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FCM, SSR and CAPS analysis of intergeneric somatic hybrid plants between Changshou kumquat and Dancy tangerine

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Abstract. Flow cytometry (FCM), simple sequence repeats (SSR) and cleaved amplified polymorphic sequence (CAPS) were used to characterize the ploidy level and the nuclear and cytoplasmic compositions of two intergeneric somatic hybrids of Changshou kumquat (*Fortunella obovata*), leaf parent, and Dancy tangerine (*Citrus reticulata*), suspension parent. FCM showed that both somatic hybrids were tetraploids. One out of two SSR primer pairs was able to detect polymorphisms between the fusion parents, and the somatic hybrids showed additive banding profiles, showing them to be allotetraploid hybrids with inherited nuclear DNA from both parents. CAPS showed that 4 and 1 polymorphic loci were obtained for parental chloroplast and mitochondrial genomes, respectively. The somatic hybrid plants had the same banding patterns as the suspension parent for all of the polymorphic chloroplast markers, and their mitochondrial banding patterns approximated those of the suspension parent with loss of some mtDNA fragments. The present research plays a pivotal role in understanding the relationship between nuclear and cytoplasmic compositions and the likely field performance of somatic hybrids in the future.

Keywords: Allotetraploid; Citrus; cpDNA; mtDNA; Somatic hybrids.

Introduction

Somatic hybridization via protoplast fusion paves the way for circumventing some reproductive barriers in citrus traditional breeding, such as polyembryony (nucellar embryos), female and/or male sterility, high degrees of heterozygosity, inconsistencies in flowering period, and sexual incompatibility. Since the first somatic hybrid of trifoliate orange (*Poncirus trifoliata*) and sweet orange (*Citrus sinensis*) was produced by Ohgawara et al. (1985), more than 250 combinations of citrus somatic hybrids have been produced worldwide, including sexually incompatible and compatible ones (Grosser et al., 2000; Grosser and Chandler, 2003; Liu et al., 2004). The somatic hybrids hold great potential for citrus cultivar improvement because some of them can be tried as rootstocks, and others can be experimented on for scion cultivars. Several of them have taken on some desirable traits and will be possibly integrated into breeding programs. In addition to producing somatic hybrids with potential for cultivar improvement, somatic hybridization is useful in the study of nuclear-cytoplasmic interactions (nuclear-nuclear, nuclear-cytoplasmic and cytoplasmic-cytoplasmic), owing to its unique advantages over conventional methods, such as the partial transfer of desirable genome (genes) from donor species, the transfer of cytoplasmic factors without changing the nuclear background, and a combination of cytoplasms from different sources, which can not be completed by cross hybridization due to maternal inheritance of cytoplasmic genomes. Nuclear-cytoplasmic interactions and the final existence fate of the nuclear and cytoplasmic genomes derived from the interactions have certain effects on the growth, development, and field performance of the somatic hybrids. Cheng et al. (2003) reported that abnormal shoot growth of intergeneric somatic hybrids between sweet orange and kumquat was concurrent with loss of mtDNA from one parent. A correlation between mtDNA compositions and field parameters was detected in potato somatic hybrids (Lossl et al., 2000). Studies on nuclear-cytoplasmic interactions depend on reference to detailed somatic hybrid compositions gleaned from morphological, cytological, and genetic markers. Some citrus somatic hybrids have been analyzed using these markers. Previously isozyme, RAPD (randomly amplified polymorphic DNA), and RFLP (restriction fragment length polymorphism) were the main methods used to identify nuclear and cytoplasmic compositions. With the development of new markers, such as SSR (simple sequence repeats) and CAPS (cleaved amplified polymorphic sequence), hybridity identification became rapid and easy. SSR and CAPS have been employed in the verification of some citrus somatic hybrids and in the clarification of the maternal inheritance of some true citrus fruit trees (Liu et al., 2002; Medina-Urrutia et al., 2004; Abkenar et al., 2004; Xu et al., 2004; Guo et al., 2004). In the present paper these two markers, coupled with flow cytometry (FCM), were used to investigate the ploidy level and the nuclear and cytoplasmic constitutions of two intergeneric somatic hybrids of *Citrus reticulata* and *Fortunella obovata* with the intention of providing information for further evaluation of their agronomic performance.

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Materials and Methods

Plant Materials

The intergeneric somatic hybrids derived from protoplast electrofusion between Dancy tangerine (*Citrus reticulata*) and kumquat (*Fortunella obovata*) were planted in the citrus orchard of Huazhong Agricultural University (Liu and Deng, 2000). Dancy tangerine callus was maintained in the MT basal medium supplemented with sucrose 30 g l^{-1} and solidified with 0.7% agar (pH 5.8). The kumquat trees derived from in vitro seedlings were also planted in the same orchard as the somatic hybrids.

Ploidy Analysis of the Plants by FCM

Ploidy of the regenerated plants was determined by FCM according to Liu et al. (2003) with minor modifications. The leaves were chopped with a sharp razor blade and incubated in 0.5 ml nuclei extraction buffer (Partec HR-A) for 3 min, followed by filtering with 30 μm Partec Celltrics™ and staining with 1 ml Partec HR-B solution for 2 min. The fluorescence of the samples was measured on a Partec Flow Cytometer (PA-I, Münster, Germany) equipped with a high-pressure mercury lamp. Leaves from diploid Changshou kumquat were used as the standard control, and the relative fluorescence intensity of the regenerated plants was compared with the control.

Genomic DNA Extraction

Total DNA was extracted from callus of Dancy, leaves of Changshou kumquat, and the somatic hybrids by CTAB extraction buffer as described by Liu et al. (2002). The resultant DNA pellet precipitated with isopropanol was dissolved in 500 μl of TE buffer (10 mmol l^{-1} Tris-HCl + 1 mmol l^{-1} EDTA). The DNA quality and concentration were analyzed by electrophoresis and spectrophotometry (UV1601 spectrophotometer, Shimadzu, Japan), respectively, followed by dilution to 25 μg μl^{-1} with TE and storage at -20ºC for subsequent analysis.

SSR Analysis

SSR analysis was performed as described by Liu et al. (2002) with minor modifications. Two primer pairs (TAA15 and TAA27) were used in the present research. PCR reactions contained 50 ng genomic DNA, 1.5 mmol l^{-1} $MgCl_2$, 0.2 mmol l^{-1} dNTPs, 1.0 U *Taq* DNA polymerase, and 0.1 μmol l^{-1} of each primer pair. PCR amplifications were carried out on a Peltier-200 thermocycler (PTC-200, MJ Research, Waltham, MA) using the following program: 1 cycle of 94ºC for 10 min, 32 cycles of 1-min denaturing at 94ºC, 40-sec annealing at 55ºC, 2-min elongation at 72ºC, followed by a 10-min extension at 72ºC. After loading buffer was added to the amplified products, they were denatured at 94ºC for 4 min and then analyzed on 6% (w/v) denaturing polyacrylamide gels (Liu et al., 2002). Silver staining was conducted according to the protocol provided by the manufacturer (Promega USA).

CAPS Analysis

PCR amplification was performed with 2 chloroplast (*trn*H-*trn*K, *trn*K-*trn*K) and 3 mitochondrial (*nad1* exon B-C, *18S-5S* rRNA, *nad4* exon1-2) universal primers in a PTC-200 thermocycler according to the report of Xu et al. (2004). The reaction cocktail (50 μl) contained 100 ng genomic DNA, 2.0 mmol l^{-1} $MgCl_2$, 0.2 mmol l^{-1} dNTPs, 2.5 U *Taq* DNA polymerase, and 0.2 μmol l^{-1} of each primer pair. The amplification programs were the same as those for the SSR analysis. After amplification 6 μl of the PCR products were digested with 5U of restriction enzymes for 3-4 h, followed by electrophoresis on 2% agarose gels for 2-3 h at 2.5 v cm^{-1}. The gels were stained with ethidium bromide (0.5 μg ml^{-1}) and photographed under UV light.

Results

FCM Analysis

The genome size of citrus is small with 382 Mb (Arumuganathan and Earle, 1991). It has been shown by FCM that mean nuclear DNA content of the species categorized as "true citrus fruit trees," which encompasses the genera *Fortunella* and *Citrus*, was 0.81pg/2C (Kayim et al., 1998). In our research the diploid kumquat plant was used as a standard, and its relative fluorescence in the FCM was compared with that of the somatic hybrids. The value of the fluorescence intensity corresponding to the curve peak of the standard kumquat was adjusted to 48.89 (Figure 1A). FCM showed that the somatic hybrids had only one peak in addition to a noise peak on the left of the histograms. The relative fluorescence intensity values corresponding to the peak of somatic hybrids were 95.75 and 96.51, respectively (Figure 1B and C), nearly twofold the standard. Previous work has demonstrated that the two parents are diploids with 18 chromosomes (2n=2x=18) while the somatic hybrids are tetraploids possessing 36 chromosomes (Liu and Deng, 2000). Therefore, in combination with the FCM analysis herein, the somatic hybrids could be confirmed as true tetraploids.

SSR Analysis

Totally two pairs of SSR primers were chosen to identify the somatic hybrids. Of the two pairs only one (TAA15) could distinguish Dancy tangerine from kumquat, and it was employed to identify the nuclear DNA of the somatic hybrids. The banding pattern of the fusion parents and the somatic hybrids derived from a sequence gel electrophoresis of the amplification products demonstrated that the somatic hybrids had additive banding patterns contributed by the two fusion parents (Figure 2). No novel bands absent in the fusion parents were detected, and there was no band loss.

CAPS Analysis

CAPS was employed to identify the cytoplasmic constitutions of the somatic hybrids. When the PCR products derived from amplification by universal primers were di-

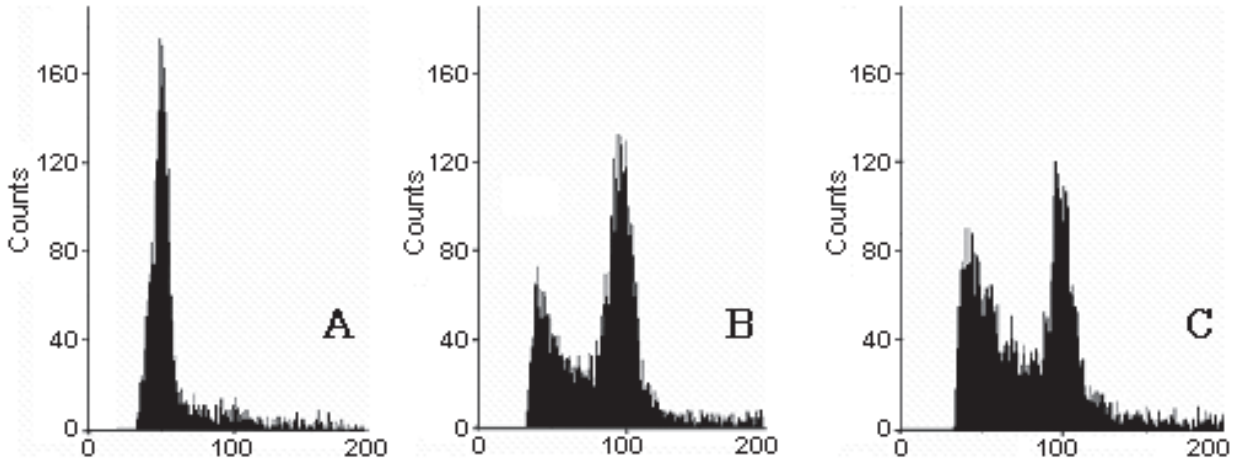

Figure 1. Flow cytometric histograms of ploidy analysis of the parent (Changshou kumquat) and the fusion-derived somatic hybrids. A, Changshou kumquat as control; B and C are the somatic hybrids No. 1 and 2, respectively.

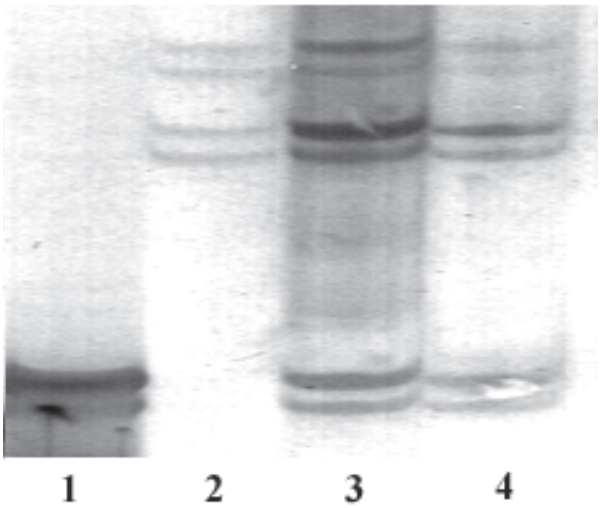

Figure 2. Analysis of the nuclear composition of the somatic hybrids by SSR. Lanes from 1-4 are banding patterns of the regenerated plants and fusion parents produced by primer TAA15. Lane 1, Changshou kumquat; 2, Dancy tangerine; 3 and 4 are the regenerated plants No. 1 and 2, respectively.

gested with the restriction endonucleases, some polymorphic loci were found between the fusion parents. In the present research 4 cpDNA and 1 mtDNA polymorphic primer/enzyme combinations were obtained. The polymorphic cpDNA markers were *trn*K-*trn*K/*Hind*III, *trn*K-*trn*K/*Hae*III, *trn*K- *trn*K/*Ras*I and *trn*K-*trn*K/*Mob*I, and the polymorphic mtDNA marker was *18S-5S*/*Hind*III. As far as the cpDNA was concerned, all of the polymorphic markers produced the banding profiles identical to the suspension parent, Dancy tangerine, and that the bands specific to the leaf parent, kumquat, were not detected (Figure 3). It is suggested that the somatic hybrids got their cpDNA only from the embryogenic parent. In the mtDNA banding profile the somatic hybrids were similar to Dancy tangerine, but some bands specific to Dancy tangerine were absent, possibly meaning that recombination has occurred in the somatic hybrids (Figure 4).

Discussions

For the nuclear and cytoplasmic compositions of citrus somatic hybrids some common conclusions can be made based on the reports so far. As far as tetraploid somatic hybrids are concerned, with the exception of those morphologically identical to the leaf parent, their nuclear DNA were derived from both fusion parents (Guo et al., 2004; Lofty et al., 2003; Medina-Urrutia et al., 2004; Fu et al., 2003) in agreement with the results in this report. This is mainly detected by chromosome counting, FCM analysis, and molecular characterization via RAPD, RFLP, or SSR. The somatic plants between Changshou kumquat and Dancy tangerine in the present paper are analyzed five years after being produced. The ploidy of the somatic hybrids is the same as before, indicating that they are stable without chromosome elimination. Compared with the nuclear DNA, the cytoplasmic components, chloroplast and mitochondrial DNA, had relatively complex inheritance modes. As for chloroplast, uniparental random transmission has been predominantly detected in many citrus protoplast fusion combinations, regardless of whether they are intergeneric or interspecific. By random transmission this means that in a given combination the somatic hybrids may get their cpDNA from either the suspension parent or from the leaf parent. For example in three tetraploid somatic hybrids of Page and Murcott, CAPS showed that two were identical to Murcott and one to Page (Guo et al., 2004). However, such inheritance patterns cannot be the case in other combinations. Chances are that, in some combinations, cpDNA of the suspension parents is transmitted to the somatic hybrids while in others cpDNA from the leaf parents is integrated, as revealed by Medina- Urrutia et al. (2004). The cpDNA transmission pattern in the present research was a case of the latter. In addition to the uni-parental transmission pattern, one case of co-existence of cpDNA has also been reported by Moreira et al. (2000). The underlying reason for this phenomenon has not been determined. It is possibly a temporary status derived from either incomplete or ongoing sorting out of cpDNA from the fu-

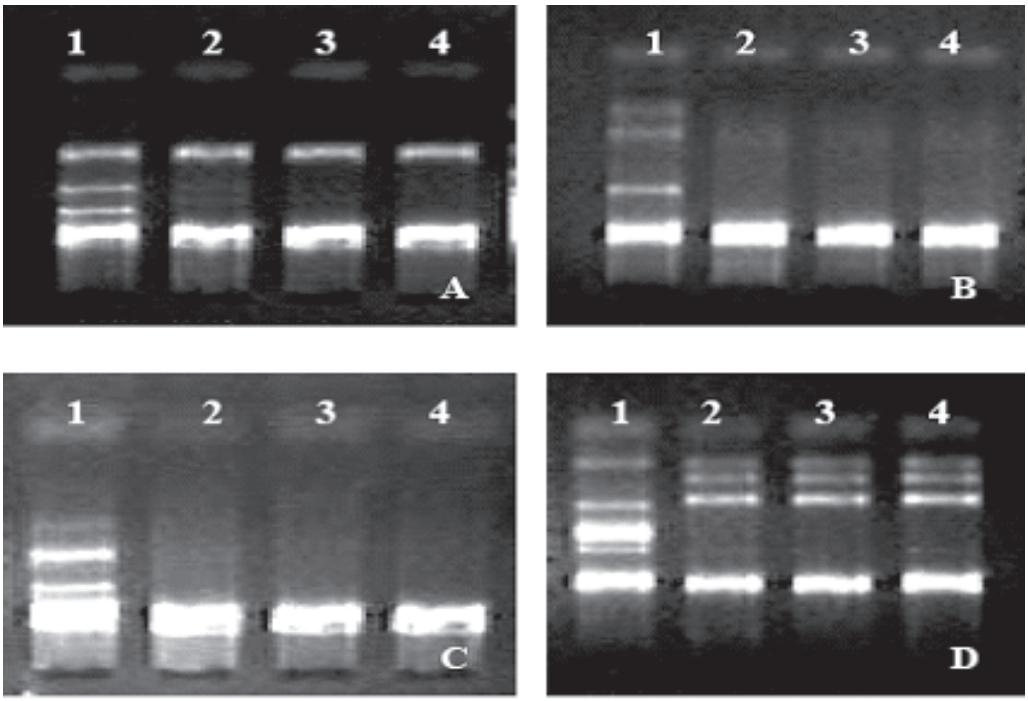

Figure 3. CpDNA banding profiles of the regenerated plants and the fusion parents as revealed with polymorphic primer/enzyme combinations *trn*K-*trn*K/*Hae*III (A), *trn*K-*trn*K/*Hind*III (B), *trn*K-*trn*K/*Ras*I (C) and *trn*K-*trn*K/*Mob*I (D), respectively. Lanes 1-4 are Changshou kumquat, Dancy tangerine, and the regenerated plants No. 1 and 2, respectively.

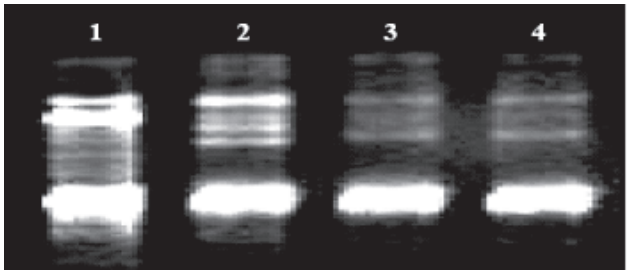

Figure 4. MtDNA banding profile of the regenerated plants and the fusion parents as revealed with polymorphic primer/enzyme combination *18S-5S*/*Hind*III. Lanes 1-4 are Changshou kumquat, Dancy tangerine, and the regenerated plants No. 1 and 2, respectively.

sion parent. As for the mtDNA, nearly all of the somatic hybrids get theirs from the suspension parents (Moreira et al., 2000; Cheng et al., 2003; Medina-Urrutia et al., 2004; Xu et al., 2004; Guo et al., 2004; Liu et al., 2004). In addition, recombination of mtDNA has also been observed in some combinations, such as *Severinia buxifolia* or *Atlantia monophylla* + tangelo (Motomura et al., 1995).

Enormous progress has been made in citrus somatic hybridization, and a large number of somatic hybrids have been obtained all over the world, which greatly enlarges the current germplasms and makes possible citrus cultivar improvement. Some citrus somatic hybrids set fruits with good quality that are expected to be released as cultivars. For example, fruits of the somatic hybrid of Nova and Succari had good external color and pleasant flavor. In addition, the fruits peel easily and are commercially seedless (Grosser et al., 2000). For the time being emphasis in citrus somatic hybridization has been placed on combinations between citrus species and/or between citrus and some genera categorized in the group of true citrus fruit trees like trifoliate orange, kumquat, and *Microcitrus* (Grosser et al., 2000; Grosser and Chandler, 2003, 2004; Medina-Urrutia et al., 2004). Several somatic hybrids have been obtained using kumquat (*Fortunella* spp.) as one fusion parent, such as *F. obovata* (Liu and Deng, 2000; De Carvalho Costa, 2003), *F. crassifolia* (Deng et al., 1992; Grosser and Chandler, 2004), *F. japonica* (Ollitrault et al., 1996; Takami et al., 2004), and *F. hindsii* (Miranda et al., 1997). Since kumquat is thought to be cold tolerant and is now regarded as a candidate for resistance to citrus tristeza virus (CTV) and citrus canker (Mestre et al., 1997; Grosser and Chandler, 2004), the somatic hybrids derived from fusion between citrus and kumquat will be of interest for producing cold-hardy, canker, and/or CTV-tolerant germplasms. Final performance of the somatic hybrids depends on the field evaluation of their agronomic traits. As mentioned above, it has been documented that nuclear and cytoplasmic compositions could affect the growth and development of somatic hybrids. So their identification is of significance for understanding the field performance of these hybrids in the long run. Cheng et al. (2003) found that the somatic hybrids of sweet orange and *F. crassifolia* showed phenotypic abnormality and shoot dieback. Detailed work on mtDNA demonstrated that the mtDNA bands underwent loss prior to shoot dieback while the nuclear and chloroplast genomes were stable during the

course. It is necessary to know if such a phenomenon will occur in the somatic hybrids involving Changshou kumquat as a fusion parent and to find the possible cause for this abnormality. The present research will contribute to this. Since the hybrid plants herein are new, they have grown normally so far. More work is needed to investigate the growth and development of somatic hybrids in the future.

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長壽金柑和丹西紅橘屬間體細胞雜種植株的 FCM, SSR 和 CAPS 分析

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採用 FCM, SSR 和 CAPS 鑒定 2 株長壽金柑（葉肉親本）和丹西紅橘（懸浮系親本）屬間體細胞雜種植株的倍性和核質組成。FCM 分析證明 2 株體細胞雜種植株為四倍體。2 對 SSR 引物中有 1 對在融合親本中具有多型性，該引物對擴增結果表明 2 株體細胞雜種具有相加性帶型，說明它們的核 DNA 來自於融合雙親。CAPS 分析結果表明在所用的通用引物/限制性酶組合中，葉綠體具有 4 個多態型位點，線粒體中具有 1 個多態型位點。在所有具有多態型的葉綠體和線粒體標記中，體細胞雜種的葉綠體帶型與懸浮親本一致，線粒體也與懸浮親本相似，但出現了帶的丟失。本研究將有利於在將來了解體細胞雜種田間表現與核質組成的關係。

關鍵詞：異源四倍體；柑桔；葉綠體 DNA；線粒體 DNA；體細胞雜種。