Isolation of a Second S-Locus-Related cDNA from Brassica oleracea: Genetic Relationships Between the S Locus and Two Related Loci

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ABSTRACT

Self-incompatibility in Brassica oleracea is controlled by the highly polymorphic S locus. Isolation and subsequent characterization of the S-locus-glycoprotein (SLG) gene, which encodes the S-locus-specific glycoprotein (SLSG), has revealed the presence of a self-incompatibility multigene family. One of these S-locus-related genes, SLR1, has been shown to be expressed. In this study we present the isolation and preliminary characterization of a second expressed S-locus-related sequence, SLR2. Through restriction fragment length polymorphism (RFLP) linkage analysis we demonstrate that the SLR1 and SLR2 loci reside approximately 18.5 map units apart in one linkage group that segregates independently of the S locus. The identification of a second SLR gene expressed in stigmas suggests that loci unlinked to the S locus may play a role in the self-incompatibility response, or in pollination in general.

SELF-INCOMPATIBILITY (SI) has evolved in many plant species as a means of creating genetic diversity through the promotion of out crossing (De Nettancourt 1977). In Brassica species, SI is manifested by a specific cell to cell interaction between a pollen grain and the surface of a stigma papillar cell which inhibits normal pollen tube growth at the stigma surface, thereby preventing fertilization. Transmission genetics has revealed the specificity of the SI recognition mechanism to be controlled by one highly polymorphic locus, designated S, for which greater than 50 naturally occurring alleles have been identified (Thompson 1957; Ockendon 1974, 1982). An incompatible cross results if the same S allele is active in pollen and stigma.

Genes linked to the S locus have been isolated from a number of different S-allele homozygotes of Brassica oleracea (Nasrallah et al. 1985, 1987; Lalonde et al. 1989; Trick and Flavell 1989; Chen and Nasrallah 1990). These genes, designated S-locus glycoprotein (SLG) genes, encode S-locus-specific glycoproteins (SLSG), the stigma molecules believed to be involved in the discrimination of self-pollen. When SLG cDNAs, were used as probes against blots of restriction enzyme digested Brassica genomic DNA, it was evident that SLG was just one of a number of related sequences which we refer to as the S-multigene family. The S-multigene family is comprised of genes that are genetically linked to the S locus, such as the SLG gene, and other genes that reside elsewhere in the genome (Nasrallah et al. 1985; Nasrallah, Yu and Nasrallah 1988; Dwyer et al. 1989; Chen and Nasrallah 1990). Genes that exhibit sequence similarity to the SLG genes, but that are genetically unlinked to the S locus, are designated S-locus-related (SLR) genes. The expression of one of these genes, the SLR1 gene, has been demonstrated (Lalonde et al. 1989; Trick and Flavell 1989). Interestingly, in contrast to the high degree of polymorphism exhibited by SLG alleles, SLR1 alleles are extremely conserved in all S genotypes examined to date, and since SLR1 is genetically unlinked to the S locus, it is not likely to be involved in determining SI specificity (Lalonde et al. 1989).

Because of the existence of these multiple related sequences, the task of determining that a DNA sequence isolated by homology to previously identified SLG sequences is derived from the S locus is not straightforward. Another complicating factor is the extensive polymorphism of SLG alleles. While the SLG genes from a number of S alleles (e.g., the S6, S13, S14, S22, S38 and S29 alleles) can be readily identified by virtue of their hybridization to an SLG gene-specific probe (Nasrallah, Yu and Nasrallah 1988) and of the reactivity of their glycoprotein products with MAb H8, a monoclonal antibody raised against S6 SLG purified from stigmas and specific for SLSG (Kandasamy et al. 1989), the SLG genes from other more diverged S alleles are more difficult to identify. This latter class of alleles, exemplified by the S2, S3 and S15 self-incompatibility alleles, produces SLSG that are not recognized by MAb H8 (Kandasamy et al. 1989). We refer to this class as CRM+ (cross-reacting material

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negative) in contrast to the CRM\(^+\) immunologically cross-reactive class. In addition, full length SLG-cDNA probes derived from the CRM\(^+\) class hybridize only weakly, and the corresponding SLG gene-specific probes not at all, to genomic DNA extracted from plants homozygous for CRM\(^-\) alleles (LALONDE et al. 1989; CHEN and NASRALLAH 1990). Sequence comparisons of SLG genes isolated from different S-allele homozygotes have revealed approximately 90% nucleotide identity overall between alleles within the CRM\(^+\) class (NASRALLAH and NASRALLAH 1989), and 70% nucleotide identity between the CRM\(^+\) alleles and S2, a CRM\(^-\) allele (CHEN and NASRALLAH 1990).

In view of the divergence between the two classes of alleles, and in order to understand the evolution of the S-multigene family, we were interested in characterizing SLG and related genes from plants homozygous for alleles of the CRM\(^-\) class. In this paper, we describe the isolation of an S-related cDNA sequence from the S2 and S3 genotypes, and the corresponding genomic sequence from a strain homozygous for the S5 CRM\(^+\) allele. These DNA sequences are highly homologous to a DNA sequence recently described as the SLG sequence of an S\(_5\) homozygote (SCUTT et al. 1990). We demonstrate through genetic analysis that these sequences are derived, not from the S locus, but from a previously unidentified S-locus-related gene which we designate SLRP. In addition, we show that the SLR1 and SLR2 genes reside in a single linkage group that segregates independently of the S locus.

**MATERIALS AND METHODS**

**Plant material:** Brassica oleracea lines homozygous for the S\(_3\), S\(_4\), S\(_{13}\), S\(_{16}\), S\(_{22}\), and S\(_{29}\) self-incompatibility alleles were of the variety acephala (kale), while the lines homozygous for the S\(_2\), S\(_3\), and S\(_{29}\) alleles were of the varieties italicca (broccoli), capitata (cabbage), and alboglabra (Chinese kale), respectively. All plant material was originally obtained from the Gene Bank Facility at Wellsbourne, United Kingdom, by the generous donation of D. J. OCKENDON, with the exception of the homozygous S\(_{14}\) cabbage variety, which was developed at Cornell University. All strains were propagated by self-pollination of unopened buds prior to the onset of the self-incompatibility response, corresponding to a stage three to four days before anthesis.

**Determination of incompatibility phenotype:** The incompatibility phenotype of the F\(_2\) progeny of a cross between the S\(_6\) and S\(_{14}\) homozygotes was determined by crosses to tester homozygous strains and by diallel analysis. Pollen-free mature stigmas were pollinated with isolated dehisced anthers. After 12–24 hr, stigmas were excised, stained with decolorized aniline blue and examined by fluorescence microscopy to determine the extent of pollen-tube growth (MARTIN 1959).

**Isolation of cDNA and genomic clones:** Sigma cDNA libraries were constructed from strains homozygous for the S\(_2\) and S\(_3\) alleles according to the method of \_\_\_\_\_\_ (1983) as described previously (NASRALLAH et al. 1987). Briefly, poly(A\(^+\)) mRNA was selected with oligo(dT)-cellulose from total RNA prepared as described below, and cDNA was generated by reverse transcription. The addition of EcoRI linkers was followed by ligation to Agt10 vector arms and in vitro packaging using the Gigapack system (Stratagene, San Diego, California). Screening of the libraries was as described in the text: and resulted in the identification of a new class of SLG-related sequences which are the subject of this paper. For detailed restriction analysis, insertions from selected recombinant phage were subcloned into plasmid vectors. The plasmids from the S\(_3\) and S\(_{14}\) libraries that contained the longest inserts of 1570 and 1572 bp, respectively, were designated pBO2R2 and pBO5R2.

Construction of an EMBL4 genomic library of DNA isolated from the S\(_6\) genotype and isolation of recombinant clones containing regions of homology to SLG cDNA was previously described (NASRALLAH, YU and NASRALLAH 1988). In the present study, appropriate clones were identified as follows. DNA was isolated from the EMBL4 clones, digested to completion with EcoRI and subjected to DNA gel blot hybridization. Two recombinant libraries from other S-multigene family members, an S\_1/EcoRI fragment corresponding to the 3' terminal 298 bp of the pBO2R2 DNA sequence shown in Figure 2 was used as a probe. A 4.3-kb EcoRI/Xhol fragment from one clone that hybridized strongly to this probe was identified and subcloned into pBluescript vectors (Stratagene) for detailed restriction analysis.

**Sequence analysis:** Overlapping restriction fragments were subcloned into the M13 vectors mp18 and mp19 (YANISCH-PERRON, VIERA and MESSING 1985). Single-stranded DNA templates were sequenced by the dideoxynucleotide chain termination method (SANGER, NICKLEN and COULSON 1977) using the standard Sequenase kit protocol (United States Biochemical, Cleveland, Ohio).

**Plant DNA isolation and DNA gel blot hybridization:** Two methods were used for the isolation of genomic DNA. For the survey of S-allele homozygous strains, nuclei were isolated from leaf tissue, lysed, and the resulting high molecular weight DNA was purified by CsCl centrifugation (BINGHAM, LEWIS and RUBIN 1981). For the genetic analysis of restriction fragment length polymorphisms, a DNA minipreparation procedure based on a rapid phage DNA extraction method was used to process tissue from the parental plants and the F\(_2\) progeny. Either 10 \(\mu\)g of CsCl purified DNA, or the amount of DNA extracted from approximately 300 mg leaf tissue by the minipreparation procedure was restricted with EcoRI and size fractionated on a 0.9% (w/v) agarose gel. Capillary transfer (SOUTHERN 1975) of the DNA to Genescreen Plus membranes (DuPont-New England Nucl. Clear, Boston, Massachusetts) was followed by prehybridization and hybridization at 65°C in 10% (w/v) dextran sulfate, 300 nm sodium phosphate (pH 7.0), 10 mm EDTA, 5% (v/v) SDS, and carrier DNA at 150 mg/ml. Radiolabeled probes were prepared by the random primed labeling reaction of FEINBERG and VOGELSTEIN (1983) using a commercially available kit (Boehringer Mannheim, Indianapolis, Indiana). Washes were at 65°C in 0.3 M sodium chloride, 40 mm Tris (pH 7.8), 2 mm EDTA, and 0.5% (w/v) SDS. The probes used were: (1) SLG-6, an SLG cDNA isolated from the S\(_3\) homozygote (NASRALLAH et al. 1987); (2) an SLG-specific probe derived from the 3' untranslated region of SLG cDNAs isolated from either the S\(_6\) or S\(_{14}\) homozygous genotype (NASRALLAH, YU and NASRALLAH 1988); (3) SLR1, a cDNA probe specific for the SLR1 gene (LALONDE et al. 1989), (4) the cDNA inserts of pBO2R2 and pBO5R2; and (5) a fragment corresponding to the 264 bp at the 3' end of the pBO2R2 DNA sequence shown in Figure 2.

**RNA isolation and RNA gel blot hybridization:** Total RNA was isolated from stigmas collected 1 day before anthesis using an SDS-proteinase K extraction procedure.
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Isolation of SLR2:

SLG-related cDNA clones from S2 and S3 homozygotes and analysis of DNA gel blot hybridization patterns: In order to isolate SLG and SLG-related sequences from the B. oleracea CRM+ alleles, we constructed cDNA libraries from poly(A+) RNA isolated from stigmas of an S2 homozygous strain and an S5 homozygous strain. Screening of the cDNA libraries was performed according to one of two methods. In one method, to avoid reclining the highly abundant SLRI sequences, the cDNA library was subjected to a differential hybridization procedure in which duplicate plaque lifts were probed separately with an SLG cDNA probe derived from the CRM+ S5 allele and an SLRI probe. This hybridization procedure led to the identification of two classes of clones. Class I clones showed greater signal intensity when hybridized with the SLRI probe. Class II clones showed greater signal intensity when hybridized with the SLG probe. In another method, an SLG probe derived from a CRM+ allele was used under reduced stringency as previously described (Chen and Nasrallah 1990), and also led to the identification of class I and class II clones.

Several cDNA inserts from each class were subcloned into plasmid vectors for further characterization. Restriction maps of representative class I clones were similar to the highly conserved SLR1 cDNA described previously (Lalonde et al. 1989) and were not characterized further. The restriction maps of class II clones were essentially identical, with the exception of variable length due to truncation at the 5' end as a result of incomplete reverse transcription during library construction. S2 and S3 derived class II cDNA clones that contained the longest inserts were designated pBO2R2 and pBO5R2 respectively and were used in all subsequent experiments.

The radioactively labeled cDNA inserts of pBO2R2 and pBO5R2 produced identical hybridization patterns in blot analyses of restriction enzyme digested Brassica DNA, and could therefore be used interchangeably (data not shown). In Figure 1, these patterns are compared to those obtained with an SLG cDNA probe derived from the S5 genotype. As pre-
Ss and SI5 DNA suggested a more complex situation at the SLR2 locus. On the other hand, bp probe in these genotypes and is consistent with shown in Figure 1C, this probe identified only one SZ2, cDNAs and for an SLR2 genomic clone isolated from more than one hybridizing restriction fragment in the region fragment demonstrates the specificity of the 264 bp strongly hybridizing EcoRI restriction fragment in S14, the pB02R2 and pBO5R2 probes produced hybridization patterns with a complexity similar to that observed with the SLG probe. However, in contrast to the result obtained with the SLG-6 probe, equivalent intensities of hybridization were obtained in the CRM+ and CRM− genotypes when hybridized with the pBO2R2 and pBO5R2 probes (Figure 1B). This result provided the first indication that the pBO2R2 and pBO5R2 cDNAs were derived from a gene other than SLG. In keeping with previous nomenclature, we will subsequently refer to this gene as SLR2.

The complexity of the hybridization pattern shown in Figure 1B precluded the direct identification of a restriction fragment that contained the SLR2 gene. In previous work we had demonstrated that S-multigene family members could be differentiated at the level of DNA blot hybridization using gene-specific probes derived from the 3′ untranslated termini of the relevant cDNAs (NASRALLAH, Yu and NASRALLAH 1988; DWYER et al. 1989; CHEN and NASRALLAH 1990). We therefore used a similar 3′-derived probe that contained a region corresponding to the 264 bp at the 3′ terminus of the pBO2R2 sequence in Figure 2. As shown in Figure 1C, this probe identified only one strongly hybridizing EcoRI restriction fragment in S14, S29, S59 and S58 DNA. Hybridization of a single restriction fragment demonstrates the specificity of the 264 bp probe in these genotypes and is consistent with homozygosity at the SLR2 locus. On the other hand, more than one hybridizing restriction fragment in the S5 and S15 DNA suggested a more complex situation that will be addressed later (see DISCUSSION).

DNA and amino acid sequence analysis: We obtained DNA sequence information for the SLR2 cDNAs and for an SLR2 genomic clone isolated from the S6 genotype as described in MATERIALS AND METHODS. The 1570-bp SLR2 cDNA insert of pB02R2 contained a complete open reading frame as shown in Figure 2. The sequence encoded 439 amino acids starting with an in-frame translation initiation codon (ATG). Typical eukaryotic polyadenylation signals (AATAAA) and poly(A) tail were found downstream of the termination codon (TAA). The 1329-bp coding region of the SLR2 genomic sequence contained an open reading frame of 434 amino acids starting with an in-frame initiation codon but was truncated at the 3′ end and missing six carboxy-terminal amino acids and an in-frame termination codon (Figure 2). The two SLR2 sequences were approximately 99% identical at the DNA level (not shown) and at the amino acid level (Figure 2). With the exception of one gap introduced to optimize the alignment, the sequences were co-linear, indicating that the SLR2 gene lacked intervening sequences, a feature shared with other expressed members of the Brassica S-multigene family (NASRALLAH, Yu and NASRALLAH 1988; LALONDE et al. 1989; CHEN and NASRALLAH 1990).

The two SLR2 sequences presented in this paper were also 99% identical to the sequence isolated from homozygous S5 B. oleracea plants by SCUTT et al. (1990). Our SLR2 genomic sequence isolated from the S6 genotype exhibited only four amino acid substitutions (shown by boxes in Figure 2) when compared to the SCUTT et al. sequence: isoleucine was substituted for leucine at position 24, proline for threonine at position 65, serine for arginine at position 235, and glycine for valine at position 236.

**Analysis of SLR2 gene expression in CRM+ and CRM− genotypes:** Expression of the SLR2 gene in the S2 and S5 genotypes, members of the CRM+ class of self-incompatibility alleles, was demonstrated by the isolation of cDNA clones. To determine if the SLR2 gene was expressed in the CRM+ class of self-incompatibility alleles, RNA blot analysis was utilized. Since the 264-bp SLR2 3′-probe hybridized to only one restriction fragment on a blot of DNA isolated from the CRM+ genotypes (Figure 1C), hybridization to RNA isolated from those genotypes would indicate expression of the SLR2 gene. Total stigma RNA isolated from plants homozygous for either the S5, S15, S6 or S14 allele was hybridized with the 264-bp SLR2 3′-probe. A hybridization signal was observed in each genotype examined (Figure 3A), indicating that the SLR2 gene was expressed in the CRM+ genotypes as well as the CRM− genotypes.

To verify that each lane contained equal amounts of RNA, the blot used to create Figure 3A was rehybridized with an SLR1-specific cDNA probe. Since the SLR1 transcript was previously shown to accumulate in equivalent amounts in all B. oleracea S genotypes tested to date (LALONDE et al. 1989), the hybridization signal obtained with the SLR1-cDNA probe was used as a measure of the amount of stigma RNA present in each lane of the blot. The SLR1 hybridization signal intensity was roughly equivalent for each genotype examined (Figure 3B). Thus, the variation in hybrid-
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and S1.l homozygotes were selected as the parental strains and nine representative F2 progeny. DNA isolated from parental (P) plants homozygous for either the S6 or S14 allele and from nine representative F2 progeny was restricted with EcoRI and subjected to DNA blot hybridization with radiolabeled probes. Shown are segregation patterns observed following hybridization with probes specific for SLG (A), SLR1 (B), and SLR2 (C). Panel D indicates the pollination phenotype of each plant: 6.6 designates an S6 homozygous plant; 14.14 an S14 homozygous plant; and 6.14 a heterozygous S6S14 plant.

determined by pollination assays. Phenotype assignments were also correlated to the pattern of S6- and S14-specific SLG polymorphisms as revealed by protein immunoblot analysis with MAb H8, a monoclonal antibody specific for SLG (data not shown). In all cases, the genotype of each of the three loci in the progeny plants could be determined as homozygous for the allele carried by the S6 parent, homozygous for the allele carried by the S14 parent, or heterozygous, based on the pattern of bands seen in each of the F2 lanes.

The data obtained from examining all 32 F2 progeny supported two previous findings: (1) The absence of recombinants between the SLG and SLR1 genes. To examine the possible linkage of SLR2 to either of the two previously defined loci, restriction fragment length polymorphism (RFLP) analysis was performed. For this analysis we used a population of 32 F2 plants derived a cross between two homozygotes of the CRM+ class because the specificity of the 264-bp SLR2 3′-probe was clearly established for these genotypes (Figure 1C). The S6 and S14 homozygotes were selected as the parental genotypes. In addition to being homozygous at the S locus, these strains were inbred, leading to the establishment of a homozygous condition at the SLR1 and SLR2 loci as well.

Figure 4 shows the RFLP patterns for the parental strains and nine representative F2 progeny. A single hybridizing restriction fragment and distinctive EcoRI RFLP was evident between the two parents (lanes S6 and S14) when EcoRI restricted genomic DNA was hybridized with probes specific for SLG (Figure 4A), SLR1 (Figure 4B), or SLR2 (Figure 4C). Figure 4D indicates the pollination phenotype of each plant as

DISCUSSION

We have identified a second transcribed S locus related gene, designated SLR2. The identification of
this gene was based on DNA sequence information and on genetic data. First, sequence analysis of the recombinant clones described in this paper showed that highly homologous (>99% identical) SLR2 sequences were isolated from Brassica strains homozygous for the divergent CRM− and CRM+ S alleles. Sequence comparisons also indicated that the cDNA sequence recently isolated by Scutt et al. (1990) from the S5 genotype was derived, not from the S locus as reported, but from the SLR2 locus and is allelic to the sequences presented here. Second, and most importantly, RFLP analysis of segregating F2 plants established that the SLR2 gene was distinct from the SLG and SLR1 genes. To circumvent the difficulty imposed by the presence of multiple cross-hybridizing sequences, we utilized probes specific for each gene to demonstrate that the SLR2 gene segregated independently of the S locus and was located approximately 18.5 map units away from the SLR1 locus.

In addition to SLR2 sequence comparisons between different genotypes, it is interesting to consider the relationship of SLR2 to other members of the S multigene family. As judged by blot analysis of Brassica DNA (Figure 1B), the genome of B. oleracea contains a number of sequences related to SLR2. The SLR2 hybridization pattern was similar in complexity to that obtained using an SLG cDNA probe (Figure 1, A and B). The patterns obtained with the two probes differed however, and several restriction fragments were identified by the SLR2 cDNA probe but not by the SLG cDNA probe. It therefore appears that the number of S-related sequences within the genome may be larger than previously anticipated (Dwyer et al. 1989). It should also be noted that the SLR2 hybridization pattern was quite different from the simple pattern of one predominant hybridizing restriction fragment typically obtained with the SLR1 cDNA probe (Lalonde et al. 1989). The SLR2 gene is therefore evolutionarily more closely related to other members of the S gene family than the SLR1 gene. In support of this conclusion, the SLR2 DNA sequences shared approximately 73% nucleotide identity with SLG-6 (Nasrallah et al. 1987), and less than 65% nucleotide identity with the highly conserved SLR1 sequence (Lalonde et al. 1989).

Significantly, the relationship of SLR2 to SLG differs in the CRM+ and CRM− subset of self-incompatibility genotypes. When SLR2 was compared to the SLG sequence isolated from the S5 genotype (Chen and Nasrallah 1990), the two sequences were found to share over 90% nucleotide identity. It is noteworthy that the 264-bp probe derived from the 3′ untranslated region of the SLR2 cDNA acted as a gene specific probe in the CRM+ genotypes, but hybridized to more than one restriction fragment in the CRM− S5 and S15 genotypes (Figure 1C). While the presence of two hybridizing restriction fragments in the S15 genotype may reflect a heterozygous condition at the SLR2 locus, the presence of three hybridizing restriction fragments in the S5 genotype cannot be easily explained as a heterozygous condition (Figure 1C). This complex pattern may be due to the presence of an EcoRI restriction site within one of the genomic regions identified by the probe, or alternatively, it is possible that the two CRM+ genotypes examined contain related sequences that cross-hybridize with the 264-bp SLR2 3′-probe, in which case the probe may only be considered specific for the SLR2 gene in genotypes belonging to the CRM+ class.

We have shown that RNA blot analysis, and in two cases by cDNA cloning, that all of the Brassica genotypes examined contained a transcribed SLR2 gene. Differences in hybridization intensity to the 264-bp SLR2 3′-probe were noted however, and were most pronounced between the CRM− and CRM+ genotypes. The significance of these differences is not understood, but may reflect a true difference in transcriptional activity of the SLR2 gene in the CRM+ and CRM− genotypes. For example, reduced SLR2 transcription might arise if the SLR2 gene competes less successfully with the highly expressed CRM+ SLG genes for a limiting amount of transcribing protein factors. Alternatively, the increased hybridization signal intensity observed in the CRM− genotypes may be due to the presence of additional expressed genes that are recognized by the 264-bp SLR2 3′-probe (see above discussion).

The role that the putative SLR2 gene product plays in the interaction between pollen and stigma has not been identified. The fact that this gene segregates independently of the S locus indicates that, like the SLR1 gene, it cannot be a determinant of self-incompatibility specificity. Both SLR genes may operate in a support role to allow the functioning of the SLG gene in specific pollen recognition. This possibility will be tested in forthcoming studies aimed at identifying the SLR2 protein product and at defining its temporal and spatial distribution during flower development.

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