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Molecular and cytological evidence of deletions in alien chromosomes for two monosomic addition lines of *Brassica campestris-oleracea*

J. Hu and C.F. Quiros

Department of Vegetable Crops, University of California at Davis, Davis, CA 95616, USA

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Summary. A series of RFLP and isozyme markers were followed in the progenies of two alien addition lines of *Brassica campestris-oleracea*. One of the lines, carrying the C genome chromosome 4 as the alien chromosome, was surveyed for six markers. Fifty-four percent of the plants carrying alien chromosomes displayed all the expected makers, whereas the rest had one to five markers missing. The second line for C genome chromosome 5 displayed a similar behavior when surveyed for three markers. All three markers were transmitted together in 46% of the plants carrying alien chromosomes, whereas the rest carried only one or two of the markers. The loss of markers was associated with reduced chromosome size caused by deletions. The observed chromosome deficiencies permitted deletion analysis for a rough physical mapping and ordering of the markers on the two C genome chromosomes. The deletions observed may represent another mechanism for molding the chromosomes of the *Brassica* genomes during their evolution.

Key words: RFLP – Deletion mapping – Genome evolution

Introduction

The generation of chromosome addition lines in *Brassica* has opened new horizons for studying the genetics and evolution of this complex genus. A series of these lines representing the *B. oleracea* (C genome, n=9) and *B. nigra* (B genome, n=8) genomes has been developed (Quiros et al. 1989). *B. campestris-oleracea* addition lines were extracted from the amphidiploid species *B. napus* (AC genomes, n=19) and from 'Hakuran', an artificially synthesized *B. napus* (McGrath 1989). The C genome chromosomes extracted from these lines have been mapped with a series of RFLP and isozyme markers (McGrath 1989; Hosaka et al. 1990).

The objective of this work was to determine the fidelity of two alien C genome chromosome in the progenies of monosomic addition lines. This determination was done on the basis of chromosome morphology and by surveying markers known to be present on these chromosomes.

Material and methods

Interspecific aneuploids

A series of *B. campestris* alien addition lines for two *B. oleracea* chromosomes was used in this study. The development of these lines has been reported elsewhere (Quiros et al. 1987; Hosaka et al. 1990; McGrath 1989). In order to generate sufficient alien addition plants for this study, interspecific aneuploids of chromosome numbers ranging from 2n=21 to 2n=24 were backcrossed to the diploid recipient parent *B. campestris* (B233) from two to five times (Table 1).

Addition line 4. A progeny of 98 plants derived by selfing a monosomic addition line (2n=21) for C genome chromosome 4 was used to follow up six markers previously located on this chromosome. The alien chromosome of this line was extracted from the natural amphidiploid *B. napus* (Quiros et al. 1987).

Addition line 5. Three progenies derived from selfing three monosomic addition lines for C genome chromosome 5, extracted from either 'Hakuran', a synthesis *B. napus*, (McGrath 1989) or natural *B. napus* (Table 1), were used to follow up three chromosome 5 RFLP markers. Two additional progenies, resulting from crossing a double monosomic addition line (2n=22) and a hyperploid carrying four alien C genome chromosome to *B. campestris* (B233), were studied. These derived from *B. napus* (Table 1).

Table 1. Progenies used for determining the genotypes of two C genome chromosomes present in *B. campestris-oleracea* alien addition lines

Line	Family	2n of parent	*N*	No. back-crosses to *B. campestris*	Origin
4	89B256	21	98	4	*B. napus*
5	89B405	24	28	2	*B. napus*
5	89B259	21	41	3	'Hakuran'
5	89B406	22	28	4	*B. napus*
5	89B386	22	22	4	*B. napus*
5	89B381	21	15	6	*B. napus*

Chromosome markers

Three isozyme loci, *6pgd1*, *6pgd2*, and *Pgm-2* (Quiros et al. 1987), and six DNA clones were used as chromosome markers. Clones pBN33, pBN113, pBN127, pBSIL9, and pCOT46 were isolated from a cDNA library of *B. napus* embryos (Harada et al. 1988). Clone pB547 was obtained from a *B. napus* genomic library (Hosaka et al. 1990). In previous studies (Quiros et al. 1987; McGrath 1989; Hosaka et al. 1990; McGrath et al. 1990), these markers were located on C genome chromosomes 4 and 5 as follows (order arbitrary):

Chromosome 4: *6pgd2, Pgm-2,* pBN113, pBN127, pBSIL9, and pB547
Chromosome 5: *6pgd1*, pBN33, pCOT46.

Enzyme electrophoresis

The isozyme markers for 6-phosphogluconase-dehydrogenase (6PGDH) and phosphoglucomutase (PGM) were determined by horizontal starch electrophoresis of leaf crude extracts (Quiros et al. 1987, 1988).

Southern analysis

DNA was isolated by the method described by Tanksley et al. (1988). Two to five gram of freshly harvest young leaf tissue was placed in a mortar and grounded into fine powder, after adding a spoonful of sand and about 20 ml of liquid nitrogen. The powder was the transferred into a 50-ml plastic tube along with extraction buffer (350 m*M* sorbitol, 100 m*M* trizma, and 5 m*M* EDTA, pH 8.2). The slurry was lysed at 65 °C for 20 min in the presence of 1% sarkosyl, 1 *M* NaCl, 0.2% CTAB (hexadecyl trimethyl ammonium bromide), and 25 m*M* EDTA. An equal amount of chloroform was then added to the lysate, mixed well, and the two phases were separated by centrifugation at 2,000 rpm in a desktop centrifuge for 20 min. The aqueous phase was transferred to a new tube and mixed with an equal volume of isopropanol to precipitate the DNA. After centrifuging for 5 min, the pellet was washed with 70% ethanol and dried in a vacuum oven for 1 hour. The pellet was then resuspended in 0.75 ml TE buffer (10 m*M* TRIS-HCl, pH 8.0, 1 m*M* EDTA). The concentration of the isolated DNA was determined by comparing band intensity with known lambda DNA controls on a minigel.

Three to five micrograms of genomic DNA was digested with EcoRI and electrophoresed in a 0.8% agarose gel containing 0.05% ethidium bromide in TRIS-acetate-EDTA buffer (TAE buffer) (Maniatis et al. 1982). After running the gels at 25 V for 15–20 h, the DNA was transferred into an Amersham Hybond-N nylon membrane following the manufacturer's protocol.

The probes were labelled with ^{32}P-dCTP using the random primer oligolabelling method (Feinberg and Vogelstein 1983 a, b). Hybridization was made in a Rubbermaid drawer organizer box (15 × 20 cm) in 25 ml of hybridization buffer (5 × SSPE, 5 × Denhardt's solution, 0.5% SDS, and 500 μg/ml sonicated fish DNA) for 18–20 h at 65 °C. After washing with 100 ml of 2 × SSPE and 1% SDS at room tempeature for 30 min, and then with 100 ml of 0.2 × SSPE and 1% SDS at 65 °C for the same length of time, the membrane was air dried and wrapped with saran wrap. It was then exposed to Kodak X-ray film at −80 °C for 1–3 days. Membranes were reprobed after the radiolabelled probes were removed by strip washing in 0.1 × SSC and 0.1 SDS at 92 °C for 1–2 h.

Cytological examination

Flower buds were collected by mid-morning and fixed in 1 part propionic acid:3 parts 95% ethanol. After 24 h the buds were washed with 50% ethanol and tranferred into 70% ethanol. The anthers were squashed in a drop of 1% acetocarmine on a slide and observed with a Zeiss universal microscope equipped with photomicrography. Chromosome counts and karyotypes were done at diakinesis of approximately 20 cells with well-spread chromosomes. Pictures were taken at 1,250 × with Kodak Technical Pan film.

Results

Survey of markers of C genome chromosome 4

As a first step, we screened at the seedling stage a progeny of 98 plants for the presence of *B. oleracea* chromosome 4-specific isozymes *Pgm-2* and *6pgdh2*. The prescreening was done for an early elimination of putative diploids in the progeny and to focus the Southern analysis only on plants known to carry C genomes markers. This resulted in the elimination of 74 plants that were negative for the *B. oleracea* isozymes. The transmission to the entire progeny of at least one chromosome 4 isozyme marker was 24%. Among the 24 plants surveyed for the RFLP markers, six different genotypes for the alien chromosome were observed. Fifty-four percent of these plants carried the six expected markers, whereas the rest displayed the loss of one to five markers (Table 2). Figure 1 shows the genotypes for four RFLP markers representative of the C genome chromosome 4.

Chromosome number 5

Because the smaller number of markers available for this chromosome, all 139 plants from the five progenies studied were tested for the three RFLP markers (Table 3). Forty-three plants displayed at least one marker for the *B. oleracea* alien chromosome. The transmission of at least one chromosome 5 marker among progenies, however, was quite different. Considering first those from the 2n = 21 parents, family 89B259 from 'Hakuran' had a transmission of 27%, whereas in 89B381 from natural *B. napus* it was only 7%. The transmission in the families derived from the two double monosomic additions was

between 14 and 21%. However, this value increased to 79% in the family derived from the 2n = 24 plant.

Approximately 47% of the plants carrying the C genome chromosome 5 displayed the three expected markers. The rest bore only one or two markers (Fig. 2). A plant derived from family 89B381 carried only the isozyme marker *6pgd1*.

Cytological survey

For line 4, 16 of the 24 plants positive for the C genome chromosome markers were subjected to chromosome counts. As expected, these plants were found to have 21 chromosomes. No diploids carrying C genome-specific markers were observed. Closer examination of the alien

Table 2. Genotypes for C genome alien chromosome 4 for two isozyme and four RFLP markers

pBN113	*Pgm-2*	pBN127	PB547	pBSIL9	*6pgdh2*	No. plants	Percent
+	+	+	+	+	+	13	54.1
+	+	+	+	+	−	2	8.3
−	+	+	+	+	+	1	4.2
−	−	−	+	+	+	1	4.2
−	−	−	−	−	+	6	25.0
−	+	−	−	−	−	1	4.2

Table 3. Genotypes and transmission of C genome chromosome 5 for three RFLP markers in five progenies

Markers pCOT46	pBN33	*6pgdh1*	No. plants per family					Total	Percent
			89B259	381	386	406	405		
+	+	+	5	0	3	5	7	20	46.5
−	+	+	1	0	0	1	8	10	23.3
+	+	−	2	0	0	0	1	3	7.0
−	−	+	0	1	0	0	0	1	2.3
+	−	−	3	0	0	0	6	9	20.9
No. plants in progeny			41	15	22	28	28	139	
% Transmission of alien chromosome			27	7	14	21	79	31	

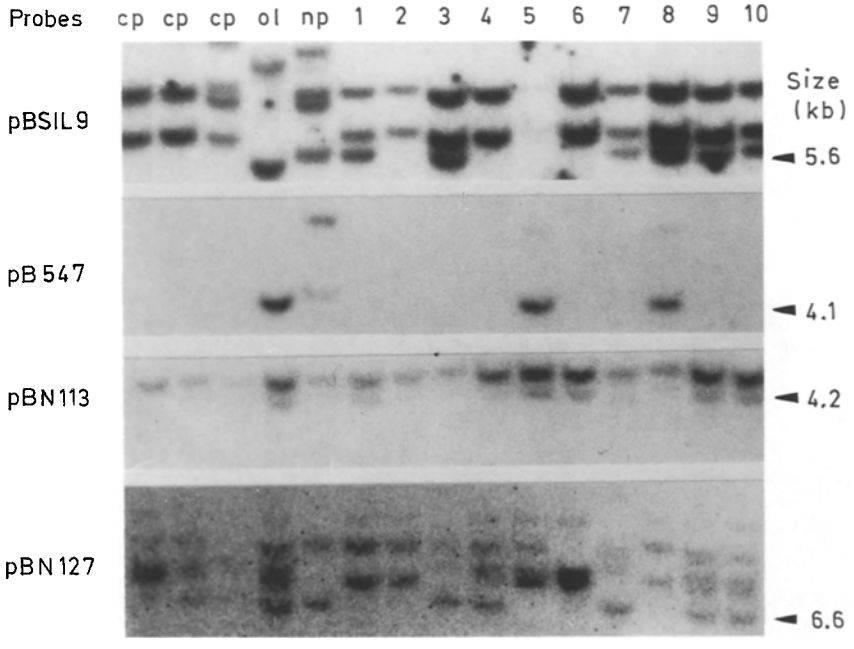

Fig. 1. EcoRI RFLPs for four probes of representative alien addition (cp-ol) plants (*lanes 1* and *3–10*) for line 4 (89B256). Each of these have at least one *B. oleracea*-specific marker. *Lane 2* corresponds to a diploid segregant in the progeny. Parentals: *B. campestris* (cp); *B. oleracea* (ol); *B. napus* (np)

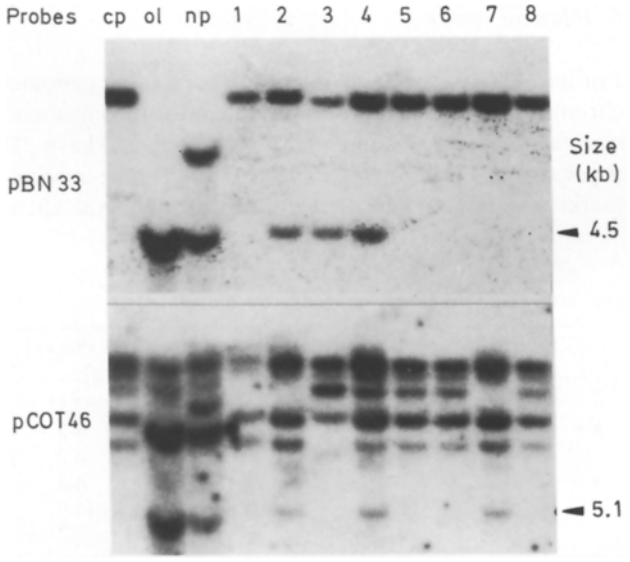

Fig. 2. EcoRI RFLPs for two probes of representative alien addition (cp-ol) plants (*lanes 2, 3, 4,* and *7*) for line 5 (family 89B259). Each of these have at least one *B. oleracea*-specific marker. The rest of the lanes correspond to diploid segregants in the progeny. Parental: *B. campestris* (cp); *lane 2: B. oleracea* (ol); *lane 3: B. napus* (Hakuran, np)

chromosome for three different genotypes revealed size reduction, indicating the occurrence of deficiencies. The plants carrying only the marker *6pgdh2* had a reduced version of the alien chromosome, with a missing arm. On the other hand, the plant carrying only the marker *Pgm-2* had a slightly smaller alien chromosome than those carrying all the expected markers (Fig. 3a).

A similar situation was observed for the plants carrying the markers of alien chromosome 5. As expected, all the plants analyzed cytologically carried alien chromosomes. The plant positive only for the *B. oleracea 6pgdh1* marker carried a smaller chromosome than those carrying any two or all three markers (Fig. 3b).

Determination of the order of the markers on the chromosomes

Putting together the marker transmission data with the chromosome observations, it was possible to determine tentatively the order of the markers (Figs. 3a,b). For chromosome number 4, five of the six genotypes could be explained by single terminal deletions. The genotype carrying only the marker *Pgm-2* could be explained by one interstitial and two terminal deletions (Figs. 3a).

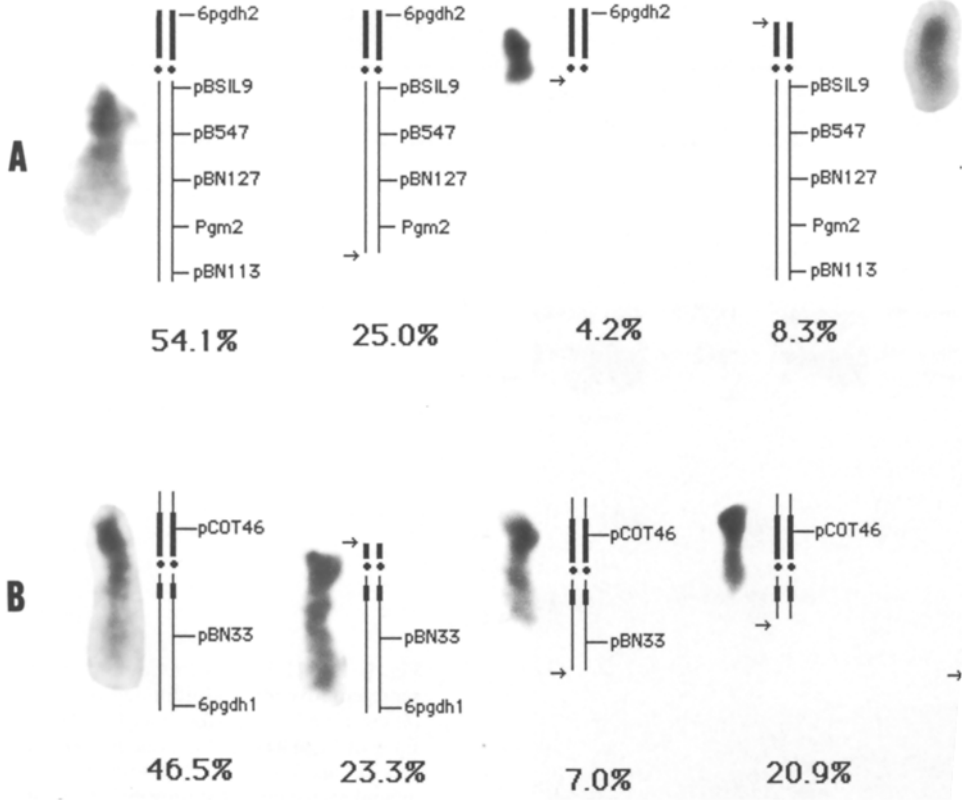

Fig. 3A and B. Karyotype and markers for two C genome chromosomes and some of their derivatives extracted from addition lines. The percent transmission is shown for each chromosome type. A: chromosome 4, B: chromosome 5

Four of the six genotypes displayed simultaneously the markers pB547 and pBSIL9; they can therefore be assumed to be located next to each other. Three genotypes had pBN127 and *Pgm-2* as well as the two markers mentioned above, thus indicating proximity of all four markers. The concurrent absence of pBN113, *Pgm-2*, and pBN127 in one genotype (89B256-16) indicates that they may be located next to each other and that pBN127 must be distal to pB547 and pBSIL9. The sole absences of pBN113 on one hand and *6pgdh2* on the other indicate that these must be at opposite ends of the group. The fact that the chromosome carrying *6pgdh2* as the sole marker displayed a large deletion, spanning most of the long arm of chromosome 4 (Fig.3a), indicates that the other five markers map on the missing arm. The presence of *Pgm-2* in the absence of all other markers can be explained by two terminal deletions.

For chromosome number 5, pBN33 must be located in the center since this marker never showed up alone.

Discussion

The present study was prompted by previous observations of sporadic loss of expected markers in some of the addition lines. At the initial stages of development of these lines, the presence of specific alien chromosomes monitored by isozyme loci. In line 4 we had observed previously the separation of the syntenic loci *Pgm-2* and *6pgdh2*. When adding RFLP as markers, we noticed that line 5, derived from *B. napus* (89B381), differed from 'Hakuran' line 5 (89B259) by the absence of pBN33. However, we found this marker by retracing progenies for four generations (89B405). Thus, it was lost during early development of this line.

The loss of markers in the alien chromosome of the addition lines is probably due to deletions. The occurrence of these deletions in both chromosome 4 and 5 indicates that they may be widespread in all the chromosomes in the genome. However, since these two are the largest chromosomes in the genome, they may be most prone to deletions. A similar phenomenon has been reported for alien addition and substitution lines of wheat (Kota and Dvorak 1988). The absence of C genome-specific markers in the diploid progenies suggests that intergenomic recombination was not a factor causing the chromosome deletions.

The mechanism responsible for the deletions remains unkown. It is tempting to speculate that the deletions may be result of intergenomic recombination. However, we did not observed any *B. oleracea* markers in diploids derived from the addition lines to support this possibility. The deletions seem to be mostly terminal for the two chromosomes studied. If these take place at random, the addition lines will provide as an added benefit the possibility to physically map marker by deletion analysis. Although cytologically the *Brassica* chromosomes are not optimal for detailed study, at least a rough physical location of the markers can be accomplished.

It can be argued that the lack of complete fidelity of the alien chromosomes may be an obstacle to the maintenance of the addition lines. However, the relatively high frequency of simultaneous transmission of all the markers, 54% for chromosome 4 and 46% for chromosome 5, indicate that this is not an unsurmountable task. Based on the results of this study, it is estimated that progenies of more than 25 individuals from alien addition lines provide enough plants carrying intact alien chromosomes. These will be identified by monitoring the plants with markers appropriately spaced on the chromosome.

It evident that the number of backcrosses to the diploid parent has an effect on the rate of transmission for the chromosome markers. For example, family 89B381 for chromosome 5, with the lowest transmission of markers, had the highest number of backcrosses, thus giving more opportunity for deletions to occur.

Another factor that may play a role in the transmission is genetic background of the alien chromosome. For example, the 'Hakuran'-derived line had 27% transmission, a value well above that of the majority of the other families. The highest transmission value, observed for family 89B405, may be explained by the presence of chromosome 5 in disomic condition in the parental plant, which had $2n = 24$ chromosomes.

Chromosomal duplications and translocations have played an important role in repatterning the chromosomes of the *Brassica* genomes (Quiros et al. 1988; Hosaka et al. 1990; McGrath et al. 1990). Our present findings indicate that deletions may be a third type of aberration involved in the molding of *Brassica* chromosomes during their evolution. Survival of homozygous individuals for delected chromosome segments is likely to be tolerated due to the duplicated nature of the *Brassica* genomes. These mechanisms affecting chromosomal architecture in conjunction with aneuploidy have been instrumental in the origin of the *Brassica* genomes.

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