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Random chloroplast segregation and mitochondrial genome recombination in somatic hybrid plants of *Diplotaxis catholica+Brassica juncea*

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Abstract Detailed molecular analysis of the somatic hybrid plants of *Diplotaxis catholica+B. juncea* indicated random chloroplast segregation. One of the five hybrid plants analyzed derived its chloroplasts from *D. catholica* and two hybrids had chloroplasts of *B. juncea* origin. Two hybrid plants maintained mixed population of chloroplasts. The mitochondrial (mt) genomes of the fusion partners had undergone recombinations. Occurrence of fragments specific to both the parents in *Hin*dIII digestion followed by *atp* 9 probing, as in hybrid DJ5, provided evidence for intergenomic mitochondrial recombination between *D. catholica* and *B. juncea*. Similar mt genome organization in two hybrids (DJ3 and DJ6) suggested that intergenomic recombination may be preferred at specific sites. Hybrid DJ1 had about 70% similarity to *D. catholica* in mt genome organization. mt genomes of hybrids DJ2, 3, 5, and 6 differed from *B. juncea* by 14.3–28%. The significance of these novel mt genome organizations in developing novel male sterility systems is discussed.

Key words Random chloroplast segregation · Mitochondrial genome recombination · *Diplotaxis catholica* · *Brassica juncea* · Somatic hybrid

Abbreviations *mt* Mitochondrial

Introduction

In the majority of sexually reproducing plants, inheritance of organelles is uniparental as the cytoplasm of the embryo is derived from the egg. In parasexual crosses achieved by protoplast fusion, on the other hand, the resulting hybrid cell contains nuclei and cytoplasm of both the fusion partners. In regenerants from fusion products, therefore, any of the following theoretical possibilities can be realized as far as cytoplasmic genomes are concerned: (1) the parental genomes segregate to homogeneity during cell division, (2) both the parental genomes are retained as a mixed population, and (3) the parental genomes recombine to generate novel genomic constitutions (Kumar and Cocking 1987). The segregation of chloroplasts is independent of mitochondrial segregation as analyzed in the plants regenerated from cell fusion products (Galun et al. 1982). Since mitochondrial (mt) genomes of *Brassica* parasexual hybrids generally undergo recombinations and plasmagenes are known to influence important characteristics such as floral morphology and male sterility (Belliard et al. 1979; Kao et al. 1992; Kirti et al. 1995a), information on organelle genome constitution of somatic hybrids/cybrids is helpful in framing their utilization in crop improvement.

We have earlier reported the synthesis and characterization of somatic hybrid plants of *Diplotaxis catholica+Brassica juncea* (Kirti et al. 1995b). Analysis of five hybrids using a mitochondrial probe for cytochrome C oxidase subunit I (*cox* I) revealed that the mitochondria were of *B. juncea* origin in three, of *D. catholica* origin in one, and of recombinant type in the fifth hybrid. To utilize these hybrids effectively in developing novel cytoplasmic male sterility systems, we have extended the molecular analysis further by using additional mitochondrial probes, especially for those mitochondrial genes which are known to be involved in rearrangements/recombinations and subsequent expression of male sterility (Vedel et al. 1994). It was found that all five hybrids had recombined mt genomes. Two hybrids also had chloroplasts of both the parents as a mixed population. The results of this investigation are reported here.

Materials and methods

Twenty-eight somatic hybrid plants were regenerated and transferred to the field. Of these, five (DJ1, 2, 3, 5, and 6) were randomly se-

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lected for detailed molecular analysis. These hybrid plants were characterized on the basis of intermediate morphology, chromosome number and the Southern hybridization pattern of the nuclear rDNA probe pTA71 (Kirti et al. 1995b). Hybrids DJ1, 2, 3, and 6 possessed 2n=54 ($AABBD^CD^C$), the sum of parental chromosome numbers of *B. juncea* (2n=36, AABB) and *D. catholica* (2n=18, D^CD^C), thereby showing that they are symmetric somatic hybrids. Hybrid plant DJ5 had 2n=45 and is therefore asymmetric ($AABBD^C$) (Kirti et al. 1995b). These hybrids were analyzed in detail by Southern analysis to confirm the origin of organelle genomes. The origin of chloroplasts in the hybrids was traced using probes *psb* A (Zurawski et al. 1982) and *psb* D (Alt et al. 1984). Constitution of mt genomes in the hybrids was investigated using the mt probes 26 s rRNA (Dale et al.1984), 5s-18 s rRNA (Chao et al. 1983), *atp* 6 (Dewey et al. 1985a), and *atp* 9 (Dewey et al. 1985b). Details of the protocols employed in DNA isolation, restriction analysis, electrophoresis, and blotting were described earlier (Kirti et al. 1995a). Standard protocols were used in Southern hybridizations (Maniatis et al. 1982). Nick translation of probes was carried out using the Du-Pont NEN (USA) kit, following the manufacturer's instructions. The percentage difference in the restriction fragment pattern generated by polymorphic mt gene probes was derived from the equation

$$PD = \frac{Fab \times 100}{(Fa + Fb)}$$

where Fa and Fb are the number of scorable bands in individuals a and b, respectively, and Fab is the number of bands that differ between two fragment profiles (Gilbert et al. 1990).

Results and discussion

Origin of chloroplast genomes in somatic hybrids

Chloroplast gene probes *psb* A and *psb* D were used in combination with restriction enzymes *Hin*dIII, *Bgl*II and *Bam*HI. Of the six probe-enzyme combinations, one (*psb* D-*Bam*HI) combination differentiated the fusion partners. *B. juncea* was characterized by a 9.8-kb *Bam*HI fragment hybridizing to the probe and *D. catholica* by a 5.9-kb fragment (Fig. 5). The hybrid DJ1 possessed the *D. catholica*-specific 5.9-kb fragment. Hybrids DJ3 and DJ6 had the *B. juncea*-specific 9.8-kb fragment. Interestingly, hybrids DJ2 and DJ5 possessed fragments characteristic of both *B. juncea* and *D. catholica*. These observations suggested that DJ1 possessed chloroplasts of *D. catholica* origin, DJ3 and DJ6 possessed chloroplasts of *B. juncea* origin, while DJ2 and DJ5 carried chloroplasts of both parents.

Chloroplast segregation has often been reported to be biased in *Brassica* somatic hybrids. Jourdan et al. (1989) and Earle et al. (1992) observed that in somatic hybrids of *Raphanus* with *B. oleracea* and *B. napus*, *Brassica* chloroplasts were preferentially retained. In somatic hybrids of *B. napus* with related diploid species, biased segregation was in favor of *B. napus* chloroplasts. Cell type could not be established as a causative factor for biased segregation (Sundberg et al. 1991; Walters et al. 1992) but nuclear DNA content and size of the cells of the fusion partners were attributed a role in influencing biased segregation as they affect the number and DNA content of plastids (Butterfass 1989). Ploidy level of the fusion partners and selective replication of a plastid genome in a particular nuclear environment have similarly been implicated as contributing significantly to biased segregation (Bonnett and Glimelius 1983; Earle et al. 1992). Our observations on the descent of chloroplasts from the fusion parents to the hybrid plants are at variance with the conclusions derived so far. Of the two fusion partners, *B. juncea* is an amphidiploid and *D. catholica* is a diploid. This difference in ploidy did not affect chloroplast segregation in somatic hybrids; it appeared to be fairly random even though the sample was small. The observation of mixed chloroplasts in somatic hybrids is interesting because it is rarely detected in cell fusion experiments (Fluhr et al. 1983; Flick et al. 1985; Gleba et al. 1985; Thomzik and Hain 1988).

The pattern of chloroplast segregation has been correlated with the degree of genetic relatedness of the fusion partners (Sundberg and Glimelius 1991). Chloroplast segregation was more random in hybrid cell populations obtained from fusion of related species. Perl et al. (1991) reported that in the interspecific somatic hybrids of *Solanum*, chloroplast transmission, estimated from the restriction fragment profile of chloroplast DNA, was related to the degree of phylogenetic relatedness of the fusion partners. In the present study, the fusion partners, *B. juncea* and *D. catholica*, are phylogenetically distant. Based on restriction fragment profiles, considerable divergence has been reported between their chloroplast genomes (Warwick and Black 1991; Pradhan et al. 1992). Our observation of chloroplast mixture and random chloroplast segregation in the hybrid plants indicated that replication of the two genomes against the nuclear background of the hybrid was synchronous and there was no negative interaction or bias leading to selective elimination of one of the genomes despite the predicted chloroplast genome divergence of the fusion partners.

In symmetric somatic hybrids between *Lycopersicon esculentum* and *L. pennellii*, Bonnema et al. (1992) reported random segregation of chloroplasts to either parental types. As the asymmetry increased with the partial presence of the *L. pennellii* nuclear genome, the chloroplast segregation became more non-random to the extent that a majority of hybrids possessed chloroplasts of *L. esculentum* origin. In contrast, in the present study, mixed chloroplasts were retained in the asymmetric hybrid DJ5 as well as in one of the symmetric hybrids (DJ2), while in other symmetric hybrids, chloroplasts were of either parental type. This further suggested the absence of negative interaction between the nuclear and chloroplast genomes in the fusion products.

Constitution of the mt genome in somatic hybrids

Unlike the segregation of chloroplasts in somatic hybrids, segregation of mitochondria to homogeneity is very rare and mt genome rearrangements/recombination occur frequently. We reported earlier mt DNA recombination in one of the five *D. catholica*+*B. juncea* somatic hybrid plants based on a limited analysis using the *cox* I probe (Kirti et al. 1995b). Since recombination is viewed as the rule governing mt genome organization in somatic hybrids in *Cru-*

Table 1 Hybridization profile of polymorphic mitochondrial probes used to characterize somatic hybrids (DJ1-6) of *Diplotaxis catholica* (*D.c.*)+*Brassica juncea* (*B.j.*)

Probe	Enzyme	Fragment (kb)	B.j.	D.c.	DJ1	DJ2	DJ3	DJ5	DJ6
atp 9	*Hin*dIII	6.6	+	–	–	+	–	+	–
		3.5	–	–	+	–	+	–	+
		2.3	–	+	–	+	–	+	–
		1.5	–	+	+	+	+	–	+
	*Bam*HI	5.5	+	+	+	+	+	+	+
		1.1	–	+	+	+	+	+	+
	*Bgl*II	20.0	+	–	–	+	–	+	–
		10.0	–	+	+	+	+	–	+
		4.8	–	+	+	+	+	+	+
5s-18S rRNA	*Bam*HI	12.4	+	–	–	+	+	+	+
		11.3	+	+	+	+	+	+	+
		8.9	–	+	–	–	–	–	–
		6.4	–	–	–	+	–	–	–
		2.8	+	+	+	+	+	+	+
*Cox*I[a]	*Hin*dIII	5.5	+	–	–	+	+	+	+
		3.5	–	–	–	+	–	–	–
		2.8	–	+	+	–	–	–	–
	*Bgl*II	9.0	–	+	+	–	–	–	–
		4.8	–	–	–	+	–	–	–
		4.5	+	–	–	+	+	+	+
	*Eco*RV	9.4	–	+	+	–	–	–	–
		7.0	+	–	–	+	+	+	+

[a] From Kirti et al. 1995b

ciferae (Pelletier et al. 1988), these hybrids were subjected to a more detailed analysis using different combinations of four mt probes (*atp* 6, *atp* 9, 26 s rRNA and 5s-18 s rRNA) with restriction enzymes *Hin*dIII, *Bam*HI, *Eco*RI, *Eco*RV and *Bgl*II. The probes *atp* 6 and 26 s rRNA did not distinguish between the parents in any of the enzyme combinations studied. Polymorphism was detected with probes *atp* 9 and 5s-18 s rRNA. The Southern hybridization pattern of the probes which detected polymorphism in different enzyme combinations is summarized in Table 1. The probe 5s-18 s rRNA distinguished between the parents only in combination with *Bam*HI. In this combination, *B. juncea* was characterized by a 12.4-kb fragment while *D. catholica* had a specific 8.9-kb fragment (Fig. 1). In three somatic hybrids (DJ3, DJ5, and DJ6), the hybridization pattern was similar to that of *B. juncea*. Hybrid DJ2 possessed a novel 6.4-kb fragment, in addition to the 12.4-kb *B. juncea*-specific fragment. The hybrid DJ1 lacked the characteristic fragments of both the parents.

The probe *atp* 9 detected polymorphism between the parents in all three enzyme combinations employed. It hybridized to two different fragments in *D. catholica* and to one fragment in *B. juncea* in all the enzyme digestions. Of the two *Bam*HI fragments of 5.5 kb and 1.1 kb in size that hybridized with *atp* 9 in *D. catholica*, the larger fragment was also present in *B. juncea* (Fig. 2). All the somatic hybrids had the hybridization pattern of *D. catholica*. In the case of *Hin*dIII digestion, a 6.6-kb fragment characterized *B. juncea* whereas two smaller fragments, 2.3 kb and 1.5 kb in size, were specific to *D. catholica*. The hybrid DJ2 possessed the characteristic fragments of both the parental species. The absence of the *D. catholica*-specific 1.5-kb fragment in DJ5 distinguished it from DJ2. The presence of a novel 3.5-kb fragment and *D. catholica*-specific 1.5-kb fragment characterized the other three hybrids DJ1, DJ3, and DJ6 (Fig. 3). The hybridization pattern of *atp* 9 to the *Bgl*II digests revealed the presence of a *B. juncea*-specific 20-kb fragment in hybrids DJ2 and DJ5. Of the two *D. catholica*-specific fragments, the 4.8-kb fragment was present in all the hybrids while the 10.0-kb fragment was not present in DJ5 (Fig. 4).

These observations revealed the presence of recombinant mt genomes in all five somatic hybrids. In the hybrid DJ2, recombination was extensive; it was detected, in addition to the *cox* I region, in *atp* 9 and 5s-18 s rRNA regions also. Variation in the hybridization profile was most striking in the *atp* 9 gene region. Three distinct hybridization profiles of *atp* 9 were revealed in *Hin*dIII digestion through the presence of (1) two *D. catholica*-specific and one *B. juncea*-specific fragment as in hybrid DJ2, (2) one *D. catholica*-specific and one *B. juncea*-specific fragment as in DJ5, and (3) one *D. catholica*-specific and one novel fragment as in DJ1, 3, and 6. This suggested the occurrence of extensive recombination in the region surrounding *atp* 9. A high frequency of recombination in the *atp* 9 region has also been observed in intergeneric hybrids of *Brassicaceae* (Landgren and Glimelius 1994).

The percentage difference in the hybridization profile based on probes *atp* 9, *cox* I, and 5s-18 s rRNA indicated that none of the hybrids possessed a parental mt genome (Table 2). The maximum difference was observed between DJ1 and *B. juncea*. There was no difference between hybrids DJ3 and DJ6. Hybrid DJ5 differed the least from *B. juncea* and hybrid DJ1 from *D. catholica*.

These observations are relevant to the utilization of these somatic hybrids in developing novel male sterile lines of *B. juncea*. It is well known that flower morphology, stamen dysfunction, and male sterility are controlled by nuclear mitochondrial interactions (Belliard et al. 1979; Breiman and Galun 1990; Kirti et al. 1995a). A mt genome con-

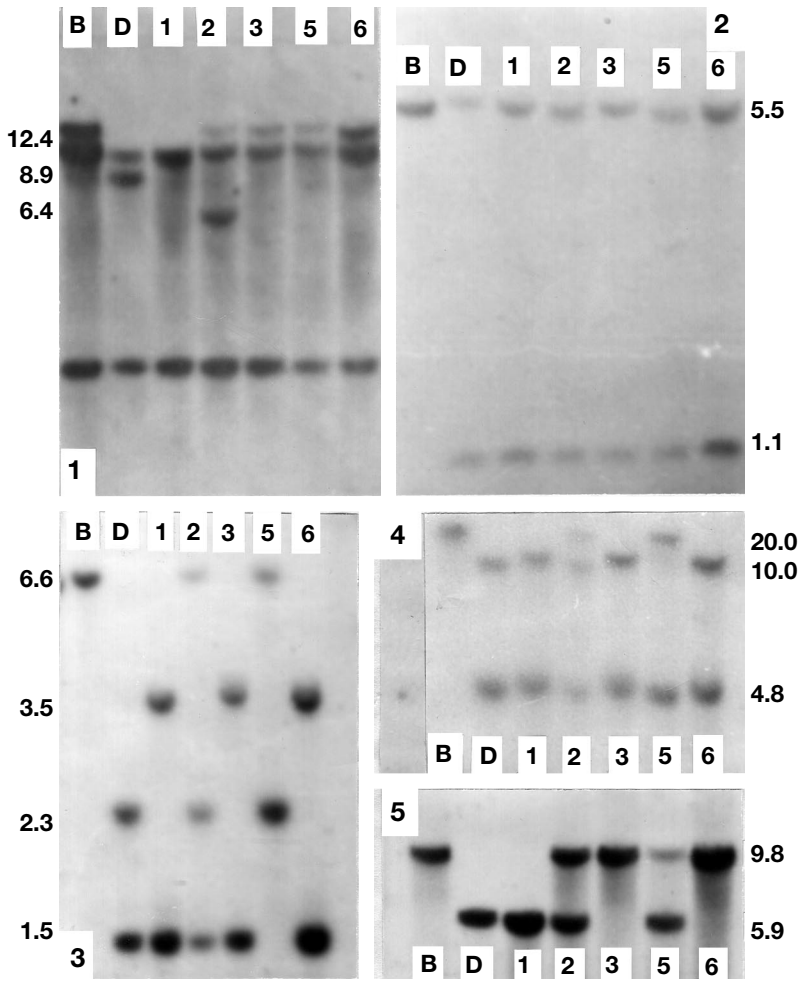

Fig. 1 *Bam*HI digest of total DNA of *Brassica juncea* (*B*), *Diplotaxis catholica* (*D*) and hybrids DJ1 (*1*), DJ2 (*2*), DJ3 (*3*), DJ5 (*5*), and DJ6 (*6*) hybridized with the 5s-18s rRNA probe

Fig. 2 *Bam*HI digests as in Fig. 1 hybridized with the *atp* 9 probe

Fig. 3 *Hin*dIII digests of total DNA probed with *atp* 9 (lanes as in Fig. 1)

Fig. 4 *Bgl*II digests of total DNA probed with *atp* 9 (lanes as in Fig. 1)

Fig. 5 *Bam*HI digests of total DNA hybridized with the chloroplast-specific probe for *psb* D

Table 2 Percentage difference in the hybridization profile based on polymorphic mitochondrial gene probes used to characterize somatic hybrids of *Diplotaxis catholica* (*D.c.*)+*Brassica juncea* (*B.j.*)

	D.c.	DJ1	DJ2	DJ3	DJ5	DJ6
B.j.	71.4	70.0	28.0	33.3	14.3	33.3
D.c.		21.7	42.9	41.7	50.0	50.0
DJ1			55.6	39.1	56.5	30.4
DJ2				28.6	14.3	28.6
DJ3					25.0	0.0
DJ5						28.0

tribution of the alien parent limited to the extent of causing stamen dysfunction and male sterility would be of great advantage because the reduced level of nuclear-mitochondrial interactions (Galun 1993) would subsequently allow easier fertility restoration. The alloplasmic male sterile line developed from the sexual hybrid involving *D. catholica* as the female parent suffers from a number of floral abnormalities like petaloid anthers and non-opening of flowers (K. R. Shivanna, personal communication). Since the somatic hybrids presently analyzed carry reconstituted mt genomes with differential contributions from the alien parent, these are expected to yield male sterile lines with varying floral characteristics; hybrids with mitochondrial genomes more similar to that of *B. juncea* may give rise to a usable male sterility system.

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