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A Functional *S* Locus Anther Gene Is Not Required for the Self-Incompatibility Response in *Brassica oleracea*

Martine Pastuglia,[a] Véronique Ruffio-Châble,[b] Valérie Delorme,[a,1] Thierry Gaude,[a] Christian Dumas,[a] and J. Mark Cock[a,2]

[a]Reproduction et Développement des Plantes, UMR 9938 CNRS-INRA-ENSL, Ecole Normale Supérieure de Lyon, 46 allée d'Italie, 69364 Lyon Cedex 07, France

[b]Amélioration des Plantes, Institut National de la Recherche Agronomique—Domaine de la Motte, BP29, 35650 Le Rheu Cedex, France

The self-incompatibility (SI) response in Brassica involves recognition of self-pollen by the papillar cells of the stigma and is mediated by the products of genes localized at the *S* (self-incompatibility) locus. Two *S* locus genes, *SRK* and *SLG*, are thought to encode components of a receptor complex present in the female partner. The putative gene product of *SLA*, a third *S* locus–linked gene that is expressed specifically in anthers, is a candidate for the male component of the SI recognition system. The identification of a mutant *SLA* allele, interrupted by a large insert resembling a retrotransposon, in self-compatible *Brassica napus* initially suggested that *SLA* played an essential role in the SI response. In this study, we have characterized an *SLA* allele from a self-compatible *B. oleracea* var *acephala* line and show that it too is interrupted by a large insert. However, analysis of seven *B. oleracea* var *botrytis* lines exhibiting both self-compatible and self-incompatible phenotypes showed that these lines carry an *S* allele very similar or identical to that of the *B. oleracea* var *acephala* line and that the *SLA* gene is interrupted by an insert in all seven lines. The insertion of the putative retrotransposon was shown to interfere with gene expression, with no *SLA* transcripts being detected by RNA gel blot analysis in a self-incompatible *B. oleracea* var *botrytis* line carrying an interrupted *SLA* gene. These data indicate that a functional *SLA* gene is not required for the SI response in Brassica.

INTRODUCTION

During compatible pollination in Brassica, pollen grains adhere to the papillar cells of the stigma, hydrate, and then germinate to emit a pollen tube. The pollen tube grows down through the transmitting tissue carrying the male gametes, which fertilize an ovum in an ovary at the base of the pistil. In self-incompatible plants, self-pollen grains are recognized by the papillar cells, and the pollination process is rapidly blocked, usually before the hydration or germination stage (reviewed in Nasrallah et al., 1994b; McCubbin and Kao, 1996).

Self-incompatibility (SI) in Brassica is controlled by a single, highly polymorphic locus, the *S* locus, of which there are >50 alleles (Ockendon, 1974, 1982). Rejection of self-pollen occurs when the same *S* allele is expressed in both pollen and stigma. The effectiveness of the SI response varies, depending on which *S* alleles are present and the dominance/recessivity relationships that exist between different *S* alleles. Based on these phenomena, *S* alleles have been broadly classified into two groups, depending on whether they tend to be dominant or codominant and confer a strong SI response (class I) or whether they tend to be recessive and confer a weak SI response (class II).

The *S* locus has a complex molecular structure. A number of genes have been shown to be genetically linked to the *S* locus: *SLG* (for *S* locus glycoprotein; Nasrallah et al., 1985, 1987), *SRK* (for *S* locus receptor kinase; Stein et al., 1991), *SLA* (for *S* locus anther; Boyes and Nasrallah, 1995), *298* and *299* (Boyes et al., 1997), and SLL_1 and SLL_2 (for *S* locus–linked genes; Yu et al., 1996). Moreover, pulse field gel electrophoresis analysis suggests that the polymorphic nature of the *S* locus may extend to a region of up to 570 kb surrounding the *S* locus genes (Boyes and Nasrallah, 1993). As a result of this complex structure and the fact that several of the *S* locus genes have been implicated in the SI response, the term "*S* haplotype" is used rather than "*S* allele" to describe different allelic forms of the *S* locus (Boyes and Nasrallah, 1993).

Two *S* locus genes, *SLG* and the *SRK*, are thought to be involved in perception of self-pollen by the stigma. Both genes are highly polymorphic, and both are expressed specifically at the surface of mature stigmas. Low levels of mRNA

[1] Current address: Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, United Kingdom.

[2] To whom correspondence should be addressed. E-mail mark.cock@ens-lyon.fr; fax 33-4-72-72-86-00.

for these two genes are also present in anthers (Sato et al., 1991; Stein et al., 1991), but the protein products of the genes have not been detected in this tissue (Delorme et al., 1995b; Stein et al., 1996). Sequence data are available for several different alleles of both *SLG* and *SRK*. These genes exhibit the high level of polymorphism expected for genes involved in the SI response, and moreover, the different alleles of *SLG* and *SRK* can be divided into two classes based on sequence similarities, which correspond closely with the two classes defined on the basis of phenotype. Further evidence that *SRK* is involved in the SI response has come from the identification of self-compatible Brassica plants that also possess mutated *SRK* genes (Goring et al., 1993; Nasrallah et al., 1994a).

SLG encodes an abundant, secreted glycoprotein (Nasrallah et al., 1985, 1987), whereas *SRK* encodes a membrane-spanning protein kinase with a predicted extracellular domain that shares extensive sequence identity (~90%) with SLG (Stein et al., 1991; Delorme et al., 1995b). *SRK* is a member of the large receptor-like kinase (RLK) gene family that has been identified in plants (Becraft et al., 1996; Braun and Walker, 1996; Hervé et al., 1996; Schulze-Muthe et al., 1996; Swarup et al., 1996; Wang et al., 1996). The different gene products encoded by members of the RLK superfamily are thought to function as receptors based on their similarity to animal receptor kinases, but this has yet to be demonstrated experimentally. The *SRK* gene product is perhaps the best characterized member of this family. Monoclonal antibodies (MAbs) raised against a peptide based on the SRK sequence detected a 120-kD protein in stigma extracts, and this protein has been shown to be glycosylated and anchored in the plasma membrane (Delorme et al., 1995b; Stein et al., 1996). The kinase domain of SRK has been expressed in *Escherichia coli* and has been shown to have protein serine/threonine kinase activity (Goring and Rothstein, 1992). A soluble, truncated form of SRK (eSRK for extracellular form of SRK) has also been detected in stigma extracts (Giranton et al., 1995). This protein, which is produced as a result of alternative splicing of *SRK* transcripts, lacks the membrane-spanning and kinase domains of SRK and therefore closely resembles SLG.

Current models describing the pollen–pistil recognition step of the SI response propose that SRK, present in the cell membrane of papillar cells on the stigma surface, binds to a pollen-borne ligand and initiates a protein phosphorylation cascade that leads to the SI response. SLG and eSRK are thought to modulate signal transduction via SRK. Identification of a ligand bound by SRK would greatly facilitate testing of these models. To date, none of the proteins encoded by members of the RLK superfamily has been shown to bind a ligand (hence the term receptor-like kinase), although in the case of SRK, two candidate ligands have been proposed in the literature.

The first candidate is a small protein (PCP7 for 7-kD pollen coat protein) present on the surface of Brassica pollen (in the pollen coat). It has been shown to interact with SLG in vitro (Doughty et al., 1993). It is thought that PCP7 or a related protein might therefore bind to SRK, either directly as a result of SRK's similarity with SLG or indirectly as a complex incorporating SLG. However, no PCP has yet been shown to bind specifically to the gene products of a particular *S* haplotype, and although a cDNA clone has been isolated for one member of the *PCP* gene family, no members of this gene family have yet been shown to be linked to the *S* locus (Stanchev et al., 1996).

A second candidate for the male component of the SI recognition system is the product of the *SLA* gene, which was originally identified in the S_2 haplotype, where it is located just downstream of the SLG_2 gene (Boyes and Nasrallah, 1995). *SLA* encodes two complementary transcripts that accumulate specifically in anthers. One of the transcripts contains two small open reading frames (ORFs), and it has been proposed that a peptide encoded by *SLA* might interact, either directly or indirectly, with SLG and SRK to initiate the SI response (Boyes and Nasrallah, 1995). A second *SLA* allele was shown to be interrupted by a large insertion resembling a retrotransposon in a self-compatible *B. napus* line, suggesting a correlation between the presence of a functional *SLA* gene and SI (Boyes and Nasrallah, 1995). *SLA* was not detected in a number of other *S* haplotypes, and it has been suggested that this might be due to a high level of polymorphism between *SLA* alleles (Boyes and Nasrallah, 1995), as would be expected for a gene involved in the SI response.

In this study, we have characterized the *SLA* gene from the self-compatible *B. oleracea* line P57Sc and have shown that it also contains a large insertion resembling a retrotransposon. However, the structure of the *SLA* gene in a number of *B. oleracea* var *botrytis* lines, which carry the same *S* haplotype as P57Sc but exhibit different levels of SI, indicated that a functional *SLA* gene is not required for the SI response.

RESULTS

Cloning of *SLG* and *SLA* from a Self-Compatible *B. oleracea* Line

The cloning and characterization of a cDNA clone, denoted CG15, from the *B. oleracea* line P57Sc has been described previously (Gaude et al., 1993). Based on sequence similarity to previously characterized *SLG* genes, CG15 was thought to correspond to the *SLG* gene of the P57Sc line (SLG_{Sc}; Gaude et al., 1993).

In this study, an 880-bp BamHI fragment from the 5′ end of the CG15 cDNA was used to probe 10^6 plaque-forming units of a P57Sc genomic library. Subcloning and DNA sequence analysis of phages, which hybridized with the probe, revealed that they included one clone with 100% sequence identity to CG15 and a second clone, denoted CG514, that was highly similar but not identical to CG15. A 10.5-kb region of the latter clone was sequenced and shown to share

extensive similarity with the *SLG/SLA* region of the S_2 haplotype (Figure 1). Based on this similarity, the two genes carried by the CG514 clone have been designated SLG_{Sc} and SLA_{Sc}, and hence, the gene represented by CG15 is no longer denoted SLG_{Sc}, as was originally proposed (Gaude et al., 1993). We now propose that CG15 corresponds to a second, *S* locus–linked gene and may represent an alternative transcript of the SRK_{Sc} gene encoding a truncated form of SRK analogous to the eSRK protein identified in the S_3 haplotype (Giranton et al., 1995).

Comparison of the *SLG* and *SLA* Genes of the S_{Sc} and S_2 Haplotypes

Alignment of the *SLG/SLA* gene region of the *S* locus of the P57Sc line (S_{Sc}) with the corresponding region of the S_2 haplotype showed that the two sequences are highly similar, apart from an insertion of 4826 bp in SLA_{Sc} that is described in more detail below. SLG_2 has been shown to encode two alternative transcripts, which encode the soluble SLG_2 protein and a membrane-anchored form of SLG_2, $mSLG_2$, respectively. The SLG_{Sc} gene possesses both of the exons present in the SLG_2 gene and therefore potentially encodes both SLG and mSLG proteins. The deduced amino acid sequences of SLG_{Sc} and SLG_2 share 98.2% similarity. In both the S_{Sc} and S_2 haplotypes, *SLA* is located directly downstream of *SLG* (Figure 1). Boyes and Nasrallah (1995) reported the sequences of cDNAs corresponding to two overlapping transcripts of the SLA_2 gene: a spliced transcript that was transcribed from the same strand as SLG_2 and that contains two ORFs and an unspliced transcript that was transcribed from the opposite strand. Sequences corresponding to both of these transcripts are present in SLA_{Sc} and exhibit 99.6% nucleotide similarity with the spliced and unspliced transcripts of SLA_2 (Figure 1). The two ORFs in the spliced transcript are identical between the two alleles, except that the eighth codon of ORF2 is an alanine codon (GCG) in SLA_2 and an arginine codon (CGC) in SLA_{Sc}.

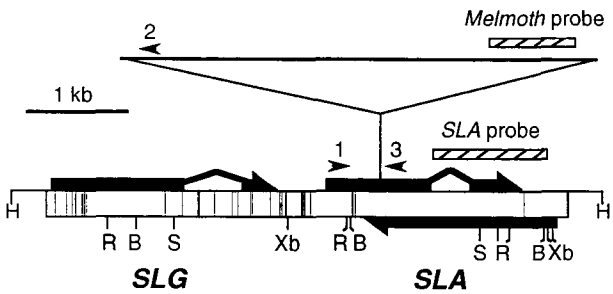

Figure 1. Structure of the *SLG/SLA* Gene Region in the Self-Compatible *B. oleracea* Line P57Sc Carrying the S_{Sc} Haplotype.

Sequences corresponding to transcribed regions in the S_2 haplotype are shown as half-arrows appearing above the line if the transcript runs from left to right and below the line if the transcript runs from right to left. The open box corresponds to the region of the S_{Sc} haplotype that has been sequenced in this study. Nucleotides differing from the corresponding region of the S_2 haplotype are indicated by vertical lines in the box. A 4826-bp insert found in SLA_{Sc} but absent from SLA_2 is marked by a triangle. Arrowheads mark the positions of three oligonucleotides, OSLA1, OSLA2, and OSLA3 (represented by 1, 2, and 3, respectively). An *SLA*-specific DNA probe (*SLA* probe) and a probe for the 4826-bp element inserted in the *SLA* gene (*Melmoth* probe) are shown as hatched bars. H, HindIII; R, EcoRI; B, BamHI; S, SalI; Xb, XbaI. The EMBL accession number for the SLG_{Sc}/SLA_{Sc} sequence is Y12321.

The 4826-bp insertion in SLA_{Sc} contains all the structural features of a retrotransposon (Figure 2; reviewed in Grandbastien, 1992). The insertion contains two long terminal repeats (LTRs) that differ by 7 bp at a single position 32 bp from the 3′ end. The LTRs are flanked by a duplicated 6-bp sequence of the *SLA* gene resembling the direct repeats found at sites of transposon insertion. Between the two LTRs is a region of 4432 bp that is flanked by sequences resembling sites involved in priming reverse transcription of known retrotransposons (Figure 2A). These sites consist of an 11-bp region identical to the initiator methionine tRNA from Arabidopsis at the 5′ end of the internal domain and a purine-rich sequence at the 3′ end.

The 4432-bp internal domain contains two long ORFs (Figure 2B). ORF1 is predicted to encode an 1132–amino acid polypeptide that includes regions similar to conserved motifs found in retrotransposons (Figure 2B): an RNA binding site involved in positioning the tRNA during the initiation of reverse transcription, a protease domain implicated in the endoproteolytic cleavage of the polypeptide, an integrase region necessary for the integration of the retroelement in the host genome, and a reverse transcriptase domain. ORF2 is located 64 bp downstream of the end of ORF1 and is predicted to encode a 253–amino acid protein with an RNase H domain. Comparison of the sequence of this element, which we have named *Melmoth* (Maturin, 1820), with previously identified retrotransposons showed that it is most closely related to the *Ty1/copia* family of retroelements (Figure 2B).

A DNA fragment from within the *Melmoth* element (see Figure 1) was used to probe a DNA blot of restriction endonuclease–digested genomic DNA of the *B. oleracea* var *acephala* and *B. oleracea* var *botrytis* (cauliflower) lines used in this study. The results indicated the presence of between one and three copies of the *Melmoth* sequence per haploid genome, depending on the genotype (data not shown). These results suggest that the *Melmoth* element is not replicating at a high frequency in the genome of the lines analyzed, and it is possible that the element is an inactive member of the *Ty1/copia* family. However, low copy number does not necessarily indicate that an element is inactive; the maize retrotransposon *Bs1*, which is present at only two to three copies in the genome, is nonetheless active because it was isolated after a transposition event (Jin and Bennetzen, 1989). On the other hand, the 7-bp difference between the two

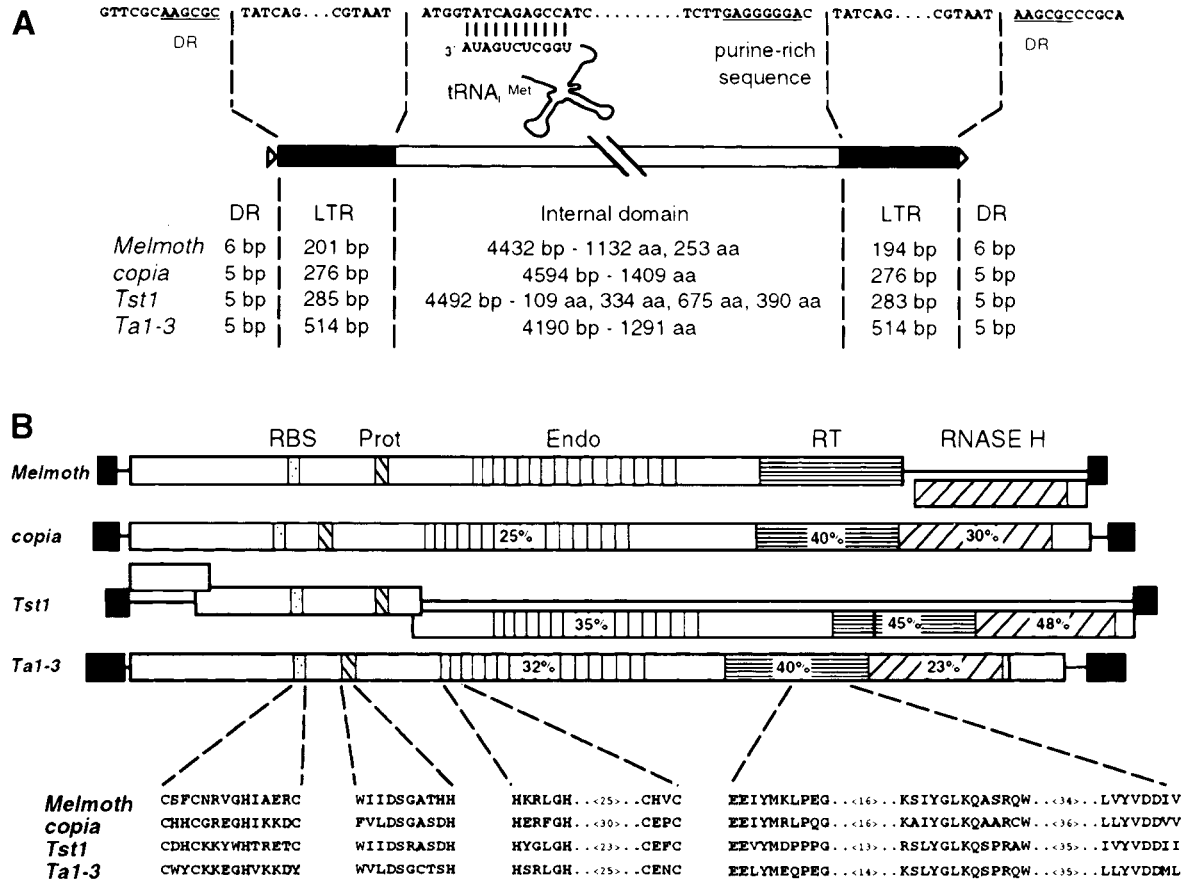

Figure 2. Sequence Similarity between the *Melmoth* Element Inserted in SLA_{Sc} and Retrotransposons of the *Ty1/copia* Family.

(A) Schematic representation of the *Melmoth* element that interrupts *SLA* in the S_{Sc} haplotype. The diagram shows details of the sequences at the borders of the different domains and compares the sizes of the domains with the corresponding regions of retrotransposons of the *Ty1/copia* family. Dashed lines indicate the positions of the different domains and sequences relative to the diagram. The lengths of the internal domains in base pairs (bp) and the lengths of the ORFs within this region in amino acid residues (aa) are indicated for each element. A 6-bp duplication flanking the *Melmoth* element and resembling the direct repeats (DR) found at the integration sites of transposons is underlined. Conserved sequences corresponding to priming sites for initiation of reverse transcription are shown. These consist of an 11-bp region adjacent to the left LTR, which is identical to the 3′ end of the tRNA initiator ($tRNA_i$) of Arabidopsis (Akama and Tanifuji, 1989) and a purine-rich sequence at the 5′ end of the right LTR (underlined). Triangles, closed boxes, and the open box represent direct repeats, LTRs, and the internal domain, respectively. **(B)** Comparison of the organization of ORFs in the *Melmoth* element with those of the *copia*, *Ta1-3*, and *Tst1* retrotransposons. The positions of conserved regions corresponding to the RNA binding site (RBS), the protease site (Prot), the endonuclease region (Endo), the reverse transcriptase region (RT), and the RNase H region are indicated by variously marked boxes. Percentage of amino acid similarity of the *copia*, *Tst1*, and *Ta1-3* sequences compared with *Melmoth* for the different domains is indicated. Blocks of conserved sequence within each region are shown with invariant amino acids boxed. Their position relative to the diagram is indicated with dashed lines. Black boxes represent LTRs. *copia* is from Drosophila (Mount and Rubin, 1985), *Tst1* is from potato (Camirand and Brisson, 1990), and *Ta1-3* is from Arabidopsis (Voytas and Ausubel, 1988).

LTRs of *Melmoth* indicate that its insertion into *SLA* was not recent. The mechanism of transposition of yeast and insect retrotransposons has been well characterized and involves the formation of a terminally redundant, circular RNA molecule and hence the production of retrotransposons with identical LTRs. Assuming that *Melmoth* is mobilized by a similar mechanism, the difference between the two LTRs is likely to have been acquired subsequent to its insertion into the *SLA* gene. It remains to be determined whether this difference prevents subsequent transpositions of this element.

SLG_{Sc} and SLA_{Sc} Are Linked to the *S* Locus

To demonstrate that SLG_{Sc} and SLA_{Sc} are linked to the *S* locus, we analyzed an F_2 population of 23 plants resulting from a cross between the homozygous P57Sc line and a line homozygous for the S_{3a} haplotype. The segregation of the two *S* haplotypes was followed by immunoblot analysis of stigma proteins separated by isoelectric focusing. A battery of antibodies raised against both class I and class II SLG proteins that had been developed in our Lyon laboratory for the purpose of determining the *S* haplotype of Brassica plants (Delorme et al., 1995a) was used for this analysis. Segregation of SLA_{Sc} was followed by polymerase chain reaction (PCR) amplification of SLA_{Sc} genomic DNA sequences by using oligonucleotides OSLA1 and OSLA2 (see Figure 1 for the positions of the oligonucleotides relative to the *SLA* sequence). Figure 3 shows that an SLA_{Sc} PCR product was only amplified from genomic DNA of F_2 progeny carrying the S_{Sc} haplotype, indicating that SLA_{Sc} is linked to the *S* locus in the P57Sc line.

Analysis of Self-Compatible and Self-Incompatible *B. oleracea* var *botrytis* Lines Carrying Transposon-Mutated *SLA* Genes

To investigate further the role of *SLA* in SI, we analyzed a number of *B. oleracea* var *botrytis* lines also carrying class II *S* haplotypes. The origins and SI phenotypes of the *B. oleracea* var *botrytis* lines are described in Table 1. Surprisingly, despite the fact that the different lines exhibited different levels of SI, several lines of evidence indicate that all of the *B. oleracea* var *botrytis* lines carry the S_{Sc} haplotype. MAb 157-35-50, an antibody raised against a peptide corresponding to the N terminus of the SLG protein of the P57Sc line (Gaude et al., 1993), recognizes SLG proteins from a range of class II *S* haplotypes, and closely related alleles can be distinguished based on the isoelectric points of the immunodetected proteins when separated by isoelectric focusing (Delorme et al., 1995a).

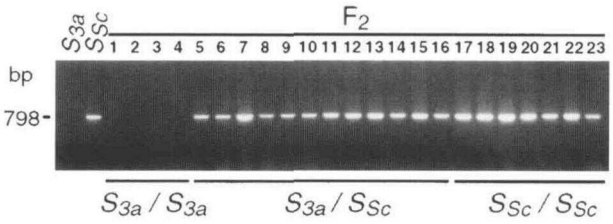

Figure 3. SLA_{Sc} Is Genetically Linked to the *S* Locus.

DNA was extracted from two parental plants, P57Sc (S_{Sc}) and a plant homozygous for the S_{3a} haplotype (S_{3a}), and from 23 F_2 progeny (F_2) descended from the two parental plants. PCR amplification was performed using oligonucleotides OSLA1 and OSLA2 (see Figure 1), and the PCR products were separated on an agarose gel and stained with ethidium bromide. The number at left indicates the length of the PCR products in base pairs. Segregation of the two *S* haplotypes in the F_2 population was followed by immunoblot analysis of stigmatic SLG proteins separated by isoelectric focusing (Delorme et al., 1995a), and the *S* haplotypes carried by the different progeny are indicated below the lanes.

Table 1. Origins and SI Phenotypes of the *B. oleracea* var *botrytis* Lines Used in This Study

Line	Type	Number of Generations of Self-Pollination	Pollen Tube Mean Score[a]			Phenotype[b]
			Mean	Min.	Max.	
11035	Autumn	5	0.1	0.0	0.3	SI
24062	Autumn	14	0.3	0.3	0.3	SI
41865	Autumn	9	0.1	0.0	0.3	SI
17144	Autumn	6	0.9	0.0	1.7	SI
40443	Autumn	7	0.7	0.0	1.7	SI
14518	Winter	4	4.9	4.7	5.0	SC
22495	Winter	9	5.4	4.0	6.8	SC

[a] Pollen tube mean scores were determined by aniline blue staining of pistils 24 hr after pollination. Three pistils were scored at one time, and two to five triplets of pistils were scored for each plant. Nine scores were defined: 0, 0 tubes; 1, 1 or 2 tubes; 2, 3 to 5 tubes; 3, 6 to 9 tubes; 4, 10 to 14 tubes; 5, 15 to 25 tubes; 6, 26 to 50 tubes; 7, 51 to 100 tubes; and 8, >100 tubes. The mean score was determined from all of the pistils tested for a particular plant; minimum (Min.) and maximum (Max.) scores refer to the lowest and highest scores, respectively, obtained for a triplet of pistils of a particular genotype.
[b] The SI phenotype based on pollen tube mean scores: SI, self-incompatible; SC, self-compatible.

Figure 4A shows that the pattern of stigma glycoproteins detected by MAb 157-35-50 in extracts from seven *B. oleracea* var *botrytis* lines was highly similar to that of the P57Sc line but markedly different from that of the two lines carrying the closely related S_2 and S_5 haplotypes. This result suggested that all of the *B. oleracea* var *botrytis* lines carry the S_{Sc} haplotype, and this hypothesis was supported by DNA sequence analysis of PCR products from the *B. oleracea* var *botrytis* lines. A 1018-bp region corresponding to nucleotides 317 to 1335 of the CG15 cDNA and including regions identified as highly variable in *SLG* sequences (Nasrallah et al., 1987) was amplified using the PCR from genomic DNA of each of the *B. oleracea* var *botrytis* lines and cloned into a plasmid vector. The DNA sequences of all seven fragments were identical to the corresponding region of the CG15 cDNA from P57Sc (data not shown). In addition, in reciprocal genetic crosses, pollen of the *B. oleracea* var *botrytis* lines 11035 and 40443 was rejected by stigmas of the P57Sc line, whereas pollen from P57Sc was compatible on stigmas of these two lines. These results indicate that the female part of the SI recognition system is functional in the P57Sc line and that it recognizes pollen from the 11035 and 40443 lines as "self," that is, as carrying the same *S* haplotype. Taken

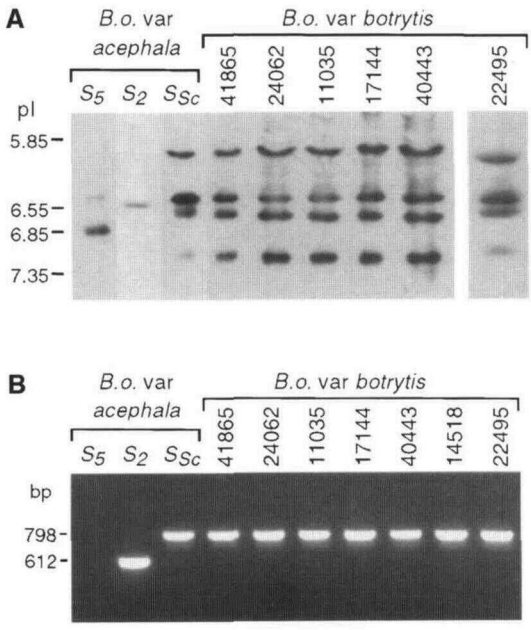

Figure 4. Comparison of *B. oleracea* var *botrytis* Lines with *B. oleracea* var *acephala* Lines Carrying Different *S* Haplotypes.

(A) Immunoblot analysis of proteins in stigma extracts of different lines of *B. oleracea* var *acephala* and *B. oleracea* var *botrytis*. Proteins were separated by isoelectric focusing and probed with MAb 157-35-50 as the primary antibody. Stigmas were from *B. oleracea* var *acephala* lines homozygous for the S_5, S_2, and S_{Sc} (the P57Sc line) haplotypes and from the *B. oleracea* var *botrytis* lines 41865, 24062, 11035, 17144, 40443, and 22495. The positions of the pI standards are shown at left.

(B) PCR analysis of *SLA* in different *B. oleracea* lines. Genomic DNA was extracted and PCR amplification was performed with three oligonucleotides corresponding to SLA_{Sc} sequences (OSLA1, OSLA2, and OSLA3; see Figure 1). The PCR products were separated on an agarose gel and stained with ethidium bromide. A 798-bp PCR product is expected if the *SLA* gene contains an insert in the same position as that in SLA_{Sc} (amplification with oligonucleotides OSLA1 and OSLA2), whereas a 612-bp product is expected if an *SLA* gene with no insert is present (amplification with oligonucleotides OSLA1 and OSLA3). DNA was from *B. oleracea* var *acephala* lines homozygous for the S_5, S_2, and S_{Sc} (the P57Sc line) haplotypes and from the *B. oleracea* var *botrytis* lines 41865, 24062, 11035, 17144, 40443, 14518, and 22495. Numbers at left indicate the lengths of PCR products in base pairs.

together, these results indicate that the *B. oleracea* var *botrytis* lines used in this study carry the same *S* haplotype as does P57Sc.

Because the different *B. oleracea* var *botrytis* lines exhibited different levels of SI, we were interested in determining whether their *SLA* genes would be interrupted by an insertion. Therefore, PCR amplification was performed using oligonucleotides OSLA1, OSLA2, and OSLA3 (see Figure 1 for oligonucleotides). This combination of oligonucleotides was predicted to give a 798-bp PCR product in the presence of an *SLA* gene carrying the putative retrotransposon and a 612-bp PCR product if an uninterrupted *SLA* gene were provided as template. PCR amplification from genomic DNA of each of the *B. oleracea* var *botrytis* lines resulted in a product of 798-bp indicating that all seven carried an *SLA* gene interrupted by the putative retrotransposon (Figure 4B). The fact that the size of the PCR products amplified from DNA of the seven *B. oleracea* var *botrytis* lines was the same as that amplified from DNA of the P57Sc line (Figure 4B) indicated that the putative retrotransposon was inserted at the same position in the *SLA* genes of all of these lines. The smaller 612-bp amplification product obtained with S_2 DNA is consistent with the absence of an insert in the SLA_2 gene. No amplification product was obtained from DNA of the S_5 haplotype. This is consistent with the observation of Boyes and Nasrallah (1995) that this haplotype did not contain sequences that hybridized with an SLA_2 probe.

The experiment shown in Figure 4B demonstrated that all of the *B. oleracea* var *botrytis* lines, including the strongly self-incompatible lines 11035, 24062, and 41865, carried an interrupted *SLA* gene. It was therefore important to determine whether the insertion interfered with expression of *SLA*. In the S_2 haplotype, which contains a wild-type *SLA* gene lacking an inserted retrotransposon sequence, Boyes and Nasrallah (1995) showed that the 1.56-kb spliced *SLA* transcript accumulates in young anthers, with a maximum abundance in 7- to 9-mm-long flower buds (which correspond to the bicellular and tricellular stages of microspore development), and that the 2.17-kb antisense unspliced transcript accumulates later and is most abundant when the flower buds are >9 mm long (anthers containing tricellular microspores and mature pollen). The increase in abundance of the unspliced transcript is concurrent with a decrease in abundance of the spliced transcript. The site of insertion of the putative retrotransposon in SLA_{Sc} corresponds to a region that is transcribed in both directions in SLA_2. The corresponding site in SLA_2 is 198 bp from the 3′ end of the unspliced transcript and 492 bp from the 5′ end of the spliced transcript. An insertion in this region would be expected to perturb synthesis of both of the transcripts, particularly of the spliced transcript that is thought to encode a peptide product (Boyes and Nasrallah, 1995).

We used RNA gel blot analysis to assay for the presence of *SLA* transcripts in different Brassica lines (Figure 5). Poly(A)$^+$ RNA was extracted from anthers at developmental stages corresponding to the stages when either the spliced or the unspliced transcripts were most abundant in the S_2 haplotype studied by Boyes and Nasrallah (1995). An RNA gel blot was probed with a DNA fragment from a region of the SLA_{Sc} gene corresponding to a region that is transcribed in both directions in SLA_2. Bands corresponding to both the spliced and unspliced transcripts were detected in anther poly(A)$^+$ RNA from an S_2 homozygous line, but neither *SLA* transcript was detected in anthers from either P57Sc or the

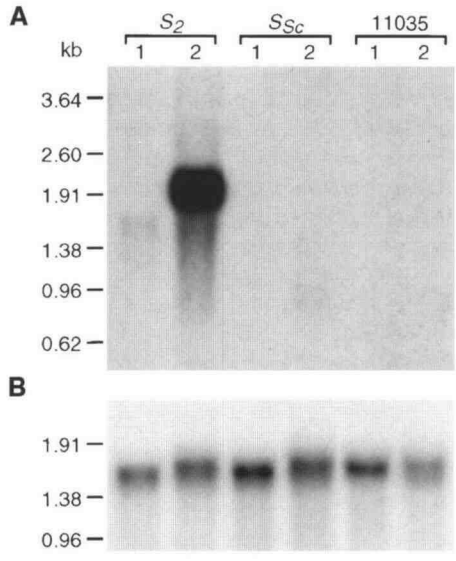

Figure 5. RNA Gel Blot Analysis of *SLA* Transcription.

Each lane corresponds to 16 μg of poly(A)$^+$ RNA from anthers of a line homozygous for the S_2 haplotype (S_2), the P57Sc line (S_{Sc}), or *B. oleracea* var *botrytis* line 11035 (11035) at either the unicellular/bicellular stages of microspore development (1) or at the tricellular stage (2).

(A) The RNA gel blot was probed with a 1260-bp EcoRV-BamHI fragment from SLA_{Sc} (*SLA* probe in Figure 1), and the autoradiograph was exposed for 10 days.

(B) The same RNA gel blot was probed with a Brassica actin cDNA probe. The autoradiograph was exposed for 6 hr.

Numbers at left indicate the lengths of RNA molecular markers in kilobases.

self-incompatible *B. oleracea* var *botrytis* line 11035. The two *SLA* transcripts were therefore not detected in lines carrying an SLA_{Sc} allele with an inserted *Melmoth* element, indicating that the presence of this element prevents expression of the *SLA* gene. The absence of detectable transcripts in the self-incompatible *B. oleracea* var *botrytis* line 11035 indicates that *SLA* function is not required for the SI response.

SLA Is a Single-Copy Homozygous Gene in the Brassica Lines Used in This Study

Other possible explanations for the self-incompatible phenotype of some of the *B. oleracea* var *botrytis* lines are either that they were heterozygous at the *SLA* locus or that *SLA* is a member of a functionally redundant gene family, with only one active member being required for the SI response. All seven of the *B. oleracea* var *botrytis* plants were derived from lineages that had undergone several generations of self-fertilization (Table 1); therefore, they were highly likely to be homozygous at the *SLA* locus. To confirm this, the seven *B. oleracea* var *botrytis* plants were self-fertilized, and the segregation of *SLA* was followed in the progeny. Genomic DNA was prepared from the progeny, and the *SLA* gene structure was analyzed by PCR amplification using oligonucleotides OSLA1, OSLA2, and OSLA3. All of the progeny analyzed (a total of 117 representing between 10 and 29 progeny per line, depending on the line) carried the *SLA* allele interrupted by an insertion (Table 2). These data provide strong evidence that the parental plants were homozygous at the *SLA* locus; for example, a probability of homozygosity of >99% was calculated for the three strongly self-incompatible *B. oleracea* var *botrytis* lines 11035, 24062, and 41865 (assuming a normal Mendelian segregation of *SLA* alleles).

Moreover, there is no evidence that *SLA* is part of a gene family. Boyes and Nasrallah (1995) showed that *SLA* is a single-copy gene in the genome of a line homozygous for the S_2 haplotype. We have confirmed this result and have shown that *SLA* is also a single-copy gene in P57Sc and the seven *B. oleracea* var *botrytis* lines. Figure 6 shows that a DNA probe derived from the *SLA* gene (see Figure 1) detected a single hybridizing fragment per lane at low stringency in a gel blot of restriction endonuclease–digested genomic DNA from the P57Sc line, the S_2 homozygous line, and the seven *B. oleracea* var *botrytis* lines. No hybridizing fragment was detected in DNA of the class I S_3 haplotype. The *SLA* probe detected a 3.8-kb fragment in HindIII-digested genomic DNA from P57Sc and all seven *B. oleracea* var *botrytis* lines, again supporting the hypothesis that these lines carry the same *S* haplotype.

Table 2. PCR Detection of *SLA* in Progeny Issued from Self-Fertilization of the Seven *B. oleracea* var *Botrytis* Plants Used in This Study

Line[a]	Phenotype[b]	Number of Progeny Tested	Number of PCR-Positive Progeny[c]	Probability That Parent Was Homozygous (%)
41865	SI	29	29	99.97
24062	SI	17	17	99.2
11035	SI	17	17	99.2
17144	SI	10	10	94.4
40443	SI	15	15	98.7
14518	SC	13	13	97.6
22495	SC	16	16	99.0

[a] Genomic DNA was extracted from each of the plants tested, and a fragment of SLA was amplified by using oligonucleotides OSLA1, OSLA2, and OSLA3 (see Figure 1).

[b] The SI phenotype based on the pollen tube mean scores (see Table 1): SI, self-incompatible; SC, self-compatible.

[c] A 798-bp product was amplified from genomic DNA of these plants, indicating the presence of an *SLA* gene interrupted by an insertion.

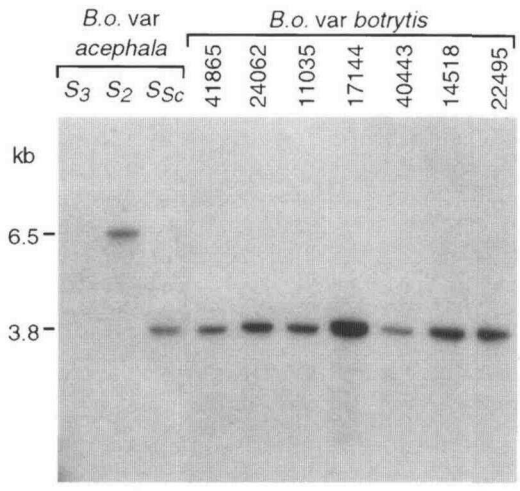

Figure 6. DNA Blot Analysis of the *SLA* Gene.

DNA gel blot of HindIII restriction endonuclease–digested genomic DNA from *B. oleracea* var *acephala* lines homozygous for the S_3, S_2, and S_{Sc} (the P57Sc line) haplotypes and from the *B. oleracea* var *botrytis* lines 41865, 24062, 11035, 17144, 40443, 14518, and 22495. A 1260-bp EcoRV-BamHI fragment from SLA_{Sc} (*SLA* probe in Figure 1) was used as a probe. Numbers at left indicate the lengths of hybridizing fragments in kilobases.

DISCUSSION

In this study, we have characterized an allele of the *S* locus–linked gene *SLA* from the S_{Sc} haplotype carried by the *B. oleracea* var *acephala* line P57Sc. This allele, SLA_{Sc}, was shown to be highly similar to the SLA_2 allele from the S_2 haplotype (Boyes and Nasrallah, 1995), except that it contained a large insertion of 4826 bp that resembled retrotransposons of the *Ty1/copia* family (Figures 1 and 2). Analysis of the structure of SLA_{Sc} provided conflicting evidence concerning the role of *SLA* in the SI response. Plants homozygous for the S_{Sc} locus have been shown to exhibit a self-compatible phenotype (Gaude et al., 1993). The presence of an insertion in the *SLA* gene of this haplotype therefore provided a correlation between the self-compatible phenotype and the presence of a mutated *SLA* gene. However, the high degree of similarity between SLA_{Sc} and SLA_2 is not consistent with the proposed highly polymorphic nature of *SLA* (Boyes and Nasrallah, 1995). Genes involved in the recognition step of the SI response are expected to be highly polymorphic, with a different allele existing for each *S* haplotype. Boyes and Nasrallah (1995) proposed that *SLA* was not detected in haplotypes other than S_2 (and the closely related *B. napus* haplotype S_{2W}) because a high level of polymorphism prevented cross-hybridization of probes. However, comparison of the S_2 and S_{Sc} haplotypes showed that the nearby *SLG* genes had diverged to a greater extent (98.6% nucleotide similarity) than had the two *SLA* genes (99.6% nucleotide similarity), with *SLA* exhibiting a very low level of polymorphism (Figure 1).

To further investigate the role of *SLA* in the SI response, we analyzed a number of *B. oleracea* var *botrytis* lines carrying S_{Sc} or a very closely related *S* haplotype but exhibiting different degrees of self-fertility (Figures 3 and 4, and Table 1). All of the lines analyzed carried a single-copy homozygous *SLA* gene that was interrupted by an insertion (Figure 6). No *SLA* transcripts were detected in anthers of *B. oleracea* var *botrytis* line 11035, which nonetheless exhibited a strong SI response. These data strongly indicate that a functional *SLA* gene is not required for the SI response in *B. oleracea*.

The *S* locus is predicted to encode both the male and female components of the SI recognition system, and several studies have been initiated recently to identify the gene encoding the pollen component by analysis of the regions flanking the *SLG* and *SRK* genes of different *S* haplotypes. Boyes et al. (1997) analyzed the regions downstream of the SLG_8 and SLG_{13} alleles of *B. campestris* and *B. oleracea*, respectively, for sequences that exhibited haplotype specificity and anther-specific expression in a search for functional homologs of SLA_2. No such sequences were found. This supports our hypothesis, based on the high degree of similarity between SLA_2 and SLA_{Sc}, that *SLA* exhibits a low level of polymorphism and indicates that *SLA* is not present at the *S* locus in all *S* haplotypes.

In the same study, Boyes et al. (1997) identified two novel genes, *298* and *299*, downstream of SLG_8 in *B. campestris*. These genes were not characterized in detail, but the fact that both genes are expressed in leaves as well as floral organs and that gene-specific probes hybridize equally strongly with DNA from lines carrying the S_8, S_2, or S_{13} haplotypes argue against these genes being involved in determining the specificity of the SI response. Similarly, Yu et al. (1996) characterized two novel genes, SLL_1 and SLL_2, located between *SLG* and *SRK* in both the 910 and A10 *S* haplotypes of *B. napus*. The DNA sequence of SLL_1, and probably also of SLL_2, is 100% conserved between the two *S* haplotypes, indicating that neither gene is involved in determining the specificity of the SI response (although one or both of these genes may play a more general role in SI). Taken together, the analyses of *SLA*, *298*, *299*, SLL_1, and SLL_2 have revealed that genes exhibiting a low level of polymorphism are present at the *S* locus close to the high polymorphic *SLG* and *SRK* genes and that, at least in the case of *SLA*, localization at the *S* locus does not necessarily imply a role in the SI response.

Based on the low level of polymorphism of SLL_1 and SLL_2 compared with *SLG* and *SRK*, Yu et al. (1996) proposed a model in which the former were inserted into the *S* locus after the divergence of the different *S* haplotypes. SLL_1 and SLL_2 would then have spread from one *S* haplotype to another by homologous recombination. The fact that *SLA* is present in the two closely related S_2 and S_{Sc} haplotypes but was not detected in the S_5, S_3, or S_{3a} haplotype (Boyes and Nasrallah, 1995; this work) indicates that *SLA* may similarly

have been inserted into the *S* locus during the divergence of the different *S* haplotypes, perhaps into the common ancestor of S_{Sc} and S_2. We cannot, however, rule out the possibility that *SLA* has been deleted from the other *S* haplotypes during evolution or that it has been transferred from S_2 to S_{Sc} or vice versa by a recombination event. The insertion of *Melmoth* into *SLA* presumably occurred after the divergence of the different *S* haplotypes in Brassica but before the divergence of *B. oleracea* and *B. napus*.

Interestingly, a retrotransposon-like sequence has also been identified at the *S* locus in *Nicotiana alata* (Royo et al., 1996). *N. alata* possesses a gametophytic SI system in which pollen rejection is mediated by stigmatic *S* RNases. There is no known sequence homology between the *S* locus genes of gametophytic and sporophytic SI systems. However, in both cases, sequence polymorphism between alleles is known to extend for several kilobases into the regions surrounding the *S* locus genes, indicating that there have been extensive rearrangements in these regions of the respective genomes (Clark et al., 1990; Coleman and Kao, 1992; Yu et al., 1996; Boyes et al., 1997). It will be interesting to determine whether the presence of putative retrotransposons at two different *S* loci is coincidental or whether their presence is related in some way to the unusual structure of *S* loci.

Boyes and Nasrallah (1995) have described an *SLA* allele (SLA_{2w}) from *B. napus* var Westar that, like SLA_{Sc}, contains a large insertion within the transcribed part of the gene. Although no sequence data were provided, the description of this allele indicates that it strongly resembles the SLA_{Sc} allele described above. The *B. napus* allele is >99% identical to SLA_2, apart from a large insert of 4827 bp (SLA_{Sc} contains an insert of 4826 bp). In particular, the two ORFs are highly conserved between SLA_{2w} and SLA_{Sc}, with only one amino acid difference between each ORF. No *SLA* transcripts were detected in *B. napus* plants carrying SLA_{2w} by RNA blot analysis (Boyes and Nasrallah, 1995).

Evidence has been available for some time indicating that the S_{Sc} haplotype is present in the genome of *B. napus* var Westar. Robert et al. (1994) have described a full-length cDNA from this variety that has a nucleotide sequence 100% identical to that of the CG15 cDNA isolated from the *B. oleracea* P57Sc line (Gaude et al., 1993). Robert et al. (1994) named the cDNA SLG_{WS2}; however, based on our analysis of the P57Sc line, this cDNA, like CG15, most likely corresponds to an alternative transcript of *SRK* (Giranton et al., 1995; see above). Moreover, we have analyzed 10 self-compatible commercially available varieties of *B. napus* by sequencing of PCR-amplified fragments and have shown that all 10 carry sequences identical to the CG15 cDNA, indicating that S_{Sc} or a very closely related *S* haplotype is common in commercial varieties of *B. napus* (V. Delorme and J.M. Cock, unpublished data). Based on these data, it seems likely that the mutated *SLA* gene identified by Boyes and Nasrallah (1995) is part of an *S* haplotype closely related to the S_{Sc} haplotype in *B. napus,* and for the reasons described above, it therefore seems unlikely that the self-compatible nature of Westar is related to the presence of a nonfunctional *SLA* gene. However, the fact that an *S* haplotype closely related to S_{Sc} is widespread in self-compatible *B. napus* is interesting, considering that this haplotype is associated with a self-compatible phenotype in some lines of *B. oleracea* (Gaude et al., 1993; this study). In addition, the S_{Sc} haplotype carried by the *B. oleracea* P57Sc line is particularly interesting in that genetic crosses indicate that the male component of the SI response is defective. Further work will be aimed at determining the molecular basis of the self-compatible phenotype associated with this haplotype.

METHODS

Plant Material, Genetic Crosses, and Determination of Incompatibility Phenotype

The self-compatible *Brassica oleracea* var *acephala* line P57Sc has been described previously (Gaude et al., 1993). An F_2 population obtained by self-fertilization of an S_{3a}/S_{Sc} F_1 hybrid (the parental lines are described by Delorme et al. [1995a] and Gaude et al. [1993], respectively) was used to demonstrate linkage of *SLA* with the *S* locus. The incompatibility phenotypes of the F_2 progeny were determined by self-pollination and by crosses to tester plants, using previously described procedures (Delorme et al., 1995b). Of the seven *B. oleracea* var *botrytis* lines used in this study, five were commercial and open-pollinated varieties, either Lecerf (41865) or Automne malouin tardif (11035, 17144, 40443, and 14548), and two were noncommercial and open-pollinated varieties (24062 and 22495). The incompatibility phenotype was determined by aniline blue staining of pollinated pistils, as previously described (Ruffio-Châble et al., 1997).

Genomic DNA Cloning and DNA Sequencing

The SLG_{Sc}/SLA_{Sc} gene region was isolated from a genomic library constructed with DNA of the P57Sc line. The construction and screening of the library were as described previously (Delorme et al., 1995b). Restriction endonuclease–digested fragments of isolated λ clones were subcloned into pBluescript II SK+ (Stratagene, La Jolla, CA) and sequenced by using the dideoxynucleotide chain termination method (Sanger et al., 1977) either with an automatic sequencer (Applied Biosystems, Foster City, CA) or with a T7 DNA polymerase sequencing kit (Pharmacia Biotech, Uppsala, Sweden). Sequence data were analyzed by using Lasergene sequence analysis software (DNASTAR, London, UK). The results of comparisons are expressed as percentage similarity, which is calculated as follows: 100 times the number of matched amino acids divided by the sum of the length in amino acids of the aligned region plus the number of gaps introduced to optimize the alignment.

Protein Extraction, Electrophoretic Analysis, and Immunodetection of Proteins

Protein extraction, separation of proteins by isoelectric focusing or SDS-PAGE, electrotransfer onto nitrocellulose membranes, and detection of antigen with antibodies were as described previously (Gaude

et al., 1991, 1993). Monoclonal antibody (MAb) 157-35-50 has been described previously (Giranton et al., 1995).

DNA Extraction, Polymerase Chain Reaction Amplification, and DNA Gel Blots

Genomic DNA for DNA gel blot analysis and polymerase chain reaction (PCR) experiments was extracted as follows. Two-centimeter-square pieces of leaf were ground to a powder in liquid nitrogen and incubated at 65°C in 500 μL of extraction buffer (extraction buffer is 0.1 M Tris, pH 8, 50 mM EDTA, 100 mM NaCl, 1% SDS, and 9 μM 2-mercaptoethanol). After the addition of 165 μL of 3 M potassium acetate and 500 μL of a phenol–chloroform mixture (1:1), the samples were agitated for 5 min, incubated for 10 min on ice, and centrifuged for 15 min at 18,000*g* at 4°C. The aqueous fraction was mixed with 750 μL of isopropanol and incubated for 2 hr at room temperature. Genomic DNA was recuperated by centrifugation and washed with 70% ethanol before being resuspended in 100 μL of water.

When the DNA was prepared for PCR analysis, samples of tissue from a Brassica plant that did not contain the template sequence for the PCR (leaves of a *B. oleracea* var *acephala* plant homozygous for the S_3 haplotype) were harvested after every fifth test sample. These intercalated control samples underwent the same extraction and PCR amplification steps as the test samples to check that there was no cross-contamination between samples. The oligonucleotides used for PCR amplification were OSLA1 (5′-GAAGCCGACCCGTTTGGA-3′), OSLA2 (5′-GAGAGAAGGCGGAGGTGA-3′), and OSLA3 (5′-GTCCTGGCGTTTGAGAGC-3′), which correspond to SLA_{Sc} sequences, and SG2 (5′-GGCCTGCAGCAGCATTCAATCTGAC-3′) and SG22 (5′-TGGAACCCTCAAAATCT-3′), which correspond to CG15 (Gaude et al., 1993).

For DNA blot analysis, 10 μg of genomic DNA was digested with the HindIII, fractionated on 0.8% agarose gels, and transferred to nylon membranes (Hybond N^+; Amersham) under alkaline conditions. A 1260-bp EcoRV-BamHI restriction fragment of *SLA* and a 860-bp EcoRV restriction fragment of the insertion in *SLA* were used as probes for *SLA* and the *Melmoth* element, respectively. The probes are positioned with respect to the *SLG/SLA* region of the S_{Sc} haplotype in Figure 1. Radiolabeled DNA probes were prepared by using a random priming DNA labeling kit (Boehringer Mannheim). Filters were prehybridized and hybridized at 42°C in 50% formamide, 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.5% SDS, 0.1% Ficoll, 0.1% PVP, 0.1% BSA, and herring sperm DNA (100 μg mL^{-1}). Identical results were obtained after washing at either low (30 min in 6 × SSC, 0.1% SDS at 50°C) or high (30 min in 0.1 × SSC, 0.1% SDS at 50°C) stringency.

RNA Gel Blot Analysis

Developmental stages of anthers were determined by fluorescence microscopy observation of 4′,6-diamidino-2-phenylindole–stained microspores and approximately correlated with bud length. Immature anthers containing unicellular and bicellular microspores were dissected from either 5- to 9-mm flower buds for *B. oleracea* var *acephala* plants or from 3- to 6-mm flower buds for *B. oleracea* var *botrytis* plants, which have proportionally smaller flowers. Similarly, anthers containing tricellular microspores were dissected from 9- or 6-mm flower buds of *B. oleracea* var *acephala* or *B. oleracea* var *botrytis* plants, respectively. Total RNA extraction was as described by Cock et al. (1997). Poly(A)$^+$ RNA was purified by hybridization with magnetic beads coated with oligo(dT)$_{25}$ (Novagen, Inc., Madison, WI), and 16 μg of poly(A)$^+$ RNA was separated in each lane of a formaldehyde gel before being transferred to a nylon filter for hybridization. Preparation of radiolabeled probes and hybridization conditions were as described by Pastuglia et al. (1997).

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