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Characterization of *Brassica* *S*-haplotypes lacking *S*-locus glycoprotein[1]

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Abstract Self-incompatibility (SI) in *Brassica* is regulated by a single multi-allelic locus, *S*, which contains highly polymorphic stigma-expressed genes, *SLG* and *SRK*. While SRK is shown to be the determinant of female SI specificity, SLG is thought to assist the function of SRK. Here we report that the *SLG* genes of self-incompatible S^{18} and S^{60} homozygotes of *Brassica oleracea* have an in-frame stop codon and a 23 bp deletion resulting in a frame-shift, respectively. The finding that these *SLG* genes do not encode functional SLG proteins suggests that SLG is not essential for SI. The possible role of SLG in SI was discussed.

Key words: Self-incompatibility; SLG; In-frame stop codon; Frame-shift; SRK

1. Introduction

Many hermaphrodite species of flowering plants possess a self-incompatibility (SI) system, which prevents self-fertilization by inhibiting germination of self-pollen on the stigmatic surface or growth of self-pollen tubes in the style [1]. This is the result of a cell–cell recognition event between pollen and the pistil. In *Brassica*, this self/non-self recognition is sporophytically controlled by the polymorphic *S*-locus. That is, the SI phenotype of the pollen is determined by the two *S*-haplotypes carried by its parent. If either of these two *S*-haplotypes matches one of the two *S*-haplotypes carried by the pistil, the pollen is recognized as self-pollen and its germination is inhibited. SLG (the *S*-locus glycoprotein) was the first *S*-haplotype specific protein to be identified in the pistil of *Brassica* [2]. SLG is synthesized in the stigma just before anthesis, at the timing coincident with the acquisition of SI by the pistil in flower development. SLG accumulates in the wall of the stigma papillar cell, which contacts directly with pollen grains and pollen tubes. These characteristics of SLG, coupled with the *S*-haplotype specific sequence polymorphism [3,4], have been regarded as evidence suggesting that SLG plays an important role in the *S*-haplotype specific recognition of pollen by the stigma [5].

More recently, another *S*-locus gene expressed in the stigma was identified; it encodes a putative transmembrane receptor protein kinase, termed SRK (*S*-locus receptor kinase) [6]. The extracellular domain of SRK is presumed to serve as the ligand binding domain, and it has been named S domain because of its sequence similarity with SLG. Like SLG, SRK also exhibits *S*-haplotype specific sequence polymorphism. SRK is localized in the plasma membrane of stigma papillar cells [7]. Recently, it was shown that introduction of SRK^{28} could confer S^{28}-haplotype specificity in the stigma, suggesting that SRK is a female determinant of SI specificity [8]. A currently favored model for the mechanism of SI interactions proposes that SLG and its SRK of the same *S*-haplotype form an active complex, which then interacts with a pollen ligand, a male determinant of SI specificity, to trigger a signal transduction cascade, leading to the SI response [9]. Such a male determinant gene was found in the *S*-locus, designated as *SCR* by Schopfer et al. [10] and *SP11* by Suzuki et al. [11]. *SCR*/*SP11* gene is expressed in both tapetum cells and microspores [12] and encodes a small putative secreted protein with highly diverged structures between different *S*-haplotypes except almost conserved eight cysteine residues [13]. It is postulated that SCR/SP11 protein is a ligand of SLG and/or SRK because it shows similarity to PCP-A1 [14], a small SLG binding protein in pollen coat, although direct binding of SCR/SP11 protein to SLG and/or SRK has not been determined.

While the involvement of SRK and SCR/SP11 in SI recognition is obvious, the results from several recent reports have actually raised questions about the involvement of SLG in SI. We recently reported that SLG was not detected in some normal self-incompatible lines of *Brassica oleracea*, including S^{18} and S^{60} homozygotes, by an immunoblot analysis using anti-SLG antibody [15]. However, it has not been known whether these two self-incompatible lines lack SLG, or produce an undetectable but sufficient amount of SLG for its function in SI. Here we report isolation and examination of their *SLG* genes, SLG^{18} and SLG^{60}. These *SLG* genes do not encode functional SLG because of premature truncation of SLG proteins due to an in-frame stop codon and a frame-shift, respectively, suggesting that SLG is not essential to SI. The possible role of *SLG* in the function and evolution of SI is discussed.

2. Materials and methods

2.1. Plant materials and pollination analysis

The S^{18} and S^{60} tester lines of *B. oleracea* were provided by Drs. Astley and Ockendon. The population segregating for S^{60} and S^{2-b}

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Abbreviations: SI, self-incompatibility; RACE, rapid amplification of cDNA ends

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was derived from an F_1 plant with S^{60}/S^{2-b} genotype, which was generated by a cross between the S^{60} tester line and a commercial broccoli cultivar 'Ryokurei' (S^{18}/S^{2-b}). A selfed population of commercial cultivar 'Swing' (S^{18}/S^{2-b}) was used for a population segregating for S^{18} and S^{2-b}. Observation of pollen tubes using UV fluorescence microscopy was performed as described by Nakanishi and Hinata [16].

2.2. Southern and Northern blot analysis

Isolation of genomic DNA and poly(A)$^+$ RNA and Southern and Northern blot analyses were performed as described previously [17]. PCR products amplified using a primer pair (GGTTACGACCTCAAAACAGG and TCCGGTCCAAATCACACAAC) [15] from an SLG^6 clone, an SLG^{2-b} clone and an *SLR1* clone were used as class I *SLG*, class II *SLG* and *SLR1* probes, respectively.

2.3. Cloning of DNA fragments

DNA fragments corresponding to the hybridized bands were recovered from agarose gels using a gel extraction kit (Qiagen) and cloned into the *Eco*RI or *Hin*dIII site of pZerO-1 (Invitrogen). Positive clones for the SLG^6 probe were isolated by the standard colony hybridization procedure using DIG labeling and detection system with NBT/BCIP (Boehringer Mannheim).

2.4. 3′ and 5′ rapid amplification of cDNA ends (RACE) analysis

3′ RACE was performed with a primer specific for SLG^{18} (GGCGACTGTAGACAGGAGCA), a primer specific for SRK^{18} (GGCGATTGTCGACCGGAGTG), a primer specific for SLG^{60} (ATCCGTCAAGCGGGGAAATCACT) and a primer specific for SRK^{60} (ATCCGTCAAGCGGGGACTTG) according to standard protocols. The nucleotide sequence of the 5′ region of SRK^{60} was obtained by 5′ RACE using the first gene specific primer (TTTCGAGCTTGTAATAGTA) and the second gene specific primer (ACTTTGCCACAAGAATTCAT) with the 5′ RACE System (Gibco BRL) according to the manufacturer's protocol. RACE products were cloned into the pCR2 plasmid vector (Invitrogen).

2.5. Sequencing and sequence analysis

DNA sequencing was carried out by the dye-terminator method using PRISM™ 377 (Perkin Elmer). The nucleotide sequences of RACE products were determined as consensus sequences of at least three independent clones. Sequence analyses were performed by Genetyx ver. 10 (Software Kaihatsu, Tokyo, Japan).

2.6. Measurement of pollen adhesion

Pollen–stigma adhesion forces were measured essentially as described by Luu et al. [18]. The force was shown as the acceleration necessary to release all the pollen grains from the stigma. Three S^{60} homozygotes and three S^{2-b} homozygotes from a segregating population for S^{60} and S^{2-b} were pollinated with S^{25} pollen. Nine flowers were used for each plant.

3. Results

3.1. Normal SI phenotype in the S^{18} and S^{60} homozygotes

In self-incompatible species of *Brassica*, germination of self-pollen is specifically inhibited on the stigma. When the S^{18} and S^{60} tester lines of *B. oleracea* were self-pollinated using open flowers, no self-pollen was found to germinate on either S^{18} or S^{60} stigmas (Fig. 1A,C). On the other hand, pollen tubes were fully developed when S^{25} pollen was crossed to S^{18} and S^{60} stigmas (Fig. 1B,D) and S^{18} and S^{60} pollen was crossed to S^{25} stigmas (data not shown). This suggests that the SI phenotype of S^{18} and S^{60} was normal.

3.2. Characterization of SLG and SRK genes of the S^{18} and S^{60} haplotypes

Based on the degree of sequence similarity between SLGs/SRKs, *Brassica S*-haplotypes have been classified into class I and class II. Class I SLGs and the S domain of class I SRKs exhibit approximately 65% sequence identity to class II SLGs and the S domain of class II SRKs, whereas within each class, SLGs and the S domain of SRKs exhibit approximately 80% sequence identity. When the genomic DNA of S^{18} and S^{60} homozygotes was digested with *Hin*dIII and *Eco*RI, respectively, and analyzed by Southern blot analysis using SLG^6 (an

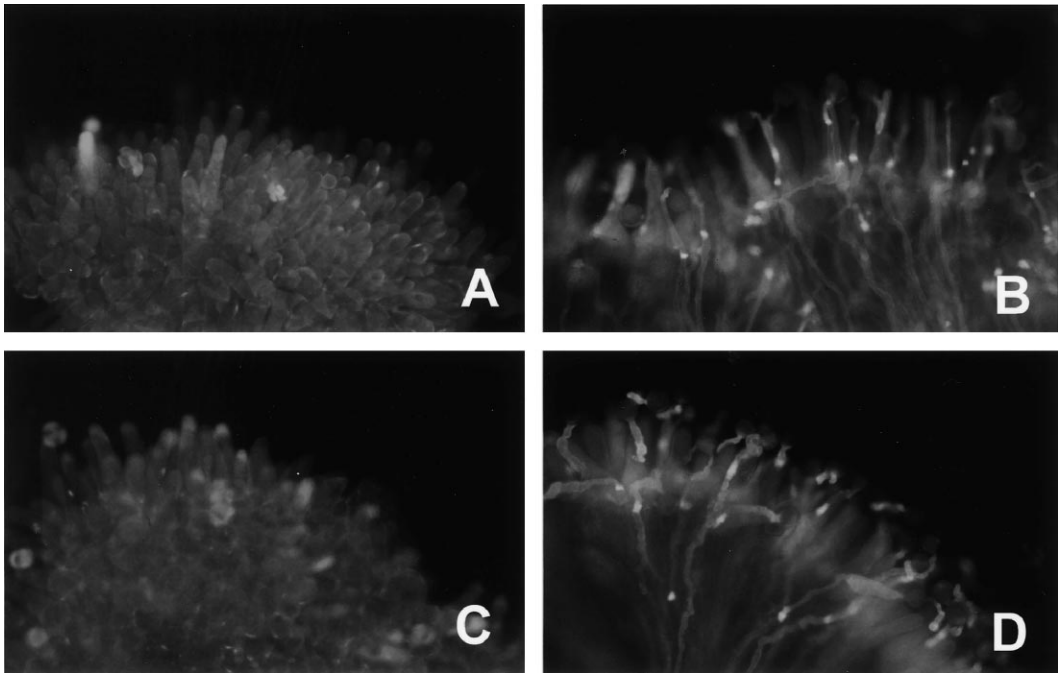

Fig. 1. Pollen tube growth 24 h after pollination. (A) Self-pollination of an S^{18} stigma. (B) An S^{18} stigma pollinated with S^{25} pollen. (C) Self-pollination of an S^{60} stigma. (D) An S^{60} stigma pollinated with S^{25} pollen. Pollen tube development was specifically inhibited in self-pollination in both S^{18} and S^{60}. Pollen tubes were stained with aniline blue and observed using UV fluorescence microscopy. Pollen grains in (A) and (C) were washed off during the staining procedure.

SLG of class I *S*-haplotype) as a probe, two hybridizing DNA fragments were detected for each homozygote (Fig. 2A,C). In Southern blot analysis of populations segregating for S^{18} and S^{2-b} haplotypes, and for S^{60} and S^{2-b} haplotypes, 7.5 and 4.2 kb fragments of S^{18}, and 3.0 and 2.5 kb fragments of S^{60} were found to cosegregate with the S^{18} and S^{60} haplotypes, respectively (Fig. 2C,D). When cDNA of SLG^{2-b} (an *SLG* of a class II *S*-haplotype) was used as a probe, no DNA fragments cosegregating with the S^{18} and S^{60} haplotype were observed (data not shown). Cloning and sequence analysis of the two DNA fragments detected in the S^{18} genotype revealed that the 7.5 kb fragment contained a highly conserved sequence found in the 3′ untranslated region of the *SLG* gene of all class I *S*-haplotypes studied [19], suggesting that it contained SLG^{18}. 3′ RACE analysis using a primer specific to the 4.2 kb fragment revealed that a transcript corresponding to the 4.2 kb DNA fragment encodes a kinase domain, suggesting that the 4.2 kb fragment contained the S domain of SRK^{18}. No RACE products corresponding to the 7.5 kb DNA fragment of S^{18} had a kinase domain. Using similar analysis, we determined that the 3.0 kb DNA fragment of the S^{60} homozygote contained SLG^{60} and the 2.5 kb DNA fragment contained the S domain of SRK^{60}.

3.3. No functional SLG protein is required for SI in the S^{18} and S^{60} homozygotes

Deduced amino acid sequences of SRK^{18} and SRK^{60} showed high degrees of similarity to that of SRK^{6}: 82.7% identity between SRK^{6} and SRK^{18}, and 76.4% identity between SRK^{6} and SRK^{60}. They also had typical characteristics of SRK, including three hypervariable regions in the S domain, which are thought to be involved in the determination of the self/non-self recognition specificity, the 12 conserved cysteine residues in the S domain, and the amino acid residues conserved among protein kinases in the kinase domain (Fig. 3). Northern blot analysis showed that SRK^{18} and SRK^{60} were expressed in the stigma (Fig. 4). These results suggest that SRK^{18} and SRK^{60} are functional. In contrast, even though SLG^{18} and SLG^{60} showed high degrees of nucleotide sequence similarity to SLG^{6}, 88.3% and 84.4% sequence identity, respectively, neither could encode a complete SLG protein (Fig. 5A,B). In SLG^{18}, an in-frame stop codon was found in the region corresponding to the hypervariable region I. In SLG^{60}, there was a deletion of 23 bp, and, as a result, a frame-shift generated stop codons. The possible open reading frames of SLG^{18} and SLG^{60} (651 bp and 297 bp, respectively) cannot encode the hypervariable regions nor the 12 conserved cysteine residues. Therefore, it is unlikely that these two SLG proteins are functional. It is possible that our previous failure to detect an SLG protein in the S^{18} and S^{60} homozygotes by immunoblot analysis was due to instability of the aberrant SLG mRNA and truncated SLG protein. Indeed, Northern blot analysis revealed that considerably lower levels of SLG transcripts were found in S^{18} and S^{60} than in S^{13}, which produced an abundant functional SLG protein (Fig. 4). Together with the normal SI phenotype of the S^{18} and S^{60} homozygotes, these results suggest that no functional SLG protein is required for SI in these homozygotes.

3.4. Examination of involvement of SLG in pollen adhesion

SLR1 is an SLG-like soluble protein, which is synthesized at a high level in the stigma [20]. *SLR1* is not linked to the *S*-locus and an antisense experiment has demonstrated that it is dispensable for SI [21]. Since SLG and SLR1 share very similar properties, it is possible that they have a common or overlapping function. Luu et al. [18] suggested that SLG and SLR1 are involved in pollen adhesion to the stigmatic surface. Because of the possibility that other factors, in addition to SLG, might also contribute to the strength of pollen, we used a population segregating for S^{60} and S^{2-b} for testing the effect of the amount of SLG on the strength of pollen adhesion. Unlike the S^{60} haplotype, the S^{2-b} haplotype produces abundant SLG [22]. The acceleration necessary for releasing all the pollen grains from the stigma, a measure of the strength of pollen adhesion [18], was 4230 ± 610 g (*S*60) and 4560 ± 840 g (*S*2-b). ANOVA indicated that there is no significant difference in pollen–stigma adhesion force between S^{60} and S^{2-b} homozygotes ($F = 3.086 < F(1, 48, 0.05)$) and among three S^{60} homozygotes ($F = 1.478 < F(2, 48, 0.05)$), although significant difference at the 5% level ($F = 3.636 > F(2, 48, 0.05)$) was observed among three S^{2-b} homozygotes. On the other hand, the expression level of SLR1 was similar among S^{13}, which produces abundant SLG, S^{18}, and S^{60} (Fig. 4), suggesting that the absence of SLG does not affect the expression level of *SLR1*. Our results thus do not support the involvement of SLG in pollen adhesion.

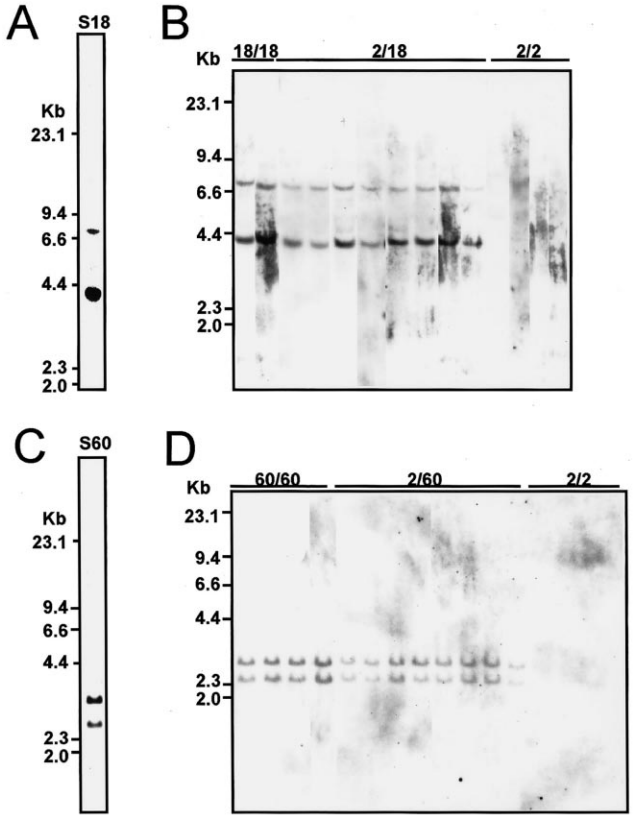

Fig. 2. Detection of *SLG*-like gene in the segregating populations. An S^{18} homozygote (A); an S^{60} homozygote (C); a population segregating for S^{18} and S^{2-b} (B); and a population segregating for S^{60} and S^{2-b} (D). The *S* genotypes determined by test crossing are shown above each lane. Genomic DNA was digested with restriction enzymes, *Hin*dIII (A and B) and *Eco*RI (C and D), and the filters were hybridized with SLG^{6} probe.

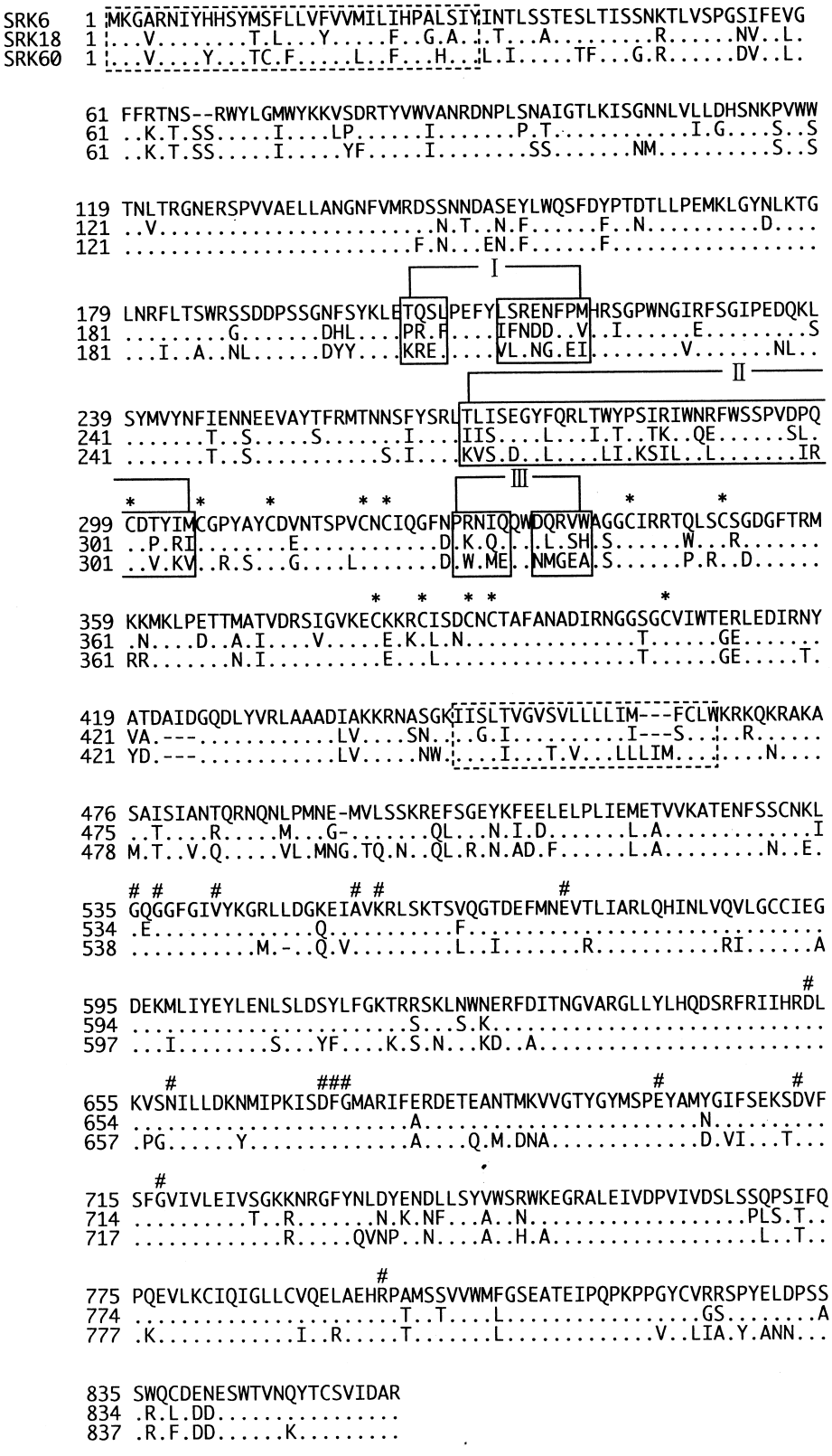

Fig. 3. Comparison of amino acid sequences of SRK^{18} and SRK^{60} with SRK^{6}. The putative signal peptide and transmembrane domain are boxed with dashed lines. Boxes with solid lines represent the three hypervariable regions (I, II and III), two of which, I and III, are separated into two parts by conserved amino acid residues. * shows the position of the conserved cysteine residue in the S-multigene family. Conserved amino acid residues in protein kinase are indicated by # [6].

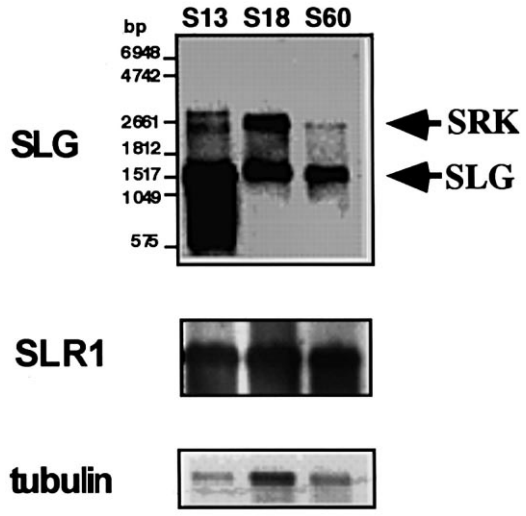

Fig. 4. Expression of *SLG*, *SRK* and *SLR1* in the S^{18} and S^{60} haplotypes. Poly(A)$^+$ RNA was extracted from S^{13}, S^{18} and S^{60} stigmas at 1 and 2 days before flower opening and probed with SLG^6 (SLG), *SLR1* (SLR1) or *Arabidopsis thaliana* α-tubulin (tubulin). 1 μg of poly(A)$^+$ RNA was loaded in each lane. Upper and lower arrowheads indicate positions of *SRK* and *SLG*, respectively.

4. Discussion

4.1. SLG is not essential to SI function in Brassica

Okazaki et al. [15] revealed that SLG is not detected in some *S*-haplotypes in *B. oleracea* including the S^{18} and S^{60} haplotypes. In this study, Southern blot analysis of the S^{18} and S^{60} homozygotes revealed that only two genes exhibiting high homology to the class I *SLG*, one of which is of *SRK* and the other is of *SLG*, exist in each homozygote. They all were linked to the *S*-locus and no gene exhibiting high homology to the class II *SLG* was associated with either the S^{18} and S^{60} genotype. While both SRK^{18} and SRK^{60} have typical structures of SRK and are expressed in the stigma, SLG showed some abnormality. In addition to the observation that amounts of transcripts of the *SLG* genes of S^{18} and S^{60} were considerably lower than those of typical class I *SLG* genes, these *SLG* genes had an in-frame stop codon in the coding region and a 23 bp deletion resulting in a frame-shift, respectively, and did not encode functional SLG proteins. These results indicate that the S^{18} and S^{60} homozygotes do not produce functional SLG protein. Taking into account these results and the observation that both the S^{18} and S^{60} homozygous lines showed normal SI, we conclude that SLG is not essential for SI in these two homozygotes.

This conclusion is most likely valid for other *S*-haplotypes of *B. oleracea* and *Brassica rapa* because of the following reasons. First, SLGs of some genetically distinct *S*-haplotypes (for example, S^{23} and S^{29} in *B. oleracea*, and S^8 and S^{46} in *B. rapa*) have been shown to be very similar in their sequences [17]. Second, SLGs of different self-incompatible lines with the same *S*-haplotype (for example, S^2 and S^{2-b} in *B. oleracea*) have been found to be significantly different in their sequences, even in the hypervariable regions, which is thought to be important for *S*-haplotype specificity [22]. Third, *SLG* appears to be deleted in the S^{24} haplotype of *B. oleracea* [15]. Fourth, the amount of SLG produced does not correlate with the strength of SI in class II *S*-haplotypes [23]. Previous attempts to change the SI phenotype of *Brassica* plants by the introduction of the *SRK* or *SLG* gene of a different *S*-haplotype often led to reduced expression of the endogenous *SRK* and *SLG* genes in transgenic plants, due to sense cosuppression by the *SLG* and/or *SRK* transgenes. As a result, the transgenic plants became self-compatible [24,25]. With the evidence we have presented in this work showing that SLG is not essential in SI, we could also conclude that down regulation of *SRK* is most likely the reason for the breakdown of SI in these transgenic plants. That is, SRK plays a central role in the SI reaction. Consistent with this, Takasaki et al. [8] showed that introduction of an SRK^{28} transgene of *B. rapa* can confer the ability to reject S^{28} pollen.

4.2. Possibility of involvement of SLG in SI as an accessory molecule and as a co-evolutionary partner of SRK

Although it is thought that SLG is not essential to SI, whether or not the function that SLG is thought to have is required for SI remains to be argued. For example, it is possible that a functionally redundant gene might be compensating the absence of SLG in the S^{18} and S^{60} haplotypes. One potential candidate is *SRK* because it could produce a soluble S domain protein from an unspliced mRNA product due to the presence of stop codons in the first intron [26]. In the 3′ RACE analyses of SRK^{18} and SRK^{60}, we found that expression of such a transcript is very low (data not shown). Alternatively, the SLG-like protein in the stigma, such as SLR1, might have a common or overlapping function with SLG and compensate for the absence of SLG. However, our observation does not support the idea that SLG functions in pollen adhesion together with SLR1.

Takasaki et al. [8] observed that an SLG^{28} transgene together with SRK^{28} enhanced the strength of SI, suggesting some involvement of SLG in SI. However, the observation that the *SRK* transgene alone can confer *S*-haplotype specificity suggests that SLG of the same *S*-haplotype is not necessarily required to express female SI specificity. This means that SLG functions not as a primary molecule of SI recognition but as an accessory molecule. Such a function of SLG might be a stabilizer of SRK [10]. We observed a strong SI phenotype in S^{18}, S^{24} and S^{60} haplotypes in *B. oleracea*, which produce no functional SLG protein, suggesting that SLG is not required even as an accessory molecule for SI in these haplotypes. It has been claimed that SLG is especially similar to SRK of the same *S*-haplotype [6] and that the high similarity between SLG and SRK is important for the SI function [8]. The degree of similarity between them is actually quite different among *S*-haplotypes. The highest is 99.8% of S^{45} of *B. rapa* [27] and the lowest is 75.9% of S^{12} of *B. rapa* [28]. In this context, it can be speculated that the degree of requirement of SLG as an accessory molecule of SI signal transduction is different between *S*-haplotypes. No SLG is required for SRK^{18}, SRK^{24} and SRK^{60} of *B. oleracea* to conduct SI reaction while SLG^{28} of *B. rapa* helps its function. Analysis of interactions between SRK, SCR/SP11 and SLG could be a clue to understand the mechanism of such differences.

In the evolution of *Brassica* SI system, *SLG* might have been generated by a duplication of the S domain of *SRK* [29], or an *SLG*-like gene such as *SLR1* might have been translocated into the *S*-locus and evolved into a distinct gene [18]. In the latter case, the *SLG*-like gene might have

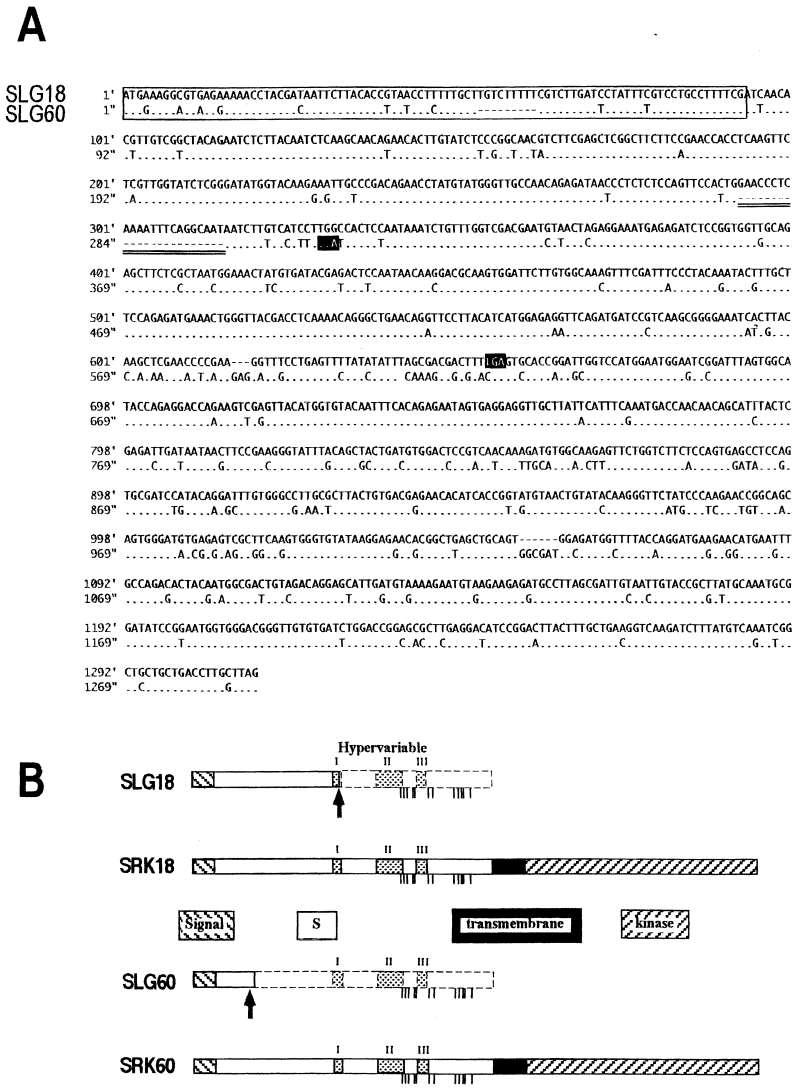

Fig. 5. The structures of SLGs and SRKs of S^{18} and S^{60}. (A) The nucleotide sequences of SLG^{18} and SLG^{60}. The in-frame stop codon in SLG^{18} and the first stop codon after the 23 bp deletion (underline) in SLG^{60} are highlighted. The regions corresponding to the putative signal sequences are boxed. The TGA sequences at the ends of sequences correspond to the stop codons in the normal *SLG*. (B) The signal sequences and kinase domains are indicated by hatched boxes. The S and transmembrane domains are shown by open and closed boxes, respectively. Shaded boxes in the S domains are the hypervariable regions I, II and III. The positions of the 12 conserved cysteine residues are shown as short vertical lines. The regions not translatable in SLG^{18} and SLG^{60} are represented by dashed lines. Arrowheads indicate the positions of the in-frame stop codon in SLG^{18} and the 23 bp deletion in SLG^{60}.

become more similar to the S domain of *SRK* gene by sequence exchange with *SRK* through gene conversion [4]. In both cases, *SLG* could contribute to the evolution of *SRK* through accumulation of mutations in *SLG* and gene conversion between *SLG* and *SRK*. This would provide more opportunities to evolve new recognition specificity. Consistent with this hypothesis, Kusaba et al. [22] observed that *SLG* genes have accumulated more amino acid substitutions than have *SRK* genes.

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