

# Male Sterility and Fertility Restoration in Crops

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**Abstract:** Plant male sterility refers to the failure in the production of fertile pollen. It occurs spontaneously in natural populations and may be caused by genes encoded in the nuclear (genic male sterility; GMS) or mitochondrial (cytoplasmic male sterility; CMS) genomes. The exploitation of male sterility systems has enabled the commercialization of heterosis in many crops. This feature has great agronomic value for the production of hybrid seeds, since it prevents self-pollination without the need of emasculation which is time-consuming and cost-intensive. CMS has been widely used in crops, such as corn, rice, wheat, citrus, and several species of the family Solanaceae and Cruciferous. Mitochondrial genes determining CMS have been uncovered in a wide range of plant species. The modes of action of CMS have been classified in terms of the effect they produce in the cell, which ultimately leads to a failure in the production of fertile pollen. The interaction of chimeric mitochondrial genes with nuclear genes causes CMS, which may be restored by *restorer of fertility* (Rf) genes. Male fertility can be restored by nuclear-encoded genes, termed restorer-offertility (Rf) factors. CMS from wild plants has been transferred to species of agronomic interest through somatic hybridization. Somatic hybrids have also been produced to generate CMS depends upon recombination of the mitochondrial genomes of two parental plants or by separating the CMS cytoplasm from the nuclear Rf alleles. As a result, somatic hybridization can be used as a highly efficient and useful strategy to incorporate CMS in breeding programs.

**Keywords:** Self- incompatibility, mitochondrial gene, somatic hybrid, genetic recombination, Fertility restoration

## I. INTRODUCTION

Male reproductive development in plants involves several major developmental stages in series and along several cell lineage pathways, which include specification of stamen primordia, production of sporogenous cells, development of tapetum and microspore mother cells (MMCs), meiosis, formation of free haploid microspores, degeneration of tapetum and release of mature pollen grains. Arrest of any of these steps can result in male sterility (MS), the failure to produce or release functional pollen grains. The phenotypic manifestations of MS may range from the complete absence of male organs, abnormal sporogenous tissues, to the inability of anther to dehisce or of pollen to germinate on compatible stigma (8).

Commercialization of any hybrid crop can only be achieved if reasonably priced technical solutions to hybrid seed production are available. In vegetable and rice, hybrid seed production was first attempted using chemical hybridizing agent in the 1970s, but this approach was no longer used after MS systems became available. In order for an MS system to be workable for hybrid seed production, it must meet the following prerequisites: (1) complete and stable MS during hybrid seed production; (2) no substantial negative effect on MS and hybrid plants; (3) ability to multiply MS seeds through an intermediate genetic line (maintainer) or under particular environmental conditions; (4) ability to fully achieve fertility in hybrids. Therefore, although a number of MS systems have been generated during the past 40 years, only those that met these requirements were adopted in hybrid production.

Numerous CMS systems with different cytoplasm/nucleus combinations have been generated through backcross breeding. The cytoplasm and nucleus of CMS lines may originate from two different species, two different subspecies (*indica* × *japonica*), or two cultivars (*indica* × *indica*)

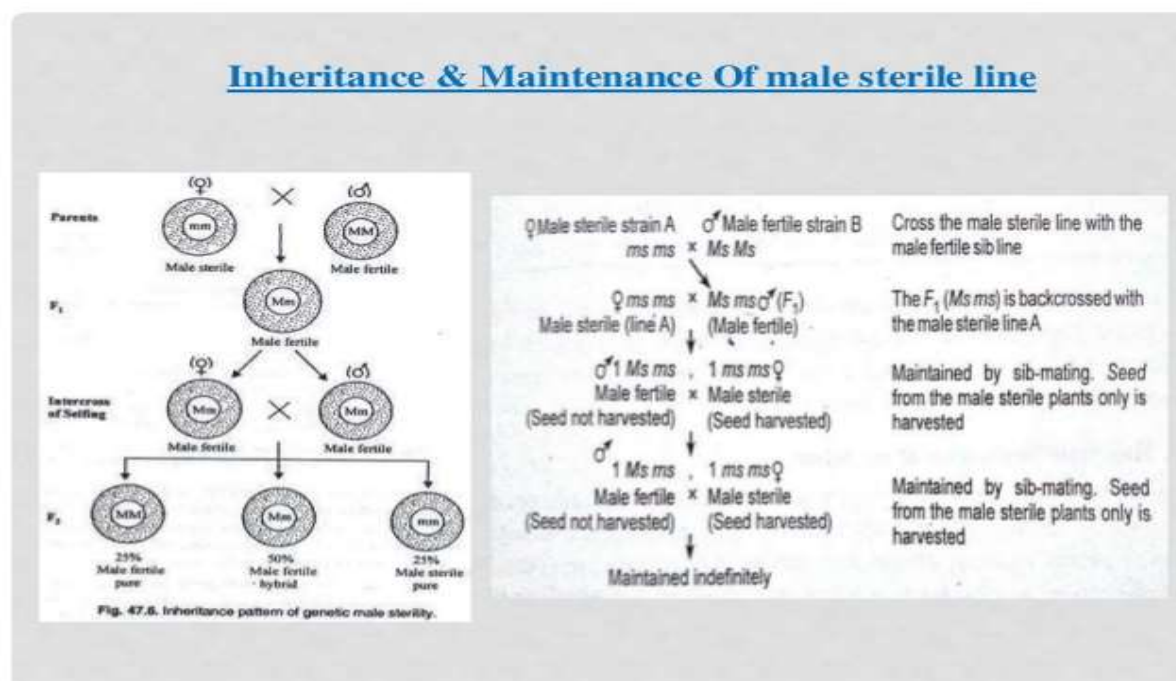
In CMS, the production of non-functional pollen is maternally inherited and conditioned by cytoplasmic (mitochondrial) genes coupled with nuclear genes (Figure 1B). The CMS condition has been reported in more than 300 plant species. In natural populations, CMS could be responsible for the existence of gynodioecy, a breeding system in which females (male sterile) and hermaphroditic individuals coexist in a population. Thus, two or more different mitotypes exist within the same species. There are commonly two alternative mitotypes in a single population, one normal (usually designated N) and the inducer of male sterility (designated S). The S mitotype interacts with a pair of nuclear alleles: a restorer-of-fertility (if dominant usually designated Rf) and a sensitive (if recessive usually designated rf) allele. In the simplest genetic model, six possible mitotype-genotype combinations are possible, only one of which leads to a male sterile phenotype (76).

Although identifying genes and pathways is necessary in order to understand the underlying mechanisms in the development of the male reproductive system, not all MS mutations have practical use in hybrid crop production. This paper aims to analyse different MS systems that have been explored in hybrid seed production and summarize the latest understanding of their genetics, biochemistry, Molecular mechanism and fertility restoration through somatic hybridization.

## II. MALE STERILITY in BREEDING PROGRAMS

Male sterility, in the broadest sense, refers to the failure in the production of dehiscent anthers, functional pollen, or viable male gametes. Although Darwin acknowledged the evolutionary importance of male sterility (9), its utility was initially ignored in breeding programs. When the potential of hybrid vigour as a breeding tool was identified, male sterility was incorporated in crop species and represented a significant step in genetic improvement programs towards the study of the influence of cytoplasm on

plant development. The concept of hybrid vigour or heterosis is related principally to yield gains of hybrid lines or cultivars given their superiority in characters like biomass, adaptability, fertility, and biotic or abiotic stress tolerance compared to their parental lines (4). A 'hybrid' can be defined as any offspring of a cross between two genetically unlike individuals. For example, the yield of hybrids obtained by crossing different lines of *Brassica napus* (rapeseed) is 30% higher than the average of their parental lines. However, the creation of hybrid crops is not a simple procedure from a technical point of view since producing hybrid seeds of self-pollinating plants requires emasculation (i.e., removing functional pollen grains to prevent self-pollination). Until the mid-twentieth century, this technique involved manual work or chemical treatments, making it costly, inefficient, and harmful to the environment. In this sense, the use of male sterility reduces the cost of hybrid seed production for several reasons. It avoids hand emasculation and pollination, accelerating the hybrid breeding programs and allowing the large-scale production of hybrid seeds and the commercial exploitation of hybrid vigour.



The male sterile condition includes both genic (GMS) and cytoplasmic (CMS) male sterility. The first one is caused only by genes encoded in the nuclear genome (24). The second one is caused by mitochondrial genes that

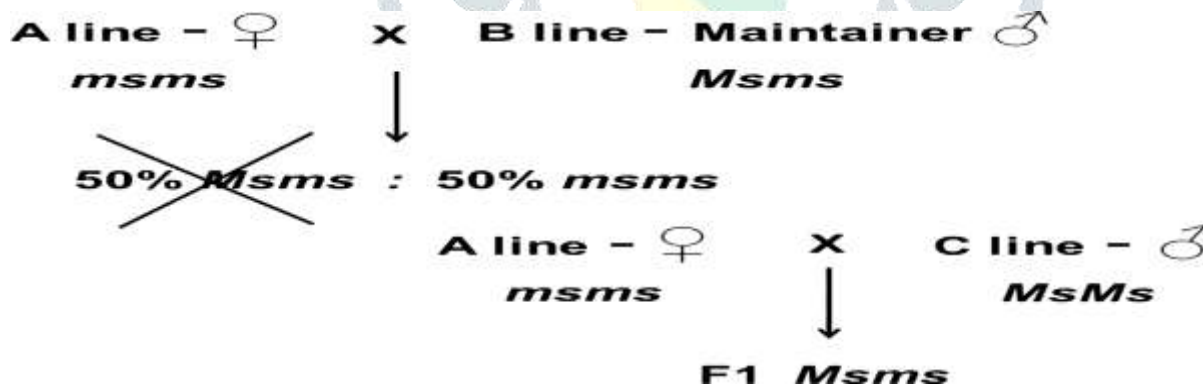


Figure 1A. Hybrid seed production using GMS system

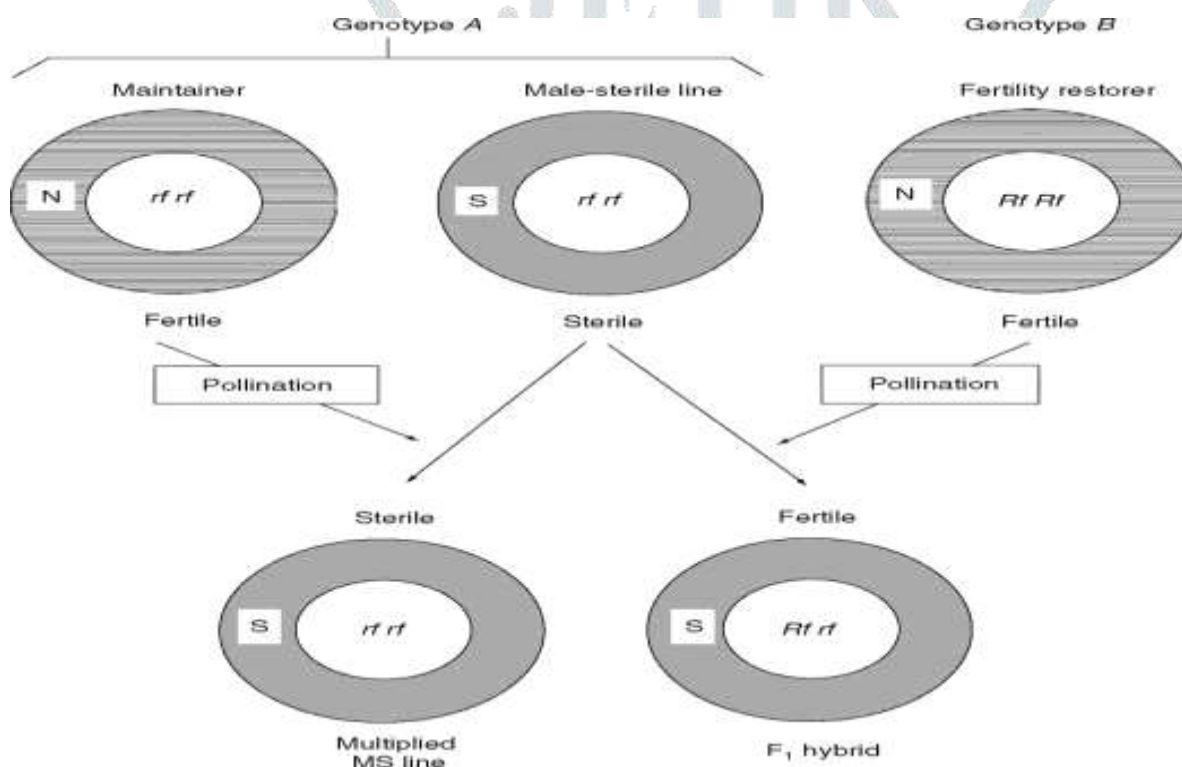
directly or indirectly affect nuclear gene functions. In GMS, nuclear Male sterility (Ms) genes control the male sterility condition without the influence of cytoplasmic sequences (Figure 1A.). In the simplest genetic model, there are three possible genotypes for the nuclear locus Ms, in which the male sterile phenotype is conditioned by recessive ms alleles. A Mendelian inheritance pattern can be observed, in which the offspring of a male sterile genotype (female line) could be entirely male fertile or segregate 50% male sterile: 50% male fertile depending on whether the parental line (male fertile) is homozygous or heterozygous, respectively (Figure 1A.). The use of GMS in plant breeding and hybrid seed production involves three different lines: i) a male sterile (female parent), ii) a maintainer, and iii) a restorer (male parent) line. The male sterile line is maintained using pollen of a maintainer line, which presents identical genotype (isoline), except for the presence of a dominant Ms allele.

However, the perpetuation of the male sterile (female parent) presents a difficulty: the Segregation obtained in the cross with the maintainer line implicates an additional step of selecting the male sterile phenotype (identification and removal of heterozygotes) for hybrid seed production (Figure 1A). The inefficiency in maintaining the male sterile line had initially restricted the use of GMS in hybrid seed production of crop species in which CMS had not been found or engineered (12). At present, the discovery of environment sensitive genic male sterility (EGMS) has overcome this drawback by eliminating the need of a maintainer line (43).

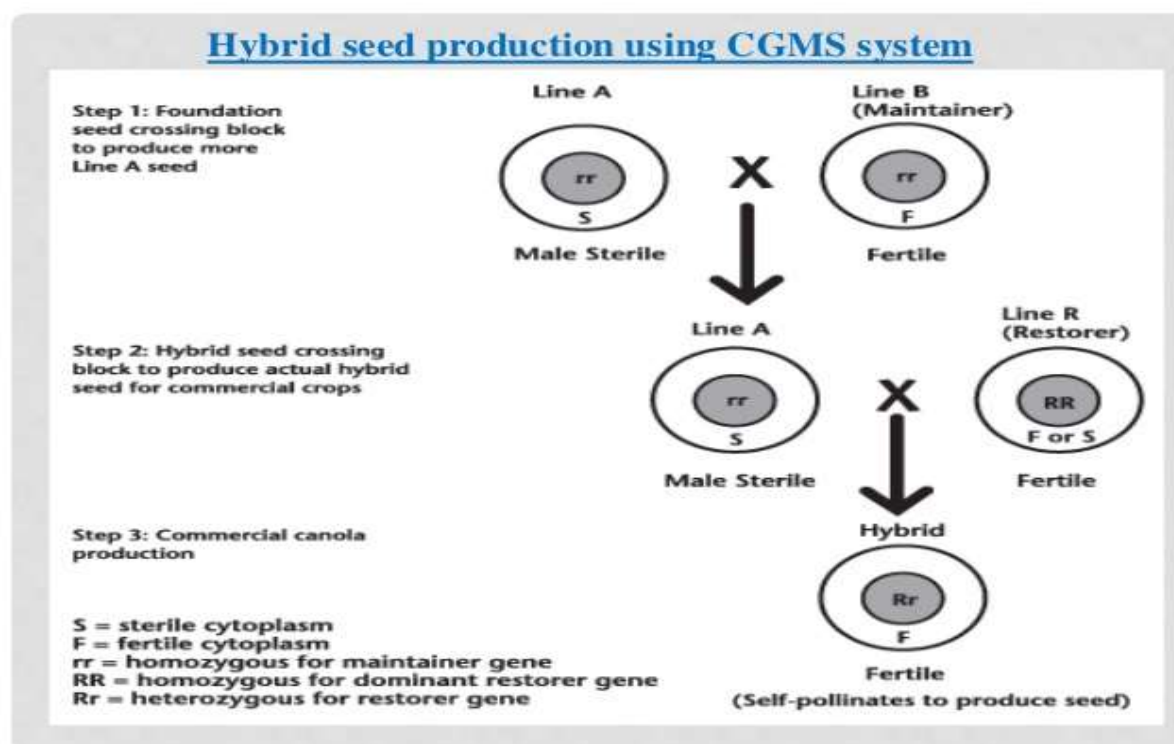
In this system, the male sterile phenotype is reversible in response to changes in environmental cues like day length and temperature; and two conditions can be differentiated: i) restrictive, in which the *ms ms* genotype exhibits male sterility, and ii) permissive, in which this genotype is male fertile (12). By cultivating under permissive conditions, the male sterile *ms ms* line can be propagated by self-pollination.

In CMS, the production of non-functional pollen is maternally inherited and conditioned by cytoplasmic (mitochondrial) genes coupled with nuclear genes (Figure 1B). The CMS condition has been reported in more than 300 plant species (49). In natural populations, CMS could be responsible for the existence of gynodioecy, a breeding system in which females (male sterile) and hermaphroditic individuals coexist in a population. Thus, two or more different mitotypes exist within the same species. There are commonly two alternative mitotypes in a single population, one normal (usually designated N) and the inducer of male sterility (designated S). The S mitotype interacts with a pair of nuclear alleles: a restorer-of-fertility (if dominant usually designated *Rf*) and a sensitive (if recessive usually designated *rf*) allele. In the simplest genetic model, six possible mitotype-genotype combinations are possible, only one of which leads to a male sterile phenotype (Figure 1B).

The offspring of the male sterile line (female line) could be entirely male sterile, entirely male fertile, or segregate 50% male sterile: 50% male fertile, depending on whether the male fertile parent is homozygous recessive, homozygous dominant, or heterozygous for the nuclear restorer-of-fertility locus, respectively (Figure 1B). Similar to GMS, the breeding value of CMS depends on the management of three different lines: i) male sterile (female parent), ii) maintainer, and iii) restorer (male parent). The male sterile line is perpetuated through crosses with the maintainer line, which is isogenic and differs only in the presence of the N-cytoplasm. In contrast to GMS, the cross between the male sterile and the maintainer lines produces only male sterile offspring (Figure 1B). Furthermore, the maintainer line can be propagated by self-pollination. Finally, for those crops whose seeds are harvested and commercialized, the male fertility needs to be restored in F<sub>1</sub> hybrids. The restorer line has dominant restorer-of-fertility alleles *Rf* and produces fertile F<sub>1</sub> hybrids. As the cytoplasm is maternally inherited, the mitotype of the restorer line is irrelevant (Figure 1B)







**Figure 1B. Hybrid seed production using CMS system**

*Genetic models for male sterility in plants and its utilization in breeding programs.*

Letters within small circles indicate nuclear genes;

Letters within big circle indicate cytoplasmic genes.

A. Genic male sterility (GMS) is conditioned by nuclear recessive ms alleles.

B. Cytoplasmic male sterility (CMS) is expressed when the sterile cytoplasm S is coupled with recessive non-functional nuclear restorer of fertility rf alleles.

A dash (-) indicates that the cytoplasm can be N (normal) or S (inductor of male sterility)

The use of CMS lines to generate hybrids was first known in maize and it has been increasingly applied to major food crops such as wheat and rice, and also in others important cereals, vegetables, legumes, oilseeds, industrial, and ornamental species like sorghum, Brassicaceae, onion, carrot, sugar beet, sunflower, soybean, pear millet, common bean, cotton, pepper and petunia (40). It is important to acknowledge that, in general, very few sources of CMS have been used in plant breeding, situation that conduces to the development of hybrids with a narrow genetic diversity. This limitation can be illustrated by the episode of the Southern Corn Leaf Blight of 1970 in United States. Upon the discovery of CMS-T (CMS-Texas) in maize in 1952, this genetic system was widely adopted by the hybrid seed corn industry of the United States during the 1960s. By 1970, the CMS-T was part of the genetic background of 75-90% hybrid cultivars grown in this country. This CMS-T cytoplasm conditioned the susceptibility to Southern corn leaf blight, disease that destroyed 15% of the maize production in 1970-1971 (5). After this epidemic, CMS-T was no longer used in maize hybrid breeding programs and today other tools are preferred by breeders for maize hybrid seed production. This example verifies the need to diversify stable sources of CMS, by identifying a variety of cytoplasmic genes producing male sterility phenotypes along with their corresponding nuclear-encoded restorer-of-fertility genes and by improving our understanding of the co-evolution of these genetic systems. Alternate CMS/Rf systems were established in rice, maize, sunflower, wheat, and Brassica in search of genetic variability and resistance to pathogens and abiotic stresses. For instance, more than 70 CMS lines were reported in wheat and sunflower (28). Modern genetic tools for studying mitochondrial genome dynamics and its interaction with nuclear genes are offering new experimental frameworks to move forward on these challenges (37).

In addition to the agronomic importance of CMS in hybrid seed production, it is also used in Citrus to achieve seedless fruit production (53). Furthermore, CMS is a feature governed by nuclear-cytoplasmic interactions and it constitutes a valuable model to increase our understanding of the cross-talk between both genomes (14). In fact, the mutations responsible for CMS provided means to demonstrate the role of the mitochondrion in reproductive development.

### III. MOLECULAR MECHANISMS RESPONSIBLE for CMS

The CMS phenotype has arisen spontaneously many times in natural populations. It originates through spontaneous mutations that involve rearrangements of the mitochondrial genome (mtDNA). In general, these mutations result from intragenomic homologous or non-homologous recombination events that create new open reading frames (ORFs) (14). Shandu et al. (2007) managed to reproduce the appearance of CMS in fertile plants after repressing the expression of the nuclear gene Msh1 that is involved in recombination surveillance in plant mitochondria. The rearrangements that cause CMS may be in low stoichiometry in plant

mitochondria but can increase their concentration through sub stoichiometric shifting allowing the expression of the CMS phenotype (54). A few studies indicated the existence of the CMS ORF in fertile lines though at extremely low concentrations.

The mechanisms that explain the condition of CMS are far from being fully understood, mainly because each CMS system seems to be unique in terms of the mitochondrial genes associated with the male sterility condition (17). In fact, there are CMS lines highly used in breeding programs, in which a specific restorer line has been developed, but the identity of the gene responsible of the male sterile condition remains unknown (52). To date, several modes of action for CMS genes have been described, namely, energy deficiency, cytotoxic proteins (18), aberrant programmed cell death and retrograde signalling from mitochondria that affects nuclear pathways. Retrograde signals from the mitochondria can involve nuclear miRNAs that provoke CMS as they are regulators of pollen development. However, the exact relationship between the candidate CMS gene and the observed phenotype has not been assessed in the majority of the cases. In addition, the mechanisms of action of the restorer-of-fertility loci are poorly understood, but most Rf genes encode pentatricopeptide repeat (PPR) proteins involved in diverse mitochondrial pathways. For instance, Rf system can act by modifying CMS transcripts or decreasing the accumulation of toxin proteins.

Identifying the molecular basis of CMS requires the use of different strategies. One of them is to search for the gene or genes responsible for CMS by comparing mtDNAs directly. Proposing a candidate CMS gene through the examination of plant mtDNA sequences is particularly challenging in these highly rearranged genomes. However, there are cases in which the gene proposed as a candidate is almost the only difference between the mitochondrial sequences of the normal and the CMS lines. In these cases, the mutation or rearrangement that gave rise to the CMS phenotype has been likely a very recent event in the mtDNA. Alternatively, a fertile and a CMS lines can be combined through somatic hybridization producing a male-sterile plant with a chimeric mitochondrial genome. If limited homologous recombination gives rise to a mtDNA with few regions from the CMS parent, candidate genes for CMS could be proposed. When rearrangements of the mtDNA create numerous new ORFs, the identification of the CMS candidate requires a differential expression assay and/or a segregation analysis (29). In general, the mitochondrial ORFs identified as CMS candidates share some characteristics: i) all causal genes for CMS are encoded in the mtDNA; ii) most ORFs are chimeric formed by a region of a known mitochondrial gene and an new sequence as a result of recombination (29); iii) new ORFs are co-transcribed with known mitochondrial genes and iv) the resulting proteins generally have transmembrane domains.

One of the most deeply studied cases is the CMS-Wild Abortive line (CMS-WA) that has been widely exploited in rice breeding. Through the examination of transcripts by RNA blotting, the CMS-associated transcript was identified revealing that it is a chimeric ORF that encodes a protein of 352 residues (wa352c) with three transmembrane domains (49). Its implication in CMS and its mitochondrial localization was confirmed by transforming the nuclear genome of rice and *Arabidopsis thaliana* with a construct that carries the candidate ORF and a mitochondrial transit signal provoking a CMS phenotype. In addition, its interaction with the mitochondrial COX11 protein, encoded in the nucleus, was confirmed by yeast two-hybrid assays. The mitochondrial wa352c is constitutively expressed in rice CMS-WA, but it accumulates specifically in the mitochondria of anther cells where it interacts with COX11 to prevent its function in the degradation of hydrogen peroxide, leading to programmed cell death and pollen abortion. Its origin and evolution were studied in detail by comparing different wild and cultivated lines. The formation of wa352c involved homologous and non-homologous recombination and sub stoichiometric shifting giving rise to protogenes that finally resulted in the ORF responsible of CMS. A restorer line for this CMS system was developed a long time before characterizing the gene responsible for male sterility (29).

Another example comes from the male sterile somatic hybrid *Brassica juncea* + *Moricardia arvensis*, in which the mitochondrial orf108, identified as responsible for CMS, is co-transcribed with the gene *atp1*. In the presence of the restorer-of-fertility allele, the transcript of *atp1* is monocistronic, after separating the gene *atp1* from the orf108 (45). The mechanism by which the orf108 causes male sterility has not been accurately confirmed. It is possible that the orf108 translates into a cytotoxic protein or it prevents the normal translation of *atp1*. Another case of CMS in *B. juncea* involves the hau line.

The CMS mitochondrial orf288 was identified by expression assays and it was analysed at the protein level. The CMS protein represses the growth of *E. coli*, pointing to a possible cytotoxic effect (21). Subsequent analysis using *A. thaliana* transformants could detect the exact stage in which the orf288 is involved, and the sites responsible for cytotoxicity. Transcript analysis detected differences in the expression of nuclear genes involved in the development of the anther and, thus, proposed a mechanism of retrograde regulation for orf288 (33).

#### IV. CMS and MITOCHONDRIAL RNA EDITING

Gene expression in plant organelles is substantially affected by post-transcriptional processing events, like intron splicing and RNA editing. In RNA editing, cytidines are changed to uridines (C-to-U) in specific RNA positions, called editing sites. These editions more frequently take place in diverse positions of mitochondrial mRNAs that are well conserved across angiosperms. Since these C-to-U changes can generally alter organellar protein products, through the creation of novel start/stop codons or changing the membranebound properties of the proteins translated from edited RNA precursors, RNA editing is essential for plants because it allows the synthesis of functional organellar proteins that are crucial for plant and seed development (16, 48).

Deficient RNA editing in plant mitochondria can induce male-sterile phenotypes because abnormal proteins are synthesized, impairing mitochondrial function. RNA editing has been associated with some CMS systems. In one of the best studied rice CMS

systems, two *atp6* genes are present in the mitochondria of CMS-Boro II, N-*atp6* and B-*atp6*. Whereas N-*atp6* is a normal *atp6* gene, B-*atp6* is similar to N-*atp6* but fused to an additional downstream ORF named *orf79*. The accumulation of B-*atp6* products in microspores affects pollen fertility because it impairs ATP synthase activity in mitochondria. However, two nuclear-encoded restorer-of-fertility factors, RF1 and RF2 (24), are responsible for suppressing the expression of B-*atp6* transcripts, which are processed into two smaller transcripts that are efficiently edited and translated into normal polypeptides (31). Conversely, when such nuclear restorer genes are absent in the nuclear background, unprocessed B-*atp6* transcripts are poorly edited and associated with male sterility (19).

In addition, RNA editing has the potential to be used as a tool for male-sterility induction. For instance, CMS has been induced in tobacco plants by introducing a nuclear transgene, an unedited *atp9* from wheat targeted to mitochondria. In this case, ATP synthases were impaired due to the competition between mitochondrial-encoded ATP9 and nuclear-encoded mitochondrial-targeted ATP9 synthesized from unedited *atp9* transcripts, since the editing machinery only acts in plant organelles. The male fertility of transgenic tobacco plants was restored by suppressing the expression of the transgenic *atp9* through an antisense strategy (51). With a similar approach, male-sterile phenotypes have been induced using transgenic and unedited *orfB* and *nad3* genes (66).

## V. CYBRIDIZATION AS A TOOL to BUILD CMS PLANTS

As the CMS phenotype is very useful in plant breeding, it is often transferred to the crop of interest from natural populations or created *de novo* in the laboratory. CMS can be experimentally induced through intraspecific, interspecific or intergeneric crosses, protoplast fusions, or genetic engineering (45). Somatic hybridization by protoplast fusion is a technique that combines somatic cells from two different cultivars, species, or genera of plants with the aim of regenerating novel germplasm. It basically consists of four steps (Figure 2): i) protoplast isolation of two parental species by lysis of the cell wall; ii) fusion of both cells aided by an electrical or chemical impulse; iii) regeneration of hybrid calli and plants; and iv) selection of the somatic hybrid lines of interest (26).

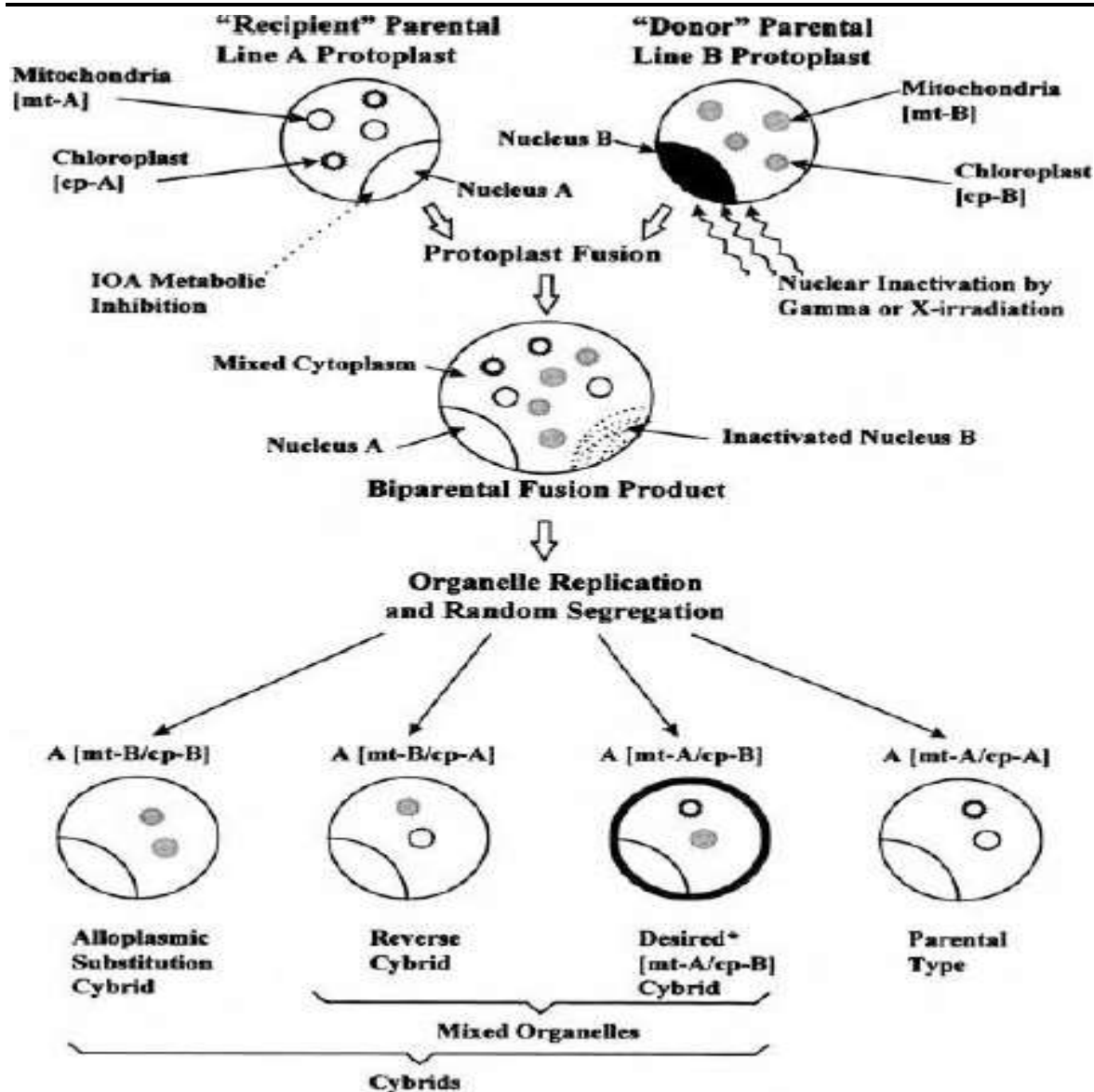
The fusion of protoplasts can be symmetric or asymmetric depending on the nature of the genetic contribution (nuclear and cytoplasmic) of the parents involved. It is symmetric when the contribution of both parental genomes is equivalent. That is, both nuclei are involved in the fusion and are part of the nuclear genome of the resulting somatic hybrid. In order to limit the genetic contribution of one of the parents, the nucleus of one of them (the donor) can be inactivated using radioactivity. This gives rise to an asymmetrical protoplast fusion that results in a somatic hybrid with the complete genome of the receptor and fragments of the donor's genome (39).

First, protoplasts are isolated from mesophyll cells by enzymatic reactions and donor protoplasts may be irradiated to inactivate the nucleus (indicated by a cross). Second, chemical or electrical protoplast fusions give rise to somatic hybrid cells and calli. Third, hybrid plants are regenerated *in vitro*. Fourth, cybrid plants of interest are selected.

A cybrid (Cytoplasmic hybrid) is a special type of asymmetric somatic hybrid in which its nuclear genome comes from a single parent while the cytoplasmic genomes are inherited from both parents but follow different fates (Figure 2). After successive cell divisions, the chloroplast tends to be uniparental with some exceptions while the mitochondrial genome is recombinant, containing segments of both parental mtDNAs (58). Due to the composition of the nuclear genome, cybrids are the most attractive in breeding programs. The fact that the nuclear genome is completely from a parent guarantees the integrity of the cultivar (22). In addition, cytoplasmic hybrids often show CMS and are a valuable tool in plant breeding.

Somatic hybridization represents a powerful tool for transferring genomes or genomic fragments of wild plants with useful agronomic characteristics to commercial crops. Protoplast fusion eludes the drawbacks of pre- and post-zygotic barriers of sexual hybridization and combines sexually incompatible germplasms between crops and even between phylogenetically distant plants (39). It also allows the transfer of desirable traits encoded by the plastid or mitochondrial genomes of an uncultivated variety to a commercial crop. Examples of the use of somatic hybridization to transfer desirable mitochondrial-encoded features include resistance to citrus canker caused by *Xanthomonas citri* (32), improved tolerance to salinity and CMS (see below). In addition, somatic hybridization allows the replacement of the cytoplasm of a cultivar in a single step, which is extremely efficient, taking into account the traditional method requiring several backcrosses to introduce exogenous cytoplasm into crops (34). Finally, somatic hybridization has been used in fundamental science for studying nuclear-cytoplasm composition and DNA methylation patterns (10), as well as to investigate the recombination pathways that take place between donor and recipient mtDNAs (37).





CP, chloroplast; MT, mitochondria; Nu, nucleus.

Figure 2. Schematic production of a cybrid plant by protoplast fusion of donor and recipient plants.

#### VI. Cybrid Production: Strategies to Produce cms Lines through Protoplast Fusion

Using somatic hybridization to transfer or create de novo the CMS feature has many advantages when compared to sexual reproduction. The classic transfer of characters through sexual hybridization is not always favourable because other genes than those responsible for CMS are simultaneously transmitted, leading to unwanted results (46). Cybridization by protoplast fusion has become a highly valuable method to introduce or generate the CMS condition by using different strategies.

In the first strategy, cybridization per se is the process by which the CMS phenotype is induced. During cybrid production, the mitochondrial genome results recombinant, containing segments of both parental mtDNAs while the nuclear content is engineered to be of a single parent (39). In some cases, the donor parent in the cybridization experiment is male fertile but presents a mitochondrial ORF responsible for CMS and a restorer-offertility allele in its nuclear genome. In the resulting cybrid, the CMS ORF is now in a different nuclear background that lacks the restorer allele and the cybrid exhibits the CMS phenotype. For instance, the intergeneric somatic hybrid between *M. arvensis* and *B. juncea* is male sterile due to the mitochondrial-encoded orf108 obtained from *M. arvensis*. However, *M. arvensis* is male fertile because of a nuclear restorer factor that cleaves the transcript containing the orf108 and atp1 (2).

Alternatively, CMS can be generated by the formation of chimeric ORFs through intergenomic recombination of the mtDNA in somatic hybrids (45). For example, the fusion of protoplasts from *B. napus* and *Isatis indigota* gave rise to a plant with low pollen viability. Through the analyses of the parental and hybrid mitochondrial genomes, a recombinant cox2 gene was identified as the candidate gene for CMS (22, 23). Also, a CMS phenotype was created through a protoplast fusion experiment between the Solanaceae, *Nicotiana tabacum* and *Hyoscyamus niger* (54). This feature has likely originated from the homologous recombination events that took place between the parental mtDNAs.

Another strategy involves the transfer of CMS from wild plants into cultivars. As mentioned above, valuable features encoded in the organellar genomes can be transferred directly to crops through cybridization assays. Breeding programs have taken advantage of the fact that CMS has arisen spontaneously in wild species, such as in *B. napus* 'Polima' (11), in cultivars of radish (*Raphanus*

*sativus* cv. Ogura and cv. Kosena (33), in *Citrus inshui* cv. Satsuma (47) and in *Nicotiana suaveolens* (15). The CMS feature has been incorporated directly or indirectly into breeding programs via somatic hybridization (46). For instance, the CMS phenotype observed in the wild plant *R. sativus* cv. Kosena was transferred to *B. napus* through asymmetric protoplast fusion (37). The mitochondrial genome of the somatic hybrid SW18 was sequenced and compared to the parental mtDNAs. Through comparative genomics, the mitochondrial-encoded orf125 derived from *R. sativus* cv. Kosena was identified as responsible for the CMS condition. Alternatively, the CMS could be transferred indirectly. First, the CMS condition is transferred to a crop of interest through sexual intergeneric hybridization followed by several backcrosses and it is incorporated into breeding programs using protoplast fusion experiments. For example, CMS was transferred from *R. sativus* cv. Ogura to *B. napus* by sexual intergeneric hybridization. The resulting cultivar showed CMS but also chlorosis due to nuclear-chloroplast incompatibility. To overcome this, the chloroplast of *R. sativus* was replaced by that of *B. napus* through an intraspecific somatic hybridization between a CMS and a fertile line of *B. napus*. Finally, breeding programs of the genus *Citrus* took advantage of valuable features of two cultivars through symmetric somatic hybridization experiments. The cultivar *C. inshui* cv. Satsuma that exhibits CMS and lacks seeds was combined with the cultivar *Citrus grandis* HBP of high commercial quality but with abundant seeds (32). Even though it is not clear whether CMS is a cytoplasmic-based feature in *C. inshui* cv. Satsuma, its mtDNA in the nuclear background of *C. grandis* resulted in CMS. Transcriptomic analyses showed that miRNA regulatory networks may be involved in the citrus floral development and retrograde regulation in nuclear-cytoplasmic interactions in *Citrus* CMS (34).

## VII. CONCLUSION

Studies discussed above suggest that CMS in lines of different origin may have common molecular mechanisms. A similar resemblance may also be found among Rf genes. On the other hand, multiple ways of fertility restoration through evolution of different Rf genes are also evident. Further detailed and integrated studies are expected to clarify the evolutionary relationships of CMS and Rf genes. Such studies would also contribute to establishing efficient F1 hybrid breeding systems in most of the vegetable crops. Plant breeding programs are in constant need of new sources of CMS lines to avoid a narrow, susceptible genetic background. Often, wild plants contain CMS cytoplasm but are male fertile due to the presence of restorer-of-fertility genes in their nuclear genomes. Therefore, CMS cytoplasm is usually discovered by genetic crossing or somatic hybridization that separates the CMS cytoplasm from the nuclear Rf alleles. Interestingly, the fully sequenced mitochondrial genomes of most angiosperms contain ORFs with typical features described for CMS genes. Those ORFs may be able to induce the CMS phenotype but are likely suppressed by nuclear regulators. Thus, it is probable that diverse angiosperm mitochondria may reveal the presence of CMS genes when moved to a novel nuclear background through somatic hybridization assays. This study will give further way will be a powerful tool to uncover CMS/Rf systems to incorporate in plant breeding programs.

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