Molecular Characterization of *S* Locus Genes, *SLG* and *SRK*, in a Pollen-Recessive Self-Incompatibility Haplotype of *Brassica rapa* L

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Manuscript received December 1, 1997
Accepted for publication April 6, 1998

ABSTRACT

In Brassica species that exhibit self-incompatibility, two genes, *SLG* and *SRK*, at the *S* locus are involved in the recognition reaction with self and non-self pollen. From a pollen-recessive S^{29} haplotype of *Brassica rapa*, both cDNA and genomic DNA clones for these two genes were isolated and characterized. The nucleotide sequence for the S domain of SRK^{29} showed a high degree of similarity with that of SLG^{29}, and they belong to Class II type. RNA gel blot analysis showed that the transcript of SLG^{29} consisted of the first and second exons, and no other transcript containing any part of the intron sequence was detected. Because no transmembrane domain was encoded by the second exon of SLG^{29}, SLG^{29} was designated a secreted type glycoprotein. *SLG*s of two other pollen-recessive haplotypes, S^{40} and S^{44}, of *B. rapa* also had a similar structure to that of SLG^{29}. Previously, SLG^{2} from a pollen-recessive haplotype, S^{2}, of *Brassica oleracea* was found to produce two different transcripts, one for the secreted type glycoprotein and the other for a putative membrane-anchored form of *SLG*. Therefore, the nature of these *SLG*s from pollen-recessive haplotypes of *B. rapa* is different from that of SLG^{2} of *B. oleracea*.

SELF-INCOMPATIBILITY is a mechanism by which many flowering plants prevent self-fertilization and promote outbreeding. The self-incompatibility system in Brassica is controlled sporophytically by alleles at a single locus called the *S* locus (Bateman 1955). More than 30 *S* alleles have been identified in *Brassica rapa* (Nou *et al.* 1993) and more than 40 *S* alleles in *Brassica oleracea* (Ockendon 1974). Because the activity of *S* alleles is controlled sporophytically, codominance and dominance relationships influence the ultimate phenotype of stigma and pollen. The following observations have been made about the dominance relationships between *S* alleles: (1) Codominance is common; (2) dominance/recessiveness is frequent in pollen; (3) dominance relationships are different between stigma and pollen; and (4) dominance relationships are nonlinear (Thompson and Taylor 1966; Ockendon 1975; Visser *et al.* 1982; Hatakeyama *et al.* 1998).

Results from molecular analyses have revealed that the Brassica *S* locus consists of at least two physically linked genes expressed in stigma papillae (Boyes and Nasrallah 1993). One of the genes is the *S* locus glycoprotein (*SLG*) gene, which encodes a secreted glycoprotein (Nasrallah *et al.* 1987; Takayama *et al.* 1987), and the other is the *S* locus receptor kinase (*SRK*) gene, which encodes a receptor protein kinase with an extracellular domain (S domain) that shares a high degree of sequence similarity with *SLG* (Stein *et al.* 1991; Watanabe *et al.* 1994). Several lines of evidence suggest that these two genes are involved in the recognition reaction of self-incompatibility (Shiba *et al.* 1995; Conner *et al.* 1997). Because the *S* locus contains multiple genes, *S* alleles are referred to as *S* haplotypes (Nasrallah and Nasrallah 1993). In addition, there are three *SLG*-related genes (*SLR1*, *SLR2*, and *SLR3*), which are unlinked to the *S* locus but show sequence similarity with *SLG* (Lalonde *et al.* 1989; Trick and Flavell 1989; Scutt *et al.* 1990; Boyes *et al.* 1991; Isogai *et al.* 1991; Watanabe *et al.* 1992; Yamakawa *et al.* 1993; Cock *et al.* 1995; Tantikanjana *et al.* 1996; Watanabe *et al.* 1997). *SLR2* shows a high degree of sequence similarly to *SLG*s isolated from pollen-recessive haplotypes (Tantikanjana *et al.* 1996; Watanabe *et al.* 1997).

Based on the degree of sequence similarity among *SLG*s and dominance relationships among their corresponding *S* haplotypes, Nasrallah and Nasrallah (1993) have classified *SLG*s into two groups, Class I and Class II. The *SLG*s in Class I all correspond to haplotypes that show dominance in pollen, whereas Class II *SLG*s correspond to haplotypes that show recessiveness. Molecular investigations on S^{2} haplotype in Class II have revealed that the SLG^{2} gene produces two transcripts that differ at their 3′ ends (Tantikanjana *et al.* 1993). One transcript encodes the expected secreted glycoprotein, while the other encodes a putative membrane-anchored form that is not found in Class I S^{6} haplotype. This unusual feature of the *SLG* of the S^{2} haplotype is

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thought to be related to its pollen-recessive nature (Chen and Nasrallah 1990; Stein *et al.* 1991; Tantikanjana *et al.* 1993). So far, SLG^2 is the only *SLG* gene from a Class II haplotype that has been studied in detail, although *SLG* and *SRK* genes from several Class I haplotypes of *B. rapa* have been studied (Watanabe *et al.* 1994; Yamakawa *et al.* 1994; Yamakawa *et al.* 1995; Suzuki *et al.* 1995; Matsushita *et al.* 1996) and of *B. oleracea* (Stein *et al.* 1991; Delorme *et al.* 1995).

In a previous study, we determined the dominance relationships between 24 *S* haplotypes of *B. rapa* by examining pollen tube behavior in diallel crosses. We have classified these *S* haplotypes into codominant (CD), dominant/recessive (DR), and recessive (R) groups on the pollen side (Hatakeyama *et al.* 1998). *S* haplotypes belonging to the CD group are codominant to each other. The members of the DR group are either codominant or recessive to those of the CD group as well as to others within the DR. The members of the R group are generally recessive to those of the other two groups. Here, we report a study of a set of *SLG* and *SRK* genes from one of the pollen-recessive haplotypes (R), S^{29}, of *B. rapa* to determine whether *SLG* and *SRK* genes from a pollen-recessive haplotype exhibit any unique features.

MATERIALS AND METHODS

Plant materials and determination of *S* genotype: All *S* homozygous lines of *B. rapa* L used in this study were described by Hatakeyama *et al.* (1998). The F_2 plants used for RFLP analysis were obtained from an F_1 heterozygous individual by bud pollination. The *S* genotype of the F_2 progeny was determined by reciprocal test-pollinations between each F_2 plant and the two parental lines (Hatakeyama *et al.* 1998).

Reverse-transcriptase PCR (RT-PCR) and cloning of PCR products: Stigmas of S^{29}, S^{40}, and S^{44} homozygotes were collected from buds at 2–3 days before anthesis. Poly(A)$^+$RNA was isolated with a Fast Track mRNA Isolation Kit (Invitrogen, San Diego, CA). The first strand cDNA was synthesized from 1 μg of poly(A)$^+$RNA using the *Not*I-$(dT)_{18}$ adapter primer with the T-Primed First Strand Kit (Pharmacia LKB, Uppsala, Sweden). PCR reactions contained total cDNAs in 100 μl with Class II-specific primer (PS3; Nishio *et al.* 1996) corresponding to the 5′ terminal region of the SLG^2 ORF (Chen and Nasrallah 1990), the adapter primer, and *Taq* DNA polymerase (TaKaRa Shuzo, Shiga, Japan). PCR was performed as described in Nishio *et al.* (1996) with minor modifications. The PCR condition was 35 cycles for 1 min at 94°, 2 min at 50°, and 3 min at 72° with a DNA thermal cycler (Perkin Elmer/Cetus Corp., Norwalk, CT). For the 3′ end amplification of SLG^{29} cDNAs, R-6 primer (Figure 4; 5′-GAGGACGACG CAGATGAGCT-3′) was used as a 5′-primer.

Amplification of the intron sequence of the SLG^{40} and SLG^{44} genes was performed with a forward primer (R-7 in Figure 4; 5′-AGTCAGTGAGTTCACACTCG-3′) located 828 bp downstream of the initiation codon of SLG^{29} and a reverse primer (5′-CGTCTACGTGGCCAATTGA-3′) complementary to the sequence of the C-terminal region of the SLG^{40} cDNA (Figure 7A). Genomic DNA extracted from young leaves of S^{40} and S^{44} haplotypes was used as a template. PCR was carried out as described by Nishio *et al.* (1996). The amplified products were cloned into the pCRII plasmid vector using the TA cloning kit (Invitrogen).

Isolation of cDNA and genomic clones: An S^{29} stigma cDNA library was constructed in lambda ZAPII (Stratagene, La Jolla, CA) as described by Matsushita *et al.* (1996). Approximately 2×10^5 plaques were screened using digoxigenin-labeled (dig-labeled) PCR-amplified fragments, pRT26 and pRT37 (see results for details of these two clones), as probes. Prehybridization and hybridization were performed as described by Watanabe *et al.* (1994). Filters were washed twice with 0.2× SSC, 0.1% sodium dodecyl sulfate (SDS) at 65° for 20 min. *In vivo* excision of the inserts was performed from positive clones by following the manufacturer's protocol.

For the isolation of SRK^{29}, an S^{29} stigma cDNA library was constructed in lambda gt10 (Stratagene) as described by Watanabe *et al.* (1994) because we could not obtain positive clones from the cDNA library constructed in lambda ZAPII. Approximately 4×10^5 plaques were screened using dig-labeled SLG^{29} cDNA and a 0.7-kb *Eco*RI fragment of the SRK^9 cDNA that corresponded to the kinase domain (Watanabe *et al.* 1994) as a probe. Filters hybridized with both SLG^{29} cDNA and the 0.7-kb fragment of SRK^9 cDNA were washed twice in 0.2× SSC, 0.1% SDS at 65° for 20 min or in 0.5× SSC, 0.1% SDS at 65° for 20 min, respectively. An insert was subcloned from a cross-hybridized phage into the pCRII plasmid vector (Invitrogen).

An S^{29} genomic library was constructed in the bacteriophage vector lambda GEM11 (Promega, Madison, WI) as described by Suzuki *et al.* (1995). A total of 7.0×10^5 recombinants were screened with dig-labeled SLG^{29} cDNA. For isolation of an SRK^{29} genomic clone, an additional probe, a kinase-encoding domain of the SRK^{29} cDNA, was also used. Putative SLG^{29}-containing recombinant clones were identified by their intense hybridization signal to the probe. The SLG^{29}-homologous regions from the genomic clones were subcloned into the pCR II plasmid vector.

DNA sequencing, DNA gel blot analysis, and RNA gel blot analysis: DNA sequencing was carried out by the dideoxynucleotide chain termination method (Sanger *et al.* 1977) using a model 373 DNA sequencer (Applied Biosystems, Foster City, CA). Sequence data were analyzed using DNASIS software (Hitachi Software Engineering, Yokohama, Japan). The homology search was performed using the FASTA program (Pearson and Lipman 1988).

For DNA gel blot analysis, genomic DNA was extracted from 3 g of young leaves by the procedure of Murray and Thompson (1980). The procedures for gel electrophoresis and blot hybridization were performed as described in Watanabe *et al.* (1994) with minor modifications. A full-length SLG^{45} cDNA (K. Hatakeyama, T. Takasaki, M. Watanabe and K. Hinata, unpublished results) was used as a probe for DNA gel blot analysis of 24 *S* haplotypes. Filters were washed twice in 0.2× SSC, 0.1% SDS at 65° for 20 min.

For RFLP linkage analysis, genomic DNA isolated from parental plants, an F_1 plant and F_2 plants was digested with several restriction enzymes. After hybridization with the full-length pRT37 clone or a 1.0-kb *Sac*I-fragment of the pRT26 clone or 1.7-kb *Bam*HI-*Xba*I fragment of the SRK^{29} cDNA which contains 456 bp of the S domain and 692 bp of the kinase catalytic domain, filters were washed twice in 0.1× SSC, 0.1% SDS at 65° for 20 min.

For RNA gel blots, poly(A)$^+$RNA was extracted from stigmas and anthers of flower buds at 1 day before anthesis with the Micro FastTrack mRNA isolation kit (Invitrogen). After denaturation in glyoxal, 1 μg of mRNA was subjected to electrophoresis on 1% (w/v) agarose gel and transferred to a Nytran nylon membrane by blotting with 20× SSC. The blots were prehybridized and hybridized as described in Watanabe *et*

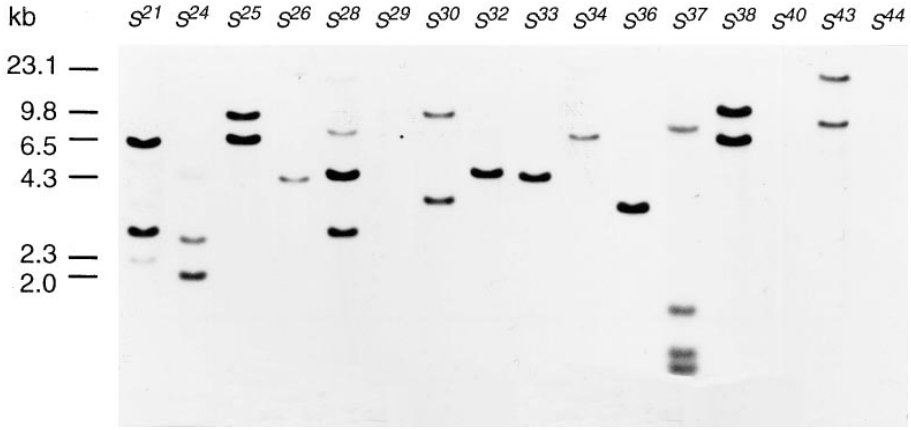


Figure 1.—DNA gel blot analysis of 16 different *S* haplotypes of *B. rapa*. Two micrograms of genomic DNA were digested with *Eco*RI and separated on 0.8% agarose gels. After transfer to a nylon membrane, the blots were hybridized with the SLG^{45} cDNA probe. S^{29}, S^{40}, and S^{44} are pollen-recessive haplotypes. DNA size markers, in kb, are shown on the left.

al. (1994) and washed twice in 0.1× SSC, 0.1% SDS at 65° for 20 min. To check the integrity of RNA, the RNA blot was probed with a part of the genomic clone of the beta subunit of the mitochondrial ATP synthase gene of *Nicotiana plumbaginifolia* (Boutry and Chua 1985). After hybridization at 65°, the filter was washed twice with 0.5× SSC and 0.1% SDS at 65° for 20 min.

RESULTS

Isolation of *SLG*-like sequences from S^{29} haplotype: We first performed DNA gel blot analysis of 24 *S* haplotypes of *B. rapa* to ascertain whether *S* haplotypes in the CD and DR groups had Class I *SLG*. To detect Class I sequences, we probed with an SLG^{45} cDNA, the predicted amino acid sequence of which is 78.7% identical to the polypeptide encoded by Class I SLG^{9} cDNA (Watanabe *et al.* 1994; K. Hatakeyama, T. Takasaki, M. Watanabe and K. Hinata, unpublished results). Figure 1 shows a representative result obtained from 16 of the 24 haplotypes. The SLG^{45} cDNA probe hybridized very strongly to genomic DNA fragments from 20 of the 24 *S* haplotypes. The hybridization pattern was unique for each *S* haplotype, as expected. However, we were unable to detect a strong hybridization signal from genomic DNA of the 4 other *S* haplotypes (*i.e.*, S^{29}, S^{31}, S^{40}, and S^{44}), all of which are pollen-recessive. Similar results were obtained when SLG^{8} cDNA (Yamakawa *et al.* 1994) and SLG^{9} cDNA (Watanabe *et al.* 1994) from pollen-dominant *S* haplotypes were used as probes (Watanabe, Okazaki, Suzuki and Hinata, unpublished results).

To isolate the *SLG*-homologous sequences from the S^{29} haplotype, RT-PCR was conducted using the Class II-specific primer; two clones, pRT26 and pRT37,

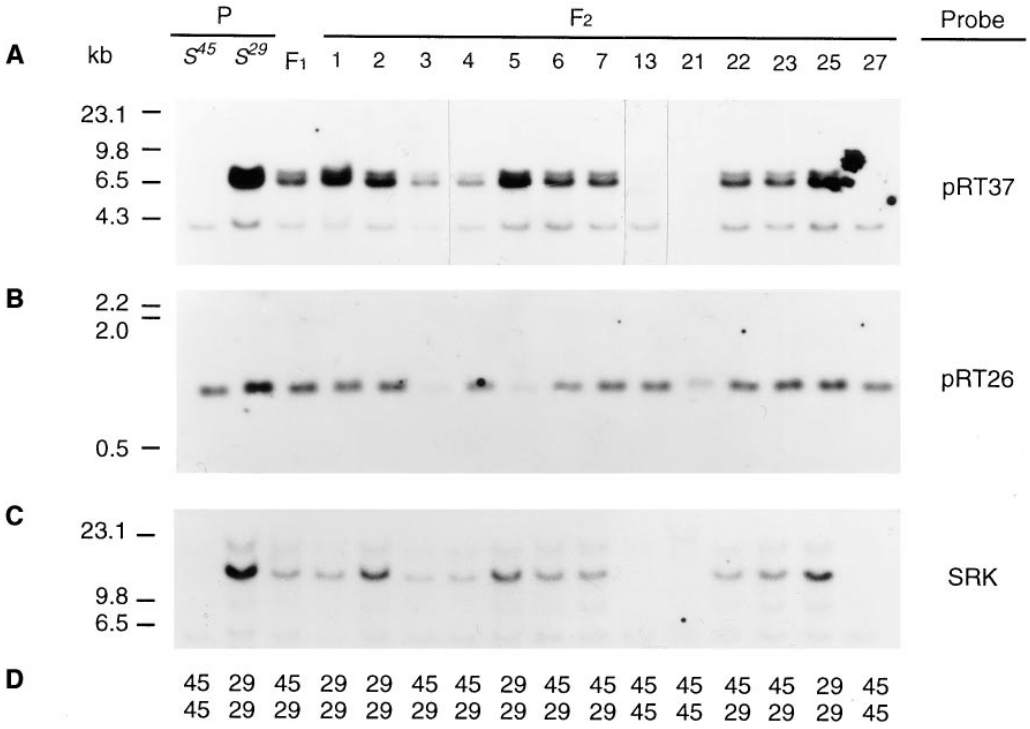


Figure 2.—RFLP linkage analysis of an F_2 population segregating for S^{45} and S^{29} self-incompatibility haplotypes. DNA isolated from parental (P) plants homozygous for either the S^{45} or S^{29} haplotype, their F_1 heterozygotes and 13 F_2 progeny was digested with both *Eco*RI and *Eco*RV (A) or digested with *Sac*I (B), or *Bam*HI (C), and analyzed by blot hybridization. Segregation patterns observed after hybridization with probes for pRT37 clone (A), pRT26 clone (B), and SRK^{29} cDNA (C) are shown. The incompatibility phenotype of each plant was determined by pollination tests (D). DNA size markers, in kb, are shown on the left.

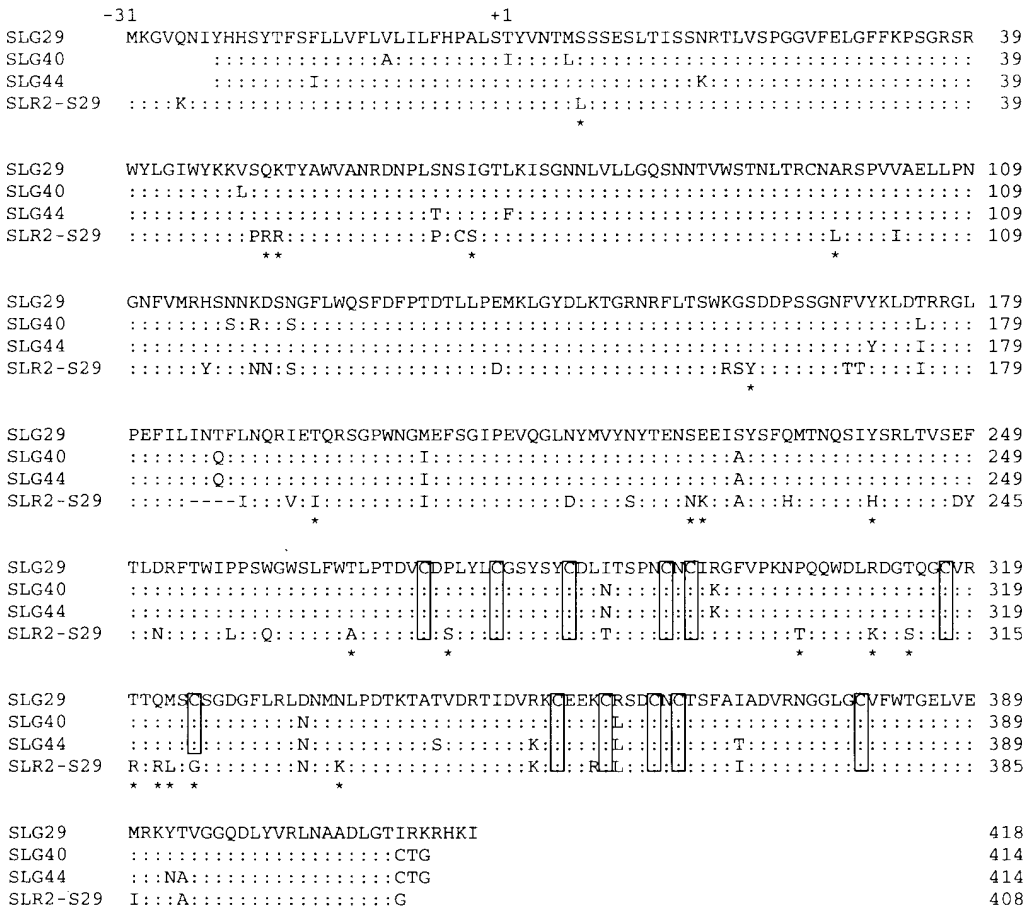


Figure 3.—Alignment of predicted amino acid sequences of three Class II *SLG*s and *SLR2-S*^{29} from *B. rapa*. Colons and dashes indicate identical amino acid residues and gaps introduced to optimize the alignment, respectively. Boxed residues represent twelve conserved cystein residues among Class II SLGs. Asterisks below the sequence indicate twenty amino acid residues that differ between the Class II SLG and SLR2 families. Seven residues corresponding to the primer sequence at the N-terminal end of *SLG*^{40} and *SLG*^{44} are omitted.

each containing a DNA insert with the expected size of *ca.* 1.6 kb, were obtained. The sequences of the two fragments were different (88.1% identity). Database searches revealed that the nucleotide sequence of the pRT37 clone showed the highest identity, 95.3%, with that of *SLG*^{5} isolated from the pollen-recessive *S*^{5} haplotype (Scutt and Croy 1992). On the other hand, the sequence of the pRT26 showed the highest identity, 94.4% with those of *SLR2* genes (Boyes *et al.* 1991).

Cloning and sequence analysis of the *SLG*^{29} gene of *S*^{29} haplotype: Genetic linkage to the *S* locus of the gene corresponding to one of the two clones, pRT37, was examined by RFLP analysis of 13 plants from an F_2 progeny segregating for *S*^{45} and *S*^{29} haplotypes. When genomic DNA was hybridized with the pRT37 clone, two bands of 6.0 and 6.8 kb were detected only in plants carrying the *S*^{29} haplotype (Figure 2A; plants 1, 2, 3, 4, 5, 6, 7, 22, 23, and 25). The intensities of the 6.0- and 6.8-kb bands observed in plants 3 and 4 were much weaker than observed in the other F_2 plants due to lower amounts of DNA loaded. The genomic DNA fragments detected by the pRT37 clone correlated perfectly with *S*^{29} haplotype. Therefore, we concluded that pRT37 corresponds to the *SLG*^{29} gene. Genetic linkage analysis for the other clone, pRT26, is described below.

A full-length *SLG*^{29} cDNA clone was obtained from an *S*^{29} stigma cDNA library by using the pRT37 clone as a probe; this clone was completely sequenced. The *SLG*^{29} cDNA encodes a polypeptide of 449 amino acids that begins, as in other *SLG*s, with a signal peptide sequence of 31 residues (Figure 3). There are six potential sites of *N*-glycosylation (N-X-S or N-X-T) distributed throughout the protein. Twelve conserved cysteine residues present in the C-terminal region of all *SLG*s were also found in this protein. An additional cysteine residue was found in the N-terminal region of the protein. The deduced amino acid sequence of *SLG*^{29} shows a higher degree of similarity with Class II *SLG*s than with Class I *SLG*s. For example, there is 93% identity with the Class II *SLG*^{2} protein (Chen and Nasrallah 1990), but only 66% identity with the Class I *SLG*^{9} (Watanabe *et al.* 1994).

We used this *SLG*^{29} cDNA clone to detect Class II sequences in a DNA gel blot analysis of the 24 *B. rapa S* haplotypes. This probe showed a strong hybridization signal with the genomic DNA from only the four pollen-recessive *S* haplotypes (data not shown).

A genomic clone corresponding to *SLG*^{29} was obtained from an *S*^{29} genomic library by using the *SLG*^{29} cDNA as a probe. Alignment of the genomic sequence and the cDNA sequence revealed the presence of a 1640-bp intron in the region encoding the C-terminal part of

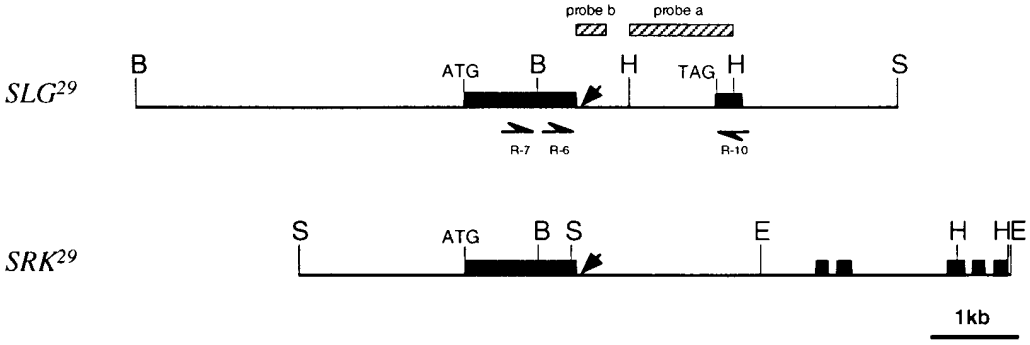


Figure 4.—Restriction map of the subcloned regions of the *SLG*[29] and *SRK*[29] genomic clones. The exon of each gene is indicated by filled black boxes. The initiation codon of each gene (ATG) and stop codon of the *SLG*[29] gene (TAG) are shown. Arrowheads represent an in-frame TAG codon of each gene. The position and orientation of the PCR primers are indicated by horizontal arrows. The striped boxes above the *SLG*[29] indicate the region used as probes in Figure 6. B, *Bam*HI; H, *Hin*dIII; E, *Eco*RI; S, *Sac*I. The GenBank data library accession numbers for the *SLG*[29] genomic clone and the *SRK*[29] cDNA sequences are AB008190 and AB008191, respectively.

the S domain (Figure 4). This intron interrupts the 1321-bp open-reading frame of 440 amino acids, and the last eight amino acids are encoded by the second exon (one amino acid is encoded by the first and second exon). As has been found for *SLG*[2] (Tantikanjana *et al.* 1993), the only intron of the *SLG*[29] gene contains an in-frame stop codon (TAG) that could be used to produce an alternative transcript. Unlike the *SLG*[2], however, the second exon of *SLG*[29] does not encode a transmembrane domain, and the only protein product predicted from the *SLG*[29] sequence is a secreted type glycoprotein.

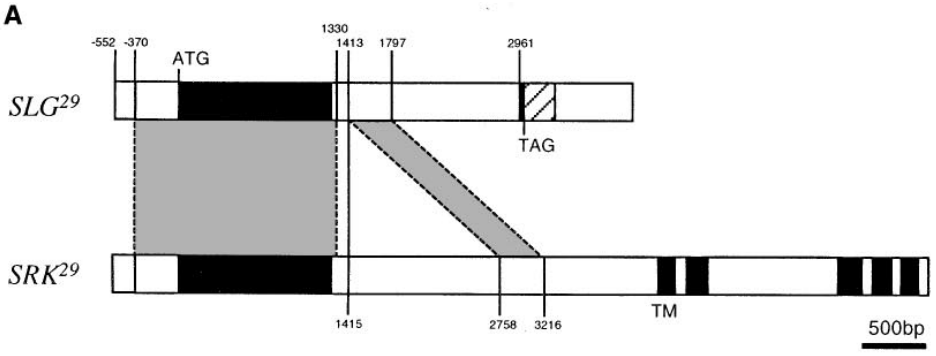
Cloning and sequence analysis of the *SLR2-S*[29] cDNA: We used the pRT26 clone to isolate cDNA from what appears to be the *SLR2* gene from the *S*[29] line. We designate this gene as *SLR2-S*[29]. Upon digestion of genomic DNA with *Sac*I and hybridization with a 1.0-kb *Sac*I-fragment of pRT26 (Figure 2B), a 1.0-kb fragment was detected in *S*[45] and *S*[29] homozygous parents and in all of the F_2 progeny. This suggested that the region that hybridized to the pRT26 probe did not cosegregate with the *S*[29] haplotype.

We then used the pRT26 clone to probe the aforementioned cDNA library. Two positive clones containing the ATG initiation codon were obtained, and the longer insert was selected for sequence analysis. This cDNA clone encodes a polypeptide of 439 amino acids that begins with a signal peptide sequence of 31 residues (Figure 3). There are six potential sites of *N*-glycosylation distributed throughout the protein. The deduced amino acid sequence shows the high degree of similarity (99.8% identity) with that of the *SLR2*-C636 gene isolated from *B. rapa* (Watanabe *et al.* 1997). However, *SLR2-S*[29] did not contain a 4-bp frame-shifting insertion at its C-terminal region, as had been found for *SLR2-S*[8] (Tantikanjana *et al.* 1996) and *SLR2*-C636 (Watanabe *et al.* 1997) isolated from *B. rapa.* Eleven of the 12 conserved cysteine residues are present in the C-terminal region of the cDNA clone, as in other *SLR2*. The seventh conserved cysteine (counted from the N-terminal end) is changed to glycine. These results suggest that pRT26 corresponds to the *SLR2* gene.

Cloning and sequence analysis of the *SRK*[29] gene of *S*[29] haplotype: A full-length *SRK*[29] cDNA clone was isolated by using as probes the *SLG*[29] cDNA and the 0.7-kb *Eco*RI-fragment of the *SRK*[9] cDNA that encodes the kinase domain (Watanabe *et al.* 1994). Using methods similar to those described for the isolation of *SLG*[29], we identified a genomic clone corresponding to *SRK*[29]. The *SRK*[29] genomic clone was subcloned into a plasmid vector, and the DNA sequences were determined. Comparison of the cDNA and the genomic DNA sequences of *SRK*[29] revealed that the subcloned region of the genomic clone lacked the seventh exon (Figure 4). As in other *SRK*s, the first intron of the *SRK*[29] contains an in-frame stop codon (TAG). Hanks and Quinn (1991) have shown that protein kinases have 11 conserved subdomains in which 15 invariant or nearly invariant amino acid residues are located. These subdomains and all the 15 conserved residues are present in *SRK*[29]. The sequences in subdomains VI (HRDLKASN) and VIII (GTYGYMSPE) suggest that *SRK*[29] is likely to have serine/threonine kinase activity.

To confirm the linkage between the *SRK*[29] gene and the *S* locus, RFLP analysis was performed on the same F_2 progeny used in the linkage analysis of the *SLG* gene. When the genomic DNA was digested with *Bam*HI and hybridized with the *Bam*HI-*Xba*I fragment of the *SRK*[29] cDNA, an intense band of 12 kb was identified in the *S*[29] but not in the *S*[45] homozygous parent (Figure 2C). A perfect correlation was observed between the presence of the *SRK*[29] band and the *S*[29] haplotype in 13 plants of the F_2 family segregating for *S*[45] and *S*[29]. These results indicate that *SLG*[29] and *SRK*[29] were linked to the *S* locus.

Genomic structural similarities between *SLG*[29] and *SRK*[29]: Comparison of the genomic sequences of the *SLG*[29] and *SRK*[29] genes revealed a region of sequence similarity, which extends from 370 bp upstream of the ATG codon of the S domain to 4 bp downstream of the



B

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                BOX I                BOX II              BOX III              BOX IV
SLG29  -210 TT GAC-GAATGATA CAA GTTTGT GGAAG TGAATTAATCG ATAGGG TGAAAAAGTCATGGA CT
SLG2   -209 TT GACTGAATGATA CAA GTTTGT GGAAG TGACTTAATCG TAGGGA TGAAAAAGTCATGGA CT
SRK29  -247 TT GAA-GAATGATA CAT ATTTGT GGAAG TGAACTAATCG ATGGGA TGAAAAAGTCATGGA CT
SRK2   -213 TT GAA-GAATGATA CAT ATTTGT GGAAG TGAATTAAACG ATGGGA TGAAAAAGTCATGGA CT
            ** **  ******** **   ***** ***** ***  *** **    **  ***************  **

                BOX V
SLG29  -149 ATGGAAGAACACAAC ATTTTGCTTGCCTGCT AGGTTCGTCATAATAATTTAGTTTC-AAATTTTCT--
SLG2   -147 ATGGA---ACTCAAC ATTTTGCTTGCCAGTT AGGTTCGTCATAATAGTTTAATTCG-AA-TTTTCTTG
SRK29  -186 ATGGAA---CACATC ATTTTGCTTGCCTGCT ATGTTCGTCATAATAAATTAATTTCCAA-TTTTCTCG
SRK2   -152 ATGGAA---CACATC ATTTTGCTTGCCTGCT AGGTTCGTCATACTAAATTAATTTCCAA-TTTTCTCG
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C

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SLG29  ATGCGGAAATACACCGTCGGTGGTCAAGATCTTTACGTCAGATTGAATGCTGCTGATCTAGgttagttta  1330
SRK29  ::CA::::::T:G::::::::::::::::::::::::::::::::::::::::::::::::::::::::  1327

SLG29  cttctgt----ccggatga---ggaagtctccctatttttttccgatccaaatatttgacgggtcttaaaa  1392
SRK29  t:ctct:aaaa:taa:cacatc:::ta:::atg:g:ca:caataa:tt:c:ct::::tcc::a-:g:gg:  1396

SLG29  atcttttctacttttttttttaacaacaaaatcttttctactttctatgaatttgttaagttaaaaag--t  1461
SRK29  :gtc:cc:::t:::aaaa:|::tc:tc:c-t::aa:::g:::::::c:::::::::::::::::::aaa:  2806
                         1415 \ 2758
SLG29  tttctagtgcgaaatataaaactgat---taacgggaaggctgaataaaatttccaatccaagttgtttt  1528
SRK29  ::::a:::::::::::::c:::::::gat:::::a:::t::::g:a::::::::::::::::::::::::  2876

SLG29  tat-----ttta----------------------------ttct--ataaaa------------------  1545
SRK29  :t:gtttt::::acacctccaaagtcgttttttgtttgt::::ca::::::ctgagattgtttatattat  2946

SLG29  ------------------ttttaaaa---taactaaaattaaattaattgtatataaaaatatattcaa  1593
SRK29  atatatatcactaatttttt:::::::aaa:::::::::::-::g:::::::::::::c::::g::::t::  3015

SLG29  taaaattataaactttgtgttacatatataatttgttttgaaatgcaaaggaaatagtacttgcacatac  1663
SRK29  ::::::g:::::::::::::::::::::::-------:a:::::::::::::::::::::::::c::::::c:t  3078

SLG29  tttagcattgaacaaaa--ctgcttaaataagagtttaaaattaatcattttaaacatttatttcaaaat  1731
SRK29  ::::::::c::::::::aa:::::g:::::::::::::::::::::::::::::c:::cat:::::::::  3148

SLG29  tcaaataataaatgtgagtcattcgtccaaataagta-attaaacaaaaa-gcattagttaatagaat--  1797
SRK29  :::::c::::::::::::c:::c:a::::::g:::::t::a:::t::gc:taa:ac::::::::::::aa  3218

SLG29  -------------cccctatat---attat-ttga-----gaag-------cattgcaa----------  1827
SRK29  aatagtataattgacagt:::::cgaaac::a::::tagcta:::gtatgatt::c::::aaacctataa  3288
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Figure 5.—Comparison of the genomic sequences of the *SLG*[29] and *SRK*[29] genes. (A) Schematic representation of the sequenced regions of the *SLG*[29] and *SRK*[29] genomic clones. The shaded boxes represent the protein coding regions in both *SLG*[29] and *SRK*[29]. The striped box corresponds to the untranslated region of the *SLG*[29] gene. The initiation and stop codons of the *SLG*[29] gene are shown. The transmembrane domain (TM) of the *SRK*[29] is indicated under the diagram. The stippled areas between the two diagrams indicate the region showing high similarity. The sequences are numbered relative to the ATG initiation codon of each gene. (B) Nucleotide sequences of the 5′ flanking region of the *SLG*[29] and *SRK*[29] genes aligned with those of *SLG*[2] and *SRK*[2]. Dashes represent gaps introduced to optimize the alignment. Asterisks indicate nucleotides that are conserved in all four sequences. The boxed sequences show regions corresponding to the five boxes that were identified previously by Dzelzkalns *et al.* (1993). The sequences are numbered from the translation initiation codon of each gene. (C) Nucleotide sequence comparison of the first intron of the *SLG*[29] and *SRK*[29] genes. Colons represent nucleotide identities and dashes represent gaps introduced to optimize the alignment. Exon sequences are shown by uppercase letters and intron sequences by lowercase letters. Boxed sequence indicates the in-frame stop codon. The *SRK*[29] intron sequences (1415–2758) that do not show sequence similarity to the *SLG*[29] sequences are omitted. The sequences are numbered relative to the ATG initiation codon of each gene.

TABLE 1

Comparison of the amino acid sequences of different domains of SRK[29] and some other Brassica SRKs

SRK[a]	(class)	Receptor domain (S domain)	Transmembrane domain	Juxtamembrane domain	Kinase domain	C-terminal domain
SRK[29]	(II)	100.0[b] (66.0)[c]	100.0 (45.4)	100.0 (37.7)	100.0 (72.0)	100.0 (59.5)
SRK[2]	(II)	94.2 (66.4)	90.9 (40.9)	92.0 (38.9)	95.5 (70.3)	97.4 (57.1)
SRK[9]	(I)	66.0 (100.0)	45.5 (100.0)	37.7 (100.0)	72.0 (100.0)	59.5 (100.0)
SRK[8]	(I)	68.0 (75.1)	45.5 (64.0)	39.6 (79.6)	67.9 (82.5)	52.3 (63.6)
SRK[6]	(I)	66.7 (76.2)	54.5 (80.0)	51.9 (89.6)	71.2 (88.7)	54.5 (84.1)

[a] SRK[2] and SRK[6] are from *B. oleracea* (Stein *et al.* 1991). SRK[9] and SRK[8] are from *B. rapa* (Watanabe *et al.* 1994; Yamakawa *et al.* 1995).
[b] Data are represented as percent identity of other SRKs relative to SRK[29].
[c] Data are represented as percent identity of other SRKs relative to SRK[9].

in-frame stop codon (at position 1330) in the first intron (Figure 5A). In the S domain, the sequence identity is 84%, and in the 5′ flanking region, the sequence identity is 71%. The five conserved elements (box I to V) previously identified in the 5′ flanking region by Dzelzkalns *et al.* (1993) were also found in these two genes (Figure 5B). This finding suggests that the *SLG*[29] and *SRK*[29] genes may have an expression pattern similar to other *SLG*s and *SRK*s. The promoter regions of the *SLG*[29] and *SRK*[29] genes are more similar to those of Class II *SLG*[2] and *SRK*[2] genes (79.8 and 83.7% identity, respectively) than to those of Class I *SLG*[9] and *SRK*[9] genes (53.9 and 54.0% identity, respectively).

The alignment of the sequences at the 3′ end of the S domain is shown in Figure 5C, with the nucleotides numbered from the ATG initiation codon in each gene. When the first introns of the *SRK*[29] and *SLG*[29] genes were compared, only a 384-bp region (from position 1413 to 1797 in *SLG*[29]) located 87 bp downstream of the in-frame stop codon showed 84% sequence identity, albeit several small deletions/insertions were observed in this region (Figure 5, A and C). The sequence similarity for the rest of the intron (from position 1798 to 2960 in *SLG*[29]) was less than 50%.

The sequence encoding the receptor (S domain), juxtamembrane, transmembrane, kinase and C-terminal domains of *SRK*[29] were compared with the corresponding domains of other *SRK*s, and the results are shown in Table 1. Very low similarity was observed for the juxtamembrane and transmembrane domains between Class I and Class II types of SRKs.

Expression of *SLG* and *SRK*: Because the *SLG*[29] gene had an in-frame stop codon in the intron, it could potentially produce two transcripts that differed at their 3′ ends. One transcript (type I) would contain only the first exon and the other (type II) would contain both the first and second exons (as is the case for the *SLG*[29] cDNA). To examine this possibility, we hybridized stigma and anther poly(A)$^+$RNA to probes expected to be specific for each transcript (see Figure 4). Using the full-length *SLG*[29] cDNA as a probe, a strong band of *ca.* 1.6 kb was observed only in the stigma (Figure 6A). A 1.2-kb *Hin*dIII fragment (probe a in Figure 4) from the *SLG*[29] genomic clone was used to detect type II expression. This probe detected a *ca.* 1.6-kb band in stigmas and none in anthers (Figure 6B). A *ca.* 1.0-kb fragment corresponding to the 5′ end of the first intron of the *SLG*[29] genomic clone (probe b in Figure 4) was amplified by PCR and used as a probe to detect type I expression. This probe detected only a very weak signal in stigmas and none in anthers, even after overexposure (Figure 6C). Furthermore, RT-PCR was performed to look for transcripts that contained the first intron of the *SLG*[29] gene. Poly(A)$^+$RNA isolated from *S*[29] stigmas was reverse transcribed and amplified with a 20-bp oligonucleotide primer (R-6 in Figure 4) located 1050 bp

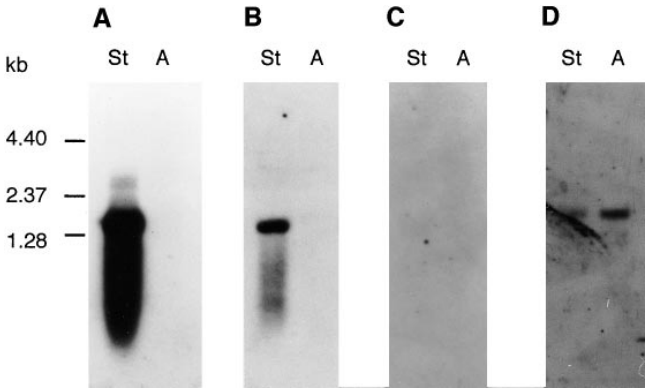

Figure 6.—RNA gel blot analysis of transcripts of the *SLG*[29] and *SRK*[29] genes. One microgram of poly(A)$^+$RNA isolated from stigma (St) and anther (A) was loaded in each lane. RNA blots were hybridized with probes of the following construction: (A) a full-length *SLG*[29] cDNA; (B) the clone containing a part of the intron and the second exon as illustrated in Figure 4 (probe a); (C) the clone containing the 3′ part of the first exon and a part of the intron as illustrated in Figure 4 (probe b); (D) a gene encoding beta subunit of the mitochondrial ATP synthase. RNA size markers, in kb, are shown on the left.

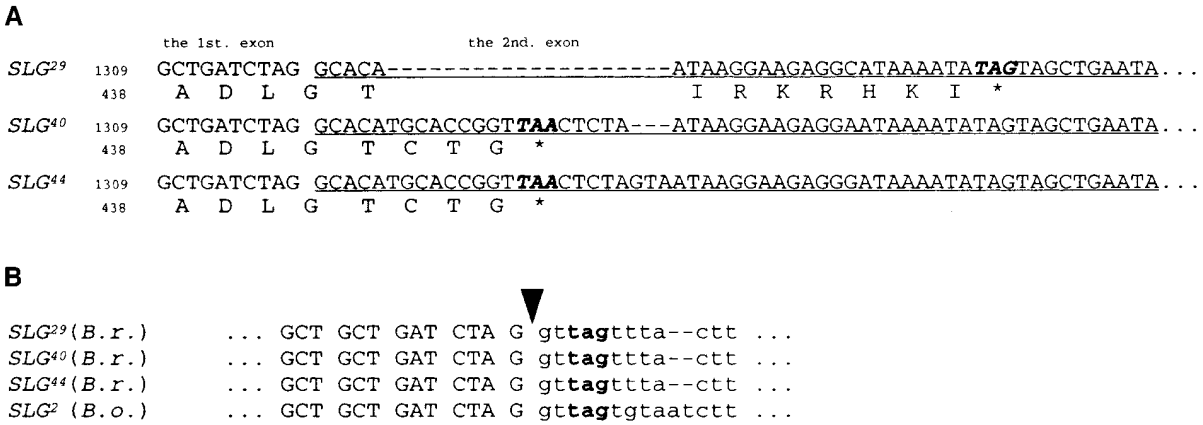

Figure 7.—Comparison of the 3′ terminal regions between pollen-recessive *SLG* genes. (A) Alignment of nucleotide and deduced amino acid sequences at the 3′ terminus between cDNA of SLG^{29}, SLG^{40}, and SLG^{44}. The stop codon of each *SLG* cDNA clone is indicated by bold italics. Sequence encoded by the second exon of the *SLG* gene is underlined. Gaps indicated by dashes are introduced to optimize nucleotide sequence alignment. Sequences are numbered relative to ATG initiation codon of each cDNA. (B) DNA sequence around the first exon/intron junction in the SLG^{29}, SLG^{40}, and SLG^{44} and the SLG^{2} of *B. oleracea* (Tantikanjana *et al.* 1993). Exon sequences are shown by uppercase letters and intron sequences by lowercase letters. Gaps indicated by dashes are introduced to optimize nucleotide sequence alignment. The exon-intron junctions are indicated by an arrowhead. The in-frame stop codons as indicated by bold type letters are present in all genes.

downstream of the translation initiation codon of SLG^{29} and a 3′ oligo (dT) primer. Thirty positive clones were isolated by hybridization with an SLG^{29} cDNA probe. PCR analysis was performed on these clones by using the SLG^{29} forward primer (R-6) and a type II-specific primer (R-10 in Figure 4) complementary to the sequence of the second exon of the SLG^{29} gene. We found that all positive clones corresponded to the type II SLG^{29} transcripts (data not shown). These results suggested that only the type II transcript, consisting of the first and second exons, was produced from the SLG^{29} gene, and that the SLG^{29} gene was expressed mainly in the stigma.

In addition to the SLG^{29} transcript, a band of *ca.* 3.0 kb was observed in the stigma after long exposure, when the full-length SLG^{29} cDNA was used as a probe (Figure 6A). On the basis of the length of the transcript and the intensity of the band, this band was ascribed to the SRK^{29} transcript.

Gene structure of other Class II *SLG*s of *B. rapa*: *SLG* cDNAs were also amplified from stigma poly(A)$^+$ RNA of two other pollen-recessive haplotypes, S^{40} and S^{44}, by using the PS3 primer and an oligo (dT) primer. Alignment of SLG^{29}, SLG^{40} and SLG^{44} cDNA sequences of *B. rapa* revealed that the sequences of the 3′ terminal regions of SLG^{40} and SLG^{44} cDNA were very similar to those of the second exon of SLG^{29}, as shown in Figure 7A. On the basis of the results of the cDNA sequence analysis, the SLG^{40} and SLG^{44} genes were predicted to contain an intron that interrupted the 1321-bp ORF, as did SLG^{29}. To confirm this prediction, amplification of the first intron sequence from S^{40} and S^{44} haplotype genomic DNA was performed (see materials and methods). DNA sequences of approximately 2.0-kb amplified fragments were determined. As found for SLG^{29}, the amplified products from both S^{40} and S^{44} haplotypes contained an in-frame stop codon following the GT motif at the 5′ end of the amplified fragment (Figure 7B), indicating that the *SLG* genes of pollen-recessive haplotypes of Brassica have in common an intron at their C terminus.

DNA sequence analysis of cDNA clones showed that the predicted amino acid sequences of SLG^{40} and SLG^{44} had strong similarity with that of SLG^{29} (96.3% and 95.9% identity, respectively) and contained all of the 12 conserved cysteine residues (Figure 3). However, the amino acid residues (TCTG) encoded in the second exon of SLG^{40} and SLG^{44} were different from those (TIRKRHKI) of SLG^{29}, because of a *ca.* 20-bp deletion or insertion in the sequences of the second exon (Figure 7A).

DISCUSSION

We have characterized three *SLG* genes, SLG^{29}, SLG^{40}, and SLG^{44}, from pollen-recessive haplotypes of *B. rapa* in this experiment. These three *SLG* genes, belonging to the Class II *SLG*, all contain an intron at their C terminus. In contrast, none of the Class I *SLG* genes so far reported contains an intron. The nucleotide sequences of the second exon of the three *SLG* genes are highly conserved, except for a *ca.* 20-bp deletion/insertion (Figure 7A). This deletion/insertion provided different amino acid sequences at the C terminus of SLG^{29} relative to the other two. In SLG^{40} and SLG^{44}, a specific amino acid sequence, TCTG, was found in the C-terminal region (Figures 3 and 7A). This sequence was also found in the *SLG* from another pollen-recessive haplotype, S^5 in *B. oleracea* and self-compatible *Brassica*

napus, although it was not determined whether or not these four amino acids are encoded by the second exon (Scutt and Croy 1992; Robert *et al.* 1994). The structure of the *SLG* genes observed here seems to be typical of Class II haplotypes of Brassica species. However, it is different from that of the SLG^2 gene isolated from a pollen-recessive haplotype of *B. oleracea*, in which the second exon encodes the transmembrane and a part of the cytoplasmic domain (Tantikanjana *et al.* 1993). Tantikanjana *et al.* (1993) suggested that the existence of a membrane-anchored form of SLG might be involved in a leaky self-incompatibility phenotype or the pollen-recessive nature of this haplotype. Our data demonstrated that an unusual structure of SLG^2 was not the sole determinant of its pollen-recessive nature.

In a previous article, we showed, based on pollination results, that 24 *S* haplotypes in *B. rapa* could be classified into three groups: codominant (CD), dominant/recessive (DR), and recessive (R) (Hatakeyama *et al.* 1998). DNA gel blot analysis demonstrates, without exception, that the *SLG* and *SRK* genes isolated from the R group belong to Class II and those from the CD and DR groups belong to Class I (Figure 1). In the S^{29} haplotype of the R group also, the deduced amino acid sequence of the S domain of SRK^{29} is highly similar to that of SLG^{29} (93%), as is true in many *SLG*/*SRK* gene pairs. Both SLG^{29} and the S domain of SRK^{29} show a high degree of sequence similarity (89.8% to 95.7% identity) to the SLGs of S^2 and S^5, which have been classified as Class II, whereas they show less than 70% sequence identity to Class I SLGs. Furthermore, the transmembrane, juxtamembrane, kinase, and C-terminal domains of SRK^{29} are also divergent from those of Class I SRK. In particular, the juxtamembrane and transmembrane domains show the lowest similarity (37.7% and 45.5% identity, respectively) to those of Class I SRK^9 (Table 1). Similar trends have been also observed in the detailed analysis of SLG^2 and SRK^2 isolated from the pollen-recessive S^2 haplotype in *B. oleracea* (Chen and Nasrallah 1990; Stein *et al.* 1991). The feature that pollen-recessive haplotypes have Class II *SLG* and *SRK* genes and the others have Class I seems to be common in Brassica species.

Dominance relationships among haplotypes differ between stigma and pollen expression: for example, pollen-recessive haplotypes, S^{29}, S^{40}, and S^{44}, are codominant in the stigma to many *S* haplotypes (Hatakeyama *et al.* 1998). Pollen (but not stigma) expression correlates well with class type, with all Class II haplotypes examined showing pollen-recessivity. A current model of self-incompatibility in Brassica (Nasrallah and Nasrallah 1993) is that the SLG/SRK complex recognizes an unidentified pollen ligand, which is encoded at the *S* locus. A pulsed-field gel electrophoresis analysis of genomic DNA from S^6 (Class I) and S^2 (Class II) haplotypes of *B. oleracea* has revealed extensive between-class differences across the entire *S* locus region (Boyes and Nasrallah 1993). The complete association between Class II and pollen-recessivity suggests that some of the differences that distinguish the classes may correspond to the subregion that encodes the hypothesized pollen ligand.

The SLG^{29} and SRK^{29} genes have an in-frame stop codon, TAG, following the conserved GT motif at the 5′ end of the first intron. This in-frame stop codon was also found in the intron sequences of the SLG^{40} and SLG^{44} genes. This stop codon could be used to produce a truncated SLG-like protein from an alternative transcript that retains the first intron. The presence of transcripts of the *SRK* gene that retain a part or the full-length of the first intron has been reported previously (Stein *et al.* 1991; Giranton *et al.* 1995; Suzuki *et al.* 1996). In the case of SLG^{29}, however, the transcript that consists of the first and the second exons was predominantly detected, whereas the alternative transcript was undetectable. Further studies are needed to examine the role of the type I transcripts, which consist of the first exon and a part of the intron.

In dendrograms reconstructed using the neighbor-joining method from the base substitutions observed in *SLG*, *SRK*, and *SLG*-related sequences (Hinata *et al.* 1995; Uyenoyama 1995; Kusaba *et al.* 1997), Class II *SLG* and *SLR2* cluster together. A cDNA clone corresponding to *SLR2* was isolated in addition to the PCR-amplified cDNA corresponding to SLG^{29} from the S^{29} haplotype. The deduced amino acid sequence of $SLR2\text{-}S^{29}$ showed a high degree of sequence similarity (more than 85% identity) to that of three Class II *SLG*s, SLG^{29}, SLG^{40}, and SLG^{44} (Figure 3). Class II *SLG* and *SLR2* likely share a common ancestor. When we aligned the deduced amino acid sequences of Class II *SLG*s and *SLR2* that had been isolated previously (Chen and Nasrallah 1990; Boyes *et al.* 1991; Tantikanjana *et al.* 1996; Watanabe *et al.* 1997) with the four clones we isolated, we found that twenty amino acids differed between the Class II *SLG* and *SLR2* families (asterisks in Figure 3). Some of these differences, scattered throughout the amino acid sequence of Class II *SLG* and *SLR2*, may possibly reflect the different functions of the two families.

In *B. rapa*, the several *SLG* genes from Class I *S* haplotypes that have been isolated (Watanabe *et al.* 1994; Yamakawa *et al.* 1994; Matsushita *et al.* 1996; Nishio *et al.* 1996; Kusaba *et al.* 1997) show pairwise sequence identity ranging from 78 to 98%. We observed sequence identity levels among the Class II *SLG*s, SLG^{29}, SLG^{40}, and SLG^{44}, in excess of 95%; this apparent increase in similarity may imply that the time since divergence among haplotypes within Class II is less than that within Class I. Based on results of their simulation model, in which alleles interacted codominantly in the style and formed a dominance hierarchy in the pollen (SSIdomcod), Schierup *et al.* (1997) argued that loss because of drift occurs more easily for pollen-recessive than for pollen-dominant alleles, resulting in lower expected life

span for recessive alleles. Our observation of lower divergence among pollen-recessive Class II *SLG*s may be consistent with this theoretical finding.

The authors thank Professor Teh-hui Kao, Pennsylvania State University, for his critical reading of the manuscript and help in correcting the English. We are grateful to Professor Nam-Hai Chua, The Rockefeller University, for providing us with a genomic clone of the beta subunit of the mitochondrial ATP synthase gene of *Nicotiana plumbaginifolia*. We thank Go Suzuki, Tohoku University, for his helpful comments and criticisms. This work was supported in part by Grants-in-Aid for Special Research on Priority Areas (nos. 07281102 and 07281103; Genetic Dissection of Sexual Differentiation and Pollination Process in Higher Plants) from the Ministry of Education, Science, Culture and Sports, Japan.

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Communicating editor: M. K. Uyenoyama