Transfer of the Petaloid-Type CMS in Carrot by Donor-Recipient Protoplast Fusion

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The CMS trait of petaloid-type flowers in carrot was tried to transfer to fertile varieties by donor-recipient protoplast fusion. X-irradiated protoplasts of the carrot CMS line, 31A, and iodoacetamide-treated protoplasts of 5 different fertile varieties were used as a cytoplasmic donor and recipients, 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 mitochondrial DNA analyses of the fusion products revealed that all of the plants investigated had nuclei derived from Z1 and rearranged mtDNAs. After crossing with fertile varieties including K5, the petaloid male sterility of the fusion products was maternally inherited in the F_1 and F_1B_1 generations. Other agronomic traits of the progenies were similar to those of the parental fertile line. The results indicate that the petaloid CMS trait can be transferred effectively to another carrot line by using two-step donor-recipient protoplast fusion.

KEY WORDS: *Daucus carota*, carrot, petaloid-type CMS, donor-recipient protoplast fusion.

Introduction

In carrot (*Daucus carota* L.), F_1 breeding has arrested our attention because some carrot F_1 hybrids between the lines having high combining ability showed uniformity in productivity, quality and nutrient value (KATSUMATA 1985; KAUL 1988). In commercial carrot F_1 seed production, two types of cytoplasmic male sterility (CMS) are being used as female parents; one is a brown anther-type whose flowers possess brown anthers with aborted pollen grains, and the other is a petaloid-type whose stamens are transformed into petal-like structure. Compared with the brown anther-type, the petaloid-type CMS is more practical because of its environmental stability (TIMIN and DOBRUTSUKAYA 1981).

Previously, we have reported the successful transfer of the CMS trait with brown anthers to a fertile variety by donor-recipient protoplast fusion (TANNO-SUENAGA *et al.* 1988). This method is advantageous in that it shortens the time required for backcrossing by conventional breeding (TANNO-SUENAGA *et al.* 1988, KEMBLE *et al.* 1988). Also, modification of cytoplasmic traits could be expected through recombination between the parental mitochondrial DNAs and/or random segregation of chloroplasts in the fusion products (ICHIKAWA *et al.* 1989, MALIGA 1986).

In this report, we 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 fertile variety was used as the recipient in the succeeding donor-recipient protoplast fusion.

Materials and Methods

Plant materials: A petaloid CMS line of *Daucus Carota* L., 31A, was used as cytoplasmic donor, and a fertile variety, K5, was the recipient in the donor-recipient protoplast fusions. Z1, which is a regenerated plant obtained from protoplast fusion between 31A and K5, was used as the recipient in the succeeding fusions. Z1 showed the same agricultural characteristics as K5, except that it was male sterile with brown anthers. The seeds of 31A and K5 were provided by Kyowa Seed Co.

Donor-recipient protoplast fusion: Protoplasts were isolated separately from suspension cells of 31A and K5. Prior to fusion, 31A and K5 protoplasts were treated with 75 krad X-ray and 15mM iodoacetamide (IOA), respectively. Both protoplasts were then mixed in a 1:1 ratio and fused with polyethylene glycol (PEG). After the fusion treatment, the protoplasts were cultured in MS medium containing 0.5mg/l 2,4-D. The detailed methods for protoplast isolation, fusion, and culture were described by ICHIKAWA *et al.* (1987).

The protoplasts isolated from a 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.

Investigation of male fertility in progeny of a CMS cybrid: A petaloid CMS cybrid, A-5-11, was crossed with fertile lines, K5 and AM. Three plants from A-5-11 $\times$ K5 and seven plants from A-5-11 $\times$ AM were provided for segregation analysis on male sterility.

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 counting chromosome numbers and satellite chromosome observations. The methods of pretreatment, fixation, and staining were the same as those described by NISHIBAYASHI and KAERIYAMA (1986).

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 SalI, EcoRI, BamHI, XbaI, and PstI restriction enzymes for mtDNA restriction pattern analyses. The mtDNAs were isolated according to the procedure of ICHIKAWA *et al.* (1987). The isolated mtDNAs were separated by electrophoresis in 0.7% agarose gel, and stained with ethidium bromide.

Results

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

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

To introduce the CMS trait of 31A into the fertile varieties, five different protoplast fusions were carried out combining 31A as donor and five fertile varieties 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 fertile or male sterile with

brown anthers (Table 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 genomes in these plants, their mtDNA restriction patterns were analyzed following digestion with 5 different endonucleases, BamHI, SalI, EcoRI, XbaI, and

Table 1. Anther phenotype of fusion derived plants between 31A (petaloid CMS) and 5 fertile varieties

Donor	Recipient	Anther phenotype			Total No. of plants
		Fertile	Brown anthers	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

Fig. 1. Flower morphology of parental plants and the cybrids. ^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.

PstI. 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. 1c). As shown in Fig. 2, when digested with the 5 endonucleases, Z1 had unique 2.1kb SalI, 6.9kb EcoRI, and 26.0kb and 2.2kb XbaI fragments, which were not detected in K5, and a SalI fragment at 5.3kb specific to K5 was absent in Z1. When digested with BamHI or PstI, 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 progeny when backcrossed with 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 recipients 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. 1d) and 2 plants showed male sterility with brown anthers. A male sterile plant with petaloid stamens, A-5-11, set seeds after crossing with K5 and AM. Ten F₁ plants were grown to determine the mode of inheritance concerning the male sterility. As a result, all the F₁ plants were male sterile with petaloid stamens, indicating that the male sterility trait of the fusion product with petaloid stamens was maternally inherited.

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

Fig. 2. BamHI, SalI, EcoRI, XbaI, and PstI restriction fragment patterns of mtDNAs from Z1 and K5. White dots indicate novel fragments found in Z1 and K5.

31A chromosomes might have been eliminated completely from the fusion products. We further investigated the number of satellite chromosomes of 31A, Z1, and their fusion products to confirm the origin of nuclear genome of the fusion products; photographs of the metaphase chromosomes are shown in Fig. 3. The satellite chromosome numbers were different between 31A and Z1. 31A had two satellite chromosomes (Fig. 3a), whereas Z1 and K5 had one (Fig. 3b, d). All the fusion products analyzed had one satellite chromosome (Fig. 3c).

4. *MtDNA* analysis

We analyzed mtDNAs of 31A, Z1 and their 15 fusion products, including a plant having brown anthers, with *SalI* digestion (Fig. 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 cybrids.

Fig. 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.

Fig. 4. *Sal*I 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 (petaloid), A-4-7 (petaloid), A-4-10 (petaloid), A-4-11 (petaloid), A-4-13 (petaloid), A-4-14 (petaloid), A-4-37 (petaloid), A-5-5 (brown anthers), A-5-7 (petaloid), A-5-11 (petaloid), A-5-12 (brown anthers), A-5-21 (petaloid), A-5-25 (petaloid), A-6-15 (petaloid), A-6-19 (petaloid), lane 17 : Z1 (recipient with brown anthers).

Discussion

In our previous reports on carrot cybridization, we described a large number of cybrids possessing various rearranged mtDNAs that were formed by donor-recipient protoplast fusions (ICHIKAWA *et al.* 1987, TANNO-SUENAGA *et al.* 1988). When 31A, a CMS line with petaloid stamens, was used as cytoplasmic donor, however, we failed to recover cybrids with petaloid stamen 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, we were successful in forming cybrids with petaloid stamens. These results imply the successful cybridization may depend on the combination of parental varieties or lines in the protoplast fusion.

Z1 plant had the same morphological characteristics and number of satellite chromosomes as K5, except that it was male sterile and had brown anthers. The male sterility was segregated in the progenies of Z1 when backcrossed with K5. IZHAR *et al.* (1983) reported that male sterile somatic hybrids in petunia segregated sterile and male fertile progenies. They considered that phenomenon was attributed 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 $\times$ K5 and the fact that both male fertile and sterile plants were produced through protoplast fusion between 31A and K5 indicate that the cytoplasm of these plants may be heteroplasmic for the 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.

We 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. 2). During callus or protoplast culture, extensive mtDNA alterations have been reported in a wide range of species, such as maize (GENGENBACH *et al.* 1981, UMBECK and GENGENBACH 1983, EARLE *et al.* 1987), potato (KEMBLE and SHEPARD 1984), *Nicotiana* (LI *et al.* 1988), wheat (RODE *et al.* 1987; HARTMANN *et al.* 1989), sugar beet (BREARS *et al.* 1989), *B.campestris* (SHIRZADEGAN *et al.* 1989), and rice (CHROWDHURY *et al.* 1989). Two reasons could be mentioned for the occurrence of such novel mtDNA restriction fragments in Z1 (Fig. 2). One is that the novel fragments were caused by the intermolecular recombinations after 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.

Endonuclease restriction patterns of mtDNAs from most of cybrids with petaloid stamens resembled to that of 31A (Fig. 4). However, cybrids, A-5-11 and A-5-12, showed unique restriction patterns which were different from those of the parental lines, 31A and Z1. These observations were consistent with the results of Southern hybridization analysis (TANNO-SUENAGA and IMAMURA in press), where the mitochondrial genomes of A-5-11 and A-5-12 were indicated to be rearranged. The flower phenotypes of A-5-11 and A-5-12 were male sterile with petaloid stamens and brown anthers, respectively. One simple assumption is that the genes responsible for the CMS petaloidy trait encoded by mitochondrial genome could not be transferred to A-5-12, and as a result, the brown anther phenotype have appeared in the cybrid. 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 fertile line (BOESCHORE *et al.* 1985; YOUNG and HANSON 1987). Similarly as in petunia, the carrot cybrid not exhibiting petaloidy formed during cybridization would play an important role in the investigation of the 'CMS genes'.

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ニンジンにおける非対称細胞融合によるペタロイド型 CMS の導入

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ニンジンのペタロイド型細胞質雄性不稔 (CMS) を非対称細胞融合を用いて、可稔系統に導入する実験を行った。融合には、X 線を照射したニンジンの CMS 系統、31 A を細胞質供与体として、ヨードアセトアミド処理した 5 つの可稔系統をその受容体として用いた。この融合実験から 58 個体が再分化したが、ペタロイドの花をもつ個体は全く得られなかった (Table 1)。すなわち、すべての再分化個体は、可稔または褐変化した葯をもつ雄性不稔であった。そこで、ペタロイド型の花をもつサイブリッドを作出するために、31 A と可稔系統 K 5 の間の融合処理によって得られた褐変化した葯をもつ Z 1 を細胞質受容体に用いて、X 線を照射をした 31 A のプロトプラストと融合した。この非対称細胞融合により 41 の再分化個体が得られ、花を着けた。このうちの 39 個体はペタロイド型の雄蕊をもつ CMS で、2 個体は褐変化した葯をもつ雄性不稔だった (Fig. 1)。染色体の核型とミトコンドリア DNA の制限酵素分析結果 (Fig. 2, Fig. 3, Fig. 4) から、得られた再分化個体はすべて、Z 1 に由来した核をもち、又両親とは異なる再編成した形のミトコンドリアをもつことが分かった。これらの融合産物に見られるペタロイド型の雄性不稔性は、K 5 を含む可稔系統との交配の後 F_1 及び $F_1 B_1$ 世代に母性遺伝した。以上の結果は、ペタロイド型 CMS が 2 段階の非対称細胞融合を使うことによって、他のニンジンの系統に効率良く導入することが可能であることを示す。