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Development of a new *S* locus haplotyping system based on three tightly linked genes in the *S* locus controlling self-incompatibility in radish (*Raphanus sativus* L.)

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ABSTRACT

To develop an efficient and reliable haplotype identification system of the *S* locus controlling self-incompatibility (SI) in radish (*Raphanus sativus* L.), polymorphic sequences of the *SLL2* and *SP6* genes, which were located at each border of the *S* core regions, were used together with those of the *SRK* gene, the female determinant of SI. Partial sequences of the *SP6* and *SRK* genes were isolated from 35 diverse breeding lines that showed differential self-incompatibility responses. A total of 25 *SP6* and 29 *SRK* alleles were isolated in this study, and 29 *SLL2* alleles isolated from the previous study were included for the *S* haplotyping. Whereas the phylogenetic tree of the *SP6* alleles showed species-specific clustering patterns, the phylogenetic tree of the *SRK* alleles revealed intergeneric pairing of some *SRK* alleles, although the others showed species-specific clustering. Among 35 breeding lines, 13 had combinations of the unique *SLL2*, *SRK*, and *SP6* alleles, but the others shared a common allele of either *SLL2* or *SP6* genes. Except for two breeding lines that contained the same *SLL2*, *SRK*, and *SP6* alleles, all breeding lines were shown to harbor unique haplotypes that consisted of different combinations of *SLL2*, *SRK*, and *SP6* alleles. When 73 additional diverse breeding lines were analyzed by this new *S* locus haplotyping system, the haplotypes of all breeding lines were clearly identified. In addition, eight new combinations that contained different combinations of the *SLL2*, *SRK*, and *SP6* alleles were identified.

1. Introduction

Self-incompatibility (SI) is a general genetic mechanism that limits self-fertilization in many angiosperms, and promotes genetic diversity of populations to increase the potential to survive adverse environmental conditions. Approximately 40% of angiosperm species were estimated to possess the SI systems (Igic et al., 2008). In most cases, the SI is controlled by a single locus, called *sterility* (*S*), with multiple alleles (Stone and Goring, 2001; Watanabe et al., 2012), but the SI systems controlled by two unlinked loci are also known to be widespread in the grass Poaceae family (Shinozuka et al., 2010).

While the SI systems are generally classified into heteromorphic and homomorphic SI, the majority of them belong to homomorphic SI systems. Heteromorphic SI, in which fertilization occurs between flowers with differential lengths of styles and stamens, has been reported in limited species, such as primula (Stevens and Murray, 1982). On the other hand, homomorphic SI occurs in species with the same flower morphology. Depending on the inheritance patterns of pollen SI phenotypes, the homomorphic SI is further classified into gametophytic and sporophytic SI systems. While pollen SI phenotypes are determined by the *S* locus genotypes of haploid pollen grains in gametophytic SI, the *S* genotypes of diploid mother plants producing pollen determine pollen SI phenotypes in sporophytic SI (Stone and Goring, 2001; Watanabe et al., 2012).

The gametophytic SI is considered to be more widely distributed in angiosperm, such as Rosaceae and Solanaceae families, and genes encoding S-RNase and S-locus F-box are revealed as female and male determinants of SI, respectively (Franceschi et al., 2012). Meanwhile, the sporophytic SI system has been extensively studied in *Brassica* species (Sobotka et al., 2000; Takayama and Isogai, 2003; Tantikanjana et al., 2010; Watanabe et al., 2012). The gene encoding *S*-locus glycoprotein (SLG) was first isolated from the *S* locus and considered as a putative SI determinant (Nasrallah et al., 1985), but its role in SI responses has been controversial, since the gene coding for *S* receptor kinase (SRK) was revealed as the genuine female determinant (Takasaki et al., 2000). Later, a small-sized gene encoding a cysteine-rich protein was found to be the male determinant of SI responses. This gene, independently identified by Schopfer et al. (1999) and Takayama et al.

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(2000), is generally referred to as *S*-locus cysteine-rich protein (*SCR*)/*S* locus protein 11(*SP11*) or *SP11/SCR*.

The SI systems have not only been an ideal model for studying the evolution of reproductive mechanisms of flowering plants, but they have also been used as an important means of production of F_1 hybrid seeds in *Brassica* species and radish (*Raphanus sativus* L.). Since SI suppresses self-fertilization, seeds of F_1 hybrids can be economically produced by cross-fertilization of parental lines, without laborious emasculation. However, for efficient deployment of the SI systems in F_1 hybrid seed production, specific *S* haplotypes of breeding lines should be identified as a prerequisite. Initially, pollination tests have been used to classify *S* haplotypes, but such processes were laborious and time-consuming, due to the existence of diverse *S* haplotypes.

It was reported that at least 50 and 100 different *S* haplotypes might exist in *B. rapa* (Nou et al., 1993) and *B. oleracea* (Ockendon, 2000), respectively. Nucleotide sequences of the *SLG* or *SRK* alleles have been obtained from more than 40 *S* haplotypes of *Brassica* species, and the *SLG* and *SRK* alleles were largely classified into class I and class II groups, based on sequence similarity (Nasrallah et al., 1991; Sato et al., 2002). Phylogenetic analysis of the *SLG* and *SRK* sequences showed clear separation of two classes and interspecific pairing of alleles, implying the origination of diverse *S* haplotypes before the speciation of *Brassica* species (Sato et al., 2002; Lim et al., 2002; Okamoto et al., 2004).

To replace laborious pollination tests, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methods have been developed on the basis of polymorphic sequences of the radish and *Brassica SLG* or *SRK* genes (Brace et al., 1993, 1994; Nishio et al., 1994, 1996, 1997; Sakamoto et al., 2000; Lim et al., 2002). However, there were some disadvantages in the PCR-RFLP methods. First, it was difficult to design universal primer pairs for the *SLG* and *SRK* genes, due to existence of multiple alleles, and paucity of conserved regions. Second, the presence of the *S* multigene family showing homology with the *SLG* or *SRK* genes hindered specific amplification of *SLG* or *SRK* alleles (Lalonde et al., 1989; Boyes et al., 1991; Kumar and Trick, 1993; Suzuki et al., 1997). Third, the band pattern of PCR-RFLP is sometimes too complex to identify specific *S* haplotypes (Nishio et al., 1997; Lim et al., 2006). For these reasons, the PCR-RFLP methods do not seem to be suitable for the analysis of large-scale samples in breeding programs.

A new *S* haplotyping method was developed in the previous study (Kim et al., 2016) on the basis of the polymorphism of the *SLL2* gene, which was tightly linked to the *S* core region. However, identification of the *S* haplotypes of some breeding lines was difficult, due to possible recombination between the *SLL2* and *S* core regions. In this study, to improve discriminatory power, polymorphic sequences of the *SRK* kinase domains, and *SP6* gene that tightly flanks the *S* core region, together with *SLL2* gene, were used for the establishment of a more reliable *S* haplotyping system in radish.

2. Materials and methods

2.1. Plant materials

Thirty-one breeding lines that were assumed to contain different *S* haplotypes, based on pollination tests and PCR-RFLP analysis (Lim et al., 2002), were used to isolate the nucleotide sequences of *SRK* and *SP6* alleles. The DNAs of these 31 breeding lines that had been isolated in the previous study (Kim et al., 2016) were used, and Supplementary Table 1 shows detailed information about these breeding lines. Four additional breeding lines containing the novel *SLL2* alleles were included for *SRK* and *SP6* gene analysis. Forty-eight diverse breeding lines that had been used in the previous study (Kim et al., 2016) and 25 additional breeding lines were analyzed for the identification of additional *S* haplotypes. Supplementary Table 2 lists these breeding lines and their *S* haplotypes. Although detailed pedigrees of each breeding line were not available, only breeding lines known as inbred lines were selected from diverse germplasm. Breeding lines that showed apparent heterozygous genotypes of the *SLL2*, *SRK*, and *SP6* genes were excluded from the list.

2.2. PCR amplification and sequencing of the PCR products

Total genomic DNAs were extracted from leaves of a single seedling from each inbred line using cetyl trimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). The PCR amplifications were performed in a 25 μL reaction mixture containing a 0.1 μg template, 2.5 μL 10 × PCR buffer, 0.5 μL forward primer (10 μM), 0.5 μL reverse primer (10 μM), 0.5 μL dNTPs (10 mM each), and 0.25 μL polymerase mix (Advantage 2 Polymerase Mix, Clontech, Palo Alto, CA, USA). In the case of primers with lengths ranging from 19 to 24 base pairs, PCR amplification consisted of an initial denaturation step at 95 °C for 4 min, 10 cycles at 95 °C for 30 s, 65 °C (0.8 °C decreases in each cycle) for 30 s, and 72 °C for 1 min, 35 cycles at 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min, and a final 10 min extension at 72 °C. When using primers with lengths ranging from 27 to 28 base pairs, PCR amplification was performed with an initial denaturation step at 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 65 °C for 30 s, 72 °C for 3 min, and a final 10 min extension at 72 °C. For isolation of the full-length *SP6* genes, long PCR was carried out in a 50 μL reaction mixture containing 0.25 μg template, 5 μL 10 × PCR buffer, 0.2 μM forward primer, 0.2 μM reverse primer, 0.2 mM dNTPs, and 0.5 μL Taq polymerase (TaKaRa LA PCR™ kit Ver. 2.1; Takara Bio, Otsu, Shiga, Japan). Long PCR amplification was carried out with 40 cycles of 98 °C for 10 s and 68 °C for 15 min. Supplementary Table 3 lists the primer sequences used in this study.

The PCR products were visualized on 1.5% agarose gels after ethidium bromide staining. For sequencing of the PCR products, they were purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA). Sequencing reactions were performed by a specialized company (Macrogen, Seoul, Republic of Korea).

2.3. Construction of phylogenetic trees of the SRK and SP6 alleles isolated from radish and other Brassica species

The genomic DNA or cDNA sequences of *SP6*, *SLL2*, and *SRK* genes isolated from radish and *Brassica* species were aligned using BioEdit software (Hall, 1999). Large gaps in the alignment were removed using Gblocks software (Castresana, 2000), with the less stringent selection options. Phylogenetic trees were constructed by the neighbor-joining method using MEGA ver. 7 (Kumar et al., 2016). Node support of the phylogenetic tree was assessed by 1000 bootstrap replicates.

3. Results

3.1. Identification of polymorphic SP6 and SRK alleles from diverse breeding lines showing different SI responses

To overcome the difficulty in PCR amplification of *SLG* or *SRK* genes due to the presence of *SLG*- or *SRK*-homologous genes, a new *S* locus haplotyping system had been developed in the previous study (Kim et al., 2016) on the basis of polymorphic *SLL2* alleles. However, some breeding lines showing different SI responses were found to contain the same *SLL2* alleles, because of the possible recombination between the *SLL2* and SI-determining genes (data not shown). To verify any recombination between the *S* core and flanking regions, and to improve the discriminatory efficiency of *S* haplotypes, the *SP6* gene, which was located at the opposite end of the *S* core region to the *SLL2* gene (Fig. 1), was selected to obtain sequences from diverse *S* haplotypes. The positions of the *SLL2* and *SP6* genes were conserved among nine *S* haplotype sequences isolated from radish and *Brassica* species (Fig. 1), but the sequence homology began to disappear from nearby regions of the start codons of *SP6* and *SLL2* genes in the two radish *S* haplotype sequences (Fig. 1).

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