Theor Appl Genet (1989) 78: 609–612

Somatic hybridization in *Citrus:* navel orange (*C. sinensis* Osb.) and grapefruit (*C. paradisi* Macf.)

T. Ohgawara [1], S. Kobayashi [2], S. Ishii [1], K. Yoshinaga [2] and I. Oiyama [2]

[1] Research and Development Division, Kikkoman Corporation, 399 Noda, Noda City, Chiba 278, Japan
[2] Akitsu Branch, Fruit Tree Research Station, Akitsu, Hiroshima 729-24, Japan

Received March 29, 1988; Accepted June 27, 1989
Communicated by I. Potrykus

Summary. Protoplasts of navel orange, isolated from embryogenic nucellar cell suspension culture, were fused with protoplasts of grapefruit isolated from leaf tissue. The fusion products were cultured in the hormone-free medium containing 0.6 *M* sucrose. Under the culture conditions, somatic embryogenesis of navel orange protoplasts was suppressed, while cell division of grapefruit mesophyll protoplasts was not induced. Six embryoids were obtained and three lines regenerated to complete plants through embryogenesis. Two of the regenerated lines exhibited intermediate morphological characteristics of the parents in the leaf shape. Chromosome counts showed that these regenerated plants had expected 36 chromosomes (2n = 2x = 18 for each parent). The rDNA analysis using biotin-labeled rRNA probes confirmed the presence of genomes from both parents in these plants. This somatic hybridization system would be useful for the practical *Citrus* breeding.

Key words: *Citrus sinensis* Osb. – *C. paradisi* Macf. – Somatic hybridization – Embryogenesis – rDNA

Introduction

Protoplast fusion is a useful tool for overcoming the difficulty in making a conventional sexual cross. In *Citrus*, the practical application of protoplast fusion is attractive, since sexual cross between *Citrus* cultivars is limited owing to their sterility and/or polyembryony. Furthermore, parasexual hybridization of *Citrus*, which belongs to woody plants, may be valuable for shortening the breeding period because of its long juvenile phase.

Previously, we reported on the production of the intergeneric somatic hybrid plant between Trovita orange (*Citrus sinensis* Osb.) and trifoliate orange (*Poncirus trifoliata*) (Ohgawara et al. 1985). The selection system was based on the inability of mesophyll protoplasts to divide and on suppression of embryogenesis of nucellar protoplasts in the hormone-free MT medium containing 0.6 *M* sucrose. We concluded that hybrid vigor has played a significant role in the selective regeneration of amphidiploid somatic hybrid plants. Using a similar selection scheme, Grosser et al. also produced intergeneric somatic hybrid plants between the two combinations, Hamlin orange + Flying Dragon trifoliate orange (Grosser et al. 1988 a), and Hamlin orange + *Severinia disticha* (Grosser et al. 1988 b).

Recently, we reported on the application of the hybridization system to the combination between *Citrus* cultivars, F.N. Washinton navel orange + Hayashi satsuma mandarin (Kobayashi et al. 1988 a), and F.N. Washinton navel orange + Murcott Tangor (Kobayashi et al. 1988 b). The present communication describes the somatic hybridization between Bahia navel orange (*C. sinensis* Osb.) and Marsh grapefruit (*C. paradisi* Macf.). Grapefruit is considered to be derived from the sexual hybrid between *C. sinensis* and *C. grandis* (Scora et al. 1982), however, the cultivar Marsh used here has not been crossed with navel orange due to their polyembryony, male-sterility and seedlessness.

As reliable molecular markers, species-specific fragments of rDNA have been used in various somatic hybridization (Uchimiya et al. 1983; Ohgawara et al. 1985; Ozias-Akins et al. 1986; Tabaeizadeh et al. 1986; Pental et al. 1986; Robertson et al. 1987). We applied here a high resolution and simple rDNA analysis using rRNA probes labeled with photobiotin for the hybrid identification between these closely related *Citrus* cultivars.

Materials and methods

Plant materials and protoplast isolation. Nucellar callus induced from navel orange (*C. sinensis* Osb.) cultivar Bahia (Kobayashi et al. 1984) was maintained by suspension culture in Murashige and Tucker (1969) medium (MT) containing 10 mg/l 6-benzylaminopurine (BA). Prior to isolation of protoplasts, the cells were cultured in a hormone-free liquid MT, as in the case of Trovita orange described previously (Ohgawara et al. 1985). The protoplasts were isolated by the method of Vardi et al. (1982). For protoplast isolation of grapefruit (*C. paradisi* Macf.) cultivar Marsh, mesophyll tissues obtained by ovule culture were used. Ovules excised several days after anthesis were cultured aseptically on MT containing 10 mg/l BA. The embryos that emerged from the ovules were transferred to MT supplemented with 5 mg/l gibberellic acid (GA_3) and cultured under continuous light (3000 lx) at 26 °C. Fully expanded leaves of the plants were harvested and subjected to protoplast isolation.

Protoplast fusion and culture. Protoplasts of navel orange and grapefruit were fused with PEG by the method of Uchimiya (1982) with modifications (Ohgawara et al. 1985). The fusion-treated protoplasts were cultured in 2 ml of hormone-free MT containing 0.6 *M* sucrose and 0.6% Sea Plaque agarose (FMC) at a cell density of 10^5/ml in 60 × 15 mm plastic dishes. The plates were maintained under continuous light (700 lx) at 26 °C. After 1 month, 0.5 ml hormone-free liquid MT containing 0.15 *M* sucrose was added to each plate and the plates were kept under 3000 lx light intensity. The embryoids obtained were transferred to hormone-free MT supplemented with 10 m*M* galactose and 5 m*M* glutamine and cultured for a further month. Cotyledonary embryoids thus obtained were transferred and then cultured on MT with 10 mg/l GA_3.

Observation of chromosome number. Root tips of regenerated plants pretreated with 8-hydroxyquinoline (2 m*M*) for 20 h at 10 °C were fixed in ethanol:acetic acid (3:1) for 24 h, and then stained with lacto-propionil orcein for 3 h according to the method of Oiyama (1981).

Analysis of rDNA. Total DNAs were extracted from leaves of parents and regenerated plants using the CTAB micro-preparation method (Rogers and Bendich 1985). DNAs digested with restriction enzymes were separated on agarose gels and blotted onto nitrocellulose filters. rRNA was prepared from roots of trifoliate orange seedlings by the one-step extraction method (Laulhere and Rozier 1976), and purified through two cycles of sucrose gradient centrifugation (Oono and Sugiura 1980). The purified rRNA was denatured and labeled with photobiotin (Forster et al. 1985). A mixture of 2 µg 25 S + 17 S rRNA and 2 µg photobiotin (BRL) was irradiated for 20 min at 0 °C. Then denatured yeast tRNA (200 µg) was added to the solution and precipitated with ethanol. Nitrocellulose blots were wetted with 50 ng/ml biotinylated rRNA solution in 5 × SSC and 50% formamide and incubated overnight at 40 °C. They were then washed four times with 5 × SSC-50% formamide and twice with 2 × SSC. Streptavidin-alkaline phosphatase conjugate and dyes (BRL) were used for colorimetric detection.

Results and discussion

Cell suspension protoplasts of navel orange were fused with leaf mesophyll protoplasts of grapefruit, and the fusion products were plated in dishes in hormone-free MT containing 0.6 *M* sucrose as described in 'Materials and methods'. After a month, hormone-free liquid MT containing 0.15 *M* sucrose was added to the culture. After 2–3 months culture, pale green globular embryoids were obtained among many unorganized cell masses. Kobayashi et al. (1985) reported that the nearly direct somatic embryogenesis of Trovita orange protoplasts was observed only with a combination of low cell densities and low sugar concentrations. Under these conditions, protoplasts from navel orange nucellar callus can also undergo somatic embryogenesis. In the presence of high concentrations of sucrose, however, they could not develop to embryoids. On the other hand, mesophyll protoplasts of grapefruit did not undergo cell division in the hormone-free medium.

After 2–3 months of culture, six embryoids were obtained and transferred to hormone-free MT supplemented with galactose and glutamine, which was effective for greening of the embryoids. Three of the embryoid clones developed to green cotyledonary embryoids. Within 6 months of culture, two to five plants were regenerated from each cotyledonary embryoid clone. Each regenerated plant was transplanted to soil in pots and grown in a growth chamber under 3000 lx light intensity at 26 °C (Fig. 1).

All of the plants were morphologically normal and no intraclonal variation was observed. Leaf morphology of

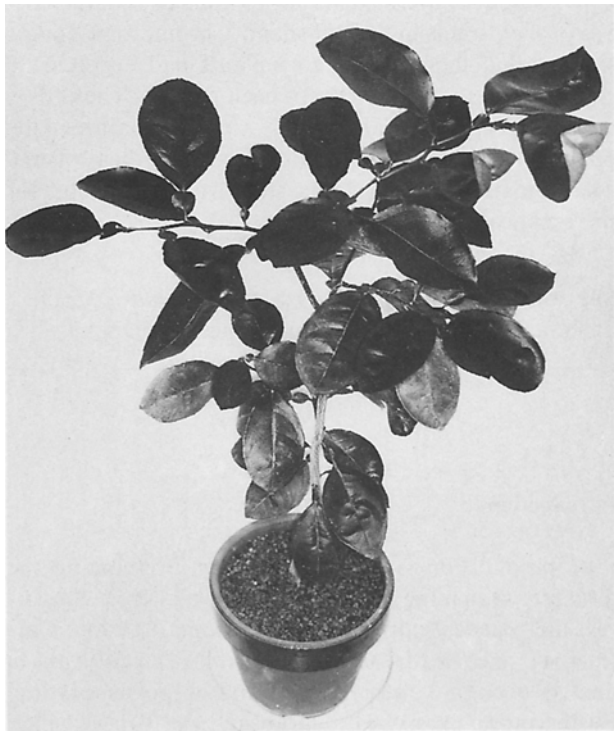

Fig. 1. A somatic hybrid plant between navel orange and grapefruit

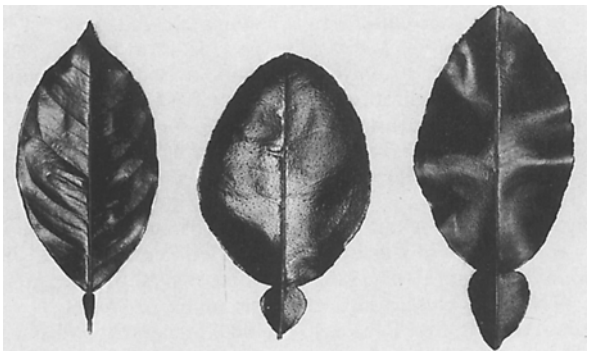

Fig. 2. Leaf morphology of navel orange (*left*), grapefruit (*right*) and a somatic hybrid (*center*)

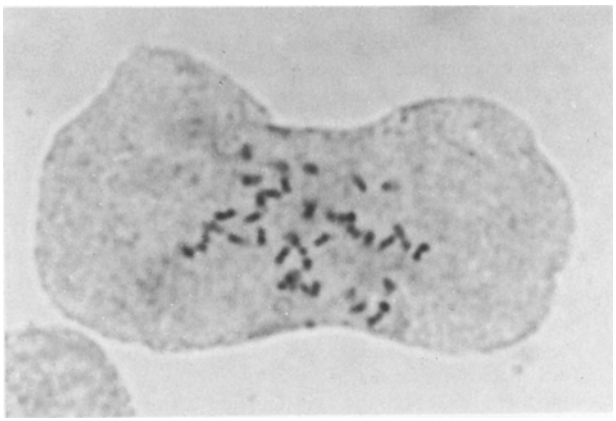

Fig. 3. Chromosomes of a somatic hybrid plant (2n = 4x = 36)

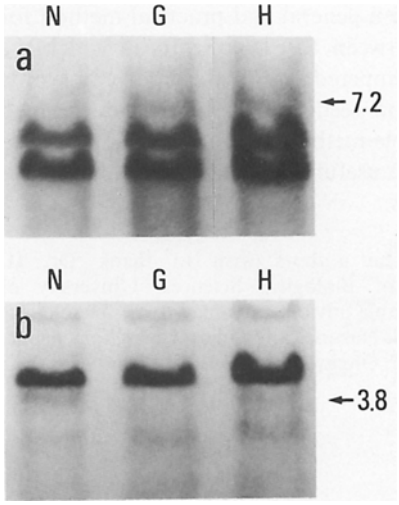

Fig. 4 a and b. Photographs of blot hybridization of biotin-labeled 25s + 17s rRNA to restriction endonuclease digests of total DNA. N: navel orange; G: grapefruit; H: somatic hybrid. **a** EcoRI-digested total DNA (2.5 μg) was loaded in each well. Electrophoresis was performed in 0.8% agarose gel at 30 V for 15 h. *Arrow* indicates unique band (7.2 kbp) present in grapefruit and somatic hybrid. **b** SacI-digested total DNA (2.5 μg) was loaded. Electrophoresis was performed in 1.2% agarose gel at 30 V for 15 h. *Arrow* indicates unique band (3.8 kbp) present in navel orange and somatic hybrid

the regenerated plants was compared with their parents. As shown in Fig. 2, navel orange has small petiole wings, while grapefruit has large petiole wings. Two out of three regenerated lines exhibited intermediate characteristics of both parents in the size and shape of petiole wings (Fig. 2). Another line closely resembled grapefruit. Chromosome counts revealed that the regenerated plants exhibiting intermediate morphology had the expected amphidiploid chromosome number of 36 (2n = 2x = 18 for each parent) (Fig. 3), while another plant similar to grapefruit had 18 chromsomes. From these results, two out of three regenerated lines were assumed to be somatic hybrid plants between navel orange and grapefruit.

To confirm the hybridity of the regenerated plants, rDNA analysis was carried out. Total DNAs extracted from the parents and the regenerated plants were digested with various endonucleases and hybridized with biotin-labeled rRNA probes.

The non-isotopic detection system gave higher resolution than the autoradiographic detection system (Ohgawara 1989). The cleavage patterns of rDNA from navel orange and grapefruit with the restriction endonucleases were very similar. This may be explained by the fact that grapefruit is closely related taxonomically to navel orange (Scora et al. 1982). No restriction enzyme was obtained that produced the marker fragments simultaneously for both parents. Among the restriction enzymes tested, EcoRI yielded a 7.2-kbp fragment specific for grapefruit, and SacI yielded a 3.8-kbp fragment specific for navel orange. The putative amphidiploid somatic hybrid plants had both EcoRI 7.2-kbp and SacI 3.8-kbp fragments (Fig. 4 a and b). These results indicate that two regenerated lines contain the genomes of both parents.

However, the regenerated plant of another line similar to grapefruit had the same rDNA patterns as those of grapefruit (data not shwon). In another fusion experiment, we also had some grapefruit-type regenerated plants among the somatic hybrids. The leaf mesophyll protoplasts did not divide in the medium used here, even if cultured with navel orange protoplasts. These results lead us to speculate that the mesophyll protoplasts of grapefruit may be induced for division and embryogenesis by the hybrid cell. The experiments to determine the reason for the regeneration of the grapefruit-type plants are in progress.

In conclusion, we were able to produce the amphidiploid somatic hybrid plants between navel orange and grapefruit. Thus, this parasexual hybridization sys-

tem will prove to be a general and practical method for creating hybrids between *Citrus* cultivars in which the sexual cross was hampered due to their polyembryony, male-sterility and low seediness. Furthermore, the high resolution and simple method of molecular analysis described here must be useful for the identification in various somatic hybrids.

Acknowledgements. The authors wish to thank Dr. H. Uchimiya, Institute of Biological Sciences, University of Tsukuba, for his valuable advice and suggestions. Thanks are also extended to Dr. S. Nasuno, Kikkoman Corp., for encouragement and to Mrs. E. Ohgawara and Mr. T. Kimura for their assistance.

References

Forster AC, McInnes JL, Skingle DC, Symons RH (1985) Non-radioactive hybridization probes prepared by the chemical labelling of DNA and RNA with a novel reagent, photobiotin. Nucleic Acids Res 13:745–761

Grosser JW, Gmitter FG, Chandler JL (1988a) Intergeneric somatic hybrid plants of *Citrus sinensis* cv Hamlin and *Poncirus trifoliata* cv Flying Dragon. Plant Cell Rep 7:5–8

Grosser JW, Gmitter FG, Chandler JL (1988b) Intergeneric somatic hybrid plants from sexually incompatible woody species: *Citrus sinensis* and *Severinia disticha*. Theor Appl Genet 75:397–401

Kobayashi S, Ikeda I, Nakatani M (1984) Induction of nucellar callus from orange (*Citrus sinensis* Osb.) ovules, and uniformity of regenerated plants. Bull Fruit Tree Res Stn E 5:43–54

Kobayashi S, Ikeda I, Uchimiya H (1985) Conditions for high frequency embryogenesis from orange (*Citrus sinensis* Osb.) protoplasts. Plant Cell Tiss Org Cult 4:249–259

Kobayashi S, Ohgawara T, Ohgawara E, Oiyama I, Ishii S (1988a) A somatic hybrid plant obtained by protoplast fusion between navel orange (*Citrus sinensis*) and satsuma mandarin (*C. unshiu*). Plant Cell Tiss Org Cult 14:63–69

Kobayashi S, Fujiwara K, Oiyama I, Ohgawara T, Ishii S (1988b) Somatic hybridization between Navel Orange and 'Murcott' Tangor In: Goren R, Mendel K (eds) Proc 6th Int Citrus Congr, Tel Aviv, pp 23–28

Laulhere JP, Rozier C (1976) One-step extraction of plant nucleic acids. Plant Sci Lett 6:237–242

Murashige T, Tucker DPH (1969) Growth factor requirements of *Citrus* tissue culture. In: Chapman HD (ed) Proc 1st Int *Citrus* Symp, vol 3. Riverside, pp 1155–1161

Ohgawara T (1989) Analysis of somatic hybrids using non-radioactive hybridization probes. Plant Tiss Cult Lett 6:35–37

Ohgawara T, Kobayashi S, Ohgawara E, Uchimiya H, Ishii S (1985) Somatic hybrid plants obtained by protoplast fusion between *Citrus sinensis* and *Poncirus trifoliata*. Theor Appl Genet 71:1–4

Oiyama I (1981) A technique for chromosome observation in root tip cells of *Citrus*. Bull Fruit Tree Res Stn D 3:1–7

Oono K, Sugiura M (1980) Heterogeneity of the ribosomal RNA gene clusters in rice. Chromosoma 76:85–89

Ozias-Akins P, Ferl RJ, Vasil IK (1986) Somatic hybridization in the gramineae: *Pennisetum americanum* (L.) K. Schum. (Pearl millet) + *Panicum maximum* Jacq. (Guinea grass). Mol Gen Genet 203:365–370

Pental D, Hamill JD, Pirrie A, Cocking EC (1986) Somatic hybridization of *Nicotiana tabacum* and *Petunia hybrida*. Mol Gen Genet 202:342–347

Robertson D, Palmer JD, Earle ED, Mutschler MA (1987) Analysis of organelle genomes in a somatic hybrid derived from cytoplasmic male-sterile *Brassica oleracea* and atrazine-resistant *B. campestris*. Theor Appl Genet 74:303–309

Rogers SO, Bendich AJ (1985) Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. Plant Mol Biol 5:69–76

Scora RW, Kumamoto J, Soost RK, Nauer EM (1982) Contribution to the origin of the grapefruit, *Citrus paradisi* (rutaceae). Syst Bot 7:170–177

Tabaeizadeh Z, Ferl RJ, Vasil IK (1986) Somatic hybridization in the gramineae: *Saccharum officinarum* L. (sugarcane) and *Pennisetum americanum* (L.) K. Schum. (pearl millet). Proc Natl Acad Sci USA 83:5616–5619

Uchimiya H (1982) Somatic hybridization between male sterile *Nicotiana tabacum* and *N. glutinosa* through protoplast fusion. Theor Appl Genet 61:69–72

Uchimiya H, Ohgawara T, Kato H, Akiyama T, Harada H, Sugiura M (1983) Detection of two different nuclear genomes in parasexual hybrids by ribosomal RNA gene analysis. Theor Appl Genet 64:117–118

Vardi A, Spiegel-Roy P, Galun E (1982) Plant regeneration from *Citrus* protoplasts: variability in methodological requirements among cultivars and species. Theor Appl Genet 62:171–176