Symmetric and Asymmetric Somatic Hybridization in Citrus: Review

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ABSTRACT

Somatic hybridization by nuclear or cytoplasmic fusions leads to three distinct possible outcomes -- [a] "symmetric" hybrid: fusion of whole nuclei, [b] "asymmetric" hybrid: fusion of parental nuclei, one or both deficient for one or more chromosome(s), and [c] "cybrid": fusion without a nuclear contribution by one parent, i.e., an extremely asymmetric hybrid. Somatic hybridization in citrus has been a powerful tool for the production of novel allotetraploid somatic genotypes that combine desirable characteristics of two parents. These hybrids open new opportunities for sexual hybridization at the tetraploid level, and for interploid crosses for the production of potentially seedless triploids, among other uses. Somatic symmetric hybrids have great potential for rootstock improvement and are excellent as breeding parents, but they may have limited direct application as scion cultivars because they typically contain the entire genomes of both fusion parents, and are likely to exhibit the desirable and undesirable traits of both parents. Asymmetric hybridization is very promising as it allows partial genome transfer and may be better tolerated than a whole-genome transfer. A brief review about symmetric and asymmetric somatic hybridization and cybridization in citrus is presented. Somatic hybridization seems to be a great tool for citrus breeding. However, the release of new improved cultivars produced by this technology is what is going to prove its effectiveness.

Index terms: protoplast fusion; hybrids; cybrids; genetic improvement; citrus breeding.

RESUMO

Hibridação Simétrica e Assimétrica em citros: Revisão

Hibridação somática por fusão nuclear ou citoplasmática apresenta três possíveis resultados -- [a] fusão simétrica de núcleos inteiros, [b] fusão assimétrica de núcleos parentais, sendo um ou ambos deficientes para um ou mais cromossomos, e [c] "cíbrido": fusão sem contribuição nuclear de um dos parentais, a qual também pode ser considerada como assimétrica. Hibridação somática em citros tem sido uma ponderosa ferramenta para produção de novos genótipos alotetraplóides somáticos, combinando características desejáveis de dois parentais. Tais híbridos oferecem possibilidades de hibridação sexual a nível tetraplóide e para cruzamentos interplóides visando produção de triplóides potencialmente sem sementes, além de outros usos. Híbridos

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simétricos somáticos apresentam grande potencial para o melhoramento de porta-enxertos e são excelentes como parentais para melhoramento genético, mas podem apresentar limitada aplicação direta como cultivares copa, uma vez que contém genomas dos dois parentais, onde tanto as características desejáveis como as indesejáveis podem ser expressas. Hibridação assimétrica é muito promissora já que permite a transferência parcial do genoma e pode ser mais tolerada do que a transferência completa do genoma doador. Uma breve revisão sobre hibridação somática simétrica e assimétrica em citros é apresentada. Hibridações somáticas se apresentam como uma importante ferramenta no melhoramento de citros. Todavia, a liberação de novos cultivares melhorados produzidos por tal tecnologia é o que comprovará sua eficiência.

Termos de indexação: fusão de protoplastos; híbridos; cíbridos; melhoramento genético; cruzamentos em citros.

INTRODUCTION

Diversity among extant *Citrus* and related genera seems to offer opportunities to create new, improved types of citrus, however, diversity among commercially used scions and rootstocks is low (Machado et al., 2005). Therefore, many programs have invested in scion and rootstock breeding, mainly focused on improvement of grapefruits (*C. paradisi* Macf.), sweet oranges (*C. sinensis* L. Osb.) and mandarins (*C. reticulata* Blanco), the most commercially important *Citrus* species (Davies & Albrigo, 1994; Louzada et al., 2002).

However, despite breeding efforts and the diversity of *Citrus* and related genera (Carvalho et al., 2005), most of the current cultivars originated from natural or induced mutations rather than from sexual breeding (Grosser & Gmitter Jr., 1990a; Grosser & Gmitter Jr., 1990b; Rouse et al., 2001; Talon & Gmitter Jr., 2008), which is considered difficult for most *Citrus* species. The prevalence of asexual embryos, the long juvenile periods, ranging on average from five to 15 years (Davies & Albrigo, 1994; Grosser & Gmitter Jr., 1999), various degrees of pollen or ovary sterility in many species, highly heterozygozity, and few important traits with single-gene inheritance patterns (Louzada et al., 2002) are some of the barriers to conventional breeding. When crosses are possible, the resulting offspring are typically variable and replete with unexpected and undesirable types, and deleterious recessive gene combinations contribute to significant inbreeding depression (Furr, 1969; Grosser & Gmitter Jr., 1999; Deng et al., 2000;). Where nucellar embryony is prevalent, particularly in sweet orange, grapefruit and lemon (Davies & Albrigo, 1994; Ollitrault et al., 2000a), the sexual embryos often die for lack of nourishment, which makes the construction of hybrid populations problematic (Grosser & Gmitter Jr., 1999; Louzada et al., 2001).

SOMATIC HYBRIDIZATION

Somatic hybridization by protoplast fusion, on the other hand, has been a powerful tool in genetic improvement (Mendes *et al*, 2001). It has overcome many problems related to *Citrus* reproductive characteristics, allowing the creation of novel genotypes (Grosser & Gmitter Jr., 1990a,b; Gmitter Jr. et al., 1992; Grosser et al., 1998, Grosser et al., 1998a,b; Grosser & Gmitter Jr., 1999).

SYMMETRIC SOMATIC HYBRIDIZATION

The first symmetric somatic hybrid of citrus was created by protoplast fusion of *C. sinensis* and *Poncirus trifoliata* (Ohgawara et al., 1985) and the production of hybrid plants between two sexually incompatible *Citrus* genera was first reported in 1988, where *C. sinensis* L. Osb. cv. 'Hamlin' protoplasts were fused with *Severinia disticha* (Blanco) Swing protoplasts (Grosser et al., 1988). After that, production of many inter- and intra-specific, inter- and intra-generic and inter-tribal symmetric somatic hybrids have been reported (Saito et al., 1993; Miranda et al., 1997; Carneiro et al., 1998; Liu & Deng, 2000; Mendes-da-Glória et al., 2000; Cabasson et al., 2001; Mendes et al., 2001; Liu & Deng, 2002; Chen et al., 2004; Guo et al., 2004; Takami et al., 2004; Xu et al., 2004; Liu et al., 2005; Xu et al., 2005; Wu et al., 2005; Guo et al., 2006; Bona et al., 2009a; Cai et al., 2009). The most important application of somatic hybridization has been the building of novel germplasm as a source of elite breeding parents for interploid crosses and for crosses at the tetraploid level (Bona et al., 2009a; Wu & Mooney, 2002), however, a few tetraploid hybrid combinations have been showing good characteristics for direct use as scions (Grosser, personal communication).

The technique is well established for *Citrus* (Grosser et al., 2000) and the somatic hybrids have been produced to improve a series of targeted horticultural traits. For example, fused protoplasts of 'Bonanza' navel orange (*C. sinensis*) with 'Red Blush' grapefruit (*C. paradisi*) regenerated plants that flowered precociously (Guo et al., 2000). Plants were obtained from protoplast fusions of 'Rangpur' lime (*C. limonia* L. Osb.) and 'Caipira' sweet orange (*C. sinensis*), to combine the drought tolerance and vigor from the 'Rangpur' lime with the blight tolerance of 'Caipira' sweet orange (Mendes-da-Gloria et al., 2000). 'Hamlin' sweet orange and 'Singapura' pummelo (*C. grandis* L. Osb.) were fused-targeting blight, Citrus tristeza virus (CTV), and *Phytophthora*-induced disease tolerance (Calixto et al., 2004). Hybrids were regenerated from fused *C. micrantha* Wester, a progenitor of lime, with sweet orange to recreate a lime-like fruit using the sweet orange as source of resistance against Witches' broom disease of lime (WBDL) (Khan & Grosser, 2004). Allotetraploid somatic hybrid plants of 'Hamlin' sweet orange (*Citrus sinensis*) + 'Montenegrina' mandarin (*Citrus deliciosa* Ten.) were produced to improve tolerance to Asiatic citrus canker (ACC), caused by *Xanthomonas axonopodis* pv. *citri* and citrus variegated chlorosis (CVC), caused by *Xylella fastidiosa* (Pavan et al., 2007). Intergeneric somatic hybrids between round kumquat (*Fortunella japonica* Swingle) and 'Morita' navel orange were produced to create breeding parents for interploid crosses aiming to release new seedless kumquat-like cultivars for Japanese market (Takami et al., 2004).

Although somatic hybridization techniques are still ignored by a number of improvement programs for most commodities, their contribution to citrus variety improvement continues to expand. Citrus is one of the few commodities where somatic hybridization is considered to be reaching its predicted potential, as somatic hybrid citrus plants from more than 500 parental combinations have been produced (Grosser & Gmitter Jr., 2009) and more than 16,000 triploid citrus genotypes were produced by interploid crosses using somatic hybrids as parents (Grosser, personal communication).

Major *Citrus* interespecific and intergeneric symmetric somatic hybridizations performed are presented in tables 1 and 2, respectively.

Table 1. *Citrus* interspecific somatic hybrids, in chronological order.

Fusion parents	References
'Washington Navel' sweet orange + 'Murcott' tangor (*C. reticulata* x *C. sinensis*)	Kobayashi, 1988
'Key' lime (*C. aurantifolia* (Christm.) Swingle) + 'Valencia' sweet orange	Grosser et al., 1989
'Bahia' sweet orange + 'Marsh seedless' grapefruit	Ohgawara et al., 1989
Citrus sudachi Hort. ex Shirai + *Citrus latifolia* Tan.	Saito et al., 1991
'Hamlin' sweet orange + Rough lemon (*C. jambhiri* Lush.) 'Valencia' sweet orange + Rough lemon 'Thompson' grapefruit + 'Murcott' tangor 'Cleopatra' mandarin (*Citrus reshni* Hort. Ex Tan.) + 'Swingle' citrumelo (*Citrus paradisi* Macf. x. *Poncirus trifoliata* L. Raf.)	Louzada et al., 1992
'Cleopatra' mandarin + Sour orange (*C. aurantium* L.) 'Cleopatra'mandarin + Rough lemon 'Cleopatra'mandarin + 'Volkameriano' lemon (*C. volkameriana* Pasq.) 'Cleopatra' mandarin + Rangpur lime	Tusa et al., 1990

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'Hamlin' sweet orange + Rangpur lime	
Sour orange + 'Volkameriano' lemon	
'Valencia' sweet orange + 'Carrizo' citrange (*Citrus sinensis* x *Poncirus trifoliata*)	Tusa et al., 1990
'Valencia' sweet orange + 'Carrizo' citrange	
'Valencia' sweet orange + 'Femminello' lemon (*C. limon* L. Burm. f.)	
Sour orange + Rangpur lime	Grosser et al., 1994
(*C. jambhiri* Lush x *C. sinensis*) + 'Sun Chu Sha' mandarin	
'Ponkan' mandarin + Rough lemon	Moriguchi et al., 1996
'Meiwa' kunquat (*Fortunella crassifolia* Swingle) + 'Dancy' madarin	Grosser et al., 1996
'Meiwa' kumquat + 'Cleopatra' mandarin	
'Succari' sweet orange + 'Dancy' mandarin	
'Succari' sweet orange + 'Minneola' tangelo (*C. reticulata* x *C. paradisi*)	
'Succari' sweet orange + 'Murcott' tangor	Mourão Filho, 1996 Mourão Filho et al., 1996
'Succari' sweet orange + 'Page' tangelo	
'Succari' sweet orange + 'Ponkan' mandarin	
'Milam' lemon hybrid + *Citrus obovoidea* hort. ex I. Takah	
'Milam' lemon hybrid + 'Swingle' citrumelo	
'Milam' lemon hybrid + 'Carrizo' citrange (*Poncirus trifoliata* x *Citrus sinensis*)	
'Cleopatra' mandarin + 'Carrizo' citrange	
Sour orange + 'Carrizo' citrange	
'Succari' sweet Orange + Sour orange	
'Succari' sweet Orange + *Citrus obovoidea*	
'Succari' sweet orange + Rough lemon	
'Cleopatra' mandarin + Rough lemon	
'Redblush' grapefruit + Rough lemon	
[*C. reticulata* x (*C. paradisi* x *C. reticulata*)] + Persian lime (*C. latifolia* Tan.)	
'Valencia' sweet orange + 'Minneola' tangelo	
'Valencia' sweet orange'+ 'Murcott' tangor	
'Valencia' sweet orange + 'Page' tangelo	
'Valencia' sweet orange + 3 USDA-CJH hybrid	Grosser et al., 1998b
'Valencia' sweet orange Rhode Red + 'Dancy' mandarin	
'Valencia' sweet orange Rhode Red + 1 USDA-CJH hybrid	
'Murcott' tangor + LB8-9	
'Murcott' tangor + 1 USDA-CJH hybrid	
'Murcott' tangor + 2 USDA-CJH hybrid	
'Redblush' grapefruit + LB8-9	
'Succari' sweet orange + 'Hirado Buntan Pink' pummelo (*C. maxima* (Burm.) Merr)	
'Succari' sweet orange + 1 USDA-CJH hybrid	
'Hamlin' sweet orange + 'Ponkan' mandarin	
'Hamlin' sweet orange + LB8-9	
'Succari' sweet orange + 'Hirado Buntan' pummelo	
'Nova' tangelo (*C. reticulata* Blanco × C. *paradisi* Mcf.) × (*C. reticulata* Blanco)+ 'Hirado Buntan pummelo'	

Continuação...

'Cleopatra' mandarin + Sour orange	Benedito, 1999
'Caipira' sweet orange + Rangpur lime	Mendes-da-Gloria et al., 2000
'Star Ruby' grapefruit + 'Corse' citron (*C. medica* Linn.) 'LAC' lemon + 'Eureka' lemon	Ollitrault et al., 2000b
'Hongju'mandarin + Rough lemon 'Kinnow' mandarin + 'Bendizao' mandarin (*Citrus succosa* Hort)	Deng et al., 2000
Rangpur lime + 'Cleópatra' mandarin	Latado et al., 2002
'Cleopatra' mandarin + 'Volkameriano' lemon Rangpur lime + 'Sunki' mandarin [*Citrus sunki* (Hayata) hort. ex Tan.] 'Valencia Ruby Blood'sweet Orange + 'Volkameriano' lemon 'Valencia Rohde Red' sweet orange+ 'Volkameriano' lemon	Costa et al., 2003
'Murcott' tangor + 'Dancy' mandarin 'Page' tangelo + 'Murcott' tangor 'Page' tangelo + 'Ortanique' tangor (*Citrus sinensis* (L.) Osbeck x *Citrus reticulata* Blanco)	Guo et al., 2004
Citrus sinensis + *Citrus micrantha* (Wester)	Khan & Grosser, 2004
'Hamlin' sweet orange + 'Indian red' pummelo 'Hamlin' sweet Orange + 'Singapura' pummelo	Calixto et al., 2004
Rough lemon + *Citrus obovoideae* Sour orange + 'Benton' citrange [(*Poncirus trifoliata* (L.) Raf. X *Citrus sinensis* (L.) Osbeck] 'Amblycarpa' mandarin 'C-35' (*Citrus limonellus* var. amblycarpa Hassk) + citrange [(*Poncirus trifoliata* (L.) Raf. X *Citrus sinensis* (L.) Osbeck] 'Amblycarpa' mandarin + 'Benton' citrange 'Amblycarpa' mandarin + 'Carrizo' citrange 'Amblycarpa' mandarin + 'Volkameriano' lemon 'Amblycarpa' mandarin + *Citrus macrophylla* (Wester) 'Murcott tangor + 'Hirado Buntan Pink' pummelo 'Murcott' tangor + 'Chandler' pummelo 'Amblycarpa' mandarin + 'Hirado Buntan Pink' pummelo 'Amblycarpa' mandarin + 'Chandler' pummelo 'Amblycarpa' mandarin + 'Ling Ping Yau' pummelo 'Amblycarpa' mandarin + 'Large Pink' pummel 'Shekwasha' mandarin (*Citrus depressa* Hay.) + 'Hirado Buntan Pink' pummelo 'Shekwasha' mandarin (*C. depressa* Hay.) + 'Chandler' pummelo 'Shekwasha' mandarin + 'Hirado Buntan Pink' pummelo	Grosser & Gmitter Jr., 2005
'Encore' mandarin + 'Valencia' sweet orange 'Encore' mandarin + 'Caffin' mandarin	Wu et al., 2005
'Changshou' kunquat (*Fortunella obovata* Swingle) + 'Dancy' mandarin	Xu et al., 2005
'Page' tangelo + Rough lemon	Guo et al., 2006

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'Amblycarpa' mandarin + 'Siamese' pummelo 'Amblycarpa' mandarin + 'Large Pink' pummelo 'Murcott' tangor + 'Siamese' pummelo 'Succari' sweet orange + 'Hirado Buntan Pink' pummelo	Ananthakrishnan et al., 2006
'Shekwasha' mandarin + 'Hirado Buntan Pink' pummelo 'Murcott' tangor + 'Hirado Buntan Pink' pummelo 'Amblycarpa'mandarin + 'Liang Ping Yau' pummelo	Grosser, 2007
'Satsuma' mandarin + 'Navel' sweet Orange	An et al., 2008
'Satsuma' mandarin + 'Page' tangelo 'Satsuma' mandarin + 'Nova' tangelo	Cai et al., 2009
'Bingtang' sweet orange + *Citrus microcarpa* Bunge	Cai et al., 2010

Table 2. *Citrus* intergeneric somatic hybrids, in chronological order.

Fusion parents	References
'Trovita' sweet orange + *Poncirus trifoliata*	Ohgawara et al., 1985
'Hamlin' sweet orange + *Severinia disticha* (Blanco) Swingle	Grosser et al., 1988
'Hamlin' sweet orange + *Citropsis gilletiana* Swingle & Kellerm. 'Cleópatra' mandarin + *Citropsis gilletiana*	Grosser & Gmitter Jr., 1990b
'Hamlin' sweet orange + *Severinia buxifolia* (Poir.) Ten. 'Cleopatra' mandarin + *Poncirus trifoliata* 'Flying Dragon'	Grosser et al., 1992
'Valencia' sweet orange + 'Meiwa' kumquat	Deng et al., 1992
'Ponkan' mandarin + *Citropsis gabunensis* (Engl.) Swingle & M.Kellerm.	Ling & Iwamasa, 1994
'Seminole' tangelo (*Citrus reticulata* Blanco × *Citrus paradisi* Macf.) + *Atalantia monophylla* (L.) DC. 'Seminole' tangelo + *Severinia buxifolia*	Motomura et al., 1995
'Succari' sweet orange + *Severinia buxifolia* 'Hamlin' sweet orange + *Severinia disticha* 'Valencia' sweet orange + *Severinia disticha* 'Cleopatra' mandarin + *Severinia disticha* 'Nova' tangelo + *Severinia disticha* 'Succari' sweet orange + *Citropsis gilletiana* 'Nova' tangelo + *Citropsis gilletiana* 'Succari' sweet orange + *Atalantia ceylanica* (Arn.) Oliv. 'Succari' sweet orange + *Feronia limonia* (L.) Swingle 'Nova' tangelo + *Citrus ichangensis* (Guill.) Swingle 'Red Marsh' grapefruit+ *Poncirus trifoliata* 'Argentine' 'Red Marsh' grapefruit+ *Poncirus trifoliata* 'Flying Dragon' 'Succari' sweet orange + 'Meiwa' kumquat 'Succari' sweet orange + *Microcitrus papuana* Winters 'Hamlin' sweet orange + *Microcitrus papuana*	Grosser et al., 1996
'Mame' kunquat + *Poncirus trifoliata*	Miranda et al., 1997

Continuação...

'Hamlin' sweet orange + *Poncirus trifoliata* 'Flying Dragon'	Grosser et al., 1998a
'Page' tangelo + *Murraya paniculata* (L.) Jack	Guo & Deng, 1998
'Red' mandarin + *Poncirus trifoliata*	Guo et al., 2002
'Newhall' sweet Orange + *Clausena lansium* Skeels	Fu et al., 2003
'Round' Kunquat + 'Morita navel' sweet Orange	Takami et al., 2004
Microcitrus papuana + Sour orange	Xu et al., 2004
'Changsha' mandarin + *Poncirus trifoliata* White grapefruit + *Poncirus trifoliata* Sour orange + *Poncirus trifoliata* 'Amblycarpa' mandarin + *Poncirus trifoliata* 'Flying Dragon' 'Amblycarpa' mandarin + *Poncirus trifoliata* 'Rubidoux' 'Murcott' tangor + *Poncirus trifoliata* 'Rubidoux'	Grosser & Gmitter Jr., 2005
'Red' tangerine + (*Citrus sinensis* x *Poncirus trifoliata*)	Guo et al., 2007

Somatic hybrids, however, may contain both desirable and undesirable traits from the fusion parents, leading to unexpected performance and may not be directly used as commercial scion cultivars (Xu et al., 2007), since such hybrids contain the genome of both parents, even though a partial or combined expression of genomes is possible, and both desirable and undesirable traits may be inherited (Grosser et al., 2000). The introduction of large amounts of exogenous genetic material along with the genes of interest may induce genetic imbalance; fruits may present undesirable characteristics such as irregular, thick skin which, to some degree, limits their utilization (Liu & Deng, 2000).

Tetraploid symmetric hybrids (2n = 4x = 36), however, are very useful in crosses with monoembryonic diploid cultivars (2n = 2x = 18 chromosomes) to generate triploid progenies (2n = 3x = 27 chromosomes), that would potentially be seedless. Furthermore, if the tetraploid somatic hybrid is monoembryonic it can be used as seed parent, eliminating the need for embryo rescue (Grosser & Gmitter Jr., 2005; Gmitter Jr. et al., 1992; Mourão Filho et al., 1996; Grosser & Chandler, 2000).

ASYMMETRIC SOMATIC HYBRIDIZATION

When genes that determine tolerance to a specific biotic or abiotic stress are predominantly carried in one or a few chromosomes, a more productive approach may be asymmetric fusion, also called gamma-fusion or donor-recipient fusion. This approach has recently been used in citrus as well (Louzada et al., 2002; Bona et al., 2009a; Chang & Jong, 2005; Liu & Deng, 2002). The asymmetric hybridization leads to addition of a fraction of an alien genome to a receptor genome (Xia, 2009). In some cases, reduced contribution from one parent allows the creation of individuals free of undesirable genetic effects sometimes associated with allopolyploidy (Xu et al., 2007) and may be better tolerated than a whole-genome transfer (Ramulu et al., 1996a,b).

In general, the frequency of chromosome elimination is subject to parental, genomic, chromosomal and genetic effects (Foster et al., 2007). Asymmetric hybrids may arise from spontaneous chromosome elimination in some distant combinations, but special treatments can be applied to increase the amount of genome elimination (Hinnisdaels et al., 1991). This may be done by X or γ radiation, which induces chromosome fragmentation prior to fusion with receptor cells. The ionizing radiation can causes fragmentation of DNA and induces both single and double-strand breakage, which leads to elimination of donor chromosomes, formation of micro-chromosomes and translocations (Deng et al., 2000; Chang & Jong, 2005; Sutarto et al., 2009).

Asymmetric hybrids have been produced in species of the genera *Medicago*, *Nicotiana*, *Lycopersicum*, *Solanum*, and between *Arabidopsis thaliana* + *Brassica napus* and rice + *Zizania latifolia* (Liu et al., 1999; Tian et al., 2002). However, the first report about the regeneration of asymmetric hybrids in citrus was published in 2002, in which the authors produced asymmetric hybrids from 'Dancy' tangerine and 'Page' tangelo by using X-rays (Liu & Deng, 2002). However, plants were recalcitrant to root in rooting inducing media and had to be grafted. In 2007, asymmetric shoots were produced by protoplast fusion of UV-irradiated 'Satsuma' mandarin protoplasts with 'Jincheng' (*C. sinensis*) by electrofusion, but also failed to root despite enormous endeavors (Xu et al., 2007).

In vitro rooting of citrus putative asymmetric hybrid plantlets was achieved for the first time by Bona et al. (2009a) who dipped the somatic hybrids regenerated shoots' stems in 3,000 mg L^{-1} IBA solution for 10 min and placed them in EME medium with 6% sucrose.

Some of the citrus asymmetric hybrids produced to date are presented in table 3.

Another type of somatic hybridization capable of generating diversity is the technique called 'Microprotoplast mediated chromosome transfer' (MMCT), which was based on the Microcell mediated chromosome transfer technique developed for mammalian cells, in which micronucleation is induced by prolonged mitotic arrest using microtubule inhibitor compounds (Fournier, 1981). In MMCT, suspension cells are treated with a spindle depolymerizing agent such as amiprophos-methyl (APM), to induce formation of multinucleated cells. After treatment with cytochalasin B (CB), an enucleation substance, high speed centrifugation is used to induce formation of some nuclei, surrounded by a thin layer of cytoplasm and plasma membrane which are pinched off, forming microprotoplasts containing one or few chromosomes. This may significantly reduce destabilization of receptor cells in somatic hybrids (Verhoeven & Ramulu, 1991; Thomas et al., 1976; Wallin et al., 1977; Yemets & Blume, 2003).

Fusions of potato microprotoplasts with tobacco and with tomato protoplasts by MMCT and transfer of small portions of the genome of potato (1-2 chromosomes) were reported by Ramulu et al., (1996a,b). In citrus, MMCT was first applied in 2002, where microprotoplasts of 'Ruby Red' grapefruit containing one to three chromosomes were fused with protoplasts of 'Succari' sweet orange, and *Swinglea glutinosa* microprotoplasts with Sour orange protoplasts (Louzada et al., 2002). Embryos and suspension cells with a few additional chromosomes were obtained, but there was no further information about the produced plants.

MMCT combined with gamma radiation was used in citrus by Bona et al. (2009b), where *S. glutinosa* microprotoplasts were irradiated with different doses of gamma-rays and fused with grapefruit, sweet orange or 'Murcott' tangor protoplasts and presence of *S. glutinosa* was detected into the genomes of the hybrids by AFLP molecular marker.

CYBRIDIZATION

After protoplast fusion, if the whole nucleus from one of the parents is lost and the cytoplasms of both parent combine, a new type of asymmetric hybrid will be produced, a cybrid. The cybrid will contain the nuclear genome of one parent with the mitochondrial genome of the other parent (Saito et al., 1994) or with recombination. Plastides may be originated from either parent (Moreira et al., 2000a,b).

The technique has been mainly applied to produce alloplasmic lines (Grosser & Chandler, 2000) which are of potential interest because cytoplasmic factors, mainly chloroplasts and mitochondria, affect traits with agronomicvalue. The chloroplast genome, for example, can affects plant photosynthesis and herbicide resistance, while mitochondria genome plays a key role in cytoplasmic male-sterility (Xu et al., 2006).

Table 3. Asymmetric somatic hybrids in chronological order.

Fusion parents	References
'Dancy' mandarin + 'Page' tangelo	Liu & Deng, 2002
'Satsuma' tangerine + 'Jincheng' (*Citrus sinensis*)	Xu et al., 2007
'Ruby Red' grapefruit + 'Succari' sweet Orange 'Ruby Red' grapefruit + 'Itaborai' sweet Orange 'Murcott' tangor + 'Natal' sweet Orange 'Murcott' tangor + 'Itaborai' sweet Orange	Bona et al., 2009a

Cybridization is a potential source of genetic variability for breeding programs; however, the practical value of cybrids was disregarded for many years because many horticultural important traits related to the genomic organelles had not yet been well understood (Grosser et al., 2000). It is currently known that CMS, which causes seedlessness, is controlled by the mitochondrial genome and was identified in 'Satsuma' (Grosser & Gmitter Jr., 2005; Guo et al., 2004). Some citrus cybrids are presented in table 4.

Obstacles associated with somatic hybridization. Despite of much research dedicated to somatic hybridization and the enormous progress achieved, there are still many limitations in using this procedure for citrus breeding. For example, orange, grapefruit, lemon, and many mandarins have highly polyembryonic seeds with many nucellar embryos. Their embryos are predominantly of maternal origin, rendering them difficult to use as female parents in conventional crosses. Furthermore, availability of monoembryonic diploid parents with high quality is limited (Grosser & Gmitter Jr., 2009). Our knowledge and genetic definition of critical agronomic traits are quite limited (Talon & Gmitter Jr., 2008).

Interploid crosses of diploid females with tetraploid male parents generates endosperm imbalance, resulting in its failure and subsequent embryo abortion, and *in vitro* embryo rescue is required (Viloria et al., 2005). If a tetraploid is used as female parent, this problem does not occur, but the availability of quality monoembryonic tetraploids is also quite limited (Grosser & Gmitter Jr., 2009).

Production of monoembryonic tetraploid somatic hybrids is difficult, because the fusion technology requires the use of an embryogenic suspension culture from a nucellar polyembryonic parent to provide the necessary totipotency. To date it has not been possible to generate embryogenic callus cultures of monoembryonic citrus types (Grosser & Gmitter Jr., 2009).

Even though symmetric somatic hybrids have great potential for rootstock improvement and are excellent as breeding parents, they may have limited direct application as scion cultivars (Louzada et al., 2002), because they contain genomes of two fusion parents where desirable and undesirable traits that may be expressed equally (Liu & Deng, 2002).

Table 4. Citrus somatic cybrids, in chronological order.

Fusion parents	References
'Valencia' sweet orange + 'Feminnelo' lemon	Tusa et al., 1990
Citrus sudachi + 'Galego' lemon *Citrus sudachi* + 'Eureka' lemon	Saito et al., 1993
Citrus unshiu Marc. + 'Washington Navel' sweet orange	Yamamoto & Kobayashi, 1995
Citrus microcarpa + Sour orange 'Cleopatra' mandarin + Sour orange 'Valencia' sweet Orange + 'Femminello' lemon	Grosser et al., 1996
'Seminole' tangelo + 'Lisboa' lemon 'Seminole' tangelo + Rough lemon	Moriguchi et al., 1996
'Star Ruby' grapefruit + LAC lemon	Ollitrault et al., 2000b
'Willowleaf' mandarin (*Citrus deliciosa* Tenore) + 'Duncan' grapefruit *Swinglea glutinosa* + Sour orange 'Willowleaf' mandarin + 'Valencia' sweet orange	Moreira et al., 2000ab
Microcitrus papuana + Sour orange *Microcitrus papuana* + Rough lemon	Xu et al., 2004
'Satsuma' mandarin + 'Hirado Buntan' pummelo	Guo et al., 2004
'Page' tangelo + Rough lemon	Guo et al., 2006
'Satsuma' mandarin' + 'Page' tangelo	Cai et al., 2009

CONCLUSIONS

Aside all difficulties, the production of tetraploid breeding parents for interploid crosses completely changed the way citrus breeding is performed today. Currently, citrus breeding can be better planned by combining in allotetraploid somatic hybrid traits of interest for the next generation of triploid scions and expect that segregation will create the genetic diversity for selection of superior genotypes (Grosser & Gmitter Jr., 2009). Somatic hybridization demands less common skills and expertise, but regardless of the many drawbacks, it still ranks as a method which has yielded good results (Singh & Rajam, 2009).

Asymmetric hybridization is very promising as it allows partial genome transfer (Liu & Deng, 2002; Trick et al., 1994; Derks & Hall, 1992), which may be better tolerated than a whole-genome transfer (Ramulu et al., 1996a,b). However, even though citrus asymmetric hybrid plantlets have been produced (Bona et al., 2009a; Liu & Deng, 2002), there was no further experimentation with such hybrids to attest their real benefit. Further studies on field survival capacity and productivity of such plantlets are necessary to validate the procedure. Although somatic hybridization, both symmetric and asymmetric, has been a great tool for citrus breeding, the release of new improved cultivar produced by this technology is what is going to prove its effectiveness (Singh & Rajam, 2009).

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