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Heterochromatic and genetic features are consistent with recombination suppression of the self-incompatibility locus in *Antirrhinum*

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Summary

Self-incompatibility (SI) is a genetic mechanism to prevent self-fertilization that is found in many species of flowering plants. Molecular studies have demonstrated that the *S-RNase* and *SLF*/*SFB* genes encoded by the single polymorphic *S* locus, which control the pollen and pistil functions of SI in three distantly related families, the Solanaceae, Scrophulariaceae and Rosaceae, are organized in a haplotype-specific manner. Previous work suggested that the haplotype structure of the two genes is probably maintained by recombination suppression at the *S* locus. To examine features associated with this suppression, we first mapped the *S* locus of *Antirrhinum hispanicum*, a member of the Scrophulariaceae, to a highly heterochromatic region close to the distal end of the short arm of chromosome 8. Both leptotene chromosome and DNA fiber fluorescence *in situ* hybridization analyses showed an obvious haplotype specificity of the *Antirrhinum S* locus that is consistent with its haplotype structure. A chromosome inversion was also detected around this region between *A. majus* and *A. hispanicum*. These results revealed that DNA sequence polymorphism and a heterochromatic location are associated with the *S* locus. Possible roles of these features in maintenance of the haplotype specificity involved in both self and non-self recognition are discussed.

Keywords: *S* haplotype, heterochromatin, genetic polymorphisms, recombination suppression, *Antirrhinum*.

Introduction

Self-incompatibility (SI) systems are widespread genetic mechanisms that prevent self-fertilization and are thought to play an important role in the diversification and dominance of angiosperms (Whitehouse, 1950; de Nettancourt, 2001). There are three known major types of SI systems, the Papaveraceae, the Brassicaceae and the Solanaceae systems, which have been extensively studied. In these systems, the SI responses are all controlled by a single polymorphic locus, termed the *S* locus (Kao and Tsukamoto, 2004; Wheeler *et al.*, 2003). In the Papaveraceae, a pistil-specific protein encoded by the *S* locus mediates pollen-tube growth inhibition through a Ca^{2+} signaling cascade involving programmed cell death (Franklin-Tong *et al.*, 2002; Thomas and Franklin-Tong, 2004). In the Brassicaceae, the two *S*-locus proteins known as SRK (*S*-locus receptor-like kinase), which is expressed in the stigma, and SCR (*S*-locus cysteine-rich)/SP11(*S*-locus pollen 11), which is expressed in pollen, have been shown to control pollen recognition and rejection through a ligand–receptor binding mechanism (Chookajorn *et al.*, 2004; Kachroo *et al.*, 2001; Takayama *et al.*, 2001). In the Solanaceae-type gametophytic self-incompatibility (GSI) system found in three plant families, Rosaceae, Solanaceae and Scrophulariaceae, the *S*-locus product in the pistil is a glycoprotein with ribonuclease activity called S-RNase, whereas the *S*-locus pollen product is one of a class of

F-box proteins called SLF (S-locus F-box) or SFB (S-haplotype-specific F-box) (Kao and Tsukamoto, 2004). The SLF male determinant physically interacts with its haplotype-specific female determinant, S-RNase, probably forming an SCF complex to control pollen rejection (Qiao *et al.*, 2004a,b; Sijacic *et al.*, 2004; Huang *et al.*, 2006). The tight linkage of the two *S*-locus genes is possibly maintained by recombination suppression, which could be involved in guaranteeing the haplotype-specific interaction of their products (Cui *et al.*, 1999; Wheeler *et al.*, 2003).

However, it is still unclear how recombination suppression occurs around the *S* locus in the species studied. Chromosome fluorescence *in situ* hybridization (FISH) analysis revealed that the *S* locus of *Papaver rhoeas* was probably located in the centromeric region of a chromosome (Wheeler *et al.*, 2003). For the solanaceous species, the *S* locus was also found to be located in the centromeric region by genetic analysis and direct FISH localization in *Petunia*, *Nicotiana* and *Lycopersicon* (Bernacchi and Tanksley, 1997; Bernatzky, 1993; Brewbaker and Natarajan, 1960; Entani *et al.*, 1999; Golz *et al.*, 2001; ten Hoopen *et al.*, 1998; Pandy, 1965). For these species, a centromeric location is considered as an important factor in *S*-locus recombination suppression (Entani *et al.*, 1999; ten Hoopen *et al.*, 1998; Wheeler *et al.*, 2003). However, the *S* locus of the rosaceous almond was deduced to lie in a region outside the centromere that lacks repetitive sequences (Ushijima *et al.*, 2001).

The *S* locus of the scrophulariaceous *Antirrhinum* was previously located in a peri-centromeric region using mitotic metaphase chromosome FISH analysis (Ma *et al.*, 2003). FISH analysis using mitotic metaphase chromosomes has a very low resolution, far lower than for meiotic pachytene chromosomes or extended DNA fibers (Cheng *et al.*, 2002). It is necessary to detect the precise chromosomal location of the *Antirrhinum S* locus using high-resolution FISH techniques to provide new cytological clues to the mechanism of recombination suppression in this organism. As a closer relative of the Solanaceae than the Rosaceae, the *S*-locus location of *Antirrhinum* might provide important information for recombination suppression and the evolutionary conservation of the *S* locus in the S-RNase-based SI systems.

In this study, several TAC (transformation-competent artificial chromosome) clones derived from the region of the *S* locus (Zhou *et al.*, 2003) were used as FISH probes. Pachytene and leptotene chromosomes, as well as DNA fibers, were employed to detect the chromosomal location of the *S* locus, to examine *S*-locus haplotype specificity and to measure the distance between the two *S*-determinant genes, respectively. Our goal is to elucidate the possible cytological features involved in maintenance and evolution of the haplotype specificity of the *S* loci involved in both self and non-self recognition.

Results

Fine detection of the S*-locus chromosomal location in* Antirrhinum

The *S* locus was previously mapped on the smallest chromosome of *A. hispanicum* by mitotic metaphase chromosome FISH (Ma *et al.*, 2003). For fine mapping of the *S* locus using a cytological approach, TAC clones (Zhou *et al.*, 2003) corresponding to S-RNase and SLF genes were used for pachytene chromosome FISH. The clone's names and their corresponding genes are listed in Table 1. We first examined the S_2 haplotype. The two genes, *S_2-RNase* and *AhSLF-S_2*, are separated by 9 kbp (revealed by sequence analysis) (Lai *et al.*, 2002), which was too close to distinguish by pachytene chromosome FISH. *S_2SLF-TAC* and *S_2RNaseR-TAC* were used to determine the location and orientation of *S_2-RNase* and *AhSLF-S_2* on the S_2S_4 chromosomes (the correlation of *S_2-RNase* and *AhSLF-S_2* with the TACs of the S_2 haplotype is illustrated in Figure 3). A centromeric repeat sequence, *CentA1*, was used to mark the centromere position and also to help identify the individual chromosomes (Zhang *et al.*, 2005). To separate the closely linked *S_2RNaseR-TAC* and *S_2SLF-TAC*, we probed early pachytene chromosomes (Figure 1). The FISH results showed that these two clones are indeed very close to each other, and located at a distal position on the short arm of chromosome 8, two-thirds of the arm length away from the centromere. It was also found that the *S_2RNaseR-TAC*, representing the *S_2-RNase*, was closer to the centromeric region based on analysis of more than 20 pachytene cells. Signals of the *S*-locus-derived TAC clones were not detected in the centromeric region defined by *CentA1*. These results show that the *S* locus is located away from the centromere. This is quite different from the centromeric localization of some solanaceous *S* loci observed in metaphase chromosome FISH analyses (Entani *et al.*, 1999; Golz *et al.*, 2001; ten Hoopen *et al.*, 1998).

To further examine the proposed centromeric localization of the solanaceous *S* locus, digoxigenin-labeled *PhS_3-RNase-TAC* was hybridized to pachytene chromosomes of the S_3S_3 haplotype of *Petunia hybrida* (Figure S1). The nucleolus organizer region (NOR) sequence was biotin-

Table 1 *S*-locus TAC clones used in FISH analyses of *A. hispanicum*

TAC clones	*S-Rnase*	*SLF*	Origin
S_2RNaseR-TAC	–	–	This study
S_4RNase-TAC	+	–	This study
S_5RNase-TAC	+	–	Zhou *et al.* (2003)
S_2SLF-TAC	–	+	This study
S_4SLF-TAC	–	+	This study
S_5SLF-TAC	–	+	This study

Absence (–) and presence (+) of the *S*-locus genes.

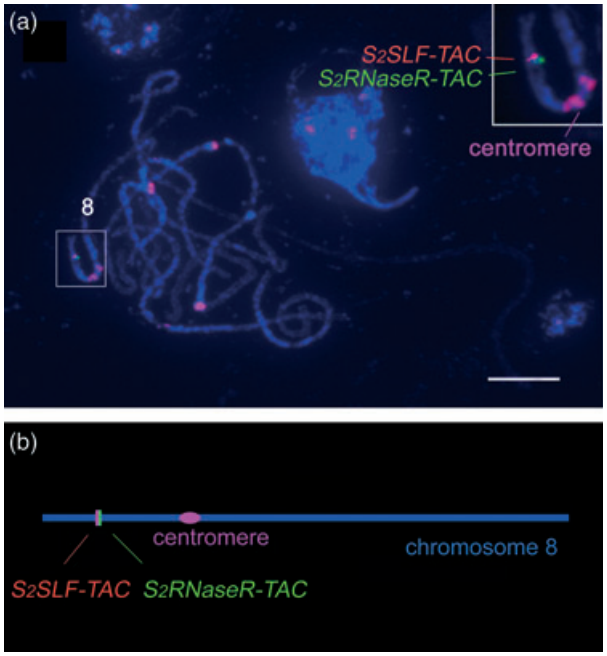

Figure 1. Sub-chromosomal localization of the *S* locus in *A. hispanicum*.
(a) Biotin-labeled S_2*SLF-TAC* containing *AhSLF-*S_2 (red signal) and digoxigenin-labeled S_2*RNaseR-TAC* containing S_2*-RNase* (green signal) were hybridized to early pachytene chromosomes of the S_2S_4 haplotype of *Antirrhinum hispanicum*. The gap between these two TAC clones was 26 kbp (Zhou *et al.*, 2003). A centromeric tandem repeat sequence, *CentA1*, was biotin-labeled to assign the chromosomes, except for the signal indicating S_2 *SLF-TAC* (red signals, see also Zhang *et al.*, 2005). The image of the short arm of chromosome 8 is enlarged and shown in the upper right corner. Scale bar = 5 μm.
(b) Schematic representation of the sub-chromosomal location of the TAC clones derived from the *S* locus.

labeled to assign this NOR region to chromosome 3 (Gerlach and Bedbrook, 1979). The major constriction of this chromosome is lightly stained with DAPI. The *CP100-TAC* containing *CP100*, which is closely linked to the *S-RNase* in the Solanaceae (Gebhardt *et al.*, 1991; ten Hoopen *et al.*, 1998; Harbord *et al.*, 2000; McCubbin *et al.*, 2000; Golz *et al.*, 2001), was also selected and used as a biotin-labeled FISH marker (red signal, Figure S1). The results show that the *S* locus of *P. hybrida* is localized on the short arm of chromosome 3, just on the border of major constriction. This is consistent with its centromeric localization proposed previously (Entani *et al.*, 1999; ten Hoopen *et al.*, 1998). However, although linked to the *PhS*$_3$*-RNase*, the *CP100-TAC* appeared distant from the centromere, which is not consistent with the results found in *Nicotiana alata* (Golz *et al.*, 2001), which is a relative but belongs to another genus of the Solanaceae. Unfortunately, the TAC containing *PhS*$_3$*-SLF* showed too much non-specific hybridization signal when used as a FISH probe (data not shown), and the relative positions of the *PhS*$_3$*-RNase* and *PhS*$_3$*-SLF* could not be determined. Nevertheless, these results indicate that the relative positions of the *S* locus and the centromere appeared to be different between *Antirrhinum* and the solanaceaous species.

To further confirm these results in *Antirrhinum*, the S_4*RNase-TAC* and S_4*SLF-TAC* and the S_5*RNase-TAC* and S_5*SLF-TAC* from *A. hispanicum* were probed to the S_4S_5 or S_1S_5 chromosomes, respectively (data not shown). They produced two doublet signals with similar distance and orientation to those of the S_2*RNaseR-TAC* and S_2*SLF-TAC* on the S_2S_4 chromosomes. These results indicated that the S-RNase and SLF genes were relatively close to each other on the short arm of chromosome 8 in *Antirrhinum*.

Determination of the S*-haplotype specificity of the TAC clones derived from the* S *locus*

To examine the *S*-haplotype specificity of S_2, S_4 and S_5 TAC clones, we probed them to the leptotene chromosomes before synapsis. The S_2*RNaseR-TAC* and S_2*SLF-TAC* were labeled with digoxigenin and biotin, respectively, and hybridized together to S_2S_4 and S_2S_5 leptotene chromosomes (Figure 2). On the S_2S_4 chromosomes, they showed two pairs of signals. But on the S_2S_5 chromosomes, only one pair of signals could be detected. The signals of the S_2*RNaseR-TAC* and S_2*SLF-TAC* on leptotene chromosomes could not be separated as clearly as those on the early pachytene chromosomes. The chromatin of this region was more compact at the leptotene stage than at the pachytene stage, indicating heterochromatic characteristics.

The S_4*RNase-TAC* and S_4*SLF-TAC* and the S_5*RNase-TAC* and S_5*SLF-TAC* of *A. hispanicum* were also probed to S_2S_4 and S_2S_5 leptotene chromosomes, respectively. On the S_2S_4 chromosomes, the S_4*RNase-TAC* and S_4*SLF-TAC* showed two pairs of signals at the same positions as those of the S_2*SLF-TAC* and S_2*RNaseR-TAC*, and the red and green signals always overlapped, showing the proximity of the S_4*RNase-TAC* and S_4*SLF-TAC* (Figure 2a). On S_2S_5 leptotene chromosomes, the S_5*RNase-TAC* and S_5*SLF-TAC* showed only one pair of signals located at positions different from those of S_2*SLF-TAC* and S_2*RNaseR-TAC* (Figure 2b), and there was a small distance between the S_5*RNase-TAC* and the S_5*SLF-TAC*. The physical distance between the S_5*RNase-TAC* and S_5*SLF-TAC* was estimated to be larger than that between the S_2*SLF-TAC* and S_2*RNaseR-TAC*. These results indicate that the leptotene FISH hybridization signals of S_2 and S_4 haplotypes cross-hybridized more with each other without obvious *S*-haplotype specificity, whereas those of the S_2 and S_5 haplotypes showed strong *S*-haplotype specificity with only faint cross-hybridization.

Estimation of the physical distance between S-RNase *and* AhSLF *in the* S_4 *and* S_5 *haplotypes of* A. hispanicum

To measure the distance between S_4*RNase-TAC* and S_4*SLF-TAC*, and between S_5*RNase-TAC* and S_5*SLF-TAC*, on the

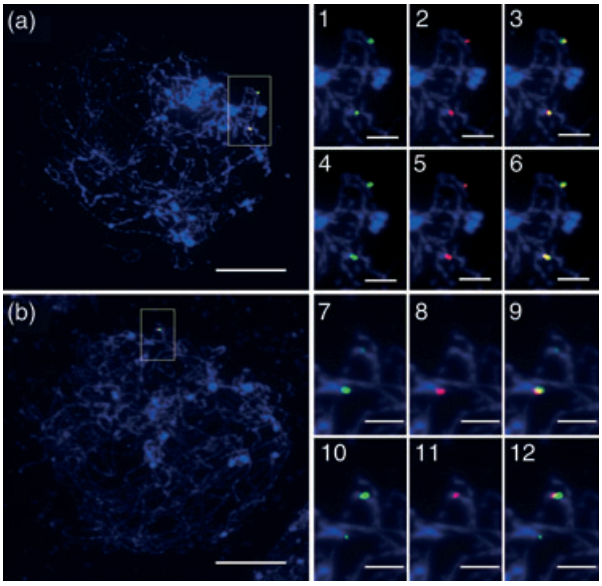

Figure 2. Determination of the haplotype specificity of the TAC clones from the *S* locus of *A. hispanicum*.
(a) Digoxigenin-labeled *S_2RNaseR-TAC* (green signal) and biotin-labeled *S_2SLF-TAC* (red signal) were used to probe *S_2S_4* leptotene chromosomes. *S_2RNaseR-TAC* and *S_2SLF-TAC* show two pairs of signals here. Enlarged images of the hybridization results are shown on the right: (1) *S_2RNaseR-TAC* (green); (2) *S_2SLF-TAC* (red); (3) merged *S_2RNaseR-TAC* (green) and *S_2SLF-TAC* (red). Digoxigenin-labeled *S_4RNase-TAC* containing *SRNase-S_4* (green signal) and biotin-labeled *S_4SLF-TAC* containing *AhSLF-S_4* (red signal) were used to probe *S_2S_4* leptotene chromosomes: (4) *S_4RNase-TAC* (green); (5) *S_4SLF-TAC* (red); (6) merged *S_4RNase-TAC* (green) and *S_4SLF-TAC* (red). These show two pairs of signals at the same positions as *S_2RNaseR-TAC* and *S_2SLF-TAC* (3). The red and green signals are overlapping, indicating the proximity of *S_4RNase-TAC* and *S_4SLF-TAC*.
(b) *S_2RNaseR-TAC* and *S_2SLF-TAC* were used to probe *S_2S_5* leptotene chromosomes, and show only one pair of signals. Enlarged images of the hybridization results are shown on the right: (7) *S_2RNaseR-TAC* (green); (8) *S_2SLF-TAC* (red); (9) merged *S_2RNaseR-TAC* (green) and *S_2SLF-TAC* (red). Digoxigenin-labeled *S_5RNase-TAC* containing *SRNase-S_5* (green signal) and biotin-labeled *S_5SLF-TAC* containing *AhSLF-S_5* (red signal) were used to probe *S_2S_5* leptotene chromosomes: (10) *S_5RNase-TAC* (green); (11) *S_5SLF-TAC* (red); (12) merged *S_5RNase-TAC* (green) and *S_5SLF-TAC* (red). They show only one pair of signals, which localizes to positions different from those of *S_2SLF-TAC* and *S_2RNaseR-TAC* (9). The distance between *S_5RNase-TAC* and *S_5SLF-TAC* was estimated to be larger than that of *S_2RNaseR-TAC* and *S_2SLF-TAC*. Scale bar = 5 μm in (a) and (b), and 1 μm in (1)–(12).

chromatin of their corresponding *S* haplotypes, we hybridized them together to the DNA fiber generated from *S_2S_5* interphase cells (Figure 3). The *S_2RNaseR-TAC* and *S_2SLF-TAC* were hybridized to DNA fibers of the *S_2* haplotype as a control, where they are known to be separated by 26 kbp. The signals for the *S_2SLF-TAC* and *S_2RNaseR-TAC* unambiguously showed their actual size, and the gap between the two clones showed very low cross-hybridization. However, the signals between *S_4RNase-TAC* and *S_4SLF-TAC* or between *S_5RNase-TAC* and *S_5SLF-TAC* showed considerable cross-hybridization and could not be separated easily, indicating that they contained much more repetitive sequence than the TAC clones from the *S_2* haplotype. The *S_4* haplotype had mostly green dots at one end and red dots at the other. These represent the *S_4RNase-TAC* and *S_4SLF-TAC*, respectively. The dots between these TACs were probably caused by shared repetitive sequences located on both sides. The distance between the *S_4RNase-TAC* and *S_4SLF-TAC* could be deduced from the total length of the fiber FISH signals minus the length of the *S_4RNase-TAC* and *S_4SLF-TAC*, and was estimated to be approximately 50 kbp. As for the *S_5* haplotype, it showed scattered green and red dots, with one end biased toward green, the other biased toward red. The starts or ends of *S_5RNase-TAC* and *S_5SLF-TAC* could not be delimited. Thus, only a maximum distance between the *S_5RNase-TAC* and *S_5SLF-TAC* could be deduced from the total length of the fiber FISH signals minus the length of the *S_5RNase-TAC* and *S_5SLF-TAC*, and was estimated to be approximately 100 kbp. These results show that the *S-RNase* and *AhSLF* genes in the *S* haplotypes are closely linked to each other, with variable abundances of repetitive sequences, and their physical separation ranged from 9 to approximately 100 kbp.

An inversion was detected between self-compatible A. majus *and self-incompatible* A. hispanicum *around the* S *locus*

Antirrhinum majus is a self-compatible (SC) species in the *Antirrhinum* genus. To investigate the cytological features of the *S* locus in *A. majus*, we first cloned *AmSLF-like 1*, similar to *AhSLF*, from *A. majus* based on amino acid sequence homology (Figure S2). It diverged earlier than AhSLF-S_1, S_2, S_4 or S_5 (Figure S3), and shared 95% identity with the AhSLFs at the amino acid level. The expression pattern of *AmSLF-like 1* was determined by RT-PCR and Western blot analyses (data not shown) and was found to be similar to that of *AhSLF* (Lai *et al.*, 2002; Zhou *et al.*, 2003), suggesting that *AmSLF-like 1* is specifically expressed in pollen. However, we were unable to obtain an S-RNase sequence from *A. majus* by a similar approach. Thus, it is unclear whether a functional *S-RNase* gene is present in *A. majus*.

To compare the *S*-locus regions in the SI and SC *Antirrhinum*, the TAC clones from *A. hispanicum* were probed to the chromosomes of *A. majus*. The *S_2RNaseR-TAC* and *S_2SLF-TAC* were selected to probe early pachytene chromosomes of *A. majus*. Surprisingly, although the two TAC clones were located in a similar region of chromosome 8, they had an inverted orientation compared with that on the chromosome of *A. hispanicum* (Figure 4a).

S-locus-linked TAC clones containing *CYC* and *RAD* markers (Schwarz-Sommer *et al.*, 2003; Zhang *et al.*, 2005) were used to determine the inversion boundary. The *CYC-TAC* showed two hybridization signals, one in the *S*-locus region and the other in the long arm of chromosome 8. Based on genetic linkage evidence (Schwarz-Sommer *et al.*, 2003), the short-arm signal most likely contained the *CYC* gene, whereas the long-arm signal probably did not. The

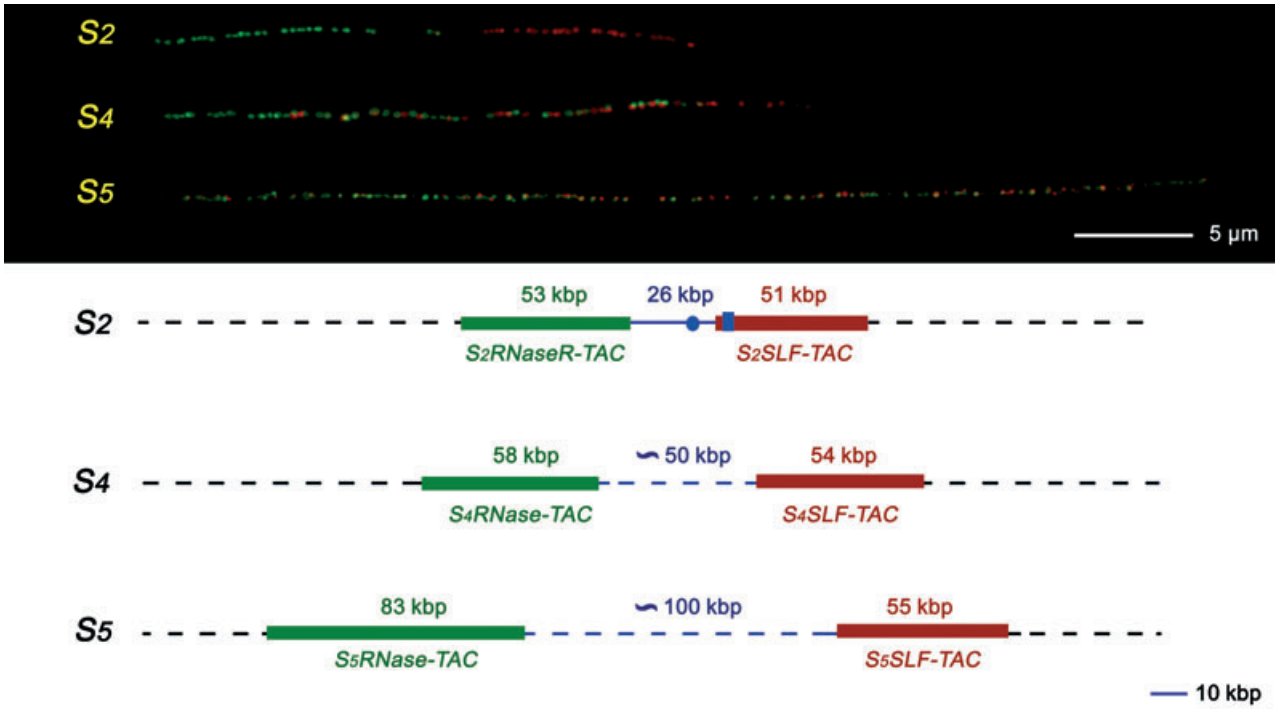

Figure 3. Estimation of the physical distances between *S-RNase* and *SLF* in three *S* haplotypes of *A. hispanicum*.
The top panel shows the representative results for FISH on the extended DNA fibers of the three *S* haplotypes of *A. hispanicum* using TAC probes as follows: (S_2) S_2*RNaseR-TAC* (green) and S_2*SLF-TAC* (red); (S_4) S_4*RNase-TAC* (green) and S_4*SLF-TAC* (red); (S_5) S_5*RNase-TAC* (green) and S_5*SLF-TAC* (red). The bottom panel shows a schematic representation of the physical distances between the TAC clones in three *S* haplotypes. The S_2*-RNase* (blue circle, not present in S_2*RNaseR-TAC*) and *AhSLF-*S_2 (blue rectangle, present in S_2*SLF-TAC*) are shown. The 26 kbp distance between S_2*RNaseR-TAC* and S_2*SLF-TAC* was used as a control distance. The estimated maximum physical distances between S_4*RNase-TAC* and S_4*SLF-TAC* and between S_5*RNase-TAC* and S_5*SLF-TAC* were deduced from the fiber FISH result.

S_2*RNase-TAC* located between the S_2*RNaseR-TAC* and S_2*SLF-TAC*, containing S_2*-RNase*, was used to designate the *S* locus. The order of the three signals on the chromosomes of *A. majus* (Figure 4b,c) was different from that of *A. hispanicum* (Figure 4d,e). The fact that the fully sequenced *S*-locus TAC clones did not contain *CYC* and *RAD* (Zhou *et al.*, 2003) demonstrated that *CYC-TAC* and *RAD-TAC* were outside the *S* locus. The physical distance between the *CYC-TAC* and *RAD-TAC* was 0.41 ± 0.04% of the total length of chromosome 8 in *A. majus*, and 2.4 ± 0.2% in *A. hispanicum*, based on measurement of three different chromosome samples. These results show that the chromosome fragments containing *RAD* and the *S* locus are inverted in relation to each other in *A. majus* and *A. hispanicum* (Figure 4f).

The S *locus is localized to a highly heterochromatic region*

When pachytene chromosomes were stained with DAPI, the brightly stained regions correspond to the heterochromatic domains, and were highly consistent among different cells (Zhang *et al.*, 2005). The DAPI staining pattern at pachytene stage showed that there were five heterochromatic domains on the short arm of chromosome 8 (Zhang *et al.*, 2005). If numbered from first to fifth from the short-arm end, the *S*-locus signal was located in the 2nd heterochromatic domain (Figure 5a,b). This was apparent in the relatively lightly stained distal domains where the *S*-locus region could be easily identified on the DAPI-stained chromosomes even without the *S*-locus FISH markers.

On the more stretched leptotene chromosomes, the *S*-locus-derived TACs were found to occupy the borderline of the heterochromatin and euchromatin of both S_2S_4 and S_2S_5 chromosomes (Figure 5c,d), indicating that the *S* locus is located in a less condensed region of the second heterochromatic domain.

As the *S* locus is located in a highly heterochromatic region in the different *Antirrhinum* species, the DNA elements might be in a highly methylated state around this region. To investigate this, we selected several repetitive DNA elements from the *S*-locus region and further checked their methylation status. Transposon *Tam3-like* and retrotransposon *copia-like* were identified in the *S* locus by comparing the *S*-locus DNA sequence with that in the NCBI database (Zhou *et al.*, 2003). We examined the methylation status of these transposable elements (TEs) to investigate epigenetic modification in the *S* locus. *Tam3-like* and *copia-like* were used to probe genomic DNA digested by *Hap*II/

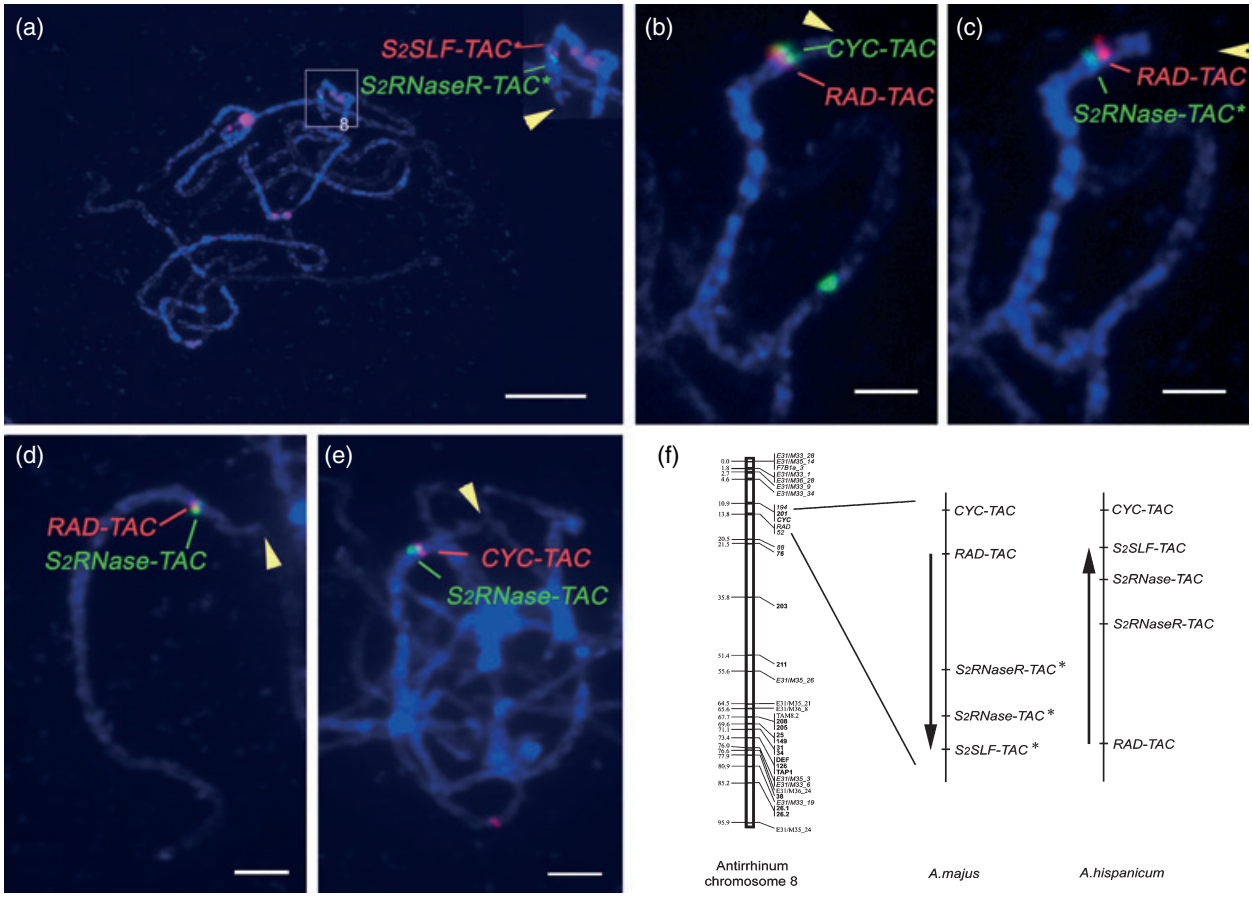

Figure 4. The *S*-locus region is inverted in *A. majus* relative to *A. hispanicum*.
(a) In *A. majus*, digoxigenin-labeled *S_2RNaseR-TAC* (green signal) and biotin-labeled *S_2SLF-TAC* (red signal) probes localize to a similar *S*-locus region containing *S_2RNaseR-TAC* and *S_2SLF-TAC* in *A. hispanicum*, with *S_2SLF-TAC* farther from the telomere.
(b) TACs containing *CYC* and *RAD* were used to determine the inversion boundary. Biotin-labeled *RAD-TAC* (red signal) and digoxigenin-labeled *CYC-TAC* (green signal) were used for *A. majus*. *CYC-TAC* showed two hybridization signals, but only the one at the *S*-locus region represents *CYC*, and is located closer to the telomere.
(c) Digoxigenin-labeled *S_2RNase-TAC*, which is located between *S_2RNaseR-TAC* and *S_2SLF-TAC*, and contains *SRNase-S_2*, was used to identify the *S*-locus region (green signal). In *A. majus*, biotin-labeled *RAD-TAC* (red signal) localized closer to the telomere.
(d) In *A. hispanicum*, digoxigenin-labeled *S_2RNase-TAC* (green signal) localized closer to the telomere than biotin-labeled *RAD-TAC* (red signal).
(e) *CYC-TAC* was biotin-labeled (red signal) for *A. hispanicum*, and localized closer to the telomere than digoxigenin-labeled *S_2RNase-TAC* (green signal).
(f) Schematic representation of the TAC clones around the *S*-locus region in SI *A. hispanicum* and SC *A. majus*. The arrows indicate the orientation and extension of the inverted fragments.
*These TACs isolated from SI *Antirrhinum* hybridized to the chromosomes of SC *A. majus*. The arrangements of the three signals on the chromosomes of SC (b, c) and SI (d, e) *Antirrhinum* indicate that the chromosome fragments containing *RAD* and the *S* locus were inverted relative to each other. The yellow arrowheads indicate the end of the short arm of chromosome 8. Scale bar = 5 μm in (a), and 1 μm in (b)–(e).

*Msp*I (isoschizomers with different DNA methylation sensitivity). Most of the *Tam3-like* and *copia-like* TE copies were heavily methylated (Supplementary Figure S4a), consistent with the usual features of TE (Lippman *et al.*, 2004).

To examine the methylation status of coding genes in the *S* locus, we used *AhSLF* as a representative. As all the 5′ UTR regions of *AhSLF* genes share the same restriction sites, the 5′ UTR region of *AhSLF-S_2* was chosen to probe genomic DNA digested by *Hap*II/*Msp*I and *Bst*NI/*Psp*GI (Supplementary Figure S4b). The size of resultant bands showed that all the restriction sites in the *AhSLF* sequence were fully digested, suggesting that *AhSLF* lacks DNA methylation. We also tested other *S*-locus genes, the S-RNase genes, but the probe used appeared to contain unknown repetitive sequences that generated smeared Southern hybridization signals that could not be analyzed (data not shown).

To examine whether small RNA is involved in epigenetic modification of the *S* locus, Northern blot hybridization was conducted. It showed that the *Tam3-like* and *copia-like* had their corresponding 25 nt small interfering RNAs without tissue specificity (Figure S4c), and these small RNAs were probably involved in regulation of this region.

For *AhSLF-S_2*, no small RNA corresponding to the *AhSLF-S_2* promoter was detected (Figure S4c), and no microRNA or

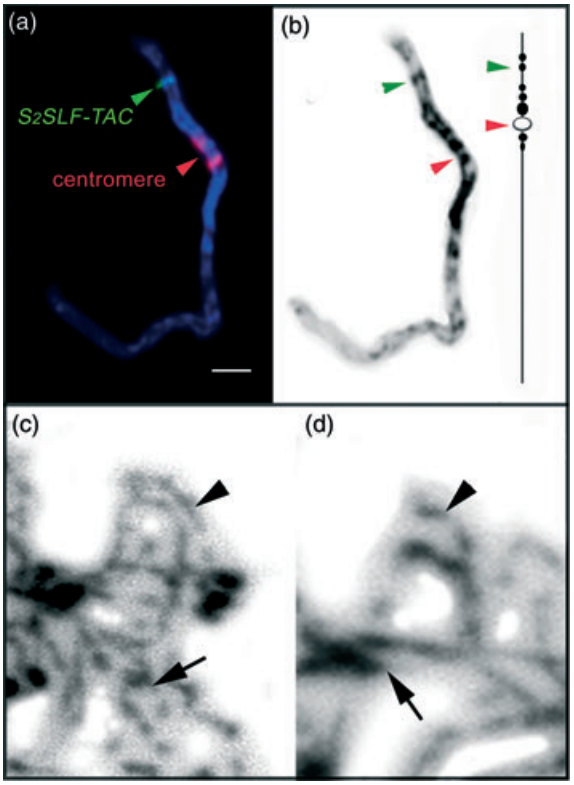

Figure 5. The *S* locus is located in a highly heterochromatic region.
(a) At the pachytene stage, the *S*-locus signal (digoxigenin-labeled *S_2SLF-TAC*, green) was located in the second heterochromatic domain of chromosome 8. The centromere was probed using the centromeric repeat sequence *CentA1* (red signal). Scale bar = 5 μm.
(b) The DAPI-stained chromosome 8 shown in (a) was converted to a black-and-white image to enhance the visibility of the distribution of euchromatin and heterochromatin. An ideogram of the relationship of the *S* locus and DAPI-bright regions is shown on the right.
(c) Black-and-white image of the DAPI staining pattern corresponding to Figure 2(1–6). The arrow and arrowhead indicate the *S*-locus position for the two *S* haplotypes, S_2 and S_4, without discriminating the *S* haplotypes. Both the arrow and arrowhead show the border of the heavily and lightly stained regions.
(d) Black-and-white image of the DAPI staining pattern corresponding to Figure 2(7–12). The arrow and arrowhead indicate the *S*-locus position for the S_2 and S_5 haplotypes respectively. As in S_2S_4 chromosomes, both the arrow and arrowhead show the border of the heavily and lightly stained regions in the S_2S_5 chromosomes. In the S_2, S_4 and S_5 haplotypes analyzed, the *S* locus is located at the border of the heterochromatin and euchromatin of the various leptotene *S* haplotypes.

small interfering RNA could be detected corresponding to the known *S*-locus sequences in small RNA databases such as the NCBI microRNA registry (http://www.sanger.ac.uk/Software/Rfam/mirna) and the siRNA database (http://sirna.cgb.ki.se/). These results indicate that the *S*-locus coding genes are probably not regulated by small RNA.

To investigate whether histone modification is associated with the *S*-locus gene, we performed a ChIP (chromatin immuno-precipitation) experiment with anti-H3 K4me2 antibody using nuclei isolated from leaf tissues (Figure S4d). Mock experiments using pre-immunized rabbit serum served as the non-specific binding control. Precipitated DNA was analyzed by semi-quantitative PCR. ChIP-PCR analyses of *Actin* and *Tam3-like* sequences were included as positive and negative controls, respectively. Primers for the promoter region of *AhSLF*, S_2*-RNase* and S_4*-RNase* were designed to produce fragments of 332, 179 and 332 bp, respectively. It was observed that the promoters of these genes had an H3 K4me2 positive modification. Because the H3 K4me2 modification is generally associated with euchromatin (Sims *et al.*, 2003), this suggests that the chromatin regions of these genes retain euchromatic status in the heterochromatic region in the leaves despite being pollen- or stylar-specific genes.

Discussion

The S*-loci are not always located in the centromeric region among different S-RNase-based SI families*

Our results have shown that the *S* locus of *A. hispanicum* is located outside the centromeric region on the short arm of chromosome 8 despite the fact that *S* loci are believed to lie in the centromeric region in several other S-RNase-based SI species (Bernacchi and Tanksley, 1997; Bernatzky, 1993; Brewbaker and Natarajan, 1960; Entani *et al.*, 1999; Golz *et al.*, 2001; ten Hoopen *et al.*, 1998; Pandy, 1965). The *S* locus of *P. hybrida* was confirmed to be located in the centromeric region using pachytene chromosome FISH (Entani *et al.*, 1999; ten Hoopen *et al.*, 1998). These findings suggest that the relative positions of the *S* locus and the centromere differ between the Solanaceae and Scrophulariaceae, although both possess the S-RNase-based SI system. Moreover, the relative positions of the pollen and stylar *S* components and the centromere appear to be different between *N. alata* and *A. hispanicum,* but similar between *N. alata* and self-compatible *A. majus*, despite its self-compatibility. In *P. hybrida*, the relative positions of the PhS_3*-RNase* and PhS_3*-SLF* were not resolved. In *N. alata*, the pollen *S* component was postulated to be close to the centromere based on frequent associations of the pollen-part mutations (PPMs) and centromeric fragments (Golz *et al.*, 2001), which appears to be quite common for several other S-RNase-based self-incompatible species (Brewbaker and Natarajan, 1960).

The *S* locus of *N. alata* and *P. hybrida* resides in the centromeric region (Entani *et al.*, 1999; Golz *et al.*, 2001; ten Hoopen *et al.*, 1998), indicating that it is prone to breaking and duplicating with the centromere to produce a centromeric fragment, in which the functional centromere guarantees the stable inheritance of the chromosome fragment. The PPMs of these species were mostly generated by duplication of a small centromeric fragment containing the pollen *S* gene (Brewbaker and Natarajan, 1960; Golz *et al.*,

1999, 2001). In addition, the relatively short distance between the *S* locus and the centromere in *Antirrhinum* suggests that it would also be possible to generate PPM by an additional centromeric fragment in this species. Consistently, we have found a trisomic PPM in *A. hispanicum* (unpublished data). Thus, it is likely that the frequent generation of centromeric fragments in the PPMs is based on close linkage of the pollen *S* gene and the centromere.

Rosaceae apparently have the same SI mechanism as members of the Solanaceae and Scrophulariaceae (Kao and Tsukamoto, 2004). However, recently, some differences have been noted (Sonneveld *et al.*, 2005; Ushijima *et al.*, 2004; Yamane *et al.*, 2003). Although the precise chromosomal localization and chromatin structure of the *S* locus is not clear in the Rosacecae, it is rich in repetitive sequences (Ushijima *et al.*, 2003) and was recently reported to be of simple genomic structure and suggested to reside outside the centromeric region (Ushijima *et al.*, 2001). As no PPM containing the centromeric fragment has ever been described in the Rosaceae, its *S* locus might be located distant from the centromere, and not prone to being duplicated on a stably inherited centromeric fragment. Thus, it could be deduced that the chromosomal localization of the *S* locus appears to be different in the Solanaceae, Scrophulariaceae and Rosaceae, even though they share similar S-RNase-based SI systems.

Interestingly, the chromosome regions containing the *S* locus are inverted between *A. majus* and *A. hispanicum*. This inversion is seen for all three *S* haplotypes of *A. hispanicum* studied here. In general, SI is thought to be an ancestral state (Igic *et al.*, 2003). Although the genus *Antirrhinum* contains both SI and SC species, it is not clear how the SC type evolved from the SI type. Nevertheless, several scenarios including deletion/point mutations in the *S* gene and mutations outside the *S* gene (modifiers) have been described to account for the origin of SC in SI species (Hancock *et al.*, 2005; Huang *et al.*, 1994; Kao and Tsukamoto, 2004; Kondo *et al.*, 2002; Kowyama *et al.*, 1994; Royo *et al.*, 1994). It is not clear whether the inversion could be associated with self-compatibility of *A. majus*. Further examination of more SC and SI species in *Antirrhinum* would help to clarify this issue.

The S *locus is located in a highly heterochromatic region in* Antirrhinum

Our findings have revealed that the *S* locus of *Antirrhinum* is located in a pronounced heterochromatic region. The heterochromatic localization of the *Antirrhinum S* locus is consistent with the fact that it contains abundant repetitive sequences, a feature shared with other S-RNase-based self-incompatible species in the Solanaceae (Coleman and Kao, 1992; Entani *et al.*, 1999; Matton *et al.*, 1995). In *Antirrhinum*, many retro-elements or transposons were identified in the sequenced *S*-locus-derived TACs, and account for most of the predicted genes of the *S* locus (Zhou *et al.*, 2003).

The possible centromeric localization and the presence of repetitive sequences of the solanaceous *S* locus suggest that it probably resides in heterochromatin. Although not localized to the centromeric region, the *S* locus of *Antirrhinum* has a similar position in a condensed chromatin environment. The *S* loci of other SI systems also contain various transposable elements, such as those found in the Brassicaceae and Papaveraceae (Cui *et al.*, 1999; Wheeler *et al.*, 2003), implying that they are also likely to be heterochromatic. Localization in a heterochromatic environment of low recombination is common to the *S* locus of most, if not all, SI species, providing a genetic mechanism for recombination suppression.

Haplotype polymorphisms of the S*-locus*

The differences in length and gene arrangements between *S* haplotypes in *Antirrhinum* are still unclear. In estimating the sizes of *S* haplotypes, we found that, although tightly genetically linked, the physical distances between the *S-RNase* and *AhSLF* of respective *S* haplotypes vary widely. The distance between the *Antirrhinum* S_2*-RNase* and *AhSLF-*S_2 is only approximately 9 kbp, with only one transposon-like sequence predicted in this region (Lai *et al.*, 2002). By contrast, several large insertions were predicted to have occurred between the *S-RNase* and *AhSLF* in the S_4 and S_5 haplotypes compared with the S_2 haplotype (Zhou *et al.*, 2003), and the gaps between the *S-RNase* and *AhSLF* for these two haplotypes are much longer and estimated to be approximately 50 and 100 kbp, respectively (Figure 3). In addition, these regions are not as simple as that between the S_2*-RNase* and *AhSLF-*S_2, and are rich in retroelements and transposons, as revealed by both fiber FISH analyses in this study and DNA sequence analyses by Zhou *et al.* (2003). The length of this region of the S_5 haplotype was almost double that of the S_4 haplotype, and was rich in repetitive sequences as deduced from the more mixed signals beyond the TACs in our fiber FISH analyses. Consistent with this, the leptotene chromosome FISH result clearly displayed distinct hybridization signals for the different haplotypes, indicating different sequence compositions (Figure 2). The regions between the *S-RNase* and *AhSLF* genes have not been fully sequenced in the S_4 and S_5 haplotypes, so the exact repetitive element organization in these regions is still unknown. Although it was noted that the density of repetitive elements is low in the 40 kbp region containing PhS_3*-RNase* compared with other regions of the *S* locus in *P. hybrida* (our unpublished data), it is not clear whether the distribution of repetitive elements is unique to *P. hybrida*. However, owing to the duplications, insertions and deletions (Zhou *et al.*, 2003), repetitive element compositions and arrangements appear not to be uniform among different *S* haplotypes, despite their similarities in *Antirrhinum*. This feature appears to be

present widely in other SI species (Cui *et al.*, 1999; Suzuki *et al.*, 1999; Ushijima *et al.*, 1998; Wheeler *et al.*, 2003).

Haplotype polymorphisms of the *S* locus have been identified previously in *Brassica* (Cui *et al.*, 1999; Shiba *et al.*, 2003) and *Prunus* (Entani *et al.*, 2003; Ushijima *et al.*, 2001, 2003). Despite a rather smaller and simpler *S*-locus region, the Rosaceae displayed a similar variation in *S*-haplotype genomic structure. The rosaceous *S*-locus region not only showed *S*-haplotype sequence diversity, but also varied greatly in the extent of its *S*-locus region between different *S* haplotypes (Entani *et al.*, 2003; Ushijima *et al.*, 2001, 2003). Aside from the species possessing GSI systems, the *S* locus of *Brassica*, which encodes a sporophytic SI (SSI) system, also displayed *S*-haplotype polymorphisms (Cui *et al.*, 1999; Shiba *et al.*, 2003). Taken together, genomic haplotype polymorphisms are characteristic for the *S* loci.

Possible mechanisms for maintaining S*-haplotype structural diversity*

The *S* loci known in eukaryotic organisms might share some similar mechanisms to maintain their haplotype structures during evolution. It has been thoroughly investigated that large genomic structural differences, including repetitive sequences, haplotype-specific intergenic sequences and gene arrangements between homologous chromosomes, may contribute to the recombination suppression that is intrinsic to recognition loci (May and Matzke, 1995; O'hUigin, 1995; Nasrallah, 2002).

The well-studied plant disease resistance (*R*) locus shares several features with *S* loci, such as clustered duplicated genes and repetitive sequences (Meyers *et al.*, 1998; Wei *et al.*, 2002). The *Mi* gene in tomato is localized at the border of the heterochromatin (Zhong *et al.*, 1999). Furthermore, the mating-type locus in yeast is heterochromatic (reviewed by Haber, 1998), and the major histocompatibility complex (MHC) locus in mammals is probably heterochromatic, with repetitive sequences and low recombination levels (Singer *et al.*, 1983; Walsh *et al.*, 2003), although no direct evidence exists as yet. The large heterochromatic region could block recombination structurally within the centromere, despite the coding regions that are present within it (Saffery *et al.*, 2003; Yan *et al.*, 2005).

In conclusion, sequence polymorphisms and the highly condensed and extensive heterochromatic region are always associated with the *S* locus in *Antirrhinum*. These features appear to be shared by the *S* loci of other SI systems that possess two *S* determinants, and perhaps are common to recognition loci in yeast, animal and plant organisms. They could also contribute to the regional recombination suppression, retention of point mutations and accumulation of retro-element insertions that are required for maintaining recognition specificity while promoting diversification.

Experimental procedures

Plant materials

A. majus (stock 75), *A. hispanicum* and *P. hybrida* plants were grown in a greenhouse environment as previously described (Lai *et al.*, 2002; Robbins *et al.*, 2000; Xue *et al.*, 1996). Young buds were collected for meiotic chromosome preparation. Leaf tissue was harvested for nuclei preparation, genomic DNA and small RNA isolation, and flower tissue was also used for small RNA isolation.

Screening of TAC library

The TAC libraries from *A. hispanicum* and *P. hybrida* have been described previously (Qiao *et al.*, 2004a; Zhou *et al.*, 2003). For the S_1S_5 library, clones of 384-well plates were imprinted onto a 15 cm plate using a VP384 pin replicator (V&P Scientific, http://www.vp-scientific.com) and inoculated onto LB agar medium containing kanamycin (25 mg l^{-1}). After incubation at 37°C overnight, bacteria 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 primers specific for marker genes. When a specific PCR product was detected in one or more pools, the ten 384-well plates of the positive pool were individually re-screened with the primer pair, and positive 384-well plates were identified. Finally, the positive clone was identified by PCR screening in a row and column combination.

Chromosome preparation

Immature *Antirrhinum* flower buds (length 1.5–3.0 mm) were harvested and fixed in Carnoy's solution (ethanol/glacial acetic acid 3:1). Microsporocytes at meiosis stage were squashed in an aceto-carmine solution according to the method described by Wu (1967). Slides were frozen in liquid nitrogen. After coverslip removal, slides were dehydrated through an ethanol series (70%, 90%, and 100%) prior to use in FISH.

Chromosome fluorescence in situ hybridization

Chromosome FISH and fiber FISH were performed according to published protocols (Jackson *et al.*, 1998; Jiang *et al.*, 1995). BAC/TAC DNA was isolated using a standard alkaline extraction procedure (Sambrook *et al.*, 1989) and labeled with either biotin-11-dUTP or digoxigenin-16-dUTP (Roche, http://www.roche.com) by nick translation. Chromosomes were counterstained with 4′,6-diamidino-phenylindole (DAPI) in an anti-fade solution (Vector Laboratories, http://www.vectorlabs.com). Chromosomes and FISH signal images were captured with an Olympus BX61 fluorescence microscope (http://www.olympus-global.com/) coupled to an Apogee KX85 CCD camera. Grey-scale images were captured for each color channel, and then merged using Image-Pro Plus (IPP) software (Media Cybernetics, http://www.mediacy.com). Pachytene chromosome lengths were measured using IPP software.

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Centromeric localization of the *S*-locus of *P. hybrida*.

Figure S2. Amino acid sequence alignment of AmSLF-like1 and AhSLFs from *Antirrhinum*.

Figure S3. A phylogenetic tree of AmSLF-like1 and AhSLFs.

Figure S4. Epigenetic modification of the *S*-locus in *Antirrhinum*. DNA blot hybridization of *Tam3-like* (**a**) and *AhSLF-S_2* (**b**) to genomic DNA of *S_2S_5 A. hispanicum*. Small RNA northern blot of *Tam3-like*, *Copia-like* and *AhSLF-S_2* promoter (**c**). An H3K4me2 modification of the *S*-locus genes (**d**).

This material is available as part of the online article from http://www.blackwell-synergy.com.

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