Production of Somatic Hybrid Plants through Protoplast Fusion in Citrus

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Citrus is one of the most important fruit-bearing trees in the world. Polyembryony and sterility often cause serious problems in citrus breeding. Few or no zygotic seedlings are produced when polyembryonic cultivars are used as maternal parents, because nucellar embryos restrict and often abolish zygotic embryo development prior to seed maturation[1].

Protoplast fusion provides an alternative way of producing hybrids for species which can not be crossbred. Many inter- and intra-generic plants have been created by this technique[2]. The application of this technique to citrus would be of great value for the improvement of this kind of fruit tree.

Here, we report the production of some somatic hybrid plants in the *Rutaceae* family, although some of the results have already been reported elsewhere[8,11].

Materials and methods

1) *Plant materials*

Nucellar calli were induced from Trovita orange (*Citrus sinensis* Osb.) and from F. N. Washington navel orange (*C. sinensis* Osb. var. *brasiliensis* Tanaka) as described previously[5]. About 1 g of the calli were suspended in a 40 m*l* liquid Murashige and Tucker (MT)[9] medium supplemented with 5% sucrose and 10 mg/*l* 6-benzylaminopurine (BA) without auxin in a 125 m*l* Erlenmeyer flask. The cultures were maintained on a rotary shaker at 110 rpm, and kept at 25°C under 16 hr/day illumination with cool fluorescent light (2,000 lux). Serial transfer of callus was done every 2 weeks. Seeds of *Poncirus trifoliata*, Hayashi satsuma mandarin (*C. unshiu* Marc.), Troyer citrange and Murcott tangor were germinated in a pot containing Vermiculite. Plants (nucellar seedlings) were grown under the same environmental conditions as described in the callus culture. About 10 fully expanded leaves were harvested from 2-month-old plants.

2) *Protoplast isolation*

Prior to isolation of the protoplasts from suspention-cultured cells, 2-week-old cells were transferred to a hormone-free MT liquid medium (denoted to MT basal medium). After subculture in the same medium for 2 weeks, the cells were collected and subjected to protoplast isolation using the procedure described previously[6].

In the case of the nucellar seedlings, leaves were rinsed with 70% ethanol, immersed in a solution containing 0.5% sodium hypochlorite plus 0.1% Tween 20 for 20 min and washed twice with sterile distilled water. The leaves were then cut into about 2 mm wide strips with a razor blade and floated on a pretreatment solution (pH 5.8) containing 1 mM MES, 0.6 M mannitol and 1/2 strength of MT macro elements for 1 hr. About 0.6 g leaves were incubated in a Petri dish with 8 m*l* enzyme solution (pH 5.8), which consisted of pretreatment solution supplemented with 3% Cellulase Onozuka R-10 and 0.3% Macerozyme R-10. The incubation was carried out at 25°C on a rotary shaker (45 rpm) for 16 hr. The cell and enzyme mixture was filtered through a nylon mesh (58 μm pore openings), washed twice with 0.6 M mannitol by centrifugation

at 110 × g for 2 min. Then, protoplasts were resuspended in the same solution.

3) Protoplast fusion and culture

Protoplast fusion was carried out at 5 combinations, that is Trovita orange plus *P. trifoliata*, Trovita orange plus Troyer citrange, Trovita orange plus Hayashi satsuma mandarin, F. N. Washington naval orange plus Hayashi satsuma mandarin, and F. N. Washington naval orange plus Murcott tangor. Protoplasts of two cultivars were adjusted to a density of 10^6 cells/ml, mixed together at an equal volume, and fused with the aid of polyethylene glycol (PEG) by the method of Uchimiya[15]. PEG was diluted with 0.6 M mannitol-50 mM $CaCl_2$ and removed by centrifugation at 150 × g for 5 min. Protoplasts were washed twice with 0.6 M mannitol, and once with MT basal medium containing 0.6 M sucrose by centrifugation at 100 × g for 2 min. These protoplasts (10^5 cells/ml) were cultured in 3 ml medium, which consisted of MT basal medium containing 0.6 M sucrose and 0.6% agarose (Sea Plaque, LMT, Marine Colloids) in a Falcon Petri dish (60 × 15 mm). The plates were sealed with Parafilm and maintained under 16 hr/day illumination with cool fluorescent light (500 lux) at 25°C. After 25th day, 0.5 ml MT basal medium was added to the protoplast culture, and plates were transferred to under 3,000 lux light intensity.

4) Plant regeneration

Green embryoids (0.5–1 mm diameter) derived from protoplasts were transferred to MT basal medium containing 500 mg/l malt extract, 40 mg/l adenine and 0.8% agar. They developed into cotyledonary embryoids after about 1 month. Cotyledonary embryoids developed into whole plants within 6 months of culture when transferred to a MT agar medium containing 1 mg/l gibberellic acid.

5) Observation of chromosome number

Ten root tips of regenerated plants pretreated with 8-hydroxy-quinoline (2 mM) for 20 hr at 10°C were fixed in a mixed solution of ethanol: acetic acid (3:1) for 24 hr, and then stained with lacto-propionil orcein for 3 hr according to Oiyama[12].

6) Analysis of leaf oil and peroxidase isozyme

Leaf oils were extracted from leaves of parents and those of regenerated plants, and then analysed by a gas chromatography according to the method described previously[7]. Analysis of peroxidase isozyme was carried out by isoelectric focusing as described previously[7].

7) Analysis of ribosomal RNA genes (rDNA)

DNAs were extracted from leaves of parents and those of regenerated plants according to the method of Rogers et al.[13]. DNAs were subjected to restriction endonuclease digestion, and followed by agarose electrophoresis and blot-hybridization with biotin-labeled rDNA fragments as probe. rDNA fragments were prepared from recombinant plasmid pRR217 (kindly provided by Dr. K. Oono) which contained the whole rRNA gene sequences of rice[14], and then labeled with biotin using biotin-11-dUTP and nick-translation reagent kit (Bethesda Res. Lab., USA).

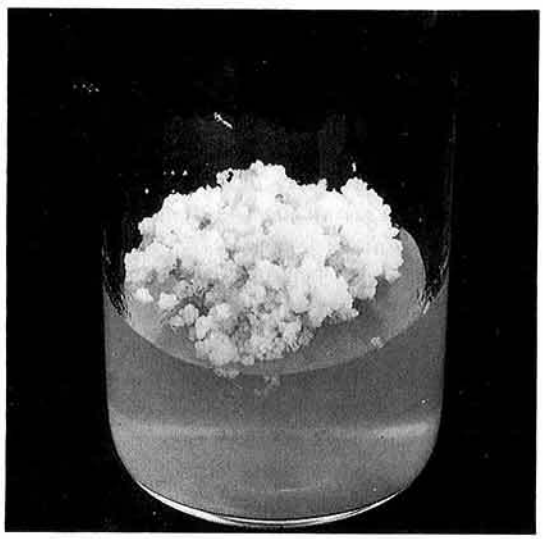

Plate 1. Nucellar callus induced from the ovule of Trovita orange

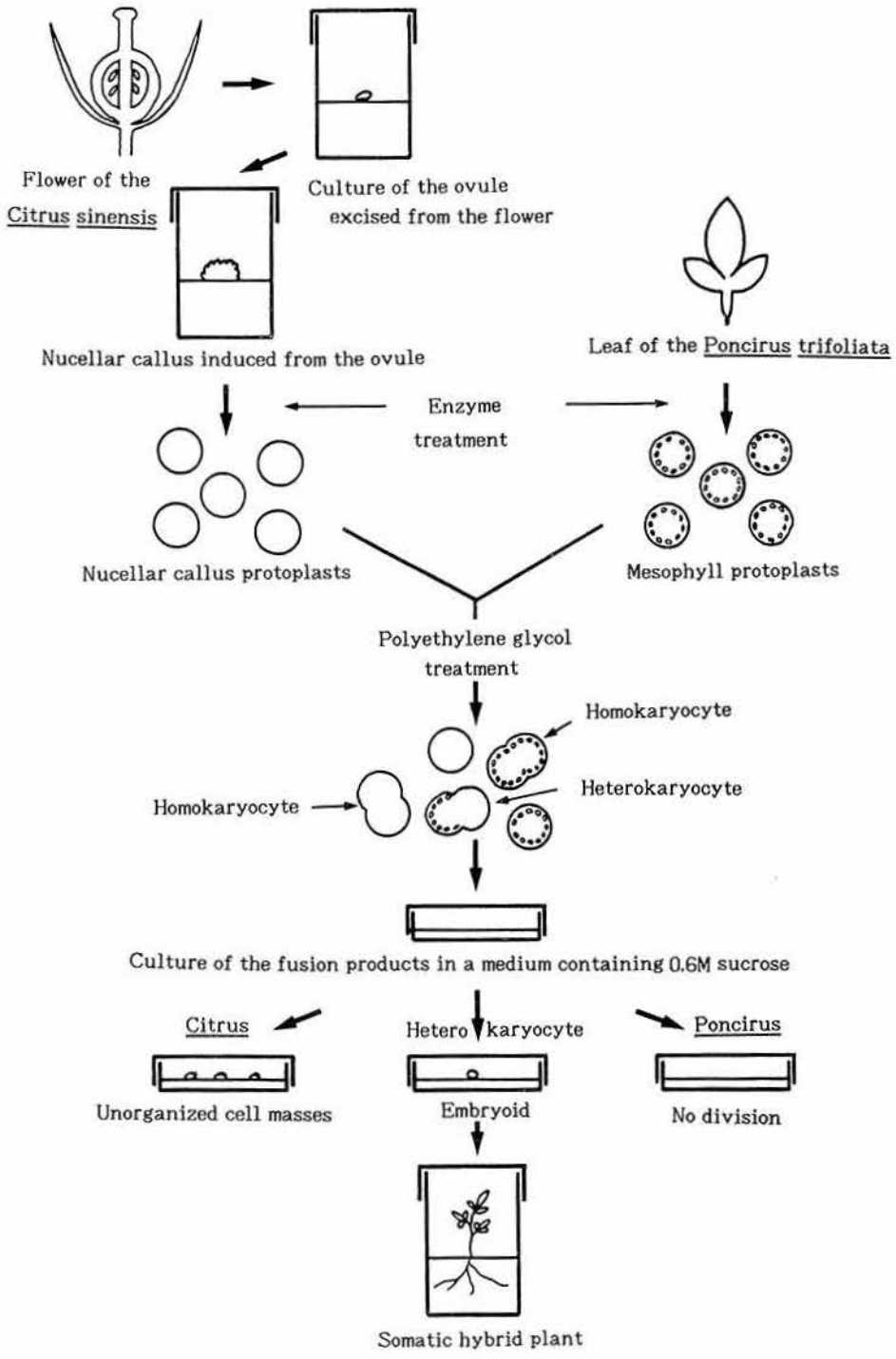

Fig. 1. An outline of somatic hybridization between Trovita orange and *Poncirus trifoliata*

Visualization of the probe-target DNA hybrid was carried out using streptavidin-alkaline phosphatase conjugate, NBT (nitro blue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt).

Results and discussion

The nucellar callus was induced from ovules of unpollinated flowers[5] (Plate 1). It has been known that the nucellar callus induced from polyembryonic cultivars has a high potential to regenerate embryos, eventually the whole plants[10]. Taking this advantage, Vardi et al.[17] and we[4] have been successful to regenerate the whole plants from protoplasts of nucellar callus of some citrus cultivars. Therefore, we used the nucellar callus as a partner of somatic hybridization.

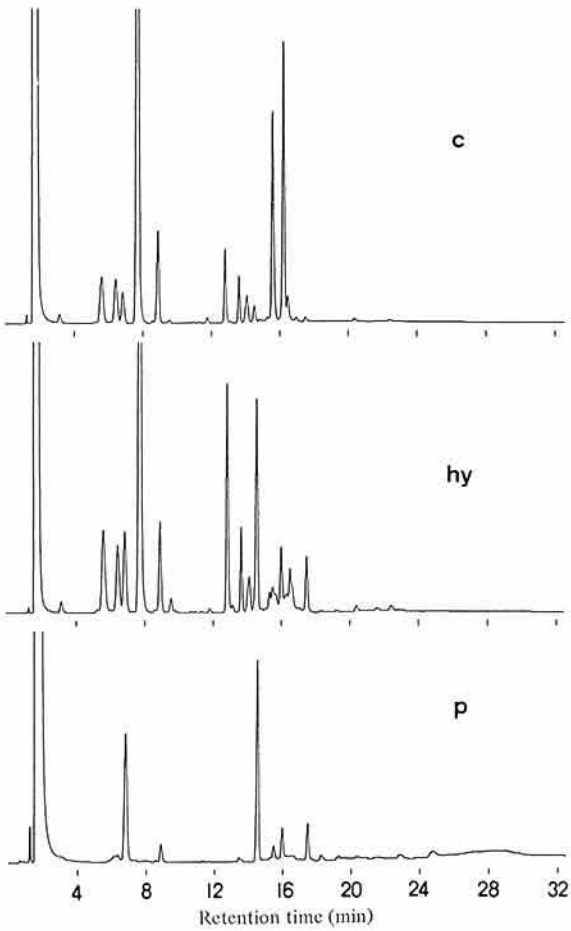

Fig. 2. Gas chromatograms of leaf oil of Trovita orange (c), somatic hybrid (hy) and *P. trifoliata* (p)

An outline of somatic hybridization is drawn in Fig. 1. Protoplasts of the nucellar callus (Plate 2A) had an ability to divide, proliferate and develop to green embryoids in a MT basal medium containing 0.25 M mannitol[6]. However, in the cultural condition of this study, most of the protoplasts produced unorganized cell masses, and only a few occasionally developed into embryoids. Under the same conditions, mesophyll protoplasts of nucellar seedlings (Plate 2B) never divided. After the fusion treatment, heterokaryons (Plate 2D) 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. About 50 days after culturing, many white unorganized cell masses and green embryoids were formed in the Petri dishes (Plate 2E). These embryoids developed into the whole plants (Plate 2F). In the following combinations, Trovita orange plus *P. trifoliata*, Trovita orange plus Troyer citrange, Trovita orange plus Hayashi satsuma mandarin, and F. N. Washington navel orange plus Murcott tangor, only hybrid cells developed into embryoids in the presence of high concentrations of sucrose. But in the combination of F. N. Washington navel orange plus Hayashi satsuma mandarin, embryoids were formed not only from hybrid cells, but also from navel orange protoplasts.

A plant obtained by the protoplast fusion between Trovita' orange and *P. trifoliata* had characteristics of both parents. Leaves were trifoliate like *P. trifoliata*, and their size, thickness and smoothness resembled those of Trovita orange (Plate 2G). A chromosome number of 36 was counted in the root tip of the plants (Plate 2H). Both parents have a chromosome number 2n=18. In view of the intermediate leaf morphology and chromosome number, this plant must be somatic hybrid (amphidiploid).

The isozyme analysis and the restriction endonuclease analysis of rDNA have been

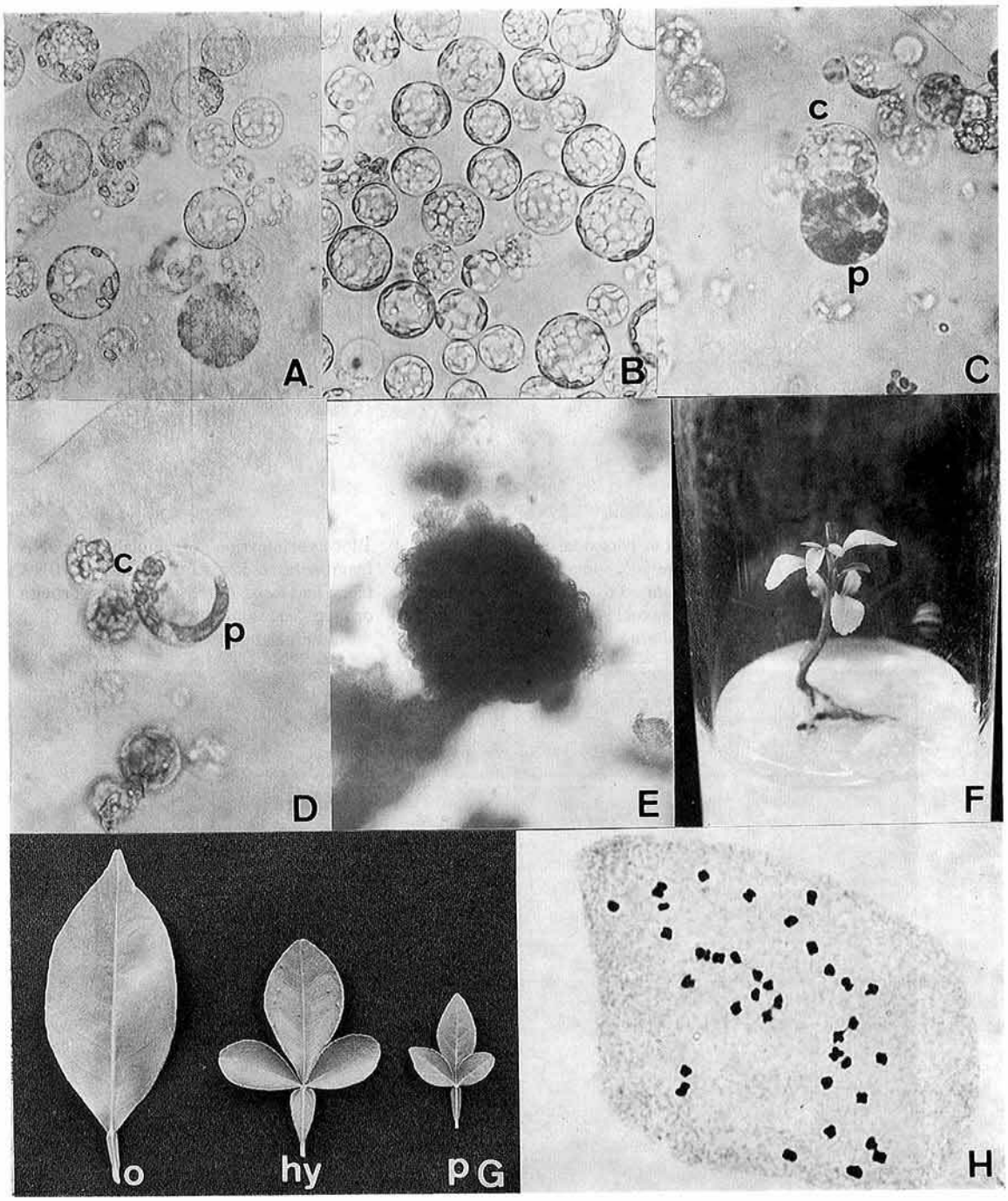

Plate 2. Somatic hybridization between Trovita orange and *P. trifoliata*
A : Freshly isolated protoplasts from Trovita orange nucellar callus,
B : Mesophyll protoplasts of *P. trifoliata*,
C : Adhered protoplast (c: Trovita orange, p: *P. trifoliata*),
D : Heterokaryon,
E : An embryoid derived from a heterokaryon,
F : A plant regenerated from an embryoid,
G : Leaf morphology of a somatic hybrid plant and parents (o: Trovita orange, hy: somatic hybrid, p: *P. trifoliata*),
H : A somatic hybrid plant had 36 chromosomes.

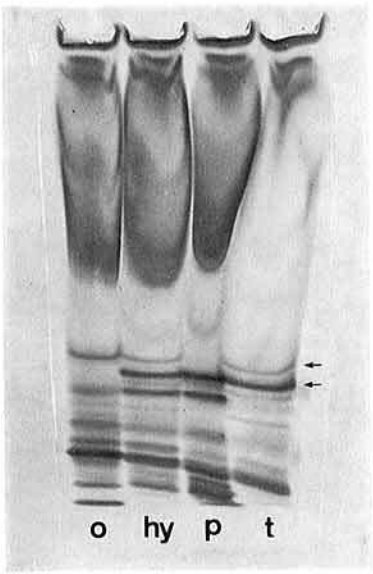

Plate 3. Isoelectric focusing profiles of peroxidase from roots of Trovita orange (o), somatic hybrid (hy), *P. trifoliata* (p) and Troyer citrange (t) (a sexual hybrid of *C. sinensis* and *P. trifoliata*)

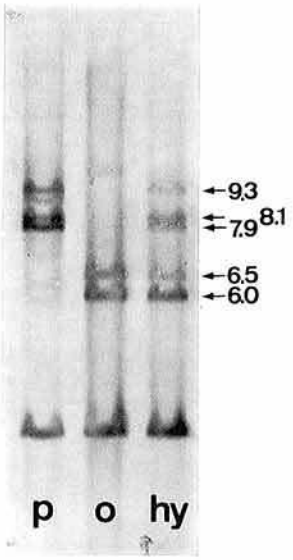

Plate 4. Blot-hybridization of biolabeled-rDNA fragments to *Eco* RI digested total DNA from leaves of *P. trifoliata* (p), Trovita orange (o) and somatic hybrid (hy) Numerals indicate kbp.

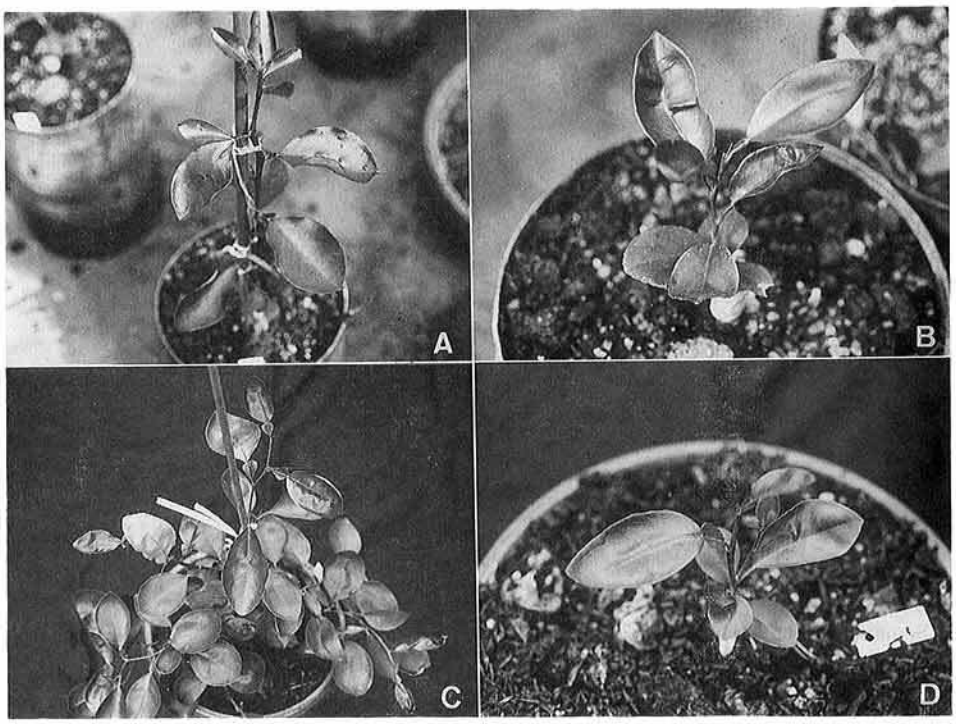

Plate 5. Somatic hybrid plants obtained in the following combinations
A : Trovita orange plus Troyer citrange,
B : Trovita orange plus Hayashi satsuma mandarin,
C : F. N. Washington navel orange plus Hayashi satsuma mandarin,
D : F. N. Washington navel orange plus Murcott tangor.

employed for the identification of somatic hybrid[3,16]. Thus, we employed the leaf oil-, isozyme- and rDNA-analysis in the regenerated plant and parents for the further confirmation of the somatic hybrid. Gas chromatogram of leaf oil of the regenerated plant was different from those of both parents (Fig. 2). The zymograms of peroxidase showed that the regenerated plant had the specific band of Trovita orange and that of *P. trifoliata* (Plate 3). Among the restriction endonuclease tested, *Eco* RI was shown to be the best enzyme for discriminating between rDNA fragments of Trovita orange and those of *P. trifoliata*. Clear and specific rDNA fragments originating from nuclear DNA of Trovita orange were 6.0 and 6.5 kbp, while those of *P. trifoliata* were 7.9, 8.1, 8.9 and 9.3 kbp. The regenerated plant had all of these fragments (Plate 4). These results indicated that the regenerated plant was somatic hybrid. In the other combinations, the regenerated plants (Plate 5) were also confirmed to be somatic hybrid (amphidiploid) by the analysis of rDNA and the observation of chromosome number.

In conclusion, we produced some somatic hybrid plants in the *Rutaceae* family by protoplast fusion. Such hybrid plants would be useful for the practical citrus breeding programs.

Summary

Somatic hybrid plants were obtained by protoplast fusion in the *Rutaceae* family. Protoplasts isolated from nucellar calli and from leaves of nucellar seedlings were fused by the PEG method. The fusion products were cultured in a Murashige and Tucker medium containing 0.6 M sucrose. In this medium, some colonies developed into the whole plants through embryogenesis. Almost all of the plants were shown to be somatic hybrid, which were proven by the restriction endonuclease analysis of nuclear ribosomal DNA. The chromosome number of the hybrid plants was 36, which was the sum of the parents (2n=18).

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