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RNase-Based Self-Incompatibility: Puzzled by *Pollen S* [W]

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Many plants have a genetically determined self-incompatibility system in which the rejection of self pollen grains is controlled by alleles of an *S* locus. A common feature of these *S* loci is that separate pollen- and style-expressed genes (*pollen S* and *style S*, respectively) determine *S* allele identity. The long-held view has been that *pollen S* and *style S* must be a coevolving gene pair in order for allelic recognition to be maintained as new *S* alleles arise. In at least three plant families, the Solanaceae, Rosaceae, and Plantaginaceae, the *style S* gene has long been known to encode an extracellular ribonuclease called the S-RNase. *Pollen S* in these families has more recently been identified and encodes an F-box protein known as either SLF or SFB. In this perspective, we describe the puzzling evolutionary relationship that exists between the *SLF*/*SFB* and *S-RNase* genes and show that in most cases cognate pairs of genes are not coevolving in the expected manner. Because some *pollen S* genes appear to have arisen much more recently than their *style S* cognates, we conclude that either some *pollen S* genes have been falsely identified or that there is a major problem with our understanding of how the *S* locus evolves.

In three plant families, the Solanaceae, Rosaceae, and Plantaginaceae (formerly Scrophulariaceae; Albach et al. 2005), self-incompatibility (SI) is controlled by extracellular ribonucleases (S-RNases) in the style that are the products of an *S* locus characterized by large numbers of alleles (Takayama and Isogai, 2005). For many years the pollen counterpart of the S-RNases (pollen S) was unknown. However, a series of recent articles identified candidate genes in all three families (Lai et al., 2002; Entani et al., 2003; Ushijima et al., 2003; Ikeda et al., 2004; Sijacic et al., 2004) and provided evidence for involvement of these genes in the SI response (Ikeda et al., 2004; Sijacic et al., 2004; Qiao et al., 2004a, 2004b; Ushijima et al., 2004; Sonneveld et al., 2005). The studies implicated genes encoding an F-box protein, involved in selecting targets for ubiquitination, as being pollen S in all three families. In the Solanaceae and Plantaginaceae, this F-box protein is called SLF and in the Rosaceae it is called SLF by some groups (Entani et al., 2003) and SFB by others (Ushijima et al., 2003; Ikeda et al., 2004). For simplicity, however, we will refer to the pollen S proteins from the Solanaceae and Plantaginaceae as SLF and from the Rosaceae as SFB. Commentaries heralding this exciting development appeared in many scientific journals, including this one (McClure, 2004), and it appeared that we were closer than ever to understanding RNase-based SI.

However, many more questions than answers have been raised by our as yet limited understanding of *pollen S*. Rather than leading to elucidation of a single conserved SI mechanism among families that use S-RNases, subsequent studies have identified mechanistic differences between the Rosaceae and Solanaceae in the way incompatible pollen is rejected (Sonneveld et al., 2005; Hauck et al., 2006). Uncertainty surrounds the mechanism of SLF action in the Solanaceae (McClure, 2006), and even the components that comprise the ubiquitination complex of which SLF is a part appear different between the Solanaceae and Plantaginaceae (Hua and Kao, 2006; Huang et al., 2006). In this perspective, we examine another aspect of the SLF/SFB story: the puzzling evolutionary relationships between SLF/SFB alleles and their putative cognate S-RNases. In at least some cases, the putative *pollen S* genes are evolving far differently than expected for this self-recognition locus. Either some *pollen S* genes have been falsely identified, or there is a major problem with our understanding of how *pollen S* evolves.

POPULATION GENETICS OF THE *S* LOCUS

To understand why currently identified *pollen S* genes are puzzling requires an appreciation of the evolutionary processes that act on the *S* locus and explain its unusually high levels of allelic polymorphism. In the SI systems described here, haploid pollen grains are rejected when they express an *S* allele that matches either of those expressed in the diploid style. This simple genetic system prevents self-fertilization and limits cross-fertilizations among related plants, avoiding the deleterious effects of close inbreeding. As Wright (1939) pointed out, a rare *S* allele will experience strong selection to increase in frequency

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because it has increased mating opportunities relative to common *S* alleles. This type of selection, called frequency-dependent selection, is responsible for the maintenance of dozens of *S* alleles in populations of SI plants (reviewed in Lawrence, 2000).

A further consequence of frequency-dependent selection is that *S* alleles persist in populations for periods of time much longer than those of alleles of more standard genes (Charlesworth and Guttman, 1997). Allelic polymorphism persists because any allele that becomes rare in a population through random genetic drift is likely to subsequently increase in frequency due to selection, rather than to decline further in frequency toward extinction. Frequency-dependent selection acting on the *S* locus is expected to preserve polymorphism over tens of millions of years, far longer than the average lifetime of a species (Takahata, 1990; Vekemans and Slatkin, 1994).

The predicted effects of frequency-dependent selection on the *S* locus have largely been confirmed from analysis of S-RNase sequences. For instance, in addition to their large numbers, S-RNase alleles from the Solanaceae are highly polymorphic: amino acid identity of alleles drawn from the same obligately heterozygous individual is often <50% (Ioerger et al., 1990; Richman et al., 1995). Much of this diversity is found in hypervariable regions. Various tests of the magnitude and nature of the changes in these regions have shown it to be due to selection favoring amino acid replacement, as one would expect if selection favors the creation of new specificities (Ioerger et al., 1991; Ishimizu et al., 1998; Takebayashi et al., 2003; Igic et al., 2007; Vieira et al., 2007). Moreover, there is direct transgenic evidence in the Solanaceae showing that changing amino acids within hypervariable regions alters the allelic identity of an S-RNase (Matton et al., 1999). However, there is abundant polymorphism throughout the *S-RNase* gene, save for a relatively few sites that are conserved to protect enzymatic function or proper protein conformation. So while some of the extreme sequence polymorphism is due to selection for different specificities, much of it has accrued due to the great age of allelic lineages. Another piece of evidence for the great age of S-RNase alleles is shared ancestral polymorphism. An allele from one species is often more closely related to an allele from another species or genus than to other alleles within its own species (Ioerger et al., 1990). This is evidence that the common ancestor of these species already possessed much of the extant allelic diversity and passed it down to its descendents. For instance, most of the S-RNase allele polymorphism in the Solanaceae was present in a common ancestor that is estimated to have existed 30 to 40 million years ago (Paape et al., 2008). Furthermore, genealogies also point to a single evolutionary origin for the S-RNases in the Rosaceae, Solanaceae, and Plantaginaceae, suggesting that RNase-based SI was the ancestral state in the majority of eudicots (Igic and Kohn, 2001; Steinbachs and Holsinger, 2002).

In all SI systems that have been characterized at the molecular level, the determinants of pollen and stylar specificity are encoded by a pair of genes at the *S* locus, with one specifying stylar and the other pollen specificity. To maintain the functional relationship between any pair of pollen and stylar genes, recombination is suppressed around the *S* locus. Accordingly, the creation of a new *S* allele requires coordinate changes to both genes. How this happens is unknown, although some plausible evolutionary pathways have been described (Newbigin and Uyenoyama, 2005). Whatever the route, the strong expectation is that the pollen and stylar genes should coevolve. That is, *SLF* or *SFB* alleles that control pollen recognition are expected to show the same signature of long-term frequency-dependent selection seen in the *S-RNases*: abundant sequence polymorphism, evidence for positive selection, long coalescence times of extant alleles, and shared ancestral polymorphism. In addition, the genealogies of cognate pollen and stylar alleles are expected to be largely concordant, reflecting a long coevolutionary history. In the Brassicaceae, which possess an SI system not involving S-RNases, these theoretical expectations of pollen and stylar loci have largely been confirmed: both pollen and style genes at the *S* locus show extreme polymorphism, long coalescence times, and evidence of positive selection, and there is good concordance in the genealogies of pairs of pollen and style alleles (Sato et al., 2002: Takebayashi et al., 2003).

WHAT'S PUZZLING ABOUT THE SLF AND SFBs?

Perhaps the clearest example of where the SLFs have failed to meet these evolutionary expectations is from *Antirrhinum hispanicum* (Plantaginaceae), a wild relative of the snapdragon. This example is significant because the first *SLF* allele to be described came from this family. This allele is Ah *SLF-*S_2, the *SLF* allele associated with the S_2 allele (Lai et al., 2002). Ah *SLF-*S_2 was identified based on its proximity to the *A. hispanicum* S_2*-RNase* gene and pollen-dominated expression pattern. Later it was shown that Ah SLF-S_2 and S-RNase interact in yeast two-hybrid and coimmunoprecipitation assays and that expressing Ah *SLF-*S_2 in transgenic plants produces a pollination phenotype that is consistent with some models of how *pollen S* functions (Qiao et al., 2004a, 2004b). However, there were two major surprises when the sequences of the Ah *SLF* alleles associated with the S_1, S_4, and S_5 alleles became available (Zhou et al., 2003). The first was the extremely high (97 to 99%) pairwise amino acid identity among the four SLF alleles (Figure 1A). The second was that the few amino acid differences that did exist were scattered throughout the sequence, not clustered into a hypervariable region as they are in the S-RNases. By comparison, the S-RNases associated with these four *Antirrhinum* S alleles appear just as expected, with pairwise amino acid identities ranging from 36 to 51%. Levels of nonsynonymous (amino acid–changing, Ka) and synonymous (silent, Ks) substitutions in the DNA sequence are both more than an order of magnitude lower at the *Antirrhinum SLF* locus than at the cognate *S-RNase* locus (Table 1; Xue et al., 1996; Zhou et al., 2003; Sassa et al., 2007; Wheeler and Newbigin, 2007). The high level of identity among the *Antirrhinum*

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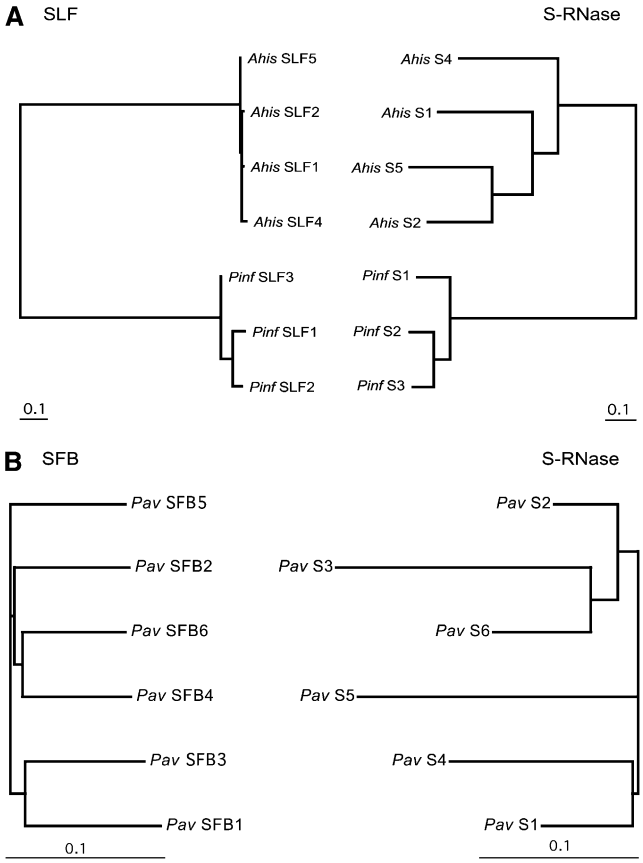

Figure 1. Gene Genealogies of S-RNases and Their Proposed SLF or SFB Cognates.

(A) Genealogies for all available SLFs and their corresponding S-RNases from the Plantaginaceae (*Antirrhinum*) and Solanaceae (*Petunia*).

(B) Same analysis for SFBs and their corresponding S-RNases from *P. avium* (Rosaceae). Branch lengths are proportional to substitutions per site. There are too few alleles and/or too little phylogenetic structure to formally test whether the phylogenetic relationships for S-RNases and their proposed cognate genes are concordant. However, terminal branch lengths for the *A. hispanicum* SLF alleles are markedly shorter than those of the corresponding S-RNase alleles. Phylogenies could not be reconstructed for *S* locus genes from the Maloideae because of uncertainty regarding which *SFBB* genes encode pollen specificity (Sassa et al., 2007). Topologies were obtained by maximum likelihood using a GTR + G + I nucleotide substitution model in *PAUP** (Swofford, 2000), and TreeMap v2.0 (available at http://www.it.usyd.edu.au/~mcharles/) was used to scale corresponding S-RNase and SLF/SFB phylogenies. Trees were redrawn using FigTree (available at http://evolve.zoo.ox.ac.uk/software/figtree). Supplemental Table 1 online contains accession numbers for all the sequences used.

SLF alleles is indicative of polymorphisms with a much shorter evolutionary history than that of the *S-RNase* (Figure 1A). Equating the *Antirrhinum SLF* gene with *pollen S* means that in *Antirrhinum*, *pollen S* must evolve in a radically different way than the *S-RNase* it interacts with.

By contrast, the Rosaceae *SFBs* provide some of the best evolutionary and other evidence of a gene that is coevolving with an *S-RNase* gene and that regulates pollen SI phenotype. Many *SFB* alleles have been cloned from species in the genus *Prunus* (apricots, peaches, and almonds), which belongs to the Amygdaloideae subfamily of the Rosaceae, and sequence variability among them is quite similar to that of the corresponding *S-RNases* (Table 1, Figure 1B). In addition, statistical tests find strong evidence that sequence polymorphism among *SFB* alleles is due to selection (Ikeda et al., 2004; Nunes et al., 2006). While the amount of divergence among pollen and style alleles is similar, there is unfortunately little phylogenetic structure to either the *Prunus S-RNase* or *SFB* alleles. Therefore, no strong inference can be made as to whether style and pollen genes share the same evolutionary history (Nunes et al., 2006). Finally, loss-of-function mutations in *Prunus SFB* are associated with pollen-part self-fertility (Ushijima et al., 2004; Sonneveld et al., 2005, Hauck et al., 2006). Intriguingly, *Prunus* also provides examples of *S-RNases* from different species that are identical or nearly identical in sequence but are associated with *SFBs* that differ by as many as 12 amino acid replacements (Šurbanovski et al., 2007).

The generally encouraging story to emerge from analyses of SI in *Prunus* becomes a bit more curious when recent evidence from another subfamily of Rosaceae, the Maloideae (apples and pears, among others), is considered. Whereas each *Prunus S* allele has just one linked *SFB* gene, in the Maloideae, each allele has two to three related *SFB* genes (Sassa et al., 2007). Called *SFBB*, for *S locus F-box brothers*, levels of polymorphism across

Table 1. Rates of Synonymous and Nonsynonymous Substitutions per Site from S-RNases and Corresponding SLF/SFB

	S-RNase		SLF/SFB/SFBB	
	Ks	Ka	Ks	Ka
A. hispanicum	1.118	0.506	0.034	0.0092
P. inflata	0.386	0.152	0.159	0.0502
Maloideae	0.251	0.218	0.233	0.1560
P. avium	0.239	0.138	0.247	0.1138

Loci compared are from *A. hispanicum* (Plantaginaceae), *P. inflata* (Solanaceae), subfamily Maloideae (Rosaceae; two haplotypes from *Malus domestica* and two from *Pyrus pyrifolia*), and *P. avium* (Rosaceae, subfamily Amygdaloideae). For the Maloideae, values are averaged across SFBB sequences (see text). Ka (replacement) and Ks (silent) values were estimated using DNASP v4.20 (Rozas et al., 2003). Supplemental Table 1 online contains accession numbers for all the sequences used.

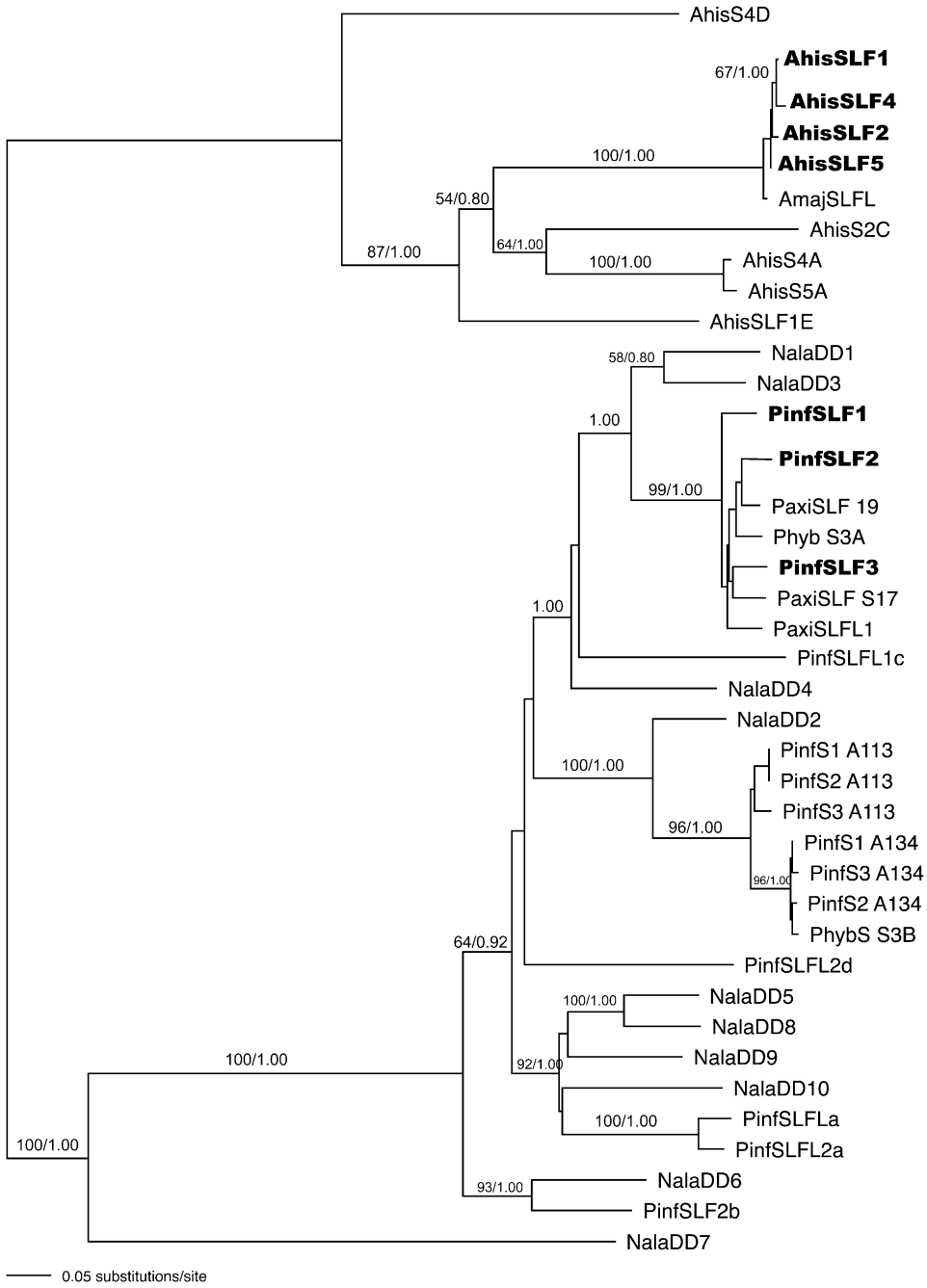

Figure 2. Maximum Likelihood Tree of *SLF* and *SLF-Like* Genes from the Plantaginaceae and Solanaceae.

Plantaginaceae sequences are from *A. hispanicum* (Ahis) and *A. majus* (Amaj), and Solanaceae sequences are from *Nicotiana alata* (Nala), *Petunia axillaris* (Paxi), *P. hybrida* (Phyb), and *P. inflata* (Pinf). Genes considered to encode pollen S are indicated in boldface. The phylogeny was constructed using PAUP (Swofford, 2000), and ModelTest v3.0 (Posada and Crandall, 1998) was used to select the most likely nucleotide substitution model (GTR + G + I). The results from MrBayes 3.1 (http://mrbayes.csit.fsu.edu/manual.php/) produced a topology consistent with the maximum likelihood tree (GTR + G + I nucleotide substitution model; the analysis was run for 1,000,000 generations, sampling every 500th generation). Numbers on branches represent bootstrap support (PAUP; Swofford, 2000) and Bayesian posterior probabilities (calculated using MrBayes3; Ronquist and Huelsenbeck, 2003), respectively. Both methods yielded trees with similar topologies. Supplemental Table 1 online contains accession numbers for all the sequences used.

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these genes are similar to each other and only somewhat lower than those of their cognate *S-RNases*. It is thus unclear which *SFBB* locus is involved in pollen specificity, and Sassa et al. (2007) suggest that they may act in concert to determine mating type. Testing this hypothesis will be difficult, however, because all of the Maloideae that have been examined are trees. Transgenic approaches aimed at testing the effects of loss-of-function and gain-of-function mutations on pollination phenotype, while feasible (Broothaerts et al., 2004), may take some time.

Fitting in between the Rosaceae *SFBs* and the Plantaginaceae *SLFs* in terms of nucleotide variation are the Solanaceae *SLFs*, which are all from the genus *Petunia* (Figure 1A; Sijacic et al., 2004). Despite the fact that *S-RNases* of the Solanaceae generally have higher levels of nucleotide polymorphism, implying a greater age, than those of *Prunus* or the Maloideae, levels of nucleotide polymorphism among the *Petunia SLFs* are lower than those of the *SFBs* (Table 1). As with the *Antirrhinum* SLFs, variable amino acid positions are not clustered into distinct hypervariable regions but distributed over the sequence. Similar types of binding and transgenic experiments to those performed with Ah *SLF-S_2* have also been done with the *Petunia SLF* alleles and have produced results that are consistent with expectations for *pollen S* (Sijacic et al., 2004; Hua and Kao, 2006; Hua et al., 2007).

Attempts at identifying *SLF* orthologs in solanaceous species other than *Petunia* have highlighted one further evolutionary difference between this gene and the *S-RNase* gene (Wheeler and Newbigin, 2007). Phylogenetic clustering of S-RNases relative to nearly all other plant RNases points to a single common origin for S-RNase–based SI (Igic and Kohn, 2001; Steinbachs and Holsinger, 2002). In particular, aside from a few genes that appear to be derived from the *S* locus itself (relic S-RNases; Golz et al., 1998), S-RNases from the Plantaginaceae and Solanaceae are each other's closest known relatives, as are S-RNases from the two subfamilies of Rosaceae from which S-RNases have been cloned. By contrast, the *SLF* or *SFB* genes in each taxa are more closely related to other *F-box protein* genes in the same taxon, usually also found linked to the *S* locus, than to the *SLF* and *SFB* genes of other taxa (Figure 2; Entani et al., 2003; Ushijima et al., 2003; Wheeler and Newbigin, 2007). This introduces a further complication into the SLF/SFB story: embedded in the *S* locus are many pollen-expressed *F-box protein* genes that are related to the *SLFs* and *SFBs*. These genes are called *SLF-likes*. Although some *SLF-likes* are closely related to the *SLFs* (Figure 2), they are generally thought not to function in SI because alleles from different *S* genotypes show little or no sequence variation, and when expressed in transgenic plants these genes do not alter the pollination phenotype (Entani et al., 2003; Ushijima et al., 2003; Wang et al., 2003; Zhou et al., 2003; Hua et al., 2007; Sassa et al., 2007; Wheeler and Newbigin, 2007). The close intrafamily relationships between the *SLF/SFB* and *SLF-like* genes again implies an origin for the *SLF/SFB* alleles that is much younger than that of the corresponding *S-RNase* alleles.

CONCLUSIONS

If the molecular data supporting the role of *SLF/SFB* genes as encoding pollen S are accepted, then it is obvious that we must abandon, at least in some taxa, our expectations for how frequency-dependent selection will affect sequence polymorphism and coevolution with the *S-RNase* locus. Given that coevolution of pollen and stylar genes occupies a central place in theories of the *S* locus and that conclusions based wholly or in part on this expectation are to be found in much of the literature on RNase-based SI systems, abandoning this theory is not going to be easy.

An obvious first consequence of uncoupling the histories of pollen and style genes is that our methods for finding *pollen S* in additional taxa will need to be revised. Most attempts at identifying *pollen S* have been based on analyzing the sequences of pollen-expressed genes at the *S* locus. Those genes with high levels of polymorphism are considered *pollen S* candidates and less polymorphic genes are rejected. For instance, Takebayashi et al. (2003) rejected the *S* locus–linked, pollen-expressed gene *48a* as a candidate for *pollen S* based on its low level of polymorphism and lack of evidence of positive selection. However, as the *Antirrhinum* example shows, abundant sequence polymorphism can no longer be considered a reliable diagnostic feature of *pollen S*. Compounding the problem is the fact that most taxa have many pollen-expressed *F-box protein* loci at or near the *S* locus (Sassa et al., 2007; Wheeler and Newbigin, 2007), all of which must be considered candidates until functional tests demonstrate otherwise.

There are several possible ways to remove the requirement for coevolution of pollen and stylar determinants and allow *pollen S* lineages to be much younger than *S-RNase* lineages. Potentially, it is the existence of paralagous genes, as is the case for the *SLF* and *SFB* loci but not the *S-RNase* locus, that is responsible for eradicating evidence of frequency-dependent selection. For instance, it is possible that gene conversion between *SLF* alleles or among related *F-box protein* loci homogenizes these sequences except for whatever variation is needed to specify allelic identity. Alternatively, any of the *F-box protein* paralogs at the *S* locus (*SLFs*, *SFBs*, and *SLF-likes*) may also be able to encode pollen S, so that over time new genes are recruited to this function. Although the selective constraints that permit these and other conceivable scenarios to take place are not readily apparent, it should at least be possible to distinguish them by the predictions they make about how sequence variation is distributed over *SLF* and *SFB* alleles and their *SLF-like* paralogs.

Supplemental Data

The following material is available in the online version of this article.

Supplemental Table 1. Gene Names and Accession Numbers for the F-Box Proteins (SLF, SLF-Like, SFB, and SFBB) Used in the Analyses Shown in Table 1 and Figures 1 and 2.

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