Determining the Physical Limits of the Brassica S Locus by Recombinational Analysis

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A genetic analysis was performed to study the frequency of recombination for intervals across the Brassica S locus region. No recombination was observed between the S locus glycoprotein gene and the S receptor kinase gene in the segregating populations that we analyzed. However, a number of recombination breakpoints in regions flanking these genes were identified, allowing the construction of an integrated genetic and physical map of the genomic region encompassing one S haplotype. We identified, based on the pollination phenotype of plants homozygous for recombinant S haplotypes, a 50-kb region that encompasses all specificity functions in the S haplotype that we analyzed. Mechanisms that might operate to preserve the tight linkage of self-incompatibility specificity genes within the S locus complex are discussed in light of the relatively uniform recombination frequencies that we observed across the S locus region and of the structural heteromorphisms that characterize different S haplotypes.

INTRODUCTION

The self-incompatibility or S locus of Brassica is a complex, multigene, multiallelic locus that is responsible for the specificity of the rejection of self-pollen at the stigma surface. The chromosomal region containing the S locus is a gene-rich segment in which the polymorphic genes known to be required for self-incompatibility (SI) are interspersed with genes whose functions apparently are unrelated to SI (Boyes and Nasrallah, 1995; Yu et al., 1996; Conner et al., 1998; Letham and Nasrallah, 1998). Although it is not known how many genes within the S locus complex are required for determining the specificity of the SI response in stigma and pollen, current models require, at minimum, the action of three S locus genes: the two stigma-specific genes, SLG (for S locus glycoprotein) and SRK (for S receptor kinase), already known to be required for the ability of the epidermal (papillar) cells of the stigma to recognize and inhibit self-pollen (reviewed in Nasrallah et al., 1994a), as well as an unidentified gene that would impart an SI specificity on pollen grains. Such a “pollen specificity” gene would be expressed during pollen development and would encode a pollen-borne molecule (presumably a ligand for SRK) that, when delivered to the stigma surface, would induce receptor activation and initiation of a signal transduction cascade within the stigmatic papillar cell that prevents the hydration and germination of self-pollen.

The physical distance between SLG and SRK has been estimated by pulsed-field gel electrophoresis (PFGE) for a number of S haplotypes in different Brassica spp and was found to vary greatly, from as little as a few kilobases in haplotypes of Brassica campestris (syn B. rapa) to as much as several hundred kilobases in haplotypes of B. oleracea (Boyes and Nasrallah, 1993; Yu et al., 1996; Boyes et al., 1997). Significantly, recombinants between SLG and SRK have not been reported, despite the substantial physical distances that can separate the two genes. Tight genetic linkage of genes that code for the specificity of SI in pistil and pollen is expected, because SI in Brassica segregates as a single Mendelian locus. In view of the extensive rearrangements and sequence divergence observed in intraspecific comparisons of different S haplotypes, as well as the occurrence of repetitive sequences (Boyes and Nasrallah, 1993; Boyes et al., 1997), it has been suggested that the frequency of recombination events, or the recovery of recombinant haplotypes, might be suppressed within the S locus region. Several complex plant loci have been subjected to detailed recombinational analysis, including the R (Walker et al., 1995) and the Rp1 (Richter et al., 1995) complexes of maize, and the DM3 cluster of disease resistance genes in lettuce (Meyers et al., 1998). In the latter case, strong recombinational suppression was observed over a 3.5-Mb region
(Meyers et al., 1998). Similar recombinational suppression might explain how the S locus complex is inherited as a single genetic unit and how S haplotypes have been maintained since their origin more than 20 to 40 million years ago before speciation in the genus Brassica (Boyes et al., 1997; Charlesworth and Awadalla, 1998).

We conducted a recombinational analysis of the S locus region to study the frequency of recombination for the interval spanning SLG/SRK and for flanking intervals, to derive genetic and physical maps of the region, and to evaluate the possible phenotypic consequences of intralocus recombinants. This analysis required the identification of a series of molecular markers located at various distances from the SLG and SRK gene pair. We previously had generated molecular markers for a 510-kb segment of the S locus region (Boyes et al., 1997; Conner et al., 1998). Additional molecular markers were obtained using a strategy based on differential display of RNA prepared from bulked segregants. These markers were used for restriction fragment length polymorphism (RFLP) analysis of large populations segregating for the S locus. The identification of recombinant S haplotypes allowed us to construct an integrated genetic and physical map of an ~1000-kb chromosomal region and to define a 50-kb segment that must encompass all SI specificity functions in the S haplotype that we analyzed.

RESULTS

Isolation of Novel S Locus-Linked Markers by Differential Display of Anther Transcripts

Differential display analysis was conducted using anther RNA in conjunction with bulked segregants to isolate additional markers from the S locus region. This analysis, which is described in detail in Casselman et al. (1998), used a B. oleracea population of 110 F2 plants segregating for the S2 and S6 haplotypes. For each F2 plant, S genotype was determined by RFLP analysis and SI phenotype by pollination assays. Poly(A)+ RNA isolated from anthers of homozygous S2 and S6 individuals was pooled to create an S2 pool and an S6 pool. Generating pools from an F2 population effectively homogenizes background unlinked polymorphisms found between the parental S2S2 and S6S6 lines (see Methods).

In this screen, 25 cDNAs were isolated that identified RFLPs between DNA from S2 and S6 homozygotes. These cDNAs hybridized to DNA from both parental lines and therefore were not S haplotype specific. A preliminary analysis of F2 plants segregating for the S2 and S6 haplotypes revealed that 22 of the cDNAs segregated away from SLG and SRK in at least one of 34 plants. Two cDNAs, DD70 and DD26, recombined away from SLG and SRK at a frequency of 1 of 76. The remaining cDNA, DD15, and its corresponding gene (designated SPA [S locus, expressed predominantly in anthers] based on its predominant expression in anthers [Casselman et al., 2000]) segregated perfectly with SLG and SRK in 110 F2 plants.

Genetic Analysis of the S Locus Region

To determine whether recombination events between SLG and SRK could be detected in relatively large populations of segregating plants, we analyzed 400 B. oleracea F2 plants segregating for the S2 and S6 haplotypes. Previous work has shown that SLG and SRK are separated by as much as 350 and 220 kb of DNA in the S2 and S6 haplotypes, respectively (Boyes and Nasrallah, 1993). Nevertheless, RFLP analysis demonstrated the absolute linkage of SLG2 with SRK2 and of SLG6 with SRK6, and no recombinants were observed in the 800 chromosomes screened.

To investigate in detail the frequency of recombination, not only between SLG and SRK but also in other intervals across the chromosomal region that spans the S locus, we used a B. campestris population of 509 plants segregating for S6 (a haplotype for which we already had generated a partial long-range physical map) and S2, a previously described nonfunctional haplotype with a defective SRK gene (Nasrallah et al., 1994b; see Methods). The population, consisting of 509 F2 plants, was homozygous for mod, a recessive mutation at a locus unlinked to the S locus that causes self-compatibility (Ikeda et al., 1997). Homozygosity for the mod mutation allows for the production of large numbers of seed by unassisted self-pollination and circumvents the need for the laborious method of manual pollination in immature buds that is required to generate seed in self-incompatible strains. However, because mod is epistatic to the S locus, evaluation of pollination phenotype in plants carrying any recombinant S haplotypes identified in this population requires backcrossing into a MOD/MOD genetic background.

Earlier studies have shown that the SLG6 (Dwyer et al., 1991) and SLG10 genes (Watanabe et al., 1997) share only 75% sequence identity, indicating that the S6 and S10 haplotypes are quite diverged from each other and probably derived from distinct ancestral haplotypes. The overall divergence of these haplotypes was further reflected in our observation that the majority of restriction enzymes that we tested produced RFLPs between the two haplotypes for each of the markers used in this study. One such enzyme, HindIII, was used for the recombinational analysis described here. Genomic DNA prepared from each of the 509 plants was digested with HindIII, and gel blots of these digests were first hybridized with a probe that detects both SLG and SRK to determine the S genotype of the plants. A typical blot is shown in Figure 1A. Of the 509 plants analyzed, 138 were S8S8, 260 plants were S6S10 heterozygotes, and 111
were \( S_8 S_7 \), which represents a close fit to the expected 1:2:1 segregation ratio (\( P \) value of \( \approx 0.2 \)). No recombination events between SLG and SRK were detected in this population.

Five DNA markers that span the \( S \) locus region subsequently were used as probes on the blots: DD70 and DD26,

which were generated in the differential display screen described above; \( c31 \) and \( y14 \), which were isolated in a Brassica and Arabidopsis comparative mapping study (Conner et al., 1998); and \( wg5A1 \), which was mapped to the vicinity of SLG in a \( B. \) oleracea mapping study (Camargo et al., 1997). Plants in which the marker recombined with SLG/SRK were identified by inspection of the RFLP patterns, as shown for marker DD26 in Figure 1B. This RFLP analysis allowed us to determine the order of the markers relative to SLG/\( \delta \)SRK. For example, the plants that were recombinant for marker \( c31 \) were a subset of the plants recombinant for DD26, indicating that these two markers are on the same side of the \( S \) locus and that \( c31 \) is closer to SLG/\( \delta \)SRK than DD26. For each of the five markers, map distances to SLG/\( \delta \)SRK were calculated based on the number of recombinants detected in the segregating population. Only the total number of plants that produced unambiguous data was considered. Map distances in centimorgans (\( cM \)) are 1.1 (11 of 505 recombinants) for DD70, 0.7 (seven of 509 recombinants) for DD26, 0.2 (two of 499 recombinants) for \( c31 \), 0.2 (two of 507 recombinants) for \( y14 \), and 0.4 (two of 268 recombinants) for \( wg5A1 \).

Of the above five markers, the markers closest to SLG/\( \delta \) and SRK/\( \delta \) were \( c31 \) and \( y14 \), and each detected two recombination events. To locate these recombination breakpoints more precisely, we used markers derived from the \( \lambda \) contigs of the \( S_8 \) haplotype (Boyes et al., 1997; Conner et al., 1998), starting with markers from the ends of the contigs and followed sequentially by markers progressively closer to SLG/\( \delta \) and SRK/\( \delta \). The location of the markers is shown on the map in Figure 2A. Because the original DNA blots no longer produced strong hybridization signals, and because it was not necessary to examine the entire population, five to six progeny from each of the recombinant plants were grown, DNA was prepared from individual progeny plants, and new blots were generated. In this manner, the recombination breakpoints were located as follows: breakpoint A between markers s298 and sBH2.0 at a distance of 7 to 20 kb 3’ of SLG/\( \delta \); breakpoint B between markers SLL1 and SLL2/s299 at a distance of 2 to 4 kb 3’ of SLG/\( \delta \); breakpoint C between markers s33-13 and s40.2-1.7 at a distance of 8 to 19 kb 3’ of SRK/\( \delta \); and breakpoint D between markers s400 and \( y14 \) at a distance of 48 to 70 kb 3’ of SRK/\( \delta \). The location of each of the breakpoints within the \( \lambda \) contig and a genetic map of the \( S_8 \) haplotype and flanking regions are shown in Figure 2. An example of plants homozygous for the recombinant haplotype resulting from the crossover event at breakpoint A is shown in Figure 3; these plants are homozygous for the SLG/\( \delta \) and SRK/\( \delta \) alleles but carry the sBH2.0 allele derived from the \( S_7 \) haplotype.

A second \( F_2 \) population of 260 plants segregating for the \( S_8 \) and \( S_7 \) haplotypes (Ikeda et al., 1997) also was analyzed. Initial RFLP analysis indicated that one plant from this population contained a recombination breakpoint between marker \( y14 \) and SLG/\( \delta \)SRK/\( \delta \). Subsequent analysis of progeny produced by selfing this plant confirmed the recombination

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**Figure 1.** Segregation Analysis of \( F_2 \) Plants Segregating for the \( S_8 \) and \( S_7 \) Haplotypes.

The results of segregation analysis of SLG/SRK and of marker DD26 in a representative sample of 19 \( F_2 \) plants are shown. Gel blots were prepared with HindIII-digested genomic DNA isolated from individual \( F_2 \) plants.

**A** Gel blot probed with the SLG probe. Restriction fragments marked with an asterisk correspond to the \( S \) genes derived from the \( S_7 \) locus; those marked with a circle correspond to the SLG (20-kb fragment) and SRK (5.0-kb fragment) alleles derived from the \( S_8 \) locus. The genotypes shown above each lane were determined by RFLP analysis, using the SLG probe (see Methods).

**B** Gel blot probed with the DD26 probe. The blot in (A) was stripped and reprobed with DD26. Restriction fragments marked with an asterisk correspond to the DD26 allele found in the \( S_7 \) parental line, and the restriction fragment marked with a circle corresponds to the DD26 allele present in the \( S_8 \) parental line. The arrow marks an individual that is homozygous for the SLG/SRK alleles derived from the \( S_7 \) locus but heterozygous at the DD26 locus. The genotype of this individual resulted from a recombination event between SLG and SRK on the one hand and DD26 on the other hand. Numbers at right indicate molecular lengths in kilobases.
event and located this fifth recombination breakpoint (E) to a region between markers s40-2-1.7 and s338 at a distance of 19 to 43 kb of SRK₈, as shown in Figure 2A.

**Physical Analysis of the S Locus Region**

To correlate genetic distances to physical distances for markers not contained within the λ contigs, we performed PFGE analysis. Two sources of DNA were used in this study: large-size genomic DNA from the S₈ homozygous strain and two overlapping BAC clones isolated from a large-insert library of S₈ DNA by hybridization of a BAC library to the single-copy s298 probe (see Methods).

Figures 4A and 4B and Table 1 show the PFGE analysis of Brassica genomic DNA. Markers wg5A1 and y14, which are located to one side of the S locus, hybridized with a 110-kb EagI fragment and, along with marker s338, with a 97-kb BssHII fragment. Therefore, no more than 97 kb separated wg5A1 and s338. Located to the other side of the S locus, the DD70 and DD26 markers hybridized with a 660-kb NotI fragment and a 250-kb SgrAI fragment, as well as with 560- and 240-kb Sfil fragments. The 560-kb Sfil fragment most likely is due to partial digestion of the DNA; incomplete digestion of Brassica DNA previously has been observed with Sfil (Sadowski et al., 1996; Conner et al., 1998). We conclude that DD70 and DD26 reside no farther apart than 240 kb.

The physical distances separating markers c31 and y14 from each other and from the ends of the λ contigs were determined by PFGE analysis of two overlapping BAC clones, C16 and F15. These clones together span at least 180 kb of DNA from the S locus region, as shown in Figure 5. The 120-kb insert in BAC C16 includes the S domain of SRK₈ and extends at least as far as marker c31. The 110-kb insert in BAC F15 includes 18 kb of DNA 3' of SLG₈ and extends to marker y14 but does not include marker wg5A1.

BAC C16 DNA was doubly digested with NotI (which releases the insert from the BAC vector) in combination with Apal, BssHII, EagI, or MluI. Based on hybridization of PFGE blots with c31, s16 (the end marker from the λ contig that includes DNA 3' of SLG₈), s13, DD15, and sBH2.0 (Table 1), we conclude that c31 is no more than 52 kb from marker s16 (and no more than 124 kb from SLG₈/SRK₈).

For restriction analysis of BAC F15, NotI could not be used to release the insert because of the presence of a NotI site within the insert. Instead, double digest analysis was conducted using Sfil, which cuts only to one side of the insert, leaving the 7-kb BAC vector attached to one of the restriction fragments and therefore requiring adjustment of the observed fragment sizes (Table 1). We conclude, based on
the data summarized in Table 1, that markers y14 and s338 are no farther apart than 40 kb.

Recombination Frequencies across the S Locus Region

A compilation of the physical distances (Table 1) and the genetic distances (Figure 2B) estimated from our analysis of the $S_f^b/S_f^a$ segregating populations allowed a calculation of kilobase-to-centimorgan ratios for intervals across the S locus region. The 118-kb region between markers s16 and s338, which includes SLG$_8$ and SRK$_8$, was found to exhibit a ratio of 410 kb/cM. To one side of SLG$_8$/SRK$_8$, the interval between DD26 and c31 exhibited a ratio of 490 to 1090 kb/cM. To one side of c31, the interval between DD70 and DD26 a ratio of ≈625 kb/cM. To the other side of c31, the interval between markers s400 and wg5A1 was found to exhibit a ratio of ≈433 kb/cM. These ratios are shown on the integrated genetic and physical map of the $S_8$ haplotype in Figure 5.

Pollination Phenotype of S Locus Recombinants

We were interested in determining whether the SI response was compromised in plants carrying recombinant S haplotypes, as might be expected if the recombination events resulted in the uncoupling of coadapted recognition genes. Four of the five recombinant S haplotypes identified in this study, namely, those resulting from recombination events at breakpoints A, B, D, and E (see Figure 2), contained the functional SLG$_8$ and SRK$_8$ gene pair and therefore were informative. Recombinant C was homozygous for the S genes of the nonfunctional $S_f^b$ haplotype and was not analyzed further. Plants homozygous for each of the $A$, $B$, $D$, and $E$ recombinant S haplotypes (which we designate recombinants A, B, D, and E) and carrying the wild-type MOD allele were generated (see Methods). The pollination phenotype of these plants was determined by performing a series of self-pollinations and reciprocal pollinations to the $S_8S_8MODMOD$ nonrecombinant parental strain. In addition, reciprocal pollinations to a strain that is cross-compatible with the $S_8S_8MODMOD$ nonrecombinant parental strain were performed to verify that stigma and pollen of the recombinants functioned normally. As shown in Table 2, plants homozygous for each of the $A$, $B$, and $D$ recombinant haplotypes were self-incompatible; they also retained the $S_8$ specificity, because they were reciprocally incompatible with nonrecombinant $S_8S_8MODMOD$ plants. In contrast, plants homozygous for recombinant haplotype E were self-compatible; they produced 50 to 100 pollen tubes per stigma when self-pollinated, which is a pollen tube count that although lower than the >300 pollen tubes produced in cross-pollinations, nevertheless resulted in the production of a full seed set. Reciprocal pollinations with the nonrecombinant $S_8S_8MODMOD$ nonrecombinant parental strain indicated that the partial breakdown of SI in these recombinants was due to an alteration in the pollination response of the stigma and not that of pollen (Table 2). Thus, a gene required for a full SI response in the stigma is present between recombination breakpoints D and E.

As stated earlier, the $S_8$ and $S_{12}$ haplotypes are highly diverged; consequently, the $S_{12}$ haplotype is not expected to carry factors that contribute to $S_8$ specificity. Therefore, our results place the limits of the segment of the S locus region that contains the $S_8$ specificity genes at 2 to 4 kb downstream of SLG$_8$ and 46 to 70 kb downstream of SRK$_8$, that is, between recombination breakpoints B and D. The chromosomal region’ of SLG$_8$ that flanks recombination breakpoint B is gene rich, as determined by a previously reported comparative analysis of the Brassica $S_8$ haplotype and its homeolog in Arabidopsis (Conner et al., 1998), as well as by direct sequencing of the Brassica region (Suzuki et al., 1999; J. B. Nasrallah, unpublished data). A list of these genes/open reading frames along with their location relative to SLG$_8$ is given in Table 3. Some of these genes are expressed in stigma and/or anthers, but all may be unambiguously excluded as having a role in SI specificity because they reside beyond recombination breakpoint B. The previously
described SLL1 transcriptional unit (Yu et al., 1996) resides closer to SLG8 than recombination breakpoint B. However, SLL1 lacks S haplotype-associated polymorphism (Yu et al., 1996), is apparently not present in the S8 haplotype (Suzuki et al., 1999), and therefore can be discounted as functioning in SI specificity.

**DISCUSSION**

The isolation of markers located at varying genetic and physical distances from SLG/SRK has allowed us to conduct a recombinational and physical analysis of the S locus and flanking regions. In our analysis of plants segregating for the S8 and Sf2 haplotypes, we examined a region of the S8 haplotype of B. campestris that spanned 740 to 1000 kb and 1.46 cM. Five of the recombination breakpoints that we identified mapped to an ~150-kb genomic region containing the SLG/SRK gene pair and flanking DNA, all of which is represented on two overlapping BAC clones.

These recombination events allowed us to place limits on the chromosomal region that encodes S8 specificity. We infer, based on the pollination phenotype of plants homozygous for the recombinant S haplotypes, that the full complement of genes that constitute the S8 specificity–encoding haplotype lies within a 46- to 70-kb region stretching from recombination breakpoint B to breakpoint D. Furthermore, we predict the presence of a gene required for SI in the stigma between recombination breakpoints D and E, based on the self-compatible phenotype of plants homozygous for recombinant haplotype E in which the region beyond breakpoint E is derived from the Sf2 haplotype. This gene might contribute to specificity in the SI response, or, perhaps more likely in view of the only partial breakdown of SI in plants homozygous for recombinant haplotype E, this gene might fulfill a non–S haplotype–specific function required for SI that is not provided by the defective Sf2 haplotype. Most significantly, plants homozygous for recombinant haplotypes B and E did not exhibit a modified pollen phenotype. Thus, the pollen determinant(s) of SI must reside within the ~50-kb segment delineated by these two recombination breakpoints.

By correlating genetic distances to physical distances, we derived estimates of the numbers of DNA base pairs per recombination unit for different intervals in the region. Segments of the genome with suppressed recombination are expected to exhibit large kilobase-to-centimorgan ratios rel-

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**Figure 4. Physical Linkage of Markers in the S Locus Region.**

Genomic DNA prepared from S8 homozygotes was digested with BssHII and EagI (A) and with NotI and SfiI (B) and then separated by PFGE.

(A) The PFGE blot was probed sequentially with wg5A1, y14, and s338. Fragments that hybridized with all probes are indicated with asterisks and circles. Probe s338 did not hybridize with an EagI fragment on this blot because the fragment was too small to be retained in the gel under the running conditions used. Less intense bands presumably are due to nonspecific hybridization.

(B) The PFGE blot was probed sequentially with DD70 and DD26. Digestion of genomic DNA with SfiI often results in partial digestion, resulting in the two bands observed in the Sfi digest. The hybridization signal seen in the compression zone (c.z.) corresponds to undigested DNA often resulting from digestion with NotI and SfiI. The asterisks, circles, and plus signs mark restriction fragments that hybridized with both probes.

Numbers between the gels indicate molecular length markers in kilobases.
ative to regions with more frequent recombination events. However, at the level of resolution of this study, and in the \( S_8 \)/\( S_{10} \) segregating population that we analyzed, we detected no significant differences in kilobase-to-centimorgan ratios across the region spanning the \( S_8 \) locus: the 123-kb region immediately surrounding \( SLG_8 \)/\( SRK_8 \) and spanned by the \( S_8 \) contigs did not exhibit a kilobase-to-centimorgan ratio dramatically larger than the neighboring intervals. Indeed, kilobase-to-centimorgan ratios across the region are comparable to the average values of 500 to 700 kb/cM deduced for the entire genome in Brassica (Sadowski et al., 1996; Camargo et al., 1997). The frequency of crossovers that we detected can be contrasted with the strong recombinational suppression associated with the structural heteromorphism of the Chlamydomonas mating-type locus (Ferris and Goodenough, 1994) and the low recombination frequencies that have been reported for some other well-characterized complex loci in plants, such as the 3.5-Mb region spanned by the DM3 cluster of disease resistance genes in lettuce (Meyers et al., 1998).

Despite our inability to detect recombinational suppression in the \( S_8 \)/\( S_{10} \) segregating population, it is premature to rule out suppression of crossover events or reduced recovery of recombinant haplotypes as mechanisms for maintaining the integrity of the \( S \) locus complex over time. Different results may be obtained in the analysis of populations segregating for other \( S \) haplotypes. Indeed, the extensive sequence polymorphisms and structural heteromorphisms that characterize the \( S \) locus predict that populations heterozygous for different combinations of \( S \) haplotypes might exhibit drastically different recombination frequencies, because variable degrees of structural and sequence divergence can lead to variability in the extent of chromosome pairing. For example, analysis of the maize \( R p1 \) region has demonstrated that certain \( R p1 \) haplotypes can be meiotically stable in particular heterozygous combinations but highly recombinogenic in others (Sudupak et al., 1993). In addition, genetic background can have a significant effect on frequencies of recombination in a particular region of the genome (Timmermans et al., 1997; Palmer et al., 1998), and recombination values for the \( S \) locus may vary with the Brassica sp or cultivar being investigated.

Furthermore, there may be a direct correlation between the physical size of an \( S \) haplotype and the occurrence or extent of recombinational suppression. Indeed, in our analysis of the \( B. \) oleracea \( S_8 \) and \( S_8 \) haplotypes, in which \( SLG \) and \( SRK \) are separated by 200 kb, we did not observe crossover events between the two genes in 800 chromosomes screened, suggesting a lower than average recombination frequency. In the case of the \( S_8 \) haplotype analyzed here, the \( SLG \) and \( SRK \) genes are separated by only 13 kb as determined by restriction analysis of BAC clones (C. Schopfer, M.E. Nasrallah, and J.B. Nasrallah, submitted manuscript). It is possible that recombination is not suppressed in physically compact \( S \) haplotypes such as \( S_8 \). In these situations, maintenance of the \( S \) locus complex would be a direct consequence of the small physical extent of the locus, because intralocus crossover events would be

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<td>sBH2.0</td>
<td>BAC C16d</td>
<td>115</td>
<td>97</td>
<td>68</td>
<td>56</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>y14</td>
<td>BAC F15f</td>
<td>40g</td>
<td>123g</td>
<td>31</td>
<td>12</td>
<td>65g</td>
<td>103g</td>
<td>49</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>s338</td>
<td>BAC F15f</td>
<td>40g</td>
<td>123g</td>
<td>89</td>
<td>21</td>
<td>65g</td>
<td>103g</td>
<td>49</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a The DNA used for PFGE was either \( S_8 \) genomic DNA or BAC DNA.

b Numbers indicate the lengths of restriction fragments in kilobases. Only informative restriction fragments are shown.
c DD70 and DD26 hybridized with two restriction fragments in the SfiI digest, presumably due to partial digestion.
d BAC C16 DNA was doubly digested with NotI and each of the indicated restriction enzymes.
e Probe s13 hybridized with two restriction fragments in the Apal digest, indicating that the s13 sequence contains an Apal restriction site.
f BAC F15 DNA was doubly digested with SfiI and each of the indicated restriction enzymes.
g Numbers represent the observed length minus 7 kb, which corresponds to the length of the BAC vector included in these fragments.
rare (e.g., recombination between SLG and SRK is expected to occur at a frequency of approximately one of 1700 in the S8 haplotype). Alternatively, recombinational suppression might not have been detected in our study because it operates only in the 20- to 25-kb segment that includes the SLG8/SRK8 gene pair, or only in the SLG/SRK intergenic region that may be enriched for sequences that are highly diverged between the S8 and Sf2 haplotypes. Analysis of other complex sex-determining loci has revealed that the unit of linkage disequilibrium can vary dramatically in size, from 800 kb in the mating-type locus of Chlamydomonas to as little as 5 kb in the mating-type locus of Coprinus cinereus (May and Matzke, 1995).

Irrespective of how variable recombination frequencies at the S locus prove to be, recombination events, even if they occur at low frequency, can have dramatic consequences. In particular, recombination events that result in the shuffling of polymorphic domains between SLG and SRK alleles (Kusaba et al., 1997; Awadalla and Charlesworth, 1999) can contribute to the generation of novel SI specificities, similar to the role that recombination events apparently played in the evolution of another class of plant recognition genes, the disease resistance genes (Richter et al., 1995; Parniske et al., 1997; McDowell et al., 1998). On the other hand, recombination events that disrupt the complex of SI genes would produce nonfunctional recombinant S haplotypes and compromise the SI system. However, the persistence of SI in natural populations suggests that the spread of nonfunctional S haplotypes is restricted in some manner. One likely scenario is that the self-fertility associated with recombinant S haplotypes would lead to increased homozygosity for deleterious alleles and consequently to the reduced fitness of selfed progeny relative to outcrossed progeny. In the long term, such inbreeding depression would effectively lead to the elimination of nonfunctional recombinant S haplotypes from natural populations.

Table 2. Phenotypic Analysis of Plants Homozygous for Recombinant S8 Haplotypes

<table>
<thead>
<tr>
<th></th>
<th>S8S4</th>
<th>AA</th>
<th>BB</th>
<th>DD</th>
<th>EE</th>
<th>S4S4</th>
</tr>
</thead>
<tbody>
<tr>
<td>S8S8</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>++</td>
</tr>
<tr>
<td>AA</td>
<td>—</td>
<td>—</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>BB</td>
<td>—</td>
<td>ND</td>
<td>—</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>DD</td>
<td>—</td>
<td>ND</td>
<td>ND</td>
<td>—</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>EE</td>
<td>++</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>S4S4</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

*AA, BB, DD, and EE designate plants homozygous for the recombinant S haplotypes A, B, D, and E, respectively.

b (–), <10 pollen tubes per stigma; (+), 50 to 100 pollen tubes per stigma; (++), >300 pollen tubes per stigma.

Table 5. Integrated Physical and Genetic Map of the Chromosomal Region Encompassing the S8 Haplotype.

Map distances outside of the A contig (hatched box) were compiled from PFGE analysis of S8S8 genomic DNA and of two BAC clones. The kilobase-to-centimorgan ratios were calculated from the estimated physical distances and the genetic distances. Above the map are segments of the region contained in the restriction fragments shown in Figure 4: the 660-kb NotI restriction fragment (which includes markers DD70, DD26, and s338), the 240-kb SfiI restriction fragment (which includes markers DD70 and DD26), and the 97-kb BssHII restriction fragment (which includes markers wg5A1, y14, and s338). Below the map are shown the positions of the inserts in BAC F15 and BAC C16. Arrows at the end of the restriction fragments and of the BAC clones indicate that the segments/inserts extend an unknown distance in the direction of the arrow. Recombination breakpoints are marked by X’s, and the positions of the A to E recombination breakpoints are shown. The exact location of breakpoints between DD70 and DD26 and between DD26 and c31 is not known.

Figure 5. Integrated Physical and Genetic Map of the Chromosomal Region Encompassing the S8 Haplotype.
Table 3. S Locus–Linked Open Reading Frames That Do Not Function in SI Specificity

<table>
<thead>
<tr>
<th>Approximate Distance 3' of SLG_8 (kb)</th>
<th>Sequence Similarity to Known Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2</td>
<td>None [SLL2/s299; Yu et al., 1996; Conner et al., 1998]</td>
</tr>
<tr>
<td>4.4</td>
<td>Clp protease [s298; Conner et al., 1998; Letham and Nasrallah, 1998]</td>
</tr>
<tr>
<td>14</td>
<td>Neuropeptide FMRFamide precursor isolog</td>
</tr>
<tr>
<td>19</td>
<td>Seven-in-absentia isolog [cX; Conner et al., 1998]</td>
</tr>
<tr>
<td>24</td>
<td>Methionyl-tRNA formyltransferase</td>
</tr>
<tr>
<td>27</td>
<td>None [s68-1.2s; Conner et al., 1998]</td>
</tr>
<tr>
<td>33</td>
<td>None [DD15/SPA; Casselman et al., 2000]</td>
</tr>
<tr>
<td>42</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>50</td>
<td>Fructokinase [c17; Conner et al., 1998]</td>
</tr>
<tr>
<td>54</td>
<td>Serine/threonine kinase^a</td>
</tr>
<tr>
<td>57</td>
<td>Calmodulin [c20; Conner et al., 1998]</td>
</tr>
</tbody>
</table>

^a cDNAs isolated from Brassica. ^b cDNAs isolated from Arabidopsis (Conner et al., 1998).

METHODS

Plant Material

Brassica oleracea var alboblabra plants homozygous for the S_2 haplotype as well as B. oleracea var acephala plants homozygous for the S_6 haplotype were derived from the Gene Bank Facility (Wellesbourne, UK), courtesy of D.J. Ockendon. B. campestris plants homozygous for the S_9 haplotype were derived from a wild population in Oguni, Japan, and were obtained from K. Hinata (Research Institute of Seed Production Co., Sendai, Japan). The parents were crossed to produce self-incompatible heterozygous offspring, as previously described (Hinata and Prakash, 1984). Self-compatibility in plants homozygous for the S_9 haplotype were derived from a wild population in Oguni, Japan, and were obtained from K. Hinata (Research Institute of Seed Production Co., Sendai, Japan). Self-compatibility in plants homozygous for the S_9 haplotype was selected along with self-compatible S_9 plants, and nine S_9 individuals of a segregating F_2 population and pooled to create an S_9 bulk and an S_8 bulk. All 240 primer combinations provided in the RNAImage kits were used, potentially displaying a large proportion of cellular transcripts.

DNA Probes

Probes generated from the differential display screen described above are preceded by DD and include DD70, DD26, and DD15. Probes derived from the S contigs are preceded by s and include s16, s13, sBH2.0, s298, s33-13, s40-2-1.7, s338, and s400. Markers c31 and y14 originated from a Brassica/Arabidopsis comparative mapping study (Conner et al., 1998). Marker wg5A1 was generated in a B. oleracea mapping study (Camargo et al., 1997). The SL1 marker is a fragment of SSL1 (Yu et al., 1996) that was amplified from B. napus (cv Westar) genomic DNA by using primers specific to the intronless 3' end of the gene. The s299/SSL2 (Yu et al., 1996; Conner et al., 1998) marker is a cDNA isolated from a XUNIZAP library by using the corresponding genomic fragment from the S contigs. The SLG probe used to determine the genotypes of the segregating populations was derived from the S_9 haplotype. This probe hybridizes with S locus genes from the S_9, S_8, and S_6 haplotypes. All DNA fragments were produced by polymerase chain reaction amplification of insert from intact plasmid. Primers were specific either to the plasmid or to the insert itself. Fragments were labeled with phosphorus-32 by using a random-primed DNA labeling kit (Boehringer Mannheim).

DNA Gel Blot Analysis

For conventional DNA gel blot analysis, DNA was prepared using a minipreparation procedure, digested with HindIII, and subjected to electrophoresis on 0.8% (w/v) agarose gels. For pulsed-field gel electrophoresis, high molecular weight genomic DNA was isolated by preparing nuclei from Brassica/Arabidopsis (Conner et al., 1998). Marker wg5A1 was generated in a B. oleracea mapping study (Camargo et al., 1997). The SL1 marker is a fragment of SSL1 (Yu et al., 1996) that was amplified from B. napus (cv Westar) genomic DNA by using primers specific to the intronless 3' end of the gene. The s299/SSL2 (Yu et al., 1996; Conner et al., 1998) marker is a cDNA isolated from a XUNIZAP library by using the corresponding genomic fragment from the S contigs. The SLG probe used to determine the genotypes of the segregating populations was derived from the S_9 haplotype. This probe hybridizes with S locus genes from the S_9, S_8, and S_6 haplotypes.

For the S locus, a total of 540 seeds were sown. Leaf tissue was collected from each plant for DNA extraction.

Differential Display Screen

Differential display was performed using the RNAImage kit from the GenHunter Corporation (Nashville, TN). To isolate anther-expressed sequences that might represent candidates for the pollen SI specificity factor, we performed differential display by using anther RNA (Casselman et al., 1998). Poly(A)^+ RNA was prepared from anthers dissected from buds 5 to 7, 7 to 9, and >9 mm in length, corresponding, respectively, to the binucleate, a mix of binucleate and trinucleate, and trinucleate stages of microspore development (Boyce and Nasrallah, 1995). Equal amounts of RNA from each stage were pooled. Additionally, the buds were collected from six S_6 S_7 and nine S_6 S_6 individuals of a segregating F_2 population and pooled to create an S_2 bulk and an S_6 bulk. All 240 primer combinations provided in the RNAImage kits were used, potentially displaying a large proportion of cellular transcripts.
After electrophoresis, DNA was transferred to GeneScreen Plus (DuPont-New England Nuclear) nylon membranes by capillary action in a denaturing solution. Prehybridization and hybridization of the membranes were performed at 65°C in 10% (w/v) dextran sulfate, 330 mM sodium phosphate, pH 7.0, 10 mM EDTA, and 5% (w/v) SDS. Generally, membranes were washed at 65°C in 0.4% (w/v) SDS and 2 × SSC (1 × SSC is 0.1 M NaCl and 0.015 M sodium citrate), although some probes required higher stringency washes.

Construction of a Bacterial Artificial Chromosome Library from the B. campestris S₈ Homozygote and Analysis of Overlapping Bacterial Artificial Chromosome Clones from the S Locus Region

A large-insert library of B. campestris S₂S₈ DNA was constructed in the pBeloBAC vector according to Woo et al. (1994). A series of overlapping clones derived from the S locus region were isolated by hybridization with the single-copy s298 probe (Conner et al., 1998).

DNA was prepared from two of the positive bacterial artificial chromosome (BAC) clones, C16 and F15, by using a modified alkaline lysis minipreparation protocol. After digestion, the DNA was resolved with PFGE by using a CHEF-DRII apparatus on 1% (w/v) 0.5 × Tris-borate-EDTA agarose gels. Digested BAC DNA was run at 150 V with a pulse-switching interval ramped from 5 to 120 sec over 24 hr at 14°C. DNA was transferred to nylon membranes as described above. Membranes were hybridized and washed as given above.

Generation and Analysis of Recombinant S Haplotypes

The recombinant S haplotypes A, B, and D were identified in mod-mutant plants. For phenotypic analysis, the recombinant S haplotypes were introgressed into a MOD background as follows. For each recombinant S haplotype, the original F₂ plant carrying the recombinant haplotype was selfed and F₃ plants homozygous for the recombinant S haplotype were identified by restriction fragment length polymorphism (RFLP) analysis. Two homozygous recombinant F₃ plants were selected and backcrossed to a nonrecombinant S₂S₂MODMOD plant. The resulting progeny plants were selfed by bud pollination to generate a population of plants segregating for the recombinant S haplotype and for the mod locus. Plants homozygous for the recombinant S haplotype and carrying at least one copy of the MOD allele were identified by RFLP analysis, as described by Ikeda et al. (1997).

To determine pollination phenotype, we pollinated stigmas and 3 hr after pollination we fixed and processed them for staining with de-colored aniline blue (Kho and Baer, 1968). Pollen tube development was monitored by fluorescence microscopy.

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## Determining the Physical Limits of the Brassica S Locus by Recombinational Analysis

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