Theor Appl Genet (1989) 78: 741–747

Protoplast-fusion-mediated transfer of organelles from *Microcitrus* into *Citrus* and regeneration of novel alloplasmic trees *

A. Vardi [1], P. Arzee-Gonen [2], A. Frydman-Shani [1], S. Bleichman [2], and E. Galun [2]

[1] Institute of Horticulture, Agricultural Research Organization, The Volcani Center, Bet Dagan 50250, Israel
[2] Department of Plant Genetics, The Weizman Institute of Science, Rehovot 76100, Israel

Received June 1, 1989; Accepted June 27, 1989
Communicated by R. Hagemann

Summary. Iodoacetate-treated *Citrus* protoplasts from embryogenic nucellar calli of Sour orange (*C. aurantium*) or from Rough lemon (*C. jambhiri*) were fused with γ-irradiated protoplasts from a related genus, *Microcitrus*. The fused protoplasts were cultured to obtain colonies and micro-calli. Micro-calli derived from these two fusion combinations were isolated, propagated and differentiated into embryos, which subsequently regenerated trees having the morphology of Sour orange or Rough lemon. These intergeneric fusions resulted in mitochondria with novel DNA, indicating recombination between the chondriomes of *Citrus* and *Microcitrus*. Chloroplast DNA analyses of fusion-derived embryos indicated that they contained the chloroplasts of either fusion-partner or a mix of these chloroplasts. Later plastome analyses of leaves from fully differentiated plants showed that cybrids having Rough lemon morphology had either Rough lemon or *Microcitrus* chloroplast DNA, indicating complete sorting out of chloroplasts. Likewise, sorting out of *Microcitrus* chloroplasts was detected in a cybrid plant having Sour orange morphology.

Key words: *Citrus* – *Microcitrus* – Cybrids – Chloroplast DNA – Mitochondrial DNA

Introduction

Concerted interactions between the nuclear genome and the chloroplast genome (plastome) as well as between the nuclear genome and the mitochondrial genome (chondriome) are required to establish metabolic functionality and morphogenetic integrity in plants. Thus, the exchange of endogenous organelles by alien ones may disturb these interactions and lead to malfunctions and malformations (reviews: Galun and Aviv 1983, 1986). On the other hand, the establishment of alloplasmonic crop plants by our "donor-recipient" protoplast-fusion procedure (Zelcer et al. 1978) or its modifications was reported by numerous authors to result in cultivars with potential breeding advantages (e.g. Sidorov et al. 1981; Pelletier et al. 1983; Aviv et al. 1984; Flick et al. 1985; Barsby et al. 1987; Galun et al. 1987, 1988; Imamura et al. 1987; Kumar and Cocking 1987; Morgan and Maliga 1987; Robertson et al. 1987; Thomzik and Hain 1988).

The tribe *Citrinae* ("True Citrus Fruit Trees") of the Rutaceae includes the cultivated genus *Citrus*, a cultivated genus of lesser economic importance, *Fortunella*, a genus used as rootstock, *Poncirus* and three additional genera: *Eremocitrus*, *Clymenia* and *Microcitrus* (Swingle and Reece 1967). Among these six genera, *Microcitrus* is rather distinct. It is native to Australia and southeastern New Guinea and its vegetative and fruit characteristics differ substantially from those of *Citrus*. This distinction is also expressed in the unique amylase-isozyme composition (Esen and Scora 1977) and in the unique chloroplast DNA restriction patterns (Green et al. 1986).

In the following work we sought an answer to the question of whether or not chloroplasts and/or mitochondria from *Microcitrus* could replace these organelles in two *Citrus* rootstocks: Sour orange and Rough lemon. A positive answer could furnish novel cybrid trees with potential breeding value.

Materials and methods

Protoplast sources

Nucellar calli of Sour orange (*Citrus aurantium* L.) and Rough lemon (*Citrus jambhiri* Lush.) were obtained and maintained as

* Contribution from the Agricultural Research Organization, The Volcani Center, Bet Dagan, 50250 Israel. No. 2663-E, 1989 series

described previously (Vardi et al. 1982). The embryogenic callus line of *Microcitrus* sp. was the same as the one described by Vardi et al. (1986). The source of feeder-protoplasts was a non-embryogenic callus line derived originally from ovules of the hybrid Poorman × *Poncirus trifoliata* (PPT).

Isolation, pre-fusion treatment and plating of protoplasts

Protoplasts were isolated from calli by the methods detailed previously (Vardi et al. 1987; Vardi and Galun 1989). Donor protoplasts (i.e. *Microcitrus*) and feeder protoplasts (i.e. PPT) were washed once with a wash solution (Vardi et al. 1987) and exposed to 50–60 krad. After irradiation, the protoplasts were washed two more times with the same wash solution. Recipient protoplasts were washed once after release from maceration fluid, suspended for 20 min in wash solution containing 0.25 m*M* (recrystallized) iodoacetate and then washed three times by centrifugation in wash solution.

Feeder layer, protoplast fusion and plating

Feeder protoplasts were plated at 10^5 per ml in BM solidified with 0.6% agar to constitute a bottom layer of 2 ml per 5-cm (diameter) petri dish, as described elsewhere (Vardi and Raveh 1976; Vardi et al. 1987). Equal amounts of recipient (Rough lemon or Sour orange) and donor (*Microcitrus*) protoplasts at a final concentration of $5 \cdot 10^5$ per ml were mixed, a drop (ca. 0.3 ml) of this mixed suspension was placed on a petri dish between two adjacent polyethylene-glycol fusion-solution drops and the fusion was induced as detailed (Vardi et al. 1987). After dilution with $Ca(NO_3)_2$ (pH 10.5) and washings, the fused protoplasts were suspended in BM containing 0.3 *M* sucrose and 0.3 *M* mannitol; to each 2.0 ml of this suspension, 1.5 ml of the same BM with sucrose and mannitol, but containing 1.2% agar (maintained at 42 °C), was added and the mixture was plated over the feeder layer.

Isolation of colonies, induction of embryos and regeneration of plants

Isolation of colonies, induction of embryos and regeneration of plants was performed as detailed by Vardi and Galun (1989). Briefly, petri dishes containing a layer of fused protoplasts over a layer of feeder protoplasts were maintained at 25° ± 2 °C in low light (fluorescent tubes, approx. 2000 lx). When colonies reached a diameter of 1–2 mm, they were individually transferred to BM with 4% sucrose. The resulting calli were induced to form embryos by transfer to BM containing 2.0% glycerol and devoid of sucrose. Embryos were individually transferred to plates containing solidified BM with malt extract (0.15%) and sucrose (4%). When the cotyledonary leaves expanded, the plantlets were transfered into tubes containing solidified BM with sucrose (2%) and naphthaleneacetic acid (0.05 ppm) to cause axis elongation and root formation. When roots had elongated another transfer was made, into Jiffy turf-pots, and ultimately the plants with a well-developed root system were acclimatized in the greenhouse.

Analyses of organelle content

Chondriomes were characterized by restriction-pattern analysis of mitochondrial DNA (mtDNA). Calli derived from individual fusion products were given identification numbers and induced to differentiate into embryogenic callus. The latter served for mtDNA analysis as well as for the further regeneration of plants (as indicated above). MtDNA restriction profiles were obtained as described previously (Vardi et al. 1987). The mtDNA from each fusion-derived cybrid was digested with *Sal*I, *Bam*HI or *Pst*I. Thus, three restriction profiles were obtained for each mtDNA sample.

The characterization of plastome content was performed in two stages. The first stage was as noted for chondriome characterization. The second stage was at the differentiated plant level. The characterization at both stages was based on the restriction profiles of chloroplast DNA (ctDNA). For the ctDNA analysis at the first stage, the embryogenic calli were suspended in BM containing 2% glycerol. This resulted in the establishment of green suspensions derived from individual fusion products. Ten to 20 grams (fresh weight) of cells served for each ctDNA restriction-profile analysis. The analyses were performed as described by Green et al. (1986). The ctDNA was digested with *Pst*I, separated by gel-electrophoresis, blotted on nitrocellulase paper and hybridized to a radiolabelled cloned *Pst*I fragment (Ps2A) of *Nicotiana tabacum* ctDNA. For ctDNA analysis at the second stage, 1–2 g leaf samples were harvested from individual fusion-derived plants. The method of Mettler (1987) was employed and the respective blots were hybridized with radiolabelled Ps2A, as previously described (Green et al. 1986).

Results

Two fusion combinations were performed. In the first combination, γ-irradiated *Microcitrus* (donor) protoplasts were fused with iodoacetate-treated (recipient) Rough lemon (*C. jambhiri*) protoplasts (MR). In the second combination, γ-irradiated *Microcitrus* protoplasts were fused with iodoacetate-treated (recipient) Sour orange (*C. aurantium*) protoplasts (MS). Control platings of unfused γ-irradiated *Microcitrus* protoplasts did not result in colonies. Iodoacetate-treated recipient protoplasts from either Rough lemon or Sour orange, which were not fused with donor protoplasts, succumbed within 24 h after plating. When donor protoplasts were mixed with recipient protoplasts, but not fused, no colonies were obtained.

From the MR fusion, we analyzed the derivatives of nine colonies. When these colonies produced micro-calli, parts of each such callus were transferred to glycerol-containing medium. This transfer initiated embryogenesis. Several embryos from each original micro-callus were regenerated to plants (as described in "Materials and methods"). Altogether, 60 MR plants were thus rooted in Jiffy turf-pots. All the regenerated plants had the morphology of Rough lemon (Fig. 1 A) and none showed the characteristics of *Microcitrus* (Fig. 1 B). Note the great difference in leaf blade and spininess: Rough lemon leaves are ovate and large (length > 5 cm), while *Microcitrus* leaves are lanceolate and small (length < 2 cm); *Microcitrus* shoots contain conspicuous spines in each leaf axil, while only occasional spines develop on Rough lemon shoots. No plants with intermediate morphology (between *Microcitrus* and Rough lemon) were encountered. All analyzed root tips or young leaf tips retained the typical *Citrus* chromosome count (2n = 18).

From the MS fusion 60, micro-calli were isolated, propagated and later transferred to regeneration. Over 200 MS plants were rooted in Jiffy turf-pots. As observed

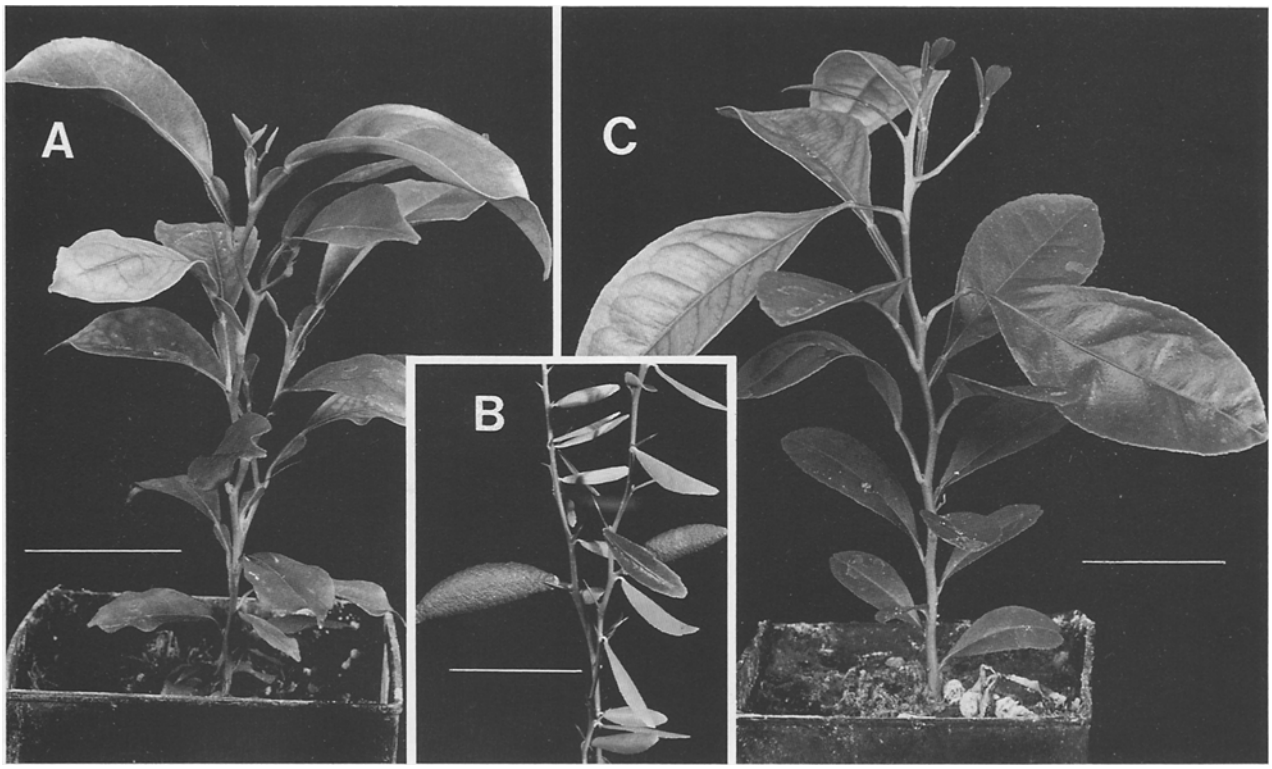

Fig. 1 A–C. Morphology of fusion-derived plants and of the donor plant (*Microcitrus* sp). **A** – a plant regenerated from the fusion of a γ-irradiated *Microcitrus* protoplast with an iodoacetate-treated Rough-lemon protoplast; **B** – a branch from *Microcitrus*-bearing fruits; **C** – a plant regenerated from the fusion of a γ-irradiated *Microcitrus* protoplast with iodoacetate-treated Sour-orange protoplast. The *bars* represent 3 cm

in the MR-derived plants, none of the plants had *Microcitrus* morphology; all the MS plants resembled Sour orange (Fig. 1 C) and all tested root tips retained the diploid chromosome number of *Citrus*.

Since none of the iodoacetate-treated (recipient) protoplasts of either Rough lemon or Sour orange, which were not fused, resulted in colonies, we assumed that all the regenerated MR and MS plants were indeed fusion-derived. To verify this assumption, we analyzed the mtDNAs from the presumptive cybrids. Three and eight samples from MR and MS, respectively, served for these analyses and every mtDNA analysis was based on restriction profiles obtained, respectively, after digestion with *Pst*I, with *Bam*HI or *Sal*I. Figures 2 and 3 demonstrate restriction profiles after *Pst*I and *Bam*HI digestions, respectively (digestion with *Sal*I is not shown). None of these mtDNA restriction profiles was identical to either of the respective mtDNA profiles of the original fusion partners. Most mtDNA samples revealed novel mtDNA fragments in addition to the fusion-partners' fragments (i.e. mtDNA fragments of *Microcitrus* and Sour orange in MS). Moreover, at least some of the fusion-partners' specific fragments were missing from the profiles of the fusion-derived mtDNA samples. This excluded the possibility that the analyzed mtDNA samples represented mixtures of *Microcitrus* and *Citrus* chondriomes. The most plausible explanation for the observed novel mtDNA restriction profiles is that donor and recipient chondriomes came in contact in the fused protoplasts and exchanged mtDNA components. Similar phenomena were encountered in *Nicotiana* interspecific fusions and were traced to mtDNA recombination (Galun and Aviv 1986). Since the fusion-derived regenerated plants had the recipients' morphology and were diploid, but none had mtDNA which was identical to that of the recipient, we assumed that these plants were indeed cybrids. This assumption was further supported by the mtDNA restriction-profiles of some presumptive cybrids (e.g. MR6, MR8, MR9, MS14), which were almost identical to the mtDNA profiles of *Microcitrus* (the donor).

To substantiate our assumption, we analyzed the ctDNA of the apparent cybrids. Samples for ctDNA analysis from the first stage (see "Materials and methods") showed that some of them provided restriction profiles (by Southern blot hybridization) which were identical to either donor (MR6, MR8, MS4) or recipient (MR7, MR9) profiles, while most samples provided profiles with both recipient and donor fragments (Fig. 4). Obviously, MS and MR ctDNAs having typical *Microcitrus* profiles indicated that these ctDNAs represent cybrids. Since no novel fragments were observed, the samples which showed both donor and recipient ctDNA fragments

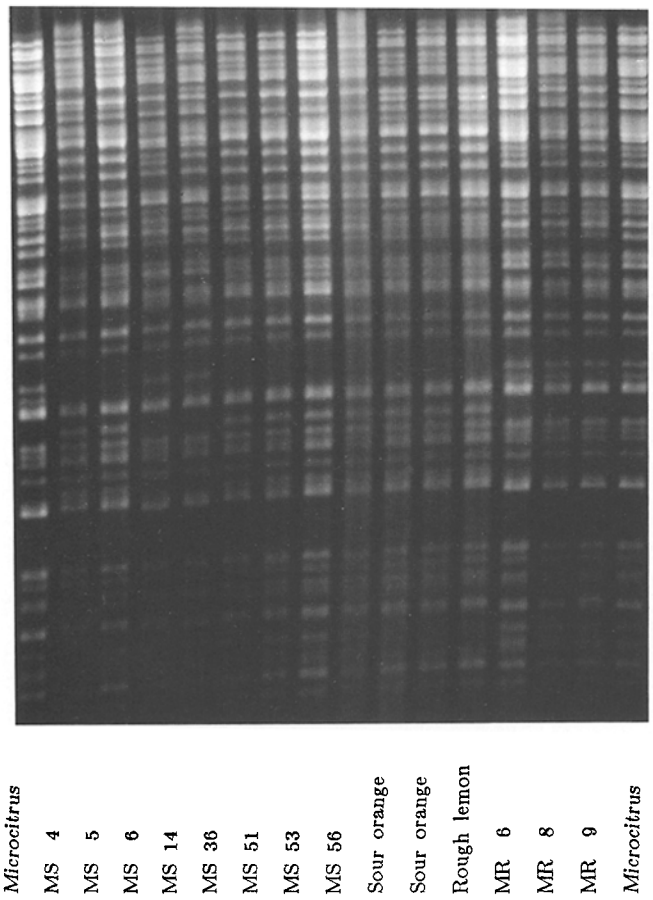

Fig. 2.

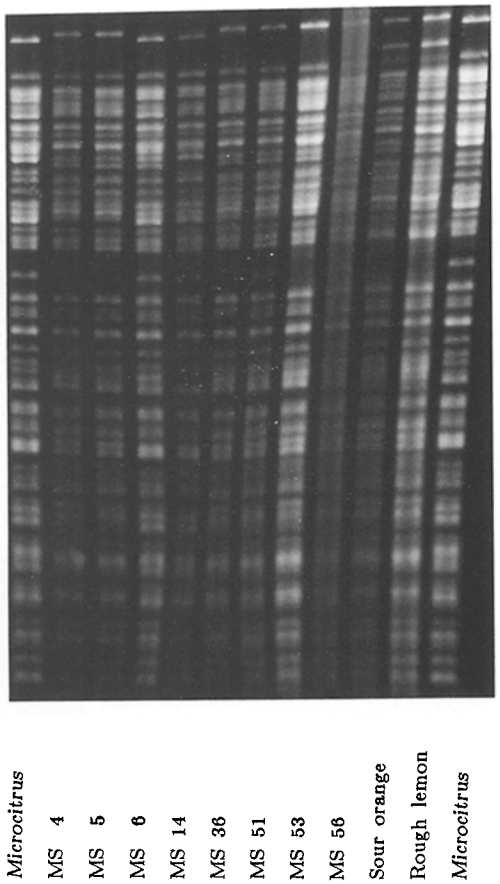

Fig. 3.

Fig. 2. Mitochondrial DNA restriction profiles from *Microcitrus*, Sour orange, Rough lemon, and cybrids derived from MS and MR fusions. *Pst*I digests (2 µg DNA per slot) were fractionated on an agarose gel and stained with ethidium bromide

Fig. 3. Mitochondrial DNA restriction profiles from *Microcitrus*, Sour orange, Rough lemon, and cybrids derived from MS fusions. *Bam*HI digests (2 µg DNA per slot) were fractionated on an agarose gel and stained with ethidium bromide

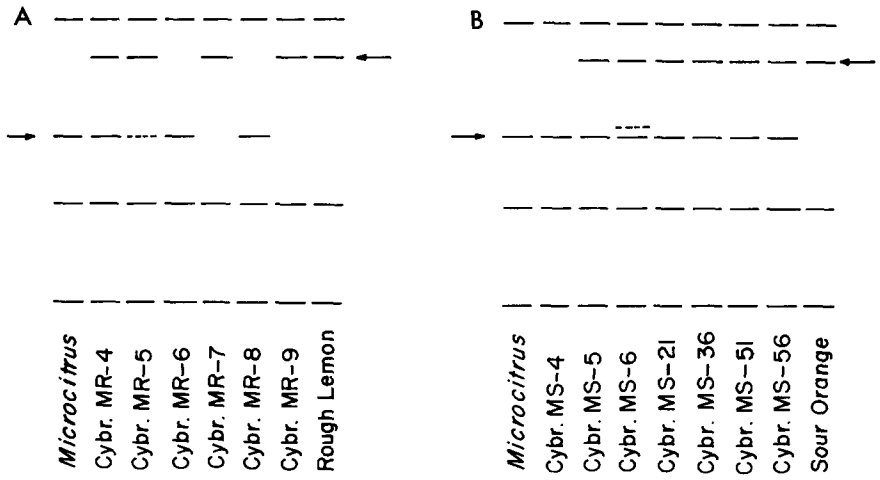

Fig. 4 A and B. Schematic summary of Southern blot hybridizations from *Microcitrus*, Rough lemon, Sour orange, and cybrids derived from MR (**A**) and MS (**B**) fusions. Blots with *Pst*I-digested ctDNA were hybridized with a radiolabelled *Pst*I fragment (Ps2A) from *Nicotiana tabacum* ctDNA

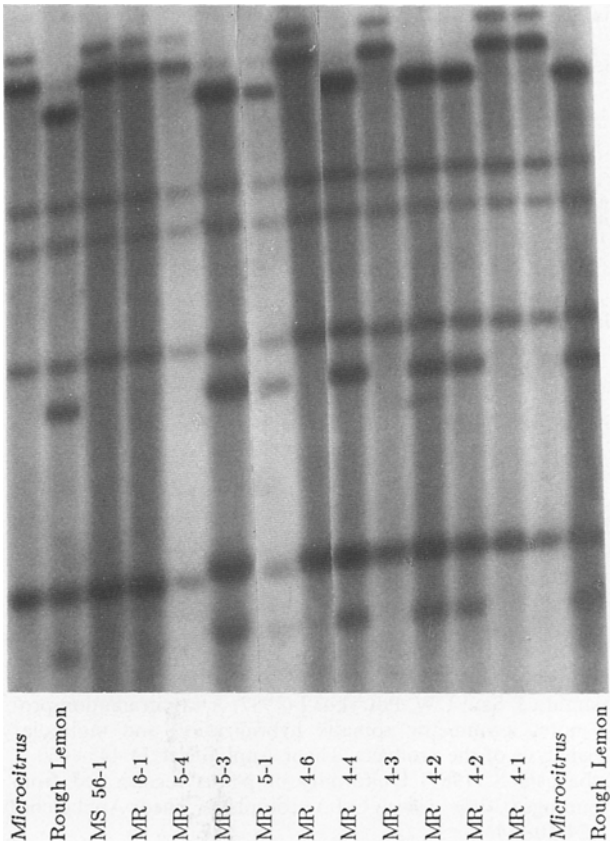

Fig. 5. Southern blot hybridization of ctDNA from *Microcitrus*, Rough lemon and cybrids derived from MS and MR fusions. Blots with *Pvu*II-digested ctDNA were hybridized with a radiolabelled *Pst*I fragment (Ps2A) from *N. tabacum*. The *Pvu*II restriction profile of Rough lemon ctDNA is identical to that of Sour orange (not shown)

could represent mixed tissues or indicate that sorting out of chloroplasts was not completed. We therefore analyzed the ctDNA from leaves of regenerated plants (Fig. 5). These analyses indicated that in all the tested leaves, sorting out of chloroplasts was indeed completed: all the samples had either the recipient's or the donor's ctDNA restriction profiles. Interestingly, among the five MR4 plants, the leaves of three had *Microcitrus* ctDNA and the leaves of two (MR 4-2 and MR 4-4) had Rough lemon ctDNA. We conclude that plants containing ctDNA which was identical to that of the donor (*Microcitrus*) but with morphological features of the recipient should be defined as cybrids.

Discussion

Citrus was the first arboraceous genus in which efficient procedures were established to regenerate functional plants from isolated protoplasts (Vardi et al. 1975, 1982; Kobayashi et al. 1983; Grosser and Chandler 1987; Kobayashi 1987; Sim et al. 1988; Vardi and Galun 1988). These procedures paved the way to protoplast-fusion-derived plants in which orange protoplasts constituted one of the fusion partners, while protoplasts from either *Poncirus trifoliata* (Ohgawara et al. 1985; Grosser et al. 1988 a) or *Severinia disticha* (Grosser et al. 1988 b) were the other fusion partner. Analyses of the nuclear traits of plants derived from these two fusion combinations indicated that somatic hybrids were obtained. No information was supplied on the organelle compositions of these somatic hybrids.

We previously reported (Vardi et al. 1987) on the production of *Citrus* cybrids by the utilization of the donor-recipient protoplast-fusion procedure. The cybrid trees had the nuclear-coded features of the recipient fusion-partners and chondriome components from the donors. In these combinations, the transfer of chloroplasts from the donors to the cybrids could not be analyzed because the respective ctDNAs had identical restriction profiles (Green et al. 1986). In the present study we used recipients and donors which differ substantially in both their plastomes and chondriomes, as revealed by the analysis of their ctDNA and mtDNA restriction profiles.

The iodoacetate treatment of recipient protoplasts was efficient: no colonies were formed after plating of unfused Rough lemon or Sour orange protoplasts, and none of the fusion-derived plants were identical in both nuclear-coded traits and organelle composition to the recipient plants. Indeed, fusion-derived mtDNA samples revealed restriction profiles which were not identical to either of the fusion partners. It should be noted that sorting out of chondriomes is probably an extended process. For example, Aviv and Galun (1987) found that in the first sexual progeny of *Nicotiana* cybrids, the individual plants varied in respect to their mtDNA restriction profiles, while the plants of the next sexual generation were identical to their maternal parents. Thus, in a given cybrid plant there may be more than one chondriome population. Consequently, if *Microcitrus* chondriome components induce male sterility, the very same plant may bear both male-fertile and -sterile flowers.

Sorting out of chloroplasts in *Citrus* cybrids seems to be a quite rapid process. Most ctDNA extracts obtained from the embryogenic calli, derived from fused protoplasts, had both recipient and donor ctDNA fragments. However, the ctDNAs from leaves of regenerated cybrid plants provided restriction profiles which were identical to either the donor's profile or the recipient's profile. Thus, sorting out of chloroplasts was completed in the leaves. In several of the cybrids, sorting out was completed already at the embryogenic callus stage (e.g. MS4, MR6, MR8).

Our study indicated that *Microcitrus* chloroplasts could be transferred into diploid cybrids having *Citrus*

(Rough lemon or Sour orange) nuclear-coded morphology. On the other hand, in certain cybrids (e.g. MR9), the recipients' chloroplasts were retained while *Microcitrus* chondriome components were transferred into these cybrids. Chloroplast transfer by the donor-recipient protoplast-fusion procedure was thus independent from the transfer of chondriome components. In this respect, *Citrus* cybrids are similar to cybrids of annual genera (Galun et al. 1988).

Morphological identity of the cybrids, with the respective recipient fusion-partners, does not exclude the possibility that some nuclear DNA was transferred from *Microcitrus* into the cybrids' nuclear genomes causing some genetic variability. Such variability could be useful for breeding.

One of the purposes of this study was to explore the possibility of utilizing cybridization for *Citrus* breeding. We showed that, indeed, chloroplasts and chondriome components could be transferred into *Citrus* cultivars from an alien genus. The novel *Citrus* cybrids will be analyzed for breeding traits. More specifically, the chondriome components from *Microcitrus* could induce male sterility. Male sterility in *Citrus* fruit cultivars (with potential parthenocarpy) is of immense economical importance – it may lead to seedless fruits. Thus, if indeed male sterility is revealed among the cybrids produced in this study, a novel and efficient means to breed seedless *Citrus* fruits could be explored.

Acknowledgements. This study was supported by the United States – Israel Binational Agricultural Research and Development Fund (BARD) Grant NO. I-468-82, and the Leo and Julia Forschheimer Center for Molecular Genetics. E.G. holds the Irene and David Schwartz Chair for Plant Genetics.

References

Aviv D, Galun E (1987) Chondriome analysis in sexual progenies of *Nicotiana* cybrids. Theor Appl Genet 73:821–825

Aviv D, Arzee-Gonen P, Bleichman S, Galun E (1984) Novel alloplasmic *Nicotiana* plants by "donor-recipient" protoplast fusion: cybrids having *N. tabacum* or *N. sylvestris* nuclear genomes and either or both plastomes and chondriomes from alien species. Mol Gen Genet 196:244–253

Barsby TL, Chuong PV, Yarrow SA, Wu S-C, Coumans M, Kemble RJ, Powel AD, Beversdorf WD, Pauls KP (1987) The combination of polima cms and cytoplasmic triazine resistance in *Brassica napus*. Theor Appl Genet 73:809–814

Esen A, Scora RW (1977) Amylase polymorphism in *Citrus* and some related genera. Am J Bot 64:305–309

Flick CE, Kut SA, Bravo JE, Gleba YY, Evans DA (1985) Segregation of organelle traits following protoplast fusion in *Nicotiana*. Bio/Technol 3:555–560

Galun E, Aviv D (1983) Cytoplasmic hybridization. In: Evans DA, Sharp WR, Ammirato PV, Yamada Y (eds) Handbook of plant cell culture, vol I. Macmillan, New York, pp 358–392

Galun E, Aviv D (1986) Organelle transfer. Meth Enzymol 118:595–611

Galun E, Aviv D, Breiman A, Fromm H, Perl A, Vardi A (1987) Cybrids in *Nicotiana, Solanum* and *Citrus:* isolation and characterization of plastome mutants, pre-fusion treatments, selection and analysis of cybrids. In: Wettstein D von, Chua N-H (eds) Plant molecular biology. Plenum Press, New York, pp 199–207

Galun E, Perl A, Aviv D (1988) Protoplast-fusion mediated transfer of male-sterility and other plasmone-controlled traits. In: Application of plant cell and tissue culture, Ciba Foundation Symp 137. Wiley, Chichester, pp 97–112

Green RM, Vardi A, Galun E (1986) The plastome of *Citrus:* physical map, variation among *Citrus* cultivars and species and comparison with related genera. Theor Appl Genet 72:170–177

Grosser JW, Chandler JL (1987) Aseptic isolation of leaf protoplasts from *Citrus, Poncirus, Citrus* × *Poncirus* hybrids and *Severinia* for use in somatic hybridization experiments. Sci Hortic 31:253–258

Grosser JW, Gmitter FG Jr, Chandler JL (1988a) Intergeneric somatic hybrid plants of *Citrus sinensis* cv Hamlin and *Poncirus trifoliata* cv Flying Dragon. Plant Cell Rep 7:5–8

Grosser JW, Gmitter FG Jr, Chandler JL (1988b) Intergeneric somatic hybrid plants from sexually incompatible woody species: *Citrus sinensis* and *Severinia disticha.* Theor Appl Genet 75:397–401

Imamura J, Saul UW, Potrykus I (1987) X-ray irradiation promotes asymmetric somatic hybridization and molecular analysis of the products. Theor Appl Genet 74:445–450

Kobayashi S (1987) Uniformity of plants regenerated from orange (*Citrus sinensis* Osb.) protoplasts. Theor Appl Genet 74:10–14

Kobayashi S, Uchimiya H, Ikeda I (1983) Plant regeneration from "Trovita" orange protoplasts. Jpn J Breed 33:119–122

Kumar A, Cocking CE (1987) Protoplast fusion: a novel approach to organelle genetics in higher plants. Am J Bot 74:1289–1303

Mettler IJ (1987) A simple and rapid method for minipreparation of DNA from tissue cultured plant cells. Plant Mol Biol Rep 5:346–349

Morgan A, Maliga P (1987) Rapid chloroplast segregation and recombination of mitochondrial DNA in *Brassica napus* cybrids. Mol Gen Genet 209:240–246

Ohgawara T, Kobayashi S, Ohgawara E, Uchimiya H, Ishii S (1985) Somatic hybrid plants obtained by protoplast fusion between *Citrus sinensis* and *Poncirus trifoliata.* Theor Appl Genet 71:1–4

Pelletier G, Primard C, Vedel F, Chetrit P, Remy R, Rouselle R, Renard M (1983) Intergeneric cytoplasmic hybridization in Cruciferae by protoplast fusion. Mol Gen Genet 191:244–250

Robertson D, Palmer JD, Earle ED, Mutschler MA (1987) Analysis of organelle genomes in a somatic hybrid derived from cytoplasmic male-sterile *Brassica oleracea.* Theor Appl Genet 74:303–309

Sidorov VA, Menczel L, Nagy F, Maliga P (1981) Chloroplast transfer in *Nicotiana* based on metabolic complementation between irradiated and iodoacetate-treated protoplasts. Planta 152:341–345

Sim G-E, Loh C-S, Goh C-J (1988) Direct somatic embryogenesis from protoplasts of *Citrus mitis* Blanco. Plant Cell Rep 7:418–420

Swingle WT, Reece PC (1967) The botany of *Citrus* and its wild relatives. In: Reuter W, Webber HJ, Batchelor LD (eds) The citrus industry, vol I. The University of California, Berkeley, pp 190–430

Thomzik JE, Hain R (1988) Transfer and segregation of triazine tolerant chloroplasts in *Brassica napus*. Theor Appl Genet 76:165–171

Vardi A, Galun E (1988) Recent advances in protoplast culture of horticultural crops: Citrus. Sci Hortic 37:217–230

Vardi A, Galun E (1989) Isolation and culture of *Citrus* protoplasts. In: Bajaj YSP (ed) Plant protoplasts and genetic engineering I. Springer, Berlin Heidelberg New York, pp 147–159 (Biotechnology in agriculture and forestry, vol 8)

Vardi A, Raveh D (1976) Cross feeder experiments between tobacco and orange protoplasts. Z Pflanzenphysiol 78:350–359

Vardi A, Spiegel-Roy P, Galun E (1975) Citrus cell culture: isolation of protoplasts, plating efficiencies, effect of mutagens and regeneration of embryos. Plant Sci Lett 4:231–236

Vardi A, Spiegel-Roy P, Galun E (1982) Plant regeneration from *Citrus* protoplasts: variability in methodological requirements among cultivars and species. Theor Appl Genet 62:171–176

Vardi A, Hutchison DJ, Galun E (1986) A protoplast-to-tree system in *Microcitrus* based on protoplasts derived from a sustained embryogenic callus. Plant Cell Rep 5:412–414

Vardi A, Breiman A, Galun E (1987) *Citrus* cybrids: production by donor-recipient protoplast-fusion and verification by mitochondrial-DNA restriction profiles. Theor Appl Gent 75:51–58

Zelcer A, Aviv D, Galun E (1978) Intespecific transfer of cytoplasmic male sterility by fusion between protoplasts of normal *Nicotiana sylvestris* and x-ray irradiated protoplasts of male-sterile *N. tabacum*. Z Pflanzenphysiol 90:397–407