Application of Cleaved Amplified Polymorphic Sequence Method for Analysis of Cytoplasmic Genome among *Aurantioideae* Intergeneric Somatic Hybrids

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ABSTRACT. Somatic hybridization allows the creation of new patterns of nuclear, mitochondrial and chloroplastic association. It is therefore necessary to master cytoplasmic molecular markers to determine the genetic origin of both organelles of plantlets obtained from protoplasts fusion. In the case of *Citrus* and related genera, only southern blot hybridization and restriction fragment-length polymorphism (RFLP) techniques were used for this task until now. Here, we describe the use in the *Aurantioideae* subfamily, of a simple and non labeling cleaved amplified polymorphic sequence (CAPS) technique, to determine the cytoplasmic genome origin of intergeneric somatic hybrids. Mitochondrial and chloroplastic universal primers previously selected for population genetic studies in *Quercus* by Demesure et al. (1995) are used with some modifications. The variability of cytoplasmic genome among somatic fusion partners is detected by coupling amplification and restriction reactions. Digested DNA fragments are analyzed by agarose gel electrophoresis (PCR-RFLP). This technique has been applied for the analysis of the cytoplasmic constitution of somatic hybrids arising from intergeneric, intersubtribal and intertribal combinations. Systematic transmission of the mitochondria from protoplasts isolated from embryogenic callus parents was confirmed.

Somatic hybridization is a way of increasing genetic variability of the gene pools, not only by overcoming sexual incompatibility or sterility, but also by combining nuclear, chloroplastic and mitochondrial genomes in new patterns. In *Citrus* this technique has major applications for ploidy manipulation for the creation of seedless triploid scion hybrids (Grosser, et al., 1992; Ollitrault et al., 1998a, 2000a) and to cumulate resistance traits for rootstock breeding (Grosser et al., 1996a, 1998; Ollitrault et al., 1998b). Interesting traits of tolerance for biotic and abiotic stresses are also present in distant genera such as *Glycosmis*, *Murraya*, *Triphasia* or *Clausena*, that display sexual incompatibility with *Citrus* (Iwamasa et al., 1988). The important progress in somatic hybridization has made it possible to bypass these incompatibility barriers (Grosser et al., 1996b; Guo and Deng, 1998, 1999; Hidaka et al., 1992). At this level of genetic distance, the nucleocytoplasmic interaction should have a strong impact in plant development. The characterization of mitochondrial and chloroplastic genomes, as well as the nuclear genome, are essential for further genetic studies.

Nuclear diversity is very high at the intergeneric level and the nuclear genome of the somatic hybrids can be rapidly explored at the earlier steps of plant development by varied molecular markers including isozymes, restriction fragment-length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPD) or single tagged microsatellite Sequence (STMS). Contrary to the nuclear genome, cytoplasmic DNA sequences are highly conserved for both chloroplast (Palmer and Stein, 1986) and mitochondria (Schuster et al., 1990). Moreover, cytoplasmic genome

analyses have shown a lack of polymorphism in various species complexes like *Citrus* (Vardi et al., 1987) and *Quercus* (Demesure et al., 1995), especially between more closely related species. However, polymorphic noncoding sequences are observed in both organelles DNA (Palmer, 1987; Palmer et al., 1988), flanking the highly conserved coding regions. This polymorphism is due to alteration of genes arrangement or some substitutions, additions or deletions during evolution. Taberlet et al. (1991) and Demesure et al. (1995) take advantage of this polymorphism to develop polymerase chain reaction (PCR) cytoplasmic markers. They define universal primers in highly conserved sequences allowing for amplification of flanking noncoding regions. They also demonstrate their efficiency to display polymorphism in height taxa. Cytoplasmic genome analyses among *Aurantioideae* plants were done principally by Southern blot hybridization (e.g., Grosser et al., 1996b; Kobayashi et al., 1991; Vardi et al., 1987). This method is powerful in the detection of polymorphism but it is expensive, time consuming and requires a higher fresh weight of plant tissues than PCR techniques. A preliminary work using mitochondrial and chloroplastic universal primers described by Demesure et al. (1995), coupling PCR and RFLP techniques was developed (Luro and Ollitrault, 1996). This method proved more efficient for cpDNA polymorphism detection than for mtDNA.

In the present study, the same standard set of primers was tested to amplify homologous segment of mtDNA and cpDNA from *Citrus aurantifolia*, 'Carrizo' citrange and three wild genera related to *Citrus*: *Clausena excavata*, *Triphasia trifolia*, and *Murraya paniculata*. A study of combination of amplification and restriction reaction with various endonucleases was conducted to detect polymorphism between Mexican lime (*Citrus aurantifolia*) and these four genotypes involved in protoplast fusion experiments.

Some results concerning using this technique among the true

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Citrus group for parental chloroplasts segregation study between corresponding somatic hybrids have been previously published in a synthetic review (Ollitrault et al., 2000b). Here we present the detailed methodology. This technique designated as cleaved amplified polymorphic sequence (CAPS) is applied here for the first time for cytoplasmic characterization of intergeneric somatic hybrids. A similar method has also been recently used in *Citrus* for cpDNA phylogenetic analysis (Nicolosi et al., 2000, Ollitrault et al., 2000c).

Materials and Methods

PLANT MATERIAL. DNA was extracted from leaves of grafted trees of Mexican lime (*Citrus aurantifolia* (Chrism.) Swing) and 'Carrizo' citrange, nucellar seedlings of *Triphasia trifolia* (Burm. F.) P. Wils, *Murraya paniculata* (L.) Jack. and zygotic seedlings of *Clausena excavata* (Burm.F.). Nucellar origin of *Triphasia trifolia* seedlings was confirmed by isozyme analysis.

TOTAL DNA EXTRACTION. DNA was extracted as described by Risterucci et al. (2000), from 500 mg of fresh material.

SOMATIC HYBRID ANALYSIS. Somatic hybrids were obtained by electrofusion of embryogenic nucellar callus-derived protoplasts of Mexican lime with nucellar organogenic callus-derived protoplasts of 'Carrizo' citrange, androgenetic-embryogenic callus-derived protoplasts of *Clausena excavata* and leaf-derived protoplasts of *Triphasia trifolia* and *Murraya paniculata*. Hybrid status of the nuclear genome of the regenerated plants or embryos have been previously demonstrated by isozymes and microsatellites analyses (Froelicher, 1999). Ploidy evaluation was done by flow cytometry by the same author. The cytoplasmic genomes of two somatic hybrid plants or embryos of each combination are characterized by CAPS in the present study.

CAPS CONDITIONS. The 13 pairs of universal cytoplasmic primers tested are as described by Demesure et al. (1995) The primers and PCR conditions selected are described in Table 1.

DNA AMPLIFICATION. The PCR reaction mixture (25 µL) contained 67 mM Tris-HCl (pH 8.8), 16 mM (NH₄)₂SO₄, 0.01%

Tween 20, 0.2 µM of each primer and 300 µM of dNTP (Eurobio), 1.5 or 2 mM MgCl₂, 0 or 4% glycerol (depending on the primer pair used, Table 1), 0.5 unit of Taq DNA polymerase (Eurobio) and 50 ng of sample DNA. The mixture was covered with a drop of mineral oil, and the reaction was performed in a DNA thermal cycler (model PTC-100; MJ Research), programmed for an initial denaturing cycle of 4 min at 94 °C then 30 cycles of 45 s denaturation at 92 °C, 45 s annealing at 55 or 58 °C (depending on the primer pair used, Table 1), 3 min elongation at 72 °C and a final step of 10 min at 72 °C to complete the synthesis of DNA strands.

DNA RESTRICTION. Amplified DNA fragments were digested using four- to six-base recognition restriction endonucleases (*Dra*I, *Alu*I, *Bsp* 143-I, *Hae* III, *Rsa*I, *Eco*R-I, *Mva*I, *Hinf*I, *Hind* III, *Ava* II and *Ama* 87-I) (Eurogentec or Amersham), in a final volume of 25 µL containing 1× specific buffer (Eurogentec or Amersham) for each restriction enzyme, 5 unit endonuclease and 15 µL amplification product. Reaction medium was incubated for 3 h at 37 °C.

DNA ANALYSIS. Native and digested amplification products were separated by electrophoresis in 1.8% agarose gel with TBE 1× during 5 h and then visualized by UV fluorescence after staining with ethidium bromide (3 µg·mL⁻¹). Sizes of separated fragments were estimated by comparison with the DNA ladder 1 kb (0.5 to 10 kb) (Sigma).

Results

CAPS METHOD DEVELOPMENT. Among the 13 pairs of universal mitochondrial and chloroplastic primers described by Demesure et al. (1995) and that we tested under different concentrations of MgCl₂ and glycerol, we have obtained amplifications with five primer pairs: three specific for the chloroplast genome (*psaA/trnS*, *trnM/rbcL*, and *trnH/trnK1*) and two for the mitochondria genome (*nad41/nad42* and *nad42/nad44*). Other chloroplast amplification primers were searched by comparing the primer sequences to the *Zea mays*, *Oryza sativa*, and *Nicotiana tabacum*

Table 1. PCR conditions for cpDNA and mtDNA analysis on *Citrus* with the 7 pairs of universal primers derived from Demesure et al. (1995).

Primer 1	Primer 2	Observed length (bp) in <i>Citrus</i>	Annealing temp (°C)	MgCl ₂ (mM)	Glycerol (µL)
Chloroplast primers					
<i>psaA</i> [PSI (P 700 apoprotein A1)] 5'-ACTTCTGGTTCCGGCGAACGAA-3'	<i>trnS</i> [tRNA-Ser(GGA)] 5'-AACCACTCGGCCATCTCTCCTA-3'	3 054	58.0	2	0
<i>trnM</i> [tRNA-Met (CAU)] 5'-TGCTTTTCATACGGCGGAGT-3'	<i>rbcL</i> [RuBisCo large subunit] 5'-GCTTTAGTCTCTGTTGTGG-3'	3 000	58.0	2	0
<i>trnH</i> [tRNA-His (GUG)] ² 5'-ACGGGAATTGAACCCGCGCA-3'	<i>trnK2</i> [tRNA-Lys (UUU) exon 2] 5'-CAACGGTAGAGTACTCGGCTTTTA-3'	4 072	55.0	1.5	1
<i>trnC</i> [tRNA-Cys (GCA)] ³ 5'-CCAGTCAAATCYGGGTGYC-3'	<i>trnD</i> [tRNA-Asp (GUC)] 5'-GGGATTGTAGTTCATTGGT-3'	3 500	55.0	2	0
<i>trnT</i> [tRNA-Thr (GGU)] ⁴ 5'-CCCTTTAACTCAGTGGTAG-3'	<i>trnD'</i> [tRNA-Asp (GUC)] 5'-ACCAATTGAACATAATCCC-3'	1 850	55.0	2	0
Mitochondrial primers					
<i>nad 41</i> : <i>nad4</i> exon 1 5'-CAGTGGGTGGTCTGGTATG-3'	<i>nad 42</i> : <i>nad4</i> exon 2 5'-TCATATGGGCTACTGAGGAG-3'	2 036	58.0	1.5	1
<i>nad 42'</i> : <i>nad4</i> exon 2 5'-TGTTTCCCGAAGCGACACTT-3'	<i>nad 44</i> : <i>nad4</i> exon 4 5'-GGAACACTTTGGGGTGAACA-3'	3 054	55.0	2	1

²New combination, compare to Demesure et al. (1995).

³*trnC* is degenerated for two different bases, compare to Demesure et al. (1995).

⁴*trnT* sequence is reversed, compare to Demesure et al. (1995).

Table 2. Universal primer pairs and restriction enzymes tested to detect cytoplasmic DNA polymorphism between some genera among *Aurantioideae* subfamily. For the same primer pair and endonuclease (the same line in the table), identical numbers indicate identical cleaved amplified polymorphic sequence profiles, and different numbers indicate cytoplasmic polymorphism between the individuals.

Primer pair endonuclease	Cleaved amplified polymorphic sequence				
	<i>Citrus aurantifolia</i>	Carrizo citrange	<i>Triphasia trifolia</i>	<i>Murraya paniculata</i>	<i>Clausena excavata</i>
TrnH/trnK2	Dra-I	1	1	1	2
	Ava-II	1	1	2	4
	EcoR-V	1	1	1	2
	Mva-I	1	1	2	4
	Hae-III	1	1	2	3
	BamH-I	---	1	1	2
TrnT/trnD	Alu-I	1	1	2	3
	Bsp143-I	1	2	3	4
	Dra-I	1	2	1	1
TrnC/trnD	Ama87-I	1	2	3	4
	Alu-I	1	2	3	---
	Hae-III	1	2	3	---
	Rsa-I	1	2	2	---
	EcoR-I	1	2	3	---
psaA/trnS	Hind-III	1	1	2	1
	EcoR-I	1	1	1	2
TrnM/rbcL	Mva-I	1	1	1	2
nad41/nad42	Hinf-I	1	1	2	3
	Mva-I	1	1	1	2
nad42/nad44	Ava-II	1	1	2	2
	Hinf-I	1	1	1	2

²Not determined.

sequences obtained from the Organelle Genome Megasequencing Program (OGMP: <http://megasun.bch.unmontreal.ca>). Primers of two pairs defined by Demesure et al. (1995), one that gives a product for citrus (*trnH/trnK1*) and the other that does not (*trnK1/trnK2*), have been combined, which provides the *trnH/trnK2* pair of primers that amplify a longer fragment. The *trnC* primer was degenerated for two bases which allowed us to obtain a product in association with the primer *trnD*. The *trnT* primer was reversed allowing us to obtain a product associated with the *trnD'* primer (Table 1).

The PCR amplification conditions have been optimized by adding 4% (v/v) of glycerol in the case of three primer pairs (Table 1). This effect had been previously described by Lu and Negre (1993). We also enhanced the efficiency and the specificity of PCR amplification by decreasing $MgCl_2$ concentration from 2 to 1.5 mM in the case of *nad41/nad42* and *trnH/trnK2*. The effect of low $MgCl_2$ concentrations on PCR amplification improvement is known (Larzul, 1993). Furthermore, we slightly modified annealing temperature for *trnM/rbcL* and *nad41/nad42* and optimized it for the three new primer pairs (*trnH/trnK2*; *trnC/trnD* and *trnT/trnD'*) (Table 1).

The new PCR conditions determined for *Aurantioideae* permitted amplification of a single product per primer pair. No polymorphism was detected between the somatic fusion partners which are at the origin of intergeneric hybrids studied here. Various endonucleases were used to digest the different amplified fragments. Table 2 presents different primer pair/restriction enzyme combinations showing polymorphisms between parents. No polymorphism was observed with this technique in mitochondrial products among genera belonging to the *Citrus* group (*Citrus aurantifolia* and 'Carrizo' citrange which is known to have the sweet orange cytoplasmic organelles). In contrast, both

cpDNA and mtDNA of *Citrus* can be differentiated from those of less related genera (*Clausena*, *Murraya* and *Triphasia*). Examples of plastid CAPS obtained with *trnT/trnD'* primers coupled with *Alu-I* or *Bsp143-I* and mitochondrial CAPS obtained with *nad41/nad42* primers coupled with *Hinf-I* are provided respectively in Figs. 1 and 2.

CYTOPLASMIC GENOME ORIGIN OF INTERGENERIC SOMATIC HYBRIDS. Two somatic hybrid plants or embryos from each combination were examined in this study. The cpDNA and mtDNA

Fig. 1. Chloroplast genome cleaved amplified polymorphic sequence analysis of somatic hybrid parents. Electrophoresis on 1.8% agarose gel of fragments amplified by *trnT/trnD'* and digested with *Alu-I* (1–5) and *Bsp143-I* (6–10). Lane 1, 6 = mexican lime; lane 2, 7 = 'Carrizo' citrange; lane 3, 8 = *Triphasia trifolia*; lane 4, 9 = *Clausena excavata*; lane 5, 10 = *Murraya paniculata*.

Fig. 2. Mitochondrial genome cleaved amplified polymorphic sequence analysis of somatic hybrid parents. Electrophoresis on 1.8% agarose gel of fragments amplified by *nad41/nad42* and digested with *Hinf*-I. Lane 1 = Mexican lime; lane 2 = 'Carrizo' citrange; lane 3 = *Triphasia trifolia*; lane 4 = *Murraya paniculata*; lane 5 = *Clausena excavata*.

analyses indicated that each of the eight somatic hybrids showed unilateral transmission

for both organelle genomes. No plantlet or embryo contained a mix of parental chloroplast or a mix of parental mitochondrial DNA (Table 3 and Fig. 3). This fact suggested that one of the parental chloroplast or mitochondria was eliminated from the heterocaryon during embryogenesis and plant development.

In the case of somatic hybrids arising from electrofusion between Mexican lime and 'Carrizo' citrange, cpDNA fragments detected were identified as those of 'Carrizo' citrange (Table 3). For somatic hybrids produced by combining two embryogenic callus-derived protoplasts of Mexican lime and *Clausena excavata*, both individuals contained cpDNA of this latter parent. In contrast there was segregation of mtDNA from both parents between the two hybrids (Fig. 3, Table 3). The four regenerants from the electrofusion of Mexican lime protoplasts with leaf-derived protoplasts of *Triphasia trifolia* and *Murraya paniculata* possessed the same CAPS patterns as those of Mexican lime for both plastome and chondriome (Table 3). These results have been confirmed with all primer pair/endonuclease combinations displaying polymorphisms, between *Triphasia trifoliata* and Mexican lime, and between Mexican lime and *Murraya paniculata* (Table 2).

Discussion

The CAPS method developed here efficiently determined the origin of cytoplasmic genomes in *Aurantioideae* somatic hybrids, especially when more distantly related genera were involved in their production. This was the case for the combinations of *Citrus*

aurantifolia with *Clausena excavata*, *Triphasia trifolia* and *Murraya paniculata*. For somatic hybrids arising from more closely related genotypes such as *Citrus aurantifolia* + Carrizo citrange, it was not possible to determine the origin of mtDNA, due to a lack of polymorphism between the two parents. Similar results were reported for mitochondrial fragments of *Quercus robur* species complex also analyzed by CAPS (Demesure et al., 1995). The lower level of polymorphism observed for mtDNA probably is a result of the lower point mutation rate for this DNA (Wolfe et al., 1987). It will be necessary to increase the set of mitochondrial primers and restriction enzymes in order to detect a few polymorphisms among more closely related *Citrus* genera belonging to the true *Citrus* group. The development of SCAR from polymorphic RFLP should also be an interesting way to develop PCR tools for mitochondrial genome analysis in citrus somatic hybrids.

According to the results previously reported by various authors dealing with the somatic fusion between leaf-derived protoplasts and embryogenic-derived ones (Cabasson et al., 2001; Grosser et al., 1996b; Kobayashi et al., 1991; Ollitrault et al., 1996), cytoplasmic genome analysis of the resulting somatic hybrids showed a consistent transmission of mtDNA from the embryogenic parent. This result was also observed in our study for somatic hybrids of Mexican lime with *Murraya paniculata* and *Triphasia trifolia*. These results corroborate the hypothesis that the acquisition of mitochondria from embryogenic cells by leaf-derived protoplasts is a prerequisite for subsequent plant regeneration via somatic embryogenesis in *Citrus* (Grosser et al., 1996b). Furthermore, other works showed that some cybrids or somatic hybrids underwent recombination/rearrangement of mtDNA, suggesting that the whole intact mtDNA genome from the callus parent may not be essential. Nevertheless, these cases of mtDNA recombination/rearrangement remain scarce in symmetric (Moreira et al., 2000a, 2000b; Morigushi et al., 1997; Motomura et al., 1995) and asymmetric somatic hybridization (Vardi et al., 1987 and 1989; Li and Deng, 1997). In our study, no cytoplasmic genome recombination events were detected among the somatic hybrids we investigated. However multipoint analysis with RFLP markers is more powerful to detect such events than the present CAPS method.

Regarding the chloroplast genome, it has been previously shown that cpDNA could have random uniparental segregation from either one or the other parent (Grosser et al., 2000; Kobayashi et al., 1991). There is only one publication (Moreira et al., 2000b)

Table 3. Cytoplasmic genome origin of intergeneric somatic hybrids.

Intergeneric combination ²	Somatic hybrid individual	Cleaved amplified polymorphic sequence profile	
		cpDNA	MtDNA
Lime x 'Carrizo' citrange	1	'Carrizo' citrange	— ³
	2	'Carrizo' citrange	—
Lime x <i>Clausena excavata</i>	1	<i>C. excavata</i>	<i>C. excavata</i>
	2	<i>C. excavata</i>	Lime
Lime x <i>Triphasia trifolia</i>	1	Lime	Lime
	2	Lime	Lime
Lime x <i>Murraya paniculata</i>	1	Lime	Lime
	2	Lime	Lime

²Primer pairs and restriction enzymes are used as follows: lime x 'Carrizo' citrange = *trnC/trnD* with *Alu*-I, *Hae*-III, *Rsa*-I or *Eco*R-I; *trnT/trnD* with *Bsp*143-I or *Dra*-I (cpDNA); lime x *Clausena excavata* = *trnT/trnD* with *Alu*-I; *psaA/trnS* with *Hind*-III or *Eco*R-I (cpDNA); *nad41/nad42* with *Hinf*-I; *nad42/nad44* with *Ava*-II or *Hinf*-I (mtDNA); lime x *Triphasia trifolia* = *trnH/trnK2* with *Ava*-II; *psaA/trnS* with *Hind*-III; *trnT/trnD* with *Alu*-I or *Bsp*143-I; *trnC/trnD* with *Alu*-I, *Hae*-III, *Rsa*-I, *Eco*R-I or *Ama*87-I (cpDNA); *nad41/nad42* with *Hinf*-I and *nad42/nad44* with *Ava*-II (mtDNA); lime x *Murraya paniculata* = *trnM/rbcL* with *Mva*-I and *trnC/trnD* with *Hae*-III (cpDNA); *nad41/nad42* with *Hinf*-I or *Mva*-I (mtDNA).

³No polymorphism.

1 2 3 4 5 6 7 8 9 10 11 12 M

Fig. 3. Cytoplasmic genome cleaved amplified polymorphic sequence analysis of somatic hybrids between Mexican lime and *Clausena excavata*. Electrophoresis on 1.8% agarose gel of fragments amplified by *psaA/trnS* (1–8) (cpDNA) and *nad41/nad42* (9–12) (mtDNA), and digested with *Hind*-III (1–4); *Eco*R-I (5–8) and *Hinf*-I (9–12). Lane 1, 5, 9 = Mexican lime; lane 2, 6, 10 = *Clausena excavata*; lane 3, 7, 11 = first hybrid; lane 4, 8, 12 = second hybrid. Lane M = DNA ladder 1 kb.

reporting the addition of chloroplastic parental genome for a combination between ‘Succari’ sweet orange and *Citropsis gilletiana*. In our study, uniparental transmission was the rule, and the very low number of plants analysed for each combination should explain that no segregation was observed.

In the case of somatic hybrids of Mexican lime with *Murraya* and *Triphasia*, the cpDNA of all tested plantlets originated from the lime. It will be interesting to investigate a fairly large number of hybrids to know if chloroplastic genome can also proceed from *Murraya* or *Triphasia* parents.

Interestingly, in the case of somatic hybridization between Mexican lime and *Clausena excavata*, and as we can forecast when both parental protoplasts originated from embryogenic callus lines, the mtDNA of the two somatic hybrids originated from either one or the other parent. Therefore, such protoplast fusion combinations can generate more diversified nuclear/chloroplastic/mitochondrial associations than the leaf + callus types. For instance, four cytoplasmic constitutions associated with one tetraploid hybrid nucleus have been obtained from *C. paradisi* + *C. deliciosa* callus derived protoplast fusion (our unpublished data). This material will allow the study of interactions between nuclear and cytoplasmic genomes and their effects on phenotypic traits expression. Tusa et al. (2000) suggest from cybrid evaluation that specific mechanisms of resistance against Mal Secco could be active in these genotypes. Mandarin and sweet orange cybrids in the field at the University of Florida Citrus Research and Education Center are showing significant variation in agronomically important traits including fruit maturity date and seed content, indicating that cybridization is a potential source of genetic variation for citrus cultivar improvement (Grosser et al., 2000). Such information will promote cytoplasmic manipulation for cultivar creation through symmetric fusion between leaf and embryogenic-derived protoplasts which easily enables control of organelle transmission (Morigushi et al., 1996; Grosser et al., 2000), or by asymmetric fusion (Vardi et al., 1987, 1989). In conclusion, the described CAPS method works efficiently with *Citrus*. It does not require radioactive labeling and is efficient for early characterization of somatic hybrid cytoplasms because it requires small quantities of plant material. This technique should facilitate various studies on *Aurantioideae* cytoplasmic genome.

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