A Pollen Coat Protein, SP11/SCR, Determines the Pollen *S*-Specificity in the Self-Incompatibility of *Brassica* Species[1]

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Many flowering plants have evolved self-incompatibility (SI) systems to prevent inbreeding. In the Brassicaceae, SI is genetically controlled by a single polymorphic locus, termed the *S*-locus. Pollen rejection occurs when stigma and pollen share the same *S*-haplotype. Recognition of *S*-haplotype specificity has recently been shown to involve at least two *S*-locus genes, *S*-receptor kinase (*SRK*) and *S*-locus protein 11 or *S*-locus Cys-rich (*SP11/SCR*). *SRK* encodes a polymorphic membrane-spanning protein kinase, which is the sole female determinant of the *S*-haplotype specificity. *SP11/SCR* encodes a highly polymorphic Cys-rich small basic protein specifically expressed in the anther tapetum and in pollen. In cauliflower (*B. oleracea*), the gain-of-function approach has demonstrated that an allele of *SP11/SCR* encodes the male determinant of *S*-specificity. Here we examined the function of two alleles of *SP11/SCR* of *B. rapa* by the same approach and further established that SP11/SCR is the sole male determinant of SI in the genus *Brassica* sp. Our results also suggested that the 522-bp 5′-upstream region of the S_9-*SP11* gene used to drive the transgene contained all the regulatory elements required for the unique sporophytic/gametophytic expression observed for the native *SP11* gene. Promoter deletion analyses suggested that the highly conserved 192-bp upstream region was sufficient for driving this unique expression. Furthermore, immunohistochemical analyses revealed that the protein product of the *SP11* transgene was present in the tapetum and pollen, and that in pollen of late developmental stages, the SP11 protein was mainly localized in the pollen coat, a finding consistent with its expected biological role.

Self-incompatibility (SI) prevents self-fertilization and promotes out-crossing in hermaphrodite seed plants (Nettancourt, 1977). In most species the self/non-self recognition in SI is controlled by a single multi-allelic locus termed the *S*-locus. The *S*-locus is expected to contain at least two separate polymorphic genes, one determining the female and the other the male *S*-haplotype specificity. Numerous attempts have been made to identify these genes in several families that possess SI, e.g. Brassicaceae, Solanaceae, and Papaveraceae (McCubbin and Kao, 2000).

In the Brassicaceae, three highly polymorphic genes have been identified at the *S*-locus: *SLG* (for *S*-locus glycoprotein), *SRK* (for *S*-locus receptor kinase), and *SP11/SCR* (for *S*-locus protein 11 or *S*-locus Cys-rich). *SLG* encodes an abundant, secreted glycoprotein located in the cell wall of the papillar cell of the stigma (Takayama et al., 1987; Kandasamy et al., 1989). *SRK* encodes a membrane-anchored Ser/Thr protein kinase containing an extracellular domain, which shares extensive sequence similarity with SLG (Stein et al., 1991). SRK is expected to span the plasma membrane of the papillar cell. Earlier loss-of-function experiments using an antisense *SLG* gene demonstrated that *SLG* and/or *SRK* encoded the female determinant of SI; however, the precise role of each gene was not determined (Shiba et al., 1995, 2000). Recent gain-of-function experiments have provided conclusive evidence that SRK is the sole determinant of the *S*-haplotype specificity of the stigma (Takasaki et al., 2000). These experiments have also demonstrated that SLG is not required for the *S*-haplotype specificity, but may nonetheless play a role in enhancing the SI response. However, how this is accomplished is not yet known.

SP11/SCR is the third polymorphic gene at the *S*-locus to be discovered. *SP11* was first identified as an anther-expressed S_9-haplotype specific gene in an *SLG/SRK* flanking region of S_9-haplotype of *Brassica rapa* (Suzuki et al., 1999), and a different allele of the same gene (but named *SCR*) was independently identified in the corresponding region of S_8-haplotype of *B. rapa* (Schopfer et al., 1999). To date, 22 alleles of *SP11/SCR* have been identified in *B. rapa*, cauliflower, and oilseed rape, all of which encode proteins characteristic of novel pollen coat protein (PCP) family proteins (small, basic Cys-rich proteins; Bi et al., 2000; Schopfer et al., 1999; Suzuki et al., 1999; Takayama et

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al., 2000b; Watanabe et al., 2000). The fact that *SP11/SCR* determines the *S*-haplotype specificity of pollen was first demonstrated in cauliflower by a gain-of-function experiment (Schopfer et al., 1999). In this experiment, pollen of the transgenic plants carrying SCR_6 transgene from the S_6-haplotype of cauliflower was shown to acquire the S_6-haplotype specificity. In *B. rapa* we also demonstrated the biological role of SP11/SCR using a pollination bioassay (Takayama et al., 2000b). In this bioassay, recombinant S_9-SP11 (SP11 protein of the S_9-haplotype) was shown to elicit an SI response in the papillar cells in an *S*-haplotype-specific manner (i.e. the response was observed only when the protein was applied to the papillar cells of the same S_9-haplotype), resulting in the inhibition of cross-pollen hydration.

In this work we independently used the gain-of-function approach to further confirm the role of *SP11/SCR* in *B. rapa*. We report the results of the analyses of two lines of transgenic plants, one carrying S_8-*SP11* cDNA driven by the promoter of the S_9-*SP11* gene, and the other carrying S_9-*SP11* genomic DNA, including the promoter region. S_8-*SP11* and S_9-*SP11* transgenes were expressed at high levels in some of their respective transgenic plants and the pollen of these plants was shown to acquire the corresponding *S*-haplotype specificity. These results together with the previously reported transformation experiment in cauliflower conclusively establish that *SP11/SCR* is the sole male determinant of SI in the genus *Brassica*.

The transformation experiments also revealed that the 522-bp 5′-upstream region of the S_9-*SP11* gene used to drive the S_8-*SP11* transgene contained all the regulatory elements required for the unique sporophytic/gametophytic expression pattern of *SP11/SCR* that we had previously observed by in situ hybridization (Takayama et al., 2000b). Furthermore, promoter-deletion analyses suggested that the 192-bp 5′-upstream region, which is highly conserved in all of the *SP11/SCR* alleles examined, defines the minimum promoter sequence necessary for driving this unique expression. Immunohistochemical analyses using an antibody for recombinant S_8-SP11 protein showed that the protein product of the S_8-*SP11* transgene in transgenic plants was located in the tapetum and pollen, and was mainly localized in the pollen coat at late developmental stages, consistent with its expected biological role. This is the first demonstration that SP11/SCR protein is present on the surface of the pollen grain.

RESULTS

Promoter Sequences of *SP11* Alleles

We have previously shown that the *SP11* gene is specifically expressed in the tapetal cell of the anther at early developmental stages, as well as in the microspore at late developmental stages (Takayama et al., 2000b). To identify the promoter sequence elements required for this dual sporophytic/gametophytic expression we first compared the nucleotide sequences of the 5′-flanking region of five alleles of *SP11*, three from S_9, S_8, and S_{12} haplotypes of *B. rapa* (designated S_9-*SP11*, S_8-*SP11*, and S_{12}-*SP11*, respectively) and two from S_{910} and S_{A14} haplotypes of oilseed rape (designated S_{910}-*SP11* and S_{A14}-*SP11*, respectively). These sequences are highly conserved (69.4%–88.0% identity) in the SP11 promoter from −200 to −1 bp, but less conserved in the region upstream beyond −200 bp (Fig. 1). From database searches, no common repeat or palindromic sequences were found in the 5′-flanking region of these *SP11* alleles.

Analyses of Promoter Deletions of *SP11*

To identify and characterize *cis*-regulatory elements involved in promoter strength and specificity, transient expression analyses were performed using promoter deletion-β-glucuronidase (GUS) constructs. The GUS expression pattern for each of the constructs in the anther tapetum and pollen is presented in Figure 2A.

The entire 522-bp S_9-*SP11* 5′-flanking region drove high levels of GUS expression in anthers and pollen, but did not drive any expression in petals, sepals, or pistils (data not shown). Progressive deletions from

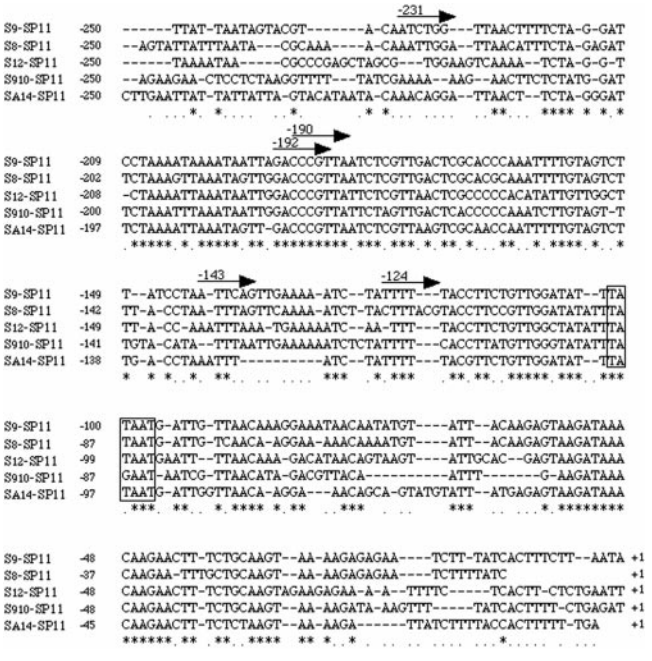

Figure 1. Alignment of the nucleotide sequences of the promoter region of S_9-*SP11*, S_8-*SP11*, S_{12}-*SP11*, S_{910}-*SP11*, and S_{A14}-*SP11*. The open box represents the putative TATA box. The arrows indicate the 5′ endpoints of the truncated promoter constructs used in the promoter analysis. Identical and conserved sequences are indicated by asterisks and periods, respectively. The position of the translation start site is assigned +1.

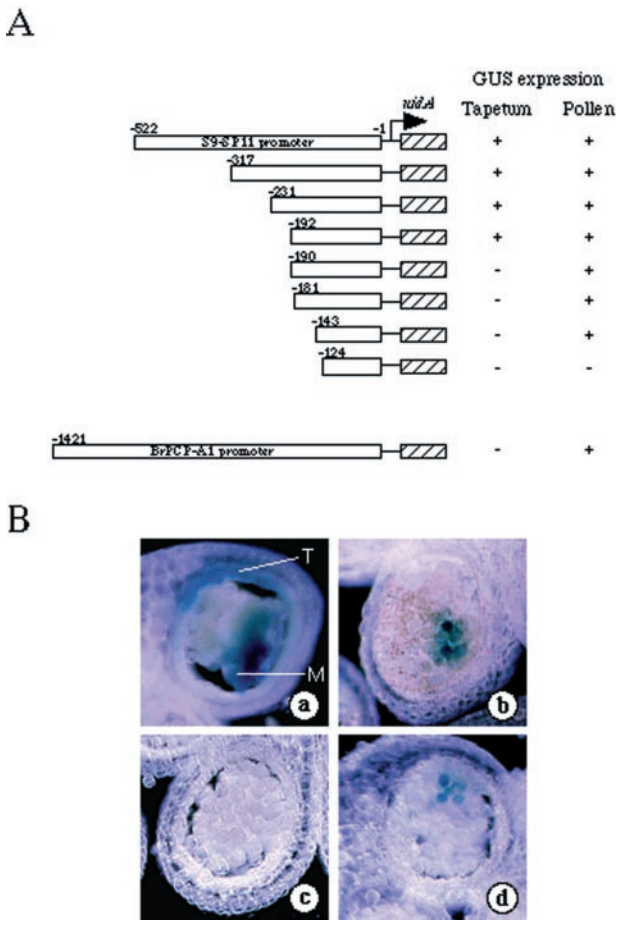

Figure 2. Deletion analyses of the *SP11* promoter region. A, *SP11* promoter deletion constructs and summary of GUS staining results. Numbers denote the 5′ most positions of the truncated promoters relative to the translation initiation codon (ATG) of the S_9-*SP11* gene. The GUS expression of each promoter construct in the tapetum and pollen is represented by + (positive) or − (negative). B, Representative GUS staining results of transient promoter-*gus* fusion analyses. Cross-sections of anthers that had been bombarded with 317-bp *SP11* promoter-*gus* (a), 143-bp *SP11* promoter-*gus* (b), 124-bp *SP11* promoter-*gus* (c), and 1,421-bp *BrPCP-A1* promoter-*gus* (d). T and M represent tapetum and microspore, respectively.

the 5′ end to −317, −231, and −192 bp did not affect the promoter activity in tapetum or pollen (Fig. 2B, a). Further deletions removing the region between −190 to −143 bp resulted in GUS expression in pollen only (Fig. 2B, b). The smallest construct that contained the 5′-flanking region up to position −124 showed no detectable GUS expression in tapetum or pollen (Fig. 2B, c). In the control experiment, the 5′-flanking region of the *BrPCP-A1* gene, which has previously been shown to be expressed gametophytically in pollen (Takayama et al., 2000b), exhibited GUS expression only in pollen (Fig. 2B, d). These results suggested that the 5′-flanking region up to −192 bp is sufficient to direct gene expression in tapetum and pollen, and that the region from −124 to 190 is involved in the specific expression in pollen.

Transformation of *B. rapa* Plants with S_8-*SP11* and S_9-*SP11* of *B. rapa*

We constructed two transformation vectors, pSLJS8-SP11 and pSLJS9-SP11 (Fig. 3), to introduce S_8-*SP11* cDNA and S_9-*SP11* genomic DNA, respectively, into SI *B. rapa* cv Osome, a heterozygote of S_{52} and S_{60} haplotypes. Seven transgenic plants with the S_8-*SP11* transgene and 29 transgenic plants with the S_9-*SP11* transgene were obtained by using the *Agrobacterium*-mediated transformation procedure described previously (Shiba et al., 2000). These transgenics were morphologically indistinguishable from the wild-type cv Osome. We chose three plants (T66, T67, and T74) containing the S_8-*SP11* transgene and two plants (T161 and T254) containing the S_9-*SP11* transgene for further analyses, all of which strongly expressed the GUS marker gene (data not shown). One GUS-negative plant carrying the S_8-*SP11* transgene (T59) was used as a negative control. The presence of both *SP11* transgenes was confirmed by PCR amplification of the *SP11* gene and by DNA-blot analysis using a full-length S_8-*SP11* and S_9-*SP11* cDNA probe (data not shown).

Expression Analyses of S_8-*SP11* and S_9-*SP11* Transgenes in Transgenic Plants

To confirm expression of the transgenes in the transgenic plants we performed RNA gel-blot analyses (Fig. 4). All three GUS-positive S_8-*SP11* transgenic lines, T66, T67, and T74, produced S_8-*SP11* mRNA, whereas the GUS-negative control line, T59, did not. The expression levels of the S_8-*SP11* mRNA in the former three transgenic lines were comparable with that in S_8-homozygote. Both GUS-positive S_9-*SP11* transgenic lines, T161 and T254, produced similar

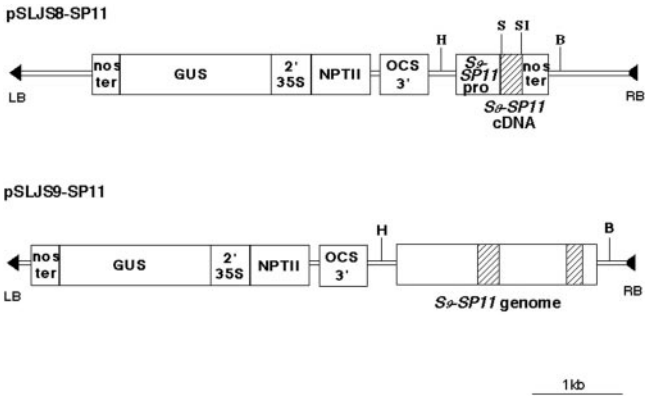

Figure 3. Schematic maps of the *SP11* transgenes. The coding regions of the *SP11* genes are indicated by hatched boxes. S_9-*SP11* pro, S_9-*SP11* promoter; Nos ter, nopaline synthase terminator; 2′35S, divergently transcribed 2′ and cauliflower mosaic virus 35S promoters; Npt II, neomycin phosphotransferase gene; OCS3, octopine synthase 3′ end; RB and LB, right and left borders of the T-DNA, respectively; H, *Hin*dIII restriction digest site; S, *Sma*I site; SI, *Sac*I site; B, *Bam*HI site.

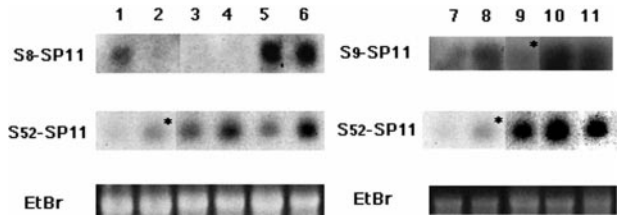

Figure 4. RNA-blot analysis of transcript levels of the endogenous S_{52}-*SP11* gene and the S_8-*SP11* and S_9-*SP11* transgenes. Blots containing total RNA from anthers at stage 7 (see "Materials and Methods") were hybridized with an S_8-*SP11* probe (lanes 1–6, top), an S_9-*SP11* probe (lanes 7–11, top), and an S_{52}-*SP11* probe (middle). Bottom, The ethidium bromide staining of rRNA. Lanes 1 through 11 contain anther RNA isolated from a *B. rapa* S_8 homozygote (lanes 1 and 7); an S_9 homozygote (lanes 2 and 8); an $S_{52}S_{60}$ heterozygote (recipient of the transgenes, lanes 3 and 9); S_8-*SP11* transformants T66 and T74 (lanes 5 and 6, respectively); T59, a GUS-negative S_8-*SP11* transformant (lane 4); and S_9-*SP11* transformants T161 and T254 (lanes 10 and 11, respectively). The asterisks indicate cross-hybriding bands due to high sequence similarity between S_9-*SP11* and S_{52}-*SP11* (Takayama et al., 2000b).

amounts of S_9-*SP11* mRNA, as did the S_9-homozygote. We also detected endogenous S_{52}-*SP11* mRNA in these transgenic plants, confirming the absence of cosuppression between the endogenous S_{52}-*SP11* gene and both *SP11* transgenes.

To confirm the expression of the *SP11* transgene products at protein level we performed immunohistochemical analyses utilizing antibodies produced against the recombinant S_8-SP11 protein. In the S_8-*SP11* transgenic line T66, strong signals for S_8-SP11 protein were detected in the tapetal cells of anther and in the pollen grains (Fig. 5, a and c). In control experiments no signal was detected in these organs of the wild-type plant throughout their developmental stages (Fig. 5, b and d).

Localization of the SP11 Protein in the Pollen Grains of Transgenic Plants

The localization of the SP11 protein in pollen grains at late developmental stages was further confirmed by immunoelectron-microscopic analyses. When sections of pollen grains of the S_8-*SP11* transgenic line T66 were treated with the anti-S_8-SP11 antibody and gold-conjugated anti rabbit IgG, the gold particles were mainly observed on the surface of pollen, especially in the pollen coat (Fig. 6a). In a control experiment pollen sections of the host strain ($S_{52}S_{60}$) showed very little labeling (Fig. 6b). Treatment of these sections with preimmune serum also produced very little labeling (data not shown).

Examination of SI Phenotypes of Transgenic Plants

To investigate the effect of the introduced *SP11* transgenes on the SI phenotype of the transgenic plants, pollination tests were reciprocally performed with three lines of *B. rapa*; S_8 homozygotes, S_9 homozygotes, and $S_{52}S_{60}$ heterozygotes (host strain). Pollen germination and pollen tube penetration in the style were examined under UV-fluorescence microscopy following staining with aniline blue. Pollen from all three lines of S_8-*SP11* transgenic plants (T66, T67, and T74) and two lines of S_9-*SP11* transgenic plants (T161 and T254) was incompatible with the stigma of the $S_{52}S_{60}$ host strain. In addition, pollen from the three lines of S_8-*SP11* transgenic lines was

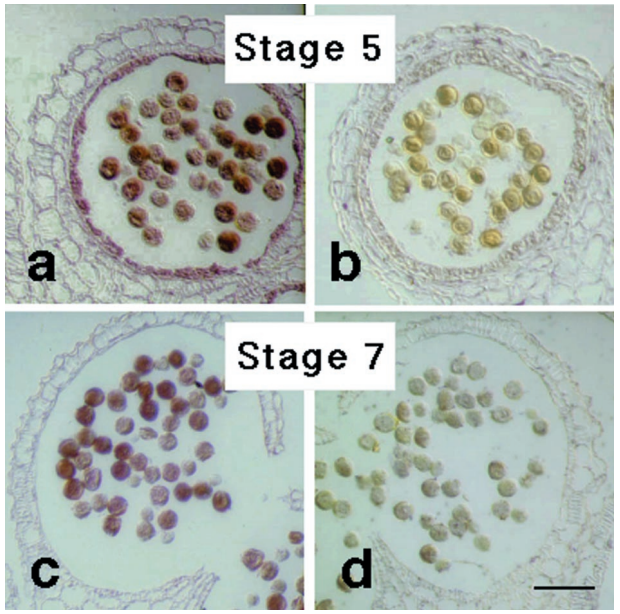

Figure 5. Immunolocalization of S_8-SP11. Anther sections derived from stage 5 and stage 7 flower buds (see "Materials and Methods") were immunostained with the anti-S_8-SP11 antibody. a, T66 line (S_{52} S_{60} /S_8-*SP11*) at stage 5; b, wild type (S_{52} S_{60}) at stage 5; c, T66 line at stage 7; d, wild type at stage 7. Scale bars = 50 μm.

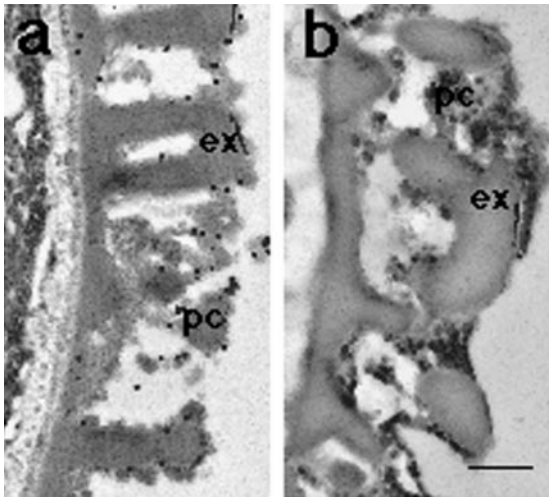

Figure 6. Immunoelectron microscopy of pollen grains. Immunogold localization of pollen grains treated with the anti-S_8-SP11 antibody and 20-nm gold-conjugated anti-rabbit IgG. a, T66 line ($S_{52}S_{60}$/ S_8-*SP11*); b, wild-type plant. ex, Exine; pc, pollen coating. Scale bar = 1 μm.

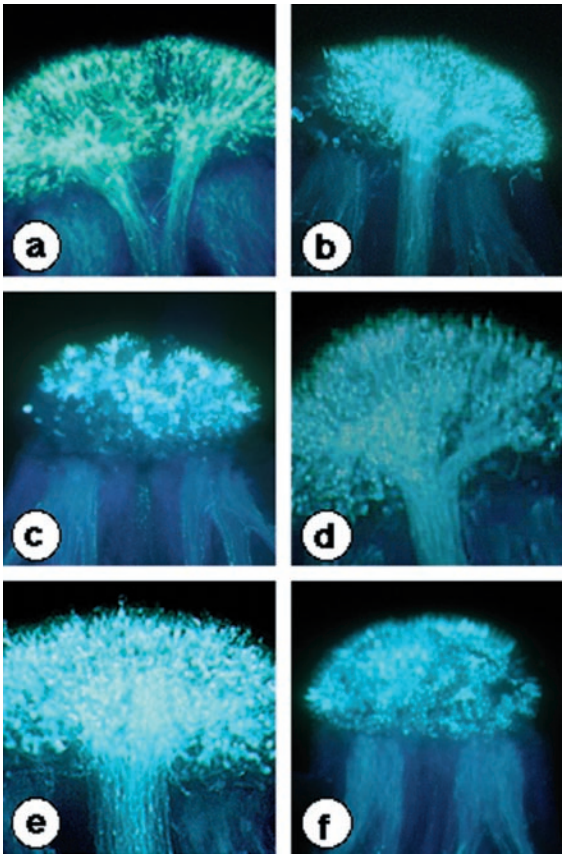

Figure 7. Representative results of pollination tests. Photographs were obtained by UV fluorescence microscopy. a, Cross-pollination of an S_8S_8 stigma with wild-type pollen ($S_{52}S_{60}$); b, cross-pollination of an S_9S_9 stigma with wild-type pollen; c, cross-pollination of an S_8S_8 stigma with transgenic pollen from T66 ($S_{52}S_{60}$ */S_8-SP11*); d, cross-pollination of an S_9S_9 stigma with transgenic pollen from T66; e, cross-pollination of an S_8S_8 stigma with transgenic pollen from T161 ($S_{52}S_{60}$ */S_9-SP11*); f, cross-pollination of an S_9S_9 stigma with transgenic pollen from T161.

incompatible with the stigma of S_8 homozygotes, whereas the wild-type host pollen was fully compatible with S_8 stigmas (Fig. 7, c and a). The pollen of these S_8-*SP11* transgenic plants was compatible with an unrelated *S* haplotype, S_9 (Fig. 7d), indicating that the incompatibility observed with S_8 homozygotes did not result from a reduced viability of pollen. For the two lines of S_9-*SP11* transgenic plants, their pollen was incompatible with S_9 stigmas (Fig. 7f), yet compatible with S_8 stigmas (Fig. 7e). No phenotypic alterations were observed in the stigmas of all these S_8-*SP11* and S_9-*SP11* transgenic plants (data not shown).

To ensure that the pollen behavior observed in the above pollination tests reflected the plant incompatibility phenotype, we examined the ability of these plants to produce seeds following reciprocal crosses. When pollen from the three S_8-*SP11*-expressing transgenic lines was used to pollinate S_8 stigmas, no seeds were produced (Fig. 8A); however, silique and ample seeds were produced when pollen from these three transgenic lines was used to pollinate S_9 stigmas (Fig. 8B). Moreover, the stigma phenotype of the S_8-*SP11* transgenic plants was the same as that of the wild-type host plants (data not shown). For the two S_9-*SP11*-expressing lines, no seeds were produced when their pollen was used to pollinate S_9 stigma (Fig. 8B) and again, silique and ample seeds were produced when their pollen was used to pollinate S_8 stigmas (Fig. 8A). These results demonstrate that SP11 is the determinant of the *S*-haplotype specificity of pollen in SI recognition.

DISCUSSION

In this report we introduced two *SP11* transgenes, S_8-*SP11* and S_9-*SP11*, into SI *B. rapa* $S_{52}S_{60}$ heterozygotes to examine the effect of the transgenes on SI behavior. Pollen from S_8-*SP11* and S_9-*SP11* transformants was shown to acquire the S_8- and the S_9-haplotype specificity, respectively. Our results, together with the previously reported SCR gene transformation in cauliflower (Schopfer et al., 1999), have definitively established that SP11/SCR is the sole male determinant of SI in the genus *Brassica*.

Our previous in situ hybridization analysis has revealed that *SP11/SCR* is expressed in the tapetal cells at early developmental stages and in the microspores at late stages (Takayama et al., 2000b). This sporophytic/gametophytic expression pattern is

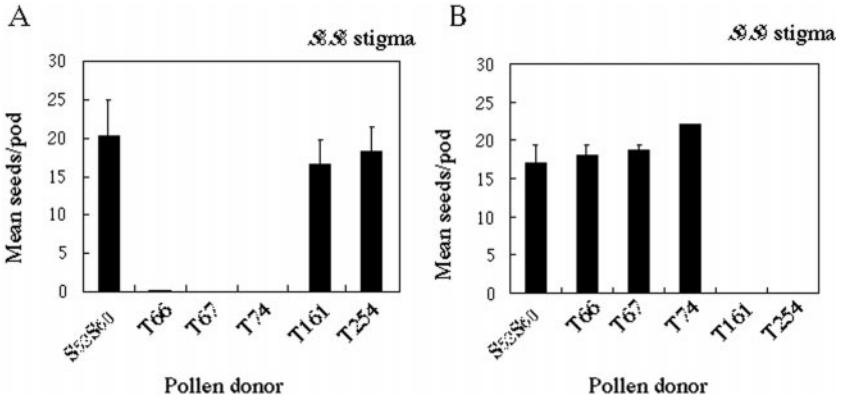

Figure 8. Seed set analyses. Data represent the mean number of seeds per pod after cross-pollination. A, S_8S_8 pistils pollinated with transgenic pollen; B, S_9 S_9 pistils pollinated with transgenic pollen. T66, T67, and T74, $S_{52}S_{60}$ /S_8-*SP11*; T161 and T254, $S_{52}S_{60}$ /S_9-*SP11*. Error bars indicate ±SE.

quite unique to *SP11/SCR* when compared with genes encoding other PCP family of proteins, PCP-A and PCP-B classes (Doughty et al., 1998, 2000; Takayama et al., 2000a), and can explain why SI phenotype of pollen is sporophytically determined in *Brassica* sp. In this study we have analyzed the 5′-upstream sequences of five alleles of the *SP11* gene from *B. rapa* or oilseed rape. Alignment of these sequences suggested that the immediate upstream region within 200 bp of the coding region is highly conserved; these sequences share 69.4% to 88.0% identity with one another. However, no common repeat or palindromic sequence that could potentially play a role in regulation of gene expression was identified within this region. The region beyond 200 bp upstream of the coding region is not conserved among these alleles. This region does not appear to have any conserved sequence element either. In the promoter region of the *PCP1* gene, the first reported *PCP-A* class gene from cauliflower, two direct repeats (TTTTAGATTATAAA) and a putative pollen-specific element (CTTAAATTAGA), were identified (Stanchev et al., 1996). However, these sequences were not found in the 5′-flanking region of the *SP11* gene.

Although no known regulatory element was found in the promoter region of the *SP11* gene, the analysis of the S_8-*SP11* gene transformants indicated that the 522 bp of the S_9-*SP11* promoter region used to drive the transgene is sufficient to drive proper *SP11* gene expression. Thus, this region must contain all the regulatory elements required for the unique expression of the *SP11* gene. Transient expression analyses using truncated *SP11* promoter-*gus* fusions allowed us to examine the active elements involved in this promoter region. Our results revealed that the region around −192 bp contains the element(s) required for GUS expression in the tapetum, and that the region between −124 and −143 represents the minimal promoter region for pollen expression. Tissue-specific expression is thought to be the result of combinatorial regulation by specific sets of regions present in the promoter. For example, the tapetum and pollen expression of *Bcp1* was controlled by different cis-acting elements that act in conjunction with common cis-acting elements to confer the expression in the tapetum or pollen (Xu et al., 1993). Our results also suggest that the sporophytic and gametophytic expression patterns of the *SP11/SCR* gene are controlled by different cis-regulatory elements.

Immunolocalization analyses of S_8-SP11 protein revealed that this protein was specifically localized to the pollen coat when pollen grains reached the trinucleate stage. The pollen coat is the outermost layer of a pollen grain and makes the initial contact with the stigma surface during sexual reproduction. Therefore, the localization of SP11 is consistent with its biological role as an *S*-determinant. Although the pollen coating has been considered to be derived principally from the tapetal cells that line the anther locule, the origin of SP11 protein in the pollen coat (whether derived from the tapetal cells and/or the pollen grain) remains unclear (Doughty et al., 1998). Further transformation experiments introducing the *SP11* gene under the control of a pollen-specific or tapetum-specific promoter will likely provide the answer to this question.

MATERIALS AND METHODS

Plant Material

Brassica rapa (syn. *campestris*) S_8 and S_9 homozygotes were established from spontaneous populations at Oguni in Japan as previously described (Hinata and Nishio, 1978). *B. rapa* cv Osome (Takii Seed Co., Kyoto), a commercial F_1 hybrid of the $S_{52}S_{60}$ haplotype (Takasaki et al., 1997), was used as the recipient of transgenes. S_8, S_9, and S_{52} belong to class I *S*-haplotypes and S_{60} belongs to class II *S*-haplotypes (Hatakeyama et al., 1998; Takasaki et al., 2000). The dominance/recessive relationships among S_8, S_9, S_{52}, and S_{60} are as follows. S_{52} is codominant with S_9 and dominant over S_8 and S_{60} in the stigma; S_9 is dominant over S_8 and S_{60} in the stigma; S_8 is dominant over S_{60} in the stigma; S_{52} is codominant with S_9 and S_8 and is dominant over S_{60} in pollen; S_9 and S_8 are dominant over S_{60} in pollen. *Agrobacterium tumefaciens* strain EHA105 was a kind gift of Prof. Atsuhiko Sinmyo (Nara Institute of Science and Technology, Ikoma, Japan).

Transient Expression Vectors and Promoter Deletion Constructs

A cassette containing the *gus* coding region followed by the nopaline synthase polyadenylation signal from pBI221 (CLONTECH, Palo Alto, CA) was used to construct promoter-*gus* fusions. The 522-bp S_9-*SP11* 5′-flanking region was obtained from a P1-derived artificial chromosome clone, E89 (Suzuki et al., 1997), and the 1,421-bp fragment containing the 5′-flanking region of the *BrPCP-A1* gene (Takayama et al., 2000b) was isolated from genomic DNA of *B. rapa* S_{12} homozygote. These fragments were reamplified by PCR using specific primers designed to add *Hin*dIII and *Bam*HI restriction sites to the 5′ and 3′ ends, respectively (for the amplification of the 5′-flanking region of the S_9-*SP11* gene: sense primer 5′-GAAGCTTGGTACATTAACTATGTCT-3′, antisense primer 5′-GGATCCGCGAGTCAACGAGATTAACGGGTC-3′; for the amplification of 5′-flanking region of the *BrPCP-A1* gene: sense primer 5′-GAAGCTTCTACACTAGATCAATGGCAA-3′, antisense primer 5′-CCCGGGAACCGTGTTTTTCATCTTAG-3′). The amplified fragments were subcloned into pBI221 to yield the 522-bp *SP11* promoter-*gus* and 1,421-bp *PCP-A1* promoter-*gus* constructs, respectively.

A series of deletion constructs was generated from the 522-bp *SP11* promoter-*gus* construct by Exonuclease III and religation, following the protocol supplied with the kilo-sequence deletion kit (Takara, Shiga, Japan). All seven constructs were sequenced to confirm the absence of any possible mutation introduced during PCR. All plasmid

DNA was prepared using the Plasmid Midi kit (Qiagen, Valencia, CA), following the manufacturer's instructions.

Particle Bombardment

Developmental stages of anther were classified according to bud length as previously described (Takayama et al., 2000b). Immature buds at developmental stage 5 (bud length: 4–5 mm) from *B. rapa* S_9 homozygote were cut transversely into approximately 1-mm sections. Thirty sections per plate (100 mm in diameter) were placed onto a filter paper (Toyo Roshi, Tokyo) immersed in sterilized water. Plasmid DNA (approximately 0.5 μg) was precipitated onto 1.0-μm gold particles (Bio-Rad, Hercules, CA) according the manufacturer's instructions. Bombardments were performed using a Biolistic particle acceleration device (PDS 1000/He, Bio-Rad). Three shots were performed per plate. One day after bombardment, the sections from each plate were incubated for 16 h at 37°C in 50 mM sodium phosphate buffer, pH 7.0, containing 1 mg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (Wako, Osaka). The samples were embedded in 2% (w/v) agar and then cut into 150-μm sections with a Microslicer (D. S. K., Osaka), and were observed by microscopy (Axiophot 2, Zeiss, Jena, Germany).

Construction of S_8-*SP11* and S_9-*SP11* Transgenes

The full length S_8-*SP11* cDNA (Takayama et al., 2000b) was amplified by PCR using *SP11*-specific primers 5′-CCCGGGATGAAATCGGCTGTTTATGC-3′ (underlined sequence indicating the incorporated *Sma*I site) and 5′-GAGCTC GATAGCATTTGCTAACAC-3′ (underlined sequence indicating the incorporated *Sac*I site), and inserted into pGEM vector (Promega, Madison, WI). The chimeric gene, consisting of the 522-bp promoter region of S_9-*SP11*, the coding region of S_8-*SP11* cDNA and the nopaline synthase transcription terminator, was inserted into binary vector, pSLJ1006 (Jones et al., 1992), to create pSLJS8-SP11. A 2.3-kb fragment of S_9-*SP11* genomic DNA was amplified by PCR using the E89 DNA (Suzuki et al., 1997) as a template and primers that had been designed based on the 5′-non-coding sequence of the S_9-*SP11* gene (5′-GAAGCTCCAGTACACCTGCTCAGTCATAGATG-3′, with the underlined sequence indicating the incorporated *Hin*dIII site) and the 3′-non-coding sequence of the S_9-*SP11* gene (5′-GAAGCTCGTTCACATGGATCAACATCTACCGG -3′, with the underlined sequence indicating the incorporated *Hin*dIII site). The DNA fragment obtained was digested with *Hin*dIII and inserted into the corresponding sites of binary vector, pSLJ1006 (Jones et al., 1992), to create pSLJS9-SP11.

Transformation

The plasmids pSLJS8-SP11 and pSLJS9-SP11 were electroporated into *A. tumefaciens* strain EHA105 (Hood et al., 1993). The hypocotyl transformation of *B. rapa* cv Osome with *Agrobacterium* harboring pSLJS8-SP11 or pSLJS9-SP11 and the subsequent regeneration of transgenic plants was performed as previously described (Shiba et al., 2000). To ascertain the presence of the *SP11* transgenes, leaf pieces from the transgenic plants were examined for GUS staining using 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid and was analyzed by PCR amplification using *SP11*-specific primers.

RNA Gel-Blot Analyses

Total RNA was isolated from stage 7 anthers (bud length: 7–10 mm) using Isogen (Nippon Gene, Toyama, Japan). Fifteen micrograms of total RNA was electrophoresed on a 1.2% (w/v) agarose/formamide gel and transferred to a Gene-screen Plus membrane (DuPont/NEN, Boston). The membrane was hybridized at 60°C for 12 h with random-prime-^{32}P-labeled S_8-, S_9-, and S_{52}-*SP11* gene probes specific for the coding region of the mature protein and the 3′-non-coding region. After hybridization, the membrane was washed in 0.1× SSPE, 2% (w/v) SDS at 60°C for 30 min and exposed on x-ray film (Amersham Pharmacia Biotech, Piscataway, NJ). Equal loading of total RNA was assessed by ethidium bromide staining of rRNA bands.

Immunocytochemistry

To produce recombinant S_8-SP11 protein, the cDNA of mature S_8-*SP11* coding region was amplified using specific primers (5′-CGGAATTCCATCGAAGGTCGTCAAGAACTGGAAGCTAATCT-3′, with the underlined sequence indicating the incorporated *Eco*RI site, and 5′-GCTCGAGT CTAACACGATTTACAGTCACAG-3′, with the underlined sequence indicating the incorporated *Xho*I site), and inserted into pGEX-5X-3 vector (Amersham Pharmacia). This construct was transformed into *Escherichia coli* strain BL21. The induction and purification of the recombinant glutathione *S*-transferase (GST)-S_8-SP11 fusion protein was performed according to the manufacturer's protocol. This fusion protein was used to immunize rabbits. To remove non-specific antibodies that might react with the GST-domain of the fusion protein, the crude serum was first absorbed with *N*-hydroxysuccinilamide-activated Sepharose resin (Amersham Pharmacia Biotech) to which the GST-S_9-SP11 fusion protein had been covalently attached (Takayama et al., 2000b). The S_8-SP11-specific antibody was then affinity purified on the same resin with the GST- S_8-SP11 fusion protein also attached to it.

Anthers at developmental stages 5 and 7 were collected and 10 μm of Paraplast (Sigma, St. Louis) sections were prepared as described by Doughty et al. (1998). Prior to incubation with the primary antibody, sections were blocked with 1% (w/v) non-fat dry milk (NFM) and 1% (v/v) goat serum in Tris-buffered saline (TBS) at 37°C for 30 min. The purified S_8-SP11 antibody was diluted 1:100 in TBS supplemented with NFM and goat serum. Following incubation with the primary antibody at 37°C for 1 h, sections were washed with 0.05% (v/v) Tween 20 in TBS (TBST, pH 7.4). The secondary antibody, alkaline phosphatase-conjugated goat anti-rabbit IgG (Biocell Research Laborato-

ries, Cardiff, UK), was diluted 1:100 in TBST. Sections were incubated with the secondary antibody at 37°C for 1 h, then washed in TBST and distilled water. Detection of the alkaline phosphatase activity was performed using 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate, according to the manufacturer's instructions (Sigma). In control experiments, the primary antibody was either omitted or replaced with preimmune rabbit serum.

Immunoelectron Microscopy

Immunoelectron microscopy was performed as described by Seo et al. (2000) except that anthers of the late developmental stage 7 were cut in halves and fixed with 4% (v/v) paraformaldehyde and 0.1% (v/v) glutaraldehyde in cacodylate buffer (pH 7.4) under vacuum. After dehydration, the tissues were embedded in Resin LR White (London Resin Co., London) at −20°C. Ultra-thin sections were incubated with the anti-S_8-SP11 antibody (1:100 dilution in PBS-NFM) overnight at 4°C and then incubated with 20-nm (in diameter) gold-conjugated goat anti-rabbit IgG (dilution 1:100 in PBS buffer, Biocell Research Laboratories) for 1 h at 25°C. After immunolabeling, the sections were stained with uranyl acetate. As a control, specimens were incubated with preimmune rabbit serum. Samples were observed in a Hitachi transmission electron microscope (H-7100; Hitachi, Tokyo).

Pollination Assay

Fresh flowers collected at the day of anthesis were used for the pollination assay. Hand-pollinated pistils were cut at the peduncle, stood on 1% (w/v) solid agar, and kept at 20°C for 6 h. After fixation for 2 h in ethanol:acetic acid (3:1), the pistils were softened in 1 N NaOH at 60°C for 1.5 h, and stained with 0.01% (w/v) decolorized aniline blue for 2.5 h in 2% (w/v) K3PO4. Pistils were gently squashed onto a microscopic slide glass by placing the cover glass over the pistils. Samples were examined under a fluorescence microscope (Axiophot 2, Zeiss) with an excitation filter of 395 nm and an emission filter of 420 nm (Dumas and Knox, 1983).

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