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Transfer of the CMS trait in *Daucus carota* L. by donor-recipient protoplast fusion

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Summary. X-irradiated protoplasts of *Daucus carota* L., 28A1, carrying cytoplasmic male sterile (CMS) cytoplasm and iodoacetamide-treated protoplasts of a fertile carrot cultivar, 'K5', were fused with polyethylene glycol (PEG), and 73 plants were regenerated. Twenty-six randomly chosen regenerated plants had non-parental mitochondrial DNA (mtDNA) as revealed by XbaI restriction fragment patterns, and all of the plants investigated had diploid chromosome numbers. Of the 11 cybrid plants that showed mtDNA fragment patterns clearly different from those of the parents, 10 plants showed male sterility with brown or red anthers, and one plant possessed partially sterile yellow anthers. The mtDNA fragment patterns of the ten cybrid plants with male sterile flowers resembled that of a CMS parent, 28A1; and four fragments were identified that were common between the sterile cybrid plants and 28A1, but absent from the partially sterile cybrid plants and a fertile cultivar, 'K5'. The results indicated that the CMS trait of the donor was efficiently transferred into the cybrid plants by donor-recipient protoplast fusion.

Key words: *Daucus carota* L. – Cytoplasmic male sterility – Donor-recipient protoplast fusion – Cybrids – Mitochondrial DNA

Introduction

Since cytoplasmic male sterility (CMS) was first documented in *Daucus carota* L. by Welch and Grimball (1947), it has been considered a useful trait in commercial carrot F_1 seed production. Two types of CMS flowers have been observed: the brown anther type (Welch and Grimball 1947; Braak and Kho 1958; Banga et al. 1964) and the petaloid type (Thompson 1961; McCollum 1966). As 8–10 years of backcrossing is required to introduce CMS into desired carrot varieties or lines, there is an urgent need to develop more efficient procedures for this process. One possibility is the transfer of cytoplasm from a carrot CMS line into a commercial fertile variety by protoplast fusion.

The successful transfer of the CMS trait to fertile lines by protoplast fusion has been achieved in *Nicotiana, Brassica,* and *Petunia* (Belliard and Pelletier 1978; Zelcer et al. 1978; Aviv et al. 1980; Izhar et al. 1983; Menczel et al. 1983; Pelletier et al. 1983; Yarrow et al. 1986; Barsby et al. 1987a, b; Menczel et al. 1987; Morgan and Maliga 1987; Chuong et al. 1988).

Donor-recipient protoplast fusion based on metabolic complementation is an efficient procedure for the selective formation of cybrids (Sidorov et al. 1981; Morgan and Maliga 1987; Ichikawa et al. 1987; Vardi et al. 1987). Exposure of the donor protoplasts to an overdose of lethal X- or gamma-irradiation stimulates the selective elimination of donor chromosomes from the fusion products, and chemical pretreatment of the recipient protoplasts with iodoacetamide prevents cell division. As a result, only the metabolically complementary cells are capable of developing into plantlets after fusion treatment. Furthermore, in such a donor-recipient protoplast fusion method, there is no need to make use of a selectable cytoplasmic marker of the donor during protoplast culture after fusion treatment.

In this report, we describe male sterility and flower characteristics in carrot cybrids derived from donor-recipient protoplast fusion between cytoplasmic male sterile and fertile plants. We also discuss the correlation between male sterility in the cybrid plants and their mtDNA restriction endonuclease patterns.

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Materials and methods

Plant materials

A CMS line of *Daucus carota*, 28A1 (brown anther type), and a fertile cultivar of *D. carota*, 'K5', were used as cytoplasmic donor and recipient, respectively. The seeds of the line and cultivar were provided by the Kyowa Seed Co.

Pretreatments and fusion of protoplasts

Protoplasts of each parent were isolated from suspension cells derived from hypocotyls, as described by Ichikawa et al. (1987). The protoplasts of the cytoplasmic donor (28A1) were X-irradiated at 60 Krad (1 Krad/min), and those of the recipient ('K5') were treated with 15 m*M* iodoacetamide at room temperature for 10 min. The dosages of each pretreatment had been determined to be lethal to protoplasts in our preliminary experiments (Ichikawa et al. 1987).

After each treatment, the protoplasts were rinsed once with washing solution (0.5 *M* mannitol, 0.1% MES, pH 5.7), mixed in a 1:1 ratio, and fused with polyethylene glycol (PEG) according to the method of Kao and Michayluk (1974). Subsequent to the fusion treatments, the protoplasts were cultured in MS medium containing 0.3 *M* sorbitol and 0.1 mg/l 2,4-D at 25°C.

The X-irradiated and the iodoacetamide-treated protoplasts were cultured separately with and without PEG treatment to investigate their cell division and plant regeneration ability. The mixtures of both pretreated protoplasts were also cultured without PEG treatment. For details of protoplast culture, see Ichikawa et al. (1987).

The colonies derived from heteroplasmic protoplast fusions were transferred onto hormone-free MS medium (Murashige and Skoog 1962) for plant regeneration. Those regenerated plants with well-developed roots were transferred into Magenta boxes (Magenta Co, USA) containing sterilized soil supplemented with MS inorganic salt components. After 1–2 months, the box covers were replaced with plastic bags, and several pin holes were punched in the plastic bags every 3–6 days for 1–2 months. During this period, the plants were acclimatized to an environment of low humidity and transferred into a greenhouse. Plants grown for a few weeks in the greenhouse were then transplanted into the field.

MtDNA restriction endonuclease analysis

Twenty-six plants were randomly chosen from the 73 transplants for mtDNA restriction pattern analysis. The mtDNA isolation procedure has been described previously (Ichikawa et al. 1987). MtDNAs isolated from both suspension cells of parental plants and the 26 randomly chosen regenerated plants derived from protoplast fusion were digested with XbaI restriction enzyme for at least 4 h and then treated with 0.05 mg/ml RNase A at 37°C for 30 min. The DNA fragments were separated by electrophoresis in 0.7% agarose gel and stained with ethidium bromide.

Chromosome counting

Suspension cells induced from the regenerated plants were incubated in a 0.2% colchicine solution at 4°C for 4 h and fixed with 3:1 ethanol-acetic acid for 10 min. They were then macerated with the following enzyme solution: 4% Cellulase RS, 1% Pectolyase Y-23, 7.5 m*M* EDTA, pH 4.0 (Nishibayashi and Kaeriyama 1986). They were rinsed twice with water, spread on glass slides, and stained with aceto-orcein for microscopic observation.

Investigation of flower morphology and male fertility

Of the 26 regenerated plants selected for mtDNA restriction endonuclease analysis, 11 were found to have fragment patterns differing from those of the parents. Consequently, their flower morphology and male fertility were investigated. Petal shape, anther color, and stamen filaments were observed microscopically. Male fertility was evaluated by determining the percentage of functional pollen grains. About 1,000 pollen grains were stained by 1% cotton blue solution. Those which showed normal shape and good stainability by the cotton blue solution were judged to be functional. In addition, 35 anthers were collected from each of the regenerated plants and their parents for counting the number of functional pollen grains per anther. The pollen grains were suspended in 15 μl 10% sucrose solution and counted using a hemocytometer.

Results

Cybrid formation by protoplast fusion

No colony formation was observed in cultures of X-irradiated donor (28A1) protoplasts, iodoacetamide-treated recipient ('K5') protoplasts, and their mixed protoplasts lacking the fusion treatment. Some colonies were formed through the homoplasmic fusion of X-irradiated protoplasts (about 0.2% plating efficiency), but these colonies showed no morphogenic responses on hormone-free MS medium. No colonies formed from the homoplasmic fusion of iodoacetamide-treated protoplasts. In contrast, when a 1:1 mixture of the X-irradiated and iodoacetamide-treated protoplasts were fused with PEG, colonies were formed at about a 2.0% plating efficiency. These colonies regenerated plantlets via embryogenesis after being transferred to hormone-free MS medium. Although some of the regenerated plantlets were normal in their morphological development, most of them showed abnormal growth and produced some secondary embryos from the stems. The secondary embryos tended to develop into morphologically normal plants. Plants that did develop normally were obtained from the regenerated plantlets at a frequency of about 10%.

Analysis of mtDNA restriction endonuclease patterns and chromosome number of cybrids

Figure 1 shows the XbaI restriction fragment patterns of mtDNA from 28A1 (lane 1), 'K5' (lane 11) and the regenerated plants derived from protoplast fusion (lanes 2–10). 28A1 and 'K5' showed patterns distinguishable from each other. Out of the 26 regenerated plants, 11 plants showed fragment patterns clearly different from that of the recipient, and 15 plants showed a pattern similar to that of 'K5'. Lanes 2–7 in Fig. 1 show the fragment patterns from 6 of these 11 plants, and lanes 8–10 show those from 3 of the latter 15 plants. All the regenerated plants examined had at least one novel frag-

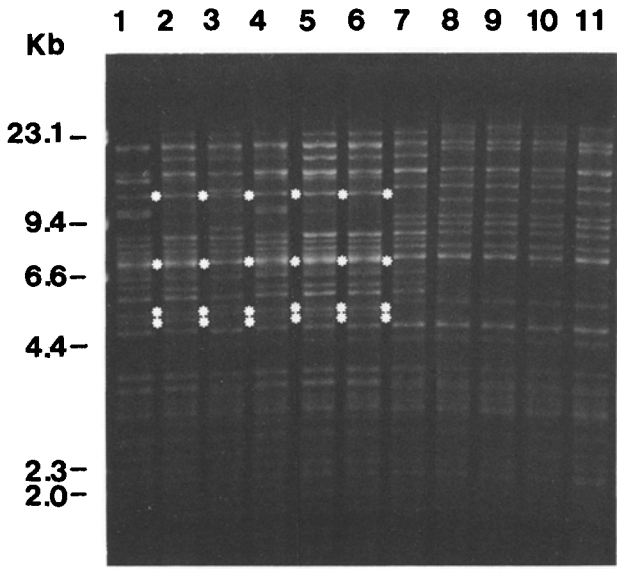

Fig. 1. XbaI restriction endonuclease analysis of mtDNAs from 28A1 (CMS), K5 (fertile) and their cybrid plants. *Lane 1:* 28A1 (CMS); *lane 2:* A-7-33 (sterile); *lane 3:* A-2-30 (sterile); *lane 4:* A-2-18 (sterile); *lane 5:* A-1-20 (sterile); *lane 6:* E-2-5 (sterile), *lane 7:* A-5-18 (partially sterile), *lane 8:* D-2-14, *lane 9:* E-1-11, *lane 10:* E-2-14, *lane 11;* K5 (fertile). *White dots* indicate those fragments common between the sterile cybrid plants and 28A1 that were not present in the partially sterile cybrid plant and K5

Table 1. Characterization of regenerated plants derived from protoplast fusion between 28A1 (CMS) and K5 (fertile)

Plant no.	Chromosome no.	mtDNA fragment pattern	Male fertility
A-2-18	18	Recombinant	Sterile
A-7-33	18	Recombinant	Sterile
A-5-18	18	Recombinant	Partially sterile
D-1-20	18	Recombinant	–
D-2-25	18	Recombinant	–
E-2-14	18	Recombinant	–

ment that was absent from both parents and lacked a few fragments characteristic of the parents. Most of the other fragments from the regenerated plants could be observed in either 28A1 or 'K5'. These novel fragment patterns were considered to have resulted from recombination between homologous mtDNA regions of the parental plants.

The chromosome number of each regenerated plant was also studied. All the plants investigated were found to possess diploid chromosomes ($2n = 18$) (Table 1) indicating that the chromosomes of donor cells might be eliminated completely from the fused cells during their developmental process. The regenerated plants investigated were therefore classified as cybrids. The 11 cybrid plants whose mtDNA fragment patterns clearly differed from that of the recipient were subsequently investigated for flower morphology and male fertility.

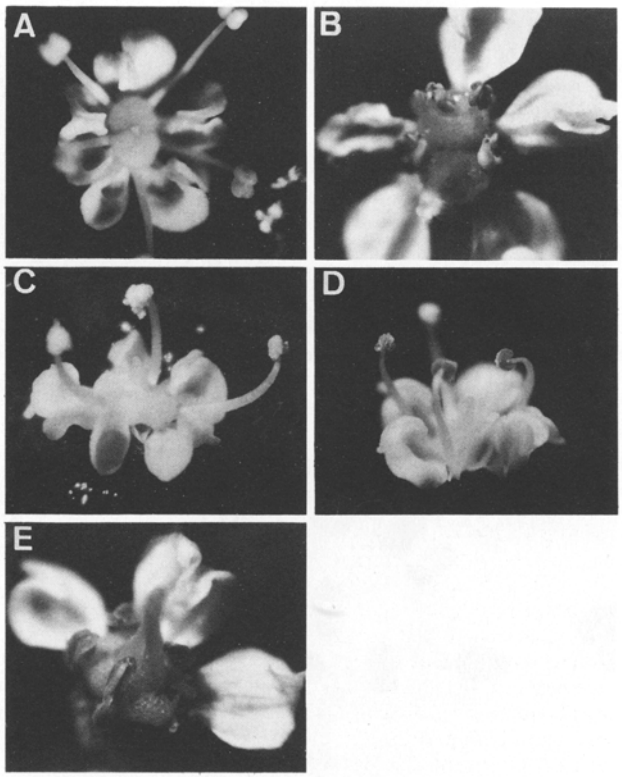

Fig. 2A–E. Flower morphology of parental plants and cybrid plants. **A** K5 (fertile) with yellow anthers and normal filaments; **B** 28A1 (CMS) with brown anthers and stunt filaments; **C** A-5-18 with red anthers and normal filaments; **D** D-2-15 with red anthers and normal filaments; **E** E-1-16 with brown anthers and stunt filaments

Flower morphology and male fertility of the cybrid plants

The flower morphologies of the parental plants and their cybrid plants are shown in Fig. 2. The fertile parent, 'K5', possessed yellow anthers with normal filaments (Fig. 2A), and the CMS parent, 28A1, had brown anthers with stunt filaments (Fig. 2B). The flowers of their cybrid plants were classified morphologically into three types: yellow anthers with normal filaments (Fig. 2C), red anthers with normal filaments (Fig. 2D), and brown anthers with stunt filaments (Fig. 2E). The red anthers with normal filaments were a specific phenotype observed only in the cybrid plants. The petals of the cybrid plants showed a normal shape and a color identical to those of both parents.

Table 2 shows male fertility and the flower morphology of the parents and the cybrid plants. The CMS parent, 28A1, shed no pollen grains, while the fertile parent, 'K5', shed about 7.8×10^2 functional pollen grains per anther (Fig. 3A). The brown or red anthers of cybrids A-2-34, A-7-33, D-2-15, E-1-3, E-1-16, and E-2-5 contained no pollen grains, and A-2-18, A-2-25, A-2-30 and A-8-3 had non-functional pollen grains that were abnor-

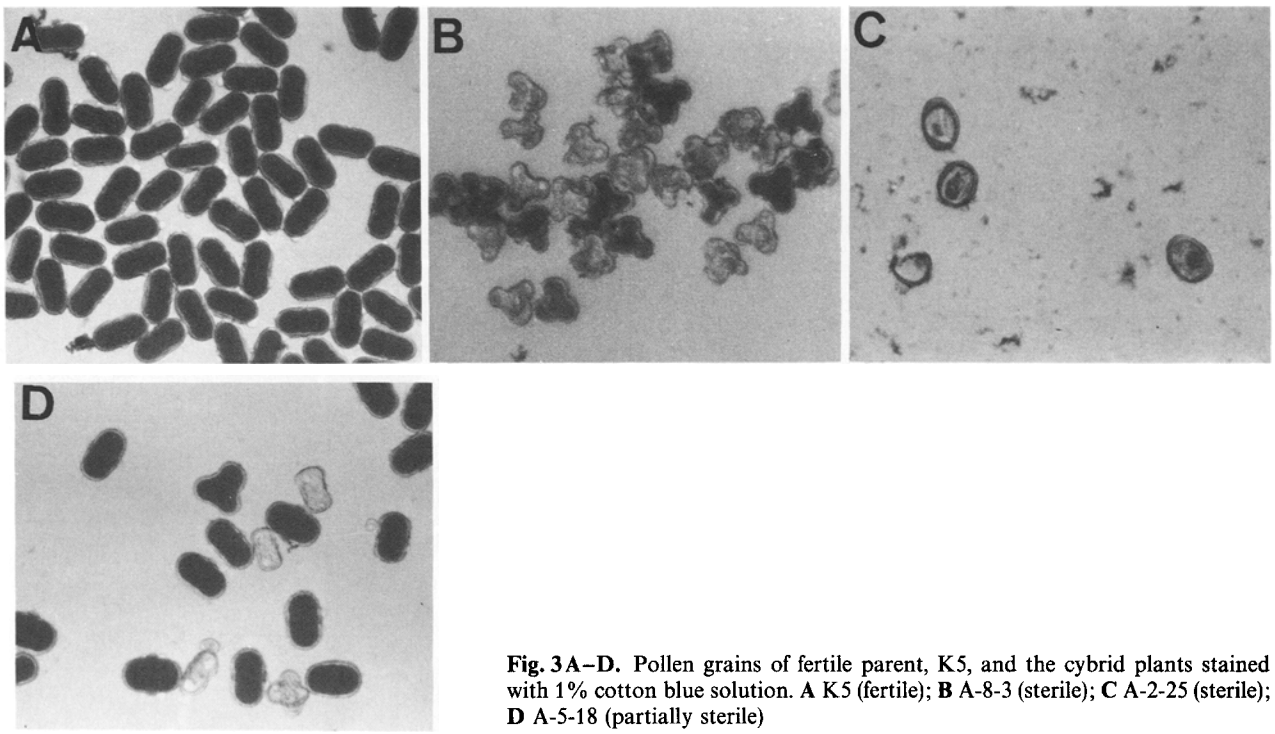

Fig. 3A–D. Pollen grains of fertile parent, K5, and the cybrid plants stained with 1% cotton blue solution. **A** K5 (fertile); **B** A-8-3 (sterile); **C** A-2-25 (sterile); **D** A-5-18 (partially sterile)

Table 2. Male fertility and flower morphology of 28A1 (CMS), K5 (fertile) and their cybrid plants

Line	Male fertility	% of functional pollen grains	No. of functional pollen grains/anther	Pollen shape	Anther color	Stamen filaments[a]
Parents						
28A1	Sterile	0	0	–	Brown	s
K5	Fertile	96.9	7.8×10^2	Normal	Yellow	n
Cybrids						
A-2-18	Sterile	0	0	Abnormal	Brown	s
A-2-25	Sterile	0	0	Normal	Brown	s
A-2-30	Sterile	0	0	Normal	Brown	s
A-2-34	Sterile	0	0	–	Brown	s
A-5-18	Partially sterile	42.5	2.2×10^2	Normal	Yellow	n
A-7-33	Sterile	0	0	–	Brown	s
A-8-3	Sterile	0	0	Abnormal	Red	n
D-2-15	Sterile	0	0	–	Red	n
E-1-3	Sterile	0	0	–	Brown	s
E-1-16	Sterile	0	0	–	Brown	s
E-2-5	Sterile	0	0	–	Brown	s

[a] n and s indicate normal and stunt filaments, respectively

mally shaped (Fig. 3B) or unstained by the cotton blue solution (Fig. 3C). This indicated that they were male sterile. They were able to set seeds by backcrossing with the fertile parent, 'K5'. On the other hand, the cybrid plant with yellow anthers (A-5-18) shed some functional pollen grains (Fig. 3D) and set a few seeds. The clone A-5-18 was therefore classified as partially male sterile.

While those cybrid plants with mtDNA fragment patterns similar to that of 28A1 were fully sterile, partial sterility was observed in a cybrid plant (A-5-18) whose mtDNA fragment pattern was clearly different from those of both parents. In addition, four common fragments, which were absent in the partially sterile cybrid plants and 'K5', were also identified in the sterile cybrid plants and 28A1 (Fig. 1).

Discussion

The data presented here show that somatic protoplast fusion between X-irradiated CMS donors and

iodoacetamide-treated recipients can result in the selective formation of male sterile cybrids in carrot.

All of the 26 regenerated plants investigated had recombinant mtDNA types, and of the six plants analyzed, all were diploids. Treatment with X-irradiation proved effective in eliminating donor chromosomes in these experiments, an observation consistent with our earlier work (Ichikawa et al. 1987). Some workers have demonstrated that a complete elimination of donor chromosomes has not always been attained by X- or gamma-irradiation in experiments on cybrid formation (Zelcer et al. 1978; Aviv et al. 1980; Menczel et al. 1983; Medgyesy et al. 1985; Bates et al. 1987; Barsby et al. 1987b; Menczel et al. 1987; Sidorov et al. 1987). Factors affecting chromosome elimination from fusion products treated by X-irradiation have not yet been specified.

As indicated in Table 1, all but one of the 11 cybrid plants investigated were found to be male sterile on flowering, and their mtDNA restriction patterns were relatively similar to that of the CMS parent (lanes 2–6 in Fig. 1). On the other hand, a cybrid plant (A-5-18) showed partial male sterility and carried an mtDNA fragment pattern different from those of both parents. These results support the idea that the genes responsible for the CMS trait in donor plants might be transferred into male sterile cybrid plants, and that the CMS genes could not be transferred into the partially sterile cybrid plant. We are planning to investigate the genetic segregation of male sterility in the progenies to confirm the maternal inheritance of the transferred trait in a future study.

Our results are in agreement with the findings of Galun et al. (1982) and Aviv and Galun (1987) who reported that mtDNA restriction patterns of male sterile and male fertile cybrids are similar to those of the CMS donor and the fertile recipient, respectively. The existence of this correlation means that putative male sterile cybrids can be selected from among a large number of cybrid plants at an early stage of plant regeneration using the mtDNA analysis.

Four XbaI fragments that were common to both male sterile cybrids and the CMS parent were missing in the partially sterile plant and the fertile parent. In *Petunia,* two common BglI fragments of mtDNA have been observed within CMS cybrids (Boeshore et al. 1985), and very recently a CMS-related gene of *Petunia* was identified from the fragment (Young and Hanson 1987). However, further investigations are required to determine whether these four mtDNA fragments found in the male sterile carrot cybrids are directly related to the CMS traits.

The introduction of carrot CMS by conventional backcrossing usually takes 8–10 years. However, using the protopolast fusion methods, it can be achieved within about 16 months (from protoplast fusion to flowering). Thus, we conclude that this technique is an effective method for transferring the CMS trait and can be applied in the practical breeding of carrots.

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