Intergeneric Somatic Hybrids between *Citrus* and *Atalantia* or *Severinia* by Electrofusion, and Recombination of Mitochondrial Genomes

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Summary

Two intergeneric hybridizations through protoplast fusion between the embryogenic callus cells of 'Seminole' tangelo (*Citrus reticulata* Blanco × *C. paradisi* Macf.) and leaf cells of Indian atalantia (*Atalantia monophylla* DC.) or Chinese box-orange (*Severinia buxifolia* (Poir.) Tenore) were performed to overcome the genetic incompatibility. We were successful in regenerating whole plants of 16 somatic hybrids between 'Seminole' tangelo and Indian atalantia, and 46 somatic hybrids between 'Seminole' tangelo and Chinese box-orange, and hybridity was confirmed by Southern blot hybridization with rDNA. The mitochondrial DNA (mtDNA) of the somatic hybrids between 'Seminole' tangelo and Indian atalantia was identical to that of 'Seminole' tangelo, when *rrn* 26 and *cox* I were used as probes. However, when *atp* A was used as a probe, a 'Seminole' tangelo-specific fragment was found missing besides an Indian atalantia-specific fragment. The band pattern obtained by chloroplast DNA (cpDNA) analysis was identical to that of Indian atalantia. On the contrary, somatic hybrids between 'Seminole' tangelo and Chinese box-orange showed the mtDNA band pattern identical to that of 'Seminole' tangelo, when *atp* A and *cox* I were used as probes. However, analysis by *rrn* 26 probe showed the presence of a part of a Chinese box-orange-specific band in addition to a 'Seminole' tangelo-specific band. The cpDNA of the somatic hybrids was identical to that of Chinese box-orange. These results suggest the occurrence of mitochondrial recombination between parents.

Key Words : *Citrus*, *Atalantia monophylla*, *Severinia buxifolia*, intergeneric somatic hybrid, mitochondrial recombination, electrofusion, genetic cross-incompatibility.

Introduction

Since Melchers *et al.* (1978) had succeeded in the production of intergeneric somatic hybrids between potato and tomato by protoplast fusion, there have been many trials of somatic hybridization to introduce desirable

traits from remote species, especially in the sexually incompatible combinations.

In Citrinae, Ohgawara *et al.* (1985) first reported the somatic hybrid between 'Trovita' orange (*Citrus sinensis* Osb.) and *Poncirus trifoliata* (L.) Raf. by protoplast fusion. This system was based on the capacity of callus cells to generate plants via embryogenesis. While many intergeneric somatic hybrids using this system were reported since then, the derivation of the entire plant from fused protoplasts was relatively limited to a few cases ; e.g. *C. sinensis*+*Severinia disticha* (Blanco) Swing. (Grosser *et al.* 1988) , *C. sinensis*+*Citropsis gilletiana* Swing. & M. Kell. (Grosser and Gmitter 1990) , *C. reticulata* Blanco+*Citropsis gilletiana* (Grosser *et al.* 1990) , *C. sinensis*+*Severinia buxifolia* (Poir.) Tenore (Grosser *et al.* 1992) , *C. aurantifolia* (Christm.) Swing.+*Feroniella lucida* (Scheff.) Swing. or *Swinglea glutinosa* (Blanco) Merr. (Takayanagi *et al.* 1992) , *C. sinensis*+*Murraya paniculata* (L.) Jack. (Shinozaki *et al.* 1992) , *C. sinensis*+*Atalantia ceylanica* (Arn.) Oliv. (Louzada *et al.* 1993) and *C. reticulata*+*Citropsis gabunensis* (Engl.) Swing. & M. Kell. (Ling and Iwamasa 1994). These regenerated somatic hybrids served as the useful materials both for basic research and breeding.

As part of the initiative to expand the genetic resources available to *Citrus* breeding, we have investigated the possibility of somatic hybridization between *Citrus* and other Aurantioideae species, which are sexually incompatible to *Citrus* (Takayanagi *et al.* 1992, Shinozaki *et al.* 1992). Indian atalantia (*Atalantia monophylla*) is a member of Near-Citrus Fruit Trees, and is expected to possess dwarf character, as this plant grafted onto *Citrus* rootstock shows a slow initial growth (Bitters *et al.* 1969). Chinese box-orange (*Severinia buxifolia*) is a member of Primitive-Citrus Fruit Trees, and is resistant to *Phytophthora citrophthora* (R. E. Smith et E. H. Smith) Leonian (Broadbent 1969).

Here, we report the production of new somatic hybrid plants of 2 combinations between 'Seminole' tangelo and Indian atalantia or Chinese box-orange by the electrofusion and the uniqueness of hybrid plants in their genome composition.

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Materials and Methods

Plant materials

An embryogenic callus derived from the immature embryo of 'Seminole' tangelo (*C. reticulata* Blanco $\times$ *C. paradisi* Macf.) was used for preparing callus protoplasts. This callus line has been maintained in the solidified Murashige and Skoog's (1962) (MS) medium containing 0.2 M sucrose and 50 μ M kinetin.

Indian atalantia (*Atalantia monophylla* DC.) and Chinese box-orange (*Severinia buxifolia* (Poir.) Tenore) were grown in a greenhouse at 25 ~ 35 °C without supplemental lighting and were used for making leaf protoplasts.

Protoplast isolation

The protoplasts were prepared by the method of Hidaka and Omura (1992). Before isolation, the callus cultures were transferred from the solid MS medium to the liquid MS medium and shaken on an orbital shaker (140 rpm) under 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 with a 2-week interval. The cultures were then transferred to a liquid MS medium containing 0.1 M galactose and 0.1 M sorbitol and pre-cultured before protoplast preparation for 1 ~ 2 weeks.

For isolation of mesophyll protoplasts of Indian atalantia and Chinese box-orange, 2 ~ 4 fully expanded leaves were cut into small pieces (about 2 mm $\times$ 2 mm) with a razor blade after sterilization with 70 % ethanol and 0.5 % sodium hypochlorite. Before maceration, their tissues were pre-incubated with half strength MS liquid medium supplemented with 0.7 M mannitol on the orbital shaker (50 rpm) for about 1 hr at 25 ± 1 °C. Tissues were then macerated in a medium (pH 5.6) containing half strength MS medium with 0.3 % Macerozyme R-10, 3.0 % Cellulase Onozuka R-10, 1 mM 2-(*N*-morpholino) ethanesulfonic acid (MES), 0.35 M mannitol and 0.35 M sorbitol at 25 ± 1 °C for 16 hr.

Protoplasts were filtered through a layer of Miracloth (Calbiochem, USA) and centrifuged 3 times at 100 $\times$ g for 10 min in a fusion solution containing 0.35 M mannitol, 0.35 M sorbitol and 0.25 mM calcium chloride.

Protoplast fusion

A protoplast mixture which contains 6 $\times$ 10⁵ callus protoplasts and 1.7 $\times$ 10⁶ mesophyll protoplasts was prepared, and both protoplasts were electrically fused by the procedure of Hidaka *et al.* (1995). After fusion, the mixture was kept still for at least 10 min before transferring to 10 ml tubes and centrifuging at 100 $\times$ g for 10 min. The supernatant was discarded and the fusion products were resuspended at a density of 2 $\times$ 10⁵ cells in a 0.25 % Gelrite (Kelco, Merck & Co. Inc. USA) solution containing high sugars (0.15 M sucrose and 0.45 M galactose) and 0.05 % glutamine. Then they were

solidified in 5 ml of double strength MS liquid medium which was supplemented with 0.15 M sucrose, 0.45 M glucose and 0.05 % glutamine in a plastic Petri dish (60 mm diameter, 15 mm high) for selection of the somatic hybrids by the high sugar concentration medium as reported by Ohgawara *et al.* (1985).

Protoplast culture

The protoplast cultures were incubated under 16 hr photoperiod (warm-white fluorescent lamps, 2 ~ 4 μ E m⁻² · sec⁻¹) for 3 weeks, and then transferred to moderate light conditions (20 ~ 30 μ E m⁻² · sec⁻¹). Embryoids were transplanted onto a 0.2 % Gelrite-solidified MS medium containing 0.1 M galactose, 0.1 M sorbitol and 1 μ M gibberellic acid (GA₃) in 20 $\times$ 100 mm culture tubes. Regenerated shoots were transplanted to rooting medium (0.2 % Gelrite-solidified MS medium containing 0.1 M galactose, 0.1 M sorbitol, 10 nM GA₃ and 100 nM naphthaleneacetic acid). Shoots which did not form roots on this medium, were directly grafted on root stocks of 'Troyer' citrange (*C. sinensis* $\times$ *Poncirus trifoliata*). The *in vitro* plantlets were acclimatized by the method of Hidaka and Kajiura (1989).

DNA isolation

Total DNA was extracted from the callus of 'Seminole' tangelo and the leaves of Indian atalantia, Chinese box-orange and the regenerated plants according to the sodium dodecylsulfate (SDS) method by Dellaporta *et al.* (1983).

Southern blot hybridization

Five μ g DNA was digested with the following endonucleases: *Bam* HI, *Dra* I, *Eco* RI, *Pst* I, *Pvu* II and *Xho* I. The digests were electrophoresed in 0.8 % agarose gels and transferred to nylon membrane (Hybond-N⁺, Amersham, UK) with 0.4 M NaOH solution. The blots were probed with digoxigenin (DIG)-labeled 7.7 kbp *Eco* RI fragment from rice-rDNA kindly provided by Dr. F. Takaiwa (Takaiwa *et al.* 1984) for the confirmation of hybridity. For mitochondrial DNA (mtDNA) analysis, *atp* A (1.4 kbp), *rrn* 26 (4.4 kbp) fragments from pea and *cox* I (1.5 kbp) fragment from sugar beet (kindly provided from Dr. T. Mikami, Hokkaido Univ.) were used as probes. For chloroplast DNA (cpDNA) analysis, pTB7 (10.9 kbp), pTB19 (12.3 kbp) and pTB28 (7.3 kbp) derived from *Bam* HI fragments of *Nicotiana tabacum* cpDNA (Sugiura *et al.* 1986) were used as probes. Labeling and hybridization were carried out according to the manufacturer's instructions (DIG-AMPPD system, Boehringer Mannheim GmbH).

Results

Protoplast fusion between 'Seminole' tangelo and Indian atalantia

Following the electrofusion of protoplasts of 'Seminole' tangelo and Indian atalantia, 16 shoots were regener-

ated. All shoots were directly grafted onto seedlings of 'Troyer' citrange and the recovered plants showed the intermediate leaf morphology of the both parents (Fig. 1).

Southern blot hybridization probed with rDNA, showed that all 7 plants examined (coded as SA-1 ~ SA-7) had specific bands derived from both parents (Fig. 2), suggesting that those were the somatic hybrids.

The composition of mtDNA was analyzed by *atpA*, *coxI* and *rrn26* probes. When *atpA* was used as a probe after digestion with *Eco*RI, all plants showed a 2.2 kbp fragment specific to Indian atalantia while a 2.9 kbp fragment specific to 'Seminole' tangelo was missing (data not shown). Some plants (SA-3, SA-5 and SA-6) lost an about 5.0 kbp *Pvu*II fragment derived from 'Seminole' tangelo (Fig. 3a). All plants had a 2.4 kbp Indian atalantia specific *Xho*I fragment, and lost a 5.5 kbp *Xho*I fragment derived from Indian atalantia. Moreover, the sizes of larger *Xho*I fragments of these seven plants were different from each other, suggesting that the recombination of mtDNA occurred at the site near the *atpA* gene (Fig. 3b). SA-3, SA-5 and SA-6 lost a 4.4 kbp

*Dra*I fragment specific to 'Seminole' tangelo, while SA-6 had a novel *Dra*I fragment of 2.8 kbp (Fig. 3c). When *rrn26* and *coxI* were used as probes, all plants showed band profiles identical to those of 'Seminole' tangelo (data not shown).

The cpDNA of regenerated plants was analysed by pTB7, pTB19 and pTB28. All plants showed band pattern identical to that of Indian atalantia (data not shown).

Protoplast fusion between 'Seminole' tangelo and Chinese box-orange

Forty-six plantlets were regenerated from the fused

Fig. 1. Leaf morphology of somatic hybrids and parents. Numbers from left to right are: (1); *Atalantia monophylla*, (2); SA-1, (3); 'Seminole' tangelo, (4); SS-1, (5); deformed line derived from cell fusion between 'Seminole' tangelo and *Severinia buxifolia*, (6); resembling line to *Severinia buxifolia* derived from cell fusion between 'Seminole' tangelo and *Severinia buxifolia*, (7); *Severinia buxifolia*.

Fig. 2. Southern blot hybridization of *Bam*HI digests of total DNA to rice-rDNA probe. Lanes from left to right: (1) 'Seminole' tangelo, (2) SA-1, (3) SA-2, (4) SA-3, (5) SA-4, (6) SA-5, (7) SA-6, (8) SA-7, and (9) *Atalantia monophylla*. Arrows indicate the bands specific to parents.

Fig. 3. Southern blot hybridization of restriction endonuclease digests of total DNA to *atpA* probe. (a) *Pvu*II digestion, (b) *Xho*I digestion, (c) *Dra*I digestion. Lanes from left to right: (1) 'Seminole' tangelo, (2) SA-1, (3) SA-2, (4) SA-3, (5) SA-4, (6) SA-5, (7) SA-6, (8) SA-7, and (9) *Atalantia monophylla*.

protoplasts. They were rescued by grafting onto the rootstocks of 'Troyer' citrange seedling. Leaf shape in 36 plants was intermediate of both parents. However, 8 plants developed deformed leaves and the remaining 2 plants resembled Chinese box-orange (Fig. 1).

Three (coded as SS-1 ~ SS-3) out of 36 plants, which showed an intermediate morphology of both parents, were selected for further analysis. Southern blot hybridization with rDNA probe revealed that the plants

Fig. 4. Southern blot hybridization of *Bam* HI digests of total DNA to rice-rDNA probe. Lanes from left to right ; (1) 'Seminole' tangelo, (2) SS-1, (3) SS-2, (4) SS-3, and (5) *Severinia buxifolia*. Arrows indicate the bands specific to parents.

Fig. 5. Southern blot hybridization of restriction endonuclease digests of total DNA to *rm26* probe. (a) *Dra* I digestion, (b) *Pst* I digestion. Lanes from left to right ; (1) 'Seminole' tangelo, (2) SS-1, (3) SS-2, (4) SS-3, and (5) *Severinia buxifolia*.

had specific fragments derived from both the parents, suggesting hybrid nature of these plants (Fig. 4).

When *rm26* was used as a probe for mtDNA analysis after digestion of the total DNA with *Dra* I, all 3 plants showed a 27.1 kbp fragment specific to Chinese box-orange besides the fragments of 'Seminole' tangelo (Fig. 5a). All plants had a 7.0 kbp *Pst* I fragment specific to 'Seminole' tangelo, while SS-1 had one more 13.1 kbp *Pst* I fragment specific to Chinese box-orange (Fig. 5b). The other mitochondrial probes, *atp* A and *cox* I, showed the fragment profiles identical to those of 'Seminole' tangelo (data not shown).

CpDNAs probed by pTB7, pTB19 and pTB28 exhibited the same fragment pattern as those of Chinese box-orange (data not shown).

Discussion

There are several reports of obtaining intergeneric somatic hybrids in Citrinae (Grosser *et al.* 1988, Grosser and Gmitter 1990, Grosser *et al.* 1990, Grosser *et al.* 1992, Takayanagi *et al.* 1992, Shinozaki *et al.* 1992, Louzada *et al.* 1993, Ling and Iwamasa 1994). Grosser *et al.* (1992) produced the somatic hybrids between *Citrus* and *Severinia buxifolia*, as the latter showed good immunity to many *Citrus* diseases (Swingle and Reece 1967). However, there were no reports so far regarding production of somatic hybrids between *Citrus* and *Atalantia monophylla*. *Atalantia monophylla* can be grafted with *Citrus*. Although the growth of the scion is slow at an early stage, it improves gradually (Bitters *et al.* 1969). The feature suggests the possibility of its use as a dwarf rootstock. In this study, the efficiency of protoplast fusion system for producing the somatic hybrids of species, which were sexually incompatible, had been verified. The regenerated plants are likely to provide a potentially useful source for *Citrus* breeding.

In *Citrus* protoplast fusion, chloroplast genomes were known to be transmitted from one or both parents without recombination (Vardi *et al.* 1989, Kobayashi *et al.* 1991). Vardi *et al.* (1989) analyzed the cpDNA in cybrids between *Microcitrus* and *Citrus* at the fused-embryogenic callus stage and the differentiated plant leaf stage. Samples from the first stage showed that some of the cybrids were identical to the donor or recipient parent, while most samples provided profiles having fragments specific to both parents. However, the samples from the second stage had either donor or recipient parent's cpDNA profiles. In this experiment, cpDNAs of all regenerated plants were similar to those of the mesophyll parent. The results suggest that the sorting out of chloroplasts in Citrinae seems to be a quite rapid process (Vardi *et al.* 1989) and that there was no recombination of chloroplast genomes by protoplast fusion.

On the contrary, there were many reports regarding the recombination of mitochondrial genomes of somatic hybrids (Nagy *et al.* 1981, Galun *et al.* 1982, Boeshore *et*

al. 1985, Aviv *et al.* 1984, Chetrit *et al.* 1985, Aviv and Galun 1987), and the direct evidence for intergenomic recombination was presented by *Petunia* and *Brassica* somatic hybrids (Rothenberg *et al.* 1985, Rothenberg and Hanson 1987, Robertson *et al.* 1987). Vardi *et al.* (1989) also showed the recombination of mtDNA in the cybrids between *Microcitrus* and *Citrus*. Aviv and Galun (1987) found that in the first sexual progeny of *Nicotiana* cybrids, the individual plants varied in their mtDNA restriction profiles, while the plants of the next sexual generation were identical to their maternal parent. Based on this, Vardi *et al.* (1989) speculated that the sorting out of mtDNA was a slow process. On the other hand, it has been reported that mtDNAs were kept stable during protoplast fusion in many trials (Kobayashi *et al.* 1991, Saito *et al.* 1993). Kobayashi *et al.* (1991) reported that all somatic hybrids between navel orange (*C. sinensis*) and 'Murcott' tangor had only the mtDNA derived from the callus parent. Although they used 11 mtDNA probes, only mtDNA fragments from callus parent were observed, suggesting that somaclonal variation in mtDNA band patterns were rare during the cell culture in *Citrus*. Our results, therefore, suggested that the mtDNA recombination had occurred in the intergeneric somatic hybrids. Are the features of mtDNA recombination in Citrinae limited to the intergeneric combinations? Further studies are necessary to elucidate this point.

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