Duplicated *SP11* genes produce alternative transcripts in the *S*¹⁵ haplotype of *Brassica oleracea*

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Self-incompatibility (SI) discriminating self and non-self pollen is regulated by *S*-locus genes in *Brassica*. In most *S* haplotypes, a set of three highly polymorphic genes, *SLG*, *SRK*, and *SP11*, is located at the *S*-locus region. In the present study, we found duplicated *SP11* genes, *S*¹⁵-*SP11a*, *S*¹⁵-*SP11b*, and *S*¹⁵-*SP11b'*, in the self-incompatible *S*¹⁵ haplotype of *B. oleracea*. RNA gel blot and reverse transcription polymerase chain reaction (RT-PCR) analyses showed that two different sizes of *SP11* transcripts were specifically detected in anther tissues: a 0.65-kb transcript corresponded to *S*¹⁵-*SP11a* (an exon-1 region of *S*¹⁵-*SP11b* was also co-transcribed in some cases), and a 1.4-kb transcript contained the duplicated three genes, *S*¹⁵-*SP11a*, *S*¹⁵-*SP11b*, and an exon-1 region of *S*¹⁵-*SP11b'*, all three of which were connected to intergenic spacer regions.

Key words: alternative transcript, *Brassica oleracea*, gene duplication, self-incompatibility (SI), *S* locus protein 11 (*SP11*)

INTRODUCTION

Many flowering plants have mechanisms to avoid self-fertilization. One such sophisticated mechanism is self-incompatibility (SI), which is defined as the inability of a fertile hermaphrodite plant to produce zygotes after self-pollination. In the *Brassica* SI system, self-pollen is rejected at the surface of the papilla cells due to recognition of self and non-self pollen. This highly regulated system is sporophytically controlled by a single *S* locus with multiple alleles (Bateman 1955). At the *Brassica* *S*-locus region, three highly polymorphic genes, *SRK* (a gene for *S* receptor kinase), *SLG* (a gene for *S* locus glycoprotein), and *SP11/SCR* (a gene for *S* locus protein 11 or *S* locus cysteine-rich protein), are located (Suzuki et al. 1999; Takayama et al. 2000). The *SLG* gene encodes a secreted glycoprotein (Takayama et al. 1987). The *SRK* gene encodes a transmembrane receptor-like kinase, which consists of an extracellular SLG-like domain (*S*

domain), a transmembrane domain, and a cytoplasmic kinase domain (Stein et al. 1991). Both *SLG* and *SRK* are specifically expressed in stigma tissues (Stein et al. 1991; Watanabe et al. 1994). Based on a gain-of-function experiment, it has been demonstrated that *SRK* is a female determinant of the SI recognition (Takasaki et al. 2000). For *SLG*'s function, it has been reported that *SLG* enhanced the SI reaction in *SRK*⁹-introduced transgenic *Brassica* (Takasaki et al. 2000), and co-expression of *SLG* stabilized *SRK* in transgenic plants (Dixit et al. 2000). *SP11/SCR* encodes a small cysteine-rich protein (Schopfer et al. 1999; Suzuki et al. 1999), and was specifically expressed in anther tapetum and immature pollen (Takayama et al. 2000; Shiba et al. 2001; Iwano et al. 2002). From gain-of-function and bioassay experiments, it has been demonstrated that *SP11/SCR* is a male determinant of the SI recognition (Schopfer et al. 1999; Takayama et al. 2000; Shiba et al. 2001). In addition, recent studies showed that *SP11/SCR* was released from a self-pollen coat, and could bind *SRK* on the stigma surface to activate its kinase in an allele-specific manner (Kachroo et al. 2001; Takayama et al. 2001; Iwano et al. 2002).

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To date, more than 50 *S* haplotypes have been identified in *Brassica* species (Nou et al. 1993; Ockendon 2000; Ruffio-Chable and Gaudé 2001). Based on the sequence diversity of *SI* genes, *S* haplotypes are classified into class I and class II; the amino acid sequence similarity of *SLG* and *SRK* is almost 65% between classes and 80–90% within classes (Watanabe et al. 2001; Watanabe et al. 2003). This classification has also been applied to highly polymorphic *SP11/SCR*, because *SP11/SCR* seems to have co-evolved with *SLG* and *SRK* (Watanabe et al. 2000, Shiba et al. 2002). As for the *SI* phenotype, class-I *S* haplotypes are generally dominant over class-II *S* haplotypes on the pollen side (Thomson and Taylor 1966; Hatakeyama et al. 1998b). In general, class-I *S* haplotypes exhibit a strong *SI* phenotype, and class-II *S* haplotypes show a weak or leaky *SI* phenotype (Nasrallah and Nasrallah 1993, Cabrillac et al. 1999).

In *B. oleracea*, three class-II *S* haplotypes (S^2 , S^5 , and S^{15}) were identified (Thompson and Taylor 1966), and two different forms of *SLG* (*SLGA* and *SLGB*) were associated with these class-II *S* haplotypes (Cabrillac et al. 1999; Miede et al. 2001). *SLGA* encodes both soluble and membrane-anchored forms (Tantikanjana et al. 1993; Cabrillac et al. 1999). *SLGB* encodes a soluble form (Cabrillac et al. 1999), which has also been characterized in class-II *S* haplotypes of *B. rapa* (Hatakeyama et al. 1998a). It has been reported that two groups of the S^2 haplotypes could be distinguished depending on whether *SRK* was associated with *SLGA* or *SLGB* (Miede et al. 2001), and that S^5 haplotype carried only *SLGB* (Cabrillac et al. 1999). It is noteworthy that both *SLGA* and *SLGB* were identified in the self-compatible *B. oleracea* line, P57Sc, carrying the S^{15} haplotype, and was thought to have evolved via a duplication event (Gaudé et al. 1993; Cabrillac et al. 1999). Although *SLG* is not necessary to recognize self-pollen in the *SI* system (Okazaki et al. 1999; Takasaki et al. 2000; T Suzuki et al. 2000; G Suzuki et al. 2003), the duplication which occurred in the S^{15} haplotype is interesting from the viewpoint of the evolutionary history of the *SI* genes and its weak *SI* phenotype. In this study, we characterized the genomic organization and transcription of the *SP11* gene in the self-incompatible S^{15} haplotype of *B. oleracea*, and found that duplicated S^{15} -*SP11* genes produced a long alternative transcript containing three *SP11* sequences.

MATERIALS AND METHODS

Plant materials and pollination test. *B. oleracea* S^{15} homozygote, which was classified as a class-II *S* haplotype, was used. The self-incompatible line of S^{15} was derived from inbred line #102 (NongWoo Seed Co., Seoul, Korea) and inbred line TCS15-S (Takii Seed Co. Ltd, Kyoto, Japan).

Cloning of S^{15} -*SP11*. Total DNA was extracted from young leaf tissues of *B. oleracea* according to Park et al. (2002). To determine the genomic structure of *SP11s*, a set of polymerase chain reaction (PCR) primers (5'-GCGAAAATCTTATATACTCATAAG-3' and 5'-TTCGT-TGATCAATTATGATT-3') designated from the 5'- and 3'-ends of the coding region of S^{60} -*SP11*, respectively, was used (Shiba et al. 2002). In the case of the determination of the 5'-upstream region of *SP11*, another set of PCR primers (C2SP11pro-2F: 5'-CGCAGGAAAAGAAGAAT-TGGAT-3', C2SP11pro-2R: 5'-CAGGGCATTTCGAAGCT-CCCAC-3') was used. The 3'-end of the *SP11* was determined by 3'-rapid amplification of the cDNA ends (RACE) method (Shiba et al. 2002). To analyze the transcript of *SP11*, reverse transcription (RT)-PCR and 5'-RACE were performed. In the RT-PCR, a set of primers (S15-SP11F: 5'-ACTAGATGTGGGAGCTTGGAATG-3', S15-SP11R: 5'-CTCGAGTTCGTTGATCAATTATGATT-3'), which corresponded to the 2nd exon and 3'-untranslated region (UTR), respectively, were used. PCR condition and 5'-RACE were done according to Shiba et al. (2002). The amplified fragments were subcloned into the vector pGEM-T Easy (Promega, Madison, USA), and sequenced by a dideoxy-chain termination method (Watanabe et al. 2000). Sequence data were analyzed with GENETYX (Software Development, Tokyo, Japan) and DNASIS (Hitachi Software, Tokyo, Japan).

RNA gel blot analysis. Isolation of total RNA, electrophoresis of denatured RNA samples and blotting to a nylon membrane (Hybond N⁺; Amersham-Pharmacia) were performed as described by Shiba et al. (2002). Five µg of total RNA was loaded in each lane. 32 P-labeled S^{15} -*SP11a* probe was prepared as described above. After hybridization of each probe, membranes were washed in 0.1x SSPE, 0.1% SDS at 65°C for 30 min, and exposed on X-ray film. Equal loading of total RNA was assessed by ethidium bromide staining of rRNA bands.

RESULTS AND DISCUSSION

Identification and characterization of duplicated *SP11* genes in the S^{15} haplotype. Two kinds of genomic fragments (0.4 and 0.9 kb in length) containing S^{15} -*SP11* were amplified from genomic DNA of the *B. oleracea* S^{15} homozygote by polymerase chain reaction (PCR) with a set of primers, which could specifically amplify class-II *SP11* genes (Shiba et al. 2002). We cloned and sequenced the two fragments, and identified that the 0.4-kb fragment contained one *SP11* gene (S^{15} -*SP11a*), and the 0.9-kb fragment contained two *SP11* genes (S^{15} -*SP11a* and S^{15} -*SP11b*). The two amplified fragments were derived from the same genomic region, because they contained the same S^{15} -*SP11a* sequences. Thus, we found that two *SP11* genes, S^{15} -*SP11a* and S^{15} -*SP11b*, occurred

in tandem with the same orientation at the *S*-locus region of the *S*¹⁵ haplotype (Fig. 1). From the comparison of the nucleotide sequence of *S*¹⁵-*SP11a* with that of *S*¹⁵-*SP11b*, the sequence identities of the regions corresponding to exon 1, intron 1, and exon 2 were 65.2%, 100%, and 98.5%, respectively. The low similarity observed in exon 1 was due to the deletion in *S*¹⁵-*SP11b*, but not sequence diversity between *S*¹⁵-*SP11a* and *S*¹⁵-*SP11b*, as described below. The N-terminal hydrophobic amino acid sequences of *S*¹⁵-*SP11a* and *S*¹⁵-*SP11b* should be corresponded to their putative signal peptides. Eight cysteine residues, which were characteristics of other *SP11*s, were also conserved in *S*¹⁵-*SP11a* and *S*¹⁵-*SP11b* (Fig. 1). Furthermore, the 5'-upstream region of *S*¹⁵-*SP11a* was amplified

and sequenced; a putative TATA-box element and sequences similar to the 5'-promoter region of class-II *S*⁶⁰-*SP11* were identified (Shiba et al. 2002, Fig. 1, Fig. 2), suggesting that *S*¹⁵-*SP11a* was normally transcribed in anthers. A 239-bp intergenic region, separating *S*¹⁵-*SP11a* and *S*¹⁵-*SP11b*, did not show significant homology to the 5'-promoter regions of *S*¹⁵-*SP11a* and other class-II *SP11*s, indicating that this intergenic region did not contain the promoter elements for *S*¹⁵-*SP11b*.

Expression of *SP11* in the *S*¹⁵ haplotype. In order to analyze transcription of the duplicated *SP11* genes in the *S*¹⁵ haplotype, RNA gel blot with an *S*¹⁵-*SP11a* probe was performed. Two kinds of *SP11* transcripts (1.4 kb

Fig. 1. Nucleotide sequence and deduced amino acid sequence of the *SP11* genes in the *S*¹⁵ haplotype of *B. oleracea*. The coding regions are shown in uppercase letters. The non-coding regions are shown in lowercase letters. Nucleotides are numbered from the transcription start site (a in boldface), which was determined in a 5'-RACE experiment. A putative TATA box is denoted in underlined boldface letters. Direct AACATC repeats observed at the position of insertion is denoted in underlined italic letters. The enclosed C with square is conserved cysteine residue in other *SP11*s. The deduced signal peptide of *SP11* is denoted in underlined boldface letters. The nucleotide sequence of *S*¹⁵-*SP11* has been deposited in GenBank, EMBL, and DDBJ databases (accession no. AB176545).

S15-SP11a (B.o.)	CGCAGGAAAAAGCAAGAAATTGGATGAGGAAGGT <u>ACACACA</u> CGAATAAGGTGTGACCCGA	-81
S60-SP11 (B.r.)	CGCAGG-AAAAG-AAG-AATTGGATGAGGAAGTT <u>ACACACA</u> CGAATAAGGTGTGACCCGA	-86
S15-SP11a (B.o.)	<u>ATTG</u> TTTACGTGTAATAATAGGCAATTATGTGCTAGATGCG-----AAGAAAAACA <u>TATAT</u>	-26
S60-SP11 (B.r.)	<u>ATTG</u> TTTACGTGTAATAATAGGCAATTAAGTGCAAGATGTGAAGGAAAAAATA <u>TATAT</u>	-26
S15-SP11a (B.o.)	<u>ATA</u> GTAATACAAACAATAACATTCT	-1
S60-SP11 (B.r.)	<u>ATA</u> GTAATCCAAGCAATACCATTCT	-1

Fig. 2. The 5'-promoter region of S^{15} -SP11a is significantly similar to that of class-II S^{60} -SP11. Nucleotide sequences corresponding to 5'-upstream regions of S^{15} -SP11a (class II) of *B. oleracea* and S^{60} -SP11 (class II) of *B. rapa* are aligned. Nucleotides are numbered from the transcription start site. A putative TATA box and inverted sequence homologs of the CAAT motif and the *LAT52/56* box are denoted in underlined boldface letters. B.o., *Brassica oleracea*; B.r., *Brassica rapa*.

and 0.65 kb in length) were specifically expressed in anther tissues, and were developmentally up-regulated from 3 to 6 days before anthesis (Fig. 3). The amount of the 0.65-kb transcript was significantly higher than that of the 1.4-kb transcript.

Furthermore, we characterized the SP11 transcripts by sequencing analysis of reverse transcription (RT)-PCR and 5'-rapid amplification of the cDNA ends (5'-RACE) products. The amplified SP11 transcripts were classified into two groups based on the length of the products. In the first group of the ~0.65-kb transcript, only an S^{15} -SP11a region was transcribed, and the intron 1 was spliced out (Fig. 4). The length of the 3'-untran-

srated region (UTR) sequence varied among the cDNA clones; interestingly, the exon-1 region of S^{15} -SP11b, encoding the signal peptide, was also co-transcribed in some cases (Fig. 4). These ~0.65-kb S^{15} -SP11a transcripts might have been mainly transcribed, as shown in the RNA gel blot analysis (Fig. 3). In fact, the cDNA sequence identical to S^{15} -SP11a has been reported as *BoSP11-15* (Sato et al. 2003). In the second group of the 1.4-kb transcript, both full-length S^{15} -SP11a and S^{15} -SP11b were co-transcribed, and their intron-1 regions were spliced out (Fig. 4). The 1.4-kb transcripts contained the region corresponding to the exon-1 region of another SP11 as the 3'-UTR sequence which was also the

Fig. 3. Two types of SP11 transcripts observed in the S^{15} haplotype. RNA gel blot analysis of S^{15} -SP11 in an SI line of the S^{15} homozygote of *B. oleracea*. The total RNAs of anther (A), stigma (S), and leaf (L) were used. Developmental stages of the anther were classified as follows: stage 2, 6–7 days before anthesis; stage 3, 5–6 days before anthesis; stage 4, 4–5 days before anthesis; stage 5, 3–4 days before anthesis; stage 6, 3 days before anthesis. The bottom gel of each blot shows ethidium bromide (EtBr)-stained rRNA bands.

Fig. 4. Schematic representation of the S^{15} -SP11 transcripts observed in sequencing analysis of RT-PCR and 5'-RACE products. Filled and open boxes represent exon-1 (without 5'-UTR) and exon-2 (without 3'-UTR) regions, respectively. Arrows indicate direction of transcription of the SP11 genes.

case of the ~0.65-kb transcripts (Fig. 4), suggesting that the third *SP11* gene (*S*¹⁵-*SP11b'*) was located 182-bp downstream of *S*¹⁵-*SP11b* with the same orientation (Fig. 1). The exon 1 and a part of the intron 1 of *S*¹⁵-*SP11b'* observed in the 1.4-kb transcript were completely identical to those of *S*¹⁵-*SP11b*, suggesting that a duplication event occurred to produce *S*¹⁵-*SP11b* and *S*¹⁵-*SP11b'* (described below). The existence of the full-length *S*¹⁵-*SP11b'* in the *S*¹⁵ genome was not confirmed in this study.

Transcripts containing only *S*¹⁵-*SP11b* could not be observed in our RT-PCR and 5'-RACE analyses. This may have been due to the lack of promoter elements in the intergenic region between *S*¹⁵-*SP11a* and *S*¹⁵-*SP11b* as described above. The *S*¹⁵-*SP11b* might have been a pseudogene whose transcript is only transcribed as the 1.4-kb mRNA with *S*¹⁵-*SP11a*. Similarly, *S*¹⁵-*SP11b'* might also have been a pseudogene, because there were no promoter elements in the intergenic region between *S*¹⁵-*SP11b* and *S*¹⁵-*SP11b'*. Thus, we concluded that the SI response in the *S*¹⁵ haplotype was regulated by the *SRK*¹⁵ and *S*¹⁵-*SP11a*.

Duplication and/or conversion occurring in the *S*¹⁵-locus region. Sequence similarity within the *S*¹⁵-*SP11* region was analyzed by Harr plot (Fig. 5). The plot clearly showed the long stretch of similar sequences within the *S*¹⁵-*SP11* region, suggesting that gene duplication events occurred in the *S*¹⁵ haplotype. This finding is quite interesting because duplicated *SLGs* were observed in a self-compatible *S*¹⁵ line (Cabrillac et al. 1999). The 611-bp region encompassing from the exon-1 of *S*¹⁵-*SP11a* to the intron-1 of *S*¹⁵-*SP11b* was quite similar to the 554-bp region encompassing from the exon-1 of *S*¹⁵-*SP11b* to the intron-1 of *S*¹⁵-*SP11b'*; only three nucleotide differences were observed between the two sequences except for a 57-bp insertion associated with direct AACATC repeats in the 611-bp region (Fig. 1). In the *S*¹⁵ haplotype, a 502-bp region of 100% sequence identity is shared by *SRK*¹⁵ and *SLGA*¹⁵ (Cabrillac et al. 1999), suggesting that the two genes have been involved in a gene conversion event. Similarly, the quite similar ca. 600-bp sequences observed in the *SP11* region might have been produced by recent duplication of the *SP11* genes or gene conversion of the duplicated *SP11* genes, as discussed by Suzuki et al. (1997) and Cabrillac et al. (1999). These evidences indicated that the gene duplication and/or conversion events have frequently occurred in the *S*¹⁵ haplotype. The duplicated *SP11* genes in the *S*¹⁵ haplotype were shown to produce 1.4-kb alternative transcript containing full-length *S*¹⁵-*SP11a*, *S*¹⁵-*SP11b*, and a part of *S*¹⁵-*SP11b'*. Although there remains the possibility that this alternative transcript might be able to produce functional *SP11* protein as observed in certain transcripts containing short open reading frames (Campalans et al. 2004; Casson et al. 2002), this unusual form of transcript

Fig. 5. Duplication in the *S*¹⁵-*SP11* region. Sequence similarities within the *S*¹⁵-*SP11* region is shown by Harr plot which was made with DNASIS V3.7 software. Within the plot, each dot represents a match of >90% within a window of 10 nucleotides.

is expected to be non-functional. Generally, the plants with class-II *S*-haplotype are known to exhibit weak or leaky SI phenotype. In *B. oleracea*, some *S*¹⁵ homozygous lines (e.g. P57Sc) with complete self-compatible phenotype have been generated from SI founder plants (Gaude et al. 1993). It should be an interesting experiment to analyze the relationship between the amount of alternative *SP11* transcript and SI phenotype in these lines.

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