Coevolution of the *S*-Locus Genes *SRK*, *SLG* and *SP11/SCR* in *Brassica oleracea* and *B. rapa*

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ABSTRACT

Brassica self-incompatibility (SI) is controlled by *SLG* and *SRK* expressed in the stigma and by *SP11/SCR* expressed in the anther. We determined the sequences of the S domains of 36 *SRK* alleles, 13 *SLG* alleles, and 14 *SP11* alleles from *Brassica oleracea* and *B. rapa*. We found three *S* haplotypes lacking *SLG* genes in *B. rapa*, confirming that *SLG* is not essential for the SI recognition system. Together with reported sequences, the nucleotide diversities per synonymous and nonsynonymous site (π_S and π_N) at the *SRK*, *SLG*, and *SP11* loci within *B. oleracea* were computed. The ratios of π_N:π_S for *SP11* and the hypervariable region of *SRK* were significantly >1, suggesting operation of diversifying selection to maintain the diversity of these regions. In the phylogenetic trees of 12 *SP11* sequences and their linked *SRK* alleles, the tree topology was not significantly different between *SP11* and *SRK*, suggesting a tight linkage of male and female SI determinants during the evolutionary course of these haplotypes. Genetic exchanges between *SLG* and *SRK* seem to be frequent; three such recent exchanges were detected. The evolution of *S* haplotypes and the effect of gene conversion on self-incompatibility are discussed.

SELF-INCOMPATIBILITY (SI) in Brassica is controlled by a set of closely linked genes at the *S* locus, called the *S* haplotype. These genes have multiple alleles and are expressed either in the stigma or in the pollen. Stigma cells inhibit pollen tube growth to prevent self-fertilization when the expressed *S* specificity of the pollen matches that of the stigma. In Brassica, self-recognition specificity of the pollen is determined sporophytically. It depends on the *S* haplotype of the pollen parent rather than on that of the pollen grain itself. About 50 *S* haplotypes in *Brassica oleracea* (Ockendon 2000) and 30 in *B. rapa* (Nou *et al.* 1993) have been identified so far.

The first *S*-locus gene to be isolated, *SLG* (*S*-locus glycoprotein), encodes a secreted protein, which localizes in the wall of stigma papillar cells (Nasrallah *et al.* 1988). The *SLG* alleles are classified into two groups, class I and class II, on the basis of their nucleotide sequences (Nasrallah *et al.* 1991). Subsequently, the *SRK* gene (*S*-locus receptor kinase) was isolated (Stein *et al.* 1991). SRK is a membrane protein consisting of an extracellular domain (S domain), which is similar in sequence to SLG, a single-pass transmembrane domain, and a cytoplasmic domain with protein kinase activity. The coding region of *SRK* is 2.6 kb in length and is partitioned by six introns. Loss of the function of SRK was found to result in a breakdown of SI (Goring *et al.* 1993; Nasrallah *et al.* 1994). S domain sequences of *SRK* alleles are similar to *SLG* sequences in the same class (Cabrillac *et al.* 1999). Class II *S* haplotypes show a pollen-recessive phenotype. Introduction of the *SRK* gene alone was found to confer a new *S*-haplotype specificity in the stigma (Takasaki *et al.* 2000). Consequently, SRK is considered to be an indispensable factor in the stigma for both SI recognition and response leading to the rejection of self-pollen. The determinant of *S*-haplotype specificity in pollen has recently been identified by two groups of researchers. This gene has been called *S*-locus protein 11 (*SP11*) by Suzuki *et al.* (1999) and *S*-locus cysteine-rich protein (*SCR*) by Schopfer *et al.* (1999). The deduced amino acid sequences of *SP11* in *B. rapa* have been shown to be highly divergent except for the presence of conserved cysteine residues (Wata-

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NABE *et al.* 2000). It is considered that recognition of SP11 by SRK results in inhibition of self-pollen germination and pollen tube growth.

KUSABA *et al.* (1997) sequenced >30 *SLG* alleles from *B. oleracea* and *B. rapa* and subjected them to phylogenetic analysis with other reported sequences. They found a high extent of intraspecific variation and interspecific similarity between *SLG* alleles and confirmed that the divergence of *SLG* alleles predates the speciation of *B. oleracea* and *B. rapa*. They also demonstrated that recombination or gene conversion plays a major role in the course of the evolution of *SLG* genes. This observation was confirmed by a further study by AWADALLA and CHARLESWORTH (1999).

KUSABA *et al.* (1997) also observed a striking sequence similarity (97.5% identity in their amino acid sequences) between *SLG* genes from *S-8* and *S-46* haplotypes in *B. rapa*. However, the S domains of the *SRK* genes in these haplotypes are not as similar to each other (85.6% amino acid identity) as are the *SLG* genes, suggesting that *SLG* is not essential for self-recognition (KUSABA and NISHIO 1999). Further evidence suggests that *SLG* is not crucial for the SI phenotype: a nonsense mutation and frameshift that eliminated function were found in *SLG-18* and *SLG-60* in *B. oleracea* (SUZUKI *et al.* 2000). Furthermore, the *SLG* gene in *B. oleracea S-24* appears to have been deleted, having not been detected in a DNA gel-blot analysis (OKAZAKI *et al.* 1999). On the other hand, transgenic studies have shown that the function of SLG is to intensify the strength of SI (TAKASAKI *et al.* 2000).

In this article, we report the following new sequences: the S domain sequences of 21 *BoSRK* alleles (*SRK* in *B. oleracea*), 15 *BrSRK* alleles (*SRK* in *B. rapa*), 14 *BoSP11* alleles (*SP11* in *B. oleracea*), 11 *BoSLG* alleles (*SLG* in *B. oleracea*), and 2 *BrSLG* alleles (*SLG* in *B. rapa*). Together with previously reported sequences of *SLG*, *SRK*, and *SP11* (NASRALLAH *et al.* 1988; STEIN *et al.* 1991; KUSABA *et al.* 1997; SCHOPFER *et al.* 1999; SUZUKI *et al.* 1999; TAKAYAMA *et al.* 2000; WATANABE *et al.* 2000), a total of 25 *BoSRK*, 34 *BoSLG*, 19 *BoSP11*, 18 *BrSRK*, 21 *BrSLG*, and 18 *BrSP11* alleles were used for the analysis. This data set includes the majority of *S* haplotypes known in *B. oleracea* and *B. rapa*. We herein discuss the mode of evolution of *S* haplotypes, focusing in particular on the coevolution of male and female SI determinants and the molecular mechanisms affecting the diversity of SI genes.

MATERIALS AND METHODS

DNA sources and sequencing: Forty-five *S* homozygous lines of *B. oleracea* L. provided by D. Astley (Horticulture Research International, Warwick, UK) and 15 homozygous lines of *B. rapa* L. maintained at Tohoku University were used as plant materials. *SLG* and the *SRK* alleles that have a short first intron were amplified by PCR using genomic DNA as a template. *SP11* and those *SRK* alleles having a long first intron were amplified by RT-PCR from anther and stigma RNA, respectively. Genomic DNA was prepared from young leaves by the method of ROGERS and BENDICH (1985), and RNA was extracted from stigmas and anthers of the *S* homozygotes using Isogen (Nippongene) or Micro-Fast track mRNA isolation kit (Invitrogen, San Diego). The primers used for the amplification of *SLG* were the pair PS5 (5′-ATGAAAGGCGTAAGAAA AACCTA-3′) and PS15 (5′-CCGTGTTTTATTTTAAGAGAAAG AGCT-3′; NISHIO *et al.* 1996) or the pair PS22 (5′-ATCGATG GGATGAAAAAGTCATCG-3′; SAKAMOTO *et al.* 1998) and PS15. The primers for amplification of the S domain of *SRK* were the pair PK7 (5′-ATGCAAGGTGTACGATACATCTATCA TCATTCTTAC-3′) and PK8 (5′-GATCAGAAGAAGCAGAACAG TAACTCCAACAGTC-3′) or the pair of PK7 and PK9 (5′-CCT TGTCCGAGTTTGTTACAGTTGGAGAAATTTTCGG-3′; NISHIO *et al.* 1997). The conditions for PCR and the cloning of the PCR products have been reported previously (NISHIO *et al.* 1997). For RT-PCR of the S domain of *SRK*, the first strand cDNA was synthesized with SuperScriptII RT (GIBCO BRL, Gaithersburg, MD) using the primer PK8 or PK9, and PCR amplification was carried out under the same conditions as for genomic DNA using the pairs of primers PK7 + PK8 and PK7 + PK9. For the amplification of *SP11*, the primer pair of pSP11-1 (ATGAAATCTGCTATTTATGCTTTATTATG) and *Not*I(dT)18 (Amersham Pharmacia Biotec) was used for the first cycle of RT-PCR, and the pair pSP11-2 (TTCATATTCA TCGTTTCAAGTC) and RT-1 (ACTGGAAGAATTCGCGGC) was used in the second cycle.

The nucleotide sequences of the PCR products were determined with PRISM377 (Perkin-Elmer ABI). To eliminate errors that may have occurred during the PCR process, three independent clones obtained from the same plant were sequenced. The DNA sequence data were analyzed with the Genetyx version 10 program (Software Development, Tokyo).

DNA blot analysis: DNA gel blotting was performed as described by OKAZAKI *et al.* (1999) except for the probe and the washing conditions. Two micrograms of genomic DNA was digested with *Eco*RI or *Hin*dIII, electrophoresed on agarose gel, and transferred to a nylon membrane. The membranes were hybridized with a mixture of S domain probes of *BrSRK-32*, *BrSRK-33*, and *BrSRK-36*, which were amplified from plasmids by PCR. After hybridization, the membrane was washed twice in 0.5× SSC, 0.1% SDS at 65° for 20 min.

Phylogenetic analysis: Sequences were aligned by using CLUSTAL W (THOMPSON *et al.* 1994) and modified manually. Synonymous and nonsynonymous sites and differences were counted by using an algorithm of the modified Nei and Gojobori method (NEI and KUMAR 2000) with $R = 1$. Phylogenetic analyses were performed by using programs of Neighbor, DNAML (PHYLIP version 3.5; FELSENSTEIN 1993), PROTML (MOLPHY version 3.2; ADACHI and HASEGAWA 1994), and PAML (YANG 2000).

RESULTS

Deletion of the *SLG* gene in some *S* haplotypes: Only one band was detected in the DNA blot analysis of *Hin*dIII and *Eco*RI fragments of *BrS-36*, *BrS-32*, and *BrS-33* homozygotes with the bulked *SRK* probe (Figure 1), while two bands, corresponding to *SRK* and *SLG*, have been found in most other *S* haplotypes (OKAZAKI *et al.* 1999). Since these three haplotypes maintain distinct SI specificities, *SLG* is unlikely to play any essential role in SI recognition (KUSABA and NISHIO 1999; OKAZAKI *et al.* 1999; SUZUKI *et al.* 2000).

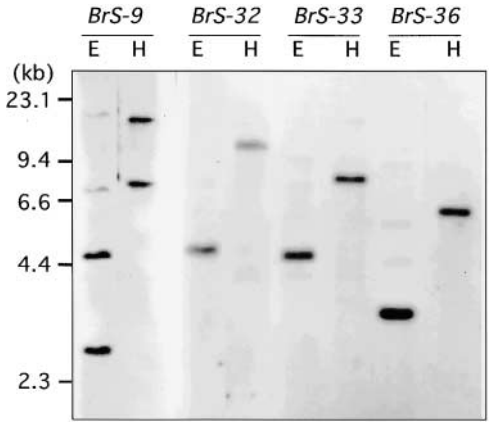

FIGURE 1.—DNA blot analysis of genomic DNA isolated from *BrS-9*, *BrS-32*, *BrS-33*, and *BrS-36* homozygotes. E, *Eco*RI; H, *Hin*dIII. DNA sizes are shown on the left.

The *BrSRK-36* had extremely high similarity to *BoSRK-24* [93.5% identity in amino acids (aa)]. The hypervariable regions (HVRs), which are considered to be important for recognition specificity of SLG and SRK (KUSABA *et al.* 1997; NISHIO and KUSABA 2000), were identical except for two amino acid residues, and the *SP11* sequences were also similar (96.8% identity in aa). Additionally, the *BoS-24* haplotype lacks the *SLG* gene (OKAZAKI *et al.* 1999). Therefore, *BoS-24* and *BrS-36* are likely to have been derived from a single ancestral haplotype and the *SLG* gene in the haplotype was probably deleted in the common ancestor of *B. oleracea* and *B. rapa*. The *BrS-32* homozygote also showed only one band, suggesting the deletion of *SLG*. Since *BrS-32* has an *SRK* sequence similar to *BrSRK-36* (88.5% identity in aa), *BrS-32* most likely originated from the same ancestor as *BoS-24* and *BrS-36*. However, this DNA blot analysis indicated that *BrS-33*, which is distantly related to *BrS-32*, *BrS-36*, and *BoS-24* in their *SRK* sequences, has also lost the *SLG* gene. This implies that the *SLG*-deletion event occurred independently at least twice.

Sequence diversity of *SRK*, *SLG*, and *SP11* in each species: The average proportion of identical amino acids per site among all pairwise comparisons of class I *S*-locus sequences was 80% in 28 *BoSRK*s, 79% in 21 *BrSRK*s, 82% in 34 *BoSLG*s, and 79% in 21 *BrSLG*s. In *SLG* sequences, the highest extent of amino acid sequence identity was 99.5% between *BoSLG-23* and *BoSLG-29* in *B. oleracea* and 98.2% between *BrSLG-43* and *BrSLG-46* in *B. rapa*. Likewise, the highest similarity in the S domain of *SRK* sequences was 89.9% in *B. oleracea* (*BoSRK-23* and *BoSRK-29*) and 88.5% in *B. rapa* (*BrSRK-32* and *BrSRK-36*). KUSABA and NISHIO (1999) showed that the high extent of similarity between some *SLG* genes was due to homogenization by genetic exchanges among alleles. To investigate whether or not a similar number of exchanges occurred among *SRK* alleles, we applied the method of KUSABA *et al.* (1997)—in which topologies of neighbor-joining (NJ) trees using nucleotide sequences of the hypervariable regions I, II, III, and the C-terminal variable region were compared—to all available *SRK* sequences from *B. oleracea* and *B. rapa*. However, no evidence of frequent recombination was detected among *SRK* alleles. In contrast to the relatively high extent of homology among *SRK* or *SLG* amino acid sequences, *SP11* sequences, responsible for the *S* specificity of the pollen, showed extraordinary diversity. As shown in Figure 2, although six cysteine residues are conserved among the sequences, the remaining residues show extensive variation, including frequent insertions and deletions (indels). Since the inclusion of many indels reduces the number of comparable amino acid sites, we selected six sequences (*BoSP11-7*, *-18*, *-24*, *-29*, *-39*, and *-64*) whose alignment requires a relatively small number of indels. The highest amino acid identity among the six sequences was 41.2%, between *BoSP11-39* and *BoSP11-64*.

The nucleotide diversities per synonymous and nonsynonymous site (π_S and π_N) at the *SRK*, *SLG*, and *SP11* loci within *B. oleracea* were computed. The values π_S and π_N are the average numbers of synonymous or nonsynonymous nucleotide differences per site between two randomly chosen sequences. Because the extent of sequence difference of *SLG* and *SRK* at both the amino acid and nucleotide levels varies along the coding region (HINATA *et al.* 1995), the sequence was divided into two subregions: the HVR and the remaining conserved region (CR), as designated by KUSABA *et al.* (1997). Table 1 shows π_S and π_N in the HVR, CR, and entire gene (ALL) of the *SRK* and *SLG* loci separately. For *SP11*, since the number of comparable sites is small, the division of the sequence into subregions is not useful. Therefore, we calculated π_S and π_N for the entire region only. The ratio (γ) of π_N:π_S for each region is also shown (Table 1).

To estimate the γ-value for each region, we used the modified Nei and Gojobori method (NEI and KUMAR 2000). To calculate the parameter R in the modified method, where R is defined as $\alpha/2\beta$, we estimated the ratio of transitional (α) to transversional (β) substitutions by maximum-likelihood methods by using the PAML (YANG 2000). We estimated the ratio, α/β, under the maximum-likelihood topology, using the sites at the third codon positions of the entire gene. The ratio, α/β, in *SRK* was almost 2 and that in *SP11* was 1.5. Because the total number of the third codon positions is small in *SP11*, we decided to use the ratio 2, setting R equal to 1. We also applied the unbiased Nei and Gojoboris method to our data. This actually reduced the γ-value, but the tendency did not change.

It is clear that π_N in HVR is significantly greater than that in CR for both *SRK* and *SLG*, and the γ-value in HVR of *SRK* and *SLG* and in *SP11* exceeds unity. Under the neutral theory of molecular evolution (KIMURA 1968, 1983), γ of a particular gene depends on the strength of functional constraints imposed on the prod-

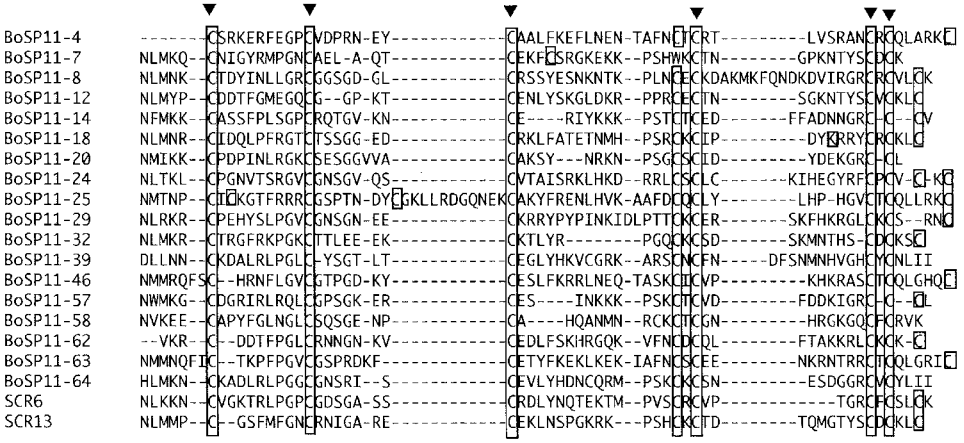

FIGURE 2.—Multiple sequence alignment of SP11 amino acid sequences in *B. oleracea*. Cysteine residues are in boxes, and conserved cysteine residues are indicated by solid triangles.

uct of the gene. However, γ does not exceed one, unless mutations are selectively advantageous. In other words, if γ in a gene or a part of a gene is significantly larger than one, this indicates an operation of balancing selection or Darwinian (positive natural) selection in these regions.

To examine whether or not these γ-values are significantly larger than unity, we calculated $D = \pi_N - \pi_S$, computed the variance of D by bootstrap samplings with 1000 replications, and applied the Z test (NEI and KUMAR 2000, p. 55). The result showed that D was significantly larger than zero in *SP11* and in the HVRs of both *SRK* and *SLG* ($P < 0.01$, Table 1). The observation of $\gamma > 1$ suggests the operation of Darwinian selection or balancing selection at *SP11* and at the HVRs of *SRK* and *SLG*. A similar result was obtained for *B. rapa*.

Phylogenetic analysis of *SRK* and *SP11*: To examine whether or not the linkage between *SP11* and *SRK* is tight, we compared phylogenetic relationships of these genes. For both *B. oleracea* and *B. rapa*, nucleotide sequences from the two loci in 26 different haplotypes (13 for each species) are available. However, as mentioned, reliable alignment among all available *SP11* amino acid or nucleotide sequences is difficult to achieve due to the large number of indels. We therefore used six *BoSP11* alleles for further phylogenetic analysis (Table 1, Figure 2). In addition to these six *BoSP11* alleles, we chose six other alleles from *B. rapa* (*BrSP11-36*, *SP11-45*, *SP11-49*, *SP11-47*, *SP11-41*, *SP11-46*), which are seemingly closely related to *BoSP11* and therefore can be aligned with each other with a relatively small number of indels (data not shown).

TABLE 1

The average number of synonymous (π_S) and nonsynonymous (π_N) differences per site of *SRK*, *SLG*, and *SP11* alleles in *B. oleracea*

Genes[a]	Regions	π_S	π_N	π_N/π_S
BoSRK (28)	HVR	0.219 (41)	0.299 (130)	1.37[b]
	CR	0.173 (232)	0.071 (719)	0.41
	All	0.181 (280)	0.110 (872)	0.61
BoSLG (34)	HVR	0.226 (42)	0.292 (129)	1.29[c]
	CR	0.158 (230)	0.061 (715)	0.39
	All	0.168 (280)	0.099 (866)	0.59
BoSP11 (6)	All	0.321 (35)	0.505 (115)	1.57[b]

[a] The numbers of sites are in parentheses.

[b] The ratio is greater than unity with significance at the 0.1% level.

[c] The ratio is greater than unity with significance at the 1% level.

Figure 3A shows the NJ tree (SAITOU and NEI 1987) estimated on the basis of both synonymous and nonsynonymous differences among 12 *SP11* sequences. It is clear that six major lineages are among them: lineage I (*BoSP11-24*, *BrSP11-36*), lineage II (*BrSP11-45*, *BrSP11-49*), lineage III (*BoSP11-64*, *BrSP11-41*, *BoSP11-39*), lineage IV (*BoSP11-7*, *BrSP11-46*, *BrSP11-47*), lineage V (*BoSP11-29*), and lineage VI (*BoSP11-18*). Lineages I–IV have significant bootstrap support ($P > 0.99$). This pattern did not change even when we used synonymous or nonsynonymous differences separately (Figure 3, B and C), although the bootstrap probabilities for the synonymous tree became a bit smaller. The relationship among the six lineages was not resolved at the root of the tree. This low resolution at the root might be due to either the small number of compared sites or the possible large number of recurrent substitutions or frequent recombination/gene conversions.

Phylogenetic trees of the 12 *SP11* alleles and their linked *SRK* alleles were constructed separately by using the deduced amino acid sequences. First, maximum-likelihood analysis (PROTML in the MOLPHY version 2.3; ADACHI and HASEGAWA 1994) was applied (Figure 4). The topologies of these *SP11* and *SRK* trees were a little different from each other. In the following, we call the tree topology of *SRK* genes Tree 1 and that of *SP11* genes Tree 2 (Figure 4). To test the significance of topological differences between the two trees, likelihood values were calculated for both genes. Because the topology of the best tree, which shows the maximum-likelihood value among different topologies, was differ-

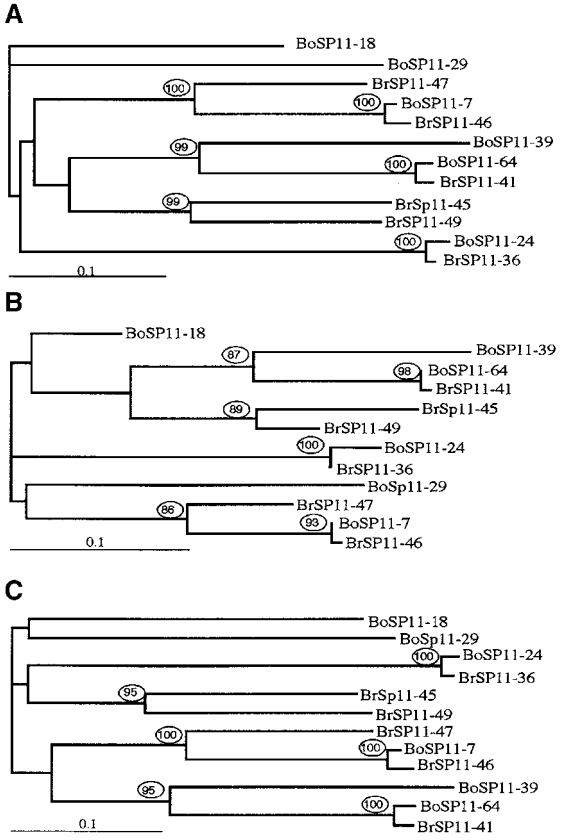

FIGURE 3.—Neighbor-joining tree of selected *SP11* sequences. One thousand bootstrap trials were performed and the bootstrap values are shown in ovals. The scale shows the number of nucleotide differences per site. The reason for the selection of *SP11* sequences is shown in the text. (A) NJ tree based on all nucleotide changes. (B) NJ tree based on synonymous nucleotide changes. (C) NJ tree based on nonsynonymous nucleotide changes.

ent between *SRK* and *SP11*, we also calculated the likelihood value of the *SRK* gene tree under the assumption of the Tree 2 topology and vice versa. For each tree, the difference of the likelihood values between the best and alternative trees and its standard error were calculated (KISHINO and HASEGAWA 1989; Table 2). There

TABLE 2

Comparison of likelihood values of different gene trees between *SRK* and *SP11*

	SRK		*SP11*	
Tree[a]	ln *L*	Difference in ln *L*	ln *L*	Difference in ln *L*
1	−3585.5	Best	−1205.8	−8.5 ± 6.8
2	−3572.6	−12.9 ± 11.2	−1197.3	Best

Likelihood values were calculated using the JTT matrix model. The substitution model, however, is adjusted so that the equilibrium frequencies are the data frequencies.

[a] The topology of Trees 1 and 2 are Tree 1: ((((*BoS-7,BrS-46*), *BrS-47*),(((*BoS-24,BrS-36*),*BoS-29*),((*BoS-39,*(*BoS-64,BrS-41*)), (*BrS-45,BrS-49*)))),*BoS-18*); Tree 2: (((((*BoS-7,BrS-46*),*BrS-47*), (*BoS-39,*(*BoS-64,BrS-41*))),*BoS-18*),(*BoS-29,*((*BoS-24, BrS-36*), (*BrS-45,BrS-49*)))).

were no significant differences between the maximum-likelihood values of the two topologies for both the *SP11* and *SRK* sequences, and therefore the hypothesis that the topologies of phylogenetic trees supported by these two loci were the same was not rejected. To address whether heterogeneity in amino acid substitution rate affects the conclusion, we carried out a similar analysis using PAML (YANG 2000). A gamma distribution was used for the rate heterogeneity model and the shape parameter of the distribution was determined to fit the data. Even after taking heterogeneity of substitution rate into consideration, the hypothesis of the same topology of the two gene trees (*SP11* and *SRK*) was not rejected.

If the topology is the same, one may consider whether or not the divergence time of each operational taxonomic unit (OTU) is the same. Indeed, in the study of parasites and host coevolution, there are several such kinds of discussions (HUELSENBECK *et al.* 1997, 2000). In these studies, the nucleotide or amino acid sequences of the same genes or proteins are available from both hosts and parasites (*e.g.*, mitochondrially encoded COI genes in HUELSENBECK *et al.* 1997), permitting direct comparison of maximum-likelihood estimates of external

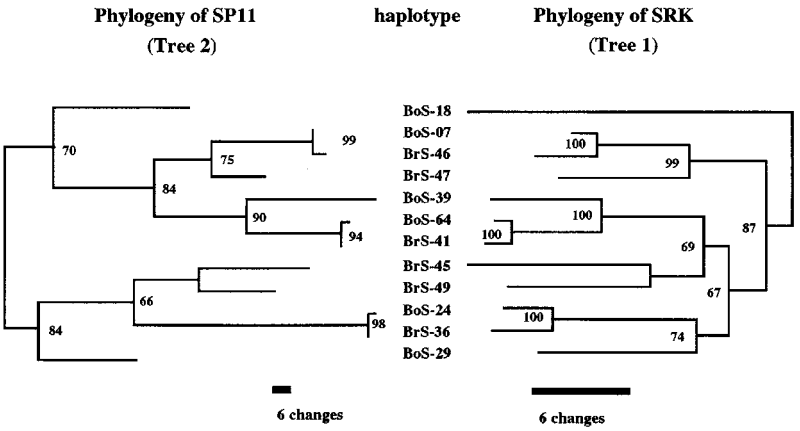

FIGURE 4.—The phylogenetic relationship of *SRK* and *SP11* genes on a single haplotype. Number shown at each node indicates the bootstrap value of the OTU cluster connecting at the node. The scale of each tree is indicated by a thick bar. Note that these are unrooted trees.

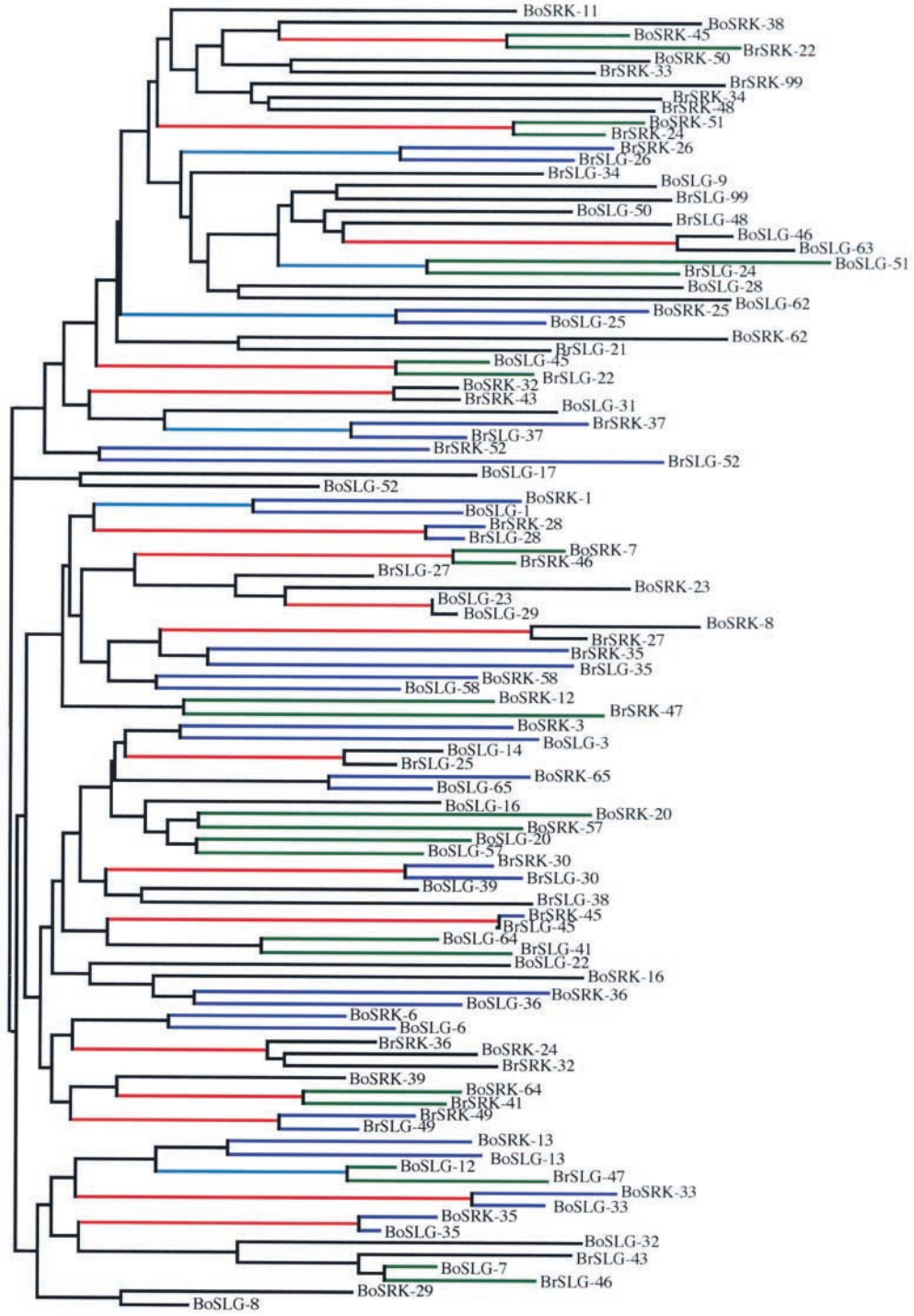

FIGURE 5.—Phylogenetic relationships of *SRK* and *SLG* sequences in *B. oleracea* and *B. rapa*. The NJ tree based on synonymous substitutions was constructed. Internal branches that are highly supported are in red ($P > 0.99$) and in light blue ($0.99 > P > 0.95$). External branches and OTUs in blue indicate haplotypes in which *SRK* and *SLG* are more closely related to each other than to *SRK* and *SLG* alleles in the other haplotypes. External branches and OTUs in green indicate that *SRK* or *SLG* alleles are more closely related to *SRK* or *SLG* alleles in an interspecific pair of haplotypes than to those in the other haplotypes.

branch length between host and parasites. However, in our case, divergence time of different genes is compared. It is obvious that the amino acid substitution rate is different between two genes (Table 1, Figure 4) and therefore we cannot compare the maximum-likelihood estimate of external branches, as in the case of host-parasite coevolution, to examine whether the divergence time of genes is the same or not. Furthermore, synonymous substitutions were not used because of large standard errors due to the small number of compared sites in *SP11*. Therefore to evaluate whether the divergence time of each gene at *SRK* and *SP11* on a single haplotype is similar, we examined the correlation of external branch lengths. If the two genes on each pair of haplotypes diverged at the same time, there would be a correlation between *SRK* and *SP11* external branch lengths. Since we do not know the true tree, we estimated branch lengths under both topologies (Tree 1 and Tree 2) separately by using PROTML (ADACHI and HASEGAWA 1994). As we expected, the correlation of branch lengths between the two genes was quite high ($r = 0.91 \pm 0.05$ for Tree 1 and $r = 0.79 \pm 0.04$ for

TABLE 3

Synonymous divergences for *SRK* and *SLG* genes in five particular pairs of haplotypes

Haplotype pair[a]	*SRK*[b]	BP[c]	*SLG*[b]	BP[d]
BoS-45:*BrS-22*	0.054 ± 0.017	0.999	0.040 ± 0.013	0.996
BoS-51:*BrS-24*	0.040 ± 0.013	0.999	0.12 ± 0.02	0.911
BoS-7:*BrS-46*	0.031 ± 0.012	1.00	0.036 ± 0.013	0.584
BoS-12:*BrS-47*	0.14 ± 0.02	0.704	0.045 ± 0.014	0.978
BoS-64:*BrS-41*	0.054 ± 0.015	0.990	0.078 ± 0.018	0.868

[a] *BoS* and *BrS* show haplotypes in *B. oleracea* and *B. rapa*, respectively.

[b] These columns show the synonymous nucleotide divergence at each locus.

[c] The bootstrap probability (1000 replications) of supporting the *SRK* gene cluster in a phylogenetic analysis (Figure 6).

[d] The bootstrap probability (1000 replications) of supporting the *SLG* gene cluster in a phylogenetic analysis (Figure 6).

Tree 2). This observation shows that the SP11 and SRK genes on a single haplotype seem to have diverged at the same time.

Phylogenetic relationship and tracing gene conversion between *SRK* and *SLG*: Regarding the generation of diversified haplotypes, the involvement of the frequent duplication of the S domain of *SRK* and gene conversion between *SRK* and *SLG* has been pointed out (Goring *et al.* 1993; Watanabe *et al.* 1994; Tantikanjana *et al.* 1996). However, these conclusions are based on a limited number of samples. Thus, here we reexamined the phylogenetic relationship between *SLG* and *SRK* on the basis of an extensive number of samples (49 *SRK* and 55 *SLG* sequences).

Since amino acids are likely to be a target of diversifying selection, only synonymous changes were used for the phylogenetic analysis (Figure 5). Among 43 haplotypes for which both *SLG* and *SRK* were sequenced, 18 cases show that *SLG* and its linked *SRK* are more closely related to each other than to their alleles from different haplotypes. Furthermore, of these, 10 (*BrS-26*, *BoS-25*, *BrS-37*, *BoS-1*, *BrS-28*, *BrS-30*, *BrS-45*, *BrS-49*, *BoS-33*, and *BoS-35*) showed close relationships between *SLG* and *SRK* that were significantly supported by high bootstrap probability (95% bootstrap support, Figure 5). The number of synonymous changes per site between a pair of *SRK* and *SLG* genes on the same haplotype ranges from 0.004 ± 0.004 (*BrSRK-45*:*BrSLG-45*) to 0.088 ± 0.020 (*BoSRK-1*:*BoSLG-1*) with an average of 0.045 ± 0.014. Compared with the minimum divergence (0.022 ± 0.010) observed in interspecific comparisons between *B. oleracea* and *B. rapa* (*BoSRK-32* and *BrSRK-43*), the relatively small synonymous changes between *SRK* and *SLG* suggest relatively recent conversion, including conversion after the species divergence, of *SLG* by *SRK* or vice versa.

Five pairs of *S* haplotypes (*BoS-45*:*BrS-22*, *BoS-51*:*BrS-24*, *BoS-7*:*BrS-46*, *BoS-12*:*BrS-47*, and *BoS-64*:*BrS-41*) show that haplotypes from different species, *B. oleracea* and *B. rapa*, are closely related to each other: *BoSRK* genes are closely related to *BrSRK* genes and *BoSLG* genes are closely related to *BrSLG* genes (Figure 5, Table 3). Among these five, three pairs (*BoS-45*:*BrS-22*, *BoS-7*:*BrS-46*, and *BoS-64*:*BrS-41*) showed comparable levels of nucleotide divergence at *SRK* and *SLG* ($P > 0.05$). Taking the average of *SRK* and *SLG* divergence for each pair, we compared these averages with the minimum synonymous divergence (0.022 ± 0.010) of the two species. The divergences of two pairs, 0.047 ± 0.010 between *BoS-45*:*BrS-22* and 0.034 ± 0.009 between *BoS-7*:*BrS-46*, were not significantly different from the minimum ($P > 0.05$). These observations indicate that each of these haplotype pairs is likely to have diverged from a common ancestor at the time of species divergence.

The relationship between *BoS-64* and *BrS-41* was somewhat different from those of other pairs. In both nucleotide and amino acid sequences, *BrSRK-41* is quite similar to *BoSRK-64* over the entire coding region (95.1% identity in aa and 96.8% identity in DNA). This close relationship was supported by phylogenetic analysis ($P = 0.99$, Figure 5). The nucleotide sequence from position 669 to 1115 (from the ATG initiation codon) in *BrSLG-41* is highly similar to those of *BrSRK-41* and *BoSRK-64* (0 and 2.4% difference in DNA, respectively), but not to *BoSLG-64* (9.2% difference in DNA). The remaining region of *BrSLG-41* is highly similar to that of *BoSLG-64* (2.5% difference, Figure 6), but distantly related to that of *BrSRK-41* (13.9% difference). This was also reflected in a relatively low bootstrap probability of the branch of *BoSLG-64* and *BrSLG-41* in Figure 5 ($P = 0.868$). Partial but high identity observed between *BrSLG-41* and *BrSRK-41* might be caused by convergent evolution with some natural selection. However, because the highly homologous segment in *BrSLG-41* involves synonymous sites as well as nonsynonymous sites, gene conversion is more likely than convergence due to natural selection.

BoS-51:*BrS-24* shows large divergence in *SLG* compared with a close relationship in *SRK*, and *BoS-12*:*BrS-47* shows the opposite pattern, namely, large divergence in *SRK* compared to *SLG* (Table 3). In the former case, *BoSRK-51* and *BrSRK-24* are closely related through the entire coding region. Nucleotide differences were detected at only 18 among 1152 sites (1.6%). However, the relationship between *BoSLG-51* and *BrSLG-24* is complicated. In some regions, *BoSLG-51* is almost identical to *BoSRK-51* or *BrSRK-24*, but in others, *BrSLG-24* is almost identical to *BoSRK-51* or *BrSRK-24*. This suggests that segmental transfer between *SRK* and *SLG* has occurred not only once but several times. In the case of *BoS-12* and *BrS-47*, *BrSRK-47* and *BoSRK-12* are relatively distantly related (Table 3). In fact, 33 and 25 unique nucleotides are not shared with the other three sequences in *BrSRK-47* and *BoSRK-12*, respectively. In nucleotide position 457–729, the *BrSRK-47* sequence is quite similar to *BrSLG-47* and *BoSLG-12* (0 and 2.2%

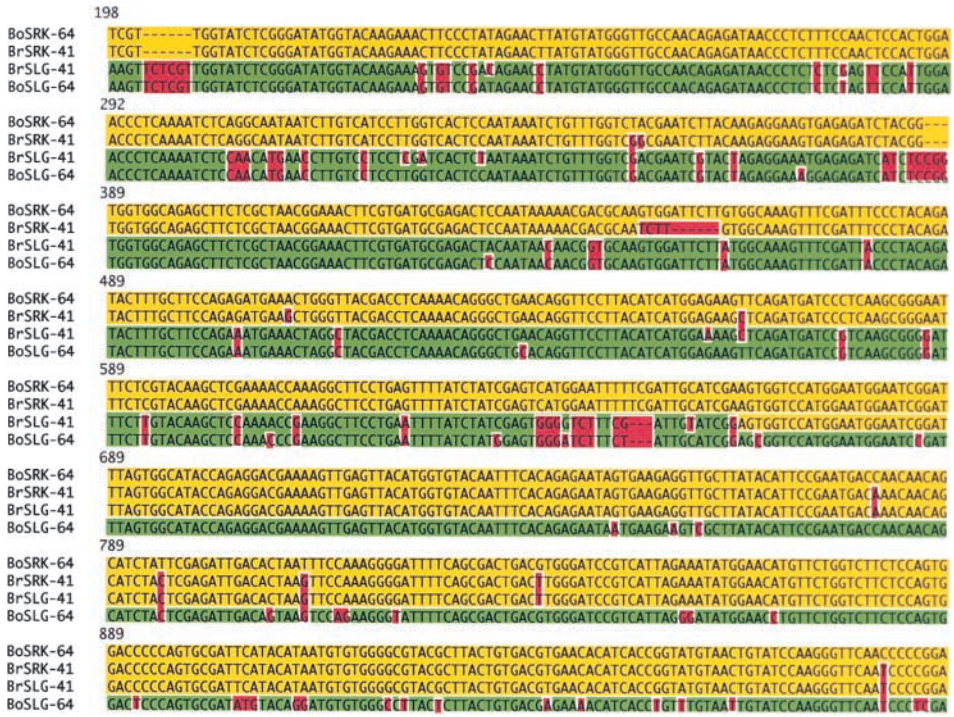

FIGURE 6.—Comparison of nucleotide sequences between the S domain of *SRK* and *SLG* of *BoS-64* and *BrS-41*. Nucleotides different from those in *BoSRK-64* are shown in red boxes.

difference, respectively) but not to *BoSRK-12* (4.4% difference), while *BrSRK-47* is similar to *BoSRK-12* from position 809 to 1014 (0.5% difference) but not to *BrSLG-47* (10.7% difference). This fact suggests that segmental transfer between *BrSLG-47* and *BrSRK-47* has occurred.

DISCUSSION

Diversity of *S*-locus genes: Knowledge of the synonymous nucleotide diversity (π_S) at other neutral loci may help to distinguish among the possible processes that may have enhanced the rate of nonsynonymous substitution in the *S*-locus genes. Balancing selection, including diversifying selection, makes π_S at *SRK* or *SLG* much larger than that at the neutral loci due to relatively longer persistence time of alleles at these loci, whereas positive selection makes π_S rather small due to selective sweep. If the π_S at *SRK* and *SLG* is significantly larger than π_S at other unlinked loci, balancing selection is plausible. Although there has been no systematic analysis of nucleotide diversity at loci unlinked to the *S* locus in Brassica species, a π_S value >10% seems unusually large (Table 1). In a relatively closely related species, *Arabidopsis thaliana*, nucleotide diversity in different genomic regions ranges from 0.5 to 1.8% (AGUADE 2001). To understand the reason for the relatively large synonymous nucleotide diversity in Brassica, we need nucleotide sequence information for other neutral loci in the species.

In the present study, *SRK*, *SLG*, and *SP11* alleles in class I *S* haplotypes were compared. Although the frequency of the class II *S* haplotypes is high in Brassica vegetables, the number of functionally distinct haplotypes is few—three in *B. oleracea* (CABRILLAC *et al.* 1999). There are a limited number of nucleotide sequence data of the class II *S* haplotypes (CABRILLAC *et al.* 1999). Through comparison of *SLG* sequences, an ancient divergence between class I and class II has been inferred (KUSABA *et al.* 1997). SCHIERUP *et al.* (2001) found greater sequence diversity in *A. lyrata SRK* alleles than in Brassica *SRK* alleles, suggesting more ancient diversification of *A. lyrata* alleles. The class I *SRK* sequences newly determined in the present study did not enlarge remarkably the sequence diversity of the class I alleles, supporting the finding of SCHIERUP *et al.* (2001).

Coevolution of *SP11*, *SRK*, and *SLG*: In the phylogenetic analysis of *SP11* and the S domain of *SRK*, the hypothesis that the topology is the same between the *SP11* tree and the *SRK* tree was not rejected. A positive correlation in divergence time between the *SRK* and *SP11* alleles was suggested by comparison of branch lengths in the two trees. This phylogenetic relationship between the *SRK* and *SP11* alleles likely suggests strong linkage disequilibrium of these two genes in the *S* locus. Recent studies on *S*-locus structure have demonstrated that the distance between *SP11* and *SRK* and the orientation of these genes are highly variable among different *S* haplotypes (TAKAYAMA *et al.* 2000). The structural diversity of *S* locus likely discourages recombination between these genes. Functional interactions between *SRK* and *SP11* may also have contributed to the coevolution of these genes.

Since *SRK* and *SLG* genes do not fall into separate clusters in the gene genealogy, genetic exchange between the two loci seems to play a significant role in the diversification of *S* haplotypes. This pattern of molecular

evolution contrasts with the pattern observed in human *MHC* (*HLA*) class I genes. In both cases, diversified alleles are favored and selection operates to maintain extensive polymorphism in a population. However, in *HLA*, a reciprocally monophyletic relationship between different loci is observed (Gu and Nei 1999) and this suggests infrequent exchanges between different loci. Further information is necessary to understand the molecular mechanism facilitating such frequent exchanges between different loci in the *S*-locus complex.

The role of gene conversion in SI gene diversity: Dixit *et al.* (2000) showed that a self-compatible mutant line in *B. oleracea* that lacked *SLG* synthesized a wild-type level of *SRK* transcripts but failed to produce the SRK protein, suggesting that SLG plays some role in stabilizing the SRK protein. Kusaba *et al.* (2001) found that self-incompatible *A. lyrata* has *SRK* but lacks *SLG*, suggesting the dispensability of *SLG* in SI. Our finding of three distinct SI haplotypes lacking *SLG* supports the latter view. On the other hand, it has been verified that *SRK* plays an essential role in self-recognition and *SLG* may enhance the process (Takasaki *et al.* 2000). The role of SRK and SLG in the SI recognition system must be different (Charlesworth 2000; Dickinson 2000). Therefore, it is likely that the evolutionary forces operating and the resulting patterns of nucleotide substitutions in the HVRs of *SRK* are different from those of *SLG*. However, in practice, a similar diversification pattern between *SRK* and *SLG* (Table 1) was observed. We suggest that this similar pattern is mainly due to gene conversion between *SRK* and *SLG*. Gene conversion might have occurred so frequently that it masked the natural evolutionary forces acting on *SLG*.

In disease resistance genes, gene conversion plays a role in maintaining paralogs and in generating new specificities (Michelmore and Meyers 1998). In this study, we showed three examples of apparent gene conversion detected because of their high similarity in long stretches in the genes. Also, we observed 18 haplotypes in which *SRK* and *SLG* sequences are more closely related to each other than to alleles in different haplotypes (Figure 5). It has been suggested that the presence of SLG highly similar to SRK promotes strong SI (Takasaki *et al.* 2000). Gene conversion from *SRK* to *SLG* may help to maintain a strong SI phenotype.

In the analysis of *BrS-47*, gene conversion from *SLG* to *SRK* can be speculated. Replacement of *SRK* sequence with *SLG* sequence may change the recognition specificity in stigma and result in self-compatibility. The region from 457 to 729 in *BrSRK-47*, which is the putative converted region, contains HVR1. However, the HVR1 sequence in *BrSRK-47* has only one synonymous nucleotide difference from *BoSRK-12*. The recognition specificity of *BrSRK-47* was found to be the same as that of *BoSRK-12* in our investigation (Y. Sato, R. Fujimoto, K. Toriyama and T. Nishio, unpublished data), as shown between *SRK-46* in *B. rapa* and *SRK-7* in *B. oleracea* (Kimura *et al.* 2002). These observations suggest that the gene conversion from *BrSLG-47* to *Br-SRK-47* may have happened but did not influence the recognition specificity of SRK. Alternatively, some mutation in *SRK* might have been repaired by the *SLG* sequence. Gene conversion may have played a role in resetting the variation between *SRK* and *SLG*.

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