Evolutionary Aspects of the *S*-Related Genes of the Brassica Self-Incompatibility System: Synonymous and Nonsynonymous Base Substitutions

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ABSTRACT

In the Brassicaceae, self- *vs.* nonself-recognition in self-incompatibility is controlled by sporophytic *S*-alleles. Haplotypes specifying both *SRK* (*S*-receptor kinase) and *SLG* (*S*-locus glycoprotein) are considered to play an important role in the recognition reactions. We compared the nucleotide sequences of SRK^9*(Bc)* and SRK^6*(Bo)*. The number of nonsynonymous substitutions per site (P_n) was lower, constrained, in the kinase than the receptor domain, while the numbers of synonymous substitutions (P_s) in the two domains were largely comparable. Pairwise values for P_s and P_n were calculated among 17 operational taxonomic units, including eight *SLG*s, the receptor domains of two *SRK*s, four *SRA*s (*S*-related A) and three *SRB*s (*S*-related B), which have high homologies with each other. The values of P_s and P_n of *SLG* were mostly comparable to those of the receptor domain of *SRK*. Dendrograms constructed on the basis of P_n and P_s indicated that *SRA* differentiated first, followed by *SRB*. The differentiation of *SLG* alleles is one of prerequisite factors for the establishment of self-incompatibility, and the allelic differentiation has occurred more than tens of million years ago.

IN the Brassicaceae, self-incompatible plants have a self/nonself recognition system that is sporophytically controlled by multiple alleles at what appears by classical genetics to be a single locus (*S*). Recent studies have revealed that *SLG* (*S*-locus glycoprotein) and *SRK* (*S*-receptor kinase) are both in linkage with *S*-alleles and are best considered as a haplotype (BOYES and NASRALLAH 1993; NASRALLAH and NASRALLAH 1993). It was also pointed out that there are other clones, *SRA* (*S*-related A) and *SRB* (*S*-related B), that have high homology to *SLG* but segregate at a different locus from *S*. All of these genes express proteins mainly in mature stigma tissue, in which the self-incompatibility reaction occurs (reviewed by HINATA *et al.* 1993). Their evolutionary relationships are important points to be considered for the maintenance of the *S*-allele polymorphism in this family.

As for *S-RNase* genes in solanaceous plants, which express gametophytic self-incompatibility, transformation experiments have indicated that style *S-RNase* plays a direct role in the recognition reaction between pollen tubes and style (LEE *et al.* 1994; MURFETT *et al.* 1994). In a study of pairwise comparison between *S-RNase*s cloned from various species in the Solanaceae, CLARK and KAO (1991) pointed out that *S-RNase*s have accumulated many synonymous and nonsynonymous base substitutions. They also showed that the level of amino acid constraint was significantly heterogeneous among different regions of the gene, with some regions being highly constrained and others appearing to be virtually unconstrained. These studies have contributed much toward the understanding of gametophytic self-incompatibility. In the case of sporophytic self-incompatibility, however, only TRICK and HEIZMANN (1992) have pointed out that *SLG* and *SRA* in *B. oleracea* have accumulated many nonsynonymous base substitutions. More extensive evolutionary analyses are needed to understand the self-incompatibility in this family.

We have cloned and sequenced SLG^9, SRK^9 and some other genes in *B. campestris* (WATANABE *et al.* 1994). In this report, we compared the nucleotide sequences between *S*-related genes, including *SLG, SRK, SRA* and *SRB,* and discussed their variability and relationships with respect to synonymous and nonsynonymous base substitutions.

MATERIALS AND METHODS

The DNA sequences used are shown in Table 1.

Nucleotide sequence alignments were constructed by first performing an amino acid alignment and then constructing the nucleotide alignment to maximize similarity. Synonymous and nonsynonymous substitutions per site were calculated by using MEGA software (KUMAR *et al.* 1993) and other packages that compute synonymous and nonsynonymous differences. These values are represented as P_s and P_n, respectively.

RESULTS

Synonymous and nonsynonymous changes in the receptor and kinase domains of *SRK*s: P_s and P_n between SRK^9*(Bc)* and SRK^6*(Bo)* were calculated for the receptor

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TABLE 1

DNA sequences used

DNA sequence	Original notation and reference[a]
SLG genes	
SLG^{8} (*B. campestris*)	S^{8}, DWYER *et al.* (1991) (X55274)
SLG^{9} (*B. campestris*)	S^{9}, WATANABE *et al.* (1994) (D30050)
SLG^{12} (*B. campestris*)	S^{12}, YAMAKAWA *et al.* (1994)
SLG^{6} (*B. oleracea*)	S^{6}, NASRALLAH *et al.* (1987) (Y0026)
SLG^{13} (*B. oleracea*)	S^{13}, DWYER *et al.* (1991) (X55275)
SLG^{29} (*B. oleracea*)	S^{29}-2, TRICK and FLAVELL (1989) (X16123)
SRK genes	
SRK^{9} (*B. campestris*)	WATANABE *et al.* (1994) (D30049)
SRK^{6} (*B. oleracea*)	STEIN *et al.* (1991) (M76647)
SRB-like genes	
SRB^{2} (*B. oleracea*)	*SLR2*, BOYES *et al.* (1991) (X57673)
SLG^{2A} (*B. oleracea*)	CHEN and NASRALLAH (1990)
SLG^{5} (*B. oleracea*)	S^{5}, SCUTT and CROY (1992) (X65814)
SRA genes	
SRA^{1} (*B. campestris*)	NS^{1}, ISOGAI *et al.* (1991) (X58440)
SRA^{3} (*B. campestris*)	NS^{3}, YAMAKAWA *et al.* (1993) (D11108)
SRA-S^{63} (*B. oleracea*)	$S^{63}1$ (*BS63-1*), TRICK (1990) (X52089)
SRA-S^{22} (*B. oleracea*)	*SLR1*, LALONDE *et al.* (1989) (S44181)
SLG-like genes from *B. napus*	
SLG^{14} (*B. napus*)	*A14*, GORING *et al.* (1992a) (Z11725)
SLG^{91} (*B. napus*)	*910*, GORING *et al.* (1992b) (Z11724)

[a] Parentheses indicate data base accession number.

and kinase domains separately (Table 2). The values of P_s for these two domains were 0.208 and 0.217, indicating that they have similar potentials for variability. However, P_n was as low as 0.048 in the kinase domain, while it was 0.120 in the receptor one. The amino acid sequence at the kinase domain appears to be constrained, probably reflecting the maintenance of enzymatic function, while the receptor domain has accumulated nonsynonymous substitutions at a rate as high as that of *SLG*s, as will be shown later.

Comparison between *SLG*, *SRA*, *SRB* and the receptor domains of *SRK*: P_s and P_n were calculated pairwise among 17 *S*-related OTUs (operational taxonomic units).

The values of P_s between *SLG* alleles ranged from 0.14 to 0.35, and of P_n from 0.06 to 0.15 (Table 3).

TABLE 2

Comparison of synonymous (P_s) and nonsynonymous (P_n) substitutions per site on the receptor and kinase domains of *SRK*^{9} (*B. campestris*) and *SRK*^{6} (*B. oleracea*)

	Substitutions per site	
	P_s	P_n
Receptor domain	0.208 (283)	0.120 (1016)
Kinase domain	0.217 (186)	0.048 (681)

Numbers in parentheses indicate number of sites.

These values were mostly comparable to those of the receptor domains of *SRK*s.

The values of P_s and P_n between *SRB*s were a little lower than those of *SLG*s. In this comparison, *SRB*^{2}, *SLG*^{2A} and *SLG*^{5} showed high sequence homology, and they were grouped as *SRB* in this context.

The values of P_s and P_n between *SRA* alleles were the lowest observed.

P_s and P_n are shown for all pairwise comparisons in Figure 1. The P_s value was generally higher than that of the corresponding P_n. The correlation coefficient between P_s and P_n for intralocus comparisons within *SLG*s, *SRA*s and *SRB*s was as high as 0.785. The ratio of P_n to P_s seemed to be very similar among alleles of *SLG*s, *SRA*s and *SRB*s. However, the ratio of P_n to P_s was lower in interlocus than in intralocus comparisons, indicating that the pattern of variation differs within and between loci. The standard errors of P_n were <11% of P_n values and those of P_s were <15% of P_s, when P_n or P_s exceeds 0.1.

Neighbor-joining dendrograms (SAITOU and NEI 1987) of 17 *S*-related OTUs were constructed on the bases of P_s and P_n (Figure 2a, b). Very similar figures were obtained for P_s and P_n, though a little difference was observed among the *SLG*s. In either calculation, both trees show three major clusters, corresponding to *SLG*, *SRB* and *SRA* sequences. *SLG*^{2} and *SLG*^{5}, pollen-recessive alleles of *SLG*, were included in the *SRB* cluster. The *SRA* cluster appears to have diverged first from the *SLG* and *SRB* clusters, followed by the differentiation of the *SRB* and *SLG* clusters. The receptor domain of *SRK* clustered with the *SLG* group.

The differentiation of *SLG* alleles does not reflect the differentiation of species (Figure 2). The *SRA* sequences show lower allelic differentiation compared to *SLG* and alleles within species group together.

Highly variable regions: P_s and P_n were calculated at different positions along the sequences within window sizes of 60 bp for eight *SLG*s, three *SRB*s and two *SRA*s, respectively (Figure 3 a–c). Other calculations with larger window sizes gave similar results.

As shown in Figure 3a for *SLG*, synonymous, as well as nonsynonymous, substitutions were distributed unevenly through the sequence. P_s was high at the signal peptide, V1, and 3′-terminal regions. Three P_n peaks were observed at sites 600 (V1), 810 (V2) and 930 (V3)

TABLE 3

Synonymous (upper right) and nonsynonymous (lower left) substitution per site calculated by Jukes-Cantor method

	SLG^{8} *Bc*	*SLG*^{9} *Bc*	*SLG*^{12} *Bc*	*SLG*^{6} *Bo*	*SLG*^{13} *Bo*	*SLG*^{29} *Bo*	*SLG*^{14} *Bn*	*SLG*^{91} *Bn*	*SRK*^{9}RD *Bc*	*SRK*^{6}RD *Bo*	*SRB*^{2} *Bo*	*SLG*^{2A} *Bo*	*SLG*^{5} *Bo*	*SRA*^{1} *Bc*	*SRA*^{3} *Bc*	*SRA-S*^{63} *Bo*	*SRA-S*^{6} *Bo*
SLG^{8}*Bc*		0.26	0.26	0.19	0.18	0.18	0.19	0.30	0.28	0.22	0.74	0.76	0.76	0.87	0.89	0.87	0.86
SLG^{9}*Bc*	0.13		0.25	0.23	0.28	0.18	0.22	0.29	0.03	0.20	0.70	0.70	0.75	0.92	0.89	0.92	0.91
SLG^{12}*Bc*	0.12	0.13		0.22	0.27	0.21	0.23	0.19	0.29	0.26	0.72	0.65	0.69	0.96	0.92	0.90	0.87
SLG^{6}*Bo*	0.10	0.11	0.13		0.18	0.14	0.14	0.24	0.25	0.11	0.72	0.75	0.77	0.86	0.85	0.82	0.85
SLG^{13}*Bo*	0.06	0.12	0.12	0.09		0.16	0.21	0.30	0.30	0.20	0.77	0.76	0.82	1.02	1.00	0.97	0.98
SLG^{29}*Bo*	0.11	0.10	0.12	0.10	0.10		0.18	0.26	0.20	0.17	0.69	0.71	0.76	0.90	0.87	0.91	0.90
SLG^{14}*Bn*	0.12	0.13	0.16	0.10	0.11	0.13		0.28	0.25	0.18	0.74	0.82	0.80	0.80	0.79	0.81	0.81
SLG^{91}*Bn*	0.13	0.15	0.10	0.13	0.13	0.13	0.15		0.30	0.25	0.72	0.70	0.77	0.94	0.91	0.96	0.92
SRK^{9}*Bc*RD*	0.13	0.01	0.14	0.12	0.13	0.11	0.13	0.15		0.20	0.68	0.68	0.74	0.89	0.87	0.90	0.89
SRK^{6}*Bo*RD	0.12	0.12	0.15	0.06	0.11	0.13	0.13	0.14	0.12		0.69	0.72	0.76	0.88	0.87	0.87	0.87
SRB^{2}*Bo*	0.22	0.23	0.22	0.23	0.22	0.24	0.23	0.23	0.23	0.22		0.16	0.15	1.02	1.01	1.03	1.02
SLG^{2A}*Bo*	0.21	0.21	0.21	0.21	0.20	0.22	0.21	0.22	0.21	0.20	0.07		0.12	1.07	1.08	1.11	1.08
SLG^{5}*Bo*	0.21	0.23	0.22	0.22	0.21	0.22	0.21	0.23	0.23	0.22	0.08	0.06		1.01	0.98	1.05	1.04
SRA^{1}*Bc*	0.29	0.27	0.27	0.27	0.27	0.27	0.29	0.27	0.28	0.28	0.33	0.33	0.33		0.06	0.08	0.08
SRA^{3}*Bc*	0.29	0.27	0.27	0.28	0.27	0.27	0.30	0.28	0.28	0.28	0.34	0.33	0.34	0.02		0.11	0.11
SRA-S^{63}*Bo*	0.28	0.26	0.27	0.27	0.26	0.26	0.28	0.27	0.27	0.27	0.33	0.33	0.33	0.04	0.04		0.01
SRA-S^{6}*Bo*	0.28	0.27	0.28	0.27	0.27	0.27	0.28	0.27	0.28	0.28	0.33	0.33	0.33	0.04	0.05	0.01	

* Receptor domain was calculated.

in *SLG*, and the peak values of P_n were mostly comparable to those of P_s, especially in V2 and V3. Thus, the whole sequence was divided into C1, V1(604–627), C2, V2(855–870), V3(871–982), and C3, as shown in Figure 3a, in which C and V represent conserved and variable regions, respectively. In the C1 region, N-linked glycosylated sites and tryptophane residues were conserved fairly well. Between V2 and V3, cysteine residues were conserved. In the C3 region, cysteine residues were conserved and P_n was comparatively low. The conserved regions may be important for maintaining the function of this protein and the variable regions for the allelic polymorphism.

The variation at *SRB* showed patterns similar to *SLG*, but P_n was higher in V1 than V2 and V3 (Figure 3b). The differences between *SRB* and *SLG* occurred more frequently at the 3′-terminal half, and these sites seemed to characterize the *SRB* sequences.

Fewer nonsynonymous substitutions occurred in *SRA*, and the variation spectrum was different from *SLG* and *SRB* (Figure 3c). Differences between *SRA* and *SLG* appeared to be distributed more evenly along the sequences.

DISCUSSION

According to Nou *et al.* (1993), more than 100 *S* alleles may exist in *B. campestris*. Stigmatic *SLG* proteins from 30 *S* homozygotes showed different isoelectric points (pI). The high variability of *SLG* observed here

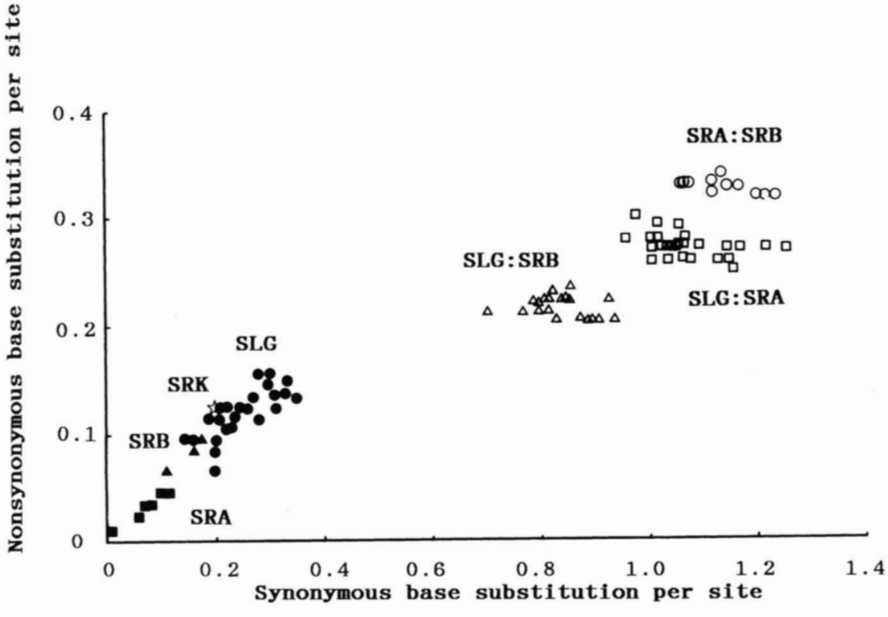

FIGURE 1.—Relationships between P_s and P_n in pairwise comparison of intralocus (denoted as *SLG*, ●; *SRA*, ■; *SRB*, ▲; *SRK*, ☆) and interloci (*SLG:SRA*, □; *SLG:SRB*, △; *SRA:SRB*, ○).

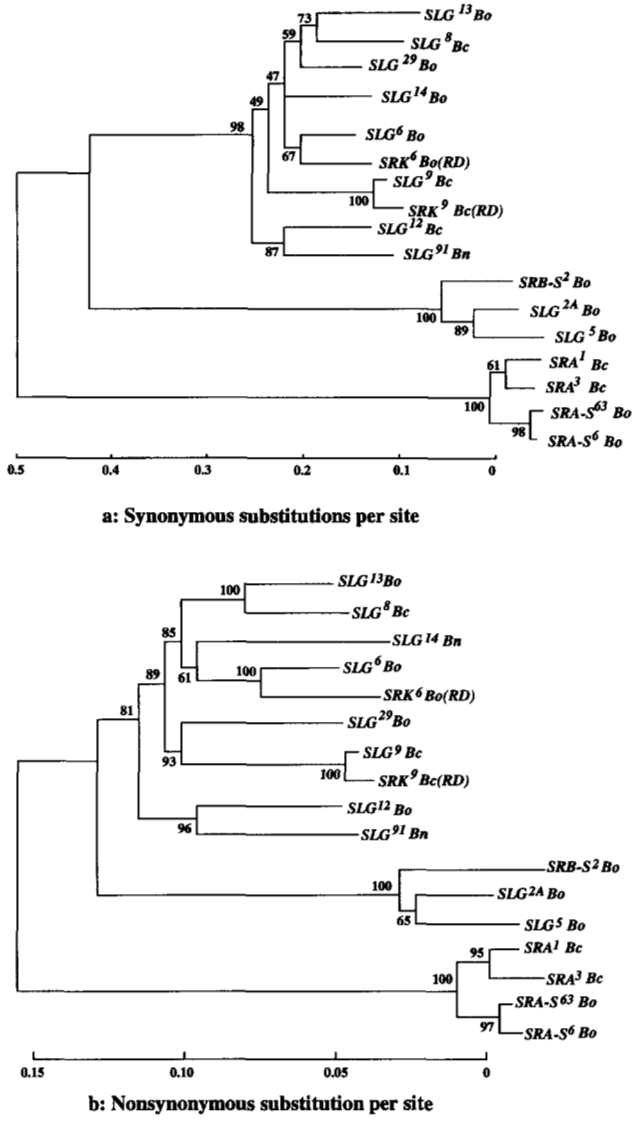

FIGURE 2.—Dendrograms of OTUs constructed by neighbor-joining method based on P_s (a) and P_n (b).

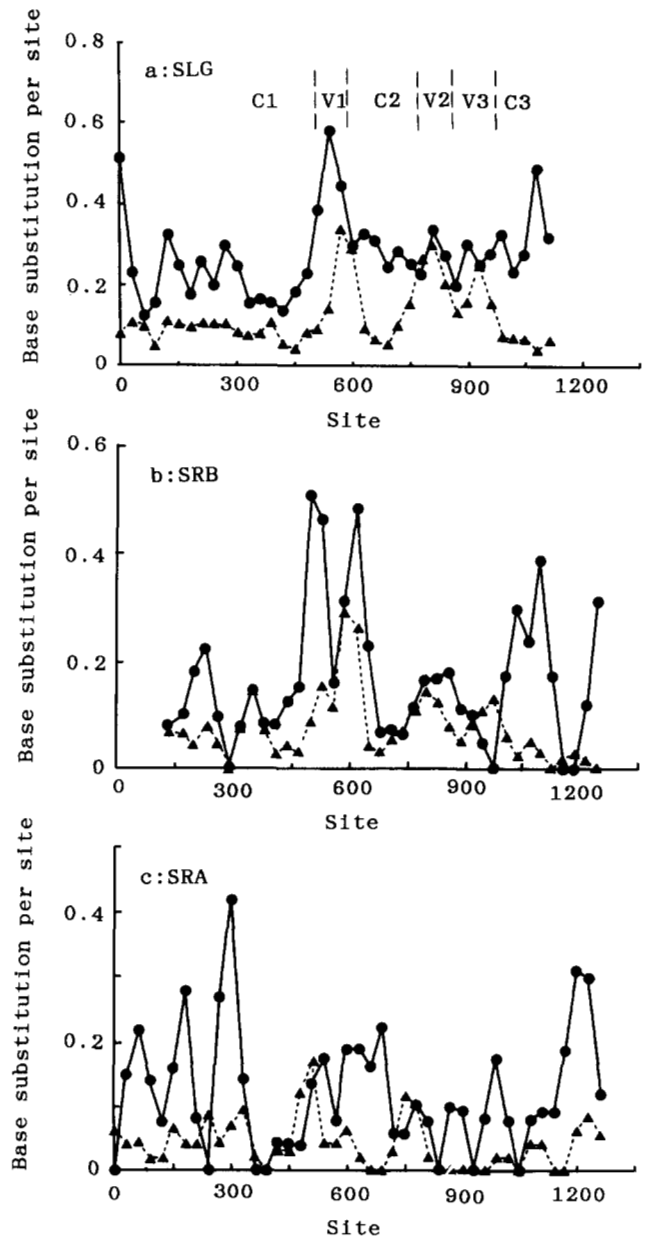

FIGURE 3.—P_s (●) and P_n (▲) along the sequence of eight *SLG* (a), two *SRB* (b) and two *SRA* (*SRA*^{1} and *SRA*^{3}) (c), calculated on 60-bp window size at 30-bp sliding intervals. Conserved (C1–C3) and variable (V1–V3) regions are indicated in Figure 3a.

may explain the polymorphism of the *SLG* protein. Although the estimates of pI values based on amino acid composition of each *SLG* did not always coincide well with the actual pI values, the relative order of the estimated and actual pI values showed full agreement for those proteins whose pI values were known.

High correlations between P_n and P_s were observed in allelic comparison within the *SLG, SRB* and *SRA* loci. This may indicate that the nucleotide changes of alleles occurred in a similar fashion in every locus, though the magnitude may differ. On the other hand, the ratio of P_n to P_s was higher in within-locus comparisons. This may indicate either that the modes of nonsynonymous substitutions are different among loci, or that the nonsynonymous substitutions are approaching saturation.

The dendrogram indicates that the *SRA* locus differentiated from the other *S*-related genes first, and its allelic differentiation occurred rather recently. *SRA* does not play a role in the recognition reactions, but its abundant production in the stigma may indicate that it contributes to the pollination process (WATANABE *et al.* 1992).

After the divergence of *SRA, SRB* appears to have differentiated from *SLG* and *SRK*. According to BOYES *et al.* (1991), the *SRB* locus is located near *SRA*. It has been shown that *SRB* has high homology to *SLG*^{5}*Bo* and *SLG*^{2A}*Bo*, both of which were cloned from stigmas as pollen-recessive *SLG*s (CHEN and NASRALLAH 1990; SCUTT and CROY 1992). These genes differ from other *SLG*s by many nucleotide differences in the 3′ half of

the sequences. One possibility is that the recessive *SLG* has appeared recently after *SLG* allelic differentiation. Further studies are needed on this group of genes.

Lastly, *SLG* and *SRK* appear to have differentiated among *S*-alleles. Together, as a haplotype, they play a role in the recognition reaction in self-incompatibility (NASRALLAH and NASRALLAH 1993). Analysis of the S^9 genotype in *B. campestris* showed that the receptor domain of *SRK* and the corresponding *SLG* have as high as 98% nucleotide identity (WATANABE *et al.* 1994). These authors considered that certain mechanisms act to maintain similarity among the sequences, though the mechanisms are unknown. P_s and P_n within the receptor domains of *SRK*s were mostly comparable to those of *SLG*s. Nonsynonymous substitutions in the kinase domain of *SRK* seemed to be constrained, as in many plant enzymes. Compared to the kinase domain, a very high level of nonsynonymous base substitutions has accumulated in the *SLG* and *SRK*-receptor domains, which play a direct role in the recognition of self and nonself. Substitutions may result in the production of novel *S*-allele classes, and such mutants may be preferentially kept in the population. The relationships between *SLG* and the corresponding receptor domain of *SRK*, and between the receptor and kinase domains should be studied. It could be the case that the expression of self-incompatibility in the Brassicaceae was established when the interaction between *SLG* and *SRK* was formed.

Allelic differentiation of *SLG* as well as *SRK* appears to have occurred at a very ancient time, suggesting that self-incompatibility may have been established before the differentiation of species in this family. According to MULLER (1981), only one fossil record from the upper Miocene of France has been published for the family Brassicaceae. The upper Miocene occurred 5–11 million years ago. If the diversification of *SLG* arose 10 million years ago, and if we take mean values of P_s and P_n of *SLG* to be 0.22 and 0.12, respectively, then the rate of molecular change would be estimated to be 11×10^{-9}/site/year for synonymous substitutions and 6×10^{-9}/site/year for nonsynonymous substitutions. These values are very high compared to those of other plant genes (WOLFE *et al.* 1987; BOUSQUET *et al.* 1992). The fossil record may indicate only the existence of species but not always their origins. If the time of establishment of the self-incompatibility were 50 million years ago, the rates would be 2.2×10^{-9}/site/year for synonymous and 1.2×10^{-9}/site/year for nonsynonymous substitutions. An interesting question is whether the origin of this form of self-incompatibility predates or postdates the origin of the Brassicaceae.

In contrast, the differentiation of alleles of *SRA* appears to have occurred rather recently, because the variation among alleles was inferred to have occurred after the species divergence.

In the *S-RNase* system of the Solanaceae, P_s and P_n in the variable region have been estimated as 0.4–0.7 and 0.2–0.6, respectively (CLARK 1993). These values are somewhat higher than the values for the Brassicaceae herein calculated. Self-incompatibility may have been independently established in the Brassicaceae at a more recent time than in the Solanaceae.

The variable sites occur unevenly along the length of the sequence. Although the reason is unknown, tryptophane residues were observed to be well conserved in the C1 region. High levels of nonsynonymous substitution within *SLG* were found in the V1, V2, and V3 regions. The latter two variable regions are located around the first cysteine-conserved cluster. These regions may affect the conformation of this molecule. An interesting open question concerns whether such conformational changes can explain the existence of more than 100 *S* alleles. Some of the conserved regions may be important to the process of recognition.

Allelic differentiation of the haplotype of *SLG* and *SRK* may play an important role in the establishment of self-incompatibility in these taxa. The self-incompatibility thus established may have played an important role in the species differentiation of this family.

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