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Review

The molecular biology of self-incompatibility systems in flowering plants

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Abstract

Self-incompatibility is a common mechanism by which flowering plants can exert some control over the process of fertilization. Typically, the self-incompatibility response involves the recognition and rejection of self-incompatible pollen which leads to a block in self-fertilization and, as a consequence, promotes outcrossing. In recent years, considerable progress has been made in the molecular understanding of several self-incompatibility systems. Interestingly, a common mechanism for self-incompatibility is not employed by all flowering plants, but in fact quite diverse mechanisms have been recruited for the rejection of self-incompatible pollen. In this review, the recent advances in the self-incompatibility systems of the *Solanaceae, Papaveraceae*, and *Brassicaceae* will be described as well as some of the molecular work that is emerging for the Poaceae and the heteromorphic self-incompatibility systems.

Abbreviations: ARC1 – arm repeat containing 1; HVa – hypervariable domain a; HVb – hypervariable domain b; KAPP – kinase associated protein phosphatase; *mod* – modifier locus; PS – positive selection; SLA – *S* locus anther; SBP – *S* binding protein; SLG – *S* locus glycoprotein; SRK – *S* receptor kinase; SCR – *S* locus cysteine rich; SLSG – *S*-locus-specific glycoprotein

Introduction

Upon pollen–pistil interactions, many flowering plants have the inherent capability to recognize their own pollen and subsequently prevent self-fertilization. This inherited phenomenon, which promotes outcrossing, is termed self-incompatibility. A generalized definition of self-incompatibility is "the inability of a fertile hermaphrodite seed plant to produce zygotes after self-pollination" (de Nettancourt, 1977). In a typical compatible pollination, the pollen grains land on the stigma, at the top of the pistil, hydrate and germinate to form a pollen tube. The pollen tube then penetrates the stigma and grows down the style of the pistil to eventually reach and fertilize the ovule. The mechanism of self-incompatibility involves a number of stages which begin with the production of the pollen and pistil component(s) of self-incompatibility. These components are involved in the recognition process leading to the inhibition of self-incompatible pollen. The site of inhibition differs depending on the self-incompatibility system and can lead to the pollen grain failing to adhere, hydrate, or germinate on the stigma surface, or if pollen germination occurs, pollen tube growth can be inhibited by either the stigma or style.

A variety of self-incompatibility systems have evolved in flowering plants. Based on morphological differences, these systems have been classically divided into two groups, homomorphic and heteromorphic self-incompatibility. The flowers of homomorphic species have the reproductive organs in close proximity to each other. In contrast, heteromorphic species have morphologically distinct flowers with the reproductive organs in different positions within the flower. Self-incompatibility systems have also been classified as being either gametophytic or sporophytic. In gametophytic self-incompatibility, the phenotype of the pollen is determined by its own haploid genotype,

while in sporophytic self-incompatibility, the pollen phenotype is determined by the genotype of the pollen parent.

Gametophytic homomorphic self-incompatibility is employed by a number of families, however, only a few have been studied at the molecular level. The most extensively studied families are the *Solanaceae* (including genera such as *Nicotiana, Petunia, Solanum* and *Lycopersicon*) and the *Papaveraceae*. Despite both being gametophytic homomorphic self-incompatibility systems, these two families have evolved quite different mechanisms of self-incompatibility. Other families that have been studied to a lesser extent are the *Rosaceae* and the *Poaceae*. Sporophytic homomorphic self-incompatibility is not as widespread as gametophytic homomorphic self-incompatibility and is found in families such as the *Brassicaceae* and the *Asteraceae*. Molecular analysis of this type of pollen rejection system has been largely studied in the *Brassicaceae*. Progress in understanding the molecular biology of heteromorphic self-incompatibility systems has been much slower and not much is known about how self-incompatible pollen is rejected in these systems.

Molecular analysis of self-incompatibility systems has focused on identifying and characterizing the pollen and pistil components of the self-incompatible response as well as the other proteins and events that lead to pollen rejection. In this review, we will describe the molecular and cellular biology of different self-incompatibility systems and discuss recent advances in the well studied gametophytic and sporophytic homomorphic self-incompatibility systems.

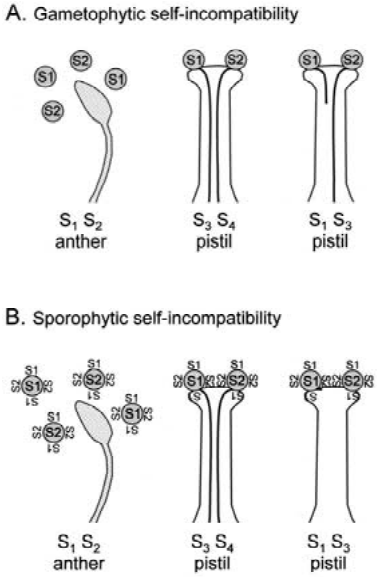

Figure 1. Genetic control of self-incompatibility. *(A)* Gametophytic self-incompatibility. The phenotype of the pollen is determine by its own haploid genotype. Pollen rejection occurs when the *S* allele of the pollen matches either allele present in the pistil and inhibition of pollen tube growth generally occurs in the upper part of the style for the Solanaceae self-incompatibility system. In the example shown, S_1 pollen grains from an S_1S_2 plant will not germinate on an S_1S_3 pistil but S_2 pollen grains are fully compatible with the S_1S_3 pistil. *(B)* Sporophytic self-incompatibility. The phenotype of the pollen is determine by the diploid genotype of the parent plant and pollen rejection occurs at the stigma surface. Pollen grains are rejected if the pistil and the pollen parent have an *S* allele in common. In the example shown, both S_1 and S_2 pollen grains from an S_1S_2 plant will not germinate on the S_1S_3 pistil because the S_1 allele is present in both plants. Both pollen grains are fully compatible on the S_3S_4 pistil.

Self-Incompatibility in the *Solanaceae*

In the *Solanaceae*, the gametophytic homomorphic self-incompatibility system is characterized by the arrest of the incompatible pollen tube within the upper third part of the style. The walls of the incompatible pollen tube becomes thinner, callose deposition becomes irregular, the tip of the tube swells, tube growth slows and the tip may erupt (de Nettancourt, 1977). The self-incompatibility trait is controlled by the multi-allelic locus called the *S* locus. Pollen rejection occurs when the *S* allele of the haploid pollen corresponds to one of the *S* alleles present in the pistil (Figure 1A). If the pistil carries different *S* alleles to the pollen, then the growing pollen tube is not blocked and is allowed to fertilize the plant (Figure 1A). While the classical genetics studies indicated this self-incompatibility trait is controlled by the single *S* locus, molecular studies are showing that there are in fact at least two tightly linked loci, one controlling self-incompatibility in the style and a second controlling self-incompatibility in the pollen. The stylar *S* gene product has been extensively characterized, while the pollen *S* gene has not yet been identified.

Stylar S protein

Style proteins associated with pollen rejection in the Solanaceae were first described by Bredemeijer and Bass (1981). These proteins, called *S* proteins, were observed to co-segregate with the self-incompatibility phenotype. Anderson et al. (1986) then went on to isolate the first cDNA encoding an *S* protein. This *S* protein was a small basic glycoprotein which se-

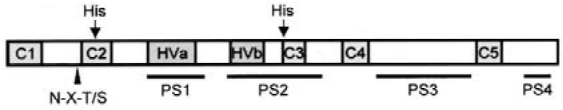

Figure 2. Schematic representation of the S-RNase. The five conserved regions, C1 to C5, and the two hypervariable regions, HVa and HVb, identified by Ioerger et al. (1991) are shown. The four variable regions, PS1 to 4, identified by Ishimizu et al. (1998) are also shown. The conserved N-glycosylation site and the two histidines required for RNase activity are marked by arrows.

gregated with the S_2 allele of *Nicotiana alata*. The corresponding cDNA was predicted to encode a 32-kDa protein with a putative signal peptide at its N-terminus. The gene encoding this S_2 protein exhibited characteristics which are consistent with a function in self-incompatibility. The expression was developmentally regulated and accumulation of the mRNA was correlated with the onset of self-incompatibility. Within the style, the mRNA was detected throughout the transmitting tract, where the pollen rejection process occurs (Anderson et al., 1986; Cornish et al., 1987). The *S* proteins were subsequently shown to possess ribonuclease activity and were then referred to as S-RNases (Kawata et al., 1988; McClure et al., 1989).

The cloning of the cDNA for the *Nicotiana alata* S-RNase and the identification of the N-terminal conserved sequence have since then lead to the isolation and sequencing of a number of S-RNases corresponding to different *S* alleles in several species of *Solanaceae* including *Petunia* (Ai et al., 1990; Clark et al., 1990), Solanum (Xu et al., 1990; Kaufman et al., 1991), and *Lycopersicon* (Tsai et al., 1992). S-RNases similar to that found in the *Solanaceae* have also been identified in other families with gametophytic self-incompatibility systems such as the *Rosaceae* and *Scrophulariaceae* (Sassa et al., 1993; Xue et al., 1996). The similarity of the S-RNases suggests that these families employ a similar molecular mechanism of self-incompatibility.

S-RNase structure

The *S* alleles of the *Solanaceae* species encoding the S-RNase are highly divergent, with amino acid sequence identities ranging from 38 to 98% (Ioerger et al., 1990; Clark and Kao, 1991; Tsai et al., 1992). The degree of amino acid sequence divergence within a species is similar to the degree of amino acid sequence divergence between species. In some cases, an *S* allele shows higher sequence identity to *S* alleles from other species rather than to *S* alleles within that same species (Ioerger et al., 1990; Robbins et al., 2000).

Despite the high sequence diversity, the S-RNases contain a number of conserved regions. There are five highly conserved regions designated C1 – C5 (Figure 2; Ioerger et al., 1991). C1, C4, and C5 contain mostly hydrophobic amino acids and may be involved in forming the core structure of the *S* protein (Kao and McCubbin, 1996). Analysis of *S* allele sequences from *Rosaceae* revealed that all the features and characteristics of the predicted protein are similar to the *Solanaceae* S-RNase except that the C4 region was lacking (Sassa et al., 1993, 1994; Boothaerts et al., 1995). The C4 region was also missing in the *Antirrhinum* (*Scrophulariaceae*) S-RNase sequences (Xue et al., 1996). The C2 and C3 regions show sequence similarity to the fungal T2 RNase (Kawata et al., 1988). The sequence identity to the fungal RNase is as low as 24% and is confined to the C2 and C3 regions (McClure et al., 1989; Ishimizu et al., 1995). There are also two histidine residues in the active site of the T2 RNase which are required for the RNase activity. These histidines are conserved in the S-RNases, one in the C2 region and the other in the C3 region (Figure 2), and are also required for S-RNase activity (Kawata et al., 1988; McClure et al., 1989; Royo et al., 1994; Ishimizu et al., 1995; Parry et al., 1997).

The S-RNase contains between 7 and 10 cysteine residues in conserved positions, and a number of these cysteines form disulfide bonds to stabilize the tertiary structure of the protein (Tsai et al., 1992; Ishimizu et al., 1996; Oxley and Bacic, 1996; Parry et al., 1998). All S-RNases are also glycoproteins with up to five potential *N*-glycosylation sites. The *N*-glycosylation site closest to the C2 region is conserved in all S-RNases identified to date (Figure 2, Ioerger et al., 1991; Kao and McCubbin, 1996; Oxley et al., 1996).

Outside the conserved regions, the *S* alleles show a high level of variability, and potentially important variable domains which may play a role in *S* allele specificity have been identified by different authors. Ioerger et al. (1991) identified two areas exhibiting the highest levels of variability and were referred to as "hypervariable domains" HVa and HVb (Figure 2). Due to the high sequence diversity and the hydrophilic nature of these regions, they are thought to be involved in determining *S* allele specificity (Ioerger et al., 1991; Sims et al., 1993). In contrast to the restricted areas of the HVa and HVb region, Zurek et al. (1997) referred to all sequences outside the conserved regions as variable (Figure 2). Therefore, any region or re-

gions outside the conserved domains may play a role in determining specificity. Recently, Ishimizu et al. (1998) identified four different regions, called PS1 to PS4 ('positive selection'), which may function as the determinant of *S* allele specificity (Figure 2).

Biological role for S-RNase in self-incompatibility

Early evidence for a biological role for S-RNases in self-incompatibility was provided by McClure et al. (1990), where they showed that pollen rRNA was degraded after incompatible but not compatible pollinations. This suggested that self-incompatibility in the *Solanaceae* acts through a cytotoxic mechanism, and growth inhibition of self-incompatible pollen tube is due to the loss of RNA and the resulting inability to synthesize the proteins required for pollen tube growth. Gray et al. (1991) provided further evidence to support the role of S-RNase in pollen rejection when they showed that the S-RNase was able to enter pollen tubes grown *in vitro*. The cytotoxic effect is not permanent as it has been shown that incompatible pollen tubes can recover from the S-RNase induced growth inhibition (Lush and Clarke, 1997).

Plant transformation studies have since confirmed that active S-RNases are required in the pistil for the allele specific inhibition of incompatible pollen tubes. Down-regulation of the S_3-RNase gene in S_2S_3 *Petunia inflata* plants by an antisense S_3-RNase transgene rendered the transgenic plants incapable of rejecting S_3 pollen (Lee et al., 1994). Conversely, the expression of the S_3-RNase transgene in a S_1S_2 *Petunia inflata* plants conferred the ability to reject S_3 pollen (Lee et al., 1994). Similarly, Murfett et al. (1994) introduced the *Nicotiana alata* S_{A2} gene into a *Nicotiana langsdorffii* × self-compatible *Nicotiana alata* hybrid and found that these transgenic plants showed allele specific rejection of S_{A2} pollen. Thus, S-RNases are necessary and sufficient in the pistil for the recognition and rejection of incompatible pollen tubes. Huang et al. (1994), using a mutant S_3-RNase gene, was able to demonstrate that ribonuclease activity of S-RNases is central to their role in self-pollen rejection. The conserved histidine in the C3 region of the S_3-RNase gene was substituted with an asparagine, rendering the S-RNase inactive. Normal expression of the mutant S_3-RNase gene in S_1S_2 *Petunia inflata* was unable to confer on the transgenic plant the ability to reject S_3 pollen. These experiments, in conjunction with the observation that a self-compatible line, *Lycopericum pervianum*, produces an inactive S-RNase (Royo et al., 1994), suggests that the self-incompatible process requires the ribonuclease activity of the S-RNase.

S allele specificity of S-RNases

In plants and animals, the carbohydrate moiety of glycoproteins is important for many biological interactions and is involved in recognition systems. Several functions have been proposed for the glycosylation groups on the S-RNase. The N-glycan side chains may have a function determining the overall structure of the S-RNase molecule or they may function in non-allelic interactions with the pollen *S* product (Woodard et al., 1992; Ishimizu et al., 1999). It was also speculated that the allelic specificity of the S-RNase maybe determined, at least in part, by the *N*-glycan side chains (Woodard et al., 1989, 1992; Boothaerts et al., 1991). This was proven not to be the case, since transgenic S_1S_2 *Petunia inflata* plants expressing a non-glycosylated S_3-RNase were able to fully reject S_3 pollen (Karunanandaa et al., 1994). Thus, any role for the *N*-glycan side chains still remains to be identified.

Since the S-RNase contains both conserved as well as variable regions, it only follows that the conserved regions are involved in a common function such as observed for the C2 and C3 regions, and the variable regions would be involved in a unique function such as *S* allele-specific recognition. A number of studies using chimeric S-RNase constructs attempted to define which variable regions were important for defining *S* allele specificity. Kao and McCubbin (1996) constructed functional chimeric S_3-RNase genes, with either both the HVa and HVb regions or only the HVa region replaced by the corresponding region from the S_1-RNase, and introduced these into S_2S_2 *Petunia inflata* plants. The transgenic plants failed to reject both S_1 and S_3 pollen but not S_2 pollen. Thus, the HV regions were necessary but not sufficient for determining *S* allele specificity.

In a more extensive study by Zurek et al. (1997), using the S_{C10} and S_{A2} alleles of *Nicotiana alata*, a number of chimeric constructs were produced which sampled all regions outside of the conserved regions in the S-RNase. The chimeric S-RNases, all of which had normal RNase activity, were introduced into a *Nicotiana langsdorffii* × self-compatible *Nicotiana alata* hybrid. While the control S_{C10}-RNase or S_{A2}-RNase transgenic plants were able to reject the S_{C10} or S_{A2} pollen, respectively, none of the resulting chimeric

S-RNase transgenic plants could rejected the S_{A2} or S_{C10} pollen. Zurek et al. (1997) concluded that the *S* allele specificity was not localized to the hypervariable regions but scattered throughout the protein.

In the study by Matton et al. (1997) using *Solanum chacoense*, instead of swapping the HV regions, they substituted four amino acids in the HV regions of the S_{11} allele for the corresponding amino acids in the S_{13} allele. The S_{11} and S_{13}-RNase differ by only 10 amino acids, three of which are found in the HVa region and one in the HVb region (Saba-El-Leil et al., 1994). $S_{12}S_{14}$ *Solanum chacoense* plants expressing the chimeric S_{11} gene acquired the ability to reject S_{13} pollen. Therefore, Matton et al. (1997) concluded that the HV regions are not only necessary but sufficient for *S* allele specificity. Matton et al. (1999) went on to show that if three of the four amino acids in the HV regions of the S_{11}-RNase were substituted with the corresponding amino acids from the S_{13}-RNase, the resulting S-RNase gained dual specificity in pollen rejection. The expression of this S-RNase in transgenic plants caused the rejection of both S_{11} and S_{13} pollen.

The conclusions of each experiment, though different, do not completely contradict each other. All three experiments indicated that the hypervariable regions are necessary for allelic specificity. However, the conclusions differed on whether the hypervariable regions alone were sufficient for *S* allele specificity. Kao and McCubbin (1996) and Zurek et al. (1997) suggested that they are not sufficient and may require additional regions throughout the protein while the results from Matton et al. (1997) suggested that the hypervariable regions were sufficient for allelic recognition. One notable point to be made is that the level of amino acid sequence identity between the *Solanum chacoense* S_{11} and S_{13} alleles (95%) is not typical of other S-RNase pair-wise comparisons which tend to be much lower (Ioeger et al., 1990). Therefore, regions conserved between the S_{11} and S_{13} S-RNases may also be involved in *S* allele specificity. As pointed out by Verica et al. (1998), amino acids outside the HV regions may also have a potential role in determining *S* allele specificity and these hypervariable amino acids are identical in the S_{11} and S_{13} -RNase. An indication of the regions that determine *S* allele specificity will likely come from solving the tertiary structure of the S-RNase.

The secondary structure of the S-RNases are predicted through molecular models, to be similar to the known secondary structure of the fungal RNase Rh (Kurihara et al., 1992; Ishimizu et al., 1998; Parry et al., 1998). A molecular model of the S_3-RNase from *Lycopersicon peruvianum* predicted that the HVa and HVb regions form a continuous surface on one side of the S-RNase (Parry et al., 1998). In a second model, the four PS regions, which contain mainly basic amino acids, are predicted to form two clusters on the surface of the molecule (Ishimizu et al., 1998). The PS1 and PS2 regions which contain the hypervariable regions HVa and HVb (Figure 2) form one cluster, and the PS3 and PS4 regions form the second cluster. The two clusters corresponds to two sites on opposite ends of the surface of the tertiary structure of RNase Rh, which flank the substrate binding site of the molecule. Each model places the hypervariable regions on the surface of the molecules and contends that these surface sites, as well as others, may interact with other molecules such as the pollen *S* product. The final determination of the region of *S* allele specificity will most likely be revealed following the identification of the pollen *S* component.

Pollen S protein

Observations that S-RNases are also expressed in developing pollen led to the speculation that the same *S* gene may encode both the pollen and pistil component of gametophytic self-incompatibility (Dodds et al., 1993). However, the loss-of-function and gain-of-function studies did not have any effect on the pollen self-incompatible phenotype. Furthermore, it was recently demonstrated that transgenic plants expressing the sense or antisense S_3-RNase gene under the control of a pollen specific promoter did not show any changes in the pollination phenotype (Dodds et al., 1999). Finally, in a self-compatible cultivar of *Pyrus serontina*, the self-incompatible response was observed to be lost in the pistil, but not in the pollen, and there was a corresponding deletion of S_4-RNase gene (Sassa et al., 1997). Therefore, the pollen *S* product is encoded by a separate gene.

The pollen *S* gene in the *Solanaceae* has not been identified, but there are several predictions that can be made regarding this gene. It must be genetically tightly linked to the S-RNase gene (*S* locus) and its allelic sequences should display a highly level of diversity. The encoded protein is also predicted to interact with the pistil S-RNase in an allele specific manner which would ultimately lead to inhibition of an incompatible pollen tube. Support for this latter prediction was given by McCubbin et al. (1997) where they showed that the expression of an inactive S_3-

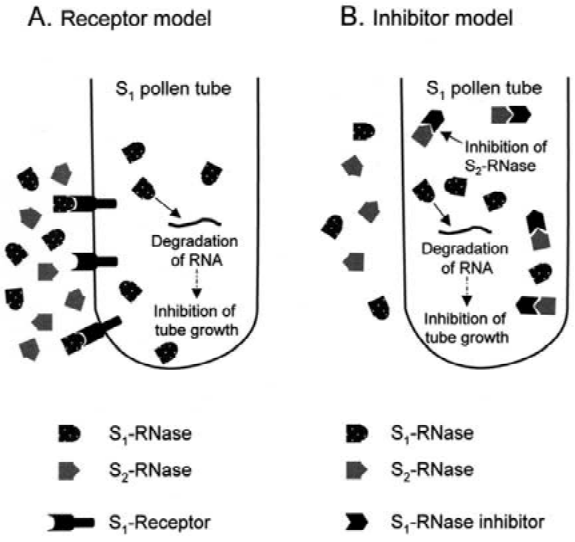

Figure 3. Models for *S* allele specific inhibition of pollen tube growth in the *Solanaceae*. As the S_1 pollen tube grows down the S_1S_2 style, pollen RNA becomes degraded by the S_1-RNase and this leads to an inhibition of pollen tube growth. The S_2-RNase produced by the style has no effect. In the receptor model *(A)*, the S_1-Receptor on the growing pollen tube allows only the S_1-RNase to enter the pollen tube. In the inhibitor model *(B)*, both S-RNases enter the pollen tube but the S_2-RNase activity is inhibited by the S_1-RNase inhibitor which is predicted to inhibit all S-RNases except the S_1-RNase.

RNase in S_2S_3 *Petunia inflata* rendered the plants incapable of rejecting S_3 pollen but had no effect on the plants ability to reject S_2 pollen. The authors suggested that the inactive transgenic S_3-RNase had a dominant negative effect on the endogenous S_3-RNase by competing for the binding to a common molecule, which they predicted is the pollen S_3-product.

Attempts to identify the pollen *S* product using a number of different approaches have so far been unsuccessful. Golz et al. (1999) screened for mutations that affect only the pollen component of self-incompatibility in *Nicotiana alata*, and Harbord et al. (2000) screened for T-DNA insertions in the *S* locus region of *Petunia hybrida*. Dowd et al. (2000) used RNA differential display and subtractive hybridization to identify 13 pollen expressed *S* linked genes in *Petunia inflata*. Li et al. (2000) used chromosome walking to generate a genetic map of the *Nicotiana alata S* locus region. The map includes the S-RNase gene and a pollen expressed sequence which is suggested to be a candidate for the pollen *S* gene. However, it still remains to be determined if any of these genes are indeed involved in the rejection of self-incompatible pollen.

Models for S allele specific pollen rejection

There are currently two models for gametophytic self-incompatibility, the receptor model and the inhibitor model (Figure 3; Thomas and Kirch, 1992; Kao and McCubbin, 1996). The receptor model predicts that the pollen *S* product is a transmembrane receptor which allows extracellular S-RNase to enter the pollen tube in an *S* allele-specific manner (Figure 3A). The uptake of the allele-specific S-RNase would result in pollen RNA degradation leading to inhibition of pollen tube growth. The inhibitor model predicts that the pollen *S* product is a general S-RNase inhibitor that resides within the growing pollen tube (Figure 3B). The model proposes that all the S-RNases freely enter the pollen tube, and the pollen *S* product binds and inhibits all S-RNases except the allele-specific S-RNase, allowing the allele-specific S-RNase to degrade the pollen tube RNA resulting in pollen inhibition.

Based on what is known about the self-incompatibility system in *Solanaceae*, it is difficult to rule out either of these models as there is evidence in the literature to support both models. Support for the receptor model comes from the study by McCubbin et al. (1997) where an inactive S_3-RNase had a dominant-negative effect on the wild-type S_3-RNase suggesting that the inactive S_3-RNase and the wild-type S_3-RNase are competing for binding to a pollen *S* product such as a receptor. The inhibitor model does not predict binding between the pollen *S* product and its allele-specific S-RNase.

In the *Solanaceae*, the phenomenon of competitive interactions has been observed where self-incompatibility breaks down if more that one *S* allele are present in the haploid pollen grain (Brewbaker and Natarajan, 1960). For example, an S_1S_2 plant would typically produce haploid pollen grains carrying either the S_1 or the S_2 allele. If one of these genes became duplicated and haploid pollen grains are produced carrying both the S_1 and S_2 alleles, then self-incompatibility breaks down. The style produces the two different S-RNases, one from each *S* allele, and is capable of rejecting pollen carrying two copies of the same *S* allele, but cannot reject the S_1S_2 pollen. This can be explained by the inhibitor model since both the S_1 pollen inhibitor and the S_2 pollen inhibitor are present in the same pollen tube and would inhibit the S_2-RNase and the S_1-RNase, respectively (Golz et al., 2000). However, Golz et al. (2000) also pointed out that the competitive interaction could be explained by the receptor model if the pollen *S* receptor is mul-

timeric and in the example described above, the S_1 and S_2 receptors form inactive heteromers.

Support for the inhibitor model comes from searches for mutations that only affect the pollen *S* gene (Golz et al., 1999, 2000). If the pollen *S* product encodes an inhibitor, one would predict that loss-of-function mutations would not be recovered because there would be no inhibition of the S-RNases and consequently these mutations would be lethal. In keeping with this prediction, searches for self-compatible pollen mutants have only identified duplications of the *S* locus region which are likely causing competitive interactions (Golz et al., 1999, 2000). Further support for the inhibitor model was shown by Luu et al. (2000) who demonstrated that S-RNase uptake by growing pollen grains occured in a non-allele specific manner. Validation of the predictions of either model will likely have to wait for the identification of the pollen *S* product.

Modifiers of self-incompatibility

The *S* locus undoubtedly produces the pollen and pistil determinants of self-incompatibility. However, there is genetic evidence that factors other that the *S* proteins are required to carry out the self-incompatible response. These genes, referred to as modifier genes (Mather, 1943; Martin, 1968; de Nettancourt, 1977), have been placed into three groups depending on how they affect the determinants of allelic specificity (McClure et al., 2000). The group I factors would affect the expression of the *S* genes such as transcription factors. The group II and III factors would affect the function of the *S* proteins, with the group II factors being specific to the rejection of incompatible pollen, and the group III factors having a more general role in pollen–pistil interactions.

There are several examples where crossing *S* alleles from one background to another background causes the loss or gain of self-incompatibility (Ai et al., 1991; Bernatzy et al., 1995; McClure et al., 2000). In all of these studies, the S-RNase was expressed before and after the crosses indicating that some other factor was affecting the self-incompatibility response. Recently, McClure et al. (1999) identified a small asparagine rich 8.6-kDa protein, called HT, that was shown to be required for pollen rejection. Reduction of HT transcripts by an antisense HT transgene in self-incompatible plants disrupted the self-incompatible response. The HT gene is expressed in the styles of self-incompatible *Nicotiana alata* and not self-compatible *Nicotiana plumbaginifolia*. The accumulation of HT transcript lags behinds that of the S-RNase but does correlate with the onset of self-incompatibility (McClure et al., 1999). HT protein was not seen to bind to S-RNase (McClure et al., 2000). Therefore, how HT actually functions along with the S-RNases to mediate self-incompatibility remains to be determined.

The self-incompatible response may also involve protein phosphorylation. A calcium-dependent protein kinase, NAK-1, isolated from *Nicotiana alata* pollen tubes was found to phosphorylate *Nicotiana alata* S-RNases *in vitro* (Kunz et al., 1996). Whether or not phosphorylation of the stylar S-RNases has a significant function on the self-incompatible response is yet to be determined.

Self-Incompatibility in the *Papaveraceae*

As in the *Solanaceae*, the gametophytic homomorphic self-incompatibility system in the *Papaveraceae* is genetically controlled by a single polymorphic loci, also termed the *S* locus (Lawrence et al., 1978). As well, the phenotype of the pollen is determined by the *S* allele present in the haploid pollen genome as seen for the Solanaceae (Figure 1A). However, there are no further similarities between the self-incompatibility systems of each family. During the self-incompatibility response of *Papaveraceae*, inhibition of pollen tube growth occurs at the stigma surface, before or immediately after germination (Franklin-Tong, 1992). Molecular studies with *Papaver rhoeas*, have indicated that the pistil *S* product, as well as the mechanism for self-pollen recognition and rejection are very different from that of *Solanaceae*.

Stigmatic S protein

The development of a system using *in vitro* grown pollen tubes to study the self-incompatibility response in *Papaver rhoeas* led to the identification of a small protein (approximately 15 kDa) in the stigma which inhibited pollen tubes in an *S* allele specific manner (Franklin-Tong et al., 1988, 1989; Foote et al., 1994). Early on, it was found that the stigmatic *S* protein was not an RNase (Franklin-Tong et al., 1991). The level of RNase activity in the mature stigmas of *Papaver* were found to be approximately 200-fold less than the S-RNase activity observed in the styles of *Nicotiana alata*. In addition, allele specific inhibition of pollen

tube growth occurred in the presence of the stigmatic *S* protein regardless of the presence or absence of any RNase activity (Franklin-Tong et al., 1991).

Foote et al. (1994) cloned the cDNA encoding the stigmatic S_1 protein by using N-terminal sequence information derived from purified stigmatic S_1 protein. The predicted protein had a signal peptide at the N-terminus, and the mature protein of 120 amino acids was largely hydrophilic with a short hydrophobic region in the N-terminus. The S_1 gene is developmentally regulated with increasing expression levels correlating with expression of the self-incompatibility phenotype (Foote et al., 1994). The subsequent cloning of other *S* alleles for the stigmatic *S* gene has shown that there is a high degree of polymorphism with the predicted amino acids sequences sharing between 51 and 63% sequence identity (Walker et al., 1996; Kurip et al., 1998). The variable regions are interspersed throughout the amino acid sequence with conserved blocks of amino acids in between (Kakeda et al., 1998). *N*-Glycan side chains are found on some of the stigmatic *S* proteins, but they are not involved in allelic specificity. Recombinant non-glycosylated *S* proteins were found to be capable of inhibiting pollen tube growth in vitro in a *S* allele specific manner (Foote et al., 1994; Walker et al., 1996). There is a low level of sequence similarity between the *Papaver* stigmatic *S* proteins and the N-terminal region of the *Brassica S* proteins, the *S* locus glycoprotein and the *S* receptor kinase (Walker et al., 1996). Also, a family of predicted genes in *Arabidopsis*, called SPH, shows sequence identity to the *Papaver* stigmatic *S* proteins (Ride et al., 1999).

Despite the sequence diversity, the secondary structure of the *Papaver* stigmatic *S* proteins are thought to be identical with four cysteine residues at conserved positions which may function in forming the tertiary structure (Figure 4; Walker et al., 1996; Kurip et al., 1998). Mutation of one of the cysteine residues disrupts *S* protein activity (Figure 4; Kakeda et al., 1998). The predicted structure consists of six β strands followed by two α helices, all linked by seven hydrophilic loops (Figure 4). Recently, several amino acids in loop 6 were shown to be essential for the function of the stigmatic *S* protein (Kakeda et al., 1998). Among the different stigmatic *S* protein amino acid sequences examined by the authors, there was only one variable amino acid in loop 6 (Figure 4). Substitution of the variable amino acid in the stigmatic S_1 protein for that of the stigmatic S_3 protein resulted in a loss of S_1 pollen tube growth inhibition. However, allelic specific inhibition of S_3 pollen tube growth also did not occur. Substitution of conserved amino acids in loop 6 also resulted in a loss of stigmatic *S* protein activity. Amino acid substitutions in the other six loops (loops 1–5, 7), did not abolish the stigmatic *S* protein activity (Kakeda et al., 1998). Therefore, the hydrophilic loop 6 is critical for the inhibitory activity of the stigmatic *S* protein on incompatible pollen, and the variable amino acid in this loop is not sufficient for allele specific inhibition of pollen tube growth.

Pollen S protein

To initiate the pollen rejection process, the stigmatic *S* protein is predicted to interact in an allelic-specific manner with the pollen *S* product which is thought to be a receptor. In an attempt to identify the pollen *S* product, Hearn et al. (1996) carried out a series of binding experiments which resulted in the isolation of a 70–120-kDa glycoprotein called SBP (*S* binding protein). SBP is found in the plasma membrane of mature pollen. Recently, it was demonstrated that SBP binding to the stigmatic *S* protein is essential for pollen tube growth inhibition (Jordan et al., 1999). Mutations in the stigmatic *S* protein which resulted in loss of activity also shows reduced binding to SBP (Jordan et al., 1999). The interaction between SBP and the stigmatic *S* protein is not allele specific suggesting that SBP is not the pollen *S* receptor. SBP may act as an accessory receptor interacting in a non-allelic manner with the conserved residues of the stigmatic *S* protein and facilitating the interaction of the stigmatic *S* protein with the putative pollen *S* receptor (Hearn et al., 1996; Jordan et al., 1999). However, one still cannot rule out the possibility that SBP is the putative pollen *S* receptor (Jordan et al., 1999).

Mechanisms involved in the self-incompatibility response

Studies have shown that a number of events are involved in the *Papaver* self-incompatibility response. The metabolic inhibitors, actinomycin D and tunicamycin, were found to overcome the inhibitory effects of the stigmatic *S* protein suggesting that both transcription and glycosylation are involved in the self-incompatibility response (Franklin-Tong et al., 1990). A number of pollen genes were found to be 'up' or 'down' regulated during the self-incompatibility response, and new proteins were found to be synthesized in the incompatible pollen (Franklin-Tong et al., 1990, 1992).

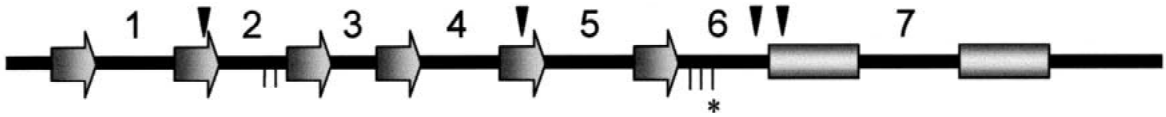

Figure 4. Schematic representation of the *Papaver* stigmatic *S* protein. The predicted secondary structure of the stigmatic *S* protein consists of six β-strands ( ), two α-helixes ( ), and seven hydrophilic loops (numbered 1–7). There are also four conserved cysteine residues (▼), and mutation of the third cysteine residue results in inactivation of the stigmatic *S* protein. The position of the amino acids which may be involved in SBP binding are indicated by lines. The asterix denotes the position of the variable amino acids residues in loop six that is essential for stigmatic *S* protein function.

Much insight has been gained into the nature of the signal transduction pathway involved the self-incompatibility response of *Papaver rhoeas*. The initial step leading to pollen tube growth inhibition is associated with an increase in calcium levels within the pollen tube (Franklin-Tong et al., 1993, 1995, 1997). The stigmatic *S* protein was shown to be capable of eliciting a rapid increase in calcium levels in incompatible pollinations which was then followed by pollen tube growth arrest (Franklin-Tong et al., 1995). The self-incompatible response produces a calcium wave which occurs subapically in the shank of the pollen tube (Franklin-Tong et al., 1997). Therefore, calcium appears to function as a secondary messenger in the self-incompatibility signaling pathway. The rapid increase in pollen tube calcium levels was found to be induced by inositol-1,4,5, triphosphate (IP_3) (Franklin-Tong et al., 1996). Therefore, the increase in calcium during the self-incompatible response may be mediated by IP_3, and thus implicating the phosphoinositol signaling pathway in the self-incompatibility response (Franklin-Tong et al., 1996).

The self-incompatible response also involves protein phosphorylation. Increased phosphorylation levels of two pollen proteins, a 26- and a 68-kDa protein, referred to as p26 and p68, respectively, occurs as a result of the self-incompatible reaction (Rudd et al., 1996, 1997). A rapid increase in phosphorylation of p26 in pollen extracts was only observed in the presence of the incompatible stigmatic *S* protein (Rudd et al., 1996). Upon separation of the pollen extracts into soluble and microsomal fractions, two different 26-kDa phosphoproteins were identified, p26.1 and p26.2. Serine/threonine phosphorylation of each phosphoprotein is calcium dependent, however, only p26.1 phosphorylation seems to also be calmodulin dependent. It was also determined that p26.1 and not p26.2 shows an increase in phosphorylation due to the self-incompatible response. Recently, it was reported that p26 is a pyrophophatase and phosphorylation results in down regulation of its activity (Jordan et al., 2000a). Serine /threonine phosphorylation of p68 is calcium independent and occurs some time after phosphorylation of p26 (Rudd et al., 1997). p68 phosphorylation may be involved in later stages of the signaling pathway, which could result from a 'second wave' of calcium-independent signaling or it may be involved in another signaling pathway all together (Rudd et al., 1997).

Programmed cell death is also thought to be involved in the *Papaver* self-incompatibility response leading to pollen tube growth inhibition. Jordan et al. (2000b) reported that in the presence of the stigmatic *S* protein, the nuclear DNA of incompatible pollen, but not compatible pollen was cleaved. Fragmentation of nuclear DNA is one of the main features of programmed cell death (Cohen, 1993). Lastly, Geitmann et al. (2000) has recently reported that incompatible pollen tubes also undergo rapid alterations in the actin cytoskeleton and these changes are not due to a general growth arrest, but are specific to the self-incompatibility response.

Model for self-incompatible pollen inhibition

The proposed model for the self- incompatibility response in *Papaver rhoeas* is illustrated in Figure 5 (Franklin et al., 1994; Rudd et al., 1997). As the incompatible pollen tube is growing along the stigmatic surface, the stigmatic *S* protein is predicted to interact with the pollen *S* protein in an *S* allele specific manner. The unidentified pollen *S* product is thought to be a membrane receptor in the pollen tube and may in fact be SBP; nevertheless, SBP is involved in the interaction. Binding of the stigmatic *S* protein to the pollen *S* receptor then triggers a signal transduction cascade in the pollen tube. The signal transduction pathway is thought to involve two phases, a calcium-dependent signaling pathway followed by a calcium-independent signaling pathway. In the first

step, there is a rapid increase in calcium levels which may be mediated by IP_3. This is then followed by an increase in phosphorylation of specific proteins, such as p26, by calcium dependent protein kinases. During this early phase, rearrangements of the actin cytoskeleton are also occurring, though it is not known if this is caused by the calcium signaling (Geitmann et al., 2000). Subsequently, the calcium independent signaling pathway occurs and there is an increase in phosphorylation of other proteins. These events may lead to gene activation and ultimately may result in programmed cell death and the inhibition of pollen tube growth.

Self-Incompatibility in the *Poaceae*

Poaceae, the grass family, is the fourth largest family of flowering plants and contains up to 700 genera (Watson, 1990). Ludgvist (1956) was the first to describe that the gametophytic homomorphic self-incompatibility in the grasses was genetically controlled by two loci, *S* and *Z*. Both loci are unlinked and multi-allelic. Gametophytic self-incompatibility is present in at least 16 genera and the two loci system have so far been identified in eight species of grasses (Connor, 1979; Bauman et al., 2000). Inhibition of self-fertilization is rapid and occurs at the stigma surface (de Nettancourt, 1977). Each allele confers a particular specificity and this results in a degree of incompatibility (Figure 6). Matching of alleles at only one locus in the interacting pollen and pistil does not result in pollen rejection. Inhibition only occurs when the interacting pollen and pistil shares the same *S* and *Z* alleles.

The self-incompatibility genes

In the previously described gametophytic systems, the single genetic *S* locus has turned out to be comprised of at least two tightly linked genes, one encoding the pollen *S* protein and the second encoding the pistil *S* protein. A similar prediction has also been made for the self-incompatibility system in grasses except for the complexity of having two loci, *S* and *Z*. To get pollen rejection, both the *S* and *Z* alleles need to match in both the pollen and pistil. Therefore, it is thought that each loci is comprised of at least two genes, the stigmatic *S* gene and pollen *S* gene, and the stigmatic *Z* gene and the pollen *Z* gene. Attempts to identify the *S* and *Z* genes have so far been unsuccessful. Li et al. (1994) isolated and sequenced a putative pollen gene of *Phalaris coerulescens* named Bm2. The Bm2 gene was predicted to encode a protein with a thioredoxin domain in the C-terminus and a proposed allelic specificity domain in the N-terminus (Li et al., 1994). Bm2 is expressed only in the later stages of the developing pollen, and the C-terminus was shown to have thioredoxin activity (Li et al., 1994, 1995). As well, a self-compatible mutant was found to have reduced levels of thioredoxin activity (Hayman and Richter, 1992; Li et al., 1996). These findings and the fact that Bm2 was found to segregate with the *S* locus, lead to the suggestion that Bm2 may be the pollen *S* gene for *Phalaris coerulescens* (Li et al., 1994). However, recent experiments have provided evidence to the contrary. The high levels of Bm2 mRNA observed in mature pollen of *Phalaris coerulescens* were not detected in other self-incompatible species of grass (Li et al., 1997). Also, the Bm2 homologues isolated from other self-incompatible grass species, as well as other alleles of *Phalaris coerulescens* were predicted to encode a protein approximately half the size of Bm2 corresponding to the thioredoxin region (Langridge et al., 1999; Bauman et al., 2000). Finally, recombination has been detected between Bm2 and the *S* locus (Bauman et al., 2000). Therefore, Bm2 is not the pollen *S* gene, but it has been suggested that Bm2 may still have a function in pollen-pistil interactions (Bauman et al., 2000).

Modifiers of self-incompatibility

Analysis of a self-compatible mutant from *Phalaris coerulescens* demonstrated the existence of a third locus, called the *T* locus, which affects self-incompatibility (Hayman and Richter, 1992). The *T* locus is not closely linked to either the *S* or *Z* loci, and mutations only affect the pollen side of the self-incompatible response, rendering the pollen compatible to all pistils (Hayman and Richter, 1992). Similarly in a self-compatible mutant of *Lolium penne*, Thorogood and Hayward (1991) identified a locus that again only affects the pollen self-incompatibility. These loci are likely modifiers of self-incompatibility as described for the Solanaceae.

Mechanisms of self-incompatibility

The molecular mechanism of self-incompatibility in the grasses is largely unknown. However, the fact that two loci are involved increases the complexity of the

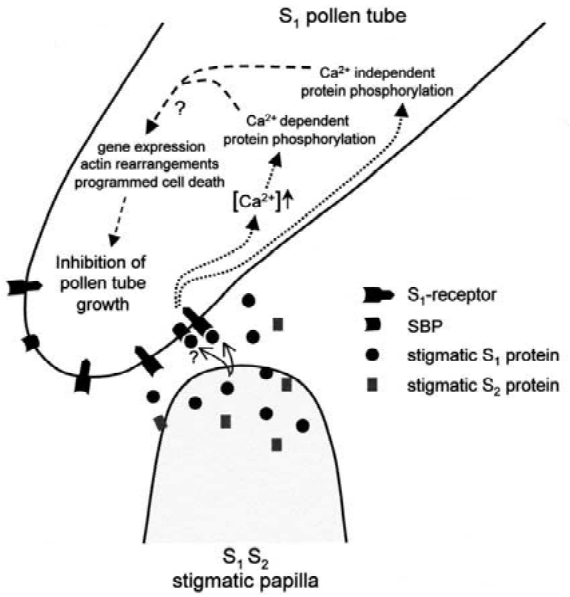

Figure 5. Proposed model for the self-incompatible response in *Papaver rhoeas*. Upon pollen–stigma interaction, the secreted stigmatic S_1 protein interacts with the pollen S_1-receptor on the pollen tube. Whether SBP represents the pollen *S* receptor or is required for this initial step is not known. The binding of the stigmatic S_1 protein to the receptor triggers a signaling cascade within the pollen tube. The initial step in the response involves an increase in calcium levels, followed by calcium-dependent and calcium-independent protein phosphorylation. This may lead to the expression of pollen 'response' genes, rearrangements of the cytoskeleton, and programmed cell death, and ultimately lead to pollen tube growth inhibition.

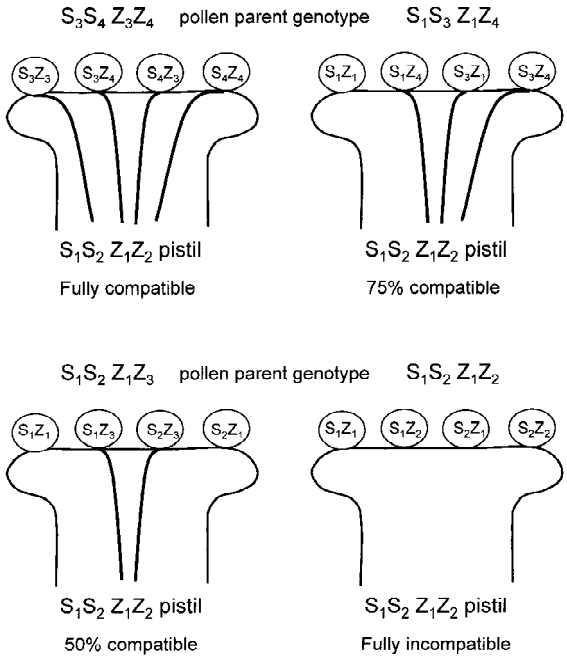

Figure 6. Pollination in the grasses. Pollen–pistil interactions between plants heterozygous at the *S* and *Z* alleles results in a degree of compatibility. For example, pollination of a S_1S_2 Z_1Z_2 plant with pollen from a S_1S_3 Z_1Z_4 plant is 75% compatibility. The S_1Z_1 pollen is incompatible because both the S_1 and Z_1 alleles are present in the pistil.

inhibitory response. Both the *S* and *Z* loci are expected to each produce two proteins, and interactions are expected to occur between the pollen and stigma *S* proteins, and the pollen and stigma *Z* proteins. As well, there may be cross interactions between the *S* and *Z* proteins. Protein phosphorylation and calcium uptake have been implicated in the self-incompatible response of *Secale cereale* (rye) (Wehling et al., 1994). Using an *in vitro* germination assay, pollen germinated in the presence of incompatible stigma extracts were found to have a higher level of protein phosphorylation when compared to treatment with compatible stigma extracts. Protein kinase inhibitors were also shown to disrupt the self-incompatible response (Wehling et al., 1994). A calcium channel blocker also inhibited the self-incompatible response suggesting that the self-incompatibility response may involve a calcium-dependent signal transduction pathway (Wehling et al., 1994).

Self-Incompatibility in the *Brassicaceae*

Research on the self-incompatibility system in *Brassicaceae* has been largely carried out using the *Brassica* genus with species such as *Brassica oleracea*, *Brassica campestris* (syn. *rapa*) and *Brassica napus*. In this sporophytic homomorphic self-incompatibility system, the incompatible pollen grain is rapidly inhibited at the stigma surface. The recognition and rejection of the self-incompatible pollen is genetically controlled by a single multi-allelic *S* locus, and the phenotype of the haploid pollen is determined sporophytically by the diploid parent (Bateman, 1955). Therefore, pollen rejection occurs when the pistil and the pollen parent plant share the same *S* allele (Figure 1B). The sporophytic nature of this self-incompatibility system has been hypothesized to occur by the deposition of the pollen *S* protein in the outer coat of the pollen grain during pollen development by the diploid anther tapetum (de Nettancourt, 1977). It has also been shown in *Brassica oleracea* that the majority of *S* alleles exhibit a relationship of dominance, codominance or recessiveness in hetero-

zygous plants in a non-linear arrangement (Thompson and Taylor, 1966). A few alleles, called pollen recessive alleles, were found to always be recessive to other *S* alleles in the pollen (Thompson and Taylor, 1966). As in other self-incompatibility systems, the *Brassica S* locus has proven to be a very complex region consisting of a number of genes (Cui et al., 1999; Suzuki et al., 1999; Casselman et al., 2000). However, for the *Brassica* self-incompatibility system, the identity of the both the pollen and pistil *S* genes have been determined.

Pistil S protein

Early studies on *Brassica* self-incompatibility identified an abundant stigma-specific glycoprotein called S-locus-specific glycoprotein (SLSG) and the inheritance of different isoforms of SLSG correlated with the different *S* alleles (Nasrallah and Wallace, 1967; Nasrallah et al., 1972; Hinata and Nisho, 1978). Differential screening of a mature self-incompatible stigma cDNA library from *Brassica oleracea*, lead to the isolation of a candidate cDNA (Nasrallah et al., 1985) for which the predicted amino acid sequence was subsequently found to share high sequence identity to peptide sequences derived from *Brassica campestris* SLSGs (Takayama et al., 1987). Analysis of this gene, designated the *S* locus glycoprotein (SLG) gene, showed that the predicted protein contained a N-terminal signal peptide, several potential *N*-glycosylation sites, two of which are conserved, three hypervariable regions, and 12 conserved cysteine residues in its C-terminus (Figure 7A, Dwyer et al., 1991; Kusaba et al., 1997). The SLG protein was demonstrated to be localized to the cell wall of the stigma papillae at the top of the pistil (Kandasamy et al., 1989).

A number of SLG alleles have been sequenced from different *Brassica* species and comparison of the sequences has revealed a high level of sequence identity at both the DNA and amino acid level (Goring and Rothstein, 1996). The SLG alleles can been grouped into two classes, class I and II, based on sequence identity and strength of the self-incompatibility response (Thomas and Taylor, 1966; Nasrallah et al., 1991). The class I alleles are generally dominant to other alleles and show a strong self-incompatibility phenotype, while the class II alleles are recessive to other alleles and exhibit a weaker self-incompatibility phenotype. Analysis of the class I and II SLG genes revealed that while the class I genes are intronless, the class II SLG genes possess one intron. Furthermore, the Class II alleles, S_2 and S_{15}, were found to produce both a soluble SLG and a membrane-anchored form called mSLG (Figure 7B, Tantikanjana et al., 1993; Cabrillac et al., 1999). A second SLG gene was also present at the S_{15} allele which only encoded a soluble SLG (Cabrillac et al., 1999).

The identification of SLG subsequently lead to the isolation of a second *S* locus gene, the *S* receptor kinase (SRK) gene (Stein et al., 1991; Goring and Rothstein, 1992). The predicted amino acid sequence for SRK consisted of a signal peptide, followed by an extracellular domain with high sequence identity to SLG, a transmembrane domain and a kinase domain. Similarly to the SLG, the predicted extracellular or receptor domain of SRK contained the putative *N*-glycosylation sites, the three hypervariable regions, and the conserved cysteine residues (Figure 7A, Stein et al., 1991; Goring and Rothstein, 1992; Galvin et al., 1994).

SRK is a functional serine/threonine kinase localized to the plasma membrane of the stigma papillae (Goring and Rothstein, 1992; Stein and Nasrallah, 1993; Delmore et al., 1995; Stein et al., 1996). Using a insect cell/baculovirus system, further evidence was recently provided indicating that SRK is indeed an integral membrane protein with the N-terminus or receptor domain positioned outside of the cell (Letham et al., 1999; Giranton et al., 2000). In this system, SRK was shown to be constitutively autophosphorylated in the absence of pollen or stigma proteins/extracts, and appeared to exist as a homooligomer (Giranton et al., 2000). Oligomeric complexes with SRK were also detected *in planta* (Giranton et al., 2000). The class I SRK_3 gene was found to encode a truncated or soluble SRK corresponding to the extracellular domain of SRK_3 (eSRK) as well as a full length SRK (Figure 7B, Giranton et al., 1995).

Several of the SLG/SRK pairs that have been characterized show higher levels of amino acid sequence identity to each other than to SLGs and SRKs from other S alleles (Goring and Rothstein, 1996; Nasrallah, 1997). However, there are examples of SLGs from different S alleles sharing higher amino acid sequence identities to each other than to their corresponding SRKs (Kusuba et al., 1997, 1999). For example, the amino acid identity between the *Brassica campestris* S_8 and S_{46} alleles is as high as 97.5%. These observations led to the suggestion that SLG and SRK do not have the same *S* allele specificity and they bind dif-

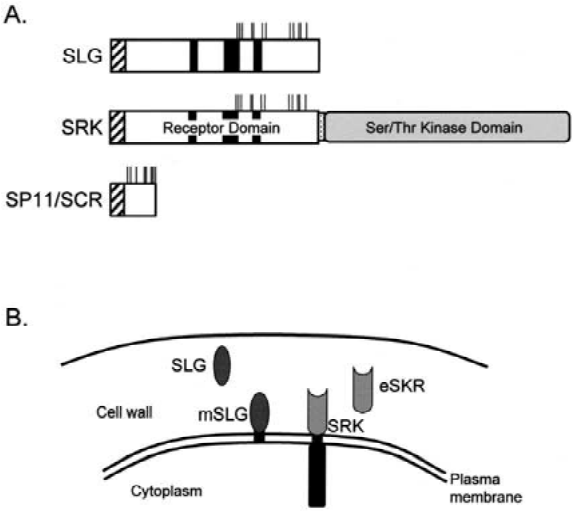

Figure 7. (A) Schematic representation of the *Brassica S* locus proteins. SLG and SRK are associated with self-incompatibility in the pistil while SP11/SCR represents the pollen *S* protein. Vertical lines represent conserved cysteines found within each class of proteins. The features of SLG are also found in the receptor domain of SRK, and in addition to the conserved cysteines, there are three hypervariable regions shown by black boxes. The hatched box at the beginning of all three proteins denotes the signal peptide. The putative SRK receptor domain is separated from the kinase domain by a predicted transmenbrane domain. *(B)* Illustration of the different forms of SLG and SRK encoded by their respective genes.

ferent sites on the pollen ligand (Kusuba et al., 1997, 1999).

SLG and SRK in the self-incompatibility response in the pistil

Over the years, several studies suggested evidence for a role for SLG in *Brassica* self-incompatibility. Transgenic studies demonstrated a correlation between the reduction in SLG expression and the loss of the self incompatible phenotype (Toriyama et al., 1991; Shiba et al., 1995; Takasaki et al., 1999). However, due to the high sequence similarity between SRK and SLG, the transgene induced cosuppression could also have reduced SRK expression; thus making it difficult to attribute the loss of self-incompatibility to a particular *S* gene (Connor et al., 1997). A self-compatible *Brassica oleracea* line was found to have a mutation in an unlinked gene leading to a loss of *SLG* gene expression, but not *SRK* gene expression (Nasrallah et al., 1992). There were also several arguments against the requirement of SLG in the self-incompatible response. Self-compatible plants expressing high levels of SLG and self-incompatible lines expressing low levels of SLG have been identified (Gaude et al., 1993, 1995). Therefore, there was no correlation between SLG expression and self-incompatibility. Furthermore, the SLG gene in the *Brassica oleracea* S_{24} line was found to be deleted (Okazaki et al., 1999).

Evidence for the involvement of SRK in *Brassica* self-incompatibility included the identification of self-compatible lines of *Brassica* carrying non-functional or mutated SRK alleles (Goring et al., 1993; Nasrallah et al., 1994). In addition, transformation of the self-incompatible *Brassica napus* line W1 with a kinase-inactive SRK_{910} resulted in a partial breakdown of self-incompatibility (Stahl et al., 1998). In one transgenic line, the kinase-inactive SRK_{910} was likely acting as a dominant negative mutant interfering with the wild-type SRK_{910}, possibly by forming inactive heterodimers (Stahl et al., 1998).

Recently, a series of gain-of-function experiments have helped to clarify the roles of SLG and SRK in the self-incompatibility reponse. Cui et al. (2000) demonstrated that the transformation of self-compatible *Brassica napus* cv. Westar with BAC clones carrying both the SLG_{910} and SRK_{910} genes dominantly conferred the self-incompatibility trait on the pistil. Takasaki et al. (2000) conclusively showed that SRK is the primary determinant of self-incompatibility in the pistil. They transformed the self-incompatible S_{60} *Brassica campestris* line with the SRK_{28} or SLG_{28} transgene and showed that only the SRK_{28} transgenic plants were capable of rejecting S_{28} pollen resulting in very little seed production. They also observed that plants expressing both the SLG_{28} and SRK_{28} transgenes exhibited a stronger self-incompatible phenotype and produced less seeds. Thus, SLG_{28} appeared to enhance the activity of SRK_{28}.

Silva et al. (2001) also found that SRK is the primary determinant of self-incompatibility in the pistil; however, no enhancing role was detected for SLG. The SRK_{910} and the SLG_{910} cDNAs, previously isolated from the self-incompatible *Brassica napus* W1 line (Goring and Rothstein, 1992; Goring et al., 1992a), were separately transformed into self-compatible *Brassica napus* cv. Westar. The SRK_{910} transgenic Westar plants gained the ability to efficiently reject W1 pollen while the SLG_{910} transgenic Westar plants were fully compatible to W1 pollen. Double transgenic plants expressing both SLG_{910} and SRK_{910} did not shown any significant decrease in the mean number of seeds produced following a W1 pollination when compared to the SRK_{910} only transgenic plants. Therefore, the presence and expression

of SLG_{910} did not enhance the ability of the SRK_{910} transgenic plants to reject W1 pollen. The difference in the two studies regarding the role of SLG may be attributed to the different systems used or to the levels of transgene expression achieved in each experiment. The level of SRK transgene expression observed by Takasaki et al. (2000) was 32–35% of a heterozygous S_{28} plant, while the expression levels observed by the Silva et al. (2001) were similar to a heterozygous W1 plant. However, both studies concluded that SRK is both necessary and sufficient for the self-incompatibility trait in the pistil.

The role of SLG in the self-incompatibility response still appears to be unclear. Schopfer et al. (1999) suggested that SLG may function in stabilizing SRK. Perhaps allele-specific SLGs are not required for such a role as long as other related proteins are present. In the gain-of-function experiments described above, there were other endogenous SLG genes present in the transgenic lines. Takasaki et al. (2000) crossed the SRK_{28} transgene into lines with different endogenous SLGs and found that the closer in sequence identity that the SLG had to SRK_{28}, the higher the enhancing effect. These enhancing effects are not large since SRK_{28} (in the presence of the S_{60} allele) reduced seed levels to 1.9 seeds/pod compared to a compatible pollination of approximately 15 seeds/pod, and the addition of the SLG_{28} transgene further reduced seed levels to 0.3 seeds/pod (Takasaki et al., 2000). Silva et al. (2001) used the self-compatible *Brassica napus* cv. Westar which carries the non-functional S_{A10} allele and has high levels of SLG_{A10} expression (Goring et al., 1993). Therefore, since SRK has not been tested in a background completely absent of SLGs, it is possible that SLG plays a general but non-specific role in self-incompatibility. A very different role for SLG has also being proposed where it is thought to be required for pollen adhesion and have a more general role in compatible pollen-pistil interactions (Luu et al., 1999).

The pollen S gene

Similarly to the *Solanaceae* self-incompatibility system, the *Brassica* pollen *S* gene was predicted to be tightly linked to the *S* locus, exhibit a high degree of polymorphism, and be expressed in the anther/pollen. A number of approaches were used to identify the pollen *S* protein without success until recently. A small (approximately 7 kDa) cysteine-rich pollen coat protein, PCP7/PCP-A1, which belongs to a large family of PCP genes, was found to interact with SLG (Doughty et al., 1993, 1998; Hiscock et al., 1995; Stanchev et al., 1996, Takayama et al., 2000b). PCP-A1 was however ruled out as a candidate for the pollen *S* component because its interaction with SLG was non-*S* allele-specific and the PCP gene was not linked to the *S* locus (Doughty et al., 1993, 1998). Another candidate for the pollen *S* gene was SLA (*S* locus anther). SLA is linked to the *S* locus and encodes for a small protein expressed specifically in the anther (Boyes and Nasrallah, 1995). However, further analysis of the SLA gene showed that both a self-incompatible and a self-compatible *Brassica oleracea* line carried a mutant SLA allele (Pastuglia et al., 1997). Therefore, a functional SLA was not required for the self-incompatible response.

A number of groups took the approach of mapping and sequencing the *S* locus region to find the pollen *S* gene (Yu et al., 1996; Boyes et al., 1997; Conner et al., 1998; Suzuki et al., 1999; Cui et al., 1999; Casselman et al., 2000). This led to the recent identification of a gene that exhibited all the requirements for the pollen *S* gene (Schopher et al., 1999; Suzuki et al., 1999; Takayama et al., 2000a). Suzuki et al. (1999) identified a number of genes in the *Brassica campestris* S_9 locus region including one anther expressed gene called SP11 which was predicted to encode a cysteine rich protein like the PCP family. Takayama et al. (2000) went on to show that SP11 encoded the pollen *S* product. They identified the SP11 gene from three other *Brassica campestris S* alleles and found that SP11 was highly polymorphic and showed that SP11 protein produced in *E. coli* could elicit an allele-specific self-incompatibility response. The SP11 gene was found to be expressed at earlier stages in the anther tapetum surrounding the developing microspores, consistent with the sporophytic nature of self-incompatibility. The SP11 gene was also found to be gametophytically expressed at later stages in the microspores (Takayama et al., 2000a). Watanabe et al. (2000) cloned an additional 14 SP11 alleles and found that the predicted amino acids sequences were highly divergent with the exception of eight conserved cysteines (Figure 7A).

By sequencing the region between the SLG_8 and SRK_8 genes from *Brassica campestris*, Schopher et al. (1999) also identified the same gene, a polymorphic gene expressed in the anther and predicted to encode a cysteine-rich protein, and named it *S* locus cysteine-rich (SCR) gene. The mature SCR protein is predicted to be hydrophilic with a size of 8.4–8.6 kDa and contains eight conserved cysteine residues. The SCR

transcript was found to be absent in a pollen self-incompatibility mutant and transformation of a SCR_6 transgene into the *Brassica oleracea* S_2 line resulted in the transgenic pollen being rejected by S_6 pistils. Thus, both loss-of-function and gain-of-function studies demonstrated that SCR is required for determining the pollen self-incompatibility trait.

The signal transduction pathway

Pollen rejection is proposed to involve a signaling pathway in the stigmatic papillae mediated by SRK (Figure 8A). SRK is thought to be activated upon binding of the pollen ligand, SP11/SCR, to its extracellular domain. However, whether SP11/SCR binds to SRK in an allele-specific interaction leading to SRK activation must still be determined. If SLG plays a general role in self-incompatibility, it may function to shuttle the pollen ligand across the cell wall to the plasma membrane-associated SRK, and form a complex with SRK. The activation of SRK may involve dimerization, as seen for the animal receptor kinases, followed by SRK autophosphorylation to activate the downstream signaling pathway (Goring and Rothstein, 1996).

Beyond the proteins involved in initiating the response very little else is known about the molecules that participate in the signaling cascade. While the deposition of callose had been associated with the self-incompatibility response, it was found not to be necessary for the rejection of self-incompatible pollen (Sulaman et al., 1997). As well, there were no changes in cytosolic calcium levels or changes to the cytoskeleton associated with the *Brassica* self-incompatibility response (Dearnaley et al., 1997, 1999). Phosphatase inhibitors were found to disrupt the self-incompatible response suggesting that protein phosphatases are involved (Scutt et al., 1993). As well, the kinase-associated protein phosphatase (KAPP) which is part of the CLV1 signaling pathway was found to interact with the SRK kinase domain *in vitro* (Braun et al., 1997). Analysis of a modifier (*mod*) locus that leads to a breakdown in *Brassica* self-incompatibility resulted in the isolation of a gene predicted to encode an aquaporin-related protein (Ikeda et al., 1997). This gene was linked to the *mod* locus and showed considerably reduced levels of mRNA in *mod/mod* plants. However, recently Fukai et al. (2001) demonstrated that there is natural variation in the RNA levels for this aquaporin-like gene and that the RNA levels were unrelated to the self-incompatible status of the plant. Thus, the *mod* locus is likely another gene nearby.

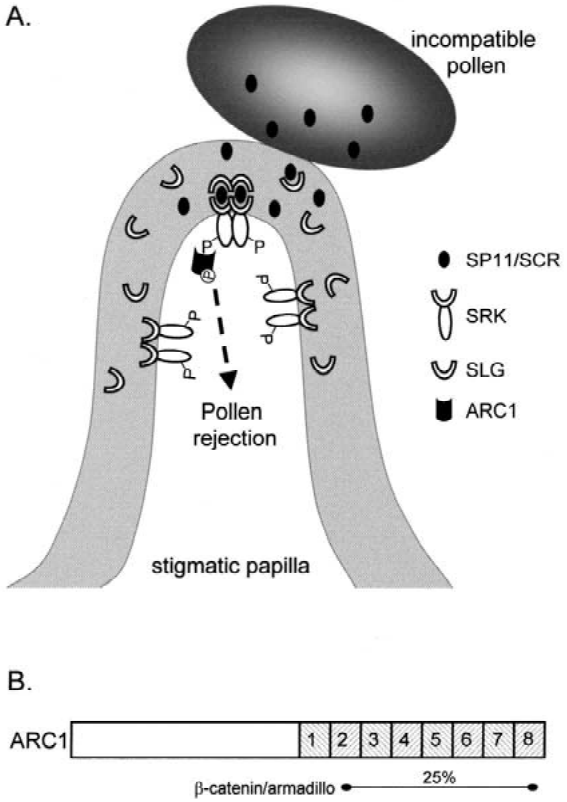

Figure 8. Model for the recognition and rejection of self-incompatible pollen in *Brassica*. *(A)* Pollen–pistil interaction results in the interaction of the pollen ligand, SP11/SCR, with SRK. SLG may be involved in this initial interaction, but in a non-allelic manner. This results in the activation of SRK which then initiates a signaling pathway in the stigmatic papilla ultimately leading to pollen rejection. The initial step of the signaling pathway involves ARC1 which is predicted to bind and become phosphorylated by SRK. However, the function of activated ARC1 inside the papilla is unknown. *(B)* Schematic representation of the ARC1 protein. ARC1 is 661 amino acids in length and contains eight arm repeats (numbered boxes) in the C-terminal region. This region also contains sequence identity to the β-catenin/armadillo family as shown.

Through a yeast two-hybrid system, three proteins which interact with the kinase domain of SRK_{910} have been isolated (Bower et al., 1996; Gu et al., 1998). Two of these proteins, THL1 and THL2, belong to the thioredoxin h family and bind specifically to the kinase domain of SRKs, though the interaction does not appear to be phosphorylation dependent (Bower et al., 1996). Experiments with THL1 have shown that the nature of the interaction involves the redox activity of THL1 since amino acid substitutions of

the cysteines in the active site of THL1 abolish the interactions with the SRKs (Mazzurco et al., 2001). In addition, a cysteine located at the end of the SRK transmembrane domain is also required for this interaction (Mazzurco et al., 2001). Recently, THL1 was shown to be an inhibitor of SRK autophosphorylation *in vitro* (Cabrillac et al., 2001). This inhibitory effect was alleviated by the addition of the allele-specific pollen coat proteins. Thus, THL1/2 were proposed to be acting as negative regulators of SRK in the absence of the pollen ligand, SP11/SCR. The inhibitory effect of the THL1/2 on SRK is only overcome with the addition of the corresponding SP11/SCR ligand (Cabrillac et al., 2001).

The third protein isolated from the yeast two-hybrid screen, named ARC1 for arm repeat containing, is a novel gene expressed only in the stigmatic papillae cells at the top of the pistil. The C-terminal region of the predicted ARC1 protein contains up to eight arm repeats (Figure 8B). Arm repeats are degenerate 42-amino acid motifs that are found in a number of structural and signaling molecules and function in mediating protein–protein interactions. The sequence diversity found in arm repeats determines the binding specificity of the protein while the regions outside of the arm repeats determine protein function (Hatzfeld, 1999). The arm repeat region of ARC1 specifically interacts with SRK kinase domains in a phosphorylation dependent manner and ARC1 is phosphorylated by the SRK_{910} kinase domain *in vitro* (Gu et al., 1998). Recent transgenic studies have shown that ARC1 is required for the *Brassica* self-incompatibility response (Stone et al., 1999). Reduction of ARC1 mRNA levels in self-incompatible W1 plants by an ARC1 antisense transgene led to a partial breakdown in the self-incompatible response in the pistil and a significant number of seeds were produced. The most affected stages in pollen rejection were the early stages of the self-incompatibility response resulting in the adherance and hydration of a large number of pollen grains (Stone et al., 1999).

Following SRK activation, ARC1 is thought to bind to the SRK kinase domain and promote the signal from SRK (Figure 8A). This interaction may result from phosphorylation or conformational changes in SRK and involve ARC1 binding to a phosphoserine or phosphothreonine docking site in the SRK kinase domain. The interaction between SRK and ARC1 may result in the phosphorylation and activation of ARC1. While ARC1 is involved in the self-incompatibility reaction, how ARC1 functions in the signaling pathway is not known. Activated ARC1 may then act as an adaptor and interact with the next molecule of the signaling pathway. Ultimately, based on the analysis of the ARC1 antisense transgenic plants, the signaling pathway involving ARC1 appears to act at the early stages of the self-incompatibility response leading to a block in pollen hydration and germination (Stone et al., 1999).

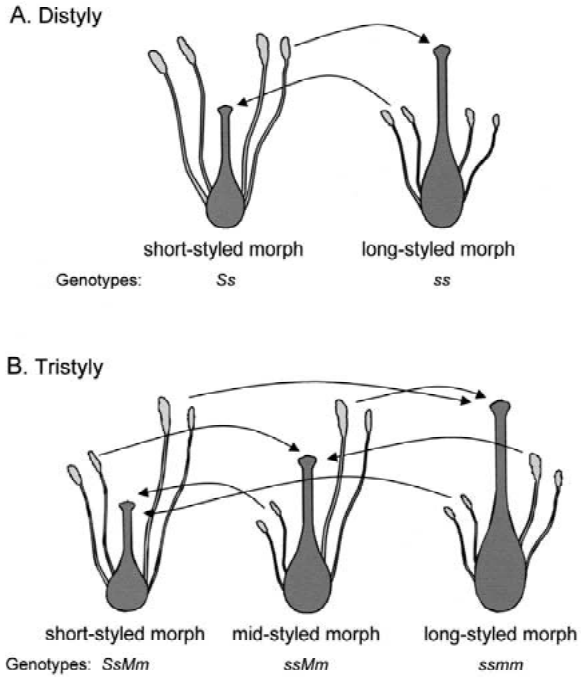

Figure 9. Morphological polymorphism in heteromorphic plants. *(A)* The two floral morphs of distyly and *(B)* the three floral morphs of tristyly are illustrated. Compatible pollinations between the different morphs are indicated by arrows. Examples of genotypes for each floral morph are indicated underneath.

Heteromorphic self-incompatibility

Heteromorphic self-incompatibility is less widespread than homomorphic self-incompatibility and has been described in 25 families of angiosperms (Barrett and Curzan, 1994). Unlike homomorphic self-incompatible species, the flowers of heteromorphic self-incompatible species are morphologically distinct. The differences, termed heterostyly, lie in the length of the style and anther within each flower, and have been subdivided into two groups, distyly and tristyly (Figure 9). Distyly consists of two morphs, long- and short-styled morphs, and tristyly consists of three morphs, long-, mid-, and short-styled morphs

(Figure 9). Heterostyly is sporophytic in nature, and genetically controlled in distyly by one diallelic locus (*S*,*s*), and in tristyly by two diallelic loci (*S*, *s* and *M*, *m*). The dominant *S* allele determines the short-styled morph in both distyly and tristyly and is epistatic to *M* in tristyly (Lewis and Jones, 1992). There are also a few species in which the long-style morph is dominant (Lewis and Jones, 1992). Compatible or legitimate pollination occurs between anther and stigmas of the same height between morphs of the distylic and tristylic species (Figure 9). Self and intramorph pollinations are termed illegitimate and result in reduced or no seed production. Inhibition of pollen tube growth may occur at a number of sites within the pistil including the stigma, style, and ovaries. The site of inhibition usually depend upon the type of morphs and the various polymorphisms of the pollen and stigma (Dulberger, 1992).

In addition to the style–stamen length polymorphism, there are other morphological polymorphisms closely associated with the incompatibility mechanism (Dulberg, 1992; Barrett and Curzan, 1994). In distylic species, there is also the stylar-pollen incompatibility, and differences in pollen size (de Nettancourt, 1977; Lewis and Jones, 1992). Lewis and Jones (1992) reported that there is no evidence for the separation of floral polymorphisms such as pollen size from incompatibility. Thus, the *S* locus in distyly is thought to consist of a number of tightly linked genes that are inherited together and has been referred to as the *S* supergene (Dorwick, 1956; Lewis and Jones, 1992).

Studies on heterostyly have focused mainly on the genetics of the system and very little is known about the molecular mechanisms governing floral polymorphisms and self-incompatibility. Attempts have been made to identify proteins involved in self-incompatibility in distylic species (Golynskaya et al., 1974; Wong et al., 1994; Anthanasiou et al., 1997). Isoelectric focusing was used to identify anther and style specific proteins of the long-styled and short-styled morphs in *Averrhoa carambola* (Wong et al., 1994) and various species of *Tunera* (Anthanasiou and Shore, 1997). Wong et al. (1994) reported that the number of proteins in the style and stamen increased as the floral reproductive organs matured. They also identified three proteins which were specific to the floral organs of each morph, two of these proteins bind concanavalin A.

Anthanasiou and Shore (1997) identified three style proteins and two pollen proteins specific to the short-styled morph of *Turnera*. Further analysis suggested that the loci encoding these morph specific proteins were linked to the distyly locus. Shore and co-workers have since cloned and sequenced the genes encoding the short-specific style and pollen proteins. Based on sequence identity to published sequences, the style and pollen proteins appear to be polygalacturonases (Anthanasiou and Shore, pers. commun.). Through the use of western blot analysis with polyclonal antibodies generated against the style fusion protein, they have determined that the style proteins are also unique to the short-styled morph of two additional species, *Turnera joelii* and *Turnera grandiflora* (Tamari, Khosravi and Shore, pers. commun.). Using immunocytochemistry, the style proteins were detected throughout the transmitting tract of short-styled but not long-styled plants from five *Tunera* species that were studied (Khosravi, 2000). These experiments suggest that a style polygalacturonase is only expressed in the transmitting tissue of short styled plants and may have a role in the self-incompatibility response in short styled morphs. However, this hypothesis requires further confirmation through plant transformation experiments.

Conclusions

In recent years, exciting progress has been made towards understanding the molecular basis of several self-incompatibility systems. Several *S* locus genes have been identified and information is starting to emerge on how the encoded *S* proteins function to cause the rejection of self-incompatible pollen. However, there still is much to be discovered about processes involved in these self-incompatibility systems. Research on these very important systems not only will lead to a greater understanding of how flowering plants can control a basic process such as fertilization, but it will also shed light on the processes used by plants for cell–cell communication.

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