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REVIEW: PART OF A SPECIAL ISSUE ON SEXUAL PLANT REPRODUCTION

S-RNase-based self-incompatibility in *Petunia inflata*

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• *Background* For the Solanaceae-type self-incompatibility, also possessed by Rosaceae and Plantaginaceae, the specificity of self/non-self interactions between pollen and pistil is controlled by two polymorphic genes at the *S*-locus: the *S-locus F-box* gene (*SLF* or *SFB*) controls pollen specificity and the *S-RNase* gene controls pistil specificity.
• *Scope* This review focuses on the work from the authors' laboratory using *Petunia inflata* (Solanaceae) as a model. Here, recent results on the identification and functional studies of *S-RNase* and *SLF* are summarized and a protein-degradation model is proposed to explain the biochemical mechanism for specific rejection of self-pollen tubes by the pistil.
• *Conclusions* The protein-degradation model invokes specific degradation of non-self S-RNases in the pollen tube mediated by an SLF, and can explain compatible versus incompatible pollination and the phenomenon of competitive interaction, where SI breaks down in pollen carrying two different *S*-alleles. In Solanaceae, Plantaginaceae and subfamily Maloideae of Rosaceae, there also exist multiple *S*-locus-linked *SLF/SFB-like* genes that potentially function as the pollen *S*-gene. To date, only three such genes, all in *P. inflata*, have been examined, and they do not function as the pollen *S*-gene in the *S*-genotype backgrounds tested. Interestingly, subfamily Prunoideae of Rosaceae appears to possess only a single *SLF/SFB* gene, and competitive interaction, observed in Solanaceae, Plantaginaceae and subfamily Maloideae, has not been observed. Thus, although the cytotoxic function of S-RNase is an integral part of SI in Solanaceae, Plantaginaceae and Rosaceae, the function of SLF/SFB may have diverged. This highlights the complexity of the S-RNase-based SI mechanism. The review concludes by discussing some key experiments that will further advance our understanding of this self/non-self discrimination mechanism.

Key words: Competitive interaction, *Petunia inflata*, Plantaginaceae, protein degradation, Rosaceae, self-incompatibility, *S*-locus F-box protein, Solanaceae, S-RNase, ubiquitination.

INTRODUCTION

Flowering plants (angiosperms) represent the most diverse group of land plants, and most of them have perfect flowers, with the male reproductive organ (anther) and the female reproductive organ (pistil) located in close proximity. As self-fertilization results in inbreeding, the outcome of which is deleterious to the long-term survival of any population, flowering plants have adopted various strategies to circumvent the strong tendency to self-fertilize so as to promote out-crossing and generate genetic variability within a species. Self-incompatibility (SI) is one such strategy widely adopted by flowering plants (de Nettancourt, 2001) and it has long been considered responsible for their explosive success (Whitehouse, 1950). SI allows the pistil to distinguish between genetically related (self) and genetically unrelated (non-self) pollen; self pollen is rejected, either on the stigmatic surface or during its tube growth in the style, whereas non-self pollen is accepted for fertilization. In the simplest cases, self/non-self recognition between pollen and the pistil is controlled by one highly polymorphic locus, named the *S*-locus, with variants of the locus referred to as 'haplotypes' and designated S_1, S_2, S_3, etc.

To date, extensive molecular studies have been carried out on five of the families that possess SI, all of which have a single-locus SI system. The results have revealed that (*a*) flowering plants have adopted different SI mechanisms to accomplish the same goal, and (*b*) for each mechanism, two separate genes at the *S*-locus control pollen and pistil functions in SI. Based on the chemical nature of the pollen and pistil *S*-genes, three of these five families, Solanaceae, Rosaceae and Plantaginaceae, are thought to employ a similar SI mechanism, whereas Brassicaceae and Papaveraceae each employ different mechanisms (Takayama and Isogai, 2005). This paper focuses on the Solanaceae mechanism, and particularly the results obtained in the authors' laboratory using *Petunia inflata* as a model. Recent reviews of this mechanism can be found in Kao and Tsukamoto (2004), Sims (2007), Franklin-Tong (2008), Hua *et al.* (2008), McClure (2009) and Chen *et al.* (2010).

For the Solanaceae-type SI, pollen behaviour is determined by its own *S*-genotype (i.e. pollen is recognized as self-pollen and rejected by the pistil only if its *S*-haplotype is identical to one of the two *S*-haplotypes carried by the pistil). Two tightly linked genes at the *S*-locus have been identified and shown to be involved in SI, the *S-RNase* gene controlling pistil

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specificity (Lee *et al.*, 1994; Murfett *et al.*, 1994) and the *S-locus F-box* (*SLF* or *SFB*) gene controlling pollen specificity (Entani *et al.*, 2003; Yamane *et al.*, 2003; Qiao *et al.*, 2004*b*; Sijacic *et al.*, 2004; Ushijima *et al.*, 2004; Sonneveld *et al.*, 2005; Sassa *et al.*, 2007). In this type of SI, the rejection of self-pollen occurs during the growth of self-pollen tubes in the style, around the time when growth shifts from the autotrophic phase to the heterotrophic phase (Herrero and Hormaza, 1996).

S-RNASE: THE PISTIL SPECIFICITY DETERMINANT

The *S-RNase* gene was first identified in *Nicotiana alata* by Anderson *et al.* (1986) after its allelic products had been identified based on their showing *S*-haplotype-specific differences in molecular mass and isoelectric point. S-RNase exhibits properties expected of the protein functioning as the pistil specificity determinant for the following reasons. S-RNase is a pistil-specific protein, and it is initially synthesized in the transmitting cells of the style and then secreted into the extracellular space of the transmitting tract. S-RNase is most abundant in the upper third segment of the style, a location coinciding with the site of growth arrest of self-pollen tubes after incompatible pollination (Ai *et al.*, 1990). S-RNase is present at very low levels in immature pistils, which do not exhibit SI, but is abundantly present in mature pistils, accounting for up to 10 % of total pistil protein (Roalson and McCubbin, 2003), when SI is fully functional. S-RNase shows a high degree of allelic sequence diversity, with the most divergent pair sharing only approx. 38 % amino acid sequence identity (Tsai *et al.*, 1992), as expected of a protein functioning in self/non-self recognition. The function of the *S-RNase* gene in SI was definitively established from gain-of-function and loss-of-function experiments conducted in transgenic plants (Lee *et al.*, 1994; Murfett *et al.*, 1994), which showed that S-RNase is necessary and sufficient for the pistil to recognize and reject self-pollen. Specifically, the S_3-*RNase* gene of *P. inflata* was introduced into plants of S_1S_2 genotype and it was found that the transgenic plants that produced S_3-RNase from the transgene acquired the ability to reject S_3 pollen. When an antisense S_3-*RNase* gene was introduced into plants of S_2S_3 genotype to suppress the production of S_3-RNase, the transgenic plants lost the ability to reject S_3 pollen, but still retained the ability to reject S_2 pollen (Lee *et al.*, 1994). Thus, the *S-RNase* gene alone determines pistil specificity in SI.

S-RNase was so named because it has ribonuclease activity (McClure *et al.*, 1989; Broothaerts *et al.*, 1991; Singh *et al.*, 1991), which was shown to be essential for rejection of self-pollen (Huang *et al.*, 1994). Thus, the biochemical mechanism of rejection of self-pollen tubes by the pistil most likely involves degradation of pollen RNA in incompatible pollen tubes. S-RNases are glycoproteins with various numbers of N-linked glycan chains; however, the carbohydrate moiety is not required for their RNase activity or recognition function (Karunanandaa *et al.*, 1994). Thus, the allelic specificity determinant of S-RNase resides in its protein backbone. Comparison of the sequences of S-RNases from different solanaceous species revealed five conserved regions, named C1 to C5, and two hypervariable regions, named HVa and HVb (Ioerger *et al.*, 1991). The crystal structure of an S-RNase of *N. alata* revealed that the HVa and HVb regions are located on the outside surface of the molecule, suggesting that they are likely to be involved in interactions with allelic products of *SLF* (Ida *et al.*, 2001). However, domain-swapping experiments showed that these hypervariable regions are necessary but not sufficient for the allelic specificity of S-RNases of *P. inflata* (Kao and McCubbin, 1996) and of *N. alata* (Zurek *et al.*, 1997). Thus, it remains unknown which amino acid residues of S-RNase are involved in *S*-allele specificity.

S-LOCUS F-BOX PROTEIN: THE POLLEN SPECIFICITY DETERMINANT

Identification of the gene encoding the pollen specificity determinant turned out to be much more difficult than the identification of the *S-RNase* gene. Attempts to identify pollen/pollen tube proteins that showed *S*-haplotype-specific differences in molecular mass and/or isoelectric point did not result in any potential candidate, nor did attempts to identify pollen/pollen tube proteins that interact with S-RNase (Dowd *et al.*, 2000). RNA differential display was used to identify pollen-specific genes that are tightly linked to the *S*-locus (McCubbin *et al.*, 2000*a*); however, none of the genes identified were deemed as good candidates because they all showed low degrees of allelic sequence diversity at the amino acid sequence level (Wang *et al.*, 2003). Interestingly, two of the genes found through RNA differential display encode F-box proteins and were later identified as SLF-like proteins (Hua *et al.*, 2007). Ultimately, it was through genomic sequencing of the *S*-locus that *SLF* was identified, first in *Antirrhinum* (Plantaginaceae) (Lai *et al.*, 2002) and then in two rosaceous species (Entani *et al.*, 2003; Ushijima *et al.*, 2003) and *P. inflata* (Wang *et al.*, 2004). In *P. inflata*, a BAC library of S_2S_2 genotype was constructed (McCubbin *et al.*, 2000*b*), and screening of the library using the S_2-*RNase* gene as a probe, followed by chromosome walking, resulted in an 881-kb contig. Sequencing of a 328-kb region of this contig that included the S_2-*RNase* gene revealed that it was rich in repetitive sequences and that the majority of the predicted genes with sequence similarities to known genes in the database encoded retrotransposons or polyproteins, with the only exception being *PiSLF* (*P. inflata SLF*), located 161 kb downstream from the S_2-*RNase* gene (Wang *et al.*, 2004). *PiSLF* exhibited several characteristics expected of the pollen *S*-gene, including *S*-haplotype-specific RFLP, developing pollen- and mature pollen/pollen tube-specific expression, and the deduced amino acid sequences of the S_1, S_2 and S_3 alleles showing approx. 10 % pair-wise sequence diversity.

As stated earlier, expression of an allele of *S-RNase* different from the two alleles of *S-RNase* present in the pistil of transgenic plants confers on the pistil the *S*-allele specificity of the introduced *S-RNase* (Lee *et al.*, 1994). Similarly, one might expect that, if *PiSLF* is the pollen *S*-gene, expression of an additional allele of *PiSLF* different from the allele of *PiSLF* present in pollen grains of transgenic plants would lead to gain of the *S*-allele specificity of the introduced *PiSLF* in the transgenic pollen. The competitive interaction phenomenon (Fig. 1A), discovered from earlier genetic

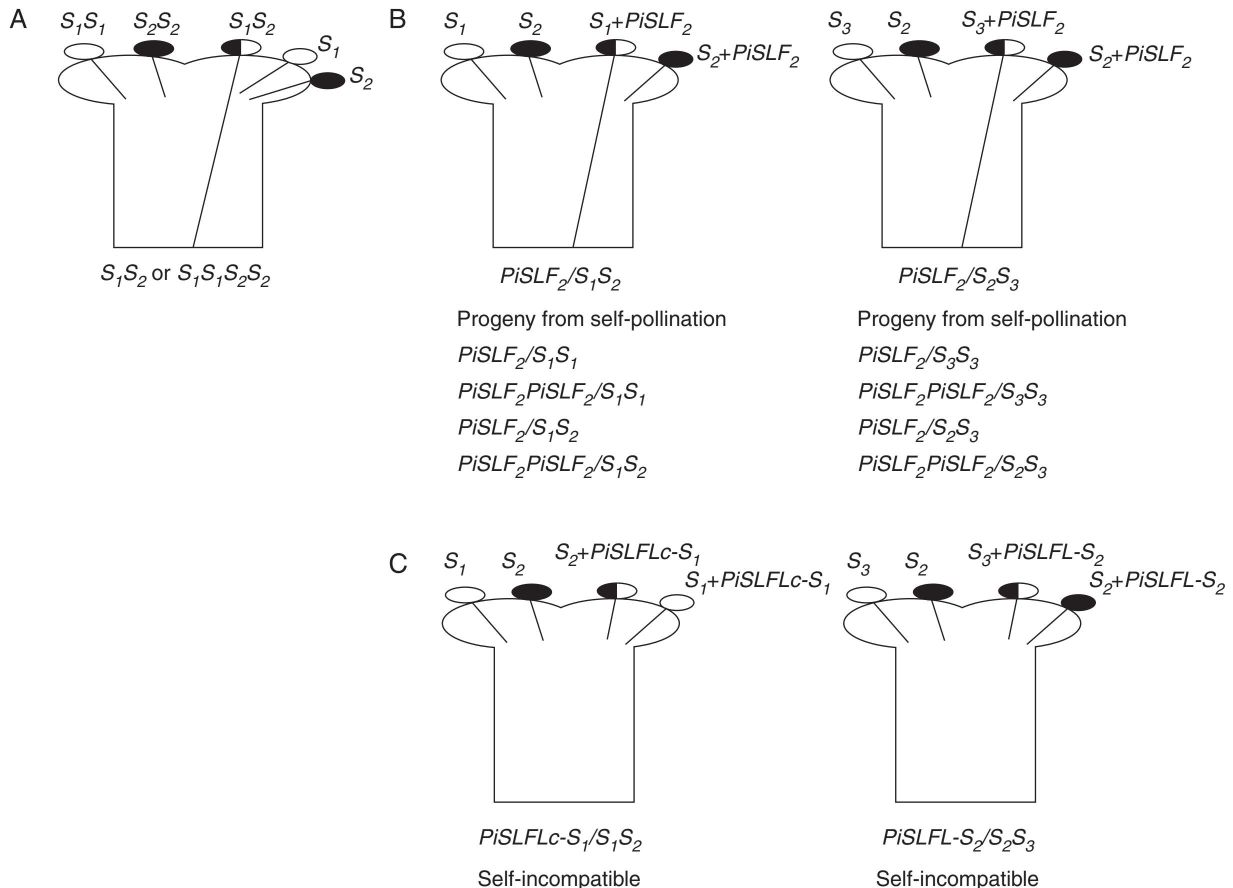

FIG. 1. Competitive interaction and its use in establishing the function of *PiSLF* and assessing potential function of *PiSLF-like* genes in SI. (A) Competitive interaction. For a diploid self-incompatible plant of S_1S_2 genotype, S_1 and S_2 pollen are rejected by the S_1S_2 pistil due to matching of *S*-haplotypes. A tetraploid plant of $S_1S_1S_2S_2$ genotype produces three *S*-genotypes of pollen and, whereas S_1S_1 and S_2S_2 pollen are rejected by S_1S_2 and $S_1S_1S_2S_2$ pistils, S_1S_2 (heteroallelic) pollen is not. (B) Breakdown of SI in S_1S_2 and S_2S_3 transgenic plants caused by expression of *PiSLF*$_2$ in pollen. The left panel shows self-pollination of a transgenic plant *PiSLF*$_2$/S_1S_2. The plant produces four different genotypes of pollen and, based on inheritance of the *PiSLF*$_2$ transgene and the *S*-genotypes in the progeny, only S_1 pollen carrying the *PiSLF*$_2$ transgene was accepted by the pistil. The right panel shows self-pollination of a transgenic plant *PiSLF*$_2$/S_2S_3. Progeny analysis suggests that the *PiSLF*$_2$ transgene caused breakdown of SI in S_3 pollen, but not in S_2 pollen. That *PiSLF*$_2$ caused breakdown of SI in S_1 and S_3 pollen, but not in S_2 pollen, is consistent with the prediction made by competitive interaction, that pollen carrying two different *S*-alleles fails to function in SI. (C) Testing potential function of *PiSLF-like* genes. The left panel shows self-pollination of an S_1S_2 transgenic plant carrying the S_1-allele of a *PiSLF-like* gene, *PiSLFLc*. The transgenic plant remained self-incompatible, suggesting that *PiSLFLc-S*$_1$ did not cause breakdown of SI in S_2 pollen. The right panel shows self-pollination of an S_2S_3 transgenic plant carrying the S_2-allele of either *PiSLFLb* or *PiSLFLd* (labelled as *PiSLFL-S*$_2$). The resulting transgenic plants remained self-incompatible, suggesting that neither *PiSLFLb-S*$_2$ nor *PiSLFLd-S*$_2$ caused breakdown of SI in S_3 pollen.

studies in solanaceous species including *P. inflata* (Brewbaker and Natarajan, 1960; Entani *et al.*, 1999; de Nettancourt, 2001), provided the basis for predicting the outcome of such an experiment. It was found that diploid pollen carrying two different pollen *S*-alleles (heteroallelic pollen, e.g. resulting from duplication of the entire set of chromosomes in a diploid plant with two different *S*-haplotypes) failed to function in SI, whereas diploid pollen carrying two copies of the same pollen *S*-allele (homoallelic pollen) functioned normally in SI. Although the biochemical basis of the phenomenon was unknown, taking advantage of competitive interaction, the function of *PiSLF* in SI was ascertained (Sijacic *et al.*, 2004). The prediction was that if *PiSLF* was the pollen *S*-gene, then the expression of two different alleles of *PiSLF* in a pollen grain should cause breakdown of SI. The S_2-allele of *PiSLF* (*PiSLF*$_2$) was introduced into S_1S_1, S_1S_2 and S_2S_3 plants, and the presence of *PiSLF*$_2$ in S_1 and S_3 pollen grains, but not in S_2 pollen grains, was found to cause the breakdown of SI, as predicted by competitive interaction and confirmed through progeny analysis. For example, self-pollination of a *PiSLF*$_2$/S_2S_3 transgenic plant resulted in S_2S_3 and S_3S_3 progeny plants, but no S_2S_2 progeny plants, and all progeny plants carried the *PiSLF*$_2$ transgene (Fig. 1B). A biochemical model for competitive interaction will be presented in the next section.

BIOCHEMICAL BASIS FOR SPECIFIC REJECTION OF SELF-POLLEN

With the identification of the *S-RNase* and *SLF* genes, a model, based on the biochemical properties of the protein products of these genes, their locations within the pollen tube and the interactions between different allelic products of these two proteins, has been proposed to explain *S*-haplotype-specific rejection of self-pollen tube growth by the pistil in *P. inflata*. A model, which is likely to be applicable to other solanaceous

species, *Antirrhinum* (Plantaginaceae) and the Maloideae subfamily of Rosaceae, will be proposed, followed by a discussion of the possible differences in the function of SLF/SFB in the Prunoideae subfamily of Rosaceae.

An SLF selectively detoxifies its non-self S-RNases by mediating their degradation inside pollen tubes

Luu *et al.* (2000) and Goldraij *et al.* (2006) showed that uptake of S-RNase by growing pollen tubes is not *S*-haplotype-specific, because both self and non-self S-RNases were found inside pollen tubes after pollination (e.g. both S_1-RNase and S_2-RNase are taken up by S_1 pollen tubes growing in an S_1S_2 style). This is an important finding, as it rules out any model that invokes specific uptake of self S-RNase by a pollen tube as being responsible for rejection of self-pollen tubes (Kao and McCubbin, 1996). However, Luu *et al.* (2000) observed that all S-RNases were localized in the cytoplasm of the pollen tube, whereas Goldraij *et al.* (2006) observed that most, if not all, S-RNases were initially sequestered in a vacuole-like compartment, which was later specifically disrupted in incompatible pollen tubes, releasing the sequestered S-RNases into the cytoplasm. Regardless of whether S-RNases are taken up directly into the cytoplasm or into a vacuolar compartment, they must eventually enter the cytoplasm in order to exert their cytotoxic activity, as it has been shown that the RNase activity of S-RNase is required for its function (Huang *et al.*, 1994) and that pollen rRNA is degraded after incompatible pollination, but not after compatible pollination (McClure *et al.*, 1990). Interestingly, when S_2-RNase and S_3-RNase of *P. inflata* were expressed as green fluorescent protein (GFP) fusion proteins in their respective self and non-self pollen, all the ectopically expressed S-RNases were compartmentalized in both self and non-self pollen tubes, and direct expression of S-RNase in pollen had no effect on its viability or SI behaviour (Meng *et al.*, 2009). Whether the S-RNase-containing compartments observed by the authors are the same as those observed by Goldraij *et al.* (2006) remains to be determined, but the results lend additional support to the proposal that the cytoplasm of the pollen tube is the site of the cytotoxic action of S-RNase. This is also consistent with the cytoplasmic localization of SLF (Hua *et al.*, 2007; Meng *et al.*, 2009).

Although both self and non-self S-RNases are taken up by a pollen tube, only self S-RNase exerts its cytotoxic activity to inhibit the growth of the pollen tube. The biochemical properties of PiSLF have allowed the authors to begin to elucidate the molecular basis for this observation. Most F-box proteins are components of SCF (Skp1-Cullin-F-box) complexes, which are a type of E3 ubiquitin ligase complex. This protein-degradation system uses E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme) and E3 (ubiquitin ligase) to transfer polyubiquitin chains to target proteins for degradation by the 26S proteasome (Hershko and Ciechanover, 1998; Moon *et al.*, 2004; Smalle and Vierstra, 2004). The F-box protein component of an SCF complex recognizes and interacts with a specific set of proteins to result in their specific degradation (Tyers and Jorgensen, 2000). Since SLF contains an F-box domain at its N-terminus, it is reasonable to hypothesize that SLF is a component of an SCF complex, and that SLF mediates specific degradation of all its non-self S-RNases inside a pollen tube. This can explain why only self S-RNase functions inside a pollen tube, and results consistent with this model are described below.

First, SLF is a component of either a canonical SCF complex or a novel E3 ubiquitin ligase complex. A conventional SCF complex consists of four proteins, Skp1, Cullin, a RING-HC finger protein (Rbx1) and an F-box protein (Tyers and Jorgensen, 2000; Moon *et al.*, 2004). Cullin serves as a bridge, binding Rbx1 through its C-terminal domain and Skp1 through its N-terminal domain. The F-box protein interacts with Skp1 through its F-box domain, and interacts with specific substrates with a separate protein–protein interaction domain, which may contain WD40-repeats, leucine-rich repeats, etc. (Cenciarelli *et al.*, 1999). However, no recognizable protein–protein interaction domain has been identified within the C-terminal part of SLF. In *Antirrhinum*, the SLF-containing complex is thought to be a conventional SCF complex, which contains a novel Skp1-like protein, SSK1 (SLF-interacting SKP1-like1) (Huang *et al.*, 2006; Zhao *et al.*, 2010). *SSK1* is specifically expressed in pollen, but is encoded by a monomorphic gene. Interestingly, SSK1 has a unique seven to nine amino-acid tail consisting of a disordered coil at the C-terminus, located downstream from the conventional C-terminal residues 'WAFE' found in most plant Skp1 homologues (Gagne *et al.*, 2002; Risseeuw *et al.*, 2003). Since no other Skp1-like proteins interact with $AhSLF_2$, the unique C-terminal tail of SSK1 may be important for the interaction. In *P. inflata*, PiSLF is thought to be a component of a novel E3 ubiquitin ligase complex that contains PiCUL1-G and PiSBP1 (*P. inflata* S-RNase Binding Protein1, a RING-HC protein), but does not contain Skp1 or Rbx1 (Hua and Kao, 2006). In this complex, PiSBP1 rather than Skp1 is thought to organize PiCUL1-G and PiSLF since, like Skp1, PiSBP1 interacts with the Cullin component (PiCUL1-G) and the F-box component (PiSLF) of the putative complex, and like Rbx1, interacts with an E2. The fact that PiSBP1 is three times the size of PiRBX1 (Hua and Kao, 2006) lends support for this dual role. However, since PiSBP1 is expressed in all tissues examined (Hua and Kao, 2006), it may have a more general role than just in pollination. For example, yeast two-hybrid and *in vitro* pull-down assays showed that NaSBP1, SBP1 of *N. alata*, interacted with the C-terminal domain of two arabinogalactan proteins in the extracellular matrix of the pistil, suggesting that SBP1 might have a role in trafficking of endocytic cargo (Lee *et al.*, 2008).

Second, Liu *et al.* (2009) developed a style-by-style method for quantification of S-RNase levels in *Solanum chacoense* (Solanaceae), and they reported that the S-RNase levels measured in unpollinated styles were approximately equal to those measured in incompatibly pollinated styles, but the S-RNase levels measured in compatibly pollinated styles were lower by up to 30 %. These results provide *in vivo* support for the notion that non-self S-RNases are degraded in compatible pollen tubes, whereas self S-RNase is not degraded in incompatible tubes. Moreover, *in vitro*, cell-free protein ubiquitination and degradation assays have been developed for *P. inflata*, and it has been shown that S-RNases were ubiquitinated and degraded in pollen tube extracts (Hua and Kao, 2006). The observed degradation and ubiquitination of

S-RNase was not *S*-haplotype-specific, but this could be because the *in vitro* systems did not completely mimic *in vivo* conditions. For *Antirrhinum*, the physical interaction between AhSLF-S_2 and S-RNase was demonstrated by pull-down, yeast two-hybrid, and co-immunoprecipitation, although allelic specificity was not shown. Upon compatible pollination, the extent of ubiquitination increased and the growth of pollen tubes was inhibited after treatment with a proteasomal inhibitor, suggesting that non-self S-RNases may be degraded by the ubiquitin-26S proteasome pathway (Qiao *et al.*, 2004*a*, *b*). Since lysine residues are normally the attachment sites for polyubiquitin chains, each of the 20 lysine residues of S_3-RNase of *P. inflata* was mutated to arginine, either one at a time or several at a time, and the rate of degradation of all the resulting lysine-to-arginine mutant proteins assayed in the *in vitro* system. It was found that changing six lysine residues near the C-terminus to arginines had the most significant effect on the extent of ubiquitination and rate of degradation of the mutant S-RNases (Hua and Kao, 2008). Interestingly, an alignment of the amino acid sequences of 32 S-RNases from several solanaceous species showed that two of these six lysines are among the most highly conserved lysines of S-RNases (Hua and Kao, 2008).

Third, *in vitro* protein-binding assays showed that a PiSLF interacts with its non-self S-RNases more strongly than with its self S-RNase (Hua and Kao, 2006). For example, when equal amounts of GST-tagged S_1-RNase and S_3-RNase were separately assayed for their interactions with His-tagged $PiSLF_1$, GST:S_3-RNase pulled down more His:$PiSLF_1$ than did GST:S_1-RNase, i.e. $PiSLF_1$ interacted more strongly with its non-self S-RNase, S_3-RNase, than with its self S-RNase, S_1-RNase.

Biochemical basis for preferential non-self interaction between SLF and S-RNase

It may seem counterintuitive that non-self interactions between S-RNase and SLF are favoured over self-interactions, as self-interactions are usually viewed as between a key and a matching lock. However, SLF and S-RNase could have evolved in such a way that the overall strength of interaction is reduced when they are derived from the same *S*-haplotype. Thus, any S-RNase that interacts strongly with an SLF would suffer the fate of degradation, and only when an S-RNase has acquired a specificity domain that matches the specificity domain of SLF would it escape degradation.

To understand the biochemical basis for the differential interactions between an SLF and its self and non-self S-RNases, first six *PiSLF-like* genes of *P. inflata* and three alleles of *PiSLF* were compared (McCubbin *et al.*, 2000*a*; Wang *et al.*, 2003; Hua *et al.*, 2007). The *PiSLF-like* genes share a number of properties with *PiSLF*. They are tightly linked to the *S*-locus, show *S*-haplotype-specific RFLP, are specifically expressed in pollen, and their deduced amino acid sequences are 50–54 % identical with PiSLF. However, when three of these *PiSLF-like* genes (one from S_1-haplotype and two from S_2-haplotype) were expressed in pollen of transgenic S_1S_2 or S_2S_3 plants, none of them caused the breakdown of SI function in their respective heteroallelic pollen grains (Fig. 1C). An *in vitro* protein-binding assay was also used to show that these PiSLF-like proteins either did not interact with S-RNase, or interacted with S-RNase much more weakly than did $PiSLF_2$.

Comparison of the amino acid sequences of three allelic variants of PiSLF and ten PiSLF-like proteins (some of which are allelic variants) revealed three PiSLF-specific regions. PiSLF was then divided into three functional domains, named FD1, FD2 and FD3, each of which contains one of the PiSLF-specific regions (Hua *et al.*, 2007). *In vitro* protein-binding assays using various truncated forms of $PiSLF_2$ containing one or two domains showed that FD2 interacted with S_3-RNase the strongest, even more strongly than did full-length $PiSLF_2$, and that addition of FD1 or FD3 to FD2 reduced the strength of interaction. As FD2 is relatively conserved among the three allelic variants of PiSLF, it may be the primary region for the interaction between PiSLF and S-RNase. FD1 and FD3 each contain one of the two variable regions of PiSLF, and they may negatively regulate the strong interaction between FD2 and S-RNase. Results from *in vitro* protein-binding assays using chimeric proteins between $PiSLF_1$ and $PiSLF_2$, with their FD1 and FD3 swapped, suggested that FD1 and FD3 together determine the strength of interaction with S-RNase. For example, a chimeric protein with FD1 and FD3 from $PiSLF_1$ and FD2 from $PiSLF_2$ interacted with S_2-RNase as strongly as did full-length $PiSLF_1$, whereas a chimeric protein with FD1 and FD3 from $PiSLF_2$ and FD2 from $PiSLF_1$ interacted with S_2-RNase as weakly as did full-length $PiSLF_2$. Thus, FD1 and FD3 together appear to constitute the *S*-allele-specificity determinant of PiSLF. Our lab proposes that during self-interactions, matching of FD1 and FD3 of a PiSLF and the specificity domain of its self S-RNase weakens the otherwise strong interaction between FD2 and S-RNase. How this may occur is not known at the biochemical level.

Biochemical model for compatible and incompatible pollinations and competitive interaction

The proposed protein-degradation model can explain the outcome of compatible and incompatible pollinations and, most importantly, the phenomenon of competitive interaction (Hua *et al.*, 2008). In the case of incompatible pollination, for example, pollination of an S_1S_2 style by S_2 pollen (Fig. 2A), both S_1-RNase and S_2-RNase are taken up by the S_2 pollen tube during penetration into the style, and the strong interaction between SLF_2, produced in the cytoplasm of the S_2 pollen tube, and S_1-RNase (a non-self S-RNase for SLF_2) would result in the ubiquitination and degradation of S_1-RNase. However, S_2-RNase (self S-RNase for SLF_2) would not be ubiquitinated or degraded, and would thus degrade pollen RNA to result in inhibition of tube growth. In the case of compatible pollination, for example, pollination of an S_1S_2 style by S_3 pollen (Fig. 2B), the strong interaction between SLF_3 and S_1-RNase and S_2-RNase (both non-self S-RNases for SLF_3) inside the S_3 pollen tube would result in their ubiquitination and degradation, thus allowing the S_3 pollen tube to circumvent the toxic effect of S-RNase. In the case of competitive interaction, for example, pollination of an S_2S_3 pistil by heteroallelic S_2S_3 pollen (Fig. 2C), SLF_2 and SLF_3 would preferentially interact with their respective

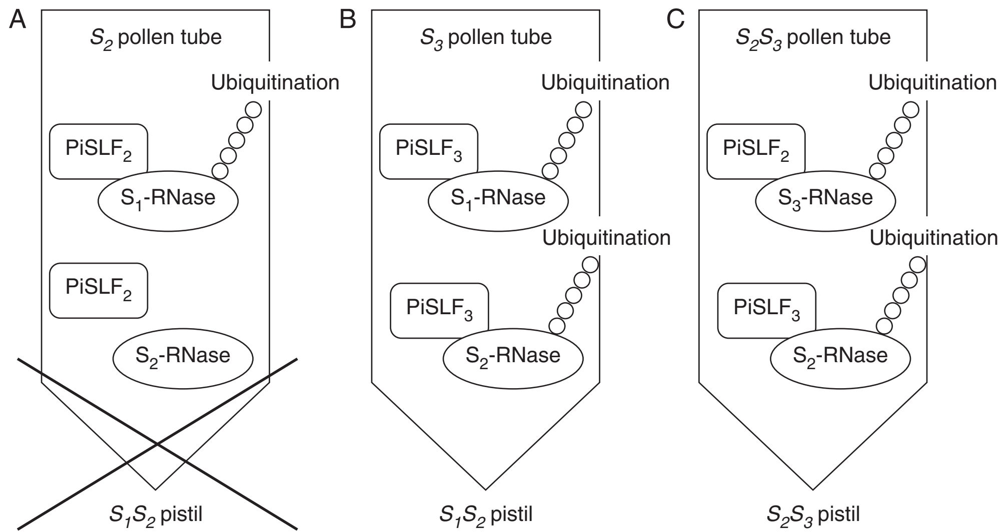

Fig. 2. Biochemical model for compatible and incompatible pollinations and competitive interaction. (A) Incompatible pollination. An S_2 pollen tube produces PiSLF$_2$, and as it is growing in an S_1S_2 pistil, it takes up S$_1$-RNase and S$_2$-RNase from the style. PiSLF$_2$ preferentially interacts with S$_1$-RNase (a non-self S-RNase) to mediate its ubiquitination and degradation. S$_2$-RNase (self S-RNase) is not affected and can exert its cytotoxic function in degrading pollen RNA. As a result, growth of the S_2 pollen tube is arrested. (B) Compatible pollination. An S_3 pollen tube produces PiSLF$_3$ and, as it is growing in an S_1S_2 pistil, it takes up S$_1$-RNase and S$_2$-RNase from the style. PiSLF$_3$ interacts strongly with S$_1$-RNase and S$_2$-RNase (non-self S-RNases), and mediates their ubiquitination and degradation. Thus, the S_3 pollen tube is able to grow through the style to effect fertilization. (C) Competitive interaction. An S_2S_3 heteroallelic pollen tube produces both PiSLF$_2$ and PiSLF$_3$ and, as it is growing in an S_2S_3 pistil, it takes up S$_2$-RNase and S$_3$-RNase from the style. PiSLF$_2$ preferentially interacts with S$_3$-RNase to mediate its ubiquitination and degradation, and PiSLF$_3$ preferentially interacts with S$_2$-RNase to mediate its ubiquitination and degradation. As a result, the S_2S_3 pollen tube is able to grow through the style to effect fertilization.

Table 1. *Mechanistic differences for S-RNase-based SI between subfamily Prunoideae of Rosaceae and Solanaceae, Plantaginaceae and subfamily Maloideae*

Family	Subfamily	Competitive interaction	Defects in SLF/SFB	Potential number of genes for pollen specificity	Proposed function of SLF/SFB
Rosaceae	Prunoideae	No*	SC†	Single	Protect self S-RNase from being degraded
Rosaceae	Maloideae	Yes	Not reported	Multiple	Mediate degradation of non-self S-RNases
Solanaceae		Yes	Not reported	Multiple	Mediate degradation of non-self S-RNases
Plantaginaceae		Yes	Not reported	Multiple	Mediate degradation of non-self S-RNases

* Huang *et al.* (2008) reported the only case of competitive interaction in tetraploid *Prunus pseudocerasus*, but the breakdown of SI could have been caused by mutations in other SI-related genes (Tao and Iezzoni, 2010).

† SC (self-compatible) indicates breakdown of SI in pollen.

non-self S-RNases (S$_3$-RNase for SLF$_2$ and S$_2$-RNase for SLF$_3$) in the cytoplasm of the S_2S_3 pollen tube, and as a result, both S$_2$-RNase and S$_3$-RNase would be ubiquitinated and degraded. Thus, heteroallelic S_2S_3 pollen would be compatible with the S_2S_3 pistil. The fact that heteroallelic pollen, regardless of the two *S*-haplotypes it carries, is universally accepted by pistils of any *S*-genotype can be explained by our model, which predicts that the two allelic products of SLF produced in heteroallelic pollen together would mediate the ubiquitination and degradation of all S-RNases.

Mechanistic differences for S-RNase-based SI in subfamily Prunoideae of Rosaceae

Although Solanaceae, Rosaceae and Plantaginaceae all employ S-RNase as the pistil determinant and SLF/SFB as the pollen determinant, how SLF/SFB functions in SI interactions between pollen and pistil may not be entirely conserved (Table 1). First, the above-mentioned competitive interaction phenomenon is observed in Solanaceae, Plantaginaceae and the subfamily Maloideae of Rosaceae (Crane and Lewis, 1942; Golz *et al.*, 1999, 2001; Adachi *et al.*, 2009; Xue *et al.*, 2009), but not in the subfamily Prunoideae of Rosaceae (Hauck *et al.*, 2006*b*; Tsukamomo *et al.*, 2006). [Huang *et al.* (2008) reported the only case of competitive interaction in a self-compatible line of tetraploid *Prunus pseudocerasus* (Prunoideae), but Tao and Iezzoni (2010) raised the possibility that the breakdown of SI was caused by mutations in other SI-related genes.] Second, defects in *SLF* of Solanaceae and Plantaginaceae most likely lead to pollen rejection by pistils of any *S*-genotype, as such mutants have never been found among pollen-part self-compatible mutants. In contrast, mutations in *SLF/SFB* of Prunoideae result in breakdown of SI (Ushijima *et al.*, 2004; Tsukamoto *et al.*, 2006; Yamane and Tao, 2009). These findings suggest that, in contrast to the proposed function of SLF

of Solanaceae, Plantaginaceae and subfamily Maloideae of Rosaceae in mediating degradation of non-self S-RNases, the function of SLF/SFB in subfamily Prunoideae of Rosaceae may be to specifically protect self S-RNase from being degraded. Thus, loss-of-function of SLF/SFB in this subfamily results in the inability of pollen tubes to protect self S-RNase, as well as all non-self S-RNases, from being degraded.

Another significant difference between Solanaceae, Plantaginaceae and subfamily Maloideae of Rosaceae, and subfamily Prunoideae of Rosaceae is the copy number of *SLF/SFB* (Sassa *et al.*, 2010). In Prunoideae, although there are additional F-box genes linked to the *S*-locus of *Prunus*, they exhibit much lower allelic sequence diversity compared with *SLF/SFB* and are not thought to be involved in SI (Ushijima *et al.*, 2003). On the contrary, in Maloideae, Sassa *et al.* (2007) identified two highly similar F-box genes (87·5 % identity in their deduced amino acid sequences) of apple, named *SFBB*s (*S-locus F-box brothers*), and proposed that both encode the pollen specificity determinant. Most recently, 20 additional *SFBB-like* genes (some of which may be alleles of the same gene) were isolated from screening a BAC library of an *S*-heterozygote of apple, suggesting that the *SLF/SFB* gene family in Maloideae is even larger than initially thought (Minamikawa *et al.*, 2010). In Plantaginaceae, three *AhSLF-like* genes were identified and their deduced amino acid sequences are 38–54 % identical with that of *AhSLF-S_2* (Zhou *et al.*, 2003). In *P. inflata*, as stated earlier, six *PiSLF-like* genes have been identified and they share similar properties with *PiSLF* (McCubbin *et al.*, 2000*a*; Wang *et al.*, 2003; Hua *et al.*, 2007). In *N. alata*, ten genes encoding SLF-related proteins were identified, and all of them are linked to the *S*-locus. Seven of these ten genes are specifically expressed in pollen and at least three are located in the same chromosomal segment as the pollen *S*-allele (Wheeler and Newbigin, 2007). It is interesting that, in these species, there exist a number of additional F-box genes that are tightly linked to the *S*-locus and possess other properties expected of the pollen *S*-gene. To date, functional studies of only three of the *SLF-like* genes from these species (*PiSLFLb-S_2*, *PiSLFLc-S_1* and *PiSLFLd-S_2* of *P. inflata*) have been reported (Hua *et al.*, 2007), and none of them cause breakdown of SI in S_3 pollen (in the case of *PiSLFLb-S_2* and *PiSLFLd-S_2*) or S_2 pollen (in the case of *PiSLFLc-S_1*) of transgenic plants (Fig. 1C). It will be enlightening to examine the possible function of more *SLF-like* genes in SI by a similar transgenic approach.

CONCLUSIONS AND FUTURE PROSPECTS

The discovery in the mid-1980s that the *S-RNase* gene (initially simply referred to as the pistil *S*-gene) encoded the pistil determinant of the Solanaceae SI system (Anderson *et al.*, 1986) marked the dawn of the molecular genetic/biochemical studies of this type of SI mechanism. Since then, there have been several major breakthroughs that led to new directions of research and enhanced our understanding of the SI mechanism. The demonstration that S-RNases have RNase activity (McClure *et al.*, 1989) and that the RNase activity is required for the function of S-RNase (Huang *et al.*, 1994) provided clues as to how the pistil might inhibit growth of self-pollen tubes at the biochemical level. The finding that uptake of S-RNases by the pollen tube is not *S*-haplotype-specific (Luu *et al.*, 2000; Goldraij *et al.*, 2006) suggested that specific rejection of self-pollen tubes by the pistil most likely lies in differential fates of self and non-self S-RNases inside the pollen tube. Finally, the recent identification of the *SLF/SFB* gene as the pollen determinant (Lai *et al.*, 2002; Entani *et al.*, 2003; Ushijima *et al.*, 2003; Qiao *et al.*, 2004*a*, *b*; Sijacic *et al.*, 2004; Ushijima *et al.*, 2004; Wang *et al.*, 2004; Sonneveld *et al.*, 2005; Tsukamoto *et al.*, 2005, 2006; Hauck *et al.*, 2006*a*; Vilanova *et al.*, 2006; Sassa *et al.*, 2007) allowed the formulation of new hypotheses and models for the biochemical basis of *S*-haplotype-specific rejection of pollen tubes.

The *S-RNase* gene has been very extensively studied over the past quarter century, but there are still some aspects of S-RNase that remain unknown. For example, what specific amino acid residues of each S-RNase determine its *S*-allele specificity? What is the role, if any, of the glycan chain(s)? What is the mechanism for the uptake of S-RNases by pollen tubes? How might S-RNases sequestered in a vacuole-like compartment in the pollen tube be specifically released into the cytoplasm of an incompatible pollen tube to exert their cytotoxic effect?

The transgenic approach used to establish the function of *SLF* in *P. inflata* (Sijacic *et al.*, 2004) and *Antirrhinum* (Qiao *et al.*, 2004*b*) is a robust assay for testing whether a candidate for the pollen *S*-gene is indeed involved in controlling pollen specificity, provided that competitive interaction has been observed in the species under study. The *S*-locus-linked *SLF-like* genes, that share similar properties with *SLF* but whose function has not been examined, will be good candidates for this *in vivo* functional assay. This assay is based on whether a particular F-box protein produced in the pollen from a transgene can cause breakdown of SI in transgenic pollen, which, based on our model, reflects whether the F-box protein can interact with the S-RNases taken up into the transgenic pollen tubes to mediate their degradation. For example, if the S_1-allelic variant of SLF, when expressed in S_2 pollen, causes breakdown of SI in S_2 pollen, and if the S_1-allelic variant of an SLF-like protein fails do so, the results would be interpreted to mean that this SLF-like protein, unlike SLF_1, cannot interact with S_2-RNase to mediate its degradation. As more and more SLF-like proteins of the S_1-haplotype are tested, one can compare the sequences of those that do not cause breakdown of SI in S_2 pollen with the sequences of those that do, if any, and SLF_1 to identify any amino acid residues that might be responsible for the functional and biochemical differences between these two groups of F-box proteins.

The *in vivo* functional assay can also be used for structure/function studies to identify specific amino acids and domains of SLF that are involved in a particular function. For example, one can address the question of whether the F-box domain of SLF is required for its function by expressing a truncated SLF in pollen carrying a different *S*-allele and examining whether the truncated SLF can cause competitive interaction in transgenic pollen. Another key question about SLF is how the *S*-specificity is determined. FD1 and FD3 of PiSLF have been identified as the putative *S*-specificity determinant from

in vitro protein-binding assays. One can test this model by expressing chimeric SLF proteins between two allelic variants in transgenic plants of the appropriate *S*-genotype, and examining the effect of the chimeric proteins on the SI behaviour of transgenic pollen. For example, based on our model, the chimeric protein containing FD1 and FD3 from $PiSLF_2$ and FD2 from $PiSLF_3$ would possess S_2-allele specificity and would cause breakdown of SI in S_3 pollen (heteroallelic), but not in S_2 pollen (homoallelic), when introduced into plants of S_2S_3 genotype. Once a particular domain is found to possess a particular function, site-directed mutagenesis can be used to narrow down specific amino acids involved.

The tenet of the protein-degradation model proposed by our lab is that an SLF protein preferentially interacts with all its non-self S-RNases in the pollen tube to mediate their ubiquitination and ultimate degradation. This model predicts that absence of SLF in pollen would lead to the inability of the pollen to detoxify any S-RNase (self or non-self), and as a result, the pollen would be rejected by pistils of any *S*-genotype. One direct approach to test this prediction is to use the methodology of RNA interference (RNAi) to suppress the production of SLF in pollen, and to determine whether the transgenic pollen becomes incompatible with pistils that should normally recognize it as non-self pollen. For example, if our model is correct, then suppression of SLF_1 in S_1 pollen would result in the inability of the transgenic pollen to detoxify any S-RNase that it takes up, and consequently, the transgenic pollen would be rejected by pistils of any *S*-genotype. The same approach can be used to examine the involvement of all other genes that have been implicated in SI. For example, if PiSBP1 is indeed an essential component of the PiSLF-containing E3 ligase complex, suppression of its production in pollen would result in the inability of the E3 ligase complex to be assembled and consequently, the inability of PiSLF to mediate degradation of non-self S-RNases. The prediction is that the transgenic pollen, in which the expression of *PiSBP1* is suppressed, would be rejected by pistils of any *S*-genotype.

In conclusion, tremendous progress has been made towards understanding the Solanaceae SI system since the cloning of the *S-RNase* gene was first reported many years ago, but there remain many key questions, as any new discovery invariably leads to new questions and new avenues of research. The availability of robust *in vivo* functional assays for *S-RNase* and *SLF* in *P. inflata* has made this species a good model system, and it would be of interest to determine how much of the information obtained is applicable to other taxa possessing the Solanaceae-type SI system, particularly those in subfamily Prunoideae of Rosaceae.

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