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Fertile fruit trees obtained by somatic hybridization: navel orange (*Citrus sinensis*) and Troyer citrange (*C. sinensis* × *Poncirus trifoliata*)

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Summary. Nucellar cell suspension protoplasts of navel orange (*Citrus sinsensis* Osb.) were chemically fused with mesophyll protoplasts of Troyer citrange (*C. sinensis* × *Poncirus trifoliata*) and cultured in hormone-free Murashige and Tucker medium containing 0.6 *M* sucrose. Two types of plant were regenerated through embryogenesis. One type showed intermediate mono- and difoliate leaves and the other types was identical to Troyer citrange. The regenerated plants with intermediate morphology were demonstrated by chromosome counts and rDNA analysis to be amphidiploid somatic hybrids. Five clones of these somatic hybrids were grafted in the field. After 4 years, they set flowers having a morphology intermediate between those of the two parents. The pollen grains showed high stainability and sufficient germinability, and were larger than those of Troyer citrange. The fruits of the somatic hybrids were large and spherical with thick rinds. Most of them contained seeds with normal germinability. These results indicate that somatic hybridization is a useful tool for *Citrus* breeding.

Key words: Somatic hybrids – Navel orange – Troyer citrange – Plant breeding

Introduction

The first successful somatic hybridization of woody plants was achieved for a combination of Trovita orange (*Citrus sinsensis* Osb.) and trifoliate orange (*Poncirus trifoliata*) by Ohgawara et al. in 1985. Since then, the protoplast fusion technique has been applied to produce Rutaceae hybrids, which cannot be obtained by sexual crossing because of incompatibility (Grosser et al. 1988; 1990a) or male and female sterility (Kobayashi et al. 1988; Ohgawara et al. 1989). The somatic hybrids obtained are amphidiploids and have morphological characteristics intermediate between those of their parents. *Citrus* tetraploids themselves have no economic value due to characteristics such as thick rinds, but they are of interest as breeding material, because when crossed with diploids, they are able to produce triploids that are potentially useful since they lack seeds (Soost and Cameron 1975). Therefore, fertility of amphidiploid somatic hybrid plants is desirable for integration of the somatic hybridization technique into *Citrus* breeding programs, except in the case of direct use of somatic hybrids as rootstock (Grosser et al. 1990b).

Recently, somatic hybrid plants obtained from a fertile combination between Trovita orange and trifoliate orange flowered and set fruit, and their fertility was demonstrated (S. Kobayashi et al. in press). In the present experiment, we produced somatic hybrids between navel orange with male and female sterility, and Troyer citrange, which is a sexual hybrid of navel orange and trifoliate orange, and we examined the fertility of the somatic hybrids.

Materials and methods

A cell suspension culture of nucellar callus induced from navel orange (*C. sinensis* Osb.) cv Bahia (Kobayashi et al. 1984) was used for protoplast isolation, according to the procedure of Ohgawara et al. (1989). Seeds of Troyer citrange were germinated in a pot containing vermiculite. Plants (nucellar seedlings) were grown in a growth chamber (16-h photoperiod, 3,000 lx, 26 °C). Mesophyll protoplasts were isolated using the method described previously (Ohgawara et al. 1985). Navel orange and Troyer citrange protoplasts were mixed and fused using a PEG method (Uchimiya 1982) with modifications (Ohgawara et al. 1985). Following fusion, the protoplasts were cultured in hor-

mone-free Murashige and Tucker (1969) medium (MT) containing 0.6 *M* sucrose and 0.6% Sea Plaque agarose. After 1 month, 0.5 ml of hormone-free liquid MT containing 0.15 *M* sucrose was added. Embryoids obtained were regenerated according to the procedure described previously (Ohgawara et al. 1989). Chromosomes were observed by root-tip cytology and counted according to the method of Oiyama (1981). The nuclear genomes of the regenerated plants were analyzed using rRNA (Uchimiya et al. 1983) with modification, as described previously (Ohgawara et al. 1989). Total DNAs were extracted and digested with restriction enzymes, then separated using agarose gel electrophoresis. Pollen stainability was checked by staining 1,000 pollen grains with acetocarmine. Pollen germinability was determined by a germination test on agar medium (2% agar and 20% sucrose) at 25 °C for 20 h. About 500 pollen grains were examined from two plates.

Results and discussion

The somatic hybridization system we established between Trovita orange and trifoliate orange (Ohgawara et al. 1985) was also applied to the combination between navel orange and Troyer citrange. Twenty clones of regenerated plants were obtained through embryogenesis. Among the regenerated plants, two types of morphological characteristics were observed. Regenerated plants from 17 clones had intermediate mono- and difoliate leaves, as shown in Fig. 1. These leaves were broad, like those of previous amphidiploid somatic hybrids, whereas three clones had trifoliate leaves like Troyer citrange.

Chromosome counts showed that morphologically putative hybrids had the expected amphidiploid chromosome number of 36 ($2n = 2x = 18$ for each parent), whereas the regenerated plants of the Troyer citrange type had 18 chromosomes. These results indicated that 17 clones were somatic hybrids and 3 were not. For confirmation, nuclear rRNA gene analysis using a biotin-labeled probe was applied. Since Troyer citrange is a sexual hybrid of navel orange and trifoliate orange, almost all the rDNA fragments of navel orange were present in Troyer citrange. Among the restriction enzymes tested, EcoRV produced marker fragments for each parent: an 11.5-kbp framgent specific for navel orange and 6.8-kbp and 5.8-kbp fragments for Troyer citrange. The putative somatic hybrid plants had both specific fragments for each parent (Fig. 2). rDNA analysis demonstrated that the regenerated plants morphologically intermediate between the two parents were somatic hybrids between navel orange and Troyer citrange. However, the other regenerated plants having Troyer citrange morphology showed a rDNA pattern identical with that of Troyer citrange. This result suggested that the mesophyll protoplasts of Troyer citrange were regenerated through embryogenesis. Previously, we obtained a similar result for somatic hybridization between navel orange and grapefruit (Ohgawara et al. 1989).

Fig. 1. Leaf morphology of navel orange (*left*), somatic hybrid (*center*), and Troyer citrange (*right*)

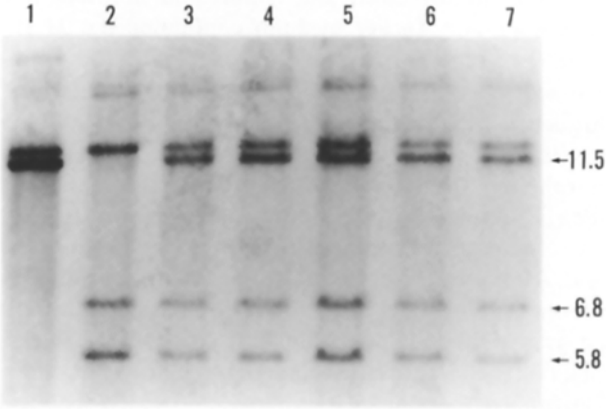

Fig. 2. Blot hybridization of biotin-labeled 25S+17S rRNA to EcoRV digests of total DNA. *1* navel orange, *2* Troyer citrange, *3–7* somatic hybrids. Electrophoresis was performed in 0.7% agarose gel at 30 V for 16 h. *Arrow* indicates unique band (11.5 kbp) present in navel orange and unique bands (6.8 kbp and 5.8 kbp) present in Troyer citrange

In 1985, five clones of the somatic hybrids were top-grafted onto 7-year-old nucellar seedlings of satsuma mandarin. After 4 years, these somatic hybrids flowered and their flower morphology was intermediate between those of the parents (Fig. 3). The pollen grains of the somatic hybrids showed high stainability and sufficient germinability, and their diameter was larger than that of Troyer citrange (Table 1). The diameter of the pollen corresponded to that of 4x Trovita orange or somatic hybrid plants between Trovita orange and trifoliate orange (S. Kobayashi et al. in press). The fruits of the somatic hybrids were large like navel orange and had a spherical

Fig. 3. Flower morphology of navel orange (*left*), somatic hybrid (*center*), and Troyer citrange (*right*)

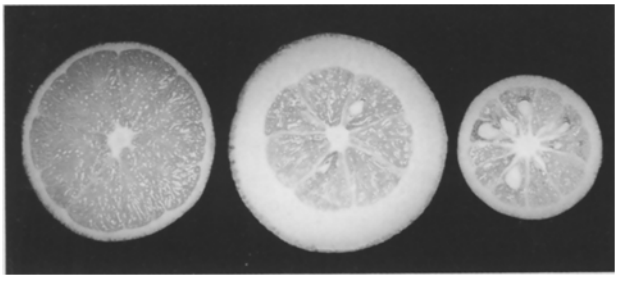

Fig. 4. Cut fruits of navel orange (*left*), somatic hybrid (*center*), and Troyer citrange (*right*)

Table 1. Comparison of pollen characteristics in somatic hybrids (SH-1, 2, 4, 9, and 15) and Troyer citrange (TC)

Plant	Pollen grain		
	Stainability (%)	Germinability (%)	Diameter (μm)
SH-1	90.5	47.9	41.3 ± 2.53
SH-2	90.1	38.8	41.6 ± 2.82
SH-4	84.8	36.9	41.9 ± 2.53
SH-9	88.4	44.7	41.3 ± 2.56
SH-15	85.3	35.2	41.7 ± 2.49
TC	86.2	65.9	33.6 ± 1.38

shape, but the rind was extremely thick like that of the typical tetraploid (Fig. 4). Most of the fruits contained seeds that had normal germinability. This indicated that the male and female sterility of navel orange was overcome by the fertility of Troyer citrange, and that at least one sterile cultivar can be used to create amphidiploid breeding materials, which produce triploids in crosses with diploids. Since Troyer citrange has inherited the cold hardiness and resistance to tristeza virus from trifoliate orange, these amphidiploid somatic hybrid plants are expected to be useful materials for *Citrus* breeding.

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