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Protein Interactions in S-RNase-Based Gametophytic Self-Incompatibility

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1. Introduction

With well over 200,000 documented species (Mora et al., 2011) flowering plants (angiosperms) are among the most successful taxa on the planet. A major reason for the success of the angiosperms is self-incompatibility, a genetic and biochemical barrier to inbreeding that promotes outcrossing and diversity in populations. Plants exhibiting self-incompatibility have the ability to recognize (species-specific) pollen as being "self" or "non-self", with self (incompatible) pollen being rejected and non-self (compatible) pollen being accepted. S-RNase-based Gametophytic Self-Incompatibility (GSI) has been characterized in the Solanaceae, Rosacaeae, and Plantaginaceae (McClure et al., 2011; Meng et al. 2011; Chen et al., 2010; Sims & Robbins 2009), with the genetic, physiological and molecular basis of this form of GSI described in detail. To date, over a dozen different proteins have been identified that function in different parts of the GSI response; most of these have been tested for protein interactions with other GSI response pathway proteins. The two key recognition proteins: S-RNase (the style-expressed recognition component) and SLF (the pollen-expressed recognition component) interact with each other, and with other components of a putative SCF^{SLF} E3 ubiquitin ligase complex. Recently Kubo et al., 2010 demonstrated the existence of multiple SLF variant classes. Multiple S-RNase and SLF alleles are present in breeding populations (Richman et al., 1995, 1996, 2000), and it now seems probably that collaborative interaction of different SLF alleles and classes with different S-RNase alleles governs self/non-self recognition in GSI. In this review, I summarize the genetic basis of GSI, describe the different proteins identified that are thought to function in the GSI pathway, and describe what is known with regard to protein interactions underlying the function of self-incompatibility. Most of the work discussed here comes from studies in the Solanaceae and Plantaginaceae. Gametophytic self-incompatibility has also been studied extensively in the Rosaceae (e.g. Sassa et al., 2010). Work that demonstrates possible differences in the mechanism of GSI in Solanaceae/Plantaginaceae versus Rosaceae will be discussed as appropriate.

2. Genetic studies of gametophytic self-incompatibility

The first description of gametophytic self-incompatibility was by none other than Charles Darwin. As Darwin (1891) observed:

"....protected flowers with their own pollen placed on the stigma never yielded nearly a full complement of seed; whilst those left uncovered produced fine capsules, showing that pollen from other plants must have been brought to them, probably by moths. Plants growing vigorously and flowering in pots in the green-house, never yielded a single capsule; and this may be attributed, at least in chief part, to the exclusion of moths."

Since Darwin's observation, self-incompatibility systems in general, and GSI in particular, have interested both molecular and evolutionary biologists. As an example of self/non-self recognition, GSI presents interesting challenges in terms of molecular interactions, how recognition specificity is determined, and what types of sequences determine allelism. In terms of population genetics, evolutionary biologists have investigated questions of how GSI haplotypes are established and maintained over evolutionary time (Kohn, 2008).

2.1 The genetic basis of S-RNase-based gametophytic self-incompatibility

Early studies (de Nettancourt, 1977; Linskens 1975, Mather, 1943) demonstrated that self/non-self recognition was encoded by a single genetic locus, the S-locus, with pistil and pollen recognition components (termed "pistil-S" and "pollen-S", respectively). Both pistil-S and pollen-S have multiple alleles, such that a given S-locus recognition phenotype is now termed a S-locus haplotype. The S-locus ribonuclease (S-RNase) is pistil-S, and the S-locus F-box protein (SLF; SFB in Rosaceae) has been demonstrated to be pollen-S (Sijacic et al., 2004). During pollination, a match between S-RNase and SLF haplotypes results in pollen rejection (incompatibility). Lack of a match results in pollen acceptance (compatibility) and fertilization (see Figure 1). Recognition specificity in GSI is a cell-autonomous response, in that rejection or acceptance is specific to individual pollen tubes, and is not an "all or none" phenomenon. This can be demonstrated by the existence of "half-compatible" pollinations (Figure 1). In this case, pollen tubes expressing a SLF-specificity matching the S-RNase in the style are rejected, while other pollen tubes in the same style, with no haplotype match, grow normally and function for fertilization and seed set.

2.2 Tetraploidy results in self-compatibility due to competitive interaction

An intriguing aspect of GSI is that tetraploidy, in heterozygous individuals, leads to self-compatibility (Figure 1). In this case, heteroallelic, diploid pollen (e.g. S1-SLF/S2-SLF) is compatible on either a tetraploid style ($S_1S_1S_2S_2$) or a diploid style (S_1S_2). Haploid pollen (e.g. S_1 or S_2) remains incompatible on tetraploid styles (Figure 1). This phenomenon has been termed "competitive interaction" (de Nettancourt 1977). Competitive interaction is only observed in situations where the parent plant was heterozygous for S-locus haplotype. Tetraploids that are homozygous at the S-locus (homozygous plants can be obtained by bud-pollination), do not show competitive interaction, but remain self-incompatible. Competitive interaction is most likely the cause of GSI breakdown (compatibility) in induced pollen-part mutants (Golz et al., 1999, 2001). In mutants induced by radiation, Golz et al. (1999, 2001) showed that GSI breakdown was associated with partial duplications of S-haplotypes, in which the compatible pollen presumably phenocopied the heteroallelic condition found in tetraploids. Competitive interaction has been used as a test for the identity of pollen-S (Kubo et al., 2010; Sijacic et al., 2004), since transgenic plants, having diploid, heteroallelic pollen (two different SLF haplotypes) are self-compatible (Figure 1 and sections below).

3. Pistil-S and pollen-S

Although the genetic "identities" of pistil-S and pollen-S have been known for many years, the identification of specific proteins corresponding to these entities has been a more recent phenomenon. The S-locus ribonuclease (S-RNase) was initially cloned in 1986 (Anderson et al., 1986) with its identity as pistil-S confirmed eight years later (Lee et al., 1994, Murfett et al., 1994). Identification of SLF as pollen-S is far more recent. SLF was first identified by chromosomal walking (Entani et al., 2003; Lai et al., 2002; Wang et al., 2004) and subsequently confirmed as pollen-S using a competitive-interaction assay (Sijacic et al., 2004). As will be explained, the molecular nature of pollen-S appears to be far more complex than originally envisioned.

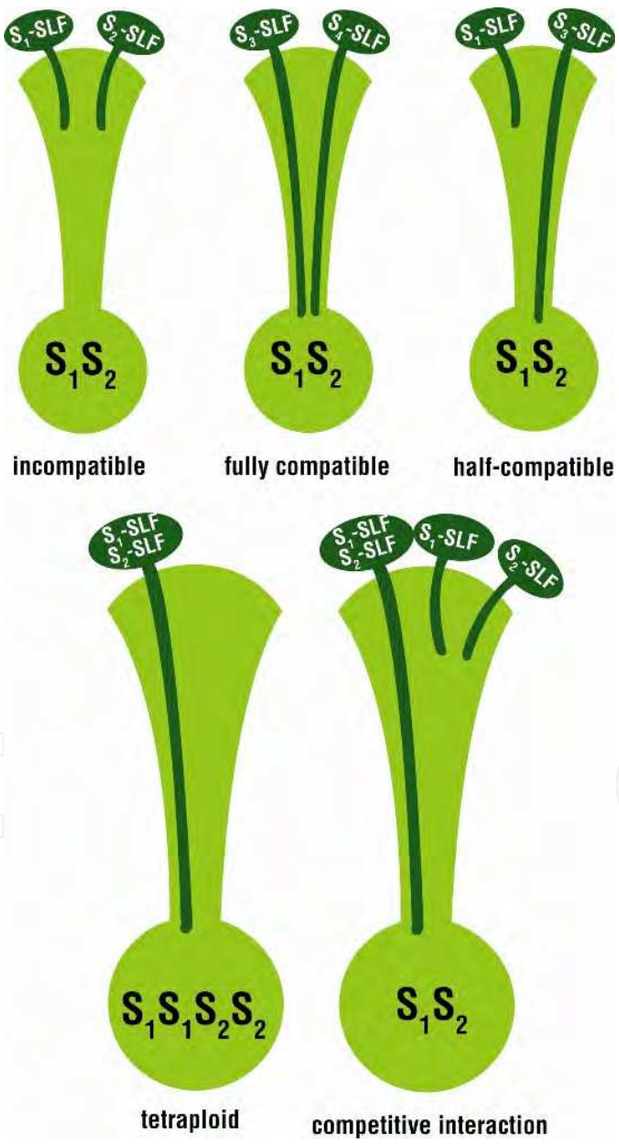

Fig. 1. **Genetic basis of gametophytic self-incompatibility.**

Figure 1 illustrates different types of pollinations with styles and pollen expressing different haplotypes at the S-locus. In an incompatible pollination (top left), a match of haplotypes between pollen and style results in incompatibility. No match of S-locus haplotypes (top, middle) results in full compatibility. A "half-compatible" cross results when half of the pollen carries a S-locus haplotype matching that of the style, but the other pollen is not matching. In this case, only the "matching" pollen tubes are rejected. The lower portion of the figure illustrates GSI breakdown in tetraploids (left figure) by competitive interaction. The figure at lower-right illustrates how competitive interaction can be used in transgenic assays to demonstrate that a particular gene (in this case, SLF) is pollen-S.

3.1 S-RNase is the pistil recognition component of GSI

The ability to selectively inhibit the growth of self pollen is determined in the style by a S-locus encoded ribonuclease known as the S-RNase. The S-RNase was first identified as a highly-expressed stylar protein that co-segregated with specific S-haplotypes (Anderson et al., 1986). The S-RNase gene is expressed at high levels late during development of the pistil (Clark et al., 1990), and encodes a secreted protein that accumulates to high levels in the transmitting tract of the style (Ai et al., 1990; Anderson et al., 1989). Comparative sequence analysis showed that S-RNase alleles have a high degree of sequence polymorphism, but that the polymorphism is not evenly distributed across the protein. Overall amino acid sequence identity can be less than 50% between allleles (Ioerger et al., 1991; Sims, 1993; Richman et al., 1995). Detailed sequence comparisons showed that S-RNase proteins have five highly conserved domains and two adjacent hypervariable domains, HVa and HVb (Ioerger et al., 1991; Sims, 1993). Although much of the sequence variability among S-RNase alleles is found in the two hypervariable regions, other portions of the protein are variable as well (Figure 2). The conserved domains C2 and C3 contain two histidine residues, His31 and His91, that along with Lys90 make up the catalytic site of the ribonuclease (Ida et al., 2001; Ishimizu et al., 1995). (Note that in different S-RNase alleles, the exact positions of these concerved amino acids vary by one or two positions).

Transgenic gain-of-function and loss-of-function experiments gave conclusive evidence that the S-RNase was the style-recognition component of GSI. Murfett et al. (1994) used a gain-of function approach, where the S_{A2}-RNase of *Nicotiana alata* (under control of a strong style-specific promoter) was transferred to a regenerable *N. lansgsdorfii* x *N. alata* hybrid. The transgenic plant remained compatible when pollinated with S_{C10} pollen from *Nicotiana alata*, but now showed the ability to reject S_{A2} pollen. Lee et al. (1994) used an antisense approach to down-regulate the *Petunia inflata* S_3-RNase in a S_2S_3 background. Plants with reduced levels of S_3-RNase were no longer capable of inhibiting S_3 pollen, although the transgenic plant showed otherwise normal GSI behavior. Lee et al. (1994) also used a gain-of-function approach, in which the S_3-RNase of *Petunia inflata* was transferred to a plant of the S_1S_2 genotype. Transgenic plants expressing the S_3-RNase at levels comparable to endogenous S-RNase had acquired the ability to reject S_3 pollen. These plants continued to reject S_1 and S_2 pollen, but set seed capsules when pollinated at an immature bud stage where the S-RNase is expressed at minimal levels (Clark et al., 1990; Lee et al., 1994). In these experiments, only the style recognition was altered. Pollen recognition specificity was not affected, confirming that a separate gene product from the S-RNase encoded the "pollen-S" component.

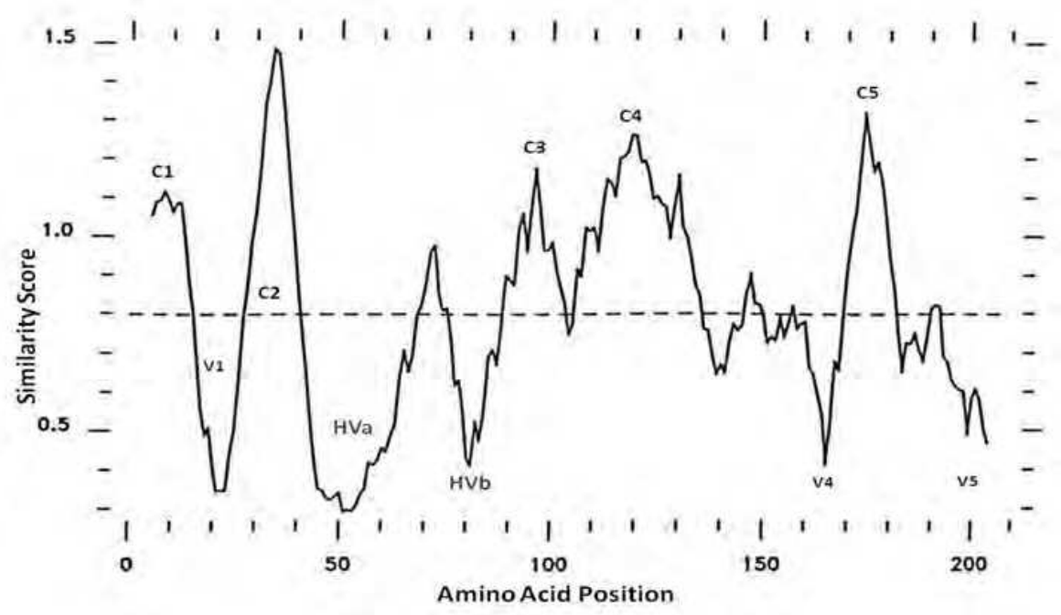

Fig. 2. **Graphical depiction of amino acid alignment among Solanaceae S-RNase alleles.** Amino acid sequences for eighteen S-RNase alleles were aligned using PlotSimilarity. The dotted line shows the average similarity score across the protein. Peaks above the line represent conserved regions (labeled C1 through C5). Valleys below the line represent more variable regions. Amino acids in hypervariable regions HVa and HVb were shown to be sufficient for determining S-RNase recognition specificity (after Sims, 1993).

Further work either analyzing spontaneous mutants (Royo et al., 1994) or using transgenic plants (Huang et al.,1994) demonstrated that ribonuclease activity of the S-RNase was required for pollen rejection. Royo et al. (1994) cloned and sequenced the S-RNase allele from a self-compatible S_cS_c accession of *Lycopersicon peruvianum* (now '*Solanum peruvianum*', http://solgenomics.net). The S_c allele sequence showed a change at amino acid 33 from the conserved histidine to asparagine. No other sequence changes were observed, and the authors concluded this change was correlated with both the loss of RNase activity in S_cS_c styles and with self-compatibility. In related work, Huang et al. (1994) used *in vitro* mutagenesis to construct a H93N variant of the *P. inflata* S_3-RNase, and introduced that construct in the S_1S_2 background. Unlike the results obtained when the wild-type S_3-RNase was transferred (Lee et al., 1994), the H93N S_3-RNase was unable to reject S_3 pollen. Reinforcing the critical role of ribonuclease activity in S-RNase function were earlier experiments indicting that degradation of pollen-tube RNA was associated with self-incompatibility. In those experiments (McClure et al., 1990) pollen RNA was labeled *in vivo* by watering plants with a solution containing ^{32}P-orthophosphate, then used for compatible or incompatible pollinations. Pollen tube RNA was degraded following incompatible pollination, but was not degraded following compatible pollination.

3.1.1 S-RNase recognition specificity is determined by hypervariable domains

Experiments investigating the basis for allele specificity in the S-RNase protein have focused on the role of the hypervariable regions. In one approach, Zurek et al. (1997) constructed

chimeric S-RNase genes having different combinations of S_{A2} and S_{C10} conserved and variable domains, then expressed the chimeric proteins in transgenic plants. Although the transgenic styles had ribonuclease activity levels equivalent to self-incompatible controls, none of the chimeric S-RNase constructs could reject S_{A2} or S_{C10} pollen. By contrast, Matton et al. (1997) took advantage of two S-RNase allles in *Solanum chacoense* that were closely related in sequence, to make more limited alterations. The S_{11}- and S_{13}-RNase alleles of *S. chacoense* differ by only 10 amino acids, three of which are found in HVa and one in HVb. Matton et al. (1997) used *in vitro* mutagenesis to change the four S_{11} residues in the HVa and HVb regions to those found in S_{13}, then expressed the altered allele transgenically in the $S_{12}S_{14}$ background. Pollination with S_{11} and S_{13} pollen demonstrated that changing only these residues changed the recognition specificity of the transferred S-RNase from S_{11} to S_{13}. In an extension of this experiment (Matton et al., 1999), changing only two residues in HVa plus the HVb residue resulted in a "dual-specificity" S-RNase that retained the ability to reject S_{11} pollen while acquiring the ability to also reject S_{13} pollen. These experiments demonstrated that, at least for these two alleles, amino acid sequences in the hypervariable regions determine allelic specificity. The protein crystal structure has been determined for the S_{F11} S-RNase of *Nicotiana alata* (Ida et al., 2001). The two hypervariable regions are located on the surface of the S_{F11} S-RNase and readily accessible to solvent (Ida et al., 2001). These regions include all four of the equivalent residues to those targeted in the mutagenesis experiments of Matton et al. (1997, 1999). Another potential basis for allele specificity might be variability in carbohydrate modification of S-RNases, which are glycoproteins (Woodward et al., 1989). This does not appear to be the case, however as as elimination of the glycosylation site has no effect on the ability of S-RNase to reject pollen (Karunanandaa et al.,1994).

3.1.2 Both self and non-self S-RNases are imported into pollen tubes in vivo

Immunolocalization experiments, either using traditional TEM (Luu et al., 2000) or fluorescently-tagged antibodies hybridized to paraffin-embedded sections (Goldraij et al., 2006) demonstrate that both incompatible and compatible S-RNases are imported into pollen tubes. The authors of these two studies reached different conclusions about the location of S-RNase inside pollen tubes following compatible or incompatible pollinations. Luu et al., (2000) working with self-incompatible potato (*S. chacoense*), fixed pollinated styles, 18 hr post-pollination, with 0.5% glutaraldehyde, followed by embedding, hybridization with anti-S_{11} antibody and 20 nm gold-labeled secondary antibody, and visualization via TEM. S_{11}-RNase was taken up into both compatible and incompatible pollen tubes, and labeling was seen primarily in pollen-tube cytoplasm, with little labeling in the pollen-tube vacuole. Goldraij et al. (2006), working with *Nicotiana*, hybridized anti-S-RNase antibodies along with anti-callose, anti-aleurain (marker for vacuolar lumen) and anti-vPPase (marker for vacuolar membrane) to fixed, paraffin-embedded sections, then visualized fluorescence using confocal microscopy. These authors concluded that S-RNase was initially sequestered in a vacuolar compartment in pollen in both compatible and early-stage (16 hr) incompatible pollen tubes, but that this compartment broke down at later stages (36 hr) of incompatible pollinations, releasing S-RNase into the pollen-tube cytoplasm.

3.1.3 The cytotoxic model for pollen rejection

Current models for pollen rejection in GSI propose a cytotoxic mechanism, where RNA degradation in incompatible pollen tubes reduces protein synthesis resulting in a slowing or cessation of pollen tube growth and failure of incompatible pollen tubes to reach the ovary. This model is based on observations outlined in the previous sections, but is not without its caveats. S-RNases are imported into pollen tubes, and at least in self-incompatible pollinations (Goldraij et al., 2006) are freely distributed in the pollen-tube cytoplasm. The ribonuclease activity of S-RNases is required for pollen tube rejection, and generalized RNA degradation is associated with self-incompatibility, but not with cross-pollination. The ability to reject incompatible pollen tubes is also dependent on a threshold level of S-RNase expression and accumulation in the style. Both developmental (Clark et al., 1990; Shivanna, 1969) and transgenic assays (Lee et al., 1994; Murfett et al., 1994, Zurek et al., 1997) show that styles expressing the S-RNase at low-to-moderate levels are incapable of rejecting otherwise incompatible pollen. S-RNases, like other T2 ribonucleases, show no obvious substrate specificity, at least *in vitro* (Singh et al., 1991).

The cytotoxic model, while attractive and consistent with the majority of current evidence, cannot, however, fully explain some other observations. Grafting experiments (Lush & Clarke, 1996) where upper regions of incompatible styles were grafted onto compatible styles, and pollinated, showed that incompatible pollen tubes could recover, growing out of the incompatible style into the compatible style. Also, Walles & Han (1998) using conventional TEM, observed intact polysomes in incompatible pollen tubes after pollination. Last, there is little correlation between overall ribonuclease activity found in different styles with the level of self-incompatibility (Clark et al., 1990; Singh et al., 1991; Zurek et al., 1997), although it should be assumed that non S-RNase ribonucleases likely contribute to overall style RNase levels.

3.2 SLF is the pollen-recognition component of GSI

Although the S-RNase was identified and cloned early on, it took an additional 18 years before the S-locus F-box protein (SLF) was conclusively identified as "pollen-S". Even today, what, functionally constitutes "pollen-S" is not fully understood; recent work suggests that different SLF variants may act collaboratively to recognize S-RNases. Additionally, several other proteins are involved and/or required for recognition (see sections below).

Even before SLF was identified and cloned, the majority of experimental evidence pointed to pollen-S as an inhibitor of S-RNase activity in compatible pollen. Tetraploidy is associated with the breakdown of self-incompatibility in those cases where the parental diploid plant was heterozygous for two different S-locus haplotypes, but not when the parent plant was homozygous (Chawla et al., 1997; de Nettancourt, 1977; Entani et al., 1999). In early studies, Brewbaker & Natarajan (1960) induced pollen-part mutants of *Petunia* using irradiation (pollen part mutants are self-compatible, fertile as pollen parents, and show normal GSI behaviour in the style). Pollen-part mutants were obtained only when the irradiated parent was heterozygous, and were associated with centric chromosome fragments. Golz et al (1999, 2001) revisited this work, carrying out mapping and cytological analyses of pollen-part mutants of *Nicotiana alata* induced by gamma radiation. In all cases, the pollen-part mutants were associated with apparent duplications of part or all of the S-locus, either as

centric chromosome fragments or as translocations. Luu et al. (2000), and later Goldraij et al. (2006) showed that both compatible and incompatible S-RNase proteins were imported into pollen tubes. Together, these results discredited a model where pollen-S was a "gatekeeper" preventing import of non-self S-RNases and suggested that compatible pollinations result from the specific inhibition of imported S-RNase proteins. According to models based on the results just described, pollen-S was an inhibitor of all S-RNases, except its own cognate S-RNase. Thus, compatible pollinations resulted from pollen-S inhibiting the action of any non-self S-RNase, while incompatible pollinations resulted from the inability of pollen-S to inhibit a co-evolved S-RNase.

3.2.1 Predictions for pollen-S

Prior to the actual isolation of pollen-S, there was a relative consensus with regard to the properties expected of this protein. Genetic studies had indicated that there was little or no recombination between pistil-S and pollen-S (de Nettancourt 1977), so both genes were expected to be tightly linked. That linkage, together with the observation that S-RNase alleles had diverged prior to speciation in the Solanaceae (some S-RNase alleles are more similar across species than within species) resulted in the assumption that S-RNase sequences and pollen-S sequences should be co-evolved. That is, most researchers fully expected that the degree of polymorphism among pollen-S alleles should be roughly equivalent to the polymorphism observed among S-RNase alleles (Kao & McCubbin, 1996). Pollen-S was also thought to interact directly with the S-RNase, with that interaction resulting in the inhibition of the action of the S-RNase in compatible pollinations. The sections below will illustrate that these assumptions were only partially correct.

3.2.2 Genetic and physical mapping of the S-Locus

The first attempt at mapping the S-locus was carried out by Tanksley and Loaiza-Figueroa (1985) who mapped the S-locus to chromosome I of *Lycopersicon peruvianum* using enzyme-linkage. RFLP mapping in potato (Gebhardt et al., 1991) demonstrated that chromosome I of tomato and potato were homeologous, and that the S-locus mapped to the same location in potato as in tomato. The S-locus was physically mapped in *Petunia hybrida* by fluorescence in-situ hybridization (FISH), using T-DNA inserts linked to the S-locus (ten Hoopen et al., 1998). Those experiments showed that in *Petunia hybrida*, the S-locus was located in a sub-centromeric region of chromosome III. Mapping of linked RFLP markers demonstrated synteny of the S-locus across four species in the Solanaceae (*Lycopersicon peruvianum, Nicotiana alata, Petunia hybrida* and *Solanum tuberosum*). Entani et al. (1999) carried out similar FISH experiments, but used cDNAs and genomic clones of the S-RNase instead of linked T-DNA inserts. Like ten Hoopen et al. (1998), Entani et al. (1999) found that the S-RNase gene was found in a subcentromeric region of chromosome III of *Petunia hybrida*. Both Li et al. (2000) and McCubbin et al. (2000) used RNA differential display to identify pollen-expressed cDNAs linked to the S-locus. Although not realized at the time, both of these differential display experiments identified cDNAs that would later turn out to true pollen-S genes. Part of the failure to recognize that these linked cDNAs did, in fact, encode pollen-S was the high degree of sequence identity between cDNAs isolated from different haplotypes as compared to the polymorphism previously observed for S-RNase alleles.

3.2.3 Gene walking identified pollen-S

The large amount of repetitive DNA sequences flanking S-RNase genes (Coleman & Kao, 1992; Matton et al., 1995), together with the subcentromeric location of the S-locus (ten Hoopen et al., 1998; Entani et al., 1999) were originally thought to preclude a map-based cloning approach for isolation of pollen-S (Kao & McCubbin 1996). Indeed, some of the early efforts to clone pollen-S involved T-DNA tagging (Harbord et al., 2000) yeast two-hybrid screens (Sims & Ordanic, 2001) or other protein-interaction methods (Dowd et al., 2000). Although these approaches provided important information regarding S-RNase-based GSI, none of them resulted in the identification of pollen-S. The first indication that pollen-S might be cloned using a map-based approach came from the work of Ushijima et al. (2001) who constructed an ~200 kb cosmid contig around the S-locus of *Prunus dulcis* (almond). When these authors carried out Southern blots with cosmid clones spanning the contig, with genomic DNA of different S-locus haplotypes, they observed that a ~70 kb region in the center of the contig was highly polymorphic across haplotypes, whereas either end of the contig showed a high degree of sequence similarity across haplotypes. This "island of polymorphism" presumably resulted from the known lack of recombination at the S-locus and was taken as defining the physical limits of the S-locus in *Prunus dulcis.*

Lai et al. (2002) were the first to report the isolation of the S-locus F-box gene, which would turn out to be pollen-S. Screening of a BAC library from *Antirrhinum hispanicum* with the S_2-RNase identified a 63 kb BAC clone, which was then fully sequenced. Of several putative ORFs identified, most were retrotransposons, however, the 'gene-11' ORF, when used to screeen a cDNA library, identified a pollen-expressed F-box protein, termed AhSLF-S_2. AhSLF-S_2 was located 9 kb downstream of the S_2-RNase gene, and appeared to be allele-specific, making it a good candidate for pollen-S. Similarly, Ushijima et al. (2003) sequenced the 70 kb region of *Prunus dulcis*, and identified a pollen-expressed, haplotype-specific, F-box gene, which they termed SFB. Using S-locus-specific cDNAs previously generated, and starting with BACs known to contain the S-RNase gene, Wang et al. (2004) identified an 881 kb contig surrounding the S-locus in *Petunia inflata.* Sequencing and analysis of a 328 kb region of this contig identified several genes, one of which, PiSLF, was highly similar to the F-box genes isolated from *Antirrhinum* and *Prunus*. Two previously identified S-linked F-box genes *A113* and *A134* (McCubbin et al., 2000) mapped outside of the 881 kb region.

3.2.4 Competitive interaction showed that SLF was pollen-S

The identity of SLF as pollen-S was confirmed by taking advantage of the phenomenon of competitive interaction in heteroallelic pollen (see section 2.2 and Figure 1). Sijajic et al., transferred the S_2-allele of SLF (PiSLF$_2$, but see nomenclature change in section 5.x, below) into a S_1S_1 line of *Petunia inflata*. First generation transgenic plants segregated two types of pollen, haploid S_1 pollen and heteroallelic S_1(PiSLF$_2$) pollen.. Self-pollination of the the S_1S_1(PiSLF$_2$) plant produced large fruits, indicating that the trangenic plant, formerly self-incompatible, was now self-compatible. Similarly, when S_1S_1(PiSLF$_2$) pollen was used to pollinate a non-transformed S_1S_1 plant, fruit set showed that the S_1S_1(PiSLF$_2$) pollen behaved as compatible pollen. Conversely, pollination of S_1S_1(PiSLF$_2$) styles with pollen from a non-transformed S_1S_1line produced no seed capsules, demonstrating that the loss of self-incompatibility in the

transgenic plant was confined to the pollen. Analysis of progeny resulting from self-pollination demonstrated that all of the progeny inherited the transgene. Similar results were reported by Qiao et al. (2004b) who transferred the Ah-SLF_2 gene of *Antirrhinum hispanicum* into $S_{3L}S_{3L}$ *Petunia hybrida.* This experiment was conducted with two variations. In the first variation, a clone containing both Ah-S_2-RNase and Ah-SLF_2 was transferred to the host plant. Transgenic plants expressing both the *A. hispanicum* S-RNase and SLF were self-compatible, with the change in compatibility again confined to the pollen. Analysis of progeny confirmed that all inherited both the S_2-RNase and Ah-SLF_2. The change to the self-compatible phenotype was dependent on expression of the transgenes. In two individuals, neither the S_2-RNase transgene nor the endogenous S_{3L}-RNase was expressed at detectable levels, most likely due to co-suppression. Both of these progeny were completely self-incompatible. In the second variation, the Ah-SLF_2 cDNA alone, under control of the pollen-specific LAT52 promoter, was introduced into the $S_{3L}S_{3L}$ line. As above, the transgenic plants were self-compatible on the pollen side, but displayed normal self-incompatibility behavior when used as the style parent. These conversions of self-incompatibility to compatibility following pollen-specific expression of the SLF transgene is a direct phenocopy of the competitive interaction effect seen in heteroallelic pollen in tetraploid heterozygotes. One remarkable aspect of the work reported by Qiao et al (2004b) is that the *Antirrhinum* SLF protein can apparently cause competitive interaction in a completely different species.

3.2.5 SLF proteins appear to have different evolutionary history than S-RNases

Although many of the key predictions for the properties of pollen-S are indeed found for the SLF proteins (pollen-expression, interaction with S-RNases, competitive interaction), a surprising and confusing finding was the distinct lack of polymorphism among SLF proteins, together with the existence of multiple SLF-related proteins, originally termed SLFL (SLF-like) proteins. As will be discussed below (see section 8), many of these SLFL proteins may turn out to be true SLFs. Another confusing finding was apparent differences in the functional characteristics of SLF proteins in Solanaceae and Plantaginaceae compared with the equivalent SFB proteins in Rosaceae.

The first SLF proteins identified (Ah-SLF_1, Ah-SLF_2, Ah-SLF_4, Ah-SLF_5 in *Antirrhinum*) share 97% to 99% amino acid sequence identity. By contrast the related *Antirrhinum* S-RNase proteins share only 38% to 53% amino acid identity by pairwise BLASTp. Similarly, if not quite so dramatically, SLF proteins from *Petunia inflata* share ~ 90% amino acid sequence identify, while the corresponding S-RNase proteins share only about 70% amino acid sequence identity. Phylogenetic comparisons (e.g. Newbigin et al., 2008) present an even more striking picture. S-RNase sequences appear to be an ancient lineage; in the Solanaceae, S-RNases from one species are often more similar to a S-RNase from a different species than to other S-RNases within the same species. By contrast SLF sequences from an individual species cluster together. In addition, while the variability across S-RNases is clustered in variable and hypervariable regions (Figure 2), the variability across SLF alleles appears to be uniformly distributed across the protein. Because recombination between style and pollen recognition specificities is rarely if ever observed (de Nettancourt, 1977) the traditional assumption has been that pistil-S and pollen-S (S-RNase and SLF) have co-evolved and share the same evolutionary history. The actual observations, however appear to contradict

that notion. One potential solution to this dilemma is that pollen-S may actually be comprised of multiple SLF protein variants, not a single SLF (see section 8).

4. Interaction assays identified other pollen proteins with roles in GSI

S-RNase and SLF are the pistil and pollen recognition components of GSI, however several other proteins with presumed or demonstrated roles in GSI have been identified and studied. Some of these proteins were first identified by protein-interaction screens with S-RNase or SLF, in other cases, a presumed role in GSI has been demonstrated using protein interaction assays. Figure 3 summarizes the interactions of pollen-expressed proteins with the S-RNase, specifics of these interactions are discussed below.

4.1 SBP1

Sims and Ordanic (2001) identified PhSBP1 (S-ribonuclease binding protein) in a yeast two-hybrid screen of a pollen cDNA library from S_1S_1 *Petunia hybrida.* The bait protein used for the screen was the N-terminal half of the S_1-RNase, containing domains C1 to C3 (see Figure 2). In subsequent pairwise interaction assays, PhSBP1 interacted with the same N-terminal construct of the S_3-RNase, and with subdomains (C2-HVa-HVb-C3, HVa-HVb) of both S_1- and S_3-RNases.

SBP1 did not show interaction with the C-terminal regions of either S-RNase (C4-V4-C5-V5 in Figure 2) nor with an unrelated bait protein, P53. ScSBP1 was isolated from *Solanum chacoense* (O'Brien et al., 2004), using a yeast two-hybrid screen with the HVa+HVb regions of the S_{11}-Rnase and the S_{13}-RNase as bait. Both the S_{11} and S_{13} baits interacted with ScSBP1, however a full-length S-RNase with a single amino acid change (H144L) at one of the active-site histidines failed to interact with SBP1. Similarly Hua & Kao (2006) used a partial bait (HVa-HVb-C3) of the *Petunia inflata* S2-RNase to screen a two-hybrid library and isolated PiSBP1. Similar to other reports (O'Brien et al., 2004; Sims & Ordanic, 2001) PiSBP1 did not interact with full-length S-RNase, with non-specific controls, or importantly, with a non-S-locus ribonuclease. Hua & Kao (2006) further showed that SBP1 interacted with $PiSLF_2$ and $PiSLF_1$ in both two-hybrid and pull-down assays, as well as with Cullin-1 and PhUBC1 (Sims, unpublished), an E2 conjugation enzyme protein from *Petunia hybrida*. Lee et al. (2008) used C-terminal domains of the style-transmitting-tract proteins TTS and 120K to screen a pollen two-hybrid library from *Nicotiana alata*, and also pulled out NaSBP1 from this screen. All of these reports (Hua & Kao, 2006; Lee et al., 2008; O'Brien et al., 2004; Sims & Ordanic 2001) showed that SBP1 was not pollen-specific, but was expressed in all tissues examined. SBP1 is non-allelic as well, as SBP1 isolated from S_1S_1 and S_3S_3 lines of *Petunia hybrida* are 100% identical. The SBP1 protein has two identifiable protein domains, a coiled-coil domain in the center of the sequence and a C-terminal RING-HC domain. RING-HC domains are characteristic of E3 ubiquitin ligases (Freemont 2000), and SBP1 has E3 ubiquitin ligase activity *in vitro* (Hua and Kao, 2008).

4.2 SSK1

Huang et al. (2006) used the *Antirrhinum hispanicum* SLF protein Ah-SLF_2 to screen a pollen yeast-two-hybrid library, and identified a SKP1-like protein that they named SSK1. AhSSK1

interacted with both Ah-SLF$_2$ and Ah-SLF$_5$ but not with proteins identified as SLF paralogs (Zhou et al., 2003). AhSSK1 futher interacted with a Cullin-1 protein. Sequence and phylogenetic analyses showed that AhSSK1 was related to, but distinct from canonical SKP1 proteins. In particular, AhSSK1 differed at several internal residues and also has a 7-residue C-terminal "tail" that extends beyond the "WAFE" sequence that terminates most plant SKP1 proteins (Huang et al., 2006; Zhao et al, 2010). Zhao et al. (2010) showed that SSK1 almost certainly plays a critical role in self-incompatibility. Using AhSSK1 as a guide, they isolated PhSSK1 from *Petunia hybrida.* PhSSK1, similar to AhSSK1 interacts with both SLF and Cullin-1 from *Petunia.* To directly test the role of PhSSK1, Zhao et al. (2010) used a RNAi construct of PhSSK1 to down-regulate this gene in $S_{3L}S_{3L}$ *Petunia hybrida.* When transgenic plants showing reduced levels of PhSSK1 in pollen were used as the pollen parent in crosses to S_1S_1 or S_vS_v *P. hybrida*, no seed capsules were produced. Conversely, when these same lines were used as pollen parent to a line defective for S-RNase expression (S_oS_o) normal seed capsules were produced, suggesting that down-regulation of SSK1 specifically affected cross-compatibility in the self-incompatibility response.

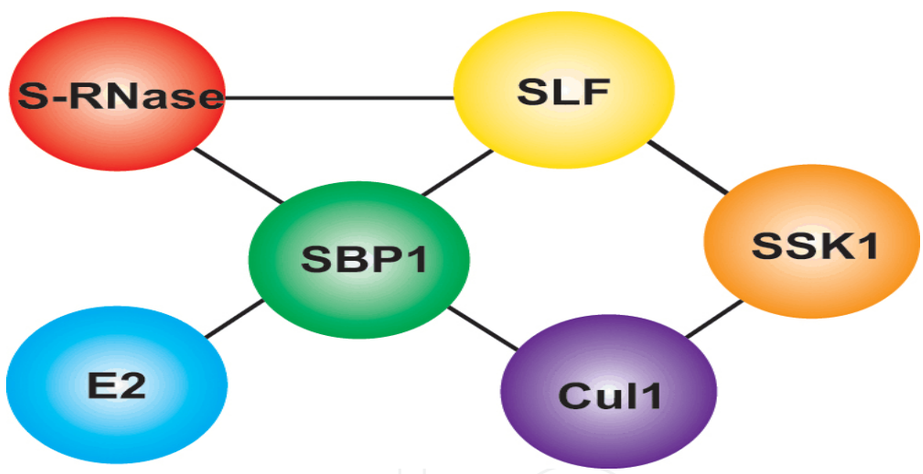

Fig. 3. **Protein interactions of pollen-expressed proteins in GSI.**
Lines between individual proteins indicate protein interactions that have been observed by yeast two-hybrid or pull-down assays. See the text for details.

5. Protein interactions between SLF and S-RNase proteins

S-locus F-box proteins were first cloned by chromosome-walking experiments to identify pollen-expressed proteins tightly linked to the S-RNase. Further studies examined the interaction between SLF proteins and S-RNases in detail. Qiao et al. (2004a) examined the interaction of the *Antirrhinum* Ah-SLF-S_2 protein with S-RNases using pull-down (His-tag), yeast two-hybrid, and co-immunoprecipitation assays. Pull-down assays demonstrated that the C-terminal portion of Ah-SLF-S_2 (lacking the F-box domain) interacted with S-RNase from style extracts of *Antirrhinum hispanicum*. The N-terminal F-box domain was incapable

of interacting with S-RNase, and the full-length SLF protein could not be tested as it could not be expressed in *E. coli*. Similarly, the C-terminal portion of Ah-SLF-S_2 interacted with a full-length (lacking the signal peptide) S-RNase construct in yeast two hybrid assays; neither the F-box domain nor the full-length SLF protein showed interaction in the two-hybrid assays. Both of these assays also showed that Ah-SLF-S_2 interacted with different S-RNases, without any apparent allelic specificity. Co-immunoprecipitation assays where extracts from pollinated styles were immunoprecipitated using anti-Ah-SLF-S_2 antibody, then blotted with anti-S-RNase antibody showed that Ah-SLF-S_2 interacted with both S_2-and S_4-RNase *in vivo.* Qiao et al., (2004a) also tested interaction of Ah-SLF paralogs (Zhou et al., 2003) with S-RNase proteins. The SLF paralogs were identified as pollen-expressed SLF-like genes linked to the S-locus but distant from S-RNase or Ah-SLF-S_2. Similar SLF-like genes linked to the S-locus but outside of the core S-RNase-SLF contig had previously been identified in *Petunia inflata* (McCubbin et al., 2000; Wang et al., 2003). No interaction was observed between Ah-SLF-S_2 and any of the SLF paralogs. Recent data (see section 8) indicates the SLF-like genes (SLF paralogs) may, however be true SLF proteins, that recognize a subset of S-RNases rather than all S-RNases.

Hua and Kao (2006) also tested interactions between SLF and S-RNase allles in *Petunia inflata* using pull-down assays with His-tagged SLF and GST-tagged S-RNase constructs expressed in bacteria. These assays showed that both PiSLF$_1$ and PiSLF$_2$ interacted with the HVaHVbC3domain of the S_2-RNase, but that the non-self interactions (PiSLF$_1$:S_2-RNase) were far stronger than the self interactions (PiSLF$_2$:S_2-RNase) interactions. Similarly the reciprocal interactions of S_1- and S_2-RNase domains with His-tagged PiSLF$_1$ while showing some interaction in both cases, were far stronger for the non-self pairs than the self-pairs. Sims et al., (2010) used a fluorogenic substrate for β-galactosidase to quantify the strength of two-hybrid interactions between different domains of the S_1- and S_3-RNase of *Petunia hybrida* with SLF-S1 from *P. hybrida.* Similar to the results obtained by Hua & Kao (2006), both self and non-self S-RNases interacted with *P. hybrida* SLF-S1, but the interaction appeared stronger for the non-self interactions compared with the self-interactions.

One of the critical questions in GSI is that of how SLF and S-RNase alleles recognize each other as self versus non-self. This question has recently become even more complicated (see section 8) as it appears that proteins originally identified as SLF-like (SLFL), and not involved in GSI, may in fact be true SLF proteins. Chromosome-walking, differential display or degenerate PCR-cloning approaches (McCubbin et al., 2000; Wang et al., 2003,2004; Wheeler & Newbigin 2007; Zhou et al., 2003) in the Solanaceae and Plantaginaceae identified a number of SLF-like genes (SLFL) that were linked to the S-locus. These genes were thought not to be involved in self-incompatibility interactions specifically, since they did not show interaction with known S-RNases nor did they exhibit competitive interaction in transgenic assays (Hua et al., 2007; Meng et al., 2011). Hua et al., (2007) attempted to identify domains of SLF proteins that governed allelic specificity by iterative pairwise comparisons of SLF proteins with SLFL proteins. These comparisons identified three "SLF-specific" regions SR1, SR2 and SR3. Based on this identification, these authors then divided the SLF proteins into three domains: FD1, containing the F-box and SR1 (amino acids 1-110), FD2 (amino acids 111-259, including the SR2 region) and FD3 (amino acids 260-389, including SR3). Domain-swapping experiments, in which different chimeric proteins were

tested for the ability to interact with the S_3-RNase in pull-down assays suggested that FD2 was the domain primarily responsible for SLF-S-RNase interactions. Testing chimeric constructs between $PiSLF_2$ and PiSLFLb-S_2 (a SLF-like protein in the same S_2 haplotype as $PiSLF_2$) failed to demonstrate functionality of the FD2 domain *in vivo* (Fields et al., 2010). That is, neither chimeric protein showed competitive interaction in transgenic assays. Given that most SLFL proteins now appear to be bona fide SLF variants (see section 8), the long-term significance of these assays is unclear. The different SLFL proteins used for sequence comparisons represent different classes of SLF variants (section 8) so that the "SLF-specific" domains identified may represent regions that are more similar within a particular SLF-variant class.

6. A role for ubiquitination in gametophytic self-incompatibility

The observed protein interactions described above, together with the properties of SLF, SBP1 and SSK1 all suggest that recognition of self versus non-self in gametophytic self-incompatibility involves ubiquitination pathways. Pollen-S (SLF), is an F-box protein, and F-box proteins are know the be the recognition component of SCF E3 ubiquitin ligases (Cardozo & Pagano, 2004; Hua et al., 2008; Sijacic et al., 2004). SBP1 (Hua & Kao 2006; Sims & Ordanic 2001; Sims et al., 2010) is a RING-HC protein. RING proteins are E3 ubiquitin ligases (Deshaies & Joazeiro 2009; Freemont 2000), and SBP1 has E3 ubiquitin ligase activity *in vitro* (Hua et al., 2007; Sims unpublished). AhSSK1 (Huang et al., 2006) and PhSSK1 (Zhao et al., 2010) are SKP1-like proteins (SKP1 is a scaffold component of SCF E3 ligases). Pollen extracts have been shown to ubiquitinate S-RNase proteins, albeit in an allele-independent manner (Hua & Kao 2006). Together, these results have lead to the proposal that a non-canonical SCF^{SLF}-like complex acts to recognize and ubiquitinate S-RNases, leading to the inhibition of S-RNase activity in compatible pollen tubes. This complex is proposed to differ from a canonical SCF complex, because neither SKP1 orthologues (Hua & Kao 2006; Huang et al., 2006; Zhao et al., 2010) or RBX1 (Hua & Kao 2006) interact with SLF or Cullin. Instead either (or both) SBP1 and SSK1 have been proposed to replace RBX1 and/or SKP1 in this complex (Sims 2007; Hua et al., 2008; Sims & Robbins 2009; Zhao et al., 2010). According to the simplest version of this model, recognition of non-self S-RNases by the SCF^{SLF} complex would lead to polyubiquitination and degradation of S-RNase by the 26S proteasome complex (Sims 2007; Hua et al., 2008). One prediction of the SCF^{SLF} ubiquitin ligase complex model is that down-regulation of SLF, SBP1 or SSK1 should render all pollen tubes incompatible, regardless of genotype. To date, down-regulation of SLF or SBP1 has not been reported. Down-regulation of PhSSK1 (Zhao et al., 2010) does, however, result in a switch from compatibility to incompatibility, in accordance with this model. Figure 4 summarizes the structure of the proposed SCF^{SLF} ubiquitin ligase complex.

7. Style-expressed proteins with roles in GSI

Several style-expressed proteins have been shown to either be required for pollen rejection, or to interact with S-RNase or SBP1 in different assays. Cruz-Garcia et al., (2005) immobilized the S_{c10}-RNase from *Nicotiana alata* on an Affi-Gel column, then tested the ability of different proteins from style extracts to bind to the immobilized S-RNase. NaTTS, Na120K and NaPELPIII stylar proteins all bound to the S_{c10}-RNase in a specific manner. All three of these

are previously-characterized proteins that are secreted into the transmitting tract of the style and that interact with pollen tubes. Biochemical data suggests that these proteins and the S-RNase may form a complex that is taken up into pollen tubes. In an extension of these experiments, Lee et al., (2008) used the C-terminal domains of the TTS and the 120 K proteins in yeast two-hybrid screens of pollen cDNA libraries. In addition to interaction with SBP1 (see section 4.2) a putative cysteine protease, NaPCCP, interacted with both TTS and 120K. Figure 5 summarizes observed protein interactions of the style proteins.

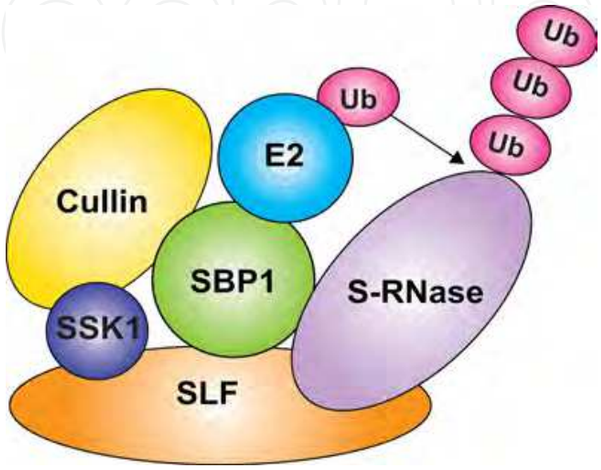

Fig. 4. **Proposed SCF^{SLF} ubiquitin ligase complex.**
Components of a proposed SCF^{SLF} complex are illustrated digrammatically. Specific contacts between components are based on protein-interaction assays summarized in Figure 3.

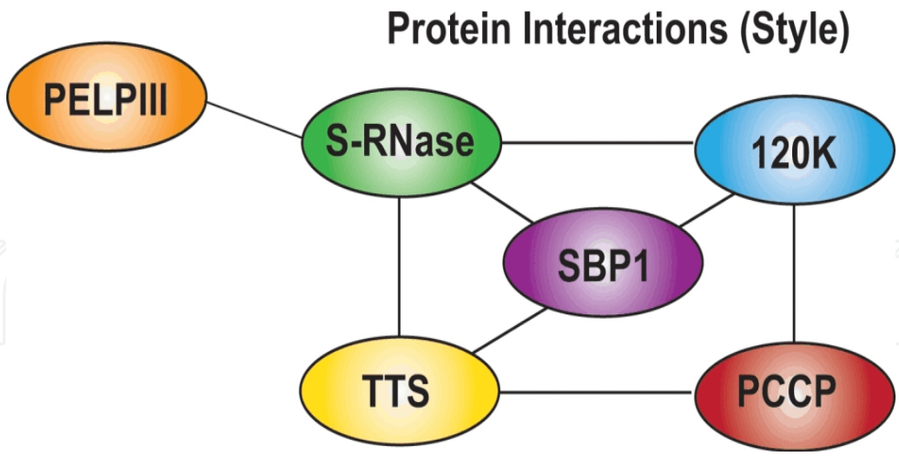

Fig. 5. **Interactions of the style proteins TTS, 120K and PELPIII with S-RNase and the pollen-expressed proteins SBP1 and PCCP.**

Two proteins, the 120k protein and a small asparagine-rich protein HT-B are required for the ability to reject pollen tubes in GSI (Hancock et al., 2005; McClure et al., 1999; O'Brien et al., 2002). Down-regulation of these genes by antisense (McClure et al., 1999) or RNAi (Hancock et al., 2005; O'Brien et al., 2002) resulted in an inability to block pollen tube

growth, even though S-RNase proteins were expressed at normal levels. The 120K protein interacts with S-RNase and may be imported into pollen tubes in a complex with S-RNase and other style proteins (Cruz-Garcia et al., 2005). HT-B is also imported into pollen tubes (Goldraij et al., 2006), but whether in a complex with S-RNase and other style proteins, or separately, is not known.

8. Collaborative non-self recognition by SLF proteins in GSI

Although SLF proteins fulfill many of the expectations of pollen-S, different lines of evidence suggested that the nature of pollen-S may be more complicated than previously thought. First, as described, the evolutionary history of SLF proteins appeared far different than that of the linked S-RNase proteins, with the SLF proteins having evolved more recently and showing no evidence of co-evolution with S-RNase proteins as previously expected (Newbigin et al., 2008). Further, multiple SLF-like genes had been identified in *Petunia, Antirrhinum* and *Nicotiana* which appeared to be linked to the S-locus if not as close to the S-RNase as was SLF. It was unclear how the high degree of sequence identity among different SLF proteins could account for the ability to recognize and inhibit multiple different S-RNase alleles in populations.

Kubo et al. (2010) cloned additional SLF alleles from *Petunia hybrida*. [It should be noted here that *Petunia hybrida* is an "artificial" species created in the 19th century by crossing *Petunia axillaris* x *Petunia integrifolia. Petunia inflata* has been viewed either as synonymous with, a subspecies of, or very closely related to *Petunia integrifolia* (Stehmann et al., 2009). Thus S-locus haplotypes found in *Petunia hybrida* should be identical to those in either of the progenitor species.] When the SLF protein from *Petunia hybrida* S_7 haplotype was sequenced, it was found to be identical with the previously isolated SLF from *Petunia axillaris* S_{19}. What was striking, however was that the two S-RNases in these lines (S_7 versus S_{19}) were substantially different, having only 45% amino acid sequence identity. Reciprocal pollinations between S_7 and S_{19} confirmed that these two lines indeed had separate S-locus haplotypes. These results suggested that additional genes beyond SLF might constitute pollen-S. Further testing of SLF-S_7 showed that it could not cause competitive interaction in S_5S_7, S_7S_{11} or S_5S_{19} plants, but that it did show competitive interaction in S_7S_9 plants as well as in S_5S_{17} plants (Kubo et al., 2010 and supplemental material). Thus it appeared that individual SLF proteins could cause competitive interaction (i.e. act as pollen-S) with a limited subset of S-RNase alleles. Further analysis of proteins previously identified as SLF-like genes showed that these too, reacted with different subsets of S-locus haplotypes to cause competitive inhibition. Protein interaction assays demonstrated that there was direct correlation between SLF-S-RNase interaction and the ability to show competitive interaction in diploid heteroallelic pollen. Additional sequence comparisons demonstrated that SLF proteins could be grouped into at least six subclasses. Because Wheeler & Newbigin (2007) identified at least 10 different SLF-like genes in *Nicotiana*, and because not all of the previously-identified SLFL genes from *Petunia inflata* were included in the comparative sequence analysis of Kubo et al. (2010), it is possible that more than six SLF subclasses are present. As a result of this analysis, a new nomenclature for SLF proteins has been proposed. The original SLF isolates (e.g. $PiSLF_1$, $PiSLF_2$ etc.) have been renamed as the SLF1 variant class. Thus $PiSLF_1$ has been renamed S_1-SLF1, $PiSLF_2$ is now S_2-SLF1 and so on.

Genes previously identified as encoding SLFL proteins now comprise SLF2, SLF3, SLF4, SLF5, SLF6 and possibly additional SLF classes. The general nomenclature is thus $S_{haplotype\ ID}$-SLF(class).

These results led Kubo et al. (2010) to propose a "Collaborative Recognition" model for the interaction of SLF variants with different S-RNases. According to this model, different SLF variants can recognize separate but partially overlapping subsets of S-RNases. Thus S_7-SLF1 reacts with S_{17}-RNase and S_9-RNase, but not with S_{11}- or S_{19}-RNase, S_7-SLF2 reacts with S_9-, S_{11}- and S_{19}-RNases, but not with S_{17}-RNase. One important area of future research (see below) will be to further dissect the complexities of protein interactions between different SLF variants and S-RNase proteins.

9. Models for pollen recognition and rejection in GSI

At present, two different, but non-exclusive models have been proposed to explain the mechanism of pollen acceptance versus pollen-rejection in gametophytic self-incompatibility. Both models presume that incompatible pollen tubes are rejected via the cytotoxic action of the S-RNase, and that self-compatibility (or cross-compatibility) results from inhibiting S-RNase action, consistent with the presumed role of pollen-S as an inhibitor. Where the models differ is in the primary mechanism for S-RNase inhibition, as well as the "default" condition of pollination. One model proposes that pollen-S (SLF) acts via the SCF^{SLF} E3 ubquitin ligase complex to polyubiquitinate S-RNases, resulting in degradation by the 26 S proteasome complex. In this model, self-incompatibility (pollen rejection) would be the default pathway, unless the S-RNase is inhibited. The alternative model proposes that S-RNase imported into pollen tubes is sequestered in a vacuolar-like compartment. In this model, the default pathway is compatibility, unless SLF-S-RNase recognition leads to a breakdown of the compartment and release of the S-RNase.

9.1 The ubiquitination-degradation model

Much of the evidence for this model comes from protein-interaction assays, along with the known characteristics of the interacting proteins. Pollen-S (SLF), is an F-box protein, the recognition component of SCF E3 ubiquitin ligases (Cardozo & Pagano, 2004; Sijacic *et al.* 2004; Hua *et al.* 2008). SBP1 (Sims & Ordanic 2001; Hua & Kao 2006; Patel 2008, Sims *et al.*, 2010) is a RING-HC protein. RING proteins are E3 ubiquitin ligases (Freemont 2000, Deshaies & Joazeiro 2009), and SBP1 has E3 ubiquitin ligase activity *in vitro* (Hua *et al.* 2007; Sims unpublished). AhSSK1 (Huang *et al.* 2006) and PhSSK1 (Zhao *et al.* 2010) are SKP1-like proteins (SKP1 is a scaffold component of SCF E3 ligases). Pollen extracts have been shown to ubiquitinate S-RNase proteins, albeit in an allele-independent manner (Hua & Kao 2006). Together, these results have lead to the proposal that a non-canonical SCF^{SLF}-like complex acts to recognize and ubiquitinate S-RNases, leading to the inhibition of S-RNase activity in compatible pollen tubes. This complex is proposed to differ from a canonical SCF complex, because neither SKP1 orthologues (Hua & Kao 2006; Huang *et al.* 2006; Zhao et al 2010) or RBX1 (Hua & Kao 2006) interact with SLF or Cullin. Instead either (or both) SBP1 and SSK1 have been proposed to replace RBX1 and/or SKP1 in this complex (Sims 2007; Hua *et al.* 2008; Sims & Robbins 2009; Zhao *et al.* 2010). According to the simplest version of this model, recognition of non-self S-RNases by the SCF^{SLF} complex would lead to

polyubiquitination and degradation of S-RNase by the 26S proteasome complex (Sims 2007; Hua et al., 2008). One prediction of the SCF^{SLF} ubiquitin ligase complex model is that down-regulation of SLF, SBP1 or SSK1 should render all pollen tubes incompatible, regardless of genotype. To date, down-regulation of SLF or SBP1 has not been reported. Down-regulation of PhSSK1 (Zhao *et al.* 2010) does, however, result in a switch from compatibility to incompatibility, in accordance with this model.

Although the ubiquitination-degradation model is attractive, several predictions of this model remain untested, and other predictions may (depending on interpretation) be contradicted by current evidence. The pattern of ubiquitination of the S-RNase *in vivo* is not known. Because K48-linked or K63-linked polyubiquitination, or monoubiquitination leads to different cellular outcomes for the tagged proteins, it will be important to determine what ubiquitination patterns occur in reponse to SLF:S-RNase interaction. Also, it is not clear whether large-scale degradation of S-RNase proteins occurs in compatible pollinations. The high level of secreted extracellular S-RNase that accumulates in the transmitting tract make it challenging to monitor the level of S-RNase proteins in pollinated styles. As stated earlier, the degradation model predicts that inactivation or down-regulation of SCF^{SLF} E3 ubiquitin ligase components should result in pollen rejection. This prediction appears to be sustained in the case of SSK1. Different SFB mutants characterized in the Rosaceae, however (Marchese et al., 2007; Sonneveld et al., 2005; Ushijima et al., 2004; Vilanova et al., 2006), all of which either truncate or delete the SFB protein, are self-compatible. Although these data (along with some other differences between Solanaceae/Plantaginaceae versus Rosaceae) have been interpreted as suggesting that GSI has a different mechanistic basis in these taxa, there is also a large degree of similarity in how GSI functions in Solanaceae/Plantaginaceae versus Rosaceae (e.g., S-RNase, F-box proteins) such that it may be premature to make a definitive judgement on that point (McClure et al., 2011). Figure 6 summarizes the basic ubiquitination-degradation model.

9.2 The sequestration model

Evidence for this model comes primarily from the work of Goldraij et al. (2006), who reported that S-RNase was sequestered in a vacuolar compartment in compatible pollinations. These authors fixed and paraffin-embedded pollinated styles of *Nicotiana alata*, then hybridized sections to antibodies for callose (pollen-tube cell wall marker), S_{c10}-RNase, 120K protein, HT-B, aleurain (vacuolar lumen marker) or vPPase (vacuolar membrane marker). They concluded that in a compatible pollination, S-RNase inside pollen tubes remained in a ribbon-like vacuole bounded by the 120K protein. HT-B levels in compatible pollinations were low or undetectable. In later stages of incompatible pollinations, conversely, S-RNase appeared to be released into the cytoplasm of pollen tubes, and HT-B levels remained higher than in compatible pollinations (Goldraij et al., 2006; McClure et al., 2011). This model is consistent with the results of RNAi down-regulation of HT-B and 120K, which prevent rejection of incompatible pollen. What this model only incompletely explains is the required S-RNase::SLF interaction leading to compatibility or incompatibility. Both genetic evidence and the protein-interaction data summarized in previous sections show that S-RNase and SLF must interact to determine self/non-self recognition. If S-RNase is sequestered in a vacuolar compartment, however and SLF is cytoplasmic, it is not clear how

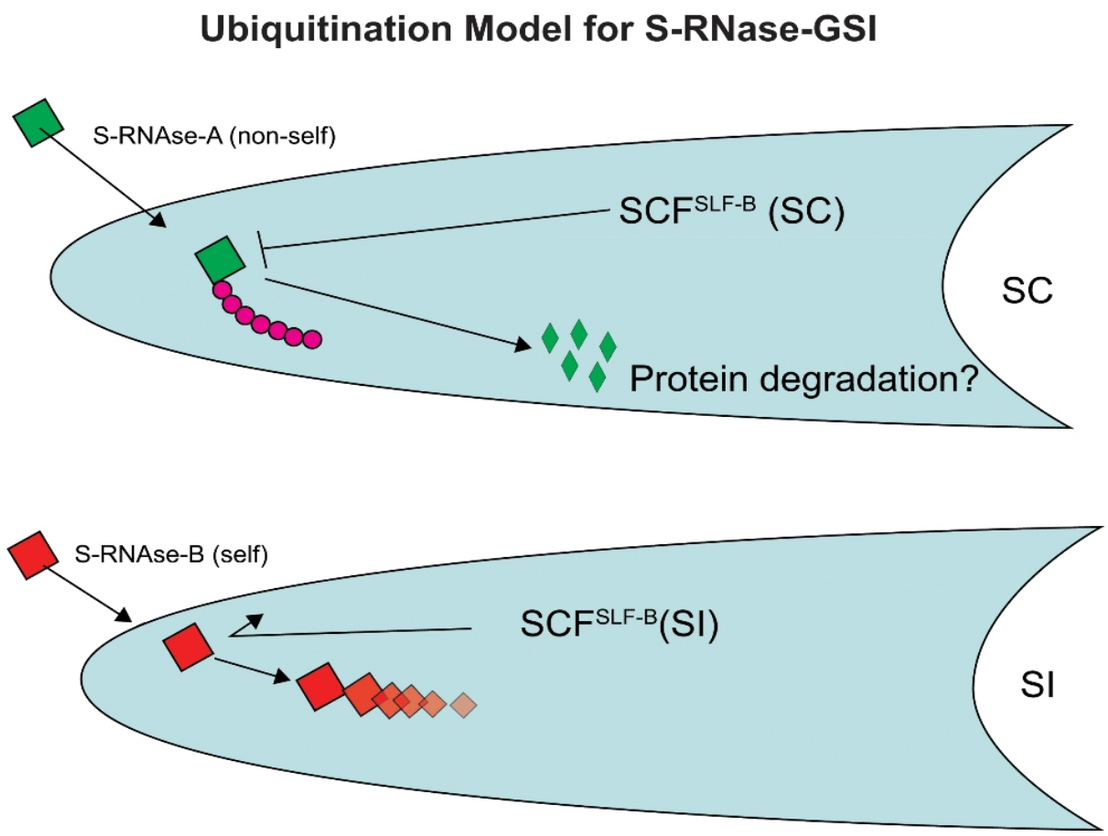

Fig. 6. **Ubiquitination-degradation model for gametophytic self-incompatibility.** According to this model, both non-self (S-RNase-A) and self (S-RNase-B) proteins are imported into pollen tubes (the mechanism of import is not defined, but probably does not involve a specific receptor). In compatible (non-self) pollen tubes a SCF^{SLF} E3 ubiquitin ligase complex targets ths S-RNase for polyubiquitination and degradation. In self-incompatible (self) pollen tubes, the SCF^{SLF} complex is incapable of targeting the S-RNase, which acts to degrade pollen tube RNA and inhibit protein synthesis and growth.

this interaction can take place. McClure et al. (2011) suggest that a small amount of S-Rnase may be able to escape the vacuolar compartment, possibly by retrograde transport, to interact with the SCF^{SLF} complex. In the case of an incompatible pollination, this interaction presumably leads to stabilization of HT-B, breakdown of the vacuolar compartment and release of the S-RNase. Figure 7 summarizes the essential aspects of the sequestration model.

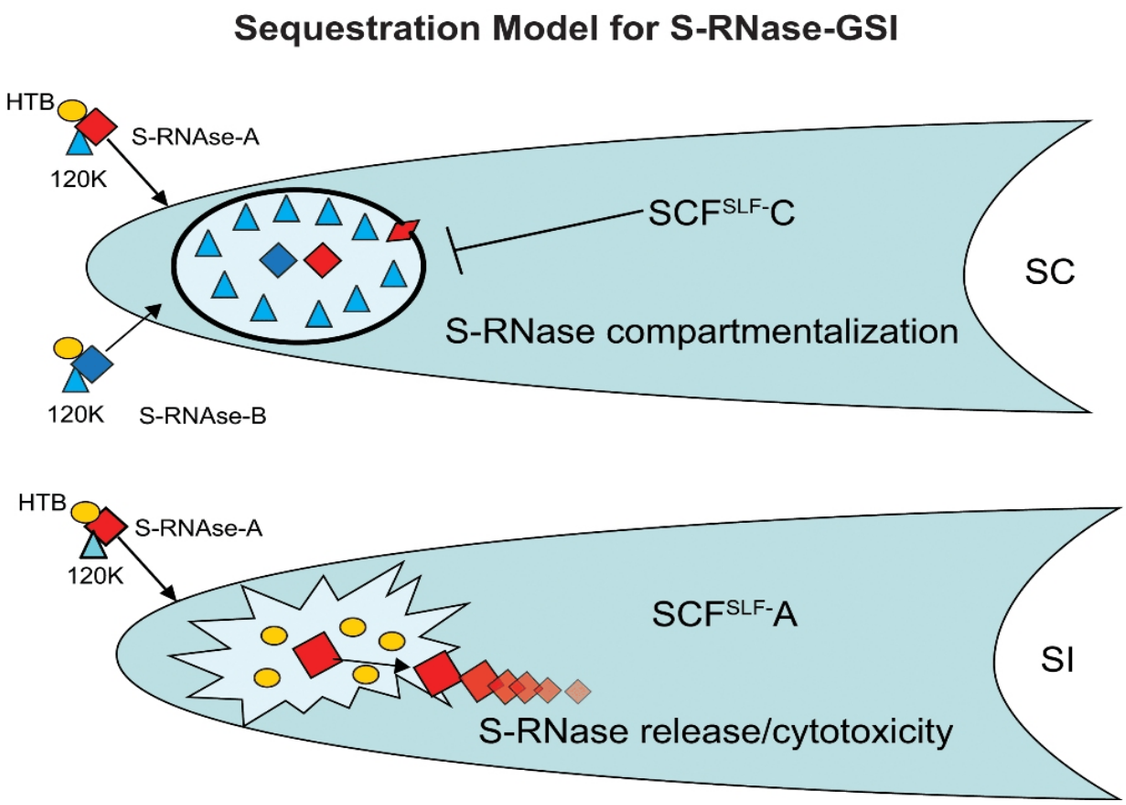

Fig. 7. **Sequestration model for gametophytic self-incompatibility.**
According to the sequestration model, S-RNases are imported into pollen tubes via an exocytotic mechanism, possibly in a complex with other style proteins (the complex shown with HT-B and 120K is speculative). S-RNase remains sequestered in a compatible pollination; in an incompatible pollination the vacuolar compartment breaks down releasing the S-RNase.

10. Current questions and future research

Although tremendous progress has been made in identifying genes and proteins involved in gametophytic self-incompatibility and in understanding much of the basic molecular biology of this phenomenon, many questions remain, and additional research is needed on nearly all aspects of GSI. In particular the collaborative recognition model raises the question of what is the exact nature of pollen-S? Do single SLF proteins interact one-on-one with individual S-RNases or can multiple SLFs interact simultaneously? Given the high degree of sequence identity with any specific class of SLF variants, what constitutes an allele? What protein interactions are required to make a determination of self versus non-self? If the sequestration model is correct, how do SLF and S-RNase even make contact? What is the specific role of ubiquitination in GSI interactions? Are S-RNases polyubiquitinated and degraded, or do different patterns of ubiquitination result in directing S-RNases to (or keeping them in) a membrane-bound compartment? What other proteins are needed for GSI interactions? Investigations not addressed in this review have

suggested that proteins such as NaStEP (Busot et al., 2008) or Sli (Hosaka & Hanneman, 1998) may act as modifiers of the GSI response. What is the molecular basis for the quantitative, reversible, breakdown of GSI known as pseudo-self-compatibility, or PSC (Flaschenreim & Ascher 1979a, 1979b; Dana & Ascher 1985, 1986a, 1986b). What is the mechanism of uptake of S-RNases and other proteins into pollen tubes? Do Solanaceae/Plantaginaceae and Rosaceae really differ in fundamental mechanisms of GSI? More refined protein-interaction assays, suh as those using bi-molecular fluorescence complementation (Gehl et al., 2009), robust transgenic experiments, more complete information on genes and gene families involved in gametophytic self-incompatibility should all prove valuable in addressing these questions.

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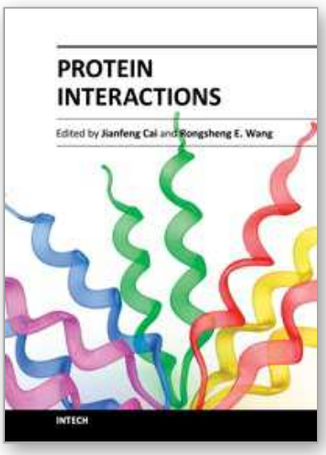

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Protein interactions, which include interactions between proteins and other biomolecules, are essential to all aspects of biological processes, such as cell growth, differentiation, and apoptosis. Therefore, investigation and modulation of protein interactions are of significance as it not only reveals the mechanism governing cellular activity, but also leads to potential agents for the treatment of various diseases. The objective of this book is to highlight some of the latest approaches in the study of protein interactions, including modulation of protein interactions, development of analytical techniques, etc. Collectively they demonstrate the importance and the possibility for the further investigation and modulation of protein interactions as technology is evolving.

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