Botanical Studies (2008) 49: 295-300.

Analysis of mitochondrial genomes in *Citrus* interspecific somatic hybrids produced by protoplast fusion

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(Received December 14, 2006; Accepted April 9, 2008)

ABSTRACT. Somatic hybrids produced via protoplast fusion are valuable germplasm for citrus improvement. Herein, four randomly selected six-year-old interspecific somatic hybrid plants between Bonanza sweet orange (*Citrus sinensis* Osbeck) and rough lemon (*C. jambhiri*), were thoroughly analyzed. Flow cytometry and nuclear SSR analysis confirmed them as true tetraploid somatic hybrids. Chloroplast SSR analysis showed the random inheritance nature of chloroplast DNA. PCR amplification of mitochondrial genome by universal primer pair showed that the somatic hybrids had the specific band from rough lemon (the leaf parent) while all samples shared a common band. CAPS analysis by further *Tas*I restriction endonuclease cut of the PCR products, however, revealed the band specific to Bonanza orange, the embryogenic callus parent, was also present in all these analyzed hybrids. Further sequencing of the common band and searching for restriction endonuclease recognition sites well explained the banding pattern by CAPS analysis. It was concluded that mitochondrial recombination in citrus somatic hybrids occurred as revealed by CAPS analysis and DNA sequencing.

Keywords: *Citrus*; Cleaved amplified polymorphic sequence (CAPS); Cytoplasmic genome; DNA sequencing; Simple sequence repeat (SSR); Somatic hybrids.

INTRODUCTION

Somatic hybridization has been an effective and successful tool for plant improvement (Davey et al., 2005) and is still being applied in several crops such as cotton (Sun et al., 2004), potato (Trabelsi et al., 2005), rapeseed (Wang et al., 2005) and wheat (Chen et al., 2004). The somatic hybridization technique is especially useful in circumventing the natural polyembryony and pollen/ovule sterility of perennial citrus crops. During the past two decades, numerous somatic hybrids have been produced and evaluated for cultivar improvement (Guo et al., 2004a; Grosser and Gmitter, 2005).

These novel germplasm resources are also valuable for plant somatic cell genetics research by molecular markers. Molecular analysis could not only help us understand the nucleus-nucleus, nucleus-cytoplasm, and cytoplasm-cytoplasm interaction between both fusion parents, but it could also be conducive to the correlation of phenotypic performance or specific traits with the concrete nuclear and cytoplasmic composition of these novel hybrids. For their molecular evaluation, RAPD (random amplified polymorphic DNA) and RFLP (restriction fragment length polymorphism) were previously widely applied, but newly developed simpler and more efficient molecular markers such as SSR (simple sequence repeat), CAPS (cleaved amplified polymorphic sequence), and chloroplast SSR are currently utilized (Lotfy et al., 2003; Guo and Grosser, 2005; Takami et al., 2005; Wu et al., 2005). Genetic information of plant mitochondria has been the subject of intensive research work (Cheng et al., 1997; Cheng and Dai., 2000; Huang et al., 2003; Lo et al., 2003; Wang et al, 2007). Herein, we report the molecular characterization of somatic hybrids between sweet orange and rough lemon by novel markers where recombination of the mitochondria genome was revealed for the first time by CAPS analysis and DNA sequencing.

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MATERIALS AND METHODS

Plant materials

The protoplast fusion and regeneration of somatic hybrid plants between Bonanza sweet orange (*Citrus sinensis* Osbeck) and rough lemon (*C. jambhiri*) were described and detailed by Guo and Deng (2000). These plants are six-years-old and are maintained in the germplasm field of the National Citrus Breeding Center, Huazhong Agricultural University, Wuhan.

Flow cytometry analysis

Ploidy analysis of these plants was conducted by Partec flow cytometry (D-48161 Münster Germany). Approximately 1 cm^2 of young leaf was chopped in a plastic Petri dish containing 0.4 ml Partec HR-A buffer (Partec high resolution nuclei extraction solution). After being filtered, the samples were stained with 0.8 ml of HR-B buffer (Partec high resolution DAPI staining solution), and the relative fluorescence of total DNA was measured. Each histogram was generated by analyzing at least 3000-5000 nuclei.

DNA extraction, nuclear SSR and chloroplast SSR analysis

Total DNA was extracted following a modified CTAB procedure (Cheng et al., 2003b). SSR analysis of nuclear genome was conducted using primer pairs TAA1 (primer 1: 5′-GAC AAC ATC AAC AAC AGC AAG AGC-3′; primer 2: 5′-AAG AAG AAG AGC CCC CAT TAG C-3′), and TAA3 (primer 1: 5′-AGA GAA GAA ACA TTT GCG AGC-3′; primer 2: 5′-GAG ATG GGA CTT GGT TCA TCA CG-3′) (Kijas et al., 1997). One pre-screened chloroplast SSR primer pair SPCC1 (primer 1: 5′-CTT CCA AGC TAA CGA TGC-3′; primer 2: 5′-CTG TCC TAT CCA TTA GAC AAT G-3′) was also applied to reveal chloroplast inheritance (Cheng et al., 2005). SSR and chloroplast SSR reaction conditions were according to Cheng et al. (2005). The products were analyzed on 6.0% (w/v) denaturing polyacrylamide gels, then the gels were silver-stained according to the protocol of the technical manual on silver sequence DNA staining reagents (Promega, Madison, Wis.).

Mitochondrial DNA analysis by CAPS

For CAPS analysis, one prescreened mitochondrial universal primer pair, i.e. 18S rRNA-5S rRNA (forward: 5′-GTG TTG CTG AGA CAT GCG CC-3′, reverse: 5′ -ATA TGG CGC AAG ACG ATT CC-3′) (Cheng et al., 2003a) was selected, and PCR reaction was performed in a PTC-200 thermocycler (Bio-Rad). The PCR reaction mixture (50 µl) consisted of 67 mM Tris-HCl (pH 8.8), 16 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 0.2 µM of each primers and 300 µM of dNTP, 1.5 to 2.5 mM $MgCl_2$, 1.5 unit of *Taq* DNA polymerase and 100 ng of sample DNA. The amplification parameters were: 1 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, a final step of 10 min at 72°C, then storage at 4°C. DNA digestion: 5 to 8 µl of the PCR products were digested with 5 units of the *Hinf*I, *Msp*I and *Tas*I restriction endonucleases (MBI Fermentas) at 37°C or 65°C for 4 h. For DNA analysis, the digested DNA samples were electrophoresed in 2.0% agarose gel with 1× TBE and 5 µg/ml ethidium bromide at 2 V/cm for 3 to 4 h, then photographed under UV light.

DNA sequence analysis

The common band of the PCR products generated by mitochondrial universal primer pair 18S rRNA-5S rRNA was extracted with an EZNA Gel Extraction Kit (Omega Biotek, Doraville, GA). The DNA samples obtained were ligated into the pMD 18-T Vector (Takara Biotech, Dalian, China) and transformed into *Escherichia coli* strain DH5α, and then sequenced by the United Gene Company (Shanghai, China). DNA sequence comparison was analyzed by using ClustalW Multiple Sequence Alignment (dot.imgen.bcm.tmc.edu). Number and sites of restriction endonuclease *Tas*I cut of these sequence samples were compared.

RESULTS

These somatic hybrid plants had been growing in the field for more than six years. They were verified to be tetraploids by chromosome counting (Guo and Deng, 2000). To evaluate their ploidy stability, flow cytometry analysis was conducted, and they were confirmed as stable tetraploids (Figure 1).

The nuclear origin of these regenerates was determined by SSR analysis with two primer pairs (TAA1, TAA3), which could distinguish both parents. All the four analyzed plants had specific bands from both parents (Figure 2). Flow cytometry and nuclear SSR analysis confirmed these plants were true allotetraploid somatic hybrids.

To reveal the cytoplasmic genome composition of these hybrid plants, chloroplast SSR and mitochondria CAPS analysis were conducted. Chloroplast SSR analysis indicated that primer pair SPCC1 distinguished the parents well. Among the four analyzed plants, No. 2 hybrid had the chloroplast DNA from Bonanza orange, and that of Nos. 1, 3 and 4 was from rough lemon (Figure 3), suggesting chloroplast DNA was randomly inherited in these hybrids.

Mitochondria CAPS analysis of these four hybrid plants with mitochondria primer pair, i.e. 18S rRNA-5S rRNA was performed. Polymorphism was detected even without

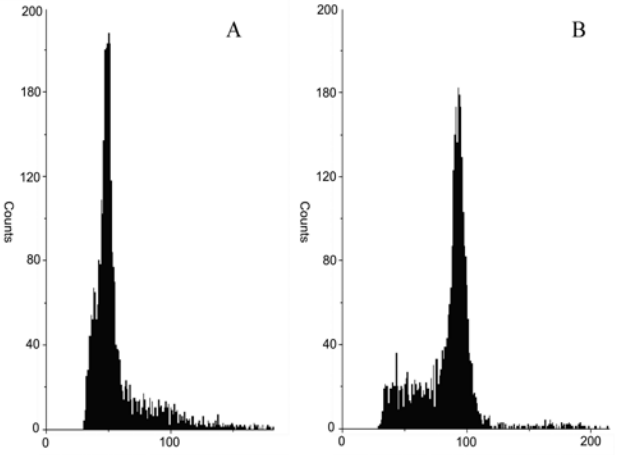

Figure 1. Ploidy determination by flow cytometry analysis. A: diploid control (leave mixture of Bonanza sweet orange and rough lemon); B: tetraploid somatic hybrids between sweet orange and rough lemon.

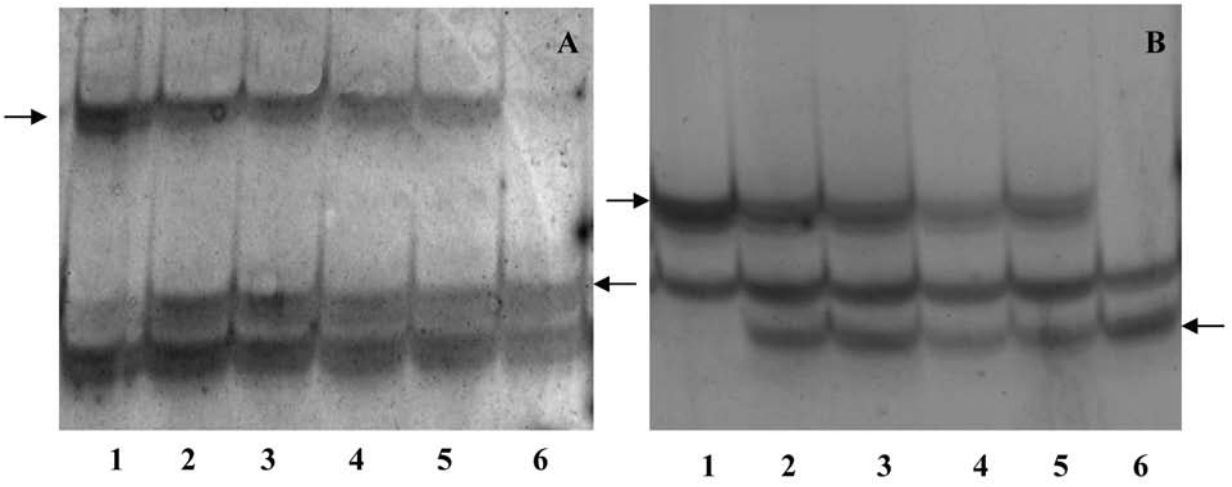

Figure 2. SSR analysis of nuclear genome in somatic hybrids and their parental genotypes. Lane 1: rough lemon, 2-5: somatic hybrids No. 1 to No. 4, 6: Bonanza navel orange. Primer pair for A: TAA1, and B: TAA3. Arrows indicate specific bands.

restriction endonuclease digestion, where the banding pattern of these somatic hybrids was identical to rough lemon, the leaf parent; meanwhile all samples shared a common band of about 1,100 bp (Figure 4A). After the PCR product was further digested with restriction endonuclease *Tas*I, the band specific to Bonanza sweet orange, the embryogenic callus parent, was revealed and was present in all four analyzed hybrid plants (Figure 4B).

To further explain the mtDNA banding pattern following *Tas*I cut, the approximately 1,100 bp common band was extracted and sequenced for both parents and somatic hybrids No. 1 and No. 2. It showed that the band is 1,069 bp in size for Bonanza navel orange, somatic hybrids No. 1 and No. 2 while that for rough lemon is 1,064 bp. There are some single nucleotide mutations in these sequences. Searching for *Tas*I recognition site of 5′-^AATT-3′ in the DNA sequence revealed that Bonanza navel orange, somatic hybrids No. 1 and No. 2 had three recognition sites producing four fragments of 81 bp, 412 bp, 173 bp and 403 bp while rough lemon, with one

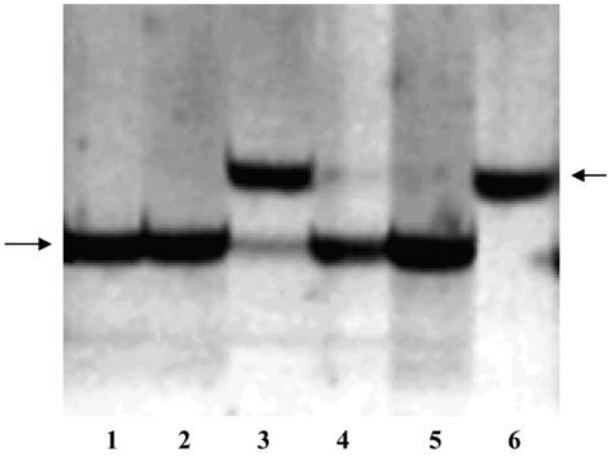

Figure 3. Chloroplast SSR analysis by chloroplast primer pair SPCC1. Lane 1: rough lemon, 2-5: somatic hybrids No. 1 to No. 4, 6: Bonanza navel orange. Arrows indicate specific bands.

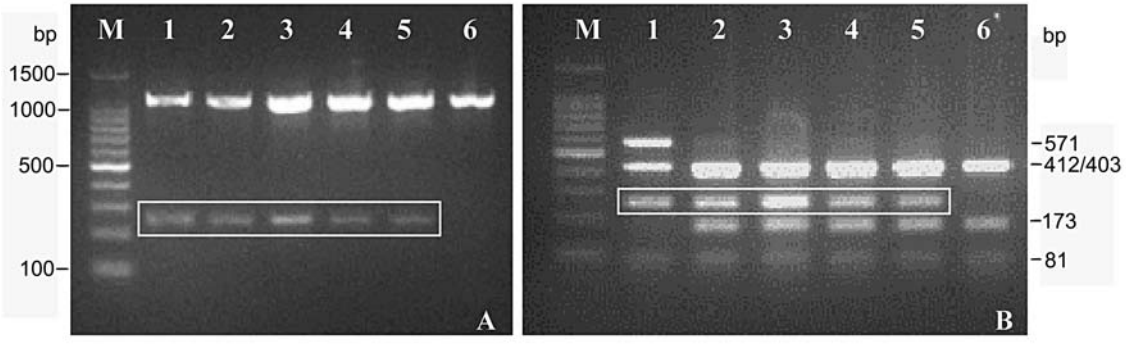

Figure 4. Mitochondrial DNA analysis using universal primer pair *18S* rRNA-*5S* rRNA. M: 100 bp DNA ladder, Lane 1: rough lemon, 2-5: somatic hybrids No. 1 to No. 4, 6: Bonanza navel orange. A: before restriction endonuclease *Tas*I cut; B: after restriction endonuclease *Tas*I cut. The boxed bands in Figure 4A are the same as the boxed bands in Figure 4B. The size of DNA ladder (left of Figure 4A) and that of the specific bands after *Tas*I cut (right of Figure 4B) were indicated.

Table 1. DNA sequence analysis of the approximately 1100 bp common band for both parents and two hybrids after *Tas*I restriction endonuclease cut.

Fragment Nos.	Sample Nos. and size (bp)			
	Rough lemon	Somatic hybrid No. 1	Somatic hybrid No. 2	Bonanza navel orange
Common band	1064	1069	1069	1069
Fragment 1	81	81	81	81
Fragment 2	412	412	412	412
Fragment 3	571	173	173	173
Fragment 4	/	403	403	403

*Tas*I recognition site mutated, had two recognition sites producing three fragments of 81 bp, 412 bp and 571 bp, respectively (Table 1; DNA sequences not shown). The sequencing information explained the banding pattern of CAPS analysis well since the 412 bp and 403 bp fragments were too close in size to be apart, and they appeared as one band by agarose gel electrophoresis (Figure 4B). DNA sequencing combined with CAPS analysis suggested that mtDNA recombination occurred in these somatic hybrids.

DISCUSSION

Nuclear and cytoplasmic inheritance analysis was conducted on these plants produced by somatic hybridization. In our previous research, primer pairs showing good polymorphism for nuclear SSR, chloroplast SSR and CAPS analysis were screened (Cheng et al., 2002, 2003a, 2005), some of which showed good results in this research. Compared with previously routinely used RFLP analysis with labeled probes, CAPS is simpler, more rapid, and less expensive (Bastia et al., 2001; Guo et al., 2004b). Meanwhile, the results by CAPS technique were proved reliable and agreeable to that of RFLP (Cheng et al., 2002). Furthermore, compared with CAPS analysis, chloroplast SSR is even more convenient and efficient since enzyme cutting following PCR reaction is not needed (Provan et al., 2001; Cheng et al., 2005).

Herein, DNA sequencing revealed novel information for mitochondrial genome variation in citrus somatic hybrids. DNA sequencing combined with searching for restriction endonuclease recognition sites explained the banding pattern of CAPS analysis. DNA sequencing can provide direct and reliable genetic information while CAPS is based on fragment length polymorphism and much work as well as money is needed to screen for restriction endonucleases that can produce polymorphic fragments. Since DNA sequencing is currently quite cheap and convenient, for CAPS analysis, it might be better to sequence the common band and search for restriction endonuclease recognition sites that could produce polymorphic fragments before conducting restriction endonuclease cutting and agarose gel electrophoresis, thus circumvent the tedious and expensive work of screening for restriction endonucleases. DNA sequencing combined with further CAPS analysis could also be done in confirmation of each other.

The fusion model of ”embryogenic callus protoplasts + mesophyll protoplasts” was widely adopted in citrus protoplast fusion. Previous molecular analysis on many citrus somatic hybrids revealed that their mitochondrial genome was usually non-randomly inherited from their corresponding embryogenic callus parent while their chloroplast genome was randomly inherited (Guo et al., 2004a). The result presented herein was consistent with this rule for chloroplast genome; but for the mitochondrial genome, the result of this fusion was an exception since mitochondrial recombination was revealed in all four analyzed hybrid plants. This is also the first report on mitochondrial recombination in citrus somatic hybrids as revealed by CAPS markers and DNA sequencing. Mitochondrial recombination was only revealed previously by RFLP analysis in a few plants from three intergeneric (Motomura et al., 1995; Cheng et al., 2003a) and two interspecific fusions (Moriguchi et al., 1997; Olivares-Fuster et al., 2005) in citrus.

For mitochondrial interaction after protoplast fusion at the subcellular level, by fusing protoplasts containing either green fluorescent protein or MitoTracker-labelled mitochondria, Sheahan et al. (2005) reported the phenomenon of massive mitochondrial fusion (MMF) which leads to near-complete mixing of the mitochondrial population within 24 h. MMF appears specific to dedifferentiation, since it also occurs in mesophyll protoplasts of *Arabidopsis* and *Medicago* but not in protoplasts from already dedifferentiated cells such as tobacco BY-2 or callus cultures. These results by Sheahan et al. (2005) allow a clearer interpretation of how novel mitochondrial genotypes develop following cell fusion. Such research is also currently underway in our group to reveal the mitochondria interaction at the subcellular level following various kinds of protoplast fusion in citrus, which may explain why dominantly non-random mitochondrial inheritance and a few novel mitochondrial genotypes occurred, following cell fusion in citrus.

Acknowledgements. The research was financially supported by the National Natural Science Foundation of China (Nos. 30771481, 30571288), the Ministry of Education of China (Nos. 705037, 20060504014, IRT0548), and the 863 High Technology Program of MOST (No. 2006AA100108).

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柑橘種間體細胞雜種的線粒體基因組分析

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原生質體融合創造的體細胞雜種對柑橘改良具有重要價值。本文選取 4 株 6 年生的朋娜臍橙 (*Citrus sinensis* Osbeck) + 粗檸檬 (*C. jambhiri*) 種間體細胞雜種植株進行遺傳分析。倍性流式細胞儀和核基因組 SSR 分析表明它們是真正的異源四倍體雜種。葉綠體 SSR 分析表明其葉綠體基因組為隨機遺傳。其線粒體基因組的特異 PCR 擴增表明，這些體細胞雜種除共有一條 1,100 bp 左右的譜帶外，還出現了葉肉親本粗檸檬的特異帶。PCR 產物的進一步 *Tas*I 限制性酶切揭示，葉肉親本的特徵帶無變化，但共有帶在所有樣品中均被酶切，而且體細胞雜種的帶型與懸浮系親本朋娜臍橙相同。共有帶的 DNA 測序和 DNA 序列的 *Tas*I 限制性酶切位點檢索很好地解釋了線粒體基因組的 CAPS 分析帶型結果。這是 CAPS 分析和 DNA 測序揭示柑橘體細胞雜種線粒體基因組重組的首例報導。

關鍵詞：柑橘；CAPS；胞質基因組；DNA 測序；簡單序列重複；體細胞雜種。