BIOCHEMICAL AND FUNCTIONAL STUDIES OF S-RNASE-BASED SELF-INCOMPATIBILITY IN PETUNIA INFLATA

A Dissertation in

Plant Physiology

by

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Self-incompatibility (SI) allows the female reproductive tissue, pistil, to distinguish between self- and non-self pollen during sexual reproduction in flowering plants. In simplest cases, this self/non-self recognition is controlled by a highly polymorphic locus, named the \( S \)-locus. If the \( S \)-haplotype of pollen is also carried by the pistil, the pollen is recognized by the pistil as self-pollen and rejected. If the \( S \)-haplotype of pollen is different from both \( S \)-haplotypes carried by the pistil, the pollen is recognized as non-self pollen and its tube is allowed to grow through the pistil to effect fertilization. Thus, SI allows flowering plants to avoid inbreeding and generate genetic diversity in the offspring. My thesis research focuses on the type of SI that has so far been found in the Solanaceae and two other families, and I have used \textit{Petunia inflata}, a wild species of petunia, as a model. The Kao Lab has identified the \( S-RNase \) gene and the \( PiSLF \) (\textit{P. inflata} \textit{S}-locus \textit{F-box}) gene as the genes that control pistil and pollen SI function, respectively. The overall goal of my thesis research is to study how \( S-RNase \) and \( PiSLF \) interact inside a pollen tube to result in specific growth inhibition of self-pollen tubes.

There are several clues to the biological functions of \( S-RNase \) and \( PiSLF \). First, the RNase activity of \( S-RNase \) is essential for its function in growth inhibition of self-pollen tubes. Second, \( PiSLF \) is a member of a large family of proteins, named F-box proteins, and a typical F-box protein is a component of an E3 ligase complex, named SCF, which consists of Skp1, Cullin 1, F-box protein and Rbx 1. Third, a typical SCF complex, along with two other proteins, E1 and E2, catalyzes the attachment of poly-ubiquitin chains to a subset of proteins through specific recognition by the F-box protein. The poly-ubiquitin chains allow the target proteins to be recognized by the 26S proteasome and degraded. Based on these biochemical clues, I have designed experimental approaches utilizing a variety of techniques to study the biochemical mechanism of \( S-RNase \)-based SI.

In Chapter 2, I describe the use of various protein-protein interaction assays to show that \( PiSLF \) is not a typical F-box protein, as the \( PiSLF \)-containing complex consists of only two other proteins, Cullin 1 and \( PiSBP1 \), with \( PiSBP1 \) possibly playing the dual role of Skp1 and Rbx1. This finding suggests that \( PiSLF \) is likely involved in ubiquitin-mediated protein degradation. Further \textit{in vitro} binding experiments showed that a \( PiSLF \) interacted with its non-self \( S-RNases \).
(produced by different S-haplotypes) much more strongly than with its self S-RNase, and an S-RNase interacted with its non-self PiSLFs much more strongly than with its self PiSLF. This preferential binding with non-self S-RNases would allow the PiSLF-containing complex to specifically target non-self S-RNases for ubiquitination and degradation, but allow the self S-RNase to exert its RNase activity to degrade pollen RNAs. This finding thus provides a biochemical explanation for why a pistil only rejects its self-pollen tubes during SI interactions. I then developed a cell-free ubiquitination and degradation system using extracts of in vitro germinated pollen tubes, and showed that S-RNases were ubiquitinated and degraded via the ubiquitin-26S proteasome protein degradation pathway in vitro, albeit not in an S-haplotype-specific manner.

Extensive sequencing of the S-locus region in Antirrhinum and several species in the Rosaceae family, all of which possess S-RNase-based SI, has revealed the existence of additional F-box genes at the S-locus. In Chapter 3, I describe the identification of genes encoding four PiSLF-like proteins that share many properties with PiSLF, and present both in vitro and in vivo comparative studies of PiSLF-like proteins and PiSLF. The results showed that none of the PiSLF-like proteins interacted with S-RNases to any significant degree, or functioned in SI, suggesting that PiSLF has a unique function in SI. Sequence comparison between PiSLF and these PiSLF-like proteins has revealed three domains that are specific to PiSLF. I used various chimeric proteins between PiSLF1 and PiSLF2, and between PiSLF2 and one of the PiSLF-like proteins, to show that one of the domains is responsible for the strong interaction with non-self S-RNases, and the other two domains together specifically suppress the interactions between PiSLF and its self S-RNase. This finding provides the biochemical basis for why a PiSLF preferentially interacts with its non-self S-RNases as described in Chapter 2.

To further test the involvement of ubiquitin-26S proteasome-mediated protein degradation in SI, I studied whether any of the 20 lysine residues in S3-RNase of P. inflata might be targets for ubiquitination. In Chapter 4, I report the finding that six lysine residues near the C-terminus, when changed to arginines, significantly reduced ubiquitination and degradation of the mutant S3-RNase, GST:S3-RNase (K141-164R), in pollen tube extracts. I further showed that GST:S3-RNase (K141-164R) had similar RNase activity as GST:S3-RNase, suggesting that their
degradation was not likely caused by an ER-associated protein degradation pathway that removes mis-folded proteins. Finally, I showed that PiSBP1 (P. inflata S-RNase Binding Protein 1), the RING-HC subunit of the PiSLF (P. inflata SLF)-containing E3-like complex identified in Chapter 2, could target S-RNase for ubiquitination in vitro. All these results suggest that ubiquitin-26S proteasome-dependent degradation of S-RNase is likely an integral part of the S-RNase-based SI mechanism.

Two biochemical models, a degradation model and a sequestration (compartmentalization) model, have recently been proposed to explain the S-RNase-based SI mechanism. In the first part of Chapter 5, I provide a critical evaluation of these two models in view of the results I have obtained in my thesis research, and discuss why the model invoking specific degradation of non-self S-RNases via the ubiquitin-26S proteasome pathway can better explain some key aspects of SI. I have developed several projects and collaborated with three other graduate students in the Kao Lab to further test the validity of this model. In the second part of Chapter 5, I discuss the preliminary results from these projects, as well as future directions.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>A. hispanicum</td>
<td>Antirrhinum hispanicum</td>
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<td>AhSLF</td>
<td>Antirrhinum hispanicum S-locus F-box protein</td>
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<tr>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
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<tr>
<td>bp</td>
<td>base pair</td>
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<tr>
<td>C</td>
<td>Celsius</td>
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<td>$^{32}$P</td>
<td>$^{32}$P Adenosine 5’-triphosphate, alpha</td>
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<td>PMC</td>
<td>Pollen mother cell</td>
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<td>Simple modular architecture research tool</td>
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CHAPTER 1

Introduction
Most flowering plants (angiosperms) produce bisexual flowers, in which both the female (pistil) and male (anther) reproductive organs are located in close proximity. This creates a strong tendency for them to self-pollinate, resulting in inbreeding. Inbreeding often leads to reduced fitness in the progeny (e.g., increased susceptibility to disease and stress). During the evolution of flowering plants, had they not adopted various mechanisms to prevent self-pollination and promote out-crossing, there would not have been such a highly diverse plant kingdom on earth, more than 60% of which is dominated by angiosperms.

Flowering plants have adopted many different strategies to prevent self-pollination. For example, some species produce two different morphological types of flower, one with a long pistil and short stamen and the other with a short pistil and long stamen. In order for pollination to be successful, pollen not only must come from genetically unrelated individuals, but also from an anther that is of the same height as the pistil of the flower being pollinated (de Nettancourt, 2001). This type of inbreeding-preventing mechanism is referred to as heteromorphic self-incompatibility (SI). Another example is homomorphic SI, where the success of pollination is determined by the genetic identity of the pollen and pistil, and not by any morphological characters, as plants possessing this type of SI produce the same morphological type of flower within each species. In this case, genetically related (self) pollen is rejected by the pistil and only genetically unrelated (non-self) pollen is able to effect fertilization. Both homomorphic and heteromorphic SI are pre-zygotic reproductive barriers that prevent self-pollen/self-pollen tubes from delivering the sperm cells to the ovary for fertilization. Since this thesis deals with the study of a homomorphic SI mechanism, hereafter, the term SI refers to this type of SI.

1.1 Major Types of SI Mechanisms

SI is first classified into sporophytic and gametophytic types based on the difference in the genetic basis of pollen SI behavior. For sporophytic SI, the pollen behavior is determined by the genotype of the pollen-producing plant, whereas for gametophytic SI, the pollen behavior is determined by the genotype of the pollen itself. To date, extensive molecular studies have been conducted in the Brassicaceae family which possesses sporophytic SI and in the Solanaceae, Rosaceae, Plantaginaceae (formerly known as Scrophulariaceae) and Papaveraceae families, all
of which possess gametophytic SI (Kao and Tsukamoto, 2004). In all these families, a highly polymorphic locus, named the S-locus, controls the outcome of pollination. Earlier genetics studies suggested that the pollen and pistil functions in SI are independently controlled by separate genes that are tightly linked at the S-locus. Because there are more than one polymorphic gene at the S-locus, variants of the S-locus are referred to “haplotypes”, and variants of an S-locus gene are referred to “alleles”. In the Brassicaceae, the gene controlling pollen SI specificity, S-locus cysteine-rich protein (SCR)/S-locus protein-11 (SP11), and the gene controlling pistil SI specificity, S-locus receptor kinase (SRK), have been identified. The interaction between SCR/SP11 and its cognate SRK triggers a signal transduction cascade in the stigmatic papilla, starting with phosphorylation of SRK and eventually resulting in inhibition of germination of self-pollen on the stigmatic surface (for a review, see Kachroo et al., 2002).

Of the four GSI families mentioned above, all except the Papaveraceae employ the S-RNase-based mechanism (Kao and Tsukamoto, 2004), with the S-RNase gene controlling pistil specificity (Lee et al., 1994; Murfett et al., 1994) and the S-locus F-box gene (SLF) controlling pollen specificity (Qiao et al., 2004b Sijacic et al., 2004; Sonneveld et al., 2005). Degradation of pollen RNA is thought to be responsible for growth inhibition of incompatible pollen tubes. However, how SLF and S-RNase mediate S-haplotype-specific inhibition of pollen tube growth remains to be investigated. The mechanism employed by the Papaveraceae involves a small stigmatic S-protein (~120 amino acids) and an as yet unidentified pollen specificity determinant (Thomas and Franklin-Tong, 2004). The interaction between an S-protein and its cognate pollen specificity determinant (likely serving as the receptor) is thought to result in a cascade of biochemical events, including a transient increase in the intracellular calcium concentration, phosphorylation and dephosphorylation, to result in program cell death of incompatible pollen. The discussion below pertains to the S-RNase-based SI mechanism, using solanaceous species, particularly Petunia inflata, as a model.

1.2 Cellular Functions of Pollen and Stigma

Pollen development initiates from a primordial diploid cell within the anther. The division of the primordial cell produces a tapetal initial cell and a pollen mother cell (PMC). The tapetal
initial cell develops into an inner anther layer, named tapetum, which provides nutrients and signaling molecules for the development of microspores. The PMC undergoes meiosis to produce four haploid cells, named tetrad, surrounded by the callose wall. The four haploid cells are released, after the callose wall is digested by the callase produced and secreted from the tapetum, to become unicellular microspores. After this stage, the microspore undergoes an asymmetric mitotic division to generate a bicellular microspore, which contains a large vegetative cell and a small generative cell, which is engulfed within the vegetative cell. In most flowering plants, including *Petunia inflata*, the bicellular microspore develops into mature pollen grain by further dehydration. However, in some flowering plants, including *Arabidopsis thaliana*, the small generative cell is further divided into two sperm cells inside the vegetative cell to produce a tricellular pollen grain (McCormick, 1993). In *P. inflata*, anther/pollen development has been divided into five different stages: stage 1 anthers contain microspores in the tetrad configuration; stage 2 anthers contain free unicellular microspores; stage 3 anthers contain mostly bicellular microspores; stage 4 anthers contain exclusively bicellular microspores; and stage 5 anthers contain mature pollen grains (Lee et al., 1996). These five pollen developmental stages correlate well with the flower bud size: from stage 1 to stage 5 anthers, the flower bud sizes (in length) are less than 0.5 cm, 0.5 to 1.0 cm, 1.0 to 1.5 cm, 1.5 to 2.0 cm, and 2.0 and 2.5 cm, respectively (Figure 1.1; Lee et al., 1994).

Based on the presence or absence of the viscous surface secretion (which contains proteins, lipids, polysaccharides, and pigments), mature stigmas are classified into two types, dry and wet stigmas (Edlund et al., 2004). The stigma of *P. inflata* belongs to the wet type. When matured pollen is released from the anther, the pollen grains are delivered onto the stigma surface for germination by biotic (insects, bees, birds, etc.) and abiotic (wind, water, etc.) vectors. In *P. inflata*, the growth of pollen tubes in a pistil can be divided into two different phases, the autotrophic slow growth phase and the heterotrophic accelerated growth phase, similar to the growth of bicellular pollen tubes in other species (Herrero and Hormaza, 1996; Lubliner et al., 2003). The rejection of self-pollen tubes occurs at the transition from the autotrophic to the heterotrophic phase (Herrero and Hormaza, 1996). At this stage, the generative cell in the bicellular pollen tube undergoes mitosis to produce two sperm cells. Compatible pollen tubes continue to grow down the style to reach the ovary and enter into the embryo sac. In the
embryo sac, the tip of the pollen tube bursts to release the two sperm cells into the embryo sac for double fertilization, one fusing with the egg cell to give rise to the diploid embryo, and the other fusing with the diploid central cell to producing the triploid endosperm.

1.3 S-RNase: the Female SI Determinant

S-RNase is a pistil-specific protein, and all its allelic variants are abundantly present in mature pistils, accounting for 1 to 10% of the total pistil protein (Roalson and McCubbin, 2003). Thus, it is relatively easy to identify and purify each allelic variant for biochemical characterization. The S-haplotype-specific differences of this protein were first demonstrated by the differences in the molecular mass from analyzing total pistil proteins of different S-genotypes by SDS-polyacrylamide gel electrophoresis (Bredemeijer and Blaas, 1981). Subsequent cloning and sequencing of cDNAs for this protein from different S-haplotypes of *Nicotiana alata*, *P. inflata* and other solanaceous species has further revealed the high degree of S-allele sequence polymorphism (Anderson et al., 1986; Ioerger et al., 1991; Tsai et al., 1992). The S-allelic variants of this protein were initially named S-allele associated proteins, or S-proteins, but renamed S-Rnases after the finding that they have RNase activity (McClure et al., 1989; Singh et al., 1991).

The tissue-specific and temporal expression patterns of the *S-RNase* gene are well correlated with the manifestation of SI by the pistil during flower development. Self-pollen tubes are usually inhibited in the upper third segment of the mature style. This is the region where most of S-RNase molecules are produced. Furthermore, the levels of both the *S-RNase* transcript and the S-RNase are very low in immature pistils, which are not able to reject self-pollen. Both the transcript and protein levels dramatically increase in pistils one day before flower opening, and the timing of the increase coincides with the transition from self-compatibility to SI during the maturation of the pistil (Roalson and McCubbin, 2003). Thus, S-RNase was considered a strong candidate for the pistil-specificity determinant of SI (Clarke and Newbigin, 1993). This was confirmed by both gain-of-function and loss-of-function experiments (Lee et al., 1994; Murfett et al., 1994). In *P. inflata*, *S1S2* plants were transformed with the *S3-RNase* gene, and the transgenic plants that expressed normal levels of *S3-RNase* acquired the ability to reject *S3*...
pollen. Conversely, when the level of S3-RNase in S2S3 transgenic plants was suppressed by the introduction of an antisense S3-RNase gene, the plants lost the ability to reject S3 pollen but still retained the ability to reject S2 pollen. The results of both experiments taken together suggested that the S-RNase gene alone is necessary and sufficient to control the SI specificity in the pistil (Lee et al., 1994).

Comparisons of the deduced amino acid sequences of a large number of alleles of the S-RNase gene of the Solanaceae have revealed the existence of five conserved domains, named C1 through C5, and two hypervariable regions, named Hva and HVb (Ioerger et al., 1991; Tsai et al., 1992). Two histidines, one in the C2 region and the other in the C3 region, are required for RNA catalysis, and replacing either one resulted in the loss of RNase activity and the inability of the mutant S-RNase to function in SI (Huang et al., 1994). Thus, the RNase activity of the S-RNase is essential for its function in rejecting self-pollen tubes. All S-Rnases that have been characterized so far are glycoproteins with various numbers of N-linked glycan chains, raising a question as to whether S-allele-specificity of the S-RNase resides in the protein backbone and/or the glycan chain(s). Site-directed mutagenesis was used to replace the codon for the only N-linked glycan site (asparagine-29) of P. inflata S3-RNase with a codon for aspartic acid, and the resulting non-glycosylated S3-RNase produced in transgenic plants was found to function normally in SI (Karunanandaa et al., 1994). Thus, S-allele-specificity of S-RNase is determined entirely by the protein sequence, with the two hypervariable regions being the most likely candidate for the S-allele specific function. Furthermore, the crystal structure of an S-RNase of N. alata shows that both regions are located on the surface of the protein molecule (Ida et al., 2001). However, the domain swapping experiments carried out on S-Rnases of P. inflata and N. alata showed that they are necessary but not sufficient for determining S-allele specificity (Kao and McCubbin 1996; Zurek et al., 1997). What additional region(s) are required remain(s) to be identified.

1.4 Competitive Interaction

Studies of pollen-part self-compatible mutants (which retain normal pistil function in SI) have revealed that they all resulted from duplication of part or the entire S-locus, with the duplicated
region existing as a centric fragment or attached to a chromosome (Golz et al., 2001). Further mutagenesis studies have shown that the loss of the pollen SI function always occur in pollen grains where the S-haplotype of the duplicated region is different from that of the endogenous S-locus (Golz et al., 2001). A similar phenomenon has also been observed in tetraploid plants that carry two different S-haplotypes (de Nettancourt, 1977). For example, when a diploid self-incompatible plant of \( S_1S_2 \) genotype is converted to a tetraploid plant of \( S_1S_1S_2S_2 \) genotype, the plant becomes self-compatible due to breakdown of the pollen function in SI (Figure 1.2). Among the three S-genotypes of the pollen produced, \( S_1S_1 \) and \( S_2S_2 \) behave normally in SI, but \( S_1S_2 \) fails to be rejected by the pistil of the tetraploid plant or the parent diploid plant. Thus, SI breaks down in heteroallelic pollen but not in homoallelic pollen.

The phenomenon of the breakdown of SI in heteroallelic pollen is referred to as competitive interaction as it was thought that different pollen S-alleles interact competitively to weaken the SI function of both alleles, thereby rendering the pollen unable to be rejected by otherwise incompatible pistils (de Nettancourt, 2001).

1.5 S-locus F-box Protein: the Pollen SI Determinant

Several lines of evidence suggest that S-RNase cannot control the pollen function in SI. First, self-compatible mutants that are defective in either pollen function (pollen-part mutant) or pistil function (pistil-part mutants) have been identified (de Nettancourt, 2001). Second, some pollen-part mutants carrying a duplicated S-locus region do not contain the S-RNase gene (Golz et al., 1999, 2001). Third, deletion of \( S_4-RNase \) gene in a cultivar of Pyrus serotina (a species of the Rosaceae family) causes the loss of SI function in the pistil but not in pollen (Sassa et al., 1997). The pollen S-gene is expected to have several characteristics to allow it to function as pollen S-specificity determinant. First, it should be specifically expressed in pollen or pollen tubes. Second, it should display S-haplotype-specific restriction fragment length polymorphism (RFLP), reflecting its S-allele sequence polymorphism. Thus, it is able to specifically interact with different S-alleles of S-RNase gene. Third, it should be tightly linked to the S-RNase gene in order for the two genes to be transmitted as a single gene unit to maintain the SI function. Fourth, allelic products of the pollen S-gene should interact with allelic products of the S-RNase
gene in an $S$-haplotype-specific manner. These features were used to design experimental approaches to identify the pollen $S$-gene. In $P. \text{inflata}$, RNA differential display and subtractive hybridization were used to identify 13 pollen-expressed genes that showed $S$-haplotype-specific RFLP (Dowd et al., 2000; McCubbin et al., 2000). From the analysis of 1205 F2 plants, segregating for $S_1$ and $S_2$ haplotypes, nine of these 13 genes were found to be tightly linked to the $S$-$RNase$ gene (Wang et al., 2003). However, none of the nine $S$-linked pollen-expressed genes were deemed likely to be the pollen $S$-gene because their allelic sequence diversities are very low, less than 5.1% (McCubbin et al., 2000; Wang et al., 2003).

The approach of direct sequencing of the flanking regions of the $S$-$RNase$ gene was used to successfully identify the pollen $S$-gene. In all three families that have so far been found to possess $S$-$RNase$-based SI, sequencing of $S$-locus regions has revealed the existence of a strong candidate for pollen $S$-gene, which was named $SLF$ ($S$-locus F-box) or $SFB$ ($S$-locus F-box) because the deduced amino acid sequences contain an F-box domain at the N-terminus (Lai et al., 2002; Entani et al., 2003; Ushijima et al., 2003; Wang et al., 2004). For example, by sequencing a 63-kb $S$-locus region containing $S_2$-$RNase$ in Antirrhinum hispanicum, Lai et al. (2002) identified an $A. \text{hispanicum} S$-locus F-box gene ($AhSLF$) located ~9-kb downstream of the $S_2$-$RNase$ gene. They further cloned three additional alleles $AhSLF-S_1$, $AhSLF-S_4$ and $AhSLF-S_5$, and compared the sequences of these four alleles. Surprisingly, $AhSLF$ exhibits a very low degree of allele sequence diversity, e.g., $AhSLF-S_1$ and $AhSLF-S_2$ are 97% identical in their deduced amino acid sequences (Lai et al., 2002; Zhou et al., 2003). In Prunus dulcis (Rosaceae), sequencing of an ~70-kb $S$-locus region of $S_c$ haplotype also identified an $S$-locus F-box gene, named $PdSFB$, which shows $S$-allele sequence polymorphism (Ushijima et al., 2003). In Prunus mume, sequencing of ~64.0-kb and 62.5-kb $S$-locus regions of $S_7$ and $S_7$ haplotypes, respectively, identified $PmSLF$ as the pollen $S$-locus F-box gene (Entani et al., 2003). In solanaceous species, the $S$-locus is located near a subcentromeric region where recombination is suppressed (Entani et al., 1999). Using nine $S$-linked marker genes mentioned above for chromosome walking in the $S$-locus of $P. \text{inflata}$ has revealed that the size of the $S$-locus region where recombination is suppressed exceeds 4.4 Mb (Wang et al., 2004). A 328-kb contig that contains the $S_2$-$RNase$ gene was constructed from screening an $S_2S_2$ BAC library using the full-length $S_2$-$RNase$ cDNA as a probe, followed by chromosome walking (Wang et al., 2004).
Complete sequencing of this contig has identified the *P. inflata* SLF (*PiSLF*) located ~161 kb downstream of the *S2-RNase* gene. *S1* and *S3* alleles of *PiSLF* were also cloned by RT-PCR and these three alleles of *PiSLF* are 89-90% identical in their deduced amino acid sequences (Sijacic et al., 2004).

Since the *SLF/SFB* genes mentioned above are closest to *S-RNase* in their respective *S*-locus regions, and since they show *S*-allele sequence polymorphism, they were considered good candidates for the pollen *S*-genes. The results from *in vivo* functional analysis in transgenic plants and from analysis of pollen-part self-compatible mutants in rosaceous species have confirmed that these genes are indeed the pollen-*S* genes. For example, Sijacic et al. (2004) took advantage of the competitive interaction phenomenon to ascertain whether *PiSLF* is the pollen *S*-gene. They introduced *PiSLF2* into plants of *S1S1*, *S1S2* and *S2S3* genotypes and examined whether expression of *PiSLF2* in pollen of the transgenic plants would cause breakdown of pollen *SI* function in heteroallelic situation but not in homoallelic situation. The expression of *PiSLF2* was found to render *S1S1*, *S1S2* and *S2S3* transgenic plants self-compatible through the specific breakdown of *SI* in *S1* and *S3* pollen, but not in *S2* pollen (Figure 1.3). Based on competitive interaction, *S1* and *S3* pollen that carry a duplicated *S2*-allele of the pollen *S*-gene should fail to function in *SI*, whereas *S2* pollen that carries a duplicated *S2*-allele of the pollen *S*-gene should function normally in *SI*. Thus, the results of the transgenic experiments have confirmed that *PiSLF* is the pollen *S*-gene. Using a similar transgenic approach, Qiao et al. (2004b) introduced *AhSLF-S2* into *Petunia hybrida* plants of *S3S3* genotype and showed that the expression of *AhSLF-S2* caused breakdown of the *SI* function in *S3* pollen. However, since an efficient transformation and regeneration system was not available for *Antirrhinum*, testing the function of an *Antirrhinum SLF* gene in a heterologous species did not allow Qiao et al. (2004b) to examine whether the breakdown of the *SI* function is specific to heteroallelic pollen. Thus, conclusive evidence that *AhSLF* is the pollen *S*-gene has yet to be obtained.

Studies of naturally occurring and radiation-generated pollen-part mutants in the Solanaceae suggested that deletion of the pollen *S*-gene may be lethal because all such self-compatible mutants resulted from duplication of the *S*-locus region containing the pollen *S*-allele (de Nettancourt, 2001; Golz et al., 1999; 2001). However, in the Rosaceae, several pollen-part
mutants have been found to be caused by mutation in the SLF/SFB gene or deletion of the entire gene (e.g., see Sonneveld et al., 2005). These results confirm that SLF/SFB is the pollen S-gene in the Rosaceae, and also suggest that the biochemical functions of SLF/SFB in the Rosaceae and SLF in the Solanaceae may be different.

1.6 S-Locus F-box-Like Proteins

In the rosaceous species and Antirrhinum, sequence analysis of their S-locus regions has also revealed the presence of additional F-box genes that are located in the vicinity of SLF/SFB. For example, AhSLF-S3, AhSLF-S4D and AhSLF-S1E, were identified within 51-, 75- and 71-kb of the S-locus regions of S3, S4 and S1 haplotypes, respectively, and their deduced amino acid sequences are 38-54% identical with that of AhSLF-S2 (Zhou et al., 2003). PdSLF was sequenced as a PdSFB-like gene (Ushijima et al., 2003). PmSLF1, PmSLF2, and PmSLF3 were identified as PmSLF-like genes (Entani et al., 2003). Perhaps because of the large size of the S-locus (>4.4 Mb), sequencing of the 328-kb S-locus region of P. inflata only identified the PiSLF gene but not any PiSLF-like gene. However, two of the S-linked markers identified by RNA differential display, A113 and A134, encode PiSLF-like proteins with 45-50% sequence identity to PiSLF2 (McCubbin et al., 2000; Wang et al., 2003). Judging from the numbers of the SLF/SFB-like genes found within the S-loci of other species, e.g., three PmSLF-like genes in ~64.0-kb and 62.5-kb S-locus regions of S7 and S1 haplotypes, respectively, of P. mume (Entani et al., 2003), it is most likely that there are other PiSLF-like genes located in the S-locus of P. inflata. The SLF/SFB-like genes show high sequence identities to the SLF/SFB genes; however, whether they play any role in determining pollen SI specificity remains to be studied.

1.7 Modifier Genes

In addition to the pistil SI determinant, S-RNase, and the pollen SI determinant, SLF/SFB, other genes located outside the S-locus, named modifier genes, are also required for the full manifestation of the SI response. The existence of the modifier genes was first established from genetic studies of self-compatible mutants or self-compatible relatives of self-incompatible species (de Nettancourt, 2001). Based on how these modifier genes may function in SI, they
are classified into three groups. Group 1 directly controls the expression of the pollen or pistil SI determinant, Group 2 affects pollen tube rejection but does not have a wider role in pollination; Group 3 affects not only pollen tube rejection but also pollen-pistil interactions (McClure et al., 2000; Cruz-Garcia et al., 2003).

Some examples of the modifier genes are described below. From the study of a self-compatible mutant of Petunia axillaris, a group-1 modifier gene has been implicated in the expression of $S_{13}$-RNase gene; the breakdown of the pistil function in this self-compatible mutant was attributed to defect in this gene (Tsukamoto et al., 1999). In Nicotiana alata, a 120-kD protein, identified based on its interaction with S-RNase (McClure et al., 2000; Cruz-Garcia et al., 2005), and HT-B, identified based on its presence in the pistil of self-incompatible N. alata but not in that of self-compatible N. plumbaginifolia (McClure et al., 1999), are required for the pistil function in SI but not required for the expression of the S-RNase gene. Thus, the genes encoding these two proteins are Group 2 modifier genes (McClure et al., 1999; Hancock et al., 2005). It is curious that all the modifier genes identified so far affect the pistil function in the SI response. In P. hybrida, a ubiquitously expressed protein, named S-RNase-binding protein 1 (PhSBP1), has been isolated based on its interaction with S-RNase in the yeast two-hybrid assay (Sims and Ordanic, 2001). Since PhSBP1 is also expressed in the pollen and pollen tube, it will be interesting to determine whether it plays any role in the SI function in pollen.

1.8 Earlier Models for S-RNase-Based SI

The findings that the RNase activity of S-RNase is essential for its function in rejecting the growth of self-pollen tubes (Huang et al., 1994) and that rRNA was degraded in self-pollen tubes (McClure et al., 1990) strongly suggest that S-RNase must pass through the plasma membrane of the transmitting cell and the cell wall and plasma membrane of the pollen tube to reach the cytoplasm of the pollen tube. It is not known how S-RNase enters the cytoplasm of the pollen tube. Two different models, a receptor model and an inhibitor model, were proposed, prior to the identification of the pollen S-gene, to explain how S-RNase, once in the cytoplasm, specifically rejects the growth of self-pollen tubes (Haring et al., 1990; Thompson and Kirch, 1992; Kao and McCubbin, 1996). The receptor model proposes that the product of the pollen
S-gene is localized on the plasma membrane or cell wall of the pollen tube, and that each allele variant interacts specifically with its self S-RNase, thereby selectively taking up the self S-RNase into the cytoplasm of the pollen tube. Two versions of the inhibit model were proposed. Both predict that the product of the pollen S-gene is located in the cytoplasm of the pollen tube, and that each allelic variant specifically inhibits its non-self S-RNases, thereby only allowing its self S-RNase to exert cytotoxic activity. The two versions differ in the predicted nature of the pollen S-gene product: according to the "simple" inhibitor model, the pollen S-gene product possesses both the S-specificity and inhibitor functions, whereas according to the "modified" inhibitor model, the pollen S-gene product only possesses the S-specificity function and a general inhibitor is involved in the inhibition of non-self S-RNases. Both versions predict that the interactions of an allelic product of the pollen S-gene with its self S-RNase are thermodynamically favored over the interactions with its non-self S-RNases (for review see Kao and Tsukamoto 2004). This prediction was based on the assumption that (1) self-interactions are through the matching allelic-specific domains of a pollen S-allele product and its self S-RNase, whereas non-self interactions are through a domain common to all pollen S-allele products and a domain common to all S-RNases, and (2) evolution of the SI mechanism has selected for matching allelic products of the male and female S-genes to recognize and interact with each other. Since the outcome of self-interactions in SI is inhibition of pollen tube growth, the inhibitor model predicts that self-interactions between the allelic-specific domains render self S-RNase immune to inhibition by the RNase-inhibition domain of the matching pollen S-allele product, as in the case of the simple inhibitor model (Kao and Tsukamoto, 2004), or by a general RNase inhibitor, as in the case of the modified inhibitor model (Luu et al., 2001). Immunolocalization experiments have shown that S-RNase is localized in both self and non-self pollen tubes, suggesting that uptake of S-RNases by pollen tubes is not S-allele-specific (Luu et al., 2000; Goldraij et al., 2006). Thus, the receptor model is not likely to be valid. However, it remains to be determined whether the simple or modified inhibitor model is valid.

1.9 Model Invoking Ubiquitin-26S Proteasome-Mediated Protein Degradation

Most of the F-box proteins whose functions have been characterized so far are components of a type of multi-subunit E3 ubiquitin ligase complex, named SCF (Skp1-Cul1-F-box), which is
composed of Skp1, Cullin-1, F-box protein and Rbx1, and which, along with E1 ubiquitin activating enzyme and E2 ubiquitin conjugating enzyme, is involved in ubiquitin-mediated protein degradation by the 26S proteasome (for reviews see Cardozo and Pagano, 2004; Moon et al., 2004; Smalle and Vierstra, 2004). The existence of a predicted F-box domain at the N-terminus of SLF/SFB suggests that SLF/SFB may be a component of an SCF complex. Thus, the inhibitor model has been modified to take into account the potential function of SLF/SFB in the ubiquitin-26S proteasome mediated protein degradation pathway (Qiao et al., 2004a; Sijacic et al., 2004). That is, each allelic variant of the pollen S-gene specifically mediates the ubiquitination and degradation of its non-self S-RNases, rather than inhibition of the RNase activity of its non-self S-RNases.

1.10 Homo-Tetramer Model to Explain Competitive Interaction

All the models described above predict that a pollen S-allele product interacts with its self S-RNase more strongly than with its non-self S-RNases. For example, the model that invokes ubiquitination and degradation of S-RNases proposes that, in the case of self-pollination, the strong interaction between an SLF/SFB and its self S-RNase would result in the self S-RNase not being degraded, whereas in the case of compatible pollination, the lack of interaction between an SLF/SFB and its non-self S-RNases would result in the degradation of non-self S-RNases. However, none of these models can explain the phenomenon of competitive interaction. For example, if a heteroallelic pollen tube of S1S2 genotype has penetrated into an S1S2 pistil and taken up S1- and S2-RNases, SLF1 would preferentially interact with S1-RNase and SLF2 would preferentially interact with S2-RNase. As a result, neither S1-RNase nor S2-RNase would be degraded and thus they would inhibit the growth of this heteroallelic pollen tube; this predicted outcome is precisely the opposite of what is observed. This conundrum led Luu et al. (2001) to propose, based on the modified inhibitor model, that the active form of the pollen S-allele product is a homo-tetramer. In the case of incompatible pollination, a homo-tetramer interacts with its self S-RNase and protects it from inhibition by a general RNase inhibitor. In heteroallelic pollen, using the example given above, SLF1 and SLF2 would form a hetero-tetramer, which would be unable to interact with either S1-RNase or S2-RNase. As a result, neither S-RNase would be protected from inhibition by the general RNase inhibitor, and thus this
heteroallelic pollen tube would be compatible with $S_1S_2$ pistils. However, no biochemical data supporting this model have been reported yet.

1.11 Hypotheses and Objectives

The overall goal of this thesis research is to understand how PiSLF interacts with S-RNase to elicit $S$-haplotype-specific inhibition of pollen tube growth. Since PiSLF is an F-box protein, we hypothesize that it is a component of an SCF complex, which specifically targets non-self S-RNases for ubiquitination in compatible pollen tubes. Poly-ubiquitinated S-RNases are then recognized and degraded by the 26S proteasome. Objective 1 is to address this hypothesis. I will first isolate the known components of the conventional SCF complex (Skp1, Culin-1 and Rbx1) and examine whether they form a complex with PiSLF. If not, I will use PiSLF as bait to identify all possible interacting proteins. The components I have identified will provide a clue as to whether PiSLF is a component of a typical SCF or an atypical SCF. I will also determine whether self and/or non-self S-RNases are ubiquitinated and degraded in pollen tubes using both in vivo and in vitro assays. All the results obtained from the experiments under this objective are described in Chapter 2, except that the in vivo ubiquitination and degradation assays of S-RNase will be discussed in Chapter 5.

Since there are a large number of F-box proteins in plants, this raises a question as to whether PiSLF is unique in its function in SI. We hypothesize that PiSLF is unique among F-box proteins in its function in SI, and I will address this hypothesis in Objective 2. I will first isolate pollen-expressed F-box genes that are tightly linked to the $S$-locus, show allelic sequence diversity, and share sequence similarity with PiSLF. I will then use these newly isolated PiSLF-like genes, as well as two such genes, $A113$ and $A134$, that had previously been identified in the lab (McCubbin et al., 2000; Wang et al., 2003) for biochemical and functional studies. If none of the PiSLF-like genes are likely involved in SI, I will compare their deduced amino acid sequences with those of three alleles of PiSLF to identify PiSLF-specific regions, and then examine the roles of these regions in interactions with S-RNase to reveal their unique functions in SI. If one of the PiSLF-like genes is shown to function in SI as well, I will examine whether
the function of PiSLF in SI requires that it form a complex with this F-box protein. All the results obtained from the experiments under Objective 2 are described in Chapter 3.

Ubiquitinated proteins usually contain many lysine residues with some of them playing a major role in targeting the proteins for ubiquitination through the formation of an iso-peptide bond between their ε-amino group and the C terminal glycine residue of ubiquitin (for a review, see Hochstrasser 2006). If S-RNase is indeed ubiquitinated and degraded in a pollen tube, I hypothesize that one or more lysine residues must play a major role in this process. To address this hypothesis, in Objective 3, I will use site-directed mutagenesis to change all the lysine residues of S3-RNase, one or more at a time, and examine the effect of these lysine-to-arginine changes on the ability of the mutant S3-RNases to be ubiquitinated and degraded in vitro. The results obtained under Objective 3 are described in Chapter 4.

In Chapter 5, I will present a new biochemical model to explain S-RNase-based SI based on the results obtained in my thesis research. I will also discuss a number of future experiments, built upon my results, to further advance the understanding of this type of SI mechanism. Some primary results have been obtained and will be described.
At Stage I, a pollen mother cell undergoes meiosis to produce a tetrad, which is enclosed within a callose wall. At Stage II, the enzyme callase, secreted from the tapetum of the anther, digests the callose wall to release the microspores into the anther cavity. Following migration of the nucleus and enlargement of the microspore, each uninucleate microspore undergoes one round of mitosis to become an immature pollen grain, which contains a vegetative cell and a generative cell completely enclosed within the vegetative cell (Stage III). After dehydration (Stage IV), mature pollen is formed at Stage V. When mature pollen lands on the surface of the stigma, it is re-hydrated and germinates to produce a pollen tube. The generative cell undergoes another round of mitosis to generate two sperm cells during the growth of the pollen tube.
Figure 1.2 Model for Competitive Interaction in Heteroallelic Pollen.

(A) Self-pollination of a diploid self-incompatible plant of $S_1S_2$ genotype. This plant produces haploid pollen of either $S_1$ or $S_2$ haplotype, and the growth of both pollen tubes in the diploid $S_1S_2$ pistil is inhibited because both $S_1$ and $S_2$ haplotypes are carried by the pistil.

(B) Self-pollination of a tetraploid plant of $S_1S_1S_2S_2$ genotype, which is derived from genome duplication of an $S_1S_2$ self-incompatible diploid plant. This tetraploid plant produces three $S$-genotypes of diploid pollen, $S_1S_1$, $S_2S_2$, and $S_1S_2$. The $S_1S_2$ pollen (heteroallelic pollen) fails to be rejected by the pistil, whereas both the $S_1S_1$ and $S_2S_2$ pollen (homoallelic pollen) behave normally in SI and are rejected by the pistil. As a result, this tetraploid plant is self-compatible.
Figure 1.3 Transgenic Approach to Assess the Function of PiSLF in SI (Modified from Sijacic et al., 2004).

The S-genotypes of the pollen produced by each transgenic plant are indicated at the top of the figure. The predicted S-genotypes of the progeny produced from self-pollination, and the inheritance of the transgene in the progeny are indicated at the bottom of the figure.

(A) Self-pollination of an $S_1S_1$ transgenic plant carrying one copy of the PiSLF$_2$ transgene. (B) Self-pollination of an $S_2S_3$ transgenic plant carrying one copy of the PiSLF$_2$ transgene.
CHAPTER 2

Identification and Characterization of Components of a Putative

*Petunia* S-Locus F-Box-Containing E3 Ligase Complex Involved in

S-RNase-Based Self-Incompatibility

The work described in this chapter has been published in Hua and Kao, Plant Cell 18: 2531-2553, 2006.
2.1 Introduction

As discussed in Chapter 1, a current model for the function of SLF is predicated on the assumption that SLF is a component of a canonical SCF complex (Qiao et al., 2004a,b; Sijacic et al., 2004; Huang et al., 2006). This model predicts that an SLF interacts with its self and non-self S-RNases differently so that only non-self S-RNases are ubiquitinated and degraded by the 26S proteasome. As a first step toward testing the validity of this model in *P. inflata*, I set out to identify potential components of the putative SCF$_{PiSLF}$ complex. The results suggest that a Cullin-1 (named PiCUL1-G), a RING finger protein (named PiSBP1) and PiSLF are likely to be components of a novel E3 ligase complex, with PiSBP1 playing the roles of Skp1 and Rbx1 in the canonical SCF complex. An *in vitro* binding assay was also used to examine the interactions between S-RNases and PiSLFs, and the results suggest that S-RNases interact with their non-self PiSLFs to a greater extent than with their self PiSLF, and that PiSLFs exhibit a similar binding property for S-RNases. Finally, I used pollen tube extracts and purified recombinant S$_1$, S$_2$ and S$_3$-RNases, as well as both glycosylated and de-glycosylated forms of S$_3$-RNase purified from pistils, to show that all non-glycosylated S-RNases were degraded via the 26S proteasome pathway, and that the recombinant S-RNases were ubiquitinated.

2.2 Methods

2.2.1 Plant Material

The five *S*-genotypes of *Petunia inflata* used in this study, *S$_1$S$_1$*, *S$_1$S$_2$*, *S$_2$S$_2$*, *S$_2$S$_3$* and *S$_3$S$_3$*, were identified by Ai et al. (1990).

2.2.2 cDNA Library Screening

The *S$_1$* and *S$_2$* pollen cDNA libraries of *P. inflata*, constructed in λZAPII vector (Stratagene, La Jolla, CA), were described by Skirpan et al. (2001). The *ASK1* and *ASK2* cDNA clones of *Arabidopsis* were radiolabeled with $^{32}$P using the Ready-To-Go DNA Labeling kit (GE Healthcare, Piscataway, NJ), and used as probes for screening the *S$_1$* pollen cDNA library as
described by Mu et al. (1994) except for the following modifications. The nitrocellulose membranes (Millipore, Bedford, MA) were prehybridized in 10% (w/v) dextran sulfate, 1M NaCl, 1% (w/v) SDS and sonicated salmon sperm DNA (200 μg/mL) for 2 hr, hybridized in the same buffer plus the 32P-labeled ASK1 and ASK2 cDNA probes overnight, and washed in 2× SSC, 0.1% SDS twice for 20 min each and then in 1× SSC, 0.1% SDS for 30 min. All the manipulations were carried out at 52°C allowing ASK1 and ASK2 cDNA probes to cross-hybridize with the homologous cDNAs of P. inflata. cDNAs of the positive clones were excised from the λZAPII vector and re-circulated to form pBluescript SK- phagemid DNA according to the manufacturer’s protocol (Stratagene). Plasmid DNA was purified by the NucleoSpin® Plasmid kit (Clontech, Palo Alto, CA) using the procedure recommended by the manufacturer. The cDNA library screenings using PiSK1, Cullin-1, AtRBX1 cDNA probes were similarly carried out.

2.2.3 Yeast Two-Hybrid Protein-Protein Interaction Assay

The coding sequences for PiSK1, PiSK2, PiSK3, PiSLF1, PiSLF2, PiSLF2(FB), PiSLF2(CTD), PiCUL1-G, and PhUBC1 were cloned in-frame to the coding sequence of the GAL4-binding domain (BD) in pGBD-C1. The coding sequences for seven Arabidopsis ASKs (ASK1, ASK4, ASK5, ASK9, ASK11, ASK13 and ASK16), PiSK1, PiSK2, PiSLF1, PiSLF2, PiRBX1, PiSBP1, and PiSBP1(Δcoiled-coil) were fused in-frame to the GAL4-activation domain (AD) in pGAD-C1. For testing the interaction between a pair of proteins, the corresponding pGAD-C1 and pGBD-C1 constructs were co-transformed into Saccharomyces cerevisiae SFY526 (Clontech), and the transformants were plated out on synthetic dropout (SD) medium without Trp and Leu to select cells in which both BD and AD fusion proteins co-expressed. Six independent transformants were streaked together as a dot on the filter paper and then assayed using the X-gal filter lift method (Breeden and Nasmyth, 1985). For β-galactosidase activity quantitative assay, six independent colonies were separately inoculated in 5 mL of SD medium lacking Leu and Trp. The cultures growing at the mid-log phase were used for β-galactosidase activity assay according to Miller (1972). Relative β-galactosidase activities were calculated using the methods described in Skirpan et al. (2001).
2.2.4 Yeast Two-Hybrid Library Screening

PiSK1, PiSLF2, PiSLF2(FB) and PiSLF2(CTD) were used as baits in the library screening. Yeast HF7C (Clontech) cells were transformed with 0.1 μg of each bait construct in pGBD-C1, and the transformants were subsequently transformed with 500 μg of DNA isolated from the S2 pollen yeast two-hybrid library (in pGAD424) previously constructed by Skirpan et al. (2001). Colonies that produced interacting proteins were selected on SD medium without Leu, Trp, and His, but with 3-aminotriazole (10 mM) when PiSK1 was used as bait. The positive colonies were further confirmed by X-gal filter lift assay as described under “Yeast Two-Hybrid Protein-Protein Interaction Assay”. To recover the prey plasmid from each positive colony, plasmid DNA was isolated and transformed into E. coli HB101, and the transformants were plated out on Leu-Amp+ M9 agar medium. Plasmid DNA was purified from the E. coli cultures by the NucleoSpin™ Plasmid kit and sequenced using GAL4 AD forward primer (5’-TACCACTACATGGATG-3’) and reverse primer (5’-TGAGATGGTGCGCAGCAG-3’). 6×10^6, 3×10^6, 1.4×10^6 and 1.7×10^6 yeast colonies were screened using PiSK1, PiSLF2, PiSLF2(FB) and PiSLF2(CTD) as baits, respectively.

2.2.5 DNA Sequence Analysis

All DNA sequencing was carried out at the Nucleic Acid Facility of The Pennsylvania State University. Nucleotide sequences were assembled and analyzed using DNA Strider 1.2.1. Database searches were run on the BLASTx program at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST/). Alignments of amino acid sequences were carried out using ClustalW (http://www.ebi.ac.uk/clustalw/), gonnet250 protein weight matrix was selected, and the gap opening and extension parameters were 10 and 0.05, respectively. Alignments were shaded using Boxshade, version 3.21 (http://www.ch.embnet.org/software/BOX_form.html). The protein interaction motifs were detected by SMART (http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1).
2.2.6 DNA and RNA Gel Blot Analyses

Genomic DNA was purified from young leaves of S1S1, S1S2, S2S2, S2S3 and S3S3 P. inflata plants using Plant DNAzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's procedure. Genomic DNA (15 μg) from each tissue was digested overnight by EcoR I or Xba I; the digests were separated on a 0.7% (w/v) agarose gel and transferred to a charged nylon membrane, Biodyne® B (Pall, Pensacola, FL). Total RNA was purified from different tissues of S2S2 plants by TRIzol reagent (Invitrogen), and 20 μg from each tissue was electrophoresed on a 1% (w/v) agarose/formaldehyde gel and blotted onto a Biodyne® B membrane as previously described (Sijacic et al., 2004). The probe was 32P-labeled PiSBP1 cDNA, which was obtained by PCR amplification of the yeast two-hybrid clone of PiSBP1 using the GAL4 AD forward and reverse primers as described under “Yeast Two-Hybrid Library Screening”, and by radiolabeling with 32P using the Ready-To-Go DNA Labeling kit. Pre-hybridization, hybridization, and washing of the membranes were carried out as described by Skirpan et al. (2001).

2.2.7 In Vitro Binding Assays

The coding sequences of PiSLF1, PiSLF2, PiFBP2411, S1-RNase, S2-RNase, RNase X2, PiCUL1-G, PhUBC1, and PiSLF2(CTD) were separately cloned in-frame behind the sequence for the (His)6:T7 tag in vector pET28 (Novagen, Madison, WI). The coding sequences of PiSBP1, S1-RNase, S2-RNase, S3-RNase, RNase X2, S1(HVabC3), and S2(HVabC3) and S3(HVabC3) were separately cloned as in-frame fusions to the GST coding sequence in vector pGEX-5X-1 (GE Healthcare). The recombinant proteins were expressed in BL21 Codon Plus Escherichia coli (Stratagene) and purified as described in Skirpan et al. (2001). To examine the binding between a GST fusion protein and a (His)6 T7 fusion protein, the GST fusion protein (0.5 - 1.0 μg) bound to 30 μL of Glutathione Sepharose 4 Fast Flow resin (GE Healthcare) was incubated with the (His)6 T7 fusion protein (0.5 - 1 μg) in 500 μL of the binding buffer (50 mM Tris-Cl, pH 7.4, 100 mM NaCl, 5 mM MgCl2, 1 mM DTT, and 0.01% NP-40) for 1 hr at 25°C. After binding, the resin was washed 3 times with binding buffer, the bound proteins were eluted by boiling in 30 μL 2× SDS reducing sample buffer for 5 min, and separated by SDS-PAGE. The presence of bound proteins was analyzed by protein gel blotting as described under “Protein
Gel Blot Analysis. For the assays that used different amounts of (His)_6 T7:PiSLF1 to bind its self and non-self S-RNases, equal amounts of GST:S1-RNase and GST:S2-RNase (or GST:S3-RNase) were separately bound to 250 μL of Glutathione Sepharose 4 Fast Flow resin, and the resin with either bound GST:S1-RNase or bound GST:S2-RNase (or GST:S3-RNase) was equally divided into eight aliquots. Each aliquot was equilibrated with 500 μL of the binding buffer, and to each two aliquots were added 2, 4, 6, or 8 μL of a stock of purified (His)_6 T7:PiSLF1. The binding reaction was carried out at 25°C for 90 min and analysis of the bound proteins was carried out as described above.

2.2.8 Purification of S3-RNase from Pistils

S3-RNase was purified from pistils of a _P. inflata_ plant of S3S3 genotype as previously described (Lee et al., 1994). Briefly, 30 pistils were collected and ground with 1 mL of extraction buffer (50 mM Tris-HCl, pH 8.5, 10 mM EDTA, 1 mM phenylmethylsulphonyl fluoride [PMSF], 1 mM CaCl2, and 1 mM dithiothreitol). After centrifugation at 12,000 × g for 10 min to remove tissue debris, the supernatant was filtered through a 0.45 μm MILLEX-GV filter (Millipore) and the filtrate was chromatographed on a Mono-S column (HR 5/5) equilibrated with 50 mM sodium phosphate (pH 6.0) using fast protein liquid chromatograph (FPLC) (GE Healthcare). The bound proteins were eluted with a linear gradient of 0-500 mM NaCl in the same buffer at a flow rate of 0.5 mL/min. The eluted proteins were monitored at A280nm with the sensitivity of the detector set to 0.1 absorbance-unit-full-scale. The fractions containing S3-RNase were determined by SDS-PAGE and confirmed by immunoblotting using an anti-S3-RNase antibody. Protein concentrations were determined using a Bio-Rad (Hercules, CA) protein assay kit with BSA as the standard.

2.2.9 Protein Gel Blot Analysis

Proteins were resolved by 10% polyacrylamide gels and blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore). To visualize the amount of proteins loaded in each lane, a duplicate gel was stained with Coomassie blue, or the membrane was stained with Ponceau S before immunoblotting. The primary antibodies used were an anti-T7 tag monoclonal antibody.
(1:10,000; Novagen), an anti-(His)$_6$ tag antibody (1:2,000; Novagen), an anti-GST antibody (1:200, Oncogene Research Products, San Diego, CA) and an affinity purified anti-S$_3$-RNase antibody (1:1000). The antiserum for S$_3$-RNase had previously been raised in rabbits against a synthetic peptide, DGDKFVSFLKDRIV (corresponding to amino acids 48 to 62 of S$_3$-RNase in the hypervariable region HVa). The mono-specific antibody against S$_3$-RNase was purified using GST:S$_3$(HV$_a$bC3), which encodes amino acids 47 to 97 of S$_3$-RNase, following the procedure of Bar-Peled and Raikhel (1996). After the blots had been incubated with the secondary antibody of peroxidase-linked sheep anti-mouse IgG (1:10,000; GE Healthcare), the immunoreactive proteins were visualized with Supersignal® West Pico Chemiluminescent Substrate KIT (Pierce, Rockford, IL). Alternatively, the immunoreactive proteins were detected with a Bio-Rad AP-Conjugate Substrate Kit after the blots had been incubated with alkaline phosphatase conjugated goat anti-mouse IgG (1:5,000; Calbiochem, La Jolla, CA).

2.2.10 Preparation of Pollen Tube Extracts

Fresh pollen was collected from S$_1$S$_1$, S$_2$S$_2$, and S$_3$S$_3$ plants, and separately germinated in vitro for 3 hr in the pollen germination medium described in Lee et al. (1996). Pollen tubes were harvested by centrifugation at 16,000 × g for 1 min and quickly frozen in liquid nitrogen before storage at -80°C. The pellets were homogenized in a 1.5 mL microfuge tube with a pestle and extracted with 500 μL pollen tube extraction buffer (50 mM Tris-HCl, pH 7.4, 5 mM MgCl$_2$, 2 mM DTT, and 1 mM PMSF). After centrifugation at 20,000 × g at 4°C for 30 min, the supernatants were aliquoted and stored at -80°C. The total protein concentration of each extract was determined by the Bio-Rad protein assay kit.

2.2.11 In vitro Degradation and Ubiquitination Assays

For the degradation assay of bacterially expressed proteins, 0.3 μg of GST, GST:S$_1$-RNase, GST:S$_2$-RNase, GST:S$_3$-RNase, or GST:RNase X2 were incubated with 10 μg of total pollen tube extracts (quantified based on the protein concentration) in a final volume of 30 μL of ubiquitin reaction buffer (50 mM Tris-HCl, pH 7.4, 2 mM ATP, 2 mM DTT, 5 mM MgCl$_2$, ~4 μg creatine phosphokinase [Calbiochem], 10 mM creatine phosphate [Calbiochem], and 1 mM
PMSF) for 1 hr at 30°C. The reaction was stopped by the addition of 7 μL 5x SDS reducing sample buffer, and the mixture was heated at 95°C for 5 min. The proteins were resolved on two duplicate 10% reducing SDS polyacrylamide gels and then transferred to PVDF membranes (Millipore). S3-RNase (0.1 μg), purified from pistils, and its de-glycosylated form (0.1 μg) were similarly analyzed for degradation except that the incubation time was 1.5 hr. The deglycosylation reaction was carried out at 37°C using PNGase-F (New England Biolabs, Ipswich, MA) according to the manufacturer’s protocol.

For the ubiquitination assay, 0.5 μg of GST:S2-RNase, GST:S3-RNase, GST:RNase X2 or GST were incubated with 5 μg of total S2 pollen tube extracts and 1 μg/μL ubiquitin (Boston Biochem, Cambridge, MA) or (His)6 ubiquitin in a final reaction volume of 30 μL of ubiquitin reaction buffer at 30°C. A (His)6 ubiquitin construct was made by digesting pET26Ub (Gohara et al., 1999) with NdeI and BamHI to release the DNA fragment encoding Saccharomyces cerevisiae ubiquitin, and cloning the fragment at the NdeI and BamHI sites in pET28. The recombinant ubiquitin protein was expressed and purified as described under “In Vitro Binding Assays”. At various time points during the assay, the reaction was stopped, and GST, GST fused proteins, and their respective ubiquitinated forms were purified by binding to the Glutathione Sepharose 4 Fast Flow resin in the GST-binding buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 1 mM DTT, 0.4% [w/v] Triton X-100, and 1 mM PMSF) for 30 min at room temperature. The resin was washed 3 times in the GST-washing buffer (50 mM Tris-HCl, pH 7.4, 500 mM NaCl, 10 mM EDTA, 1 mM DTT, and 0.4% [w/v] Triton X-100), and the bound proteins were eluted by heating at 95°C for 5 min in 30 μL of 2.5× SDS reducing sample buffer. The eluted proteins were separated on 10% reducing SDS polyacrylamide gels and transferred to a PVDF membrane for immunoblotting.

2.3 Results

2.3.1 Isolation and Characterization of Three Skp1 Genes of P. inflata

To isolate Skp1 genes of P. inflata, I used cDNAs for ASK1 and ASK2, two well-characterized Arabidopsis Skp1s, as probes to screen an S2S3 pollen cDNA library under low stringency
hybridization conditions. Screening of $3 \times 10^5$ pfu resulted in four independent clones, and sequencing revealed that they all corresponded to the same gene. The longest cDNA was 681 bp, with a 468-bp open reading frame. The deduced amino acid sequence was 80% and 83% identical with the amino acid sequences of ASK1 and ASK2, respectively, suggesting that this cDNA encodes a Skp1. The corresponding gene was thus named $PiSK1$ ($P$. inflata $Skp1$). Genomic gel blot analysis revealed that, under low stringency hybridization conditions, $PiSK1$ hybridized to at least three additional genomic fragments of $P$. inflata (Figure 2.1). To isolate other $Skp1$ genes homologous to $PiSK1$, I used the full-length $PiSK1$ cDNA as a probe to screen $3 \times 10^6$ pfu of an $S_2S_2$ pollen cDNA library under low stringency hybridization conditions. Twenty-two positive clones were isolated, and sequencing revealed that six encoded $PiSK1$ and the other 16 corresponded to two $PiSK1$ homologues. These two genes were named $PiSK2$ and $PiSK3$. Alignment of the amino acid sequences of $PiSK1$, $PiSK2$ and $PiSK3$ with those of ASK1, ASK2 and a human Skp1 is shown in Figure 2.2. Pair-wise amino acid sequence identities between these three $P$. inflata Skp1 proteins range from 90% to 92%, which is higher than the 79% amino acid sequence identity between ASK1 and ASK2.

I next used the yeast two-hybrid assay to examine whether $PiSK1$, $PiSK2$ and $PiSK3$ interact with $PiSLF$. The coding sequences of $PiSK1$, $PiSK2$ and $PiSK3$ were inserted into a yeast two-hybrid bait vector, pGBD-C1 (James et al., 1996), and the coding sequence of $PiSLF_2$, the product of $S_2$-allele of $PiSLF$, was inserted into a prey vector, pGAD-C1 (James et al., 1996). No positive interactions were observed between any of these three $PiSK$s and $PiSLF_2$ (Figure 2.3). The yeast two-hybrid assay was also carried out using $PiSK1$ and $PiSK2$ in pGAD-C1, and $PiSLF_1$ ($S_1$-allele of $PiSLF$) and $PiSLF_2$ in pGBD-C1. Again, no interactions were observed in any of the four possible combinations of $PiSK$s and $PiSLFs$ (Table 2.1). To ascertain whether these three $PiSK$s are bona fide Skp1s, I used $pGBD-C1$-$PiSK1$ as bait to screen an $S_2S_2$ pollen prey library previously constructed in pGAD424 (Skirpan et al., 2001). Twenty independent colonies were isolated under high stringency screening. PCR fingerprinting and sequencing revealed that these 20 clones represented seven different genes, and the deduced amino acid sequences of all of them contained an F-box domain at the N-terminus. β-galactosidase activity assays showed that all these seven F-box proteins interacted strongly with $PiSK1$, $PiSK2$ and $PiSK3$, the results for two of these F-box proteins, named
PiFBP23 and PiFBP2011 (*P. inflata* F-Box Protein 23 and 2011, respectively), are shown in Figure 2.3. The observation that all the interacting proteins of PiSK1 isolated from the yeast two-hybrid screen are F-box proteins suggests that PiSK1 and its homologues, PiSK2 and PiSK3, are *bona fide* Skp1 proteins. None of the genes encoding these seven F-box proteins are likely linked to the *S*-locus, because no restriction fragment length polymorphism (RFLP) was observed when cDNAs for these genes were used as probes in genomic gel blot analysis of *S1S1, S1S2, S2S2, S2S3*, and *S3S3* genotypes (Figure 2.4).

### 2.3.2 PiSLF Does Not Interact with Arabidopsis Skp1 Proteins

The observation that PiSLF2 did not interact with PiSK1, PiSK2 or PiSK3 suggested that Skp1 might not be a component of the complex containing PiSLF. To address this possibility, I first examined whether there is any Skp1 that interacts with PiSLF2. Since the complete genome sequence of *Petunia* was not available, I tested the interactions of PiSLF2 with the *Arabidopsis* Skp1s. The *Arabidopsis* genome sequence predicts the existence of 19 *Skp1* genes, *ASK1* through *ASK19* (Farras et al., 2001; Gagne et al., 2003; Zhao et al., 2003), which, based on phylogenetic studies, have been classified into seven subgroups (Zhao et al., 2003). I chose eight members (*ASK1, 4, 5, 9, 11, 13, 16, 18*), with at least one from each subgroup, to test whether their encoded proteins interact with PiSLF2. The yeast two-hybrid assay showed that none of these seven ASKs interacted with PiSLF2 (Figure 2.5). As a positive control for the interacting protein of PiSLF2, a protein, named PiSBP1 (*P. inflata* SBP1) (see the following section for detail), showed specific interaction with PiSLF2 in the same yeast strain, SFY526 (Figure 2.5). Because Skp1 interacts with F-box proteins through their F-box domain, I also used the yeast two-hybrid assay to examine whether any of these seven *Arabidopsis* ASKs interacts with PiSLF2(FB), the F-box domain (amino acids 1 to 49) of PiSLF2. Again, no interactions were observed again.

### 2.3.3 Identification of PiSBP1 as an Interacting Partner of PiSLF

To further examine the possibility that PiSLF might interact with other Skp1(s) present in the pollen tube, I used PiSLF2, PiSLF2(FB) and PiSLF2(CTD), which contains the C-terminal
domain (amino acids 50 to 389), as baits to separately screen the S2\S2 pollen prey library; 10, 13, and 7 positive colonies were isolated for \textit{pGBD-C1-PiSLF2}, \textit{pGBD-C1-PiSLF2(FB)} and \textit{pGBD-C1-PiSLF2(CTD)}, respectively. Restriction digestion and sequence analysis revealed that the cDNAs contained in all the positive clones corresponded to the same gene whose deduced amino acid sequence was 98% identical with that of \textit{PhSBP1} (\textit{P. hybrida} S-RNase-Binding Protein 1). \textit{PhSBP1} had previously been identified by Sims and Ordanic (2001) from yeast two-hybrid screens using as baits truncated \textit{P. hybrida} S-RNases (containing the two hypervariable regions, \textit{HVa} and \textit{HVb}, and the conserved region \textit{C3}, but missing the conserved regions \textit{C1}, \textit{C2}, \textit{C4} and \textit{C5}, defined in Ioerger et al., 1991). A \textit{PhSBP1} homologue was identified in \textit{S. chacoense} using the same approach (O’Brien et al., 2004). Because the amino acid sequence of the protein found to interact with \textit{PiSLF2} is almost completely identical with that of \textit{PhSBP1}, the protein was named \textit{PiSBP1} (\textit{P. inflata} SBP1). The interaction between \textit{PiSLF2} and \textit{PiSBP1} was further confirmed by the findings that co-expression of \textit{PiSLF2} (as a bait) and \textit{PiSBP1} (as a prey) in the yeast strain SFY526 promoted the expression of $\beta$-galactosidase and that presence of either one alone did not cause the expression of $\beta$-galactosidase (Figure 2.5). An alignment of the amino acid sequences of \textit{PiSBP1}, \textit{PhSBP1} and \textit{ScSBP1} (\textit{S. chacoense} SBP1) is shown in Figure 2.6. Amino acid sequence analysis by SMART showed that all these three proteins have two protein-protein interaction domains: a coiled-coil region between amino acids 183 and 227, and a RING-HC domain between amino acids 289 and 323.

To determine whether \textit{PiSLF1} also interacts with \textit{PiSBP1}, \textit{pGAD-C1-PiSBP1} was separately co-transformed with \textit{pGBD-C1-PiSLF1} and \textit{pGBD-C1-PiSLF2} into yeast. The interactions of \textit{PiSBP1} with \textit{PiSLF1} and \textit{PiSLF2} were quantified by an ONPG (o-nitrophenyl-$\beta$-D-galactoside) assay of the $\beta$-galactosidase activity produced by the colonies (Figure 2.7A). Comparable activities were detected for the interactions of \textit{PiSBP1} with \textit{PiSLF1} and \textit{PiSLF2}, whereas only background activities were detected in all the negative controls.

The interaction between \textit{PiSBP1} and \textit{PiSLF2} was further confirmed by an \textit{in vitro} binding assay. The coding sequence of \textit{PiSBP1} was cloned into an expression vector, pGEX-5X-1, to produce a glutathione S-transferase (GST) fusion protein, GST:PiSBP1, and the coding sequence of \textit{PiSLF2} was cloned into another expression vector, pET28, to produce a (His)$_6$ and T7 tagged...
protein, (His)\_6\_T7\_PiSLF\_2. To determine whether PiSBP1 interacts with other F-box proteins, I also cloned the coding sequence of PiFBP2411 (one of the seven F-box proteins found to interact with PiSK1) into pET28 to produce (His)\_6\_T7\_PiFBP2411. As shown in Figure 2.7B, (His)\_6\_T7\_PiSLF\_2 was detected only when both the GST:PiSBP1-bound resin and this protein were present in the reaction mixture. Similarly, (His)\_6\_T7\_PiFBP2411 was detected only when both the GST:PiSBP1-bound resin and this protein were present in the reaction mixture. Thus, the interaction of PiSBP1 with PiSLF is not specific to the F-box protein involved in SI.

### 2.3.4 Genomic Complexity and Expression Pattern of PiSBP1

To determine whether PiSBP1 exhibits S-specific RFLP, I used the full-length PiSBP1 cDNA as a probe for genomic gel blot analysis of five different S-genotypes, S\_1\_S\_1, S\_1\_S\_2, S\_2\_S\_2, S\_3\_S\_3, and S\_3\_S\_3, of P. inflata (Figure 2.8A). The same hybridizing fragment was detected in XbaI digests of all the genotypes (no XbaI recognition sequence is present in the PiSBP1 cDNA), and the same two hybridizing fragments were detected in EcoRI digests of all the genotypes (one EcoRI recognition sequence is present in the PiSBP1 cDNA). Thus, it is likely that PiSBP1 is a single-copy gene and unlinked to the S-locus. RNA gel blot analysis showed that PiSBP1 was expressed in all the tissues examined (Figure 2.8B). All these results are similar to those reported for PhSBP1 by Sims and Ordanic (2001). Interestingly, the relative expression level of PiSBP1 in anthers of different developmental stages and in pollen/pollen tubes is very similar to that of PiSLF (Sijacic et al., 2004). The expression level peaked in stage 3 and stage 4 anthers, after the completion of meiosis of pollen mother cells to produce microspores (Lee et al., 1996), and significantly reduced in mature pollen and in vitro cultured pollen tubes (Figure 2.8B).

### 2.3.5 PiSBP1 Interacts with S-RNases but Not with an S-Like RNase

Since both PhSBP1 and ScSBP1 had been shown by the yeast two-hybrid assay to interact with truncated S-RNases containing the two hypervariable regions (HV\_a and HV\_b) and the conserved region C\_3 (Sims and Ordanic, 2001; O’Brien et al., 2004), I examined whether PiSBP1 also interacts with the corresponding region of S\_1- and S\_2-RNases of P. inflata. The sequences for this region of S\_1-RNase (amino acid residues 47 to 97) and S\_2-RNase (amino acid residues 46 to
were used to make two bait constructs, \( pGBD-C1-S_1(HV_{ab}C3) \) and \( pGBD-C1-S_2(HV_{ab}C3) \), both of which were separately co-transformed with \( pGAD-C1-PiSBP1 \) into yeast. The ONPG assay showed that PiSBP1 interacted with the truncated S1- and S2-RNases (Figure 2.9A). Similar to what was reported by Sims and Ordanic (2001) for PhSBP1 and by O’Brien et al. (2004) for ScSBP1, when the mature S1- and S2-RNases were used as baits, no interaction with PiSBP1 was detected by the yeast two-hybrid assay. Hereafter, the terms “S1-RNase”, “S2-RNase”, and “S3-RNase” refer to the mature form of these S-RNases without the leader peptide, i.e., amino acid residues 1 to 200 for S1-RNase, 1 to 199 for S2-RNase, and 1 to 200 for S3-RNase.

The failure to detect any interaction between PiSBP1 and S1- and S2-RNases by the yeast two-hybrid assay could be because S-RNase did not fold properly in the yeast cell, as it has eight conserved cysteines involved in four intra-molecular disulfide bonds (Ishimizu et al., 1996; Oxley and Bacic, 1996). So, I used the in vitro binding assay, as described in Figure 2.7B, to re-examine the interaction. The coding sequences of S1- and S2-RNases were cloned into pET28 to produce (His)_6:T7:S1-RNase and (His)_6:T7:S2-RNase, respectively. As a control, the coding sequence (without the signal peptide; amino acid residues 1 to 193) of RNase X2 of \( P.\ inflata \) (Lee et al., 1992) was also cloned into pET28 to produce (His)_6:T7:RNase X2. RNase X2, an S-like RNase, is 43% identical with S1- and S2-RNases, and it was used to ascertain whether any interaction observed with either S-RNase is specific to S-RNase. The results showed that PiSBP1 interacted with both S1- and S2-RNases, but not with RNase X2 (Figure 2.9B).

2.3.6 S-RNases Interact with Non-Self PiSLFs to a Greater Extent than with Self PiSLFs, and PiSLFs Show a Similar Binding Property for S-RNases

The in vitro binding assay described above was used to examine whether a PiSLF interacts with its self S-RNase and/or non-self S-RNases, and if it interacts with both, whether there is any difference in the extent of the interaction. The coding sequences of S1-, S2- and S3-RNases were fused in-frame to the GST coding sequence in expression vector pGEX-5X-1 to produce GST:S1-RNase, GST:S2-RNase and GST:S3-RNase, respectively. The coding sequence of RNase X2 (without the signal peptide) was similarly fused to the GST coding sequence to
produce GST:RNase X2. The coding sequence of PiSLF1 was cloned into pET28 to produce (His)6:T7:PiSLF1. I first showed that both PiSLF1 and PiSLF2 interacted with S1- and S2-RNases, but not with RNase X2 (Figure 2.10), suggesting that PiSLFs specifically interact with S-RNases. To examine whether an S-RNase interacts with its self and non-self PiSLFs to different extents, equal amounts of (His)6:T7:PiSLF1 and (His)6:T7:PiSLF2 were separately incubated with the same amount of resin-bound GST:S2-RNase, and the bound proteins were detected by an anti-(His)6 antibody. As shown in Figure 2.11A, the intensity of the (His)6:T7:PiSLF1 band was much stronger than that of the (His)6:T7:PiSLF2 band. No binding was detected between GST and either (His)6:T7:PiSLF1 or (His)6:T7:PiSLF2 (Figure 2.11A). These results suggest that S2-RNase interacts with its non-self PiSLF to a greater extent than with its self PiSLF. To further confirm this finding, I carried out the in vitro binding assay in a single reaction mixture containing GST:S2-RNase and equal amounts of (His)6:T7:PiSLF1 and (His)6:T7:PiSLF2 (Figure 2.11B). As these two (His)6 T7 tagged proteins can be clearly separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; see “Control” lanes), I was able to assess the relative intensity of these two protein bands. Consistent with the results shown in Figure 2.11A, the intensity of the (His)6:T7:PiSLF1 band was much stronger than that of the (His)6:T7:PiSLF2 band.

I next examined whether PiSLFs have a similar binding property for S-RNases, using the truncated S1- and S2-RNases that had been shown to interact with PiSBP1 (Figure 2.9A). Purified GST:S1(HVabC3) and GST:S2(HVabC3) fusion proteins were used along with (His)6:T7:PiSLF2 in the in vitro binding assay. As shown in Figure 2.11C, (His)6:T7:PiSLF2 interacted with both truncated S-RNases, but the intensity of the (His)6:T7:PiSLF2 band was stronger in the binding reaction containing GST:S1(HVabC3) than in that containing GST:S2(HVabC3). Moreover, (His)6:T7:PiSLF1 interacted with both truncated S-RNases, but the intensity of the (His)6:T7:PiSLF1 band was stronger in the binding reaction containing GST:S2(HVabC3) than in that containing GST:S1(HVabC3) (Figure 2.11D). These results suggest that both (His)6:T7:PiSLF1 and (His)6:T7:PiSLF2 interact with their respective truncated non-self S-RNase to a greater extent than with their respective truncated self S-RNase.
To determine whether the interaction between S-RNase and PiSLF is specific to PiSLF, I examined whether (His)_6 T7.PiFBP2411 interacts with GST:S1(HVabC3) and GST:S2(HVabC3) in the *in vitro* binding assay. PiFBP2411 was chosen because I had shown that both PiFBP2411 and PiSLF2 interacted with PiSBP1 (Figure 2.7B). No interactions were observed between (His)_6 T7.PiFBP2411 and either of these two truncated S-RNases (Figure 2.11D), suggesting that S-RNases most likely interact specifically with PiSLFs.

I carried out additional *in vitro* binding assays to further assess the relative extent of binding between PiSLF1 and S1-, S2- and S3-RNases. In one assay, equal amounts of GST:S1-RNase and GST:S2-RNase were separately incubated with four different amounts of (His)_6 T7.PiSLF1, with each incubation in duplicates. Increasing binding of (His)_6 T7.PiSLF1 to both GST:S1-RNase and GST:S2-RNase was observed as the amount of (His)_6 T7.PiSLF1 was increased (Figure 2.11E). For all the amounts examined, (His)_6 T7.PiSLF1 interacted with its non-self S-RNase, GST:S2-RNase, to a greater extent than with its self S-RNase, GST:S1-RNase. Most importantly, at the lowest amount used (2 μL), there was very little binding with GST:S1-RNase, but significant binding with GST:S2-RNase. Another assay was similarly carried out except that a different preparation of (His)_6 T7.PiSLF1 was used and that GST:S3-RNase, instead of GST:S2-RNase, was used as a non-self S-RNase. The results (Figure 2.11F) were similar to those obtained in Figure 2.11E. For all the amounts examined, (His)_6 T7.PiSLF1 interacted with its non-self S-RNase, GST:S3-RNase, to a greater extent than with its self S-RNase, GST:S1-RNase, and again at the lowest amount used (2 μL), there was very little binding of (His)_6 T7.PiSLF1 with GST:S1-RNase, but significant binding with GST:S3-RNase.

### 2.3.7 PiSBP1 Interacts with S-RNase and PiSLF Differently

Since PiSBP1 contains a coiled-coil region (amino acid residues 183 to 227; Figure 2.6) that could potentially be involved in protein-protein interactions, I examined whether this region is required for PiSBP1 to interact with PiSLF and S-RNase. The sequence for the coiled-coil region was deleted from the full-length *PiSBP1* cDNA to produce *PiSBP1*(Δcoiled-coil), which was cloned into pGAD-C1. *pGAD-C1-PiSBP1*(Δcoiled-coil) was separately co-transformed with *pGBD-C1-S1(HVabC3)*, *pGBD-C1-S2(HVabC3)*, and *pGBD-C1-PiSLF2* into yeast for the two-
hybrid assay. The results showed that PiSBP1Δcoiled-coil interacted with S1(HVabC3) and S2(HVabC3), but not with PiSLF2 (Figure 2.12). Thus, the coiled-coil region of PiSBP1 is either directly or indirectly required for its interaction with PiSLF2, but is not required for its interaction with S-RNase, suggesting that S-RNase and PiSLF interact differently with PiSBP1.

2.3.8 PiSBP1 Interacts with a Cullin-1 and an E2 Ubiquitin Conjugating Enzyme

I isolated cDNAs for Cullin-1 and Rbx1 of P. inflata to examine whether they are components of the complex containing PiSLF. A partial Cullin-1 cDNA clone, previously isolated during our attempts to identify S-linked pollen-expressed genes by RNA differential display (Joseph A. Verica and Teh-hui Kao, unpublished results; McCubbin et al., 2000), was used as a probe to screen 3 × 10⁵ pfu of the S₁S₁ pollen cDNA library and 6 × 10⁵ pfu of the S₂S₂ pollen cDNA library. Full-length cDNA clones for this gene, named PiCUL1-C, were isolated from the S₂S₂ library, and full-length cDNA clones for another Cullin-1 gene, named PiCUL1-G, were isolated from both cDNA libraries. PiCUL1-C and PiCUL1-G shared 80% amino acid sequence identity, and among the five Arabidopsis Cullins (Moon et al., 2004), they are most similar to ATCUL1 (83 and 78% identity, respectively). Since PiCUL1-C and PiCUL1-G are quite similar in sequence, I chose PiCUL1-G for all subsequent studies. Screening of 6 × 10⁵ pfu of the S₂S₂ pollen cDNA library, using the full-length cDNA for an Arabidopsis Rbx1, AtRBX1 (Lechner et al., 2002) as a probe, resulted in the isolation of one class of cDNA clones. The corresponding gene was named PiRBX1 whose deduced amino acid sequence is 87% identical to that of AtRBX1.

The yeast two-hybrid assay showed that there were no interactions between PiCUL1-G and PiRBX1 (Figure 2.13A), or between PiRBX1 and PiSLF2 (Figure 2.13A). The findings that PiRBX1 did not interact with either PiCUL1-G or PiSLF2, and that PiSBP1 interacted with PiSLF2 (Figures 2.5, 2.7 and 2.13A) raised a possibility that PiSBP1, but not PiRBX1, is the RING protein that brings E2 into the complex containing PiSLF. To address this possibility, I examined whether PiSBP1 interacts with PiCUL1-G and PhUBC1, an E2 of P. hybrida. The yeast two-hybrid assay showed that PiSBP1 interacted with both PiCUL1-G and PhUBC1 (Figure 2.13A). These interactions were further examined by the in vitro binding assay. The
coding sequences of PiCUL1-G and PhUBC1 were cloned into pET28 to produce (His)_{6}:T7:PiCUL1-G and (His)_{6}:T7:PhUBC1, respectively. The results of the binding assay showed that GST:PiSBP1 interacted with both (His)_{6}:T7:PiCUL1-G (Figure 2.13B) and (His)_{6}:T7:PhUBC1 (Figure 2.13C). The interactions of PiSBP1 with a Cullin-1 and an E2 conjugating enzyme suggest that in the complex containing PiSLF, PiSBP1 plays the role of Rbx1 in a canonical SCF complex.

2.3.9 S-RNases Are Degraded via the 26S Proteasomal Pathway in a Non-S-Specific Manner in Pollen Tube Extracts

To examine whether pollen tubes contain a ubiquitin-26S proteasome pathway that degrades S-RNases, I developed a cell-free system using extracts of *in vitro* germinated pollen tubes and purified GST:S-RNases or native S-RNases. First, GST:S_{1}-RNase and GST:S_{2}-RNase were separately incubated with extracts of *in vitro* germinated S_{2} pollen tubes at 30°C for 1 hr in the absence or presence of MG132 (40 μM), a specific 26S proteasome inhibitor (Lee and Goldberg, 1998; Smalle and Vierstra, 2004). The proteins were then separated by SDS-PAGE, and an anti-GST antibody was used to detect GST:S_{1}-RNase and GST:S_{2}-RNase by protein gel blot analysis (Figure 2.14A). Neither GST:S_{1}-RNase nor GST:S_{2}-RNase was detectable after 1 hr incubation in the absence of MG132. However, in the presence of MG132, the amount of each protein after 1 hr incubation was approximately the same as that prior to the incubation.

The disappearance of both GST:S-RNase bands, as assayed by the anti-GST antibody, could be due to removal of the GST tag from the fusion proteins by proteolytic cleavages. To rule out this possibility, I carried out an additional degradation assay using both the anti-GST antibody and an anti-S_{3}-RNase antibody to examine the fate of GST:S_{3}-RNase in S_{2} pollen tube extract. As shown in Figure 2.14B, the GST:S_{3}-RNase band, detected by both antibodies prior to incubation, was not detectable by either antibody after 1 hr incubation in the absence of MG132, confirming that the S-RNase part of the fusion protein was degraded. As was observed in Figure 2.14A, the disappearance of the GST:S_{3}-RNase band was prevented when MG132 was included in the extract. To further confirm these results, I also carried out degradation assays
on GST:S1-RNase and GST:S2-RNase using S1 pollen tube extract. The same results were obtained.

Since native S-RNases are glycosylated, but the bacterially expressed GST:S-RNases are not, I examined whether S3-RNase purified from pistils of S3S3 genotype was also degraded in pollen tube extracts. The purified native S3-RNase was separately incubated with S1, S2 and S3 pollen tube extracts, and the fate of the protein was analyzed by protein gel blotting using the anti-S3-RNase antibody. The results (left panel of Figure 2.14C) showed that the native S3-RNase was not degraded to a significant extent, if at all, after 90 min incubation in the absence of MG132. However, when MG132 was present, a faint band (indicated with a darkened arrow) was detected in all three pollen tube extracts after the same length of incubation. Since this protein band was not detectable prior to the incubation and had a lower molecular mass than the native S3-RNase band, I hypothesized that it might correspond to the de-glycosylated S3-RNase. That is, a small amount of S3-RNase might have been de-glycosylated and then degraded during the incubation in the pollen tube extracts without MG132.

To test this hypothesis, S3-RNase purified from the pistils was treated with Peptide:N-glycosidase F (PNGase F) to remove the N-linked glycan chain attached to Asn-29 (Karunananda, 1994), and then incubated with S2 or S3 pollen tube extract. The results of the degradation assay are shown in the right panel of Figure 2.14C. The native S3-RNase was completely de-glycosylated by PNGase F (as shown in the “0 min” lane), and the de-glycosylated S3-RNase band was virtually non-detectable after 90 min incubation in either S2 or S3 pollen tube extract in the absence of MG132. However, the intensity of the de-glycosylated S3-RNase band after 90 min incubation in the presence MG132 was comparable to that at 0 min.

When a GST fusion protein is produced in E. coli, the GST moiety of some fusion protein molecules is cleaved off around the Factor Xa cleavage site. This was also the case for GST:S1-RNase, GST:S2-RNase and GST:S3-RNase (Figures 2.14A and 2.14B). Under the assay conditions where non-glycosylated S-RNases were completely degraded, GST molecules released from these recombinant S-RNases were not degraded. To further confirm that the degradation of non-glycosylated S-RNases in our in vitro system was not caused by general
proteolytic cleavages, and to assess whether the degradation was specific to S-RNases, recombinant GST and GST:RNase X2 were examined, along with GST:S3-RNase, in S2 pollen tube extract. As shown in Figure 2.14D, GST was not degraded, whereas GST:RNase X2 was degraded in a similar 26S-proteasome-dependent manner as was GST:S3-RNase. The findings that neither GST nor the native glycosylated S3-RNase was degraded suggest that the degradation of non-glycosylated S-RNases was not caused by general proteases present in the pollen tube extracts. However, the degradation observed in our in vitro system was not S-specific, nor was it specific to S-RNases.

2.3.10 S-RNases Are Ubiquitinated by Pollen Tube Extracts in a Non-S-Specific Manner

Since the bacterially expressed non-glycosylated GST:S-RNases were degraded to similar extents in pollen tube extracts as was the de-glycosylated native S3-RNase, I decided to use GST:S2-RNase and GST:S3-RNase to examine whether degradation of S-RNase resulted from ubiquitination. Recombinant GST and GST:RNase X2 were used as controls to assess whether ubiquitination was restricted to the protein moiety (but not to the GST tag) and was specific to S-RNases. Using GST tagged S-RNases and RNase X2 also allowed us to simplify the isolation of their ubiquitinated forms, if any, by affinity purification from among many other ubiquitinated proteins in pollen tube extracts (Figure 2.15). The ubiquitination assay was carried out in a similar manner as the “degradation” assay except for the following modifications. First, 30 μg of ubiquitin or (His)6 ubiquitin was added to S2 pollen tube extract before incubation. Second, after incubation, GST:S2-RNase GST:S3-RNase, GST:RNase X2, GST, and any products derived from them were isolated by Glutathione Sepharose 4 Fast Flow resin. The bound proteins were eluted and separated by two duplicated reducing SDS-polyacrylamide gels, and protein gel blotting was carried out separately using the anti-GST antibody and an anti-ubiquitin or the anti-(His)6 antibody.

As shown in Figure 2.16A, after 5 min incubation in the S2 pollen tube extract at 30°C, the anti-GST antibody detected three discrete protein bands with molecular masses higher than that of GST:S2-RNase, and all the bands remained clearly visible after 10 min incubation. One of these bands (indicated with an asterisk in Figures 2.16A and 2.16B) was also detected by the
anti-ubiquitin antibody at the 5-min and 10 min time points (Figure 2.16B). Based on the estimated molecular masses of the three bands (67, 93 and 110 kD) detected by the anti-GST antibody, I attributed them to be GST:S2-RNase (50 kD) conjugated with two, five and seven ubiquitins (with total molecular masses of 67, 92.5 and 109.5 kD, respectively). After 1 hr incubation, the two bands with higher molecular masses had disappeared, but not the one possibly corresponding to GST:S2-RNase conjugated to two ubiquitins. This ubiquitinated GST:S2-RNase might have escaped degradation because the number of conjugated ubiquitin subunits was fewer than the minimum of four required for recognition by the 26S proteasome (Thrower et al., 2000). Moreover, the intensity of a protein band (indicated with a black dot in Figure 2.16A) with a lower molecular mass than GST:S2-RNase became more and more visible from the 5-min time point onward. This band could correspond to degradative product(s) of GST:S2-RNase.

(His)$_6$ ubiquitin was used in the ubiquitination assay of GST, GST:S3-RNase and GST:RNase X2 in the S$_2$ pollen tube extract (Figures 2.16C and 2.16D). Using (His)$_6$ ubiquitin allowed us to detect the ubiquitinated proteins by the anti-(His)$_6$ antibody (Figure 2.16D), and to compare the results with those obtained from the use of the anti-ubiquitin antibody (Figure 2.16B). The results of GST:S3-RNase and GST:RNase X2 were similar to those of GST:S2-RNase; after 10 min incubation in the pollen tube extract, discrete bands with molecular masses higher than that of the respective GST fusion protein were detected by both the anti-GST antibody (Figure 2.16C) and the anti-(His)$_6$ antibody (Figure 2.16D). In contrast, no ubiquitinated forms of GST were detected, confirming that ubiquitination of the GST fusion proteins occurred exclusively in the proteins that were fused to GST. However, similar to the results of the degradation assay, the ubiquitination observed in our in vitro system was not S-specific, nor was it specific to S-RNases.

2.4 Discussion

The recent identification of the S-Locus F-box gene as the pollen S-gene has opened up opportunities to investigate the biochemical mechanism of S-RNase-based SI. As the RNase activity of S-RNases is required for their function in SI (Huang et al., 1994) and rRNA of pollen tubes may be degraded after incompatible pollination (McClure et al., 1990), S-RNases are
thought to act as cytotoxic molecules to inhibit the growth of self-pollen tubes through RNA degradation. Both Luu et al. (2000) and Goldraij et al. (2006) have shown that uptake of S-RNases by pollen tubes is not S-allele specific. Prior to the identification of SLF as the pollen S-gene, one of the models proposed that the pollen S-allele product encodes a cytosolic RNase inhibitor, which specifically inhibits the RNase activity of all non-self S-RNases and renders them non-functional inside a pollen tube (Kao and McCubbin, 1996; Golz et al., 2001). Since most F-box proteins are involved in ubiquitin-mediated protein degradation, this model has now been modified to state that a pollen S-allele product mediates degradation of its non-self S-RNases, but not that of its self S-RNase, inside a pollen tube (Qiao et al., 2004a; Sijacic et al., 2004; Huang et al., 2006). According to this model, pollen S-allele products regulate the stability, rather than the RNase activity, of S-RNases. In this work, I have begun to address the biochemical role of the SLF of P. inflata.

2.4.1 The Complex That Contains PiSLF Is Not a Canonical SCF Complex

If PiSLF functions as a conventional F-box protein, it would be expected to be a component of an SCF complex. In this work, I have obtained several lines of evidence to suggest that the PiSLF-containing complex is not a canonical SCF complex, and is instead a novel E3 ligase complex containing a Cullin-1 protein (PiCUL1-G), a RING-HC protein (PiSBP1), and PiSLF, but not containing Skp1 or Rbx1. First, I have isolated cDNAs for three Skp1s of P. inflata and shown that none of them interact with PiSLF2 in the yeast two-hybrid (Figure 2.3). When PiSK1, one of these Skp1s, was used as bait in yeast two-hybrid library screening, all seven classes of prey proteins identified were F-box proteins, suggesting that PiSK1, and most likely its homologues, PiSK2 and PiSK3, are bona fide Skp1s. Second, the Arabidopsis genome is predicted to encode 19 Skp1s, but none of the seven Skp1s representing all seven subgroups interact with PiSLF2 in the yeast two-hybrid assay. Third, PiCUL1-G interacts with PiSBP1, but not with PiRBX1 (Figures 2.13A and 2.13B). Fourth, PiSLF1 and PiSLF2 interact with PiSBP1 (Figure 2.7), but not with PiRBX1 (Figure 2.13A). Fifth, PiSBP1 interacts with an E2 conjugating enzyme (Figure 2.13C). Interestingly, the expression of both PiSLF and PiSBP1 peaks during pollen development and declines significantly in mature pollen and in vitro.
germinated pollen tubes (Figure 2.8B; Sijacic et al., 2004). It is possible that the protein produced in developing microspores is retained in mature pollen and pollen tubes.

I have concluded that, in the PiSLF-containing complex, PiSBP1 likely plays the roles of Skp1 and Rbx1 of a canonical SCF complex. This is because PiSBP1, like Skp1, bridges the Cullin-1 component (PiCUL1-G) and an F-box protein (PiSLF), and, like Rbx1, interacts with the Cullin-1 component and E2. PiSBP1 (335 amino acids) is ~3 times the size of PiRBX1 (116 amino acids), so it could potentially interact with more proteins than does PiRBX1. Variants of the SCF complex have been reported that contain some but not all of the typical components (Willems et al., 2004). For example, an SCF-like complex in humans consists of Skp1, an F-box protein (Ebi), a RING-HC protein (Siah-1) and an adaptor protein (SIP) that bridges Siah-1 and Skp1 (Matsuzawa and Reed, 2001). In this complex, Siah-1 plays the roles of Cullin-1 and Rbx1, and interestingly Siah-1 (298 amino acids) is also larger in size than Rbx1. Another example is the E2F1 transcription factor, which can be ubiquitinated by multiple ROC-Cullin ligases that do not contain Skp1 (Ohta and Xiong, 2001).

Moreover, in Drosophila melanogaster, Sina, a RING-HC protein, forms an E3 ligase complex with Phyllopod (Phyl) and Ebi to regulate the degradation of a transcription repressor protein, Tramtrack (Ttk) (Li et al., 2002). In this complex, Ebi interacts with Sina and the substrate, Ttk. Sina and Phyl can mediate degradation of Ttk, but Ebi promotes more efficient degradation of Ttk, perhaps by stabilizing the Sina-Phyl-Ttk complex (Boulton et al., 2000; Li et al., 2002). Since SBP1 could potentially function as a single-subunit E3, the finding by Sims and Ordanic (2001) that PhSBP1 interacted with S-RNases posed a conundrum as to why two E3s (PhSBP1 and the then predicted SLF-containing SCF complex) would be involved in ubiquitination of S-RNases (McClure, 2004). Our finding that PiSBP1 is a component of a novel E3 ligase complex containing PiSLF, and that PiSBP1 likely assumes the roles of Skp1 and Rbx1 of a canonical SCF complex would provide a solution to this conundrum. Moreover, since PiSBP1 alone interacts with S-RNases, the PiCUL1-G-PiSBP1-PiSLF complex may function in a similar fashion as the Sina-Phyl-Ebi complex in that PiCUL1-G-PiSBP1 could mediate basal level degradation of all S-RNases, but PiSLF would promote efficient degradation.
of specific S-RNases (see “2.4.2 S-RNases Are Degraded via a Ubiquitin-26S Proteasomal Pathway” below).

Our finding that Skp1 is not a component of the PiSLF-containing complex contradicts the results for AhSLF of *A. hispanicum*. Qiao et al. (2004a) used an anti-AhSLF2 antibody to pull down protein complexes that contained AhSLF2 from mixtures of pollen and stylar proteins under both compatible and incompatible conditions. They found proteins that cross-reacted with an anti-ASK1 antibody or with an anti-ATCUL1 antibody. Huang et al. (2006) subsequently used yeast two-hybrid screens to identify an Skp1-like protein, named AhSSK1, which interacted with the F-box domain of AhSLF2. Thus, the AhSLF-containing complex of *A. hispanicum* may have a different subunit composition from that of the PiSLF-containing complex of *P. inflata*. In this context, it is interesting to note that the *SLF* of the Solanaceae functions differently at the mechanistic level from the *SLF/SFB* of the Rosaceae, as all the pollen-part self-compatible mutants characterized so far in the Solanaceae resulted from duplication, but not deletion, of the pollen *S*-allele (*SLF*) (Golz et al., 1999, 2001), whereas those in the Rosaceae resulted from defects (deletion or frame-shift mutations) in *SLF/SFB* (Ushijima et al., 2004; Sonneveld et al., 2005). Moreover, in the Solanaceae, pollen carrying two different functional *S*-alleles (i.e., *SLF* alleles) fails to function in SI due to competitive interaction (Golz et al., 2001; Sijacic et al., 2004), whereas in sour cherry (*Prunus cerasus*) of the Rosaceae, such heteroallelic pollen has been shown to function normally in SI (Hauck et al., 2006). Thus, it appears that even though all these three families employ F-box proteins and S-RNases as the male and female determinants, respectively, the biochemical mechanisms of SI are likely to have diverged.

### 2.4.2 S-RNases Are Degraded via a Ubiquitin-26S Proteasomal Pathway

If the PiSLF-containing E3 ligase complex is involved in degradation of S-RNases in pollen tubes, one would expect the degradation to be via the ubiquitin-mediated 26S proteasomal pathway. Previously, Qiao et al. (2004a) addressed the role of this protein degradation pathway in SI of *Antirrhinum* by examining the effect of 26S proteasome inhibitors on *in vitro* growth of pollen tubes, in the presence of either compatible or incompatible stylar extracts. They found
that the inhibitors had no effect on the growth inhibition of pollen tubes by incompatible stylar extracts, but inhibited the growth of pollen tubes in compatible stylar extracts by about 50%. Qiao et al. (2004a) further showed that protease inhibitors had no significant effect on the growth of compatible pollen tubes, and thus they concluded that protein degradation by the 26S proteasome is required for growth of compatible pollen tubes. The ubiquitin-mediated 26S proteasomal pathway is thought to be involved in many developmental processes. For example, more than 5% of the Arabidopsis proteome is related to this pathway (Smalle and Vierstra, 2004). Thus, inhibitors of the 26S proteasome will likely affect a plethora of biochemical events during pollen tube growth, complicating the interpretation of the results.

Another approach to address the role of the 26S proteasome in SI is to compare the amounts of S-RNase in pistils after compatible pollination with those after incompatible pollination at various times post pollination, up to the time when the growth of incompatible pollen tubes is inhibited in the style. For example, Qiao et al. (2004) pollinated \( S_2S_5 \) pistils of Antirrhinum with compatible or incompatible pollen, isolated total proteins from pistils collected at eight different time points (up to 60 hr) after compatible or incompatible pollination, and determined the amounts of \( S_2 \)-RNase. They detected a lower amount of \( S_2 \)-RNase in compatibly pollinated pistils than in incompatibly pollinated pistils 36 hr post pollination, and they interpreted the results to mean that \( S_2 \)-RNase was degraded in compatible pollen tubes. Goldraij et al. (2006) used a similar approach to measure the amounts of \( S_{C10} \)-RNase of \( N. \) alata in \( S_{C10}S_{C10} \) pistils that had been pollinated with \( S_{C10} \) or \( S_{105} \) pollen, but they did not detect any significant difference in the amounts of \( S_{C10} \)-RNase between compatibly and incompatibly pollinated pistils up to 48 hr post pollination. Since S-RNase is abundant in the style and since it is very likely that not all the S-RNase molecules are taken up by pollen tubes, it is difficult, if not impossible, to know precisely how much of the total amount determined from pollinated pistils is contributed by the S-RNase inside pollen tubes. This may be a reason why Qiao et al. (2004) and Goldraij et al. (2006) have reached opposite conclusions about whether S-RNase is degraded in compatible pollen tubes.

Goldraij et al. (2006) also dissected pollen tubes from \( S_{A2}S_{A2} \) pistils pollinated with compatible or incompatible pollen, and determined the amounts of \( S_{A2} \)-RNase in soluble and
membrane fractions of both compatible and incompatible pollen tubes. They concluded that there was no significant difference in the amounts of S_{A2}-RNase between the soluble or membrane fractions of compatible and incompatible pollen tubes. Accurate determination of the amount of S-RNase inside pollen tubes by this approach requires isolation of intact pollen tubes completely free of contaminating style tissue, which is abundant in S-RNase. This may be technically difficult, as the pollen tube preparations of Goldraij et al. (2006) also contained transmitting tract cells and extracellular matrix material of the style tissue. Another potential problem of using pollinated pistils or dissected pollen tubes to examine the fate of S-RNase is that, if the great majority of S-RNase taken up by a pollen tube is indeed sequestered in a vacuolar compartment and not subject to degradation, as suggested by Goldraij et al. (2006), it would be difficult to accurately assess the degradation of a small amount of S-RNase among a large amount of sequestered S-RNase.

Here, I have developed a cell-free system to examine whether S-RNases are degraded in compatible pollen tubes, and if so, whether this is mediated by the ubiquitin/26S proteasomal pathway. The system involves the use of extracts of in vitro germinated pollen tubes and exogenously added GST:S-RNases or native S-RNases purified from pistils. As the ubiquitin-mediated 26S proteasome degradation machinery is active in pollen tube extracts, I was able to use this cell-free system to show that the bacterially expressed GST:S_{1}-RNase, GST:S_{2}-RNase, GST:S_{3}-RNase and GST-RNase X2, but not GST alone, were degraded by S_{2} pollen tube extracts (Figures 2.14A and 2.14B and 2.14D). Moreover, I found that the degradation was dependent on the activity of the 26S proteasome, as MG132, a specific inhibitor of the 26S proteasome, completely inhibited the degradation (Figures 2.14A, 2.14B and 2.14D). I also showed that GST:S_{2}-RNase, GST:S_{3}-RNase and GST-RNase X2, but not GST alone, were rapidly ubiquitinated in this cell-free system (with the addition of ubiquitin or [His]_{6} ubiquitin) (Figure 2.16). The time course of ubiquitination of GST:S_{2}-RNase suggested that the molecules conjugated with four or more ubiquitins were subsequently degraded (Figure 2.16A and 2.16B). The use of the native glycosylated S_{3}-RNase in the degradation assay led us to the discovery that de-glycosylation was required for the degradation of the native S_{3}-RNase (Figure 2.14C). It will be interesting to determine why this is the case in our in vitro system, and whether this is
also true \textit{in vivo}. One possible explanation is that the N-linked glycan chain of the native \( S_3 \)-RNase might mask the lysine residue(s) essential for ubiquitination.

All the results from the degradation and ubiquitination assays taken together suggest that \( S \)-RNases are degraded via the ubiquitin-mediated 26S proteasomal pathway. However, for \( S_1 \), \( S_2 \) and \( S_3 \) pollen tubes tested, no \( S \)-specific degradation was observed because both self and non-self \( S \)-RNases were degraded in their extracts. It is possible that our \textit{in vitro} system cannot duplicate the \( S \)-specific degradation that exists \textit{in vivo}. For example, as stated earlier, PiCUL1-G-PiSBP1 might act as an E3 ligase complex to mediate non-specific degradation of all \( S \)-RNases, and an allelic variant of PiSLF might promote the degradation of its non-self \( S \)-RNases to confer allelic specificity to the E3 ligase complex containing PiCUL1-G, PiSBP1 and PiSLF. If this scenario is true and if the PiCUL1-G-PiSBP1 complex predominates over the PiCUL1-G-PiSBP1-PiSLF complex in the pollen tube extracts (e.g., due to the dissociation of PiSLF from the complex during extraction), I would expect to see degradation of both self and non-self \( S \)-RNases. \textit{In vivo}, the PiCUL1-G-PiSBP1-PiSLF complex would predominate, resulting in specific degradation of non-self \( S \)-RNases. That is, two different E3 ligase complexes may operate in the ubiquitin-26S proteasome pathway to regulate the amount of \( S \)-RNases in the cytoplasm of the pollen tube: one for specific degradation of non-self \( S \)-RNases and the other for non-specific degradation of all \( S \)-RNases.

RNase X2 was also ubiquitinated and degraded in our \textit{in vitro} system (Figures 2.14D, 2.16C and 2.16D). This is not unexpected given that ubiquitin-mediated protein degradation is involved in regulating the stability of many proteins. Also, many different E3 ligases are used in this process, for example, \( \sim \)1,300 genes of the \textit{Arabidopsis} genome are annotated as encoding components of E3 ligases (Smalle and Vierstra, 2004). Since RNase X2 did not interact with PiSLFs (Figure 2.10) or PiSBP1 (Figure 2.9B), it would seem likely that an E3 ligase different from the PiSLF-containing complex mediated the ubiquitination and degradation of RNase X2 in the pollen tube extract. However, I cannot rule out the possibility that ubiquitination and degradation of \( S \)-RNases in our \textit{in vitro} system was not mediated by the PiSLF-containing complex either.
It would be interesting to reconstitute the PiCUL1-G-PiSBP1 complex and the PiCUL1-G-
PiSBP1-PiSLF1 (or PiSLF2) complex in vitro, and then compare their interactions with self and
non-self S-RNases. The reconstituted complexes could also be used in conjunction with
purified E1, E2, ubiquitin, and self or non-self S-RNases to determine whether self and/or non-
self S-RNases is (are) ubiquitinated. Ultimately, all the results obtained in vitro will have to be
confirmed by in vivo approaches. If, in vivo, the PiCUL1-G-PiSBP1-PiSLF complex indeed
mediates specific degradation of non-self S-RNases via the preferential binding of a PiSLF with
its non-self S-RNases, it will be interesting to investigate the biochemical basis for the binding
difference between a PiSLF and its self and non-self S-RNases. For example, is the binding
difference due to difference in the binding affinity, difference in the binding stoichiometry, or
other reasons?

2.5 Accession Numbers

All the sequence data obtained in this work have been deposited with the EMBL/GenBank data
libraries under the following accession numbers: PiSBP1 (DQ250022), PiSK1 (DQ250014),
PiSK2 (DQ250015), PiSK3 (DQ250013), PiCUL1-C (DQ250016), PiCUL1-G (DQ250017),
PiRBX1 (DQ250021), PiFBP23 (DQ250018), PiFBP2011 (DQ250019), PiFBP2411
(DQ250020). The accession numbers of the sequence data used in this chapter are as follows:
ASK1 (AAM45019), ASK2 (AAC14445), ATCUL1 (CAC85264) and AtRBX1 (Q940X7) from
Arabidopsis, PhSBP1 (AAF28357) from Petunia hybrida, Sc SBP1 (AAS76633) from Solanum
chacoense, human Skp1 (AAH09839), CUL1 (NP_003583) and Rbx1 (NP_055063), PiSLF1
(AAS79484), PiSLF2 (AAS79485), S1-RNase (AAA33726), S2-RNase (AAG21384) and RNase
X2 (S28611) from Petunia inflata.
Figure 2.1 Genomic Southern Analysis of the Complexity of the PiSK1 Gene.

Genomic DNA (15 μg) isolated from each of the five different S-generotypes was digested with 35 units of Xba I overnight at 37°C. The digested DNA samples were electrophoresed on a 0.7% agarose gel. The blot was hybridized with the $^{32}$P labeled PiSK1 cDNA probe at 55 °C. The arrows indicate the homologues of PiSK1. The S-generotypes of the plants used are: lane a, $S_1S_1$; lane b, $S_1S_2$; lane c, $S_2S_2$; lane d, $S_2S_3$; and lane e, $S_3S_3$. 
Figure 2.2 Alignment of Amino Acid Sequences of PiSK1, PiSK2 and PiSK3 of P. inflata, ASK1 and ASK2 of Arabidopsis, and a Skp1 of human.

For a given site, the amino acid that is present in at least three of the sequences is highlighted in dark shading, and the amino acid(s) that is (are) similar to the residue in dark shading is (are) highlighted in gray shading. The black dots show the amino acid residues important for the interactions between the human Skp1 and an F-box protein, Skp2 (Schulman et al., 2000; Zheng et al., 2002).
PiFBP2011 and PiFBP23 are F-box proteins that are likely encoded by genes unlinked to the S-locus. The bait vector is pGBD-C1 and the prey vector is pGAD-C1; p53 is a tumor suppressor and was used here as a negative control. Six colonies of yeast SFY526 co-expressing a pair of bait and prey proteins were streaked on filter paper for β-galactosidase activity assay. The paper was incubated in an X-gal containing solution (Breeden and Nasmyth, 1985) for 1 hr at 30°C.
Figure 2.4 Genomic DNA Gel-blot Analysis of PiFBP Genes for $S$-haplotype-specific Restriction Fragment Length Polymorphism.

Genomic DNA (15 μg) isolated from each plant was digested with EcoRI, XbaI, or HindIII as indicated. The DNA digests were separated by 0.7% agarose gels. Each blot was hybridized with the $^{32}$P labeled cDNA probe indicated at the top of the autoradiogram at 65 °C. The $S$-genotypes of the plants used are labeled as in the legend to Figure 2.1.
Figure 2.5 Yeast Two-Hybrid Assay of Interactions Between PiSLF$_2$ and Eight ASKs, as well as PiSBP1.

ASK1, ASK4, ASK5, ASK9, ASK11, ASK13, ASK16, and ASK18 are eight Skp1-like proteins. PiSBP1 was included as a positive control, and pGBD-C1 and p53 were included as negative controls. β-galactosidase activity assay was performed as described in the legend to Figure 2.3, except that the filter paper was incubated for 5 hr at 30°C.
Figure 2.6 Alignment of the Deduced Amino Acid Sequences of PiSBP1 of *P. inflata*, PhSBP1 of *P. hybrida* and ScSBP1 of *S. chacoense*.

At a given site, the amino acid that is present in at least two of the three sequences is highlighted in dark shading, and the amino acid that is similar to the residue in dark shading is highlighted in gray shading. The RING-HC finger motif is enclosed in the box region, with the asterisks indicating the cysteine-histidine signature of the motif. The predicted coiled-coil region is overlined.
Figure 2.7 Analyses of Interactions of PiSBP1 with PiSLF1 and PiSLF2.

(A) Yeast two-hybrid assay. PiSBP1 (in prey vector pGAD-C1) along with three bait constructs containing PiSLF1, PiSLF2 or p53, and with the bait vector pGBD-C1, were separately co-transformed into the yeast strain SFY526, and the transformants were used for the assay. PiSBP1 (in pGAD-C1) alone was also transformed into SFY 526 (indicated as NONE). The β-galactosidase activities shown are the mean ± SD values measured from six independent yeast transformants.

(B) In vitro binding assay. PiSLF2 and PiFBP2411 (an F-box protein encoded by a gene unlinked to the S-locus) were expressed as (His)6:T7:PiSLF2 and (His)6:T7:PiFBP2411, respectively, and the purified proteins were separately incubated with GST:PiSBP1-bound Glutathione Sepharose 4 Fast Flow resin. As negative controls in this figure and in Figures 2.9B, 2.10, 2.11C, 2.11D, 2.13B, 2.13C, the same amount of GST:PiSBP1-bound resin as used in each binding assay was incubated without any (His)6:T7 tagged protein, and GST-bound resin was incubated with the (His)6:T7 tagged protein, as indicated, under the same conditions as those used in each binding assay. The bound proteins (indicated with an arrow) were eluted and analyzed by immunoblotting using an anti-(His)6 antibody. Input lanes in this figure and in Figures 2.9B, 2.10, 2.11C, 2.11D, 2.13B, 2.13C contain a fraction of the (His)6:T7 tagged protein, as indicated, used in the binding assay.
Figure 2.8 Genomic Hybridization and Expression Pattern of PiSBP1.

(A) DNA gel blot analysis. Genomic DNA (15 μg) isolated from each S-genotype indicated was digested with EcoR I or Xba I, and the blot was hybridized with the full-length PiSBP1 cDNA.

(B) RNA gel blot analysis. Each lane contains 20 μg of total RNA isolated from the tissue indicated, and the blot was hybridized with the full-length PiSBP1 cDNA. The anther stages are defined by flower-bud size as previously described (Lee et al., 1996). Ethidium bromide staining of the gel used in blotting shows equal loadings of the RNA samples.
Figure 2.9 Analyses of Interactions of PiSBP1 with Full-Size and Truncated S₁- and S₂-RNases.

(A) Yeast two-hybrid assay. The assay was carried out as described in the legend to Figure 2.4A. S₁(HVabC3) and S₂(HVabC3) are truncated S₁-RNase and S₂-RNase, respectively, each containing the two hypervariable regions and the conserved C₃ region. All the negative controls are as described in the legend to Figure 2.7A.

(B) In vitro binding assay. The interactions between GST:PiSBP1 and purified (His)₆ T₇ tagged S₁-RNase, S₂-RNase and RNase X₂ were analyzed as described in the legend to Figure 2.7B. The bound proteins (indicated with an arrow) were detected by immunoblotting using an anti-T₇ antibody. The band above the (His)₆ T₇ tagged protein in each input lane is an *E. coli* protein that co-purified with the tagged protein.
Purified (His)$_6$T7:PiSLF$_2$ and (His)$_6$T7:PiSLF$_1$ were separately incubated with GST:S$_1$-RNase-, GST:S$_2$-RNase-, GST:RNase X$_2$-, and GST-bound Glutathione Sepharose 4 Fast Flow resin. The bound proteins were eluted and analyzed by immunoblotting using an anti-PiSLF$_3$ antibody, which cross-reacts with PiSLF$_1$ and PiSLF$_2$, indicated as a darkened arrow in top panels. The PiSLF antibody was raised in rabbits against a synthetic peptide corresponding to the last 14 amino acids at the C-terminal end of PiSLF$_3$. The open arrows indicate cross-reacting bacterial proteins that co-purified with the GST tagged proteins. The “input” lanes were used as positive controls for the (His)$_6$T7 tagged proteins, and the lanes containing GST:S$_1$-RNase, GST:S$_2$-RNase and GST:RNase X$_2$ alone were used as negative controls to show that there were no cross-reacting proteins of the sizes similar to those of the (His)$_6$T7 tagged proteins. Bottom panels: Ponceau S staining of the immunoblots before immunoblotting. The single asterisk indicates the GST:S$_1$-RNase, GST:S$_2$-RNase and GST:RNase X$_2$ bands, and the double asterisks indicate the GST band.
Figure 2.11 In Vitro Binding Assay of Interactions of PiSLFs with S1-, S2-, and S3-RNases.

(A) Interactions of PiSLF1 and PiSLF2 with S2-RNase. Equal amounts of purified (His)6:T7:PiSLF1 and (His)6:T7:PiSLF2 were assayed for their interactions with GST:S2-RNase, as described in the legend to Figure 2.7B, except that two independent reactions were carried out to assess each interaction. Reaction mixtures containing GST-bound resin and the (His)6:T7
tagged protein, as indicated, were used as negative controls. Each input lane contains 10% the amount of the (His)_6 T7 tagged protein, as indicated, used in the binding reaction; note that the intensities of the (His)_6 T7:PiSLF1 and (His)_6 T7:PiSLF2 bands are approximately equal. Top panel: immunoblot. The arrow indicates the (His)_6 T7:PiSLF1 band and the (His)_6 T7:PiSLF2 band detected by the anti-(His)_6 tag antibody. Bottom panel: Ponceau S staining of a part of the blot shown in the top panel before immunoblotting to reveal equal amounts of GST:S2-RNase used in the binding assay. The single asterisk indicates the GST:S2-RNase band, and the double asterisks indicate the GST band.

(B) Competition between PiSLF1 and PiSLF2 for interactions with S2-RNase. Equal amounts of purified (His)_6 T7:PiSLF1 and (His)_6 T7:PiSLF2 were mixed and used with GST:S2-RNase in the binding assay. Purified (His)_6 T7:PiSLF1 and (His)_6 T7:PiSLF2 were loaded in the "Control" lanes to mark their respective positions in the gel. The reaction mixture containing GST-bound resin and both (His)_6 T7 tagged proteins served as a negative control. The input lane contains 10% the amount of the mixture of the two (His)_6 T7 tagged proteins used in the binding reaction, note that the intensities of the (His)_6 T7:PiSLF1 and (His)_6 T7:PiSLF2 bands are approximately equal. (His)_6 T7:PiSLF1 (upper band) and (His)_6 T7:PiSLF2 (lower band) were detected by the anti-(His)_6 tag antibody.

(C) Interactions of PiSLF2 with truncated S1- and S2-RNases. Purified (His)_6 T7:PiSLF2 was tested for its interaction with GST:S1(HV_abC3) and GST:S2(HV_abC3). The binding assay and negative controls were carried out as described in the legend to Figure 2.7B, except that GST:S1(HV_abC3)-bound and GST:S2(HV_abC3)-bound resins were used. The two truncated S-RNases are described in the legend to Figure 2.9A. Top panel: immunoblot. The arrow indicates the (His)_6 T7:PiSLF2 band detected by the anti-(His)_6 tag antibody, the other bands with lower molecular masses cross-reacted with the antibody and were detected only after a long exposure of the blot. Bottom panel: Ponceau S staining of the blot shown in the top panel before immunoblotting; the single asterisk indicates the GST:S1(HV_abC3) and GST:S2(HV_abC3) bands, and the double asterisks indicate the GST band.

(D) Interactions of PiSLF1 with truncated S1- and S2-RNases. Except for the use of (His)_6 T7 tagged PiSLF1 and PiFBP2411, the binding assay and negative controls were carried out as described in (C). The top and bottom panels are as described in (C), except that the arrow indicates the (His)_6 T7:PiSLF1 and (His)_6 T7:PiFBP2411 bands.

(E) Binding differences between PiSLF1 and S1- and S2-RNases. Different amounts of purified (His)_6 T7:PiSLF1 were used with equal amounts of GST:S1-RNase and GST:S2-RNase in separate binding assays, as described in the legend to Figure 2.7B. The top two panels are immunoblotting against the anti-(His)_6 tag antibody and the bottom two panels are Ponceau S staining of the same blots shown in the top panels before immunoblotting.

(F) Binding differences between PiSLF1 and S1- and S3-RNases. The assays were carried out as described in (E) except that a different batch of purified (His)_6 T7:PiSLF1 and a different GST tagged non-self S-RNase, GST:S3-RNase, were used.
Figure 2.12 Role of the Coiled-Coil Region of PiSBP1 in Interactions with PiSLF2 and Truncated S-RNases.

PiSBP1(Δcoiled-coil), a truncated PiSBP1 without the coiled-coil region, was tested for interactions with PiSLF2 and two truncated S-RNases (described in Figure 2.9A) by the yeast two-hybrid assay. PiSBP1 was included as a positive control, and pGBD-C1 and p53 were included as negative controls. β-galactosidase activity assay was performed as described in the legend to Figure 2.3 except that the filter paper was incubated for 5 hr at 30°C.
Figure 2.13 Interactions of PiSBP1 with PiCUL1-G and PhUBC1.

(A) Yeast two-hybrid assay showing that PiSBP1, but not PiRBX1, interacts with PiCUL1-G, PhUBC1 (an E2 ubiquitin conjugating enzyme of *P. hybrida*) and PiSLF2. β-galactosidase activity assay was performed as described in the legend to Figure 2.3 except that the filter paper was incubated for 5 hr at 30°C.

(B) *In vitro* binding assay of the interaction between PiSBP1 and PiCUL1-G. Purified (His)_6 T7:PiCUL1-G was tested for its interaction with GST:PiSBP1 in the binding assay as described in the legend to Figure 2.7B. Top panel: immunoblot. The bound (His)_6 T7:PiCUL1-G was detected by the anti-T7 tag antibody and is indicated with an arrow. The other bands with lower molecular masses cross-reacted with the antibody and were detected only after a long exposure of the blot. Bottom panel: Coomassie staining of a duplicate gel of that used in immunoblotting. The asterisk indicates the GST:PiSBP1 band and the double asterisks indicate the GST band.

(C) *In vitro* binding assay of the interaction between PiSBP1 and PhUBC1. Purified (His)_6 T7:PhUBC1 was used along with GST:PiSBP1 in the assay as described in the legend to Figure 2.7B. The bound (His)_6 T7:PhUBC1 was detected by an anti-T7 tag antibody. Top panel: immunoblot. The arrow indicates the (His)_6 T7:PhUBC1 band. Bottom panel: Coomassie staining of a duplicate gel of that used in immunoblotting. The asterisk indicates the GST:PiSBP1 band and the double asterisks indicate the GST band.
Figure 2.14 Degradation Assay of Bacterially Expressed GST, GST:S₁-RNase, GST:S₂-RNase, GST:S₃-RNase and GST:RNase X2, as well as S₃-RNase Purified from Pistils, by Extracts of S₁, S₂ or S₃ Pollen Tubes.

(A) GST:S₁-RNase and GST:S₂-RNase. Purified GST fusion proteins (0.3 μg each) were separately incubated with extracts of *in vitro* germinated S₂ pollen tubes either in the absence or in the presence of 40 μM MG132 (a specific inhibitor of the 26S proteasome) for 1 hr at 30°C. An anti-GST antibody was used to detect GST:S₁-RNase and GST:S₂-RNase (indicated with an arrow), as well as GST (indicated with double asterisks).

(B) GST:S₃-RNase. Purified GST:S₃-RNase (0.3 μg) was used in the assay as described in (A), except that both anti-GST and anti-S₃-RNase antibodies were used to detect GST:S₃-RNase. The arrow indicates GST:S₃-RNase; the single asterisk indicates a cross-reacting protein present in the reaction mixture; the double asterisks indicate GST.

(C) Native glycosylated S₃-RNase and its de-glycosylated form. S₃-RNase (0.1 μg) purified from pistils of S₃S₃ genotype (left panel) and an equal amount of purified de-glycosylated S₃-RNase (right panel) were separately incubated with extracts of S₁, S₂ and S₃ pollen tubes either in the absence or in the presence of MG132 (40 μM) for 90 min at 30°C. The anti-S₃-RNase antibody was used to detect both glycosylated (indicated with an open triangle) and de-glycosylated (indicated with solid arrows) S₃-RNase. Single and double asterisks indicate cross-reacting proteins in the reaction mixture. A longer exposure time was used for the blot shown in the left panel (5 min) than that shown in the right panel (30 s).

(D) GST:S₃-RNase, GST:RNase X2 and GST. Each purified protein (0.3 μg) was used in the assay as described in (A). Left panel: immunoblot. The arrows indicate GST:S₃-RNase or GST:RNase X2, and the double asterisks indicate GST. Right panel: Ponceau S staining of the blot shown in the left panel before immunoblotting.
(His)$_6$:Ub(K48R), expressed and purified from *E. coli* BL21(DE3)pLysS (Novagen), was used in the assay. The replacement of Lys-48 by Arg in Ub(K48R) blocked the formation of poly ubiquitin chains but still allowed the attachment of mono ubiquitin units to target proteins. Five micrograms of S2 pollen tube extract were incubated in 30 μL ubiquitin reaction buffer (50 mM Tris-HCl, pH 7.4, 2 mM ATP, 2 mM DTT, 5 mM MgCl$_2$, ~4 μg creatine phosphokinase [Calbiochem], 10 mM creatine phosphate [Calbiochem], 1 mM PMSF) for 1 hr at 30°C in the presence or absence of 30 mg of (His)$_6$:Ub(K48R). The reactions were stopped by the addition of 7 μL 5 x SDS reducing sample buffer and subsequent heating at 95°C for 5 min. The samples were analyzed by immunoblotting using an anti-(His)$_6$ tag antibody. The strong smear signals detected by the antibody indicate that many proteins in the pollen tube extract were ubiquitinated.

Figure 2.15 Ubiquitination Assay of Total Protein in S2 Pollen Tube Extract.
Figure 2.16 Ubiquitination Assay of GST, GST:S2-RNase, GST:S3-RNase and GST:RNase X2 by Extracts of S2 Pollen Tubes.

(A), (B) Time course of ubiquitination and degradation of GST:S2-RNase. GST:S2-RNase (0.5 μg) was used in each reaction containing ubiquitin, and the reaction was stopped at different time points as indicated. The anti-GST antibody was used in the protein gel blot shown in (A) and an anti-ubiquitin antibody was used in the blot shown in (B). The open triangles indicate GST:S2-RNase and the filled triangle indicates GST; the black dot indicates the degradative products of GST:S2-RNase; the solid arrows indicate three ubiquitinated forms of GST:S2-RNase with two, five or seven ubiquitin subunits; the asterisks indicate GST:S2-RNase conjugated with five ubiquitin subunits that was detected by both anti-GST and anti-ubiquitin antibodies.
Ubiquitination assay of GST, GST:S₃-RNase and GST:RNase X2. Purified GST, GST:S₃-RNase and GST:RNase X2 (0.5 μg each) were used in the assay as described in (A) and (B), except that the reaction contained (His)₆ ubiquitin and was analyzed only at the 10-min time point. Each reaction mixture was then divided equally and electrophoresed in two duplicated gels. The transferred membranes were immunoblotted with the anti-GST antibody (C) and the anti-(His)₆ antibody (D). Top panels, immunoblot. Bottom panels, Ponceau S staining of the blots shown in the top panels before immunoblotting.
Table 2.1 Results of yeast two-hybrid assay of possible interactions between PiSLF₁/PiSLF₂ and PiSK₁/PiSK₂ proteins

<table>
<thead>
<tr>
<th>Prey</th>
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<td>PiSLF₁</td>
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*N indicates no interaction was detected; the number of “N” in each cell represents the number of times the assay was carried out.
CHAPTER 3

Comparison of *Petunia Inflata* S-Locus F-Box Protein (PiSLF) with PiSLF-Like Proteins Reveals Its Unique Function in S-RNase-Based Self-Incompatibility

The work described in this chapter has been published in Hua et al., Plane Cell, 19: 3593-3609, 2007.

Xiaoying Meng, another graduate student in the lab, made the construct *pBI LAT52-PiSLFLd-S2:GFP*, raised the transgenic plants *S2S3/pBI LAT52-PiSLFLb-S2:GFP* and *S2S3/pBI LAT52-PiSLFLd-S2:GFP*, and performed most of the pollination assays. Her data are presented in part of Figure 3.3, Figure 3.4C, Figure 3.5B and 3.5D, Figure 3.6, and part of Figure 3.7. I designed all the experiments and performed all the other experiments.
3.1 Introduction

SI in the Solanaceae, Rosaceae and Plantaginaceae families employs the S-RNase as the pistil specificity determinant (Lee et al., 1994; Murfett et al., 1994; Xue et al., 1996) and the S-locus F-box (abbreviated SLF or SFB) protein as the pollen specificity determinant (Lai et al., 2002; Entani et al., 2003; Ushijima et al., 2003; Qiao et al., 2004a,b; Sijacic et al., 2004; Sonneveld et al., 2005; Tsukamoto et al., 2006). Both the S-RNase and SLF genes are located at the highly polymorphic S-locus. If the S-haplotype of pollen matches one of the two S-haplotypes of the pistil, the pollen is recognized by the pistil as self-pollen and the growth of its tube in the style is inhibited. Pollen that carries a different S-haplotype than those carried by the pistil is recognized as non-self pollen and is accepted for fertilization. S-RNases are secreted into the intercellular space of the transmitting tract of the pistil, and are taken up by pollen tubes in a non-S-haplotype-specific manner as shown by immunocytochemical experiments (Luu et al., 2000; Goldraij et al., 2006). The observations that the RNase activity of S-RNases is essential for their function in rejection of self-pollen tubes (Huang et al., 1994) and that pollen rRNAs were degraded upon incompatible pollination but not compatible pollination (McClure et al., 1990), have led to a hypothesis that only self-S-RNase is able to exert its cytotoxic action inside a pollen tube. Recent identification of SLF of Petunia inflata (PiSLF) as the pollen specificity determinant (Sijacic et al., 2004) has allowed us to examine this hypothesis.

Most of the F-box proteins whose functions have been characterized so far are components of a type of multi-subunit E3 ubiquitin ligase complex, named SCF (Skp1-Cullin-F-box), which is composed of Skp1, Cullin-1, F-box protein and Rbx1, and which, along with E1 ubiquitin activating enzyme and E2 ubiquitin conjugating enzyme, is involved in ubiquitin-mediated protein degradation by the 26S proteasome (for reviews see Cardozo and Pagano, 2004; Moon et al., 2004; Smalle and Vierstra, 2004). In Chapter 2, I have reported that PiSLF is likely a component of a novel E3 ligase complex, which also contains Cullin-1 and a RING-HC protein, named PiSBP1 (P. inflata S-RNase-Binding Protein 1), a homologue of Ph (P. hybrida) SBP1 (Sims and Ordanic, 2001), but does not contain Skp1 or Rbx1 (RING-HC protein). Since PiSBP1 is approximately three times the size of Rbx1, it may play the roles of Skp1 and Rbx1. Whether this PiSLF-containing complex is involved in mediating ubiquitination and degradation
of S-RNases in compatible pollen tubes remains to be determined. However, I have used cell-
free systems to show that S-RNases are ubiquitinated and degraded in pollen tube extracts via the
26S proteasome pathway, although neither ubiquitination nor degradation was S-haplotype-
specific. I have also used an in vitro binding assay to show that an allelic product of PiSLF
interacts with its non-self S-RNases more strongly than with its self S-RNase, and that an S-
RNase interacts with its non-self PiSLFs more strongly than with its self-PiSLF. Thus, it is
possible that a PiSLF-containing complex specifically mediates ubiquitination of non-self S-
RNases in a pollen tube to target them for degradation.

F-box proteins constitute a large family of proteins in plants, e.g., the Arabidopsis genome
encodes more than 700 F-box proteins (Gagne et al., 2002; Risseeuw et al., 2003), and multiple
F-box genes have been identified at the S-loci of all three families that possess S-RNase-based SI
(McCubbin et al., 2000; Entani et al., 2003; Ushijima et al., 2003; Wang et al., 2003; Zhou et al.,
2003; Sassa et al., 2007). The functions of none of these S-locus-linked SLF-like genes have
been determined. Recently, Sassa et al. (2007) proposed that two highly similar F-box genes
(87.5% identity in their deduced amino acid sequences) of apple (Rosaceae), that are located at
the S-locus, are specifically expressed in pollen and show allelic polymorphism, may both
encode the pollen specificity determinant.

In this chapter, I wished to examine whether PiSLF is unique in its function in SI, and if so,
what features of PiSLF distinguish it from other F-box proteins that are similar in sequence and
share other properties with PiSLF. I first identified S1- and/or S2-alleles of four PiSLF-like
(PiSLFL) genes and showed that at least three of them are tightly linked to the S-locus. I used
an in vivo functional assay, with the help of another graduate student, Xiaoying Meng, to show
that none of these three S-locus-linked PiSLFs control pollen function in SI. I then used an in
vitro binding assay to show that all the four newly identified PiSLFLs, as well as one of the two
previously identified PiSLFLs whose genes are linked to the S-locus (McCubbin et al., 2000;
Wang et al., 2003), either failed to interact, or could not compete with PiSLF2 for interaction,
with S3-RNase (a non-self S-RNase for all of them). Comparison of the deduced amino acid
sequences of three allelic variants of PiSLF (PiSLF1, PiSLF2 and PiSLF3; Sijacic et al., 2004),
the four newly identified PiSLFLs, and the two previously identified PiSLFLs revealed three
PiSLF-specific regions (SRs), named SR1, SR2 and SR3. I further divided PiSLF into three function domains (FDs), FD1, FD2 and FD3, which contain SR1, SR2, and SR3, respectively, and used in vitro binding assays to show that FD2, conserved among PiSLF1, PiSLF2 and PiSLF3, is primarily responsible for the strong interaction between an allelic product of PiSLF and its non-self S-RNases. This interaction is negatively modulated by FD1 and FD3, divergent among PiSLFs, and the effect is much greater on self-interactions than on non-self interactions between PiSLF and S-RNase.

3.2 Methods

3.2.1 Plant Material

$S_1S_1$, $S_1S_2$, $S_2S_2$, $S_2S_3$, $S_3S_3$ and $S_1S_3$ genotypes of P. inflata were used in this study, and were identified by Ai et al. (1990).

3.2.2 cDNA Library Screening

The $S_1$ and $S_2$ pollen cDNA libraries used were constructed by Skirpan et al. (2001), and library screening was carried out as described in Chapter 2 except for the use of PiSLF$_2$(CTD) as a probe.

3.2.3 DNA and RNA Gel Blot Analyses

Genomic DNA was isolated from young leaves using Plant DNAzol reagent (Invitrogen) according to the manufacturer’s protocol. Genomic DNA (10 to 15 μg) was digested overnight by various restriction enzymes as indicated. DNA blotting and hybridization were carried out as described in Chapter 2, except that the temperatures for the low- and high-stringency hybridization conditions were 55°C and 65°C, respectively. RNA gel blot analysis was performed as described in Chapter 2 and hybridization was performed under high-stringent conditions. The DNA probes used for both genomic DNA blotting shown in Figure 3.1 and RNA blotting shown in Figure 3.2 were obtained by PCR using a T3 primer (5'-ATTAACCC
TCACTAAAGGGA-3') and a T7 primer (5'-TAATACGACTCACTATAGGG-3') to amplify the corresponding cDNAs inserted in pBluescript SK- vector (Stratagene). The DNA probes used for genomic DNA blotting shown in Figure 3.3 were obtained by PCR using specific primers, listed in Table 3.1, to amplify cDNAs corresponding to the genes shown in Figure 3.3.

3.2.4 RT-PCR Analysis

Total RNA was isolated from anthers at different developmental stages, mature pollen, pollen tubes, ovaries, styles, and leaves of an S2S2 plant as described in Chapter 2. Each RNA sample (5 μg) was used for cDNA synthesis in the presence (RT+) and absence (RT–) of Power Script™ Reverse Transcriptase according to the manufacturer’s manual (Clontech). The RT+ products (0.2 μl each) were used for PCRs to amplify the coding sequences of PiSFLd-S2 and PiSLF2. The gene-specific primers used are shown in Table 3.1. As controls, PCRs were also carried out for each RT+ and RT- product using primers specific to a gene encoding actin: 5'-GGCATCAC ACTTTCTACAATGAGC-3' (forward) and 5'-GATATCCACATCACATTTC ATGAT-3' (reverse). All PCRs were performed as described in Wang et al. (2003) except for the following modifications: the annealing temperature for all three genes was 55 °C; the times of extension were 90 s for PiSFLd-S2 and PiSLF2, and 60 s for Actin all at 72 °C; the reactions were carried out for 30 cycles for PiSFLd-S2 and PiSLF2 and 20 cycles for Actin. The amplified products (20 μl each) were subjected to electrophoresis on 1% (w/v) agarose gels, and the gels were stained with ethidium bromide.

3.2.5 Generation of Ti-Plasmid Constructs and Plant Transformation

Full-length cDNA for PiSLF2 was subcloned into pGEM®-T Easy Vector (Promega), and an Nco I restriction site was introduced at the 5’-end and a Not I site was introduced at the 3’-ends by PCR. The 1.177-kb Nco I-Not I fragment containing the full-length PiSLF2 cDNA was released from pGEM®-T Easy Vector and used to replace the 0.72-kb Nco I-Not I fragment of the GFP coding sequence in pLAT-GFP (Dowd et al., 2006). The resulting construct, pLAT52-PiSLF2, contains PiSLF2 driven by the LAT52 pollen-specific promoter of tomato (Twell et al., 1990). The GFP coding sequence was re-amplified and a 39-nucleotide linker, encoding a 13-
amino-acid, (Ala)\(_3\)(Gly)\(_{10}\), peptide linker, was inserted in front of the start codon of GFP by PCR using \(pLAT-GFP\) as template. A \(N\ot I\) restriction site was then introduced at both ends of the PCR product. The \(N\ot I\) fragment was inserted into the \(pLAT52-PiSLF\) to produce \(pLAT52-PiSLF:GFP\). The 2.553-kb \(Sal\ I-Pst\ I\) fragment, containing the \(LAT52\) promoter, \(PiSLF\), 39-nucleotide linker and the \(GFP\) coding sequence, was released from \(pLAT52-PiSLF:GFP\) and inserted into the \(Sal\ I\) and \(Pst\ I\) sites of \(pBluescript\) \(SK\) (Stratagene) to generate \(pBluescript\) \(LAT52-PiSLF:GFP\). A \(Sac\ I\) site was introduced at the 3’ end of the \(LAT52-PiSLF:GFP\) insert. The \(Sal\ I-Sac\ I\) fragment was released from \(pBluescript\) \(LAT52-PiSLF:GFP\) and used to replace the \(Sal\ I-Sac\ I\) fragment in \(pBI101\) (Clontech), which contains the GUS coding sequence, to yield \(pBI\) \(LAT52-PiSLF:GFP\). The \(pBI\) \(LAT52-PiSLFLc-S1:GFP\), \(pBI\) \(LAT52-PiSLFLb-S2:GFP\), and \(pBI\) \(LAT52-PiSLFLd-S2:GFP\) constructs were similarly generated using cDNAs for \(PiSLFLc-S1\), \(PiSLFLb-S2\), and \(PiSLFLd-S2\) respectively. The schematics for all the constructs are shown in Figure 3.3. All the Ti plasmid constructs were separately electroporated into \(Agrobacterium\) \(tumefaciens\) strain LBA4404 (Invitrogen), and the \(Agrobacterium\)-mediated transformation was performed according to the procedure described previously (Lee et al., 1994).

### 3.2.6 Visualization of Pollen Tube Growth in Pollinated Pistils

Twenty hours post pollination, pollinated pistils (without ovaries) were fixed, macerated, and stained with 0.1% aniline blue dye according to the method described by Holden et al. (2003). Pollen tubes within different segments of the entire pistil (stigma plus style) were visualized with a Nikon Eclipse 90i epifluorescence microscope and recorded by a camera. All the images of pollen tube growth within a pistil were integrated by Adobe\textsuperscript{\textregistered} Photoshop\textsuperscript{\textregistered} CS2.

### 3.2.7 Sequence Analysis

Amino acid sequences were aligned by ClustalW (http://www.ebi.ac.uk/clustalw/). A modified normed-variability-index method (Kheyr-Pour et al., 1990) was used to identify the PiSLF-specific regions. For each alignment site, PiSLF\(_1\), PiSLF\(_2\) and PiSLF\(_3\) were used separately as a reference sequence for comparison with all the other aligned sequences. If the amino acid residue of a sequence being compared with the reference sequence was the same as that of the
reference sequence, an index number of -1 was assigned. If it was not, an index number of 1 was assigned. The total index value for each alignment site was calculated by summing up all the index numbers of the sequences compared. To better visualize regions that are specific to \textit{PiSLF}, a sliding window (a 60-alignment-site window with a 6-alignment site slide) analysis of the total index value was performed and the value for each window was plotted against the first alignment site of that window. The variable regions among \textit{PiSLF}\textsubscript{1}, \textit{PiSLF}\textsubscript{2}, and \textit{PiSLF}\textsubscript{3} were determined by the normed-variability-index analysis as described by Kheyr-Pour et al. (1990). To identify the regions of \textit{PiSLF} that are under positive selection, \(\text{Ka}/\text{Ks}\) values were calculated for pairwise comparisons among \textit{PiSLF}\textsubscript{1}, \textit{PiSLF}\textsubscript{2} and \textit{PiSLF}\textsubscript{3} using the K-estimator 6.1 software package (Comeron, 1999) with a 180-nucleotide window and an 18-nucleotide slide.

3.2.8 Expression and Purification of Recombinant Proteins

The DNA fragments containing coding sequences for all the full-length and truncated genes described in the text and listed in Table 3.2 were obtained by PCR using the primers listed in Table 3.1, and the fragments were separately subcloned in-frame behind the sequence for the (His)\textsubscript{6}:T7 tag in vector \textit{pET28} (Novagen), or in-frame behind the GST coding sequence in vector \textit{pGEX-5X-1} (GE Healthcare). The DNA fragments containing coding sequences for the chimeric fusion proteins shown in Figure 3.14 and Figure 3.15 were obtained by overlap PCR using the primers listed in Table 3.1, and the fragments were separately subcloned into \textit{pET28} (Novagen) in-frame behind the (His)\textsubscript{6}:T7 tag. All the recombinant proteins were expressed at 18\(^\circ\)C in BL21 Codon Plus \textit{E. coli} (Stratagene) and purified using HIS-Select\textsuperscript{TM} HF Nickel Affinity Gel (Sigma), or Glutathione Sepharose\textsuperscript{TM} 4 Fast Flow affinity beads (GE Healthcare), according to the respective manufacturer's procedure.

3.2.9 \textit{In vitro} Protein Binding and Competition Assays

The \textit{in vitro} protein-binding assay was performed using GST:S\textsubscript{3}-RNase attached to glutathione Sepharose\textsuperscript{TM} 4 Fast Flow affinity beads, and (His)\textsubscript{6}:T7 tagged proteins, as described in Chapter 2, except for the use of a modified binding buffer (50 mM Tris-HCl [pH 7.5], 5 mM MgCl\textsubscript{2}, 150 mM NaCl, 2 mM DTT, 0.01% NP40, 5% glycerol). All the (His)\textsubscript{6}:T7 tagged proteins were first
tested to ensure that they did not interact with GST under the same binding-assay conditions. The competition assay was similarly performed except for the following modifications. The amount of GST:S3-RNase bound to the GST beads was decreased to one twentieth of the amount used for the protein-binding assay, and equal amounts of (His)$_6$T7 tagged proteins, (His)$_6$T7:PiSLF$_2$ and (His)$_6$T7:PiSLFLb-S$_2$, were used in the same binding reaction. After 90 min incubation in the competition binding buffer [50 mM Tris-HCl (pH 7.5), 5 mM MgCl$_2$, 250 mM NaCl, 2 mM DTT, 0.01% NP40, 5% glycerol] at room temperature with gentle rotating, the beads were thoroughly washed four to five times with binding buffer. The bound proteins were eluted and analyzed by immunoblotting as was done in the protein-binding assay.

3.2.10 Immunoblot Analysis

Immunoblot analysis of the (His)$_6$T7 tagged proteins was performed as described in Chapter 2. Total pollen tube extracts used in Figure 3.4A were prepared as described in Chapter 2 and the total proteins of stage 5 anthers used in Figure 3.4B were extracted with the same extraction buffer described by Lee et al. (1994), except that both extract buffers contained 1% protease inhibitor cocktail (Sigma). GFP and its fusion proteins were detected by a rabbit anti-GFP antibody (Abcam).

3.3 Results

3.3.1 Isolation of $PiSLFL$ Genes from $S_1$ and $S_2$ Pollen cDNA Libraries of Petunia inflata

I was interested in identifying pollen-expressed F-box genes whose amino acid sequences share significant degrees of similarity with those of $PiSLF$ alleles outside the F-box domain. Thus, I used cDNA for PiSLF$_2$(CTD) (340 amino acids), which does not contain the N-terminal 49 amino acids of the F-box domain, as a probe to screen previously constructed pollen cDNA libraries of $S_1$- and $S_2$-haplotypes (Skirpan et al., 2001). Under low-stringency hybridization conditions, six positive clones were obtained from screening 6 x 10$^6$ plaques of the $S_1$ cDNA library, and nine positive clones were obtained from screening 9 x 10$^6$ plaques of the $S_2$ cDNA library. Sequencing of all these clones revealed that some of them corresponded to three
previously identified genes: *PiSLF* (Sijacic et al., 2004; Wang et al., 2004) and two *S*-locus-linked *PiSLFL* genes, *A113* and *A134* (McCubbin et al., 2000; Wang et al., 2003). The remaining clones corresponded to four "new" genes based on their sequences and genomic DNA blotting (see next section). One class of cDNA clones identified from the *S*<sub>i</sub> library and one class of cDNA clones identified from the *S*<sub>2</sub> library were 93.7% identical in their coding sequences and 88.6% identical in their deduced amino acid sequences. Thus, they were considered allelic of the same gene, and named *PiSLFLa-S*<sub>i</sub> and *PiSLFLa-S*<sub>2</sub>. The other classes of cDNA clones, one isolated from the *S*<sub>i</sub> library and two from the *S*<sub>2</sub> library, were 66.4 to 69.1% identical in their coding sequences, and they were designated *PiSLFLb-S*<sub>2</sub>, *PiSLFLc-S*<sub>i</sub> and *PiSLFLd-S*<sub>2</sub>. The pairwise nucleotide sequence identities of these *PiSLFL* genes and three alleles of *PiSLF* are shown in Table 3.3, and the pairwise sequence identities for their deduced amino acid sequences are shown in Table 3.4. The *PiSLF* genes show 67.8 to 71.5% identity with the three *PiSLF* alleles (Sijacic et al., 2004) in their coding sequences and 47.4 to 54.6% identity with these *PiSLF* alleles in their deduced amino acid sequences.

### 3.3.2 Assessing Linkage of Four *PiSLFL* Genes to the *S*-Locus

Genes that are linked to the *S*-locus typically show *S*-haplotype-specific sequence differences, and when they are used as probes in genomic DNA blot analysis, they often show *S*-haplotype-specific restriction fragment length polymorphism (RFLP). Genomic DNA blotting was carried out using the cDNAs for *PiSLFLa-S*<sub>i</sub>, *PiSLFLa-S*<sub>2</sub>, *PiSLFLb-S*<sub>2</sub>, *PiSLFLc-S*<sub>i</sub> and *PiSLFLd-S*<sub>2</sub> as probes. *PiSLFLa-S*<sub>i</sub> and *PiSLFLa-S*<sub>2</sub> showed the same monomorphic hybridization pattern (Figure 3.1A), confirming that they are allelic. *PiSLFLb-S*<sub>2</sub>, *PiSLFLc-S*<sub>i</sub> and *PiSLFLd-S*<sub>2</sub> corresponded to different genes as they showed different *S*-haplotype-specific RFLPs (Figures 3.1B, 3.1C, and 3.1D).

I next examined whether *PiSLFLa-S*<sub>2</sub>, *PiSLFLb-S*<sub>2</sub> and *PiSLFLd-S*<sub>2</sub> are located in an 881-kb contig containing *S*<sub>2-RNase* (Wang et al., 2004). I used PCR primers specific to each gene to separately amplify eight BAC clones that collectively span this contig. No DNA fragments were obtained for any of these three genes. Since *S*<sub>2-RNase* is located ~250 kb from the proximal end of the 881-kb contig, the results of PCR suggest that *PiSLFLa-S*<sub>2</sub>, *PiSLFLb-S*<sub>2</sub> and
PiSLFLd-S2 are located at least 250 kb away from S2-RNase. In contrast, PiSLF2 is located within this contig, ~161 kb downstream of S2-RNase (Wang et al., 2004). Since the S-locus region of P. inflata where recombination is suppressed exceeds 4.4 Mb (Wang et al., 2004), I further examined whether these genes could still be linked to the S-locus, but at greater distances from S-RNase than is PiSLF.

Our lab previously generated a population of 1205 F2 plants segregating for S1- and S2-haplotypes, and used them to ascertain whether the genes identified from the search of S-linked pollen-expressed genes were tightly linked to the S-locus (Wang et al., 2003). Recombination was detected between some of the genes and S-RNase in nine F2 plants. The S-RNase gene and its flanking chromosomal regions in three of the recombinant plants are graphically shown in Figure 3.1E. In plant N43 (S2S2) and N142 (S1S1), recombination occurred between gene 3.16 and S-RNase, and in plant P85 (S1S2), a double crossover occurred, one between 3.16 and S-RNase and the other between G221 and S-RNase. The genetic distances between these two marker genes, 3.16 and G221, and S-RNase are 0.17 cM and 0.08 cM, respectively (Wang et al., 2003). To assess how tightly PiSLFLb-S2, PiSLFLc-S1, and PiSLFLd-S2 are linked to the S-locus, their cDNAs were used as probes in genomic blotting analysis of these three recombinant plants. PiSLFLa was not included because it did not show S-specific RFLP (Figure 3.1A). All three PiSLFL genes hybridized to their respective S2-specific fragments in N43 and P85, and to their respective S1-specific fragments in N124 (Figure 3.1F for PiSLFLb-S2, Figure 3.1G for PiSLFLc-S1, and Figure 3.1H for PiSLFLd-S2), consistent with the S-genotypes of these three recombinant plants determined by the hybridization patterns of S-RNase. These results suggest that these three PiSLFL genes lie within the S-locus region delimited by 3.16 and G221, and are all tightly linked to S-RNase. Although I could not establish whether PiSLFLa is tightly linked to the S-locus, since it is similar in sequence to PiSLF, it was also included, along with the other three PiSLFL genes, in the analyses described below.

3.3.3 Four PiSLFL Genes are Specifically Expressed in Pollen and Pollen Tubes

The RNA blotting results showed that PiSLFLa-S1, PiSLFLb-S2, and PiSLFLc-S1 were all expressed in pollen and in vitro germinated pollen tubes, but not in any of the vegetative and
female reproductive tissues examined (Figures 3.2A and 3.2B). During anther development, the transcripts of these genes were first detected in stage 3 anthers, reached the highest level in stage 4 and/or stage 5 anthers, and declined in mature pollen. The transcripts were also detected at low levels in pollen tubes. The tissue-specific expression pattern and the expression pattern during anther development are similar to those of PiSLF and two previously identified PiSLFL genes, A113 and A134 (McCubbin et al., 2000; Sijacic et al., 2004). Furthermore, the results from Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) showed that PiSLFLd-S2, like PiSLF2, was expressed in stages 3 to 5 anthers, pollen and pollen tubes, but not in stages 1 to 2 anthers, leaves, styles or ovaries (Figure 3.2C).

3.3.4* Assessment of SI Function of PiSLFLb-S2, PiSLFLc-S1, and PiSLFLd-S2

We chose PiSLFLb-S2, PiSLFLc-S1 and PiSLFLd-S2 to examine whether they control pollen function in SI. As stated above, these three genes share a similar tissue-specific expression pattern with PiSLF (Figure 3.2) and they are also tightly linked to S-RNase, albeit at greater distances from S-RNase than PiSLF. We used the same in vivo approach as that described by Sijacic et al. (2004) to ascertain whether PiSLFLb-S2, PiSLFLc-S1, and PiSLFLd-S2 function in SI. This approach was based on the phenomenon, termed competitive interaction, that pollen carrying two different pollen S-alleles fails to function in SI (de Nettancourt, 2001). In a previous study of PiSLF, our lab showed that expression of a PiSLF2 transgene in pollen of S1S1, S1S2 and S2S3 transgenic plants caused the breakdown of SI function in S1 and S3 pollen that carried the transgene (i.e., heteroallelic pollen), but not in S2 pollen that carried the transgene (i.e., homoallelic pollen) (Sijacic et al. 2004).

In making the transgene constructs for PiSLFLb-S2, PiSLFLc-S1, and PiSLFLd-S2, we fused the coding sequence for a Green Fluorescent Protein (GFP) in-frame to the last codon of each gene, and used a pollen-specific promoter of tomato, LAT52 (Twell et al., 1990), to express each transgene. The resulting transgene constructs, pBI LAT52-PiSLFLb-S2:GFP, pBI LAT52-
PiSLFLc-S1:GFP, and pBI LAT52-PiSLFLd-S2:GFP (Figure 3.3) were introduced into S2S3, S1S2, and S2S3 plants, respectively. The reason for using the LAT52 promoter was because I had previously shown that pollen of S2S3 transgenic plants carrying LAT52-PiSLF2:GFP produced strong GFP signals, whereas pollen of S2S3 transgenic plants carrying the same PiSLF2:GFP coding sequence driven by the PiSLF2 promoter did not produce any detectable signal. Thus, using the LAT52 promoter would facilitate detection of the GFP fusion proteins by an anti-GFP antibody.

The transgenic plants that carried a single copy of LAT52-PiSLF2:GFP, LAT52-PiSLFLb-S2:GFP, LAT52-PiSLFLc-S1:GFP, or LAT52-PiSLFLd-S2:GFP were identified by genomic blotting. Pollen was collected from each “single-copy” transgenic plant, germinated in vitro, and examined for the expression of the GFP-fused protein. Those plants found to produce ~50% pollen tubes with GFP fluorescence were further analyzed by protein gel blotting using an anti-GFP antibody. Two plants each of the LAT52-PiSLF2:GFP, LAT52-PiSLFLb-S2:GFP and LAT52-PiSLFLc-S1:GFP transgenic lines (Figure 3.4A) and four plants of the LAT52-PiSLFLd-S2:GFP transgenic line (Figure 3.4B) produced comparable levels of the GFP-fused proteins in pollen or stage 5 anthers. The results of genomic blotting for the 10 transgenic plants analyzed in Figure 3.4 are shown in Figure 3.5.

The 10 transgenic plants mentioned above were further analyzed for their SI behavior. Both transgenic plants that carried Lat52-PiSLF2:GFP (named S2S3/PiSLF2:GFP-5 and -10; Figure 3.4A) showed the same SI behavior as the previously reported S2S3 transgenic plants that expressed a single copy of the PiSLF2 transgene (Sijacic et al., 2004). Both transgenic plants were completely self-compatible, setting large-sized fruits with seed numbers comparable to those obtained from normally compatible pollinations. Moreover, their pollen was compatible with pistils of S2S3 wild-type plants, but their pistils were incompatible with pollen of S2S3 wild-type plants, suggesting that the pollen SI function, but not the pistil SI function, of these two transgenic plants was affected. We raised 41 plants from seeds obtained from pollination of a wild-type S2S3 plant by S2S3/PiSLF2:GFP-5, and determined their S-genotypes by PCR using primers specific to S2-RNase and to S3-RNase. The results of representative plants are shown in Figure 3.4C. The S3-RNase-specific fragment was detected in all 41 plants, and the S2-RNase-
specific fragment was detected in 22 of the 41 plants, indicating that 22 plants were of $S_2S_3$ genotype and 19 were of $S_3S_3$ genotype (in an approximately 1:1 ratio). The absence of progeny with $S_2S_2$ genotype suggests that both $S_2$ pollen and $S_2$ pollen carrying the transgene produced by $S_2S_3/PiSLF_2:GFP$-5 functioned normally in SI and were rejected by the $S_2S_3$ pistil. Furthermore, when pollen from each of the 41 progeny plants was germinated in vitro, ~50% of the pollen tubes produced by each plant were found to show GFP fluorescence under an epifluorescent microscope (the result of one progeny plant is shown in Figure 3.6), suggesting that all the progeny plants carried one copy of the transgene inherited from $S_2S_3/PiSLF_2:GFP$-5. This finding, coupled with the finding that only $S_2S_2$ and $S_3S_3$ genotypes were present in the progeny, suggests that only the $S_3$ pollen carrying the transgene was accepted by the wild-type $S_2S_3$ pistil for fertilization. Thus, the expression of $PiSLF_2:GFP$ caused the breakdown of SI function in $S_3$ pollen (heteroallelic pollen) but not in $S_2$ pollen (homoallelic pollen). Similar results were obtained from the analysis of the progeny between a wild-type $S_2S_3$ plant (female) and $S_3S_3/PiSLF_2:GFP$-10 (male). We thus concluded that fusion of the GFP to the C-terminus of PiSLF2 does not affect its function in SI.

In contrast to $S_2S_3/PiSLF_2:GFP$-5 and -10, self-pollination of the two $PiSLFLb-S_2$ transgenic plants, the two $PiSLFLc-S_1$ transgenic plants, and the four $PiSLFLd-S_2$ transgenic plants analyzed in Figure 3.4 did not result in any fruit set (>10 pollinations for each transgenic plant). When we used pollen from each transgenic plant to pollinate wild-type plants with the same S-genotype, e.g., pollen from $S_2S_3/PiSLFLd-S_2:GFP$-30 was used to pollinate wild-type $S_2S_3$ plants, no fruits were obtained either (>10 pollinations for each transgenic plant). To rule out the possibility that the $PiSLFLb-S_2$, $PiSLFLc-S_1$, and $PiSLFLd-S_2$ transgenes might have affected the function of pollen/pollen tubes produced by their respective transgenic plants, we carried out crosses that were expected to be compatible with these transgenic plants, e.g., crosses between a wild-type $S_3S_3$ plant (female) and $S_3S_3/PiSLFLc-S_1:GFP$-20 (male). Large-sized fruits characteristic of compatible pollination were obtained in all cases. We examined the inheritance of the transgene in each progeny by PCR analysis, using a primer specific to the transgene and a primer specific to the GFP coding sequence (see Table 3.1 for the primers used), and found that the transgene was transmitted to approximately half of the progeny. For example, of the 55 progeny plants from the pollination mentioned above, 27 were found to
inherit the transgene. Thus, expression of \( \text{PiSLFLb-S2}, \text{PiSLFLc-S1}, \text{and PiSLFLd-S2} \) in transgenic pollen did not affect the normal function of pollen/pollen tubes.

We further compared the extent of pollen tube growth in pistils of a wild-type \( S_2S_3 \) plant 20 hr after pollination by transgenic plants \( S_2S_3/\text{PiSLFLd-S2-GFP-30} \) and \( S_2S_3/\text{PiSLF-S2-GFP-5} \). For \( S_2S_3/\text{PiSLFLd-S2-GFP-30} \), the growth of almost all pollen tubes was stopped in the upper segment of the pistil, similar to what was observed for pollination of the wild-type \( S_2S_3 \) plant by another wild-type \( S_2S_3 \) plant (Figures 3.7A and 3.7B), whereas for \( S_2S_3/\text{PiSLF-S2-GFP-5} \), almost all pollen tubes reached the bottom of the style, similar to what was observed for a compatible cross between the wild-type \( S_2S_3 \) plant and a wild-type \( S_1S_1 \) plant (Figures 3.7C and 3.7D). These results further confirmed the SI behavior of the transgenic plants determined by the pollination experiments. Thus, all the results taken together suggest that none of the three \( \text{PiSLFL} \) genes examined caused breakdown of SI function in heteroallelic pollen, and we concluded that \( \text{PiSLFLb, PiSLFLc, PiSLFLd} \) do not control pollen function in SI.

### 3.3.5 Analysis of Interactions between \( \text{PiSLFL} \) Proteins and \( S_3 \)-RNase by an \textit{in vitro} Binding Assay

I next examined whether the observation that \( \text{PiSLFLb-S2, PiSLFLc-S1, and PiSLFLd-S2} \) did not cause the breakdown of SI in heteroallelic pollen was due to the inability of these three \( \text{PiSLFL} \)s to interact with \( S \)-RNases. I also included two other \( \text{PiSLFL} \)s, \( \text{PiSLFLa-S2} \) and \( \text{S2-A134} \), as well as \( \text{PiSLF2} \) (as control) in the binding assay. The other previously identified \( \text{PiSLFL, A113} \), was not included because there is no sequence difference between its \( S_1 \) and \( S_2 \) allelic variants.

All the F-box proteins used in the assay were expressed as \( \text{(His)_6-T7-tagged proteins in Escherichia coli} \). The \( \text{(His)_6-T7} \) tagged proteins were purified and assayed for interactions with GST-\( S_3 \)-Nase, representing a non-self \( S \)-RNase for \( \text{PiSLF2} \) and all the \( \text{PiSLFL} \)s tested. The amounts of GST-\( S_3 \)-RNase bound to GST beads used in all the binding reactions were in large excess over that of each \( \text{(His)_6-T7} \)-tagged protein to ensure that the binding was not limited by the amount of GST-\( S_3 \)-RNase (Figure 3.8). After the binding reactions, the \( \text{(His)_6-T7} \)-tagged proteins were eluted and detected by an anti-(His)_6 tag antibody. Since the amounts of the \( \text{(His)_6-T7} \)-tagged proteins used in the same assay were not the same, to compare their binding
differences, the amount of each bound protein was normalized against the input amount (both of which were quantified by ImageQuant5.2 program).

Figure 3.9A shows the results of the binding assay for (His)$_6$T7:PiSLFLa-S$_2$ and (His)$_6$T7:PiSLF$_2$. Two different concentrations of these two proteins were used, and for each concentration, the amount of (His)$_6$T7:PiSLFLa-S$_2$ was approximately twice that of (His)$_6$T7:PiSLF$_2$. At the high concentration, (His)$_6$T7:PiSLF$_2$ interacted with GST:S$_3$-RNase, as expected; however, no binding was detected for (His)$_6$T7:PiSLFLa-S$_2$ even though its input amount was twice that of (His)$_6$T7:PiSLF$_2$. At the low concentration (20% that of the high concentration), a weak binding could still be detected for (His)$_6$T7:PiSLF$_2$.

I carried out a similar binding assay for (His)$_6$T7:PiSLFLb-S$_2$ and (His)$_6$T7:PiSLFLc-S$_1$. At each concentration, the amounts of (His)$_6$T7:PiSLFLb-S$_2$ and (His)$_6$T7:PiSLFLc-S$_1$ were 3 and 10 times, respectively, that of (His)$_6$T7:PiSLF$_2$. At the high concentration, no binding with GST:S$_3$-RNase was detected for (His)$_6$T7:PiSLFLb-S$_2$, whereas binding was detected for (His)$_6$T7:PiSLFLc-S$_1$, but the amount bound was much less than that detected for (His)$_6$T7:PiSLF$_2$, even though the amount of (His)$_6$T7:PiSLFLc-S$_1$ used in the assay was 10 times that of (His)$_6$T7:PiSLF$_2$ (Figure 3.9B). At the low concentration, a weak binding to GST:S$_3$-RNase could still be detected for (His)$_6$T7:PiSLF$_2$, but no detectable binding was observed for (His)$_6$T7:PiSLFLc-S$_1$ (Figure 3.9B). The interactions of (His)$_6$T7:PiSLFLb-S$_2$ and (His)$_6$T7:PiSLFLc-S$_1$ with GST:S$_3$-RNase were further examined along with (His)$_6$T7:PiSLFLd-S$_2$ and (His)$_6$T7:S$_2$-A134, and the results are shown in Figure 3.9C. At each concentration, the amounts of (His)$_6$T7:PiSLFLb-S$_2$, (His)$_6$T7:PiSLFLc-S$_1$, (His)$_6$T7:PiSLFLd-S$_2$ and (His)$_6$T7:S$_2$-A134 were approximately 30, 40, 8, and 5 times, respectively, that of (His)$_6$T7:PiSLF$_2$. At the high concentration, (His)$_6$T7:PiSLFLb-S$_2$, even at a higher relative amount to (His)$_6$T7:PiSLF$_2$ (30 to 1) than that (3 to 1) used in the assay shown in Figure 3.9B, did not interact with GST:S$_3$-RNase. At a higher relative amount to (His)$_6$T7:PiSLF$_2$ (40 to 1) than that (10 to 1) used in the assay shown in Figure 3.9B, the amount of (His)$_6$T7:PiSLFLc-S$_1$ bound to GST:S$_3$-RNase was similar to that detected for (His)$_6$T7:PiSLF$_2$ at the high concentration, but slightly less at the low concentration. At both high and low concentrations, the amounts of (His)$_6$T7:PiSLFLd-S$_2$ bound to GST:S$_3$-RNase
were similar to the amounts of (His)₆·T₇·PiSLF₂ bound, even though the amount of (His)₆·T₇·PiSLFLd·S₂ used was 8 times that of (His)₆·T₇·PiSLF₂ at each concentration. At the high concentration, the amount of (His)₆·T₇·S₂-A₁₃₄ bound to GST·S₃·RNase was approximately one fifth that of (His)₆·T₇·PiSLF₂ bound, even though the amount of (His)₆·T₇·S₂-A₁₃₄ used in the assay was 5 times that of (His)₆·T₇·PiSLF₂. At the low concentration, no bound (His)₆·T₇·S₂-A₁₃₄ could be detected.

The results of the binding assays shown in Figures 3.9A, 3.9B, and 3.9C indicate that (1) three of the five PiSLFLs examined interact to varying extents with S₃·RNase, with (His)₆·T₇·PiSLFLd·S₂ interacting the strongest, and (2) all these interactions are weaker than the interactions between PiSLF₂ and S₃·RNase. To further confirm that PiSLF₂ interacts with S₃·RNase much more strongly than does (His)₆·T₇·PiSLFLd·S₂, I performed a competition assay in a single reaction that contained equal amounts of (His)₆·T₇·PiSLF₂ and (His)₆·T₇·PiSLFLd·S₂, and an amount of GST·S₃·RNase that was 1/20 that used in all the other binding assays. A significantly larger amount of (His)₆·T₇·PiSLF₂ was bound to GST·S₃·RNase than was (His)₆·T₇·PiSLFLd·S₂, confirming that PiSLF₂ interacts with S₃·RNase much more strongly than does PiSLFLd·S₂.

### 3.3.6 Sequence Comparison among PiSLFs and PiSLFLs

The deduced amino acid sequences of three alleles of *PiSLF, PiSLF₁, PiSLF₂* and *PiSLF₃*, were aligned with those of *PiSLFLa-S₁, PiSLFLa-S₂, PiSLFLb-S₂, PiSLFLc-S₁, PiSLFLd-S₂, S₁(S₂)-A₁₁₃, S₃-A₁₁₃, S₁-A₁₃₄, S₂-A₁₃₄*, and *S₃-A₁₃₄* by ClustalW (Figure 3.10). To identify the regions that are specific to PiSLF, I first used the sequence of PiSLF₁ as a reference, and compared each amino acid with the corresponding amino acid of each of the other sequences in the alignment (Figure 3.10). An index number of -1, or +1, was assigned to the amino acid of the sequence being compared with PiSLF₁ if it was identical to, or different from, the reference amino acid of PiSLF₁. After all the sequences had been compared with the PiSLF₁ sequence, the index numbers for each site of the alignment were summed up to obtain the index value for that site. A sliding window analysis of the index value was then performed (using a window of 60 sites and a 6-site slide), and the value for each window was plotted against the starting site of
that window. The process was repeated using PiSLF$_2$ and then PiSLF$_3$ as a reference sequence. 

The plots for these three allelic variants of PiSLF are very similar, and they all contain three major peaks (Figure 3.11A). The regions delimited by these three peaks represent PiSLF-specific regions, and they were named SR1 (from sites 62 to 114), SR2 (from sites 184 to 196), and SR3 (from site 268 to 305) (Figure 3.10). Secondary structure predictions revealed that all three regions contained loop structures (Figure 3.10), which could potentially be involved in protein-protein interactions.

I next analyzed the amino acid sequences of PiSLF$_1$, PiSLF$_2$ and PiSLF$_3$ (see the alignment in Figure 3.12) using the normed variability index (Kheyr-Pour et al., 1990) to identify the most divergent regions (Figure 3.11B). Two regions, named Va and Vb (for Variable a and Variable b, respectively), were identified, and interestingly they are contained within SR1 and SR3, respectively (Figure 3.12). To examine whether the Va and Vb regions are under positive selection during evolution, the ratios of Ka (non-synonymous nucleotide substitutions) to Ks (synonymous nucleotide substitutions) were calculated for each pairwise comparison of the coding sequences of these three alleles of PiSLF (Figure 3.11C). The results show that the nucleotide sequence for the Va region is possibly under positive selection.

### 3.3.7 Dissecting the Biochemical Function of Three Different Regions of PiSLF$_2$

Based on the three PiSLF-specific regions (Figures 3.10 and 3.11) and preliminary biochemical characterization of the GST:S$_3$-RNase-binding properties of 11 truncated forms of PiSLF$_2$ (Table 3.2), I divided PiSLF$_2$ into three functional domains, named FD1, FD2 and FD3, which contain SR1, SR2 and SR3, respectively, (Figure 3.13A; Figure 3.12). I then generated five (His)$_6$ T7 tagged truncated constructs, each containing one or two of the FDs. All except the construct containing FD1 alone were successfully expressed in *E. coli*. Each (His)$_6$ T7 tagged protein was purified and tested for interactions with GST:S$_3$-RNase, as described in the previous section.

Both (His)$_6$ T7:PiSLF$_2$(FD2) and (His)$_6$ T7:PiSLF$_2$(FD3) interacted with GST:S$_3$-RNase, however, FD2 interacted more strongly than did FD3, and most notably, even more strongly than did the full-length protein (compare lanes b, c and f of Figure 3.13B and compare bars b, c and f
FD2+FD3 interacted with GST:S3-RNase to a lesser extent than did FD2 alone (compare lanes b and e of Figure 3.13B and compare bars b and e of Figure 3.13C), but still to a greater extent than did the full-length protein (compare lanes e and f of Figure 3.13B and compare bars e and f of Figure 3.13C). Finally, FD1+FD2 interacted with GST:S3-RNase less strongly than did FD2 alone and FD2+FD3 (compare lanes b, d and e of Figure 3.13B and compare bars b, d and e of Figure 3.13C), but more strongly than did the full-length protein (compare lanes d and f of Figure 3.13B and compare bars d and f of Figure 3.13C). All these results taken together suggest that (1) FD2 of PiSLF2 is the primary region for interactions with S3-RNase, and (2) the interactions between FD2 and S3-RNase may be negatively modulated by FD1 and FD3, with FD1 exerting a greater effect.

### 3.3.8 Contribution of FD2 of PiSLF2 to Strong Interactions with a Non-Self S-RNase

FD2 of PiSLF2 [PiSLF2(FD2)] contains SR2, one of the three PiSLF-specific regions (Figures 3.10 and 3.11A, Figure 3.12), and the results of the binding assay shown in Figure 3.13 suggest that FD2 is the primary region for interaction with S3-RNase, a non-self S-RNase of PiSLF2. Thus, I further examined the role of PiSLF2(FD2) by a domain-swapping approach. Since PiSLFb-S2 did not interact with S3-RNase (Figures 3.9B and 3.9C), I swapped FD2 of PiSLF2 and the corresponding domain of PiSLFb-S2 to generate two (His)_6:T7 tagged chimeric proteins, (His)_6:T7:PiSLF2(FD1):PiSLFb-S2(FD2):PiSLF2(FD3) and (His)_6:T7:PiSLFb-S2(FD1):PiSLF2(FD2):PiSLFb-S2(FD3) (Figure 3.14A). I then determined whether these two chimeric proteins could interact with GST:S3-RNase, and if so, what effect the domain swapping had on the binding properties of PiSLF2 and PiSLFb-S2. Similar to what was previously observed, (His)_6:T7:PiSLF2 interacted strongly with GST:S3-RNase, but there was no detectable interaction between (His)_6:T7:PiSLFb-S2 and GST:S3-RNase (lanes a and b of Figure 3.14B, bars a and b of Figure 3.14C). Replacing FD2 of PiSLF2 with the corresponding domain of (His)_6:T7:PiSLFb-S2 greatly reduced the extent of interactions with GST:S3-RNase (compare lanes a and c of Figure 3.14B and compare bars a and c of Figure 3.14C). Most interestingly, replacing the corresponding FD2 of PiSLFb-S2 with FD2 of PiSLF2 conferred on the chimeric PiSLFb-S2 the ability to interact with GST:S3-RNase (compare lanes b and d of Figure 3.14B and compare bars b and d of Figure 3.14C). All these results confirm that FD2 of PiSLF2 plays
an important role in the interactions with \( S_3 \)-RNase. However, the findings that the chimeric PiSLF\( \text{c} \) could still interact with GST: \( S_3 \)-RNase (lane c of Figure 3.14B; bar c of Figure 3.14C) and that FD3 alone could interact with \( S_3 \)-RNase (lane c of Figure 3.13B and bar c of Figure 3.13C) would suggest that some amino acids in FD3 also contribute to the interactions.

### 3.3.9 FD1 and FD3 Together Determine the Specificity of PiSLF in Its Interaction with \( S_3 \)-RNase

The results of the binding assay for the full-size PiSLF\( \text{c} \) and its various truncated forms showed that addition of FD1 or FD3 to FD2 reduced the interaction between FD2 and \( S_3 \)-RNase (Figures 3.13B and 3.13C). Moreover, sequence comparison among three allelic variants of PiSLF revealed that FD1 and FD3 each contained one of the two variable regions of PiSLF (Figure 3.11B, Figure 3.12). Thus, I hypothesized that FD1 and FD3 might differentially control the interactions between a PiSLF and its self and non-self \( S \)-RNases through blocking the interactions between FD2 and \( S \)-RNases more strongly under self-interactions than under non-self interactions. That is, FD1 and FD3 might determine allelic specificity of PiSLF such that the interactions between matching specificity determinants of PiSLF and \( S \)-RNase would significantly weaken the general interactions between FD2 and \( S \)-RNase. To examine this possibility, I made four constructs for expressing chimeric proteins between PiSLF\( \text{c} \) and PiSLF\( \text{c} \) by swapping either FD1 alone or both FD1 and FD3 (Figures 3.15A and 3.15D), and assessed the effects of the domain swapping on the interactions of PiSLF\( \text{c} \) and PiSLF\( \text{c} \) with \( S_2 \)-RNase under both non-self and self-interaction conditions.

Consistent with our previous finding that a PiSLF interacted with its non-self \( S \)-RNases much more strongly than with its self \( S \)-RNase (Hua and Kao, 2006), PiSLF\( \text{c} \) interacted much more strongly with \( S_2 \)-RNase than did PiSLF\( \text{c} \) (compare lanes a and b in Figures 3.15B and 3.15E, bars a and b in Figures 3.15C and 3.15F). When FD1 of PiSLF\( \text{c} \) was replaced with FD1 of PiSLF\( \text{c} \), the chimeric protein interacted with \( S_2 \)-RNase much more strongly than did PiSLF\( \text{c} \) (compare lanes b and c in Figure 3.15B, bars b and c in Figure 3.15C). One interpretation of this finding is that the negative effect of FD1 of PiSLF\( \text{c} \) on the self-interaction between PiSLF\( \text{c} \) and \( S_2 \)-RNase was alleviated when this FD1 was replaced with FD1 of PiSLF\( \text{c} \), a non-self PiSLF for \( S_2 \)-RNase.
When FD1 of PiSLF1 was replaced with FD1 of PiSLF2, the chimeric protein still interacted with S₂-RNase to a similar extent as did PiSLF1 (compare lanes a and d in Figure 3.15B, bars a and d in Figure 3.15C), suggesting that FD1 alone is not sufficient to negatively regulate the strong general interactions between FD2 of a PiSLF and its self S-RNase, and that FD3 may cooperate with FD1 in this regulatory function.

Indeed, when both FD1 and FD3 of PiSLF1 were replaced with the corresponding domains of PiSLF2, the chimeric protein behaved like PiSLF2 in that its interaction with S₂-RNase was as weak as the self-interaction between PiSLF2 and S₂-RNase (compare lanes b and f in Figure 3.15E, and bars b and f in Figure 3.15F). Conversely, when FD1 and FD3 of PiSLF2 were replaced with the corresponding regions of PiSLF1, the chimeric protein behaved like PiSLF1 in that it interacted with S₂-RNase to a similar extent as the non-self interaction between PiSLF1 and S₂-RNase (compare lanes a and e in Figure 3.15E, and bars a and e in Figure 3.15F). These results also suggest that FD2 is unlikely to contribute to the specific interaction between PiSLF and S-RNase because the chimeric PiSLF1 protein, containing FD2 of PiSLF2 (see e of Figure 3.15D), still retained the strong binding affinity of PiSLF1 for S₂-RNase (i.e., non-self interactions, compare lanes a and e in Figure 3.15E, and bars a and e in Figure 3.15F). Moreover, the chimeric PiSLF2, containing FD2 of PiSLF1 (see f of Figure 3.15D), still interacted with S₂-RNase as weakly as did PiSLF2 (i.e., self-interactions, compare lanes b and f in Figure 3.15E, and bars b and f in Figure 3.15F).

### 3.4 Discussion

In this work, I have identified four F-box genes of *P. inflata*, PiSLFLa, b, c, and d, which share several properties with *PiSLF*. Their deduced amino acid sequences are similar to those of three alleles of *PiSLF* our lab previously studied (e.g., 47.6 to 54.4% identical to PiSLF2, Table 3.4); they are specifically expressed in pollen/pollen tubes, all except PiSLFLa show S-haplotype-specific RFLP and have been shown to be tightly linked to the S-locus. I have used these four *PiSLFL* genes, along with the two previously identified S-locus-linked *PiSLFL* genes, A113 and A134, to investigate whether *PiSLF* is unique in its function in SI, and if so, what properties/features of PiSLF confer on it the unique function.
I, with the help of another graduate student, Xiaoying Meng, have used the same approach that our lab previously used to establish the function of *PiSLF* in SI to examine whether three of the four *PiSLFL* genes identified in this work, *PiSLFLb*, *PiSLFLc* and *PiSLFLd*, play a similar role in SI. We first used *LAT52:* *PiSLF2:*GFP as a control to show that fusion of GFP to the C-terminal end of *PiSLF2* did not affect its function in SI. That is, expression of *PiSLF2:*GFP in *S2S3* transgenic plants caused the breakdown of SI function in *S3* pollen carrying the transgene (heteroallelic pollen) but not in *S2* pollen carrying the transgene (homoallelic pollen), the same results as were previously obtained with *PiSLF2* (Sijacic et al., 2004). We then showed that *PiSLFLb-S2:*GFP, *PiSLFLc-S1:*GFP, and *PiSLFLd-S2:*GFP did not cause the breakdown of SI function in heteroallelic pollen, even though their proteins were produced to comparable levels in respective transgenic plants as the protein produced from *PiSLF2:*GFP in the control *S2S3* transgenic plants. Thus, none of these three *PiSLFL* genes play a role in *S*-specificity of pollen.

All the earlier models on the S-RNase-based SI mechanism, proposed prior to the identification of the pollen *S*-gene, predicted that the interactions of an allelic product of the pollen *S*-gene with its self S-RNase were thermodynamically favored over the interactions with its non-self S-RNases (for review see Kao and Tsukamoto 2004). This prediction was based on the assumption that (1) self-interactions are through the matching allelic-specific domains of a pollen *S*-allele product and its self S-RNase, whereas non-self interactions are through a domain common to all pollen *S*-allele products and a domain common to all S-RNases; and (2) evolution of the SI mechanism has selected for matching allelic products of the male and female *S*-genes to recognize and interact with each other. Since the outcome of self-interactions in SI is inhibition of pollen tube growth, these models also predict that self-interactions between the allelic-specific domains render self S-RNase immune to inhibition either by the RNase-inhibition domain of the matching pollen *S*-allele product (Kao and Tsukamoto, 2004) or by a general RNase inhibitor (Luu et al., 2001; see below).

After the pollen *S*-gene was identified, these earlier models were modified to take into account the potential function of SLF/SFB in mediating ubiquitination and degradation of S-RNases (Qiao et al., 2004b; Sijacic et al., 2004; Hua and Kao, 2006). That is, self-interactions would result in the inability of self S-RNase to be ubiquitinated and degraded, whereas non-self
interactions would result in ubiquitination and degradation of non-self S-RNases. However, if self-interaction is thermodynamically favored over non-self interactions, and if self-interactions result in protection of self S-RNase from being degraded, these predictions cannot explain the phenomenon of competitive interaction. For example, if a heteroallelic pollen tube producing both PiSLF1 and PiSLF2 has penetrated into an S1S2 pistil and taken up S1- and S2-RNases, PiSLF1 would preferentially interact with S1-RNase and PiSLF2 would preferentially interact with S2-RNase. As a result, neither S1-RNase nor S2-RNase would be degraded and thus they would inhibit the growth of this heteroallelic pollen tube; this predicted outcome is precisely the opposite of what is observed. This conundrum led Luu et al. (2001) to propose a general inhibitor model. They hypothesized that a pollen S-allele product forms a homo-tetramer, which interacts with its self S-RNase and protects it from inhibition by a general RNase inhibitor. Using the example of heteroallelic pollen given above, PiSLF1 and PiSLF2 would form a hetero-tetramer, which would be unable to interact with either S1-RNase or S2-RNase. As a result, both S-RNases would be inhibited by the general RNase inhibitor, and this heteroallelic pollen tube would be compatible with S1S2 pistils. However, no biochemical data supporting this model have been reported yet.

In Chapter 2, I used an in vitro binding assay to compare the interactions of an allelic product of PiSLF with its self and non-self S-RNases, and the interactions of an S-RNase with its self and non-self PiSLFs. Contrary to our previous prediction, I found that a PiSLF interacted with its non-self S-RNases more strongly than with its self S-RNase, and similarly, an S-RNase interacted with its non-self PiSLFs more strongly than with its self PiSLF. This unexpected finding provides an explanation for competitive interaction. Again, using the example given above, PiSLF1 would preferentially interact with S2-RNase and PiSLF2 would preferentially interact with S1-RNase to mediate their degradation. If there is any PiSLF1 or PiSLF2 molecule that binds its self S-RNase, the complex might rapidly dissociate, because self-interactions are much weaker than non-self interactions, allowing the dissociated S-RNase to bind its non-self PiSLF and be degraded. As a result, both S1- and S2-RNases would be degraded and thus this heteroallelic pollen tube would be accepted by the S1S2 pistil.
Our model for the biochemical basis of competitive interaction, and SI interactions in general, is further supported by the *in vitro* binding results of PiSLFLb-S2, PiSLFLc-S1, and PiSLFLd-S2 (Figures 3.9B, 3.9C, and 3.9D). For example, when PiSLFLb-S2 was introduced into S2S3 plants, the heteroallelic pollen produced PiSLF3 and PiSLFLb-S2. PiSLF3 would mediate the degradation of S2-RNase, but PiSLFLb-S2 would not be able to mediate the degradation of S3-RNase, because it does not interact with S3-RNase (Figures 3.9B and 3.9C). As a result, PiSLFLb-S2 would not alter the SI behavior of heteroallelic pollen produced by S2S3 transgenic plants, which was precisely what we observed. All the PiSLFLs examined in this work either failed to interact with S3-RNase (i.e., PiSLFLa-S2 and PiSLFLb-S2, Figures 3.9A, 3.9B, and 3.9C) or interacted with S3-RNase much more weakly than did PiSLF2 (i.e., PiSLFLc-S1, PiSLFLd-S2, and S2-A134; Figures 3.9B, 3.9C, and 3.9D). Thus, under the normal situation when pollen only carries a single allele of PiSLF, none of these PiSLFLs would be able to compete with this allelic product of PiSLF for binding to any of its non-self S-RNases.

I have further investigated our hypothesis that the unique function of PiSLF in SI is due in large part to the fact that it has co-evolved with S-RNase to allow their allelic products to interact more strongly between non-matching alleles than between matching alleles. I first identified three PiSLF-specific regions, SR1, SR2 and SR3, which are divergent in the PiSLFLs studied here (Figures 3.10 and 3.11). After initial study using various truncated forms of PiSLF2 to dissect its functional domains, I divided PiSLF2 into three domains, FD1, FD2 and FD3, with each containing one of three PiSLF-specific regions (Figure 3.13A, Figure 3.12, Table 3.2), and examined the contributions of each domain to the interactions with S3-RNase. Using various truncated forms of PiSLF2, with one or both FD domains deleted, I found that FD2 plays a major role in the strong interactions of PiSLF2 with S3-RNase, a non-self S-RNase (Figure 3.13), whereas FD1 and FD3, each containing one of the two variable regions, appear to negatively modulate the interactions (Figure 3.13). The role of FD2 is further supported by the finding from the domain-swapping experiment that when it replaced the corresponding domain of PiSLFLb-S2, it conferred on the chimeric protein the ability to interact with S3-RNase, although the interaction was not as strong as that between PiSLF2 and S3-RNase (Figure 3.14).
Most importantly, the results of the domain-swapping experiment involving swapping FD1 alone, or both FD1 and FD3, between PiSLF1 and PiSLF2 (Figure 3.15) have revealed a functional role of these two domains in controlling the interactions between a PiSLF and its self and non-self S-RNases. Specifically, I have shown that the interactions of PiSLF1 and PiSLF2 with S2-RNase are completely reversed after their FD1 and FD3 have been swapped. That is, replacing FD1 and FD3 of PiSLF1 with the corresponding domains of PiSLF2 renders the chimeric protein to behave as PiSLF2 (the protein that contributes FD1 and FD3 to the chimeric protein) in that it interacts with S2-RNase as weakly as does PiSLF2. Conversely, replacing FD1 and FD3 of PiSLF2 with the corresponding domains of PiSLF1 renders the chimeric protein to behave as PiSLF1 (the protein that contributes FD1 and FD3 to the chimeric protein) in that it interacts with S2-RNase as strongly as does PiSLF1. Thus, FD1 and FD3 appear to be the prime candidates for the allelic specificity determinant of PiSLF.

3.5 Accession Numbers

The new sequence data reported in this chapter can be found in the GenBank data library under the following accession numbers: *PiSLFLa*-S1 (EF614190), *PiSLFLa*-S2 (EF614189), *PiSLFLb*-S2 (EF614188), *PiSLFLc*-S1 (EF614191), *PiSLFLd*-S2 (EF614187). The accession numbers for the previously identified sequence data used in this chapter are as follows: PiSLF1 (AAS79484), PiSLF2 (AAS79485), PiSLF3 (AAS79486), S1(S2)-A113 (AAR15911), S1-A134 (AAR15914), S2-A134 (AAR15915), S3-A134 (AAR15916) from Petunia inflata.
Figure 3.1 Genomic DNA Gel Blot Analysis of *PiSLFL* Genes for *S*-Haplotype-Specific Restriction Fragment Length Polymorphism and Linkage to the *S*-Locus.

Genomic DNA (15 μg) isolated from each plant was digested with *Xba* I, or *EcoR* I as indicated in (B) and in (F). The DNA digests were separated by 0.7% agarose gels. Each blot was hybridized with the 32P labeled cDNA probe indicated at the top of the autoradiogram at 65 °C. The *S*₁-specific fragments are indicated with gray arrows; the *S*₂-specific fragments are indicated with blank arrows; the *S*₃-specific fragments are indicated with black arrows. For (A), (B), (C) and (D), the *S*-genotypes of the plants used are: lane a, *S*₁*S*₁; lane b, *S*₁*S*₂; lane c, *S*₂*S*₂; lane d, *S*₂*S*₃; lane e, *S*₃*S*₃; lane f, *S*₃*S*₃. The small dark triangles in (B) and (F) indicate a fragment that
cross-hybridized with PiSLFLb-S2; this fragment is similar in size to the $S_1$-specific fragment of PiSLFLb indicated with gray arrows.

(A) The blot was first hybridized with cDNA for PiSLFLa-S1 and after autoradiography, the hybridized PiSLFLa-S1 probe was stripped off and the blot was rehybridized with cDNA for PiSLFLa-S2.

(B), (C), (D) Each blot was hybridized with the respective cDNA probe as indicated.

(E) Schematic representation of recombination between two markers, 3.16 and G221, and the $S$-RNase gene in three recombinant plants, N43, N124 and P85. $S_1S_1$ and $S_2S_2$ are wild-type plants. Chromosomal DNA of $S_1$-haplotype is marked in gray and chromosomal DNA of $S_2$-haplotype is marked in white. The $S$-genotypes of the recombinant plants, determined by genomic blotting results using $S$-RNase as a probe (Wang et al., 2006), are: N43, $S_2S_2$; N142, $S_1S_1$; P85, $S_2S_2$.

(F), (G), (H) Each blot was hybridized with the respective cDNA probe as indicated.
Figure 3.2 Expression Patterns of Four PiSLFL Genes Determined by RNA Gel Blot or RT-PCR.

Total RNA was extracted from various tissues, and anthers at five different developmental stages, of an $S_1S_1$ plant (A) and an $S_2S_2$ plant (B and C). Anther stages are defined by flower-bud size as described in Lee et al. (1996).

(A) The RNA samples (10 μg per lane) were electrophoresed on 1.25% formamide-agarose gels, and each blot was hybridized with $^{32}$P labeled cDNA for one of the three PiSLFL genes, as
indicated. Equal loading of RNA samples was assessed by ethidium bromide staining of the ribosomal RNAs separated on a 1% agarose gel in (A), or by ethidium bromide staining of the gel used in blotting in (B). (C) RT-PCR products of PiSLFLd-S2 and PiSLF2 were electrophoresed on 1% agarose gels and visualized by ethidium bromide staining. To demonstrate equal amounts of RNA used for amplification of PiSLFLd-S2 and PiSLF2, RT-PCR was performed on each RNA sample to amplify the Actin gene. To exclude contamination of genomic DNA in the RNA samples, RT-PCR was also performed using primers for Actin gene on each sample in the absence of reverse transcriptase (RT-). PCR of genomic DNA of S2S2 genotype was used to demonstrate the size of the genomic DNA band.
Figure 3.3 Schematics of the Ti-Plasmid Constructs Used in Plant Transformation Experiments.

The name of each gene or sequence element is indicated under each construct and the restriction sites used for subcloning and digestion of genomic DNA for blotting analysis are indicated above each construct. The fragment used as probe in DNA-blotting analysis is marked with a broken line in each construct. The region between the right (RB) and the left border (LB) is integrated into transgenic plants. NOS: the gene encoding nopaline synthase; pro: promoter; ter: transcription terminator; NPT II: the gene encoding neomycin phosphotransferase II (conferring kanamycin resistance).
Figure 3.4 Functional Analysis of Three *PiSLFL* Genes and *PiSLF2* in Transgenic Plants.

(A) Protein gel blot showing that similar levels of proteins were produced from the *PiSLF2:GFP*, *PiSLFLb-S2:GFP* and *PiSLFLc-S1:GFP* transgenes in six transgenic lines. Top panel, immunoblot (IB) of total pollen tube proteins. The single asterisk indicates *PiSLFLb-S2:GFP*, *PiSLF2:GFP* or *PiSLFLc-S1:GFP*; the double asterisks indicate the cleaved GFP tag. Bottom panel, Ponceau S staining of the blot shown in the top panel before immunoblotting to reveal equal loading of total proteins.

(B) Protein gel blot showing that similar levels of proteins were produced from four independent *PiSLFLd-S2:GFP* and one *PiSLF2:GFP* transgenic plants. Top panel, immunoblot (IB) of total proteins from stage 5 anthers. The single asterisk indicates *PiSLFLd-S2:GFP* or *PiSLF2:GFP*. Bottom panel, Ponceau S staining of the blot shown in the top panel before immunoblotting to reveal equal loading of total proteins.

(C) PCR genotyping of the T1 progeny from pollination of a wild-type S2S3 plant by pollen of S2S3/*PiSLF2:GFP-5*. Genomic DNA (~500 ng) isolated from 16 progeny plants and from three wild-type plants (S1S1, S2S2, and S3S3) was amplified by primers specific to S2-RNase (top panel) or S3-RNase (bottom panel). The S2-RNase-specific PCR fragment is indicated with a single asterisk and the S3-RNase-specific PCR fragment is indicated with double asterisks.
Figure 3.5 Genomic DNA Gel Blots Showing Independent Transgenic Lines That Contain a Single Insert of (A) *PiSLF₂*:GFP, (B) *PiSLFLb*:GFP, (C) *PiSLFLc*:GFP, or (D) *PiSLFLd*:GFP.

Each lane contains restriction digests of 15 μg genomic DNA isolated from the transgenic plant indicated, or from one of the two wild-type plants (*S₁S₂* and *S₂S₃*). The restriction enzyme used for each blot is indicated at the bottom of the autoradiogram. cDNAs for *PiSLF₂*(CTD), *PiSLFLb*, *PiSLFLc*, and *PiSLFLd*, indicated in Figure 3.3, were used as probes in (A), (B), (C), and (D), respectively. On each blot, the fragments that contain the transgene are indicated with small white triangles.
Figure 3.6 Bright Field (Top) and Fluorescence (Bottom) Images of Representative Pollen Tubes Produced by a Progeny Plant From the Cross Between a Wild-Type $S_2S_3$ Plant and the Transgenic Plant $S_2S_3/PiSLF_2:GFP-5$.

Pollen was germinated in a pollen germination medium, as described in Lee et al. (1996), for 2 hr with gentle shaking at 30 °C, and the pollen tubes were observed under an epifluorescent microscope. Note that 11 of the 20 pollen tubes show GFP fluorescence.
Figure 3.7 Fluorescence Images of Pollen Tubes in Pistils of a Wild-Type $S_2S_3$ Plant 20 hr Post-Pollination with Pollen From (A) Transgenic Plant $S_2S_3/PiSLF$-$dS_2$:GFP-30, (B) Wild-Type $S_2S_3$ Plant, (C) Transgenic Plant $S_2S_3/PiSLF_2$:GFP-5, (D) Wild-Type $S_1S_1$ plant.

Pollen tubes (PT) were stained with aniline blue and visualized under an epifluorescent microscope. Note that most pollen tubes shown in (A) and (B) were stopped in the upper segment of the pistil (indicated with a black arrow), whereas most pollen tubes in (C) and (D) grew through the entire pistil to reach the ovary (not shown). PG: pollen grain.
Figure 3.8 Ponceau S Staining of the Immunoblots Containing Binding Assays Conducted at High Concentrations to Show That the Amount of GST:S3-RNase Used in Each Binding Reaction was in Large Excess over the (His)6:T7 Tagged Protein.

(A), (B), (C) show the part of immunoblots in Figures 3.8A, 3.8B, and 3.8C, respectively, that contains binding assays conducted at the high concentration (left panel). The same amount of GST:S3-RNase shown was also used in all the binding assays conducted at low concentrations of each (His)6 T7 tagged protein. For each (His)6 T7 tagged protein, one tenth the input amount assayed at the high concentration is shown (right panel). The single asterisks indicate GST:S3-RNase and the dark triangles indicate the predicted position where the (His)6 T7 tagged proteins migrate on each gel.
Figure 3.9 *In vitro* Binding Assay for Interactions Between PiSLFL Proteins and S3-RNase.

PiSLFLa-S2, PiSLFLb-S2, PiSLFLc-S1, and PiSLFLd-S2 were expressed as (His)$_6$T7 tagged proteins, and the purified proteins were incubated separately with GST:S3-RNase-bound Glutathione Sepharose beads. PiSLF3 was similarly expressed and purified for use as control for binding to GST:S3-RNase. The bound proteins were eluted and analyzed by immunoblotting using an anti-(His)$_6$ antibody. Each input lane contains one tenth the amount of the (His)$_6$T7-tagged protein used in the binding assay at the high concentration. The bound (His)$_6$T7 tagged proteins are indicated with small dark triangles. All the other cross-reacting bands may correspond to *E. coli* proteins that co-purified with the recombinant proteins used in the assay.

**(A)** Binding assay for PiSLFLa-S2.

**(B)** Binding assay for PiSLFLb-S2 and PiSLFLc-S1. H, binding assay carried out at the high concentration of the indicated (His)$_6$T7 tagged proteins; L, binding assay carried out at the low concentration, which is one fifth the high concentration.

**(C)** Binding assay for PiSLFLb-S2, PiSLFLc-S1, PiSLFLd-S2, and S2-A134.

**(D)** Assay for competition between PiSLF2 and PiSLFLd-S2 for binding to GST:S3-RNase. Equal amounts of (His)$_6$T7:PiSLF2 and (His)$_6$T7:PiSLFLd-S2 were incubated with GST:S3-RNase in the same reaction. GST:S3-RNase amount was used ~1/20 of that in (A), (B) and (C).
Figure 3.10 Alignment of the Deduced Amino Acid Sequences of Three Alleles of *PiSLFs* and Six *PiSLFL* Genes (Some with Multiple Alleles).

Amino acids that are conserved at a given site are highlighted in dark shading; amino acids that are similar to the conserved residues are highlighted in grey shading. The regions of *PiSLF* predicted by the PROFsec program (http://cubic.bioc.columbia.edu/predictprotein/) to assume α-helix, β-sheet and loop secondary structures (with a higher than 82% of the expected average accuracy) are indicated by black, gray and blank bars, respectively. The F-box domain predicted by SMART (http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1) and the three *PiSLF*-specific regions, SR1, SR2 and SR3, are indicated by black lines above the aligned sequences.
Figure 3.11 Sequence Analysis of PiSLF and PiSLFL Proteins.

(A) Plots of window-averaged variability index values calculated using PiSLF1, PiSLF2 and PiSLF3 as reference sequences. The alignment in Figure 3.10 was used for sequence comparison between each allelic variant of PiSLF and all the PiSLFL sequences. The window-averaged variability index value for each site of the alignment was obtained by a sliding window analysis (using a 60-amino acid window and a 6-amino acid slide) as described in the text. The peaks, named SR1, SR2 and SR3, represent PiSLF-specific regions.

(B) Plot of window-averaged norm variability index calculated based on the alignment of the amino acid sequences of PiSLF1, PiSLF2 and PiSLF3 shown in Supplemental Figure 6. The index for each sliding window (60-amino acid window; 6-amino acid slide) was calculated according to the method of Kheyr-Pour et al. (1990). The peaks, named Va and Vb, represent two regions of variability among the three PiSLFs.

(C) Plots of Ka/Ks calculated based on pairwise comparison of the nucleotide sequences of PiSLF1, PiSLF2 and PiSLF3. A sliding window was used at a 180-bp window and an 18-bp slide. The numbers refer to amino acid residues as shown in (B).
Figure 3.12 Alignment of the Deduced Amino Acid Sequences of Three Allelic Variants of *PiSLF*.

The alignment was performed as described in Figure 3.10. The three PiSLF-specific regions, SR1, SR2, and SR3, are indicated by black lines above the aligned sequences. The two variable regions, Va and Vb, are boxed with dash lines. FD1 and FD3 are separated by FD2, which is highlighted in red.
Figure 3.13 Roles of Three Separate Regions of PiSLF2 in Tts Interactions with S3-RNase.

(A) Schematic representation of five truncated versions of PiSLF2 and the full-length protein. Each was expressed as a (His)6-T7 tagged protein in E. coli. The amino acid residues that demarcate the F-box domain and the three PiSLF-specific regions, SR1, SR2 and SR3, are indicated.

(B) In vitro assay for interactions between GST-S3-RNase and five (His)6-T7 tagged truncated PiSLF2 and the full-length PiSLF2. The assay was performed as described in the legend to Figure 3.9.

(C) Quantification of the binding results shown in (B). The intensity of each bound band as well as the input band was quantified by ImageQuant5.2 (GE Healthcare). The relative bound amount for each (His)6-T7 tagged protein used in the assay was calculated as the percentage of the total input amount.
Figure 3.14 Analysis of the Biochemical Function of FD2 of PiSLF2 by Domain Swapping.

(A) Schematic representation of (His)_6 T7:PiSLF2, (His)_6 T7:PiSLFLb-S2, and their two chimeric proteins with the FD2 region swapped between them. The amino acid residue numbers demarcating FD1, FD2, and FD3 are shown (refer to Figure 3.10 for actual amino acid residues).

(B) In vitro binding assay for the interaction of (His)_6 T7:PiSLF2, (His)_6 T7:PiSLFLb-S2, and the two chimeric proteins with GST:S3-RNase. The binding assay was performed as described in the legend to Figure 3.9.

(C) Quantification of the binding results shown in (B). The quantification was performed as described in the legend to Figure 3.13C.
Figure 3.15 Analysis of the Biochemical Function of FD1 and FD3 of PiSLF2 by Domain Swapping.

(A) Schematic representation of (His)$_6$ T7:PiSLF$_1$, (His)$_6$ T7:PiSLF$_2$, and two chimeric proteins with the FD1 region swapped between them. The amino acid residue numbers demarcating FD1, FD2, and FD3 are shown (refer to Supplemental Figure 6 for actual amino acid residues).

(B) In vitro binding assay for the interaction of GST:S2-RNase with (His)$_6$ T7:PiSLF$_1$, (His)$_6$ T7:PiSLF$_2$, and the two chimeric proteins shown in (A). The binding assay was performed as described in the legend to Figure 3.9.

(C) Quantification of the binding results shown in (B). The quantification was performed as described in the legend to Figure 3.13C.
(D) Schematic representation of two chimeric proteins with FD1 and FD3 swapped between (His)_6 T7:PiSLF_1 and (His)_6 T7:PiSLF_2. The amino acid residue numbers are the same as shown in (A).

(E) *In vitro* binding assay for the interaction of (His)_6 T7:PiSLF_1, (His)_6 T7:PiSLF_2, and the two chimeric proteins from (D) with GST:S_2-RNase. The binding assay was performed as described in the legend to Figure 3.9.

(F) Quantification of the binding results shown in (E). The quantification was performed as described in the legend to Figure 3.13C.
### Table 3.1 List of PCR primers used in this study

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<th>Constructs for (His)$_6$:T7 fusion proteins</th>
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<td>PiSLF$_2$(FD1):PiSLFLb-S$_2$(FD2)</td>
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<td>Reverse Primer Sequence</td>
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<td>pET28 PiSLF₁(81-297)</td>
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<td>Constructs</td>
<td>Forward Primer Sequence</td>
<td>Reverse Primer Sequence</td>
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<td>-------------------------</td>
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<tr>
<td>Constructs for (His)$_6$:T7 fusion proteins</td>
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<tr>
<td>pET28 PiSLF2(50-260) (FD1 without F-box domain, FD2)</td>
<td>CCGGATCTCTGATCAATCGCAAAACAAACAC</td>
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<tr>
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GFP Reverse
Table 3.2  List of recombinant proteins involved in this study

**(His)**6:T7 tagged proteins expressed produced (17 total)

<table>
<thead>
<tr>
<th>Protein Name</th>
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<tr>
<td>(His)6:T7:PiSLF2</td>
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<tr>
<td>(His)6:T7:PiSLFLα-S2</td>
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<tr>
<td>(His)6:T7:PiSLFLβ-S2</td>
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<tr>
<td>(His)6:T7:PiSLFLc-S1</td>
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<td>(His)6:T7:PiSLFLd-S2</td>
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<tr>
<td>(His)6:T7:S2-A134</td>
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<tr>
<td>(His)6:T7:PiSLF2(FD2)</td>
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<tr>
<td>(His)6:T7:PiSLF3(FD3)</td>
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<tr>
<td>(His)6:T7:PiSLF3(FD1+FD2)</td>
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<tr>
<td>(His)6:T7:PiSLF3(FD2+FD3)</td>
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<tr>
<td>(His)6:T7:PiSLF2(FD1):PiSLFLβ-S2(FD2):PiSLF2(FD3)</td>
</tr>
<tr>
<td>(His)6:T7:PiSLF2(FD1):PiSLFLβ-S2(FD3)</td>
</tr>
<tr>
<td>(His)6:T7:PiSLF2(FD1):PiSLF1(FD2)</td>
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<td>(His)6:T7:PiSLF2(FD1):PiSLF1(FD3)</td>
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<td>(His)6:T7:PiSLF2(FD1):PiSLF2(FD2):PiSLF1(FD3)</td>
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<tr>
<td>(His)6:T7:PiSLF2(FD1):PiSLF2(FD3):PiSLF3(FD3)</td>
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**(His)**6:T7 tagged proteins produced for initial study but not mentioned in detail in the paper (11 total)

<table>
<thead>
<tr>
<th>Protein Name</th>
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<tbody>
<tr>
<td>(His)6:T7:PiSLF2(81-160)</td>
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<td>(His)6:T7:PiSLF2(111-230)</td>
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<tr>
<td>(His)6:T7:PiSLF2(244-389)</td>
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<td>(His)6:T7:PiSLF2(81-297)</td>
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<td>(His)6:T7:PiSLF2(81-389)</td>
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<td>(His)6:T7:PiSLF2(50-179) (FD1 without F-box domain + partial FD2)</td>
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<td>(His)6:T7:PiSLF2(50-191) (FD1 without F-box domain + partial FD2 with SR2)</td>
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<td>(His)6:T7:PiSLF2(50-260) (FD1 without F-box domain + FD2)</td>
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<td>(His)6:T7:PiSLF2(50-297) (FD1 without F-box domain + FD2 + SR3)</td>
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<td>(His)6:T7:PiSLF2(50-316) (FD1 without F-box domain + FD2 + partial FD3 with SR3)</td>
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<td>(His)6:T7:PiSLF2(50-389) (FD1 without F-box domain + FD2 + FD3)</td>
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<td>Table 3.2  List of recombinant proteins involved in this study (continued)</td>
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Table 3.3  Percent pairwise nucleotide sequence identities between the coding regions of *PiSLF* and *PiSLFL* genes

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<th>PiSLFLa-S₁</th>
<th>PiSLFLb-S₂</th>
<th>PiSLFLc-S₁</th>
<th>PiSLFLd-S₂</th>
<th>Si(S₂)-A₁₁₃</th>
<th>S₁-A₁₃₄</th>
<th>S₂-A₁₃₄</th>
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Table 3.4  Percent pairwise sequence identities between deduced amino acid sequences of *PiSLF* and *PiSLFL* genes

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<th>PiSLFLd-S2</th>
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CHAPTER 4

Identification of major lysine residues of S3-RNase of

*Petunia inflata* involved in ubiquitin-26S proteasome-mediated
degradation *in vitro*

The work described in this chapter has been accepted for publication in Plant Journal, 2008.
4.1 Introduction

As discussed in Chapter 1, S-RNase-based Self-incompatibility (SI) mechanism is controlled by the highly polymorphic S-locus. Self pollen, which carries an S-haplotype matching one of the two S-haplotypes carried by the diploid pistil, suffers growth arrest during its tube growth in the pistil, whereas non-self pollen, which carries an S-haplotype not present in the pistil, is accepted and its tube grows down through the style to effect fertilization in the ovary. How a pistil distinguishes between self and non-self pollen, and how this recognition results in growth inhibition of self pollen tubes have been very intensively studied over the past two decades. It is now known that two polymorphic genes at the S-locus, S-RNase and S-locus F-box (abbreviated SLF in the Solanaceae and Plantaginaceae, and SLF or SFB in the Rosaceae), encode the specificity of the pistil and pollen, respectively, in SI interactions (Lee et al., 1994; Murfett et al., 1994; Lai et al., 2002; Entani et al., 2003; Ushijima et al., 2003; Qiao et al., 2004b; Sijacic et al., 2004; Sonneveld et al., 2005; Tsukamoto et al., 2006).

S-RNase is produced in the transmitting cells of the pistil and secreted into the transmitting tract, and immunolocalization experiments have shown that S-RNase is taken up by both self and non-self pollen tubes in a non-S-haplotype-specific manner (Luu et al., 2000; Goldraij et al., 2006). It has also been shown that the RNase activity of S-RNase is required for its function in SI (Huang et al., 1994). Why self S-RNase, but not non-self S-RNase, can exert cytotoxic activity inside a pollen tube to elicit the SI response is still unclear. Two models have recently been proposed to address the specific growth inhibition of self pollen tubes.

One model proposes that, although both self and non-self S-RNases are taken up into the cytoplasm of a pollen tube as reported by Luu et al. (2000), all non-self S-RNases are degraded through the ubiquitin-26S proteasome pathway; therefore, only self S-RNase can function inside a pollen tube (Qiao et al., 2004a; Sijacic et al., 2004; Ushijima et al., 2004; Sonneveld et al., 2005; Sims, 2007, Chapter 2). AhSLF, the SLF of Antirrhinum hispanicum (Plantaginaceae), interacts with an atypical SKP1-like protein, named AhSSK1 (Huang et al., 2006), but not with any of the Arabidopsis SKP1-like proteins (ASKs). AhSSK1 also interacts with a Cullin 1-like protein, suggesting that it may bring AhSLF into an SCF-like complex which, in conjunction
with ubiquitin-activating enzyme (E1) and ubiquitin-conjugating enzyme (E2), targets S-RNase for ubiquitination and degradation by the 26S proteasome (Qiao et al., 2004a; Huang et al., 2006). In *Petunia inflata*, although no AhSSK1-like protein has been found to interact with PiSLF (*P. inflata* SLF) by yeast-two hybrid library screening, a RING-HC protein, named PiSBP1 (*P. inflata* S-RNase Binding Protein 1), was found to interact with PiSLF, PiCUL1-G (a Cullin 1-like protein) and S-RNases. PiSBP1 is a homologue of PhSBP1 (*P. hybrida* SBP1), which was identified by Sims and Ordanic (2001) as an S-RNase-interacting protein from yeast two-hybrid library screening. Moreover, neither SKP1-like proteins of *P. inflata* nor any ASK of Arabidopsis interact with PiSLF, suggesting that the PiSLF-containing complex may be a novel E3 ligase complex (Chapter 2). However, it is also possible, as suggested by Sims (2007), that an atypical SCF$^\text{SLF}$ E3-like complex, contains SLF, SBP1 (replacing RBX1), Cullin 1 and SSK1 (replacing SKP1), functions in S-RNase-based SI. Although whether an E3-like complex is indeed involved in this type of SI is yet to be determined, S-RNase has been clearly shown to be ubiquitinated and degraded by the 26S proteasome in pollen tube extracts, albeit not in an S-haplotype-specific manner (Chapter 2). Moreover, *in vitro* binding assays have revealed that non-self interactions between PiSLF and S-RNase are stronger than self interactions, suggesting that this preferential interaction may allow the putative PiSLF-containing E3-like complex to specifically mediate ubiquitination and degradation of non-self S-RNases (Chapter 2, Chapter 3).

The other model is based on immunolocalization of S-RNase in pollen tubes within compatibly- and incompatibly-pollinated pistils (Goldraij et al., 2006). It proposes that both self and non-self S-RNases taken up by a pollen tube are initially sequestered in a vacuolar compartment, and that the compartment remains intact in the case of compatible pollination, whereas the compartment is disrupted in the case of incompatible pollination, releasing the sequestered S-RNase (both self and non-self) into the cytoplasm to inhibit the growth of self pollen tubes (Goldraij et al., 2006). This model also invokes a protein, HT-B, which is required for SI in *Nicotiana alata* (McClure et al., 1999) and *Solanum chacoense* (O'Brien et al., 2002). HT-B is produced in the pistil and taken up by the pollen tube in a non-S-specific manner, and its steady-state level is lower in compatible pollen tubes than in incompatible pollen tubes (Goldraij et al., 2006). Thus, the model further proposes that HT-B is required for the disruption of the
vacuolar compartment, and its degradation in compatible pollen tubes allows the compartment to remain intact.

The major difference between the two models discussed above lies in the predicted outcome of the S-haplotype-specific interaction between SLF and S-RNase in the cytoplasm. The model invoking the ubiquitin-26S proteasome-mediated degradation pathway predicts that non-self interactions, but not self interactions, result in ubiquitination and degradation of S-RNases, whereas the model invoking compartmentalization predicts that non-self interactions, but not self interactions, result in the degradation of HT-B (McClure, 2006).

A polyubiquitin chain is usually attached to the ε-amino group of a lysine residue in a substrate through an isopeptide bond with the C terminal glycine residue of ubiquitin (Smalle and Vierstra, 2004; Hochstrasser, 2006). The polyubiquitin chain serves as a recognition signal for the 26S proteasome to target the substrate for degradation (Thrower et al., 2000; Hochstrasser, 2006). If a lysine residue targeted for ubiquitination is changed to an arginine, which has similar biochemical properties but does not have an ε-amino group, the site will not be ubiquitinated. If all the lysines of a protein targeted for ubiquitination are changed to arginines, the protein will evade degradation by the 26S proteasome. For solanaceous S-RNases, Qin et al. (2005) showed that replacing a conserved lysine residue, located in the conserved region C4 (Ioerger et al., 1991), with arginine in S11-RNase of S. chacoense did not affect its function, ruling out the possibility that this lysine residue alone serves as the target for ubiquitination. Since S-RNase contains more lysine residues, e.g., S1-, S2- and S3-RNases of P. inflata contain 18, 19 and 20 lysine residues, respectively, a more comprehensive and systematic approach will be required to identify the target sites for ubiquitination. Thus, I set out to examine the role of each of the 20 lysine residues of S3-RNase in ubiquitination and degradation of S3-RNase using the cell-free system I previously developed (Chapter 2). I made a total of 24 mutant forms of GST:S3-RNase for the ubiquitination and degradation assays, and identified six lysine residues near the C-terminus that when changed to arginines significantly reduced ubiquitination and degradation of the mutant protein GST:S3-RNase (K141-164R).
The ubiquitin-26S proteasome pathway was also adopted by endoplasmic reticulum-associated protein degradation (ERAD) to remove mis-folded proteins in eukaryotic cells (Romisch, 2005; Meusser et al., 2005). To address the possibility that the observed degradation of GST:S3-RNase and its various mutant forms studied in this work might have been mediated by ERAD, I used an in-gel RNase activity assay to show that both purified GST:S3-RNase (K141-164R) and GST:S3-RNase had a similar RNase activity and thus were unlikely to be mis-folded.

Lastly, I examined whether PiSBP1 could act as an E3 to mediate ubiquitination of S-RNase. PiSBP1 is a potential component of the PiSLF-containing E3-like complex and it interacts with S-RNases in a non-S-haplotype-specific manner (Hua and Kao, 2006), as do PhSBP1 (Sims and Ordanic, 2001) and ScSBP1 of Solanum chacoense (O'Brien et al., 2004). I used in vitro reconstitution to demonstrate that PiSBP1, in conjunction with E1 and E2, could mediate ubiquitination of Strep:(His)6-tagged S3-RNase.

4.2 Methods

4.2.1 Generation of Constructs for Protein Expression

To generate all the constructs for the lysine mutants of S3-RNase, the coding sequence of mature S3-RNase (without the leader peptide) was cloned in phase behind the GST coding sequence in pGEX-5X-1 (GE Healthcare), and overlapping PCR was used to generate the constructs for the GST:S3-RNase mutants that contained one or more lysine-to-arginine changes (Table 4.1) using appropriate primers listed in Table 4.2. All the mutant constructs were confirmed by sequencing. The coding sequence for wheat UBA1 was cloned into pGEX-5X-1 in phase behind the GST coding sequence to generate pGEX-UBA1, the coding sequence of the mature S3-RNase was used to replace the coding sequence for yEsa1 in pST70Trc3-STR:(His)6:nyEsa1x3 (Song Tan, unpublished results) to generate pST70Trc3-STR:(His)6:S3-RNase. GST:PiSBP1, (His)6:T7:PhUBC1, and (His)6:Ub were described in Chapter 2.
4.2.2 Expression and Purification of Recombinant Proteins and Purification of Non-Glycosylated S3-RNase

GST:S3-RNase and its mutants, GST:UBA1, GST:PiSBP1, Strep:(His)6:S3-RNase, (His)6:T7:PhUBC1, and (His)6:Ub were expressed in E. coli and purified as described in Chapter 2. A transgenic P. inflata plant of S2S2 genotype carrying a mutant S3-RNase gene with the codon for asparagine-29 replaced with a codon for aspartic acid was used for the purification of non-glycosylated S3-RNase as described in Karunanandaa et al. (1994).

4.2.3 In Vitro Degradation and Ubiquitination Assays

The in vitro degradation and ubiquitination assays in S2 pollen tube extracts using GST:S3-RNase and its mutants as substrates were performed as described in Chapter 2. The in vitro assay for ubiquitination of Strep:(His)6:S3-RNase was performed in the same reaction buffer except for the use of purified GST:UBA1 (E1), (His)6:T7:PhUBC1 (E2), GST:PiSBP1 (E3) and (His)6:Ub.

4.2.4 In-gel RNase Activity Assay

GST:S3-RNase and GST:S3-RNase (K141-164R) were expressed and bound to Glutathione Sepharose beads as described in Chapter 2. The proteins were eluted at 95°C in 2×SDS sample buffer without 1% β-mercaptoethanol for 2-3 min, and the eluted proteins were separated by 10% SDS-polyacrylamide gels containing 300 μg/ml of torula yeast RNA. The RNase activity assay was performed as described by Blank et al. (1982).

4.2.5 Protein Gel Blot Analysis

Proteins were separated by 10% SDS-polyacrylamide gels under reducing or non-reducing conditions (i.e., with or without 1% β-mercaptoethanol in the sample buffer), and then blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore). The primary antibodies used were an anti-(His)6 antibody (1:2,000; Novagen), an anti-GST antibody (1:2000; Oncogene Research Products), an anti-Strep antibody (1:2000; Qiagen), and an affinity-purified anti-S3-
RNase antibody (1:1500) prepared as described in Chapter 2. The immunoreactive proteins were visualized as described in Chapter 2.

4.3 Results

4.3.1 C-terminal Lysine Residues Play a Major Role in Targeting Degradation of S₃-RNase in Pollen Tube Extracts

I previously used GST-tagged S-RNases, expressed in *Escherichia coli*, for ubiquitination and degradation assays in pollen tube extracts, and the results showed that both self and non-self S-RNases were ubiquitinated and degraded via the 26S proteasome pathway (Chapter 2). To determine whether any of the 20 lysine residues in S₃-RNase might be ubiquitinated, I carried out site-directed mutagenesis on GST:S₃-RNase to examine the role, if any, of each lysine residue in this biochemical process. The PCR primers used in making 24 mutant constructs are listed in Table 4.2, and these mutant forms of GST:S₃-RNase (listed in Table 4.1), having one or more lysines replaced with arginines, were examined in the *in vitro* degradation assay.

I first focused on the N-terminal two-thirds of S₃-RNase that contains 12 of the 20 lysine residues (K20 to K133; Figure 4.1). I generated 17 mutant constructs of GST:S₃-RNase by replacing one or more of the lysine codons with CGT for arginine, and produced six single-lysine mutants (K20R, K39R, K51R, K73R, K92R, and K133R), three double-lysine mutants (K51R, K58R; K73R, K77R; and K114R, K116R), two quadruple-lysine mutants (K51-77R, and K73-82R), one quintuple-lysine mutant (K51R, K73-82R), one sextuple-lysine mutant (K51-82R), and four multiple-lysine mutants that contain 7, 9, 11, and 12 lysine-arginine mutations, respectively (see Table S2 for the names of these four mutants and the specific lysine residues mutated). All these GST:S₃-RNase mutants were compared with GST:S₃-RNase for the extent of degradation after 1 hr incubation in *S₂* pollen tube extracts. All the 17 lysine residues when mutated singly, or in combination with one or more of the others, had either no effect or only modest effect on the degradation of GST:S₃-RNase. The results of GST:S₃-RNase (K114R, K116R), GST:S₃-RNase (K20-92R) and GST:S₃-RNase (K20-133R) are shown in Figure 4.2.
I next examined the role of the eight lysine residues (K141, 144, 150, 155, 161, 164, 167, 196) in the C-terminal one-third of S3-RNase by progressively introducing two or more lysine-to-arginine changes to the GST:S3-RNase (K20-133R) construct to make six additional mutant constructs (Table 4.1). Degradation of GST:S3-RNase (K20-144R), GST:S3-RNase (K20-150R), and GST:S3-RNase (K20-155R) were modestly affected (results not shown), whereas degradation of GST:S3-RNase (K20-164R), GST:S3-RNase (K20-167R), and GST:S3-RNase (K20-196R) are significantly affected to similar extents (Figure 4.2). Since additional replacement of K167 by arginine in GST:S3-RNase (K20-167R), and additional replacement of both K167 and K196 by arginines in GST:S3-RNase (K20-169R), did not result in further reduction in the extent of degradation, I infer that the last two lysine residues, K167 and K196, may not significantly contribute to the instability of GST:S3-RNase.

The results of the degradation assay mentioned above suggested that different lysine residues might contribute differently to the instability of S3-RNase. I reasoned that if ubiquitination indeed determines the fate of S-RNase in pollen tubes, the lysine residues that play a major role in this process would likely be among those that are more highly conserved among S-RNases. To examine this possibility, I aligned the deduced amino acid sequences of 17 S-RNase alleles, including S3-RNase, of P. inflata (Wang et al., 2001), along with the deduced amino acid sequences of 15 S-RNase alleles of other solanaceous species, six of N. alata (S2-, S3-, S6-, S42-, SCl6R-, and Sf11), four of P. hybrida (S1-, S3-, S1X- and SB1), and five of S. chacoense (S2-, S3-, S11-, S12-, and S114), and counted the number of S-RNase sequences that contain each of the 20 lysine residues of S3-RNase. The results are graphically shown in Figure 4.3. The 20 lysine residues were distributed throughout S3-RNase, and six (K51, K92, K114, K116, K144, and K155) were present in more than half of the sequences compared, with all but K51 and K92 located in the C-terminal half of the protein. Five of these most highly conserved lysine residues were clustered in two regions: K92, K114 and K116 were located in the middle part of the protein, and K144 and K155 (plus K161 which was present in 14 of the 32 sequences compared) were located in the C-terminal one-third of the protein. Since GST:S3-RNase (K20-133R) contained lysine-to-arginine mutations of K92, K114 and K116, and since degradation of this mutant was not significantly affected (Figure 4.2), these three highly conserved lysine residues might not play a major role, if any, in the degradation of S-RNase. However, introducing six additional lysine-
to-arginine mutations (K141R, K144R, K150R, K155R, K161R and K164R) to GST:S3-RNase (K20-133R) significantly reduced the extent of degradation of the resulting mutant protein, GST:S3-RNase (K20-164R) (Figure 4.2). Thus, K144, K155 and/or some of the other lysines in this region may play a major role in the degradation of S3-RNase.

I thus further examined the role of all these six lysine residues by making the GST:S3-RNase (K141-164R) construct and comparing the rate of degradation of GST:S3-RNase (K141-164R) with those of GST:S3-RNase (K20-133R) and GST:S3-RNase (Figure 4.4a and b). After 10 min incubation in S2 pollen tube extracts, ~62% of the input GST:S3-RNase and ~59% of the input GST:S3-RNase (K20-133R) were degraded, whereas ~44% of the input GST:S3-RNase (K141-164R) was degraded. After 20 min incubation, the percentage of the input protein that remained for GST:S3-RNase (K141-164R) was nearly twice that for GST:S3-RNase and GST:S3-RNase (K20-133R). These results further support the notion that these six C-terminal lysine residues (K141, 144, 150, 155, 161, 164) are more important than the other lysine residues in controlling the degradation of S3-RNase in vitro.

To further establish the role of these residues in the degradation of S3-RNase, I compared the degradation of GST:S3-RNase (K141-164R) and GST:S3-RNase in S2 pollen tube extracts over an 80-min period. An anti-GST antibody was used to detect each protein that remained after 20, 40, 60 and 80 min incubations (left panel of Figure 4.4c). Approximately 92% of the input GST:S3-RNase was degraded after 20 min incubation and more than 96% was degraded after 40 min incubation. In contrast, only ~65% of the input GST:S3-RNase (K141-164R) was degraded after 20 min incubation, and ~17% of the input protein still remained after 40 min incubation. At the end of 80 min incubation, ~3% of the input GST:S3-RNase (K141-164R) remained, and ~1% of the input GST:S3-RNase remained. To rule out the possibility that the anti-GST antibody might react with GST:S3-RNase (K141-164R) and GST:S3-RNase differently, I also used an anti-S3-RNase antibody, raised against a synthetic peptide of HVa that is present in both GST:S3-RNase and GST:S3-RNase (K141-164R) (Figure 4.1, Chapter 2), to detect each protein in a separate assay (right panel of Figure 4.4c). Similar to the results obtained with the use of the anti-GST antibody, degradation of GST:S3-RNase (K141-164R) was slower than that of
GST:S3-RNase (e.g., 21% of the input GST:S3-RNase [K141-164R] remained compared with ~7% of the input GST:S3-RNase remaining after 40 min incubation).

4.3.2 Reduction in the Rate of Degradation of GST:S3-RNase (K141-164R) is Correlated with Reduction in Ubiquitination

To examine whether replacement of the six lysine residues in GST:S3-RNase (K141-164R) affected the rate of protein degradation by reducing the extent of ubiquitination, I carried out a ubiquitination assay (Chapter 2) using similar amounts of GST:S3-RNase, GST:S3-RNase (K141-164R), and GST:S3-RNase (K20-133R) in S2 pollen tube extracts containing (His)6 ubiquitin. Since numerous pollen proteins were ubiquitinated in this assay (Chapter 2), I further purified the ubiquitinated GST-tagged proteins from the reaction mixture by Glutathione Sepharose 4 Fast Flow resin.

Consistent with my previous finding with GST:S3-RNase (Chapter 2), strong discrete bands with molecular masses higher than that of GST:S3-RNase were detected by an anti-(His)6 antibody after 5 min incubation, and these bands became more prominent after 10 min incubation (Figure 4.5a). These results suggest that GST:S3-RNase was ubiquitinated. For GST:S3-RNase (K141-164R), very faint bands corresponding to the discrete bands observed with GST:S3-RNase were detectable only after 10 min incubation (Figure 4.5a), suggesting that the extent of ubiquitination of this protein was significantly reduced. Thus, replacing the six lysines at the C-terminus with arginines significantly affected the ability of GST:S3-RNase to be ubiquitinated; this is consistent with the reduced rate of degradation of GST:S3-RNase (K141-164R). For GST:S3-RNase (K20-133R), the intensity of the bands corresponding to the discrete bands observed with GST:S3-RNase were stronger than that of GST:S3-RNase (K141-164R), consistent with the notion that the lysine residues in the C-terminal one-third of S3-RNase are the major ubiquitination sites. However, since ubiquitination of GST:S3-RNase (K141-164R) was not completely abolished and since GST:S3-RNase (K20-133R) did not appear to be ubiquitinated to the same extent as GST:S3-RNase, some of the 12 lysine residues in the N-terminal two-thirds of S3-RNase might also serve as ubiquitination site(s). The polyubiquitin chains attached there might not be as efficient as those attached to the C-terminal six lysine

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residues in targeting the protein for degradation by the 26S proteasome. This would be consistent with the suggestion by Petroski and Deshaies (2003) that the context of a lysine residue could affect the attachment of a polyubiquitin chain by E3, and/or the efficiency of the 26S proteasome in unfolding the ubiquitinated substrate for degradation.

To confirm that the degradation of GST:S3-RNase (K141-164R) and GST:S3-RNase (K20-133R) in S2 pollen tube extracts was mediated by the 26S proteasome, as is the case for GST:S3-RNase (Hua and Kao, 2006), I performed a degradation assay in the presence or absence of MG132, a 26S proteasome inhibitor. In order for the degradation to be clearly observed, similar amounts of GST:S3-RNase, GST:S3-RNase (K141-164R) and GST:S3-RNase (K20-133R) used in the reactions were approximately one-fifth those of the respective proteins used in the assays shown in Figure 4.4c and Figure 4.5a. I used both the anti-GST antibody (upper panel of Figure 4.5b) and the anti-S3-RNase antibody (lower panel of Figure 4.5b) to detect the proteins. The results with the anti-GST antibody show that, after 60 min incubation, all three GST-tagged S3-RNases (indicated with an arrowhead) were degraded to similar extents in S2 pollen tube extracts and that the degradation was completely inhibited in the presence of MG132. These results suggest that the degradation of GST:S3-RNase (K141-164R), albeit slower than that of GST:S3-RNase, was also mediated by the 26S proteasome. For GST:S3-RNase and GST:S3-RNase (K141-164R), similar results were obtained with the anti-S3-RNase antibody. However, this antibody did not cross-react with GST:S3-RNase (K20-133R), most likely because the 15-amino-acid peptide (see Figure 4.1) against which the antibody was raised contains two of the lysines (K51 and K58) that were changed to arginines in GST:S3-RNase (K20-133R).

Taking together the results of the ubiquitination and degradation assays, I concluded that (1) the six lysine residues mutated in GST:S3-RNase (K161-164R) are likely the major sites of ubiquitination in S3-RNase; (2) absence of these lysine residues reduces, but does not completely block, ubiquitination of GST:S3-RNase (K141-164R) in S2 pollen tube extracts; (3) ubiquitination allows S3-RNase to be recognized and degraded by the 26S proteasome.
4.3.3 *E. coli*-Expressed GST:S₃-RNase and GST:S₃-RNase (K141-164R) Have RNase Activity

In eukaryotes, various endoplasmic reticulum-associated protein degradation (ERAD) pathways remove mis-folded proteins via ubiquitination and the 26S proteasome (Romisch, 2005; Meusser *et al.*, 2005). Thus, to rule out the possibility that the ubiquitin-26S proteasome-dependent degradation of GST:S₃-RNase and its various lysine-to-arginine mutants was caused by ERAD of the abnormally folded GST-tagged proteins, I assessed the RNase activity, a key biochemical property of S-RNase, of GST:S₃-RNase and GST:S₃-RNase (K141-164R).

I used an in-gel RNase activity assay (Blank *et al.*, 1982) to ascertain whether GST:S₃-RNase and GST:S₃-RNase (K141-164R) were able to degrade RNA. Since S-RNases contain intramolecular disulfide bonds, which are required for their activity, β-mercaptoethanol (1%) or DTT (100 mM) normally used in denaturing SDS-polyacrylamide gels was not included in the sample buffer. Two different amounts of each protein were used in the assay, and in all the sample lanes, a blank band of similar mobility was observed after the gel was stained for RNA with toluidine blue (indicated with a gray arrow in Figure 4.6a), indicating that the RNA imbedded in the gel was degraded by an RNase which had migrated to this position. To determine whether GST:S₃-RNase and GST:S₃-RNase (K141-164R) were responsible for the degradation of RNA in their respective gel lanes, approximately one-third the amount of GST:S₃-RNase or GST:S₃-RNase (K141-164R) used in Figure 4.6a was similarly electrophoresed and immunoblotted with the anti-S₃-RNase antibody. A protein band with a similar mobility as that of the blank band observed in Figure 4.6a was detected (marked with a gray arrow in Figure 4.6b). In addition, several extra bands, including a major one (marked with a black arrow in Figure 4.6b), were also detected. The lower intensity of the bands detected by the anti-S₃-RNase antibody in the GST:S₃-RNase (K141-164R) lanes compared to the bands detected in the corresponding GST:S₃-RNase lanes (Figure 4.6b) suggested that the amount of GST:S₃-RNase (K141-164R) used for the RNase activity assay was less than that of GST:S₃-RNase, consistent with the weaker RNase activity band observed for the former in Figure 4.6a.
Under non-reducing conditions, GST and GST fusion proteins are known to form homodimers through disulfide bonds (Parker et al., 1990; Ji et al., 1992; Maru et al., 1996). Since the molecular mass of a GST dimer (58 kD) is close to that of GST:S3-RNase (50 kD), the band showing the RNase activity in Figure 4.6a could be a GST dimer, formed after the GST tag had been cleaved from the GST fused S-RNases. Consequently, the S3-RNase or S3-RNase (K141-164R) released might also form a dimer. Since the molecular mass of an S3-RNase or S3-RNase (K141-164R) dimer is also close to that of GST:S3-RNase or GST:S3-RNase (K141-164R), the RNase activity band in each sample could also be contributed by the dimeric form of S3-RNase or S3-RNase (K141-164R). To rule out these possibilities, I performed an in-gel RNase activity assay of GST:S3-RNase (using the same preparation as used in Figure 4.6a and b, but having been kept at 4°C for two extra days in order to generate more free S3-RNase from proteolytic cleavage), non-glycosylated S3-RNase, and GST (Figure 4.6c). The non-glycosylated S3-RNase was purified from pistils of a transgenic S2S2 plant that carried a mutant S3-RNase gene with the codon for asparagine-29 replaced by a codon for aspartic acid (Karunananda et al., 1994). Two RNase activity bands were detected in both GST:S3-RNase samples. The stronger upper band was attributed to GST:S3-RNase as it had a similar mobility as the lower major band detected by the anti-S3-RNase antibody in Figure 4.6b. The weaker bottom band was attributed to free S3-RNase, as it had a similar mobility as the RNase activity band (indicated with an open arrow) detected in the non-glycosylated S3-RNase sample. No RNase activity band was detected in the GST samples. Since proteins stained weakly with toluidine blue, the significant amounts of GST and free GST released from GST:S3-RNase were detected (marked with arrowheads). A protein band (indicated with a black arrow) with a higher molecular mass than that of GST:S3-RNase was also detected in the GST:S3-RNase samples, and I attributed it to dimeric GST:S3-RNase. Finally, in this assay, no dimeric S3-RNase with RNase activity could be detected in the non-glycosylated S3-RNase sample. Taking together all the results shown in Figure 4.6, I concluded that the monomeric, but not the dimeric forms, of GST:S3-RNase and GST:S3-RNase (K141-164R) had RNase activity, suggesting that these two proteins were unlikely mis-folded and degraded via ERAD.
4.3.4 An E3 Ubiquitin-Ligating Enzyme Targets Ubiquitination of S-RNase

Since PiSBP1, a RING-HC protein, interacted with S-RNases in a non-S-haplotype-specific manner and with PhUBC1, an E2-like protein of *P. hybrida* (Hua and Kao, 2006), I examined whether PiSBP1 by itself might act as an E3, and, in conjunction with E1 and E2, mediate ubiquitination of S3-RNase. I used GST-tagged ubiquitin-association 1 of wheat (GST:UBA1) as E1 (Hatfield *et al.*, 1990), (His)6:T7:PhUBC1 as E2, GST:PiSBP1, Strep:(His)6:S3-RNase as substrate, and a (His)6-tagged ubiquitin ([His]6:Ub). All these recombinant proteins were expressed in *E. coli*, purified and used for the ubiquitination assay. An anti-(His)6 antibody was first used to detect any ubiquitinated Strep:(His)6:S3-RNase by protein gel blotting (Figure 4.7a). When all the proteins were included in the reaction, strong discrete bands having molecular masses higher than that of Strep:(His)6:S3-RNase (~28 kD) were detected (Lanes 6 and 7). These bands were not detected in the reactions without (His)6:Ub (lane 1), GST:PiSBP1 (lane 3), (His)6:T7:PhUBC1 (lane 4), or GST:UBA1 (lane 5), suggesting that the discrete bands were ubiquitin-modified proteins and that GST:PiSBP1 functioned as an E3. Some discrete bands were also detected in the reaction without Strep:(His)6:S3-RNase (Lane 2). Since E2 and E3 may be autoubiquitinated, particularly when the substrates of E3 are absent (Hochstrasser, 2006), these bands could result from autoubiquitination of (His)6:T7:PhUBC1 and GST:PiSBP1.

To confirm that at least some of the bands detected in lanes 6 and 7, but not in lane 2, corresponded to ubiquitinated Strep:(His)6:S3-RNase, duplicate samples from all the reactions carried out in Figure 4.7a were immunoblotted with an anti-Strep tag antibody specific to Strep:(His)6:S3-RNase (Figure 4.7b). Two distinct bands (marked with triangles) similar in mobility to two of the discrete bands detected in lanes 6 and 7 of Figure 4.7a were detected only when all the components were present in the reaction (lanes 6 and 7 of Figure 4.7b), confirming that Strep:(His)6:S3-RNase was ubiquitinated. Taking together the results shown in Figure 4.7, I concluded that PiSBP1 can mediate ubiquitination of S3-RNase *in vitro*. 
4.4 Discussion

Any model for the biochemical mechanism of S-RNase-based SI must address how the growth of a pollen tube is specifically affected by self S-RNase but not by any non-self S-RNase, as uptake of S-RNase into pollen tubes is not S-haplotype-specific (Luu et al., 2000; Goldraij et al., 2006). One current model, based on the assumption that SLF functions as a conventional F-box protein, hypothesizes that the mechanism involves ubiquitin-26S proteasome-dependent degradation of S-RNase (Qiao et al., 2004a; Sijacic et al., 2004; Ushijima et al., 2004, Sonneveld et al., 2005; Sims, 2007, Chapter 2).

One way to test this model would be to compare the levels of S-RNase between pollen tubes in compatibly- and incompatibly-pollinated pistils, as one would expect to see less S-RNase molecules in the former than in the latter. However, since S-RNase is highly abundant in the transmitting tract of the style, accounting for 1 to 10% of the total pistil protein (Roalson and McCubbin, 2003), it is difficult, if not impossible, to detect any difference in the level of S-RNase between compatible and incompatible pollen tubes within the high background. Goldraij et al. (2006) circumvented this problem by examining the S-RNase level in pollen tubes isolated from compatibly- and incompatibly-pollinated pistils, and they found no significant difference. Moreover, Goldraij et al. (2006) found that most S-RNase molecules remained compartmentalized in a compatible pollen tube, whereas most S-RNase molecules are initially sequestered but released into the cytoplasm at later stages of pollination in an incompatible pollen tube. Thus, even if the small number of S-RNase molecules in the cytoplasm of a compatible pollen tube are degraded, the resulting difference in the total amount of S-RNase between compatible and incompatible pollen tubes might not be clearly detected. Two additional factors may further complicate the interpretation of the results from the two approaches discussed above. First, the amount of S-RNase in the pistil varies substantially (up to 20-fold) from flower to flower even in the same plant (Qin et al., 2006). Second, during pollen tube growth in the style, uptake of S-RNase presumably is a continuous process, and thus there may be a steady-state level of S-RNase, even if degradation does occur.
To overcome the potential problems mentioned above, I previously established a cell-free system using pollen tube extracts and purified recombinant and native S-RNases to show that S-RNase was ubiquitinated and degraded by the 26S proteasome in pollen tube extracts (Chapter 2). This *in vitro* system may not completely mimic the *in vivo* situations as both self and non-self S-RNases are degraded. However, additional results supporting the model have been obtained. First, a RING-HC protein, SBP1, was identified to interact with S-RNase in *P. hybrida*, *S. chacoense*, and *P. inflata* (Sims and Ordanic, 2001; O'Brien et al., 2004; Chapter 2). Second, AhSLF and PiSLF interact with S-RNase of their respective species, and with SSK1 and SBP1, respectively, potential components of the putative SLF-containing E3 ligase complex (Huang et al., 2006; Sims, 2007; Chapter 2). Third, non-self interactions between PiSLF and S-RNase are stronger than self interactions (e.g., PiSLF2 interacts with S1-RNase and S3-RNase more strongly than with S2-RNase), suggesting that the PiSLF-containing E3-like complex could specifically mediate ubiquitination and degradation of non-self S-RNases, allowing only self S-RNase to exert cytotoxic action (Chapter 2, Chapter 3).

However, before the SLF-containing E3 complex is reconstituted and shown to have the predicted biochemical property, it remains possible that the ubiquitination and degradation of S-RNase observed in pollen tube extracts is regulated by a general proteolytic machinery, ERAD, which utilizes the 26S proteasome to degrade mis-folded proteins (Romisch, 2005; Meusser et al., 2005). In this work, I have further provided evidence to suggest that ERAD was not likely responsible for ubiquitination and degradation of S-RNase. First, I identified six lysine residues (K141, 144, 150, 155, 161, 164) near the C-terminal one-third of S3-RNase (Figure 4.1) as the major sites for ubiquitination, because when they were changed to arginines, the ubiquitination and degradation of the mutant S3-RNase, GST:S3-RNase (K141-164R), in pollen tube extracts were significantly reduced (Figure 4.2, Figure 4.4 and Figure 4.5a). If GST:S3-RNase were regulated by ERAD due to its mis-folding, one would expect that additional lysine residues in the N-terminal two-thirds of S3-RNase would play a similar role in its degradation as the six lysine residues near the C-terminus. Second, I showed that GST S3-RNase (K141-164R) had similar RNase activity as GST S3-RNase (Figure 4.6a), further suggesting that neither protein is mis-folded. Third, I showed that PiSBP1 acts as an E3 targeting S3-RNase for ubiquitination *in vitro* (Figure 4.7). PiSBP1 shows only 4 and 9% amino acid sequence identity with Hrd1p and
Doa10p, respectively, the only major ERAD-related E3s identified so far in yeast (Romisch, 2005), suggesting this ubiquitination is not likely related to ERAD. Moreover, since PiSBP1 is the RING-HC subunit of the PiSLF-containing E3-like complex, the finding that it has an E3 activity would also suggest that ubiquitination and degradation of S-RNase are mediated by this E3-like complex and not by ERAD in vivo.

In conclusion, this work has provided further support for our previous conclusion that S-RNase is degraded in pollen tube extracts via a specific ubiquitin-26S proteasome pathway (Chapter 2; Chapter 3). PiSLF may be responsible for specific degradation of non-self S-RNases in the pollen tube through the stronger non-self interactions between PiSLF and S-RNase (Chapter 2). A future challenge will be to reconstitute the PiSLF-containing E3-like complex in vitro and ascertain whether this complex mediates specific ubiquitination and degradation of non-self S-RNases. Ultimately, whether control of degradation of S-RNase is an integral part of the S-RNase-based SI mechanism will have to be investigated using in vivo approaches.

4.5 Accession Numbers

The accession numbers of the sequence data used in this chapter are S3-RNase (AAA33727) and PiSBP1 (ABB77434) of P. inflata; S2-RNase (AAB40027), S3-RNase (AAB07492), S6-RNase (AAB40028), S2-RNase (AAA87045), S10-RNase (AAA87046), and S11-RNase (Q7SID5) of N. alata; S1-RNase (AAA60465), S3-RNase (AAA60466) Sx-protein (AAA33729), and SB1-RNase (BAA76513) of P. hybrida; S2-RNase (CAA40216), S3-RNase (CAA40217), S11-RNase (AAA50306), S12-RNase (AAD56217), and S14-RNase (AAF36980) of S. chacoense.
Figure 4.1 Deduced Amino Acid Sequence of Mature S₃-RNase of *P. inflata*.

C1, C2, C3, C4 and C5 indicate the five conserved regions, and HVa and HVb indicate the two hypervariable regions (Ioerger *et al.*, 1991). The 20 lysine residues are shaded in gray, and the residue number of each lysine is shown.
Figure 4.2 Protein Gel Blot Analysis of Degradation of GST:S₃-RNase and Representative Lysine Mutants in S₂ Pollen Tube Extracts.

Approximately 0.3 μg each of the purified GST:S₃-RNase and six lysine-to-arginine mutants, as indicated, were separately incubated in S₂ pollen tube extracts for 1 h at 30°C. An anti-GST antibody was used to detect the GST fusion proteins in the degradation assay (top panel) and the input control (bottom panel). The single asterisk indicates a cross-reacting band due to long exposure of the immunoblot necessary to visualize the low intensity of the undegraded protein bands in the control and some of the mutants. The arrow indicates the undegraded GST-fused S₃-RNase and its lysine-to-arginine mutants.
Figure 4.3 Graphic Representation of the Degree of Conservation of the 20 Lysine Residues of S$_3$-RNase among 32 Solanaceous S-RNases.

The number of 32 S-RNases of various solanaceous species that contain each of the 20 lysine residues of S$_3$-RNase was plotted against the lysine residues. The broken line represents half of the 32 S-RNases that contain the lysine residues indicated. The lysine residues located in the HVa, HVb, C3 and C4 regions are marked.
Figure 4.4 Protein gel blot analysis of the effect of the lysine-to-arginine changes on degradation of GST:S3-RNase (K141-164R) and GST:S3-RNase (K20-133R) in S2 pollen tube extracts.

(A) Approximately 1 μg each of purified GST:S3-RNase, GST:S3-RNase (K141-164R) and GST:S3-RNase (K20-133R) were separately incubated in S2 pollen tube extracts for 10 and 20 min at 30°C. Two independent experiments, indicated as Repeat 1 and Repeat 2, were performed. An anti-GST antibody was used to detect the undegraded GST fusion proteins.
(B) Quantification of the undegraded GST:S-RNases shown in (A). The intensity of each protein band was quantified by ImageQuant5.2 (GE Healthcare), and the relative undegraded protein was calculated as the percentage of the total input amount.

(C) Comparison of the extent of degradation of GST:S3-RNase and GST:S3-RNase (K141-164R) in S2 pollen tube extracts after different lengths of incubations at 30°C. An anti-GST antibody (left panel) and an anti-S3-RNase antibody (right panel) were used to detect the undegraded GST fusion proteins. The gray arrows indicate GST:S3-RNase (K141-164R), and the dark arrows indicate GST:S3-RNase. The single and double asterisks indicate protein bands that cross-reacted with the anti-GST antibody and the anti-S3-RNase antibody, respectively.
Figure 4.5 Protein Gel Blot Analysis of Ubiquitination and Degradation of GST:S3-RNase, GST:S3-RNase (K141-164R) and GST:S3-RNase (K20-133R) in S2 Pollen Tube Extracts.

(A) Ubiquitination of GST:S3-RNase, GST:S3-RNase (K141-164R) and GST:S3-RNase (K20-133R) after 5 and 10 min incubation in the presence of (His)_6 ubiquitin. An anti-(His)_6 antibody was used to detect the ubiquitinated GST-tagged proteins. The arrow indicates the
non-ubiquitinated GST-tagged proteins, which cross-reacted with the anti-(His)$_6$ antibody due to the large amount of loading.

(B) Degradation of GST:S$_3$-RNase, GST:S$_3$-RNase (K141-164R) and GST:S$_3$-RNase (K20-133R) in the absence or presence of 40 μM MG132. The arrows indicate the undegraded GST-tagged proteins detected by an anti-GST antibody (upper panel) or an anti-S$_3$-RNase antibody (lower panel). The single asterisk indicates GST; the double asterisks indicate a protein band that cross-reacted with the anti-S$_3$-RNase antibody. The triangles indicate possible degradation products of GST:S$_3$-RNase and GST:S$_3$-RNase (K141-164R).
Figure 4.6 In-Gel RNase Activity Assay of GST:S₃-RNase and GST:S₃-RNase (K141-164R).

(A) RNase activity gel. The gray arrow indicates the RNase-activity bands of GST:S₃-RNase or GST:S₃-RNase (K141-164R). The arrowheads indicate the GST dimer stained by toluidine blue.

(B) Approximately one third the amount of GST:S₃-RNase and GST:S₃-RNase (K141-164R) used in (A) were separated by non-reducing SDS-polyacrylamide gels and immunoblotted with an anti-S₃-RNase antibody. The gray arrow indicates monomeric GST:S₃-RNase and GST:S₃-RNase (K141-164R), and the dark arrow indicates dimeric GST:S₃-RNase and GST:S₃-RNase (K141-164R).

(C) RNase activity gel showing that the monomer, but not the dimer, of GST:S₃-RNase has RNase activity. The gray arrow indicates monomeric GST:S₃-RNase, the dark arrow indicates dimeric GST:S₃-RNase, and the blank arrow indicates non-glycosylated S₃-RNase. The arrowheads indicate the GST dimer stained by toluidine blue.
Figure 4.7 Assay for E3 Ubiquitin Ligase Activity of PiSBP1.

GST:PiSBP1 was assayed for its ability to ubiquitinate Strep:(His)$_6$:S$_3$:RNase in the presence of GST:UBA1 (E1), (His)$_6$:T7:PhUBC1 (E2), and (His)$_6$:ubiquitin (lanes 6 and 7). The reactions with one of the five components absent were carried out as controls (lanes 1 to 5). The samples were immunblotted with an anti-(His)$_6$ antibody (A) or an anti-Strep antibody (B). The bands corresponding to the ubiquitinated Strep:(His)$_6$:S$_3$:RNase are indicated by a vertical line in (A) and by small triangles in (B). Single asterisks indicate Strep:(His)$_6$:S$_3$:RNase; the double asterisks indicate (His)$_6$:T7:PhUBC1; the triple asterisks indicate (His)$_6$:ubiquitin. The blot in (B) was exposed five times longer than the blot in (A). The reason that more bands were detected in (A) than in (B) might be because the anti-(His)$_6$ antibody was more sensitive than the anti-Strep antibody.
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### Table 4.2 List of primers for site-directed mutagenesis of \( \text{GST:S}_3\text{-RNase} \)

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CHAPTER 5

New Biochemical Model for S-RNase-Based SI and Future Perspectives
5.1 Introduction

As addressed in Chapter 1, prior to the identification of SLF/SFB as the pollen S-gene, several models had been proposed to explain the biochemical mechanism of S-RNase-based SI, but all of them are either unable to explain competitive interaction, a well-documented genetic phenomenon in S-RNase-based SI, or lack biochemical evidence to support the model. During my Ph.D. thesis research, another model was presented by Goldraij et al. (2006), based on the observation of cellular localization of S-RNase in the pollen tube growing in a compatibly or incompatibly pollinated pistil. This model was subsequently significantly revised (McClure, 2006), but without any additional supporting data. In this chapter, I first review this new model and its revised version, and then discuss why neither can explain both the genetics of SI and the breakdown of SI in the competitive interaction situation. Therefore, to further understand the S-RNase-based SI mechanism, I propose a new biochemical model based on the results I have obtained from my thesis research (Chapter 2 to Chapter 4). In the second part of this chapter, I discuss the status of several ongoing projects in the lab that I have developed during my research, and outline future directions for each project.

5.2 Sequestration Model for S-RNase-based SI

Goldraij et al. (2006) recently performed a more detailed analysis of the localization of S-RNases in *in vivo* grown pollen tubes than did Luu et al. (2000). The results showed that in *Nicotiana alata*, S-RNases were taken into pollen tubes after both compatible (cross) and incompatible (self) pollinations, and most, if not all, S-RNase molecules were initially (up to 16 hr post-pollination) sequestered in a vacuolar compartment of the pollen tube. Later on (36 hr post-pollination), the S-RNase molecules remained sequestered in the compartment after compatible pollination, but were released into the cytoplasm of the pollen tube after incompatible pollination as a result of the disruption of the compartment. Based on these results, Goldraij et al. (2006) presented a model to explain why S-RNase has a specific cytotoxic effect in self pollen tubes, resulting in their growth inhibition, but not in non-self pollen tubes. The model predicts that the sequestration of S-RNases in a compatible pollen tube, rather than the degradation of S-RNase, allows the pollen tube to grow through the style for pollination, and that the disruption of the S-
RNase-containing compartment in an incompatible pollen tube releases S-RNases into the cytoplasm to result in growth inhibition of the pollen tube (Goldraij et al., 2006; McClure, 2006).

Using immunolocalization and immuno-blot analysis, Goldraij et al. (2006) further observed that a non-S-specific protein, named HT-B, appeared to be preferentially degraded in the compatible pollen tube. Since expression of HT-B in the pistil is required for SI (McClure et al., 1999) and since the S-RNase-containing compartment is maintained in the pollen tube of transgenic plants expressing an antisense HT-B gene (Goldraij et al., 2006), it was concluded that degradation of HT-B is required for maintaining the sequestration of S-RNases in the compatible pollen tube (Goldraij et al., 2006; McClure, 2006). However, neither the cause-and-effect relationship between the presence of HT-B and the disruption of the compartment was established, nor was any biochemical evidence presented to support the notion that HT-B is preferentially degraded in the compatible pollen tube. Goldraij et al. (2006) further invoked a hypothetical pollen protein (PP, possibly a protease) responsible for the degradation of HT-B. Whether there is such a protease to modulate the stability of HT-B and whether the specific interaction between SLF and S-RNase regulates the function of this "protease" remain to be experimentally demonstrated.

The timing of the disruption of the S-RNase-containing compartment seems to correlate with morphological changes of incompatible pollen tubes in a style (Goldraij et al., 2006). However, this disruption event does not seem to correlate with the timing of rRNA degradation, presumably caused by the cytoplasmically-located S-RNase, in incompatible pollen tubes within a style (McClure et al., 1990). McClure et al. (1990) used metabolic labeling to uniformly label pollen rRNAs with 32P, and used the radiolabeled pollen to pollinate compatible and incompatible pistils. In theory, if no rRNA degradation occurs in a pollen tube, the radioactive intensity of 28S RNA should be stronger than that of 18S RNA because of its larger size. This was what McClure et al. (1990) observed from styles 46 hr after pollination with compatible pollen. In contrast, they observed that, in styles 12 hr after incompatible pollination, the amount of 28S RNA was significantly less than that of 18S RNA, suggesting that the 28S RNA was degraded in incompatible pollen tubes (Figure 2b in McClure et al., 1990). Thus, it is difficult to explain why rRNA is degraded in an incompatible pollen tube 12 hr post-pollination.
when the S-RNase-containing compartment still remains intact (McClure et al., 1990; Goldraij et al., 2006). One explanation is that some S-RNase molecules are not targeted to the compartment when they are taken up by the pollen tube. These cytoplasmically-located S-RNase molecules can account for the RNA degradation observed in incompatible pollen tubes at the time when the S-RNase-containing compartment is essentially intact.

Most critically, this sequestration or disruption model for S-RNase-based SI cannot explain all the SI phenomena in this system (Figure 5.1). Goldraij et al. (2006) proposed that the interaction between SLF and its self S-RNase would prevent the degradation of HT-B by the hypothetical protease, allowing HT-B to cause the disruption of the compartment and release of the S-RNase into the cytoplasm to inhibit the growth of self pollen tubes. However, this model cannot explain the competitive interaction phenomenon. For example, if heteroallelic pollen, containing both $SLF_1$ and $SLF_2$, is used to pollinate an $S_1S_2$ pistil, based on this model, the interaction between the two SLF proteins and their corresponding self S-RNases would stabilize HT-B and thus lead to the disruption of the compartment to release both $S_1$-RNase and $S_2$-RNase into the cytoplasm. This would result in the inhibition of the $S_1S_2$ heteroallelic pollen tube by the $S_1S_2$ pistil (Figure 5.1A). However, this is precisely the opposite of what is observed, because as discussed in Chapter 1, competitive interaction between two different pollen S-alleles renders heteroallelic pollen compatible to pistils of any S-genotype. Perhaps recognizing this problem, McClure (2006) has subsequently modified this model to make it compatible with the competitive interaction phenomenon without providing supporting data. The revised model proposes that SLF interacts with its non-self S-RNase, rather than with itself S-RNase as originally proposed, to result in the degradation of HT-B. In this case, if the same heteroallelic pollen mentioned above (containing $SLF_1$ and $SLF_2$) is used to pollinate an $S_1S_2$ pistil, the interaction between $SLF_1$ and $S_2$-RNase would result in degradation of HT-B, so would the interaction between $SLF_2$ and $S_1$-RNase, and thus, as the original model by Goldraij et al. (2006) predicts, the degradation of HT-B would render both $S_1$-RNase and $S_2$-RNase to remain sequestered in the compartment (Figure 5.1B). This revised model can explain the competitive interaction phenomenon, but it fails to explain the “normal” case of SI. For example, when $S_I$ pollen is used to pollinate an $S_I$ pistil, both $S_1$-RNase and $S_2$-RNase are taken into the $S_I$ pollen tube non-specifically and would be sequestered in the same compartment (Goldraij et al., 2006),
and according to the revised model (McClure, 2006), SLF₁ would interact with its non-self S-RNase, S₂-RNase, to result in the degradation of HT-B and consequent stabilization of the compartment containing both S₁-RNase and S₂-RNase. Thus, based on the revised sequestration model, S₁ pollen would be accepted by an S₁S₂ pistil, because neither S₁-RNase nor S₂-RNase would be released to inhibit the growth of the S₁ pollen tube (Figure 5.1C). This is obviously not the case.

5.3 New Biochemical Model for S-RNase-Based SI

Several lines of evidence suggest that some, if not all, of the S-RNase molecules (including self and non-self S-RNases) taken into a pollen tube are cytoplasmically localized. First, SLF appears to be localized in the cytoplasm of the pollen tube, as revealed by immunolocalization of AhSLF₂ (Wang and Xue, 2005) and by localization of the GFP fluorescence of PiSLF₂:GFP in transgenic pollen tubes germinated in vitro (Hua et al., unpublished results). Second, if the stability of HT-B is indeed controlled by the interactions between SLF and its self or non-self S-RNase (Goldraij et al., 2006; McClure, 2006), it would seem that some S-RNase molecules should be present in the cytoplasm in order for their interactions with SLF to take place. Third, as discussed above, in incompatible pollen tubes growing in Nicotiana alata styles, the timing of rRNA degradation observed by McClure et al. (1990) is earlier than that of the disruption of the S-RNase-containing compartment observed by Goldraij et al. (2006), suggesting that some S-RNases are not compartmentalized. Fourth, although Goldraij et al. (2006) could not detect S-RNase in the cytoplasm of compatible pollen tubes or the degradation of S-RNase, they pointed out that they could not rule out the existence of some S-RNases in the cytoplasm nor the degradation of small amounts of S-RNase. Qiao et al. (2004a) reported the observation of degradation of S-RNase in pistils under compatible pollination conditions in Antirrhinum hispanicum, however, judging from the results shown in Figure 10, the extent of degradation did not appear to be as significant as the authors claimed. Fifth, Huang et al. (2006) recently used yeast two-hybrid screens to identify a Skp1-like protein that interacts with the F-box domain of AhSLF₂, suggesting that AhSLF is likely to target the ubiquitination of S-RNase in the cytoplasm of pollen tubes by acting in a putative SCF<sub>AhSLF</sub> complex.
To date, it remains unclear how S-RNase enters the cytoplasm of a pollen tube. McClure (2006) adopted the retrograde transport pathway of Ricin A to explain the entrance of S-RNase into the pollen tube cytoplasm. During its expression, Ricin A re-translocates from the ER to the cytosol via the Endoplasmic Reticulum Associated Protein Degradation (ERAD) pathway (Di Cola et al., 2001). However, this mechanism does not seem a plausible one for the uptake of S-RNase into the cytoplasm of a pollen tube for the following reasons. First, the amino acid sequence identity between S3-RNase of Petunia inflata and Ricin A is only 3%, with no significant clusters of similar amino acids. Second, S-RNase is expressed and secreted from the transmitting cell of a pistil and enters the cytoplasm of a pollen tube; whereas cytoplasmic localization of Ricin A results from its retrograde movement from the ER during its maturation in the same cell where it is expressed (Di Cola et al., 2001). Third, most Ricin A-like cytotoxins contain very few lysine residues; this presumably allows them to evade degradation through the ubiquitin-26S proteasome-mediated ERAD pathway so that they can exert their cytotoxic function in the cytoplasm. For example, only two of the 267 amino acid residues of Ricin A are lysines (Hazes and Read, 1997; Di Cola et al., 2005). In contrast, S-RNases contain much higher percentages of lysine residues. For example, the lysine residues in S3-RNase of P. inflata comprise 10% of the total amino acid residues (20 lysine residues out of the 200 total amino acid residues, Chapter 4). Fourth, ubiquitin-26S-mediated S-RNase degradation does not seem to be related to ERAD (see Chapter 4 and also discussion below), further suggesting that a completely different mechanism from retrograde transportation is responsible for the uptake of S-RNase into the cytoplasm of pollen tubes. This mechanism remains to be determined.

Although the sequestration model, be it in the original form or in the revised version, is flawed, its prediction that the majority of S-RNase molecules, including both self and non-self S-RNases, are sequestered in a pollen tube may be valid. If so, any change in the small amount of cytoplasmically-localized S-RNase in a pollen tube at different time points post-pollination would be difficult to detect due to the large amount of the total S-RNase, either sequestered in the pollen tube or present in the style. Furthermore, even if some S-RNase molecules are degraded within a pollen tube during compatible pollination, the pistil still continues to synthesize additional molecules of S-RNase and delivers them to the pollen tube. This may
explain why Goldraij et al. (2006) could not observe significant differences in S-RNase levels within pollen tubes between compatible and incompatible pollinations. Further complicating this type of experimental approach, S-RNase levels have been found to vary over a wide range in pistils from different flowers of the same plant, e.g., up to 20-fold differences were observed in *Solanum chacoense* (Qin et al. 2006). This may explain why the results pertaining to S-RNase level reported to date, obtained via either direct detection from a pollinated style (Qiao et al., 2004a) or dissection of pollen tubes from a pollinated style (Goldraij et al., 2006), show very high variations, judging from the data presented in Figure 10C of Qiao et al. (2004b) and Figure S7 of Goldraij et al. (2006), making the interpretation of the results difficult. Therefore, in order to detect any changes in S-RNase levels in pollen tubes after compatible pollination, a more accurate method should be established.

I have developed a cell-free system, based on similar systems commonly used to study the fate of proteins targeted by the ubiquitin-26S proteasome pathway in yeast and mammalian cells (Verma et al., 1997; Jiang et al., 2005), and I have used it to demonstrate that S-RNases, including *E. coli*-expressed recombinant S-RNases and native deglycosylated S-RNases, are degraded through the ubiquitin-26S proteasome pathway in pollen tube extracts, albeit not in an *S*-allele-specific manner (Chapter 2 and Chapter 4). In Chapter 4, I further showed that the *E. coli*-expressed GST:S-RNases used for the degradation and ubiquitination assays had RNase activity and were thus functional. Furthermore, via a site-directed mutagenesis assay, I have found that the C-terminal lysine residues play a major role in targeting the ubiquitination of S-RNase in pollen tube extracts. These results suggest that S-RNases, used for the degradation and ubiquitination assays in pollen tube extracts, are folded normally and that ubiquitin-26S proteasome-mediated S-RNase degradation is likely an integrated part of the SI mechanism.

Using two independent protein-protein interaction assays, yeast two-hybrid and *in vitro* binding, I have identified a putative PiSLF-containing complex, which contains PiSLF, PiSBP1 and PiCUL1-G. No Skp1-like proteins were found to interact with the predicted F-box domain located at the N-terminus of PiSLF. Instead, the interactions of PiSBP1 with PiSLF and PiCUL1-G likely result in the formation of a novel E3-like complex (Chapter 2). Two homologues of PiSBP1, PhSBP1 (Sims and Ordanic, 2001) and ScSBP1 (O'Brien et al., 2004),
had been shown to interact with S-RNase, before I showed that PiSBP1 interacts with both the male (PiSLF) and female (S-RNase) SI determinants (Chapter 2). Since SBP1 contains a RING-HC domain at its C-terminus and since the SBP1 gene does not show any sequence polymorphism in different S-genotypes, Sims and Ordanic (2001) proposed that it could serve as a general inhibitor to inactivate S-RNase non-specifically by targeting S-RNase for ubiquitination and degradation. This raised a question as to why two different putative E3 ligases, SCFSLF and SBP1, would exist in the pollen tube to affect the function of S-RNase (McClure, 2004). My finding that PiSBP1 and PiSLF work together in the same E3-like complex has provided a possible answer. The in vitro reconstitution experiment showing that PiSBP1 alone acts as an E3 ligase to ubiquitinate S-RNase further suggests that the PiSLF-containing complex is an E3-like complex in vivo and that the ubiquitination of S-RNase is most likely independent of ERAD (Chapter 4). More significantly, the results from my in vitro binding assays between PiSLFs and S-RNases showed that a PiSLF interacts with its non-self S-RNase much more strongly than with its self S-RNases, and vice versa. This finding suggests that the PiSLF-containing complex specifically targets non-self S-RNase for ubiquitination and subsequent degradation by the 26S proteasome, and leaves self S-RNase active to perform its cytotoxic function in vivo (Chapter 2 to Chapter 4).

The fact that PiSLF and S-RNase prefer to interact with their non-self partners is intriguing. It is known that SLF and S-RNase are tightly linked at the S-locus. Within the S-locus, in addition to SLF, several SLF-like genes have also been identified (McCubbin et al., 2000; Entani et al., 2003; Ushijima et al., 2003; Wang et al., 2003; Zhou et al., 2003; Sassa et al., 2007; Chapter 3). From both in vivo transgenic experiments and in vitro protein-protein interaction assays, we found that, unlike PiSLF2, three of the six PiSLF-like genes identified within the P. inflata S-locus were unable to break down the pollen SI function in transgenic plants and all these six PiSLF-like proteins, except A113 (which was not studied because its S1 and S2 allelic variants are identical in sequence), either failed to interact or interacted with S-RNase much more weakly than did PiSLF2. These results strongly suggest that PiSLF is unique in its function in determining the pollen SI behavior (Chapter 3). Confirmation of this unique function of PiSLF led me to further investigate three PiSLF-specific regions, classified into three functional domains (FD1, FD2 and FD3). From domain-swapping experiments between
PiSLF and a PiSLF-like protein, which does not interact with S-RNase, and also between PiSLF1 and PiSLF2, I showed that (1) FD2, which functions as the S-RNase binding domain (SBD), is the primary region for the interaction between PiSLF and S-RNase, and (2) FD1 and FD3, working together as the S-RNase binding regulating domain (SBRD), negatively regulate the interaction between PiSLF and its self S-RNase. Thus, the results from these domain-swapping experiments can explain why a PiSLF interacts with its non-self S-RNase much more strongly than with its self S-RNase (Chapter 3).

Based on the results of the *in vitro* biochemical experiments and the *in vivo* transgenic experiments, I present here a new biochemical model to explain S-RNase-based SI (Figure 5.2). This model addresses three different scenarios: incompatible pollination, compatible pollination and competitive interaction, and all the interactions depicted occur in the cytoplasm of the pollen tube.

In the case of incompatible pollination (Figure 5.2A), a PiSLF would interact with its self S-RNase through the S-RNase-binding-regulating domain (SBRD). This allele-specific interaction would block the strong general interaction between S-RNase and the S-RNase-binding domain (SBD) of PiSLF. The self S-RNase, either in its free form or in association with PiSLF, would degrade pollen RNA to result in the growth inhibition of self-pollen tubes.

In the case of compatible pollination (Figure 5.2B), there is no specific interaction between SBRD of PiSLF and any of its non-self S-RNases because there is no matching between their allele-specific domains. This would expose the SBD domain of PiSLF to interact strongly with all non-self S-RNases. This strong interaction would result in the formation of a stable PiSLF-S-RNase complex, and allow the PiSLF-containing E3 ligase to efficiently ubiquitinate non-self S-RNases to target them for degradation. Thus, the growth of non-self pollen tubes is not inhibited.

In the case of competitive interaction (Figure 5.2C), two different PiSLFs and their respective self-S-RNases are present in the same pollen tube. Each PiSLF would either interact with its non-self S-RNase through its SBD domain to form a stable PiSLF-S-RNase complex, or
recognize its self S-RNase through its SBRD domain to make an unstable complex. Because of the strong interaction between an S-RNase and the SBD domain of its non-self PiSLF, the non-self PiSLF in the same pollen tube would out-compete self PiSLF to form a stable PiSLF-S-RNase complex. Ultimately, all the PiSLF-S-RNase complexes in the heteroallelic pollen tube would form between one of the PiSLFs and its non-self S-RNase. This would result in ubiquitination and degradation of all S-RNases as in the case of compatible pollination.

5.4 Conclusions

PiSLF has been conclusively shown to determine the pollen SI specificity in *Petunia inflata* (Sijacic et al., 2004). Biochemical studies I carried out in my thesis research have shown that PiSLF preferentially interacts with its non-self S-RNases and forms a novel E3-like complex, suggesting that it specifically targets non-self S-RNase for ubiquitination and degradation and thus allows the growth of non-self pollen tubes but not that of self pollen tubes (Chapter 2). I have further shown that PiSLF contains two protein-protein interaction domains, SBD and SBRD, which contribute differently to its interaction with self and non-self S-RNases. This finding provides a biochemical explanation for the preferential binding of a PiSLF with its non-self S-RNases (Chapter 3). The ubiquitination and degradation assays I carried out on S-RNases using pollen tube extracts have demonstrated that S-RNase is regulated by the ubiquitin-26S proteasome pathway, most likely mediated by the PiSLF-containing E3-like complex in vivo. Thus, ubiquitin-26S proteasome-mediated S-RNase degradation is an integral part of the S-RNase-based SI mechanism (Chapter 4).

However, mechanistic details of S-RNase-based SI appear to be different in the Solanaceae, Rosaceae, and Plantaginaceae. In *Antirrhinum* (Plantaginaceae), AhSLF may be a component of a classical SCF complex (Qiao et al., 2004a; Huang et al., 2006). Alternatively, both PiSLF and AhSLF may function in a similar non-conventional SCFSLF complex which contains SLF, SBP1 (replacing RBX1), SSK1 (replacing SKP1) and Cul1 (Sims 2007). In the Rosaceae, the *SLF/SFB* may function completely differently because deletion of *SFB* in sweet cherry (*Prunus avium*) renders pollen compatible with the pistil, i.e., resulting in breakdown of SI (Sonneveld et al., 2005) whereas deletions of *SLF* in the Solanaceae are most likely “lethal”, rendering pollen tube growth inhibition by pistils of any *S*-genotype (Golz et al., 2001). Thus, although all three
families utilize S-RNase as the female SI determinant, the pollen S-gene, *SLF/SFB*, may function differently to control the fate of S-RNase in a pollen tube. Further biochemical and molecular studies of the S-RNase-based SI mechanism, particularly in the Rosaceae and *Antirrhinum*, are required to address these potential differences. Furthermore, how S-RNase enters the cytoplasm of a pollen tube remains to be determined.

5.5 Ongoing Projects in Collaboration with other Graduate Students in the Lab

5.5.1 Loss-of-Function Study of *PiSLF*<sub>2</sub> and *PiSBP1*

The transgenic experiments carried out so far in the lab have clearly shown that expression of *PiSLF*<sub>2</sub> or *PiSLF*<sub>2</sub>:GFP in transgenic pollen specifically breaks down the SI behavior of heteroallelic pollen and not that of homoallelic pollen (Sijacic et al., 2004; Chapter 3). In addition, I have shown that PiSLF<sub>2</sub> may be part of an E3-like complex which targets non-self S-RNase for ubiquitinn-26S proteasome mediated degradation by preferentially binding to non-self S-RNase, and thus allowing successful pollination by non-self pollen (Chapter 2). Functional comparison between PiSLF<sub>2</sub> and PiSLF-like proteins has revealed that PiSLF<sub>2</sub> may have a unique function in determining the pollen S-specificity (Chapter 3). To further confirm the aforementioned experimental results, it would be interesting to determine what effect suppression of the expression of PiSLF<sub>2</sub> has on the SI behavior of S<sub>2</sub> pollen. We hypothesize that loss of PiSLF<sub>2</sub> function in S<sub>2</sub> pollen would render the transgenic pollen incompatible with pistils of any S-genotype, because no PiSLF<sub>2</sub>-containing complex could mediate the degradation of any cytoplasmically-localized non-self S-RNases.

As described in Chapter 2, I have also shown that the PiSLF-containing complex may be a novel E3-like complex which does not contain Skp1 or RBX1 components of a typical SCF complex. Instead, PiSBP1 is the RING-finger protein component that interacts with PiSLF, PiCUL1-G, and S-RNase. Furthermore, I have shown that PiSBP1 can act as a mono-subunit E3 to ubiquitinate S-RNases *in vitro* in a non-S-specific manner, further suggesting that PiSBP1 may be involved in SI (Chapter 4). Thus, we hypothesize that loss of *PiSBP1* function in pollen would also render the pollen incompatible with pistils of all S-genotypes.
In recent years, the approach of RNA silencing has been commonly used to suppress the function of a target gene in various cells/tissues. Interestingly, the transcripts of 15 of the genes involved in small RNA pathways could not be detected in mature pollen of Arabidopsis (Pina et al., 2005). It is not known whether this finding means that the RNAi approach is not effective in suppressing pollen-expressed genes, as protein products of these 15 genes may still be present in mature pollen. Nonetheless, RNA silencing in pollen may be achieved by stable transformation with an RNAi construct employing a pollen-specific promoter, such as the LAT52 promoter, that is active at earlier stages of pollen development when the transcripts of the 15 genes mentioned above could be detected (Pina et al., 2005). Our finding that the transcripts of both PiSLF2 and PiSBP1 peak in immature pollen (Sijacic et al., 2004; also see Chapter 2 and Chapter 3) suggests that it may be possible to down regulate the expression of PiSLF2 or PiSBP1 in pollen using an RNAi strategy. However, not all RNAi constructs may produce small interfering RNA (siRNA) to efficiently down regulate the target gene. Our lab has tried an RNAi construct using 735 bp (from nucleotide 187 to 920) of the PiSLF2 coding sequence driven by the LAT52 promoter, but failed to reduce the transcript of PiSLF2 in the pollen of the transgenic plants (Sijacic et al., unpublished result). I have designed a new PiSLF2 RNAi construct using 325 bp of the 3'-untranslated region (UTR) of PiSLF2 and 441 bp of the 3' part of the PiSLF2 coding sequence (nucleotides 730 to 1170). In animals, the 3'-UTR of a gene is known to be targeted by small interfering RNA (siRNA) more efficiently than the other regions (Bermingham et al., 2006). I have also designed a PiSBP1 RNAi construct in a similar fashion, using 192 bp of the 3'-UTR and 630 bp of the 3' part of the coding sequence (nucleotides 382 to 1011). Transformation and functional analyses of transgenic plants carrying the PiSLF2 RNAi construct and the PiSBP1 RNAi construct are being carried out by two graduate students, Allison Fields and Xiaoying Meng, respectively.

5.5.2 Over-Expression of PiSBP1 In Vivo

Since PiSBP1 acts as an E3 ligase in vitro to ubiquitinate S-RNase, and most E3s also down regulate their own protein levels by autoubiquitination when their target proteins are absent (Chapter 4), it may also be possible to down regulate the protein level of PiSBP1 in pollen or pollen tubes by over-expressing PiSBP1. In this case, over-expression of PiSBP1 in pollen may
cause a similar phenotype as that caused by suppression of PiSBP1 such that the transgenic pollen would be incompatible with pistils of any S-genotype. On the other hand, expression of PiSBP1 by the strong pollen-specific promoter, LAT 52, could produce high levels of PiSBP1 in pollen. In this case, we hypothesize that over-production of PiSBP1 may enhance the basal ubiquitination of S-RNase in pollen tubes, rendering the transgenic pollen to be accepted by pistils of any S-genotype, as our model predicts that ubiquitin-26S mediated S-RNase degradation is required for compatible pollination.

In this project, I have designed an over-expression construct of PiSBP1 that includes the LAT 52 promoter and the coding sequence of GFP fused to the 3’-end of the coding sequence of PiSBP1; this will allow us to more easily monitor the expression of the transgene. Transformation and functional analyses of the transgenic plants carrying this construct are being carried out by Xiaoying Meng.

5.5.3 In Vivo Functional Study of S3-RNase (K141-164R)

From the mutagenesis study in which the lysine residues of S3-RNase were changed to arginine residues, I have found that six lysine residues, K141-K164, near the C-terminal end of S3-RNase play a major role in targeting S3-RNase for ubiquitination in pollen tube extracts (Chapter 4). Since several lines of evidence obtained from my thesis research suggest that ubiquitin-26S proteasome mediated S-RNase degradation is an integral part of the S-RNase-based SI mechanism (Chapter 4), we hypothesize that over-expression of S3-RNase (K141-164R) in the pistil would lead to rejection of any pollen by significantly lowering the extent of ubiquitination and degradation of S3-RNase in the pollen tube.

To test this hypothesis, I changed the codon for each of the six C-terminal lysine residues (K141 to K164) in pBS-GS3(N29D) (Karunanandaa et al., 1994) to CGT (encoding arginine) to generate pBS-GS3(N29D, K141-164R). pBS-GS3(N29D) contains a mutant form of S3-RNase with the codon for the only N-glycosylation site, asparagine-29, replaced by a codon for aspartic acid. This change does not affect the SI function of S3-RNase in vivo (Karunanandaa et al., 1994). The reason for choosing CGT for the arginine codon is that it is the most frequently
used codon for arginine in *Arabidopsis thiana* and *Petunia inflata* based on statistical analysis of the codon usage of all known complete protein coding genes (http://www.kazusa.or.jp/codon/).

The absence of the glycan chain in S3-RNase (N29D) and S3-RNase (N29D, K141-164R) renders their molecular mass smaller than that of the wild-type S3-RNase, allowing easy separation of the former two proteins from the latter by SDS-PAGE (Karunananda et al., 1994). In collaboration with Xiaoying Meng, we introduced pBS-GS3(N29D, K141-164R) into S2S2 and S3S3 plants, and as a control, we also introduced pBS-GS3(N29D) into S2S2 and S3S3 plants. We have identified two S2S2/S3-RNase (N29D, K141-164R) transgenic plants, from a total of 30, that showed expression of S3-RNase (N29D, K141-164R), and identified 13 S2S3/S3-RNase (N29D, K141-164R) transgenic plants, from a total of 164, that showed expression of S3-RNase (N29D, K141-164R). We have also identified one S2S2/S3-RNase (N29D) and seven S3S3/S3-RNase (N29D) transgenic plants that showed expression of S3-RNase (N29D).

The expression levels of S3-RNase (N29D, K141-164R) and S3-RNase (N29D) varied significantly from plant to plant. Although we could not rule out the possibility that the different expression levels of either transgene in some transgenic plants may be due to different copy numbers of the transgene, the finding that different cuttings from the same transgenic plant, S2S2/S3-RNase (N29D), also showed very different expression levels of S3-RNase (N29D), suggests that the expression of S-RNase in the pistil may be sensitive to epigenetic regulation. This finding is consistent with the finding by Qin et al. (2006) that the expression level of S-RNase in *Solanum chacoense* varied up to 20-fold from flower to flower within a plant. Furthermore, the average expression level of S3-RNase (N29D, K141-164R) is much lower than that of S3-RNase (N29D).

Analyses of the transgenic plants are being carried out by Xiaoying Meng with help from Allison Fields and Ning Wang.

### 5.5.4 Study of Ubiquitination of S-RNase *In Vivo*

My *in vitro* biochemical results suggest that S-RNase may be ubiquitinated in compatible pollen tubes. However, these results are yet to be confirmed by *in vivo* evidence mainly due to the
difficulty in determining the fate of a presumably small amount of cytoplasmically-localized S-RNase molecules from the large amount that are sequestered in the pollen tube or present in the transmitting tract of the pistil. Qiao et al. (2004b) reported that S-RNase molecules that were immunoprecipitated by an anti-S-RNase antibody from extracts of compatibly pollinated pistils, but not from extracts of incompatibly pollinated pistils, also cross-reacted with an anti-ubiquitin antibody. Thus, they concluded that S-RNase is ubiquitinated in compatible pollen tubes. However, due to the low specificity of the anti-ubiquitin antibody used and probably of the anti-S-RNase antibody as well (the authors did not show the specificity of either antibody), a very high background was also detected by the anti-ubiquitin antibody (Figures 9A and 9B in Qiao et al. 2004). Thus, it is not clear whether the two extra bands detected in compatibly pollinated pistils, but not in incompatibly pollinated pistils, were ubiquitinated S-RNases.

In my in vitro ubiquitination assay, I found that the anti-(His)6 antibody I used was specific to the (His)6:Ubiquitin (Figure 2.16D in Chapter 2). Furthermore, I affinity-purified the anti-S3-RNase antibody that had been previously raised in the lab and showed that the purified antibody was specific to S3-RNase of P. inflata (Figures 2.14B and C in Chapter 2). Therefore, if I use the anti-S3-RNase antibody to immunoprecipitate S3-RNase from the pistils that have been compatibly or incompatibly pollinated by pollen expressing (His)6:Ubiquitin, I may be able to use the anti-(His)6 antibody to assess whether S3-RNase is ubiquitinated, since any poly-ubiquitin chain attached to S3-RNase will also contain (His)6:Ubiquitin. In order to express (His)6:Ubiquitin in pollen, I have made a pBI-LAT52-(His)6:Ubiquitin construct, and Xiaoying Meng and another graduate student, Ning Wang, have carried out transformation and analyses of the transgenic plants raised. So far, we have obtained several transgenic plants that show expression of (His)6:Ubiquitin in pollen.

Furthermore, the results of my in vitro degradation assay using native S3-RNase as the substrate suggest that deglycosylation of S-RNase may be required for efficient ubiquitin-26S proteasome mediated degradation in pollen tubes (Figure 2.14C in Chapter 2). Since S3-RNase (N29D), a non-glycosylated S3-RNase, functions as well as the native glycosylated S3-RNase in vivo (Karunananda et al., 1994; also see Section 5.5.3), I reasoned that using S3-RNase (N29D) may help us detect more ubiquitinated S3-RNase molecules in compatible pollen tubes and make...
the results of this experiment more clear-cut than using the native S3-RNase. Xiaoying Meng has raised several lines of S2S2/S3-RNase (N29D) and S3S3/S3-RNase (N29D) transgenic plants, and Ning Wang has produced S3-RNase (N29D) transgenic plants in other S-genotype backgrounds.

5.5.5 Real-Time Cellular Localization of S-RNase in Pollen Tubes

Goldraij et al. (2006) showed that S-RNase is compartmentalized in pollen tubes, after both compatible and incompatible pollinations, at early stages of tube growth in the pistil, and that the compartment is disrupted later in pollen tubes after incompatible pollination but not in pollen tubes after compatible pollination. Since immunolocalization of S-RNase in pollen tubes inside a pistil requires fixation, this may result in artifacts. GFP has been used as a reporter protein to monitor real-time cellular localization of many proteins with success, and thus I have decided to use it to examine the cellular localization of S-RNase in self and non-self pollen tubes. Since S-RNase is taken up by pollen tubes and exerts its cytotoxic action in the cytoplasm of self-pollen tubes, my strategy is to use the LAT52 promoter to transiently and stably express GFP-tagged, mature S-RNase (without the leader peptide) in both self and non-self pollen tubes. I will then examine cellular localization of the GFP-tagged S-RNase, and determine whether the GFP-tagged S-RNase will inhibit the growth of self pollen tubes and whether its level in non-self pollen tubes is less than that in self pollen tubes.

The preliminary results obtained from both the transient expression experiments and transgenic plants showed that 1) expression of S2-RNase:GFP in both non-self (S1) and self (S2) pollen tubes produced similar intensities of GFP fluorescence; 2) S2-RNase:GFP was sequestered in both S1 and S2 pollen tubes; 3) both S1 and S2 pollen producing S2-RNase:GFP are compatible with S2S2 and S1S1 pistils, respectively, suggesting that pollen tube-expressed S2-RNase:GFP is not toxic to either self or non-self pollen; 4) the movement of the S2-RNase:GFP-containing compartment in pollen tubes was completely stopped by a microtubule toxin, rhizoxin, suggesting that it is localized in the Golgi apparatus, since the Golgi apparatus is associated with microtubules (for a review to see Thyberg and Moskalewski, 1999).
S-RNase is an N-glycosylated protein, S2-RNase:GFP does not contain a leader peptide and since the Golgi apparatus is the major location for glycosylation of N-glycosylated proteins, the glycosylation process may be the cause of S2-RNase:GFP's localization in the Golgi apparatus. This cellular localization blocks the cytotoxic function of S2-RNase:GFP in the cytoplasm of a pollen tube. Thus, both self and non-self pollen tubes expressing S2-RNase:GFP are still healthy and behave like wild type pollen. The same results were obtained by Kirch et al., (1996) from pollen tube-expressed S-RNase. To address whether glycosylation causes the sequestration of S-RNase expressed in pollen tubes and also determine whether the C-terminally-attached GFP tag has any effect on the function of S-RNase in pollen tubes, I further modified this project as follows. First, I made a construct containing S2-RNase and an N-terminal GFP tag, \( pBI-LAT52-GFP:S2-RNase \), to address the possible positional effect of the GFP tag on the function and localization of S2-RNase in a pollen tube. Second, our lab had previously showed that the N-glycan chain is not required for the function of S3-RNase (Karunanandaa et al., 1994) and my \textit{in vitro} degradation data showed that the degradation of non-glycosylated S3-RNase is much more efficient than the glycosylated S3-RNase in pollen tube extracts (Chapter 2). Therefore, I designed three constructs, \( pBI-LAT52-S3-RNase(N29D) \), \( pBI-LAT52-S3-RNase(N29D):GFP \), and \( pBI-LAT52-S3-RNase:GFP \) to address whether the N-glycan chain, which is attached to N29 in S3-RNase, leads to the potential sequestration of S3-RNase and affects its cytotoxic function in pollen tubes and also to see whether the GFP tag has any effects on its function in a pollen tube. Xiaoying Meng and Ning Wang will help me to finish this project and we will have equally-contributed to this project.

5.5.6 \textit{In Vivo} Functional Assay of SBD and SBRD Domains of PiSLF

As described in Chapter 3, I concluded from the results of the \textit{in vitro} binding assay that PiSLF may contain two functional domains, SBD and SBRD, which contribute differently to the interaction between PiSLF and its self and non-self S-RNases. According to my hypothesis, SBD is primarily responsible for the strong interaction between PiSLF and S-RNase, whereas SBRD contributes to allelic specificity of a PiSLF. Moreover, the specific interaction between SBRD of a PiSLF and the matching S-allele-specific domain of its self S-RNase blocks the otherwise strong interaction between the SBD domain and S-RNase. The SBRD domain is
divided into two subdomains, an N-terminal FD1 domain and a C-terminal FD3 domain, and the SBD domain is in the middle of PiSLF, named FD2 (Chapter 3). One way to test the proposed biological roles of FD1, FD2 and FD3, is to swap each of the domains between two different allelic variants of PiSLF and determine what allelic specificity the chimeric proteins display.

To date, three alleles of PiSLF have been cloned in the lab. PiSLF2 was identified from direct sequencing of a 328-kb S-locus region containing S2-RNase, and it is the only PiSLF allele whose function in SI has been established in vivo (Sijacic et al., 2004, Chapter 3). PiSLF1 and PiSLF3 were isolated by RT-PCR (Sijacic et al., 2004), and before using them for making chimeric constructs with PiSLF2, we need to first confirm their in vivo function using the same approach as that used to assess the function of PiLF2. For example, the in vivo function of PiSLF1 will be tested by transforming the construct pBI-LAT52-PiSLF1:GFP into S2S3 and S1S2 plants.

To simplify the description of the proposed domain-swapping experiments, I will use the chimeric proteins made from exchanging the domains between PiSLF1 and PiSLF2 as an example to explain the experimental design. In vitro binding results showed that exchange of the SBRD (FD1 and FD3) domain between PiSLF1 and PiSLF2 precisely reversed the interaction of PiSLF1 and PiSLF2 with S2-RNase, i.e., the strong interaction between a chimeric protein and S2-RNase is determined by the allelic variant of PiSLF that contributes the SBRD domain but not by the allelic variant that contributes the SBD domain (Figure 3.15 in Chapter 3). Furthermore, the comparison between PiSLF2 and PiSLF-like proteins suggests that the strong binding between PiSLF and S-RNase may be necessary for the unique function of PiSLF. Therefore, I hypothesize that the chimeric protein PiSLF1(FD1):PiSLF2(FD2):PiSLF1(FD1) (abbreviated as F121) will have a similar S-haplotype specificity as PiSLF1, and PiSLF2(FD1):PiSLF1(FD2):PiSLF2(FD1) (abbreviated as 212) will have a similar S-haplotype specificity as PiSLF2.

I have made pBI-LAT52-F121:GFP and pBI-LAT52-212:GFP constructs, and Allison Fields will introduce both constructs separately into S2S3 and S1S2 plants to analyze the function of these two chimeric proteins in controlling the pollen SI behavior in vivo. I have also made the
following constructs for three other chimeric proteins, $\text{PiSLF}_3(\text{FD}1):\text{PiSLF}_2(\text{FD}2):\text{PiSLF}_3(\text{FD}1)$ (abbreviated as F323), $\text{PiSLF}_2(\text{FD}1):\text{PiSLF}_3(\text{FD}2):\text{PiSLF}_2(\text{FD}1)$ (abbreviated as 232), and $\text{PiSLF}_2(\text{FD}1):\text{PiSLF}_3(\text{FD}2):\text{PiSLF}_3(\text{FD}1)$ (abbreviated as 233). Ning Wang will raise transgenic plants carrying these constructs and examine the function of these three chimeric proteins in controlling the pollen SI behavior \textit{in vivo}.
Figure 5.1 Inability of the Sequestration Model to Explain S-RNase-Based SI.

The black and gray dots indicate intact and degraded HT-B, respectively. F1 and F2 indicate SLF1 and SLF2, respectively; S1 and S2 indicate S1-RNase and S2-RNase, respectively. PP indicates a hypothetical pollen protein proposed by Goldraji et al. (2006). During the uptake of S-RNase by the pollen tube, some S-RNase molecules may directly enter into the cytoplasm of the pollen tube to interact with SLFs in an S-haplotype-specific manner. The S-genotype of the pollen is shown at the top of the figure, and the S-genotype of the pistil is shown at the bottom of the figure.

(A) The original sequestration model (Goldraji et al., 2006) fails to explain the competitive interaction phenomenon. If the preferential interaction between an SLF and its self S-RNase results in the stabilization of HT-B (by evading the hypothetical protease (PP)-mediated degradation) and causes the disruption of the S-RNase containing compartment, both S1-RNase and S2-RNase would be released into the cytosol to degrade RNA, resulting in the inhibition of the heteroallelic pollen tube. This is precisely opposite of what is observed.

(B) The modified sequestration model (McClure, 2006) can explain the competitive interaction phenomenon. The preferential interaction between an SLF and its non-self S-RNase (i.e., PiSLF1 with S2-RNase and PiSLF2 with S1-RNase) results in the degradation of HT-B by PP. Thus, the S-RNase-containing compartment is maintained and both S1-RNase and S2-RNase remain sequestered. The heteroallelic S1S2 pollen is compatible with the S1S2 pistil, as predicted by competitive interaction.

(C) The modified sequestration model (McClure, 2006) fails to explain incompatible pollination. Both S1-RNase and S2-RNase are taken up by the S1 pollen tube. The preferential interaction between SLF1 and S2-RNase would not lead to the disruption of the S-RNase-containing compartment, as addressed in (B), and both S1-RNase and S2-RNase would remain sequestered.
Thus, this model predicts that $S_i$ pollen would not be rejected by an $S_jS_2$ pistil, an outcome precisely the opposite of what is known about SI.
Figure 5.2 A New Biochemical Model to Explain S-RNase-based SI.

(A) A graphical representation of S-RNase molecules in an incompatible pollen tube. An $S_1$-RNase molecule either interacts weakly with its self PiSLF, PiSLF$_1$, indicated by the same gray color, through the SBRD domain, or exists as a free form, in the cytoplasm of an $S_1$ pollen tube.
Both forms of $S_1$-RNase have active RNase activity and would degrade pollen RNA to result in growth inhibition of the $S_1$ pollen tube.

**(B)** A graphical representation of S-RNase molecules in a compatible pollen tube. An $S_1$-RNase molecule interacts strongly with its non-self PiSLF, PiSLF$_2$, indicated by gray color of lesser intensity, through the SBD domain to form a stable $S_1$-RNase-PiSLF$_2$ complex. This complex further interacts with PiSBP1, PiCUL1-G and a potential unidentified SSK1-like protein (Sims, 2007) to form an E3-like complex, which mediates ubiquitination of $S_1$-RNase and subsequent degradation by the 26S proteasome.

**(C)** A graphical representation of the interaction between S-RNases and PiSLFs in a heteroallelic pollen tube. Two different S-RNases, $S_1$-RNase and $S_2$-RNase, are taken up by a heteroallelic pollen tube containing two different pollen $S$-alleles, PiSLF$_1$ and PiSLF$_2$. Both $S_1$-RNase and $S_2$-RNase preferentially interact with the SBD domain of their corresponding non-self PiSLF proteins, PiSLF$_1$ and PiSLF$_2$, respectively, to form stable S-RNase-PiSLF complexes, as depicted in **(B)**. They may form unstable S-RNase-PiSLF complexes through the SBRD domain of their corresponding self PiSLF as depicted in **(A)**. However, this complex, if formed, will likely dissociate because the strong interaction between an S-RNase and the SBD domain of its non-self PiSLF will out-compete the weak interaction with its self PiSLF, allowing the S-RNase to form a stable S-RNase-PiSLF complex with its non-self PiSLF.
REFERENCES


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Publications Resulting from Ph.D. Study


