Highly divergent sequences of the pollen self-incompatibility (*S*) gene in class-I *S* haplotypes of *Brassica campestris* (syn. *rapa*) L.

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Abstract Self-incompatibility (SI) enables flowering plants to discriminate between self- and non-self-pollen. In *Brassica*, SI is controlled by the highly polymorphic *S* locus. The recently identified male determinant, termed SP11 or SCR, is thought to be the ligand of *S* receptor kinase, the female determinant. To examine functional and evolutionary properties of *SP11*, we cloned 14 alleles from class-I *S* haplotypes of *Brassica campestris* and carried out sequence analyses. The sequences of mature SP11 proteins are highly divergent, except for the presence of conserved cysteines. The phylogenetic trees suggest possible co-evolution of the genes encoding the male and female determinants.

Key words: *Brassica*; Co-evolution; Phylogenetic analysis; Self-incompatibility; *SP11*/*SCR*; *SRK*

1. Introduction

Many flowering plants possess self-incompatibility (SI) which promotes outbreeding and maintains genetic diversity. SI prevents self-fertilization by rejecting pollen from plants with the same genotype. The SI system in *Brassica* is controlled sporophytically by multiple alleles at a single locus, termed the *S* locus [1], and it involves cell–cell communication between male (pollen) and female (stigma). On the female side, two genes located at the *S* locus encode SLG (*S* locus glycoprotein) and SRK (*S* receptor kinase), both of which are glycoproteins. SLG is a secreted protein abundantly expressed in the papillar cell of the stigma surface [2–4], and SRK is a membrane-spanning receptor-like serine/threonine protein kinase whose extracellular domain (termed the S domain) is highly similar to SLG [5,6]. Recent gain-of-function experiments have demonstrated that SRK alone determines *S* haplotype-specificity of the stigma, while SLG enhances the recognition reaction of SI [7]. SRK is thought to function as a receptor for the pollen ligand, which is encoded by a gene also located at the *S* locus. Binding of SRK to the male *S* determinant would elicit a signal transduction pathway involving a kinase cascade in the papillar cell, leading to the rejection of self-pollen.

Unlike the female *S* determinant, the male *S* determinant had long remained elusive. Although several anther-specific genes were found to be located in the flanking regions of *SLG* and/or *SRK*, none of them has turned out to encode the male *S* determinant [8–15]. In an attempt to identify the male *S* determinant, pollen coat proteins (PCPs), which are thought to be involved in pollen–stigma interactions, were extensively characterized. Using a pollination bioassay system, it was found that a pollen coat fraction containing PCP-A1 (PCP-class A1), a small cysteine-rich basic protein which specifically binds SLGs [16,17], could induce an SI response in the stigma in an *S* haplotype-specific manner [18]. This suggests the possibility that the male *S* determinant is a member of the PCP-A1 family.

Recently, sequence analyses of the *S* locus genomic region of *Brassica campestris* containing *SRK* and *SLG* led to the identification of an anther-specific gene, termed *SP11* or *SCR*, which is located in close proximity to the *SRK* gene and encodes a small cysteine-rich basic protein similar to PCP-A1 [13,19]. Hereafter, the term *SP11* will be used to indicate the gene, and *SP11*/*SCR* will be used when also referring to the alleles reported by Schopfer et al. [19]. Two different approaches have been used to show that the *SP11* gene indeed encodes the male *S* determinant. First, using a gain-of-function approach, *Brassica* plants transformed with the *SP11*/*SCR* gene of a new *S* haplotype were found to acquire the new *S* haplotype on the pollen side, but not on the stigma side ([19], H. Shiba, unpublished results). Second, recombinant SP11 protein produced in *Escherichia coli* was used in a pollination assay to show that it could induce the SI response in the stigma in an *S* haplotype-specific manner [20]. Furthermore, the *SP11* gene was found to be expressed in the tapetum of the anther, a site consistent with sporophytic control of *Brassica* SI [20].

To date, six *SP11*/*SCR* alleles have been isolated and characterized in *B. campestris* and *Brassica oleracea* [13,19,20]. Because of the high degree of sequence conservation among

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the signal peptide sequences of these class-I SP11/SCR proteins, we reasoned that other class-I alleles of the *SP11* gene could be amplified by PCR using the primer designed based on the signal peptide region and an oligo-dT primer. Here we report the cloning of 14 novel alleles of *SP11* from *B. campestris* by using the PCR method, and comparison of the deduced amino acid sequences. We also discuss the variability of the *SP11* sequences and co-evolution of the genes encoding the male and female *S* determinants of *Brassica* SI.

2. Materials and methods

2.1. Plant materials

The 24 *S* homozygotes, containing a total of 20 *S* haplotypes, of *B. campestris* used in the present study have previously been reported by Nou et al. [21–23] and Takasaki et al. [24]. The dominance relationships between these *S* haplotypes have been partially determined by Takasaki et al. [24] and Hatakeyama et al. [25].

2.2. Cloning and sequencing of SP11 alleles

Anthers were collected from buds at stage 3 and stage 4, as defined in Suzuki et al. [13]. Isolation of poly(A)$^+$ RNA was performed using a Micro-FactTrack mRNA isolation kit (Invitrogen, San Diego, CA, USA) as described in Suzuki et al. [13]. The RNA samples were reverse-transcribed to synthesize first-strand cDNA by using a First-Strand cDNA synthesis kit (Amersham–Pharmacia, Uppsala, Sweden), and the single-stranded cDNA was used as a template for PCR amplification with a set of primers, SP11-F1 (5′-ATGAAATCNGNTNTTTATGCTTTATTATGTTTC-3′) designed based on the conserved signal peptide coding region of four alleles of *SP11* (S^8, S^9, S^{12} and S^{52}; [13,20]) and *Not*I-d(T)$_{18}$ primer (Amersham–Pharmacia). PCR was performed with *ExTaq* DNA polymerase (TaKaRa Shuzo, Shiga, Japan) for 42 cycles using a DNA thermal cycler, TP3000 (TaKaRa Shuzo), with denaturation for 30 s at 94°C, annealing for 30 s at 45°C and extension for 1 min at 72°C, followed by a final extension for 5 min. The amplified PCR products were cloned into pCR2.1 plasmid vector (Invitrogen). In order to select clones that contained *SP11*, nested PCR was performed with a set of primers, SP11-F2 (5′-TTTATGCTTTATTATGTTTCATATTCATC-3′) designed based on the signal peptide coding region conserved in the same four alleles of *SP11* described above and *Not*I-dT (5′-AACTGGAAGAATTCGCGGCCGCAGGAAT-3′). Inserts in the plasmid vector were sequenced by the dideoxy chain-termination method using model 373S, 377 and 310 DNA sequencer (PE Biosystems, Foster City, CA, USA). More than three independent positive clones were completely sequenced for each *S* haplotype.

2.3. Pulsed-field gel electrophoresis (PFGE)

The megabase DNA embedded in agarose plugs was prepared from young leaf tissue by a rapid method [26,27]. Subsequent digestion with restriction enzymes was performed overnight in the appropriate digestion buffer (TaKaRa Shuzo) supplemented with bovine serum albumin to a final concentration of 0.1 mg/ml. The agarose plugs containing digested DNA were loaded on 1% SeaKem GTG agarose (FMC, Rockland, ME, USA) in 0.5×TBE. The gel was run with a CHEF-DR II apparatus (Bio-Rad, Hercules, CA, USA) at 5.1 V/cm and 14°C with switching times ramped from 30 to 50 s for 24 h. After electrophoresis, the gel was exposed to UV light (60 mJ) with GS Gene Linker UV Chamber (Bio-Rad) and transferred to a nylon membrane overnight by a standard DNA blotting method. Hybridization was carried out in 5×SSC, 0.5% blocking reagent (Roche Diagnostics GmbH, Mannheim, Germany), 0.1% sodium *N*-lauroyl sarcosinate and 0.02% sodium dodecyl sulfate (SDS) at 65°C. After hybridization with digoxigenin (dig)-labeled *SP11* PCR probes, which were amplified by PCR from each *SP11* cDNA clone using a set of primers, SP11-F1 and *Not*I-dT, the filter was washed twice in 0.2×SSC and 0.1% SDS at 65°C for 20 min. The dig-labeled SLG^{45} PCR probe, amplified by PCR from SLG^{45} cDNA [28], was also used for hybridization and, in this case, the washing condition was 0.5×SSC and 0.1% SDS at 65°C. In the case of S^{52} haplotype, the blot was hybridized with the dig-labeled SLG^{52}, SRK^{52}-KD [7,24] and *SP11-52* [20]. The detection of the hybridized probe was carried out as described in Matsuda et al. [29].

2.4. Sequence analysis, sequence comparison and construction of phylogenetic trees

Sequence data were analyzed by DNASIS software (Hitachi Software Engineering, Yokohama, Japan). The deduced amino acid sequences of 14 SP11 proteins were aligned together with six other previously reported sequences. Because the extent of diversity among the sequences is high, the alignment was carried out manually. Among the 20 sequences, cysteine residues appear to be relatively conserved. Therefore, these residues were kept invariable in the alignment. Based on this alignment, the number of amino acid differences for each pair of proteins was calculated. In the calculation, any sites that contain a gap were excluded. The neighbor-joining tree was constructed based on this number of differences [30]. To evaluate clustering patterns, a bootstrap probability for each cluster was calculated with 1000 times resampling. Because of the lack of appropriate outgroup sequences, the root of the tree was determined by the mid-point method.

3. Results

3.1. Amplification of the SP11 gene from 18 different S haplotypes

When we initiated the work to isolate the *SP11* gene from

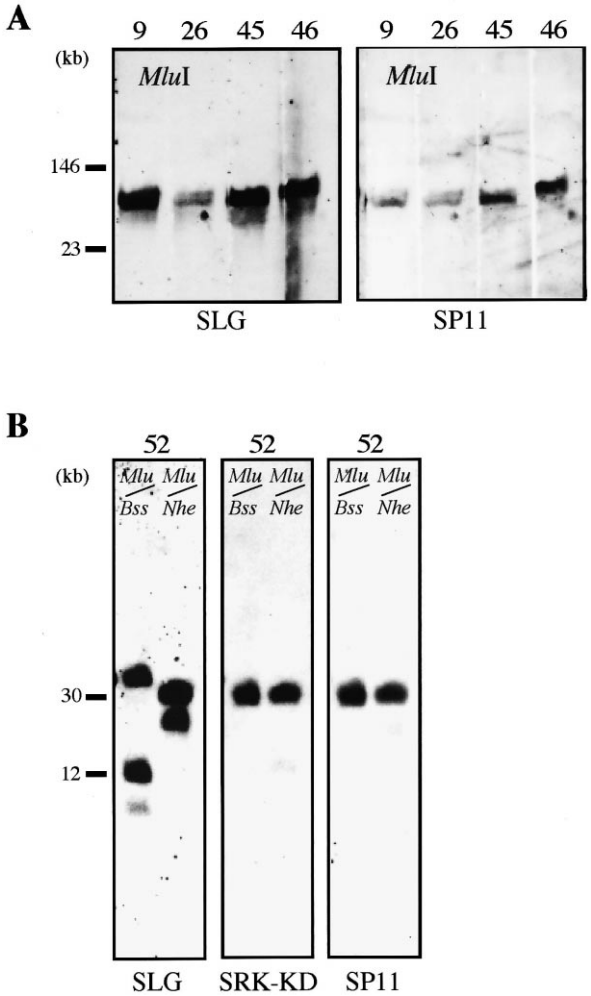

Fig. 1. PFGE gel blot analysis of five *S* haplotypes of *B. campestris*. High molecular weight genomic DNA was digested with *Mlu*I (A), or *Mlu*I along with *Bss*HII or *Nhe*I (B), and separated on a CHEF gel. The DNA was transferred to a membrane filter and hybridized with *SLG*, SRK-KD or *SP11* probes. The numeral above each lane indicates the *S* haplotype number. DNA size markers are shown on the left in kb.

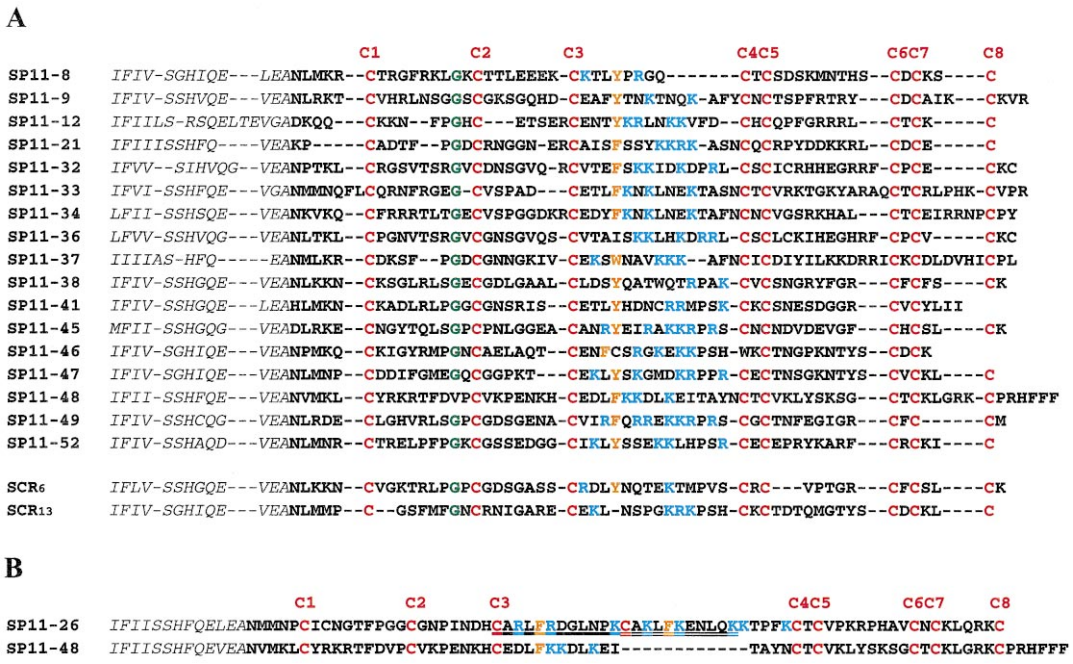

Fig. 2. Multiple alignment of predicted amino acid sequences of 19 SP11/SCR proteins from *Brassica* species (A). Alignment of predicted amino acid sequences of SP11-26 and SP11-48 (B). Gaps (hyphens) were introduced to optimize the manual alignment. The amino acids of the putative signal peptide for each protein are shown in italics. The eight conserved cysteine residues are shown in red letters. The conserved glycine residue between C1 and C2 is shown in green. The yellow and blue letters between C3 and C4 are the conserved aromatic amino acid residue and polar amino acid residues, respectively. The underlined amino acid residues indicate the estimated duplication in SP11-26. All the nucleotide sequences have been deposited in GenBank, EMBL and DDBJ databases (accession numbers AB039754–AB039767).

different *S* haplotypes using reverse transcription-PCR (RT-PCR), the nucleotide sequences of only four alleles of *SP11* were available to us (*SP11* from S^8, S^9, S^{12} and S^{52} haplotypes; [13,20]). These four *S* haplotypes belong to class-I *S* haplotypes based on the classification of *SLG*s/*SRK*s. An oligonucleotide primer (SP11-F1), designed based on the signal peptide region conserved among the four SP11 proteins, was used in conjunction with an oligo-dT primer (*Not*I-d$(T)_{18}$), to amplify other alleles of *SP11* from class-I *S* haplotypes. To ensure specific amplification of *SP11* by this set of primers, RT-PCR was first conducted on anther cDNA of S^8, S^9, S^{12} and S^{52} haplotypes. A single band around 500–600 bp was amplified for each *S* haplotype (data not shown). After the PCR products were cloned, positive clones were selected by PCR using a set of nested primers (SP11-F2 and *Not*I-dT) and sequenced. Without exception, the nucleotide sequences of all the clones obtained from the same *S* haplotype were identical, except for the primer sequence and the length of the 3′ non-coding region. Furthermore, for each *S* haplotype, the sequence of *SP11* obtained was identical to that of previously isolated *SP11* cDNA [13,20], except for the primer sequence and the polyadenylation site. We therefore concluded that the two sets of primers described above could specifically amplify alleles of *SP11*.

We applied this RT-PCR procedure to additional 15 class-I *S* haplotypes (S^{21}, S^{26}, S^{28}, S^{32}, S^{33}, S^{34}, S^{36}, S^{37}, S^{38}, S^{41}, S^{45}, S^{46}, S^{47}, S^{48}, S^{49}). A single band around 400–600 bp was specifically amplified in each *S* haplotype except for the S^{36} haplotype, in which two distinct bands were amplified (data not shown). Again, for each *S* haplotype, the nucleotide sequences of positive clones of *SP11* were identical, except for the primer sequence and the length of the 3′ non-coding region, as was the case for S^8, S^9, S^{12} and S^{52}. The nucleotide sequence of *SP11* from the S^{28} haplotype (strain 11-6j) was completely identical to that from S^9 haplotype; it had previously been reported that these two strains were incompatible with each other, and had the same *S* haplotype [23]. Furthermore, we used two different strains for S^{34} (9-1j and S-5j) and three different strains for S^{45} (11-12j, 19-5j and 29-5j), and we did not detect any sequence difference in either the coding or 3′ non-coding region between different strains of the same *S* haplotype.

We also attempted to amplify *SP11* from S^{29} and S^{60} haplotypes, which are classified as class-II *S* haplotypes [24,31]. However, no band was amplified, even when re-amplification was performed with a set of nested primers, SP11-F2 and *Not*I-dT (data not shown).

Table 1
Restriction fragments identified by PFGE gel blot analysis of the 12 *S* haplotypes

S haplotype	Length of hybridized band[a]	
	SLG^{45} probe	*SP11* probe
S^9	76	76
S^{21}	102	102
S^{25}	104	104
S^{26}	78	78
S^{33}	68	68
S^{34} (S-5j)	84	84
S^{37}	78	78
S^{41}	102	102
S^{45} (11-12j)	75	75
S^{46}	90	90
S^{47}	68	68
S^{48}	60	60

The code number in brackets shows the strain number.
[a]Numbers indicate the length of restriction fragments (kb).

3.2. Physical location of SP11 at the S locus

Physical linkage of the amplified *SP11* gene to the *S* locus was examined in 12 *S* haplotypes by gel blot analysis, following separation of DNA by PFGE. Dig-labeled SLG^{45} PCR probe used in this analysis can detect both class-I *SLG* and/or *SRK* [28,31]. To detect *SP11* that is highly polymorphic between different *S* haplotypes, each amplified *SP11* clone was used as a probe to hybridize with the genomic DNA digest of the same *S* haplotypes. In the case of digestion with *Mlu*I, both the SLG^{45} and *SP11* probes detected a single band of 60–104 kb in all *S* haplotypes examined except S^{52} (Fig. 1A and Table 1). Thus, we concluded that *SLG*/*SRK* and *SP11* are clustered within a 60 kb to a 104 kb *S* locus region in these 11 *S* haplotypes.

In the case of S^{52} haplotype, the *SLG* probe hybridized with two different ca. 50 kb fragments that contained SLG^{52} and SRK^{52}, respectively (data not shown). Because the SLG^{52} *Mlu*I fragment and the SRK^{52} *Mlu*I fragment were of similar size, we used double digestion with *Mlu*I and *Bss*HII, or with *Mlu*I and *Nhe*I, to determine the linkage between SLG^{52}, SRK^{52} and *SP11-52*. SRK^{52}-KD probe, detecting the kinase domain region of SRK^{52}, hybridized with a 30 kb *Mlu*I/*Nhe*I fragment, which also hybridized with the *SP11-52* probe (Fig. 1B). Moreover, the 30 kb *Mlu*I/*Bss*HII fragment, which hybridized with both SRK^{52}-KD and *SP11-52* probes, did not hybridize with SLG^{52} probe. These results suggest that both *SP11-52* and the region encoding the kinase domain of SRK^{52} are located on the same 30 kb *Mlu*I/*Bss*HII fragment, but the S domain region of SRK^{52} is not (Fig. 1B). Therefore, *SP11-52* is located within 30 kb downstream of SRK^{52}.

All these results suggest that *SP11*, *SLG* and *SRK* are tightly linked in the *S* locus region. Based on the fact that the PCR products were amplified using a set of specific primers for *SP11*, and that the gene in each *S* haplotype that hybridized to the PCR product obtained from the same haplotype was physically linked to the *S* locus, we concluded that the amplified PCR products must correspond to different alleles of *SP11*.

3.3. Primary structural features of SP11

In order to identify the primary structural features of SP11, the predicted amino acid sequences of all the amplified *SP11* clones, except for 11 amino acid residues derived from the primer sequence (SP11-F1), were aligned with those of previously reported *SP11*/*SCR* alleles [13,19,20] (Fig. 2). The amino acid sequence of the signal peptide is more highly conserved than that of the mature protein. Eight cysteine residues are conserved in the mature protein of all SP11s except SP11-41 and SP11-46. For both SP11-41 and SP11-46, the eighth (C8) cysteine residue is absent, and for SP11-46, the fourth (C4) cysteine is replaced with tryptophan. The number of amino acid residues between most pairs of adjacent conserved cysteine residues is variable. The exceptions are C4–C5 and C6–C7: a single amino acid is located between these two pairs of cysteines in all SP11s. In the region between C1 and C2, a glycine residue is conserved among all SP11s except SP11-48. In the region between C3 and C4, one aromatic amino acid residue is conserved among all SP11s except SP11-36 and SCR_{13}. Furthermore, more than two polar amino acid residues (lysine or arginine) are located in the region between C3 and C4 of all SP11s except SP11-8 and SCR_{13}. These common primary structural features would be important for the tertiary structure of SP11. The estimated p*I* value of SP11 varies from 7.8 to 9.3. The estimated molecular mass of SP11 varies from 5.7 kDa to 8.0 kDa. The potential *N*-glycosylation sites are not conserved in all SP11s. SP11-26 contains the largest number of amino acid residues, due to duplication of 11 amino acid residues in the C3–C4 region (Fig. 2B). Pairwise identities of the amino acid sequences calculated from the alignment range from approximately 30% to 55%, except that SP11-32 and SP11-36 show 74% identity.

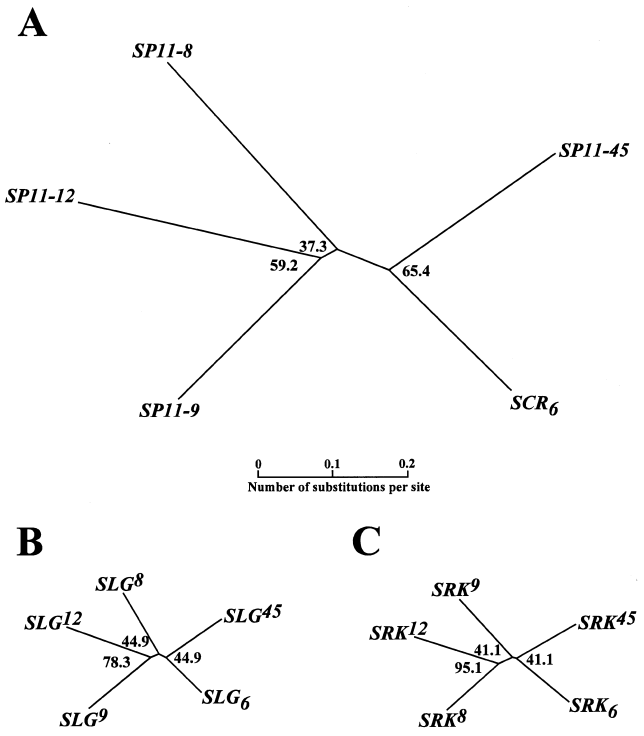

Fig. 3. Neighbor-joining phylogenetic tree of *SP11*/*SCR* (A), *SLG* (B) and *SRK* (C). Bootstrap probabilities for clusters are shown as percentages. The bar under the tree shown in (A) represents the number of amino acid substitutions per site. GenBank accession numbers: SLG_6, Y00268; SRK_6, M76647; SCR_6, AF195625; SLG^8, X55274; SRK^8, D38563; *SP11-8*, AB035504; SLG^9, D30050; SRK^9, D30049; *SP11-9*, AB022078; SLG^{12}, D84505; SRK^{12}, D38564; *SP11-12*, AB035503; SLG^{45}, AB012105; SRK^{45}, AB012106; *SP11-45*, AB039763.

3.4. Evolutionary aspects of male and female determinants of SI

In order to understand the evolutionary relationship between the male and female determinants of *Brassica* SI, separate phylogenetic trees were constructed using the sequences of mature SP11/SCR and the S domain of SRK, as well as SLG, from five *S* haplotypes (*B. campestris*: S^8, S^9, S^{12} and S^{45}; *B. oleracea*: S^6) whose sequences are all available. The clustering patterns of the three trees are similar (Fig. 3). For example, the S^6 haplotype always clusters with the S^{45} haplotype, albeit the supporting bootstrap probabilities are not always significant. As evident from Fig. 3, the lengths of external branches in the SP11/SCR tree are approximately three times those in other trees. Since the number of amino acids compared in SP11/SCR (38) is much smaller than in SRK (393) and SLG (395), standard errors could be larger. However, this difference is significant ($0.05 > P > 0.001$) and might

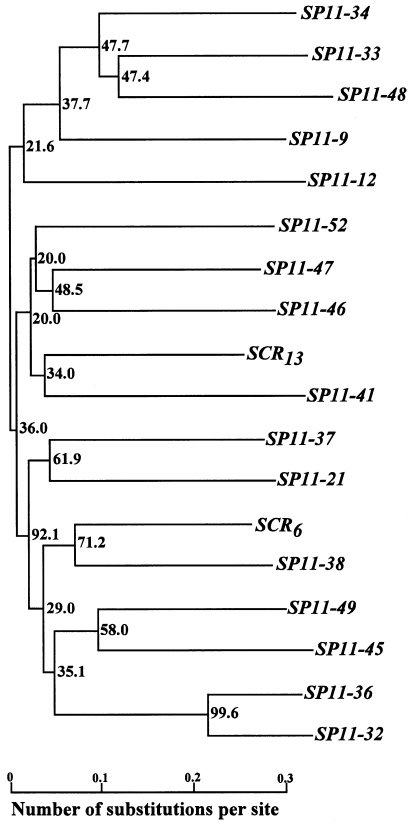

Fig. 4. Neighbor-joining phylogenetic tree of 18 alleles of *SP11*/*SCR* of *Brassica* species. Bootstrap probabilities for clusters are shown as percentages. The bar under the tree represents the number of amino acid substitutions per site.

indicate a diversifying selection operating on *SP11*. There might be acceleration of non-synonymous substitutions in *SP11* by selection. Or this might be due to relaxation of functional constraints on this gene. To distinguish between these two possibilities, dn/ds (non-synonymous differences per site/synonymous differences per site) ratios were calculated. This ratio for a particular pair of alleles of *SP11*/*SCR* showed that it (1.96) is significantly higher than one ($P < 0.05$). However, this is not the case for all pairs of alleles, as the ratio for distantly related pairs is less than one (0.68).

Fig. 4 shows the phylogenetic relationship among 18 alleles of *SP11*/*SCR*. SP11-8 and SP11-26 were not included in the tree construction, because their sequences are either shorter or longer than the others and their inclusion would require introduction of many gaps in the alignment. Although the bootstrap probability of each node is not significantly high, three important features were revealed. First, the *trans*-specific mode of polymorphism is apparent as in the case of *SRK* and *SLG* [32]. Although we do not know the pattern of polymorphism at other non-*S* loci, this *trans*-specific mode suggests operation of balancing selection. Second, the branching pattern of *SP11*/*SCR* is similar to that of *SLG* and *SRK* [33], suggesting that each lineage accumulates specific changes. Third, the molecular clock seems to be kept in *SP11*/*SCR*, though we do not have an appropriate outgroup sequence, by and large external branch lengths are similar to each other.

4. Discussion

We have shown in this report that RT-PCR amplification with *SP11*-specific primers (SP11-F1, nested SP11-F2 and oligo-dT) can be used to isolate the male *S* determinant gene, *SP11*, of class-I *S* haplotypes of *B. campestris*. In the case of S^{36} haplotype, although two distinct bands were observed, no sequence variability was observed among 23 *SP11-36* clones examined. This might be due to exclusion of false positive clones by nested PCR with a set of primers (SP11-F2 and *Not*I-dT). Because of the high degree of sequence conservation in the signal peptide coding region of the *SP11*/*SCR* gene of different species within the genus *Brassica*, the method of amplification employed in this work should be applicable to class-I *S* haplotypes of other *Brassica* species as well. However, since we failed to use these primers to amplify *SP11* from two class-II *S* haplotypes (S^{29} and S^{60}), the nucleotide sequences of *SP11* alleles from class-I *S* haplotypes are likely to be highly divergent from those of class-II *S* haplotypes. This is consistent with the results from DNA gel blot analysis [19]. The classification of *S* haplotypes, class I and class II, is related to the dominant/recessive SI phenotype in the pollen without exception [25,34]. The relationship of the classes and SI phenotype cannot be explained without hypothesis of co-evolution of male *S* gene (*SP11*/*SCR*) and female *S* gene (*SRK*), because the definition of the classes is due to the dominant/recessive relationship in the pollen and the sequence difference of the *SRK*s [34], which determine the stigma SI phenotype and not the pollen phenotype [7]. From our finding indicating the high sequence difference of *SP11* between the class-I and class-II *S* haplotypes, we conclude that the sequence difference of *SP11*/*SCR*s between two classes affects the dominance/recessive SI phenotype of the pollen, and the sequence difference of *SRK*s between the classes is merely the result of the co-evolution between male (*SP11*/*SCR*) and female (*SRK*) *S* determinants.

We used PFGE analysis to confirm the physical linkage of the amplified *SP11* alleles to *SRK* and *SLG*. The results show that *SP11* is located in the vicinity of *SLG* and *SRK* in all 12 *S* haplotypes examined. This tight linkage between the male and female SI genes is undoubtedly important for maintaining the SI system and allowing their co-evolution.

For three of the *S* haplotypes studied (S^9, S^{34} and S^{45}), we cloned and sequenced *SP11* from two or three different strains. Both the coding and 3′ non-coding regions of *SP11* are completely identical between different strains of the same *S* haplotype. Previously, the sequences of *SLG* from three strains of S^{24} haplotype of *B. campestris* were found to be completely identical in both the coding and 3′ non-coding regions [35]. In contrast, for two *S* haplotypes of *B. oleracea*, the coding sequences of *SLG* and *SRK* from one strain are not completely identical to their corresponding sequences of another strain [36]. To confirm whether the complete conservation of *SP11* is necessary for expression of *S* haplotype-specificity in *Brassica* species, it would be necessary to compare more sequences of *SP11* from *B. campestris* or *B. oleracea* plants of different origins but having the same *S* haplotype.

Except for some completely or mostly conserved amino acid residues, most of the amino acid residues of SP11 are quite variable. It would be of interest to identify which of the variable amino acid residues in SP11 are important for *S* haplo-

type-specificity. Having information on the tertiary structure of the protein would help this identification.

The topology of the phylogenetic tree of *SP11/SCR* is very similar to that of *SLG* and the S domain of *SRK*, except that the branching pattern in *SP11-8*, *SP11-9* and *SP11-12* is slightly different from that of the S domain of *SRK*. Thus, it appears that the three SI-related genes have similar evolutionary histories. That is, *SP11* appears to have co-evolved with *SRK* and *SLG*. To the best of our knowledge, this is the first evidence of co-evolution between plant genes.

In order to determine more precisely whether *SP11* has co-evolved with *SRK* or *SLG*, it would be necessary to confirm whether the interaction between SP11 and SRK elicits SI signal transduction, and to identify which regions of SRK and SLG interact with SP11. Such information on ligand–receptor binding would likely reveal why the branch length of *SP11/SCR* is about three times that of *SLG* and *SRK*.

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