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GENETIC TRANSFORMATION AND HYBRIDIZATION

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Molecular characterization of cytoplasmic and nuclear genomes in phenotypically abnormal Valencia orange (*Citrus sinensis*) + Meiwa kumquat (*Fortunella crassifolia*) intergeneric somatic hybrids

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Abstract Organelle DNA inheritance of four 10-year-old somatic hybrid trees between Valencia orange [*Citrus sinensis* (L.) Osbeck] and Meiwa kumquat (*Fortunella crassifolia* Swingle) was analyzed by cleaved amplified polymorphic sequence (CAPS) and restriction fragment length polymorphisms (RFLPs). Five chloroplast (cp) and three mitochondrial (mt) universal primer pairs were amplified, but no polymorphisms were detected. When the polymerase chain reaction products were digested by 15 restriction enzymes, four polymorphic cpDNA-CAPS and two mtDNA-CAPS markers were found. Both the cpDNA and mtDNA in the somatic hybrids were derived from Valencia orange (the embryogenic suspension parent). Genomic DNA of the somatic hybrids and corresponding parents was digested by five restriction endonucleases and hybridized with one chloroplast probe (*Rbc*L-*Rbc*L) and nine mitochondrial probes (*cox*I, *cox*II, c*ox*III, c*ob*, *atp*A, *tyr*, *pro*I, *atp*6 and *atp*9). The results indicated that three hybrid plants shared one strong cpDNA band with both parents and that the remaining one plant had two additional novel bands besides the shared band, while their mtDNA was identical to that of Valencia orange plus non-parental bands. When data on the mtDNA banding patterns were combined with observations on phenotypic performance in the field, it was found that the more complex mtDNA banding pattern coincided with increased vigor of the plant. The stability of the organelle genomes was studied by extracting the genomic DNA of one hybrid plant at monthly intervals for 1 year and then analyzing it using RFLPs. Before the dieback of the shoots, two fragments of the mtDNA were lost while the cpDNAs remained stable. Ploidy analysis by flow cytometry showed that all of the hybrids were stable tetraploids. Four simple sequence repeat primer pairs were applied to detect microsatellite alleles of the four hybrid plants, both parents and the 12 DNA samples from one plant. The results showed that all hybrids had biparental bands uniformly, which indicated that they had the same nuclear background. These results suggest that the mtDNA pattern is correlated with the phenotypic abnormality of Valencia and kumquat somatic hybrid plants and that nuclear-cytoplasm incompatibility may be the cause of dieback.

Keywords Citrus · Somatic hybrids · CpDNA · MtDNA · Mitochondria loss

Abbreviations *CAPS*: Cleaved amplified polymorphic sequence · *CpDNA*: Chloroplast DNA · *MtDNA*: Mitochondrial DNA · *PCR*: Polymerase chain reaction · *RFLP*: Restriction fragment length polymorphism · *SSR*: Simple sequence repeats

Introduction

Protoplast fusion has circumvented problems encountered in conventional citrus breeding and has been a useful tool for citrus cultivar improvement efforts (Grosser et al. 2000). To date, about 200 kinds of citrus somatic hybrids have been produced in the world (Grosser et al. 2000), including more than 60 intergeneric combinations (Guo and Deng 2001). Protoplast fusion has made it possible to combine cytoplasm traits in higher plant species in which chloroplasts and mitochondria are inherited maternally in sexual hybridization. It is believed that cytoplasmic genomes control some valuable agronomic traits. For instance, cpDNA may play a role in the inheritance of some disease resistance (Guo et al. 2000), and mtDNA directly relates to cytoplasmic male sterility (Kumar and Cocking 1987). The organelle genomes of citrus somatic hybrids and cybrids have been intensively studied during recent years (Cabasson et al. 2001; Guo et al. 2002; Moreira et al. 2000a, b). Previous studies in

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Citrus interspecific and intergeneric somatic hybrids and cybrids revealed that mtDNA is inherited from the embryogenic parent, while cpDNA is randomly inherited from either parent (Grosser et al. 1996a; Guo and Deng 2001; Guo et al. 2002; Kobayashi et al. 1991; Miranda et al. 1997; Moreira et al. 2000a; Ohgawara et al. 1994; Ollitrault et al. 2002; Saito et al. 1993). Recombination of the mitochondrial genome was also found in some cases (Moreira et al. 2000b; Moriguchi et al. 1997; Motomura et al. 1995), and the co-existence of cpDNA from both parents was revealed in one case (Motomura et al. 1996). For genetic incompatibility reasons, some intergeneric fusions could not regenerate into plantlets, and some regenerated plants grew abnormally. The intergeneric somatic hybrid between Valencia orange and Meiwa kumquat is one of the most typical examples of abnormal growth (Deng et al. 1992).

In the investigation reported here, we analyzed the organelle genomes of the intergeneric somatic hybrids between Valencia orange and Meiwa kumquat using molecular markers in the expectation that the results may explain the severe dieback of the hybrid plants in the field.

Materials and methods

Plant materials, primer pairs and probes

Protoplast fusion and regeneration protocols were as reported previously by Deng et al. (1992). Somatic hybrid plants of Valencia orange with Meiwa kumquat were transplanted to the field in 1990. Embryogenic callus of Valencia orange has been maintained in vitro since 1988. The primer pairs for CAPS and SSR amplification were synthesized by Sangon (Shanghai). The c*ox*I, c*ox*II, c*ox*III, c*ob* and *atp*A probes were kindly provided by Dr. C.D. Moreira (University of Florida), and the *tyr*, *pro*I, *atp*6 and *atp*9 probes were provided by Dr. F.D. Zhang (Huazhong Agricultural University). The chloroplast probe was *Rbc*L-*Rbc*L PCR products were extracted from a 2.0% agarose gel using a DNA gel extraction kit (Sangon).

Genomic DNA extraction

DNA extraction was performed according to Cheng et al. (2001). Between 5 g and 8 g of fresh healthy leaves from four somatic hybrid plants, Meiwa kumquat plant, was harvested, ground in liquid nitrogen and incubated with CTAB extraction buffer at 65°C for 1 h. The genomic DNA of Valencia orange was isolated from fresh callus following the same procedure. After RNA was removed, the DNA was extracted with water-saturated ether and NaCl, precipitated with isopropanol and then dissolved in TE buffer.

CAPS analysis

Organelle DNA was amplified using five chloroplast and three mitochondrial universal primer pairs (Tables 1, 2). The PCR reactions were conducted in a PTC-200 thermocycler in reaction mixtures (50 µl) of 0.2 m*M* dNTPs, 1.5 m*M* $MgCl_2$, 2 U *Taq* DNA polymerase (Biostar, Toronto, Ont.) and corresponding 1× reaction buffer, 0.2 µ*M* of each primer and 100 ng of sample DNA. The amplification program consisted of one initial denaturing cycle at 94°C for 3 min; 32 cycles of 1 min (denaturing) at 94°C, 40 s (annealing) at 55°C, 2 min (elongation) at 72°C; and one final cycle of 10 min at 72°C, after which the sampled were stored at 4°C. Aliquots of 5 µl of the PCR reactions were run on a 1.8% agarose gel, and then, 5–8 µl of the PCR products was digested with 5 U of restriction enzymes (MBI, Lithuania), including *Alu*I, *Bsu*RI, *Hin*I, *Hin*6I, *Taq*I, *Msp*I, *Eco*RI, *Hind*III, *Bam*HI, *Dra*I, *Pst*I, *Bsp*143I, *Csp*6I, *Bsh*1236I and *Tas*I. The digested DNA samples were electrophoresed on a 2.0% agarose gel with 1× TAE and 5 µg/ml ethidium bromide at 2.5 V/cm for 2-3 h, then photographed under UV light.

RFLP analysis

Approximately 10 µg of genomic DNA was used for each enzyme digestion. Five restriction enzymes (*Eco*RI, *Hind*III, *Bam*HI, *Dra*I and *Pst*I) were used to digest the DNA samples. The digested products were then electrophoresed on a 0.8% agarose gel and the DNA subsequently blotted onto a Hybond-N+ membrane (Amersham, Piscataway, N.J.) for 16–20 h in an alkali-downward capillary blotting procedure according to the manufacturer's instructions (Hybond-N+ nylon membrane manual 1998). Probe labeling, hybridization and stringency washing were based on the procedure of Feinberg and Vogelstein (1983) with minor modifications. The probes were labeled with d(CTP)-[^{32}P]. Hybridization was performed in tubes at 65°C overnight, and the membranes were washed in a high-stringency solution (0.1× SSC, 0.1% SDS) at 65°C for 4 h followed by exposure to X-ray film at –80°C overnight to a week before development of the film.

Ploidy analysis

Ploidy analysis was carried out in a Partec flow cytometry (D-48161 Münster, Germany) according to the protocol described by Miranda et al. (1997) with minor modifications. Approximately 1 cm^2 of young leaf was chopped in a plastic petri dish containing 0.4 ml Partec HR-A buffer. After being filtered, the samples were stained with 0.8 ml of HR-B buffer and the relative fluorescence of total DNA measured. Each histogram was generated by the analysis of at least 3,000–5,000 nuclei.

SSR analysis

SSR analysis was conducted according to the procedure of Kijas et al. (1997) with minor modifications. Approximately 100 ng of genomic DNA, 0.2 m*M* dNTPs, 1.5 m*M* $MgCl_2$, 0.5 U *Taq* DNA polymerase (Biostar) and corresponding 1× reaction buffer and 0.2 µ*M* of each primer pair were mixed well in a total reaction volume of 20 µl. PCR conditions were the same as those for the CAPS analysis. The products were analyzed on 6.0% (w/v) denaturing polyacrylamide gels, and the gels were silver-stained according to the protocol of the technical manual on silver sequence DNA staining reagents (Promega, Madison, Wis.).

Results and discussion

Analysis of mitochondrial and chloroplast genomes by CAPS and RFLPs

Five chloroplast universal primer pairs were used in this study (Table 1). While every primer pair amplified bands satisfactorily, they did not reveal any polymorphism on agarose gels. When the PCR products were digested with 15 restriction endonucleases, cpDNA polymorphism was observed in four primer pair/enzyme combinations – *Trn*D-*Trn*T/*Msp*I (Fig. 1), *Rbc*L-*PSA*I/*Msp*I, *Rbc*L-*PSA*I/*Taq*I, and *Trn*H-*Trn*K/*Taq*I. All of the specific bands of the four

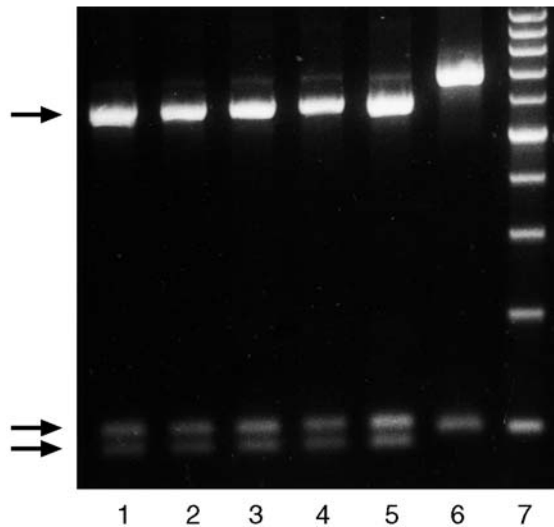

Fig. 1 CpDNA banding pattern amplified using the universal primer pair of *Trn*D-*Trn*T and digested by *Msp*I. *Lanes*: *1* Valencia orange, *2–5* somatic hybrids, *6* Meiwa kumquat, *7* 200-bp DNA ladder. Characteristic bands of the hybrids were identical to those of the Valencia orange embryogenic suspension

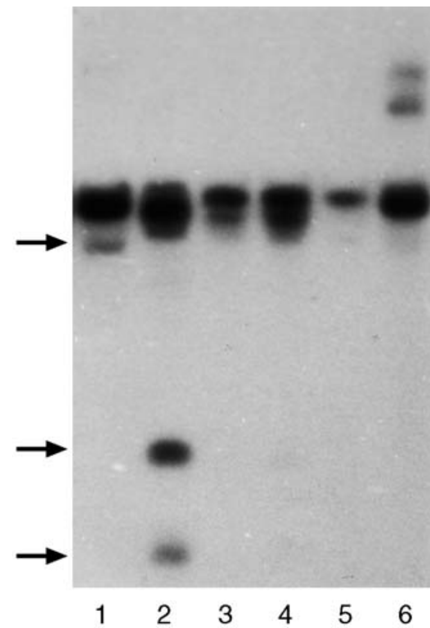

Fig. 2 CpDNA banding pattern analyzed by RFLP. Genomic DNA was digested by *Eco*RI and hybridized with the probe of the *Rbc*L-*Rbc*L-amplified fragment. *Lanes*: *1* Valencia orange, *2–5* somatic hybrids, *6* Meiwa kumquat. Novel bands in *lane 2* indicate that variable cpDNA exists among the hybrid plants

somatic hybrid plants were uniformly identical to the embryogenic Valencia orange suspension parent. No polymorphism was detected in other primer pair/enzyme combinations, which indicated that the amplified regions of *Rbc*L-*Rbc*L and *Trn*K-*Trn*K were conserved. After being extracted from the agarose gel, the amplified fragment of *Rbc*L-R*bc*L was used as a probe to hybridize with *Eco*RI-digested genomic DNA samples. The results showed that the four somatic hybrid plants tested and both parents shared a strong band, which further confirmed the conservation of the *RbcL-RbcL* region that was revealed by CAPS. Moreover, one of the four hybrid plants had two novel non-parental bands (Fig. 2), which indicated that

Table 1 CpDNA universal primer sequences, size of amplified fragments and references

Primer 1	Primer 2	Fragment length (in basepairs)	References
*Rbc*L 5′-ATGTCACCACAAA-CAGAAACTAAA-GCAAGT-3′	*Rbc*L 5′-CTTCACAAGCAGCA-GCTAGTTCA-GGACTCC-3′	1,381	Hiratsuka et al. 1989
*Rbc*L 5′-TTTGGTGGAGGAACT-TTAGGACA-CCCTTGGGG-3′	*PSA*I 5′-GCAATT-GCCGGAAATACTAAGC-3′	3,350	Morton and Clegg 1993
*Trn*H 5′-ACGGGA-ATTGAACCCGCGCA-3′	*Trn* K 5′-CCGAC-TAGTTCCGGGTTCGA-3′	1,750	Nicolosi et al. 2000
*Trn*D 5′-ACCAA-TTGAACTACAATCCC-3′	*Trn*T 5′-CTAC-CACTGAGTTAAAAGGG-3′	1,600	Nicolosi et al. 2000
*Trn*K 5′-AACC-CGGAACTAGTCGGATG-3′	*Trn*K 5′-TCAAT-GGTAGAGTACTCGGC-3′	2,569	Hiratsuka et al. 1989

Table 2 MtDNA universal primer sequences, size of amplified fragments and references

Primer 1	Primer 2	Fragment length (in basepairs)	References
*nad*1 exon B 5′-GCATTA-CGATCTGCAGCTCA-3′	*nad*1 exon C 5′-GGAGCTCGATTAGTTTCTGC-3′	1,184	Demesure et al. 1995
18S rRNA 5′-GTGTTGCT- GAGACATGCGCC-3′	5S rRNA 5′-ATATGGCGCAAGACGATTCC-3′	1,177	Al-Janabi et al. 1994
nad4 exon 1 5′-CAGTGG- GTTGGTCTGGTATG-3′	nad4 exon 2 5′-TCATATGGGCTACTGAGGAG-3′	2,100	Demesure et al. 1995

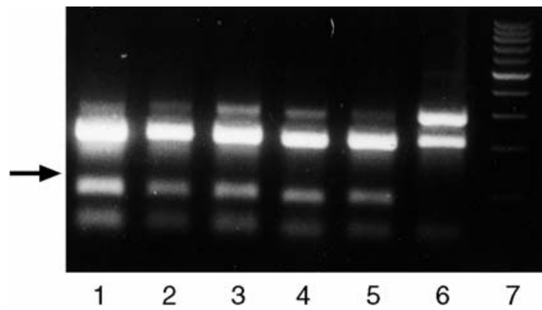

Fig. 3 MtDNA banding pattern amplified using the universal primer pair of *nad*1-*nad*1 and digested by *Tas*I. *Lanes*: *1* Valencia orange, *2–5* somatic hybrids, *6* Meiwa kumquat, *7* 200-bp DNA ladder. Characteristic bands of the hybrids were identical to those of the Valencia orange embryogenic suspension

varied plastid DNA existed among the individual plants that originated from this fusion. This would cover the broader possibility of amplified plastid DNA sequences in the mitochondria or the transfer of plastid sequences to the nuclear genome.

MtDNA universal primer pairs (Table 2) were also used to detect the mtDNA composition of the somatic hybrid plants. The enzymes and procedures were the same as those used in the cpDNA analysis. Among all the primer pair/enzyme combinations tested, polymorphisms were detected only in the combinations of *nad*1-*nad*1/*Tas*I (Fig. 3) and *nad*4-*nad*4/*Taq*I. The results showed that the mitochondrial genomes of the hybrids came uniformly from the embryogenic suspension parent Valencia orange. To obtain more detailed information on the mtDNA, nine mitochondria-specific probes (see Materials and methods) and the *nad*1-*nad*1 PCR product of kumquat were labeled and hybridized with membranes blotted with DNA samples digested by *Eco*RI, *Hin*dIII, *Bam*HI, *Dra*I and *Pst*I respectively. Character-istic bands of both fusion parents were detected in most of the restriction endonuclease/probe combinations such as *Hin*dIII/*atp*A, *Eco*RI/*nad*1-*nad*1, *Hin*dIII/*atp*9, *Eco*RI/*atp*A, *Eco*RI/*tyr,* *Hin*dIII/*cox*II, *Eco*RI/*pro*I and *Pst*I/*cox*II. No polymorphism was found when the membranes were hybridized with the *cob* and *26s* probes. The results showed that differences in mtDNA did exist between kumquat and Valencia orange and that the regions of the *atp*, c*ox*, *tyr* and *pro* genes were variable while the regions of *cob* and *26s* were conserved. All of the bands characteristic of the somatic hybrids were either identical to those of Valencia orange or novel non-parental bands. When *Hin*dIII-digested genomic DNA was hybridized with the *atp*9 and *cox*II probes, all of the hybrids uniformly had one band, which was identical to that of Valencia orange (Fig. 4). The results of RFLP analysis were in line with those of the CAPS analysis. When *Eco*RI-digested genomic DNA was hybridized with probes of *atp*A, *tyr*, *pro*I, and *nad*1-*nad*1, one of the four somatic hybrids tested always had more bands (one to three bands) than the others (Fig. 5, lane 2). Conversely, another sample always had fewer bands (Fig. 5, lane 5). The appearance of novel bands in the RFLP analysis indicated that varied mtDNA patterns existed among the individual plants regenerated from this fusion.

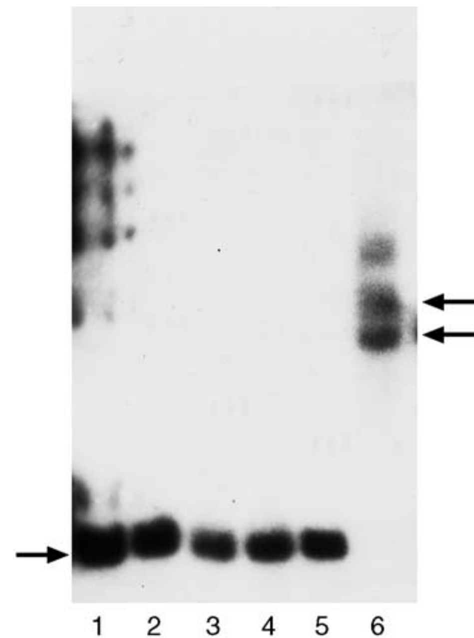

Fig. 4 MtDNA banding pattern analyzed by RFLP. Genomic DNA was digested by *Hin*dIII and hybridized with the *CoxII* probe. *Lanes*: *1* Valencia orange, *2–5* somatic hybrids, *6* Meiwa kumquat. Characteristic bands of the hybrids were identical to those of the Valencia orange embryogenic suspension

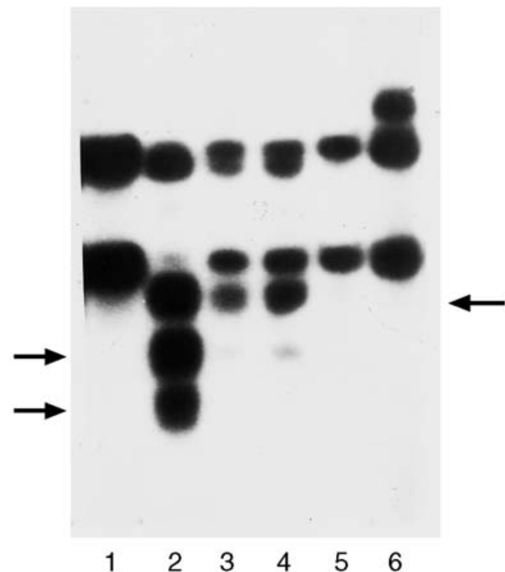

Fig. 5 MtDNA banding pattern analyzed by RFLP. Genomic DNA was digested by *Eco*RI and hybridized with the *atp*A probe. *Lanes*: *1* Valencia orange, *2–5* somatic hybrids, *6* Meiwa kumquat. Novel mtDNA bands appeared in *lanes 2–4*, and the copy number of the *atpA* gene was different among the four individuals (*lanes 2–5*), which might result from amplification of alternative mitochondrial genome configurations or mtDNA recombination. Both have been documented to occur in plant mitochondrial genomes

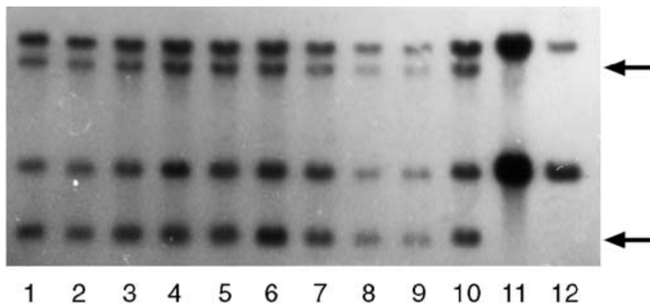

Fig. 6 The instability of mtDNA in one somatic hybrid plant (*lane 2* in Figs. 1, 2, 3, 4 and 5) analyzed by RFLPs with the enzyme/probe combination of *Hin*dIII/*atp*A, *Lanes 1–12* Samples were harvested on the first day of every month from 1 December 2000 to 1 November 2001. Shoots of the plant exhibited serious dieback in October 2001, and a loss of mtDNA fragments was detected (*arrows* on 1 September sample)

RFLP analysis of cytoplasmic genomes in 12 DNA samples extracted from one hybrid plant at monthly intervals for 1 year

The somatic hybrid plants between Valencia orange and Meiwa kumquat grew less vigorously and their shoots died back annually (Shi et al. 1998) compared to other somatic hybrids between *Citrus* species and Meiwa kumquat which grew normally (Grosser et al. 1996b). In order to prove whether the banding pattern of organelle DNA was associated with the phenotypic performance of the plants and to detect the stability of the organelle DNA, we harvested leaves of the plant with the most bands (lane 2 in Figs. 2 and 5) on the first day of each month from 1 December 2000 to 1 November 2001 for DNA extraction. The 12 samples were then analyzed by RFLPs using the restriction enzyme/probe combinations of *Hin*dIII/*atp*A, *Bam*HI/*atp*9, *Eco*RI/*tyr, Dra*I/*cox*II, *Eco*RI/*Rbc*L-*Rbc*L, *Hin*dIII/*Rbc*L-*Rbc*L, *Bam*HI/*Rbc*L-*Rbc*L and *Dra*I/*Rbc*L-*Rbc*L, No cpDNA differences were detected, while mtDNA differences were found with the enzyme/probe combination of *Hin*dIII/*atp*A, in which two bands were lost in the two samples taken during the last 2 months (Fig. 6). Field observation showed that the plant underwent serious shoot dieback from October 2001 onwards, with about two-thirds of the canopy dying.

Nuclear genome background confirmation by flow cytometry and SSR analysis

As organelles are semi-autonomous in eukaryotes, nuclear and organelle genomes must be coordinated in their expression. Thus, any attempt to address the adaptive significance of mtDNA variants should consider the importance of nuclear-cytoplasmic interaction (Nigro 1994). In order to reveal the nuclear background of the somatic hybrid plants, we carried out ploidy analysis by flow cytometry using diploid Valencia orange as a control. The results showed that all of the plants were stable tetraploids (Fig. 7). Flow cytometry is a reliable method for ploidy analysis, and the results represent the actual ploidy level (Miranda et al. 1997). Identification of microsatellite alleles in the hybrid plants, the parental plants and the 12 samples for the one hybrid plant discussed above was conducted. Four microsatellites (TAA1, TAA15, TAA27, TAA52) described in Kijas et al. (1997) were analyzed, and all of the samples displayed a biparental specific banding pattern (Fig. 8). Combining the results of ploidy and SSRs analysis, it was confirmed that all of the hybrid plants had the same nuclear background and that nuclear composition apparently did not correlate with the phenotypic abnormality in this fusion.

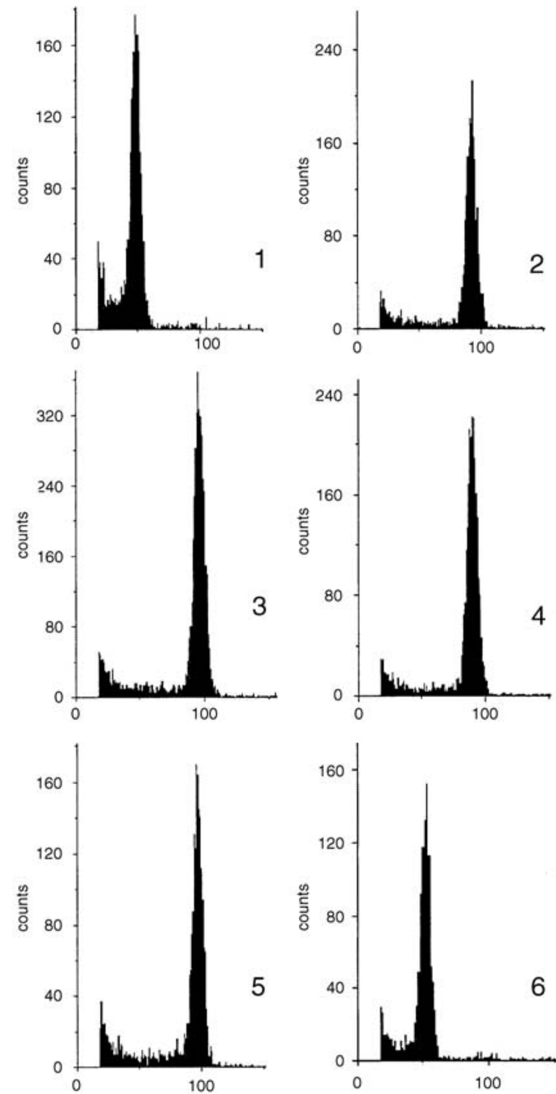

Fig. 7 Ploidy analyses by Partec flow cytometry. *1* Valencia orange, *2–5* somatic hybrids, *6* Meiwa kumquat. Diploid Valencia orange was used as a control; the results revealed that all somatic hybrids had the same ploidy level (tetraploid), while the two parents were diploid

That novel cpDNA bands appeared in these hybrid plants upon RFLP analysis was an interesting result, in

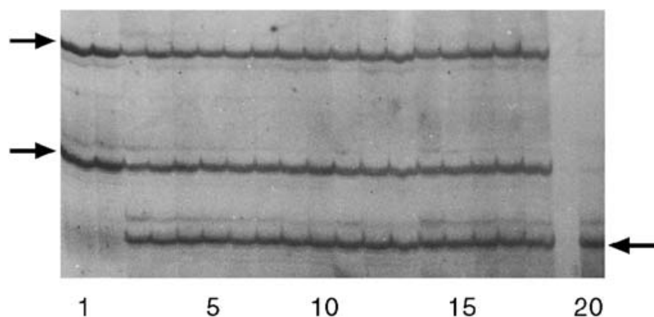

Fig. 8 Silver-stained polyacrylamide gel of microsatellite. TAA15 alleles were amplified within the somatic hybrids, parents and the 12 samples of 1 December 2000 to 1 November 2001. *Lanes*: *1*, *2* Valencia orange, *3–6* the four tested somatic hybrids, *7–18* the 12 samples of 1 December 2000 to 1 November 2001, *19, 20* Meiwa kumquat (19 failed to amplify in PCR). All hybrids had stable biparental alleles

that plastid recombination has been rarely observed in protoplast fusion (Medgyesy et al. 1985). The reasons for our results could be either that plastid DNA recombination did occur or that some plastids were inserted into the mitochondrial genomes during protoplast fusion. The molecular structure of cpDNA is a circle with two inverted repeats, a formation that can prevent cpDNA rearrangement. To date, cpDNA recombination has virtually never been observed in citrus somatic hybrids in the absence of selection for recombinants. Comparing the cpDNA banding patterns with those of mtDNA by RFLP analysis, we found that the cpDNA composition in one hybrid plant was as complex as that of their mtDNA (compare Fig. 2 with Fig. 5). Therefore, it seems that the latter explanation for the non-parental cpDNA bands could be much more reasonable, namely, that the mitochondrial genomes contain some insertions of cpDNA. It has been reported that in *Arabidopsis* some mtDNA was recruited from the chloroplast genome (Gray et al. 1999). In addition, except for the advantages of CAPS analysis mentioned previously (Guo et al. 2002; Ollitrault et al. 2002), the outcome of this study also displayed its inherent disadvantage that copy numbers of amplified regions in CAPS can not be obtained.

This is the first report on mtDNA fragment loss in mature plants of citrus somatic hybrids. We observed that the banding patterns of organelle DNA could be related to their phenotypic growth performance in the field – the more complex the banding pattern of organelle DNA composition, the better the growth of the individual plant. The two plants corresponding to lanes 2 and 5 in Figs. 2 and 5 were the two extremes; the height, trunk and canopy size of the plant in lane 5 was just about one-third those of the plant in lane 2. In comparing the two plants, we found that the more similar the organelle DNA banding pattern was to Valencia orange, the more severe was the dieback problem.

The cytological and molecular evidence available suggests that the nucleus controls the copy number and transcription of organelle genes (Mackenzie and McIntosh 1999). Herein, we suggest that the variation in both mtDNA and cpDNA copy number was the results of an interaction of a novel nuclear-cytoplasm complex. Differences in mtDNA and cpDNA copy number in individual plants and the loss of mtDNA fragments before the dieback of shoots implied the genetic instability of mtDNA, which could be a reasonable interpretation for the abnormal growth and differences in growth vigor among the individual hybrid plants. Organelles in higher plants are directly related to energy metabolism such as photosynthesis and oxidative phosphorylation. A point mutation in mtDNA could cause any gene to be less than 100% active, and at the whole organism level, such a mutation could cause serious symptoms (Scheffler 2000); alterations in mitochondrial distribution and morphology are associated with a variety of pathological conditions (Yaffe 1999).

In conclusion, the results of the present investigation indicate that the mtDNA pattern was correlated with the phenotypic abnormality of Valencia and kumquat somatic hybrid plants and that the instability of mtDNA was one possible reason for their dieback. We have more somatic hybrids, such as the hybrid plants between *Citrus reticulata* and citrange, which have the same annual dieback problem (W.W. Guo, unpublished data). Further investigation by means of molecular techniques on this hybrid will be helpful in elucidating the relationship between mtDNA pattern and the phenotypic abnormality of some citrus somatic hybrids.

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