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Production of Somatic Hybrids between Satsuma Mandarin (*Citrus unshiu*) and Navel Orange (*Citrus sinensis*) by Protoplast Fusion

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We have regenerated allotetraploid plants that are interspecific somatic hybrids between *Citrus sinensis* Osbeck cv. Yoshida navel orange and *Citrus unshiu* Marc cv. Okitsu satsuma mandarin. Protoplasts isolated from 'Yoshida' leaves were chemically fused with callus-derived protoplasts from 'Okitsu'. After 6 months of culture, 102 plants were obtained. These hybrids were identified by differential leaf morphology, DNA fluorescence intensity, and DNA analysis. Ploidy analysis via the flow cytometry revealed that 15 of the 102 plants were tetraploids, with the rest being diploids that morphologically resembled their mesophyll parent. SRAP analysis confirmed that 9 of the tetraploid plants were allotetraploid somatic hybrids. These will be utilized as a possible pollen parents for improving seedy citrus cultivars, e.g., ponkan, mandarin, lemon and kumquat, in order to produce triploid seedless hybrids.

Keywords: allotetraploid, protoplast, satsuma mandarin, somatic hybrid, SRAP

Although members of the citrus species are some of the most important fruit-bearing trees in the world, polyembryony and sterility often cause serious problems in their breeding. Few or no zygotic seedlings result when polyembryonic cultivars are used as maternal parents because their nucellar embryos deter and often prevent zygotic embryo development prior to seed maturation (Button and Kochba, 1977). Protoplast fusion is an alternative way to produce hybrids from species that cannot be crossbred: many inter- and intra-generic plants have been created by this technique (Gleba and Syntik, 1984). Somatic hybridization plays an integral role in programs aimed at developing and improving citrus cultivars. Since successful protoplast fusion was first reported in citrus (Ohgawara et al., 1985), citrus somatic hybrids and cybrids have been obtained by combining sexually compatible and incompatible parents (Grosser and Gmitter, 1990). Most frequently, such interspecific somatic hybrids have been achieved by fusing protoplasts from nucellar-derived embryogenic callus or suspension cultures (embryogenic parent) with non-regenerable mesophyll protoplasts (leaf parent), either via electrofusion (Kunitake, 2000; Olivares-Fuster et al., 2005; Cai et al., 2007) or in the presence of polyethylene glycol (PEG) (Grosser and Chandler, 2000; Khan and Grosser, 2004; Wu et al., 2005; Ananthakrishnan et al., 2006)

Our study objective was to produce somatic hybrid plants from economically valuable cultivars of navel orange and Satsuma mandarin. Such hybrids are difficult to obtain through conventional methods. Although both cultivars have high-quality fruit, sexual hybrid plants have not previously been possible because of their polyembryony, complete or partial male-sterility, and low seediness.

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MATERIALS AND METHODS

Plant Materials

Our parent materials included satsuma mandarin cv. Okitsu (*Citrus unshiu* Marc.), the leading cultivar in Korea, and navel orange cv. Yoshida (*C. sinensis* Osb.). Nucellar embryogenic calli were induced from unfertilized immature mandarin ovules, as described by An et al. (2004). These calli were then maintained by subculturing in an MT medium (Murashige and Tucker, 1969) that was supplemented with 5% (w/v) sucrose and 500 mg L^{-1} malt extract, and solidified with 0.8% (w/v) agar at pH 5.8 (adjusted with 1N KOH before autoclaving). In addition, plantlets regenerated directly from the immature ovules of navel orange (see An et al., 2004) were grown *in vitro*. They were sub-cultured monthly on an MT medium containing 0.2 μM kinetin, 0.1 μM NAA, and 0.8% (w/v) agar. Their fully expanded young leaves were used for isolating mesophyll protoplasts.

Protoplast Isolation

Protoplasts of 'Okitsu' satsuma mandarin were isolated directly from nucellar-derived embryogenic callus cultures according to the method of Grosser and Gmitter (1990), with some modifications. The calli were gently squashed and incubated with a mixture of the enzyme solution -1% (w/v) Cellulase Onozuka RS (Yakult Pharmaceutical, Japan), 0.5% (w/v) Macerozyme R10 (Yakult Pharmaceutical), 0.5% (w/v) Driselase (Sigma, USA), 24 mM $CaCl_2$, 0.92 mM NaH_2PO_4, 6.15 mM 2MES, 0.7 M mannitol (pH 5.6 with 1N KOH) – and a 0.7 M BH_3 medium (Grosser and Gmitter, 1990). This calli-enzyme mixture was incubated on a rotary shaker (50 rpm) for 14~16 h at 29°C to liberate the protoplasts. Leaves of 'Yoshida' navel orange were cut into 0.5~1.0-mm-wide strips, then incubated in a 100 mL flask with 10 mL of enzyme solution containing 1% (w/v) Cellu-

lase Onozuka RS, 1% (w/v) Macerozyme R10, 0.2% (w/v) Pectolyase Y23 (Kyowa Chemical, Japan), 24 mM $CaCl_2$, 0.92 mM NaH_2PO_4, 6.15 mM MES, and 0.7 M mannitol, under the same conditions as for the calli digestion. To isolate and purify these protoplasts, the cell-enzyme mixtures were filtered through a 45-m stainless steel sieve to remove undigested cell clumps and debris. All materials were then centrifuged at 100 *g* for 10 min. The supernatant was removed with a Pasteur pipet and the pellet was gently resuspended in 5 mL of a 25% (w/v) sucrose solution. Afterward, 2 mL of a 13% (w/v) mannitol solution was slowly added directly on top of the sucrose layer, avoiding any mixing. The tube was centrifuged at 100 *g* for 10 min. Viable protoplasts that usually collected in a band at the interface between the two layers were transferred to another centrifuge tube, then washed twice with a 0.6 M BH_3 medium. Both satsuma mandarin and navel orange protoplasts were separately suspended in the 0.6 M BH_3 medium and their densities were adjusted to 1×10^6 cells mL^{-1}.

Protoplast Fusion and Culture

Protoplast fusion was carried out using the PEG. Approximately equal volumes of protoplasts from each parental source were mixed, then suspended in 10 mL of a 0.6 M BH_3 medium and centrifuged at 100 *g* for 10 min. The supernatant was discarded and the pellet re-suspended in the same medium at three times the volume of the pellet. The protoplast mixture was dispensed at four drops per Petri dish, followed by the addition of four drops of PEG solution (40% PEG 6000 in a solution containing 0.3 M glucose and 66 mM $CaCl_2$) on top of the protoplast mixture. This was allowed to stand for 15 min. Afterward, four drops of a 0.6 M BH_3 medium were added to opposite sides of the PEG-treated protoplast mixtures, which were then allowed to stand for 15 min. The fusion products were washed twice, each time with 20 drops of a 0.6 M BH_3 medium, at 10-min intervals. The washed mixture of fused protoplasts was cultured in a liquid 0.6 M BH_3 medium and placed in a clear plastic box. Heterocaryons were identified by the presence of starch bodies from the callus-derived protoplasts and chloroplasts from the leaf protoplasts within the same cells.

Plant Regeneration

For somatic embryo induction, microcalli derived from fused protoplasts were transferred to an MT medium that was supplemented with 5% (w/v) sucrose and 1500 mg L^{-1} malt extract, and solidified with 0.8% (w/v) agar. To promote shoot formation, the cotyledonary embryos were transferred to an MS (Murashige and Skoog, 1962) medium containing 3% (w/v) sucrose and 3 M gibberellic acid. When the regenerated plantlets were about 10 cm tall, they were washed in tap water and planted in plastic pots containing an autoclaved commercial horticulture nursery medium. These plantlets were maintained for 1 month at 26±1°C and 95±5% relative humidity, under low fluorescent light. After acclimatization, plantlets were transferred to the greenhouse.

Flow Cytometry Analysis

Ploidy of the regenerated plants was determined by flow cytometry (FCM) analysis. Fluorescence of samples was measured on a Partec Flow Cytometer (PA-I; Germany) equipped with a high-pressure mercury lamp. Leaves and calli were chopped, then incubated in 0.5 mL of nuclei extraction buffer (High Resolution DNA Kit Type P, Solution A; Partec) for 1 min, followed by filtering with 30-m Partec Celltrics™ and staining for 2 min with 1 mL of Partec HR-B solution. The diploid 'Yoshida' navel orange was used as the control, against which the relative fluorescence intensity of our regenerated plants was compared.

SRAP Analysis

To identify the allotetraploids among our regenerated plants, we used a simple marker, sequence-related amplified polymorphism (SRAP), for DNA analysis. Total DNA was extracted from the 14 tetraploid and 2 diploid plants that had been regenerated via protoplast fusion, using G-spin™ IIp with the plant genomic DNA extraction kit (iNtRon, Korea). The 20 µL PCR mixtures contained 250 µM dNTP, 1.5 mM $MgCl_2$, 1.0 unit of *Taq* DNA Polymerase (Promega, USA), 50 pmole of either forward (f35: 5-TGAGTCCAAACCGGGAG-3) or reverse (r55: 5-GACTGCGTACGAATTTCG-3) primers, 10 mM Tris-HCl (pH 9.0), 40 mM KCl, and 25 ng of template DNA. PCR amplification was conducted in a TaKaRa PCR Thermal Cycler (TaKaRa, Japan) under the following conditions: an initial denaturation at 94°C for 5 min, then five cycles of 1 min of denaturing at 94°C, 1 min of annealing at 35°C, and 2 min of elongation at 72°C. Over the next 35 cycles, the annealing temperature was increased to 50°C, with a final elongation at 72°C for 10 min (Sun et al., 2006). The PCR products were analyzed by electrophoresis in 1.2% (w/v) SeaKem LE Agarose gels (Cambrex, USA) with 0.5 µg mL^{-1} of ethidium bromide.

RESULTS AND DISCUSSION

Previous experiments with embryogenic calli showed that protoplasts could not be isolated directly from callus cultures because of the incomplete maceration of cell walls and bursting of the isolated protoplasts. Such studies involved pre-treated calli or suspension cultures (Ohgawara et al., 1985; Ling et al., 1989; Kunitake et al., 1991). Here, however, we were able to obtain many viable protoplasts of satsuma mandarin (Fig. 1A) from callus tissue cultured on an MT medium containing 5% (w/v) sucrose and 500 mg L^{-1} malt extract. Protoplast yields were usually 2×10^6~7×10^6 cells per gram of callus.

After the fusion treatment, heterocaryons (Fig. 1C) were easily distinguished microscopically from other cells because of the existence of a colorless part from the cultured cell partner and a green portion from the mesophyll partner. The first mitotic divisions occurred after 7~10 d of culture (Fig. 1D). Individual colonies, consisting of as many as 15~30 cells, were observed after 21~30 d of culture. When a cell mass colony was observed, we added new media to the Petri dish. These colonies grew rapidly into friable, embryogenic and nodular calli that closely resembled the original calli (Fig. 1G). Differentiated calli were transferred onto an MT medium supplemented with 5% (w/v)

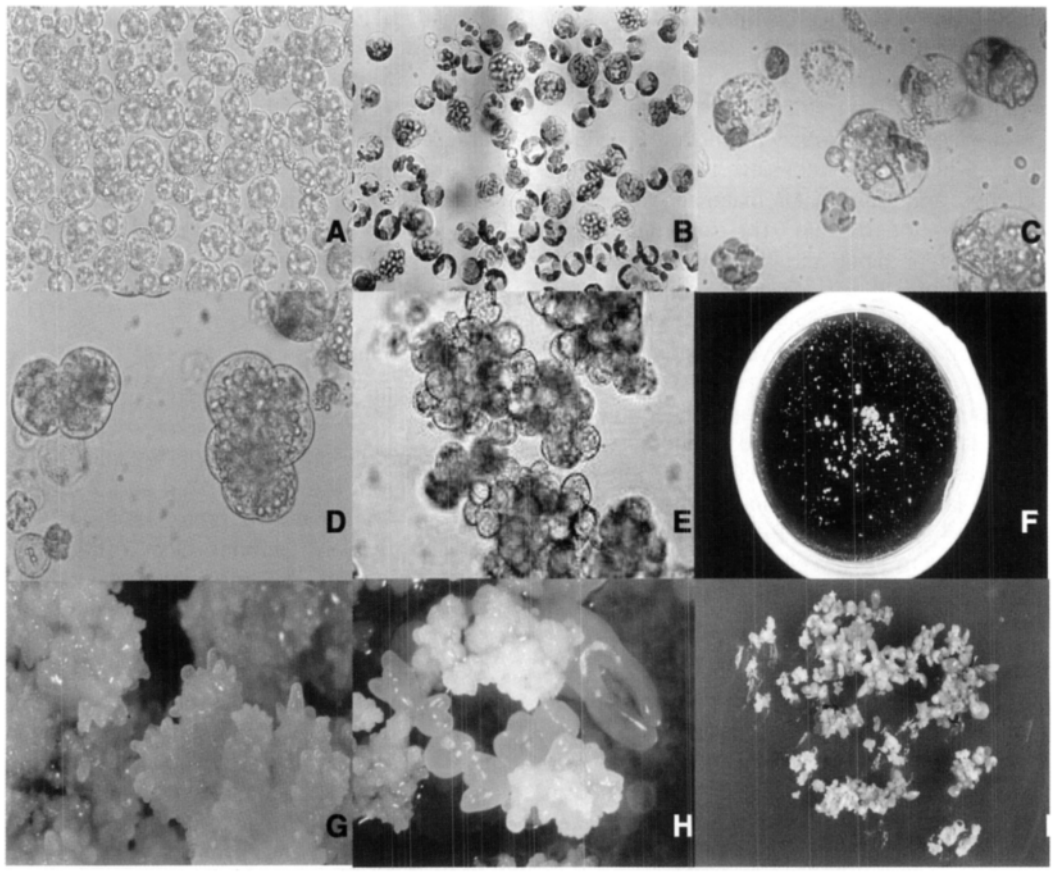

Figure 1. Somatic embryogenesis via fusion between protoplast derived from embryogenic callus of satsuma mandarin and protoplast isolated from leaf of navel orange. **A,** protoplasts from embryogenic callus; **B,** mesophyll protoplasts; **C,** fused protoplasts; **D,** divided cell; **E,** micro-colony; **F,** differentiated microcalli; **G,** differentiated nodular callus; **H,** globular, torpedo, and heart-shaped embryos; **I,** cotyledonary embryo.

sucrose and 1500 mg L^{-1} malt extract to promote embryogenesis after 30~40 d of culture.

Hidaka and Omura (1989) have suggested that somatic embryogenesis of nucellar calli from citrus species can be stimulated when sucrose in the medium is replaced by certain other sugars or sugar alcohols, e.g., galactose, lactose, raffinose, maltose, or glycerol (see also Button, 1978; Kochba et al., 1982; and Ben-Hayyim and Neumann, 1983). However, for satsuma mandarin cultivars, embryos can be induced from 'Tokumori' wase, 'Okitsu' wase, and 'Miyagawa' wase only when lactose is added as a carbon source; no embryoid formation has previously been observed in MT media containing sucrose, glucose, fructose, or galactose (Kunitake et al., 1991). Those earlier results differ from ours. At 15 to 30 d after the embryogenic callus was transplanted to the embryogenesis medium, calli were developed to globular, torpedo, heart-shaped, and cotyledonary embryos (Fig. 1H, I).

To regenerate plantlets, we transferred these cotyledonary embryos onto a germination medium containing GA_3. These embryos developed into whole plants (Fig. 2) that were morphologically normal. The maturation, germination, and conversion of somatic embryos into plants are difficult tasks during somatic embryogenesis (Sutton and Polonenko, 1999). For woody species especially, the efficiency of conversion is relatively low, which hampers the application of a somatic embryogenesis system for purposes of commercialization (Merkle et al., 2003). However, Moon et al. (2006) have achieved the highest conversion rate,

Figure 2. Regenerated hybrids from 'Okitsu' satsuma mandarin + 'Yoshida' navel orange.

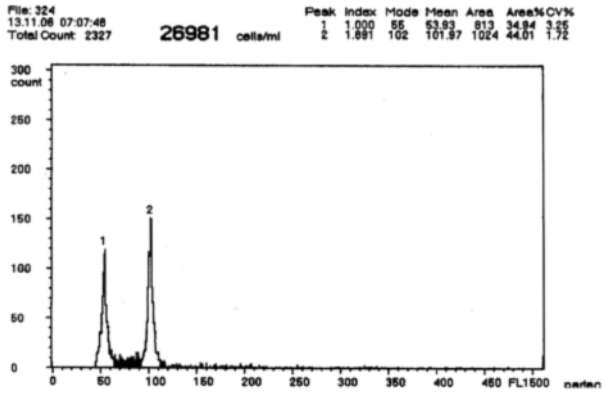

Figure 3. Flow cytometry analysis of one plant regenerated after cell fusion. Histogram shows peak for control diploid parent (1) at approximately channel 50, and peak for tetraploid hybrid (2) at approximately channel 100.

Figure 4. Tetraploid plant (left) regenerated from fusion of 'Okitsu' satsuma mandarin + 'Yoshida' navel orange, and diploid plant (right).

35%, via somatic embryogenesis in *Oplopanax elatus*, a valuable medicinal woody plant. Here, we obtained 102 putative somatic hybrids between 'Okitsu' satsuma mandarin and 'Yoshida' navel orange. Previously, Ohgawara et al. (1985) established a technique for somatic hybridization that fused the callus protoplast of 'Trovita' orange with the mesophyll protoplast of trifoliate orange. That research group developed a unique method for hybrid selection, i.e., after fusion by PEG treatment, the products were cultured in a hormone-free MT medium containing a high concentration of sucrose. Under such conditions, homocaryons fused from callus protoplasts and unfused callus protoplasts could divide but mesophyll protoplasts never did. Therefore, we can assume that our 102 regenerated plants were not only hetero- and homo-hybrids but also diploids regenerated from callus protoplasts. These regenerated plants continued to grow under greenhouse conditions.

To reveal the nuclear background of those regenerated plants, we carried out ploidy analysis by flow cytometry (Fig. 3), using the diploid 'Yoshida' navel orange as a control. FCM is a technique that allows for the precise determination of nuclear DNA content and ploidy level by using DNA-specific fluorochromes to stain isolated nuclei and monitoring their relative fluorescence intensity (Lucretti et al., 1992). Out of 102 plants, 15 were identified as being tetraploid, with the rest being diploid and morphologically similar to navel orange (Fig. 4). These ploidy determinations by flow cytometry were then confirmed by DNA analysis.

We also performed SRAP analysis to see whether 14 of those tetraploids were allotetraploids or autotetraploids; the 1 remaining tetraploid could not be analyzed. Primer f35/r55 amplified specific bands from both parents; 9 of 14 tetraploids were allotetraploid while 5 were autotetraploid (Fig. 5). Furthermore, all tetraploids had the typical leaf morphology of tetraploid citrus plants, being broader, thicker, and darker green than those of the diploids (Fig. 6). These

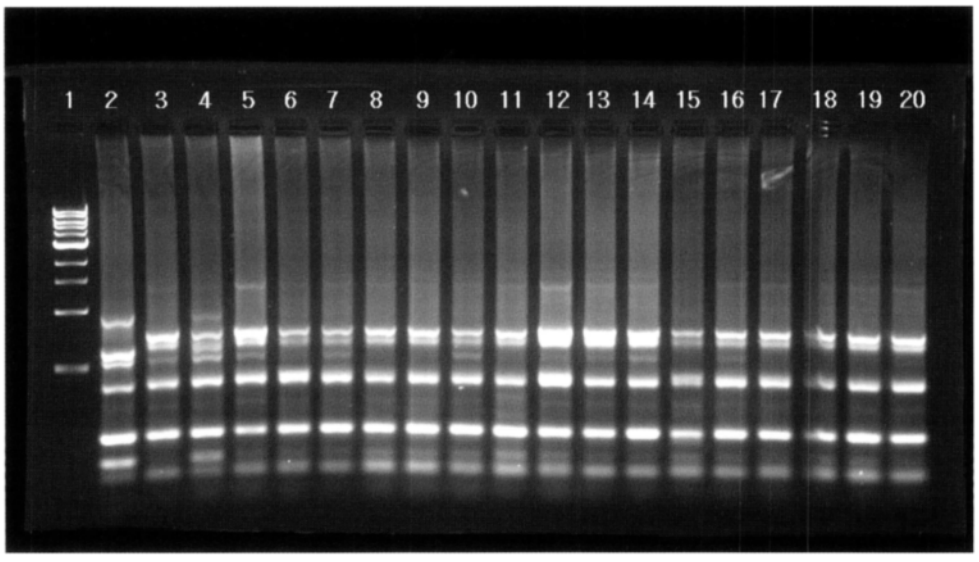

Figure 5. SRAP patterns for regenerants and both parental genotypes (primer f35/r55). 1: DNA ladder; 2: 'Okitsu' satsuma mandarin; 3: 'Yoshida' navel orange; 4: DNA mixture of satsuma mandarin and navel orange; 8-12, 15, 16, 18, and 19: allotetraploids; 5-7, 17, and 20: autotetraploids; 13 and 14: diploids regenerated from protoplast fusion.

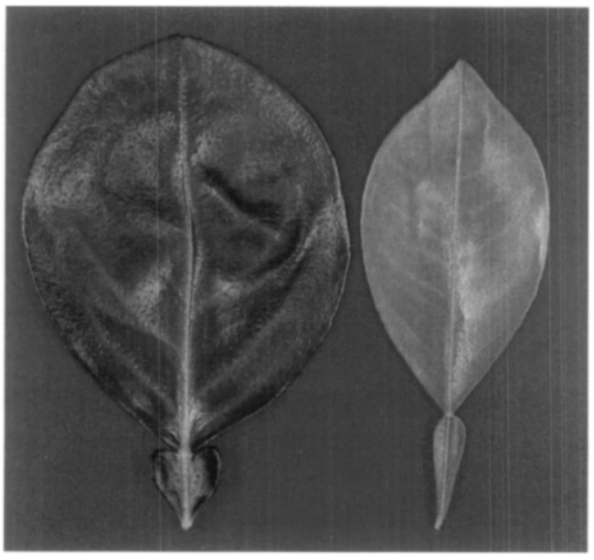

Figure 6. Leaf morphology of somatic hybrid (left) and diploid plant (right).

allotetraploid somatic hybrids also grew very vigorously.

In contrast to our observation, Wu and Mooney (2002) have reported that autotetraploid plants derived from colchicine-treated embryogenic calli show stunted growth. It is possible that our protoplast fusion hybrids had more regular cell divisions and, hence, grew faster because they were allotetraploids. In all, we produced 9 allotetraploid, 5 autotetraploid, and 87 diploid plants that morphologically resembled the navel orange. It is not clear why mesophyll parent-type plants are able to be regenerated. Other researchers have demonstrated, via RFLP analysis with the cytoplasmic genomes of mesophyll regenerants, that all are cybrids, inheriting their mitochondrial DNA (mtDNA) from the corresponding embryogenic callus parents (see Grosser et al., 1996; Moriguchi et al., 1996; Tokunaga et al., 1999; Liu and Deng, 2000; Guo et al., 2006). Because mtDNA may control cytoplasmic male sterility (CMS) (Kumar and Cocking, 1987), and because the satsuma mandarin has been verified to be of the CMS type (Yamamoto et al., 1997), the mesophyll regenerants from a combination that uses embryogenic callus suspension cultures of a male-sterile cultivar as the fusion partner probably will be male-sterile and will bear seedless fruits. Therefore, it is truly a novel event to achieve a seedless diploid cybrid *Citrus* via standard symmetrical protoplast fusion.

In the *Citrus* genus, satsuma mandarin and navel orange are typically of the male and/or female sterile type, such that genetic interchange and rearrangement cannot be realized by sexual crosses. However, the cell fusion technique efficiently solves this problem, with the somatic hybrids of satsuma mandarin + navel orange being fertile, thereby, enabling researchers to obtain triploid plants by using those hybrids as the pollen parents (Kobayashi et al., 1995). Thus, the somatic hybrids reported here will probably be fertile, and can be utilized as a possible pollen parent to improve seedy cultivars, including those of the lemon, kumquat, ponkan, and mandarin.

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