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Diversification and Alteration of Recognition Specificity of the Pollen Ligand SP11/SCR in Self-Incompatibility of Brassica and Raphanus [W]

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The recognition specificity of the pollen ligand of self-incompatibility (SP11/SCR) was investigated using *Brassica rapa* transgenic plants expressing *SP11* transgenes, and *SP11* of *Raphanus sativus S-21* was found to have the same recognition specificity as that of *B. rapa S-9*. In a set of three *S* haplotypes, whose sequence identities of *SP11* and *SRK* are fairly high, *R. sativus S-6* showed the same recognition specificity as *Brassica oleracea S-18* and a slightly different specificity from *B. rapa S-52*. *B. oleracea S-18*, however, showed a different specificity from *B. rapa S-52*. Using these similar *S* haplotypes, chimeric SP11 proteins were produced by domain swapping. Bioassay using the chimeric SP11 proteins revealed that the incompatibility response induction activity was altered by the replacement of Region III and Region V. Pollen grains of Brassica transgenic plants expressing chimeric *SP11* of the *B. oleracea SP11-18* sequence with Region III and Region V from *B. rapa SP11-52* (chimeric *BoSP11-18*[*52*]) were partially incompatible with the *B. rapa S-52* stigmas, and those expressing the *R. sativus SP11-6* sequence with Region III and Region V from *B. rapa SP11-52* (chimeric *RsSP11-6*[*52*]) were completely incompatible with the stigmas having *B. rapa S-52*. However, the transgenic plant expressing chimeric *RsSP11-6*(*52*) also showed incompatibility with *B. oleracea S-18* stigmas. These results suggest that Regions III and Region V of SP11 are important for determining the recognition specificity, but not the sole determinant. A possible process of the generation of a new *S* haplotype is herein discussed.

INTRODUCTION

The molecular mechanism of pollen-pistil recognition in self-incompatibility has been intensively studied in *Brassica oleracea* and *Brassica rapa*. The pollen-pistil recognition takes place by the interaction between membrane-spanning Ser-Thr protein kinase in the stigma, i.e., *S*-locus receptor kinase (Stein et al., 1991; Takasaki et al., 2000), and low–molecular weight Cys-rich protein in pollen, i.e., *S*-locus protein 11(SP11)/*S*-locus Cys-rich protein (SCR) (Schopfer et al., 1999; Suzuki et al., 1999). The genes encoding these molecules, i.e., *SRK* and *SP11*/*SCR* (referred to as *SP11* hereafter), are located in the *S* locus, and another molecule encoded in the *S* locus is *S*-locus glycoprotein (SLG), the function of which in self-incompatibility is still controversial (Gaude et al., 1995; Okazaki et al., 1999; Dixit et al., 2000; Suzuki et al., 2000; Takasaki et al., 2000; Silva et al., 2001). The alleles of *SRK*, *SP11*, and *SLG* are transmitted to the progeny as one set named *S* haplotype. On the basis of the nucleotide sequence similarity of *SLG* and *SRK*, *S* haplotypes fall into two classes, class I and class II (Nasrallah et al., 1991). The identities of the deduced amino acid sequences of SLG and the S domain of SRK are >72% in both classes and <70% between the two classes (Chen and Nasrallah, 1990; Nishio and Kusaba, 2000). The class-II *S* haplotypes are generally recessive to class-I *S* haplotypes in pollen and express weaker self-incompatibility than the class-I *S* haplotypes (Nasrallah et al., 1991).

The numbers of *S* haplotypes in *B. oleracea* and *B. rapa* have been estimated to be 50 and >100, respectively (Nou et al., 1993; Ockendon, 2000). Nucleotide sequence analyses of *SRK*, *SP11*, and *SLG* of many *S* haplotypes have revealed that *B. oleracea* and *B. rapa* have pairs of *S* haplotypes in common, the amino acid identities of SRK and SP11 between the pairs being >90% (Sato et al., 2002, 2003). These interspecific pairs have been proved to possess the same recognition specificity (Kimura et al., 2002; Sato et al., 2003). Intergeneric pairs have also been identified between Brassica and Raphanus, the identity of amino acid sequences being lower than that of the interspecific pairs (Okamoto et al., 2004). The recognition specificity of the intergeneric pairs has not been investigated.

The recognition mechanism between SP11 and SRK has been fairly well elucidated (Kachroo et al., 2001; Takayama et al., 2001), but the regions of these recognition molecules important for recognition specificity have not been identified. Sato et al. (2003) have assigned six regions to SP11, Region I to Region VI, on the basis of conserved Cys residues, and considered Regions

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Table 1. Amino Acid Identity of SP11 and S Domain of SRK between *S* Haplotypes Used in This Study

Pairs of *S* Haplotypes	Amino Acid Identity	
	SP11	SRK
RsS-21 versus *BrS-9*	89.5	91.0
RsS-6 versus *BoS-18*	70.9	88.3
RsS-6 versus *BrS-52*	69.1	86.6
BoS-18 versus *BrS-52*	58.2	87.6
The highest in intraspecies[a]	57.1	87.3

[a] The highest amino acid identity in *B. oleracea*.

III, V, and VI to be more important for recognition specificity than Regions I, II, and IV, because amino acid sequences in Regions III, V, and VI are conserved between the interspecific pairs. Determining the solution structure of the SP11 protein of *B. rapa S-8*, Mishima et al. (2003) have identified a hypervariable region, which they considered to be important for recognition specificity. This hypervariable region corresponds to Region IV, which has been considered to be unimportant by Sato et al. (2003). Recently, Chookajorn et al. (2004) have shown that the specificity of SP11 can be altered by the substitution of four continuous amino acid residues in Region V.

In this study, we investigated the recognition specificity of an intergeneric pair between *Brassica* and *Raphanus* using transgenic plants. We also compared the recognition specificity of a set of three *S* haplotypes, the shared amino acid identities of which are fairly high, in *Raphanus sativus*, *B. oleracea*, and *B. rapa*. Chimeric *SP11* genes were produced by swapping the sequences between a set of *S* haplotypes, and the recognition specificity of chimeric SP11 proteins was investigated using a bioassay with recombinant SP11 proteins and a pollination test with transgenic plants expressing the chimeric *SP11* genes. The regions of the SP11 protein important for the recognition specificity and the process of generation of a new *S* haplotype are herein discussed.

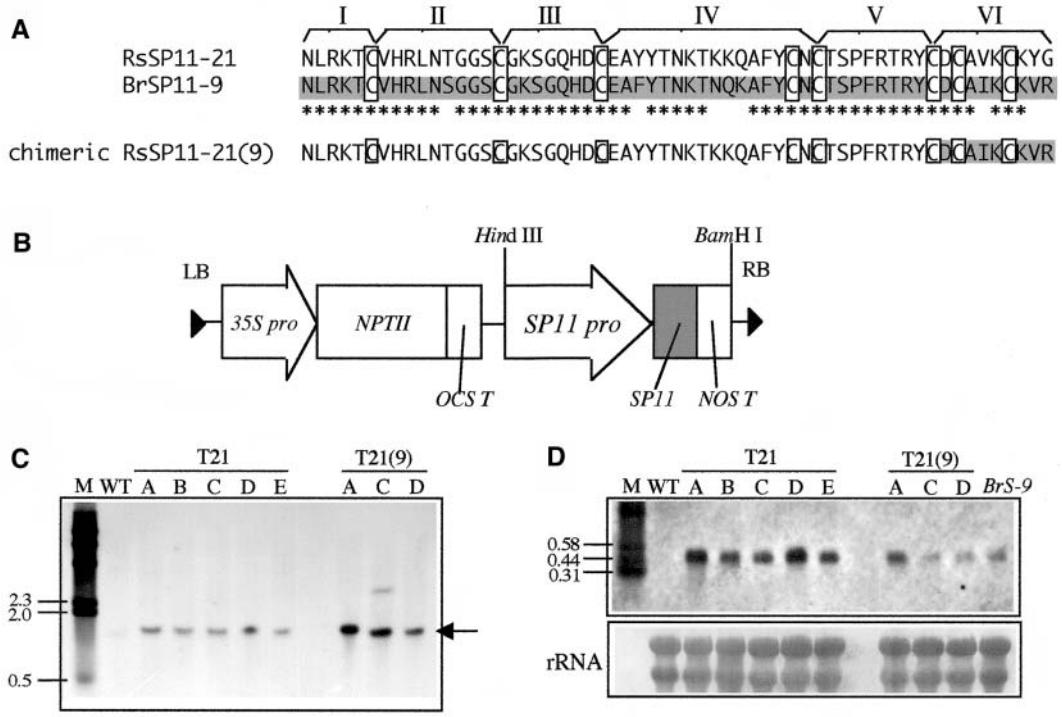

Figure 1. *B. rapa* Transgenic Plants Expressing *RsSP11-21* and Chimeric *RsSP11-21*(*9*).

(A) Deduced amino acid sequences of RsSP11-21 and BrSP11-9 (shaded background). Boxes indicate Cys residues, and asterisks show the same amino acid residues between RsSP11-21 and BrSP11-9. The sequence of chimeric RsSP11-21(9) used for the plant transformation is also shown.

(B) The T region of the vector used for *B. rapa* transformation. The coding region of *SP11* is indicated by a shaded box. *SP11 pro*, *BrSP11-46* promoter; *NOS T*, terminator of nopaline synthase gene; *35S pro*, *Cauliflower mosaic virus* 35S promoter; *NPTII*, neomycin phosphotransferase gene; *OCS T*, terminator of octopine synthetase gene; RB, right border; LB, left border.

(C) DNA gel blot analysis of the transgenes. After electrophoresis and transfer to a membrane, DNA digested with *Bam*HI and *Hind*III was hybridized with a probe of a mixture of the coding regions of *RsSP11-21* and chimeric *RsSP11-21*(*9*). The arrow indicates the 1.5-kb band of the transgenes. DNA size markers in kb are shown to the left. M, molecular marker; WT, nontransgenic *BrS-52/S-60* plant. T21, transgenic plants carrying wild-type *RsSP11-21*; T21(9), those carrying chimeric *RsSP11-21*(*9*).

(D) RNA gel blot analysis of the transgenes. The same probe as in the DNA gel blot analysis was used. RNA size markers in kb are shown to the left. *BrS-9*, a *BrS-9* homozygote.

Table 2. Incompatibility of Pollen Grains of *B. rapa* Transgenic Plants Expressing *RsSP11-21* or Chimeric *RsSP11-21* with *BrS-9* Stigmas

Pollen Donors	*S* Haplotypes	Transgenes	Stigmas	
			BrS-9	*BrS-46*
T21-A	*BrS-52/S-60*	*RsSP11-21*	−	++
T21-B	*BrS-52/S-60*	*RsSP11-21*	−	++
T21-C	*BrS-52/S-60*	*RsSP11-21*	−−	+
T21-D	*BrS-52/S-60*	*RsSP11-21*	−	++
T21-E	*BrS-52/S-60*	*RsSP11-21*	−−	+
T21(9)-A	*BrS-52/S-60*	Chimeric *RsSP11-21*(*9*)	−	+
T21(9)-C	*BrS-52/S-60*	Chimeric *RsSP11-21*(*9*)	+−	++
T21(9)-D	*BrS-52/S-60*	Chimeric *RsSP11-21*(*9*)	−	+
Wild type	*BrS-52/S-60*		+	++
BrS-9			−	++

The indices are as follows: −−, completely incompatible (no or few germinating pollen grains on a stigma); −, incompatible (>30 pollen grains on a stigma and <5 pollen tubes penetrating papilla cells); +−, partially incompatible (5 to 30 pollen tubes penetrating papilla cells); +, compatible (30 to 100 pollen tubes penetrating papilla cells); ++, completely compatible (>100 pollen tubes penetrating papilla cells).

RESULTS

The Same Recognition Specificity between *R. sativus S-21* and *B. rapa S-9*

We investigated the recognition specificity of an intergeneric pair between *Raphanus* and *Brassica*, in which amino acid identities of SRK and SP11 are lower than those of the interspecific pairs between *B. oleracea* and *B. rapa* (Sato et al., 2003). The most similar pair between *Raphanus* and *Brassica* was a pair of *S-21* in *R. sativus* (*RsS-21*) and *S-9* in *B. rapa* (*BrS-9*), the amino acid identity of SP11 being 89.5% and that of the S domain of SRK being 91.0% (Table 1) (Okamoto et al., 2004). Different amino acid residues between SP11 of *RsS-21* (RsSP11-21) and that of *BrS-9* (BrSP11-9) are located in Regions II, IV, and VI (Figure 1A). Our trial to investigate the recognition specificity of this intergeneric pair by a bioassay using recombinant SP11 proteins failed because of the low yield of the recombinant RsSP11-21 protein synthesized by *Escherichia coli*. The yield of the RsSP11-21 protein was about one-tenth of that of the BrSP11-9 protein. Our preliminary experiment using several chimeric *SP11* genes produced by DNA shuffling (Zhao and Arnold, 1997) between *RsSP-21* and *BrSP11-9* suggested that the difference of the efficiency of producing recombinant SP11 protein was because of the amino acid residues in Region VI. A bioassay of BrSP11-9 using *R. sativus* stigmas was also unsuccessful, because of the difficulty of the treatments of the recombinant proteins on the small stigmas of *R. sativus* and unstable results of control experiments.

Because of the difficulties of the bioassay using the recombinant SP11 proteins, we constructed genes of a 1-kb sequence of *BrSP11-46* promoter, cDNA of *SP11*, and *NOS* terminator, in which the *SP11* cDNA was wild-type *RsSP11-21* or chimeric *RsSP11-21*(*9*) composed of Region I to Region V of *RsSP11-21* and Region VI of *BrSP11-9*, and introduced them into *B. rapa* cv Osome (a *BrS-52/S-60* heterozygote) (Figures 1A and 1B). Five independent transgenic plants carrying wild-type *RsSP11-21* (T21-A, T21-B, T21-C, T21-D, and T21-E) and three independent transgenic plants carrying chimeric *RsSP11-21*(*9*) [T21(9)-A, T21(9)-C, and T21(9)-D] were obtained. DNA gel blot analysis after digestion with a mixture of *Bam*HI and *Hin*dIII showed a 1.5-kb band in all the transgenic plants (Figure 1C). Expression of wild-type *RsSP11-21* and chimeric *RsSP11-21*(*9*) was detected in all the transgenic plants by RNA gel blot analysis, but the expression levels in T21(9)-C and T21(9)-D were low (Figure 1D). The pollen grains of the transgenic plants expressing wild-type *RsSP11-21* or chimeric *RsSP11-21*(*9*) were pollinated onto the stigmas of homozygotes of *BrS-9* and *BrS-46* (Table 2, Figure 2). The pollen grains of all the transgenic plants were incompatible with the *BrS-9* stigmas, but were compatible with the *BrS-46* stigmas. This result indicates that *RsSP11-21* has the same recognition specificity as *BrSP11-9*.

Recognition Specificity of a Set of *S* Haplotypes in Raphanus and in Two Species of Brassica

Comparison of the nucleotide sequences of *SRK* and *SP11* in many *S* haplotypes of *B. oleracea*, *B. rapa*, and *R. sativus* revealed a set of three fairly similar *S* haplotypes, *RsS-6*, *BoS-18*, and *BrS-52* (Table 1). Interspecific hybrid plants, which have been successfully used for the test of recognition specificity of *S* haplotypes between *B. oleracea* and *B. rapa* (Kimura et al., 2002; Sato et al., 2003), were produced to investigate the recognition

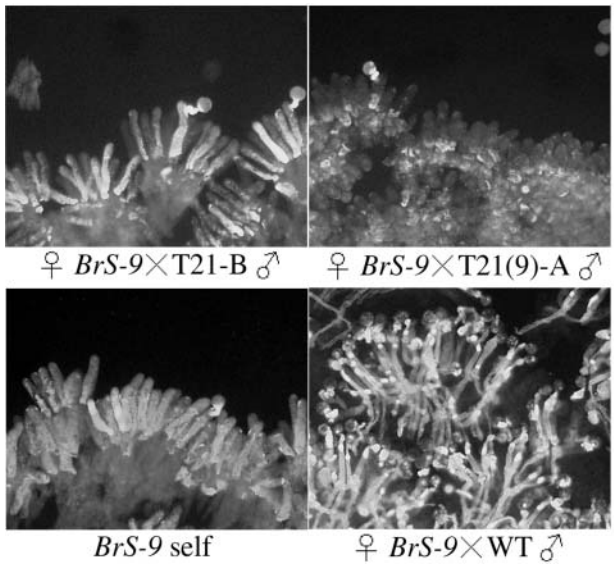

Figure 2. Pollen Tube Growth from the Pollen Grains of the *B. rapa* Transgenic Plants Carrying *RsSP11-21* and Chimeric *RsSP11-21*(*9*) in the Stigma of a *BrS-9* Homozygote.

The *BrS-9* homozygote and the wild-type plant of *BrS-52/S-60* heterozygote (WT) were crossed as the controls. The pollen grains of the transgenic plants [T21-B and T21(9)-A] were incompatible with the stigmas of *BrS-9*.

Table 3. Pollination Tests of *BoS-18* and *BrS-52* Using Interspecific Hybrids Having *BoS-18/BrS-60* and *BrS-52/BoS-15*

	Stigmas	
Pollen Donors	*BoS-18/BrS-60*	*BrS-52/BoS-15*
BoS-6	++	++
BoS-8	++	++
BoS-18	−−	++
BrS-8	++	++
BrS-9	++	+
BrS-36	++	++
BrS-45	++	++
BrS-52	++	−

The indices are as follows: −−, completely incompatible (no or few germinating pollen grains on a stigma); −, incompatible (>30 pollen grains on a stigma and <5 pollen tubes penetrating papilla cells); +, compatible (30 to 100 pollen tubes penetrating papilla cells); ++, completely compatible (>100 pollen tubes penetrating papilla cells).

specificity of *BoS-18* and *BrS-52*. *BoS-18* and *BrS-52* homozygotes were crossed with *BrS-60* and *BoS-15* homozygotes, respectively, in which *BoS-15* and *BrS-60* are class-II *S* haplotypes. A hybrid between *BoS-18* and *BrS-60* was an amphidiploid plant (*BoS-18/BrS-60*), whereas that between *BrS-52* and *BoS-15* was a diploid plant (*BrS-52/BoS-15*). The latter was sterile like diploid interspecific hybrids between these species reported so far, the sterility of which is caused by inability of chromosome pairing in meiosis. Therefore, only the stigmas of the hybrids were used for the pollination test.

Pollen grains of *BoS-6*, *BoS-8*, *BoS-18*, *BrS-8*, *BrS-9*, *BrS-36*, *BrS-45*, and *BrS-52* homozygotes were pollinated onto the stigmas of the interspecific hybrid plants (Table 3). When pollinated onto the stigmas of *BoS-18/BrS-60*, only pollen grains of *BoS-18* were incompatible. The pollen grains of *BrS-52* were completely compatible with the stigmas of *BoS-18/BrS-60*. When pollinated onto the stigmas of *BrS-52/BoS-15*, only pollen grains of *BrS-52* were incompatible, whereas those of *BoS-18* were completely compatible with the stigmas of *BrS-52/BoS-15*. These results indicate that *BoS-18* and *BrS-52* have different recognition specificity. The inability of the *BrS-52* stigmas to recognize BoSP11-18 as self was also shown by a pollination test using *B. rapa* transgenic plants carrying a *BoSP11-18* transgene as the control experiment for the test of a chimeric gene (data are shown later in Table 5).

The recognition specificity of *RsS-6* was investigated using transgenic plants of *B. oleracea* carrying an *RsSP11-6* transgene. The *RsSP11-6* gene under the *BrSP11-46* promoter was introduced to *B. oleracea* cv Green Comet, heterozygotes of *BoS-39/S-15* or *BoS-2/S-15* (Figure 3A), the pollen phenotype of which is *S-39* or *S-2*, respectively, because *S-2* of *B. oleracea* is dominant to *S-15* in pollen (Thompson and Taylor, 1966) and *S-39* is also dominant to *S-15* (data not shown). Three independent transgenic plants (T6-A, T6-B, and T6-C) were obtained. DNA gel blot analysis detected a 2.1-kb band in the transgenic plants after double-digestion with *Eco*RI and *Hin*dIII (Figure 3B). The expression of the transgene was observed in all the transgenic plants using RNA gel blot analysis (Figure 3C). Pollen grains of the three transgenic plants expressing *RsSP11-6* were incompatible with the stigmas of the *BoS-18* homozygote and the *BoS-18/BrS-60* interspecific hybrid (Figure 4, Table 4). For the pollination test of *BrS-52*, the *BrS-52/BoS-15* interspecific hybrid was used to avoid the interspecific barrier. The pollen grains of all the transgenic plants were partially incompatible with those of the *BrS-52/BoS-15* interspecific hybrid. These results indicate that RsSP11-6 is recognized as self by the *BoS-18*

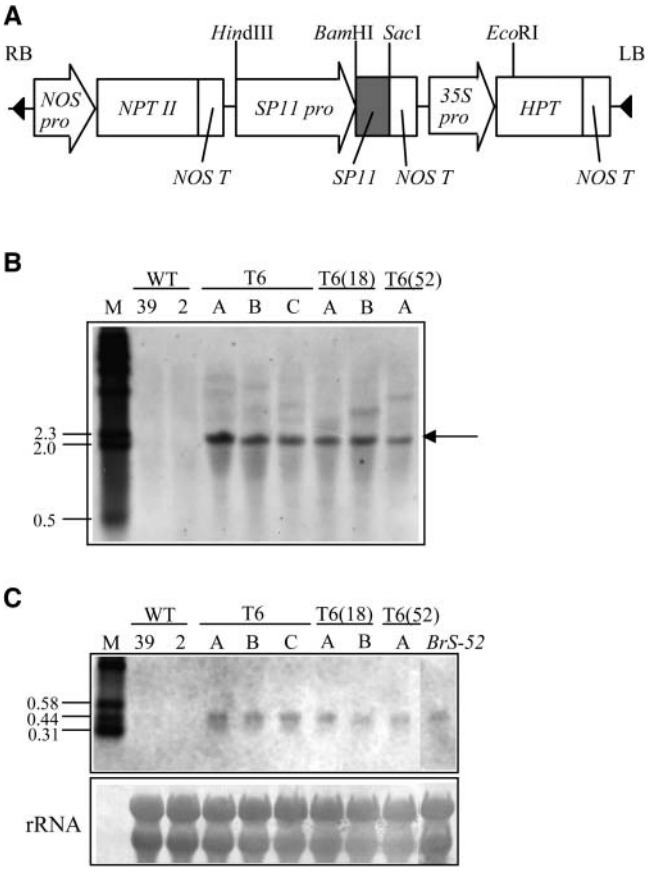

Figure 3. *B. oleracea* Transgenic Plants Expressing *RsSP11-6*, Chimeric *RsSP11-6*(*18*), and Chimeric *RsSP11-6*(*52*).

(A) The T region of the vector used for *B. oleracea* transformation. The coding region of *SP11* is indicated by a gray box. *SP11 pro*, *BrSP11-46* promoter; *NOS T*, nopaline synthase gene terminator; *35S pro*, *Cauliflower mosaic virus* 35S promoter; *NPTII*, neomycin phosphotransferase gene; *HPT*, hygromycin phosphotransferase gene; RB, right border; LB, left border.

(B) DNA gel blot analysis of the transgenes. DNA was digested with *Eco*RI and *Hin*dIII, and probed with a mixture of the coding regions of *RsSP11-6* and *BrSP11-52*. The arrow indicates the 2.1-kb band of the transgenes. DNA size markers in kb are shown to the left. M, molecular marker; WT, nontransgenic *BoS-39/S-15* heterozygote (39), *BoS-2/S-15* heterozygote (2); T6, transgenic plants carrying wild-type *RsSP11-6*; T6(18), those carrying chimeric *RsSP11-6*(*18*); T6(52), those carrying chimeric *RsSP11-6*(*52*).

(C) RNA gel blot analysis of the transgenes. The same probe was used as in the DNA gel blot analysis. RNA size markers in kb are shown to the left. *BrS-52*, a *BrS-52* homozygote.

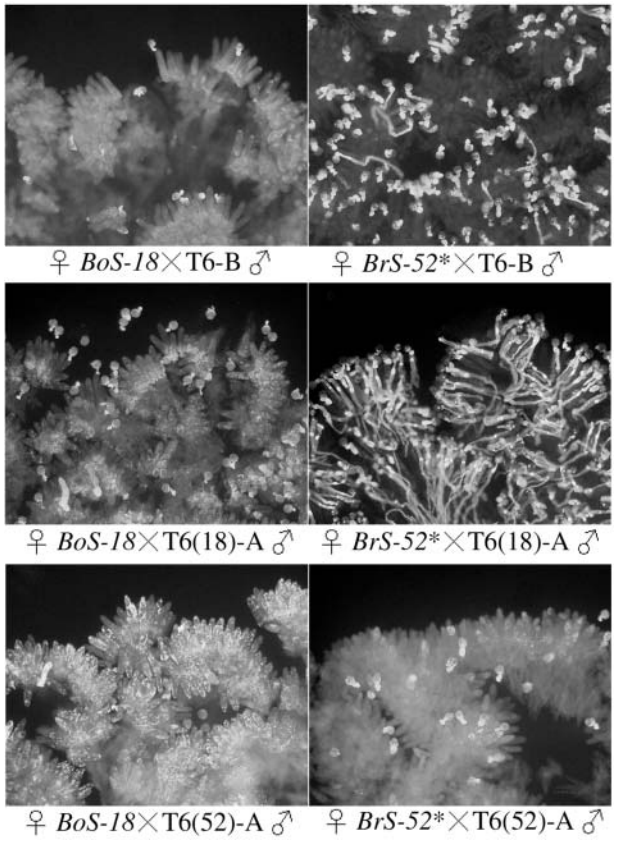

Figure 4. Pollen Tube Growth from the Pollen Grains of the Transgenic Plants Carrying *RsSP11-6*, Chimeric *RsSP11-6*(*18*), and Chimeric *RsSP11-6*(*52*) in the Stigmas of the *BoS-18* Homozygote (*BoS-18*) and the *BrS-52*/*BoS-15* Interspecific Hybrid (*BrS-52**).

Pollen grains of the transgenic plants carrying *RsSP11-6* (T6-B) were incompatible with the *BoS-18* stigmas, but only partially incompatible with the stigmas having *BrS-52*. The transgenic plants carrying chimeric *RsSP11-6*(*18*) [T6(18)-A] were incompatible with *BoS-18*, but compatible with *BrS-52**. Those carrying chimeric *RsSP11-6*(*52*) [T6(52)-A] were incompatible with the stigmas of both *BoS-18* and *BrS-52**.

stigmas and as a little different from BrSP11-52 by the stigmas having *BrS-52*.

Analysis of the Regions Important for Recognition Specificity of BrSP11-52 by Bioassay Using Recombinant SP11 Proteins

Using *BoS-18* and *BrS-52*, which are similar but have different recognition specificity, chimeric SP11 proteins were produced by domain swapping to investigate the regions important for the recognition specificity. Seven chimeric SP11s [52(II-1), 52(II-2), 52(III), 52(IV-1), 52(IV-2), 52(V-1), and 52(V-2)] were produced to have the sequence of BrSP11-52 with short segments of Region II, III, IV, and V from BoSP11-18 (Figure 5A). Regions I and Regions VI were not replaced because of the high similarity between *BoSP11-18* and *BrSP11-52*. Recombinant proteins of the seven chimeric SP11s, wild-type BrSP11-52, and wild-type BoSP11-18 were applied onto the surface of the stigmas of *S-52* homozygotes in *B. rapa* and compatible pollen grains were pollinated. The levels of incompatibility induced by the recombinant proteins were rated with indices, i.e., 1 (incompatible) to 4 (compatible), based on the number of pollen tubes penetrating the stigma. The results of this bioassay were not as stable as those of the pollination test using transgenic plants; therefore, the bioassay was performed for 23 d using 55 to 75 flowers for each treatment in total. Averaged indices of BrSP11-52 and BoSP11-18 were 1.7 and 2.5, respectively (Figure 5B). Replacements of Region III and Region V reduced significantly the incompatibility response induction activity in the *BrS-52* stigmas, whereas those of Region II and Region IV did not. The recombinant proteins having Region II or Region IV of BoSP11-18 induced significantly higher incompatibility response than BoSP11-18. These results suggest that Region III and Region V of BrSP11-52 are more important for the recognition specificity than Region II and Region IV.

The BoSP11-18 sequence was replaced with the sequences of BrSP11-52 in Region III and Region V [18(III,V), Figure 5A], and the incompatibility response induction activity of 18(III,V) was tested by the bioassay using 54 stigmas of the *BrS-52* homozygotes. Significantly higher activity inducing the incompatibility response than BoSP11-18 was observed (Figure 5B).

Alteration of the Recognition Specificity of SP11 by Swapping Region III and Region V

The bioassay revealed Region III and Region V of SP11 to be important for the recognition specificity. To confirm this finding, we investigated the recognition specificity of chimeric SP11s using the plant transformation and pollination tests. Chimeric *BoSP11-18*(*52*), in which Region III and Region V of BoSP11-18 were replaced by those of BrSP11-52, was produced (Figure 6), and chimeric *BoSP11-18*(*52*) and wild-type *BoSP11-18* were introduced into *B. rapa S-60* homozygotes. Five independent transgenic plants carrying chimeric *BoSP11-18*(*52)* [T18(52)-A, T18(52)-B, T18(52)-F, T18(52)-G, and T18(52)-I] and seven independent transgenic plants carrying wild-type *BoSP11-18* (T18-A, T18-B, T18-C, T18-D, T18-E, T18-F, and T18-G) were obtained. DNA gel blot analysis after double-digestion with *Bam*HI and *Hin*dIII detected a 1.5-kb band in all the transgenic plants, except for 18-G, in which a 3.0-kb band was detected (Figure 7A). The expression of the transgenes in the transgenic plants was confirmed by RNA gel blot analysis except for T18(52)-B, T18-D, and T18-G (Figure 7B). The expression of the transgene was not detected in T18(52)-B, and the levels of the expression in T18-D and T18-G were low. T18(52)-F was sterile and DNA gel blot analysis showed T18-B to have an additional band. Therefore, the transgenic plants of T18(52)-A, T18(52)-G, T18(52)-I, T18-A, T18-C, T18-E, and T18-F were used for pollination tests. When the pollen grains of T18(52)-A, T18(52)-G, T18(52)-I were pollinated onto the stigmas of *BrS-52*, they showed partial incompatibility (Table 5). The pollen grains of T18-A, T18-C, T18-E, and T18-F were compatible with the stigmas of *BrS-52*.

Chimeric *SP11* genes of *RsSP11-6* were produced by replacing Region III and Region V of *RsSP11-6* with the sequence of

Table 4. Incompatibility of Pollen Grains of *B. oleracea* Transgenic Plants Expressing *RsSP11-6* with the Stigmas of *BoS-18* and *BrS-52*

			Stigmas			
Pollen Donors	*S* Haplotypes	Transgene	*BoS-18*	*BoS-18/BrS-60*	*BrS-52/BoS-15*	*BoS-8*
T6-A	*S-39/S-15*	*RsSP11-6*	−−	−−	+−	++
T6-B	*S-2/S-15*	*RsSP11-6*	−−	−−	+−	++
T6-C	*S-2/S-15*	*RsSP11-6*	−	−	+−	++
WT1	*S-39/S-15*		++	++	++	++
WT2	*S-2/S-15*		++	++	++	++

The indices are as follows: −−, completely incompatible (no or few germinating pollen grains on a stigma); −, incompatible (>30 pollen grains on a stigma and <5 pollen tubes penetrating papilla cells); +−, partially incompatible (5 to 30 pollen tubes penetrating papilla cells); ++, completely compatible (>100 pollen tubes penetrating papilla cells).

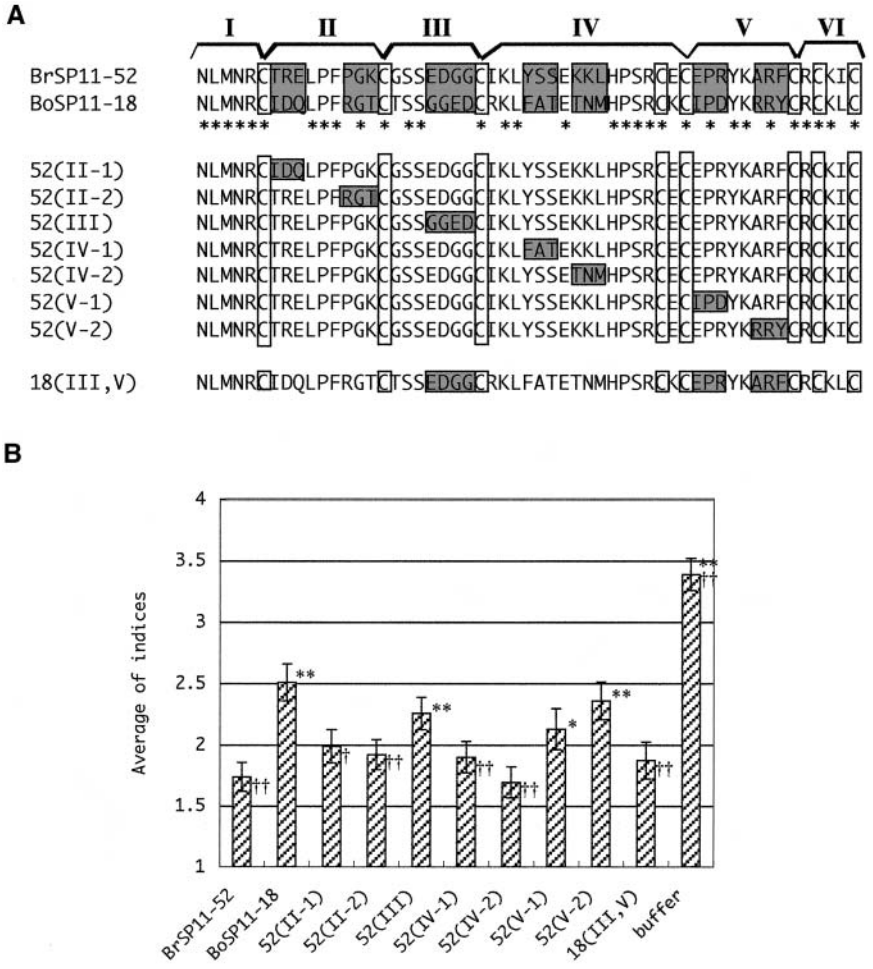

Figure 5. Sequences of Chimeric SP11s and Analysis of Recognition Specificity of the Chimeric SP11 Proteins by Bioassay.

(A) The chimeric SP11-52s [52(II-1), 52(II-2), 52(III), 52(IV-1), 52(IV-2), 52(V-1), and 52(V-2)] were produced by replacing the *BrSP11-52* sequence with small segments of the *BoSP11-18* sequence. A chimeric SP11-18 [18(III,V)] was produced by replacing Region III and Region V of the *BoSP11-18* sequence with the sequence of *BrSP11-52*. The white and gray boxes indicate the conserved Cys residues and the amino acid sequences that were replaced, respectively.

(B) The bioassay of incompatibility response inducing ability of recombinant proteins of chimeric SP11s using the stigmas of *S-52* homozygotes in *B. rapa*. The level of incompatibility was rated with the indices as follows: 1, the pollen tubes penetrating into the stigma being <10; 2, those being 10 to 30; 3, those being from 30 to 100; 4, those being >100. The values are the means ± SE of 54 to 75 stigmas. ** and * represent significant differences from an incompatible control, BrSP11-52, at 1 and 5% levels, respectively. †† and † represent significant differences from a compatible control, BoSP11-18, at 1 and 5% levels, respectively.

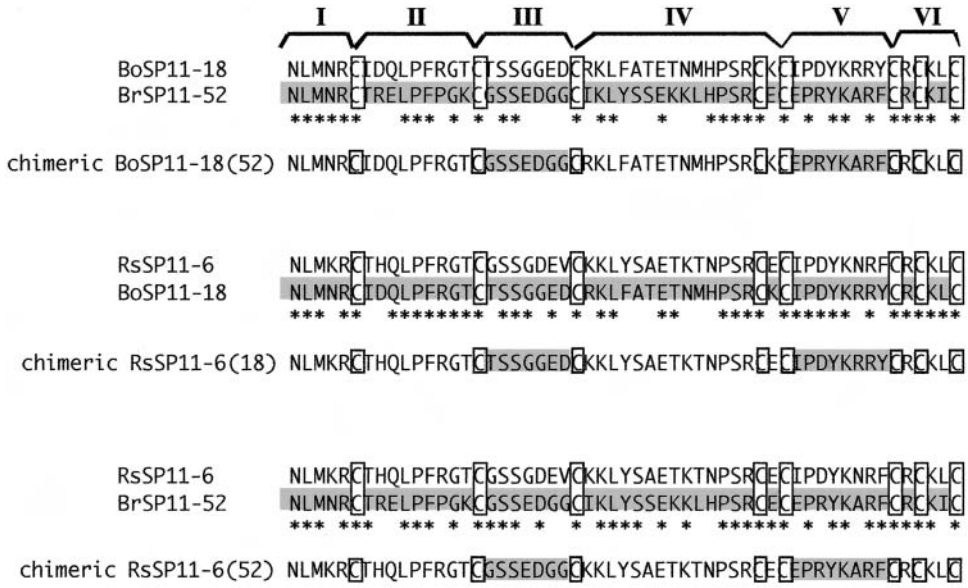

Figure 6. Deduced Amino Acid Sequences of BoSP11-18, BrSP11-52, RsSP11-6, and Chimeric SP11s Used for Brassica Transformation.

Boxes indicate the conserved Cys residues, and asterisks show the same amino acid residues between two sequences.

BoSP11-18 [chimeric *RsSP11-6*(*18*)] or *BrSP11-52* [chimeric *RsSP11-6*(*52*)] (Figure 6). These genes were introduced into *B. oleracea* cv Green Comet, *BoS-39/S-15* or *BoS-2/S-15* heterozygote. The independent transgenic plants obtained were two with chimeric *RsSP11-6*(*18*), i.e., T6(18)-A and T6(18)-B, and one with chimeric *RsSP11-6*(*52*), i.e., T6(52)-A. DNA gel blot analysis detected 2.1-kb bands after double-digestion with *Eco*RI and *Hin*dIII in the transgenic *B. oleracea* plants (Figure 3B). RNA gel blot analysis showed the expression of the *SP11* transgenes in all the transgenic plants (Figure 3C). The expression levels of the *SP11* transgenes in the transgenic plants were as high as that of the endogenous *SP11* in a *BrS-52* homozygote. T6(18)-A and T6(18)-B carrying chimeric *RsSP11-6*(*18*) were incompatible with the stigmas of the *BoS-18* homozygote and the interspecific hybrid of *BoS-18*/*BrS-60*, although completely compatible with the hybrid of *BrS-52*/*BoS-15* (Figure 4, Table 6). T6(52)-A carrying chimeric *RsSP11-6*(*52*) was incompatible with the stigmas of *BrS-52*/*BoS-15*, *BoS-18*, and *BoS-18*/*BrS-60*.

DISCUSSION

Regions of SP11 Important for the Recognition by SRK

In this study, *BrS-9* and *RsS-21*, which are an intergeneric pair of *S* haplotypes having highly similar *SP11* and *SRK* alleles, were demonstrated to have the same recognition specificity of pollen-stigma interaction. It was also found that *RsS-6* is incompatible with *BoS-18* and partially incompatible with *BrS-52*. Alignment of the deduced amino acid sequences of BrSP11-9 and RsSP11-21 indicates that there are differences of amino acid residues in Region II, Region IV, and Region VI but not in Region III and Region V. Higher sequence polymorphism in Region IV than those in the other regions has been observed in the interspecific pairs of *S* haplotypes between *B. oleracea* and *B. rapa* (Sato et al., 2003). This observation may suggest that Region IV is not as important as Region III and Region V, which are conserved between the SP11 proteins in the interspecific pairs and the intergeneric pair. However, between *BoS-18* and *RsS-6*, which have the same recognition specificity, there was no biased distribution of different amino acid residues in the SP11 sequences.

The results of the bioassay using chimeric SP11 proteins suggested that Region III and Region V are important for the recognition of BrSP11-52. This inference was supported by the results of the pollination tests using transgenic plants having chimeric *SP11* genes, where the pollen grains of the transgenic plants carrying chimeric *RsSP11-6*(*52*) and chimeric *BoSP11-18*(*52*) were incompatible and partially incompatible, respectively, with the stigmas having *BrS-52*, whereas those of the transgenic plants carrying chimeric *RsSP11-6*(*18*) and wild-type *BoSP11-18* were compatible with the *BrS-52* stigmas. Using a bioassay with recombinant proteins, Chookajorn et al. (2004) have found that replacing only four continuous amino acid residues is sufficient to alter the recognition specificity from *S-6* to *S-13* in *B. oleracea*. These four amino acid residues are located in Region V. On the other hand, the pollen grains of the transgenic plants carrying chimeric *RsSP11-6*(*52*) were also incompatible with the stigmas of *BoS-18*. This result suggests that other regions of RsSP11-6 may also be important for the recognition by the *BoS-18* stigmas.

Mishima et al. (2003) have elucidated the solution structure of the SP11 proteins, in which Region III forms an α-helix and Region V corresponds to the region from β2 to β3. Region III and Region V are arranged close to each other by disulfide linkage (Takayama et al., 2001; Mishima et al., 2003). The lower half of

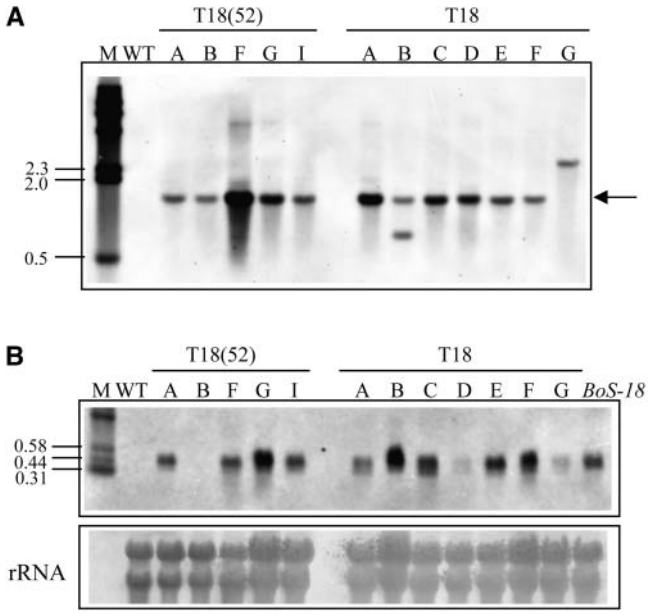

Figure 7. Analysis of *B. rapa* Transgenic Plants Expressing Chimeric *BoSP11-18*(*52*) and *BoSP11-18*.

(A) DNA gel blot analysis of the transgenes. DNA was digested with *Bam*HI and *Hin*dIII, and probed with a mixture of *BoSP11-18* and chimeric *BoSP11-18*(*52*). The arrow indicates the 1.5-kb band of the transgenes. DNA size markers in kb are shown to the left. M, molecular marker; WT, nontransgenic *BrS-60* plant; T18(52), transgenic plants carrying chimeric *BoSP11-18*(*52*); T18, those carrying wild-type *BoSP11-18*.

(B) RNA gel blot analysis of the transgenes. The same probe as in the DNA gel blot analysis was used. RNA size markers in kb are shown to the left. *BoS-18*, a *BoS-18* homozygote.

Region II is also located close to both Region III and Region V. The tertiary structure formed by these regions may determine the recognition specificity of the SP11 proteins. On the other hand, the loop in Region IV is located opposite the loop formed by Region V in the SP11 proteins. The loop in Region IV is the hypervariable region of the SP11 protein, which has been considered to serve as a specific binding site for SRK by Mishima et al. (2003). This study indicated that Region IV is not as important as Region III and Region V for determining the recognition specificity. It can be inferred that amino acid variations may be accumulated in Region IV because of its low importance of this region in the recognition function.

Evolution of *S* Haplotypes

The pair of *BrS-9* and *RsS-21* can be considered to have been derived from the same ancestral *S* haplotype, and the amino acid differences between these *S* haplotypes, which would have arisen after the divergence of these two genera, is not considered to have contributed to the alteration of recognition specificity. Although *BoS-18*, *BrS-52*, and *RsS-6* are also considered to have been derived from the same ancestral *S* haplotype, remarkably lower sequence similarities between these *S* haplotypes than those between *BrS-9* and *RsS-21* may suggest that diversification of *BoS-18*, *BrS-52*, and *RsS-6* had occurred before the divergence of these genera. If the sequence differences between these *S* haplotypes arose after the divergence of the genera and the species, the sequence similarity between *BrS-52* and *BoS-18* would be higher than that between *RsS-6* and *BrS-52* or between *RsS-6* and *BoS-18*. However, the similarities of nucleotide sequences and deduced amino acid sequences between *BrS-52* and *BoS-18* were lowest in those between these three *S* haplotypes. Furthermore, *BrS-52* and *BoS-18* have different recognition specificity, indicating that they are different from the interspecific pairs of *S* haplotypes identified so far (Sato et al., 2003).

The consensus sequences of the *SP11* and *SRK* alleles of *RsS-6*, *BoS-18*, and *BrS-52*, which can be regarded as the sequences of the putative ancestral *S* haplotype (*S-X*), were determined by selecting the nucleotides that are present in more than two sequences in the alignment of these three *S* haplotypes. The consensus amino acid sequences of SP11 and the S domain of SRK, except three amino acids for SP11 and five amino acids for SRK, were deduced. The sequence similarities of RsSP11-6, BoSP11-18, and BrSP11-52 to the consensus amino acid sequence of SP11, i.e., SP11-X, were 87.3, 78.2, and 72.7%, respectively, and those of RsSRK-6, BoSRK-18, and BrSRK-52 to the consensus sequence of the S domain of SRK, i.e., SRK-X, were 92.8, 93.8, and 92.3%, respectively (Figures 8A and 8B). The relationships between these sequences suggest that *RsSP11-6* is closest to the putative ancestral *SP11* sequence and that *BrSP11-52* is most distantly related. The lowest amino acid sequence similarity between BoSP11-18 and BrSP11-52 among the four SP11 sequences including SP11-X and the different recognition specificity between BoSP11-18 and BrSP11-52 suggest that *BoSP11-18* and *BrSP11-52* have evolved so as to acquire different recognition specificity.

Table 5. Incompatibility between *B. rapa S-52* and the Transgenic *B. rapa* Plants Expressing Chimeric *BoSP11-18*(*52*) or *BoSP11-18*

			Stigmas	
Pollen Donors	*S* Haplotype	Transgenes	*BrS-52*	*BrS-46*
T18(52)-A	*BrS-60*	Chimeric *BoSP11-18*(*52*)	+−	++
T18(52)-G	*BrS-60*	Chimeric *BoSP11-18*(*52*)	+−	++
T18(52)-I	*BrS-60*	Chimeric *BoSP11-18*(*52*)	+−	++
T18-A	*BrS-60*	*BoSP11-18*	+	++
T18-C	*BrS-60*	*BoSP11-18*	+	++
T18-E	*BrS-60*	*BoSP11-18*	+	++
T18-F	*BrS-60*	*BoSP11-18*	+	++
Wild type	*BrS-60*		+	++
BrS-52			−	++

The indices are as follows: −, incompatible (>30 pollen grains on a stigma and <5 pollen tubes penetrating papilla cells); +−, partially incompatible (5 to 30 pollen tubes penetrating papilla cells); +, compatible (30 to 100 pollen tubes penetrating papilla cells); ++, completely compatible (>100 pollen tubes penetrating papilla cells).

Table 6. Incompatibility of Pollen Grains of *B. oleracea* Transgenic Plants Expressing Chimeric *RsSP11-6*(*18*) and Chimeric *RsSP11-6*(*52*) with the Stigmas of *BoS-18* and *BrS-52*

Pollen Donors	*S* Haplotypes	Transgenes	Stigmas			
			BoS-18	*BoS-18/BrS-60*	*BrS-52/BoS-15*	*BoS-8*
T6(18)-A	*S-2/S-15*	Chimeric *RsSP11-6*(*18*)	−	−	++	++
T6(18)-B	*S-2/S-15*	Chimeric *RsSP11-6*(*18*)	−	−	++	++
T6(52)-A	*S-39/S-15*	Chimeric *RsSP11-6*(*52*)	−−	−−	−	++
WT1	*S-39/S-15*		++	++	++	++
WT2	*S-2/S-15*		++	++	++	++

The indices are as follows: −−, completely incompatible (no or few germinating pollen grains on a stigma); −, incompatible (>30 pollen grains on a stigma and <5 pollen tubes penetrating papilla cells); ++, completely compatible (>100 pollen tubes penetrating papilla cells).

Several models of the generation of a new *S* haplotype have been presented and discussed (Matton et al., 1999; Uyenoyama and Newbigin, 2000; Uyenoyama et al., 2001; Chookajorn et al., 2004). The relationship of *BoS-18*, *BrS-52*, and *RsS-6* would provide interesting information to facilitate understanding of the mechanism of the *S* haplotype diversification. Their relationships of cross-incompatibility, i.e., complete incompatibility between *BoS-18* and *RsS-6*, partial incompatibility between *BrS-52* and *RsS-6*, and compatibility between *BoS-18* and *BrS-52*, suggest that in the diversification of *S* haplotypes between *BoS-18* and *BrS-52*, *BoS-18* has maintained the incompatibility with *RsS-6* and that *BrS-52* has diverged more, resulting in lowering of its incompatibility with *RsS-6*. This process of *S* haplotype diversification supports the model presented by Chookajorn et al. (2004), in which gradual sequence modification maintaining the recognition specificity of SP11 and SRK is considered to

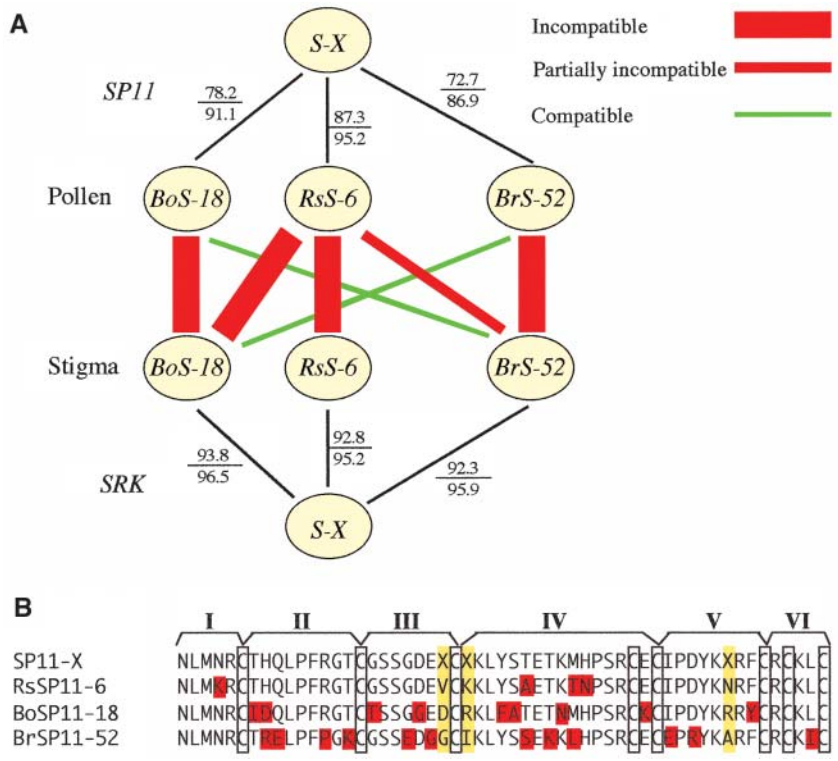

Figure 8. Relationships of the Three *S* Haplotypes *RsS-6*, *BoS-18*, and *BrS-52*.

(A) Schematic representation of the relationships. *S-X* shows the putative ancestral *S* haplotype. Deduced amino acid identities and nucleotide identities of *SP11* and *SRK* of *BoS-18*, *RsS-6*, and *BrS-52* to those of *S-X* are shown on and under the bars, respectively. The thick and thin red bars show complete and partial incompatibility, respectively. The green bars show compatibility.

(B) Comparison of deduced amino acid sequences of SP11s. White boxes show the conserved Cys residues. Red boxes show amino acid residues different from those of SP11-X, and yellow boxes show the position in which the amino acid residues of SP11-X were not assumed.

contribute to the generation of a new *S* haplotype. Matton et al. (1999) have proposed that the generation of a new *S* haplotype passed through an intermediate stage in which it had dual specificity. *RsSP11-6* can be regarded as dual-specific *SP11* in a broad sense. Although the dual-specific *S* haplotypes would be eliminated from a population (Uyenoyama and Newbigin, 2000), it can be inferred that reproductive isolation may have contributed to the maintenance of *RsSP11-6* in the population. The coexistence of a dual-specific *S* haplotype and other *S* haplotypes incompatible with it in a natural population has not been observed in any plant species so far.

The strength or stability of self-incompatibility is an important genetic trait for F1 hybrid breeding of Brassicaceae vegetables. Strength of self-incompatibility is controlled by the *S* locus as well as by genetic background. Weak incompatibility by the *S* locus may be caused by low affinity of SP11 and SRK, which was represented as partial incompatibility between the chimeric SP11 proteins and the wild-type SRK protein in this study. Alteration of strength of self-incompatibility might have occurred in the evolution of the *S* haplotypes.

METHODS

Plant Materials

Homozygotes of *S-8*, *S-9*, *S-32*, *S-36*, *S-45*, *S-46*, *S-52*, and *S-60* in *Brassica rapa* and those of *S-6*, *S-8*, and *S-18* in *Brassica oleracea* were used as plant materials. The *S* haplotypes in these species have been numbered in England and Japan independently (Nou et al., 1993; Ockendon, 2000). Interspecific hybrids between *B. oleracea* and *B. rapa* were raised by ovary culture according to Inomata (1977). *B. rapa* cv Osome (Takii Seed, Kyoto, Japan), which is a heterozygote of *S-52* and *S-60*, and an *S-60* homozygote derived from Osome were used for the transformation of *B. rapa*. For the transformation of *B. oleracea*, a broccoli cultivar, Green Comet (*S-39*/*S-15* or *S-2*/*S-15*) (Takii Seed), was used.

Construction of the *SP11* Genes for Transformation of Brassica

The mature protein region of the wild-type *SP11* gene was amplified from *SP11* cDNAs of *BoSP11-18* (Sato et al., 2002), *RsSP11-6*, and *RsSP11-21* (Okamoto et al., 2004) using primers shown in Supplemental Tables 1 and 2 online. The signal peptide region of *SP11* was amplified from *BrSP11-9* and *BrSP11-52* using a primer pair of 9-ATG-*Bam*HI and 9-S-R and a pair of 52-ATG-*Bam*HI and 52-S-R, respectively. The sequence of *BrSP11-9* was used for the construction of *RsSP11-21* and that of *BrSP11-52* was used for the construction of *BoSP11-18* and *RsSP11-6*. The PCR product of the signal peptide region was mixed with that of the mature protein region. The mixture was used as a template of PCR with a primer pair of 9-ATG-*Bam*HI and 21RS for *RsSP11-21*, that of 9-ATG-*Bam*HI and 9RS for *RsSP11-21*(*9*), and that of 52-ATG-*Bam*HI and 18RS for *RsSP11-6* and *BoSP11-18*. The PCR products were cloned to the pGEM-T vector. Each clone that had the expected sequence was selected by nucleotide sequencing. The coding region of *SP11* was ligated to the 1.0-kb sequence of the *BrSP11-46* promoter (Sato et al., 2003), and inserted into the binary vector pSLJ491 (Jones et al., 1992) for the transformation of *B. rapa*, and into the position of the ubiquitin promoter and the spinach (*Spinacia oleracea*) *GPAT* gene of the binary vector used in rice (*Oryza sativa*) transformation (Ariizumi et al., 2002) for the transformation of *B. oleracea*.

Plant Transformation

The constructs were introduced into *Agrobacterium tumefaciens* strain EHA105 for *B. rapa* transformation, and into EHA101 for *B. oleracea* transformation. Hypocotyls of *B. rapa* and flower stems of *B. oleracea* were used as explants. Adventitious shoots from transformed cells were selected by kanamycin for *B. rapa* and by hygromycin for *B. oleracea*.

DNA Gel Blot Analysis

Total DNA was isolated from a leaf by DNeasy plant mini kit (Qiagen USA, Valencia, CA). Two micrograms of DNA were digested with appropriate restriction endonucleases, electrophoresed on a 1.0% (w/v) agarose gel, and transferred to a nylon membrane (Nytran N) (Schleicher and Schuell, Dassel, Germany). Hybridization was performed in 5× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) containing 1.0% blocking reagent (Boehringer Mannheim, Mannheim, Germany), 0.1% sodium-*N*-lauroyl sarcosinate, and 0.02% SDS at 65°C. The membrane was washed twice in a solution consisting of 0.1× SSC and 0.1% SDS at 65°C for 20 min. A digoxigenin-labeled probe of cDNA was prepared by PCR with PCR DIG labeling mix (Boehringer Mannheim). DNA bands hybridized with the digoxigenin-labeled probe were detected following the supplier's instructions (Boehringer Mannheim).

RNA Gel Blot Analysis

Total RNA was isolated from anthers by ISOGEN (Nippongene, Tokyo, Japan). After denaturation in glyoxal, 12 μg RNA was subjected to electrophoresis on a 1% agarose gel in 10 mM sodium phosphate buffer, pH 7.0, and transferred to Nytran N. Hybridization was performed using a digoxigenin-labeled cDNA probe in 5× SSC containing 1.0% blocking reagent (Boehringer Mannheim), 0.1% sodium-*N*-lauroyl sarcosinate, and 0.02% SDS at 65°C. The membrane was washed twice in 0.1× SSC containing 0.1% SDS at 65°C for 20 min.

Pollination Test

On the day of anthesis, flowers were cut off from the plants and placed on an agar plate. After emasculation, the stigmas were covered with a layer of pollen grains. The pollinated flowers were kept at 20°C for 6 h. The pistils were immersed in 1 N NaOH for 1 h at 50°C, stained with aniline blue (0.1% aniline blue in 0.1 M K_3PO_4), and mounted in 50% glycerol. Pollen tubes were observed under an ultraviolet light fluorescence microscope. The evaluation of self-incompatibility was conducted using indices based on the number of pollen tubes penetrating stigma papilla cells. The indices are as follows: $--$, completely incompatible, i.e., no or few germinating pollen grains observed on a stigma with no pollen tube penetrating a papilla cell; $-$, incompatible, i.e., >30 germinating pollen grains on a stigma and <5 pollen tubes penetrating papilla cells; $+-$, partially incompatible, i.e., >5 and <30 pollen tubes penetrating papilla cells; $+$, compatible, i.e., 30 to 100 pollen tubes penetrating papilla cells; $++$, completely compatible, i.e., >100 pollen tubes penetrating papilla cells. Pollination tests were conducted using >12 flowers in each cross-combination, and the most frequent index in each cross-combination was represented.

Production of Chimeric *SP11* Genes and Recombinant Proteins

Chimeric *SP11* genes were constructed for a bioassay and the transformation of Brassica. Primers and methods used for the construction of the chimeric *SP11* genes are shown in Supplemental Tables 1 and 2 online. For example, in the production of chimeric *RsSP11-6*(*18*), an *RsSP11-6* cDNA clone was used as a template of 1st PCR and the PCR product amplified using the primer pair of 6-S-F and 6III-18R was mixed

with the PCR product amplified using three primers of 6III-18F, 52V-2, and 18RS. The mixture was used as a template of 2nd PCR using a primer pair of 6-S-F and 18RS. Each PCR product of chimeric *SP11* was cloned into the pGEM-T vector (Promega, Madison, WI), and a clone of each chimeric *SP11* was selected by determining the nucleotide sequence.

The plasmids of 52(II-1), 52(IV-2), and 52(V-2) were digested with *Bam*HI and *Pst*I, and digested inserts were cloned into the pQE30 vector (Qiagen). The plasmids of the other clones were digested with *Bam*HI and *Sal*I, and the inserts were cloned into the pQE30 vector. The pQE30 DNA with each chimeric *SP11* was introduced into *E. coli* M15. The synthesized protein of SP11, which was tagged with 6× His at the N-terminal, was purified using nickel-nitrilotriacetic acid agarose (Qiagen).

Bioassay of Recombinant SP11 Proteins

The stigma was treated with a 0.5 μL solution of 300 ng/μL of the recombinant SP11 protein. After being air-dried, the stigma was pollinated by the pollen grains of a compatible *S* haplotype, i.e., *BrS-32*, *BrS-36*, or *BrS-46*. The levels of incompatibility were rated with indices different from those used for the tests of the transgenic plants and the interspecific hybrids, because the incompatibility exhibited by this assay was weak. The indices of the number of pollen tubes penetrating the stigma are as follows: 1, <10; 2, 10 to 30; 3, 31 to 100; and 4, >100. Means and standard errors of the indices were calculated, and significance of the difference of the means between the treatments and the control experiment was analyzed by *t* test.

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