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Absence of mitochondrial and chloroplast DNA recombinations in *Brassica napus* plants regenerated from protoplasts, protoplast fusions and anther culture

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Summary. Over 400 *Brassica napus* plants regenerated from individual protoplasts, from protoplast fusions and from anther culture were analysed for chloroplast and mitochondrial genome rearrangements by restriction fragment length polymorphisms. None were detected, attesting to the fidelity of the tissue culture procedures employed. In the majority of protoplast fusion products, the cytoplasmic organelles had completely sorted out at the callus stage but three regenerated plants possessed mixed parental populations of mitochondrial genomes and one regenerant contained mixed chloroplast genomes. In all four examples, the cytoplasmic genome sorted out in planta in favor of one parental type which was faithfully maternally transmitted to progeny.

Key words: *Brassica* – Mitochondrial DNA – Chloroplast DNA – Protoclone – Protoplast fusion – Anther culture

Introduction

Protoplast fusion techniques are being used by ourselves (Barsby et al. 1987a, b, c; Yarrow et al. 1986) and others (Menczel et al. 1987; Pelletier et al. 1983) to transfer desirable agronomic traits such as cytoplasmic male sterility (*cms*) and cytoplasmic triazine resistance (*ctr*) to rapeseed and canola (*B. napus* and *B. campestris*). *Cms* is encoded on mitochondrial DNA (mtDNA) and *ctr* is encoded on chloroplast DNA (cpDNA). Whereas the other groups have reported extensive mtDNA recombination predominating in their limited number of protoplast fusion products (Chetrit et al. 1985; Menczel et al. 1987; Morgan and Maliga 1987; Vedel et al. 1986) evidence for such a phenomenon was not detected in our numerous *B. napus* cybrids (Barsby et al. 1987a, b, c; Yarrow et al. 1986).

Using the rapid, non-destructive, restriction fragment length polymorphism (RFLP) cytoplasm classification assay for *Brassica* mtDNA and cpDNA (Kemble 1987) we have used up to 10 different enzymes to analyse in excess of 300 regenerated plants from our *B. napus* protoplast fusion system, over 50 *B. napus* protoclones (plants regenerated from individual protoplasts) and 27 *B. napus* anther culture derived plants to address the frequency at which cytoplasmic DNA recombination may occur in our well defined and repeatable tissue culture systems. Consequently, this study represents the most thorough of such investigations to date.

Materials and methods

Plant material

Plants of *B. napus* (2n = 4x = 38) regenerated from protoplasts, from protoplast fusion products and from anther culture were used.

Protoclones. Plants were regenerated from protoplasts of the Canadian spring varieties Regent and Westar and from the Swedish spring variety Olga according to Barsby et al. (1986). One protoplast derived Olga callus was maintained on B5 medium (Gamborg et al. 1968) as an embryogenic line. At various times up to 6 months after protoplast isolation, plants were recovered by transferring embryos to fresh B5 media without hormones.

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Table 1. Number and type of regenerated *B. napus* plants and restriction enzymes used in this study

Lines used	No. individual plants analysed	Cytoplasmic DNA analysed	No. restriction enzymes used
Protoclones:			
cv. Regent	28	mt and cp	10[a]
cv. Westar	2	mt and cp	10[a]
cv. Olga	8	mt and cp	10[a]
Olga embryogenic line	17	mt and cp	10[a]
Protoplast fusion regenerants:			
various	334	mt and cp	1 to 7[b]
Anther culture:			
cv. Regent	7	mt	4[c]
cv. Tower	2	mt	4[c]
cv. Triton	8	mt	4[c]
cv. Westar	10	mt	4[c]

[a] *Bam* HI, *Bcl* I, *Eco* RI, *Hin* dIII, *Hpa* I, *Pst* I, *Pvu* II, *Sal* I, *Xba* I, *Xho* I
[b] *Eco* RI and up to 6 others of those listed above
[c] *Bam* HI, *Eco* RI, *Hin* dIII, *Xba* I

Protoplast fusion regenerants. Plants were recovered from various fusion experiments involving many different Canadian spring and European winter lines most of which exhibited *cms* and/or resistance to the triazine group of herbicides. The *cms* lines employed were of the *Polima (pol), Ogura (ogu)* and *napus (nap)* types. Male fertile lines used carried either *campestris* (*cam*) or *nap* cytoplasms; the latter exhibiting a male fertile phenotype due to nuclear male fertility restorer genes. The construction of these protoplast fusion regenerants has recently been described by Barsby et al. (1987a, b, c) and Yarrow et al. (1986).

Anther culture. Plants were derived from anther culture of four different Canadian spring varieties according to Lichter (1981).

Extraction and analysis of cytoplasmic DNA

MtDNA and cpDNA was extracted from leaves of individual regenerated plants and analysed by the RFLPs produced after agarose gel electrophoresis as described by Kemble (1987).

Results

Table 1 summarizes the extent of the study. CpDNAs and mtDNAs from a total of 55 individual protoclones and 334 individual plants from protoplast fusion experiments were analysed to assess the degree of organellar DNA recombination. In addition, mtDNA from a total of 27 individual plants regenerated from anther culture was also investigated.

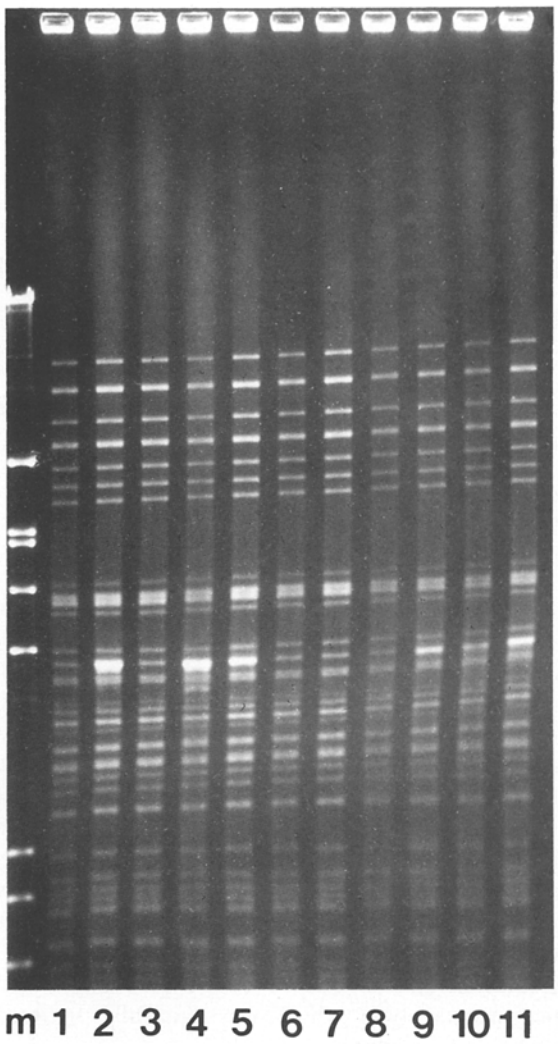

Fig. 1. *Eco* RI restriction enzyme analyses on 1% agarose gel of mtDNA from: *lane 1 B. napus* cv. Regent plant germinated from certified seed; and *lanes 2 to 11* ten individual protoclone plants regenerated from Regent protoplasts. *Lane m* contains size marker fragments produced by independent fragmentation of lambda DNA with *Eco* RI and *Hae* III. The additional low molecular weight nucleic acid fragments visible in some lanes (positions indicated by *small arrows)* are mtRNAs described previously (Kemble et al. 1986b)

Protoclones

Regardless of the restriction enzyme used (Table 1) all produced mtDNA and cpDNA RFLP profiles identical to those obtained from control plants germinated from certified seed stocks. Figure 1 shows the uniform *Eco* RI mtDNA RFLP profiles obtained from a representative sample of 10 individual Regent protoclones. Similarly, the lack of variation in the *Hin* dIII cpDNA RFLP profiles from the same 10 individual Regent protoclones is shown in Fig. 2. It is noteworthy that

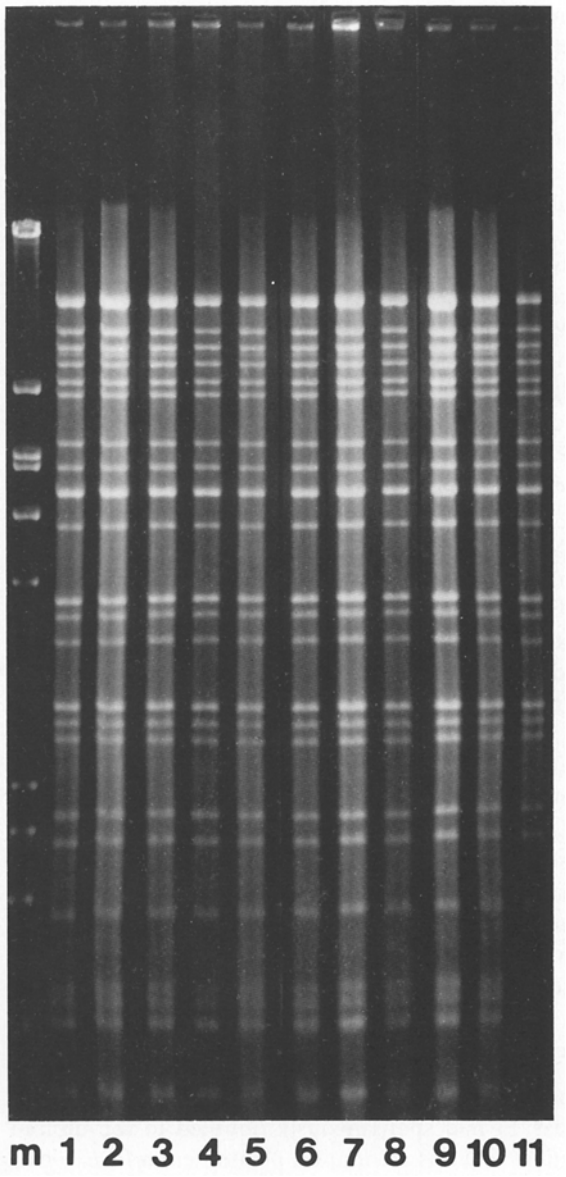

Fig. 2. *Hin* dIII restriction enzyme analyses on 1% agarose gel of cpDNA from the same plants as shown in Fig. 1. Labelling as in Fig. 1

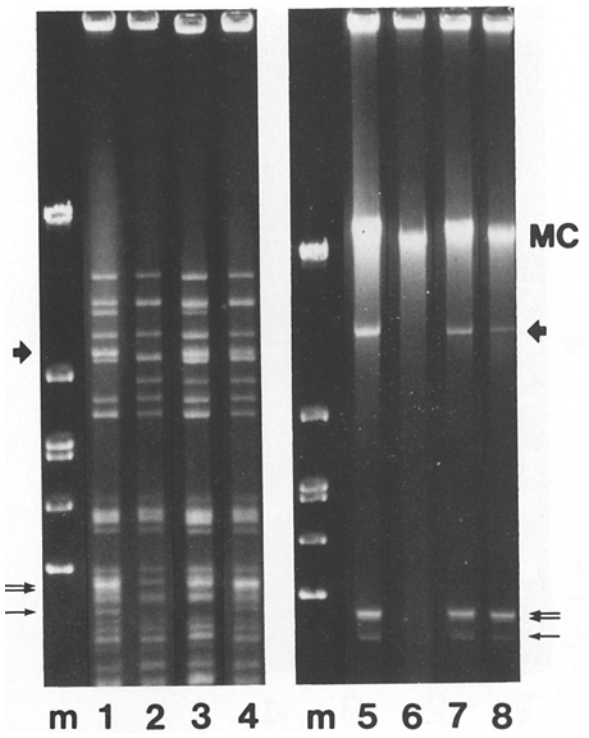

Fig. 3. *Eco* RI fragmented (*lanes 1 to 4*) and native (*lanes 5 to 8*) mtDNA, on 1% agarose gel, from plants of: *lanes 1 and 5, ctr* parental protoplast donor line; *lanes 2 and 6, nap* parental protoplast donor line; *lanes 3 and 7,* protoplast fusion regenerant M1 at the 3–4 leaf stage of development; and *lanes 4 and 8,* M1 at flowering. The *large arrows* indicate the position of the native 11.3 kb mitochondrial plasmid in *lanes 5, 7 and 8* and its largest *Eco* RI fragment in *lanes 1, 3 and 4.* MC refers to the position of the unfragmented mitochondrial chromosome in *lanes 5 to 8.* All other labelling as in Fig. 1

even the Olga embryogenic line which had been maintained in vitro did not give rise to alterations in mtDNA or cpDNA.

Protoplast fusion regenerants

Of the 334 individual plants analysed, 330 produced mtDNA and cpDNA RFLP profiles identical to those of the parental lines used in the fusion experiment. Again, this was irrespective of the restriction enzyme used. Many of these plants were either self-pollinated or pollinated with a maintainer to increase seed of each line. All plants analysed from the seed generation also exhibited parental-type cytoplasmic DNA RFLPs. In addition, several plants were raised from cuttings of the original regenerants. These also possessed parental type mtDNA and cpDNA.

When analysed at the 3–4 leaf stage of development, the 4 other protoplast fusion regenerants exhibited a mixed population of parental cytoplasmic organelles (Table 2). Figure 3 shows the example of plant M1 (lanes 3 and 7) which possessed a mtDNA RFLP profile equivalent to an equimolar mix of its *ctr* parental line (lanes 1 and 5) and its *nap* parental line (lanes 2 and 6). Subsequently 6 mtDNA extractions were obtained from individual leaves from different heights and radial locations on the main stem of the plant. All leaf RFLP profiles were identical to the earlier sample (lanes 3 and 7) indicating that the plant indeed possessed a mixed population of mitochondrial genomes and the mtDNA profiles were not the result of combining sectors in which the mitochondria had

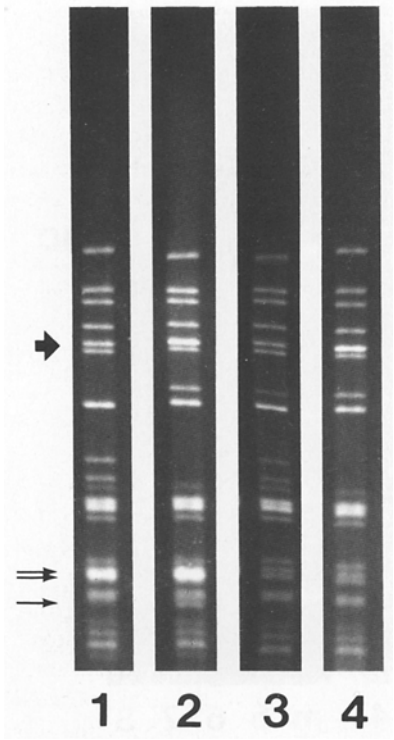

Fig. 4. *Eco* RI restriction enzyme analyses on 1% agarose gel of mtDNA from plants of: *lane 1, pol* parental protoplast donor line; *lane 2, ctr* parental protoplast donor line; *lane 3*, protoplast fusion regenerant M2 at the 3–4 leaf stage of development; and *lane 4*, M2 at flowering. Labelling as in Fig. 3

Table 2. Protoplast fusion regenerants which exhibited mixed organelle populations in planta

Plant code	Cytoplasmic DNA classification					
	Pre-flowering		At flowering		F_1 generation[a]	
	mt	cp	mt	cp	mt	cp
M1	*ctr-nap*	*nap*	*nap*	*nap*	*nap*	*nap*
M2[b]	*ctr-pol*	*ctr*	*ctr*	*ctr*	*ctr*	*ctr*
M3[b]	*ctr-pol*	*ctr*	–[c]	–[c]	–	–
M4	*pol*	*ctr-pol*	*pol*	*ctr*	*pol*	*ctr*

[a] All plants were crossed, as females, with cv. Regent, as a male, to produce F_1 progeny. 6 to 12 plants of each F_1 set were analysed to determine their cytoplasmic DNA classification
[b] M2 and M3 were regenerated from the same callus
[c] This regenerant died before flowering

already sorted. However, as the plant continued to develop, the relative contribution of *ctr* mitochondrial genomes decreased in younger leaves until, at flowering, only the *nap* mtDNA RFLP profile was observed (lanes 4 and 8). In this case, the *nap* mitochondria had obtained and maintained the 11.3 kb plasmid originally present in the *ctr* mitochondrial population. The transfer of this extrachromosomal plasmid between mitochondria of different protoplast populations by protoplast fusion has been described in detail elsewhere (Kemble et al. 1988).

Confirmation that the mitochondrial genomes had sorted out during development in planta in favor of the parental *nap* type was obtained by analysing 12 F_1 plants from M1 with 5 different restriction enzymes. All possessed unaltered *nap* mtDNA and cpDNA profiles (Table 2).

Figure 4 shows details of plant M2 which, early in its development, possessed a mixed parental mitochondrial genome population (lane 3) of *pol* (lane 1) and *ctr* (lane 2). At the stage of flowering, the mitochondrial genome population had sorted in planta in favor of *ctr* (lane 4). F_1 progeny of M2 possessed mtDNA and cpDNA RFLP profiles which were indistinguishable from the parental *ctr* profiles (Table 2).

Plant M3 which was regenerated from the same callus as M2 also possessed a mixed *ctr-pol* mitochondrial genome population early in its development. Unfortunately, this plant did not survive to maturity. Plant M4 was different in that it contained one mitochondrial genome population but a mixed chloroplast genome population at the 3–4 leaf stage of development. By the time of flowering the chloroplast genomes had sorted to a single parental type. This plant was a true stable cybrid, as were the F_1 progeny derived from it, since it possessed unaltered mtDNA from its *pol* parent and unaltered cpDNA from its *ctr* parent (Table 2).

Anther culture

Of the 27 plants analysed (Table 1), 14 were haploid whereas 13 had spontaneously doubled to the diploid condition which is a common phenomenon in *B. napus* (Lichter 1981).

Regardless of the plant ploidy level or the restriction enzyme used, all regenerants produced mtDNA RFLP profiles identical to those in control plants grown from certified seed. CpDNA analyses were not performed on anther culture regenerants.

Discussion

All 5 of the *B. napus* protoplast fusion derived cybrid plants of Pelletier et al. (1983) possessed non-parental mitochondrial genomes suggesting that inter- or intra-mtDNA recombination had occurred (Chetrit et al. 1985; Vedel et al. 1986). As in the present study, these researchers were able to extract mtDNA of sufficient quantity and quality to be fragmented with restriction enzymes and be visualized by ethidium bromide

staining after electrophoresis. Hence the entire mitochondrial genomes could be assessed for rearrangements. Menczel et al. (1987) and Morgan and Maliga (1987) have reported that 1 out of 22 and 6 out of 7, respectively, *B. napus* plants regenerated from protoplast fusion experiments contained non-parental mitochondrial genomes. However, the degree of rearrangements could not be accurately evaluated because total cellular DNA was extracted from the plants and a 5.5 kb radish mtDNA clone used as a hybridization probe. Consequently, only a very small region of the mitochondrial genome was analysed. Furthermore, definitive interpretations as to whether the altered mitochondrial genomes were due to inter- or intragenomic recombination could not be made because their *cms* parental line exhibited mtDNA rearrangements in culture in the absence of any fusogen (Morgan and Maliga 1987). This is reminiscent of our earlier study on *S. tuberosum* – *S. brevidens* somatic hybrids where almost all regenerated plants possessed non-parental mitochondrial genomes (Kemble et al. 1986 a) but up to 15% of *S. tuberosum* protoclones displayed rearranged mitochondrial genomes in the complete absence of any protoplast fusion system (Kemble and Shepard 1984). In addition, mtDNA recombination has been implied in protoplast fusions involving tobacco (Aviv and Galun 1986; Aviv et al. 1984; Belliard et al. 1979; Galun et al. 1982; Medgyesy et al. 1985 b; Nagy et al. 1981, 1983), petunia (Boeshore et al. 1983; Clark et al. 1986; Rothenberg et al. 1985), carrot (Matthews and Widholm 1985) and the Gramineae (Ozias-Akins et al. 1987; Tabaeizadeh et al. 1987).

In contrast, the definitive study described here, in which entire organelle genomes were analysed, indicates no rearranged mitochondrial genomes in any *B. napus* protoclones regenerated by the system of Barsby et al. (1986) or in any *B. napus* plants regenerated from the protoplast fusion systems of Barsby et al. (1987 a, b, c) or Yarrow et al. (1986). It is not possible to categorically state that mitochondrial genome rearrangements do not occur in these systems but simply that no examples have yet been recovered. However, considering the very large number of regenerated plants analysed, if significant mtDNA rearrangements do occur, they must do so at a very low frequency.

It may be argued that the three main cytoplasms used in this study, *pol, nap* and *ctr*, are not as susceptible to mtDNA rearrangement as *ogu* cytoplasm which was employed by Pelletier et al. (1983) and Morgan and Maliga (1987). However, three cybrids carrying *ogu* mitochondria were included in the present study. All possessed a mtDNA RFLP profile identical to control *ogu* cytoplasm lines which had not been subjected to tissue culture. Consequently, we suggest that the degree of mtDNA rearrangements obtained in *B. napus* plants regenerated from protoplasts or protoplast fusion products is independent of cytoplasmic type and may be dependent on the tissue culture system employed. A study of the differences in the tissue culture system employed by the different research groups may enable the factors controlling the phenomenon to be defined.

In common with numerous other studies we did not detect any cpDNA rearrangements in any of our regenerated plants. There has, however, been one report of cpDNA recombination in *Nicotiana* (Medgyesy et al. 1985 a).

Four of the 334 plants regenerated from protoplast fusion experiments possessed a mixed parental population of either chloroplast or mitochondrial genomes indicating that organelle genome sorting out is not always completed in callus cells at the time of plant regeneration. The mixtures were not caused by chimeras and, in all 4 examples, the organelle genomes sorted in planta towards one parental genome type which was stably inherited through seed generations. Thus, even though the time that the two parental organelle genomes were together in the same tissue was obviously much longer than in cases where organelle sorting was completed at the callus stage, no recombination between the genomes was observed. The presence of two organelle genomes in the plants did not adversely affect the plant morphology or rate of development. Mixed chloroplast populations, some of which sorted in planta, have been reported in protoplast regenerants of *Medicago* (Rose et al. 1986) and *Nicotiana* (Aviv et al. 1984; Flick et al. 1985; Fluhr et al. 1983, 1984; Gleba et al. 1984, 1985, Glimelius et al. 1981; Iwai et al. 1981; Sidorov et al. 1981).

The comparatively small study of anther culture derived plants showed no evidence for mitochondrial genome rearrangement regardless of ploidy level. This is in complete agreement with Rode et al. (1985) who did not detect mtDNA rearrangements in their study of anther culture derived wheat lines.

In conclusion, this study clearly demonstrates that mitochondrial and chloroplast genome rearrangements are not the usual consequence of tissue culture. Regenerated plants can be included in breeding programs with a high degree of confidence that their cytoplasmic organelles will remain unchanged. Although it may be postulated that altered mitochondrial genomes could be beneficial, for example as potential new forms of *cms*, there remains a much stronger argument for ensuring that tissue culture systems suit already existing conventional hybridization schemes, and faithfully reproduce the mitochondrial input. The importance of good tissue culture systems cannot be underestimated.

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