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**BASIC STUDIES ON TRANSFER OF CYTOPLASMIC MALE
STERILITY BY MEANS OF CYTOPLASMIC HYBRIDIZATION
IN CARROT (*Daucus Carota* L.)**

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INTRODUCTION

Our current attention has been focused on 'somatic cell engineering', because it may enable us to widen the range of genetic variation and to shorten the time required for raising new varieties. In the conventional plant breeding, introduction of desired genes was performed through sexual reproduction within the species that are crossable each other, which limits the range of genes available for a given variety. Furthermore, a long period of time for selection and purification is necessary to incorporate the desired genes into the variety. Recent advances in genetic engineering and *in vitro* manipulation of cells may offer a useful complement to traditional procedures of plant breeding, and provide an additional tool to create gene pools hitherto not available in nature.

Discovery of a method for the enzymatic isolation of protoplasts by Cocking³¹⁾ have paved the way for tremendous progress in somatic cell engineering because the lack of cell wall makes access of foreign genes easier. To apply the protoplasts in practical plant breeding, however, it is prerequisite to establish a culture method for regeneration of complete plants from isolated protoplasts with high reproducible efficiency. Up to date, such a regeneration system has been developed in a number of species⁹⁾. At present, the techniques, which are considered to be promising for practical application in crop improvement, are as follows; 1) somaclonal variation to increase genic diversity, 2) somatic hybridization to transfer nuclear or cytoplasmic genes, 3) transformation to directly introduce foreign genes, and so on.

Somatic hybridization provides a method for breaking the barrier of sexual incompatibility between distantly related species^{124,125)}, and simultaneously, for mixing cytoplasmic genomes of the parents^{16,19,97)}. Introduction of agronomically important traits encoded by nuclear genomes through somatic hybridization have been attempted by many researchers^{17,52,61,63,72,99,137,138)}. However, most of the intergenic or interspecific fusion products failed to regenerate functional plants probably due to a various level of genetic incompatibilities. To overcome this problem, asymmetric somatic hybridization using radioactive irradiation has been proposed by Dudits *et al.*³⁹⁾. This method enabled us to transfer only a part of the nuclear genomes to a desired plant by partially eliminating chromosomes of the irradiated cells from fused cells^{13,39,40,55,56,66,68,131,133,146)}.

For effective selection of hybrid cells, it is generally required to use the cells with selection markers such as amino acid analog or antibiotics resistance, nitrate reductase deficiency, nutrient requirement and albino mutant. However, these mutants only exist in a few limited species. A selection method of only somatic hybrids without use of such selection markers was demonstrated by Sidorov *et al.*¹³⁰⁾, in which somatic hybrids could be selected based on metabolic complementation between X-irradiated donor cells and iodoacetate-treated recipient cells. This method also made it possible to produce cybrids, which possess only one parental nuclear genomes and both parental organelle genomes, through complete elimination of chromosomes of donor cells by X-irradiation.

Cytoplasmic male sterility (CMS), which causes pollen abortion, is a widespread phenomenon in over 140 species, and maternally inherited. This trait is agronomically valuable for F₁ seed production⁵⁹⁾, and is considered to be encoded by mitochondrial genomes. Zelcer *et al.*¹⁵⁰⁾ have first transferred the CMS to a fertile plant by irradiating X-ray to CMS protoplasts prior to protoplast fusion in tobacco. To date, CMS transfer using donor-recipient protoplast fusion has been achieved in *Nicotiana*^{3,4,45,80,81,97,103,150)}, *Brassica*^{11,12,77,101,106,147)}, and *Oryza sativa*^{1,148)}.

Carrot (*Daucus carota* L.) is an important crop which is cultivated in all over the world, but most of the modern varieties lack uniformity in productivity, quality and nutrient value⁷⁵⁾. Recently, F₁ breeding have arrested our attention because some carrot F₁ hybrids between the lines having high combining ability showed higher yield and more uniform quality. The carrot CMS was first reported by Welch and Grimball¹⁴⁵⁾, and at present, two types of the CMS are used as female parents in carrot F₁ breeding; one is brown anther type whose flowers possess brown anthers with aborted pollen grains, and the other is petaloid type whose stamens are transformed into petal-like structure. To introduce these CMS traits into a desired carrot variety, however, 7-8 years of repeated back-crossings are necessary because of the biennial trait, and laborious emasculation of anthers is needed because of the small flowers (2-4 mm in diameter). Therefore, direct transfer of the CMS in carrot through cytoplasmic hybridization has been urgently required by breeders, but such trials have not been succeeded yet.

Cytoplasmic hybridization or cybridization also provides useful materials for searching for the DNA regions coding cytoplasmic traits such as CMS and herbicide resistances, because the mixture of cytoplasms of the parents generates novel compositions of organelle genomes in fused cells. In petunia, both CMS and fertile somatic hybrid plants were created by protoplast fusion between CMS and fertile lines. They carried novel mitochondrial genomes that arose from recombination of the parental lines^{19,120)}. The comparison of the mitochondrial genomes between CMS and fertile somatic hybrids made it possible to identify a mitochondrial DNA region correlated with the CMS^{18,60,149)}. Therefore, analysis of cytoplasmic genomes of cytoplasmic hybrids or cybrids, and their progenies may contribute to identification of the cytoplasmic genes, and to study on

molecular or evolutionary genetics of higher plant organelle genomes.

In this thesis, I describe (I) an efficient method of carrot protoplast culture, (II) successful formation of cybrids between *D. carota* and the related wild species by donor-recipient protoplast fusion and their characterization, (III-1) transfer of brown anther-type CMS to a fertile carrot cultivar through the protoplast fusion based on metabolic complementation, and their mtDNA analysis, (III-2) transfer of petaloid-type CMS in carrot by the donor-recipient protoplast fusion, (IV) composition of mitochondrial genomes in carrot cybrids and their sexual progenies, and finally, I discuss the correlation between carrot petaloid CMS and mitochondrial genes.

I. ESTABLISHMENT OF AN EFFICIENT METHOD OF PROTOPLAST CULTURE IN *DAUCUS*

Introduction

Since totipotency of plant cells was first documented by Steward *et al.*¹³⁵, regeneration of complete plants from cultured cells has been reported in a number of species. Establishment of a method for regeneration of whole plants from protoplasts with high reproducible efficiency is essential for crop improvement using somatic hybridization.

In *Daucus carota*, plant regeneration from protoplasts was first demonstrated by Grambow *et al.*⁵⁴, and Kameya and Uchimiya⁷¹. In their reports, it was also described that regeneration of carrot plantlet occurs through embryogenesis from colonies. The embryo formation can be easily controlled by adding or omitting 2,4-dichlorophenoxyacetic acid (2,4-D) into medium^{96,132,140}. Furthermore, it was reported that several other hormones or chemicals could promote the embryogenesis^{2,24,26,85,88,118,123,140}. Biochemical and molecular-biological studies on carrot embryogenesis were extensively performed to understand the morphogenetic and physiological process during embryogenesis, and recently, carrot embryonic gene, namely DC8, has been identified by Franz *et al.*⁴⁸. However, efficient method for differentiation from the somatic embryos has not been developed yet. Here, I establish a system for carrot plant regeneration from protoplasts with high reproducible frequency.

Materials and methods

1. Plant materials

Carrot cultivars with high combining ability, K5, NS and 35B were used. These seeds were provided by Kyowa Seed Co.

2. Protoplast isolation and culture

Hypocotyls of about 1 week old seedling were sterilized with sodium hypochlorite solution containing 3% active chloride for 15 min after treatment by 70% ethanol for 2 min. The sterilized hypocotyls were rinsed twice in sterilized water and inoculated on MS medium¹⁰⁸ containing 1.0 mg/l 2,4-D for callus induction. The calli induced from each variety were suspended in liquid MS medium containing 0.5 mg/l 2,4-D. The suspension cells were subcultured every 7-10 days for transferring 1 ml packed cells in 25 ml of fresh MS medium. Protoplasts were isolated from the suspension cells 3-4 days after subculture.

Several combinations of the following enzymes were used for protoplast isolation ; Driselase (Kyowa Hakko Co.), Pectolyase Y-23 (Seishin Pharmaceutical Co.), Cellulase YC (Yakult Co.), Cellulase R-10 (Yakult Co.), and Cellulase RS (Yakult Co.). The enzyme solutions contained 0.5 M mannitol and 0.1% 2-(N-mopholino) ethane sulfonic acid (MES), and pH was adjusted to 5.7. The suspension cells were treated with the enzyme solutions at 25°C shaking for 1-6 hr.

The suspension cells and protoplasts isolated from the suspension cells were separated in size by passing through 20, 37, 63, 105, 297, and 500 μm stainless steel meshes. The isolated protoplasts were rinsed twice with a washing solution (0.5 M mannitol and 0.1% MES, pH 5.7) by 1000 rpm centrifugation for 5 min, and cultured in liquid MS medium containing 0.1-0.5 mg/l 2,4-D and 0.3 M sorbitol at 25°C. For embryo formation, colonies formed from the protoplasts were transferred into the liquid MS medium with decreased concentration of sorbitol and no 2,4-D added. The embryos developed from the colonies were transferred onto the MS medium solidified by 0.2% Gelrite for plant regeneration.

3. Acclimatization of the regenerated plants

The regenerated plants with well developed roots were transferred into Magenta boxes (Magenta Co.) containing sterilized soil supplemented with inorganic salts of MS medium. 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 in growth chamber at 25°C, and finally transferred into a greenhouse. Plants grown for a few weeks in the greenhouse were transplanted into pots or a field.

Results and discussion

1. Enzyme solution for protoplast isolation

Protoplasts of cv. K5 were isolated from the suspension cells using various combinations of enzymes (Table I-1). As shown in Fig. I-1, enzyme solutions 1, 2 and 3 gave higher yield of protoplasts. About 2×10^6 protoplasts/g fresh weight were obtained by using these solutions. Among them, solution 1 produced

Table I-1. Enzyme components of solutions used for isolation of *Daucus carota* cv. K5 protoplasts.

Solution No.	Enzyme components
1	1% Driselase, 0.5% Cellulase YC, 0.01% Pectolyase Y-23
2	1% Cellulase, 0.01% Pectolyase Y-23
3	1% Cellulase YC, 0.2% Macerozyme, 0.01% Pectolyase Y-23
4	1% Driselase, 0.5% Cellulase YC
5	1% Driselase
6	1% Cellulase YC
7	1% Cellulase R10

* Enzyme solution contained 0.5 M mannitol and 0.1% MES (pH.5.7).

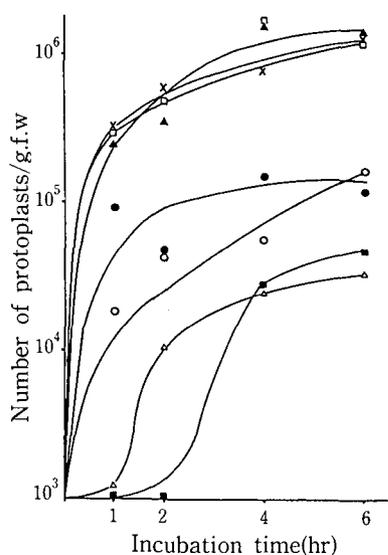


Fig. I-1. Comparison of number of *Daucus carota* cv. K5 protoplasts isolated with different enzyme solutions.

—□—; solution 1 (1% Driselase, 0.5% Cellulase YC and 0.01% Pectolyase Y-23), —▲—; solution 2 (1% Driselase, 0.01% Pectolyase Y-23), —×—; solution 3 (1% Cellulase YC, 0.2% Macerozyme and 0.01% Pectolyase Y-23), —●—; solution 4 (1% Driselase, 0.5% Cellulase YC), —○—; solution 5 (1% Driselase), —△—; solution 6 (1% Cellulase YC), and —■—; solution 7 (1% Cellulase R10). All the solutions contain 0.5 M mannitol and 0.1% MES (pH 5.7).

protoplasts with higher viability repeatedly. However, since in a separate experiment, Cellulase RS gave a higher yield when used instead of Cellulase YC (data not shown), thereafter, an enzyme solution containing 0.5% Cellulase RS instead of 0.5% Cellulase YC of solution 1 was used for carrot protoplast isolation.

2. Screening of suspension cells

Nomura *et al.*¹¹³⁾ have reported that small size of carrot protoplasts were rich in cytoplasm and showed higher embryogenic ability. Since the suspension cells used for protoplast isolation were not a uniform population in size, the cells of cv. NS were first separated according to the size to select such embryogenic protoplasts. The suspension cells were separated into following sizes using stainless meshes; 20–37 μm , 37–63 μm , 63–105 μm , 105–297 μm , 297–500 μm , and larger than

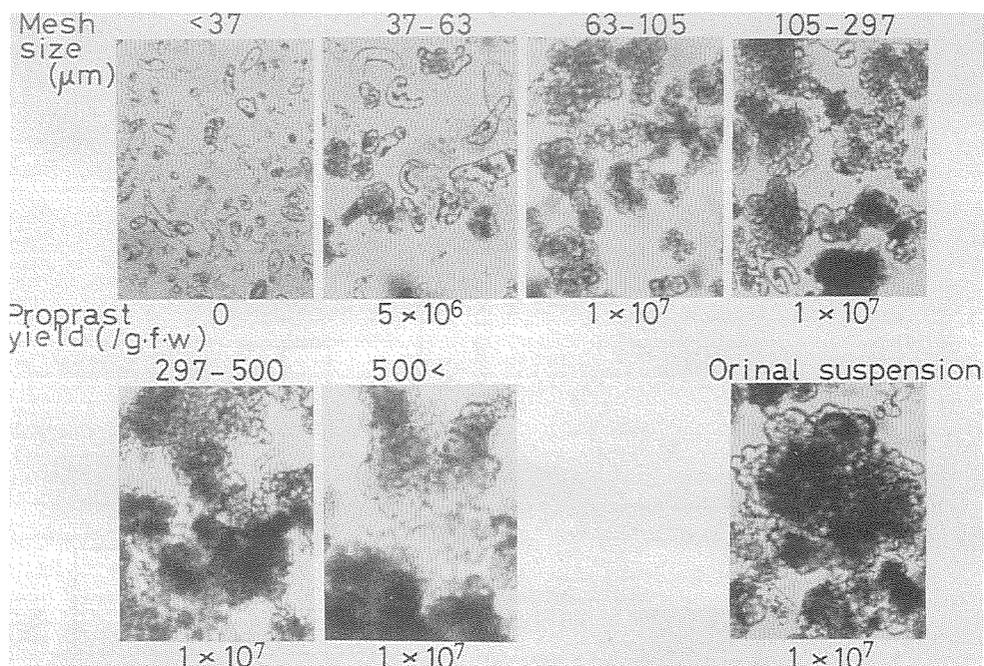


Fig. I-2. Comparison of number of carrot protoplasts isolated from of different sizes of suspension cell aggregates. The carrot variety used here is NS.

500 μm (Fig. I-2). Each size of cells gave about 1×10^7 protoplasts/g fresh weight after enzyme treatment, indicating that cell size did not affect the protoplast yield. On the other hand, the smallest size of cell aggregates (20-37 μm) gave the biggest size of protoplasts (average 24.4 μm), and larger sizes of cell aggregates (37-63, 63-105 and >105 μm) gave smaller sizes of protoplasts (average 16.0, 17.0 and 21.0 μm), respectively (Fig. I-3). These results indicated that the small size of protoplasts which had a high embryogenic capacity could be selected by screening the larger sizes of cell aggregates.

3. Screening of protoplasts

It was investigated whether the small size of protoplasts have high cell division and embryogenic ability as shown by Nomura *et al.*¹¹³⁾. Protoplasts of cv. 35B were separated into each size by sieving them using stainless meshes. There was a tendency that the larger size of protoplasts had vacuolated cytoplasm (data not shown), and that the smaller size of protoplasts had abundant cytoplasm with few vacuoles. They were separately cultured at a density of 1×10^5 protoplasts/ml in MS medium containing 0.1 mg/l 2,4-D for 6 weeks, and the number of colonies formed was counted. As shown in Fig. I-4, smaller protoplasts obtained from cells of 63-105 μm and 105-297 μm gave 3-5 times more colonies and embryos, compared with those obtained from non-separated suspen-

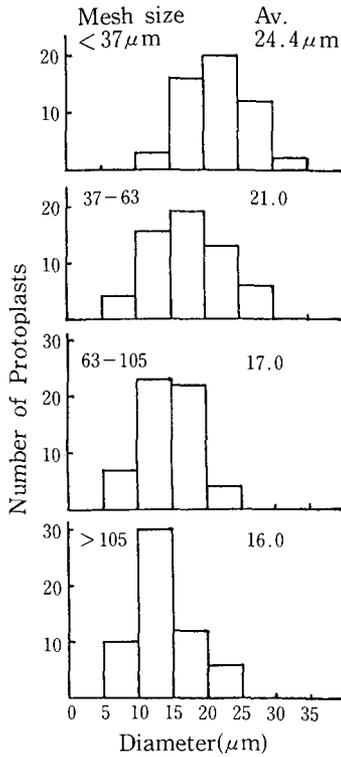


Fig. I-3. Distribution of sizes of *Daucus carota* cv. 35B, protoplasts isolated from of different sizes of suspension cell aggregates.

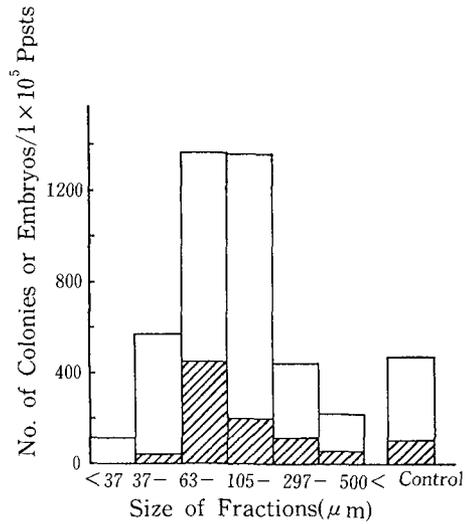


Fig. I-4. Number of colonies (□) or embryos (▨), derived from carrot protoplasts separated into each size and non-separated protoplasts (control).

sion cells (control).

These results indicated that smaller protoplasts isolated from larger cell aggregates had higher cell division and embryogenic ability, compared with larger ones with vacuolated cytoplasm isolated from smaller cell aggregates.

4. Plant regeneration via embryogenesis

a) Effect of colony and embryo density

It is generally observed that most of the cultured protoplasts do not divide or proliferate without contact with the other cells, and also in overcrowding. Colonies or embryos could not also develop into plantlets when a density of the population is not adequate. Therefore, precise control of colony or embryo density during each developmental stage is necessary for plant regeneration.

Protoplasts cultured at a density of 1×10^5 protoplasts/ml formed $1-2 \times 10^3$ colonies/ml about 6 weeks after the culture. After subculturing into MS medium 2,4-D omitted every 2 weeks for 2-3 months, the colonies have developed into torpedo embryos through globular and heart shape stages, but they did not

further develop into plantlets. When the density was, however, gradually decreased, the torpedo embryos grew into abnormal plantlets. Fig. I-5 shows the adequate density at each developmental stage ; 1×10^3 /ml at globular stage, 1×10^2 /ml at heart stage, and 1-10/ml at torpedo stage. The abnormal shoots from the torpedo embryos showed no rooting in the same medium even if the density was reduced. This result suggested that reduction of colony or embryo density promoted the development of embryos, but did not promote the differentiation from embryos.

b) Effect of osmolality of medium

Since it is considered that osmolality of the medium is an important factor to facilitate formation of shoots and roots, adequate osmolality of medium was examined at each developmental stage.

Osmolality of the medium used for protoplast culture was 500 mOsm, and at this osmolality, the normal rooting was not observed. Torpedo embryos were formed with high efficiency when the osmolality was reduced to 200 mOsm from 500 mOsm, and further differentiation was observed when the osmolality was reduced to 50 mOsm from 200 mOsm (Fig. I-5).

These results were summarized as follows ; a) colony or embryo density has to be gradually decreased during each developmental stage, and b) at the same time, osmolality of medium has to be reduced by decreasing the concentration of sorbitol in the medium.

Rooting from the regenerated plantlets was further promoted when transferred onto the medium solidified by Gerlite. By combining these conditions, I obtained somatic plants at a rate of 1 plant/100 protoplasts. This rate (1%) was higher than that described by Nomura *et al.*^{11,2)}

5. Development of regenerated plants

There is no report described in detail about acclimatization of carrot somatic plants. The regenerated carrot plants were very sensitive to environmental conditions, especially, to humidity. Most plants died when transferred directly from culture bials with high humidity into soil. Two to three months were needed to completely acclimatize the regenerated plants to lower humidity conditions in a greenhouse, but still 30% plants were finally died during this process. Therefore, the final rate of normally-developed plants from protoplasts was about 7×10^{-3} .

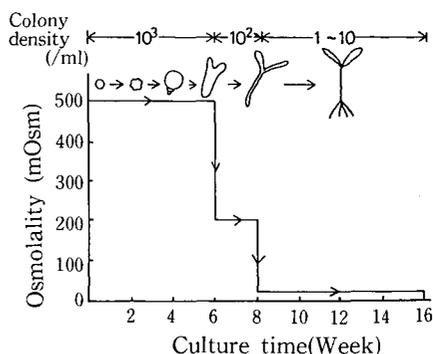


Fig. I-5. Adequate osmolality of medium and colony or embryo density at each developmental stage of carrot embryos. The carrot cultivar used here was NS.

The results presented here showed that somatic carrot plants were obtained at a frequency of 1 plant/140 protoplasts by employing the adequate culture conditions at each developmental stage. This rate was considered to be high enough to apply the protoplasts in somatic cell engineering.

II. PRODUCTION OF CYTOPLASMIC HYBRIDS IN *DAUCUS* BY DONOR-RECIPIENT PROTOPLAST FUSION

Introduction

It has been known that some agronomically important traits such as triazine resistance or cytoplasmic male sterility are encoded by chloroplast or mitochondrial genomes, respectively⁸²⁾. Successful introduction of the maternally inherited traits through organelle transfer by cell fusion has been reported in *Nicotiana*^{3,16,49,53,103,109)}, *Petunia*^{19,30)} and *Brassica*^{27,116,147)}. By analyzing the fate of transferred organelles in those hybrids or cybrids, 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, can be created by cell fusion.

Radioactive irradiation on 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^{100,150)}, cybrids are formed through protoplast fusion between irradiated cells and iodoacetate-treated cells by metabolic complementation in tobacco¹³⁰⁾.

In this Chapter, I produced cytoplasmic hybrids in *Daucus* for the first time by donor (*D. carota* ssp. *gummifer* and *D. capillifolius*)-recipient (*D. carota*) protoplast fusion. The mitochondrial genomes of cytoplasmic hybrids possessed unique restriction fragment patterns distinct from both parental mtDNAs.

Materials and methods

1. Plant materials

Suspension cultures of *D. carota* ssp. *gummifer* and *D. carota* cv. NS and 35B were initiated from calli derived from seedling hypocotyls and maintained every 2 weeks by transferring the cells in fresh MS medium containing 0.5 mg/l 2,4-D with shaking (60rpm) at 25°C. The seeds of *D. carota* ssp. *gummifer* was provided by Agricultural Research Station, USDA, and *D. carota* cv. 35B and NS by Kyowa Seed Co. Suspension cells of *D. capillifolius* were provided by Dr. Y. Kameya, Tohoku University. This *D. capillifolius* suspension cell line had lost its regeneration capacity.

2. Protoplast treatments with X-irradiation and iodoacetamide (IOA)

The isolated protoplasts of *D. capillifolius* were washed twice with washing solution (0.5 M mannitol, 0.1% MES, pH 5.7), and suspended in the same solution with a few drops of enzyme solution (1% Driselase, 0.5% Cellulase RS, 0.01% Pectolyase Y-23, 0.5 M Mannitol, and 0.1% MES, pH 5.7) added to avoid cell wall formation during X-irradiation. The protoplasts adjusted to a density of 1×10^6 /ml in the solution were X-irradiated with a total dosage of 5, 10, 20, 30 and 40 krad (1 krad/min) using X-irradiation machine (Softex Co. type M-80W). The protoplasts irradiated with each dosage were rinsed once with the washing solution and separately cultured in liquid MS medium with 0.1% 2,4-D at a density of 1×10^5 /ml. The number of colonies formed from the irradiated protoplasts was counted 6 weeks after the culture.

On the other hand, protoplasts of *D. carota* cv. NS (1×10^6 /ml of washing solution) were treated with 1.0, 2.5, 5.0 and 10.0 mM iodoacetamide (IOA) for 10 min at room temperature in dark. The each treated protoplasts were rinsed twice with the washing solution and cultured in the same medium with 0.1 mg/l 2,4-D or no hormone. The plating efficiencies were determined by counting the colonies formed 6 weeks after the culture.

3. Donor-recipient protoplast fusion

Protoplasts of *D. capillifolius* and *D. carota* ssp. *gummifer* (1×10^6 /ml) were X-irradiated with a total dosage of 60 krad (1 krad/min). Protoplasts of *D. carota* cv. NS and 35B (1×10^6 /ml) were inactivated by incubation in 15 mM IOA. The IOA-treated protoplasts of NS and 35B were washed twice and mixed with X-irradiated *D. carota* ssp. *gummifer* or *D. capillifolius* protoplasts in a 1 : 1 ratio at a density of 1×10^6 protoplasts/ml. Protoplast fusion was followed to Kao and Michayluk⁷³.

The fused protoplasts were cultured in MS supplemented with 0.3 M sorbitol and 0.1 mg/l 2,4-D at a density of 1.25×10^5 protoplasts/ml at 25°C. After about 1 month culture, the plates containing fusion-derived colonies were gradually substituted with MS plus 0.1 M sorbitol without 2,4-D. The resultant somatic embryos were then transferred to 2-fold diluted MS containing 0.2% Gerlite for further growth. Subsequently large enough plantlets were transplanted into soil. The detail procedure was described in Chapter I.

4. Isolation of mtDNAs

The cell suspensions initiated from hypocotyl-derived callus of each plant were used for mitochondrial DNAs (mtDNAs) analysis. Five to 10 g of cell suspensions were incubated with the enzyme solution for 3-4 hr. The macerated cells were collected by centrifugation, washed once with the washing solution, and resuspended in CP lysis buffer (0.4 M Sorbitol, 0.1 M Tris, 1 mM EDTA, 0.1% albumin, 0.3% 2-mercaptoethanol, pH 8.0)³⁴ using 2 ml ice cold buffer per gram

tissue. The macerated cells were ruptured by a blender at 15000 rpm for half second. 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 $\mu\text{g/g}$ fresh weight DNase I (TAKARA Co.) and 10 mM 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 Salsosyl buffer (1% N-lauryl sarcosine in 50 mM Tris-HCl, 20 mM EDTA, pH 8.0) with 50 $\mu\text{g/ml}$ Proteinase K and incubated at 37°C for 1 hr. 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.

5. Restriction endonuclease analysis of mtDNA

The mtDNAs (1-2 μg) of somatic hybrids and their parental lines were digested to completion with restriction enzymes, *SalI* and *XbaI*, according to the supplier's instructions for at least 4 hr at 37°C. The restriction fragments were separated in 0.7% agarose gels and stained with ethidium bromide.

6. Chromosome counting

Suspension cells of *D. capillifolius* (no regeneration potential) and *D. carota* cv. NS and 35B, and root tips of their hybrid plants were incubated with 2 mM 8-hydroxyquinoline, then fixed in ethanol : acetic acid (3 : 1), and treated with an enzyme solution containing 4% Cellulase RS, 1% Pectolyase Y-23, 7.5 mM KCl and 7.5 mM EDTA, pH 4.0⁽¹¹⁾. They were gently spread on slide glass and stained with aceto-orcein for microscope observation.

Results

1. Effect of X-irradiation on cell division and colony formation

D. capillifolius protoplasts were X-irradiated with various dosages and cultured for 6 weeks. As shown in Fig. II-1, the number of colonies per plate was

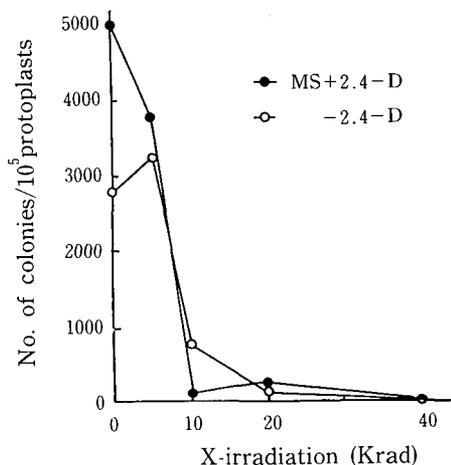


Fig. II-1. Effect of X-irradiation dosage on colony formation of *D. capillifolius* protoplasts.

clearly reduced when the protoplasts were exposed to more than 10 krad. No colony was observed for 40 krad exposure, indicating that the protoplasts irradiated at 40 krad were completely inactivated.

Microscopically, the irradiated protoplasts were observed to repair cell walls 1-2 days after the culture, but no cell division occurred (Fig. II-2). Furthermore, FDA-staining revealed that the cells irradiated with 60 krad X-ray were alive until about 2 weeks after the culture (data not shown). These results indicate that *Daucus* protoplasts were not dead immediately after irradiation and remained for several days.

Galun and Raveh⁵⁰ reported that decrease in ploidy level of protoplasts took place after the irradiation. The data presented here suggested that exposure to X-ray of more than 40 krad was indispensable to completely eliminate the chromosomes of cytoplasmic donor cells.

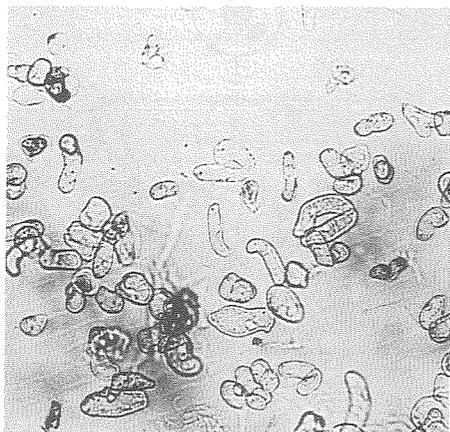


Fig. II-2. Cells of *D. capillifolius* on 6 days after X-irradiation to protoplasts at 60 krad.

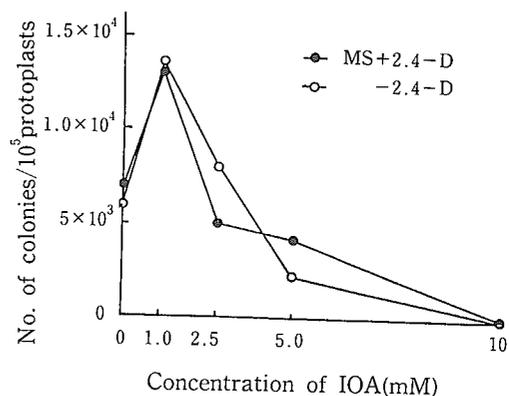


Fig. II-3. Effect of iodoacetamide (IOA) on colony formation of *D. carota* cv. 35B protoplasts.

2. Effects of IOA treatments on cell division and colony formation

Metabolic inhibitor, IOA, was used to inactivate the protoplasts of *D. carota* cv. K5. Fig. II-3 shows colony formation after 6 days in culture. No colony formation was observed for 10 mM IOA treatment. From this result, it was identified that at least 10 mM IOA was required to prevent the colony formation.

3. Selection of putative cybrids

In order to obtain *Daucus* cybrids, donor-recipient protoplast fusion combining X-irradiation and IOA treatment was schemed as shown in Fig. II-4.

As control experiments, the X-irradiated protoplasts of wild species (*D. carota* ssp. *gummifer* and *D. capillifolius*) or IOA-treated protoplasts of the cultivated species (*D. carota* cv. NS and 35B) were cultured separately with or without polyethylene glycol (PEG) treatment. Furthermore, the mixtures of X-

irradiated and IOA-treated protoplasts were cultured without PEG treatment (Fig. II-4). Neither formed colonies except when the X-irradiated protoplasts were cultured after PEG-treatment. The colonies formed through fusion between X-irradiated protoplasts lacked the regeneration ability (data not shown). In contrast, following PEG-treatment of mixed parental protoplasts, many colonies were obtained from them.

All the colonies obtained from fusion combination of *D. carota* ssp. *gummifer* and *D. carota* cv. NS formed abnormal plants without roots by transferring them to MS without 2,4-D. However, colonies obtained from two independent protoplast fusion experiments between *D. capillifolius* and *D. carota* cv. 35B grew into normal plants through somatic embryogenesis. The regenerated plants had the same leaf morphology as the parental *D. carota* cv. 35B, while the characteristics of leaves from those regenerated plants were clearly different from those of the other parental *D. capillifolius* (Fig. II-5).

Chromosome numbers were counted in cell lines of *D. capillifolius*, *D. carota* cv. 35B and their several regenerated plants (Fig. II-6, Table II-1). The chromosome number of *D. carota* cv. 35B cells used in the experiments was 17 ($2x-1$). One chromosome might be deleted from the genome in the cells during the

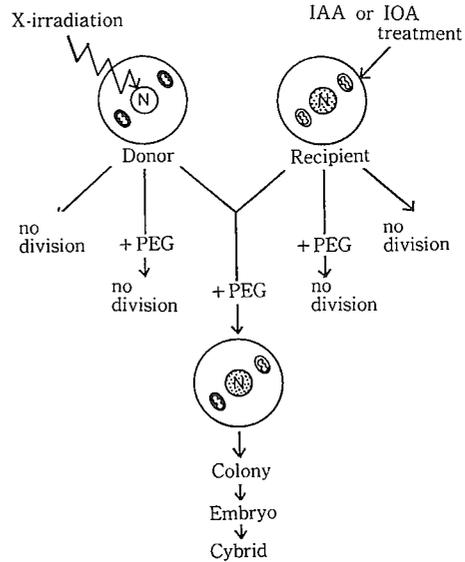


Fig. II-4. Schematic representation of selective formation of cybrid plants based on X-irradiation and metabolic inhibitor-treatment.

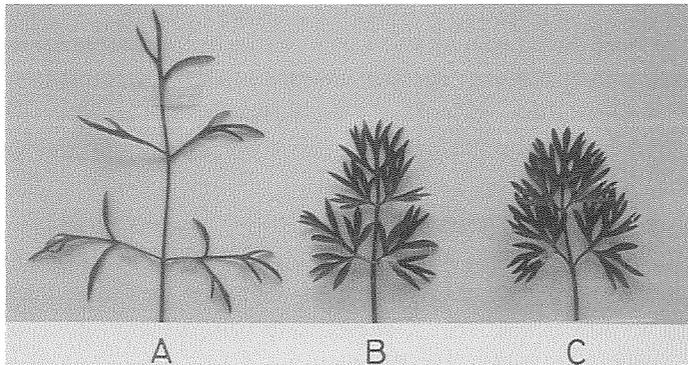


Fig. II-5. Leaf characteristics of *D. capillifolius*, *D. carote* cv. 35B and their cybrid plants. A *D. capillifolius*; B cybrid B-2-20; C *D. carota* cv. 35B.

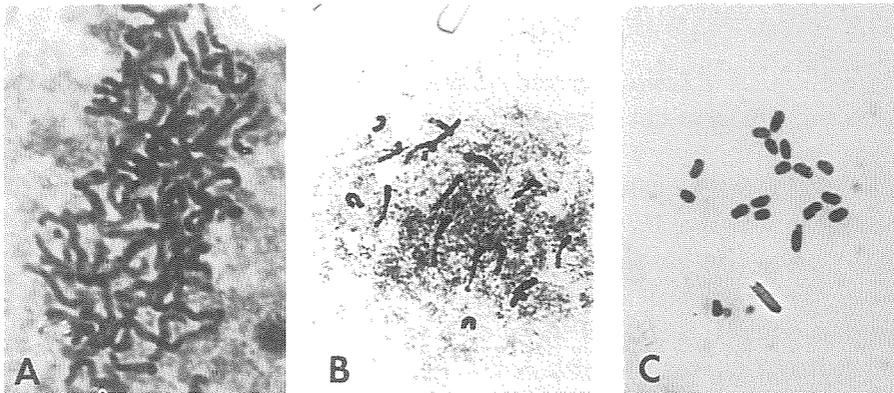


Fig. II-6. Chromosomes of *Daucus capillifolius* and *D. carota* cell cultures, and their cybrid plants. **A** *D. capillifolius* cell line ; **B** cybrid plant A-27 ; **C** *D. carota* var. 35B cell line.

Table II-1. Characterization of cybrid plants between *D. capillifolius* and *D. carota* var. 35B 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	<i>D. carota</i>	17	rearranged: I-a
A-27	<i>D. carota</i>	17	rearranged: I-a
A-31	<i>D. carota</i>	17	rearranged: I-a
A-39	<i>D. carota</i>	17	rearranged: I-a
A-59	<i>D. carota</i>	34	rearranged: I-b
B-1-2	<i>D. carota</i>	—	rearranged: II-a
B-1-8	<i>D. carota</i>	—	rearranged: II-b
B-1-9	<i>D. carota</i>	—	rearranged: II-c
B-2-15	<i>D. carota</i>	—	rearranged: II-a
B-2-20	<i>D. carota</i>	—	rearranged: II-c
B-3-1	<i>D. carota</i>	—	rearranged: II-c

culture¹⁴). 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 II-1, the chromosome numbers of five regenerated plants were counted. Four plants (A-22, A-27, A-31 and A-39) possessed 17 ($2x-1$), like the *D. carota* cv. 35B cell line, and the other one (A-59) had 34 ($4x-2$), twice as many.

These morphological and chromosomal observations strongly suggested that the regenerated plants, which were produced by protoplast fusion between *D. capillifolius* and *D. carota* cv. 35B, possessed chromosomes of only one of the fusion parents, *D. carota* cv. 35B, and the chromosomes derived from *D. capillifolius* cells were eliminated from the fused cells during protoplast culture. However, it could not be denied that some segments of chromosomes of the donor,

D. capillifolius might have translocated into nuclear genome of the fusion products.

Chromosomes of fusion-derived colonies between *D. carota* ssp. *gummifer* and *D. carota* cv. NS were not observed.

4. Restriction endonuclease analysis of mtDNAs from asymmetric fusion products between *D. carota* ssp. *gummifer* and *D. carota* cv. NS

Different restriction patterns of mtDNAs were detected between the parental cell lines, *D. carota* ssp. *gummifer* and *D. carota* cv. NS by *Xba*I restriction endonuclease analysis (Fig. II-7, lanes 1 and 5). Three fusion products which formed primary shoots, A-4-20, A-2-25, and A-7-6, were selected for mtDNA analysis using the same enzyme. The *Xba*I-digested patterns of mtDNAs from the fusion products were distinctly different from those of the parents (lanes 2-4 in Fig. II-7). In A-4-20, 6 of the 19 fragments larger than 1.0 kb in size were unique and not present in either parent. A-2-25 had 3 unique fragments not present in both parents and 18 fragments common to either parent. In both

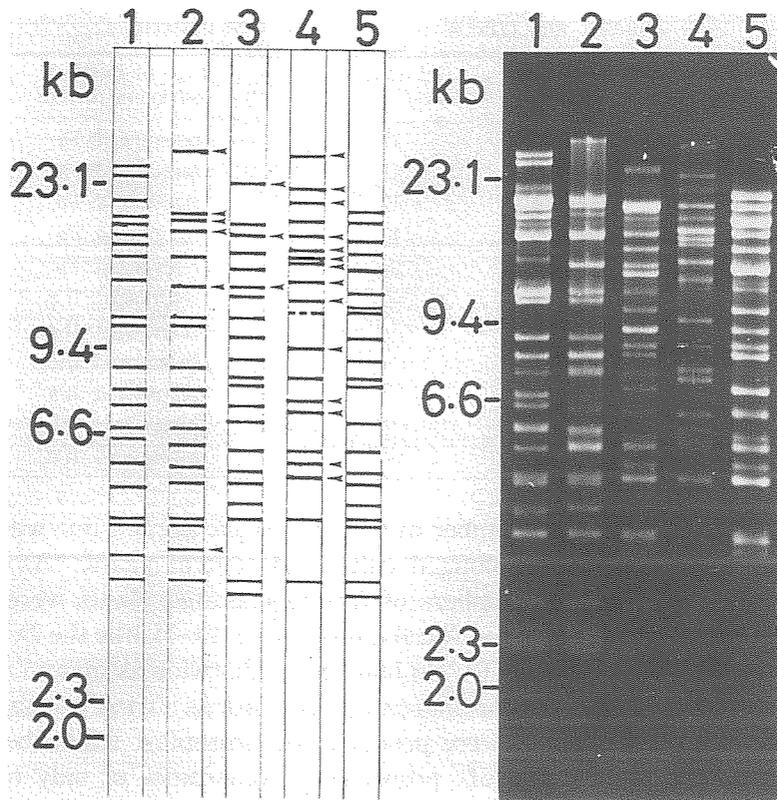


Fig. II-7. *Xba*I restriction endonuclease analysis of mtDNAs from *D. carota* ssp. *gummifer* (lane 1), *D. carota* var. NS (5) and their cybrid plants, A-4-20, A-2-25, A-7-6 (2, 3 and 4)

fusion products, A-4-20 and A-2-25, most of the fragments were common to the parents, and some fragments specific to either parent were missing in them. However, most of the restriction fragments of A-7-6 were novel ones absent in the parents. This indicates that an intensive rearrangement or recombination had occurred in this fusion product.

The comparison of mtDNA restriction patterns between fusion products and the parents showed that an extensive rearrangement of mtDNA could be caused by protoplast fusion, and that the fusion products obtained from this combination were cytoplasmic hybrids. However, since chromosomes of these 3 cytoplasmic hybrids have not been observed, it was not identified whether they were real cybrids possessing recipient nuclei.

5. Restriction endonuclease analysis of mtDNAs from putative cybrids between *D. capillifolius* and *D. carota* cv. 35B

The restriction fragment patterns of mtDNAs from *D. capillifolius* cell line, *D. carota* cv. 35B and their fusion-derived plants were compared (Fig. II-8). Different restriction patterns were detected between the mtDNAs from both parents by digesting with *Sal*I and *Xba*I (lanes 1 and 7 in Fig. II-8). The

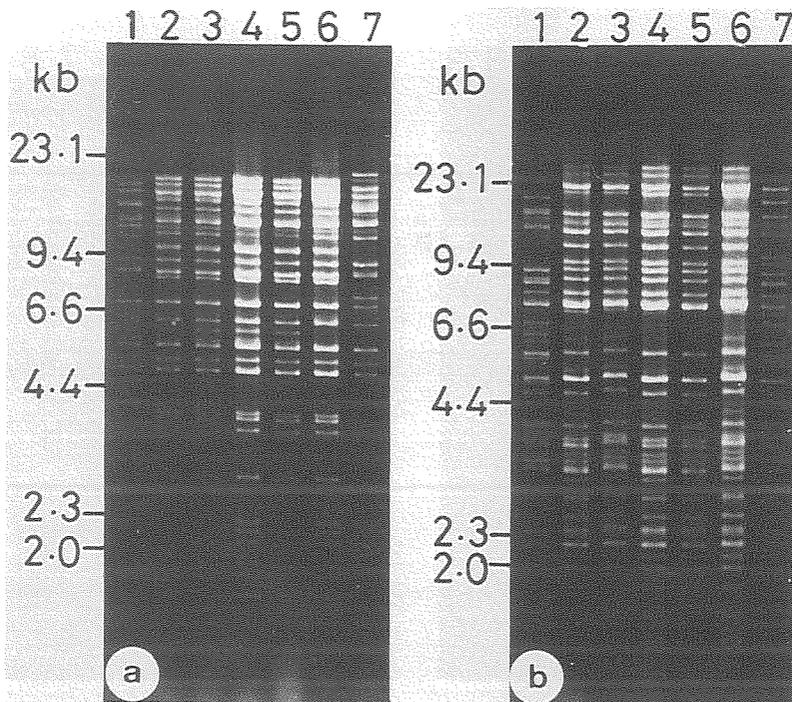


Fig. II-8. *Sal*I (a) and *Xba*I (b) restriction endonuclease analysis of mtDNAs from *D. capillifolius*, *D. carota* cv. 35B and their cybrid plants in experiment A. *D. capillifolius* (lane 1), cybrid plants: A-22 (2), A-27 (3), A-31 (4), A-39 (5) and A-59 (6), and *D. carota* cv. 35B (7).

restriction patterns of mtDNAs from the fusion-derived plants, A-22 (lane 2), A-27 (3), A-31 (4) and A-39 (5) were identical to each other, and that of mtDNA from A-59 plant (6) was a little different from those of the former mtDNAs. MtDNAs from those five putative cybrids possessed unique restriction patterns compared to those of their parental cell lines. For example, as shown in Fig. II-8a, seven unique fragments of *SalI* digests not present in both parental mtDNAs, 5 fragments of only *D. carota* cv. 35B 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 cytoplasmic hybrids between *D. capillifolius* and *D. carota* cv. 35B, and that mtDNA sequences characteristic for *D. capillifolius* were introduced into *D. carota* cv. 35B plants by protoplast fusion after X-irradiation of *D. capillifolius* protoplasts.

A total of 25 regenerated plants were analyzed 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 II-1), though the two patterns were nearly identical. The possibility that all the plants in this experiment were originated from one clone is 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

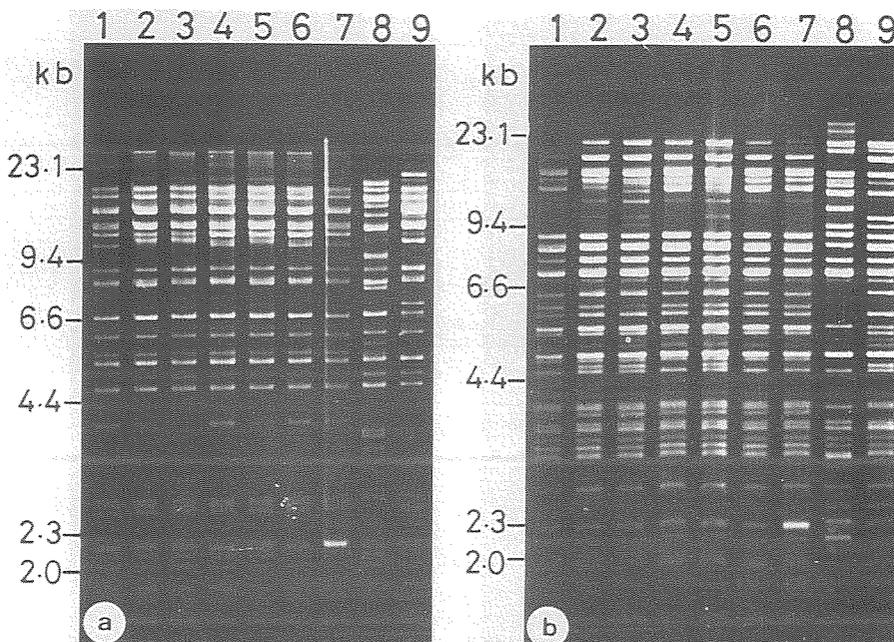


Fig. II-9. *SalI* (a) and *XbaI* (b) restriction endonuclease analysis of mtDNAs from *Daucus capillifolius*, *D. carota* cv. 35B, and their cybrid plants in experiment B. *D. capillifolius* (lane 1), cybrid plants: B-1-2 (2), B-1-8 (3), B-1-9 (4), B-2-15 (5) and A-39 (from experiment A) (8), and *D. carota* cv. 35B (9).

colonies in the other plates.

All the regenerated plants possessed an identical leaf morphology to that of *D. carota* cv. 35B plants. The mtDNAs of putative cybrids derived from each plate were analyzed (Fig. II-9, Table II-1). Although their restriction patterns resembled each other, they were classified into three types which are different from those of fusion-derived plants from the experiment A. The restriction fragment patterns of fusion-derived plants from experiment B (lanes 2-7 in Fig. II-9) were more similar to that of *D. capillifolius* than that of the plants from experiment A. MtDNAs of three putative cybrids, B-1-2 (lane 2), B-1-8 (3) and B-1-9 (4), derived from the same plate, had slightly different *SalI* and *XbaI* restriction patterns from each other. The *SalI* and *XbaI* restriction patterns of B-2-20 (6), derived from an another plate, completely coincided with those of B-1-9 (4). These results show that an identical restriction patterns of mtDNAs observed between the cytoplasmic hybrids can be acquired not only by fusion products derived from a single clone, but also by fusion products derived from distinct clones.

Discussion

In this Chapter, I reported the successful formation of cytoplasmic hybrids in *Daucus* for the first time by protoplast fusion between X-irradiated *Daucus* wild species, *D. capillifolius* (donor), and iodoacetamide-treated *D. carota* cultivars (recipient), and characterized the mtDNA of parents and their cytoplasmic hybrids.

In case of the regenerated plants after protoplast fusion between *D. capillifolius* and *D. carota* cv. 35B, about 10% of them showed the same leaf characteristics and the same mtDNA restriction patterns as *D. carota* cv. 35B, suggesting that the plants might be escaped plants of *D. carota* cv. 35B from the selection systems. However, about 90% of the regenerated plants showed the characteristics of putative cybrids, 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 patterns of mtDNAs from the fusion-derived plants were compared to those of the both parents. MtDNA recombinations were detected in *Nicotiana* cybrids derived from the asymmetric fusions between gamma- or X-irradiated and iodoacetate-treated protoplasts^{3,109}. I also observed some novel fragments in the regenerated plants in addition to the fragments corresponding to those of either one parental mtDNA or both parental mtDNAs (Fig. II-7, 8 and 9).

Matthew and DeBonte⁹³ have pointed out the stability of mitochondrial and plastid DNA restriction patterns from *Daucus* cell cultures. Also in this experiment, the mtDNA restriction patterns of the *D. carota* suspension cell lines did not change over a subculture of one year (data not shown). Moreover, the plants

regenerated from protoplasts treated with 5 mM iodoacetamide before culture bore the same mtDNA restriction pattern as plants which originated from seeds (data not shown). MtDNAs of *D. capillifolius* cell lines derived from protoplasts that escaped lethal X-irradiation and possessed no regeneration potential could not be distinguished from that of a cell line initiated from seeds (data not shown). Matthews and DeBonte⁹³⁾ also pointed out the stability of carrot organelle DNAs in cell suspension cultures maintained for 10 years and in regenerated plants derived from a cell culture. From these results, it was concluded that the novel mtDNA band patterns of the fusion products in this study were the results of cytoplasmic hybridization and interspecific mtDNA recombination.

MtDNA recombinations have been observed in a somatic hybrid of *D. capillifolius* and *D. carota*⁹⁴⁾ and in *D. carota* somatic hybrids⁷⁹⁾. 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 cytoplasmic hybrids of *D. capillifolius* and *D. carota* cv. 35B obtained here, the mtDNA restriction fragment patterns in experiment A and B were relatively dissimilar, but similar within each experiment. Only two types of mtDNA restriction patterns were found in a total of 25 cytoplasmic hybrids 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 mitochondrial genome. On the other hand, the mtDNA restriction patterns of fusion-derived plants between *D. carota* ssp. *gummifer* and *D. carota* cv. NS were distinctly different from the parents and from each other, and showed abnormal plant morphology. This result does not exclude the existence of the 'hot spot', but suggests that the degree of mtDNA rearrangement may affect the plant development or morphology. The correlation remains to be examined.

In tobacco^{3,16,49,109)}, petunia^{19,30)} and *Brassica*²⁷⁾, extensive diversity of mtDNA rearrangements were observed. Direct repeat elements are present within some plant mtDNAs and rearrangements in mtDNAs are thought to occur through the elements^{86,115)}. There should be some relationships between the degree of mtDNA recombinations in cybrids and the number or structure of the repeat elements within those mtDNAs.

III. CMS TRANSFER IN *DAUCUS CAROTA* L. BY DONOR-RECIPIENT METHOD

III-1. TRANSFER OF THE BROWN ANTHHER-TYPE CMS

Introduction

Since cytoplasmic male sterility (CMS) was first documented in *Daucus carota* L. by Welch and Grimball¹⁴⁵⁾, it has been considered as a useful trait in com-

mercial F_1 seed production. Two types of CMS flowers have been observed : the brown anther type^{10,20,145}) and petaloid type^{95,139}). As 8-10 years of backcrossing are required to introduce CMS into desired varieties or lines, there is an urgent need to develop a more efficient procedure 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*^{5,15,80,150}), *Petunia*⁶⁹), *Brassica*^{11,12,29,101,106,116,147}), and rice^{1,148}).

Donor-recipient method is an efficient procedure for the selective formation of cybrids, because only the metabolically complemented cells are capable of developing into plantlets after protoplast fusion^{106,130,142}). Furthermore, in such a donor-recipient method, there is no need to make use of a selectable cytoplasmic marker of the donor during protoplast culture after fusion treatment.

In this section, I describe male sterility and flower characteristics in carrot cybrids derived from donor-recipient method to transfer cytoplasmic male sterility of brown anther type. I also discuss the correlation between male sterility and mtDNA restriction endonuclease patterns in the cybrid plants.

Materials and methods

1. Plant materials

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

2. Pretreatments and fusion of protoplasts

Protoplasts of each parent were isolated from suspension cells derived from hypocotyls. The details were described in Chapter I.

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 mM iodoacetamide at room temperature for 10 min. The dosages of each pretreatment had been determined to be lethal to protoplasts in the previous experiment (Chapter II).

After each treatment, the protoplasts mixed in a 1 : 1 ratio were fused according to the method described in Chapter II. 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 fusion treatment using PEG. For details of the protoplast culture, see Chapter I.

The colonies derived from heteroplasmic protoplast fusions were transferred onto hormone free MS medium for plant regeneration. Those regenerated plants with well developed roots were transferred into soil and acclimatized to outside environment (Chapter I).

3. MtDNA restriction endonuclease analysis

To screen the real cybrids, twenty-six plants were examined for mtDNA restriction pattern analysis.

The procedures of mtDNA isolation and endonuclease digestion were described previously (Chapter II). *Xba*I-digested mtDNAs of parental plants and the 26 regenerated plants were observed under UV light after separation by electrophoresis and staining with ethidium bromide.

4. Southern hybridization analysis

MtDNAs from the 2 fusion-derived plants (A-5-18 and A-7-33) and their parental lines, 28A1 and K5, were digested with *Xba*I endonuclease and separated by electrophoresis. The digested mtDNAs in a gel were transferred to a nylon membrane (Hibond-N, Amersham Co.) after the denature in alkali solution (0.5 N NaOH, 1.5 M NaCl). Seven clones containing mitochondrial genes shown in Table III-1-1 were used as probes. Hybridization were performed by using the 'Nonradioactive DNA Labeling and Detection Kit' of Boehringer Mannheim Co.. The detailed processes of labeling probes, hybridization, and immunological detection followed the procedures given in the kit protocol.

Table III-1-1. Mitochondrial gene probes used in this study

Gene	Protein	References
<i>cox</i>	Cytochrome c oxidase subunit I	ISSAC <i>et al.</i> ⁶⁷⁾
<i>coxI</i>	Cytochrome c oxidase subunit II	FOX and LEAVER ⁴⁷⁾
<i>coxII</i>	Cytochrome c oxidase subunit III	HIESEL <i>et al.</i> ⁶⁴⁾
<i>cob</i>	Apocytochrome b	DAWSON <i>et al.</i> ³²⁾
<i>atpA+atp9</i>	F1-F0 ATPase subunits A and 9	MORIKAMI and NAKAMURA ¹⁰⁷⁾
<i>rrn18</i>	18S rRNA	MORIKAMI and NAKAMURA unpublished
<i>rrn26</i>	26S rRNA	MORIKAMI and NAKAMURA unpublished

5. Chromosome counting

Suspension cells prepared from the regenerated plants were pretreated and stained according to the procedure described in Chapter I for microscopic observation.

6. Investigation of flower morphology and male sterility

Out of the 26 regenerated plants selected for mtDNA 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, and stamen filaments were observed microscopically. Pollen fertility was evaluated by determining the percentage of functional pollen grains. About 1000 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 from their parents for counting the number of functional pollen grains per anther. The pollen grains were suspended in 15 μ l of 10% sucrose solution and counted using a hemocytometer.

Results

1. Cybrid formation by donor-recipient method

No colony formation was observed in the 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 2.0% plating efficiency. These colonies regenerated plantlets via embryogenesis after being transferred to hormone-free MS medium. Although some of the regenerated plantlets from primary embryos showed morphologically normal development, most of them were abnormal in growth and produced some secondary embryos from the abnormal shoots. The secondary embryos tended to develop into morphologically normal plants. The total efficiency of plants that did develop normally via primary and secondary embryos were about 10%.

2. MtDNA analysis of the putative cybrids

Figure III-1-1 shows the *Xba*I restriction fragment patterns of mtDNAs from 28A1 (lane 1), K5 (lane 11) and the regenerated plants derived from protoplast fusion (lanes 2-10). The fragment patterns of 28A1 and K5 were 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 were similar to K5 in the pattern. Lanes 2-7 in Fig. III-1-1 show the fragment patterns from 6 of these 11 plants, and lanes 8-10 show those from 3 out of the latter 15 plants. All the regenerated plants examined had at least one novel fragment that was absent from both parents and lacked a few fragments characteristic of the

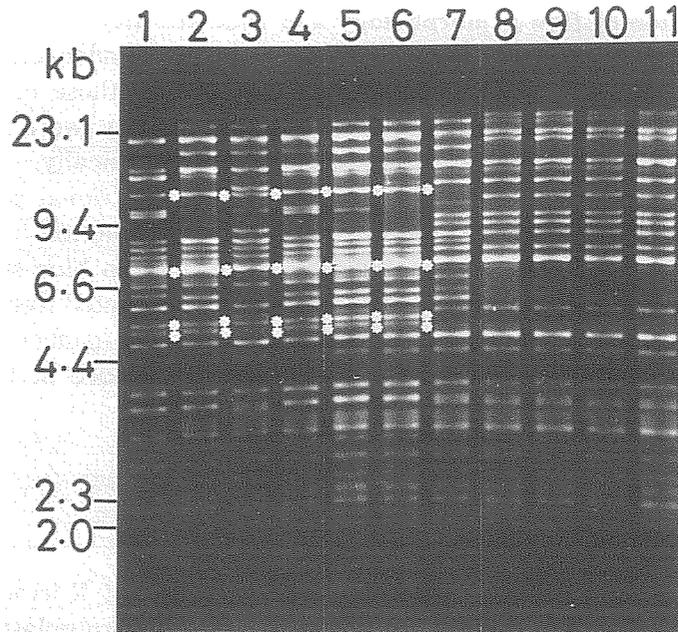


Fig. III-1-1. *Xba*I restriction endonuclease analysis of mtDNAs from 28Al (CMS), K5 (fertile), and their cybrid plants.

Lane 1 : 28Al (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 (partial sterile) ; lane 8 : D-2-14 (fertile) ; lane 9 : E-1-11 (fertile) ; lane 10 : E-2-14 (fertile) ; lane 11 : K5 (fertile). White dots indicate those fragments common between the sterile cybrid plants and 28Al that were not present in the partially sterile cybrid plant and K5.

parents. Most of the other fragments from the regenerated plants could be observed in either 28Al or K5. Although these novel fragment patterns were considered to have resulted from recombination between homologous mtDNA regions of the parental plants, the possibilities that reorganization, stoichiometric change or sorting out of mtDNAs have occurred during the culture process could not be denied.

MtDNAs from two putative cybrids whose pollen fertility is different from each other, A-5-18 (partial male sterile) and A-7-33 (male sterile), were hybridized with 7 different heterologous mitochondrial genes. Fig. III-1-2 shows the hybridization patterns of *Xba*I-digested mtDNAs from 28A (lane 1), A-5-18 (2), A-7-33 (3), and K5 (4). The patterns of *coxII*, *cob*, *rrn26*, and *atpA + atp9* were the same ; *coxII* hybridized at 8.2 kb *Xba*I fragment (Fig. III-1-2a), *cob* at 2.4 kb (Fig. III-1-2b), *rrn26* at 4.1 kb and 2.7 kb (Fig. III-1-2c), and *atpA + atp9* at 6.2 kb and 2.9 kb (Fig. III-1-2d). Hybridization patterns of *coxI*, *coxIII* and *rrn18* were different between the parents.

coxI (Fig. III-1-2e) ; MtDNA from 28Al hybridized at 6.2 kb *Xba*I-fragment (lane 4), while that from K5 hybridized at 8.2 kb fragment (lane 1). The fusion-

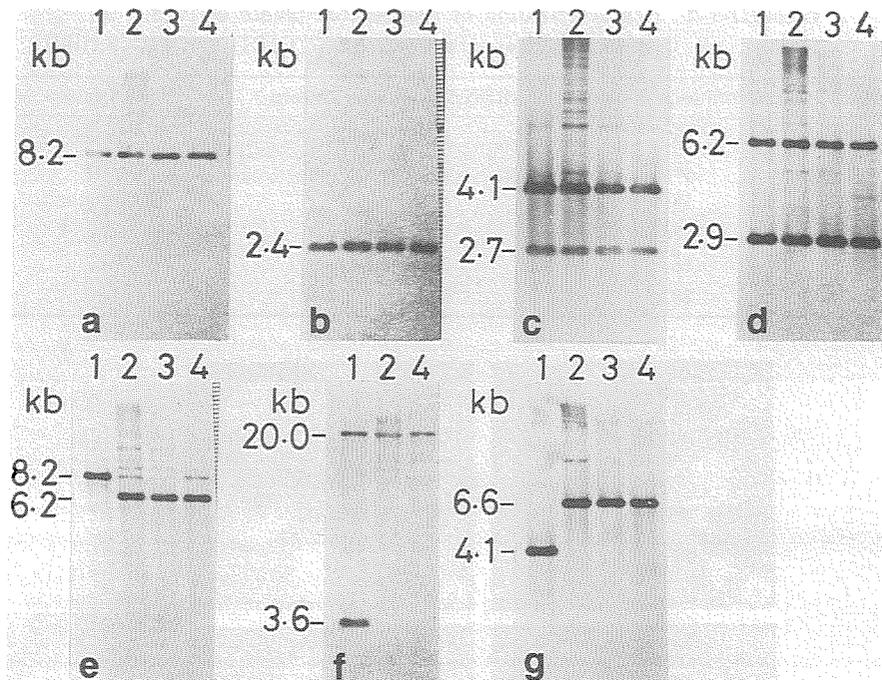


Fig. III-1-2. Southern hybridization of 7 mitochondrial genes to 2 cybrid plants and their parental lines, K5 (fertile) and 28A1 (CMS). The mitochondrial DNAs were digested with *Xba*I endonuclease. Lane 1 : K5, lane 2 : A-5-18 (partial male sterile), lane 3 : A-7-33 (male sterile), and lane 4 : 28A. The gene probes used were *coxII* of *Zea mays* (a), *cob* of *Zea mays* (b), *rrn26* of *Oenothera* (c), *atpA+atp9* of *pea* (d), *coxI* of *Zea mays* (e), *coxIII* of *Oenothera* (f), and *rrn18* of *pea* (g).

derived plants (lanes 2 and 3) showed the pattern identical to that of 28A.

coxIII (Fig. III-1-2f) ; K5 (lane 1) contained 20.0 kb and 3.6 kb hybridization fragments, while 28A1 (lane 4) and the fusion-derived plants A-5-18 lacked the 3.6 kb fragment.

rrn18 (Fig. III-1-2g) ; A-5-18 and A-7-33 showed the hybridization patterns at 6.6 kb identical to that of 28A (lanes 2, 3 and 4). Homologous region to *rrn18* of K5 was located in 4.1 kb fragment which was different from those of 28A1 and their fusion products (lane 1).

These results revealed that no rearrangement within the mitochondrial genes examined here occurred in these two plants.

3. Chromosome number of the putative cybrids

The chromosome number of each regenerated plant was also studied. All the plants investigated were found to possess diploid chromosomes ($2n=18$) (Table III-1-2), indicating that the chromosomes of donor cells might be eliminated completely from the fused cells during their developmental process or that

Table III-1-2. 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	Rearranged	Sterile
A-7-33	18	Rearranged	Sterile
A-5-18	18	Rearranged	Partially sterile
D-1-20	18	Rearranged	—
D-2-25	18	Rearranged	—
E-2-14	18	Rearranged	—

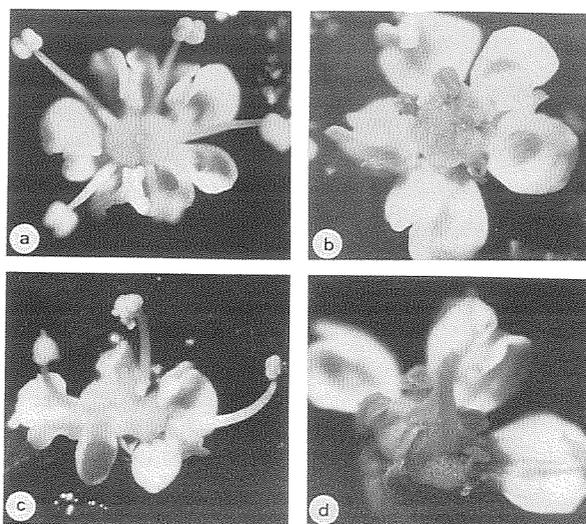


Fig. III-1-3. Flower morphology of parental plants and cybrid plants. **a** K5 (fertile) with yellow anthers and normal filaments ; **b** 28A1 (CMS) with brown anthers and stunt filaments ; **c** A-5-18 with yellow anthers and normal filaments ; **d** E-1-16 with brown anthers and stunt filaments.

some segments of chromosomes of donor cells might be translocated into the recipient chromosomes. The regenerated plants investigated were therefore classified as cybrids or segmental somatic hybrids. Considering plant morphology, the diploid plants were identified as cybrids having elements of cytoplasmic genomes from both 28A1 and K5. The 11 fusion-derived cybrids whose mtDNA fragment patterns clearly differed from that of the recipient were subsequently investigated for flower morphology and male sterility.

4. Flower morphology and male sterility of the cybrids

The flower morphologies of the parental plants and their cybrid plants are shown in Fig. III-1-3. The fertile parent, K5, possessed yellow anthers with normal filaments (Fig. III-1-3a), and the CMS parent, 28A1, had brown anthers

Table III-1-3. Pollen fertility and flower morphology of 28A1(CMS) and, K5(fertile) and their cybrid plants

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

* n and s indicate normal and stunt filaments, respectively.

with stunt filaments (Fig. III-1-3b). The flowers of their cybrid plants were classified morphologically into 2 types : yellow anthers with normal filaments (Fig. III-1-3c) and brown anthers with stunt filaments (Fig. III-1-3d). The petals of the cybrid plants showed a normal shape and a color identical to those of both parents.

Table III-1-3 shows male sterility and the flower morphology of the parents and the cybrids. The CMS parent, 28A1, shed no pollen grains, while the pollen fertile parent, K5, shed about 7.8×10^2 functional pollen grains per anther (Fig. III-1-4a). The brown 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

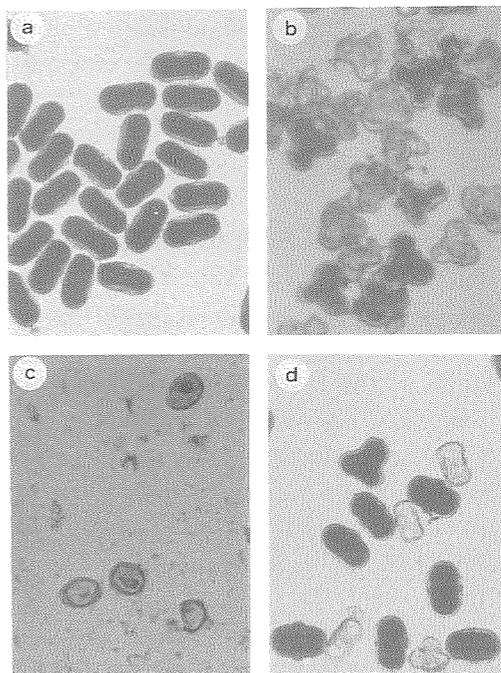


Fig. III-1-4. Pollen grains of fertile parent K5, and the cybrid plants stained with 1% cotton blue solution. **a** K5 (fertile) ; **b** A-8-3 (sterile) ; **c** A-2-25 (sterile) ; **d** A-5-18 (partially sterile).

-18, A-2-25, A-2-30 and A-8-3 had non-functional pollen grains that were abnormally shaped (Fig. III-1-4b) or unstained with the cotton blue solution (Fig. III-1-4c). This indicated that they were male sterile. They were able to set seeds by backcrossing with the male fertile parent, K5. On the other hand, the cybrid with normal anthers (A-5-18) shed some functional pollen grains (Fig. III-1-4d) and set a few seeds. The clone A-5-18 was therefore classified as partially male sterile.

While those cybrids with mtDNA fragment patterns similar to that of 28A1 were fully sterile, partial sterility was observed in a cybrid (A-5-18) whose mtDNA fragment pattern was clearly different from those of both parents. However, the hybridization patterns of A-5-18 to 7 heterologous mitochondrial gene probes were identical to those of CMS parent, 28A1, and CMS cybrid, A-7-33 (Fig. III-1-2). In addition, from the restriction fragment patterns of the cybrid plants, four common fragments, which were absent in the partially male sterile cybrids and K5, were also identified in the male sterile cybrids and 28A1 (Fig. III-1-1).

Discussion

The data presented here show that 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 rearranged mtDNAs, and of the six plants analyzed, all were diploids. Treatment with X-irradiation proved effective in eliminating donor chromosomes in these experiments, showing an observation consistent with the earlier work (Chapter II). Some workers have demonstrated that complete elimination of donor chromosomes has not always been attained by X- or gamma-irradiation in experiments on cybrid formation^{5,12,13,98,101,102,131,150}. Factors affecting chromosome elimination from fusion products treated by X-irradiation have not yet been specified.

As indicated in Table III-1-2, all but one of the 11 cybrids 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. III-1-1). The results of the restriction fragment pattern analysis are in agreement with the findings of Galun *et al.*⁴⁹, and Aviv and Galun⁶ who reported that mtDNA restriction patterns of male sterile and male fertile cybrids are similar to those of the CMS donor and the male fertile recipient, respectively. The existence of this correlation means that male sterile cybrids can be selected from among a large number of fusion-derived plants at an early stage of plant regeneration using the mtDNA analysis.

On the other hand, a cybrid (A-5-18) showed partial male sterility and carried a mtDNA organization different from those of the parents. These results sup-

port the idea that the genes responsible for the CMS trait of brown anther type in donor plant might be transferred into male sterile cybrids, and that the partially sterile cybrid might be caused by inadequate introduction of donor cytoplasm. However, CMS-associated fragments containing mitochondrial genes could not be identified by the Southern hybridization analysis using 7 heterologous mitochondrial gene probes.

Four *Xba*I 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 *Bgl*II fragments of mtDNA have been observed within CMS cybrids¹⁸⁾, and a CMS-related gene of *Petunia* was identified from the fragment¹⁴⁹⁾. Further investigations are required to determine whether these four mtDNA fragments found in the male sterile cybrids are directly related to the CMS of brown anther type.

The introduction of carrot CMS through conventional backcrossings usually takes eight to ten years. However, using the protoplast fusion, it can be achieved within about 16 months (from protoplast fusion to flowering). Thus, it is concluded that this technique is an effective method for transferring the CMS trait and can be applied in the practical breeding of carrots.

III-2. TRANSFER OF THE PETALOID-TYPE CMS

Introduction

In carrot, both types of CMS, the brown anther type and petaloid type, are being used for F₁ seed production. The CMS flower of the petaloid type is characterized by 5 stamens, which are transformed into petal-like structures⁹⁵⁾. Further, the petaloid CMS has been reported to be environmentally more stable than the brown anther-type CMS²⁵⁾.

In Section III-1, I have reported the successful transfer of the CMS trait with brown anthers to a male fertile cultivar by donor-recipient protoplast fusion. This method is advantageous in that it overcomes natural barrier for sexual hybridization between species and shortens the time required for backcrossing by conventional breeding⁷⁶⁾. Also, modification of cytoplasmic traits could be expected through both recombinations between the parental mtDNAs and/or random segregation of chloroplast in the fusion products^{49,92)}.

Here I describe successful formation of cybrids exhibiting petaloid CMS, which were produced only when a regenerated plant from a protoplast fusion between a petaloid CMS line and a male fertile variety was used as the recipient in the succeeding donor-recipient protoplast fusion.

Materials and methods

1. Plant materials

A petaloid CMS line of *Daucus carota* L., 31A, was used as cytoplasmic donor, and a male fertile cultivar, K5, was as the recipient in the donor-recipient protoplast fusion. Z1, which was a regenerated plant obtained from protoplast fusion between 31A and K5, was used as the recipient in the succeeding fusions. Z1 showed the same characteristics as K5, except that it was male sterile with brown anthers.

2. Donor-recipient protoplast fusion

Protoplasts were isolated separately from suspensions of 31A and K5 cells. Prior to fusion, 31A and K5 protoplasts were treated with 75 krad X-ray and 15 mM iodoacetamide (IOA), respectively. Both protoplasts were then mixed in a 1 : 1 ratio and fused with PEG. After the fusion treatment, the protoplasts were cultured in MS medium containing 0.5 mg/l 2,4-D. The detailed methods for protoplast isolation, fusion, and culture were described in Chapters I and II.

The protoplasts isolated from suspension cells of Z1 were fused with X-irradiated protoplasts of 31A. The methods for protoplast pretreatment, fusion, and culture were the same as those used in the protoplast fusion between 31A and K5.

3. Investigation of male sterility and other agricultural characteristics in progeny of a CMS cybrid

The petaloid CMS cybrid, A-5-11 was crossed with male fertile cultivars, K5 and AM. Three plants from A-5-11 × K5, 7 plants from A-5-11 × AM and 20 plants from (A-5-11 × K5) × K5 (F₁B₁) were provided for the examination of inheritance of male sterility. The F₁B₁ plants were also used for comparison of the other agricultural characteristics with K5.

4. Chromosome analysis

Root tip cells of the parental lines, 31A and K5, and suspension cells of Z1, and the fusion products between 31A and Z1 were subjected to chromosome counting and satellite chromosome observations. The methods of pretreatment, fixation, and staining are described in Chapter II.

5. MtDNA endonuclease restriction analysis

MtDNAs of 31A, K5, Z1, and the fusion products between 31A and Z1 were isolated from the suspension cells and digested with *Sal*I, *Eco*RI, *Bam*HI, *Xba*I, and *Pst*I for restriction pattern analyses.

Results

1. Donor-recipient protoplast fusions between petaloid CMS line, 31A and fertile cultivars

The flower morphologies of the parental plants and their fusion-derived plants are shown in Fig. III-2-1. The fertile parent, K5, possessed normal anthers with filaments (Fig. III-2-1a), while the CMS parent, 31A, had petal-like stamens lacking anthers and filaments (Fig. III-2-1b).

To introduce the CMS trait of 31A into the male fertile cultivars, five different protoplast fusions were carried out combining 31A as donor and five male fertile cultivars as recipient. Through these experiments, 58 plants were regenerated and bore flowers, but no plant with petaloid flowers was observed. All the regenerated plants were male fertile or male sterile with brown anthers

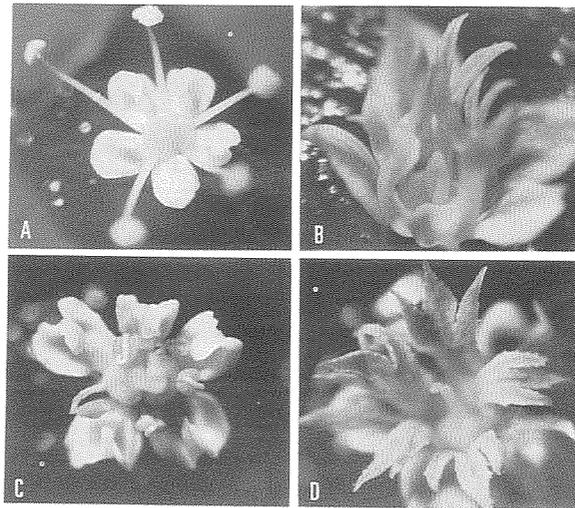


Fig. III-2-1. Flower morphology of parental plants and the cybrid plants. **A** K5, fertile variety ; **B** 31A, CMS line of petaloid type ; **C** Z1, a male sterile fusion product between 31A and K5 with brown anthers ; **D** cybrid plant, A-5-11, with petaloidy, produced by the donor-recipient protoplast fusion between 31A and Z1.

Table III-2-1. Anther phenotypes of fusion-derived plants between 31A (petaloid CMS) and 5 fertile cultivars

Donor	Recipient	Anther phenotypes			No. of plants
		Fertile	Brown anther	Petaloid	
31A	K5	8	9	0	17
31A	NS	1	4	0	5
31A	35B	6	4	0	10
31A	IPCH	21	2	0	23
31A	IP4	3	0	0	3

(Table III-2-1).

In the fusion experiment between 31A and K5, 17 regenerated plants were obtained. Among them, 9 plants showed male sterility with brown anthers and the rest were fertile. To identify the mitochondrial genome in the plants, their mtDNA restriction patterns were analyzed following digestion with 5 different endonucleases, *Bam*HI, *Sal*I, *Eco*RI, *Xba*I, and *Pst*I. All the plants analyzed showed patterns identical to those of the recipient, K5 (data not shown), except for one fusion product with brown anthers, Z1 (Fig. III-2-1c). As shown in Fig. III-2-2, when digested with the 5 endonucleases, Z1 had unique 2.1 kb *Sal*I, 6.9 kb *Eco*RI, and 26.0 kb and 2.2 kb *Xba*I fragments, which were not detected in K5, and a *Sal*I fragment at 5.3 kb specific to K5 was absent in Z1. When digested with *Bam*HI or *Pst*I, however, the restriction patterns of Z1 were identical to those of K5. In spite of the modification of mtDNA, the male sterility of Z1 was segregated in the progenies backcrossed with K5.

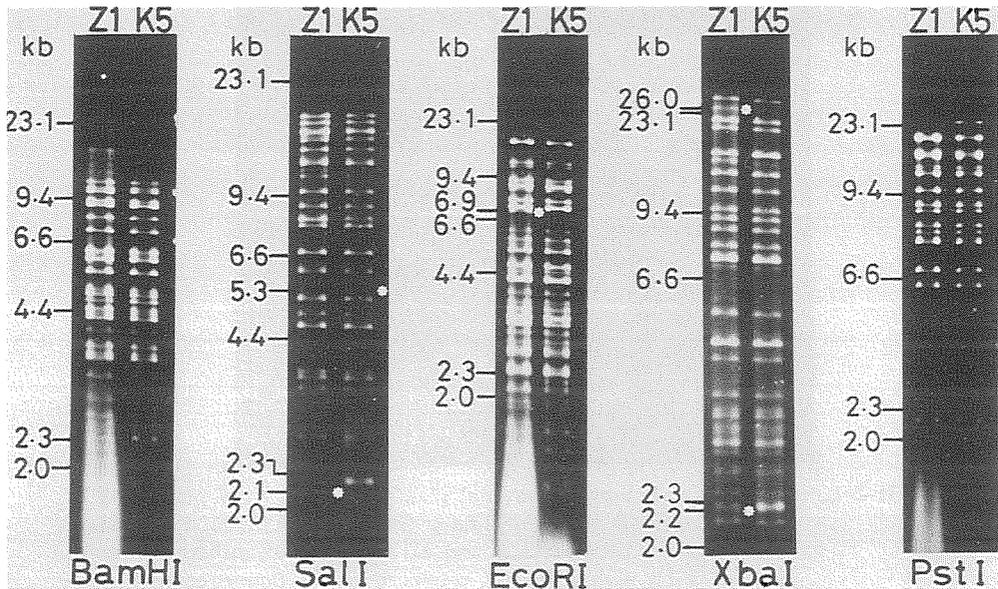


Fig. III-2-2. *Bam*HI, *Sal*I, *Eco*RI, *Xba*I, and *Pst*I restriction fragment patterns of mitochondrial DNAs from Z1 and K5. White dots indicate novel fragments found in Z1 and K5.

2. Transfer of petaloid CMS by donor-recipient protoplast fusion between 31A and a regenerated plant, Z1

The protoplasts isolated from the Z1 suspension cells were pretreated with a lethal dose of IOA and fused as recipient with X-irradiated 31A protoplasts. From this fusion experiment, 41 regenerated plants were obtained. Among them, 39 plants were male sterile with petaloid stamens (Fig. III-2-1d) and 2 plants showed male sterility with brown anthers. A male sterile plant with petaloid

Table III-2-2. Root length and root weight of F_1B_1 plants of cybrid, A-5-11, and K5 (control).

	Average of root length (cm)	Average of root weight (g)
(A-5-11 x K5) x K5 (24*)	21.0	178.0
K5 (control) (5*)	17.9	178.0

*indicates the number of plants examined.

stamens, A-5-11, set seeds after crossing with K5 and AM. Ten F_1 plants were grown to examine the transmission of male sterility. As a result, all the F_1 plants were male sterile with petaloid stamens, indicating a possibility that the male sterility of the fusion product with petaloid stamens was maternally inherited. The plants from A-5-11 x K5 were further backcrossed with K5 to examine the agricultural characteristics in progeny of the cybrid. Table III-2-2 shows root length and root weight of the F_1B_1 plants and K5. The results of t-test showed that no difference was found between the F_1B_1 plants and K5. The male sterility was observed in all the F_1B_1 plants.

3. Cytological analysis

The chromosome numbers were studied for 13 randomly chosen fusion products between 31A and Z1. All of them had a diploid chromosome number ($2n=18$), suggesting that the 31A chromosomes might have been eliminated completely from the fusion products. I further investigated the number of satellite chromosomes of 31A, Z1, and their fusion products to confirm the constitution of genome of the fusion products; photographs of the metaphase chromosomes are shown in Fig. III-2-3. The satellite chromosome number was different in 31A and Z1. 31A had two satel-

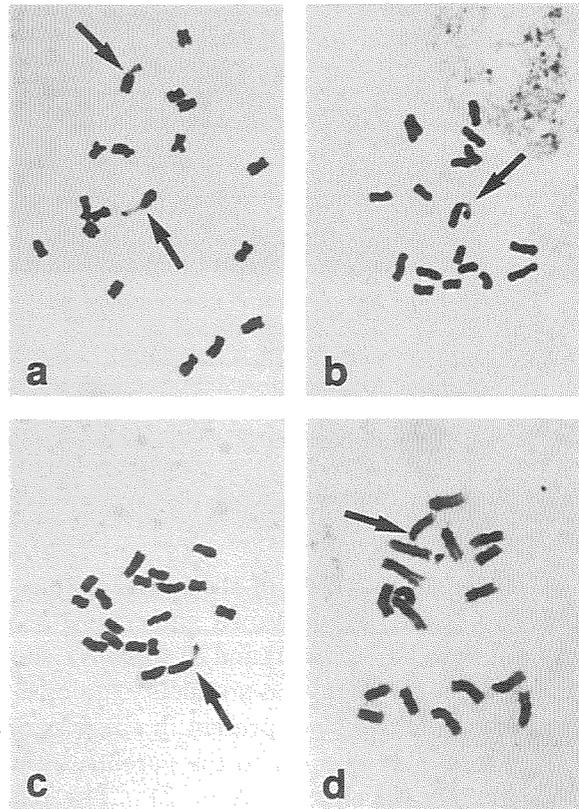


Fig. III-2-3. Chromosomes of 31A (a), Z1 (b), cybrid A-4-10 between 31A and Z1 (c) and a fertile variety, K5 (d). Arrows indicate satellite chromosomes.

lite chromosomes (Fig. III-2-3a), whereas Z1 and K5 had one (Fig. III-2-3b, d). All the fusion products analyzed had one satellite chromosome (Fig. III-2-3c). These results indicated that the nuclear marker of Z1 and the fusion products between 31A and Z1 was the same as that of K5.

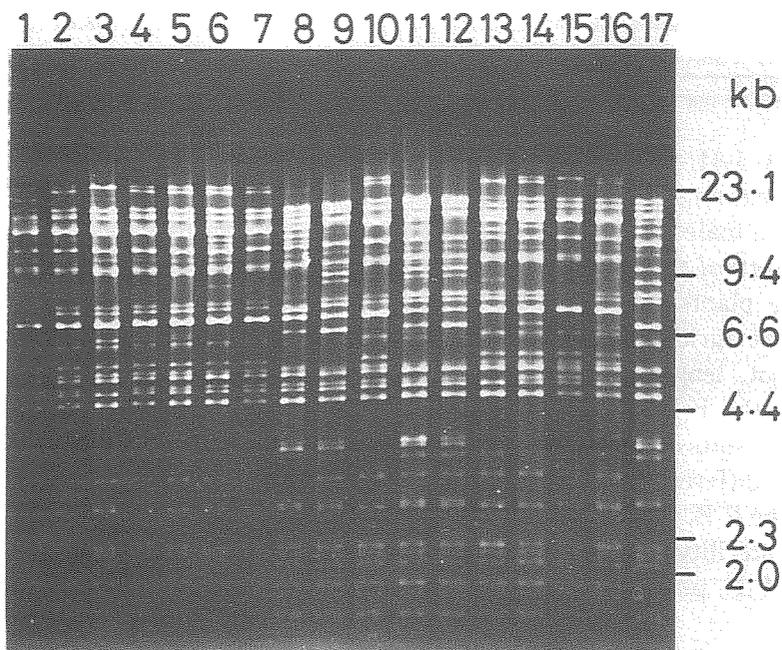


Fig. III-2-4. *Sal* restriction endonuclease analysis of mtDNAs from parental lines 31A, Z1, and their cybrids.

lane 1 : 31A (CMS donor), lane 2-16 : cybrid A-4-5 (petaloidy), A-4-7 (petaloidy), A-4-10 (petaloidy), A-4-11 (petaloidy), A-4-13 (petaloidy), A-4-14 (petaloidy), A-4-37 (petaloidy), A-5-5 (brown anthers), A-5-12 (brown anthers), A-5-21 (petaloidy), A-5-25 (petaloidy), A-6-15 (petaloidy), and A-6-19 (petaloidy), lane 17 : Z1 (recipient with brown anthers).

4. MtdNA analysis

I analyzed mtDNAs of the parental lines and their 15 fusion products, including a plant having brown anthers, with *SalI* digestion (Fig. III-2-4). The parental lines, 31A (lane 1) and Z1 (lane 17), showed patterns distinguishable from each other. All the fusion products examined (lanes 2-16) had at least one novel fragment, which was not present in either parent, and a few fragments had been deleted that were specific to the parents, although most of the other fragments were common to either 31A or Z1. The results indicated that all the fusion products, despite their flower phenotypes, possessed various rearranged types of mtDNA which might have been resulted from mtDNA recombination.

From the cytological and mitochondrial analyses, it was revealed that all the fusion products between 31A and Z1 were cytoplasmic hybrids or cybrids.

Discussion

In the previous reports on various cybrids, I described a large number of cybrids possessing various rearranged mtDNAs that were formed by donor-recipient method (Chapter II, and Chapter III, Section III-1). When 31A, a CMS line with petaloid stamens, was used as cytoplasmic donor, however, I failed in recover cybrids with petaloidy even from protoplast fusions using five different varieties, including K5, as recipients. Only when Z1, which was a regenerated plant obtained through the protoplast fusion between 31A and K5, was used as the recipient, I was successful in forming the petaloidy cybrids. These results imply that the successful hybridization of carrot may depend on the combination of parental cultivars or lines in the protoplast fusion.

Z1 plant has the same morphological characteristics and number of satellite chromosomes as K5, except that they were male sterile and had brown anthers. The male sterility was segregated in the progenies of Z1 when backcrossed with K5. Izhar *et al.*⁶⁹⁾ reported that male sterile somatic hybrids in petunia segregated male sterile and fertile progenies. They considered that the segregation occurred due to the somatic or gametic sorting out of heteroplasmic fused protoplasts or tissues during flower development and anther formation. The result of a test cross of Z1 × K5 and the fact that both male fertile and sterile plants were produced through protoplast fusion between 31A and K5 indicates that the cytoplasm of these plants may be heteroplasmic for male sterile factor. After the protoplast fusion between 31A and Z1, the cytoplasm of the fusion products might have been stabilized, because most of the regenerated plants obtained from this fusion were male sterile with petaloid stamens.

I have observed unique mtDNA restriction fragment patterns in Z1, which were slightly different from those of K5, although most of the fragments were common to those of K5 (Fig. III-2-2). During callus or protoplast culture, extensive mtDNA alterations have been reported in a wide range of species, such as maize^{41,51,141)}, potato⁷⁸⁾, *Nicotiana*⁸⁴⁾, wheat^{62,119)}, sugar beet²¹⁾, *B. campestris*¹²⁸⁾, and rice²⁸⁾. Two reasons could be mentioned for finding such novel mtDNA restriction fragments in Z1 (Fig. III-2-2). One is that the novel fragments were caused by the intermolecular recombinations followed by the protoplast fusion, and the other is that alterations were induced during the protoplast culture. Further studies are required to clarify the features of Z1.

Through the protoplast fusion described here, I have obtained 2 male sterile cybrids with brown anthers, which possess rearranged mtDNAs. One simple assumption is that the genes responsible for the CMS petaloidy encoded by mitochondrial genome could not be transferred to the cybrids, and as a result, the brown anther phenotype have appeared in the cybrids. In petunia, the CMS-associated mtDNA region, namely *S-pcf*, has been identified by analysis of both CMS and fertile somatic hybrid lines that were created by fusing protoplasts from

a CMS line with those from a male fertile line^{18,149}. Similarly as in petunia, the carrot cybrids not exhibiting petaloidy formed during cytoplasmic hybridization would play an important role in investigation of the 'CMS genes'.

IV. COMPOSITIONS OF MITOCHONDRIAL GENOMES IN CYBRIDS AND THEIRPROGENIES

Introduction

In protoplast fusion, parental cytoplasmic organelles, which are maternally inherited in most crops, can be combined in one cell. The fate of the organelle genomes in the fused cells has been studied. In many cases, parental chloroplast were segregated randomly and the resulting hybrid plants possessed only one type of the parental chloroplasts⁹². Recombination of chloroplast DNA was infrequent^{97,98}, although widespread intra- and intermolecular recombination and reorganization of mitochondrial genomes in somatic hybrids have been observed. These hybrids carry chimeric mitochondrial genomes composed of DNA sequences or regions derived from the both parents^{30,120,121,122}. Somatic cell fusion, therefore, makes it possible to alter the traits encoded by the mitochondrial DNAs.

Through the process of mitochondrial reorganization following somatic cell hybridization between the CMS line and a male fertile line, both CMS and male fertile hybrids or cybrids could be produced in tobacco^{16,49}, petunia¹⁹, and rice¹. In petunia, by comparing mtDNA restriction fragments from male sterile somatic hybrids with those from male fertile somatic hybrids, which were produced by protoplast fusion between a CMS line and a male fertile line, a DNA arrangement associated with CMS was identified and sequenced^{18,149}.

In Chapter III, Section III-2, I have produced carrot cybrids exhibiting petaloid CMS and genic male sterility with brown anthers by donor-recipient method. In this chapter, I analyze the composition of mitochondrial genomes in those cybrids and their progenies by Southern hybridization with heterologous mitochondrial genes, and compare the hybridization patterns of petaloid cybrids with those of a cybrid exhibiting brown anthers. Results of the hybridization analysis showed that a hybridizing fragment which is characteristic of petaloid-CMS cybrids and the CMS parent was absent in a cybrid with brown anthers.

Materials and methods

1. Plant materials

Sixteen cybrids with petaloid stamens and one cybrid with brown anthers (A-5-12), which showed novel restriction fragment patterns of mtDNAs, were selected for mtDNA analysis. Those cybrids were produced by the donor-recipient protoplast fusion between a petaloid CMS line, 31A, and the male sterile line

with brown anthers, Z1, as described in Chapter III, Section III-2.

The progenies of petaloid CMS cybrid, A-5-11 × K5 and A-5-11 × AM, were also provided for mtDNA analysis.

2. Southern hybridization analysis

MtDNAs of the 17 cybrids and their parents were isolated according to the method described in Chapter II. They were double-digested with *Eco*RI and *Sal*I restriction enzymes, and the digested mtDNAs in a gel were transferred to a nylon membrane filter after denature treatment.

Southern hybridizations were performed using the 'Nonradioactive DNA Labeling and Detection Kit' as described in Chapter III, Section III-1. The three clones containing 7 mitochondrial genes, *atp6* of *Oenothera*¹²⁶⁾, *rrn18 + rrn5 + nad5* of *Oenothera*²³⁾, and *coxI + rps13 + nad1* of *Oenothera*¹²⁷⁾ were used as probes in addition to the clones shown in Table III-1-1. The entire recombinant plasmids harboring mitochondrial genes were used as probes.

Results

1. Southern hybridization patterns of cybrids to heterologous mitochondrial genes

In Chapter III, Section III-2, all the cybrids analyzed showed unique mtDNA endonuclease restriction patterns, which were different from those of their parents, and diploid chromosomes (2x=18) derived from Z1. By crossing with fertile lines, the male sterility was found to be maternally inherited.

To investigate the composition of mitochondrial genomes of the cybrids and to determine whether the correlation between hybridization patterns and the stamen phenotypes existed, twelve heterologous mitochondrial genes were used as probes and hybridized them to the *Sal*I-*Eco*RI double digested mtDNAs of the cybrids with petaloid stamens or brown anthers and their parents.

Fig. IV-1 shows the hybridization patterns of the 17 cybrids (lanes 2-18) and their parental lines, 31A (lane 1), Z1 (lane 19), and a fertile variety, K5 (lane 20). The cytoplasms of 31A and Z1 could not be distinguished by hybridization with the heterologous genes, *rrn18*, *rrn26*, and *rrn18 + rrn5 + nad5* (Fig. IV-1a, b, c). Hybridization patterns obtained from the seven other clones containing eight mitochondrial genes heterologous to the mtDNAs of the cybrids were as follows.

atp6 (Fig. IV-1d) ; All the cybrids except A-5-11 (lane 13) and A-5-12 (lane 14) hybridized at the 9.0 kb fragment specific to 31A. One cybrid, A-4-37, (lane 11) was unique in having a novel 13.3 kb fragment, which was not present in either parent. Cybrids A-5-11 (lane 13) and A-5-12 (lane 14) were characterized by the presence of both the novel 13.3 kb fragment and the 14.5 kb fragment specific to Z1.

coxI (Fig. IV-1e) ; Except for cybrid A-5-12 (lane 14), all the cybrids investi-

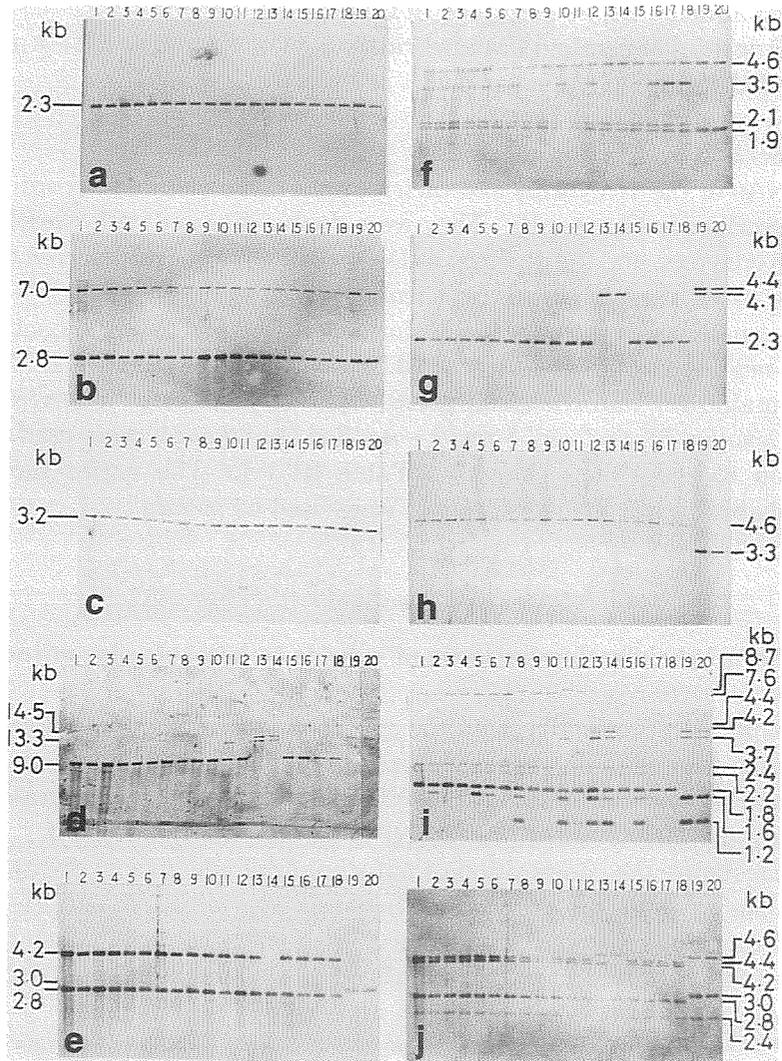


Fig. IV-1. Southern hybridizations of 13 mitochondrial genes to 17 cybrid plants and their parental lines, 31A (CMS), Z1 (male sterile with brown anthers), and cv. K5 (fertile).

Lane 1 : 31A, lane 2 : cybrid A-4-5, lane 3 : cybrid A-4-7, lane 4 : cybrid A-4-10, lane 5 : cybrid A-4-13, lane 6 : cybrid A-4-14, lane 7 : cybrid A-4-25, lane 8 : cybrid A-4-28, lane 9 : cybrid A-4-32, lane 10 : cybrid A-4-35, lane 11 : cybrid A-4-37, lane 12 : cybrid A-5-7, lane 13 : cybrid A-5-11, lane 14 : cybrid A-5-12, lane 15 : cybrid A-5-21, lane 16 : cybrid A-5-25, lane 17 : cybrid A-6-15, lane 18 : cybrid A-6-19, lane 19 : Z1, lane 20 : K5. The gene probes used were *rrn18* of pea (a), *rrn18 + rrn5 + nad5* of *Oenothera* (b), *rrn26* of *Oenothera* (c), *atp6* of *Oenothera* (d), *coxI* of *Zea mays* (e), *coxII* of *Zea mays* (f), *coxIII* of *Oenothera* (g), *cob* of *Zea mays* (h), *atpA + atp9* of pea (i), and *coxI + rps13 + nad1* of *Oenothera* (j).

gated showed hybridization patterns identical to that of 31A. A-5-12 was characterized by the absence of hybridization at 4.2 kb.

coxII (Fig. IV-1f) ; The hybridization patterns of 31A and Z1 could be distinguished from each other, and 2 fragments at 4.6 kb and 1.9 kb were common. Four cybrid plants, A-4-28 (lane 8), A-4-38 (lane 11), A-5-11 (lane 13), and A-5-12 (lane 14) were unique in having no 3.5 kb fragment specific to 31A.

coxIII (Fig. IV-1g) ; Cybrids A-5-11 (lane 13) and A-5-12 (lane 14) were characterized by the absence of hybridization at the 2.3 kb fragment specific to 31A and at 4.4 kb fragment specific to Z1.

cob (Fig. IV-1h) ; all the cybrids contained a hybridization fragment identical to 31A at 4.6 kb.

atpA+atp9 (Fig. IV-1i) ; 31A was characterized by the hybridization to 7.6 kb, 2.4 kb, 2.2 kb and 1.8 kb fragments, of which 2.4 kb and 1.8 kb fragments were conserved in all the cybrids. Z1 was characterized by the hybridization at 4.4 kb, 4.2 kb, 3.7 kb, 1.6 kb and 1.2 kb. The cybrids were classified into 5 types by the hybridization patterns ; 1) A-4-5 (lane 2), A-4-13 (lane 5), A-4-14 (lane 6), A-5-21 (lane 15), and A-6-15 (lane 17) were unique in having all the four hybridization fragments specific to 31A and a 1.6 kb fragment specific to Z1. 2) A-4-28 (lane 8) and A-4-37 (lane 11) hybridized to a novel fragment at 8.7 kb, 2.4 kb, 2.2 kb and 1.8 kb fragments specific to 31A, and 3.7 kb, 1.6 kb and 1.2 kb fragments specific to Z1. 3) A-5-7 (lane 12) and A-5-25 (lane 16) were characterized by either 1.2 kb or 1.6 kb fragment specific to Z1 in addition to all the four fragments specific to 31A, respectively. 4) A-5-11 (lane 13) and A-5-12 (lane 14) showed the same hybridization patterns, that is, five hybridization fragments specific to Z1 at 4.4 kb, 4.2 kb, 3.7 kb, 1.6 kb and 1.2 kb and two hybridization fragments specific to 31A at 2.4 kb and 1.8 kb were observed in them. 5) The remaining 6 cybrids showed the same hybridization pattern as that of 31A (lanes 3, 4, 7, 9, 10 and 18).

coxI+rps13+nad1 (Fig. IV-1j) ; Cybrids A-5-11 and A-5-12 were characterized by the absence of the 4.4 kb fragment specific to 31A and presence of the 4.6 kb fragment specific to Z1. Judging from the hybridization with *coxI* alone (Fig. V-1e), the 4.2 kb and the 2.8 kb fragments were the results of hybridizations of the *coxI* gene in this clone. The other cybrids showed the same hybridization patterns as that of 31A.

The results obtained from these Southern hybridization analyses and the anther phenotypes of the individual cybrid plants used for the analyses are summarized in Table IV-1. The cybrids that showed hybridization patterns distinguishable from the parental lines were A-4-5, A-4-13, A-4-14, A-4-28, A-4-37, A-5-7, A-5-11, A-5-12, A-5-21, A-5-25, and A-6-15. These results might indicate that the intermolecular recombination or reorganization have occurred in regions containing mitochondrial genes derived from the parental lines. The remaining 6 cybrids showed hybridization patterns the same as those of 31A.

All the hybridization patterns of Z1 (lane 19 shown in Fig. IV-1) were

Table IV-1. Summary of hybridization patterns of the carrot cybrids and their anther phenotypes.

Plant No.	Probes										Anther phenotype	
	<i>atp6</i>	<i>coxI</i>	<i>coxII</i>	<i>coxIII</i>	<i>cob</i>	<i>atpA+atp9</i>	<i>coxI+rps13+ nad1</i>	<i>rrn18</i>	<i>rrn26</i>	<i>rrn18+rrn5+nad5</i>		
A-4-5	31A*	31A	31A	31A	31A	31A	R**	31A	ND***	ND	ND	Petaloid
A-4-7	31A	31A	31A	31A	31A	31A	31A	31A	ND	ND	ND	Petaloid
A-4-10	31A	31A	31A	31A	31A	31A	31A	31A	ND	ND	ND	Petaloid
A-4-13	31A	31A	31A	31A	31A	31A	R	31A	ND	ND	ND	Petaloid
A-4-14	31A	31A	31A	31A	31A	31A	R	31A	ND	ND	ND	Petaloid
A-4-25	31A	31A	31A	31A	31A	31A	31A	31A	ND	ND	ND	Petaloid
A-4-28	31A	31A	R	31A	31A	31A	R	31A	ND	ND	ND	Petaloid
A-4-32	31A	31A	31A	31A	31A	31A	31A	31A	ND	ND	ND	Petaloid
A-4-35	31A	31A	31A	31A	31A	31A	31A	31A	ND	ND	ND	Petaloid
A-4-37	R	31A	R	31A	31A	31A	R	31A	ND	ND	ND	Petaloid
A-5-7	31A	31A	31A	31A	31A	31A	R	31A	ND	ND	ND	Petaloid
A-5-11	R	31A	R	R	31A	31A	R	R	ND	ND	ND	Petaloid
A-5-12	R	R	R	R	31A	31A	R	R	ND	ND	ND	Brown anthers
A-5-21	31A	31A	31A	31A	31A	31A	R	31A	ND	ND	ND	Petaloid
A-5-25	31A	31A	31A	31A	31A	31A	R	31A	ND	ND	ND	Petaloid
A-6-15	31A	31A	31A	31A	31A	31A	R	31A	ND	ND	ND	Petaloid
A-6-19	31A	31A	31A	31A	31A	31A	31A	31A	ND	ND	ND	Petaloid

31A* : Hybridization pattern identical to the CMS line, 31A, R** : Hybridization pattern of rearranged type, ND*** : Not distinguishable.

identical to those of K5 (lane 20), which is a parental variety of Z1, as reported in the fusion experiment (Chapter III, Section III-2). These results suggest that rearrangement of mtDNA in Z1 might not have occurred, at least in the regions covered by the heterologous mitochondrial gene probes used.

Cybrids A-5-11 and A-5-12 showed the same hybridization patterns, except when *coxI* gene was used as a probe. Their stamen phenotypes, however, were different from each other, that is, A-5-11 showed petaloidy, while A-5-12 bore flowers with the same brown anthers as Z1 (Table IV-1). These results imply that the mtDNA sequence related to the petaloid CMS in A-5-12 might be eliminated during the process of mtDNA rearrangement. A comparison of the *coxI* hybridization patterns between A-5-11 and A-5-12 suggests that the absence of 4.2 kb fragment may correlate with conversion of the anther phenotype from petaloidy to brown anthers due to nuclear gene(s) (Fig. IV-1e).

2. Southern hybridization analysis of mtDNAs from the progenies of a CMS cybrid

EcoRI-SalI digested mtDNAs of the 10 progenies of petaloid CMS cybrid, A-5-11, were hybridized with 6 clones containing 8 heterologous mitochondrial genes. When *coxI*, *coxIII*, and *atp6* were used as probes, no difference was detected among parental cybrid A-5-11 and the 10 progenies (Fig. IV-2a, b, c). However, unique hybridization patterns which were different from those of A-5-11, were detected in the progenies when *coxII*, *atpA+atp9*, and *coxI+rps13+*

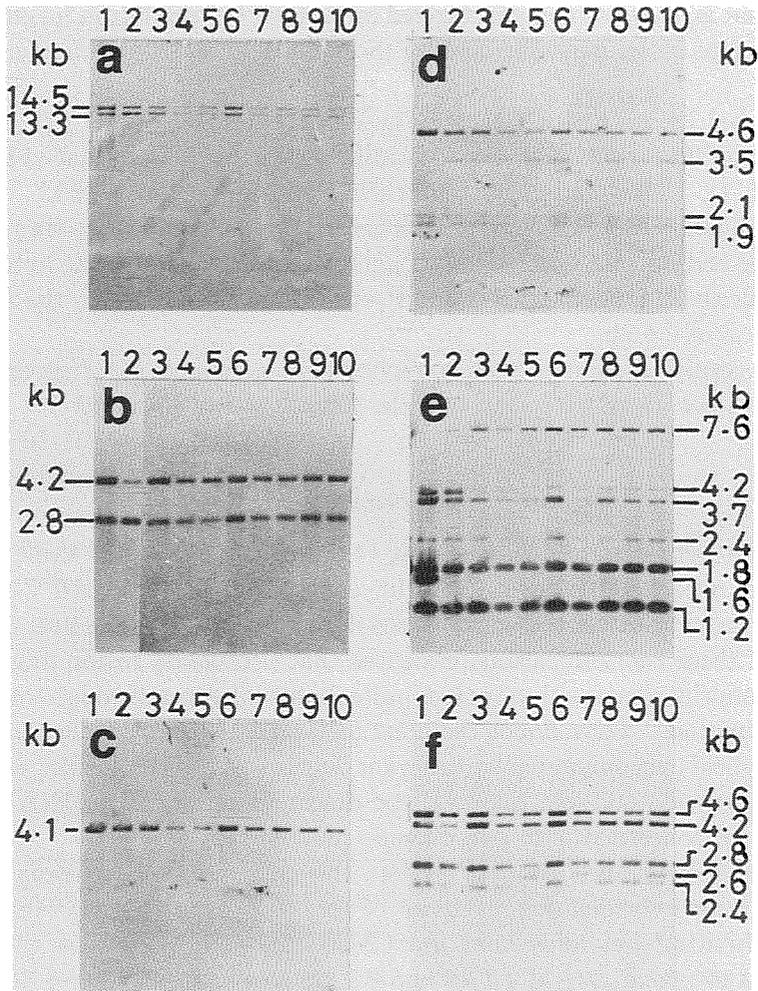


Fig. IV-2. Southern hybridization of 8 mitochondrial genes to CMS cybrid A-5-11 (lane 1) and progenies of A-5-11 \times K5 (lanes 2-4) and A-5-11 \times AM (lanes 5-11). Lane 1 : A-5-11, lane 2 : BC-1, lane 3 : BC-2, lane 4 : BC-3, lane 5 : F₁-1, lane 6 : F₁-2, lane 7 : F₁-3, lane 8 : F₁-4, lane 9 : F₁-5, lane 10 : F₁-6, lane 11 : F₁-7. The probes were *atp6* of *Oenothera* (a), *coxI* of *Zea mays* (2), *coxIII* of *Oenothera* (c), *coxII* of *Oenothera* (d), *atpA* + *atp9* of pea (e), and *coxI* + *rps13* + *nad1* of *Oenothera* (f).

nad1 genes were used as probes (Fig. IV-2d, e, f). The results were as follows.

coxII (Fig. IV-2d) ; Only F₁-3 (lane 7) showed the same hybridization pattern as that of 31A. The other 9 progenies were unique in having a 3.5 kb hybridization fragment, which was absent in the parental cybrid, A-5-11, but present in 31A (Fig. IV-1f).

atpA + *atp9* (Fig. IV-2e) ; All the progenies showed novel hybridization

patterns, that is, they were characterized by a novel hybridization fragment at 7.6 kb with various stoichiometries and by the absence of a 1.6 kb fragment specific to A-5-11.

coxI + *rps13* + *nad1* (Fig. IV-2f) ; Three progenies, F₁-2 (lane), F₁-4 (lane 6) and F₁-7 (lane 10) showed different hybridization patterns from that of the female parent, A-5-11. They had a novel hybridization fragment at a 2.6 kb, which was absent in A-5-11, 31A, Z1 and K5. This 2.6 kb hybridization fragment was judged to be coding *rps13* and/or *nad1* genes from the result of the hybridization with *coxI* alone (Fig. IV-2a). F-2 and F₁-4 were characterized by the absence of hybridization at 2.4 kb.

These results indicated that mitochondrial genomes of offsprings of the cybrids were not identical to those of the female parental cybrid.

Discussion

Southern hybridization showed that 11 out of the 17 cybrids had exclusively reorganized mitochondrial genes of the parental lines, 31A and Z1, and the remaining six cybrids possessed mt genes derived from the CMS line, 31A. The presence of these reorganizations indicated that protoplast fusion might modify the mitochondrial genome that include mitochondrial genes through intermolecular recombination. Thus, donor-recipient method which can produce male sterile or fertile cybrids accompanied by different degrees of intermolecular recombination, could provide suitable material for determining which mtDNA region or sequences are concerned with cytoplasmic male sterility.

As described in the 'Results' section, the petaloid CMS phenotype can be correlated with hybridization patterns of the *coxI* gene (Fig. IV-1e). All the cybrids exhibiting petaloidy and the CMS line, 31A, possessed two regions homologous to maize *coxI* at 4.2 kb and 2.8 kb, whereas cybrid A-5-12, which is male sterile with brown anthers, lacked the 4.2 kb hybridization fragment. Furthermore, the hybridization patterns of A-5-11 and A-5-12 with 14 heterologous mitochondrial genes other than *coxI* were identical despite the difference of anther phenotypes. The simplest assumption, therefore, is that the 4.2 kb fragment contains a modified *coxI* gene, and that this gene product might be related to the differentiation of stamen primordia. This possibility was supported by the same result of the hybridization with *coxI* to a male fertile cybrid, which was produced by the protoplast fusion using a male fertile cultivar, 35B, as donor and 31A as recipient (data not shown).

It has been reported that rearrangements within or adjacent to coding regions of the mtDNA were associated with CMS phenotypes in some species^{7,8,35,37,90,129,149}. In *Sorghum*, the mitochondria from a CMS line possessed a variant form of the *coxI* gene that arose from at least two rearrangements. This novel *coxI* gene synthesized a *coxI* polypeptide with a significantly larger molecular weight than

that of the male fertile line. This polypeptide has been supposed to interfere with the formation and release of functional pollen⁸⁾.

The data do not exclude the possibility that other mtDNA regions or sequences are involved with the CMS-associated regions. Further investigations will be required to determine whether or not the 4.2 kb fragment, which might be correlated with CMS, is directly related to the petaloid CMS phenotype in the carrot.

The results from cytological and mtDNA analyses described in Chapter III, Section III-2 indicated that the CMS cybrids were resulted from the introduction of mtDNA regions at least coding for CMS of donor plant into the recipient plants. The CMS trait of the cybrids was maternally inherited by crossing with two male fertile cultivars. This indicated that the CMS-coding region in the CMS cybrid was transferred to the progeny through the female parent. However, hybridization analysis with the heterologous genes revealed that the mtDNA of the progenies was not identical to those of the parental CMS cybrid. This suggested three possibilities ; 1) mitochondrial genomes of the cybrid were chimeric in one mitochondrion, in one cell, or in one plant, 2) stoichiometric changes have occurred in mitochondrial genome of the progeny, 3) reorganizations or rearrangements of mitochondrial genome have occurred in the progenies. Only from the data presented here, either speculations cannot be excluded.

Aviv and Galun⁶⁾ reported that variation of mitochondrial genome found in progeny of *Nicotiana* cybrids continued until the third generation, suggesting the instability of mitochondrial genome in the recombinants. Furthermore, stoichiometric changes of mtDNA restriction fragments have been observed when a cytoplasm was transferred to a foreign nuclear background through backcrossings in sorghum⁷⁾, bean⁸⁹⁾, and maize⁴³⁾. The data presented here suggested that the transmission of rearranged mitochondrial genomes in cybrids might be unstable.

Further analysis of mitochondrial genomes in cybrids and their progenies will play an important role in elucidation of mitochondrial genome organization and differentiation in higher plants.

GENERAL DISCUSSION

There are several major approaches to the introduction of foreign genes into plant cells. The transfer of isolated genes can be accomplished by using the transformation systems based upon *Agrobacterium*, or direct plasmid uptake into protoplasts through chemical and physical treatments or with help of microinjection and/or microinjectil bombardment. In addition to production of transgenic plants, there is a need to manipulate uncharacterized or complicated traits. For these purposes, the methods of cell fusion or organelle transfer offer an alternative solution.

The results presented here demonstrated for the first time successful formation of carrot cybrids, which possessed the nuclei of one parent and novel cytoplasm derived from the both parents, through donor-recipient method. Additionally, two types of carrot CMS, brown anther-type and petaloid-type, were directly transferred to a male fertile variety in a shorter time using this method. The genetical and chromosomal analyses showed that the transferred CMS was maternally inherited after backcrossings with the male fertile parent, and that the nuclei of the fusion products were derived from the recipient parent. At present, the CMS cybrids, which have been produced in this study, are being utilized in a F₁ breeding programme, and new F₁ varieties will be released in near future.

The mitochondrial DNA analyses revealed that the cybrids possessed unique mitochondrial genomes, which were different from those of the parents. Mitochondrial genomes of higher plants are more complex in structure than those of fungi or mammalian^{38,42,70,82,83,104,110,136,143,144}. Palmer and Shields¹¹⁵ reported that those complex feature occurred via intra- or intermolecular recombination between direct repeat sequences contained in *Brassica campestris* mtDNAs. The similar multipartite genomic organization via a homologous recombination between repeated sequences were reported in maize^{44,86,87}, wheat¹¹⁷, spinach¹³⁴, *B. hirta*¹¹⁴, sugar beet²², radish⁹¹, sunflower¹²⁹, rice¹⁰⁵, and *Petunia hybrida*⁴⁶. Mitochondria of somatic hybrid or cybrid were often different from those of their parents, that is, they showed novel mtDNA restriction fragment patterns, which were different either from the parental and the mixture of the parental patterns. Rearrangements of mtDNAs through cytoplasmic hybridization have been reported in *Solanaceae*, *Umbelliferae*, *Cruciferae*, *Graminae*, *Leguminosae*, and *Ruaceae*⁶⁵. Belliard *et al.*¹⁶ suggested that the rearrangements of mtDNAs observed in somatic hybrids were attributed to intermolecular recombination between two mtDNAs. MtDNA alterations observed in the carrot cybrids may be due to such intermolecular recombination between the parental mtDNAs. Chloroplast DNAs of *Daucus* species has been analyzed by DeBonte *et al.*³³. They compared the restriction fragment patterns among four species and observed few variation among them. Based on the result of their report, the chloroplast genome of fusion products was not investigated in this study.

Furthermore, it was found that the mitochondrial genomes in cybrids were genetically unstable (Chapter IV). The progenies (R₁) of the cybrids had different composition of mitochondrial genes from that of the female parent. This suggested the possibilities that reorganizations or sorting out of mitochondrial genome, or stoichiometric changes of fragments homologous to mitochondrial genes have occurred through reproduction. Further investigations are however required to determine the mechanism.

It is generally considered that CMS is encoded by mitochondrial genome. In *petunia*, mtDNA regions related to CMS were detected by comparison of mtDNAs

between male fertile and CMS somatic hybrids^{18,149}). Also in this study, male fertile and CMS cybrids were produced. Southern blot analysis probed with heterologous mitochondrial genes revealed that the regions containing *coxI* gene might be associated with the petaloid CMS. Further analysis will be needed to identify the carrot CMS gene(s).

The progress of this study will bring a great advantage in practical breeding, and contribute to elucidate the molecular mechanism related to male sterility.

ABSTRACT

In this study, I report successful cybrid formation, transfer of cytoplasmic male sterility (CMS) in carrot (*Daucus carota* L.) by donor-recipient protoplast fusion, and their morphological, cytological and molecular natures. The thesis consist of 4 chapters, and the results are summarized as follows.

Chapter I

An efficient method of plant regeneration from carrot protoplasts was determined. Namely ; 1) enzyme solution consisting of 1% Driselase, 0.5% Cellulase RS and 0.01% Pectolyase Y-23 was most effective for protoplast isolation, 2) larger cell aggregates (>105 μm) gave smaller protoplasts (average 16.0 μm), which showed high embryogenic ability, 3) decrements of colony or embryo density, from $1 \times 10^5/\text{ml}$ to $1 \times 10^2/\text{ml}$ at heart stage and from $1 \times 10^2/\text{ml}$ to 1–10/ml at torpedo stage, and osmolality of medium, from 500 mOsm to 200 mOsm at heart stage and from 200 mOsm to 50 mOsm at torpedo stage were essential for normal plant regeneration, 4) for acclimatization of regenerated plants, it was important to gradually decrease the humidity in culture bials by making pin holes every 3–6 days for 2–3 months. The overall efficiency was 1 plant/140 protoplasts.

Chapter II

To produce cybrids in *Daucus*, conditions for donor-recipient protoplast fusion were investigated. Prior to the donor-recipient protoplast fusion between *D. carota* and the related wild species, effects of pretreatments by X-ray and metabolic inhibitors, iodoacetamide (IOA), on carrot protoplasts were examined. Exposure to over 40 krad X-ray and treatment with >10 mM IOA for 10 min prevented colony formation completely. These results indicated that X-irradiation more than 40 krad and treatment of more than 10 mM IOA are required to completely eliminate the nuclear genome of donor cells, and to prevent the metabolic activity of recipient cells, respectively.

Protoplasts of *D. capillifolius* and *D. gummifer* isolated from each suspension cells were X-irradiated over lethal dose (60 krad) just prior to protoplast fusion. Protoplasts of *D. carota* cell line were treated with 15 mM iodoacetamide and

fused with the X-irradiated protoplasts. The regenerated plants were obtained from protoplast fusion between *D. gummifer* and *D. carota*, and between *D. capillifolius* and *D. carota*. The regenerated plants from the fusion between *D. capillifolius* (chromosome number above 60) and *D. carota* (chromosome number 17) possessed chromosome number 17 (2x-1) or 34 (4x-2) and an identical leaf morphology to *D. carota*. Their mitochondrial DNAs (mtDNAs) were analyzed with restriction endonucleases. Novel restriction fragments of mtDNA, not present in mtDNA digests from both parents, were observed in both fusion products between *D. gummifer* and *D. carota*, and between *D. capillifolius* and *D. carota*. These results indicated successful formation of cytoplasmic hybrids or cybrids between *D. carota* and the related wild species.

Chapter III, Section III-1

X-irradiated protoplasts of *D. carota*, 28A1, carrying cytoplasmic male sterile (CMS) cytoplasm of brown anther type and iodoacetamide-treated protoplasts of a male fertile cultivar, K5, were fused with polyethylene glycol (PEG) and 73 regenerated plants were obtained. Twenty-six randomly chosen regenerated plants had non-parental mtDNAs as revealed by *Xba*I restriction fragment patterns, and all of the plants investigated had diploid chromosome numbers. Out of 11 regenerated plants that showed mtDNA fragment patterns clearly different from those of the parents, 10 plants showed male sterility with brown anthers and one plant possessed partially male sterile anthers. The mtDNA fragment patterns of the ten plants with male sterile flowers resembled that of a CMS parent, 28A1, and four fragments were identified that were common between the male sterile plants and 28A1, but absent from the partially male sterile plants and a male 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.

Chapter III, Section III-2

The CMS trait of petaloid-type flowers was tried to transfer to fertile cultivars by donor-recipient method. X-irradiated protoplasts of the carrot CMS line, 31A and iodoacetamide-treated protoplasts of 5 different fertile cultivars were used as a cytoplasmic donor and recipient, respectively. Fifty-eight plants were regenerated from the fusion experiments, and no plant with petaloid flowers was observed. All the regenerated plants were fertile or male sterile with brown anthers. To produce cybrids with petaloid-type flowers, Z1, which is a regenerated plant obtained from protoplast fusion between 31A and K5, was used as a cytoplasmic recipient and fused with X-irradiated protoplasts of 31A. From this donor-recipient protoplast fusion, 41 fusion products were regenerated and bore flowers. Among them, 39 plants were CMS with petaloid stamens and 2 plants were the same male sterile with brown anthers as Z1. Chromosomal and mtDNA

analyses of the fusion products revealed that all of the plants investigated had nuclei derived from Z1 and rearranged mtDNAs. After crossing with fertile cultivars including K5, the petaloid male sterility of the fusion products was maternally inherited in the F₁ and F₁B₁ generations. Other agronomic traits of the progenies were similar to those of the parental fertile line.

The results indicate that petaloid CMS trait can be transferred effectively to another carrot line by using donor-recipient method.

Chapter IV

Mitochondrial genomes of 17 cybrids and the progenies were analyzed by Southern hybridization using different heterologous mitochondrial gene probes. The cybrids were produced by donor-recipient protoplast fusion between a petaloid CMS line, 31A, and a fusion product with brown anthers, Z1. All the cybrids analyzed exhibited petaloidy except one, which had brown anthers (A-5-12) (Chapter III, Section III-2). From Southern hybridization analyses, it was revealed that eleven plants out of the 17 cybrids possessed rearranged mitochondrial genomes of the parental lines. The remaining six cybrids had the same mitochondrial gene composition as the CMS parent, 31A.

Furthermore, a comparison of the hybridization patterns between the 16 cybrids with petaloid stamens and the cybrid with brown anthers, A-5-12, revealed that the hybridization patterns probed with maize cytochrome c oxidase subunit I (*coxI*) could be correlated with the anther phenotypes. Cybrids with petaloid stamens and the CMS parent, 31A, possessed two *coxI*-homologous fragments at 4.2 kb and 2.8 kb when digested with *EcoRI* and *SalI*, whereas A-5-12 lacked the homologous region at 4.2 kb. This result suggests that the 4.2 kb fragment might be associated with the petaloid CMS phenotype.

The CMS cybrid was crossed with two male fertile varieties, and mitochondrial genomes of the 10 progenies were analyzed by Southern hybridization with mitochondrial gene probes. Despite the CMS trait of cybrid was maternally inherited in the progenies, their mitochondrial genomes were different from that of the parental CMS cybrid. This indicated that the mitochondrial genome of cybrids was genetically unstable.

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