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Physical distances between *S*-locus genes in various *S* haplotypes of *Brassica rapa* and *B. oleracea*

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Abstract In *Brassica*, self-incompatibility genes *SLG* (for *S*-locus glycoprotein) and *SRK* (for *S*-receptor kinase) are located in the *S*-locus complex region with several other *S*-linked genes. The *S* locus is a highly polymorphic region: polymorphism has been observed not only in sequences of *SLG* and *SRK* but also in the location of the *S*-locus genes. In order to compare the physical location of the *S*-locus genes in various *S* haplotypes, we used six class-I *S* haplotypes of *B. rapa* and seven class-I *S* haplotypes of *B. oleracea* in this study. DNA gel blot analysis using pulsed-field gel electrophoresis (PFGE) showed that the physical distances between *SLG* and *SRK* in *B. rapa* are significantly shorter than those in *B. oleracea* and that the sizes of *Mlu*I and *Bss*HII fragments harboring *SLG* and *SRK* are less variable within *B. rapa* than within *B. oleracea*. We concluded that several large genomic fragments might have been inserted into the *S*-locus region of *B. oleracea* after allelic differentiation of *S*-locus genes.

Key words *Brassica rapa* (syn. *campestris*) · *B. oleracea* · PFGE · *S*-locus · Self-incompatibility

Introduction

The self-incompatibility (*S*) locus regulates self/non-self discrimination between pollen and stigma in higher plants. Recent molecular genetic studies have revealed that the *S*-locus complex region in *Brassica* contains several genes, including *SLG* (for *S*-locus glycoprotein), *SRK* for *S*-receptor kinase), *SLA* (for *S*-locus anther), *SLL1* (for *S*-locus linked gene 1), *SLL2* (for *S*-locus linked gene 2), *SAE1* (for *S*-locus anther expressed gene 1), and so on (Stein et al. 1991; Boyes and Nasrallah 1995; Yu et al. 1996; Boyes et al. 1997; Letham and Nasrallah 1998; Suzuki et al. 1999; Watanabe et al. 1999). Because of the complex nature of the *S*-locus with multiple genes, ‘‘*S* allele” is referred to as ‘‘*S* haplotype” (Boyes and Nasrallah 1993; Nasrallah and Nasrallah 1993). Nou et al. (1993) estimated that more than 100 haplotypes presently exist in *Brassica rapa* (syn. *campestris*). This allelic differentiation is considered to have occurred over a period of ten million years (Hinata et al. 1995).

Among the *S*-locus genes, *SLG* and/or *SRK* are thought to be related to the self-incompatibility (SI) recognition as stigmatic receptors. *SLG* encodes a secreted glycoprotein (Takayama et al. 1987), while *SRK* encodes a membrane-spanning, receptor-like kinase, whose extracellular domain (S domain) is similar to SLG (Stein et al. 1991). On the basis of the structure of *SLG* genes, *S* haplotypes have been classified into two groups, class I and class II. The class-I *SLG*s have no introns, but class-II *SLG*s have an intron near the 3′-end of the genes (Tantikanjana et al. 1993; Hatakeyama et al. 1998b). The level of DNA sequence divergence among reported *SLG* alleles ranges from 10% to 20% within the class, whereas a divergence of almost 30% has been noted between class-I and class-II *SLG*s (Nasrallah and Nasrallah 1993). Most class-I *S* haplotypes belong to a pollen-dominant SI phenotype, while class-II *S* haplotypes are pollen-recessive.

Physical distances between *SLG* and *SRK* were first estimated in S^6 (class I) and S^2 (class II) haplotypes of *Brassica oleracea* by pulsed field gel electrophoresis (PFGE) gel blot analysis; the distance between the two genes is less than 220 kb for the S^6 haplotype and less than 350 kb for the S^2 haplotype (Boyes and Nasrallah 1993). Furthermore, long-range restriction maps for the *S* locus of S^2 (class II) and S^{13} (class I) haplotypes of *B.*

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oleracea and an S^8 (class I) haplotype of *B. rapa* were constructed from the results of PFGE analysis and lambda DNA contigs (Boyes et al. 1997). These maps showed that the estimated distances between *SLG* and *SRK* are within 266 kb in the S^2 haplotype, 55 kb in the S^{13} haplotype, and 20 kb in the S^8 haplotype. In the S^{910} (class I) haplotype of self-incompatible *B. napus* (having the *B. rapa S*-locus), a long polymerase chain reaction (PCR) was used for cloning the region between SLG^{910} and SRK^{910} (Yu et al. 1996), and the actual distance between the two genes was found to be 25 kb. In an S^9 haplotype (class I) of *B. rapa*, a 76-kb *Mlu*I fragment containing both SLG^9 and SRK^9 was cloned into an artificial chromosome vector (Suzuki et al. 1997), and the physical distance between SLG^9 and SRK^9 was found to be approximately 13 kb (Suzuki et al. 1999). Comparison of these analyses suggests the highly polymorphic nature of the *S*-locus. However, information on *S*-locus organization is restricted to within these several *S* haplotypes. Further genomic analysis of more *S* haplotypes is needed to elucidate the *S*-locus organization.

Twelve genes, including pollen *S*-gene candidates (*SAE1* and *SP11*), were identified in the 76-kb *SLG/SRK* region of the S^9 haplotype of *B. rapa* (Suzuki et al. 1999; Watanabe et al. 1999). Among them, *SP4* (*S*-locus protein 4) and *SP7 (S*-locus protein 7) are located at the termini of the region, and both are single-copy genes. *SP4* encodes novel protein, and *SP7* encodes protein similar to yeast ASF1 (anti-silencing function 1). Although it has not yet been determined whether these genes are located in or out of the *S*-locus, it is of interest to clarify the physical linkage and physical distance between *SP4/SP7* and *SLG/SRK* with regard to the structure and evolution of the *S*-locus complex. In the study reported here, we used six *S* haplotypes of *B. rapa* and seven *S* haplotypes of *B. oleracea* to estimate physical distances between the *S*-locus genes (*SLG*, *SRK*, *SP4*, and *SP7*) by PFGE analysis. Differences in the *S*-locus organization between *B. rapa* and *B. oleracea* are discussed.

Materials and methods

Plant materials

Plant materials used were homozygotes of S^8, S^9, S^{45}, S^{46}, S^{47}, and S^{48} haplotypes of self-incompatible *Brassica rapa* (Hatakeyama et al. 1998c) and homozygotes of S^3, S^6, S^{14}, S^{16}, S^{18}, S^{20}, and S^{29} haplotypes of self-incompatible *Brassica oleracea* (Brace et al. 1994). All these *S* haplotypes belong to class I.

Pulsed-field gel electrophoresis (PFGE) gel blot analysis

Megabase DNA embedded in agarose plugs was prepared from young leaf tissue of *Brassica* plants by the rapid method described by Suzuki et al. (1997). Subsequent digestion with infrequently cutting restriction enyzmes was performed overnight in digestion buffer (TaKaRa shuzo, Shiga, Japan) supplemented with bovine serum albumin (BSA) to a final concentration of 0.1 mg/ml. The agarose plugs containing the digested DNA were washed once in 0.5 × TBE (1 × TBE: 89 m*M* TRIS, 89 m*M* boric acid, 2 m*M* EDTA) and loaded onto a 1% SeaKem GTG agarose gel (FMC, Rockland, Me.) in 0.5 × TBE. The DNA was electrophoresed with a CHEF-DR II apparatus (Bio-Rad, Hercules, Calif.) at 170 V and 14°C with a switching interval ramped from 30 to 50 s for 24 h, or from 50 to 70 s for 28 h. After electrophoresis, the gel was exposed to UV light for 3 min, and DNA was transferred to nylon membranes (Nytran; Schleicher & Schuell, Dassel, Germany). Hybridization was carried out in 5 × SSC, 0.5% blocking reagent (Boehringer Mannheim, Mannheim, Germany), 0.1% sodium *N*-lauroyl sarcosinate, and 0.02% SDS, at 65°C. Digoxigenin (dig)-labeled probes were prepared by random primed DNA labeling using a digoxigenin DNA labeling kit (Boehringer Mannheim). The membrane was washed twice in 0.3 × SSC, 0.1% SDS at 65°C for 20 min each. The detection of the hybridized probe was carried out according to the instruction manual of the DIG Luminescent Detection Kit (Boehringer Mannheim) with CSPD (Tropix, Bedford, Mass.) as the substrate. The SLG^{45} probe was a 0.6-kb dig-labeled fragment amplified by PCR from SLG^{45} cDNA (Hatakeyama et al. 1998a) using primers, GGTTACGACCTCAAAACAGG and TCCGGTCCAA ATCACACAAC (Okazaki et al. 1999). *SP4* and *SP7* probes were prepared from corresponding cDNA clones (Suzuki et al. 1999). The *SP4* gene is locatd 35 kb downstream of the SLG^9 gene in the S^9 haplotype, while the *SP7* gene is located 15 kb upstream of the SRK^9 gene in the S^9 haplotype (Fig. 1; Suzuki et al. 1999).

Conventional DNA gel blot analysis

Genomic DNA gel blot analysis using conventional electrophoresis was conducted to detect bands shorter than 23 kb. The megabase DNA embedded in agarose plugs was digested with enzymes and subjected to electrophoresis in 0.8% agarose gel. Blotting, hybridization and detection of DNA were performed as described above.

Results

Conventional DNA gel blot analysis of *SLG* and *SRK*

To estimate physical distances between the *S*-locus genes in various *S* haplotypes, we isolated the megabase

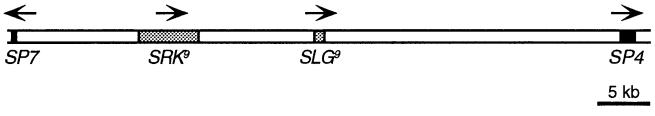

Fig. 1 Physical locations of SLG^9, SRK^9, *SP4*, and *SP7* in the 76-kb *S*-locus region of the S^9 haplotype which wre cloned into the PAC vector (Suzuki et al. 1997, 1999). *Arrows* indicate the direction of transcription of the genes

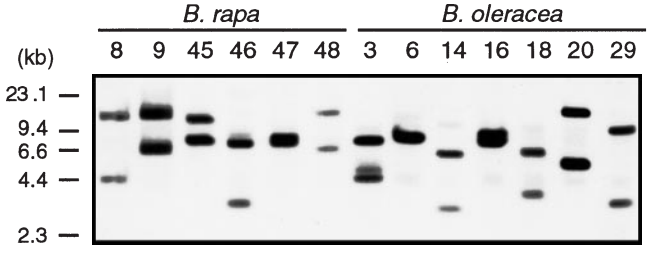

Fig. 2 Conventional DNA gel blot analysis of 13 different *S* haplotypes of *B. rapa* and *B. oleracea*. Genomic DNA was digested with *Hin*dIII, separated on 0.8% agarose gel, and transferred to a nylon membrane. The SLG^{45} probe detected fragments corresponding to *SLG* and *SRK* in each *S* haplotype. The *number above* each *lane* indicates the *S* allelic number of each *S* homozygous plant. DNA size markers are shown on the *left* in kilobases (kb)

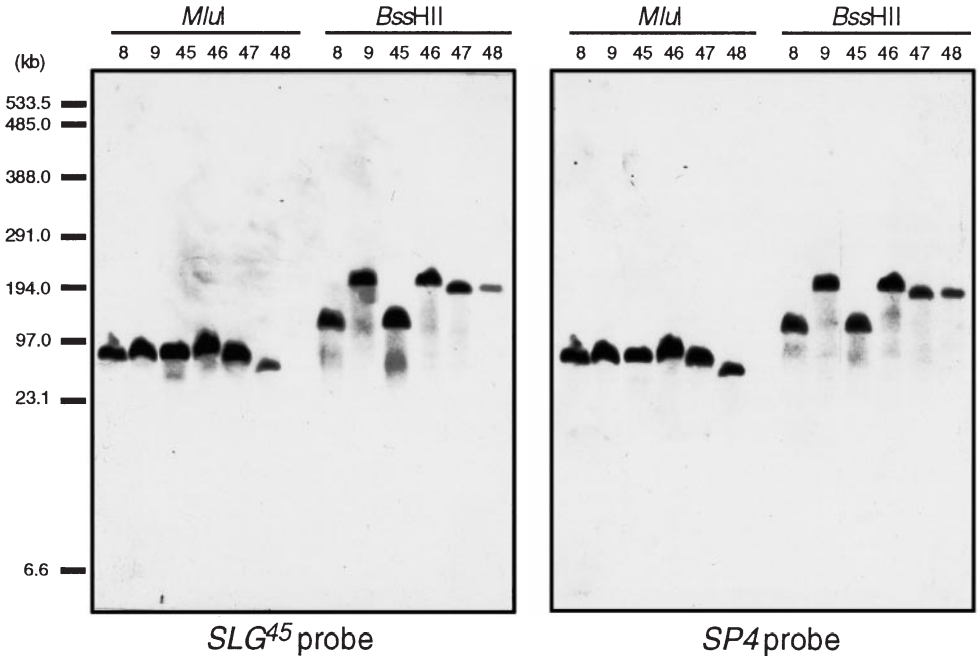

Fig. 3 PFGE gel blot analysis of *B. rapa*. High-molecular weight genomic DNA was digested with *Mlu*I and *Bss*HII and then separated on a CHEF gel. The DNA was transferred to a membrane and hybridized with the SLG^{45} and *SP4* probes. The *number above* each *lane* indicates the *S* allelic number of each *S* homozygous plant. The probes are shown *below* the panels. DNA size markers are indicated on the *left* in kilobases (kb)

Table 1 Restriction fragments identified by PFGE gel blot analysis of the six *S* haplotypes in *B. rapa*

Enzyme	*Mlu*I			*Bss*HII			*Nhe*I			*Not*I		
Probe	SLG^{45}	*SP4*	*SP7*	SLG^{45}	*SP4*	*SP7*	SLG^{45}	*SP4*	*SP7*	SLG^{45}	*SP4*	*SP7*
S^8	75[a]	75	75	130	130	ND[c]	20	2.3	37	610	610	610
S^9	76[b]	76[b]	76[b]	210	210	210	21/15	4.1	17	600	600	600
S^{45}	75	75	75	130	130	ND	65/52	2.3	ND	610	610	610
S^{46}	90	90	90	210	210	210	74	2.3	74	640	640	640
S^{47}	68	68	68	190	190	190	45/22	20	45	600	600	600
S^{48}	60	60	60	190	190	190	23	2.3	23	50	550	50

[a] Numbers indicate the length of restriction fragments (in kilobases)
[b] Precise length of cloned fragments (E89 clone of the S^9 haplotype; Suzuki et al. 1999)
[c] ND, Not determined

DNAs from six class-I *S* haplotypes (S^8, S^9, S^{45}, S^{46}, S^{47}, and S^{48}) of self-incompatible *B. rapa* and seven class-I *S* haplotypes (S^3, S^6, S^{14}, S^{16}, S^{18}, S^{20}, and S^{29}) of self-incompatible *B. oleracea*. The dig-labeled SLG^{45} probe, which corresponds to the middle part of the SLG^{45} gene (Hatakeyama et al. 1998a), was used to detect *SLG* and *SRK* genes because it is able to hybridize with both *SLG* and *SRK* genes in various class-I *S* haplotypes (Hatakeyama et al. 1998b). Conventional gel blot analysis of *Hin*dIII-digested DNA using the SLG^{45} probe revealed that the 13 *S* haplotypes have both *SLG* and *SRK* (Fig. 2). The two bands in each lane correspond to *SLG* and *SRK*, respectively, and a single band observed in the S^{47} (*B. rapa*) and S^6 (*B. oleracea*) haplotypes was considered to be due to the overlap of two fragments of the same size because two bands were obtained in the case of digestion with other enzymes (data not shown, see the results of *Nhe*I digestion shown in Tables 1 and 2).

Physical distances between *S*-linked genes in *B. rapa*

We used four restriction enzymes, *Bss*HII, *Nhe*I, *Not*I, and *Mlu*I in the PFGE gel blot analysis. *Nhe*I digested the *Brassica* genome more frequently than did *Mlu*I and *Bss*HII, and *Not*I digested it less frequently than did *Mlu*I and *Bss*HII (observation of the PFGE gel stained by ethidium bromide; data not shown). Results of the PFGE gel blot analysis of *B. rapa S* haplotypes after *Mlu*I and *Bss*HII digestion using the SLG^{45} and *SP4* probes are shown in Fig. 3, and the fragment sizes detected in this analysis are summarized in Table 1. Bands smaller than 20 kb were analyzed by conventional DNA gel blot analysis (Table 1).

The SLG^{45}, SP4, and *SP7* probes detected 60- to 90-kb single *Mlu*I fragments in all six *S* haplotypes of *B. rapa*. The *Mlu*I fragment of the S^9 haplotype, which hybrdidized with the SLG^{45}, *SP4*, and *SP7* probes, corresponds to the 76-kb E89 region (Suzuki et al. 1997,

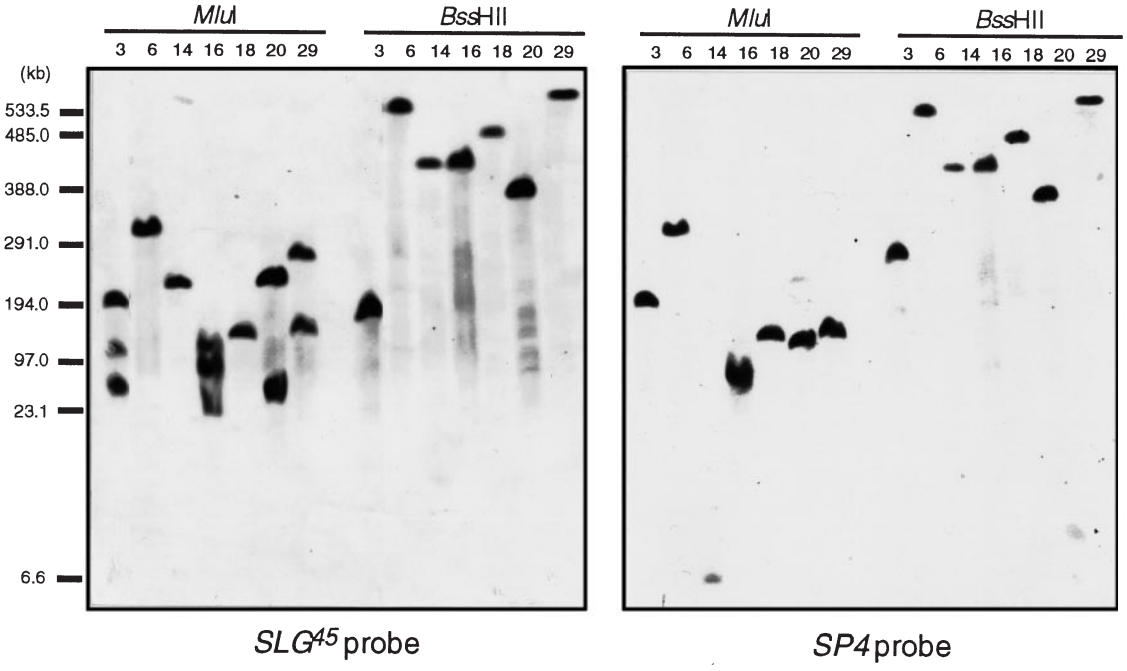

Fig. 4 PFGE gel blot analysis of *B. oleracea*. High-molecular weight genomic DNA was digested with *Mlu*I and *Bss*HII and then separated on a CHEF gel. The DNA was transferred to a membrane and hybridized with the SLG^{45} and *SP4* probes. The *number above* each lane *lane* indicates the *S* allelic number of each *S* homozygous plant. The probes are shown *below* the panels. DNA size markers are indicated on the *left* in kilobases (kb)

Table 2 Restriction fragments identified by PFGE gel blot analysis of the seven S hyplotypes in *B. oleracea*

Enzyme	*Mlu*I			*Bss*HII			*Nhe*I			*Not*I		
Probe	SLG^{45}	*SP4*	*SP7*	SLG^{45}	*SP4*	*SP7*	SLG^{45}	*SP4*	*SP7*	SLG^{45}	*SP4*	*SP7*
S^3	195/105/52[a]	195	105	170	280	120	27/18/13	5.3	ND[b]	> 780	> 780	40
S^6	330	330	330	540	540	140	75/23	6.8	ND	> 780	> 780	> 780
S^{14}	230	6.5	230	420	420	105	37/26	20	ND	> 780	> 780	> 780
S^{16}	115/72/21	72	115	420	420	120/67	52/32	5.3	32	> 780	> 780	> 780
S^{18}	130	130	25	485	485	485	52/32	5.3	32	> 780	> 780	> 780
S^{20}	240/45	120	240	380	380	120	35/12/6	5.3	65	> 780	> 780	> 780
S^{29}	280/140	140	280	>560	>560	>560	65/20	20	65	> 780	> 780	> 780

[a] Numbers indicate the length of restriction fragments (in kilobases)
[b] ND, Not determined

1999). These three probes detected the same bands in the S^8, S^{45}, S^{46}, S^{47}, and S^{48} haplotypes, suggesting that the *SLG*, *SRK*, *SP4*, and *SP7* genes are clustered within the region of 60–90 kb in the *B. rapa* genome. In these six *S* haplotypes, the sizes of the *Bss*HII fragment, including *SLG*, *SRK*, and *SP4*, were 130 kb (for S^8 and S^{45}), 190 kb (for S^{47} and S^{48}), and 210 kb (for S^9 and S^{46}). Most of the *S* haplotypes in *B. rapa* had a 600-650 kb *Not*I fragment including the *S* locus, while a significantly short *Not*I fragment (50 kb) including both *SLG* and *SRK* genes was observed in the S^{48} haplotype. The detection of the same fragment by the SLG^{45} and *SP7* probes and the detection of different bands by the *SP4* probe were obtained in the S^{46} haplotype with *Nhe*I digestion, the S^{47} haplotype with *Nhe*I digestion, and the S^{48} haplotype with *Nhe*I and *Not*I digestion.

Physical distances between *S*-linked genes in *B. oleracea*

Results of the PFGE gel blot analysis for *S*-linked genes in *B. oleracea* are shown in Fig. 4 and Table 2. *Mlu*I-digested DNA of the S^6, S^{14}, and S^{18} haplotypes showed single bands from 130 to 330 kb by the SLG^{45} probe, suggesting that both *SLG* and *SRK* genes were located in the same *Mlu*I fragment. In the other haplotypes (S^3, S^{16}, S^{20}, and S^{29}), two or three *Mlu*I fragments were detected by the SLG^{45} probe, suggesting that *SLG* and *SRK* were on different *Mlu*I fragments. In the PFGE analysis after *Bss*HII digestion using the SLG^{45} probe, single bands (170–420 kb) were observed in the S^3, S^{16}, and S^{20} haplotypes. From these results, physical distances between *SLG* and *SRK* in *B. oleracea* were estimated within 170, 330, 230, 420, 130, and 380 kb in the S^3, S^6, S^{14},

S^{16}, S^{18} and S^{20} haplotypes, respectively. The distance btween *SLG* and *SRK* in the S^{29} haplotype of *B. oleracea* could not be estimated in the present study because the single band obtained in the S^{29} haplotype with *Bss*HII appeared in a compression zone (> 560 kb) and was not separated under the present conditions. The fragments containing both *SLG* and *SRK* were relatively longer in *B. oleracea* than in *B. rapa*.

The *S*-locus complex region including other *S*-linked genes, *SP4* and *SP7*, was also longer in *B. oleracea* than in *B. rapa*. That the fragments were found to hybridize with all three probes only in two cases (S^6 with *Mlu*I, and S^{18} with *Bss*HII) clearly indicates the huge *S* region in *B. oleracea*. All the *Not*I fragments except for the 40-kb fragment detected in the S^3 haplotypes with the *SP7* probe were found in the compression zone (> 780 kb), supporting this indication. In the case of *Mlu*I or *Bss*HII digestion of all seven *S* haplotypes of *B. oleracea*, the existence of the same fragments detected by the SLG^{45} and *SP4* probes indicated the physical linkage between *SLG/SRK* and *SP4* and, similarly, the existence of the same fragments detected by the SLG^{45} and *SP7* probes indicated the physical linkage of *SLG/SRK* and *SP7*. The *SP4* and *SP7* genes, which were located at the both ends of the 76-kb region of the S^9 haplotype of *B. rapa*, are concluded to be tightly linked to the *SLG* and *SRK* genes in *B. oleracea*.

Discussion

We performed PFGE gel blot analysis of six *S* haplotypes in *B. rapa* and seven *S* haplotypes in *B. oleracea*. Physical distances between *SLG* and *SRK* in *B. rapa* were estimated to be significantly shorter than those in *B. oleracea*. It may be concluded that the *S*-locus complex region, including *SLG*, *SRK*, *SP4*, and *SP7*, is larger in *B. oleracea* than that in *B. rapa*. In addition, the *Mlu*I and *Bss*HII fragments containing *SLG* and *SRK* are similar in size within *B. rapa* but variable in *B. oleracea*. These results suggest that several large genomic fragments were inserted into the *S*-locus region of *B. oleracea* rather than that DNAs were deleted from the *B. rapa* *S* locus. It has been inferred that allelic differentiation of SI occurred before species divergence within the genus (Dwyer et al. 1991; Hinata et al. 1995; Uyenoyama 1995), and a striking sequence similarity of *SLG/SRK* between *B. rapa* and *B. oleracea* has been reported (Kusaba et al. 1997; Kusaba and Nishio 1999). Therefore, the large insertion into the *S*-locus region of *B. oleracea* might have occurred independently in different *S* haplotypes after *S*-allelic diversification. Boyes et al. (1997) speculated that the shorter physical distances between *S*-linked markers in the *B. rapa* S^8 haplotype than in the *B. oleracea* S^{13} haplotype might reflect the smaller genome size of *B. rapa* relative to *B. oleracea*. The insertions into *B. oleracea* could have occurred in the whole genome and might have contributed to the differentiation of *B. rapa* and *B. oleracea*.

In *B. rapa*, the *Mlu*I fragments containing both *SLG* and *SRK* were found to be 60–90 kb in length. These fragments can be easily cloned in artificial chromosome vectors, such as PAC (P1-derived artificial chromosome) and BAC (bacterial artificial chromosome), by using a previously described method (Suzuki et al. 1997). The 76-kb *Mlu*I fragment containing the *S* locus of the S^9 haplotype of *B. rapa* has been found to contain 12 genes, in addition to *SLG* and *SRK* (Suzuki et al. 1999; Watanabe et al. 1999). Two genes, *SP4* and *SP7*, out of the 12 *S*-linked genes were found to be physically linked to *SLG* and *SRK* in all the *S* haplotypes analyzed in this study. In addition, the observations that the SLG^{45} and *SP7* probes detected the same fragment but that the *SP4* probe detected different bands in three *S* haplotypes (S^{46}, S^{47}, and S^{48}) of *B. rapa* would indicate that the physical distance between *SP4* and *SLG/SRK* might be longer than that between *SP7* and *SLG/SRK* in these *S* haplotypes, as in the case of the S^9 haplotype. Although the precise location and orientation of the 12 *S*-linked genes are not known in all but the S^9 haplotype, it may be said that these 12 genes are located near *SLG/SRK* in both *B. rapa* and *B. oleracea*. Therefore, the cloning of the *Mlu*I fragments in several different *S* haplotypes will reveal the allelic sequence differences of *SP11* and *SAE1* which are pollen *S*-gene candidates of the 12 *S*-linked genes (Suzuki et al. 1999; Watanabe et al. 1999). Because allelic divergences of the *SP11* and *SAE1* genes have been suggested to be too high to detect alleles in different *S* haplotypes by DNA gel blot analysis (Suzuki et al. 1999; Watanabe et al. 1999), the cloning of the *Mlu*I fragment followed by DNA sequence analysis is one possible strategy to determine the alleles of *SP11* and *SAE1* in several different *S* haplotypes.

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