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REVIEW

Cytoplasmic male sterility-based hybrids: mechanistic insights

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Abstract

Main conclusion **A comprehensive understanding of the nucleocytoplasmic interactions that occur between genes related to the restoration of fertility and cytoplasmic male sterility (CMS) provides insight into the development of hybrids of important crop species. Modern biotechnological techniques allow this to be achieved in an efficient and quick manner.**

Abstract Heterosis is paramount for increasing the yield and quality of a crop. The development of hybrids for achieving heterosis has been well-studied and proven to be robust and efficient. Cytoplasmic male sterility (CMS) has been explored extensively in the production of hybrids. The underlying mechanisms of CMS include the role of cytotoxic proteins, PCD of tapetal cells, and improper RNA editing of restoration factors. On the other hand, the restoration of fertility is caused by the presence of restorer-of-fertility (Rf) genes or restorer genes, which inhibit the effects of sterility-causing genes. The interaction between mitochondria and the nuclear genome is crucial for several regulatory pathways, as observed in the CMS–Rf system and occurs at the genomic, transcriptional, post-transcriptional, translational, and post-translational levels. These CMS–Rf mechanisms have been validated in several crop systems. This review aims to summarize the nucleo-mitochondrial interaction mechanism of the CMS–Rf system. It also sheds light on biotechnological interventions, such as genetic engineering and genome editing, to achieve CMS-based hybrids.

Keywords Cytoplasmic male sterility · Restorer-of-fertility genes · CMS · Rf · Nucleo-mitochondrial interaction · Hybrid development

Introduction

The inability of plants to produce viable pollen, functional male gametes, and dehiscent anthers is termed male sterility (Frank 1989; Mariani et al. 1990). The two major kinds of male sterility are caused by nuclear genes and interactions between nuclear and mitochondrial genes. CMS (cytoplasmic male sterility) is caused by the latter interaction, while GMS (genic male sterility) is caused by nuclear genes alone. Environmentally sensitive genic male sterility (EGMS) systems have also been studied at the genetic and molecular levels to understand cytoplasmic and nuclear interactions in plants (Chen and Liu 2014). The non-viability of pollen may be due to homeotic changes in the developing plant, such as the conversion of stamens into petals or carpels and the degeneration of stamens, anthers or tapetal cells (Chase and Gabay-Laughnan 2007).

Mitochondrial function is dependent on the coordinated interaction between the nuclear and mitochondrial genomes

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and an assortment of transcription factors and cofactors. CMS has been found in more than 150 plant species, and the interaction between the nucleus and mitochondria has been elucidated (Leaver et al. 1988). The transcription of numerous mRNAs for mitochondrial proteins occurs in the nucleus; these mRNAs are then translated by cytoplasmic ribosomes and distributed to mitochondrial compartments. In addition, post-transcriptional and translational mechanisms contribute to mitochondrial activities (Cannino et al. 2007). Interestingly, the mitochondrial genome also controls certain nuclear arrangements, activities, and expression patterns. This process has been termed retrograde communication (Liu and Butow 2006), which exemplifies the association between two organelles. Despite the corporeal distinction between mitochondrial and nuclear genomes, they play a prominent role in each other's regulatory mechanisms. Interactions between mitochondrial and nuclear genes are regulated in both anterograde and retrograde manners (Fujii and Toriyama 2008). A perfect example of this kind of regulation is the interaction of the CMS genes which cause mitochondrial defects (Bentolila et al. 2002) and their corresponding Rf (restorer of fertility) genes. The inheritance of matrilineal cytoplasmic genes usually occurs through ovules, while autosomal genes are passed through ovules and pollen (Budar et al. 2003). While the mitochondrial genes responsible for CMS are unrelated in sequence, they possess certain unique features that make them distinguishable and may regulate the expression of certain nuclear genes (Chase and Gabay-Laughnan 2007). The Rf genes, on the other hand, are nuclear and affect several mitochondrial and plastid functions related to anterograde regulation (Leon et al. 1998).

In most cases, the CMS-inducing gene is an open reading frame (ORF) that is chimeric in nature and translates into unique proteins which interfere with mitochondrial function and pollen development (Wise and Pring 2002). One theory suggests that these unique proteins are hydrophobic in nature and that they alter mitochondrial regulation, leading to pollen abortion (Andrés et al. 2007). The expression of these ORFs occurs due to the presence of the mitochondrial promoter, as they are transcribed along with other mitochondrial proteins (Chase and Gabay-Laughnan 2007). In some species of the Brassicaceae family, the proteins translated from these ORFs are cytotoxic in nature (Jing et al. 2012). Most plants that carry a CMS-determining ORF simultaneously possess normal respiratory genes (Yang and Zhang 2007; Yang et al. 2008). The ORFs possess a functional copy of the mitochondrial respiratory gene, as the CMS-related genes are gain-of-function mutations. These gain-of-function mutations, which disrupt mitochondrial respiratory gene regulation and function, result in female and male sterility and sometimes have damaging effects, such as stunted plant growth and striping. Cytoplasmic male sterility, corresponding to mitochondrial ORFs, is likely a gain-of-function mutation, except for certain loss-of-function mutations, which are not lethal in nature (Newton et al. 2004; Chase 2007). Phenotypically, CMS causes abnormal reproductive growth of the plant as well as homeotic changes (Carlsson et al. 2008). Several genes are associated with these homeotic mutations and belong to the MADS-box class of transcription factors expressed in cells that eventually develop into flowers. Respiratory mutations associated with these abnormalities are numerous and are concomitant with respiratory chain components (Chase 2007; Touzet and Meyer 2014). CMS caused by *orfs* has been shown to impart partial (semi)sterility and is attributed to the heterozygous nature of the plant, which has a dominant and recessive allele of the gene (Shinjyo 1969; He et al. 1996; Kim et al. 2007; Yamamoto et al. 2008; Bhattacharya et al. 2023).

The expression of CMS can be suppressed by proteins encoding fertility-related genes. Restorer fertility genes are usually dominant in nature, with a few instances of recessiveness, loss of function, and overdominance also observed (Horn et al. 2014). These genes are nuclear genes that target mitochondria-associated CMS genes by impeding their accumulation in organelles (Hanson and Bentolila 2004), leading to the restoration of fertility in plants (Bucher 1961). These Rf genes convene partial or complete restoration of pollen production to plants carrying the respective CMS-inducing cytoplasm. Fertility restoration systems can be sporophytic in nature (diploid), whereby all pollen produced by the plant is functional irrespective of the genotype. These systems may also be gametophytic (haploid), and only pollen possessing the restorer allele is viable (Horn 2006). The Rf genes have been mostly found to encode a protein containing a pentatricopeptide repeat (PPR) motif and a mitochondrial transit peptide (Kazama and Toriyama 2003). The PPR proteins consist of a tandem array of 35 amino acid motifs (Small and Peeters 2000; Lurin et al. 2004; Geddy and Brown 2007). The Rf proteins mostly belong to the P-class (35 canonical P motifs) of PPRs and are responsible for the cleavage of sterility-associated RNAs, while Rfs belonging to the PLS-class (P and PPR-like (L,S motifs)) of PPRs are also known (Dahan and Mireau 2013; Melonek et al. 2016). Such PPRs have been found in cereals and legumes, participating in the cleavage of cytotoxic mitochondrial sterility-related transcripts and facilitating the restoration of fertility in sterile plants (Zhang et al. 2020). PPR proteins are usually involved in post-transcriptional RNA processing in the mitochondria and chloroplasts, which are involved in plant development (Saha et al. 2007). PPRs are usually large proteins that bind to the corresponding single-stranded RNA in a sequence-specific manner. Owing to its large size, it is difficult to determine the exact site of binding. However, significant evidence has shown that PPRs bind to RNA in a parallel orientation via a combinatorial amino acid recognition mechanism with specificity associated with

amino acids at positions 6 and 1`. In silico analysis provides a better understanding of the function of PPR, but identifying the exact site of natural binding and off-target effects remains challenging (Barkan et al. 2012; Manna 2015). It has also been demonstrated that the binding of these PPRs to the RNA segment induces structural reorganization of the RNA, thereby leading to the possibility of RNA processing as binding sites become exposed (Prikryl et al. 2011).

With the increasing demand for food globally, hybrid production will provide economic benefits in crop production as well as in land conservation. Heterosis or hybrid vigour has been exploited in several crops, such as *O. sativa, H. vulgare, B. napus*, *C. cajan*, and *G. max,* among major cereals and legumes. Heterosis is the phenomenon wherein the progeny of two separate parental lines exhibit characteristics superior to those of either of the parents. Hybrid crops possess superior quality traits, such as increased yield and better adaptability to abiotic and biotic stresses. In approximately 1920, maize became the first commercialized hybrid seed crop. Hybrid seed production has since been extended to several other crops, such as rice, rapeseed, and sorghum (Girke et al. 2012; Li et al. 2015a; Kim and Zhang 2018; Liao et al. 2021). The development of hybrids includes cross-pollinating parental lines with superior and diverse traits. Therefore, in the case of self-pollinating crops, such as maize, rice, cotton, sorghum, and a variety of vegetables, male-sterile female plants are indispensable (Eckardt 2006). A three-line hybrid system consisting of a CMS line, a restorer line, and a maintainer line has been successfully adopted for hybrid seed production (Kim and Zhang 2018).

Several approaches, including chemical and manual methods such as emasculation, have been adopted to develop male-sterile female plants. However, with the advent of new techniques, such as genetic engineering and genome editing, there has been exponential development in the field of hybrid technology. Molecular approaches provide insight into the mechanism of sterility and the restoration of fertility, allowing large-scale hybrid production. This review focuses on exploring the utility of the CMS system in hybrid production and on defining the interactions between CMS and Rf components in plants.

Whole mitochondrial genome sequencing, multicentric origin, and diversification of CMS-associated genes

As mentioned earlier, CMS genes are chimeric in nature and originate from recombination events that occur between mitochondrial genes and the flanking sequences (He et al. 2020). While these recombination events contribute toward complexity of the genomic structures, they also help maintain genomic stability along with increasing genetic variation (Tuteja et al. 2013). Such homologous rearrangements lead to expression of novel *orfs* which also cause CMS and are known as CMS-associated genes (Kazama and Toriyama 2016). Next-generation sequencing methods, such as whole mitochondrial genome sequencing, have been employed in various plant species such as *Arabidopsis, B. vulgaris, O. sativa, T. aestivum, Z. mays, B. napus*, and *R. sativus* to uncover CMS-associated genes and also unravel their evolutionary diversification (Chen and Liu 2014).

Mitochondrial genome studies have conclusively demonstrated that most of these mitochondrial genes developed during recombination events belong to the mitochondrial electron transfer chain (mtETC), viz, *cox1*, *atp8*, and *atp6*. Sequence characterization of CMS genes has been done in species such as *O. sativa, H. annuus, B. napus, B. juncea, B. tournefortii, R. sativus, S. bicolor, T. aestivum, P. vulgaris, C. annuum, D. carota, C. cajan*, and *B. vulgaris* (Chen and Liu 2014, Toriyama et al. 2021). These studies have extensively demonstrated that *orfs* and their variants in CMS lines encode small proteins and sequences of unknown origin (SOU).

Comparative studies have identified genomic reorganization and candidate gene selection allowing for comprehension of mitochondrial gene flow. In a study by Fujii et al. 2010, two mitochondrial genomes, LD-CMS and CW-CMS derived from *O. sativa* L. ssp. *indica* and *O. rufipogon* Griff., respectively, were compared to previously sequenced Nipponbare derived from *O. sativa* L. ssp. *Japonica*. The study led to the identification of unique rearrangements and the determination of novel genetic structures (Fujii et al. 2010). In *C. cajan*, mitochondrial genome sequencing of 3 *C. cajan* line and wild relative, *C. cajanifolius* led to identification of rearrangements, no-coverage regions, and chimeric CMS ORFs. This study also identified partial subunits of several components of the respiratory chain complex including *atp1*, *nad4*, *rps4*, *nad5*, and *atp9*. These orfs are also found to contain transmembrane domains and have been observed in wild abortive CMS rice as well (Tuteja et al. 2013).

In rice, the most well-characterized CMS-associated gene is *orf79* of Boro Taichung (BT)-type CMS. Similar to the study by Fujii et al. (2010) which used standard cultivars like Nipponbare for determination of novel *orfs*, a study by Kazama and Toriyama (2016) sequenced Boro-type mitochondria and identified unique *orfs* as compared to Nipponbare, and found that *orf79* is associated only with the former. An abundance of B-*atp6* molecules were also reported implying towards its association with *orf79* (Kazama and Toriyama 2016). In rice, most of the genes conferring CMS are homologous to *orf79* and are co-transcribed with *atp6*. De novo assembly of over 590 mitochondrial genomes of wild (*O. rufipogon* Griff.) and Asian cultivated (*O. sativa* L.) rice revealed 16 haplotypes of *atp6-orf79*-like structures and 11 *orf79* alleles. They were also able to trace back the

origins of these *atp6-orf79*-like structures demonstrating a complex multicentric pattern of geographical distribution. A distinct gene exchange between *O. rufipogon* and *O. sativa* implied toward the high level of diversification (He et al. 2020). Similarly, comparative analysis of mitochondrial genomes of *hau*-CMS line, *B. juncea*, which is the maintainer counterpart, and normal type line, J163-4, revealed high rearrangement patterns in *hau*-CMS. *Orf288* was found to be the CMS-associated gene of *hau*-CMS in *B. juncea*. This chimeric *orf288* gene was also observed to contain a *nad5* sequence which is same as that of *orf263* reported in *B. tournefortii* (Heng et al. 2014). Studies like that of He et al. (2020) and Heng et al. (2014) help understand the evolutionary different between mitotypes of a species. Whole-genome sequencing of the CMS line (138A) and maintainer line (138B) in *C. annuum* L. indicated the presence of recombination and rearrangements in mitochondrial genome structure. Comparative analysis also helped identify novel *orfs* (*orf300a* and *orf314a*) in unique regions which are strong candidates for conferring CMS in 138A (Wang et al. 2019a).

Inherent mechanisms of CMS

The mechanism of sterility-conferring genes and their associated restorer-of-fertility genes have been explored extensively in various crops, and several theories have been proposed. One theory suggests that the interaction between anther-specific gene products and CMS genes in some way triggers mitochondrial dysfunction. It is also suggested that this restriction may be due to natural selection of mitochondrial genes (Chase 2007). Mutations in the mitochondria affecting the female reproductive system are readily removed because the mitochondria are maternally inherited. However, if the mutation compromises the male system, then it is passed on to further generations, which eventually contributes to male sterility (Chase and Gabay-Laughnan 2007). Interestingly, CMS may occur spontaneously in breeding lines due to mutagenesis as a result of broad crosses or interspecific exchange of nuclear and cytoplasmic genomes (Dalvi et al. 2010; Islam et al. 2014). In the case of Cytoplasm-T of maize, CMS cytoplasm has been obtained in normal breeding lines (Dewey et al. 1987). Plants with CMS and their associated restorer-of-fertility genes are often indistinguishable from normal plants. Therefore, CMS cytoplasm can be maintained for generations until the restorer of the fertility gene is removed due to mutation or segregation. Backcrosses and interspecific hybridization can result in the development of CMS-inducing cytoplasm. A highly successful cross was observed between two separate *Helianthus* species, which gave rise to CMS-inducing PET1 cytoplasm (Schnable and Wise 1998).

Previously, four major models of the CMS mechanism were identified: the energy deficiency model, cytotoxicity model, aberrant programmed cell death (PCD) model, and retrograde regulation model (Chen and Liu 2014) (Fig. 1). An overview of CMS induction in various crop species is given in Table 1.

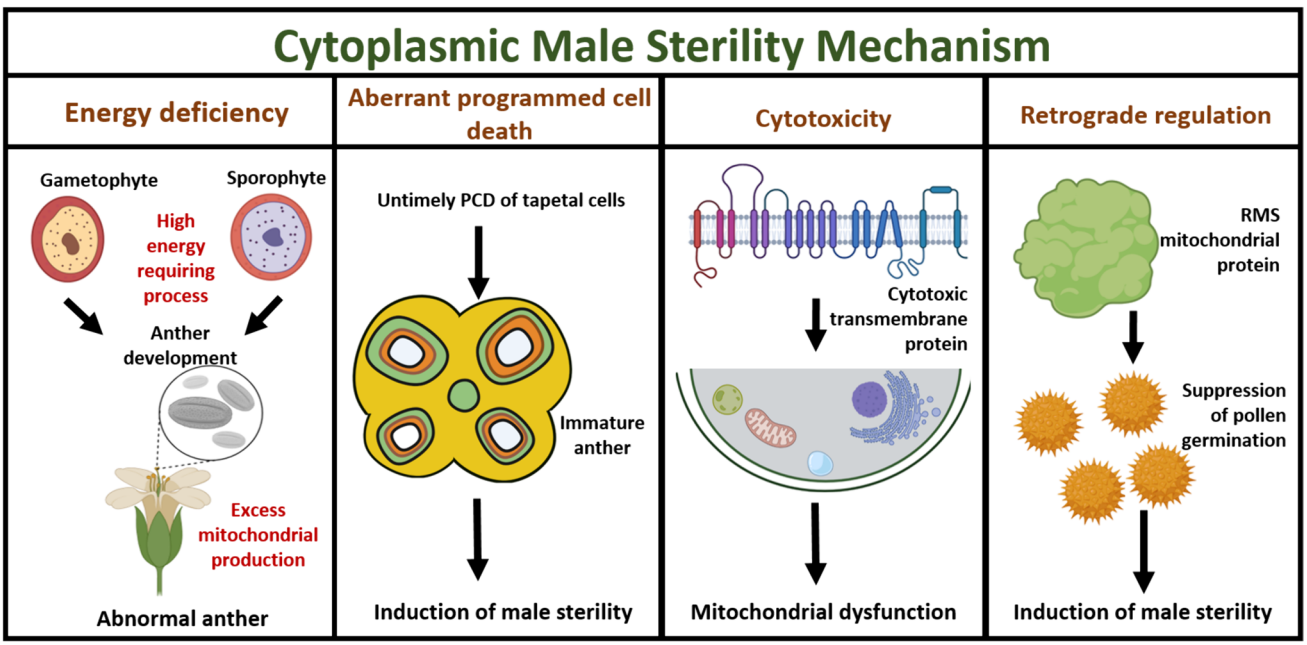

Fig. 1 Cytoplasmic male sterility (CMS) mechanism. Pictorial representation of the prominent models of the mechanism of CMS. (i) Energy-deficiency model, (ii) aberrant PCD, (iii) cytotoxicity model, and (iv) retrograde regulation

Table 1 Summary of crop-specific genes exploited to study four models of the CMS mechanism, viz., energy deficiency, cytotoxicity of the CMS protein, programmed cell death of tapetal cells, and retrograde regulation model

CMS mechanism	Gene	Crop species	CMS type	References
Energy deficiency	*orf147*	*Cajanus cajanifolius*	A4 cytoplasm	Bhatnagar-Mathur et al. 2018
	orf346	*Brassica napus*	Nsa CMS	Sang et al. 2021
	atp6 sequence	*Foeniculum vulgare*	–	Palumbo et al.2020
	DEGs	*Nicotiana tabacum* and *N. suaveolens*	*sua*-CMS	Liu et al. 2020
	orf165	*Capsicum annuum* L	CMS-14A	Wen et al. 2021
	orf138a and orf154a	*Brassica oleracea*	Ogura CMS	Zhong et al. 2021
Aberrant programmed cell death of tapetal cells	DAPS	*Capsicum annuum* L	–	Guo et al. 2017
	–	*Aegilops kotschyi* and *Ae. juvenalis*	*A. kotschyi* and *A. juvenalis* cytoplasm	Liu et al. 2018a
	orfH522	*Helianthus annuus*	PET1-cytoplasm	Nizampatnam et al. 2009
	–	*Aegilops uniaristata*	MU-CMS	Liu et al. 2018b
	–	*Glycine max*	N8855-derived CMS	Ding et al. 2019a
	ERD15, NAC81, NRP-A/B, and *VPE*	*Nicotiana tabacum* and *N. suaveolens*	*sua*-CMS	Liu et al. 2020
	orf224	*Brassica napus*	pol-CMS	Wang et al. 2021a
	atp6	*Zea mays*	C-type CMS	Yang et al. 2022
	DEGs	*Cajanus cajanifolius*	AKCMS11	Saxena et al. 2020
Cytotoxicity of CMS protein	*T-urf13*	*Zea mays* L	T-CMS	Dewey et al. 1986; Rhoads et al. 1995
	orfH79	*Oryza sativa*	HL-CMS	Yi et al. 2002
	orf138	*Brassica* and *Raphanus* sp.	Ogura CMS	Duroc et al. 2005
	orf79	*Oryza sativa*	Boro II cytoplasm	Wang et al. 2006, Kazama et al. 2008
	orf288	*Brassica juncea*	*hau*-CMS	Jing et al. 2012, Heng et al. 2018
	orf147	*Cajanus cajanifolius*	A4 cytoplasm	Bhatnagar-Mathur et al. 2018
	orf346	*Brassica napus*	Nsa CMS	Sang et al. 2021
	orfH522	*Helianthus annuus*	PET1-cytoplasm	Nizampatnam et al. 2009
Retrograde regulation model	*RMS*	*Oryza sativa*	CW-CMS	Fujii and Toriyama 2009; Suketomo et al. 2020

CMS due to energy deficiency in anthers

The energy deficiency model, proposed by Warmke and Lee in 1978, states that CMS genes modify mitochondrial functions and hamper pollen development. The development of gametophytic and sporophytic anther cells is a highly energy-demanding process. This energy can be met either by increasing the production of mitochondria or by increasing the activity of the mitochondrion. Rapid division of mitochondria was observed in maize CMS-T, which eventually led to mitochondrial dysfunction and insufficient ATP for anther development (Dewey et al. 1987). Structural analysis of various CMS proteins has demonstrated and significantly supported this theory, as most CMS proteins are transmembrane in nature and affect ATP synthesis (Warmke and Lee 1978; Horn et al. 1996). In addition, CMS proteins are chimeric in nature and have been found to be associated with portions of other mitochondrial proteins, such as NAD7, COXIV, and ATP, in *C. cajanifolius* that are involved in respiratory pathways (Saumitou-Laprade et al. 1994; Chase and Gabay-Laughnan 2007). Similarly, the novel chimeric *orf346* gene in oilseed rape co-transcribes with the *nad3* and *rp212* genes, resulting in the accumulation of reactive oxygen species and reduced ATP content, eventually leading to mitochondrial dysfunction (Sang et al. 2021). This finding is consistent with the energy-deficiency model for pollen abortion. Another gene, *atp6*$^{-}$, has been established to be a key component of the F_o portion of ATP synthase and is also involved in CMS. An *atp6*$^{-}$-like sequence was found when mitochondrial DNA (mtDNA) of fertile and sterile lines of fennel were examined to identify candidate CMS genes. The *atp6*$^{-}$ mutant was detected in the mtDNA of the CMS line, while the *atp6*$^{+}$ mutant was detected in the male-fertile line. This finding suggested that *atp6*$^{-}$, a translated mRNA that reduces ATP synthesis, could drive essential developmental and cellular processes but not high-energy-demanding

processes such as anther/pollen development (Palumbo et al. 2020). Structural and physiological analysis demonstrated that a lack of F_1F_0-ATPase subunit transcripts leads to ATP deficiency in flower buds, which corroborates the energy deficiency model (Liu and Yang 2020). In another study, comparative transcriptome analysis of the tobacco *sua*-CMS msZY revealed that pollen abortion occurs before sporogenous cell differentiation, and several differentially expressed genes (DEGs) corresponding to these characteristics were identified (Liu et al. 2020). Through comparative transcriptomic analysis, Wen et al. (2021) demonstrated the role of *orf165* in conferring CMS in pepper, which was attributed to the abnormal functioning of energy metabolism genes. The ATP content in the flowers of CMS-14A (a sterile male line) was significantly lower than that in the flowers of the maintainer line, and H^+-ATPase activity was enhanced, leading to an imbalance in energy supply and eventually microspore abortion (Wen et al. 2021). Similarly, in another report, the transmembrane proteins ORF138a and ORF154a were found to impact the transcript levels of genes involved in energy metabolic activity, especially those involved in the electron transport complex, causing CMS in Ogura-CMS cabbage (Zhong et al. 2021). This evidence allows for the hypothesis that CMS is related to energy deficiencies in developing anthers.

CMS due to programmed cell death (PCD) of tapetal cells

The aberrant PCD model emphasizes the interaction between gametophytic and sporophytic cells for controlled PCD of tapetal cells. Male sterility is attained due to incorrect or untimely PCD of tapetal cells.

Proteomic analysis of the CMS line and maintainer line in pepper demonstrated the role of protein species involved in pollen exine formation (Guo et al. 2017). The tapetal cells in the CMS line of pepper exhibited vacuolation, cell death, and damaged microspores. The key differentially abundant protein species (DAPS) identified were malate dehydrogenase (MDH), pyruvate dehydrogenase (PDH), aldehyde dehydrogenase (ALDH), acetyl-CoA synthetase (ACS), and pyruvate decarboxylase (PDC), which are involved in the tricarboxylic acid (TCA) cycle and PCD in tapetal cells (Qui et al. 2016). Another study with two isonuclear alloplasmic male-sterile lines (IAMLS) of wheat, K87B1-706A and Ju87B1-706A, from the cytoplasm of *A. kotschyi* and *Ae. juvenalis* revealed that aberrant PCD progression in the tapetum hindered microspore development (Liu et al. 2018a). PCD was attributed to varying levels of antioxidant enzyme transcripts, *SOD, CAT*, *APX* (superoxide dismutase, catalase and ascorbate peroxidase) and ROS-scavenging enzymes. Similarly, U87B1-706A, with the *Aegilops uniaristata* cytoplasm, has been shown to induce CMS due to PCD of tapetal cells, owing to ROS activity, leading to abnormal pollen exine and shrunken microspore (Liu et al. 2018b). Metabolomic studies pertaining to ROS stress and the antioxidant enzyme activity of the N8855-derived cytoplasm of soybean revealed the downregulation of flavonoids, phenolamides, oxidized glutathione, CAT, and POD. The stimulation of ROS bursts causes tapetal PCD and consequently pollen abortion in the soybean CMS line (Ding et al. 2019a). Active PCD progression has been observed in the *sua*-CMS line msZY of tobacco. The upregulation of the *ERD15, NAC81, NRP-A/B*, and *VPE* genes involved in the PCD pathway was detected in the flower buds of msZY. Heat shock proteins (HSPs), which negatively regulate PCD, were correspondingly downregulated. Overall, alterations in the expression of these genes cause PCD and anther abortion in tobacco CMS (Liu et al. 2020). In pigeonpea, a total of 3167 DEGs were identified, 1432 of which were upregulated in the CMS line and 1390 of which were downregulated in the restorer line. Among these genes, 34 homologous pigeonpea genes were identified, among which the *EMS1, MS1, and ARF17* genes are involved in pollen development. Some of these DEGs were also involved in tapetal development, the TCA cycle, and ROS bursts, suggesting their potential role in pollen development and subsequent CMS in pigeonpea (Saxena et al. 2020). In addition, the function of the *orfH522* mitochondrial gene of the PET1 CMS system in sunflower was validated in tobacco. Although the transgenic plants were morphologically similar to the fertile plants, microscopic studies confirmed the ablation of tapetal cells and PCD at the meiosis stage in the anthers (Nizampatnam et al. 2009). The *pol*-CMS of oilseed rape is the most widely studied CMS system. The sterility-inducing gene *orf224* causes anther abnormalities and disintegration, eventually leading to early PCD of sporogenous cells and subsequently to abnormal differentiation of the tapetum and microspore mother cells (Wang et al. 2021a). In the maize C-type CMS variety Yu87-1A, CMS is conferred by the mitochondrial gene *atp6c*. The protein causes reduced activity and quantity of the F_1F_o-ATP synthase complex, triggering an ROS burst and subsequent PCD of tapetal cells (Yang et al. 2022).

Transcriptome analysis of Ogura cabbage lines revealed the role of *orf135a* and *orf154a* in inducing CMS. Although this was partially attributed to energy deficiency, the alteration of genes associated with energy metabolism reportedly caused increased ATP synthesis, leading to abnormal proliferation of tapetal cells that hindered the development of haploid microspores as well as PCD of tapetal cells (Zhong et al. 2021).

CMS due to cytotoxic proteins

The cytotoxicity model suggests that the proteins associated with CMSs directly kill cells or are cytotoxic in nature.

Sequencing studies have suggested that CMS DNA encodes 10–35 kDa proteins (Kim and Zhang 2018). Subcellular localization studies of CMS products have demonstrated that they are located in the inner mitochondrial membrane, which consists of transmembrane domains with hydrophobic regions (Levings III and Brown 1989; Levings 3rd 1993). This causes mitochondrial dysfunction and subsequent male abortion. Furthermore, some of these proteins form polymers in the membrane, causing electrolyte leakage, thereby proving to be cytotoxic to *E. coli* and yeast and impairing their growth (Hu et al. 2014). This phenomenon has been observed with the URF13 protein of maize, which forms a tetramer and is a pore-forming receptor (Rhoads et al. 1995). The ORF138 protein, which is responsible for CMS in the Ogura CMS of *Brassica* and *Raphanus* species, forms oligomers in the bacterial membrane owing to the hydrophilic and hydrophobic moieties of the protein, eventually inhibiting bacterial growth (Duroc et al. 2005). The cytotoxic nature of CMS proteins has been extensively observed in other plants and CMS lines, such as *orf79* in the Boro II cytoplasm of rice (Wang et al. 2006; Kazama et al. 2008), *orfH79* in rice (Yi et al. 2002), *orfH522* in the PET1 cytoplasm of sunflower (Nizampatnam et al. 2009), *orf288* in the *hau*-CMS of *B. juncea* (Jing et al. 2012; Heng et al. 2018), *orf147* in the A_4 cytoplasm of *C. cajanifolius* (Bhatnagar-Mathur et al. 2018), and *orf346* in the Nsa CMS of oilseed rape (Sang et al. 2021). Transgenic chickpea strains generated by expressing *orf147* of *C. cajanifolius* exhibited partial sterility, implying the cytotoxic nature of the peptide (Bhattacharya et al. 2023). Although the cytotoxic nature of these peptides has been duly demonstrated in bacterial systems, the underlying mechanism of their cytotoxicity in their respective plant species and their impact on sterility are still unexplored. Apart from being cytotoxic in nature, some of these peptides are also known to cause energy deficiency (Sang et al. 2021) and ablation of tapetal cells induced by PCD (Nizampatnam et al. 2009), which directly induces CMS. CMS proteins in the tapetum cause abnormal mitochondrial function, such as ATP synthesis, as well as aberrant/premature PCD, resulting in CMS. The translation of CMS proteins in the membrane causes reactive oxygen species (ROS) bursts and, therefore, untimely PCD (Hu et al. 2014). The reason for the accumulation of ROS and eventual PCD remains unknown.

CMS due to the retrograde-regulated male sterility protein

Furthermore, the retrograde model of CMS suggests the role of the RETROGRADE-REGULATED MALE STERILITY (RMS) mitochondrial protein, which suppresses pollen germination, leading to sterility (Zubko 2004; Chen and Liu 2014). These proteins are regulated by retrograde signaling from the mitochondria. RMS proteins are essentially non-PPR proteins encoding Rf genes. The coevolution of CMS–Rf across various cytoplasm types in different crops is a well-studied phenomenon. In the CW-type CMS of rice, a nuclear RETROGRADE-REGULATED MALE STERILITY (RMS) for the *Rf17* gene was identified. The reduced expression of *Rf17* achieved through RNA interference (RNAi) led to the restoration of fertility, while the overexpression of the gene caused pollen lethality. The RMS gene can be regulated through retrograde signaling. Positional cloning of *Rf17* limited it to two possible candidates, *PPR2* and *ORf11 (RMS).* Although RMS does not encode a PPR, it encodes a protein containing a segment similar to ACPS (acyl-carrier protein synthase). Expression studies showed that reduced levels of *PPR2* mRNA had no effect on seed setting or pollen fertility. On the other hand, RMS overexpression caused pollen lethality. The RMS protein is not considered to be responsible for post-transcriptional RNA modification of CMS-associated genes. However, loss of RMS function facilitates fertility induction (Fujii and Toriyama 2009). This finding was functionally validated through CRISPR/Cas9, wherein mutations in *RMS* and *PPR2* were introduced. Fertility restoration was observed in RMS mutants with unaltered PPR2 expression. Fertility restoration through CRISPR/Cas9 mutation of *RMS* could aid in the development of restorer lines (Suketomo et al. 2020).

Improper RNA editing causes CMS

Another perspective put forward for the induction of CMS is defects in post-transcriptional RNA editing of mitochondrial transcripts. These are essential for regulation, and RNA editing usually occurs in the coding regions of mitochondrial transcripts. In the case of CMS-associated genes, RNA editing is regulated by the restorer-of-fertility genes, which helps in the restoration of fertility. RNA editing through the cleavage and degradation of CMS genes causes the restoration of fertility (Chen et al. 2017). Incomplete RNA editing or a lack of RNA editing causes the development of CMS in crop species (Hu et al. 2013). This phenomenon has been observed in several cereals such as maize (Gallagher et al. 2002) and legumes such as soybean (Jiang et al. 2011) as well as other plant species (Stone et al. 2017), wherein inefficient editing has caused the formation of truncated proteins or unexpected changes in the transmembrane structure of the ATP9 protein. Although RNA editing is known to cause changes in CMS lines, it is not always the cause of CMS. RNA editing in plants is a result of nucleocytoplasmic interactions and, therefore, can be utilized for studying mitochondrial gene functions and structure (Horn et al. 2014).

Crosstalk between sterility and restoration

The mechanism of fertility restoration involves suppressing the expression of CMS-related genes or counteracting their deleterious effects. Similar to that of CMS, the restoration of fertility is a complex phenomenon that occurs at various molecular levels. Restoration may occur at the genomic, post-transcriptional, translational, post-translational, or metabolic level (Fig. 2). An excellent example of restoration at the genomic level has been demonstrated in *Phaseolus vulgaris* (common bean), wherein the presence of the nuclear gene *Fr*, the mitochondrial transcript *PVS*, is lost. Site-specific excision occurs after the transcript is converted to its normal fertile form (Johns et al. 1992). At the transcriptional level, mitochondrial and chloroplast transcripts undergo several modifications, such as splicing, editing, and cleavage (Schmitz-Linneweber and Small 2008). This is similar to the previously mentioned modification of CMS genes through improper RNA editing. RNA editing normally involves the conversion of cytidine to uracil or cytidine to uridine in the chloroplast and mitochondria by PPR proteins (Fujii and Small 2011). For example, in the A3 cytoplasm of sorghum, editing of the cytidine and uracil sites in the *orf107* transcript by the restorer gene *Rf3 occurs*. The edited *orf107* is then degraded rapidly in fertility-restored plants (Tang et al. 1999). The role of RNA editing in pollen development has also been observed for the rice PPR protein PPR756, which causes amino acid changes in three major mitochondrial proteins involved in the electron transport chain, viz, *atp6, ccmC,* and *nad7*. Loss of PPR756 function was also shown to induce nonviable pollen and abortive seeds, which further validated its role in pollen development as well as the restoration of fertility (Zhang et al. 2020). Most PPR Rfs suppress the activity of CMS-causing genes through post-transcriptional activities such as cleavage, splicing, and degradation of CMS transcripts. However, they also function and require other cofactors for mRNA processing (Chen and Liu 2014). An example of translational regulation of CMS transcripts has been observed in the Ogura CMS system of rapeseed, wherein reversion to male fertility was due to the PPR-B fertility restorer (also known as *Rfo*). In this process, *Rfo* binds to the coding sequence of the CMS-causing mRNA *orf138*, inhibiting the translation of the transcript into the CMS protein. This is the first observed mechanism of restoration at the translational level (Wang et al. 2021b).

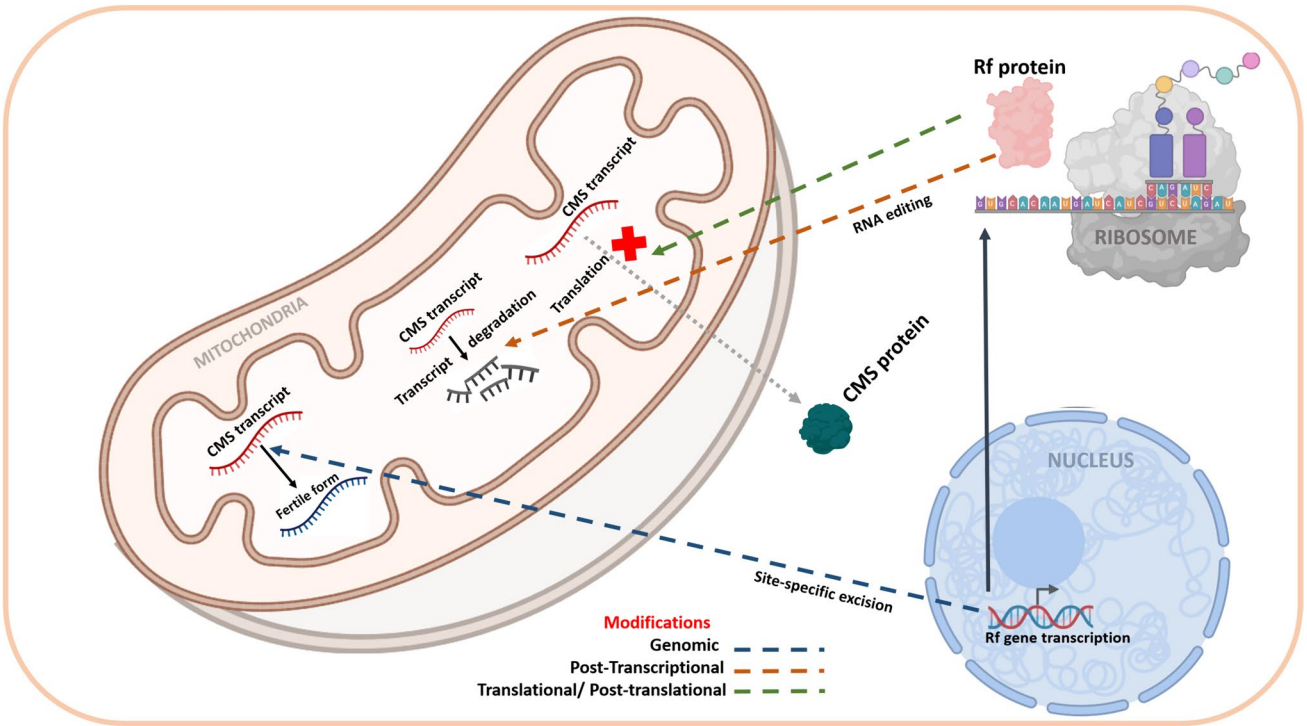

Fig. 2 CMS–Rf interaction mechanism. The interaction between CMS and Rf genes occurs at various molecular levels, including the genomic, post-transcriptional, translational, and post-translational levels. CMS is induced via the modification of CMS and Rf components at these levels. At the genomic level, Rf converts the CMS transcript to the fertile form through site-specific excision. At the post-transcriptional level, the Rf protein degrades the CMS transcript via RNA editing, hence inhibiting its translation into the cytotoxic CMS protein. Finally, at the translational and post-translational levels, the Rf protein inhibits the translation of the CMS transcript by binding to the transcript

A similar mechanism has been observed in the A4 cytoplasm of *Cajanus cajanifolius,* wherein the protein encoding *Rf3* binds to the CMS-causing *orf147* transcript, thereby leading to the restoration of fertility. This study also predicted the exact binding site of the RNA and protein complex using homology modeling (Bhattacharya et al. 2024, preprint).

In some cases, the size of the CMS transcript does not change, suggesting that the modifications are controlled at the translational or post-translational level (Kazama and Toriyama 2003; Komori et al. 2004; Deng et al. 2012). In Brassica, maize, common bean, etc., the CMS-associated *orf* is never altered. However, there is an alteration in the amount of the ORF protein that accumulates (Chen and Liu 2014). At the metabolic level, various enzymes and metabolic pathways are involved in the restoration of fertility (Liu et al. 2001; Bibi et al. 2014; Wesołowski et al. 2015). In CMS-C-type maize, *Rf4* is one of the major fertility restoration genes, and its function in fertility restoration is associated with tricarboxylic acid (TCA) cycle-related genes. Another development study and subsequent molecular analysis led to the identification of differentially expressed genes (DEGs). A total of 7,125 DEGs were found to be involved in various developmental pathways, such as pollen development, pollen tube development, pollen tube growth, gametophyte development and metabolic pathways, such as glycolysis, the pentose phosphate pathway and the tricarboxylic acid (TCA) pathway. (Liu et al. 2018c). Similarly, in the HL-CMS type of rice, *Rf6* was found to be associated with the function of hexokinase 6 (*OsHXK6*), which induces the processing of aberrant CMS-associated transcripts, thereby restoring fertility (Huang et al. 2015). Most of the research pertaining to CMS and its associated mechanism is correlated with anther tapetum, as the regulation of mitochondrial function impairs its organization (Kubo et al. 2011).

Although the individual mechanisms of CMS and its corresponding fertility restoration have been demonstrated through numerous models and mechanisms, nucleocytoplasmic interaction studies have revealed opportunities to elucidate the underlying mechanisms associated with these two organelles. Although the perception lies in the individual activities of the two components, which in turn affect each other, another perspective is the indirect activity of Rfs in the restoration of fertility. Multiple reports have indicated the direct binding of *Rfs* to CMS-associated transcripts at various molecular levels, as described above. In others, Rfs were observed to exist in RNA-free and soluble states, which suggests their diverse functions as well as the possibility of other cofactors contributing to fertility restoration (Gillman et al. 2009). However, there is still much to be explored regarding indirect means of plant restoration. Among the many molecular mechanisms identified, restoration is also theorized to involve compensatory or repair mechanisms. The repair mechanism involves complete removal or inactivation of the sterility factor at the transcriptional or translational level. In certain cases, the translated protein can also be truncated, rendering its expression unresolved. In the case of the compensation mechanism, modification of innate cellular metabolism occurs. There is no direct effect on sterility factors or their structures (Touzet et al. 2014).

In WA-type CMS rice, fertility restoration is caused by two major restorers, *Rf3* and *Rf4*. This restoration is known to be governed by a sporophytic mechanism; however, the genetic basis of this mechanism is not fully understood. An elite BT-type restorer of rice, C418, was shown to restore fertility in the WA-type CMS rice line. In C418, the gene responsible for fertility restoration was *Rf4*; however, this gene conferred only partial fertility restoration. This led to findings that fertility restoration was governed by a single dominant gene, as was confirmed by the low competitiveness of the pollen grains of *rf4* compared to those of *Rf4*. In the testcross F_1 generation plants, the *Rf4* gene was observed to demonstrate preferential fertilization, which had less effect on the WA-type CMS line due to the dosage effect leading to partial fertility. Considering the lack of restorer lines in *japonica* rice, the findings of this study have elucidated the underlying genetic mechanism of fertility restoration and subsequent generation of three-line hybrids of *Japonica* species (Zhang et al. 2022a).

While most of the Rfs identified have been found to belong to the PPR class of proteins, there have also been reports of Rfs encoding non-PPR proteins. These include aldehyde dehydrogenase, glycine-rich proteins, acyl-carrier proteins, and peptidases. These systems act at the metabolic level instead of on the CMS genes, as is the case for the PPR. Studies relating to their structure have shown that the repeats are composed of two antiparallel helices, one of which is highly positive. The positive side is known to assist in RNA binding. The CMS/Rf system is generally highly specific. Apart from RNA processing and expression, Rf proteins are involved in RNA stabilization, editing, 5'-3' RNA cleavage, intron splicing, and mRNA splicing (Gaborieau et al. 2016).

In the case of CW-CMS in rice, the nuclear restoration of the fertility gene *Rf17* is an RMS gene that encodes a mitochondrial protein of unknown function. *Rf17* was found to encode a protein that is homologous to the acyl-carrier protein, causing metabolic changes in the mitochondria and restoring fertility (Fujii and Toriyama 2009). The restoration gene *Rf2* of LD-CMS encodes a glycine-rich domain (GRP) and is known to directly interact with CMS-associated genes, leading to fertility restoration. This phenomenon has also been observed in other CMS varieties of rice, such as LD-CMS (*Rf2*) (Itabashi et al. 2011) and HL-CMS (*Rf5* and *Rf6*) (Hu et al. 2012). These GRPs hinder the translation of CMS-associated *orfs* and are known to function in the absence of PPR proteins (Gaborieau et al. 2016). The

restoration of fertility proteins has also been shown to result in the expression of a non-PPR, aldehyde dehydrogenase, which aids in reducing oxidative stress caused by CMS genes. These genes play a role in anther development in the T-CMS of maize (Liu et al. 2001) and *Beta vulgaris* (sugar beet). Anthers containing *bvORF20* encode a mitochondrial-targeting protein that binds to the *orf* of sugar beet, which is *preSatp6*. Contrary to existing studies of the CMS–Rf interaction, where Rf binds to CMS mRNA, the interaction between bvORF20 and preSatp6 is protein−protein binding (Kitazaki et al. 2015). The mitochondrial-targeting protein prevents the homo-oligomerization of the preSATP6 protein through a post-translational mechanism and, hence, restores fertility in sugar beet (Gaborieau et al. 2016).

Technological advances in hybrid production using the CMS–Rf system

A wide array of technological advancements aimed at hybrid crop production has occurred. A few of the milestone technologies are mentioned below (Fig. 3).

Protoplast fusion

Protoplast fusion for the development of 'cybrids' (Pelletier and Budar 2007) was exploited at the beginning of the 1990s for cytoplasmic male-sterile *Brassica* species. The *Brassica* species is able to respond to biotechnological approaches such as genetic engineering, somatic hybridization, in vitro culture, and species crosses with relative ease. Naturally occurring CMS systems have been found in only a few genera of the Brassicaceae family. The first case was observed in *B. rapa* (Okhawa and Shiga 1981), followed by *B. napus* (Fu 1981). In India, spontaneously occurring CMS was observed in *B. juncea* (Rawat and Anand 1979). However, later, it was discovered that this was a result of intergeneric hybridization between two genera of Brassica, *B. tournefortii* and *B. juncea* (Pradhan et al. 1991). In an early study carried out by Earle and Dickinson (1995), protoplast fusion of *Ogura-type* CMS of *B. oleracea* with fertile CMS of *B. napa.* or *B. rapa.* was done to obtain horticulturally improved vegetable lines. The resultant cybrids showed no chlorosis, as was previously observed in Ogura CMS, under low temperatures. The study emphasized the broader implications of their findings for the development of CMS systems in other crops, especially the Brassicaceae family (Earle and Dickinson 1995). Commercial production of cold-tolerant cauliflower, broccoli, and cabbage has been developed now by major seed companies such as Syngenta, Bejo Zaden BV, and Rijk Zwaan. Thereafter, novel CMS *B. oleracea* were produced by transferring 'Anand' cytoplasm originally derived from *B. tournefortii* via protoplast fusion of *B. rapa* and *B. oleracea.* The presence of 'Anand' mtDNA and its segregation pattern are associated with CMS (Cardi and Earle 1997). The recovery of the genetic backgrounds of parents through backcrossing is highly time-consuming, which is why protoplast fusion has gained much leverage as a technique. The transfer of CMS has been duly carried

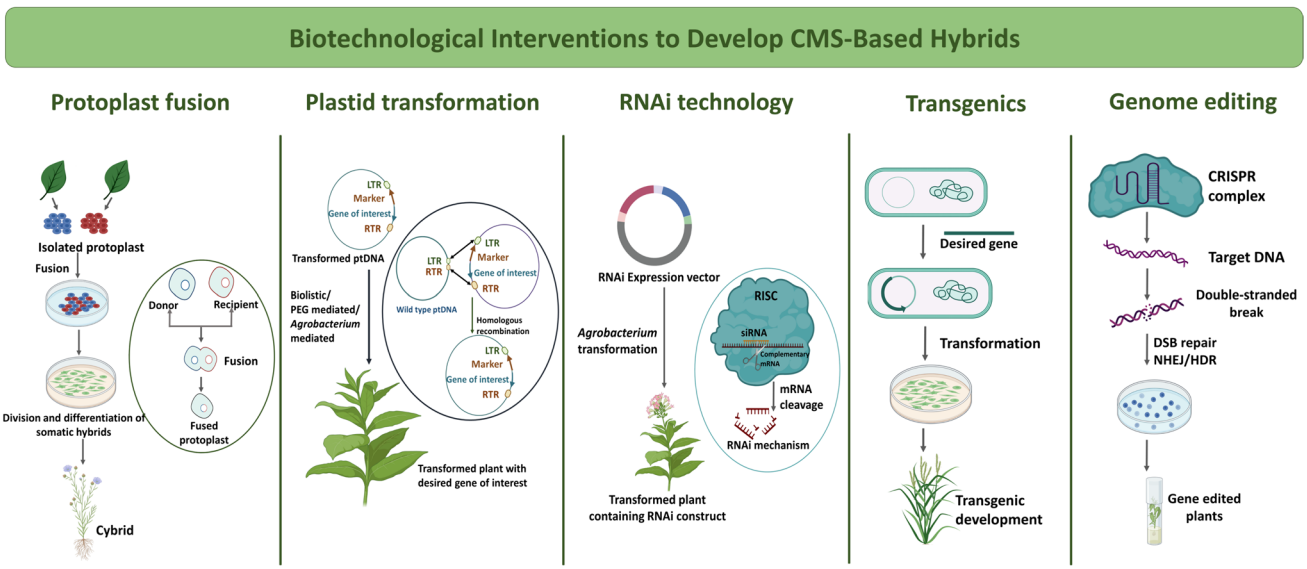

Fig. 3 Biotechnological interventions to develop CMS-based hybrids. The development of CMS-based hybrid systems has been carried out in different crop systems. Techniques such as protoplast fusion and plastid transformation have been proven efficient initially; however, their use is controversial. Therefore, modern biotechnological interventions have been developed to overcome these drawbacks. Technologies such as RNAi, transgenics, and, more recently, genome editing are being explored for the development of CMS-based hybrids

out in species such as carrot, *Brassica*, *Nicotiana*, and *Petunia* using symmetric fusion, asymmetric fusion, and cytoplast-protoplast fusion for the recovery of organelle-encoded traits (Guo et al. 2004). In this regard, seedlessness is an important market trait for citrus cultivars and has been achieved via the cybridization of the CMS type in the seedless Satsuma mandarin (*Citrus unshiu*). Several cybrids have since been developed in similar citrus cultivars, such as Juman Satsuma with Yuzu (*Citrus junos*) and Eureka (*C. limon*), which are seedless cultivars (Guo et al. 2013). Such traits have immense commercialization potential; therefore, organelle manipulation through protoplast fusion is a promising avenue. Similar studies have been carried out in other *Brassica* species. Protoplast fusion for novel CMS has been explored in *B. napus* (inap CMS), wherein CMS was developed by generating somatic hybrids of *Isatis indigotica* (Chinese woad) through recurrent backcrossing. CMS developed due to the conversion of tetradynamous stamens to carpelloid structures. The development of the female reproductive structure thereby led to a lack of pollen and, hence, male sterility. After pollination with *B. napus*, *I. indigotica* demonstrated normal morphology (Kang et al. 2017).

While protoplast fusion for the generation of cybrids in sexually incompatible crops has been utilized extensively, it is associated with insufficient progeny fertility and deformed gametes and seeds. In addition, genomic incompatibilities often have an impact on the integration of desired traits in symmetric hybrids. This can be overcome using asymmetric hybrids instead, wherein only a portion of the genome is transferred, minimizing the chances of undesired integration (Wang et al. 2013). Henceforth, asymmetric protoplast fusion for the generation of cybrids has been developed in different species to create CMS. The Ogura-type CMS line was obtained by asymmetric protoplast fusion between *B. oleracea* (fertile) and *R. sativus* (fertile). The Ogura-type CMS line is one of the most extensively studied lines in *R. sativus*. The mtDNA of the designated protoplast fusion-derived CMS (PDC) line was found to be similar to that of Ogura-type CMS mtDNA and varied from that of the parental lines. This phenomenon has been attributed to stoichiometric shifts within the mitochondrial genomes of different genotypes, leading to heteroplasmic mtDNA (Motegi et al. 2003). In another study, celeriac (*Apium graveolens* var. *rapaceum*) suspension cell protoplasts were used as acceptors, and mesophyll protoplasts of carrot, coriander, and white celery were used as donors for the fusion experiment. After selective staining and fusion of the respective protoplasts via electroporation and polyethylene glycol (PEG) treatment, the candidate hybrid shoots were obtained. The resultant cybrids obtained were found to be a result of fusion between carrot and celeriac. However, the frequency of occurrence of cybrids is low, and further examination is needed to improve the technique (Bruznican et al. 2021). Nevertheless, asymmetric protoplast fusion has opened avenues for obtaining novel CMS lines.

Plastid transformation

Since mitochondria and plastids are both maternally inherited, plastid transformation has also been exploited for the generation of CMS (Pelletier and Budar 2007). The first male-sterile plant identified through this method was *N. tabacum*, in which the specific role of the β-ketothiolase gene (*phaA*) was studied. By exploring the chloroplast genome, CMs can be generated by examining genes that hinder plastid metabolic pathways such as those involved in pollen development (Pelletier and Budar 2007). Polyhydroxybutyrate (PHB) synthesis occurs due to the sequential metabolic activity of β-ketothiolase (*phaA* gene), acetoacetyl-CoA reductase (*phaB* gene), and PHB synthase (*phaC* gene). The expression of the *Acinetobacter Pha* operon results in the synthesis of PHB, which in turn leads to stunted growth of the plant and male sterility. Nonetheless, plastid transformation using the *phaA* gene driven by the *psbA* promoter resulted in male sterility in tobacco without any other side effects (Oscar Ruiz and Daniell 2005). The role of β-ketothiolase in sterility has been further examined in relation to light. The β-ketothiolase gene (*phaA*) driven by the psbA promoter was inserted into the chloroplast genome, and its effects under continuous illumination and a particular photoperiod were investigated. *psbA* mRNA translation is known to be regulated by light via the 5' UTR in tobacco plastid (Staub and Maliga 1994). Under continuous illumination, reversibility of sterility was observed, and phenotypically viable pollen was produced. This is the first report of an engineered CMS system in plants (Oscar Ruiz and Daniell 2005).

Plastid transformation is a comparatively newer technology and, therefore, like any recent technology, has certain shortcomings. This technology lacks efficient protocols for all crops and selection markers and has reduced transgene expression in non-plastids and extended the length of time required for the recovery of plants (Ahmad et al. 2016). In addition, the expression of foreign proteins has been shown to induce male sterility, causing yellow leaves and stunted growth. While inducible systems have been developed to reduce damage caused by foreign proteins, they are costly and complex (Maliga 2004; Yu et al. 2020). Nevertheless, studies based on this topic have gradually increased in recent years (Kuchuk et al. 2006); therefore, there is a great scope for further exploitation in this field for the development of transgenic CMS crops.

Transgenics and RNAi technology

To overcome global food security issues, genetic engineering of various cereal and legume species is being explored. Owing to the stringent regulations associated with the development of genetically modified (GM) crops or transgenics, gene-containment strategies concerning maternal inheritance, male sterility, and seed sterility have been adopted for the development of GM crops (Daniell 2002). Transgenic male-sterile plants can be developed through RNA interference (RNAi) technology or transcriptional gene silencing. Several genes related to anther development have been identified in crop species and have been directly exploited via RNA interference to induce male sterility (Mansoor et al. 2006). Induction of male sterility can also be achieved by expression of the silencing construct in maize driven by an inducible promoter (Greenland et al. 1998).

The identification of nuclear *Rf17* against the *RETROGRADE-REGULATED MALE STERILITY (RMS)* protein in Chinese wild rice (CW)-type CMS in rice suggested a role for *RMS* in inducing pollen abortion. Suppression of the expression of *RMS* via retrograde signaling was observed to induce the restoration of fertility in haploid pollen (Fujii and Toriyama 2009). Initially, engineered male sterility has been developed in model crop species such as *A. thaliana* and *N. tabacum.* A system was developed using *Agrobacterium tumefaciens*-mediated gene transfer, after which several reports of GMS were reported. However, this approach was not applicable to CMS. In a study carried out in a model plant system, tobacco, an unedited and edited sequence of the wheat *atp9* mitochondrial gene, was fused with the *coxIV* subunit of yeast for the generation of transgenics. When targeted into the mitochondria, nuclear-encoded ATP synthase subunit 9 (*atp9*) was hypothesized to impair organelle activity and cause CMS. The transgenic tobacco, which contained unedited *atp9*, generated completely sterile or partially sterile plants. The control plants containing edited atp9 genes were considered fertile plants (Hernould et al. 1993). This study essentially utilized a transgenic mitochondrial gene to induce male sterility and provided a basis for further study of the RNA editing process in plants. The transgenic induction of male sterility was also successful in tobacco and tomato plants via the use of RNAi for the expression of *Msh1*. The nuclear gene is responsible for the suppression of mitochondrial recombination in *A. thaliana,* and its disruption causes CMS. Induced male sterility was found to be maternally inherited and associated with normal female fertility in both plants (Sandhu et al. 2007). In addition to the generation of transgenic male-sterile plants via RNAi technology, plastid transformation is another technique that has been adapted as described previously (Ruiz and Daniell 2005).

In the CMS-WA of rice, the CMS-causing gene *WA352* was identified, and the corresponding protein interacts with the nuclear-encoded mitochondrial protein COX11, which functions in peroxide metabolism. The accumulation of WA352 in the tapetum inhibits COX11 and subsequently causes PCD and pollen abortion. RNAi of COX11 with a tapetum-specific promoter was shown to cause CMS (Luo et al. 2013).

In the wild abortive (WA) cytoplasm of *Oryza sativa,* the gene transcript *orfB* has been found to be associated with CMS. Dose-dependent insertion of unedited *orfB* into the genome of male-fertile rice plants demonstrated the generation of male-sterile plants. Furthermore, the downregulation of unedited *orfB* through RNAi led to the restoration of fertility, suggesting that *orfB* functions as a CMS-inducing factor in wild rice species (Chakraborty et al. 2015).

In addition, RNAi technology-based CMS induction in tobacco using *atp9* gene from *Boehmeria nivea* (L.) Gaudich exhibited plants with partial sterility. The study conclusively implied strong association of the *atp9* gene with CMS (Liu and Yang 2020).

Genome editing

Mitochondrial gene target modification has tremendous potential for functional validation of CMS-causing genes. While several studies have been carried out to generate CMS lines in crops using various biotechnological techniques, genome editing has recently been used. Considering the specificity and cost-effectiveness of this technology, genome editing can be a useful tool in CMS-based hybrid system development. TALEN-mediated mitochondrial genome editing has been used to restore fertility in *O. sativa* and *B. napus* by knocking down two CMS-associated genes (*orf79* and *orf125*) (Kazama et al. 2019). More recently, mitoTALENs have been developed that integrate a mitochondrial signal peptide into TALEN, thereby eliminating the need for transformation of the organelle of interest and allowing its integration into the nucleus. MitoTALENs targeting mitochondrial *orf352* in CMS lines of rice led to the loss of function of the gene, generating plants with viable pollen (Omukai et al. 2021). Although complete restoration of fertility was not observed in the present study, as the seeds were not set, this study was significant for understanding the functions of genes and their phenotypic outcomes. This study demonstrated the usefulness of mitoTALENs for functional validation of mitochondrial-associated genes in hybrid studies involving the CMS–Rf system. In a related study in CMS-WA rice, mitoTALEN-mediated targeting of the CMS-associated *WA352* gene led to the development of mutants which demonstrated pollen viability as well as seed setting (Zhou et al. 2024). The stable regulation of sterility and fertility is crucial for a robust CMS breeding pipeline. Efforts have been made to develop a system to achieve an inducible hybrid system within plants which could help in

achieving fertility and sterility transitions in plants. The Brassicaceae family is one of the most widely studied families for CMS. The use of mitoTALENs in *Ogura* CMS has successfully demonstrated this transition in the *Ogura* CMS broccoli hybrid. A mitoTALEN was used to knockout CMS-causing *ORF138* from a CMS-derived hybrid of broccoli, YX, resulting in restoration of fertility and subsequently also developing a cold-sensitive male-sterile line. In addition, a stable inheritance of the mitoTALENs-mediated *ORF138* depletion was also observed. This line can, therefore, be directly used for commercial purposes in hybrid breeding programs (Xu et al. 2024). Apart from CMS-associated genes, mitoTALEN-mediated editing has been used for targeting conserved mitochondrial genes such as the *nad9* gene which encodes a major component of the respiratory chain. The knockout plants demonstrated male sterility and also showed an inducible system wherein the introduction of a functional Nad9 protein restores fertility (Forner et al. 2023).

The restoration of fertility in Chinese cabbage has been explored using CRISPR/Cas9. Interestingly, the seed-setting rate of these mutants was greater than that of transgenics obtained through RNAi-mediated knockout. This technique would allow for the development of artificial restorer lines for use in breeding (Suketomo et al. 2020). In a hybrid study utilizing CRISPR/Cas9 and a conventional backcross, a hybrid glutinous rice variety was developed by knocking out *OsWaxy* in 209B (maintainer line), which has a reduced amylose content compared to that of its parent. Subsequently, one generation of hybridization and two generations of backcrossing of 209A (a sterile female parent) with WX209B (a male parent) led to the generation of the glutinous CMS line WX209A. This study paved the way for achieving low-amylose, hybrid glutinous rice varieties in a short duration of time (Wang et al. 2019b). In a similar study, maintainer line edits were generated for the genes *TGW6* (grain weight) and *Wx* (amylose content) using CRISPR/Cas9. Hybridization of the glutinous maintainer mutant with the CMS line 209A generated glutinous CMS lines. Mutations in *TGW6* and *Wx* generated maintainer lines of rice with enhanced yield and reduced amylose content (Han et al. 2018).

In a hybrid study in rice, an artificial CMS gene generated through CRISPR/Cas9 technology resulted in a *CYP703A3*-deficient male-sterile mutant (9311-3A), which was subsequently transformed with the pollen fertility restoration gene *CYP703A3* and the pollen lethality gene *orfH79* to develop maintainer 9311-3B. In addition, sterile lines were crossed with restorer lines to investigate heterosis (Song et al. 2021). In another study in rice, the functionality of the fertility restoration gene *OsRf19* was confirmed by knockout using CRISPR/Cas9 in the restorer line 9311(FA)R. Complete sterile plants were obtained that followed the Mendelian ratio of segregation in subsequent generations (Jiang et al. 2022). These knockout mutants facilitated the confirmation of the findings and further use of the identified genes.

In tomato, using a mitoTALEN vector, the association between *orf137* and CMS was studied by targeting the *orf137* coding region. The obtained plants exhibited a wild-type phenotype with viable pollen and mature seeds, suggesting the restoration of fertility (Kuwabara et al. 2022). This study also provided sufficient evidence of the double-strand break repair mechanism mediated through homologous recombination, as observed in other crop species.

In maize, CMS-C is one of the most widely studied CMS systems (Beckett 1971), with *Rf4* being the dominant restoration of fertility genes (Kheyr-Pour et al. 1981). Unlike most restorers of fertility genes, which are mitochondrial targets, *Rf4* has been found to contain a nuclear localization signal and does not interact with mitochondria directly. This finding suggested that *Rf4* indirectly impacts mitochondria through upstream and downstream triggers, possibly through the induction of other mitochondrial proteins. In addition, *Rf4* is an essential gene involved in tapetal development (Chaubal et al. 2000). The variation in *Rf4* has been determined through CRISPR/Cas9 HDR editing across restoring and non-restoring inbred lines. Positional cloning demonstrated that *Rf4* is a bHLH transcription factor with a nuclear localization signal. The alteration in *Rf4* restoration is altered by a single amino acid substitution, F187Y, within the conserved bHLH domain. The generation of isogenic lines through CRISPR/Cas9 revealed that editing of the F187Y line caused sterility, suggesting that this isoform has the ability to restore fertility (Jaqueth et al. 2020).

Multiple genes responsible for a single trait can be targeted via the use of multigene editing vectors. Mutations in homologues of genes in putative male sterility families, via knockout using CRISPR/Cas9, resulted in complete male sterility in maize. This study combined phenotypic and genotypic analyses to determine the function of each gene homologue and shed light on the underlying mechanism of anther and pollen development in this crop (Liu et al. 2022).

Recent studies have explored the prospects of target sequence modifications in the mitochondrial genome (mtDNA) using CRISPR/Cas9. In a study by Chang et al. 2023 in tobacco, mitoCas9 was successfully transported into the mitochondria, leading to selective cleavage of the mtDNA of the CMS-associated mtatp9 gene and, subsequently, male sterility (Chang et al. 2023).

Genome editing using TALENs and CRISPR/Cas9 for developing CMS mutant lines has been successful in crops and has shown remarkable progress in the last decade. This technology can, therefore, facilitate the production of CMS lines and accelerate the establishment of hybrid platforms for major crop species. In addition, owing to the evolving notion of fewer risk factors for genome editing, a wider array

of applications of CMS hybrid platforms and crop improvement has been proposed.

Conclusion and future prospects

Considering the availability of advanced biotechnological tools and techniques, the identification of CMS-associated genes and their corresponding restorers of fertility genes has accelerated tremendously in the last few decades. The establishment of various mechanisms that emphasize nucleocytoplasmic interactions has enabled researchers to explore hybrid development in numerous crop species.

CMS allows circumvention of conventional methods of emasculation, which are extremely tedious and time-consuming, for the development of hybrid progenies that possess significant advantages over their respective parents (Duvick 1959). The three-line hybrid system comprising a sterile line (A), restorer line (R) and maintainer line (B) provides a rather sophisticated platform for ensuring hybrid vigour (Zhang et al. 2022b). While conventional techniques using the three-line system may take more than 10 years to obtain hybrids, using newer precision genetics tools such as CRISPR/Cas in many cases takes only a year to produce transgene-free CMS mutants. Numerous monocots, such as rice, maize, sorghum, pearl millet, and wheat and dicots, such as sunflower, *Brassica*, soybean, and pigeonpea, have been explored for stable cytoplasmic male-sterile lines. Similarly, the associated restorer-of-fertility genes and the interactive mechanism underlying these genes have been determined. Considering the diverse ways to restore fertility genes, the cloning of *Rf* genes is imperative for deciphering the exact nucleocytoplasmic mechanism involved. This has led to the identification of PPR proteins related to the restoration of fertility functions along with non-PPR proteins that behave the same way (Uyttewaal et al. 2008).

While various methods have been employed for the development of CMS–Rf lines, several molecular and omics-based technologies can aid in gaining further perspective on hybrid development. Whole-genome sequencing of mitochondria across CMS lines in crops has revealed novel *orfs* in crops such as rice, wheat, maize, Brassica, pigeonpea, capsicum, and sorghum (Bohra et al. 2016). Similarly, whole-genome transcriptome profiling allows the examination of transcripts in reproductive organs to identify genetic factors responsible for the induction of sterility as well as the restoration of fertility. Transcript profiling has helped in the identification of genetic factors that are responsible for pollen wall formation, reactive oxygen species generation, and scavenging activity (Li et al. 2015b). Transcriptome profiling also aids in the identification of novel gene candidates and pathways associated with crop species where the regulatory mechanism of CMS is unclear. For instance, transcriptome analysis of *Dianthus spiculifolius* led to the identification of several differentially expressed genes (DEGs) strongly associated with the male regulatory pathway. It also helped in the identification of a *DsHSP70* gene whose ectopic in *A. thaliana* caused abnormalities in floral meristem development (Liu et al. 2023). Such studies also pave the way for future functional studies using gene editing technology. Further, such studies also help decipher the molecular mechanism of CMS and can serve as a model for other flowering species, such as the integrated transcriptomics and proteomics studies in tobacco (Mo et al. 2023). In addition, RNA sequencing-transcriptome profiling has enabled the combination of omics data with cellular-level techniques and has been extended to crops such as Capsicum (Wei et al. 2020), *B. rapa* (Lin et al. 2019), and maize (Zhang et al. 2021). Single-cell RNA sequencing (scRNAseq) (scRNAseq) has revolutionized the examination of cellular development and underlying mechanisms and characterized cellular heterogeneity (Gupta and Kuznicki 2020). However, owing to the cell wall structure of plant cells, scRNAseq has been limited to only root and germinal cells (Zhang et al. 2021). Transcriptome profiling of microspore cells has been performed to investigate genes that are differentially expressed in CMS lines of crops (Bohra et al. 2016). MicroRNAs (miRNAs) also play a crucial role in the development of male and female reproductive organs; therefore, the study of RNA targets through next-generation sequencing (NGS) has been employed for the identification of miRNAs between hybrids (Shen et al. 2011; Zhang et al. 2018; Nie et al. 2018; Ding et al. 2019b; Bohra et al. 2021). Studies reporting potential CMS-associated miRNAs have been conducted on plants such as *Brassica* sp., *O. sativa*, *R. sativus*, and many others (Wei et al. 2015; Zhang et al. 2016). While miRNAs form a larger and much well-studied subset of the larger set of non-coding RNAs (ncRNAs), the long non-coding RNAs (lncRNAs) and phased small interfering RNAs (phasiRNAs) are also critical elements in examining the genetic mechanism of male sterility. The ncRNAs are known key players in regulating male sterility by participating in hormone homeostasis. High-throughput sequence analysis along with degradome analysis for the identification of ncRNAs at different developmental stages would help in understanding the ncRNA-mediated regulatory network of CMS genes during anther development (Nie et al. 2023). In addition, exploring opportunities to apply CRISPR/Cas9 technology can further reveal the function of ncRNA mutants. While the above-mentioned technologies aim to investigate the occurrence of CMS at the transcript level, transcript abundance may not demonstrate variations between the A and R lines. However, at the protein level, differences can be deciphered using proteomics. Furthermore, the variations occurring in transcriptomic and proteomic data can help minimize the time required for the identification of factors and provide quicker

conclusive analysis. The mitochondrial proteome can also help in studying the nucleocytoplasmic mechanism of CMS/Rf (Bohra et al. 2016). Metabolomics-based studies allow for further exploration of mechanistic insights of CMS from the biochemical aspect. Such studies can help understand the role of plant regulating hormones, amino acid metabolites, and other regulatory enzymes in the floral development of a plant (Yang et al. 2023; Wang et al. 2024). In addition, other molecular methods, such as biochemical and microscopic analysis, such as the yeast three-hybrid system, RNA immunoprecipitation, electrophoretic mobility shift assay/filter binding assay (EMSA/FBA), electron microscopy, UV crosslinking and footprinting, and more comprehensive analysis, such as RNA affinity purification and proteome microarray, can be applied to identify CMS transcripts with relevant Rf proteins (Jazurek et al. 2016). Cellular processes, such as DNA methylation, are also known to directly regulate pollen development, carbohydrate metabolism and hormone regulation which subsequently lead to CMS development (Rahman et al. 2024).

The robust experimental conclusions arising from biotechnological interventions have led to the establishment of molecular models that significantly explain the occurrence of CMS and the restoration of fertility in crop species. Understanding mitochondrial evolution patterns would also provide insight into CMS generation and the identification of uncharacterized CMS (Li et al. 2018). Modern approaches such as transgenic development and genome editing have shortened the time span for generating CMS lines while maintaining specificity. Considering the pressing need to introduce hybrids to meet growing food demands, the development of the CMS/Rf line is a crucial step toward this goal. Although there are still gaps and hurdles, major strides have been made over the years in various crops, which will help elucidate the molecular factors responsible for CMS.

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Declarations

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