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Production of somatic hybrids between *Daucus carota* L. and *Nicotiana tabacum*

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Abstract Protoplasts of a kanamycin-resistant (KR, nuclear genome), streptomycin-resistant (SR, chloroplast genome) and chlorophyll-deficient (Al, nuclear genome) *Nicotiana tabacum* (KR-SA) cell suspension cultures or X-ray-irradiated mesophyll protoplasts of kanamycin- and streptomycin-resistant green plants (KR-SR) were fused with protoplasts of a cytoplasmic male-sterile (CMS) *Daucus carota* L. cell suspension cultures by electrofusion. Somatic hybrid plants were selected for kanamycin resistance and the ability to produce chlorophyll. Most of the regenerated plants had a normal *D. carota* morphology. Callus induced from these plants possessed 23–32 chromosomes, a number lower than the combined chromosome number (66) of the parents, and were resistant to kanamycin, but they segregated for streptomycin resistance, which indicated that *N. tabacum* chloroplasts had been eliminated. Genomic DNA from several regenerated plants was analyzed by Southern hybridization for the presence of the neomycin phosphotransferase gene (*NPT*II); all of the plants analyzed were found to contain this gene. Mitochondrial (mt) DNA was analyzed by Southern hybridization of restriction endonuclease digests of mtDNA with two DNA probes, *PKT5* and *cox*II. The results showed that the two plants analyzed possessed the mitochondria of *D. carota*. These results demonstrate that the regenerated plants are interfamilial somatic hybrids.

Key words Protoplast · Fusion · *Nicotiana tabacum* · *Daucus carota* · Interfamilial hybridization

Introduction

As somatic hybridization by protoplast fusion provides the possibility of combining genetic factors of sexually incompatible species, it is a useful method for increasing genetic variability in higher plants. Also, somatic hybrids are useful for studying cytoplasmic inheritance and rearrangements of cytoplasmic genomes after protoplast fusion. Many intra- and interspecific somatic hybrids and several inter-generic somatic hybrids have been reported (Aviv et al. 1980; Toriyama et al. 1978, 1987; Pental et al. 1986; Negrutiu et al. 1986; Sjödin and Glimelius 1989; Kameya et al. 1989; Sihachaker et al. 1989; Perl et al. 1991; Toki and Kameya 1990; Christey et al. 1991; Sproule et al. 1991). However, fusion products between remote species generally eliminate spontaneously one of the parental genomes.

In recent years one of the methods used to achieve partial genome or cytoplasm transfer has been to irradiate the donor species with a high dose of X- or gamma-rays (Dudits et al. 1987; Imamura et al. 1987; Bates et al. 1987; Gleba et al. 1988; Yamashita et al. 1989; Hinnisdaels et al. 1991; Itoh et al. 1991; Melzer et al. 1992). Dominant selectable markers have been used to obtain asymmetric somatic hybrids (Bates et al. 1987; Dudits et al. 1987). Although many somatic hybrids and cybrids have been described, few were hybrids or cybrids of remote species, such as between different families (Dudits et al. 1987). In our investigation of the nuclear and cytoplasmic traits in somatic hybrids between remote species, we have produced somatic hybrids between kanamycin-resistant (KR), streptomycin-resistant (SR) and chlorophyll-deficient (A1) *N. tabacum* (KR-SA) and cytoplasmic male-sterile (CMS) *D. carota*. Regenerated plants were analyzed at the biochemical, molecular and cytological levels.

Materials and methods

Plant and cell materials

Chlorophyll-deficient, kanamycin- and streptomycin-resistant *N. tabacum* (KR-SA) was produced as previously described (Toki et al. 1990). Kanamycin- and streptomycin-resistant green plants (KR-SR) of *N. tabacum* were maintained as axenic shoot cultures on Medium A [MS medium (Murashige and Skoog 1962) solidified with 0.8% agar] under continuous fluorescent light (4 W/m^2) at 25 °C. Callus of KR-SA was induced from surface-sterilized leaves on Medium B (Medium A plus 1 mg/l 2,4-D and 0.1 mg/l kinetin). Callus formed from the leaves was transferred to liquid Medium B (Medium B minus agar) and sub-cultured every 2 weeks.

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Callus of cytoplasmic male-sterile *D. carota* (Kanzaki et al. 1991) was induced by the same method in KR-SA and maintained in liquid MS Medium B.

Protoplast isolation, irradiation and fusion

Protoplasts of *N. tabacum* (KR-SR) were isolated from leaves by incubation in an enzyme solution containing 1.6% (w/v) Cellulase Onozuka R10 (Yakult Biochemicals), 0.3% (w/v) Macerozyme R10 (Yakult Biochemicals), 8% (w/v) mannitol and 0.1% (w/v) $CaCl_2 \cdot 2H_2O$ (pH 5.5) for 3 h at 25 °C. Protoplasts of *N. tabacum* (KR-SA) were isolated from 8-month-old cell suspension cultures, and protoplasts of *D. carota* were isolated from 1-month-old cell suspension cultures by incubation under the same conditions.

Protoplasts were filtered through a 50-μm sieve and washed twice with washing solution containing 8% (w/v) mannitol and 0.1% (w/v) $CaCl_2 \cdot 2H_2O$ with centrifugation at 80 *g* for 5 min. The protoplasts were purified by floating them in a 25% (w/v) sucrose solution followed by centrifugation at 80 *g* for 5 min. They were brought to a density of 5×10^5/ml in washing solution. The protoplasts isolated from *N. tabacum* (KR-SR) were irradiated with either 7 Krad or 10 Krad of X-rays (Fig. 1a) before cell fusion. After irradiation the protoplasts were washed once in the washing solution.

The two protoplast populations were mixed in equal proportions to give a total population density of 5×10^3/ml and fused by electrofusion using a BTX ELECTRO CELL MANIPULATOR 200.

Protoplast culture, hybrid selection and plant regeneration

After fusion the protoplasts were diluted with an equal volume of Gelrite solution (0.30% Gelrite, 3% sucrose, 5% glucose) and then distributed in plastic petri dishes containing Medium C (Table 1). During the first 2 weeks of culture, petri dishes were incubated in the dark at 25 °C (Fig. 3a). The cell suspensions were subsequently diluted in selection medium (Medium D, Table 1 (Fig. 1b)) and transferred to continuous light (4 W/m^2) at 25 °C (Fig. 3b). After 1 month of culture, visible callus was transferred to a solid selection medium (Medium E, Table 1 (Fig. 1b, Fig. 3c)); Subsequently, colonies about 1–2 mm in diameter were transferred to shooting media (Media F, G, Table 1 (Fig. 3D)). Regenerated shoots were transferred to a rooting medium (Medium G, Table 1 (Fig. 3E)).

Kanamycin or streptomycin sensitivity of regenerated plants

The calli of regenerated hybrids were induced from leaves onMedium B, then transferred to the same medium containing 50 mg/l kanamycin sulfate. Subsequently, suspension callus of regenerated hybrids were cultured in liquid Medium B (Medium B minus agar) containing 50 mg/l kanamycin sulfate or 300 mg/l streptomycin sulfate.

Cytological analysis

For chromosome analysis actively growing cell suspension cultures established from calli of regenerated plants were treated with 2 m*M* 8-quinolinol for 3 h and fixed with methanol-glacial acetic acid (3:1, v/v) for 1 h. The cells were squashed and then stained with 0.8% Giemsa, and chromosomes were counted in about 20 cells.

Analysis of DNA

Plant DNA was prepared from leaves of *D. carota*, *N. tabacum* and regenerated plants digested with *Pst*I. Southern hybridization was carried out using the digoxigenin-labeled DNA fragment of *NPT*II as the probe.

Table 1 Composition of culture media[a] (*MS* Murashige and Skoog (1962) formulation, *2,4-D* 2,4-dichlorophenoxyacetic acid, *NAA* naphthaleneacetic acid, *BAP* benzylaminopurine)

Addenda	Medium C	Medium D	Medium E	Medium F	Medium G
Mineral Salts	MS	MS	MS	MS	MS
Vitamins	MS	MS	MS	MS	MS
Sucrose (w/v)	3%	3%	3%	3%	3%
Glucose (w/v)	5%				
2,4-D	1.0 mg/l	1.0 mg/l	1.0 mg/l		
NAA				0.1 mg/l	
BAP				1.0 mg/l	
Kinetin	0.5 mg/l	0.5 mg/l	0.5 mg/l		
Kanamycin sulfate		50.0 mg/l	50.0 mg/l		
Agar (w/v)			0.8%	0.8%	0.8%

[a] pH was adjusted to 5.8

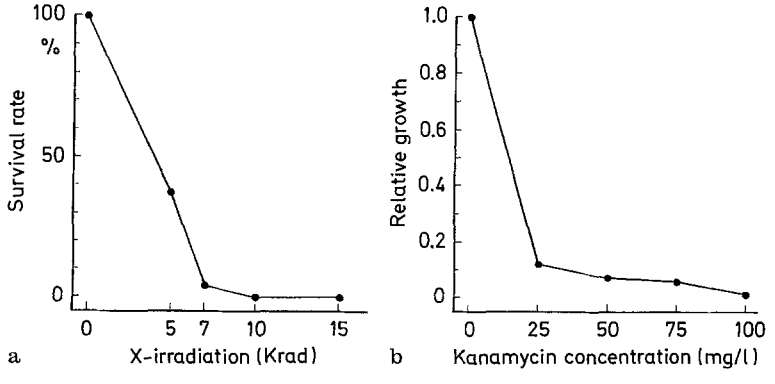

Fig. 1a, b Establishment of selective conditions to obtain somatic hybrids. **a** Effect of X-irradiation dosage on cell division of *N. tabacum* protoplasts. **b** Effect of kanamycin concentration on cell growth of *D. carota* suspension cultures. These results were measured after 2 weeks of culture

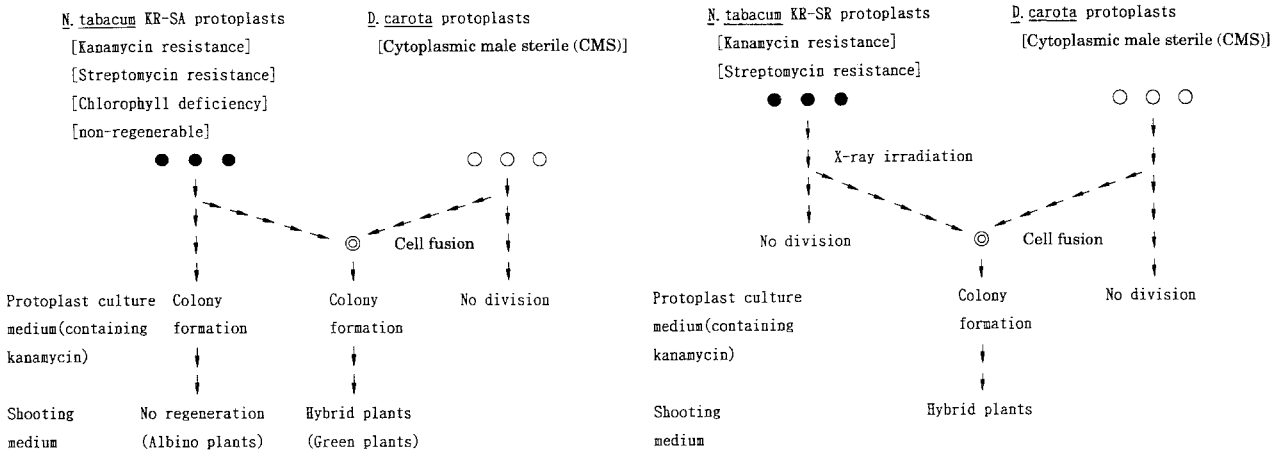

Fig. 2 Selection scheme for hybrid plants between *N. tabacum* and *D. carota*. *Left scheme* was KR-SA and *D. carota*; *right scheme* was KR-SR and *D. carota*

For mitochondrial (mt) DNA extraction, suspension calli of *D. carota*, *N. tabacum* and two regenerated plants were used. MtDNA extraction and purification were carried out by the modified methods of Kemble (1987) from 10 *g* fresh weight of the cell. The DNA fragment containing *PKT5*, a 2.0-kb *Hin*dIII fragment, isolated from CMS carrot mitochondria (Kanzaki et al. 1991) and pea *cox*II were used as probes for Southern hybridization.

Results and discussion

We have successfully produced somatic hybrid plants between *D. carota* and *N. tabacum* using the selection scheme in Fig. 2. The plants regenerated had a morphology closely resembling that of *D. carota* morphology (Fig. 3F, G). The result of nuclear DNA analysis by Southern hybridization with the *NPT*II gene as probe indicated that the plants had the *NPT*II gene (Fig. 4). Furthermore, calli induced from regenerated plants were resistant to kanamycin sulfate (Table 2, Fig. 3H). These results suggest that the regenerated plants were somatic hybrids between *D. carota* and *N. tabacum*. The somatic hybrids possessed 23–32 chromosomes; this is fewer than the additive chromosome number of 66 of the parents (Table 2). It seemed that chromosomes of *N. tabacum* were eliminated from regenerated plants during culture, which indicates that it is necessary to eliminate many chromosomes for regeneration from callus and that 23–32 chromosomes are the limiting numbers for regeneration in the interaction between the *N. tabacum* and *D. carota* genomes. Since many chromosomes were eliminated, the chromosome number may be shifted further until these chromosomes maintain stability. We suggest that further investigation of chromosome num-bers over a longer period of time is needed.

When calli induced from regenerated plants were cultured on streptomycin medium, some calli showed resistance while others did not (Table 2). This result suggests that several cell lines contained the chloroplasts of *N. tabacum* and that segregation of chloroplasts occurred. Although all of the calli induced from the regenerated plants were resistant to kanamycin, only three cell lines were resistant to streptomycin. As kanamycin was used in the present selection system

Table 2 Expression of resistance markers and chromosome number in regenerated plants

Selected lines	Clone number	Resistance[a]		Chromosome number
		Kanamycin (50 mg/l)	Streptomycin (300 mg/l)	
	H 1	+ +	+ +	23.5 ± 1.6
KR-SR	H 2	+ +	+ +	25.5 ± 2.4
(7 Krad, leaf)	H 3	−	−	−
+	H 4	+ + +	+ + +	32.0 ± 11.2
D. carota	H 5	−	−	−
(CMS,	H 6	+ +	0	29.4 ± 5.4
suspension)	H 7	+ +	0	26.0 ± 2.3
KR − SA	H 8	+ +	0	28.3 ± 1.9
(suspension)	H 9	+ +	0	26.0 ± 2.0
+	H 10	−	−	−
D. carota	H 11	+ +	0	27.7 ± 1.8
KR-SR	H 12	−	−	25.3 ± 2.9
(10 Krad)	H 13	−	−	24.2 ± 2.0
+ *D. carota*				

[a] + + +, 80–100% callus relative growth; + +, 50–80% callus relative growth; +, 30–50% callus relative growth in the presence of inhibitor; 0, no growth; −, not tested

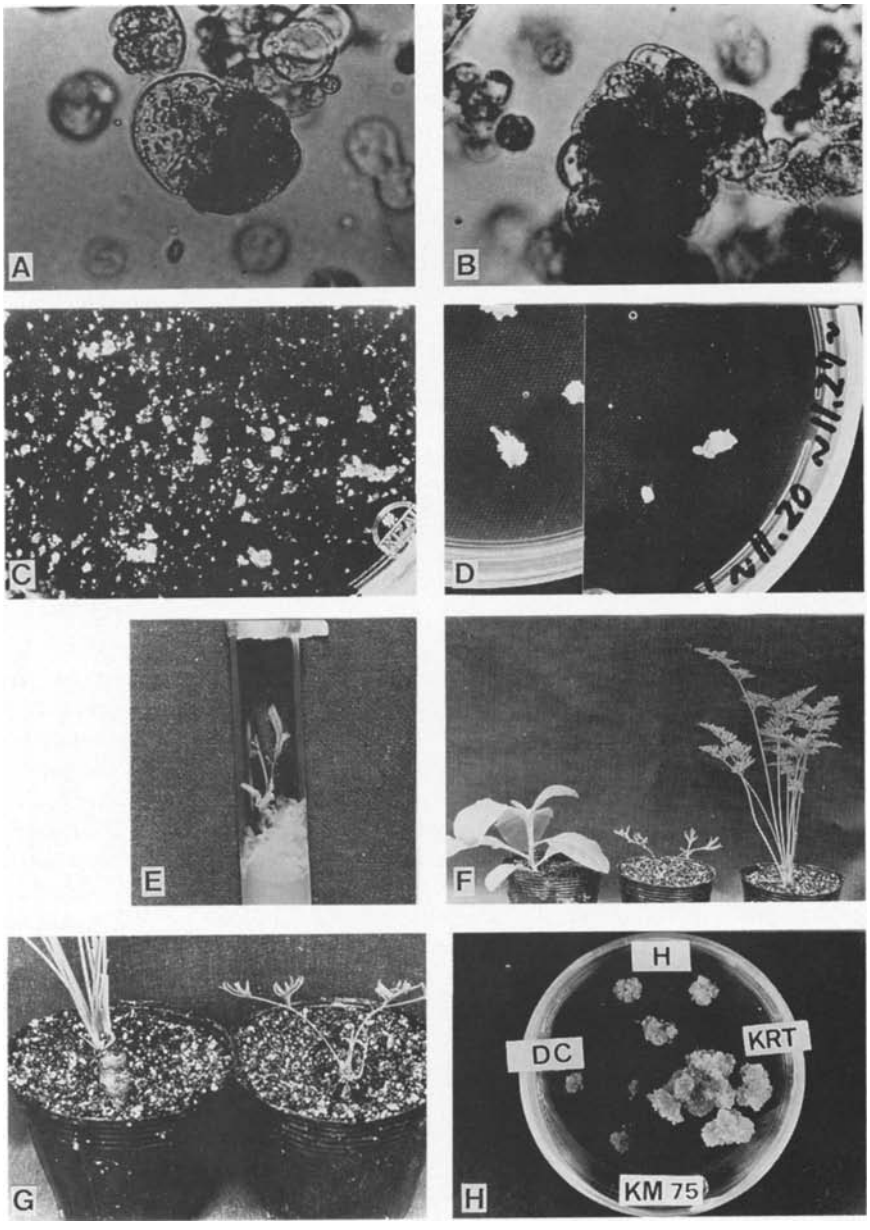

Fig. 3A–H **A, B** Multicellular microcalli after 2 weeks and 1 month of culture, **C** cell colonies after 2 months of culture on selection medium, **D** regeneration of shoots from selected callus, **E** regenerated plant, **F** (*left to right*) young plants of *N. tabacum*, a somatic hybrid and *D. carota*, **G** (*left to right*) roots of *D. carota* and of a somatic hybrid, **H** kanamycin-resistant test of *N. tabacum* (*KRT*), *D. carota* (*DC*) and a somatic hybrid (*H*). Concentration of kanamycin is 75 mg/l

while streptomycin was not, it may be suggested that positive selection is necessary for stabilizing resistance. MtDNA recombination (Young et al. 1987; Morgan and Maliga 1987; Tanno-Suenaga et al. 1988; Smith et al. 1989; Takamizo et al. 1991; Perl et al. 1991) and lack of mtDNA recombination (Yarrow et al. 1990; Christey et al. 1991) have been reported. Kao et al. (1992) reported that some regenerated plants had recombination mtDNA and others did not. Two regenerated plants which were resistant to streptomycin were used for mtDNA analysis. From the results of Southern hybridization, these plants could be seen to contain only *D. carota* mtDNA fragments (Fig. 5). In restriction patterns of mtDNA, regenerated plants showed the same restriction profile as the *D. carota* parent. These results indicate that recombinant mitochondrial genomes were not generated during protoplast fusion. Cytoplasmic male sterility has usually been used as a fusion marker (Belliard et al. 1978; Aviv et al. 1980; Menczel et al. 1983, 1987; Young et al. 1987; Barsby et al. 1987; Tanno-Suenaga et al. 1988; Christey et al. 1991). In our experiment, Southern hybridization with the *PKT5* probe was used to determine that regenerated hybrid plants also had CMS DNA.

We have used cell suspension cultures induced from chlorophyll-deficient *N. tabacum* plants in this experiment, but all of the regenerated plants were green plants. Chlorophyll-deficiency is due to a recessive gene in the nuclear genome. Perhaps these results demonstrate that the nuclear genome concerned with chlorophyll synthesis is common throughout the family.

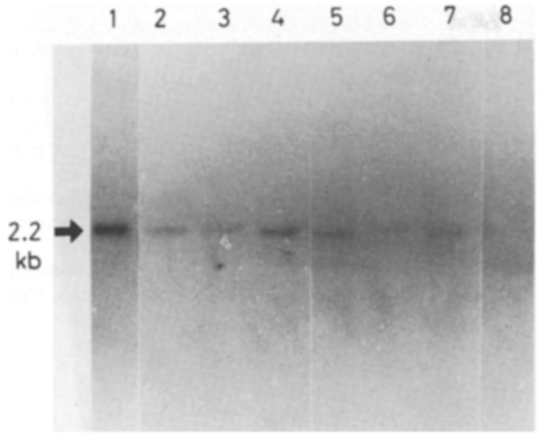

Fig. 4 Southern blot showing the presence of the neomycin phosphotransferase gene (*NPT*II, kanamycin resistance gene). Genomic DNA was digested with *Pst*I and the *NPT*II gene probe: *lane 1 N. tabacum, lanes 2–7* hybrid plants (no. H.2, 4, 6, 8, 11, 13), *lane 8 D. carota*

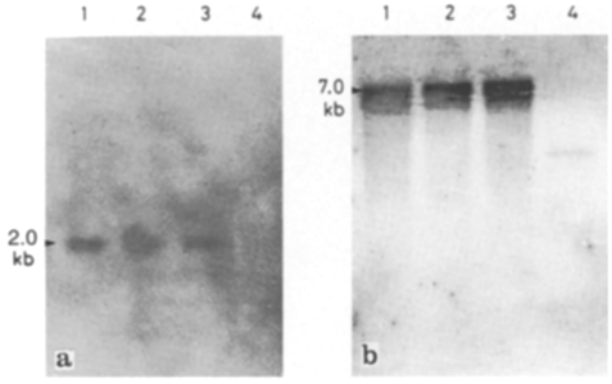

Fig. 5a, b Southern blot hybridization of mitochondrial DNA (mtDNA) from *D. carota, N. tabacum* and their hybrids. **a** MtDNA was digested with *Hin*dIII and the *PKT5* gene probe. **b** MtDNA was digested with *Hin*dIII and the *cox*I gene probe: *lane 1 D. carota, lanes 2–3* hybrid plants (H.1 and H.4), *lane 4 N. tabacum*

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