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S-Locus F-Box Proteins Are Solely Responsible for S-RNase-Based Self-Incompatibility of *Petunia* Pollen

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Self-incompatibility (SI) in *Petunia* is regulated by a polymorphic S-locus. For each S-haplotype, the S-locus contains a pistil-specific *S-RNase* gene and multiple pollen-specific *S-locus F-box* (*SLF*) genes. Both gain-of-function and loss-of-function experiments have shown that *S-RNase* alone regulates pistil specificity in SI. Gain-of-function experiments on *SLF* genes suggest that the entire suite of encoded proteins constitute the pollen specificity determinant. However, clear-cut loss-of-function experiments must be performed to determine if SLF proteins are essential for SI of pollen. Here, we used CRISPR/Cas9 to generate two frame-shift indel alleles of S_2-*SLF1* (*SLF1* of S_2-haplotype) in S_2S_3 plants of *P. inflata* and examined the effect on the SI behavior of S_2 pollen. In the absence of a functional S_2-SLF1, S_2 pollen was either rejected by or remained compatible with pistils carrying one of eight normally compatible *S*-haplotypes. All results are consistent with interaction relationships between the 17 SLF proteins of S_2-haplotype and these eight S-RNases that had been determined by gain-of-function experiments performed previously or in this work. Our loss-of-function results provide definitive evidence that SLF proteins are solely responsible for SI of pollen, and they reveal their diverse and complex interaction relationships with S-RNases to maintain SI while ensuring cross-compatibility.

INTRODUCTION

Self-incompatibility (SI) is a reproductive strategy widely used by flowering plants producing bisexual flowers to circumvent the tendency to self-fertilize, thereby promoting outcrossing to generate genetic variability (de Nettancourt, 2001). For the Solanaceae, SI is regulated by a polymorphic locus named the *S*-locus. If the *S*-haplotype of pollen matches either *S*-haplotype of the pistil, growth of the pollen tube is inhibited. In *Petunia*, the *S*-locus of each haplotype houses a single pistil-specific *S-RNase* gene (Lee et al., 1994) and a suite of pollen-specific *S-locus F-box* (*SLF*) genes (Sijacic et al., 2004; Kubo et al., 2010, 2015; Williams et al., 2014a, 2014b). The polymorphic *S-RNase* gene is solely responsible for pistil specificity in SI, as has been demonstrated by gain-of-function and loss-of-function experiments (Lee et al., 1994; Murfett et al., 1994). For example, in *P. inflata*, a wild parent of garden petunia (*P. hybrida*; Bombarely et al., 2016), expression of S_3-*RNase* (*S-RNase* of S_3-haplotype) in the pistils of S_1S_2 transgenic plants resulted in the pistil's gaining the ability to reject S_3 pollen. Conversely, expression of an antisense S_3-*RNase* gene in the pistils of S_2S_3 transgenic plants abolished their ability to reject S_3 pollen but did not affect their ability to reject S_2 pollen (Lee et al., 1994). Based on pollen transcriptome analysis, both S_2-haplotype and S_3-haplotype of *P. inflata* possess the same set of 17 *SLF* genes, named *SLF1* to *SLF17* (Williams et al., 2014a). These 17 *SLF* genes, plus one other type, have been found in eight additional *S*-haplotypes of *P. hybrida*, with the number of *SLF* genes in each of these *S*-haplotypes ranging from 16 to 18 (Kubo et al., 2015).

S-RNases may act as a cytotoxin to degrade pollen tube RNAs, as their ribonuclease activity is essential for their function in SI (Huang et al., 1994). During initial pollen tube growth in the pistil, S-RNases are taken up by the pollen tube (Luu et al., 2000; Goldraij et al., 2006); however, only self S-RNase (having an *S*-haplotype matching that of pollen) can inhibit further tube growth to the ovary. A model, named collaborative non-self recognition, was proposed to explain why self S-RNase, but not any non-self S-RNase, inhibits pollen tube growth (Kubo et al., 2010). The model predicts that, for a given *S*-haplotype, each SLF functions as the F-box protein subunit of an SCF (Skp1–Cullin1–F-box) type E3 ubiquitin ligase to mediate ubiquitination and degradation of the non-self S-RNase(s) with which the SLF interacts. Indeed, all 17 SLF proteins of S_2-haplotype and S_3-haplotype of *P. inflata* have been shown to assemble into similar SCF complexes (Li et al., 2014, 2016), with both the Skp1-like and Cullin1 components being pollen specific (named PiSSK1 and PiCUL1-P, respectively). Moreover, S-RNases expressed in *Escherichia coli* or isolated from pistils have been shown to be ubiquitinated and degraded in pollen extracts in a 26S proteasome-dependent manner (Hua and Kao, 2006; Entani et al., 2014).

The collaborative non-self recognition model further predicts that a complete suite of SLF proteins is required to detoxify all

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IN A NUTSHELL

Background: Plant inbreeding results in reduced fitness of the progeny. Since plants cannot move around to select mates, many flowering plants have adopted a reproductive strategy, self-incompatibility, that allows pistils to prevent inbreeding by rejecting genetically identical self pollen and only accepting non-self pollen for fertilization. The *Petunia* pistil uses S-RNase as a toxin to reject self pollen. Non-self pollen is thought to escape S-RNase toxicity using a suite of its own proteins, S-locus F-box (SLF) proteins, to meditate non-self S-RNase degradation. For example, pollen having the S_2-haplotype contains 17 types of SLF protein. Each type can mediate the degradation of some non-self S-RNases from pistils with *S*-haplotypes other than S_2. Collectively, the entire suite of SLF proteins can detoxify all non-self S-RNases.

Question: We wished to determine whether pollen indeed requires SLF proteins to detoxify non-self S-RNases when a pollen tube grows in a genetically compatible pistil. If an SLF is essential for detoxifying a certain S-RNase, pollen lacking this SLF should be rejected by the pistil producing this S-RNase.

Findings: We used CRISPR/Cas9 genome-editing to generate insertion/deletion mutations in the S_2-*SLF1* gene of S_2-haplotype of *Petunia inflata* to create mutant plants whose pollen did not produce a functional S_2-SLF1. Mutant S_2 pollen was rejected by pistils producing S_3-RNase or S_{13}-RNase but was still accepted by pistils producing S_7-RNase or S_{12}-RNase. We separately expressed each of the 17 SLF proteins of S_2-haplotype in pollen of various *S*-haplotypes and showed that only S_2-SLF1 could detoxify S_3-RNase, whereas in addition to S_2-SLF1, S_2-SLF2 and S_2-SLF5 could also detoxify S_7-RNase and S_{12}-RNase, respectively. Therefore, S_2-SLF1 is essential in the defense of S_2 pollen against S_3-RNase, and S_2 pollen uses multiple SLF proteins to defend against some other S-RNases as a fail-safe strategy.

Next steps: The next challenge is to understand why among the 17 SLF proteins produced by S_2 pollen, only one, or a few, can detoxify a particular S-RNase. At the biochemical level, which amino acids are responsible for enabling an SLF to interact with certain S-RNase(s), and at the structural level, how does it interact with them?

non-self S-RNases to allow cross-compatible pollination, and that none of the SLF proteins can interact with their self S-RNase, allowing it to degrade pollen tube RNAs to result in self-incompatible pollination. The role of *SLF* genes in *Petunia* has been examined using an in vivo gain-of-function approach (Sijacic et al., 2004; Hua et al., 2007; Kubo et al., 2010, 2015; Sun and Kao, 2013; Williams et al., 2014b). For example, a pollen-specific promoter of tomato (*Solanum lycopersicum*), *LAT52* (Twell et al., 1990), was used to express green fluorescent protein (GFP)-fused S_2-SLF1 in pollen of S_2S_3 transgenic plants, with the result that S_3 transgenic pollen expressing S_2-SLF1 was able to successfully pollinate S_3-carrying pistils, whereas S_2 transgenic pollen expressing S_2-SLF1 remained incompatible with S_2-carrying pistils (Hua et al., 2007). Thus, expression of S_2-SLF1 in S_3 pollen allows the transgenic pollen tube to gain the ability to detoxify S_3-RNase, suggesting that S_2-SLF1 interacts with S_3-RNase to mediate its ubiquitination and degradation. We previously used this assay to determine a total of 40 pairwise interaction relationships (indicated by brackets in Table 1) between six SLF proteins of S_2-haplotype (S_2-SLF1, -SLF3, -SLF4, -SLF5, -SLF6, -SLF8) and eight S-RNases (S_2-, S_3-, S_5-, S_{6a}-, S_7-, S_{11}-, S_{12}-, S_{13}-RNase).

To definitely establish the role of *SLF* genes in SI, it is imperative that their function also be examined by loss-of-function experiments. For example, if SLF1 of *P. inflata* is essential for SI of pollen, and if S_2-SLF1 is the only SLF of the 17 produced by S_2 pollen that can detoxify S_3-RNase, then in the absence of a functional S_2-SLF1, we would expect S_2 pollen to be rejected by normally compatible S_3-carrying pistils. We previously used the approach of artificial microRNA (amiRNA) to knock down the expression of S_2-*SLF1* in S_2 pollen and found that the S_2 transgenic pollen remained compatible with S_3-carrying pistils (Sun and Kao, 2013). These results could be interpreted to mean that SLF1 is not required for SI of pollen. However, we cannot rule out the possibility that a small amount of residual S_2-SLF1 produced in S_2 transgenic pollen, due to incomplete suppression of the transcript of S_2-*SLF1*, might be responsible for the normal SI phenotype. The results could also be interpreted to mean that at least one of the 11 SLF proteins whose interaction relationship with S_3-RNase had not been determined by the gain-of-function assay at that time might also interact with S_3-RNase. Thus, to date, definitive evidence for the role of SLF proteins in SI remains lacking.

We had succeeded inusing the polycistronic tRNA-gRNA (PTG)-based CRISPR/Cas9 genome-editing system (Xie et al., 2015) to knock out *PiSSK1* (Sun and Kao, 2018). This attainment guided our decision to use this more definitive loss-of-function approach to examine the role of the *SLF* genes of *P. inflata* in SI. In the present study, we chose S_2-*SLF1* as the target of CRISPR/Cas9, as we previously found that, among the 40 interaction relationships established between six SLF proteins and eight S-RNases, only S_2-SLF1 interacted with multiple S-RNases: S_3-, S_7-, and S_{13}-RNase (Sun and Kao, 2013). Thus, knocking out S_2-*SLF1* would allow us to study the effect on the compatibility of S_2 pollen with pistils of different *S*-haplotypes. We observed that $S_2{}^*$ pollen (denoting S_2 pollen with an indel allele of S_2-*SLF1*) carrying one of the two frame-shift indel alleles identified was rejected by S_3S_3 and $S_{13}S_{13}$ pistils, but remained compatible with S_5S_5, $S_{6a}S_{6a}$, S_7S_7, $S_{11}S_{11}$, $S_{12}S_{12}$, and $S_{16}S_{16}$ pistils. We then used gain-of-function experiments to identify 68 additional interaction relationships between the 17 SLF proteins of S_2-haplotype and nine S-RNases (the eight previously studied plus S_{16}-RNase). Based on a total of 108 interaction relationships, we showed that the SI behavior of $S_2{}^*$ pollen lacking a functional S_2-SLF1 with pistils carrying different *S*-haplotypes is entirely consistent with whether

Table 1. Summary of Genetic Interaction Relationships among 17 SLF Proteins Produced by S_2 Pollen and 9 S-RNases of *Petunia inflata*

	S_2- RNase	S_3- RNase	S_5- RNase	S_{6a}- RNase	S_7- RNase	S_{11}- RNase	S_{12}- RNase	S_{13}- RNase	S_{16}- RNase
S_2-SLF1	[—]	[+]	[—]	—	[+]	[—]	+	[+]	—
S_2-SLF2	—	—			+				—
S_2-SLF3	[—]	[—]	—	—	—	—	—	—	—
S_2-SLF4	[—]	[—]	[+]	[—]	[—]	[—]	[—]	[—]	—
S_2-SLF5	[—]	[—]	[—]	[—]	[—]	[—]	[+]	[—]	—
S_2-SLF6	[—]	[—]	[—]	[—]	[—]	[—]	[—]	[—]	—
S_2-SLF7	—	—		—	—				
S_2-SLF8	[—]	[—]	[—]	[+]	[—]	[—]	[—]	[—]	—
S_2-SLF9	—	—	—	—	—	—	—	—	—
S_2-SLF10	—	—	—	—	—	—	—	—	—
S_2-SLF11	—	—			—				
S_2-SLF12	—	—			—				
S_2-SLF13	—	—			—				—
S_2-SLF14	—	—			—				
S_2-SLF15	—	—	—	—	—	—	—	—	—
S_2-SLF16	—	—			—				
S_2-SLF17	—	—			—				

+ indicates positive interaction, determined in this study; — indicates no interaction, determined in this study; [+] indicates positive interaction, determined in previous studies; [—] indicates no interaction, determined in previous studies; blank indicates relationship not yet determined.

S_2 pollen employs S_2-SLF1 as the only SLF in detoxifying a particular S-RNase, whether S_2 pollen employs S_2-SLF1 and at least one other SLF protein in detoxifying a particular S-RNase, and whether S_2 pollen employs SLF protein(s) other than S_2-SLF1 in detoxifying a particular S-RNase. Thus, the results of CRISPR/Cas9-mediated knockout of *S_2-SLF1*, coupled with analysis of the SLF-S-RNase interaction relationships, provide definitive evidence that SLF proteins are solely responsible for SI of pollen and reveal the complexity and diversity of the interactions between SLF proteins and S-RNases.

RESULTS

Four Different Indel Alleles of *S_2-SLF1* Generated by CRISPR/Cas9-Mediated Genome Editing

To edit the coding sequence of *S_2-SLF1* in S_2S_3 plants without affecting *S_3-SLF1* (with which it shares 94% nucleotide sequence identity), we designed a guide RNA (gRNA) to target a 20-bp protospacer sequence (624 to 643 bp, counting from the start codon) of the antisense strand of *S_2-SLF1*; this protospacer (named *S_2-SLF1-PS9*) is followed by the protospacer adjacent motif (PAM), TGG (Figure 1A). The corresponding 20-bp sequence in *S_3-SLF1* contains two nucleotide differences (Figure 1A) and was not expected to be the target of the gRNA. No other potential off-target sites were found in any of the other 16 *SLF* genes of S_2-haplotype and S_3-haplotype, as their sequences differed from the protospacer sequence by more than two nucleotides (Supplemental Figure 1). The PTG fragment-containing Ti-plasmid construct (Supplemental Figure 2A) was used in Agrobacterium-mediated transformation of S_2S_3 plants.

PCR analysis of 36 plants regenerated from transformation showed that 10 carried the *35S:Cas9* transgene (Supplemental Figure 2B; sequences of all primers used in this work are listed in Supplemental Table 1). The wild-type sequence of *S_2-SLF1-PS9* contains a cleavage site of restriction enzyme *BsrG*I close to the PAM. The PCR products amplified with a pair of primers specific to *S_2-SLF1* from T_0 plants #2, #13, # 27, and #35 were resistant to digestion, suggesting a loss of this *BsrG*I cleavage site caused by genome editing (Figure 1B). Sequencing of the PCR products of these four T_0 plants revealed the exact sequence of each indel allele of *S_2-SLF1* in the targeted region (Figure 1C). Genome editing in #2 and #35 resulted in a 1-bp deletion (denoted *-1G*) and a 1-bp insertion (denoted *+1T*), respectively, causing frame-shift after the codon for Val-209 (Supplemental Figure 3). Editing in #13 and #27 resulted in 3-bp and 6-bp in-frame deletions, respectively, yielding mutated forms of S_2-SLF1 with Gln-210 (Q_{210}) deleted, and with both Q_{210} and Leu-211 (L_{211}) deleted, respectively (Supplemental Figure 3). To confirm that no off-target editing occurred in *S_3-SLF1*, we sequenced the PCR products (217 bp) amplified from leaf genomic DNA of these four T_0 plants and a wild-type S_2S_3 plant using a pair of primers specific to *S_3-SLF1* (Figure 1A). The sequences of the four T_0 plants were completely identical to the wild-type sequence (Figure 1D).

Analysis of Self-Incompatibility Behavior of S_2 Pollen Carrying One of the Two Frame-Shift Indel Alleles of *S_2-SLF1*

To examine the effect of the two frame-shift indel mutations in *S_2-SLF1* on the SI behavior of S_2* pollen, we first used pollen from T_0 plants #2/S_2*S_3 and #35/S_2*S_3 to separately pollinate the wild-type S_3S_3 plants. No fruits were set from these pollinations, and aniline blue staining of pollen tubes inside S_3S_3 pistils showed few pollen tubes in the bottom segment of the style (Figure 2A). These results suggest that S_2* pollen lacking a functional S_2-SLF1 cannot detoxify S_3-RNase; and that, if SLF proteins are required for SI of pollen, no other SLF proteins produced by S_2 pollen can interact with S_3-RNase.

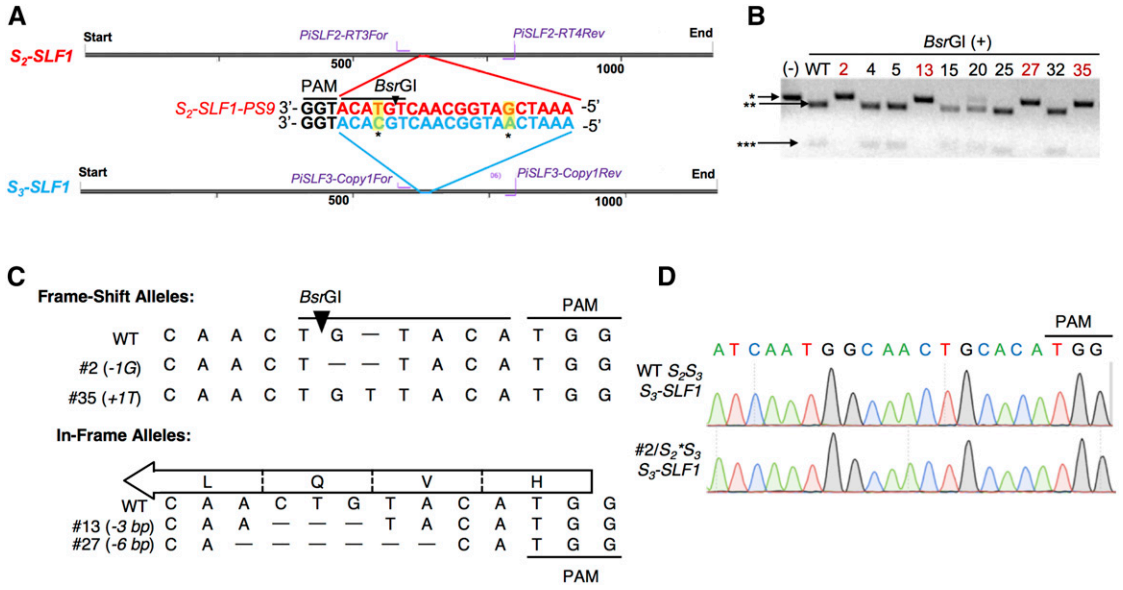


Figure 1. Generation of S_2-*SLF1* Indel Alleles by CRISPR/Cas9-Mediated Genome Editing.

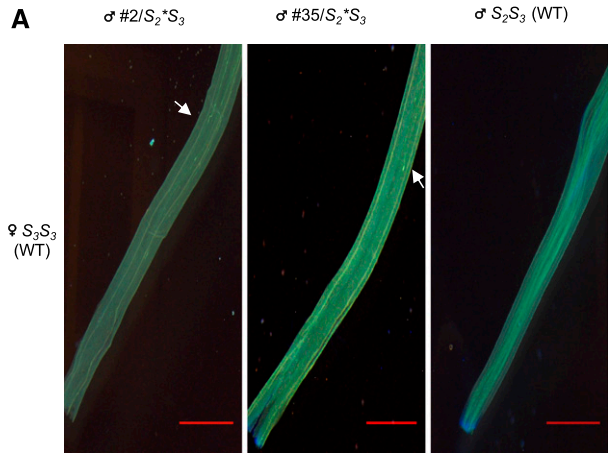
(A) Design of a gRNA specifically targeting S_2-*SLF1*. A 20-bp sequence (named S_2-*SLF1-PS9*) of the antisense strand of S_2-*SLF1* followed by the PAM motif (TGG) was chosen as the protospacer for CRISPR/Cas9; two mismatches (highlighted in yellow and indicated with asterisks) are found in the corresponding 20-bp region in S_3-*SLF1* (highlighted in blue). "Start" indicates the start codon (ATG) on the sense strand (5′ to 3′) of these two genes, and "End" indicates the stop codon (TAG). The positions of the PCR primers specific to S_2-SLF1 (*PiSLF2-RT-3For/PiSLF2-RT-4Rev*) and those specific to S_3-*SLF1* (*PiSLF3-Copy1For/PiSLF3-Copy1Rev*) are indicated by purple lines. Black triangle indicates the cleavage site of *Bsr*GI in the wild-type S_2-*SLF1* sequence.
(B) PCR-restriction enzyme digestion screen for edited S_2-*SLF1* alleles in 10 transgenic plants. (-)**:** PCR product amplified from genomic DNA of one of the transgenic plants by the S_2-*SLF1* specific primers, without digestion by *Bsr*GI. *Bsr*GI (+)**:** *Bsr*GI digestion of the PCR products amplified from genomic DNA of a wild-type S_2S_3 plant and the 10 transgenic plants. Asterisk (*) indicates the ~220-bp PCR product resistant to, or not subjected to, *Bsr*GI digestion; double asterisks (**) indicate the ~180-bp *Bsr*GI fragment; triple asterisks (***) indicates the ~40-bp *Bsr*GI fragment. The plant numbers of those T_0 plants carrying mutant S_2-*SLF1* alleles resistant to *Bsr*GI digestion are highlighted in red.
(C) Sequences of four indel alleles in the edited region of S_2-*SLF1*. The sequences shown are those of the antisense strand of S_2-*SLF1* (5′ to 3′ from left to right). The black triangle indicates the cleavage site of *Bsr*GI in the wild-type S_2-*SLF1* sequence. The open arrow indicates the direction of translation, and the encoded amino acids in the wild-type S_2-SLF1 are shown. The 3-bp in-frame deletion in plant #13 abolishes the codon 5′-CAG-3′ for Gln-210. The 6-bp in-frame deletion in plant #27 abolishes the codon for Gln-210 and disrupts the codon 5′-GTA-3′ for Val-209 and the codon 5′-TTG-3′ for Leu-211. However, as the Val codon is restored as 5′-GTG-3′, only Gln-210 and Leu-211 are deleted from the encoded protein.
(D) Sequencing chromatograms of PCR amplicons of S_3-*SLF1* from T_0 plant #2/S_2*S_3 and from a wild-type S_2S_3 plant. The sequences are those of the antisense strand from 5′ to 3′ (left to right).

We next used pollen from #2/S_2*S_3 and #35/S_2*S_3 to separately pollinate the wild-type S_7S_7 and $S_{13}S_{13}$ plants. S_3 pollen produced by these two transgenic plants should be compatible with both S_7S_7 and $S_{13}S_{13}$ pistils to yield S_3S_7 and S_3S_{13} progeny plants, respectively. As expected, all these pollinations set fruits, and we used PCR to determine the *S*-genotypes of at least 18 randomly selected plants in each progeny. If S_2* pollen remained compatible with S_7S_7 and $S_{13}S_{13}$ pistils, we would expect to obtain S_2*S_7 and S_2*S_{13} progeny plants, respectively. We identified both S_2*S_7 and S_3S_7 genotypes in the progeny from crosses with S_7S_7 pistils (Figure 2B, Supplemental Figure 4A), but we found only S_3S_{13} genotype in the progeny from crosses with $S_{13}S_{13}$ pistils (Figure 2B, Supplemental Figure 4B). Thus, S_2* pollen produced by both #2/S_2*S_3 and #35/S_2*S_3 remained compatible with S_7-carrying pistils, but it was incompatible with normally compatible S_{13}-carrying pistils.

To further examine the effects of the two frame-shift indel mutations of S_2-*SLF1* on the SI behavior of S_2* pollen, we performed bud-selfing (BS, self-pollination of immature flower buds) on #2/S_2*S_3 and #35/S_2*S_3 to obtain S_2*S_2*, plants each carrying one of the indel alleles. BS circumvents SI, because immature buds produce very low levels of S-RNases that are insufficient to inhibit growth of self-pollen tubes (Lee et al., 1994; Sun and Kao, 2013). Two S_2*S_2* plants in each progeny (#2-BS-#2 and -#8 in the progeny of #2/S_2*S_3, and #35-BS-#8 and -#10 in the progeny of #35/S_2*S_3) were identified (Supplemental Figures 5A to 5C). Using a primer pair specific to the *Cas9* transgene, we found that only #2-BS-#8 inherited the *Cas9* transgene (Supplemental Figure 5D). We used all four BS plants for subsequent analysis to assess the SI behavior of S_2* pollen in the absence of S_3 pollen. By using the three transgene-free BS plants, we could eliminate any possible complications that might be caused by the presence of the *Cas9*-containing transgene.

We used pollen from those four BS plants to pollinate the wild-type plants of 10 different *S*-genotypes: S_3S_3, S_5S_5, $S_{6a}S_{6a}$, S_7S_7, $S_{11}S_{11}$, $S_{12}S_{12}$, $S_{13}S_{13}$, $S_{16}S_{16}$, S_7S_{13}, and S_7S_{16}. For each *S*-genotype, the same results were obtained for all four BS plants (described below and summarized in Figure 3A). We used aniline

A



B

Pollen Source (T_0)	Progeny *S*-genotypes Observed	
	S_7S_7 ♀	$S_{13}S_{13}$ ♀
♂ #2/S_2*S_3	S_2S_7:S_3S_7 = 14:10 (n=24)	S_3S_{13} (n=20)
♂ #35/S_2*S_3	S_2S_7:S_3S_7 = 7: 17 (n=24)	S_3S_{13} (n=18)

Figure 2. Analysis of SI Behavior of T_0 Plants Carrying Either a 1-bp Deletion or a 1-bp Insertion Frame-Shift Allele of *S_2-SLF1*.

(A) Aniline blue staining of pollen tubes in the bottom segment of the style after a wild-type S_3S_3 plant was separately pollinated by pollen from T_0 plants, #2/S_2*S_3 and #35/S_2*S_3, and a wild-type S_2S_3 plant. White arrows indicate where growth of most pollen tubes stopped, in the case of incompatible pollinations. Scale bar = 1 mm.

(B) Progeny analysis of crosses using pollen from #2/S_2*S_3 or #35/S_2*S_3 to separately pollinate the wild-type S_7S_7 and $S_{13}S_{13}$ pistils. n indicates the number of plants in each progeny analyzed.

blue to stain and visualize pollen tubes in the pollinated pistils of all crosses. Representative results are shown in Figures 3B to 3G.

Pollinations with S_3S_3 pistils were incompatible (Figure 3B), consistent with the results obtained for T_0 plants #2/S_2*S_3 and #35/S_2*S_3 (Figure 2A). To determine whether the incompatibility of S_2* pollen with normally compatible S_3S_3 pistils was due to the inability of S_2* pollen to detoxify S_3-RNase, we also used pollen of these four BS plants to pollinate a self-compatible transgenic plant, *As-S_3/S_3S_3*, whose production of S_3-RNase in the pistil is completely suppressed by an antisense *S_3-RNase* transgene (Lee et al., 1994; Sun and Kao, 2013). Pollinations with pistils of *As-S_3/S_3S_3* were fully compatible (Figure 3C), setting normal size fruits. Therefore, S_2* pollen, which lacks a functional S_2-SLF1, was incompatible with the wild-type S_3S_3 pistils, but it was compatible with *As-S_3/S_3S_3* pistils. These results suggest the necessary presence of S_2-SLF1 for S_2 pollen to detoxify S_3-RNase.

Pollinations with S_7S_7 pistils were compatible (Figure 3D), but pollinations with $S_{13}S_{13}$ pistils were incompatible (Figure 3E), consistent with progeny analysis of the crosses using pollen from T_0 plants #2/S_2*S_3 and #35/S_2*S_3 to pollinate the wild-type S_7S_7 and $S_{13}S_{13}$ plants (Figure 2B, Supplemental Figure 4). These results together suggest that for S_2 pollen, S_2-SLF1 is not the only SLF that interacts with S_7-RNase, but that S_2-SLF1 is the only SLF that interacts with S_{13}-RNase.

All pollinations with S_5S_5, $S_{6a}S_{6a}$, $S_{11}S_{11}$, and $S_{12}S_{12}$ pistils were compatible (Figures 3A, 3F, and 3G), consistent with our previous findings that (a) S_2 pollen did not use S_2-SLF1 to interact with S_5-RNase or S_{11}-RNase; (b) S_2 pollen used S_2-SLF4 to interact with S_5-RNase, S_2-SLF8 to interact with S_{6a}-RNase, and S_2-SLF5 to interact with S_{12}-RNase (Table 1; Sun and Kao, 2013; Williams et al., 2014b). Pollinations with $S_{16}S_{16}$ pistils were also compatible (Figure 3A), suggesting that some other SLF protein(s) produced by S_2 pollen is (are) responsible for detoxifying S_{16}-RNase. All these results also suggest that the gRNA used for editing *S_2-SLF1* does not affect the other *SLF* genes, or genes involved in pollen development or fertilization.

Consistent with the results described above, pollinations with S_7S_{13} pistils were incompatible, because S_2* pollen without a functional S_2-SLF1 cannot detoxify S_{13}-RNase. Pollinations with S_7S_{16} pistils were compatible, suggesting that S_2* pollen can still use some other SLF protein(s) to detoxify S_7-RNase and S_{16}-RNase.

Interaction Relationships between 17 SLF Proteins of S_2-Haplotype and Various S-RNases

To further examine the results obtained from loss-of-function of S_2-SLF1, we used the in vivo gain-of-function assay to determine 68 additional interaction relationships (denoted + or — in Table 1) between the 17 SLF proteins of S_2-haplotype and nine S-RNases. These, together with the 40 previously determined (denoted [+] or [—] in Table 1), bring the total of interaction relationships to 108. To perform this assay, we first made transgene constructs for the 11 *SLF* genes that had not been previously studied, each fused with the coding sequence for GFP (Figures 4A and 4B). We used each construct to generate S_2S_3 transgenic plants. All the T_0 lines of S_2S_3 used in the gain-of-function assay are listed in Supplemental Table 2, and the workflow of this assay is outlined in Figure 4C. Pollen from at least three transgenic plants found to express a particular GFP-tagged SLF at high levels, based on intensity of GFP fluorescence in the pollen tubes (Supplemental Figure 6), was used to pollinate the wild-type S_2S_3 plants to examine the interaction relationship between this SLF and S_3-RNase. None of the pollinations involving pollen expressing one of the 11 SLF proteins set fruits (Supplemental Table 2). These results (illustrated in Figure 4D) suggest that none of these 11 SLF proteins interact with S_3-RNase (denoted — in the "S_3-RNase" column of Table 1). Of the six SLF proteins we previously examined, five did not interact with S_3-RNase (denoted [—] in the "S_3-RNase" column of Table 1; Hua et al., 2007; Williams et al., 2014b). Thus, among the 17 SLF proteins produced in S_2 pollen, only S_2-SLF1 interacts with S_3-RNase, consistent with the finding that S_2* pollen carrying one of the frame-shift indel alleles of *S_2-SLF1* was rejected by S_3-carrying pistils. These results also confirmed that none of the 17 SLF proteins of S_2-haplotype interact with their self S_2-RNase (denoted — or [—] in the "S_2-RNase" column of Table 1). These resultswere consistent with the predictionby the collaborative, non-self recognition model, that none of the SLF proteins of a given *S*-haplotype interact with their self S-RNase (Kubo et al., 2010).

We then examined the other 46 of the 68 additional interaction relationships, including those between the 17 SLF proteins of

A

♀ \ ♂	$S_2^*S_2^*$ (-1G/-1G)		$S_2^*S_2^*$ (+1T/+1T)	
	#2-BS-#2 (Cas9+)	#2-BS-#8 (Cas9-)	#35-BS-#8 (Cas9-)	#35-BS-#10 (Cas9-)
S_3S_3	—	—	—	—
$As\text{-}S_3/S_3S_3$	+	+	+	+
S_7S_7	+	+	+	+
$S_{13}S_{13}$	—	—	—	—
$S_{12}S_{12}$	+	+	+	+
S_5S_5	+	+	+	+
$S_{6a}S_{6a}$	+	+	+	+
$S_{11}S_{11}$	+	+	+	+
$S_{16}S_{16}$	+	+	+	+
S_7S_{13}	—	—	—	—
S_7S_{16}	+	+	+	+

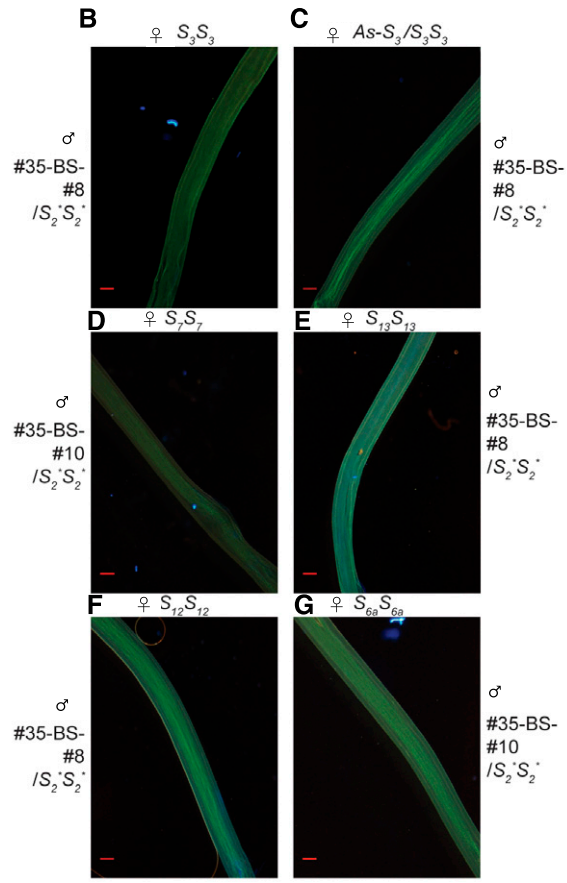


Figure 3. Analysis of SI Behavior of S_2S_2 Plants Homozygous for Either Frame-Shift Indel Allele of S_2-*SLF1*.

(A) Results of pollination using pollen from two bud-selfed (BS) progeny plants of #2/$S_2^*S_3$ (#2-BS-#2 and #2-BS-#8), and two BS progeny plant of #35/$S_2^*S_3$ (#35-BS-#8 and #35-BS-#10) to separately pollinate pistils of various *S*-genotypes. $S_2^*S_2^*$ indicates that all four BS progeny plants were S_2S_2 and homozygous for the indel allele of S_2-*SLF1* inherited from their respective T_0 plants. (Cas9+) and (Cas9-) indicate presence and absence of the *Cas9* transgene, respectively, in the BS plants. —: incompatible pollination (no fruit set); +: compatible pollination (fruit set). *As-*S_3/S_3S_3: a self-compatible transgenic plant not producing any S_3-RNase in the pistil due to expression of an antisense S_3-*RNase* gene.

(B) to **(G)** Aniline blue staining of pollen tubes in the bottom segment of the style of the pistil from each of the wild-type plants of five different *S*-genotypes, as indicated, and from a transgenic plant *As-*S_3/S_3S_3. These plants were separately pollinated with pollen from #35-BS-#8 or #35-BS-#10, as indicated. Scale bar = 0.25 mm.

S_2-haplotype and seven non-self S-RNases (S_5-, S_{6a}-, S_7-, S_{11}-, S_{12}-, S_{13}-, and S_{16}-RNase). We used pollen from the S_2S_3 transgenic plants expressing one of these 17 SLF proteins (denoted S_2-SLFn in Figures 4C and 4D) to pollinate plants of appropriate *S*-genotypes to obtain S_2S_x or S_3S_x transgenic plants (S_x being S_5, S_{6a}, S_7, S_{11}, S_{12}, S_{13}, or S_{16}). Subsequently we used pollen produced by these transgenic plants to pollinate the wild-type plants of the same *S*-genotype to test the interaction relationship between each SLF protein and S_x-RNase. The transgenic lines generated and their SI behavior are summarized in Supplemental Table 2.

Among the pollinations performed, the only ones that set fruits were (a) pollinations of the wild-type S_2S_7 plants by pollen of the S_2S_7 transgenic plants expressing S_2-SLF2:GFP (Figure 5A), and (b) pollinations of the wild-type S_2S_{12} plants by pollen of the S_2S_{12} transgenic plants expressing S_2-SLF1:GFP (Supplemental Table 2). We then randomly selected 24 progeny plants raised from compatible pollinations involving the wild-type S_2S_7 and wild-type S_2S_{12} plants and used PCR to analyze segregation of the *S*-haplotype and *SLF* transgene in each progeny. Among the progeny from pollinations of the wild-type S_2S_7 plants by pollen of the S_2S_7 transgenic plants expressing S_2-SLF2:GFP, all carried S_7-haplotype and the S_2-*SLF2:GFP* transgene (Figure 5B). The chi-square test supported the 1:1 ratio of S_2S_7:S_7S_7, and the 1:2:1 ratio of S_2S_2:S_2S_7:S_7S_7 was rejected with a P-value < 0.05 (Figure 5C). These results suggest that expression of S_2-SLF2:GFP causes breakdown of SI in S_7 transgenic pollen (Figure 5D). In the progeny from pollinations of the wild-type S_2S_{12} plants by pollen of the S_2S_{12} transgenic plants expressing S_2-SLF1:GFP, all carried S_{12}-haplotype and the S_2-*SLF1:GFP* transgene (Figure 6A). The chi-square test supported the 1:1 ratio of S_2S_{12}:$S_{12}S_{12}$, and the 1:2:1 ratio of S_2S_2:S_2S_{12}:$S_{12}S_{12}$ was rejected with a P-value <0.05 (Figure 6B). These results suggest that expression of S_2-SLF1:GFP causes breakdown of SI in S_{12} transgenic pollen.

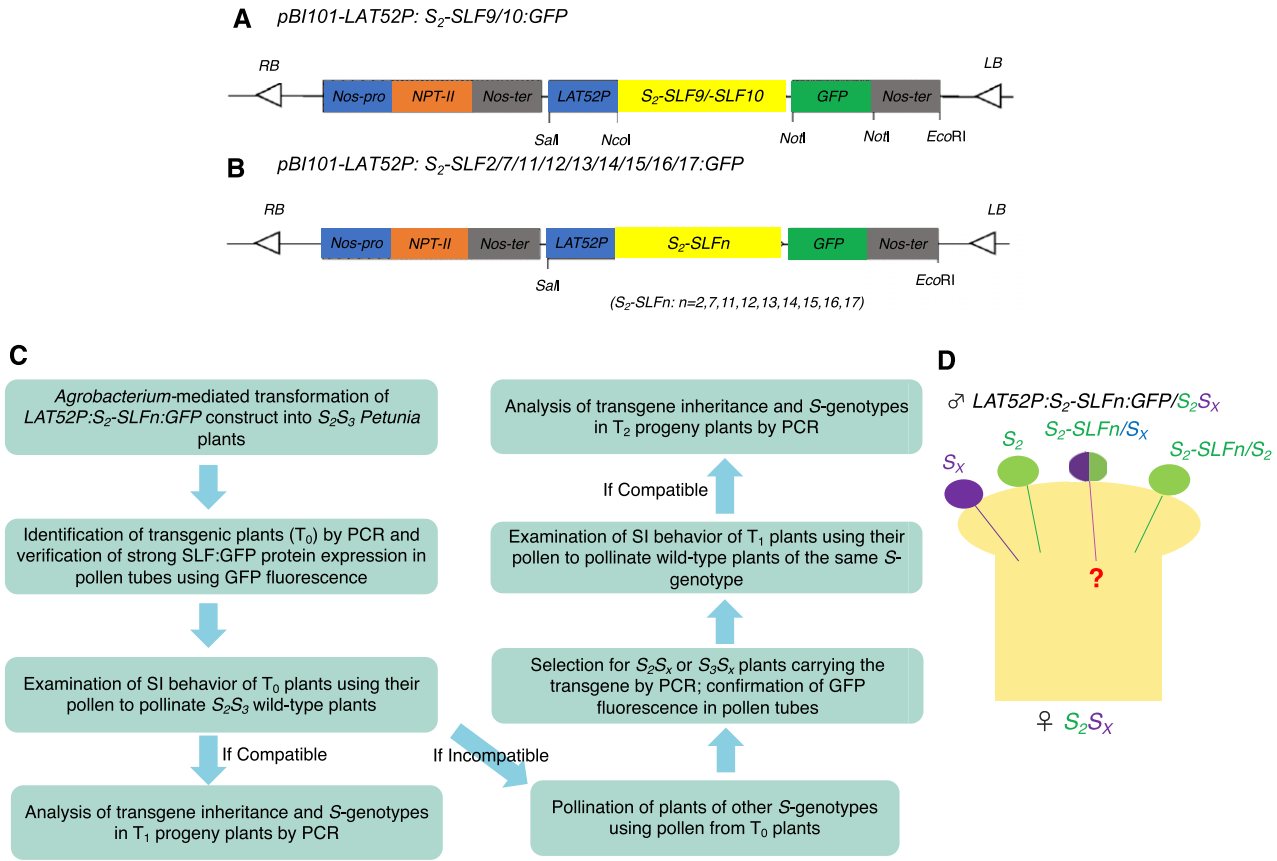
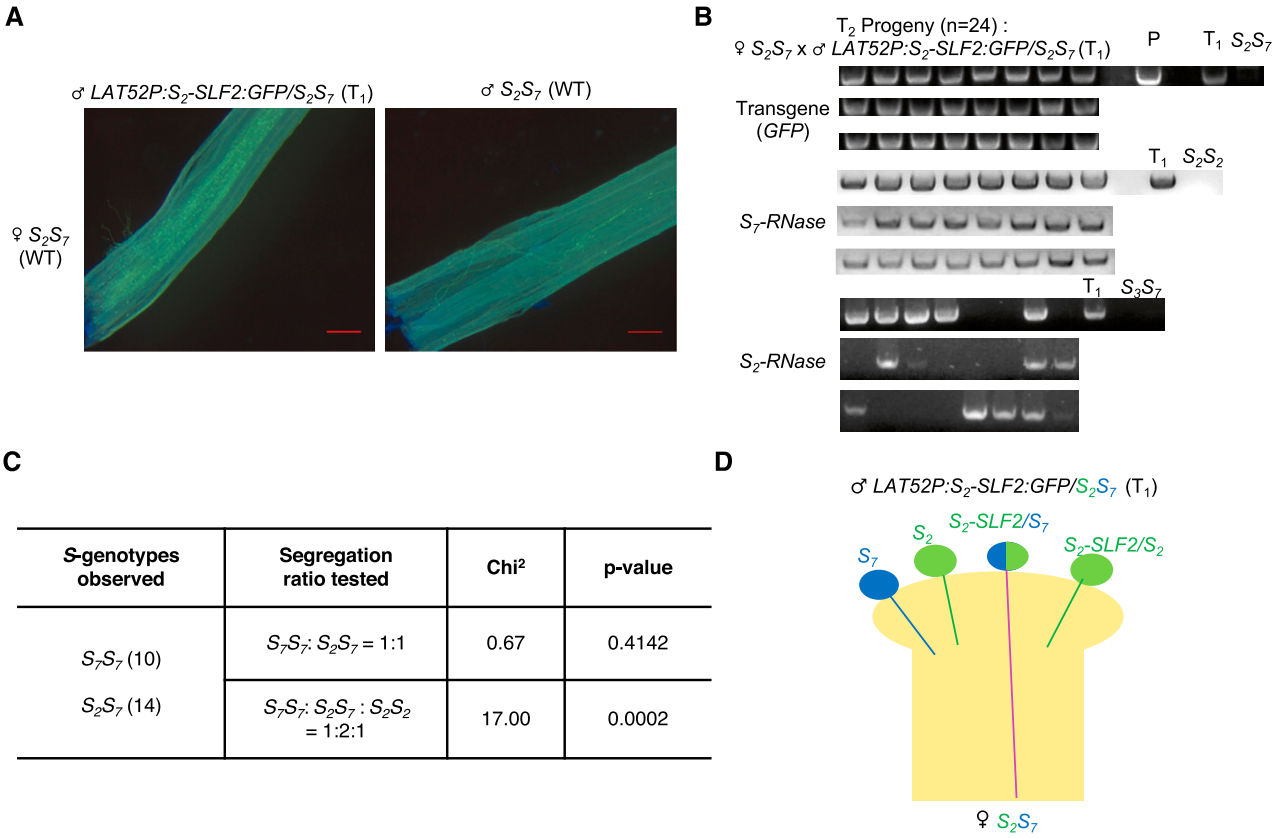


Figure 4. The in Vivo Gain-of-Function Assay Used for Determining Interaction Relationships between SLF Proteins and S-RNases.

(A) Schematic of the transgene constructs for S_2-*SLF9* and S_2-*SLF10*.
(B) Schematic of nine additional transgene constructs, each containing one of the *SLF* genes indicated (denoted S_2-*SLFn*). All constructs shown in **(A)** and **(B)** were made using Ti plasmid pBI101 as the backbone. *RB*, right border of the T-DNA; *Nos-pro*, promoter of the gene encoding nopaline synthase; *NPT-II*, gene encoding neomycin phosphotransferase II (conferring resistance to kanamycin); *Nos-ter*, transcription terminator of the gene encoding nopaline synthase; *LAT52P*: promoter of the pollen-specific *LAT52* gene from tomato; *GFP*, gene encoding green fluorescent protein; *LB*, left border of the T-DNA.
(C) Workflow of the in vivo gain-of-function assay.
(D) Graphic illustration of the genetic basis for determining interaction relationships between SLF proteins and S-RNases. The transgene construct for an *SLF* gene of S_2-haplotype, denoted S_2-*SLFn*, is introduced into S_2S_x plants, with S_x being an *S*-haplotype different from S_2. Pollen from the *LAT52P:*S_2*-SLFn:GFP/*S_2S_x transgenic plant is used to pollinate a wild-type S_2S_x plant. Among the four genotypes of pollen produced by the transgenic plant, S_2 and S_x should be rejected by the S_2S_x pistil, and S_2 carrying the transgene is expected be rejected, as the S_2-SLF_n transgene is from the same *S*-haplotype as pollen. Thus, whether or not this pollination is compatible is determined solely by the SI behavior of S_x pollen carrying the transgene. If S_2-SLFn interacts with S_x-RNase to mediate its ubiquitination and degradation in the *LAT52P:*S_2*-SLFn:GFP/*S_x pollen tube, then the pollination should be compatible, and all the progeny will inherit the transgene and carry S_x-haplotype. If S_2-SLFn does not interact with S_x-RNase, then the *LAT52P:*S_2*-SLFn:GFP/*S_x pollen tube should be rejected in the style and the pollination should be incompatible.

Thus, in addition to S_2-SLF1 and S_2-SLF5, which we previously found to interact with S_7-RNase and S_{12}-RNase, respectively, S_2-SLF2 also interacts with S_7-RNase, and S_2-SLF1 also interacts with S_{12}-RNase (Table 1). The functional redundancy employed by S_2 pollen in detoxifying S_7-RNase and S_{12}-RNase is consistent with the finding that S_2* pollen lacking a functional S_2-SLF1 remained compatible with S_7S_7 and $S_{12}S_{12}$ pistils.

As none of the other pollinations set fruits, these results, combined with the previously established 40 interaction relationships, led us to these four conclusions: (a) among the nine SLF proteins examined for their interaction relationships with S_5-RNase, S_2-SLF4 is the only one that interacts; (b) among the 10 SLF proteins examined for their interaction relationships with S_{6a}-RNase, S_2-SLF8 is the only one that interacts; (c) none of the nine SLF proteins examined for their interaction relationships with S_{11}-RNase interact with this S-RNase, and none of the 11 SLF proteins examined for their interaction relationships with S_{16}-RNase interact with this S-RNase; (d) S_2-SLF1 is the only one among the nine SLF proteins examined that interacts with S_{13}-RNase (Table 1; Sun and Kao, 2013; Williams et al., 2014b). The findings that S_2-SLF1 is not responsible for interacting with S_5-, S_{6a}-, S_{11}-, or S_{16}-RNase are consistent with the findings that S_2* pollen lacking a functional S_2-SLF1 remained compatible with S_5-, S_{6a}-, S_{11}-, and S_{16}-carrying pistils. For S_{11}-RNase and S_{16}-RNase, we would



S-genotypes observed	Segregation ratio tested	Chi²	p-value
S_7S_7 (10)	S_7S_7: S_2S_7 = 1:1	0.67	0.4142
S_2S_7 (14)	S_7S_7: S_2S_7 : S_2S_2 = 1:2:1	17.00	0.0002

Figure 5. Assessment of Interaction between S_2-SLF2 and S_7-RNase by in Vivo Gain-of-Function Assay.

(A) Aniline blue staining of pollen tubes in the bottom segment of the style after a wild-type S_2S_7 plant was self-pollinated (right) and pollinated with pollen from transgenic plant *LAT52P:S_2-SLF2:GFP/S_2S_7* (left). This transgenic plant (a T_1 plant) was obtained by pollinating a wild-type S_7S_7 plant with pollen from a T_0 transgenic plant *LAT52P:S_2-SLF2:GFP/S_2S_3*. Scale bar = 1 mm.

(B) Analysis of 24 T_2 plants resulting from the cross, S_2S_7 x *LAT52P:S_2-SLF2:GFP/S_2S_7*, shown in **(A)**. T_1 indicates genomic DNA from the T_1 plant *LAT52P:S_2-SLF2:GFP/S_2S_7*; P indicates plasmid DNA of *pBI101-LAT52P:S_2-SLF2:GFP* (as positive control for the PCR amplification of the *GFP* transgene). S_2S_7 indicates genomic DNA from a wild-type S_2S_7 plant (as negative control for the PCR amplification of the *GFP* transgene). S_2S_2 indicates genomic DNA from a wild-type S_2S_2 plant (as negative control for the PCR amplification of the *S_7-RNase* gene). S_3S_7 indicates genomic DNA from a wild-type S_3S_7 plant (as negative control for the PCR amplification of the *S_2-RNase* gene).

(C) Chi-square analysis of the *S*-haplotype inheritance in the 24 T_2 plants analyzed in **(B)**. Chi-square analysis was used to test the null hypothesis of *S*-haplotype inheritance, 1:1 ratio of S_2S_7:S_7S_7 versus 1:2:1 ratio of S_2S_2:S_2S_7:S_7S_7.

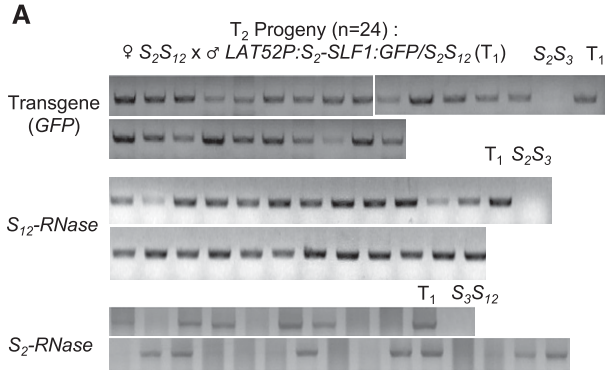
(D) Graphic illustration of interpretation of the results of progeny analysis from S_2S_7 x *LAT52P:S_2-SLF2:GFP/S_2S_7* shown in **(B)**. The observation that all progeny plants inherited the *GFP* transgene and none were S_2S_2 indicates that only the transgenic S_7 pollen carrying the *LAT52P:S_2-SLF2:GFP* transgene can effect fertilization. This result suggests that S_2-SLF2 produced in the transgenic S_7 pollen interacts with and detoxifies S_7-RNase to render the transgenic S_7 pollen compatible with the S_2S_7 pistil.

predict that at least one of the eight (for S_{11}-RNase) and at least one of the six (for S_{16}-RNase) SLF proteins yet to be examined interact with S_{11}- and S_{16}-RNase, respectively. Moreover, based on the finding that S_2* pollen was incompatible with S_{13}-carrying pistils, we would predict that none of the eight SLF proteins yet to be examined interact with S_{13}-RNase.

Analysis of Self-Incompatibility Behavior of S_2 Pollen Carrying One of the Two In-Frame Indel Alleles of *S_2-SLF1*

We also examined the in-frame indel alleles of *S_2-SLF1* identified in T_0 plants #13/S_2*S_3 and #27/S_2*S_3 (Figure 1C) for their effects on the SI behavior of S_2* pollen. We used pollen from both transgenic plants to pollinate the wild-type S_3S_3 plants. Pollinations by pollen of #13/S_2*S_3 were compatible (left panel of Figure 7A), whereas pollinations by pollen of #27/S_2*S_3 were incompatible (right panel of Figure 7A). Pollen produced by both transgenic plants was either S_3 or S_2*, and S_3 pollen should be rejected by S_3S_3 pistils. Thus, the finding of compatible pollinations between pollen of #13/S_2*S_3 and S_3-carrying pistils suggests that deletion of Q_{210} of S_2-SLF1 does not affect its ability to detoxify S_3-RNase, and the finding of incompatible pollinations between pollen of #27/S_2*S_3 and S_3-carrying pistils suggests that deletion of Q_{210} and L_{211} of S_2-SLF1 abolishes its ability to detoxify S_3-RNase.

We then used pollen of #13/S_2*S_3 and #27/S_2*S_3 to pollinate pistils of the wild-type S_3S_{13} and $S_{13}S_{13}$ plants, respectively, to



B

S-genotypes observed	Segregation ratio tested	Chi²	p-value
$S_{12}S_{12}$ (12)	$S_{12}S_{12}$: S_2S_{12} = 1:1	0	1
S_2S_{12} (12)	$S_{12}S_{12}$: S_2S_{12} : S_2S_2 = 1:2:1	12.00	0.0025

Figure 6. Assessment of Interaction between S_2-SLF1 and S_{12}-RNase by in Vivo Gain-of-Function Assay.

(A) Analysis of 24 T_2 plants resulting from the cross, S_2S_{12} x *LAT52P:*S_2*-SLF1:GFP/*S_2S_{12}. T_1 indicates genomic DNA from the T_1 plant *LAT52P:*S_2*-SLF1:GFP/*S_2S_{12}; S_2S_3 indicates genomic DNA from a wild-type S_2S_3 plant (as negative control for the PCR amplification of the *GFP* transgene and S_{12}*-RNase* gene); S_3S_{12} indicates genomic DNA from a wild-type S_3S_{12} plant (as negative control for the PCR amplification of the S_2*-RNase* gene).
(B) Chi-square analysis of the *S*-haplotype inheritance in the 24 T_2 plants analyzed in **(A)**. Chi-square was used to test the null hypothesis of *S*-haplotype inheritance, 1:1 ratio of S_2S_{12}:$S_{12}S_{12}$ versus 1:2:1 ratio of S_2S_2:S_2S_{12}:$S_{12}S_{12}$.

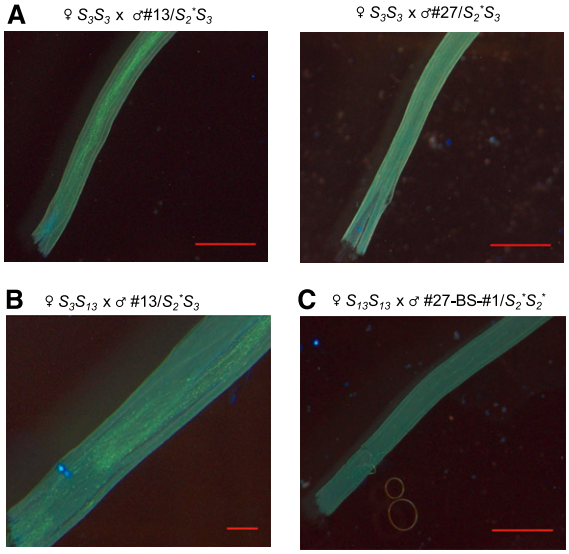
examine whether the two mutated forms of S_2-SLF1 could still detoxify S_{13}-RNase. Pollinations of S_3S_{13} pistils by pollen of #13/S_2*S_3 were compatible (Figure 7B), suggesting that deletion of Q_{210} of S_2-SLF1 does not affect its ability to interact with and detoxify S_{13}-RNase. Pollinations of $S_{13}S_{13}$ pistils by #27/S_2*S_3 were compatible, as pollen of S_3-haplotype should be compatible with $S_{13}S_{13}$ pistil. We then used PCR to determine the *S*-genotypes of 24 randomly selected progeny plants and found all of them to be S_3S_{13} (Supplemental Figure 7A); the absence of S_2S_{13} plants in the progeny suggests that S_2* pollen from #27/S_2*S_3 was incompatible with S_{13}-carrying pistils. Thus, deletion of both Q_{210} and L_{211} also abolishes the ability of S_2-SLF1 to detoxify S_{13}-RNase. To further confirm the results obtained from the crosses involving #27/S_2*S_3, we bud-selfed this plant and identified two *Cas9* transgene-free S_2*S_2* progeny plants, #27-BS-#1 and -#2 (Supplemental Figures 7B to 7E). We then used pollen of each plant to separately pollinate S_3S_3, $S_{13}S_{13}$, and *As-*S_3/S_3S_3 pistils. Pollinations of S_3S_3 and $S_{13}S_{13}$ pistils were incompatible (Figure 7C, Supplemental Figure 7F), consistent with the results obtained for #27/S_2*S_3; but pollinations of *As-*S_3/S_3S_3 pistils, which did not produce a detectable level of S_3-RNase, were compatible (Supplemental Figure 7F). Taking together all these results, we conclude that S_2-SLF1 with both Q_{210} and L_{211} deleted fails to detoxify S_3-RNase and S_{13}-RNase, while S_2-SLF1 with Q_{210} alone deleted can still interact with and detoxify both S-RNases (summary in Figure 7D).

Q_{210} and L_{211} are conserved between S_2-SLF1 and S_3-SLF1 (Supplemental Figure 3), suggesting that they are not involved in differential interactions between these two SLF proteins and S-RNases (e.g., S_2-SLF1, but not S_3-SLF1, interacts with S_3-, S_7- and S_{13}-RNases; Hua et al., 2007; Kubo et al., 2010; Wu et al., 2018). We previously used computational modeling and molecular docking to predict the interaction surface between S_2-SLF1 and S_3-RNase (Supplemental Figure 8A; Wu et al., 2018), and here we used the same approach to predict the interaction surface between S_2-SLF1 and S_{13}-RNase (Supplemental Figure 8B). The docking results show that Q_{210} and L_{211} are not located at the predicted interface between S_2-SLF1 and S_3-RNase or S_{13}-RNase. Thus, the inability of S_2-SLF1 (with both Q_{210} and L_{211} deleted) to detoxify S_3-RNase and S_{13}-RNase is unlikelycaused by direct disruption of the interaction surface between S_2-SLF1 and S_3-RNase or S_{13}-RNase, and the inability may be caused by conformational changes to S_2-SLF1, which indirectly affect the interaction.

DISCUSSION

The molecular and biochemical basis of three different SI systems have been studied extensively. Both the Brassicaceae and Papaveraceae systems involve highly specific "one-to-one" self-recognition between pollen and pistil *S*-specificity determinants, with each determinant encoded by a single polymorphic gene (Kachroo et al., 2001; Takayama et al., 2001; Wheeler et al., 2009, 2010; Iwano and Takayama, 2012; Fujii et al., 2016). In contrast, the Solanaceae system involves complex non-self recognition between pollen and pistil specificity determinants. A single polymorphic *S-RNase* gene encodes the pistil specificity determinant, as has been demonstrated by both gain-of-function and loss-of-function experiments (Lee et al., 1994), whereas multiple polymorphic *SLF* genes collectively encode the pollen specificity determinant (Kubo et al., 2010, 2015; Williams et al., 2014b). According to the collaborative non-self recognition model (Kubo et al., 2010), each SLF protein produced by pollen of a given *S*-haplotype is only capable of interacting with a subset of its non-self S-RNases. Thus, a complete suite of SLF proteins is required to detoxify all their non-self S-RNases, but not self S-RNase, to result in cross-compatible but self-incompatible pollination (Kubo et al., 2010).

The evidence for the involvement of SLF proteins in pollen specificity was obtained by the gain-of-function assay developed based on the phenomenon of "competitive interaction," which refers to the breakdown of SI in diploid pollen carrying two different *S*-haplotypes (Stout and Chandler, 1941, 1942). For example, SI breaks down in diploid S_mS_n pollen produced by a tetraploid S_mS_m



D

S_2-SLF1			210	211				Interactions with S_3-RNase	Interactions with S_{13}-RNase
WT	H	V	Q	L	P	S	I	+	+
#13 (*-3 bp*)	H	V	—	L	P	S	I	+	+
#27 (*-6 bp*)	H	V	—	—	P	S	I	—	—

Figure 7. Analysis of SI Behavior of S_2* Pollen Carrying a 3-bp or a 6-bp In-Frame Deletion Allele of *S_2-SLF1*.

(A) Pollen tube growth in the style after a wild-type S_3S_3 plant was separately pollinated with pollen from T_0 plants #13/S_2*S_3 (carrying a 3-bp deletion allele) and #27/S_2*S_3 (carrying a 6-bp deletion allele).

(B) Pollen tube growth in the style after a wild-type S_3S_{13} plant was pollinated with pollen from T_0 plant #13/S_2*S_3.

(C) Pollen tube growth in the style after a wild-type $S_{13}S_{13}$ plant was pollinated with pollen from a progeny plant, #27-BS-#1/S_2*S_2*, obtained by bud-selfing T_0 plant #27/S_2*S_3. Scale bar = 1 mm in all microscopy images of aniline blue staining in **(A)**, **(B),** and **(C)**.

(D) Effect of a single amino-acid deletion (Q_{210}) and a two-amino acid deletion (Q_{210} and L_{211}) of S_2-SLF1 on its ability to detoxify S_3-RNase and S_{13}-RNase. Seven amino acids of the wild-type S_2-SLF1 in the region where deletions occur are shown for comparison. + indicates ability to detoxify S_3-RNase and S_{13}-RNase, and — indicates inability to detoxify these two S-RNases.

S_nS_n plant, derived from a self-incompatible S_mS_n plant. The collaborative non-self recognition model can explain this interesting observation. Because S_mS_n pollen produces all SLF proteins of S_m-haplotype and all SLF proteins of S_n-haplotype, it could use (a) at least one of the SLF proteins of S_m-haplotype to interact with and detoxify S_n-RNase (a non-self S-RNase for S_m pollen), and (b) at least one of the SLF proteins of S_n-haplotype to interact with and detoxify S_m-RNase (a non-self S-RNase for S_n pollen). As a result, S_mS_n pollen tubes can detoxify both S_m-RNase and S_n-RNase, and they are thus compatible with pistils of both tetraploid $S_mS_mS_nS_n$ and diploid S_mS_n plants. Using the gain-of-function assay to identify which of the 17 SLF proteins of S_2-haplotype interact(s) with S_3-RNase, we previously found that expressing S_2-SLF1 alone in S_3 pollen was sufficient to render S_3 transgenic pollen compatible with S_3-carrying pistils (Sijacic et al., 2004; Hua et al., 2007). This finding suggests that S_2-SLF1 interacts with and detoxifies S_3-RNase in the S_3 transgenic pollen tube. It would seem counter-intuitive that gain-of-function of S_2-SLF1 in the recipient S_3 pollen actually results in loss of the SI function in S_3 pollen. We further used the gain-of-function assay to show that S_2-SLF1 also interacted with S_7-RNase and S_{13}-RNase (Table 1; Sun and Kao, 2013).

In this work, we have used a loss-of-function approach, CRISPR/Cas9-mediated genome editing, to definitively establish that SLF proteins are solely responsible for SI of pollen. Most notably, we found that S_2* pollen lacking a functional S_2-SLF1 was incompatible with normally compatible S_3-carrying pistils, but that it remained compatible with S_7- and S_{12}-carrying pistils. Moreover, we found that S_2* pollen was compatible with a transgenic plant (*As-S_3/S_3S_3*) whose production of S_3-RNase in the pistil was non-detectable, and was also compatible with pistils of immature S_2S_3 pistils that produce very low levels of S_3-RNase insufficient to inhibit S_3 pollen (Lee et al., 1994; Sun and Kao, 2013). We reasoned that if the SLF proteins are required for SI of pollen, then these results would suggest that S_2-SLF1 is the only SLF of the 17 produced by S_2 pollen that interacts with S_3-RNase. Furthermore, we reasoned that there are additional SLF protein(s) that interact with S_7-RNase and S_{12}-RNase. For S_3-RNase and S_7-RNase, we have used the gain-of-function assay to completely determine their interaction relationships with all 17 SLF proteins of S_2-haplotype. We found that none of the other 16 SLF proteins interact with S_3-RNase (Table 1), whereas one of them, S_2-SLF2, also interacts with S_7-RNase (Figure 5). For S_{12}-RNase, we have so far determined their interaction relationships with nine SLF proteins (Table 1) and found that both S_2-SLF1 and S_2-SLF5 interact with S_{12}-RNase (Figure 6). These interaction relationships established by the gain-of-function experiments are entirely consistent with the SI behavior of S_2* pollen with S_3-, S_7-, and S_{12}-carrying pistils. That is, in the absence of S_2-SLF1, S_2* pollen cannot use any other SLF proteins to detoxify S_3-RNase, but S_2* pollen can still use S_2-SLF2 to detoxify S_7-RNase and at least S_2-SLF5 to detoxify S_{12}-RNase.

The 108 pairwise interaction relationships between the 17 SLF proteins of S_2-haplotype and nine S-RNases that we have determined so far (Table 1) also reveal the complexity and diversity involving both “one-to-one” interactions (one SLF protein recognizing a particular S-RNase) and redundant interactions (at least two SLF proteins interacting with the same S-RNase). Functional redundancy of recognition molecules has been observed in other non-self recognition systems. For example, in plant-bacteria interactions, the effector proteins AvrPto and AvrPtoB produced by *Pseudomonas syringae* pv tomato DC300 function redundantly to block the pathogen-associated molecular pattern (PAMP) bacterial flagellin protein fliC produced by host plants (Kvitko et al., 2009). Functional redundancy enhances the robustness of a biological system by making it more “fail-safe” (Kitano, 2004). In the *Petunia* SI system, redundancy in the use of SLF proteins to detoxify a given non-self S-RNase could be advantageous, as this fail-safe mechanism would minimize the possibility of losing cross-compatibility in situations where mutations abolish the recognition function of certain SLF proteins (Sun and Kao, 2013). It has also been proposed that SLF proteins with overlapping specificities could gain new interaction specificity at the same time, which makes evolution of S-RNases with new specificities

possible (Fujii et al., 2016). However, evolving and maintaining SLF proteins with redundant, or overlapping, interaction specificity with S-RNases may also be evolutionarily costly and therefore may not always be favored by natural selection (Kubo et al., 2015). In cases in which a single SLF is responsible for detoxifying a particular S-RNase, the deleterious effect of loss-of-function mutations could be alleviated by (a) a decrease in the frequency of the plants whose pistils produce this S-RNase in the population, and/or (b) transmission of the mutated *SLF* gene through the female. The complexity of the interaction network between S-RNases and SLF proteins, therefore, is likely to be shaped by the evolutionary history of the *S-RNase* gene and the *SLF* gene repertoire at the *S*-locus.

We have thus far found that the largest number of SLF proteins produced by S_2-haplotype that recognize the same non-self S-RNase is two: S_2-SLF1 and S_2-SLF2 for S_7-RNase, and S_2-SLF1 and S_2-SLF5 for S_{12}-RNase (Sun and Kao, 2013; Williams et al., 2014b; this study). This is also the case for the interactions between SLF proteins and S-RNases of *P. hybrida*: PhS_5-SLF1 and PhS_5-SLF2 for PhS_9-RNase; PhS_7-SLF1 and PhS_7-SLF2 for PhS_9-RNase; and PhS_7-SLF2 and PhS_7-SLF9A for PhS_{19}-RNase (Kubo et al., 2010, 2015). It would be interesting to determine, for a given *S*-haplotype, what is the maximum number of SLF proteins that can interact with the same non-self S-RNase. The upper limit of different SLFs with overlapping specificities may be restricted by the time required for the evolution of different interaction specificity between SLF proteins and S-RNases and/or by the biochemical properties of the interaction surface of SLF proteins and S-RNases. Determining more interaction relationships between SLF proteins and S-RNases, and theoretical modeling of their interactions, will likely shed light on the question of why certain S-RNases only interact with one SLF, while others interact with more than one.

It is important to note that our laboratory previously used artificial microRNA (amiRNA) targeting S_2-*SLF1* to knock down S_2-*SLF1* in pollen and reported that S_2 pollen in which the S_2-*SLF1* transcript level was significantly reduced remained compatible with S_3-carrying and S_{13}-carrying pistils (Sun and Kao, 2013). Those results are different from our finding in this work that CRISPR/Cas9-mediated knockout of S_2-*SLF1* rendered S_2 pollen incompatible with S_3-carrying and S_{13}-carrying pistils. Accurate assessment of the level of suppression of a pollen-expressed gene is often difficult, because if a single copy of the transgene is integrated into the genome of a transgenic plant, only half of the pollen produced carries the transgene. Thus, even if an amiRNA completely suppresses the transcript level of its target gene in the half of the pollen that carries the transgene, the other half of the pollen will still produce the wild-type levels of the transcript. In this case, when equal amounts of total pollen RNA were examined, the total transcript level of the target gene will be ~50%—not ~0%, that of pollen produced by the wild-type plants. Also, in the amiRNA-mediated knockdown experiments of S_2-*SLF1*, the native (and weak) promoter of S_2-*SLF1* was used to drive the transcription of amiRNA in the generative nucleus of the pollen where the *SLF* genes are expressed (Sun and Kao, 2013). Therefore, the phenotypic difference may be due to detoxification of S_3-RNase and S_{13}-RNase by the residual S_2-SLF1 in S_2 transgenic pollen. This explanation also suggests that SLF proteins are efficient in detoxifying S-RNases in vivo, and thus it is essential to use the knockout approach to address the function of any SLF.

The generation of two in-frame deletion alleles of S_2-*SLF1* has allowed us to also address the effect of a small number of amino acid deletions on the function of S_2-SLF1 in interactions with S_3-RNase and S_{13}-RNase. Interestingly, deletion of one amino acid, Q_{210}, near the middle of the protein does not affect the ability of S_2-SLF1 to interact with and detoxify these two S-RNases. However, deletion of Q_{210} and its adjacent amino acid, L_{211}, results in the loss of the ability of S_2-SLF1 to detoxify both S-RNases (Figure 7D). The results of molecular docking show that Q_{210} and L_{211} are not located at the interface of S_2-SLF1 and S_3-RNase or S_{13}-RNase (Supplemental Figure 8; Wu et al., 2018), suggesting that their deletion most likely indirectly affects the interaction interface of S_2-SLF1 with these two S-RNases. Q_{210} and L_{211} are conserved in S_3-SLF1, which, unlike S_2-SLF1, does not interact with S_3-RNase or S_{13}-RNase (Hua et al., 2007; Kubo et al., 2010; Wu et al., 2018). These results suggest that conserved amino acids outside the interface between SLF proteins and S-RNases can also contribute to the establishment of interactions, even though they do not directly contribute to the specificity of interactions. Thus, interactions between SLF proteins and S-RNases are under strict and intricate constraints. Further structural studies of SLF-S-RNase complexes will help shed light on the amino acid residues of SLF proteins that are critical for the establishment of their interactions with S-RNases.

In summary, in this work we have extensively characterized the effect of loss-of-function of S_2-SLF1 on the SI behavior of S_2 pollen, using two frame-shift indel alleles of S_2-*SLF1* generated by CRISPR/Cas9-mediated genome editing. The results, coupled with the comprehensive analysis of interaction relationships between the 17 SLF proteins of S_2-haplotype and nine S-RNases determined via the in vivo gain-of-function assay, provide definitive evidence for the essential role of SLF proteins in the SI of pollen and lend strong support for the validity of the collaborative non-self recognition model (Kubo et al., 2010). The interaction relationships also reveal that the *Petunia* SI system has evolved complex and diverse interaction patterns between SLF proteins and S-RNases, with both "fail-safe" and "one-to-one" interactions. The results from this work and determination of more SLF-S-RNase interaction relationships will provide valuable insights for investigations into the biochemical and structural basis of differential interactions between SLF proteins and S-RNases, as well as for studies of the evolutionary dynamics of SLF repertoires and S-RNases during the long evolutionary history of this SI system.

METHODS

Plant Materials and Growth Conditions

All the *S*-haplotypes of *Petunia inflata* used in this work (S_2, S_3, S_5, S_{6a}, S_7, S_{11}, S_{12}, S_{13}, and S_{16}) were from our laboratory's genetic stock (Ai et al., 1990; Wang et al., 2001; Sun and Kao, 2013). The *As*-S_3/S_3S_3 plants were obtained by bud-selfing the previously generated *As*-S_3/S_2S_3 plants (Lee et al., 1994; Sun and Kao, 2013). *Petunia* seedlings were grown at 30°C with a light cycle of 16 h (2600 lumens cool white light, Philips 40-Watt Cool White Linear Fluorescent Light Bulbs). Mature plants (over 30-cm tall) in

individual pots were maintained in the greenhouse at Pennsylvania State University. The temperature in the greenhouse was kept at 25°C, with a light cycle of 16 h under a high-pressure sodium (HPS) light system (1080-watt PL 2000, P.L. Light Systems).

Generation and Characterization of CRISPR/Cas9-Mediated Knockout Mutants of *S_2-SLF1*

To generate the CRISPR/Cas9 construct targeting *S_2-SLF1,* the pre-tRNA and the gRNA scaffold fragments were ligated to the *S_2-SLF1-Protospacer-9* (*S_2-SLF1*-PS9) fragment, and the resulting DNA fragment was cloned into pKSE401 plasmid (Xing et al., 2014; a gift from Qi-Jun Chen; Addgene plasmid # 62202) following the protocol described previously (Xie et al., 2015; Sun and Kao, 2018). The pGTR plasmid (obtained from Yinong Yang laboratory, Pennsylvania State University) was used as a template to synthesize the tRNA-gRNA fragment (PTG gene) in two separate PCRs using Phusion DNA polymerase (Thermo Scientific). *S_2-SLF1-PS9*-F/L3AD5-R and *S_2-SLF1-PS9*-R/L5AD51-F were used to synthesize two halves of the PTG gene, and they were ligated together using the Golden Gate Assembly method. The assembled product was further amplified using S51AD5-F and S3AD5-R primers, and it was digested with *Fok*I (New England BioLabs). The *Fok*I-digested PTG fragment was ligated to *Bsa*I-digested pKSE401 to create the *PTG-S_2-SLF1-PS9*-pKSE401 construct. The binary plasmid *PTG-S_2-SLF1-PS9*-pKSE401 was transformed into *Agrobacterium tumefaciens* (LBA4404) by electroporation. Transformation of *P. inflata* of S_2S_3 genotype was performed as described previously (Meng et al., 2011). Genomic DNA from regenerated plants was extracted as described previously (Meng et al., 2011). A pair of primers was used for the identification of transgenic plants. The pair of primers (*35S*-gt-F/*Cas9*-gt-R) were flanking a 580-bp fragment, from the 3′-end of the CaMV 35S promoter to the 5′-end of *Cas9* on the *PTG-S_2-SLF1-PS9*-pKSE401 Ti plasmid.

The target region in *S_2-SLF1* was amplified with *PiSLF2*-RT-*3For/4Rev* primers using Phusion DNA polymerase. The PCR products were subjected to *Bsr*GI-HF (New England BioLabs) digestion overnight, and the digested PCR products were electrophoresed on 2% agarose gels. The PCR products that were not digested by *Bsr*GI were sequenced to determine the nature of indel mutations. The region in *S_3-SLF1* corresponding to the target region in *S_2-SLF1* was amplified with *PiSLF3-Copy1For/Rev* primers using Phusion DNA polymerase, and the PCR products were sequenced. The *S*-genotype of BS progeny plants were determined by PCR using primers *PiSLF3-Copy1For/Rev* (for *S_3-SLF1*).

Generation of Transgenic Plants Used for Gain-of-Function Assays

A total of 11 Ti plasmid constructs were made in this work for the in vivo gain-of-function assay, all of which were in the pBI101 backbone. Each construct contained the *LAT52* promoter (*LAT52P*; Twell et al., 1990) driving the expression of one SLF fused at its last codon with the coding sequence of GFP. These 11 constructs are schematically detailed in Figures 4A and 4B. Generation of the *pBI101-LAT52P:S_2-SLF9/S_2-SLF*10 constructs (Figure 4A) was as described by Hua et al. (2007). *pBI101-LAT52P:S_2-SLF2/-SLF7/S_2-SLF11/-SLF12/-SLF13/-SLF14/-SLF15/-SLF16/-SLF17* constructs (Figure 4B) were generated using the In-fusion HD Cloning Kit from Clontech (Williams et al., 2014b). Primers used for making all 11 constructs are listed in Supplemental Table 1. All Ti plasmid constructs were electroporated into *Agrobacterium tumefaciens* (LBA4404) competent cells, and subsequently they were transformed into *P. inflata* plants of S_2S_3 or S_2S_7, as described previously (Meng et al., 2011). Transgenic lines were identified using a primer pair specific to the *GFP* transgene (*GFP-001For* and *GFP-500Rev*).

Visualization of GFP Fluorescence of Pollen Tubes

Mature pollen was collected and germinated in pollen germination medium for two hours and visualized with a Nikon Eclipse 90i epifluorescence microscope, as described previously (Meng et al., 2011; Williams et al., 2014b).

Pollination Assay

Stigmas of emasculated mature flowers were manually pollinated with pollen from mature anthers. For aniline blue staining of pollen tubes, the pollinated pistils were collected 20 h after manual pollination, fixed with a mix of acetic acid with 95% ethanol (1:3), macerated with 8 M KOH, and then stained with 0.1 mg/ml aniline blue dye diluted with 1 mM KH_2PO_4 (1:20). The stained pistils were visualized under the DAPI-filtered UV light of a Nikon Eclipse 90i epifluorescence microscope.

Computational Modeling of Protein Tertiary Structures and Protein-Protein Docking Analysis

Wu et al. (2018) reported the predicted tertiary structures of S_2-SLF1 (without the first 95 amino acids that contain the F-box domain) and S_3-RNase, as well as their molecular docking. The tertiary structure of S_{13}-RNase was modeled using I-TASSER (Zhang, 2008; Yang et al., 2015), and structure refinement was similarly performed as previously described by Wu et al. (2018). Molecular docking of S_{13}-RNase (as the ligand) with S_2-SLF1 (without the first 95 amino acids, as the receptor) was similarly performed using ClusPro, as previously described (Comeau et al., 2004; Kozakov et al., 2017; Wu et al., 2018). All structures were visualized using the PyMOL molecular visualization package (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: S_2-SLF1 (AAS79485), S_3-SLF1 (AAS79486), S_2-SLF2 (KJ670474), S_2-SLF3 (EF614187), S_2-SLF4 (KF524351), S_2-SLF5 (KF524352), S_2-SLF6 (KF524353), S_2-SLF7 (EF614189), S_2-SLF8 (EF614188), S_2-SLF9 (AY363971), S_2-SLF10 (AY363974), S_2-SLF11(KJ670428), S_2-SLF12 (KJ670433), S_2-SLF13 (KJ670438), S_2-SLF14 (KJ670443), S_2-SLF15 (KJ670448), S_2-SLF16 (KJ670453), S_2-SLF17 (KJ670458), S_2-RNase (AAG21384), S_3-RNase (AAA33727), S_{6a}-RNase (AF301167), S_7-RNase (AF301168), S_{11}-RNase (AF301172), S_{12}-RNase (AF301173), S_{13}-RNase (AF301174), S_{16}-RNase (AF301176), S_5-SLF1(KC590092), S_7-SLF1 (KC590093), S_{11}-SLF1 (KC590094), and S_{13}-SLF1 (KC590095). All genes are from *Petunia inflata*.

Supplemental Data

Supplemental Figure 1. Alignment of nucleotide sequences of the protospacer region of *S_2-SLF1* and the corresponding regions of all *SLF* genes of S_2-Haplotype and S_3-Haplotype.

Supplemental Figure 2. Generation of the PTG-CRISPR/Cas9 construct targeting *S_2-SLF1,* and analysis of regenerated plants for the presence of the transgene.

Supplemental Figure 3. Alignment of deduced amino acid sequences of *S_2-SLF1*, *S_3-SLF1*, and four indel alleles of *S_2-SLF1* in the region targeted by CRISPR/Cas9.

Supplemental Figure 4. PCR analysis of *S*-Genotypes of plants in each progeny obtained from pollination of S_7S_7 and $S_{13}S_{13}$ plants by pollen from T_0 plants #2/S_2*S_3 and #35/S_2*S_3.

Supplemental Figure 5. Identification of bud-selfed (BS) progeny plants homozygous for S_2-Haplotype and for the indel alleles inherited from their respective T_0 plants #2/S_2*S_3 and #35/S_2*S_3.

Supplemental Figure 6. GFP fluorescence of pollen tubes germinated in vitro from pollen of a representative T_0 plant of each of the 12 transgenic lines generated in this study.

Supplemental Figure 7. Analysis of SI behavior of plants carrying a 6-bp In-frame deletion allele of *S_2-SLF1*.

Supplemental Figure 8. Computational modeling of interactions of S_2-SLF1 with S_3-RNase and S_{13}-RNase.

Supplemental Table 1. Primers used in this study

Supplemental Table 2. Summary of T_0 and T_1 plants used in the in vivo gain-of-function assay and their SI behavior

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AUTHOR CONTRIBUTIONS

L.S., J.S.W., S.L., and T.-h.K. designed the experiments. L.S., J.S.W., S.L., L.W., W.A.K., P.G.S., and M.D.K. performed the experiments and analysis. L.S. and T.-h.K. wrote the article.

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