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Identification and classification of S haplotypes in *Raphanus sativus* by PCR-RFLP of the S locus glycoprotein (SLG) gene and the S locus receptor kinase (SRK) gene

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Abstract Polymorphism of the S-locus glycoprotein (SLG) and S-locus receptor kinase (SRK) genes in *Raphanus sativus* was analyzed by PCR-RFLP using SLG- and SRK-specific primers. Twenty four inbred lines of *R. sativus* could be grouped into nine S haplotypes. DNA fragments of SLG alleles specifically amplified from five S haplotypes by PCR with Class-I SLG-specific primers showed different profiles upon polyacrylamide-gel electrophoresis after digestion with restriction endonucleases. The five *R. sativus* SLG alleles were determined for their nucleotide sequences of DNA fragments. Comparison of the amino-acid sequences with a reported *Brassica* SLG (S_6) showed 77–84% homology. Deduced amino-acid sequences showed 12-conserved cystein residues and three hypervariable regions which are characteristic of *Brassicsa* SLG. A DNA fragment was also amplified by PCR from two of each S haplotype with Class-II SLG-specific primers, and showed polymorphism when cleaved with restriction endonucleases. The nucleotide sequences of amplified DNA fragments of the Class-II SLG revealed about 60% similarity with those of the Class-I SLG. It is concluded that there exist both Class I and Class II S alleles in *R. sativus*, as in *Brassica campestris* and *Brassica oleracea*. PCR using SRK-specific primers amplified a DNA fragment of about 1.0 kb from seven of each S haplotype out of 24 tested. These DNA fragments showed high polymorphism in polyacrylamide-gel electrophoresis after digestion with restriction endonucleases. Nucleotide sequences of the DNA fragments amplified from the seven S haplotypes showed that the fourth and the fifth exons of SRK are highly conserved, and that there is high variation in the fifth intron, the sixth intron and seventh exon of the SRK which may be responsible for the polymorphic band patterns in PCR-RFLP analysis. The PCR-RFLP method has proven useful for the identification of S alleles in inbred lines and for listing S haplotypes in *R. sativus*. Phylogenic analysis of the SLG and SRK sequences from *Raphanus* and *Brassica* revealed that the *Raphanus* SLGs and SRKs did not form an independent cluster, but were dispersed in the tree, clustering together with *Brassica* SLGs and SRKs. Furthermore, SLGs and SRKs from *Raphanus* were both grouped into Class-I or Class-II S haplotypes. Therefore, these results suggest that the diversification of the SLG and SRK alleles occurred prior to the differentiation of the two genera *Brassica* and *Raphanus*.

Keywords *Raphanus sativus* · Self-incompatibility · SLG · SRK · PCR-RFLP

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Introduction

Self-incompatibility (SI) is one of the mechanisms promoting outbreeding that have evolved in higher plants. In many species, SI is genetically controlled by a single Mendelian locus, the S (sterility) locus that exists as multiple alleles, each of which encodes a distinct mating specificity (Bateman 1955). SI response involves cell to cell interactions between the pollen cells and the papillar cells of the stigma that result in the recognition and inhibition of pollen of genetically identical S alleles. Molecular analyses have shown the complexity of the *Brassica* S locus, which consists of at least two physically linked genes that are expressed in the stigma. Of these two genes, the S locus glycoprotein (SLG) gene encodes a secreted S glycosylated protein localized in the stigmatic papillar (Nasrallah et al. 1985), and the S locus receptor

kinase (SRK) gene encodes a receptor-like protein (Goring and Rothstein 1992; Rundle et al. 1993; Stein and Nasrallah 1993). SRK consists of an extracellular 'S' domain that shares strong homology to its corresponding SLG (Stein et al. 1991), a membrane-spanning domain, and a cytoplasmic domain with serine/threonine kinase activity. Comparison of SLG sequences from different S haplotypes revealed extensive sequence polymorphism (Nasrallah et al. 1987; Trick and Flavell 1989; Scutter and Croy 1992). Thus, SLG alleles can be divided into two classes, Class I and Class II (Nasrallah et al. 1991). The amino-acid sequence divergence among Class-I SLGs ranged from 2.5% to 20%, while that between C1ass-I and Class-II SLGs was about 30% (Kusaba et al. 1997). Class-I haplotypes have a strong self-incompatible phenotypic effect and are generally considered dominant and codominant to other S haplotypes, whereas Class-II haplotypes display a weak self-incompatible phenotypic effect and, therefore, are considered to be recessive (Nasrallah and Nasrallah 1993).

In order to identify the S haplotypes of plant material, crossing with all S tester lines and observation of pollen-tube germination or seed set are necessary. Fifty, 30, and 18 S alleles have been identified in *Brassica oleracea*, *Brassica campestris* and *Raphanus sativus*, respectively (Ockendon 1974; Nou et al. 1993; Sakamoto et al. 1998). Therefore, in *B. oleracea* as many as 50 test crosses may be needed. Because the SI phenotype is affected both by environmental factors and the physiological conditions of plants, the test crossing should be repeated several times. This is a highly time-consuming and labor-intensive procedure. Consequently, simple methods for identifying S haplotypes using polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) analysis of SLG and SRK have been developed (Brace et al. 1994; Nishio et al. 1994, 1996, 1997; Sakamoto et al. 1998). This method is expected to be useful for identification of S haplotypes in *Brassica* and *Raphanus*.

In this study, we developed primers to specifically amplify SLGs and SRKs in *R. sativus*, and used them in PCR-RFLP analysis to identify S haplotypes in the radish inbred lines. To understand evolution of the SLG and SRK alleles in the Crucifer family, we compared the deduced amino-acid sequences of SLGs and SRKs among *B. oleracea*, *B. campestris* and *R. sativus*.

Materials and methods

Plant materials

Twenty four homozygous breeding lines that belong to ten S haplotypes in *R. sativus* L. were analyzed in this study (Table 1). The S haplotypes of these lines were named by pollen-tube germination analysis. The inbred lines were developed by repeated selfing for six to seven generations, and are used as the parental lines for commercial F_1 hybrid cultivars. For segregation analysis of the F_2, a S homozygous S_1 plant (inbred #37) was crossed with a homozygous S_2 plant (inbred #50). Thirty eight F_2 progenies were generated by bud pollination of F_1 hybrid plants (S_1 S_2) and used to identify S haplotype segregation. Incompatibility genotypes of S haplotypes were determined by self-pollination and by the diallele pollination test, using fluorescence microscopy analysis of pollen-tube development. The pollen-tube germination analysis consisted of fixing styles 24 h after pollination, softening in 1M NaOH for 50 min at 60 °C, staining with aniline blue, and observing the squashed style with a fluorescence microscope (Kho and Baer 1968).

PCR-RFLP analysis

Genomic DNA was prepared from young leaves according to Nahm et al. (1997). DNA fragments were amplified with SLG- or SRK- specific primer sets (Table 2). The amplification reaction of genomic DNA was carried out in a volume of 25 µl with 25 pmol of each primer, 50 pg of template DNA, 200 µM of each dNTP, 1 unit of *Pwo* DNA polymerase (Boehringer Mannheim, Germany) and a reaction buffer containing 250 mM KCl, 100 mM Tris-HCl(pH 8.85), 50 mM $(NH_4)_2$ SO_4 and 20 mM $MgSO_4$. The PCR condition involved pre-denaturation for 5 min at 94 °C, 35 cycles of 1 min at 94 °C, 2 min at 55 °C, and 3 min at 72 °C, and an extension of 10 min at 72 °C with a thermal cycler (PTC-100, MJ Research, Inc.). PCR products were digested with *Taq*I, *Tru*91, *Msp*I, *Alu*I, *Rsa*I and *Hae*III. The digested DNA was subjected to electrophoresis in a 5% polyacrylamide gel containing TBE buffer. After electrophoresis at 150 V for 2 h, DNA bands were detected by silver staining (Promega, Madison, USA).

Determination of nucleotide sequences of the PCR products

The DNA fragments amplified with *Pwo* DNA polymerase were cloned with de-phosphorylated *Sma*I-digested pBluescript SK vector (Stratagene, USA), and the nucleotide sequences of the cloned DNA were determined with a DNA sequencer (ABI377, Perkin Elmer, USA). To avoid error, three independent clones were sequenced. Restriction fragment sizes were deduced from the nucleotide sequences by the Restriction Enzyme Analysis tool of GENESEO.

Table 1 S haplotype assignment from S tester lines and undefined S inbred lines in *R. sativus*

SLG class	S haplotype	Number of lines	Cultivar (breeding lines)
Class I	S_1	5	OH1(#37), OH2(#43), 307(#3), OS (#80), OSJ (#143)
Class I	S_2	1	OH3 (#50)
ND[a]	S_3	1	CH (#267)
Class II	S_4	1	Ulsanbancheong (#107)
Class II	S_5	7	KK1 (#262), KK2 (#5324), JYI (#5334), 23 (#18), 31 (#28), 69 (#32), Seoul Radish (#154)
Class I	S_6	2	03 (#250), 60 (#39)
Class I	S_7	1	92 (#204)
ND	S_8	2	JY2 (#5335), Samge (#5314)
ND	S_9	2	23 (#12), OS (#85)
Class I	S_{10}	1	Younghyeonbanchenong (#102)

[a] Not determined by PCR-RFLP

Table 2 Primers used for amplification of SLGs and SRKs by PCR

Primer set	Nucleotide sequence	Source	Reference
Class-I SLG	ATGAAAGGCGTAAGAAAAACCTA CCGTGTTTTATTTTAAGAGAAAGAGCT	Radish clone # 20 (17–39)[a] Radish clone # 20 (1,372–1,346)[a]	This study
Class-II SLG	CTCAAGTCCCACTGCTGCGG ATGAAAGGGGTACAGAACAT	SLG 2A (1,025–1,006) SLG 2A (1–20)	Chen and Nasrallah (1990)
SRK	TGATGAGTTTATGAATGAGGTGA GCTTTCATATTACCGGGCATCGATGA	SRK 3 (3,773–3,796) SRK 3 (4,928–4,902)	Delorme et al. (1995)

[a] Number of nucleotide sequences

Results

Design of primers

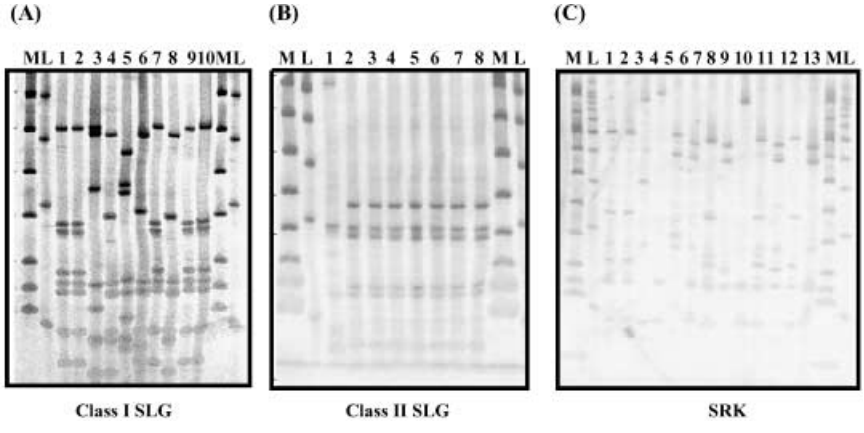
To isolate the SLG gene from *R. sativus*, the cDNA library was constructed from the stigma of radish flowers and screened with the *Brassica* SLG gene as a probe. From the screening, we isolated 14 independent clones of *R. sativus* SLG genes. To select the primer sequences, a multiple sequence alignment with the S6 SLG from *B. oleracea* (Nasrallah et al. 1985) and the 14 SLG genes of *R. sativus* was performed. The Class-I SLG-specific primer set was selected from the conserved region. The Class-II SLG-specific primer set was also designed from alignment of the *B. oleracea* S_5, S_2 and S_{15} SLG genes. For the specific amplification of SRK, primers were designed by aligning SRK genes of *B. oleracea* S_3 (Delorme et al. 1995) and S_{29} (Kumar and Trick 1994), and of *B. campestris* S_{12} (Yamakawa et al. 1995), and by depicting the most variable region between exon 4 and exon 7. Table 2 shows the nucleotide sequence of the primer sets used in this study.

PCR-RFLP analysis of SLG or SRK alleles

To identify and classify the S haplotypes from inbred lines having SI phenotypes, the PCR reaction was performed with the Class-I SLG-specific primer set. The size of the PCR product that was obtained from 10 out of 24 radish inbred lines was approximately 1,400 bp. Digestion of the PCR products with restriction endonucleases and subsequent polyacrylamide-gel electrophoresis revealed polymorphism of the amplified DNA fragments. Five types of electrophoretic profiles were found in ten inbred lines. Five inbred lines (#37, #43, #3, #80 and #143) had the same profile, two inbred lines (#250 and #39) were of another profile, and the other three lines all had different profiles (Fig. 1A). All the different S genotypes showed different electrophoretic profiles, and lines having the same S genotype showed identical profiles. Based on PCR-RFLP data and pollen-tube germination analysis, five S haplotypes were found in ten inbred lines, and were designated as S_1, S_2, S_6, S_7 and S_{10}.

Since there are two types of SLG, the primers specific to Class-II SLGs were also used for PCR. The Class-II SLG DNA fragment, having an expected size of about 1,000 bp, was amplified from 8 out of 24 plants tested.

Fig. 1A–C Polyacrylamide-gel electrophoresis of *Taq*I-digested DNA fragments, which were amplified as PCR products from inbred lines in *R. sativus*. **A** PCR-RFLP with Class-I SLG DNA fragments. *1*: #37 (S_1 S_1), *2*: #43 (S_1 S_1), *3*: #50 (S_2 S_2), *4*: #250 (S_6 S_6), *5*: #204 (S_7 S_7), *6*: #102 (S_{10} S_{10}), *7*: #3 (S_1 S_1), *8*: #39 (S_6 S_6), *9*: #80 (S_1 S_1), and *10*: #143 (S_1 S_1). **B** PCR-RFLP with Class-II SLG DNA fragments. *1*: #107 (S_4 S_4) *2*: #262 (S_5 S_5) *3*: #5324 (S_5 S_5), *4*: #5334 (S_5 S_5), *5*: #18 (S_5 S_5), *6*: #28 (S_5 S_5), *7*: #32 (S_5 S_5), and *8*: #154 (S_5 S_5). **C** PCR-RFLP with SRK DNA fragments. *1*: #37 (S_1 S_1), *2*: #43 (S_1 S_1), *3*: #50 (S_2 S_2), *4*: #250 (S_6 S_6), *5*: #204 (S_7 S_7), *6*: #5335 (S_8 S_8), *7*: #3 (S_1 S_1), *8*: #12 (S_9 S_9), *9*: #39(S_6 S_6), *10*: #80 (S_1 S_1), *11*: #85 (S_9 S_9), *12*: #143 (S_1 S_1), and *13*: #160 (S_8 S_8). Brackets represent the S genotype of inbred lines. *M*: pUBCM21 digested with *Hpa*II, *Dra*I and *Hin*dIII. *L*: 100-bp ladders



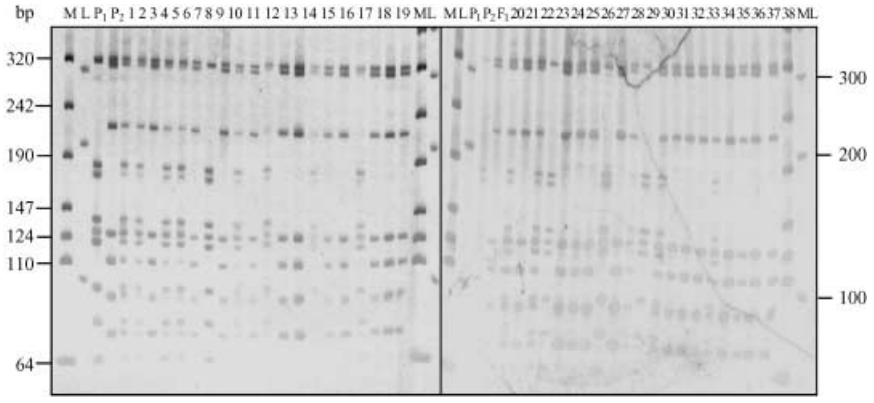


Fig. 2 Analysis of a small F_2 population segregating for S_1 (P_1) and S_2 (P_2) haplotypes. Genotypes assigned by pollen-tube germination analysis were as follows. Lanes 2, 5, 7, 9, 11, 12, 14, 15, 17, 18, 19, 20, 23, 24, 25, 27, 31, 32, 33, 35, 36, 37, 38: S_2 S_2. Lanes 6, 22, 26, 29: S_1 S_1. Lanes 1, 3, 4, 8, 10, 13, 16, 21, 28, 30, 34: S_1 S_2. P_1, P_2, and F_1 refer to parent 1 (S_1 S_1), parent 2 (S_2 S_2), and their hybrid (S_1 S_2), respectively. *M*: pUBCM21 digested with *Hpa* II, *Dra* I, and *Hin*dIII. *L*: 100-bp ladders

Table 3 Sizes of restriction fragments of the PCR products of Class-I SLGs, Class-II SLGs and SRK in *R. sativus*

S alleles	Expected sizes of restriction fragments (bp)		
SLG (Class)	*Taq*I	*Msp*I	*Nde*II
S_1 SLG (I)	309, 181, 167, 138, 129, 123, 98, 81, 64, 46	809, 234, 154, 88, 32, 19	382, 327, 263, 192, 78, 63, 31
S_2 SLG (I)	319, 303, 221, 129, 111, 91, 76, 32, 25, 14, 9	586, 227, 223, 155, 88, 32, 19	475, 358, 270, 93, 71, 63
S_4 SLG (II)	197, 194, 177, 121, 91, 82, 77, 51, 32	356, 200, 161, 153, 131, 21	398, 184, 165, 140, 84, 40, 11
S_5 SLG (II)	227, 188, 176, 129, 121, 82, 48, 43, 74	508, 435, 82	296, 197, 180, 90, 70, 55, 48, 45, 19, 17, 14, 11
S_6 SLG (I)	297, 188, 186, 130, 123, 120, 100, 91, 76, 25	534, 388, 273, 90, 51	347, 290, 270, 140, 93, 82, 63, 51
S_7 SLG (I)	270, 221, 216, 129, 121, 106, 91, 88, 46, 32, 25	844, 233, 161, 107	702, 337, 243, 63
S_{10} SLG (I)	298, 189, 188, 130, 123, 121, 98, 88, 82, 25	538, 429, 99, 88, 78, 59, 51	430, 334, 272, 191, 63, 52
SRK (Class)	*Taq*I	*Tru*9I	*Alu*I
S_1 SRK (I)	463, 221, 154, 129, 94, 50, 22, 22	371, 352, 220, 96, 41, 26, 16, 15, 12, 6	424, 289, 201, 190, 51
S_2 SRK (I)	741, 291, 93, 21	369, 238, 189, 96, 89, 82, 42, 26, 15	434, 268, 190, 152, 51, 34, 17
S_6 SRK (I)	846, 129, 95, 50, 22, 22	378, 275, 250, 185, 64, 6, 6	463, 258, 197, 169, 43, 34
S_7 SRK (I)	455, 374, 191, 50, 33, 22, 22	584, 409, 122, 32	423, 281, 190, 169, 50, 34
S_8 SRK ([a])	424, 378, 129, 97, 51, 22, 22	371, 244, 218, 126, 122, 42	677, 190, 169, 87
S_9 SRK ([a])	433, 372, 146, 129, 22, 22, 11	522, 375, 184, 47, 7	434, 257, 197, 169, 44, 34
S_{10} SRK (I)	443, 303, 164, 132, 61, 22	367, 230, 226, 224, 47, 31	682, 197, 169, 43, 34

[a] Not determined

Polyacrylamide-gel electrophoresis of DNA fragments cleaved with restriction endonucleases showed polymorphism of the PCR-amplified DNA fragments. PCR-RFLP band patterns revealed that eight inbred lines were grouped int two haplotypes, which were designated as S_4 and S_5 (Fig. 1B).

PCR using an SRK-specific primer set produced a single DNA fragment from 15 out of 24 inbred lines tested. By PCR-RFLP analysis using several restriction endonucleases, the SRK alleles of the 15 inbred lines were classified into seven S haplotypes (Fig. 1C). S inbred lines, #5335, #5314, #12, #85 and # 160, were amplified only with the SRK-specific primer set. Three lines (#5335, #5314 and #160) and two lines (#12 and #85) grouped together, and they were designated as S_8 and S_9, respectively. The result of grouping the S haplotypes of the inbred lines with the SRK allele by PCR-RFLP was consistent with that made by the pollen-tube germination analysis (data not shown). The sizes of the DNA fragments after digestion with restriction endonuclease were obtained by the sequence data (Table 3). They corresponded exactly with the sizes estimated from the electrophoretic mobility of DNA fragments in the polyacrylamide gel (Fig. 1C).

Analysis of S genotypes in the F_2 segregating population by PCR-RFLP

To investigate the S haplotypes of a segregating population, 38 F_2 plants were raised by self-pollinating heterozygous plants, which were derived from the cross between S_1 and S_2 homozygotes. With the Class-I SLG-specific primer set and the SRK-specific primer set, a single DNA fragment was generated in the parental lines the heterozygous F_1 and 38 F_2 plants (data not shown). Electrophoretic profiles based on digestion of the PCR products with *Taq*I revealed that the Class-I SLG-specific primer set can assort 38 F_2 progenies into three S genotypes: S_1 S_1 and S_2 S_2 homozyotes, and the S_1 S_2 heterozygote (Fig. 2). In the segregating population, the S haplotypes identified by PCR-RFLP analysis with the Class-I SLG-specific primer set matched with those identified with the SRK-specific primer set without

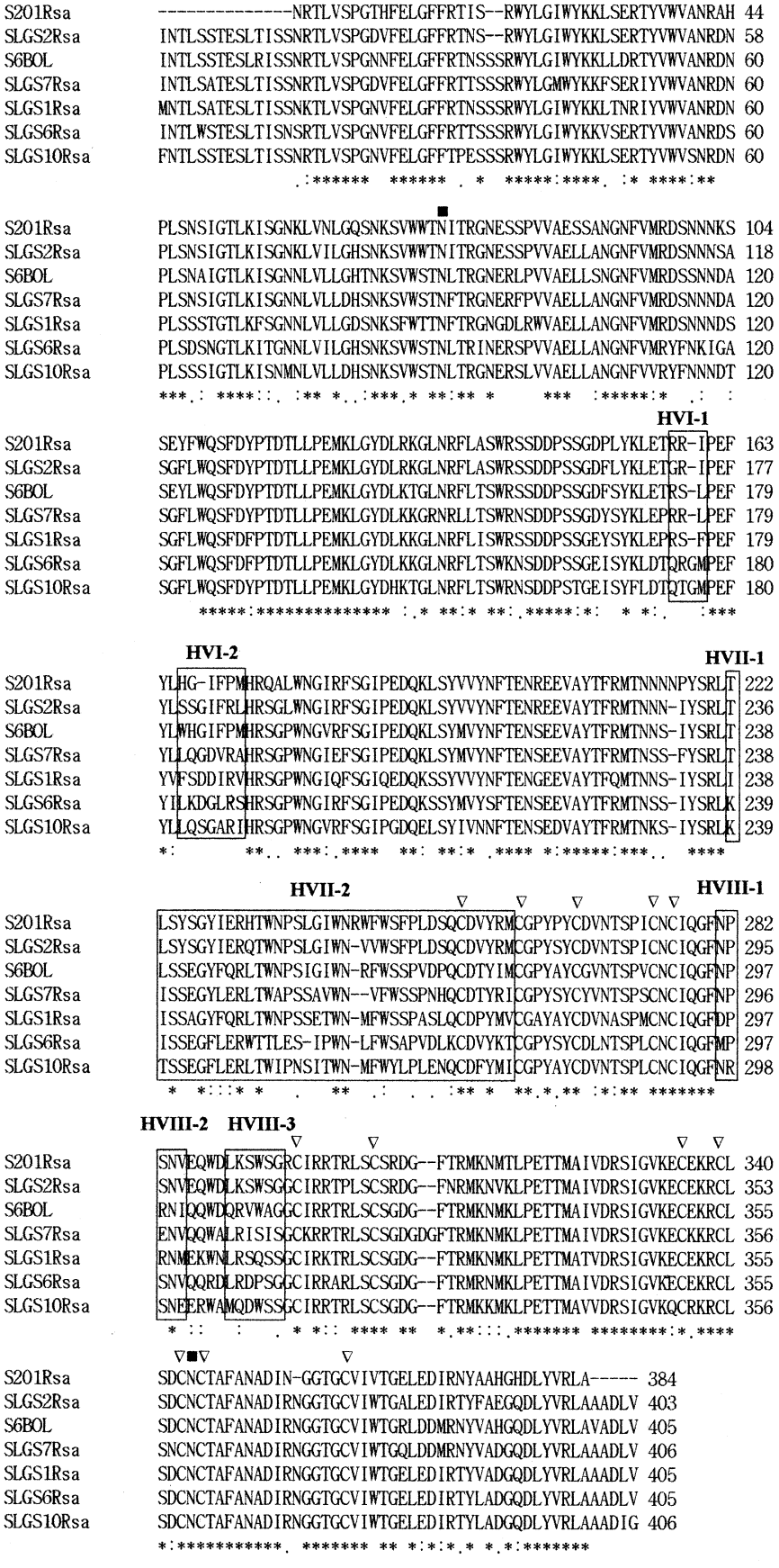


Fig. 3 Alignment of predicted amino-acid sequences of SLGs from S_1 (SLGS1*Rsa*), S_2 (SLGS2*Rsa*), S_6 (SLGS6*Rsa*), S_7 (SLGS7*Rsa*) S_{10} (SLGS10*Rsa*), 201 (S201*Rsa*) in *R. sativus*, and S_6 (SLG6*Bol*) in *B. oleracea*. The amino-acid sequences of S201*Rsa* and SLG6*Bol* were from Nikura and Matsuura (1997) and Nasrallah et al. (1987), respectivly. *Filled rectangles and open triangles* indicate the positions of the potential N-linked glycosylation sites and conserved cystein residues, respectively. Boxed regions represent the hypervariable regions I (SLGS10*Rsa* 173–188 aa), II (238–274 aa), and III (296–310 aa), respectively. *Asterisks* indicate conserved amino-acid residues. *Dashes* indicate gaps introduced to maximize alignment. The sequences reported in this paper have been submitted to Genebank (Accession nos. AY052572-AY052576)

Fig. 4 Multiple alignment of the nucleotide sequences of the DNA fragments amplified with the SRK-specific primer set. *Boxes* indicate 4th, 5th, 6th and 7th exons of the SRK. SRKS1*Rsa* (AY052579), SRKS2*Rsa* (AY052580), SRKS6*Rsa* (AY052581), SRKS7*Rsa* (AY052582), SRKS8*Rsa* (AY052583), SRKS9*Rsa* (AY052584), and SRKS10*Rsa* (AY052585) are the PCR products from S_1, S_2, S_6, S_7, S_8, S_9, and S_{10} homozygotes, respectively. The nucleotide sequence of SRK3*Bol* was from Delorme et al. (1995). *Dashes* indicate gaps introduced to maximize alignment

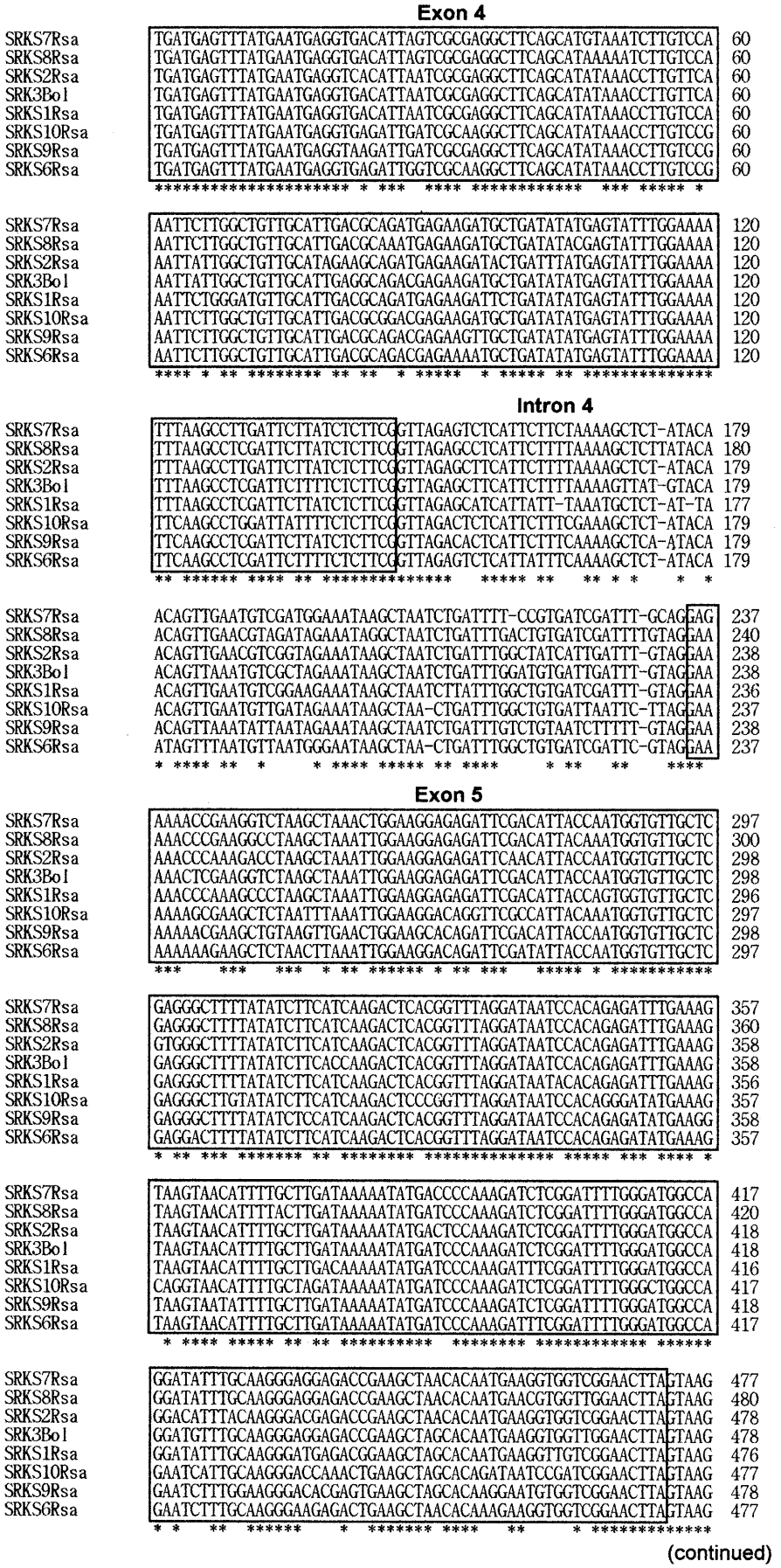


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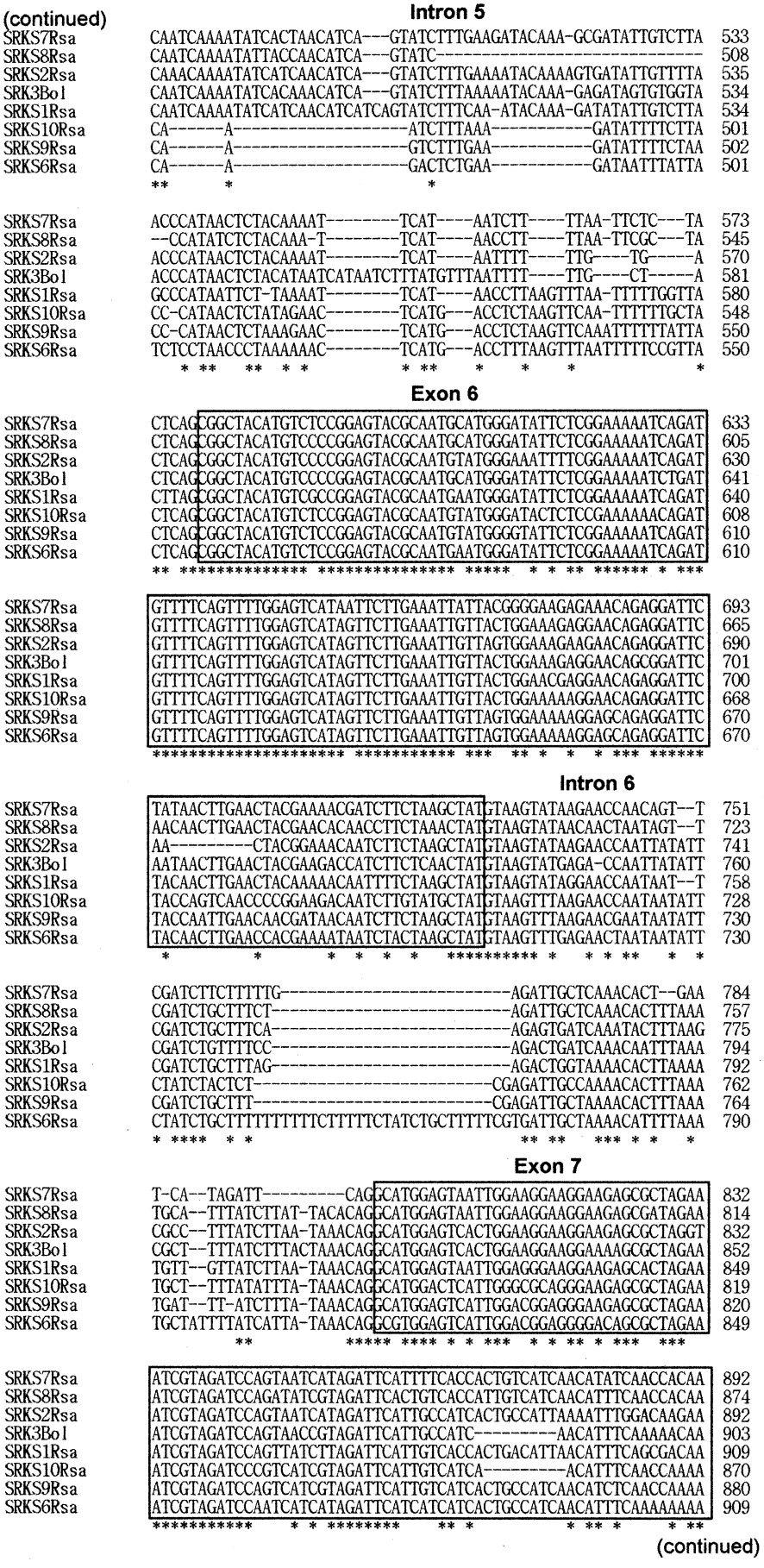
Fig. 4 Legend see page 1258



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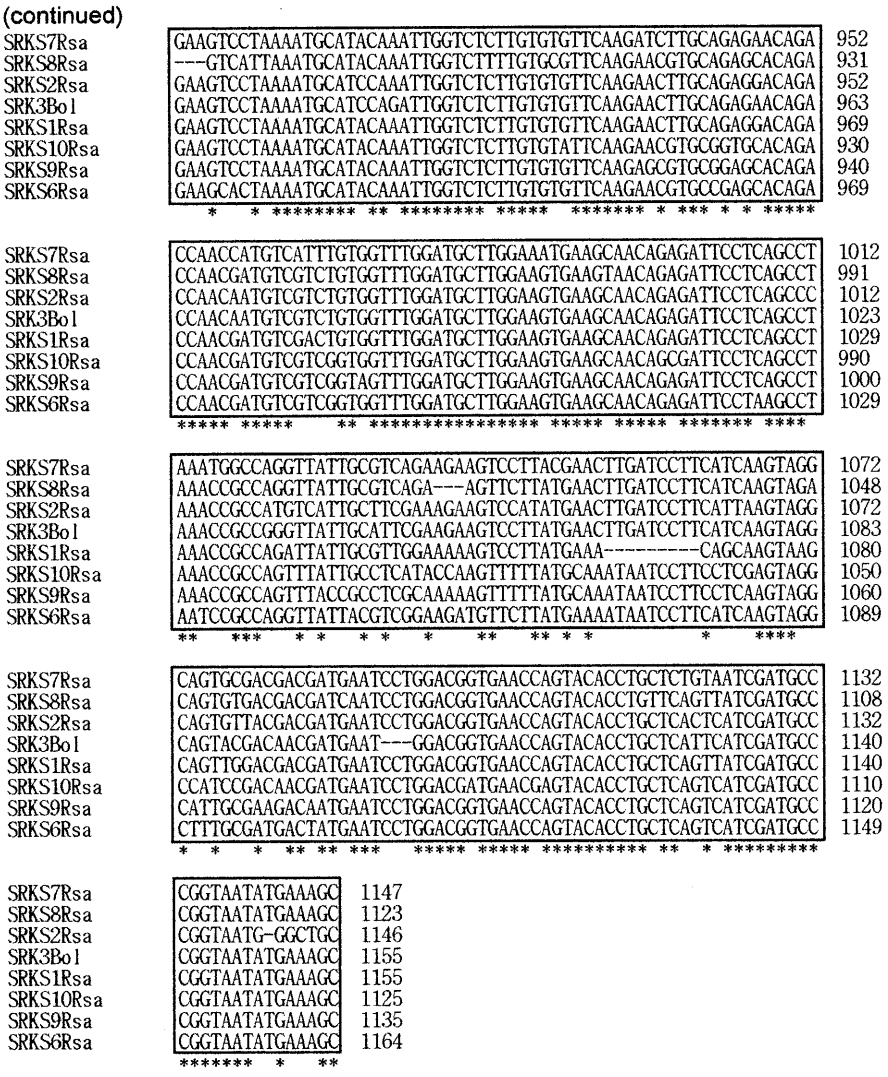

exception. The assigned S genotypes based on electrophoretic profiles coincided with those identified by pollen-tube germination analysis after diallele pollinations (data not shown).

Comparison of nucleotide sequences and deduced amino-acid sequences of SLG and SRK alleles

Nucleotide sequences of the DNA fragments amplified with the Class-I SLG-specific primer set were determined for the five S haplotypes and compared with those of the SLG gene in SLG_6 *Bol* (the SLG of the S_6 haplotype in *B. oleracea*) to show similarities from 77% to 82%. Similarities with those from *R. sativus* ranged from 84% to 88%. The deduced amino-acid sequences revealed characteristics unique to the SLG proteins. They had the 12 conserved cystein residues, the potential N-glycosylation sites, and three hypervariable regions as shown with the SLG protein in *B. oleracea* (Fig. 3). The level of amino-acid sequence similarity to SLG_6 *Bol* ranged from 77% to 84%, not significantly lower than those among *R. sativus* SLGs (from 71% to 89%). These results suggest that all of these DNA fragments are from SLGs.

The partial nucleotide sequences of the PCR products from the two S haplotypes that were amplified with the Class-II SLG-specific primer set were also determined and compared with that reported for SLG_2 *Bol* (the SLG of the S_2 haplotype in *B. oleracea*) to show 90–92% homology. Similarities between the nucleotide sequences from the *R. sativus* lines showed 90% homology. The amino-acid sequence of the Class-II SLG in *R. sativus* showed 85–87% homology with that of SLG_2 *Bol*. The amino-acid sequence of the Class-II SLG revealed about 60% similarity with those of the Class-I SLG in *R. sativus*, as is the case for the Class-I and Class-II SLGs in *Brassica*. It is concluded that both Class I and Class II exist in the S haplotypes of *R. sativus*, as in *B. oleracea* and *B. campestris*.

The nucleotide sequences of the PCR products amplified with the SRK-specific primer set were determined for seven S haplotypes. The DNA fragments had four regions

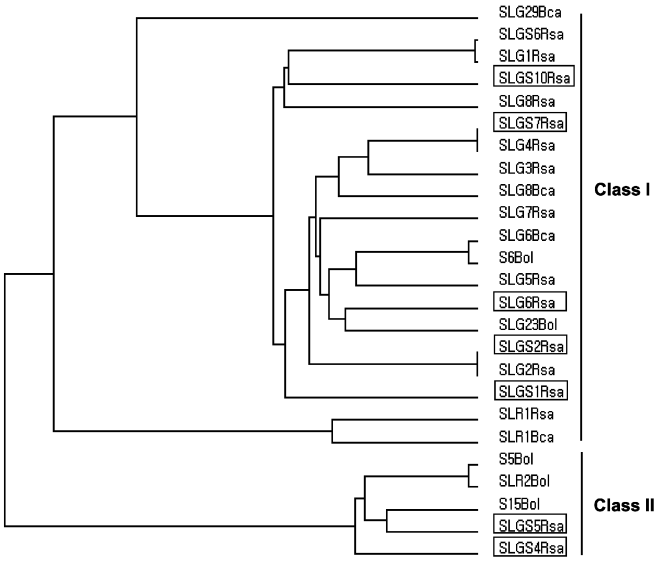

Fig. 5 Phylogenic tree of SLG sequences. Deduced amino-acid sequences of the putative mature protein regions of SLGs were used for the analysis. The aligned amino-acid sequences are as follows: SLG1*Rsa* (AB009677), SLG2*Rsa* (AB009678), SLG3*Rsa* (AB009679), SLG4*Rsa* (AB009680), SLG5*Rsa* (AB009681), SLG6*Rsa* (AB009682), SLG7*Rsa* (AB009684), SLG8*Rsa* (AB009683) and SLR1*Rsa* (AB009874) were isolated from *R. sativus*. SLG6*Bca* (M36301), SLG29*Bca* (AB008190), SLG8*Bca* (X55274) and SLR1*Bca* (Z26914) were from *B. campestris*. S6*Bol* (X03170), S5*Bol* (X51637), S15*Bol* (Y18261), SLG23*Bol* (AB013719) and SLR2*Bol* (Y18259), were isolated from *B. oleracea*. The phylogenic tree was generated by the algorithm from EMBL (Heidelberg)

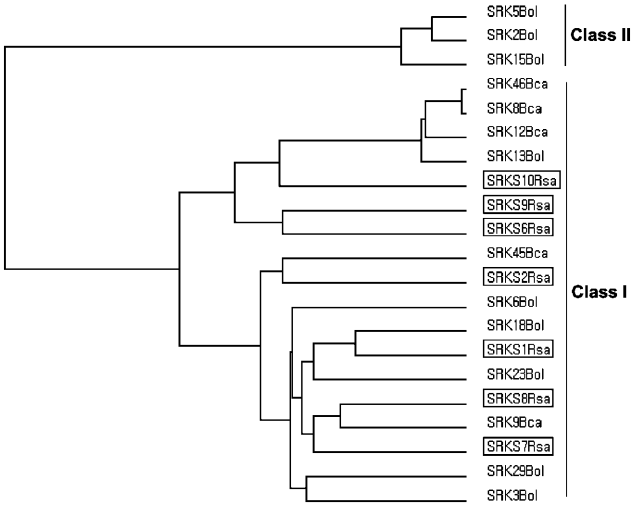

Fig. 6 Phylogenic tree of SRK sequences. Nucleotide sequences from the DNA fragment amplified with SRK-specific primer set were used for the analysis. The aligned amino-acid sequences are as follows: SRK29*Bol* (Z30211), SRK3*Bol* (X79432), SRK18*Bol* (BAA92836), SRK13*Bol* (AB02440), SRK5*Bol* (Y18259), SRK15*Bol* (Y18260), SRK2*Bol* (BAA83746) and SRK23*Bol* (BAA83746) were isolated from *B. oleracea*. SRK45*Bca* (AB012106), SRK46*Bca* (AB013718), SRK12*Bca* (D38564), SRK8*Bca* (D38563) and SRK9*Bca* (D30049) were from *B. campestis*. The phylogenic tree was generated by the algorithm from EMBL (Heidelberg)

sharing homologies in the fourth, fifth, sixth and seventh exons of SRK_3 *Bol* (the SRK of the S_3 haplotype in *B. oleracea*), and the amino-acid sequences deduced from them were similar to those that all of the SRK_3 *Bol*, suggesting that all of the SRKs were the fragments of SRK alleles. Comparison of the nucleotide sequences of SRK fragments of the seven S haplotypes isolated here with those already reported revealed high polymorphisms in the fifth introns, sixth introns and seventh exon (Fig. 4). The variation in these regions might have contributed to the polymorphism of DNA fragment sizes in PCR-RFLP analysis.

A phylogenic tree

A phylogenic tree of SLGs was constructed using the deduced amino-acid sequences of *R. sativus* SLGs and the amino-acid sequences of SLGs and SLR previously reported from *B. oleracea*, *B. campestris* and *R. sativus* (Fig. 5). They were clustered into two groups as described previously (Kusaba et al. 1997); a Class-I SLG group and a Class-II SLG group. The Class-II SLG group from *R. sativus* and from *B. oleracea* included SLR2. SLGs from *R. sativus* did not cluster independently and were dispersed in the tree, but rather clustered with SLGs from *B. oleracea* and *B. campestris*.

The phylogenic tree of the kinase domain from SRK alleles was constructed using the deduced amino-acid sequences of *R. sativus* SRKs and those previously reported for *B. oleracea* and *B. campestris*. As in the SLG phylogenic tree, SRKs from *R. sativus* were grouped into Class I and Class II (Fig. 6). Class-I SRKs from Class-I S haplotypes in *R. sativus* were clustered with Class-I SRKs in *B. oleracea* and *B. campestris*. These results supported the hypothesis that the divergence of Class I and Class II should have occurred before the differentiation of the genera *Brassica* and *Raphanus*.

Discussion

By selecting primers from the conserved region of SLG alleles *Raphanus* and *Brassica*, we were able to amplify SLG and SRK alleles from *R. sativus*. We assigned 24 inbred lines of *R. sativus* to ten S haplotypes based on PCR-RFLP analysis (except for S_3) and pollen-tube germination analysis. However, the S_3 haplotype identified by pollen-tube germination analysis in this study was not amplified with either SLG- or SRK-specific primer sets. SLG and SRK from the S_3 haplotype probably contain different nucleotide sequences in the primer annealing site. Therefore, based on nucleotide sequences from a number of SLG and SRK alleles, it seems necessary to develop another SLG- and SRK-specific primer set for amplifying the unclassified S haplotypes.

In order to identify the S genotypes in 38 F_2 plants, both analysis of PCR-RFLP and pollen-tube germination analysis were carried out. Brace et al. (1994) showed that selective amplification of one allele relative to an-

other in heterozygotes could have occurred. However, in this study, all the F_2 plants were amplified with the Class-I SLG-specific primer set or the SRK-specific primer set, and then after digestion with restriction endonucleases their PCR products showed a polymorphism depending on their genotypes. The lack of preferential amplification with the SLG- or the SRK-specific primer sets may be due to the high sequence homology at the sites of the primers. Therefore, SLG- or SRK-specific primer sets, developed in this study, are expected to be useful in S genotype identification and seed purity testing. In addition, based on the analysis of the F_2 population, it was shown that the S_1 SLG plant has the S_1 SRK allele; therefore, PCR-RFLP analysis of either the SLG or SRK allele is adequate for identification of the S haplotypes in F_2 segregating plants.

SLG alleles from *R. sativus* had high homology with the SLG from *B. oleracea* and *B. campestris*. Five SLG alleles amplified with the Class-I SLG-specific primer set and two SLG alleles amplified with the Class-II SLG-specific primer set were sequenced and compared with SLG alleles from *Brassica*. The deduced amino-acid sequences of the five Class-I SLG alleles in *R. sativus* had the 12 conserved cystein residues and three hypervariable regions characteristic of *Brassica* SLG proteins (Kusaba et al. 1997). The structural conservation of these cystein residues and hypervariable regions in *Raphanus* and *Brassica* may be suggestive that the regions play important roles in SI function. The phylogenic tree was constructed using deduced amino-acid sequences of the Class-I and Class-II SLGs of *Raphanus* and *Brassica*. They clustered into two groups, a Class-I group and a Class-II group. The SLGs of *Raphanus* did not form a unique cluster, but dispersed in the tree, often clustering with the SLGs of *Brassica*, as was the case in the phylogenic tree of SRKs from *Brassica* and *Raphanus*. In addition, SLG and SRK of the same S haplotypes belonged to the same class. This observation suggested that Class-I and Class-II group divergence occurred first, and then the SLG and SRK diverged. Furthermore, it showed that Class-I and Class-II groups exist both in *Raphanus* and *Brassica*. This observation suggested that divergence of the Class-I and Class-II S haplotype occurred prior to the differentiation of the genera *Brassica* and *Raphanus*.

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