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Somatic hybrid plants obtained by protoplast fusion between *Citrus sinensis* and *Poncirus trifoliata*

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Summary. Somatic hybrid plants of *Rutaceae* were obtained by protoplast fusion between *Citrus sinensis* Osb. ('Trovita' orange) and *Poncirus trifoliata*. Protoplasts isolated from embryogenic cells of *C. sinensis* and from leaves of *P. trifoliata*, and the culture of fusion products in the presence of high concentrations of sucrose were essential requirements for the selection of hybrids. Green globular embryoids derived from protoplasts resulted in the regeneration of trifoliate plants. Other morphological characters of these plants were intermediate between both parents. The chromosome number in one of the hybrid plants was 36, which was the sum of *C. sinensis* (2n = 18) and *P. trifoliata* (2n = 18). EcoRI restriction analysis of rDNA confirmed the presence of parental nuclear DNAs in the hybrid.

Key words: *Citrus* – *Poncirus* – Embryogenesis – rDNA analysis – Somatic hybrid

Introduction

Recent advances in somatic cell fusion technology made it possible to create several inter- and intrageneric plants (Pelletier and Chupeau 1984). The application of this technology to *Citrus* would be of great value of the improvement of this important fruit bearing tree.

Recently it has been possible to regenerate plants from the protoplasts of certain *Citrus* cultivars (Vardi and Spiegel-Roy 1982; Kobayashi et al. 1983). Nevertheless, the successful production of somatic hybrids in the *Rutaceae* family has not yet been demonstrated. Here we report the first case of parasexual hybridization in *Rutaceae* using *Citrus sinensis* and *Poncirus trifoliata*.

Materials and methods

Plant materials

About 1 g nucellar callus of *C. sinensis* Osb. ('Trovita' orange) was inoculated into 50 ml of liquid culture medium consisting of Murashige and Tucker (MT) basal medium formula (1969), in which 10 mg/l 6-benzyl aminopurine only was used as phytohormone. The culture was maintained at 25 °C under continuous light (1,300 lux). Serial transfer of the callus was done every two weeks. Seeds of *P. trifoliata* were germinated in a pot containing Vermiculite. Plants were grown in a growth chamber kept at 26 °C under 16 h/day illumination with a cool fluorescent light (3,000 lux). A nutrient solution containing 1/400 strength Hyponex was supplied to each pot once a week. Leaves were harvested from 2 month-old-plants bearing about five fully expanded leaves.

Protoplast isolation

Prior to isolating the protoplasts from *C. sinensis*, two-week-old cells, obtained after serial dilution, were transferred to the hormone-free MT liquid medium. After a further 2 weeks, callus was again transferred to the same medium used for the previous culture. On the 5th day, cells were collected and subjected to protoplast isolation using the procedure of Vardi and Spiegel-Roy (1982).

In the case of *P. trifoliata*, 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 1 mM MES (pH 5.8) solution containing 0.6 M mannitol and MT macro elements for 1 h. About 0.6 g leaves were incubated in a petri dish with 20 ml enzyme solution (pH 5.8) containing 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 h. The cell and enzyme mixture was filtered through two layers of Miracloth and centrifuged (110 × g; 2 min). The protoplasts were then washed twice with 0.6 M mannitol by centrifugation (110 × g; 2 min).

Cell fusion and protoplast culture

C. sinensis and *P. trifoliata* protoplasts (Fig. 1 a, b, respectively) were mixed and fused by the method of Uchimiya (1982). Ten

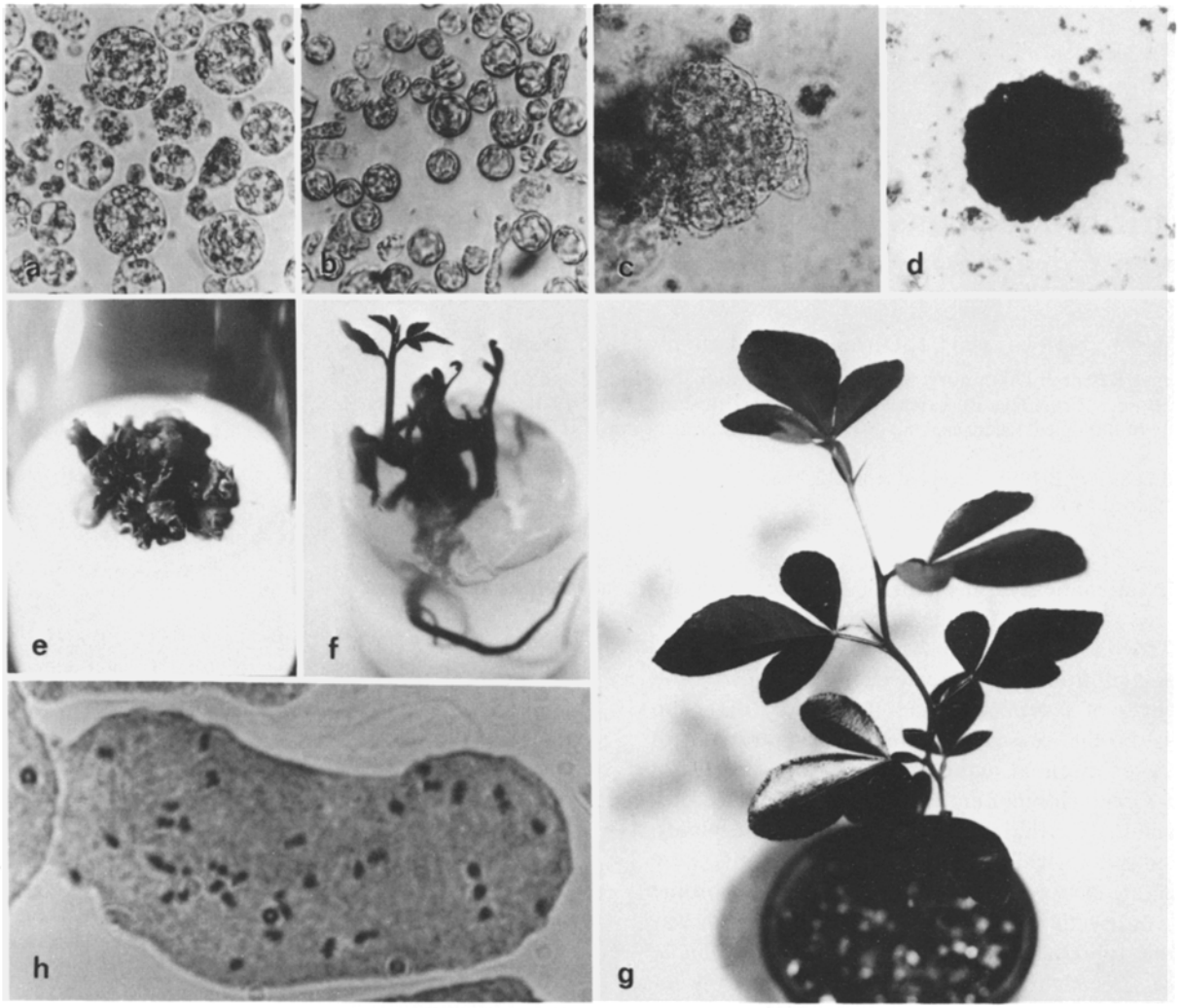

Fig. 1. Freshly isolated protoplasts from suspension cultures of *C. sinensis* Osb. ('Trovita' orange) (×400) (**a**); Mesophyll protoplasts of *P. trifoliata* (×400) (**b**); Formation of a cell mass (×400, 15 day) (**c**); Globular embryoid (×75, 5 weeks) (**d**); Embryoid (×1, 4 months) (**e**); Young plantlet (×0.8, 6 months) (**f**); The regenerated plant (×0.5, 9 months) (**g**); Chromosomes in the regenerated plant (×1,000) (**h**)

minutes after the addition of polyethylene glycol, PEG was diluted with 0.6 M mannitol-50 mM $CaCl_2$, and removed by centrifugation (170×g; 5 min). Protoplasts were washed twice with 0.6 M mannitol, and once with hormone-free MT liquid medium containing 0.6 M sucrose by centrifugation (170×g; 5 min). These protoplasts (10^5 cells/ml) were cultured in a liquid droplet (0.5 ml) of the hormone-free MT medium containing 0.6 M sucrose in a Falcon petri dish (60×15 mm). The plate was sealed with Parafilm and maintained under continuous light (1,700 lux) at 25 °C. After 3 weeks, two volumes of a melted agar (45 °C) MT medium containing 5% sucrose and 1.2% agar – free of phytohormones – was added to the protoplast culture.

Observation of chromosome number

Root tips of regenerated plants were treated with 8-hydroxyquinoline (2 mM) at 10 °C for 18 h. Following the rinse of root tips with distilled water, tissues were fixed in ethanol : acetic acid (3 : 1) at 4 °C for 24 h and stained with lactopropionic orcein (1%) to enable microscopic observation of chromosomes.

Analysis of rDNA

Preparation of nuclear DNAs, endonuclease digestion, electrophoresis and DNA-RNA hybridization were carried out according to the method of Uchimiya and co-workers (1983).

Results and discussion

The present investigation was carried out to examine the feasibility of somatic cell hybridization in the *Rutaceae* family. We have chosen two species, *C. sinensis* and *P. trifoliata* for the following reason. Protoplasts

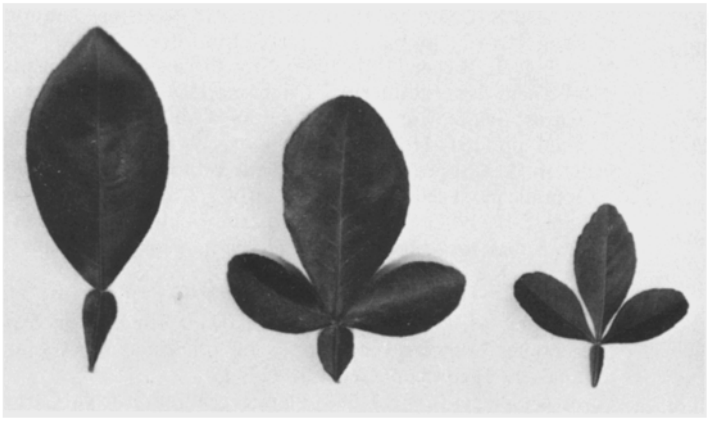

Fig. 2. Leaf morphology of a somatic hybrid and parents. *Left: C. sinensis* Osb. ('Trovita' orange). *Middle:* a somatic hybrid. *Right: P. trifoliata*

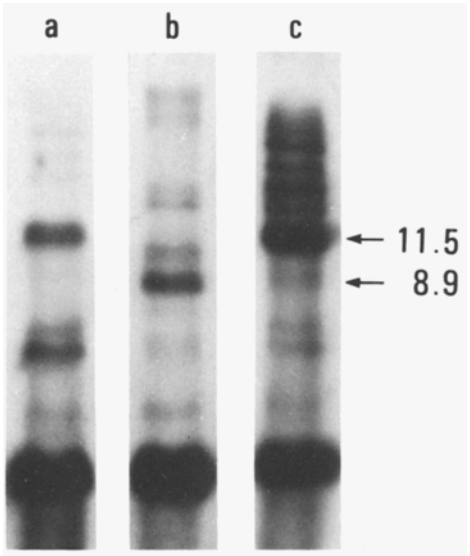

Fig. 3. Autoradiography of Southern image hybridization of ^{32}P-labelled-25s+17s rRNA to EcoRI digests of nuclear DNA from seeds of *C. sinensis* (*a*), *P. trifoliata* (*b*), and from embryoids of somatic hybrid (*c*). Numerals indicate kbp

of *C. sinensis* Osb. ('Trovita' orange) nucellar callus have been shown to be capable of resuming cell divisions and eventually developing into plants through embryogenesis (Kobayashi et al. 1983). We were unable to induce cell divisions from leaf protoplasts of *P. trifoliata* under the same culture conditions as *C. sinensis* (unpublished results). Sexual hybridization between two species resulted in the production of fertile hybrids whose leaves were trifoliate. Therefore, somatic hybrids of these combination can be confirmed by regenerating plants possessing trifoliate leaves.

During the course of this investigation we found that only protoplasts isolated from cells of *C. sinensis* grown in hormone free MT medium were capable of resuming division after PEG treatment. After the fusion treatment, the first cell division occurred on the 8th day of culture, and some cell masses were observed after 15 days (Fig. 1 c). Some of them formed tightly packed cell masses with a dense cytoplasm. In solidifying the culture medium and reducing sucrose concentration to approximately 0.3 M, globular embryoids were developed from small cell clusters (Fig. 1 d). Mesophyll protoplasts of *P. trifoliata* never divided under the same conditions. Nucellar protoplasts of *C. sinensis* alone or mixed culture with *P. trifoliata* protoplasts did not produce embryoids under the same conditions. A similar sucrose concentration (> 0.256 M) is known to suppress the embryogenesis of nucellar callus of *C. sinensis* (Kochba et al. 1982).

The growth of green globular embryoids was promoted by the addition of malt extract (500 mg/l) and adenine sulfate (40 mg/l) in hormone free MT medium (Fig. 1 e). Many cotyledonary embryoids were produced from one of embryoids in this medium. When these cotyledonary embryoids were transferred to MT medium containing 10 mg/l gibberellic acid (GA_3) with only as phytohormone, roots and leaflets were produced (Fig. 1 f) within 5–6 months of culture.

Plants derived from the same embryoid were morphologically uniform and had characteristics of both parents (Fig. 1 g). Leaves were trifoliate like *P. trifoliata,* and their size, thickness and smoothness ressembled those of *C. sinensis* (Fig. 2). A chromosome number of 36 was counted in the root tip of these plants (Fig. 1 h). Both parents have a chromosome number $n = 18$. Based on the intermediate leaf morphology and chromosome number, this somatic hybrid must be amphidiploid.

For the further confirmation of the somatic hybrid, we employed the restriction endonuclease analysis of rDNA in the somatic hybrid and parents. Among the restriction enzymes tested, EcoRI was shown to be the best enzyme for discriminating rDNA fragments of *C. sinensis* and *P. trifoliata.* A clear and specific rDNA fragment originated from nuclear DNA of *C. sinensis* was 11.5 kbp, while that of *P. trifoliata* was 8.9 kbp. EcoRI digested nuclear DNA of the hybrid contained

both 11.5 and 8.9 kbp fragments (Fig. 3). These results indicate that the somatic hybrid plant contained nuclear information of both parents.

In addition to this hybrid plant we obtained at least 10 plants from different fusion products. Every plant was morphologically similar to this hybrid plant.

The application of the procedure employed in this investigation for the creation of somatic hybrid plants in other *Citrus* cultivars is in progress.

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