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Genetic features of a pollen-part mutation suggest an inhibitory role for the *Antirrhinum* pollen self-incompatibility determinant

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Abstract Self-incompatibility (SI), an important barrier to inbreeding in flowering plants, is controlled in many species by a single polymorphic *S*-locus. In the Solanaceae, two tightly linked *S*-locus genes, *S-RNase* and *SLF* (*S-locus F-box*)/*SFB* (*S-haplotype-specific F-box*), control SI expression in pistil and pollen, respectively. The pollen *S*-determinant appears to function to inhibit all but self S-RNase in the Solanaceae, but its genetic function in the closely-related Plantaginaceae remains equivocal. We have employed transposon mutagenesis in a member of the Plantaginaceae (*Antirrhinum*) to generate a pollen-part SI-breakdown mutant *Pma*1 (Pollen-part mutation in *Antirrhinum*1). Molecular genetic analyses showed that an extra telocentric chromosome containing *AhSLF-S*$_1$ is present in its self-compatible but not in its SI progeny. Furthermore, analysis of the effects of selection revealed positive selection acting on both SLFs and SFBs, but with a stronger purifying selection on *SLF*s. Taken together, our results suggest an inhibitor role of the pollen *S* in the Plantaginaceae (as represented by *Antirrhinum*), similar to that found in the Solanaceae. The implication of these findings is discussed in the context of *S*-locus evolution in flowering plants.

Keywords Self-incompatibility · *Antirrhinum* · *SLF* · Pollen-part mutation · S-RNase

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Introduction

Self-fertilization in numerous species of angiosperms is prevented by a signaling and response mechanism termed self-incompatibility (SI; de Nettancourt 2001). In many cases, SI is genetically controlled by a single polymorphic *S*-locus. Based on genetic and molecular analyses, SI systems have been classified into several types (Takayama and Isogai 2005). In the Solanaceae, Plantaginaceae, and Rosaceae, self-pollen tube growth is arrested within the style when the *S*-haplotype carried by pollen matches either of the two *S*-haplotypes present in pistil (so-called gametophytic SI; Franklin-Tong and Franklin 2003; McClure and Franklin-Tong 2006).

Significant progress has been made over the past two decades in identifying the pollen- and stigma-specific factors involved in this type of SI response. Two tightly linked *S*-locus genes, *S-RNases* (Anderson et al. 1986; Lee et al. 1994; Murfett et al. 1994) and *SLFs/SFBs* (*SLF* in the Solanaceae and Plantaginaceae or *SFB* in the Rosaceae; Lai

et al. 2002; Entani et al. 2003; Ushijima et al. 2003; Qiao et al. 2004a; Sijacic et al. 2004; Wang et al. 2004), control the expression of SI in style and pollen, respectively. However, how their products recognize each other and trigger the arrest of self-incompatible pollen tubes is not yet fully understood. While the function of the stylar S-RNase as a cytotoxin to selectively inhibit self pollen has been established (McClure et al. 1990), both genetic and molecular evidence suggest that the role of the pollen *S* determinant differs among the Solanaceae, Plantaginaceae and Rosaceae (Newbigin et al. 2008).

A number of self compatible (SC) pollen part mutants (PPMs) have been identified in the Solanaceaea, generally derived from the duplication of a small ''centric'' chromosomal fragment carrying the *S*-haplotype (Livermore and Johnstone 1940; Brewbaker and Natarajan 1960; Pandy 1965; Golz et al. 1999, 2001). Likewise, tetraploids derived from SI diploids are often self-fertile in the Solanaceae (Stout and Chandler 1941, 1942; Entani et al. 1999; Mable 2004). An inhibitor model has been proposed in which the pollen *S* determinant is assumed to be an S-RNase inhibitor which is capable of inhibiting all S-RNases as they enter the tube—except that of the corresponding *S*-haplotype (Dodds et al. 1996; Kao and McCubbin 1996; Luu et al. 2000). In this model, the pollen *S* protein with two functions, one of conferring S-specificity and the other of inhibiting non-self S-RNases (Kao and Tsukamoto 2004). While the model predicts the deletion of the pollen *S*-locus would be lethal, self compatibility following the formation of PPMs is explained by the expression of two different pollen *S*-haplotypes in the same pollen grain leading to the inhibition of multiple S-RNases. In agreement with this model, introduction of an additional *SLF* locus leads to the loss of the ability of the pistil to reject self pollen in both petunia and *Antirrhinum* (Qiao et al. 2004a; Sijacic et al. 2004).

Recent molecular analyses of SLF function in the Solanaceae also support the inhibitor model, showing that the S-RNase can be ubiquitinated and is possibly degraded via the ubiquitin/26S proteasome pathway (Qiao et al. 2004b; Hua and Kao 2006, 2008). Since SLF itself interacts with S-RNase and participates in an E3 ubiquitin ligase complex, it was proposed to be the direct inhibitor of S-RNase (Hua and Kao 2006; Huang et al. 2006; Hua et al. 2007). By contrast, no SI breakdown mutants had ever been reported to be associated with the presence of a centric fragment in the Rosaceae (de Nettancourt 2001), and polyploids do not appear to be SC (Hauck et al. 2002, 2006b; Mable 2004). Since the deletion of *SFB* gene results in pollen-part SC (Ushijima et al. 2004; Sonneveld et al. 2005; Hauck et al. 2006a; Tsukamoto et al. 2006; Vilanova et al. 2006), a general inactivation mechanism has been proposed with the SFB conferring specificity by protecting self S-RNases from this general inactivation (Ushijima et al. 2004; Sonneveld et al. 2005; Hauck et al. 2006a).

Early genetic studies in *Antirrhinum* (Plantaginaceae) revealed that, as in the Solanaceae, heteroallelic diploid pollen was also associated with breakdown of SI (Straub 1941). In addition, the *Cycloidea* (*Cyc*), a gene controlling floral symmetry in *Antirrhinum* (Luo et al. 1996), was shown to be ca. 3 cM distant from the *S*-locus (Brieger 1935). More recently, an *SLF* gene was identified to be tightly linked to *S-RNase* in *Antirrhinum* (Lai et al. 2002), and its introduction into SI *Petunia hybrida* led to SI breakdown (Qiao et al. 2004a). To examine further the role of pollen *S* in *Antirrhinum*, we have performed a transposon mutagenesis screen of SI *Antirrhinum* lines. A PPM (*Pma*1, Pollen-part mutation in *Antirrhinum*1) arising from this screen has been identified and shown to carry a functional *SLF* copy. These data confirm that, as in the Solanaceae, the pollen *S* determinant of *Antirrhinum* acts as an S-RNase inhibitor.

Materials and methods

Plant material

Antirrhinum majus, *A. hispanicum* lines and a family of their hybrid progeny (D285) with *S*-haplotypes of S_1S_2, S_4S_5, S_2S_5, and S_1S_4, respectively, were grown under standard glasshouse conditions as described previously (Xue et al. 1996).

Transposon mutagenesis

Self-incompatible interspecific hybrids from the D285 family were grown at 15° to maximize transposon activity (Carpenter et al. 1987). About 6,500 progeny were grown under standard greenhouse conditions. For each plant, a minimum of five flowers were self-pollinated to examine pollination behavior.

Pollination studies and harvest of pollen tubes

Before anthesis, flowers were emasculated and pollinated. Pistils were harvested at given time points post-pollination. For pollen tube growth analysis, pistils were stained with aniline blue essentially as previously described (Martin 1959).

Statistical methods

The chi square test was used to determine whether the number of progeny from crosses of both $S_2S_5 \times Pma1$ and $Pma1 \times S_2S_5$ were uniformly distributed, since random

segregation should lead to an equal number of progeny with particular SI pheno- or genotypes. A value of $P < 0.05$ was regarded as indicating that a segregation ratio differed significantly from expectation.

Fluorescence in situ hybridization

The Fluorescence in situ hybridization (FISH) protocol and probes used were as described by Yang et al. (2007).

Molecular techniques

Total RNA and genomic DNA were prepared as previously described (Lai et al. 2002). The *S*-locus linked *Cyc* gene (Luo et al. 1996) was used as a probe to determine the *S*-haplotypes (Xue 2000). Following genomic DNA digestion with *Eco*RI and hybridization with the *Cyc* gene, RFLPs (restriction fragment length polymorphism) were detected showing the linkage of the four *S*-haplotypes segregating in the D285 family. Their sizes for S_1, S_2, S_4, and S_5 were 6.1, 5.5, 5.6, and 6.8 kbp in length, respectively. Pistil and pollen cDNAs were generated using the SuperScript reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and a poly-dT primer, and applied as template to amplify *S-RNase* and *SLF*, respectively. The primers for *AhSLF*s were G11j and 3′ UTR region-specific primers corresponding to *AhSLF*-S_1, S_2, S_4, and S_5 as previously described by Lai et al. (2002) and Zhou et al. (2003). The specific primers corresponding to S_1, S_2, S_4, and S_5-*RNase* were described in Xue et al. (1996). RT-PCR products were resolved by gel electrophoresis and sequenced to confirm their identity.

Evolutionary analysis

Working in PAML4, two modified branch-site models, A and B were applied to construct two LRTs (Likelihood Ratio Tests) for evolutionary selection analysis of *SLF*s and *SFB*s (Zhang et al. 2005). For the first test the null hypothesis was the site model M1a, with model A as the alternative hypothesis and the χ^2 ($df = 2$) was used to perform the test; the null hypothesis for the second test was the branch-site model A and the alternative hypothesis is model B, with $\omega_2 = 1$ fixed and the χ^2 ($df = 1$) was used to perform the test.

Results

Transposon mutagenesis of the interspecific SI hybrids of *Antirrhinum*

Antirrhinum majus lines carrying active transposons are SC owing to the presence of non-functional *S*-locus, *Sc*. To combine a functional *S*-locus and active transposons. SI lines of *A. hispanicum* were crossed with *A. majus* to introduce the active *S*-locus through partial introgression. In the F3 family resulting there were four groups of SI plants, A, B, C, and D, with *S*-haplotypes S_1S_2, S_4S_5, S_2S_5, and S_1S_4, respectively. These SI plants and their progeny were used previously to demonstrate that S-RNases are involved in stylar expression of SI in *Antirrhinum* (Xue et al. 1996). The *S*-linked *Cyc* gene was used as a probe to identify distinctive RFLP patterns among these four *S*-haplotypes, which were then used as markers for specific *S*-haplotypes (Xue 2000), particularly because we had difficulty in obtaining an S_1-*RNase* sequence for S_1 haplotype (Xue et al. 1996). For transposon mutagenesis, plants from the four SI groups (the 'D285 family') were grown at 15°C to maximize transposition frequency, and following intercrossing the four cross-compatible SI groups, seed was harvested. To screen for functional mutations of the *S*-locus, 6,500 plants derived from 450 seed capsules from the intercrosses were raised and their SI phenotypes were determined by self-pollination. If *S*-locus (or related loci) function were altered, seed set would be expected in the selfed progeny. Incompatible or compatible outcomes were determined as follows: plants were classified as SI if first five self-pollinated flowers failed to set seed; if there was some seed set but no further seeds were set following selfing a further five flowers, plants were classified as SI. Lines setting seed consistently following selfing were regarded were further investigated. Over 70 plants were thus identified by the screen to be capable of setting seed variably (between 1 and ca. 200 seeds per capsule), indicating a possible change from SI to SC (data not shown). Average seed set per capsule between compatible crosses in *A. majus* is between 100 and 200. The SI phenotype of the parental lines is extremely stable, for they have generated no seed while being vegetatively propagated for more than 15 years in the greenhouse. Mutant plants capable of selfing were termed IC (incompatibility changed) lines and used for further analysis.

Genetic analysis of the pollen SC mutant *Pma*1

Among the IC lines, we identified a plant (IC209[4]) carrying a dominant mutation only affecting the pollen-part function. Named *Pma*1, this line was classified as a true SC, since it set seed in ≥30% of pollinations, and more than half of the progeny resulting set seeds. Self-pollination of the *Pma*1 progeny (family V168) showed the SC phenotype to have been transmitted to nearly all of the progeny plants (Table 1 and Supplemental Fig. 1), confirming that the change from SI to SC was heritable. Both S_1S_4 and S_4S_4 plants were present in the progeny (in a segregation ratio of 2:1) while no S_1S_1 was detected, indicating that S_4 function

Table 1 Haplotypes and phenotypes of selfed progeny of *Pma*1 (S_1S_4)

No. of plants	*S*-haplotype	*S*-phenotype
9	S_4S_4	SC
15	S_1S_4	SC
5	S_4S_4	SI[a]
7	S_1S_4	SI[a]

[a] SI denoted here is not a typical SI response during which the pollen tube growth is arrested within the top one-third of the transmitting track. In almost all the plants all the pollen tubes grew to the base of the style. Thus, the *S*-phenotype was determined by pollen tube growth rather than seed set

was disrupted in *Pma*1 lines. Because *Pma*1 was identified in the first generation of crosses, it must have carried a dominant mutation (s).

To determine whether lack of seed set in the remaining SI progeny of the V168 family resulted from inhibition of pollen tube growth, as in normal SI reaction, or from some other defect of pollination and fertilization, patterns of pollen tube growth were investigated using aniline blue staining (Fig. 1). In the progeny that failed to produce seed, self-pollen tubes grew normally through the transmitting tissue and entered the ovary—in clear contrast to the typical SI response where the pollen tube growth is arrested within the top one-third of transmitting track. This 'normal' pollen tube growth by apparently SI pollen pointed to these tubes being able to overcome the classical SI barrier for self fertilization, located in by the pistil. For this reason these plants were classified as SC. Why these pollination fail to set seed is unclear; certainly unbalanced crosses between different ploidies can result in aberrant endosperms (Burton and Husband 2000), but here both sperms, eggs and central cells carry the duplicated chromosome fragment (see below).

To further examine the nature of the mutation in the *Pma1* lines, reciprocal test crosses were carried out (Table 2). These experiments revealed the *Pma*1 to carry a mutation affecting only the pollen function of the *S*-locus—because it was compatible when used as a male in crosses to SI stocks with identical *S*-haplotypes (S_1S_4), but incompatible as a female.

To discover whether *Pma*1 was linked to the *S*-locus, plants carrying *Pma*1 were outcrossed with SI plants with unrelated *S*-haplotypes, either as female (family V165) or male (family V166) parents (Table 3; Fig. 2). The SC progeny carried either S_1 or S_4, suggesting that *Pma*1 is not linked to the *S*-locus and segregated randomly. This was further confirmed by using the Chi square test to compare the cross progeny with a theoretically uniform distribution. *P* values were obtained of 0.21 and 0.83 for families V165 and V166, respectively, suggesting that random segregation could not be rejected (see "Materials and methods"). Although no SC S_2S_4 progeny were found in the V165 family, or SI S_2S_4 progeny in the V166 family, our subsequent pollination tests on larger families identified these two haplotypes in the progeny of similar outcrosses (data not shown).

*Pma*1 carries an extra telocentric chromosome containing the S_1-locus

The presence of an additional copy of the *S*-haplotype is a common reason for SI breakdown in the Solanaceae (Brewbaker and Natarajan 1960; Pandy 1965; Golz et al. 1999, 2001), and early genetic studies in *Antirrhinum* identified an association between heteroallelic diploid pollen and pollen-part SI breakdown (Straub 1941). A plausible explanation for the random segregation of *Pma*1 was thus that the mutation involved a chromosomal duplication of S_1, allowing heteroallelic S_4 pollen to be accepted, but not homoallelic S_1 pollen. If this was the case, S_4S_4 plants in compatible progeny derived from the outcrossed families would still reject S_1 pollen because of the presence of a duplicated S_1 haplotype. To test this, homozygous S_4 progeny genotyped using *Cyc* from *Pma*1 were backcrossed as females to S_1S_4 SI plants. No seed was set, even after extensive crosses, and controls involving

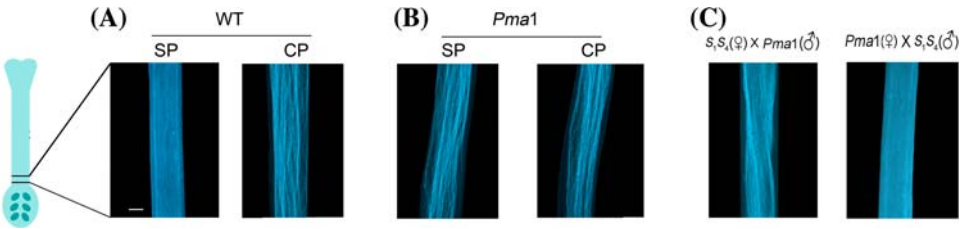

Fig. 1 Pollen tube growth after self- and compatible-pollination in wild type and *Pma*1 styles. Wild type (S_1S_4) (**a**) and *Pma*1 (**b**) styles were pollinated with self pollen (self-pollination, *SP*) and S_2S_5 pollen (compatible-pollination, *CP*), respectively. The basal parts of the style were used for staining pollen tubes with aniline blue. In normal SI plants, the pollen tubes are arrested in the top third of the style after selfing but reached the base of the style 72 h after crossing. In *Pma*1, both self pollen and S_2S_5 pollen tubes reached the base of the styles. **c** Reciprocal crosses between *Pma*1 and a wild type S_1S_4 plant; pollen from *Pma*1 grew normally in S_1S_4 style while the growth of pollen tube from S_1S_4 was arrested in the top third of the *Pma*1 style. The *scale bar* represents 300 μm

Table 2 Results of reciprocal crosses of *Pma*1 with tester plants

Sex of *Pma*1	*S* haplotype of tester	
	S_1S_4	S_2S_5
♀	−	+
♂	+	+

All these four pollination tests have been carried out using at least ten *Pma*1 lines with similar results. +, compatible cross; −, incompatible cross. The progeny of those compatible pollinations underwent subsequent test crosses (refer to Table 3 for detail)

Table 3 *S*-haplotypes and phenotypes from backcrosses and outcrosses of *Pma*1

Parental genotypes	No. of plants	*S*-haplotypes	*S*-phenotype
S_1S_4 × *Pma*1			
	16	S_1S_4	SC
	14	S_4S_4	SC
S_2S_5 × *Pma*1			
	5	S_1S_5	SC
	2	S_1S_5	SI
	4	S_1S_2	SC
	4	S_1S_2	SI
	3	S_4S_5	SC
	7	S_4S_5	SI
	5	S_2S_4	SI
*Pma*1 × S_2S_5			
	1	S_1S_5	SC
	2	S_1S_5	SI
	2	S_1S_2	SC
	1	S_1S_2	SI
	2	S_4S_5	SC
	1	S_4S_5	SI
	1	S_2S_4	SC

outcrosses between these plants as females and SI plants with S_2S_5 haplotypes produced normal seed set (data not shown). These apparently homozygous S_4 plants must thus also carry a functional S_1 haplotype (termed S_1^d). However, DNA blot analysis using the *S*-linked *Cyc* marker failed to detect this duplication (Fig. 2), either because the S_1-linked *Cyc* copy was deleted or fragmentation occurred between the *Cyc* and the *S*-loci.

To resolve this apparent paradox we adopted a FISH strategy. *SLF* has been identified as the pollen *S* determinant in *Antirrhinum*, so a biotin-labeled probe (S_1*SLF-TAC;* red signal) incorporating the *AhSLF-S*$_1$ sequence was hybridized to *Pma*1 chromosomes (Fig. 3; Supplemental Fig. 2). However, this probe hybridizes to S_1- and S_4-haplotypes with similar signal intensity (Yang et al. 2007); for this reason the centromeric tandem repeat sequence,

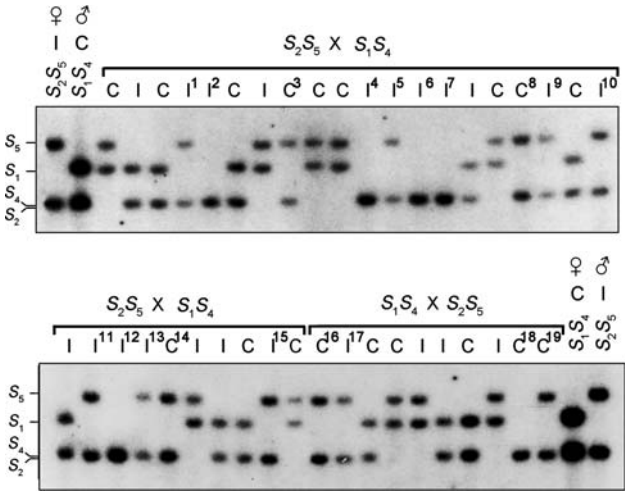

Fig. 2 *S*-haplotype determination of outcrossed populations of *Pma*1. The progeny were derived from two outcrosses, S_2S_5 × *Pma*1 (S_1S_4) (V165 family) and *Pma*1 (S_1S_4) × S_2S_5 (V166 family). Genomic DNA from these progeny was digested by *Eco*RI and then hybridized by a *Cyc* probe. The hybridizing fragments representing four *S* haplotypes (S_1, S_2, S_4, and S_5) are indicated on the *left*. The sequential superscripts represent progeny with S_4 haplotype which were subjected to further expression analysis shown in Fig. 4. I, SI progeny; C, SC progeny

CentA1, was digoxigenin-labeled to identify centromeres (green signal). Thus, the S_1*SLF-TAC* showed three distinct signals on interphase nuclei of *Pma*1, indicating that the nucleus of *Pma*1 plants contain an additional *S*-haplotype besides the two normal ones (Fig. 3a). Importantly, at mitotic metaphase FISH showed that the additional *S*-locus of *Pma*1 is located on a small telocentric chromosome (the 'endogenous' *S*-locus is carried on chromosome 8; Fig. 3b). This small chromosome was supernumery to the 16 chromosomes of the diploid *Antirrhinum* genome. To determine its origin, we analyzed the pachytene chromosomes of *Pma*1 (Fig. 3c) and discovered this supernumery chromosome to pair with the short arm of chromosome 8, forming a fork-shaped trivalent at pachytene stage. We also found that the *CentA1* hybridization signal on this chromosomal fragment was weaker than that of chromosome 8. Taken together these data indicated the telocentric chromosome to be derived from the short arm of chromosome 8, having fragmented at a point within the centromeric region.

The expression of additional *AhSLF-S*$_1$ locus in *Pma*1 is associated with SC in its progeny

To determine whether an additional copy of *AhSLF-S*$_1$ was expressed in *Pma*1, the progeny of *Pma*1 outcrossed to S_2S_5 plants were analyzed using RT-PCR. Since *SLF-S*$_1$ expression would be detectable in all SC and SI lines carrying the endogenous S_1-haplotype, expression of an

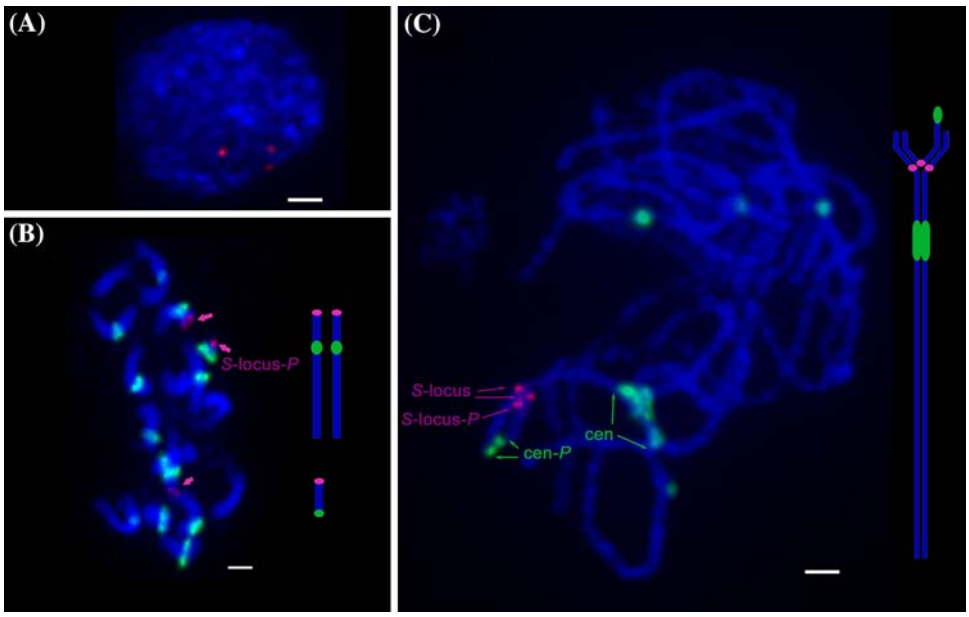

Fig. 3 Detection of a centric fragment in *Pma*1. **a** FISH using S_1*SLF-TAC* detects three signal origins in the interphase nucleus. Biotin-labeled S_1*SLF-TAC* containing *AhSLF-*S_1 (*red signal*) was used to hybridize the chromosomes of *Pma*1. **b** *Pma*1 has an additional S_1-locus on the telocentric chromosome other than chromosome 8. The extra S_1-locus was detected as in (**a**) but by mitosis metaphase FISH. Centromeric tandem repeat sequence, *CentA1*, was digoxigenin-labeled to locate the centromeres (*green signal*). The *red arrows* indicate the *S*-locus. The "*S*-locus-*P*" represents the additional telocentric chromosome in *Pma*1. A schematic representation of the three metaphase chromosomes hybridized with the S_1*SLF-TAC* is shown on the *right*. **c** Detection of synapsis of the extra chromosome involving in the telotrisomic nucleus. FISH analysis of the meiotic pachytene trivalent chromosomes of *Pma*1 was carried out using both S_1*SLF-TAC* and *CentA1* (*left*). A schematic representation of the synapsis of the trivalent chromosomes is shown on the *right*. The *bars* represent 1 μm

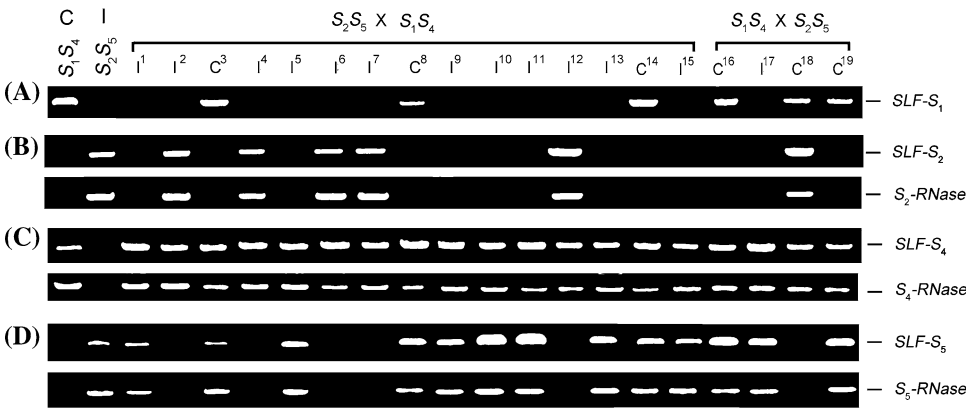

Fig. 4 Expression of *AhSLF-*S_1 in *Pma*1 progeny. **a**, **b**, **c**, and **d** represent the expression of S_1-, S_2-, S_4-, and S_5-haplotypes in selfed progeny of *Pma*1 bearing S_4 haplotype detected by RT-PCR analysis with specific primers for *AhSLF-*S_1, *AhSLF-*S_2 and S_2-RNase, *AhSLF-*S_4 and S_4-*RNase*, *AhSLF-*S_5 and S_5-*RNase*, respectively. The *first* and *second lanes* represent the parental lines, namely, compatible *Pma*1 (S_1S_4) and an incompatible plant carrying (S_2S_5); the remaining *lanes* are the progeny of S_4-haplotype from the families V165 and V166. *Superscripts* correspond to those labeled in Fig. 2. The *S*-phenotypes are indicated on the *top* of the *panel*

extra *SLF-*S_1 could only be feasible in progeny carrying the S_4-haplotype. As shown in Fig. 4, *SLF-*S_1 expression occurred in all the SC, but not SI progeny carrying the S_4-haplotype from the families V165 and V166—demonstrating expression of an additional *AhSLF-*S_1 to be closely linked to SC in *Pma*1 progeny.

SLF and SFB proteins appear to be under different selective pressures

Recent reports have indicated mechanistic differences in the operation of SI to occur among the three S-RNase-based families (Newbigin et al. 2008). To address this

problem from an evolutionary perspective, we performed tests for selection on an assembly of *SLF* sequences from *Antirrhinum* and *Petunia* known to encode pollen S proteins, together with a set of *Prunus SFB*s sequences which had already been shown to be under a strong positive selection (Ikeda et al. 2004). Conventional calculation of Ka/Ks ratios have been reported not to show strong positive selection on SLFs (Newbigin et al. 2008), but since the Ka/Ks test assumes a single selection ratio among all sites and all lineages, positive selection upon a few sites would be obscured by far more frequent negative selection (Zhang et al. 2005). Thus, two modified branch-site specific models were implemented in PAML4, which allow the determination of selective pressure variation (the ω ratio) among both sites and lineages (Zhang et al. 2005). The phylogenetic tree constructed based on the maximum likelihood method is shown in Supplemental Fig. 3 and considers the *Prunus* lineage as the 'foreground branch' and all other branches of the tree as background branches. Parameter estimates under model A suggested that 37% of sites were conserved across all lineages with $\omega_0 = 0.218$, whereas as high as 46% of sites were under a strong positive selection along the *Prunus* branch ($\omega_2 = 35.204$; Table 4). Comparison of Model with the model M1a (Nearly Neutral) using a likelihood ratio test (LRT; Zhang et al. 2005) showed differences of $2\Delta = 8.79 \times 2 = 17.58$, with $P < 0.001$ and $df = 2$. However, the ω_1 for the background lineages was fixed at one, which seemed biologically unlikely. Model B was considered more realistic with the assumption that both foreground and background branches could undergo positive selection. Parameter estimates under model B point to 13% of sites being under a weak positive selection in both SLFs and SFBs ($\omega_1 = 1.259$), whereas 48% of sites were under an even stronger positive selection in the *Prunus* branches ($\omega_2 = 38.883$; Table 4). The LRT comparing the branch-site model B and the corresponding null model gave differences of $2\Delta = 7.12 \times 2 = 14.24$, with $P < 0.001$ and $df = 1$. Model B thus fitted the data significantly better than the null model, and indicated that both SLFs and SFBs appear to under positive selection, with most sites ($p_1 + p_2 + p_3 > 50\%$ in Model B) in SFBs being under the positive selection and the overwhelming number of sites ($p_0 + p_2 > 70\%$ in Model B) in SLFs being under purifying selection. Moreover, for those sites under selection, the ω value was much higher for SFBs than for SLFs. Sensitivity analyses clearly established that the LRTs and qualitative results of the ML parameter estimation were sufficiently robust to withstand different initial values, and tree topology obtained through distinct methods (data not shown). Taken together, these results indicated that both SLFs and SFBs are under positive selection—but under different selective pressures.

Discussion

SC in *Pma1* results from an additional *S*-locus

The dominant pollen-part SC mutation *Pma*1 in *A. hispanicum* carries a stably inheritable, telocentric fragment containing an S_1-locus. This locus is functional because its selfed $S_1^d S_4 S_4$ progeny rejected $S_1 S_4$ pollen. Our data show *Pma*1 to behave similarly to PPMs associated with centric fragments in the Solanaceae previously described (Brewbaker and Natarajan 1960; Pandy 1965; Golz et al. 1999, 2001), and therefore demonstrate the role of the pollen *S* determinant to be functionally conserved in the Solanaceae and Plantaginace. The relative short interval between the *S*-locus and the long centromeric region of chromosome 8 in

Table 4 Different branch-site tests for positive selection in SLFs/SFBs

Tests	*l*	Estimates of parameters*	LRT statistics (*P* values)
Test 1			
M1a(Nearly Neutral)	−10,609.29	$p_0 = 0.684$, ($p_1 = 0.316$)	17.58**
		$\omega_0 = 0.222$	
Model A	−10,600.50	$p_0 = 0.370$, $p_1 = 0.167$, ($p_2 = 0.319$, $p_3 = 0.144$, $p_2 + p_3 = 0.463$)	
		$\omega_0 = 0.218$, **$\omega_2 = 35.204$**	
Test 2			
Model A ($\omega_2 = 1$ fixed)	−10,604.61	$p_0 = 0.423$, $p_1 = 0.194$, ($p_2 + p_3 = 0.383$)	14.24**
		$\omega_0 = 0.213$	
Model B	−10,597.49	$p_0 = 0.387$, $p_1 = 0.134$, ($p_2 = 0.356$, $p_3 = 0.123$, $p_2 + p_3 = 0.479$)	
		$\omega_0 = 0.251$, **$\omega_1 = 1.259$, $\omega_2 = 38.883$**	

* Parameters indicating positive selection are presented in bold type. Those in parentheses are presented for information only but are not free parameters. Estimates of κ range from 2.10 to 2.19 among the models

** Statistically significant at $P < 0.001$

Antirrhinum (Yang et al. 2007) may promote fragmentation of the short arm of chromosome 8 during transposon mutagenesis, leading to the generation of *Pma*1. By contrast, no additional pollen-*S* gene containing a centric fragment has ever been detected in the Rosaceae (de Nettancourt 2001), perhaps because the Rosaceae *S*-locus is located well outside the centromeric region (Ushijima et al. 2001) and thus may not be susceptible to being included in a stably inherited centric fragment (Yang et al. 2007).

The function of the pollen self-incompatibility determinant

Genetic evidence from the Solanaceae supports an inhibitor model in which the pollen *S* determinant functions to inhibit all but self S-RNase. In the Rosaceae, by contrast, duplication of *S*-haplotype do not always lead to SC and the presence of a general inhibitor for S-RNase has been proposed (Ushijima et al. 2004; Sonneveld et al. 2005; Hauck et al. 2006a). This apparent mechanistic distinction was also reflected by different levels of sequence diversity in the pollen S candidates among these families. Sequence diversity of SFBs in the Rosaceae (70–80% amino acid sequence identity; Ikeda et al. 2004; Vaughan et al. 2006) is much higher than that of SLFs in the Solanaceae (ca 90% amino acid sequence identity; Sijacic et al. 2004). This divergence is consistent with these pollen S proteins having different roles. The pollen S proteins of the Solanaceae appear to have at least two roles: one to inhibit all S-RNases, and another to recognize self S-RNases, pointing to a requirement for high level of specificity. In the Rosaceae, the pollen S protein appears solely to protect self S-RNase from a general S-RNase inhibition, which may be carried out by another polypeptide. Thus, the evolutionary pressure for conservation of this protein may be less than for the Solanaceae pollen S determinant, resulting in a higher substitution rate. Our analysis suggested that while positive selection was acting on both SLFs and SFBs (Table 4), more sites were under positive selection in *SFB*s. However, most *SLF* sites were under purifying selection- pointing to a role for SLFs in the Solanaceae as inhibitors of S-RNases.

Consistent with the inhibitory model held to operate in the Solanaceae, the *Pma*1 line identified in *Antirrhinum* carried an additional copy of S_1-haplotype, indicating that the pollen *S* product also functions as an inhibitor in the Plantaginaceae. Although the inhibitory role of *Antirrhinum* SLFs has been confirmed by molecular evidence (Qiao et al. 2004a), the low sequence divergence in the four known *Antirrhinum* SLFs (ca 97% amino acid sequence identity; Zhou et al. 2003) has thrown doubt on their pollen *S* function. Analyses of the molecular evolution of these proteins have suggested that both Ka and Ks values of *Antirrhinum* SLFs are lower than those of other functional S-RNases (Newbigin et al. 2008). Nevertheless, the corresponding Ka/Ks ratios are reasonably similar (0.27 for SLFs; 0.44 for S-RNases) and, since only four haplotypes of *SLF*s from *Antirrhinum* are available, we cannot exclude the possibility that the low diversity is due to a small sample size. Further genetic and molecular analyses are thus required to establish whether the high level of similarity of *Antirrhinum* SLFs results from sampling a small population, or other sequences altogether are responsible for S-RNase recognition and inhibition (Newbigin et al. 2008; Sassa et al. 2007).

It remains unclear how the S-RNases in Solanaceae and Plantaginaceae are inhibited at a molecular level. Two prevalent models have been proposed. One suggested that the SLF protein participates in an SCF complex that targets the S-RNase for degradation (Qiao et al. 2004b; Hua and Kao 2006; Hua et al. 2007). This inference is supported by the identification of SSK1, a SKP1-like protein that interacts with the SLF in both *Antirrhinum* and *Petunia* (Huang et al. 2006, and our unpublished data). There is also evidence that an untypical adaptor protein SBP1 forms an unorthodox E3 ligase complex (Hua and Kao 2006). The central question, however, surrounds the specificity of this ubiquitination, because there are no data showing differences between self and non-self pollen tubes (Qiao et al. 2004b; Hua and Kao 2006). Perhaps both classes of pollen tubes are ubiquitinated, but differentially; for example mono- and poly-ubiquitination can lead to different fates in other systems, since it has been proposed that it is the type of ubiquitination that determines whether a protein is degraded (Pickart 2001; Hicke and Dunn 2003; Mukhopadhyay and Riezman 2007). Recently, a further complexity has been added at a cytological level for S-RNase, which has been reported as being sequestered to the vacuole, rather than being degraded following ubiquitination in *Nicotiana* (Goldraij et al. 2006). The role of SLF in this model is still speculative. In addition, it has been suggested that the pollen S probably functions as a multimer and heteromers are assumed not to be functional (McClure 2006). Thus, our data could also be explained by this model.

Some recent investigations suggested that the exact role of the pollen *S* is still far from clear. The evolutionary analyses of SLF/SFB and S-RNase have challenged the role of SLFs/SFBs as male *S*-determinants since topologies of phylogenetic trees generated by SLF-related sequences and corresponding S-RNases are different, pointing to different evolutionary histories (Wheeler and Newbigin 2007; Newbigin et al. 2008). Moreover, multiple *S*-locus F-box genes (termed *S*-locus F-box brothers; SFBBs) have been reported to participate in SI in the Maloideae, a subfamily of Rosaceae (Sassa et al. 2007).

We still could not exclude the possibility that the failure to recover the pollen *S* in Solanaceae and Plantaginaceae is due to a functional redundancy or some other unknown reasons. The molecular identity for the pollen *S* thus remains equivocal, and a clear understanding of the molecular basis of S-RNase inhibition in these three families will help to resolve this question.

'Telotrisomy' may play an important part in *S*-locus evolution

Although duplication of centric fragments is common in PPMs in the Solanaceae and Plantaginaceae (de Nettancourt 2001 and this study), its significance to the evolution of the *S*-locus has been unexplored. Transition through a SC intermediate has been proposed as a necessary step in the origin of new *S*-haplotypes, although the nature and the evolutionary process leading to the formation of this SC intermediate remain undetermined (Uyenoyama et al. 2001; Newbigin and Uyenoyama 2005). We suggest that duplication of centric fragments carrying elements of the *S*-locus could, at least partly, fulfill this function and serve as an important step in the transition from SI to SC. Loss of the ability of SLFs to inhibit S-RNases would clearly be fatal, and it is difficult to see how sufficient mutations could be accumulated to generate favorable new *S*-alleles before being swept out of the population by purifying selection. However, in the presence of an additional *S*-locus, sequence diversification could continue under neutral selection while selection operates every generation after its segregation, allowing a purifying selection for the inhibition conservation and diversifying selection for a new functional allele. Being new, the allele would be favored in the population and spread in a negative frequency-dependent manner (Wright 1939). Further, unfavorable alleles could be maintained in the population in the form of telotrisomies, allowing 'evolutionary time' for the formation of more favorable alleles. The presence of an additional *SLF* copy would thus provide not only an opportunity for diversification, but also act as a reserve for new intermediate alleles. Duplication events of this type may thus constitute a major feature of the evolution of S-alleles in the Solanaceae and Plantaginaceae where deletion of the pollen S-determinant is lethal. Of course, centric fragments, once formed, may subsequently be incorporated into the 'normal' chromosome complement (Golz et al. 2001), resulting in their consistent inheritance in further generations.

In conclusion, our findings provide both genetic and molecular evidence to suggest an inhibitory function for the pollen *S* in *Antirrhinum*, and thus a conserved SI mechanism in the Solanaceae and Plantaginaceae. Breakdown of SI in the presence of an *S*-locus containing telocentric chromosome suggests that chromosomal duplication may play a part in the diversification of the *S*-locus.

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