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Polymorphism of the kinase domain of the *S*-locus receptor kinase gene (*SRK*) in *Brassica oleracea* L.

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Abstract DNA polymorphism of the *S*-locus receptor kinase gene (*SRK*) participating in self-incompatibility in *Brassica* was analyzed by PCR-RFLP and nucleotide sequencing. In the screening of primers for specific amplification of polymorphic DNA fragments of *SRK*, the best combination was that of a forward primer (PK1) having the nucleotide sequence of the second exon of S^6 *SRK* and a reverse primer (PK4) having the complementary nucleotide sequence of the fifth exon of S^6 *SRK*. PCR using this primer pair amplified DNA fragments of 0.9–1.0 kb from 36 *S* haplotypes out of 42 tested. These DNA fragments showed high polymorphism in polyacrylamide-gel electrophoresis after digestion with restriction endonuclease(s): 25 types were found in a double digestion with *Mbo*I and *Afa*I. Nucleotide sequencing of the DNA fragments amplified from five *S* haplotypes showed that the third, fourth, and fifth exons of *SRK* are highly conserved, and that there are high variations of the second and third introns of *SRK*, which produced polymorphism of the band pattern in PCR-RFLPs. Another forward primer (PK5) having the nucleotide sequence of the second exon, which is derived from S^2 *SRK*, amplified DNA fragments of almost the same region of *SRK* from 27 *S* haplotypes in combination with PK4. Although *SRK* alleles of the class-II *S* haplotypes were not amplified, all of the class-I *S*-haplotypes were amplified with a primer mixture of PK1, PK4 and PK5. The DNA fragments of both *SRK* alleles in *S* heterozygotes, or a 1:1 mixture of the genomic DNA of different *S* homozygotes, were amplified without exception, suggesting the usefulness of these primers for the identification of *S* heterozygotes. The DNA fragment sizes obtained by digestion with restriction endonucleases served as markers for the identification of *S* haplotypes.

Key words *Brassica oleracea* · Self-incompatibility · *SRK* · DNA polymorphism · PCR-RFLP

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Introduction

Self-incompatibility is the mechanism which prevents self-fertilization by inhibiting pollen-tube growth of self-pollen on the stigma surface or in the style-transmitting tissue. Many angiosperm species employ this system to maintain genetic diversity in a population. *Brassica* has a sporophytic self-incompatibility system which has been intensively studied at the molecular level. This system is controlled by a single *S* locus with multiple alleles. The *Brassica S* locus is a gene complex having at least two genes participating in the recognition of self and non-self in the stigma: the *S*-locus glycoprotein gene (*SLG*) and the *S*-locus receptor kinase gene (*SRK*); and it is believed that a gene(s) determining the *S* specificity of pollen is also present in this gene complex. Therefore, the term '*S* haplotype' is used instead of '*S* allele', which has long been employed (Nasrallah and Nasrallah 1993).

For molecular genetic study of self-incompatibility, accurate identification of the *S* haplotype of the plant material used is indispensable to prevent confusion and to ensure that results obtained by different workers are comparable. From the nucleotide sequence similarity of the *SLG* of different *S* haplotypes, they can be classified

into two types, class I and class II, and it is known that dominant *S* haplotypes belong to class I and that recessive *S* haplotypes belong to class II (Nasrallah and Nasrallah 1993). Identification of the *S* haplotype is also required in the breeding of cruciferous vegetables, because F_1 hybrid seeds are produced using a self-incompatibility system. In order to identify the *S* haplotype of plant material, crossing with all *S* tester lines and observation of pollen tube growth or seed set are necessary. Fifty alleles and 30 alleles have been identified in *Brassica oleracea* and in *Brassica campestris*, respectively (Ockendon 1974; Nou et al. 1993), and therefore in *B. oleracea* as many as 50 test crosses may be needed. Because the self-incompatibility phenotype is influenced both by environmental factors and the physiological condition of a plant, the test crossing should be repeated several times. This is a highly time-consuming procedure.

We recently developed a simple method for identifying *SLG* alleles, which is called PCR-RFLP. This consists of specific amplification of *SLG* alleles, using the polymerase chain reaction (PCR) with a pair of *SLG*-specific primers, and electrophoretic analysis of the PCR products after cleavage with one or more restriction endonucleases (Brace et al. 1993; Nishio et al. 1994), and is expected to be useful for the identification of *S* haplotypes in *Brassica*.

The gene (*SRK*) for *S*-locus receptor kinase is also known to be highly polymorphic (Stein et al. 1991; Delorme et al. 1995). This gene comprises an *S* domain homologous to *SLG*, a transmembrane domain, and a protein kinase domain. Specific amplification of *SRK* using PCR can also provide a method for the identification of *S* haplotypes.

In the present study, we found a set of primers which gives amplification of DNA fragments of *SRK* from class-I *S* haplotypes in *B. oleracea*. PCR-RFLP analysis using these primers showed a high polymorphism of the kinase domain of *SRK*. Nucleotide sequencing of the amplified DNA fragments suggested that the polymorphism of the DNA fragments is due to considerable variation of the second and third introns of *SRK*. In *S* heterozygotes, the DNA fragments of both *SRK* alleles were amplified, suggesting the usefulness of these primers for identification of the *S* genotype of plant material. The molecular method described here is an important step in the development of a more reliable and rapid method to identify *S* haplotypes than the usual test crossing method.

Materials and methods

Materials

S tester lines homozygous for *S* haplotypes in *B. oleracea* L. (Ockendon 1974) were used as plant material. Plant DNA was prepared from leaf tissue according to Rogers and Bendich (1985).

Amplification of DNA fragments of *SRK*

Primers having nucleotide sequences at various *SRK* sites (see Fig. 1) were used for the amplification of *SRK* DNA fragments with PCR. Plant genomic DNA (1 µl, approximately 20 ng) was mixed with a pair of primers, 20 pmoles each, 1 µl of 10× buffer (500 mM KCl, 100 mM Tris-HCl pH 8.3, 15 mM $MgCl_2$, 0.01% gelatin), 0.8 µl of dNTP mixtures (each 5 nmoles), 0.05 µl of *Taq* DNA polymerase (*Takara Taq* 0.25 units), and distilled water to make a final volume of 10 µl. PCR involved 30 cycles of 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C with a thermal cycler (Model 9600, ABI).

Electrophoretic analysis of cleaved DNA fragments

PCR products were digested with *Hin*fI, *Alu*I, and a mixture of *Mbo*I and *Afa*I. The digested DNA was subjected to electrophoresis in a 5% polyacrylamide gel containing TBE buffer. After electrophoresis at 100 V for 1 h, DNA bands were detected with silver staining (Daiich Chemicals, Japan).

Determination of nucleotide sequences of the PCR products

The DNA fragment amplified with *Taq* DNA polymerase was cloned with a TA cloning kit (Invitrogen), and the nucleotide sequence of the cloned DNA was determined with a DNA sequencer (Pharmacia). To avoid errors that may have occurred during the PCR process, three independent clones were sequenced.

Linkage analysis of *SLG* and *SRK*

A plant homozygous for S^{22} was crossed with a plant of broccoli cv 'Ryokurei' (Sakata Seed Co. Japan). An F_1 plant having the genotype $S^{22}S^{18}$ was selected and its anthers were cultured in vitro according to Sato et al. (1989). *SLG* and *SRK* of the plants developed from microspores were analyzed with PCR-RFLP. In the analysis of *SLG*, a primer pair of PS5 and PS15 (Nishio et al. 1996) was used for PCR and the PCR products were digested with *Mbo*I.

Results

Kinase domain of *SRK*

For the specific amplification of *SRK* DNA, primers matching various sites of S^6 *SRK* were screened (Fig. 1). The pair PK1 and PK4 was found to be the best combination of primers for the amplification of polymorphic DNA fragments of *SRK* alleles. The nucleotide sequence of PK1 was taken from the transmembrane domain (1375–1399) of S^6 *SRK* (Stein et al. 1991). PK4 is a reverse primer having a nucleotide sequence corresponding to the fifth exon (2002–2023). PCR using this primer pair gave a single DNA fragment from 0.9 to 1.0 kb in 36 *S* haplotypes among 42 tested (Table 1). Two bands of 0.8 kb and 1.0 kb were amplified from the S^{50} homozygote. In homozygotes of S^2, S^5, S^{15} and S^{24}, no amplification was observed, and only a small amount of DNA was amplified from S^{18} and S^{46}.

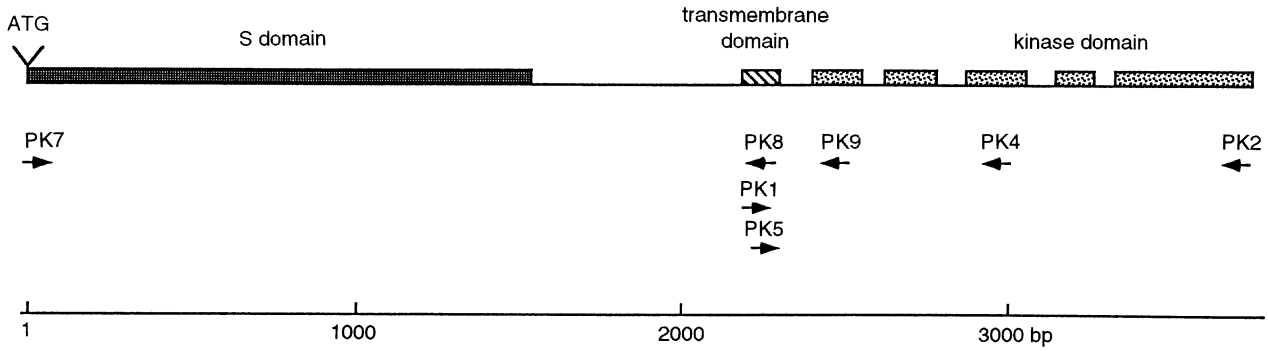

Fig. 1 The sites of nucleotide sequences used as primers for specific amplification of *SRK*. *Arrows* indicate the primers. *Shaded boxes* show exons:

PK1: 5′-CTGCTGATCATGTTCTGCCTCTGG-3′
PK2: 5′-ATATTGAATCACTCAGCTATCATA-3′
PK4: 5′-CAATCCCAAAATCCGAGATCT-3′
PK5: 5′-AGACAAAAGCAAGCAAAAGCA-3′
PK7: 5′-ATGCAAGGTGTACGATACATCTATCATCATTCTTAC-3′
PK8: 5′-GATCAGAAGAAGCAGAACAGTAACTCCAACAGTC-3′
PK9: 5′-CCTTGTCCGAGTTTGTTACAGTTGGAGAAATTTTCGG-3′

Digestion of the PCR products with restriction endonucleases and subsequent polyacrylamide gel electrophoresis revealed polymorphism of the amplified DNA fragment (Fig. 2A). From several restriction endonucleases tested, *Hin*fI and *Alu*I were found to be the most suitable, giving DNA fragments of various sizes from many *S* haplotypes. Double digestion with *Mbo*I and *Afa*I also gave high polymorphism of the PCR products. The sizes of the DNA fragments estimated from their electrophoretic mobilities are shown in Table 2. Electrophoretic profiles of the PCR products from 36 *S* haplotypes after digestion with *Hin*fI were classified into 20 types, those with *Alu*I into 23 types, and those with *Mbo*I and *Afa*I into 25 types. Three haplotypes, S^{14}, S^{16}, and S^{20}, showed the same electrophoretic profile after digestion with *Alu*I, but they had profiles which differed from each other after digestion with *Hin*fI. Thirty *S* haplotypes can be identified by combining the three analyses using these four restriction endonucleases. The other nine *S* haplotypes had counterparts always showing the same electrophoretic profiles. These haplotypes fell into three groups, 1: S^{13} and S^{32}, 2: S^{22} and S^{58}, and 3: S^{4}, S^{7}, S^{28}, S^{31} and S^{60}, members of which belonged to the same type in the analyses using the three enzyme combinations.

The second best combination of primers was that of PK5 (3659–3680 of S^2 *SRK*, Tantikanjana et al. 1993) and PK4. A DNA fragment having the expected size was amplified from 27 *S* haplotypes with this primer pair. These haplotypes included S^{18}, S^{24} and S^{46}, which gave little or no amplification with PK1 + PK4. However, the DNA fragments of S^2, S^5, and S^{15}, which are

Table 1 Amplification of DNA fragments of *SRK*s from *S* tester lines in *B. oleracea*

Lines	PK1 + PK4	PK5 + PK4	PK7 + PK8	PK7 + PK9
S^1	+[a]	+	−	−
S^2	−[b]	−	−	−
S^3	+	−	+	+
S^4	+	+	−	−
S^5	−	−	−	−
S^6	+	+	+	+
S^7	+	+	−	−
S^8	+	+	+	+
S^9	+	−	−	−
S^{11}	+	−	−	−
S^{12}	+	−	−	−
S^{13}	+	+	−	−
S^{14}	+	+	−	−
S^{15}	−	−	−	−
S^{16}	+	+	+	+
S^{17}	+	+	−	−
S^{18}	±[c]	+	+	+
S^{20}	+	+	−	−
S^{22}	+	+	−	−
S^{24}	−	+	−	−
S^{25}	+	+	−	−
S^{28}	+	+	−	−
S^{29}	+	−	+	−
S^{31}	+	+	−	−
S^{32}	+	+	−	−
S^{33}	+	−	−	−
S^{35}	+	−	−	−
S^{36}	+	+	+	+
S^{38}	+	−	−	−
S^{39}	+	−	+	+
S^{46}	±	+	−	−
S^{50}	+	+	−	−
S^{51}	+	−	−	+
S^{52}	+	+	−	−
S^{57}	+	−	−	−
S^{58}	+	+	−	+
S^{60}	+	+	−	−
S^{61}	+	−	−	−
S^{62}	+	+	+	−
S^{63}	+	+	−	−
S^{64}	+	+	−	−
S^{65}	+	+	−	−

[a] +: amplified
[b] −: not amplified
[c] ±: a small amount of DNA was amplified

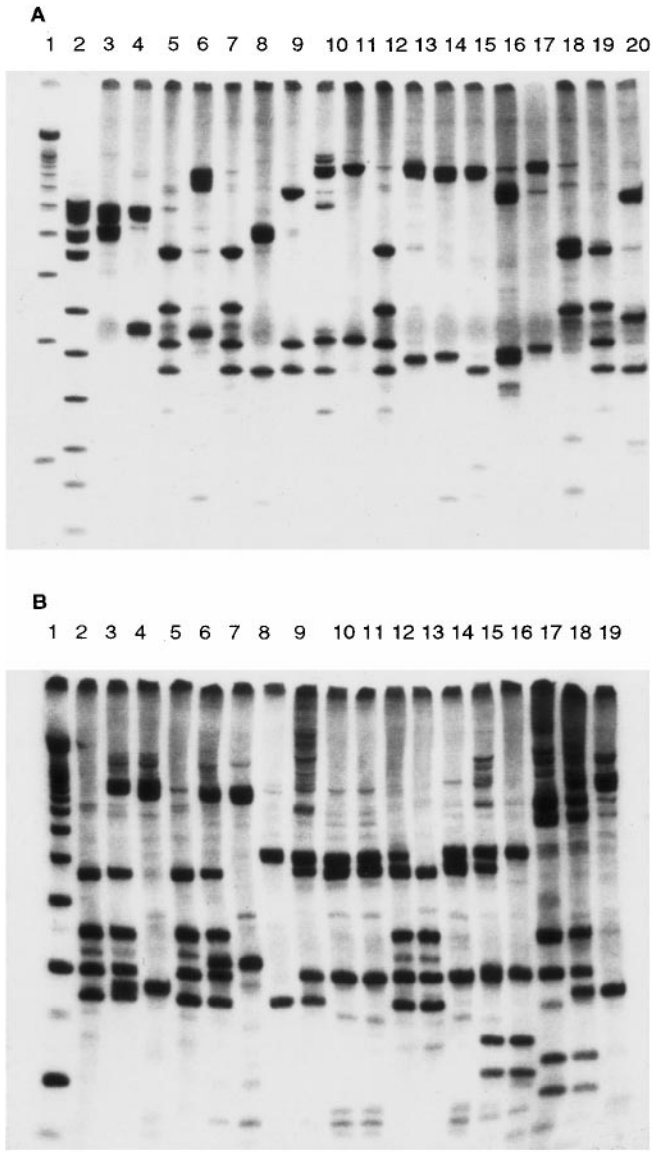

Fig. 2 PCR-RFLP analysis of *SRK* DNA fragments of *S* homozygotes (**A**) and *S* heterozygotes (**B**) using primers of PK1 + PK4 and the restriction endonucleases *Mbo*I + *Afa*I. **A** *1*: 100-bp ladder, *2*: pUC18/*Hap*II, *3*: S^1S^1, *4*: S^3S^3, *5*: S^4S^4, *6*: S^6S^6, *7*: S^7S^7, *8*: S^8S^8, *9*: S^9S^9, *10*: $S^{11}S^{11}$, *11*: $S^{12}S^{12}$, *12*: $S^{13}S^{13}$, *13*: $S^{14}S^{14}$, *14*: $S^{16}S^{16}$, *15*: $S^{17}S^{17}$, *16*: $S^{20}S^{20}$, *17*: $S^{22}S^{22}$, *18*: $S^{25}S^{25}$, *19*: $S^{28}S^{28}$, *20*: $S^{29}S^{29}$. **B** *1*: 100-bp ladder, *2*: S^7S^7, *3*: S^7S^{14}, *4*: $S^{14}S^{14}$, *5*: S^7S^7, *6*: S^7S^6, *7*: S^6S^6, *8*: S^8S^8, *9*: S^8S^{36}, *10*: $S^{36}S^{36}$, 11: $S^{36}S^{36}$, *12*: $S^{36}S^{31}$, *13*: $S^{31}S^{31}$, *14*: $S^{36}S^{36}$, *15*: $S^{36}S^{35}$, *16*: $S^{35}S^{35}$, *17*: $S^{50}S^{50}$, *18*: $S^{50}S^{14}$, *19*: $S^{14}S^{14}$

class-II *S* haplotypes, were not amplified with PK5 + PK4 (Table 1). In S^{50}, which showed two DNA fragments with PK1 + PK4, only the shorter 0.8-kb DNA fragment was amplified. Electrophoretic profiles of the PCR products digested with the restriction endonucleases were similar to those of the DNA fragments amplified with PK1 + PK4, suggesting that this primer pair amplifies almost the same region of the gene as PK1 + PK4. The three primers, PK1, PK4 and PK5, were mixed for PCR amplification of the *SRK* fragments, and gave almost the same electrophoretic profiles in the PCR-RFLP analysis as did each primer pair.

The nucleotide sequences of the PCR products amplified with PK1 + PK4 from five *S* haplotypes, S^{13}, S^{31}, S^{32}, S^{50} and S^{58}, were determined. The four DNA fragments from S^{13}, S^{31}, S^{32} and S^{58} had four regions homologous to the second, third, forth and fifth exon of S^3 *SRK*, respectively, in common (Fig. 3) and the amino-acid sequences deduced from them were similar to S^6 *SRK*, suggesting that all of them are the fragments of *SRK* alleles. While the nucleotide sequences of the four exons were highly conserved, those of the second intron and the third intron were much more variable. It is considered that the variation of these regions contributed largely to the polymorphism of DNA fragment sizes in the PCR-RFLP analysis. The DNA fragments amplified from S^{13} and S^{32}, which showed the same electrophoretic profiles by the PCR-RFLP analyses using the three enzyme combinations, had the same nucleotide sequence. Nucleotide sequencing of the two DNA fragments amplified from S^{50} revealed that both are homologous to S^3 *SRK*, but the shorter one has a deletion extending from the 3′ end of the second exon to the second intron, which results in a frameshift. It is considered that the longer DNA fragment is of S^{50} *SRK* while the shorter one is a pseudo gene.

S domain of *SRK*

For specific amplification of the *S* domain of *SRK*, three primers were used. These were the forward primer (PK7), which is located at the translation initiation site, and the reverse primers, located at the transmembrane domain (the second exon) (PK8) or the third exon (PK9) (Fig. 1). DNA fragments of about 2.2 kb and 2.4 kb, which is consistent with the S^3 *SRK* sequence (Delorme et al. 1995), were amplified with PK7 + PK8 and PK7 + PK9, respectively, and the PCR products showed polymorphism in the electrophoresis after digestion with *Mbo*I (data not shown). However, DNA fragments were amplified only from 11 haplotypes (Table 1) including S^3, S^6 and S^{29} *SRK*, the nucleotide sequences of which have already been published (Stein et al. 1991; Kumar and Trick 1994; Delorme et al. 1995).

S heterozygotes

The DNA polymorphism of six *S* heterozygotes was analyzed with PCR-RFLP using PK1 + PK4. All the plants tested showed amplification of both *SRK* alleles in the *S* heterozygotes (Fig. 2B). The genomic DNA of pairs of *S* homozygotes from ten *S* haplotypes, S^1, S^3, S^4,

Table 2 Restriction fragment sizes of the PCR product of *SRK*s estimated from the mobilities in a polyacrylamide gel[a]

Lines	*Mbo*I + *Afa*I	*Alu*I	*Hin*fI
S^1	0.47, 0.41	0.44, 0.28, 0.16, 0.05	0.62, 0.14, 0.09, 0.05 a
S^3	0.47, 0.21, 0.21	0.35, 0.30, 0.16, 0.05	0.24, 0.22, 0.14, 0.10, 0.09, 0.07, 0.05
S^4	0.34, 0.24, 0.19, 0.16 a[b]	0.39, 0.18, 0.17, 0.12 b	0.44, 0.21, 0.12, 0.09, 0.06, 0.05 b
S^6	0.67, 0.21	0.36, 0.33, 0.16, 0.05	0.62, 0.14, 0.09, 0.05 a
S^7	0.34, 0.24, 0.19, 0.16 a	0.39, 0.18, 0.17, 0.12 b	0.44, 0.21, 0.12, 0.09, 0.06, 0.05 b
S^8	0.40, 0.40, 0.16	0.38, 0.35, 0.16, 0.05	0.62, 0.14, 0.09, 0.05 a
S^9	0.55, 0.19, 0.16	0.39, 0.31, 0.17	0.24, 0.21, 0.18, 0.12, 0.09, 0.06, 0.05
S^{11}	0.68, 0.20, 0.16 d	0.46, 0.43, 0.17	0.53, 0.24, 0.14, 0.09, 0.05
S^{12}	0.70, 0.20	0.39, 0.25, 0.16, 0.05	0.62, 0.14, 0.09, 0.05 a
S^{13}	0.34, 0.24, 0.19, 0.16 a	0.39, 0.18, 0.17, 0.16 c	0.63, 0.12, 0.09, 0.06, 0.05 c
S^{14}	0.70, 0.18 b	0.65, 0.16, 0.05 a	0.80, 0.09, 0.05
S^{16}	0.70, 0.18 b	0.65, 0.16, 0.05 a	0.38, 0.25, 0.14, 0.09, 0.05
S^{17}	0.70, 0.16	0.43, 0.30, 0.16, 0.05	0.62, 0.11, 0.09, 0.05
S^{20}	0.55, 0.18, 0.17	0.65, 0.16, 0.05 a	0.62, 0.14, 0.09, 0.05 a
S^{22}	0.70, 0.19 c	0.65, 0.16, 0.05 a	0.62, 0.14, 0.09, 0.05 a
S^{25}	0.36, 0.34, 0.24	0.30, 0.18, 0.17, 0.12, 0.09	0.44, 0.28, 0.12, 0.09, 0.05
S^{28}	0.34, 0.24, 0.19, 0.16 a	0.39, 0.18, 0.17, 0.12 b	0.44, 0.21, 0.12, 0.09, 0.06, 0.05 b
S^{29}	0.51, 0.22, 0.16	0.39, 0.30, 0.16	0.65, 0.13, 0.09, 0.05
S^{31}	0.34, 0.24, 0.19, 0.16 a	0.39, 0.18, 0.17, 0.12 b	0.44, 0.21, 0.12, 0.09, 0.06, 0.05 b
S^{32}	0.34, 0.24, 0.19, 0.16 a	0.39, 0.18, 0.17, 0.16 c	0.63, 0.12, 0.09, 0.06, 0.05 c
S^{33}	0.59, 0.16, 0.07	0.65, 0.16, 0.05 a	0.40, 0.26, 0.14, 0.09, 0.05
S^{35}	0.38, 0.19, 0.13, 0.11	0.29, 0.25, 0.19, 0.16, 0.05	0.62, 0.20, 0.09
S^{36}	0.38, 0.35, 0.19	0.65, 0.16, 0.05 a	0.62, 0.14, 0.09, 0.05 a
S^{38}	0.47, 0.19, 0.16, 0.10, 0.07	0.41, 0.17	0.53, 0.12, 0.11, 0.09, 0.06, 0.05
S^{39}	0.70, 0.20	0.65, 0.16, 0.05 a	0.62, 0.14, 0.09, 0.05 a
S^{50}	0.54, 0.50, 0.25, 0.19, 0.12, 0.10	0.37, 0.31, 0.25, 0.19, 0.16, 0.05	0.44, 0.43, 0.31, 0.24, 0.10, 0.09
S^{51}	0.70, 0.18 b	0.37, 0.28, 0.16, 0.10	0.62, 0.14, 0.09, 0.05 a
S^{52}	0.52, 0.21, 0.16	0.39, 0.30, 0.16, 0.08	0.62, 0.14, 0.09, 0.05 a
S^{57}	0.70, 0.18 b	0.65, 0.16, 0.05 a	0.38, 0.25, 0.09, 0.09, 0.06, 0.05
S^{58}	0.70, 0.19 c	0.65, 0.16, 0.05 a	0.62, 0.14, 0.09, 0.05 a
S^{60}	0.34, 0.24, 0.19, 0.16 a	0.39, 0.18, 0.17, 0.12 b	0.44, 0.21, 0.12, 0.09, 0.06, 0.05 b
S^{61}	0.52, 0.18, 0.14	0.38, 0.28, 0.16, 0.05	0.62, 0.10, 0.09, 0.05
S^{62}	0.37, 0.23, 0.16, 0.12	0.40, 0.28, 0.17	0.41, 0.25, 0.09, 0.08, 0.05
S^{63}	0.68, 0.20, 0.16 d	0.39, 0.30, 0.17, 0.09, 0.08	0.49, 0.21, 0.12, 0.09, 0.06, 0.05
S^{64}	0.52, 0.19, 0.15	0.39, 0.29, 0.16, 0.05	0.62, 0.14, 0.09, 0.05 a
S^{65}	0.49, 0.30, 0.20	0.70, 0.16, 0.05	0.49, 0.25, 0.10, 0.07, 0.05
S^{18}[c]	0.65, 0.18, 0.07	0.46, 0.16	0.62, 0.15, 0.09
S^{24}[c]	0.33, 0.19, 0.18	0.65, 0.16	0.62, 0.14, 0.09
S^{46}[c]	0.54, 0.19, 0.16	0.41, 0.31, 0.16	0.41, 0.25, 0.15, 0.10, 0.09

[a] Combination of primers was PK1 + PK4
[c] Combination of primers was PK5 + PK4
[b] Letters (a–d) in the Table show the groups, members of which have indistinguishable electrophoretic profiles each other

S^6, S^8, S^9, S^{12}, S^{14}, S^{17} and S^{20}, were mixed in a 1:1 ratio to make a DNA sample equivalent to an *S* heterozygote, and used as a template for PCR. Without exception, all the DNA mixtures of the 45 possible combinations gave amplification of both *SRK* alleles.

For linkage analysis between *SLG* and *SRK*, PCR-RFLP analysis was performed on a segregating population of 184 plants obtained from an anther culture of an $S^{18}S^{22}$ heterozygote. Sixty two plants had S^{18} *SLG* and 122 plants had S^{22} *SLG*. All plants with S^{18} *SLG* had S^{18} *SRK* , which is amplified with PK5 + PK4, and those with S^{22} *SLG* had S^{22} *SRK*, which is amplified with PK1 + PK4. There was no recombinant between *SLG* and *SRK*.

Discussion

The DNA fragments amplified with the primers PK1 + PK4 had the conserved regions of the second, third, fourth and fifth exons of *SRK*, and were polymorphic enough to show many different electrophoretic profiles after digestion with restriction endonucleases. In a segregating population, the *S* haplotype identified by PCR-RFLP analysis with the PK1 + PK4 or PK5 + PK4 primers corresponded to that identified with *SLG*-specific primers without exception. These observations suggest that the DNA fragments amplified with PK1 + PK4 and PK5 + PK4 were of *SRK* genes.

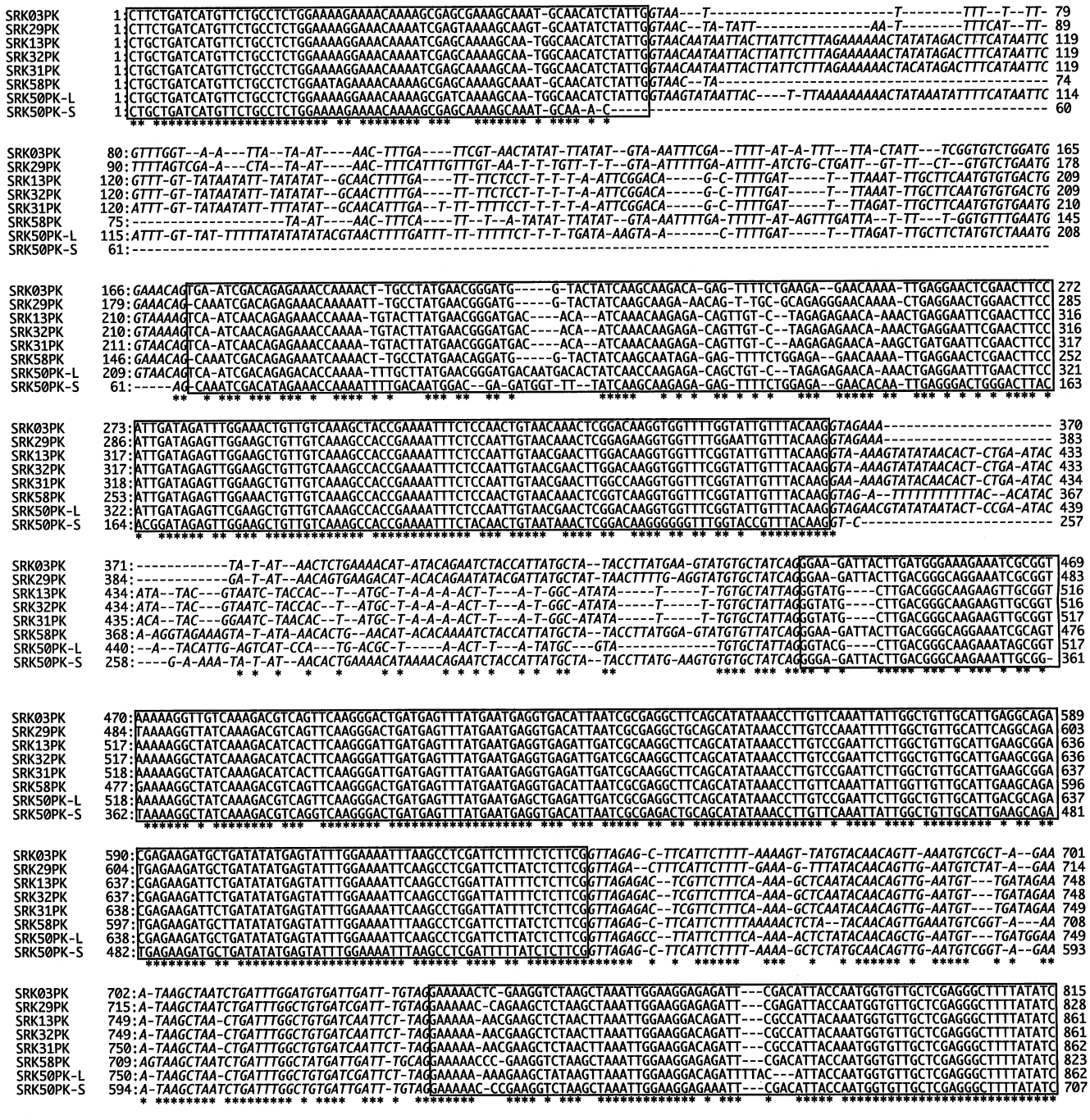

Fig. 3 Nucleotide sequences of DNA fragments amplified from five *S* homozygotes with PK1 + PK4. *Boxes* indicate the 2nd, 3rd, 4th and 5th exon of *SRK*. SRK13PK, SRK31PK, SRK32PK and SRK58PK are the PCR products from S^{13}, S^{31}, S^{32} and S^{58} homozygotes, respectively. SRK50PK-L is the longer DNA fragment of the two PCR products from the S^{50} homozygote, and SRK50PK-S is the shorter one. The nucleotide sequences of S^3 *SRK* (SRK03PK) and S^{29} *SRK* (SRK29PK) were from Delorme et al. (1995) and Kumar and Trick (1994), respectively

The *S* domain of *S* receptor kinase is highly polymorphic and considered to be a binding site of an *S*-specific pollen ligand in the recognition of self and non-self (Nasrallah and Nasrallah 1993). In attempts to specifically amplify the *S* domain of *SRK* by PCR, DNA fragments were amplified only from 11 *S* haplotypes. The difficulty of specific amplification of the *S* domain

would be due to the long size of the first intron of some *SRK* alleles (Nasrallah and Nasrallah 1993) or else to high variation of DNA sequence at the site used as a primer.

The primer pair PK1 and PK4 was the best combination for the amplification of polymorphic DNA fragments from *SRK* genes of many *S* haplotypes. The *S* haplotypes in which DNA fragments of *SRK* were not amplified with PK1 + PK4 were the class-II *S* haplotypes, S^2, S^5 and S^{15}. The inability to amplify class-II *SRK* DNA may be due to either the low homology with the primer sequences at the corresponding sites or to the long third intron (Tantikanjana et al. 1993). The other *S* haplotypes used in this work showed polymorphic bands in genomic Southern-hybridization analysis using an S^6 *SLG* cDNA clone as a probe, suggesting that all of them are class-I haplotypes (data not shown). *SRK* DNA fragments were amplified with PK1 + PK4 in all of them except S^{18}, S^{24} and S^{46}, but *SRK* fragments of these *S* haplotypes were amplified with another forward primer, PK5, instead of PK1. By combining three primers, PK1, PK5 and PK4, *SRK* fragments of all the class-I *S* haplotypes were amplified.

When comparing the nucleotide sequences of *SRK* fragments of the five *S* haplotypes reported here with those already reported, high polymorphism of the second intron and the third intron was found. The polymorphism of these regions probably contributed largely to the different electrophoretic profiles observed in PCR-RFLP analysis. Twenty five, 23, and 20 types were distinguished by PCR-RFLP using *Mbo*I + *Afa*I, *Alu*I, and *Hin*fI, respectively, and a combination of enzyme treatments enabled the identification of 30 out of 42 *S* haplotypes tested. The class-I *SRK* fragments were classified into 33 types by PCR-RFLP using the set of PK1, PK4 and PK5. This result suggests that analysis of DNA polymorphism of the *SRK* fragments from the second exon (transmembrane domain) to the fifth exon (the middle of kinase domain) is useful for the identification of *S* haplotypes.

The preferential amplification of one *SLG* allele in an *S* heterozygote was observed in PCR with the class-I *SLG*-specific primers, PS1 + PS2 (Nishio et al. 1994). This preferential amplification may be caused by the difference in DNA homology of the template DNA to the primer sequences between different *SLG* alleles. The lack of observed preferential amplification with PK1 + PK4 may be due to low sequence variation at the sites of the primers. In the breeding of cruciferous vegetables, testing the heterozygosity of a plant is important. The combination of PK1 and PK4 is expected to be useful in this test.

It is considered that both *SLG* and *SRK* participate in self-recognition of pollen and stigma in *Brassica* (Nasrallah et al. 1992, 1994). If recombination between *SLG* and *SRK* did occur, the progeny might show a breakdown of the self-incompatibility phenotype. However, no recombinant between *SLG* and *SRK* was obtained in the screening using 184 plants from anther culture of an *S* heterozygote. In addition to these plants, we have tried to find a recombinant using a total of about 100 other plants obtained from anther culture of other *S* heterozygotes in *B. oleracea* and *B. campestris*, but without success. These results suggest that the tight linkage between *SLG* and *SRK* is hardly ever, or never, broken at meiosis. The low possibility of recombination between *SLG* and *SRK* indicates that analysis of either *SLG* or *SRK* is adequate for the identification of *S* haplotypes.

Some *S* haplotypes showed the same band pattern in PCR-RFLP using PK1 + PK4. Although the choice of restriction endonucleases may help to distinguish these *S* haplotypes, DNA fragments amplified from S^{13} and S^{32} with PK1 + PK4 cannot be distinguished, because they have the same nucleotide sequence. The inability of distinguishing some *S* haplotypes is the major problem of PCR-RFLP using PK1, PK4 and PK5. These *S* haplotypes may be distinguished by the PCR-RFLP analysis of *SLG* (Nishio et al. 1996). Combining the analysis of *SLG* and *SRK* should increase the number of cases in which the identification of *S* haplotypes in *Brassica* can be achieved by these methods.

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