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Cybrid Plants Produced by Electrofusion Between Satsuma Mandarin (*Citrus unshiu*) and Yuzu (*C. junos*) or Lemon (*C. limon*), and Recombination of Mitochondrial Genomes

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Abstract

Electrofusion was conducted to use the embryogenic callus cells of satsuma mandarin (*Citrus unshiu* cv. 'Juman' unshiu) and mesophyll cells of yuzu (*C. junos*) and lemon (*C.limon* cv. 'Eureka' lemon). Two plants were regenerated from the combination of satsuma mandarin and yuzu, and one plant from that of satsuma mandarin and lemon. The plants had 18 chromosomes ($2n = 2 \times = 18$ in each parent), and showed the same nuclear rDNA fragment pattern as that of the mesophyll parent. The mitochondrial DNA analysis showed the presence of specific yuzu or lemon bands in addition to specific satsuma mandarin bands. From these results with morphological evidence, the regenerated plants were considered as cybrids with recombined mitochondrial DNA.

Introduction

Seedlessness caused by male and/or female sterility and parthenocarpy (Yamamoto *et al.*, 1995) is one of the most important characteristics in *Citrus*. In many higher plants, it is known that male sterility is caused by nuclear-cytoplasmic interaction (Kaul, 1988), and that mitochondrial genomes encode genes related to male sterility (Leaver and Gray, 1982; Whitfeld and Bottomly, 1983). In citrus , satsuma mandarin (*Citrus unshiu*) and 'Encore'(*C. nobilis* × *C. deliciosa*) probably have male sterile cytoplasm (Iwamasa, 1966; Yamamoto *et al.*, 1992). Therefore, citrus cybrids having sterile cytoplasm are considered to be useful tools for studying the mechanism of sterility.

Vardi *et al.* (1987) first succeeded in producing *Citrus* cybrids having *Citrus* nuclear genome and *Poncirus* or *Microcitrus* cytoplasmic genome by a donor-recipient protoplast fusion system. Saito *et al.* (1993) produced cybrids using nucellar callus cells of *C. sudachi* and mesophyll cells of *C. aurantifolia* or *C. limon* by standard symmetric fusion procedure. Moriguchi *et al.* (1996) reported a high frequency induction of cybrids between 'Seminole' tangelo (*C. reticulata* Blanco × *C. paradisi* Macf.) and 'Lisbon' lemon (*C. limon*), and 'Hazzara (Anbohar)' and rough lemon (*C. jambhili*). However, only one report has been made of cybrid which probably has sterile cytoplasm (Yamamoto and Kobayashi, 1995; cybrid having *C. sinensis* nuclear genome and *C.unshiu* cytoplasmic genome).

We report here the production of cybrids having cytoplasmic genome of *C. unshiu* and nuclear genome of *C. junos* or *C. limon*, and mitochondrial recombination in these cybrids.

Materials and Methods

1. Plant materials

An embryogenic callus derived from nucellar embryo of satsuma mandarin (*C. unshiu*) was used as the source of protoplasts.This callus line has been maintained in a solidified Murashige and Skoog (MS)(1962) medium containing 50 $g \cdot l^{-1}$ sucrose and 10 $mg \cdot l^{-1}$ kinetin. Callus was suspended in a liquid MS medium supplemented with 10 $mg \cdot l^{-1}$ kinetin. Serial transfer of the callus was done every two weeks. Seeds of yuzu (*C. junos* Sieb.ex Tanaka) and lemon (*C.limon*) were germinated in vermiculite and grown in a greenhouse at 25~35 ℃ without supplemental lighting. Three fully expanded leaves were harvested from the plants for protoplast isolation

2. Protoplast fusion and plant regeneration

Protoplasts were isolated from both suspension-cultured cells and leaves by the method of Hidaka and Omura (1992), and suspended in 0.35 M mannitol, 0.35 M sorbitol and 0.25 mM calcium chloride. Their densities were adjusted to $5 \times 10^5 \cdot ml^{-1}$ and $1.5 \times 10^6 \cdot ml^{-1}$, respectively.

Electrofusion was carried out using a model SSH-2 electrofusion apparatus and FTC-34D5 electrode (Shimadzu Co., Ltd., Kyoto, Japan). Callus and mesophyll protoplasts were mixed in equal volumes, and 4 ml of the mixture was transferred to a 60 mm diameter plastic petri dish where fusion was induced. The electrical parameters used in this study were as follows: AC field, 1 MHz, 125 $V \cdot cm^{-1}$, 60 sec.; DC field, 1,250 $V \cdot cm^{-1}$ square-wave 50 micro sec. in duration, 5 times at 0.5 sec intervals.

The treated protoplasts were transferred to 15 m*l* tubes and pelleted by centrifugation at 100 × g for 10 min. The supernatant was discarded and the fusion products were resuspended at a density of 2×10^5 cells$\cdot ml^{-1}$ in a 0.25% gellan gum solution containing 0.15 M sucrose, 0.45 M glucose and 0.05% glutamine. Five m*l* of the suspension was transferred to 60 mm plastic petri dishes, then 5 m*l* of double strength MS liquid medium supplemented with 0.15 M sucrose, 0.45 M glucose and 0.05% glutamine was added for solidification.

Green embryoids derived from the protoplasts were moved to hormone-free MS medium containing 500 $mg \cdot l^{-1}$ malt extract, 40 $mg \cdot l^{-1}$ adenine, 5% maltose and 0.5% gellan gum. Cotyledonary embryoids which developed were transferred to a MS medium containing 0.05 $mg \cdot l^{-1}$ α-naphthaleneacetic acid, 5% maltose, and 0.5% gellan gum for shooting and rooting.

3. Determination of chromosome number

Root tips from regenerated plants pretreated with 8-hydroxyquinoline (2 mM) for 20 hr at 10 ℃ were fixed in a mixed solution of ethanol and acetic acid (3:1) for 24 hr, and then stained with lacto-propionic orcein for 3hr according to Oiyama (1981).

4. DNA analysis

Total DNA was extracted from leaves according to Rogers and Bendich (1985). One microgram of DNA, digested with restriction endonucleases, was separated on 1 % agarose gel, then transferred to nitrocellulose filter or nylon membrane according to Southern (1975).

DNA fragments prepared from the following recombinant plasmids were used as probes: plasmid pRR 217 containing whole nuclear rDNA sequences of rice (Takaiwa *et al.*, 1984), plasmid pTBa 1 containing *Nicotiana tabacum* cpDNA fragment (Sugiura *et al.*, 1986), and mtDNA clones containing *Pst* I or *Sac* I fragments of *Brassica campestris* mtDNA (Palmer and Shields, 1984). Plasmid pRR 217, pTBa 1 and mtDNA clones were provided by Drs. K. Oono, M. Sugiura and J. D. Palmer, respectively.

Probe labellings and detections for rDNA and cpDNA analysis were carried out using the ECL method (Amersham), and those for mtDNA analysis were done using the DIG-AMPPD system (Boehringer Mannheim).

Results and Discussion

About 5 month after electrofusion, about 60 green globular embryoids appeared, and developed into 2 whole plants (coded as JY1 and JY2) from the combination of satsuma mandarin and yuzu. From the combination of satsuma mandarin and lemon, fifteen green globular embryoids appeared after 4 months and developed into one plant (coded as JL1). Leaf morphology of all the regenerated plants was similar to that of the respective mesophyll parent (**Fig. 1a** and **1b**), and chromosome counts showed that the plants had diploid chromosome number of 18 (2n=2 × =18 in each parent).

Southern blot analysis with a rice rDNA probe showed that all 3 plants had the same rDNA banding patterns observed in yuzu or lemon (respective mesophyll parent) (**Fig. 2**), whereas cpDNAs of the

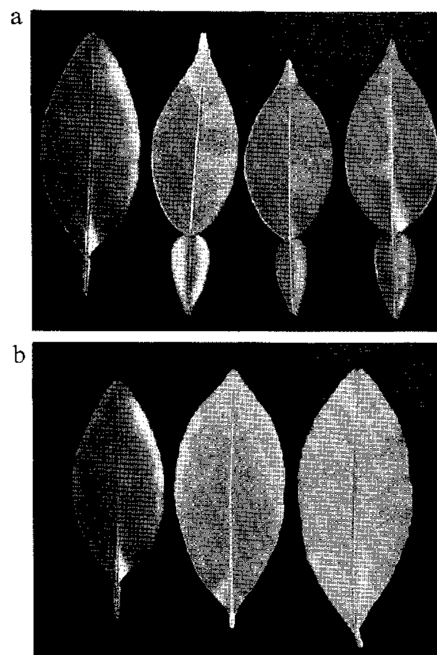

Fig. 1 Leaf morphology of the regenerated plants drived from protoplast fusion between satsuma mandarin and (a) yuzu (JY1, 2) or (b) lemon (JL1).

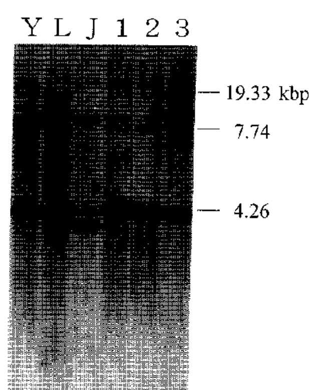

Fig. 2 Southern blot hybridization of total DNA to a rice rDNA probe after digestion with *Dra* I .
Y: yuzu, L: lemon, J: satsuma mandarin, 1,2: JY1 and JY2, 3: JL1.

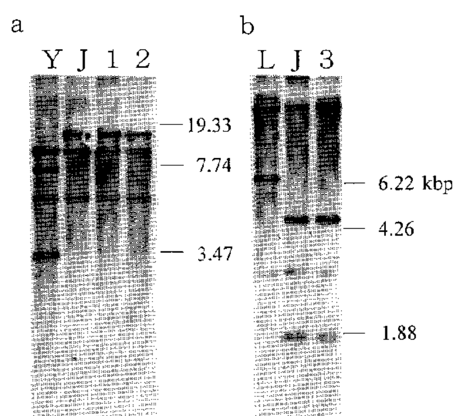

Fig. 3a, b Southern blot hybridization of total DNA to a cpDNA fragments after digestion with (a) *Sac* I or (b) *Pst* I digestion.
Y: yuzu, L: lemon, J: satsuma mandarin, 1,2: JY1 and JY2, 3: JL1.

regenerated plants were identical to that of satsuma mandarin (callus parent) (**Fig. 3a, b**). These results indicate that the regenerated plants were cybrids having yuzu or lemon nuclear genome and satsuma mandarin cytoplasmic genomes.

Moriguchi *et al.* (1996) reported that a specific cell line, parental combination, or both can increase the efficiency of inducing cybrids in *Citrus*. In this experiment, only cybrids were produced from the combination of satsuma mandarin (cv. juman unshiu) and yuzu or lemon. However, Hidaka and Omura (1992) obtained only somatic hybrids following fusion of satsuma mandarin (cv. saruwatari) and yuzu or rough lemon. These results suggest that the selection of cell line is important for inducing an interspecific cybrid.

For mtDNA analysis we used 4 mtDNA probes: P 4.8, P 9.7, S 8.3 and S 11.8 scattered throughout the mitochondrial genome of *B.campestris*. The analysis with P 4.8 or P 9.7 probe after digestion with

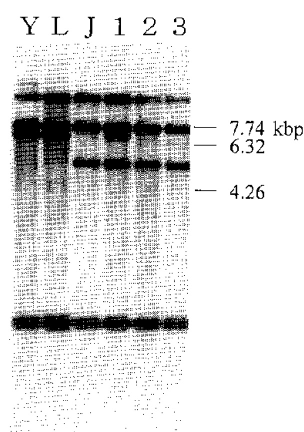

Fig. 4 Southen blot hybridization of total DNA to mtDNA S 8.3 probe after digestion with *Hind* Ⅲ.
Y: yuzu, L: lemon, J: satsuma mandarin, 1,2: JY1 and JY2, 3: JL1.

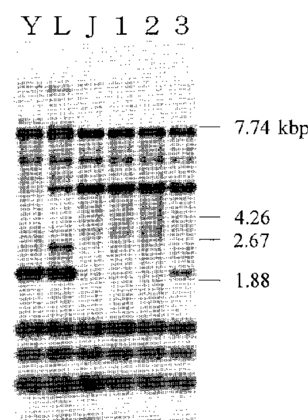

Fig. 5 Southen blot hybridization of total DNA to mtDNA S 11.8 probe after digestion with *Hind* Ⅲ.
Y: yuzu, L: lemon, J: satsuma mandarin, 1,2: JY1 and JY2, 3: JL1.

Hind Ⅲ showed that all the regenerated plants had the same mtDNA banding pattern as those of satsuma mandarin (data not shown). However, when S 8.3 was used as a probe, all regenerated plants had Hind Ⅲ fragments specific to satsuma mandarin, while JY1 had one more fragment specific to yuzu (**Fig. 4**). When S11.8 was used as a probe, the fragments of JY1 and JY2 were identified as those of satsuma mandarin, but JL1 showed the presence of part of a lemon specific fragment in addition to satsuma mandarin specific fragments (**Fig. 5**). The occurrence of recombination of mitochondrial genomes is thus apparent in JY2 and JL1.

Saito *et al.* (1993) suggested that the mitochondria of the callus parent might play an important role in *Citrus* embryogenesis; they observed that the regenerated somatic hybrids and cybrids had only mitochondrial DNA of the callus parent. Indeed, many reports on protoplast fusion in *Citrus*

have indicated that mtDNA from the callus parent was preferentially transmitted to somatic hybrids (Kobayashi *et al.* 1991; Motomura *et al.* 1995) and cybrids (Saito *et al.* 1994; Yamamoto and Kobayashi 1995; Grosser *et al.* 1996; Moriguchi *et al.* 1996).

In contrast, mtDNA recombination/ rearangement was observed in intergeneric somatic hybrids (Motomura *et al.* 1995) and interspecific cybrids (Moriguchi *et al.* 1997). In this study, we also showed the recombination of mitochondrial genomes. There were no plants which had the mitochondrial genome only from the mesophyll parent and had lost that genome from the callus parent. Thus, it is considered that the whole intact mitochondrial genome from the callus parent is not essential, but that it has an important function in cell division and plant regeneration.

In many higher plants such as rice (Kadowaki *et al.* 1986) and maize (Kemble *et al.* 1980), a strong relationship between cytoplasmic-male-sterility (cms) and mitochondrial genome has been reported. In citrus also, cms most likely relates to mitochondrial genome.

In this study, we produced citrus cybrids having intact or recombined mitochondrial genome derived from *C.unshiu* which is though to have sterile cytoplasm. These cybrids may thus be useful as a material for studying the function of cytoplasm.

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