The Dominance of Alleles Controlling Self-Incompatibility in *Brassica* Pollen Is Regulated at the RNA Level

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Self-incompatibility (SI) in *Brassica* is controlled sporophytically by the multiallelic S-locus. The SI phenotype of pollen in an S-heterozygote is determined by the relationship between the two S-haplotypes it carries, and dominant/recessive relationships often are observed between the two S-haplotypes. The S-locus protein 11 (SP11, also known as the S-locus cysteine-rich protein) gene has been cloned from many pollen-dominant S-haplotypes (class I) and shown to encode the pollen S-determinant. However, SP11 from pollen-recessive S-haplotypes (class II) has never been identified by homology-based cloning strategies, and how the dominant/recessive interactions between the two classes occurred was not known. We report here the identification and molecular characterization of SP11s from six class II S-haplotypes of *B. rapa* and *B. oleracea*. Phylogenetic analysis revealed that the class II SP11s form a distinct group separated from class I SP11s. The promoter sequences and expression patterns of SP11s also were different between the two classes. The mRNA of class II SP11, which was detected predominantly in the anther tapetum in homozygotes, was not detected in the heterozygotes of class I and class II S-haplotypes, suggesting that the dominant/recessive relationships of pollen are regulated at the mRNA level of SP11s.

INTRODUCTION

Many species of hermaphrodite plants have evolved mechanisms to prevent self-fertilization. Self-incompatibility (SI) is one physiological means of avoiding self-fertilization through recognition of self-pollen in or on the female pistil. Classic genetic analyses have revealed the presence of two major types of homomorphic SI systems, gametophytic and sporophytic (de Nettancourt, 1977). Although the recognition of self-pollen is controlled genetically by a single highly polymorphic locus called the S-locus in both of these systems, the SI phenotype of pollen (gametophyte) is determined by its own S-haplotype in the gametophytic system, whereas in the sporophytic system, the SI phenotype is controlled by the S-haplotypes of the diploid parent (sporophyte).

The majority of the members of the cruciferous plant genus *Brassica* possess a strong sporophytic SI system. Thus, the SI phenotype of pollen as well as stigma is determined by relationships between the two S-haplotypes carried by its parent (Bateman, 1955). In other words, a codominant or a dominant/recessive relationship between the two S-haplotypes influences the ultimate SI phenotype of both pollen and stigma (Thompson and Taylor, 1966). The following observations have been made about dominance relationships among S-haplotypes: (1) codominance is common; (2) dominance/recessiveness is frequent in pollen; (3) dominance relationships among stigmas are different from those among pollen; and (4) dominance relationships are nonlinear (Thompson and Taylor, 1966; Ockendon, 1975; Visser et al., 1982; Hatakeyama et al., 1998a).

Recent molecular studies have revealed that the S-locus of *Brassica* encodes three highly polymorphic molecules that are involved in SI recognition: S-receptor kinase (SRK), S-locus protein 11 (SP11, also known as S-locus cysteine-rich protein [SCR]), and S-locus glycoprotein (SLG). SRK is a membrane-spanning serine/threonine receptor kinase predominantly present at the papilla cell membrane that functions as the sole determinant of the SI phenotype of stigma (Stein et al., 1991; Delorme et al., 1995; Takasaki et al., 2000). SP11/SCR is a small cysteine-rich basic protein predominantly present at the pollen coat that functions as the sole determinant of the SI phenotype of pollen (Schoof et al., 1999; Suzuki et al., 1999; Takayama et al., 2000a; Shiba

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Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.010378.
SLG is a secreted glycoprotein predominantly present in the papilla cell wall that, although not involved directly in the determination of the SI phenotype, enhances the SI response by an unknown mechanism (Nasrallah et al., 1987; Takayama et al., 1987; Kandasamy et al., 1989; Kishinonishizawa et al., 1990; Takasaki et al., 2000). Now that the determinants of both stigma and pollen have been identified, it is important to analyze the connection between the expression or activities of these determinants and dominance relationships. Recently, Hatakeyama et al. (2001) investigated whether the stigmatic determinant of SRK was involved in determining the dominance relationships of stigma using five S-homozygotes carrying an SRK28 transgene. They showed that the dominance relationship between the SRK28 transgene and each of the endogenous S-haplotypes was identical to that between the S28-haplotype and the respective endogenous S-haplotype. Moreover, in the S28S43-homozygote carrying the SRK28 transgene, in which the S43 phenotype in the stigma was masked by the presence of SRK28, the transcript level of SRK28 was found to be much lower than that of SRK43. These results suggest that the dominant/recessive relationships between S-haplotypes in the stigma are determined by SRK itself, but not as a result of its relative transcription level.

Regarding dominance relationships in pollen, the pollen determinant SP11/SCR from pollen-recessive S-haplotypes has not been identified, and how the relationships are determined is not known. However, it has been suggested that pollen-recessive S-haplotypes have a set of SLG and SRK called class II, whereas pollen-dominant S-haplotypes have a different set called class I (Nasrallah and Nasrallah, 1993). To date, class II SLGs have been found only in pollen-recessive S-haplotypes, which include S2g, S40, S44, and S60 haplotypes of B. rapa (syn. campestris) (Hatakeyama et al., 1998a; Takasaki et al., 2000) and S2, S5, and S1-haplotypes of B. oleracea (Chen and Nasrallah, 1990; Scutt and Ory, 1992; Cabrillac et al., 1999). The pairwise sequence identity among class II SLGs is in excess of 86%, whereas that between class I and class II SLGs is only ~60 to 70%. The extracellular domains of pollen-dominant and pollen-recessive SRKs also can be placed into these two classes in a similar manner. These analytical results on stigmatic S-locus proteins suggest that class II S-haplotypes have an origin quite different from that of class I S-haplotypes (Uyenoyama, 1995; Kusaba et al., 1997; Schierup et al., 2001). In accordance with this assumption, attempts to isolate SP11s from class II S-haplotypes based on their sequence similarity with class I SP11s have been unsuccessful (Watanabe et al., 2000; Kusaba et al., 2001).

We analyzed the SRK28 flanking region of the class II S60 haplotype of B. rapa and succeeded in identifying the allelic S60-SP11. Based on their similarity to this sequence, we also identified the class II SP11 alleles from the S2g, S40, and S44 haplotypes of B. rapa and the S5- and S1-haplotypes of B. oleracea. These SP11 alleles from class II S-haplotypes showed relatively greater sequence similarity with each other than with SP11 alleles from class I S-haplotypes, as was the case with SLG and SRK. In situ hybridization analysis revealed that class II SP11 was expressed predominantly in anther tapetum in the homozygote of the class II S-haplotype. In contrast, the expression of the class II SP11 was found to be greatly suppressed in the heterozygote of class I and class II S-haplotypes, suggesting that the dominant/recessive relationship in pollen is determined by the expression level of SP11.

RESULTS

Cloning and Sequence Analysis of S60-SP11

We previously amplified 18 alleles of SP11 from class I S-haplotypes of B. rapa by reverse transcription–polymerase chain reaction (RT-PCR) using a primer designed from the conserved signal peptide region and an oligo(dT) primer (Watanabe et al., 2000). However, the same combination of primers failed to amplify allelic SP11 from any class II S-haplotypes. Various attempts to amplify the class II SP11 genomic fragment using primers designed from the conserved 5’ and 3’ noncoding regions of class I SP11 alleles also failed (data not shown), suggesting that the entire genomic sequences of SP11s are rather different between class I and class II S-haplotypes. This idea was supported by the observation that DNA gel blot analysis using class I SP11 probes detected no or only a weak hybridization signal with DNA of any class II S-haplotype of B. oleracea and B. rapa (Schopfer et al., 1999; A. Ito and M. Watanabe, unpublished results).

Therefore, we decided to adopt a genome-walking strategy to identify SP11 from a class II S-haplotype. The genomic organization of the S-locus has been analyzed intensively in class I S-haplotypes of B. rapa (S2, S5, and S1-haplotypes [Schopfer et al., 1999; Suzuki et al., 1999; Takayama et al., 2000a]) and B. napus (S10 and S40-haplotypes [Cui et al., 1999; Brugière et al., 2000]). Although the relative positions of the three S-locus genes are different in different S-haplotypes, these genes are located within an ~30-kb genomic region. Because our preliminary analysis of the class II S60-haplotype of B. rapa also suggested that both SLG60 and SRK60 were localized within a 35-kb mul digested genomic fragment (see below), we decided to analyze the S-locus region of this S-haplotype.

We first cloned the SRK60 cDNA from stigmas of the S60-homozygote by PCR and used it as a probe to isolate a genomic SRK60 λ clone (see Methods). The SRK60 genomic clone, named N4p4, was ~12 kb in size and contained ~3.4 kb of the upstream side of SRK60 (Figure 1A). Using an end probe of N4p4, we next isolated a clone, designated Bp120, that extends ~17 kb on the upstream side of SRK60 (Figure 1A).

Nucleotide sequence analysis of Bp120 revealed the presence of a short open reading frame (ORF) encoding a putative cysteine-rich SP11 protein ~6.5 kb upstream of...
Recessive SP11 in Brassica SI

Another ORF encoding a putative signal peptide was found just upstream of this ORF. RT-PCR analysis using primers based on the putative signal peptide and an oligo(dT) primer revealed that this genomic region is expressed in anther tissue and produces a secreted cysteine-rich small basic protein, as shown in Figure 2. Because this gene is located close to SRK, is expressed in anther tissue, and encodes a SP11/SCR-type pollen coat protein (PCP) with the distinguishing characteristic position of the fourth cysteine residue (Doughty et al., 1998, 2000; Schopfer et al., 1999; Suzuki et al., 1999; Takayama et al., 2000a, 2000b), we determined that this was an allelic SP11 of the S$_{60}$-haplotype of B. rapa and designated it S$_{60}$-SP11.

Figure 1. Genomic Structure of the SRK Flanking Region of the S$_{60}$-Haplotype of B. rapa and the DNA Sequence of S$_{60}$-SP11.

(A) Genomic organization of the SRK flanking region of the S$_{60}$-haplotype. Thick bars represent the $\lambda$ phage clones covering the S$_{60}$-SP11/SRK$_{60}$ region. Arrows indicate the direction of transcription of each gene. Exons are indicated by solid boxes, and introns are indicated by dips.

(B) Nucleotide sequence of S$_{60}$-SP11 and its deduced amino acid sequence. The noncoding regions are shown in lowercase letters, with the intron splice donor/acceptor sequences (gt and ag in boldface letters) demarcating the intron. The coding regions are shown in uppercase letters, with the underlined TAA sequence indicating the stop codon. Nucleotides are numbered from the transcription start site (a in boldface), which was determined in a 5' rapid amplification of cDNA ends experiment. A putative TATA box and inverted sequence homologs of the CAAT motif and the LAT52/56 box are denoted in underlined boldface letters.
Detailed RT-PCR analysis revealed the presence of two types of S60-SP11 transcripts in anther with ORFs of 285 and 279 bp, both of which could be produced from S60+ SP11 by alternative splicing (Figure 3A). The former (the AL[+] form) encodes a 95-amino acid protein and is expected to produce a 66-amino acid mature form of S60+ SP11 after removal of the putative 29-amino acid hydrophobic signal peptide. The latter (the AL[-] form) encodes a 93-amino acid protein in which the predicted cleavage site of signal peptide in the former is deleted; it is expected to produce a 61-amino acid mature form of S60-SP11 after removal of a putative 32-amino acid signal peptide at an alternative cleavage site (Figure 3A). Although the presence of the alternative form of SP11 has not been detected in class I S-haplotypes (Watanabe et al., 2000), the genomic organization of SP11s having an intron close to the 3' end of the signal peptide coding region is common to both classes (Takayama et al., 2000a).

Biological Activity of Recombinant S60-SP11

To confirm that S60-SP11 works as the pollen determinant of the S60-haplotype of B. rapa, we prepared a recombinant S60-SP11 protein and tested its biological activity using a pollination bioassay system (see Methods). The results are summarized in Table 1. When the stigma of S60 S60-homozygote was pretreated with recombinant S60-SP11 and then pollinated with compatible pollen (from S12 S12-homozygote), the penetration of compatible pollen tubes into stigma was inhibited in a dose-dependent manner. Because the recombinant S60-SP11 did not affect the compatible pollination when applied on the stigma of S12S12-homozygote, this inhibitory effect of S60-SP11 was not caused by the toxicity of this protein but rather by an S-haplotype–specific induction of the SI response. These results clearly demonstrated that S60-SP11 was an allelic SP11 of the S60-haplotype of B. rapa.

Cloning and Sequence Analysis of SP11s from Other Class II S-Haplotypes of B. rapa and B. oleracea

Despite the polymorphic nature of SP11s, the nucleotide sequences of class I alleles are highly conserved in the region coding for the signal peptide (Watanabe et al., 2000). Suspecting that this region also might be conserved within class II SP11s, we attempted to amplify SP11 cDNAs from other class II S-haplotypes of B. rapa using an oligonucleotide primer (SP11-60-F3) based on the signal peptide region of S60-SP11 and an oligo(dT) primer (NotI-dT). The PCR products were amplified further using a set of nested primers (SP11-60-F4 and NotI-dT). Each of the tested class II S-homozygotes, S29 S29, S40 S40, and S44 S44-homozygotes, gave a single band at ~400 to 450 bp, whereas the class I S-homozygotes, S9 S9, S9 S9, and 12 S12-homozygotes, exhibited no amplification (data not shown). The full-length cDNA of each class II SP11 was obtained by the 5' rapid amplification of cDNA ends strategy. The deduced amino acid sequences of each class II SP11 are shown in Figure 2. As with S60-SP11, two types of transcripts (AL[+] and AL[−] forms) were obtained in S29- and S40-SP11 that presumably were generated by alternative splicing, as shown in Figure 3B. Because this alternative splicing site is conserved among all of the class II SP11s analyzed, the presence of two types of SP11 transcripts might be a common feature of class II S-haplotypes. However, because to date we have not detected the AL(−) form from the S44-haplotype, a more careful analysis should be performed before making this conclusion.

DNA gel blot analysis showed that each of these class II SP11 alleles of B. rapa was present as a single-copy gene (Figure 4A). Polymorphisms of the restriction fragment length suggested a high degree of sequence diversity in the vicinity of the SP11s. Using HindIII restriction fragment length polymorphism, the genetic linkage between SP11s and the corresponding S-haplotypes was confirmed in S29 S40-heterozygote progeny (Figure 4B). Pulsed-field gel
electrophoresis (PFGE) gel blot analysis suggested that each SP11, together with its cognate SLG and SRK, was localized within a 30- to 35-kb mlul-digested genomic fragment in the S_{29}, S_{44}, and S_{60}-haplotypes of B. rapa (Figure 4C).

We also identified the S_{29} and S_{29}-SP11 genomic sequences of B. oleracea from commercial F1 hybrid cultivars containing the class II S_{29} or S_{29}-haplotypes (Sakamoto et al., 2000). In the case of S_{29}-SP11, a completely identical genomic sequence was obtained from two independent commercial broccoli cultivars, Three Main (S_{29}S_{13}) and Ryokurei (S_{29}S_{18}), supporting its identity. The deduced amino acid sequences of S_{29} and S_{29}-SP11 are aligned with those from B. rapa in Figure 2.

The amino acid sequences of SP11s corresponding to their putative signal peptides are highly conserved among class II S-haplotypes (amino acid identity, ~82.8 to 100%), as is the case with class I, although the actual sequences are rather different between class I and class II. In contrast, the mature protein region of class II SP11s is polymorphic (~50.0 to 93.7%), although like the mature regions of class I SP11s, it features hydrophilic and basic (pI ~8.1 to 8.5) properties. All eight cysteine residues are conserved in an arrangement that is characteristic of SP11/SCR. Two other residues that are conserved among most of the class I SP11s, a glycine residue between C1 and C2 and an aromatic amino acid residue between C3 and C4 (Watanabe et al., 2000), also are conserved among all of the class II SP11s (Figure 2).

**Molecular Phylogenies of the SP11s of Class I and Class II S-Haplotypes**

A phylogenetic tree of class I/class II SP11 sequences (Figure 5) was constructed with the deduced amino acid sequences of the mature protein region using the program PROTMOL in MOLPHY (Adachi and Hasegawa, 1994). The class II SP11s form a distinct cluster from class I SP11s, as do stigmatic SLGs and SRKs, consistent with the proposal that class II S-haplotypes have a different origin than do class I S-haplotypes (Hinata et al., 1995; Kusaba et al., 1997). The amino acid sequence identities among class II SP11s are 63.2 to 94.6%, rather high compared with those of class I SP11s, which range from 19.5 to 76.1%. These observations suggest that the coalescence time of class II SP11 alleles is shorter than that of class I and/or that the

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**Figure 3. Alternative Transcripts of Class II SP11s.**

**A** Exon-intron structure and alternative transcripts of S_{60}-SP11. The alternative splicing produces S_{60}-SP11 proteins with or without an alanine-leucine (AL) in the sequence. They are designated AL(+) and AL(-), respectively. Coding regions are shown in uppercase letters, and the amino acid residues they encode are shown below. Introns are shown in lowercase letters, and the intron splice donor/acceptor sequences (gt and ag) are shown in boldface letters. The expected signal peptide cleavage sites are indicated by arrows.

**B** Alignment of the exon/intron junction of six class II SP11s. The intron splice donor/acceptor sequences (gt and ag) and an alternative possible acceptor sequence (AG) are shown in boldface letters. The alternative transcripts were detected experimentally in the S_{60}, S_{29}, S_{44}, S_{60}, S_{29}, and S_{44}-haplotypes, but not in the S_{44}-haplotype, of B. rapa. The S_{29}- and S_{44}-haplotypes of B. oleracea were not tested.
Numbers in parentheses indicate the number of pollination events examined.

- The Plant Cell

### Table 1. Effect of the Pretreatment of Stigma with Recombinant S<sub>40</sub>-SP11 on Cross-Pollination

<table>
<thead>
<tr>
<th>Pretreatment of Stigma</th>
<th>Recombinant S&lt;sub&gt;40&lt;/sub&gt;-SP11 (pmol per Stigma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S&lt;sub&gt;40&lt;/sub&gt;-stigma × S&lt;sub&gt;40&lt;/sub&gt;-pollen</td>
<td>100 (45)</td>
</tr>
<tr>
<td>compatible pollination (%)</td>
<td>0+ (24)</td>
</tr>
<tr>
<td>5</td>
<td>33+ (33)</td>
</tr>
<tr>
<td>0.5</td>
<td>38+ (29)</td>
</tr>
<tr>
<td>0.05</td>
<td>82+ (22)</td>
</tr>
<tr>
<td>0.005</td>
<td>100 (21)</td>
</tr>
</tbody>
</table>

Numbers in parentheses indicate the number of pollination events examined.

*P* < 0.01 with Fisher’s exact probability test compared with the Tween 20–treated control.

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The promoter region of S<sub>40</sub>-SP11, which can affect gene expression, is shown in lowercase letters in Figure 1B. A putative TATA box is located ~30 bp upstream of where the 5’ end of the mRNA begins. Inverted sequence homologs of the CAAT motif and the LAT52/56 box, both of which have been found in the promoter of the anther/pollen-expressed respiratory chain complex I genes (Zabaleta et al., 1998), were identified in the promoter sequence of S<sub>40</sub>-SP11 (Figure 1B). However, no other known regulatory elements, common repeats, or palindromic sequences were found.

Class II SP11 Transcripts Are Regulated Recursively by Class I S-Haplotypes

The promoter region of S<sub>40</sub>-SP11, which can affect gene expression, is shown in lowercase letters in Figure 1B. A putative TATA box is located ~30 bp upstream of where the 5’ end of the mRNA begins. Inverted sequence homologs of the CAAT motif and the LAT52/56 box, both of which have been found in the promoter of the anther/pollen-expressed respiratory chain complex I genes (Zabaleta et al., 1998), were identified in the promoter sequence of S<sub>40</sub>-SP11 (Figure 1B). However, no other known regulatory elements, common repeats, or palindromic sequences were found.

We previously analyzed the promoter sequences of class I SP11s and found that the immediate upstream region within ~200 bp of the coding region is highly conserved among different class I S-haplotypes. Using promoter deletion analysis, we further demonstrated that the region between −124 and −192 is essential for the sporophytic/gametophytic expression of class I SP11s (Shiba et al., 2001). However, the promoter sequence of S<sub>40</sub>-SP11 showed little homology and no motif conservation with that of class I SP11s.

To determine the temporal and spatial expression pattern of the class II SP11, we first performed RNA gel blot analysis. Total RNAs were prepared from the anthers at several developmental stages and from the stigma and the leaf tissue of a *B. rapa* S<sub>40</sub>-SP11 homoygote. A full-length S<sub>40</sub>-SP11 cDNA was used as a probe. As shown in Figure 6A, transcripts were detected only in anther, with the highest expression at an early stage of development, when the tapetal cells were fully intact (stage 5), and little expression at late stage, when tapetal degradation was virtually completed (stage 7). This is in contrast to the expression of the class I SP11s, which continues at a high level throughout these developmental stages of the anther (Takayama et al., 2000a).

In situ hybridization analysis showed that S<sub>40</sub>-SP11 was expressed predominantly in tapetal cells of the anther at early stages (Figure 7A). However, the gametophytic expression of S<sub>40</sub>-SP11 was not detected in pollen at late developmental stages (Figure 7B), a time when class I SP11s are known to be expressed (Schopfer and Nasrallah, 2000; Takayama et al., 2000a). This finding suggests that the gene is expressed either weakly or not at all in pollen. Control experiments using a sense S<sub>40</sub>-SP11 probe showed no signal at any stages (Figures 7C and 7D).

Next, we analyzed the expression of S<sub>40</sub>-SP11 in a heterozygote of class I and class II S-haplotypes, the S<sub>40</sub>S<sub>40</sub>-heterozygote of *B. rapa*, to gain insight into the mechanisms of dominance/recessiveness. RNA gel blot analysis showed no expression of S<sub>40</sub>-SP11 in the anther (mixture of stages 5, 6, and 7) of S<sub>40</sub>S<sub>40</sub>-heterozygote, whereas strong expression was observed in the anther of S<sub>40</sub>S<sub>40</sub>-homozygote (Figure 6B). A similar result was obtained in S<sub>40</sub>-SP11, which showed clear expression in anther tissue of the S<sub>40</sub>S<sub>40</sub>-homozygote but no expression in the S<sub>40</sub>S<sub>40</sub>-heterozygote, another class I/class II heterozygote (Figure 6C).

In situ hybridization analysis revealed that the expression
Recessive SP11 in Brassica SI

DISCUSSION

Structural Features on SP11s of Class II S-Haplotypes

In this work, we have identified SP11 from four class II S-haplotypes of B. rapa and two class II S-haplotypes of B. oleracea. Class II SP11s show several similarities with class I SP11s: (1) they are small, secreted proteins with conserved putative signal peptides; (2) their mature proteins show S-haplotype–specific polymorphisms in spite of their common hydrophilic and basic properties; (3) like other PCPs, they have eight conserved cysteine residues whose positioning is characteristic of SP11s but different from other PCPs; and (4) they have a glycine residue between C1 and C2 and an aromatic residue between C3 and C4 that are conserved.

However, the amino acid sequence alignment revealed extensive differences between SP11s from class I and class II. In contrast, pairwise identities among the products of class II alleles were relatively high, resulting in a tight cluster in the phylogenetic tree. This relatively high similarity of class II SP11s gives some insight into the amino acid residues that determine the S-haplotype specificities of SP11s, because all four of the class II S-haplotypes of B. rapa and two of the class II S-haplotypes of B. oleracea have been shown to be cross-compatible by reciprocal crosses within each species (Hatakeyama et al., 1998a; Cabrillac et al., 1999).

Conservation of the eight cysteine residues suggests a common three-dimensional protein structure of SP11s that is stabilized by intramolecular disulfide bonds, similar to the...
A

\[ \begin{array}{c}
S_{60}\text{-}SP11 \\
EtBr
\end{array} \]

B

\[ \begin{array}{c}
S_{60}\text{-}SP11 \\
EtBr
\end{array} \]

C

\[ \begin{array}{c}
S_{52}\text{-}SP11 \\
EtBr
\end{array} \]

Figure 6. RNA Gel Blot Analyses of the Class II SP11s.

(A) RNA gel blot analysis of \( S_{60}\text{-}SP11 \) in an \( S_{60}\text{-}S_{60} \)-homozygote of \( B. \text{rapa} \). The total RNAs of anther (A), stigma (S), and leaf (L) were used. Numbers represent developmental stages of anther classified by bud sizes, where 5 = 4 to 5 mm, 6 = 5 to 7 mm, and 7 = 7 to 10 mm in length (Takayama et al., 2000a).

(B) RNA gel blot analysis of \( S_{60}\text{-}SP11 \) and \( S_{60}\text{-}SP11 \) in an \( S_{60}\text{-}S_{60} \)-homozygote (60) and an \( S_{60}\text{-}S_{60} \)-heterozygote (52) of \( B. \text{rapa} \). The same amount of total RNA of anthers (mixture or stages 5 to 7) was loaded in each lane. Similar results were obtained on three independent \( S_{60}\text{-}S_{60} \)-homozygotes and \( S_{60}\text{-}S_{60} \)-heterozygotes, and the results of a representative experiment are shown.

(C) RNA gel blot analysis of \( S_{60}\text{-}SP11 \) in an \( S_{60}\text{-}S_{40} \)-homozygote (40) and an \( S_{60}\text{-}S_{60} \)-heterozygote (H) of \( B. \text{rapa} \). The same amount of total RNA of anthers (stages 5 to 7, mixture) was loaded in each lane. The blot shown is representative of two independent experiments. The bottom gel of each blot shows ethidium bromide (EtBr)-stained rRNA bands.

defensin family of antimicrobial proteins (Broekaert et al., 1995). In proteins of this family, such as defensin A and MGD-1, positively charged and hydrophobic side chains, which are positioned so that they are exposed to the molecular surface and stabilized by the backbone structure, are supposed to generate bactericidal potency and Gram-positive specificity (Comet et al., 1995; Yang et al., 2000). In class II SP11s, N-terminal amino acid sequences before C1 and the single amino acid spacer between C4 and C5 are well conserved. The C-terminal sequences after C6 are conserved completely among all of the class II SP11s. Therefore, it is evident that at least one connecting region of C1-C2, C2-C3, C3-C4, or C5-C6 is involved in the determination of S-haplo-
type specificity in SP11s, although the involvement of the conserved region cannot be excluded completely.

Hydropolarity analysis of the class II SP11s does not suggest a hydrophilic (surface-exposed) structure in the C3-C4 region (data not shown), which has been suggested for class I SP11s (Schopfer and Nasrallah, 2000). However, this region does contain a conserved aromatic amino acid residue and more than one basic amino acid residue, which have been shown to be involved in the bactericidal potency of defensins (Yang et al., 2000). Three other regions, C1-C2, C2-C3, and C5-C6, are highly divergent and contain two or three amino acid residues that are completely variable across the four S-haplotypes of \( B. \text{rapa} \) (and also between the two S-haplotypes of \( B. \text{oleracea} \)). We speculate that some of these four connecting regions are involved in the specific interaction between SP11 and its cognate SRK.

Evolutionary Implications

The phylogenetic tree of SP11s confirmed that the class II genes form a monophyletic group separating from a cluster of class I genes, as observed in SRKs and SLGs (Hinata et al., 1995; Uyenoyama, 1995; Schierup et al., 2001). The sequence identity among class II SP11s is relatively high. According to the evolutionary model of \( Brassica \) SI proposed by Uyenoyama (2000), class II S-haplotypes are expected to invade populations at lower rates and decline to extinction at higher rates than class I S-haplotypes. This predicts a lower survival rate of a newly arisen mutation and less divergence among alleles in class II than class I. The most diverged pair of alleles (\( B. \text{rapa} \) S40-SP11 and S60-SP11) reveals a synonymous divergence rate per site of 0.111 ± 0.049. Using 0.5 × 10^{-8} per site per year (Li, 1997) as a representative of the synonymous nucleotide substitution rate in plant nuclear genes, the divergence time of class II SP11s is estimated at 10 ± 5 million years ago. This estimate is consistent with the previous estimate, 6.4 ± 0.2 million years ago, based on SRK and SLG sequences (Uyenoyama, 1995).

The diversification of S-haplotypes in \( Brassica \) has been suggested to predate the speciation of \( B. \text{rapa} \) and \( B. \text{oleracea} \) (Dwyer et al., 1991; Hinata et al., 1995). The phylogenetic tree of SP11s (Figure 5) showed that class II SP11s also diverged before speciation of these species. Among six SP11 alleles from \( B. \text{rapa} \) and \( B. \text{oleracea} \), the most closely related interspecific pair of alleles is \( B. \text{rapa} \) S40-SP11 and \( B. \text{oleracea} \) S52-SP11, which are identical at the 46 synonymous sites. Although the number of synonymous sites is small, the presence of an allele pair identical at the synonymous sites implies that \( B. \text{rapa} \) and \( B. \text{oleracea} \) are closely related. No synonymous changes at 46 sites gives an upper limit of species divergence time of 2.6 million years with a 95% confidence limit, based on the assumption of 0.5 × 10^{-8} per site per year as a synonymous nucleotide substitution rate. This species divergence is much younger than ex-
Recessive SP11 in Brassica SI

We expected, which has tentatively been estimated as on the order of 10 million years ago (Uyenoyama, 1995). However, an alternative hypothesis, such as the introgression of S-haplotypes (Schierup et al., 1998), also must be considered before drawing any conclusion on the evolutionary trail of these S-haplotypes.

The conservation in the sequence of class II SP11s allowed us to calculate the levels of synonymous and nonsynonymous nucleotide substitutions for the coding regions of mature proteins, which was impossible to do in class I SP11s because of their great diversity and consequent ambiguous alignment (Watanabe et al., 2000). The high $P_n/P_s$ ratio (3.58) for the mature coding region of class II SP11s indicates an involvement of diversifying selection. In fact, when we align six SP11s in amino acid sequences among 63 comparable sites, 28 sites are identical between distinct alleles. On the other hand, some amino acid residues are highly variable among six SP11s (Figure 2), suggesting that these sites are targets of the selection and probably functionally important for SI determination.

Recently, probable orthologs of Brassica SP11 have been identified from two S-haplotypes of Arabidopsis lyrata (Kusaba et al., 2001). A. lyrata belongs to the tribe Arabeae (Price et al., 1994) and is thought to have diverged from Brassica species $\sim$15 to 20 million years ago (Yang et al., 1999), and its putative SRKs have shown more variability than those of Brassica. In accord with this, two putative SP11s obtained from A. lyrata showed great divergence, including their predicted signal sequences (Kusaba et al., 2001), which are fairly conserved in Brassica SP11s. This evidence shows that the origin of SP11s existed before the divergence of Brassicaceae from Arabideae. Recently, Schierup et al. (2001) analyzed 11 putative SRKs (Aly13) of A. lyrata together with Brassica class I and class II SRKs. Although the phylogeny was unrooted, the Brassica class II SRK cluster shares an ancestor with one cluster of Aly13. If we can determine whether any A. lyrata SP11 shares an ancestor with Brassica class II SP11, we may be able to infer the origin of the dominant/recessive expression system.

**Dominant/Recessive Relationships**

Class II $S_{60'}$-SP11 exhibited an exclusively sporophytic expression pattern in the anther tapetum and little or no gametophytic expression in the pollen, which is in contrast to the sporophytic/gametophytic expression pattern of class I SP11s (Schopfer and Nasrallah, 2000; Takayama et al., 2000a). This characteristic expression pattern of class II SP11 was

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**Figure 7.** Analysis of $S_{60}$- and $S_{52}$-SP11 Expression in Anther Using in Situ Hybridization.

Anther sections (stages 5 and 7) of an $S_{60}S_{60}$-homozygote or an $S_{60}S_{52}$-heterozygote were hybridized with $S_{60}$-SP11 or $S_{52}$-SP11 antisense riboprobes or their sense riboprobes.

(A) and (B) $S_{60}S_{60}$-homozygote hybridized with an antisense $S_{60}$-SP11 probe.

(C) and (D) $S_{60}S_{60}$-homozygote hybridized with a sense $S_{60}$-SP11 probe.

(E) and (F) $S_{60}S_{60}$-heterozygote hybridized with an antisense $S_{60}$-SP11 probe.

(G) and (H) $S_{52}S_{60}$-heterozygote hybridized with an antisense $S_{52}$-SP11 probe.

Bar in (H) = 50 μm for (A) to (H).
confirmed by in situ analysis of $S_{29}$-SP11 expression in the $S_{29}S_{29}$-homozygote, which also showed purely sporophytic expression and no signal in pollen (data not shown). As expected given the difference in the expression pattern of class I and class II SP11s, the promoter sequences of SP11s showed little homology between these two classes.

RNA gel blot and in situ analyses demonstrated that the sporophytic expression of class II SP11s was suppressed in the class I/class II heterozygotes, suggesting that the dominant/recessive relationship between class I and class II S-haplotypes in the determination of pollen phenotype is generated at the level of SP11 transcription. This is in contrast to the dominant/recessive relationship in the stigma, which is determined by SRK itself, but not by virtue of its relative expression level (Hatakeyama et al., 2001).

To our knowledge, there is no other demonstration that the dominant/recessive relationship between multiallelic genes is determined by their expression level. The mechanisms of this epigenetic phenomenon, in which an allelic class II SP11 is suppressed in the presence of another allelic class I gene, remain to be revealed. One hypothetical explanation is the “enhancer imbalance” model, in which dominant class I SP11s have higher binding affinities for limiting transcription factors and the sequestration of critical transcription factors by class I SP11 enhancers results in the silencing of class II SP11s. Another possible explanation is that the selective hypermethylation of recessive class II SP11s blocks the binding of RNA polymerase II transcription factors, as demonstrated to occur during the development of a number of plant and animal species (Eden and Cedar, 1994).

Another epigenetic occurrence known as “nucleolar dominance” might be similar to the dominant/recessive phenomenon seen between class I and class II SP11s (Frieman et al., 1999). Nucleolar dominance describes the phenomenon in which rRNA genes inherited from only one parent are transcribed in interspecies hybrids or allotetraploids. This event was first described in plants (Navashin, 1934) and then in insects, amphibia, and mammals (Reeder, 1985; Chen and Pikaard, 1997). Although nucleolar dominance is a transcriptional event and was shown to be controlled at the level of RNA polymerase I transcription (Chen and Pikaard, 1997), the mechanisms that discriminate between parental sets of rRNA genes remain obscure. In interspecies hybrids between *Xenopus laevis* and *X. borealis*, experimental data support the enhancer imbalance model (Reeder and Roan, 1984), although this model does not fit observations with hybrids between *B. rapa* and *B. oleracea* (Frieman et al., 1999). The involvement of DNA methylation and histone modification in the mechanism for the enforcement of nucleolar dominance also has been suggested (Chen and Pikaard, 1997). The dominance of class I over class II SP11s was very clear and does not require an enforcement process (i.e., newly formed F1 heterozygotes of class I and class II S-haplotypes exhibited clearly distinguishable dominant/recessive relationships). This study demonstrates that these relationships are determined at the steady state mRNA level. This newly found silencing system should provide a promising model for studies of general epigenetic phenomena.

**METHODS**

**Plant Materials**

The eight S-haplotypes of *Brassica rapa* ($S_{34}$, $S_{29}$, $S_{19}$, $S_{29}$, $S_{44}$, $S_{39}$, and $S_{46}$-haplotypes) used in this study have been described (Takayama et al., 1987; Nou et al., 1993; Hatakeyama et al., 1998a, 1998b; Takasaki et al., 1999). The commercial F1 hybrid lines used in this study have been described (Takayama et al., 1997). The dominance of class I over class II $S_{29}$-haplotypes exhibited clearly distinguishable dominant/recessive SRK$^{60}$-homozygote, classified previously as $S_{18}$-heterozygote, $B. rapa$ Osome ($S_{34}$-$S_{29}$-heterozygote), and $B. oleracea$ Three Main ($S_{19}$-$S_{29}$-heterozygote) were obtained from Takii Seed Co. (Kyoto, Japan). $B. oleracea$ Ryokurei ($S_{19}$-$S_{29}$-heterozygote) and $B. oleracea$ Kinkei 201 ($S_{19}$-$S_{29}$-heterozygote) were obtained from Sakata Seed Co. (Hatakeyama et al., 1998b; Takasaki et al., 1999; Sakamoto et al., 2000). All of the S-genotypes of plant materials used in this study were checked through direct cloning of SLG genes by polymerase chain reaction (PCR) using class I SLG-specific primers (Bartec et al., 1993) and class II SLG-specific primers (Nishio et al., 1996).

**Cloning of SRK$^{60}$ cDNA**

Stigmas of the $S_{34}$-$S_{34}$-homozygote were collected from buds at 2 to 3 days before anthesis. Total RNA was isolated using Isogen (Nippon Gene, Toyama, Japan). Approximately 20 μg of RNA was subjected to first-strand cDNA synthesis using Superscript II (GIBCO) with an oligo(dT)$_{18}$ primer. The partial sequence of SRK$^{60}$ (~2.4 kb) was amplified by PCR using a set of primers (5'-ATGAAGGGGTACAGAACAT-3' [Nishio et al., 1996] and 5'-GTCTCTGAAAYACAAKACGRC-CAAT-3') designed from the conserved extracellular domain of class II SLG and the conserved kinase domain of SRK, respectively. The 5' and 3' rapid amplification of cDNA ends (RACE) cloning strategies were used to reveal the entire cDNA sequence of SRK$^{60}$. The genetic linkage of cloned SRK$^{60}$ cDNA as the probe and obtained selfed F2 progeny of an $S_{34}$-$S_{46}$-heterozygote.

**Screening of the Genomic Library**

Genomic DNA from the *B. rapa* $S_{34}$-$S_{46}$-homozygote was digested partially with Sau3AI and fractionated on a cesium chloride density gradient. The fraction containing 9- to 23-kb fragments was ligated with λDASH II vector (Stratagene). To isolate the genomic clone containing SRK$^{60}$, we screened 1.6 × 10$^6$ plaque-forming units under standard conditions using an SRK$^{60}$ cDNA as the probe and obtained one phage clone designated N4p4. The insert DNA was amplified by PCR with LA Taq DNA polymerase (TaKaRa, Shiga, Japan) using specific primers corresponding to the vector (EMBL3R, 5'-CTTGGACGCACTGCGAAG-3'; EMBL3R, 5'-GATCCACTGCT-TTCTCGGACGAGTGTCG-3') and then fragmented using HydroShear (GeneMachines, San Carlos, CA). Fragments ranging from 1.5 to 3 kb were treated with T4 DNA polymerase (TaKaRa) and subcloned.
into pBluescript SK+ (Stratagene) for sequence analysis. To isolate flanking regions of \( \text{SRK}_{60} \), we screened 3 \times 10^6 plaque-forming units with both end probes of the N4p4 clone. The end probes were amplified by specific primers designed from both end sequences. One clone covering the 18.5-kb upstream region of \( \text{SRK}_{60} \) was isolated and designated Bp120. The insert DNA was amplified by PCR and sequenced as described above.

**Cloning of Class II SP11s from Brassica Species**

Total or poly(A)+ RNAs were extracted from anthers of \( S_{60}S_{60} \), \( S_{39}S_{39} \), \( S_{29}S_{29} \), and \( S_{29}S_{29} \)-homozygotes of \( B. \) rapa and reverse transcribed to synthesize first-strand cDNA as described (Suzuki et al., 1999; Takayama et al., 2000a). An \( S_{60}-\text{SP11} \) cDNA fragment was amplified by PCR using a primer (5'-ATGAGATCTGCTTTTATGTTCC-3') designed from the genomic sequences of Bp120 and the oligo(dT)\(_{18}\) primer and subcloned into the vector pGEM-T Easy (Promega). The 5’ end of \( S_{60}-\text{SP11} \) was determined by 5’ RACE. Other class II SP11s of \( B. \) rapa were amplified using the primers SP11-60-F3 (5’-ACATTTCATAACATTACACTATGTG-3’) and NotI-dT (Watanabe et al., 2000). The PCR products were amplified further using a nested primer, SP11-60-F4 (5’-ATACACTACTTAGTGTGTTACTATT-3’) and the NotI-dT primer. The amplified PCR products were cloned into the vector PCR2.1 (Invitrogen, Carlsbad, CA) and sequenced. The 5’ end of the SP11s was determined by 5’ RACE.

Genomic DNA was extracted from leaves of \( S_{60}S_{60} \), \( S_{39}S_{39} \), \( S_{29}S_{29} \), and \( S_{29}S_{29} \)-heterozygotes of \( B. \) oleracea as described (Edwards et al., 1999). The genomic structures of SP11s were determined by PCR using a set of primers (5’-GGCGAAATCTTATATACTGACAG-3’ and 5’-CTCGTTGATCAATTATGATT-3’) designed from the 5’ and 3’ ends of the coding region of \( \text{SRK}_{60} \), respectively. The fragments obtained were subcloned into the vector pGEM-T Easy and then sequenced.

**Preparation of Recombinant \( S_{60}-\text{SP11} \)**

The mature coding region of the \( S_{60}-\text{SP11} \) cDNA (AL[+] form) was amplified using the primers 5’-GATCCCTAGTGAGGAGCGTG-3’ and 5’-CTCGAGTTCGTTGATCAATTATGATT-3’ and cloned between the BamHI and XhoI sites of pGEX-6P-3 vector (Amersham Pharmacia). This construct was transformed into the Escherichia coli BL21 strain. The induction and purification of the glutathione S-transferase fusion protein were performed according to the manufacturer’s protocol. The recombinant \( S_{60}-\text{SP11} \) with the five-aminoc acid N-terminal extension (GPLGSL) was obtained after cleavage with PreScission Protease (Amersham Pharmacia). The recombinant \( S_{60}-\text{SP11} \) was reduced once in the presence of DTT and then refolded in the presence of both oxidized and reduced forms of glutathione (1:10). One major oxidized form of recombinant \( S_{60}-\text{SP11} \) was purified to homogeneity by reverse phase HPLC.

**Pollination Bioassay**

The pollination bioassay was performed as described (Takayama et al., 2001). Stigmas were treated with 0.5 \( \mu \)L of a recombinant \( S_{60}-\text{SP11} \) solution containing 0.05% Tween 20 and air dried for 1 hr. After cross-pollination, the stigmas were kept at 20°C for 6 hr, and penetration of pollen tubes into each stigma was observed after aniline blue staining, as described previously (Shiba et al., 2000). Typically, >100 pollen tubes penetrated the stigma in a 0.05% Tween 20–treated control. When the number of penetrating pollen tubes was reduced to <10 in the test, pollination was judged to become incompatible.

**DNA Gel Blot Analysis**

Total DNA was extracted from young leaves by the cetyltrimethylammonium bromide method (Murray and Thompson, 1980). The extracted DNA (1 \( \mu \)g) was digested with EcoRI, BamHI, or HindIII and separated by electrophoresis on a 0.8% agarose gel. DNA fragments were transferred onto nylon membranes, hybridized with digoxigenin-labeled probes, washed, and detected as described (Watanabe et al., 1999). Digoxigenin-labeled SP11 cDNA probes were prepared by PCR amplification with a specific primer set. The detection of the hybridized probe was performed as described (Matsuda et al., 1996).

**PFGE Gel Blot Analysis**

MegaBase DNA embedded in agarose plugs was prepared from young leaf tissue of \( S_{60}S_{60} \), \( S_{39}S_{39} \), and \( S_{29}S_{29} \)-homozygotes by the rapid method (Tanaka et al., 1993; Liu and Whittier, 1994). DNA digestion with mluI, pulsed-field gel electrophoresis (PFGE) of digested DNA, DNA transfer to a nylon membrane, hybridization with digoxigenin-labeled probes, and detection of the hybridized probes were performed as described (Watanabe et al., 2000) except that the membranes were washed twice in 0.1 \times \( \text{SSC} \) (1 \times \( \text{SSC} = 0.15 \text{M NaCl and 0.015 M sodium citrate} \)) and 0.1% SDS at 68°C for 20 min. The digoxigenin-labeled SLG29 probe and \( \text{SRK}_{60} \) kinase domain probe (Hatakeyama et al., 1998b) were used under the same conditions used for hybridization to SP11.

**Sequence Analysis of Class II SP11s**

Deduced amino acid sequences of six class II SP11s were aligned together with previously reported class I \( S_{29} \) and \( S_{13}-\text{SP11s} \) (Takayama et al., 2000a). Eight cysteine residues were kept invariant in the alignment. Based on this alignment, the following analyses were performed. Any sites that contained a gap were excluded from the analysis. The phylogenetic tree was constructed using the program PROTMOL in MOLPHY version 2.3 (option JTT-F; Adachi and Hasegawa, 1994). To evaluate the clustering patterns, bootstrap probability for each cluster was calculated with 1000 times of resampling. For the calculation of synonymous and nonsynonymous differences per site, taking account of transition/transversion bias, we used the modified Nei and Gojobori method with \( \delta = 1.0 \) (Nei and Kumar, 2000).

**RNA Gel Blot Analysis**

Total RNA was isolated from several tissues of \( B. \) rapa using Isogen. Each 10 \( \mu \)g of total RNA was electrophoresed on a 1.2% (w/v) agarose/formamide gel and transferred to a nylon membrane (Hybond N+, Amersham-Pharmacia). The membrane was hybridized at 65°C for 12 hr with \( ^{32} \text{P}-\text{labelled} \) \( S_{60}, S_{39}, \) and \( S_{29}-\text{SP11} \) probes specific for their respective coding regions of the mature proteins and the 3’ noncoding regions. After hybridization, the membrane was washed...
in 0.1 x SSPE (1 x SSPE is 0.115 M NaCl, 10 mM sodium phosphate, and 1 mM EDTA, pH 7.4) and 0.1% (w/v) SDS at 65°C for 30 min and exposed on x-ray film. Equal loading of total RNA was assessed by ethidium bromide staining of rRNA bands.

In Situ Hybridization

The anthers at developmental stage 5 (bud length, 4 to 5 mm) and stage 7 (7 to 10 mm) were collected from a B. rapa S60/S60-homozygote and an S60/S60-heterozygote. Digoxigenin-labeled sense and antisense RNA probes of S60-SP11 and S32-SP11 (coding region of mature protein) were prepared using the SP6/T7 digoxigenin RNA labeling kit (Roche Diagnostics, Mannheim, Germany). In situ hybridization to 10-μm-thick Paraplast (Sigma) sections of paraformaldehyde-fixed anthers was performed according to the method described previously (Doughty et al., 1998).

Accession Numbers

The GenBank accession numbers for S60-SP11, S32-SP11, S52-SP11, S42-SP11, S7-S-SP11, and S5-S-SP11 are AB067446, AB067449, AB067450, AB067451, AB067447, and AB067448, respectively.

ACKNOWLEDGMENTS

We thank Dr. Katsuori Hatakeyama (Research Institute of Seed Production) and Dr. Koji Sakamoto (Takii Seed Company) for generous gifts of a B. rapa S60/S60-homozygote and a commercial cultivar (Ku-kaï), respectively. We thank Kazuiko Iwasaki, Hanae Sugita, Taduru Ueda, and Hiroko Sato for technical assistance. This work was supported in part by Grants-in-Aid for Special Research on Priority Areas B (No. 11238025) to A.I., B (Nos. 11238201 and 11460001) to M.W., and C (No. 13206052) to H.S. from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, by a grant from the Research for the Future Program (No. JSPS-RFTF 00L01605) to S.T. from the Japan Society for the Promotion of Science, and by a grant from the Mitsubishi Foundation to A.I.

Received August 28, 2001; accepted October 30, 2001.

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The Dominance of Alleles Controlling Self-Incompatibility in *Brassica* Pollen Is Regulated at the RNA Level

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*Plant Cell* 2002;14;491-504; originally published online February 19, 2002;
DOI 10.1105/tpc.010378

This information is current as of September 14, 2017