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Chapter - 4

Production of Somatic Hybrids and Cybrids

Richa Bora

Abstract

Breeders have successfully used genetic diversity within the species in their quest to enhance crops. Large efforts have been made to expand the current gene pool of crops since the variability present in a breeding population may not be adequate for modern plant breeding goals. New traits have primarily been introduced through sexual crosses between genotypes within or between closely related species. Only closely related species or even unrelated organisms may exhibit a number of desirable and agronomically noteworthy features. There are currently techniques for transmitting genes across sexual boundaries and over significant taxonomic distances thanks to the quick development of somatic cell genetics. Somatic hybridization is useful for altering and enhancing polygenic features in addition to transferring unidentified genes. Additionally, because the hybrid cell has a mixture of the two fusion partners, somatic hybridization makes it possible to alter the genetic makeup of organelles. Somatic hybridization, also known as the creation of hybrid plants through the fusion of the protoplasts of two distinct plant species or varieties, is the process by which such hybrids are created. Therefore, somatic hybridization is only an option when both of the following two conditions are met: totipotency of the separated protoplasts and large-scale protoplast isolation. Somatic hybridization, which creates inter-specific and inter-generic hybrids, is a crucial tool for plant breeding and crop development. It is advantageous for asexual, sterile plants as well as those that are sexually incompatible with other species of plants.

Keywords: Cybrids, hybridization, protoplast culture, protoplast fusion, somatic hybrids.

Introduction

Somatic hybridization and cybridization is a novel approach in crop improvements and plant breeding through the development of inter-specific

and inter-generic hybrids. Protoplast fusion of two different genotypes represents the finest technique followed by selection of somatic hybrids and regeneration of a fully viable hybrid plant (Evans and Bravo, 1988). The protoplasts of two different plants or species or varieties are isolated and fused together to produce hybrids. This phenomenon is known as somatic hybridization.

Somatic hybridization also includes combination of cytoplasmic organelles from two different parents resulting in nuclear-cytoplasmic combinations. The fusion of nucleus of one parent and the extra-nuclear genomes of another parents lead to the formation of cybrids and the phenomenon to obtain cells or plants through this process is called cybridization.

This chapter deals with the various techniques for production of somatic hybrids and cybrids involving a series of interdependent series. Figure 1 shows the process of isolation and culture of protoplasts. Regeneration of a complete plant from the hybrid cell is dealt with in figure 3. Others related aspects are discussed in the succeeding chapters.

1. Somatic hybridization

1.1 Protoplast isolation

Protoplast isolation has been described from from mesophyll cells of *in vivo* and *in vitro* developed plants, aseptic seedlings, embryogenic and non-embryogenic organisms, male and female gametes, cotyledons, hypocotyls, and suspension cultures. The tissue that is most frequently used to isolate protoplasts is the young leaves from aseptic shoot cultures that are cultivated *in vitro*. The flexible arrangement of the mesophyll cells in the leaves allows the enzymes to easily reach the cell wall. It takes at least two enzymes to isolate protoplasts, such as pectinase to dissolve the central lamella that connects the nearby cells and cellulose to break down the cell walls and release the protoplasts. Hemicellulase may be necessary for some tissues in addition to cellulose and pectinase in order to release protoplasts. These enzymes' activity is influenced by pH and temperature. The enzyme mixture is adjusted to have a pH between 4.7 and 6.0. Although the tissues are typically incubated in enzyme solutions at 25 to 30°C because the higher temperatures may harm the cells, the enzymes have their peak activity between 40 and 50°C. In order to isolate and cultivate the isolated protoplasts because they are osmotically fragile, a range of ionic and nonionic solutes are utilised as osmoticum. Mannitol, a sugar that is metabolically inert, is the most widely used osmotic stabiliser and can be

found in concentrations of 450–800 mM. The most often used purification method is filtration followed by centrifugation. The protoplast preparation is contaminated with undigested cells, tissues, and debris of overdigested broken cells, which must be removed before their culture and manipulation.

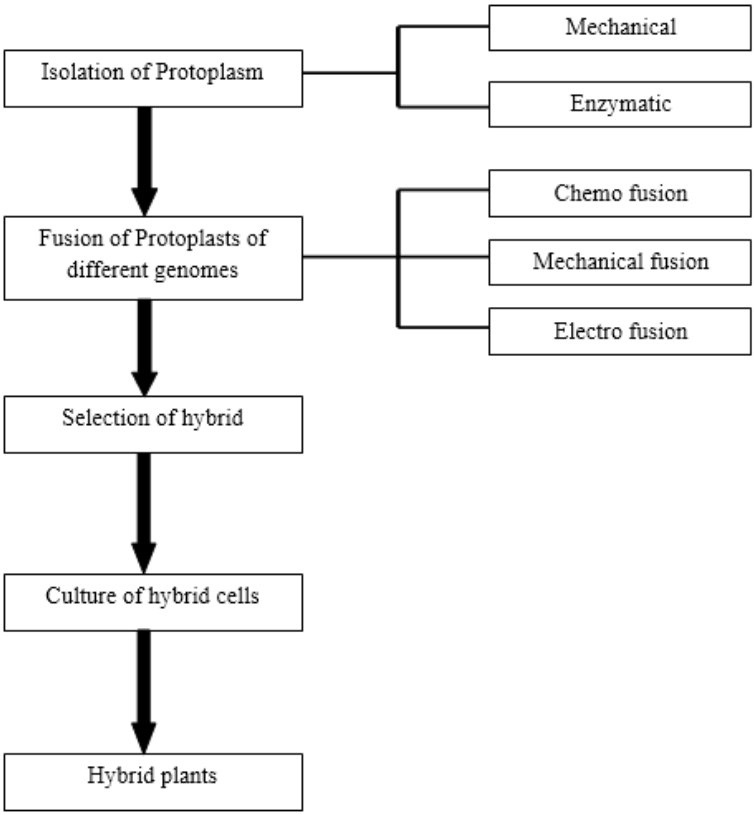

Fig 1: Schematic representation of production of hybrid cells via protoplast fusion

1.2 Protoplast fusion

Bringing newly isolated protoplasts into close proximity and holding them there for a few minutes causes them to merge. The most popular methods for fusing plant protoplasts are electrical stimulation and chemical fusion using PEG (polyethylene glycol). PEG is commonly used as a fusogen of plant protoplasts because it causes frequent heterokaryon production while being quite nontoxic to plant cells. The original PEG approach and the high Ca2+ high pH treatment are frequently combined. Homokaryons are fused protoplasts with two nuclei from the same parent, while heterokaryons are those with two nuclei from different parents. A

hybrid cell is created when the heterokaryons' nuclei merge during culture. The three main drawbacks of the chemical fusion method are: (1) the fusogen must be eliminated before culture; (2) it forms random and many cell aggregates; and (3) it is hazardous to various cell systems. Additionally, electrofusion is a quick (completes in 15 min), synchronous, practical method that allows for the fusion of specific protoplasts because the treatments are comparatively nontoxic to protoplasts. Compared to chemical fusion, electrical fusion is more repeatable and frequently produces higher fusion frequencies. Protoplasts for electrofusion should be of high quality since those of lower quality rupture during the process and release salts that alter the conductivity of the fusion mixture. The osmolarity of the fusion mixture and the strength, length, and number of DC pulses must all be optimized for effective electrofusion.

1.3 Protoplast culture

Numerous techniques, such as hemocytometer, similar to those used for cell culture, have been used to culture freshly isolated protoplasts and after fusion treatment. Under ideal culture conditions, protoplasts synthesize a wall within 24 hours and lose their distinctive spherical shape. Generally, a proper somatic cell wall is required for the cell to function. In protoplast cultures, the period of time required for the first cell to divide depends on the species, genotype, source of the protoplasts, the technique used to isolate them, their viability, the composition of the culture media, and the culture conditions.

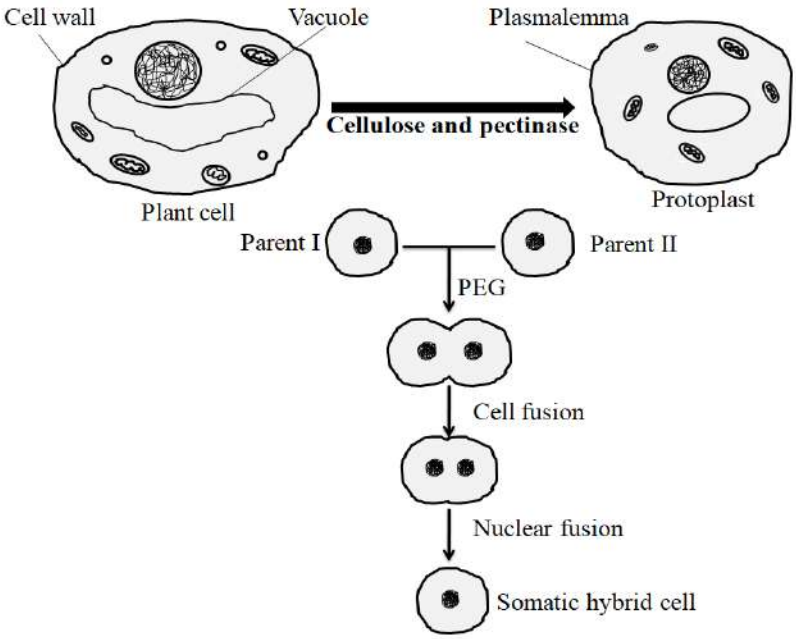

Fig 2: Production of somatic hybrid cells

1.4 Selection of somatic hybrids

The fusion mixture that results from chemical fusion is typically a mixture of heterokaryons, homokaryons, parental types, and other nuclear cytoplasmic combinations. Desirable fusion products always occur less frequently than their parental types. The population of hybrid cells has been chosen or enriched using a variety of techniques. Biochemical mutants, antibiotic, and herbicide resistance are among those that are employed rather regularly. Until now, albino mutants and mutants without nitrate reductase have been the most commonly used biochemical mutants.

1.5 Plant regeneration

Organogenesis or embryogenesis is two processes that enable plant regeneration from newly isolated protoplasts or after their union. Takebe *et al*., (1971) provided the first account of plant regeneration from isolated *Nicotiana tabacum* protoplasts.

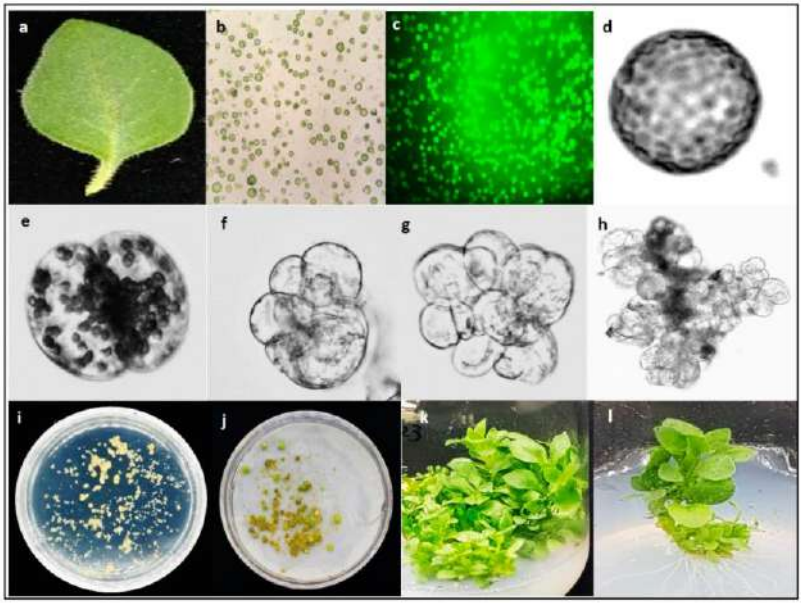

Fig 3: Protoplast isolation and shoot regeneration from a protoplast-derived callus of *Petunia hybrida* cv. Mirage Rose. (a) Fully expanded *in vitro* leaf used for protoplast isolation, (b) protoplasts isolated from leaf mesophyll cells, (c) protoplasts stained with fluorescein diacetate, (d) status of a single protoplast on day 1 in liquid culture, (e) first cell division on day 3, (f) second cell division on day 5, (g) third cell division on day 7, (h) microcolony formation, (i) microcalli formation from the microcolonies, (j) callus proliferation from microcalli, (k) shoot induction from the callus, and (l) *in vitro* rooted shoots derived from protoplasts. (Kand *et al*.,2020)

2. Genetic consequences of protoplast fusion

Protoplast fusion at plasmodesmata level is non-specific, and fusion of cells between species, genera, families, or even kingdoms is not prevented. In order to introduce beneficial genes from wild species into modern cultivars of crop plants, a variety of wide crosses between sexually incompatible parents have been attempted using cell fusion.

Thorough cytological and biochemical examinations of somatic hybrid cell lines and the plants formed from them have shown that hybrid cells can produce symmetric hybrid plants that include the complete nuclear genomes of both fusion partners. Asymmetric hybrids, on the other hand, have the whole nuclear genome of one parent and only a portion of the nuclear genome of the other parent because slow elimination of one partner's chromosomes over several cell cycles is more common. The third groups of plants produced from fused protoplasts are known as cybrids, which have at

least a few extra-nuclear genes from alien species and just one partner's nuclear genome.

The fate of the nuclear genome in the course of somatic hybridization largely depends on three factors:

1) The number and type of parental cells participating in fusion;
2) Genomic segregation during the first division of the fusion product; and
3) Chromosome segregation and/or rearrangement during colony formation and/or plant regeneration.

Consequently, a variety of genetic recombinants may appear in a mass protoplast fusion experiment with various frequencies.

Table 1: Examples of inter-specific and inter-generic somatic hybrids

Sl. No.	Somatic hybrids	References
Inter-specific somatic hybrids		
1.	*Brassica napus* + *B. juncea*	Sjodin and Glimelius (1988b)
2.	*B. napus* + *B. nigra*	Sjodin and Glimelius (1988a,b)
3.	*B. napus* + *B. oleracea*	Jourdan *et al*., (1989)
4.	*Citrus sinensis* + *C. limon*	Tusa *et al*., (1990)
5.	*C. sinensis* + *C. paradisi*	Ohgawara *et al*., (1989)
6.	*Lycopersicon peruvianum* + *L. pennellii*	Adams and Quiros (1985)
7.	*Oryza sativa* + *O. brachyantha*	Hayashi *et al*., (1988a)
8.	*O. sativa* + *O. officinalis*	Hayashi *et al*., (1988a)
9.	*Solanum melongena* + *S. integrifolium*	Kameya *et al*., (1990)
10.	*S. melongena* + *S. nigrum*	Guri and Sink (1988b)
11.	*S. melongena* + *S. ethiopicum*	Daunay *et al*., (1993)
12.	*S. tuberosum* + *S. circaeifolium*	Mattheij *et al*., (1992)
Inter-generic somatic hybrids		
1.	*Brassica campestris* + *Barbarea vulgaris*	Oikarinen and Ryoppy (1992)
2.	*B. carinata* + *Camelina sativa*	Narasimhulu *et al*., (1994)
3.	*B. juncea* + *Moricandia arvensis a*	Kirti *et al*., (1992b)
4.	*B. napus* + *Arabidopsis thaliana*	Forsberg *et al*., (1994)
5.	*B. napus* + *B. tournefortii*	Liu *et al*., (1995)
6.	*C. reticulata* + *Citropsis gabunensis*	Ling and Iwamasa (1994)
7.	*Oryza sativa* + *Echinochola oryzicola*	Terada *et al*., (1987)

8.	*Solanum lycopersicoides* + *Lycopersicon esculentum*	Hossain *et al*., (1994)
9.	*Solanum tuberosum* + *Lycopersicon pimpinellifolium*	Okamura (1988)

3. Cybridization

In sexual hybridization, only the female parent typically contributes the plastid and mitochondrial genomes, but in somatic hybridization, both parents' extranuclear genomes are joined. As a result, the latter method of plant crossing presents a rare chance to research how the cytoplasmic organelles interact. Plants with new nuclear/plastid/mitochondria genome combinations are produced through interparental recombination of mitochondrial genomes and separate assortment of chloroplasts and mitochondria after cell fusion. A plant is referred to as cybrid if its nuclear genome is primarily derived from one of the fusion partners and has at least some alien organelle genomes that are derived from the other fusion partner.

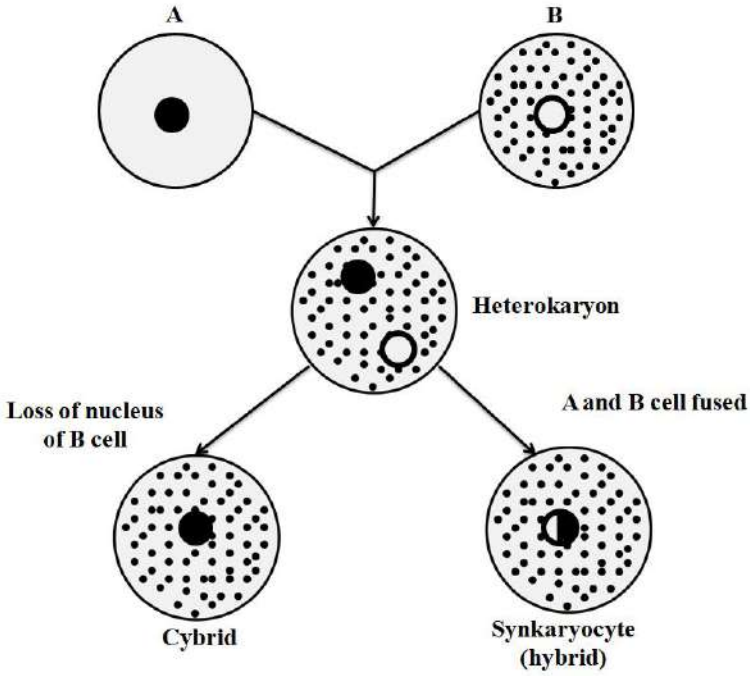

Fig 4: Production of cybrids

Extranuclear genomes contain the genetic coding for some desirable characteristics, including cytoplasmic male sterility (CMS), specific disease

tolerance, and herbicide resistance. Conventionally, alloplasmic lines those with the nucleus of one parent in the cytoplasm of the other parent are created by mating the two parents, with the cytoplasm-donor parent serving as the female parent. This is followed by several backcrosses, with the recipient parent serving as the recurrent pollinator. This process takes a long time, possibly several years. The fact that alloplasmic transfer may only be carried out between species that are sexually compatible is another disadvantage of this technique. Additionally, this method forbids combining two cytoplasmically controlled traits that exist in various plants.

i) **Techniques for creating hybrids:** Cybrids may develop through the fusing of the complete protoplasts of two parents in an experiment, such as

   a) Fusion of a normal protoplast with an enucleate protoplast;
   b) Fusion between a normal protoplast and a protoplast containing non-viable nucleus;
   c) Elimination of one of the nuclei after heterokaryon formation; or
   d) Selective elimination of chromosomes at a later stage.

Although cybrids without a nuclear fusion control have been created using this method, it is feasible to increase the likelihood of recovering the intended cybrids by inactivating the donor parent's nucleus using X-irradiation of its protoplasts (5–30 kR) prior to fusion. Organelle genomes do not appear to be negatively or mutagenically affected by these treatments, most likely because they are abundantly present in each plant cell. As a result, only the protoplasts of the recipient parent with the cytoplasmic genome of either or both parents are able to divide and regenerate plants when the donor plant's irradiation protoplasts are fused with the recipient plant's normal protoplasts and fusion products are cultivated. However, when creating hybrids from irradiated donor protoplasts, it is possible to incorporate the donor parent's nuclear DNA fragments into the recipient genome. After all, this is the most widely used technique for breeding asymmetric hybrids. It's likely that adding more selection pressure in favour of the recipient parent's nuclear genome will improve the likelihood of cybrid production using this strategy.

ii) **A few illustrations of practical cell-fusion-produced hybrids:** In tobacco, petunia, rice, and *Brassica* species, hybridization has been utilised to successfully produce intergeneric and interspecific transfer of cytoplasm.

4. Advantages of somatic hybridization

i) It results in production of a reproducible high frequency of heterokaryon.

ii) Low cell toxicity.

iii) Production of reduced bi-nucleate heterokaryon.

iv) Since PEG-induced fusion is non-specific, it can be used to a variety of plants.

5. Limitations of somatic hybridization

Somatic hybrids were originally thought to be extremely beneficial for crop improvement. However, the preliminary results are not very positive. Techniques for the selection and management of somatic hybrid cells and the regeneration of hybrid plants from them are now restricted to a small number of exceptional circumstances where they can be easily controlled in culture. Agronomically significant plants cannot currently be produced as somatic hybrids. To overcome the pre-fertilization barrier to sexual incompatibility or any genomic incompatibility was the primary goal of protoplast fusion and somatic hybridization. As a result, it would be unquestionably expected that protoplast fusion would result in very wide crosses and resolve a number of issues with regard to crop improvement. However, it is almost impossible to cross two closely related but sexually incompatible species of plants.

There are just a few inter-specific somatic hybridizations that result in plants that are either sexually compatible or incompatible because of natural reproductive isolation. Another restriction of somatic hybridization is the removal of chromosomes from the hybrid cell in specific wide crossovers. Delightful hybrids are thus no longer accessible. Even though various efforts have been made to enhance the proportion of fused cells, somatic hybridization still has this constraint. Last but not least, there is no standardized technique that can be used for all material for hybrid identification, selection, and isolation at the culture level.

6. Applications of somatic hybridization

The creation of symmetric hybrids with the whole nuclear genomes of both parents is the most typical goal of somatic hybridization. Hybrid vigour frequently promotes somatic hybrid recovery after protoplast fusion. A novel somatic hybrid occasionally serves as a direct improvement to a cultivar. The creation of a germplasm as a source of superior breeding parents for many sorts of traditional crossings involving numerous distinct crop species is the most significant use of somatic hybridization. In particular, it has increased

the possibility of producing intergeneric combinations that maximise genetic diversity. To gain access to genes that give disease resistance, numerous somatic hybrids have been created. Somatic cybridization is the process of fusing the mitochondrial or chloroplast genome of one parent with the nuclear genome of the other parent. The donor-recipient approach and cytoplast-protoplast fusion are two ways to create hybrids, although they can also develop naturally by intraspecific, interspecific, or intergeneric symmetric hybridization. Somatic cybridization experiments focus mostly on transferring the cytoplasmic male sterility (CMS) to aid traditional breeding. Incomplete asymmetric somatic hybridization, which is similar to somatic cybridization, also offers the chance for the transfer of nuclear genome fragments, such as one or more intact chromosomes from one parent who is the donor into the intact genome of a second parent who is the recipient.

Somatic hybrids are typically employed to transfer beneficial genes, such as genes for disease resistance, abiotic stress resistance, or genes with industrial use. Somatic hybridization reduces the amount of time needed for cytoplasm transfer from 6-7 years to just one year. Additionally, this technique enables cytoplasm transfer across species that cannot coexist. Somatic hybridization also overcomes the incompatibility barriers in sexual recombination at the inter-specific or inter-gene levels. On the other hand, somatic hybridization by protoplast fusion has proved a potent tool for genetic advancement (Mendes *et al*, 2001).

i) Disease and insect resistance: Numerous disease resistance genes (such those for the potato virus X, club rot disease, and tobacco mosaic virus, for example) could be effectively transferred from one species to another. For instance, resistance to pests and diseases like TMV and spotted wilt virus has been bred into tomato plants. To transfer the bacterial blight resistance trait from wild Oryza meyeriana L. to Oryza sativa L. ssp. Japonica, asymmetric somatic hybridization was used. (Yan *et al*.,2004). The possibility of partial genome transfer, which may be more tolerable than whole-genome transfer, makes asymmetric hybridization particularly attractive. Similar to how traits from "wild" Nicotiana species have been utilised to improve crop species in other agricultural species, tobacco has included traits from at least 13 other species.

ii) Environmental tolerance and wider adaptation: By removing the constraints of sexual incompatibility, *in vitro* fusing of protoplasts creates a path for the development of distinctive hybrid plants. Through somatic hybridization, the genes for cold, frost, and salt

tolerance could be successfully introduced, for example, the insertion of the cold tolerance gene in tomatoes. By fusing ditch reed and rice protoplasts using an electrofusion process for salt tolerance, somatic hybrids were created. The method has been used in the horticultural sector to develop new hybrids that produce more fruit and are more disease-resistant. When citrus protoplasts were fused with other closely related citrinae species, viable hybrid plants were successfully created (Motomura T, et.al, 1997).

iii) Germplasm diversification: The genomes of two species are combined during somatic hybridization via protoplast fusion, which is then used to convey monogenic or polygenic characteristics (Liu *et al*.,2005). By mixing the cytoplasmic genomes of multiple species or cultivars, it also produces unique genotypes. This method resulted in the generation of several somatic hybrid plants that were interspecific, intergeneric, intertribal, and even interfamilial. There are a sizable number of reports on somatic hybrids involving grasses available. Protoplast fusion somatic hybridization can be a useful strategy for grass genetic improvement (Xiang *et al*.,2010).

iv) Overcoming barriers of sexual incompatibility: By using traditional breeding techniques, it is impossible to have sexual relations between two different species (inter-specific) or two separate genuses (inter-generic). Sexual incompatibility hurdles are surmounted by somatic hybridization. Examples:

Pomato (*Solanopersicon*, a novel genus) was produced by the fusion of tomato (*Lycopersicon esculentum*) and potato (*Solanum tuberosum*) protoplasts.

To enhance the crop, interspecific fusion of the four rice species *Oryza brachyantha*, *O. elchngeri*, *O. officinalis*, and *O. perrieri* could be carried out.

v) Transfer of cytoplasm: Certain plants have cytoplasmically regulated genetic characteristics. This includes a few forms of male sterility as well as drug and herbicide resistance. A excellent method for quickly transferring the appropriate cytoplasm is through hybridization. In agriculturally useful plants, hybrids are crucial for the transmission of antibiotic and herbicide resistance, as well as cytoplasmic male sterility (CMS). CMS has been transferred successfully in rice by hybridization. There have been generated *Brassica raphanus* hybrids with the nucleus of *B. napus*,

chloroplasts of *B. campestris* resistant to atrazine, and male sterility from *Raphanus sativas*. The cytoplasmic genes and their roles have been studied with the aid of somatic hybridization. In actuality, plant breeding operations successfully utilise the knowledge. To create a distinct nuclear-cytoplasmic genetic mix, mitochondria and chloroplasts will combine through protoplast fusion. Plants that are still in their juvenile stage might undergo somatic hybridization. Innovative plants will result from protoplast transformation (with features like nitrogen fixing by adding external DNA) followed by somatic hybridization.

7. Conclusion

Somatic hybridization has evolved from the academic stage to field applications, making it a promising method for introducing foreign genes, such as polygenic characteristics, into crop plants. Somatic hybridization has the unique ability to combine different nuclear and/or cytoplasmic organelles, which increases the gene pool's diversity. Symmetrical hybrids, which are plants with the whole nuclear genomes of both parents, may develop from the hybrid cells. However, there are frequently different mixes of nuclear and cytoplasmic genomes as a result of interactions between the two parents' genomes. Prior to plant regeneration, the nuclear genome of one of the parents may be partially or entirely destroyed throughout subsequent cell cycles, resulting in the creation of an asymmetric hybrid or cybrid, respectively. The impact of somatic hybridization has been greatly increased by asymmetric somatic hybridization, which transfers a portion of the donor parent's gene, and by cybridization, which transfers cytoplasmic features. Furthermore, it is well known that protoplast fusion can be exploited to produce valuable bridging material for breeding schemes.

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forms, and each organelle can harbour many copies of the genome. Sequenced plastid genomes range from 1,000 kbp in size; they often (but not always) contain two inverted repeats, which separate large and small single copy regions. Plastid genomes are often rich in adenine and thymine (AT) residues, a hallmark of reductive evolution also seen in mitochondrial genomes and those of bacterial endosymbionts. Some plastid genomes have abundant non-coding DNA; others are full of self-splicing introns. Some dinoflagellates have their genome spread across a sea of mini-circles and, recently, certain green algae have been found to have a plastid genome comprised of multiple linear chromosomes that form hairpin structures.

Plastid genome encodes depends on the genome, but there are common themes. All plastid genomes have been shaped by reductive evolution. A large fraction of the cyanobacterium derived genes needed for plastid function now reside in the nucleus, having migrated there by a process known as endosymbiotic gene transfer (EGT). Consequently, most plastid proteins are imported post-translationally. Plastid genomes nevertheless typically encode at least some of their own information processing machinery, including ribosomal RNAs, ribosomal proteins, tRNAs, and a bacterial RNA polymerase — though land plants also have a nuclear-encoded plastid RNA polymerase. In addition, not surprisingly, plastid genomes encode many components of the photosynthesis apparatus, such as photosystem II and I proteins (e.g., the well-known psbA gene coding for the D1 unit of photosystem II) as well as cytochrome b6f, which mediates electron transfer between the two photosystems. Nevertheless, there are lineage-specific differences in plastid genomes, for example, transfer of the gene encoding the small subunit of RuBisCO (rbcS) to the nucleus in the green lineage, and the presence of genes coding for phycobilisome proteins in red algae and glaucophytes. Plastid genomes are retained in almost all secondarily non-photosynthetic eukaryotes as well.

Mitochondrial genome: Occurrence and decryption

The term proto-mitochondrial denotes the already reduced genome of the last common ancestor (proto-mitochondrion) of extant mitochondria, assuming that mitochondria have a single evolutionary origin mitochondria. On the basis of SSU rRNA data, mitochondria have been associated specifically with the α-proteobacterial subdivision that contains such obligate intracellular parasites as Rickettsia, Ehrlichia, and Anaplasma, grouped together into the taxonomic assemblage Rickettsiaceae. The only complete α- proteobacterial genome sequence available at present, and the most