

The F-Box Protein AhSLF-S₂ Controls the Pollen Function of S-RNase–Based Self-Incompatibility

Hong Qiao,^{a,1} Fei Wang,^{a,1} Lan Zhao,^{a,1} Junli Zhou,^a Zhao Lai,^a Yansheng Zhang,^a Timothy P. Robbins,^b and Yongbiao Xue^{a,2}

^aInstitute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100080, China

^bPlant Science Division, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, LE12 5RD, United Kingdom

Recently, we have provided evidence that the polymorphic self-incompatibility (S) locus-encoded F-box (SLF) protein AhSLF-S₂ plays a role in mediating a selective S-RNase destruction during the self-incompatible response in *Antirrhinum hispanicum*. To investigate its role further, we first transformed a transformation-competent artificial chromosome clone (TAC26) containing both AhSLF-S₂ and AhS₂-RNase into a self-incompatible (SI) line of *Petunia hybrida*. Molecular analyses showed that both genes are correctly expressed in pollen and pistil in four independent transgenic lines of petunia. Pollination tests indicated that all four lines became self-compatible because of the specific loss of the pollen function of SI. This alteration was transmitted stably into the T1 progeny. We then transformed AhSLF-S₂ cDNA under the control of a tomato (*Lycopersicon esculentum*) pollen-specific promoter LAT52 into the self-incompatible petunia line. Molecular studies revealed that AhSLF-S₂ is specifically expressed in pollen of five independent transgenic plants. Pollination tests showed that they also had lost the pollen function of SI. Importantly, expression of endogenous SLF or SLF-like genes was not altered in these transgenic plants. These results phenocopy a well-known phenomenon called competitive interaction whereby the presence of two different *pollen S* alleles within pollen leads to the breakdown of the pollen function of SI in several solanaceous species. Furthermore, we demonstrated that AhSLF-S₂ physically interacts with PhS₃-RNase from the *P. hybrida* line used for transformation. Together with the recent demonstration of PiSLF as the pollen determinant in *P. inflata*, these results provide direct evidence that the polymorphic SLF including AhSLF-S₂ controls the pollen function of S-RNase–based self-incompatibility.

INTRODUCTION

Self-incompatibility (SI) in the Solanaceae, Scrophulariaceae, and Rosaceae is controlled by S-RNases that have been thought to act as S-allele–specific cytotoxins that inhibit the growth of pollen bearing an S-allele matching to either of the style S-alleles (McClure et al., 1990; Gray et al., 1991; McCubbin and Kao, 2000). However, a distinct gene controlling the pollen specificity known as *pollen S* is postulated to interact with S-RNases to accomplish the pollen rejection or acceptance (Golz et al., 1999, 2001).

Recently, S-locus F-box (SLF) proteins have been shown to be potential candidates for *pollen S* in *Antirrhinum hispanicum*, a member of the Scrophulariaceae (Lai et al., 2002; Zhou et al., 2003), several species of the Rosaceae (Entani et al., 2003; Ushijima et al., 2003; Yamane et al., 2003), and also the Solanaceae (Kao and Tsukamoto, 2004; Sijacic et al., 2004). As

predicted for *pollen S*, these S-linked genes are polymorphic with a pollen-specific expression pattern. Moreover, we have found that AhSLF-S₂ physically interacts with S-RNases in a nonallele-specific fashion probably through a proposed SCFAhSLF-S₂ (Skp1/Cullin or CDC53/F-box) complex that targets S-RNase destruction during compatible rather than incompatible response (Qiao et al., 2004).

In certain species of Nicotiana and petunia, heteroallelic S-allele duplications have been found to be associated with pollen-part self-compatible mutants (PPM) (Brewbaker and Natarajan, 1960; Pandey, 1965; Golz et al., 1999; reviewed in de Nettancourt, 2001). The genetic behavior of these mutants could be explained if *pollen S* products act as an inhibitor of all S-RNases except for its cognate S-RNase (Golz et al., 1999). It is known that tetraploids derived from self-incompatible diploid plants often lose the pollen function of SI through a competitive interaction between two heteroallelic S-alleles within the diploid pollen but remain cross-incompatible as females with normal self-related haploid pollen (Crane and Lewis, 1942; Stout and Chandler, 1942; Lewis, 1947; reviewed in de Nettancourt, 2001). In *Nicotiana glauca*, Lewis et al. (1999) have isolated PPMs with γ -ray radiation that specifically affects the SI phenotype of pollen but not the SI phenotype of the style. These PPMs are predominantly associated with centric fragments that carry a duplicated copy of an S-allele. Furthermore, S-allele duplications lacking duplicated S-RNases still produce PPMs (Golz et al.,

¹These authors contributed equally to this work.

²To whom correspondence should be addressed. E-mail ybxue@genetics.ac.cn; fax 86-10-6253-7814.

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2001), showing that the *pollen S* duplication alone is capable of generating pollen compatibility. These observations provide a test for the validity of a *pollen S* candidate in an S-RNase-based SI system through genetic transformation (Kao and Tsukamoto, 2004). In fact, Sijacic et al. (2004) have recently used this approach to demonstrate that PiSLF determines the pollen-self-incompatibility in *P. inflata*.

In this study, we transformed an Antirrhinum transformation-competent artificial chromosome (TAC) clone containing both *AhS₂-RNase* and *AhSLF-S₂* into a self-incompatible line of *Petunia hybrida*. In a separate experiment, we transformed the *AhSLF-S₂* cDNA driven by a pollen-specific promoter into the same line. In both cases, the introduced genes were correctly expressed in the reproductive tissues of transgenic petunia plants that significantly all became self-compatible. Pollination tests showed that they had specifically lost the pollen function of SI and phenocopied the competitive interaction because endogenous *SLF* or *SLF*-like genes maintained normal expression in these transgenic plants. Moreover, we detected a physical interaction between *AhSLF-S₂* and *PhS₃-RNase*. Taken together, these results provide direct evidence that *AhSLF-S₂* mediates the pollen function of SI in the S-RNase-based SI species.

RESULTS

Generation and Analysis of Petunia Plants Transformed with TAC26

To test the validity of the *pollen S* candidate *AhSLF-S₂*, we created the *S*-allele duplication by genetic transformation of a self-incompatible line of *P. hybrida*, a member of the Solanaceae. In the initial experiment, a TAC26 clone containing both *AhSLF-S₂* and *AhS₂-RNase* genes (Figure 1A) was transformed into an *S₃S₃* self-incompatible line of *P. hybrida* (Robbins et al., 2000) via an *Agrobacterium tumefaciens*-mediated approach. Sequence analysis of the TAC insert of ~55 kb revealed that this genomic region mainly contains repetitive sequences except for two genes, *AhSLF-S₂* and *AhS₂-RNase* (Lai et al., 2002; Zhou et al., 2003). The TAC clone was used to transform petunia via an *Agrobacterium*-mediated approach, resulting in seven hygromycin-resistant T0 lines. DNA gel blot analysis of their leaf genomic DNA digested with *HindIII* or *BamHI*, using a *Hyg* fragment as probe (Figures 1B and 1C), showed that lines a, d, and r had a single copy insertion of the hygromycin transgene, lines b and m two copies, line l three copies, and line g four copies. As a negative control, no hybridization signal was detected in the untransformed wild-type line.

To determine whether an intact insert was transferred into the genome, further DNA gel blot analysis was performed using both *AhSLF-S₂* (Figures 1D and 1E) and *AhS₂-RNase* (Figures 1F and 1G) cDNA fragments as probes. Out of the seven lines, four lines (b, g, l, and m) had both complete genes, indicated by the two predicted hybridizing bands of 5-kb *HindIII* and 6.2-kb *BamHI* fragments using *AhSLF-S₂* as a probe and 5-kb *HindIII* and 12.5-kb *BamHI* fragments using *AhS₂-RNase* as a probe (Xue et al., 1996; Lai et al., 2002), suggesting that these four lines contain an intact copy(s) of the TAC26 insert. These results indicated that the genomic region encompassing both *AhSLF-S₂*

and *AhS₂-RNase* had been successfully integrated into the petunia genome in the four independent transformants. The *S*-haplotype of the primary transformants was confirmed using *PhS₃-RNase* fragment as a probe (Figure 1H). Notably, none of the single hygromycin copy insertion lines (a, d, and r) had intact target genes (Figures 1B to 1G). This might be caused by an incomplete transfer of the T-DNA as occasionally observed for *Agrobacterium*-mediated transformation (Liu et al., 2000).

We examined the expression of *AhSLF-S₂* and *AhS₂-RNase* in the four transgenic lines with both target genes in detail (b, g, l, and m). RNA transcripts from *AhSLF-S₂* (Figure 2A) and *AhS₂-RNase* (Figure 2B) were detected by RT-PCR in mature anther and pistil, respectively. PCR products were exclusively detected in lanes with reverse transcriptase added (+ lanes) and the positive control of the TAC26 plasmid (lane 11 in Figures 2A and 2B) containing both *AhSLF-S₂* and *AhS₂-RNase*. No product was detected in lanes without reverse transcriptase (– lanes) and the negative control of cDNA from the untransformed *S₃S₃* petunia plant, suggesting no genomic DNA contamination in the cDNA used. An RT-PCR reaction with *tubulin* primers was used as a control for RNA loading.

To further confirm normal expression of the transgenes, *AhSLF-S₂* expression was examined in anther by immunoblot analysis using an antibody against the C-terminal region (Qiao et al., 2004) and was only detected in the transgenic lines at a similar level to that in Antirrhinum (Figure 2C). We also examined S-RNase expression in pistil using an antibody against *AhS-RNase* (Qiao et al., 2004) (Figure 2D). The immunoblot showed that *AhS₂-RNase* (~29 kD) was also detected in each transgenic line, proving that it is expressed and processed normally in styles. The endogenous *PhS₃-RNase* (~24 kD) present in the untransformed petunia was detected in three lines (lines b, g, and l) except one (line m). Why *PhS₃-RNase* was not detected in line m was not clear, but it could be because of gene silencing of the transgenes (Fagard and Vaucheret, 2000). In fact, two copies of the transgenes were detected in line m (see above). However, it might not be because of cosuppression because *AhS₂-RNase* and *PhS₃-RNase* show a low degree of nucleotide sequence identities (48.9%; Xue et al., 1996; Robbins et al., 2000). Furthermore, lower levels of RNA transcripts of *PhS₃-RNase* were detected in this line (Figure 2B, middle panel). No expression of the two Antirrhinum genes was detected in vegetative tissues in the transgenic plants (data not shown). Taken together, these results clearly showed that both *AhSLF-S₂* and *AhS₂-RNase* are expressed in the majority of the transgenic plants in the same tissues as that observed in Antirrhinum.

Phenotypic Analysis of TAC26 Primary Transformants

We did not observe any obvious morphological change of the transgenic plants compared with that of the untransformed plants. To test the pollination behavior of the transformants, they were self- or cross-pollinated. All of the pollinations were performed at the open flower stage, and the results are shown in Table 1. After self-pollination, all four transgenic lines lost their SI behavior and became self-compatible (SC). They set variable-sized capsules with an average seed number from 75.2 to 241 per capsule, comparable to a fully compatible pollination in

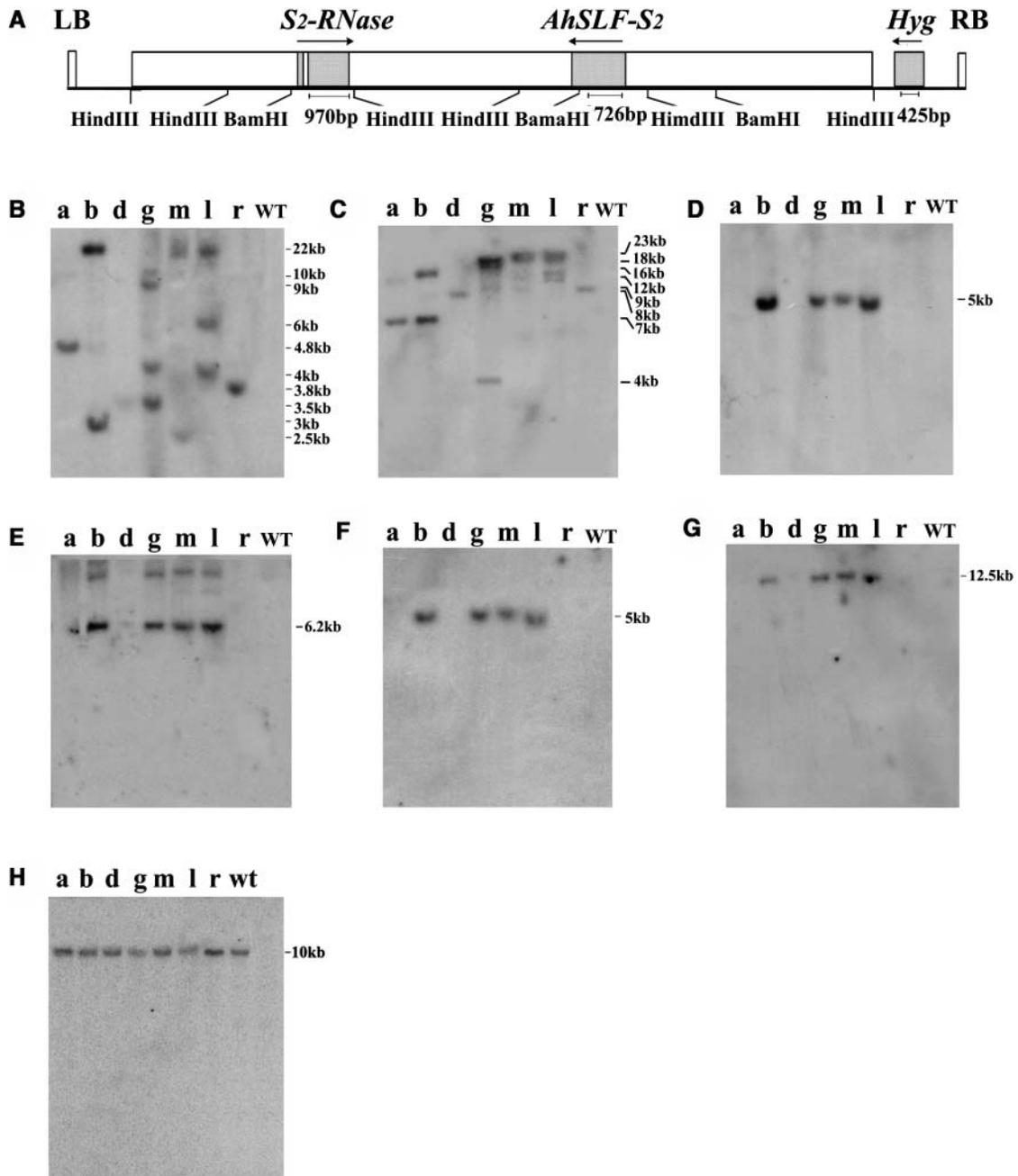


Figure 1. Molecular Analysis of *TAC26* Transgenic Petunia Plants.

(A) A schematic structure of *TAC26*. Probes for *AhS₂-RNase* and *AhSLF-S₂* used are also indicated and *Hind*III and *Bam*HI sites with the genomic region covered by *TAC26*. LB, left border; RB, right border of the T-DNA; *Hyg*, hygromycin resistance gene.

(B) to (G) DNA gel blot analysis of the T₀ transgenic lines a, b, d, g, m, l, r, and the untransformed control (WT). Leaf DNA (5 μg) was digested with *Hind*III ([B], [D], and [F]) or *Bam*HI ([C], [E], and [G]) and was blotted and probed with the *Hyg* fragment ([B] and [C]), *AhSLF-S₂* ([D] and [E]) and *S₂-RNase* ([F] and [G]).

(H) DNA gel blot of *Hind*III-digested leaf DNA was probed with a *PhS₃-RNase* cDNA fragment.

Sizes of the markers ([B] and [C]) and the hybridizing bands ([D] to [H]) are indicated in kilobase pairs.

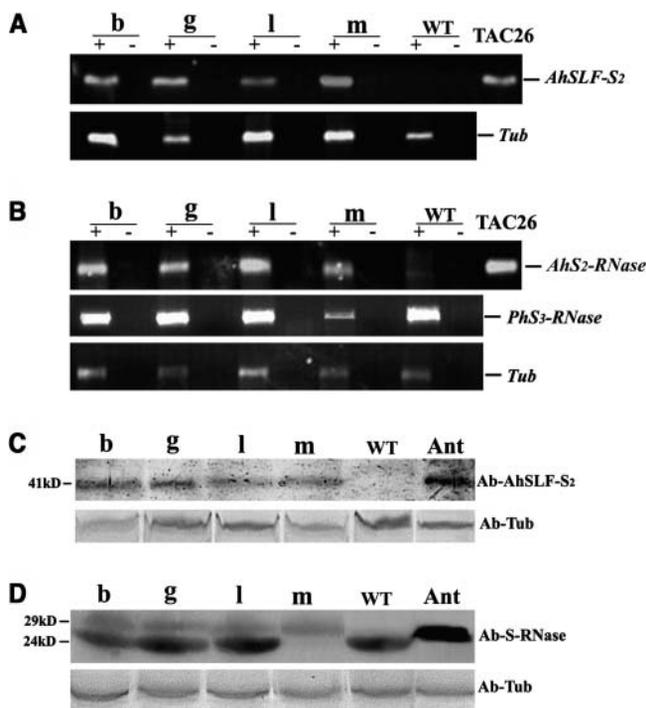


Figure 2. Expression Analysis of *AhSLF-S₂* and *AhS₂-RNase* in the Transgenic Petunia Lines.

(A) and (B) RT-PCR analysis of RNA isolated from pollens or styles, with (+) or without (-) reverse transcriptase in the synthesis of cDNA.

(A) Top panel, RT-PCR analysis of RNA isolated from pollen of the transgenic lines b, g, l, m, and *S₃S₃* wild-type petunia plant using specific primers of *AhSLF-S₂*. *TAC26* plasmid (*TAC26*) was used as a positive control. Bottom panel, RT-PCR analysis of *tubulin* for loading control.

(B) Top panel, RT-PCR analysis of RNA isolated from styles of transgenic lines b, g, l, m, and the wild type using specific primers of *AhS₂-RNase*, and the *TAC26* plasmid (*TAC26*) was used as a positive control. Middle panel, RT-PCR analysis of RNA isolated from styles of the transgenic lines using specific primers of *PhS₃-RNase*. Bottom panel, RT-PCR analysis of *tubulin* for loading control.

(C) and (D) Immunoblot detection of *AhSLF-S₂* or *S-RNases*.

(C) Top panel, detection of *AhSLF-S₂* from total pollen proteins of transgenic lines b, g, l, m, and wild-type *S₃S₃* petunia plant and *Antirrhinum* of *S₂S₄* (*Ant*) by polyclonal antibody against *AhSLF-S₂*. Bottom panel, detection of *tubulin* for loading control.

(D) Top panel, detection of *AhS₂-RNase* (~29 kD) and endogenous *S₃-RNase* (~24 kD) from total style proteins of transgenics lines b, g, l, m, wild-type *S₃S₃* petunia plant and *Antirrhinum* (*Ant*) by polyclonal antibody against *AhS-RNases*. Bottom panel, detection of *tubulin* for loading control. Sizes of the detected protein bands are indicated in kilodaltons.

petunia. To further test whether the conversion of SI into SC is because of the complete loss of the *S*-locus function or the loss of male or female component function, the transgenic plants were reciprocally crossed with an untransformed petunia line either as pollen donor or acceptor (Table 1). First, we pollinated the untransformed *S₃S₃* plants using the four transgenic lines as pollen donors. As a control, we self-pollinated the untransformed *S₃S₃* plants, and no single seed set was obtained, suggesting that they maintain normal SI function in their pollen and pistil.

After being pollinated by each transgenic line, they set middle-sized capsules, and the average seed number per capsule they set ranged from 98.4 to 135.2, also comparable to a fully compatible pollination. We also pollinated the four transgenic lines using *S₃S₃* wild-type plants as pollen donor. The results showed that three lines (b, g, and l) set no seed, except line m, which set full-size capsules with an average seed number of 213.5. The abnormal SI behavior of line m is consistent with our observation that it expressed very low levels of the endogenous *PhS₃-RNase* (Figure 2C). Thus, it should accept *S₃* pollen. The above data showed that the transformation of *TAC26* into petunia leads to the breakdown of the pollen function of self-incompatibility, a phenocopy of competitive interaction, suggesting that the *TAC26* clone contains the *pollen S*.

Molecular and Phenotypic Analysis of the Transgenic Progeny

To determine whether the alteration of self-incompatibility induced by expressing *TAC26* is heritable, the transgenic T1 progeny were raised from the seeds obtained from self-pollinations of two lines, b and l. Three T1 plants were obtained from line b. The *TAC26* insert was inherited stably, as detected by DNA gel blot hybridization with *hyg*, *AhSLF-S₂*, and *AhS₂-RNase* fragments as probes (Figures 3A to 3C). Compared with the T0 primary transgenic line, no apparent recombination or independent segregation occurred for the transgenes. Seven T1 plants were obtained from line l and were also found to inherit the transgenes stably (Figures 3D to 3F).

To examine the transgenes expression in the progeny, RT-PCR analysis was performed. As shown in Figures 4A and 4B, the seven progeny from line l all expressed *AhSLF-S₂* and *AhS₂-RNase* in pollen and style, respectively. As for the three progeny from line b, only one (b3) expressed *AhSLF-S₂* normally

Table 1. Pollination Behaviors of the *TAC26* T0 Transgenic Petunia Plants

Plant ^a	Self-Pollination ^b	Cross-Pollination	
		WT Female ^c	WT Male ^d
b	15/16 (88.4 ± 9.7)	10/10 (105.7 ± 23.5)	0/12 (0)
g	15/15 (241.0 ± 39.5)	11/12 (105.2 ± 21.9)	0/18 (0)
l	15/17 (75.2 ± 10.2)	9/10 (135.2 ± 35.2)	0/16 (0)
m ^e	11/12 (170.0 ± 15.5)	7/7 (98.4 ± 32.2)	13/13 (213.5 ± 32.4)
WT	0/35 (0)	- ^f	-

^a b, g, l, and m are four transgenic lines, and WT is the wild-type *S₃S₃* plant.

^b Data are represented as mature capsules/total pollination sets (average seeds per capsule ± SD).

^c The pollen from the transgenic plants were used to pollinate mature styles from the wild-type *S₃S₃* plants.

^d The pollen from the wild-type plants were used to pollinate mature styles from the transgenic plants.

^e Plants with low expression of endogenous *PhS₃-RNase*.

^f Not applicable.

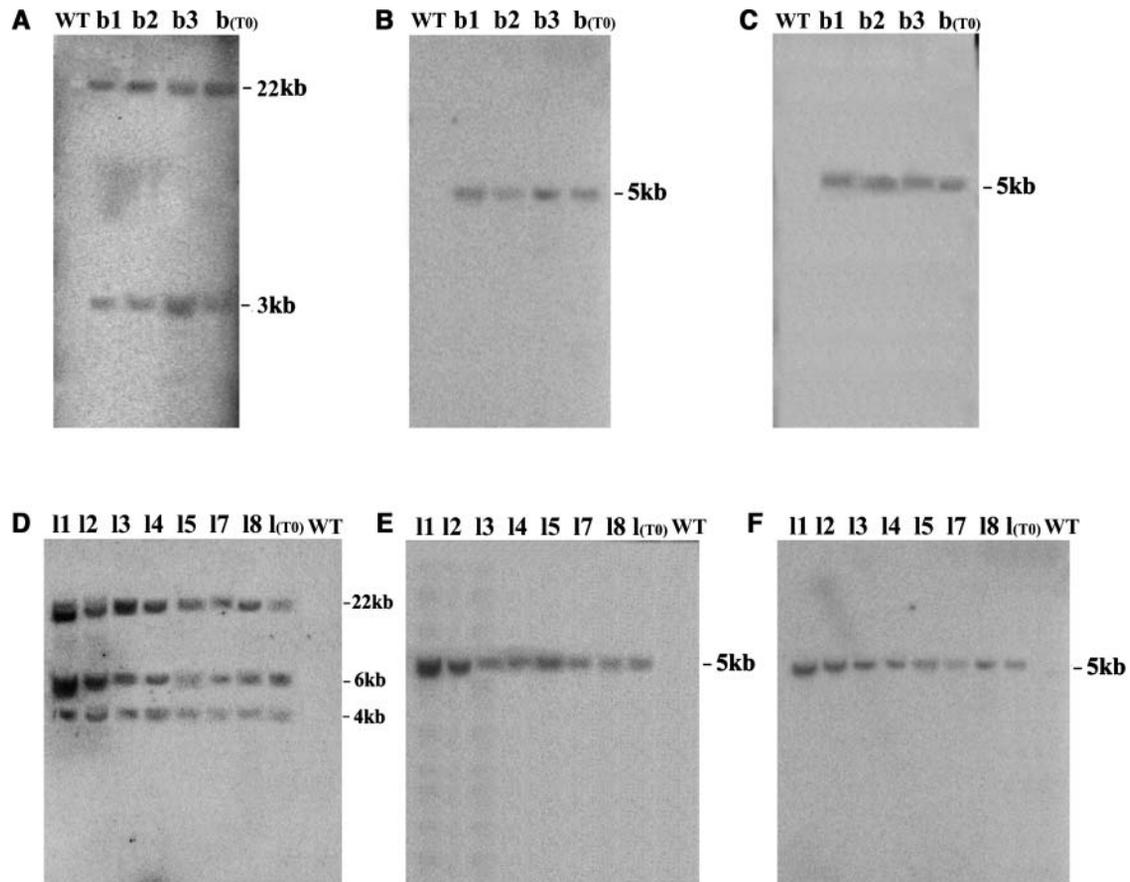


Figure 3. Molecular Analysis of DNA from T1 Progeny of the *TAC26* Transgenic Petunia Lines.

(A) to (C) Genomic DNA gel blot analysis of the T1 progeny (b1 to b3), T0 transformant b, and the untransformed control. Leaf DNA digested by *Hind*III was blotted and probed with the *Hyg* fragment (A), *AhSLF-S₂* (B), and *AhS₂-RNase* (C), respectively.

(D) to (F) DNA gel blot analysis of the T1 progeny (I1 to 5, 7, and 8), T0 transformant, and the untransformed control. Leaf DNA digested by *Hind*III was blotted and probed with the *Hyg* fragment (D), *AhSLF-S₂* (E), and *AhS₂-RNase* (F), respectively. Sizes of the hybridizing fragments are indicated in kilobase pairs.

(Figures 4A and 4C). No or very low expression of *AhS₂-RNase* was detected in the T1 siblings b1 and b2 despite of the presence of their RNA transcripts (Figures 4B and 4D), compared with that in b3, which had a normal *AhS₂-RNase* expression. Furthermore, protein gel blot analysis showed that AhSLF-S₂ was detected in all the seven line I progeny (Figure 4C). AhS₂-RNase was detected normally in five plants from line I (Figure 4D, progeny 1, 2, 3, 7, and 8) consistent with the lower levels of expression observed in the RT-PCR (Figure 4B). It was also found that endogenous *PhS₃-RNase* was not expressed in the three I progeny (I2, 3, and 7) consistent with the absence of RNA transcripts detected in these lines (Figure 4B). In addition, genomic PCR analysis showed that these progeny contained no *Sv* allele used initially to develop S₃S₃ line from S₃Sv plant (Robbins et al., 2000; data not shown). The reason why AhSLF-S₂, AhS₂-RNase, or endogenous PhS₃-RNase could not be detected in some progeny is not clear, but some posttranscriptional gene silencing could lead to this. It has been known that gene silencing triggered by a transgene can lead to the mRNA degradation of the transgene and/or endogenous gene with no or

low expression of the protein, rendering the protein undetectable by antibody (Fagard and Vaucheret, 2000).

To investigate whether the alteration of SI to SC displayed by the primary transgenic lines was transmitted into the progeny, pollination tests were performed on the progeny plants of lines b and I (Table 2). After self-pollination, all seven progeny from line I (I1 to 5, 7, and 8) and one progeny b3 from line b still maintained their self-compatibility and set variable-sized capsules. However, the two progeny b1 and b2, in which AhSLF-S₂ was undetectable using an AhSLF-S₂ antibody (Figure 4C), set no capsule. The self-incompatibility may be restored as a result of the loss of AhSLF-S₂ expression in pollen. Furthermore, the wild-type plants pollinated by the seven I progeny and the b3 set large capsules, demonstrating that the alteration of SI to SC is stably transmitted into the progeny. By contrast, the wild-type plants set no seed after being pollinated by two b1 and b2 progeny. This was consistent with the results of self-pollination test of these two plants described above.

Finally, the T1 progeny were pollinated using wild-type S₃S₃ plants as pollen donor. The result showed that three b progeny

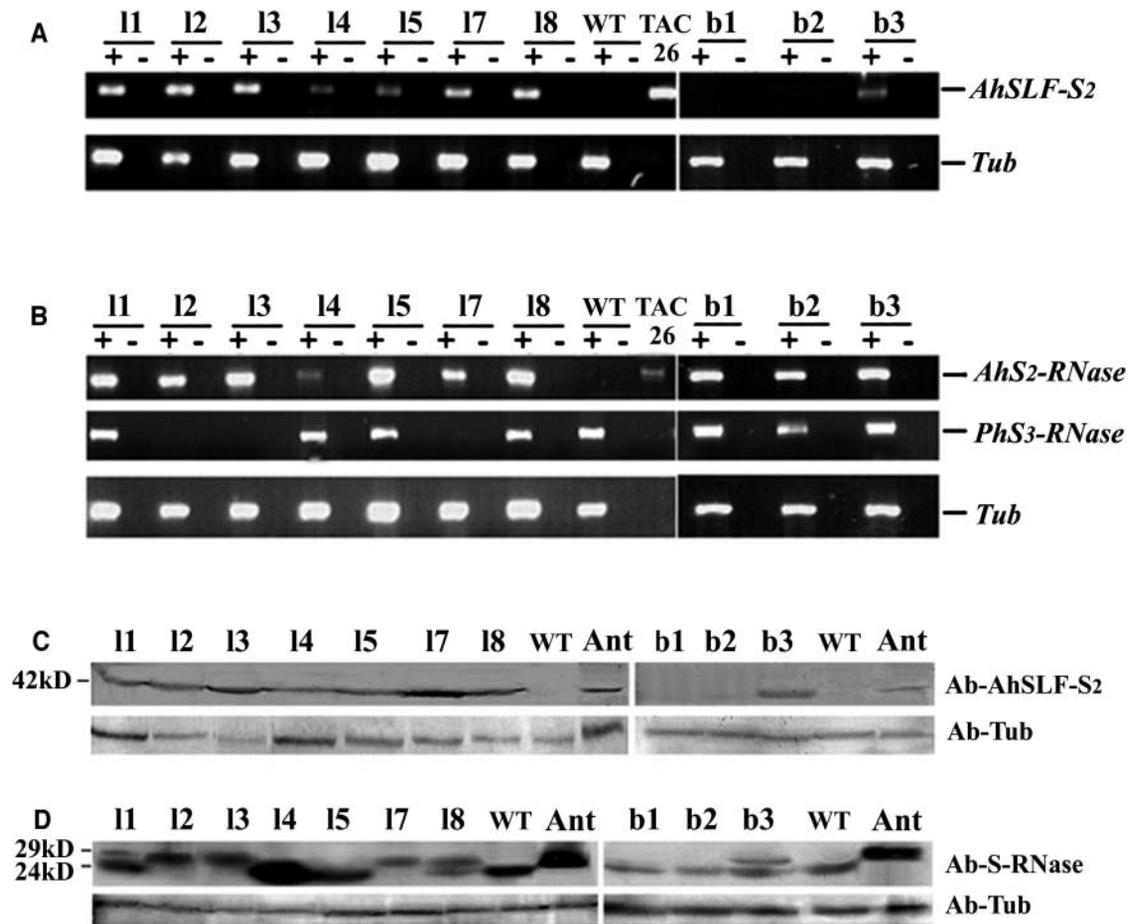


Figure 4. Expression Analysis of *AhSLF-S₂* and *AhS₂-RNase* in the Transgenic Petunia T1 Progeny.

(A) and **(B)** RT-PCR analysis of RNA isolated from pollen or style, with (+) or without (–) reverse transcriptase in the synthesis of cDNA.

(A) Top panel, RT-PCR analysis of RNA isolated from pollen of the transgenic T1 lines I1, 2, 3, 4, 5, 7, and 8, lines b1 to 3, and wild-type plant using *AhSLF-S₂*-specific primers with *TAC26* plasmid as a positive control. Bottom panel, RT-PCR analysis of *tubulin* for loading control.

(B) Top panel, RT-PCR analysis of RNA isolated from styles of the transgenic progeny using *AhS₂-RNase*-specific primers. Middle panel, RT-PCR analysis of RNA isolated from styles of the transgenic progeny using specific primers of *PhS₃-RNase*. Bottom panel, RT-PCR analysis of *tubulin* for loading control.

(C) and **(D)** Immunoblot detection of *AhSLF-S₂* or S-RNases.

(C) Top panel, detection of *AhSLF-S₂* from total pollen proteins of the transgenic lines I1 to 5, 7, and 8, b1 to 3, wild-type plant, and Antirrhinum (Ant) by polyclonal antibody against *AhSLF-S₂*. The bottom panel is tubulin for loading control.

(D) Top panel, detection of *AhS₂-RNase* (~29 kD) and endogenous *S₃-RNase* (~24 kD) from total style proteins of the transgenic lines I1 to 5, 7, and 8, b1 to 3, wild-type plant, and Antirrhinum (Ant) by polyclonal antibody against S-RNases. Bottom panel, detection of tubulin for loading control.

maintain normal SI function in styles, consistent with the normal expression of endogenous *PhS₃-RNase*, and out of seven I progeny, four plants (I1, 4, 5, and 8) maintain normal style SI function, whereas three plants (I2, 3, and 7) lost their normal style function, consistent with the result that no endogenous *PhS₃-RNase* transcript or protein could be detected (Figures 4B and 4D). These results also suggest that expression of *AhS₂-RNase* is not related to the conversion of SI to SC in the transgenic lines because I4 and I5 progeny lost the expression of *AhS₂-RNase* in their styles but maintained the SC trait as with those still having *AhS₂-RNase* in styles, such as I1, 8, and b3. Thus, these results

further indicate that the *TAC26* encodes the pollen component of self-incompatibility response.

Expression of *AhSLF-S₂* in Pollen Causes the Breakdown of Pollen Self-Incompatibility in Transgenic Petunia

Because no predicted genes except *AhSLF-S₂* and *AhS₂-RNase* are found in the genomic region covered by the *TAC26* (Lai et al., 2002; Zhou et al., 2003) and S-RNases do not function in pollen (Kao and McCubbin, 1996), the alteration of the pollen function of SI in these transgenic lines is very likely because of the presence

Table 2. Pollination Behaviors of the *TAC26* Transgenic Petunia T1 Progeny

Plant ^a	Self-Pollination ^b	Cross-Pollination	
		WT Female ^c	WT Male ^d
I1	9/11 (89.1 ± 19.6)	10/10 (102.0 ± 12.3)	0/12 (0)
I2 ^e	10/10 (222.7 ± 42.5)	11/12 (117.5 ± 20.8)	13/13 (201.5 ± 23.7)
I3 ^e	10/10 (79.9 ± 10.98)	9/10 (109.7 ± 24.4)	11/11 (176.5 ± 18.3)
I4 ^f	7/8 (186.43 ± 29.44)	7/7 (91.4 ± 8.6)	13/13 (0)
I5 ^f	8/9 (104.0 ± 16.1)	11/12 (196.5 ± 18.2)	0/16 (0)
I7 ^e	11/11 (225.5 ± 40.8)	10/10 (230.7 ± 21.2)	8/8 (207.9 ± 20.0)
I8	10/10 (202.1 ± 35.3)	14/14 (230.6 ± 21.2)	0/18 (0)
b1 ^{f,g}	0/17 (0)	0/18 (0)	0/12 (0)
b2 ^{f,g}	0/15 (0)	0/21 (0)	0/14 (0)
b3	10/12 (70.9 ± 14.0)	12/12 (260 ± 22.7)	0/16 (0)
WT	0/25 (0)	– ^h	–

^a b, g, I, and m are four transgenic lines, and WT is the wild-type S₃S₃ plant.

^b Data are represented as mature capsules/total pollination sets (average seeds per capsule ± SD).

^c The pollen from the transgenic plants were used to pollinate mature styles from the wild-type S₃S₃ plants.

^d The pollen from the wild-type plants were used to pollinate mature styles from the transgenic plants.

^e Plants with low or undetectable expression of PhS₃-RNase.

^f Plants with low or undetectable expression of introduced AhS₂-RNase.

^g Plants with low or undetectable expression of introduced AhSLF-S₂.

^h Not applicable.

of *AhSLF-S₂*. To confirm this and further exclude the possibility that the repetitive or unknown sequences flanking *AhSLF-S₂* could somehow influence the phenotype, we constructed a transformation vector, *pBIAhSLF-S₂* (Figure 5A). To express *AhSLF-S₂* in pollen, we used a tomato (*Lycopersicon esculentum*) pollen-specific promoter *LAT52* (Twell et al., 1991) to drive the expression of *AhSLF-S₂* and introduced the construct into the S₃S₃ homozygotes of *P. hybrida* by Agrobacterium-mediated transformation. Five kanamycin-resistant T0 lines were generated. DNA gel blot analysis of leaf genomic DNA digested with *HindIII* or *EcoRI* was performed on these plants using *AhSLF-S₂* cDNA as a probe (Figures 5B and 5C). The results showed that two lines (WQ-2 and WQ-5) had one copy and the other three lines contained three copies of the *AhSLF-S₂* transgene and that all five plants were independently transformed. The S₃S₃ genotype of the transgenic lines was also confirmed using *PhS₃-RNase* cDNA as a probe (Figure 5D).

To examine the expression of *AhSLF-S₂*, RT-PCR analysis was conducted. Pollen cDNA from the five transgenic lines and the wild-type S₃S₃ plants with or without reverse transcriptase were used. As shown in Figure 6A, the PCR products of ~700 bp were exclusively detected in the five transgenic lines and Antirrhinum, whereas the wild-type S₃S₃ petunia showed no product using *AhSLF-S₂* specific primers. To further confirm the normal expression of the *AhSLF-S₂* transgene, total protein extracts from the pollen of the five transgenic lines were subjected to immunoblot analysis using the antibody against the AhSLF-S₂ C-terminal region (Qiao et al., 2004). The result showed that its expression could be detected in the transgenic lines (Figure 6B) at a similar size (~41 kD) to that observed in Antirrhinum, whereas no similar protein could be detected in the pollen of wild-type petunia plant. To exclude the possibility of abnormal expression of the endogenous *PhS₃-RNase*

in pistil, total protein extracts from pistil were examined using the polyclonal antibody against Antirrhinum S-RNases (Qiao et al., 2004). The result showed that the expression level of PhS₃-RNase in transformed lines was similar to that of the wild-type line (Figure 6C). These results showed that the *AhSLF-S₂* transgene is expressed in pollen in the five independent transgenic lines.

As with the lines transformed by *TAC26*, no obvious morphological change of the *AhSLF-S₂* transgenic plants was observed compared with the untransformed plant. The self-incompatibility phenotype of all *AhSLF-S₂* transgenic plants was examined by a series of self- and cross-pollinations (Table 3). To check whether the expression of *AhSLF-S₂* could change the SI behavior as observed in the *TAC26* transgenic plants, the five transgenic lines were self-pollinated, and all of them lost their SI and became SC. They also set variable-sized capsules with an average seed number of 49.0 to 98.7, slightly lower than a normal compatible pollination. The reason why these lines set lower numbers of seeds than that of the *TAC26* lines is not clear, and perhaps it is related to the different promoters used or physiological conditions of the plants. The fruit size was not related to the insert copy number. To observe how many seeds were set after pollination by compatible S-genotype pollen, we pollinated these plants using homozygous Sv pollen and found they also set a similar number of seeds (~60 to 80 seeds per capsule). To exclude the possibility that the breakdown of SI was because of the change in style function, we pollinated mature style of the wild-type S₃S₃ plant using the transgenic lines as pollen donor. As shown in Table 3, they also set fruits, and the average seed number per capsule set ranged from 177.2 to 213.2. The wild-type plants maintain their SI rigorously because 34 flowers were self-pollinated and none could set seed. We also pollinated the five transgenic lines using the wild-type plant as pollen donor and

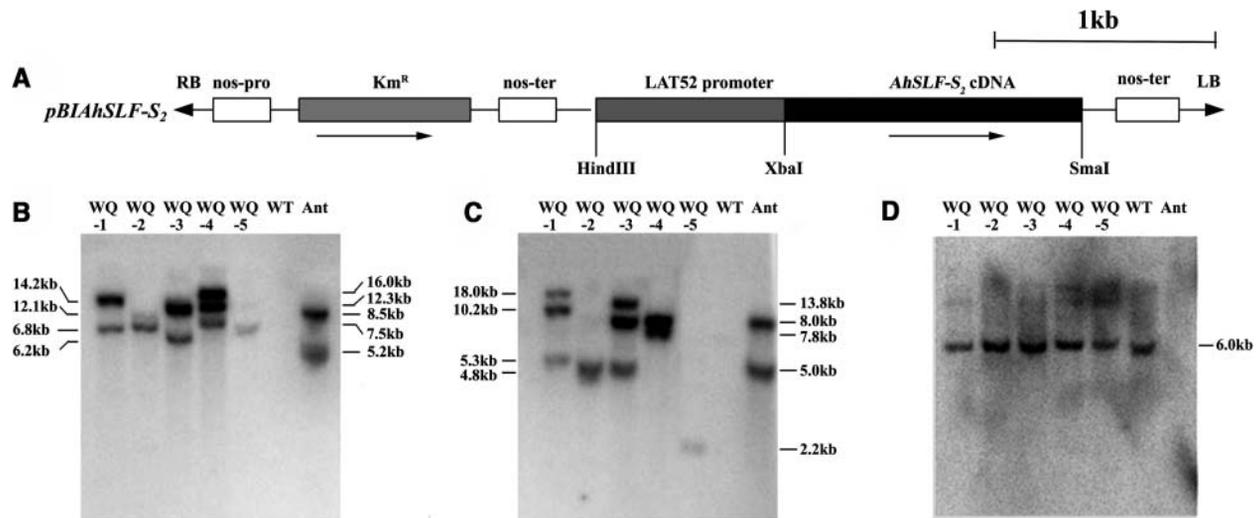


Figure 5. Molecular Analysis of *AhSLF-S₂* Transgenic Petunia Plants.

(A) A schematic diagram of *pBIAhSLF-S₂*. RB and LB, right and left borders of the T-DNA; *Km^r*, kanamycin resistance (neomycin phosphotransferase) gene. Several restriction sites also are indicated.

(B) and (C) DNA gel blot analysis of the transgenic lines WQ-1, WQ-2, WQ-3, WQ-4, and WQ-5 and the untransformed control (WT). Leaf genomic DNA was digested with *Hind*III (B) or *Eco*RI (C) was blotted and probed with *AhSLF-S₂*. Leaf DNA of an *S₂S₄* Antirrhinum line (Ant) was as a positive control.

(D) Leaf DNA digested by *Eco*RI from the transgenic lines was blotted and probed with *PhS₃-RNase*.

found that no fruit was set. These results clearly demonstrate that the styles of the transgenic lines maintain the normal SI function, but they had lost the pollen SI function. Taken together, these results directly show that the expression of *AhSLF-S₂* in pollen leads to the loss of the S-locus pollen function, and the *AhSLF-S₂* gene alone could control the pollen function of S-RNase-based self-incompatibility.

The Physical Interaction between *AhSLF-S₂* and *PhS₃-RNase*

The above results indicated that *P. hybrida* contains an *AhSLF-S₂* ortholog and that *AhSLF-S₂* is capable of physically interacting with *P. hybrida* *S₃-RNase*. To examine the possibility of the physical interaction between *AhSLF-S₂* and *PhS₃-RNase*, we used a yeast two-hybrid screening procedure. Our previous work showed that only C-terminal *AhSLF-S₂* interacts physically with *AhS-RNases* in yeast (Qiao et al., 2004). Thus, we also used the C-terminal *AhSLF-S₂* to test whether it is able to interact physically with *PhS₃-RNase* in yeast. The C-terminal *AhSLF-S₂* was introduced into *pGBKT₇* vector and expressed as a fusion to *GAL4* DNA binding domain (BD), whereas *AhS₂-RNase* and *PhS₃-RNase* were introduced into *pGADT₇* vector and expressed as a fusion to transcriptional activating domain (AD) as described previously (Qiao et al., 2004). The AD: *AhS₂-RNase* or AD: *PhS₃-RNase* was transformed into yeast AH109 in combination with BD: *AhSLF-S₂-C*, respectively. As shown in Figure 7A, transformed yeast cells by AD: *AhS₂-RNase*/BD: *AhSLF-S₂-C* and AD: *PhS₃-RNase*/BD: *AhSLF-S₂-C* grew well on both -Leu/-Trp and -Leu/-Trp/-His/-Ade media, indicating that a physical interaction had occurred between *AhSLF-S₂-C* and *PhS₃-RNase*. Yeast transformed with the control plas-

mids AD: *PhS₃-RNase* and *pGBKT₇* or BD: *AhSLF-S₂-C* and *pGADT₇* did not grow (Figure 7A). Furthermore, the β -galactosidase reporter gene activity was detected and appeared to be positive in yeast cells cotransformed with AD: *PhS₃-RNase* and BD: *AhSLF-S₂-C* as with the positive controls AD: *AhS₂-RNase* and BD: *AhSLF-S₂-C* (Figure 7B), showing that *AhSLF-S₂* and *PhS₃-RNase* physically interact in yeast cells.

To further confirm this interaction, a pull-down assay was performed using a recombinant *AhSLF-S₂* protein fused with an N-terminal His-tag (Figure 7C). The purified His-*AhSLF-S₂-C* fusion proteins were incubated with the style extracts from wild-type *S₃S₃* *P. hybrida* plant. After washing with buffer, the nickel-nitrilotriacetic acid agarose (Ni-NTA) resin-bound proteins were assayed by SDS-PAGE and examined by immunoblot analysis with the polyclonal *AhS-RNase* antibody (Qiao et al., 2004). As shown in Figure 7C, a specific protein of \sim 24 kD, similar to that detected in the wild-type *S₃S₃* *P. hybrida* style, was also detected by the antibody when using the His-*AhSLF-S₂-C* fusion protein with the style extracts. The \sim 24-kD protein was not detected when using the style extract only with the resin, indicating that the C-terminal part of *AhSLF-S₂* physically interacts with *PhS₃-RNase* in vitro, similar to the interaction observed previously for Antirrhinum *AhSLF-S₂* and S-RNases (Qiao et al., 2004).

Expression of *AhSLF-S₂* Does Not Interfere with the Expression of Endogenous *SLF* or *SLF*-Like Genes in Pollen

To test for the normal expression of an endogenous *SLF* gene in these transgenic plants, we designed two degenerate primers based on two conserved regions of the *SLF* proteins (Figure 8A)

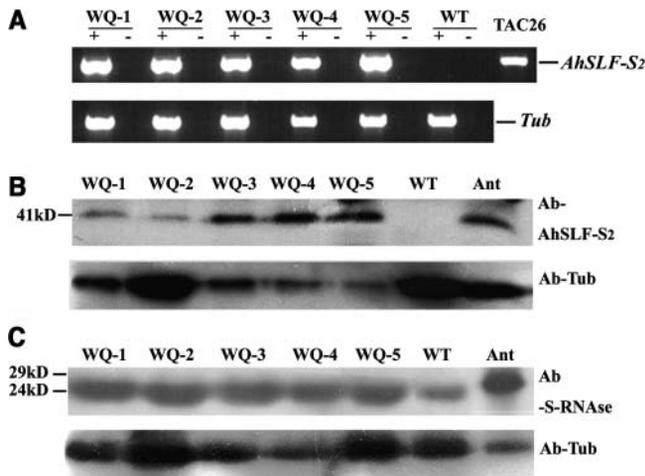


Figure 6. Expression Analysis of *AhSLF-S₂* Transgenic Petunia Plants.

(A) RT-PCR analysis of RNA isolated from pollen of the *AhSLF-S₂* transgenic lines WQ-1, WQ-2, WQ-3, WQ-4, and WQ-5 and wild-type plant, using specific primers of *AhSLF-S₂* with (+) or without (-) reverse transcriptase in the synthesis of cDNA. The *TAC26* plasmid (lane 13) was used as a positive control (top panel). RT-PCR analysis of *tubulin* for loading control (bottom panel).

(B) Top panel, immunoblot detection of AhSLF-S₂ from total pollen protein of the transgenic plants WQ-1, WQ-2, WQ-3, WQ-4, and WQ-5 and wild-type S₃S₃ petunia by polyclonal antibody against AhSLF-S₂. Bottom panel, detection of tubulin for loading control. Antirrhinum pollen protein (Ant) was used as a positive control.

(C) Immunoblot detection of PhS₃-RNase from total style proteins of the transgenic lines of WQ-1, WQ-2, WQ-3, WQ-4, and WQ-5 and wild-type plant by polyclonal antibody against AhS-RNase (top panel). Detection of tubulin for loading control (bottom panel). Antirrhinum style protein (Ant) was used as a positive control.

and subsequently cloned two *SLF* genes from mature pollen of S₃S₃ *P. hybrida*. Their full-length cDNA were obtained by rapid amplification of cDNA ends (RACE). Amino acid comparison revealed that the first gene named *PhSLF-S₃A* has ~90% amino acid identity to PiSLF-S₁, S₂, and S₃ (Sijacic et al., 2004) (Figure 8A), similar to the identity observed for the three *P. inflata* alleles, which have been demonstrated as the pollen determinant genes in *P. inflata* (Sijacic et al., 2004). The second gene, named *PhSLF-S₃B*, has ~96% amino acid identity to PiA134-S₁, S₂, and S₃, which are tightly linked to the *S*-locus in *P. inflata* (Wang et al., 2003). Sequence alignment showed that AhSLF-S₂ shares ~30% amino acid identity to PhSLF-S₃A and PhSLF-S₃B. RT-PCR analysis showed that these two genes also are specifically expressed in pollen (data not shown). Although further work is required to formally demonstrate that PhSLF-S₃A and PhSLF-S₃B are orthologs of PiSLF and PiA134, respectively, their sequence similarities strongly suggest that they are functionally related.

To examine whether these two endogenous *SLF* genes are expressed normally in pollen of the transgenic plants, RT-PCR were conducted using specific primers of these two genes. Ten T1 *TAC26* plants and five *AhSLF-S₂* cDNA transgenic plants all expressed *PhSLF-S₃A* and *PhSLF-S₃B* normally in

pollen (Figures 8B and 8C), showing that the expression of *AhSLF-S₂* does not interfere with the normal expression of the endogenous *SLF* or their related genes in transgenic plants and the compatibility phenotype observed in the transgenic plants could not be caused by the loss of expression of the endogenous *SLF* genes.

DISCUSSION

AhSLF-S₂ Controls the Pollen Function of S-RNase-Based Self-Incompatibility

The finding that the allelic *SLF* genes in several species from three families with S-RNase-based self-incompatibility are expressed specifically in pollen suggests that they encode the elusive *pollen S* function (Kao and Tsukamoto, 2004). Consistent with this view, we have shown that AhSLF-S₂ physically interacts with S-RNases albeit without allelic specificity (Qiao et al., 2004). In this study, the breakdown of the pollen component of self-incompatibility by expressing *AhSLF-S₂* in self-incompatible petunia provides direct evidence that it controls the pollen function of the S-RNase-based self-incompatible reaction. In fact, Sijacic et al. (2004) have demonstrated that *PiSLF-S₂* also controls the pollen self-incompatibility in *P. inflata*. Together, these results show that these polymorphic *SLF* proteins determine the pollen self-incompatibility in both the Solanaceae and Scrophulariaceae and possibly in the Rosaceae as well.

Two different models, the gatekeeper model and the inhibitor model, have been proposed to explain how S-RNases function inside the pollen tube (Thompson and Kirch, 1992; Kao and McCubbin, 1996). The inhibitor model predicts that the *pollen S* products are cytosolic RNase inhibitors, with each allelic product specifically inhibiting the RNase activity of all nonself S-RNases but not that of its cognate self S-RNase. In the gatekeeper model, the *pollen S* presumably encodes an allele-specific receptor for S-RNases whereby only self S-RNases would be taken up into

Table 3. Pollination Behaviors of the *AhSLF-S₂* Transgenic Petunia Plants

Plant ^a	Self-Pollination ^b	Cross-Pollination	
		WT Female ^c	WT Male ^d
WQ-1	12/14 (56.6 ± 23.4)	8/8 (174.0 ± 30.6)	0/12 (0)
WQ-2	11/11 (69.8 ± 13.5)	11/11 (190.9 ± 26.3)	0/18 (0)
WQ-3	20/24 (49.0 ± 16.2)	9/10 (177.2 ± 16.7)	0/16 (0)
WQ-4	34/37 (50.3 ± 27.1)	7/7 (213.2 ± 36.4)	0/12 (0)
WQ-5	30/33 (98.7 ± 21.1)	13/13 (200.0 ± 24.3)	0/14 (0)
WT	0/34 (0)	- ^e	-

^a WQ-1 to -5 are five transgenic lines, and WT is the wild-type S₃S₃ plant.

^b Data are represented as mature capsules/total pollination sets (average seeds per capsule ± SD).

^c The pollen from the wild-type plants were used to pollinate mature styles from the wild-type S₃S₃ plants.

^d The pollen from the wild-type plants were used to pollinate mature styles from the transgenic plants.

^e Not applicable.

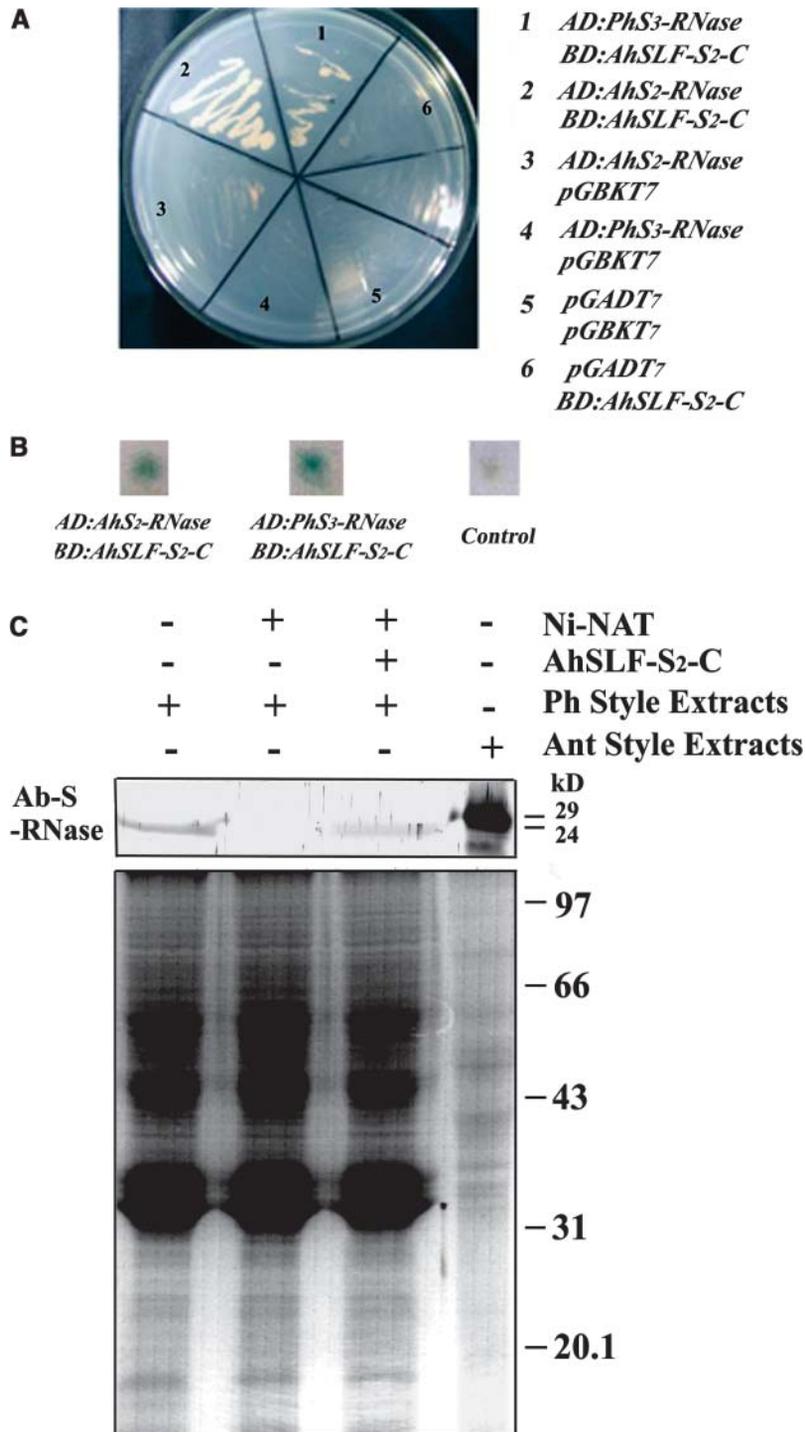


Figure 7. Physical Interaction between AhSLF-S₂ and PhS₃-RNase.

(A) Yeast cells containing various combinations of BD and AD fusions were tested for their growth on -Leu/-Trp/-His/-Ade dropout media. Plasmid pGBKT₇ with various AD:constructs and plasmid pGADT₇ with various BD:constructs were used as negative controls.

(B) The strains were grown further to test for expression of the β-galactosidase reporter gene.

(C) A pull-down assay for the physical interaction between AhSLF-S₂ and PhS₃-RNase. Ni-NTA resin and the purified fusion proteins of His-AhSLF-S₂-C were incubated with the style extract of the S₃S₃ line of *P. hybrida* (Ph Style Extracts). Bound proteins were pulled down with Ni-NTA resin, eluted with the lysis buffer, separated by 12% SDS-PAGE, transferred to membranes, and analyzed by immunoblotting using the anti-S-RNase antibody (top panel). Style extracts from Antirrhinum (S₂S₅) (Ant Style Extracts) were also included as a control. Input style total protein was used as a positive control (bottom panel). Molecular mass markers are indicated in kilodaltons.

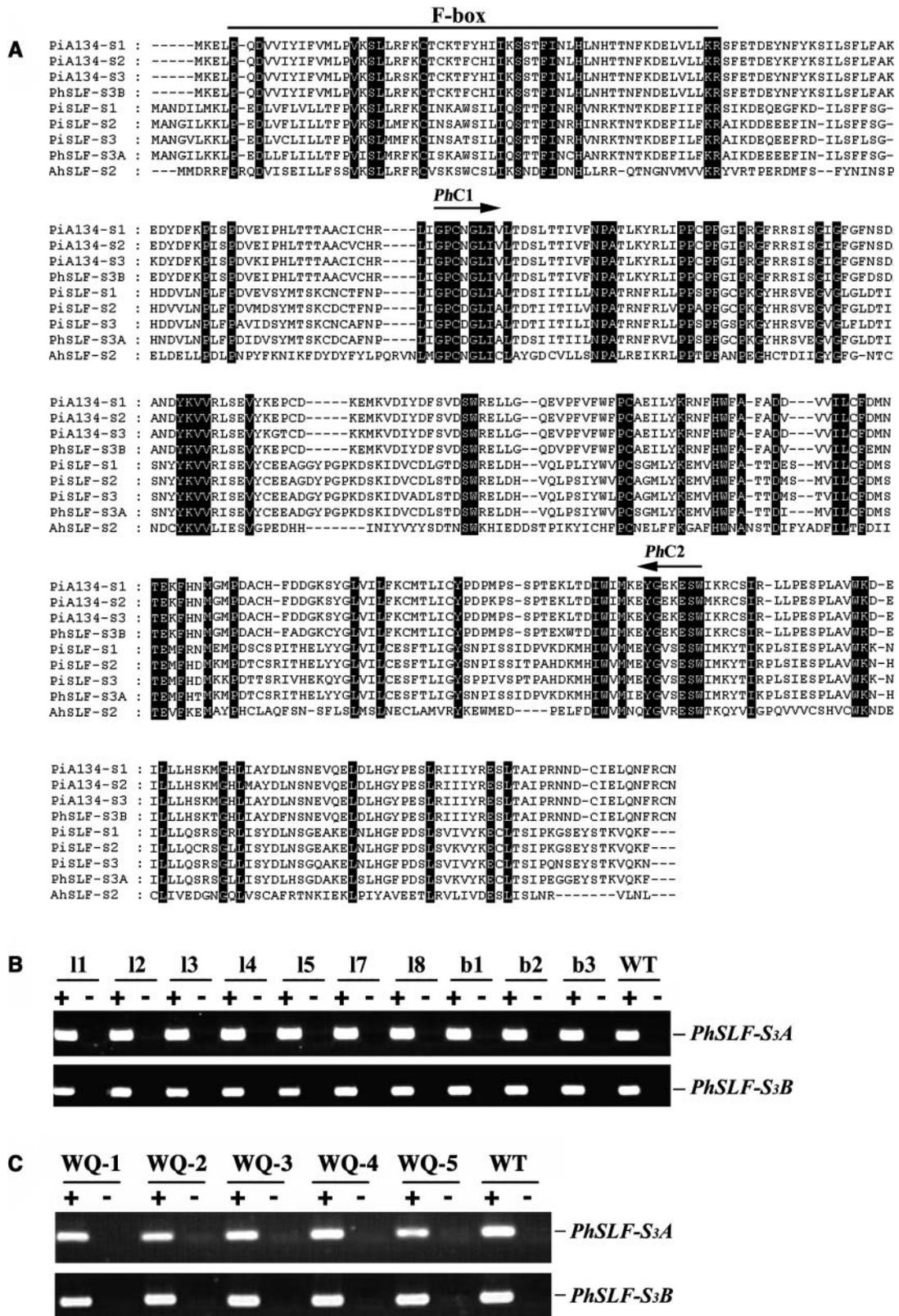


Figure 8. Amino Acid Sequence Alignment of Predicted SLF Polypeptides from *Antirrhinum* and *Petunia* and Expression Analysis of Endogenous SLF Genes in Transgenic *Petunia* Plants.

the cytoplasm of a pollen tube. Several lines of evidence support the inhibitor model (reviewed in Kao and Tsukamoto, 2004). Luu et al. (2000) used immunocytochemistry to clearly show that there is unspecific uptake of S-RNases by the pollen tube, thus providing a strong support for the inhibitor model. This model can explain a well-known phenomenon termed competitive interaction, which refers to a breakdown of pollen function in SI caused by the presence of two heteroallelic S-haplotypes in pollen (Crane and Lewis, 1942; Stout and Chandler, 1942; Lewis, 1947; reviewed in de Nettancourt, 2001). Based on recent results (Qiao et al., 2004; Sijacic et al., 2004; this study), we propose a modified inhibitor model to explain how AhSLF-S₂ recognizes self- or nonself-S-RNase and leads to self-incompatibility or compatibility in the S-RNase-based self-incompatible reaction. After self-S-RNase and nonself-S-RNase are taken up into the growing pollen tube, AhSLF-S₂ interacts with both S₁- and S₂-RNases. The interaction of AhSLF-S₂ with self S₂-RNase is somehow not effective in forming a functional SCF complex to ubiquitinate S-RNase, thus allowing its action in inhibiting the pollen tube growth. By contrast, the interaction of AhSLF-S₂ with nonself S₁-RNases produces a functional SCF complex that in turn leads to its subsequent ubiquitination and destruction by the 26S proteasome.

Thus, in our transformation experiments, AhSLF-S₂ or PhSLF-S₂ recognizes endogenous PhS₃-RNase or introduced AhS₂-RNase as a nonself S-RNase and inhibits its RNase activity, which leads to the successful growth of the pollen tube in pistil, a phenocopy of the well-known phenomenon of competitive interaction. This model also provides a possible explanation to an apparently contradictory observation of the domain-swapping experiments in petunia (Kao and McCubbin, 1996), *Nicotiana* (Zurek et al., 1997), and *Solanum* (Matton et al., 1997). The swapping of hypervariable regions and other domains between alleles results in the production of a chimeric ribonuclease. In petunia and *Nicotiana*, the chimeric S-RNases generated between two significantly diverged alleles were unable to reject self-pollen. It is likely that the alteration of their structure compared with the native S-RNases are so gross that it renders them to behave as nonself S-RNases, and the pollen could grow through the styles because the chimeric S-RNases were degraded as nonself S-RNases. In *Solanum*, a so-called dual-specificity chimeric S-RNase, named S_{11/13}-RNase, was generated between two highly similar alleles (Matton et al., 1997; Luu et al., 2001), and this would produce two very similar S-RNases apart from the S₁₁- and S₁₃-allele specificity domains. Plants that produce the S_{11/13}-RNase reject both S₁₁ and S₁₃ pollen, showing that the chimeric S-RNase is not degraded in the pollen tube. In addition, the pistil of

transgenic plants that produce the S_{11/13}-RNase also is able to reject the pollen from tetraploid plants of S₁₁S₁₁S₁₃S₁₃ genotype (Luu et al., 2001), suggesting that the S_{11/13}-RNase is still functional within the diploid pollen. The hybrid S_{11/13}-RNase would be recognized as a self RNase by either of the *pollen S* products present in the pollen because of its little alteration in three-dimensional conformation; subsequently, it would not be degraded, thus preventing the pollen tube growth. This possibility could be tested after cloning the *SLF-S₁₁* and *-S₁₃* genes in *Solanum chacoense* and using a genetic transformation approach.

AhSLF-S₂ Could Determine Both Allelic Specificity and S-RNase Inhibitory Activity

The breakdown of the pollen self-incompatibility by AhSLF-S₂ in petunia and the fact that AhSLF-S₂ is an F-box protein suggests that it could control both S-allele specificity and inhibition of S-RNase activity. Kao and Tsukamoto (2004) consider that the putative *pollen S* contains two domains, an S-allele-specific domain and an inhibitor domain. The S-RNase also contains two separated functional domains, an S-allele specificity domain and a catalytic domain. In the case of self-interaction, the S-allele-specific domain of *pollen S* interacts with S-allele-specific domain of self S-RNase by virtue of the match between the paired domains, leaving the catalytic domain of S-RNase active. However, in the case of nonself interaction, the inhibitor domain of pollen S interacts with the catalytic domain of the S-RNase in the absence of a match between their S-allele specificity domains, thus inhibiting the RNase activity of S-RNase.

It is known that S-RNases show a high degree of allelic sequence diversity and domain-swapping experiments have demonstrated that S-allele specificity is scattered throughout the molecule and not restricted to specific sites in both petunia and *Nicotiana* (Kao and McCubbin, 1996; Zurek et al., 1997). Nevertheless, in some cases, S-RNase alleles share very high similarity, for example, S₁- and S₁₁-RNase in *Solanum tuberosum* (Kaufmann et al., 1991) and S₁₁- and S₁₃-RNases in *S. chacoense* (Despres et al., 1994; Saba-el-Leil et al., 1994) all show >95% homology and yet they are phenotypically distinct. Despite the low degree of polymorphism of four *AhSLF* alleles (~95% identity) (Zhou et al., 2003) and three *PiSLF* alleles (~90% identity) (Sijacic et al., 2004) compared with *PdSFB* and *PmSLF* alleles (~68 to 80% similarity) (Entani et al., 2003; Ushijima et al., 2003; Yamane et al., 2003), the alteration of the pollen function by AhSLF-S₂ clearly shows that this Antirrhinum F-box gene is capable of determining male specificity.

Figure 8. (continued).

(A) Alignment of the predicted polypeptide sequences from the SLF family. Antirrhinum, AhSLF-S₂ (CAC33010); *P. inflata*, PiA134-S₁ (AAR15914), PiA134-S₂ (AAR15915), PiA134-S₃ (AAR15916), PiSLF-S₁ (AY500390), PiSLF-S₂ (AY500391), and PiSLF-S₃ (AY500392); *P. hybrida*, PhSLF-S₃A (AY639403) and PhSLF-S₃B (AY639402). PhC1 and PhC2 indicate the conserved regions that were used to design degenerate primers for cloning *PhSLF-S₃A* and *PhSLF-S₃B*. The F-box domain is also indicated.

(B) and **(C)** RT-PCR analysis of RNA isolated from pollen of the *TAC26* and *AhSLF-S₂* transgenic lines and wild-type plant, using specific primers of *PhSLF-S₃A* and *PhSLF-S₃B* with (+) or without (–) reverse transcriptase in the synthesis of cDNA. *Tubulin* control was the same as in Figures 4A and 6A.

Although two hypervariable regions have been described in the SFB/SLF proteins from the Rosaceae as in the case of S-RNase alleles (Ushijima et al., 2003; Ikeda et al., 2004; Kao and Tsukamoto, 2004), amino acid differences among four AhSLF-S alleles are scattered throughout the proteins, including the F-box domain (Lai et al., 2002; Zhou et al., 2003), which is similar to the three PiSLF alleles in *P. inflata* (Sijacic et al., 2004). It is likely that these minor allelic differences could possibly lead to the change of the higher-order conformation of the protein and eventually affect the outcome of their interaction with S-RNases. The amino acid similarity between AhS₂-RNase and PhS₃-RNase is as low as 36.9%, but AhSLF-S₂ does appear to physically interact with PhS₃-RNase (Figure 7), indicating the structural and functional conservation between AhSLF-S₂ and the endogenous ortholog of *P. hybrida*.

Recently, we have shown that AhSLF-S₂ interacts with Ask-1 and Cul-1 like proteins and the 26S proteasome pathway is required for the inhibition of S-RNase during a compatible pollination (Qiao et al., 2004). These findings indicated that, as a possible substrate of AhSLF-S₂, nonself S-RNase is ubiquitinated by SCF^{AhSLF-S₂} complex and degraded through the 26S proteasome pathway. It remains to be determined what differences lead to self S-RNase stability in contrast with nonself S-RNase being degraded. In addition, most known sub-

strates of the SCF complexes in animals and plants need to be phosphorylated before being recognized by the F-box protein (Hershko and Ciechanover, 1998). Kunz et al. (1996) found that S-RNases could be phosphorylated by a calcium-dependent protein kinase from pollen tube without allelic specificity. Thus, it needs to be tested whether the phosphorylation condition will affect the degradation of S-RNases in vivo. The ubiquitin/26S proteasome has been found to participate in other mating systems, such as in yeast and sporophytic SI (Hicke and Riezman, 1996; Stone et al., 2003), and its possible participation in S-RNase-based SI systems suggests the conservation and importance of the ubiquitin/26S proteasome pathway in the operation of a diverse range of breeding systems.

Functional and Evolutionary Implications for the SLF Proteins in S-RNase-Based SI Families

The SLF sequences are extensively diverged among the three families Solanaceae, Scrophulariaceae, and Rosaceae (Kao and Tsukamoto, 2004), but they possibly maintain a similar three-dimensional structure because of the presence of several conserved domains. Our results and that obtained in *P. inflata* (Sijacic et al., 2004) suggest that the SLF sequences closest to S-RNases are functionally conserved. However, it has been

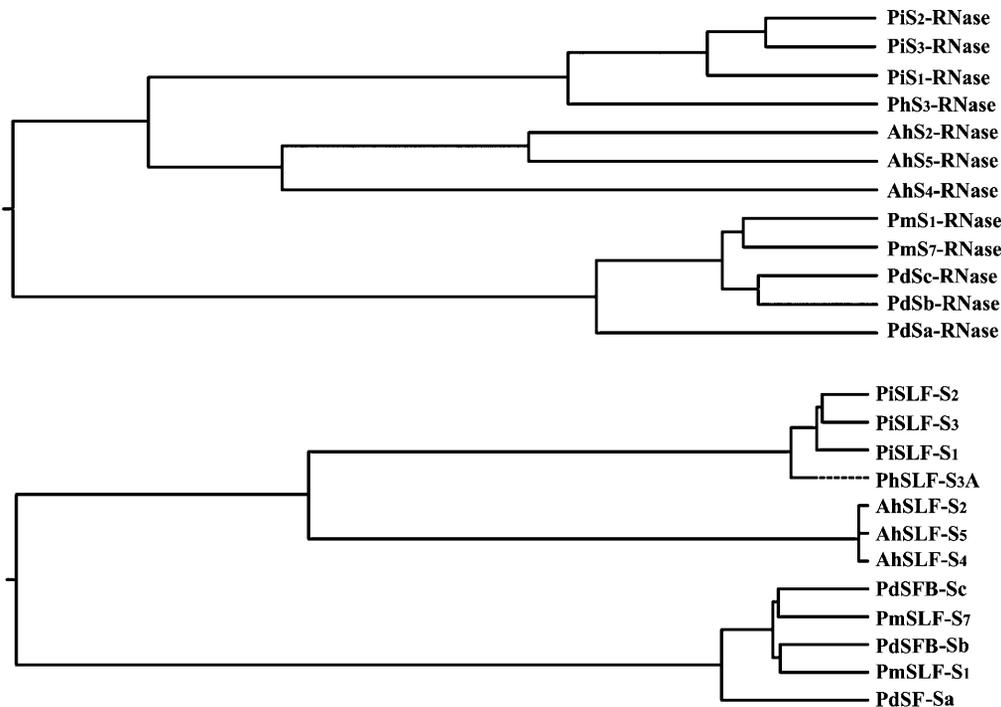


Figure 9. Schematic Representations of the Phylogenetic Relationships for Predicted SLF and S-RNase Polypeptides.

The phylogenetic relationships are shown for the S-RNases (top) and SLFs (bottom), respectively. Antirrhinum, AhSLF-S₂, AhSLF-S₄ (CAD56661), AhSLF-S₅ (CAD56664), AhS₂-RNase (CAC33020), AhS₄-RNase (Q38717), and AhS₅-RNase (CAA65318); *P. inflata*, PiSLF-S₁, PiSLF-S₂, PiSLF-S₃, PiS₁-RNase (S20989), PiS₂-RNase (AAG21384), and PiS₃-RNase (AAA33727); *P. hybrida*, PhSLF-S₃A and PhS₃-RNase (AJ271065); *Prunus dulcis*, PdSFB-Sa (BAC65206), PdSFB-Sb (BAC65207), PdSFB-Sc (BAC65201), PdSa-RNase (BAA95317), PdSb-RNase (T12078), and PdSc-RNase (T12076); *P. mume*, PmSLF-S₁ (BAC66622), PmSLF-S₇ (BAC66623), PmS₁-RNase (BAC56115), and PmS₇-RNase (BAC56116).

found that clusters of F-box genes are present in the S-locus of *Antirrhinum* (Zhou et al., 2003) and several other solanaceous and rosaceous species (Entani et al., 2003; Ushijima et al., 2003; Wang et al., 2003). Although the paralogous copies of AhSLF-S₂ have been found not to physically interact with S-RNases (Qiao et al., 2004), it remains unclear if they have any roles in other yet-defined processes. As a recognition locus, the disease resistance *R* locus in plants also is characterized by gene clusters. For example, the tomato *Pto* gene belongs to a complex locus consisting of a tightly linked cluster of five to seven genes. *Pto* confers resistance to *Pseudomonas syringae* (Martin et al., 1993), whereas the tightly linked paralog *Fen* confers sensitivity to an organophosphate insecticide (Martin et al., 1994; Loh and Martin, 1995). In other cases, potato *Gpa2* and *Rx1* share 88.7% homology as paralogs, but they confer resistance to the potato cyst nematode *Globodera pallida* and potato virus X, respectively (van der Vossen et al., 2000). Further functional analysis of these S-locus paralogs will provide a better understanding of their functions in plant development as well as the origin and evolution of the S-RNase-based self-incompatibility.

The functional conservation of AhSLF-S₂ in *P. hybrida* has important implication for the evolution of S-RNase-based self-incompatibility. Previous analyses have found that S-RNases from the Scrophulariaceae, Solanaceae, and Rosaceae are most likely monophyletic (Xue et al., 1996; Igic and Kohn, 2001). The Solanaceae and Scrophulariaceae belong to the subclass Asteridea, whereas the Rosaceae are in the subclass Rosidea. Together they comprise roughly three-quarters of dicot families (Cronquist, 1981). That the expression of AhSLF-S₂ from the Scrophulariaceae is able to change the pollen behavior of *P. hybrida* from the Solanaceae further supports a common origin of gametophytic SI system in these two distantly related families. Figure 9 represents the schematic diagrams of the phylogenetic relationships for several pollen determinant SLF and possible pollen determinant SLF proteins and S-RNases in the three families. A similar topology was observed for them, supporting the notion that they are coevolved. Nevertheless, it is unclear how this coevolution occurs. Further comparative analyses of the S-locus structures from members of the three families will reveal their origin and evolution.

METHODS

Plant Materials and Transformation

The self-incompatible line of S₃S₃ *Petunia hybrida* and SI lines of *Antirrhinum hispanicum* have been described previously (Xue et al., 1996; Robbins et al., 2000). *Antirrhinum TAC26* was obtained from a *TAC* genomic DNA library constructed using pTAC as described previously (Liu et al., 2000; Zhou et al., 2003). A pollen-specific promoter *LAT52* from tomato (*Lycopersicon esculentum*) was used to drive the expression of AhSLF-S₂ (Twell et al., 1991). *Hind*III and *Xba*I double digested *LAT52* fragment was ligated to pBI101.1 where the β-glucuronidase gene has been removed previously and then *Xba*I and *Sma*I double digested AhSLF-S₂ full-length coding region was ligated to pBILAT52. The plasmid *TAC26* and pBIAhSLF-S₂ were electroporated into *Agrobacterium tumefaciens* C58 and LBA4404, respectively. Transformation of leaf strips of *P.*

hybrida of S₃S₃ genotype with *Agrobacterium* and regeneration of transgenic plants were performed as previously described (Lee et al., 1994; Harbord et al., 2000). For *TAC26* transformation, leaf disks of *P. hybrida* with S₃S₃ genotype were infected with *Agrobacterium* by the cocultivation method on MS medium supplemented with 6-benzylaminopurine (2.0 mg L⁻¹) and naphthalene acetic acid (0.2 mg L⁻¹). Shoots were regenerated on fresh MS medium supplemented with hygromycin (50 μg mL⁻¹) and carbenicillin (500 μg mL⁻¹). Regenerated shoots were transferred to hormone-free MS medium containing the same concentrations of antibiotics to induce root formation. For pBIAhSLF-S₂ transformation, shoots were regenerated on fresh MS medium supplemented with kanamycin (100 μg mL⁻¹) and carbenicillin (500 μg mL⁻¹).

DNA Gel Blotting Analyses

Genomic DNA isolation was performed as described previously (Xue et al., 1996). DNA (5 μg) was digested, separated on 0.8% agarose gel, and transferred onto Hybond N⁺ (Amersham, Buckinghamshire, UK) membrane. Prehybridization, hybridization, and washing of the blot were performed as recommended by the manufacturers. Probes were labeled with ³²P by random priming using the Prime-a-Gene labeling system (Promega, Madison, WI).

RT-PCR Analysis

Total RNA was prepared as previously described (Lai et al., 2002) and was digested with DNase I (TaKaRa, Dalian, China). Reverse transcriptase (Invitrogen, Carlsbad, CA) was used to synthesize the first strand cDNA. The RT-PCR was performed using specific primers of AhSLF-S₂, AhS₂-RNase, and *P. hybrida* Sv- and S₃-RNase as previously described (Robbins et al., 2000; Zhou et al., 2003). Specific primers of PhSLF-S₃A used for RT-PCR were as follows: 5'-CTGATGGTTATCCTGGTCC-3' (forward) and 5'-CAGCTCGTGCCTAAT ACGAC-3' (reverse). Specific primers of PhSLF-S₃B used for RT-PCR were as follows: 5'-CAAG-GAACCTTGACAAAAG-3' (forward) and 5'-GTTGGACTACTAGG-CATTGG-3' reverse).

Immunoblot Analysis

For protein blot analysis, style or pollen was homogenized in SDS-loading buffer (0.2 M Tris-HCl, pH 6.8, 0.5 M DTT, 4% SDS, and 25% glycerol), boiled, and centrifuged. Proteins were separated in 12% Tris-Tricine gels (Schägger and von Jagow, 1987) and blotted onto Nitrobind (Micron Separations, Westborough, MA) using a Bio-Rad Transblot SD wet electroblotting apparatus (Hercules, CA). Blots were treated with the rabbit AhS-RNase (1:1000) or AhSLF-S₂ antiserum (1:1000) (Qiao et al., 2004) and a mouse monoclonal anti-tubulin Ab (Sigma, St. Louis, MO), and immune complexes were detected using alkaline phosphatase-conjugated secondary antibodies and nitroblue tetrazolium 5-bromo-4-chloro-3-indolyl phosphate (Harlow and Lane, 1988) or a chemiluminescent immunodetection system (Invitrogen).

Yeast Two-Hybrid Cotransformation Assay

P. hybrida S₃-RNase open reading frame lacking signal peptide was cloned into pGADT7 (AD) (Clontech, Palo Alto, CA), and the construction of C-terminal regions of AhSLF-S₂ and AhS₂-RNase was described before. The BD and AD vectors were cotransformed into AH109 and grown on -Leu/-Trp medium containing 2% agar at 30°C for 4 to 5 d. The clones were further grown on -Leu/-Trp/-His/-Ade medium containing 2% agar at 30°C for 3 to 4 d to test interaction.

For liquid β-galactosidase assay, yeast clones grown for 48 h at 30°C were transferred onto filter paper, and the clones were lysed in liquid nitrogen for 1 min, and then 5 mL of Z buffer (60 mM Na₂HPO₄, 40 mM

NaH₂PO₄, 10 mM KCl, and 1 mM MgSO₄, pH 7.0) containing β-mercaptoethanol (27 μL/10 mL) was added and incubated at 30°C (for <8 h) and checked periodically for the appearance of blue color.

Pull-Down Assay

Recombinant fusion proteins His-AhSLF-S₂C were expressed in *Escherichia coli* BL21 (DE3) strain (Promega) after induction by 0.1 mM isopropyl-D-thio-galactopyranoside (Sigma) for 3 h at 37°C and purified on Ni-NTA (Pharmacia Biotech, Piscataway, NJ) following the manufacturer's recommendation (Sigma, P6611). Ten micrograms of recombinant protein was coupled to 100 μL of a 50% suspension (v/v) of beads in equilibration buffer for 20 min at 4°C. After prewashing, style extracts were transferred to a clean tube containing His-AhSLF-S₂C coupled to the Ni-NTA resin and incubated overnight with constant rotation at 4°C. Subsequently, the beads were washed more than five times with ice-cold 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 1 mM EDTA buffer, and then 50 μL of SDS-PAGE sample buffer was added to each sample. Bound proteins were dissociated by heating at 37°C for 1 h and resolved by SDS-PAGE in 12% gels as described above.

Pollination

All of the pollinations were performed using open flowers. Except for self-pollination, anthers were removed from the flower of the plant serving as female recipient before dehiscence to prevent self-pollination. Pollination was done by collecting pollen from the male donor of the cross and applying it directly to the stigma of the female recipient. Pollinated flowers were covered with paper bags, and a positive result was scored as the formation of a mature capsule with seeds.

Cloning and Sequence Analysis of *PhSLF* Genes

Degenerate primers *PhC1* and *PhC2* were used to clone partial cDNA sequences of *PhSLF-S₃A* and *-S₃B* from *S₃S₃ P. hybrida* pollen cDNA. *PhC1* was 5'-(T/C)TIATIGGCCITG(T/C)(A/G)A(T/C)GG-3', and *PhC2* was 5'-CCA(C/G)A(T/C)TCII(T/A)I(T/A)CICC-3'. The first strand pollen cDNA was synthesized using forward primer 5'-AAGCAGTGGTATCAACGCA-GAGTGGCCATTATGGCCGGG-3' and reverse primer oligo(dT)₁₇ following the manufacturer's recommendation (Invitrogen). For *PhSLF-S₃A*, 3'RACE was conducted using the primer 5'-CGCACGAGCTGTAT-TATGGC-3' and the primer 5'-GTACGGTGAAGCGAGTCTTGG-3'; 5'RACE was conducted using the primer 5'-GGACCAGGATAACCAT-CAG-3' and the primer 5'-GAGGACTCTGAAGTTTCTGG-3'. For *PhSLF-S₃B*, 3'RACE was conducted using the primer 5'-CCAATGCC-TAGTAGTCCAAC-3' and the primer 5'-CGGTGAAAGGAGTCTTGG-3'; 5'RACE was conducted using the primer 5'-CCGCTGATGGAACGTCT-GAAAC-3' and the primer 5'-GGCATGGTGGGATTAGTCTG-3'.

BLASTp and BLAST2 (<http://www.ncbi.nlm.nih.gov/BLAST/>) and ClustalW (<http://www.ebi.ac.uk>) were used for DNA sequence analysis and the alignment. The phylogenetic tree was generated with ClustalW using a neighbor-joining feature from the DNASTAR package. The GenBank accession numbers for and are, respectively.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY639403 (*PhSLF-S₃A*) and AY639402 (*PhSLF-S₃B*).

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