Production of New Allotetraploid and Autotetraploid Citrus Breeding Parents: Focus on Zipperskin Mandarins

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Abstract. **Somatic hybridization through protoplast fusion has proven to be a valuable technique in citrus for producing unique allotetraploid breeding parents that combine elite diploid selections. Many citrus somatic hybrids are now flowering and being used in interploid crosses to generate triploid hybrids that produce seedless fruit, a primary objective of citrus breeding programs. Most of the early somatic hybrids produced for mandarin improvement combined sweet oranges with mandarins, because the performance of sweet oranges in tissue/protoplast culture generally exceeds that of most mandarin selections. However, a high percentage of triploid progeny from interploid crosses using sweet orange + mandarin somatic hybrids as the tetraploid parent produce fruit that are difficult to peel. We report nine new allotetraploid somatic hybrids and five new autotetraploids from somatic fusion experiments involving easy-peel mandarin parents. These tetraploids can be used in interploid crosses to increase the percentage of seedless triploid progeny producing easy-to-peel fruit. Ploidy level of the new tetraploids was determined by flow cytometry and their genetic origin by expressed sequence tag–simple sequence repeat marker analysis.**

Somatic hybridization in citrus using the embryogenic suspension protoplast plus leaf protoplast fusion model can be considered a reliable technique, because hundreds of allotetraploid somatic hybrids have been produced for both scion and rootstock improvement (Grosser and Gmitter, 1990, 2005; Grosser et al., 2000). Somatic cybrids are a byproduct of the technique, and diploid cybrids have been produced from more than 50 parental combinations (Guo et al., 2004a). New somatic hybrids rarely have direct use as improved cultivars (Guo et al., 2004b); and the most important application of somatic hybridization is the building of novel germplasm as a source of elite breeding parents for various types of conventional crosses, including interploid crosses for triploid production (Grosser and Gmitter, 2009). In fact, citrus somatic hybridization has proven to be a key tool to generate allotetraploid breeding parents for use in interploid crosses to generate seedless triploids for mandarin, grapefruit/pummelo, and acid-fruit (lemon/lime) improvement (Grosser and Gmitter, 2005, 2009; Viloria and Grosser, 2005). The Citrus Research and Education Center (CREC) interploid crossing program has to date used 16 allotetraploid somatic hybrid parents for triploid mandarin hybrid production, resulting in several thousand triploid hybrids. However, 13 of the 16 hybrids used in such crosses have been somatic hybrids of sweet orange with a mandarin-type parent (Grosser and Chandler, 2004; Grosser and Gmitter, 2009; Grosser et al., 1992, 1998; Mourao et al., 1996). This was the result of the excellent performance of sweet orange regarding embryogenic culture establishment and performance in our protoplast culture system. Triploid hybrids recovered from crosses of mandarin with autotetraploid sweet oranges (Gmitter et al., 1991) or the mentioned somatic hybrids have recently begun to fruit, and although several have exhibited good fruit quality, most are difficult to peel. This is attributed for the most part to the sweet orange component of the parentage. Of the 16 somatic hybrid parents used, only one was produced from two easy-peel parents ('Nova' + 'Osceola'), and a few triploid hybrids from crosses involving this somatic hybrid flowered and fruited for the first time this year. Another allotetraploid somatic hybrid of easy-peel parents, 'Encore' mandarin hybrid + 'Caffin' Clementine mandarin, was recently reported by Wu et al. (2005). We recently reported the development of autotetraploid 'Ponkan' using an in vitro colchicine treatment (Dutt et al., 2009a).

In addition to seedlessness, there are a number of other important breeding objectives for fresh mandarin improvement. Successful fresh fruit mandarin cultivars are generally soft fruit with a narrow harvest window and limited shelf life in the marketplace. Therefore, objectives include expanding the season of availability (both early and late), improved shelf life, and improved disease resistance. Satsumas are generally the earliest maturing mandarins in the marketplace; however, they do not perform well in subtropical climates such as the primary citrus-growing areas of Florida. Combining Satsuma with better adapted germplasm at the triploid level should provide Florida growers good opportunities to compete in the early-season market. Satsumas are considered to be among the most cold-hardy citrus scions (Deng et al., 2008), and inclusion of Satsuma germplasm in subsequent triploids should improve their cold-hardiness. However, Satsuma is susceptible to citrus scab disease caused by the fungus *Elsinoe fawcettii* Bitancourt & Jenk. This disease causes ugly scab-like blemishes on fruit, reducing their attractiveness for the fresh market. Selected hybrids with scab-resistant parents such as 'Willowleaf' mandarin could minimize or eliminate this problem (Wu et al., 2005). Thus, the focus of our somatic fusion program for scion improvement shifted to the

production of new allotetraploid hybrids that combine elite diploid easy-peel parents. We report the use of established stable embryogenic cultures of Satsumas and W. Murcott, also known as Nadorcott (Nadori, 2006) as the source of totipotent parents in somatic fusions with selected easy-peel leaf parents to regenerate allotetraploid somatic hybrid plants from nine parental combinations. In addition, we report on regenerated autotetraploid plants from five of the parents. All of these tetraploids should have great value for subsequent use in our interploid breeding program to generate improved seedless triploid easy-peel mandarin hybrids for the fresh market.

Materials and Methods

Explant for protoplast isolation. Embryogenic suspension cultures of 'Guoqing No.1', 'Okitsu Wase', and 'Miyagawa Wase' Satsumas (*Citrus unshiu* Marc.) and 'W. Murcott' hybrid tangor [purported (*C. reticulata* Blanco × *C. sinensis* L. Osbeck) × *C. reticulata* Blanco] were initiated from unfertilized ovule-derived friable embryogenic callus cultures maintained in the citrus embryogenic callus collection of the University of Florida's CREC (Grosser and Gmitter, 1990). The 'W. Murcott' callus line was initiated at the CREC, the Satsuma lines at NISA (Republic of Korea), and the 'Guoqing No.1' line was kindly provided by Dr. Wenwu Guo (Huazhong Agricultural University, Wuhan, P.R. China). Suspension-derived protoplasts were isolated from 6-month-old to 3-year-old suspension cultures maintained in H+H medium on a 2-week subculture cycle with protoplasts isolated during Days 4 to 12 (Grosser and Gmitter, 1990).

To provide sterile explant for leaf protoplast isolation, fruits of 'W. Murcott' tangor, 'Furr' mandarin hybrid [irradiated selection of 'Clementine' mandarin (*C. reticulata* Blanco) × 'Murcott' tangor (purported *C. reticulata* Blanco × *C. sinsensis* L. Osbeck)], 'Willowleaf' mandarin (*C. deliciosa* Tenore), 'Ponkan' mandarin [*C. reticulata* Blanco, 'Fremont' ('Clementine' × 'Ponkan')], 'Osceola' tangelo ['Clementine' × 'Orlando' tangelo (*C. reticulata* Blanco × *C. paradisi* Macf.)], 'FG#303' ['Lee' tangelo ('Clementine' × 'Orlando') × 'Murcott' tangor], 'FG#304' ['Lee' × 'Nova ('Clementine' × 'Orlando')], and UF-03B {'Fortune' tangerine [purported 'Clementine' × 'Dancy' (*C. reticulata* Blanco)] × 'Murcott'} were washed thoroughly and surface-disinfected in 50% Clorox regular bleach (3% sodium hypochlorite) for 30 min, followed by a rinse in sterile water, then fruit was sprayed with 95% ethanol. Fruits were cut with a sharp knife at the equatorial zone, avoiding the core where seeds are embedded. Fruits were twisted with each half in opposite directions until separated to allow for removal of intact seeds. Seeds were germinated and seedlings were maintained in Magenta vessels GA-7 (Magenta Corporation, Chicago, IL) containing 50 mL RMA medium (Grosser and Gmitter, 1990) and

sealed with Nescofilm®. Tender, expanded leaves from the cultured nucellar seedlings were used for protoplast isolation. Shoots of the seedlings were subcultured as needed to provide a continuous source of sterile leaves.

Protoplast isolation and fusion. Protoplasts were isolated from the parental suspension cultures in a 2.5:1.5 (v:v) mixture of 0.7 M BH3 protoplast culture medium and enzyme solution according to Grosser and Gmitter (1990). Sterile leaves from in vitro-cultured nucellar seedlings were feather-cut with a sharp scalpel and incubated overnight (including a 25-min vacuum infiltration) in a 8:3 (v:v) mixture of 0.6 M BH3 protoplast culture medium and modified enzyme solution (Grosser and Gmitter, 1990). Modification of the enzyme solution included the elimination of Pectolyase Y-23 and the doubling of Onozuka RS cellulose and Macerozyme R-10 from 1% to 2%. This modification was necessary because commercial suppliers of Pectolyase Y-23 changed fungal sources, and the new product no longer facilitated citrus protoplast isolation. Protoplasts from both suspension and leaf sources were purified by passage through a 45-µm stainless steel mesh screen and then by centrifugation on 25% sucrose and 13% mannitol gradient (Grosser and Gmitter, 1990).

Our highly successful model method of fusing embryogenic suspension culture-derived protoplasts of one parent with leaf-derived protoplasts of the second parent was used in all experiments (Grosser and Gmitter, 1990; Grosser et al., 2000). Fusions were conducted in 60 × 15-mm polystyrene petri dishes using our standard 40% polyethylene glycol volumetric plating method (Grosser and Gmitter, 1990). After fusion, protoplasts were cultured in a 1:1 (v:v) mixture of 0.6M BH3 and 0.6M EME protoplast culture media (Grosser and Gmitter, 1990), and petri dishes sealed with Nescofilm® were maintained in plastic boxes under low light in a tissue culture room.

Plant regeneration and ploidy analysis. Regenerating embryogenic calli and embryoids were transferred to solid EME medium containing 50 g·L^{-1} maltose (Perez et al., 1998) for somatic embryo induction according to Grosser and Gmitter (1990). Regenerated embryos were cultured over 0.22-mm cellulose acetate membrane filters placed on fresh plates of EME-maltose solid medium to normalize and enlarge the embryos (Niedz et al., 2002). After 4 weeks, embryos were cultured for one passage on 1500 embryo enlargement medium followed by one or two passages on B+ embryo germination medium (Grosser and Gmitter, 1990). Large embryos that failed to germinate, often exhibiting abnormal shapes, were sliced horizontally with a sharp scalpel and transferred directly to DBA3 medium for adventitious shoot induction (Deng et al., 1992). Recovered adventitious shoots and shoots from germinated embryos with poor root systems were cut and rooted on RMAN medium in Magenta boxes (Grosser and Gmitter, 1990). Germinated embryos with good roots were also transferred to RMAN

for plantlet enlargement. Recovered rooted plantlets were screened for ploidy level using a tabletop Partec flow cytometer (Model D-48161; Münster, Germany) as previously described (Khan and Grosser, 2004).

Micrografting to accelerate whole plant recovery. Commercially available Carrizo citrange (*C. sinensis* Osb. × *Poncirus trifoliata* L. Raf.) rootstock and experimental Tetrazyg Orange #16 {['Nova' + Hirado Buntan pummelo] somatic hybrid × ['Cleopatra' mandarin (*C. reticulata* Blanco) + *P. trifoliata*] somatic hybrid} rootstock (Grosser et al., 2003) were grown in 38-welled containers in commercial potting soil. Both rootstocks exhibit trifoliate leaves, so it is easy to identify and remove unwanted rootstock sprouts from the young trees in their early development. Shoot tips from recovered in vitro tetraploid plantlets ≈5 mm long with leaves were excised and maintained in a moist environment until grafting to prevent desiccation. Decapitated rootstocks ≈100 mm in height were cut vertically, just right of center, ≈2 to 3 mm deep. Tetraploid shoot tips were trimmed to form a "V" cut also ≈2 to 3 mm in length. Scion shoot tips were then inserted into the vertical slits in the rootstocks. The graft union was carefully wrapped with Nescofilm® (Dutt et al., 2009b), and the micrografts were covered snugly with micropipet tips to provide a mini-humidity dome (Skaria, 2000). After 2 to 3 weeks in a heavily shaded greenhouse, pipet tips were removed to allow shoot growth. After 2 additional weeks, plants were transferred to normal greenhouse conditions and subsequently transferred to larger pots as necessary.

Expressed sequence tag–simple sequence repeat primers and genotyping procedure for somatic hybrid and autotetraploid verification. The eight expressed sequence tag–simple sequence repeat (EST-SSR) primers, CX6F04, CX6F18, CX6F29, CX5F57, CX0010, CX0035, CX2007, and CX2021, used in this study, along with the genotyping procedure, were previously developed (Chen et al., 2006, 2008a). In brief, fluorescent polymerase chain reaction products were run on a Genetic Analyzer 3130xl (Applied Biosystems, Foster City, CA) to generate chromatographic files. The alleles of each EST-SSR marker in the regenerated plants were scored from the files using the GeneMark software (SoftGenetics, State College, PA). An allotetraploid somatic hybrid is defined only when two donor parents have distinct alleles and the alleles in the progeny at any locus are the combination of the alleles from the parents (in an addition fashion). A locus with four distinct alleles in two parents can simultaneously define the allele parental source and tetraploid level in such a progeny. The ploidy effect, represented by the height of a peak (like a in aaab from aa + ab genotype parents), can be supportive, not confirmative.

Results

After protoplast isolation, fusion, and culture, ploidy analysis through flow cytometry

was conducted on regenerating plantlets to identify tetraploids. All recovered tetraploid plantlets were micrografted to vigorous trifoliate-leaved rootstocks to expedite whole plant recovery and growth. Tetrazyg rootstock candidate 'Orange #16' proved to be ideal for micrografting, because it produces 30 nucellar seed per fruit, and the seedlings are vigorous with thick, sturdy stems (Grosser et al., 2003). Micrografting success was over 90% on both Carrizo citrange and tetrazyg Orange #16. These rootstocks also provide the advantage of having trifoliate leaves, facilitating the identification and removal of rootstock sprouts.

DNA was isolated from leaves of recovered tetraploid plants when they reached a minimum height of 10 cm. The genetic identity of the recovered tetraploids was determined by EST-SSR analysis. Somatic hybrid plants were recovered from the nine parental combinations listed in Table 1. Somatic hybrids were easily validated by the presence of alleles unique to each parent at any of the tested loci (Table 2). More than two alleles at any of the tested loci also validated the polyploid nature of the plant. An unexpected finding was that the SSR profiles of the Satsuma embryogenic callus lines did not completely match the allelic profiles of Satsuma parent-type leaf DNA. At loci where this occurred, there was always an allele in common with true Satsuma, suggesting that these callus lines are of zygotic origin. This happened previously with an embryogenic callus line of 'Page' tangelo ['Minneola' tangelo (*C. reticulata* Blanco × *C. paradisi* Macf.) × 'Clementine' mandarin (*C. reticulata* Blanco)] also used to make somatic hybrids (Chen et al., 2008b). Thus, although these Satsuma embryogenic callus lines were initiated from undeveloped ovules, such ovules may still contain zygotic embryos that possibly have a higher propensity to form embryogenic callus. This information indicates that the genetic origin of all new embryogenic callus lines should be validated by SSR or some other appropriate analysis to validate their true-to-typeness. Somatic hybrids produced using these Satsuma callus lines therefore contain genomic contributions of unidentified origin.

Autotetraploid plants were also recovered from the five parental combinations listed in Table 3, also validated by EST-SSR analysis (Table 4). We previously reported the use of EST-SSR analysis to validate citrus somatic hybrids for rootstock improvement (Chen et al., 2008b); this is the first report using the technique to validate citrus somatic hybrids for scion improvement.

Three of the five autotetraploids, namely 'Furr', 'Fremont', and 'FG#304', were regenerated from the leaf-protoplast parent, which is unexpected because unfused citrus leaf protoplasts do not typically regenerate. These autotetraploids are expected to contain the mitochondria (mt) genome of the embryogenic culture parent, because citrus somatic hybrids and diploid cybrid plants exhibiting the leaf-parent morphology always exhibit the mt genome of the embryogenic culture parent (Cabasson et al., 2001; Grosser et al.,

Table 1. Parental combinations of somatic fusions resulting in the production of new allotetraploid somatic hybrid citrus plants.

Embryogenic parent	Leaf parent	Nursery vigor[z]
'Guoqing No. 1' Satsuma	'W. Murcott' tangor	H
'Okitsu Wase' Satsuma	'Willowleaf' mandarin	H
'Miyagawa Wase' Satsuma	'Willowleaf'	M
'Okitsu Wase'	'W. Murcott'	M
'Okitsu Wase'	'Ponkan' mandarin	L
'Okitsu Wase'	'Osecola' ('Clementine' × 'Orlando')	L
'Okitsu Wase'	'Furr' ('Clementine' × 'Murcott')	L
'W. Murcott' tangor	FG#303 ['Lee' ('Clementine' × 'Orlando') × 'Murcott' tangor]	M
'W. Murcott' tangor	UF-03B ('Fortune' ('Clementine' × 'Dancy') × 'Murcott' tangor	H

[z]Nursery vigor: H = high; M = medium; L = low.

Table 2. Expressed sequence tag–simple sequence repeat primers for confirmation of allotetraploid citrus somatic hybrids.[z]

Locus: plant genotype	CX2007			CX2021			CX6F29			CX5F57		Confirmative markers[y]
Okitsu	174	175		148	150		151		154		167	
Willowleaf Mandarin	177			150	152		151	154		156		3
Okitsu + Willowleaf Mandarin	**174**	**175**	**177**	148	150	**152**	151	154		**156**	167	
W Murcott	177			148	152		**153**	154		156	167	3
Okitsu + WMurcott	**174**	**175**	**177**	148	150	**152**	151	**153**	154	156	167	
Ponkan	—	—	—	148	151		153			156	166	2
Okitsu + Ponkan	—	—	—	148	150	**151**	151	**153**	**154**	156	166	
Osceola	—	—	—	148	150		151	153		156	167	2
Okitsu + Osceola	—	—	—	148	150		151	**153**	**154**	156	167	
Furr	171			148	150		151	153		156	167	3
Okitsu + Furr	**171**	174	175	148	150		151	**153**	**154**	**156**	167	
W Murcott	177			148	152		153	154		156	167	
FG303	174	177		150			151	153		156	167	3
W Murcott + FG303	**174**	177		**148**	**150**	152	151	**153**	**154**	156	167	
UF03	171			148	150		144	154		156	167	3
W Murcott + UF03	**171**	177		148	**150**	152	144	**153**	**154**	156	167	
G1 Satsuma	174	175		152			—	—	—	—	—	
WMurcott	177			148	152		—	—	—	—	—	2
G1 Satsuma + WMurcott	**174**	**175**	**177**	148	152		—	—	—	—	—	

[z]There were eight markers used but only confirmative four listed. Each parent in italic is listed once but used in all the combinations. The alleles in bold can define somatic hybrids because at least one is distinct between two parents.
[y]The number stands for how many markers validated the somatic hybrids from each combination.
— = Either not scorable (CX2007) or not ran (CX6F29 and CX5F57).

Table 3. Parental combinations of somatic fusions resulting in the regeneration of new citrus autotetraploids.

Embryogenic parent	Leaf parent	Nursery vigor[z]
'Guoqing No. 1' Satsuma	**'Furr' mandarin**[y]	H
'Guoqing No. 1' Satsuma	**'FG#304'**	M
'Guoqing No. 1' Satsuma	**'Fremont'**	M
'W. Murcott'	'Furr'	M
'Miyagawa' Satsuma	'Willowleaf' mandarin	L

[z]Nursery vigor: H = high; M = medium; L = low.
[y]Bold type indicates doubled nuclear genome of regenerated autotetraploid plants.

1996; Moreira et al., 2000a, 2000b). Because the parents used in this study are so closely related, it has been difficult to find mt genome markers that can distinguish the mt genotypes. Work is underway to develop the distinguishing mt genome markers as needed to determine the mt genome inheritance in all the new tetraploids reported in this study.

The next step in using these new tetraploid breeding parents in our scion improvement program is to accelerate their development through juvenility to encourage more rapid flowering. In addition to planting the new tetraploids in the field, propagated clonal plants of each tetraploid will be subjected to

various horticultural manipulations in efforts to induce early flowering. Once flowering, the hybrids will be used as pollen parents in our ongoing interploid hybridization program to generate seedless triploids.

In summary, somatic hybridization through protoplast fusion was used to develop nine new allotetraploid somatic hybrid and five autotetraploid mandarin breeding parents for use in interploid crosses, all from parents that exhibit the easy-peel characteristic. Use of these tetraploids in interploid hybridizations is expected to increase the percentages of seedless triploid progeny that will be easy-peelers. This new germplasm is expected to contribute

Table 4. Expressed sequence tag–simple sequence repeat primers for confirmation of autotetraploid citrus plants recovered following somatic fusion.[z]

	CX0010		CX0035		CX2007		CX2021		Confirmative markers[y]
G1 Satsuma	230		172	181	174	175	152		
Furr	**220**	230	172	181	**171**		**148**	**150**	3
G1 Satsuma + Furr	220	230	172	181	171		148	150	
FG304	**220**	230	172	181	174	**177**	**150**		3
G1 Satsuma + FG304	220	230	172	181	174	177	150		
Fremont	**220**	230	172	181	174	**177**	**148**	**150**	3
G1 Satsuma + Fremont	220	230	172	181	174	177	148	150	
WMurcott	220	230	172	181	**177**		**148**	**152**	2
Furr	220	230	172	181	171		148	150	
WMurcott + Furr	220	230	172	181	177		148	152	
Miyagawa	220	230	172	**181**	174	175	**148**	**150**	3
Willowleaf Mandarin	220	230	172		177		150	152	
Miyagawa + WillowLeaf	220	230	172	181	174	175	148	150	

[z]The parent in italic is listed once but used in the combinations below. The alleles in bold can define the autotetraploids because they are unique to the diploid parent.

[y]The number of stands for how many markers validated the autotetraploids from each combination.

substantially to our scion improvement efforts by providing opportunities for improved disease resistance and cold-hardiness and expanded fruit maturation dates in seedless selections.

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