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Analysis of chloroplast and mitochondrial DNAs in asymmetric somatic hybrids between tobacco and carrot

M. A. Smith*, A. Pay and D. Dudits**

Institute of Plant Physiology and Genetics, Biological Research Center, Hungarian Academy of Sciences, 6701 Szeged, P.O. Box 521, Hungary

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Summary. Chloroplast and mitochondrial DNAs have been examined by comparison of restriction enzyme patterns in asymmetric hybrid plants, resulting from the fusion between leaf mesophyll protoplasts of *Nicotiana tabacum* (*Solanaceae*) and irradiated cell culture protoplasts of *Daucus carota* (*Umbellifereae*). These somatic hybrids with normal tobacco morphology were selected as a consequence of the transfer of methotrexate and 5-methyltryptophan resistance from carrot to tobacco. The restriction patterns of chloroplast DNAs in somatic hybrids were indistinguishable from the tobacco parent. However, we found somatic hybrids with mitochondrial DNA significantly different from either parent, as judged by analysis of fragment distribution after restriction enzyme digestion. The possible formation of altered mitochondrial DNA molecules as the result of parasexual hybrid production between two phylogenetically highly divergent plant species will be discussed.

Key words: Somatic hybridization – Carrot – Tobacco – Mitochondrial DNA – Chloroplast DNA

Introduction

Production of heteroplasmic cells through fusion of plant protoplasts permits the construction of unique combinations of cytoplasmic traits. The results of extensive studies on organelle composition of various somatic hybrids have been recently reviewed by several authors (Galun and Aviv 1983; Izhar and Zelcer 1986; Pelletier 1987; Pelletier et al. 1988). In sofar as the chloroplast genome is concerned, the majority of somatic hybrids showed extensive segregation of parental chloroplasts (reviewed by Maliga and Menczel 1986). After interspecific protoplast fusion, *Nicotiana* cybrids with *Petunia* chloroplasts were also produced (Glimelius and Bonett 1986).

Furthermore, two *Solanaceae* species (*Nicotiana-Salpignosis*) were successfully used for protoplast-fusion-mediated chloroplast transfer (Thanh et al. 1988). Characterization of mitochondrial DNAs from somatic hybrids between species within genera, such as *Nicotiana, Petunia, Brassica* and *Daucus,* clearly revealed novel patterns of mitochondrial DNA restriction fragments indicating possible recombination of mitochondrial genomes (Belliard et al. 1979; Nagy et al. 1981; Boeshore et al. 1983; Chetrit et al. 1985; Matthews and Widholm 1985; Kemble et al. 1986). Because of the limitations in generating somatic hybrids between phylogenetically distant species, there is no experimental data on the behaviour of organelles in wide somatic hybrids. Recently the somatic incompatibility between tobacco and carrot has been overcome by irradiation-induced genomic instability. Asymmetric hybrids exhibiting tobacco morphology with coexpression and independent segregation of carrot-specific resistance traits were regenerated (Dudits et al. 1987). These hybrid plants offer unique experimental material to analyse the fate of parental organelles after fusion of protoplasts of two non-related species. Here we present data about the restriction fragment pattern in somatic hybrids which showed the presence of tobacco chloroplasts and formation of altered mitochondrial DNA in hybrid plants.

* Permanent address: Graduate Section of Biochemistry, Brigham Young University, Provo, Utah 84602, USA
** To whom correspondence should be addressed

Materials and methods

Plant materials

Described Dudits et al. (1987) the selection and regeneration of asymmetric somatic hybrids (NICA) after fusion of leaf protoplasts of tobacco and irradiated cell suspension protoplasts of carrot. The control SR1 tobacco plants and the NICA somatic hybrids were grown either in a greenhouse or were maintained aseptically on MS-P medium (Maliga 1984). Suspension cultures of carrot cell-line H47 were maintained on MS medium (Murashige and Skoog 1962) supplemented with 2.2 m*M* 2,4-dichlorophenoxyacetic acid, 0.01 m*M* methotrexate and 0.1 m*M* 5-methyltryptophan.

Organelle isolation

Organelles were isolated from protoplasts, leaf tissues or suspension cultures. SR1 tobacco and NICA protoplasts were isolated by standard procedures (Marton 1984). Carrot protoplasts (H47 line) were prepared from cell suspension cultures incubated with enzyme solution (Dudits 1984). Protoplasts were collected by centrifugation (2,000 rpm, 5 min) in a clinical centrifuge, resuspended in about 20 ml MOPS buffer (MOPS 30 m*M*, pH 7.2; Sorbitol 300 m*M*, EDTA 3 m*M*, 2-mercaptoethanol 8 m*M*, BSA 0.2%), and ruptured by two or three passages through 20 μm nylon mesh. Isolation of organelles from leaf tissues was the same as we described (Pay and Smith 1988).

Cells from suspension culture (15–25 g) were collected by filtration on 74 μm nylon mesh (Tetko, NY) and washed by resuspension in 3–4 volumes (ml/g fresh weight) of MOPS homogenizator buffer. Suspension culture tissue was again recovered by filtration over nylon and macerated as a moist paste with a pre-cooled mortar and pestle, in the presence of a few grams of quartz sand. Maceration was interrupted several times in order to remove organelles from larger tissue fragments. This was accomplished by resuspending the paste-like homogenate in the MOPS buffer filtrate, obtained during the washing step described above, and filtering over nylon mesh (20 μm). Tissue which did not pass through the filter was returned to the mortar for additional maceration. After repeating the cycle two or three times, using the same MOPS buffer filtrate for organelle removal, most of the suspension culture tissue passed through the 20 micron filter. The filtrate was centrifuged (1,475 × g, 10 min, Sorvall SS-34 rotor) and the supernatant was centrifuged (17,300 × g, 10 min, SS-34 rotor) to obtain the crude mitochondrial fraction. The resulting pellet was resuspended with a small camel hair brush in 10%–20% of the original homogenation volume of fresh MOPS buffer, and recentrifuged (1,300 × g, 10 min, SS-34rotor) to remove large organelles and additional debris. The supernatant fraction was either treated with DNase as described below, or again centrifuged (17,300 × g, 10 min, SS-34 rotor) to recover washed mitochondria and was further purified by isopycnic centrifugation in Percoll as described below.

The final purification of chloroplast and mitochondrial fractions was carried out by isopycnic Percoll centrifugation (Pay and Smith 1988).

Alternatively, chloroplast or mitochondrial fractions obtained by classical differential centrifugation techniques, as described above, were treated with DNase (Bland et al. 1985) as follows. The $MgCl_2$ concentration of the crude mitochondrial fraction, suspended in fresh MOPS buffer, was adjusted to 10 m*M*, DNase was added to 50 μg/ml and the resulting mixture was incubated at 4 °C for 30 min. EDTA was then added to 20 m*M* and the entire mitochondrial solution was underlayered with 3–4 volumes of buffer B (50 m*M* Tris, pH 8; 300 m*M* sucrose; and 20 m*M* EDTA) and centrifuged (17,300 × g, 10 min, SS-34 rotor). All procedures were carried out at 4 °C unless otherwise stated.

Organelle DNA isolation

Organelles recovered from Percoll gradients, or following DNase treatment as described above, were washed in about 5 ml of NET buffer (NaCl 150 m*M*, Tris-HCl 40 *M*, pH 7.9) and were recovered by centrifugation (12,000 × g, 3 min). Washed organelles were suspended in 0.5–1.5 ml NET buffer containing 2% sarkosyl and 50 μg/ml Proteinase K and incubated at 37 °C for 30 min, followed by 3 cycles of NET buffer-saturated phenol/chloroform (1:1), and then 3 cycles of chloroform/isoamylalcohol (24:1) extraction. Nucleic acids were precipitated by addition of two volumes of ethanol (−20 °C overnight).

Restriction enzyme analysis

Chloroplast or mitochondrial DNAs purified by phenol-chloroform deproteinization as described above, were digested with restriction endonucleases (8–15 units) according to instruction of the supplier (Boehringer Mannheim), separated by agarose (0.8%) gel electrophoresis (80 mA, 6 h) and stained with ethidium bromide.

Results

Tobacco chloroplasts in NICA (tobacco + carrot) asymmetric hybrids

Several independent hybrid plants were regenerated after fusion of tobacco leaf protoplasts and non-morphologic cell suspension protoplasts of carrot. Fusion origin of selected clones was proved by expression of various nuclear-coded resistance traits from the carrot parent (Dudits et al. 1987). The overall phenotype of these hybrids resembled tobacco, however, in a few cases morphological or developmental abnormalities were detected. Since protoplast fusion facilitated production of heteroplasmic cells, we analyzed the chloroplast population in various NICA regenerants. Restriction endonuclease digestion profiles of chloroplast DNAs generated by agarose gel electrophoresis were used for comparison. As shown by Fig. 1, HindIII digestion of chloroplast DNAs from SR1 tobacco and somatic hybrid (NICA 401) resulted in identical restriction patterns. Similar results were obtained with other hybrids using several restriction enzymes (data not shown). As a reference we show the restriction pattern of chloroplast DNA of carrot plants regenerated from the WOOlc wild carrot cell line, which served as starting material for isolation of mutants used for fusion in the present experiments. Analysis of chloroplasts DNAs from all of the somatic hybrids tested indicated the presence of tobacco chloroplasts in NICA plants.

Novel mitochondrial DNA in NICA somatic hybrids

Among the somatic hybrids which developed flower, various degrees of male sterility were observed. Self-polli-

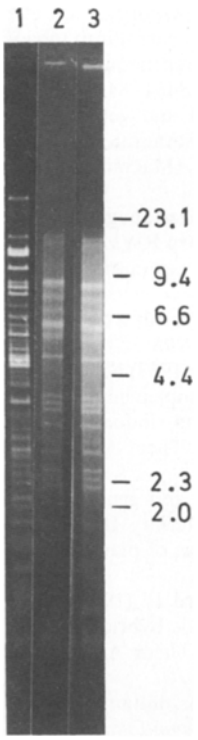

Fig. 1. Agarose gel electrophoresis of HindIII digested parental and hybrid chloroplast DNAs. *Lane 1:* carrot (WOO1c); *lane 2:* tobacco/carrot hybrid (NICA 401); *lane 3:* tobacco (SR1). Lambda HindIII restriction fragments were used for size markers

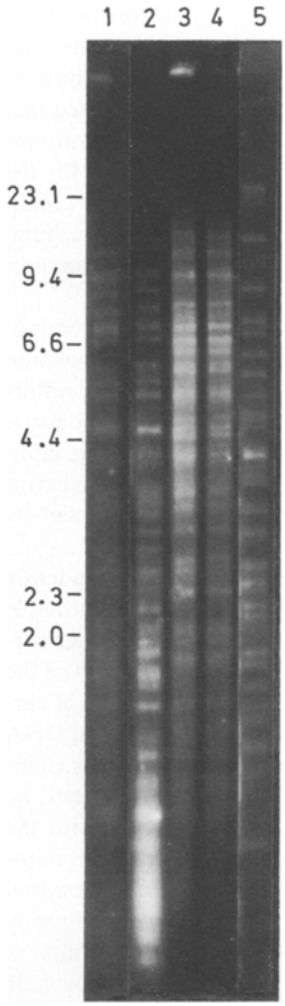

Fig. 2. Agarose gel electrophoresis of HindIII digested parental and hybrid mitochondrial DNAs. *Lane 1:* SR1 tobacco; *lanes 2–4:* tobacco/carrot hybrids (NICA-112-5D, NICA-305 and NICA 105-2, respectively); *lane 5:* carrot H47. Lambda HindIII restriction fragment were used for size markers

nation of hybrid plants grown under greenhouse conditions was prevented by formation of short filaments and abnormal anthers. These plants exhibited female fertility, and seed progeny was obtained after pollination with SR1 parental plants. Mitochondrial DNAs of hybrid plants with male sterile phenotype were characterized by restriction enzyme analysis. Figure 2 presents the distribution of HindIII mtDNA fragments from three somatic hybrids of tobacco and carrot. This comparison shows that whereas NICA 112-5D is indistinguishable from the tobacco parent, the mitochondrial DNAs from NICA 105 and NICA 305 hybrids are significantly different from either tobacco or carrot. It is interesting that these last-mentioned somatic hybrids have similar HindIII (Fig. 2) and BamHI, as well as EcoRI patterns (data not shown).

Discussion

We have previously produced asymmetric hybrid plants between two such phylogenetically distantly related plant species as tobacco and carrot (Dudits et al. 1987). The aim of this work was to introduce carrot specific-resistance markers into tobacco. The resulting hybrids showed tobacco morphology and chromosome number which was significantly lower than predicted by simple addition of parental chromosome. In the present work we analysed the organelle population in random representative of these somatic hybrid plants. Detection of tobacco chloroplasts in all the hybrids analysed is consistent with the dominance of tobacco partner in generation of these hybrids. The bi-directional sorting-out of parental chloroplast types was characteristic for large number of interspecific (Maliga and Menczel 1986), as well as for intergeneric somatic hybrids (Schiller et al. 1982). In the present case, several factors could contribute to the selective disadvantage of carrot chloroplasts in the fused cells as follow: 1. The parental carrot protoplasts were isolated from cell suspension cultures in which the chloroplast development was depressed by plant hormones and sugar content of the culture medium for several years. The parental carrot cells were unable to

differentiate into plants. The primary fusion products contained fully developed tobacco chloroplasts and only proplastids from carrot. 2. The irradiation-induced chromosome instability in the carrot nuclei resulted in a nuclear background with dominance of tobacco chromosomes which could provide selective advantages for the tobacco chloroplasts in the hybrid cells. 3. Culture conditions, such as hormonal composition of the medium, were selected on the basis of the tobacco parent requirement.

According to the restriction pattern of mitochondrial DNAs, we found hybrid plants with novel composition of chondriones. In line with previously reported results (reviewed by Pelletier et al. 1988), altered mitochondrial DNA is consistent with recombination, however, additional factors, for example somaclonal variation in extranuclear DNA should also be considered (discussed by Evans and Sharp 1986).

In the present work, we detected two independently isolated and regenerated hybrids with indistinguishable restriction patterns, each being different from either parent. The existence of 'hot spots' for rearrangement of the mitochondrial genome was suggested by analysis of carrot somatic hybrids having only two mitochondrial DNA restriction patterns in a total of 25 cybrid plants (Ichikawa et al. 1987). On the basis of restriction pattern, we could not correlate the male sterile phenotype with the mitochondrial DNA. Hybrids with tobacco-type mitochondrial DNA and hybrids with new mitochondrial DNA were found to be male sterile. This finding may show multifactoral determination for male sterility in wide somatic hybrids, in contrast to the origin of male sterile interspecific somatic hybrids.

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