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Molecular and Cytogenetic Characterization of Triploid Somatic Hybrids between 'Shogun' Mandarin and Grapefruit.

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Abstract

Somatic hybrids were produced between embryogenic callus protoplasts of 'Shogun' mandarin (*Citrus reticulata* Blanco, 2n = 2x = 18) and leaf protoplasts of grapefruit (*C. paradisi* Macf., 2n = 2x =18) by electrofusion. Hybridity of the two regenerated plants was confirmed by leaf characteristics and nuclear genome analysis using RAPD and PCR-RFLP. Flow cytometry and root-tip cell count of the two hybrids revealed a chromosome number of 27. These triploid somatic hybrids on transfer to field showed normal morphology and vigorous growth. Field performances as well as disease resistance are under investigation.

Key words: *Citrus*, electrofusion, flow cytometry, PCR-RFLP, somatic hybrid, triploid.

Introduction

Citrus is one of the commercially most important fruit trees in the world, and is grown throughout the world in tropical and subtropical areas. In addition to the fresh fruits, 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. 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 have been produced (Ohgawara *et al.*, 1989; Kobayashi *et al.*, 1991; Grosser *et al.*, 1992, 1996; Mendes *et al.*, 2001; Guo *et al.*, 2002). 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 'Shogun' mandarin and diploid leaf protoplasts of grapefruit.

Materials and Methods

Plant materials

'Shogun' mandarin (*Citrus reticulata* Blanco, 2n = 2x = 18), one of the leading cultivars in Thailand and grapefruit L-1 (*C. paradisi* Macf., 2n = 2x = 18), was used in this study. To induce nucellar calli, mature fruits were surface-sterilized and then were cut open aseptically. Seeds containing nucellar embryos were carefully dissected and placed on Murashige and Tucker (MT; 1969) medium containing 5 mgl^{-1} adenine, 500 mgl^{-1} malt extract, 30 gl^{-1} sucrose and 2 gl^{-1} gellan gum. The white and friable nucellar calli induced after 3 months of culture were maintained by subculturing on MT medium containing 10 mgl^{-1} 6-benzylaminopurine (BA), 30 gl^{-1} sucrose and 2 gl^{-1} gellan gum at 25 ℃ under continuous illumination (38 $\mu mol\ m^{-2}\ s^{-1}$).

Protoplast isolation

Protoplasts of 'Shogun' 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 gl^{-1} lactose and 2 gl^{-1} 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^{-1}) for 16 h 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 of plants which grown in a greenhouse were rinsed with 70% ethanol for 5 sec, immersed in 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 h. 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 at pH 5.7, and their densities were adjusted to $1x10^6\ ml^{-1}$.

Electrofusion

Electrofusion was carried out using a model SSH-1 electrofusion apparatus connected to a fusion chamber FTC-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 the fusion chamber. The protoplasts were aligned into short chains by applying an alternating current of 120 Vcm^{-1}, at 1MHz for 15 sec. Fusion was then induced by a direct current square pulse of 1.5 $kVcm^{-1}$ 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 gl^{-1} 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 mgl^{-1} GA_3, 30 gl^{-1} sucrose and 2 gl^{-1} 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. The leaf samples were treated for 5 min in 1ml 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 $\mu g\ ml^{-1}$ 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.

Chromosome number

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

Random amplified polymorphic DNA (RAPD) analysis

Total DNA was 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). 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 s, 37 ℃ for 2 min and 72 ℃ for 3 min in a ASTEC Program Control System 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 10 Vcm^{-1} for 15 min. The gels were stained with 10 mgl^{-1} 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 chloroplast and nuclear DNA

PCR-RFLP analysis of chloroplast (cpDNA) and nuclear DNA was performed by the methods described by Haruki *et al.* (1998) and Buiteveld *et al.* (1998), respectively. The putative somatic hybrid R1 and the both parents were used for these analyses. The primers for ribulose-1, 5-bis-phosphate carboxylase large subunit (*rbcL*) gene and ORF106 designed by Ogihara *et al.* (1991) were used for cpDNA analysis. The internal transcribed spacer (ITS) region of rDNA in the nuclear genome was amplified by PCR. 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, 0.15 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 s, 60 ℃ for 30 s 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, *Taq*I and Hap II (TOYOBO, Co. Ltd., Japan) were tested to reveal polymorphism within the putative somatic hybrid R1 and the both parents. Reaction were carried out at 37 ℃ for one hour in 10 μl solution containing 10 units of *Alu*I, *Dde*I, *Hinf*I, *Msp*I, *Nco*I, *Sau*3AI, *Scr*FI and Hap II . 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.

Results and Discussion

Application of an alternating current field at 1 MHz and 120 Vcm^{-1}, led to the formation of pearl chains consisting of 5-10 protoplasts within 15 s. 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 $kVcm^{-1}$ for 30 μs. Approximately 50% of the protoplasts were viable and this was confirmed by staining with FDA. After electrofusion, the first cell division occurred after 7 days of culture, and a lot of colonies (**Fig. 1A**) and several globular embryos (**Fig. 1B**) were observed after 30 days of culture. Some of them formed 1-5 green somatic embryos per plastic dish after 60 days of culture (**Fig. 1C**). When these somatic embryos were transferred onto 1/2 MT medium containing 1 mgl^{-1} GA_3, and 30 gl^{-1} sucrose, roots and leaflets were produced 2 months after transfer (**Fig. 1D**). 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 (**Fig. 2**). Chromosome count of root tip squashes revealed that the chromosome number of R1 and R2 was 27 (**Fig. 3A**), which was less than the sum of chromosome numbers of 'Shogun'

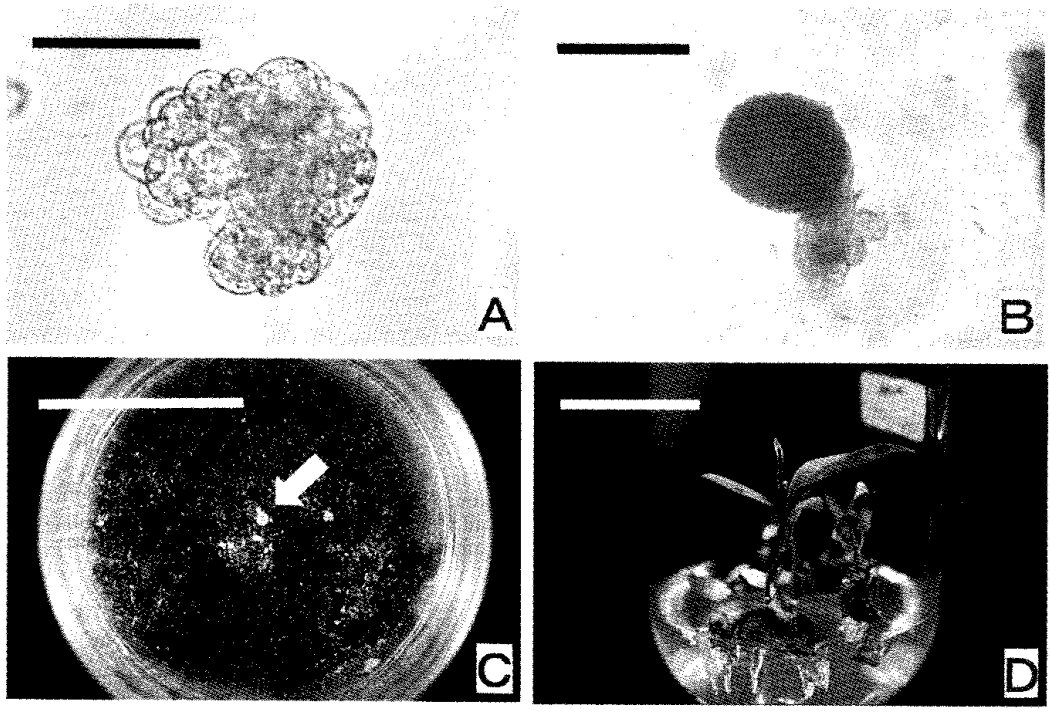

Fig. 1 Somatic hybrid plantlets derived after electrofusion between embryogenic callus protoplasts of 'Shogun'mandarin (*Citrus reticulata* Blanco) and leaf protoplasts of grapefruits (*C. paradisi* Macf.).
(A) Small colony formation after 30 days of culture. Bar = 100 μm.
(B) Globular embryo formation after 30 days of culture. Bar = 200 μm.
(C) Green embryo formation after 60 days of culture. Bar = 3 cm.
(D) Plantlet formation 2 months after transfer onto the germination medium. Bar = 5 cm.

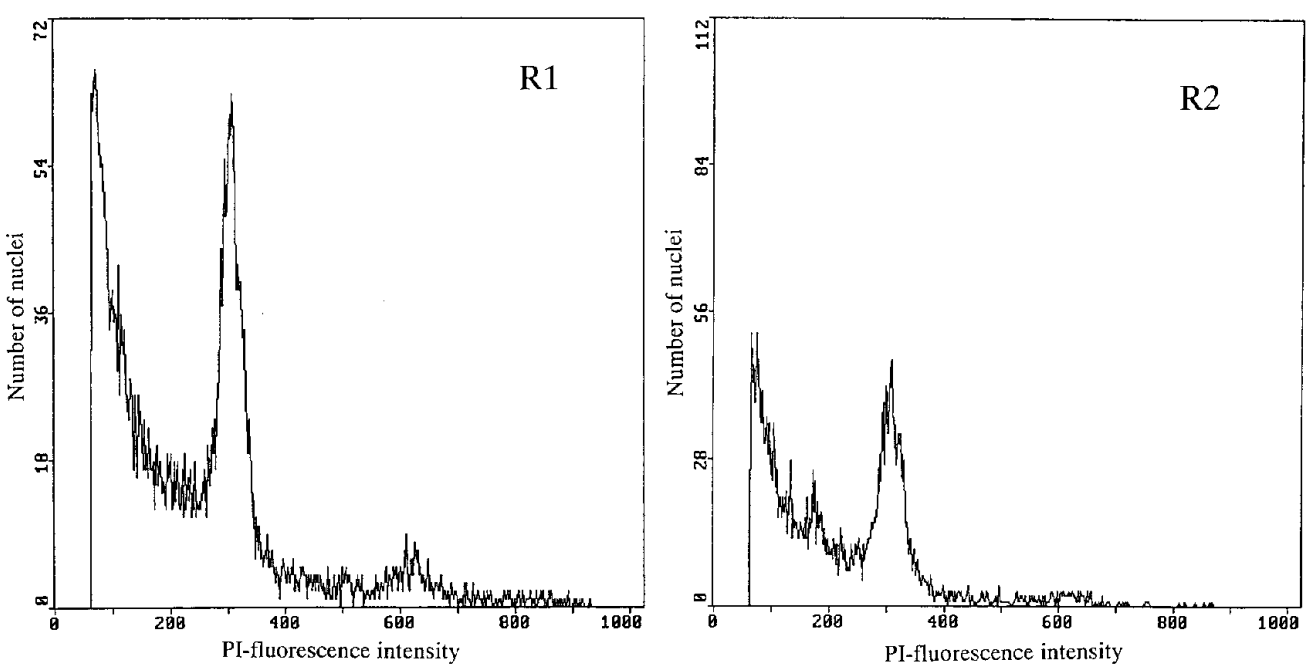

Fig. 2 Flow cytometric analysis of the regenerated plant No. 1. (R1) and No. 2 (R2).

mandarin (2n = 18) and grapefruit (2n = 18).

Flow cytometry is a reliable method for the rapid and easy determination of ploidy levels (Arumuganathan and Earle, 1991). In *Citrus* species, flow cytometry has been used for detecting triploids and for selecting somatic hybrids at early stages of culture (Ollitrault *et al.*, 1996; Tusa *et al.*, 1996; Harusaki *et al.*, 2000). In the present study, two

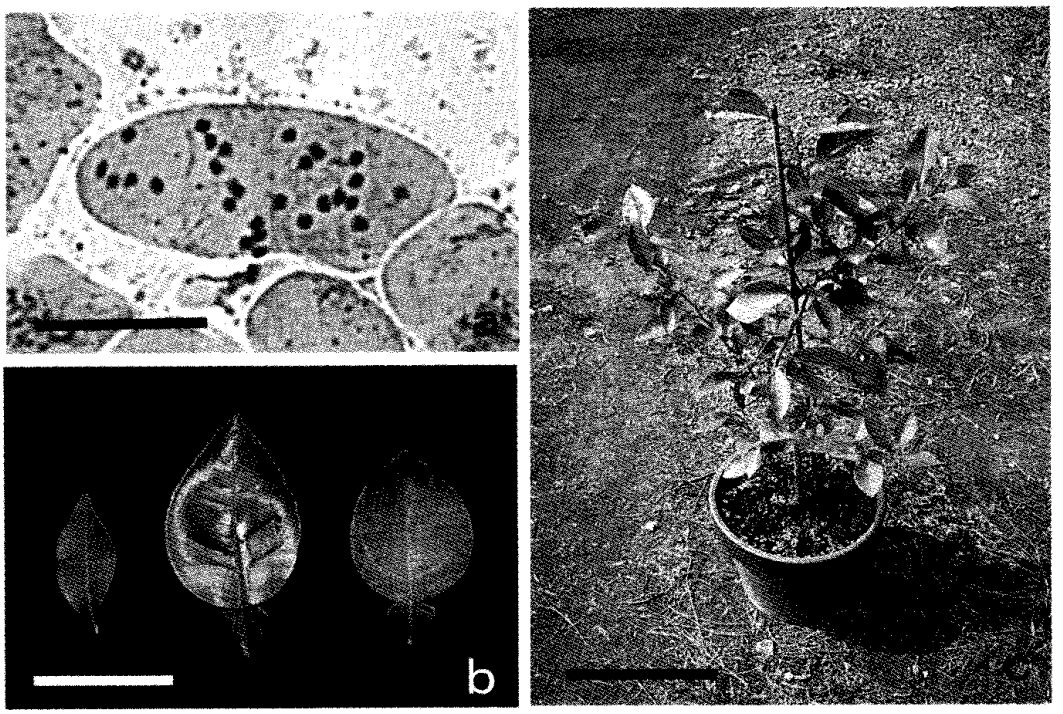

Fig. 3 Root-tip chromosome number and leaf morphology of somatic hybrid (R1) between 'Shogun' mandarin (*Citrus reticulata* Blanco) and grapefruits (*C. paradisi* Macf.).
(A) Chromosomes in a root tip cell (2n = 2x = 27). Bar = 50 μm.
(B) Leaf morphology of 'Shogun' mandarin (left), somatic hybrid (center) and grapefruit (right) Bar = 5 cm.
(C) A triploid somatic hybrid plant. Bar = 30 cm.

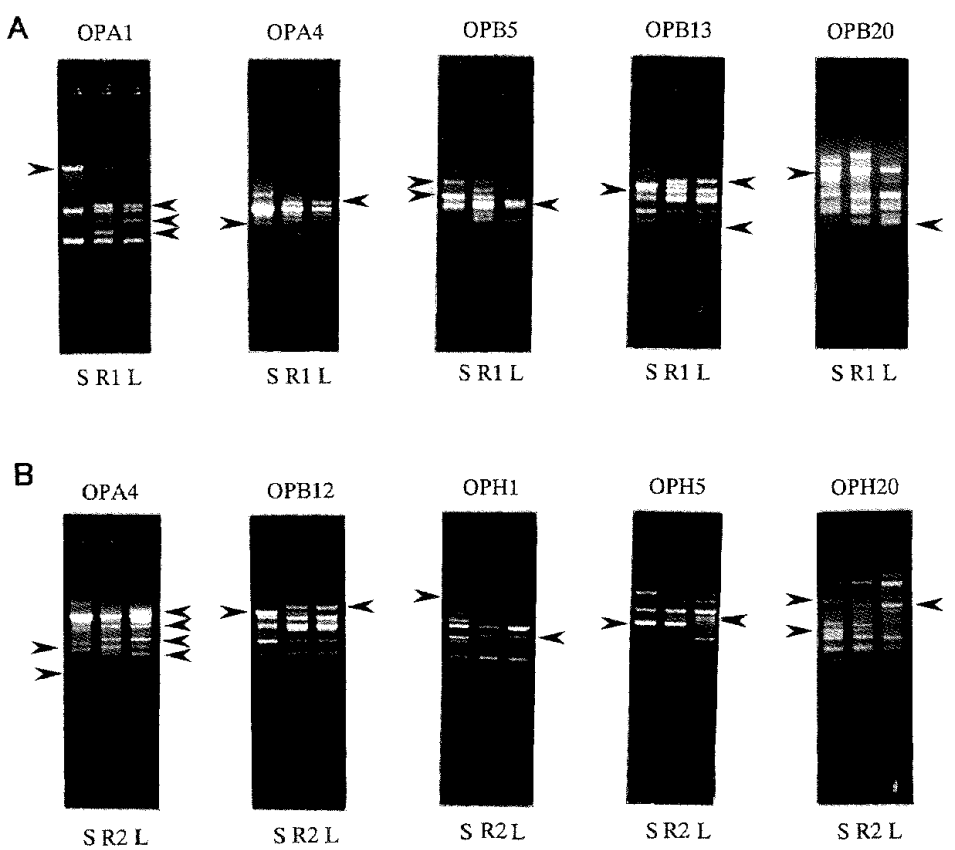

Fig. 4 Random amplified polymorphic DNA (RAPD) analysis of regenerated plants and their parents.
(A) S: 'Shogun' mandarin, R1: Regenerated plant No. 1, L: Grapefruit
(B) S: 'Shogun' mandarin, R1: Regenerated plant No. 1, L: Grapefruit

triploids, R1 and R2, were easily selected from a lot of young regenerated plants. Repeated analyses showed that they were not chimeras.

Two triploid plants, R1 and R2 showed vigorous growth and thick leaves, which were typical characters of polyploids. They also had intermediate morphology when compared to fusion parents (**Fig. 3B**). The leaf type and the size of wing 1eaf 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. Vigorous growth was observed when the somatic hybrids were grafted on to three year-trifoliate orange (**Fig. 3C**).

The somatic hybrids were further confirmed using RAPD analysis in R1, R2 and both their parents. Among the primers tested, OPA1, OPA4, OPB5, OPB13, OPB20 for R1 and OPA4, OPB12, OPH1, OPH5, OPH20 for R2 were suitable for discrimination between 'Shogun' mandarin and grapefruit. R1 and R2 plants had the fragments specific for both parents (**Fig. 4**).

RAPD analysis has been successfully employed for the identification of somatic hybrids between relatively close fusion parents (Xu *et al.*, 1993; Grosser *et al.*, 1996). As reliable molecular markers, species-specific fragments of rDNA have been used for *Citrus* somatic hybridization (Ohgawara *et al.*, 1985, 1989). However, Kobayashi *et al.* (1997) also tried to identify interspecific somatic hybrids using Southern blot hybridization with rRNA probes, but they did not find any suitable restriction enzymes for production of parent-specific fragments on the analysis of genomic DNA. In the present study, we found several efficient RAPD primers for producing parent-specific bands to identify the hybridity of R1 and R2 plants.

The R1 plant was subjected to further analysis of rRNA ITS region in nuclear genome. It was revealed by DNA analysis that R1 plant retained DNA fragments from both parents, thus confirming the somatic hybrid nature (**Fig. 5**). However, PCR-RFLP analysis of the rbcL-ORF106 region did not reveal polymorphism in both parents, when the fragments were digested with restriction enzymes. (data not shown). Therefore, we were unable to detect the origin of cpDNA. Further analyses using other primers and restriction enzymes are necessary to identify cpDNA of somatic hybrids.

In the present study, protoplast fusion using embryogenic callus cells of 'Shogun' mandarin (2n = 2x = 18) and mesophyll cells of grapefruit (2n = 2x = 18) allowed induction of triploid somatic hybrids. To date, somatic hybrids have been produced from more than 100 parental combinations in Citrinae, and chromosome numbers of those hybrids were normally the sum of those of both parents. However, there are several exceptions in *Citrus* fusion events. Grosser *et al.* (1992) reported that only triploid somatic hybrids were produced from protoplast fusion between *C. sinensis* cv. 'Hamlin' (2n = 2x = 18) and *Severinia buxifolia* (2n = 2x = 18). Miranda *et al.* (1997) also showed one pentaploid somatic hybrid (2n = 5x = 45) obtained by fusion between *Poncirus trifoliata* (2n = 2x = 18) and *Fortunella hindisii* (2n = 4x = 36). Furthermore, Guo and Deng (1999) described that hexaploid somatic hybrids regenerated from electrofusion between diploid of *Citrus sinensis* and its sexually incompatible relative, *Clausena lansium*. They reported that these phenomena are due to elimination or doubling of chromosome prior to regeneration. On the other hand, occurrence of haploid cells in diploid callus cultures has been reported in several plants such as *Vicia hajastana* and carrot (Singh *et*

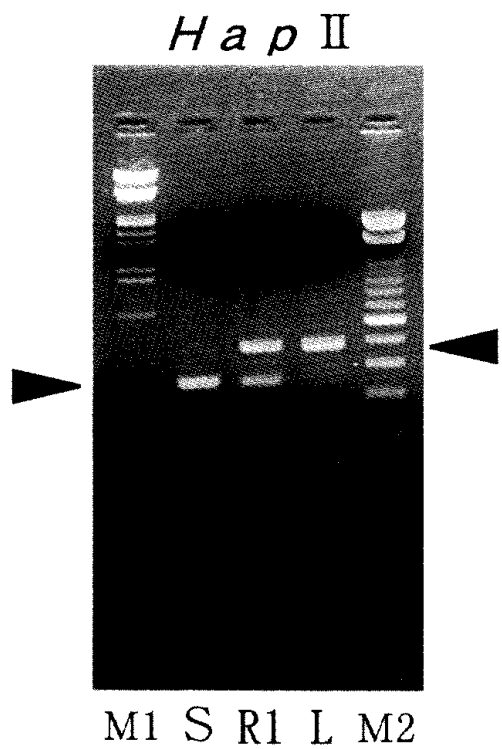

Fig. 5 Restriction pattern of the *Hap* II-digested rRNA ITS region of nuclear genomes in the regenerated plant (R1) and their parents.
M1: DNA size markers (λ /*Eco*R I +*Hin*d III), S: 'Shogun' mandarin, R1: Regenerated plant No. 1, L: Grapefruit, M2: DNA size marker (100 bp radder).
Arrows indicate the bands specific to each parents.

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 stage of embryos. D' Amato (1995) described that nuclear fragmentation (amitosis) followed by mitosis is a very important process leading to chromosome number reduction and haploidization *in vitro*. In the present study, the triploid somatic hybrids might have derived from fused cells between haploid cell in callus cultures and diploid mesophyll cell, although the mechanism involved in the occurrence of haploid cells from diploid callus cultures of citrus cultivars is still unclear.

In conclusion, we obtained vigorous triploid somatic hybrids between 'Shogun' mandarin and grapefruit. These triploids have a possibility to be immediately utilized as citrus cultivars, because of their favorable characteristics such as seedlessness and thin rind. Actually, triploid citrus cultivars, 'Oroblanco' and 'Melogold' are grapefruit-like and seedless and have the rinds equal to that of 'Marsh' grapefruit (Soost and Cameron, 1980, 1985). These somatic hybrids have been propagated for evaluating disease resistance, soil adaptability and fruit characteristics.

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