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Production of Triploid Somatic Hybrids Between Mandarin and Grapefruit through Electrofusion

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Abstract

Somatic hybrids were produced by electrofusion between embryogenic callus protoplasts of 'Syougun' mandarin and leaf protoplasts of grapefruit. Hybridity of the two plants was confirmed by leaf morphological characteristics and random amplified polymorphic DNA (RAPD) analysis. The cpDNA analysis using PCR-RFLP could not distinguish those of both parents. These plants showed normal growth and had chromosome number of 27. These unexpected triploid somatic hybrids might be derived from fused cells between haploid protoplast of embryogenic calli and diploid protoplast of leaf, because polysomaty, a mixture of haploid cells and diploid cells was observed in the lactose medium-pretreated embryogenic calli of 'Syougun' by flow cytometry analysis.

Introduction

Citrus is one of the most commercially important fruit trees in the world, and is grown throughout the world in tropical and subtropical areas. In addition to the fresh fruit itself, various products such as juice, essential oils and pectin have been produced from *Citrus* species. In conventional cross breeding of *Citrus* and closely related genera, several barriers restrict its utilization for the improvement of *Citrus* by sexual crossing. One of the major problems is polyembryony. When polyembryonic species are used as maternal parents, few or no sexual hybrids are obtained, because the nucellar embryos restrict and often abolish hybrid embryo development prior to seed maturation. Male and female sterilities in *Citrus* cultivars are also frequent limitation factors. Furthermore, heterozygosity and the long juvenility have hampered an effective improvement of *Citrus*. Therefore, protoplast fusion, which has been used as a new tool for bypassing the difficulty in making conventional sexual crosses, is potentially of great value in *Citrus* breeding. Since the first report on successful protoplast fusion in *Citrus* (Ohgawara et al., 1985), many other somatic hybrids and cybrids have been produced (Grosser et al., 1996; Tokunaga et al., 1999). Although these somatic hybrids are of great interest as breeding materials, they cannot be directly used as commercial cultivars because they possess undesirable characteristics related to tetraploidy (Kobayashi et al., 1995). On the contrary, triploids are economically useful because of their favorable characteristics such as seedlessness and thin rind (Soost and Cameron, 1980; 1985). Recently, triploid somatic hybrids were produced between several diploid cultivars and haploid 'Clementine' mandarin (Kobayashi et al., 1997; Ollitrault et al., 1998). However, this technique seems to be poorly adapted for large scale breeding programs since haploid production in polyembryogenic cultivars is difficult.

In the present study, we describe the unexpected production of triploid somatic hybrid plants between diploid embryogenic callus protoplasts of 'Syougun' mandarin and diploid leaf protoplasts of grapefruit.

Materials and Methods

Plant materials

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'Syougun' mandarin (*Citrus reticulata* Blanco), one of the leading cultivars in Thailand and grapefruit L-1 (*C. paradisi* Macf.), was used in this study. To induce nucellar calli, fruits were surface-sterilized and then were cut open aseptically. Seeds containing nucellar embryos were carefully dissected and placed on Meurashige and Tucker (MT) medium containing 5 mg/L adenine, 500 mg/L malt extract, 30 g/L sucrose and 2 g/L gellan gum. The white and friable nucellar calli induced after 3 months of culture were maintained by subculturing on MT medium containing 10 mg/L benzylaminopurine (BA), 30 g/L sucrose and 2 g/L gellan gum at 25℃ under continuous illumination (38μM/m^2sec).

Protoplast isolation

Protoplasts of 'Syougun' mandarin were prepared from embryogenic calli according to the methods of Kunitake et al. (1991). Prior to isolating the protoplasts, the embryogenic calli were transferred to MT medium containing 50 g/L lactose and 2 g/L gellan gum for preventing the bursting of protoplasts. The pretreated calli were gently squashed and incubated with an enzyme solution containing 0.3% (w/v) Cellulase Onozuka R-10 (Yakult Pharmaceutical Co. Ltd., Japan), 0.3% (w/v) Macerozyme R-10 (Yakult Pharmaceutical Co. Ltd., Japan), 0.1% (w/v) Driselase (Kyowa Pharmaceutical Co. Ltd., Japan), 1/2MT macro elements and 0.7 M sorbitol, at pH 5.7. The mixture was incubated on a rotary shaker (60 rpm/min) for 16 hr at 25℃ to liberate protoplasts. Protoplasts were collected by filtration through a nylon sieve (60 μM) and washed twice with 0.6 M mannitol solution after centrifugation (100 × g for 5 min).

In the case of grapefruit, leaves were rinsed with 70% ethanol for 5 sec, immersed in a solution containing 1 % sodium hypochlorite and 0.1 % Tween 20 for 15 min and washed twice with sterile distilled water. The leaves were then cut into about 2 mm wide strips with a razor blade and were incubated in a Petri dish with 10 mL enzyme solution containing 2 % Cellulase Onozuka RS, 0.5% (w/v) Macerozyme R-10 , 0.05 % (w/v) Pectolyase Y-23 (Seishin Pharmaceutical Co. Ltd., Japan), 10 mM $CaCl_2 \cdot 2H_2O$, 5 mM MES and 0.6 M sorbitol, at pH 5.7. The incubation was carried out at 25℃ on a rotary shaker (45 rpm) for 16 hr. The cell-enzyme mixture was filtrated through two layers of Miracloth and centrifuged (100 × g for 5 min). The protoplasts were then washed twice with 0.6 M mannitol by the same centrifugation. Both mandarin and grapefruit protoplasts were separately suspended in the fusion solution containing 1 mM $CaCl_2 \cdot 2H_2O$, 5 mM MES and 0.6 M mannitol, and their densities were adjusted to 1×10^6/mL.

Electrofusion apparatus and procedure

Electrofusion was carried out using a model SSH-1 electrofusion apparatus connected to a fusion chamber SSH-CO3 (Shimadzu Co. Ltd., Japan) with parallel stainless steel electrodes 2 mm apart. The fusion chamber was sterilized by autoclaving before use. Protoplast fusion was observed using an inverted microscope.

Protoplasts of both species were mixed at a ratio of 1 : 1, and 1 mL of the protoplast suspension was pipetted into a fusion chamber. The protoplasts were aligned into short chains by applying an alternating current of 120 V/cm, at 1MHz for 15 sec. Fusion was then induced by a direct current square pulse of 1.5 kV/cm for 30 μs. The fusion frequency was observed under a light microscope and viability of these protoplasts was assessed using fluorescein diacetate (FDA) (Widholm, 1972). After applying the fusion pulse, the protoplast suspension was kept for 10 min at room temperature. Then, the protoplasts were carefully pipetted into a glass tube and collected by centrifugation (100 × g, 2-3 min).

Protoplast culture and induction of somatic embryos

The fusion-treated protoplasts were cultured in MT medium containing 0.6 M sucrose and 2 g/L gellan gum, according to the selection method of Ohgawara et al. (1985). After 2 months of culture, several somatic embryos were transferred to 1/2 MT medium containing 1 mg/L GA_3, 30 g/L sucrose and 2 g/L gellan gum for plant regeneration.

Flow cytometry

A young leaf of Approximately 1 cm^2 was collected from each of the both parents and the regenerated plants and chopped with a razor blade for 5 min in 1mL of a buffer solution containing 1.0% (v/v) Triton X-100, 140 mM mercaptoethanol, 50 mM Na_2SO_3 and 50 mM Tris-HCl at pH 7.5, according to the preparation method of Harusaki et al. (2000). Crude samples were filtered through Miracloth and stained with 25 μg/mL propidium iodide (PI). The relative fluorescence of total DNA was measured for each nucleus with a Flow Cytometry System EPICS XL (Beckman-Coulter, Co. Ltd., Germany) equipped with an argon laser (488 nM, 15 mW).

For each sample, at least 10,000 nuclei were

counted to generate a histogram. For calibrating the scale of fluorescence, x, 2x, 3x, 4x and 5x nuclei from citrus samples were used as controls.

For evaluating of ploidy level of the pretreated embryogenic calli for protoplast isolation, 1.0 cm^3 of the calli from were used for measuring the DNA contents by the same methods in 'Syougun' mandarin and 'Ohta'ponkan.

Counting chromosome number

According to the modified methods of Oiyama (1981), root tips of *in vitro* plantlets were pretreated with 2 mM 8-hydroxyquinoline for 30 hr at 4℃ and fixed in a mixed solution of ethanol : acetic acid (3:1) for 24 hr. The root tips were then macerated in 1N HCl for 3 min at 60℃, and stained and squashed in 1% aceto-orcein.

Extraction of total DNAs

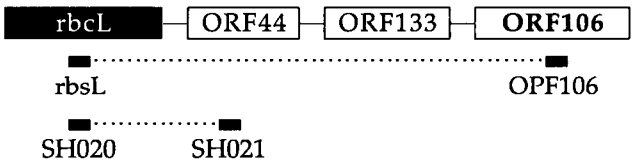
Total DNAs were extracted from plants grown in pots in parental species and the putative somatic hybrids, R1 and R2, according to the method of Doyle and Doyle (1987).

Random amplified polymorphic DNA (RAPD) analysis

PCR and electrophoresis were performed by the methods described by Williams et al. (1990) with some modifications. The reaction mixtures contained 10 mM Tris-HCl, pH 8.3; 80 mM KCl; 1.5 mM $MgCl_2$; 100 μM each dATP, dCTP, dTTP, and dGTP, 0.3 μM primer; 2.5 U *Taq* DNA polymerase and 10 ng of genomic DNA, in a total volume of 25.0 μL. Reactions were cycled 45 times at 94℃ for 30 sec, 37℃ for 2 min and 72℃ for 3min in a ASTEC Program Control Ststem PC-700. Primers of 10 nucleotides in length were purchased from Operon Technology Inc. (CA, USA). After all of the PCR cycles were completed, 5 μL of the samples were loaded on 1.0% agarose gels and subjected to electrophoresis at 10V/cm for 15 min. Then, the gels were stained with 10 mg/L of ethidium bromide and photographed under UV light (360 nM). For each combination of samples and primers, the PCR was carried out twice and only stable polymorphism was taken into account.

PCR-RFLP analysis of cpDNA

PCR-RFLP analysis of cpDNA was performed by the methods described by Haruki et al. (1997). The putative somatic hybrid R1 and the parents were used for the analysis of cpDNA. The nucleotide sequences of primers and their positions are indicated in Figure 1. The primers for ribulose-1, 5-bisphosphate carboxylase large subunit (*rbc*L) gene and ORF106 designed by Ogihara et al. (1991) were used. Other primers, SH20 and SH21 were designed, based on the nucleotide sequences of *rbc*L (Nishizawa and Hirai, 1987). The reaction mixtures contained 10 mM Tris-HCl, pH 8.3; 80 mM KCl; 1.5 mM $MgCl_2$; 100μM each dATP, dCTP, dTTP, and dGTP, 0.15 μM of each primer; 2.5 U Ampli *Taq* polymerase and 10 ng of genomic DNA, in a total volume of 25.0 μL. Reactions were cycled 30 times at 93 ℃ for 30 sec, 60 ℃ for 30 sec and 73 ℃ for 2 min in a ASTEC Program Control System PC-700. Eight restriction endonucleases, *Alu*I, *Dde*I, *Hinf*I, *Msp*I, *Nco*I, *Sau*3AI, *Scr*FI and *Taq*I (TOYOBO, Co. Ltd., Japan) were tested to reveal polymorphism within the putative somatic hybrid R1 and the both parents. Reactions were carried out at 37℃ for one hour in 10 μL solution containing 10 units of *Alu*I, *Dde*I, *Hin*fI, *Msp*I, *Nco*I, *Sau*3AI and *Scr*FI. For *Taq*I, the reaction was performed at 65 ℃, and the reaction mixture in the microfuge tubes was covered with mineral oil to prevent evaporation. After digestion, the restriction fragments were separated by electrophoresis in 2 % agarose gel and observed under UV light by the same methods as described previously.



rbcL	5'	-ATGTCACCACAAACAGAAACTAAAGCAAGT
OPF106	5'	-ACTACAGATCTCATACTACCCC
SH020	5'	-CGCGGTGGACTTGATTTTACC
SH021	5'	-GGCATATGCCAAACATGAATACC

Figure 1. Positions of pair primers: rbcL and ORF106, SH106, SH020 and SH021 represented by short solid lines.

Results and Discussion

When an alternating current field was applied at 1 MHz and 120 V/cm, pearl chains consisting of 5-10 protoplasts were formed within 15 sec. After the application of direct current pulse, the fused protoplasts rapidly became round. The fusion efficiency was approximately 3% at a field strength of 1.5 kV/cm for 30 μs. However, protoplast viability by assessing with FDA staining method decreased to approximately 50%. After the electrofusion treatment, the first cell division occurred after 7 days of culture, and a lot of colonies were observed after 30 days of culture. Some of them formed 1-5 green somatic em-

bryos per plastic dish after 60 days of culture. When these somatic embryos were transferred onto 1/2 MT medium containing 1 mg/L GA_3 and 30 g/L sucrose, roots and leaflets were produced 3 months after transfer. Finally, 36 plants each originated from different somatic embryos were obtained after acclimatization.

Flow cytometric analysis on the x, 2x, 3x, 4x and 5x controls of *Citrus*, revealed that the fluorescence intensity of these ploidy levels corresponded to 100, 200, 300, 400, and 500, respectively. Analysis of regenerated plants 1 (R1) and 2 (R2) showed that their fluorescence intensity coincided to that of the 3x control, while fluorescence intensity of the other 34 regenerated plants corresponded to that of the 2x control. Chromosome observation on root tip squashes revealed that the chromosome number of R1 and R2 was 27, which was different from the sum of chromosome numbers of 'Syougun'mandarin (2n=18) and grapefruit (2n=18).

Flow cytometry is a reliable method for the rapid and easy determination of ploidy levels (Aru-muganathan and Earle, 1991). In *Citrus* species, flow cytometry has been used for detecting triploids produced from crossing between diploid and tetraploid and for selecting somatic hybrids at early stage of culture (Tusa et al., 1996; Ollitrault et al., 1996; Harusaki et al., 2000). In the present study, two triploids, R1 and R2, could be easily selected from a lot of young regenerated plants. Repeated analyses showed that they were not a chimeric.

Two triploid plants, R1 and R2 showed vigorous growth and thick leaves, which were typical characters of polyploids. They also had intermediate morphology between the fusion parents. At the immature stage of leaves, the size of wing leaf resembled those of grapefruit. Cell division in leaf protoplasts of grapefruit was not observed under the present protoplast culture condition. Therefore, based on the division and plant regeneration capacity of both parents, leaf morphology and chromosome numbers, two regenerated plants must be triploid somatic hybrids.

For the further confirmation of these regenerated plants we employed RAPD analysis in R1, R2 and their parents. Among the primers tested, OPA1, OPA4, OPB5, OPB13, OPB20 for R1 and OPA4, OPB 12, OPH1, OPH5, OPH20 for R2 were suitable for discrimination between 'Syougun'and grapefruit L-1. R1 and R2 had the fragments specific for both parents. These results also indicate that R1 and R2 are somatic hybrids.

RAPD analysis has been successfully employed for the identification of somatic hybrids (Grosser et al., 1996; Xu et al., 1993). Kobayashi et al. (1997) identified somatic hybrids using RAPD analysis, but they did not find any suitable restriction enzymes for the production of parent-specific fragments on the analysis of ribosomal RNA genes (rDNA). In the present study, we could efficiently find several primers for producing parent-specific bands to identify the hybridity of R1 and R2.

The length of the fragment cpDNAs amplified with *rbc*L+ORF106 primers and SH20 + SH21 was about 3.2 kb and 0.57 kb, respectively. However, the polymorphisms could not be observed when the fragments were digested with all restriction enzymes tested for R1. Further analyses using other primers and restriction enzymes are necessary to identify cpDNA of R1.

In the present study, protoplast fusion using embryogenic callus cells of 'Syougun'(2n=18) and mesophyll cells of grapefruit (2n=18) allowed induction of the unexpected triploid somatic hybrids. To date, somatic hybrids have been produced from more than 100 parental combinations in Citrinae, and most of those hybrids were tetraploids derived from diploid parents. However, Grosser et al. (1992) reported that following protoplast fusion between C. *sinensis* cv. 'Hamlin' and *Severinia buxifolia* (poir.) Tenore, both diploid accessions, only triploid regenerated plants were produced, but they could not determine whether the callus line was at the haploid level at the time of protoplast isolation or chromosome elimination had taken place after fusion. In the present study, using the flow cytometry analysis, polysomaty with haploid and diploid cells was observed in the embryogenic calli pretreated on MT medium containing 50 g/L lactose for isolating protoplasts (Figure 2). Therefore, these unexpected triploid somatic hybrids might be derived from fusion between haploid protoplast of embryogenic calli and diploid protoplast of leaf.

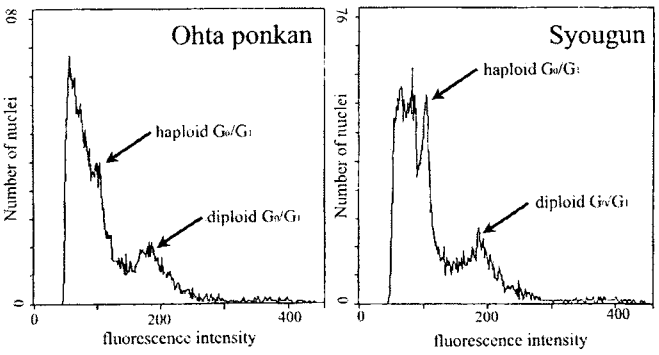


Figure 2. Flow cytometric analysis of nucellar calli pretereated by culturing on the lactose medium for 10 days.

Occurrence of haploid cells in diploid callus cultures has been also reported in several plants such as *Vicia hajastana* and carrot (Singh et al., 1972; Nuti-Ronchi et al., 1992). Nuti-Ronchi et al. (1992) showed that frequency of haploid cells in carrot suspension cultures was 24.0% in the stage of cell clusters, and was 38.8% in the sage of embryos. D' Amato (1998) described that nuclear fragmentation (amitosis) followed by mitosis is a very important process of chromosome number reduction and haploidization *in vitro*. The mechanism involved in the occurrence of haploid cells from diploid suspension cultures of Citrus cultivars is still unclear. However, it is possible to postulate that sugar starvation on the lactose medium might prevent normal cell metabolism, and then induce the haploid cells.

In the present study, somatic hybridization using haploid cells which were induced from diploid embryogenic calli allowed the direct induction of triploid hybrids. Production of triploid somatic hybrids between polyembryonic cultivars may be possible, if haploid cells could stably be induced from diploid embryogenic calli. Further studies, however, are still required to overcome several problems such as the frequency of haploid cells and the difference of reaction among each Citrus cultivars.

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