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Expression of 10 S-Class *SLF-like* Genes in *Nicotiana alata* Pollen and Its Implications for Understanding the Pollen Factor of the S Locus

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ABSTRACT

The S locus of *Nicotiana alata* encodes a polymorphic series of ribonucleases (S-RNases) that determine the self-incompatibility (SI) phenotype of the style. The pollen product of the S locus (pollen S) in *N. alata* is unknown, but in species from the related genus Petunia and in self-incompatible members of the Plantaginaceae and Rosaceae, this function has been assigned to an F-box protein known as SLF or SFB. Here we describe the identification of 10 genes (designated *DD1–10*) encoding SLF-related proteins that are expressed in *N. alata* pollen. Because our approach to cloning the *DD* genes was based on sequences of *SLF*s from other species, we presume that one of the *DD* genes encodes the *N. alata SLF* ortholog. Seven of the *DD* genes were exclusively expressed in pollen and a low level of sequence variation was found in alleles of each *DD* gene. Mapping studies confirmed that all 10 *DD* genes were linked to the S locus and that at least three were located in the same chromosomal segment as *pollen S*. Finally, the different topologies of the phylogenetic trees produced using available SLF-related sequences and those produced using *S-RNase* sequences suggests that *pollen S* and the *S-RNase* have different evolutionary histories.

SELF-INCOMPATIBILITY (SI) is a genetic mechanism found in many flowering plants that acts to reduce the negative fitness effects associated with inbreeding (de Nettancourt 2001). In the best-understood examples, SI is controlled by a single genetic S locus with many S alleles: styles reject pollen grains when both express the same S specificity. Although SI systems have evolved independently several times during the diversification of flowering plants (Matton *et al.* 1994), in three families—the Solanaceae, Plantaginaceae, and Rosaceae—the SI phenotype of the style (but not of pollen) is controlled by an extracellular ribonuclease known as the S-RNase (Anderson *et al.* 1986; McClure *et al.* 1989; Sassa *et al.* 1993; Xue *et al.* 1996), a feature that suggests a single evolutionary origin for RNase-based SI systems (Igic and Kohn 2001). Mechanistically, S-RNases are thought to act as cytotoxins that can enter both compatible and incompatible pollen tubes (Luu *et al.* 2000; Goldraij *et al.* 2006), retarding the growth of incompatible pollen tubes possibly by degrading the limited amount of ribosomal RNA that they contain (McClure *et al.* 1990).

The S-locus pollen factor, known as pollen S, until recently was unknown. The first indication of its identity came from Lai *et al.* (2002), who sequenced a 63-kb region of *Antirrhinum hispanicum* (Plantaginaceae) genomic DNA that contained the *S-RNase* gene and who found within this sequence an *F-box* gene specifically expressed in pollen. *F-box* genes were soon identified at the S loci of Petunia (Solanaceae) (Qiao *et al.* 2004a; Y. Wang *et al.* 2004; Tsukamoto *et al.* 2005), Prunus, Pyrus, and Malus (Rosaceae) (Entani *et al.* 2003; Ushijima *et al.* 2003; Sassa *et al.* 2007), and collectively these genes are now called the *S locus F-box* genes (abbreviated as SLF for the Solanaceae and Plantaginaceae and as SFB for the Rosaceae). Functionally, F-box proteins are a component of one type of E3 ubiquitin (Ub)-protein ligase, the enzyme responsible for transferring Ub from the E2 Ub-conjugating enzyme to free Lys residues on a selected protein target (Vierstra 2003). This association with protein ubiquitination has led to a model in which compatible pollen tubes are protected from S-RNase cytotoxicity by a SLF-mediated mechanism involving Ub attachment and degradation (Lai *et al.* 2002; Qiao *et al.* 2004b).

In each family, evidence exists to show that *SLF/SFB* encodes pollen S. In the Rosaceae, for example, where producing transgenic plants is difficult, statistical tests of amino acid variation between different Prunus *SFB* alleles have identified two short regions of sequence that are under balancing selection (Ikeda *et al.* 2004; Nunes *et al.* 2006). SI is one of the best-documented examples of balancing selection and the way in which it affects nucleotide sequence variation has previously been used to identify genes involved in SI (Takebayashi *et al.* 2003). Also consistent with a role for SFB in SI are

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truncations or deletions of this gene in self-compatible cultivars of fruit trees such as *Prunus avium* (sweet cherry) and *Prunus mume* (Japanese apricot) that carry mutations specifically affecting the SI phenotype of pollen (Ushijima *et al.* 2004; Sonneveld *et al.* 2005). For the Solanaceae and Plantaginaceae, transgenic experiments have been used to show that *SLF* encodes a protein that behaves in the manner expected of *pollen S* (Qiao *et al.* 2004a; Sijacic *et al.* 2004). A *SLF* transgene does not cause pollen from a transgenic plant to express a new S-allele identity, but instead causes a breakdown of SI in pollen if the *SLF* transgene is derived from an S allele that differs from the plant's own S alleles (Qiao *et al.* 2004a; Sijacic *et al.* 2004). Thus, the *SLF* transgene derived from the *Petunia inflata* S_2 allele (*PiSLF*$_2$) caused loss of SI expression in pollen carrying either the S_1 or S_3 allele but not in pollen carrying the S_2 allele (Sijacic *et al.* 2004). The ability of two different *pollen S* alleles present in the same pollen grain to "competitively interact" with each other, and thus allow the pollen tube to grow through an otherwise incompatible style, is well known, having previously been observed in tetraploids derived from self-incompatible diploids (*e.g.*, Chawla *et al.* 1997). SI plants with pollen-part mutations (pollen-part mutants, PPMs) are another example of competitive interaction between pollen S alleles. PPMs are diploid plants with an extra copy of the S locus that is present either as a small additional chromosome called a centric fragment, as a translocated fragment attached to a nonhomologous (*i.e.*, non-S-bearing) chromosome, or as a fragment attached to a homologous (S-bearing) chromosome via unequal exchange (Golz *et al.* 1999, 2001).

Genes related to *SLF/SFB* but with no known role in SI also occur in plant genomes, including those of self-compatible plants lacking an RNase-based SI mechanism (L. Wang *et al.* 2004). These genes, referred to here as *SLF-like* genes, are members of a large family of *F-box protein* genes found only in plants (L. Wang *et al.* 2004). Except for *SLF/SFB*, functions for most members of this family are unknown, even though it includes ~13% of the *F-box protein* genes (92/694) in *Arabidopsis thaliana* (L. Wang *et al.* 2004). Phylogenetic analysis divides the *SLF-like* gene family into five different classes (A, B, C, M, and S), with all *SLF/SFB* genes and some *SLF-like* genes belonging to the S class (L. Wang *et al.* 2004). The presence of *SLF-like* genes can complicate the process of identifying *SLF/SFB* genes, especially when *SLF-like* genes are pollen expressed and linked to the S locus (Entani *et al.* 2003; Ushijima *et al.* 2003; Zhou *et al.* 2003; Y. Wang *et al.* 2004; Sassa *et al.* 2007).

In this article we describe an analysis of the pollen-expressed S-class *SLF-like* genes from the solanaceous self-incompatible plant *Nicotiana alata*. A motivation for this study was to broaden the range of solanaceous plants for which information on *SLF* genes is available, as all existing sequences are from Petunia (Qiao *et al.* 2004a; Sijacic *et al.* 2004; Tsukamoto *et al.* 2005). Using degenerate primers and a reverse transcriptase PCR (RT–PCR) approach with *N. alata* pollen RNA as the template, full-length sequences for 10 S-class genes were obtained. Because potentially 1 of these 10 genes is the *N. alata SLF* ortholog, we gave them the temporary names *DD1–10*. Of the 10 *DD* genes, 7 were exclusively expressed in pollen. Polymorphisms in 7 *DD* genes were used to show linkage to the S locus: placement of individual *DD* genes with regard to the *S-RNase* gene and surrounding S-locus markers was done using the PPM plants described in Golz *et al.* (2001). Finally, phylogenetic analysis using *DD* and other S-class sequences suggests that the SLFs in the Solanaceae and Plantaginaceae do not have a long history of co-evolving with the *S-RNase* gene. We discuss the implications that this has for identifying *pollen S* in these families and for understanding the evolutionary history of the pollen and stylar genes of the S locus.

MATERIALS AND METHODS

Plant material: SI lines of *N. alata* homozygous for S_1, S_2, S_3, S_6, or S_7 were maintained as described in Anderson *et al.* (1986). The collection of *N. alata* PPMs homozygous for the S_6 allele and with mapped duplications containing the S_3 allele have been described previously (Golz *et al.* 1999, 2001). *N. alata* families segregating for four S alleles were produced by germinating $S_1S_3 \times S_6S_7$ seed and $S_2S_3 \times S_6S_7$ seed. *S* genotypes were determined by PCR amplification of S-RNase genes as described by Li *et al.* (2000).

Primer design and PCR: The primers D2 5′-YTIATIGGIC CITGYRAYGG-3′ (forward) and D4 5′-CICCRTAYTSIWTCAT NAYCC-3′ (reverse) were designed on the basis of regions conserved in *SLF* genes from the Solanaceae and Plantaginaceae. PCR was performed with ~50 ng of genomic DNA or 1 μl of cDNA template. Reactions were carried out in a final volume of 20 μl of 1× PCR buffer (Invitrogen, San Diego) containing template DNA, 0.2–0.5 μm of each primer, 0.2 mm dNTPs, 1.5 mm $MgCl_2$, and 2 units of Taq polymerase (Invitrogen) on a GeneAmp 2700 thermal cycler (Applied Biosystems, Foster City, CA). Cycling conditions were as follows: 94° for 2 min and then 32 cycles of 94°at 30 sec, 45°–65° at 30 sec, and 72° at 60 sec. Rapid amplification of cDNA ends (RACE) PCR was performed using the Smart RACE kit (CLONTECH), as described in the manufacturer's instructions.

Cloning and DNA sequencing: PCR products were purified using the QIAquick kit (QIAGEN, Valencia, CA) and ligated into the pGEM-Teasy vector (Promega, Madison, WI). Electrocompetent *Escherichia coli* (DH10B) cells were transformed and recombinant clones were selected for a PCR-based screen using standard vector specific primers (T7/Sp6). Resulting products were digested with 1 unit of either *Hae*III or *Rsa*I (Promega) and separated by gel electrophoresis. Plasmid DNA from clones with unique restriction digestion patterns was isolated and sequenced commercially (Macrogen, Seoul). Sequences of the *N. alata DD* genes have been deposited in GenBank with the accession nos. EF420251–EF4202510. Protein domain analysis was done using the SMART search tool (Letunic *et al.* 2006) (http://smart.embl-heidelberg.de/). All evolutionary analyses were performed using the Phylip package of programs (Felsenstein 2004). DNA alignments were

generated using TRANALIGN (http://bioweb.pasteur.fr/docs/EMBOSS/tranalign), based on ClustalW (v1.8) (Higgins and Sharp 1988) protein alignments of the corresponding sequences. Maximum-parsimony (MP) trees were estimated from the DNA alignment using DNAPARS with default settings. Neighbor-joining (Saitou and Nei 1987) distance trees were generated using NEIGHBOR, with a DNA distance matrix computed using DNADIST. Bootstrap analyses (Felsenstein 1985) were carried out using 1000 bootstrap pseudoreplicates.

DNA blot analysis: Genomic DNA was extracted from leaf material using the plant DNAeasy kit (QIAGEN). Leaf DNA (10 μg) was digested to completion with *Eco*RI (Promega), fractionated on an agarose gel, and transferred to Hybond N+ (Amersham, Piscataway, NJ) membrane using the alkaline blot procedure described by the manufacturer. DNA fragments were radiolabeled with [^{32}P]dCTP using the Primagene kit (Promega). Hybridizations were performed overnight at 65° in 5× SSPE, 5× Denhardt's solution, 0.5% SDS, and 100 μg/ml denatured herring sperm. After hybridization membranes were washed twice in 2× SSC and 0.1% SDS and exposed to film.

RT–PCR analysis: Total RNA was prepared using Trizol reagent (Invitrogen). DNA contamination was removed by treating isolated RNA (1 μg) with 2 units of DNase I (Invitrogen). First-strand cDNA synthesis was carried out using an oligo(dT_{17}) primer and 200 units of Superscript III (Invitrogen). Sequences of the gene-specific primers used in this experiment are shown in supplemental Table 1 at http://www.genetics.org/supplemental/.

RESULTS

Cloning and sequence analysis of candidate S-class *SLF-like* protein genes from *N. alata* pollen: Partial cDNAs for S-class *SLF-like* genes were isolated from *N. alata* pollen by a degenerate PCR approach. Primers D2 and D4 were designed to bind to sequences conserved in Antirrhinum and Petunia *SLF*s. Pollen RNAs isolated from *N. alata* plants homozygous for the S_1, S_2, S_3, S_6, or S_7 allele were reverse transcribed and used as templates. PCR products were cloned and sequenced, and conceptual translations of all six frames were compared to GenBank using BlastP. After several rounds of screening, 10 partial cDNAs with high amino acid sequence similarity to Petunia *SLF* and *SLF-like* sequences were identified and given the provisional names *DD1–DD10*. Sequence differences between the 10 partial cDNAs were 30–40% and there was ≤5% difference in the sequence of the same *DD* cDNA amplified from different pollen RNA templates (data not shown). As the *DD* cDNAs represented candidates for *N. alata SLF*, RACE was used to obtain additional 5′ and 3′ sequence information. Full-length sequences for *DD1* and *DD5–8* were obtained by RACE using S_2S_2 pollen cDNA as the template. Full-length sequences for *DD2* (S_6S_6), *DD3/DD4* (S_1S_1), and *DD9* (S_3S_3) were obtained from the indicated pollen cDNAs, and a near full-length sequence for *DD10* (missing the initiator Met codon and 5′-UTR) was obtained from S_6S_6 pollen cDNA.

Figure 1 shows conceptual translations of the 10 *DD* cDNAs aligned with representative SLF and SLF-like proteins from *P. inflata* (Solanaceae), *A. hispanicum* (Plantaginaceae) and *P. mume* (Rosaceae). DD5 and DD8 were the most closely related of the *N. alata* DD proteins with 88% amino acid similarity (78% identity), and DD3 and DD7 were the least related with 50% amino acid similarity (35% identity). The protein similarity between the DD sequences and *P. inflata* SLF-S_3 ranged from 53% (DD7) to 80% (DD3). SMART and PFAM analysis identified an N-terminal F-box domain in all *N. alata* DD proteins, consistent with them being members of the F-box protein superfamily. Other F-box proteins have diverse regions outside the F-box domain that bind to specific substrate proteins (Gagne *et al.* 2002). Potential substrate-binding motifs present in the DD proteins include the related F-box-associated domains type 1 and type 3 (FBA_1, PF07734; FBA_3, PF08268) (Figure 1). L. Wang *et al.* (2004) incorporated these domains into the four conserved C-terminal motifs (C1–C4) used to define the S-class SLF-like proteins. C1–C4 are present in the SLF/SFB proteins and in all 10 DD proteins (Figure 1).

Nucleotide sequences of the *N. alata DD* genes were aligned with sequences of the *SLF* and S-class *SLF-like* genes from *P. inflata*, *Petunia axillaris*, and *Petunia hybrida* (Solanaceae) and *A. hispanicum* (Plantaginaceae). Sequences of *SFB*s from species in the Rosaceae were also included. Figure 2 shows the MP tree produced from the DNA alignment. Bootstrap analysis provides strong support for almost all nodes, and distance trees generated using the same alignment had an identical topology (results not shown). The Solanaceae *SLF* and *SLF-like* sequences are in a single cluster, as are the Antirrhinum sequences, with nodes at the base of the Solanaceae and Plantaginaceae clusters (nodes 1 and 2) having strong bootstrap support (100%). *DD7* and *DD6* are at the base of the Solanaceae cluster and sisters to three well-supported smaller clusters (nodes 3–5). The *P. inflata SLF* sequences, *PiSLF-S1*, *PiSLF-S2*, and *PiSLF-S3*, group together in a cluster (node 6) with *PaF1*, an *SLF-like* sequence from *P. axillaris* (Tsukamoto *et al.* 2005). These sequences are sisters to the Nicotiana sequences *DD1*, *DD3*, and *DD4* (node 5). The second small cluster contains *P. hybrida* and *P. inflata SLF-like* sequences and *DD2* (node 4), and the third cluster comprises only *DD* sequences (node 3).

Expression of the *DD* genes in various *N. alata S* genotypes and tissues: To determine whether expression of a *DD* gene was restricted to pollen of a particular *S* genotype, PCR experiments were done with gene-specific primers and S_1S_1, S_2S_2, S_1S_3, S_6S_6, and S_7S_7 pollen cDNA templates (Figure 3). In each case, DNA sequence analysis and/or restriction enzyme digests were used to confirm that the correct products had been amplified (data not shown), and pollen RNA (−RT control) was used to show that the products were derived from cDNA and not from genomic DNA. Transcripts for 2 of the 10 *DD* genes were amplified from all

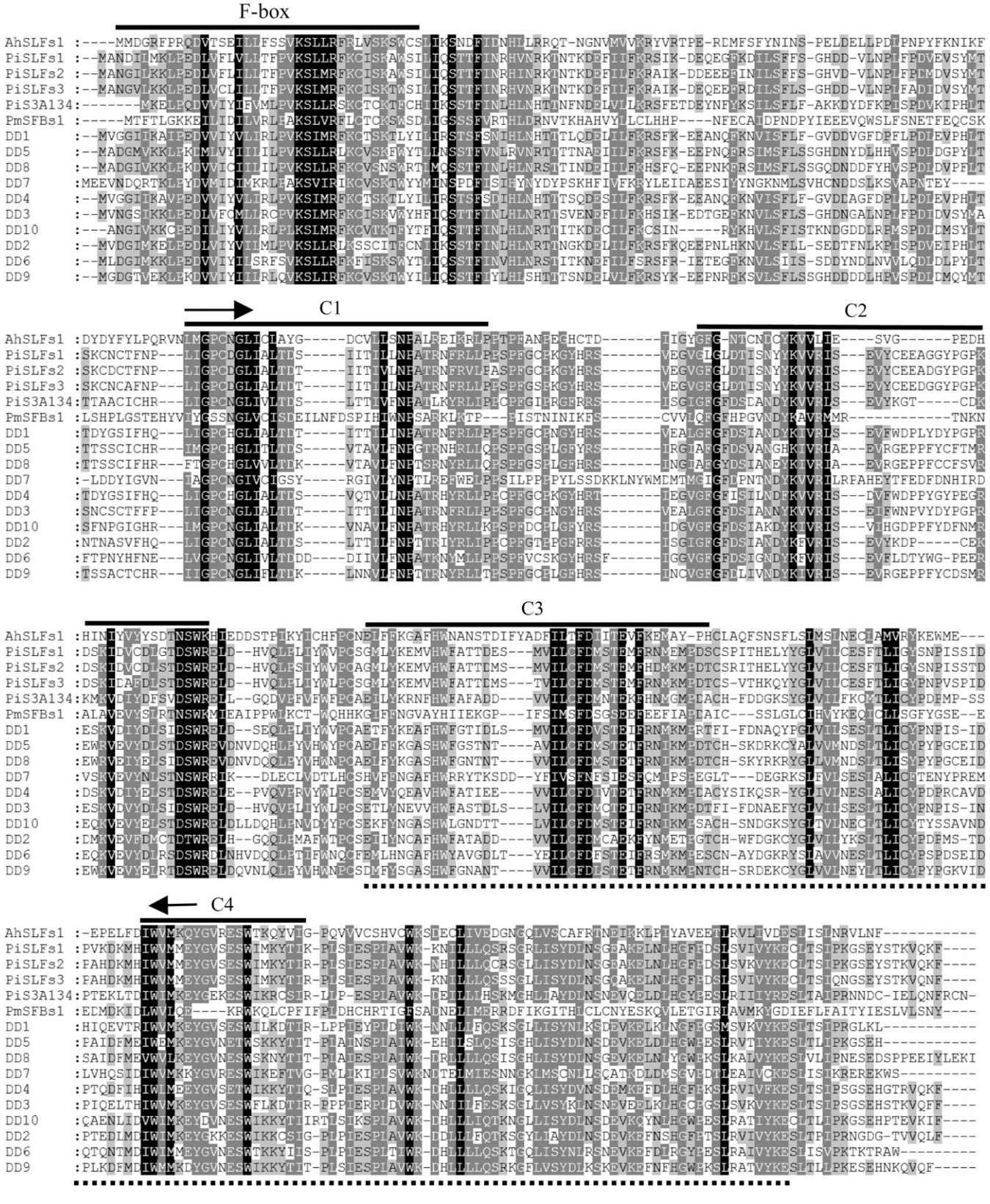

FIGURE 1.—Amino acid sequences encoded by the 10 *N. alata* DD cDNAs aligned with representative SLF-like sequences from *A. hispanicum*, *P. inflata*, and *P. mume*. Primer binding sites for degenerate primers D2 and D4 are indicated by arrows. The F-box and four conserved motifs identified in L. WANG *et al.* (2004) are indicated by solid lines. The C-terminal F-box associated domain (FBA 1 and FBA 3) is indicated by the dashed line below the alignment. Accession numbers of sequences from species other than *N. alata* are AhSLFS$_1$ (AJ515535), PiSLFS$_1$ (AY500390), PiSLFS$_2$ (AY136628), PiSLFS$_3$ (AY500392), PiS3A134 (AY363975), and PmSFBS$_1$ (BAD08320).

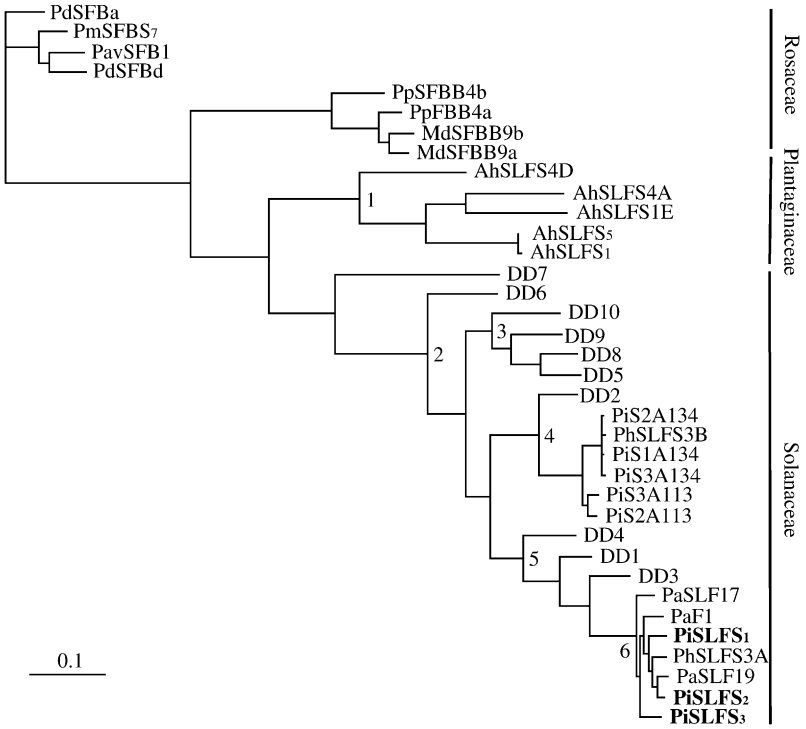

FIGURE 2.—Maximum-parsimony tree (5087 steps) derived from aligned SLF/SFB and SLF-like DNA sequences. The three Petunia sequences identified with *pollen S* are indicated by boldface type. Bootstrap values (1000 pseudoreplicates) for numbered nodes are 1 = 100%, 2 = 100%, 3 = 96%, 4 = 100%, 5 = 99%, 6 = 100%. Sequences in the tree and their accession numbers are $PaSLFS_{17}$ (AY766153), $PaSLFS_{19}$ (AY766154), PhSLFS3A (AY639403), PhSLFS3B (AY639402), PaF1 (AY766155), PiS3A113 (AY363972), PiS1A134 (AY363973), PiS2A134 (AY363974), PiS2A113 (AY363971), $AhSLFS_5$ (AJ515536), AhSLF-S1E (AJ515535), AhSLF-S4D (AJ515534), AhSLFS4A (AJ515534), $PmSFBS_7$ (AB092622), PdSFBa (AB092966), MdSFBB9b (AB270794), MdSFBB9a (AB270793), PpFBB4a (AB270797), PpSFBB4b (AB270798), PavSFB1 (PavSFB1), and PbSFBb (AB081648).

five pollen templates tested (*DD4* and *DD6*) and transcripts for another 6 *DD* genes (*DD1–DD3*, *DD7*, *DD8*, and *DD10*) were amplified from between two and four templates. For example, *DD1* transcripts were present in all pollen cDNAs except S_1S_1, and *DD2* transcripts were detectable in S_1S_1, S_6S_6, and S_7S_7 cDNAs, but not in S_2S_2 and S_1S_3 cDNAs. *DD3* was detected in S_1S_1 and S_1S_3 pollen cDNAs but could not be amplified from S_3S_3 cDNA (data not shown). The remaining two *DD* genes were more restricted in their expression and transcripts were detectable in only a single cDNA type: the S_2S_2 template for *DD5* and the S_1S_3 template for *DD9*.

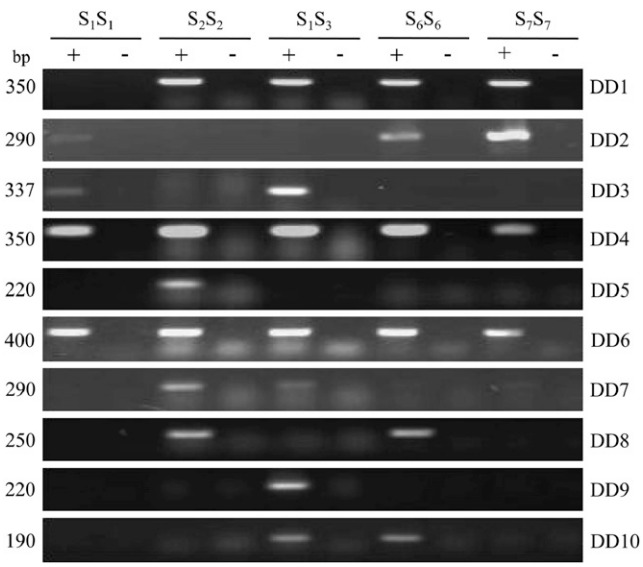

FIGURE 3.—RT–PCR was carried out using primers specific for each *DD* gene shown on the right, with pollen cDNA templates of the indicated S genotype shown at the top of the gel. "No reverse transcriptase controls" are indicated by the minus sign and the size of each PCR product in base pairs is shown at the left of each gel.

The tissue-specific expression patterns of the *DD* genes were investigated by RT–PCR using cDNA synthesized from *N. alata* pollen, style, leaf, and petal RNA. Figure 4 shows that *DD2*, *DD3*, and *DD5–9* were expressed only in pollen, that *DD1* and *DD4* were expressed in pollen and style, and that *DD10* was expressed in pollen and petal. The *DD* genes were not expressed at detectable levels in nonfloral tissues such as leaf or root (Figure 4 and data not shown).

Determining linkage between the *DD* genes and the *S* locus: Evidence of linkage between the *S* locus and individual *DD* genes was obtained using two families of *N. alata* plants in which four S alleles were segregating. *S* genotypes of progeny plants resulting from a $S_1S_3 \times S_6S_7$ cross or a $S_2S_3 \times S_6S_7$ cross were determined using allele-specific PCR of the *S-RNase* gene (results not shown). Cleavable amplified polymorphic sequence markers were designed for eight of the *N. alata DD* genes to test for cosegregation with an individual *S-RNase* allele (*e.g.*, S_3). Analysis of 18–20 plants detected no recombination between the *DD* marker and S_3*-RNase* (supplemental Table 2 at http://www.genetics.org/supplemental/), indicating that these *DD* genes are <5 cM from the *S* locus.

Mapping of the *DD* genes was also done using a collection of PPM *N. alata* plants that are homozygous for the S_6 allele and that carry chromosomal duplica-

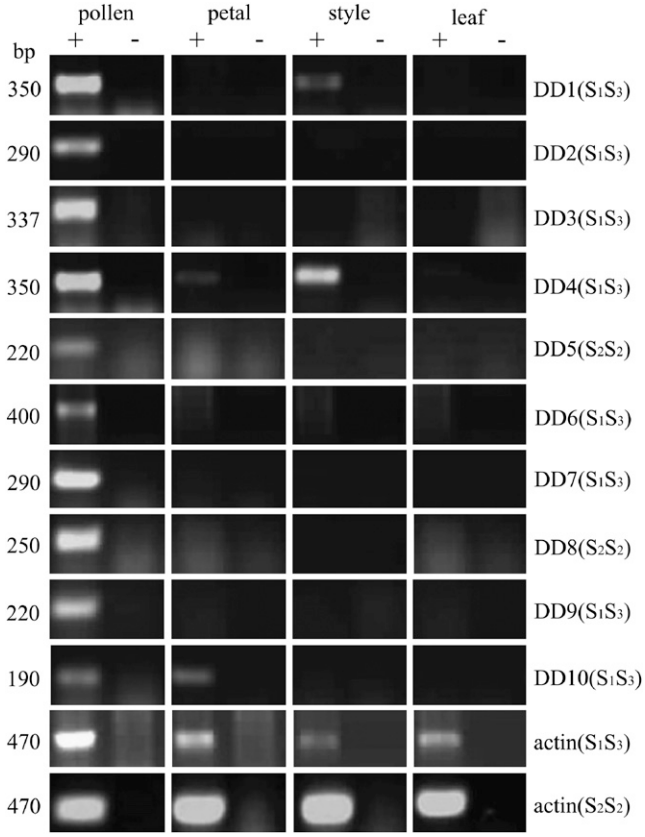

Figure 4.—Spatial expression pattern of *DD* genes. RT–PCR was performed on *N. alata* tissue indicated at the top of each gel. Primers used were specific for the gene shown at the right, and the cDNA genotype tested is indicated in parentheses. "No reverse transcriptase controls" are indicated by the minus sign, and actin-specific primers were used to confirm the presence of template. PCR product sizes in base pairs are indicated at the left.

tions of varying lengths containing an S_3 allele (dS_3). The duplications are present in the genomes of these plants as small extra chromosomes (centric fragments), segments that have been added to other chromosomes (translocations), or segments that are linked to an S_6 allele via an unequal crossover (Figure 5B). This approach to mapping genes to the *N. alata S* locus is quicker than the standard linkage-based approach (supplemental Table 2 at http://www.genetics.org/supplemental/) and provides information about relative gene order, but is suitable only for genes with S_3- and S_6-associated polymorphisms. Suitable polymorphisms were detected for *DD1–3*, *DD6*, *DD7*, *DD9*, and *DD10*. DNA blot analysis indicated that the *DD* genes were all single-copy sequences in the *N. alata* genome (Figure 5A and data not shown).

Figure 5 illustrates how the PPMs were used to determine map locations for the *DD* genes. Four PPM lines were selected for this analysis (M1-1, M1-2, M1-5, and M1-7) with Figure 5B showing the previously described order of markers (*CP100*, *48A*, *S-RNase*, and *167A*) on dS_3 in each line. *CP100*, *48A*, and *S-RNase* all map without recombination to the *S* locus and *167A* is 0.9 cM away (Li *et al.* 2000). On blots of genomic DNA from S_3S_3, S_6S_6, M1-1, M1-2, M1-5, and M1-7 (Figure 5A), the *DD1* probe detected a restriction fragment length polymorphism between S_3S_3 (*DD1–3*) and S_6S_6 (*DD1–6*). As expected, *DD1–6* was present in all M1 plants ($S_6S_6dS_3$) but *DD1–3* was present only in M1-7, placing *DD1* to the right of marker *167A* (Figure 5C). Similarly, the *DD2* probe hybridized to *DD2–3* and *DD2–6* in S_3S_3 and S_6S_6, respectively (Figure 5A). *DD2–3* was present in all M1 plants, placing this gene within the *S* locus in a chromosomal segment delimited by the left border of the translocation in M1-7 and the right border of the unequal exchange in M1-5 (Figure 5C). The other genes known to lie in this segment are *48A* and *pollen S*. Figure 5C shows *DD2* grouped with *48A* on the centromeric side of the S locus, although the placement of these markers with respect to each other and *pollen S* is arbitrary. Figure 5C summarizes the PPM mapping data. *DD7* and *DD10* are in the same chromosomal segment as *DD2*, *DD9* lies between the *S-RNase* and *167A*, and *DD1*, *DD3*, and *DD6* are farther from the S locus than *167A* (supplemental Figure 6 at http://www.genetics.org/supplemental/).

DISCUSSION

This article describes the identification and initial characterization of 10 genes expressed in *N. alata* pollen (*DD1–10*) that code for S-class SLF-like proteins, a class that includes all the known SLFs of Antirrhinum and Petunia, the SFBs of Prunus, and other clearly related proteins in these and other species with no obvious role in SI (L. Wang *et al.* 2004). The *DD* genes were all present as single-copy sequences and the 10 *DD* genes appear to represent the full complement of S-class S *SLF-like* genes expressed in *N. alata* pollen that can be recovered using the RT–PCR-based approach described here. Because none of the *DD* genes could unambiguously be identified as encoding the *N. alata* ortholog of SLF (see below), we propose to continue using this provisional naming scheme until such time as functions are identified and individual *DD* genes can be given more descriptive names.

A surprising aspect of this work was the linkage detected in small-scale experiments between 8 of the 10 *DD* genes (*DD3–DD10*) and the *S* locus (supplemental Table 2 at http://www.genetics.org/supplemental/). This potentially indicated the existence of a single cluster of *DD* genes. Clusters of *F-box protein* genes have been described in Arabidopsis by Gagne *et al.* (2002), where 35.9% of the 694 *F-box protein* genes are in arrays of two to seven members. Instead of a single cluster of *DD* genes, it could equally be true that there are many separate *SLF-like* genes, all subject to the suppressed recombination affecting the S locus and precluding the

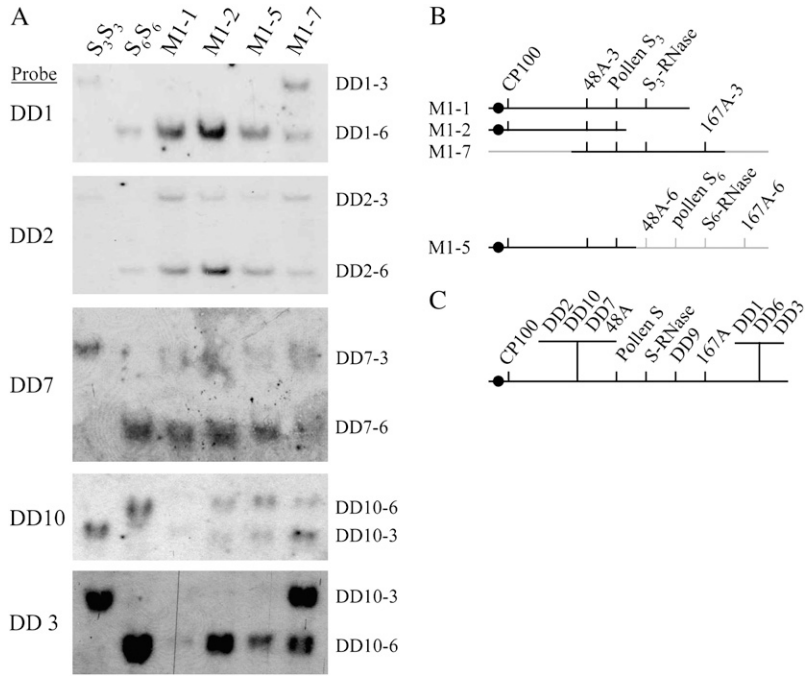

FIGURE 5.—Linkage of the *DD* genes to the S locus. (A) Representative Southern blots using probes specific for the indicated *DD* gene. The source of genomic DNA is shown at the top of each lane and the different alleles are indicated at the right. (B) Maps of dS_3 in each of the indicated PPMs. A solid dot indicates the centromere. All PPMs used in this experiment have the genotype $S_6S_6dS_3$. (C) Map of the *N. alata* S locus showing relative placement of the seven *DD* genes mapped using this approach. Placement of genes in the segment between *CP100* and *S-RNase* with respect to each other is arbitrary.

production of well-resolved genetic maps (LI *et al.* 2000; WANG *et al.* 2003). The relative order of seven of the *DD* genes with respect to each other and the surrounding S locus markers, however, could be determined using a small number of PPMs with duplicated S_3 allele segments of differing sizes (GOLZ *et al.* 2001). This analysis showed that four *DD* genes (*DD2*, *DD7*, *DD9*, and *DD10*) exist as single genes (or possibly as a cluster of a few genes) on either side of the *S-RNase* gene and within the S locus. Three of the *DD* genes (*DD1*, *DD3*, and *DD6*) were farther from the *S-RNase* gene than the marker *167A* and accordingly must be at least 0.9 cM from the S locus. In total, all 10 *DD* genes were linked to the S locus, although a lack of suitable polymorphisms for *DD4*, *DD5*, and *DD8* meant that the position of these genes relative to other S-locus markers and each other could not be determined.

Even though all the class S *SLF-like* genes identified in this study were expressed in *N. alata* pollen, they clearly cannot all be involved in SI. Some, for example, were expressed in tissues other than pollen—specifically *DD1* and *DD3*, which were also expressed in styles, and *DD10*, which had some expression in petals. Since all *SLFs* characterized to date are expressed only in pollen, these genes can be excluded from consideration as potential *SLF* orthologs. The remaining seven *DD* genes, however, appear to be expressed only in pollen and thus warrant further study. Although all the *DD* genes are at or near the *N. alata* S locus, fine-scale mapping indicates that only three—*DD2*, *DD7*, and *DD10*—are in the same region of the chromosome as *pollen S*. Since expression in petal means that *DD10* can be excluded, only *DD2* and *DD7* can still be considered possible *SLF* orthologs, with the status of *DD4*, *DD5*, and *DD8* being uncertain.

Interestingly, of these five "candidate" genes, *DD5* transcripts were amplified only from S_2S_2 pollen cDNA, and *DD2*, *DD7*, and *DD8* transcripts were amplified only from two of the five pollen cDNA templates tested (Figure 3 and data not shown). *DD4* transcripts were amplified from all five pollen templates. While this could potentially reflect a level of S-allele specificity, amplification of transcripts from some cDNA templates but not others was also seen with *DD* genes that are clearly not *pollen S* (*e.g.*, *DD9* was amplified only from S_1S_3 pollen cDNA).

Both the number of S-class *SLF-like* genes at or near the S locus and their pollen expression are worthy of comment. No such clustering of S-class *SLF-like* genes is apparent in Arabidopsis, where the 7 genes of this type are distributed across three of the five chromosomes (L. WANG *et al.* 2004). Predominant or exclusive expression of 10 S-class *SLF-like* genes in *N. alata* pollen also contrasts with the situation in Arabidopsis, where six of the seven genes are constitutively expressed and only one, *AtSFL79*, is strictly pollen expressed (L. WANG *et al.* 2004). *SLF-like* genes, however, have been found at or near the S locus in the Rosaceae (*e.g.*, see ENTANI *et al.* 2003; USHIJIMA *et al.* 2003; SASSA *et al.* 2007), the Plantaginaceae (ZHOU *et al.* 2003), and in the solanaceous species *P. inflata* (WANG *et al.* 2003). In each case, these S-locus-linked *F-box protein* genes are predominantly or exclusively expressed in pollen, just like the *DD* genes. We speculate that the number of S-class *SLF-like* genes at the S locus and their pollen expression is not due to chance but is an evolutionary outcome arising from the state of permanent heterozygosity that SI imposes.

In a process analogous to that leading to a loss of active genes on the male-determining Y chromosome of

animals (see Skaletsky *et al.* 2003), permanent heterozygosity at the S locus will reduce the effectiveness of selection against deleterious mutations occurring in genes with unrelated functions that are embedded within it (Uyenoyama 1997, 2005). As each individual S allele experiences a small effective population size, deleterious mutations in genes tightly linked to the S locus are highly likely to replace functional wild-type alleles, a process that should, over time, lead to the degeneration of most genes within the S-locus region except those with SI-specific functions. Any gene expressed in a haploid cell like a pollen grain will escape this effect because there are no genes associated with a second S allele to provide in *trans* the functions lost by mutation. Hence these genes will still be subject to selection against deleterious mutations, especially if their products contribute to the reproductive fitness of pollen. Selection may also favor expanding the number of these genes at the S locus through gene duplication in cases where higher expression levels enhance male fitness. Thus, this evolutionary scenario suggests that the cluster of *DD* genes at the *N. alata* S locus arises because enforced heterozygosity favors genes that contribute to male fitness and that through pollen expression avoid gene erosion processes.

The phylogenetic tree in Figure 2 highlights several puzzling aspects of the S-class SLF-like proteins. Most notably, the SLF/SFB proteins of the Plantaginaceae, Solanaceae, and Rosaceae do not form a monophyletic clade to the exclusion of other S-class SLF-like proteins with no role in SI, such as many of the DD proteins. Contrast can be made here to trees of S-RNases and the related S-like RNases, which are not involved in SI. Phylogenetic analysis places S-RNases and S-like RNases into one of three classes: a single monophyletic clade that contains all S-RNases from the Solanaceae, Plantaginaceae, and Rosaceae and two separate clades of S-like RNases (Igic and Kohn 2001; Steinbachs and Holsinger 2002). The apparent homology of the S-RNases leads to the conclusion that the three known RNase-based SI systems share a single evolutionary origin. In Figure 2, however, S-class SLF-like proteins are grouped not according to function but on the basis of the taxonomic relationships of the organisms from which they were derived. Thus the conclusion that the S-RNases of the Solanaceae, Rosaceae, and Plantaginaceae are homologous is not borne out by this analysis of the SLFs and SFBs. Assuming that the SLFs and SFBs are involved in SI, then the most parsimonious interpretation of Figure 2 is that their encoding genes have been independently recruited to roles in RNase-based SI in the Rosaceae, Plantaginaceae, and Solanaceae. Lack of homology implies that different mechanisms may be used to achieve the rejection of incompatible pollen in each family.

A second notable aspect of the phylogenetic reconstruction in Figure 2 is the well-resolved clade arising at node 5 that contains all the Petunia SLFs, proteins such as PaF1 (*P. axillaris* F-box protein 1) that are presumed not to be at the Petunia S locus (Tsukamoto *et al.* 2005), and two DD proteins (DD1 and DD3) that are not considered candidates for the *N. alata* SLF ortholog because their encoding genes are at least 0.9 cM from the S locus (Figure 5). Numerous phylogenetic analyses of the solanaceous S-RNases place these sequences in a well-resolved monophyletic clade that excludes S-like RNases (*e.g.*, Igic and Kohn 2001; Steinbachs and Holsinger 2002). Any RNase sequence falling into the S-RNase clade not involved in self-incompatibility (*e.g.*, one obtained from a self-compatible plant) is presumed to be derived from a nonfunctional S-RNase (relic S-RNases; Golz *et al.* 1998). For the clade in Figure 2 to be a clade of solanaceous SLFs resembling the clade of solanaceous S-RNases, DD1, DD3, and PaF1 must all be relic SLFs. Since these proteins are from self-incompatible species, it therefore becomes problematic to assign the name SLF to any protein sequence within this clade unless its function in SI has been verified experimentally. It is also notable that four of the remaining five SLF candidates—*DD2*, *DD5*, *DD7*, and *DD8*—are not in this SLF-containing clade.

The final puzzling feature of the tree is the shortness of terminal branches for SLFs in the Plantaginaceae and Solanaceae, reflecting the low levels of sequence polymorphism among SLF alleles in these two families. Notably, this feature is not seen with SFBs from the Rosaceae. The four Antirrhinum SLF alleles are identical at 97% or more of their amino acid positions and the two most divergent Petunia SLF alleles, PiSLF-S_3 and PaSLF-S_{17}, are 87% identical. These percentages of identity values are closer to those of genes with functions unrelated to self-incompatibility that also lie within the S locus (such as *48A*; Takebayashi *et al.* 2003) than they are to the S-RNases, where pairwise amino acid identities of 40–50% are common (Clark and Kao 1994). The pairwise amino acid identity in partial cDNAs of *DD1*, *DD2*, *DD4*, *DD6*, *DD7*, *DD8*, and *DD10* from different S-allele backgrounds ranges from 1 to 5%.

SI imposes a very intense form of balancing selection on the S locus, as a consequence of which high numbers of S-allele lineages are maintained over long periods of time (reviewed by Clark and Kao 1994; Richman 2000). The effect that balancing selection has had on the phylogenetic history of the *S-RNase* gene, particularly on extending the phylogenies of allelic lineages over time spans that are vastly longer than those expected under neutrality, has been demonstrated many times (see Ioerger *et al.* 1990; Richman *et al.* 1996). In the Rosaceae, identifying SFBs as the pollen S factor is based in part on agreement between the expected phylogeny of a gene under balancing selection and the observed SFB phylogenies (Ikeda *et al.* 2004; Nunes *et al.* 2006; Sassa *et al.* 2007). Conversely, genes have also been excluded from consideration as pollen S if their

phylogeny did not show the expected features (*e.g.*, Takebayashi *et al.* 2003).

Little evidence of this predicted evolutionary history is seen in the Petunia and Antirrhinum SLFs, however: the clades containing these sequences are marked by short terminal branches that quickly converged on the common ancestor of all sampled lineages. Because in the Rosaceae SFBs, sites identified as positively selected largely overlap with regions of highest sequence variability (Ikeda *et al.* 2004; Nunes *et al.* 2006), we visually inspected an alignment of SLF proteins from the Solanaceae for highly variable sites (*i.e.*, sites occupied by many different amino acids; see supplemental Figure 7 at http://www.genetics.org/supplemental/). As only one such site was found, it is probable that the sequences also lack positively selected sites likely to be responsible for defining pollen specificities.

The marked lack of concordance between S-RNase trees, which are largely consistent with well-established theories as to how genes evolve under balancing selection (Clark and Kao 1994), and the S-class SLF-like tree for the Solanaceae leads to the unsettling conclusion that the SLFs and S-RNases are not stably co-evolving partners, which previously had been assumed would be true of the pollen and stylar factors of the S locus. Two possible explanations of this situation exist: either there is no obligate relationship between the S-RNase and a particular S-class F-box protein or the SLFs do not fulfill the role of pollen S in the Solanaceae and Plantaginaceae.

Although phylogenetic reconstruction of the S-class SLF-like proteins provides little indication that the SLFs have a long history of evolving under balancing selection, other evidence is consistent with the expected behavior of pollen S. Apart from their genetic location and expression pattern, this evidence includes the binding of recombinant SLF proteins to S-RNases and the pollination behavior of transgenic plants that express SLFs in their pollen (*e.g.*, see Qiao *et al.* 2004b; Sijacic *et al.* 2004). It should, however, be noted that so far it has not been possible to demonstrate allele specificity for the SLFs (McClure 2004). Recombinant SLF proteins bind S-RNases nonspecifically, and SLF expression in transgenic plants results in a loss of SI rather than in the gain of a new S-allele specificity. Since other proteins can bind S-RNases (Sims and Ordanic 2001), and since mutations in other genes can lead to self-compatible phenotypes (*e.g.*, Ai *et al.* 1991), it remains formally possible that the properties currently used to define pollen S in the Solanaceae and Plantaginaceae are not exclusive to this factor.

The alternative explanation is that the functions of *pollen S* are transient and able to be performed by any one of the many paralagous S-class *SLF-like* genes within the S locus. Sassa *et al.* (2007) recently made a similar suggestion on the basis of their observation that the S locus in Malus and Pyrus (Rosaceae) contained two or more copies of the *SFB* gene ("*SFB brothers*" or *SFBBs*). If paralagous *SLF* genes act as proposed, then *pollen S* lineages might be expected to turn over far more rapidly than S-RNase lineages. Although this suggestion currently lacks experimental support, it is obvious that identifying which, if any, of the *DD* genes codes for pollen S in *N. alata* will help refine ideas as to why the *SLF* and *S-RNase* genes have such remarkably divergent evolutionary histories.

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