INVESTIGATION

Involvement of MLPK Pathway in Intraspecies Unilateral Incompatibility Regulated by a Single Locus With Stigma and Pollen Factors

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ABSTRACT

Plants have evolved many systems to prevent undesirable fertilization. Among these, incompatibility is a well-organized system in which pollen germination or pollen tube growth is inhibited in pistils. We previously found that a novel one-way pollen–stigma incompatibility response [unilateral incompatibility (UI)] occurred between two self-incompatible Brassica rapa plants, a Turkish line, and a Japanese cultivated hybrid variety, “Osome.” Pollen from the Turkish line is rejected on the stigma of the Osome line, but the reverse cross is compatible; such a UI phenotype closely resembles self-incompatibility (SI). The pollen factor of this UI has been genetically explained by a single locus which is different from the S-locus. In this study, we performed further genetic analyses of this intraspecies UI and showed that the stigma factor was also controlled by a single locus, and we named the loci corresponding to the stigma and pollen factors of the intraspecies UI, stigmatic unilateral incompatibility (SUI), and pollen unilateral incompatibility (PUI) loci, respectively. Interestingly, segregation analyses of SUI and PUI indicated that they are closely linked to each other and behave as a single unit. To investigate the effect of an SI-related gene, MLPK in this UI, we produced segregation lines for SUI and mlpk. A distorted segregation ratio of SUI phenotype in an mlpk background indicated involvement of MLPK in SUI, suggesting the existence of an MLPK-dependent novel pollen–stigma recognition mechanism.

KEYWORDS

Brassica rapa L unilateral incompatibility self-incompatibility pollen-stigma recognition

Flowering plants have evolved several pollen rejection mechanisms to prevent inappropriate pollination and fertilization. In such systems, pollen-pistil unilateral incompatibility (UI) is defined as a positive prefertilization barrier that results in pollen from one species being rejected on the stigma of another species, whereas a cross in the reciprocal combination is compatible (De Nettancourt 2001). In theory, this barrier prevents gene flow and is thus thought to underlie the maintenance of species identity. On the other hand, many plant species have self-incompatibility (SI) systems to prevent self-fertilization, which restricts inbreeding and generates genetic variation in a population. Recent studies of molecular mechanisms of pollen-pistil incompatibility have been focused mainly on the self-recognition system of SI (Iwano and Takayama 2012). Although molecular determinants for self-recognition from some plant species have been identified, including those of Brassica (Iwano and Takayama 2012), little information has been obtained for UI pollen-pistil recognition. Interspecies UI occurs between closely related species belonging to the same family, or cluster of families, hosting the same SI system. Incompatible pollination in UI is generally observed when an SI species is used as the stigma parent and a self-compatible (SC) species as the pollen parent. This relationship is called the “SI × SC rule,” although it cannot be applied in all cases (Lewis and Crowe 1958). In Solanaceae plants, which possess gametophytic SI, UI has also been reported in intraspecies crosses (Martin 1963).
In the SI system of Brassicaceae, self-pollen is rejected at the surface of stigmatic papilla cells after the discrimination of self-from non-self pollen. This recognition mechanism is genetically controlled by a single S locus, which contains two highly polymorphic genes, SRK (S-locus receptor kinase) and SP11/SCR (S locus protein 11 or S locus cysteine-rich protein) (Iwano and Takayama 2012). The pollen SI determinant SP11/SCR is a small cysteine-rich pollen coat protein which is expressed in sporophytic anther tapetum cells (Suzuki et al. 1999; Schopfer et al. 1999; Takayama et al. 2000b). This expression pattern governs sporophytic control of the Brassica SI system (Shiba et al. 2001; Tarutani et al. 2010). The stigma SI determinant SRK is a membrane-spanning serine/threonine receptor kinase (Stein et al. 1999; Takasaki et al. 2000). S-genotype-specific physical interaction between SP11/SCR, and a receptor domain of SRK is thought to trigger a signal cascade leading to self-pollen rejection are becoming better understood. One of these, the M-locus protein kinase (MLPK), which was isolated by positional cloning of the M locus of an SC B. rapa var. yellow sarson, is an essential positive regulator of the SI response (Murase et al. 2004). MLPK encodes a cytoplasmic serine/threonine protein kinase, which is expressed predominantly in the stigma, targets to the plasma membrane of stigmatic papilla cells, and colocalizes with SRK (Kakita et al. 2007).

It is known that the mechanism of SI is to some extent related to UI. In Solanaceae, several genetic studies have revealed that one of three factors that plays an important role in pistil-side UI mapped to the 2 locus, although the other two factors were located in different loci (Bernacchi and Tanksley 1997). A transgenic experiment using the female SI determinant S-RNase (S-ribonuclease; female determinant of SI in Solanaceae and Rosaceae) clearly demonstrated that both S gene-dependent and S gene-independent mechanisms are involved in interspecies incompatibility in Solanaceae species (Murfett et al. 1996). Furthermore, discrete pollen-side UI factors in Solanaceae members have been mapped at or near the S-locus on chromosome 1, and another has been located on chromosome 6 (Chetelat and De Verna 1991). The factor mapped to chromosome 6 has been cloned and was found to carry a pollen-expressed Cullin1 gene with high similarity to petunia SI factors (Hua and Kao 2006; Li and Chetelat 2010). In the genus Brassica, Hiscock and Dickinson (1993) reported some phenotypic correlations between SI and interspecies UI. On the other hand, recent QTL analysis showed that the S and M loci are not involved in interspecies UI between B. rapa and B. oleracea (Udagawa et al. 2010). These multiple and redundant aspects of the UI phenomenon make interpretation of experimental results difficult. Thus, for an overall understanding of the UI mechanism, it will be necessary to dissect carefully defined UI systems on both the pollen and stigma sides.

We previously reported the novel interspecies UI response that occurred between a Turkish line and a Japanese cultivated hybrid variety, Osone, in Brassica rapa (Takada et al. 2005). Pollen from the Turkish line was rejected on the stigma of some Osone-derived individuals, but crosses in the reverse combination were compatible. The physiological features of the UI closely resembled those of the SI responses in Brassica. Genetic analysis revealed that the factor controlling the pollen-side UI [pollen unilateral incompatibility (PUI)] is regulated by a single PUI locus, which is not linked to the S locus (Takada et al. 2005). Further genetic analysis of the stigma-side incompatibility [stigmatic unilateral incompatibility (SUI)] is required to isolate the PUI and SUI genes, which will lead to greater understanding of the novel pollen-stigma recognition mechanism of the interspecies UI.

Herein, based on linkage analysis using F2 progeny between the SUI/SUI homozygote and the suisui homozygote, we concluded that SUI is controlled by a single dominant locus, which is not linked to the S locus. Segregation analysis for the SUI and PUI loci revealed that these two loci are closely linked to each other. In addition, to investigate the effect of MLPK in the SUI, we produced segregating progeny

Table 1 Plant materials and their expected genotypes

| Line     | Name                  | Expected Genotype                  | Study(ies) |
|----------|-----------------------|------------------------------------|------------|
| SUI      | S9                   | SUI/SUI, pulveri, MLPK/MLPK, S9/S9  | Takada et al. 2005 |
| PUI      | S9                   | sui/sui, PUI/PUI, MLPK/MLPK, S9/S9 | Takada et al. 2005; Hatakeyama et al. 1998 |
| non-UI   | S9                   | sui/sui, pulveri, MLPK/MLPK, S9/S9 | Takada et al. 2005; Hatakeyama et al. 1998 |
| mlpk mutant | S9                  | sui/sui, pulveri, mlpk/MLPK, S9/S9 | Murase et al. 2004 |

Figure 1 Crossing scheme used to develop each segregation line.
for SUI and mlpk. The biased segregation ratio of SUI phenotype in the mlpk/mlpk background suggested an MLPK-dependent incompatibility system operates in the SUI reaction.

**MATERIALS AND METHODS**

**Plant materials**

Detailed genotypes of plant materials used in this study are shown in Table 1. Because we hypothesized that a positive interaction between female and male factors resulted in the UI reaction, the indicated genotypes of SUI, sui, PUI, and pui corresponded to the phenotype of SUI (compatible), non-SUI (compatible), PUI (compatible), and non-PUI (compatible), respectively. It has not yet been determined whether PUI is regulated sporophytically or gametophytically (see **Discussion**). For the SUI homozygous line, S90-9 and S92-12 were self-pollinated progeny of B. rapa cv. Osome (Takasaki et al. 1999; Takada et al. 2005). The original B. rapa cv. Osome line is heterozygous for S90 and S92 S-haplotype. S90 and S92 homozygous lines were selected from self-crossed progeny of B. rapa cv. Osome by using allele-specific polymerase chain reaction (PCR) and test pollination. In our previous study, S90-9 and S92-12 were characterized as possessing the SUI phenotype (Takada et al. 2005). We checked the SUI phenotype of the self-crossed progeny of S90-9 and S92-12, and established that these are both SUI homozygous lines. The non-SUI line S9 was derived from Oguni, Japan (Nou et al. 1993; Hatakeyama et al. 1998). For the S9UI line, an SUI/SUI, S9/S9 homozygous plant was selected from the F2 segregation line of S9UI line (S90-9) and non-SUI line (S9) (see Supporting Information, Figure S1). PUI homozygous lines [S9t and S9mm (where t stands for Turkey)] were obtained from self-pollinated progeny of the Turkish variety with S9t and S90 background, respectively (Nou et al. 1993; Hatakeyama et al. 1998; Takada et al. 2005). The mlpk mutant S9mm was reported by Murase et al. (2004).

**Production of segregating progeny and test pollinations**

The crossing scheme is represented in Figure 1. For the production of SUI segregating progeny termed SF2-52 (stigma side F2 line with S92 genotype) and SF2-60 (stigma side F2 line with S90 genotype), S92-12 or S90-9 was crossed with S9 to produce F1 plants, which were then self-crossed by bud pollination to produce F2 seeds (Figure 1A; Figure S1). SUI and PUI segregating progeny, termed the pollen-stigma side F2 (PSF2) line, was produced by self-bud pollination of an F1 plant, which was produced by bud pollination of S92-12 and S90-9 (Figure 1B). For production of the backcross C1F1 (backcross, F1 line), we performed bud pollination of S90-9 stigma with S9t pollen. Thereafter, the F1 plant was bud-pollinated with S9t pollen (Figure 1C). In the case of SUI and MLPK (MF2 line), SUI and S9mm were used as parental plants. Subsequently, we performed bud pollination of the F1 plant (Figure 1D). SUI, PUI phenotypes, and S-genotypes of all plants used in this study were determined by test pollination as described in Takada et al. (2005). Flower buds were numbered from the lowest bud in an inflorescence, as described in Gonai and Hinata (1971). Because the average number of flowers opening per day is three, the flower buds at stages 1′, 2′, and 3′ are expected to bloom on the next day, and stigmas of these three buds have the potential to exhibit a normal SI reaction (Gonai and Hinata 1971). Nonpollinated flower buds were cut at the peduncle and, after pollination, stood on 1% solid agar for approximately 24 hr under room conditions. Thereafter, pistils of the pollinated flowers were softend in 1 N NaOH for 1 hr at 60°C and stained with basic aniline blue (0.1M K3PO4, 0.1% aniline blue). Samples were mounted in 50% glycerol (fluorescence microscope grade) on slides and observed by UV fluorescence microscopy (Kho and Bear 1968). At least three flowers were used in each cross combination, and observations were generally replicated at least three times for each cross combination on different dates. For determination of the SI phenotype, S-homozygous plants, S9, S9t, S90, S92, and S9mm, were used. To determine the UI phenotype, PUI homozygous (S9t or S90t) and SUI homozygous (S92-12 or S90-9) plants were used in the test pollination (Takada et al. 2005).

**Seed production by test crossing using mature stigmas**

To avoid self-pollination in the bud, 1 day before opening the stamens were removed from three flowers corresponding to stages −1′, −2′, and −3′. The inflorescence was then covered with a paper bag for 24 hr. After pollination, we excised all flowers in the inflorescence with the exception of the three flowers and re-covered the inflorescence with a paper bag for approximately 3 weeks. For a comparison of the incompatibility strength, we measured the lengths of siliques and counted the number of seeds.

![Figure 2](image-url) Representative results of test pollinations and seed production. Photographs were obtained by UV fluorescence microscopy (A–C). (A) Cross-pollination of an S9t stigma with S92-12 pollen. (B) Self-pollination of S92-12. (C) Cross-pollination of an S92-12 stigma with S9t pollen. The pollen germinated, but penetration of the pollen tube was inhibited (arrowheads). Silique length of each test cross (D–H). (D) S90-9 self-cross; (E) S90-9 stigma × S9t pollen; (F) S90-9 stigma × PUI/pui heterozygous pollen; (G) S90-9 stigma × S92t; (H) S92-12 stigma × S90t. Short siliques were observed in SI and UI crosses (arrows).
Table 2 Seed Production by UI and SI Phenotypes

| Cross                      | Silique Length (cm) | Mean Number of Seeds (± SD) | n |
|----------------------------|---------------------|-----------------------------|---|
| Compatible cross           | 5.5 ± 0.5a          | 15.3 ± 2.7c                 | 6 |
| Self-incompatible cross     | 2.8 ± 0.9b          | 0.9 ± 0.9f                  | 9 |
| Unilateral incompatible cross | 3.0 ± 1.1b         | 1.2 ± 1.5d                 | 11|

Seed production phenotypes were measured by silique length and mean number of seeds (± SD). Values followed by the same letter are not significantly different at the 5% level, as determined by Tukey’s multiple comparison test.

Determination of the S genotype and MLPK genotype

The S-genotype of each plant was determined by allele-specific PCR. Total DNA was extracted from young leaf tissue of B. rapa by the procedure described by Murray and Thompson (1980). PCR was performed using Taq DNA polymerase (Takara BIO, Shiga, Japan) for 30 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 20 sec, and extension at 72°C for 30 sec. Genomic DNA of each plant was amplified using S-genotype-specific SP1I primers. For genotyping of MLPK, the functional MLPK allele was specifically amplified by using the PCR primers wtMLPK-F and wtmMLPK-R, and the mutated mlpk allele was specifically amplified by the mMLPK-F and wtmMLPK-R primers. PCR primers used in this study are listed in Table S1. The PCR product was subjected to electrophoresis on 1% agarose gel. Vacular H+-ATPase (V-ATPase) of B. rapa (Takasaki et al. 2000) was amplified as a positive control.

Statistical analysis

Statistical analyses were performed using Statistica version 6.1 software (StatSoft Inc., Tulsa, OK). Tukey’s honestly significant difference test was used to determine significant differences. Pearson’s correlation coefficient (r) values were calculated between silique length and seed number.

For the segregation analysis, a standard χ² goodness-of-fit test was performed to determine if phenotype or genotype distribution deviated from expected ratios.

RESULTS

Seed production of UI combination crosses

Our previous experiments showed that the UI phenotype in B. rapa closely resembles the SI phenotype on the stigmatic papillae cells (Takada et al. 2005). In contrast to compatible pollination, the incompatible pollen cannot germinate and/or cannot penetrate stigmatic papillae cells in the SI and UI reaction (Figure 2, A, B, and C). Moreover, the UI pollen rejection can be overcome by bud pollination, as in the case of SI. In this study, to test the effect of UI on seed production, we performed test crosses between the SUI stigma and the PUI pollen. The silique length and number of seeds were measured in each test cross. A strong correlation (r = 0.86) was detected between silique length and number of seeds (Table 2; File S1). In the SI cross (S²⁰⁹/S⁶⁰), only short siliques were obtained, and the average seed number was 0.9 (Figure 2D; Table 2), while fully developed long siliques were observed in a compatible combination (cross-pollination: S⁶⁰/PUI stigma × S⁳²/PUI pollen) (Figure 2E; Table 2). The UI cross (S⁵²/PUI stigma × S⁴⁰/PUI pollen) resulted in production of short siliques (Figure 2G and H, and Table 2), which could not be distinguished from those of the SI combination cross. There were no significant differences between SI and UI in silique length or seed number (Table 2). These results indicate that UI shows the same strength of incompatibility as SI in terms of seed production. When we crossed pollen from PUI/pui heterozygous plants (S⁵⁰/S⁴⁰, PUI/pui) (Takada et al. 2005) with mature SUI stigmas, full seed sets was observed, as occurred in the SC combination (Figure 2F). This was consistent with our previous report showing compatible behavior of PUI/pui heterozygous pollen on the SUI stigma (Takada et al. 2005).

Genetic segregation analysis of the SUI factor

The UI reaction at the pollen side is regulated by a single SUI locus, which is not linked to the S locus (Takada et al. 2005). Furthermore, in our previous report, the stigma UI phenotype was shown to occur in both the S⁰⁰/S⁰⁰ and S⁵²/S⁵² backgrounds (Takada et al. 2005), suggesting the possible independent segregation of SUI and S genotypes. In order to determine the genetic control of SUI and compatible progeny and F₁ plants were determined by test cross-pollination using PUI/PUI homozygous plants (S⁴⁰) as the pollen donor. The stigma phenotype of all the 11 F₁ plants was incompatible with the PUI pollen, indicating that the SUI trait was dominant over normal fertility (non-SUI) (Figure 3; File S2). According to χ² test results, the SUI:non-SUI segregation ratio of 146:46 fitted to the single locus

![Figure 3](image-url)
because recombination within this region is highly repressed. In order to examine the relationship between the SUI and PUI loci, we further performed linkage analysis using a segregating population derived from a plant heterozygous for both SUI and PUI (SUI/sui and PUI/pui). The F1 heterozygous plant was produced by self-bud pollination between S24/S24 (SUI/SUI, pui/pui, S60/S60) and S9/S9 (sui/sui, PUI/PUI, S0/S0) and the F2 progeny (PSF2) was produced by self-pollination of the F1 heterozygous plant (SUI/sui, PUI/pui, S2/S0) (Figure 1B). Stigma- and pollen-UI phenotypes of each plant were determined by test pollination using S4t (sui/sui, PUI/PUI, S4/S4) as a pollen donor and S60-9 (SUI/SUI, pui/pui, S60/S60) as a stigma donor. These test plants were chosen in order to discriminate between UI and SI responses. In the PSF2 progeny, all of the PUI individuals showed a non-SUI phenotype, and all of the pollen from SUI individuals was compatible on SUI tester stigma, indicating the tight linkage of the SUI and PUI loci (Figure 4A). There were no plants showing both SUI and PUI phenotypes in the 67 PSF2 plants. The phenotypic SUI non-PUI/non-SUI/PUI segregation ratio was 51·16 (Figure 4B), clearly matching to single-locus inheritance (3:1; $\chi^2 = 0.69; P > 0.05; df = 1$). For further confirmation of this linkage, we used another segregating progeny (BC1F1), which was produced by bud pollination using the heterozygous F1 (SUI/sui, PUI/pui, S0/S0) stigma and PUI homozygous (sui/sui, PUI/PUI, S60/S60) pollen (Figure 1C). Results of test crosses of 92 BC1F1 individuals revealed that the phenotypic segregation ratio of SUI/non-PUI and non-SUI/PUI was 50:42 (1:1 by $\chi^2 = 0.689; P > 0.05; df = 1$), and complete linkage was observed with the SUI allele and pui allele from S0-9, and sui allele and PUI allele from S4t (Figure 4C; File S4). A plant possessing both SUI stigma and PUI pollen, or non-SUI stigma and non-PUI pollen, was not present in the 67 F2 progeny and 92 BC1F1. Therefore, recombination between SUI and PUI was not observed. These results clearly indicate a strong linkage between the SUI and PUI loci.

**Effect of MLPK on SUI pollen rejection**

Due to the phenotypic resemblance between SI and this intraspecies UI, it is possible that the SI and UI systems use common cellular mechanisms to reject the pollen. To investigate the effect of MLPK, whose product is one of the important targets of the SI system (Murase et al. 2004), on this UI we produced segregating progeny for SUI and MLPK. F1 plants heterozygous for the SUI locus and the MLPK locus were produced by crossing an SUI plant (SUI/sui, pui/pui, S60/S60, MLPK/MLPK) and an S0mm plant (sui/sui, pui/pui, S0/S0, mlpk/mlpk) (Figure 1D). The stigma phenotype of the F1 heterozygous plant (SUI/sui, pui/pui, S0/S0, MLPK/mlpk) was incompatible with the PUI tester pollen (S60t), in the same manner as that expected with UI. In the MF2 segregation line, S-genotypes segregated as expected: S0S0, S0S60, S60S60, S60S0 = 22:34:16 (1:2:1; $\chi^2 = 1.21; P > 0.05; df = 2$) (Table 3; File S5). The overall segregation of SUI phenotype in the MF2 line did not fit a 3:1 ratio (3:1; $\chi^2 = 7.41; P < 0.01; df = 1$) but was consistent with a 9:7 ratio (9:7; $\chi^2 = 0.689; P > 0.05; df = 1$). This suggests that functional SUI and MLPK are both essential for the UI pollen rejection. The UI phenotypes of plants possessing a functional MLPK allele (MLPK/MLPK and MLPK/mlpk) segregated in a SUI/non-SUI 3:1 ratio, indicating that the SUI locus is located at a different chromosomal position from MLPK (Table 3). In the case of the nonfunctional mlpk background (mlpk/mlpk), 12 of 15 plants showed a compatible phenotype to PUI tester pollen, although the other 3 plants remained UI (Table 3). Here the altered SUI observed in most of the plants was associated with mlpk/mlpk-dependent disruption of the incompatible reaction. Thus, it is suggested that an MLPK-dependent incompatibility

**Figure 4** (A–C) SUI and PUI loci are closely linked to each other. The PSF2 segregation line was produced by a cross between $S^{0-9}$ and $S^{60}$ (as shown in Fig. 1). (A) The UI phenotypes of the stigma side and pollen side were determined by using $S^{0-9}$ as the stigma tester plant and $S^{60}$ as the pollen tester plant. SUI, Stigmatic UI; PUI, pollen UI; C (shaded boxes), compatible. (B) Segregation ratio of the PSF2 line. The SUI, PUI or non-SUI, non-PUI progeny was not found. (C) Segregation ratio of the BC1F1 line. Segregation ratio of 3:1 ($\chi^2 = 0.11; P > 0.05; df = 1$). In this progeny, the S-genotype determined by the allele-specific SP11 amplification segregated normally. The SUI phenotype and S genotype in the SF2-52 individuals segregated independently (Figure 3), indicating that the SUI locus is not linked to the S locus, which is consistent with our previous crossing experiments (Takada et al. 2005). Furthermore, the S locus-dependent segregation of the SUI phenotype was confirmed by using $S^{0-9}$ S genotype background (Figure S1, File S3). Thus, the stigma UI phenotype is genetically controlled by a single dominant SUI locus, which is not linked to the S locus.

**Linkage analysis of the SUI and PUI loci**

Our segregation analysis clearly showed that both the female and male factors of Brassica intraspecies UI are regulated by a single locus. This reminded us of the SI recognition mechanism in which SRK and SP11 (genes for female and male SI factors, respectively) are tightly linked on the S locus region and are inherited as a single segregation unit.
system may have an important role in the rejection of PUI pollen on SUI stigmas in B. rapa. Moreover, it is noteworthy that 3 plants showed SUI in the mlpk/mlpk background, indicating the possible existence of an additional signal cascade, which is MLPK-independent, in this UI system (Table 3). To confirm the interaction between MLPK and UI pollen rejection, we further selected 13 plants possessing mlpk/mlpk homozygous background in the MF1 line and checked their SUI phenotype. All plants were compatible with PUI tester pollen, apart from one that still showed SUI pollen rejection (File S6).

**DISCUSSION**

In the present genetic study, we have characterized the novel stigma-pollen interaction between SUI and PUI factors in B. rapa, which is similar to SI in the following points: (1) UI is regulated by a single locus with the tightly linked stigma and pollen factor genes SUI and PUI. (2) The incompatibility phenotype of UI on the stigma is quite similar to that of SI, and bud pollination can be used to overcome UI. (3) MLPK may be involved in the UI reaction. Although this UI is similar to SI, the SUI/PUI locus is not linked to the S locus, and there is no evidence for multiple alleles. It would be interesting to discuss the evolution of this UI, if the SUI and PUI genes could be identified.

In our series of genetic analyses, the SUI phenotype was found to be genetically controlled by a single dominant locus. The recognition and rejection of the PUI/PUI pollen specifically occurred on the stigma surface by action of the SUI determinant. In the SUI/SUI homozygous or SUI/sui heterozygous plant, the SUI gene would be expressed in stigmatic papillae cells, as in the case of SRK in the SI system. Therefore, SRK-like receptor kinase is a possible candidate for the SUI factor. In Arabidopsis, S-domain receptor kinases fall into three classes with more than 40 members (Shiu and Bleeker 2003), and the functions of this family of kinases remain largely unknown. In Brassica, at least two SRK-like genes that are not linked to the S-locus have been reported, and one of these was found to be expressed predominantly in stigma (Kai et al. 2001). It is possible that the SUI gene is one of the uncharacterized members of such a Brassica S multigene family.

The pollen determinant is regulated by a single PUI locus, and it appears to be a recessive phenotype in the heterozygous PUI/pui plant (Takada et al. 2005). However, it should be noted that there is the possibility of a pseudorecessive phenotype; if PUI is expressed gametophytically in pollen grains, the heterozygote (PUI/pui) will produce pollen grains having PUI or pui, and PUI pollen will be inhibited, whereas pui pollen will be compatible. In this case, half of the pollen grains can germinate, and their pollen tubes can penetrate the stigma, which would be judged compatible in the test pollination assay. Thus, if PUI is gametophytically regulated, its behavior will be similar to that of the recessive in the heterozygote. To date, it has been unclear whether PUI is regulated sporophytically or gametophytically. If the UI recognition and rejection system is the result of a positive interaction between stigma and a pollen-producing product, the PUI could be controlled gametophytically. As the candidate for the PUI factor in B. rapa expressed in pollen grains, several signaling molecules have been reported. Over 10 different small basic pollen coat proteins (PCPs) have been identified in Brassica pollen coat (Doughty et al. 1998; Takayama et al. 2000a). One of these PCPs, PCP-class A, 1 (PCP-A1) was reported to be expressed gametophytically in pollen grains and had the ability to bind to S-locus glycoprotein (SLG) and possibly SRK (Doughty et al. 1998, 2000). Another member of the PCPs, S locus-related glycoproteins 1-binding protein (SLR1-BP) has also been reported to have an important role in pollen adhesion to papillae cells and can physically interact with the SLG-like protein SLR1 (Takayama et al. 2000a). In view of these reports, it would interesting to determine the expression and genetic control of the PUI, as this may be different from SP11, the pollen SI factor, which is sporophytically controlled.

Interestingly, analysis of linkage between SUI and PUI clearly indicated that these two loci cosegregate and act as a one inheritance unit. There are at least three types of genomic structure in the locus: (1) non-UI in both the stigma and pollen (Japanese lines and cv. Osone non-SUI type; sui/pui); (2) stigmatic SUI and pollen non-PUI (cv. Osone SUI type; sui/pui); and (3) stigmatic non-SUI and pollen PUI (Turkish lines; sui/PUI). We have not been able to find a plant or line that has both active SUI and PUI.

To the best of our knowledge, this is the first report of novel pollen-stigma interacting factors, which are located in the same or closely linked loci, other than the SI system. Segregation analysis of SUI and MLPK clearly indicated that SI and UI share the same signal cascade in stigmatic papilla cells’ ability to reject pollen. These findings suggest a possible ligand receptor recognition system similar to that of SI in this UI. In addition, it is reported that in the genome of A. thaliana, there are some regions where SRK-like genes and several SP11-like genes, duplicated in tandem, are located in closely linked sites (Zhang et al. 2011). In our possible hypothesis of the intraspecies UI in B. rapa, SRK-like receptor kinase expressed in the SUI stigma interacts with an SP11-like peptide expressed gametophytically in PUI pollen, triggering the MLPK-involved signaling pathway to induce a rapid incompatible reaction in the papilla cells.

In the segregation analysis of SUI and MLPK, unexpectedly, we found some plants possessing mlpk/mlpk homozygous background and still showing SUI to the PUI tester pollen. This suggests that a minor MLPK-independent pollen rejection system is also involved in intraspecies UI in B. rapa. A simple explanation of this system would be that a locus other than MLPK plays a role in UI. Because the plants that we used in this work are not inbred lines or doubled haploids, they contain some degree of heterozygosity. These genetic variations might be involved in an MLPK-independent UI system. Udagawa et al. (2010) reported that functional MLPK is not needed for the intraspecies UI between B. rapa and B. oleracea. Although both inter- and intraspecies UI in Brassica represent a one-way incompatibility response in stigmatic papilla cells, it is considered possible that intraspecies UI in this study might be regulated by a partially different pollen-stigma incompatibility mechanism from the interspecies UI.

| M Genotype | S Genotype | SUI Phenotype | Expected Ratio | χ² Results |
|------------|------------|---------------|----------------|------------|
| MLPK/MLPK  | S²/S⁹      | 2             | 2              |            |
| MLPK/MLPK  | S²/S⁸      | 7             | 3              |            |
| MLPK/MLPK  | S⁹/S⁸      | 16            | 6              | 3:1        |
| MLPK/mlpk  | S²/S⁹      | 4             | 5              |            |
| MLPK/mlpk  | S²/S⁸      | 14            | 1              |            |
| MLPK/mlpk  | S⁹/S⁸      | 2             | 7              |            |
| mlpk/mlpk  | S²/S⁹      | 25            | 10             | 3:1        |
| mlpk/mlpk  | S²/S⁸      | 0             | 3              |            |
| mlpk/mlpk  | S⁹/S⁸      | 3             | 12             | 3:1        |

*P > 0.05; **P < 0.001.
In this study, we also characterized a seed production phenotype of the intraspecies UI in *B. rapa*, in which the strength of UI could not be distinguished from that of SI. If SUI and PUI lines are grown in the same field, their hybrid seeds should not be produced on the SUI line. The UI pollen rejection system described here would potentially function as a reproductive barrier between the PUI-possessing Turkish line and SUI-possessing Japanese commercial line, although it is not a complete barrier.

The SI system is widely used for production of *Brassica* hybrid seeds, and the strength of SI is an important phenotype for efficient breeding of *F₂* hybrid crops. Several genetic studies of the variation in strength of the SI response have been reported (Hatakeyama et al. 2010; Isokawa et al. 2010), suggesting that there are several genetic factors regulating SI strength in *Brassica*. From the viewpoint of incompatibility strength, UI described here has a strong incompatible response, the same as in the rigid SI phenotype. Potentially, by using this UI locus for breeding of *Brassica* crops, alternative efficient systems for *F₂* breeding could be available in the future. Identification and characterization of the molecular determinants of this *Brassica* intraspecies UI will contribute to our understanding of the molecular mechanisms of the pollen-stigma interaction and incompatibility.

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LITERATURE CITED

Bernacchi, D., and S. D. Tanksley, 1997 An interspecific backcross of *Lycopersicon esculentum* × *L. hirsutum*: linkage analysis and a QTL study of sexual compatibility factors and floral traits. Genetics 147: 861–877.

Doughty, J., S. Dixon, S. J. Hiscock, A. C. Willis, L. A. Parkin et al., 1998 PCP-A1, a defense-like *Brassica* pollen coat protein that binds the S-locus glycoproteins, is the product of gametophytic gene expression. Plant Cell 10: 1333–1347.

Doughty, J., H. Y. Wong, and H. G. Dickinson, 2000 Cysteine-rich pollen coat proteins (PCPs) and their interactions with stigmatic S (incompatibility) and S-related proteins in *Brassica*: putative roles in SI and pollination. Ann. Bot. (Lond.) 85: 161–169.

Nou, I. S., M. Watanabe, A. Isogai, and K. Hinata, 1993 Comparison of S-alleles and S-glycoproteins between two wild populations of *Brassica campestris* in Turkey and Japan. Sex. Plant Reprod. 6: 79–86.

Shih, H., S. Takayama, M. Iwano, H. Shima, M. Watanabe, T. Entani et al., 2007 Characterization of the SP11/SCR high-affinity binding site involved in self/nonself recognition in *Brassica* self-incompatibility. Plant Cell 19: 107–117.

Shiu, S., and A. Bleecker, 2003 Expansion of the receptor-like kinase/pelle gene family and receptor-like proteins in *Arabidopsis*. Plant Physiol. 132: 530–543.

Stein, J. C., B. Howlett, D. C. Boyes, M. E. Nasrallah, and J. B. Nasrallah, 1991 Molecular cloning of a putative receptor protein kinase gene encoded at the self-incompatibility locus of *Brassica oleracea*. Proc. Natl. Acad. Sci. U S A 88: 8816–8820.

Suzuki, G., N. Kai, T. Hirose, K. Fukushima, T. Nishio et al., 1999 Genomic organization of the S loci: Identification and characterization of genes in SLG/SRK region of S⁰ haplotype of *Brassica campestris* (syn. *rapa*). Genetics 153: 391–400.

Takasaki, T., K. Hatakeyama, M. Watanabe, K. Toriyama, A. Isogai et al., 1999 Introduction of SLG (S locus glycoprotein) alters the phenotype of
endogenous S haplotype, but confers no new S haplotype specificity in *Brassica rapa* L. Plant Mol. Biol. 40: 659–668.

Takasaki, T., K. Hatakeyama, G. Suzuki, M. Watanabe, A. Isogai et al., 2000 The S receptor kinase determines self-incompatibility in *Brassica* stigma. Nature 403: 913–916.

Takada, Y., T. Nakanowatari, J. Sato, K. Hatakeyama, T. Kakizaki et al., 2005 Genetic analysis of novel intra-species unilateral incompatibility in *Brassica rapa* (syn. *campestris*). L. Sex. Plant Reprod. 17: 211–217.

Takayama, S., H. Shiba, M. Iwano, A. Asano, M. Hara et al., 2000a Isolation and characterization of pollen coat proteins of *Brassica campestris* that interact with S locus-related glycoproteins 1 involved in pollen-stigma adhesion. Proc. Natl. Acad. Sci. U S A 97: 3765–3770.

Takayama, S., H. Shiba, M. Iwano, H. Shimosato, F.-S. Che et al., 2000b The pollen determinant of self-incompatibility in *Brassica campestris*. Proc. Natl. Acad. Sci. U S A 97: 1920–1925.

Takayama, S., H. Shimosato, H. Shiba, M. Funato, F.-S. Che et al., 2001 Direct ligand-receptor complex interaction controls *Brassica* self-incompatibility. Nature 413: 524–538.

Tarutani, Y., H. Shiba, M. Iwano, T. Kakizaki, G. Suzuki et al., 2010 Trans-acting small RNA determines dominance relationships in *Brassica* self-incompatibility. Nature 466: 983–986.

Udagawa, H., Y. Ishimaru, F. Li, Y. Sato, H. Kitashiba et al., 2010 Genetic analysis of interspecific incompatibility in *Brassica rapa*. Theor. Appl. Genet. 121: 689–696.

Zhang, X., L. Wang, Y. Yuan, D. Tian, and S. Yang, 2011 Rapid copy number expansion and recent recruitment of domains in S-receptor kinase-like genes contribute to the origin of self-incompatibility. FEBS J. 22: 4323–4337.

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