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Somatic hybridization in agricultural crops improvement: An environmentally amiable era in biotechnology

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ABSTRACT

Breeders have successfully taken advantage of the species' genetic variability to ameliorate yields further. Significant efforts have been made to increase the current gene pool of crops because the rich variability in a breeding population might not be sufficient for contemporary plant breeding needs and societal requirements. The main contribution of somatic hybridization to plant breeding is overcoming the barriers of sexual crossing and the possibility of transferring foreign genes between different species, genera, and families of plants, leading the field in an environmentally friendly direction and continuing to challenge the mainstream approach to biotechnology. Somatic hybridization refers to the creation of asexual hybrids by the fusion of isolated protoplasts from somatic plant cells, which are known as somatic hybrids. This kind of hybridization can be used only when two concomitant conditions are met: abundant quantities of isolated protoplasts with high vitality proportion and their totipotency. Creating a somatic hybrid includes several stages: search for a suitable explant, isolation of protoplasts, their fusion, plant regeneration, subsequent selection, and identification of somatic hybrid plants. Currently, isolated protoplasts play a crucial role in elucidating our understanding of cell biology, structure, and function of plant cells and tissues as well as in studies of gene transfer and manipulation. It is precious for plants with sexual incompatibility with other species or genera. The successful application of somatic hybridization is mainly due to the transfer of biotic and abiotic stress resistance genes from related species into crops of high economic value, such as potato, eggplant, tomato, citrus fruit, mango, banana, strawberry, wheat, etc. Protoplast fusion allows for unique gene combinations, developing new plant kinds through somatic hybridization.

Keywords: Cybrid, Cybridization, Hybrid, Incompatibility, Inter-generic, Inter-specific, Protoplast Fusion, Somatic Hybridization; Totipotency.
Article type: Review Article.

INTRODUCTION

Somatic hybridization (SH) is a cell and tissue culture approach that allows for transforming cellular genomes by protoplast fusion and the combination of not only nuclear but also organelle genes, resulting in new inter-generic and inter-specific hybrids. This process begins by fusing protoplasts from two different plant species and then selecting the desired somatic hybrid cells to regenerate hybrid plants. Somatic hybridization can be used to improve disease resistance, quality, quantity, or other characteristics. It has also been utilized to improve salt tolerance, cytoplasmic male sterility (CMS), seedless triploids, and rootstock (Moon *et al.* 2013), which may lead to commercial exploitation. This publication outlines how somatic hybridization can introduce alien genes into crop species. Subsequently, this paper describes the mechanism of somatic hybridization and its role in improving agricultural crops.

Historical perspective of somatic hybridization

Somatic hybridization is a revolutionary, environmentally friendly approach that involves the *in vitro* fusion of protoplasts to generate a hybrid cell, which is then cultured to produce a hybrid plant. In this way, biotechnologists

have opportunities to create hybrids combining two protoplasts from various distinct plant species. These hybrids are referred to as somatic hybrids. Hanstein was the first person to use the term "Protoplast" in 1880. In 1986, Pandey released a commercial cultivar generated from protoplast fusion. Zimmermann and Scheurich, in 1981, were responsible for the development of electrofusion methods. Then in the 1990s, commercial cultivars derived from protoplast fusion started to release, as a result, protoplasts present a novel opportunity for the development of novel genetically constructed cells. From protoplasts, Takebe *et al.* (1971) were able to regenerate the entire tobacco plant (Gill *et al.* 1978).

Types of somatic hybrids

Somatic hybrids can be classified into three types based on how they are developed: symmetric somatic hybrids, asymmetric somatic hybrids, and cytoplasmic hybrids, which are known as cybrids (Guo *et al.* 2013). Symmetric somatic hybridization involves the nuclear and cytoplasmic integration of both parents' genetic material. Asymmetric somatic hybridization has been applied to introduce nuclear genome fragments from one parent, the "donor," into the intact genome of another parent, the "recipient." This type of hybridization needs to be completed due to the loss of some cytoplasmic or nuclear DNA (Taski-Ajdukovic *et al.* 2006). Cybrids contain either the cytoplasmic genome of the non-nuclear parent or a combination of both parents' nuclear genomes (Bosco *et al.* 2017). In this circumstance, micro protoplast fusion with partial chromosome transfer from the micronuclear parent was recently used to produce asymmetric hybrids in sunflower (Binsfeld *et al.* 2000) and intergeneric hybrid plants in Liliaceous (Saito *et al.* 2002), demonstrating that these methods are still evolving. In this regard, asymmetric hybridization is especially intriguing since it allows for partial genome transfer, which may be simpler to administer than whole-genome transfer (Liu & Deng 2002).

Mechanism of somatic hybridization

Somatic hybridization is the mechanism by which protoplasts are isolated and fused from somatic cells and regenerated into hybrid plants. Protoplast fusion is the spontaneous or induced mixing of protoplasts from two different genomes.

Spontaneous fusion

Physical contact is the leading cause of spontaneous fusion. Due to the enzyme treatment during isolation, protoplasts fuse spontaneously. Through the fusion of protoplasts from adjoining cells, multinucleate protoplasts are created, a widespread phenomenon in various species, particularly tobacco and citrus (Guo *et al.* 2013). However, the yields of spontaneous fused protoplasts are low and uncertain, so it would be preferable to develop a more adequate technique for inducing fusion at a higher frequency.

Induced fusion

Induced fusion can be induced chemically /Chemo Fusion/ or electrically /Electrofusion/.

Chemo fusion

Polyethylene Glycol (PEG), $NaNO_3$, and calcium ions (Ca^{++}) are among the chemicals that have been used to compel protoplast fusion. An asymmetric chemo protoplast fusion was established to develop celeriac cybrids (Bruznican *et al.* 2021). Somatic hybridization was carried out through a research project in Phaseolus using protoplast chemical fusion technology (Geerts *et al.* 2008).

Electrofusion

In protoplast electrofusion, an alternating current is employed first to stimulate protoplast transfer and establish close membrane contact. Continuous pulses are then used to cause membrane disruption at the contact points. Electrofusion has increased in popularity because it is less harmful to protoplasts than chemical methods, despite the need for expensive and specialized equipment to generate alternating current fields and continuous current pulses. Electrofusion of UV-irradiated *Satsuma mandarin* protoplasts with Jincheng (*C. sinensis* Osbeck), a high-quality local cultivar, resulted in the regeneration of multiple shoot lines (Xu *et al.* 2007). Using electrofusion, a successful approach for producing and selecting interspecific somatic hybrid plants between cultivated and wild carrots was reported, using protoplasts dual-labeling and early selection of fused cells via micromanipulator. Both subspecies employed in this study have been identified by an exceptionally high regeneration ability in protoplast cultures (Mackowska *et al.* 2023).

Source material for protoplast isolation

In protoplast culture, protoplasts can be isolated from any part of the plant, including leaves, shoot apices, roots, coleoptiles, hypocotyls, petioles, embryos, pollen grains, calli, and cell suspensions; they are all potential organs for protoplast isolation. The most dependable wellspring of protoplasts are leaf mesophyll cells, from which a high number of relatively uniform cells can be extracted (Ren *et al.* 2021). Sun *et al.*'s latest study effectively shows how DNA methylation state and leaf age strictly control the totipotency competence of protoplasts, with the main emphasis being on the epigenetic state of cells during reprogramming (Sun *et al.* 2019). The advantage of protoplast isolation from seedlings is that within a few days after seed germination, protoplasts can be separated from radicles, cotyledon tissues, roots, and root hairs (Sinha *et al.* 2003a). Despite increased protoplast output from cotyledons with seedling aging, viability decreased. Simultaneous research improved protoplast isolation from this legume's cotyledons (Sinha *et al.* 2003b). Similarly, it was discovered that cotyledons from white lupin seedlings cultivated *in vitro* produced higher protoplast yield than leaves, hypocotyls, and roots (Sinha *et al.* 2003a). To date, callus cell suspension cultures have been usually used as a protoplast donor material in research, especially for intra- and inter-specific and intergeneric somatic hybridization (Grzebelus *et al.* 2012). A highly effective and accessible method was described to isolate protoplasts from callus tissue induced from rice seeds. This approach utilizes donor materials that are resource-efficient and easily propagated. It provides an advantageous and useful platform for various *in vivo* transient transfection investigations in rice (Poddar *et al.* 2020). The same in *Petunia hybrida* cv. Mirage Rose was obtained as a high callus-derived protoplast yield (Kang *et al.* 2020). Despite the reality that suspension cultures are an excellent source of protoplasts due to their high embryogenic ability, the establishment and maintenance of suspension cultures are laborious and time-consuming, ordinarily requiring several weeks to the time required for callus induction. It is worth mentioning that the isolation process of protoplasts is significantly influenced by the type of source material, the composition of the cell wall, the presence of an additional external layer, and the cementing material between the cells.

Contribution of somatic hybridization in agricultural crop improvement

Sexual reproductive barriers, such as incompatibility, prevent the transfer of some elite breeding traits like CMS, quality traits, and disease resistance through conventional breeding methods that use sexual hybridization. However, these barriers can be overcome through protoplast fusion or SH. Utilizing the genetic diversity in cultivated crops and their wild relatives can produce crops with enhanced traits and a considerable influence on sustained food security (Watanabe 2015; Oladosu *et al.* 2021). Here is a detailed discussion of the applications of somatic hybridization in a few significant crop families.

Solanaceae (tobacco, potato, tomato, eggplant)

In Patel *et al.* (2011) investigation, somatic hybridization was employed to develop a new hybrid combination of two sexually incompatible tetraploid tobacco species, *Nicotianax sanderae*, and *N. debneyi*. All somatic hybrid plants were fertile. In contrast to the parental plants of *N.* × *sanderae*, the seed progeny of somatic hybrid plants was resistant to infection by *Peronospora tabacina*. This characteristic was introduced by the wild parent, *N. debneyi* (Patel *et al.* 2011). Somatic hybrids with quality properties have been expanded, such as the production of high nicotine content (Bhatia *et al.* 2015). Somatic hybrids between a dihaploid clone of potato. *Solanum tuberosum* cv. BF15 and *S. stenotomum* were produced by electrofusion of mesophyll protoplasts, as cultivated potatoes lack resistance to bacterial wilt caused by *Ralstonia solanacearum*. According to Fock *et al.* a total of thirty hybrid plants were regenerated. They exhibited a strong vigor and showed morphological intermediate characteristics, including leaf morphology, flowers, and tuber characteristics. Flow cytometry examination revealed that 25 were tetraploids (4×; 48 chromosomes), three were hexaploids (6; 72), and two were aneuploids (< 4×; 48). Examining isoenzyme patterns for esterase and DNA simple sequence repeat (SSR) markers proved their hybrid nature as well as the analysis of chloroplast (ct) DNA microsatellites in fourteen somatic hybrids revealed that six hybrids had *S. stenotomum* ctDNA and eight had *S. tuberosum* ctDNA. Interestingly, all somatic hybrids examined demonstrated a resistance level comparable to the wild species (Fock *et al.* 2001).Yu *et al.* (2013) demonstrated the successful introduction of bacterial wilt resistance from *S. melongena* (2n = 2x = 24) to dihaploid *S. tuberosum* (2n = 2x = 24), 34 somatic hybrids were obtained, cytoplasmic genome analysis revealed that both parents' mitochondrial DNA coexisted and/or recombined in the majority of the hybrids. However, only potato chloroplast cpDNA was maintained in the hybrids, implying a compatibility between cpDNA and the cell's

nuclear genome. The pathogen inoculation assay indicated that bacterial wilt resistance was successfully transferred from eggplant to the hybrids, providing potential resistance for potato breeding against bacterial wilt (Yu *et al.* 2013). In this field, haploid plants from protoplast fusion of *S. bulbocastanum* and *S. tuberosum* were successfully produced. These hybrids have elite traits from both the parents, 11 somatic hybrids were identified out of 42 regenerates analyzed using ISSR markers, and some hybrids experienced fragment loss or gain compared to the parents, most likely due to chromosomal segment rearrangements and deletions after fusion and somaclonal variation during hybrid regeneration, since all hybrids were sterile, *in vitro* another culture was utilized for haploidization as a feasible technique to circumvent hybridization restrictions (Iovene *et al.* 2012). The barriers of sexual incompatibility are broken down by somatic hybridization. Pomato (*Solanopersicon*, a new genus) is an example and the fusion result of the protoplasts of potato, *S. tuberosum* and tomato, *Lycopersicon esculentum* (Schoenmakers *et al.* 1994).

Brassicaceae (cabbage, radish, rapeseed)

Cruciferous vegetable crops, particularly cultured varieties of *Brassica oleracea* L., are susceptible to various diseases, resulting in significant losses in production and market value impairments (Scholze *et al.* 2010). Introducing genes that resist disease is one of the primary goals of plant breeding. Somatic hybrids were created using PEG-induced symmetric and asymmetric protoplast fusions to transfer resistance to *Alternaria brassicicola*, *A. brassicae*, *Phoma lingam*, *Plasmodiophora brassicae*, and Turnip mosaic virus (TuMV) into *Brassica oleracea* var. *capitata* (cv. 'Toskama') and *botrytis* (cv. 'Korso'). Ten plants from different genera in the Brassicaceae family, including wild relatives, were chosen as resistance donors. Of the 2,189 plants (somatic hybrids, partially *in vitro* cloned) evaluated, 1,616 (73.8%) were resistant to at least one of the diseases, demonstrating that resistance transfer was generally successful (Scholze *et al.* 2010). Hansen and Earle (1995) investigated the feasibility of transmitting the Rb resistance gene from *Brassica carinata* to *B. oleracea* var. *italia* through somatic hybridization (Zubko *et al.* 2018). Somatic hybridization has been used to introduce the CMS trait into breeding programs. Due to the great utility of the CMS phenotype in plant breeding, it is frequently introduced to the crop of interest from natural populations or created *in vitro* via genetic engineering, intraspecific, interspecific, or intergeneric crosses, or protoplast fusions (Wu *et al.* 2019). Protoplast fusion could be exploited to create new nuclear cytoplasmic organelle combinations, expanding cytoplasmic variety. In *Brassica napus*, different cytoplasmic male sterility frameworks have been described, including cytoplasmic male sterility in Ogura, Tour, and Polima and cytoplasmic male sterility in Kosena radish. Somatic hybridization has been used to incorporate them into novel genotypes (Christey 2004). Molecular markers have been used to identify cybrid or hybrid fusion products that thrive in antibiotic- or herbicide-containing media (Christey 2004). After sequencing the mitochondrial genome of the somatic hybrid SW18 and comparing it to the parental mtDNAs, comparative genomics demonstrated that the mitochondrial-encoded orf125 originates from *Raphanus sativus* cv. Kosena which was identified as responsible for the CMS condition (Yamagishi & Bhat 2014).

Apiaceae (carrot, coriander, celery, parsley, fennel)

Protoplast isolation from *Daucus carota* (carrot), *Coriandrum sativum* (coriander), *Apium graveolens* (celery), *Petroselinum hortense* (parsley), and *Foeniculum vulgare* (fennel) has been reported in the family Apiaceae thus far (Dudits *et al.* 1980; Bruznican *et al.* 2021). Tan *et al.* (2009) asymmetrically fused the protoplasts of carrots treated with UV light and celery treated with IOA to regenerate 11 petaloid celery CMS plants (Tan *et al.* 2009). The most recent recovery of CMS hybrids occurred when inactivated acceptor protoplasts from celeriac and carrot UV-inactivated donor protoplasts fused. However, in this investigation, the recovery of CMS hybrids was not possible with the use of coriander or celery donor protoplasts (Bruznican *et al.* 2021). X-irradiated parsley (*Petroselinum hortense*, 2n = 22) leaf protoplasts were fused with cell culture protoplasts of a nuclear albino mutant of carrot (*Daucus carota*, 2n = 18) in an attempt to somatically transfer plant genomes of reduced size (Dudits *et al.* 1980), the assessment of chosen green tissues and regenerated plants with chromosome numbers close to the diploid chromosome number of carrot revealed the transfer of parsley genes.

Grass family Poaceae or Gramineae (maize, wheat, rice, barley and oat)

There are numerous reports on somatic hybrids, protoplast fusion-mediated somatic hybridization can be a useful tool for grass genetic improvement, following somatic hybridization of tall wheatgrass *Agropyron elongatum* with bread wheat *Triticum aestivum* cv. Jinan177 protoplasts, and regenerates were produced. All line offspring were

phenotypically comparable to the tall wheatgrass parent, while XI progeny had thinner, smoother, and softer leaves (Cui *et al.* 2009). In this regard, in bread wheat *T. aestivum*, a successful approach of somatic fusion involving protoplasts from a non-totipotent cell line suited to *in vitro* culture T1 in combination with totipotent protoplasts extracted from embryogenic calli T2 and oat, *Avena sativa* has been established (Xiang *et al.* 2010). Asymmetric somatic hybridization was used to transfer the bacterial blight resistance trait from *Oryza meyeriana*, a wild rice species, into an elite japonica rice cultivar (Dalixiang). Analysis of random amplified polymorphic DNA indicated that hybrid plants have banding patterns determined from their parental genotypes, resistance to bacterial blight pathogens was intermediate in the majority of the hybrids compared to *O. meyeriana* and *O. sativa* cv. Dalixiang, four of the hybrid lines had high bacterial blight resistance, lower than that seen in *O. meyeriana* (Yan *et al.* 2004). Bhattacharjee *et al.* (2015) used somatic hybridization to transfer cytoplasmic male sterility in rice, which is essential for hybrid breeding (Ahmadikhah & Karlov 2006).

Rutaceae (*Citrus* species)

Somatic hybridization has solved numerous problems concerning Citrus reproductive characteristics, permitting the development of new genotypes, due to apomixis and the extended juvenile period, conventional hybridization seems to be impractical in *Citrus* spp. As a result of somatic hybridization, *Citrus* rootstocks become more resistant to numerous biotic and abiotic challenges, improving fruit quality and productivity. Protoplast fusion between sweet orange and mandarin/mandarin hybrids scion cultivars was performed, and the regenerated plants were characterized based on their leaf thickness, ploidy level, and simple sequence repeat (SSR) molecular markers. Plants were successfully created only when the embryogenic parent was 'Pera' sweet orange, 15 plants were regenerated, 7 of which were tetraploid and 8 were diploid. Based on SSR molecular markers analyses, all seven tetraploid regenerated plants proved to be allotetraploids (somatic hybrids). This hybrid material could be employed as a tetraploid parent in interploid crosses for citrus scion breeding (Soriano *et al.* 2012). Guo *et al.* (2004) conducted somatic fusion experiments in *Citrus*, aiming to create seedless cybrids through the targeted transfer of CMS by fusing embryogenic callus protoplasts of one parent with leaf protoplasts of a second parent. Through symmetric somatic hybridization experiments, breeding programs for the *Citrus* genus capitalized on the favorable traits of two cultivars, *C. inshui* cv. Satsuma, which exhibits CMS and does not produce seeds, was crossed with the cultivar *C. grandis* HBP, known for its high commercial quality but abundant seeds (Guo *et al.* 2004). Intergeneric somatic hybridization between Valencia orange, *C. sinensis*, and Meiwa kumquat, *Fortunella crassifolia*, increased the offspring's vigor. When mtDNA banding pattern data was paired with observations on phenotypic performance in the field, it was discovered that the more complicated mtDNA banding pattern corresponded with enhanced plant vigour (Cheng *et al.* 2003).

SH in other important agricultural crops

In addition to the aforementioned applications, it was proposed to extend SH technology in many other horticultural crops; in soft fruit species, the protoplasts (PEG-induced) of *Rubus fruticosus* (blackberry; 2n = 4x = 28) with *R. ideaus* (raspberry, 2n = 2x = 14) were successfully fused. Colony formation on solid media was initiated for the production of several callus lines. Cytological analyses were performed on selected callus lines with hexaploid chromosome number. Two hexaploid fusion callus lines, assigned for their homogeneity in growth and ploidy level, examined by molecular cytogenetic techniques of fluorescent *in situ* hybridization (FISH) probed with ribosomal DNA (rDNA) revealed variable numbers and sizes of loci and genomic in situ hybridization (GISH) which revealed the presence of the heterokaryon within the fusion callus lines, FISH results suggest that large karyotype rearrangements occurred, including variation in chromosome number and rDNA loci translocations (Mezzetti *et al.* 2001). Different investigations have used molecular markers to characterize symmetric and asymmetric somatic hybrids between sunflower, *Helianthus annuus* and *H. maximiliani*. Both kinds of hybrids produced varying degrees of recombination between the two parental genomes, making some hybrids excellent candidates for inclusion in traditional breeding programs (Binsfeld & Schnabl 2002). Inter-generic breeding of *Fragaria* × *ananassa* and *Rubus* to create new *Phytophthora* resistance in *Fragaria* has been studied. The study exhibited the utilization of electrofusion to generate *Fragaria* × *ananassa* var. Elsanta (+) *Rubus heterocaryons* and microcalli (Geerts *et al.* 2009). The sterility barriers connected to banana species have been overcome by using SH. Assani *et al.* tetraploid plants created by the protoplast fusion of elite diploid clones with other diploids were crossed to create regenerated triploid clones (Assani *et al.* 2010).

Limitations of somatic hybridization

Although numerous studies have been conducted throughout the world that employ SH to produce superior plants, there are challenges and limitations to large-scale implementation. The most significant of such constraints is that somatic hybridization does not guarantee that plants will generate viable and fertile seeds and provides no assurance of the successful expression of a specific trait. Since somatic hybrids have both parents' genomes, they may display both desirable and undesirable traits from their fused parents, leading to unpredictable phenotypic characteristics and possibly making them unusable (Vales *et al.* 2007). Furthermore, a genetic imbalance could result from inserting a sizable amount of exogenous genetic material in addition to the genes of interest. Fruits might display undesirable characteristics like thick, uneven skin, which would limit their use (Liu & Deng 2002). It is worth mentioning that protoplast culture has been associated with genetic instability, the protocol is often time-consuming and labor-intensive, and poor plantlet regeneration efficiency *in vitro* is a restriction in many species.

CONCLUSION

Breeders have effectively exploited genetic variability within the species to advance crop development. Nevertheless, considerable efforts have been made to increase the crop gene pool because the current variability in a breeding population might need to be revised for contemporary plant breeding objectives. Once intriguing genes have been discovered and isolated, they can be transferred using transformation. However, somatic hybridization may be the preferred method if the genes responsible for most traits are unknown. In addition to facilitating the transfer of unidentified genes, somatic hybridization can also be utilized to alter and enhance polygenic traits. While progress has been made in protoplast isolation, regeneration, and genetic manipulation via somatic hybridization, significant problems remain to address. These include the inability of various protoplast species to display totipotency and genetic instability.

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