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Somatic Hybridisation of *Daucus carota* and *D. capillifolius* by Protoplast Fusion

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Summary. Protoplasts isolated from cultured cells of albino carrot *(Daucus carota)* and normal green *D.capillifolius* were fused by polyethylene glycol. Selection of somatic hybrid plants was based on the restoration of photosynthetic function in hybrids. Green plantlets selected from embryo cultures were characterized on the basis of leaf morphology. The interspecific protoplast fusion resulted in green plants with leaves which were intermediate between those of the parents. The somatic hybrids between orange rooted carrot variety and *D.capillifolius* with long white roots produced long, white and fleshy roots. The cytological analysis of parasexual hybrids revealed that the chromosome number ranged from 34 to 54. The most frequent chromosome number was 2n = 36. Hybrids were also found with 34 and 35 chromosomes. The somatic hybrid showed the same isoenzyme pattern of leaf peroxidase as *D.carota*.

Key words: Daucus - Protoplast-fusion - Somatic Hybridisation

Introduction

Genetic alteration of higher plants by parasexual means is being extensively studied using various experimental approaches. Progress in techniques of plant protoplast fusion has made it possible to hybridize somatic plant cells (Keller and Melchers 1973; Kao and Michayluk 1974; Wallin et al. 1974). Plant regeneration from cultured protoplasts has been reported for many plants (review by Gamborg 1976).

The recently available results in somatic hybridisation have been achieved with sexually compatible plants. Fusion of protoplasts has resulted in intraspecific hybrid plants between chlorophyll deficient mutants of *Nicotiana tabacum* (Melchers and Labib 1974; Gleba et al. 1975) as well as in interspecific somatic hybrids between *N.glauca* and *N.langsdorffii* (Carlson et al. 1972; Smith et al. 1976), *N.tabacum* and *N.silvestris* (Melchers 1976), and *Petunia hybrida* and *P.parodii* (Power et al. 1976). So far, somatic hybrids have been produced using only solanaceous species.

The present paper reports on the production of interspecific somatic hybrids between *Daucus carota* and *D.capillifolius*. The sexual hybrids of these species were produced and characterized by McCollum (1975). From those experiments, it is known that the parents and F_1 plants can be morphologically distinguished. In order to recover somatic hybrid embryos and plantlets, protoplasts isolated from cell cultures of albino carrot *(D.carota)* and green *D.capillifolius* plants were fused. After selection of green plants the identification of somatic hybrids was based on the differences in leaf characteristics.

Material and Methods

Seeds of carrot (*D.carota*, c.v. 'Nantaise Slendero') were treated with 0.1% ethylmethansulphonate (EMS) for 24 hrs at room temperature prior to germination. After self-pollination of the resulting plants, 2 albino carrots were selected in the M_2' generation. Cell suspension cultures established from callus tissues of albino plants were maintained on C8 I medium containing the following components (mg/l): NH_4NO_3, 1000; NH_4 citrate, 100; urea, 100; $MgSO_4 \cdot 7H_2O$, 250; $CaCl_2 \cdot 2H_2O$, 400; KH_2PO_4, 100; $NaHCO_3$, 150; micronutrients as in B5 medium (Gamborg and Eve-

leigh 1968): nicotinic acid, 1; thiamine-HCl, 10; pyridoxine-HCl, 1; m-inositol, 200; folic acid, 1; N-Z Amine type A, 250; yeast extract, 100; sucrose, 20000; glucose, 10000; 2,4-dichlorophenoxyacetic acid, 0.55; naphthaleneacetic acid, 0.9; final pH:5.6. The embryogenic capacity of the albino carrot cell lines was tested in hormone-free medium.

The seeds of *D.capillifolius* and *D.carota* × *D.capillifolius* sexual hybrid (2n = 18) were kindly provided by Dr. G.D. McCollum (U.S. Department of Agriculture, Plant Genetics and Germplasm Institute, Beltsville, Maryland, U.S.A.). Cell cultures of *D.capillifolius* were initiated from callus tissue. The cells were cultured in B5 medium (Gamborg and Eveleigh 1968) containing 1 mg/l 2,4-dichlorophenoxyacetic acid.

The isolation procedure for protoplasts from the cultured cells was the same as described previously (Dudits et al. 1976). Fusion of protoplasts was induced by polyethylene glycol (PEG Mw. 1540) according to methods described by Kao and Michayluk (1974). The PEG treated protoplasts were cultured in C8 IV medium prepared from C8 I medium with the following supplements (mg/l): L-glycine, 10; L-glutamine, 100; L-tryptophan, 10; L-cysteine, 10; L-methionine, 5; choline, 10; coconut water, 10 ml. The osmotic stabilizer was glucose (0.38 M). Naphthaleneacetic acid (0.18 mg/l) and zeatin (0.11 mg/l) were used as plant hormones.

After three weeks of culturing, small embryos and calluses had developed and were transferred from plastic petri dishes to flasks and cultured in C8 I medium lacking plant hormones.

Green, young leaves of *D.carota*, *D.capillifolius* and of somatic hybrids grown under controlled conditions were used in electrophoretic studies. For enzyme preparation the following two methods were used:

1) One-gram samples of leaves were homogenized in 2 ml of cold sucrose solution (12.5%) with DTT (0.14 mg/ml) according to Honold et al. (1966). The extract was centrifuged at 5000 g for 10 minutes and the supernatants were centrifuged at 41 300 g for 1 hour at 4°C.

2) One gram of leaves was homogenized in acetone (Srivastava and Van Huystee 1973) and was centrifuged at 5000 g for 10 minutes. The precipitate obtained on centrifugation was dissolved in Tris-citrate buffer pH = 8,3 with 17% of sucrose and 0.1% of ascorbic acid and 0.1% of cysteine (Fric 1971). After centrifugation the supernatants were centrifuged at 41 300 g for 1 hour at 4°C. (Sorvall sprespeed RC2-P automatic refrigerated centrifuge).

Prior to electrophoresis the samples were stored at -10°C. Anodic separation was conducted according to the procedures described by Davis (1964). Gel columns were composed of 7% (w/v) polyacrilamide in the separation section and 2.5% (w/v) polyacrilamide in the spacer and sample section. After electrophoresis at 4°C, o-dianisidine was used for detection of peroxidases (Mac Donald and Smith 1972).

Results

In recent experiments, the selection of somatic hybrids has been based on the restoration of photosynthetic function in albino cells after interspecific protoplast fusion. Green plantlets were selected and subsequently characterized. Despite an inhibitory effect of C8 IV culture medium on division and embryogenesis in *D.capillifolius* cells, green *D.capillifolius* plants could also develop from protoplast cultures. Therefore it was necessary to distinguish the green somatic hybrids from *D.capillifolius* plants. Identification of regenerated plants was carried out on the basis of differences in the leaf morphology. The interspecific fusion products resulted in green plants with leaves which were intermediate between those of the parents (Figs.1, 2). The intermediate leaf shape was also characteristic of the diploid F_1 generation of sexual hybrids (McCollum 1975).

As another morphological feature, differences were found in the presence or absence of, and in the shape of, leaf hairs. Unicellular, pointed hairs could be seen at the apices of the leaflets of *D.carota* and the main leaflets of *D.capillifolius* lack apical hairs. Hairs were present at the apices and edges of the leaflet of somatic hybrids and were similar to those of *D.carota*.

The characteristic root morphology of the fusion partners and somatic hybrids is shown on Fig.3. The plants of orange-rooted cultivar, 'Nantaise Slendero' have fleshy roots. *D.capillifolius* is recognizable by its long, white roots. The somatic hybrids produced long, white and fleshy roots.

The stability of the albino mutant phenotype was tested in cell and protoplast cultures. In control experiments, 7×10^6 protoplasts of an albino cell line were induced to fuse by PEG treatment (Table 1). 8×10^3 calluses, embryos and plantlets were regenerated from these cultures without the appearance of a single green revertant. After interspecific fusion, the number of green calluses and plantlets were 102 from 1.4×10^6 protoplasts. 65 plants proved to be somatic hybrids on the basis of leaf characteristics.

The diploid chromosome number is 18 for *D.carota*. The basic chromosome number of *D.capillifolius* has not been previously counted. In our experiments, the diploid chromosome number of *D.capillifolius* proved to be 18 in root meristem and cultured cells. Results of the cytological analyses of 12 somatic hybrids showed that the chromosome number ranged from 34 to 54. The most common number was 2n = 36 in the root tips of tested plants.

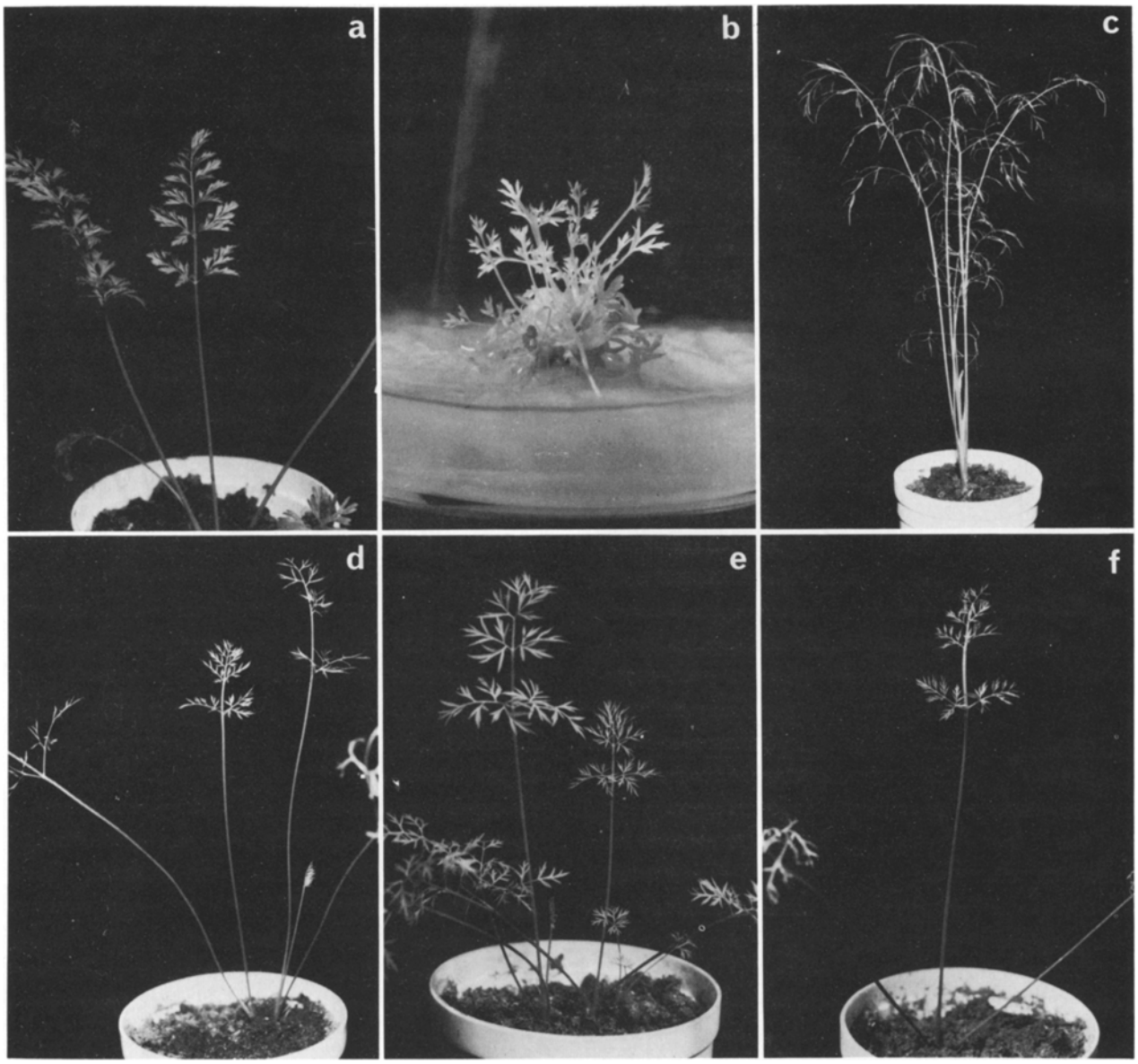

Fig.1a-f. Plants of parental species and somatic hybrids. a. *D.carota*, variety 'Nantaise Slendero b. *D.carota* albino mutant grown on culture medium, c. *D.capillifolius*, d. Somatic hybrid plant (2n = 36), e. Somatic hybrid plant (2n = 34), f. Somatic hybrid plant (2n = 36)

The addition of the two chromosome sets should give the 36 chromosomes which were determined in 5 selected plants. Hybrids with 34 chromosomes (2 plants), 35 chromosomes (1 plant), 35 chromosomes + 1 dot-like chromosome or fragment (2 plants) were also found. The 54 chromosomes in root cells of two hybrids suggest that these plants originated from triple fusion of protoplasts.

The anionic isoenzyme patterns for peroxidase appeared to be different for the two parental species (Fig.4). One slow band (R_f = 0.17) was identified in leaves of *D.carota* which was never observed in *D.capillifolius* leaves. The same band (R_f = 0.17) was found in leaves of a somatic hybrid plant. Both of the applied methods of enzyme preparation resulted in the same isoenzyme pattern.

The presented data for leaf morphology, leaf hairs and isoenzyme patterns indicate that the selected green plants originated from interspecific protoplast fusion. The presence of a *D.carota* isoenzyme band

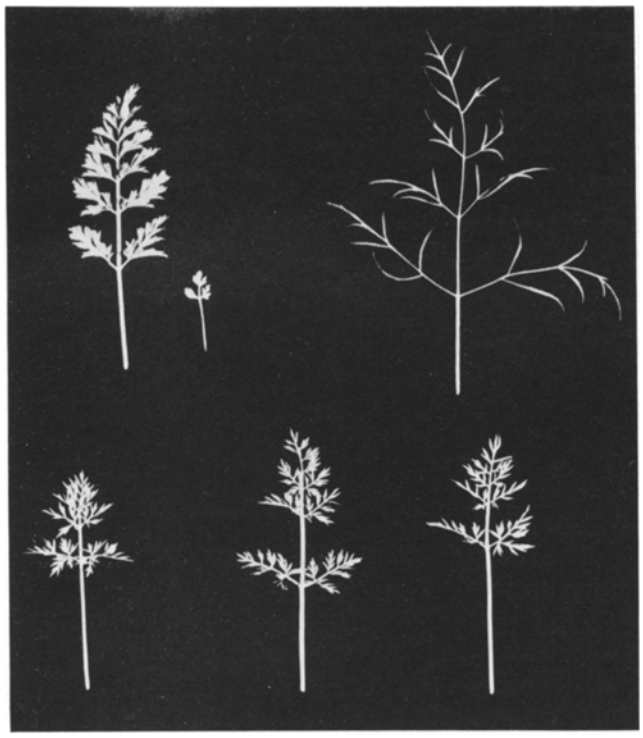

Fig.2. Leaf morphology. Top (left to right): Leaves of *D.carota*, *D.carota* albino mutant, *D.capillifolius* Bottom: leaves of different somatic hybrids

in the somatic hybrid is further evidence that the selected green plants could not have developed from homokaryon fusion of *D.capillifolius* protoplasts.

Discussion

The presented data show that the somatic hybrid plants betwenn *D.carota* and *D.capillifolius* were intermediate between the parents in overall appearance. The intermediate character was especially pronounced in leaf shape. The genes determining the long, white roots in *D.capillifolius* were found to be dominant in somatic hybrids. The observed variation in morphological traits could be correlated with the differing chromosome numbers.

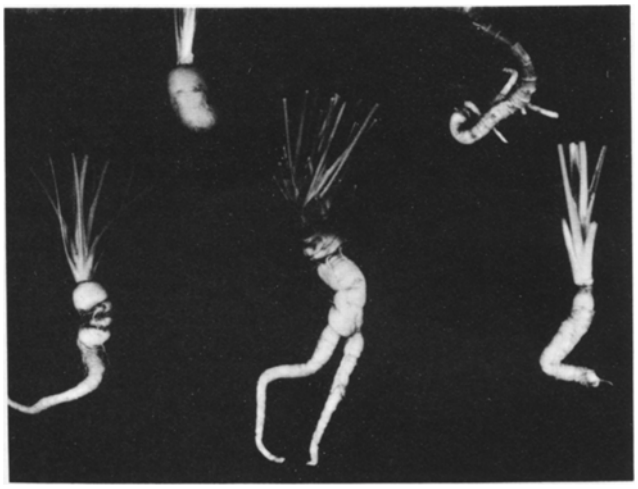

Fig.3. Root morphology. Top: Root of *D.carota* (left) and *D.capillifolius* (right). Bottom: roots of different somatic hybrids

Chromosome counts in the present work, in addition to those in earlier reports, frequently deviated from the expected values. Despite the fact that 42 chromosomes were counted in young leaves of regenerated plants after fusion of *N.glauca* and *N.langsdorffii* protoplasts (Carlson et al. 1972), the parasexual hybrids produced later by PEG treatment were found to have unusual chromosome numbers ranging from

Table 1. Number of regenerated green plantlets and calluses after intraspecific protoplast fusion of albino *D.carota*, and interspecific fusion between *D.carota* and *D.capillifolius*

Fusion experiments	Protoplast number	Calluses Embryos Plantlets	Green Calluses Plantlets	Somatic hybrid plants*
D.carota (albino) × *D.carota* (albino)	7×10^6	8×10^3	0	-
D.carota (albino) × *D.capillifolius* (green)	7.2×10^5 / 7.2×10^5	10^3	102	65

* The somatic hybrids plants were identified on the basis of leaf characteristics

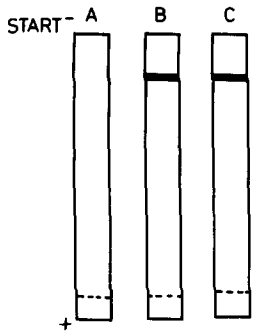

Fig.4. Diagram of poly acrylamide gel electrophoresis of peroxidase isoenzymes from leaves of *D.capillifolius* (A) somatic hybrid (B) and *D.carota* (C)

56 to 64 instead of the amphiploid number of 42 (Smith et al. 1976). Somatic hybrids with higher ploidy level were also produced in tobacco (Melchers and Labi b 1974). Fewer chromosomes than the amphiploid chromosome number were also revealed in other cases (Melchers and Labib 1974; Melchers and Sácristán 1977; Power et al. 1967).

In somatic hybrids between *D.carota* and *D.capillifolius* the departures from the expected chromosome number could be attributed to various causes:

1. different, abnormal chromosome numbers in the cell cultures used for protoplast isolation

2. loss of chromosomes during development of hybrids

3. the involvement of more than two protoplasts in the formation of the fusion product.

The embryogenic cell line of albino carrot mutant offers a potential experimental system for genetic analysis of somatic hybrids produced by protoplast fusion. This albino mutant appeared in M_2 generation and the whole seedling showed a completely white phenotype. These facts indicate that the albino character is possibly due to a nuclear gene mutation. In the somatic hybrids with mostly amphidiploid chromosome number, the *D.capillifolius* genome is dominant and the mutation in the carrot nucleus is recessive. However, *D.capillifolius* genes alone can also be responsible for the development of normal green chloroplasts.

The complementation of two recessive, non-allelic genes was demonstrated in somatic hybrids between chlorophyll deficient varieties of *Nicotiana tabacum* and between one of these varieties and a chlorophyll deficient mutant of *N.sylvestris* (Melchers and Labib 1974; Melchers 1976). The fusion of protoplasts isolated from a plastid albino mutant in one variety and a semidominant yellow gene mutant resulted in intraspecific hybrid plants of *N.tabacum* (Gleba et al. 1975). Complementation between nutritional auxotrophic mutants of the liverwort *Sphaerocarpos donnellii* was used for selection of fusion products (Schieder 1975).

In somatic hybridisation between the albino *D.carota* and the green *D.capillifolius* further studies are required in order to determine the basis of genetic control in chloroplast development.

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