Theor Appl Genet (1998) 96: 468–474

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Organization of an *Arabidopsis thaliana* gene cluster on chromosome 4 including the *RPS2* gene, in the *Brassica nigra* genome

Received: 11 July 1997 / Accepted: 9 October 1997

Abstract Genetic and physical maps, consisting of a large number of DNA markers for *Arabidopsis thaliana* chromosomes, represent excellent tools to determine the organization of related genomes such as those of *Brassica*. In this paper we report the chromosomal localization and physical analysis by pulsed-field gel electrophoresis (PFGE) of a well-defined gene complex of *A. thaliana* in the *Brassica nigra* genome (B genome n = 8). This complex is approximately 30 kb in length in *A. thaliana* and contains a cluster of six genes including *ABI1* (ABA-responsive), *RPS2* (resistance against *Pseudomonas syringae*, a bacterial disease), *CK1* (casein kinase I), *NAP* (nucleosome-assembly protein), *X9* and *X14* (both of unknown function). The *Arabidopsis* chromosomal complex was found to be duplicated and conserved in gene number at different levels in the *Brassica* genome. Linkage group B1 had the most-conserved arrangement carrying all six genes tightly linked. Group B4 had an almost complete complex except for the absence of *RPS2*. Other partial complexes of fewer members were found on three other chromosomes. Our studies demonstrate that by this approach it is possible to identify ancestrally related chromosome segments in a complex and duplicated genome, such as the genome of *B. nigra*, permitting one to draw conclusions as to its origin and evolution.

Key words Microsynteny · Genome · Gene cluster · RFLP · PFGE

Communicated by M. A. Saghai Maroof

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Introduction

Cultivated diploid *Brassica* species occupy a different genomic status on the basis of their distinct chromosome numbers: n = 8 for *B. nigra* (B genome), n = 9 for *B. oleracea* (C genome), and n = 10 for *B. rapa* (A genome). Abundant conservation of linkage arrangement for numerous chromosome segments in the three *Brassica* genomes is the rule. However, extensive genomic duplication, rearrangement of chromosome segments and the formation of new chromosomal linkages are widespread in all three genomes (Truco et al. 1996). Therefore the evolutionary history of these complex genomes is difficult to follow. Based mostly on RFLP data, our knowledge on the origin of the *Brassica* genomes can be summarized as follows: (1) The ancestral genome had less chromosomes than present-day genomes (Kowalski et al. 1994; Lagercrantz et al. 1996; Truco et al. 1996), (2) it has undergone extensive modifications by chromosomal rearrangements and changes in chromosome number in a cumulative fashion leading to the derivation of new genomes (Quiros et al. 1987; Kianian and Quiros 1992) and (3) further genomic rearrangements have taken place in derived genomes by intra- and inter-genomic recombination between homologous segments shared between different chromosomes (Quiros et al. 1994).

Chromosomal co-linearity within the *Brassica* genus has been determined with the help of F_2 linkage maps sharing common DNA probes. However, this type of map-comparison provides only a crude estimation of genome structure and organization in the species compared because of insufficient marker saturation in the existing maps (Kowalski et al. 1994; Teutenico and Osborn 1994; Truco et al. 1996). This limitation does not permit following all minor rearrangements, whose identification could facilitate understanding the possible mechanisms involved in the evolution of the different genomes. To address this problem, comparative

Fig. 1 Map of *Eco*RI restriction endonuclease sites within the *A. thaliana* gene cluster composed of six transcribed genes (Mindrinos et al. 1994). Transcripts: *1-ABI1*; *2-RPS2*; *3-CK1*; *4-NAP*; *5-X9*; *6-X14*

micro-colinearity studies have been recently initiated (Sadowski et al. 1995, 1996; Lagercrantz et al. 1996) between the maps of *Arabidopsis thaliana* and *Brassica*. The crucifer *A. thaliana* has been chosen for these studies not only because of the wealth of information gathered on its genomic structure, but also because of its small and simple genome. In a previous study we followed a 15-kb segment of five genes including the *Em*-like gene on chromosome 3 (Gaubier et al. 1993) in all three *Brassica* cultivated diploids. We found that this well-characterized single-copy DNA segment in *A. thaliana* was present in two and three copies in the different *Brassica* species, one of which conserved the *A. thaliana* array. Additionally, the physical sizes of the clusters carrying these genes were larger in *Brassica*.

In the present paper we report on the distribution and physical organization of a second gene cluster in *B. nigra*. This comprises six genes, two of them well characterized: the *RPS2* gene, which codes for resistance to *Pseudomonas syringae* (Bent et al. 1994; Mindrinos et al. 1994), and *ABII*, the abscisic acid-responsive gene (Leung et al. 1994). This gene complex located on chromosome 4 is 30-kb long in *A. thaliana*. Its physical map and sequence organization has been recently described (Mindrinos et al. 1994). We used the six genes in the cluster (Fig. 1) as probes to identify corresponding chromosomes segments in the *B. nigra* genome by RFLP and pulse-field gel electrophoresis.

Materials and methods

Genetic mapping

Plant material

An existing F_2 mapping population for *B. nigra* (83 individuals, Truco and Quiros 1994) was used for genetic linkage analysis of genes involved in the *Arabidopsis* gene complex described below.

Probes

cDNAs corresponding to five genes located within the 30-kb gene complex from *A. thaliana* (Mindrinos et al. 1994); *ABII* (ABA insensitive), *RPS2* (resistance against a bacterial disease caused by *P. syringae*), *CK1* (casein kinase I), *NAP* (nucleosome-assembly protein), *X9* (function unknown), and *X14* (function unknown) were used as probes (Fig. 1). Probes were prepared by random hexamer labeling (Feinberg and Vogelstein 1983).

RFLP analysis

DNA purification, digestion, and agarose electrophoresis were carried out as described earlier (Truco and Quiros 1994). *Eco*RI, *Eco*RV, *Bam*HI, and *Hin*dIII restriction endonucleases were used for DNA digestion. Alkaline transfer onto Zeta-Probe GT membranes and hybridization were carried out following the manufactuer's protocol (Bio-Rad). After hybridization, membranes were washed at moderate stringency: twice in 5% SDS, 20 mM $Na_2HPO_4 \times 7H_2O$ at 65°C for 15 min and twice in 2% SDS, 20 mM $Na_2HPO_4 \times 7H_2O$ at 65°C for 10 min.

F_2 linkage analysis

The F_2 linkage mapping was performed with MAPMAKER (Lander et al. 1987). Segregation ratios different from expected values (significant at $P = 0.05$ or less) were considered as distorted.

Physical mapping

Isolation of protoplasts

High-quality protoplasts from leaves of *B. nigra* were prepared using the protocol of Crucefix et al. (1987) as modified by Sadowski et al. (1996). High-quality protoplasts were obtained from 3-week old primary leaves of *B. nigra*.

DNA digestion with methylation-sensitive rare-cutting endonucleases

*Mlu*I, *Sac*II (New England Biolabs), and double *Mlu*I/*Sac*II restriction digestions were used for the digestion of isolated mega-size DNA. Digestion of the DNA plugs was carried out as described earlier (Sadowski et al. 1996).

Pulsed-field gel electrophoresis

Contour-clamped homogeneous electric field (CHEF, BioRad) gels (Chu et al. 1986) were run at 6 V/cm for 18 h with a switch time at a ramp from 15 s to 60 s at 14°C (for best resolution for restriction fragments of 20–500 kbp). DNA size markers were lambda DNA concatamers and yeast chromosomes (New England Biolabs). After separation, gels were de-purinated for 15 min in 0.25 N HCL and transferred to a Zeta-Probe GT membrane for 32–36 h with 0.4 N NaOH.

Hybridization and washing were carried out following the conditions described above.

Results

Genetic mapping

RFLP analysis

B. nigra Southern blots after hybridization with the six *A. thaliana* cDNAs revealed a simple pattern of two

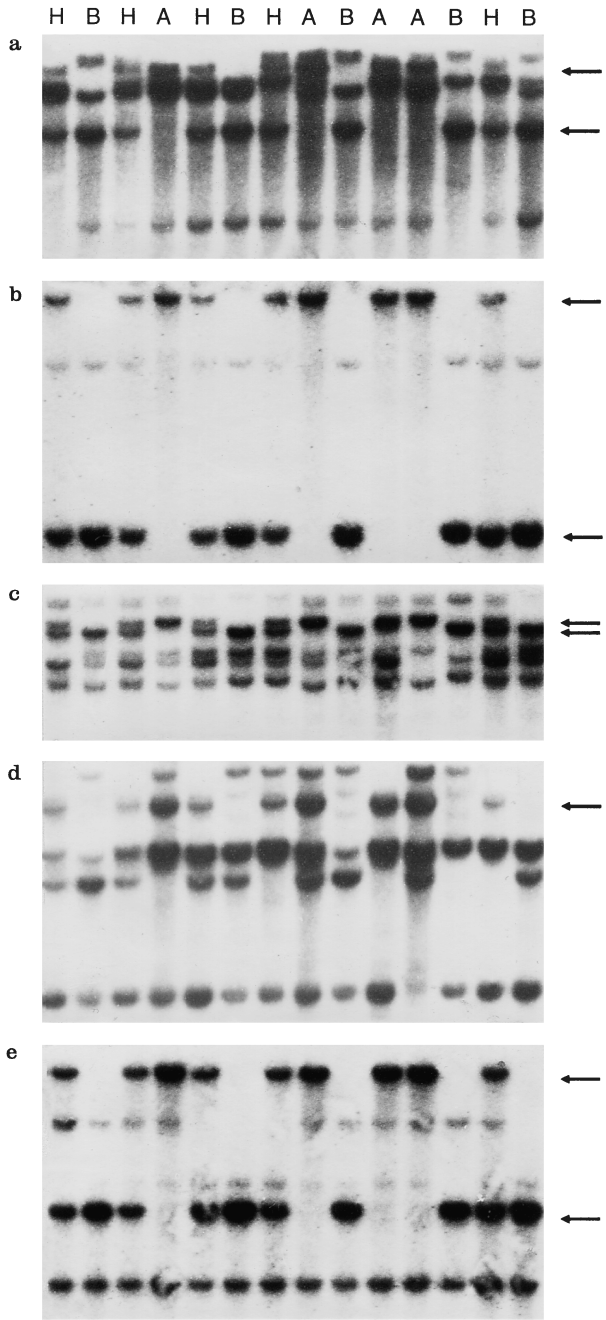

Fig. 2a–e Southern hybridization of *Eco*RI-digested DNA isolated from F_2 individuals with *ABI1* **a**, *RPS2* **b**, *CK1* **c**, *NAP* **d** and *X9* **e** as probes. The same segregation pattern (*A and B* – homozygotes, *H* – heterozygotes) of two co-dominant bands was found with these probes. Restriction fragments corresponding to the conserved segment on the B1 chromosome are indicated by *arrows*. The fast migrating co-dominant band disclosed with the NAP probe is not shown

co-dominant bands for the RPS2 probe (Fig. 2b) and complex patterns for the five other probes, including a mix of dominant and co-dominant bands (Fig. 2a, c, d, and e). These two types of hybridizing patterns were observed regardless of the restriction endonuclease used for digestion. All together 17 RFLP loci were identified in the *B. nigra* genome by the six *A. thaliana* probes. Polymorphism in the six-gene cluster appeared to be high since digestion with *Eco*RI disclosed 60% of all bands as polymorphic. All together, 82% of bands showed polymorphism after digestion with all four restriction endonucleases, *Bam*HI, *Eco*RI, *Eco*RV and *Hin*dIII, used in the study. As far as heterologous hybridization is concerned, we found that the signal intensity was rather stable. The membranes, however, required frequent monitoring during washing after hybridization to assure retention of the bound probes.

Linkage studies

Distorted segregation ratios were found for two loci, *NAPc* and *ABI1e*, out of 17 (11.76%), which is in agreement with previous mapping studies done with the same intra-species mapping population (Truco and Quiros 1994). The 17 RFLP loci were localized on five different linkage groups in *B. nigra* (Fig. 3). Gene clusters consisting of four to five members were found on two of the *Brassica* linkage groups, B1 and B4. These were the *ABI1a*, *RPS2*, *CK1a*, *NAPa*, and *X9a* complex on B1, and the *ABI1a*, *CK1b*, *NAPb*, and *X9b* complex on group B4. The gene members on B1 were tightly linked within 1.2 cM, whereas those on B4 were linked within a 3.9-cM segment. The absence of *X14* on both linkage groups, expected from the *A. thaliana* arrangement, could not be attributed to the absence of this gene, but rather to the lack of segregation of monomorphic bands observed for all the endonucleases used in this study. On the other hand, the absence of *RPS2* on B4 was real, since this probe disclosed only a single locus regardless of which of the four enzymes was used for DNA digestion (see for example Fig. 2b). The gene order in B1 and B4 shown in Fig. 3 is arbitrary for members linked at 0.0 cM. Another distinction of these two groups, in relation to the *A. thaliana* gene-cluster organization, was the presence of duplicated gene members. *ABI1* was present in duplicate on both B1 and B4 groups, 15.3 cM and 4.4 cM apart, respectively. Recombinant individuals were recovered between *ABI1d* and *ABI1b* on group B4.

In addition to the B1 and B4 clusters in *B. nigra*, three other linkage groups were also found to carry one or two other genes of the *A. thaliana* cluster. B2 had a 4.0-cM triplet consisting of two *NAP* duplicates (*NAPd* and *NAPe*) and *X14b*; B8 had two loci, *NAPc* and *ABI1e*, spaced at 13.8 cM, and B3 had a single *X14* locus (*X14a*) (Fig. 3).

Physical mapping

PFGE followed by sequential Southern hybridization of *B. nigra* DNA with all six *A. thaliana* cDNAs

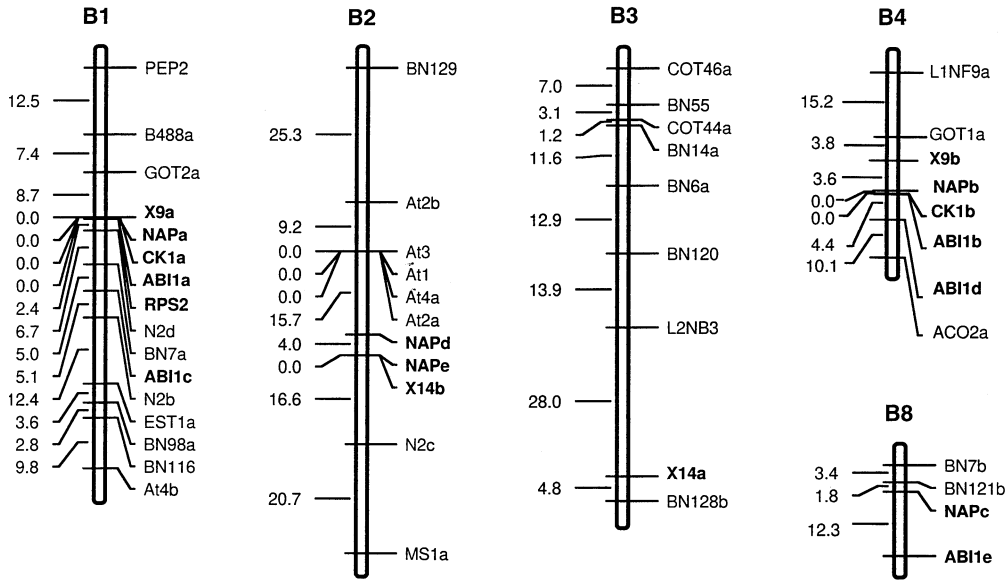

Fig. 3 Linkage map of *B. nigra* showing the corresponding loci of the *A. thaliana* chromosome-4 gene cluster (*in bold*). Genetic distances in cM are indicated at the left. The order of loci linked at 0.0 cM is arbitrary

disclosed several restriction fragments carrying from one to six gene sequences (Fig. 4). This electrophoretic approach disclosed the presence *X14* on group B1, which was not detected by linkage analysis. Therefore, B1 conserves the *A. thaliana* gene arrangement at least in respect of the number of gene members (Fig. 5).

The distribution of clones on the restriction fragments generated by PFGE is shown in Table 1. The analysis of hybridizing restriction patterns permitted us to approximate the physical distances separating the members for some of the gene clusters in *B. nigra*. This allowed us to construct the physical map of two segments corresponding to linkage groups B1 and B2. The *Bss*HII digestion (data not shown) produced a restriction fragment 290-kb long, which hybridized to all six probes. This fragment corresponded to B1, since this is the only linkage group carrying all six genes (Figs. 3, 5). The six-gene cluster, however, was included in a shorter segment of only 255 kb inside the *Bss*HII fragment, which is the minimal size for this cluster in *B. nigra*. The six genes in the cluster were mapped on different *Mlu*I fragments as follows: *ABI1* and*RPS2* on a 38-kb fragment, *CK1* on a 30-kb fragment, *NAP* on a 22-kb fragment, *X9* on a 100-kb and *X14* on a 35-kb fragment. An additional *Mlu*I restriction fragment of 5 kb which hybridized with the RPS2 probe revealed a *Mlu*I site within the *RPS2* gene (Fig. 5).

Concerning the partial complexes localized on the other groups, it was only possible to approximate the physical distance of two gene members on group B2. The presence of a 27-kb *Mlu*I/*Sac*II restriction fragment disclosed by the *NAP* and *X14* probes (Figs. 4c, 4 and 6) implies that these two genes are linked on the B2 chromosome within a distance not exceeding 27 kb.

Double-banded patterns were visible for a number of genes in the PFGE blots. For example: for *ABI1*, *RPS2*, and *CK1* (Fig. 4b, *Sac*II 145- and 115-kb bands); and for *AB1* and *RPS2* (Fig. 4 a, *Mlu*I 47- and 38-kb bands; and Fig. 4 c, *Mlu*I/*Sac*II, 47-kb and 38-kb bands). The double banding for *RPS2* is inconsistent with the RFLP data which clearly indicate that only a single copy of *RPS2* exists in the *B. nigra* genome.

Discussion

The duplicated nature of the *B. nigra* genome could be appreciated by the re-iterated gene members of the single-copy *A. thaliana* gene complex. The six *A. thaliana* loci on a single chromosome were expanded to 17 in *B. nigra* and distributed on five linkage groups that seem to represent independent chromosomes (Truco and Quiros 1994). The only apparent single-copy gene in the complex detected in *B. nigra* was *RPS2*. The double-banded pattern of the PFGE fragments disclosed by the RPS2 probe was most likely due to partial digestion resulting from methylation of the *Sac*II an *Mlu*I recognition sites, a frequently observed phenomenon in plant genomes (van Daelen et al. 1993). At this point it is not possible to tell whether the absence of multiple copies of these genes is related to a susceptibility of the individuals tested due to deletion of the *RPS2* genes on other linkage groups.

The total count of PFGE fragments arising by single and double digestion made it possible to make a correspondence of only two linkage groups, B1 and B2, to their respective physical maps (Figs. 3 and 5). This was due to the fact that the number of PFGE fragments generated was less than those expected on the basis of the number of loci disclosed by the RFLP analysis. This observation was not unforeseen for the segments

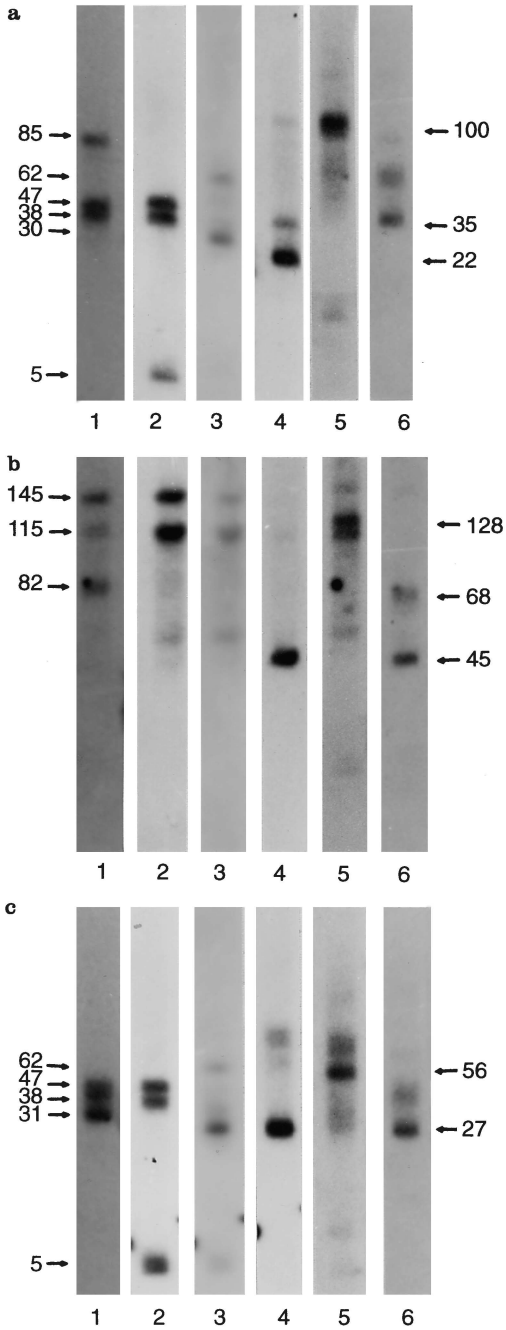

Fig. 4a–c PFGE separation and sequential hybridization fragments after *Mlu*I **a**, *Sac*II **b** and *Mlu*I/*Sac*II **c** double digestion with *ABI1* (*1*), *RPS2* (*2*), *CK1* (*3*), *NAP* (*4*), *X9* (*5*), and *X14* (*6*) probes. Bands and their sizes (in kb) are indicated by the corresponding *arrows and numbers*

on groups B3 and B8, which contain only one and two loci, respectively. However, the lack of detection by PFGE of B4, the second largest segment consisting of five loci, was unexpected. It is possible that this was caused by the conservation of *Mlu*I and *Sac*II restriction sites in the B1 and B4 segments, which will result in the overlapping of fragments of similar sizes. In particular, the *NAP* probe revealed fewer bands for all three PFGE digests than those detected by RFLP analysis. The absence of shorter PFGE bands than those observed for B1 suggests that the B4 chromosomal segment carrying copies of the *ABI1*, *CK1*, *NAP* and *X9* genes is at least as long as its corresponding segment on the B1 chromosome. Faint PFGE bands, especially for *X9*, could not be scored reliably. These may represent sequences of lower homology to the probe used in the hybridization.

Of the five *B. nigra* linkage groups carrying members of the gene complex, only group B1 conserved the *A. thaliana* gene number. Not much can be said at this point on gene-order conservation, because of the complete linkage of some members failing to produce recombinants by F_2 linkage analysis. Group B4 carried four of the six *A. thaliana* genes except for the absence of *RPS2*. It is possible that the *X14* gene was also present, but lack of polymorphism in the parents of the F_2 population precluded its disclosure. The similarity of the chromosome segments in groups B1 and B4, conserving most of the loci from *Arabidopsis*, indicated that they are ancestrally related. This is further supported by the size-similarity of the restriction fragments, which may have precluded their resolution by PFGE. A similar phenomenon has been observed in soybean by Funke et al. (1993) due to the conservation of rare-cutting enzyme sites. Lagercrantz et al. (1996), also observed higher structural conservation of two groups out of three for a 1.5-Mb contig from *A. thaliana*. We failed, however, to find the widespread triplication of loci in *B. nigra* reported by them. For example, sequences *ABI1* and *NAP* disclosed five loci each. In addition to the two main groups, which were conserved, the other partial smaller segments found on B2, B3 and B8 carried just a few members of the *A. thaliana* chromosome-4 complex. It remains to be seen whether these were derived from the ancestral groups carrying complete, or almost complete, complexes.

The extended size of the six-gene cluster in B1 of *Brassica* compared with the size of this cluster in *A. thaliana* is in agreement with the genome-size difference of these two species, 468 Mb for the former and 145 Mb for the latter (Arumuganathan and Earle 1991). The same size-differential range was observed for another gene complex on chromosome 3 of *A. thaliana*, which was also duplicated in two and three chromosomes of the diploid cultivated *Brassica* species (Sadowski et al. 1996). This observation does not agree with the conjecture that, in general, gene spacing is similar for *A. thaliana* and *B. nigra* (Lagercrantz et al. 1996).

The distribution of segments carrying duplicated complexes sharing structural features agrees with our hypothesis on the origin of the cultivated *Brassica* genomes by amphiploidy. This hypothesis assumes an ancestral genome of 5–7 chromosomes, which differentiated into several related genomes with similar chromosome numbers (Truco et al. 1996). These diverged

Table 1 Sequences contained in specific PFGE fragments after single and double digestions

Enzyme	Fragment size (kb)	Sequences	Figures
*Bss*HII	290	ABI1, RPS2, CK1, NAP, X9, X14	(Data not shown)
*Mlu*I	100	X9	(Fig. 4 a, ln 5)
*Mlu*I	85	ABI1	(Fig. 4 a, ln 1)
*Mlu*I	62	CK1	(Fig. 4 a, ln 3)
*Mlu*I	47	ABI1, RPS2	(Fig. 4 a, ln 1, 2)
*Mlu*I	35	NAP, X14	(Fig. 4 a, ln 4, 6)
*Mlu*I	30	CK1	(Fig. 4 a, ln 3)
*Mlu*I	22	NAP	(Fig. 4 a, ln 4)
*Mlu*I	5	RPS2	(Fig. 4 a, ln 2)
*Sac*II	145	ABI1, RPS2, CK1	(Fig. 4 b, ln 1, 2, 3)
*Sac*II	128	X9	(Fig. 4 b, ln 5)
*Sac*II	115	ABI1, RPS2, CK1	(Fig. 4 b, ln 1, 2, 3)
*Sac*II	115	X9	(Fig. 4 b, ln 5)
*Sac*II	82	ABI1	(Fig. 4 b, ln 1)
*Sac*II	68	X14	(Fig. 4 b, ln 6)
*Sac*II	45	NAP, X14	(Fig. 4 b, ln 4, 6)
*Mlu*I/*Sac*II	62	CK1	(Fig. 4 c, ln 3)
*Mlu*I/*Sac*II	56	X9	(Fig. 4 c, ln 5)
*Mlu*I/*Sac*II	47	ABI1, RPS2	(Fig. 4 c, ln 1, 2)
*Mlu*I/*Sac*II	38	ABI1, RPS2	(Fig. 4 c, ln 1, 2)
*Mlu*I/*Sac*II	31	ABI1	(Fig. 4 a, ln 1)
*Mlu*I/*Sac*II	27	NAP, X14	(Fig. 4 c, ln 4, 6)
*Mlu*I/*Sac*II	27	CK1	(Fig. 4 c, ln 3)
*Mlu*I/*Sac*II	5	RPS2	(Fig. 4 c, ln 2)

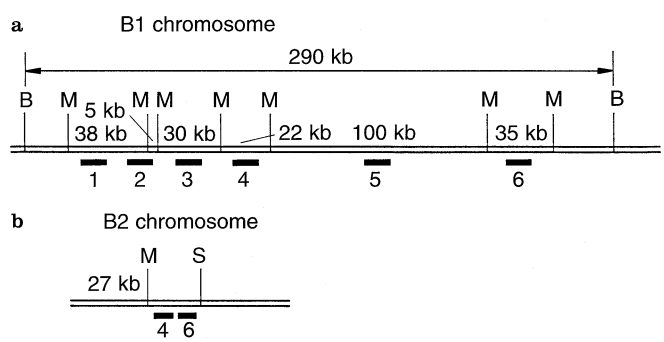

Fig. 5 Physical map of the B1 chromosome cluster (**a**) and the B2 chromosome partial cluster (**b**). *B* = *Bss*HII, *M* = *Mlu*I, *S* = *Sac*II. *1* = *ABI1*, *2* = *RPS2*, *3* = *CK1*, *4* = *NAP*, *5* = *X9*, and *6* = *X14*

from each by geographical isolation, which provided the opportunity for changes in chromosomal structure due mainly to the accumulation of translocations. By hybridization, these genomes were combined together in amphiploids, so originating the genomes of the diploid *Brassica* species. In the process, aneuploidy and secondary chromosomal rearrangements further modified the genomes resulting in the present-day *Brassica* genomes of n = 8, 9 and 10 (Truco et al. 1996). This hypothesis explains the existing level of locus duplication and sporadic locus triplication observed in the RFLP maps of *Brassica*. Further, it makes it unnecessary to invoke an ancestral genome of less than four chromosomes, which does not exist in the *Cruciferae*, and the synthesis of three ancestral genomes by hexaploidy (Lagercrantz et al. 1996), which is not a common natural event in the family.

The postulated mechanism of genomic synthesis in *Brassica* has occurred again at a higher hierarchy in the existing cultivated amphidiploid species, *B. napus* (2n = 4x = 38, A and C genomes), *B. carinata* (2n = 4x = 34, B and C genomes) and *B. juncea* (2n = 4x = 36 A and B genomes). These species have arisen by a second cycle of amphiploidization involving the A, B and C genomes. Therefore, our hypothesis on the origin of the *Brassica* genomes is simply based on existing genome molding events observed today in *Brassica* species, such as translocations (Quiros et al. 1988; Kianian and Quiros 1992), amphiploidy and aneuploidy (U 1935).

Acknowledgements We are indebted to Jinguo Hu and Vincent D'Antonio for technical assistance to Frederick Ausubel for providing the *A. thaliana* DNA clones. Research supported by USDA grant # 92-01568 to C.F.Q. and in part by KBN (Poland) grant #5 PO6A 016 11 to J.S.

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