAGTGGCA CCCCT.TCAT CCATGGGCGT CATCGATGTG
C11c      GTGGCACCCC GTGAGTGGCA CCCCTTTCAT CCATGGGCGT CATCGATGTG
Bm20-3    .TGGCACCCC GTGAGTGGCA CCCCTTCCAT CCATGGGCGT CATCGATGTG

          401                                                       450
                    *           ** *    *   *                       *
C17e      CTTGGTTCAA CACATGCATG TTTTTTATTT AAATAATCAC TATGACATTC
C6b       CTTGGTTCAA CACATGCATG TTTTTTATTT AAATAATCAC TATGACATTC
B7b       CTTGGTTCAA CACATGCATG TTTTTTATTT AAATAATCAC TATGACATTC
Bm20b     CTTGGTTCAA CACATGCATG TTTTTTATTT AAATAATCAC TATGACATTC
Bm20d     CTTGGTTCAA CACATGCATG TTTTTTATTT AAATAATCAC TATGACATTC
B5f       CTTGGTTCAA CACATGCATG TTTTTTATTT AAATAATCAC TATGACATTC
A15b      CTTGGTTCAA CACATGCATG .CT.TTAT.T A.ATAATCAC TATGACATTC
C11c      CTTGGTTCAA CACATGCATG TTTTTTATTT AAATAATCAC TATGACATTC
Bm20-3    CTTGGTTCAA AACATGCATG TTTTTTATTT AAATAATCAC TATGACATTT

          451                                                       500
                         *          *** * * **          *
C17e      CATATTTATT TTCTTTGATT TGCAAACC.. .......... ..........
C6b       CATATTTATT TTCTTTGATT TGCAATCT.. .......... ..........
B7b       CATATTTATT TTCTTTGATT TGCAATCTTC TTGATAAATA ATGTATTTTG
Bm20b     CATATTTATT TTCTTTGATT TGCAATGCTT CTTG...... ..........
Bm20d     CATATTTATT TTCTTTGATT TGCAATCTTC TTG....... ..........
B5f       CATATTTATT TTCTTTGATT TGCAATCTTC TTGATAAATA ATGTATTTTG
A15b      CATATTTATT TTCTTTGATT TGCAATCTTC TTGATAAATA ATGTATTTTG
C11c      CATATTTATT TTCTTTGATT TGCAATCTTC TTGATAAATA ATGTATTTTG
Bm20-3    CATATTTATT TTCTTAGATT TGCAATCTTC TTGATAAATA ATG.ATTTTG
```

```
          501                                                        550
                          *                                   *
C17e      .......... .......... .......... .......... ..........
C6b       .......... .......... .......... .......... ..........
B7b       TTTGTCTCAC AACAAGCTTC TTGTATGGAC TCAGTTTCTC CTTTGGAGTA
Bm20b     .......... .......... .......... .......... ..........
Bm20d     .......... .......... .......... .......... ..........
B5f       TTTGTCTCAC AACAAGCTTC TTGTATGGAC TCAGTTTCTC GTTTGGAGTA
A15b      TTTGTCTCAC AACAAGC.TC TTGTATGGAC TCAGTTTCT. ..........
C11c      TTTGTCTCAC AACAAGCTTC TTGTATGGAC TCAGTTTCTC C.........
Bm20-3    TTTGTCTCAC AACAAGCGTC TTGTATGGAC TCAGTTT... ..........

          551                                                        600
                                                        *
C17e      .......... .......... .......... .......... ..........
C6b       .......... .......... .......... .......... ..........
B7b       CCAAGTTCCT AATGTATAAT GATTACATTT GATTTGGTCA TACCCTTTTA
Bm20b     .......... .......... .......... .......... ..........
Bm20d     .......... .......... .......... .......... ..........
B5f       CCAAGTTCCT AATGTATAAT GATTACATTT GATTTGGTCG TACCCTTTTA
A15b      .......... .......... .......... .......... ..........
C11c      .......... .......... .......... .......... ..........
Bm20-3    .......... .......... .......... .......... ..........

          601                                                        650
                                    *
C17e      .......... .......... .......... .......... ..........
C6b       .......... .......... .......... .......... ..........
B7b       AAGGTTTTTG GTACATAGGT GCTATTTTTT TGTAATACGC ATTGCCGCGT
Bm20b     .......... .......... .......... .......... ..........
Bm20d     .......... .......... .......... .......... ..........
B5f       AAGGTTTTTG GTACATAGGT GCTA.TTTTT TGTAATACGC ATTGCCGCGT
A15b      .......... .......... .......... .......... ..........
C11c      .......... .......... .......... .......... ..........
Bm20-3    .......... .......... .......... .......... ..........

          651                                   683
C17e      .......... .......... .......... ...
C6b       .......... .......... .......... ...
B7b       GTGTTCTTGT CTGATCAGCT TTC..... ...
Bm20b     .......... .......... .......... ...
Bm20d     .......... .......... .......... ...
B5f       GTGTTCTTGT CTGATCAGCT TTCAAGATGA TTG
A15b      .......... .......... .......... ...
C11c      .......... .......... .......... ...
Bm20-3    .......... .......... .......... ...
```

Fig 4.3 Alignment of the DNA sequences of several members of the B7b-family. Positions at which sequence variation occurs are indicated by asterisks.

```
  1 CAACATCAAGAATCTATCGAAGATGGCGCAAAGTCCGAAGAATGCGACTGTGGCGGTACT 60
                        M  A  Q  S  P  K  N  A  T  V  A  V  L  13

 61 CCTTATCGCCGTCATGTTGGCTGCTATGTCGGCACCAATGGTGACCGCCGCCCAAGGCTG 120
    L  I  A  V  M  L  A  A  M  S  A  P  M  V  T  A  A  Q  G  C  33

121 TTGGGAACAATGCATCCAAACATGCAAGGGAGGTAACGATGTTTGCACCCCAAAGTGCAA 180
    W  E  Q  C  I  Q  T  C  K  G  G  N  D  V  C  T  P  K  C  N  53

181 TGAATTTTGTAAAAATCAGGCCGCTGCGATAAGGTATTTTGCCGCTGCCACTGGTAAATT 240
    E  F  C  K  N  Q  A  A  A  I  R  Y  F  A  A  A  T  G  K  L  73

241 GAAGGAAGCCAAAACAGCCTCACCCGAAAAGGCCACCACCCTCAAGAAAGAAGCCGACGA 300
    K  E  A  K  T  A  S  P  E  K  A  T  T  L  K  K  E  A  D  E  93

301 GTACTTAGCCACCGCAAAAAAGTACAACGACGCTGCTGGCACCCCGTGAGTGGCACCCCT 360
    Y  L  A  T  A  K  K  Y  N  D  A  A  G  T  P  *                108

361 TTCATCCATGGGCGTCATCGATGTGCTTGGTTCAACACATGCATGTTTTTTATTTAAATA 420
                                                  ------

421 ATCACTATGACATTCCATATTTATTTTCTTTGATTTGCAAACCTCCTTGATAAATAATGT

481 ATTTTGTTTGTCTCACAACAAGCTTCTTGTATGACTCAGTT 521
```

Fig 4.4 DNA and deduced protein sequence of clone C17e. Lines indicate putative start codons. The dotted line indicates a putative polyadenylation signal. Cysteine residues are highlighted in boldface letters.

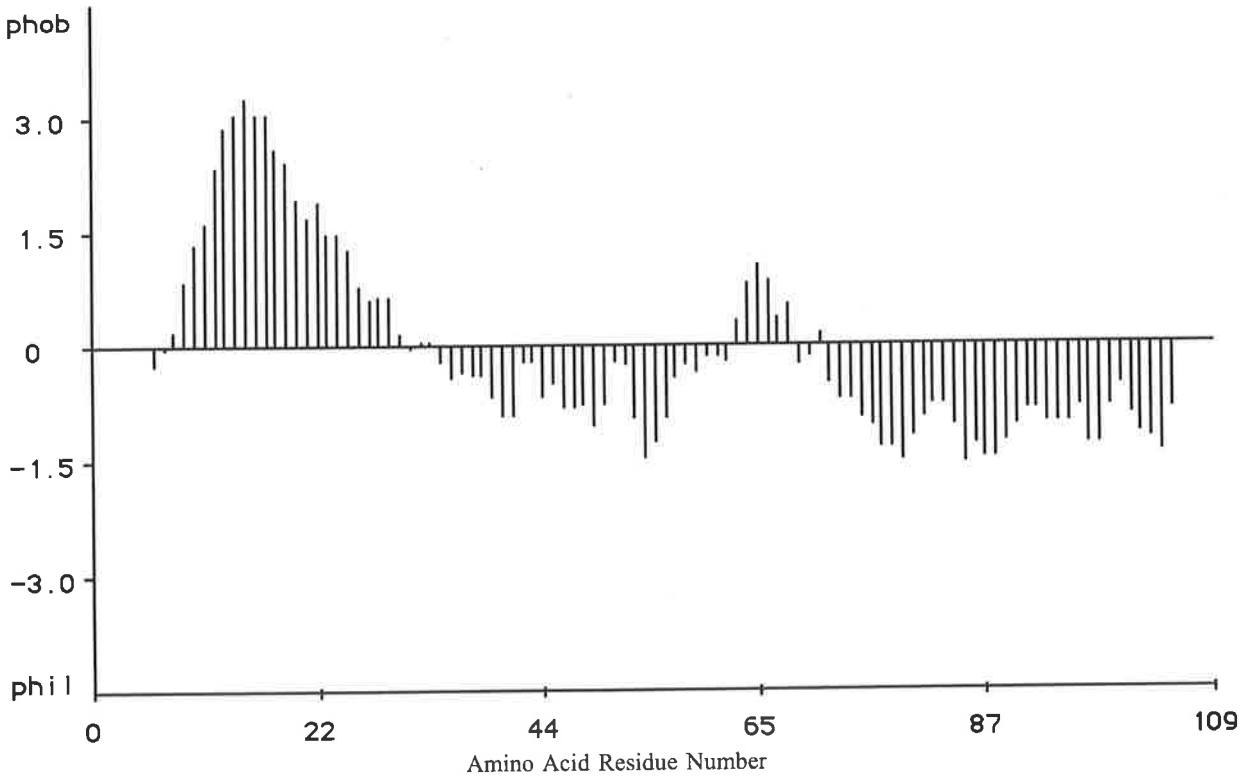


Fig 4.5 Hydrophobicity plot of the deduced protein sequence of C17e. The hydropathy profile was calculated using an 11 Amino-Acid-Residue Window. The plot was generated using the MacMolly program. phob stands for hydrophobic, phil for hydrophilic.

```
          1                                                    50
C6b       MAQSPKNATV AVLLIAVMLA AMSAPMVTAA QGCWEQCIQT CKGGNDVCTP
B7b       .......... .......MLA AMSAPMVTAA QGCWEQCIQT CKGGNDVCTP
Bm20b     .......... .......MLA AMSAPMVTAA QGCWQQCIQT CKGGNDVCTP
Bm20d     .......... .......MLA AMSAPMVTAA QGCWQQCIQT CKGGNDVCTP
C17e      MAQSPKNATV AVLLIAVMLA AMSAPMVTAA QGCWEQCIQT CKGGNDVCTP

          51                                                  100
C6b       KCNEFCKNQA AAIRYFAAAT DKLKEAKTAS PEKATTLKKE ADEYLATAKK
B7b       KCNEFCKNQA AAIRYFAAAT DKLKEAKTAS PEKATTLKKE ADEYLATAKK
Bm20b     KCNEFCKNQA AAIRYFAAAT DKLKEAKTAS PEKATTLKKE ADEYLATAKK
Bm20d     KCNEFCKNQA AAIRYFAAAT DKLKEAKTAS PEKATTLKKE ADEYLATAKK
C17e      KCNEFCKNQA AAIRYFAAAT GKLKEAKTAS PEKATTLKKE ADEYLATAKK

          101
C6b       YNDAAGTP
B7b       YNDAAGTP
Bm20b     YNDAAGTP
Bm20d     YNDAAGTP
C17e      YNDAAGTP
```

Fig. 4.6 Alignment of the deduced protein sequences of members of the B7b-family. Differences in the protein sequences are highlighted in boldface letters.

the initiation codon in plant mRNAs (Lütcke et al. 1987). It is therefore likely that the initiation of translation starts at this ATG.
The deduced protein sequence shows several features. As can be seen from the hydrophobicity plot (Fig. 4.5) the protein has a hydrophobic region at the N-terminal, which suggests that this domain represents a signal peptide (von Heijne 1985).
The protein contains an unusual cysteine-motif, $C\text{-}X_3\text{-}C\text{-}X_3\text{-}C\text{-}X_6\text{-}C\text{-}X_3\text{-}C\text{-}X_3\text{-}C$. The significance of this motif is unclear since database searches did not reveal any sequences homology to known genes, neither at the DNA nor at the protein level.
A comparison of the deduced protein sequences of those clones, which contain an ORF shows that the proteins are highly conserved. The main difference, apart from amino acid exchanges at positions 35 and 71, is that the sequences of B7b, Bm20b and Bm20d are shorter by 17 amino acids compared to C6b and C17e. Whether this truncation is due to the cDNAs being incomplete or whether these shorter proteins represent complete sequences is currently unknown.

4.3 Discussion

Genes expressed during late pollen development are thought to be important for pollen germination, pollen tube growth and/or pollen-stigma interaction (Mascarenhas 1988). However the B7b-family, which was the focus of this study, might be an example of genes with a function unrelated to those just mentioned.

Two plants were identified which did not express the B7b-family. The pollen of neither of these two plants showed any phenotypical defects in pollen germination, pollen tube growth or any other aspect of pollen function, except that both plants were self-fertile mutants. This aspect justified further analysis, during which it is was demonstrated that the lack of B7b-transcripts in the two plants was correlated with the absence of the corresponding DNA sequences. Moreover, examination of a large number of plants from the Adelaide population, as well as plants from other populations, identified more plants which did not contain the B7b family. Among them were not only self-fertile mutants, but also self-incompatible plants, suggesting that the absence of the gene-family and a self-fertile phenotype are likely to be independent phenomena. The results further indicated that this family seemed to be inherited as if it constituted a single gene and that plants could be either homozygous, hemizygous or null for the gene-family. A segregation analysis was designed to address the mode of inheritance of the B7b-family specifically, that is to clarify whether the family was encoded by the nuclear genome, extrachromosomally or even by a pollen transmitted virus.

Since it has been reported for barley and maize that both mitochondrial and plastid inheritance is strictly maternal (Mogensen 1988; Faure et al. 1993 and references therein) it was decided to use a plant that did not contain the gene-family as the female parent of the mapping population, and a plant which was suspected to be heterozygous for the gene-family as the male parent. A significant deviation from the expected 1:1 ratio of "family containing" to "family negative" plants in the offspring would then indicate that the B7b-family was not encoded by the nuclear genome. However, the result obtained showed a ratio in agreement with Mendelian inheritance. (Of course, if the male parent had been homozygous for the family, 100% penetration would have been expected for the F1.)

The finding that the B7b-family seemed to be inherited as a single locus implies that the family members are closely linked on one chromosome. Gene-families organised in clusters are not uncommon; numerous examples have been described in animal systems. Some gene-families are arranged in tandem repeats, such as the genes for rRNA and histones (Long and David 1980), others are simply closely linked such as the β-globin genes in mammals (Maniatis et al. 1980) and the Kallikrein genes in rats (Wines et al. 1991). In plants examples of tandemly organised gene families include the 18, 5.8 and 25S rRNA genes (Long and David, 1980 and references therein; Barker et al. 1988), the 5S rRNA genes (Sastri et al. 1992), the genes coding for the small subunit of Rubisco in *Mesembryanthemum crystallinum,* of which 5 were arranged in a tandem array spanning 20 kb (DeRocher et al. 1993) and the *Pto/Fen* gene-family with 6-8 members (Martin 1994). Examples of closely linked gene-families are the B-hordein genes, which consisted of at least 13 members (Lehfer et al. 1993) and the α-amylase genes (Muthukrishnan et al. 1984).

At present information on the genomic organisation of the B7b-family is limited, and future analysis of genomic clones will be required to determine whether a repeat unit can be identified. However, a comparison of the banding pattern of plants 1-3, 25A-6-2 and 25A-11-2 (Fig. 4.1) with those of related plants of the Adelaide population indicated that the organisation of the family on one chromosome differs from the organisation of the family on the other chromosome. Also, the banding pattern obtained with DNA from plants of the Adelaide population differed from those obtained with plants of other populations, with up to 5 bands at different positions (data not shown). This, and the fact that some chromosomes have completely lost this gene-family, raises questions about the evolution and stability of the region of the chromosome where the family is located.

In general, gene-families that are organised in clusters are thought to have evolved by gene-duplication and unequal cross-overs (Ohno 1970). It is possible that unequal cross-overs were responsible for the differences in banding patterns observed in Southern blots probed with B7b. More difficult to explain is the complete

absence of the family in some plants. If a major deletion in the chromosome carrying the family had occurred, one would expect deleterious effects for the plant and its offspring. An examination of the chromosomes of plants heterozygous or null for the family under a light microscope did not reveal any visible differences (D. Hayman, pers. comm., 1995).

Several studies have demonstrated the occurrence of intrachromosomal recombination between closely linked DNA repeats in plants (Peterhans et al. 1990; Assaad and Signer 1992; Swoboda et al. 1994; Puchta et al. 1995). In all the cited studies, researchers transformed plants with constructs which contained two overlapping non-functional regions of a detectable marker gene (neomycin phosphotransferase or β-glucoronidase) separated by a hygromycin resistance gene. Intrachromosomal recombination events which resulted in restoration of the marker gene were then detected either directly by histochemical staining or indirectly by growing plants on neomycin-containing medium, and confirmed by Southern blot analysis. These recombination events were observed in all plant tissues from seed stage to flowering stage, and recombination frequencies ranged from 10^{-6} to 10^{-7} events/genome (Swoboda et al. 1994).

One could speculate that, provided there are homologous sequences flanking the gene-cluster, an intrachromosomal cross-over might have led to the deletion of the complete gene-family. On the condition that the event took place in a tissue which gave rise to gametes, the deletion would be inherited. Since plants null for the family have been identified in unrelated populations, it is likely that such an event occurred either early during the evolution of *Phalaris* or happened more than once, which would indicate a certain instability of this region of the chromosome.

Analysis of the DNA sequences flanking the B7b-family, together with the analysis of the corresponding region in chromosomes which have lost the gene family, may provide the data necessary to substantiate this hypothesis.

An alternative explanation would be that the results of Southern blot hybridisations performed here do not reflect the total complexity of the family. There might be allelic and/or non-allelic members of the gene-family which are too different from B7b to cross-hybridise. One could speculate that plants which appeared to be null for the B7b-family might contain only members or alleles which share low sequence identity with B7b and are therefore not detectable under the hybridisation conditions used. An example of members of gene-families which do not cross-hybridise was presented in a study by Pang et al. (1988) on the 12S globulin seed storage protein-family. The analysis of four genes of the family revealed that two were closely related and cross-hybridised, whereas the other two showed only 60-75% nucleic acid identity to each other and the first two, and did not cross-hybridise with any of the other genes. An example of extreme allelic diversity was provided in a

study by Rivers et al. (1993) on the self-incompatibility gene in a natural populations of *L. peruvianum*. It was demonstrated that the alleles identified in these populations were so diverse that many of them did not cross-hybridise under moderately stringent conditions.

The presence of additional members or diverse alleles of B7b could be tested by hybridisation experiments at low stringency.

Sequence analysis

During the course of the cross-hybridisation analysis (see Chapter 3), it became clear that members of the gene-family could be subgrouped depending on the lengths of their inserts. Sequence analysis was carried out, firstly to gain information concerning the possible function of these genes, and secondly to investigate whether the clones represented different genes or pseudogenes. For this analysis, nine cDNA clones which showed differences in insert length were chosen as representatives, and it was possible to identify a putative open reading frame in 5 of the clones. Interestingly, it turned out that the majority of the sequence variations at the DNA level, especially the 15 bp insertion, resided in the 3' untranslated region and therefore did not affect the putative protein sequence. Since the 5' sequences were missing in all of the cDNA clones which did not contain the complete ORF, it seems likely that the reverse transcription of their corresponding transcripts was incomplete.

Sequence comparison revealed that the protein sequences were highly conserved. Analysis of further cDNAs will be required to resolve whether this conservation was a coincidence or is a significant feature of these proteins.

Considering that database searches failed to show any significant homology to known sequences, either at the protein or at the DNA level, the B7b-genes may represent a new family of pollen-specific genes. However, an interesting possibility is suggested by a study reported by Turcich et al. (1994). They characterised a pollen-specific cDNA clone, Tpc70 from *Tradescantia paludosa* which shows features similar to B7b. Tpc70 encoded a small peptide of 73 amino acids, whose N-terminal region was suggestive of a signal peptide. The protein contains a cysteine motif which is almost identical to that of B7b. (Tpc70: C-X_3-C-X_3-C-X_8-C-X_3-C-X_3-C, B7b: C-X_3-C-X_3-C-X_6-C-X_3-C-X_3-C.). However, these two features are the only similarity between the proteins. Whether Tpc70 represents a gene-family was not mentioned in the publication.

One could speculate that the cysteine motif present in the protein might be of significance, and it is possible that the spacing of the cysteines and the 3 dimensional structure of the protein, rather than the intervening sequences, are relevant for protein function, as for instance in zinc-finger (Lewin 1987) and ring-finger proteins (von Arnim and Deng, 1993). Plant defensins are another example of proteins which are

characterised by a very conserved cysteine motif, but where other amino acids are variable (Broekaert et al. 1995). Furthermore, their three dimensional structure is identical and interestingly, closely resembles that of insect defensins (Broekaert et al. 1995).

It seems curious that such an abundantly transcribed gene-family might not be required by the pollen, and this raises questions about the possible role of these genes. Assuming that "family-negative" plants do not contain any B7b-family members, the gene-family can play neither an essential nor an advantageous role in aspects like pollen germination, tube growth or pollen-stigma interaction. This is for two reasons. a) Family-negative plants did not show any phenotypical defects in pollen germination and pollen tube growth. b) If there was a significant selective advantage for pollen expressing the B7b-family, such as faster pollen tube growth, this should have been detected in the segregation analysis through an increased number of plants containing the gene-family in the F1. Therefore only two possibilities, which are not necessarily mutually exclusive, remain: that the family-negative plants contain diverse members of the family which perform the function; or that the B7b-family is not involved in the physiological activities related to pollen germination and pollen-stigma interaction.

Evidence for B7b representing a pollen-expressed transposon has not been obtained. Unlike PREM-1, a putative retrotransposon which is primarily transcribed during pollen development in maize (Turcich and Mascarenhas 1994), B7b-family members contain an open reading frame. In addition, the results of the segregation and sequence analyses argue against B7b being a transposable element.

One could contemplate whether B7b represents a new family of disease resistance genes. That proteins putatively involved in pathogen resistance are expressed in pollen has been demonstrated in a few studies. For instance, *Bet v1* represents a family of allergenic proteins which are constitutively expressed at high levels in pollen of *Betula verrucosa* and related trees of the *Fagales* family (Breiteneder et al. 1989; Valenta et al. 1991b). The deduced protein sequence showed 55% sequence identity to a gene involved in the disease resistance response in pea (Breiteneder et al. 1989). Chitinases seemed to be expressed in mature pollen of poplar trees (Clarke et al. 1994). Other studies showed that sporophytic susceptibility or resistance to certain fungi can be predicted by measuring the effect of fungal toxins on pollen tube length (Laughnan and Gabay 1973; Darakov 1995; for review see Hormaza and Herrero 1992). These findings indicate the expression of resistance genes in growing pollen tubes.

Future research is required to answer the questions posed by this intriguing gene-family.

Chapter 5 Analysis of Protein Phosphorylation in Pollen

5.1 Introduction

Protein phosphorylation plays a central role in the regulation of cellular activities in all living systems. An estimated 1 to 3% of functional eukaryotic genes encode protein kinases. In the last few years reversible phosphorylation, that is the competing and complementary action of protein kinases and protein phosphatases, has been implicated in a wide variety of biochemical processes. These range from carbohydrate transport across bacterial cell membranes (Meadow et al. 1990) to the intracellular transduction of developmental signals mediated by hormones and differentiation and growth factors (Ulrich and Schlessinger 1990; Nishizuka 1992). Cell cycle control depends critically on the concerted action of a number of cytoplasmic and nuclear protein kinases (Lewin 1990; Murray and Hunt 1993), and several are able to induce oncogenic transformation in mammalian cells when mutated or improperly expressed.

Protein kinases are usually subdivided in two main classes: those that transfer phosphate to serine and/or threonine and those that transfer phosphate to tyrosine (Taylor et al. 1992). A few kinases appear to have dual specificity and phosphorylate serine/threonine and tyrosine residues (Lindberg et al. 1992).

Protein kinases are structurally diverse in terms of size, subunit structure, subcellular localisation and substrate specificity. They also vary in the mechanisms by which they are activated. The majority of protein kinases are highly regulated enzymes that are switched on and off, either directly or indirectly, in response to specific signals as diverse as hormones, cAMP, Ca^{2+}, or binding of a ligand (Taylor et al. 1992). In spite of this considerable diversity there are significant similarities among most of the known protein kinases, indicating that they are evolutionary related and have evolved from common ancestral origins (Hanks et al. 1988).

Protein tyrosine kinases have been reviewed by van der Geer et al. (1994), protein serine/threonine kinases by Edelman et al. (1987) and protein serine/threonine phosphatases by Shenolikar (1994). Further reviews are by Hunter (1987, 1995), Hanks et al. (1988), Taylor et al. (1992).

5.1.1 Protein kinases in plants

The number of protein kinases identified in plant systems has increased markedly since the first plant protein kinase was cloned in 1989 (Lawton et al. 1989). Protein phosphorylation is now thought to play a role in responses to a diverse range of

signals, including hormones, light, pathogen invasion, temperature stress and nutrient deprivation (Stone and Walker 1995). Reversible phosphorylation also controls the activities of several plant metabolic and regulatory enzymes (reviewed by Huber et al. 1994).

Many of the protein kinases found in plants are similar to those identified in yeast or animal systems, as for instance the glycogen synthase kinase-3 (GSK-3) (Pay et al. 1993; Jonak et al. 1995), the casein kinase II (CK II) families, cyclin-dependent kinases and the mitogen-activated protein (MAP) kinases (Stone and Walker 1995). MAP kinases, also called extracellular signal regulated kinases, are serine/threonine protein kinases which are unique in that they are activated by phosphorylation on both threonine and tyrosine residues (reviewed by Jonak et al. 1994). MAP-kinases are important mediators of signal transduction from the cell surface to the nucleus, leading to gene expression and thereby control of many aspects of cell physiology and proliferation (reviewed by Karin and Hunter 1995; Herskowitz 1995 and references therein).

MAP-kinase homologs have been isolated from several different plants species (Duerr et al. 1993; Strafstrom et al. 1993; Wilson et al. 1993; Decroocq-Ferrant et al. 1995a) where they are members of small gene families (Mizoguchi et al. 1993) and have been implicated in the regulation of cell division (Jonak et al. 1993). Not much is known about the pathway which leads to activation of MAP-kinases in plants. However, a recent study by Mizoguchi et al. (1994) suggested that MAP-kinases may be activated by auxin, in a manner similar to that seen for various mitogens in animal systems.

It seems possible, although it remains to be proven, that the MAP-kinase cascades proposed in yeast and animals are also conserved in plants, since homologs of other members of the MAP kinase signal transduction pathway have also been isolated from plants. Examples are *NPK2* (Shibata et al. 1995), a MAP kinase kinase homolog and *NPK1* (Banno et al. 1993), a MAP kinase kinase kinase homolog.

At present, evidence for the existence of protein tyrosine kinases in plants remains elusive, nor is there clear evidence for second-messenger-regulated protein kinases, such as the cyclic nucleotide-dependent protein kinases and the calcium-phospholipid-dependent protein kinases (Stone and Walker 1995), although second messengers have been shown to be important for signal transduction in plants. Calcium dependent phosphorylation seems to be primarily catalysed by calcium-dependent calmodulin-independent protein kinases (CDPKs). Their catalytic domain is typical of serine/threonine protein kinases, whereas their regulatory domain is homologous to the calcium-binding sites of calmodulin (reviewed by Roberts and Harmon 1992; Poovaiah and Reedy 1993). So far this new type of protein kinase,

where the kinase domain is fused to a calmodulin like region, has only been found in plants.

Another example of plant protein kinases which have no analogue in animal systems is the receptor-like protein kinase (RLK) family. Although RLKs have structural features similar to receptor tyrosine kinases (RTK) in animal systems, their catalytic domains, which share a high degree of homology, are serine/threonine- rather than tyrosine-specific (Walker 1994). The extracellular domains of the RLKs are very divergent and have been classified into different groups accordingly (reviewed by Walker 1994). One group is characterised by having an extracellular S-domain and was first described in the context of the analysis of the self-incompatibility genes in *Brassica* (Chapter 1). However, the presence of RLKs with S-domains in monocots and dicots (Walker 1994) indicates that they may have a general function for these proteins.

The second group of RLKs contains leucine rich repeats in the extracellular domain, and members of this group have been identified in *Arabidopsis* (Chang et al. 1992) and *Petunia* (Mu et al. 1994). At present neither the ligands of the RLKs nor the molecules involved in the signal transduction downstream have been identified.

5.1.2 Protein phosphorylation in pollen

Little is known about protein phosphorylation in pollen. Polya et al. (1986) demonstrated Ca^{2+}-dependent and -independent protein kinase activity in germinated pollen from *Nicotiana alata.* Franklin-Tong et al. (1992) observed rapid and transient phosphorylation of several proteins during *in vitro* germination of *Papaver rhoeas* pollen challenged with self- and cross-stigma extracts. They found distinct differences in the phosphorylation profile, depending on the incompatibility reaction.

A number of protein kinases are expressed in pollen. Some are pollen-specific, such as *PRK1*, a receptor-like kinase with leucine rich repeats isolated from *Petunia*, and CDPK, a Ca^{2+}-dependent calmodulin-independent protein kinase isolated from *Zea mays* (see Chapter 1), whereas other protein kinases are expressed in other tissues as well. For instance, *NTF3*, a MAP-kinase homolog from tobacco and *PRK6*, a member of the *shaggy/zeste-white 3* family were also expressed in mature pollen (Wilson et al. 1993; Decroocq-Ferrant et al. 1995b).

As many researchers have not included pollen in their expression studies of the protein kinases isolated, it is difficult to estimate the exact number of protein kinases expressed in pollen.

Rationale

In the differential screening for pollen-specific genes, in this study, three of the clones isolated showed significant homology to protein kinases. The catalytic domain of A12a showed high homology to both *Fen*, a protein kinase from tomato which renders plants sensitive to the insecticide fenthion (Martin et al. 1994) and *Pto*, a protein kinase which confers resistance to *Pseudomonas syringae* pv. *tomato* (Martin et al. 1993). Bm7d showed high homology to MAP-kinases, and the partial sequence of B4e is homologous to calcium-dependent protein kinases (Chapter 3).
The expression of at least 3 protein kinases in the pollen of *Phalaris*, and the recent study by Wehling et al. (1994) where they proposed that protein phosphorylation was involved in the self-incompatibility reaction in rye, were behind the decision to further investigate the role of protein phosphorylation in pollen germination, pollen-stigma interaction and SI.

5.2 Results

5.2.1 Analysis of basic protein phosphorylation in pollen from self-incompatible and self-fertile mutant plants

In their analysis of basic phosphorylation of pollen proteins from rye, Wehling et al. (1994) found that three proteins failed to be phosphorylated in pollen of one of their self-fertile mutants. Therefore, the first question addressed in the present study was whether any of the *Phalaris* mutants showed a similar phenomenon.
Following the protocol by Wehling et al. (1994), basic protein phosphorylation of ungerminated pollen grains from a range of mutants at the S-, Z- and T-loci was analysed and compared to wildtype controls (Fig 5.1 A to C). No evidence was found for failed phosphorylation of any proteins in any of the mutants tested and, although slight variation in the overall phosphorylation activity was observed, it was not possible to correlate this unambiguously with a mutant phenotype.
It should be mentioned that the quality of the pollen from mutants was always lower on average, with a higher number of aborted empty grains compared to the wildtype. This factor might have influenced the variations observed.

5.2.2 Analysis of protein phosphorylation during pollen germination

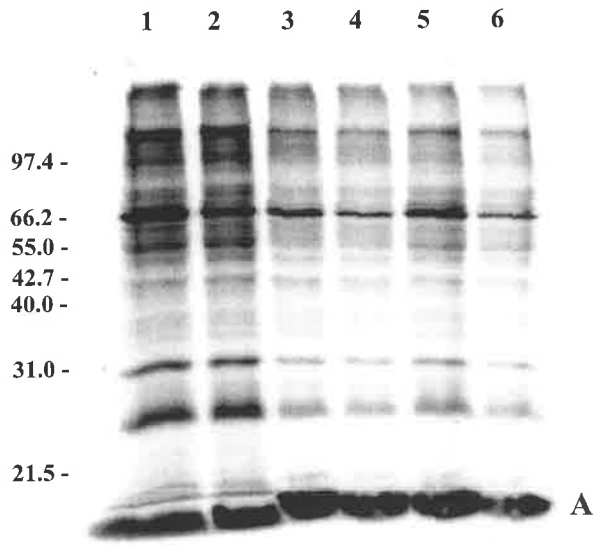
All previous studies on protein kinase activity in pollen grains or germinating pollen tubes either employed methods involving partial or complete protein purification (for instance, Polya et al. 1986) or used pollen which was no longer alive (for instance, Wehling et al. 1994).

Fig 5.1 Autoradiograms of *in situ* phosphorylated pollen proteins separated by SDS-PAGE. Assay was performed after Wehling et al. (1994).

A Comparison of basic protein phosphorylation of pollen from a self-incompatible plant and two self-fertile S-mutants. Protein phosphorylation was examined 60 and 90 sec after the start of the reaction.
Lane 1 and 2: pollen proteins from a self-incompatible plant (60 sec, 90 sec)
Lane 3 and 4: pollen proteins from the S-complete mutant.
Lane 5 and 6: pollen proteins from a S_2-pollen-only mutant.

B Comparison of basic protein phosphorylation of pollen from a self-incompatible plant and a self-fertile Z-mutant. Protein phosphorylation was examined 60, 80 and 120 sec after the start of the reaction.
Lane 1 to 3: pollen proteins from a self-incompatible plant
Lane 4 to 6: pollen proteins from a Z_2-pollen-only mutant

C Comparison of basic protein phosphorylation of pollen from a self-incompatible plant, a self-fertile T- and a S-mutant. Protein phosphorylation was examined 60, 90 and 120 sec after the start of the reaction.
Lane 1 and 2: pollen proteins from a T-mutant (60 sec, 90 sec)
Lane 3 to 5: pollen proteins from a S_2-pollen-only mutant (60, 90, 120 sec)
Lane 6 to 8: pollen proteins from a self-incompatible plant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97.4 -
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42.7 -
40.0 -
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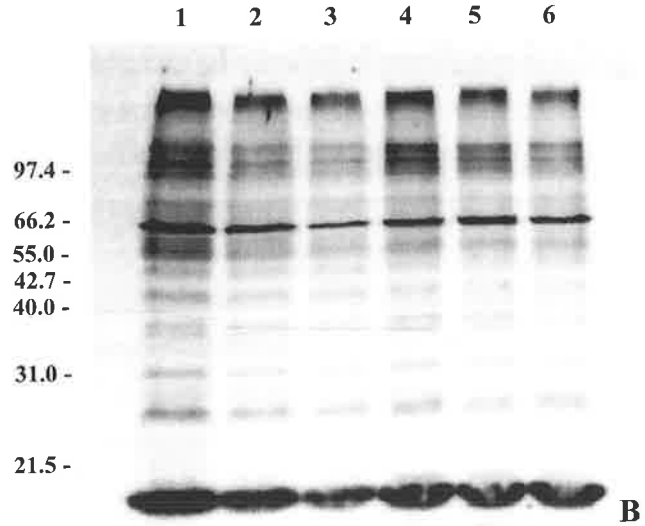
A


1 2 3 4 5 6
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55.0 -
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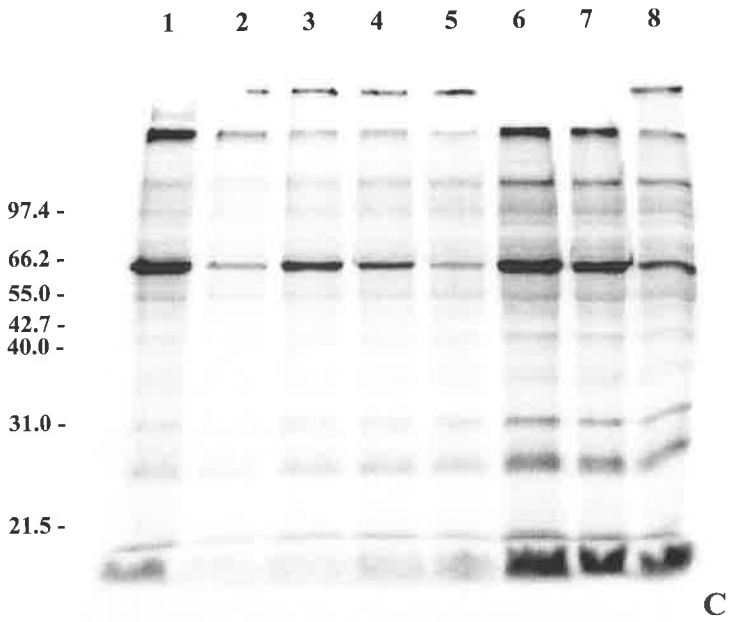
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42.7 -
40.0 -
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21.5 -
C

It was therefore decided to investigate the possibility of observing protein phosphorylation in pollen during germination and pollen tube growth *in vitro*. A liquid pollen germination medium suitable for *Phalaris* pollen was established and used for this analysis. Pollen from a self-incompatible plant was collected, weighed and germinated for various periods at room temperature. Fig. 5.2 shows the growth of pollen tubes in germination medium over a period of 14 min. Fig. 5.3 shows the corresponding phosphorylation profile. These results show that it is possible to observe protein phosphorylation during pollen germination and pollen tube growth.

In their study, Wehling et al. (1994) found that pollen germinated *in vitro* in the presence of stigma eluate contained higher phosphorylation activity than pollen germinated without eluate, and that the activity was highest when self-stigma eluate was used. Therefore, the next question addressed was whether stigma eluate would have a similar effect on the phosphorylation activity of *Phalaris* pollen assayed in germination medium. Pistils were dissected from self-incompatible plants and incubated in germination medium. As a first step, pollen tube growth was monitored at different dilutions of self- and cross-stigma eluate to determine a concentration which visibly affected but did not significantly inhibit pollen tube growth and could be used for the phosphorylation assay. Once a suitable dilution was determined, protein phosphorylation of pollen germinated in the presence of stigma eluate was assayed. Fig. 5.4 shows an example of the results obtained. As can be seen, no differences in the phosphorylation profile, nor in the phosphorylation activity, were detectable.

5.2.3 The S-protein as a substrate for protein phosphorylation

Since the study by Wehling et al. (1994) provided indications that protein phosphorylation was involved in the self-incompatibility reaction, the question arose whether the S-protein itself was a possible target for phosphorylation. Analysis of the protein sequence of the S_1-allele for putative phosphorylation sites using the Prosite program of the Winsconsin GCG-package indicated the presence of two sites for cAMP-and cGMP-dependent protein kinases, four for casein kinase II and two for protein kinase C. Due to allelic variation, the two putative phosphorylation sites for cAMP-and cGMP-dependent protein kinases were missing in the S_2- and S_4-allele. On the other hand, the S_2-allele contained two more phosphorylation sites for protein kinase C than the S_1-allele, whereas the putative phosphorylation sites for casein kinase II were conserved among the two alleles. The S_4 -allele has only been partially sequenced and no difference in phosphorylation sites were found in the partial sequence. It is important to regard this result as theoretical however, since there is no

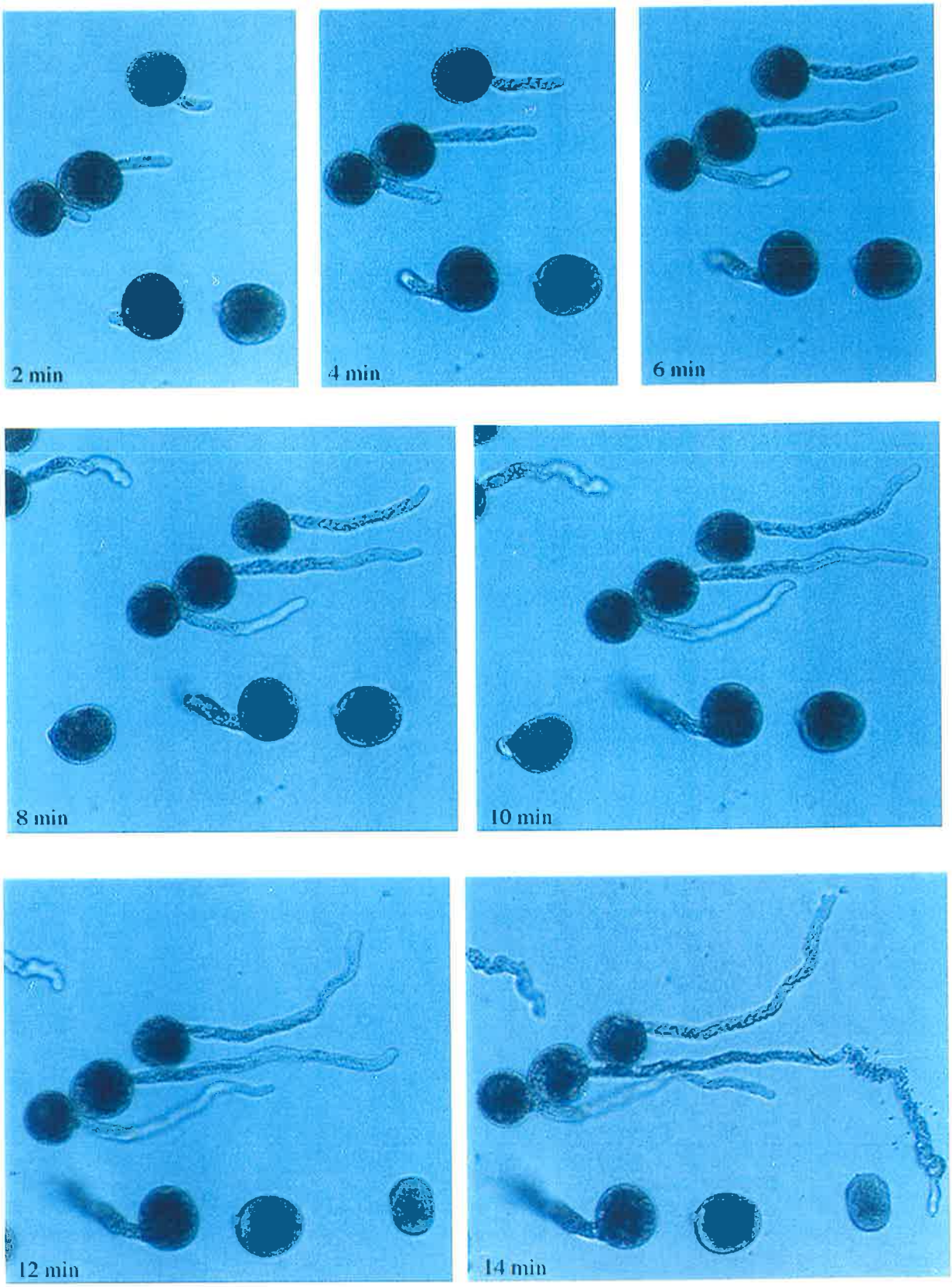


Fig. 5.2 Photographs are arranged sequentially and depict pollen germination in germination medium at two minute observation intervals

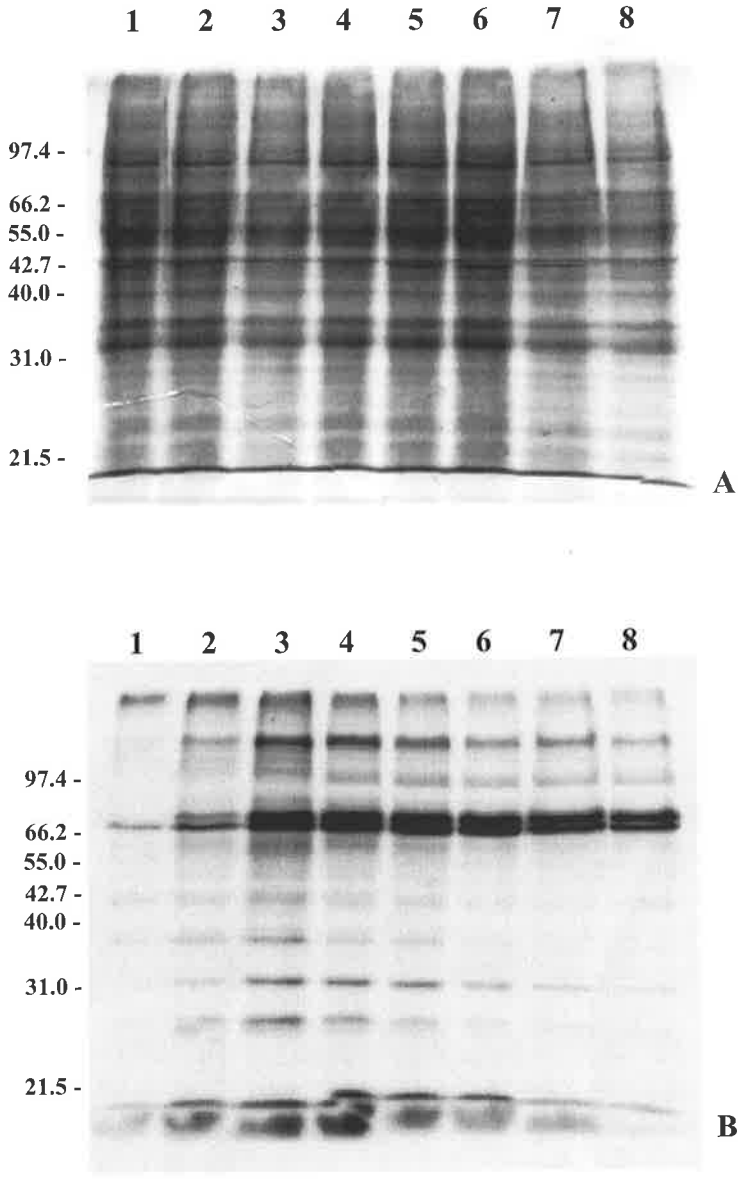


Fig. 5.3 Analysis of Protein Phosphorylation during Pollen Germination.
The phosphorylation reaction was started by adding [γ-^{33}P]-ATP to the germinating pollen grains and terminated one minute later. Pollen proteins were separated by SDS-PAGE and the dried gel exposed to X-ray film.

Time course with 2 min intervals (from left to right).
Lane 1: germination medium and γ-^{33}P-ATP were added simultaneously
Lane 2: γ-^{33}P-ATP was added 2 min after addition of germination medium
Lane 3: γ-^{33}P-ATP was added 4 min after addition of germination medium etc.

A shows the stained protein gel, **B** the corresponding autoradiogram.
Protein size marker is in kilodalton (kDa).

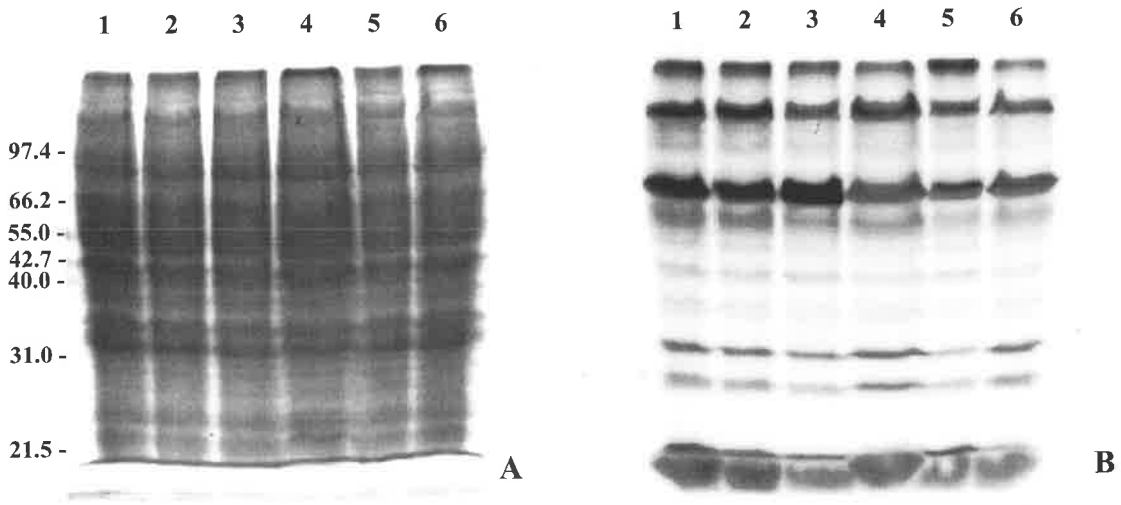


Fig. 5.4 Analysis of Protein Phosphorylation during Pollen Germination in the Presence of Stigma Eluate. Pollen was germinated for 4 minutes.
A displays the protein gel, **B** the corresponding autoradiogram.
Protein size marker is in kilodalton.

Lane 1: pollen from $S_{1.2}$ $Z_{1.1}$ germinated with self stigma eluate
Lane 2: pollen from $S_{1.2}$ $Z_{1.1}$ germinated with cross stigma eluate
Lane 3: pollen from $S_{1.2}$ $Z_{1.1}$ germinated without stigma eluate
Lane 4: pollen from $S_{1.2}$ $Z_{2.2}$ germinated with cross stigma eluate
Lane 5: pollen from $S_{1.2}$ $Z_{2.2}$ germinated with self stigma eluate
Lane 6: pollen from $S_{1.2}$ $Z_{2.2}$ germinated without stigma eluate

clear evidence for the presence of either cAMP-and cGMP-dependent protein kinases or protein kinase C in plants (see 5.1).

In order to analyse whether the S-protein was phosphorylated, several preliminary experiments were carried out. Firstly, the S-protein of the pollen was detected immunologically by Western blotting. For the immunodetection experiment, pollen from plants with different S-genotypes was used to include all possible variations in putative phosphorylation sites which may result from the allelic differences at the protein sequence level. However, superimposition of the autoradiogram of the exposed membrane and the immunodetection indicated that there was no correlation of phosphorylated proteins with the S-protein (Fig. 5.5 A, B). This result was independent of the assay system used. Secondly, immunoprecipitation was employed to enrich the S-protein after the assay to increase the sensitivity of the assay and therefore exclude the possibility that the phosphorylation signal was missed due to low protein concentration. As is shown in Fig 5.6, no evidence for phosphorylation of the S-protein was obtained. The same result was obtained when the experiment was carried out with pollen in germination medium.

Although the results of the immunoprecipitation experiments were negative, they cannot exclude the possibility that the S-protein is phosphorylated *in vivo* during the interaction with the stigma. To investigate this, the immunoprecipitation experiment was repeated in the presence of extracted total stigma protein. There were two reasons for using total stigma protein. Firstly, previous experiments with stigma eluate in germination medium had indicated that the basic phosphorylation of pollen proteins is not influenced by stigma eluates (see above). The second reason was to ensure that the stigmatic components which triggered the SI response were present in the reaction, since it is conceivable that these components are not diffusible and therefore absent in stigma eluate. As can be seen in Fig. 5.7 A-D, there is no evidence for the phosphorylation of the S-protein in response to either self- or cross-stigma protein.

5.3 Discussion

In this chapter the role of protein phosphorylation in pollen germination and the self-incompatibility reaction in *Phalaris* was investigated. Basic protein phosphorylation of pollen from self-incompatible plants and self-fertile (sf) mutants of *Phalaris* was examined to determine whether the self-compatible phenotype correlated with altered phosphorylation of any proteins. Included in this analysis were the S-complete, S-pollen-only, Z-pollen-only and T-mutants. All T-mutants of the Adelaide population are pollen-only, that is the mutation affects only the SI specificity of the pollen, whereas it is retained in the pistil (Hayman and Richter 1992). In contrast to the results obtained by Wehling et al. (1994), evidence for failed

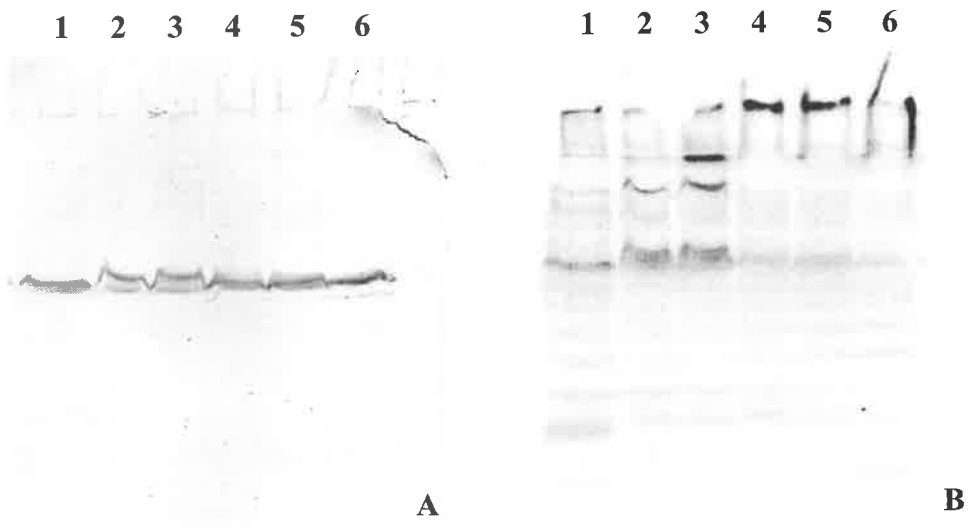


Fig. 5.5 Immunodetection of the S-protein and Analysis of Pollen Protein Phosphorylation. Pollen was germinated in germination medium, except for the pollen in lane 1 which was assayed after Wehling et al. (1994). Phosphorylation was examined after 4 min of germination, except for lane 1: 90 sec, and lane 5: 5 min.

A : Immunoblot

B : Autoradiogram of the membrane exposed after immunodetection

The two figures are aligned.

Lane 1: equal amounts of pollen with alleles $S_{1.4}$, $S_{2.2}$, $S_{1.1}$

Lane 2 and 3: $S_{1.2}Z_{1^{o}.1^{o}}$, Lane 4: $S_{1.4}Z_{1.3}$, Lane 5: $S_{2.2}Z_{1.2}$, Lane 6: $S_{1.1}Z_{1.2}$

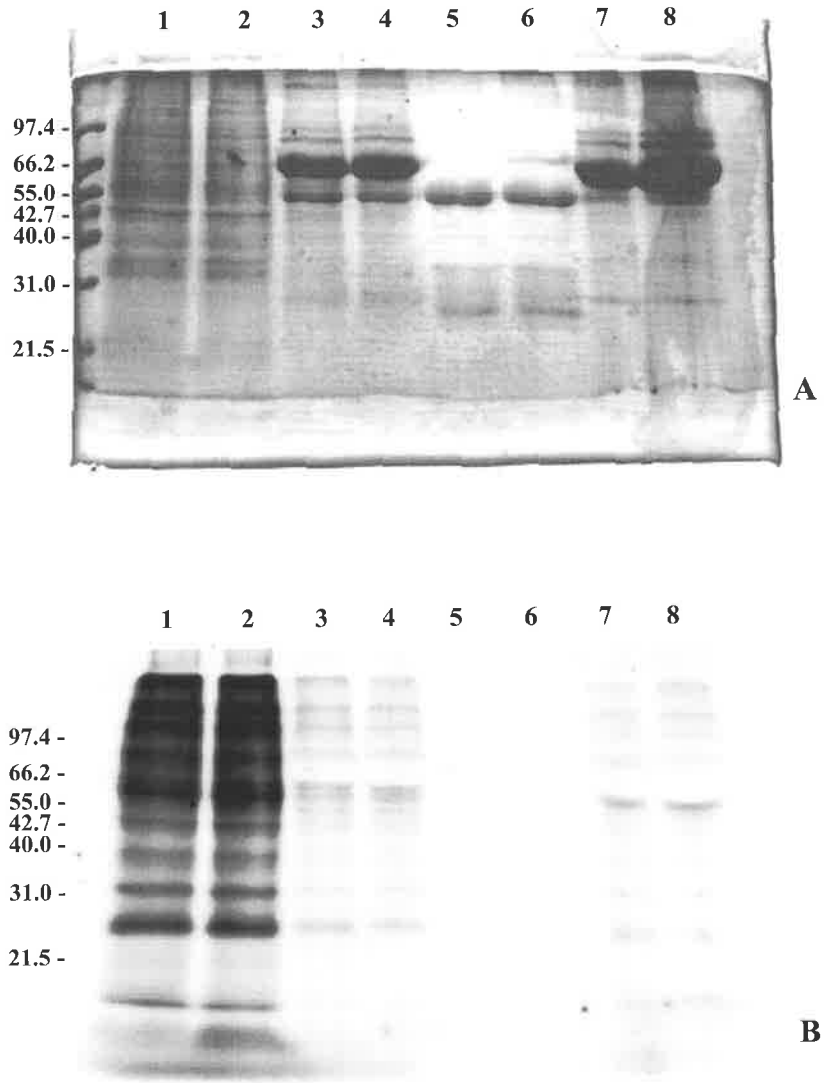
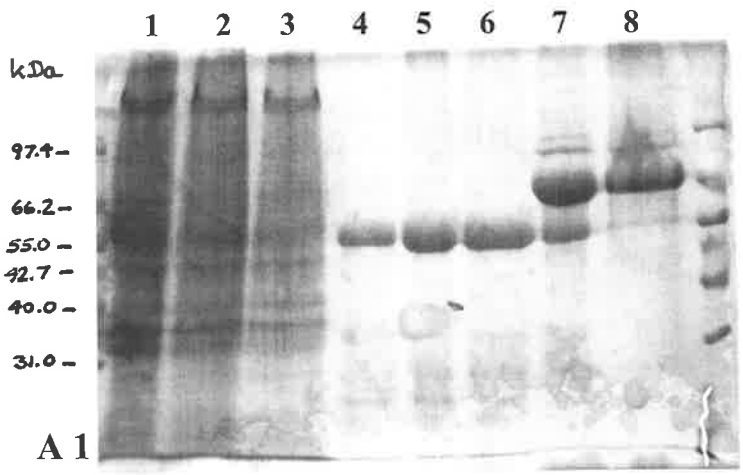


Fig 5.6 Immunoprecipitation of the S-protein after the Protein Phosphorylation Assay. The assay was performed after Wehling et al. (1994).
A shows the protein gel, **B** the corresponding autoradiogram.
Phosphorylation was assayed 60 and 90 sec after the start of the reaction.
Protein size marker is in kilodalton.
Lane 1 and 2: control: pollen pellet after the phophorylation reaction (60 sec and 90 sec)
Lane 3 and 4: control: immunoprecipitate after incubation with pre-immuneserum
Lane 5 and 6: immunoprecipitate after incubation with Anti-S serum
Lane 7 and 8: control: supernatant after immunoprecipitation

Fig 5.7 Analysis of S-protein Phosphorylation in Pollen Germinated in the Presence of Stigma Proteins.
The S-protein was immunoprecipitated after the phosphorylation reaction. Pollen was germinated for 4 min.
A1 and B1 display the protein gels, A2 and B2 the corresponding autoradiograms.

A1, A2: Lane 1 to lane 3: controls: pellets of pollen germinated in the presence of no, self- and cross- stigma proteins, respectively .
Lane 4 to lane 6: immunoprecipitates of pollen germinated in the presence of no, self- and cross- stigma proteins, respectively .
Lane 7: control: supernatant after immunoprecipitation.
Lane 8: control: immunoprecipitate after incubation with pre-immuneserum.

B1, B2: Lane 1 to lane 3: immunoprecipitates of pollen germinated in the presence of no, self- and cross- stigma proteins, respectively .
Lane 4: control: immunoprecipitate from stigma proteins.
Lane 5: control: supernatant after immunoprecipitation
Lane 6: control: immunoprecipitate after incubation with pre-immuneserum
Lane 7: control: stigma proteins after phosphorylation reaction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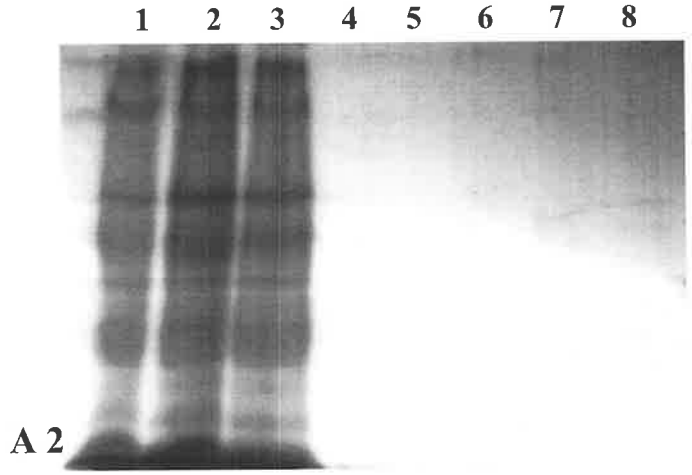
A 1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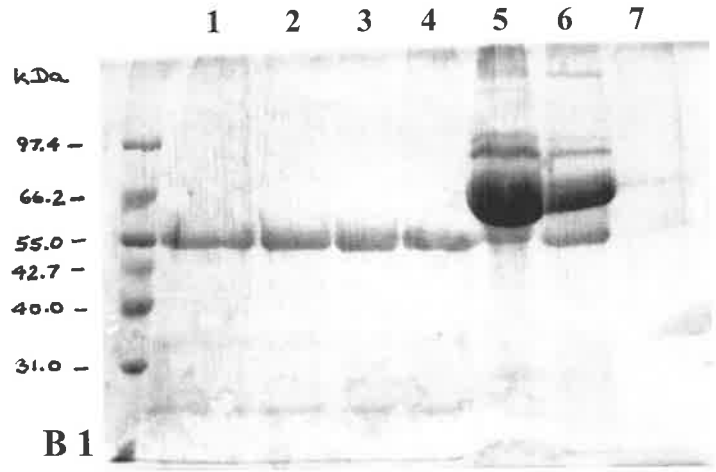
A 2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kDa
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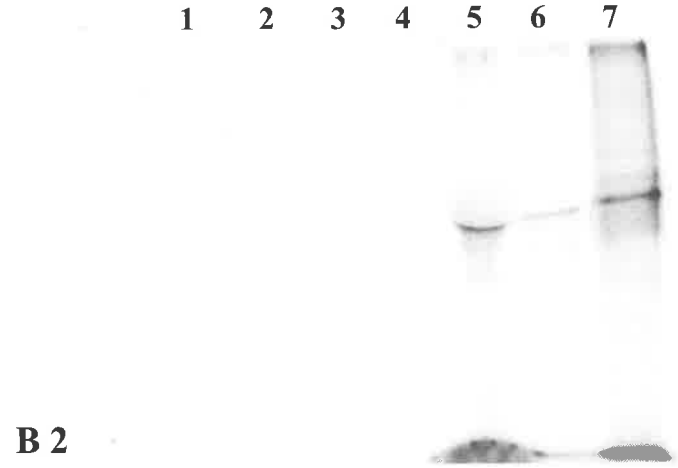
1 2 3 4 5 6 7
B 2

phosphorylation of pollen proteins was not found in any of the *Phalaris* mutants. The mutation of the self-fertile plant which was used in the analyses of Wehling et al. (1994) has not been mapped. It is conceivable that this mutation is localised at a locus different from the SI loci identified in *Phalaris* .

The overall pattern of the phosphorylated pollen proteins of *Phalaris* differed from that obtained in rye. In *Phalaris* a large number of proteins appeared to be phosphorylated in addition to the major proteins of 26, 31, 64 and more than 100 kDa. It is unknown whether these differences were due to species-specific variations or the experimental design, such as exposure times of the protein gels.

Wehling et al. (1994) tried to simulate the *in vivo* situation as closely as possible to study protein phosphorylation. However, the phosphorylation reaction was performed in an artificial buffer system. Here it was observed that pollen would not germinate in the buffer used by Wehling et al. (1994) and it was important to find out whether it was feasible to observe pollen protein phosphorylation directly and omit the artificial buffer system. This study demonstrated that it was indeed possible to monitor transient protein phosphorylation of pollen grains germinating in medium *in vitro*. Phosphorylation activity was found to be highest around 4 minutes after the start of pollen germination. Some proteins, such as the ca. 66 kDa proteins, seemed to be phosphorylated throughout the time course, whereas others were predominantly phosphorylated early during pollen tube development. A comparison of the pattern obtain here with those from the previous experiments indicated an overall similarity of phosphorylated proteins, which suggests that there was no dramatic difference between the two assay systems. However, it was not possible to reproduce the effects of stigma eluate on protein phosphorylation described by Wehling et al. (1994), which might suggest that either the "germination medium assay" was not sensitive enough or that the experimental conditions require further refinement.

What is the nature of the proteins which are phosphorylated during pollen tube growth? In Chapter 3 three cDNA clones which showed sequence homology with protein kinases were identified and their transcripts detected in pollen. Two of the clones, A12a and B4e, can be considered pollen-specific, whereas Bm7d is also expressed during anther development. One could speculate that Bm7d is one of the phosphorylated proteins, since MAP-kinases are activated by phosphorylation (see 5.1.1). The target proteins of A12a and B4e might constitute others. Enzymes, such as the mitochondrial pyruvate dehydrogenase, have been shown to be regulated by reversible phosphorylation in plants (Huber et al. 1994) and might represent some of the phosphorylated proteins here.

From the results obtained it seems unlikely that the S-protein itself is a target for phosphorylation during pollen germination. Nor was any evidence obtained for the phosphorylation of the S-protein in response to stigma proteins. These finding suggest that signal transduction involved in SI might be more complex than the simple phosphorylation of the S-protein. At present it is not possible to discount that protein kinase activity might be involved further downstream in the self-incompatibility signal cascade, perhaps in a similar fashion to the *Papaver* system, for which transient phosphorylation of pollen proteins in response to self-stigma extract has been described (Franklin-Tong et al. 1992).

Future studies, in which inhibitors directed towards different types of protein kinases are added to the growing pollen tubes, might provide information on which of the proteins are phosphorylated by, for instance, serine/threonine specific protein kinases. Ultimately, the phosphorylated proteins would have to be isolated and sequenced to answer the question of their nature entirely.

One limitation in this type of study was the lack of an optimal *in vitro* germination system for the pollen of *Phalaris*, as grass pollen does not generally germinate well *in vitro* (Heslop-Harrison 1979a). Although the germination medium used here was optimised for *Phalaris* pollen, several bursting pollen tubes were always observed, usually 10-15 min after adding the pollen to the germination medium, and pollen tube length was highly variable. Although there were clear differences in the effect of stigma eluate or total stigma proteins on pollen tube growth compared to control pollen germinated without stigma extract, in no case was this effect genotype dependent. A similar unspecific effect was observed by Franklin-Tong et al. (1988). However, in their study the inhibitory effect of self-stigma eluate on pollen tube length was more pronounced than that of cross-stigma eluate.

In conclusion, for future studies it will be necessary to further optimise the germination system, for instance, by testing whether PEG is a more suitable osmoticum than sucrose and prevents early bursting of pollen tubes. Experiments which involve stigma protein will require further refinement, such as using different fractions of the stigma proteins, to a point that genotype specific retardation of pollen tube growth can be observed. Once such a bioassay system is established it will be possible to investigate where in the cascade of events protein phosphorylation and SI are connected.

Chapter 6 General Discussion

The objective of this work was to analyse pollen-specific genes and their contribution to pollen germination, pollen tube growth and specifically to the self-incompatibility reaction. One of the aims targeted was the isolation of the Z-gene. A similar strategy to that which identified the S-gene failed to isolate Z. At present the most likely reason for the failure of the attempt to isolate Z is the methodology applied. To detect sequence divergence by using differential screening, sequences must have a minimum of 10% mismatches and these mismatches should not be clustered (see Chapter 3). It is possible that the sequence variation between the Z-alleles used in this study does not fulfil these requirements. It is now known that the isolation of the S-gene was serendipitous, since the S-alleles are not sufficiently different to allow their isolation by this technique (Li et al. 1994). One could speculate that the low sequence divergence between alleles, as demonstrated for the S-gene and indicated for the Z-gene by the results of this study, is a feature of two-locus SI systems. This phenomenon certainly contrasts with the single-locus systems, for which high degrees of sequence divergence seem to be the rule (Ioerger et al. 1990).

The results obtained in this study give clues to possible future approaches to isolating Z. One factor which needs to be taken into consideration is the high variability among the plants of the Adelaide population, in spite of their relatedness. The results of the Northern blot experiments showed variation between pollen from different plants in expression levels of the transcripts analysed. The RFLP analyses showed polymorphic patterns with all clones tested. Since self-incompatible plants are highly outbreeding, this high degree of polymorphism is not surprising. However such a high level of polymorphism is problematic, potentially leading to a high number of false positives, when one tries to isolate a single gene. To generate more isogenic plants, several approaches are theoretically feasible. One could try to generate doubled haploid plants from microspores, as has been achieved with barley (Luckett and Smithard 1992). Alternatively, one could attempt to overcome the self-incompatibility mechanism. Overcoming SI by bud-pollination, as described in other systems (de Nettencourt 1977), has not succeeded for *Phalaris* (David Hayman, pers. comm. 1993) and the generation of homozygous plants through forced selfing under high temperature conditions (Wricke 1978; Wilkins and Thorogood 1992) has not worked with *Phalaris* (data not shown). However, other means might prove successful, such as exposing flowering plants to elevated $C0_2$ concentrations, as has been done in practical breeding of *Brassica campestris* and *Raphanus sativus* (Hinata et al. 1993) .

Alternatively, mutagenesis could be carried out to generate deletion-mutants of Z. From results obtained in our laboratory, it is likely for two reasons that the self-fertile phenotype of the mutants of the Adelaide population is due to point mutations. Firstly, the sequence of the S-complete mutant was shown to have 4 single base changes, of which 3 resulted in amino acid substitutions (Li et al. 1994). The S-pollen-only mutant analysed showed no differences in sequence compared to that of the wild-type (X. Li, pers. comm., 1994). Secondly, if there was a deletion mutant among the Z-mutants analysed in this study, it should have been detected by the analyses performed. Since there was no evidence found for missing or truncated transcripts in this work (apart from B7b which was demonstrated not to be linked to any of the SI-loci), the likelihood of any of our Z-mutants having a major deletion is small. Z mutations caused by deletions of the gene would have the advantage that they are easier to detect molecularly than point mutations. However large deletions tend to have not only deleterious effect for the plant but might complicate the identification of the transcription unit responsible for the self-compatible phenotype if several genes are deleted. A possible compromise might be to use mutagens such as diepoxybutane, that has been successfully used to generate deletions of only a few kilobases long in *Drosophila*. (Ashburner 1989).

The above mentioned approaches have the disadvantage that they are very time-consuming. A faster alternative involves the use of the differential display technique (Liang and Pardee 1992). The method is sensitive enough to detect low and clustered allelic variations and could be successful, provided that enough controls are run to counteract the high degree of polymorphism. An advantage of using differential display would also be that it would not be necessary to incorporate the assumptions made here, such as that Z is pollen-specific and that the Z-transcript is of relatively high abundance in pollen. Of course, the differential display technique could be combined with any of the suggestions made above. For these reasons, the differential display technique might be the method of choice in future.

Finally, the isolation of Z could be attempted by assuming that S and Z form a heterodimer. However, preliminary experiments in this direction have not provided unambiguous evidence for the formation of a heterodimer, although bands of higher molecular weight than expected for the S-protein itself have been detected (data not shown). S protein homo-multimers have been observed following the expression of this protein in *E. coli* (X. Li, pers.comm., 1994) and could be the explanation for the high molecular weight bands observed.

On the other hand, the investigation of the thioredoxin reductase of *Phalaris* would be very interesting, since the S-protein contains a conserved domain with sequence homology to thioredoxin h. This domain was able to catalyse the reduction of the disulfide bonds in insulin and act as a substrate for *E. coli* thioredoxin reductase

(Li et al. 1995). Therefore, the isolation of the thioredoxin reductase of *Phalaris*, its genomic location and interaction with the S-protein is not only of importance for the complete description of upstream or downstream events in the self-incompatible reaction, but thioredoxin reductase itself might be a possible candidate for the Z-gene.

Besides the isolation of the Z-gene, the second aim of this work was to identify and characterise pollen-specific genes. The data obtained from the transcriptional and sequence analyses of 18 pollen-expressed cDNA clones provide information on a wide range of physiological activities of the pollen grain which occur once it contacts the stigma.

A16e, the clone which shows homology to fructose-1,6-biphosphate aldolase, provides information on pollen metabolism. It will be interesting to study the regulation of this gene and also to determine if *Phalaris* pollen produces ethanol as was shown for pollen from tobacco (Bucher et al. 1995). It would be intriguing if the tricellular pollen grains of *Phalaris*, which germinate and extrude pollen tubes within minutes of contact with the stigma or germination medium, showed a similar respiration to the bicellular grains of tobacco, which take at least half an hour to germinate (L.-H. Ji, pers. comm. 1995).

C7c, the *LAT52* homolog, is an example of a gene which is likely to be involved in aspects of pollen hydration and tube germination as demonstrated for tomato (Muschietti et al. 1994).

Protein kinases are a universal component in the control of a large number of developmental phenomena and their presence and importance in pollen has been supported by the results of this study. Two of the pollen-specific protein kinases isolated here, A12a and B4e, are very abundantly expressed in pollen, and from this observation alone it can be surmised that they are necessary for pollen funtion. In case of A12a, further analysis will show whether this clone represents a new pollen-specific protein kinase. The presence of a pollen-specific calcium-dependent protein kinase, to which B4e shows homology, has already been demonstrated in maize (Estruch et al. 1994), and it has been suggested that this protein kinase has a crucial role in pollen germination and pollen tube growth, possibly regulating the activity and structure of the cytoskeleton.

Bm7d, another clone encoding a protein with homology to protein kinases is, to my knowledge, the first MAP-kinase shown to be expressed in the pollen of a grass and only the second MAP-kinase isolated from grasses (Huttly and Phillips 1995). The only other MAP-kinase shown to be expressed in pollen, ntf3, was isolated from tobacco (Wilson et al 1993). The expression of MAP-kinases in pollen from monocots and dicots suggests a general importance for this particular type of kinases in pollen function. It is possible that a signal cascade via MAP-kinase leads to the modulation of gene expression, similar to what has been shown in animals and yeast.

The observation made here that protein phosphorylation takes place during pollen germination was anticipated from previous research (Polya et al.1986; Wehling et al. 1994). However, the experimental system designed in this study, which allows the monitoring of protein phosphorylation during pollen tube growth in germination medium, might allow a more direct investigation of the protein kinases involved and the proteins phosphorylated. As demonstrated, a significant number of proteins are phosphorylated during pollen tube growth. This finding again supports the assumption of the importance of protein phosphorylation during pollen tube growth. It was further shown that the S-protein was not among the phosphorylated proteins, and additional study is necessary to find the connection, if any, between self-incompatibility and protein phosphorylation.

An intriguing finding of this study is B7b which represents a class of genes with unusual features. The apparent absence of these genes in some plants suggests that they might be expendable during pollen germination and tube growth. Alternatively, members of this gene-family are so variable that they do not cross-hybridise. Although they are abundantly expressed in pollen of *Phalaris*, their function is currently elusive. Further studies on the presence and expression of this particular family in *Phalaris,* as well as in other plants, might provide clues to its possible role in pollen.

Six of the cDNA clones isolated in this study were also used in Southern blot hybridisation with DNA from barley, and all of them gave a hybridisation signal (data not shown). Due to the relatedness of the grasses as a phylogenetic group (Moore et al. 1995), it is likely that the further study of the genes isolated here will provide an insight into pollen germination and tube growth not only in *Phalaris* itself, but also for the agriculturally important grasses. That *Phalaris* can be used as a representative for grasses is also supported by the characterisation of C7c, which showed high homology to the pollen-specific clones identified in maize and rice.

On the other hand, it should be possible to determine the exact function of the new genes identified in *Phalaris* by taking advantage of already established transformation systems in the related grasses, rye and barley (Castillo et al. 1994; Funatsuki et al. 1995). Promotor analyses of the pollen-specific genes of *Phalaris* might provide tools for the generation of male-sterile crop plants, which are important in breeding programs.

Appendix A

Results of the Screen for Pollen-Specificity

	root	leaf	seedling	seed	young anther	
Am1a	-	-	-	-	-	Am1a
b	-	-	-	-	-	b
c	+	+-	-	+-	-	c
d	-	-	-	-	-	d
e	+	+-	-	+-	-	e
f	+	+-	-	+-	-	f
Am2a	-	-	-	-	-	Am2a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
Am3a	-	-	-	-	-	Am3a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
Am4a	++	+	++	+-	++	Am4a
b	-+	+	-	+-	-	b
c	+	+	-	-	-	c
d	-	+-	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
Am5a	-+	-	-	-	-	Am5a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	+-	-	-	-	d
e	-	+-	-	-	-	e
f	-	-	-	-	-	f
Am6a	+	+-	-	+-	-	Am6a
b	+	+	-	+-	-	b
c	+	+-	-	+-	-	c
d	+	+-	-	+-	-	d
e	+	+	+-	+-	-	e
f	++	+-	++	+-	++	f
A1a	-+	+-	-	+-	-	A1a
b	+	+-	-	+-	-	b
c	+	+-	-	-	-	c
d	-	-	-	-	-	d
e	+-	+-	-	-	-	e
f	+-	+	-	-	-	f

Results of the Screen for Pollen-Specificity - continued

	root	leaf	seedling	seed	young anther	
A2a	-	-	-	-	-	A2a
b	-	+ -	-	-	-	b
c	+ +	+	+ +	+ -	+ +	c
d	-	-	-	-	-	d
e	+	-	+	-	+ +	e
f	-	-	-	-	-	f
A3a	+ +	+	+ +	+ -	+ +	A3a
b	+ +	+	+ +	+ -	+ +	b
c	+	+ -	-	+ -	+	c
d	+ +	+ -	+	+ -	+	d
e	+ +	+ -	+	+ -	+	e
f	+ +	+	+	+ -	+	f
A4a	+	+ -	+ +	-	+	A4a
b	+	+ -	-	-	-	b
c	+	+ -	-	-	-	c
d	+	+ -	-	-	-	d
e	+	+	-	-	-	e
f	+	+	-	-	-	f
A5a	- +	+ -	-	-	-	A5a
b	+ -	+ -	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	+	+	-	-	-	e
f	+	+	-	-	-	f
A6a	-	-	-	-	-	A6a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	- +	-	-	-	-	d
e	-	-	-	-	-	e
f	+	- +	-	-	-	f
A7a	+	- +	-	-	-	A7a
b	-	-	-	-	-	b
c	+	+ -	-	-	-	c
d	- +	+ -	-	-	-	d
e	+	+ -	-	-	-	e
f	-	-	-	-	-	f
A9a	+ +	-	+	-	+	A9a
b	+ +	+	+	+ -	+	b
c	+	+	-	+ -	-	c
d	+	+	-	+ -	-	d
e	- +	- +	-	-	-	e
f	+	+	-	+ -	-	f
A10a	+ -	-	-	+ -	-	A10a
b	+ -	+ -	-	+ -	-	b
c	+ -	+ -	+ +	+ -	+ +	c
d	-	+ -	-	-	-	d
e	-	-	-	+ -	-	e
f	-	+ -	-	-	-	f

Results of the Screen for Pollen-Specificity - continued

	root	leaf	seedling	seed	young anther	
A11a	- +	+ -	-	-	-	A11a
b	+	+	-	-	+ -	b
c	+	+	-	-	+ -	c
d	+	+	-	-	+ -	d
e	+	+ -	-	-	-	e
f	-	-	-	-	-	f
A12a	-	-	-	-	-	A12a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	- +	-	-	-	-	f
A13a	-	-	-	-	-	A13a
b	-	-	-	-	-	b
c	- +	- +	-	-	-	c
d	- +	- +	-	-	-	d
e	- +	-	-	-	-	e
f	- +	-	-	-	-	f
A14a	- +	-	-	-	-	A14a
b	- +	-	-	-	-	b
c	- +	-	-	-	-	c
d	- +	-	-	-	-	d
e	+	+ -	-	-	-	e
f	-	-	-	-	-	f
A15a	- +	+ -	-	-	-	A15a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	+ -	- +	-	-	-	e
f	- +	-	-	-	-	f
A16a	+	+	+	+ -	+ -	A16a
b	+	+	+	+ -	+ -	b
c	+	+	-	+ -		c
d	+	+	+	-	+ -	d
e	- +	-	- +	-	-	e
f	- +	- +	-	-	-	f
A17a	- +	- +	-	-	-	A17a
b	+	- +	-	-	-	b
c	+	- +	-	-	-	c
d	- +	-	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
A27a	-	-	-	-	+	A27a
b	- +	- +	-	-	-	b
c	+	+ -	-	-	-	c
d	-	- +	-	-	-	d
e	-	-	-	-	-	e
f	- +	-	-	-	-	f

Results of the Screen for Pollen-Specificity - continued

	root	leaf	seedling	seed	young anther	
A28a	- +	-	+ -	-	-	A28a
b	- +	-	-	-	-	b
c	- +	-	-	-	-	c
d	+	+ -	-	-	-	d
e	- +	- +	-	-	-	e
f	- +	- +	-	-	-	f
A29a	+	+	+	+ -	+	A29a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	+	- +	-	+ -	-	e
f	+ +	+	+ +	+ -	+ +	f
A30a	-	-	-	-	-	A30a
b	+-	-	-	-	-	b
c	- +	-	-	-	-	c
d	-	-	-	-	-	d
e	-	- +	-	-	-	e
f	- +	-	+ -	-	+ -	f
A31a	- +	-	+	-	+ -	A31a
b	+ +	- +	+	-	+	b
c	+	- +	-	-	-	c
d	- +	-	-	-	-	d
e	-	-	-	-	-	e
f	+	- +	-	+ -	-	f
A32a	- +	- +	+	+ -	-	A32a
b	-	-	-	-	-	b
c	-	- +	-	-	-	c
d	-	- +	-	-	-	d
e	-	- +	-	-	-	e
f	-	-	-	-	-	f
A33a	- +	-	+	-	-	A33a
b	-	-	-	-	-	b
c	- +	-	-	-	-	c
d	-	-	-	-	-	d
e	-	-	+	-	-	e
f	-	-	-	-	-	f
A34a	-	-	-	-	-	A34a
b	-	-	-	-	-	b
c	-	- +	-	-	-	c
d	-	- +	-	-	-	d
e	-	- +	-	-	-	e
f	-	-	-	-	-	f
A35a	-	- +	-	-	-	A35a
b	-	- +	-	-	-	b
c	-	- +	-	-	-	c
d	- +	-	-	-	-	d
e	-	+ -	-	-	-	e
f	-	-	-	-	-	f

Results of the Screen for Pollen-Specificity - continued

	root	leaf	seedling	seed	young anther	
A36a	-	-	-	-	-	A36a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-+	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
Bm1a	+	++	++	+	++	Bm1a
b	+	++	++	+	++	b
c	+	++	++	+	++	c
d	+	++	++	+	++	d
e	-	-	-	-	-	e
f	+	++	++	++	++	f
Bm2a	+	+	-	-+	-	Bm2a
b	+	+	-	-+	-	b
c	-	-	-	-	-	c
d	-+	-	-	+	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
Bm3a	-	-	-	-	-	Bm3a
b	-	-	-	-	-	b
c	+	-+	-	-+	-	c
d	-	-+	-	-	-	d
e	-	-+	-	-	-	e
f	-	-+	-	-	-	f
Bm4a	++	++	+	+	+	Bm4a
b	-	-	-	-	-	b
c	-+	-+	-	-+	-	c
d	-+	++	++	+	++	d
e	+	++	++	+	++	e
f	-+	-+	+	-	++	f
Bm5a	-	-+	-	-	-	Bm5a
b	+	-+	-	-+	-	b
c	+	-+	-	-+	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
Bm6a	++	++	+	+	+	Bm6a
b	-+	-	-	-	-	b
c	-	-	-	-	-	c
d	++	+	++	+	+	d
e	-+	-+	-	-+	-	e
f	-+	-+	-	-	-	f
Bm7a	-+	-+	-	-	-	Bm7a
b	-	-	-	-	-	b
c	-+	-+	-	-	-	c
d	-+	-+	-	-	-	d
e	-+	-+	-	-	-	e
f	-	-	-	-	-	f

Results of the Screen for Pollen-Specificity - continued

	root	leaf	seedling	seed	young anther	
A36a	-	-	-	-	-	A36a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	- +	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
Bm1a	+	+ +	+ +	+	+ +	Bm1a
b	+	+ +	+ +	+	+ +	b
c	+	+ +	+ +	+	+ +	c
d	+	+ +	+ +	+	+ +	d
e	-	-	-	-	-	e
f	+	+ +	+ +	+ +	+ +	f
Bm2a	+	+	-	- +	-	Bm2a
b	+	+	-	- +	-	b
c	-	-	-	-	-	c
d	- +	-	-	+	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
Bm3a	-	-	-	-	-	Bm3a
b	-	-	-	-	-	b
c	+	- +	-	- +	-	c
d	-	- +	-	-	-	d
e	-	- +	-	-	-	e
f	-	- +	-	-	-	f
Bm4a	+ +	+ +	+	+	+	Bm4a
b	-	-	-	-	-	b
c	- +	- +	-	- +	-	c
d	- +	+ +	+ +	+	+ +	d
e	+	+ +	+ +	+	+ +	e
f	- +	- +	+	-	+ +	f
Bm5a	-	- +	-	-	-	Bm5a
b	+	- +	-	- +	-	b
c	+	- +	-	- +	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
Bm6a	+ +	+ +	+	+	+	Bm6a
b	- +	-	-	-	-	b
c	-	-	-	-	-	c
d	+ +	+	+ +	+	+	d
e	- +	- +	-	- +	-	e
f	- +	- +	-	-	-	f
Bm7a	- +	- +	-	-	-	Bm7a
b	-	-	-	-	-	b
c	- +	- +	-	-	-	c
d	- +	- +	-	-	-	d
e	- +	- +	-	-	-	e
f	-	-	-	-	-	f

Results of the Screen for Pollen-Specificity - continued

	root	leaf	seedling	seed	young anther	
Bm8a	-	-	-	-	-	Bm8a
b	+	- +	-	- +	-	b
c	-	-	-	-	-	c
d	+	- +	-	- +	-	d
e	+	- +	-	- +	-	e
f	+	- +	-	- +	-	f
Bm9a	- +	- +	-	- +	-	Bm9a
b	- +	-	-	- +	-	b
c	-	-	-	- +	-	c
d	+	+	-	-	-	d
e	- +	- +	-	-	-	e
f	- +	-	-	-	-	f
Bm10a	- +	-	-	-	-	Bm10a
b	- +	-	-	-	-	b
c	+	- +	+	- +	+	c
d	+	- +	-	- +	-	d
e	-	-	-	-	-	e
f	+	- +	-	- +	-	f
Bm11a	+	- +	-	+	-	Bm11a
b	+	+-	-	+	-	b
c	-	-	-	-	-	c
d	+	+-	-	+	-	d
e	-	-	-	-	-	e
f	+	-	-	+	-	f
Bm12a	-	-	-	-	-	Bm12a
b	- +	- +	-	- +	-	b
c	- +	- +	-	- +	-	c
d	- +	- +	-	- +	-	d
e	- +	-	-	- +	-	e
f	+	- +	-	- +	-	f
Bm13a	+	- +	-	-	-	Bm13a
b	- +	-	-	-	+	b
c	+ +	+ +	+ +	+	+ +	c
d	+ +	+ +	+ +	+	+ +	d
e	+-	- +	-	-	+	e
f	+	- +	+	-	- +	f
Bm14a	+-	- +	-	-	-	Bm14a
b	-	-	-	-	-	b
c	+-	- +	-	-	-	c
d	+-	- +	-	-	-	d
e	+-	- +	-	-	-	e
f	+ +	+ +	+ +	+	+ +	f
Bm15a	+-	- +	-	- +	-	Bm15a
b	+-	- +	-	- +	-	b
c	+	+-	-	- +	-	c
d	+	+-	-	- +	-	d
e	+	- +	-	- +	-	e
f	- +	-	-	-	-	f

Results of the Screen for Pollen-Specificity - continued

	root	leaf	seedling	seed	young anther	
Bm16a	- +	- +	-	-	-	Bm16a
b	- +	- +	-	-	-	b
c	- +	- +	-	-	-	c
d	- +	- +	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
Bm17a	-	-	-	-	-	Bm17a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
Bm18a	-	-	-	-	-	Bm18a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
Bm19a	-	-	-	-	-	Bm19a
b	-	-	-	-	-	b
c	- +	- +	-	-	-	c
d	- +	- +	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
Bm20a	-	-	-	-	-	Bm20a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	+	+	-	+	e
f	-	+	+	-	+	f
B1a	+	-	-	-	-	B1a
b	+ -	-	-	-	+ -	b
c	+	+ -	-	+ -	+ -	c
d	+	+ -	-	+ -	+	d
e	+ -	-	-	-	-	e
f	-	-	-	-	+	f
B2a	+ +	+ -	-	+ -	-	B2a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	+ +	+	-	+	-	e
f	-	-	-	-	-	f
B3a	-	-	-	-	-	B3a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	+ -	-	-	-	-	e
f	+ -	-	-	-	-	f

Results of the Screen for Pollen-Specificity - continued

	root	leaf	seedling	seed	young anther	
B4a	-	-	-	-	-	B4a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
B5a	-	-	-	-	-	B5a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
B6a	+-	+	-	+-	-	B6a
b	+-	+	-	+-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	+-	+-	-	-	-	f
B7a	-	-	-	-	-	B7a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
B8a	++	++	++	+	++	B8a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	+	-	+-	-	e
f	+-	+-	-	-	-	f
B9a	+-	+-	-	-	-	B9a
b	+	+-	-	-	-	b
c	-	-	-	-	-	c
d	+-	-	-	-	-	d
e	+-	+-	-	-	-	e
f	-	-	-	-	-	f
Cm1a	+-	-	-	-	-	Cm1a
b	+-	+-	-	-	-	b
c	-	-	-	-	-	c
d	+	++	+	+-	+	d
e	+-	+	+	+-	+	e
f	+-	+	+	-	+	f
Cm2a	-	-	-	-	-	Cm2a
b	-	-	-	-	-	b
c	-	-	-	-	+	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f

Results of the Screen for Pollen-Specificity - continued

	root	leaf	seedling	seed	young anther	
Cm3a	-	-	-	-	-	Cm3a
b	++	++	+	+	++	b
c	-	+-	-	+-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	+-	-	-	-	f
Cm4a	-	+-	-	-	-	Cm4a
b	-	+-	-	-	-	b
c	+-	-	-	-	-	c
d	+-	-	-	-	-	d
e	+-	-	-	-	-	e
f	+-	+-	-	-	-	f
Cm5a	-	-	-	-	-	Cm5a
b	+	+-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	+-	+	+	+-	++	f
Cm6a	++	++	+	+	++	Cm6a
b	++	++	+	+	++	b
c	+	++	+	+	++	c
d	+-	+-	-	-	-	d
e	-	+-	-	-	-	e
f	-	+-	-	-	-	f
Cm7a	-	-	-	-	-	Cm7a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	+-	+	++	+	+	e
f	-	-	-	-	-	f
Cm8a	-	-	-	-	-	Cm8a
b	+-	++	++	+	+	b
c	-	-	-	-	-	c
d	+-	+	++	+	+	d
e	+-	++	++	+	+	e
f	+-	++	++	+	+	f
Cm9a	-	-	-	-	-	Cm9a
b	-	-	-	-	-	b
c	+-	-	-	-	-	c
d	-	-	-	-	-	d
e	+-	-	-	+-	-	e
f	+-	+-	-	+-	-	f
Cm10a	+	++	++	++	++	Cm10a
b	-	-	-	-	-	b
c	+-	-	-	-	+	c
d	-	-	-	-	-	d
e	+-	++	++	+	+	e
f	+-	-	-	-	-	f

Results of the Screen for Pollen-Specificity - continued

	root	leaf	seedling	seed	young anther	
Cm11a	+-	-	-	-	-	Cm11a
b	+-	-	-	-	-	b
c	+-	-	-	-	-	c
d	+-	-	-	-	-	d
e	+-	-	-	-	-	e
f	+-	-	-	-	-	f
Cm12a	+	++	++	++	++	Cm12a
b	-	-	-	-	-	b
c	-	-	+-	+-	-	c
d	+-	-	+	-	+	d
e	-	-	+-	-	-	e
f	-	-	+	-	-	f
Cm13a	-	-	-	-	-	Cm13a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
Cm14a	-	-	-	-	-	Cm14a
b	-	-	-	-	-	b
c	-	-	+	-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	+-	+	-	-	f
C1a	-	-	-	-	-	C1a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
C2a	-	-	-	-	-	C2a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
C3a	-	-	-	-	-	C3a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	+-	-	-	-	f
C4a	-	+-	-	+-	-	C4a
b	-	-	-	-	-	b
c	+-	+-	-	-	-	c
d	+	+-	-	+-	-	d
e	+	+-	-	+-	-	e
f	-	-	-	-	-	f

Results of the Screen for Pollen-Specificity - continued

	root	leaf	seedling	seed	young anther	
C5a	+	+	+	+ -	-	C5a
b	-	-	-	-	-	b
c	+	+ -	-	-	-	c
d	+	+ -	-	+ -	-	d
e	+	+ +	+	+ -	-	e
f	+	-	-	+ -	-	f
C6a	-	+	+	-	-	C6a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
C7a	-	-	-	-	-	C7a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
C8a	-	-	-	-	-	C8a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
C9a	-	-	-	-	-	C9a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
C10a	+	+ +	+ +	+	+ +	C10a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
C11a	-	-	-	-	-	C11a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	+ -	-	-	-	d
e	-	+ -	-	-	-	e
f	-	-	-	-	-	f
C12a	-	-	-	-	-	C12a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f

Results of the Screen for Pollen-Specificity - continued

	root	leaf	seedling	seed	young anther	
C13a	-	-	-	-	-	C13a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	+-	-	-	-	e
f	-	-	-	-	-	f
C14a	-	-	-	-	-	C14a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
C15a	-	-	-	-	-	C15a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
C16a	+	++	++	+	++	C16a
b	-	-	-	-	-	b
c	-	-	+-	-	+	c
d	-	-	-	-	-	d
e	+-	+-	+	-	+	e
f	-	-	-	-	-	f
C17a	-	-	-	-	-	C17a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
C18a	-	-	-	-	-	C18a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
C19a	-	-	-	-	-	C19a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
C20a	-	-	-	-	-	C20a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	+-	-	+-	-	d
e	++	+	-	++	-	e
f	+-	+-	-	-	-	f

Results of the Screen for Pollen-Specificity - continued

	root	leaf	seedling	seed	young anther	
C21a	-	-	-	-	-	C21a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
C22a	-	-	-	-	-	C22a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	+	+	-	-	-	d
e	+	+	-	-	-	e
f	-	-	-	-	-	f
C23a	+ -	+ -	-	-	-	C23a
b	-	-	-	-	-	b
c	+	+	-	-	-	c
d	-	+ -	-	+ -	-	d
e	-	-	-	-	-	e
f	+ -	-	-	-	-	f
C24a	-	-	-	-	-	C24a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	+	+ -	-	+ -	-	f
C25a	+ +	+	-	+ -	-	C25a
b	+	+ -	-	+ -	-	b
c	+ +	+ -	-	+ -	-	c
d	+ +	+	-	+ -	-	d
e	-	-	-	-	-	e
f	+ -	-	-	-	-	f
C26a	-	-	-	-	-	C26a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
C27a	-	-	-	-	-	C27a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
C28a	-	-	-	-	-	C28a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f

Results of the Screen for Pollen-Specificity - continued

	root	leaf	seedling	seed	young anther	
C29a	-	-	-	-	-	C29a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	++	++	-	++	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
C30a	+	+	-	+-	-	C30a
b	+	+	-	+-	-	b
c	+	+	-	+-	-	c
d	-	-	-	-	-	d
e	+	+	-	+	-	e
f	-	-	-	-	-	f
C31a	++	+	+	+	++	C31a
b	+-	+	-	+	+-	b
c	+-	+-	-	+	-	c
d	-	-	-	-	+	d
e	++	++	++	++	++	e
f	-	-	-	-	-	f
C32a	++	+	+	+	+	C32a
b	-	-	-	-	-	b
c	+	-	+	-	+	c
d	+-	+-	-	-	-	d
e	-	-	-	-	-	e
f	++	++	+	+	+	f
C33a	+-	-	-	-	-	C33a
b	+-	-	-	-	-	b
c	+	++	-	+	+	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
C34a	+	+	-	+-	-	C34a
b	+-	+	-	+-	-	b
c	+-	+	-	+-	-	c
d	+-	+-	-	+-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
C35a	+	+-	+	+	++	C35a
b	-	-	+	-	+	b
c	-	-	+	+-	++	c
d	-	-	+	+-	++	d
e	-	-	+	+-	++	e
f	-	-	+	-	+	f
C36a	-	-	-	-	-	C36a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	+	-	-	-	+	e
f	-	-	-	-	-	f

Results of the Screen for Pollen-Specificity - continued

	root	leaf	seedling	seed	young anther	
C37a	-	+	+	-	-	C37a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	++	++	+	+	-	e
f	-	-	-	-	-	f
C38a	+	-	+	-	+	C38a
b	-	-	-	+-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	+-	-	+	-	+	e
f	++	++	+-	++	-	f
C39a	-	-	-	-	-	C39a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
C40a	-	-	-	-	-	C40a
b	-	-	-	-	-	b
c	-	-	-	-	-	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	-	-	-	-	-	f
C41a	-	-	-	-	-	C41a
b	-	-	-	-	-	b
c	+	+	+	+	+	c
d	-	-	-	-	-	d
e	-	-	-	-	-	e
f	+	+	+	+	+	f

++ indicates a very strong hybridisation signal
+ indicates a strong hybridisation signal
+ - or - + indicates that the signal might be due to unspecific hybridisation
- indicates no hybridisation

Appendix B

C41b - sequence of the cDNA

```
          10          20          30          40          50
GCCCAAAGGA GGAACCAGCG GCGAAGGAGG AACCAGCAGC GAAGGAGGAG 50
CCGAAAACAC CAACAGCTGG TACACCCGAT GCCCCTGCCG AGCCAAAGAA 100
GGAGGAGCCA GCGAAGGAGG AGCCAAAGGC ACCGGAGACG CCGGCGGCCA 150
GCACAACCGA CGCTTACGCC GCACCGAAGA CTGCTGCGCC AGAGACGCCC 200
GCTACCACAA CCGAGACGCC GGCGGCCAGC ACAACCGACG CTTCCGCCGC 250
ACCGAAGACT GCTGCGCCAG AGACACCAGC GGCTACCACA ACTGAGACGA 300
CGGCGGCCAG CACAACCGAC GCTTACACCG CACCGAAGAC TGCCGAGCAG 350
GAAACACCGG CGGCCGGAAC AACTGATGCC TACCCAGGGC AGAAGCCAAG 400
GAAGATCGGT CATTTACCTC TTCCATACCT GTGAATCCAA CGTCTCGCCG 450
ATTTTCCGAG GGACCAACCA AAGTAAGTTG CCCTCACAGC TCAGCTGGTG 500
CTGGTGCTGG TGCTGGTAAC ATGGCCGNAA AATCACAATA AGGCCCATGC 550
TCCTTTTTTA TCCCCCGATT TATATTATAT CGTGTAAGTG TGTACCACTT 600
ACGCATGCAA CTACGTTTGC ACTATTACTC TACTTTTCTG TAAAGAGAAC 650
TTATGTACCT GTTGTGTAAA GAAACGGG                          678
```

C38d - sequence of the cDNA

```
          10          20          30          40          50
CGAACAAGGG CCGGCCGCTG CCCAAGTTCG GGGAATGGGA CGTCAAGAAC 50
CCGGCGTCCG CCGAGGGCTT CACCGTCATC TTCCAGAAGG CCCGCGACGA 100
CAAGAAGACC ACCGGCCCGG GGAAGTCCGG CATCCCGCCG GCATTCAGGA 150
ACGGCGACCG CTACAGCTAC GGCTCCGGCT CCGAGTTCAA GTCCGGCAAC 200
AGCTACCACC AGTACTCGAG GGTGGCGACG CCAAACGGGT CAAGAAGAGG 250
TGGTTCTTCT GCGGCAGCTT CTGATTCTGT CGCCGGGGCC GCTTCTTGCC 300
GTTGGTGGAA GAGATCTGAA ACCGTGCCGC GCCAGAACCA GAAGCACATT 350
ACCTACCTGA TGGGAGGCGA CGGTGAAAAT TTTGGCGATC GCGGTGTTTT 400
AGCTATAGTC GATTGCCGAA TCTATTGGAA AAGAATCAAT CGATGCTTAT 450
GATTCCGTTC ATATTTACGA                                  470
```

C17a - sequence of the cDNA

```
          10          20          30          40          50
CTCGAGAGCA AGCCCCTGGA GCTCCAGCGC TGCTACCAGG GCTGCAAGAA 50
GAGGTACGAA GCGGCGGTGG CGTATCTTGG CGACGCGGCG GCGGCGCTGG 100
AGAAGAGCAA GTTGGACGAC GCGAGCCTGC TGCTGGGGAC GGCGCAGGCG 150
CAGGTGAAGC TGTGCCAGAG GGGGTGCCAG GCCGTGCCGC CGCAGTGGGA 200
GCTCATCGAG CGCAACCGCA AGGTGGAAAG CCTATGCAAC GTTGCCACGG 250
CCGTTACACG GACGCTCCGG CGACACTGAG AGCTGCCTGC CATGGCCCAT 300
GTGGTAGCCG TCCATCTCTG CATGTACGGG GTGTGGTCGG CTCTGTTTCG 400
ATCCAT                                                 406
```

Cm5e - sequence of the cDNA

```
          10          20          30          40          50
CAAAAAGTAC CCACAATCTC CCTTAAGTAG ATGATACTAC TACTACTAAG 50
TTGTTCCAAG CTCTCTGCAG GATCTGGTAC TGGTAGCAGC TGTTGCGGCA 100
CTCTTCTCGT TCGTTCAGGG TTCTGGTTTT ACACATTCAA GGGGGGAAAA 150
CTGTCTCGGT CTTCTTGCTT CTCTCTCTAT CTCCGCTCGC TCATC      195
```

Bm7e - partial sequences of the cDNA

a) likely to be 5'end

```
          10          20          30          40          50
GTAAAGGATG AAGAACTCTA CCAGCTGTTC TGTGGTCCCA GTGGACCAAA 100
GGTGATGTTG AGGCTATACG AGTTGTTAGG GATCCAGATT CAAGCCTGGG 150
GAAAGGGCAT CGGATATGGT TTATTCAAAA CAAGGGAGGC TTCTTATGGG 200
TTTGTCAAAA AACGGAAGTT TAAGGTCCAA GGACCGCCTT TGAGGGTTAA 250
CCATTGCCAA GGAAGTGGTT CGGCGGCCGA GGAGGCCAAC GGTTGGGAAA 300
CCGGTT                                               306
```

b) likely to be 3'end

```
          10          20          30          40          50
ATCACGGACT TGGCTCCGAC GACACCGAGA ACACACCACC GGAGCAAGAA 50
GGCGAGAAAG TAGCCACACC CTACAATGAA TACCACCAAA TCATGGACAA 100
GATGAGACCC TGATAGTGTC TGCACCGGTT AATGTCCAAA TGTAACGCTG 150
GAACTGAGTC CAGTGTAATA GCAATGTGGT ACTTGCCTTA AGTTGGGACC 200
TCTTGCCTTC TGTACTAATG TCAAAATGAT GCTTATGTCT GTAACATCAT 250
GTACTTGACA TCATTTTTGC CTTTGTTCTC TGTGTAAGCC AGTCTCGTGC 300
CAATCAAGTA ATGGAACTAT CAGCG                           325
```

A36d - partial sequence of the cDNA

```
          10          20          30          40          50
ACCTCTATCC AAGGGAAGAA CAGAATATAA CAAGAAAGCA CCAAATTATA 50
TCAGAGCGGT GCATAACAAT TTCGCCAAAA GGAGGAGAAA TAATAGAATA 100
ATTATAAGGT AAACCAAAAC ATGTTCATGC ATCCATCAAA GATTCAGAAT 150
GCACGCGCAT GTTTTGGATG AGCGCGGCGT AGAAGCCATA GGTAGTAAAT 200
CATCTTCTTC CCTTGATTCC TCCTACCCGC AGCGAATGGT TCGACGGCGC 250
GCTTACTACA GGTAGTAGGC GAAGAAGGAG AGCACGGTGG CCCGGAGCAA 300
CGACGCC                                              307
```

A34b - sequence of the cDNA

```
          10          20          30          40          50
GCGTATGGTC GCCCCTCCTC CTTGCCACGG TTTGGGCCGC GCTCGGACGG 50
AGCGGCACCA TGATGCTGAC CATCGACGAC ATATCTCATG CGGCCAAACT 100
CCACCGCCGG ATCCTCCGCC TTGCTTGCCA CCTCGCTTCT GGGACGGGAC 150
ACCGCGGATT CTGACGAGAA GAAATGCAGC TGAATGCGGT GCCATTACCC 200
GACGAAAGCC ATCTGCAAAA TGATCACTGT TCTGTCAAAT CAGAAGTCGT 250
GATTCTATTG GAGTAATCAA AATGAACCGT GTTAAGTTGC AGGATACATC 300
AAACCTAGAG CTGTCCTTAC AAGTGTATTA CCATGTTAAT CTCATCAAGG 350
GCGAATGCAA GATTAGAGGG AACACGACAG TCGAGGAGCT CGTTGGTCTN 400
ATACAGAAAA TCCCACCTCA AATCTATGCG AATCCATTAT TTTGCTATGT 450
GTTTACAATT TCTCCGATAC ATGCAAAGGT GTCAGCAATG TAGAGGAGTT 500
TGCATGCGAT GAGTAACACA TAAATGGAAG GATCCGTTGT GCACTGGTTT 550
CGAGTAGTGA AAAAACAACG ACGATGTGCC TTGGGCCAGG AGCGACGCTT 600
TGCCAGAGGC AAGCAGGACC AACTTCTTTT GCAATGCCAA AACTGTTTTT 650
TGTTTTGTTG GAGTTGCATC CAATTTCATT TGTATCAACC GACTGGGCTG 700
ATGATGTGAC TACAGTTGGT TTGGCTGCTG TGN                  733
```

B5c - sequence of the cDNA

```
          10          20          30          40          50
GGGAGGGAAG AAGAACTTCT GAACATTTGT ATGAACTTCA GATGTGACAA 50
AATACTTGAA TTTAATGACC ATTGTTATCC GGTAACTACT TTTCCTGACC 100
TCCTTCCATA GCTTCTTCAC GTATCTTTGC TATTTCATCG AACCAATGTT 150
TATACTGATC TTCGATAAAA TCAAACTCCC CATTTGGTGC GTCTTGACTA 200
TCAGTAATAG AAATCGATGA GCAACCACTC ACCAACTCCG AACCAGGTGA 250
TGCTTGATCT ATATGGAAAA TCGAAATATT CCTTGGCCCA TCACCTAAAA 300
CATGATTGCA GGCTAGTTTA TCAATCCCCT CTTTGTACCC GCCATATTGC 350
ATAGTCTCAG AGTCGTCAAT CTCATTATAA CTCGGACTTT CGGAAGTACC 400
AACAAGTTGT ACATAAAGGT CCGACCCTTC AGAGCCCTTT GG         442
```

C7b - sequence of the cDNA

```
          10          20          30          40          50
CAGAGCCGCA GAAACAACCT CCATATACTT CAGTGCCGCA ATGCGCGTGA 50
ACTCAAAAAC TCAACCCATC GATGCAAACC AGCGTCGTAG GCAGCATCGG 100
CCTCGAAGTC ACCGATCTCC TCACCGAGCA TCATCCCTCC GACCGCGCCA 150
CCGCGGAGCC CGAGGCCTAG ACCCATGCCC ATCCCTCCCC GATGCCTTGC 200
CGGCGGCGGC ATACCCGTAC ATCGCCGGCG CTGGAGCAGC GTTGTACCCG 250
TAAGGCGGCG GAGACGATAC CCACCATATG GCTGCGGCGG ATACGCCTTG 300
TACGGTGCGC CATAAGGTAC ACTGCTCGGT GGGTATGCCA ACGGAGCCTG 350
CGGTGGTGGG TACGCGGTGA CGGGCGCCAT GGCCTTGGCC ACTGCCATAT 400
CGGGGTCAGC AGGATACTTG GCATATTGCT CTTCTCTGTT CTTCGCGGCG 450
ATCGATGGTG CGGCCAGGTC GGACGTGATG TCGCCGAACC TGTAGCTGAA 500
ATAGAGAAAC CCATGGGCGC GGCCAGAGAT TGGCCGCCGC ACCTGGTAGC 550
TCCTAGTCCT GGGGTCCCCG CCGTCATCCG CGCCGGAGAG AAGATCAGTG 600
AGAGGCACGA ACACCTCGCC GACGTCGCGG TGG                   633
```

C24e - sequence of the cDNA

```
          10          20          30          40          50
CAGAAGTGTT AATTAATCAA ATCCATTGAA TTGAAATGAT CGACCCCAAA 50
ATGTTGCATA AGCAAAATGA GTTGAGCAAA ACCAAGAAAA TTATAATCAA 100
TCCCTCCTGA TATGAGTGAC AACCCAGAAC ACACAGCCAA GGTTTTTCCT 150
TCTTCCTCTC ACCGCTGCCC GCCCAATGGT TCAAGCGAGG ATGAAAGCGG 200
CAACAACGGC GCCAGCAACA ATAGCGACGG TAGCACTGTA CCCAAGGGCA 250
GCAGAGTCCG AGTTATCAGC ATCAGCCGGA TCCTCGGTAG GCCCGCTGGC 300
GATAGGGGAT ATCGCAGCTA ATGGCGCTGG GGGTCTTGGC AGGTCGCCGG 350
CAGAAACCGA CACGCTGAAA TCCGGATTAG AAGTGGCCTG AGAGCTATTA 400
CTGGGTGGCG CAGCGCCGCA GCGGCAGGGC CAGACTTCCT TGACATCGGA 450
GCAGTGGCGG ACTCGGGGGA CTTAGCCTTC TTCGCAGCTT CGGGCGCCTT 500
TACGGGGGTC ATCACCACCG GT                              522
```

Appendix C

λ gt10 Forward primer:

5'-GCA AGT TCA GCC TGG TTA AG-3'

λ gt10 Reverse primer:

5'-TGA GTA TTT CTT CCA GGG T-3'

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