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INVITED REVIEW:

SOMATIC HYBRIDIZATION IN CITRUS: AN EFFECTIVE TOOL TO FACILITATE VARIETY IMPROVEMENT

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Summary

Citrus somatic hybridization and cybridization *via* protoplast fusion has become an integral part of citrus variety improvement programs worldwide. Citrus somatic hybrid plants have been regenerated from more than 200 parental combinations, and several cybrid combinations have also been produced. Applications of somatic hybridization to citrus scion improvement include the production of quality tetraploid breeding parents that can be used in interploid crosses to generate seedless triploids, and the direct production of triploids by haploid + diploid fusion. Applications of somatic hybridization to citrus rootstock improvement include the production of allotetraploid hybrids that combine complementary diploid rootstocks, and to combine citrus with sexually incompatible or difficult to hybridize genera that possess traits of interest for germplasm expansion. A few somatic hybrid tetraploid breeding parents have flowered, are fertile, and are being used as pollen parents to generate triploids. Several allotetraploid somatic hybrid rootstocks are performing well in commercial field trials, and show great promise for tree size control. Seed trees of most of these somatic hybrid rootstocks are producing adequate nucellar seed for standard propagation. Somatic hybridization is expected to have a positive impact on citrus cultivar improvement efforts.

Key words: citrus tissue culture; protoplast fusion; seedlessness; scion and rootstock improvement.

Introduction

During the decade of the 1980s, somatic hybridization was touted as a biotechnique that would revolutionize agricultural/plant improvement research. This prediction never materialized, and research efforts have shifted heavily to molecular-based strategies. Reasons why somatic hybridization failed to have a major impact on crop development are many, including difficulties in protoplast isolation, culture and plant regeneration in many elite crop genotypes, and the elevated ploidy levels resulting when somatic hybrid plants could be produced. Developed biotechniques are often bypassed or forgotten before they reach their full potential, and this was the case with somatic hybridization. Such techniques, however, may have great potential for specific commodities where impediments to their application are minimal, as is the case with somatic hybridization in citrus. Protoplast to plant regeneration in citrus is possible for many important citrus rootstock and scion cultivars (Vardi et al., 1982; Grosser, 1994a) and citrus-related species (Jumin and Nito, 1996), and the first somatic hybrid between *Citrus sinensis* and *Poncirus trifoliata* was obtained in 1985 by Ohgawara et al. Elevated ploidy levels (primarily tetraploid) in somatic citrus hybrids may actually have a positive impact on the horticultural performance of rootstocks (Grosser et al., 1996a; Ollitrault et al., 1998a), and they have value in specific breeding schemes (Grosser and Gmitter, 1990, 1996; Grosser et al., 1992, 1998a; Ollitrault et al., 1998b).

Increasing competition in international citrus markets and disease pressure have stimulated worldwide interest in citrus variety improvement. Targeted improvements with potential economic impact include improved fruit quality for fresh market citrus and improved disease/pest resistance in rootstocks and scions to increase production efficiency and tree longevity. Practical strategies involving applications of somatic hybridization to meet these goals have been developed and implemented. Techniques for producing somatic hybrids among elite citrus selections have advanced beyond an academic exercise, to a point where targeted combinations can be produced on a routine basis (for reviews see Kobayashi and Ohgawara, 1988; Grosser and Gmitter, 1990; Louzada and Grosser, 1994).

Somatic hybridization is now involved in five primary strategies to develop improved citrus varieties. For scion improvement, the primary strategy is to produce allotetraploid breeding parents by combining complementary elite scion varieties. Pollen from such hybrids can be used in interploid crosses with selected mono-embryonic diploid females to produce seedless triploid hybrids for

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TABLE 1

CITRUS SOMATIC HYBRIDS REGENERATED AFTER FUSION OF EMBRYOGENIC CALLUS-DERIVED PROTOPLASTS WITH LEAF-DERIVED PROTOPLASTS

Parental combination[a]	Method[b]	Goal[c]	Author
C. sinensis L. Osb. cv. Trovita + *Poncirus trifoliata* L. Raf.	PEG	2, 3	Ohgawara et al., 1985
C. sinensis L. Osb. + *C. unshiu* Marc.	PEG	1	Kobayashi et al., 1988
C. sinensis L. Osb. cv. Trovita + *C. unshiu* Marc. cv. Hayashi	PEG	1	Kobayashi and Ohgawara, 1988
C. sinensis L. Osb. cv. Trovita + [*C. sinensis* L. Osb. × *Poncirus trifoliata* L. Raf.] Troyer	PEG	1	Kobayashi and Ohgawara, 1988
C. sinensis L. Osb. cv. Trovita + *Poncirus trifoliata* L. Raf.	PEG	1	Kobayashi and Ohgawara, 1988
C. sinensis L. Osb. cv. Washington + *C. unshiu* Marc. cv. Hayashi	PEG	1	Kobayashi and Ohgawara, 1988
C. sinensis L. Osb. cv. Washington + [*C. sinensis* L. Osb. × *C. unshiu* Marc.] Murcott	PEG	1	Kobayashi and Ohgawara, 1988
C. sinensis L. Osb. cv. Navel + *C. paradisi* Macf.	PEG	1	Ohgawara et al., 1989
C. sinensis L. Osb. cv. Navel + [*C. sinensis* L. Osb. × *C. unshiu* Marc.] Murcott	PEG	1	Kobaysahi et al., 1991
C. sinensis L. Osb. cv. Bahia + [*C. sinensis* L. Osb. × *Poncirus trifoliata* L. Raf.] Troyer	PEG	2, 3	Ohgawara et al., 1991
C. sudachi Hort. cv. Shirai + *C. aurantifolia* Swing.	e^-	1	Saito et al., 1991
C. unshiu Marc. + *C. jambhiri* Lush.	e^-	3, 4	Hidaka and Omura, 1992
C. unshiu Marc. + *C. junos* Sieb. ex Tanaka	e^-	3, 4	Hidaka and Omura, 1992
C. sinensis L. Osb. cv. Trovita + *Murraya paniculata* L. Jack	e^-	2	Shinozaki et al., 1992
C. aurantifolia Swing. + *Feroniella lucida* (Sceff.) Swing.	e^-	2, 3	Takayanagi et al., 1992
C. aurantifolia Swing. + *Swinglea glutinosa* (Blanco) Merr.	e^-	2, 3	Takayanagi et al., 1992
C. jambhiri Lush. cv. Milam + *C. limonia* Osb. Kusaie	PEG	1	Tusa et al., 1992
C. jambhiri Lush. cv. Milam + *Poncirus trifoliata* Raf. cv. Flying Dragon	PEG	1	Tusa et al., 1992
C. jambhiri Lush. cv. Milam + [*C. depressa* Hay. × *Poncirus trifoliata* Raf.] cv. CNRP1	PEG	1	Tusa et al., 1992
C. jambhiri Lush. cv. Milam + *C. aurantium* L. cv. Keen Sour	PEG	1	Tusa et al., 1992
C. jambhiri Lush. cv. Milam + *C. madurensis* Lour. cv. Calamondin	PEG	1	Tusa et al., 1992
C. sinensis L. Osb. cv. Valencia + [*C. depressa* Hay. × *Poncirus trifoliata* Raf.] cv. CNRP1	PEG	1	Tusa et al., 1992
C. sinensis L. Osb. cv. Hamlin + *C. limonia* Osb. cv. Rangpur	PEG	1	Tusa et al., 1992
C. madurensis Lour. cv. Calamondin + *C. aurantium* L. cv. Keen Sour	PEG	1	Tusa et al., 1992
Lime-type cybrid diploid + *C. limon* L. Burm f. cv. Eureka	e^-	1	Saito et al., 1994
C. aurantifolia Swing. + *C. jambhiri* Lush.	e^-	4	Hidaka et al., 1995
C. aurantifolia Swing. + *C. junos* Sieb. ex Tanaka	e^-	4	Hidaka et al., 1995
[*C. reticulata* Blanco × *C. paradisi* Macf.] Seminole + *C. jambhiri* Lush.	e^-	4	Hidaka et al., 1995
[*C. reticulata* Blanco × *C. paradisi* Macf.] Seminole + *C. junos* Sieb. ex Tanaka	e^-	4	Hidaka et al., 1995
C. sinensis L. Osb. cv. Shirayanagi + *Poncirus trifoliata* L. Raf.	e^-	2	Kaneko et al., 1995
C. sinensis L. Osb. cv. Valencia + [*C. sinensis* L. Osb. × *C. unshiu* Marc.] Murcott	e^-	2	Kaneko et al., 1995
[*C. reticulata* Blanco × *C. paradisi* Macf.] Seminole + *Atalantia monophylla* DC.	e^-	3	Motomura et al., 1995
[*C. reticulata* Blanco × *C. paradisi* Macf.] Seminole + *Severinia buxifolia* (Poir.) Tenore	e^-	3	Motomura et al., 1995
C. reticulata Blanco cv. Ohta + *C. jambhiri* Lush.	e^-	1, 2	Moriguchi et al., 1996
C. deliciosa Ten. cv. Willow leaf + *C. limon* L. Burm f. cv. Eureka	e^-	1	Ollitrault et al., 1996b
C. deliciosa Ten. cv. Willow leaf + *Poncirus trifoliata* L. Raf. Cv. Pomeroy	e^-	2	Ollitrault et al., 1996b
C. deliciosa Ten. cv. Willow leaf + *C. aurantifolia* (Christm.) Swing.	e^-	1	Ollitrault et al., 1996b
C. deliciosa Ten. cv. Willow leaf + *C. sinensis* L. Osb. cv. Washington Navel	e^-	1	Ollitrault et al., 1996b
C. paradisi Macf. cv. Star Ruby + *C. aurantifolia* (Christm.) Swing.	e^-	1	Ollitrault et al., 1996b
C. deliciosa Ten. cv. Willow leaf + *Fortunella japonica* (Thumb.) Swing. cv. Marumi	e^-	1	Ollitrault et al., 1996b
C. unshiu Marc. cv. Juman + haploid clementine-type plant	e^-	1	Kobayashi et al., 1997
C. sinensis L. Osb. var. *brasiliensis* cv. Ohmishima + haploid clementine-type plant	e^-	1	Kobayashi et al., 1997
C. sinensis L. Osb. cv. Trovita + haploid clementine-type plant	e^-	1	Kobayashi et al., 1997
Poncirus trifoliata L. Raf. + *Fortunella hindsii*	e^-	2, 3	Miranda et al., 1997
[*C. reticulata* Blanco × *C. paradisi* Macf.] Seminole + *C. jambhiri* Lush.	e^-	1	Moriguchi et al., 1997
C. reticulata Blanco cv. Hazzara + *Murraya koenigii* L. Spreng.	e^-	3, 4	Motomura et al., 1997
C. reticulata Blanco cv. Ohta + *Glycosmis pentaphylla* (Retz.) Correa	e^-	3, 4	Motomura et al., 1997
C. unshiu Marc. cv. Saruwatari + *Aegle marmelos* L.	e^-	3, 4	Motomura et al., 1997
C. reticulata Blanco cv. Hazzara + *Swinglea glutinosa* (Blanco) Merr.	e^-	3, 4	Motomura et al., 1997
C. reticulata Blanco cv. Ohta + *Severinia buxifolia* (Poir.) Tenore	e^-	3, 4	Motomura et al., 1997
C. reticulata Blanco cv. Hazzara + *Severinia buxifolia* (Poir.) Tenore	e^-	3, 4	Motomura et al., 1997
[*C. reticulata* Blanco × *C. paradisi* Macf.] Seminole + *Severinia buxifolia* (Poir.) Tenore	e^-	3, 4	Motomura et al., 1997
[*C. reticulata* Blanco × *C. paradisi* Macf.] Seminole + *Atalantia monophylla* DC.	e^-	3, 4	Motomura et al., 1997
C. reticulata Blanco cv. Hazzara +*Microcitrus australis* (Planch.) Swing.	e^-	3, 4	Motomura et al., 1997
[*C. reticulata* Blanco × *C. paradisi* Macf.] Seminole + *Microcitrus australis* Swing.	e^-	3, 4	Motomura et al., 1997
[*C. reticulata* Blanco × *C. paradisi* Macf.] Page + *Murraya paniculata* L. Jack	e^-	2, 3	Guo and Deng, 1998
[*C. reticulata* Blanco × *C. paradisi* Macf.] Page + *C. jambhiri* Lush.	e^-	4	Guo et al., 1998
C. sinensis L. Osb. cv. Skagg's Bonanza + *Clausema lausium* (Lour.) Skeels	e^-	1	Guo and Deng, 1999
C. limon L. Burm f. cv. Kutdiken + *C. limon* L. Burm f. cv. Zagara Bianca	PEG	1	Koc et al., 1999
C. sinensis L. Osb. cv. Skagg's Bonanza + *C. jambhiri* Lush.	e^-	2	Deng et al., 2000
C. sinensis L. Osb. cv. Skagg's Bonanza + *C. aurantium* L. cv. Goutou	e^-	2	Deng et al., 2000
C. sinensis L. Osb. cv. Skagg's Bonanza + *C. paradisi* Macf. cv. Red blush	e^-	1	Deng et al., 2000
[*C. reticulata* Blanco × *C. paradisi* Macf.] Page + *C. sinensis* L. Osb. cv. Valencia	e^-	1	Deng et al., 2000
[*C. reticulata* Blanco × *C. paradisi* Macf.] Page + *Poncirus trifoliata* L. Raf.	e^-	2	Deng et al., 2000
C. reticulata Blanco cv. Kinnow + *C. reticulata* Blanco cv. Bendizao	e^-	1	Deng et al., 2000

Table 1. *continued*

Parental combination[a]	Method[b]	Goal[c]	Author
C. reticulata Blanco cv. Hongju + *C. aurantium* L. cv. Guotou	e^-	2	Deng et al., 2000
C. reticulata Blanco cv. Hongju + *C. jambhiri* Lush.	e^-	2	Deng et al., 2000
C. sinensis L. Osb. cv. Jingchen + [*C. sinensis* L. Osb. cv. Hamlin + *C. jambhiri* Lush.] HR	e^-	2	Deng et al., 2000
C. reticulata Blanco cv. Hongju + *Poncirus trifoliata* L. Raf.	e^-	2	Deng et al., 2000
Fortunella obovata + *C. reticulata* Blanco cv. Hongju	e^-	1	Deng et al., 2000
M. papuana [*M. australis* (Planch.) Swing. × *M. australasica* (F. Muell.)] + *C. jambhiri* Lush.	e^-	3	Deng et al., 2000
M. papuana [*M. australis* (Planch.) Swing. × *M. australasica* (F. Muell.)] + *C. reticulata* Bl.	e^-	3	Deng et al., 2000
C. reticulata Blanco cv. Hongju + [*C. sinensis* L. Osb. × *Poncirus trifoliata* L. Raf.]	e^-	2	Deng et al., 2000
C. sinensis L. Osb. cv. Newhall + *C. limon* L. Burm f. cv. Eureka	e^-	1, 2	Deng et al., 2000
C. sinensis L. Osb. cv. Newhall + [*C. reticulata* Blanco × *C. grandis* (L.) Osb. cv. Kuigan]	e^-	1, 2	Deng et al., 2000
C. sinensis L. Osb. cv. Newhall + *C. reticulata* Blanco cv. Bingtangju	e^-	1	Deng et al., 2000
C. sinensis L. Osb. cv. Caipira + *C. limonia* L. Osb. cv. Rangpur	PEG	2	Mendes-da-Gloria et al., 2000
C. deliciosa Ten. cv. Willow leaf + *C. sinensis* L. Osb. cv. Shamouti	e^-	1	Ollitrault et al., 2000b
C. paradisi Macf. cv. Star Ruby + *C. medica* L. Burm f. cv. Corse	e^-	1	Ollitrault et al., 2000b
C. limon L. Burm f. cv. LAC + *C. limon* L. Burm f. cv. Eureka	e^-	1	Ollitrault et al., 2000b
C. sinensis L. Osb. cv. Salustiana + *C. aurantium* L.	PEG	2	Olivares-Fuster, in prep.
C. aurantium L. + *C. sinensis* L. Osb. cv. Salustiana	PEG/e^-	2	Olivares-Fuster, in prep.
C. sinensis L. Osb. cv. Salustiana + *C. excelsa* Wester	PEG/e^-	1	Olivares-Fuster, in prep.
C. sinensis L. Osb. cv. Salustiana + *C. jambhiri* Lush.	PEG	2	Olivares-Fuster, in prep.
C. sinensis L. Osb. cv. Salustiana + *C. volkameriana* Ten. and Pasq.	PEG/e^-	2	Olivares-Fuster, in prep.
C. sinensis L. Osb. cv. Salustiana + *C. aurantifolia* (Christm.) Swing.	PEG/e^-	1, 2	Olivares-Fuster, in prep.
C. sinensis L. Osb. cv. Caipira + *C. reshni* Hort. ex Tanaka cv. Cleopatra	PEG	2	Mourao, pers. commun.
C. sinensis L. Osb. cv. Caipira + *C. volkameriana* Ten. and Pasq.	PEG	2	Mourao, pers. commun.
C. sinensis L. Osb. cv. Caipira + *C. jambhiri* Lush.	PEG	2	Mourao, pers. commun.
C. reshni Hort. ex Tanaka cv. Cleopatra + *C. aurantium* L.	PEG	2	Mourao, pers. commun.
C. limonia L. Osb. cv. Rangpur + *C. aurantium* L.	PEG	2	Mourao, pers. commun.

[a] Leaf-derived parental listed in second place.
[b] e^-, electrical protoplast fusion; PEG, chemical (polyethylene glycol) protoplast fusion.
[c] Goals: 1, scion improvement; 2, rootstock improvement; 3, germplasm expansion; 4, somatic hybridization methodology.

selection (Grosser and Gmitter, 1996). A second way for producing improved scions is to produce triploids directly by haploid + diploid protoplast fusion (Deng et al., 1992a; Ollitrault et al., 1997, 1998b, 2000a). Two strategies being employed for rootstock improvement are: (1) to produce somatic hybrids of complementary rootstock parents that have potential for improved disease resistance, tree size control, and horticultural performance (Grosser and Gmitter, 1990; Grosser et al., 1996a; Ollitrault et al., 1998a) and (2) to produce wide hybrids of *Citrus* with related genera to broaden the germplasm base, including sexually incompatible or difficult to hybridize citrus relatives (Louzada and Grosser, 1994; Grosser et al., 1996b; Motomura et al., 1997; Guo and Deng, 1999). A final option is the production of citrus somatic cybrids, which may have potential in both scion and rootstock improvement (Tusa et al., 2000). This review defines some basic components necessary for a successful applied somatic hybridization program in citrus, identifies the numerous somatic hybrids (Tables 1–3) and cybrids (Table 4) produced worldwide, and briefly describes the more than 100 somatic hybrids produced at the Citrus Research and Education Center (CREC, Florida) to date (Tables 5 and 6). The rationale for producing each hybrid at the CREC and information regarding the evaluation and use of each hybrid are also provided, as well as some results of the haploid + diploid somatic hybridization program from the Center for International Cooperation in Agricultural Research for Development (CIRAD, France).

Building a Broad-based Program

Citrus somatic hybrids are generally produced from the fusion of protoplasts isolated from embryogenic callus or suspension cultures of one parent with leaf-derived protoplasts of the second parent (Fig. 1A). Protoplast fusion is induced either by polyethylene glycol (PEG) (Grosser and Gmitter, 1990) or electrically (Saito et al., 1991; Hidaka and Omura, 1992; Ling and Iwamasa, 1994; Hidaka et al., 1995; Ollitrault et al., 1996b) (Tables 1–5). Regeneration of citrus somatic hybrids after fusion of two callus-type parents has proven to be a viable alternative (Table 2). The embryogenic parent provides the capacity for plant regeneration from fusion products. To produce somatic hybrids from a wide range of parental combinations, it is necessary to maintain a large collection of embryogenic cultures of the most important selections. Approximately 50 such lines are maintained in the CREC collection, the majority initiated from undeveloped ovules cultured on EBA [Murashige and Tucker (1969) MT basal medium containing 0.045 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.44 μM N^6-benzyladenine (BA)], EME (MT basal containing 0.5 g l^{-1} malt extract), or H + H (modified MT basal containing 1.55 g l^{-1} glutamine, 0.5 g l^{-1} malt extract, and 50% less KNO_3 and NH_4NO_3) media (Moore, 1985; Gmitter and Moore, 1986; Grosser and Gmitter, 1990). Callus cultures are generally maintained on both EME and H + H on a 6-wk subculture cycle, and suspension cultures are maintained in H + H on a 2-wk cycle. Large collections of embryogenic citrus cultures are also maintained in Brazil, China, France (more than 20 entries), Israel, Italy, Japan, Spain (more than 50 entries), and probably elsewhere. Embryogenic callus cultures have also been stored efficiently *via* cryopreservation, which greatly reduces the labor involved in maintaining a collection (Sakai et al., 1990; Engelmann et al., 1994; Perez et al., 1997). It is quite useful to exchange lines with other laboratories, to increase the number of available lines for fusion experiments and to recover lines lost to

TABLE 2

CITRUS SOMATIC HYBRIDS OBTAINED AFTER FUSION OF EMBRYOGENIC CALLUS-DERIVED PROTOPLASTS

Parental combination	Method	Goal[a]	Author
C. reticulata Blanco cv. Ponkan + *Citropsis gabunensis* (Engl.) Swing. cv. Gabon	e⁻	2, 3	Ling and Iwamasa, 1994
C. deliciosa Ten. cv. Willow leaf + *C. aurantifolia* (Christm.) Swing.	e⁻	1	Ollitrault et al. 1996b
C. paradisi Macf. cv. Star Ruby + *C. aurantifolia* (Christm.) Swing.	e⁻	1	Ollitrault et al., 1996b
C. limon L. Burm f. cv. LAC + *C. sinensis* L. Osb. cv. Hamlin	e⁻	1	Ollitrault et al., 1996b
C. deliciosa Ten. cv. Willow leaf + *C. sinensis* L. Osb. cv. Shamouti	e⁻	1	Ollitrault et al., 2000b
C. deliciosa Ten. cv. Willow leaf + *C. sinensis* L. Osb. cv. C1	e⁻	1	Ollitrault et al., 2000b
C. deliciosa Ten. cv. Willow leaf + *C. paradisi* Macf. cv. Star Ruby	e⁻	1	Ollitrault et al., 2000b
C. deliciosa Ten. cv. Willow leaf + *C. aurantium* L.	e⁻	1	Ollitrault et al., 2000b
C. deliciosa Ten. cv. Willow leaf + *C. limon* L. Burm f. cv. LAC	e⁻	1	Ollitrault et al., 2000b
C. paradisi Macf. cv. Star Ruby + *C. limon* L. Burm f. cv. LAC	e⁻	1	Ollitrault et al., 2000b
C. paradisi Macf. cv. Star Ruby + *C. sinensis* L. Osb. cv. C1	e⁻	1	Ollitrault et al., 2000b
C. sunki Hort. ex Tan. + [*C. sinensis* L. Osb. × *Poncirus trifoliata* L. Raf.] Carrizo	e⁻	2	Ollitrault et al., 2000b
C. sinensis L. Osb. cv. Shamouti + *C. tangerina* Hort. ex Tan. cv. Beauty	e⁻	1	Ollitrault et al., 2000b
C. sinensis L. Osb. cv. Shamouti + *C. paradisi* Macf. cv. Star Ruby	e⁻	1	Ollitrault et al., 2000b
C. sinensis L. Osb. cv. Shamouti + *C. aurantifolia* (Christm.) Swing.	e⁻	1	Ollitrault et al., 2000b
C. sinensis L. Osb. cv. Shamouti + *C. limon* L. Burm f. cv. LAC	e⁻	1	Ollitrault et al., 2000b
C. sinensis L. Osb. cv. Shamouti + *C. sinensis* L. Osb. cv. C1	e⁻	1	Ollitrault et al., 2000b
C. aurantifolia (Christm.) Swing. + *C. sinensis* L. Osb. cv. G1	e⁻	1	Ollitrault et al., 2000b
C. aurantifolia (Christm.) Swing. + *C. aurantium* L.	e⁻	2	Ollitrault et al., 2000b
C. aurantifolia (Christm.) Swing. + [*C. sinensis* L. Osb. × *Poncirus trifoliata* L. Raf.] Carrizo	e⁻	2	Ollitrault et al., 2000b
C. aurantium L. + *C. sinensis* L. Osb. cv. C1	e⁻	2	Ollitrault et al., 2000b

[a] Goals: 1, scion improvement; 2, rootstock improvement; 3, germplasm expansion.

TABLE 3

POLYPLOID SOMATIC HYBRIDS BETWEEN 11 DIPLOID CULTIVARS AND TWO HAPLOID LINES OF CLEMENTINE (H1 AND H2) IN FIELD EVALUATION AT CIRAD-FLHOR, GUADELOUPE (FWI)

Diploid genitors	H1	H2	3× hybrids	4× hybrids	>4× hybrids	Total
'Willow leaf' mandarin	*	*	21	5	0	26
'Beauty' mandarin		*	1	15	0	16
'Kinnow' mandarin	*	*	20	30	0	50
'Sunki' mandarin	*		4	2	0	6
'Murcott' tangor	*	*	1	4	4	9
'Shamouti' sweet orange	*	*	24	2	0	26
'Valencia' sweet orange	*	*	2	14	0	16
Sour orange		*	17	1	0	18
'Star Ruby' grapefruit	*	*	28	38	1	67
'Mexican' lime	*		0	4	0	4
'Marumi' Kumquat	*		1	7	0	8
Total			119	122	5	246

contamination. Suitability of cryopreserved callus as a source of quality protoplasts has been demonstrated (Olivares-Fuster et al., 2000). Protoplasts should always be isolated from embryogenic callus and suspension cultures that are in the log phase of growth (Grosser, 1994b).

As mentioned, protoplasts of the complementary parent are generally isolated from leaves, usually from nucellar seedlings or recently budded trees for monoembryonic genotypes. At the CREC, best results have been obtained from fully expanded leaves of new flush that have not completely hardened, taken from plants maintained in a growth chamber. Two to six plants of each selection are routinely maintained, rotating plants between the growth chamber and the greenhouse to promote continuous healthy flushing. Very poor results have been obtained on attempting to isolate protoplasts from greenhouse or field material. The importance of high-quality source tissue for protoplast isolation cannot be over-emphasized. The collection of plant material providing leaves should, of course, contain any selections of interest that are not amenable to embryogenic callus induction (i.e. monoembryonic clones and anything with trifoliate orange background). Once good collections of embryogenic callus and leaf-source plants are established, somatic hybridizations of numerous parental combinations can be attempted.

After regeneration, it is necessary to select the somatic hybrids and alloplasmic plants. Following fusion, protoplast suspensions contain parental, homofused, heterofused, and multifused protoplasts. Furthermore, post-fusion events such as chromosome elimination can occur during the regeneration process. Morphological characters can be useful for somatic hybrid identification (Grosser and Gmitter, 1990). However, accurate nuclear characterization is generally done by the combination of molecular (Fig. 1B) or isozyme marker analysis and ploidy evaluation. Chromosome counting can now be favorably bypassed by flow cytometry for quicker analysis (Ollitrault et al., 1996b). Cytoplasmic genome analysis is done by RFLP (Kobayashi et al., 1991; Saito et al., 1993, 1994; Ohgawara et al., 1994; Luro et al., 1995; Yamamoto and Kobayashi, 1995; Ollitrault et al., 1996b; Grosser et al., 1996d; Moriguchi et al., 1996, 1997; Moreira et al., 2000a, 2000b) or CAPS analysis (P. Ollitrault, unpublished data).

Somatic hybridization programs for citrus improvement have been developed all over the world, in some cases many years ago, and in others more recently. For example, some of the countries supporting a program based in this technology are the USA (Grosser et al., 1998a, 1998b), France (Ollitrault et al., 1996b, 1998a, 1998b, 2000a), Japan (Ohgawara and Kobayashi, 1991; Oiyama et al., 1991), China (Deng et al., 1996, 2000), Italy (Tusa et al., 1996, 2000), Brazil (Mourao, personal communication), Spain (Navarro et al., 1997), Turkey (Koc, Cukurova University, personal communication), and Costa Rica (Guevara, CIGRAS, University of Costa Rica, personal communication).

TABLE 4

CITRUS ALLOPLASMIC PLANTS

Parental combination[a]	Method	Author
C. deliciosa Ten. cv. Willow leaf + *C. sinensis* L. Osb. cv. Valencia	PEG	Luro et al., 1985; Moreira et al., 2000a
C. deliciosa Ten. cv. Willow leaf + *C. paradisi* Macf. cv. Star Ruby (callus + callus)	PEG	Luro et al., 1985; Moreira et al., 2000a
C. sinensis L. Osb. + *C. unshiu* Marc.	PEG	Kobayashi et al., 1988
Microcitrus sp. + *C. aurantium* L.	PEG/asymmt.	Vardi et al., 1989
Microcitrus sp. + *C. jambhiri* Lush.	PEG/asymmt.	Vardi et al., 1989
C. sinensis L. Osb. cv. Navel + *C. paradisi* Macf.	PEG	Ohgawara et al., 1989
C. sinensis L. Osb. cv. Valencia + *C. limon* (L.) Burm cv. Feminello	PEG	Tusa et al., 1990
C. sinensis L. Osb. cv. Navel + [*C. sinensis* L. Osb. ×*Poncirus trifoliata* L. Raf.] Troyer	PEG	Ohgawara et al., 1991
C. sudachi Hort. cv. Shirai + *C. aurantifolia* Swing.	e^-	Saito et al., 1993
C. sudachi Hort. cv. Shirai + *C. limon* (L.) Burm.	e^-	Saito et al., 1993
C. sinensis L. Osb. cv. Hamlin + *C. jambhiri* Lush.	PEG	Deng and Grosser, 1993
Lime-type cybrid callus (sudachi mitochondrial) + *C. limon* Burm. cv. Eureka	e^-	Saito et al., 1994
C. unshiu Marc. + *C. sinensis* L. Osb.	e^-	Yamamoto and Kobayashi, 1995
C. deliciosa Ten. cv. Willow leaf + *C. paradisi* Macf. cv. Duncan	e^-	Ollitrault et al., 1996b
C. microcarpa Bunge + *C. aurantium* L. cv. Keen	PEG	Grosser et al., 1996c
C. reshni Hort. ex Tan. + *C. aurantium* L.	PEG	Grosser et al., 1996c
C. sinensis L. Osb. cv. Valencia + *C. limon* (L.) Burm cv. Feminello	PEG	Grosser et al., 1996c
C. reticulata Blanco cv. Ohta + *C. jambhiri* Lush.	e^-	Moriguchi et al., 1996
[*C. reticulata* Blanco ×*C. paradisi* Macf.] Seminole + *C. limon* (L.) Burm cv. Lisbon	e^-	Moriguchi et al., 1996
[*C. sinensis* L. Osb. ×*C. unshiu* Marc.] Murcott + *C. sinensis* L. Osb. cv. Bonanza	e^-	Li and Deng, 1997
[*C. reticulata* Blanco ×*C. paradisi* Macf.] cv. Nova + *C. sinensis* L. Osb. cv. Newhall	e^-	Li and Deng, 1997
[*C. reticulata* Blanco ×*C. paradisi* Macf.] Seminole + *C. jambhiri* Lush.	e^-	Moriguchi et al., 1997
[*C. reticulata* Blanco ×*C. paradisi* Macf.] cv. Page + *C. jambhiri* Lush.	e^-	Guo et al., 1998
C. reticulata Blanco cv. Willowleaf + *C. paradisi* Macf. cv. Duncan	PEG	Moreira et al., 2000a
C. reticulata Blanco cv. Willowleaf + *C. sinensis* L. Osb. cv. Valencia	PEG	Moreira et al., 2000a
C. reticulata Blanco cv. Cleopatra + *C. aurantium* L.	PEG	Moreira et al., 2000a
C. sinensis L. Osb. cv. Hamlin + *C. reticulata* Blanco cv. Ponkan	PEG	Moreira et al., 2000a
C. sinensis L. Osb. cv. Rhode Red + *C. reticulata* Blanco cv. Dancy	PEG	Moreira et al., 2000a
Swinglea glutinosa Swing. + *C. aurantium* L.	PEG	Moreira et al., 2000a
C. deliciosa Ten. cv. Willow leaf + *C. limon* L. Burm f. cv. Eureka	e^-	Ollitrault et al., 2000b
C. deliciosa Ten. cv. Willow leaf + *C. limon* L. Burm f. cv. LAC (callus + callus)	e^-	Ollitrault et al., 2000b
C. deliciosa Ten. cv. Willow leaf + *Fortunella japonica* Swing.	e^-	Ollitrault et al., 2000b
C. paradisi Macf. cv. Star Ruby + *C. limon* L. Burm f. cv. LAC (callus + callus)	e^-	Ollitrault et al., 2000b
C. clementine Hort. ex Tan. haploid + *Fortunella japonica* Swing.	e^-	Ollitrault et al., 2000b

[a] Parental listed in second place provides the nucleus in the cybrid plants.

Somatic Hybrids in Scion Improvement

Easy-peeling fresh market citrus has received increasing attention from the consumer over the last 20 yr. 'Clementine' is presently one of the most popular fresh citrus easy-peelers, but there is an increasing demand for additional early and late ripening seedless mandarin cultivars. Seedlessness is a primary breeding objective internationally and especially in Florida, where currently the commercial mandarin hybrids are seedy. Seediness limits the domestic marketing potential of Florida fresh fruit, and totally eliminates many international marketing opportunities. Seedless varieties of similar or better quality with expanded availability would provide a huge opportunity to citrus growers. As seedlessness is a major criteria for the fresh fruit market, triploid breeding is a promising way for easy-peeler diversification (Starrantino, 1994).

Several breeding strategies were developed for triploid hybrid creation. In the early 1970s, occasional triploid progenies have been selected from conventional crosses between diploid cultivars (Esen and Soost, 1971; Geraci et al., 1975). This spontaneous natural process can be exploited with more efficiency by combining embryo rescue and triploid selection by flow cytometry (Ollitrault et al., 1996c). *In vitro* culture of triploid endosperm tissue has also enabled the recovery of triploid plants (Gmitter et al., 1990). However, this technique seems poorly adapted for large-scale breeding programs (Gmitter, personal communication). The classical method for breeding triploid citrus is crossing diploid and tetraploid genotypes (Esen and Soost, 1973; Starrantino and Recupero, 1981). In the past, low availability of tetraploid genitors limited this strategy. Today, the pool of tetraploid genitors is enriched considerably with new highly heterozygous allotetraploid genotypes obtained by somatic hybridization (Grosser et al., 1992; Kobayashi et al., 1995; Deng et al., 1996; Mourao et al., 1996; Tusa et al., 1996; Ollitrault et al., 1998b). In addition to 44 CREC somatic hybrids (Table 5), about 60 somatic hybrids with potential to serve as tetraploid parents have been developed at the international level (Tables 1 and 2).

Production of tetraploid breeding parents at the CREC. As mentioned, a primary role of somatic hybridization in scion breeding is to develop quality tetraploid breeding parents that can be used in interploid crosses to generate seedless triploids, and this has been an important objective of the somatic hybridization program at the CREC (Grosser et al., 1992, 1998a; Grosser and Gmitter, 1996; Mourao et al., 1996). Somatic hybrids produced via protoplast fusion at the CREC are described in Table 5, and the techniques used to produce and verify these hybrids are summarized in Grosser and Gmitter (1990). Many of these hybrids

TABLE 5

SUMMARY OF CONFIRMED SOMATIC HYBRID PLANTS PRODUCED BY CELL-FUSION AT THE CREC AS OF MARCH 2000

Parental combination (embryogenic parent listed first)	No. plants in soil	Evaluation for rootstock potential in progress	Primary objectives[a]
Intergeneric hybrids, sexually compatible			
1. *Citrus sinensis* cv. Hamlin + *Poncirus trifoliata* cv. Flying Dragon	>800	+	1, 2, 5
2. *C. sinensis* cv. Succari + *P. trifoliata* cv. Argentine	>250	+	1, 2, 5
3. *C. sinensis* cv. Valencia + Carrizo citrange	>75	+	2
4. *C. reticulata* cv. Cleopatra + *P. trifoliata* cv. Flying Dragon	>300	+	1, 2, 5
5. *C. reticulata* cv. Cleopatra + *P.trifoliata* cv. Argentine	>100	+	1, 2, 5
6. *C. reticulata* cv. Cleopatra + Swingle citrumelo	>300	+	1, 2, 5
7. *C. sinensis* cv. Valencia + *Fortunella crassifolia* cv. Meiwa	>100	+	7, 1, 2, 5
8. *F. crassifolia* cv. Meiwa + *C. reticulata* cv. Dancy	>40	+	7, 1, 2, 5
9. *F. crassifolia* cv. Meiwa + *C. reticulata* cv. Changsha	>30	+	7, 1, 2, 5
10. *C. sinensis* cv. Hamlin + *Microcitrus papuana*	>30	+	1, 2
11. *C. aurantium* cv. sour orange + *P. trifoliata* cv. Flying Dragon	>100	+	1, 2, 3, 5, 6
12. *C. aurantium* cv. sour orange + Carrizo citrange	>100	+	1, 2, 3, 5, 6
13. *C. paradisi* cv. Red Marsh + *P. trifoliata* cv. Flying Dragon	36	+	1, 2, 5
14. *C. paradisi* cv. Red Marsh + *P. trifoliata* cv. Argentine	>40	+	1, 2, 5
15. *C. sinensis* cv. Succari + *F. crassifolia* cv. Meiwa	>50	+	1, 2, 5, 7
16. 'Murcott' tangor + Cohen citrange (pentaploid)	3	−	1, 2, 3, 5, 6
17. 'Nova' tangelo + Cohen citrange (pentaploid)	>30	+	1
18. *C. reticulata* cv. Cleopatra + Cohen citrange (pentaploid)	>30	+	2
19. *C. reticulata* cv. Cleopatra + Carrizo citrange	>50	+	1
20. *C. sinensis* cv. Succari + *Microcitrus papauna*	>10	−	1, 2
21. *C. jambhiri* hybrid cv. Milam + Swingle citrumelo XX[b]	>35	−	1, 2, 4, 5, 6
22. *C. jambhiri* hybrid cv. Milam + Carrizo citrange	>50	+	1, 4, 6
23. *C. aurantium* cv. sour orange + *P. trifoliata* 50–7	>100	+	1, 2, 3, 5, 6
24. *C. sinensis* cv. Washington Navel + *P. trifoliata* 50–7	>100	+	1, 2, 5
25. *C. reticulata* cv. Changsha + *P. trifoliata* 50–7	>100	+	1, 2, 5
26. *C. paradisi* cv. Duncan + *P. trifoliata* 50–7	>50	+	1, 2, 5
27. *C. aurantium* cv. sour orange + Benton citrange	>50	+	2, 3, 5, 6
28. *C. sinensis* cv. Itaborai + G96 cold-hardy hybrid	>30	−	1, 2, 5, 7
29. Murcott tangor + *Microcitrus papauna*	7	−	1, 2
Intergeneric hybrids, sexually incompatible			
30. *C. sinensis* cv. Hamlin + *Severinia buxifolia*	>200	+	1, 2, 4, 5
31. *C. sinensis* cv. Hamlin + *S. buxifolia* (triploid)	>100	+	1, 2, 4, 5
32. *C. sinensis* cv. Succari + *S. buxifolia*	>30	+	1, 2, 4, 5
33. *C. sinensis* cv. Hamlin + *S. disticha* XX	4	−	1, 2, 4, 5
34. *C. sinensis* cv. Valencia + *S.disticha* XX	3	−	1, 2, 4, 5
35. *C. reticulata* cv. Cleopatra + *S. disticha* XX	2	−	1, 2, 4, 5
36. *C. sinensis* cv. Succari + *S. disticha* XX	3	−	1, 2, 4, 5
37. 'Nova' tangelo + *S.disticha* XX	2	−	1, 2, 4, 5
38. *C. sinensis* cv. Hamlin + *Citropsis gilletiana*	>150	+	2, 4, 5
39. *C. reticulata* cv. Cleopatra + *Citropsis gilletiana* XX	>40	−	2, 4, 5
40. 'Nova' tangelo + *Citropsis gilletiana*	>50	+	2, 4, 5
41. *C. sinensis* cv. Hamlin + *Atalantia ceylanica*	>20	−	1, 2, 4, 5
42. *C. sinensis* cv. Succari + *A. ceylanica*	>50	+	1, 2, 4, 5
43. *C. sinensis* cv. Succari + *Citropsis gilletiana*	>30	+	2, 4, 5
44. *C. sinensis* cv. Succari + *Feronia limonia*	>24	−	2
Interspecific hybrids, sexually compatible			
45. *C. aurantium* cv. sour orange + *C.limmettioides* Tan. cv. Palestine sweet lime	>100	+	2, 3, 6
46. *C. aurantium* cv. sour orange + *C. limonia* cv. Rangpur	>200	+	2, 3, 6
47. *C. reticulata* cv. Cleopatra + *C. aurantium* cv. sour orange	>150	+	1, 2, 3
48. *C. sinensis* cv. Succari + *C. aurantium* cv. sour orange	>40	+	2, 3, 6
49. Smooth Flat Seville + *C. jambhiri* cv. Rough lemon	>100	+	2, 3, 6
50. *C. sinensis* cv. Succari + *C. reticulata* cv. Changsha	>30	+	1, 2, 6
51. *C. jambhiri* hybrid cv. Milam + *C. reticulata* cv. Sun Chu Sha	>50	+	1, 2, 4, 6
52. *C. aurantifolia* cv. Key lime + *C. sinensis* cv. Valencia	>100	+	2, 7
53. *C. jambhiri* hybrid cv. Milam + *C. limon* cv. Femminello	>100	+	2, 4, 7
54. *C. sinensis* cv. Valencia + *C. limon* cv. Femminello	>300	+	2, 7
55. *C. sinensis* cv. Hamlin + *C. limon* cv. Femminello	>40	+	2, 7
56. *C. sinensis* cv. Hamlin + *C. jambhiri* cv. rough lemon	>200	+	2, 7
57. *C. sinensis* cv. Valencia + *C. jambhiri* cv. rough lemon	>200	+	2, 7
58. *C. sinensis* cv. Hamlin + *C. limonia* cv. Rangpur	>400	+	2, 7
59. *C. reticulata* cv. Cleopatra + *C.limonia* cv. Rangpur	>500	+	1, 2, 7
60. 'Nova' tangelo + Palestine sweet lime	>50	+	1, 2, 7
61. *C. reticulata* cv. Cleopatra + *C. jambhiri* cv. rough lemon	>200	+	1, 2, 7

Table 5. *continued*

Parental combination (embryogenic parent listed first)	No. plants in soil	Evaluation for rootstock potential in progress	Primary objectives[a]
62. *C. reticulata* cv. Cleopatra + *C. volkameriana* cv. Volkamer lemon	>200	+	1, 2, 7
63. 'Nova' tangelo + *Citrus ichangensis*	>100	+	1, 2
64. *C. sinensis* cv. Succari + *C. jambhiri* cv. rough lemon 8166	>50	+	1, 2, 5, 6
65. *C. sinensis* cv. Succari + *C. obovoidea* cv. Kinkoji	>20	+	2, 6
66. *C. jambhiri* hybrid cv. Milam + *C. obovoidea* cv. Kinkoji	>100	+	2, 6
67. *C. jambhiri* hybrid cv. Milam + *C. grandis* cv. Hirado Buntan (zygotic)	>50	+	2, 6
68. *C. paradisi* cv. Red Marsh + *C. jambhiri* cv. rough lemon 8166	>15	−	2, 6
69. *C. sinensis* cv. Succari + *C. reticulata* cv. Ponkan	>20	−	7
70. *C. sinensis* cv. Succari + 'Minneola' tangelo	>10	−	7
71. *C. sinensis* cv. Valencia + (Robinson × Temple hybrid) C. J. Hearn/USDA	6	−	7
72. 'Nova' tangelo + *C. sinensis* cv. Succari	6	−	7
73. 'Nova' tangelo + *C. grandis* cv. Hirado buntan (zygotic)	>200	+	2, 7
74. *C. sinensis* cv. Succari + *C. grandis* cv. Hirado buntan (zygotic)	>20	+	2, 7
75. *C. sinensis* cv. Hamlin + ('Clementine' × 'Minneola' hybrid AP-LB8-4)	>10	−	7
76. *C. sinensis* cv. Valencia + 'Minneola' tangelo	3	−	7
77. *C. sinensis* cv. Succari + USDA-CJH Hybrid 1[c] (Fortune × Kinnow)	6	−	7
78. *C. paradisi* cv. Thompson Pink + 'Murcott' tangor	35	−	7
79. *C. sinensis* cv. Hamlin + *C. reticulata* cv. Dancy	>20	−	7
80. *C. sinensis* cv. Rohde Red Valencia + *C. reticulata* cv. Dancy	6	−	7
81. *C. sinensis* cv. Valencia + 'Page' tangelo	>10	−	7
82. *C. sinensis* cv. Valencia + 'Murcott' tangor	21	−	7
83. *C. sinensis* cv. Hamlin + *C. reticulata* cv. Ponkan	9	−	7
84. *C. sinensis* cv. Succari + 'Page' tangelo	>10	−	7
85. *C. sinensis* cv. Succari + 'Murcott' tangor	>10	−	7
86. *C. sinensis* cv. Succari + *C. reticulata* cv. Dancy	>20	−	7
87. 'Murcott' tangor + USDA-CJH Hybrid 1 ('Fortune' × 'Kinnow')	>6	−	7
88. 'Murcott' tangor + USDA-CJH Hybrid 2 ('Wilking' × 'Valencia')	>3	−	7
89. 'Murcott' tangor + ('Clementine' × 'Minneola' hybrid AP-LB8-9)	>10	−	7
90. *C. paradisi* cv. Red Marsh + ('Clementine' × 'Minneola' hybrid AP-LB8-9)	6	−	7
91. *C. sinensis* cv. Rohde Red + USDA-CJH Hybrid 1 ('Fortune' × 'Kinnow')	6	−	7
92. *C. sinensis* cv. Itaborai + *C. medica* L. cv. Citron	10	−	2, 6
93. *C. reticulata* cv. Changsha + *C. medica* L. cv. Citron	15	−	2, 6
94. *C. sinensis* cv. Succari + Natsudaidai hybrid	3	−	2, 6
95. Natsudaidai hybrid + *C. jambhiri* cv. Rough lemon 8166	2	−	2, 6
96. 'Nova' tangelo + 'Osceola' tangelo	3	+	7
97. 'Nova' tangelo + 'Ortanique' tangor	5	+	7
98. 'Murcott' tangor + *C. sinensis* cv. Washington Navel	8	+	7
99. 'Murcott' tangor + 'Osceola' tangelo	4	−	7
100. 'Murcott' tangor + 'Ortanique' tangor	5	−	7
101. *C. paradisi* cv. Red Marsh + USDA-CJH Hybrid 2 ('Wilking' × 'Valencia')	6	−	7
102. *C. aurantium* cv. sour orange + *C. sinensis* cv. Succari	>50	+	2, 3
103. 'Murcott' tangor + 'Sunburst' tangelo	2	−	7
104. 'Murcott' tangor + ('Clementine' × 'Satsuma')	4	−	7
Intraspecific hybrids			
105. *C. sinensis* cv. Valencia + *C. sinensis* cv. Jaboticaba	6	−	7

[a] Primary objectives: 1, improved cold hardiness/tree size control; 2, improved blight field tolerance; 3, improved resistance to CTV-induced quick decline; 4, improved nematode resistance; 5, improved *Phytophthora* resistance; 6, improved adaptation to high pH calcareous soils; 7, tetraploid breeding parent for seedless triploid scion production.
[b] XX=unexpected disease susceptibility (fungal).
[c] Seed of USDA hybrids kindly provided by C. J. Hearn.

were produced for use in interploid crosses. Dr Frederick G. Gmitter, Jr has been conducting an extensive triploid-breeding program for the past few years at the CREC, producing several thousand triploid hybrids. Recently, he has been incorporating somatic hybrid tetraploid parents into this program, as they reach maturity and begin to produce adequate fertile pollen. Rather than growing somatic hybrid breeding parents in the field on their own roots, they were initially budded to Swingle citrumelo rootstock to expedite flowering. More recently, we have discovered that budding to certain somatic hybrid rootstocks (i.e. somatic hybrids of Flying Dragon trifoliate orange) accelerates flowering even more.

The first somatic hybrid used as a pollen parent to generate seedless triploids at the CREC was 'Nova' tangelo + 'Succari' sweet orange (Fig. 1E) (Grosser and Gmitter, 1996), and resulting triploid hybrids have been budded to rootstocks and planted in the field. Since 1996, several hundred additional triploids have been produced. Triploid hybrids will subsequently be selected for advanced trials on the basis of fruit quality and season of maturity. During the past crossing season, pollen obtained from somatic hybrids of 'Nova' + 'Succari', 'Pink Marsh' grapefruit + 'Murcott' tangor, 'Hamlin' sweet orange + 'Dancy' mandarin, 'Hamlin' + 'Ponkan' mandarin, 'Succari' + 'Page' tangelo, and 'Valencia' +

TABLE 6

CREC SOMATIC HYBRID SEED TREES FLOWERING AND FRUITING AS OF FEBRUARY 2000

Hybrid combination	Seed content of fruit
Hybrids for rootstock improvement	
'Hamlin' sweet orange + Flying Dragon trifoliate orange	F/M
'Cleopatra' mandarin + Flying Dragon	F
Sour orange + Flying Dragon	M
Sour orange + Carrizo	M
'Hamlin' + Rangpur	M
Sour orange + Palestine sweet lime	M
Sour orange + Rangpur	M
'Cleopatra' + Volkameriana	VF
'Cleopatra'+ rough lemon	VF
'Cleopatra' + Swingle	F
'Cleopatra' + Argentine trifoliate orange	F
Smooth Flat Seville + rough lemon	M
'Nova' tangelo + *C. ichangensis*	M
'Cleopatra' + Rangpur	Flowers, no fruit yet
'Hamlin' + rough lemon	M
'Red Marsh' grapefruit + Flying Dragon	Flowers, no fruit yet
'Succari' sweet orange + Argentine trifoliate orange	F
'Hamlin' + *Microcitrus papuana*	F
'Succari' sweet orange + *Microcitrus papuana*	Flowers, no fruit yet
Hybrids for scion improvement	
'Nova' tangelo + Succari sweet orange	VF
'Pink Marsh' grapefruit + 'Murcott' tangor	VF
'Hamlin' + LB8-4 ('Clementine' × 'Minneola')	VF
'Valencia' + ('Robinson' × 'Temple')	VF
'Hamlin' + 'Dancy' mandarin	VF
'Succari' sweet orange + 'Page' tangelo	Flowers, no fruit yet
'Valencia' sweet orange + 'Page' tangelo	Flowers, no fruit yet
'Hamlin' sweet orange + 'Ponkan' mandarin	Flowers, no fruit yet
'Succari' sweet orange + 'Hirado Buntan' zygotic pummelo[a]	Flowers, no fruit yet
'Nova' + 'Hirado Buntan' zygotic pummelo[a]	M
'Valencia' sweet orange + 'Femminello' lemon[a]	M
Milam lemon hyrid + 'Femminello'[a]	VF
'Hamlin'+ 'Femminello'[a]	M
'Valencia' + 'Key' lime[a]	F

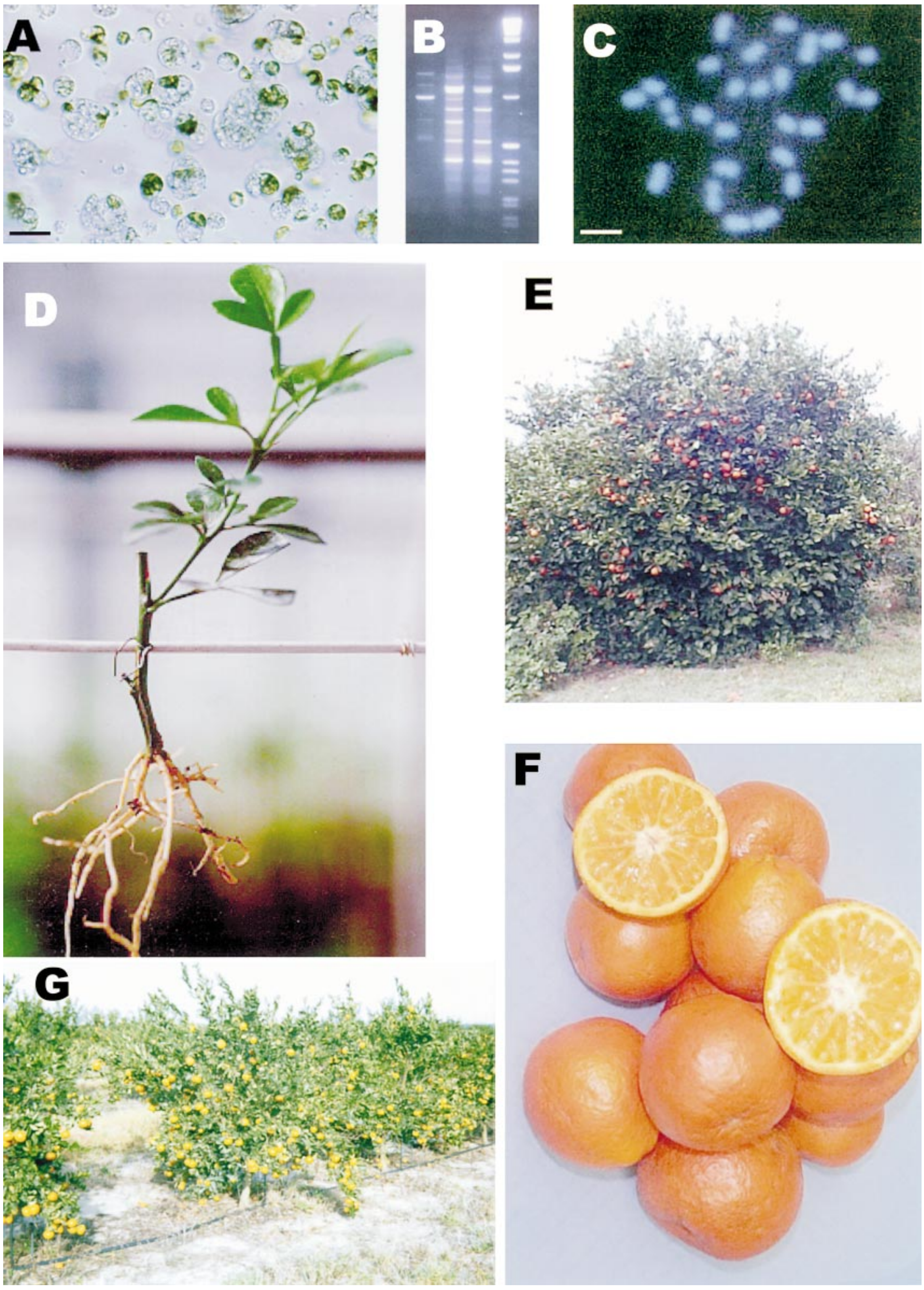
[a] Also being tested as rootstock.

M, six or more seeds per fruit; F, 2–5 seeds per fruit; VF, 0–2 seeds per fruit.

('Robinson' × 'Temple') was used in interploid crosses. Each year we expect additional somatic hybrids to flower, which will continually expand the germplasm base available for interploid crosses. CREC, as well as CIRAD, have also recently focused on developing breeding parents for developing late maturing triploids, as there are very few quality mandarins available late in the season (e.g. 'Murcott' tangor + 'Ortanique' tangor) (Grosser et al., 1998a). This research is expected to have a dramatic impact on the production of improved fresh fruit seedless mandarin hybrids during the next 5–20 yr. The team in Sicily is also using lemon/lime somatic hybrids produced at the CREC as pollen parents in interploid crosses with 'Femminello' lemon, in an effort to generate seedless lemons with improved resistance to the systemic fungal disease mal secco (Tusa et al., 1996, 2000). At the CREC, we are producing acid-fruit triploids with emphasis on improving cold-hardiness. It is also possible that a few tetraploid somatic hybrids could produce fruit with adequate quality to be released as improved cultivars in their own right. Young-tree fruit from 'Nova' + 'Succari' (Fig. 1F) has good external color, peels easily, has a very low seed count (commercially seedless) and pleasant flavor, but has variable size and sometimes inadequate juice content. Young-tree fruit from 'Pink Marsh' + 'Murcott' is juicy with excellent quality and nearly seedless, but its acid level never drops to an acceptable level. We look forward to the evaluation of fruit from additional somatic hybrids as they progress through juvenility.

Direct triploid production via haploid + diploid fusion at CIRAD. In all the above strategies, both of the parental genomes are submitted to meiotic recombination. Maximum heterozygosity in

FIG. 1. A, PEG-induced fusion of citrus embryogenic protoplasts with leaf protoplasts. B, RAPD analysis verifying a somatic hybrid combining 'Succari' sweet orange with *Feronia limonia* using Operon primer W-15; lane 1, *Feronia* ; lane 2, 'Succari' + *Feronia* ; lane 3, 'Succari' sweet orange; lane 4, DNA ladder. C, Root-tip cell of a somatic hybrid plant regenerated from haploid + diploid fusion showing the triploid chromosome number ($2n = 3\times = 27$). D, Rooted cutting of *C. deliciosa* + *P. trifoliata* somatic hybrid rootstock. E, Fruiting tree of the tetraploid 'Nova' tangelo + 'Succari' sweet orange somatic hybrid breeding parent. F, Fruit of the tetraploid 'Nova' tangelo + 'Succari' sweet orange somatic hybrid. G, Four-year-old trees of 'Ambersweet' scion budded to the 'Cleopatra' mandarin + Flying Dragon trifoliate orange somatic hybrid rootstock in a high-density planting. *Bars* : A = 90 μm; C = 5 μm.


A
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triploid progeny will be obtained from interploid crosses involving an allotetraploid parent (produced by somatic hybridization) rather than an autotetraploid parent produced by other means. Concerning triploids arising in diploid sexual crosses, it appears that only a part of parental heterozygosity is present in the $2n$ gametes producing the spontaneous triploids (Ollitrault et al., 1998b). Considering that most cultivars are generally highly heterozygous, it is clear that in most cases the selected genetic balances of the parental cultivars will be lost in triploid hybrids. Therefore, the effectiveness of selection made at the diploid level for complex characters is low and it is necessary to make final selections from large numbers of triploid hybrids.

Keeping in mind the conservation of the genetic balance of selected diploid cultivars in the triploid hybrids, CIRAD has developed a new strategy for the direct synthesis of triploid hybrids by somatic hybridization between diploid cultivars and haploid lines (Ollitrault et al., 1997, 2000a). The regeneration of haploid plants and cell lines by anther culture (Germana, 1992) or by induced gynogenesis (Oiyama and Kobayashi, 1993; Ollitrault et al., 1996a) has opened the avenue for this new breeding scheme. At the same time Kobayashi et al. (1997) have published successful results of somatic hybridization between haploid 'Clementine' and diploid 'Satsuma' mandarin, 'Navel' sweet orange, and 'Trovita' sweet orange.

At CIRAD, two haploid cell lines of 'Clementine' obtained by induced gynogenesis (Ollitrault et al., 1996a) have been combined with 11 diploid cultivars (Table 3). Somatic hybridization has been accomplished by callus–callus protoplast electrofusion for most of the combinations. Only protoplasts of kumquat cv. 'Marumi' were isolated from leaves.

As for diploid + diploid somatic hybridization, regeneration of embryos in haploid + diploid is reached in less than 2 mo. and cotyledonary embryos can be subcultured in germination medium 3–4 mo. after protoplast isolation in order to develop *in vitro* plantlets. Flow cytometry and molecular markers have been used to study the nuclear genomes of regenerated plantlets and it appears that all the polyploid plants are hybrids. Triploid (Fig. 1C) and tetraploid hybrids have been obtained for each combination as well as a few pentaploid hybrids with 'Star Ruby', 'Sunki', 'Murcott', and 'Kinnow'. Therefore, the ploidy-level diversity of the regenerated material from these diploid + haploid somatic hybridizations is much greater than that observed in diploid + diploid combinations. This result could be due to the ploidy instability in the 'haploid callus line'. Indeed, the presence of diploid and triploid cells in this callus has been verified by flow cytometry analysis (Ollitrault et al., 2000a). Furthermore, the fact that Kobayashi et al. (1997) obtained only diploids (with one of the nuclear parental genomes) or triploid somatic hybrids by combining protoplasts from diploid calluses and haploid leaves confirms that triploid cells arising from haploid + diploid fusion do not exhibit a specific nuclear genome instability during mitosis.

Some genotypes (particularly pentaploid hybrids) have been lost during acclimatization by shoot grafting. Presently, 246 polyploid hybrids are under field evaluation at CIRAD in Guadeloupe. Triploid hybrids should be exploited directly and will soon be evaluated in tropical, subtropical and Mediterranean areas. Tetraploid hybrids obtained by this method will join the pool of allotetraploids for further diploid × tetraploid sexual crosses.

Citrus is the first example of triploid hybrids obtained by somatic hybridization in fruit crops. The production of allo-triploids from somatic hybridization between haploid potato and diploid tomato has been described previously (Schoenmakers et al., 1991) while triploid somatic hybrid plants of *Nicotiana* and *Petunia* have been obtained by gametic + somatic ($n + 2n$) protoplast fusion (Pirrie and Power, 1986; Lee and Power, 1988). This method allows the synthesis of triploids in one cycle of hybridization, and provides an interesting alternative to diploid by tetraploid sexual crosses or spontaneous triploid selection. Indeed it is the only method that enables the addition, without recombination, of a haploid genome to the whole genome of a diploid cultivar. It is probable that a major part of the organoleptic quality and/or resistance traits of the diploid cultivars will be intact in the triploid hybrids produced by this method. The main limitation of this strategy is the lack of haploid lines. This could be overcome by the application of gameto-somatic hybridization mentioned by Deng et al. (1992a), who have regenerated only one chimeric plant with 18 and 19 chromosomes. This technique is currently being developed by CIRAD, and will hopefully allow the production of polymorphic triploid progenies recombining only for the haploid source. If successful, this would provide a new avenue for citrus genetics and breeding research.

Somatic Hybrids in Rootstock Improvement

The use of rootstocks is inherent to citriculture worldwide. Rootstock genotypes are generally more widely adapted and offer better disease resistance than do most scions grown on their own roots. Budding from selected mature trees onto seedling rootstocks circumvents juvenility and maintains cultivar integrity. Trees on rootstocks therefore come into production more quickly and generally produce higher quality fruit than seedling trees. However, the need for improved citrus rootstocks has never been greater. Commercial citrus rootstocks used worldwide rarely satisfy all selection criteria for a specific location, including disease and nematode resistance, cold-hardiness, adaptation to various soils and conditions, appropriate tree size, and high yields of quality fruit (Castle, 1987). In Florida alone, two diseases, citrus blight and quick-decline caused by citrus tristeza virus, combine to kill nearly 1.5 million trees per yr. Thousands more trees annually are either killed or rendered non-productive by fungal diseases (i.e. *Phytophthora*), *Diaprepes* root weevil larvae, and parasitic nematodes (including the citrus, burrowing, sting, and coffee nematodes). Severe freezes in Florida during the decade of the 1980s destroyed more than 300,000 acres of commercial citrus. Citrus rootstock status in the Mediterranean Basin has also become very critical with important abiotic constraints such as alkalinity, drought and salinity, and the spread of tristeza virus that will soon hamper the use of sour orange (which is still the most common rootstock in this region). Salinity in irrigation water is also becoming a problem in localized areas the world over. Tree size control has also recently become more important to maximize the efficiency of harvesting and emergent cold protection methodology (Parsons et al., 1991).

The technique of somatic hybridization is well-suited for citrus rootstock breeding. Indeed, it allows for the addition of the entire nuclear genomes of two complementary genitors without recombination. In this way, dominant traits can be effectively stacked in each hybrid, and there is only one hybrid from each parental combination to evaluate (assuming appropriate choices in parentage). The high level of heterozygosity of most cultivars and the high number of traits to select for confounds conventional rootstock breeding at the diploid level. Moreover, somatic hybridization opens an avenue for germplasm expansion by combining sexually incompatible species.

Somatic hybrids of complementary rootstock parents. A primary goal of the CREC citrus cultivar improvement program is to develop improved rootstocks that will provide growers with alternative rootstock choices for any given location/citriculture scheme. Part of this program is the production and evaluation of somatic hybrid rootstocks produced from the fusion of complementary rootstocks (Grosser and Gmitter, 1990; Grosser et al., 1996a, 1998b). For example, the somatic hybrid of sour orange + Carrizo citrange has the potential to combine the blight resistance of sour orange with the resistance to tristeza virus-induced quick-decline of Carrizo. All of the intergeneric somatic hybrids among sexually compatible parents and many of the interspecific somatic hybrids described in Table 5 were produced with this strategy in mind. The primary objectives for producing each of these hybrids are also provided. Recently, we have produced somatic hybrids using parents identified for superior traits (Grosser et al., 1998b). For example, trifoliate orange 50-7 was identified as a superior source of resistance to *Phytophthora nicotianae* (Graham, 1995), and Benton citrange was identified as a superior yielding rootstock that produces high-quality fruit (Castle, 1998). Several new hybrids using these selections as parents have been produced (Table 5). Complete evaluation of new rootstock candidates is a long-term, expensive endeavor that requires the cooperation of horticulturists, pathologists, nematologists, and commercial growers. Complete evaluation also requires the propagation of a few hundred uniform plants of each hybrid to conduct these tests.

Propagation of somatic hybrid rootstocks. Standard commercial citrus rootstocks are propagated via nucellar seed, which is not an option for newly produced somatic hybrids. Two alternative methods are being used at the CREC to generate the necessary populations of plants. Some somatic hybrids, especially those with trifoliate orange parentage, are amenable to tissue culture micropropagation. DBA3 shoot induction medium (MT basal medium containing 13.3 μM BA and 0.045 μM 2,4-D) (Deng et al., 1992b) was found to be superior for this application. Dissected somatic embryos of amenable hybrids generally will produce multiple shoots over several transfer cycles on this medium. Shoots obtained are easily rooted on RMAN medium [1/2 strength MT basal medium containing 0.11 μM 1-naphthaleneacetic acid (NAA) and 0.5 g l^{-1} activated charcoal] (Grosser and Gmitter, 1990). For example, more than 300 rooted plants of the sour orange + trifoliate orange 50-7 somatic hybrid were recently produced at the CREC using this system. Alternatively, a simple rooted-cutting method (Fig. 1D) is used whenever inadequate numbers of plants are produced by tissue culture. The general technique is as follows: two-node cuttings from recently hardened healthy vegetative flushes are dipped in 100% indole-3-butyric acid (or another effective root-induction substance), inserted in plastic 38-welled containers filled with commercial potting mix and placed on a mist-bed. Under optimum conditions (summer), 90–100% rooting is achieved within 3–8 wk. The technique is less efficient during the winter due to shorter days and cooler temperatures, but can still be successful if supplemental lighting and mist-bed heating pads are utilized. This technique works for all hybrids tested so far, including intergeneric somatic hybrids among sexually incompatible genera. In cases where only one or a few hybrid plants are obtained from the somatic fusion experiments, such hybrids are budded to vigorous rootstocks to provide an adequate source of vegetative material for cuttings as quickly as possible. A similar technique (Ollitrault et al., 1998a) has been used at CIRAD to produce more than a thousand plants of the *C. deliciosa* + *P. trifoliata* somatic hybrid for multilocal evaluation (Fig. 1D).

Evaluation of somatic hybrid rootstocks. Once an adequate number of plants of a particular hybrid becomes available, subsets can be budded with an appropriate scion and entered into replicated commercial field trials where they will be compared to standard commercial rootstocks for yield, fruit quality, and tree survival. CREC somatic hybrid rootstock candidates are being tested under two regimes that represent the two primary citriculture schemes in Florida: (i) trials conducted on the deep, well-drained sand ridge and (ii) trials conducted on the poorly drained flatwoods with trees grown on raised beds. Most of the somatic hybrid rootstock candidates produced are either in trials (Tables 2 and 5) or are being prepared for inclusion in trials. The oldest somatic hybrid rootstock trials have reached the age where yield and fruit quality data are being collected on an annual basis. Preliminary observations regarding the performance of somatic hybrid root-stocks in these trials are generally encouraging. Many of the somatic hybrid rootstocks are producing trees smaller than presently used commercial rootstocks (Fig. 1G). For example, trees budded to the ‘Cleopatra’ mandarin + Swingle citrumelo somatic hybrid (and several other similar somatic hybrids) are much smaller than trees on either of the parents. Yield data from a 6-yr-old somatic hybrid rootstock trial (with ‘Valencia’ sweet orange scion) demonstrated that trees budded to a somatic hybrid of sour orange + Palestine sweet lime produced as much fruit as trees on sour orange, yet the trees on the somatic hybrid were approximately half the size of the sour orange trees. The ability of tetraploid rootstocks to reduce tree size was first reported by Lee et al. (1990). Superior dwarfing and/or semidwarfing rootstocks may provide growers with some advantages regarding harvesting and cold protection (Fig. 1G).

Other subsets of somatic hybrid plants are being screened for resistance to tristeza virus-induced quick-decline, citrus blight, *Phytophthora*-induced diseases, *Diaprepes* root weevil larvae, various nematodes, and salinity, in cooperation with pathologists, entomologists, and physiologists. In most cases, resistant somatic hybrids are being identified. Screening of somatic hybrids combining CTV-induced quick-decline susceptible sour orange with resistant parents resulted in the identification of several hybrids that are resistant to this disease, including sour orange + Palestine sweet lime and sour orange + Carrizo citrange (Grosser et al., 1996d). Both of these hybrids are performing well in commercial field trials, and seed trees of these somatic hybrids are producing seedy fruit (Table 6). No commercial citrus germplasm has been identified that is resistant to feeding damage by *Diaprepes* root weevil larvae. Therefore, a range of wide intergeneric somatic hybrids was screened at the CREC in the hope that one or more of the citrus relative parents would provide resistance in the somatic hybrids. All hybrids screened were susceptible (Grosser and McCoy, 1996). We concluded that it would be extremely difficult to develop hybrids that are resistant to larval feeding, and we have since implemented a new strategy of developing hybrids that can tolerate feeding damage and regenerate healthy roots in the presence of invading fungi (*Phytophthora*-induced diseases). Results from blight, *Phytophthora*, nematode and salinity screening experiments will be published as data become available. Preliminary data from nematode greenhouse-screening experiments at the CREC look quite promising, as somatic hybrids have been identified that are significantly more resistant than commercial rootstocks to the citrus, burrowing, and coffee nematodes.

Somatic hybrid rootstocks showing the most promise to date regarding wide adaptation, tree size control, disease resistance, and good fruit quality include 'Cleopatra' mandarin + Argentine trifoliate orange (CREC), sour orange + Carrizo (CREC), and sour orange + Palestine sweet lime (CREC). The *C. deliciosa* + *P. trifoliata* hybrid (CIRAD) is showing promise for the Mediterranean region as it is immune to tristeza virus, resistant to *Phytophthora*, and is performing well on calcareous soils and under high-saline conditions.

Somatic hybrid seed trees. Simultaneous with the establishment of field trials and screening experiments, seed trees of each somatic hybrid rootstock candidate are being grown to determine if they are amenable to standard nursery propagation via nucellar seed. Such trees can also provide seed for further experimentation, and could become a nursery seed source in the event that a hybrid is released to the industry. Flowering, fruiting and seed production data to date are provided in Table 6. It must be noted that these data are preliminary and may be influenced by juvenility, environmental factors, and lack of cross-pollination. Several of the CREC somatic hybrids are now consistently producing high yields of seedy fruit. Seed from several somatic hybrids, including 'Hamlin' sweet orange + rough lemon, 'Valencia' sweet orange + 'Femminello' lemon, 'Cleopatra' mandarin + Flying Dragon trifoliate orange, 'Cleopatra' + Argentine trifoliate orange, sour orange + Carrizo, sour orange + Flying Dragon, and 'Nova' + *C. ichangensis* germinated and produced nearly 100% uniform nucellar seedlings. Sour orange + Rangpur has produced high seed yields of which 30–40% appear to be zygotic. This hybrid is therefore being utilized as a female for breeding rootstocks at the tetraploid level (a new and highly promising strategy). A high percentage of zygotic seedlings has also been observed from 'Valencia' sweet orange + rough lemon. A few other somatic hybrid rootstock candidates are producing very few seed, including 'Cleopatra' mandarin + rough lemon and 'Cleopatra' mandarin + Volkameriana. This is unfortunate because these two hybrids perform well in field trials. Overall, preliminary data suggest that many but not all of the somatic hybrids will be amenable to the standard propagation method. In the event that a low-seeded hybrid shows promise as a rootstock, alternative propagation methods are available.

Wide hybridization for rootstock germplasm enhancement. The second strategy being used for rootstock improvement is the production of somatic hybrids combining citrus with sexually incompatible or difficult to hybridize related genera (Hidaka et al., 1992; Louzada et al., 1993; Louzada and Grosser, 1994; Grosser et al., 1996b; Guo and Deng, 1998, 1999). Citrus relatives are considered to be a vast but relatively untapped reservoir of genetic diversity. The original idea was to use wide somatic hybrids directly as rootstocks, but this has met with mixed results. Trees budded onto *Citrus* + *Severinia* somatic hybrids are all showing severe nutrient deficiencies after a few years in the field. Trees budded onto *Citrus sinensis* + *Citropsis* somatic hybrids are showing a severe trunk-splitting problem just below the graft union. Trees budded onto 'Valencia' sweet orange + *Fortunella crassifolia* somatic hybrid show a bark decay disorder just below the graft union. These horticultural inadequacies may be due to genetic weaknesses, and/or altered nutritional requirements in these hybrids. More recently, we are encouraged by the field performance of trees budded to the wide somatic hybrids Succari sweet orange + *Atalantia ceylanica* and 'Nova' tangelo + *Citropsis gilletiana*. Two-year-old trees on these hybrid rootstocks at two distinct locations look promising. Additional somatic hybridization experiments may result in the production and identification of wide somatic hybrids with adequate horticultural performance. However, the real value of these wide hybrids may be to serve as parents for breeding at the tetraploid level, to further introgress genes from the related genera into more horticulturally useful hybrids. This will require adequate flowering and fertility in the wide hybrids, which has yet to be demonstrated. Graft compatibility with somatic hybrid rootstocks has been excellent, as we have so far encountered only one incompatibility problem. Three-year-old trees of 'Valencia' sweet orange budded to a somatic hybrid of 'Meiwa' kumquat + 'Dancy' mandarin are dying due to a graft incompatibility.

Wider hybridizations done at the inter-subtribe or inter-tribe levels result in great difficulties for plant or shoot regeneration associated with nuclear genome instability (Hidaka et al., 1992; Ling et al., 1994; Grosser et al., 1996b; Guo and Deng, 1998, 1999). These results reflect the limitations associated with symmetric somatic hybridization at this taxonomic level.

Somatic Cybridization

Production of diploid somatic hybrid plants containing the nuclear genome of one parent and either the cytoplasmic genome of the other parent or a combination of both parents (cybrids), has been a common approach in plant improvement. Characterization of such cybrids can determine cytoplasm inheritance and cytoplasm-coded agronomic traits, and may lead to improved selections (Kumar and Cocking, 1987). Compared to the broad success achieved in symmetric citrus somatic hybridization worldwide, only a few reports can be found pursuing the production of citrus cybrid plants by asymmetric somatic hybridization (Vardi et al., 1987, 1989; Li and Deng, 1997). The challenging methodology involved with the original donor–recipient method and the lack of information concerning cytoplasmic traits initially slowed the evolution of cybridization in citrus. But more recently, chance and nature have played an important role in this field, allowing the regeneration of several citrus alloplasmic plants as a byproduct of the application of standard somatic hybridization procedures (Table 4).

Genetic studies on the regenerated citrus somatic hybrids and cybrids after fusion of callus-derived protoplasts with leaf-derived protoplasts, demonstrated specific elimination of the embryogenic parent nuclear genome in all cybrids, the non-segregation of mitochondrial genomes (the mitochondrial genome from the embryogenic parent always prevails in cybrids, as well as complete somatic hybrids), or segregation of chloroplastic genomes (either one of the chloroplastic genomes is found both in cybrids and hybrids) (Kobayashi et al., 1991; Saito et al., 1993, 1994; Ohgawara et al., 1994; Luro et al., 1995; Yamamoto and Kobayashi, 1995; Grosser et al., 1996c; Moriguchi et al., 1996, 1997; Ollitrault et al., 1996b; Moreira et al., 2000a). Recombination of the cytoplasmic genomes has been often reported in somatic hybrids and cybrids of many plant species (Ichikawa et al., 1989), but in citrus does not seem to be as common, as only four reports can be found: Vardi et al. (1987, 1989) and Li and Deng (1997) after asymmetric somatic hybridization, and Motomura et al. (1995), Moriguchi et al. (1997), and Moreira et al. (2000b) after standard somatic hybridization.

The sole presence of the mitochondrial genome from the embryogenic parent in all regenerated cybrids and somatic hybrids suggests a critical role in plant regeneration via somatic embryogenesis (Kobayashi et al., 1991; Saito et al., 1993; Ohgawara et al., 1994; Grosser et al., 1996d; Moriguchi et al., 1996; Ollitrault et al., 1996b; Moreira et al., 2000a). In this last reference, the authors hypothesized that this may be a quantitative effect and that only cultured cells have adequate quantities of mitochondria to provide the necessary energy for somatic embryogenesis. They also verified that the number of mitochondria per embryogenic culture cell was significantly higher than that of leaf cells. This phenomenon merits further study, as do other factors including chondriome segregation, organelle recombination, and the mechanism(s) of nuclear exclusion. In the case of callus + callus protoplast fusion, it appears that both mitochondria and chloroplasts can segregate among progenies. Among 10 analyzed somatic hybrids between 'Willow leaf' mandarin and 'Star Ruby' grapefruit calluses, the same tetraploid hybrid nucleus has been found together with three of four possible cytoplasmic combinations (CIRAD/INRA, France, unpublished data). Thus, the study of material arising from callus + callus hybridizations could provide a more complete understanding of nuclear/cytoplasmic interactions than leaf + callus fusion-derived material.

The agronomic value of citrus cybrids is currently unknown because no horticulturally important traits have yet been associated with organelle genomes in citrus. The development of citrus cybrid callus should increase the range of somatic hybridization parents (Saito et al., 1994; Grosser et al., 1996d). The evaluation of citrus cybrids in the field will allow characterization of agronomic traits encoded by the cytoplasmic genome (Tusa et al., 2000). Mandarin and sweet orange cybrids in the field at the CREC are showing significant variation in agronomically important traits including fruit maturity date and seed content, indicating that cybridization is a potential source of genetic variation for citrus cultivar improvement. Efforts are under way to determine if the seedlessness trait of 'Satsuma' mandarin and 'Navel' orange can be transferred to other seedy cultivars via cybridization (X. X. Deng, personal communication).

Concluding Remarks

Somatic hybridization provides citrus geneticists with another effective tool for developing improved scion and rootstock cultivars. The technique offers a method of producing unique genetic variation, and like any other method of generating variation, some of the resulting variation is useful and some not. A broad-based program maximizes the parental combinations that can be attempted, and therefore increases the chances for success. Selection of parental combinations should be based on current information on citrus germplasm provided by entomologists, geneticists, horticulturists, physiologists, and pathologists, and studies of existing somatic hybrids in the field. Citrus somatic hybrid plants have been produced from more than 200 parental combinations, covering a wide range of germplasm. The most promising application of somatic hybridization for scion improvement is to produce quality tetraploid breeding parents that can be used in a conventional interploid-breeding program to generate seedless triploids. Several somatic hybrid-breeding parents have already flowered and are being used to father triploids in different countries. This application in citrus may subsequently serve as a model for other fruit crops, i.e. for producing bananas with improved disease resistance or improved seedless grapes. Triploid citrus hybrids are also being produced directly by haploid + diploid protoplast fusion, and resulting triploids should have a greater chance in achieving cultivar status. However, the availability of haploid parents presently limits the application of this method. For rootstock improvement, somatic hybridization is successfully being used to combine complementary rootstock germplasm in a unique fashion. Several of the somatic hybrid rootstocks produced to date are performing well in commercial field trials, and some show excellent potential for tree size control. Somatic hybridization is also successfully being used to combine *Citrus* with sexually incompatible or difficult to hybridize genera that possess traits of interest, including *Atalantia*, *Citropsis*, *Feronia*, and *Severinia*, in efforts to expand the germplasm base available for citrus rootstock improvement. More distant crosses at the inter-tribe and subtribe levels seem more difficult to achieve by symmetric somatic hybridization. These applications to rootstock improvement in citrus could also serve as a model for efforts to develop improved rootstocks for other fruit and nut crops, i.e. apples, avocados, blueberries, peaches, etc. In closing, it should be emphasized that as for all cultivar improvement programs, a successful somatic hybridization program requires a complete and long-term effort in which the *in vitro* laboratory is only the 'visible part of the iceberg'; most of the work has to be done in growth chambers, greenhouses, and the field in collaboration with geneticists, pathologists, entomologists, physiologists, agronomists, and growers for evaluation and validation before any new cultivar can be released to the industry. It will be a few more years before we understand the true value of somatic hybridization to citrus cultivar improvement, but we are optimistic from the achieved results so far, and we think that somatic hybridization will indeed have a positive impact on overall citrus cultivar improvement efforts.

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