Alteration of the Self-incompatibility Phenotype in *Brassica* by Transformation of the Antisense SLG Gene

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Self-incompatible (SI) *Brassica rapa* (syn. *B. campestris*) was transformed with an antisense SLG gene by using SLG*G* cDNA isolated from the *B. campestris* S*4* homozygote. Two transformed lines were obtained and analyzed. Northern blot and Western blot analyses revealed that endogenous SLG and SRK were greatly reduced of the transcriptional and translational levels in the transformant. Pollination experiments confirmed that their SI phenotype had broken down. In addition, the progeny with the antisense SLG gene, resulting from self- or cross-pollination of the transgenic plant, also showed the self-compatible phenotype. The breakdown of SI in the transformants was due to the change in property of the stigma and not of the pollen. These results provide strong evidence that SLG and/or SRK is implicated in the pollen-stigma recognition of SI and that they act only as stigmatic factors.

**Key words:** SLG; SRK; *Brassica rapa*; self-incompatibility; antisense RNA

The self-incompatibility (SI) system in flowering plants prevents self-fertilization and promotes out-crossing in fertile hermaphrodite seed plants. In *Brassica*, the SI reaction is sporophytically controlled by multiallelic genes at the S-locus. A molecular analysis has shown that a pair of genes, SLG and SRK, are at the S-locus. The SLG gene encodes a secreted glycoprotein that is in the wall of the papillar cell on the stigma, and the SRK gene encodes a membrane-anchored serine/threonine receptor protein kinase that has an extracellular receptor domain with high homology to SLG. Various lines of circumstantial evidence suggest that SLG and SRK function for the recognition of self pollen in the papillar cells on the stigma. Several experiments to confirm the roles of these two genes in the SI system by transforming them into self-incompatible and self-compatible *Brassica* species have so far been attempted, but no conclusive result has been obtained; in some instances, sometimes nothing occurred, and in others, the self-incompatible phenotype unexpectedly changed to being self-compatible.

Although some of the stigmatic components of the SI system have been discussed and analyzed, the S-gene products of pollen have only been superficially studied. Those genes should be at the S-locus in the neighborhood of the SLG and SRK genes. Some researchers have revealed the presence of genes at the S-locus such as SLA and SL1 and SL2, but these are not believed to be the real S-genes that determine pollen specificity in the SI system.

On the other hand, a reporter-gene analysis has indicated that an SLG promoter-GUS fusion protein was expressed not only in the stigma, but also in the anther tapetum and microspores. In addition, a transgenic *B. oleracea* into which the *B. campestris* SLG*G* genomic gene had been introduced became self-compatible due to a change in the pollen property. These results suggested the possibility that SLG might also play a role in determining the SI specificity of pollen still remaining. Considering the somewhat confusing situation just described, we conducted a transformation experiment to confirm the roles of the SLG and SRK genes and to examine the possibility that those genes would also act in pollen. In this experiment, we introduced the antisense gene of SLG, a technique that has never previously been tried in studies on the SI of *Brassica*. We are able to show that the introduced gene broke down the SI of the recipient plant as briefly communicated in our previous paper. In this paper we...
examine precisely the properties of the transformants and the heredity of the transgene. We further analyze the relationship between the transgene and the SI phenotype of the T4 and T5 progenies of the transformant. We found from this experiment that a transgenic plant of *B. rapa* (syn. *B. campestris*) could set seeds after self-pollination, that the levels of the endogenous *SLG* and *SRK* transcripts were substantially reduced, and that no *SLG* protein was detectable in the transgenic plant. These results indicate that the SI of the transformants had been broken down by the action of the antisense gene. Furthermore, the progeny obtained from self- or cross-pollination of the transformant inherited the self-compatible property with the antisense gene. A back-cross experiment reveals that the change of the SI phenotype in the transformant occurred only in the stigma and not in the pollen. These observations provide strong evidence that *SLG* and/or *SRK* are directly involved in the recognition system of SI in the stigma.

**Materials and Methods**

*Materials.* *B. rapa* L. ssp. oleifera cv. Candle, *B. campestris* L. cv. Kousaitai, and *B. campestris* L. S1 and S2 homozygotes were used (*B. rapa* and *B. campestris* were classified as the same *Brassica* species). "Candle" was presented by the Plant Research Institute (Yokohama, Japan), and "Kousaitai" was purchased from Takii Seed Co. (Kyoto, Japan). In addition, the S1 and S2 homozygotes were collected from spontaneous populations in Oguni, Japan and identified.20 *Agrobacterium tumefaciens* strain EHA10121 was obtained from Prof. Yasunori Machida (Nagoya University, Nagoya, Japan). Except for pBC8, all plasmids were obtained from Prof. Hiroshi Kamada (Tsukuba University, Tsukuba, Japan).

*Construction of the antisense *SLG* gene.* The *SLG* promoter used was approximately a 1.9-kbp 5' upstream flanking sequence of the *SLG* genomic gene (derived from the self-incompatible *B. campestris* S1 homozygote). This fragment was obtained from an *SLG* genomic DNA fragment in pBC8 by PCR amplification with a primer synthesized to the *SLG* coding sequence and an M13 reverse primer. The amplified fragment was cloned into the *EcoRI* site (blunted with T4 DNA polymerase) of pBSII SK+ (Stratagene) and designated pSLG8 pro. To truncate the CaMV 35S promoter gene down to position-90, pBI221 (Clontech) was digested with *Pst*I, blunt ended with T4 DNA polymerase, and digested with *EcoRV*. The resulting 0.8-kbp fragment was subcloned between the *PstI/EcoRV* sites of pBSII SK+ to generate p35SPro. To construct the Npt II-Hyg8 gene, pBI212 (Clontech) was first digested with *SacII*, blunt ended with T4 DNA polymerase, and digested with *HindIII*. The resulting 3.1-kbp fragment was inserted between the *EcoRV* and *HindIII* sites of pBSII SK+ to generate pNptII. A 2.2-kbp *Hyg8* fragment was excised from pBI303 by *SacI* and *XhoI*, and treated with T4 DNA polymerase to create blunt ends. This fragment was inserted into the *HindIII* site (Klenow-treated) of pNptII and designated pKH1.

To make the antisense *SLG* gene construct, pSLG8 pro was digested with *NotI*, treated with T4 DNA polymerase to create blunt ends, and digested with *BamHI*. The full sequence of *SLGs* cDNA in pUC823 was digested with *EcoRI* and then blunt ended with T4 DNA polymerase. The blunt plasmid was further digested with *BamHI*, and the resulting 1.5-kbp fragment was inserted between the *NotI* (Klenow-treated) and *BamHI* sites of pSLG8 pro to generate pANTI1. pANTI1 was digested with *SacI*, blunt ended with T4 DNA polymerase, and digested with *HindIII*. The resulting 3.5-kbp fragment was cloned between the *XhoI* (Klenow-treated) and *HindIII* sites of p35S pro to generate pANTI2. Plasmid pANTI2 was released by doubly digesting with *KpnI* (Klenow-treated) and *SacI*. This fragment was ligated between the *SacI* (Klenow-treated) and *SacI* sites of pBI121 to generate pANTI3. Finally, pKH1 was released by doubly digesting with *SacI* and *KpnI*, and treated with T4 DNA polymerase. This fragment was cloned into the *EcoRI* site (Klenow-treated) of pANTI3 to generate pANTI4. The resulting plasmid, pANTI4 was electroporated into *Agrobacterium tumefaciens* strain EHA101,21

*Plant transformation.* Self-incompatible *Brassica rapa* (syn. *B. campestris*) ssp. oleifera "Candle" F hybrids and *B. campestris* L. "Kousaitai" were used as the gene recipient plants. The hypocotyl transformation was done according to the described method with a slight modification:22 10 mM glucose was added to the MS medium supplemented with 1 mg/l of 2,4-D when the hypocotyls of *B. rapa* were co-cultivated with *A. tumefaciens*. Two independent lines with the antisense *SLG* gene were grown in a greenhouse at 25°C. Self-pollinated seeds were obtained by bud pollination. In the case of crossing with the *B. campestris* S1 and S2 homozygotes, stigmas from the S1 and S2 homozygotes were pollinated with pollen derived from the transgenic plant, and their T1 progenies were obtained by self-pollination.

*DNA gel-blot analysis.* Genomic DNA was extracted from young *Brassica* leaves as described.23 Approximately 2 μg of genomic DNA was digested with *EcoRI*, separated on 0.7% agarose gel, and transferred to a Gene-Screen Plus membrane (New England Nuclear) according to the manufacturer's protocol. The membrane was pre-hybridized in 5X SSC, 5X Denhardt and 0.5% SDS solutions and 100 mg/ml of sheared and denatured salmon-sperm...
DNA for 2 h at 42°C. They were continuously hybridized with random-primer-32P labeled SLGs cDNA comprising an EcoRI fragment derived from pUC8 cDNA\(^{10}\) or with the Npt II gene comprising an SphI fragment derived from pBII121 (Clontech) in the same buffer for 12 h at 42°C. The membrane was washed in 2X SSC and 1% SDS for 1 h at 42°C, exposed on a Bio Imaging plate (Fuji Film) for 2 h, and then analyzed by a Bio Image Analyzer (Fuji Film).

*In vivo pollen-tube growth.* Hand-polliated pistils were excised and treated by aniline blue staining for direct fluorescence microscopic observation.\(^{24}\) Stigmas were self- or cross-pollinated after removing the anthers and incubated for 6 h. Two hours after their fixation in ethanol:acetic acid (3:1), the stigmas were softened in 1 n NaOH for 1.5 h at 60°C and subsequently stained with 0.01% decolorized aniline blue in a 2% solution of K$_2$PO$_4$ for 2.5 h. Whole pistils were placed on a microscopic slide glass and gently squashed by placing a cover glass on the pistils. Callose plugs in the pollen tubes were viewed by fluorescence microscopy.\(^{25}\)

**RNA gel-blot analysis.** Poly (A)$^+$ RNA was extracted from 50 stigmas at the time when the buds were almost mature (2–4 days before anthesis) by using a Micro FastTrack purification kit (Invitrogen). Poly (A)$^+$ RNA (1.5 $\mu$g) was electrophoresed on 1.2% agarose/formamide gel and transferred to a Gene-screen Plus membrane according to the manufacturer’s protocol. The actin probe was 1.6-kbp cDNA derived from the Nicotiana tabacum actin gene. To construct the SLG$_5$ gene probe, the membrane was first pre-hybridized in 5X SSC, 5X Denhardt, and 1% SDS solutions and 100 mg/ml of sheared and denatured salmon-sperm DNA for 2 h at 60°C, before being hybridized in the same buffer for 12 h at 60°C with a random-primer-32P labeled SLG$_5$ cDNA probe as described for the DNA gel-blot analysis. After hybridization, the membrane was washed in 0.1X SSC and 2% SDS for 1 h at 60°C, exposed on a Bio Imaging plate (Fuji Film) for 2 h (short exposure) or 12 h (over exposure), and analyzed with a Fuji Imaging Analyzer (BAS2000, Fuji Film). The same amounts of mRNA loaded per lane in the RNA gel-blot analysis were confirmed by re-hybridization with an actin probe. Hybridization with the actin probe involved the membrane first being hybridized as already described and then with random-primer-32P labeled actin cDNA in the same buffer for 12 h at 60°C. The membrane was finally washed in 1X SSC and 2% SDS for 1 h at 60°C before being exposed on a Bio Imaging plate for 12 h.

**Protein immunoblot analysis.** SDS immunoblotting involved total proteins being extracted from 5 stigmas collected from open flowers by 50 mM Tris-HCl (pH 7.5). After centrifuging the crude extract at 12,000 x g for 20 min, proteins in the supernatant was measured by the method of Bradford with the Biorad reagent. Proteins (5 $\mu$g) were mixed with 2% SDS, 5% 2-mercaptoethanol, and a 15% loading buffer (a saturated sucrose solution containing 0.001% bromophenol blue). The sample was boiled for 5 min and subjected to SDS polyacrylamide gel electrophoresis on 12% resolving gel at 25 mA for 2 h, and subsequently transferred to a PVDF membrane (Millipore) by electroblotting at 0.2 A for 45 min. IEF-immunoblotting involved extracting and centrifuging total proteins with the same method as that in the SDS-immunoblot. The supernatant (15 $\mu$l) derived from 30 stigmas was subjected to a thin-layer polyacrylamide gel isoelectric focusing (IEF; pF 3.5–9.5: LKB) at 1500 V, 50 mA, and 30 W per gel for about 2 h. After that, the membrane was mounted on the gel for 30 min. These membranes had been pre-hybridized in 1% (w/v) BSA in TBS (50 mM Tris-HCl, pH 7.4, 0.2 mM NaCl) at room temperature for 1 h. A monoclonal anti SLG8 antibody\(^{10}\) was used as the primary antibody. For detection, a horseradish peroxidase-conjugated goat anti-mouse antibody was used by the standard method (ABC kit, Vectastain).

*Genomic PCR.* Genomic DNA was isolated and amplified by PCR by using SLG-specific oligonucleotide primers.\(^{26}\) The SLG-1 primer (5'-AGAACACT-TGTATCTCCGGGT-3') and SLG-2 primer (5'-AA-GGTCAGCAG(A/G)AGCCAATC-3') were located at both ends of the SLG sequences, and their PCR product was approximately 1100-bp.

PCR amplification was carried out in a 100-$\mu$l reaction solution containing 1X the reaction buffer, 0.2 mM of each dNTP, 0.1 $\mu$m of each primer, 2.5 units of Taq polymerase (Takara), and approximately 500 ng of genomic DNA. Amplification was carried out in a thermal cycler (Perkin Elmer) for 30 cycles comprising denaturation at 94°C for 1 min, reannealing at 59°C for 2 min, and extension at 72°C for 1 min. After DNA amplification, these PCR products were analyzed with the XhoI restriction enzyme because neither the SLG$_5$ nor the SRK$_5$ gene was digested in its internal sequences. Samples (10 $\mu$l) were subjected to electrophoresis on 1% agarose gel in a TAE buffer. Subsequent agarose gel electrophoresis, ethidium bromide staining and a DNA gel-blot analysis with an SLG$_5$ cDNA probe revealed a pattern characteristic of segregation and discriminated between different S haplotypes.

After electrophoresis, DNA fragments in the agarose gel were moved by capillary transfer to a Hybond-N membrane (Amersham Pharmacia) according to the manufacturer’s protocol. The blots were hybridized with digoxigenin-labeled cDNA of SLGs comprising an EcoRI fragment derived from the pUC8 cDNA by the random-primer method sug-
Fig. 1. Construction of the Antisense SLG Gene (pANTI4).
Nos pro: nopaline synthase promoter from pbI121; Nos ter: nopaline synthase terminator from pbI121; Npt II: neomycin phosphotransferase gene from pbI121; Hyg R: hygromycin-resistant gene from pbI303; RB and LB: right and left borders of T-DNA. B, BamHI; E, EcoRI; SII, SacII; X, XhoI; K, KpnI; SI, SacI. The arrow under SLG8 indicates the 5' to 3' orientation of the gene.

Fig. 2. DNA Gel-blot Analysis of the Untransformed Control (Lane 1) and the Transgenic Brussica Plant (Lane 2).
A Southern blot is shown of the genomic DNA (2 µg) of the untransformed control and of the transgenic plant that had been digested with EcoRI and hybridized with 32P-labeled cDNA of SLG (a) and Npt II (b) from pBC8. The size markers are HindIII-digested λDNA.

Transformation of self-incompatible Brussica with the antisense SLG8 gene
The antisense construct was introduced via Agrobacterium-mediated transformation.20 The kanamycin-resistant plants obtained were analyzed by genomic PCR for the Npt II gene and then hybridized with the Npt II gene probe. Of four regenerated plants from the 150 explants used, two independent lines, one generated from B. rapa Candle and one from B. rapa Kousaitai, were found to contain the transformed Npt II gene, although only one of the two did so in a consistent fashion in the progeny. Therefore, the one line generated from B. rapa Candle was studied further.

Genomic DNA of transgenic B. rapa Candle was analyzed by a DNA gel-blot analysis. Under highly stringent hybridization conditions, the transgenic plant showed a band of 4.0-kbp which hybridized with both the Npt II and SLG8 gene probes (Fig. 2). The data suggested that this transformant contained one copy of the transgene.
Specific reduction of the transcripts and proteins of SLG and SRK in the transgenic plant

To determine the level of the endogenous SLG and/or SRK transcripts in the stigma of the transgenic plant, total RNA was isolated from the stigma and analyzed by an RNA gel-blot analysis with an SLG<sub>3</sub> cDNA probe. Figure 3(a) shows that a band of approximately 1.4-kb for SLG was greatly reduced in the transgenic plant. Furthermore, a band of approximately 2.8-kb for SRK, which could be detected under the overexposed condition, was also reduced (Fig. 3(b)). A quantitative analysis showed that the endogenous SLG mRNA of the transformant was less than 25% of that of the non-transformant. We could not, however, quantify the level of the SRK transcript in the transgenic plant due to its much lower expression level. These data suggest that the expression of the endogenous SLG and SRK genes was suppressed at the mRNA level in the transgenic plant.

The stigma proteins were subjected to an SDS-immunoblot analysis to estimate the quantity of SLG protein in the transgenic plant. No SLG could be detected with the anti-SLG<sub>3</sub> monoclonal antibody (Fig. 4(a)). Similarly, an IEF-immunoblot analysis of the stigma proteins of the transformant showed no band in the range from pH 3.5 to 9.5, whereas a high level of the SLG protein band was detected in the non-transformant (Fig. 4(b)). Thus, no SLG protein was detected in the transgenic plant, confirming the results of the RNA blot analysis.

Examination of self-incompatibility phenotypes of the transgenic plant

The appearance of the transgenic plant was vegetatively and florally indistinguishable from that of the wild type. First, the transgenic plant was self-pollinated to investigate whether the introduced antisense gene affected the self-incompatibility phenotype. As shown in Fig. 5(a), the wild type showed a strong SI phenotype; no pollen tube penetrated into the stigma and callose deposits were observed on the papillar cells. In contrast, the transgenic plant had lost its SI phenotype; self pollen germinated and pollen tubes (about 50–100 tubes per stigma) penetrated into the stigma (Fig. 5(b)), although the number of pollen tubes was not as many as the case of cross-pollination (200 tubes per stigma) (Fig. 5(c) and 5(d)). The self-pollinated transgenic plant set silique and normal seeds, as in the case of cross-pollination, although the average size of the silique and the number of seeds per silique were smaller than those of the cross-pollinated plants (data not shown).

The transformant derived from B. rapa cv. Kousaitai showed the same phenotype; reduced SLG and SRK expression and breakdown of SI (data not shown). These results clearly demonstrate that the introduced antisense gene caused the change in the self-incompatibility phenotype of the transformant as well as the reduction of endogenous SLG and SRK mRNA.

Heredity of the introduced transgenic gene

To clarify the heredity of the introduced antisense gene, we obtained T<sub>2</sub> progenies by self-pollination of

![Fig. 3. RNA Gel-blot Analysis of the Untransformed Control (Lane 1) and the Transgenic Brassica Plant (Lane 2). Poly (A)<sup>+</sup> RNA (1.5 μg) from 30 stigmas was loaded into each lane and hybridized with a probe containing full-length SLG<sub>3</sub> cDNA. Poly (A)<sup>+</sup> RNA was assayed after 2 h (a) and 12 h (b) of exposure. Subsequent hybridization with the actin probe showed equivalent amounts of RNA present in each lane. RNA size markers are indicated in kb at the right.](image)

![Fig. 4. Protein Gel-blot Analysis of Protein Extracts from the Untransformed Control (Lane 1), Transgenic Brassica (Lane 2) and the B. campestris S<sub>5</sub> Homozygote (Lane 3). (a) SDS-immunoblot. Protein was extracted from 5 stigmas, after which SLG proteins were detected immunologically with the anti-SLG<sub>3</sub> monoclonal antibody. The arrowhead indicates the SLG band. Molecular weight markers are indicated in kDa at the right. (b) IEF-immunoblot. Protein was extracted from 20 stigmas, after which SLG protein was detected by the method used for the SDS-immunoblot. The arrowheads indicate the SLG bands. The pf gradient is shown at the right.](image)
primary transgenic *B. rapa* cv. Candle or by cross-pollination with tester plants of the self-incompatible *B. campestris* *S*<sub>0</sub> and *S*<sub>0</sub> homozygotes. A genomic PCR analysis of the *T*<sub>3</sub> progenies indicated that six out of seven selfed progenies, four out of twelve from cross-pollination with the *S*<sub>0</sub> homozygote, and four out of seven from cross-pollination with the *S*<sub>0</sub> homozygote, carried the transgene. These segregation data indicate that the transgenic plant had one copy of the antisense gene, which is consistent with the data from DNA gel-blotting (Fig. 2).

We further examined the effect of the transgene on the function of the endogenous *SLG* gene in the *T*<sub>3</sub> progenies. The *T*<sub>3</sub> progenies (Table 1) were obtained by self-pollination of a *T*<sub>2</sub> progeny (*S*<sub>0</sub>*S*<sub>0</sub> haplotype: *S*<sub>0</sub> indicates *S*-allele from the primary transformant) which retained a transgene and self-compatibility phenotype. To unify the *S* haplotype, we selected only *S*<sub>0</sub>*S*<sub>0</sub> heterozygotes from the *T*<sub>3</sub> progenies. A genomic PCR analysis of the transgene revealed that *T*<sub>3</sub>-13 inherited the transgene and self-compatibility phenotype, while *T*<sub>3</sub>-12 and *T*<sub>3</sub>-15 did not. Consistent with this inheritance, *T*<sub>3</sub>-13 showed the self-compatible phenotype as well as that of the primary transformed plant, while *T*<sub>3</sub>-12 and *T*<sub>3</sub>-15 showed the self-incompatibility phenotype. However, the self-compatible phenotype of *T*<sub>3</sub>-13 was not complete, and a relatively low number of pollen tubes penetrated into the self-stigma (30–50 pollen tubes per stigma). An SDS-immunoblot analysis revealed that the stigmatic expression of the SLG protein was less in *T*<sub>3</sub>-13, although its suppression level was lower than that in the original transformant (Fig. 6). These results indicate that the antisense *SLG* gene was inherited by the progenies with the ability to break down SI; however, the effect of the transgene was not stable in the progenies.

We further examined whether the inhibition of *SLG* and *SRK* would have any effects on the SI phenotype of pollen in the transgenic plants. *T*<sub>3</sub> progenies obtained by cross-pollination of the transformant with *S*<sub>0</sub> homozygotes were back-crossed with parental *S*<sub>0</sub> and the pollen-tube growth on the stigmas was examined. Table 1 shows that the stigmas of self-compatible *T*<sub>3</sub> progenies carrying the transgene were all compatible with the pollen of each parent,
while the pollen from the transgenic plants was rejected by the stigma of the self-incompatible \(^{S_r}\) homozygote. Furthermore, reciprocal crosses among the \(T_3\) progenies showed that the stigmas of self-compatible \(T_3\) progenies carrying the transgene were all compatible with the \(T_2\)-12 and \(T_2\)-15 pollen, while the pollen from transgenic plants was phenotypically identical to the \(T_2\)-12 and \(T_2\)-15 pollen. These results indicate that the antisense \(SLG\) gene was able to change the self-incompatibility phenotype of the stigma, but not of the pollen.

**Discussion**

As a strategy to confirm the involvement of the \(SLG\) and \(SRK\) genes in self-incompatibility, we used antisense RNA. The cDNA of the \(SLG_r\) gene was used as an antisense gene because multiple-sequence alignment of the published data for \(SLG\) showed that \(SLG_r\) shares more than 75% DNA sequence homology with them, except for the class II \(S\) allele.\(^{20}\) In fact, the \(SLG_r\) gene was hybridized to genomic DNA of the recipient plants under highly stringent hybridization conditions (data not shown). Therefore, antisense RNA of \(SLG_r\) was expected to inhibit the endogenous \(SLG\) gene of the recipient. We further assumed that the endogenous \(SRK\) transcript would also be inhibited by antisense \(SLG_r\) mRNA because of the high DNA sequence similarity between the \(SLG\) gene and the putative receptor domain of the \(SRK\) gene.\(^{4,12,30}\) Furthermore, the \(SLG\) and \(SRK\) genes are coordinately regulated temporally and spatially in the stigma.\(^{5,9}\) Therefore, the hybrid of the CaMV 35S truncated promoter and the \(SLG_r\) promoter was used to achieve complete inhibition of stigma \(SLG\) and/or \(SRK\).

Two transgenic plants were obtained, and one of them was further analyzed. Endogenous \(SLG\) was reduced to about 25% of the level in the control plant, and \(SRK\) was also reduced to an undetectable level. We obtained many progenies by self-pollination of the transformant and by its cross-pollination with different \(S\) lines. A pollination analysis of the progenies showed that most of the plants having the transgene were self-compatible. In one line of progenies with the antisense gene, the endogenous \(SLG\) protein had almost disappeared. Furthermore, when this plant was self-pollinated, it produced numerous seeds, as many as by the original transformant. These results confirm the participation of the genes in the self-incompatibility response.

With self-pollination of the transgenic plant, the number of invading tubes was somewhat lower than with cross-pollination, and the resulting siliques were small and seed yields were low. This is explainable by the idea that inhibition by the transgene was not complete and a few transcripts were still expressed. The incomplete rejection of pollen tubes by antisense gene expression, as described here, has also been documented for the gametophytic self-incompatibility system of *Petunia*.\(^{31}\) A similar result has also been obtained for *Brassica* by the gene silencing of \(SLG\) and \(SRK\).\(^{32}\)

The Western blot analysis revealed that the content of the endogenous \(SLG\) protein in the self-compatible \(T_3\) progeny was clearly higher than that of the original transformant, and the self-pollinated \(T_3\) plants subsequently accepted a lower number of pollen tubes and set a lower number of seeds than the original transformant. Furthermore, the self-pollinated \(T_2\) progenies obtained by self- or cross-pollination of the \(T_3\) plant also exhibited a variable degree of self-pollen acceptance. There are many reports about the reflectable phenotypes of posterity when the level of transcripts is low.\(^{32,33}\) Thus, the variety of self-incompatibility phenotypes of the \(T_3\) or \(T_2\) progeny might be ascribable to the ability of the antisense RNA transcripts, and the SI phenotypes might therefore reflect the expression level of the antisense RNA transcripts. This incomplete inhibition supports the idea that the effect was the result of the action of the antisense gene rather than an unspecified effect of the introduced gene on the expression of \(SLG\) and \(SRK\).

An analysis of the \(SLG\) promoter has indicated that it was expressed in both the pollen and stigma of *Brassica*.\(^{20}\) Interestingly, it has been suggested that the loss of endogenous \(SLG\) would also affect the pollen phenotype.\(^{5}\) Back-crossing of the self-compatible \(T_3\) progenies with parental \(S_r\) homozygotes indicated that the properties of the stigmas changed and they accepted the pollen of the parent. However, the pollen of the transformant was not accepted by the stigma of the parental homozygotes, indicating that the pollen phenotype of the transformant was not changed at all. These data were also ascertained by reciprocal pollination between self-compatible and self-incompatible \(T_3\) progeny with the \(S_r\) allele. Our pollination data clearly indicate that \(SLG\) and/or \(SRK\) are necessary to the self-incompatibility system of only the stigma, and do not work as pollen phenotype determinants.

In *Brassica*, the self-incompatibility phenotype is determined sporophytically. To date, two \(S\) locus-linked genes, \(SLG\) and \(SRK\), have been considered to be implicated in the pollen-stigma recognition of the self-incompatibility system, and some models have been presented.\(^{34}\) In this study, we have provided strong evidence that \(SLG\) and/or \(SRK\) are involved in the SI system. However, many points remain to be solved to understand the molecular mechanism of the SI system in *Brassica*. First, whether both \(SLG\) and \(SRK\) are necessary or only one is enough for SI needs to be determined. \(SRK\) is a serine/threonine kinase and should be concerned in transmitting signals into a papillar cell which would express an inhibition response to the invading pollen. If this is valid, then
what is the role of SLG? To answer this question, the functions of SRK and SLG should be separately analyzed. Furthermore, finding the pollen ligand would also be very important to answer the question of whether SLG plays a role when the ligand in the pollen is bound to the receptor domain of SRK. Some small peptides recently been found in the pollen coat through in vitro binding assay to SLG and its related proteins. 35-37 These studies might be useful to explain the role of SLG and the mechanism for pollen stigma interaction.

The molecular mechanisms leading to the inhibition of self-pollen on the stigma after SRK are still poorly understood. Recent data have indicated that an aquaporin-like gene might be necessary for the self-incompatibility response in Brassica. 38 Absorbing water from the stigma is an early event of pollination, 39 but the relationship between aquaporin and the self-incompatibility reaction is still uncertain. Furthermore, the result of a yeast two-hybrid experiment have suggested that the SRK kinase domain could interact with the thioledoxin-h-like gene and ARC1 to phosphorylate it under in vitro conditions. 40,41 These genes might be implicated in the self-incompatibility mechanism. Although we have proved the participation of SLG and SRK in the self-incompatibility response by transformation of the anti-SLG gene, further evidence is still required for a full understanding of their roles in the self-incompatibility system.

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References

17) Yu, K., Schafer, U., Glavin, T. L., Goring, D. R., and Rothstein, S. J., Molecular characterization of