Production of Somatic Hybrid and Autotetraploid Breeding Parents for Seedless Citrus Development

Jude W. Grosser, Frederick G. Gmitter, Jr., E.S. Louzada, and J.L. Chandler

Citrus Research and Education Center, University of Florida, Institute of Food and Agricultural Sciences, Department of Fruit Crops, 700 Experiment Station Road, Lake Alfred, FL 33850

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Abstract. Allotetraploid somatic hybrid plants of 'Nova' tangelo [a sexual hybrid of 'Clementine' mandarin (*C. reticulata* Blanco) × 'Orlando' tangelo (*C. reticulata* × *C. paradisi* Macf.)] + 'Succari' sweet orange (*C. sinensis* L. Osbeck), and 'Hamlin' sweet orange (*C. sinensis* L. Osbeck) + 'Dancy' tangerine (*C. reticulata*) were regenerated following protoplast fusion. 'Nova' and 'Hamlin' protoplasts were isolated from ovule-derived embryogenic callus and suspension cultures, respectively, and fused using a polyethylene glycol method with seedling leaf-derived protoplasts of 'Succari' and 'Dancy', respectively. Plants were regenerated via somatic embryogenesis, and somatic hybrids were identified on the basis of leaf morphology, root-tip cell chromosome number, and electrophoretic analysis of peroxidase and phosphoglucose mutase isozyme banding patterns. Diploid plants were regenerated from unfused protoplasts of 'Hamlin', 'Nova', and 'Succari'. Tetraploid plants of 'Hamlin' and 'Succari' were also recovered, apparently resulting from homokaryotic fusions. No 'Dancy' plants were recovered. The somatic hybrid and autotetraploid plants can be used for interploid hybridization with selected monoembryonic scions to generate improved seedless triploid tangor/tangelo cultivars. The lack of suitable tetraploid breeding parents has previously inhibited the development of quality seedless cultivars by this method.

Mandarin hybrids, e.g., tangors (sweet orange × mandarin hybrids) and tangelos (grapefruit × mandarin hybrids) are among the most commercially important fresh citrus fruit cultivars (Saunt, 1990). However, many such cultivars produce fruit with undesirably many seeds, making the development of improved seedless cultivars an important breeding objective (Grosser and Gmitter, 1990). One strategy to achieve this objective is to generate and select seedless triploids from interploid crosses (Soost and Cameron, 1969). Triploid citrus cultivars released from interploid crosses include 'Oroblanco' and 'Melogold' pummelo × grapefruit hybrids (Soost and Cameron, 1980, 1985). A major obstacle to full exploitation of this strategy has been the lack of suitable tetraploid breeding parents. Recently, tissue culture methods have been used to facilitate the development of autotetraploids (Gmitter and Ling, 1991; Gmitter et al., 1991) and allotetraploid somatic hybrids for mandarin hybrid cultivar improvement, including 'Washington' navel orange (*C. sinensis*) + 'Hayashi' satsuma mandarin (*C. unshiu*) (Kobayashi et al., 1988), 'Washington' navel orange + 'Murcott' tangor (Kobayashi

and Ohgawara, 1988), 'Trovita' sweet orange (*C. sinensis*) + 'Hayashi' satsuma mandarin (Kobayashi and Ohgawara, 1988), 'Bahia' navel orange (*C. sinensis*) + 'Marsh' grapefruit (*C. paradisi*) (Ohgawara et al., 1989), and 'Thompson Pink' grapefruit (*C. paradisi*) + 'Murcott' tangor (Grosser et al., 1992). Interspecific *Citrus* somatic hybrids have also been produced for use in lemon cultivar improvement (Tusa et al., 1990, 1992) and for rootstock improvement (Grosser et al., 1992; Louzada et al., 1992).

The objective of the research reported herein was to generate additional tetraploid somatic hybrid breeding parents for use in mandarin hybrid improvement by use of protoplast fusion to combine the genomes of 'Nova' tangelo with 'Succari' sweet orange

and 'Hamlin' sweet orange with 'Dancy' tangerine. 'Nova' is a high quality, very early ripening tangelo with good color; it was produced from a cross of 'Clementine' mandarin × 'Orlando' tangelo and released in 1964 (Hodgson, 1967). Its main drawback is fruit with high seed content. 'Succari' sweet orange is a vigorous, early ripening, sweet orange with good color and is virtually acidless at maturity with a sugar : acid ratio of 90:100:1. 'Succari' was selected as a parent for somatic hybridization because excessive acidity in progeny from crosses involving mandarins has been a breeding problem (Soost and Cameron, 1969). Unacceptably high levels of acid in hybrid citrus fruit frequently result from crosses of parents with medium acid levels (Soost and Cameron, 1975). Depending on the specific low-acid parent used, it is possible to increase percentages of hybrid progeny with low- to medium-acid fruit from crosses of low-acid × medium-acid parents. Interploid crosses using a tetraploid lemon as a seed parent usually generated seedless triploid progeny with high acid levels that were strongly influenced in the direction of the tetraploid parent (Soost and Cameron, 1969). 'Succari' sweet orange has been shown to transmit low acidity to its progeny (Barrett, 1990). The use of tetraploid breeding parents containing 'Succari' sweet orange parentage in interploid crosses should increase the percentage of triploid zygotic progeny that produce fruit with acceptable acid levels. 'Hamlin' is the major early season sweet orange grown in Florida. It is consistently productive with nearly seedless fruit that can be harvested before the danger of freezing weather in Florida; its main drawback is poor juice color at maturity. 'Dancy' is a vigorous tangerine with medium-sized, easy-to-peel fruit with good color that ripen early midseason. Use of a 'Hamlin' sweet orange + 'Dancy' tangerine somatic hybrid in interploid crosses should contribute to the development of improved early maturing seedless cultivars.

'Nova' tangelo protoplasts were isolated from an ovule-derived embryogenic callus culture initiated and maintained on MT (Murashige and Tucker, 1969) basal medium containing 500 mg malt extract, 50 g sucrose, and 8.0 g agar/liter. 'Hamlin' sweet

Fig. 1. Starch gel stained for PGM activity. (Left to right) 'Nova' tangelo (lanes 1 and 2), 'Nova' + 'Succari' somatic hybrid (lanes 3 and 4), 'Hamlin' sweet orange (lanes 5 and 6), 'Hamlin' + 'Dancy' somatic hybrid (lanes 7 and 8), 'Dancy' tangerine (lanes 9 and 10). Origin at 0 cm (not shown).

Fig. 2. Starch gel stained for PER activity. (Left to right) 'Hamlin' sweet orange (lanes 1 and 2), 'Hamlin' + 'Dancy' somatic hybrid (lanes 3 and 4), and 'Dancy' tangerine (lanes 5 and 6). Origin at 0 cm (not shown).

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Fig. 3. Leaf morphology (left to right) of: (a) 'Nova' tangelo, (b) 'Nova' + 'Succari' sweet orange somatic hybrid, (c) 'Succari', (d) tetraploid 'Succari', (e) tetraploid 'Hamlin' sweet orange, (f) aberrant diploid 'Hamlin', (g) 'Hamlin', (h) 'Hamlin' + 'Dancy' tangerine somatic hybrid, and (i) 'Dancy'

orange protoplasts were isolated from an ovule-derived embryogenic suspension culture maintained on H + H liquid medium with a 2-week subculture cycle, according to the methods of Grosser and Gmitter (1990). Leaf-derived protoplasts of 'Succari' sweet orange and 'Dancy' tangerine were isolated from nucellar seedlings as described by Grosser and Gmitter (1990). Protoplasts from each source were purified and fused using a modified polyethylene glycol (PEG) method (Grosser and Gmitter, 1990). Protoplasts of 'Nova' + 'Succari' were initially cultured in a 1:1 (v/v) mixture of BH3 and EMEP protoplast culture media, and 'Hamlin' + 'Dancy' protoplasts were cultured in either this same mixed medium or in EMEP protoplast culture medium alone (Grosser and Gmitter, 1990). Protoplast culture and plant regeneration were carried out as described by Grosser and Gmitter (1990).

Chromosome numbers of root-tip cells of regenerated plants were determined according to a modified hematoxylin staining technique (Grosser and Gmitter, 1990). Isozyme banding patterns of 'Nova' tangelo, 'Succari' sweet orange, 'Hamlin' sweet orange, 'Dancy' tangerine, and regenerated plants were analyzed using crude leaf tissue extracts sampled as in Torres et al. (1978). Peroxidase (PER) and phosphoglucose mutase (PGM) isozymes were separated using horizontal starch gel electrophoresis on 10% starch gels and the pH 5.7 histidine-citrate buffer of Cardy et al. (1981). Electrophoresis was carried out for 3 h at 4°C and constant 60 mA current, and staining recipes were from Vallejos (1983).

A total of 99 and 222 plants were recovered from fusion cultures of 'Nova' + 'Succari' and 'Hamlin' + 'Dancy', respectively. Three plants of each group exhibited typical tetraploid morphology, and all six were later confirmed to be tetraploid ($2n = 4x = 36$) by chromosome counts. Of these, one 'Hamlin' + 'Dancy' and two 'Nova' + 'Succari' somatic hybrid plants were identified on the basis of leaf isozyme analyses (Figs. 1 and 2). The 'Hamlin' + 'Dancy'

hybrid genotype for PGM (a monomeric enzyme) was FFFS, and the banding pattern produced was indistinguishable from that of 'Hamlin' (FS). The 'Dancy' genotype for PGM was FF. At PER, 'Hamlin' was determined to be FF, and 'Dancy' was FS. The banding pattern of the putative hybrid was identical to 'Dancy', and it was concluded that the hybrid PER genotype was FFFS. Therefore, on the basis of the expression of alleles from both donors, we concluded that this tetraploid was a somatic hybrid. The verification of hybridity of the 'Nova' + 'Succari' plants was straightforward. The donor genotypes for PGM were FI ('Nova') and FS ('Succari'); the hybrids produced three bands, indicating complementation of donor alleles and the FFIS genotype. One tetraploid 'Succari' and two tetraploid 'Hamlin' plants were also verified by isozyme analyses. These plants probably arose from uniparental fusions (Tusa et al., 1990). This is the first report of somatic hybrid plants of these parentages and of an autotetraploid 'Succari' sweet orange plant. We have previously obtained autotetraploid 'Hamlin' sweet orange plants following in vitro colchicine treatment of embryogenic callus (Gmitter et al., 1991) and from organogenic cultures (unpublished data). The remaining diploid plants from the 'Nova' + 'Succari' fusion cultures were identified to be either 'Nova' regenerated from unfused callus-derived protoplasts or 'Succari' regenerated from unfused leaf protoplasts. This is the 10th *Citrus* cultivar regenerated directly from leaf protoplasts, and, like the previous examples, it required coculture with embryogenic callus or suspension culture-derived protoplasts (Grosser et al., 1992; Tusa et al., 1990). All of the diploid plants recovered from the 'Hamlin' + 'Dancy' fusion cultures produced typical 'Hamlin' isozyme banding patterns, including five plants that exhibited uniform aberrant leaf morphology (Fig. 3). No 'Dancy' plants were recovered. All diploid and the tetraploid 'Hamlin' plants regenerated from cultures were initially plated on BH3/EMEP protoplast culture medium,

but the somatic hybrid and the aberrant 'Hamlin' plants were regenerated from cultures plated on EMEP protoplast culture medium. EMEP medium contains 0.6 M sucrose, a concentration that has been shown previously to inhibit sweet orange somatic embryogenesis (Grosser et al., 1988; Ohgawara et al., 1985). Use of this sucrose concentration in protoplast culture media facilitates somatic hybrid selection, particularly in fusions of sweet orange with a second parent that has poor regeneration capacity. The cause of the stable aberrant morphology in the five 'Hamlin' plants regenerated on EMEP may also have been responsible for their recovery from cultures grown in high levels of sucrose. Although these plants exhibited the normal 'Hamlin' isozyme profiles and chromosome number, more sophisticated analyses may show a genetic difference. Leaf morphology of all parents and plants regenerated from fusion cultures are shown in Fig. 3.

The percentage of regenerated plants confirmed to be somatic hybrids reported herein were among the lowest of any parental combinations attempted to date in our program. Recovery of the 'Hamlin' + 'Dancy' somatic hybrid was particularly difficult (<0.5% of regenerated plants were hybrid). Possible explanations for this phenomenon include the apparent lack of regeneration capacity contributed by 'Dancy' tangerine, and the fact that the parental types are more closely related than previously reported combinations, possibly resulting in less in vitro heterosis. Reciprocal fusions combining nucellar callus-derived protoplasts of 'Dancy' tangerine with seedling leaf-derived protoplasts of 'Hamlin' sweet orange were also performed, but no plants were recovered.

All recovered tetraploid plants have been grafted onto hybrid rootstocks and planted in the field to expedite their use as breeding parents in interploidy crosses. The first three tetraploid somatic hybrids that have surpassed juvenility to flower are adequately fertile to be used as pollen parents, namely: 'Trovita' sweet orange + *Poncirus trifoliata* (Kobayashi et al., 1991); 'Navel' orange + 'Troyer' citrange (Ohgawara et al., 1991); and 'Key' lime + 'Valencia' sweet orange (X.X. Deng, F.G.G., and J.W.G., unpublished data). The 'Trovita' sweet orange + *Poncirus trifoliata* somatic hybrid has already been used as a pollen parent with monoembryonic 'Clementine' mandarin as a seed parent to generate 85 triploid hybrid plants (Oiyama et al., 1991). We anticipate adequate fertility in the 'Nova' + 'Succari' and the 'Hamlin' + 'Dancy' somatic hybrids, and the autopolyploid sweet oranges. These, along with other tetraploid breeding lines, can be used as pollen parents in crosses with complementary seedling diploid monoembryonic selections to generate large populations of triploid zygotic progeny for selection. Although disturbed embryo : endosperm ploidy ratios associated with crosses in this direction (i.e., $2x \times 4x$) can interfere with embryo development, embryo rescue or ovule culture procedures can be used to recover triploid hybrid seedlings

from such crosses (Starrantino and Recupero, 1981).

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