Self-Incompatibility in *Petunia inflata*: The Relationship between a Self-Incompatibility Locus F-Box Protein and Its Non-Self S-RNases

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The highly polymorphic S (for self-incompatibility) locus regulates self-incompatibility in *Petunia inflata*; the S-RNase regulates pistil specificity, and multiple S-locus F-box (SLF) genes regulate pollen specificity. The collaborative non-self recognition model predicts that, for any S-haplotype, an unknown number of SLFs collectively recognize all non-self S-RNases to mediate their ubiquitination and degradation. Using a gain-of-function assay, we examined the relationships between S\(_2\)-SLF1 (for S\(_2\)-allelic product of Type-1 SLF) and four S-RNases. The results suggest that S\(_2\)-SLF1 interacts with S\(_3\)- and S\(_7\)-RNases, and the previously identified S\(_5\)- and S\(_{13}\)-RNases, but not with S\(_7\)- or S\(_{11}\)-RNase. An artificial microRNA expressed by the S\(_2\)-SLF1 promoter, but not by the vegetative cell-specific promoter, *Late Anther Tomato* 52, suppressed expression of S\(_2\)-SLF1 in S\(_2\) pollen, suggesting that SLF1 is specific to the generative cell. The S\(_2\) pollen with S\(_2\)-SLF1 suppressed was compatible with S\(_2\), S\(_3\), S\(_7\), S\(_{13}\), and S\(_{15}\)-carrying pistils, confirming that other SLF proteins are responsible for detoxifying S\(_5\) and S\(_{11}\)-RNases and suggesting that S\(_2\)-SLF1 is not the only SLF in S\(_2\) pollen that interacts with S\(_2\), S\(_7\), and S\(_{13}\)-RNases. *Petunia* may have evolved at least two types of SLF proteins to detoxify any non-self S-RNase to minimize the deleterious effects of mutation in any SLF.

INTRODUCTION

To circumvent the strong tendency to self-pollinate, many flowering plants producing bisexual flowers have adopted a reproductive strategy, called self-incompatibility (SI), by which pistils can reject self-pollen and only allow non-self pollen to effect fertilization (De Nettancourt, 2001). Despite the widespread occurrence of SI, to date our knowledge of the genes involved in self/non-self recognition between pollen and pistil, and the underlying mechanism, has largely been acquired from studies of five families: Brassicaceae, Papaveraceae, Plantaginaceae, Rosaceae, and Solanaceae (Iwano and Takayama, 2012). These studies have revealed that Brassicaceae and Papaveraceae each employ a distinct mechanism, whereas the other three families employ similar mechanisms. Thus, SI appears to have evolved multiple times in flowering plants.

For the Solanaceae, a highly polymorphic locus, termed the S-locus (SI locus), determines the outcome of pollination. Matching of the S-haplotype of pollen with either S-haplotype of the pistil results in growth inhibition of self-pollen tubes in the pistil. The S-locus contains the S-RNase gene, which regulates pistil specificity (Lee et al., 1994; Murfett et al., 1994), and multiple, but as yet unknown number of, S-locus F-box (SLF) genes that collectively regulate pollen specificity (Kubo et al., 2010). The S-RNase gene was first identified in *Nicotiana alata* based on the prediction that its allelic products are specific to pistils and divergent in sequence (Anderson et al., 1986), and it was subsequently shown to be solely responsible for regulating pistil specificity by both gain- and loss-of-function experiments (Lee et al., 1994; Murfett et al., 1994). The ribonuclease activity of S-RNase is essential for the SI function of S-RNase, as expressing a mutant form of S-RNase of *Petunia inflata*, with one of the two catalytic His residues replaced with Arg, in plants of S\(_S\)S\(_2\) genotype did not confer on pistils of the transgenic plants the ability to reject S\(_2\) pollen (Huang et al., 1994). Thus, S-RNase is most likely functions as a toxin inside pollen tubes to specifically degrade RNAs of incompatible pollen tubes to result in their growth inhibition.

Pollen specificity was initially thought to be regulated by a single SLF gene (named SFB in some rosaceous species), which was identified in *Antirrhinum hispanicum* (Plantaginaceae), *P. inflata*, and several *Prunus* species (Rosaceae) by sequencing various S-locus regions containing a particular allele of the S-RNase gene (Lai et al., 2002; Entani et al., 2003; Ushijima et al., 2003; Ikeda et al., 2004; Sijacic et al., 2004). The role of the SLF gene in *P. inflata* in regulating pollen specificity was confirmed by a gain-of-function approach (Sijacic et al., 2004). This transgenic functional assay was designed based on the observation, first made in self-incompatible *Petunia axillaris*, that SI breaks down in diploid pollen carrying two different S-haplotypes or in haploid pollen that carries a duplicated S-locus region of a different S-haplotype from that of the resident S-locus (Brewbaker and Natarajan, 1960; Entani et al., 1999; Golz et al., 2001). For example, when a self-incompatible plant of the S\(_S\)S\(_2\) genotype is converted to tetraploid, the tetraploid plant becomes...
self-compatible, as its $S_1S_2$ pollen is compatible with its $S_1S_7S_9S_9$ pistil, as well as with the $S_1S_2$ pistil of the diploid plant (see Supplemental Figures 1A and 1B online). Thus, if SLF was solely responsible for pollen specificity, introduction of its $S_7$-allele, $SLF_2$, into $S_1S_1S_7S_7$ and $S_7S_7$ plants should cause breakdown of SI in both $S_7$ and $S_9$ transgenic pollen due to the presence of two different alleles of SLF. The results of the transgenic experiments were precisely as predicted (Sijacic et al., 2004).

A protein degradation model was proposed to explain breakdown of SI in heteroallelic pollen (Hua and Kao, 2006; Hua et al., 2008). This model was formulated based on the finding that both self- and non-self S-RNases were taken up by a pollen tube (Luu et al., 2000; Goldraj et al., 2006) and based on the results from in vitro protein binding, protein ubiquitination/degradation, and yeast two-hybrid assays (Hua and Kao, 2006). For example, in the case of breakdown of SI in $S_7$ transgenic pollen carrying $SLF_2$, the model predicts that $SLF_2$ produced in $S_7$ transgenic pollen would interact with all S-RNases except its self S-RNase (thus, with $S_{2-RNase}$ but not with $S_{2-RNase}$), and $SLF_3$ produced from the endogenous gene would interact with all S-RNases except its self S-RNase (thus, with $S_{2-RNase}$ but not with $S_{2-RNase}$). The model also predicts that SLF is the F-box protein component of an E3 ubiquitin ligase (Hua and Kao, 2006; Hua et al., 2008), which, in conjunction with E1 ubiquitin activating enzyme and E2 ubiquitin conjugating enzyme, mediates ubiquitination of all S-RNases with which the SLF component interacts to result in their subsequent ubiquitination and subsequent degradation by the 26S proteasome. Thus, both $S_{2-RNase}$ and $S_{2-RNase}$ taken up by $S_7$ transgenic pollen would be degraded, resulting in breakdown of SI (i.e., allowing $S_7$ transgenic pollen to be accepted by $S_7$-carrying pistils).

However, when $SLF_2$ was later examined by a similar transgenic functional assay, unexpectedly it did not cause breakdown of SI in $S_7$ transgenic pollen (Kubo et al., 2010). Similar unexpected results were also obtained when several alleles of SLF in Petunia hybrida were examined (Kubo et al., 2010). For example, the $S_7$-allele of SLF caused breakdown of SI in both $S_7$ and $S_{17}$ transgenic pollen, but not in $S_7$, $S_{17}$, or $S_7$ transgenic pollen. All these results, on the one hand, further confirmed the involvement of SLF of Petunia in pollen specificity, but on the other hand suggested that this gene is not likely the only gene responsible for pollen specificity. This finding raised the possibility that the previously identified SLF-like genes (Hua et al., 2007) might also be involved. These genes not only share similarity in their deduced amino acid sequences with those of SLF alleles but also are tightly linked to the S-locus and specifically expressed in pollen. They were initially thought not to be involved in SI, as when the $S_7$- or $S_2$-allele of three of these genes was introduced into $S_7S_7$ or $S_7S_7$ plants of P. inflata, none caused breakdown of SI in heteroallelic transgenic pollen (Hua et al., 2007). However, a more comprehensive study on the homologs of these genes in P. hybrida has recently shown that at least two of the SLF-like genes are also involved in pollen specificity (Kubo et al., 2010). SLF has been renamed SLF1 (Type-1 SLF), and the SLF-like genes and additional such genes identified in P. hybrida have been renamed Type-2 (SLF2), Type-3 (SLF3), etc. For example, the transgenic functional assay showed that expression of $S_{7}/SLF2$ (Type-2 SLF of $S_7$-haplotype) caused breakdown of SI in $S_7$ and $S_{17}$ pollen but not in $S_7$ or $S_7$ pollen (Kubo et al., 2010). Coimmunoprecipitation results showed that, using an anti-FLAG antibody, $S_{7}/SLF2$:FLAG produced in transgenic pollen coprecipitated with $S_{7}$-RNase and $S_{17}$-R-Nase but not with $S_{7}$-RNase or $S_{7}$-RNase in style extracts (Kubo et al., 2010), thus providing further support for the involvement of non-self interactions between SLFs and S-RNases in SI responses. Moreover, the finding that $S_{7}/SLF2$ caused breakdown of SI in $S_7$ and $S_{17}$ pollen is consistent with the ability of $S_7$:SLF2 to interact with $S_{7}$-RNase and $S_{17}$-R-Nase, thus suggesting that the transgenic functional assay may be used to determine whether an SLF interacts with any non-self S-RNase.

A new protein degradation model, named collaborative non-self recognition, has been proposed, which takes into account the involvement of multiple SLF proteins in pollen specificity (Kubo et al., 2010). It proposes that for a given S-haplotype, (1) each type of SLF recognizes a subset of non-self S-RNases to mediate their ubiquitination and subsequent degradation by the 26S proteasome, (2) multiple types of SLF proteins together recognize and detoxify all non-self S-RNases to allow compatible pollination, and (3) none of the SLF proteins can interact with self S-RNase, allowing it to degrade pollen RNAs to result in incompatible pollination.

If a single SLF gene were solely responsible for pollen specificity, mutation in this gene would be lethal for pollen, as pollen tubes would be unable to detoxify any S-RNase and be incompatible with pistils of any S-genotype. However, based on the collaborative nature of multiple SLF genes, mutation in any SLF gene will affect the SI behavior of pollen only if the protein encoded by this gene is solely responsible for recognizing and detoxifying one or more non-self S-RNases. In this case, pollen will be incompatible with pistils producing this (these) non-self S-RNase(s), but will remain compatible with pistils producing all other non-self S-RNases. It seems logical that pollen of any S-haplotype would employ more than one type of SLF protein to recognize any of its non-self S-RNases to minimize the effect of mutation in any SI gene on the SI behavior of pollen. Indeed, the results obtained from a transgenic functional study of three types of SLF genes of Petunia, SLF1, SLF2, and SLF3, showed that SLF1 and SLF2 of $S_7$ and $S_{17}$ pollen all caused breakdown of SI in $S_7$ transgenic pollen, suggesting that $S_{7}$-RNase interacts with SLF1 and SLF2 of $S_7$ and $S_{17}$ pollen (Kubo et al., 2010).

To date, it is not known how many types of SLF proteins constitute the pollen specificity determinant of any S-haplotype, but there must be more than the three types that have already been shown to be involved because (1) for each of the four S-haplotypes studied, at least one of its non-self S-RNases examined did not interact with any of these three types of SLF proteins (Kubo et al., 2010) and (2) only five non-self S-RNases were examined for each S-haplotype, a small number considering that at least a total of 32 distinct S-haplotypes have been reported in P. inflata and P. hybrida and considering that there may be up to 40 more, if these haplotypes identified from P. axillaris are genetically distinct from any of the S-haplotypes identified in P. inflata or P. hybrida (Sims and Robbins, 2009). The function of any potential SLF gene in SI needs to be examined by the well-established gain-of-function transgenic assay (Sijacic et al., 2004; Hua et al., 2007; Kubo et al., 2010), and
its role in SI can only be established if it causes breakdown of SI in heteroallelic transgenic pollen carrying certain S-haplotype(s). Thus, the success of identifying any additional SLF protein involved in pollen specificity depends on whether the S-haplotypes available for testing contain at least one that encodes an S-RNase with which this SLF protein interacts.

We previously showed that expression of Type-1 SLF of S$_2$-haplotype, S$_2$-SLF1 (formerly named SLF$_3$), of P. inflorescens caused breakdown of SI in S$_2$, and S$_2$ pollen (Sijacic et al., 2004), suggesting that S$_2$-SLF1 interacts with S$_3$- and S$_5$-RNases to mediate their degradation, thereby allowing S$_2$ and S$_5$ transgenic pollen to be compatible with S$_3$- and S$_5$-carrying pistils, respectively. As a first step toward a comprehensive study of the relationship between each type of SLF involved in pollen specificity and its non-self S-RNases, in this study, we first used the transgenic functional assay to examine the relationship between S$_2$-SLF1 and four other non-self S-RNases in P. inflorescens. We examined the effect on the SI behavior of the transgenic plant, with which this SLF protein interacts.

The results from the transgenic functional assay taken together suggest that S$_2$-SLF1 interacts with S$_3$- and S$_5$-RNases (in addition to interacting with S$_2$- and S$_5$-RNases that were previously shown), but it does not interact with S$_5$- or S$_{11}$-RNase.
The sequence of amiRNA targeting to Type-6) identiﬁed by Schwab et al. (2006) to generate sequence was integrated into the coding sequence). Both amiRNAs were designed according to the amiRNA designing tool at http://wmd3.weigelworld.org/cgi-bin/webapp.cgi, following the strict base-pairing rules for using amiRNA to suppress expression of plant genes: Only one mismatch is allowed between the 2nd and 12th nucleotide of the amiRNA, but no mismatch is allowed for the 10th and 11th nucleotide, and up to four mismatches, but no more than two in a row, are allowed between the 13th and 21st nucleotide (Schwab et al., 2005). In addition, the amiRNA targeting S2-SLF1 was designed to speciﬁcally target S2-SLF1 without affecting S2-SLF1 or any of the other SLF genes (named Type-2 to Type-6) identiﬁed so far (Hua et al., 2007; Kubo et al., 2010). The sequence of amiRNA targeting S2-SLF1 was 5′-TCAATAATCGATGGCAACTG-3′, and the sequence of amiRNA targeting GFP was 5′-TTGAAGTTCACCTTGATGCCG-3′. Each sequence was integrated into the Arabidopsis miR319a stem-loop precursor by site-directed mutagenesis, following the procedure described by Schwab et al. (2006), to generate AmiS2-SLF1 and AmiGFP (see Supplemental Figure 3 online). The LAT52 promoter was inserted upstream of AmiS2-SLF1 and AmiGFP in Ti plasmids pCAMBIA1300 and pTA7001, respectively, to generate pCAMBIA1300-LAT52P-AmiS2-SLF1 and pTA7001-LAT52P-AmiGFP (see Supplemental Figure 2 online). The constructs were separately introduced into the pBI101-LAT52P-S2-SLF1:GFP**/S2S3 transgenic plant. One (pCAMBIA1300-LAT52P-AmiS2-SLF1+LAT52P-S2-SLF1:GFP**)/+S2S3 and two (pTA7001-LAT52P-AmiGFP+LAT52P-S2-SLF1:GFP**/+S2S3 double transgenic plants (designated -1 and -2) were obtained, and all were used in subsequent experiments.

We analyzed GFP ﬂuorescence of in vitro–germinated pollen tubes of the three double transgenic plants, the pBI101-LAT52P-S2-SLF1:GFP**/S2S3 transgenic plant, and a wild-type S2S3 plant. For the pBI101-LAT52P-S2-SLF1:GFP**/S2S3 transgenic plant, all the pollen tubes examined showed ﬂuorescence (Figure 1A), whereas for the wild-type S2S3 plant, none of the pollen tubes showed ﬂuorescence (Figure 1B). For the two double transgenic plants carrying LAT52P-AmiGFP, ~50% (79 of 147) and ~40% (27 of 70) of their pollen tubes showed ﬂuorescence (Figures 1C and 1D show representative pollen tubes examined for each transgenic plant), and for the double transgenic plants carrying LAT52P-AmiS2-SLF1, ~70% the pollen tubes (70 of 95) showed ﬂuorescence (Figure 1E shows representative pollen tubes examined). These results suggest that both amiRNAs were able to suppress the expression of the S2-SLF1:GFP transgene.

To more precisely determine the extent of suppression, we next used real-time PCR to quantify the levels of S2-SLF1 transcripts in total RNA isolated from stage 5 anthers (purple buds between 2.0 and 2.5 cm, containing anthers before anthesis; Mu et al., 1994) of the S2S3 transgenic plant homozygous for the S2-SLF1 transgene, all three double transgenic plants, and a wild-type S2S3 plant (Figure 2A; see Supplemental Figure 4 online). For all but the wild-type plant, the transcript level was the total transcript of the endogenous S2-SLF1 gene and S2-SLF1:GFP transgene. The level of pBI101-LAT52P-S2-SLF1:GFP**/+S2S3 (labeled as **/S2S3 in Figure 2A) was 19.01 ± 2.84 (average ± ss) times that of the wild-type S2S3 plant, and the levels of the double transgenic plant carrying LAT52P-AmiS2-SLF1 and the two double transgenic plants (-1 and -2) carrying LAT52P-AmiGFP were 7.21 ± 0.62, 6.97 ± 0.89, and 9.32 ± 0.13 times that of the wild-type S2S3 plant, respectively. If one copy of either amiRNA transgene was integrated into the double transgenic plants, the maximum level of reduction in the S2-SLF1:GFP transcript level should be 50%; thus, the ~54 to 67% reduction suggests that these three double transgenic plants most likely carried more than one copy of the respective amiRNA construct. We used real-time PCR to determine the copy numbers of the LAT52P-AmiS2-SLF1 and LAT52P-AmiGFP transgenes (see Supplemental Figure 2 online); the S2S3 double transgenic plant carrying LAT52P-AmiS2-SLF1 had four copies of the transgene, and plants -1 and -2 of the S2S3 double transgenic plants carrying LAT52P-AmiGFP both had two copies of the transgene. (The validity of this method will be discussed in a later section.) All these results suggest that both amiRNAs efﬁciently suppressed the expression of the S2-SLF1:GFP transgene in the vegetative cell of pollen.

### Table 1. Effect of S2-SLF1 on SI Behavior of S2, S17, S26, and S23 Pollen and Effect of Loss of Function of S2-SLF1 on SI Behavior of S2 Pollen

<table>
<thead>
<tr>
<th>S-Genotype of Transgenic Plant</th>
<th>Transgene</th>
<th>No. of Transgenic Plants Examined</th>
<th>SI Behavior</th>
</tr>
</thead>
<tbody>
<tr>
<td>S2S2</td>
<td>LAT52P-S2-SLF1:GFP</td>
<td>2</td>
<td>Self Incompatible</td>
</tr>
<tr>
<td>S2S17</td>
<td>LAT52P-S2-SLF1:GFP</td>
<td>2</td>
<td>Self Incompatible</td>
</tr>
<tr>
<td>S2S5</td>
<td>LAT52P-S2-SLF1:GFP</td>
<td>1</td>
<td>Self Incompatible</td>
</tr>
<tr>
<td>S2S26</td>
<td>LAT52P-S2-SLF1:GFP</td>
<td>2</td>
<td>Self Incompatible</td>
</tr>
<tr>
<td>S2S23</td>
<td>S2P-AmiS2-SLF1</td>
<td>3</td>
<td>Self Incompatible</td>
</tr>
</tbody>
</table>

**Suppression of Expression of the LAT52P-S2-SLF1:GFP Transgene by LAT52 Promoter–Driven amiRNAs Targeting Either S2-SLF1 or GFP**

Arabidopsis thaliana microRNA (miRNA) stem-loop precursors have been used to successfully silence genes in tomato and tobacco (Nicotiana tabacum) (Alvarez et al., 2006), and small RNA sequences matching miR319 have been found in P. hybrida inflorescence small RNA libraries (Tedder et al., 2009), suggesting that Arabidopsis miR319a precursor could be processed in P. inﬂata. To assess the feasibility of using amiRNA to suppress expression of the S2-SLF1 in S2 pollen, we ﬁrst used the S2S2 transgenic plant homozygous for the LAT52P-S2-SLF1:GFP transgene as the recipient of two amiRNA constructs, one targeting S2-SLF1 (628 to 648 bp of the coding sequence) and the other targeting GFP (480 to 500 bp of the coding sequence). Both amiRNAs were designed according to the amiRNA designing tool at http://wmd3.weigelworld.org/cgi-bin/webapp.cgi, following the strict base-pairing rules for using amiRNA to suppress expression of plant genes: Only one mismatch is allowed between the 2nd and 12th nucleotide of the amiRNA, but no mismatch is allowed for the 10th and 11th nucleotide, and up to four mismatches, but no more than two in a row, are allowed between the 13th and 21st nucleotide (Schwab et al., 2005). In addition, the amiRNA targeting S2-SLF1 was designed to speciﬁcally target S2-SLF1 without affecting S2-SLF1 or any of the other SLF genes (named Type-2 to Type-6) identiﬁed so far (Hua et al., 2007; Kubo et al., 2010). The sequence of amiRNA targeting S2-SLF1 was 5′-TCAATAATCGATGGCAACTG-3′, and the sequence of amiRNA targeting GFP was 5′-TTGAAGTTCACCTTGATGCCG-3′. Each sequence was integrated into the Arabidopsis miR319a stem-loop precursor by site-directed mutagenesis, following the procedure described by Schwab et al. (2006), to generate AmiS2-SLF1 and AmiGFP (see Supplemental Figure 3 online). The LAT52 promoter was inserted upstream of AmiS2-SLF1 and AmiGFP in Ti plasmids pCAMBIA1300 and pTA7001, respectively, to generate pCAMBIA1300-LAT52P-AmiS2-SLF1 and pTA7001-LAT52P-AmiGFP (see Supplemental Figure 2 online). The constructs were separately introduced into the pBI101-LAT52P-S2-SLF1:GFP**/S2S3 transgenic plant. One (pCAMBIA1300-LAT52P-AmiS2-SLF1+LAT52P-S2-SLF1:GFP**/+S2S3 and two (pTA7001-LAT52P-AmiGFP+LAT52P-S2-SLF1:GFP**/+S2S3 double transgenic plants (designated -1 and -2) were obtained, and all were used in subsequent experiments.

Gain- and Loss-of-Function of SLF
Figure 1. GFP Fluorescence of in vitro-Germinated Pollen Tubes.

For each plant, the left panel shows pollen tubes visualized under 10-s exposure to UV light, and the right panel shows pollen tubes visualized under white light. The triangles in (C) to (E) indicate pollen tubes that were observed in the bright field but did not show GFP fluorescence in the dark field. (A) S₂S₃ transgenic plant homozygous for the LAT52P:S₂-SLF1:GFP transgene. (B) Wild-type (WT) S₂S₃ plant. (C) and (D) Two S₂S₃ double transgenic plants homozygous for the LAT52P:S₂-SLF1:GFP transgene and carrying the LAT52P:AmiGFP transgene. (E) S₂S₃ double transgenic plant homozygous for the LAT52P:S₂-SLF1:GFP transgene and carrying the LAT52P:AmiS₂-SLF1 transgene.

AmiS₂-SLF1 Expressed by S₂-SLF1 Promoter, but Not by LAT52 Promoter, Suppresses Expression of Endogenous S₂-SLF1

We next examined whether expression of the endogenous S₂-SLF1 gene in the S₂S₃ double transgenic plant carrying the LAT52P:AmiS₂-SLF1 transgene was also suppressed. Real-time PCR was performed using a pair of primers that specifically amplify the endogenous S₂-SLF1 gene and not the S₂-SLF1:GFP transgene (see Supplemental Figure 4 online). The results showed that the transcript level of the endogenous S₂-SLF1 gene in this transgenic plant was comparable to those of a wild-type S₂S₃ plant and the transgenic plant homozygous for LAT52P:S₂-SLF1:GFP (Figure 2B). Both the S₂-SLF1:GFP transgene and the AmiS₂-SLF1 transgene were driven by the LAT52 promoter, which is active in the vegetative nucleus of pollen (Eady et al., 1995). During pollen development, the LAT52 promoter is active from the stage of late unicellular microspores, before pollen mitosis I in Arabidopsis and immediately after pollen mitosis I in tobacco (Twel et al., 1989, 1990; Eady et al., 1994), to the stage of pollen tube germination, whereas expression of the S₂-SLF1 gene is first detected in stage 3 anthers of P. inflata, which contain mostly bicellular microspores, and remains active in germinating pollen tubes (Mu et al., 1994; Sijacic et al., 2004). In stage 5 anthers of P. inflata, both promoters should be active in pollen. Thus, the finding that the LAT52 promoter-driven AmiS₂-SLF1 suppressed the LAT52 promoter-driven S₂-SLF1:GFP transgene, but not the endogenous S₂-SLF1 gene, raised the possibility that the S₂-SLF1 gene is expressed in the generative cell of pollen and thus its transcript could not be suppressed by amiRNA expressed in the vegetative cell.

To test this possibility, we first introduced the LAT52P:AmiS₂-SLF1 transgene into wild-type S₂S₃ plants to directly target the endogenous S₂-SLF1 gene. Five of the 13 transgenic plants obtained, pBI101-LAT52P:AmiS₂-SLF1-1, -3, -6, -8, and -17, were chosen for all subsequent analyses. We used real-time PCR to examine the transcript level of the endogenous S₂-SLF1 gene and found that the levels in these five transgenic plants were not statistically different from that of a wild-type S₂S₃ plant (Figure 3A). We then used a stem-loop real-time RT-PCR procedure (Varkonyi-Gasic et al., 2007; see Supplemental Figure 5 online) to determine whether the 21-nucleotide mature amiRNA was produced in all these transgenic plants and in the three double transgenic plants carrying either LAT52P:AmiS₂-SLF1 or LAT52P:AmiGFP. The mature amiRNA targeting S₂-SLF1 was detected in all these five S₂S₃ transgenic plants carrying LAT52P:AmiS₂-SLF1 and in the S₂S₃ double transgenic plant carrying the LAT52P:AmiS₂-SLF1 transgene; the mature amiRNA targeting GFP was also detected in the two S₂S₃ double transgenic plants carrying the LAT52P:AmiGFP transgene (Figure 3B). Thus, the failure of the amiRNA targeting S₂-SLF1 to suppress the expression of the endogenous S₂-SLF1 gene was not due to lack of production of the mature amiRNA.
We thus decided to test whether AmiS2-SLF1 driven by the promoter of S2-SLF1 could suppress the expression of the endogenous S2-SLF1 gene. We used a genomic fragment (named S2P) containing the 2577-bp sequence upstream of the translation initiation codon of S2-SLF1 to make a pBI101-S2P-AmiS2-SLF1 construct (see Supplemental Figure 2 online) and introduced it into wild-type S2S3 plants. We identified 14 transgenic plants by PCR, using a pair of primers specific to the transgene, and performed real-time PCR to measure the levels of the S2-SLF1 transcript in stage 5 anthers of all these transgenic plants and a wild-type S2S3 plant. The results for the three transgenic plants, S2P-AmiS2-SLF1/S2S3-4, -13, and -24, that showed the most significant reduction in the level of the S2-SLF1 transcript when compared with level of the wild-type S2S3 plant are shown in Figure 4A. We then used the stem-loop real-time RT-PCR procedure to show that the mature amiRNA was produced in all of them (Figure 4B).

The findings that (1) the amiRNA targeting S2-SLF1 that was produced in the vegetative cell suppressed the expression of S2-SLF1:GFP there, but not the expression of the endogenous S2-SLF1 gene, and (2) the same amiRNA expressed by the promoter of the S2-SLF1 gene suppressed the expression of the endogenous S2-SLF1 gene suggest that the S2-SLF1 gene is specifically expressed in the generative cell of pollen and that, contrary to the observation in Arabidopsis (Slotkin et al., 2009), there may be no path for transporting 21-nucleotide miRNA generated in the vegetative cell to the generative cell in Petunia pollen (see Supplemental Figure 6 online).

**S2-SLF1 Localized in Cytoplasm of Vegetative Cell of Pollen**

To examine the subcellular localization of S2-SLF1, we generated two Ti plasmid constructs using the promoter of S2-SLF1 to drive the expression of GFP and S2-SLF1:GFP. The constructs, pBI101-S2P-GFP and pBI101-S2P-SLF1:GFP (see Supplemental Figure 2 online), were separately introduced into wild-type S2S3 plants. We first examined in vitro–germinated pollen tubes of 13 transgenic plants carrying S2P-GFP and 21 transgenic plants carrying S2P-S2-SLF1:GFP for GFP fluorescence. The LAT52P:S2-SLF1:GFP***/S2S3 transgenic plant was used for comparison, and a wild-type S2S3 plant was included as negative control. As expected, pollen tubes of the LAT52P:S2-SLF1:GFP***/S2S3 transgenic plant showed strong fluorescence (Figure 5A), and no fluorescence was detected in pollen tubes of the wild-type S2S3 plant (Figure 5B). Seven of the 21 S2P:S2-SLF1:GFP transgenic plants and two of the 13 S2P-GFP transgenic plants showed visible fluorescence; the results of one such S2P:S2-SLF1:GFP transgenic plant and one such S2P-GFP transgenic plant are shown in Figures 5C and 5D, respectively. The weaker fluorescence signals
observed with the $S_2$-SLF1 promoter-driven GFP than with the $LAT52$ promoter-driven GFP is consistent with the real-time PCR results shown in Figure 2A.

We next used confocal microscopy to examine GFP fluorescence in mature pollen of these four plants. As pollen tends to autofluoresce under the conditions used to observe GFP fluorescence, we used a lambda spectral scan function of the confocal microscope to distinguish between autofluorescence/background fluorescence and GFP fluorescence. For the $LAT52P:S_2$-SLF1:GFP$^*/S_2S_3$ transgenic plant, strong GFP fluorescence was evenly distributed in the vegetative cell cytoplasm of pollen (Figure 5E), whereas for the wild-type $S_2S_3$ plant, only autofluorescence/background fluorescence was observed (Figure 5F). Like the $LAT52P:S_2$-SLF1:GFP$^*/S_2S_3$ transgenic plant, GFP fluorescence of both $S_2P:S_2$-SLF1:GFP/S_2S_3 and $S_2P$-GFP/S_2S_3 transgenic plants was also evenly distributed in the vegetative cell cytoplasm of pollen (Figures 5G and 5H).

$S_2$-SLF1 Is Not the Only SLF in $S_2$ Pollen That Interacts with $S_7^-$, $S_7^+$, and $S_{13}^-$RNases

The three transgenic plants, $S_2P:AmiS_2$-SLF1/S_2S_3-4, -13, and -24, that showed significant degrees of suppression of the endogenous $S_2$-SLF1 gene by the amiRNA targeting $S_2$-SLF1 (Figure 4) were examined for their SI behavior. We first used real-time PCR to determine the copy number of the $S_2P:AmiS_2$-SLF1 transgene carried by each of these three transgenic plants. The validity of this method was assessed by comparing the copy number of the $LAT52P:AmiS_2$-SLF1 transgene carried by each of three transgenic plants, $LAT52P:AmiS_2$-SLF1/S_2S_3-6, -8, and -17 (Figure 3A), determined by genomic DNA gel blotting with that determined by real-time PCR. Based on genomic DNA gel blotting, the copy numbers for transgenic plants -6, -8, and -17 were 1, 2, and 2, respectively (see Supplemental Figure 7 online). Real-time PCR was performed using the primer pair PiSLF3Copy1for/PiSLF3Copy1rev for $S_2$-SLF1 and the primer pair AmiSPL1Copy1for/AmiSPL1Copy1rev (see Supplemental Figure 2 online) for $AmiS_2$-SLF1, with $S_2$-SLF1 serving as internal control (as each transgenic plant carried one copy of this gene) and transgenic plant -6 serving as the control plant (as it carried one copy of the transgene based on genomic blotting). The results showed that the transgenic plants -6, -8, and -17 carried 1, 1.91 ± 0.18, and 2.10 ± 0.10 copies of the $LAT52P:AmiS_2$-SLF1 transgene, respectively, consistent with the results from genomic DNA gel blotting. Using the same real-time PCR method, we determined the copy numbers of the $AmiS_2$-SLF1 transgene for $S_2P:AmiS_2$-SLF1/S_2S_3-4, -13, and -24 to be 1, 2, and 1, respectively (Table 2). All these three transgenic plants remained self-incompatible (Table 1).

To examine whether suppression of expression of $S_2$-SLF1 in $S_2$ pollen had any effect on the ability of $S_2$ transgenic pollen to be accepted by pistils carrying $S_3$-haplotype, we pollinated

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Figure 3. Real-Time PCR Analysis of Transcript Levels of Endogenous $S_2$-SLF1 and PCR Analysis of Mature amiRNA Levels in Five $S_2S_3$ Transgenic Plants Carrying $LAT52P:AmiS_2$-SLF1.

"A" Endogenous $S_2$-SLF1 transcript levels. The primer pair, PiSLF2RT3for and PiSLF2RT4rev, was used to specifically amplify the endogenous $S_2$-SLF1 gene from the five transgenic plants, as indicated, and a wild-type $S_2S_3$ plant. Three biological replicates were performed for $LAT52P:AmiS_2$-SLF1/S_2S_3-3 and -6 and wild-type $S_2S_3$, and one biological sample was used for the other three transgenic plants. Each biological replicate/sample consisted of three technical replicates. The y axis shows the $S_2$-SLF1 transcript level of each transgenic plant relative to that of the wild-type $S_2S_3$ plant. The $S_2$-SLF1 transcript levels in all the transgenic plants are not significantly different from that of the wild-type $S_2S_3$ plant based on the Student’s t test (P value > 0.01).

(B) Mature amiRNA levels of amiRNA targeting $S_2$-SLF1 in the five $S_2S_3$ transgenic plants shown in Figure 3A and the $S_2S_3$ double transgenic plant carrying $LAT52P:AmiS_2$-SLF1 and mature amiRNA levels of amiRNA targeting GFP in two $S_2S_3$ double transgenic plants carrying $LAT52P:AmiGFP$. Two asterisks indicate plants homozygous for the $LAT52P:S_2$-SLF1:GFP transgene. One biological sample was used for each plant, and each biological sample consisted of three technical replicates. The y axis shows the mature amiRNA level of a transgenic plant relative to that of the $S_2S_3$ double transgenic plant carrying $LAT52P:AmiS_2$-SLF1. An asterisk placed above a bar means
wild-type $S_2S_3$ plants with pollen from these three transgenic plants and raised 25, 29, and 32 progeny plants, respectively. In the case of transgenic plants $S2P:AmiS_2$-SLF1/$S_2S_3$-13 and -24 (each carrying one copy of the transgene), half of the $S_2$ pollen and half of the $S_3$ pollen produced were expected to carry the transgene, but as $AmiS_2$-SLF1 produced amiRNA specifically targeting $S_2$-SLF1, the $S_2$ transgenic pollen should behave the same as wild-type $S_2$ pollen and be rejected by $S_2S_3$ pistils. We determined the S-genotypes of all the progeny plants by PCR using primers specific to $S_2$-RNase and primers specific to $S_3$-RNase and determined whether each plant had inherited the transgene by PCR using primers specific to the $S2P:AmiS_2$-SLF1 transgene. All progeny plants were $S_2S_3$ genotype and $\sim 50\%$ of the plants in each progeny had inherited the $AmiS_2$-SLF1 transgene (Table 2). For $S2P:AmiS_2$-SLF1/$S_2S_3$-13, even though it carried two copies of the transgene, in this cross, as well as in four additional crosses described below, $\sim 50\%$ of the plants in each progeny inherited the $S2P:AmiS_2$-SLF1 transgene, suggesting that the two copies were tightly linked and transmitted as a single gene unit. The results of the crosses with wild-type $S_2S_3$ plants suggest that both wild-type $S_2$ pollen and $S_3$ pollen carrying $AmiS_2$-SLF1 were accepted by $S_2S_3$ pistils. We next used pollen from $S2P:AmiS_2$-SLF1/$S_2S_3$-4, -13, and -24 to pollinate wild-type $S_2S_3$ plants and raised 24, 31, and 30 progeny plants, respectively. The results of PCR analyses showed that all these plants were $S_2S_3$ genotype and that $\sim 50\%$ of the plants in each progeny carried the transgene (Table 2), confirming that $S_2$ transgenic pollen behaved as wild-type $S_2$ pollen.

We also used pollen from these three transgenic plants to pollinate a previously generated transgenic plant $AS-S_2$-RNase/ $S_2S_3$ (carrying an antisense $S_2$-RNase gene), which did not produce any $S_2$-RNase in the pistil. Thus, $S_2$ pollen produced by the three $S2P:AmiS_2$-SLF1/$S_2S_3$ transgenic plants should be accepted by the pistils of $AS-S_2$-RNase/$S_2S_3$ to produce $S_2S_3$ plants, with $\sim 50\%$ carrying the $S2P:AmiS_2$-SLF1 transgene. For $S_2$ pollen, regardless of whether or not it carried the $S2P:AmiS_2$-SLF1 transgene and whether the transgene had any effect on the ability of $S_2$ transgenic pollen to detoxify $S_2$-RNase, it should also be accepted by the pistils of $AS-S_2$-RNase/$S_2S_3$ to produce $S_2S_3$ plants, with $\sim 50\%$ carrying the $S2P:AmiS_2$-SLF1 transgene. The results of PCR analyses of 32 plants in each progeny were indeed as expected: Both $S_2S_3$ and $S_2S_3$ genotypes were obtained in each progeny and $\sim 50\%$ of the plants from each genotype inherited the $AmiS_2$-SLF1 transgene (Table 2). All the results from the crosses described above suggest that suppression of $S_2$-SLF1 did not affect the ability of $S_2$ transgenic pollen to detoxify $S_3$-RNase.

As the results of the transgenic functional assay suggested that $S_2$-SLF1 also interacted with $S_7$- and $S_{13}$-RNases, we further examined whether $S_2$-SLF1 is the only type of SLF in $S_2$ pollen that interacts with these two non-self S-RNases. We used pollen from $S2P:AmiS_2$-SLF1/$S_2S_3$-13 and -24 to pollinate wild-type $S_2S_3$ plants and determined the S-genotypes of 60 and 47 progeny plants, respectively, and the inheritance of the $S2P:AmiS_2$-SLF1 transgene. All four possible S-genotypes, $S_2S_3$, $S_2S_7$, $S_2S_{13}$, and $S_2S_{19}$, were found in each progeny, and $\sim 50\%$ of the plants from each S-genotype had inherited the $AmiS_2$-SLF1 transgene (Table 2), suggesting that suppression of

Figure 4. Real-Time PCR Analysis of Transcript Levels of Endogenous $S_2$-SLF1 and PCR Analysis of Mature amiRNA Levels in Three $S_2S_3$ Transgenic Plants Carrying $S2P:AmiS_2$-SLF1.

Each error bar indicates se.

(A) Endogenous $S_2$-SLF1 transcript levels. $S2P$ denotes the promoter of $S_2$-SLF1. The primer pair PiSLF2RT3for and PiSLF2RT4rev was used for specific amplification of the endogenous $S_2$-SLF1 gene. Three biological replicates were performed for each transgenic plant and a wild-type $S_2S_3$ plant. An asterisk placed above a bar means that the $S_2$-SLF1 transcript level in that transgenic plant is significantly lower than that of the wild-type $S_2S_3$ plant, based on the Student's t test (P value < 0.01).

(B) Mature amiRNA levels. One biological sample was used for each plant. Each biological sample consisted of three technical replicates. The y axis shows the mature amiRNA level of a transgenic plant relative to that of the $S_2S_3$ double transgenic plant carrying LAT52P:AmiS_2-1RT3. An asterisk placed above a bar means the mature amiRNA level in that transgenic plant is significantly different from that of the wild-type $S_2S_3$ plant, based on the Student's t test (P value < 0.05).
Figure 5. Subcellular Localization of S2-SLF1.

(A) to (D) GFP fluorescence of in vitro-germinated pollen tubes of three transgenic plants, as indicated, and a wild-type (WT) S2S3 plant. For each plant, the left panel shows a dark-field image of pollen tubes under 5-s exposure to UV light, and the right panel shows a bright-field image of pollen tubes under 50-ms exposure to white light.

(E) to (H) Fluorescence and/or autofluorescence detected in mature pollen of the same three transgenic plants and wild-type S2S3 plant. For each plant, the right panels show spectral lambda scans of two selected areas of their corresponding image(s) shown in the left panel(s). (A spectra lambda scan is a spectral imaging method using 488-nm laser excitation to acquire a collection of images captured at different wavelengths, producing a complete spectrum of specimen at every pixel location.) The GFP fluorescence was identified based on its characteristic peak profile.

(A) S2S3 transgenic plant homozygous for the S2-SLF1:GFP transgene driven by the LAT52 promoter.

(B) Wild-type S2S3 plant.

(C) S2S3 transgenic plant expressing S2-SLF1:GFP driven by the promoter of S2-SLF1.

(D) S2S3 transgenic plant expressing GFP driven by the promoter of S2-SLF1.

(E) S2S3 transgenic plant homozygous for the S2-SLF1:GFP transgene driven by the LAT52 promoter. The top right panel shows background fluorescence of the area labeled “1” in the corresponding fluorescence image, and the bottom right panel shows GFP fluorescence of the area labeled “2” in the corresponding fluorescence image.

(F) Wild-type S2S3 plant. Both top and bottom right panels show autofluorescence of the areas labeled “1” and “2” in their corresponding fluorescence images.
S2-SLF1 in S2 pollen did not affect the ability of S2 transgenic pollen to detoxify S7- or S13-RNase.

As the two copies of the transgene carried by S2:AmiS2-SLF1/S2S3-13 were transmitted as a single gene unit, we calculated the efficiency of suppression for all three transgenic plants, S2:AmiS2-SLF1/S2S3-4, -13, and -24, based on each having a single copy of the transgene. In this case, 50% of the S2 pollen produced should carry the transgene, so if the amiRNA completely silences the expression of S2-SLF1 in S2 transgenic pollen, the transcript level of S2-SLF1 produced by each transgenic plant should be 50% that of the wild-type S2S3 plant. From the results of the real-time PCR analysis shown in Figure 4A, we determined the transcript levels of S2:AmiS2-SLF1/S2S3-4, -13, and -24, based on three biological replicates for each plant and three technical replicates for each biological sample, to be 0.66 ± 0.07, 0.53 ± 0.05, and 0.59 ± 0.05, respectively, with the level of the wild-type S2S3 plant set at 1. Thus, the levels of suppression were 68.67% ± 14.28% for S2:AmiS2-SLF1/S2S3-4, 93.56% ± 9.46% for S2:AmiS2-SLF1/S2S3-13, and 81.78% ± 9.51% for S2:AmiS2-SLF1/S2S3-24. Despite these high levels of suppression, particularly in the case of S2:AmiS2-SLF1/S2S3-13 and S2:AmiS2-SLF1/S2S3-24, S2 pollen carrying the S2:AmiS2-SLF1 transgene was still fully accepted by S3S3 and S13S1 plants, suggesting that S2-SLF1 is not the only SLF in S2 pollens that interacts with S3S3, S7S13, and S13S1.

Finally, we used pollen from S2:AmiS2-SLF1/S2S3-13 and -24 to pollinate wild-type S2S1 plants and analyzed 86 and 50 plants in each progeny to determine their S-genotypes and inheritance of the AmiS2-SLF1 transgene. All four possible S-genotypes, S3S3, S5S11, S2S13, and S3S3, were found in each progeny and ~50% of the plants from each S-genotype inherited the AmiS2-SLF1 transgene (Table 2). That suppression of

Table 2. Progeny Analysis of Crosses Involving Three S2P:AmiS2-SLF1/S2S3 Transgenic Plants

<table>
<thead>
<tr>
<th>Male Parent</th>
<th>Copy No.</th>
<th>Female Parent</th>
<th>Observed Ratio</th>
<th>Progeny S-Genotype</th>
<th>Expected Ratio</th>
<th>$\chi^2$</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>S2P:AmiS2-SLF1/S2S3-4</td>
<td>1.29 ± 0.09</td>
<td>S2S3</td>
<td>10:25</td>
<td>S2S3</td>
<td>1:2</td>
<td>1.00</td>
<td>0.32</td>
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<tr>
<td>S2P:AmiS2-SLF1/S2S3-4</td>
<td>1.29 ± 0.09</td>
<td>S2S2</td>
<td>8:24</td>
<td>S2S2</td>
<td>1:2</td>
<td>2.67</td>
<td>0.10</td>
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<tr>
<td>S2P:AmiS2-SLF1/S2S3-4</td>
<td>1.29 ± 0.09</td>
<td>AS-S3R/S3S3 b</td>
<td>10:24</td>
<td>S3S3</td>
<td>1:2</td>
<td>0.67</td>
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<tr>
<td>S2P:AmiS2-SLF1/S2S3-13</td>
<td>2.28 ± 0.38</td>
<td>S3S3</td>
<td>14:29</td>
<td>S3S3</td>
<td>1:2</td>
<td>0.03</td>
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<tr>
<td>S2P:AmiS2-SLF1/S2S3-13</td>
<td>2.28 ± 0.38</td>
<td>AS-S3R/S3S3 b</td>
<td>14:31</td>
<td>S3S3</td>
<td>1:2</td>
<td>0.29</td>
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<td>S2P:AmiS2-SLF1/S2S3-13</td>
<td>2.28 ± 0.38</td>
<td>AS-S3R/S3S3 b</td>
<td>8:18</td>
<td>S3S3</td>
<td>1:2</td>
<td>0.11</td>
<td>0.74</td>
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<tr>
<td>S2P:AmiS2-SLF1/S2S3-13</td>
<td>2.28 ± 0.38</td>
<td>S2S3</td>
<td>7:14</td>
<td>S2S3</td>
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*aNumber of plants that had inherited AmiS2-SLF1 transgene/total number of plants examined.

*bAS-S3R/S3S3 stands for AS-S3-RNase/S3S3.
$S_2$-SLF1 in $S_2$ pollen did not affect the ability of $S_2$ transgenic pollen to detoxify $S_5$- or $S_{11}$-RNase is consistent with the gain-of-function results showing that $S_2$-SLF1 is not the type of SLF that interacts with $S_5$- or $S_{11}$-RNase.

**DISCUSSION**

The recent discovery that pollen specificity in the S-RNase–based SI system possessed by *Petunia* is not only regulated by the SLF gene reported by Sijacic et al. (2004) (now named $SLF_1$), but also by at least two other types of SLF genes (Kubo et al., 2010), has added another layer of complexity to this already complex self/non-self recognition system. To gain a comprehensive understanding of how pollen of a given S-haplotype employs a battery of SLF proteins to defend against invasion of all non-self S-RNases to ensure cross-compatible pollination will require the identification of all SLF proteins that constitute pollen specificity and establishing the relationship between each SLF protein and all non-self S-RNases via the now well-established transgenic functional assay (Sijacic et al., 2004; Hua et al., 2007; Kubo et al., 2010; see Supplemental Figure 1 online). This is a daunting task considering that there are a large number of S-haplotypes in any self-incompatible species.

However, once an SLF protein of an S-haplotype is found to interact with a certain non-self S-RNase based on the transgenic functional assay, we can use this pair of SLF and S-RNase to begin to explore the complex network of the interactions between SLFs and S-RNases. For example, we can use a loss-of-function approach to examine whether suppression of the production of this SLF will result in the rejection of the pollen by otherwise compatible pistils producing this non-self S-RNase. If so, the result would suggest that this SLF protein is solely responsible for detoxifying this non-self S-RNase. If not, the result would suggest that at least one other SLF protein is also involved in detoxifying this non-self S-RNase. We can then test other known types of SLF proteins to see which one(s) might. If none of them can, this would suggest that there exist as yet unidentified SLF protein(s) in pollen of this S-haplotype.

In this work, we used the transgenic functional assay to examine the relationships between $S_2$-SLF1 of *P. infata* and four of its non-self S-RNases, and the results suggest that it interacts with two additional non-self S-RNases besides the two ($S_1$- and $S_2$-RNases) previously identified (but not with two other non-self S-RNases tested). We also established the approach of amiRNAs as a viable approach for efficiently suppressing the expression of SLF genes in pollen. The results suggest that SLF1 is specifically expressed in the generative cell in pollen and that, in contrast with what was observed in *Arabidopsis*, miRNA generated in the vegetative cell is not transported to the generative cell. Thus, this work further supports the collaborative non-self recognition model and provides insight into the complicated relationships between SLF proteins and S-RNases.

**Use of amiRNA to Suppress Expression of $S_2$-SLF1 in Pollen**

The approaches of long-hairpin RNAs have been commonly used in gene silencing, but almost all the reported downregulation experiments were performed in diploid sporophytic tissues (Grant-Downton et al., 2009). There are a few reports of success in using this approach to silence genes in haploid male gametophytes. Gupta et al. (2002) used long-hairpin RNAs to suppress the expression of a pollen specific gene in *Arabidopsis PTEN1* (for phosphatase and tensin homolog) and found a pollen lethal phenotype after mitosis in some of the RNA interference (RNA) lines. Takeda et al. (2006) used long-hairpin RNAs to silence the expression of another pollen specific gene in *Arabidopsis*, TCP16 (for transcription factor TCP16), and found both abnormal and normal pollen grains in the RNAi plants. Zhao et al. (2010) used long-hairpin RNAs to downregulate SLF-INTERACTING Skp1-LIKE1 of *P. hybrida* (which might encode a component of SLF-containing E3 ubiquitin ligase complexes) in pollen and concluded that the RNAi plants exhibited reduced cross-compatibility. However, Xing and Zachgo (2007) discovered pollen lethality in *Arabidopsis* RNAi lines generated via the use of long-hairpin RNAs to suppress AGAMOUS-LIKE18 (AGL18) but did not find the same phenotype in the T-DNA knockout mutant, agl18. They then generated RNAi lines to suppress genes that are not known to be expressed in pollen and showed that long-hairpin RNAs themselves, rather than silencing a specific gene, caused the pollen lethal phenotype.

The mechanism of gene silencing by long-hairpin RNAs is not completely understood, but it is generally believed that they are predominantly processed by DICER-LIKE4 (DCL4) into 21-nucleotide small interfering RNAs; one strand of the small interfering RNAs then anneals with its target transcript by base pairing, leading to the cleavage of the target transcript by ARGONAUTE1 (AGO1) (Small, 2007). However, Grant-Downton et al. (2009) showed by RT-PCR that DCL4 is not expressed during pollen development, further suggesting that using the approach of long-hairpin RNAs to silence pollen-expressed genes may not work.

miRNAs have been identified in mature pollen of *Arabidopsis* and tobacco (Grant-Downton et al., 2009). In addition to identifying the preliminary miRNA, primary miRNA, and mature miRNA, Grant-Downton et al. (2009) also found that the genes encoding two key enzymes in the miRNA pathway, DCL1 and AGO1, were expressed throughout pollen development. Moreover, the sizes of the cleavage products of miRNA target transcripts were as predicted, further indicating that the miRNAs in mature pollen are functional in silencing.

Therefore, we decided to use the approach of miRNA to design a strategy to suppress the expression of $S_2$-SLF1. We first used the LAT52 promoter, which is specifically active in the vegetative cell nucleus of microspores/pollen, to express two amiRNA constructs, one targeting $S_2$-SLF1 and the other targeting GFP of the $S_2$-SLF1::GFP transgene driven by the LAT52 promoter, and introduced the amiRNA constructs into $S_2$-transgenic plants homozygous for the LAT52:S2-SLF1::GFP transgene. This experimental design allowed us to first use GFP fluorescence in vitro–germinated pollen tubes to assess whether these amiRNA constructs were capable of suppressing the expression of the LAT52 promoter–driven transgene (Figure 1). Moreover, Slotkin et al. (2009) reported that, in *Arabidopsis*, an amiRNA driven by the LAT52 promoter successfully suppressed the expression of an enhanced GFP transgene expressed by a sperm cell–specific promoter, suggesting that miRNAs can be transported...
from the vegetative cell cytoplasm to the sperm cell to silence the target genes there. As it was not known whether SLF genes are specifically expressed in the vegetative cell and/or generative cell, we thought that if a similar mechanism existed for transporting miRNA from the vegetative cell cytoplasm to the generative cell in Petunia, using the LAT52 promoter would lead to suppression of S2-SLF1 regardless of its site of expression.

**SLF Genes Are Specifically Expressed in the Generative Cell, but There May Be No Path for Transporting miRNAs from Vegetative Cell Cytoplasm to the Generative Cell in Petunia Pollen**

Unexpectedly, we found that whereas the LAT52 promoter–driven amiRNA targeting S2-SLF1 efficiently suppressed the expression of the S2-SLF1 transgene driven by the LAT52 promoter (Figure 2A), it had no effect on the expression of the endogenous S2–LAT52 (Figure 2B and 3A). All the SLF genes of Petunia characterized so far are specifically expressed in pollen (Sijacic et al., 2004; Hua et al., 2007; Kubo et al., 2010). For S2–LAT52, its transcript is first detected in stage 3 anthers (containing microspores undergoing mitosis), peaks in stage 4 anthers (containing immature bicellular pollen), and then declines, but is still detectable in stage 5 anthers, mature pollen, and in vitro–germinated pollen tubes. In Petunia, the LAT52 promoter is active during the pollen developmental stages when the endogenous S2–LAT52 is expressed; thus, the lack of suppression by the LAT52 promoter–driven amiRNA suggests that the SLF genes are transcribed in the generative cell of microspores and mature pollen and that, contrary to the finding in Arabidopsis, there may be no path for transporting miRNAs from the vegetative cell cytoplasm to the generative cell to silence their target genes there (see Supplemental Figure 6 online). This difference may be attributed to the fact that mature pollen of Petunia is bicellular, whereas mature pollen of Arabidopsis is tricellular. Bicellular pollen and tricellular pollen might use different mechanisms to communicate between the vegetative cell and generative/sperm cell. A cytoplasmic projection at the late bicellular pollen stage between the generative cell and vegetative cell nucleus has been found in many species, including those producing bicellular or tricellular pollen. McCue et al. (2011) proposed that this kind of connection could serve as a path for transporting miRNAs from the vegetative cell cytoplasm to the generative/sperm cell in Arabidopsis. In tricellular pollen, the transport might initiate at the bicellular stage, as the sperm-specific enhanced GFP (Slotkin et al., 2009) starts to be expressed from this stage in Arabidopsis (Engel et al., 2005), but in bicellular pollen, the transport or path of transport might initiate or form after pollen germination.

We further showed that the endogenous S2–SLF1 gene was suppressed by the same amiRNA driven by the promoter of the S2–SLF1 gene (Figure 4A), suggesting that the miRNA pathway exists in the generative cell of Petunia mature pollen. As the transcript of S2–SLF1 is also detected in in vitro–germinated pollen tubes, it is likely that the expression of SLF genes of Petunia continues in the sperm cell during pollen tube growth. These results are consistent with the detection of the expression of DCL1 and the homolog of AGO1 in the sperm cell of Arabidopsis (Borges et al., 2008). Interestingly, analysis of sperm cell transcripts of Arabidopsis has shown that genes involved in the ubiquitin-mediated proteolysis pathway are enriched in the sperm cell and that the most highly expressed genes in the sperm cell encode F-box proteins (Borges et al., 2008). Using transgenic plants carrying either GFP or S2–SLF1:GFP driven by the S2–SLF1 promoter, we showed that S2–SLF1 is located in the vegetative cell cytoplasm of both mature pollen and in in vitro–germinated pollen tubes. This finding is consistent with the subcellular localization of SLF–S2 (the S2–allelic variant of an SLF in Antirrhinum) detected by immunocytochemistry (Wang and Xue, 2005). Moreover, as the LAT52:S2–SLF1:GFP transgene functions the same as the S2P:S2–SLF1 transgene in causing breakdown of SI in S1, but not S2 pollen (Sijacic et al., 2004; Hua et al., 2007), and as S2–SLF1:GFP (expressed by the LAT52 promoter) is localized in the cytoplasm of the vegetative cell (Figure 5E), it would seem logical that the endogenous S2–SLF1 is also localized in the cytoplasm of the vegetative cell. If true, this would suggest that S2–SLF1 is transcribed in the generative nucleus, the transcript is translated in the cytoplasm of the generative cell, and the protein is later transported into the cytoplasm of the vegetative cell. The cytoplasmic location of S2–SLF1 (and likely all other types of SLF proteins) is also consistent with its role in interacting with a subset of non-self S-RNases to target them for degradation, as the results from ectopic expression of S-RNase in pollen suggested that S-RNase exerts its cytotoxicity in the cytoplasm (Meng et al., 2009). In the sperm cell cDNA library of maize (Zea mays) studied by Engel et al. (2003), 8% of the sequences were predicted to encode secreted or plasma membrane–localized proteins, suggesting that secretion of proteins from generative/sperm cells into the cytoplasm is possible. However, at present, the existence of any secretory process for S2–SLF1 is entirely based on indirect evidence and remains speculative, as SLF proteins do not contain any known signal peptide or other secretion signal.

**Gain-of-Function Transgenic Assay and Loss-of-Function Studies of S2-SLF1 Further Support the Collaborative Non-Self Recognition Model and Provide Insight into Complex Relationships between SLF Proteins and S-RNases**

S2–SLF1 was the first SLF protein examined for possible function in SI, and the results from the transgenic functional assay suggested that it interacted with both non-self S-RNases tested, S1– and S3–RNases (Sijacic et al., 2004). In this work, we used the same assay to examine the relationships between S2–SLF1 and four additional non-self S-RNases, and the results suggest that S2–SLF1 interacts with two of them. The relationship between S2–SLF1 and these six non-self S-RNases further supports the prediction by the collaborative non-self recognition model (Kubo et al., 2010) that each SLF protein only interacts with a subset of non-self S-RNases. Among the SLF proteins and S-RNases whose interactions have been observed by coimmunoprecipitation, in vitro protein interaction assays, or indirectly inferred from the transgenic functional assay, S2–SLF1 interacts with the largest number of non-self S-RNases; previously,
S2-SLF2 was found to interact with three of the five non-self S-RNases tested (Kubo et al., 2010).

For Petunia, the function of the S-RNase gene in regulating pistil specificity was established through both gain- and loss-of-function experiments (Lee et al., 1994), while gain-of-function experiments have demonstrated the function of three types of S-RNase genes in pollen specificity (Sijacic et al., 2004; Kubo et al., 2010). For the S-RNase gene, an antisense S2-SRNase RNA expressed in the pistil efficiently suppressed the production of S2-SRNase (Lee et al., 1994). In this work, we used the approach of amiRNA to suppress the expression of S2-SLF1 in the generative cell of pollen and identified three transgenic plants, S2P:AmiS2-SLF1/S2S2-4, -13, and -24, that showed the most significant suppression of the S2-SLF1 transcript (Figure 4A).

As the results of the transgenic functional assay suggests that S2-SLF1 interacts with S2-, S7-, and S13-RNases (Sijacic et al., 2004; see Supplemental Figure 1 online), we examined whether suppression of S2-SLF1 would render S2 transgenic pollen tubes incapable of detoxifying these three non-self S-RNases when growing in pistils of S2S2 and S2S13 genotypes. The results from all the genetic crosses summarized in Table 2 showed that these three transgenic plants, S2 pollen carrying the S2P:AmiS2-SLF1 transgene was still fully accepted by S2S2 and S2S13 pistils. We interpret these results to mean that S2-SLF1 is not the only S-RNase gene, an antisense transcription for S2-SLF1 interacts with S3-, S7-, and S13-RNases (Sijacic et al., 2010). Employing multiple types of S2-SLF1 to recognize and detoxify any given non-self S-RNase will allow plants to minimize the loss of cross-compatibility caused by mutations in any of the S-RNase genes, as for an SI system to remain fully functional over time, it is just as important for pistils not to reject non-self pollen.

However, at this time, we cannot completely rule out the possibility that some small amounts of S2-SLF1 might still be produced in S2 transgenic pollen and be responsible for the normal SI function. To ultimately validate the collaborative nature of different SLF proteins in detoxifying the same S-RNase, we will have to identify any additional SLF protein(s) that interact with S2-, S7-, and/or S13-RNases. We could then design amiRNAs to simultaneously suppress the expression of two or more types of S-RNase genes that are involved and examine the SI behavior of transgenic pollen. As more SLF proteins are shown to be involved in pollen specificity based on their interactions with at least one non-self S-RNase, their interactions with S2-, S7-, and/or S13-RNases will be examined.

METHODS

Plant Material

S2S2, S3S3, and S5S5 genotypes of Petunia inflata were described by Ai et al. (1990). S2-, S5-, S7-, and S13-haplotypes of P. inflata were described by Wang et al. (2001), and S2S11 and S2S13 plants were generated by crosses between S2S2 and S2S11 and between S2S5 and S2S13 plants, respectively. Plants of S2S2 and S2S13 genotypes were generated by crosses between S2S2 and S2S13 plants, and plants of S2S5 and S2S13 genotypes were generated by crosses between S2S5 and S2S13 plants.

The S-genotypes of all the plants were determined by PCR using primers specific to S-RNase or SLF1 of each haplotype. The AS-S2-RNase/S5S5 transgenic plants were identified from selfed progeny of the AS-S2-RNase/S5S5 transgenic plants described by Lee et al. (1994).

Construction of AmiS2-SLF1 and AmiGFP Stem-Loop Precursors

The 423-bp stem-loop precursor AmiS2-SLF1 was constructed by integrating the miRNA sequence targeting S2-SLF1 into the miR319a stem-loop precursor on plasmid pRS300 following the protocol of Schwab et al. (2006). Primers SPL1-IImiRs, SPL1-IImiRa, SPL1-IImiR’s, SPL1-IImiR’s, SPLA, and SPLB (see Supplemental Table 1 online) corresponding to segments I, II, IV, A, and B respectively (see Supplemental Figure 3 online), were used in separate PCRs with the pRS300 template to generate fragments A-IV, III-II, and I-B. These three fragments were then fused together to yield fragment SPLA-SPLB by overlapping PCR using the primers corresponding to segments A and B. The fragment SPLA-SPLB was further amplified using SPLNcoolf and SPLNotrev as primers to introduce the Ncol and NotI restriction sites. The 423-bp stem-loop precursor AmiGFP was similarly constructed except that primers SPL9-IImiRs, SPL9-IImiRa, and SPL9-IImiR’s corresponding to segments I, II, III, and IV, along with primers SPLA and SPLB, were used to generate fragments A-IV, III-II, and I-B.

Generation of Ti Plasmid Constructs and Plant Transformation

All Ti plasmid constructs used in plant transformation are schematically shown in Supplemental Figure 2 online. pBI101-LAT52P:S2-SLF1-GFP was described by Hua et al. (2007). The 423-bp stem-loop precursor AmiS2-SLF1 was subcloned into pGEM-T Easy vector (Promega), released by Ncol-NotI digestion, and the 423-bp Ncol-NotI fragment was used to replace the 0.72-kb Ncol-NotI fragment of the GFP coding sequence in pLAT-LAT52P-GFP (Dowd et al., 2006) to yield pLAT-LAT52P-AmiS2-SLF1. pLAT-LAT52P-AmiGFP was similarly generated using the 423-bp stem-loop precursor AmiGFP. The fragment containing LAT52P-AmiS2-SLF1 was released from pLAT-LAT52P-AmiS2-SLF1 by Sall-EcoRI digestion and inserted into the Sall-EcorI-digested pBI101 and pCAMBIA1300 to yield pBI101-LAT52P-AmiS2-SLF1 and pCAMBIA1300-LAT52P-AmiS2-SLF1, respectively. The fragment containing LAT52P-AmiGFP was reamplified using pLAT-LAT52P-AmiGFP as template and SPlxholf/SPlxpserv as primers, subcloned into pGEM-T Easy vector, released by Xhol-Spel digestion, and inserted into the Xhol-Spel-digested pTA7001 to yield pTA7001-LAT52P-AmiGFP. The 2577-bp S2-SLF1 native promoter, S2P, was released from pBI101-S2P-SLF1 by Sall-Ncol digestion and used to replace the Sall-Ncol fragment of the LAT52 promoter in pLAT-LAT52P-AmiS2-SLF1 to yield pLAT-S2P-AmiS2-SLF1. pLAT-S2P-AmiS2-SLF1 was digested by Sall-EcoRI, and S2P-AmiS2-SLF1 was cut into two fragments, the 2.2-kb fragment of S2P and the 1.1-kb fragment containing the rest of S2P and AmiS2-SLF1. The 2.2-kb fragment was first inserted into Sall-EcoRIdigested pBI101; the resulting plasmid was digested by EcoRI, and the 1.1-kb fragment was ligated into it to yield pBI101-S2P-AmiS2-SLF1. pBI101-S2P-AmiGFP was similarly generated using pLAT-LAT52P-GFP. The 2577-bp S2-SLF1 native promoter, S2P, was released from pBI101-S2P-SLF1 by Sall-Ncol digestion and used to replace the Sall-Ncol fragment of the LAT52 promoter in pLAT-LAT52P-S2P-SLF1-GFP (Hua et al., 2007) to yield pLAT-S2P-S2P-SLF1-GFP. The S2P-S2P-SLF1-GFP fragment was amplified by PCR using INFUSION PBI S2Pb or INFUSION PBI NSov as primers and ligated into Sall-EcoRIdigested pBI101 using the Infusion HD cloning kit (Clontech) to yield pBI101-S2P-S2P-SLF1-GFP. All the Ti plasmids were electroporated into Agrobacterium tumefaciens (LB4404), and plant transformation was performed as described by Lee et al. (1994).
Visualization of GFP Fluorescence

Mature pollen was collected and germinated in pollen germination medium for 2 h as described by Meng et al. (2009). The pollen tubes shown in Figure 1 were visualized using a Nikon Eclipse 90i epifluorescence microscope, and the pollen tubes shown in Figures 5A to 5D were visualized using a Nikon Eclipse E600. For subcellular localization of S2-SLF1 shown in Figures 5E to 5H, mature pollen was collected and re-suspended in the pollen germination medium, and the pollen samples were visualized by a Zeiss LSM 510 META confocal microscope with a lambda spectral scan.

Real-Time PCR Analysis to Determine Levels of S2-SLF1 Transcript and Mature amiRNA, and Copy Numbers of Transgenes

Total RNA was isolated from stage 5 anthers (purple buds between 2.0 and 2.5 cm containing anthers before anthesis) using Trizol reagent (Invitrogen). For measuring the total transcript level of S2-SLF1 (endogenous plus S2-SLF1:GFP) or endogenous transcript level of S2-SLF1, 2 μg of total RNA was reverse transcribed using oligo(dT) and Smart-Script reverse transcriptase following the manufacturer’s protocol (Clontech). Real-time PCR was performed on ABI7300 (Applied Biosystems) using 50 ng of cDNA for each technical replicate and PerfeCTa SYBR Green FastMix ROX (Quanta Biosciences) following the manufacturer’s protocol, except that the initial denaturation was at 95°C for 10 min.

For analyzing mature amiRNA targeting S2-SLF1 using stem-loop real-time RT-PCR, 1 μg of total RNA was treated with DNaseI (Fermentas) and 0.0125 μM U6rev-2 primer, and Smart-Scribe reverse transcriptase following the manufacturer’s protocol (Clontech). The ΔΔ cycle threshold (Ct) method was used to calculate the ratio of target gene expression in a test plant and the control plant, the control gene Actin (LAT52P:Actin-4F for Actin, total S2-SLF1, and endogenous S2-SLF1 and the PCR efficiencies calculated from the standard curve. The raw Ct values were used for establishing the standard curves for Actin, total S2-SLF1, and endogenous S2-SLF1 in Supplemental Table 2 online.

For analyzing mature amiRNA targeting S2-SLF1 using stem-loop real-time RT-PCR, 1 μg of total RNA was treated with DNaseI (Fermentas) following the manufacturer’s protocol, and 100 ng of DNaseI-treated RNA was reverse transcribed using 0.0375 μM AmiSPL1 stem loop RT primer, 0.0125 μM U6rev-2 primer, and Smart-Script reverse transcriptase (Clontech) following the manufacturer’s protocol, except that pulsed RT reaction was employed as follows: 16°C for 30 min, followed by 60 cycles of 30°C for 30 s, 42°C for 30 s, 50°C for 1 s, and then 70°C for 15 min (Varkonyi-Gasic et al., 2007). For real-time PCR, 10 ng of cDNA was used for each technical replicate. AmiSPL1for and Universal reverse primer were used to amplify the target gene, and U6for and U6rev-2 were used to amplify control gene U6. (LAT52P:S2-SLF1:GFP) + LAT52P:AmiS2-SLF1/S2-SLF1 was used as the control plant. All other procedures for real-time PCR were the same as described above. The procedure for detecting the mature amiRNA targeting GFP was the same as described above for analyzing the mature amiRNA targeting S2-SLF1 except that AmiSPL9 stem loop RT primer and U6rev-2 primer were used in reverse transcription, and AmiSPL9for and Universal reverse primer were used to amplify the target gene.

For cophotography analysis, 100 ng of genomic DNA was used in each technical replicate. PISL3F3Copy1for/PISL3F3Copy1rev were used to amplify the control gene S2-SLF1. LAT52P:AmiS2-SLF1/S2-SLF1 was used as control plant. AmiSPL1Copy1for/AmiSPL1Copy1rev (see Supplemental Figure 2 online) were used to amplify the target gene AmiS2-SLF1, and AmiSPL9-Copy1for/AmiSPL9Copy1rev were used to amplify the target gene AmiGFP. The procedures and cycling conditions for real-time PCR were the same except that the initial denaturation was at 95°C for 10 min.

Genomic DNA Isolation and Blotting

The protocols for genomic DNA isolation and blotting were as described by Meng et al. (2011), except that the genomic DNA was digested by EcoRI. The [32P]-radiolabeled probe was obtained by PCR using SPLNcofor/SPLNnotrev as primers and pB101-LAT52P:AmiS2-SLF1 as template.

Genotyping of Progeny by PCR

The AmiS2-SLF1 transgene was amplified using SPLNcofor/SPLNnotrev as primers. The primers for genotyping S2-SLF1-haplotypes were SS-SLF1-1for/SS-SLF1-3rev, and the primers for GFP were GFPRT5for/GFPRT5rev. The cycling conditions were 95°C for 5 min for initial denaturation, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 2 min, then 72°C for 10 min. The primers for genotyping S2-SLF1-haplotypes were S2-SLF1-1for/S2-SLF1-2rev, respectively. The cycling conditions were 95°C for 5 min for initial denaturation, followed by 41 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 2 min, then 72°C for 10 min. The primers were amplifying S2-SLF1-1for/S2-SLF1-2rev and S13-SLF1-2for/S13-SLF1-3rev, respectively. The cycling conditions were 95°C for 5 min for initial denaturation, followed by 41 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 2 min, and then 72°C for 10 min.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: S2-SLF1 (AAS79485), S2-SLF1 (AAS79486), S2-SLF1 (KC590092), S2-SLF1 (KC590093), S2-SLF1 (KC590094), S2-SLF1 (KC590095), S2-SLF1 (AAC21384), and S2-SLF1 (AAC33727) from P. inflata.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Gain-of-Function Assay for Interactions between S2-SLF1 and Non-Self S-RNases.

Supplemental Figure 2. Schematic Representation of Transgene Constructs Used in Plant Transformation.

Supplemental Figure 3. Scheme for Constructing 423-bp AmiS2-SLF1 and AmiGFP Stem-Loop Precursors Using miR319a Stem-Loop Precursor.

Supplemental Figure 4. Schematic Representation of S2-SLF1:GFP Transgene and Endogenous S2-SLF1 Gene.

Supplemental Figure 5. Scheme for Amplification of Mature 21-Nucleotide amiRNA by Stem-Loop Real-Time RT-PCR Using Primers Indicated.

Supplemental Figure 6. Suppression of Expression of Vegetative Cell-Specific S2-SLF1:GFP, but Not Endogenous S2-SLF1, by Vegetative Cell Expressed amiRNA Targeting S2-SLF1 or GFP.

Supplemental Figure 7. Genomic DNA Gel Blot Analysis of Copy Numbers of Transgene.

Supplemental Table 1. List of Primer Sequences.

Supplemental Table 2. Determination of PCR Efficiencies for Real-Time PCR Primers.

Supplemental Data Set 1. Raw Ct Values and Calculations for Real-Time PCR.
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AUTHOR CONTRIBUTIONS

P.S. performed all the experiments. P.S. and T.-h.K. designed the experiments and wrote the article.

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**Self-Incompatibility in *Petunia inflata*: The Relationship between a Self-Incompatibility Locus F-Box Protein and Its Non-Self S-RNases**

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