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Originals

Analysis of cytoplasmic genomes in somatic hybrids between navel orange (*Citrus sinensis* Osb.) and 'Murcott' tangor

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Summary. Somatic hybrid plants were produced by protoplast fusion of navel orange and 'Murcott' tangor. Hybridity of the plants was confirmed by the restriction endonuclease analysis of nuclear ribosomal DNA. All of the plants (16 clones) were normal, uniform, and had the amphidiploid chromosome number of 36 ($2n=2x=18$ for each parent). The cpDNA analysis showed that each of the 16 somatic hybrids contained either one parental chloroplast genome or the other. In all cases, the mitochondrial genomes of the regenerated somatic hybrids were of the navel orange type.

Key words: Protoplast fusion – Ribosomal DNA – Chloroplast DNA – Mitochondrial DNA – Citrus

Introduction

Protoplast fusion provides an alternative way to produce hybrids from species that cannot be crossbred. In addition, it has been shown that cytoplasmic genetic diversity can be increased by protoplast fusion. Since the first report of successful protoplast fusion in *Citrus* by Ohgawara et al. in 1985, several studies have been done on the production of *Citrus* somatic hybrids (Grosser et al. 1988a, b, 1989, 1990; Kobayashi et al. 1988; Ohgawara et al. 1989) and cybrids (Vardi et al. 1987, 1989).

Determining the composition of cytoplasmic genomes, including organeller DNA recombination in the somatic hybrids/cybrids, is of great interest for the fields of cytoplasmic genetics and citrus breeding. Vardi et al. (1989) analyzed mitochondrial DNA (mtDNA) and chloroplast DNA (cpDNA) in the intergeneric cybrids between *Citrus* and *Microcitrus*. They showed that recombination of mtDNA and complete segregation of chloroplasts occurred in the cybrid plants.

In somatic hybrids, however, the organelle composition has not yet been analyzed. Here we report on the production of somatic hybrids between navel orange and 'Murcott' tangor and on their organelle compositions. Sexual hybrids between these two plants have not been obtained because of male sterility, low seediness (they are usually seedless), and polyembryony in navel oranges.

Contribution No. E-132 of the Fruit Tree Research Station

Materials and methods

Plant materials

Suspension-cultured cells of 'F. N. Washington' navel orange (*Citrus sinensis* Osb. var. *brasiliensis* Tanaka), originating from nucellus tissue, were maintained in a Murashige and Tucker (MT) (1969) liquid medium supplemented with 10 mg/l 6-benzylaminopurine (BA), as described previously (Kobayashi et al. 1985). Seeds of the 'Murcott' tangor (this variety is a tangor of unknown origin resulting from the breeding program of the U.S. Department of Agriculture) were germinated in vermiculite. Plants (nucellar seedlings) were grown in a growth chamber kept at 25 °C under 16 h/day illumination with cool fluorescent light (25 $\mu E\ s^{-1}\ m^{-2}$). Five fully expanded leaves were harvested from the 2-month-old plants.

Protoplast fusion and plant regeneration

Protoplasts were isolated from suspension-cultured cells and from leaves of nucellar seedlings, and were fused with the aid of polyethylene glycol by the method described earlier (Kobayashi et al. 1988). The protoplasts (10^5 cells/ml) were cultured in 3-ml medium, which consisted of hormone-free MT medium (BM) containing 0.6 *M* sucrose and 0.6% agarose (Sea Plaque, LMT, Marine Coloids), in Falcon dishes (60×15 mm).

Green embryoids derived from protoplasts were transferred to BM containing 500 mg/l malt extract, 40 mg/l adenine, 5% sucrose, and 0.9% agar. They developed into cotyledonary embryoids after about 1 month. Cotyledonary embryoids devel-

oped into entire plants within 3 months of culture when transferred to BM containing 10 mg/l gibberellic acid, 2% sucrose, and 0.9% agar.

Determination of chromosome number

Five root tips of regenerated plants pretreated with 8-hydroxyquinoline (2 m*M*) for 20 h at 10 °C were fixed in a mixed solution of ethanol and acetic acid (3:1) for 24 h, and then stained with lacto-propionil orcein for 3 h according to Oiyama (1981).

DNA analysis

Total DNA was extracted from leaves of regenerated plants according to the method of Rogers and Bendich (1985). Two micrograms of DNA, digested with restriction endonucleases, was subjected to agarose gel electrophoresis. After electrophoresis, the gel was treated with alkali, then blotted to a nitrocellulose filter by the Southern method. The filter was baked in vacuo for 2 h at 80 °C and preincubated 20 min at 42 °C with constant shaking in a hybridization buffer [for enhanced chemiluminescence (ECL) method, Amersham]. Then the filter was hybridized overnight at 42 °C with constant shaking in the same buffer containing the labelled probe. The hybridized blot was rinsed twice with the first washing solution (6 *M* urea, 0.4% SDS, and 0.5 × SSC) for 20min at 42 °C, followed by two rinses of the second washing solution (2 × SSC) for 5 min at room temperature. DNA fragments prepared from recombinant plasmids (pRR217, pTBal, *atp 9*, and the mtDNA library) were used as probes. Plasmid pRR217, pTBal, *atp 9*, and the mtDNA library contain whole nuclear ribosomal DNA (rDNA) sequences of rice (Takaiwa et al. 1984), a 19.6-kb BamHI fragment of *Nicotiana tabacum* cpDNA (Sugiura et al. 1986), a 2.2-kb XbaI fragment containing ATPase subunit 9 gene of *Zea mays* mtDNA (Dewey et al. 1985), and PstI or SalI fragments of *Brassica campestris* mtDNA (Palmer and Shields 1984), respectively. Plasmid pRR217, pTBal, *atp 9*, and mtDNA clones were kindly provided by Drs. K. Oono, M. Sugiura, C. S. Levings III, and J. D. Palmer, respectively. Labelling of probe DNA and visualization of the probe-target DNA hybrid were carried out by the ECL method, according to the supplier's (Amersham) instructions.

Results and discussion

Sixteen plants (clones) were regenerated from the obtained fusion products via embryogenesis. All of the plants were morphologically normal (Fig. 1) and no intraclonal variation was observed. As for morphology, leaves of the plants were lanceolate in like 'Murcott', but were broad and thick (Fig. 2). Chromosome counts showed that the plants had expected an amphidiploid chromosome number of 36 (2n = 2x = 18 for each parent) (Fig. 3).

Nuclear rDNA analysis has been employed to identify somatic hybrids (Uchimiya et al. 1983; Ohgawara et al. 1985, 1989; Robertson et al. 1987; Kobayashi et al. 1988), and it was also used to identify the somatic hybrids produced in the present study. Digestion of navel orange DNA with EcoRI produced a 6.5-kb and a faint 7.0-kb fragment hybridizing to the labelled rDNA probe, whereas digestion of 'Murcott' DNA produced a 7.0-kb

Fig. 1. A somatic hybrid plant between navel orange and 'Murcott' tangor

Fig. 2. Leaf morphology of navel orange (*left*), somatic hybrid (*center*), and 'Murcott' tangor (*right*)

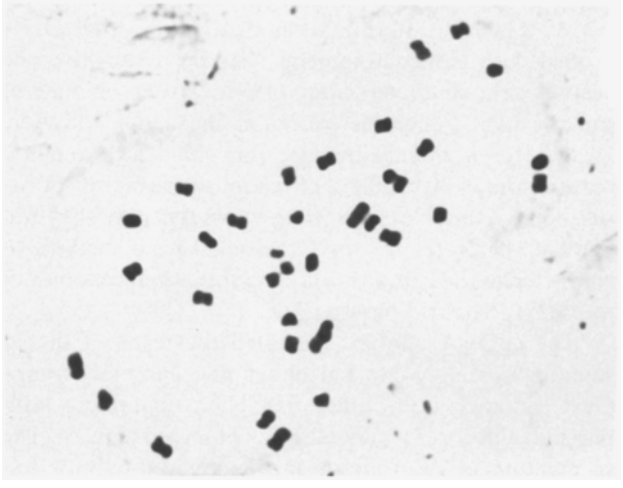

Fig. 3. Metaphase plate from one somatic hybrid plant (2n = 36)

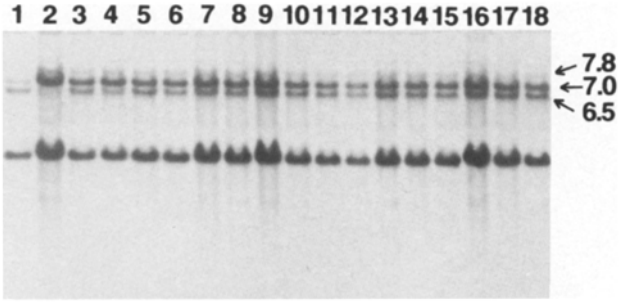

Fig. 4. Southern blot hybridization of EcoRI digests of total DNA to labelled rDNA fragments. *1:* navel orange; *2:* 'Murcott' tangor; *3–18:* somatic hybrids. Electrophoresis was performed in 0.8% agarose gel at 30 V for 14 h. *Arrows* indicate bands specific to the parental species and found in the somatic hybrids. Numerals indicate kbp

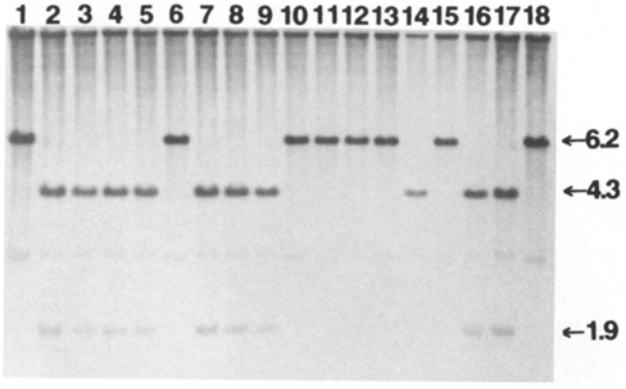

Fig. 5. Southern blot hybridization of PstI digests of total DNA to labelled cpDNA fragments. *1–18:* same as Fig. 4

and a faint 7.8-kb fragment. The DNA of the regenerated plants contained all of these fragments (Fig. 4). These results indicate that the regenerated plants were somatic hybrids (amphidiploid).

The restriction endonuclease analysis of cp/mtDNA, particularly using a small-scale method based on Southern blot hybridization analysis of digested total DNA probed with cloned fragments, has been shown to be useful for the characterization of cytoplasmic genomes of somatic hybrids/cybrids (reviewed by Galun and Aviv 1986). This is mainly because this small-scale method requires only 100–250 mg of tissue, such amount of tissue being available from young somatic hybrid/cybrid plants. Therefore, we used the small-scale method to characterize the composition of cytoplasmic genomes of the somatic hybrid plants.

The cpDNA analysis indicated that each of the 16 somatic hybrids contained either one parental chloroplast genome or the other. No plant containing both parental chloroplast genomes was observed (Fig. 5). The segregation of chloroplasts in the somatic hybrids followed approximately a 1:1 ratio (7:9). This fact suggested that one of the parental chloroplast types was randomly eliminated from the heterokaryons during its development into an entire plant. Similar chloroplast segregation has been reported in many somatic hybrid plants, although there are some exceptions (reviewed by Kumar and Cocking 1987). In citrus cybrids, while protoplast-fusion-derived embryos contained chloroplasts from both or one of the parental forms, complete segregation of chloroplasts was observed at the stage of fully differentiated plants (Vardi et al. 1989).

For mtDNA analysis, we used 11 mtDNA probes: *atp 9* from maize mtDNA and P4.8, P7.7, S10.1, P9.7, S8.3, S11.8, P5.7, P12.4, S6.2, P9.1 scattered throughout the mitochondrial genome of *B. campestris*. The *atp 9* gene region has been shown to be the intermolecular recombination site in a *Petunia* somatic hybrid (Rothenberg and Hanson 1987). The clones of P4.8 and S8.3 contain a 2-kb repeat sequence, which has been shown to be the natural intramolecular recombination site in *Brassica* (Palmer and Shields 1984) as well as one intermolecular recombination site in *Brassica* cybrids (Vedel et al. 1986). For all probes used, detected mtDNA fragments from the somatic hybrids were identified as those of naval orange. Neither plants having 'Murcott' mtDNA fragments nor both parental mtDNA fragments were observed (Fig. 6).

It has been demonstrated that some of the mtDNA sequences are homologous with DNA sequences present in chloroplast and nuclear genomes (reviewed by Schuster and Brennicke 1988). This might be one disadvantage of the small-scale method adopted in this study. However, it did not seem that mtDNA clones used in the present study hybridized to chloroplast/nuclear DNA. If mtDNA clones hybridized to chloroplast/nuclear DNA, hybridization patterns of somatic hybrids with mtDNA clones would become similar to those with cpDNA or nuclear DNA probes, albeit only partially. However, hybridization patterns with mtDNA clones differed from those with cpDNA/nuclear rDNA probes, since somatic hybrids showed both parental (rDNA), either one or the other parental (cpDNA), and only one parental (mtDNAs) fragment.

In contrast to the plastid genomes, mt genomes of most somatic hybrids/cybrids analyzed have been shown to be composed of novel mixtures of restriction fragments of both parents (reviewed by Kumar and Cocking 1987), and direct evidence for intergenomic recombination has been provided for *Petunia* and *Brassica* somatic hybrids (Rothenberg et al. 1985; Rothenberg and Hanson 1987; Robertson et al. 1987). In the present study, however, no recombination events in the mtDNA were observed. Experiments are in progress to clarify whether or not unilateral sorting-out of mitochondria occurs in other somatic hybrids from different fusion combinations, depending of the source of protoplasts (suspension

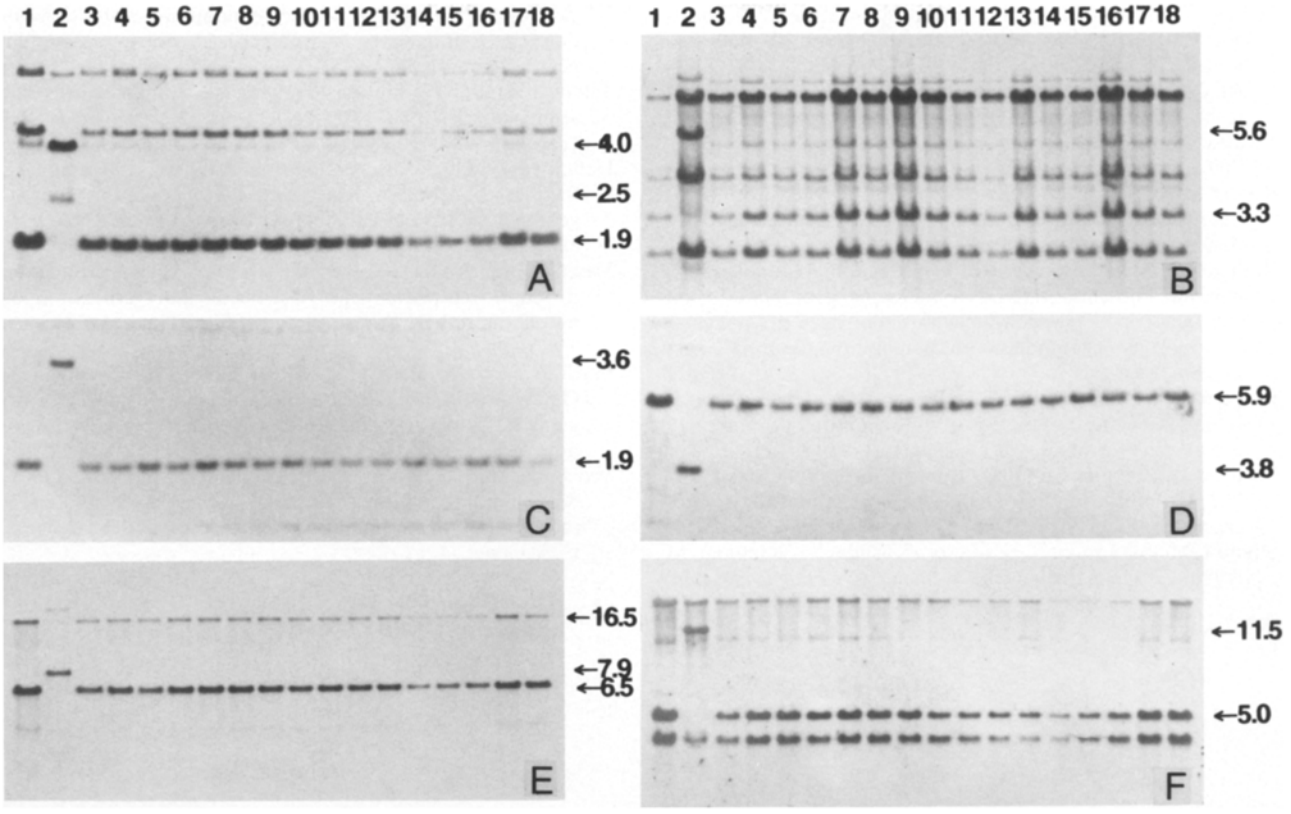

Fig. 6 A–F. Southern blot hybridization of restriction endonuclease digests of total DNA to labelled mtDNA fragments. *1–18:* same as Fig. 4. **A** HindIII digest, probe P4.8. **B** EcoRI digest, probe P9.7. **C** HindIII digest, probe S8.3. **D** XbaI digest, probe *atp 9*. **E** HindIII digest, probe P12.4. **F** EcoRI digest, probe S6.2

cultures versus leaf) or species (navel orange versus 'Murcott').

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