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Citrus cybrids: production by donor-recipient protoplast-fusion and verification by mitochondrial-DNA restriction profiles *

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Summary. Embryogenic nucellar-callus from three *Citrus* types was used for protoplast isolation. The protoplasts were fused by the 'donor-recipient' procedure by which the nuclear-division of the donor-protoplasts was arrested by gamma-irradiation and the metabolism of unfused recipient-protoplasts was transiently inhibited by iodoacetate. The following fusion combinations were performed: (1) Poorman × *Poncirus trifoliata* with Villafranca lemon; (2) Poorman × *P. trifoliata* with Sour orange; (3) Sour orange with Villafranca. Combinations 1, 2 and 3 resulted in 120 (125), 70 (5) and 19 (89) calli (regenerated plants), respectively. The mitochondrial-DNA (mtDNA) restriction profiles of Poorman × *P. trifoliata* obtained by fragmentation with *Bcl* I, *Bam* HI or *Sal* I differed from the respective profiles of Villafranca and Sour orange but no differences in mtDNA restriction profiles were detected between Villafranca and Sour orange. When Southern blots of Villafranca and Sour orange mtDNAs were hybridized with radiolabelled heterologous mtDNA probes, the mtDNAs of these two *Citrus* types could be differentiated. The fusion-derived plants from all three donor-recipient combinations had the recipients morphological (nuclear-coded) features. MtDNA restriction profiles, with and without hybridization to heterologous probes, indicated that the analysed plants were cybrids.

Key words: *Citrus* – Protoplasts – Cybrids – Mitochondrial DNA – Southern blot hybridization

Introduction

Efficient systems for embryo and tree-regeneration from protoplasts have been developed in *Citrus* (Vardi et al. 1975; Galun et al. 1977; Vardi et al. 1982; Kobayashi et al. 1983) and its related genus *Microcitrus* (Vardi et al. 1986). Such systems were all based on the capability of nucellar-callus derived protoplasts to regenerate trees via in vitro embryogenesis. These protoplast-to-tree systems paved the way to obtain novel *Citrus* types by protoplast-fusion as outlined in recent reviews (e.g. Bravo and Evans 1985; Galun and Aviv 1986). The cell-manipulation approach as an alternative to sexual-crosses for genetic studies and breeding is especially attractive in *Citrus* where sexual-reproduction and sexual-crosses have severe limitations (e.g. Cameron and Frost 1968). Thus Ohgawara et al. (1985) produced somatic hybrids between the "Trovita" orange (*C. sinensis*) and the *Citrus* root-stock *Poncirus trifoliata.*

Somatic cybrids rather than hybrids can be obtained by the donor-recipient protoplast-fusion method in which nuclear-division of the donor-protoplasts is arrested by X- or gamma-irradiation and the division of non-fused recipient-protoplasts is avoided by an antimetabolite (e.g. iodoacetate) causing transient metabolic inhibition (see Galun and Aviv 1986).

The production of specific *Citrus* cybrids is very relevant to the genetics and breeding of this genus because there is no information on "cytoplasmic" inheritance in *Citrus* while several breeding traits may be coded by the organelle-genomes. Here we report the first cases of cybrid *Citrus* trees obtained by three combinations of donor-recipient protoplast-fusions between *Citrus* cultivars.

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Materials and methods

Protoplast sources

Nucellar-calli of Sour orange (*Citrus aurantium* L.) (SO) and Poorman × *Poncirus trifoliata* (PPT) were obtained and maintained as described previously (Vardi et al. 1982). The nucellar-callus line derived from the lemon (*Citrus limon* (L.) Burn) cultivar Villafranca (VF) was obtained from the late Dr. J. Kochba and maintained as the aforementioned lines. These three callus lines were used as protoplast sources.

Isolation and plating of protoplasts

Before protoplast isolation, calli were sub-cultured at least twice, with a 2- to 3-week interval on nutrient medium (BM) of Murashige and Tucker (1969) containing 4% sucrose and lacking growth regulators as described by Vardi et al. (1982). For protoplast isolation ca 0.5 g callus tissue was placed in each tissue-culture grade 9 cm Petri dish and mixed with 10 ml maceration medium. The maceration medium consisted of 0.2% Macerozyme, 0.3% Cellulase R-10 and 0.1% Driselase, dissolved in a solution composed of half-strength BM macro-elements and containing 0.35 M sucrose and 0.35 M mannitol. The maceration solution was adjusted to pH 5.7 and filter (0.2 μm) sterilized before use. After overnight incubation (27 °C ± 1 °C) in the dark the protoplasts were isolated by sequential filtering through 50 μm and 30 μm nylon screens followed by three centrifugations (100 *g*, 5 min) in BM containing 0.3 M sucrose and 0.3 M mannitol (wash-solution).

Protoplasts which were neither irradiated nor iodoacetate-treated (see below) were suspended at a final density of 10^5 ml^{-1} BM containing 0.6% agar and were either plated without feeder-layers as 4 ml aliquots per 5 cm Petri dish or as 2 ml aliquots at a final density of 2×10^4 ml^{-1} above a feeder layer in the same Petri dishes.

Feeder and donor protoplasts

Feeder and donor protoplasts were prepared by irradiating the protoplasts immediately after their isolation. A dose of 50 to 60 krad gamma rays (cobalt-60, G.B. 150A, Atomic Energy of Canada) was required for virtually-complete division-arrest of *Citrus* protoplasts. After irradiation the protoplasts were washed twice by centrifugation in wash-solution. Feeder-protoplasts were plated as described by Vardi and Raveh (1976) in BM containing 0.3 M sucrose and 0.3 M mannitol and 0.6% agar.

Recipient protoplasts

Protoplasts which were intended to serve as recipients in fusion experiments were washed only once (after release from macerating enzymes) in wash-solution and then suspended for 20 min in wash solution containing 0.25 mM (recrystallized) iodoacetate. The protoplasts were then washed three times by centrifugation (as above) in wash solution.

Donor-recipient protoplast fusion

Equal amounts of donor and recipient protoplasts, at a final density of approximately $5 \cdot 10^5$ cells per ml, were mixed. The procedure outlined by Aviv and Galun (1985) was followed. Specifically two 0.15 ml drops of PEG solution (50% poly-ethyleneglycol 1,500 in a solution containing 10 mM $CaCl_2$, 0.1 M glucose) were placed in the center of a 5 cm plastic Petri dish. The drops were placed of few mm from each other. Then a 0.3 ml drop containing the mixture of donor and recipient protoplasts was added carefully between the two PEG drops and the three drops were caused to unite gradually. After 15 min, 4 ml of $Ca(NO_3)_2$ (pH 10.5) were added to the periphery of the PEG-protoplasts suspension drop. Then 15 min later the suspensions from two Petri dishes were collected carefully, and centrifuged (100 *g*, 5 min). The pelleted protoplasts were suspended in 2 ml liquid nutrient-medium composed of BM with 0.3 M sucrose and 0.3 M mannitol. Finally the latter suspension was mixed with 1.5 ml of nutrient medium containing 1.2% agar and the mixture was plated over the feeder layer.

Isolation of colonies and regeneration of embryos

When colonies reached a diameter of 1–2 mm they were isolated and transferred for further growth to Petri dishes containing solidified BM medium (containing 4% sucrose). When enough callus was available (i.e. colony diameter greater than 5 mm) the colony was transferred to embryo-regenerating medium. This medium contained BM, 1.0% agar and 2% glycerol rather than sucrose (Ben-Hayyim and Neumann 1983). After about 6 weeks numerous embryos were formed. The latter were individually isolated and transferred to Petri dishes containing solidified (1.0% agar) BM with 4% sucrose and 1.5 g · l^{-1} malt-extract.

Plant regeneration

Embryos which reached 7–10 mm and had normal shapes and expanded cotyledonary leaves were transferred individually to test tubes containing BM with 2% sucrose and 0.05 ppm NAA to promote axis-elongation and root-formation. Plantlets were then planted in autoclaved Jiffy turf-pots (no. 7); the pots were placed in sterile plastic containers and watered with half-strength of BM macro- and microelements. When roots started to emerge from the turf, the turf-pots with the rooted plants were transplanted into plastic containers (ca 10 × 10 × 20 cm) with a soil-Perlite mixture. The containers were covered with plastic bags and transferred to the greenhouse. The plants were acclimatized to lower humidity by gradually opening the bags and then maintained in the greenhouse without further protection.

Characterization of the mitochondrial genome

The characterization of the mitochondrial genome (the chondriome) of fusion-partners and cybrids was based on the restriction profiles of their respective mtDNAs. Both fluorography (ethidium bromide staining) and Southern blot hybridization, with heterologous mtDNA, were used to analyse the restriction profiles.

Mitochondrial DNA restriction profiles and Southern blot-hybridization. The procedures for mitochondrial isolation, mtDNA extraction, digestion with endonucleases and Southern blot-hybridization were as described previously (Galun and Aviv 1986). Suspension-cultures in BM with 2% sucrose served as the plant-material source; 10 to 20 g of filtered cells were washed once in water and one volume of cells was suspended in 4 volumes homogenization buffer (0.3 M mannitol, 3 mM dibasic EDTA, 50 mM Tris-HCl, pH 8.0; up to 0.1% BSA and 1 mM 2-mercaptoethanol were added before use). The suspended cells were passed through a French-press (3,000 lbs/in^2) and further extraction and purification were done according to Sparks and Dale (1980) with some modifications as detailed previously (Galun and Aviv 1986). Restriction-endonuclease digestion, gel-electrophoresis and Southern blot-hybridization with mtDNA probes (see below) were performed by standard procedures (see Hanson et al. 1986).

Mitochondrial DNA probes. Two heterologous probes were used in the Southern blot-hybridizations. Plasmid *pmtSylSa-8* is a *SalI* fragment of *Nicotiana sylvestris* mtDNA, cloned in pBR322 (Aviv et al. 1984). Plasmid *pZm*E1 is a 2.4 kb maize mtDNA fragment, containing the cytochrome oxidase subunit II (*COX* II) gene (Fox and Leaver 1981) cloned in pBR322. The latter plasmid was kindly provided by Dr. C. J. Leaver (Dept. of Botany, University of Edinburgh, Scotland).

Total DNA extraction. When *pZmEI* served as probe in Southern blot hybridization endonuclease-digested total DNA from leaf-tissue rather than mtDNA from suspension cultures was utilized. Total DNA was obtained from 1 to 1.5 g of young-leaves. The leaves were powdered in liquid nitrogen and the DNA extraction of Abbott et al. (1985) was followed with minor modifications as described recently (Breiman et al. 1987).

Results

Production of cybrid plants

The following donor-recipient protoplast-fusion combinations were performed: PPT (donor) with VF (recipient); PPT (donor) with SO (recipient); SO (donor) with VF (recipient).

In each case the donor protoplasts were gamma-irradiated and the recipient protoplasts were treated with iodoacetate shortly before fusion. The fused protoplasts were plated in soft agar medium over feeder layers composed of 10^5 ml^{-1} gamma-irradiated PPT protoplasts in soft agar medium. In parallel to each fusion combination the following control treatments were performed:

(1) plating of feeder-layer only (in a volume of 4 ml/5 cm Petri dish); (2) plating of unirradiated donor protoplasts at a density of 2×10^4 ml^{-1} over a feeder layer; (3) plating of unirradiated donor protoplasts at a density of 10^5 ml^{-1} (in 4 ml soft agar medium) without a feeder layer; (4) plating of irradiated donor protoplasts, at a density of 10^5 ml^{-1}, over a feeder layer; (5) plating of untreated recipient protoplasts, at a density of 2×10^4 ml^{-1}, over a feeder layer; (6) plating of untreated recipient protoplasts at a density of 10^5 ml^{-1} (in 4 ml soft agar medium), without a feeder layer; (7) plating of iodoacetate-treated recipient protoplasts, at a density of 2×10^4 ml^{-1}, over a feeder layer; (8) plating of iodoacetate-treated recipient protoplasts, at a density of 10^5 ml^{-1} (in 4 ml soft agar), without a feeder layer; (9) mixing (1 : 1) gamma-irradiated donor protoplasts with iodoacetate-treated recipient protoplasts (but without fusion) and plating at a density of 2×10^4 ml^{-1}, over a feeder layer.

In none of the controls with iodoacetate-treated recipient protoplasts were cell-divisions observed and in all the tests the untreated donor and recipient protoplasts underwent the expected cell division. When Poorman × *P. trifoliata* (PPT) protoplasts were gamma-irradiated, before plating, at a dose of 50 krad, we observed occasional cell-divisions followed by colony formation. Thus, these fusion experiments were discarded and in subsequent fusion experiments PPT donor protoplasts as well as feeder-layer PPT protoplasts were exposed to 60 krad gamma-irradiation before plating.

The number of calli and plants obtained by the three fusion combinations is summarized in Table 1. The first cell divisions were observed 2 or 3 weeks after protoplast-fusion, while cell division in untreated parental protoplasts occurred at least one week earlier. Following the procedure described in "Materials and methods," transfer of fusion-derived calli to glycerol-containing medium led to the production of highly embryogenic calli (Fig. 1). Embryos with normal morphology were isolated from such calli and plated in

Table 1. Cybrid calli and plants obtained by donor-recipient protoplast-fusion

Donor (gamma irradiated)	Recipient (IA-treated[a])	No. of calli		No. of regenerated plants
		Isolated	Retained	
PPT (Poorman × *P. trifoliata*)	Villafranca (lemon)	120	90	125
PPT (Poorman × *P. trifoliata*)	Sour Orange	70	10	5
Sour Orange	Villafranca (lemon)	19	18	89

[a] Treated before fusion with iodoacetate for transient metabolic-arrest

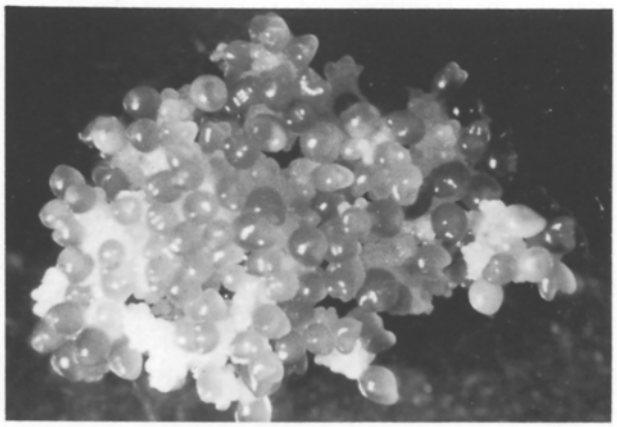

Fig. 1. Embryogenic callus obtained by the transfer of a protoplast-fusion derived colony to a glycerol-containing medium. (×7)

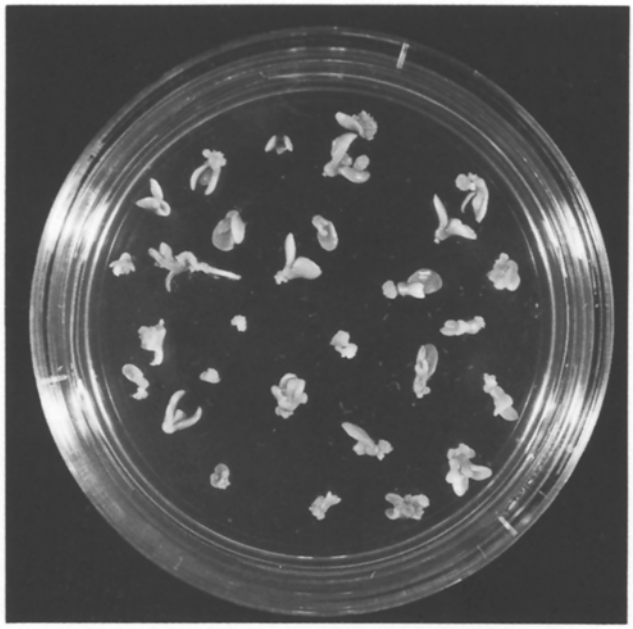

Fig. 2. Embryos derived from the fusion of gamma-irradiated PPT (donor) protoplasts with iodoacetate-treated Villafranca (recipient) protoplasts. (×1.3)

a sucrose and malt-extract containing medium which resulted in further development of the embryos (Fig. 2). In parallel, fractions of the same embryogenic calli were transferred to sucrose containing BM medium to serve as source of material for mitochondrial characterization (see below).

When the embryos, cultured in the malt-extract containing medium, developed expanded cotyledonary leaves, they were transferred to test tubes, then to Jiffy turf-pots and the plants were finally potted in a soil-Perlite mixture as described in "Materials and methods" (Fig. 3).

Plants were also regenerated from protoplasts of the fusion-partners (as well as from Shamouti orange protoplasts); the leaf-shape of these protoplast-derived plants served as morphological markers. As shown in Fig. 4, the petiole shapes of VF, SO and PPT are very different. VF has no petiole wings, SO has conspicuous but small petiole wings, while PPT has a trifoliate leaf-shape. The fusion-derived plants of all three donor-recipient combinations had leaf shapes which were identical to those of the recipients' fusion-partners.

Chromosome counts in mitosis-metaphases from leaf-tip samples of cybrid plants of all three fusion combinations showed that these plants had 18 chromosomes – thus they were diploid.

Mitochondrial DNA analyses

To characterize the chondriomes of the fusion-combination partners we isolated mtDNA from PPT, VF

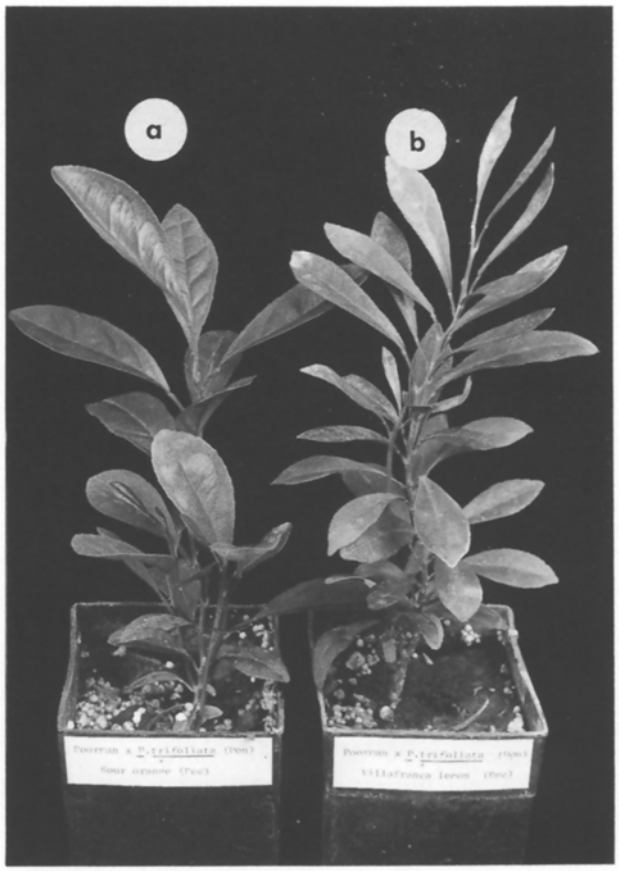

Fig. 3. Cybrid plants derived from two protoplast fusion combinations: *a* gamma-irradiated PPT (donor) with iodoacetate-treated Sour Orange (recipient); *b* gamma-irradiated PPT (donor) with iodoacetate-treated Villafranca lemon (recipient). (×0.3)

and SO. The mtDNAs were fragmented with several endonucleases electrophoresed on agarose gels and stained with ethidium bromide. As shown for *Bam* HI, *Bcl* I and *Sal* I in Fig. 5 (the restriction pattern of *Microcitrus* sp. was added for comparison), the restriction profiles of PPT mtDNA were clearly different from the respective profiles of VF and SO mtDNAs. However, there were no differences between the mtDNA restriction profiles of VF and SO. This indicated that mtDNA restriction profiles are not useful for the characterization of the chondriomes in derivatives of the fusion between SO (donor) and VF (recipient). On the other hand, when total DNA was extracted from leaf tissues and analysed by Southern blot hybridization using plasmid *pZmE1* as the radioactive probe for mtDNA the samples from SO and VF leaves provided distinctly different autoradiographic patterns (Fig. 6). Likewise, DNA samples from PPT leaves provided patterns different from those of DNA samples from SO leaves while DNA samples from PPT and VF leaves provided the same patterns (Fig. 6). We therefore char-

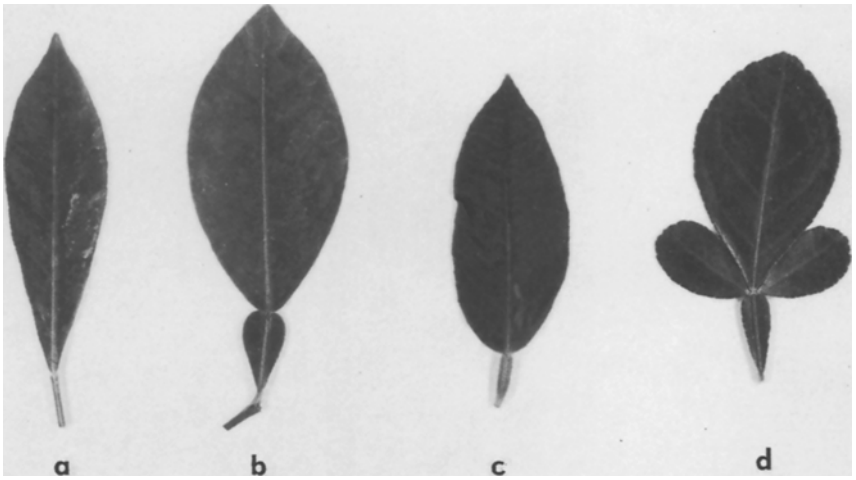

Fig. 4. Leaves from protoplast-derived plants of Villafranca lemon (*a*), Sour Orange (*b*), Shamouti orange (*c*) and PPT (*d*). Note differences in laminar shapes and petiole wings. (×0.8)

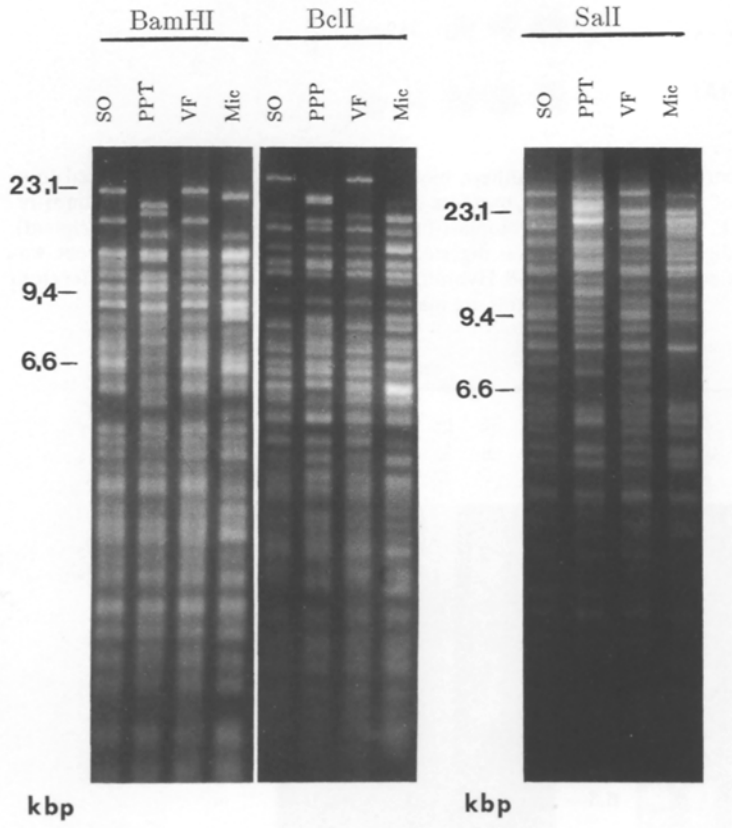

Fig. 5. Fluorograms of mtDNA restriction profiles from Sour Orange (SO), Poorman X *P. trifoliata* (PPT) and Villafranca lemon (VF). MtDNA was digested with *Bam* HI, *Bcl* I or *Sal* I, electrophoresed on a 0.8% agarose gel and stained with ethidium bromide

acterized the chondriomes of plants derived from the two fusion combinations – SO (donor) and VF (recipient) and PPT (donor) with SO (recipient) – by Southern blot hybridization, as shown in Fig. 6. The thus analysed plants from both of these fusions provided autoradiogram patterns which were identical to the respective patterns of DNAs from the donor-partners of these fusion-combinations. Since the fusion-derived plants retained the leaf morphologies of the recipient-partners of these fusion-combinations and were diploid, we concluded that all the tested plants were cybrids.

An advantage of the Southern blot hybridization for the analysis of chondriomes is the requirement of only small amounts (~ 1 g) of tissue. To obtain high-quality

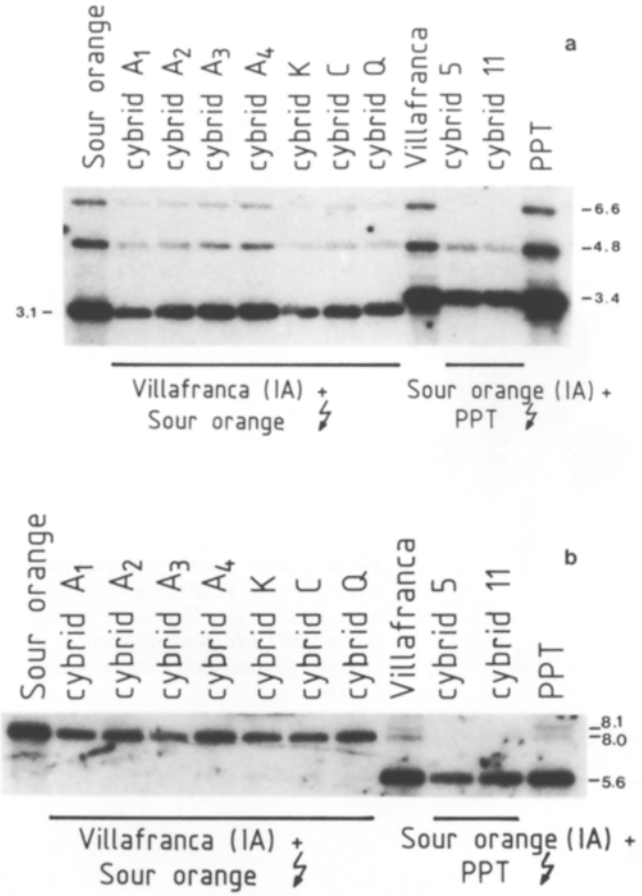

Fig. 6a, b. Southern blot hybridizations of total DNA extracted from the fusion partners and their derived cybrids of two donor-recipient protoplast-fusions: SO (donor) with VF (recipient) and PPT (donor) with SO (recipient). DNA was digested with *Bcl* I (**a**) or with *Bam* HI (**b**) and the blots were hybridized with the radiolabelled *pZmE1* plasmid

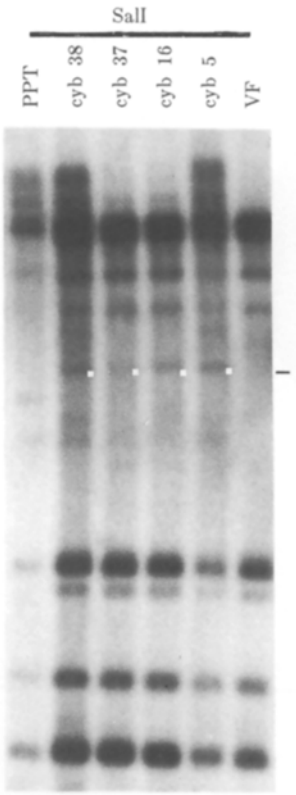

Fig. 8. Southern blot-hybridization of mtDNA extracted from the fusion-partners and four derived cybrids of the donor-recipient protoplast-fusion: PPT (donor) with VF (recipient). MtDNA was digested by *Sal* I and the radiolabelled probe was *pmtSylSa-8*. Hybridized fragments of cybrids which differ from VF fragments are marked with small white squares

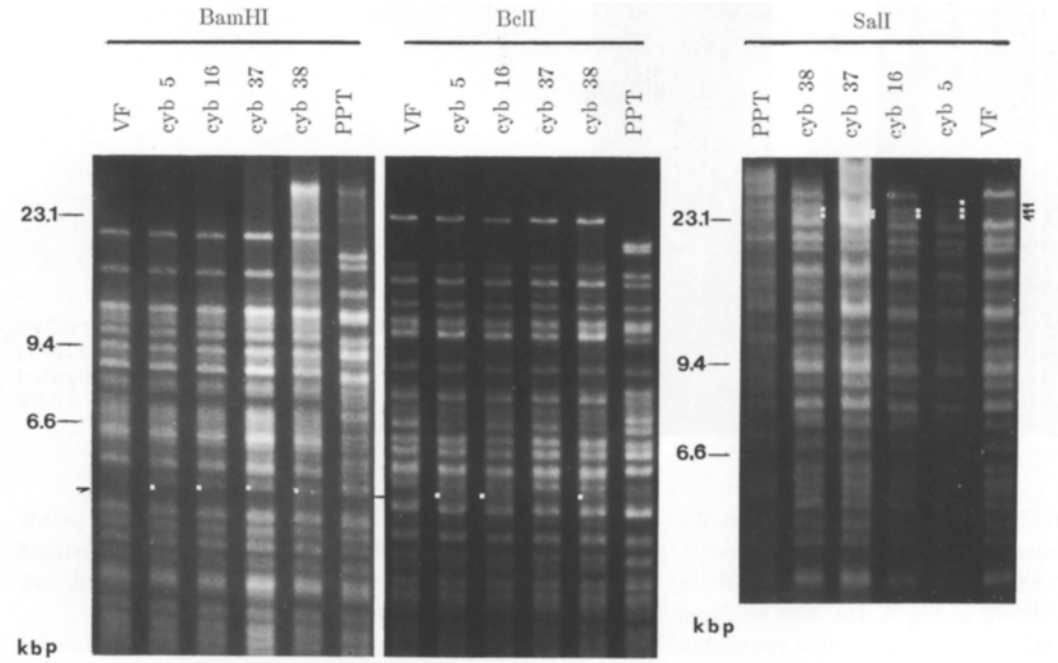

Fig. 7. Fluorograms of mtDNA restriction-profiles from two fusion partners – PPT (donor) and VF (recipient) and four cybrid derivatives. Fragments of cybrids' mtDNA which differ from VF fragments are marked with small *white squares*

fluorograms from mtDNA restriction profiles, at least 10 g of tissue are required. Such amounts were not available from individual fusion-derived young plants. We therefore used cell-suspensions derived from callus as sources for mtDNA. Small pieces (~ 10 mg) were isolated from fusion-derived calli after culture in (glycerol containing) regeneration medium, i.e. at the same time individual embryos were isolated from these calli. Since each fusion-derived cell-colony was identified by a line-number, the callus-pieces and the embryos isolated from the same line had identical identification numbers. Each fusion-derived callus-line could thus be attributed to a specific fusion-derived plant. The fusion-derived callus-lines were transferred to sucrose-containing liquid BM medium and cultured further until each line reached at least 10 g (fresh weight). Cell-suspensions were similarly established from PPT, SO and VF. Figure 7 shows the restriction profiles of 4 mtDNA samples attributed to four different fusion-derived plants which resulted from the fusion of PPT (donor) with VF (recipient). The profiles indicated that the four samples' mtDNA restriction-patterns were similar to the respective patterns of VF (the recipient) but differed from the latters' patterns in several fragments. To further verify the identification of the mtDNAs from the fusion derived samples we analysed the four samples by Southern blot analysis using a mtDNA probe (*pmSylSal*-8) from *Nicotiana* (Fig. 8). The resulting autoradiogram confirmed that the hybridization-pattern of the tested samples were similar but not identical to the pattern from the recipient partner (VF) of this fusion. Metaphase-chromosome counts confirmed that the fusion-derived plants were diploid. We therefore concluded that the fusion of PPT (donor) with VF (recipient) resulted in cybrids which contained chondriomes which were similar but not identical to those of the recipient of this fusion.

Discussion

The analysis of *Citrus* plastomes (Green et al. 1986) indicated that orange, Sour orange, lemon, grapefruit and pummelo have plastomes which cannot be differentiated by the restriction patterns of their chloroplast DNAs (i.e. the *C. aurantium* chloroplast DNA pattern). The three *Citrus* types used as fusion-partners in the present work (viz. PPT, VF, SO) belong to the above mentioned *C. aurantium* group of cultivars. Thus the characterization of cybrids' plastomes by chloroplast DNA restriction-profile cannot be used to trace organelle transfer caused by the donor-recipient protoplast-fusions among these three fusion-partners. We therefore turned to chondriomes to study such organelle-transfer. Except for a single report (Fontarnau and Hernandez-Yago 1982) which provided mtDNA restriction patterns of an orange and a lemon type (*C. limonium*) after digestion with *Sal*I, there is no previous published information on mtDNA characterization in *Citrus* cultivars. The results of this work demonstrate that good-quality restriction profiles can be obtained from callus-derived mtDNA, that such profiles as well as Southern blot hybridizations with heterologous mtDNA probes are useful tools for the characterization of chondriomes and that distinct differences can be revealed even among chondriomes of *Citrus* type belonging to the same plastome type.

The analysed cybrids from the three donor-recipient protoplast-fusion combinations had mtDNA restriction profile and Southern hybridization patterns which were not identical to the respective profiles and patterns of the recipient's mtDNAs. In two fusion-combinations the cybrids' patterns were either similar or identical to the donor's patterns and in a third fusion-combination the cybrids' patterns were similar but not identical to the recipient's pattern. However, the number of analysed cybrids constitute only a fraction of the total number of fusion-derived plants. We therefore do not draw general conclusions from our results. Previous publications (e.g. Nagy et al. 1981; Galun et al. 1982; Boeshore et al. 1985; Aviv et al. 1984; Chetrit et al. 1985; Aviv and Galun 1987; see Galun and Aviv 1986, for review) reported that plants derived from protoplast-fusion had commonly novel patterns of mtDNA restriction-profiles indicating a massive rearrangement of chondriomes derived from the fusion partners; parental-type mtDNA restriction-patterns were reported to be rare in somatic hybrids and cybrids. A notable exception was reported recently. Cybrids resulting from the fusion of X-irradiated (donor) *Nicotiana rustica* protoplasts with rhodamine 6-G treated *N. tabacum* protoplasts had mtDNA restriction profiles which were identical to either *N. rustica* or *N. tabacum* mtDNA (Aviv et al. 1986). Whether or not the treatments of the recipient protoplasts with rhodamine 6-G or with iodoacetate affect the chondriomes rearrangements still requires further study. Furthermore recent studies (D. Aviv, unpublished) indicated that the rate of chondriomes rearrangement could be determined by the fusion-partners' combination; some combinations may result in much higher rates of rearrangements than other combinations. Our results do not resolve this question but clearly show that in *Citrus* the donor-recipient protoplast-fusion technique is an efficient means of producing cybrid plants.

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