Regeneration of Intergeneric Somatic Hybrids by Electrical Fusion between *Citrus* and Its Wild Relatives: Mexican Lime (*Citrus aurantifolia*) and Java Feroniella (*Feroniella lucida*) or Tabog (*Swinglea glutinosa*)

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Summary

Electrical fusion of protoplasts was used to produce somatic hybrids between Mexican lime (*Citrus aurantifolia* Swingle) and Java feroniella (*Feroniella lucida* (Sceff.) Swingle) or tabog (*Swinglea glutinosa* (Blanco) Merr.). The fusion efficiency (rates of formation of binuclear heterokaryons) were around 15%. The fusion products were cultured and embryoids were differentiated from them. The embryoids were transferred to a medium for plantlet formation and grew into plantlets. The rDNA analysis confirmed that the differentiated plantlets from these two combinations have genomes from both parents. The results confirmed that our electrical fusion system is an effective method for producing somatic hybrids of citrus and its sexually incompatible wild relatives.

Introduction

Progress in developing higher plants resistant to pests and pathogens is often restrained by the fact that wild species which carry desirable traits cannot be cross-bred with cultivated species. To bypass sexual incompatibility, the production of somatic hybrids by protoplast fusion and the subsequent regeneration of hybrid plants is a newly elaborated alternative method to that of sexual crossing in higher plants.

Since the first somatic hybrid plants of citrus between *Citrus sinensis* and *Poncirus trifoliata* were regenerated by Ohgawara et al. (10), a number of additional somatic hybrids among *Citrus* and related genera have been reported (1, 2, 3, 7, 8, 11). Vardi et al. (16) has reported the production of cybrid citrus plants. However, most of these somatic hybrids were produced from sexually compatible species and only two sexually incompatible species; that being the regeneration of somatic

hybrid plants from *Citrus sinensis* and *Severinia dis-ticha* (2), and from *Citrus sinensis* and *Citropsis gillettiana* (3). *Severinia* and *Citropsis* belong to the subtribe Citrinae in which *Citrus* belongs also. These somatic hybrids were obtained by polyethylene glycol (PEG) mediated fusion. We have recently reported an efficient electrical fusion system which was used to produce somatic hybrid plants of *Citrus* (5). In this paper, we describe the regeneration of intergeneric somatic hybrid plants produced from cell fusion of sexually incompatible species; Mexican lime (*Citrus aurantifolia* Swingle), which belongs to the subtribe Citrinae, and Java feroniella (*Feroniella lucida* (Sceff.) Swingle) or tabog (*Swinglea glutinosa* (Blanco) Merr.), which 2 species belong to the different subtribe Balsamocitrinae. This is the first report of the somatic hybrid from the fusion between the subtribes Citrinae and Balsamocitrinae.

Materials and Methods

Plant materials

Protoplasts were isolated from callus and leaves. Embryo derived callus of Mexican lime was induced as described earlier (4). Excised embryos from young immature seeds were plated on callus

Received for publication 12 August 1991.

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induction medium. The medium was based on Murashige and Skoog (9) (MS) containing 0.2 M sucrose and 50 μ M kinetin and solidified with 0.2% Gelrite (Kelco, Merck & Co. Inc., USA). Induced callus were subcultured on the same medium and maintained with a 16-hr photoperiod (warm-white fluorescent lamps, 25 ~ 35 μ E·m⁻²·sec⁻¹) at 25° ± 1°C. These callus lines have maintained an embryogenic potential after 8 to 10 years of subculture.

Seeds of *Java feroniella* and *tabog* were germinated in Petri dishes and planted in a potting mixture. They were grown in a greenhouse at 25° ~ 35°C without supplemental lighting. Leaf protoplasts of these species were isolated from leaves of 5- to 6-year-old plants as described previously (5).

Protoplast isolation

Prior to isolation of protoplasts, the callus cultures were transferred from the solidified subculture medium to a liquid MS medium containing same components and shaken on an orbital shaker (140 rpm) with a 16-hr photoperiod (warm-white fluorescent lamps, 25 ~ 35 μ E·m⁻²·sec⁻¹) at 25° ± 1°C. They were subcultured at least 3 times every 2 weeks. The cultures were then transferred to a liquid MS medium containing 0.1 M galactose and 0.1 M sorbitol and maintained for one week or more. For the suspension culture-derived protoplasts, about 1 g of callus after 1 week of transfer were collected and macerated by the method of Vardi and Spiegel-Roy (15) with slight modification. The maceration medium (pH 5.6) contained half strength MS medium with 0.3% Macerozyme R-10 (Yakult Pharmaceutical Co. Ltd., Nishinomiya, Japan), 0.3% Cellulase Onozuka R-10 (Yakult Pharmaceutical Co. Ltd. Nishinomiya, Japan, 0.35 M mannitol and 0.35 M sorbitol. Digestion was carried out at 25° ± 1°C for 16 hr.

Five to six fully expanded leaves of *Java feroniella* and one to two leaves of *tabog* were collected and surface sterilized with 70% ethanol. The leaves were then immersed in a solution containing 0.5% sodium hypochlorite for 10 min and washed three times with sterile distilled water. The leaves were cut into small pieces (about 2 mm × 2 mm) by a sterilized razor blade and transferred into a 100 ml Erlenmeyer flask containing half strength MS liquid medium supplemented

with 0.7 M mannitol. For the pretreatment, they were shaken by an orbital shaker (140 rpm) for about 1 hr at 25° ± 1°C. After removal of the preincubation medium, the leaf pieces were incubated with 40 ml enzyme solution (pH 5.6) containing 1/2 MS medium with 0.3% Macerozyme R-10, 3.0% Cellulase Onozuka R-10, 1 mM N-morpholinoethane sulfonic acid (MES), 0.35 M mannitol and 0.35 M sorbitol. Digestion was carried out at 25° ± 1°C for 16 hr.

Protoplasts were filtered through a layer of Miracloth (Calbiochem, USA), washed and centrifuged (100 × g) 3 times with fusion solution containing 0.35M mannitol, 0.35M sorbitol and 0.25 mM calcium chloride.

Protoplast fusion

The electric fields necessary to induce protoplast fusion were generated by a power supply (RSG-1000, Hoei Sci. Co. Ltd., Tokyo, Japan). Electrical fusion was carried out using an electrode (FTC-33D5, the Shimadzu Co. Ltd., Kyoto, Japan) with 4 ml solution in a plastic Petri dish (60 mm diameter, 15 mm high).

The electrical parameters used in this experiment to induce protoplast fusion were as follows: AC field, 1 MHz, 125 V·cm⁻¹, 60 sec; DC field, 1,250 V·cm⁻¹ square-wave 50 μ sec in duration, 5 times at 1 sec intervals.

For fusion, a protoplast mixture was prepared which contained 2 ~ 6 × 10⁵ callus protoplasts and 5 ~ 50 × 10⁵ mesophyll protoplasts resuspended in the fusion solution previously described. Four ml of this mixture was transferred to a plastic Petri dish (60 mm diameter, 15 mm high) and fusion was induced. Mixtures were allowed to remain still for at least 10 min after fusion, then transferred to 10 ml tubes and pelleted by centrifugation at 100 × g for 7 min. The supernatant was discarded and the fusion products were resuspended at a density of 2 × 10⁵ cells in an MS medium with 0.6 M sucrose according to the method of Ohgawara et al. (10). After transfer of 2 ml mixture into a plastic Petri dish, an equal volume of a liquid MS medium with 1.6% agar, and 0.6% sucrose was added, mixed and allowed to solidify. The cultures were incubated with a photoperiod of 16 hr (warm-white fluorescent lamps, 2 ~ 4 μ E·m⁻²·sec⁻¹) for 3 weeks and then transferred to a higher light intensity of 20 ~ 30 μ E·m⁻²·sec⁻¹. Embryoids which de-

veloped were transplanted onto an MS medium (solidified with 0.2% Gelrite) containing 0.1 M galactose, 0.1 M sorbitol and 1 μ M gibberellic acid in 20 $\times$ 100 mm culture tubes.

DNA analysis

For DNA analysis, tissues of differentiated plantlets in culture tubes and parental callus and leaves were collected and freeze-dried. Total DNA was extracted from the samples according to the sodium dodecylsulfate (SDS) micro-preparation method of Honda and Hirai (6) with slight modification.

Thirty mg of freeze-dried tissue was homogenized with a mortar and pestle and was transferred to a 1.5 ml polypropylene microtube. Five hundred μ l of extraction buffer (100 mM 2-amino-2-hydroxymethyl-1, 3-propanediol - hydrochloric acid (Tris-HCl) pH 8.0, 50 mM ethylenediaminetetraacetic acid disodium salt (EDTA) pH 8.0, 500 mM sodium chloride (NaCl), 0.2% 2-mercaptoethanol) was then added. Small samples were ground directly with 500 μ l of extraction buffer and then transferred to a 1.5 ml microtube. The homogenate was mixed with 100 μ l of 10% SDS and incubated at 65°C for 20 min. Two hundred μ l of 5 M potassium acetate was added and the mixture was incubated on ice for 20 min. After centrifugation at 15,000 rpm for 10 min, the supernatant was transferred to a new 1.5 ml microtube, mixed with 400 μ l of isopropanol and placed at -70°C for 30 min. After the mixture was centrifuged at 15,000 rpm for 20 min, the supernatant discarded and the pellet briefly dried. The pellet was dissolved in 100 μ l of 50 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, and centrifuged at 15,000 rpm for 10 min. The supernatant was transferred to a new 1.5 ml microtube and mixed with 10 μ l of 3 M sodium acetate pH 5.2 and 100 μ l isopropanol. After incubation at -70°C for 15 min, the mixture was centrifuged at 15,000 rpm for 20 min. The pellet was rinsed with -20°C 70% ethanol and dissolved in 50 μ l TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0). The mixture was centrifuged at 15,000 rpm for 5 min and supernatant was transferred to a new 1.5 ml microtube.

After quantifying the amount of DNA, aliquots of 5 μ g from each parent and differentiated plantlets were digested separately with various restriction endonucleases (*Afa*I, *Bam*HI and *Xho*I). After

electrophoresis in 0.8% agarose gels, the digested DNA was treated with ultraviolet-light (300 nm) for 5 min and transferred to nylon membrane (Hybond-N+, Amersham, UK) with 0.4 M NaOH solution. The blots were probed with 7.7 kbp *Eco*RI fragments from rice-rDNA (14) labeled with peroxidase complexes, for visualization by the non-radioisotopic gene detection system, ECL (Amersham, UK).

Results and Discussion

Following fusion and 5 to 10 weeks of culture, small callus colonies became visible in Petri dishes. Several green embryoids developed after 12 to 14 weeks of culture. However, the number of those embryoids were around 50 per Petri dish, which is fewer than that of the combinations between *Citrus* reported (5). Embryoids were transplanted to a new medium in culture tubes for further development. Some of them formed shoots and roots, however, many shoots were deformed (Figs. 1 and 2).

DNA analysis (Figs. 3 and 4) confirmed the hybridity of the regenerated plantlets of both combinations of Mexican lime $\times$ Java *feroniella* or tabog. Among the restriction enzymes tested, *Afa*I (Fig. 3a), *Bam*HI (Fig. 3b) and *Xho*I (Fig. 3c) released specific fragments for Mexican lime, Java *feroniella* and their somatic hybrids, and *Bam*HI (Fig. 4) released specific fragments for Mexican lime, tabog and their somatic hybrids. In the Mexican lime $\times$ Java *feroniella* somatic hybrid, 2 specific fragments for each species were present between 6.5~2.3 kbp by *Afa*I. Two specific fragments for Mexican lime were present under 2.3 kbp by *Bam*HI and also 2 specific fragments for Java *feroniella* were present between 4.4~2.3 kbp by *Xho*I. In the Mexican lime $\times$ tabog somatic hybrid, 3 fragments specific for each species were present under 2.3 kbp by *Bam*HI.

We have previously described the electrical fusion system for *Citrus* (5). Sexually incompatible somatic hybrids were reported in two combinations between *Citrus* and its wild relatives so far: *C. sinensis* and *Severinia disticha* (2), and *C. sinensis* and *Citropsis gilletiana* (3). According to Swingle's classification (12), these three species belong to the subtribe Citrinae of the orange subfamily Aurantioideae. This subtribe is divided into 3 subtribal groups. *Citrus* belongs to the group of "true citrus

Fig. 1. Somatic hybrids resulting from the fusion of protoplasts from Mexican lime (*Citrus aurantifolia*) and Java feroniella (*Feroniella lucida*).

a: Mexican lime. b: Somatic hybrid from Mexican lime and Java feroniella. c: Java feroniella.

Fig. 2. Somatic hybrids resulting from the fusion of protoplasts from Mexican lime (*Citrus aurantifolia*) and tabog (*Swinglea glutinosa*). a: Somatic hybrid from Mexican lime and tabog. b: Tabog.

fruit trees". While *Citropsis* belongs to the group of "near-citrus fruit trees" and *Severinia* belongs to the group of "primitive citrus fruit trees". *Citropsis*, *Severinia* and several other relatives in Citrinae, which are potentially important for *Citrus* genetic improvement, are graft-compatible with *Citrus*, but sexually incompatible (13). Java feroniella and tabog used in our experiment belong to a different subtribe Balsamocitrinae which divided into 3 subtribal groups. Java feroniella belongs to "wood-apple group" and tabog is a member of "tabog group". *Citrus* is sexually incompatible with them, however, can be grafted on tabog and in some cases on Java feroniella (13). Tabog grows well in sandy loam and porous limestone soils and may be a source of tolerance to these conditions, and Java

Fig. 3. DNA blot hybridized to 7.7 kbp *Eco*RI fragments from rice-rDNA labeled with peroxidase complexes in a combination between Mexican lime and Java feroniella. M: Mexican lime, J: Java feroniella, MJ: somatic hybrid between Mexican lime and Java feroniella. Arrows indicate unique bands present in somatic hybrid and the parents. a: Blot with *Afa*I-digested DNA. b: Blot with *Bam*HI-digested DNA. c: Blot with *Xho*I-digested DNA.

Fig. 4. Blot with *Bam*HI-digested DNA extracted from Mexican lime, tabog and a somatic hybrid hybridized to 7.7 kbp *Eco*RI fragments from rice-rDNA labeled with peroxidase complexes. M: Mexican lime, T: Tabog, MT: Somatic hybrid between Mexican lime and tabog. Arrows indicate unique bands present in somatic hybrid and the parents.

feroniella will be a source of dwarfing rootstocks (13). However, they cannot be used directly because of severe dwarfism and cold sensitivity. Remote relatives of *Citrus* may have more potential as sources of germplasm of rootstocks for genetic improvement of *Citrus*.

The electrical fusion system described here is an efficient system for producing somatic hybrids between *Citrus* and its remote relatives as well as for producing somatic hybrids among *Citrus* spp. as described previously (5). Future work will attempt to create somatic hybrids between *Citrus* and other species of Aurantioideae, orange subfamily.

Acknowledgment

We express sincere thanks to Dr. M. A. Berhow (STA fellow) for his kind revision of manuscript.

Literature Cited

- Grosser, J.W., F.G. Gmitter and J.L. Chandler. 1988. Intergeneric somatic hybrid plants of *Citrus sinensis* cv. Hamlin and *Poncirus trifoliata* cv. Flying Dragon. Plant Cell Rep. 7: 5-8.
- Grosser, J.W., F.G. Gmitter and J.L. Chandler. 1988. Intergeneric somatic hybrid plants from sexually incompatible woody species: *Citrus sinensis* and *Severinia disticha*. Theor. Appl. Genet. 75: 397-401.
- Grosser, J.W., F.G. Gmitter, N. Tusa and J.L. Chandler. 1990. Somatic hybrid plants from sexually incompatible woody species: *Citrus reticulata* and *Citropsis gilletiana*. Plant Cell Rep. 8: 656-659.
- Hidaka, T. and M. Omura. 1989. Control of embryogenesis in *Citrus* cell culture: Regeneration from protoplasts and attempts to callus bank. Fruit Tree Res. Stn. B 16: 1-17.
- Hidaka, T. and M. Omura. 1992. Regeneration of somatic hybrid plants obtained by electrical fusion between satsuma mandarin (*Citrus unshiu*) and rough lemon (*C. jambhiri*) or yuzu (*C. junos*). Japan. J. Breed. 42. (In Press).
- Honda, H. and A. Hirai. 1990. A simple and efficient method for identification of hybrids using nonradioactive rDNA as probe. Japan. J. Breed. 40: 339-348.
- Kobayashi, S., T. Ohgawara, E. Ohgawara, I. Oiyama and S. Ishii. 1988. A somatic hybrid plant obtained by protoplast fusion between navel orange (*Citrus sinensis*) and satsuma mandarin (*C. unshiu*). Plant Cell Tissue Organ Culture 14: 63-69.
- Kobayashi, S., T. Ohgawara, E. Ohgawara, I. Oiyama and S. Ishii. 1988. Somatic hybridization between Navel orange and 'Murcott' Tangor. p.23-28. In: R. Goren and K. Mendel (eds.). Proc. 6th Int. Citrus Congr. Tel Aviv.
- Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15: 473-497.
- Ohgawara, T., S. Kobayashi, E. Ohgawara, H. Uchimiya and S. Ishii. 1985. Somatic hybrid plants obtained by protoplast fusion between *Citrus sinensis* and *Poncirus trifoliata*. Theor. Appl. Genet. 71: 1-4.
- Ohgawara, T., S. Kobayashi, S. Ishii, K. Yoshinaga and I. Oiyama. 1989. Somatic hybridization in *Citrus*: Navel orange (*C. sinensis* Osb.) and grapefruit (*C. paradisi* Macf.). Theor. Appl. Genet. 78: 609-612.
- Swingle, W.T. 1943. The botany of *Citrus* and its relatives of the orange subfamily. p. 129-474. In: H.J. Webber and L.D. Batchelor (eds.). The citrus industry. Vol. I. Univ. Calif. Press, Berkeley and Los Angeles., Calif., U.S.A.
- Swingle, W.T. and P.C. Reece. 1967. The botany of *Citrus* and its wild relatives. p.190-430. In: W. Reuther, H.J. Webber and L.D. Batchelor (eds.). The citrus industry Vol. I. Univ. Calif. Press, Berkeley, Calif., U.S.A.
- Takaiwa, F., K. Oono and M. Sugiura. 1984. The complete nucleotide sequence of a rice 17S rRNA gene. Nucleic Acids Res. 12: 5441-5448.
- Vardi, A. and P. Spiegel-Roy. 1982. Plant regeneration from *Citrus* protoplasts: Variability in methodological requirements among cultivars and species. Theor. Appl. Genet. 62: 171-176.
- Vardi, A., P. Arzee-Gonen, A. Frydman-Shani, S. Bleichman and E. Galun. 1989. Protoplast-fusion-mediated transfer of organelles from *Microcitrus* into *Citrus* and regeneration of novel alloplasmic trees. Theor. Appl. Genet. 78: 741-747.

電気融合法によるカンキツとその近縁野生種との体細胞雑種の作出：
メキシカンライム (*Citrus aurantifolia*) と、ジャワフェロニエラ (*Feroniella lucida*)
およびタボッグ (*Swinglea glutinosa*) との細胞融合

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摘 要

カンキツ類縁種の有用遺伝子を栽培種へ導入するため、カンキツとその近縁野生種2種との電気細胞融合を試み、それぞれ融合個体を得た。

一方のカンキツのプロトプラストはメキシカンライムの胚起源カルスから得、一方のプロトプラストにはジャワフェロニエラ (*Feroniella lucida*) およびタボッグ (*Swinglea glutinosa*) の葉肉から得たものを用いた。実験を通じての融合効率 (ヘテロカリオンの形成率) は15%前後であった。

融合したプロトプラストを培養したところ、緑色の胚様体 that 得られた。それらを植物体形成培地に移し、さらなる生長を図ったが、茎葉を形成するものは少なく、形成された茎葉は奇形を呈するものも多かった。

形成された植物体の一部および用いたカルス、葉から DNA を抽出し、イネ *r*DNA をプローブとして genomic southern hybridization を行ったところ、得られた個体は体細胞雑種であることが明らかになった。

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