

Applications of somatic hybridization for the improvement of horticultural crops

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ABSTRACT

Somatic hybridization (SH) using protoplast fusion is a promising tool to produce symmetrical and asymmetrical polyploidy somatic hybrids in many agricultural crops. The technique of SH could facilitate conventional breeding by providing novel lines so as to use them as elite breeding materials in conventional crosses for both scion and rootstock improvement. Further, SH can overcome those problems associated with sexual hybridization, viz., sexual incompatibility, nucellar embryogenesis, and male/female sterility. Successful exploitation of SH in horticultural crops mainly comes from the transfer of resistance genes for biotic and abiotic stresses from related species in several horticultural crops, viz., citrus, potato, brinjal, tomato, mango, avocado, banana, strawberry, pear, cherry, etc. Unlike transgenic technology, SH is not affected by legal formalities and able to transfer uncloned multiple genes. However, certain boundaries and limitations of SH restricts its use over sexual hybridization, but envisage of new genomic technologies providing better insight into the plant genomes will increase the potentiality of SH in the betterment of agriculture.

KEY WORDS: Citrus, disease resistance, genome, molecular markers, potato, somatic hybrids

INTRODUCTION

Somatic hybridization (SH) *via* protoplast fusion is an important tool for the production of interspecific and intergeneric hybrids. The technique of SH involves the fusing protoplasts of two different genomes followed by the selection of desired somatic hybrid cells and subsequent regeneration of hybrid plant. The technique provides an efficient mean of gene transfer from one species to another so as to break the crossing barriers and integration of parental nuclear and cytoplasmic genomes. SH is a multi-step process involving isolation and fusion of protoplast, culture/regeneration of the post-fusion mixture, and detection of somatic hybrids among the regenerated shoots. SH has been widely exploited in different horticultural crops to create novel hybrids with increased yield and resistance to diseases. In addition, SH has also been used for salt tolerance, quality improvement, transfer of cytoplasmic male sterility (CMS), seedless triploids and rootstock improvement (Wang *et al.*, 2013). A number of successful results of SH have been achieved in members of “model horticultural families,” i.e., *Rutaceae*, *Brassicaceae* and *Solanaceae*.

SH *via* protoplast fusion resulted in the development of both symmetric and asymmetric hybrids. “Symmetric hybrid” is a combination of diploid nuclear genomes and two maternal cytoplasmic genomes. Whereas, “asymmetric hybrids” arises due to a combination of genomes of the parents or combination of the genome of one parent and the cytoplasm of another; these are also known as cybrids. Somatic hybrids may express desirable and/or undesirable traits from the fusion parents, resulting to unpredictable phenotypes or performance and thus may not be of direct use (Xu *et al.*, 2007), since such hybrids contain the genome of both parents. There is always a possibility of inheritance of desirable and undesirable traits together even though a partial or combined expression of genomes is possible, and both (Grosser *et al.*, 2000). Further, 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 and Deng, 2000). 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. The asymmetric hybridization leads to the 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 allopolyploid (Xu *et al.*, 2007) and may be better tolerated than a whole-genome transfer.

Protoplast fusion offers an opportunities for circumventing barriers to sexual reproduction and gene transfer of nuclear and cytoplasmic genomes to enrich the gene pool of cultivated species. Moreover, protoplast fusion effectively generates novel germplasm for elite breeding of conventional crosses and promotes crop improvement in existing cultivars. Conventional breeding methods such as sexual hybridization cannot transfer some elite breeding traits (e.g., quality traits, disease resistance, and CMS) because of sexual reproductive barriers such as incompatibility, different bloom phases, male and/or female sterility, nucellar embryogenesis, and polyembryony (Aleza *et al.*, 2010). Sexual barriers can be overcome through protoplast fusion or SH, which is the fusion of protoplasts from two distinct species to form new hybrid plant possessing characteristics of both the parents (Carlson *et al.*, 1972). Applications of SH in some important horticultural crops has been discussed in details here.

POTATO (*SOLANUM TUBEROSUM* L.)

Cultivated potato (*S. tuberosum*) is an autotetraploid crop; thus its breeding requires large-scale selection in a population so as to obtain the beneficial agronomic traits pyramided in one genotype. Under these circumstances, breeding at diploid state followed by SH (so as to restore the ploidy) has been suggested as an alternative route for the production of superior cultivars. The potential benefits of SH have been demonstrated through the successful introduction of a number of desirable traits from a wild/related cultivated species into cultivated potatoes. For example, the resistance to bacterial wilt was transferred into *S. tuberosum* from *S. phureja* and *S. stenotomum* via SH (Fock *et al.*, 2001). The endosperm balance number complicates the sexual crosses in *Solanum* (Johnston *et al.*, 1980), thus to overcome such incompatibilities due to differences in ploidy and endosperm balance number, Kim *et al.* were able to produce somatic hybrids between *S. tuberosum* and *S. commersonii* (Kim *et al.*, 1993). The majority of the fusion hybrids were resistant to bacterial wilt, and some of the fertile, resistant hybrids were crossed with *S. tuberosum* and viable seeds were produced (Laferrriere *et al.*, 1999). An important objective in

S. tuberosum somatic breeding is the introduction of PVY, Colorado beetle, late blight (*Phytophthora*) and bacterial wilt resistance. Using SH, Novy and Helgeson successfully generated hybrids between PI 245939, the *S. tuberosum* clone from virus-resistant and a subsp. *tuberosum* dihaploid \times *S. berthaultii* hybrid clone ($2n=2x=24$) (Novy and Helgeson, 1994). The cytological and molecular analyses revealed that the trispecies hybrids had the expected ploidy ($2n=4x=48$). Cultivated potato lacks resistance to bacterial wilt caused by *Ralstonia solanacearum*. Iovene *et al.* (2012) successfully produced haploid plants from protoplast fusion of *S. bulbocastanum* (+) *S. tuberosum* hybrids to club elite traits from both the parents. Yu *et al.* demonstrated the successful introgression of bacterial wilt resistance from *S. melongena* ($2n=2x=24$) to dihaploid *S. tuberosum* ($2n=2x=24$) (Yu *et al.*, 2013). Whereas, Ahn and Park (1993) successfully transferred resistance to common scab from *Solanum brevidens* to *S. tuberosum* through SH technique.

TOMATO (*LYCOPERSICON ESCULENTUM*)

Lycopersicon is a relatively small genus within the large and varied *Solanaceae*. *Lycopersicon* consists of the cultivated tomato, *L. esculentum*, and eight related wild *Lycopersicon* species. The wild species of genus *Lycopersicon* have been reported to be important sources of agronomic traits, but many sexual crosses between tomato and the wild species are difficult to perform due to uni- or bilateral incompatibility and abundance of sterile F_1 hybrids. Interspecific symmetric hybrid plants were produced between cultivated tomato (*L. esculentum*) and *L. chilense*, *L. pennellii* and *L. peruvianum*. Intergeneric SH has also been possible between tomato and several species including *Nicotiana tabacum*, *S. tuberosum*, *S. tuberosum* \times *S. brevidens*, *S. lycopersicoides*, *S. muricatum*, *S. nigrum*, *S. rickii* and *S. tuberosum*. Although morphology and organellar compositions of such hybrids were extensively studied but no report is available on the transfer of agronomic traits to the somatic hybrids. The somatic hybrids of cultivated tomato and *L. peruvianum* are of particular interest since this combination is one of the few that is fertile, while the corresponding diploid sexual hybrid is reported to be sterile (Lefrancois *et al.*, 1993).

BRINJAL (*SOLANUM MELONGENA*)

In the case of eggplant (*S. melongena*), crossing barriers are a major bottleneck in transferring agronomically important traits such as resistance to insect pests and diseases from related wild species. Although interspecific protoplast fusion resulted to progenies, but combinations for which

sexual hybridization is also successful. This general rule was observed for crosses between *S. melongena* and *S. aethiopicum*, *S. melongena* and *S. torvum*, *S. melongena* and *S. khasianum*, and *S. aethiopicum* and *S. violaceum* (Tamura et al., 2002). The case of *S. melongena* and *S. sisymbriifolium* is an exception, in which SH produced a viable, albeit sterile, hybrid, whereas, sexual hybridization was not successful (Collonnier et al., 2003). Despite progress in this area, no eggplant cultivar, to date, has been produced via interspecific protoplast fusion. Initially, isozymes, then random amplified polymorphic DNAs and now inter simple sequence repeat markers are used to confirm the origin of dihaploid plants derived from *S. melongena*-*S. aethiopicum* somatic hybrids carrying *Fusarium* wilt resistance (from the *S. aethiopicum* parent) (Rizza et al., 2002).

CITRUS SPECIES

Somatic hybridization by protoplast fusion has overcome many problems related to *Citrus* reproductive characteristics, allowing the creation of novel genotypes. SH in *Citrus* resulted in rootstock's resistance to various biotic and abiotic stresses and increased yield as well as fruit quality (Soriano et al., 2012). For example, new seedless triploid *Citrus* cultivars were produced via haploid + diploid protoplast fusion and symmetric fusions of elite diploid cultivars resulted to superior allotetraploid parents which could be exploited in breeding programs (Grosser and Gmitter, 2005).

The first symmetric hybrid of citrus was created by the protoplast fusion of *Citrus sinensis* and *Poncirus trifoliata* (Ohgawara et al., 1985) while the hybrid plants between two sexually incompatible *Citrus* genera were first reported in 1988, where *C. sinensis* L. Osb. cv. "Hamlin" protoplasts were fused with *Severinia disticha* (Blanco) Swing protoplasts (Grosser and Gmitter, 2005). Later, SH became a well-established technique for *Citrus* improvement, e.g., 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 (*Citrus 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 et al., 2000). "Hamlin" sweet orange and "Singapura" pummelo (*Citrus grandis* L. Osb.) were fused-targeting blight, *Citrus tristeza virus*, and *Phytophthora*-induced disease tolerance (Calixto et al., 2004). Intergeneric somatic hybrids between round kumquat (*Fortunella japonica* Swingle) and 'Morita'

navel orange were produced to create novel parents for interploid crosses aimed to develop new seedless kumquat-like cultivars (Takami et al., 2004).

Apomixis and long juvenile period make conventional hybridization impractical in citrus spp. SH through protoplast fusion has been successfully utilized by Khan and Grosser (2004) to produce Somatic hybrids combining sweet orange (*C. sinensis*) with one of the progenitor of small fruited acid lime (*Citrus micrantha*) to impart resistance against witches broom disease in lime. Similarly, Grosser et al. (2007) developed sting nematode (*Belonolaimus longicaudatus* Rau) tolerant root stock by SH of mandarin (*Citrus reticulata* Blanco) + pummelo (*C. grandis* L. Osbeck). Somatic hybrid plants were produced from four new mandarin + pummelo parental combinations by fusing embryogenic suspension culture-derived protoplasts isolated from selected mandarins with leaf protoplasts of pummelo seedlings previously selected for tolerance to sting nematode. Asymmetric hybrids are also very promising in *Citrus* as it allows partial genome transfer, which may be better tolerated than a whole-genome transfer (Liu and Deng, 2002). They first reported the regeneration of asymmetric hybrids in citrus, in which they produced asymmetric hybrids from "Dancy" tangerine and "Page" tangelo by using X-rays. Asymmetric shoots were produced by protoplast fusion of UV-irradiated "Satsuma" mandarin protoplasts with "Jincheng" (*C. sinensis*) by electrofusion but failed to induce rooting (Xu et al., 2007). Even though *Citrus* asymmetric hybrid plantlets have been produced, there was no further experimentation with such hybrids to establish their real use.

MANGO (MANGIFERA INDICA)

Mango is one of the most popular fruit crops in tropical and subtropical regions around the globe. Mango as a crop is facing difficulties in conventional breeding due to the production of a small number of seeds, complex flowering pattern, excessive fruit drop, long juvenile phase, high heterozygosity as well as polyembryony (Iyer and Degani, 1997). SH could be handy in introducing the important traits like tolerance to biotic and abiotic stresses from popular cultivars or from wild species into mango rootstocks (Litz, 2004). Mango is one of the most recalcitrant species for *in vitro* studies due to which it lagged behind other fruit crops in the application of single cell and protoplast culture tools. However, some workers have started exploiting the SH in mango, e.g., plant regeneration from protoplasts of proembryonic masses (PEMs) of mango cv. "Amrapali" has been achieved (Ara et al., 2000). Rezazadeh et al. (2011) were the first to

obtain intraspecific somatic hybrids of mango through fusion of PEMs and leaf-derived protoplasts irrespective of target genes, for three parental combinations at cultivar level, viz., “Tommy Atkins” + “Kensington Pride,” “Keitt” + “Kensington Pride” and “Haden” + “Kensington Pride”. They isolated the protoplasts using enzymatic degradation from leaves of greenhouse-grown seedlings of cvs. “Tommy Atkins,” “Keitt” and “Haden” and from PEMs of cv. “Kensington Pride,” and flow cytometry analysis of 242 PEM revealed 41 tetraploid lines. DNA fingerprinting of tetraploid lines showed that only four lines, all resulting from “Haden” + “Kensington Pride” were somatic hybrids. By contrast, the tetraploid lines from “Keitt” + “Kensington Pride” and “Tommy Atkins” + “Kensington Pride” were autotetraploids were somatic hybrids. Such studies pave the ways for genetic improvement of mango through the production of cybrids, auto- and allotetraploid ($2n=4x=80$), haploids through haploidization ($n=2x=40$), and confirmation of other intra- and interspecific combinations.

USE OF SH IN OTHER IMPORTANT FRUIT CROPS

Besides the application of SH mentioned above, other agriculturally important traits modified by SH include chilling tolerance in *Actinidia* (Xiao et al., 2004), photoperiodical response in *Gossypium* (Sun et al., 2005), storage root formation in *Ipomoea* (Yang et al., 2009) and creation of new genome/cytoplasm combinations for generating CMS (Cai et al., 2006). (Fitter et al., 2005) demonstrated the possibility of introgressing CMS by mtDNA from a wild species. (Yamagishi et al., 2008), demonstrated usefulness of mitochondrial recombination as a tool for CMS introduction in cabbage.

Different ploidy levels and dioecious nature of *Actinidia* spp. restrict the possibilities of breeding programs, SH may allow the recovery of vigorous and fertile hybrids, permitting breeding and eventually combining genetic backgrounds within the same gender. Xiao and Han, (1997) fused protoplasts of *Actinidia deliciosa* (6x) with *Actinidia chinensis* (2x) and *A. chinensis* (2x) with *Actinidia kolomikta* (2x). Regeneration competent protoplasts of *A. deliciosa* and *A. chinensis* were isolated from cotyledon callus line, while those of *A. kolomikta*, obtained from the young fully expanded leaves of micropropagated shoots, did not regenerate.

Litz (1997) attempted SH of avocado (*Persea americana*) by means of protoplast fusion involving avocado protoplasts from embryogenic cultures with leaf protoplasts of

phytophthora root rot (PRR) resistant species. The immunity to PRR associated with different species in subgenus *Eriodaphne* is inaccessible to avocado breeders due to graft and/or sexual incompatibility barriers between *Persea* spp. in *Eriodaphne* with species in subgenus *Persea*. Litz and Litz (2000) successfully recovered somatic hybrids by fusing avocado embryonic protoplasts with non-morphogenic protoplasts of *Persea* spp. in subgenus *Eriodaphne*.

Modern banana cultivars growing in the dry monsoon areas of India, Myanmar, Thailand Philippines have been originated from the hybridization between, the diploid and triploid *Musa acuminata* (A genome) with *Musa balbisiana* (B genome) to produce progeny with AB, AAB and the ABB. SH have been used as a means to overcome the sterility barriers associated with banana spp. Assani et al., (2011) reconstituted triploid clones, when tetraploid plants generated through the protoplast fusion of elite diploid clones were crossed with other diploids. Somatic hybridization also played a considerable role in broadening the gene pool of banana.

In strawberry (*Fragaria* spp.), SH offers the possibility of genetic exchange between the diploid *Fragaria vesca* ($2n=2x=14$) and the cultivated octoploid ($2n=2x=56$) strawberry. Wallin (1997) regenerated plants from protoplast fusion of *Fragaria* × *ananassa* and *F. vesca*. Protoplasts of *Fragaria* × *ananassa* resistant to hygromycin were fused to protoplasts of *Fragaria* × *ananassa* resistant to kanamycin. Plants that deviated morphologically from the parents when tested were found to have more than 56 chromosomes (enhanced ploidy level) due to SH.

Ochatt et al. (1989) reported somatic hybrids between the sexually incompatible rootstocks of wild pear (*Pyrus communis* var. *pyraster* L.; $2n=2x=16$) and Colt cherry (*Pyrus avium* × *pseudocerasus*; $2n=4x=32$). Protoplasts of Colt cherry from suspension cultures as well as mesophyll protoplasts of wild pear were electroporated as separate populations and chemically fused to generate unique heterokaryons. The hybrids thus generated were intermediate for most morphological markers. For example, all somatic hybrid plants recovered had 58 chromosomes, equivalent to the entire complement of somatic chromosome numbers of the parents.

Henn et al. (1998) recovered fertile hybrids involving the fusion of protoplasts of *Helianthus annuus* with those of *Helianthus maximiliani*, *H. giganteus*, and *H. Nuttallii*. Other studies have characterised symmetric and asymmetric somatic hybrids between sunflower (*H. annuus*) and

H. maximiliani using molecular markers (Binsfeld and Schnabl, 2002). Both types of hybrids resulted in recombination of the two parental genomes to different levels, making some of these hybrids an excellent material for incorporation into conventional breeding programs.

Mezzetti *et al.* (2001) proposed an extension of SH technology to temperate soft fruit species, where they successfully fused the protoplasts (PEG-induced) of *Rubus idaeus* (raspberry, $2n=2x=14$) with *R. fruticosus* (blackberry; $2n=4x=28$) (Mezzetti *et al.*, 2001). Fluorescent *in situ* hybridization revealed the occurrence of large karyotic rearrangements in hybrid cells, which could be the reason for the inability to regenerate shoots from such tissues. There is an example of SH technology from fruit tree species in the family *Ebenaceae*, with hybrids between *Diospyrus glandulosa* and *D. kaki* (persimmon) having only chloroplasts from the former parent (Tamura *et al.*, 2002).

Somatic hybridization in *Mentha* species has been performed so as to modify oil composition. Such achievements may have a significant commercial value of mint in the food, especially, flavour industries. Sato *et al.* (1996) combined peppermint (*Mentha piperita* cv. Blackmint) with gingermint (*Mentha gentilis* cv. *variegata*). The somatic hybrids synthesised the major volatile oil components menthone, menthol, and linalol, the last two being from peppermint and gingermint, respectively. Later, Krasnyanski *et al.* fused protoplasts of peppermint (*M. piperita* cv. Black Mitcham) with those of spearmint (*Mentha spicata* cv. Nature Spearmint) combining high-quality oil property of spearmint with disease resistance from peppermint (Krasnyanski *et al.*, 1998).

FUTURE PROSPECTS

Protoplast fusion and SH techniques allow us to move from traditional plant breeding to asexual approaches. It provides opportunity bypassing the conventional breeding barriers through direct transfer of cytoplasmic and nuclear genome to plant cells. As compared to transgenic approaches, SH is able to broaden the germplasm base, allow the transfer of uncloned multiple genes and also able to generate products that are beyond the same legal regulations as transgene plants (Grosser and Gmitter, 2005). Further, it allows transfer of both mono- and polygenic traits (Thieme *et al.*, 2004). In the last few years, SH has been frequently used as an alternative for incompatible sexual crossing. As chromosome rearrangements are more typically observed in somatic hybrids than in their sexual counterparts though polyploidization and other genomic effects occurring

frequently (Chevre *et al.*, 1994). The molecular marker system would allow better tools to detect and follow the introgression of alien DNA in the receptor genome, helps in more detailed studies on genome stability and gives a better insight on genetic backgrounds that are responsible for hybrid selection during the SH process.

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