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The Self-Incompatibility (*S*) Haplotypes of Brassica Contain Highly Divergent and Rearranged Sequences of Ancient Origin

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In Brassica, the recognition of self-related pollen by the stigma is controlled by the highly polymorphic *S* locus that encodes several linked and coadapted genes and can span several hundred kilobases. We used pulsed-field gel electrophoresis to analyze the structure of different *S* haplotypes. We show that the S_2 and S_{13} haplotypes of *Brassica oleracea* contain extensive sequence divergence and rearrangement relative to each other. In contrast, haplotypic configuration is more conserved between *B. oleracea* S_{13} and *B. campestris* S_8, two haplotypes that have been proposed to be derived from a common ancestral haplotype based on sequence comparisons. These results support the view that extensive restructuring of the *S* locus preceded speciation in Brassica. This structural heteromorphism, together with haplotype-specific sequences, may suppress recombination within the *S* locus complex, potentially providing a mechanism for maintaining the linkage of coadapted allelic combinations of genes over time.

INTRODUCTION

Flowering plants have evolved elaborate systems for controlling mating reactions before fertilization. In many plant families, the outcome of intraspecific matings is regulated by a genetic barrier to selfing, termed self-incompatibility. In the crucifer family (the Brassicaceae), self-incompatibility is controlled by the highly polymorphic *S* locus and is manifested during the interaction of pollen grains with the papillar cells of the stigma epidermis. An incompatibility response, in which the germination of pollen and subsequent pollen tube growth are inhibited, occurs when the stigma and interacting pollen grain express a common *S* specificity (Nasrallah and Nasrallah, 1993; Nasrallah et al., 1994b).

Although genetically defined as a single locus, the *S* locus may be viewed as a master recognition locus that encodes the function(s) required for the stigma to distinguish self-related from self-unrelated pollen as well as the molecules borne by pollen that identify the pollen grain as being self or non-self. Indeed, molecular and genetic studies of the Brassica *S* locus, for which >60 allelic specificities have been identified, have indicated that the *S* locus can span several hundred kilobases of DNA (Boyes and Nasrallah, 1993) and that more than one *S* locus–encoded function is required for self-incompatibility (J.B. Nasrallah et al., 1987, 1994a; Stein et al., 1991, 1996; M.E. Nasrallah et al., 1992; Goring et al., 1993). Based on these studies, it is now known that stigmatic papillar cells carry on their surface two classes of *S* locus–encoded receptors required for self-incompatibility—the plasma membrane–anchored signaling receptor encoded by the *SRK* (for *S* receptor kinase) gene and the soluble cell wall–localized product of the *SLG* (for *S* locus glycoprotein) gene, which shares a high degree of sequence similarity with the extracellular domain of SRK. Current models of self-incompatibility in Brassica (Nasrallah et al., 1994b) postulate that pollen carries on its surface a diffusible signal, possibly a ligand for the stigmatic receptors, also encoded by an *S* locus gene. A candidate gene for this pollen function is the recently described haplotype-specific *SLA* (for *S* locus anther) gene (Boyes and Nasrallah, 1995). Upon pollination, this diffusible signal would be delivered to the stigmatic surface, subsequently activate the receptor kinase, and initiate a signaling cascade that leads ultimately to the inhibition of pollen tube development.

The above-mentioned model implies that each *S* haplotype ("allele" under classical models) encodes distinct gene products adapted for structural compatibility and that the proper functioning of the self-incompatibility system depends on the maintenance of the coadapted gene complex in a tightly linked genetic unit. Evidence in fact exists for the coevolution of *S* locus genes, at least in the case of *SLG* and *SRK*: sequence analysis has shown that *SLG* and *SRK* genes derived from the same *S* haplotype share >90% amino acid identity, although they exhibit up to 33% sequence divergence from *SLG* or *SRK* genes isolated from different haplotypes (Stein et al., 1991). In addition, recombination events between *SLG* and *SRK* have not been detected, even though the physical distance between the two genes may exceed 200 kb of DNA (Boyes and Nasrallah,

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1993). Because *SLG* and *SRK* polymorphisms are ancient and have been maintained in the population for >20 to 40 million years (Nasrallah and Nasrallah, 1993; Uyenoyama, 1995), mechanisms that allow for the maintenance of sequence similarity between the two genes and prevent the disruption of the gene complex by recombination are assumed to have acted on *S* haplotypes since the generation of the *SLG–SRK* gene pair by a gene duplication event early in the evolution of the *S* locus (Tantikanjana et al., 1993).

To gain further insight into the structure of the *S* locus and the forces that have shaped its evolution, we investigated the configuration of the *Brassica campestris* S_8 haplotype and two *B. oleracea* haplotypes—S_2, which occurs at high frequency in Brassica populations (Chen and Nasrallah, 1990), and S_{13}, which is thought to be derived from the same ancestral haplotype as the *B. campestris* S_8 haplotype based on the high degree of sequence identity exhibited by their *SLG* genes (Dwyer et al., 1991). We also show that different haplotypes within a species exhibit extensive sequence rearrangements that are likely to limit recombination in the region and maintain linkage of the genes encoding the various *S* locus functions. In addition, the results of our interspecific haplotypic comparison support the view that the structural heteromorphism characterizing the present-day *S* haplotypes was established early in the evolution of the *S* locus and before speciation in the Brassica lineage. Our results are discussed in relation to mechanisms underlying the maintenance of specific allelic combinations at complex loci in other organisms.

RESULTS

SLG Flanking Regions Are Highly Divergent in Different *S* Haplotypes

In earlier work, we established that the *S* locus region is highly polymorphic. This polymorphism is apparent not only in *SLG* sequence comparisons (Nasrallah et al., 1987; Stein et al., 1991; Nasrallah and Nasrallah, 1993) but also in the restriction fragment length polymorphisms detected among different *S* haplotypes (Nasrallah et al., 1988; Boyes and Nasrallah, 1993). To explore this polymorphism in greater detail, we compared λ genomic clones containing the *SLG* gene and flanking sequences that were isolated from the S_2 and S_{13} haplotypes of *B. oleracea* and the S_8 haplotype of *B. campestris*. The results of this comparison are shown in Figure 1 for the region downstream of *SLG*. It is striking that this region contains very few, if any, restriction sites that are

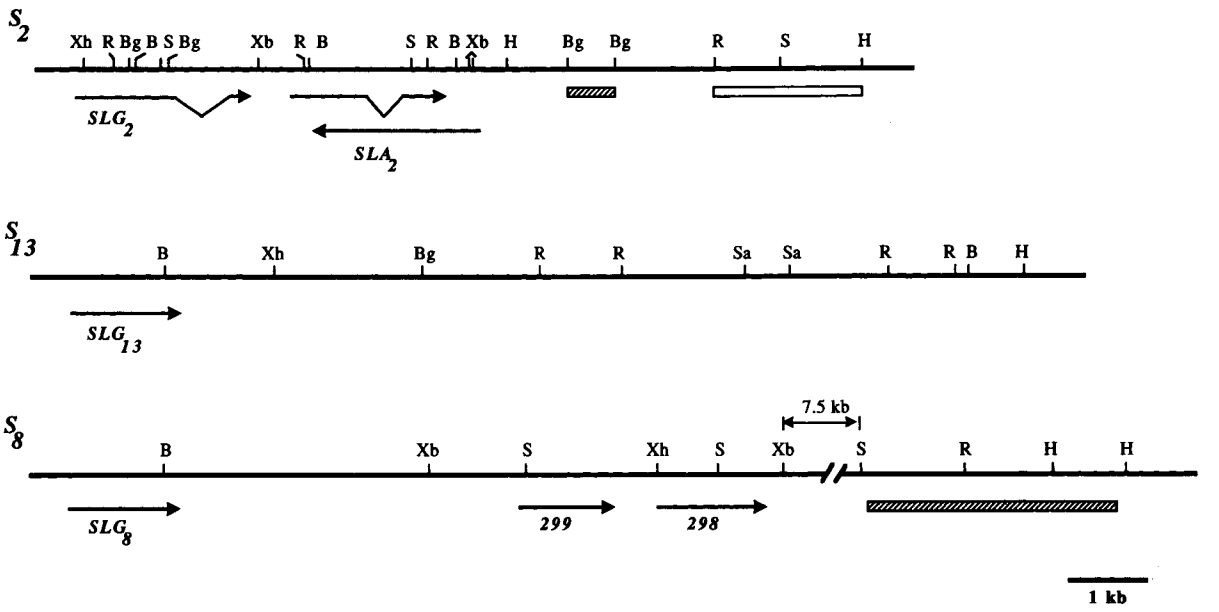

Figure 1. Restriction Maps of the Genomic DNA Downstream of the *SLG* Gene in the S_2 and S_{13} Haplotypes of *B. oleracea* and the S_8 Haplotype of *B. campestris*.

The positions and orientations of known transcriptional units are indicated by arrows beneath each map. The hatched and open boxes below the S_2 map correspond to single-copy probes described in the text. The hatched box below the S_8 map denotes the region that is identified by hybridization with the probe corresponding to the hatched box in the S_2 map. The break in the S_8 map spans 7.5 kb, as indicated. This region did not hybridize with any of the probes described in the text. The scale of the maps is given at lower right. The bent arrows delineating the SLG_2 and SLA_2 genes show the positions of the introns. B, BamHI; Bg, BglII; H, HindIII; R, EcoRI; S, SacI; Sa, SalI; Xb, XbaI; Xh, XhoI.

conserved among the three *S* haplotypes. This finding is consistent with results of earlier studies that failed to identify common restriction fragments in comparative DNA gel blot hybridization analyses using *SLG*-specific sequences as probes (Nasrallah et al., 1988; Boyes and Nasrallah, 1993).

The restriction site polymorphisms observed downstream of *SLG* were found to be correlated with a different complement of sequences within this region in each of the three haplotypes analyzed. This is demonstrated by gel blot analysis of DNA contained in the SLG_8 and SLG_{13} λ clones by using probes derived from the region downstream of *SLG* in the SLG_2 λ clone. Probes covering the entire SLG_2 downstream region shown in Figure 1, starting with the XbaI site immediately 3′ of SLG_2, were first tested by DNA gel blot analysis of Brassica genomic DNA from S_8 and S_{13} homozygotes. As previously reported (Boyes and Nasrallah, 1995), the region containing the SLA_2 sequence (from the XbaI site to the first HindIII site in Figure 1) behaved as a single-copy sequence specific for the S_2 haplotype and did not hybridize with S_8 and S_{13} DNA. The adjoining 4.8-kb HindIII fragment consisted primarily of multicopy DNA that hybridized with equal intensity to DNA from the S_2, S_8, and S_{13} homozygous genotypes. Within this region, the probes indicated by the hatched and open boxes in Figure 1 hybridized with ~10 to 15 fragments and 20 fragments, respectively, in restriction digests of DNA from the three genotypes. The remainder of the HindIII fragment contained DNA that was even more highly repetitive in the three genotypes and was not tested for representation in the SLG_8 and SLG_{13} λ clones.

As shown in Figure 1, the probe indicated by the hatched box, which is located ~4 kb 3′ of SLG_2, cross-hybridized with DNA located ~16 kb downstream of SLG_8. Furthermore, this probe failed to identify related sequences within the 12 kb of *SLG* 3′ sequences contained in the insert of the SLG_{13} λ clone. The probe indicated by the open box in Figure 1, which was derived from a region located ~6 kb downstream of SLG_2, failed to identify related sequences in cloned DNA flanking either the SLG_8 or SLG_{13} genes (Figure 1). Because gel blot analysis of S_8 and S_{13} genomic DNA demonstrated the presence of sequences homologous to this probe (see above), their absence from the SLG_8 or SLG_{13} clones appears to reflect differences in DNA structure and not simply the divergence or absence of sequences related to the two probes in the genome of plants homozygous for the S_8 or S_{13} haplotype.

Structural Diversity in *SLG* Flanking Regions Involves Nonpolymorphic and Vegetatively Expressed Sequences as Well as Haplotype-Specific Sequences

The structural differences observed downstream of *SLG* in the three haplotypes under study involve transcriptionally active sequences, as illustrated for three sequences designated SLA_2, *298*, and *299*. The positions of these sequences are shown in Figure 1. These three sequences were recently identified in our laboratory by transcriptional analysis of DNA flanking *SLG*. The SLA_2 sequence is a novel single-copy haplotype-specific sequence located ~500 bp downstream of SLG_2 (Figure 1). SLA_2 is transcribed from at least two promoters to produce complementary transcripts (shown by the divergent arrows in Figure 1) that are expressed specifically in anthers (Boyes and Nasrallah, 1995). Based on the features of the SLA_2 sequence, we have proposed that it may encode a determinant of self-incompatibility phenotype in pollen. However, we have been unable to detect SLA_2-homologous sequences by DNA gel blot hybridization in *S* haplotypes other than S_2.

In this study, we attempted to identify functional homologs of SLA_2 from the S_8 and S_{13} haplotypes by using a positional cloning strategy. We reasoned that sequences exhibiting haplotype specificity and anther-specific expression would represent candidate SLA_2 homologs. Sequences ~500 to 1000 bp downstream of *SLG* in the S_8 and S_{13} haplotypes were subcloned and used as probes against blots of anther RNA extracted from plants homozygous for the corresponding *S* haplotype. This procedure failed to identify any transcribed sequences in the interval tested (data not shown), indicating that if *SLA* functional homologs are encoded within the S_8 and S_{13} haplotypes, they occupy a different location than in the S_2 haplotype.

A similar observation was made for the *298* and *299* sequences, which are located ~7 kb downstream of SLG_8 (Figure 1). *298* and *299* occur as single-copy sequences in the Brassica genome, as illustrated in Figures 2A and 2B by restriction digests of DNA isolated from plants homozygous for the S_8, S_2, and S_{13} haplotypes. (*S* homozygotes are produced by forced selfing, as described in Methods.) All of the haplotypes analyzed hybridized with equal intensity to the *298* and *299* probes, indicating that the two sequences exhibit a high degree of conservation in plants homozygous for different *S* haplotypes. In addition, the *298* and *299* sequences are expressed in a variety of plant tissues, including stigmas, anthers, and leaves (Figure 2C). Interestingly, although located close to *SLG* in the S_8 haplotype, neither sequence was contained in the SLG_2 and SLG_{13} λ clones (Figure 1). Because *298* and *299* were found to exhibit physical linkage to the S_2 and S_{13} haplotypes (see below), we conclude that the absence of the *298* and *299* sequences from DNA immediately downstream of *SLG* in these haplotypes reflects the repositioning of these sequences to different sites within the *S* locus region.

It is evident from this comparison of λ clones that the position of several sequences within the genomic region downstream of *SLG* has not been conserved during the evolution of the three *S* haplotypes under study. To determine whether the observed differences in structure resulted from insertions or deletions of noncoding sequences or from rearrangements such as inversions or intralocus translocations, we extended our analysis to produce long-range physical maps of the three haplotypes. High-molecular-weight DNA isolated from plants homozygous for these haplotypes was subjected to

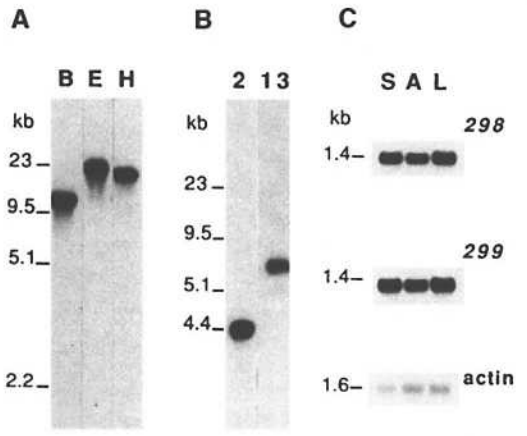

Figure 2. DNA and RNA Gel Blot Analyses of the *298* and *299* Genes.

(A) Restriction digests of DNA isolated from *B. campestris* S_8 homozygous plants were probed with a genomic fragment containing the *298* and *299* genes. Note the single-copy nature of the genes. B, BamHI; E, EcoRI; H, HindIII.

(B) DNA from *B. oleracea* S_2(2) and S_{13}(13) homozygotes was digested with EcoRI and probed as given in **(A)**.

(C) Poly(A)$^+$ RNA isolated from stigmas (S), anthers (A), and leaves (L) was hybridized sequentially with the *298* and *299* cDNA probes. Shown at bottom is the same blot probed with an actin probe as a control for the amount of RNA loaded in each lane.

Molecular length markers are indicated at left in kilobases.

multiple single and double digests with rare-cutting restriction enzymes, separated by pulsed-field gel electrophoresis (PFGE), and hybridized with the single-copy probes indicated in Figure 3 and Table 1. Based on the lengths of the restriction fragments observed, physical maps of the *S* locus region in each haplotype were generated, as shown in Figure 3. (Selected examples of the restriction fragments obtained are provided in Table 1.) Whenever possible, the location of restriction sites determined from PFGE was verified in cloned DNA. The restriction maps determined from analysis of overlapping clones in the corresponding λ contigs are shown aligned with the long-range maps in Figure 3.

SLG and *SRK* Are Separated by <20 kb in the *B. campestris* S_8 Haplotype

Initial PFGE blot analysis of the S_8 haplotype identified an 80-kb MluI fragment that hybridized with both a cDNA clone derived from the *298* sequence and the first intron of the *SRK* gene (Table 1). Because *298* and *SLG* are separated by only 7 kb in the S_8 haplotype (Figure 1), *SLG* and *SRK* in this haplotype are most likely separated by no more than 80 kb. This 80-kb estimate in the *B. campestris* S_8 haplotype, which is substantially lower than our earlier estimates of the distance between *SLG* and *SRK* in *B. oleracea S* haplotypes (Boyes and Nasrallah, 1993), prompted us to initiate a chromosomal walk in an attempt to clone the DNA between the two genes. Because of the presence of multicopy DNA interspersed with single-copy sequences within the region, at each step of the walk, single-copy restriction fragments that could be used to extend the cloned region were identified as described in Methods.

The chromosomal walk resulted in the production of two λ contigs, as shown in Figure 3. One contig was initiated at SRK_8 and extended 31.5 kb downstream to the single-copy sequence designated 40.2-1.7 (Figure 3). The second contig was initiated at SLG_8 and extended 35 kb downstream. Single-copy probes derived from the latter contig included the *298* and *299* sequences described above and the genomic DNA fragments designated SD22, BH2.0, and 6B-1.2 (Figure 3). PFGE analysis using these single-copy fragments allowed the positioning of each contig on the long-range physical map of the S_8 haplotype. The contigs are oriented such that SLG_8 and SRK_8 are transcribed in opposite orientations with their 5′ ends separated by ~15 kb of intervening DNA (Figure 3).

B. oleracea S_2 and S_{13} Haplotypes Contain Regions of Sequence Rearrangement

The long-range restriction maps of the S_2 and S_{13} haplotypes do not contain any conserved rare-cutting restriction enzyme sites (Figure 3). Together with similar results obtained in our previous comparative analysis of the *B. oleracea* S_2 and S_6 haplotypes, in which every digestion with rare-cutting restriction enzymes identified restriction fragment length polymorphisms when hybridized with *SLG* or *SRK* probes (Boyes and Nasrallah, 1993), these data suggest that extensive restriction site polymorphism characterizes the *S* haplotypes of *B. oleracea*. In addition to this restriction site polymorphism, a comparison of the long-range maps of the S_2 and S_{13} haplotypes demonstrates the presence of structural rearrangements within the *S* locus region. For example, the map of the S_2 haplotype positions the *298–299* sequences more closely to *SRK* than to *SLG*, with the minimum distance between *SLG* and *298–299* being ~250 kb (Figure 3). In contrast, in the S_{13} haplotype, *SLG* is positioned between the *298–299* sequences and *SRK*, with the maximum distance between *SLG* and *298–299* being ~55 kb (Figure 3). These results are shown in Table 1 and Figures 4A and 4B: in S_2, *298–299* and *SRK*, but not *SLG*, are located on the same 280-kb MluI fragment; and in S_{13}, *298–299* and *SLG* are contained within the same 120-kb EagI fragment, whereas *SRK* maps to an 80-kb EagI fragment.

Another example of rearrangement in the *S* locus region was provided by a fragment identified during the generation of the S_2 map. This single-copy sequence, designated J1-PX in Figure 3, was used to orient the genomic clone containing SLG_2 on the long-range restriction map of the S_2 haplotype (Figure 3). Surprisingly, this sequence was found to be genetically unlinked to the *S* locus in an F_2 population

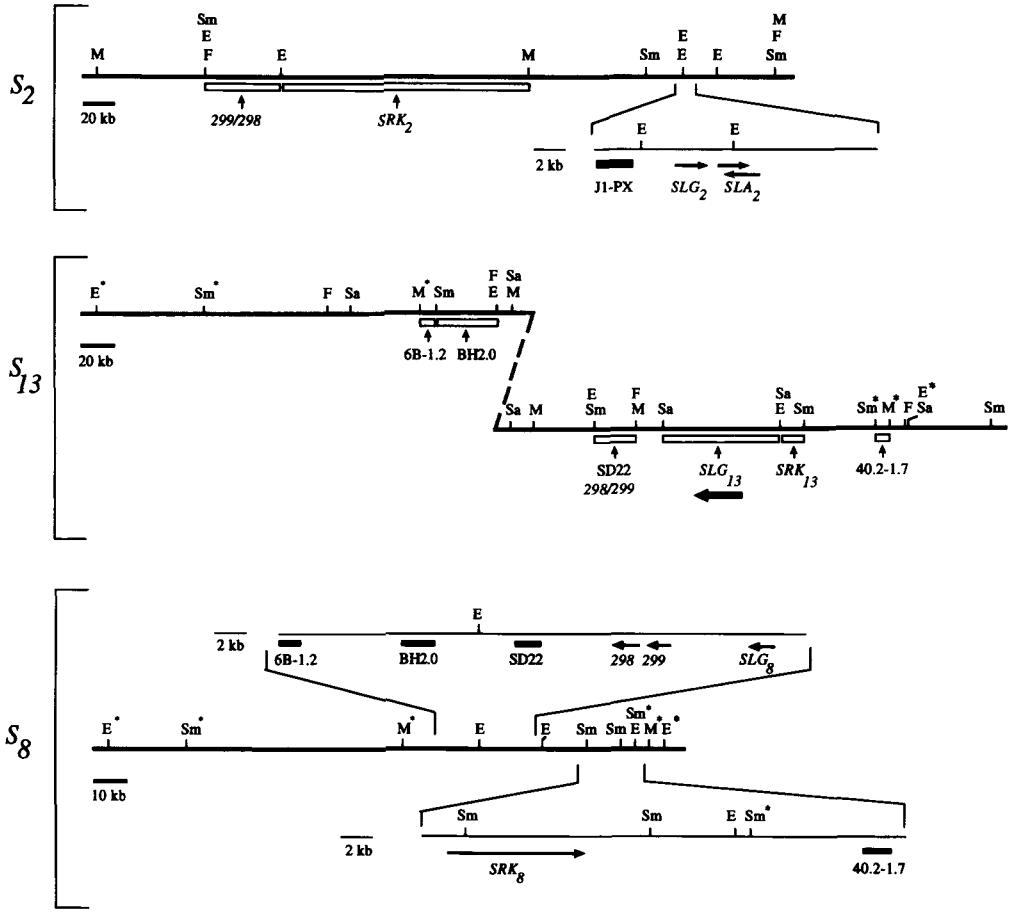

Figure 3. Long-Range Restriction Maps of the S_2 and S_{13} Haplotypes of *B. oleracea* and the S_8 Haplotype of *B. campestris*.

Thick lines represent the restriction maps derived by PFGE blot hybridization analysis. Note the 50% difference in scale in the S_8 map relative to the S_2 and S_{13} maps. The physical linkage of the two sections of the S_{13} map and the orientation of the SLG_{13} gene (bold arrow) were deduced on the basis of analysis of the deleted S_{13} haplotype in ΔS–1668 (see Results). The orientation of the 6B-1.2– and BH2.0-containing portion of the map relative to *SLG* has not been determined; however, similarity with the S_8 map suggests that they are likely to be oriented as shown. Restriction sites marked with asterisks appear to be conserved between S_8 and S_{13}. Open boxes below the maps denote intervals to which the indicated sequences map on the basis of PFGE analysis. Filled boxes show the position of the indicated probes on λ contigs. The scale of each long-range map is indicated at left in kilobases. The expanded regions of the long-range maps depicted by thin lines represent cloned DNA (scales indicated at left). The positions and orientations of known transcriptional units are indicated by arrows under the maps. E, EagI; F, FspI; M, MluI; Sa, SalI; Sm, SmaI.

segregating for the S_{13} and S_6 haplotypes, and it was not detected in total genomic DNA isolated from *B. campestris* plants homozygous for the S_8 haplotype (data not shown).

A comparison of the S_2 and S_{13} haplotypes revealed stretches of structurally conserved DNA, however. For example, in both haplotypes, as well as in a total of six *S* haplotypes analyzed to date (data not shown), the *298, 299*, and SD22 sequences occur in a cluster. In the S_{13} haplotype, all three sequences are contained within the same PFGE restriction fragments (Figure 3 and Table 1). Clustering of these sequences was also found in the S_2 haplotype: although the SD22 probe was not used in the PFGE analysis of this haplotype (and is thus not shown on the S_2 haplotypic map in Figure 3), analysis of a λ contig spanning this region (data not shown) has indicated that in S_2, the *298* and *299* sequences are separated by <1 kb of DNA, with *299* being positioned 5′ of *298* and SD22 being positioned 3′ of *298*.

B. oleracea S_{13} and *B. campestris* S_8 Haplotypes Are Similar in Structure

Several observations suggest that the S_8 and S_{13} haplotypes are closely related and are probably derived from a common

Table 1. Representative Sample of Restriction Fragment Lengths in Kilobases Identified in the S_2, S_8, and S_{13} Haplotypes by PFGE Blot Hybridization Analysis

Restriction Enzyme	*SLG*			*SRK*			*298*			SD22		6B-1.2		40.2-1.7	
	S_2	S_{13}	S_8	S_2	S_{13}	S_8	S_2	S_{13}	S_8	S_{13}	S_8	S_{13}	S_8	S_{13}	S_8
EagI	6	120	19	260	80	21	50	120	19	120	19	260	150	80	15
FspI	340	180	ND[a]	340	180	ND[a]	340	5	ND[a]	5	ND[a]	100	ND[a]	180	ND[a]
MluI	150	175	80	280	175	80	280	65	80	65	80	65	80	175	80
SalI	ND[b]	35	ND[b]	ND[b]	12	ND[b]	ND[b]	100	ND[b]	100	ND[b]	100	ND[b]	80	ND[b]
SmaI	90	140	130	280	140	8	280	140	130	140	130	150	130	75	130

[a] ND, the position of FspI sites in the S_8 haplotype was not determined.
[b] ND, the position of SalI sites in the S_2 and S_8 haplotypes was not determined.

ancestral *S* haplotype. Although isolated from different *Brassica* species, the SLG_8 and SLG_{13} DNA sequences were found to share the relatively high level of 92% nucleotide sequence identity (Dwyer et al., 1991). In addition, gene-specific fragments derived from the first intron of SRK_8 hybridize with the SRK_{13} gene under high-stringency hybridization conditions, even though *SRK* first-intron sequences usually serve as haplotype-specific probes under the same hybridization conditions.

To investigate further the relatedness of the two haplotypes, we performed a detailed PFGE blot analysis of the S_{13} haplotype with the single-copy probes used to position the contigs on the long-range physical map of the S_8 haplotype. As shown in Figure 3, we identified a number of similarities between the long-range physical maps of the S_8 and S_{13} haplotypes. Although the contigs from the S_8 haplotype span ~75 kb and the corresponding regions in the S_{13} haplotype span at least 200 kb, the order of several restriction enzyme sites (indicated by asterisks in Figure 3) was found to be conserved between the two haplotypes. The overall arrangement of sequences or blocks of sequences is also conserved, although the physical distances between these conserved blocks are expanded in S_{13} relative to S_8. For example, S_{13} sequences identified by the *298*, *299*, and SD22 probes are contained within the same 5-kb FspI fragment (Table 1) and are therefore closely associated, as they are in the S_8 haplotype. However, the region of *298*, *299*, and SD22 homology is at least 90 kb away from *SRK* in the S_{13} haplotype, whereas these sequences are within 30 kb of each other in the S_8 haplotype (Figure 3).

A similar situation was observed for the BH2.0 and 6B-1.2 sequences. In the S_8 haplotype, these sequences are located within 9 kb of each other and within 8 kb of SD22. In the S_{13} haplotype, BH2.0 and 6B-1.2 are positioned on adjacent restriction fragments and are therefore likely to be closely associated as well. However, they must be separated from SD22 sequences by an appreciable distance, because none of the restriction enzymes used in the PFGE analysis allowed us to establish the physical linkage of these two sequences to SD22 or any other sequences from the region (Figure 3).

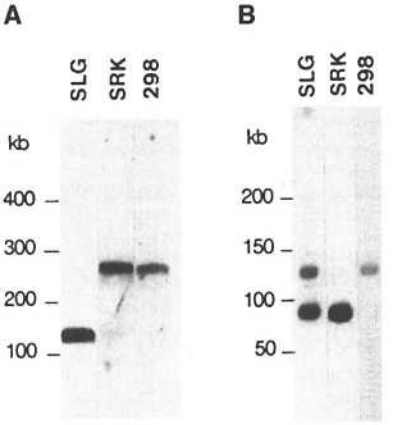

Figure 4. PFGE Analysis of the S_2 and S_{13} Haplotypes of *B. oleracea* Showing the Occurrence of Sequence Rearrangement at the *S* Locus.

(A) DNA from S_2 homozygous plants was digested with MluI and hybridized sequentially with a probe derived from a region 3′ of the SLG_2 coding region (SLG), a probe derived from the first intron of SRK_2 (SRK), and a *298* cDNA probe (298).

(B) DNA from S_{13} homozygous plants was digested with EagI and probed with an *SLG* cDNA that hybridizes with the SLG_{13} and SRK_{13} genes (SLG), with a gene-specific probe derived from the first intron of SRK_8 (SRK), and with the *298* cDNA probe (298).

Molecular length markers are indicated at left in kilobases.

A Deleted Haplotype Allows Refinement of S_{13} Haplotypic Map

PFGE analysis pointed to similarities between the S_{13} and S_8 haplotypes; however, because of the limited availability of restriction sites, this method did not allow us to determine the positioning of BH2.0 and 6B-1.2 as well as the orientation of SLG_{13} relative to SRK_{13}. Analysis of a deleted S_{13} haplotype resolved these questions and at the same time supported the relatedness of the S_{13} and S_8 haplotypes. The

deleted S_{13} haplotype was recently identified in a screen of F_1 plants derived from a cross between a mutagenized $S_{13}S_{13}$ plant and an $S_{f1}S_{f1}$ plant (Nasrallah et al., 1994a). In this screen, an F_1 plant (designated ΔS–1668) carrying this deleted haplotype was identified on the basis that it did not produce SLG_{13} protein (M.E. Nasrallah, M.K. Kandasamy, and J.B. Nasrallah, manuscript in preparation).

Figure 5 shows the results of the gel blot analysis of DNA isolated from the S_{13} and S_{f1} homozygous parents, an F_1 $S_{13}S_{f1}$ plant carrying the wild-type S_{13} haplotype, and the ΔS–1668 plant carrying the deleted S_{13} haplotype. Hybridization with an *SLG* cDNA probe revealed aberrant restriction patterns associated with the mutant S_{13} haplotype in ΔS–1668. In HindIII digests of DNA from S_{13} homozygotes, the SLG_{13} and SRK_{13} genes are contained on 21.5- and 6.5-kb restriction fragments, respectively (Figure 5). F_1 plants carrying the wild-type S_{13} haplotype had these two restriction fragments as well as the 2.2- and 2.7-kb fragments contributed by the $S_{f1}S_{f1}$ parent. In the ΔS–1668 plant, the 6.5-kb SRK_{13} fragment was maintained, as were the S_{f1}-derived restriction fragments; however, the 21.5-kb SLG_{13} fragment was replaced by a novel 3.7-kb fragment, suggesting that part of the SLG_{13}-containing restriction fragment had been removed by a deletion in the mutant haplotype.

To investigate further the structure of the mutant haplotype, we performed additional DNA gel blot studies with a probe derived from the 5′ region of *SLG* containing the first 200 bp of the *SLG* protein coding region and with a probe derived from the 3′ untranslated region of SLG_{13}. We found that the novel 3.7-kb SLG_{13}-containing fragment in ΔS–1668 hybridized with the SLG_{13} 5′ probe but not with the SLG_{13} 3′ probe (Figure 5). In addition, when hybridized with the 6B-1.2 and *299* probes (Figure 5) as well as with the *298*, SD22, and BH2.0 probes (data not shown), DNA from the ΔS–1668 plant

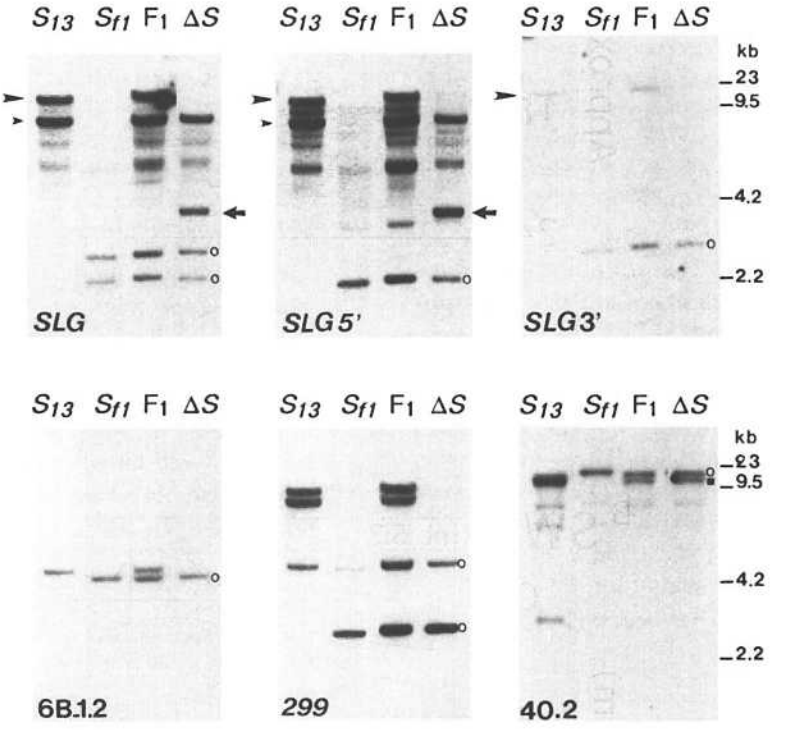

Figure 5. Structural Analysis of the Deleted S_{13} Haplotype in ΔS–1668.

DNA from an S_{13} homozygote, an S_{f1} homozygote, a control unmutagenized F_1 plant, and the ΔS–1668 F_1 plant (ΔS) carrying a deleted S_{13} haplotype was hybridized with an *SLG* cDNA probe (*SLG*), a probe derived from the 5′ region of the SLG_{13} gene (*SLG* 5′), a probe corresponding to the 3′ untranslated region of the SLG_{13} gene (*SLG* 3′), the 6B-1.2 probe, the *299* probe, and the 40.2-1.7 (40.2) probe. The hybridization patterns obtained with HindIII-digested DNA are shown for all gels, with the exception of the one at lower right. Here, BamHI-digested DNA was used because the 40.2-1.7 probe did not identify restriction fragment length polymorphisms between HindIII digests of S_{13} and S_{f1}. The large and small arrowheads indicate, respectively, the SLG_{13}-containing and SRK_{13}-containing restriction fragments derived from the S_{13} haplotype. The arrow indicates the novel 3.7-kb *SLG*-containing restriction fragment observed in ΔS–1668. The open circles denote the S_{f1}-derived restriction fragments in ΔS–1668. The filled square for the 40.2 gel denotes the S_{13}-derived restriction fragment in ΔS–1668. Molecular length markers are shown at right in kilobases.

was found to exhibit a pattern identical to that of the $S_{f1}S_{f1}$ parent and therefore lacked the S_{13}-derived restriction fragments. In contrast, hybridization of ΔS–1668 DNA with the 40.2-1.7 probe produced the hybrid pattern obtained with F_1 plants carrying the undeleted S_{13} haplotype. Thus, in ΔS–1668, a deletion with one end point located within the SLG_{13} coding region removed the 3′ end of the SLG_{13} gene as well as DNA containing the *299*, *298*, BH2.0, and 6B-1.2 sequences. Because the SLG_{13} 5′ sequences, the *SRK* gene, and 40.2-1.7 sequences were not encompassed by the deletion, we conclude that in the wild-type S_{13} haplotype, as in the S_8 haplotype, the SLG_{13} gene is oriented with its 5′ end toward SRK_{13} and that the *299*, *298*, SD22, BH2.0, and 6B-1.2 sequences are located 3′ of SLG_{13}, as shown in Figure 3.

DISCUSSION

Our analysis of the overall organization of the S_2 and S_{13} haplotypes of *B. oleracea* has revealed extensive intraspecific sequence rearrangement at the *S* locus. Sequences corresponding to several of the probes used in our study were found to occupy different locations relative to one another and to the *SLG* and *SRK* genes. Furthermore, in one instance, a sequence shown to be genetically and physically linked to *SLG* and *SRK* in one haplotype was found to be displaced to a chromosomal location unlinked to the *S* locus in plants homozygous for other haplotypes. Significantly, the occurrence of sequence rearrangements is not limited to the two haplotypes reported here, because they have been observed in preliminary analyses of other haplotypes as well (data not shown). Thus, the data are consistent with the conclusion that heteromorphism at the *S* locus was established via the accumulation of sequence rearrangements as well as of highly diverged sequences and even haplotype-specific sequences, as exemplified by the SLA_2 sequence for which no cross-hybridizing homologs have been detected in *S* haplotypes other than S_2 (Boyes and Nasrallah, 1995). Rearranged haplotypic configuration and fractured homology are expected to reduce pairing or cause the production of aberrant nonrecoverable recombination products. As a result, and by analogy to other complex loci (examples of which are discussed below), these structural features most likely suppress recombination in the region encompassing the *S* locus, thereby maintaining its genes in a complex.

Tight genetic linkage of several distinct functions, as is found at the Brassica *S* locus, is a common feature of sex-determining systems, although the size of the sex linkage group differs in different systems, ranging from small units spanning a few kilobases, as in fungal mating-type loci, to entire sex chromosomes (Charlesworth, 1991; Bölker et al., 1992; Ferris and Goodenough, 1994). It has been proposed that when several genes are required for a particular function, the maintenance of these genes in a tightly linked genetic unit is advantageous (Charlesworth, 1991, 1994). In fact, reduced frequencies of recombination have been observed not only in sex-determining regions of the genome but also in other coadapted gene complexes. For example, in the human major histocompatibility complex, a cluster of genes involved in immune recognition, recombination events have never been observed in some regions (Trowsdale, 1993). The absence or paucity of recombinants may be associated with the presence of nonhomologous sequences, as in the mating-type loci of fungi (Glass et al., 1988; Herskowitz, 1989; Bölker et al., 1992), or with the occurrence of sequence rearrangements, such as the inversions found in the *t* haplotype of the mouse that are expected to generate lethal recombination products (Silver, 1993). A combination of mechanisms may operate in some systems. Thus, both sequence rearrangement and lack of sequence similarity apparently contribute to the suppression of recombination at the mating-type locus of Chlamydomonas, which exists as two alleles, mt+ and mt−, and lies within a 640-kb region of recombinationally suppressed DNA (Ferris and Goodenough, 1994). Embedded within this region is a 190-kb sector that is rearranged extensively between the two alleles. Notably, this region also encodes sequences that are found in only one mating type and not the other (Ferris and Goodenough, 1994).

The parallels between the structural features of the Brassica *S* locus and the Chlamydomonas mating-type locus are striking and suggest that similar strategies have been adopted in the evolution of the two loci. We speculate that a mechanism for the suppression of genetic recombination over the *S* haplotype was integral to the evolution of the Brassica self-incompatibility system, which depends on the cooperative action of distinct stigma and pollen functions (Nasrallah et al., 1994b). Indeed, the suppression of recombination between *SLG* and *SRK* and throughout the locus must have been an important factor not only for maintenance of *S* haplotype integrity but also for the coevolution of these genes within each *S* haplotype. Importantly, the suppression of recombination at the *S* locus would also preclude the frequent occurrence of the unequal recombination events allowed by the high degree of sequence identity shared by *SLG* and the extracellular domain of *SRK*.

Rearranged haplotypic configuration and fractured homology may not be the only factors that impact recombination rates and the recovery of recombinants in the *S* locus region. In our analysis of the region, we found highly conserved sequences, such as *298* and *299*, located close to the polymorphic *SLG* and *SRK* genes. These sequences are expressed in vegetative tissues as well as in reproductive structures and are conserved in different *S* haplotypes. Thus, *298* and *299* are unlikely to function in determining the specificity of the self-incompatibility response. If these sequences perform an essential function in the plant, then the loss of either sequence through a recombination event would terminate the lineage of the recombinant chromosome. Their presence in the *S* locus region would thus provide an additional mechanism for selecting against *S* haplotype recombinants. We also encountered regions of

multicopy DNA interspersed with the single-copy sequences used in our PFGE analysis. Recent studies of the organization of genomic regions surrounding several active genes in maize have shown that single-copy sequences are often flanked by highly repetitive and hypermethylated DNA sequences consisting primarily of transposons (Bennetzen et al., 1994; SanMiguel et al., 1996). Because most recombination events in maize appear to involve single-copy sequences (Sudupak et al., 1993), the hypermethylation of this repetitive DNA relative to the neighboring single-copy sequences is thought to inhibit recombination (Bennetzen et al., 1994). The potential effect of repetitive sequences in limiting recombination has also been discussed for other plant loci, including the *S* RNase genes of the Solanaceae that are flanked by highly repetitive DNA (Coleman and Kao, 1992). Although the extent, nature, and methylation state of the multicopy DNA sequences that we detected in our chromosomal walk have not been investigated, such sequences could contribute to reduced recombination rates in the *S* locus region.

Clearly, it will be important to analyze the recombination frequency in the chromosomal region encompassing and flanking the *S* locus and to determine whether it differs significantly from recombination frequencies in other regions of the Brassica genome. Such an analysis would be informative not only in relation to the evolution of *S* haplotypes but also, by analogy to the Chlamydomonas mating-type locus (Ferris and Goodenough, 1994), for placing outer limits on the extent of the *S* locus, thereby defining the full complement of genes that constitute a specificity-encoding *S* haplotype. Currently, however, no estimates exist for the actual frequency of recombination in the chromosomal region containing the *S* locus and in other regions of the Brassica genome, even though Brassica linkage maps are available (Slocum et al., 1990; Song et al., 1991) and the *S* locus has been positioned on at least one such linkage map (Camargo et al., 1997). Nevertheless, the frequency of recombination in the *S* locus region must be relatively low. As indicated earlier, recombination events between *SLG* and *SRK* have not been observed even when 500 plants in a single F_2 population were analyzed (M.E. Nasrallah, unpublished observations). The absence of recombinants is especially striking in haplotypes, such as S_2 and S_6, in which the two genes are separated by substantial physical distances (Figure 3; Boyes and Narallah, 1993). A low recombination frequency would distinguish the *S* locus complex from other well-characterized plant loci that contain duplicated sequences, such as the *Rp1* (Sudupak et al., 1993), *R-r* (Robbins et al., 1991), and *R-st* (Eggleston et al., 1995) loci of maize as well as the *M* locus of flax (Ellis et al., 1995), all of which are meiotically unstable because of the frequent occurrence of unequal crossing-over events.

Another factor that is critical to our understanding of the evolution of self-incompatibility in Brassica relates to the origin of the structural heteromorphism that characterizes the *S* locus. Our previous finding that the *SLG* genes contained in the *B. oleracea* S_{13} and *B. campestris* S_8 haplotypes are more closely related to each other than to *SLG* alleles from other haplotypes within their corresponding species suggested that these two haplotypes are likely to have been derived from the same ancestral haplotype (Dwyer et al., 1991). Significantly, our long-range mapping studies and our analysis of the deleted S_{13} haplotype carried by the ΔS–1668 plant showed that the overall structure of the *B. oleracea* S_{13} haplotype is more closely related to the *B. campestris* S_8 haplotype than to the *B. oleracea* S_2 haplotype. The order of sequences detected by the probes used in this study is conserved in S_8 and S_{13}; however, the spacing of the sequences differs between the two haplotypes, perhaps reflecting a general expansion or contraction in haplotype size. Interestingly, the significantly shorter physical distances observed in the S_8 haplotype parallel the smaller estimated haploid genome size of *B. campestris* (468 to 516 Mb) relative to *B. oleracea* (599 to 662 Mb) (Arumuganathan and Earle, 1991). Interspecific shared polymorphisms have been used as evidence for diversification predating speciation (Figueroa et al., 1988; Lawlor et al., 1988). One interpretation of the interspecific conservation in overall *S* haplotypic configuration that we observed is therefore that the structural polymorphism of *S* haplotypes was established, perhaps concomitant with nucleotide sequence divergence, before speciation in the genus Brassica. The highly diverged nature of present-day *S* haplotypes would then reflect long periods of recombinational isolation resulting from the reordering of DNA segments early in the evolution of the locus.

Based on the available data, it is premature to propose a model for the evolution and structural diversification of the Brassica *S* haplotypes. However, expansion of chromosomal regions and chromosomal restructuring can be mediated by the activity of transposons (Walker et al., 1995; Wessler et al., 1995; SanMiguel et al., 1996). Interestingly, we had previously reported on a defective SLA_2 sequence in *B. napus* that contained a retroelement insertion (Boyes and Nasrallah, 1995). However, it remains to be determined what role, if any, such elements have had in the evolution of the Brassica *S* locus. Future structural characterization of several additional *S* haplotypes will no doubt shed light on this question and provide an interesting comparison to the organization and evolution of complex loci in plants and other organisms.

METHODS

Plant Materials

Plants (*Brassica oleracea* var *alboglobra* [Chinese kale]) homozygous for the S_2 haplotype, plants (*B. oleracea* var *acephala* [marrow-stem kale]) homozygous for the S_{13} haplotype, and plants (*B. campestris*) homozygous for the S_8 haplotype have been described previously (Dwyer et al., 1989, 1991; Chen and Nasrallah, 1990; Toriyama et al.,

1991). *S* homozygotes are produced by manual pollination of immature flower buds at a developmental stage before the stigma acquires the capacity to distinguish self- from non-self-pollen.

Library Construction and Chromosome Walking

Genomic libraries of S_2, S_{13}, and S_8 homozygotes were constructed with DNA that had been digested partially with Sau3AI, size fractionated, and ligated to the vector λEMBL4 or λGEM11, as described previously (Dwyer et al., 1989, 1991; Chen and Nasrallah, 1990; Toriyama et al., 1991). λ clones containing *SLG* were isolated from each of these libraries by hybridization with *SLG* cDNA probes (Dwyer et al., 1989, 1991; Chen and Nasrallah, 1990; Toriyama et al., 1991). The longest *SLG*-containing clones derived from the S_2, S_{13}, and S_8 haplotypes were described by Chen and Nasrallah (1990), Dwyer et al. (1989), and Toriyama et al. (1991), respectively, and are the clones discussed here.

Chromosome walking in the S_8 haplotype was initiated from two genomic clones: one that contained SLG_8 and another that contained SRK_8. Single-copy sequences for use as probes in each step of the walk were identified by hybridizing blots of restriction enzyme–digested DNA from λ clones with ^{32}P-labeled total Brassica genomic DNA. Fragments within the λ clones that failed to hybridize were subcloned and subsequently verified as representing single-copy sequences by hybridization with blots of Brassica genomic DNA that had been digested independently with several restriction enzymes. These single-copy sequences were then used as probes to rescreen the genomic library and isolate new overlapping clones that extended the region of cloned DNA.

Pulsed-Field Gel Electrophoresis, Standard Agarose Gel Electrophoresis, and Gel Blot Analysis

High-molecular-weight DNA for pulsed-field gel electrophoresis (PFGE) was prepared from isolated leaf protoplasts, as described previously (Boyes and Nasrallah, 1993), or from isolated plant nuclei (Zhang et al., 1995). Digestion of agarose-embedded high-molecular-weight DNA with rare-cutting restriction enzymes and size fractionation by PFGE were conducted as described previously (Boyes and Nasrallah, 1993). Gels were run at 175 V with the pulse-switching interval ramped from 5 to 50 sec over a run length of 20 hr. For standard agarose gels, DNA was prepared and processed as previously described (Stein et al., 1991).

After electrophoresis, the DNA was transferred to a GeneScreen Plus (Du Pont) membrane, hybridized with single-copy DNA probes, and exposed to x-ray film, as described previously (Boyes and Nasrallah, 1993). The probes included genomic fragments identified in the chromosomal walk described in the previous section, *298* and *299* cDNA probes, previously described *SLG*- and *SRK*-derived probes (Boyes and Nasrallah, 1993), and an SRK_8-derived probe that was obtained from the first intron of the gene. To avoid complications that might arise from gel-to-gel variation in the migration of fragments during PFGE, the long-range restriction maps were constructed from data obtained by hybridizing the same blot with all probes.

RNA Gel Blot Analysis

For RNA isolation, stigmas (including the stigma proper and part of the style) and anthers (including the anther proper and part of the filament) from flower buds at 1 to 3 days before anthesis and young leaves (including the leaf blade and vasculature) were harvested and frozen in liquid N_2. Poly(A)$^+$ RNA was isolated by using the FastTrack mRNA isolation kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The RNA was subjected to electrophoresis on 1% (w/v) agarose gels in the presence of formaldehyde, transferred to a GeneScreen membrane, hybridized, and processed as described previously (Stein et al., 1991). To perform sequential hybridizations of the same RNA gel blot with different probes, the membrane was stripped and exposed to x-ray film between probings to verify the absence of residual signal.

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