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Identification of *S* haplotypes in *Brassica* by dot-blot analysis of *SP11* alleles

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Abstract A self-incompatibility system is used for F_1 hybrid breeding in Brassicaceae vegetables. The determinants of recognition specificity of self-incompatibility in *Brassica* are SRK in the stigma and SP11/SCR in the pollen. Nucleotide sequences of *SP11* alleles are more highly variable than those of *SRK*. We analyzed the *S* haplotype specificity of *SP11* DNA by Southern-blot analysis and dot-blot analysis using 16 *S* haplotypes in *Brassica oleracea*, and found that DNA fragments of a mature protein region of *SP11* cDNA, $SP11^m$, of eight *S* haplotypes can detect only the *SP11* alleles of the same *S* haplotypes. This specificity makes these methods useful for *S* haplotype identification. Therefore, we developed two methods of dot-blot analysis for *SP11*. One is dot blotting of DNA samples, i.e. plant genomic DNA probed with labeled $SP11^m$, and the other is dot blotting of $SP11^m$ DNA fragments probed with labeled DNA samples, i.e. the *SP11* coding region labeled by PCR using a template of plant genomic DNA. The former is useful for testing many plant materials. The latter is suitable, if there is no previous information on the *S* haplotypes of plant materials.

Keywords Brassicaceae vegetables · Dot-blot analysis · F_1 hybrid breeding · Seed purity test · Self-incompatibility · *SP11/SCR*

Introduction

Molecules participating in the self-recognition reaction between pollen and the stigma in self-incompatible *Brassica* species have been intensively studied, and the gene of the *S*-specific pollen protein, *SP11/SCR* (termed *SP11* hereafter), has recently been identified (Schopfer et al. 1999; Suzuki et al. 1999). The function of the *S*-locus receptor kinase (SRK) as a recognition molecule in the stigma has been demonstrated by plant transformation experiments (Takasaki et al. 2000; Silva et al. 2001). The genes for SP11 and SRK are closely linked to each other in the *S* locus, and the two alleles are transmitted to progeny as one set, which is referred to as the *S* haplotype. The distance between the *SP11* gene and the *SRK* gene, and the orientation of these genes in the *S* locus, are different between the *S* haplotypes (Schopfer et al. 1999; Takayama et al. 2000). SP11 binds SRK in an allele-specific manner (Kachroo et al. 2001; Takayama et al. 2001).

The self-incompatibility system has been successfully used for F_1 hybrid breeding in Brassicaceae vegetables, but the breeding methodology is still based on the classical genetics developed in the 1950s (Bateman 1955; Thompson 1957). Although dominant-recessive relationships between *S* haplotypes (Thompson and Taylor 1966; Hatakeyama et al. 1998) and the characteristics of some *S* haplotypes (Thompson 1972) have been elucidated, the *S* haplotypes of breeding lines are not necessarily identified in F_1 hybrid breeding. In order to use the information on *S* haplotypes effectively in practical breeding, the identification of *S* haplotypes is required.

Among several methods for *S* haplotype identification in *Brassica*, the simplest method is PCR-RFLP analysis of *SLG*, which is homologous and linked to *SRK* (Brace et al. 1993; Nishio et al. 1994). PCR-RFLP analysis of *SRK* alleles has also been found to be useful for *S* haplotype identification (Nishio et al. 1997). *S* haplotypes in many F_1 hybrid cultivars in cabbage, broccoli, Chinese cabbage, turnips and so on, have been identified by PCR-RFLP of *SLG* and *SRK* (Sakamoto et al. 2000; Sakamoto and Nishio 2001). However, some *S* haplotypes lack the *SLG* allele (Okazaki et al. 1999) and the DNA fragment amplified from some *SRK* alleles show the same band pattern in this analysis (Nishio et al. 1997). Furthermore, PCR-RFLP is not suitable for the analysis of many

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Table 1 Nucleotide sequences of the primers used for the amplification of *SP11*^{m} DNA fragments

S haplotypes	Primer sequences	
	Forward primers (5'–3')	Reverse primers(5'–3')
SP11^{m}*-7*	AATCTGATGAAGCAGTGC	CTATTTACAATCGCAAGA
SP11^{m}*-8*	AATCTCATGAACAACTGTACG	TTACTTACACAGTACACAGCG
SP11^{m}*-12*	AATCTGATGTATCCGTGCGACG	CCAGAATTTGTGCATTCGCA
SP11^{m}*-18*	TCTGATGAATCGGTGCATCG	GTTTACAACGACAATATCGCC
SP11^{m}*-25*	GACGAATCCGTGCATCTG	GTTGACAAGTACAAACGCCG
SP11^{m}*-32*	AATCTGATGAAGCGGTGCACCCG	CTAACACGATTTACAGTCACA
SP11^{m}*-39*	GGATGCATTAAGATTGCCTGG	CAATAACAATGTCCAACATG
SP11^{m}*-57*	GGATGAAGGGGTGTGACGGTCG	CAACATCGTCCAATTTTGTC

samples used in practical breeding. Another simpler method applicable to all *S* haplotypes is required.

Nucleotide sequences of *SP11* alleles are highly variable (Watanabe et al. 2000; Sato et al. 2002). We showed that a DNA fragment of the *SP11* allele hybridizes specifically to genomic DNA of the same allele, but not to the other genomic region in *Brassica*. Using this specificity of the *SP11* alleles, we developed a simple method for *S* haplotype identification in *Brassica*.

Materials and methods

Plant materials

Sixteen *S* homozygous lines in *Brassica oleracea* (S^{5}, S^{6}, S^{7}, S^{8}, S^{9}, S^{12}, S^{13}, S^{14}, S^{15}, S^{25}, S^{29}, S^{32}, S^{57}, S^{58}, S^{60} and S^{65}) and 16 F_2 plants between an S^{18} homozygote and an S^{39} homozygote were used as plant materials. *S* haplotypes of the 16 F_2 plants were identified by PCR-RFLP analysis of *SLG* according to Nishio et al. (1996).

Southern-blot analysis

Genomic DNA was extracted from young leaf tissue by the CTAB method (Murray and Thompson 1980). The genomic DNA (3 μg) digested with *Eco*RI was subjected to electrophoresis on a 1.0% agarose gel and transferred to a nylon membrane (Nytran, Germany). Digoxigenin-labeled DNA probes were prepared by PCR using *SP11* primers (SP11-2, 5'-TTCATATTCATCGTTT-CAAGTC-3' and RT-1, 5'-ACTGGAAGAATTCGCGGC-3') (Kimura et al. 2002) and *SP11* cDNA clones (Sato et al. 2002) as templates. After denaturation by a solution containing 0.5 N NaOH and 1.5 M NaCl, the membranes were hybridized with the probes at 68 °C. After hybridization, the membranes were washed twice in a solution containing 0.1% SSC and 0.1% SDS at 68 °C for 20 min.

Dot blotting of plant genomic DNA

Genomic DNA was spotted onto nylon membranes using two types of apparatus, BIO-DOT (BioLad, USA) and Multi-pin-blotter (Atto, Japan). Since the signal peptide region is conserved well in many *S* haplotypes, two kinds of DNA probes were prepared by PCR using the *SP11* cDNA clones of eight *S* haplotypes as templates. One was the full-length of *SP11* cDNA, named the *SP11* probe. The other was the mature protein region of *SP11* DNA, named the *SP11*^{m} probe. The membranes were hybridized with these probes at 68 °C. After hybridization, the membranes were washed twice in the 0.1% SSC, 0.1% SDS solution at 68 °C for 20 min.

Dot blotting of cloned SP11 DNA

DNA fragments of the *SP11*^{m} DNA of eight *S* haplotypes were spotted on a nylon membrane using the two types of apparatus. DNA probes were prepared by PCR using plant genomic DNA and *S* haplotype-specific primers (Table 1). The PCR condition involved 45 cycles, each consisting of 30 s of denaturation at 94 °C, 30 s of annealing at 50 °C and 10 s of extension at 72 °C. The membranes were hybridized at 68 °C. After hybridization, the membranes were washed twice in a solution containing 0.05% SSC and 0.1% SDS at 68 °C for 20 min.

Results

Investigation of *S*-haplotype specificity of the *SP11* probe

S-haplotype specificity of an *SP11* probe in Southern-blot analysis has been reported (Schopfer et al. 1999). In this analysis, the probe of *SP11-8* in *Brassica rapa* detected *SP11-13* in *B. oleracea*. Since *B. rapa* S^{8} is considered to have the same recognition specificity as *B. oleracea* S^{32} (Sato et al., submitted), detection of *SP11-13* in *B. oleracea* by the *SP11-8* probe indicates insufficient specificity of the *SP11* probe for use as an *S*-haplotype-specific probe. Therefore, in the present study, we assessed the *S* haplotype specificity of different alleles of *SP11* using a different washing condition with 0.1% SSC and 0.1% SDS at 68 °C. In the analysis of 16 *S* haplotypes in *B. oleracea* using the *SP11* probe of S^{57} (*SP11-57*), a single band was observed only in an S^{57} homozygote (Fig. 1). *S* haplotype-specific detection of a single band was also observed with the probes of *B. oleracea SP11-8* and *SP11-12* (data not shown), suggest-

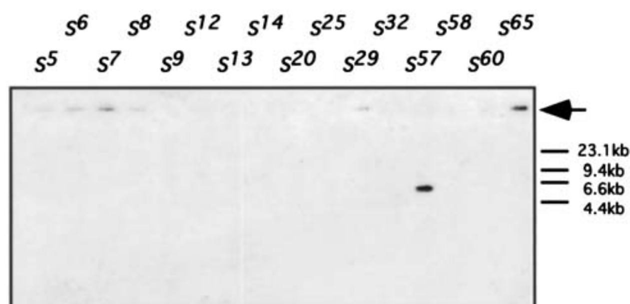

Fig. 1 Southern-blot analysis of genomic DNA of different *S* homozygotes in *B. oleracea* probed with *SP11*. *SP11-57* cDNA was used as a probe. The *arrow* indicates the position of the sample well

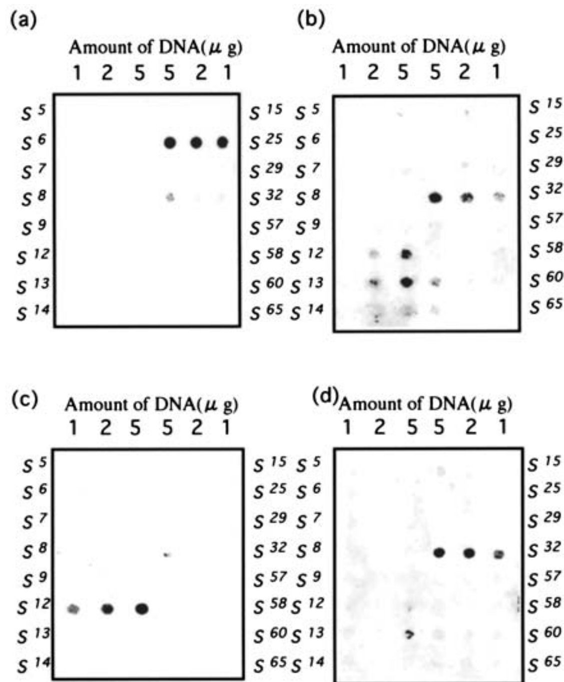

Fig. 2 Dot-blot analysis of genomic DNA of different *S* homozygotes probed with *SP11* and *SP11*^{m}. Plant genomic DNAs of 1, 2 and 5 μg were spotted on the membrane. (**a**) *SP11-25* probe, (**b**) *SP11-32* probe, (**c**) *SP11*^{m}*-12* probe, (**d**) *SP11*^{m}*-32* probe

ing that the *SP11* alleles in the genomic DNA can be detected by the *SP11* probes in an *S* haplotype-specific manner with the present washing condition.

In the dot-blot analysis with the *SP11* probe of S^{25} (*SP11-25*), a signal was observed only in an S^{25} homozygote (Fig. 2a). The *SP11* probes of S^{57} and S^{60} also detected a signal only in S^{57} and S^{60}, respectively (data not shown), suggesting a high specificity of the *SP11* probes. However, an *SP11-32* probe detected not only a signal of an S^{32} homozygote but also signals in 2- and 5-μg dots of S^{12} and S^{13} homozygotes with a little lower intensity (Fig. 2b). The *SP11* probes of other *S* haplotypes, S^{7}, S^{8}, S^{12} and S^{14}, also detected signals in different *S* haplotypes as well as the signal of the *SP11* alleles used as the probe (data not shown). These results indicate that the *SP11* probes can detect the *SP11* alleles that were used as the probe, but do not have sufficient specificity for use as *S* haplotype-specific probes for dot-blot analysis.

Since the signal peptide region of the *SP11* gene has higher similarity between different *SP11* alleles than the mature protein region, the *SP11*^{m} probe was prepared by removing the signal peptide region. In dot-blot analysis using the *SP11*^{m} probe produced from the S^{12} haplotypes (*SP11*^{m}*-12*), clear signals were detected in only the S^{12} homozygote, although a faint signal was observed in a 5-μg dot of S^{32} (Fig. 2c). The *SP11*^{m} probe produced from the S^{32} haplotype (*SP11*^{m}*-32*) detected clear signals in only the S^{32} homozygote with a faint signal in a 5-μg dot of S^{13} (Fig. 2d). The *SP11*^{m} probes of S^{7}, S^{18} and S^{39} also

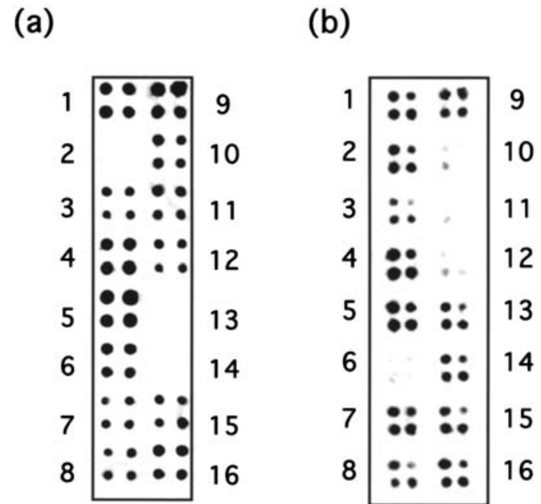

Fig. 3 Dot-blot analysis of genomic DNA in F_2 progeny of S^{18} and S^{39} homozygotes probed with *SP11*^{m}. (**a**) *SP11*^{m}*-18* probe. (**b**) *SP11*^{m}*-39* probe

detected clear signals only in the *S* haplotypes of the probes (data not shown), suggesting that the *SP11*^{m} probes can be used as *S* haplotype-specific probes for dot-blot analysis.

Identification of *S* haplotypes by dot-blot analysis

The dot-blot analysis using the *SP11*^{m} probe was applied to the identification of *S* haplotypes of seedlings. Sixteen F_2 progeny between S^{18} and S^{39} were analyzed. Signals were detected in 12 plants by the *SP11*^{m}*-18* probe and in 13 plants by the *SP11*^{m}*-39* probe. Nine plants were positive to both probes (Fig. 3). This analysis indicates that the three plants negative to the *SP11*^{m}*-39* probe are S^{18} homozygotes, the four plants negative to the *SP11*^{m}*-18* probe are S^{39} homozygotes, and the other plants are the heterozygotes of S^{18} and S^{39}. These *S* genotypes assigned by this analysis corresponded to those by the PCR-RFLP analysis of the *SLG* alleles.

DNA fragments of *SP11*^{m} amplified from eight *SP11* cDNA clones (S^{7}, S^{8}, S^{12}, S^{18}, S^{25}, S^{32}, S^{39} and S^{57}) were spotted onto a nylon membrane and hybridized with a probe labeled by PCR with the mixed primers using plant genomic DNA as a template. A signal was observed in only *SP11*^{m}*-7*, when the genomic DNA of the S^{7} homozygote was used as a template for the probe. A probe of the $S^{18}S^{39}$ heterozygote detected signals in *SP11*^{m}*-18* and *SP11*^{m}*-39* (Fig. 4). The same membrane probed with the labeled DNA samples from S^{8}, S^{12}, S^{25}, S^{32} and S^{57} homozygotes also showed signals in an *S* haplotype-specific manner (data not shown). These results suggest that two different methods of dot-blot analysis, i.e. dot blotting of DNA samples probed with labeled *SP11*^{m} DNA and dot blotting of DNA fragments of *SP11*^{m} probed with labeled DNA samples, can be used for *S* haplotype identification in *Brassica*.

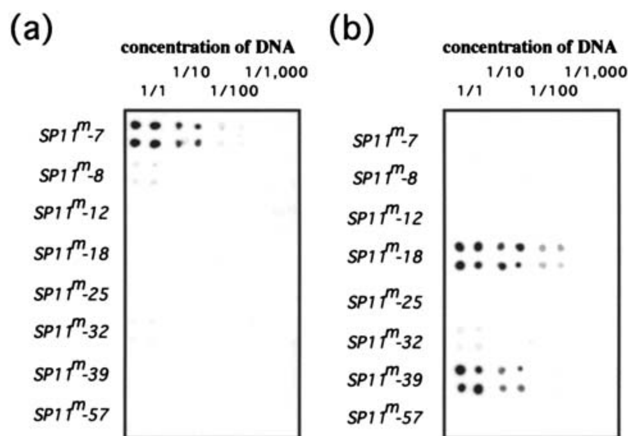

Fig. 4a, b Dot-blot analysis of *SP11*ᵐ DNA probed with labeled DNA samples. *SP11ᵐ* DNA fragments amplified by PCR were diluted to 1/1, 1/10, 1/100 and 1/1,000, and spotted on a membrane. (**a**) A DNA sample of an S^7 homozygote labeled with PCR using *SP11ᵐ*-specific primers was employed as a probe. (**b**) A DNA sample of an $S^{18}S^{39}$ heterozygote labeled with PCR using *SP11ᵐ*-specific primers was employed as a probe

Discussion

Nucleotide sequence homology between the *SP11* alleles of the *S* haplotypes used in the present study is 60.6% on the average, ranging from 45.0% to 80.8%. *SLG* and the S domain of *SRK* of these *S* haplotypes have higher sequence homology between alleles, 85.9% and 82.8% on the average, ranging from 61.2% to 93.7% and from 56.9% to 91.3%, respectively. *SLG* probes can detect *SLG* and *SRK* of different *S* haplotypes of the same class, class I or class II, in genomic Southern-blot analysis (Okazaki et al. 1999). On the other hand, the *SP11* probe detected only the same *SP11* allele in the genomic Southern-blot analysis in the present washing condition. Dot-blot analysis of genomic DNA probed with the *SP11* cDNA gave positive signals for the same *SP11* allele, but with a slightly higher background. Nucleotide sequence homology in the mature protein region, *SP11ᵐ*, between different *S* haplotypes is 53.0% on the average, ranging from 44.4% to 76.3%. The *SP11ᵐ* DNA fragment was found to be a better probe for the *S* haplotype-specific detection of *SP11* in the dot-blot analysis of genomic DNA.

We developed two methods of dot-blot analysis for *SP11*. One is dot blotting of DNA samples probed with labeled *SP11ᵐ*, and the other is dot blotting of the *SP11ᵐ* DNA fragments probed with labeled DNA samples. Since there are many *S* haplotypes in a species, for example 50 in *B. oleracea*, the two methods can be applied to two different cases. If some information on *S* haplotypes of plant materials is available, the dot blotting of DNA samples probed with labeled *SP11ᵐ* is useful for testing of many plant materials. If there is no information on *S* haplotypes, this method may require hybridization with 50 probes. In this case, the dot blotting of the *SP11ᵐ* DNA fragments probed with labeled DNA samples is applicable. However, this method is not suitable for the analysis of many plant materials.

The PCR-RFLP analyses of the *SLG* and *SRK* alleles have been successfully used for seed-purity testing of F_1 hybrid cultivars (Sakamoto et al. 2000). Because of the applicability of the dot-blot analysis to many samples, the method developed in the present study can be used instead of PCR-RFLP. With the blotting apparatus used in this study, 384 or 882 DNA samples can be spotted on a membrane. Since two replications are necessary to avoid false-positive signals, 192 or 441 plants can be analyzed. PCR and electrophoretic analysis of 192 samples are labor intensive and costly.

The dot blotting of the *SP11ᵐ* DNA can be developed for micro-array analysis. However, micro-array analysis requires special equipment for the detection of signals and is costly. There are as many as 100 *S* haplotypes in a species. Therefore, dot-blot analysis is appropriate. Use of glass, or other materials instead of a nylon membrane, may reduce the time required for hybridization and detection.

In the present study, applicability of dot-blot analysis of *SP11* alleles to *S* haplotype identification was demonstrated using 16 *S* haplotypes in *B. oleracea*. However, there may still be the possibility that there are different *S* haplotypes having highly similar *SP11* alleles. For the establishment of dot-blot analysis as a method for *S* haplotype identification, complete collections of *S* haplotypes and nucleotide sequence data of the *SP11* alleles are necessary. Almost all the *S* haplotypes in *B. oleracea*, namely 50 haplotypes, have been collected and identified by Ockendon (2000), and the *SP11* sequences have been determined in about half of them, i.e. 24 *SP11* alleles (Sato et al. 2002; Shiba et al. 2002). In *B. rapa*, the number of *S* haplotypes in a species has been estimated to be 100 (Nou et al. 1993), but only 36 *S* haplotypes have been collected and identified (Nou et al. 1993; Sakamoto and Nishio 2001) with 23 *SP11* sequences (Watanabe et al. 2000; Shiba et al. 2002). In *Raphanus sativus*, 18 *S* haplotypes have been identified (Sakamoto et al. 1998), but there is no published *SP11* sequence. We are now trying to complete the collection of the *S* haplotypes of these three important vegetable species. Since the *S* haplotypes can be easily lost by genetic drift, and because a new *S* haplotype cannot be generated by a simple mutation event (Kimura et al. 2002), urgent collection is necessary for the maintenance of the genetic resources of the Brassicaceae. The present method will make it easier to collect a large number of *S* haplotypes without confusion of naming the *S* haplotypes.

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