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Allotetraploid hybrids between *citrus* and seven related genera produced by somatic hybridization

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Abstract We have developed an efficient protoplast-fusion method to produce somatic hybrid allopolyploid plants that combine *Citrus* with seven related genera, including four that are sexually incompatible. In this paper we report the creation of 18 new allotetraploid hybrids of *Citrus*, including ten among sexually incompatible related genera, that may have direct cultivar potential as improved citrus rootstocks. All hybrids were confirmed by cytological and RAPD analyses. If fertile, the attributes of these hybrids may be amenable to further genetic manipulation by breeding at the tetraploid level. Wide somatic hybridization of *Citrus* via protoplast fusion bypasses biological barriers to the natural allopolyploidization of *Citrus*, and creates new evolutionary opportunities that would be difficult or impossible to achieve by natural or conventional hybridization.

Key words *Citrus* cultivar improvement · Germ plasm expansion · Protoplast fusion

Introduction

Citrus is particularly well suited for somatic hybridization studies for several reasons including: protoplast to plant regeneration has been achieved for numerous important cultivars (Vardi et al. 1982; Grosser 1994); sophisticated selection schemes are not required to recover somatic hybrid plants; and somatic hybrid plants have been produced from more than 100 parental combinations, including more than 80 from our program (Grosser et al. 1994 a). Three primary strategies involving somatic hybridization are being used in citrus improvement (Grosser and Gmitter 1990 a). Somatic hybridization is being used to combine complementary elite scion varieties that can be used as pollen parents in interploid sexual hybridization to produce new seedless triploid fruit varieties (Grosser et al. 1992). Examples of triploid citrus fruit varieties include the natural 'Persian lime' (Krug and Bacchi 1943) and artificially created 'Oroblanco' and 'Melogold' pummelo-grapefruit hybrids (Soost and Cameron 1980, 1985). Other strategies using somatic hybridization are designed for citrus rootstock improvement. Citrus trees are almost always budded (grafted) to seedlings of other selected citrus rootstocks that provide better general adaptation, disease resistance, cold-hardiness, and earlier fruit production (Castle 1987). One major strategy for citrus rootstock improvement is to produce and evaluate polyploid somatic hybrid rootstocks that are of interest for tree-size control to reduce harvesting costs, and which may combine positive attributes of complementary genotypes (Grosser and Gmitter 1990 a). It has been shown previously that both auto- and allo-tetraploid rootstocks can have an impact on the ultimate grafted tree size (Lee et al. 1990). Morphological changes associated with polyploidy can be tolerated in rootstocks because these changes are not expressed in diploid scion varieties grafted to them.

Another important strategy for citrus improvement, and the focus of this report, is to combine *Citrus* with related genera that possess important traits. As with many crop plants, related species/genera of domesticated citrus offer a large, but relatively untapped, reservoir of genetic diversity (Swingle and Reece 1967; Grosser and Gmitter 1990 b; Louzada and Grosser 1994). Many of the citrus relatives exist in germ plasm collections, and efforts to collect and preserve additional accessions continue today. However, most *Citrus* relatives do not have direct horticultural potential, and many are sexually incompatible with *Citrus*, so gene introgression by sexual hybridization is impossible. Somatic hybridization has become a practical means to produce intergeneric allotetraploids that have the

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potential for direct use as improved citrus rootstocks. If fertile, wide somatic hybrids may be amenable to further genetic manipulation by conventional breeding at the tetraploid level. A more remote possibility is the development of completely unique fruit types by intergeneric somatic hybridization.

The genus *Citrus* and its close relatives belong to the orange sub-family Aurantioideae, family Rutaceae, a large taxonomic group with two tribes and six sub-tribes, consisting of 33 genera and 203 species (Swingle and Reece 1967). Several close *Citrus* relatives, mostly within Tribe II, the Citreae, Subtribe 2, the Citrinae (citrus fruit trees), offer good disease and nematode resistance, as well as resistance to abiotic factors including low temperature, high salt soil content, drought, and flooding. Within *Citrus*, *C. ichangensis* (West Central and Southwest China) of the Papeda group is considered to be a wild-type *Citrus* species that could contribute cold-hardiness and resistance to *Phytophthora*-induced diseases to rootstock hybrids. Several genera are graft-compatible with *Citrus*, and sexual hybridization of *Citrus* has been successful with *Poncirus* (Central and Northern China), *Fortunella* (Southern China), *Eremocitrus* (Australia), and *Microcitrus* (Australia, New Guinea, Swingle and Reece 1967; Iwamasa et al. 1988). Although *Citrus* relatives have been poorly characterized in general, there are some examples of known valuable attributes that could contribute to citrus improvement (Bitters et al. 1969). *Poncirus* is a well-known source of resistance to citrus tristeza virus (CTV), *Phytophthora*-induced diseases, and the citrus nematode (*Tylenchulus semipenetrans* Cobb), and has been utilized in citrus rootstock improvement by conventional breeding (Castle 1987). *Microcitrus* offers resistance to drought, flooding, the burrowing nematode (*Radopholus citrophilus* Huettel, Dickson and Kaplan) (O'Bannon and Ford 1977), and *Phytophthora*-induced diseases (Carpenter and Furr 1962). *Fortunella* offers cold-hardiness with extended winter dormancy (Swingle and Reece 1967). Among the genera that are sexually incompatible with *Citrus*, *Severinia* (Southern China, Southeast Asia, the Philippines) offers cold resistance, salt and boron tolerance (Cooper 1961), resistance to *Phytophthora* (Carpenter and Furr 1962; Grimm and Hutchison 1977), and nematodes (Baines et al. 1960; Hutchison and O'Bannon 1972). *Atalantia* (Southwestern Asia) is known to perform well in wet soils, suggesting *Phytophthora* resistance, and exhibits good cold-hardiness (Bitters et al. 1969; Campbell 1979). *Citropsis* (Northwest Zaire) offers strong resistance to *Phytophthora*-induced diseases (Swingle and Reece 1967) and the burrowing nematode (Ford and Feder 1960). *Feronia* (Southwest Asia), also a member of the Tribe Citreae, is classified outside of Subtribe 2 into Subtribe 3, the *Balsamocitrinae* (the hard-shelled citroid fruit trees). *Feronia* is known to be drought tolerant and, due to its deciduous nature, it may be a source of genes for cold-hardiness (Swingle and Reece 1967).

Materials and methods

The parental combinations used to generate the 18 new wide somatic hybrids reported herein are described in Table 1. Protoplasts of the embryogenic parent were isolated from either embryogenic suspension cultures maintained on a 2-week culture cycle, or embryogenic callus cultures grown on solid medium maintained on a 6-week culture cycle (Grosser and Gmitter 1990 a). Protoplasts from leaf parents were isolated from young plants grown in either a growth chamber or greenhouse, maintained under high humidity conditions as described previously (Grosser and Gmitter 1990 a). Following protoplast purification by passage through a 45-μm stainless steel filter and centrifugation on a 25% sucrose/13% mannitol gradient, approximately equal volumes of protoplasts from each parental source were mixed and fused according to the polyethylene glycol (PEG) method (Grosser and Gmitter 1990 a). Immediately after fusion, protoplasts were cultured directly in plastic fusion Petri dishes (60×15 mm, Falcon, Lincoln Park, N.J.) in either BH3 medium, EMEP medium, or a 1:1 (v/v) mixture of both (Grosser and Gmitter 1990 a). Osmoticum reduction and somatic-embryo induction were performed as previously described (Grosser and Gmitter 1990 a). Developing somatic embryos were transferred to 1500 medium for enlargement, from which shoots were regenerated on either B+ or DBA3 medium (Deng et al. 1992). Shoots were rooted in Magenta boxes (Magenta Corp., Chicago, Ill.), and rooted plants were transferred to a commercial potting mixture and maintained at high humidity for acclimation (Grosser and Gmitter 1990 a).

Confirmation of hybridity was based on morphology, chromosome number, and molecular-marker analyses (either isozyme or RAPD analysis). The chromosome number of mitotically active meristematic root-tip cells was determined in the regenerated plants using the modified hematoxylin-staining protocol (Grosser and Gmitter 1990 a). Electrophoretic analysis of leaf isozyme banding patterns was conducted using crude leaf tissue extracts from the putative somatic hybrids and parental genotypes on horizontal mixed starch (9.85%) and agarose (0.15%) gels, with a histidine-citrate buffer system (pH 5.7). Electrophoresis was carried out for 3 h at 4°C with a 60 mA constant current. Gels were sliced and stained for peroxidase (Per), phosphogluco-mutase (Pgm), and phosphoglucose isomerase (Pgi) (Vallejos 1983). PCR analyses for RAPD markers were performed with DNA extracted from emerging shoot tips of regenerated plants and parents, using the method of Xiao, submitted. The following random primers were used: A-7, W-4, A-17, Q-20, U-14, V-02, and W-15 (Operon Technologies, Alameda, Calif.). Reaction products were electrophoresed in agarose gels (1.8%) and visualized with UV following staining with ethidium bromide.

Results and discussion

Representative leaf morphology of each class of wide hybrids and parental types is presented in Fig. 1. Leaf morphology of the hybrids is generally intermediate to that of the parental types. As expected, all of the somatic hybrid plants were tetraploid ($2n=4x=36$) according to cytological analysis. All of the somatic hybrid plants showed either complementary isozyme (data not shown) and/or PCR-RAPD (Fig. 2) banding patterns, indicating the expression of genes or the presence of DNA sequences, respectively, from each parent in a corresponding hybrid. In some cases, unique bands were amplified from somatic hybrid DNA. This is the first report of successful somatic hybridization of *Citrus* with *Microcitrus*, and the first successful hybridization of *Citrus* with *Feronia* by any method. Somatic hybrids of *Citrus* with *Citropsis* (Grosser and Gmitter 1990c; Grosser et al. 1990), *Severinia*

Table 1 Parental combinations of 18 new somatic hybrid citrus plants. Hybrids 1–7 and 18 are somatic hybrids of sexually compatible genera, but hybrids 8–17 represent sexually incompatible combinations

Embryogenic parent	Leaf parent	Hybrid plants regenerated
1. *Citrus paradisi* Macf. Red Marsh grapefruit	*Poncirus trifoliata* (L.) Raf. Argentine Trifoliate orange	33
2. *C. paradisi* Red Marsh grapefruit	*Poncirus trifoliata* (L.) Raf. Flying Dragon trifoliate orange	46
3. *Citrus sinensis* (L.) Osbeck Succari sweet orange	*Fortunella crassifolia* Swing. Meiwa kumquat	67
4. *F. crassifolia* Meiwa kumquat	*Citrus reticulata* Blanco Dancy mandarin	37
5. *F. crassifolia* Meiwa kumquat	*Citrus reticulata* Blanco Changsha mandarin	13
6. *C. sinensis* Hamlin sweet orange	*Microcitrus papuana* New Guinea wild lime	38
7. *C. sinensis* Succari sweet orange	*Microcitrus papuana* New Guinea wild lime	107
8. *C. sinensis* Succari sweet orange	*Severinia buxifolia* (Poir.) Tenore Chinese box-orange	35
9. *C. sinensis* Succari sweet orange	*Severinia disticha* (Blanco) Swing Philippine box-orange	3
10. *Citrus sinensis* Hamlin sweet orange	*S. disticha* Philippine box-orange	4
11. *Citrus sinensis* Valencia sweet orange	*S. disticha* Philippine box-orange	3
12. *C. reticulata* Cleopatra mandarin	*S. disticha* Philippine box-orange	2
13. *Nova* tangelo ['Clementine' *C. reticulata* × 'Orlando' tangelo] (*C. paradisi* × *C. reticulata*)	*S. disticha* Philippine box-orange	2
14. *C. sinensis* Succari sweet orange	*Citropsis gilletiana* Swingle & M. Kell. Gillet's cherry orange	55
15. Nova tangelo ['Clementine' × 'Orlando' tangelo']	*Citropsis gilletiana* Gillet's cherry orange	3
16. *C. sinensis* Succari sweet orange	*Atalantia ceylanica* (Arn.) Oliv. Ceylon atalantia	>150
17. *C. sinensis* Succari sweet orange	*Feronia limonia* (L.) Swing. Indian wood apple	<60
18. Nova tangelo ['Clementine' × 'Orlando' tangelo]	*Citrus ichangensis* Swing. Ichang papeda	36

(Grosser et al. 1988a; Grosser and Gmitter 1990a), *Atalantia* (Louzada et al. 1993), *Fortunella* (Deng et al. 1992), and *Poncirus* (Ohgawara et al. 1985; Grosser et al. 1988b; Louzada et al. 1992; Grosser et al. 1994 b) have been reported previously, but the creation of additional hybrids that combine selected representatives of *Citrus* and these genera is well warranted. Plants/plantlets regenerated from somatic hybridization experiments do not always exhibit adequate horticultural viability. Non-vigorous un-rooted somatic hybrid plantlets have been reported that are combinations of *Citrus* with *Feroniella*, and *Swinglea* (Takayanagi et al. 1992), and *Murraya* (Shinozaki et al. 1992). The first two hybrids of 'Hamlin' sweet orange with *Severinia buxifolia* produced by our program are performing inconsistently in commercial rootstock trials, showing a lack of vigor and negative effects on scion nutrition in some replications. Using a more vigorous selection of *S. buxifolia* which became available, the somatic hybrid reported herein with 'Succari' sweet orange is more vigorous than the previous two hybrids and, therefore, has the potential to overcome their deficiencies. This may be attributed to the much more fibrous root system observed with this hybrid as compared to the previous two. Also of interest is the fact that all of the hybrids reported herein made with *Severinia disticha* seedlings were highly susceptible to a ubiquitous fungal disorder that killed all of these plants before they reached a height of 15 cm. This susceptibility may be associated with some negative genetic interaction between the parental genomes in these hybrids, since the same result was observed in all of them, even though four *Citrus* cultivars and two *S. disticha* seedlings were used to make the hybrids. The first two *Citrus* + *Citropsis* hybrids

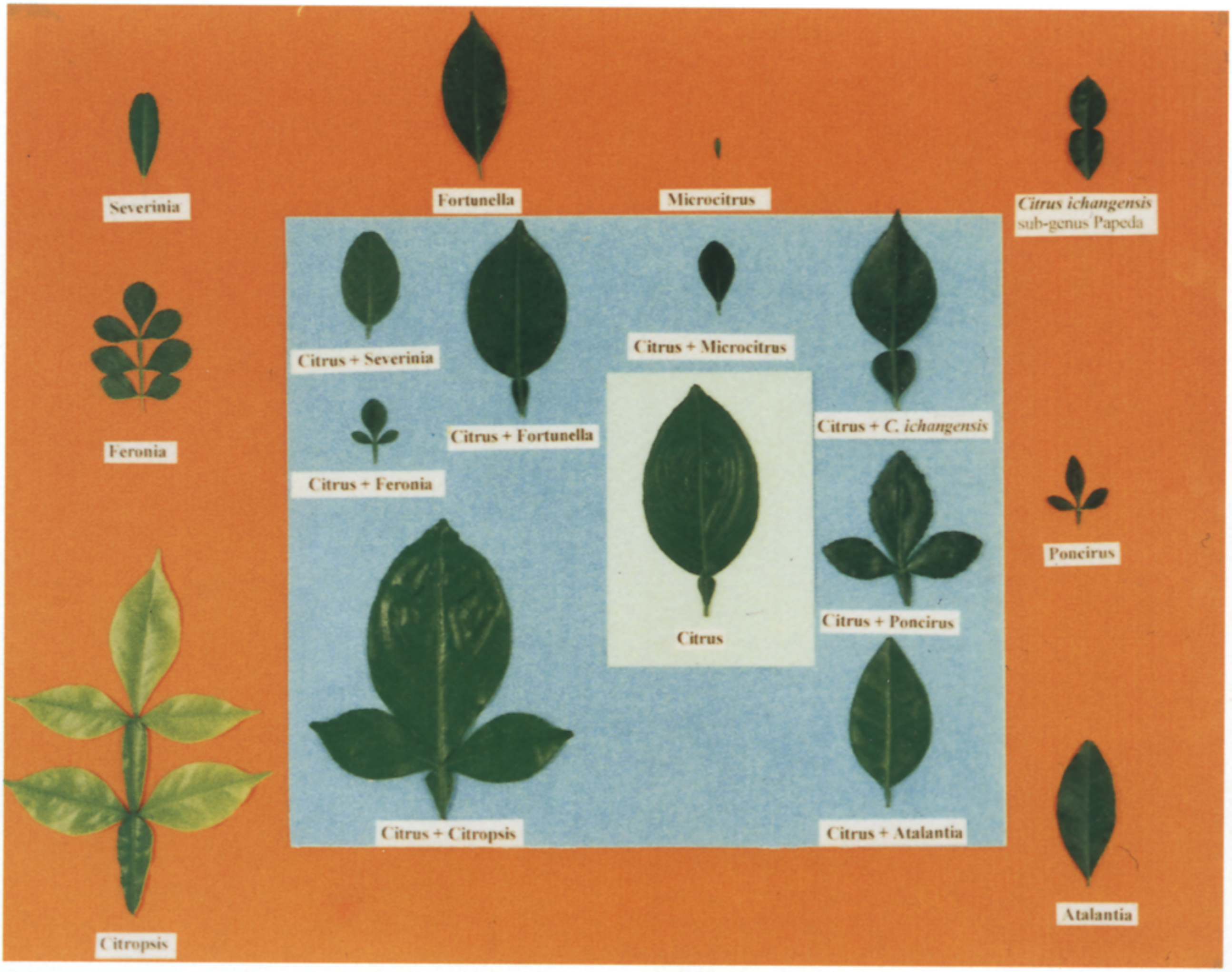

Fig. 1 Representative leaf morphology of *Citrus* and seven related genera, and corresponding intergeneric somatic hybrids with *Citrus*

produced by our program (Grosser and Gmitter 1990c; Grosser et al. 1990) also exhibited problems that are apparently not present in the 'Nova' tangelo + *Citropsis gilletiana* hybrid reported herein. The hybrid of 'Hamlin' sweet orange + *C. gilletiana* has shown severe growth splits in the main trunk. These splits eventually heal over, but they reduce the growth and vigor of the trees and make them more susceptible to herbicide damage. The Cleopatra mandarin + *C. gilletiana* hybrid has unexpectedly shown a high susceptibility to a leaf/stem fungal spotting disease that drastically reduces the vigor of the trees. It is not known if this problem is caused by a negative genomic interaction, or by negative somaclonal variation in the Cleopatra callus line used to make the hybrid. The new 'Nova' tangelo + *C. gilletiana* hybrid is also much more vigorous than the previous two *Citrus* + *Citropsis* hybrids. From our first successful hybridization of 'Hamlin' sweet orange with *Atalantia ceylanica*, only one of three hybrid plants underwent normal morphogenic development. The experiment that resulted in the successful hybridization of 'Succari' sweet orange with *A. ceylanica* reported herein produced more than 150 plants, and they are more vigorous than the previous 'Hamlin' + *Atalantia* hybrid.

Many of the original intergeneric hybrids produced in our program were made with 'Hamlin' sweet orange, due to the availability and performance of the 'Hamlin' callus/suspension line. However, citrus rootstocks are usually propagated by seed, which produce true-to-type plants because of nucellar polyembryony, and 'Hamlin' sweet orange produces very few seed. Therefore, somatic hybrids produced using 'Hamlin' may not produce enough polyembryonic seed for efficient propagation. To increase the potential for polyembryonic seed production in intergeneric hybrids of *Citrus* with *Citropsis*, *Severinia*, *Fortunella*, *Atalantia*, and *Microcitrus*, subsequent hybrids were made with the seedy 'Succari' sweet orange. These examples provide strong evidence that the production of multiple genetically distinct hybrids from any particular combination of genera can help to overcome any problems that may arise from genomic incompatibility, somaclonal variation, horticultural inadequacies, or lack of parental vigor.

Hybrids of grapefruit (*C. paradisi*) with trifoliate orange (*Poncirus trifoliata*) are called citrumelos. Swin-

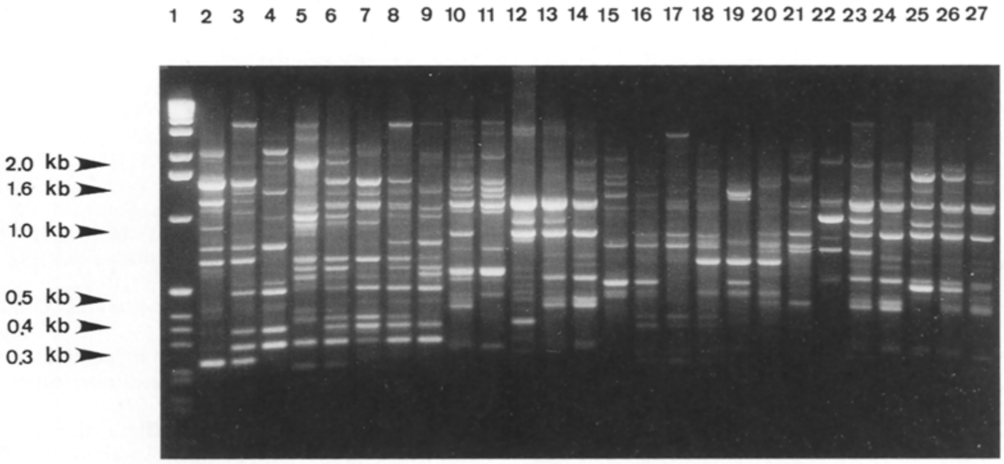

Fig. 2 RAPD patterns of wide somatic hybrids and their parents. *Lane 1* = DNA ladder. Primer identification: *lanes 2–9*, primer A-8; *lanes 10–14*, primer W-15; *lanes 15–21*, primer A-7; *lanes 22–27*, primer W-15. Genotype identification: *lane 2*, *Poncirus trifoliata* 'Argentine'; *lane 3*, *Citrus paradisi* 'Red Marsh' + *P. trifoliata* 'Argentine' somatic hybrid; *lane 4*, *Citrus paradisi* 'Red Marsh' grapefruit ; *lane 5*, *Citrus reticulata* 'Dancy' mandarin; *lane 6*, *Fortunella crassifolia* 'Meiwa' + *C. reticulata* 'Dancy' somatic hybrid; *lane 7*, *Fortunella crassifolia* 'Meiwa' kumquat; *lane 8*, *Fortunella crassifolia* 'Meiwa' + *Citrus sinensis* 'Succari' somatic hybrid; *lane 9*, *Citrus sinensis* 'Succari' sweet orange; *lane 10*, *Citrus sinensis* 'Hamlin' + *Microcitrus papuana* somatic hybrid; *lane 11*, *Microcitrus papuana*; *lane 12*, *Severinia buxifolia*; *lane 13*, *Citrus sinensis* 'Succari' + *Severinia buxifolia* somatic hybrid; *lane 14*, *Citrus sinensis* 'Succari' sweet orange; *lane 15*, 'Nova' tangelo (*C. paradisi* X *C. reticulata*); *lane 16*, 'Nova' tangelo + *Citropsis gilletiana* somatic hybrid; *lane 17*, *Citropsis gilletiana*; *lane 18*, *Citrus sinensis* 'Succari' + *Citropsis gilletiana* somatic hybrid; *lane 19*, *Citrus sinensis* 'Succari' sweet orange; *lane 20*, *Citrus sinensis* 'Succari' + *Atalantia ceylanica* somatic hybrid; *lane 21*, *Atalantia ceylanica*; *lane 22*, *Feronia limonia*; *lane 23*, *Citrus sinensis* 'Succari' + *Feronia limonia* somatic hybrid; *lane 24*, *Citrus sinensis* 'Succari' sweet orange; *lane 25*, *Citrus ichangensis* (papeda); *lane 26*, 'Nova' tangelo + *Citrus ichangensis* somatic hybrid; *lane 27*, 'Nova' tangelo

gle citrumelo has become the premier rootstock in Florida (Castle et al. 1993) because of its excellent disease resistance and yield potential. The two grapefruit + trifoliate orange hybrids reported herein were created in efforts to produce new rootstocks with the attributes of Swingle, but with the potential for tree-size reduction associated with using tetraploid rootstocks. Tree-size control is of great interest to citrus producers for reducing harvesting costs and enhancing the efficiency of cold-protection methods in *Citrus*.

From a genetic perspective, somatic hybridization parallels natural evolutionary processes. Up to 70% of all higher-plant species have been estimated to be polyploid, with the majority being allopolyploid (Gottschalk 1985; Hilu 1993). Natural allopolyploidization is generally believed to involve the fusion of unreduced gametes. Two steps are required for polyploid evolution: interspecific or intergeneric hybridization, and polyploidization. Somatic hybridization accomplishes these two steps simultaneously. The success of polyploids has been attributed to advantages conferred under various ecological conditions due to chromosome multiplicity and genome hybridity (Levin 1983; Hilu 1993). Polyploidization is a mechanism than can allow for gene exchange between species that are reproductively isolated from each other at the diploid level, due to the fertility that results from autosynthetic chromosome pairing in polyploid hybrids (De Wet 1971). Polyploidy apparently has not been a force in the evolution of *Citrus* and closely related genera, as species generally have a 2n chromosome number of 18. Polyploidy occurs infrequently in the Rutaceae (of which *Citrus* is a member), but there are examples, including *Xanthoxylum* (2n=64–72) (Moore 1973). This natural lack of polyploidy could be due at least in part to the widespread apomixis that exists in *Citrus* (nucellar polyembryony), which would minimize natural hybridization and the participation of unreduced gametes in reproduction. Somatic hybridization takes advantage of the embryogenic potential in *Citrus*, resulting from the phenomenon of nucellar polyembryony, as a means of plant regeneration following protoplast fusion, and also bypasses sexual incompatibility mechanisms that inhibit the natural wide hybridization and polyploidization of *Citrus*. Somatic wide hybridization of *Citrus* therefore offers a means of providing new evolutionary opportunities by the creation of unique allotetraploid interspecific and intergeneric somatic hybrids that may be better adapted to specific ecosystems imposing both man-made and natural selection pressures.

All of the hybrids described in Table 1, with the exception of the *Citrus* + *S. disticha* hybrids that all died, have been propagated and entered into field trials to evaluate their rootstock potential. Grafted seed trees of each hybrid are also being grown out to determine if the hybrids can be subsequently propagated via nucellar seed. Performance of these hybrids will provide valuable feedback regarding future somatic hybridization experiments. Additional somatic hybrids of *Citrus* with these and other related genera will expand further the germ plasm base contributing to citrus variety improvement. The advances made in the development and application of somatic hybridization techniques in citrus suggest a potential for parallel activities with other important plant crops, especially crops that utilize rootstocks, have a narrow cultivated germ plasm base, or where natural allopolyploidization has been inhibited by biological mechanisms. Successful applications of artificially induced polyploid evolution, as a mechanism for genetic introgression from wild to cultivated crop forms, may in turn encourage expanded efforts to preserve the germ plasm resources of wild relatives for important plants.

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