



An F-box gene linked to the self-incompatibility (*S*) locus of *Antirrhinum* is expressed specifically in pollen and tapetum

Zhao Lai¹, Wenshi Ma¹, Bin Han², Lizhi Liang¹, Yansheng Zhang¹, Guofan Hong² and Yongbiao Xue^{1,*}

¹Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100080, China (*author for correspondence; e-mail ybxue@public3.bta.net.cn or ybxue@genetics.ac.cn; ²National Center for Gene Research, Chinese Academy of Sciences, Shanghai 200233, China

Received 6 March 2001; accepted in revised form 10 December 2001

Key words: *Antirrhinum*, F-box, gametophyte, self-incompatibility, *S* locus structure

Abstract

In many flowering plants, self-fertilization is prevented by an intraspecific reproductive barrier known as self-incompatibility (SI), that, in most cases, is controlled by a single multiallelic *S* locus. So far, the only known *S* locus product in self-incompatible species from the Solanaceae, Scrophulariaceae and Rosaceae is a class of ribonucleases called S RNases. Molecular and transgenic analyses have shown that S RNases are responsible for pollen rejection by the pistil but have no role in pollen expression of SI, which appears to be mediated by a gene called the pollen self-incompatibility or *Sp* gene. To identify possible candidates for this gene, we investigated the genomic structure of the *S* locus in *Antirrhinum*, a member of the Scrophulariaceae. A novel F-box gene, *AhSLF-S₂*, encoded by the *S₂* allele, with the expected features of the *Sp* gene was identified. *AhSLF-S₂* is located 9 kb downstream of *S₂* RNase gene and encodes a polypeptide of 376 amino acids with a conserved F-box domain in its amino-terminal part. Hypothetical genes homologous to *AhSLF-S₂* are apparent in the sequenced genomic DNA of *Arabidopsis* and rice. Together, they define a large gene family, named *SLF* (*S* locus F-box) family. *AhSLF-S₂* is highly polymorphic and is specifically expressed in tapetum, microspores and pollen grains in an allele-specific manner. The possibility that *Sp* encodes an F-box protein and the implications of this for the operation of self-incompatibility are discussed.

Introduction

Self-incompatibility (SI) is a genetic mechanism to prevent self-fertilization in many flowering plants (de Nettancourt, 1977). In Solanaceae with gametophytic SI, a single multi-allelic locus, the *S* locus, controls the incompatibility reaction (de Nettancourt, 1977). Pollen rejection occurs when the *S* allele carried by pollen matches either of the two *S* alleles present in the pistil. So far, the only known product encoded by the *S* loci of the Solanaceae, Scrophulariaceae and Rosaceae is a class of ribonucleases, called S-RNases (Anderson

et al., 1986, 1989; McClure *et al.*, 1989a; Ai *et al.*, 1990; Broothaerts *et al.*, 1995; Sassa *et al.*, 1996; Xue *et al.*, 1996). Molecular and transgenic analyses have shown that S RNases mediate the stylar expression of SI without playing a role in pollen expression of SI (Huang *et al.*, 1994; Lee *et al.*, 1994; Murfett *et al.*, 1994; Dodds *et al.*, 1999). Pollen expression is controlled by a distinct gene, frequently referred to as the pollen self-incompatibility (*Sp*) gene. The bipartite structure of the *S* locus is well supported by mutational analysis of the *S* locus in several SI species including *Antirrhinum* (Lewis, 1951; Pandey, 1965; Golz *et al.*, 1999, 2000; Y. Xue, R. Carpenter, H. Dickinson and E.S. Coen, unpublished data).

The nucleotide sequence data reported will appear in the EMBL nucleotide data library under the accession numbers AJ300474 (*S2BAC*), AJ297974 (*AhSLF-S₂*) and AJ297975 (*AhSLF-S₂L*).

Understanding how S-RNases function in SI has been hampered by the lack of a cloned *Sp* product. Site-directed mutagenesis and domain swapping experiments have shown that both S-RNase activity and hypervariable domains within the S-RNases are involved in pollen rejection (Huang *et al.*, 1994; Zurek *et al.*, 1997; Matton *et al.*, 1997, 1999). It has been proposed that S-RNases act as cytotoxins that degrade pollen tube RNA and that *Sp* encodes an inhibitor or receptor for the S-RNases (McClure *et al.*, 1989a, b; Thompson and Kirch, 1992; Kao and McCubbin, 1996). Support for the inhibitor model comes from the observation that *S* allele duplications are associated with most of self-compatible (SC) mutants affecting only pollen expression of SI (pollen-part mutants) in *Nicotiana*, *Petunia* and *Antirrhinum* (Brewbaker and Natarajan, 1960; Pandey, 1965; Golz *et al.*, 1999; Y. Xue, R. Carpenter, H. Dickinson and E.S. Coen, unpublished data). This could be explained if *Sp* acts by inhibiting all S-RNases except self-S-RNase. When two alleles are present inside pollen tubes, both self- and non-self-S-RNase activities would be suppressed by action of two heteroallelic *Sp* products, allowing pollen tube growth to occur. Further support for this is that both self and non-self S-RNases are taken up by pollen tubes in both *in vitro* and *in vivo* studies (Gray *et al.*, 1991; Luu *et al.*, 2000), suggesting that S RNase uptake has no allelic specificity.

We previously showed that the *Antirrhinum* *S* locus encodes S-RNases, similar to those in the Solanaceae (Xue *et al.*, 1996). To identify possible candidates for the *Sp* gene, we have investigated the structure of *Antirrhinum* *S* locus. A bacterial artificial chromosome (BAC) library from a self-incompatible line with *S*₂*S*₄ alleles was screened with S-RNase genes, yielding a BAC clone corresponding to over 63 kb of the *S*₂ region. An F-box gene, *AhSLF-S*₂, with the expected features of the *Sp* gene, was identified 9 kb away from the *S*₂-RNase. This gene is a member of a novel family, called *SLF* (*S* locus F-box), with members in *Antirrhinum*, *Arabidopsis* and rice. A possible role for the protein encoded by *AhSLF-S*₂ in the mechanism of the incompatible reaction is discussed.

Materials and methods

Plant material

A. majus M⁷⁵ (self-compatible) and self-incompatible lines derived by interspecific crosses between *A. majus*

and *A. hispanicum*, as well as the *S* allele segregating populations, were described previously (Xue *et al.*, 1996). Two additional *S* allele segregating families with 30 progeny each (A1¹⁻³⁰ and A2¹⁻³⁰, a superscript denotes the number of plants) were generated by crossing D285⁸ (*S*₁*S*₂) with D285⁹ (*S*₄*S*₅) and D285⁴ (*S*₂*S*₅) (Xue *et al.*, 1996), respectively. Progeny *S* genotypes were determined using S-RNase probes (Xue *et al.*, 1996) and their SI phenotype confirmed by cross-pollination.

Construction and screening of the bacterial artificial chromosome library

High-molecular-weight (HMW) DNA of over 2 Mb from a self-incompatible line with *S*₂*S*₄ alleles was prepared from leaf nuclei according to Liu and Whittier (1994). The partial digestion of HMW DNA with *Hind*III, pBeloBACII vector preparation, ligation and *Escherichia coli* DH10B transformation by electroporation followed the protocol described by Shizuya *et al.* (1992). A total of 35 000 recombinant BAC clones were selected from two ligation reactions. To check the insert length, plasmid DNA from the BAC clones was prepared, restricted by *Not*I digestion and separated by pulsed-field gel electrophoresis with the CHEF-DR III System (BioRad).

A total of 35 000 BAC clones were arrayed at high density on Hybond⁺ membranes (Amersham) using BIOMECK 2000 (Beckman). On each membrane (8 cm × 12 cm), 1182 clones were spotted three times. The membranes containing the colonies were left at 37 °C overnight on LB agar medium before being treated according to Sambrook *et al.* (1989). The BAC library was screened with a mixed ³²P-labeled cDNA by Prime-a-gene Labeling System (Promega) from *S*₂ and *S*₄ RNases (Xue *et al.*, 1996). Positive clones were further screened by PCR analysis using primers corresponding to their respective full-length cDNA (Xue *et al.*, 1996).

DNA sequencing and assembly of S2BAC

The *S2BAC* was sequenced by using a shotgun approach (Bankier *et al.*, 1987). The BAC DNA was purified by cesium chloride gradient and sheared by sonication. End repair was performed by T4 DNA polymerase (Boehringer Mannheim) treatment following the manufacturer's instruction. DNA fragments were size-fractionated (2–3 kb) by low-melting-gel electrophoresis with Argorase (Boehringer Mannheim) and cloned into *E. coli* XL-1-blue electrocompetent

cells by electroporation. Recombinant clones were randomly picked. DNA templates for sequencing were isolated by using a 96-well alkaline lysis miniprep derived from the alkaline lysis miniprep (Sambrook *et al.*, 1989) and purified through a 96-well Multi-Screen filter (Millipore). Sequencing reactions were carried out using the DYEnamic ET dye terminator kit (MegaBACE, Amersham Pharmacia Biotech) and analyzed on a MegaBace 1000 sequencer. In total, 1129 reads were obtained and 63 687 bp generated with a redundancy of 11.4 (EMBL accession number AJ300474).

The *S2BAC* end sequencing reactions were performed by using SP6 (TATTTAGGTGTCCTACTATAG) and HP1 (CTCTAGAGTCGACCTGCAG) primers and the BigDye Terminator Cycle Sequencing V2.0 kit with AmpliTaq DNA polymerase, FS. The reaction products were separated on ABI 377 sequencing gel (PE Applied Biosystems).

The sequence data were assembled by Phred/Phrap software (Ewing and Green, 1998; Ewing *et al.*, 1998) and the Staden Package/Pregap/Gap4 software (Bonfield *et al.*, 1995). Correct assembly of the sequence was verified by comparison to an optical *NotI* digest pattern of the *S2BAC* clone.

Molecular techniques

Genomic DNA isolation and PCR were performed as previously described (Xue *et al.*, 1996). RNA was isolated with an RNeasy Plant Kit (Qiagen).

To clone BAC ends, the BAC plasmid DNA (1 μ g) was separately digested by several restriction enzymes (*RsaI*, *MseI*, *TaqI*, *BamHI* and *EcoRI*). After ligation with T-DNA ligase (Promega), the mixture containing 50 ng of the ligated DNA was subjected to PCR amplification with two pairs of adjacent vector primers flanking the insert. The left pairs of the primers were CCTAAATAGCTTGCGTAATCATG and TGACACTATAGAATACTCAAGCTT and the right pairs CGACCTGCAGGCATGCAAGCTT and ACTCTAGAGGATCCCCGGGTAC.

cDNA inserts were sequenced by primer walking as described above. Database searches and protein structure predications were performed using Internet accessible software (WU-Blast2.0 and Pfam) (<http://www.ebi.ac.uk/>). Protein sequences were aligned with a Clustal method from the DNASTAR package. The EMBL accession numbers for *AhSLF-S2* and *AhSLF-S2L* are AJ297974 and AJ297975, respectively.

Primers for the *S2-RNase* gene, *G2338* (ACAATC-GACATGGCTAC, nucleotides 1–17, similarly hereinafter) and *G1280* (GCTTGCCCTTTCTCAAG, 758–741), were according to Xue *et al.* (1996). *AhSLF-S2* primers were *G11e* (ATGATGGATC-GACGATTTCCG, 606–626), *G11j* (ATTATTTGACATTTGGGTTATG, 1466–1487), *G11f* (CAACAA-CGTAAGGGCACTCCC, 1937–1917), *G11m* (CATGATAGTTTTTTTTATTTC, 1807–1825) and *G11n* (GT-CAGAAGTGAACAACAG, 1962–1955). *AhSLF-S2L* primers were *G11k* (TATGGTAAAAGAATTCTT, 1362–1379) and *G11l* (TTAAAGATGTTGAACAAT, 1541–1524).

To scan the 63 kb genomic sequence for polymorphic regions, genomic DNA from several SI lines with different *S* alleles were used for PCR analysis with a total pairs of 12 primers which are available on request.

cDNA library construction

Anther cDNA libraries were constructed from an *S2S4* (A^{16}) and an *S1S5* (A^{19}) line using a SMART cDNA library Construction kit (Clontech). Each library was screened by plaque lift hybridization with a genomic fragment containing *Gene11* or *AhSLF-S2* cDNA as probes (Sambrook *et al.*, 1989). Phage cDNA clone was converted into plasmid form according to the manufacturer's instructions (Clontech).

RNA expression analysis

Total RNA (10 μ g) was subjected to RNA blot analysis (Sambrook *et al.*, 1989). RACE analysis was performed as described using *AhSLF-S2*-specific primers as described (Xue *et al.*, 1996). A 156 bp fragment amplified from the 3'-UTR of *AhSLF-S2* with primers *G11m* and *G11n* was cloned into pBluescript (Stratagene) in either orientation. Ubiquitin transcripts were amplified according to Xue *et al.* (1996). Digoxigenin-labeled sense or anti-sense RNA probes were prepared following the manufacturer's recommendation (Boehringer Mannheim). Tissue fixation and embedding, *in situ* hybridization and signal detection were essentially performed as described (Coen *et al.*, 1990; http://genome-www.stanford.edu/Arabidopsis/cshl-course/5-in_situ.html/).

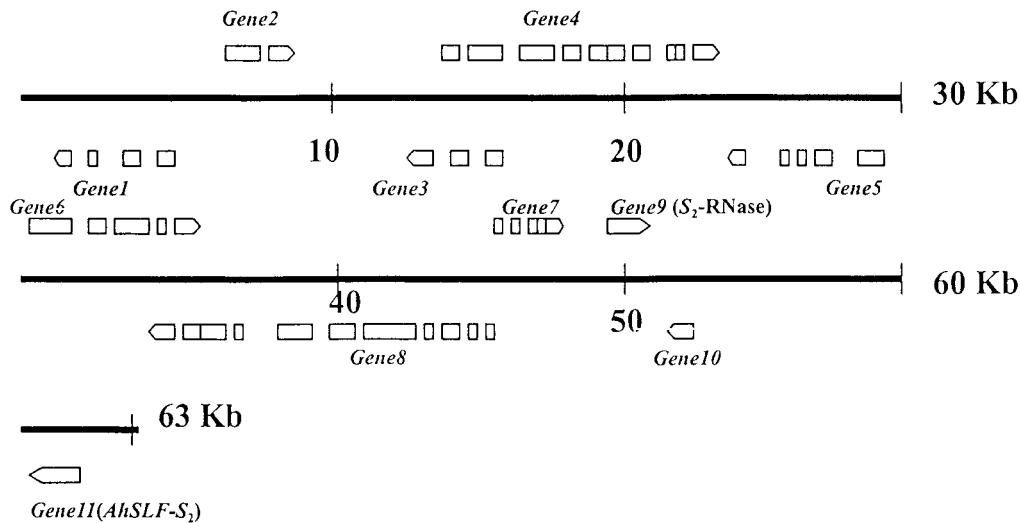


Figure 1. A schematic illustration of *S2BAC* containing part of *S2* allele. Boxes represent predicted exons with arrowed ones pointing the direction of the predicted genes (*Gene1–11*). *S2* RNase and *AhSLF-S2* are also indicated in parenthesis.

Table 1. Predicted and known genes in the *S2BAC*.

Gene	Predicted ORF (amino acids)/exons	Identity
<i>Gene1</i>	203/4	Unknown
<i>Gene2</i>	158/2	Unknown
<i>Gene3</i>	173/3	Unknown
<i>Gene4</i>	1299/15	Retroelement polyprotein (Q9XEM5 ¹)
<i>Gene5</i>	320/5	Retroelement (Q9SKS4)
<i>Gene6</i>	1455/5	Retroelement polyprotein (Q9LMV1)
<i>Gene7</i>	1504/14	Unknown
<i>Gene8</i>	151/4	Unknown
<i>Gene9</i>	156/1	<i>Antirrhinum S2</i> -RNase
<i>Gene10</i>	156/1	Transposon-like protein (Q9LIE1)
<i>Gene11</i>	376/1	AhSLF- <i>S2</i> (an F-box protein)

¹EMBL accession numbers represent similar or identical sequences to the predicted genes.

Results

Construction of a BAC library from a self-incompatible *Antirrhinum* line

To determine the genomic structure of the *S* locus and isolate additional genes involved in self-incompatibility, a BAC library from a self-incompatible *Antirrhinum* line with *S2S4* genotype was constructed. High-molecular-weight (HMW) genomic DNA was isolated from the leaf nuclei. After partial digestion with *Hind*III, the HMW DNA was cloned into *Hind*III-cut pBeloBACII (Shizuya *et al.*, 1992) and about 35 000 recombinant BAC clones selected. Pulsed-field gel electrophoresis (PFGE) analy-

sis of the inserts from 100 randomly chosen BAC clones showed that the library had an average insert length of 70 kb (data not shown). Based on the estimated *Antirrhinum* genome size (ca. 430 Mb) (Bennett and Leitch, 1995), the BAC library is equivalent to a 5× coverage of its diploid genome. The BAC clones were arrayed at high density (1152 clones per membrane) on a total of 29 Hybond⁺ membranes, with each clone duplicated three times. Screening of the library with several genes from *Antirrhinum* showed that, on average, one to five clones could be identified having the gene (data not shown), showing that the BAC library constructed had an extensive coverage of the *Antirrhinum* genome.

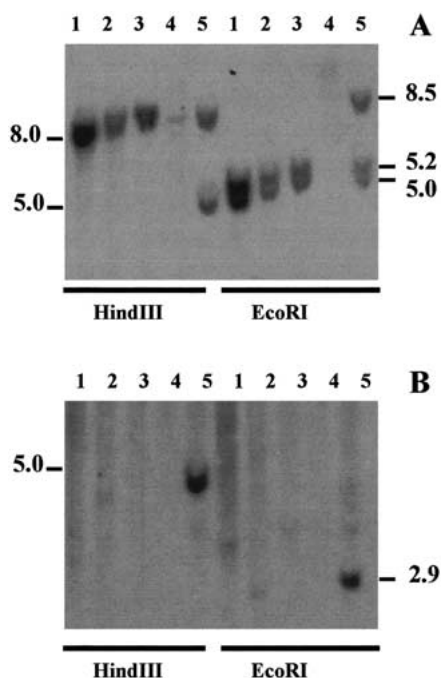


Figure 4. *AhSLF-S2* is a polymorphic gene. A. Genomic DNA (10 μg) from various *S* allele-containing lines were digested with *Hind*III and *Eco*RI. After separation by agarose gel electrophoresis and blotting, the DNA was hybridized with *AhSLF-S2* cDNA. The blot was washed at high stringency (0.1× SSC and 0.5% SDS for 30 min twice). Lanes 1–5 represent plants with *S1S4*, *S4S5*, *S1S5*, *S_cS_c* and *S1S2*, respectively. The numbers indicate the sizes of the hybridizing fragments in kilobases. B. The same blot was hybridized with the *S2* RNase cDNA.

lin, 1997), 11 genes were predicted in the sequenced region (Figure 1 and Table 1). *Gene9* corresponds to the *S2* RNase gene, although the first exon was not predicted by the software possibly because of an in-frame ATG upstream of the second exon. Apart from four predicted genes (*Gene4*, *5*, *6* and *10*) that appeared to be retrotransposons, the remaining 6 genes were not related to any of genes in the EMBL database (Table 1). One predicted gene (*Gene11*) was confirmed to be expressed in *Antirrhinum* (see below). Taken together, the results showed that the *Antirrhinum S* locus is located in a genomic region containing abundant retrotransposons, similar to the sequenced *S* locus in *Brassica* (Suzuki *et al.*, 1999; Cui *et al.*, 1999, 2000).

The *Antirrhinum S* locus contains a novel F-box gene

Self-incompatibility genes are expected to be polymorphic. To locate these genes, we scanned the *S2BAC* region from self-incompatible lines containing different *S* alleles, using PCR on genomic DNA with pairs

of primers positioned at ca. 4 kb intervals along the BAC. This showed that *Gene2*, *5*, *10* and *11*, in addition to the *S2* RNase gene, are located in polymorphic regions because their products were only detected in the lines carrying *S2* (data not shown).

To find out if any of the predicted genes are expressed in the male gametophyte, primers derived from the predicted genes were used for RACE (rapid amplification of cDNA ends) analysis of anther RNA. Anther transcripts were only detected for *Gene11* (data not shown, but see below).

To isolate its cDNA, a DNA fragment containing *Gene11* was used to screen a cDNA library made from a pool of immature and mature anthers from the *S2S4* line. One positive was identified in 10⁶ plaques and DNA sequence analysis revealed that it had the same sequence as the *Gene11* genomic region. It contained an insert of 1986 bp corresponding to a single exon (Figure 2). The cDNA detected a 2.0 kb transcript on northern blots, showing that it represented a near full-length copy (see Figure 6). The cDNA had an uninterrupted ORF of 376 amino acids (Figure 2), in agreement with the ORF predicted by GENESCAN. A notable feature was a long 5'-untranslated region (UTR) (605 bases) (Figure 2).

Database searches revealed that the ORF was similar to a large family of hypothetical proteins from sequenced *Arabidopsis* and rice genomes (Figure 3). Pfam analysis of these genes revealed a conserved F-box domain (4, 14) in their N terminal regions (Figure 3). Furthermore, three conserved domains designated C1-C3 were found outside the F-box region (Figure 3). The three regions between the conserved domains were quite divergent (Figure 3). The C-terminal parts also display extensive variability among the predicted polypeptides (Figure 3). These results indicated that the *Gene11* represents an F-box gene belonging to a gene family with members found in *Antirrhinum*, *Arabidopsis* and rice. This family of F-box genes are named *SLF* (*S* locus F-box) and the *Antirrhinum S2* allele *AhSLF-S2* (*Antirrhinum hispanicum SLF-S2*).

AhSLF-S2 is a polymorphic and allele-specific gene

To determine the genomic organization of *AhSLF-S2*, its full-length cDNA was used to probe DNA from several genotypes including a self-compatible *A. majus* line (Figure 4). The result showed that it detected an *S2* allele-specific fragment in DNA digested with either *Hind*III (5 kb) or *Eco*RI (8.5 kb) as predicted

from the *S2BAC* sequence (Figure 4). In addition, one and two fragments were detected in all of the self-incompatible lines in DNA digested with either *Hind*III (8.0 kb) or *Eco*RI (5.0 and 5.5 kb) (out of 30 plants tested), respectively, and a single weakly hybridizing fragment was detected in the self-compatible line in DNA digested with either *Hind*III (8.0 kb) or *Eco*RI (over 12 kb) (Figure 4), indicating that sequences identical or homologous to *AhSLF-S₂* are present in both the self-incompatible and compatible lines. In the self-incompatible lines, the hybridizing fragments did not show polymorphisms. Furthermore, RNA blot hybridization with the full-length *AhSLF-S₂* cDNA detected similarly sized anther-specific transcripts (2.0 kb) in both *S₂*-containing and non-*S₂*-containing lines (see Figure 6). These results indicated that either *AhSLF-S₂* is a single-copy gene with allelic variants or its homologue(s) is also an anther-specific gene.

To distinguish between these possibilities, an anther cDNA library made from the *S₁S₅* line was screened using *AhSLF-S₂* cDNA as a probe. One cDNA clone was subsequently identified. The cDNA was 1571 bp long and was named *AhSLF-S₂L* (*AhSLF-S₂*-like). This sequence had 90.5% identity to *AhSLF-S₂* at the nucleotide level and the two sequences differed mainly in their 3'-UTR regions. However, because a 2.0 kb transcript was detected in the *S₁S₅* anther (see Figure 6), the *AhSLF-S₂L* cDNA was presumably not a full-length clone, missing about 400 bases at its 5' end. *AhSLF-S₂L* was also capable of encoding an ORF of 376 amino acids showing 97.9% identity to *AhSLF-S₂*. In total, 8 amino acid changes were found between the two sequences, two at the F-box domain and 6 at the C-terminal region (see Figure 3). The substrate specificity of F-box protein is usually determined outside of the F-box region (Bai *et al.*, 1996; Skowrya *et al.*, 1997). Therefore, the location of the amino acid changes in the 3' region but not in the conserved domains suggest that *AhSLF-S₂* and *AhSLF-S₂L* may possess different substrate specificity if they indeed function as the F-box proteins. The isolation of *AhSLF-S₂L* showed that the sequences detected by *AhSLF-S₂* in self-incompatible lines represented a related but not an identical copy with a similar expression pattern to *AhSLF-S₂*. Consistent with this, an internal *Eco*RI site is present in the *AhSLF-S₂L* cDNA, which explains the two *Eco*RI fragments detected (Figure 4).

To demonstrate further the location of *AhSLF-S₂* at the *S* locus, several pairs of primers from its 3'-

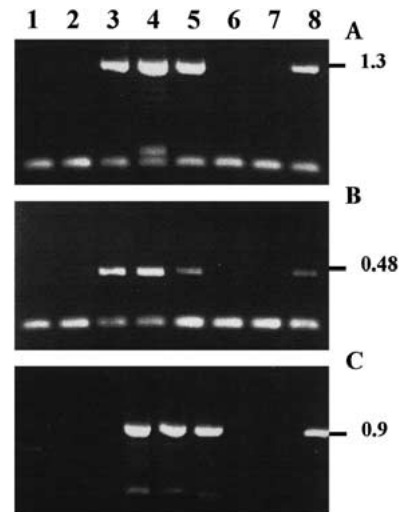


Figure 5. *AhSLF-S₂* is an allelic gene. A. PCR was performed on genomic DNA from a *S* allele-segregating population with *AhSLF-S₂*-specific primers: *G11e* and *G11f*. Lanes 1–8 represent plants with *S₁S₄*, *S₄S₅*, *S₂S₅*, *S₁S₂*, *S₂S₅*, *S₄S₅*, *S₁S₅* and *S₂S₄*, respectively. Lanes 7 and 8 represent two parental lines. The allelic PCR products are indicated with their sizes in kb. B. PCR was performed on genomic DNA from *S* allele-segregating population with *AhSLF-S₂*-specific primers: *G11j* and *G11f*. The DNA was the same as in A. C. Genomic PCR with *S₂*-RNase-specific primers: *G2338* and *G1280*. The DNA was the same as in A.

UTR regions were used for genomic PCR analysis in a population of 120 plants segregating for the *S* allele (Xue *et al.*, 1996). *S₂*-specific products were specifically detected in *S₂*-containing lines without any recombination with the *S₂*-RNase gene (Figure 5), showing that *AhSLF-S₂* maps to the *S* locus. The common amplified bands resulted from primer dimers. Further, *AhSLF-S₂*-specific probe derived its 3'-UTR region hybridized specifically to *AhSLF-S₂*-specific fragments in the plants having an *S₂* allele (data not shown). *AhSLF-S₂L*-specific primers were also used for similar analysis and the same products were detected in all the plants without allelic specificity (data not shown). Taken together, these results clearly showed that *AhSLF-S₂* is an *S₂*-specific polymorphic gene.

AhSLF-S₂ has a pollen- and tapetum-specific expression pattern

Although *AhSLF-S₂* was isolated from the anther-derived cDNA library, it was not clear if it is indeed a gametophyte-specific gene, a feature expected for the *Sp* gene. The full-length *AhSLF-S₂* specifically detected similar transcripts of 2.0 kb in the

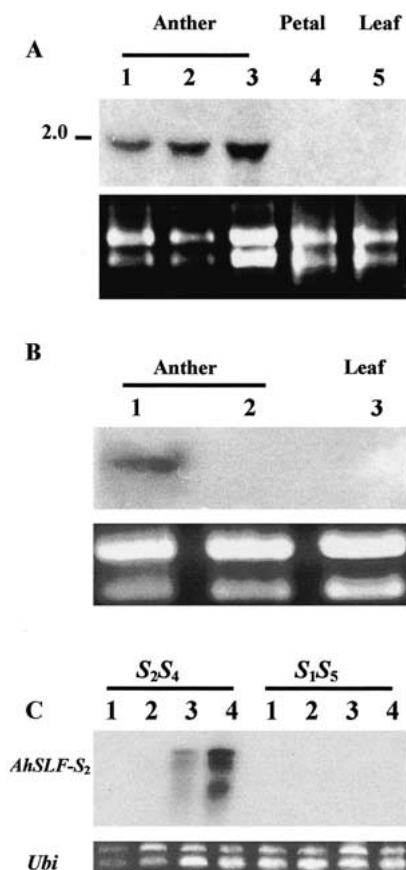


Figure 6. RNA expression analysis of *AhSLF-S2*. **A.** RNA from anther, petal and leaf of self-incompatible lines with different *S* allele combinations was hybridized with the full-length cDNA of *AhSLF-S2*. The RNA loading control was shown at the bottom part of the panel. RNA was isolated from self-incompatible plants with S_2S_4 (lanes 1, 4 and 5), S_1S_5 (lane 2) and S_2S_5 (lane 3). **B.** RNA from anther and leaf was hybridized with a carboxy-terminal probe specific to *AhSLF-S2*. Lanes 1 and 3 are RNA from S_2S_4 line and lane 2 S_1S_5 . **C.** RT-PCR analysis of *AhSLF-S2* gene expression during different stages of anther development of S_2S_4 and S_1S_5 plants. RT-PCR products were blotted and hybridized with *AhSLF-S2* cDNA as a probe. 1, anthers of less than 4 mm long; 2, anther of longer than 1 cm; 3, mature anther and 4, leaf. The multiple hybridization bands likely resulted from additional poly(A) signals or a PCR artifact. As a control, ubiquitin transcripts (*Ubi*) were amplified, separated by agarose gel electrophoresis and detected after ethidium bromide staining.

anthers from both S_2 and non- S_2 containing lines (Figure 6A), showing that *AhSLF-S2* and its homologue (*AhSLF-S2L*) are anther-specific genes. To detect *AhSLF-S2*-specific transcripts, a 3'-end probe was used in RNA blot analysis. The result showed that *AhSLF-S2*-derived transcripts were only found in anthers containing the S_2 allele, indicating that it is an S_2 anther-specific gene (Figure 6B). To determine at

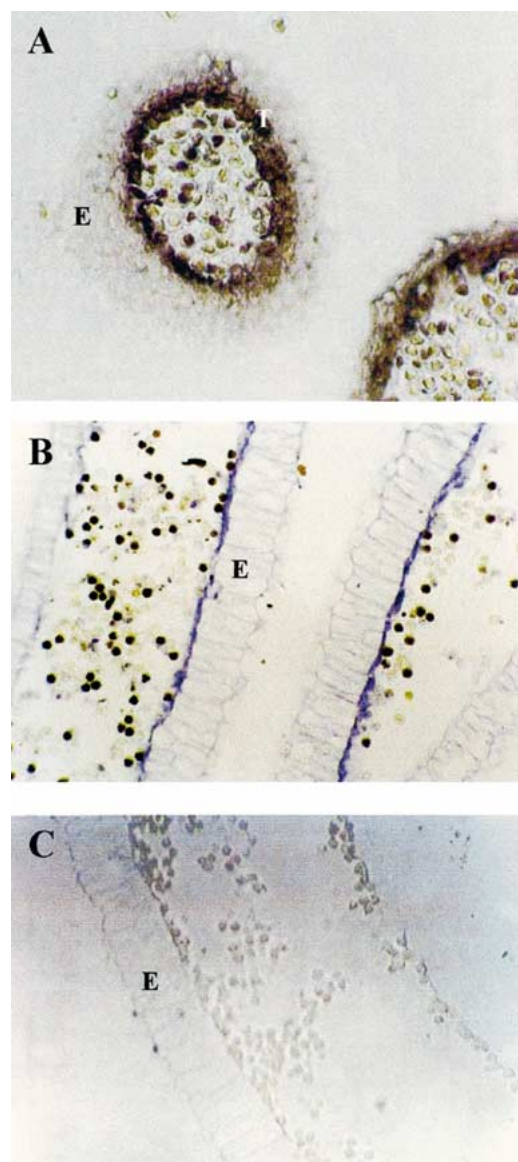


Figure 7. *AhSLF-S2* is a gametophyte-specific gene. **A.** The expression of *AhSLF-S2* in a cross-sectioned anther around the tetrad stage detected by RNA *in situ* hybridization with a 3' part anti-sense probe-specific to *AhSLF-S2*. E, epidermis; T, tapetum. The dark color shows the hybridization signal. **B.** The expression of *AhSLF-S2*: a longitudinal section of anther with well-formed pollen sac (binucleate stage). The pollen expressing *AhSLF-S2* displayed dark color. **C.** RNA *in situ* hybridization with the sense probe on a similar section as shown in B.

which stage of microspore formation *AhSLF-S₂* is expressed, RACE analysis were carried out on anther RNA from several anther developmental stages. Observations under the light microscope indicated that when the *Antirrhinum* anther length is less than 4 mm, microspores are in a premeiotic stage, and enter meiosis after this to form tetrads (data not shown). No transcripts were detected in anthers less than 4 mm, whereas they were present in the anthers longer than 4 mm (Figure 6C), suggesting that *AhSLF-S₂* is a gametophyte-specific gene. To confirm this, *in situ* RNA hybridization was done with the *AhSLF-S₂*-specific antisense probe on the anthers from the *S₂S₅* line (Figure 7). *AhSLF-S₂* expression was initially detected around the tetrad stage both in the microspores and tapetum (Figure 7A). After microspore separation (binucleate pollen) *AhSLF-S₂* transcripts were specifically found in about half of the pollen grains within the pollen sac (Figure 7B), consistent with the prediction that only *S₂*-containing pollen produced expressed it and confirming that it is a pollen- and tapetum-specific gene. No transcripts were detected using the *AhSLF-S₂*-specific sense probe (Figure 7C).

Discussion

We sequenced over 63 kb of genomic DNA sequence corresponding to the part of the *S₂* allele in *Antirrhinum*. A novel F-box gene, *AhSLF-S₂*, was identified and located 9 kb away from *S₂*-RNase gene. *AhSLF-S₂* is a pollen- and tapetum-specific gene and a member of a gene family.

AhSLF-S₂ is a candidate for the pollen self-incompatibility gene

As predicted, the *Sp* gene has to be located in the *S* locus with an allelic and gametophyte-specific expression and its product should be able to interact with S-RNases physically (Thompson and Kirch, 1992; Kao and McCubbin, 1996). Several approaches have been taken to search genes with such characteristics in solanaceous SI species. In *N. alata*, a cDNA fragment (*48A*) obtained by DD (differential display)-PCR showed linkage to the *S* locus and expressed in the pollen (Li *et al.*, 2000). *48A* was less than 0.5 cM from the *S* locus (Li *et al.*, 2000). Given that 1 cM is equivalent to over 1000 kb and recombination at the *S* locus is suppressed, it is hard to determine the physical distance separating *48A* from the S-RNase

gene. Ten pollen cDNA linked to the *S* locus in *Petunia inflata* have also been identified by transcript display in combination with RFLP (restriction fragment length polymorphism) analysis (McCubbin *et al.*, 2000a). Although it is possible that one of them encodes the *Sp* gene, further direct evidence is required. Other approaches including yeast two-hybrid screening with S RNases as baits and protein affinity-based screening did not yield any *Sp* gene candidate (Dowd *et al.*, 2000; Y. Xue, unpublished data).

It was shown that *AhSLF-S₂* is tightly linked to the S-RNase and displays corresponding allelic specificity, with no cross-hybridization and lack of PCR-amplification products when using DNA not containing *S₂* alleles (Figures 1, 4 and 5). Analysis of *AhSLF-S₂*-specific transcripts clearly showed that it is a pollen- and tapetum-specific gene (Figures 6 and 7). *AhSLF-S₂* expression also was detected in the tapetal cells around the tetrad stage and later restricted mainly to the microspores (Figure 7), the same site of expression as that seen for the pollen *S* gene (*SCR/SP11*) and *PCP-A1* in *Brassica* (Doughty *et al.*, 1998; Schopfer *et al.*, 1999; Takayama *et al.*, 2000). An *AhSLF-S₂*-related gene, *AhSLF-S₂L*, has a similar gametophytic expression pattern (Figure 6), but it showed no allelic polymorphism (Figure 4), suggesting that it does not play a direct role in SI. This finding raised a possibility that *AhSLF-S₂* and *AhSLF-S₂L* are allelic forms of the same gene and the latter is linked to none *S₂* alleles. If this is the case, *AhSLF-S₂* cannot be *Sp* in *Antirrhinum*. Further segregation experiments are required to test the linkage of *AhSLF-S₂L* to the *S* locus, for example, by using a population from a cross between *ScSc* and *S₁S₂*. If *AhSLF-S₂L* is indeed allelic to *AhSLF-S₂* both *S₁*- and *S₂*-containing progeny should have *AhSLF-S₂L*. If both genes are located in the *S* locus, it is possible that *AhSLF-S₂* represents a duplicated copy of *AhSLF-S₂L* or *vice versa*. Therefore, further work on *AhSLF-S₂L* is required to shed light on this issue. Most interestingly, *AhSLF-S₂* contains an F-box domain (Bai *et al.*, 1996) in its N-terminal region, suggesting that it likely functions by directing substrates into an ubiquitin-mediated protein degradation pathway (Skowyra *et al.*, 1997; Craig and Tyers, 1999). *AhSLF-S₂* is a member of a gene family, similar to other *S* genes (Anderson *et al.*, 1989; Stein *et al.*, 1991; Schopfer *et al.*, 1999; Takayama *et al.*, 2000). Although a direct demonstration that *AhSLF-S₂* is the pollen determinant of self-incompatibility is lacking, for example, through transformation, based on the available evidence, we believe that *AhSLF-S₂*

is a candidate for the *Sp* component of the *S* locus in *Antirrhinum*.

Possible involvement of the protein degradation pathway in self-incompatible reaction

The possibility that the *Sp* gene encodes an F-box protein has important implications in how self-incompatible reaction is accomplished in species like *Antirrhinum*. F-box proteins are known to function in several developmental controls in animal and act by bring their distinctive substrates into a protein degradation pathway mediated by ubiquitin (Bai *et al.*, 1996; Skowrya *et al.*, 1997; Craig and Tyers, 1999). In plants, F-box-containing genes have been shown to control auxin action (Ruegger *et al.*, 1998; Gray *et al.*, 1999), floral development (Ingram *et al.*, 1997), circadian rhythm (Nelson *et al.*, 2000; Somers *et al.*, 2000) and defense responses and fertility (Xie *et al.*, 1998).

In the inhibitor model it was proposed that the expression of *Sp* leads to production of an S RNase inhibitor which inhibits all other S RNase activity except self-S-RNases during self-incompatible reaction (Thompson and Kirch, 1992; Kao and McCubbin, 1996). It is not clear how the inhibition is completed. If the *S* locus-encoded F-box proteins produced inside the pollen function as *Sp*, it provides a biochemical basis for the inhibition. The F-box protein would target non-self-S RNases for breakdown and leave self-S RNases intact to function during self-incompatible reaction. In general, the regions outside the F-box protein contain domains, such as WD (tryptophan and aspartic acid) repeats or LRR (leucine-rich repeats), involved in the protein-protein interaction, which are responsible for its substrate recruitment (Craig and Tyers, 1999). No similar motifs are found in the SLF proteins apart from several well-conserved regions (Figure 3). Their roles, if any, in the protein-protein interaction between SLF and its substrate remain to be established.

Additional circumstantial lines of evidence support the involvement of an F-box protein-mediated pathway in S-RNase-based self-incompatibility. It is known that the F-box protein action requires that its substrate be phosphorylated and contains a lysine residue for ubiquitin ligation (Craig and Tyers, 1999). A pollen tube-derived Ca²⁺-dependent protein kinase (CDPK) in *N. alata* was shown to be capable of phosphorylating S RNases in a non-allele-specific fashion (Kunz *et al.*, 1996), indicating that phosphorylation of

S-RNases once inside the pollen tube could occur. A conserved lysine residue is present within the C3 domain of the S RNases (Ioerger *et al.*, 1990; Xue *et al.*, 1996). If S RNases were indeed destroyed through an F-box protein-mediated protein degradation pathway, it would provide a possible explanation for the inhibition of non-self S RNase activity in the inhibitor model.

A problem raised by the inhibitor model is how a lack of interaction between the S RNase and its linked inhibitor is maintained. The grafting of styles containing self-incompatible pollen tubes onto self-compatible styles permitted the recovery of the pollen tube growth (Lush and Clarke, 1997), suggesting that the activity of self-S RNases already present within pollen tubes can be inhibited. To explain this, Lush and Clarke suggested that S-RNase in pollen tubes is degraded or inactivated rapidly when pollen tubes cross from incompatible scions into compatible stocks (Lush and Clarke, 1997). If we assume that the destruction of self-S RNase occurs but at a slower rate than that of non-self RNases, self-S RNases would be eventually destroyed on compatible stocks, allowing the recovery of the pollen tube growth. Recently, both self- and non-self-RNase products were detected in the pollen tube during self-incompatible reaction (Luu *et al.*, 2000). This observation appeared in contrast to the final destruction of S RNases which presumably occurred in the grafting experiments. Because the detection of S RNases was done within an early time point after pollination (Luu *et al.*, 2000), the fate of S RNases was unclear during latter stages of pollen tube growth. If the *S* locus-encoded F-box protein like AhSLF-S₂ would play a role in SI, it could act through a high affinity to non-self S RNases and a low affinity to self-S RNase, resulting in a differential breakdown of S RNases allowing non-self pollen tubes to grow faster during pollination. It will be extremely interesting to investigate closely the fate of S RNases during self-incompatible and compatible reactions.

The similarities in the genomic structure of the S loci in flowering plants

Sequence analysis of the *S2BAC* identified that the *S* locus in *Antirrhinum* is located in a region rich in retrotransposons. Although mapping of the two *S2BAC* ends showed that it is part of the *S* locus, its exact physical size is not known. In *Brassica*, recombinational mapping of the *S* locus showed that it ranges from tens to hundreds of kb (Casselmann *et al.*,

2000). It is likely that the physical limits of the *S* locus in *Antirrhinum* are similar. The BAC clones overlapping with *S2BAC* (Zhao Lai *et al.*, in preparation) and a recently constructed BAC contig of over several hundred kilobases containing an *S* RNase gene in *P. inflata* (McCubbin *et al.*, 2000b) will allow similar experiments to determine the physical size of the *S* locus in these two species. Previous studies on the genomic organization of the *S* locus in several solanaceous species revealed that the *S* RNase gene is flanked by highly repetitive sequences (Coleman and Cao, 1992; Matton *et al.*, 1995; Royo *et al.*, 1996). This also seems to be the case in *Antirrhinum*. Four predicted genes homologous to retrotransposons have been found near the *S*₂-RNase gene (Figure 1). Although their copy numbers and activity have not been determined, it is likely that they represent the repetitive sequences at the *S* locus. Retrotransposons also have been identified in the *S* loci of *N. alata* and *Brassica* (Royo *et al.*, 1996; Suzuki *et al.*, 1999; Cui *et al.*, 2000). These findings indicate that the *S* locus is embedded in a genomic region rich in transposable elements. Furthermore, we have found that even inside the *S* locus DNA polymorphisms are unevenly distributed. The diverse transposons and dispersed sequence heterogeneity associated with the *S* locus provide physical bases to maintain its integrity during its evolution. In *Antirrhinum*, two genes within about 10 kb region of the *S* locus have been shown to be expressed (Figure 1), similar to the *S* locus region in *Brassica* (Cui *et al.*, 1999; Schopfer *et al.*, 1999; Takayama *et al.*, 2000). The similarities in genomic structure and gene density of the *S* loci indicate a similar mechanism for their evolution in angiosperms.

Acknowledgments

We thank Enrico S. Coen and Rosemary Carpenter for their generosity and critical comments on the manuscript, Jiayang Li for his help with BAC clone arraying, and Yunyuan Xu and Kang Chong for their help with RNA *in situ* hybridization. The project is supported by grants from the Chinese Academy of Sciences, a Distinguished Young Scholar Award from the National Natural Science Foundation of China (39825103) and the Ministry of Science and Technology of China to Y.X.

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