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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 *Brassica* 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 af-

ter 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*. Phylogenetic 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

### 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

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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 Class-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<sub>1</sub> hybrid cultivars. For segregation analysis of the F<sub>2</sub>, a S homozygous S<sub>1</sub> plant (inbred #37) was crossed with a homozygous S<sub>2</sub> plant (inbred #50). Thirty eight F<sub>2</sub> progenies were generated by bud pollination of F<sub>1</sub> hybrid plants (S<sub>1</sub> S<sub>2</sub>) 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<sub>4</sub>)<sub>2</sub> SO<sub>4</sub> and 20 mM MgSO<sub>4</sub>. 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 *TaqI*, *Tru91*, *MspI*, *AluI*, *RsaI* and *HaeIII*. 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 *SmaI*-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 GENESIO.

**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 <sub>1</sub>	5	OH1(#37), OH2(#43), 307(#3), OS (#80), OSJ (#143)
Class I	S <sub>2</sub>	1	OH3 (#50)
ND <sup>a</sup>	S <sub>3</sub>	1	CH (#267)
Class II	S <sub>4</sub>	1	Ulsanbancheong (#107)
Class II	S <sub>5</sub>	7	KK1 (#262), KK2 (#5324), JYI (#5334), 23 (#18), 31 (#28), 69 (#32), Seoul Radish (#154)
Class I	S <sub>6</sub>	2	03 (#250), 60 (#39)
Class I	S <sub>7</sub>	1	92 (#204)
ND	S <sub>8</sub>	2	JY2 (#5335), Samge (#5314)
ND	S <sub>9</sub>	2	23 (#12), OS (#85)
Class I	S <sub>10</sub>	1	Younghyeonbanchenong (#102)

<sup>a</sup> 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) <sup>a</sup> Radish clone # 20 (1,372–1,346) <sup>a</sup>	This study
Class-II SLG	CTCAAGTCCCCTGCTGCGG 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)

<sup>a</sup> 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<sub>5</sub>, S<sub>2</sub> and S<sub>15</sub> SLG genes. For the specific amplification of SRK, primers were designed by aligning SRK genes of *B. oleracea* S<sub>3</sub> (Delorme et al. 1995) and S<sub>29</sub> (Kumar and Trick 1994), and of *B. campestris* S<sub>12</sub> (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.

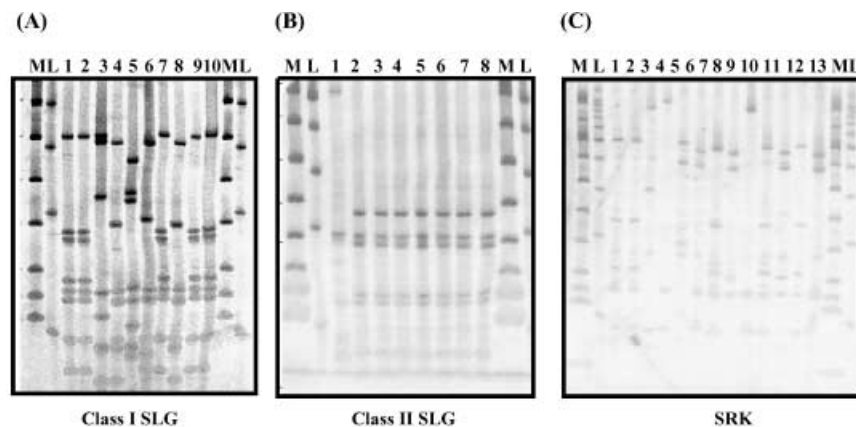
### PCR-RFLP analysis of SLG or SRK alleles

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. Di-

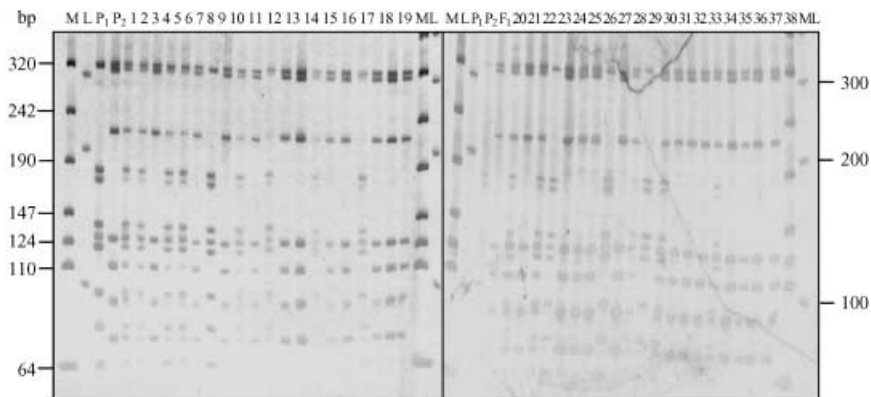
gestion of the PCR products with restriction endonucleases and subsequent polyacrylamide-gel electrophoresis revealed polymorphism of the amplified DNA fragments. Five types of electrophoretic profiles were found in ten inbred lines. Five inbred lines (#37, #43, #3, #80 and #143) had the same profile, 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<sub>1</sub>, S<sub>2</sub>, S<sub>6</sub>, S<sub>7</sub> and S<sub>10</sub>.

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 *TaqI*-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<sub>1</sub> S<sub>1</sub>), 2: #43 (S<sub>1</sub> S<sub>1</sub>), 3: #50 (S<sub>2</sub> S<sub>2</sub>), 4: #250 (S<sub>6</sub> S<sub>6</sub>), 5: #204 (S<sub>7</sub> S<sub>7</sub>), 6: #102 (S<sub>10</sub> S<sub>10</sub>), 7: #3 (S<sub>1</sub> S<sub>1</sub>), 8: #39 (S<sub>6</sub> S<sub>6</sub>), 9: #80 (S<sub>1</sub> S<sub>1</sub>), and 10: #143 (S<sub>1</sub> S<sub>1</sub>). **B** PCR-RFLP with Class-II SLG DNA fragments. 1: #107 (S<sub>4</sub> S<sub>4</sub>), 2: #262 (S<sub>5</sub> S<sub>5</sub>), 3: #5324 (S<sub>5</sub> S<sub>5</sub>), 4: #5334 (S<sub>5</sub> S<sub>5</sub>), 5: #18 (S<sub>5</sub> S<sub>5</sub>), 6: #28 (S<sub>5</sub> S<sub>5</sub>), 7: #32 (S<sub>5</sub> S<sub>5</sub>), and 8: #154 (S<sub>5</sub> S<sub>5</sub>). **C** PCR-RFLP with SRK DNA fragments. 1: #37 (S<sub>1</sub> S<sub>1</sub>), 2: #43 (S<sub>1</sub> S<sub>1</sub>), 3: #50 (S<sub>2</sub> S<sub>2</sub>), 4: #250 (S<sub>6</sub> S<sub>6</sub>), 5: #204 (S<sub>7</sub> S<sub>7</sub>), 6: #5335 (S<sub>8</sub> S<sub>8</sub>), 7: #3 (S<sub>1</sub> S<sub>1</sub>), 8: #12 (S<sub>9</sub> S<sub>9</sub>), 9: #39 (S<sub>6</sub> S<sub>6</sub>), 10: #80 (S<sub>1</sub> S<sub>1</sub>), 11: #85 (S<sub>9</sub> S<sub>9</sub>), 12: #143 (S<sub>1</sub> S<sub>1</sub>), and 13: #160 (S<sub>8</sub> S<sub>8</sub>). Brackets represent the S genotype of inbred lines. M: pUBCM21 digested with *HpaII*, *DraI* and *HindIII*. 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 *Hind*III. *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)	<i>TaqI</i>	<i>MspI</i>	<i>NdeII</i>
$S_1$ 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)	<i>TaqI</i>	<i>Tru9I</i>	<i>AluI</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

<sup>a</sup> 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 into 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 elec-

trophoretic 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 *TaqI* revealed that the Class-I SLG-specific primer set can assort 38  $F_2$  progenies into three S genotypes:  $S_1 S_1$  and  $S_2 S_2$  homozygotes, 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. 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<sub>1</sub>, S<sub>2</sub>, S<sub>6</sub>, S<sub>7</sub>, S<sub>8</sub>, S<sub>9</sub>, and S<sub>10</sub> homozygotes, respectively. The nucleotide sequence of SRK3*BoI* was from Delorme et al. (1995). Dashes indicate gaps introduced to maximize alignment

	<b>Exon 4</b>	
SRKS7 <i>Rsa</i>	TGATGAGTTTATGAATGAGGTGACATTAGTCGGCAGGCTTCAGCATGTAATCTTGTCCA	60
SRKS8 <i>Rsa</i>	TGATGAGTTTATGAATGAGGTGACATTAGTCGGCAGGCTTCAGCATGTAATCTTGTCCA	60
SRKS2 <i>Rsa</i>	TGATGAGTTTATGAATGAGGTGACATTAAATCGCGAGGCTTCAGCATATAAAACCTTGTTC	60
SRK3 <i>BoI</i>	TGATGAGTTTATGAATGAGGTGACATTAAATCGCGAGGCTTCAGCATATAAAACCTTGTTC	60
SRKS1 <i>Rsa</i>	TGATGAGTTTATGAATGAGGTGACATTAAATCGCGAGGCTTCAGCATATAAAACCTTGTCCA	60
SRKS10 <i>Rsa</i>	TGATGAGTTTATGAATGAGGTGAGATTGATCGCAAGGCTTCAGCATATAAAACCTTGTCCG	60
SRKS9 <i>Rsa</i>	TGATGAGTTTATGAATGAGGTGAGATTGATCGCGAGGCTTCAGCATATAAAACCTTGTCCG	60
SRKS6 <i>Rsa</i>	TGATGAGTTTATGAATGAGGTGAGATTGATCGCAAGGCTTCAGCATATAAAACCTTGTCCG	60
	*****	
SRKS7 <i>Rsa</i>	AATTCCTGGCTGTTCATTGACGCAGATGAGAAGATGCTGATATAGTATTTGGAAAA	120
SRKS8 <i>Rsa</i>	AATTCCTGGCTGTTCATTGACGCAGATGAGAAGATGCTGATATAGTATTTGGAAAA	120
SRKS2 <i>Rsa</i>	AATTCCTGGCTGTTCATTGACGCAGATGAGAAGATGCTGATATAGTATTTGGAAAA	120
SRK3 <i>BoI</i>	AATTCCTGGCTGTTCATTGACGCAGATGAGAAGATGCTGATATAGTATTTGGAAAA	120
SRKS1 <i>Rsa</i>	AATTCCTGGCTGTTCATTGACGCAGATGAGAAGATGCTGATATAGTATTTGGAAAA	120
SRKS10 <i>Rsa</i>	AATTCCTGGCTGTTCATTGACGCAGATGAGAAGATGCTGATATAGTATTTGGAAAA	120
SRKS9 <i>Rsa</i>	AATTCCTGGCTGTTCATTGACGCAGATGAGAAGATGCTGATATAGTATTTGGAAAA	120
SRKS6 <i>Rsa</i>	AATTCCTGGCTGTTCATTGACGCAGATGAGAAGATGCTGATATAGTATTTGGAAAA	120
	*****	
	<b>Intron 4</b>	
SRKS7 <i>Rsa</i>	TTTAAGCCTTGATTCCTTATCTCTTCGGTTAGAGTCTCATTCTTCTAAAAGCTCT-ATACA	179
SRKS8 <i>Rsa</i>	TTTAAGCCTTGATTCCTTATCTCTTCGGTTAGAGTCTCATTCTTCTAAAAGCTCTTATACA	180
SRKS2 <i>Rsa</i>	TTTAAGCCTTGATTCCTTATCTCTTCGGTTAGAGTCTCATTCTTCTAAAAGCTCT-ATACA	179
SRK3 <i>BoI</i>	TTTAAGCCTTGATTCCTTATCTCTTCGGTTAGAGTCTCATTCTTCTAAAAGCTCT-ATACA	179
SRKS1 <i>Rsa</i>	TTTAAGCCTTGATTCCTTATCTCTTCGGTTAGAGTCTCATTCTTCTAAAAGCTCT-AT-AT	177
SRKS10 <i>Rsa</i>	TTCAAGCCTGGATTTATTTCTCTTCGGTTAGAGTCTCATTCTTCTAAAAGCTCT-ATACA	179
SRKS9 <i>Rsa</i>	TTCAAGCCTGGATTTATTTCTCTTCGGTTAGAGTCTCATTCTTCTAAAAGCTCT-ATACA	179
SRKS6 <i>Rsa</i>	TTCAAGCCTGGATTTATTTCTCTTCGGTTAGAGTCTCATTCTTCTAAAAGCTCT-ATACA	179
	*****	
SRKS7 <i>Rsa</i>	ACAGTTGAATGTCGATGGAATAAGCTAATCTGATTTT-CCGTGATCGATT-GCAGGAG	237
SRKS8 <i>Rsa</i>	ACAGTTGAACGTAGATAGAAAAGCTAATCTGATTTGACTGTGATCGATTTGTAGGAA	240
SRKS2 <i>Rsa</i>	ACAGTTGAACGTAGATAGAAAAGCTAATCTGATTTGGCTATCATTTGATT-GTAGGAA	238
SRK3 <i>BoI</i>	ACAGTTAAATGTCGCTAGAAAAGCTAATCTGATTTGGATGTGATTTGATT-GTAGGAA	238
SRKS1 <i>Rsa</i>	ACAGTTGAATGTCGGAAGAAAAGCTAATCTTATTTGGCTGTGATCGATT-GTAGGAA	236
SRKS10 <i>Rsa</i>	ACAGTTGAATGTCGATGGAATAAGCTAA-CTGATTTGGCTGTGATTAATTC-GTAGGAA	237
SRKS9 <i>Rsa</i>	ACAGTTAAATATTAATAGAAAAGCTAATCTGATTTGCTGTAATCTTTTT-GTAGGAA	238
SRKS6 <i>Rsa</i>	ATAGTTAATGTTAATGGAATAAGCTAA-CTGATTTGGCTGTGATCGATT-GTAGGAA	237
	*****	
	<b>Exon 5</b>	
SRKS7 <i>Rsa</i>	AAAACCGAAGGCTTAAGCTAAATGGAAGGAGAGATTCCGACATTACCAATGGTGTGCTC	297
SRKS8 <i>Rsa</i>	AAAACCGAAGGCTTAAGCTAAATGGAAGGAGAGATTCCGACATTACCAATGGTGTGCTC	300
SRKS2 <i>Rsa</i>	AAAACCGAAGGCTTAAGCTAAATGGAAGGAGAGATTCCGACATTACCAATGGTGTGCTC	298
SRK3 <i>BoI</i>	AAAACCGAAGGCTTAAGCTAAATGGAAGGAGAGATTCCGACATTACCAATGGTGTGCTC	298
SRKS1 <i>Rsa</i>	AAAACCGAAGGCTTAAGCTAAATGGAAGGAGAGATTCCGACATTACCAATGGTGTGCTC	296
SRKS10 <i>Rsa</i>	AAAACCGAAGGCTTAAGCTAAATGGAAGGAGAGATTCCGACATTACCAATGGTGTGCTC	297
SRKS9 <i>Rsa</i>	AAAACCGAAGGCTTAAGCTAAATGGAAGGAGAGATTCCGACATTACCAATGGTGTGCTC	298
SRKS6 <i>Rsa</i>	AAAACCGAAGGCTTAAGCTAAATGGAAGGAGAGATTCCGACATTACCAATGGTGTGCTC	297
	*****	
SRKS7 <i>Rsa</i>	GAGGGCTTTTATATCTTCAATCAAGACTCAGGTTTAGGATAATCCACAGAGATTTGAAAG	357
SRKS8 <i>Rsa</i>	GAGGGCTTTTATATCTTCAATCAAGACTCAGGTTTAGGATAATCCACAGAGATTTGAAAG	360
SRKS2 <i>Rsa</i>	GTGGGCTTTTATATCTTCAATCAAGACTCAGGTTTAGGATAATCCACAGAGATTTGAAAG	358
SRK3 <i>BoI</i>	GAGGGCTTTTATATCTTCAATCAAGACTCAGGTTTAGGATAATCCACAGAGATTTGAAAG	358
SRKS1 <i>Rsa</i>	GAGGGCTTTTATATCTTCAATCAAGACTCAGGTTTAGGATAATCCACAGAGATTTGAAAG	356
SRKS10 <i>Rsa</i>	GAGGGCTTTTATATCTTCAATCAAGACTCAGGTTTAGGATAATCCACAGAGATTTGAAAG	357
SRKS9 <i>Rsa</i>	GAGGGCTTTTATATCTTCAATCAAGACTCAGGTTTAGGATAATCCACAGAGATTTGAAAG	358
SRKS6 <i>Rsa</i>	GAGGGCTTTTATATCTTCAATCAAGACTCAGGTTTAGGATAATCCACAGAGATTTGAAAG	357
	*****	
SRKS7 <i>Rsa</i>	TAAGTAACATTTTGCCTTGATAAAAAATGATCCCAAAGATCTCGGATTTTGGGATGGCCA	417
SRKS8 <i>Rsa</i>	TAAGTAACATTTTGCCTTGATAAAAAATGATCCCAAAGATCTCGGATTTTGGGATGGCCA	420
SRKS2 <i>Rsa</i>	TAAGTAACATTTTGCCTTGATAAAAAATGATCCCAAAGATCTCGGATTTTGGGATGGCCA	418
SRK3 <i>BoI</i>	TAAGTAACATTTTGCCTTGATAAAAAATGATCCCAAAGATCTCGGATTTTGGGATGGCCA	418
SRKS1 <i>Rsa</i>	TAAGTAACATTTTGCCTTGATAAAAAATGATCCCAAAGATTTTCGGATTTTGGGATGGCCA	416
SRKS10 <i>Rsa</i>	CAGGTAACATTTTGCCTAGATAAAAAATGATCCCAAAGATCTCGGATTTTGGGCTGGCCA	417
SRKS9 <i>Rsa</i>	TAAGTAATATTTTGCCTTGATAAAAAATGATCCCAAAGATCTCGGATTTTGGGATGGCCA	418
SRKS6 <i>Rsa</i>	TAAGTAACATTTTGCCTTGATAAAAAATGATCCCAAAGATTTTCGGATTTTGGGATGGCCA	417
	*****	
SRKS7 <i>Rsa</i>	GGATATTTGCAAGGGAGGAGACCGAAGCTAACACAATGAAGGTGGTTCGGAACCTTAGTAAG	477
SRKS8 <i>Rsa</i>	GGATATTTGCAAGGGAGGAGACCGAAGCTAACACAATGAAGGTGGTTCGGAACCTTAGTAAG	480
SRKS2 <i>Rsa</i>	GGATATTTGCAAGGGAGGAGACCGAAGCTAACACAATGAAGGTGGTTCGGAACCTTAGTAAG	478
SRK3 <i>BoI</i>	GGATATTTGCAAGGGAGGAGACCGAAGCTAACACAATGAAGGTGGTTCGGAACCTTAGTAAG	478
SRKS1 <i>Rsa</i>	GGATATTTGCAAGGGAGGAGACCGAAGCTAACACAATGAAGGTGGTTCGGAACCTTAGTAAG	476
SRKS10 <i>Rsa</i>	GAATCATTTGCAAGGGAGGACCAAGCTAACACAATGAAGGTGGTTCGGAACCTTAGTAAG	477
SRKS9 <i>Rsa</i>	GAATCATTTGCAAGGGAGGAGACCGAAGCTAACACAATGAAGGTGGTTCGGAACCTTAGTAAG	478
SRKS6 <i>Rsa</i>	GAATCATTTGCAAGGGAGGAGACCGAAGCTAACACAATGAAGGTGGTTCGGAACCTTAGTAAG	477
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(continued)

Fig. 4 Legend see page 1258

(continued)

**Intron 5**

SRKS7Rsa	CAATCAAAAATACCTAATCATCA---GTATCTTTGAAGATACAAA---GCGATATTGTCTTA	533
SRKS8Rsa	CAATCAAAAATATACCAACATCA---GTATC-----	508
SRKS2Rsa	CAAAACAAAATATCATCAACATCA---GTATCTTTGAAAATACAAAAGTGATATTGTTTTA	535
SRK3Bo1	CAATCAAAAATACCAAAACATCA---GTATCTTTAAAAATACAAA---GAGATAGTGTGGTA	534
SRKS1Rsa	CAATCAAAAATATCATCAACATCATCAGTATCTTTCAA---ATACAAA---GATATATTGTCTTA	534
SRKS10Rsa	CA-----A-----ATCTTTAAA-----GATATTTTCTTA	501
SRKS9Rsa	CA-----A-----GTCTTTGAA-----GATATTTTCTAA	502
SRKS6Rsa	CA-----A-----GACTCTGAA-----GATAATTTATTA	501

SRKS7Rsa	ACCCATAACTCTACAAAAT-----TCAT---AATCTT---TTAA-TTCTC---TA	573
SRKS8Rsa	--CCATATCTCTACAAA-T-----TCAT---AACCTT---TTAA-TTCGC---TA	545
SRKS2Rsa	ACCCATAACTCTACAAAAT-----TCAT---AATTTT---TTG---TG---A	570
SRK3Bo1	ACCCATAACTCTACATAATCATAATCTTTATGTTTAAATTTT---TTG---CT---A	581
SRKS1Rsa	GCCATAAATCT-TAAAAAT-----TCAT---AACCTTAAGTTTAA-TTTTGGTTA	580
SRKS10Rsa	CC-CATAACTCTATAGAAC-----TCATG---ACCTCTAAGTTCAA-TTTTTCGCTA	548
SRKS9Rsa	CC-CATAACTCTAAGAAC-----TCATG---ACCTCTAAGTTCAAATTTTATTA	550
SRKS6Rsa	TCTCTAACCCATAAAAAAC-----TCATG---ACCTTAAAGTTTAAATTTTCGGTTA	550

**Exon 6**

SRKS7Rsa	CTCAGCGGCTACATGTCTCCGGAGTACGCAATGCATGGGATATTCTCGGAAAAATCAGAT	633
SRKS8Rsa	CTCAGCGGCTACATGTCCCGGAGTACGCAATGCATGGGATATTCTCGGAAAAATCAGAT	605
SRKS2Rsa	CTCAGCGGCTACATGTCCCGGAGTACGCAATGTATGGGAAATTTTCGGAAAAATCAGAT	630
SRK3Bo1	CTCAGCGGCTACATGTCCCGGAGTACGCAATGCATGGGATATTCTCGGAAAAATCTGAT	641
SRKS1Rsa	CTTAGCGGCTACATGTCCCGGAGTACGCAATGAATGGGATATTCTCGGAAAAATCAGAT	640
SRKS10Rsa	CTCAGCGGCTACATGTCTCCGGAGTACGCAATGTATGGGATCTCTCGGAAAAATCAGAT	608
SRKS9Rsa	CTCAGCGGCTACATGTCTCCGGAGTACGCAATGTATGGGATATTCTCGGAAAAATCAGAT	610
SRKS6Rsa	CTCAGCGGCTACATGTCTCCGGAGTACGCAATGAATGGGATATTCTCGGAAAAATCAGAT	610

SRKS7Rsa	GTTTTCAGTTTTGGAGTCATAATTCTTGAAATTTACTGGGAAAGGAGAAACAGAGGATTC	693
SRKS8Rsa	GTTTTCAGTTTTGGAGTCATAGTTCTTGAAATTTACTGGAAAGGAGAAACAGAGGATTC	665
SRKS2Rsa	GTTTTCAGTTTTGGAGTCATAGTTCTTGAAATTTACTGGAAAGGAGAAACAGAGGATTC	690
SRK3Bo1	GTTTTCAGTTTTGGAGTCATAGTTCTTGAAATTTACTGGAAAGGAGAAACAGCGGATTC	701
SRKS1Rsa	GTTTTCAGTTTTGGAGTCATAGTTCTTGAAATTTACTGGAAAGGAGAAACAGAGGATTC	700
SRKS10Rsa	GTTTTCAGTTTTGGAGTCATAGTTCTTGAAATTTACTGGAAAGGAGAAACAGAGGATTC	668
SRKS9Rsa	GTTTTCAGTTTTGGAGTCATAGTTCTTGAAATTTACTGGAAAGGAGGACAGAGGATTC	670
SRKS6Rsa	GTTTTCAGTTTTGGAGTCATAGTTCTTGAAATTTACTGGAAAGGAGGACAGAGGATTC	670

**Intron 6**

SRKS7Rsa	TATAACTTGAACACGAAAACGATCTTCTAAGCTATGTAAGTATAAGAACCAACAGT--T	751
SRKS8Rsa	AACAACCTTGAACACGAAACCAACCTTCTAAGCTATGTAAGTATAACAATAATAGT--T	723
SRKS2Rsa	AA-----CTACGAAAACAATCTTCTAAGCTATGTAAGTATAAGAACAATATAT	741
SRK3Bo1	AATAACTTGAACACGAAAGACCACTTCTCAACTATGTAAGTATAGAA---CCAATATATT	760
SRKS1Rsa	TACAACCTTGAACACAAAAACAATTTCTAAGCTATGTAAGTATAGGAACCAATAAT--T	758
SRKS10Rsa	TACCAGTCAACCCCGAAGACAATCTTGTATGCTATGTAAGTTAAGAACCAATAATATT	728
SRKS9Rsa	TACCAATTGAACAACGATAACAATCTTCTAAGCTATGTAAGTTAAGAACCAATAATATT	730
SRKS6Rsa	TACAACCTTGAACCACGAAAATAATCTACTAAGCTATGTAAGTTGAGAATAATAATATT	730

SRKS7Rsa	CGATCTGCTTTTIG-----AGATTGCTCAAAACT--GAA	784
SRKS8Rsa	CGATCTGCTTTCT-----AGATTGCTCAAAACTTTTAA	757
SRKS2Rsa	CGATCTGCTTTCA-----AGAGTGATCAAATCTTAA	775
SRK3Bo1	CGATCTGTTTCC-----AGACTGATCAAACAATTTAA	794
SRKS1Rsa	CGATCTGCTTTAG-----AGACTGTTAAACAACCTTAA	792
SRKS10Rsa	CTATCTACTCT-----CGAGATTGCCAAAACCTTAA	762
SRKS9Rsa	CGATCTGCTTT-----CGAGATTGCTAAAAACTTTAA	764
SRKS6Rsa	CTATCTGCTTTT-----CGAGATTGCTAAAAACTTTAA	790

**Exon 7**

SRKS7Rsa	T-CA--TAGATT-----CAGGCATGGAGTAATTGGAAGGAAGGAAGAGCGCTAGAA	832
SRKS8Rsa	TGCA--TTTATCTTAT-TACACAGGCATGGAGTAATTGGAAGGAAGGAAGCGATAGAA	814
SRKS2Rsa	CGCC--TTTATCTTAA-TAAACAGGCATGGAGTCACTGGAAGGAAGGAAGAGCGTAGGT	832
SRK3Bo1	CGCT--TTTATCTTAACTAAACAGGCATGGAGTCACTGGAAGGAAGGAAGAGCGCTAGAA	852
SRKS1Rsa	TGTT--GTTATCTTAA-TAAACAGGCATGGAGTAATTGGAAGGAAGGAAGAGCAGTAGAA	849
SRKS10Rsa	TGCT--TTTATATTTA-TAAACAGGCATGGACTCATTGGGCGCAGGAAGAGCGCTAGAA	819
SRKS9Rsa	TGAT--TT-ATCTTTA-TAAACAGGCATGGAGTCAATTGGGCGGAGGAAGAGCGCTAGAA	820
SRKS6Rsa	TGCTATTTTATCATTAA-TAAACAGGCATGGAGTCAATTGGGCGGAGGAGAGCGCTAGAA	849

SRKS7Rsa	ATCGTAGATCCAGTAATCATAGATTCAATTTTACCACCTGTCATCAACATATCAACCACAA	892
SRKS8Rsa	ATCGTAGATCCAGATATCGTAGATTCACTGTCCACCATTTGTCATCAACATTTCAACCACAA	874
SRKS2Rsa	ATCGTAGATCCAGTAATCATAGATTCAATTTTACCACCTGTCATCAACATTTTGGACAAGAA	892
SRK3Bo1	ATCGTAGATCCAGTAACCGTAGATTCAATGGCCATC-----AACATTTCAAAAACAA	903
SRKS1Rsa	ATCGTAGATCCAGTATCTTTAGATTCAATTTGTCACCACTGACATTAACATTTCAAGGACAA	909
SRKS10Rsa	ATCGTAGATCCCGTATCGTAGATTCAATTTGTCATCA-----ACATTTCAACCACAA	870
SRKS9Rsa	ATCGTAGATCCAGTATCGTAGATTCAATTTGTCATCACTGCCATCAACATCTCAACCACAA	880
SRKS6Rsa	ATCGTAGATCCAATCATATAGATTCAATTTGTCATCACTGCCATCAACATTTCAAAAACAA	909

(continued)



Fig. 4 Legend see page 1258

(continued)

SRKS7Rsa	GAAGTCCTAAAATGCATACAAAATTGGTCTCTTGTGTGTTCAAGATCTTGCAGAGAACAGA	952
SRKS8Rsa	---GTCATTAATGCATACAAAATTGGTCTTTTGTGCGTTCAAGAACGTGCAGAGCACAGA	931
SRKS2Rsa	GAAGTCCTAAAATGCATCCTAAAATTGGTCTCTTGTGTGTTCAAGAACCTTCAGAGGACAGA	952
SRK3Bo1	GAAGTCCTAAAATGCATCCAGATTGGTCTCTTGTGTGTTCAAGAACCTTCAGAGGACAGA	963
SRKS1Rsa	GAAGTCCTAAAATGCATACAAAATTGGTCTCTTGTGTGTTCAAGAACCTTCAGAGGACAGA	969
SRKS10Rsa	GAAGTCCTAAAATGCATACAAAATTGGTCTCTTGTGTGTTCAAGAACCTTCAGAGGACAGA	930
SRKS9Rsa	GAAGTCCTAAAATGCATACAAAATTGGTCTCTTGTGTGTTCAAGAACCTTCAGAGGACAGA	940
SRKS6Rsa	GAAGTCCTAAAATGCATACAAAATTGGTCTCTTGTGTGTTCAAGAACCTTCAGAGGACAGA	969
***** ** ***** ** ***** ** ***** ** ***** ** ***** ** *****		
SRKS7Rsa	CCAACCATGTCATTTGTGGTTTGGATGCTTGGAAATGAAGCAACAGAGATTCTCAGCCT	1012
SRKS8Rsa	CCAACCATGTCGCTCTGTGGTTTGGATGCTTGGAAATGAAGCAACAGAGATTCTCAGCCT	991
SRKS2Rsa	CCAACAATGTCGCTCTGTGGTTTGGATGCTTGGAAATGAAGCAACAGAGATTCTCAGCCT	1012
SRK3Bo1	CCAACAATGTCGCTCTGTGGTTTGGATGCTTGGAAATGAAGCAACAGAGATTCTCAGCCT	1023
SRKS1Rsa	CCAACCATGTCGCTCTGTGGTTTGGATGCTTGGAAATGAAGCAACAGAGATTCTCAGCCT	1029
SRKS10Rsa	CCAACCATGTCGCTCTGTGGTTTGGATGCTTGGAAATGAAGCAACAGAGATTCTCAGCCT	990
SRKS9Rsa	CCAACCATGTCGCTCTGTGGTTTGGATGCTTGGAAATGAAGCAACAGAGATTCTCAGCCT	1000
SRKS6Rsa	CCAACCATGTCGCTCTGTGGTTTGGATGCTTGGAAATGAAGCAACAGAGATTCTCAGCCT	1029
***** ** ***** ** ***** ** ***** ** ***** ** ***** ** *****		
SRKS7Rsa	AAATGGCCAGGTTATTGGCTCAGAAGAAGTCCCTTACGAACCTTGATCCTTTCATCAAGTAGG	1072
SRKS8Rsa	AAACCGCCAGGTTATTGGCTCAGA---AGTTCCTTGAACCTTGATCCTTTCATCAAGTAGA	1048
SRKS2Rsa	AAACCGCCATGTCATTTGCTTCGAAAGAAGTCCATGAACTTGATCCTTTCATCAAGTAGG	1072
SRK3Bo1	AAACCGCCGGGTTATTGCATTTCGAAGAAGTCCCTTATGAACCTTGATCCTTTCATCAAGTAGG	1083
SRKS1Rsa	AAACCGCCAGATTATTGGCTTGGAAAAAGTCCCTTATGAAA-----CAGCAAGTAGG	1080
SRKS10Rsa	AAACCGCCAGTTTATTGCCTCATTACCAAGTTTTTATGCAAATAATCCTTCTCCAGTAGG	1050
SRKS9Rsa	AAACCGCCAGTTTACCCTCGCAAAAAGTTTTTATGCAAATAATCCTTCTCCAGTAGG	1060
SRKS6Rsa	AATCCGCCAGGTTATTACGTCGGAAGATGTTCTTATGAAAATAATCCTTTCATCAAGTAGG	1089
** *** *		
SRKS7Rsa	CAGTGCAGCAGATGAATCCTGGACGGTGAACAGTACACCTGCTGTGAATCGATGCC	1132
SRKS8Rsa	CAGTGTACGACGATCAATCCTGGACGGTGAACAGTACACCTGCTGTGAATCGATGCC	1108
SRKS2Rsa	CAGTGTACGACGATGAATCCTGGACGGTGAACAGTACACCTGCTGTGAATCGATGCC	1132
SRK3Bo1	CAGTACGACAACGATGAAT---GGACGGTGAACAGTACACCTGCTGTGAATCGATGCC	1140
SRKS1Rsa	CAGTGTACGACGATGAATCCTGGACGGTGAACAGTACACCTGCTGTGAATCGATGCC	1140
SRKS10Rsa	CCATCCGACAACGATGAATCCTGGACGGTGAACAGTACACCTGCTGTGAATCGATGCC	1110
SRKS9Rsa	CATTGCGAAGACAATGAATCCTGGACGGTGAACAGTACACCTGCTGTGAATCGATGCC	1120
SRKS6Rsa	CTTTGCGATGACTATGAATCCTGGACGGTGAACAGTACACCTGCTGTGAATCGATGCC	1149
* *		
SRKS7Rsa	CGGTAATATGAAAGC	1147
SRKS8Rsa	CGGTAATATGAAAGC	1123
SRKS2Rsa	CGGTAATG-GGCTGC	1146
SRK3Bo1	CGGTAATATGAAAGC	1155
SRKS1Rsa	CGGTAATATGAAAGC	1155
SRKS10Rsa	CGGTAATATGAAAGC	1125
SRKS9Rsa	CGGTAATATGAAAGC	1135
SRKS6Rsa	CGGTAATATGAAAGC	1164
***** * **		

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

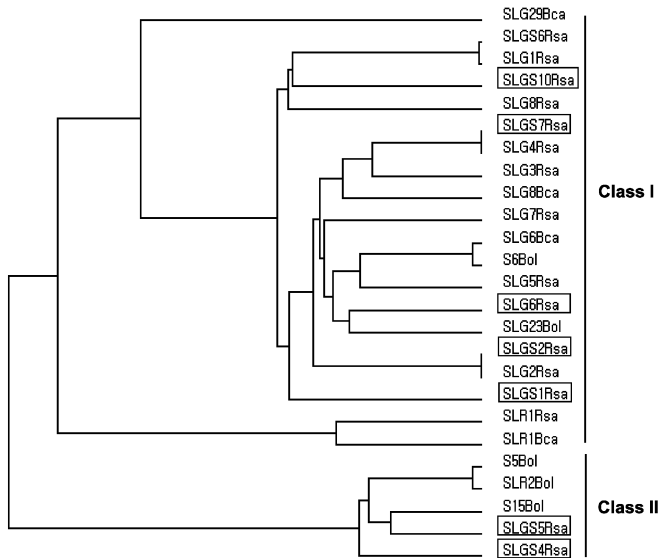
Nucleotide sequences of the DNA fragments amplified with the Class-I SLG-specific primer set were determined for the five S haplotypes and compared with those of the SLG gene in SLG<sub>6</sub> Bol (the SLG of the S<sub>6</sub> haplotype in *B. oleracea*) to show similarities from 77% to 82%. Similarities 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<sub>6</sub> 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<sub>2</sub> Bol (the SLG of the S<sub>2</sub> 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<sub>2</sub> 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





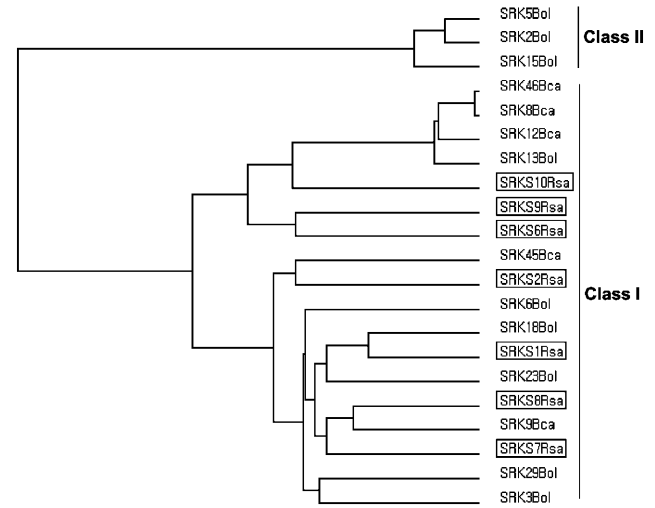
**Fig. 5** Phylogenetic tree of SLG sequences. Deduced amino-acid sequences of the putative mature protein regions of SLGs were used for the analysis. The aligned amino-acid sequences are as follows: SLG1Rsa (AB009677), SLG2Rsa (AB009678), SLG3Rsa (AB009679), SLG4Rsa (AB009680), SLG5Rsa (AB009681), SLG6Rsa (AB009682), SLG7Rsa (AB009684), SLG8Rsa (AB009683) and SLR1Rsa (AB009874) were isolated from *R. sativus*. SLG6Bca (M36301), SLG29Bca (AB008190), SLG8Bca (X55274) and SLR1Bca (Z26914) were from *B. campestris*. S6Bol (X03170), S5Bol (X51637), S15Bol (Y18261), SLG23Bol (AB013719) and SLR2Bol (Y18259), were isolated from *B. oleracea*. The phylogenetic tree was generated by the algorithm from EMBL (Heidelberg)

sharing homologies in the fourth, fifth, sixth and seventh exons of SRK<sub>3</sub> Bol (the SRK of the S<sub>3</sub> haplotype in *B. oleracea*), and the amino-acid sequences deduced from them were similar to those that all of the SRK<sub>3</sub> 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 phylogenetic tree

A phylogenetic 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 phylogenetic tree of the kinase domain from SRK alleles was constructed using the deduced amino-acid se-



**Fig. 6** Phylogenetic 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: SRK29Bol (Z30211), SRK3Bol (X79432), SRK18Bol (BAA92836), SRK13Bol (AB02440), SRK5Bol (Y18259), SRK15Bol (Y18260), SRK2Bol (BAA83746) and SRK23Bol (BAA83746) were isolated from *B. oleracea*. SRK45Bca (AB012106), SRK46Bca (AB013718), SRK12Bca (D38564), SRK8Bca (D38563) and SRK9Bca (D30049) were from *B. campestris*. The phylogenetic tree was generated by the algorithm from EMBL (Heidelberg)

quences of *R. sativus* SRKs and those previously reported for *B. oleracea* and *B. campestris*. As in the SLG phylogenetic 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<sub>3</sub>) and pollen-tube germination analysis. However, the S<sub>3</sub> 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<sub>3</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> population, it was shown that the S<sub>1</sub> SLG plant has the S<sub>1</sub> SRK allele; therefore, PCR-RFLP analysis of either the SLG or SRK allele is adequate for identification of the S haplotypes in F<sub>2</sub> 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 phylogenetic 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 phylogenetic 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*.

**Acknowledgements** This study was supported by a research grant from the Agricultural R&D Promotion Center.

## References

- Bateman AJ (1955) Self-incompatibility systems in angiosperms. III Cruciferae. *Heredity* 9:53–68
- Brace J, King GJ, Ockendon DJ (1994) Molecular approach to identification of S-alleles in *Brassica oleracea*. *Sex Plant Reprod* 7:203–208
- Chen CH, Nasrallah JB (1990) A new class of S sequences defined by a pollen recessive self-incompatibility allele of *Brassica oleracea*. *Mol Gen Genet* 222:241–248
- Delorme V, Giranton JL, Hatzfeld Y, Friry A, Heizemann P, Ariza MJ, Dumas C, Gaude T, Cock JM (1995) Characterization of the S locus genes, SLG and SRK, of the *Brassica* S<sub>3</sub> haplotype: identification of a membrane-localized protein encoded by the S-locus receptor kinase gene. *Plant J* 7:429–440
- Goring DR, Rothstein SJ (1992) The S-locus receptor kinase gene in a self-incompatible *Brassica napus* line encodes a functional serine/threonine kinase. *Plant Cell* 4:1273–1281
- Kho YO, Baer J (1968) Observing pollen tubes by means of fluorescence. *Euphytica* 17:298–302
- Kumar V, Trick M (1994) Expression of the S-locus receptor kinase multigene family in *Brassica oleracea*. *Plant J* 6:803–813
- Kusaba M, Nishio T, Satta Y, Hinata K, Ockendon D (1997) Striking similarity in inter- and intra-specific comparisons of Class-I SLG alleles from *Brassica oleracea* and *Brassica campestris*: implications for the evolution and recognition mechanism. *Proc Natl Acad Sci USA* 94:7673–7678
- Nahm SH, Yu JW, Kang BC, Kim B-D (1997) Selection of parental lines for the hot pepper mapping population using RFLP and AFLP analysis. *J Kor Soc Hort Sci* 38:693–697
- Nasrallah JB, Nasrallah ME (1993) Pollen-stigma interaction in the sporophytic self-incompatibility response. *Plant Cell* 5:1325–1335
- Nasrallah JB, Kao T-H, Goldberg ML, Nasrallah ME (1985) A cDNA encoding an S-locus-specific glycoprotein from *Brassica oleracea*. *Nature* 318:263–267
- Nasrallah JB, Kao T-H, Chen C-H, Nasrallah ME (1987) Amino-acid sequence of glycoproteins encoded by three alleles of the S-locus of *Brassica oleracea*. *Nature* 326:617–619
- Nasrallah JB, Nishio T, Nasrallah ME (1991) The self-incompatibility genes of *Brassica*: expression and use in genetic ablation of floral tissues. *Annu Rev Plant Physiol Plant Mol Biol* 42:393–422
- Nishio T, Sakamoto K, Yamaguchi J (1994) PCR-RFLP of the S locus for identification of breeding lines in cruciferous vegetables. *Plant Cell Rep* 13:546–550
- Nishio T, Kusaba M, Watanabe M, Hinata K (1996) Registration of S alleles in *Brassica campestris* L. by the restriction fragment sizes of SLGs. *Theor Appl Genet* 92:388–394
- Nishio T, Kusaba M, Sakamoto K, Ockendon D (1997) Polymorphism of the kinase domain of the S-locus receptor kinase gene (SRK) in *Brassica oleracea* L. *Theor Appl Genet* 95:335–342
- Nou IS, Watanabe M, Isogai A, Hinata K (1993) Comparison of S-alleles and S-glycoproteins between two wild populations of *Brassica campestris* in Turkey and Japan. *Sex Plant Reprod* 6:79–86
- Ockendon DJ (1974) Distribution of self-incompatibility alleles and breeding structure of open-pollinated cultivars of *Brussels sprouts*. *Heredity* 33:159–171
- Rundle SJ, Nasrallah ME, Nasrallah JB (1993) Effects of inhibitors of protein serine/threonine phosphatases on pollination in *Brassica*. *Plant Physiol* 103:1165–1171
- Sakamoto K, Kusaba M, Nishio T (1998) 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. *Mol Gen Genet* 258:397–403
- Scutter CP, Croy RRD (1992) An S<sub>5</sub> self-incompatibility allele-specific cDNA sequence from *B. oleracea* shows high homology to the SLR2 gene. *Mol Gen Genet* 232:240–246
- Stein JC, Nasrallah JB (1993) A plant receptor-like gene, the S-locus receptor kinase of *Brassica oleracea* L. encodes a functional serine/threonine kinase. *Plant Physiol* 101:1103–1106
- Stein JC, Howlett B, Boyes DC, Nasrallah ME, Nasrallah JB (1991) Molecular cloning of a putative receptor kinase gene encoded by the self-incompatibility locus of *Brassica oleracea*. *Proc Natl Acad Sci USA* 88:8816–8820
- Trick M, Flavell RB (1989) A homozygous S genotype of *Brassica oleracea* expresses two S-like genes. *Mol Gen Genet* 218:112–117
- Yamakawa S, Watanabe M, Hinata K, Suzuki A, Isogai A (1995) The sequences of S-receptor kinases (SRK) involved in self-incompatibility and their homologies to S-locus glycoproteins of *Brassica campestris*. *Biosci Biotechnol Biochem* 59:161–162