

Chapter 24

S-RNase-Based Self-Incompatibility in *Petunia*: A Complex Non-Self Recognition System Between Pollen and Pistil

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Abstract Self-incompatibility (SI) is an intraspecific reproductive barrier that allows many families of flowering plants to prevent inbreeding and promote outcrosses. Extensive studies of SI in five families during the past more than two decades have revealed three distinct SI mechanisms. This chapter focuses on the mechanism employed by the Solanaceae, using mostly results obtained from *Petunia*. We first discuss the identification of two polymorphic genes at the *S*-locus, the *S-RNase* gene, which controls pistil specificity, and the *S*-locus *F*-box (*SLF*) gene, now named *SLF1*. For several years after its identification, *SLF1* was thought to be solely responsible for pollen specificity, and biochemical models were developed based on this assumption. However, results inconsistent with this assumption were subsequently obtained, which led to the recent finding that pollen specificity is controlled by multiple, but an as yet unknown number of, polymorphic *SLF* genes located at the *S*-locus. A new model, named collaborative non-self recognition, has been proposed to explain the biochemical basis of specific inhibition of self-pollen tube growth. Based on this model, compatible pollination results from ubiquitination and subsequent degradation of non-self S-RNases collectively mediated by all SLF proteins, with each SLF responsible for detoxifying a subset of non-self S-RNases.

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We conclude this chapter with a discussion of some of the new questions raised by the finding that pollen specificity is controlled by multiple *SLF* genes.

Keywords Collaborative non-self recognition • *Petunia* • Self-incompatibility • *S*-locus F-box proteins • *S*-RNase

24.1 Introduction

In contrast to animals, plants cannot freely move about to select appropriate mates. To preserve species identity and to generate genetic diversity within species, it is imperative that plants possess mechanisms by which their female reproductive tissues, the pistils, can prevent unwanted or unsuitable pollen from delivering sperm cells to the ovary to effect fertilization. For example, many flowering plants that produce bisexual flowers have adopted a genetically controlled pre-zygotic barrier, called self-incompatibility (SI). SI involves a self/non-self recognition process between pollen and pistil. As a result of the recognition, self-pollen is rejected by the pistil to prevent inbreeding, and only non-self pollen is accepted to promote outcrosses. The specific recognition process involves the interaction between pistil specificity determinant and pollen specificity determinant, both of which are encoded by genes tightly linked at a highly polymorphic locus, named the *S*-locus. Variants of the *S*-locus are referred to as *S*-haplotypes and designated S_1 , S_2 , S_3 , etc.

SI is quite common in flowering plants, but to date our understanding of the molecular and biochemical basis is limited to 5 of the estimated more than 60 families that possess SI. Extensive studies on these 5 families since the mid-1980s have revealed three distinct mechanisms (Takayama and Isogai 2005). In this chapter, we focus on the mechanism that is employed by the Solanaceae family (and likely by two other families, the Rosaceae and Plantaginaceae) using mostly the results obtained from the study of *Petunia*. We first describe the identification and characterization of the gene that controls pistil specificity, the *S*-RNase, and then describe the identification of the first *S*-locus F-box (*SLF*) gene, now named type-1 *SLF* (or *SLF1*), that is involved in controlling pollen specificity. After the identification of *SLF1*, it was thought to be the only gene that controls pollen specificity, as the prevailing thought then was that pollen specificity must be controlled by a single polymorphic gene, just like the prior finding that pistil specificity is controlled by a single polymorphic gene, *S*-RNase. Moreover, it is already difficult to envision how a single pollen specificity gene has coevolved with a single pistil specificity gene to maintain SI over the course of millions of years since this self/non-self recognition system was established. We thus next turn to the discussion of several lines of evidence, from the evolutionary perspective and experimental data, that are inconsistent with the assumption that a single *SLF* gene is solely responsible for pollen specificity, and describe the identification of additional *SLF* genes that are involved in pollen specificity. We present the latest model, named “collaborative non-self recognition,” that explains how the polymorphic *S*-RNase gene and multiple polymorphic *SLF* genes function in SI, allowing cross-compatible pollen tubes to grow

through the pistil, but inhibiting the growth of self-pollen tubes in the pistil. Finally, we discuss some of the questions regarding the evolution, maintenance, and operation of S-RNase-based SI that were raised by the unexpected finding that pollen specificity is controlled by multiple *SLF* genes.

24.2 Identification and Characterization of the *S-RNase* Gene

To identify the gene that controls pistil specificity, pistil proteins that showed *S*-haplotype-specific differences in molecular mass and/or isoelectric point were first identified (Bredemeijer and Blaas 1981; Anderson et al. 1986). These proteins were deemed likely candidates, as allelic products of the pistil gene involved in SI were expected to be different in their amino-acid sequences such that they might be different in molecular mass or isoelectric point. This approach led to the identification of the *S-RNase* gene in *Nicotiana glauca* (Anderson et al. 1986). However, the biochemical nature of S-RNase was not known until several years later when the amino-acid sequence of the fungal RNase T2 was determined (Kawata et al. 1988) and found to share a significant degree of sequence homology between its catalytic domain and a conserved domain of several allelic variants of S-RNase (McClure et al. 1989). Subsequently, S-RNase was shown to indeed possess RNase activity in vitro (McClure et al. 1989; Broothaerts et al. 1991; Singh et al. 1991). The *S-RNase* gene has been shown to be solely responsible for pistil specificity in SI by gain- and loss-of-function experiments (Lee et al. 1994; Murfett et al. 1994). For example, in *Petunia inflata*, expression of the *S₃-RNase* gene in pistils of wild-type plants of *S₁S₂* genotype conferred on the pistils of the transgenic plants the ability to reject *S₃* pollen, whereas expression of an antisense *S₃-RNase* gene in wild-type plants of *S₂S₃* genotype led to the inability of the transgenic plants to reject *S₃* pollen, but did not affect their ability to reject *S₂* pollen (Lee et al. 1994). These experiments suggest that S-RNase is necessary and sufficient for the pistil to recognize and reject self-pollen.

The characteristics of S-RNase are also consistent with its involvement in pistil specificity. For example, S-RNase is specific to the pistil. It is first synthesized in the transmitting cell of the style and then secreted into the extracellular space of the transmitting tract. It is most abundant in the upper third segment of the style, where inhibition of incompatible pollen tubes occurs (Ai et al. 1990). Moreover, S-RNase has a high degree of allelic sequence diversity, with the highest divergent pair sharing only 38 % sequence identity (Tsai et al. 1992; McCubbin and Kao 2000), which is expected for a protein that is involved in self/non-self recognition. Sequence comparison revealed the presence of five conserved regions, C1–C5 (with C2 and C3 similar to the corresponding domains of RNase T2), and two hypervariable regions, HVa and HVb (Ioerger et al. 1991; Tsai et al. 1992; Takayama and Isogai 2005). Crystallographic analysis of a Solanaceae S-RNase showed that the protein-folding topology is typical for the RNase T2 family (Ida et al. 2001). Moreover, the results also showed that both HVa and HVb are exposed on the surface of the S-RNase protein and accessible to solvent, raising the possibility that these two regions have the allelic-specific function (Ida et al. 2001; Matsuura et al. 2001).

To further understand the function of S-RNase in SI, site-directed mutagenesis was used to replace the codon for one of the two catalytic His residues (located in C3) of S₃-RNase of *P. inflata* with an Asn codon. The mutant S₃-RNase gene was introduced into *P. inflata* plants of S₁S₂ genotype, and the transgenic plants producing this mutated S₃-RNase failed to reject S₃ pollen, suggesting that the RNase activity of S-RNase is an integral part of its function and that the biochemical mechanism of SI involves degradation of RNAs in incompatible pollen tubes (Huang et al. 1994). Moreover, as all S-RNases are glycoproteins with various numbers of N-linked glycan chains, a question was raised as to whether the recognition function of S-RNase resides in the glycan moiety, the protein backbone, or both. To address this question, the codon for the only potential N-glycosylation site of S₃-RNase was replaced with a codon for Asp, and the mutant S₃-RNase gene was introduced into wild-type plants of S₁S₂ genotype to examine whether expression of the non-glycosylated S₃-RNase could still confer on the transgenic plants the ability to reject S₃ pollen. The results showed that the non-glycosylated S₃-RNase retained the full ability to reject self-pollen, suggesting that the recognition function of S-RNase resides in its amino-acid sequence (Karunanandaa et al. 1994). Domain-swapping experiments were performed to examine the role of the two hypervariable domains in allelic specificity (Kao and McCubbin 1996; Matton et al. 1997; Zurek et al. 1997; Matton et al. 1999). Swapping HVa and HVb between S₁₁-RNase and S₁₃-RNase of *Solanum chacoense* (which differ by four amino acids in the hypervariable regions) was sufficient to switch the allelic specificity (Matton et al. 1997, 1999). However, in the case of *P. inflata* and *N. alata* S-RNases examined, the two hypervariable regions are necessary but not sufficient for allele specificity. This conclusion is consistent with the finding that two *P. inflata* S-RNases, S₆-RNase and S₉-RNase, have identical sequences in HVa and differ by only two amino acids in HVb (Wang et al. 2001; Kao and Tsukamoto 2004).

For S-RNase to inhibit growth of self-pollen tubes, it must be able to enter incompatible pollen tubes to exert its cytotoxicity. Using immunocytochemistry, Luu et al. (2000) showed that S-RNase was localized in the cytoplasm of both self- and non-self pollen tubes. In contrast, Goldraij et al. (2006), using triple antibody-labeling immunolocalization, showed that S-RNase was initially sequestered in a vacuole-like compartment of both self- and non-self pollen tubes, but the compartment was later disrupted only in incompatible pollen tubes, releasing S-RNase into the cytoplasm. Both groups are in agreement that S-RNase is taken up by self- and non-self pollen tubes; however, the mechanism of S-RNase uptake is as yet unknown.

24.3 Identification of the First *S-Locus F-box* Gene, *SLF1*

One would expect that the approach used to successfully identify S-RNase and clone the *S-RNase* gene could be used to identify the pollen specificity determinant. However, no pollen protein that show *S*-haplotype specific differences in molecular mass or isoelectric point was ever identified. Another approach, RNA differential

display, was used to identify pollen-specific genes that are tightly linked to the *S*-locus (Wang et al. 2003). This approach was based on the prediction that the gene controlling pollen specificity must be tightly linked to the *S*-RNase gene and shows allelic sequence diversity. Thirteen such genes of *P. inflata* were identified (Dowd et al. 2000; McCubbin et al. 2000). However, none of them was considered a good candidate for the pollen specificity gene because of their low allelic sequence diversity (Wang et al. 2003). Ultimately, it was through sequencing the *S*-locus region containing the *S*-RNase gene that a good candidate was first identified in *Antirrhinum hispanicum* (Plantaginaceae), which also possesses S-RNase-based SI (Lai et al. 2002). This gene is located ~9 kb downstream from the *S*₂-RNase gene, and its deduced amino-acid sequence contains an F-box motif at the N-terminus. Thus, the gene was named *AhSLF* (*A. hispanicum S*-locus *F*-box gene). Subsequently, *PmSLF*, encoding an F-box protein, was identified in *Prunus mume* of Rosaceae through genomic sequence analysis of an ~60-kb region containing *S*-RNase (Entani et al. 2003), and *SFB* (*S* haplotype-specific *F*-box gene), also encoding an *S*-locus F-box protein, was identified in another rosaceous species, *Prunus dulcis* (almond) (Ushijima et al. 2003).

In *P. inflata*, *PiSLF* (*P. inflata SLF*) was identified from sequencing a 328-kb contig of an *S*-locus region containing the *S*₂-RNase gene (Wang et al. 2004). *PiSLF* is located ~161 kb downstream from the *S*₂-RNase gene. RNA blotting results showed that *PiSLF* is specifically expressed in developing pollen, mature pollen and pollen tubes. It also shows *S*-haplotype-specific restriction-fragment length polymorphism, as expected of a gene located at the *S*-locus. The deduced amino-acid sequences of three alleles (*S*₁, *S*₂, and *S*₃) of *PiSLF* show 10.3–11.6 % allelic sequence diversity. These properties of *PiSLF* suggest that it is a good candidate for the pollen specificity gene. *PiSLF* control of pollen specificity was established by a transgenic functional assay of its *S*₂-allele, *PiSLF*₂ (Fig. 24.1a), designed based on the phenomenon named competitive interaction. This phenomenon was first observed in solanaceous species, including *P. inflata* and *P. hybrida* (Stout and Chandler 1942; Brewbaker and Natarajan 1960; Entani et al. 1999), where it was found that SI breaks down in heteroallelic pollen carrying two different *S*-alleles. For example, *S*₁*S*₂ pollen produced by a tetraploid *S*₁*S*₁*S*₂*S*₂ plant derived from self-incompatible plant *S*₁*S*₂ loses its SI function and cannot be rejected by the *S*₁*S*₁*S*₂*S*₂ pistil. Heteroallelic pollen can also result from duplication of one of the *S*-loci of self-incompatible plants carrying two different *S*-haplotypes (Golz et al. 1999). It was reasoned that if *PiSLF* controls pollen specificity, introducing *PiSLF*₂ into *S*₁*S*₁, *S*₁*S*₂, and *S*₂*S*₃ plants should cause breakdown of SI in *S*₁ and *S*₃ pollen carrying the transgene, as a result of competitive interaction between the endogenous *S*₁-allele or *S*₃-allele of *PiSLF* and the introduced *S*₂-allele. The results of these experiments were precisely as expected (Sijacic et al. 2004). For example (as shown in Fig. 24.1a), expression of a *PiSLF*₂ transgene in an *S*₂*S*₃ transgenic plant caused breakdown of SI in *S*₃ pollen (heteroallelic), but not in *S*₂ pollen (homoallelic).

As already stated, both self- and non-self S-RNases are taken up by a pollen tube, but only self-S-RNase can exert its cytotoxicity to inhibit the growth of the pollen tube. Before the discovery of the involvement of multiple *SLF* genes in pollen

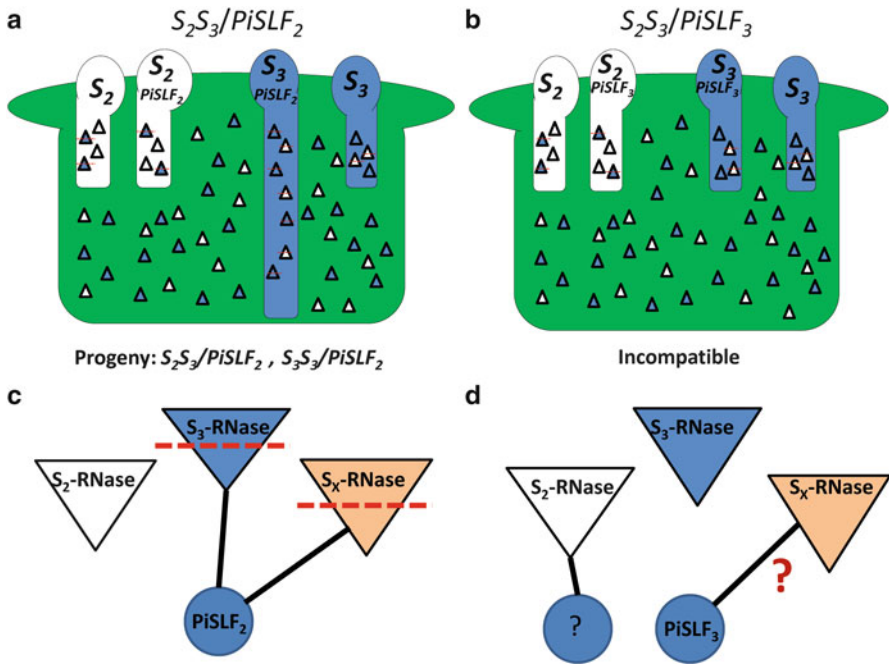


Fig. 24.1 Transgenic functional assay showing that *PiSLF* is not the sole pollen specificity gene. (a) Self-pollination of an S_2S_3 transgenic plant carrying a *PiSLF*₂ transgene. The transgenic plant produces four different genotypes of pollen as indicated. *White triangles* and *blue triangles* denote S_2 -RNase and S_3 -RNase molecules, respectively. Based on the findings that S_2S_3 and S_3S_3 , but no S_2S_2 , genotypes were present in the self-progeny and that all progeny inherited the transgene (either one copy or two copies), only S_3 pollen carrying the *PiSLF*₂ transgene was accepted by the pistil. *White and blue triangles* marked with a *broken line* across denote those S_2 -RNases and S_3 -RNases, respectively, that are inside pollen tubes but fail to exert their cytotoxicity. That *PiSLF*₂ causes breakdown of SI in S_3 , but not in S_2 , pollen is consistent with the prediction by competitive interaction that SI breaks down in pollen carrying two different *S*-alleles. (b) Self-pollination of an S_2S_3 transgenic plant carrying a *PiSLF*₃ transgene. The transgenic plant produces four different genotypes of pollen as indicated. *White triangles* and *blue triangles* denote S_2 -RNase and S_3 -RNase molecules, respectively. The transgenic plant remained self-incompatible, suggesting that all four genotypes of pollen were rejected by the pistil. *White and blue triangles* marked with a *broken line* across denote those S_2 -RNases and S_3 -RNases, respectively, that are inside pollen tubes but fail to exert their cytotoxicity. The finding that *PiSLF*₃ does not cause breakdown of SI in S_2 pollen is not consistent with the prediction by competitive interaction. (c) A protein degradation model based on the assumption that *PiSLF* is solely responsible for pollen specificity predicts that a *PiSLF* interacts with all *S*-RNases, except its self-*S*-RNase, to mediate their ubiquitination and ultimate degradation by the 26S proteasome. In the example shown, *PiSLF*₂ interacts with S_3 -RNase and S_x -RNase (with S_x denoting *S*-RNases produced by any additional haplotype except S_2) to mediate their ubiquitination and degradation, as indicated by a *broken line* across S_3 -RNase and S_x -RNase; however, *PiSLF*₂ does not interact with S_2 -RNase, its self-*S*-RNase. This model can explain the results of the transgenic functional assay shown in **a**. In S_3 pollen tubes expressing the *PiSLF*₂ transgene, *PiSLF*₃ produced by the endogenous gene interacts with S_2 -RNase, and *PiSLF*₂ produced from the transgene interacts with S_3 -RNase. As a result, both S_2 -RNase and S_3 -RNase are ubiquitinated and degraded, thus allowing the pollen tube to circumvent the toxic effect of these *S*-RNases. (d) Interpretation of the results of the transgenic functional assay shown in **b** based on the protein-degradation model shown in **c**. The failure of the *PiSLF*₃ transgene to cause breakdown of SI in S_2 pollen suggests that *PiSLF*₃ cannot interact with S_2 -RNase, a non-self *S*-RNase, raising a question of whether *PiSLF*₃ can interact with any other non-self *S*-RNases. Most importantly, this finding suggests that there must be additional protein(s) involved in pollen specificity that allow(s) S_3 pollen to interact with and detoxify S_2 -RNase to result in compatible pollination between S_3 pollen and S_2 -carrying pistils

specificity (see Sect. 24.5 below), our lab proposed a protein degradation model, based on the assumption that PiSLF (SLF1) is the sole pollen determinant, to explain why only self-S-RNase can function inside a pollen tube (Hua and Kao 2006; Hua et al. 2008). The model predicts that for pollen of a given *S*-haplotype, (1) the allelic variant of PiSLF produced interacts with all its non-self S-RNases in the cytoplasm of the pollen tubes to mediate their ubiquitination and ultimate degradation by the 26S proteasome, thus allowing non-self pollen to effect fertilization, and (2) the allelic variant of PiSLF does not interact with its self-S-RNase, allowing the self-S-RNase to degrade pollen tube RNAs to cause growth inhibition of self-pollen tubes. These predictions were largely based on the findings by Hua and Kao (2006) that bacterially expressed S-RNases were ubiquitinated and degraded via the 26S proteasomal pathway in an in vitro cell-free system, and that an allelic variant of PiSLF interacted with its non-self S-RNases more strongly than with its self-S-RNase in an in vitro protein binding assay. As most F-box proteins are components of SCF complexes, a class of E3 ubiquitin ligase involved in ubiquitin-mediated protein degradation, the protein degradation model provided a reasonable explanation for the biochemical basis of the SI process. Moreover, the following findings are consistent with this model. (1) The subcellular localization of AhSLF- S_2 (the S_2 -allelic variant of an SLF in *Antirrhinum*) detected by immunocytochemistry suggests that it is localized in the cytoplasm of pollen tubes (Wang and Xue 2005). (2) In *S. chacoense*, the level of S-RNase in compatible pollen tubes was ~30 % lower than that in incompatible pollen tubes (Liu et al. 2009). (3) Six lysine residues in S_3 -RNase of *P. inflata* were found to be necessary for targeting ubiquitination and degradation (Hua and Kao 2008).

This protein degradation model can explain why expression of a *PiSLF*₂ transgene in heteroallelic pollen causes breakdown of SI. For example, when a *PiSLF*₂ transgene is introduced into an S_2S_3 plant, it causes specific breakdown of SI in S_3 transgenic pollen (Fig. 24.1a). As shown in Fig. 24.1c, in the S_3 transgenic pollen, PiSLF₂ produced from the transgene interacts with S_3 -RNase, a non-self S-RNase, to mediate its ubiquitination and degradation. As PiSLF₃ produced by the endogenous gene interacts with S_2 -RNase, a non-self S-RNase, to mediate its ubiquitination and degradation, both S_2 -RNase and S_3 -RNase taken up by the transgenic S_3 pollen tube are detoxified, allowing the S_3 transgenic pollen tube to be compatible with the S_3 -carrying pistil.

24.4 Evidence Against *SLF1* Being Solely Responsible for Controlling Pollen Specificity

After the transgenic functional assay showing that one allele of *PiSLF*, *PiSLF*₂, behaved as precisely as expected for the gene controlling pollen specificity, it was thought that the long search for the pollen specificity gene was over. However, further analyses of this gene, now named *SLF1*, revealed puzzling results that called into question this notion.

24.4.1 Evolutionary Consideration

First, the degree of sequence diversity of *SLF1* is much lower than that of the *S-RNase* gene. For example, the allelic sequence diversity is only ~10 % for three alleles (S_1 , S_2 , and S_3) of *PiSLF*, but ranges from 19 % to 26 % for the same three alleles of *S-RNase*. Most puzzlingly, the allelic sequence diversity of four alleles (S_1 , S_2 , S_4 , S_5) of *AhSLF* (*A. hispanicum*) ranges from only 1 % to 3 %, whereas that of the same four alleles of *S-RNase* ranges from 36 % to 51 % (Zhou et al. 2003; Newbigin et al. 2008). The high degree of allelic sequence diversity observed for *S-RNase* is what would be expected for a gene that controls pistil specificity in SI interactions with pollen. During the evolution of the SI system, any new *S*-haplotype that arises in a population has a reproductive advantage over all existing haplotypes, because its frequency is lower to begin with and thus has a higher probability of successful fertilization. As such, the new *S*-haplotype will increase in its frequency in the population. Under this kind of frequency-dependent selection, there is pressure for sequence divergence in the genes that constitute *S*-haplotype specificity to generate new *S*-haplotypes. Thus, the low degree of allelic sequence diversity of the *SLF1* gene is not consistent with its presumed pollen specificity role in SI.

Second, the evolutionary history of *S-RNase* is consistent with the ancient origin of SI, but *SLF1* seemed to have evolved much more recently. Phylogenetic studies of *S-RNase* alleles from several solanaceous species revealed that polymorphism of *S-RNase* predated divergence of these species (e.g., some *P. inflata* alleles are more similar to some *N. alata* alleles than to other *P. inflata* alleles) (Ioerger et al. 1990). Allelic polymorphism of *S-RNase* was shown to exist in the common ancestor of the solanaceous species 30–40 million years ago (Paape et al. 2008). However, phylogenetic studies of *SLF1* alleles from *P. inflata* and *A. hispanicum* showed that *SLF1* has a much shorter evolutionary history (Newbigin et al. 2008).

Third, the evolution of *S-RNase* and *SLF1* seems not to be concordant. Coevolution of genes controlling pistil and pollen specificity has long been a central theory in SI. In Brassicaceae-type SI, the genes encoding pollen and style specificity determinants have been shown to be coevolved (Sato et al. 2002; Takebayashi et al. 2003). However, as stated here, *S-RNase* and *SLF1* seem to have different evolutionary histories, and moreover, genealogies of *S-RNase* and *SLF1* do not show a pattern of coevolution (Newbigin et al. 2008).

24.4.2 Experimental Data

When the same transgenic functional assay used to establish the function of *PiSLF₂* in pollen specificity was used to examine two other alleles of *PiSLF* and several alleles of *SLF1* of *P. hybrida*, the results showed that none of them behaved as expected for the pollen specificity gene. For example, as shown in Fig. 24.1b,

introduction of the S_3 -allele of *PiSLF*, *PiSLF*₃, into *P. inflata* plants of S_2S_3 genotype did not cause breakdown of SI in S_2 transgenic pollen, even though the transgenic S_2 pollen is heteroallelic (Kubo et al. 2010). Moreover, S_7 -allele of *P. hybrida* *SLF1* caused breakdown of SI in S_9 and S_{17} transgenic pollen, but not in S_5 , S_{11} , or S_{19} transgenic pollen (Kubo et al. 2010). These results, on the one hand, further confirm the involvement of *SLF1* in pollen specificity, but on the other hand, suggest that *SLF1* is not the only gene involved in pollen specificity. For example, based on the protein degradation model discussed in Sect. 24.3 earlier, the failure of a *PiSLF*₃ transgene to cause breakdown of SI in S_2 pollen suggests that *PiSLF*₃ does not interact with S_2 -RNase, a non-self S-RNase, to mediate its ubiquitination and degradation (Fig. 24.1d). This notion raises a question as to whether *PiSLF*₃ interacts with any other non-self S-RNases. Most importantly, this finding suggests that there must be additional protein(s) produced in S_3 pollen that can interact with and detoxify S_2 -RNase, as S_3 pollen is compatible with S_2 -carrying pistils.

The most definitive evidence for the involvement of additional gene(s) in pollen specificity came from the finding that the deduced amino-acid sequences of the S_7 -allele of *SLF1* of *P. hybrida* and S_9 -allele of *SLF1* of *P. axillaris* are completely identical, even though S_7 and S_9 are genetically distinct *S*-haplotypes and the deduced amino-acid sequences of their *S*-RNase alleles are only 45 % identical (Kubo et al. 2010).

24.5 Involvement of Additional *SLF* Genes in Controlling Pollen Specificity

After the identification of the first *SLF* gene, *SLF1*, it was discovered that there exist additional *F-box* genes that are tightly linked to the *S*-locus. For example, our lab identified four such *PiSLF*-like genes, named *PiSLFLa*, *PiSLFLb*, *PiSLFLc*, and *PiSLFLd* (Hua et al. 2007). Interestingly, these genes are also specifically expressed in pollen, and at least three of them show allelic-specific sequence differences. However, these *PiSLF*-like genes were initially thought not to be involved in pollen specificity, as when *PiSLFLc*- S_1 , *PiSLFLb*- S_2 , and *PiSLFLd*- S_2 were introduced into S_1S_2 , S_2S_3 , and S_2S_3 , respectively, none of them caused breakdown of SI in S_2 pollen (in the case of *PiSLFLc*- S_1) or S_3 pollen (in the case of *PiSLFLb*- S_2 and *PiSLFLd*- S_2) (Hua et al. 2007). The likely involvement of additional gene(s) in pollen specificity prompted the reexamination of these *PiSLF*-like genes, as well as additional *SLF1*-like genes of *P. hybrida* identified by the laboratory of Seiji Takayama.

Takayama's lab used PCR primers designed based on the sequences of *PiSLFLb*- S_2 , *PiSLFLc*- S_1 , and *PiSLFLd*- S_2 to identify 30 *SLF*-like genes from different *S*-haplotypes of *P. hybrida*, and classified them into five types, type 2 *SLF* (or *SLF2*) to type 6 *SLF* (*SLF6*) (Kubo et al. 2010). Within each type, the sequence identity between different alleles is high, ranging from 70.3 % to 99 %. However, the sequence identity between different types is only ~50 %. To date, *SLF2* and

SLF3 have been shown to be involved in pollen specificity as well. For example, *S₇-SLF2* (*SLF2* of *S₇* haplotype) caused breakdown of SI in *S₉*, *S₁₁*, and *S₁₉* pollen, and *S₁₁-SLF3* (*SLF3* of *S₁₁* haplotype) caused breakdown of SI in *S₇* pollen. The results also suggest that additional *SLF* genes are required for pollen specificity. For example, *SLF1*, *SLF2*, and *SLF3* of *S₇*-haplotype did not cause breakdown of SI in *S₅* pollen (Kubo et al. 2010).

24.6 Collaborative Non-Self Recognition Model

After the discovery that multiple SLF proteins are involved in pollen specificity, a modified protein degradation model, named collaborative non-self recognition, was proposed. This model proposes that, for a given *S*-haplotype, each type of SLF protein interacts with a subset of non-self S-RNases and that multiple types of SLF proteins are required to recognize the entire suite of non-self S-RNases to mediate their degradation. Similar to the original protein degradation model, the new model also predicts that none of the SLF proteins produced in pollen of a given *S*-haplotype interacts with their self-S-RNase. The prediction that an SLF interacts with a subset of its non-self S-RNases was confirmed by a co-immunoprecipitation (co-IP) experiment. This experiment showed that *S₇-SLF2:FLAG* produced in transgenic pollen coprecipitated, by an anti-FLAG antibody, with *S₉-RNase* and *S₁₁-RNase*, but not with *S₅-RNase* or *S₇-RNase*, in style extracts. Moreover, the transgenic functional assay showed that expression of *S₇-SLF2* caused breakdown of SI in *S₉* and *S₁₁* pollen, but did not cause breakdown of SI in *S₅* or *S₇* pollen (Kubo et al. 2010). Thus, the co-IP results not only provide strong support for the protein degradation aspect of collaborative non-self recognition, but also are entirely consistent with the results of the transgenic functional assay.

The collaborative non-self recognition model is graphically illustrated in Fig. 24.2. This figure depicts the outcomes of pollination of *S₅⁻*, *S₁₇⁻*, *S₁₁⁻*, and *S₉⁻*-carrying pistils by *S₅* pollen, as explained by the relationships between two types of SLF proteins produced in *S₅* pollen, *S₅-SLF1* and *S₅-SLF2*, and four S-RNases, *S₅⁻*, *S₁₇⁻*, *S₁₁⁻*, and *S₉-RNases*, based on the results obtained from the transgenic functional assay (Kubo et al. 2010). When *S₅-RNase* is taken up by the *S₅* pollen tube, none of the SLF proteins produced in *S₅* pollen can interact with their self-S-RNase, *S₅-RNase*; thus, pollen RNAs are degraded by *S₅-RNase* and pollen tube growth is inhibited. When *S₁₇-RNase* is taken up by the *S₅* pollen tube, *S₅-SLF1* interacts with *S₁₇-RNase* to mediate its ubiquitination/degradation. When *S₁₁-RNase* is taken up by the *S₅* pollen tube, *S₅-SLF2* interacts with *S₁₁-RNase* to mediate its ubiquitination/degradation. When *S₉-RNase* is taken up by the *S₅* pollen tube, both *S₅-SLF1* and *S₅-SLF2* will be able to interact with *S₉-RNase* to mediate its ubiquitination/degradation. Thus, in cross-pollinations, at least one SLF protein can interact with and detoxify a non-self S-RNase, allowing the pollen tube to grow down through the style to effect fertilization.

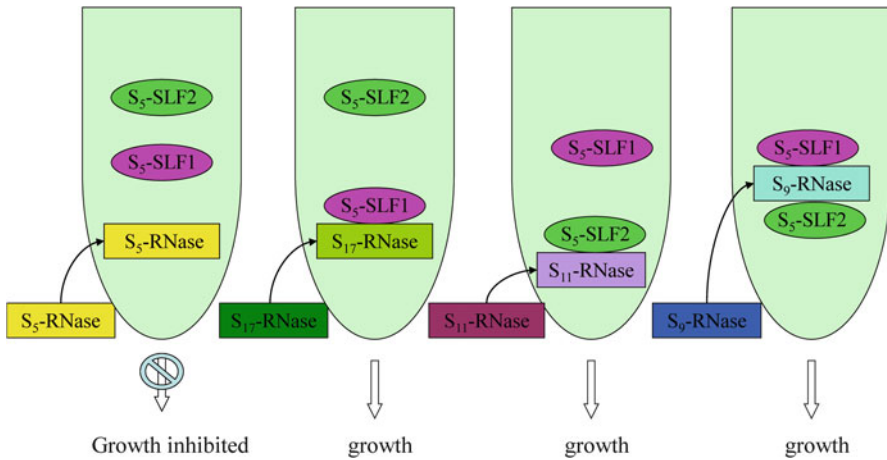


Fig. 24.2 Collaborative non-self recognition model. For pollen of a given *S*-haplotype, multiple types of SLF proteins collaboratively interact with all their non-self S-RNases, with each type of SLF only interacting with a subset of the non-self S-RNases. For the sake of simplicity, only two different types of SLF proteins, SLF1 and SLF2, are shown for S_5 pollen. When S_5 -RNase is taken up by the S_5 pollen tube (*leftmost panel*), no SLF proteins can interact with their self-S-RNase, S_5 -RNase. Pollen RNA is degraded by S_5 -RNase and pollen tube growth is inhibited. When S_{17} -RNase is taken up by the S_5 pollen tube (*second panel from left*), S_5 -SLF1 interacts with S_{17} -RNase to mediate its degradation. When S_{11} -RNase is taken up by the S_5 pollen tube (*second panel from right*), S_5 -SLF2 interacts with S_{11} -RNase to mediate its degradation. When S_9 -RNase is taken up by the S_5 pollen tube (*rightmost panel*), both S_5 -SLF1 and S_5 -SLF2 interact with S_9 -RNase to mediate its degradation. Degradation of S_{17} -RNase, S_{11} -RNase, and S_9 -RNase inside S_5 pollen tubes mediated by one or more types of SLF proteins explains compatible pollinations between S_5 pollen and S_{17} -, S_{11} -, and S_9 -carrying pistils

The collaborative non-self recognition model can explain the puzzles about the properties of *SLF1* mentioned in Sect. 24.4.1, for example, the low allelic sequence diversity of *SLF1*. As multiple types of SLF proteins act collaboratively to interact with all non-self S-RNases, each SLF only has to interact with a small number of non-self S-RNases, and thus it is unnecessary to have a high degree of allelic sequence diversity. In extreme cases, some allelic variants of an SLF can have identical amino-acid sequences, as is the case for S_7 -SLF1 and S_{19} -SLF1 of *P. axillaris* (Kubo et al. 2010). In this case, S_7 and S_{19} pollen most likely use their *SLF1* proteins (with identical sequences) to interact with the same subset of their common non-self S-RNases (i.e., not including S_7 -RNase or S_{19} -RNase). S_7 and S_{19} pollen could then use some other SLF protein(s) with allelic sequence diversity, or use different types of SLF proteins, to recognize and detoxify S_{19} -RNase and S_7 -RNase, respectively. Indeed, the degree of amino-acid sequence diversity between different types of SLF proteins is comparable to that of S-RNase, allowing different types of SLF proteins to interact with different S-RNases.

24.7 Conclusions

Molecular studies of S-RNase-based SI over the preceding more than a quarter century have revealed a number of unexpected findings, suggesting that this inbreeding-prevention genetic system is more complex than initially thought. The most unexpected findings are (1) pollen specificity comprises multiple *SLF* genes, and (2) rejection of self-pollen is the result of non-self recognition between SLFs and S-RNases, and not by specific self-interaction between an SLF and its cognate S-RNase. Thus, the mechanism of S-RNase-based SI is fundamentally different from the mechanisms of Brassicaceae and Papaveraceae SI, as each employs a single male *S*-gene and a single female *S*-gene to control SI, and both involve the interaction between products of matching alleles of the pollen and pistil *S*-genes (Iwano and Takayama 2012).

The finding that pollen specificity is controlled by multiple SLF proteins has also raised a number of interesting and challenging questions about S-RNase-based SI. We list some of the questions here. For a given *S*-haplotype, how many *SLF* genes constitute pollen specificity? Do all *S*-haplotypes employ the same number of *SLF* genes? What is the mechanism for the generation of *SLF* genes? What is the biochemical basis for the differential interactions between SLF proteins and S-RNases? Why does this SI system employ a single gene with a very high degree of allelic sequence polymorphism for pistil specificity, and multiple genes, each with a much lower degree of allelic sequence polymorphism for pollen specificity, but not vice versa? How have a single *S-RNase* gene and multiple polymorphic *SLF* genes coevolved to maintain SI?

Efforts are under way to address some of these questions. For example, our laboratory is using RNA-seq to characterize the complete pollen transcriptome of *S*₂ and *S*₃ haplotypes of *P. inflata* to identify all the F-box genes that are linked to the *S*-locus. The genes identified could then be examined by the well-established transgenic functional assay (Sijacic et al. 2004; Hua et al. 2007; Kubo et al. 2010) to see whether they can cause breakdown of SI in pollen of any non-self *S*-haplotype. As more *SLF* genes are identified, comparison of deduced amino-acid sequences of different alleles of the same type of *SLF*, and deduced amino-acid sequences of different types of *SLF* genes will likely reveal amino-acid sequences/domains of SLFs that are involved in allele specificity and in type specificity. The approach of chimeric genes could then be used to identify these sequence features. S-RNase-based SI also provides a model for F-box protein-mediated protein degradation, a key regulatory mechanism in many cellular and developmental processes in eukaryotes. Identification of most, if not all, *SLF* genes of *S*₂- and *S*₃-haplotype involved in pollen specificity and understanding of how these SLF proteins interact with their specific S-RNases will lay the foundation for studying the structural basis of interactions between F-box proteins and their substrates.

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