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Molecular characterization of the nuclear and cytoplasmic genomes of intergeneric diploid plants from cell fusion between *Microcitrus papuana* and Rough lemon

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Abstract Simple-sequence-repeat (SSR) and PCR-RFLP were employed to characterize the nuclear and cytoplasmic genomes of intergeneric diploid plants derived from symmetric fusion between *Microcitrus papuana* Swingle and Rough lemon (*Citrus jambhiri* Lush). Three out of five SSR primers distinguished the fusion parents from each other and the regenerated plants showed band profiles completely identical to the leaf parent, Rough lemon. Amplified products from the intergenic regions of cpDNA between *trn*D-*trn*T were digested with *Hae*III and *Msp*I, and those between *trn*H-*trn*K were digested with *Hin*fI, and both the regenerated plants and Rough lemon shared the same band patterns, which were different from the embryogenic parent, *M. papuana*. With mtDNA, only 2 out of 12 primer pair/restriction enzyme combinations (*nad*4 ex 1–2/*Taq*I and *nad*4 ex 1–2/*Hin*dIII) revealed polymorphisms between the fusion parents. With the former combination the regenerated plants showed the same fragment distribution as that of the embryogenic parent, *M. papuana*, whereas with the latter, a novel band absent in the fusion parents was detected in all of the regenerated plants, suggesting a possible rearrangement. The present research indicates that the plants analyzed were putative cybrids containing nuclear DNA and cpDNA from Rough lemon and mtDNA from *M. papuana*. Presumed mechanisms leading to the regeneration of diploid hybrid plants following symmetric fusion are discussed herein.

Keywords Citrus · Chloroplast DNA · Cybrids · *Microcitrus papuana* · Mitochondrial DNA

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Abbreviations *cpDNA*: Chloroplast DNA · *mtDNA*: Mitochondrial DNA · *PCR*: Polymerase chain reaction · *RFLP*: Restriction fragment length polymorphism · *SSR*: Simple sequence repeats

Introduction

Since the first citrus somatic hybrid was obtained from protoplast fusion by Ohgawara et al. (1985), more than 200 somatic hybrids have been produced worldwide (Deng and Liu 1996; Grosser et al. 2000). The majority of the somatic hybrids obtained were tetraploids resulting from additive fusion of two diploid cells. These hybrids are being evaluated as promising rootstocks for citrus industries (Gan et al. 1995; Zhou et al. 1997; Grosser et al. 1998b) and/or used as pollen parents to be crossed with diploid cultivars for production of triploids and development of new seedless cultivars (Oiyama et al. 1991; Jia and Gmitter 1993; Deng et al. 1996; Grosser et al. 1998a). Thus, somatic hybrids could significantly facilitate the genetic improvement of citrus, one of the most important fruit crops in many countries. In addition to practical breeding uses, somatic hybrids also provide unique materials for studying nuclear and cytoplasmic interactions and for creating novel combinations between nuclear and cytoplasmic genomes (mainly mitochondria and chloroplast).

Many unexpected diploid plants have been regenerated following standard symmetric fusion between diploid species (Saito et al. 1993, 1994; Grosser et al. 1996; Liu et al. 1999, 2000). These plants were called leaf-parent-type ones because they were morphologically identical to the leaf parents used in the fusion. Subsequent analyses based on molecular markers of these plants have indicated that they are actually alloplasmic hybrids or cybrids. Most of these plants arose from interspecific combinations (Deng et al. 1993, 2000; Saito et al. 1993, 1994; Yamamoto and Kobayashi 1995; Moriguchi et al. 1997). *Microcitrus* confers resistance to both biotic stresses, including burrowing nematode and *Phytophthora*, and abiotic stresses such as

drought and flood. Rough lemon (*Citrus jambhiri* Lush) is an important rootstock in many parts of the world, but it is sensitive to *Phytophythora*. Standard symmetric fusion between *Microcitrus papuana* Swingle and Rough lemon was carried out with the aim of producing a new rootstock combining the desirable agronomic traits of these two species. The resulting plants derived from the fusion were found to be diploid, morphologically identical to Rough lemon, and not tetraploid as expected (Liu et al. 1999). To our knowledge, this is the first report on regeneration of such diploid plants from cell fusion between two remote genera in perennial woody plants. Similar diploid plants have also been obtained from another intergeneric fusion combination between sour orange (*Citrus aurantium*) and *M. papuana* (Liu et al. 2000).

As mentioned above, molecular characterization of genome composition has been extensive in many interspecific citrus cybrids, but the nature of intergeneric diploid plants derived from cell fusion of remote genera is still unknown. In order to ascertain the origin of the nuclear and the cytoplasmic genomes in the hybrid plants and then to compare it with that of the interspecific somatic hybrids, two molecular markers systems, simple-sequence-repeat (SSR, or microsatellite) and PCR-RFLP and/or CAPS (cleaved amplified polymorphic sequences), were used in the present research. Another target of the present study is to provide more evidence for the mechanism of regeneration of this kind of plant (which previously mainly resulted from interspecific combinations) from standard fusion. These molecular systems have been successfully employed in phylogenetic studies (Tsumura et al. 1996; Li et al. 2000), cultivar identification (Rongwen et al. 1995; Mohanty et al. 2001), molecular diversity analysis (Sicard et al. 1999), genetic mapping (Chen et al. 1997; Kijas et al. 1997; Diwan et al. 2000) and identification of somatic hybrids (Bastia et al. 2001). Herein, we report the nuclear and cytoplasmic compositions of intergeneric diploid plants derived from protoplast fusion between *M. papuana* and Rough lemon, as revealed by these molecular marker systems.

Materials and methods

Plant materials

Greenhouse-grown plants and embryogenic cultures of *M. papuana* maintained in MT (Murashige and Tucker 1969) medium were used in the study. The leaf-parent-type plants were obtained by electrofusion between embryogenic protoplasts of *M. papuana* and leaf-derived protoplasts of Rough lemon (*C. jambhiri*) (Liu et al. 1999).

DNA extraction

Total DNA was extracted from 5–8 g of healthy fresh leaves following the SDS method modified from Xiao et al. (1995) and Shi et al. (1998), and further purified with phenol and chloroform. RNAs in the DNA preparation were removed by incubation with RNase. The resultant DNA pellet was dissolved in 400 µl TE buffer (10 m*M* Tris-HCl, 0.1 m*M* EDTA), followed by concentration assay using a UV1601 spectrophotometer (Shimadzu, Japan). The solution was then diluted to 25 ng/µl and subjected to further analysis.

Table 1 Primer pairs used for the nuclear genome analysis. Source Kijas et al. 1997

Locus	Primer sequence
TAA1	5′-GACAACATCAACAACAGCAAGAGC
	5′-AAGAAGAAGAGCCCCCATTAGC
TAA15	5′-GAAAGGGTTACTTGACCAGGC
	5′-CTTCCCAGCTGCACAAGC
TAA45	5′-GCACCTTTTATACCTGACTCGG
	5′-TTCAGCATTTGAGTTGGTTACG
TAA41	5′-AGGTCTACATTGGCATTGTC
	5′-ACATGCAGTGCTATAATGAATG
TAA52	5′-GATCTTGACTGAACTTAAAG
	5′-ATGTATTGTGTTGATAACG

SSR analysis

Primers used were based on those of Kijas et al. (1997). Their sequences are described in Table 1. Procedures for PCR amplification were modified from the protocols described by Rongwen et al. (1995) and Kijas et al. (1997). Each 20 µl reaction mixture consisted of 50 ng genomic DNA, 1× reaction buffer [67 m*M* Tris-HCl, pH 8.8; 16.6 m*M* $(NH_4)_2SO_4$; 1.5 m*M* $MgCl_2$], 0.2 m*M* each dNTP, 1.0 U *Taq* DNA polymerase and 0.1 µ*M* of each primer. A Peltier-200 thermocycler (PTC-200, MJ Research, Waltham, Mass.) was used for amplification. Thermocycling programs were based on those of Kijas et al. (1997) and Ruiz et al. (2000) with minor modification: denaturation at 94°C for 1 min, annealing at 55°C for 30 s and extension at 72°C for 1 min for 32 cycles, followed by an end cycle of 4 min at 72°C. The amplified products were mixed with an equal volume (20 µl) of loading dye (98% formamide, 0.025% bromophenol blue, 0.05% xylene cyanol), before being denatured at 94°C for 4 min. An aliquot (4 µl) of each sample was loaded on a sequencing gel containing 6% polyacrylamide, 7 *M* urea and 0.5× TBE, and run at 60 W constant power for 2 h (EC sequencing system). Silver staining was used to visualize the gels with staining kit Q4130 according to the protocol provided by the manufacturer (Promega).

PCR-RFLP analysis

For the chloroplast genome, two intergenic regions were characterized using two pairs of universal primers, *trn*H-*trn*K and *trn*D-*trn*T (Demesure et al. 1995), and a panel of restriction endonucleases were applied to the amplified products. For the mitochondrial genome, PCR primers targeted to *nad*4 exons 1–2 (Demesure et al. 1995) were used for the amplification and the resultant products were subjected to 12 restriction endonucleases.

PCR amplifications were conducted in the PTC-200 thermocycler. The reaction mixture contained 50 ng genomic DNA, 67 m*M* Tris-HCl (pH 8.8), 16.6 m*M* $(NH_4)_2SO_4$, 2.0 m*M* $MgCl_2$, 0.2 m*M* of each dNTP, 1.2 U *Taq* DNA polymerase and 0.2 µ*M* of each primer in a total volume of 25 µl. The amplification programs were those of Bastia et al. (2001). An aliquot (5 µl) of PCR product was digested with 5 U of restriction enzymes for 4–5 h, followed by electrophoresis in a 2.0% agarose gel containing 0.5 µg/ml ethidium bromide (for cpDNA and mtDNA) or in a 6% sequencing polyacrylamide gel (for mtDNA). The agarose gel was subjected to electrophoresis at 2.5 V/cm for 2–3 h and then visualized under UV, while the sequencing gel was silver stained as described above. A 100 bp ladder was used as DNA size marker (MBI Fermentas, St. Leon Rot, Germany).

Results and discussion

Morphology of the regenerated plants

In total, seven plants were regenerated from the symmetric fusion between *M. papuana* and Rough lemon. Cytological determination has demonstrated that all were diploids with 18 chromosomes (Liu et al. 1999), which was further confirmed by flow cytometric analysis (data not shown). The diploid plants were morphologically identical to the leaf parent, Rough lemon, but quite different from the embryogenic parent, *M. papuana*. They had large leaves with winged petioles with a leaf index similar to that of Rough lemon (data not shown). The trees grew very fast, similar to the growth vigor of Rough lemon (Fig. 1). This has been shown in previous reports for other diploid cybrids recovered, which were also morphologically identical to the leaf parent (Deng et al. 1993, 2000). Since the protoplasts isolated from leaves could not divide and undergo subsequent embryogenesis (Grosser and Gmitter 1990), it could be concluded that such plants were somatic hybrids, judging from their morphology.

Fig. 1 Intergeneric diploid plants derived from protoplast fusion between *Microcitrus papuana* Swingle and Rough lemon (*Citrus jambhiri*)

Nuclear genome composition

Of the five SSR primers used, three – namely TAA1, TAA15 and TAA45 – showed polymorphisms between *M. papuana* and Rough lemon. The amplified products of these polymorphic primers showed similar band patterns in the regenerated plants to those of Rough lemon (Fig. 2). Specific bands of *M. papuana* were not detected in any band profiles of the regenerated plants. These data seem to indicate that the plants inherited their nuclear genomes from the leaf parent, Rough lemon, and that the embryogenic parent, *M. papuana*, did not contribute nuclear components to the regenerated plants. This observation is in agreement with previous reports with respect to interspecific (Saito et al. 1993, 1994; Yamamoto and Kobayashi 1995; Grosser et al. 1996; Moriguchi et al. 1997; Liu and Deng 2000) and intergeneric combinations (Moreira et al. 2000a, b).

Cytoplasmic genomes composition

After amplification with the universal primers, only a single band of the same size was obtained from both fusion parents. The primer pairs, *trn*H-*trn*K and *trn*D-*trn*T, used for cpDNA characterization, gave clear and reproducible band patterns between the fusion parents after

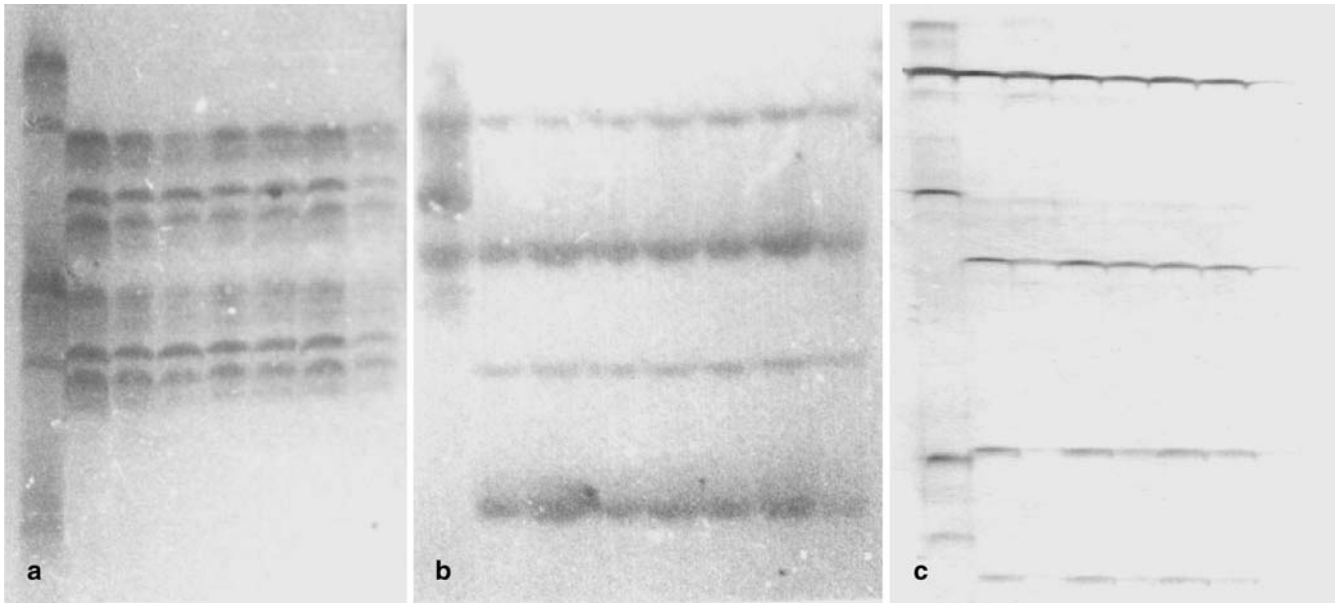

Fig. 2a–c Analysis of nuclear genome of the intergeneric diploid plants by simple sequence repeats (SSR). Lanes (from left to right): *M. papuana* Swingle, Rough lemon, and regenerated plants 1–6, respectively. The primers in **a**, **b** and **c** are TAA15, TAA45 and TAA1, respectively

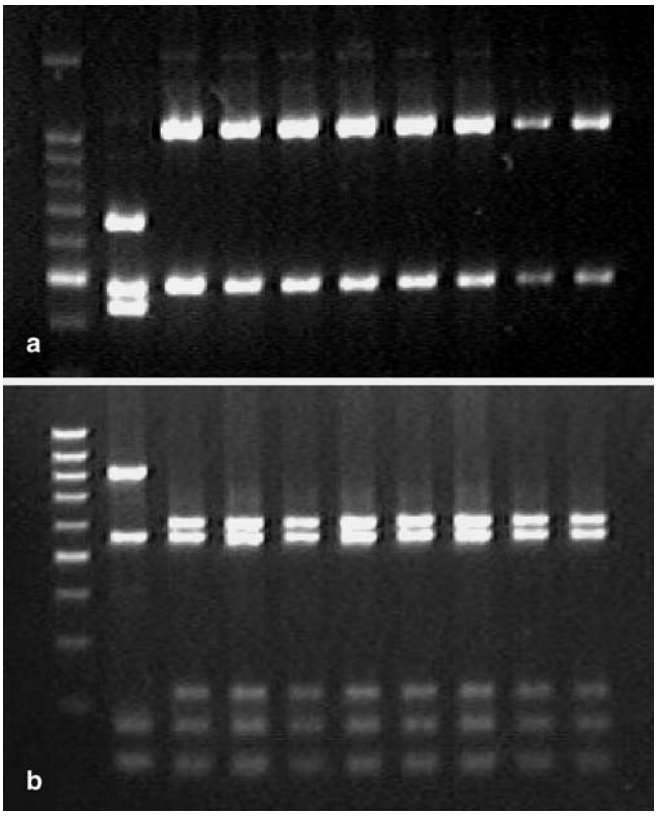

Fig. 3a, b PCR-RFLP analysis of chloroplast genome of the intergeneric diploid plants. Lanes (from left to right): DNA size marker, *M. papuana* Swingle, Rough lemon, and regenerated plants 1–6, respectively. Primer-enzyme combinations in **a** and **b** are *trn*D-*trn*T/*Hae*III, *trn*H–*trn*K/*Hin*fI, respectively

subsequent digestion with the restriction nucleases *Hin*fI, *Hae*III and *Msp*I (data not shown). PCR-RFLP of *trn*D-*trn*T with *Hae*III showed that *M. papuana* and Rough lemon had three fragments (680 bp, 480 bp and 440 bp) and two fragments (1,100 bp and 500 bp), respectively (Fig. 3a). Restriction digestion of *trn*H-*trn*K products with *Hin*fI revealed that *M. papuana* had one unique band of 830 bp and Rough lemon had two unique bands (630 bp and 200 bp), while they shared three common bands (570 bp, 190 bp and 160 bp) (Fig. 3b). This indicates that Rough lemon contained one extra *Hin*fI restriction site within the 830 bp region as compared to *M. papuana*. With both these universal primers the regenerated plants showed the same PCR-RFLP patterns as the leaf parent. No fragments specific to *M. papuana* were identified in the hybrid plants, which seems to imply that the hybrid plants acquired their cpDNA from the leaf parent. All of the regenerated plants shared similar band profiles for the above mentioned primer/enzyme combinations, indicating stable inheritance of cpDNA in the regenerated hybrid plants.

With mtDNA, only 2 of the 12 primer pair/restriction enzyme combinations (namely, *nad*4 ex 1–2/*Taq*I and *nad*4 ex 1–2/*Hin*dIII) revealed polymorphisms between *M. papuana* and Rough lemon, indicating limited variations in the intergenic regions. When the combination *nad*4 ex 1–2/*Taq*I was used, all of the fragments from *M. papuana* were detected in the regenerated plants (Fig. 4a). For the other primer pair/enzyme combination, a novel band absent in the fusion parents were detected in all of the regenerated plants (Fig. 4b), indicating possible rearrangements in some genomic regions as described in citrus hybrids by Moreira et al. (2000a, b) and Moriguchi et al. (1997), and in potato (Harding and Millam 2000), tobacco (Galun et al. 1982), petunia (Rothenberg et al. 1985), carrot (Matthews and Widholm 1985) and oil rapeseed (Chetrit et al. 1985). Results of SSR analyses, in conjunction with PCR-RFLP, confirmed the nature of the intergeneric diploid plants

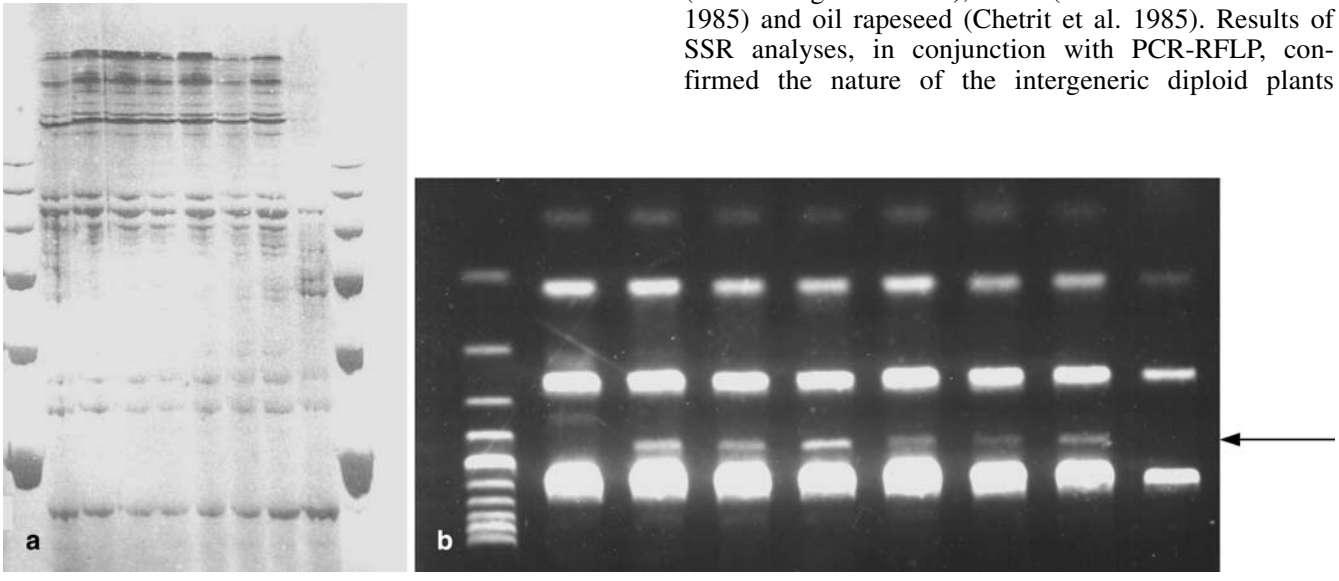

Fig. 4 Analysis of mitochondrial genome of the intergeneric diploid plants by PCR-RFLP with primer-enzyme combinations *nad*4 ex 1–2/*Taq*I (**a**) and *nad*4 ex 1–2/*Hin*dIII (**b**). **a** Lanes (from left to right): DNA size marker, *M. papuana* Swingle, regenerated plants 1–6, Rough lemon, and DNA size marker, respectively. **b** Lanes (from left to right): DNA size marker, *M. papuana*, regenerated plants 1–6, Rough lemon, respectively. The *arrowhead* shows the novel band present in the hybrid plants

derived from cell fusion between *M. papuana* and Rough lemon as true cybrids.

The present research showed that all of the intergeneric diploid plants inherited their cpDNA and mtDNA from Rough lemon and *M. papuana*, respectively, similar to previous reports for different interspecific combinations (Saito et al. 1993, 1994; Yamamoto and Kobayashi 1995; Moriguchi et al. 1997; Liu and Deng 2000). It has been shown elsewhere that almost all of the citrus hybrids or cybrids derived from protoplast symmetric fusion received their mtDNA from the embryogenic parents involved in the fusions (Kobayashi et al. 1991; Saito et al. 1993, 1994; Yamamoto and Kobayashi 1995; Grosser et al. 1996; Moriguchi et al. 1997; Liu and Deng 2000; Moreira et al. 2000a, b). Only Moreira et al. (2000a, b) and Moriguchi et al. (1997) detected mtDNA rearrangements in cybrids derived from symmetric fusion events. Moreira et al. (2000b) attributed the formation of such novel fragments to post-fusion mtDNA rearrangements. In contrast with mtDNA, random segregation of cpDNA was reported, not only in citrus somatic hybrids but also in other crops such as potato (Cardi et al. 1999; Harding and Millam 2000), tomato (Derks et al. 1991), etc. For some combinations cpDNA similar to the leaf parent was detected, whereas for other combinations cpDNA of the hybrids might be inherited from the embryogenic parent, similar to the results herein. In some cases, even plants derived from the same fusion combination may have different modes of inheritance for cpDNA, with some possessing cpDNA from one fusion parent and others from another parent. Interestingly, both parental cpDNA types were detected in 14 somatic hybrids derived from fusion between *Citropsis gilletiana* and Succari sweet orange (Moreira et al. 2000b), which does not happen so often in citrus somatic hybrids derived from cell fusion.

To date, diploid cybrid plants have been regenerated from nearly 30 fusion combinations in *citrus*. However, the exact mechanism for this phenomenon has not been explained as yet. Three hypotheses have been offered: first, an asynchronous mitotic cycle and different division speed leads to chromosome elimination of the embryogenic parent (Saito et al. 1994). If this were the case, the conclusion could be drawn that the elimination was embryogenic parent-oriented, which means that only chromosomes from the embryogenic parent were lost. Another hypothesis involves a failure in nuclear fusion following protoplast fusion and subsequent sorting out of the embryogenic parent's nucleus. Finally, uptake of mtDNA from the embryogenic parent could initiate the division and subsequent embryogenesis of leaf-derived protoplasts. It is well known that embryogenesis is a process needing a large quantity of energy. Since mitochondria are the cell power plants, the uptake of mitochondria from the embryogenic parent could meet the energy needs for embryogenesis. However, the question persists to understand why mainly mtDNA of the embryogenic parent was detected in the regenerated plants. Moreira et al. (2000a) demonstrated that the amount of mtDNA present in suspension cells was about four times that present in the leaf-derived cells, which implied a preferential transmission and detection owing to the relative abundance of this organelle in embryogenic cells. Therefore, consistent transfer of mtDNA from the embryogenic parent to the regenerated plants was observed. Molecular characterization of the diploid plants suggested that mtDNA might play a significant role in regeneration of such plants (Saito et al. 1993; Grosser et al. 1996). The result of the present research fully complements previous work concerning interspecific combinations. In addition, molecular analyses concerning mtDNA of all citrus cybrids derived from symmetric cell fusion, to some degree, support the last hypothesis mentioned above. However, further work is still needed to acquire more detailed and more convincing evidence for this unique phenomenon in citrus somatic hybridization.

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