The Molecular and Genetic Bases of S-RNase-Based Self-Incompatibility

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INTRODUCTION

The majority of flowering plants produce perfect flowers that contain both the male and female reproductive organs in close proximity; consequently, they would have a strong tendency to self-fertilize if there were no mechanisms to prevent them from doing so. Because inbreeding can result in reduced fitness in the progeny, hermaphroditic plants have adopted a variety of reproductive strategies, including self-incompatibility (SI), by which inbreeding is prevented and outcrosses are promoted. SI allows the pistil of a flower to distinguish between genetically related (self) and unrelated (non-self) pollen. This self/non-self recognition results in the inhibition of germination of self-pollen on the stigmatic surface or the inhibition of growth of self-pollen tubes in the style. Thus, SI is a prezygotic reproductive barrier by which incompatible pollen/pollen tubes are prevented from delivering the sperm cells to the ovary to effect double fertilization.

SI can be classified into homomorphic and heteromorphic types based on whether it is associated with floral polymorphism. In species that exhibit homomorphic SI, all individuals produce the same type of flower and the outcome of pollination depends only on the genetic identity of the male and female partners. In contrast, species that exhibit heteromorphic SI produce two or three different flower morphologies (e.g., a flower with short anthers and long style or a flower with long anthers and short style). For successful pollination, pollen must come from genetically unrelated individuals whose anthers are of the same height as the style of the flower being pollinated. To date, much of what we know about the molecular basis of SI has been deduced from studies of homomorphic SI, which will be the focus of this review. A monograph by de Nettancourt (2001) provides a comprehensive treatise on SI, including a discussion of the heteromorphic type.

For homomorphic SI (hereafter referred to as SI), self/non-self discrimination between pollen and pistil is determined by one or more polymorphic loci, and this type of SI is further classified into gametophytic and sporophytic types based on the genetic control of pollen behavior. To date, four of the families that exhibit gametophytic SI (GSI), Solanaceae, Rosaceae, Scrophulariaceae, and Papaveraceae, and one of the families that exhibit

sporophytic SI (SSI), Brassicaceae, have been studied extensively at the molecular level (Table 1). A single polymorphic locus, termed the S-locus, controls the SI response in all five of these families. As described below, other loci often are required for the full manifestation of the SI response, but by definition, the S-locus determines the specificity of the response. It is now known that two separate genes at the S-locus control male and female specificities. Thus, the term "haplotypes" is used to describe variants of the S-locus, whereas the term "alleles" is used to describe variants of an S-locus gene.

For the four GSI families, SI occurs when the S-haplotype of the pollen matches either of the two S-haplotypes carried by the pistil. That is, the SI phenotype of the pollen (gametophyte) is determined by its own S-genotype. For the SSI family, in the simplest case, SI occurs when the pollen-producing parent shares one or both S-haplotypes with the pistil. That is, the SI phenotype of the pollen is determined by the S-genotype of its diploid parent. For SSI, complex relationships often exist between the different S-haplotypes of the pollen and pistil parents. One S-haplotype could be dominant over or recessive to another, or it could interact with another to result in mutual weakening or in an entirely new S-haplotype specificity (Thompson and Taylor, 1966).

THREE TYPES OF SI MECHANISMS

During the past two decades, much progress has been made in identifying and characterizing the S-locus genes that control the specificity of the SI interaction in the five families mentioned above. Comparisons of the S-locus genes expressed in the pistil among the different families have revealed three biochemically distinct mechanisms (Table 1). The Solanaceae, Rosaceae, and Scrophulariaceae use the same mechanism, the Papaveraceae uses another, and the Brassicaceae uses a third. For the Solanaceae and Papaveraceae mechanisms, the gene that controls female specificity has been identified; these genes were named the S-RNase gene and the S-gene, respectively. Our understanding of the Solanaceae mechanism has progressed further, with the recent identification of a promising candidate for the male specificity gene. The Solanaceae mechanism involves S-RNase–mediated degradation of RNA in self-pollen tubes. The Papaveraceae mechanism is mediated by a signal transduction cascade in pollen that involves a number of known components of signal transduction (e.g., Ca^{2+}, phosphoinositides, protein kinases, and phosphatases). For the SSI mechanism found in the Brassicaceae, both the gene that controls male specificity, S-locus cysteine-rich protein (SCR)/S-locus protein-11 (SP11),

Table 1. Summary of Three Types of SI Mechanisms

Plant Family	Type of SI	Genetic Locus	Female Determinant	Male Determinant	Mechanism
Solanaceae, Rosaceae, Scrophulariaceae	GSI	*S*-locus	*S-RNase*	*SLF/SFB*?	S-RNase–mediated degradation of pollen tube RNA
Papaveraceae	GSI	*S*-locus	*S*-gene	Unknown	S-protein–mediated signaling cascade in pollen
Brassicaceae	SSI	*S*-locus	*SRK*	*SCR/SP11*	Receptor-kinase–mediated signaling in stigma

and the gene that controls female specificity, *S*-locus receptor kinase (*SRK*), have been identified. The SI response is mediated via a signal transduction cascade in the stigmatic papilla, which is elicited by the interaction of a pollen-borne ligand, SCR/SP11, and SRK, a receptor kinase in the stigmatic papilla.

The discussion below focuses on the Solanaceae type of SI. For a recent review of the Brassicaceae type of SI, see Kachroo et al. (2002); for a recent review of the Papaveraceae type of SI, see Thomas et al. (2003).

The Solanaceae type of SI was first discovered in *Nicotiana sanderae* (East and Mangelsdorf, 1925), and to date, this type of SI has been studied at the molecular level in four genera of the Solanaceae (*Lycopersicon*, *Nicotiana*, *Petunia*, and *Solanum*), three genera of the Rosaceae (*Malus*, *Prunus*, and *Pyrus*), and one genus of the Scrophulariaceae (*Antirrhinum*). The rejection of self-pollen occurs during pollen tube growth in the style, and the timing of the rejection coincides with the transition of pollen tube growth from the slow ("autotrophic") growth phase to the accelerated ("heterotropic") growth phase (Herrero and Hormaza, 1996). The increase in the growth rate is presumed to result from the increased acquisition of nutrients provided by the pistil tissue. Interestingly, several critical cellular events also occur around the time of this transition (e.g., mitotic division of the generative cell to give rise to two sperm nuclei).

FEMALE SPECIFICITY DETERMINANT: THE *S-RNASE* GENE

The search for the female determinant of SI was based on the prediction that the gene encoding it must exhibit allele-specific sequence differences and must be expressed in the pistil. Pistil-specific proteins that showed allele-specific differences in molecular mass and/or isoelectric point were first identified in *Nicotiana alata* (Bredemeijer and Blass, 1981), and the first sequence of such a protein was deduced from the cloning and sequencing of the corresponding cDNA (Anderson et al., 1986). These proteins were initially named *S*-allele–associated proteins or S-proteins, and the gene was named the pistil *S*-gene. Similar approaches were used to identify S-proteins and to isolate their cDNAs from other solanaceous species (Ai et al., 1990; Clark et al., 1990) and several rosaceous species (Sassa et al., 1993; Ishimizu et al., 1996).

Sequence comparisons of solanaceous S-proteins have revealed five conserved regions and two hypervariable regions (Ioerger et al., 1991; Tsai et al., 1992). The presence of distinct conserved regions has made it possible to clone genomic and cDNA fragments for S-proteins by PCR. This is particularly useful

for population and evolutionary studies of SI (Richman et al., 1995; Wang et al., 2001) and to genotype plants (Tao et al., 1999). For rosaceous species, the ability to identify *S*-genotypes by this rapid approach is particularly useful because it circumvents the time-consuming and labor-intensive pollination tests. Most notably, this approach led Xue et al. (1996) to clone cDNAs for the homologs of S-proteins in *Antirrhinum hispanicum* and to discover that this species exhibits the same type of SI mechanism as the solanaceous and rosaceous species. Phylogenetic analysis of S-proteins has suggested that the Solanaceae-type mechanisms used by the species in these three distantly related dicot families share a common origin, and that this mechanism might be exhibited by the ancestor of ~75% of all dicots (Igic and Kohn, 2001). At present, it is not known whether the Solanaceae mechanism is exhibited by any other dicot families. This question can be addressed by using PCR to ascertain whether orthologs of S-proteins are present in any other dicot families.

Biochemical and Structural Characteristics of S-Proteins/S-RNases

The biochemical nature of S-proteins was revealed when the sequence of RNase T_2 of *Aspergillus oryzae* (Kawata et al., 1988) was determined and found, unexpectedly, to share sequence similarity with S-proteins (McClure et al., 1989). This finding led to the subsequent confirmation that S-proteins have RNase activity in vitro (McClure et al., 1989; Singh et al., 1991). Significantly, two regions of RNase T_2, each of which contains a catalytic His, are now known to be present in all S-proteins and to correspond to two of the five conserved regions identified by Ioerger et al. (1991). Thus, S-proteins have been renamed S-RNases and the gene has been renamed the *S-RNase* gene. S-RNases do not appear to have any substrate specificity in vitro (Singh et al., 1991). RNase T_2 and S-RNases have been placed in a large family of RNases, named the T_2/S-RNase family, which also includes S-like RNases and relic S-RNases (Green, 1994; Golz et al., 1998). S-like RNases do not exhibit allelic sequence diversity, and they have been identified from both self-incompatible and self-compatible species of the Solanaceae, Rosaceae, and Scrophulariaceae as well as from self-compatible species of several other families. Relic S-RNases have been identified from both self-incompatible and self-compatible species of the Solanaceae, Rosaceae, and Scrophulariaceae, and they are more similar to S-RNases than to S-like RNases and, like S-RNases, are specific to the pistil. However, relic S-RNases, like S-like RNases, do not exhibit allelic sequence

polymorphism. Relic *S-RNase* genes could have been derived from the *S-RNase* gene as a result of gene duplication, followed by translocation to other loci. Despite their sequence similarity to S-RNases, relic S-RNases and S-like RNases are unlikely to play a role in SI, and to date, the physiological function of most of them remains unknown.

The role of the *S-RNase* gene in the SI interaction has been established via transgenic experiments (Lee et al., 1994; Murfett et al., 1994). These experiments showed that introduction of a new allele of the *S-RNase* gene into transgenic plants was sufficient to confer on the plants the ability to reject pollen carrying the same allele as the introduced *S-RNase* gene. Conversely, suppression of the expression of an endogenous allele of the *S-RNase* gene by the antisense RNA approach abolished the ability of the transgenic plants to reject the pollen carrying the affected allele.

To understand the biochemical mechanism of S-RNase–mediated self-rejection, it is imperative to know whether the RNase activity of S-RNases is an integral part of their function. Site-directed mutagenesis was used to replace the codon for one of the two catalytic His residues of S_3-RNase of *Petunia inflata* with an Asn codon, and transgenic plants that produced S_3-RNase without RNase activity failed to reject S_3 pollen (Huang et al., 1994). Consistent with this result is the finding that a self-compatible accession of *Lycopersicon peruvianum* produced a catalytically inactive S-RNase with one of the active-site His residues replaced with Arg (Kowyama et al., 1994; Royo et al., 1994). Because the RNase activity of S-RNases is required for pollen rejection, it is reasonable to infer that the degradation of RNA by S-RNases inside self-pollen tubes results in growth inhibition. McClure et al. (1990) obtained results consistent with this prediction. They showed that pollen tube rRNA was degraded after self-pollination but not after cross-pollination. However, the design of the experiments leaves open the possibility that the observed degradation of RNA is a consequence, but not the cause, of growth inhibition of self-pollen tubes.

S-Allele–Specificity Determinant of S-RNases

Attempts have been made to identify the region (or regions) of S-RNases that determines *S*-allele specificity (i.e., regions that are involved in the interaction with the pollen *S*-allele products). Because S-RNases are glycoproteins that vary in the number and position of N-linked glycan chains, the *S*-allele–specificity determinant could reside, a priori, in the carbohydrate moiety and/or the protein backbone. To address this question, a non-glycosylated S_3-RNase of *P. inflata* (with the Asn residue for the only *N*-glycosylation site replaced with Asp) was produced in transgenic plants, and the SI behavior of the pistil was examined. The nonglycosylated S_3-RNase was found to have RNase activity similar to that of the wild-type S_3-RNase and to function as well as the wild-type S_3-RNase in rejecting S_3 pollen (Karunanandaa et al., 1994). Thus, the *S*-allele–specificity determinant of S-RNases resides in their protein backbone.

One notable feature of S-RNases is their high degree of allelic sequence diversity. For example, the two most divergent solanaceous S-RNases share only 38% sequence identity (Tsai et al., 1992). There are a large number of variable sites scattered throughout the protein; however, the most highly variable sites are clustered in two regions, named HVa and HVb. These two hypervariable regions were identified initially from comparison of solanaceous S-RNases (Ioerger et al., 1991; Tsai et al., 1992). They were found subsequently to be the hypervariable regions of *Antirrhinum* S-RNases as well (Xue et al., 1996) and to correspond to two of the four regions of rosaceous S-RNases for which evidence of positive selection has been found (Ishimizu et al., 1998). The crystal structures of a solanaceous S-RNase and a rosaceous S-RNase show that both HVa and HVb regions are exposed on the surface of the protein and accessible to solvent (Ida et al., 2001; Matsuura et al., 2001). Together, HVa and HVb are considered the most likely candidates for the determinant of *S*-allele specificity.

Domain-swapping experiments have been performed to ascertain the role, if any, of HVa, HVb, and other regions of S-RNases in *S*-allele specificity (Kao and McCubbin, 1996; Matton et al., 1997; Zurek et al., 1997). For each chimeric *S-RNase* gene, the bulk of the sequence was from one allele of the *S-RNase* gene, with the sequence of the region to be examined contributed by another allele. Transgenic plants that produce each chimeric S-RNase then were examined for their ability to reject pollen of the two alleles used in the chimeric construct. When pairs of S-RNases with a high degree of sequence diversity (e.g., 74.1% amino acid identity between S_1- and S_3-RNases of *P. inflata*) were used in the domain swapping, none of these domains alone could bestow on the chimeric protein its unique *S*-allele specificity. Moreover, the specificity of the allele used as the backbone of the chimeric *S-RNase* gene was abolished (Kao and McCubbin, 1996; Zurek et al., 1997). Because all of these chimeric S-RNases retained normal RNase activity, their failure to reject self-pollen was attributed to the loss of the recognition function.

When two S-RNases of *Solanum chacoense*, S_{11}-RNase and S_{13}-RNase, that share 92% amino acid sequence identity (with only 10 dissimilar amino acids) were used for the construction of chimeric *S-RNase* genes, it was found that the HVa and HVb regions together were sufficient to confer on the chimeric S-RNases the new allelic specificity (Matton et al., 1997). That is, when the amino acids of HVa and HVb of S_{11}-RNase were changed to those of S_{13}-RNase, transgenic plants that produced this chimeric S-RNase rejected S_{13} pollen but not S_{11} pollen. It should be noted, however, that domain-swapping experiments can only address the role of those amino acids exchanged that differ between the two proteins. Thus, the results of Matton et al. (1997) cannot rule out the involvement of amino acids outside of HVa and HVb that are conserved between S_{11}-RNase and S_{13}-RNase (Verica et al., 1998). The finding that two *P. inflata* S-RNases, S_6-RNase and S_9-RNase, have identical sequence in HVa and differ by only two amino acids in HVb (Wang et al., 2001) suggests that amino acids outside of these two hypervariable regions likely are involved in the determination of *S*-allele specificity.

MALE-SPECIFICITY DETERMINANT

Classic genetic studies showed that the pollen and pistil functions in SI could mutate independently to result in either

pollen-part or pistil-part self-compatible mutants (de Nettancourt, 2001). This finding strongly suggests that separate genes control these two functions. In all of the transgenic experiments performed to ascertain the function of the *S-RNase* gene described above, the pistil function, but not the pollen function, was affected by the manipulation of the *S-RNase* gene, consistent with the notion that the S-RNase gene does not control male specificity. Most directly, Sassa et al. (1997) showed that the S_4-*RNase* gene was deleted in a self-compatible cultivar of *Pyrus serotina* (Japanese pear) and that this deletion affected the pistil function but not the pollen function. During the past few years, a flurry of research activities have been directed toward the identification of the gene that controls male specificity, the pollen *S*-gene.

S-Locus Linked Genes

One approach to identifying the pollen *S*-gene is to search for pollen-expressed genes that exhibit *S*-haplotype–specific restriction fragment length polymorphism, because the pollen *S*-gene is expected to show a significant degree of allelic sequence diversity. A number of such genes have been identified in *N. alata* and *P. inflata* by RNA differential display and subtractive hybridization (Dowd et al., 2000; Li et al., 2000; McCubbin et al., 2000). Recombination analysis has been performed to determine if each of these genes is tightly linked to the *S-RNase* gene. Because recombination at the *S*-locus is suppressed as a result of its centromeric location (Entani et al., 1999), a large number of plants segregating for *S*-haplotypes are needed to accurately assess the linkage to the *S-RNase* gene. Nine of the pollen-expressed genes of *P. inflata* (Wang et al., 2004) and one of the pollen-expressed genes of *N. alata* (Li et al., 2000) were found to be tightly linked to the *S-RNase* gene. The nucleotide sequences of different alleles of each of these genes were obtained to assess the allelic sequence diversity. The deduced amino acid sequences of all of these genes exhibit very low allelic sequence diversity. Also, analysis of the nucleotide sequence variation of *48A* of *N. alata* has revealed no evidence of positive selection, which is expected of the *S*-locus genes involved in SI (Takebayashi et al., 2003). In the case of *P. inflata*, chromosome walking through the S_2-locus region has shown that the nine genes that are tightly linked to the *S-RNase* gene are located at least ∼180 kb upstream or at least ∼700 kb downstream from the *S-RNase* gene and could be as far as 4 Mb away (T. Tsukamoto, Y. Wang, K.-W. Yi, A.G. McCubbin, and T.-h. Kao, unpublished data). Thus, none of the pollen-expressed genes identified by this approach is likely to be the pollen *S*-gene, and their allelic sequence polymorphism may result simply from their tight genetic linkage to the highly polymorphic *S*-locus.

The S-Locus F-Box Gene: A Candidate for the Male-Specificity Gene

Another approach to identifying the pollen *S*-gene is based on the prediction that the pollen *S*-gene must be very tightly linked to the *S-RNase* gene. Recombination in the chromosomal region between these two genes would inevitably result in the breakdown of SI by generating different *S*-haplotype specificities

for pollen and pistil, but such recombinant genotypes have never been observed (de Nettancourt, 2001). Lai et al. (2002) sequenced a 63-kb region containing the S_2-*RNase* gene of *A. hispanicum* and identified 10 additional open reading frames (ORFs); 4 encode retrotransposons and only 1 of the remaining ORFs is expressed in the anther (tapetum and pollen). This gene, located ∼9 kb downstream of the S_2-*RNase* gene, encodes an F-box–containing protein and was named *AhSLF* (*A. hispanicum* *S*-locus F-box gene). cDNA encoding a homolog of AhSLF-S_2 was isolated from a line of S_1S_5 genotype, and its deduced amino acid sequence is 97.9% identical to that of AhSLF-S_2. However, it is not clear from that report whether *AhSLF-S_2* and its homolog, named *AhSLF-S_2L*, are allelic.

Entani et al. (2003) and Ushijima et al. (2003) also attempted to identify the pollen *S*-gene by sequencing the *S*-loci of *Prunus mume* (Japanese apricot) and *Prunus dulcis* (almond), respectively. An ∼70 kb chromosomal region of *P. dulcis* that contains the S^c-*RNase* gene is considered the functional region of the *S*-locus (Ushijima et al., 2001) based on the following findings. First, genomic blot analysis showed that the sequence of this region is highly divergent between different *S*-haplotypes, whereas the sequences flanking this region are similar between different *S*-haplotypes. Second, a self-compatible mutant with a chromosomal deletion spanning this region was defective in both pollen and pistil functions. Sequencing of this ∼70-kb region revealed 10 ORFs in addition to the S^c-*RNase* gene. As with the *Antirrhinum* S_2-locus, some of the ORFs encode retrotransposons. Only two of the other ORFs are expressed in pollen, and interestingly, both encode F-box proteins. One of the F-box genes, named *SFB* (*S*-haplotype–specific F-box gene), exhibits a similarly high level of allelic sequence diversity as the *S-RNase* gene. The pairwise amino acid sequence identities of S^a-, S^b-, S^c-, and S^d-alleles range from 68.4 to 76.4%, and those of the same four alleles of the *S-RNase* gene range from 55.6 to 77.1% (Yamane et al., 2003). For the other F-box gene, named *PdSLF* (*P. dulcis* *S*-locus F-box gene), the deduced amino acid sequences of S^c- and S^d-alleles are 95.1% identical. *SFB* is a good candidate for the pollen *S*-gene, because (1) it is linked physically to the *S-RNase* gene, located within 30 kb of the *S-RNase* gene in the four *S*-haplotypes studied; (2) it is expressed specifically in pollen; and (3) it shows a high level of allelic sequence diversity. By contrast, *PdSLF*, despite its physical linkage to the *S-RNase* gene and its pollen expression, is considered unlikely to be the pollen *S*-gene because of the low degree of allelic sequence diversity. It should be noted that *PdSLF* was so named to emphasize that it shows approximately the same low level of allelic sequence diversity as *AhSLF* identified in *Antirrhinum* (Lai et al., 2002) rather than to indicate that *PdSLF* is an ortholog of *AhSLF*. In fact, both *SFB* and *PdSLF* are <25% identical to *AhSLF* in their deduced amino acid sequences.

Entani et al. (2003) identified four F-box genes in a 62.5-kb region of the *S*-locus of *P. mume* that contains the S_7-*RNase* gene; three of them also are located in a 64-kb region of the *S*-locus containing the S_1-*RNase* gene. The F-box gene closest to the *S-RNase* gene was named *SLF*; it is expressed in pollen and shows a high level of allelic sequence diversity. The amino acid sequence identities of the S_1-, S_7-, and S_9-alleles of *SLF*

range from 77.8 to 81.3%. Thus, *SLF* of *P. mume* is most likely the ortholog of *SFB* of *P. dulcis*. Two alleles of *SLF* in another rosaceous species, *P. avium* (sweet cherry), have been cloned by PCR, and their deduced amino acid sequences are 79.5% identical (Yamane et al., 2003). Hereafter, *SLF/SFB* will be used to indicate the F-box gene that is the prime candidate for the pollen *S*-gene. The other three F-box genes identified in *P. mume* were named *SLFL1*, *SLFL2*, and *SLFL3*, all of which, like *PdSLF* of *P. dulcis*, show much lower degrees of allelic sequence diversity than *SLF/SFB*. For example, the deduced amino acid sequences of the S_1- and S_2-alleles of *SLFL1* are 92.5% identical.

An alignment of the amino acid sequences of all nine SLF/ SFBs of the three *Prunus* species is shown in Figure 1. The F-box motif is located at the N terminus, and it is relatively conserved among these proteins. To identify regions of variability, the Normed Variability Index (NVI; as defined by Ioerger et al., 1991) was calculated for each site of the alignment, and the NVI of each

site was averaged with that of its neighbors in a sliding window of size 7. A window-averaged plot of NVI over the length of SLF/ SFB identified two very prominent variable regions, named HVa and HVb, which correspond approximately to the variable regions A and B, respectively, identified from the sequence comparison of four *P. dulcis* SFBs (Ushijima et al., 2003). These two regions together contain 8 of the 13 most variable sites, and the HVa region in particular shows the highest peak of window-averaged NVI. The presence of these hypervariable regions is consistent with the potential role of SLF/SFB in determining male specificity.

Most F-box proteins are involved in ubiquitin-mediated protein degradation. This system uses E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase) to catalyze the formation of polyubiquitin chains on specific substrates for degradation by the 26S proteasome (Bai et al., 1996). An F-box protein is a component of one class of E3, called SCF, which also consists of Skp1, cullin (Cul1), and a RING-HC
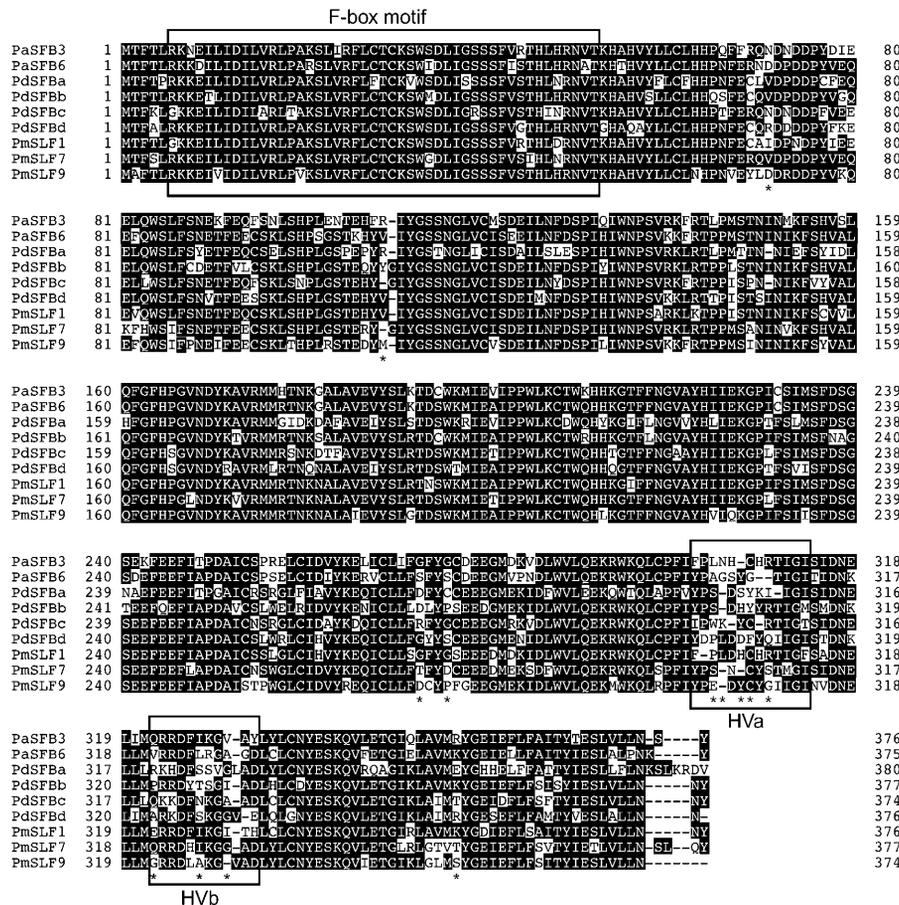
Figure 1. Alignment of Deduced Amino Acid Sequences of SLF/SFBs of Three *Prunus* Species.

The sequences were aligned by DNASIS version 3.5 (Hitachi Software Engineering Co. Ltd., Tokyo, Japan). The two variable regions, HVa and HVb, were identified as described in the text. The 13 most variable residues (with NVI scores > 0.1016) are indicated with asterisks. For each site, the most frequent residue is shown on a dark background in each sequence. The names of the proteins are indicated by the two-letter abbreviation of the species name (Pa, *P. avium*; Pd, *P. dulcis*; Pm, *P. mume*) followed by the protein name (SFB or SLF) and the identity of the *S*-allele. Amino acid residue numbers of the first and last amino acids in each row of sequence are indicated at left and right, respectively.

finger protein (Rbx1) (Tyers and Jorgensen, 2000). The F-box motif interacts with Skp1, and a separate region interacts with specific substrates. The substrate-interacting region may contain WD40 repeats, Leu-rich repeats, other protein–protein interaction modules, or unrecognized motifs (Cenciarelli et al., 1999). The SLF/SFB does not contain any recognizable protein–protein interaction motif, although the two hypervariable regions could be involved in recognition.

The pairwise amino acid sequence identities of these nine SLF/SFBs are shown in Table 2. Interestingly, several alleles are more similar to some alleles of other species than to the other alleles of the same species. For example, PdSFBb (product of the S^b-allele of *P. dulcis*) is more similar to PaSFB3 (product of the S_3-allele of *P. avium*) and PaSFB6 (product of the S_6-allele of *P. avium*) (77.2 and 78.2%, respectively) than to the product of any of the other three *P. dulcis* alleles (69.0 to 76.4%). This finding is reminiscent of the phylogenetic relationships of S-RNases of *Prunus* species, in which some S-RNases of one species are more similar to S-RNases of other species than to those of the same species (Ushijima et al., 1998).

Our laboratory has also identified a pollen-expressed F-box gene in close proximity to the S_2-RNase gene of *P. inflata* and two additional pollen-expressed F-box genes that are linked physically, but at much greater distances, to the S-RNase gene (X. Wang, A.G. McCubbin, S. Huang, Y. Wang, and T.-h. Kao, unpublished results). For *A. hispanicum*, sequencing of a more extended S_2-locus region and of S-locus regions of three other haplotypes has revealed that this species also contains multiple F-box genes, including *AhSLF*, at the S-locus (Zhou et al., 2003). However, contrary to the high degree of allelic sequence diversity of the rosaceous *SLF/SFB*, the amino acid sequences of four alleles (S_1, S_2, S_4, and S_5) of *AhSLF* share >97% sequence identity. (*AhSLF-S_2L*, previously reported by Lai et al. [2002], has been shown to be the S_1-allele of *AhSLF* from this later work.)

Thus, all three families that use the RNase-mediated SI mechanism have multiple F-box genes that are linked physically to the S-RNase gene. Moreover, the only protein-coding genes (except for those that encode retrotransposons) located in close proximity of the S-RNase gene that are common to all three of these families are the F-box genes. It is unlikely that these findings are purely coincidental. Nonetheless, the role of *SLF/SFB* in SI must be addressed by in vivo approaches, as has been accomplished for the S-RNase gene.

OTHER GENES THAT MODULATE THE SI RESPONSE

The specificity of the SI interaction is determined by the *S-RNase* gene and the pollen *S*-gene; however, classic and molecular genetic studies have revealed the existence of genes at other loci that are required for the full manifestation of the SI response. For example, attempts to restore SI to cultivated self-compatible species by introgression of the S-locus from their self-incompatible wild relatives have not been successful (Martin, 1961; Bernatzky et al., 1995). Transferring the S-RNase gene alone into self-compatible species failed to confer on them the ability to reject self-pollen (Murfett et al., 1996; Chung et al., 1999; McClure et al., 2000; Kondo et al., 2002). Moreover, some self-compatible plants with defects in pistil function were found to carry a conditionally functional S-RNase gene that, when introgressed into a self-incompatible background, functioned normally (Ai et al., 1991).

These genes, which are located outside of the S-locus but are required for the SI response, are collectively called modifier genes. One possible role of the modifier genes is to regulate the expression of the S-locus genes. For example, Tsukamoto et al. (1999, 2003) found that several pistil-part self-compatible plants identified from a natural population of self-incompatible *Petunia axillaris* carried a defective S_{13}-haplotype (designated S_{13}^{sps}), which did not produce any transcript of the S_{13}-RNase gene. They further showed that self-incompatible progeny homozygous or heterozygous for the functional S_{13}-haplotype were obtained from self-pollination of the self-compatible plants that carried the $S_{13}^{sps}S_{15}$ genotype. Thus, the S_{13}-RNase gene was not defective, and its expression was suppressed by a modifier in the self-compatible background. Tsukamoto et al. (2003) proposed that the modifier gene could encode either a suppressor of the expression of the S_{13}-RNase gene or an activator required for the expression of an active suppressor of the expression of the S_{13}-RNase gene. Interestingly, the modifier gene did not affect the expression of the S_1- or S_{15}-RNase gene carried by these self-compatible plants. The identity of the modifier gene is unknown.

One approach that has been used to identify the modifier genes is to search for pistil- or pollen-expressed genes that are expressed in a self-incompatible species but not in closely related self-compatible species. McClure et al. (1999) identified a gene, named *HT-B*, that is expressed in the pistil of self-incompatible *N. alata* but not in that of self-compatible *N.*

Table 2. Percent Pairwise Sequence Identities of *S*-Locus F-Box Proteins of *Prunus*

	PaSFB6	PdSFBa	PdSFBb	PdSFBc	PdSFBd	PmSLF1	PmSLF7	PmSLF9
PaSFB3	79.5	68.8	77.2	78.5	76.6	81.9	78.1	76.2
PaSFB6		69.3	78.2	76.5	78.0	80.3	80.1	78.8
PdSFBa			69.0	70.1	68.4	70.7	71.1	68.5
PdSFBb				75.6	76.4	80.6	79.3	77.3
PdSFBc					75.8	80.3	80.5	74.9
PdSFBd						80.1	76.9	76.0
PmSLF1							81.3	77.8
PmSLF7								80.2

plumbaginifolia. Homologs of *HT-B* also have been identified in two other genera of the Solanaceae, *Lycopersicon* and *Solanum* (Kondo et al., 2002; O'Brien et al., 2002). Suppression of the expression of *HT-B* by antisense RNA and/or RNA interference led to the loss of *S*-haplotype–specific rejection of pollen in both *N. alata* and *S. chacoense* (McClure et al., 1999; O'Brien et al., 2002). Also, a cultivar of tomato (*Lycopersicon esculentum*) was found to carry a defective *HT-B* in addition to not producing any S-RNase (Kondo et al., 2002). *HT-B* encodes a protein that contains a stretch of 20 Asn and Asp residues (the ND domain) near the C terminus (Cruz-Garcia et al., 2003). Database searches have not yielded any insight into the possible function of HT-B. Because the transcript or protein level of the *S-RNase* gene was not affected in the antisense and RNA interference transgenic plants, *HT-B* is not required for the expression of the *S-RNase* gene. No direct interaction between HT-B and S-RNases has been detected. A current hypothesis is that HT-B, perhaps working in conjunction with other pistil proteins, is required for the uptake of S-RNases into pollen tubes (Cruz-Garcia et al., 2003). *HT-B* is the first modifier gene of GSI to be cloned, and the elucidation of its role in SI will contribute to a better understanding of the mechanism of S-RNase–based SI.

Another approach to identifying the modifier genes is to isolate pistil and pollen proteins that interact with S-RNases. A pollen protein of *Petunia hybrida* that interacts with the N-terminal part of S-RNases containing the HVa and HVb regions has been identified by the yeast two-hybrid protein–protein interaction assay (Sims and Ordanic, 2001). This protein, named PhSBP1, is a novel protein and contains a RING-HC motif at its C terminus. RING-HC proteins also are involved in ubiquitin-mediated protein degradation. If *PhSBP1* plays a role in SI, it is more likely to be a general one and not as the *S*-allele–specificity determinant, because *PhSBP1* is expressed in other tissues, it does not show any allelic sequence polymorphism, and the interaction between its protein product and S-RNases is not allele specific.

Affinity chromatography has been used to identify pistil proteins that interact with S-RNases of *N. alata* (McClure et al., 2000; Cruz-Garcia et al., 2003). Interestingly, three of the five proteins identified to date are pistil glycoproteins that have previously been implicated in pollen tube growth. A 120-kD protein, first identified in *N. alata*, has been shown to be taken up into the cytoplasm of pollen tubes (Lind et al., 1996). TTS, first identified in *N. tabacum*, has been shown to be associated with the pollen tube membrane (Cheung et al., 1993; Wu et al., 1995). PELPIII, first identified in *N. tabacum*, has been shown to be associated with the callose wall and callose plugs of pollen tubes (de Graaf et al., 2003). Because these proteins are located in the extracellular space of the transmitting tract tissue and their interactions with S-RNases are not allele specific (at least in vitro), they are thought to play a role in facilitating the uptake of S-RNases into pollen tubes (Cruz-Garcia et al., 2003). In addition to these three glycoproteins, an 11-kD protein with sequence similarity to a class of copper binding proteins called phytocyanins, as well as S-RNase itself, also were found to interact with S-RNases. It is not clear what role the 11-kD protein might have, nor is it known whether S-RNases form dimers or higher multimers in vivo.

It should be noted that because none of these proteins that interact with the S-RNase is specific to SI, they are likely to be required for some other physiological processes as well. Thus, mutations in their genes could be lethal and not recoverable from genetic screens of self-compatible mutants.

MODELS FOR S-RNASE–MEDIATED SI RESPONSE

Because the RNase activity of S-RNases is essential for their function in SI, it is generally accepted, though not yet demonstrated definitively, that the degradation of pollen tube RNAs by the self S-RNase results in the growth inhibition of self-pollen tubes in the style. Two different models (and their modified versions) have been put forward to explain how S-RNases might mediate the specific degradation of self-pollen tube RNA (Thompson and Kirch, 1992; Kao and McCubbin, 1996). The receptor model predicts that the specificity lies in the uptake of S-RNases into a pollen tube: the self S-RNase, but not any non-self S-RNase, would be taken up into the cytoplasm of a pollen tube. Thus, this model predicts that the products of pollen *S*-alleles are membrane-bound or cell wall–bound receptors that serve as gatekeepers so that only the matching (self) S-RNase is allowed to enter a pollen tube. The inhibitor model, in its simplest form, predicts that the products of pollen *S*-alleles are cytosolic RNase inhibitors, with each allelic product specifically inhibiting the RNase activity of all non-self S-RNases but not that of the self S-RNase.

One approach to assess the validity of these two models is to determine if the uptake of S-RNases into a pollen tube is *S*-haplotype specific, as predicted by the receptor model, or if both self and non-self S-RNases are taken up by a pollen tube, as predicted by the inhibitor model. Luu et al. (2000) used immunocytochemistry to localize S_{11}-RNase of *S. chacoense* in pistils (of $S_{11}S_{13}$ genotype) that had been pollinated with incompatible or compatible pollen. They found that S_{11}-RNase was present in the cytoplasm of both self-pollen tubes (S_{11} genotype) and non-self pollen tubes of S_{12} and S_{13} genotypes. This finding supports the inhibitor model and suggests that if the uptake of S-RNases by pollen tubes requires a receptor (or a receptor complex), it most likely recognizes some common features of S-RNases.

Simple Inhibitor Model

One way that *S*-haplotype–specific inhibition of S-RNases could be accomplished is depicted in Figure 2A. It is reasonable to predict that S-RNases contain two separate functional domains, an *S*-allele–specificity domain, which is unique to each S-RNase, and a catalytic domain, which is common to all S-RNases. As described above, the chimeric S-RNases of *P. inflata* and *N. alata* produced in the domain-swapping experiments (Kao and McCubbin, 1996; Zurek et al., 1997) all retained RNase activity, even though they did not retain the original *S*-allele specificity or gain a new one, suggesting the existence of two separate domains. Similarly, the RNase inhibitors produced by pollen *S*-alleles also could contain two separate domains, an RNase-inhibitor domain and an *S*-allele–specificity domain.
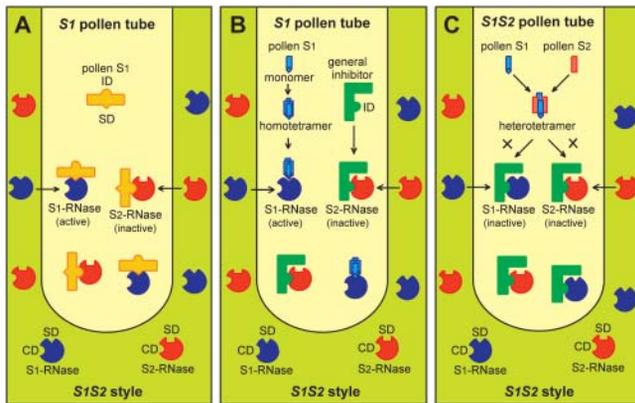
Figure 2. Models for *S*-Haplotype–Specific Inhibition of Pollen Tube Growth and Competitive Interaction.

(A) Simple inhibitor model. Both S_1- and S_2-RNases are taken up by an S_1 pollen tube, but only S_1-RNase is active in degrading pollen RNA because the pollen S_1-allele product specifically inhibits the RNase activity of S_2-RNase. Binding of S_1-RNase to the pollen S_1-allele product through their matching *S*-allele–specificity domains (SD) blocks binding of the inhibitor domain (ID) of the pollen S_1-allele product to the catalytic domain (CD) of S_1-RNase. Interaction between S_2-RNase and the pollen S_1-allele product is through the CD of the former and the ID of the latter, thus rendering S_2-RNase inactive.

(B) Modified inhibitor model. This model differs from the simple inhibitor model in several respects: pollen *S*-allele products are homotetramers and they only contain the *S*-allele–specificity domain; and a general RNase inhibitor is responsible for the inhibition of the RNase activity of S-RNases. The *S*-haplotype–specific inhibition of the RNase activity of S-RNases is achieved in a manner similar to that described for **(A)**.

(C) Competitive interaction based on the modified inhibitor model. Pollen S_1-allele and S_2-allele products mainly form heterotetramers, which cannot bind the *S*-allele–specificity domain of either S_1-RNase or S_2-RNase. As a result, the RNase activities of both S_1- and S_2-RNases are inhibited by the general RNase inhibitor.

In all of these models, additional pistil proteins that are required for the SI response are not depicted.

As depicted in Figure 2A, when a pollen tube of S_1 genotype is growing in a pistil of S_1S_2 genotype, the pollen S_1-allele product interacts with S_1- and S_2-RNases differently. In the case of self-interaction, the *S*-allele–specificity domain of the pollen S_1-allele product would interact with the *S*-allele–specificity domain of S_1-RNase by virtue of the match between the paired domains; the RNase activity of S_1-RNase would not be affected in such an interaction. However, in the case of non-self interaction, the RNase-inhibitor domain of the pollen S_1-allele product would interact with the catalytic domain of S_2-RNase in the absence of the match between their *S*-allele–specificity domains; such an interaction would inhibit the RNase activity of S_2-RNase. This model is predicated on the assumption that the interaction between the *S*-allele–specificity domains of a pollen *S*-allele product and its cognate S-RNase is stronger than the interaction between the RNase-inhibitor domain of the pollen *S*-allele product and the catalytic domain of its cognate S-RNase. Moreover, binding to one domain of the S-RNase precludes binding to the other domain. Thus, binding of a pollen *S*-allele

product to the *S*-allele–specificity domain locks the S-RNase into its active form.

This inhibitor model can explain a well-known phenomenon termed competitive interaction, which refers to a breakdown of pollen function in SI caused by the presence of two *S*-loci of different haplotypes in the pollen. This occurs when the entire *S*-locus, or a critical part of it, is duplicated in diploid plants that carry two different *S*-haplotypes or when diploid SI plants (carrying two different *S*-haplotypes) become tetraploid (de Nettancourt, 2001). *S*-locus duplication can be generated by γ-ray irradiation, with the duplicated region existing as a free centric fragment or as a translocated chromosomal segment. Among the pollen grains produced by such pollen-part self-compatible mutants, only those whose resident *S*-locus is of a different *S*-haplotype from that of the extra *S*-locus fail to function in SI. For example, if an S_1S_2 plant carries an extra S_1-locus, the only pollen grains that will not be rejected by the pistil upon self-pollination are S_2 pollen grains that carry the duplicated S_1-locus. Golz et al. (1999, 2001) found that in some cases the duplicated region lacked the *S-RNase* gene but contained markers that flank the *S*-locus, consistent with the belief that the duplicated pollen *S*-allele causes competitive interaction. Pollen carrying two different pollen *S*-alleles may be referred to as heteroallelic pollen (Luu et al., 2001). The inhibitor model predicts that when two different pollen *S*-alleles are expressed in the same pollen grain, their products together inhibit the RNase activity of all S-RNases, thus resulting in the breakdown of SI.

Modified Inhibitor Model

A modified inhibitor model was proposed by Luu et al. (2001) to explain the SI behavior of a dual-specificity chimeric S-RNase they had generated (Matton et al., 1999). This S-RNase, named $S_{11/13}$-RNase, is a chimeric protein between S_{11}- and S_{13}-RNases of *S. chacoense* that exhibits both S_{11}- and S_{13}-allele specificities. Plants that produce the $S_{11/13}$-RNase rejected both S_{11} and S_{13} pollen. Interestingly, pistils of transgenic plants that produce the $S_{11/13}$-RNase were completely incompatible with pollen from tetraploid plants of $S_{11}S_{11}S_{13}S_{13}$ genotype, suggesting that the diploid pollen of $S_{11}S_{13}$ genotype produced by the tetraploids was rejected by the $S_{11/13}$-RNase. Luu et al. (2001) further showed that diploid pollen of $S_{11}S_{13}$ genotype was compatible with pistils producing monospecific S_{11}- and S_{13}-RNases, as would be expected from competitive interaction. Thus, the dual-specificity $S_{11/13}$-RNase behaves differently from its two corresponding monospecific S-RNases in its ability to recognize and reject diploid pollen carrying two different *S*-alleles. This finding also suggests that both pollen *S*-alleles are expressed in diploid pollen and rules out the possibility that the breakdown of SI caused by competitive interaction is attributable to silencing of the expression of the two pollen *S*-alleles.

The modified inhibitor model predicts that (1) the active form of pollen *S*-allele products is a homotetramer; (2) the pollen *S*-allele products contain only the *S*-allele–specificity domain; and (3) a general RNase inhibitor is responsible for the inhibition of S-RNases. According to this model (Figure 2B), the general inhibitor would bind and inactivate all S-RNases, unless an

S-RNase were bound to its cognate pollen S-allele product through their matching S-allele–specificity domains. When a pollen grain expresses two different alleles of the pollen S-gene, the products would mainly form heterotetramers, which could not efficiently bind either cognate S-RNase; as a result, the general inhibitor would inhibit the RNase activity of both cognate S-RNases (Figure 2C). In fact, this model also predicts that the heterotetramers could not bind any other S-RNases. Thus, pollen tubes carrying two different pollen S-alleles would be compatible with pistils of any S-genotype. In the case of the dual-specificity S-RNase, because it could still bind the heterotetramers formed by the two pollen S-allele products, binding of the general inhibitors to the S-RNase would be blocked. This would explain the SI behavior of the dual-specificity S-RNase.

Potential Role of SLF/SFB in SI

If SLF/SFB indeed encodes the male-specificity determinant, each allelic product could interact with its cognate and non-self S-RNases differently, so that only non-self S-RNases are degraded by the 26S proteasome. This would be consistent with the inhibitor model, except that it is the stability, and not the RNase activity, of S-RNases that is regulated by the SI interaction. The S-allele–specific degradation could be accomplished if the interaction between the S-allele–specificity domains of an SLF/SFB and its cognate S-RNase would some-

how prevent ubiquitination of the self S-RNase and the lack of interaction between the S-allele–specificity domains of an SLF/SFB and non-self S-RNases would result in ubiquitination of the latter.

One could take advantage of the competitive interaction phenomenon to examine the function of SLF/SFB, as illustrated in Figure 3. Here, the S_1-allele of SLF/SFB (S_1-SLF/SFB) is used to transform S_1S_2 plants and the pollen of the resulting transgenic plants is used to pollinate an S_1S_2 tester plant. If SLF/SFB is the pollen S-gene, S_2 pollen that carries the S_1-SLF/SFB transgene will be compatible with S_1S_2 pistils because of competitive interaction. Thus, this pollination will be compatible and result in two different genotypes in the progeny, S_1S_2+ S_1-SLF/SFB and $S_2S_2+S_1$-SLF/SFB. That is, all of the progeny should inherit the transgene and no S_1S_1 genotype will be obtained in the progeny. Importantly, the latter result will serve as an internal control for the breakdown of SI attributable to competitive interaction. Moreover, the $S_1S_2+S_1$-SLF/SFB progeny will be compatible with S_1S_2 pistils and the $S_2S_2+S_1$-SLF/SFB progeny will be compatible with S_2S_2 pistils.

If SLF/SFB is confirmed to be the pollen S-gene, it would be of interest to determine what effect the suppression of its expression has on SI behavior. If SLF/SFB contains both the S-allele–specificity domain and the inhibitor domain, as predicted by the simple inhibitor model, the absence of this protein would render the pollen unable to inhibit any S-RNases. For
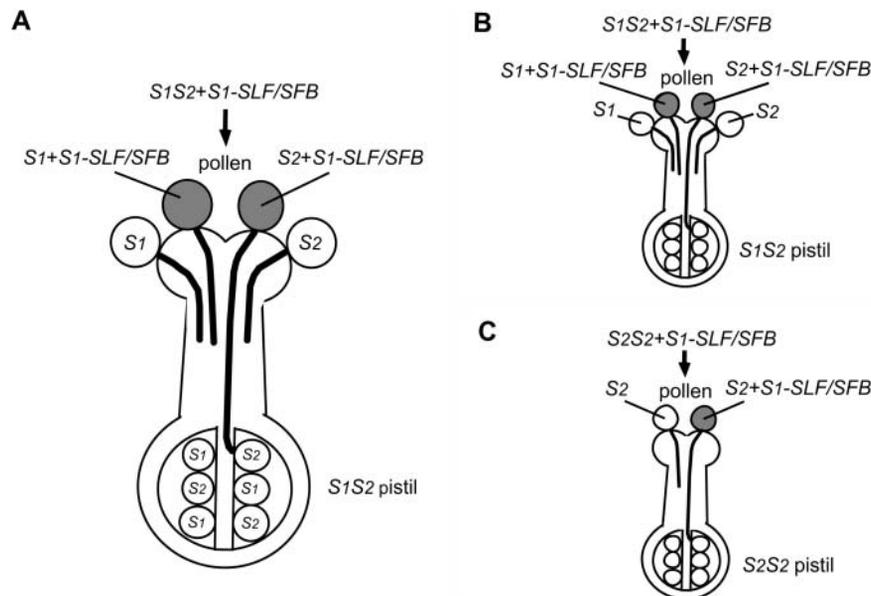

Figure 3. Scheme of a Transgenic Approach to Ascertain the Function of SLF/SFB in SI.

(A) Pollination of an S_1S_2 pistil with pollen from an S_1S_2 transgenic plant that carries an S_1-SLF/SFB transgene. The pollination will be compatible if SLF/SFB is the pollen S-gene. Among the pollen produced by the transgenic plant, S_2 pollen carrying the S_1-SLF/SFB transgene will be compatible with the S_1S_2 pistil because of competitive interaction, whereas S_1 pollen, S_2 pollen, and S_1 pollen carrying the S_1-SLF/SFB transgene will be rejected by the pistil. Two S-genotypes will be produced in the progeny, $S_1S_2+S_1$-SLF/SFB transgene and $S_2S_2+S_1$-SLF/SFB transgene.
(B) SI behavior of the progeny with the genotype of $S_1S_2+S_1$-SLF/SFB transgene. Such progeny should be compatible with an S_1S_2 pistil because S_2 pollen carrying the transgene will not be rejected by the pistil as a result of competitive interaction.
(C) SI behavior of the progeny with the genotype of $S_2S_2+S_1$-SLF/SFB transgene. For the same reason given in **(B)**, such progeny should be compatible with an S_2S_2 pistil.
Dark circles indicate pollen grains that carry the transgene.

example, if the antisense S_1-allele of *SLF/SFB* were introduced into S_1S_1 plants, half of the pollen would express the transgene and the other half would not. The former should be incompatible with pistils of any *S*-genotype, whereas the latter should behave normally in SI. Thus, if pollen from the transgenic plants were used to pollinate S_1S_1 and S_2S_2 plants, only the S_2S_2 plants would set seed and none of the progeny would carry the transgene. Moreover, the transgene should transmit normally through pollen in crosses with plants that do not produce functional S-RNase. However, if SLF/SFB contains only the *S*-allele–specificity domain, as predicted by the modified inhibitor model, suppression of the expression of *SLF/SFB* would render the pollen unable to block the action of the general inhibitor on any S-RNases. As a result, the pollen not producing SLF/SFB would be compatible with pistils of any *S*-genotypes. Therefore, in the crosses described above, both S_1S_1 and S_2S_2 plants would set seed, with all of the S_1S_1 progeny carrying the antisense transgene and half of the S_2S_2 progeny carrying the antisense transgene.

FUTURE PERSPECTIVES

Since the discovery of the *S-RNase* gene almost two decades ago, much of what we have learned about the Solanaceae type of SI is limited to this female determinant of the SI interaction. The recent identification of the *SLF/SFB* gene very likely will change the landscape of research in this type of SI. The most urgent task, in the short run, is to determine, by in vivo approaches, whether *SLF/SFB* encodes the male-specificity determinant of SI. If *SLF/SFB* is confirmed to be the pollen *S*-gene, this will open new avenues of research and bring us closer to an understanding of the mechanism of *S*-haplotype–specific inhibition of pollen tube growth. Questions can be asked regarding whether SLF/SFB functions as a conventional F-box protein in mediating the specific degradation of all non-self S-RNases or whether it functions in some unexpected manner. With the genes that encode both the male and female determinants in hand, we also could address one of the most perplexing questions about any type of SI systems: how did the male and female specificity genes coevolve to maintain SI? The fact that multiple F-box genes are linked to the *S-RNase* gene in all three families that exhibit the Solanaceae-type SI also raises questions about the physiological functions of the non-SI–related F-box genes that are linked to the *S*-locus and about the evolutionary relationships among the various *S*-linked F-box genes.

Although it is important to focus on how *S*-haplotype specificity is determined, we also should keep in mind that additional proteins are required for the full manifestation of the SI response. Because most of the candidate proteins identified to date do not appear to be specific to the SI system, understanding how they function in SI will likely have implications for other developmental processes.

Accession Numbers

The GenBank accession numbers for the sequences shown in Figure 1 are AB096857 (PaSFB3), AB096858 (PaSFB6), AB092966 (PdSFBa), AB092967 (PdSFBb), AB079776 (PdSFBc), AB081648 (PdSFBd), AB092621 (PmSLF1), AB092622 (PmSLF7), and AB092645 (PmSLF9).

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