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Structural and transcriptional analysis of *S*-locus F-box genes in *Antirrhinum*

Received: 5 April 2003 / Accepted: 7 July 2003 / Published online: 9 August 2003
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Abstract A class of ribonucleases termed S-RNases, which control the pistil expression of self-incompatibility, represents the only known functional products encoded by the *S* locus in species from the *Solanaceae*, *Scrophulariaceae* and *Rosaceae*. Previously, we identified a pollen-specific F-box gene, *AhSLF* (*S* locus F-box)-*S*₂, very similar to *S*₂-RNase in *Antirrhinum*, a member of the *Scrophulariaceae*. In addition, *AhSLF*-*S*₂ also detected the presence of its homologous DNA fragments. To identify these fragments, we constructed two genomic DNA libraries from *Antirrhinum* self-incompatible lines carrying alleles *S*₁*S*₅ and *S*₂*S*₄, respectively, using a transformation-competent artificial chromosome (TAC) vector. With *AhSLF*-*S*₂-specific primers, TAC clones containing both *AhSLF*-*S*₂ and its homologs were subsequently identified (*S*₂*TAC*, *S*₅*TACa*, *S*₄*TAC*, and *S*₁*TACa*). DNA blot hybridization, sequencing and segregation analyses revealed that they are organized as single allelic copies (*AhSLF*-*S*₂, -*S*₁, -*S*₄ and -*S*₅) tightly linked to the *S*-RNases. Furthermore, clusters of F-box genes similar to *AhSLF*-*S*₂ were identified. In total, three F-box genes (*AhSLF*-*S*₂, -*S*₂*A* and -*S*₂*C*) in *S*₂*TAC* (51 kb), three

(*AhSLF*-*S*₄, -*S*₄*A* and -*S*₄*D*) in *S*₄*TAC* (75 kb), two (*AhSLF*-*S*₅ and -*S*₅*A*) in *S*₅*TACa* (55 kb), and two (*AhSLF*-*S*₁ and -*S*₁*E*) in *S*₁*TACa* (71 kb), respectively, were identified. Paralogous copies of these genes show 38–54% identity, with allelic copies sharing 90% amino acid identity. Among these genes, three (*AhSLF*-*S*₂*C*, -*S*₄*D* and -*S*₁*E*) were specifically expressed in pollen, similar to *AhSLF*-*S*₂, implying that they likely play important roles in pollen, whereas three *AhSLF*-*SA* alleles showed no detectable expression. In addition, several types of retroelements and transposons were identified in the sequenced regions, revealing some detailed information on the structural diversity of the *S* locus region. Taken together, these results indicate that both single allelic and tandemly duplicated genes are associated with the *S* locus in *Antirrhinum*. The implications of these findings in evolution and possible roles of allelic *AhSLF*-*S* genes in the self-incompatible reaction are discussed in species like *Antirrhinum*.

Keywords Self-incompatibility · Evolution · *S* locus · *Antirrhinum* · F-box

Sequence data from this article have been deposited with the EMBL/GenBank databases under accession numbers AJ300474, AJ515534, AJ515536 and AJ515535

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Introduction

In flowering plants, a widely used mechanism to prevent inbreeding is known as self-incompatibility (SI), which is, in most cases, controlled by a single multi-allelic locus, the *S* locus (de Nettancourt 2001). Recent biochemical and molecular studies have identified genes encoded by the *S* locus in several plant families. In the *Brassicaceae*, two genes known as *SRK* expressed in the stigma and *SCR/SP11* expressed in pollen (Stein et al. 1991; Schopfer et al. 1999; Suzuki et al. 1999) belong to one haplotype and have been shown to control pollen recognition through a ligand-receptor binding mechanism (Kachroo et al. 2001; Takayama et al. 2001). In the *Papaveraceae*, a pistil-specific gene (*S*₁) is encoded by the *S* locus (Foote et al. 1994), and a sophisticated mechanism involving Ca²⁺ signaling is involved in pollen tube growth inhibi-

tion (Wheeler et al. 2001). In self-incompatible species from the *Solanaceae*, *Scrophulariaceae* and *Rosaceae*, a group of ribonucleases termed S-RNases are encoded by their respective *S* loci and control self-pollen recognition and rejection by the pistil (Anderson et al. 1986; McClure et al. 1989; Ai et al. 1990; Broothaerts et al. 1995; Sassa et al. 1996; Xue et al. 1996; McCubbin and Kao 2000).

However, because of the elusive identity of pollen *S* gene (*Sp*), it is not clear how S-RNase functions during the self-incompatible reaction. Nevertheless, several recent studies have indicated that S-RNases likely interact with an inhibitor inside the pollen tube to accomplish pollen recognition and growth inhibition (Golz et al. 1999, 2001; Luu et al. 2001). In *Nicotiana* and *Petunia*, *S* allele duplications have been found to be associated with pollen-part self-compatible mutants (Pandy 1965; Golz et al. 1999). The genetic behavior of these mutants can be explained if *Sp* acts as an inhibitor of all S-RNases except its cognate S-RNase (Golz et al. 2000). This model is further supported by the finding that S-RNase uptake by the pollen tube has no allelic specificity (Luu et al. 2000). Recently, Luu et al. (2001) have found that pollen containing two different *S* alleles is accepted by *S*₁₁/*S*₁₃ style in *Solanum chacoense*, but is rejected by a chimeric *S*_{11/13} RNase, indicating that *Sp* genes must be expressed in diploid heteroallelic pollen, and proposed that *Sp* consists of two components, a general S-RNase inhibitor and an *S*-allele-specific product blocking the inhibitory action on self-RNases. Several groups have attempted to identify the *Sp* genes in *Nicotiana glauca*, *Petunia inflata*, *Antirrhinum* and two *Prunus* species (Li et al. 2000; McCubbin et al. 2000; Lai et al. 2002; Entani et al. 2003; Ushijima et al. 2003). Although none of these candidate *Sp* have been demonstrated to play a role in SI, interestingly, Lai et al. (2002) identified a pollen-specific F-box gene, *AhSLF-S*₂, located 9 kb away from *S*₂-RNase, indicating a possible involvement of a protein degradation pathway in the self-incompatible reaction in species like *Antirrhinum*. In the same study, genomic DNA fragments homologous to *AhSLF-S*₂ were also detected but their relationships were not clear (Lai et al. 2002). Recently, several pollen-expressed F-box genes similar to *AhSLF-S*₂ have also been found in the *S* locus of both almond (*Prunus dulcis*) and Japanese apricot (*Prunus mume*) with a haplotype-specific polymorphism, indicating that they are good *Sp* candidates (Entani et al. 2003; Ushijima et al. 2003).

As shown by their presence in a wide range of flowering plants and phylogenetic analyses of *S-RNases*, the *S* loci of the *Solanaceae*, *Scrophulariaceae* and *Rosaceae* likely share a common ancestor and probably predate eudicot diversification (Igic and Kohn 2001; Steinbachs and Holsinger 2002). This ancient origin may eventually have resulted in a complex genomic structure of the *S* locus. In fact, initial genomic structural analysis of the *S* locus revealed that it is located in a region consisting of repetitive sequences (Coleman and Kao 1992; Royo et al. 1996; Lai et al. 2002; Entani et al. 2003; Ushijima et al. 2003). As yet, limited information is

available on the genomic constituents of the *S* locus in S-RNase-based self-incompatible species.

To study the genomic structure of the *S* locus and the relationship between *AhSLF-S*₂ and its homologues, we have constructed two genomic DNA libraries of *Antirrhinum* with different alleles using a transformation-competent artificial chromosome (TAC) vector. We identified TAC clones containing *AhSLF-S*₂ and its homologues and subsequently demonstrated that they are single allelic genes. DNA sequence analysis revealed the presence of several different types of retroelements and transposons near *S-RNase* genes. Furthermore, clusters of F-box genes homologous to *AhSLF-S*₂ were detected and their expression and genomic organization analyzed. The implications of the similarity between *SLF* genes and some F-box genes in *Arabidopsis* and tomato and the possible functions for *AhSLF-S* gene in the SI reaction in species like *Antirrhinum* are discussed.

Materials and methods

Plant materials

Self-incompatible lines derived from an interspecific cross between *Antirrhinum hispanicum* and *Antirrhinum majus*, as well as their growth conditions were described previously (Xue et al. 1996). Two SI lines with *S*₁*S*₅ and *S*₂*S*₄ alleles were further crossed to generate a progeny population of 100 plants segregating for four *S* alleles.

Construction and screening of TAC library

High-molecular-weight (HMW) DNA of over 2 Mb from two SI lines with *S*₂*S*₄ and *S*₁*S*₅ alleles was prepared from leaf nuclei according to Liu and Whitter (1994). Partial digestion of HMW-DNA with *Hind*III, TAC vector preparation, ligation and transformation of *Escherichia coli* DH10B by electroporation followed the method described by Liu et al. (2000). The *Hind*III-digested TAC plasmid DNA was sized-fractionated with a field inversion agarose gel electrophoresis to check the insert length. A total of ca. 77,925 recombinant clones were selected and stored in 384-well plates.

For each library, the clones of one 384-well plate were imprinted onto a 15 cm plate with a VP384 pin (V&P Scientific, San Diego, Calif.) and inoculated onto LB agar medium containing kanamycin (25 mg/l). After incubation at 37°C overnight, the bacteria on the 15 cm plate were collected for plasmid preparation. Plasmid DNA from ten 384-well plates was mixed as a pool for PCR screening. The TAC library was screened with the primers specific for *S*₂, *S*₄ and *S*₅-RNase (Xue et al. 1996), and *G11E* and *G11D* (Lai et al. 2002). Once a specific PCR product was detected in one or more pools, the ten 384-well plates of the positive pool were individually screened with the primer pair again. A positive 384-well plate was subsequently identified. Finally, the positive clone was identified by PCR screening in a row and column combination. *S*₂- (*G2338* and *G1222*), *S*₄- (*G3169* and *G1224*), and *S*₅-RNase primers (*G2339* and *G1481*) were according to Xue et al. (1996). *AhSLF-S*₂ primers (*G11E*, *G11D*, *G11f* and *G11j*) were described by Lai et al. (2002). The 3' UTR region-specific primers were as follows: *AhSLF-S*₅ (CGGAGTGTCCGGTGCATCATAG), *AhSLF-S*₄ (ACTTAACCAACTCCGATTGAA) and *AhSLF-S*₁ (TCATAATTTAAACCCGCCACC).

Sequencing and assembling of TAC clones

TAC clone sequencing and assembly was carried out as previously described by Lai et al. (2002).

Southern and northern blotting analyses

Genomic DNA isolation was performed as previously described (Xue et al. 1996). DNA (10 μ g) was digested, separated on a 0.8% agarose gel and transferred onto Hybond N⁺ (Amersham, Piscataway, N.J.) membrane. Prehybridization, hybridization and washing of the blot were performed as recommended by the manufacturer. Total RNA was extracted from different tissues using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). RNA samples were separated on 1% agarose/formaldehyde gels and transferred to Hybond N⁺. Prehybridization, hybridization and washing of the blot were performed as recommended by the manufacturer. Probes were labeled with ³²P by random priming using the Prime-a-Gene labeling system (Promega, Madison, Wis.).

Reverse transcription-PCR analysis

Total RNA was prepared as previously described and was digested with DNase I (Takara, Kyoto, Japan). Reverse transcriptase (RT) (Invitrogen, Carlsbad, Calif.) was used to synthesize first strand cDNA. RT-PCR primers were designed from the full-length coding sequences of F-box genes.

Sequence annotation and computational analysis

Genescan and FgeneSH softwares were used for gene prediction (<http://www.ncgr.ac.cn>). BLASTx, BLASTp and BLAST2 (<http://www.ncbi.ac.cn>) and WU-BLAST2 and CLUSTALW (<http://www.ebi.ac.uk>) were used for DNA sequence analysis. A Dotter

program (<http://www.cgr.ki.se>) was used for comparative analysis of TAC insert sequences. The phylogenetic tree was generated with CLUSTALW using a neighbor-joining feature (<http://www.ebi.ac.uk/>).

Results

Isolation of genes homologous to *AhSLF-S₂*

An F-box gene, *AhSLF-S₂*, about 9 kb away from the *S₂-RNase* gene was previously identified and its homologues detected in *Antirrhinum* (Lai et al. 2002). However, it was not clear whether these represent allelic or duplicated copies of the same gene. To resolve these possibilities, we constructed two genomic DNA libraries from self-incompatible lines of *S₂S₄* and *S₁S₅* genotypes using the vector TAC7 (Liu et al. 1999). The two libraries had 39,936 and 38,016 clones, respectively; both had an estimated average insert length of 70 kb (data not shown), and were equivalent to 6 and 5.8 times the haploid genome of *Antirrhinum*. Initially, we suspected that *AhSLF-S₂* homologues should be tightly linked to *S-RNase* genes as had been found for *AhSLF-S₂*. Thus, we isolated TAC clones containing *S-RNases* using a pooled PCR method in which plasmid DNA was individually prepared from each 384-well plate and the plasmid DNA from ten 384-well plates was pooled together for PCR screening with gene-specific primers. In total, we identified one TAC clone containing *S₂-RNase* (*S₂-RNase-TAC*), four containing *S₄-RNase* (*S₄RNaseTACa-d*) and

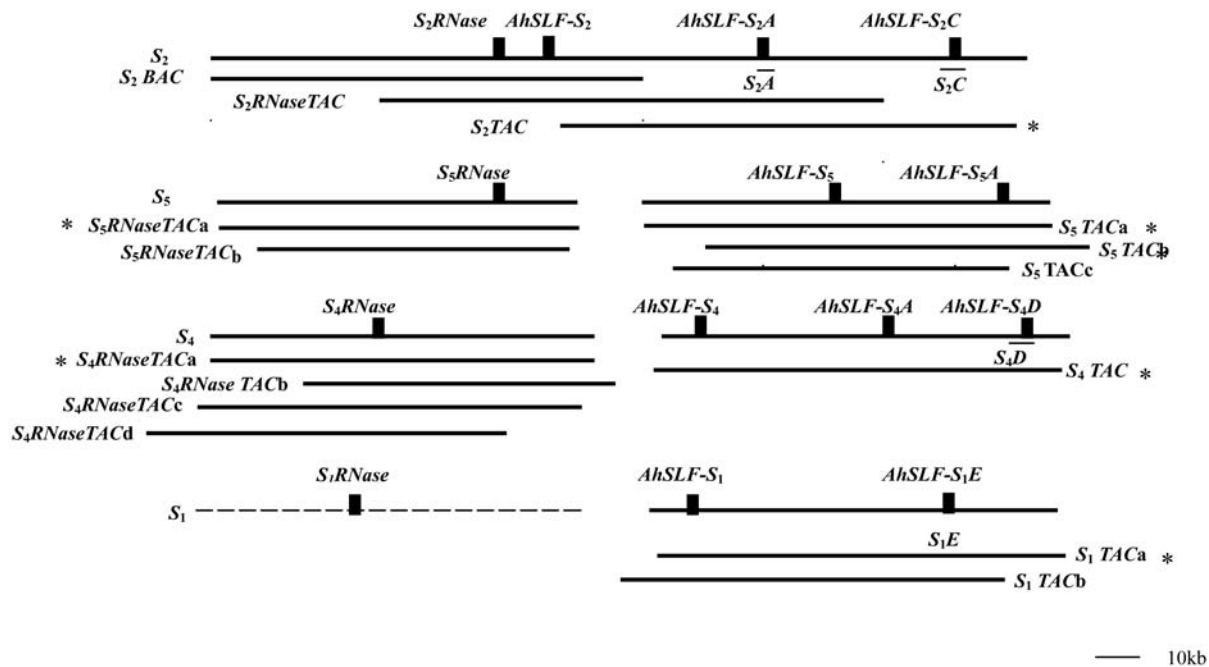


Fig. 1 Transformation-competent artificial chromosome (TAC) physical maps corresponding to four *S* alleles. The sequenced region corresponding to *S₂* allele represents 110 kb in length, and *S₂-RNase* and *AhSLF-S₂* are separated by ca. 9 kb. In the TAC contigs corresponding to the three other alleles, the physical

distance between *S-RNase* and *S* TAC clones is not known. TAC clones corresponding to the *S*-locus F-box (*SLF*) regions are aligned based on homology. Expressed or predicted *AhSLF* genes are indicated as *solid bars*. The probes used in DNA blot analysis are shown as *black bars*. * Completely sequenced TAC clones

Fig. 2A, B Amino acid sequence alignment of predicted SLF polypeptides. **A** Deduced sequences of AhSLF-S alleles were compared. **B** Sequences were derived from *Antirrhinum* (*AhSLF-S₂*, *-S_{2C}*, *-S_{4D}* and *-S_{1E}*), *Prunus dulcis* (*PdSFBa*, *PdSFBb* and *PdSLFc*), *Prunus mume* (*PmSLF-S₁*, *PmSLF-S₇* and *PmSLFL_{1-S₇}*) and *Arabidopsis* (*At4g12560* and *At4g22390*)

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AhSLF-S1 : MMDRRFPRQDVISEILLFSSVKSLLRFRVSKSWCSLIKSNDFIDNHLRRQTNGNVMVVKRYVRTPERDMFSFYNNINSPELDEL : 85
AhSLF-S2 : MMDRRFPRQDVISEILLFSSVKSLLRFRVSKSWCSLIKSNDFIDNHLRRQTNGNVMVVKRYVRTPERDMFSFYNNINSPELDEL : 85
AhSLF-S4 : MMDRRFPRQDVISEILLFSSVKSLLRFRVSKSWCSLIKSNDFIDNHLRRQTNGNVMVVKRYVRTPERDMFSFYNNINSPELDEL : 85
AhSLF-S5 : MMDRRFPRQDVISEILLFSSVKSLLRFRVSKSWCSLIKSNDFIDNHLRRQTNGNVMVVKRYVRTPERDMFSFYNNINSPELDEL : 85

AhSLF-S1 : LPDLNPFYFKNIKFDYDYFYLQQRVNLMGPCNGLICLAYGDCVLLSNPALREIKRLPPTPFANPEGHCTDIIGYGFNTCNDCYR : 170
AhSLF-S2 : LPDLNPFYFKNIKFDYDYFYLQQRVNLMGPCNGLICLAYGDCVLLSNPALREIKRLPPTPFANPEGHCTDIIGYGFNTCNDCYR : 170
AhSLF-S4 : LPDLNPFYFKNIKFDYDYFYLQQRVNLMGPCNGLICLAYGDCVLLSNPALREIKRLPPTPFANPEGHCTDIIGYGFNTCNDCYR : 170
AhSLF-S5 : LPDLNPFYFKNIKFDYDYFYLQQRVNLMGPCNGLICLAYGDCVLLSNPALREIKRLPPTPFANPEGHCTDIIGYGFNTCNDCYR : 170

AhSLF-S1 : VVLTIESVGPEDHHINIYVYSDTNSWKHIEDDSTPIKYKCHFCPCNELFFKGAFHWNANSTDFYADFILTFDIITTEVFKEMAYPH : 255
AhSLF-S2 : VVLTIESVGPEDHHINIYVYSDTNSWKHIEDDSTPIKYKCHFCPCNELFFKGAFHWNANSTDFYADFILTFDIITTEVFKEMAYPH : 255
AhSLF-S4 : VVLTIESVGPEDHHINIYVYSDTNSWKHIEDDSTPIKYKCHFCPCNELFFKGAFHWNANSTDFYADFILTFDIITTEVFKEMAYPH : 255
AhSLF-S5 : VVLTIESVGPEDHHINIYVYSDTNSWKHIEDDSTPIKYKCHFCPCNELFFKGAFHWNANSTDFYADFILTFDIITTEVFKEMAYPH : 255

AhSLF-S1 : CLAQFNSPFLSMLNECLAMVRYKEMMBPELFDIWMNQYGVRESWTQYVIGPQVVCSSHVCWKNDECLIVBDGNGQLVSCA : 340
AhSLF-S2 : CLAQFNSPFLSMLNECLAMVRYKEMMBPELFDIWMNQYGVRESWTQYVIGPQVVCSSHVCWKNDECLIVBDGNGQLVSCA : 340
AhSLF-S4 : CLAQFNSPFLSMLNECLAMVRYKEMMBPELFDIWMNQYGVRESWTQYVIGPQVVCSSHVCWKNDECLIVBDGNGQLVSCA : 340
AhSLF-S5 : CLAQFNSPFLSMLNECLAMVRYKEMMBPELFDIWMNQYGVRESWTQYVIGPQVVCSSHVCWKNDECLIVBDGNGQLVSCA : 340

AhSLF-S1 : FRTNEIKKLPYAVEETLRVLIVDESLSLNRVLE : 376
AhSLF-S2 : FRTNEIKKLPYAVEETLRVLIVDESLSLNRVLE : 376
AhSLF-S4 : FRTNEIKKLPYAVEETLRVLIVDESLSLNRVLE : 376
AhSLF-S5 : FRTNEIKKLPYAVEETLRVLIVDESLSLNRVLE : 376

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A

two containing *S₅-RNase* (*S₅RNaseTACa*, *b*) (Fig. 1). DNA blot hybridization and sequencing analysis of *S₄-RNaseTACa* and *S₅-RNaseTACa* revealed that they did not contain *AhSLF-S₂* homologues, indicating that the latter are not as close to *S-RNases* as is the case in the *S₂* haplotype (J. Zhou, F. Wang and Y. Xue, MS in preparation).

To further identify *AhSLF-S₂* homologues, we postulated that they should be highly similar in sequences, based on the cross DNA hybridization (Lai et al. 2002), therefore two primers (*G11E* and *G11D*) were designed based on the coding region and used to amplify the TAC libraries with the pooled PCR method. In total, five clones were obtained from the *S_{1S₅}* library and two clones from the *S_{2S₄}* library. To classify them, 1 kb PCR products amplified by the primers from each clone were sequenced. The results showed they share over 97% identity and can be organized into four different groups, with two groups from each SI line. In the five clones from the *S_{1S₅}* library, two distinct sequences were found, represented by three (*S₅TACa-c*) and two (*S₁TACa, b*) TAC clones with identical sequence, respectively (see Fig. 1). The two clones from the *S_{2S₄}* library (*S₂TAC* and *S₄TAC*) were different from each other, but the sequence of *S₂TAC* was identical to that of *AhSLF-S₂* (see Fig. 1). These results suggest that *AhSLF-S₂* homologous sequences from the four groups of the TAC clones are alleles representing four *S* haplotypes (also see below).

Gene content and structure of the *SLF* regions

To investigate the structure of the *SLF* regions, four TAC clones (*S₂TAC*, *S₅TACa*, *S₄TAC* and *S₁TACa*) were selected and fully sequenced. Their insert lengths were 51, 55, 75 and 71 kb, respectively (see Fig. 3). The total sequenced region combining *S₂BAC* (Lai et al. 2002) and *S₂TAC* is 110 kb in length. Sequence analysis of *S₂TAC* revealed that it has a copy of *AhSLF-S₂*. Based on linkage analysis, *S₄TAC*, *S₁TACa* and *S₅TACa* represent *S₄*, *S₁* and *S₅* alleles, respectively (see Fig. 4). However, *S₄TAC* and *S₅TACa* did not contain *S₄-* and *S₅-RNase* genes (see Fig. 1), confirming that the distances between *AhSLF-S₂* homologues and *S-RNases* in these two alleles are much larger than those in *S₂*.

To examine gene content in the four TAC sequences, gene prediction analysis revealed that, on average, two-to-three predicted genes were found to be homologous to *AhSLF-S₂* in each sequenced region, in addition to transposable elements (see Fig. 1 and Table 1). In *S₂TAC*, two additional *SLF* genes (*SLF-S_{2A}* and *S_{2C}*) were predicted. Three *SLF* genes were detected in *S₄TAC*, two in *S₅TACa* and two in *S₁TACa*. To reveal the identity among *AhSLF-S* genes, we aligned the predicted polypeptide sequences of the four *AhSLF-S* genes (Fig. 2A). The results showed that *AhSLF-S₂* and its homologues, termed *AhSLF-S₁*, *S₄* and *S₅*, share more than 97% identity at the amino acid level, indicating that they are allelic (Fig. 2A). *AhSLF-S_{2A}* and its homologues share more than 90% identity and appear to be allelic (data not shown), and were named *AhSLF-S_{4A}* and *S_{5A}*. However, the other three predicted genes in this region have only 42–48% identity either between themselves or with

Table 1 Predicted and known genes in the S_1 , S_2 , S_4 and S_5 transformation-competent artificial chromosome (TAC)

Gene	Predicted ORF(aa)/exons	Identity
<i>S₂TAC</i>		
<i>Gene1</i>	376/1	<i>AhSLF-S₂</i>
<i>Gene2</i>	283/1	Retroelement (AAM74265)
<i>Gene3</i>	1,002/5	Retroelement (Q94LN5)
<i>Gene4</i>	554/2	Unknown
<i>Gene5</i>	414/1	<i>AhSLF-S_{2A}</i>
<i>Gene6</i>	384/1	<i>AhSLF-S_{2C}</i>
<i>S₁TAC</i>		
<i>Gene1</i>	789/9	Retroelement (Q9FZN9)
<i>Gene2</i>	400/4	Retroelement (Q9ZUF5)
<i>Gene3</i>	336/2	Unknown
<i>Gene4</i>	384/1	<i>AhSLF-S_{1E}</i>
<i>Gene5</i>	355/3	Retroelement (Q9AU17)
<i>Gene6</i>	133/1	Unknown
<i>Gene7</i>	355/1	<i>AhSLF-S_{1A}</i>
<i>Gene8</i>	141/1	Unknown
<i>Gene9</i>	376/1	<i>AhSLF-S₁</i>
<i>Gene11</i>	194/1	Retroelement (AAM74265)
<i>Gene12</i>	1,155/4	Retroelement (Q94LN5)
<i>S₄TAC</i>		
<i>Gene1</i>	374/1	<i>AhSLF-S_{4D}</i>
<i>Gene2</i>	215/1	Unknown
<i>Gene3</i>	391/1	<i>AhSLF-S_{4A}</i>
<i>Gene4</i>	373/2	Unknown
<i>Gene5</i>	775/2	Retroelement (Q94LN5)
<i>Gene6</i>	427/1	Retroelement (Q94LN5)
<i>Gene7</i>	119/1	Retroelement (AAM74265)
<i>Gene8</i>	376/1	<i>AHSLF-S₄</i>
<i>Gene9</i>	557/2	Transposon (Q95L53)
<i>Gene10</i>	808/1	Transposon (AAM74372)
<i>S₅TAC</i>		
<i>Gene1</i>	404/1	<i>AhSLF-S_{5A}</i>
<i>Gene2</i>	363/1	Unknown
<i>Gene3</i>	1,157/5	Retroelement (Q94LN5)
<i>Gene4</i>	250/1	Retroelement (AAM74265)
<i>Gene5</i>	376/1	<i>AhSLF-S₅</i>
<i>Gene6</i>	245/1	Retroelement (Q9AU17)
<i>Gene7</i>	1,341/1	Retroelement (Q94E95)

AhSLF-S, indicating that they are not allelic copies. These were named *AhSLF-S_{2C}*, *-S_{4D}* and *-S_{1E}* (Fig. 2B, Table 2). In addition, within the same haplotype, predicted SLF proteins have about 38–54% identity, indicating that their duplications were ancient. Together, these results showed

Table 2 The identity of amino acid sequence of predicted *S* locus F-box (SLF) proteins. Sequences were derived from *Antirrhinum* (*AhSLF-S₂*, *-S_{2C}*, *-S_{4D}* and *-S_{1E}*), *Prunus dulcis* (*PdSFBa*, *PdSFBb*

AhSLF-S ₂	AhSLF-S _{2C}	AhSLF-S _{4D}	AhSLF-S _{1E}	PdSFBa	PdSFBb	PdSLFc	PmSLF-S ₁	PmSLF-S ₇	PmSLFL _{1-S7}	
–	42.0	37.7	44.1	15.2	16.2	21.5	18.1	18.6	20.7	AhSLF-S ₂
	–	41.7	47.1	17.6	17.8	22.7	19.9	17.2	22.1	AhSLF-S _{2C}
		–	44.9	16.3	15.0	25.1	17.6	19.3	23.5	AhSLF-S _{4D}
			–	18.7	15.9	22.6	21.0	20.7	22.1	AhSLF-S _{1E}
				–	67.4	20.0	69.1	69.2	19.7	PdSFBa
					–	17.8	79.8	78.2	17.8	PdSFBb
						–	19.1	19.9	92.2	PdSLFc
							–	81.1	18.1	PmSLF-S ₁
								–	19.6	PmSLF-S ₇
									–	PmSLFL _{1-S7}

that paralogous *SLF* genes in each allele are organized as clusters, and that *AhSLF-S₂* and *-S_{2A}* and their homologues appear to be allelic.

To examine the relationship of the known *SLF* genes in the *S* locus, polypeptides from *Antirrhinum* and two roaceous species were aligned. The similarity between *SLF* genes in *Antirrhinum* and *Rosaceae* is very low, ranging from 15 to 25%, indicating they have been separated for an extremely long time (Fig. 2B, Table 2). Nevertheless, the conserved amino acids in their F-box domains suggest that they all belong to the same F-box family. In addition, all of them have some conserved regions besides the F-box domain that are also evident in similar predicted polypeptides from *Arabidopsis* (Fig. 2B).

Many retroelements or transposons were identified in the sequenced genomic regions covered by these TAC clones (Table 1). In total, 5, 2, 5 and 4 predicted genes of *S₁-*, *S₂-*, *S₄-* and *S₅-TAC*, respectively, represented retroelements or transposons. The remainder of the predicted genes in these sequenced regions have no identity to any known genes in the EMBL database.

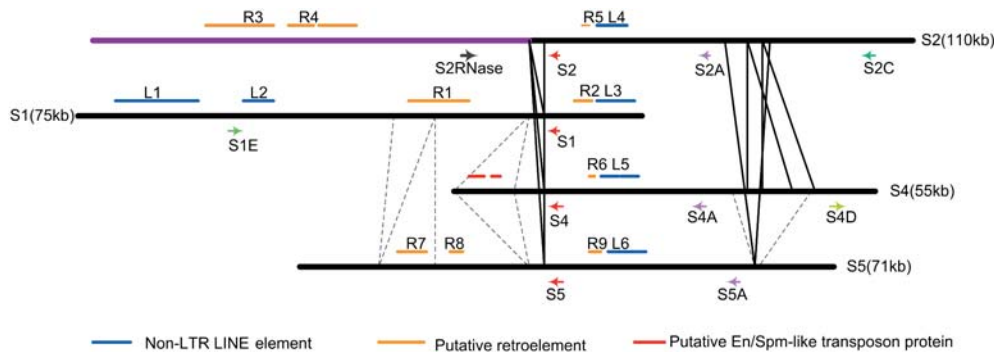
To classify the predicted retroelements and transposons, BLASTx was used to compare these sequences in detail. In total, six non-LTR LINE-like elements were identified, named *L₁–L₆* (Table 3, Fig. 3). These elements are characterized by the presence of a polyprotein, a 3' poly (A) signal, and a 5 bp target site insertion signature (Table 3). Additionally, nine predicted retroelements (*R₁–R₉*) showed no typical LTRs or non-LTR features (Fig. 3), and likely represent aberrant or scrambled retrotransposons. Finally, two putative transposon proteins were predicted in *S₄TAC*, with 57% amino acid identity to *TNP2*-like (Nacken et al. 1991), and 56% identity to *En/Spm*-like transposon (Frey et al. 1989), respectively (Table 1). These results showed that the *SLF* regions are enriched with both non-LTR retrotransposons, consistent with its location near the centromere (Ma et al. 2002) and a feature shared with other RNase-based self-incompatible species (Ushijima et al. 1998; Entani et al. 1999).

To identify the structural relationship between the *SLF* regions, we used a Dotter program to conduct a detailed pairwise comparative analysis. Despite the overall nucle-

and *PdSLFc*) and *Prunus mume* (*PmSLF-S₁*, *PmSLF-S₇* and *PmSLFL_{1-S7}*)

Table 3 Predicted non-LTR LINE-like elements in the four TAC clones

Gene	Predicted ORF(aa)	Length of poly(A)	Insertion signature (direct repeat)
<i>S</i> ₁ TAC			
<i>Gene1</i> (L1)	789	20	gatag
<i>Gene2</i> (L2)	400	18	tgacc
<i>Gene12</i> (L3)	1,255	24	ggaag
<i>S</i> ₂ TAC			
<i>Gene3</i> (L4)	1,002	16	agtga
<i>S</i> ₄ TAC			
<i>Gene5</i> (L5)	1,202	26	cttcc
<i>S</i> ₅ TAC			
<i>Gene3</i> (L6)	1,157	24	cttcc

**Fig. 3** Genomic structural comparisons of the *SLF* regions in *Antirrhinum*. The *S*₂ allele region was derived by combining both *S*₂BAC (Lai et al. 2002) and *S*₂TAC sequences. Non-LTR LINE elements, putative retroelements and En/Spm-like transposon protein are indicated with lines of different colors. The genes are

shown with red, purple or green lines; arrows transcriptional direction. Regions of duplication/deletions occurring between the *S*₂ allele and *S*₁, *S*₄ or *S*₅ alleles are indicated by solid lines, and those between *S*₁, *S*₄ and *S*₅ alleles are indicated by dashed lines

otide similarity over the most regions, several major duplications and insertion/deletions (indels) were identified (Fig. 3). Comparing *S*₂TAC with *S*₁TACa, *S*₄TAC or *S*₅TACa, a notable difference among the four regions sequenced was related to a 2 kb sequence near the left end of *S*₂TAC, which was deleted in all the other three TAC clones, suggesting that insertions have occurred between *S*-RNase and *AhSLF-S* genes in these three regions. Another variable region was a 5 kb sequence of *S*₂TAC that was deleted in *S*₅TACa, and also a 2 kb sequence of *S*₂TAC (29–31 kb) was repeated in *S*₄TAC. Several differences were also identified when comparing *S*₁TACa, *S*₄TAC and *S*₅TACa. Reciprocal deletions of 5 kb (42–47 kb) and 8 kb sequence (52–60 kb) of *S*₅TAC were detected between *S*₁TACa and *S*₅TACa (Fig. 3). As a result, retroelements *R*₁ and *R*₈ were predicted in *S*₁TACa and *S*₅TACa, respectively, but *R*₈ of *S*₅TACa was 6 kb smaller than *R*₁ of *S*₁TACa. Also, an extra retroelement (*R*₇) was detected in *S*₅TACa but not in *S*₁TACa due to deletion of a 12 kb sequence in *S*₁TACa. A second variable region corresponded to an 8 kb sequence of *S*₄TAC, which was deleted in *S*₁TACa and *S*₅TACa or vice versa. Subsequently, the two putative transposons were predicted only in *S*₄TAC. A third apparent difference was detected between *S*₄TAC and *S*₅TACa. A 10 kb region of *S*₄TAC was deleted in *S*₅TACa. Two non-LTR LINE-like

elements (*L*₁ and *L*₂) were predicted in *S*₁TACa only, and in this region *S*₁TACa has no overlap with other TAC clones, indicating a breakdown of synteny. Interestingly, this region is located to the right of the non-allelic *AhSLF* genes, implying that the latter likely resulted from unequal crossovers. Taken together, in addition to the occurrence of retrotransposition events, indels and duplications are also associated with the *S* locus region, indicating that these events have played important roles in its evolution.

The close linkage of *SLF* genes to the *S* locus

Although the genomic structures of the *SLF* regions showed some variations among *S* haplotypes, the linkage of *AhSLF-S*₂ to *S*₂-RNase indicated that the other *AhSLF-S* alleles are also probably linked to the *S* locus. To determine their relationship with the *S* locus, the sequences of the four *AhSLF-S* genes were aligned (data not shown). The coding regions were highly homologous, but differences were observed in the 3'-UTR regions. Thus, we designed a common upstream primer (*G11j*) from the conserved coding region and individual downstream primers generated from the 3'-UTR regions of each *AhSLF-S* gene. Subsequently, the four pairs of gene-

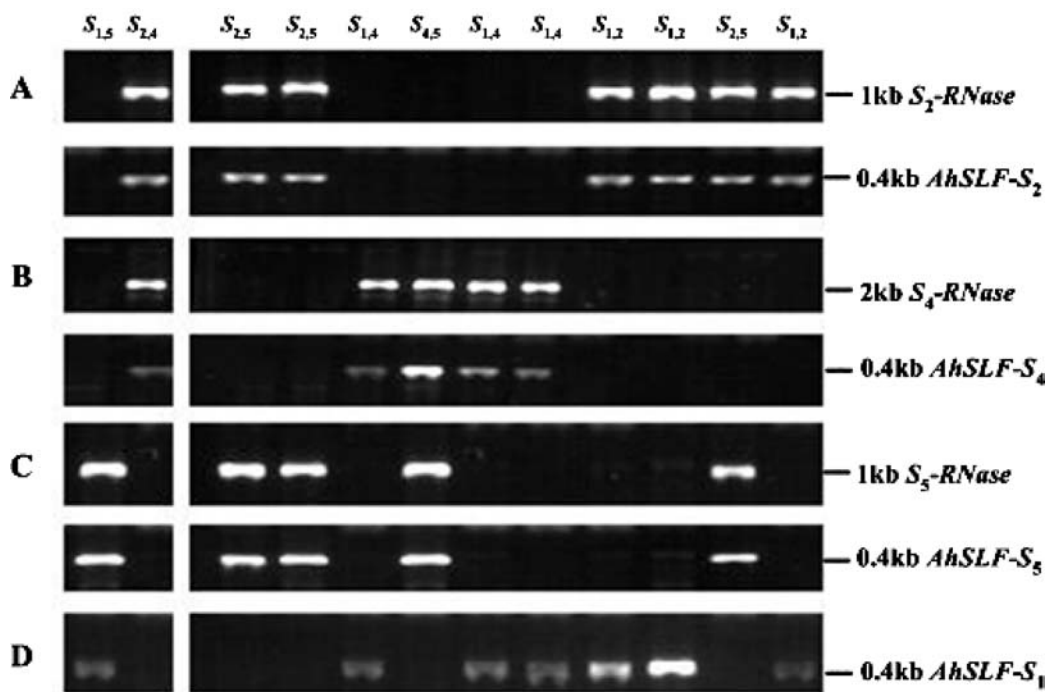


Fig. 4A–D *AhSLF-S* genes are tightly linked to their respective *S-RNase* genes. PCR was performed on genomic DNA from an *S* allele segregating population with gene-specific primers. The sizes of amplified products are indicated on the right. Two lanes on the left represent the parental plants. The ten lanes on the right

represent progeny plants. The *S* genotypes are indicated on the top of the panel. **A–D** PCR amplification with specific primers for *S*₂-*RNase* and *AhSLF-S*₂, *S*₄-*RNase* and *AhSLF-S*₄, *S*₅-*RNase* and *AhSLF-S*₅ and *AhSLF-S*₁, respectively

specific primers were used to analyze the linkage between *S-RNase* and *AhSLF-S* genes.

We generated a population of over 100 progeny segregating for four *S* alleles by crossing two self-incompatible *S*₁*S*₅ and *S*₂*S*₄ lines. Genomic DNA of 100 progeny and parental plants was used for PCR analysis with specific primers for *S*₂-, *S*₄- and *S*₅-*RNase* and the four pairs of primers for *AhSLF-S* genes. Representative PCR results are shown in Fig. 4. *S*₂, *S*₄ and *S*₅ genotypes were determined using *S-RNase*-specific primers and *S*₁ was inferred by absence of the PCR products of the other three *S-RNases* (Fig. 4). The PCR products representing *S*₅-, *S*₄- and *S*₂-*RNases* were about 1 kb, 2 kb and 1 kb, respectively (Fig. 4). By using *AhSLF-S* gene-specific primers, we detected an absolute correlation between *AhSLF-S* alleles and their respective *S-RNases*, showing that they are tightly linked to each other. The results of χ^2 test ($n=3, \chi^2=1.55, P<0.05$) showed that the four genes were segregated as 1:1:1:1 and perfectly correlated to *S-RNase* gene segregation, implying that they transmit as a single locus. In all the plants inferred to have *S*₁ genotype, a specific product of 0.4 kb was detected using *AhSLF-S*₁ primers. These results clearly show that *AhSLF-S* genes are tightly linked to the *S* locus, and possibly inherited as a haplotype together with *S-RNase* genes. Therefore, we performed a phylogenetic analysis based on the deduced amino acid sequences of the *AhSLF-S* genes. The topology of the phylogeny of *AhSLF-S* agreed with that of three *Antirrhinum S-RNases* (data not shown), sug-

gesting that these two genes are under similar selection pressure.

SLF genes are specifically expressed in pollen

Because the newly identified *SLF* genes were predicted, it was not clear whether they are expressed. To investigate their expression, primers derived from *AhSLF-S* alleles *AhSLF-S*_{2A}, -*S*_{2C}, -*S*_{4D} and -*S*_{1E} were used for RACE analysis. The results showed that these *AhSLF-S* alleles are specifically expressed in pollen containing either *S*₁*S*₅ or *S*₂*S*₄ alleles (data not shown), similar to *AhSLF-S*₂ (Lai et al. 2002). RACE products matching the genomic sequences of *AhSLF-S*_{2C}, -*S*_{4D} and -*S*_{1E} were obtained and sequenced, and the predicted proteins have 384, 374 and 384 amino acids, respectively (see Fig. 2B). However, no RACE products were detected for *AhSLF-S*_{2A} in any tissue (data not shown), indicating either that it is expressed to levels below the current detection limit or that it is not expressed under the conditions tested.

To further confirm the expression of these genes, RT-PCR analysis was conducted using primers derived from their full-length coding regions. The templates consisted of genomic DNA, or cDNA synthesized with or without RT (reverse transcriptase) from RNA extracted from leaf, stigma, petal and pollen (with *S*₁*S*₅ or *S*₂*S*₄ alleles). As shown in Fig. 5, PCR products of ca. 1.1 kb were exclusively detected in pollen derived from both *S*₁*S*₅ and

Fig. 5 Expression of *AhSLF-S₂C*, *-S₄D* and *-S₁E*. cDNA were synthesized with (+) or without (-) reverse transcriptase (RT) from total RNA from leaf, stigma, petal, and pollen (*S₁S₅* and *S₂S₄* alleles). As a control, PCR was also performed on genomic DNA (*gDNA*). The full-length coding regions of about 1.1 kb were detected with the specific primers for each of the three genes. *Tubulin* cDNA was amplified as a control

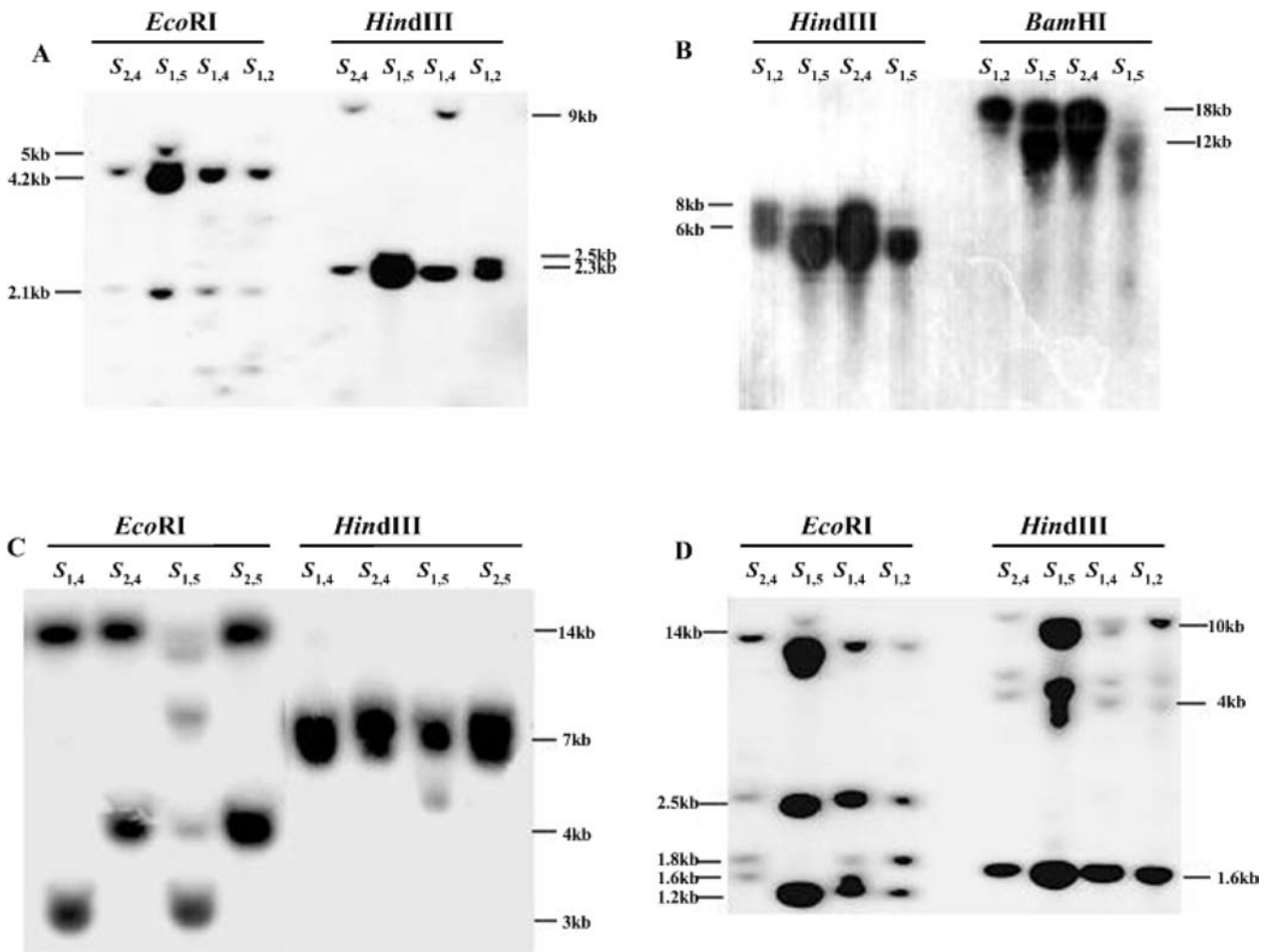
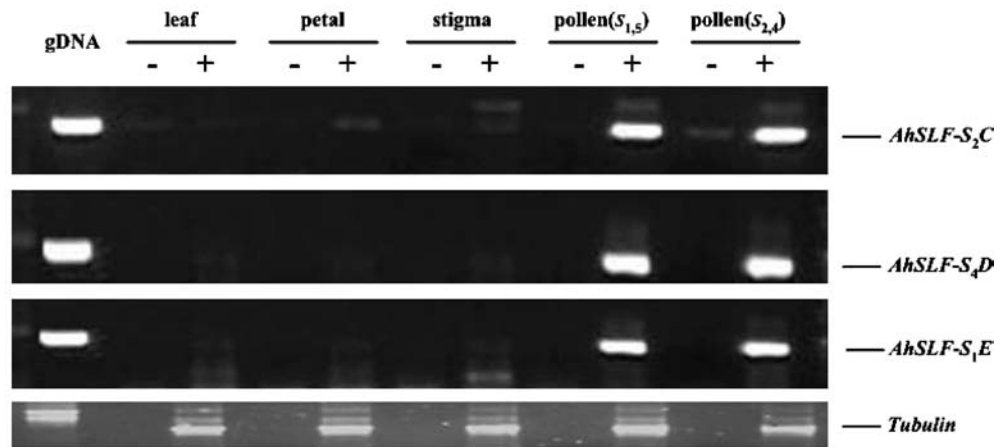


Fig. 6A–D Genomic organization of *AhSLF* genes. Genomic DNA from various *S* alleles containing lines was digested with *Hind*III, *Eco*RI or *Bam*HI and separated by agarose gel electrophoresis. The genotypes of the plants are indicated on the top. The numbers

indicate the sizes of hybridizing fragments in kb. Probes used in **A–D** were the predicted coding sequences derived *AhSLF-S₂A*, *-S₂C*, *-S₁E* and *-S₄D*, respectively

S_2S_4 alleles, consistent with the presence of similar allelic transcripts. DNA sequencing of the 1.1 kb PCR products further confirmed that they were derived from *AhSLF-S_2C*, S_4D and S_1E or their alleles (data not shown, but see Fig. 6). Similarly sized fragments were detected in genomic DNA, showing that these genes are intronless, consistent with the predicted gene models. These results indicate that *Antirrhinum* pollen expresses several *SLF* genes with a pattern similar to that of *AhSLF-S_2* (Lai et al. 2002). In addition, the absence of *AhSLF-S_2A* expression in pollen rules out the possibility that it encodes Sp.

Genomic organization of the *SLF* genes

To reveal the genomic organization of the *SLF* genes, we performed genomic DNA blot analysis using the predicted coding region of *AhSLF-S_2A*, S_2C , S_4D and S_1E as probes (Fig. 6). Hybridization to *AhSLF-S_2A* detected a 2.5 kb *Hind*III fragment in S_2 -containing lines, as predicted from the S_2TAC sequence. In addition, *Hind*III fragments of 9 kb and 2.3 kb were detected in S_4 -containing, and in S_5 - and S_1 -containing lines, respectively; also consistent with the predicted *Hind*III fragments from their respective TAC sequences (Fig. 6A). However, *Eco*RI digestion revealed no RFLP except in S_5 -containing lines. These results demonstrated that *AhSLF-S_2A* alleles are present in all the *S*-haplotypes as single copy genes.

AhSLF-S_2C hybridized to an 8 kb *Hind*III fragment in S_2 -containing lines, representing the S_2 allele (Fig. 6B). A specific 18 kb *Bam*HI fragment was detected in S_2 - and S_1 -containing lines. A 12 kb *Bam*HI fragment was detected in both S_1S_5 and S_2S_4 lines, likely representing both the S_5 and S_4 alleles. Based on the fact that there is neither a *Hind*III nor a *Bam*HI recognition site in the coding region of *AhSLF-S_2C*, the results indicate that *AhSLF-S_2C* is a single copy gene that also has allelic copies.

AhSLF-S_1E displayed no polymorphism in *Hind*III-digested DNA (Fig. 6C). However, a 3 kb *Eco*RI fragment was detected in S_1 -containing lines as predicted from S_1TACa sequence. In addition, another 4 kb *Eco*RI fragment was detected in all the S_2 -containing lines. Therefore, these two fragments likely represent S_1 and S_2 alleles, respectively. Other *Eco*RI or *Hind*III fragments showed no clear polymorphism between lines but are likely derived from S_4 and S_5 alleles. It appeared that *AhSLF-S_1E* is also a single copy gene.

Hybridization of *Hind*III-digested genomic DNA to *AhSLF-S_4D* detected no allele-specific fragments except in S_5 -containing lines (Fig. 6D). In *Eco*RI-digested DNA, a fragment of 14 kb was detected in lines containing S_4 , as predicted from the S_4TAC sequence. Another 10 kb *Eco*RI fragment was detected in the S_5 -containing lines and a 1.2 kb fragment in S_1 -containing lines, likely representing S_5 and S_1 alleles, respectively. The 2.5 kb fragments showed no polymorphism in these lines. In addition, the 1.8 and 1.6 kb *Eco*RI fragments showed no linkage to any

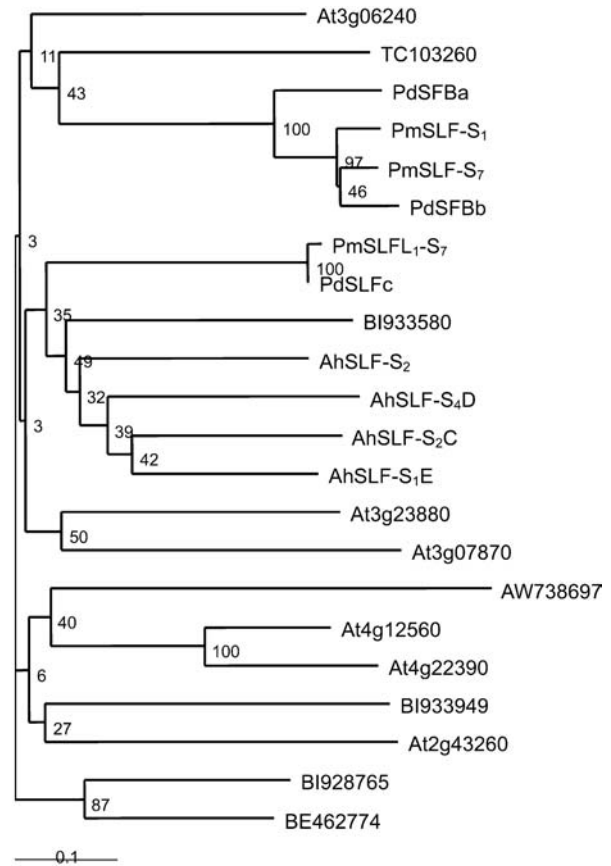


Fig. 7 A phylogenetic tree of predicted *SLF* polypeptides. The sequences were from *Antirrhinum* (*AhSLF-S_2*, *AhSLF-S_2C*, *AhSLF-S_4D* and *AhSLF-S_1E*), *Prunus dulcis* (*PdSFBa*, *PdSFBb* and *PdSLFc*), *Prunus mume* (*PmSLF-S_1*, *PmSLF-S_7* and *PmSLFL_1-S_7*), tomato (*AW738697*, *BE462774*, *BI928765*, *BI933580*, *BI933949* and *TC103260*) and *Arabidopsis* (*At2g43260*, *At3g06240*, *At3g07870*, *At3g23880*, *At4g12560* and *At4g22390*)

haplotype. This result suggested that *AhSLF-S_4D* is present in more than one copy, suggesting that it has been duplicated in the genome.

Taken together, the genomic organization of *AhSLF-S_2A*, $-S_2C$ and $-S_1E$ suggested that they are all single copy genes. In contrast, *AhSLF-S_4D* is divergent and there is more than one copy in the genome.

F-box genes similar to *SLF* are found in *Arabidopsis* and tomato

To examine the relationships of *SLF* genes, a phylogenetic analysis was performed based on deduced amino acid sequences from several available F-box genes or ESTs from *Arabidopsis* and tomato together with SLFs from *Antirrhinum* and *Rosaceae* (Fig. 7). The result showed that *SLF* genes are more closely related to each other, including an EST sequence from tomato (*BI933580*) expressed specifically in flowers. Although the functions of all of these F-box genes are still

unknown, the results indicated that *SLF* genes belong to a very large F-box gene family with a wide distribution.

Discussion

By detailed molecular studies, we have identified four clusters of F-box genes tightly linked to the *S* locus in *Antirrhinum*. Importantly, *AhSLF-S₂* and its homologs are present as single allelic genes with pollen-specific expression, supporting the possibility that they play a role in SI. In addition, gene duplications and the associations of non-LTR LINE-like elements and indels with the *S* locus region have provided some insights into its evolution.

Both allelic and tandemly repeated genes are associated with the *S* locus

It is well known that the *S* locus possesses a large number of alleles (de Nettancourt 2001). For example, over 37 alleles have been genetically identified in *Oenothera organensis* (Emerson 1939), 32 alleles in *Papaver rhoeas* (Lawrence et al. 1993), and over 40 alleles in *Physalis crassifolia* of the *Solanaceae* (Richman et al. 1996). Nevertheless, relatively little is known about how they were actually generated over time. The disease resistance *R* locus is another well-studied recognition locus in plants (Martin et al. 1993; Hammond-Kosack et al. 1998; Ellis et al. 2000a). *R*-locus-encoded genes have been found to occur either as simple (single allelic series) or, more often, complex loci consisting of duplicated genes (Ellis et al. 2000b). In both cases, similar evolutionary processes, including unequal crossing-over, gene conversion and diversifying selection, appear to contribute to overcoming rapid pathogen variations (Michelmore and Meyers 1998; Ellis et al. 2000a). The *Antirrhinum S* locus region appears to contain both types of gene organization, indicating that the evolutionary forces displayed by the *R* locus also are operating. So far, over 200 S-RNases, organized as allelic genes, have been identified in a range of different species (de Nettancourt 2001). In addition, our results have shown that clusters of paralogous *AhSLF* genes are closely associated with the *S-RNases*, a feature also shared by the rosaceous SLF genes (Entani et al. 2003; Ushijima et al. 2003). However, it is not clear whether these similarities between the *S* and *R* loci are intrinsic to their specific roles in terms of recognition. The mate recognition locus in yeast and the major histocompatibility complex (MHC) locus in animals also have similar features (May and Matzke 1995; O'hUigin 1995), supporting the view that they are intrinsic properties of recognition loci.

Structural diversity of the *S* locus region

The genomic region containing the *S* locus appears to be extremely diverse. Previous studies in several RNase-based self-incompatible species have shown that their *S* loci contain repetitive sequences (Coleman and Kao 1992; Royo et al. 1996; Lai et al. 2002; Entani et al. 2003; Ushijima et al. 2003), providing a structural basis for recombination suppression. Recently, Ma et al (2002) also found that the *S* locus of *Antirrhinum* is located in the pericentromeric region. Molecular studies have revealed that this region consists of abundant retroelements in several plant species, including *Arabidopsis* and rice (Copenhaver et al. 1999; Cheng et al. 2002). Consistent with this, we have found several types of retroelements, such as LINE-like protein elements and putative retrotransposons, as well as putative transposons associated with *S-RNases* in the *S* locus region in *Antirrhinum* (Fig. 3). In fact, these features have also been found to be associated with the *R* locus (Wei et al. 2002). For example, two nested complexes of transposable elements and a 45 kb tandem repeat region have been described in the *Mla* resistance locus in barley. The retroelements in this region belong to LTR retrotransposons and non-LTR LINE-like elements, which are thought to have played a role in recombination suppression over time (Duret et al. 2000; Fu et al. 2002; Rizzon et al. 2002).

Although it is difficult to estimate the actual time at which transposition events occurred in the *S* locus region due to the limited information available, what appears to be certain is that transposable elements and indels have played important roles in generating the structural diversity of the *S* locus region. Our data revealed that both the duplicated gene organization and dynamic genomic structures have contributed to the birth and persistence of allelic diversity of the *S* locus.

Are *AhSLF* genes capable of encoding Sp products?

An important unresolved issue in S-RNase-based SI is how S-RNases function inside the pollen tube. Two models, the gatekeeper model and the inhibitor model, have been proposed to explain this (Wheeler et al. 2001). Based on the evidence currently available, the inhibitor model is more favored. Although several *Sp* candidates have been isolated (Li et al. 2000; McCubbin et al. 2000; Lai et al. 2002; Entani et al. 2003; Ushijima et al. 2003), none have yet been assigned a role, if any, in SI. Nevertheless, the pollen-expressed *SLF* genes with haplotype-specific polymorphisms in *P. dulcis* and *P. mume* represent good candidates for *Sp* (Entani et al. 2003; Ushijima et al. 2003).

Interestingly, we have identified a cluster of three F-box genes near the *S₂-RNase* gene within a 70 kb region in *Antirrhinum*; two of these genes appeared to be expressed specifically in pollen, similar to the situation in the *S* locus in *P. dulcis* and *P. mume* (Entani et al. 2003; Ushijima et al. 2003). This may simply be fortuitous for

the S_2 allele because much larger physical separations occur between *S-RNase* and *AhSLF* gene clusters in the other three alleles (Figs. 1, 3). *AhSLF-S₂* represents the closest gene to an *S-RNase* identified so far in *Antirrhinum*. We also compared the putative amino acid sequences of *AhSLF-S* and *SLF* genes from *P. dulcis* and *P. mume* (see Table 2), but the similarity between them was very low (15–25%). Thus, it is difficult to conclude which kind of F-box gene in almond is more similar to *AhSLF-S*. So far, we have not found *SLF* genes with high polymorphism in *Antirrhinum*. There are two possibilities: either there are no high polymorphic *SLF* genes in *Antirrhinum* or, different from the two rosaceous species, such polymorphic *SLF* are located further from the *S-RNases*. Further work to close the gaps between *S-RNase*-containing TAC clones and *AhSLF* regions in other three *S* haplotypes may help resolve this point in *Antirrhinum*.

Intriguingly, it is not clear whether *AhSLF-S* genes with such high identity to each other are capable of encoding Sp. Recently, Luu et al. (2001) have proposed a model for Sp consisting of two components, a general *S-RNase* inhibitor (RI) capable of inhibiting any *S-RNase*, and an *S* allele-specific product that maintains the activity of a specific *S-RNase* inside the pollen tube by blocking RI binding. Whether *AhSLF-S* genes could encode such a general *S-RNase* inhibitor awaits further investigation. However, *AhSLF-S* is different from a recently described petunia protein (PhSBP₁) that interacts with *S-RNase* (Sims and Ordanic 2001) because the latter appears to be expressed ubiquitously.

In addition, several *SLF* genes are also detected near the *S₂-RNase* gene. The absence of expression of *AhSLF-SA* alleles in pollen ruled out their role in the self-incompatible reaction. However, it is unclear what roles other pollen-specific *SLF* genes could play. It is possible that the paralogous *AhSLF* genes represent ancient duplications of the *AhSLF-S* gene that occurred during early angiosperm diversification, leaving other *AhSLF* genes near the border of the *S* locus but playing no role in SI response. However, it is not clear why the linkage to the *S* locus is needed if *AhSLF-S* products indeed act as general RNase inhibitors. It would make sense if they simply played a general role in other unknown aspects of pollen growth, their location near the *S-RNase* genes being simply fortuitous. As a result, the observed diversity of *AhSLF* genes would have occurred because of a “hitchhiking” effect due to suppressed recombination in the *S* locus region. Another possibility is that the association of *S-RNase* and *AhSLF-S* derives from a pre-existing linkage of ancestral *S*-like RNase and *SLF* genes during earlier angiosperm formation. As discussed by Luu et al. (2000), RNase-based SI could be derived through a step-wise process. In this scenario, a pre-existing ancestral linkage between an inhibitor and an *S*-like RNase was maintained to allow inhibition—and thus pollen survival—because of the free-entry of *S* RNases into the pollen tube. Eventually, a factor (presumably Sp) attenuating or abolishing this inhibition would have been selected for due to the advantage of self-pollen rejection.

Transgenic experiments to test the function of the *AhSLF-S₂* gene in *Antirrhinum* together with detailed yeast two-hybrid screening and biochemical studies on *AhSLF-S* are clearly required to address these issues.

Acknowledgements We are grateful to E.S. Coen and R. Carpenter for providing *Antirrhinum* plants and constant support and Y. Liu for help in constructing TAC library. We also thank J. Huang, H. Qiao and H. Wang and, in particular, an anonymous reviewer for their helpful comments on the manuscript. The work was supported by the Chinese Academy of Sciences and the National Natural Science Foundation of China (39825103).

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