Theor Appl Genet (1996) 92:388–394

T. Nishio · M. Kusaba · M. Watanabe · K. Hinata

Registration of *S* alleles in *Brassica campestris* L by the restriction fragment sizes of *SLGs*

Received: 7 September 1995 / Accepted: 6 October 1995

Abstract Polymorphism of *SLG* (the *S*-locus glycoprotein gene) in *Brassica campestris* was analyzed by PCR-RFLP using *SLG*-specific primers. Nucleotide sequences of PCR products from 15 *S* genotypes were determined in order to characterise the exact DNA fragment sizes detected in the PCR-RFLP analysis. Forty-seven lines homozygous for 27 *S*-alleles were used as plant material. One combination of primers, PS5 + PS15, which had a nucleotide sequence specific to a class-I *SLG*, gave amplification of a single DNA fragment of approximately 1.3 kb from the genomic DNA of 15 *S* genotypes. All the DNA fragments showed different electrophroetic profiles from each other after digestion with *Mbo*I or *Msp*I. Different lines having the same *S* genotype had an identical electrophoretic profile even between the lines collected in Turkey and in Japan. Another class-I *SLG*-specific primer, PS18, gave amplification of a 1.3-kb DNA fragment from three other *S* genotypes in combination with PS15, and the PCR product also showed polymorphism after cleavage with the restriction endonucleases. Genetic analysis, Southern-hybridization analysis, and determination of the nucleotide sequences of the PCR products suggested that the DNA fragments amplified with these combinations of primers are class-I *SLG*s. Expected DNA fragment sizes in the present PCR-RFLP condition were calculated from the determined nucleotide sequence of *SLG* PCR products. A single DNA fragment was also amplified from six *S* genotypes by PCR with a combination of primers, PS3 + PS21, having a nucleotide sequence specific to a class-II *SLG*. The amplified DNA showed polymorphisnm after cleavage with restriction endonucleases. The cleaved fragments were detected by Southern-hybridization analysis using a probe of S^5 *SLG* cDNA, a class-II*SLG*. Partial sequencing revealed a marked similarity of these amplified DNA fragments to a class-II *SLG*, demonstrating the presence of class-I and class-II *S* alleles also in *B. campestris*. The high *SLG* polymorphism detected by the present investigation suggests the usefulness of the PCR-RFLP method for the identification of *S* alleles in breeding lines and for listing *S* alleles in *B. campestris*.

Key words *Brassica campestris* · *S* alleles · *SLG* · PCR-RFLP · DNA polymorphism

Communicated by G. Wenzel

T. Nishio (✉) · M. Kusaba
Institute of Radiation Breeding, NIAR, MAFF, Ohmiya, Naka-gun, Ibaraki 319-22, Japan

M. Watanabe · K. Hinata
Faculty of Agriculture, Tohoku University, Aobaku, Sendai 981, Japan

Introduction

The self-incompatibility system has been successfully used for the seed productioin of F_1 hybrid cultivars in the cruciferous vegetables, cabbage, Chinese cabbage, broccoli, cauliflower and radish. The specificity of pollen-stigma interaction in self-incompatibility is controlled by multiple alleles of the *S* locus. More than 40 *S* alleles have been identified in *Brassica oleracea* (Ockendon 1974) as well as 30 *S* alleles in *Brassica campestris* (Nou et al. 1993). Since the stability of the self-incompatibility phenotype of some *S* alleles, and the dominance relationship between them, have both been characterized, the identification of *S* alleles in a breeding line is required for raising the efficiency of the selection of parental lines having stable self-incompatibility.

The *S* alleles have been arbitrarily numbered by plant geneticists. If one inbred line is compatible to all the other lines having defined *S* alleles, a new *S* number is provided for this line. Since each plant geneticist has a different series of *S* tester lines, the information on the characters of each *S* allele is of little use for breeders having different *S* tester lines.

Recently, the molecular biology of self-incompatibility has clarified S-glycoprotein, *S* locus-specific glyco-

protein, which was found as a candidate for a recognition molecule in the stigma (Nishio and Hinata 1982), and a gene encoding S-glycoprotein, *SLG*, was isolated (Nasrallah et al. 1985). A gene for the S receptor protein kinase, *SRK*, having an extracellular domain similar to *SLG* was also found at the *S* locus (Stein et al. 1991). Nucleotide sequences of *SLG* and *SRK* for several *S* alleles have been reported (Trick and Flavell 1989; Chen and Nasrallah 1990; Dwyer et al. 1991; Goring and Rothstein 1992; Watanabe et al. 1994; Yamakawa et al. 1994). For a better understanding of the self-incompatibility system, a worldwide standardization of the names of the *S* alleles is essential.

Identification of *S* alleles is time-consuming. Large numbers of test crossing are necessary. Because the self-incompatibility phenotype is influenced both by environmental factors and the physiological condition of plants, pollination tests need to be repeated several times. One reliable method for the identification of *S* alleles in *Brassica* is the electrophoretic analysis of S-glycoprotein (Hinata and Nishio 1981). Another method is Southern-hybridization analysis of plant genomic DNA using *SLG* DNA as a probe (Nasrallah et al. 1985). However, these methods require skillful biochemical techniques. Recently, a simple method for detecting *SLG* polymorphism has been developed (Brace et al. 1993, 1994; Nishio and Sakamoto 1993; Nishio et al. 1994). This method involves amplification of *SLG* DNA by PCR (polymerase chain reaction) with a pair of specific primers, and electrophoretic analysis of PCR products after cleavage with restriction endonucleases. The use of polyacrylamide-gel electrophoresis and restriction endonucleases which recognize tetranucleotide sequences has made it possible to detect higher levels of polymorphism (Nishio et al. 1994).

We have now improved primers for the specific amplification of *SLG*s, and found *S* alleles with class-II *SLG*s in *B. campestris*. By determining the nucleotide sequences of *SLG*s amplified with specific primers, we have calculated the expected sizes of DNA fragments cleaved with three restriction endonucleases. These sizes corresponded exactly with those estimated from the mobility of the DNA fragments in the polyacrylamide gel. In this paper, we provide details of the improved method of PCR-RFLP of *SLG*s and of the sizes of DNA fragments used as markers for registering *S* alleles in *B. campestris*.

Materials and methods

Materials

The forty-seven *S* homozygous lines, 27 *S* genotypes, in *B. campestris* L., shown in Table 1, were used as plant material. The S genotypes of these lines were named by Nou et al. (1993). All combinations of 15 F_2 progenies between S^{24} and S^{25} homozygotes were crossed, and the *S* genotypes of these plants were identified by observing pollen-tube growth in the stigmas 1 day after pollination.

PCR-RFLP analysis

Genomic DNA was prepared from young leaves according to Rogers and Bendich (1985). DNA fragments were amplified by PCR with

Table 1 *B. campestris* S-homozygous lines used as plant material

S allele	Lines
S^{21}	50-9T, 82-9T
S^{22}	51-6J, 53-10T
S^{24}	27-1T, 1-1J, S-12J
S^{25}	38-7J
S^{26}	2-1J
S^{27}	S-10J
S^{28}	11-6J, 12-1J, S-9J
S^{29}	7-5J
S^{30}	32-14T
S^{31}	42-5T
S^{32}	16-13T
S^{33}	20-2T
S^{34}	9-1J
S^{35}	6-12T, 7-12T, 46-12T, 51-7T, 52-7T, 87-2T, 33-5J
S^{36}	9-14J, 42-7J
S^{37}	45-9T
S^{38}	22-7J
S^{39}	23-3J, 51-5J
S^{40}	83-5T
S^{41}	7-6J
S^{43}	S-8J
S^{44}	12-2J, 36-8J, 39-9J
S^{45}	11-12J, 19-5J
S^{46}	1-6J
S^{48}	45-3T
S^{49}	82-3T
S^{99}	58-5J

Table 2 Primers used for amplification of *SLG*s by PCR

Primers	Nucleotide sequence	Source[a]
PS3	ATGAAAGGGGTACAGAACAT	*SLG2A*(1–20[b])
PS4	CTAACCTAGATCAGCAGCAT	*SLG2A*(1326–1307)
PS5	ATGAAAGGCGTAAGAAAAACCTA	*SLG*-8(1–23)
PS15	CCGTGTTTTATTTTAAGAGAAAGAGCT	*SLG*-6(1336–1310)
PS18	ATGAAAGGTGTACGAAACATCTA	*SLG*-9(1–23)
PS21	CTCAAGTCCCACTGCTGCGG	*SLG*-2A(1025–1006)
PSA[c]	AGAACACTTGTATCTCCCGGT	
PSB[c]	CAATCTGACATAAAGATCTTG	

[a] *SLG2A*: Chen and Nasrallah (1990), *SLG-8*: Dwyer et al. (1991) *SLG-6*: Nasrallah et al. (1987), *SLG-9*: Watanabe et al. (1994)

[b] Number from translation initiation sites

[c] Primers designed by Brace et al. (1993)

SLG-specific oligonucleotide primers, as shown in Table 2. Plant genomic DNA (2.5 µl, approximately 50 ng) was mixed with a pair of primers, 50 pmoles, 2.5 µl 10 × buffer (500 mM KCl, 100 mM Tris-HCl pH 8.3, 15 mM $MgCl_2$, 0.01% gelatin), 2 µl dNTP mixture (each 5 nmoles), 0.1 µl *Taq* DNA polymerase (Takara *Taq* 0.5 units), 17.9 µl distilled water to give a final volume of 25 µl. The PCR condition was 30 cycles of 1 min at 93 °C, 2 min at 55°C, and 3 min at 72 °C with a thermal cycler (Atto, Tokyo), expect for the combination of primers PS3 + PS4 and PSA + PSB where the annealing temperatures were 50 °C and 58 °C, respectively.

The PCR products were analyzed by agarose-gel electrophoresis with TBE buffer, and those containing only one DNA fragment of the expected size were digested with *Mbo*I, *Afa*I or *Msp*I. For digestion with *Mbo*I and *Afa*I, 2.5 µl of PCR product was mixed with 0.5 µl of 10 × buffer (750 mM KCl, 150 mM Tris-HCl pH 7.5, 92.5 µM $MgCl_2$, 10 mM dithiothreitol), 1 unit of the restriction endonuclease, and distilled water to give a final volume of 5 µl. In *Msp*I treatment, the 10 × buffer was replaced with a low-salt buffer (250 mM NaCl, 50 mM Tris-HCl pH 7.5, 92.5 mM $MgCl_2$, 10 mM dithiothreitol). The mixture was incubated at 37 °C for 2 h, and subjected to electrophoresis on a 5% polyacrylamide gel containing TBE buffer. After electrophroesis at 100 V for 1 h, the DNA fragments were visualized by silver staining (Daiichi Pure Chemicals, Tokyo).

The experiment was repeated three times using DNA samples prepared from three different seasons.

Southern-hybridization analysis

Methods for Southern-hybridization and the detection of DNA fragments were those described previously (Nishio et al. 1994). The DNA probes used in this analysis were cDNA clones of S^6 *SLG* (Nasrallah et al. 1985), S^5 *SLG* (Nasrallah unpublished), and S^{28} *SLG* in *B. campestris* (Watanabe et al. 1994). A cloned PCR product amplified from S^{29} genomic DNA with primers PS3 + PS21 was also used as a probe.

Cloning of PCR product and determination of nucleotide sequence

For the determination of nucleotide sequences, *SLG*s were amplified with *Pfu* DNA polymerase instead of *Taq* DNA polymerase. DNA fragments amplified with PS5 + PS15 or PS18 + PS15 were ligated with pUC18 using a 'Sure clone ligation kit'(Pharmacia). Competent cells of HB101 were transformed with the ligation mixture, and *SLG*-positive clones were selected by colony hybridization using cDNA of S^6 *SLG*. The plasmid having the insert of the expected size was isolated, and the nucleotide sequence of the insert DNA was determined with a DNA sequencer (Pharmacia). Restriction fragment sizes were deduced from the nucleotide sequences.

Results

A single approximately 1.3-kb DNA fragment was amplified from 15 of the 27 *S* genotypes by PCR with the class-I *SLG*-specific primers, PS5 + PS15. The nucleotide sequence of PS5, which is a revised primer of PS1 reported earlier (Nishio et al. 1994), was taken from the 5′ terminal region of the open reading frame of the S^8 *SLG* in *B. campestris* (Dwyer et al. 1991). PS15 is a revised primer of PS2(Nishio et al. 1994). The nucleotide sequence of PS15, which was taken from the 3′ untranslated region of the S^6 *SLG* in *B. oleracea*, was very similar to that of the S^8 *SLG* in *B. campestris* in the corresponding region, showing only one nucleotide difference at the point near the 5′ end. The S^8 (= S^{43} in Table 1) *SLG* DNA fragment amplified with these primers was expected to be 1333 bp. There was no detectable variation in the size of the amplified DNA fragments between the 15 *S* genotypes.

Digestion of the PCR products with *Mbo*I and subsequent polyacrylamide-gel electrophoresis revealed high polymorphism of the amplified DNA fragments (Fig. 1). All different *S* genotypes showed mutually differing electrophoretic profiles. Independent lines having the same *S* genotype showed identical profiles even between the lines collected in Turkey and in Japan. The apparent sizes of DNA fragments of the S^{43} homozygote cleaved with *Mbo*I were 400, 375, 268, 180, and 63 bp, which

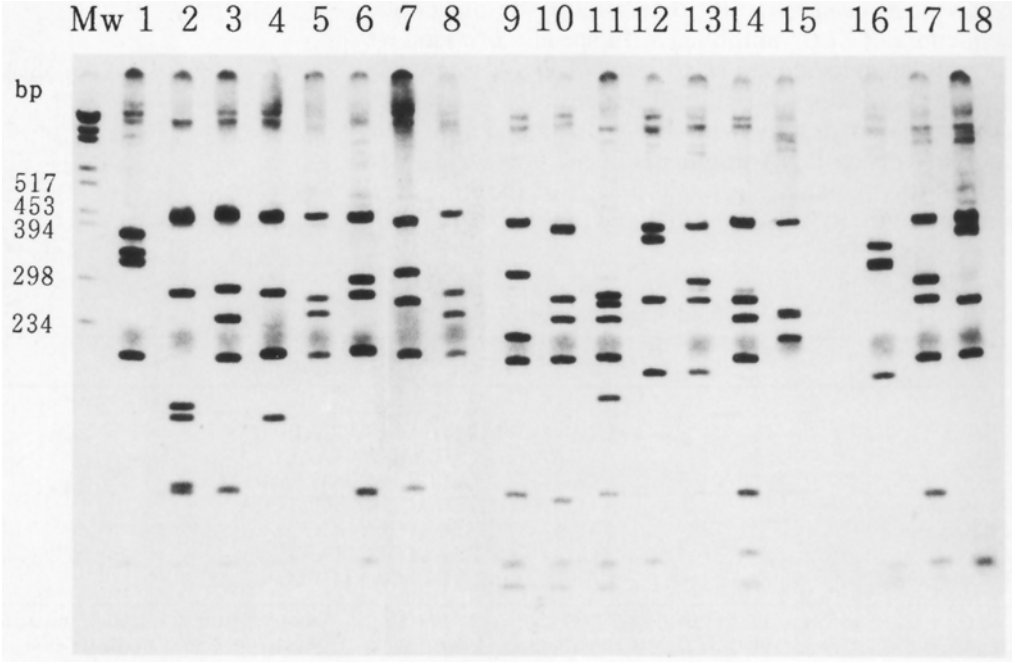
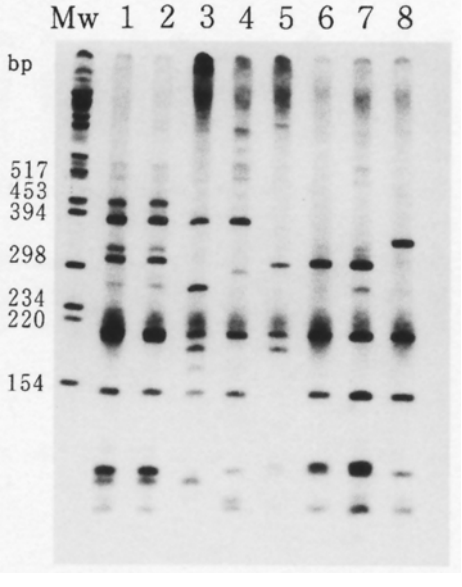


Fig. 1 Polyacrylamide-gel electrophoresis of PCR products after cleavage with *Mbo*I. *SLG* DNA was specifically amplified by PCR from the genomic DNA of *S* homozygotes in *B. campestris* with primers PS5 and PS15 (*lanes 1–16*) and with PS18 and PS15 (*lanes 17–19*). *Lane 1*, S^{21}; *2*, S^{24}; *3*, S^{25}; *4*, S^{26}; *5*, S^{27}; *6*, S^{30}; *7*, S^{34}; *8*, S^{35} (33-5J); *9*, S^{37}; *10*, S^{38}; *11*, S^{41}; *12*, S^{43}; *13*, S^{46}; *14*, S^{48}; *15*, S^{99}; *16*, S^{28}; *17*, S^{45}; *18*, S^{49};

coincided with the DNA fragment sizes expected from a published nucleotide sequence (Dwyer et al. 1991). Likewise, the fragment size of S^{24} corresponded with those expected from the published nucleotide sequence of the S^{12} ($=S^{24}$) *SLG* (Yamakawa et al. 1994). Digestion with *Afa*I or *Msp*I also showed high polymorphism of the PCR products. While *Afa*I fragments of S^{43} and S^{48} showed the same electrophoretic profile, *Msp*I digestion gave different electrophoretic profiles for all 15 *S* genotypes.

Since there are two types of *SLG* in *B. oleracea*, the primers specific to the class-II *SLG*s were used for PCR. Cass-II specific primers, PS3 and PS4, gave amplification of two 1.3-kb DNA fragments, one of which was inferred to be *SLR*2 (Boyes et al. 1991) from the cleaved fragment sizes. The other 1.3-kb DNA fragment was polymorphic and was detected only in the lines negative to class-I *SLG* primers, suggesting that this fragment is a class-II *SLG* (Nishio et al. 1994). Most of the *S* genotypes amplified the *SLR*-2 DNA having the same electrophoretic profile, but S^{40} did not show the bands of *SLR*2. S^{32} and S^{33} homozygotes also had a partially different PCR product profile from *SLR*2. The presence of *SLR*2 DNA fragments made it difficult to identify the polymorphism of class-II *SLG*s. Therefore, several class-II *SLG*-specific primers having a nucleotide sequence different from *SLR*2 at the 3′ end were tested for specific amplification of class-II *SLG*s. A combination of PS3 and PS21 resulted in the amplification of a single DNA fragment having the expected size, approximately 1.0 kb, in six *S* genotypes, S^{29}, S^{31}, S^{35}, S^{39}, S^{40}, and S^{44}. Polyacrylamide-gel electrophoresis after digestion with *Mbo*I or *Msp*I revealed polymorphism of the amplified DNA fragments, though S^{29} and S^{39} showed identical profiles to S^{31} and S^{40}, respectively (Fig. 2).

Fig. 2 *Mbo*I fragments of PCR products amplified from *S* homozygotes in *B. campestris* with primers PS3 and PS21. *Lane 1*, S^{29}; *2*, S^{31}; *3*, S^{35} (6-12T); *4*, S^{35} (51-7T); *5*, S^{35} (52-7T); *6*, S^{39}; *7*, S^{40}; *8*, S^{44}



Seven S genotypes did not amplify DNA fragments with either class-I-specific primers or class-II *SLG*-specific primers. Since the nucleotide sequence of S^9 ($=S^{28}$) *SLG* differs from that of PS5 at the 3′ end (Watanabe et al. 1994), an S^9 *SLG*-specific primer (PS18) was synthesized and used for PCR. A combination of PS18 and PS15 resulted in amplification of a 1.3-kb DNA in three *S* genotypes S^{28}, S^{45} and S^{49} homozygotes. These three *S* genotypes each showed different electrophoretic profiles of DNA fragments after cleavage with *Mbo*I or *Msp*I (Fig. 1). The apparent sizes of cleaved fragments of the S^{28} PCR product coincided with those expected from the nucleotide sequence of S^9 (Watanabe et al. 1994).

Beacuse of difficulty in the specific amplification of *SLG*s from genomic DNA of S^{22}, S^{32}, S^{33}, and S^{36}, we analyzed the PCR products amplified with a primer pair PSA + PSB, which was designed by Brace et al. (1993) for the amplification of *S*-related DNA fragments. These primers have a sequence similarity with *SLG*, *SRK*, *SLR*2 and *SLR*I (Lalonde et al. 1989) and, therefore, these four DNA fragments are expected to be amplified. The size of the amplified DNA fragment was 1150 bp, as might have been anticapted. However, electrophoretic profiles of *Mbo*I digests showed that some S-related DNA fragments were amplified and others were not. *SLR*2-specific fragments were detected in 14 *S* genotypes, but not in the other 13 *S* genotypes. The restriction fragments of *SLG*s were not found in some *S* genotypes. These unexpected results may be due to preferential amplification of some DNA fragments among *S*-related sequences. S^{22}, S^{32}, S^{33}, and S^{36} showed mutually differing electrophoretic profiles. Although there were minor variations of electrophoretic profiles among different lines having the same *S* genotype, the DNA band patterns can be used as markers for the identification of *S* alleles until specific primers for *SLG*s or *SRK*s of these *S* alleles are found.

S^{35} homozygotes gave a complicated result. One line, 33-5J, amplified a class I-like DNA fragment with PS5 + PS15, while 6-12T, 51-7T and 52-7T gave a class II-like DNA fragment with PS3 + PS21. These three DNA fragments amplified with PS3 + PS21 showed mutually different electrophoretic profiles after digestion with *Mbo*I. The other three lines, 7-12T, 46-12T and 87-2T, did not amplify DNA with either class I-specific or class II-specific primers. DNA fragments amplified with PSA + PSB from these seven lines of S^{35} homozygotes showed different *Mbo*I fragment patterns from each other. In our preliminary examination, the line collected in Japan, 33-5J, which has a class-I SLG, was partially compatible with other lines of the S^{35} homozygote collected in Turkey. Cross-compatibility between these S^{35} homozygotes should be re-examined.

Southern-hybridization analysis using a probe of S^6 *SLG* cDNA, a class-I *SLG*, detected the PCR products obtained with PS5 + PS15 (Fig. 3a). The DNA frag-

ments amplified with PS18 + PS15 also showed homology to S^6 *SLG* cDNA. S^{28} SLG cDNA of *B. campestris* used as a probe gave the same result as S^6 *SLG*, suggesting that PCR products of S^{28}, S^{45}, and S^{49} amplified with PS18 + PS15 are class-I *SLG*s like the DNA fragments amplified with PS5 + PS15. Those amplified with PS3 + PS21 showed a low density of the bands in this analysis. The S^5 *SLG*, a class-II *SLG* in *B. oleracea*, was used as a probe in Southern-hybridization analysis, and showed homology to the DNA fragments amplified with PS3 + PS21 from the genomic DNA of S^{29}, S^{31}, S^{35}, S^{39}, S^{40} and S^{44} homozygotes. The density of the bands was low in the DNA fragments amplified with the class I-specific primers PS5 + PS15 and PS18 + PS15 (Fig. 3b). The cloned S^{29} PCR product used as a probe showed the same result as the S^5 *SLG*.

Fifteen F_2 progenies of S^{24} and S^{25} homozygotes were analyzed by PCR-RFLP using the primers PS5 and PS15. The assigned *S* genotypes based on the electrophoretic profile coincided with those identified by pollen-tube behavior after diallele pollination (Fig. 4).

DNA fragments amplified from 15 *S* homozygotes with the class-I *SLG*-specific primers PS5 + PS15 and PS18 + PS15 were inserted in pUC18 and sequenced. Similarities of the nucleotide sequences of these DNA fragments to that of the S^6 *SLG* in *B. oleracea* ranged from 86% to 92%. The deduced amino-acid sequences showed the usual characteristics of S-glycoproteins. The similarity of the amino-acid sequences to S^6 *SLG* was especially high at the conserved regions of the *SLG*. They had in common a cysteine-rich region and the potential N-glycosylation sites of the S-glycoproteins. These results suggest that all these DNA fragments are *SLG*s. The details of the comparison of the nucleotide sequences will be published elsewhere

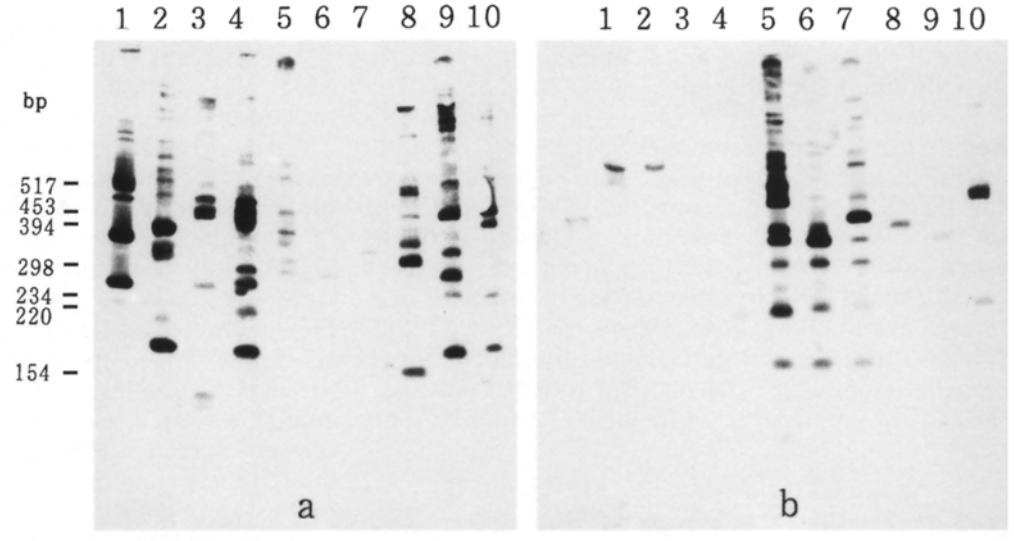


Fig. 3 Southern-hybridization analysis of PCR products using S^6 *SLG* cDNA (**a**) and S^5 *SLG* cDNA (**b**) as a probe. *Lane 1*, S^6 *SLG* cDNA; *2*, S^{21}; *3*, S^{24}; *4*, S^{25}, S^{29}; *6*, S^{40}; *7*, S^{44}; *8*, S^{28}; *9*, S^{45}; *10*, S^{49}. *Lanes 2–4* were DNA fragments amplified with PS5 + PS15, *lanes 5–7* with PS3 + PS21, and *lanes 8–10* with PS18 + PS15

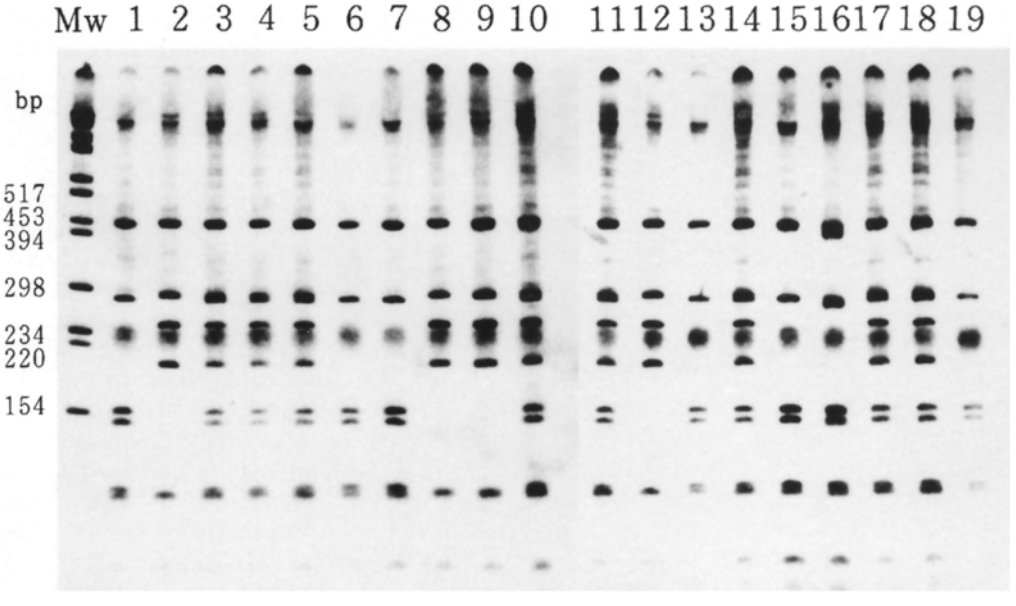


Fig. 4 *Mbo*I fragments of PCR products amplified from S^{24} and S^{25} homozygotes, their F_1 and F_2 plants, with primers PS5 + PS15. *Lane 1*, S^{24} homozygote; *2*, S^{25} homogygote; *3* and *4* F_1; *5–19*, F_2. Genotypes assigned by pollination experiment were as follows, *6, 7, 13, 15, 16, 19*: $S^{24}S^{24}$; *8, 9, 12*: $S^{25}S^{25}$; *5, 10, 11, 14, 17, 18*: S^{24} S^{25}

Table 3 Expected restriction fragment sizes of PCR products of class-I *SLG*s in *B. campestris*

S alleles	Expected restriction fragment sizes		
	*Mbo*I	*Afa*I	*Msp*I
S^{21}	384, 336, 318, 191, 64, 52	443, 375, 229, 163, 135	605, 233, 207, 161, 88, 32, 19
S^{24a}	429, 270, 148, 140, 98, 92, 64, 52, 43	375, 266, 223, 174, 163, 135	289, 275, 245, 200, 155, 107, 33, 32
S^{25}	429, 279, 238, 191, 92, 64, 52	507, 266, 223, 177, 172	809, 233, 155, 148
S^{26}	432, 270, 196, 191, 140, 64, 52	443, 375, 229, 163, 135	790, 200, 161, 88, 51, 33, 22
S^{27}	429, 261, 238, 191, 89, 64, 52, 12	375, 266, 220, 177, 166, 132	564, 245, 233, 152, 110, 32
S^{28a}	363, 330, 318, 176, 63, 52, 12	467, 266, 205, 200, 165, 11	797, 152, 149, 109, 75, 32
S^{30}	438, 284, 270, 191, 92, 64	375, 266, 217, 177, 172, 132	564, 245, 227, 155, 148
S^{34}	432, 296, 261, 191, 92, 64, 9	375, 266, 229, 177, 135, 82, 81	537, 275, 233, 161, 88, 32, 19
S^{35}	429, 270, 238, 191, 92, 64, 52	429, 375, 223, 177, 132	296, 268, 245, 233, 155, 107, 32
S^{37}	429, 296, 218, 191, 92, 64, 52	375, 266, 229, 177, 163, 132	564, 245, 233, 161, 88, 32, 19
S^{38}	405, 270, 238, 191, 89, 64, 52, 24	395, 375, 220, 163, 132, 48	809, 227, 158, 107, 32
S^{41}	270, 261, 238, 194, 159, 92, 64, 52, 12	248, 235, 223, 195, 166, 143, 132	564, 391, 213, 110, 32, 32
S^{43a}	426, 382, 270, 180, 64, 11	375, 266, 223, 174, 132, 82, 81	561, 233, 207, 155, 88, 38, 32, 19
S^{45}	432, 284, 261, 191, 92, 64, 12	375, 312, 266, 206, 166, 11	1194, 110, 32
S^{46}	426, 290, 270, 180, 92, 64, 11	375, 266, 223, 174, 132, 82, 81	447, 233, 207, 155, 114, 88, 38, 32, 19
S^{48}	432, 270, 238, 191, 92, 67, 52	569, 510, 208, 40, 15	275, 245, 234, 233, 155, 91, 58, 32, 19
S^{49}	432, 376, 254, 191, 64, 12, 7	375, 312, 266, 206, 166, 11	794, 245, 155, 110, 32
S^{99}	432, 244, 243, 218, 92, 64, 52	375, 312, 266, 229, 163	567, 245, 233, 161, 88, 51

[a] DNA fragment sizes of S^{24}, S^{28}, and S^{43} were estimated from the published nucleotide sequences in Yamakawa et al. (1994), Watanabe et al. (1994), and Dwyer et al. (1991), respectively

(Kusaba et al, in preparation). From these sequence data, expected DNA fragment sizes of *Mbo*I, *Afa*I, and *Msp*I digests were obtained (Table 3). They corresponded exactly with the sizes estimated from the electrophoretic mobility of DNA fragments in the polyacrylamide gel.

The partial nucleotide sequence of the PCR product amplified from the S^{29} homozygote with PS3 + PS21 was also determined. Five hundered base pairs from the initiation codon had a high similarity, 93.8%, to the S^2 *SLG* in *B. oleracea*, a class-II *SLG* (Chen and Nasrallah 1990). The similarity to the S^6 *SLG* in *B. oleracea*, a class-I *SLG* (Nasrallah et al. 1987), however, was the only 77.0%.

Discussion

From the following evidence, the DNA fragments amplified by PCR with the combination of primers PS5 + PS15 and PS18 + PS15 are considered to be class-I *SLG*s:

(1) The sizes of DNA fragments cleaved by restriction endonucleases coincided with those expected from the nucleotide sequences of *SLG*s in S^{24}, S^{28}, and S^{43}.

(2) Southern-hybridization analysis showed homology of the PCR products to the S^6 *SLG* cDNA clones from *B. oleracea*.

(3) Typing of the electrophoretic profiles of the cleaved DNA fragments co-segregated with the *S* alleles in the genetic analysis of F_2 progenies.

(4) The nucleotide sequences of the PCR products obtained from 15 *S* homozygotes had a high similarity to that of the S^6 *SLG*, and the deduced amino-acid sequences had the usual characteristics of S-glycoproteins.

(5) The 3′ untranslated region including PS15 is highly specific to *SLG*s in the *S* gene family (Nasrallah et al. 1988) and, therefore, *SRK* cannot be amplified with PS15.

DNA fragments amplified with PS3 + PS21 showed homology to S^5 *SLG* cDNA, a class-II *SLG*, in Southern-hybridization analysis, and the nucleotide sequence of the amplified S^{29} DNA had a high similarity to the S^2 *SLG* in *B. oleracea*. These fragments were amplified only from *S* genotypes which were negative to class-I *SLG*-specific primers except in the case of S^{35} homozygotes. They also showed polymorphism after digestion with a restriction endonuclease. These results suggest that these DNA fragments are a class-II *SLG* or *SRK*. In this investigation, the presence of class-I and class-II *S* alleles was demonstrated also in *B. campestris*. S^{29}, S^{31}, S^{39}, S^{40}, and S^{44} were class-II *S* alleles.

Although PSA and PSB, designed by Brace et al. (1993), gave amplification of *S*-related DNA fragments from all the *S* genotypes, it was difficult to determine whether or not the amplified DNA fragments were from the *S* locus. *SLG*, *SRK*, *SLR1*, and *SLR2* can be amplified simultaneously from *Brassica* genomic DNA with this primer pair, but in fact only some of them were amplified probably owing to preferential amplification. The primers designed in this work, PS5, PS15 and PS18, were highly specific and gave amplification of only *SLG*s. Therefore, more reliable results can be obtained with these primers for the identification of *S* alleles, though the *SLG*s of some *S* genotypes were not amplified by them. Another advantage of these primers over PSA + PSB is that *S* heterozygotes amplify both *SLG*s as shown in our previous paper (Nishio et al. 1994). In the present study, three sets of primers were separately used in PCR. Practically, however,

mixed primers consisting of PS5, PS18, PS15, PS3 and PS21 can be used. The mixed primers gave almost identical results in PCR-RFLP analysis as each primer pair, though traces of non-specific bands were detected in PCR products amplified with the mixed primers.

The sizes of DNA fragments which can be obtained by digestion of *SLG* PCR products with three restriction endonucleases, *Mbo*I, *Afa*I and *Msp*I, were shown to be markers for the identification of *S* alleles. These sizes are based on the nucleotide sequences of *SLG* PCR products and, therefore, there would be no experimental errors in their values. These data can be useful in inferring the *S* alleles of inbred lines in *B. campestris*. Since the number of *S* alleles in *B. campestris* was estimated to be about 100 (Nou et al. 1993), the possibility that different *S* alleles have *SLG*s giving the same electrophoretic profiles in PCR-RFLP with these three restriction endonucleases would be very small. We propose to list *S* alleles with the band sizes in PCR-RFLP, and/or the nucleotide sequences of *SLG* PCR products, for the purpose of exchange of data between plant geneticists regarding the characteristics of each *S* allele, and for the efficient use of *S* alleles in the breeding of cruciferous vegetables.

Acknowledgments We express our sincere thanks to Dr. J. B. Nasrallah for providing cDNA clones of S^6 and S^5 *SLG*s. This work was supported by a grant from the Agency of Science and Technology, Japan.

References

Boyes DC, Chen CH, Tantikanjana T, Esch JJ, Nasrallah JB (1991) Isolation of a second *S*-locus-related cDNA from *Brassica oleracea*: genetic relationships between the *S* locus and two related loci. Genetics 127:221–228

Brace J, Ockendon DJ, King GJ (1993) Development of a method for the identification of *S* alleles in *Brassica oleracea* based on digestion of PCR-amplified DNA with restriction endonucleases. Sex Plant Reprod 6:133–138

Brace J, King GJ, Ockendon DJ (1994) A molecular approach to the 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

Dwyer KG, Balent MA, Nasrallah JB, Nasrallah ME (1991) DNA sequences of self-incompatibility genes from *Brassica campestris* and *B. oleracea*: polymorphism predating speciation. Plant Mol Biol 16:481–486

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

Hinata K, Nishio T (1981) Con A-peroxidase method: an improved procedure for staining S-glycoproteins in cellulose-acetate electrofocusing in Crucifers. Theor Appl Genet 60:281–283

Lalonde BA, Nasrallah ME, Dwyer KG, Chen CH, Barlow B, Nasrallah JB (1989) A high conserved *Brassica* gene with homology to the *S*-locus-specific glycoprotein structural gene. Plant Cell 1:249–258

Nasrallah JB, Kao TH, Goldberg ML, Nasrallah ME (1985) A cDNA clone encoding an *S*-locus-specific glycoprotein from *Brassica oleracea*. Nature 318:263–267

Nasrallah JB, Kao TH, Chen CH, Goldberg ML, 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, Yu SM, Nasrallah ME (1988) The self-incompatibility genes of *Brassica oleracea*: expression, isolation and structure. Proc Natl Acad Sci USA 85:5551–5555

Nishio T, Hinata K (1982) Comparative studies on S-glycoproteins purified from different *S*-genotypes in self-incompatible *Brassica* species. I. Purification and chemical properties. Genetics 100:641–647

Nishio T, Sakamoto K (1993) PCR-RFLP of *S*-locus for the identification of breeding lines in *Brassica*. Jpn J Breed (Suppl 1):274

Nishio T, Sakamoto K, Yamaguchi J (1994) PCR-RFLP of *S* locus for identification of breeding lines in cruciferous vegetables. Plant Cell Rep 13:546–550

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 Brussel sprouts. Heredity 33:159–171

Rogers SO, Bendich AJ (1985) Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. Plant Mol Biol 5:69–76

Stein JC, Howlett B, Boyes DC, Nasrallah ME, Nasrallah JB (1991) Molecular cloning of a putative receptor protein kinase gene encoded at 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

Watanabe M, Takasaki T, Toriyama K, Yamakawa S, Isogai A, Suzuki A, Hinata K (1994) A high degree of homology exists between the protein encoded by *SLG* and the *S* receptor domain encoded by *SRK* in self-incompatible *Brassica campestris* L. Plant Cell Physiol 35:1221–1229

Yamakawa S, Shiba H, Watanabe M, Shiozawa H, Takayama S, Hinata K, Isogai A, Suzuki A (1994) The sequences of S-glycoproteins involved in self-incompatibility of *Brassica campestris* and their distribution among Brassicaceae. Biosci Biotech Biochem 58:921–925