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Isolation and Characterization of a Class I *SLG* Gene from Chinese Cabbage (*Brassica campestris*)

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In *Brassica* species, self-incompatibility in the recognition reaction between self and non-self pollens is determined by two genes, *SLG* and *SRK*, at the S locus. We have cloned and characterized a genomic DNA fragment containing a complete open reading frame of the *SLG* gene from Chinese cabbage. The genomic clone, named *BcSLG2*, was found to possess the region that shares a homology of 77% in amino acid identity with the *SLG46* gene of *Brassica campestris*. Northern blot analysis revealed that the *BcSLG2* gene expression is restricted to the pistil of Chinese cabbage flower. *In situ* hybridization showed that in the pistil, the gene is expressed predominantly in the stigmatic tissue. Much lower expression in the tapetum was also detectable at an immature stage of the flower development. Southern blot hybridization with the *BcSLG2* DNA probe showed polymorphism in the *SLG* gene organization of the Chinese cabbage plants. These results will provide valuable information in understanding the S gene complex of the Chinese cabbage plants.

Introduction

Self-incompatibility is a well-known genetic mechanism of flowering plants by which the female reproductive organ, the pistil, recognizes and rejects self pollens, whereas nonself pollen is accepted for fertilization (Bateman, 1955; Heslop-Harrison and Shivannah, 1977). This allows bisexual flowers to circumvent the tendency for self-fertilization and promotes outbreeding (de Nettancourt, 1977).

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Self-incompatibility in *Brassica* is controlled sporophytically by a single polymorphic genetic locus called the S locus. Molecular analyses revealed that the S locus consists of at least two linked genes expressed in stigma papillae (Boyes and Nasrallah, 1993). One of the gene is the S locus glycoprotein (SLG) gene, and the other is the S locus receptor kinase (SRK) gene (Nasrallah *et al*., 1987; Stein *et al*., 1991). The *SLG* gene encoding glycoprotein is known to share a high degree of homology with the extracellular domain of the *SRK* gene, suggesting that both work together cooperatively to recognize the pollen components (Nasrallah, 1997; Watanabe *et al*., 1994). Identification of the pollen component in *Brassica* has been reported very recently (Schopfer *et al*., 1999). The pollen component named SCR was found to be a small protein containing cysteine-rich residues. SRK contains a kinase domain at the cytoplasmic region, indicating that the gene product undergoes a signal transduction pathway to make the self-incompatible reaction possible (Heldin, 1995; Stein *et al*., 1991). In a recent publication, the component that directly interacts with SRK has been discussed and one of the components found so far is an arm repeat protein called ARC1. ARC1 was found to bind directly to the kinase domain of SRK in a yeast two-hybrid experiment, indicating that the protein is a downstream effector for the kinase (Gu *et al*., 1998; Stone *et al*., 1999). Because the *SLG* and *SRK* genes in *Brassica* exist as multiples alleles, the alleles are expressed as S haplotypes, which are classified into two different types, class I and class II (Nasrallah and Nasrallah, 1993). The SLGs in class I show strong activity in self-incompatibility on the dominance scale, allowing 0–10 pollens to germinate on the stigma surface, whereas the SLGs in class II show a very leaky phenotype in which 10–30 pollens can develop the tubes.

Chinese cabbage is one of the major vegetable crops in Korea. Various cultivars of the crop plants used in

agriculture, however, are cultivated without the molecular understanding that can provide valuable information for the pollen recognition reaction by the S proteins among them. In this study, we have cloned and studied an *SLG* gene from Chinese cabbage. The gene, named *BcSLG2*, was found to belong to class I and to be organized at a polymorphic basis among Chinese cabbage lines. This will help in understanding the structure and organization of the complex S locus of the plants.

Materials and Methods

Plant materials Chinese cabbage plants (*Brassica campestris* L. ssp. *pekinensis*) grown in a greenhouse were used for all DNA and RNA work. Plant materials were kindly provided by the Choong-Ang Seed company and the Chinese cabbage lines were Musso (733), Hiratsuka (734), Misung No. 2 (735), Kyoto No. 2 (736), Cheongbang (737), and Kyoto No. 3 (738).

Polymerase chain reaction Two primers (S: 5′-atgaaa ggcgtaagaaaaaccta-3′ AS: 5′-ccgtgtttattttaagagaaagagct-3′) derived from *SLG46* (Kusaba *et al*., 1997) were designed and used in PCR amplification to clone the *SLG* gene. The PCR was performed using genomic DNA isolated from *B. campestris* leaves under the following condition: step 1: 94°C (5 min), 55°C (5 min), 72°C (5 min), 1 cycle; step 2: 94°C (1 min), 54.2°C (2 min), 72°C (1 min), 32 cycles; step 3: 72°C (5 min), 1 cycle. Reaction components for the PCR included 20 ng of the template DNA, 200 μmol of dNTP, 20 pM of each primer, 5 μl of 10× reaction buffer, and 1 unit of *Taq* polymerase (Takara, Japan). The PCR products were cloned with the pGEMT-easy vector (Promega).

DNA sequencing *BcSLG2* sequencing was carried out basically by the dideoxynucleotide chain termination method (Sanger *et al*., 1977) using an automatic sequencer (ABI310, Perkin Elmer). The homology search was performed using the FASTA program.

Northern blot analysis Total RNAs were isolated by the guanidium thiocyanate method using Tri-reagent (Molecular Research Center). Twenty-five micrograms of the total RNA was separated on a 1.3% agarose gel and transferred onto a nylon membrane with 10× SSPE transferring buffer. The membrane blot was then hybridized with radioactively labeled probes at 60°C in a solution containing 0.5 M NaH_2PO_4 (pH 7.2), 1 mM EDTA, 1% BSA, and 7% SDS for 20 h at 60°C (Church and Gilbert, 1984). After the hybridization, the membrane blot was washed with 1× SSPE and 0.5% SDS three times for 10 min each at 45°C and was then autoradiographed with an X-ray film.

***In situ* hybridization** An *in situ* hybridization experiment was performed by the method described previously (Chung *et al*., 1998). For this experiment, Chinese cabbage flowers were embedded in paraffin and sliced into 8-μm sections with a rotary microtome. The sections were then attached to glass slides and hybridized with either antisense or sense RNA probes labeled with digoxigenin-dUTP. Hybridization was performed at 48°C overnight in a humid chamber. The slides were washed in 4× SSPE, 5 mM DTT for 5 min at 50°C, treated with 25 mg/ml RNase A in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 500 mM NaCl at 37°C for 30 min, and then washed in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 500 mM NaCl, 5 mM DTT twice for 15 min each at 37°C.

Southern blot analysis The total genomic DNA was extracted from the leaves of the six different cultivars of *B. campestris* (Guillemaut and Marechal-Drouard, 1992; Lodhi *et al*., 1994) and digested with *Eco*RI. After electrophoresis, the gel was rinsed briefly with distilled water and depurinated at 0.25 N HCl for 15 min at room temperature. Thereafter, the gel was immersed in 0.4 N NaOH for 90 min at room temperature with shaking. The denatured DNAs were transferred onto a Hybond N^+ nylon membrane (Amersham) with 0.4 N NaOH transfer buffer. At the end of transfer the membrane was rinsed with a membrane wash buffer (0.2 M Tris-HCl, pH 7.5, 2× SSC) and prehybridized with hybridization buffer (5× SSC, 70 mM Na_2HPO_4, 30 mM Na_2HPO_4, 2.5 mM EDTA, 0.6% SDS adjusted to pH 7.2) at 55°C for 4 h. The membrane was hybridized with an [α-^{32}P]dCTP-labeled *BcSLG2* DNA probe in the same buffer at 55°C for 24 h. After hybridization, the membrane blot was washed in 2× SSC, 0.1% SDS at 55°C for 30 min; and 1× SSC, 0.1% SDS at 55°C for 30 min. The final wash was done with 0.5× SSC , 0.1% SDS at 55°C for 30 min, and then the membrane was exposed to X-ray film.

Results and Discussion

Isolation and sequence analysis of the *BcSLG2* gene Using two oligoprimers designed from the *SLG46* sequence of *B. campestris* (Kusaba *et. al*., 1997), a class I *SLG* was successfully amplified from Chinese cabbage by PCR. Genomic DNAs isolated from six different cultivars of Chinese cabbage were used as templates for PCR. Among them, interestingly, only 738 (Kyoto No. 3)

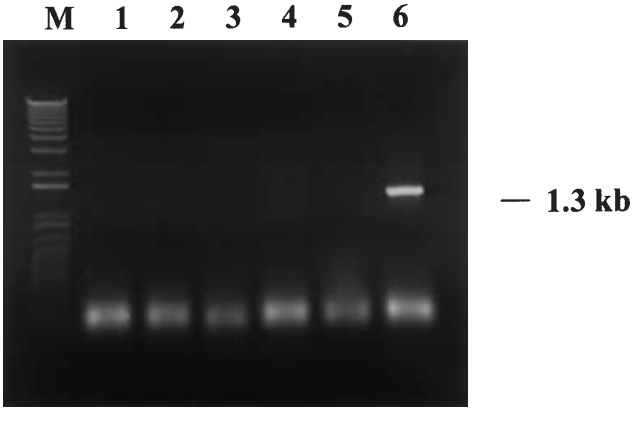

Fig. 1. PCR cloning of *BcSLG2*. M, molecular DNA marker; 1, 733 (Musso); 2, 734 (Hiratsuka); 3, 735 (Misung); 4, 736 (Kyoto No. 2); 5, 737 (Cheongbang); 6, 738 (Kyoto No. 3). Genomic DNA isolated from each Chinese cabbage plant was used as a template.

Fig. 2. Deduced amino acid alignment of BcSLG2. Boxed regions and shaded C represent the hypervariable regions and conserved C-terminal cystein residues, respectively.

line gave a specific amplification with an approximately 1.3-kb DNA fragment, indicating that the two primers specifically recognize the DNA sequence contained in the particular plant (Fig. 1). This result suggests that the 738 line contains an *SLG* gene homologue to the *SLG46* gene and this can be separated from the other five cultivars by using the specific primers. In order to determine the complete sequence, the amplified genomic DNA fragment was subcloned into a plasmid vector and sequenced. The sequence analysis revealed that the genomic clone named *BcSLG2* is 1338-bp long and contains a single open reading frame of 437 amino acid residues without any part of introns. A database search showed homologies to various SLGs of the *Brassica* family, especially with 397 amino acid residues that are located between the 23rd aa and the 418th aa (Fig. 2). Among them, the best homology occurred for SLG29 (Trick and Flavell, 1989) and SLG13 of *B. oleracea* (Nasrallah *et al.*, 1987) with 80% identity in amino acids for both cases. As expected in *B. campestris*, however, the deduced amino acid sequence of BcSLG2 fits best to that of SLG46 with 77% identity. This result suggested that the gene is indeed the *SLG* of Chinese cabbage and is the *SLG46* homologue. In *Brassica*, *SLG*s have been classified into two groups, class I and class II, on the basis of the degree of sequence similarity among *SLG*s and dominance relationships among their corresponding S haplotypes (Nasrallah and Nasrallah, 1993). All the *SLG*s in class I correspond to haplotypes that show dominance in pollen, whereas class II *SLG*s correspond to haplotypes that show recessiveness (Nasrallah *et al.*, 1991). All *SLG*s that are found to be homologous to the *BcSLG2* were found in the class I group, suggesting that the gene is a class I *SLG* of Chinese cabbage. This is also supported by the fact that the Chinese cabbage plant shows high self-incompatibility in the selfing test (data not shown). Sequence comparison between BcSLG2 and SLG46 revealed that three hypervariable regions are recognized: 187–193 aa, 243–279 aa, and 301–315 aa (Fig. 2). The hypervariable region II, which is the longest of the three regions, contained 37 amino acids and showed 54% identity with SLG46, whereas the hypervariable region III showed 33%. The hypervariable region I of BcSLG2, surprisingly, contains an amino acid residue completely different from that of SLG46. Interestingly, the hypervariable regions between SLG46 of *B. campestris* and SLG13 of *B. oleracea* showed high similarity except for region I, despite them being different plant species. It has been postulated that the hypervariable regions, but not necessarily all of them, are involved in determining the S specificity of SLG and SRK (Kusaba *et al.*, 1997).

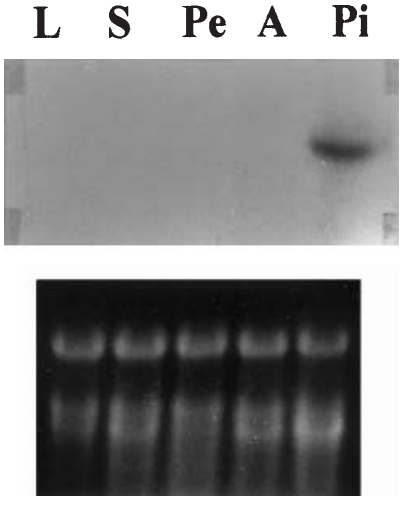

Fig. 3. Northern blot hybrization of *BcSLG2*. Twenty-five micrograms of total RNA isolated from each organ of a mature flower at anthesis was used in the hybridization. L, leaf; S, sepal; Pe, petal; A, anther; Pi, pistil.

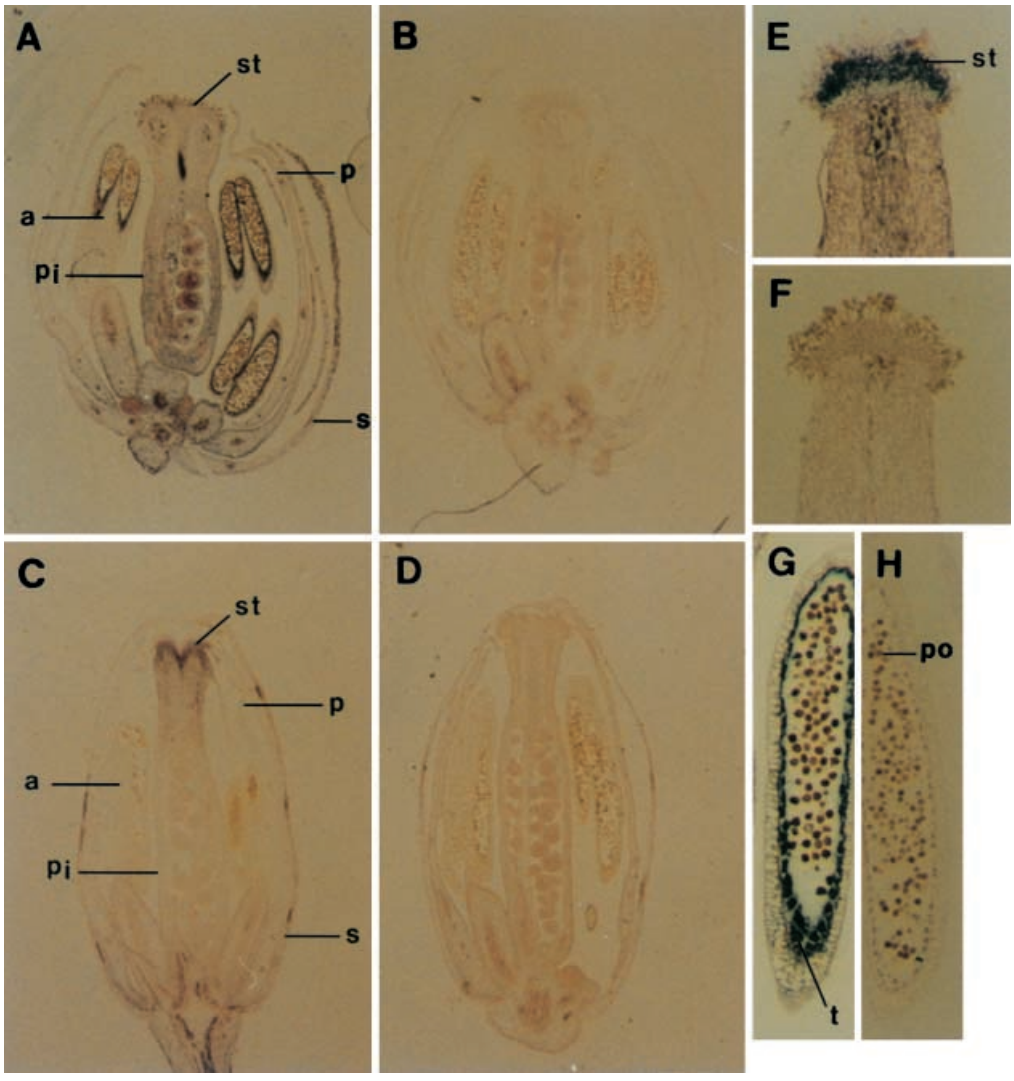

Fig. 4. *In situ* localization of *BcSLG2* transcript in a Chinese cabbage flower. A and B, antisense and sense control probe hybridizations, respectively, with flowers at 3 d prior to anthesis. C and D, antisense and sense control probe hybridizations, respectively, with flowers at 1 d prior to anthesis. E and F, antisense and sense control probe hybridizations, respectively, with pistil from the flower at anthesis. G and H, antisense and sense control probe hybridizations, respectively, with anther from the flower at 3 d prior to anthesis. a, anther; s, sepal; st, stigma; p, petal; pi, pistil; po, pollen; t, tapetum.

This could be confirmed by inter- and/or intraspecific crosses among the plants.

***BcSLG2* gene expression in the Chinese cabbage plant** *BcSLG2* gene expression was studied by Northern blot analysis and *in situ* hybridization. The *BcSLG2* genomic clone containing the full coding sequence was used as a template to generate a probe for the hybridization. For the Northern blot analysis, mature flowers were harvested and separated into four different whorl organs, sepal, petal, anther, and carpel. The total RNA was then isolated from each organ and used in the experiment. Twenty-five micrograms of total RNA was run on an agarose gel and transferred onto a nylon membrane. The membrane blot was then hybridized with an [α-^{32}P]dCTP-labeled *BcSLG2* DNA probe. The RNA blot hybridization revealed that the gene expression is restricted to the pistil organ (Fig. 3). No expression of *BcSLG2* was detected in the vegetative organ, such as the leaf. The detailed expression pattern of the gene was analyzed by an *in situ* hybridization experiment (Fig. 4). A digoxigenin-labeled antisense RNA probe was generated from the *BcSLG2* DNA template and used in the experiment. The Chinese cabbage flower was sliced to 8-μm thickness and attached onto glass slides. The RNA probe was then directly hybridized onto the tissue sections. The *in situ* hybridization showed that the gene is predominantly expressed at high level in the stigmatic

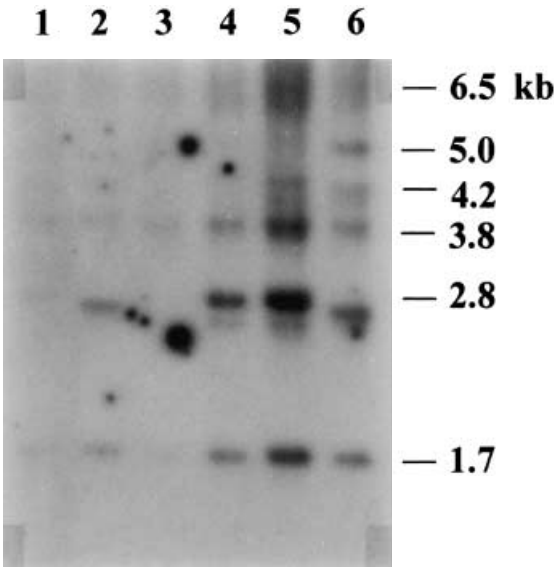

Fig. 5. Southern blot hybridization of the *BcSLG2* gene. 1, 733 (Musso); 2, 734 (Hiratsuka); 3, 735 (Misung); 4, 736 (Kyoto No. 2); 5, 737 (Cheongbang); 6, 738 (Kyoto No. 3).

tissue of the mature flower (Fig. 4E). When the flower development is at an immature stage, however, the gene expression was also found in the tapetum of the anther (Fig. 4G). Expression of the *SLG* gene in the anther of *Brassica* has already been discussed (Heslop-Harrison, 1975) but it is still not conclusive that the gene product is directly involved in male gametogenesis. It is possible that the gene product is required for the process in determining allele specificity which occurs during pollen–pistil interaction. The *BcSLG2* gene expression was not detected during the later stage of flower development (data not shown).

Southern blot analysis To study the genomic complexity of the *BcSLG2* gene and to investigate polymorphism of the *SLG* genes existing in the Chinese cabbage plants, Southern blot hybridization was performed (Fig. 5). The probe derived from the *BcSLG2* DNA template was applied onto a nylon membrane carrying the genomic DNAs isolated from leaves of six different Chinese cabbage lines. The Southern blot hybridization showed that the *BcSLG2* DNA probe hybridized to several DNA bands, indicating that the *SLG* gene in Chinese cabbage plants forms a multigene family. The DNA probe hybridized to at least one common DNA fragment (3.8 kb) in all plants. Longer-exposed film showed an additional common DNA fragment appeared on 1.7 kb (data not shown). Thus, the 733, 734, and 736 plant lines showed similar patterns of genomic organization, whereas the 735, 737, and 738 plant lines showed polymorphism different from the other three lines. The polymorphic pattern between the 737 and 738 plants was very similar except for the additional DNA band which appeared at 5.0 kb in the 738 plant which is specific and different from all the other plants. This suggests that the 738 plant possesses a much more complex genomic organization of the *SLG* gene than the other five Chinese cabbage plants. In that regard, the 735 plant appeared to be less complex in genomic organization. These results suggest that the plants may behave differently in the self-incompatible reaction. This could be supported by cross-combination to produce F1 hybrid seeds based on the polymorphic pattern of the *SLG* genes appearing on the Southern blot analysis. Therefore, further analysis will help in understanding the organization of the complex S locus and its behavior in the self-incompatible reaction.

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