

# Six New Somatic Citrus Hybrids and Their Potential for Cultivar Improvement

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**Abstract.** Protoplasm culture following polyethylene glycol (PEG) -induced fusion resulted in the regeneration of somatic hybrid plants from the following six parental combinations: *Citrus sinensis* (L.) Osbeck cv. Hamlin + *Severinia buxifolia* (Poir.) Tenore (Chinese box-orange); *C. reticulata* Blanco cv. Cleopatra + *Poncirus trifoliata* (L.) Raf. cv. Flying Dragon; *C. reticulata* cv. Cleopatra + Swingle citrumelo (*C. paradisi* Macf. × *P. trifoliata*); *C. sinensis* cv. Hamlin + *C. jambhiri* cv. Rough lemon; *C. sinensis* cv. Valencia + *C. jambhiri* cv. Rough lemon; and *C. paradisi* cv. Thompson + 'Murcott' tanger (purported hybrid of *C. reticulata* × *C. sinensis*). Diploid plants were regenerated from nonfused embryogenic culture-derived protoplasts of 'Cleopatra' mandarin and 'Hamlin' and 'Valencia' sweet orange, and from nonfused leaf-derived protoplasts of Rough lemon and 'Murcott'. Regenerated plants were classified according to leaf morphology, chromosome number, and isozyme analyses. All of the somatic hybrids reported herein are tetraploid ( $2n = 4x = 36$ ), with the exception of the 'Hamlin' + *S. buxifolia* hybrid, which was unexpectedly found to have a chromosome number of  $2n = 27$ . These six new somatic hybrids have potential in citrus scion and rootstock improvement for commercial use.

Techniques for producing somatic hybrids of *Citrus* by protoplasm fusion are well developed, and strategies for applying the techniques to citrus scion and rootstock improvement have been outlined (Grosser and Gmitter, 1990a, 1990b, 1990c). Numerous citrus somatic hybrids have been reported, including interspecific combinations (Grosser et al., 1989; Kobayashi et al., 1988a, 1988b; Ohgawara et al., 1989; Tusa et al., 1990), intergeneric combinations between sexually compatible species (Deng et al., 1991; Grosser and Gmitter 1990a, 1990b, 1990c; Grosser et al., 1988a; Ohgawara et al., 1985), and intergeneric combinations between sexually incompatible species (Grosser and Gmitter, 1990a, 1990b, 1990c; Grosser et al., 1988b, 1990). Citrus somatic cybrids have also been reported (Vardi et al., 1987, 1989).

One strategy for citrus rootstock improvement is the production of hybrids that combine *Citrus* species with graft-compatible, but sexually incompatible, related genera that possess traits of interest (Grosser and Gmitter, 1990a, 1990b, 1990c). Citrus relatives represent a largely untapped reservoir of genetic diversity, but, in general, are horticulturally unacceptable for direct use as citrus rootstock (Swingle and Reece, 1967). Somatic hybridization with *Citrus* could minimize or eliminate problems of inadequate horticultural performance. Previously, we have reported on the production of somatic hybrid plants from three such parental combinations: *Citrus sinensis* cv. Hamlin + *Severinia disticha* (Blanco) Swing. (Grosser et al., 1988b); *Citrus sinensis* cv. Hamlin + *Citropsis gilletiana* Swingle & M. Kel. (Grosser and Gmitter, 1990c); and *Citrus reticulata* + *Citropsis gilletiana* (Grosser et al., 1990). The attempted somatic hybridization of *Citrus sinensis* cv. Hamlin + *S. buxifolia* is another example of efforts to apply this strategy. *Severinia buxifolia* possesses several traits desirable for rootstock improvement, including cold hardiness, salt and B tolerance (Cooper, 1961), and resistance to *Phytophthora* (Hutchison and Grimm, 1973;

Graham, 1990) and the citrus (*Tylenchulus semipenetrans* Cobb.) and burrowing (*Radopholus citrophilus* Huettel, Dickson & Kaplan) nematodes (Hutchison and O'Bannon, 1972).

A second strategy for citrus rootstock improvement is the production of somatic hybrids from existing complementary germplasm (Grosser and Gmitter, 1990a, 1990b). The attempted somatic hybridizations of Cleopatra mandarin + Swingle citrumelo, Cleopatra mandarin + Flying Dragon trifoliolate orange, 'Valencia' sweet orange + Rough lemon, and 'Hamlin' sweet orange + Rough lemon represent efforts in this direction. Cleopatra mandarin is an important rootstock in Florida and elsewhere because it is cold-hardy, resistant to citrus tristeza virus, and moderately tolerant of citrus blight (Castle, 1987). Trees grafted on Cleopatra are moderately vigorous but are susceptible to foot and root rot (caused by *Phytophthora parasitica* Dast.) (Graham, 1990) and are not precocious. Size of fruit from Cleopatra trees is often inadequate for the fresh-fruit market. The Cleopatra + Swingle citrumelo and Cleopatra + Flying Dragon somatic hybrids (Table 1) have the potential to compensate for some of the deficiencies of Cleopatra via complementation. Both Swingle and Flying Dragon are resistant to *P. parasitica*. Flying Dragon can positively influence scion precocity and cold hardiness and has potential for reducing tree size (Castle, 1987). Reduced tree size is of interest for high-density plantings to increase productivity and the effectiveness of cold protection methods and reduce harvesting costs. The Swingle parentage may have a positive influence on fruit size.

Somatic hybridization of 'Valencia' + Rough lemon and 'Hamlin' + Rough lemon was attempted to generate vigorous rootstock with improved cold hardiness and tolerance to citrus blight from *Citrus sinensis* (sweet orange). Citrus blight is considered the most significant pathological problem of citrus in Florida and Brazil (Derrick et al., 1990). Vigorous rootstock, such as Rough lemon (*Citrus jambhiri* Lush), Volkamer lemon (*C. volkameriana* Ten. & Pasq.), Rangpur lime (*C. limonia* Osbeck), and Palestine sweet lime (*C. limettioides* Tan.), enjoyed great popularity in Florida in the past, but their suscep-

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Abbreviations: PEG, polyethylene glycol; PER, peroxidase; PGI, phosphoglucose isomerase; PGM, phosphoglucomutase.

Table 1. *Citrus* plants regenerated following protoplasm fusion.

Embryogenic parent	Leaf parent	Somatic hybrid plants <sup>z</sup>	Plants from embryogenic parent	Plants from leaf parent
<i>C. sinensis</i> 'Hamlin' <sup>y</sup>	<i>S. buxifolia</i> <sup>x</sup>	56	0	0
<i>C. reticulata</i> Cleopatra <sup>w</sup>	<i>P. trifoliata</i> Flying Dragon	164	3	0
<i>C. reticulata</i> Cleopatra <sup>w</sup>	Swingle citrumelo	204	2	0
<i>C. sinensis</i> 'Valencia' <sup>y</sup>	<i>C. jambhiri</i> Rough lemon	11	1	3
<i>C. sinensis</i> 'Hamlin' <sup>y</sup>	<i>C. jambhiri</i> Rough lemon	12	19	10
<i>C. paradisi</i> 'Thompson' <sup>w</sup>	'Murcott' tangor	19	0	28

<sup>z</sup>The total number of plants recovered, not necessarily the number of plants regenerated from individual fusion events.

<sup>y</sup>Embryogenic suspension culture.

<sup>w</sup>Nonembryogenic callus culture inste:

<sup>x</sup>Embryogenic callus culture.

tibility to 'cold and blight has reduced their use considerably (Castle, 1987).

One strategy for the application of somatic hybridization to citrus scion improvement involves the development of allotetraploid breeding parents via somatic hybridization for use in diploid × tetraploid hybridizations to generate seedless triploid hybrids. Soost and Cameron (1969) have previously demonstrated that triploid hybrids are seedless yet capable of being fruitful. Tangors (sweet orange × mandarin hybrids) and tangelos (grapefruit × mandarin hybrids) are important fresh fruit citrus varieties grown and consumed internationally (Saunt, 1990). However, most cultivars within these groups are seedy, and the development of improved seedless cultivars is an important breeding objective. The attempted somatic 'hybridization of *Citrus paradisi* 'Thompson' + 'Murcott' tangor represents our initial efforts to develop quality tetraploid breeding parents.

### Materials and Methods

**Protoplasm isolation.** Protoplasts of Flying Dragon, Swingle citrumelo, Rough lemon, and 'Murcott' were isolated from leaves of young nucellar seedlings maintained in a growth chamber (16-h photoperiod, 300  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  light intensity, 26 to 30°C) according to the procedure of Grosser and Chandler (1987). Protoplasts of *S. buxifolia* were isolated from a seedling-derived, nonembryogenic friable callus culture initiated and maintained on Murashige and Tucker (1969) basal medium containing (on a per liter basis) 0.55 mg 2,4-dichlorophenoxyacetic acid (2,4-D), 0.22 mg kinetin, 20 ml coconut H<sub>2</sub>O (Gibco Labs, Grand Island, N.Y.), and 50 g sucrose (designated MTC), according to Grosser and Gmitter (1990a). Protoplasts of *Citrus sinensis* cv. Hamlin, *C. sinensis* cv. Valencia, *Citrus reticulata* cv. Cleopatra, and *Citrus paradisi* cv. Thompson were isolated from nucellar-derived habituated embryogenic callus cultures maintained on either liquid or semisolid EME or H + H medium, as described by Grosser and Gmitter (1990a). In most cases, protoplasts were further purified by centrifugation on a 25% sucrose-13% mannitol gradient (Tusa et al., 1990; Grosser and Gmitter, 1990a).

**Protoplasm fusion and culture.** We generally produced the somatic hybrids by chemically inducing fusion of totipotent *Citrus* protoplasts, isolated from embryogenic cultures of one parent, with protoplasts isolated from seedling leaves or nonembryogenic callus cultures of the second parent (see Table 1). About equal volumes of purified protoplasts from each parental combination were mixed and fused using the modified polyethylene (PEG) method of Menczel et al. (1981) as described by Grosser and Gmitter (1990a). The 'Hamlin' + *S. buxifolia* and 'Thompson' + 'Murcott' somatic hybrids were

generated using 8000 M.W. PEG (Sigma, St. Louis), but the other hybrids were generated using 1500 M.W. PEG (Aldrich, Milwaukee). The latter has been shown to provide excellent results in somatic fusion experiments (Chand et al., 1988).

After fusion, protoplasts were cultured directly in fusion dishes (60 × 15 mm; Falcon, Lincoln Park, N.J.) in eight to 20 drops of either BH3 protoplasm culture medium, EME protoplasm culture medium, or a 1 BH3 : 1 EME medium (v/v), as described by Grosser and Gmitter (1990a). Osmoticum reduction, embryo enlargement, embryo germination, in vitro rooting, and plantlet acclimation were carried out as described by Grosser and Gmitter (1990a).

**Cytology.** Chromosome numbers of mitotically active root and leaf meristem cells from regenerated plants were determined using modifications of the hematoxylin staining protocol of Sass (1958) as described by Tusa et al. (1990) for roots and Gmitter et al. (1990) for leaves. A minimum of three cells per plant for three somatic hybrid plants from each parental combination were examined.

**Electrophoresis of leaf isozymes.** Leaf isozymes were separated in mixed starch (10%) and agarose (0.15%) gels with the pH 5.7 histidine-citrate buffer of Cardy et al. (1981). A constant 60 mA current was run through the gels for 3 h at 4°C. Gels were sliced and stained for the activity of peroxidase (PER, E. C.1.11.1.7), phosphoglucumutase (PGM, E. C.2.7.5.1), and phosphoglucose isomerase (PGI, E. C.5.3.1.9). All isozyme analyses were repeated at least three times to ensure accurate interpretation.

### Results and Discussion

The pedigrees of plants recovered after protoplasm fusion and culture are summarized in Table 1. Somatic hybrid plant recovery was generally of equal or greater efficiency than plant recovery from unfused parental protoplasts. In all cases, somatic hybrid leaf morphology was intermediate to that of the parents (Fig. 1). No morphological variability was observed in any of the somatic hybrid populations. Plant recovery was more efficient from nonfused 'Hamlin' protoplasts isolated from embryogenic callus cultures than from embryogenic suspension cultures. Only a few plants were regenerated from nonfused Cleopatra protoplasts isolated from an embryogenic callus culture. Somatic hybrid embryo proliferation was evident during culture for both the Cleopatra + Flying Dragon and Cleopatra + Swingle somatic hybrids, contributing to the large number of plants regenerated.

An unexpected result was the recovery of Rough lemon and 'Murcott' plants from nonfused leaf protoplasts. Recently, plants were regenerated from nonfused leaf protoplasts of 'Femmi-

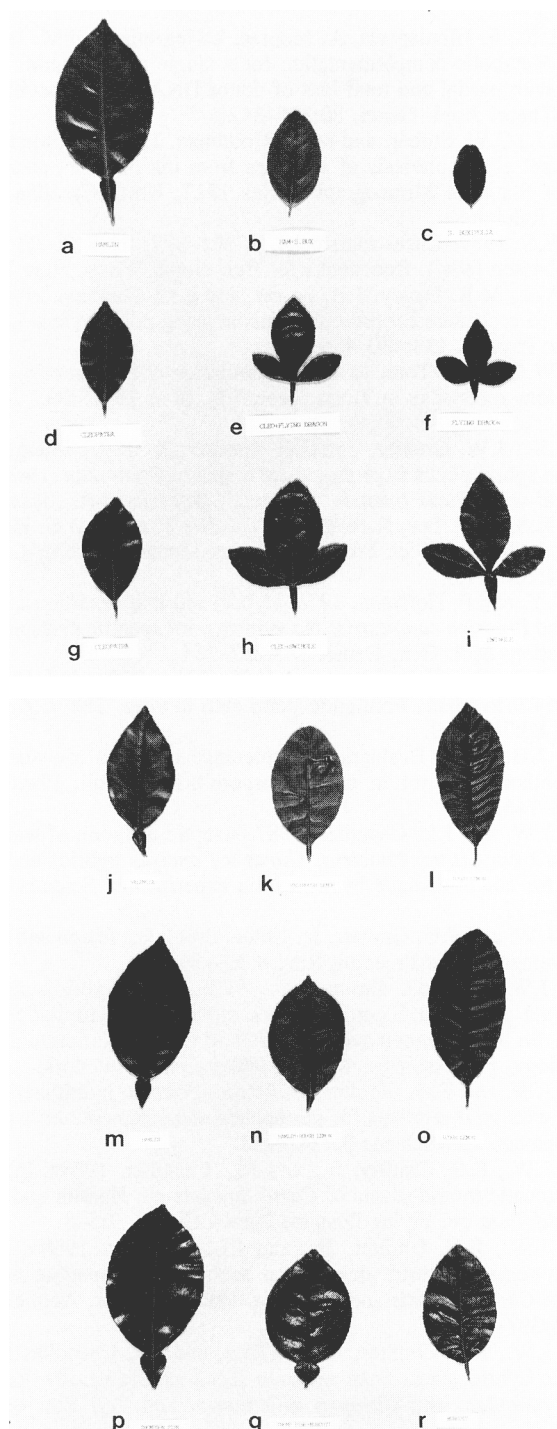


Fig. 1. Leaf morphology of: (a, m) 'Hamlin' sweet orange, (h) 'Hamlin' sweet orange + *Severinia buxifolia* somatic hybrid, (c) *S. buxifolia*, (d, g) Cleopatra mandarin, (e) Cleopatra + Flying Dragon trifoliate orange somatic hybrid, (f) Flying Dragon, (h) Cleopatra + Swingle citrumelo somatic hybrid, (i) Swingle, (j) 'Valencia' sweet orange, (k) 'Valencia' sweet orange + Rough lemon somatic hybrid, (l, o) Rough lemon, (n) 'Hamlin' + Rough lemon somatic hybrid, (p) 'Thompson' grapefruit, (q) 'Thompson' + 'Murcott' tangor somatic hybrid, (r) 'Murcott'.

nello' lemon (Tusa et al., 1990), 'Marsh' grapefruit (Ohgawara et al., 1989), and 'Duncan' grapefruit (J.W.G., unpublished

data). These results indicate that future research requiring protoplasm for plant regeneration will not necessarily be limited to genotypes capable of producing nucellar-derived friable embryogenic callus cultures.

Chromosome counts revealed that, with the exception of the 'Hamlin' + *S. buxifolia* somatic hybrid plants, all of the examined somatic hybrid plants were tetraploid ( $2n = 4x = 36$ ), as expected. All 10 of the 'Hamlin' + *S. buxifolia* plants examined from the population of 56 morphologically uniform somatic hybrids, including plants recovered from separate fusion petri dishes, were found to have a chromosome number of  $2n = 27$  (Fig. 2). Information from efforts to determine whether these plants were true triploids ( $2n = 3x = 27$ ) or aneuploids ( $2n = 27$ ) was suggestive, but 'not conclusive.' True triploidy could have resulted from haploid + diploid cell fusion or from single genome elimination. However, attempts to determine the chromosome number of the parental 'Hamlin' suspension culture and the *S. buxifolia* callus line were unsuccessful. The *S. buxifolia* callus line may have been at the haploid level at the time of protoplasm isolation. This callus line was grown with a high level of 2,4-D, a synthetic auxin associated with chromosome number instability in other plant tissue culture systems (Nickell and Torrey, 1969; Torrey, 1977). It is unlikely that the 'Hamlin' suspension culture was haploid, because three other tetraploid somatic hybrids were generated using protoplasts of this same line (Grosser and Gmitter, 1990c). Aneuploidy could have resulted from random or specific chromosome elimination. Chromosome elimination has not been reported in any other *Citrus* somatic hybrids, including tetraploid somatic hybrid plants regenerated from fusions of 'Hamlin' with *S. disticha* (Grosser et al., 1988b), but random chromosome elimination has been reported in fusion products of extremely wide intergeneric parental combinations (Gleba and Hoffman, 1978; Kao, 1977). Random chromosome elimination presumably induced by gamma irradiation has been reported in somatic hybrid plants (Agoudgil et al., 1990; Wijbrandi et al., 1990). It both parental callus lines were indeed diploid at the time of protoplasm fusion, the fact that all of the 'Hamlin' + *S. buxifolia* somatic hybrid plants examined were morphologically uniform with a chromosome number of  $2n = 27$ , including plants regenerated from separate fusion events, suggests specific chromosome elimination. If chromosome elimination was random, plants with variable chromosome counts and morphology should have been observed.

Complementarily of parental band patterns was observed among the somatic hybrids (Fig. 3). PGM and PER are monomeric enzymes, and somatic hybrids produced only those bands found in both parents. PGI is a dimeric enzyme; the 'Thompson'

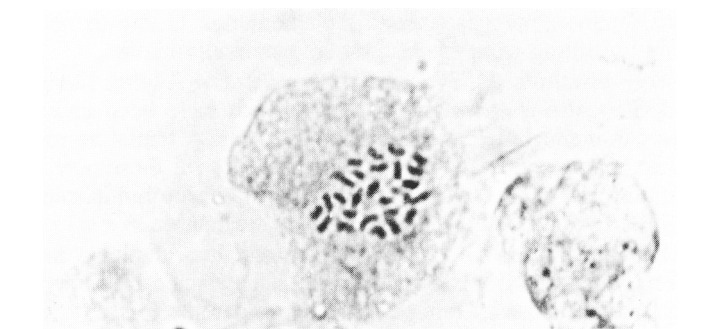


Fig. 2. Root tip squash prepared from a 'Hamlin' + *Severinia buxifolia* somatic hybrid, showing a chromosome number of  $2n = 27$  ( $\times 900$ , oil immersion).

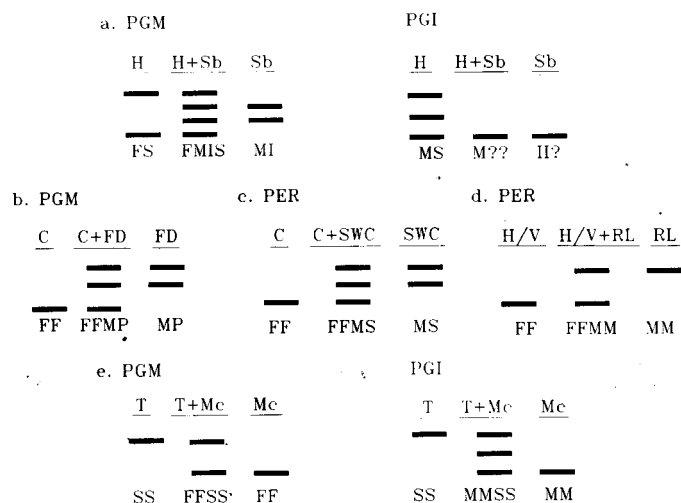


Fig. 3. Schematic diagrams and genotypes of leaf isozyme banding patterns from parents and somatic hybrids following starch gel electrophoresis: (a) 'Hamlin' (H-I) + *Severinia buxifolia* (Sb); (b) Cleopatra (C) + Flying Dragon (FD); (c) Cleopatra (C) + Swingle (SWC); (d) 'Hamlin' (H) or 'Valencia' (V) + Rough lemon (RL); (e) 'Thompson' (T) + 'Murcott' (Mc). Origin of migration is at top of diagrams.

grapefruit + 'Murcott' tanger hybrid exhibited the heterodimer form (FS) as well as both homodimers (FF and SS, Fig. 3e). Moore and Castle (1988) reported that Swingle citrumelo was FS at the PER locus used in this study. Results of repeated electrophoresis and evaluation of the Cleopatra mandarin + Swingle citrumelo hybrid and parents indicated that the correct genotype for Swingle citrumelo at PER is MS (Fig. 3c).

Isozyme analyses were conducted on 16 of the 'Hamlin' + *S. buxifolia* somatic hybrid plants and all had identical zymogram profiles. All four parental PGM bands (F, M, I, S) were visible in the zymogram of the 'Hamlin' orange + *S. buxifolia* hybrid. The zymotype of 'Hamlin' at PGI was MS, and *S. buxifolia* appeared to be II. The somatic hybrid produced what appeared to be a single band at the M position, although resolution was not sufficient to rule out the possibility that the single band was, in fact, a compressed triplet pattern resulting from the small difference in mobility between the M and I allele products. These results indicate that the 'Hamlin' chromosome that harbors the S allele of PGI was lost or the gene was silenced, but all four homologous chromosomes harboring the PGM locus were retained in the somatic hybrid. Therefore, the observed chromosome number  $2n = 27$  probably did not result from the fusion of diploid with haploid protoplasts; rather, isozyme analyses suggest that this phenomenon is due to aneuploidy resulting from specific chromosome elimination.

The 'Hamlin' + *S. buxifolia*, Cleopatra + Flying Dragon and Cleopatra + Swingle somatic hybrids have been entered into commercial field trials to evaluate their potential as rootstock. Development of propagation methods for these new somatic hybrids via rooted cuttings and in vitro shoot multiplication of each hybrid is required for extensive evaluation.

The 'Thompson' + 'Murcott' somatic hybrid plants have been grafted onto rootstock that may induce scion precocity to expedite their use as tetraploid breeding parents for scion improvement. Diploid 'Murcott' plants regenerated from leaf protoplasts have been grafted onto these rootstock to assess phenotypic stability of mature trees.

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