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The Self-Incompatibility Phenotype in Brassica Is Altered by the Transformation of a Mutant *S* Locus Receptor Kinase

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The self-incompatible (SI) *Brassica napus* line W1, which carries the *910 S* allele, was transformed with an inactive copy of the *910 S* locus receptor kinase (*SRK*) gene. Two transformed lines were analyzed based on their heritable ability to set self-seed. The first line was virtually completely self-compatible (SC), and reciprocal pollinations with the original W1 line demonstrated that only the stigma side of the SI phenotype was altered. An analysis of the expression of endogenous *SRK-910* demonstrated that the mechanism of transgene action is via gene suppression. Furthermore, the expression of the *S* locus glycoprotein gene present in the *910* allele (*SLG-910*), *SLG-A10*, which is derived from a nonfunctional *S* allele, and an *S* locus–related gene were also suppressed. When the transgene was crossed into another SI line carrying the *A14 S* allele, it was also capable of suppressing the expression of the endogenous genes and of making this line SC. The second transgenic line studied was only partly SC. In this case as well, only the stigma phenotype was affected, although no gene suppression was detected for endogenous *SRK-910* or *SLG-910*. In this line, the expression of the transgene most likely was causing the change in phenotype, and no effect was observed when this transgene was crossed into the other SI line. Therefore, this work reinforces the hypothesis that the *SRK* gene is required, but only for the stigma side of the SI phenotype, and that a single transgene can alter the SI phenotype of more than one *S* allele.

INTRODUCTION

Brassica napus is an amphidiploid plant that is normally self-compatible (SC), whereas its presumptive progenitors, *B. oleracea* and *B. rapa*, are diploid self-incompatible (SI) species (Downey and Rakow, 1987). Self-incompatibility in *Brassica* spp is of the sporophytic type in which the pollen parent genotype determines the phenotype of the pollen. Many SI alleles have been identified genetically, and pollen derived from a plant that carries the same allele as that present in the stigma is rejected (Bateman, 1955; Ockendon, 1974). These alleles are generally codominant with each other, and self-incompatibility is dominant over self-compatibility (Ockendon, 1974, 1982).

SI alleles can be transferred into *B. napus* from the progenitor species via interspecific crosses, with the resulting individuals being repeatedly backcrossed to the *B. napus* parent, thus generating SI lines (Goring et al., 1992a, 1992b). The two SI lines used in this study are called W1 and T2. W1 carries the *910 S* allele (Goring et al., 1992a), and T2 carries the *A14 S* allele (Goring et al., 1992b). Other than the region involved in self-incompatibility, these lines are virtually isogenic pairs, with the original SI lines used as the recurrent parent (for these lines, W1 is similar to Westar and T2 is similar to Topas).

The recognition of self-pollen and its subsequent rejection involve a very rapid process. For strong *S* alleles, like the *910* allele, very little of the pollen will even hydrate, thus preventing subsequent germination. The pollen that does germinate forms tubes that grow for a short distance and virtually never penetrate the stigma surface. Given the sporophytic nature of *Brassica* spp self-incompatibility, it has been postulated that some factor is produced in the tapetum or premeiotically in the diploid meiocytes of the parental plant (Pandey, 1958) and that this factor is present on the outer surface of the pollen exine and is recognized by something on the stigma surface that prevents the normal pollen germination and fertilization processes from occurring (de Nettancourt, 1977).

Two genes have been found to be associated with the SI phenotype. The first to be discovered codes for the *S* locus glycoprotein (SLG), which is expressed primarily in stigma

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papilla cells, although some transcript has been detected in anther tissue (Kandasamy et al., 1989). The sequences of the *SLG* gene from different *S* loci vary considerably (80 to 90% similarity), which is reasonable for a gene that encodes a product that might be involved in self-recognition. The second gene codes for a protein called the *S* locus receptor kinase (SRK) (Stein et al., 1991). This is a protein with a receptor-like domain very similar in sequence to the SLG, a transmembrane domain and a kinase domain that has been shown to have serine/threonine kinase activity (Goring and Rothstein, 1992). The receptor portion has some similarity in sequence to immunoglobulin-like receptor domains in animal cells (Glavin et al., 1994) and shows a similar sequence heterogeneity between genes derived from different alleles as that seen for *SLG* (Glavin et al., 1994). This gene is also expressed in the stigma papilla cells (Stein et al., 1991; Goring and Rothstein, 1992). There are a variety of *SRK*-like genes in *Brassica* spp (Kumar and Trick, 1994) as well as genes similar to the *SLG* genes that have been shown to be unlinked to self-incompatibility (Lalonde et al., 1989). The functions of most of these genes are unknown, although the expression of one of these has been correlated with wound and pathogen response (Pastuglia et al., 1997).

The following hypothesis for self-recognition has been formulated based on these experimental observations and on what is known about receptor kinase interactions with their specific ligands in mammalian cells. The SRK protein would form a receptor complex in the stigma papilla cells that recognizes an allele-specific ligand present on the pollen surface. The SLG protein either would be involved in bringing the ligand into contact with the SRK protein or would serve as part of the receptor complex. The pollen ligand is thought to be a protein that is encoded by a separately linked gene and that is recognized only by the receptor complex encoded by the same allele. Alternatively, in theory, the ligand could be SLG itself activated in the pollen by the binding of another molecule (Stein et al., 1991; Goring and Rothstein, 1992, 1996).

Although this hypothesis is reasonable, a number of aspects of this model have not been experimentally demonstrated. The involvement of the SRK and SLG in self-incompatibility has been supported by their linkage to the *S* locus and also by an analysis of plants selected for self-compatibility, with neither *SLG* (Nasrallah et al., 1992) nor *SRK* (Nasrallah et al., 1994) being expressed in these plants at normal levels. In addition, SC *B. napus* has an *S* allele with an *SRK* gene having a 1-bp deletion that would encode a truncated protein product (Goring et al., 1993), and transformation of a *B. campestris SLG* gene into *B. oleracea* was found to disrupt the SI phenotype in some lines (Toriyama et al., 1991). However, whereas the DNA sequence diversity of the *SRK* and *SLG* genes implies that these are most likely involved in self-recognition, this has not been demonstrated biochemically, nor has the pollen ligand been identified. Finally, the process by which recognition of pollen carrying the same allele leads to its rejection has not been delineated, although it has been demonstrated that *SRK* binds to thioredoxin-h, and this might be part of the signal transduction process (Bower et al., 1996).

We wished to develop a transgene that would prevent the functioning of SRK. In mammalian cells, considerable work has been done with tyrosine receptor kinases. In these cases, the binding of the specific ligand leads to the dimerization of the receptor kinase and thus stimulates kinase activity, which in turn leads to the change in phenotype through a variety of signal transduction processes (Ullrich and Schlessinger, 1990). In a number of cases, the expression of a mutant receptor kinase turned out to prevent the functioning of the wild-type receptor kinase. For example, in mouse, several mutant forms of the c-Kit receptor tyrosine kinase exist. One particular mutation, W^{41}, produces normal levels of the c-Kit protein, but with reduced autophosphorylation activity due to a point mutation in the kinase domain (Reith et al., 1990). Because $W^{41}/+$ heterozygous mice show a stronger dominant phenotype compared with other loss-of-function c-Kit mutations, the W^{41} mutation was proposed to prevent signaling by the functional c-Kit protein because of the formation of defective heterodimers (Reith et al., 1990).

Previously, we had shown that a single amino acid substitution mutation in the kinase domain of the SRK prevented kinase activity in vitro (Goring and Rothstein, 1992). Therefore, we made the same mutation in *SRK-910* and transformed this mutant gene into the W1 line carrying this allele. Two SC transgenic lines were analyzed in detail and found to be affected only in the stigma side of the SI phenotype. In one case, the presence of the transgene led to the suppression of the expression of the *SRK-910* and *SLG-910* genes as well as the corresponding genes present in other alleles. In the other case, the expression of the endogenous genes was not significantly affected, and in this case, it is likely that the expression of the mutant *SRK* was having an effect. This work strongly supports the importance of SRK for the stigma side of the SI phenotype but at the same time does not exclude the involvement of the SLG in the SI pathway.

RESULTS

Chimeric Genes and Plant Transformation

The kinase coding region of the *SRK-910* gene was modified so that the codon at position 557 now codes for an alanine instead of a lysine. This substitution had earlier been shown to prevent kinase activity completely when it was expressed in *Escherichia coli* (Goring and Rothstein, 1992). Mutated *SRK-910* was then remade so that the only difference between the wild-type and mutant versions was this substitution. Our assumption was that this mutant version would be able to be assembled into a receptor complex, but the deficiency in kinase activity would prevent it from functioning.

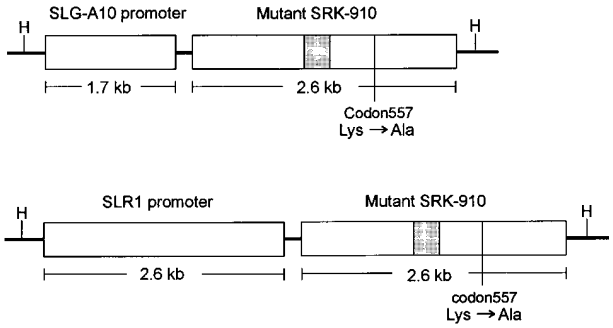

Figure 1. Schematic Representation of the Transgene Constructs.

Shaded areas designate the transmembrane domain. H, HindIII site.

Two regulatory regions were used to express the mutant *SRK-910*, as shown in Figure 1. The first used the promoter from the *SLG-A10* gene, which is expressed at very high levels in stigma tissue (Goring et al., 1992b). The second was the promoter from the *S* locus–related *SLR1* gene, which is expressed at even higher levels in stigmas (Franklin et al., 1996) and had been shown to work well in transgenic plants.

These chimeric genes were transformed into the SI cultivar W1, which carries the *910* allele, and the resulting transformants were analyzed for their ability to set self-seed. Of the 25 transformants that were transformed with the *SLG-A10* mutant *SRK* construct, only one showed a heritable increase in the production of self-seed. Of the 11 *SLR1* mutant *SRK*-transformed lines tested, two showed an increase in the amount of self-seed, although only one of these did so in a consistent fashion in the progeny. Therefore, one line transformed with each of the two constructs was studied further.

Phenotype of the Lines Transformed with the Mutant *SRK* Gene

The transformed lines were analyzed for their ability to set self-seed, the level of fertility when cross-pollinated with the W1 line, and the ability of the pollen to germinate and form pollen tubes. The *A10* mutant *SRK*-transformed line had only a slightly lower level of seed set as the SC line Westar had when it was self-pollinated. Of the 17 individuals that carried the transgene tested over two generations, the average self-seed set was 19 per pod compared with ~29 per pod for Westar, as shown in Table 1. The W1 line, derived from an introgression of the *S* locus from *B. rapa* into Westar, in general shows a noticeably weaker vigor compared with Westar. This could account in part for the overall inability of these transgenic W1 plants to be fully restored to the fertility of Westar. There were 11 individual plants derived from the original transgenic line tested in which the transgene was no longer present because of segregation, and all of these were completely SI, demonstrating that the change in phenotype was caused by the transgene. When reciprocal crosses were done with the W1 line, pollen from W1 was able to fertilize the transgenic line, yielding an average of 22 seeds per pod. However, pollen from the transgenic line was rejected by the W1 line with virtually no seed set (Table 1).

An analysis of pollen germination and tube growth corresponded exactly with the seed set results, with self-pollen and pollen from W1 both being able to germinate on the transgenic line and their pollen tubes having normal growth. Using fluorescence microscopy of aniline blue–stained tissues, Figures 2A and 2C show that this transgenic line, when crossed with W1 pollen, closely resembles the typical SC response seen in Westar (Figure 2E). In contrast, pollen from the transgenic line would not germinate on W1 stigmas (Figures 2B and 2D), and these pollinations led to the typical production of excess callose on the stigma surface, which one normally sees in an incompatible response as in W1 selfed (Figure 2F). Therefore, only the stigma side of the SI phenotype was affected by the transgene, with no noticeable change in the pollen phenotype.

The second transgenic line with the *SLR1* mutant *SRK* gene did not show a complete restoration of self-fertility. The average seed set, although much higher than for the SI W1 line, was ~25% of that seen for the SC Westar line (Table 1). Therefore, in this case, there was only a partial breakdown in the SI phenotype. Crosses between this line and

Table 1. Average Seed Set per Pod for Two Transgenic Lines When Selfed or Reciprocally Crossed with W1

Female × Pollen	Westar (Self)	W1 (Self)	*A10* Mut *SRK*[a] (Self)	W1 × *A10* Mut *SRK*	*A10* Mut *SRK* × W1	*SLR1* Mut *SRK*[a] (Self)	W1 × *SLR1* Mut *SRK*	*SLR1* Mut *SRK* × W1
Seeds/pod	29.0	0.2	19.4[b]	0.1	21.6	7.4[c]	1.5	5.8
No. of pods	98	85	164	35	40	61	64	65
No. of plants	18	4	17	4	7	6	14	10

[a] *A10* and *SLR1* mutant (Mut) *SRK* plants are in a W1 background.

[b] Values involving the *A10* mutant *SRK* plants represent combined data from first and second selfed generation descendants of the original transgenic plant.

[c] Values involving the *SLR1* mutant *SRK* plants represent data from first selfed generation descendants of the original transgenic plant.

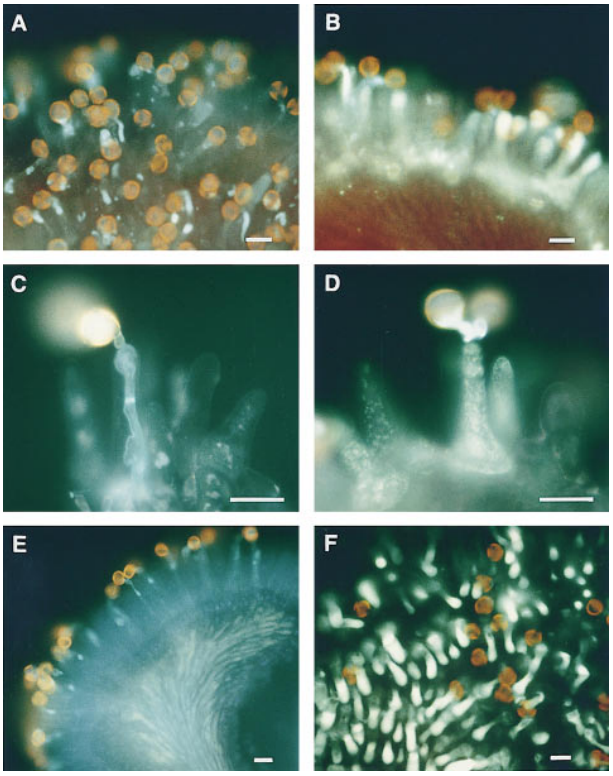

Figure 2. Analysis of Pollinated *B. napus* Stigma Tissue by Using Fluorescence Microscopy.

(A) Stigma of an *A10* mutant *SRK*-transformed plant pollinated by W1.
(B) Stigma of a W1 plant pollinated by the *A10* mutant *SRK* transgenic W1 line.
(C) Enlarged view of stigma papilla cells shown in **(A)**.
(D) Enlarged view of stigma papilla cells shown in **(B)**.
(E) Stigma of a Westar plant pollinated by a transgenic line demonstrating an SC response.
(F) Stigma of a W1 self-pollinated plant demonstrating an SI response.
(A) to **(F)** represent 16-hr postpollinated tissue stained with aniline blue and visualized under UV light to detect callose accumulation. Bars = 50 μm.

W1 again demonstrated that only the stigma phenotype was affected, with seed being formed only when the W1 pollen was used to fertilize the transgenic line and the reciprocal cross giving minimal seed set (Table 1). In this case, when either self-pollen or W1 pollen was applied to stigmas of the transgenic line, a strong callose reaction occurred, and only a small percentage of pollen would germinate and form pollen tubes (data not shown). This supports the notion that this line is intermediate in phenotype between SC and SI lines in the stigma, with the pollen phenotype not being affected.

The phenotypes of the seed pods produced after self-pollination for the SC line Westar, for the original SI line W1, for the W1 line transformed with the *A10* mutant *SRK* transgene, and for the W1 line transformed with the *SLR1* mutant *SRK* transgene are shown in Figures 3A to 3D. As can be seen, the fully developed pods for the W1 line transformed with the *A10* mutant *SRK* transgene (Figure 3C) are very similar to those seen in the SC line Westar (Figure 3A). The pods produced by the *SLR1* mutant *SRK* transgene are consistently reduced in size and seed content (Figure 3D).

Detection of the Mutant *SRK* Transgenes by DNA Gel Blot Analysis

To determine the presence and approximate copy number of the *A10* mutant *SRK* and *SLR1* mutant *SRK* transgenes, DNA blots of HindIII-digested genomic DNA from progeny derived from each of the two transgenic lines were probed with the SLG-910 cDNA, which cross-hybridizes with *SRK-910* and the transgenes (Figure 4). Because these two lines were no longer segregating, they were presumably homozygous for the transgene. All of the progeny derived from the *A10* mutant *SRK* transgenic line produced the expected 4.5-kb band, whereas the progeny from the *SLR1* mutant *SRK* line yielded a fragment of 5.4 kb. By comparing the relative intensity of these bands to the 3.6- and 6.4-kb bands representing the single-copy haploid genome of the *SRK-910* and *SLG-910* genes in WI, we estimate one copy per haploid genome for the *A10* mutant *SRK* transgene and approximately five copies per haploid genome for the *SLR1* mutant *SRK* transgene. The low-stringency wash conditions also enabled the detection of the *A10 S* locus genes present

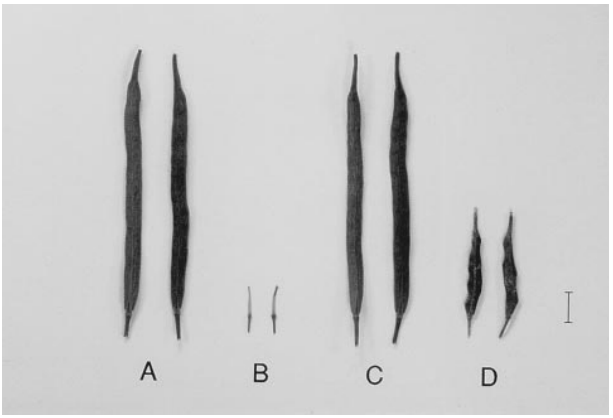

Figure 3. Comparison of Mature Self-Seeded *B. napus* Pods of Two Transgenic Lines and Two Control Plants.

(A) *B. napus* cv Westar (SC).
(B) *B. napus* cv W1 (SI).
(C) W1 carrying the *A10* mutant *SRK* transgene.
(D) W1 carrying the *SLR1* mutant *SRK* transgene.
Bar = 1 cm.

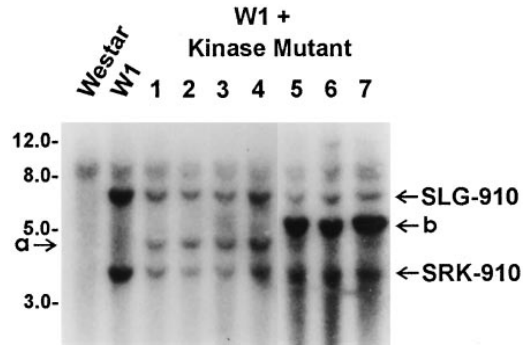

Figure 4. Genomic DNA Gel Blot Analysis of the Transgenic Lines of *B. napus* cv W1.

DNA was restricted by HindIII to excise the transgene. Lanes 1 to 4 represent plants containing the *A10* mutant *SRK* transgene (the 4.5-kb fragment is indicated by the letter a), and lanes 5 to 7 contain the *SLR1* mutant *SRK* transgene (the 5.4-kb fragment is indicated by the letter b). The blot was probed with the SLG-910 cDNA. The numbers at left indicate the positions of 1-kb DNA ladder bands (Bethesda Research Laboratories).

in Westar (*SRK-A10* and *SLG-A10* are on 9.6- and 8.4-kb fragments, respectively) due to cross-hybridization.

Expression of the *SLG* and *SRK* Genes in the *A10* Mutant *SRK* Line

To determine the mechanism by which the *A10* mutant *SRK* transgene was affecting the phenotype, the levels of the *SRK-910* and *SLG-910* transcripts present in floral buds were analyzed by using reverse transcription–polymerase chain reaction (RT-PCR) and RNA gel blot analyses, respectively (Figures 5 and 6). It is clear from these results that the mutant *SRK* transgene is working via cosuppression, with both the *SLG* and *SRK* transcripts being virtually completely absent. The receptor portion of the *SRK-910* gene is 90% similar in sequence to the *SLG-910* coding region, and this is clearly sufficient to induce the cosuppression effect. As predicted, individual plants having lost the transgene through segregation were observed to express close to wild-type levels of each affected transcript (data not shown).

In *B. napus*, there is also the nonfunctional *A10* allele present in the a genome (Goring et al., 1993) (the *910* allele was originally derived from *B. rapa* and is thus in the c genome). The level of the *SLG-A10* transcript was also analyzed using RNA gel blot hybridization (Figure 6). It is clear that this transcript was also present at very low levels. *SRK-910* is 85% similar in DNA sequence to the *SLG-A10* coding region. Therefore, the transgene suppresses the expression of *SLG-910* and *SRK-910* as well as *SLG-A10*, which is present at the *S* locus in the other genome of *B. napus.*

Brassica spp also contain *SLR* genes that have been shown to be unlinked to the *S* locus, and it has been demonstrated that an antisense gene that prevents *SLR1* expression level has no effect on the SI phenotype (Franklin et al., 1996). One such example is the highly conserved *SLR1* gene, which has only 66% sequence homology with the receptor domain of the *SRK-910* transgene. RNA gel blot hybridization was performed using the SLR1-A29 cDNA probe (Goring et al., 1992b). The abundant SLR1 transcript was affected, but the level of suppression was not as absolute as with the SLG-910 and SLG-A10 transcripts (Figure 6).

Effect of the *SLR1* Mutant *SRK* Transgene on Expression of the *SLG* and *SRK* Genes

The expression of *SLG-910* and *SRK-910* was analyzed in the line carrying the *SLR1* mutant *SRK* transgene. In this case, there was no significant decrease in the expression of these genes, as seen in Figures 7A and 7B. The mutant *SRK* transgene is expressed (see Figure 7C). However, even though the *SLR1* promoter used is expressed at high levels (Franklin et al., 1996), transgene expression could not be detected on an RNA gel blot (data not shown) and is likely in a range similar to that of wild-type *SRK-910* (Figure 7B). Therefore, in this case, cosuppression is not likely to be the mechanism involved in the partial breakdown of the SI phenotype. Instead, the expression of the transgene and presumably the production of the mutant SRK-910 protein

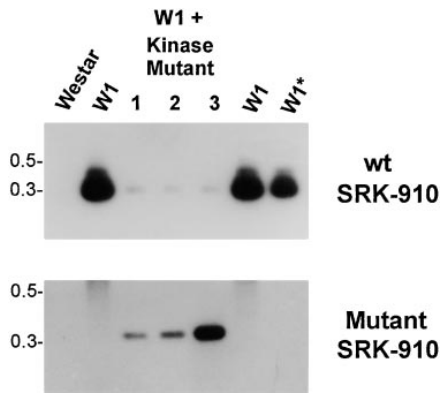

Figure 5. RT-PCR Analysis of Transcripts of the *A10* Mutant *SRK* Transgenic W1 Line.

RNA samples were reverse transcribed and then amplified for 25 cycles with gene-specific primers. The three individual W1 plants carrying the *A10* mutant *SRK* transgene are labeled 1, 2, and 3. The designations at right indicate the copy of the *SRK* gene amplified by PCR. Blots were probed with the *SRK-910* gene. The numbers at left indicate the positions of the 1-kb DNA ladder (Bethesda Research Laboratories). W1* denotes a self-seeded progeny from a transgenic plant in which the transgene was lost through segregation. wt, wild type.

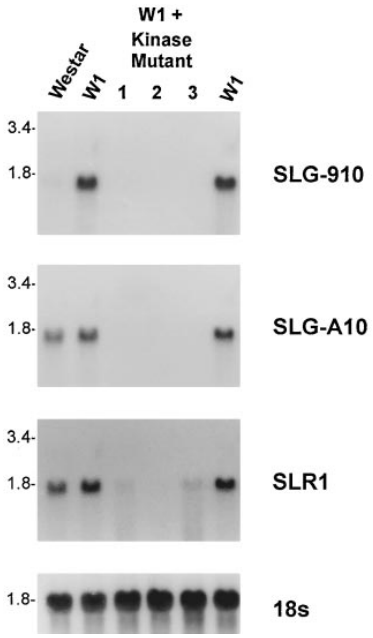

Figure 6. RNA Gel Blot Analysis of the *A10* Mutant *SRK* Transgenic W1 Line.

The three individual W1 plants carrying the *A10* mutant *SRK* transgene are labeled 1, 2, and 3. The *B. napus* cDNA probes are indicated at right. The positions of the 18S (1.8-kb) and 25S (3.4-kb) rRNA bands are marked at left.

must have an effect, although the change in phenotype is not complete.

Transfer of the Mutant *SRK* Transgenes into a Line with a Different Functional *S* Allele

B. napus line T2 carries the *A14 S* allele through introgression into the line Topas (Goring et al., 1992b). Progeny from the original transgenic line carrying the *A10* mutant *SRK* chimeric gene were used as a pollen donor in a cross with T2. Progeny from this cross would be expected to have the transgene, the *910 S* allele present in the W1 line, and the *A14 S* allele found in T2 all present in a heterozygous state. This indeed turned out to be the case when these were analyzed for their presence by PCR and by their segregation into the subsequent generation (data not shown). As shown in Table 2, these plants set self-seed at a high rate only slightly lower than the SC line Topas and much higher than is seen for the T2 × W1 hybrid line. This line, which is heterozygous for the *A14* and *910 S* alleles, produces somewhat greater seed set than either the T2 or W1 parental lines. The first T2-backcrossed generation (BC-1) of these hybrids was also analyzed for self-seed set as well as for seeding rates when reciprocally crossed with T2 (see Table 3). The BC-1 generation plants containing the transgene exhibited a strong SC stigma phenotype indicated by the high seed set values, whereas the plants without the transgene were clearly SI. The pollen of the BC-1 plants was ineffective in fertilizing the T2 line.

Expression of *SLG-A14* was analyzed, and its transcript was present at very low levels, as was the *SLG-910* in the original hybrid plants (Figure 8). As expected, transcript levels of both of these genes were restored in hybrid plants that had lost the kinase mutant transgene (Figure 8). *SLG-A14* is 83% similar in its coding sequence to *SRK-910.* Therefore, not only can the *A10* mutant *SRK* transgene prevent the functioning of the *910 S* allele, but it can also prevent the phenotypic expression of a different functional allele.

To determine whether the *SLR1* mutant *SRK* transgene was operating in an allele-specific manner, this transgene also was crossed into the SI line T2. In contrast to the *A10* mutant *SRK* transgene, the seed set phenotype of this hy-

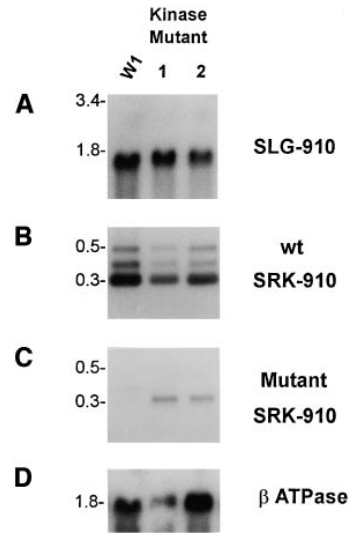

Figure 7. RNA and RT-PCR Analyses of the *SLR1* Mutant *SRK* Transgenic W1 Line.

The two individual W1 plants carrying the *SLR1* mutant *SRK* transgene are labeled 1 and 2.
(A) and **(D)** RNA gel blots probed with the *B. napus* gene indicated at right.
(B) and **(C)** Blots containing cDNA PCR amplified with primers specific for the gene indicated at right and probed with the *SRK-910* gene.
In **(B)**, the 0.3-kb band represents the amplified fragment of the mature RNA transcript, whereas the larger fragments are due to incomplete splicing of one or both of two small introns. The positions of the 18S (1.8-kb) and 25S (3.4-kb) rRNA bands are marked on the RNA blots, whereas the 1-kb DNA ladder (Bethesda Research Laboratories) bands are marked on the cDNA gel blots. wt, wild type.

Table 2. Average Self-Seed Set per Pod for Two Transgenic Lines Crossed into T2

Plant Line	Topas	T2	T2 × W1	*A10* Mutant *SRK*[a]	*SLR1* Mutant *SRK*[a]
Seeds/pod	25.2	0	3.5	23.1	2.6
No. of pods	70	61	64	40	145
No. of plants	7	7	7	6	10

[a] *A10* and *SLR1* mutant *SRK* plants are in a T2 × W1 hybrid background.

brid line showed no increase in self-seed values when compared with an equivalent T2 × W1 hybrid line lacking this transgene (Table 2). Analysis of the expression levels of *SRK-A14* and *SLG-A14* (Figures 9A and 9B) revealed no significant effect on the *A14* allele by the presence of this *SLR1* mutant *SRK* transgene. Similarly, the expression of *SRK-910* and *SLG-910* was not affected in these plants (data not shown). Finally, using RT-PCR, the expression of this transgenic copy of *SRK-910* was detected at roughly equal abundance in these hybrids as in the original transgenic line (data not shown).

DISCUSSION

The SI phenotype requires the specific recognition and subsequent rejection of pollen derived from a plant carrying the same *S* allele as that present in the female plant. The role of the *SRK* gene as the receptor that recognizes a pollen factor has been implied by the structure of the SRK protein (Stein et al., 1991; Glavin et al., 1994), by analysis of several SC lines in which this gene is downregulated (Nasrallah et al., 1994) or altered in the coding region (Goring et al., 1993), and by its linkage to the *S* locus (Stein et al., 1991; Goring and Rothstein, 1992). Furthermore, the variability in the coding sequence in this gene from different alleles is consistent with its role in allele-specific recognition. Recently, it has been shown that transformation of *SRK* gene constructs into *B. oleracea* led to the suppression of *SRK* and *SLG* expression and the loss of the self-incompatibility phenotype (Conner et al., 1997). The change in the SI phenotype caused by the transformation of the mutant *SRK* gene into *B. napus* described here strongly supports this hypothesis.

It is clear that the effect of the transgene is only on the stigma side of the SI phenotype. This is true in the *A10* mutant *SRK*-transformed line, in which the expression of both the *SLG* and *SRK* genes is reduced to very low levels, as well as in the *SLR1* mutant *SRK* plants, in which the transgene does not effect endogenous gene expression of either gene. Therefore, it seems likely that both *SRK* and the *SLG* only function in the stigma and that there must be some other gene involved in the pollen phenotype. This is supported further by some preliminary results in which an *SRK* antisense gene was tested by using a promoter expressed only in tapetal tissue. Surprisingly, one line was SC, but only in the stigma part of the phenotype (R. Stahl and S. Rothstein, unpublished results). This is presumably due to the site of insertion of the transgene and supports the notion that SRK is only involved in the stigma part of the phenotype.

There are a wide array of transgenes that have been found to suppress the expression of endogenous genes (reviewed in Flavell, 1994). The mechanism of this suppression has been found to be post-transcriptional in most studies, although in some cases it may be due to modifications in the gene itself, such as methylation (Jorgensen, 1990). In the case of the *A10* mutant *SRK*-transformed line, the transgene suppresses the expression of the endogenous *SRK-910* gene but also suppresses expression of the *SLG-910* gene and genes from other alleles. These range from 83 to 100% in similarity to the transgene. A wide array of *SLG*- and *SRK*-like genes have been found in *Brassica* spp (Lalonde et al., 1989; Kumar and Trick, 1994) as well as in other species. For example, the *SLR1* gene is related in sequence to *SLG*, but it has a different chromosomal location. It has been shown that a marked decrease in its expression due to the

Table 3. Average Seed Set per Pod for a Segregating First Backcross Generation

Female × Pollen	*A10* Mutant *SRK*[a] (Self)		*A10* Mutant *SRK* × T2	
Transgene	+	−	+	−
A14 S allele	+	+	+	+
Seeds/pod	21.7	2.2	21.7	2.8
No. of pods	44	40	36	28
No. of plants	7	6	6	5

[a] *A10* mutant *SRK* plants were derived from a backcross between the introgressed hybrid generation and the T2 parental line. The hybrids were heterozygous for the transgene, the *A14 S* allele, and the *910 S* allele. The backcrossed plants showed random segregation for the two alleles; however, only those containing the *A14 S* allele were considered here. (+) and (−) indicate the presence or absence, respectively, of the corresponding genes at left in the backcrossed female plants.

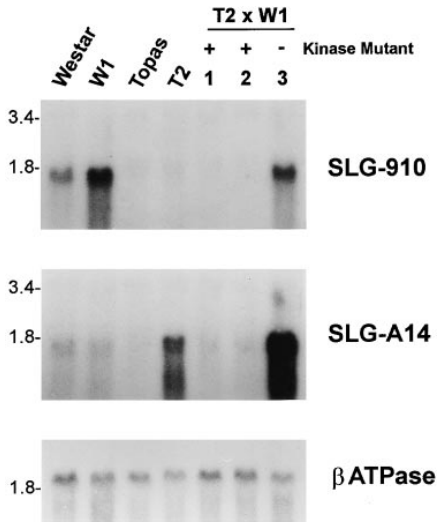

Figure 8. RNA Gel Blot Analysis of the T2 × W1 Hybrid Carrying the *A10* Mutant *SRK* Transgene.

The three individual hybrid plants are labeled 1, 2, and 3; (+) and (−) indicate the presence or absence of the transgene. The *B. napus* cDNA probes are indicated at right. The positions of the 18S (1.8-kb) and 25S (3.4-kb) rRNA bands are marked at left.

transformation of an antisense gene does not lead to a noticeable change in phenotype (Franklin et al., 1996). On the other hand, the *SRK*-like genes that are present in other places in the genome have been postulated to have a function in development (Kumar and Trick, 1994) or in wound response and pathogen defense (Pastuglia et al., 1997). It has been hypothesized that for multigene families, there must be a mechanism in place that protects the entire set of genes from suppression during genetic crosses (Flavell, 1994). However, this is clearly not the case here, where a single transgene can decrease the expression of an entire set of genes having considerable sequence heterogeneity. Even the *SLR1* gene, which is only 66% similar in sequence, showed considerable decrease in expression, although not as complete as that seen for the more similar *SLG* and *SRK* genes.

A dominant-negative mutation is one in which the expression of the mutant gene affects the functioning of the wild-type gene product. Generally, this occurs when the wild-type protein functions as a multimer and in which the assembly of the mutant monomer with the wild-type prevents function. The simplest explanation of the breakdown of self-incompatibility in the *SLR1* mutant *SRK* line is that the mutant *SRK* gene product is functioning in this fashion. This is supported by the observation that this transgene failed to have an effect on plants carrying a different *S* allele. One would not expect multimers to form between proteins derived from different alleles. Whether this also implies that SRK works as a dimer, as do tyrosine receptor kinases in animal cells, requires additional biochemical analysis of this protein.

In conclusion, the mutant *SRK* transgene can prevent the self-incompatibility phenotype. The mechanism of its action in one of the transgenic lines is clearly via cosuppression; in the other, the most likely explanation is that self-compatibility is caused by the expression of the mutant protein. In either case, only the female side of the self-incompatibility phenotype is affected, even when the expression of both *SLG* and *SRK* is reduced to very low levels. This strongly supports the hypothesis that another gene besides these must be responsible for the pollen phenotype. It is interesting that a single transgene can suppress the expression of a wide array of related genes and prevent the functioning of multiple *S* alleles. Given the large number of *SLG*- and *SRK*-related genes, the possibility is raised that this type of suppression could have arisen naturally, thus creating cryptic *S* alleles.

During the evolution of the *Brassica* spp, this ability to suppress the self-incompatibility phenotype would have been very advantageous on occasion. For example, the generation of the allotetraploid species *B. napus* involved an interspecific cross between a *B. oleracea* and a *B. rapa* individual, both of which are SI species. The ability of the newly formed allotetraploid species to efficiently set self-seed would have been crucial in this initial speciation stage, and suppression of *SLG* and *SRK* gene expression is a simple mechanism by which this could have occurred. Later, gene

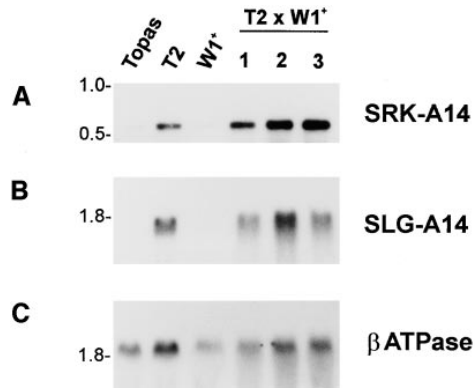

Figure 9. RNA and RT-PCR Analyses of the T2 × W1 Hybrid Carrying the *SLR1* Mutant *SRK* Transgene.

The three individual hybrid plants carrying the *SLR1* mutant *SRK* transgene are labeled 1, 2, and 3.
(A) Gel blot containing the cDNA PCR product amplified with primers specific for the *SRK-A14* gene and probed with the same gene.
(B) and **(C)** RNA gel blots probed with the genes indicated at right. The positions of the 18S (1.8-kb) rRNA bands are marked on the RNA gel blots; the 1-kb DNA ladder (Bethesda Research Laboratories) bands are marked on the cDNA blot. $W1^+$ denotes a W1 plant carrying the *SLR1* mutant *SRK* transgene.

mutation such as that seen in extant lines (Goring et al., 1993) could have occurred to ensure the maintenance of the SC phenotype. Finally, it is likely that the *A10* mutant *SRK* transgene can suppress the SI phenotype for virtually any *Brassica* spp *S* allele, and this should prove useful for the development of commercially valuable lines in several *Brassica* spp.

METHODS

Plant Transformation and the Analysis of Progeny

The self-incompatible (SI) *Brassica napus* cv W1 carrying the *910 S* locus (Goring et al., 1992a) was used as the recipient of the two transgene constructs. The 2.8-kb W1 cDNA containing the coding region and polyadenylation sequence of the *S* locus receptor kinase *SRK-910* gene had been previously modified by polymerase chain reaction (PCR) mutagenesis to introduce a lysine-to-alanine substitution at codon 557 (Goring and Rothstein, 1992). This fragment was ligated to the 1.7-kb regulatory region of the *S* locus glycoprotein *SLG-A10* gene from *B. napus* subsp *oleifera* (Goring et al., 1993) and the 2.6-kb promoter region on the *SLR1* gene from *B. napus* (Franklin et al., 1996). The assembled chimeric genes were inserted as HindIII fragments into the plant transformation vector pALL TKREP (Pioneer Hi-Bred, Johnston, IA). Plant transformations were performed using *Agrobacterium tumefaciens*–mediated methods (Moloney et al., 1989).

Selected individual plants exhibiting a self-compatible (SC) phenotype were studied in detail, and self-progeny from up to three successive generations were studied. Both transgenes were introduced into a second SI *B. napus* cultivar, T2, carrying the *A14* allele (Goring et al., 1992b) through an interspecific cross with the transgenic W1 line (as male). Backcrossed generations were made to the T2 parent (as female) to study the segregation of SI phenotype versus genotype in the progeny. The transgenes and the *S* locus alleles were screened by PCR, using gene-specific primers in addition to DNA gel blot analysis. The SI *B. napus* progenitor cultivars Westar (Agriculture Canada, Saskatoon, Canada) and Topas (Svalof AB, Savlov, Sweden) were used as controls. All plants were grown in growth cabinets under controlled conditions providing 16 hr of light at 18°C and 8 hr of dark at 15°C. Pollination bags were applied 2 days before anthesis for both self- and cross-pollination. When required, forced pollinations were made on emasculated premature flowers to circumvent the expression of the SI phenotype; otherwise, cross-pollinations were performed 2 days after emasculation onto fully open flowers. Self-incompatibility was tested by measuring seed set and by fluorescence microscopy of 16-hr postpollinated stigmas stained with aniline blue (Kho and Baer, 1968). Pollen tube growth was also observed in detail by laser scanning confocal microscopy under autofluorescing conditions.

Genomic DNA Analysis

Genomic DNA was extracted from floral bud or young leaf tissue by using a rapid alkali boiling method (Klimyuk et al., 1993) for screening by PCR or by the method of McGarvey and Kaper (1991), except that the cetyltrimethylammonium bromide concentration was increased to 3% for DNA gel blot analysis. Approximately 6 to 8 μg of DNA was digested with HindIII, size fractioned through a 0.7% agarose gel, and transferred to a Biodyne B membrane (Pall, East Hills, NY) in 10 × SSC (1 × SSC is 150 mM NaCl and 15 mM sodium citrate). DNA was cross-linked to the membrane by using the UV Stratalinker (Stratagene, La Jolla, CA). Prehybridization and hybridization steps were performed as described previously (Goring et al., 1992b). Membranes were washed for 30 min at a final stringency of 0.2 × SSC and 0.1% SDS at 60°C. Full-length purified cDNA fragments labeled by random priming (Feinberg and Vogelstein, 1983) were used as probes.

RNA Analysis

Total RNA was purified from 5- to 6-mm-long floral buds, using the guanidinium isothiocyanate phenol chloroform extraction method of Ausubel et al. (1987). Yields of RNA were quantified by UV spectrophotometry. Ten micrograms of each RNA sample was fractioned through a 1.2% agarose–formaldehyde gel and transferred to a Biodyne B membrane, as was done previously. Cross-linking and hybridization of RNA filters were the same as was done for the genomic blots. Final washing conditions consisted of 30 min in 0.2 × SSC and 0.1% SDS at 65°C. Equal loading of RNA samples was demonstrated by rehybridization of the membranes, using either the *18S* rRNA or the β-*ATPase* genes isolated from *B. napus* as probes.

The analysis of the low-abundance *SRK-910* and *SRK-A14* genes, including the transgenes, required reverse transcription (RT) followed by PCR amplification before blotting and probing, as was done for the genomic gel blots. One microgram of DNase I–treated total RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories), according to the supplier's instructions. For the *910* allele, the two different first-strand primers were designed to terminate at the introduced mutation site, thereby creating the specificity between the transgenic and endogenous copies of the *SRK-910* gene. The sequence of the wild-type *SRK-910*–specific primer consisted of 5′-CCGACGTTTTTGATAGCCTTTT-3′, whereas the mutant *SRK-910* primer read 5′-CCGACGTTTTTGATAGCCTTGC-3′. The first-strand primer for the *SRK-A14* gene was composed of 5′-TATACAACCAGTCCCACCAT-3′. Half of each cDNA sample was amplified for 25 cycles with the addition of a gene-specific upstream primer: 5′-TCTGCTTCTTATGATCATGTTC-3′ for *SRK-910* or 5′-AAAGGGATCTTTCCAGCGT-3′ for *SRK-A14.* Each cycle typically consisted of 20 sec at 94°C, 15 sec at 57°C, and 25 sec at 72°C, and 5 or 10% of the PCR product was subjected to gel electrophoresis and DNA gel blot analysis. After hybridization, membranes were washed in 0.1 × SSC and 0.1% SDS at 65°C. Control lanes included an RNA sample without reverse transcriptase and a genomic DNA positive to demonstrate primer specificity.

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