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Selection of *Daucus* cybrids based on metabolic complementation between X-irradiated *D. capillifolius* and iodoacetamide-treated *D. carota* by somatic cell fusion

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Summary. Protoplasts of *Daucus capillifolius* isolated from a suspension culture (chromosome number above 60) were X-irradiated over lethal dose (60 krad) just prior to fusion. Protoplasts from *D. carota* cell line (chromosome number 17) were treated with 15 m*M* iodoacetamide and fused with the X-irradiated protoplasts. Putative cybrid plants were regenerated on Murashige and Skoog medium (MS) lacking 2,4-D. The regenerated plants possessed chromosome numbers of 17 (2n-1) or 34 (4n-2) and an identical leaf morphology to *D. carota*. Their mitochondrial DNAs (mtDNAs) were analysed with restriction endonucleases. Novel restriction fragments, not present in mtDNA digests from both parents, were observed in mtDNAs of regenerated plants. These results indicate successful formation of cybrids between *D. capillifolius* and *D. carota* by protoplast fusion.

Key words: *Daucus* – Protoplast fusion – Cybrids – Mitochondrial DNA recombination

Introduction

It has been known that some agronomically important traits such as triazine resistance or cytoplasmic male sterility are encoded on chloroplast or mitochondrial genomes respectively (Steinback et al. 1981; Leaver and Gray 1982). Successful introduction of the maternally inherited traits through organelle transfer by cell fusion have been reported in *Nicotiana* (Belliard et al. 1979; Galun et al. 1982; Nagy et al. 1983; Aviv et al. 1984; Gleba et al. 1985; Menczel et al. 1986), *Petunia* (Boeshore et al. 1983; Clark et al. 1986) and *Brassica* (Pelletier et al. 1983; Chetrit et al. 1985; Yarrow et al. 1986). Analysing the fate of transferred organelles in those hybrid or cybrid plants, preferential elimination of one of the parental chloroplasts and recombination of mtDNAs have been observed. Accordingly, the plants carrying novel organelle combinations, which cannot be observed in the sexual hybrids by crossing, can be created by cell fusion.

Irradiation of protoplasts is useful for cytoplasmic organelle transfer through protoplast fusion. As the irradiation treatment prevents protoplast colony formation and the irradiated nucleus is eliminated from a fused cell (Zelcer et al. 1978; Menczel et al. 1982), cybrids are formed through protoplast fusion between irradiated cells and iodoacetate treated cells by metabolic complementation in tobacco (Sidorov et al. 1981).

In this report, we produced carrot cybrid plants for the first time by donor (*D. capillifolius*)-recipient (*D. carota*) protoplast fusion. The mitochondrial genomes of cybrid plants possessed unique restriction fragment patterns distinct from patterns of both parental mtDNAs.

Materials and methods

Plant material

Suspension culture of *D. carota* L. was initiated from calli derived from seedling hypocotyls and maintained every 2 weeks by transferring 1 ml packed cells in 25 ml of fresh Murashige and Skoog (1962) medium (MS) containing 0.5 mg/l 2,4-D with shaking (60 rpm) at 25 °C. Suspension cells of *D. capillifolius* Gilli. were kindly provided by Dr. Y. Kameya and maintained in the same way as the *D. carota* cell line. This *D. capillifolius* suspension cell line had lost its regeneration capacity.

Protoplast isolation, inactivation, fusion and culture

Protoplasts were prepared from 3- to 5-day-old subcultures of *D. carota* or *D. capillifolius* by incubation in 1% Driselase, 0.5% Cellulase "Onozuka" RS and 0.01% Pectolyase Y-23 dissolved in 0.5 *M* mannitol and 0.1% 2-(N-molpholino)ethane sulfonic acid (MES) at pH 5.7. Incubation was carried out at 25 °C for 4 h with occasional shaking. Protoplasts were purified by passage through 37 and 20 μm stainless steel sieves, pelleted (100 *g* for 3 min), and washed twice with 10 ml of washing solution (0.5 *M* mannitol and 0.1% MES, pH 5.7).

Protoplasts of *D. carota* (10^6/ml of washing solution) were inactivated by incubation in 15 m*M* iodoacetamide for 10 min at room temperature. The protoplasts were washed twice after this treatment. Protoplasts of *D. capillifolius* (2×10^6/ml of washing solution) were X-irradiated with a total dosage of 60 krad (1 krad/min). Iodoacetamide-treated *D. carota* protoplasts were mixed with X-irradiated *D. capillifolius* protoplasts in a 1:1 ratio at a density of 10^6/ml and fused according to the method of Kao and Michayluk (1974). The fused protoplasts were cultured in MS supplemented with 0.3 *M* sorbitol and 0.1 mg/ml 2,4-D at a density of 1.25×10^5/ml at 25 °C. After about 1 month of culture, the plates containing fusion-derived colonies were gradually substituted with MS plus 0.1 *M* sorbitol without 2,4-D. Then the resultant somatic embryos were transferred to 2-fold diluted MS containing 0.2% Gelrite for further growth. Subsequently large enough plants were transplanted into soil.

Isolation of mtDNA from carrot suspension cultures

The cell suspensions of fusion-derived plants were prepared from their shoot segments; 5–10 g of cell suspensions were incubated with enzyme solution, as above descrived. Protoplasts were collected by centrifugation, washed once with 0.5 *M* mannitol plus 0.1% MES (pH 5.7), and resuspended in CP lysis buffer (DeBonte and Matthews 1984) using 2 ml ice-cold buffer per g tissue. The protoplasts were ruptured by a blender at 15,000 rpm for 5 s 3 times. Nuclei and cell debris were sedimented from the cell extract by centrifugation at 3,000 *g* for 5 min. The supernatant was centrifuged at 6,000 *g* for 5 min, and this step was repeated once. Mitochondrial pellet was collected from the supernatant by centrifugation at 15,000 *g* for 5 min. The mitochondrial fraction was resuspended in DNase buffer (0.3 *M* sucrose, 0.05 *M* Tris-HCl, pH 7.5) with 10 μg/g fresh weight DNase I (TAKARA) and 10 m*M* $MgCl_2$, and incubation at 4 °C for 30 min.

The mitochondrial suspension was layered on 5 ml of shelf buffer [0.01 *M* Tris-HCl, 0.02 *M* EDTA, 0.6 *M* sucrose (pH 7.2)] and centrifuged at 15,000 *g* for 10 min; this step was repeated once to remove DNase I completely. The pellet was resuspended in Sarkosyl buffer [1% N-lauryl sarcosine in 50 m*M* Tris-HCl, 20 m*M* EDTA (pH 8.0)] with 50 μg/ml Proteinase K and incubated at 37 °C for 1 h. MtDNA was isolated by phenol-chloroform extraction and precipitated by ethanol in the presence of 0.3 *M* sodium acetate. The average yield of mtDNA was about 50 μg from 10 g fresh weight of suspension cells.

Restriction endonuclease analysis of mtDNA

MtDNA (1–2 μg) was digested to completion with restriction enzymes according to the supplier's instructions for at least 4 h at 37 °C. The restriction fragments were separated on 0.5% agarose gels and stained with ethidium bromide.

Chromosome counting

Suspension cells of *D. capillifolius* (no regeneration potential) or *D. carota,* and root-tips of cybrid plants were incubated with 2 m*M* 8-hydroxyquinoline, then fixed in ethanol: acetic acid (3:1), and treated with an enzyme solution containing 4% Cellulase "Onozuka" RS, 1% Pectolyase Y-23, 7.5 m*M* KCl and 7.5 m*M* EDTA, pH 4.0 (Nishibayashi and Kaeriyama 1986). They were gently spread on slide glass and stained with aceto-orcein for microscope observation.

Results

Effect of X-irradiation or iodoacetamide treatment on colony formation

D. capillifolius protoplasts were X-irradiated with various dosages, and cultured for about 1 month. Numbers of colonies per plate were counted. As shown in Fig. 1 a, usually more than 40 krad exposure to X-ray

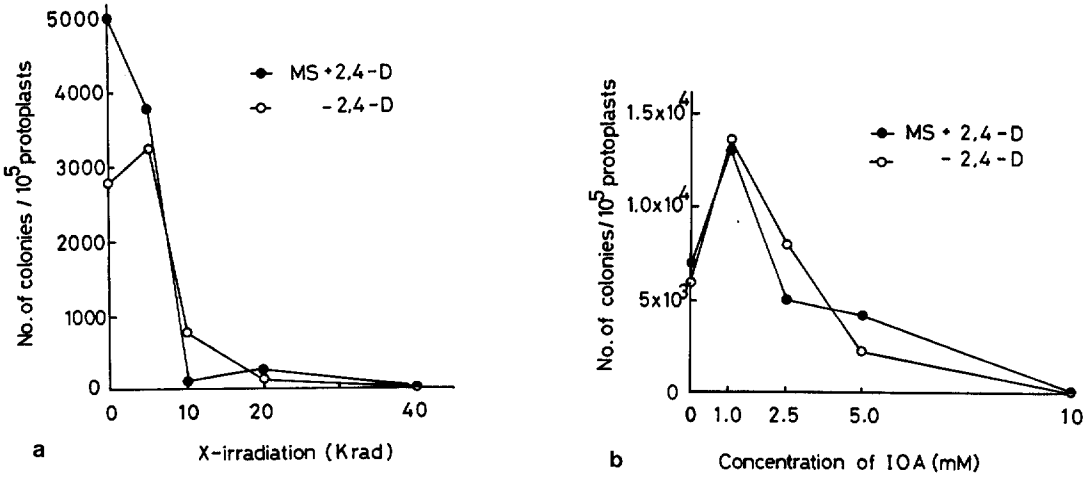

Fig. 1 a, b. Establishment of selective conditions to obtain *Daucus* cybrids. **a** Effect of X-irradiation dosage on colony formation of *D. capillifolius* protoplasts. **b** Effect of iodoacetamide (IOA) concentration on colony formation of *D. carota* protoplasts. Protoplasts were cultured for 1 month at a density of 10^5/ml in a total volume of 4 ml MS with or without 0.1 mg/l 2,4-D

inhibited colony formation in our experiments. *D. carota* protoplasts were treated with different concentrations of iodoacetamide and cultured for 1 month. No protoplast division was observed for the 10 m*M* iodoacetamide treatment (Fig. 1 b). From these results, pre-fusion treatment of protoplasts were performed as 60 krad exposure to donor (*D. capillifolius*) and 15 m*M* iodoacetamide treatment to recipient (*D. carota*).

Selection of putative cybrids

As control experiments, the X-irradiated protoplasts or iodoacetamide-treated protoplasts were cultured separately with or without polyethylene glycol (PEG) treatment. Furthermore the mixtures of both kinds of protoplasts were cultured without PEG treatment. Neither formed colonies. In contrast, following PEG-treatment of mixed parental protoplasts, many colonies were obtained from two independent experiments. By transferring them to MS without 2,4-D, the colonies grew into plants through somatic embryogenesis. The regenerated plants had the same leaf morphology as the parental *D. carota* plants, while the characteristics of leaves from those regenerated plants were clearly different from those of the other parental *D. capillifolius* (Fig. 2).

Chromosome numbers were counted in cell lines of *D. carota, D. capillifolius* and several regenerated plants (Fig. 3, Table 1). The chromosome number of *D. carota* suspension cells used in the experiments was 17 (2n−1). One chromosome might be deleted from the genome in the cells during the culture (Bayliss 1975). The chromosome number of the *D. capillifolius* cell line was over 60, presumably because of the prolonged culture of several years. As shown in Table 1, the chromosome numbers of five regenerated plants were counted. Four

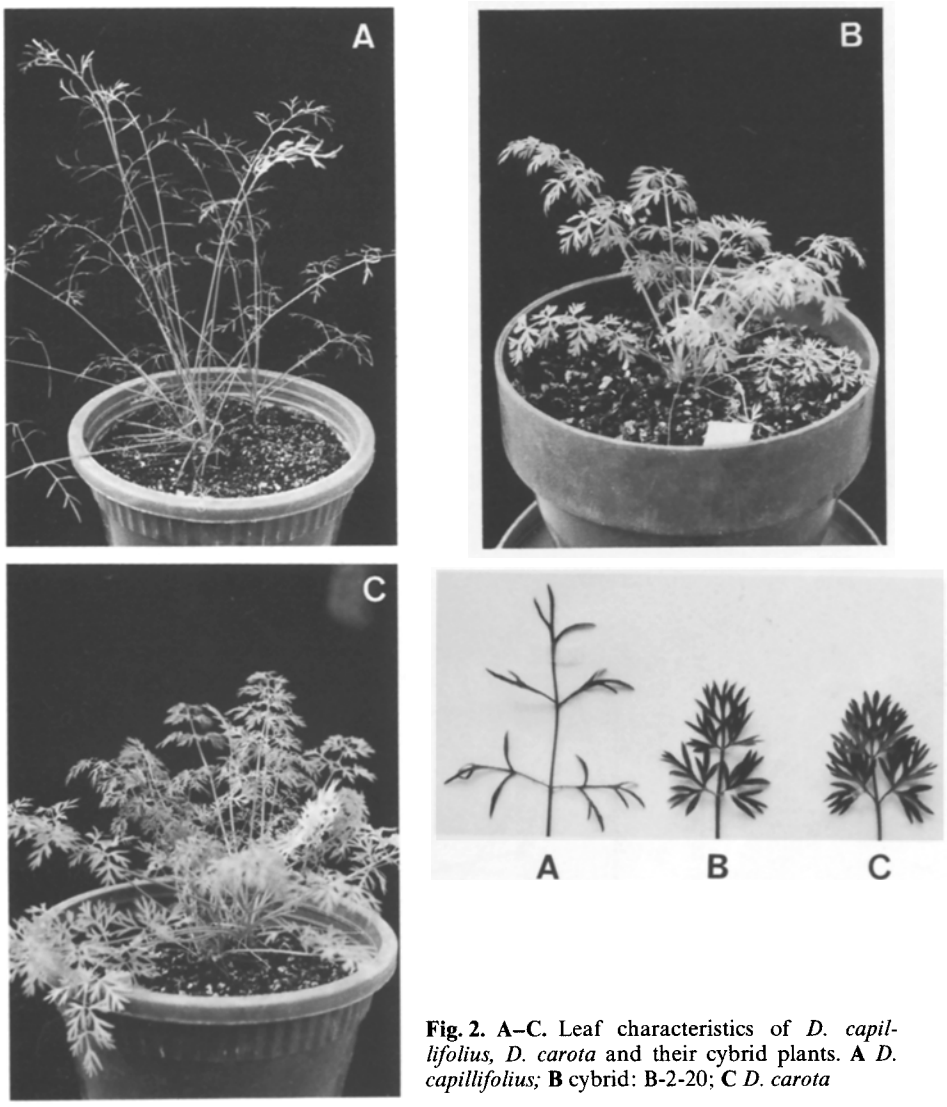

Fig. 2. A–C. Leaf characteristics of *D. capillifolius, D. carota* and their cybrid plants. **A** *D. capillifolius;* **B** cybrid: B-2-20; **C** *D. carota*

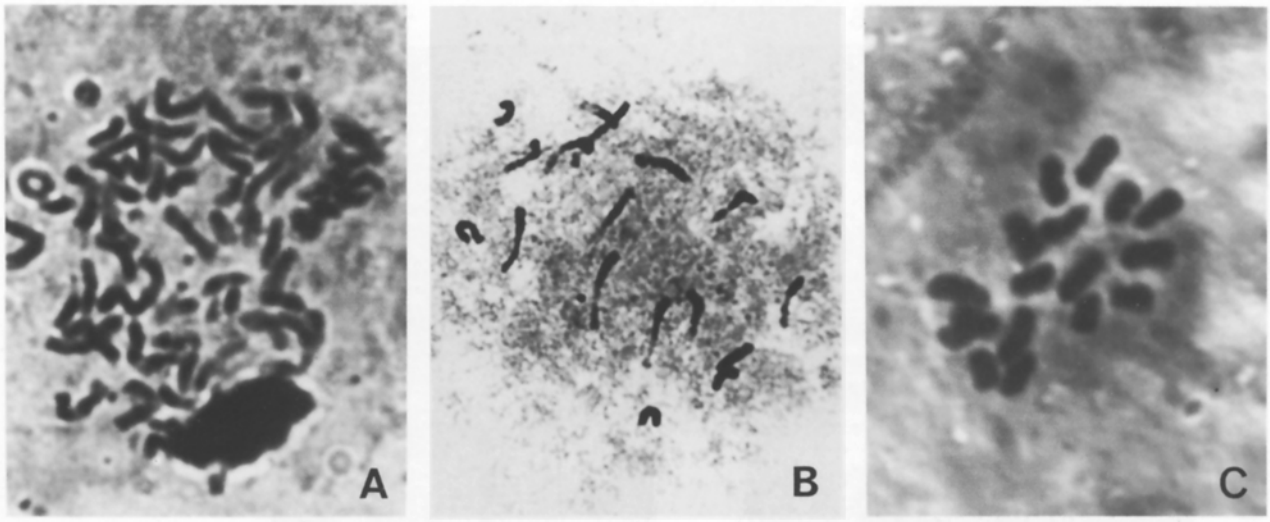

Fig. 3A–C. Chromosomes of *D. capillifolius* and *D. carota* cell cultures, and their cybrid plant. **A** *D. capillifolius* cell line; **B** cybrid plant: A-27; **C** *D. carota* cell line

Table 1. Characterization of cybrid plants with respect to leaf morphology, chromosome number and type of mtDNA restriction pattern

Plant no.	Leaf morphology	Chromosome no.	Type of mtDNA restriction pattern
A-22	*D. carota*	17	recombinant: I-a
A-27	*D. carota*	17	recombinant: I-a
A-31	*D. carota*	17	recombinant: I-a
A-39	*D. carota*	17	recombinant: I-a
A-59	*D. carota*	34	recombinant: I-b
B-1-2	*D. carota*	–	recombinant: II-a
B-1-8	*D. carota*	–	recombinant: II-b
B-1-9	*D. carota*	–	recombinant: II-c
B-2-15	*D. carota*	–	recombinant: II-a
B-2-20	*D. carota*	–	recombinant: II-c
B-3-1	*D. carota*	–	recombinant: II-c

plants (A-22, A-27, A-31 and A-39) possessed 17 chromosomes, like the *D. carota* cell line, and the other one (A-59) had 34 (4n-2), twice as many. These morphological and chromosomal observations strongly suggested that the regenerated plants possessed chromosomes of only one of the fusion parents, *D. carota,* and the chromosomes derived from *D. capillifolius* cells were eliminated from the fused cells during protoplast culture.

Restriction endonuclease analysis of mtDNAs from putative cybrids

Prior to the mtDNA analysis of regenerated plants derived from protoplast fusion, the restriction fragment patterns of mtDNAs extracted from both parental cell lines were compared (Fig. 4). Different restriction patterns were detected between the mtDNAs from both parents by Sal I and Xba I restriction endonuclease analysis. MtDNAs of the fusion-derived plants were extracted and analysed by the same restriction enzymes (Fig. 4). The restriction patterns of mtDNAs from A-22, A-27, A-31 and A-39 were identical to each other, and that of mtDNA from A-59 plant was a little different from those of the former mtDNAs. MtDNAs from those five cybrid plants possessed unique restriction patterns compared to those of their parental cell lines. For example, as shown in Fig. 4a, 7 unique fragments of Sal I digests not present in both parental mtDNAs, 5 fragments of only *D. carota* and 1 of only *D. capillifolius* were observed in mtDNAs from the fusion-derived A-22, A-27, A-31 and A-39 plants. These results indicate that A-22, A-27, A-31, A-39 and A-59 are cybrids between *D. carota* and *D. capillifolius,* and that mtDNA sequences characteristic for *D. capillifolius* were introduced into *D. carota* plants by protoplast fusion after X-irradiation of *D. capillifolius* protoplasts.

A total of 25 cybrid plants were analysed for their mtDNAs in the first fusion experiment (experiment A). The mtDNA restriction fragment patterns of those plants were classified into two types, namely I-a and I-b type (Table 1), though the two patterns were nearly identical. The possibility that all the cybrids in this experiments were originated from one clone seems to be very low, but could not be excluded completely. Therefore, in the second experiment (experiment B), the fusion-derived colonies in each plate were cultured into plants separately from the colonies in the other plates. All the regenerated plants possessed an identical leaf morphology to that of *D. carota* plants. The

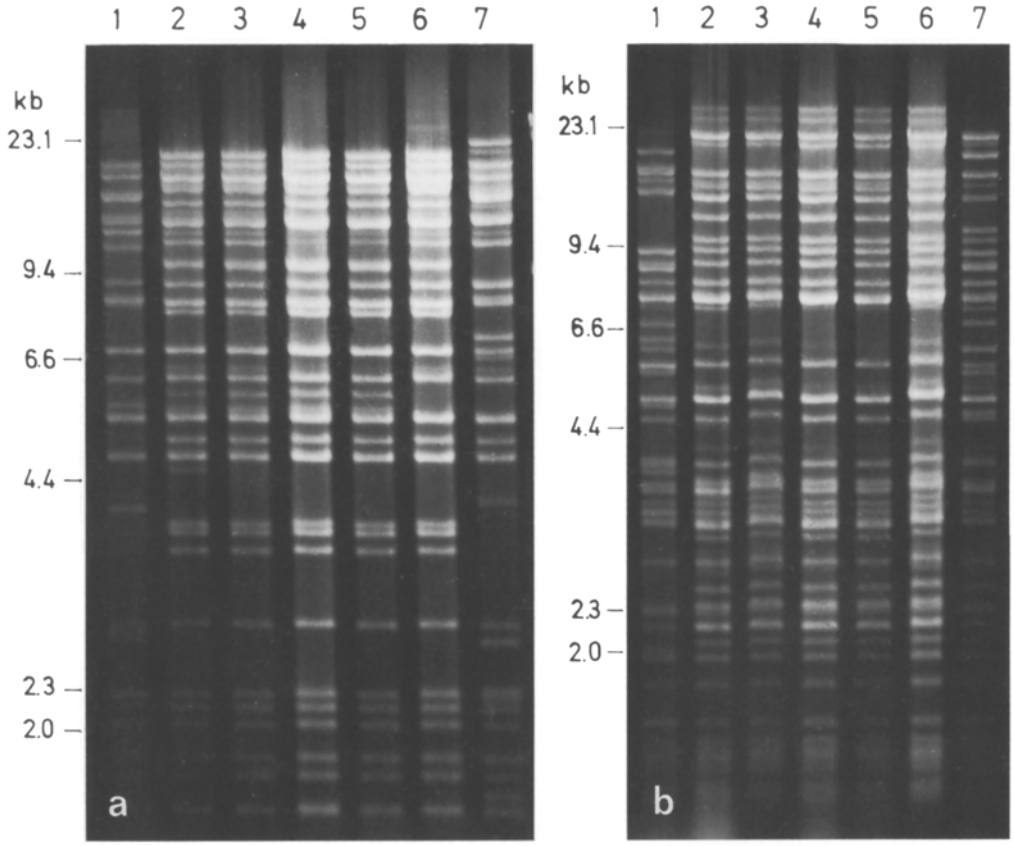

Fig. 4. **a** Sal I and **b** Xba I restriction endonuclease analysis of mtDNAs from *D. capillifolius, D. carota* and their cybrid plants in experiment A. *D. capillifolius* (*lane 1*), cybrid plants: A-22 (*2*), A-27 (*3*), A-31 (*4*), A-39 (*5*) and A-59 (*6*) and *D. carota* (*7*)

mtDNAs of putative cybrids derived from each plate were analysed (Fig. 5, Table 1).

Although their restriction patterns resembled each other, they were classified into three types which are different from those of cybrids from the experiment A. The restriction fragment patterns of cybrids from experiment B were more similar to that of *D. capillifolius* than that of cybrids from experiment A. MtDNAs of three cybrids, B-1-2, B-1-8 and B-1-9, derived from the same plate, had slightly different Sal I and Xba I restriction patterns from each other. The Sal I and Xba I restriction patterns of B-2-20, derived from an another plate, completely coincided with those of B-1-9. These results show that an identical restriction patterns of mtDNAs observed between cybrids can be acquired not only by cybrids derived from a single clone, but also by cybrids derived from distinct clones.

Discussion

In this paper, we reported the successful formation of carrot cybrid plants for the first time by protoplast fusion between iodoacetamide-treated *D. carota* (recipient) and X-irradiated *D. capillifolius* (donor), and characterized the mtDNA of parents and their cybrid plants. About 10% of regenerated plants after the fusion treatments showed the same leaf characteristics and the same mtDNA restriction patterns as *D. carota,* suggesting that the plants might be escaped plants of *D. carota* from the selection systems. However, about 90% of the regenerated plants showed the characteristics of cybrid plants, indicating that the selection systems based on metabolic complementation could be useful for the introduction of cytoplasmic factors which usually exhibit maternal inheritance.

Restriction endonuclease fragment pastterns of mtDNAs from the carrot cybrid plants were compared to those of the both parents. MtDNA recombinations were detected in *Nicotiana* cybrid plants derived from the asymmetric fusions between γ- or X-irradiated and iodoacetate-treated protoplasts (Nagy et al. 1983; Aviv et al. 1984). We observed some novel fragments in the cybrid plants in addition to the fragments corresponding to those of either one parental mtDNA or both parental mtDNAs.

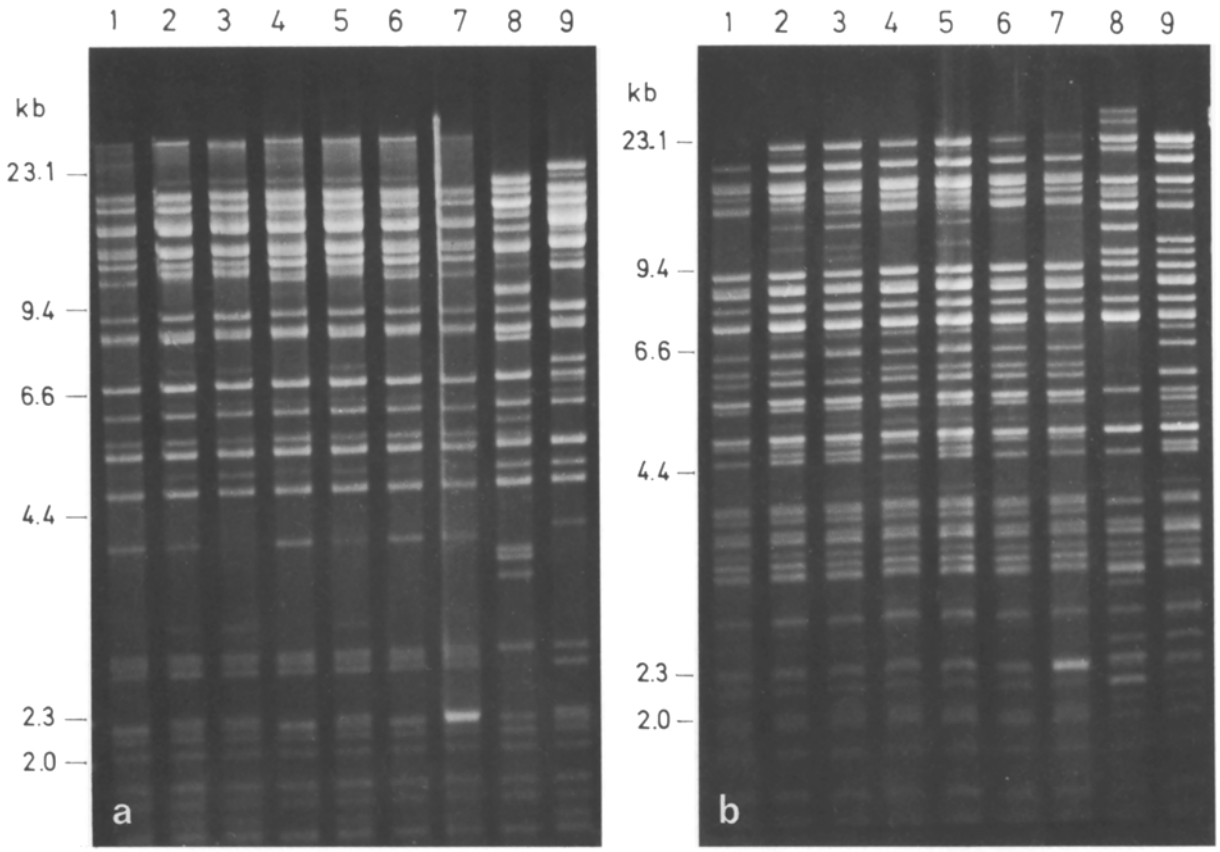

Fig. 5. **a** Sal I and **b** Xba I restriction endonuclease analysis of mtDNAs from *D. capillifolius, D. carota* and their cybrid plants in experiment B. *D. capillifolius* (*lane 1*), cybrid plants: B-1-2 (*2*), B-1-8 (*3*), B-1-9 (*4*) B-2-15 (*5*), B-2-20 (*6*), B-3-1 (*7*) and A-39 (from experiment A) (*8*), and *D. carota* (*9*)

The mtDNA restriction patterns of the *D. carota* suspension cell lines did not change over a subculture of 1 year (data not shown). Moreover, the carrot plants regenerated from protoplasts treated with 5 m*M* iodoacetamide before culture bore the same mtDNA restriction pattern as plants which originated from seeds (unpublished data). MtDNAs of *D. capillifolius* cell lines derived from protoplasts that escaped lethal x-irradiation and possess no regeneration potential could not be distinguished from that of a cell line initiated from seeds (unpublished data). Matthews and DeBonte (1985) also pointed out the stability of carrot organelle DNAs in cell suspension cultures maintained for 10 years and in regenerated carrot plants derived from a cell culture. From these results, it was concluded that the novel mtDNA band patterns of carrot cybrid plants in this study were the results of cytoplasmic mixing and interspecific mtDNA recombination.

MtDNA recombinations have been observed in a somatic hybrid of *D. carota* and *D. capillifolius* (Matthews and Widholm 1985) and in *D. carota* somatic hybrids (Kothari et al. 1986). In the latter case, although a new mtDNA restriction pattern was observed in one fusion combination, the mtDNAs of all the somatic hybrids were identical to each other. In our case, the mtDNA restriction fragment patterns in experiments A and B were relatively disimilar but very similar within each experiment. Only two types of mtDNA restriction patterns were found in a total of 25 cybrid plants in experiment A, and the restriction patterns were classified into three slightly different types in a total of 9 cybrids obtained from experiment B. These results strongly suggest the existence of 'hot spots' for recombination of the mitochondrial genome.

In contrast to the lack of diversity in carrot mtDNA rearrangements, the patterns of mtDNA recombinations in somatic hybrid or cybrid plants of tobacco (Belliard et al. 1979; Galun et al 1982; Nagy et al. 1983; Aviv et al. 1984), petunia (Boeshore et al. 1983; Clark et al. 1986) and *Brassica* (Chetrit et al. 1985) are more varied. Direct repeat elements are present within some plant mtDNAs and rearrangements in mtDNAs are thought to occur through the elements (Lonsdale et al. 1984; Palmer and Shields

1984). There should be some relationships between the degree of mtDNA recombinations in cybrids or somatic hybrids and the number or structure of the repeat elements within those mtDNAs.

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