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CURRENT PERSPECTIVE ESSAY

S-RNase and *SLF* Determine *S*-Haplotype–Specific Pollen Recognition and Rejection

Self-incompatibility (SI) is a genetically determined system for recognition and rejection of self-pollen and pollen from closely related plants. Recognition specificity is controlled by the *S*-locus. This locus is complex in the sense that it contains multiple genes: one controls specificity on the pistil side (pistil *S*), another on the pollen side (pollen *S*). It is also highly polymorphic; the *S*-specificity genes in different haplotypes have highly divergent sequences, giving rise to a set of proteins having a similar function but distinctive recognition characteristics.

S-RNase–based SI occurs in the Solanaceae, Rosaceae, and Scrophulariaceae. S-RNase itself controls specificity on the pistil side. S-RNases were discovered in the mid-1980s (Bredemeijer and Blaas, 1981; Anderson et al., 1986), but it took eight more years to provide direct evidence that they are the genes that determine *S*-specificity in the pistil (Lee et al., 1994; Murfett et al., 1994). It has recently been determined that *SLF* (*S*-locus F-box) genes control specificity on the pollen side. *SLF* genes were first reported in the Scrophulariaceae (Lai et al., 2002). At first, *SLF* was not thought to be sufficiently polymorphic to be pollen *S*, the determinant of *S*-specificity in pollen. However, highly polymorphic *SLF* genes were described in Rosaceae in 2003 and early 2004 (Entani et al., 2003; Ushijima et al., 2003; Ikeda et al., 2004). Recent months have seen publication of transformation experiments definitively showing that SLF determines specificity on the pollen side in *Petunia inflata* (Sijacic et al., 2004). These results are supported by transformation experiments showing that the Antirrhinum *SLF_2* gene is implicated in SI, although it was not possible to directly address *S*-specificity in this system (Qiao et al., 2004b). The compressed time scale between identification of *SLF* and definitive proof that it is pollen *S* is a testament to the hard work and skill of the laboratories involved in research in all three S-RNase–based SI systems.

The identification of the *S*-specificity determining genes on both the pollen and pistil sides brings closure to the first phase of research into the molecular basis of S-RNase–based SI. This is an excellent time to evaluate our understanding of the system, as research moves toward a focus on the biochemistry and physiology of pollen recognition and rejection. This essay describes only the most recent results related to identification of pollen *S* and highlights select facts that will shape research in the near future. For a more comprehensive review, please see Kao and Tsukamoto (2004).

THE FUNDAMENTALS

S-RNase–based SI is widely distributed among the angiosperms (Igic and Kohn, 2001; Kao and Tsukamoto, 2004). Fundamentally, it is a mechanism for controlling pollination. It can be described as genetic interaction between the sporophyte and the gametophyte that prevents inbreeding. It is a typical single-locus gametophytic SI system—compatibility is determined by the *S*-haplotype of pollen, the haploid male gametophyte. Pollen is rejected when its *S*-haplotype is the same as either of the *S*-haplotypes in the diploid pistil. Conversely, pollen with any *S*-haplotype not present in the pistil is compatible (de Nettancourt, 2001). This is the origin of the cumbersome but necessary term *S*-specific pollen rejection (or *S*-allele–specific pollen rejection). S-RNase is also implicated in interspecific pollen rejection, but the mechanisms are distinct from SI (Murfett et al., 1996). Thus, S-RNase–based systems are the most widespread and diverse pollen rejection systems known.

The genetics of SI are simple. The challenge is to understand how the genetic interaction between the pollen and the pistil is manifested at the biochemical level. Necessarily, the first priority was to identify the biochemical determinants of *S*-specificity. The approaches used had to be tied to the requirements imposed by the biology and genetics of SI. Putative specificity determinants must meet three criteria: linkage to the *S*-locus, polymorphism between different *S*-haplotypes, and expression in the pollen or pistil.

S-RNASE DETERMINES *S*-SPECIFICITY IN THE PISTIL

S-RNases, the determinants of *S*-specificity in the pistil, were identified by searching for proteins that met these three requirements. The principle was simple: perform controlled crosses to determine the *S*-specific pollen rejection phenotypes of a family of plants and analyze for cosegregating proteins. In practice, however, this was difficult. It took years to identify plant materials with protein bands that clearly cosegregated with *S*-specific pollen rejection (Bredemeijer and Blaas, 1981). Anderson et al. (1986) ultimately obtained N-terminal sequence from a glycoprotein expressed in the *Nicotiana alata* style that cosegregated with the ability to reject S_2-pollen. Numerous similar clones soon were obtained from other *S*-haplotypes, species, and families. Sequence analysis led to the discovery that these polymorphic proteins have ribonuclease activity; thus, they were labeled S-RNases (McClure et al., 1989).

It took eight additional years to show definitively that *S-RNase* genes met the requirements for the determinants of *S*-specificity in the pistil. Linkage to the *S*-locus was determined by genetic analyses showing no recombination between *S-RNase* genes and the pollen rejection phenotype (Murfett et al., 1994). *S-RNase* gene sequences, and the surrounding genomic regions, proved to be highly

polymorphic between different *S*-haplotypes (Anderson et al., 1989; Ioerger et al., 1990, 1991). S-RNase expression was restricted to the pistil, and the proteins were secreted into the extracellular matrix forming the path from the stigma to the ovary (Cornish et al., 1987; Anderson et al., 1989). Although these data provided strong correlative evidence that S-RNase was the determinant of *S*-specificity in the pistil, they were not definitive. Articles published in 1994 provided direct evidence that S-RNase is the product of the pistil *S* gene. They showed that expression of the *N. alata* S_{A2}-*RNase* gene or the *P. inflata* S_3-*RNase* gene caused transgenic plants to gain the ability to reject S_{A2}- or S_3-pollen, respectively (Lee et al., 1994; Murfett et al., 1994).

The basic mechanism of S-RNase–based SI was outlined in the early 1990s. Gray et al. (1991) showed that S-RNase can gain access to the pollen cytoplasm and act as a potent translational inhibitor. A tracer experiment using ^{32}P-labeled pollen showed that pollen RNA is degraded after incompatible but not compatible pollinations (McClure et al., 1990). Finally, Huang et al. (1994) showed that ribonuclease activity is required for pollen rejection. The basic model that emerged is that each *S*-haplotype encodes a unique S-RNase protein that is secreted into the transmitting tract extracellular matrix. An interaction between S-RNase and the pollen-side specificity factor, pollen S protein, determines whether the pollination is compatible or incompatible. In incompatible pollinations, S-RNase gains access to the pollen tube cytoplasm where its ribonuclease activity causes a cytotoxic effect; in a compatible pollination, this cytotoxic effect is evaded.

F-BOX PROTEINS DETERMINE *S*-SPECIFICITY IN POLLEN

The search for the pollen *S* gene began shortly after the *S-RNase* gene was cloned. It quickly became obvious that the pollen S protein is not as abundant as S-RNase and different approaches would be needed. Ultimately, pollen *S* gene candidates were successfully identified by large-scale genomic DNA sequencing in the vicinity of *S-RNase* genes. This approach succeeded because it was tied directly to the three strict requirements mentioned earlier: candidates must be linked to the *S-RNase* gene, highly polymorphic, and expressed in pollen. However, proving that a candidate gene is pollen *S* was not a trivial matter because the only known phenotypes associated with it are pollen tube growth inhibition in SI and a subtle genetic interaction between *S*-haplotypes that occurs in heteroallelic pollen.

The tight genetic linkage between pollen *S* and *S-RNase* provided an obvious route to identifying candidates. However, SI species are not always well suited for genetic experiments, and various SI systems have different strengths and weaknesses. Essential contributions were made by researchers working in Antirrhinum, Nicotiana, Petunia, and Prunus (reviewed in Kao and Tsukamoto, 2004). In Nicotiana and Petunia, historically the best systems for S-RNase studies, a great deal of effort was required to accumulate a collection of pollen-expressed markers linked to *S-RNase* genes (Li et al., 2000; McCubbin et al., 2000a, 2000b; Wang et al., 2003). In spite of the difficulties, Wang et al. (2003) mapped 13 markers and *S-RNase* in a large *P. inflata* population segregating for S_1- and S_2-haplotypes. Nine markers and the *S-RNase* gene occurred in a 0.25-centimorgan region and could not be separated by recombination. BAC clones were isolated to allow construction of a physical map. Although contigs amounting to 4.4 Mb were constructed from the S_2-haplotype, only one contig contained more than a single marker, revealing that the *S*-locus is physically very large in Petunia. Ultimately, the pollen expressed genes closest to the *P. inflata* S_2-*RNase* gene were identified in an 881-kb contig by cDNA selection and direct sequencing of 328 kb surrounding the S_2-*RNase* gene (Wang et al., 2004). In other species, the *S*-locus proved to be more compact. Markers defining the boundaries of the *S*-locus in *P. dulcis* turned out to be only 72 kb apart (Ushijima et al., 2001). In *Antirrhinum* and *Prunus mume*, genomic contigs spanning only ~65 kb proved to contain pollen *S* candidates (Lai et al., 2002; Entani et al., 2003). Genes that are physically close to the *S-RNase* gene all fulfill the first requirement for a pollen *S* candidate; they are linked to the *S*-locus. Applying the additional requirements of polymorphism and expression in pollen allowed the best candidates to emerge.

Results from genomic sequencing studies showed that the *S*-locus region contains multiple F-box protein genes. Unfortunately, expression in pollen is common; therefore, this is not a discriminating test for pollen *S* candidates. Sequence polymorphism proved to be a better way to distinguish among candidates. The polymorphic F-box protein genes so identified (i.e., pollen *S*) are referred to as SLF_x (*S*-locus F-box from haplotype X) genes in Antirrhinum, Petunia, and *Prunus mume* by some authors (Lai et al., 2002; Qiao et al., 2004b, 2004a; Sijacic et al., 2004), others refer to them as *SFB* genes (Ushijima et al., 2003, 2004; Ikeda et al., 2004).

The Antirrhinum AhSLF_2 gene was the first *SLF* gene reported (Lai et al., 2002). It was shown to be expressed specifically in pollen, but the polymorphism was not as great as was expected for pollen *S*. AhSLF_2 probes revealed restiction fragment length polymorphisms in different *S*-haplotypes but that the degree of cross-hybridization was high, suggesting less sequence polymorphism than *S-RNase* genes (Lai et al., 2002).

Studies in Prunus strongly pointed to *SLF* genes as the best pollen *S* candidates because their sequence polymorphism is comparable to *S-RNase*. This set of Prunus pollen *S* candidates (referred to as *SLF* in *P. mume* and *SFB* in *P. dulcis*) showed *S*-haplotype–specific hybridization patterns similar to those obtained with *S-RNase* probes (Entani et al., 2003; Ushijima et al., 2003). Moreover, sequence analyses of 13 of these genes showed a pattern of conserved and variable domains consistent with a role in recognition as well as evidence of positive selection in so-called hypervariable regions (Ikeda et al., 2004). Ushijima et al. (2004) also analyzed Prunus pollen-part mutants having mutations that only affect *S*-haplotype specificity in pollen while leaving pistil functions intact. The mutant *P. avium* S_4'-haplotype expressed

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a truncated *SFB*$_4$′ gene missing hypervariable regions present in the wild-type *SFB*$_4$ gene. In *P. mume*, the mutant S_f-haplotype contained an *SFB*$_f$ gene with a large insertion such that the predicted polypeptide would be truncated as well. Evidence for positive selection and the presence of mutated versions of *SFB* genes in pollen-part mutants strongly supports a connection between *SLF* and pollen *S*. Together, the data from Antirrhinum and Prunus provided strong correlative evidence (i.e., close linkage to *S-RNase*, expression in pollen, and polymorphism between *S*-haplotypes) that *SLF* (or *SFB*) and pollen *S* were the same gene. What remained lacking was a functional test.

The definitive functional test is a transformation experiment allowing an *SLF* gene to be directly tested for an *S*-haplotype–specific effect on pollination. Functional studies in *P. inflata* have now provided definitive proof that one of the F-box protein genes is indeed pollen *S* (Sijacic et al., 2004), and a recent study in Antirrhinum also directly implicated an F-box protein gene in SI but did not formally address haplotype specificity (Qiao et al., 2004b). These functional studies of pollen *S* candidates are much more subtle than those routinely applied to plant genes. A thorough grasp of how pollen behaves in S-RNase–based systems and how *S*-specificity is expressed in pollen is needed to understand the transformation strategies and, in particular, the separate tests to implicate a gene in SI and to show that it determines *S*-specificity.

Notwithstanding the fact that *S*-haplotype–specific pollen rejection is the defining feature of SI, it is essential to grasp the fact that pollen's resistance to the cytotoxic effect of S-RNase is also fundamental in S-RNase–based SI. For example, although S_1-pollen is susceptible to the cytotoxic effect of S_1-RNase, it is also resistant to all other S-RNases. Thus, although a pollen *S*-haplotype is rejected by one self-S-RNase, it is resistant to all nonself-S-RNases. The popular models to explain this behavior invoke some type of S-RNase inhibitor, and the transformation strategies to test pollen *S* candidates were strongly influenced by these models (Kao and Tsukamoto, 2004). The simple inhibitor model combines the *S*-specific recognition function (i.e., the genetic definition of pollen *S*) and the S-RNase inhibitor function into a single molecule. A modified inhibitor model places these two functions in separate molecules; pollen *S* determines *S*-specificity, and a separate inhibitor prevents the cytotoxic activity of S-RNases (Luu et al., 2001).

The inhibitory function of pollen *S* is supported by mutational studies that suggest that loss of the pollen *S* gene is lethal to the pollen. Golz et al. (1999) (2001) examined radiation-induced pollen-part mutants in *N. alata.* All the mutants could be explained by duplication of all or part of the *S*-locus such that pollen effectively became heterozygous. The absence of mutants with loss of pollen *S* in the Golz et al. (1999) (2001) studies is easily explained if it is an essential gene. However, the Ushijima et al. (2004) article discussed earlier adds a significant new twist. Their study of Prunus pollen-part mutants shows that at least one class of pollen *S* mutation is viable. Significantly, the pollen-part mutants do not appear to have lost pollen *S* entirely. The mutant forms of both SFB$_4$′ and SFB$_f$ retain the N-terminal (i.e., F-box containing) portion of the protein, whereas the C-terminal variable portions were removed. So far as is known, these pollen-part mutants behave as universal pollinators; they retain the ability to pollinate other *S*-haplotypes but they no longer show rejection by their cognate S-RNase. It is possible to speculate that the N-terminal part of the protein functions in resistance to S-RNase (i.e., compatibility) and the C-terminal portion functions in *S*-haplotype specific rejection. This interpretation is consistent with the simple inhibitor model, but the modified model is not excluded because it is still possible that a separate inhibitor exists. The Prunus results (Ushijima et al., 2004) open the door for a detailed dissection of pollen *S* function in Petunia or Solanum where in vitro mutagensis and transformation can be performed. However, such dissection is only meaningful if the two activities (recognition of self-RNase and inhibition of nonself-RNases) definitively have been shown to reside in the same molecule.

Although the details of how pollen resists the effects of nonself-S-RNase are not resolved, and the two inhibitor models make different predictions, it's clear that well-designed pollen *S* transformation experiments had to account for the possibility that pollen *S* is an essential gene (i.e., it may function to prevent the cytotoxic action of S-RNase). In particular, antisense or RNA interference approaches were not viable. Thus, Sijacic et al. (2004) and Qiao et al. (2004b) introduced pollen *S* candidates into pollen already expressing a functional pollen *S* gene. This approach, though successful, is complicated by the manner in which pollen *S* genes interact when two different *S*-haplotypes are expressed in the normally haploid pollen tube.

Heteroallelic pollen may arise from duplications of the *S*-locus, as described earlier (Golz et al., 1999, 2001), or in tetraploids. When an SI S_1S_2 plant is converted to an $S_1S_1S_2S_2$ tetraploid, SI is lost (de Nettancourt, 1977). The defect only affects pollen; the tetraploid pistil continues to reject S_1- and S_2-pollen. Moreover, breakdown only occurs in S_1S_2 diploid (i.e., heteroallelic) pollen; S_1S_1 and S_2S_2 pollen is rejected normally on both the diploid S_1S_2 or tetraploid $S_1S_1S_2S_2$ pistil (Table 1). Thus, the S_1- and S_2-haplotypes interact in such a way that *S*-specific pollen rejection is abolished.

Sijacic et al. (2004) and Qiao et al. (2004b) used the heteroallelic pollen effect to test pollen *S* candidates. However, because of the way specificity is expressed, two separate tests are needed to implicate a candidate in SI and to demonstrate specificity. Expressing a candidate pollen *S* gene in SI pollen with a different *S*-haplotype (i.e., producing heteroallelic pollen) will result in pollen that cannot be rejected. This approach can implicate a candidate in SI, but it is inherently nonspecific. *S*-specificity is only demonstrated with the additional test of expressing the candidate in conjunction with the same *S*-haplotype. It is remarkable that pollen *S* transformation results appeared in the literature only one year after it became clear from work in Antirrhinum, Petunia, and Prunus that *SLF* genes were the best candidates for pollen *S* (Lai et al., 2002; Entani et al., 2003; Ushijima et al.,

Table 1. *S*-Haplotype Interactions in Pollen

Interaction	Sporophyte *S*-Haplotype	Pollen *S*-Haplotypes	RNase Rejection (Incompatibility)	RNase Resistance (Compatibility; i.e., Pollination)
Normal haploid pollen	S_1S_2	S_1-	S_1-RNase	All except for S_1-
		S_2-	S_2-RNase	All except for S_2-
Diploid pollen (heteroallelic pollen effect)	$S_1S_1S_2S_2$	S_1S_1-	S_1-RNase	All except for S_1-
		S_2S_2-	S_2-RNase	All except for S_2-
		S_1S_2-	None	All
Nonspecific transgene experiments	S_1S_1:*SLF*$_2$	S_1-	S_1-RNase	All except for S_1-
		S_1:*SLF*-	None	All
S-specific transgene experiments	S_1S_2:*SLF*$_2$	S_1-	S_1-RNase	All except for S_1-
		S_2-	S_2-RNase	All except for S_2-
		S_2:*SLF*$_2$-	S_2-RNase	All except for S_2-
		S_1:*SLF*$_2$-	None	All

The *S*-haplotypes of hypothetical diploid, tetraploid, and transformed sporophytes are shown. Pollen *S*-haplotypes produced by these sporophytes are shown in combination with *SLF* transgenes where appropriate. Normal haploid pollen: In gametophytic SI, pollen is rejected in pistils expressing a matching S-RNase, but it is resistant to the effects of all other S-RNases (i.e., nonself-S-RNase). Diploid pollen: An $S_1S_1S_2S_2$ tetraploid is shown. SI breakdown occurs when two different pollen *S*-haplotypes (i.e., heteroallelic pollen) are present. Nonspecific transgene experiments: An S_1S_1 plant with an *SLF*$_2$ transgene. S_1-pollen behaves normally. Pollen expressing the *SLF2* transgene shows breakdown in SI similar to heteroallelic pollen. *S*-specific transgene experiment: An S_1S_2 plant with an *SLF*$_2$ transgene. Haploid pollen without the transgene behaves normally. When the *SLF*$_2$ transgene segregates with the S_1-haplotype, breakdown of SI occurs. When the transgene segregates with the S_2-haplotype, pollen behaves normally and is similar to S_2S_2 pollen derived from the tetraploid. The experiment addresses *S*-specificity because breakdown occurs when the transgene occurs with one haplotype but not the other.

2003; Kao and Tsukamoto, 2004; Wang et al., 2004).

In *P. inflata*, the Pi*SLF*$_2$ gene was transformed into an S_1S_1 background (Sijacic et al., 2004). The resulting phenotype, SI breakdown on the pollen side, was exactly as predicted. Only pollen-part function was disrupted, and significantly, breakdown only occurred in S_1-pollen expressing the Pi*SLF*$_2$ transgene. Qiao et al. (2004b) performed similar experiments with the Antirrhinum Ah*SLF*$_2$ gene. Two Ah*SLF*$_2$ gene constructs were transformed into SI *P. hybrida* S_3S_3. The Antirrhinum gene behaved as expected, and SI breakdown on the pollen side was observed. Thus, in two systems, *SLF* transgenes expressed in conjunction with a different *SLF* gene (i.e., the *SLF* gene already present at the native *S*-locus) caused a breakdown in SI on the pollen side. This is completely consistent with predictions based on the behavior of the *N. alata* pollen-part mutants and the heteroallelic pollen effect (Table 1). However, as noted earlier, the heteroallelic pollen effect is inherently nonspecific; any two different pollen *S* genes cause SI to breakdown. Moreover, breakdown of SI would be expected after disrupting any gene (i.e., other than pollen *S*) implicated in pollen SI function.

S-specificity can best be demonstrated by the absence of SI breakdown in the one case where the *SLF* transgene is from the same *S*-haplotype as the native *SLF* gene. Therefore, Sijacic et al. also transformed the Pi*SLF*$_2$ gene into an S_2S_3 background. Breakdown of pollen-part function was observed again. However, all the self-progeny were either S_2S_3 or S_3S_3 and carried the transgene. This is explained as just described; SI breaks down when the Pi*SLF*$_2$ transgene is expressed in pollen with the S_3-haplotype. The absence of S_2S_2 progeny shows that the effect of the Pi*SLF*$_2$ transgene is *S*-haplotype specific: it only interferes with other *S*-haplotypes (i.e., S_1 or S_3) and has no effect on the S_2-haplotype (Table 1). As noted, Qiao et al. (2004b) transformed the Ah*SLF*$_2$ gene into *P. hybrida* S_3S_3 to test its function in SI. Because it is not possible to examine the effect (or lack of effect) of the transgene on the native Antirrhinum S_2-haplotype, this approach precludes testing for *S*-specificity. Nonetheless, the *S*-haplotype–specific effect of Pi*SLF*$_2$ is definitive. *SLF* is pollen *S*.

S-RNASE–BASED SI IS LIKELY TO BE LINKED TO UBIQUITINATION

The most important players in S-RNase–based SI are now known; the field is at a turning point. Research will now shift toward understanding the biochemistry and physiology of *S*-specific pollen rejection. Until now, only the end points were known. At the pistil end, S-RNase is secreted into the transmitting tract extracellular matrix; at the other end, pollen RNA is degraded or protected, resulting in incompatibility or compatibility, respectively. Identification of pollen S as an F-box protein suggests that the specificity step, the interaction between pollen S (SLF) and pistil S (S-RNase), is linked to ubiquitination.

F-box proteins comprise the F in the SCF (Skp1 Cullin F-box) E3 ubiquitin ligase complex that tags proteins for subsequent degradation (Sullivan et al., 2003). The SCF complex catalyzes the final step in synthesis of a polyubiquitin chain on specific target

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proteins that become substrates for the 26S proteasome. F-box proteins can act as adaptors, conferring specificity to the E3 complex by binding specific target proteins (although additional proteins also may be required). Complexes with distinct F-box proteins are designated with a superscript, such as SCF^{PiSLF2}. Plants have an unusually large repertoire of F-box protein genes (Gagne et al., 2002; Smalle and Vierstra, 2004), and ubiquitination has been linked to a wide variety of processes, including sporophytic SI (Stone et al., 2003).

Some have proposed that SCF^{SLF} functions as an inhibitor of nonself-S-RNase by tagging it for degradation (Qiao et al., 2004a, 2004b; Sijacic et al., 2004; Ushijima et al., 2004). This simple model is attractive and may well be correct, but other models are possible. F-box proteins play roles apart from protein degradation. For example, the yeast F-box protein Rcy1p localizes to areas of polar growth and is required for recycling membrane proteins (Galan et al., 2001). Ubiquitination has been likened to phosphorylation as a signaling mechanism; it has roles in many cellular functions, including membrane traffic, transcriptional regulation, translation, and DNA repair (Weisman, 2001; Smalle and Vierstra, 2004). If protein degradation is the critical process in S-RNase–based SI, it must be determined if the simple nonself-S-RNase degradation model is adequate or whether a more complex model is needed.

Experiments to test aspects of this model have been initiated. The *S*-specificity step must, by definition, involve an interaction between SLF and S-RNase. Qiao et al. (2004a) reported important pull-down and immunoprecipitation results that show a direct interaction between *A. hispanicum* $AhSLF_2$ and S-RNase and presented evidence that $AhSLF_2$ forms a complex with Skp1- and cullin-like proteins. Thus, SLF interacts with S-RNase and probably participates in an E3 ubiquitin ligase complex. Importantly, there is no biochemical evidence for an *S*-specific interaction; $AhSLF_2$ bound both self- and nonself-S-RNases as well as S_3-RNase from *P. hybrida* (Qiao et al., 2004a, 2004b). By definition, the interaction between S-RNase and SLF determines *S*-specificity (compatibility). Thus, either the interactions between SLF and self- versus nonself-S-RNases have different outcomes, perhaps, differential activation of an SCF^{SLF} complex, or the experimental conditions for *S*-specific interaction have not been found. Only further research will resolve these questions.

Although these experiments represent a considerable advance, some of the conclusions reached by Qiao et al. (2004a) need to be confirmed in other systems before they are generally accepted. For example, a straightforward prediction of the simple model is that levels of S-RNase should decrease after compatible but not incompatible pollination. Qiao et al. tested this by comparing the amount of immunostained S-RNase in whole style extracts after self- and cross-pollination and concluded that S-RNase levels decrease after compatible pollination. However, this has not been observed in other studies. Immunolocalization studies showed large amounts of S-RNase inside both compatible and incompatible *Solanum chacoense* pollen tubes (Luu et al., 2000). Furthermore, Gray et al. (1991) found no evidence for S-RNase degradation using ^3H-labeled S_2-RNase and in vitro–grown *N. alata* pollen tubes. The hypotheses (Qiao et al., 2004a) that S-RNase is the substrate of the SCF^{SLF} complex and that it is subsequently degraded by the 26S proteasome are of central importance, but it is also important to be cautious and test them in other systems. An SCF^{SLF} complex may not be the only E3 ubiquitin ligase that binds to S-RNase. Sims and Ordanic (2001) described a putative RING E3 protein from *P. hybrida* that binds S-RNase. Unlike SLF, the RING E3 is expressed in nonsexual tissues and, thus far, only has been shown to bind to S-RNase fragments in the yeast two hybrid system. It is not yet known whether the RING E3 is directly involved in SI or whether it interacts with SCF^{SLF}. The cautions aside, it should be emphasized that Qiao et al. (2004a) initiated important experiments investigating the biochemistry and physiology underlying pollen rejection. Such studies are badly needed, and it is very important to extend their experiments to other systems.

We now know the identity of pistil *S* and pollen *S* and we can begin to move on to studies of the biochemistry and physiology of S-RNase–based SI. Sijacic et al. (2004) showed that *SLF* is the pollen *S* gene, and Qiao et al. (2004a) (2004b) showed that SLF binds S-RNase. The door is open for investigating the specificity of this interaction using standard domain-swap and mutagenesis techniques. Such studies may clarify coevolution of the *S-RNase* and *SLF* genes. The possible roles of *SLF*-like genes located at the *S*-locus should be tested. Identification of the substrate(s) of SCF^{SLF} will help determine whether the simple model involving degradation of nonself-S-RNase needs to be modified. Possible candidates include S-RNase, SLF, and non-S-RNase factors required for pollen rejection, as well as factors that are as yet unknown. Studies of potential SCF^{SLF} substrates and the origin of specificity are obvious research goals for the immediate future. However, they alone will not provide a comprehensive model of S-RNase–based SI.

ISSUES ARISING

There are essential aspects of S-RNase–based SI of which we are still totally ignorant. There are also established facts that are typically left out of popular illustrations of SI. It is worthwhile to bring some of these to the forefront so that they can contribute to a more comprehensive model.

The mechanism of S-RNase uptake is an area of glaring ignorance. Transmitting tract cells secrete S-RNase into the extracellular matrix. SLF is very likely to be a cytoplasmic protein; the *S*-specificity step, where SLF and S-RNase interact, therefore probably occurs in the pollen tube cytoplasm. This is also the final site of S-RNase action because incompatible pollen tube RNA is degraded there. Thus, it is important to define the route from the extracellular matrix to the cytoplasm. The only report of S-RNase uptake describes nonspecific uptake of both self- and nonself-S-RNase into pollen tubes (Luu et al., 2000). S-RNase was reported to be in the pollen tube cytoplasm. However, its exact location is difficult to assess because sections were prepared to

preserve antigenicity rather than membrane integrity, and established markers for cellular compartments were not reported. Although pollen tube growth is associated with massive fusion of vesicles at the tip, there is also a return flow (Taylor and Hepler, 1997). Therefore, pollen tubes are probably active in endocytosis; perhaps, this is the initial route of S-RNase entry. It is noteworthy that endocytosis and intracellular trafficking are mediated by ubiquitination (Hicke and Dunn, 2003). Membrane proteins may be monoubiquitinated or polyubiquitinated as a signal for incorporation into endocytic vesicles and subsequent sorting. There is precedent for involvement of an F-box protein in yeast membrane recycling (Galan et al., 2001). Clearly, if S-RNase is initially taken up into a membrane-bound compartment, then it would not have immediate access to either SLF or RNA in the cytoplasm. Thus, the question of whether S-RNase is initially taken up into a membrane-bound compartment or whether it somehow gains access directly to the cytoplasm is an area that needs to be addressed.

It is also unclear how the specificity inherent in SI is manifested at the biochemical level. S-RNase mutagenesis experiments are informative but they are seldom discussed in relation to models of SI. Zurek et al. (1997) reported domain-swap experiments in which portions of S_{A2}- and S_{C10}-RNase from *N. alata* were exchanged. Nine chimeric S-RNases were tested; although all were active ribonucleases, none were capable of causing *S*-specific pollen rejection. Thus, the chimeric S-RNases were capable of acting as cytotoxins but could not be properly recognized. Kao and McCubbin (1996) reported similar results in *P. inflata*. Because one of the *N. alata* chimeras was later shown to be active in an interspecific pollen rejection system (Beecher and McClure, 2001) and the evidence suggests that S-RNase uptake is nonspecific, it is unlikely that the chimeras fail to cause pollen rejection because they are not taken up by pollen tubes. At a minimum, the domain-swap experiments show that the *S*-specificity function of S-RNase is more labile than its enzymatic function. Perhaps, they also suggest that *S*-specificity and cytotoxic activity are separable functions on the pollen side. In the absence of self-S-RNase recognition, pollen may follow a default pathway that leads to compatibility. Conversely, recognition of self-S-RNase by SLF could either activate a pathway leading to incompatibility or deactivate a compatibility pathway.

Finally, although it is known that factors other than the specificity determinants are required for SI, it is not known how many such factors exist or how they function. If SCF^{SLF}-mediated ubiquitination and subsequent protein degradation are key elements of S-RNase–based SI, then any factor whose continued presence is required for pollen rejection is a possible target. HT-B is the only factor other than S-RNase and SLF that has been identified with certainty (McClure et al., 1999). It is a small Asn-rich protein secreted into the transmitting tract matrix in Nicotiana, Lycopersicon, and Solanum (McClure et al., 1999; Kondo et al., 2002a, 2002b; O'Brien et al., 2002). Antisense and RNA interference experiments show that HT-B is required for pollen rejection, but its function is unknown (McClure et al., 1999; O'Brien et al., 2002). Other factors acting on both the pollen and pistil side of the SI reaction probably exist. For example, substrates for SCF E3 ubiquitin ligases usually require prior activation. A protein kinase that phosphorylates S-RNase has been identified, but it is not known whether it has a specific function in SI (Kunz et al., 1995). Without better knowledge of the factors required for S-RNase–based pollen rejection, it is not possible to propose a comprehensive model.

Identification of the pollen *S* gene is the most exciting development in S-RNase–based SI in recent memory. We have a solid foundation from which to proceed, but there are more questions than answers. What is the true mode of SLF-mediated inhibition of nonself-S-RNase: stability, access to the cytoplasm, or something else? What are the roles of non-S-RNase factors, such as HT-B? What determines the specificity of the S-RNase–SLF interaction? What is the connection between S-RNase–SLF interaction and pollen tube growth? What are the constraints on coevolution of *S-RNase* and *SLF* genes? Some of these questions can be addressed easily and others cannot. Good tools for cell biological studies relating to S-RNase uptake and the physiology of growth inhibition are needed. A faithful in vitro pollen rejection system would be a boon. One thing, however, is clear: we are much closer to understanding S-RNase–based SI than ever.

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REFERENCES

Anderson, M.A., et al. (1986). Cloning of cDNA for a stylar glycoprotein associated with expression of self-incompatibility in *Nicotiana alata*. Nature **321,** 38–44.

Anderson, M.A., McFadden, G.I., Bernatzky, R., Atkinson, A., Orpin, T., Dedman, H., Tregear, G., Fernley, R., and Clarke, A. (1989). Sequence variability of three alleles of the self-incompatibility gene of *Nicotiana alata*. Plant Cell **1,** 483–491.

Beecher, B., and McClure, B.A. (2001). Effects of RNases on rejection of pollen from *Nicotiana tabacum* and *N. plumbaginifolia*. Sex. Plant Reprod. **14,** 69–76.

Bredemeijer, G.M.M., and Blaas, J. (1981). S-Specific proteins in styles of self-incompatible *Nicotiana alata*. Theor. Appl. Genet. **59,** 185–190.

Cornish, E.C., Pettitt, J.M., Bonig, I., and Clarke, A.E. (1987). Developmentally controlled expression of a gene associated with self-incompatibility in *Nicotiana alata*. Nature **326,** 99–102.

de Nettancourt, D. (1977). Incompatibility in Angiosperms. (Berlin: Springer-Verlag).

de Nettancourt, D. (2001). Incompatibility and Incongruity in Wild and Cultivated Plants. (Berlin: Springer-Verlag).

CURRENT PERSPECTIVE ESSAY

Entani, T., Iwano, M., Shiba, H., Che, F.S., Isogai, A., and Takayama, S. (2003). Comparative analysis of the self-incompatibility (S-) locus region of *Prunus mume*: Identification of a pollen-expressed F-box gene with allelic diversity. Genes Cells **8,** 203–213.

Gagne, J.M., Downes, B.P., Shiu, S.-h., Durski, A.M., and Vierstra, R.D. (2002). The F-box subunit of the SCF E3 complex is encoded by a diverse superfamily of genes in Arabidopsis. Proc. Natl. Acad. Sci. USA **99,** 11519–11524.

Galan, J.-M., Wiederkehr, A., Seol, J.H., Haguenauer-Tsapis, R., Deshaies, R.J., Riezman, H., and Peter, M. (2001). Skp1p and the F-box protein Rcy1p form a non-SCF complex involved in recycling of the SNARE Snc1p in yeast. Mol. Cell. Biol. **21,** 3105–3117.

Golz, J.F., Oh, H.-Y., Su, V., Kusaba, M., and Newbigin, E. (2001). Genetic analysis of *Nicotiana* pollen-part mutants is consistent with the presence of an S-ribonuclease inhibitor at the S-locus. Proc. Natl. Acad. Sci. USA **98,** 15372–15376.

Golz, J.F., Su, V., Clarke, A.E., and Newbigin, E. (1999). A molecular description of mutations affecting the pollen component of the *Nicotiana alata* S locus. Genetics **152,** 1123–1135.

Gray, J.E., McClure, B.A., Bönig, I., Anderson, M.A., and Clarke, A.E. (1991). Action of the style product of the self-incompatibility gene of *Nicotiana alata* (S-RNase) on *in vitro*-grown pollen tubes. Plant Cell **3,** 271–283.

Hicke, L., and Dunn, R. (2003). Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. Annu. Rev. Cell Dev. Biol. **19,** 141–172.

Huang, S., Lee, H.-S., Karunanandaa, B., and Kao, T.-h. (1994). Ribonuclease activity of *Petunia inflata S* proteins is essential for rejection of self-pollen. Plant Cell **6,** 1021–1028.

Igic, B., and Kohn, J.R. (2001). Evolutionary relationships among self-incompatibility RNases. Proc. Natl. Acad. Sci. USA **98,** 13167–13171.

Ikeda, K., Igic, B., Ushijima, K., Yamane, H., Hauck, N.R., Nakano, R., Sassa, H., Iezzoni, A.F., Kohn, J.R., and Tao, R. (2004). Primary structural features of the *S* haplotype-specific F-box protein, SFB, in *Prunus*. Sex. Plant Reprod. **16,** 235–243.

Ioerger, T.R., Clark, A.G., and Kao, T.-h. (1990). Polymorphism at the self-incompatibility locus in Solanaceae predates speciation. Proc. Natl. Acad. Sci. USA **87,** 9732–9735.

Ioerger, T.R., Gohlke, J.R., Xu, B., and Kao, T.-h. (1991). Primary structural features of the self-incompatibility protein in Solanaceae. Sex. Plant Reprod. **4,** 81–87.

Kao, T.-h., and McCubbin, A.G. (1996). How flowering plants discriminate between self and non-self pollen to prevent inbreeding. Proc. Natl. Acad. Sci. USA **93,** 12059–12065.

Kao, T.-h., and Tsukamoto, T. (2004). The molecular and genetic bases of S-RNase-based self-incompatibility. Plant Cell **16** (suppl.), S72–S83.

Kondo, K., Yamamoto, M., Itahashi, R., Sato, T., Egashira, H., Hattori, T., and Kowyama, Y. (2002a). Insights into the evolution of self-compatibility in *Lycopersicon* from a study of stylar factors. Plant J. **30,** 143–153.

Kondo, K., Yamamoto, M., Matton, D.P., Sato, T., Masashi, H., Norioka, S., Hattori, T., and Kowyama, Y. (2002b). Cultivated tomato has defects in both S-RNase and HT genes required for stylar function of self-incompatibility. Plant J. **29,** 627–636.

Kunz, C., Chang, A., Faure, J.-D., Clarke, A.E., Polya, G.M., and Anderson, M.A. (1995). Phosphorylation of style *S*-RNases by Ca2+-dependent protein kinases from pollen tubes. Sex. Plant Reprod. **9,** 25–34.

Lai, Z., Ma, W., Han, B., Liang, L., Zhang, Y., Hong, G., and Xue, Y. (2002). An F-box gene linked to the self-incompatibility (S) locus of *Antirrhinum* is expressed specifically in pollen and tapetum. Plant Mol. Biol. **50,** 29–42.

Lee, H.-S., Huang, S., and Kao, T.-h. (1994). *S* proteins control rejection of incompatible pollen in *Petunia inflata*. Nature **367,** 560–563.

Li, J.-H., Nass, N., Kusaba, M., Dodds, P.N., Treolar, N., Clarke, A.E., and Newbigin, E. (2000). A genetic map of the *Nicotiana alata* S locus that includes three pollen-expressed genes. Theor. Appl. Genet. **100,** 956–964.

Luu, D.-T., Xike, Q., Morse, D., and Cappadocia, M. (2000). S-RNase uptake by compatible pollen tubes in gametophytic self-incompatibility. Nature **407,** 649–651.

Luu, D.-T., Qin, X., Laublin, G., Yang, Q., Morse, D., and Cappadocia, M. (2001). Rejection of S-heteroallelic pollen by a dual-specific S-RNase in *Solanum chacoense* predicts a multimeric SI pollen component. Genetics **159,** 329–335.

McClure, B.A., Gray, J.E., Anderson, M.A., and Clarke, A.E. (1990). Self-incompatibility in *Nicotiana alata* involves degradation of pollen rRNA. Nature **347,** 757–760.

McClure, B.A., Haring, V., Ebert, P.R., Anderson, M.A., Simpson, R.J., Sakiyama, F., and Clarke, A. (1989). Style self-incompatibility gene products of *Nicotiana alata* are ribonucleases. Nature **342,** 955–957.

McClure, B.A., Mou, B., Canevascini, S., and Bernatzky, R. (1999). A small asparagine-rich protein required for S-allele-specific pollen rejection in *Nicotiana*. Proc. Natl. Acad. Sci. USA **96,** 13548–13553.

McCubbin, A.G., Wang, X., and Kao, T.-h. (2000a). Identification of self-incompatibility (*S*-) locus linked pollen cDNA markers in *Petunia inflata*. Genome **43,** 619–627.

McCubbin, A.G., Zuniga, C., and Kao, T.-h. (2000b). Construction of a binary bacterial artificial chromosome library of *Petunia inflata* and the isolation of large genomic fragments linked to the self-incompatibility (S-) locus. Genome **43,** 820–826.

Murfett, J., Atherton, T.L., Mou, B., Gasser, C.S., and McClure, B.A. (1994). S-RNase expressed in transgenic *Nicotiana* causes S-allele-specific pollen rejection. Nature **367,** 563–566.

Murfett, J.M., Strabala, T.J., Zurek, D.M., Mou, B., Beecher, B., and McClure, B.A. (1996). *S* RNase and interspecific pollen rejection in the genus *Nicotiana*: Multiple pollen rejection pathways contribute to unilateral incompatibility between self-incompatible and self-compatible species. Plant Cell **8,** 943–958.

O'Brien, M., Kapfer, C., Major, G., Laurin, M., Bertrand, C., Kondo, K., Kowyama, Y., and Matton, D.P. (2002). Molecular analysis of the stylar-expressed *Solanum chacoense* asparagine-rich protein family related to the HT modifier of gametophytic self-incompatibility in *Nicotiana*. Plant J. **32,** 1–12.

Qiao, H., Wang, H., Zhao, L., Zhou, J., Huang, J., Zhang, Y., and Xue, Y. (2004a). The F-box protein AhSLF-S_2 physically interacts with S-RNases that may be inhibited by the ubiquitin/26S proteasome pathway of protein degradation during compatible pollination in *Antirrhinum*. Plant Cell **16,** 582–595.

Qiao, H., Wang, F., Zhao, L., Zhou, J., Lai, Z., Zhang, Y., Robbins, T.P., and Xue, Y. (2004b). The F-box protein AhSLF-S_2 controls the pollen function of S-RNase-based self-incompatibility. Plant Cell **16,** 2307–2322.

Sijacic, P., Wang, X., Skirpan, A.L., Wang, Y., Dowd, P.E., McCubbin, A.G., Huang, S., and Kao, T.-h. (2004). Identification of the pollen determinant of S-RNase-mediated self-incompatibility. Nature **429,** 302–305.

Sims, T.L., and Ordanic, M. (2001). Identification of a S-ribonuclease-binding protein in *Petunia hybrida*. Plant Mol. Biol. **47,** 771–783.

Smalle, J., and Vierstra, R.D. (2004). The ubiquitin 26S proteasome proteolytic pathway. Annu. Rev. Plant Biol. **55,** 555–590.

Stone, S.L., Anderson, E.M., Mullen, R.T., and Goring, D.R. (2003). ARC1 is an E3 ubiquitin ligase and promotes the ubiquitination of proteins during the rejection of self-incompatible *Brassica* pollen. Plant Cell **15,** 885–898.

Sullivan, J.A., Shirasu, K., and Deng, X.-W. (2003). The diverse roles of ubiquitin and the 26S proteasome in the life of plants. Nat. Rev. Genet. **4,** 948–958.

Taylor, L.P., and Hepler, P.K. (1997). Pollen germination and tube growth. Annu. Rev. Plant Physiol. Plant Mol. Biol. **48,** 461–491.

Ushijima, K., Sassa, H., Dandekar, A.M., Gradziel, T.M., Tao, R., and Hirano, H. (2003). Structural and transcriptional analysis of the self-incompatibility locus of almond: Identification of a pollen-expressed F-box gene with haplotype-specific polymorphism. Plant Cell **15,** 771–781.

Ushijima, K., Sassa, H., Kusaba, M., Tao, R., Tamura, M., Gradziel, T.M., Dandekar, A.M., and Hirana, H. (2001). Characterization of the *S*-locus region of almond (*Prunus dulcis*): Analysis of a somaclonal mutant and a cosmid contig for an *S*-haplotype. Genetics **158,** 379–386.

Ushijima, K., Yamane, H., Watari, A., Kakehi, E., Ikeda, K., Hauck, N.R., Iezzoni, A.F., and Tao, R. (2004). The *S* haplotype-specific F-box protein gene, *SFB*, is defective in self-compatible haplotypes of *Prunus avium* and *P. mume*. Plant J. **39,** 573–586.

Wang, Y., Tsukamoto, T., Yi, K.-w., Wang, X., Huang, S., McCubbin, A.G., and Kao, T.-h. (2004). Chromosome walking in the *Petunia inflata* self-incompatibility (*S*-) locus and gene identification in an 881-kb contig containing S_2-*RNase*. Plant Mol. Biol. **54,** 727–742.

Wang, Y., Wang, X., McCubbin, A.G., and Kao, T.-h. (2003). Genetic mapping and molecular characterization of the self-incompatibility (*S*) locus in *Petunia inflata*. Plant Mol. Biol. **53,** 565–580.

Weisman, A.M. (2001). Themes and variations on ubiquitylation. Nat. Rev. Mol. Cell Biol. **2,** 169–178.

Zurek, D.M., Mou, B., Beecher, B., and McClure, B. (1997). Exchanging sequence domains between S-RNases from *Nicotiana alata* disrupts pollen recognition. Plant J. **11,** 797–808.