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Polymorphism of the *S*-locus glycoprotein gene (*SLG*) and the *S*-locus related gene (*SLR1*) in *Raphanus sativus* L. and self-incompatible ornamental plants in the Brassicaceae

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Abstract The *S*-locus glycoprotein gene, *SLG*, which participates in the pollen-stigma interaction of self-incompatibility, and its unlinked homologue, *SLR1*, were analyzed in *Raphanus sativus* and three self-incompatible ornamental plants in the Brassicaceae. Among twenty-nine inbred lines of *R. sativus*, eighteen *S* haplotypes were identified on the basis of DNA polymorphisms detected by genomic Southern analysis using *Brassica SLG* probes. DNA fragments of *SLG* alleles specifically amplified from eight *S* haplotypes by PCR with class I *SLG*-specific primers showed different profiles following polyacrylamide gel electrophoresis, after digestion with a restriction endonuclease. The nucleotide sequences of the DNA fragments of these eight *R. sativus SLG* alleles were determined. Degrees of similarity of the nucleotide sequences to a *Brassica SLG* (S^6*SLG*) ranged from 85.6% to 91.9%. Amino acid sequences deduced from these had the twelve conserved cysteine residues and the three hypervariable regions characteristic of *Brassica* SLGs. Phylogenetic analysis of the SLG sequences from *Raphanus* and *Brassica* revealed that the *Raphanus* SLGs did not form an independent cluster, but were dispersed in the tree, clustering together with *Brassica* SLGs. These results suggest that diversification of the *SLG* alleles of *Raphanus* and *Brassica* occurred before differentiation of these genera. Although *SLR1* sequences from *Orychophragmus violaceus* were shown to be relatively closely related to *Brassica* and *Raphanus SLR1* sequences, DNA fragments that are highly homologous to the *Brassica SLG* were not detected in this species. Two other ornamental plants in the Brassicaceae, which are related more distantly to *Brassica* than *Orychophragmus*, also lacked sequences highly homologous to *Brassica SLG* genes. The evolution of self-incompatibility in the Brassicaceae is discussed.

Key words Stigma glycoprotein · *Orychophragmus violaceus* · *Cheiranthus cheiri* · *Iberis amara* · Evolution

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Introduction

Self-incompatibility (SI), a mechanism that prevents self-fertilization by inhibiting pollen tube growth from self-pollen grains, is known to be widespread in the Brassicaceae, including the economically important genera *Brassica* and *Raphanus*. SI in these genera is of the sporophytic type and is controlled by a single multiallelic locus, the *S* locus (Bateman 1955). Molecular genetic analyses have shown that the *S* locus consists of at least two genes: the *S*-locus glycoprotein gene (*SLG*) and the *S*-locus receptor kinase gene (*SRK*). The *SLG* gene encodes a highly polymorphic secretory glycoprotein synthesized predominantly in the stigma (Nasrallah et al. 1987). The *SRK* gene encodes a transmembrane protein, consisting of an intracellular serine-threonine protein kinase and an extracellular domain which shares a high degree of sequence similarity with *SLG* (Stein et al. 1991). *S* haplotypes in *Brassica* can be classified into two groups, class I and class II. Class I SLGs show about 65% sequence similarity to class II SLGs. Class I haplotypes are known to be pollen-dominant to class II haplotypes. Forty-eight S haplotypes have been identified in *B. oleracea* (Brace et al. 1994) and thirty in *B. campestris* (Nou et al. 1993). Okazaki and Hinata (1984) identified twenty *S* haplotypes in *Raphanus sativus*, another genus of the Brassicaceae. *S*-locus glycoproteins were also detected by isoelectric focusing in *R. sativus* (Okazaki and Hinata 1984), suggesting that genes similar to *Brassica SLG* are present in *Raphanus*.

In *Brassica*, two *SLG*-like genes, *SLR1* and *SLR2*, have been identified that are unlinked to the *S* locus. *SLR1* encodes a secreted glycoprotein in the papilla cell wall of the stigma (Lalonde et al. 1989; Trick and Flavell 1989; Umbach et al. 1990; Isogai et al. 1991). *SLR2*, which is also predicted to encode a secreted glycoprotein (Boyes et al. 1991), exhibits a high degree of sequence similarity to class II *SLG*. The function of *SLR1* and *SLR2* is unknown. In view of the high conservation of their sequences and their floral-specific expression in both self-incompatible and self-compatible strains, these two *S*-locus-related genes are believed to play a fundamental role in the events of pollination in *Brassica* (Lalonde et al. 1989; Boyes et al. 1991). A homologue of *SLR1*, designated as *AtS1*, exists in *Arabidopsis* (Dwyer et al. 1992), but there is no homologue of *SLR2* in *Arabidopsis*. *SLR1* and its homologues are considered to have differentiated from other *S*-related genes first, and the differentiation of *SLR1* within species appears to have occurred rather recently – after speciation in the genus *Brassica* (Hinata et al. 1995; Uyenoyama 1995).

We analyzed *SLG* alleles in breeding lines of *R. sativus* using genomic Southern hybridization and PCR-RFLP analysis (Nishio et al. 1996) to identify *S* haplotypes, and determined the nucleotide sequences of eight class I *SLG* alleles. We also determined the nucleotide sequences of *SLR1* in *R. sativus* and in some self-incompatible ornamental plants in the Brassicaceae in order to understand the distribution of *SLG* homologues in the Brassicaceae and the phylogenetic relationships among these taxa.

Materials and methods

Plant materials

Twenty-nine inbred lines of *R. sativus* L. (twenty-seven lines of var. Daikon, Japanese radish, and the two lines of radish) and three ornamental plants in the Brassicaceae (*Orychophragmus violaceus* O. E. Schulz; wallflower, *Cheiranthus cheiri* L.; and candytuft, *Iberis amara* L. cv. White pinnacle) were used as plant materials. The inbred lines in *R. sativus* were developed through six or seven generations of selfing, and are used as the parental lines of commercial F_1 hybrid cultivars. The *R. sativus* lines and the ornamental plants were provided by the Takii Seed Company (Kyoto, Japan).

DNA isolation and Southern analysis

Genomic DNA was prepared from young leaves according to Rogers and Bendich (1985). Five micrograms of DNA were digested with a restriction endonuclease and subjected to electrophoresis on a 0.8% (w/v) agarose gel and transferred to a nylon membrane (Hybond-N, Amersham) with 10 × SSC. After hybridization with digoxygenin-labeled S^{12}*SLG* (a class I *SLG*) and S^{5}*SLG* (a class II *SLG*) probes (Kusaba et al. 1997), the nylon membrane was washed under high stringency conditions (0.1 × SSC, 0.1% SDS, 68° C) and used for immunological detection of digoxygenin-labeled DNA according to the manufacturer's instructions (Boehringer Mannheim). CDPstar (Boehringer Mannheim) was used as the substrate of alkaline phosphatase, and the chemi-luminescent signal was recorded with the Lumino CCD system (Atto, Tokyo).

PCR-RFLP analysis and cloning of PCR products

PCR-RFLP analysis was conducted as described previously (Nishio et al.1994). The primers used in this analysis included PS-5 and PS-15 (Nishio et al. 1996), and PS-22 (5′-ATCGATGGGATGAAAAAGTCATCG-3′), a forward primer corresponding to the nucleotide sequence of the promoter region of S^{13}*SLG* (Dzelzkalns et al. 1993). To amplify DNA fragments of the *SLR1* homologues, a second set of primers was designed. A forward primer, PS-24 (5′-ATGAGAGGTGTAATACCAAAC-3′), and a reverse primer, PS-25 (5′-GAGATAAAGATCTTGACCTC-3′), were derived from the signal and C-terminal sequences of an *SLR1* from an S^{29} homozygote (Trick and Flavell, 1989), respectively. The amplified products were cloned into the pCRII vector using the TA cloning system (Invitrogen, Calif.).

Sequence analysis

The nucleotide sequences of the cloned DNAs were determined with a DNA sequencer (Prism 377, Perkin-Elmer ABI). To avoid errors that may have occurred during the PCR process, three independent clones obtained from the same inbred line were sequenced. Sequence comparisons of *SLG* and *SLR1* homologues were conducted with Genetyx-Mac Version 8.0 (Software Development, Tokyo). The phylogenetic trees were generated by the neighbor-joining method (Saitou and Nei 1987).

Results

Southern analysis of genomic DNA from *R. sativus*

Twenty-one of the *R. sativus* inbred lines showed two to four bands after *Hin*dIII digestion of genomic DNA and probing with class I *SLG* (Fig. 1); lines were classified into thirteen types based on their band patterns. These lines were considered to be homozygotes of class I *S* haplotypes. The remaining eight inbred lines showed no signals when tested with the class I probe, and showed three or four bands in an analysis using the class II

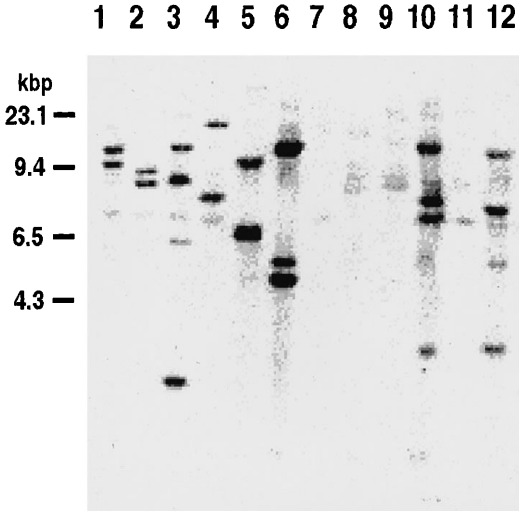

Fig. 1 Southern blot analysis of the genomic DNAs of inbred lines of *Raphanus sativus* digested with *Hin*dIII. The class I *SLG* (*B. oleracea* S^{6}) clone was used as a probe. DNA size markers are shown (in kb) on the left. Lanes: 1, S^{1}; 2, S^{2}; 3, S^{3}; 4, S^{4}; 5, S^{5}; 6, S^{6}; 7, S^{9}; 8, S^{10}; 9, S^{11}; 10, S^{12}; 11, S^{13}; 12, S^{14}

Table 1 *S* haplotypes assigned to the parental inbred lines of F_1 cultivars in *R. sativus*

S haplotype	*SLG* class	Number of lines	F^1 cultivars
S^1	Class I	3	Ball Cross, Kanpaku, Shinhasshu
S^2	Class I	4	Relish Cross, YR Kurama, YR Tengu
S^3	Class I	2	YR Kurama, Tama Winter
S^4	Class I	2	Kanpaku, YR Kurama
S^5	Class I	1	
S^6	Class I	1	
S^7	Class I	1	Easter
S^8	Class I	1	Champion
S^9	Class II	3	Relish Cross, Ryokuki
S^{10}	Class II	1	Oshin
S^{11}	Class II	1	Tama Winter, Akizumari
S^{12}	Class I	1	
S^{13}	Class II	2	
S^{14}	Class I	1	
S^{15}	Class I	2	Oshin, April Cross
S^{16}	Class II	1	
S^{17}	Class I	1	
S^{18}	Class I	1	Champion

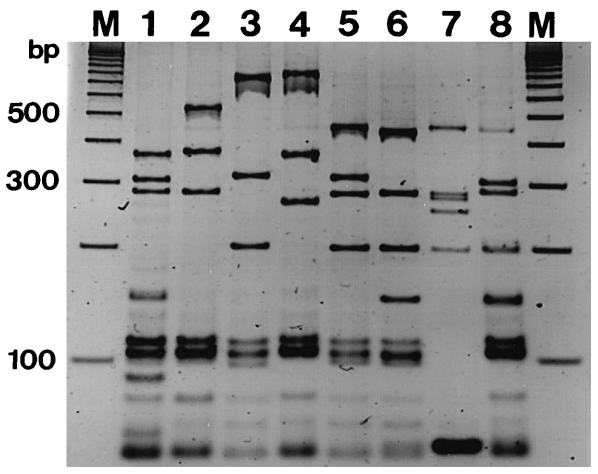

Fig. 2 Polyacrylamide gel electrophoresis of PCR products after cleavage with *Mbo*I. *SLG* DNA was specifically amplified by PCR from the genomic DNA of *S* homozygotes of *R. sativus* with primers PS5 and PS15 (lanes 1–6, 8) and with PS22 and PS15 (lane 7). Lanes: 1, S^1; 2, S^2; 3, S^3; 4,S^4; 5, S^5; 6, S^6; 7, S^7; 8, S^8

probe. They were classified into five types, considered to represent class II *S* haplotypes.

Based on band pattern in genomic Southern blots, eighteen *S* haplotypes (S^1 to S^{18}) were assigned to the twenty-nine inbred lines (Table 1). Although pollination tests have not been carried out in all cross combinations, all crosses between lines having different band patterns tested so far have been compatible, and those between lines having the same band pattern incompatible (data not shown).

PCR-RFLP analysis of class I SLG alleles

Primers PS5 and PS15, designed for specific amplification of class I *SLG* alleles in *Brassica*, were found to be useful for the PCR amplification of *SLG* alleles from *Raphanus* DNA. DNA fragments of 1.3 kb were amplified from seven class I *S* haplotypes, S^1–S^6, and S^8. Digestion of the PCR products with *Mbo*I and subsequent polyacrylamide gel electrophoresis revealed polymorphism of the amplified 1.3-kb DNA fragments (Fig. 2). Different *S* haplotypes showed distinct band patterns, and all lines having the same *S* haplotype had the same pattern. PS22, corresponding to the DNA sequence of the promoter region of *SLG*, was used instead of PS5 as a forward primer for PCR. A single DNA fragment of about 1.5 kb was amplified from one *S* haplotype, *S7*, in which no DNA fragment was amplified with PS5 and PS15.

Comparison of deduced amino acid sequences of class I SLGs

Nucleotide sequences of the DNA fragments amplified from the seven *S* haplotypes with PS5 and PS15 and the fragment amplified from S^7 with PS22 and PS15 were determined. They were highly homologous to the *SLG* gene of SLG^{6Bol} (the *SLG* of the S^6 haplotype in *B. oleracea*), a class I *SLG* allele (Nasrallah et al. 1987). The degree of nucleotide sequence similarity to SLG^{6Bol} of the DNA fragments amplified from *R. sativus* ranged from 85.6% to 91.9% in the coding region of the mature *SLG* protein. Similarities among the sequences from the *R. sativus* lines ranged from 84.7% to 91.6%.

Deduced amino acid sequences showed characteristic features of *Brassica* SLG proteins. The twelve cysteine residues present in the *Brassica* SLGs were conserved in all of the *R. sativus* sequences, and the three hypervariable regions reported by Kusaba et al. (1997) were also observed (Fig. 3). There were only two conserved potential N-glycosylation sites, as shown by comparison with SLGs of *B. oleracea* and *B. campestris*. The level of amino acid sequence similarity to SLG^{6Bol} ranged from 77.8% to 87.4%, not significantly lower than the similarities of *B. oleracea* SLGs to SLG^{6Bol} (from 77.8% to 88.6%). These results suggest that the amplified DNA fragments were indeed derive from *R. sativus SLG* alleles.

A phylogenetic tree was constructed using the deduced amino acid sequences of *R. sativus* SLGs together with previous reported SLGs from *B. oleracea* and *B. campestris* (Kusaba et al. 1997) (Fig. 4). SLGs from *R. sativus* did not form an independent cluster. Although SLG^{4Rsa} and SLG^{3Rsa} (the SLGs of S^4 and S^3 haplotypes in *R. sativus*, respectively) made up a pair with a relatively high bootstrap value (74.4%), the other SLGs in *R. sativus* were dispersed in the tree, forming clusters with SLGs from *B. oleracea* and *B. campestris*. SLG^{7Rsa} paired with SLG^{35Bca}, and SLG^{1Rsa} with SLG^{34Bca}.

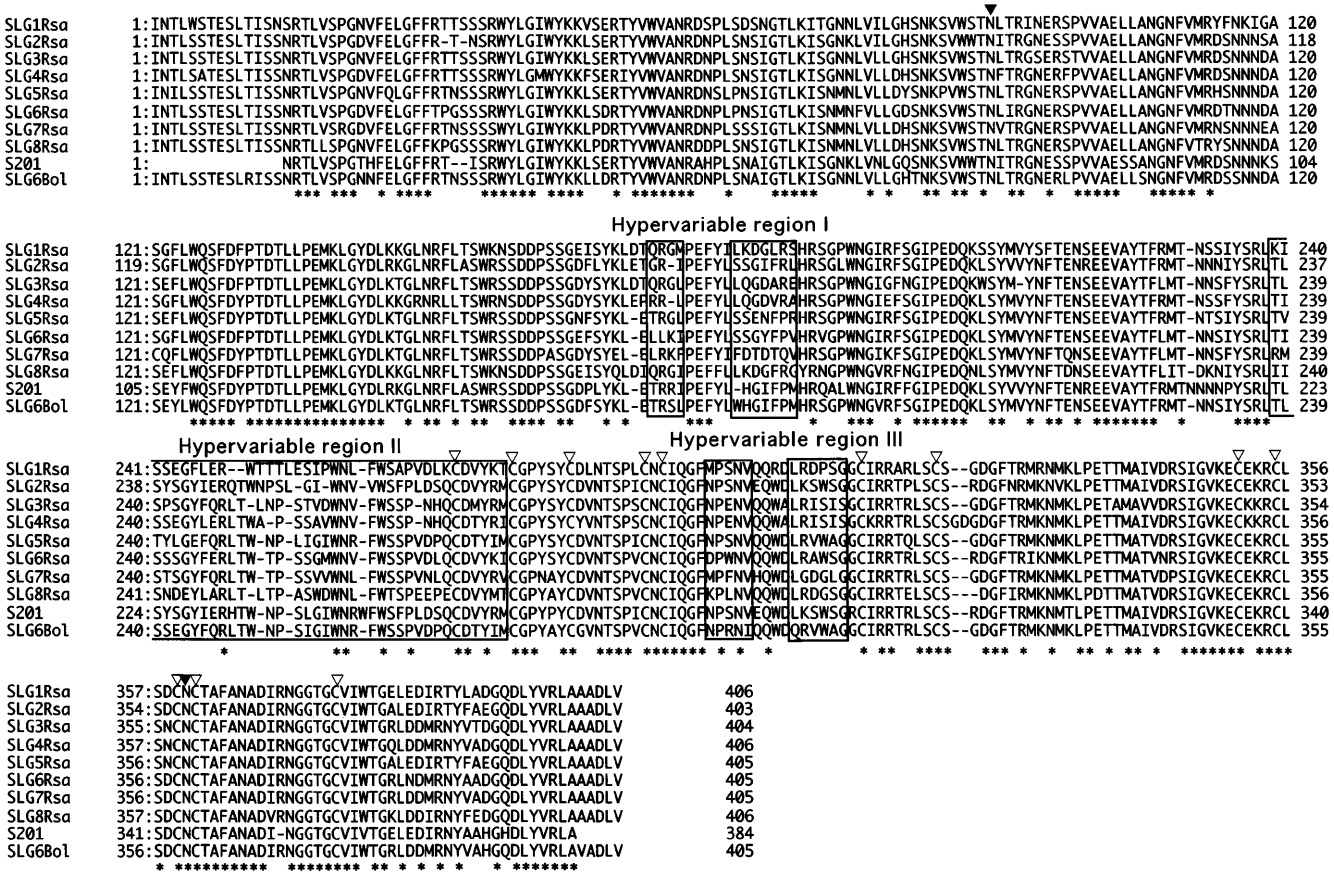

Fig. 3 Alignment of predicted amino acid sequences of SLGs from *R. sativus* S^1, S^2, S^3, S^4, S^5, S^6, S^7, S^8, and *B. oleracea* S^6. The SLG sequence of S201 in *R. sativus* (Niikura and Matsuura 1997) was also included. *Filled* and *open triangles* indicate the positions of potential N-linked glycosylation sites and conserved cysteine residues, respectively. *Boxed regions* represent the hypervariable regions. *Asterisks* indicate conserved amino acid residues. The sequences reported in this paper have been deposited in the GenBank database (Accession Nos. AB009677-AB009684 and AB009872-AB009874)

Analysis of SLG homologues in ornamental plants in the Brassicaceae

In the three brassicaceous ornamental plants, *Cheiranthus cheiri*, *Iberis amara* and *Orychophragmus violaceus*, pollen germination and pollen tube growth were inhibited after self-pollination, while the pollen grains from the same anther germinated well and pollen tubes penetrated into stigma tissue after crossing and bud pollination (data not shown). These observations confirm the expression of self-incompatibility in these species. However, some *O. violaceus* plants were self-compatible. In PCR amplifications from the genomic DNA of these ornamental plants, no DNA fragment was amplified with the class I *SLG*-specific primers PS5 + PS15 or PS22 + PS15. Genomic Southern analysis of these plants using the class I or class II *SLG* probes under high stringency conditions did not detect hybridizing bands (data not shown). Under lower stringency conditions, many bands were detected in each lane. These results indicate that there are no sequences that are highly homologous to *SLG* in these species.

In order to investigate phylogenetic relationships among *Brassica*, *Raphanus* and these ornamental plants, the *SLR1* genes, which are present both in *Brassica* and *Arabidopsis* (*AtS1*) (Dwyer et al. 1992), were analyzed. A DNA fragment of 1.3 kb was amplified by PCR with a primer pair specific for *SLR1* (PS24 and PS25) from *B. oleracea*, *R. sativus*, *O. violaceus*, *C. cheiri*, and *A. thaliana*, but not from *I. amara*. In the PCR-RFLP analysis of five individual plants of each species using these primers, there was no variation in band pattern within species, suggesting low polymorphism of this DNA in these species. Nucleotide sequences of the DNA fragments amplified from *R. sativus* (S^1), *O. violaceus* and *C. cheiri* showed 91.5%, 79.8% and 76.0% similarity, respectively, to the *SLR1* gene of *B. oleracea* (S^{29}) (Trick and Flavell 1989). The twelve cysteine residues conserved among SLR1s in *B. oleracea*, *B. campestris*, and *A. thaliana* were also conserved in the sequences of *R. sativus*, *O. violaceus* and *C. cheiri*. Similarities of the deduced amino acid sequences from *R. sativus*, *O. violaceus* and *C. cheiri* to SLR1 of *B. oleracea* (S^{29}) were 86.0%, 70.7%, and 64.8%, respectively.

A phylogenetic tree of the SLR1 homologues in *R. sativus*, *O. violaceus*, and *C. cheiri* and SLG related sequences was constructed from the deduced amino acid sequences using the neighbor-joining method (Fig. 5). The SLR1 homologues in *R. sativus*, *O. violaceus* and

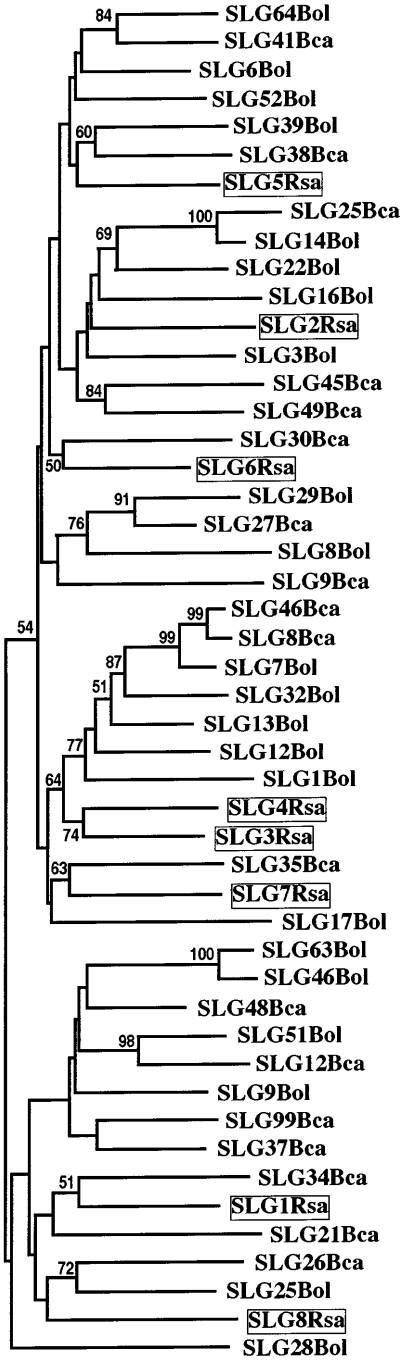

Fig. 4 Neighbor-joining tree of class I SLG sequences. Deduced amino acid sequences of putative mature protein regions of SLGs were used for the analysis. Class II SLG (*B. oleracea* S^2) was used as an outgroup. The numbers beside the branches are the bootstrap values (%) for 1000 repeats

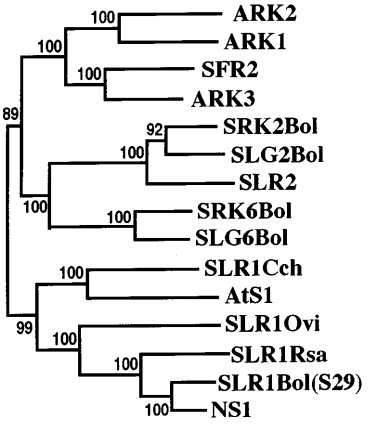

Fig. 5 Neighbor-joining tree of SLG-related sequences in Cruciferae. Amino acid sequences of putative mature protein regions, omitting the sequence corresponding to the PCR primers, were used for the analysis. RLK1 (Walker 1993) was used as an outgroup. The numbers beside the branches are the bootstrap values (%) with 1000 repeats. SLR1Rsa (*R. sativus*), SLR1Ovi (*O. violaceus*), SLR1Cch (*C. cheiri*), NS1 (*B. campestris*), and AtS1 (*A. thaliana*) are SLR1 homologues. ARK1, ARK2 and ARK3 are derived from *A. thaliana*. The other sequences, including SFR2, are derived from *B. oleracea*

C. cheiri, and *Brassica* SLR1s, including NS1 in *B. campestris* (Isogai et al. 1991), formed a single cluster with a high bootstrap value (99.7%), suggesting that all DNA fragments amplified with the primer pair PS24 + PS25 were derived from *SLR1* genes. The group including ARK1 (Tobias et al. 1992), ARK2, and ARK3 (Dwyer et al. 1994) from *Arabidopsis thaliana* and SFR2 from *B. oleracea* (Pastuglia et al. 1997) clustered with the group of class I and class II SLGs and SRKs from *B. oleracea* (89% bootstrap value). SLR1 from *B. oleracea* paired with *B. campestris* SLR1 (NS1), and *R. sativus* SLR1 formed a group with these two SLR1s. *O. violaceus* SLR1 formed a cluster with those of *Brassica* and *Raphanus*, while *C. cheiri* SLR1 formed a pair with *Arabidopsis* SLR1 (AtS1).

Discussion

Because *S*-locus glycoproteins have been identified in *R. sativus* (Okazaki and Hinata 1984), it was expected that *SLG* alleles homologous to those of *Brassica* would be present also in *Raphanus*. Genomic Southern blot analyses using class I and class II *SLG* probes from *Brassica* showed that there are both class I- and class II-homologous sequences in *R. sativus*. We assigned twenty-nine inbred lines of *R. sativus* to eighteen *S* haplotypes based on their band patterns in genomic Southern analyses, assuming that each distinct band pattern corresponds to a distinct *S* haplotype. Although this assumption has not been verified in *R. sativus*, the results of crossing experiments carried out so far have supported it.

SLG alleles that are highly homologous to *Brassica SLG* alleles were found in *R. sativus*. *SLG* alleles were specifically amplified by PCR from eight of the 13 class I S haplotypes tested, using class I *SLG* primers. The deduced amino acid sequences of the eight *SLG* alleles in *R. sativus* had the twelve conserved cysteine residues and the three hypervariable regions characteristic of *Brassica* SLG proteins (Kusaba et al. 1997). The similarity in the

structure of the SLG proteins in *Raphanus* and *Brassica* underlines the importance of these cysteine residues and the hypervariable regions for the function of SLG in self-incompatibility. The phylogenetic tree constructed using the deduced amino acid sequences of SLGs in *R. sativus*, together with those reported in *Brassica* so far, showed that *R. sativus* SLG did not form a unique cluster, but were dispersed in the tree, often clustering with *Brassica* SLGs. This indicates that the diversification of *SLG* alleles predates the differentiation of the genera *Brassica* and *Raphanus*.

The genus *Raphanus* belongs to the tribe *Brassiceae* but not to the subtribe *Brassicinae*. Because inter-generic hybrids between *R. sativus* and *B. oleracea* can be obtained (Karpechenko 1927), these two species are considered to be closely related. Analysis of restriction fragment sizes of chloroplast DNA in Brassiceae have suggested a close relationship between *Brassica* and *Raphanus* (Yanagino et al. 1987; Warwick and Black 1997). On the other hand, *Orychophragmus*, which did not seem to have any sequences highly homologous to *SLG* in its genome, was considered to be a member of the subtribe Moricandiinae in the tribe Brassiceae; however, Gomez-Campo (1980) has claimed that *Orychophragmus* should be excluded from the tribe Brassiceae. The phylogenetic analysis of *SLRl* sequences in this study is compatible with these general assignments: *Raphanus* was the genus most closely related to *Brassica* and *Orychophragmus* was more distantly related to them. Because the *SLR1* gene appears to exist in the genome as a single-copy gene and sequence variation within species is not high, it is expected that sequence analysis of *SLR1* genes can provide important information for the understanding of the phylogenetic relationships among genera within the Brassicaceae.

That DNA sequences highly homologous to a class I or class II *SLG* gene are present in *Raphanus* but not in *Orychophragmus* may provide insight into the evolution of the *S* locus in the Brassicaceae. We consider two possible interpretations of this result: (i) the *SLG*/*SRK* system of self-incompatibility arose after the divergence of *Orychophragmus* from the Brassicinae and Raphaninae and before the divergence between the Brassicinae and Raphaninae; and (ii) the *SLG*/*SRK* system of self-incompatibility occurs throughout the family Brassicaceae, but with class I and class II S-alleles restricted to the Brassicinae and Raphaninae.

All *SLG* and *SRK* alleles reported so far can be classified into two groups, class I and class II. Genomic Southern blot analysis of *S* tester lines in *B. oleracea* indicates that all 45 *S* haplotypes examined in this species belong to either class I or class II (K. Okazaki et al. unpublished results). We have demonstrated this pattern in *R. sativus* as well. In contrast, *Orychophragmus* does not appear to maintain close homologues of class I and class II alleles, even while maintaining self-incompatibility. If class I and class II alleles define the *SLG*/*SRK* system of SI, this SI system would appear to have originated after the divergence of *Orychophragmus* from Brassicinae and Raphaninae and before the divergence of Brassicinae from Raphaninae.

The second interpretation we consider is that self-incompatibility in *Orychophragmus* may be mediated by the *SLG*/*SRK* system but through classes of *S*-alleles other than I or II. Our genomic Southern blot analysis of brassicaceous self-incompatible plants outside the Brassicinae and Raphaninae revealed sequences homologous to Brassica class I and class II SLG probes under low but not high stringency conditions. The restriction of class I and class II alleles to *Brassica* and *Raphanus* may be consistent with a bottleneck hypothesis (Richman et al. 1996): from a population with highly variable *S* haplotypes including the class I and the class II haplotypes, only the class I and the class II were transmitted to Brassicinae and Raphaninae, because the size of population was extremely reduced at a particular stage of evolution. Diversification of *SLG* and *SRK* alleles among class I and class II occurred before the differentiation of genera in these two subtribes. The latter explanation is supported by the observation that, in the Brassicaceae, there are many genera having SI systems with sporophytic control and the rejection of self-pollen grains at the stigma surface, like that in *Brassica* (Bateman 1955). However, more detailed analysis of *SLG* and *SRK* alleles using many genera in the Brassicaceae is needed to understand the evolutionary process that gave rise to *S* haplotypes and the SI system in the Brassicaceae.

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