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Dominance Relationship between Two Self-Incompatible *Brassica campestris* Haplotypes in Response to CO_2 Gas

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In *Brassica*, self-incompatibility (SI) can be overcome by CO_2 application, an effective method for obtaining numerous inbred lines for F_1 commercial seed. We previously reported two different S-alleles of *Brassica campestris*, S^{733} and S^{734}, with extremely different degrees of susceptibility to this gas. In the current study, we raised a cross-population between those two genetic lines, and analyzed their reaction level of self-incompatibility to CO_2 ($RLSICO_2$). Here, all 40 of our progeny from the F_1 cross-population were susceptible, maintaining high values of $RLSICO_2$. This suggests that the susceptible line, S^{734}, is dominant to the insusceptible line, S^{733}. We also generated an F_2 selfing-population of each crossed progeny, $S^{733}♀ S^{734}♂$ and $S^{733}♂ S^{734}♀$, to assess the $RLSICO_2$ of each individual. PCR-RFLP analysis was performed to determine the S-genotype of the F_2 population. The S^{734} allele segregated in a theoretical ratio of the dominant trait, and the $RLSICO_2$ was consistent with the dominance relationship. Therefore, we have now demonstrated that high $RLSICO_2$ in *B. campestris* is controlled by a dominant gene.

Keywords: dominant, PCR-RFLP, reaction level of self-incompatibility, S locus receptor kinase

Self-incompatibility (SI) is a genetic barrier that prevents plants from inbreeding. In *Brassica*, this action is mediated by genes present at the S-locus (Watanabe et al., 2000b). Among them, two genes (*SLG* and *SRK*) that are mainly expressed in the pistil plus a pollen-expressed gene (*SCR/SP11*) have been intensively studied as the stigmatic S determinants and the pollen ligand, respectively (Stein et al., 1991; Delorme et al., 1995; Heldin, 1995; Suzuki et al., 1999; Takasaki et al., 2000; Watanabe et al., 2000a; Iwano et al., 2003). Although functioning of *SLG* in SI is still unclear, Dixit et al. (2000) have suggested that it might be required to stabilize a major component, SRK, and facilitate its accumulation to physiologically relevant levels in the stigma. Thus, SI mainly depends on the interaction between SRK and SCR/SP11. This initial recognition step then activates the SRK-mediated signaling pathway, recruiting ARC1, an E3 ubiquitin ligase (Stone et al., 2003).

SI that occurs in *Brassica* after self-pollination can be overcome by gas treatment, in which approximately 4% (w/v) CO_2 is introduced into a closed chamber containing the plants (Nakanishi et al., 1969; Nakanishi and Hinata, 1973; Dhaliwal et al., 1981; Palloix et al., 1985; Niikura and Matsuura, 2000; Lee et al., 2001; Kwun et al., 2004). However, the mechanism by which SI is overcome in this way is not yet understood. For now, researchers have demonstrated that this response is correlated with the genetic backgrounds of various *Brassica* alleles (Nakanishi and Hinata, 1975; Niikura and Matsuura, 2000). We previously examined two *Brassica campestris* lines -- S^{733} and S^{734} -- and found that their CO_2 susceptibility is accompanied by a morphological alteration in the papillar cells of the susceptible line, S^{734} (Lee et al, 2001; Kwun et al., 2004), whereas the non-susceptible line, S^{733}, shows no such change. This result supports an earlier conclusion that the effectiveness of CO_2 gas in eliminating SI varies among genetic lines. In the previous work, Niikura and Matsuura (2000) discovered a genetic relationship between two *Raphanus sativus* plants with low and high reaction levels of self-incompatibility to CO_2 ($RLSICO_2$). Using a cross-population of both susceptible (high $RLSICO_2$) and non-susceptible (low $RLSICO_2$) lines, they concluded that the former trait is controlled by a recessive gene.

Here, we generated a crossing progeny of two separate genetic lines of *B. campestris*, S^{733} and S^{734}, and investigated the dominance relationship of their F_1 progeny in response to CO_2 gas treatment. We also studied the heritability of high $RLSICO_2$ in the F_2 self-progeny.

MATERIALS AND METHODS

Plant Materials

The *B. campestris* lines used in this study were Class-I types of S-alleles, S^{733} (Musou) and S^{734} (Hiratsuka), as previously reported (Lee et al., 2001). These S^{733} and S^{734} lines are non-susceptible and susceptible, respectively, to CO_2. To produce F_1 cross-progeny, we reciprocally cross-pollinated the two parental lines using each line separately as pollen donors. Thus, 40 F_1 populations containing either $S^{733}♀ S^{734}♂$ or $S^{733}♂ S^{734}♀$ were produced. In all, 104 F_2 selfing-populations were generated by selfing our F_1 plants.

PCR-RFLP Analysis

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Abbreviations: RLSI, reaction level of self-incompatibility; $RLSICO_2$, reaction level of self-incompatibility to CO_2; SI, self-incompatibility; SLG, S-locus glycoprotein; SRK, S-locus receptor kinase

Genomic DNAs were isolated from the young leaves of parents and every cross-progeny, as described by Dellaporta et al. (1983). To differentiate the S-alleles of those progeny, an SRK-specific primer set (forward, 5'GTGAAGAGGATGAAATTGCCAG3'; and reverse, 5'CAATACCGAAACCACCTTGTCC3') was developed to amplify the genomic DNA fragment containing the coding region of SRK. Amplification was carried out in a 20 μL volume containing 25 pmol of each primer; 50 pg of template DNA; 200 μM of dNTP; 1 unit of *Taq* polymerase; and a reaction buffer composed of 500 mM KCl, 100 mM Tris-HCl (pH 9), 1% Triton X-100, and 15 mM $MgCl_2$. PCR conditions included pre-denaturation for 5 min at 94°C; then 35 cycles of 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C; with a final extension of 10 min at 72°C. The resulted PCR products from each parental line were then sequenced to confirm that the DNA fragment indeed contained the SRK coding sequence. In addition, the DNA fragment was digested with *Alu*I, *Hae*III, and *Mse*I to locate any polymorphic bands between the S^{733} and S^{734} alleles. The digested DNA was electrophoresed at 100 V for 1 h in a 3% agarose gel containing TBE buffer. Afterward, the DNA bands were detected by staining with ethidium bromide.

Evaluations of $RLSICO_2$

Three self-pollinated flowers collected at anthesis from every cross-progeny were placed on an agar medium. These samples were exposed to 4% CO_2 for 12 h at 25°C in a carbon dioxide incubator under high humidity. The pistils were then collected and fixed in an ethanol: acetic acid (3:1) solution for approximately 4 to 6 h before being softened overnight in 1 M NaOH at 4°C. Afterward, the samples were washed in distilled water, stained with 0.05% aniline blue in 0.05 M sodium phosphate, squashed, and sealed with a cover glass. Their signals were visualized with a Zeiss fluorescence microscope. Values for $RLSICO_2$ were classified into five categories, based on the number of pollen tubes that penetrated into the stigmas: (1) 0 to 4, (2) 5 to 7, (3) 8 to 10, (4) 11 to 20, or (5) >20 (Niikura and Matsuura, 2000).

RESULTS

Relationship between *Brassica* S-Alleles and $RLSICO_2$

We have previously reported two different genotypes of *B. campestris*, S^{733} and S^{734}, that belong to Class I of SI (Lee et al., 2001; Kwun et al., 2004). Here, these two differed in their response to treatment with 4% CO_2, with such exposure eliminating self-incompatibility in our S^{734} plants, but not in those of S^{733} (Fig. 1A). This suggested two different genetic backgrounds for SI. To differentiate our S^{733} and S^{734} alleles at the molecular level, we isolated the SRK gene from each type of plant, and registered their sequences in GenBank (Accession numbers DQ397538 and DQ397539 for 733 SRK and 734 SRK, respectively). Comparison and alignment of those sequences indicated that the two S-genes differed at the nucleotide level (data not shown). We further digested the SRK products from both plants with *Alu*I, *Hae*III, and *Mse*I restriction enzymes to detect polymorphic DNA bands (Fig. 1B). Our results suggested that the two carry sequence diversity in the S-gene, showing polymorphism. We later exploited this as an advantage in identifying

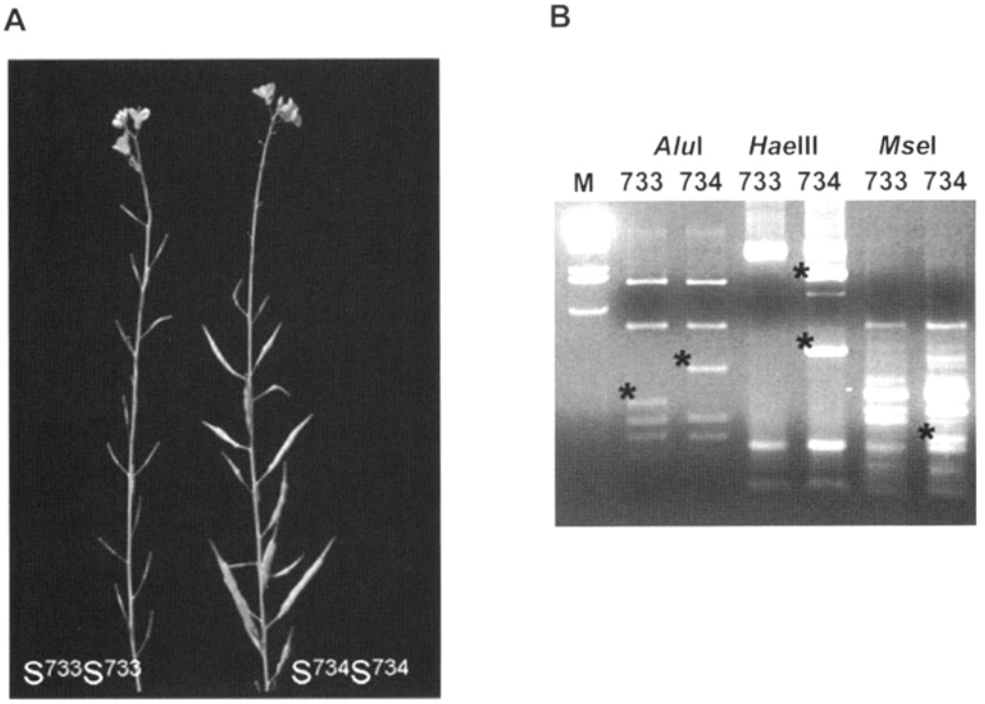

Figure 1. S^{733} and S^{734} self-pollinated plants after CO_2 treatment (**A**). Only S^{734} plant was susceptible to gas. (**B**) Restriction enzyme digestion of *SRK* clone isolated from genomic DNA of each plant. Three restriction enzymes showed polymorphism between S^{733} (733) and S^{734} (734) genes; polymorphic bands are marked with asterisk.

Table 1. $RLSICO_2$ of individual plants in the F_1 cross-population.

S-genotype / Plant	S^{733} ♀ S^{734} ♂		S^{733} ♂ S^{734} ♀	
	RLSI	$RLSICO_2$	RLSI	$RLSICO_2$
1	1	5	3	5
2	1	5	2	5
3	1	5	1	5
4	1	5	1	5
5	2	5	1	5
6	1	5	1	5
7	1	5	2	5
8	3	5	2	5
9	1	5	2	5
10	1	5	1	5
11	2	5	1	5
12	1	5	1	5
13	1	5	1	5
14	1	5	1	5
15	1	5	1	5
16	1	5	1	5
17	1	5	2	5
18	1	5	2	5
19	1	5	2	5
20	2	5	1	5

Reaction level, based on number of pollen tubes: (1) 0-4, (2) 5-7, (3) 8-10, (4) 11-20, (5) >20.

the S-genotype of the segregation progeny from the two parental lines. First, we determined the $RLSICO_2$ of the F_1 cross-population to understand the genetic relationship between the S-allele and $RLSICO_2$ in *Brassica*. To do so, we generated F_1 cross-progeny between the S^{733} and S^{734} plants, using each line separately as pollen donors. Afterward, each F_1 cross-progeny was tested for $RLSICO_2$ (Table 1). This was accomplished by self-pollinating individual plants from the S^{733}♀S^{734}♂ and S^{733}♂S^{734}♀ progenies and examining them via fluorescence microscopy (Fig. 2). Of the 20 progenies from each cross, 95% maintained strong SI (Table 1), as had been observed in the parental line (Fig. 2A, C; Table 1). This again confirmed our previous determination that the two S-alleles belong to the Class-I type of self-incompatibility (Lee et al., 2001). After the CO_2 gas treatment, however, all of the progeny showed high values of $RLSICO_2$, allowing more than 20 pollen tubes to grow (Fig. 2B, D; Table 1). These results suggest that high $RLSICO_2$ in *Brassica campestris* is controlled by a dominant gene (designated as R hereafter) that we believe is likely linked to the S^{734} allele because all of our F_1 populations from S^{733}♀S^{734}♂ and S^{733}♂ S^{734}♀ were CO_2-susceptible (Table 2).

Genetic Analysis of $RLSICO_2$

We studied the inheritance of high $RLSICO_2$ with the F_2 selfing-populations derived from each F_1 cross-progeny -- S^{733}♀S^{734}♂ and S^{733}♂ S^{734}♀. Using *Hae*III restriction enzyme (Fig. 1B), we performed PCR-RFLP to determine the genotype of the segregation population, as depicted for a few

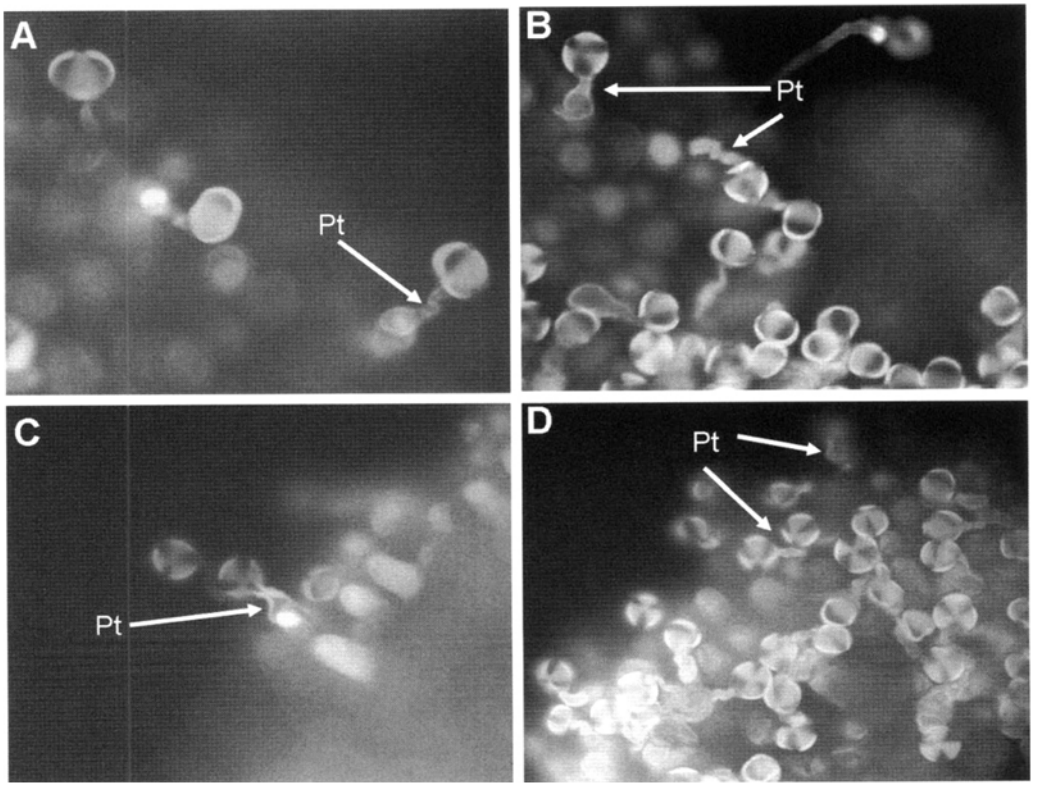

Figure 2. Microscopic observation of self-pollinations. **A** and **B**, S^{733}♀ S^{734}♂ F_1 progeny from cross between S^{733} and S^{734} parents. **C** and **D**, S^{733}♂ S^{734}♀ F_1 progeny from cross between S^{733}♂ and S^{734}♀ parents. **A** and **C**, before CO_2 treatment. **B** and **D**, after treatment. Following exposure to CO_2, all F_1 progenies in **B** and **D** self-pollinated, with high $RLSICO_2$ values and formation of more than 20 pollen tubes (Pt).

Table 2. Average reaction levels of self-incompatibility for S^{733}, S^{734}, and the F_1 progeny.

S-genotype	$S^{733}S^{733}$ (rr)	$S^{734}S^{734}$ (RR)	$S^{733}♀$ $S^{734}♂$ (Rr)	$S^{733}♂$ $S^{734}♀$ (Rr)
NPD[a]	20	20	20	20
RLSI[b]	1	1	1.2	1.45
$RLSICO_2$[c]	1	5	5	5

[a]Number of plants determined.
[b]Reaction Level of Self-incompatibility.
[c]Reaction Level of Self-incompatibility to CO_2 gas.
Reaction level: (1) 0-4, (2) 5-7, (3) 8-10, (4) 11-20, (5) >20 pollen tubes.

lines in Figure 3. Among the 56 F_2 plants from our $S^{733}♀$ $S^{734}♂$ selfing-populations, 15 and 12, respectively, showed S^{733} and S^{734} homozygous genotypes while 29 plants were heterozygous (Table 3a). A similar pattern of segregation was obtained from 48 $S^{733}♂$ $S^{734}♀$ selfing-populations, where 11 and 12 plants exhibited S^{733} and S^{734} homozygous genotypes, respectively, and 25 plants were of the heterozygous genotype (Table 3b). This suggested that the F_2 population segregates in a 1:2:1 Mendelian ratio (Table 3a, b). Furthermore, our fluorescence examination for $RLSICO_2$ revealed that 41 of the $S^{733}♀$ $S^{734}♂$ selfing-populations maintained a high $RLSICO_2$ while 15 had low $RLSICO_2$ values (Table 4). Similarly, 37 and 11 of the $S^{733}♂$ $S^{734}♀$ selfing-populations maintained high and low $RLSICO_2$, respectively (Table 4). Both cases fit into a dominant phenotype ratio of 3 (high $RLSICO_2$) to 1 (low $RLSICO_2$) (Table 4). We also confirmed this dominant relationship by matching the genotype, via PCR-RFLP, in which all F_2 progeny with low $RLSICO_2$ carried the homozygous S^{733}-genotype.

DISCUSSION

SI is an elaborate genetic mechanism that prevents self-fertilization in flowering plants. In studying the effect of CO_2 in the *Brassica* family, Nakanishi et al. (1969) have found that self-pollination is achieved when the gas concentration is over 0.3%, reaching a maximum efficiency at 4.5%, then declining. This procedure is currently the best method for achieving self-fertilization in Chinese cabbage, although

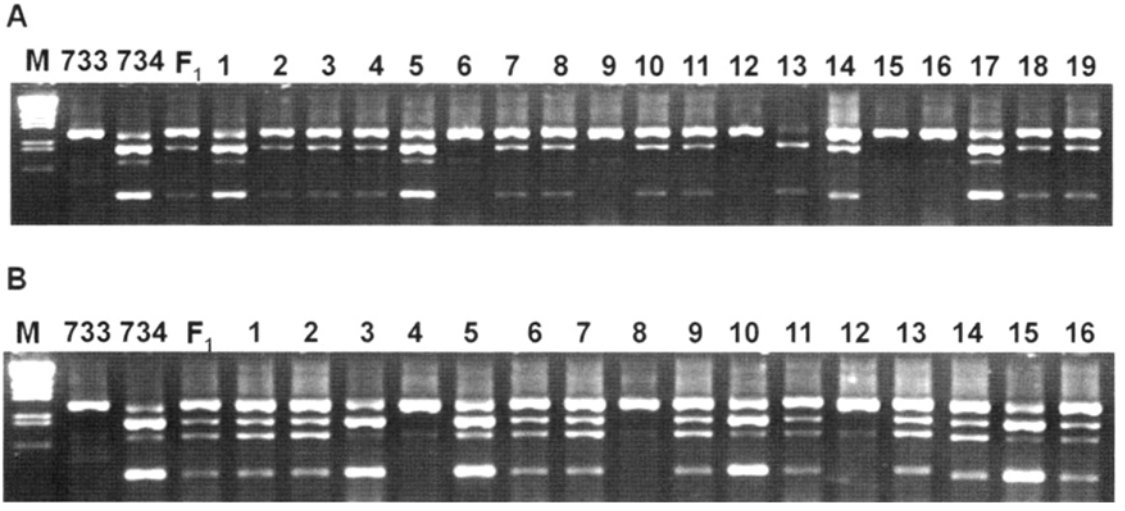

Figure 3. PCR-RFLP analysis of F_2 selfing-population. *SRK* genomic DNA fragment amplified with *SRK*-specific primer was digested with *Hae*III. **A** and **B**, PCR-RFLP results of $S^{733}♀$ $S^{734}♂$ and $S^{733}♂$ $S^{734}♀$ -selfing populations, respectively. 733 and 734, *Hae*III digestion of corresponding *SRK* clone isolated from S^{733} and S^{734} plants, respectively. F_1, *Hae*III digestion of *SRK* clone isolated from F_1 cross-progeny of $S^{733}♀$ $S^{734}♂$ (**A**) and $S^{733}♂$ $S^{734}♀$ (**B**). Results indicate that F_2 populations segregated at 1:2:1 ratio.

Table 3a. Genotype segregation and average of $RLSICO_2$ in $S^{733}♀$ $S^{734}♂$ selfing-population.

S-genotype	$S^{733}♀$ $S^{733}♀$ (rr)	$S^{733}♀$ $S^{734}♂$ (Rr)	$S^{734}♂$ $S^{734}♂$ (RR)	Total	Goodness of fit		
					Ratio	χ^2	P
Observed	15	29	12	56	1:2:1	0.39	0.70-0.90
Calculated	14	28	14	56			
Average of $RLSICO_2$	1.8	5	5				

Table 3b. Genotype segregation and average of $RLSICO_2$ in $S^{733}♂$ $S^{734}♀$ selfing-population.

S-genotype	$S^{733}♂$ $S^{733}♂$ (rr)	$S^{733}♂$ $S^{734}♀$ (Rr)	$S^{734}♀$ $S^{734}♀$ (RR)	Total	Goodness of fit		
					Ratio	χ^2	P
Observed	11	25	12	48	1:2:1	0.12	0.90-0.95
Calculated	12	24	12	48			
Average of $RLSICO_2$	1.7	5	5				

Table 4. F_2 segregation for the reaction level of self-incompatibility to CO_2.

	In the cross S^{733} ♀ S^{734} ♂					
	RLSICO_2		Total	Goodness of fit		
	High	Low		Ratio	χ^2	P
Observed	41	15	56	3 :1	0.095	0.70-0.90
Calculated	42	14	56			
	In the cross S^{733} ♂ S^{734} ♀					
Observed	37	11	48	3 :1	0.11	0.70-0.90
Calculated	36	12	48			

other methods (e.g., treatment with NaCl, $CaCl_2$, or KCl) are available for maintaining inbred lines. We reported previously that a high CO_2 concentration causes structural alterations on papillae cell surfaces (Lee et al., 2001). In the study described here, we obtained and compared the coding sequence for *SRK* from two genetically different *Brassica* lines, S^{733} and S^{734}. Our analysis revealed that the two *SRK* clones differ at the nucleotide level (data not shown), thereby suggesting separate genetic backgrounds for SI. Furthermore, our sequence analysis allowed us to identify polymorphic DNA fragments from enzyme digestion of the *SRK* clones (Fig. 1B). Thus, we utilized a PCR-RFLP strategy to determine the genotype for the S-allele in this genetic trial.

We generated F_1 and F_2 populations for the cross-progeny between S^{733} and S^{734} plants and determined their RLSICO_2. Our research relied on the fact that these two lines differ in their ability to overcome SI upon CO_2 exposure (Lee et al., 2001). Therefore, the determination of RLSICO_2 among these progeny would allow us to understand the dominant relationship between the S-alleles and RLSICO_2. Here, all of the F_1 plants from both $S^{733}S^{734}$and $S^{734}S^{733}$ cross-populations showed high RLSICO_2, such that SI was eliminated upon treatment with 4% CO_2 (Fig. 2; Table 1). Based on this, we could conclude that the S^{734} plants are genetically dominant. Evaluating the segregating F_2 population for each F_1 cross-progeny from $S^{733}S^{734}$and $S^{734}S^{733}$ also confirmed this dominant relationship (Table 3 and 4). Using PCR-RFLP, we identified the genotype of each F_2 progeny (Fig. 3) and calculated a segregation ratio of 1:2:1 (RR:Rr:rr), which fit the observed RLSICO_2 values when R was dominant (Table 3). Taken together, we propose that the RLSICO_2 in *B. campestris* is controlled by a dominant allele (R) in the S^{734} haplotype genetic background.

Our study results here do not coincide with those of Niikura and Matsuura (2000), who reported that the high RLSICO_2 in radish (*R. sativus*) is controlled by a recessive gene. However, this distinction does demonstrate that the regulation of RLSICO_2 in separate plant species might develop independently. Previous research showed that SI in *Brassica* depends upon a recognition between the SRK expressed in the stigmas and the SP11/SCR that is present on the surfaces of pollen grains (Hiscock and McInnis, 2003). This initial recognition step activates an SRK-mediated signaling pathway in the pistil that results in the rejection of self pollen. Here, we discovered that our two *B. campestris* lines possess sequence diversity in *SRK*. However, we could not conclude that this is responsible for the RLSICO_2. We previously observed that only the susceptible line, S^{734}, carries a severe structural alteration of the papillar cells when CO_2 is applied, and that a number of pistil genes, including *SLG*, are regulated by that gas (Lee et al., 2001; Kwun et al., 2004). Our present study results indicated that this susceptibility is genetically dominant to non-susceptibility. Furthermore, we have now learned that isolation of the R gene will be necessary in order to unveil the mechanism for RLSICO_2 in *B. campestris*.

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