Advances in Research on Cytoplasmic Male Sterility Phenotypes and Mechanisms: Postprint

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Abstract

Special open reading frames (ORFs) within the plant mitochondrial genome cause plants to either fail to produce male gametes or produce male gametes that cannot fertilize normally, a phenomenon known as cytoplasmic male sterility (CMS). The characteristic of stable male gamete abortion in CMS materials has long played an important role in the commercial production of hybrid varieties, effectively reducing seed production costs and improving hybrid purity. With deepening research into the CMS phenomenon, new CMS materials have been continuously developed through various approaches, and the associated sterility genes have been gradually mapped and cloned. This article first outlines current research on CMS gene evolution and the identification of commonly used CMS materials and their associated CMS genes, then summarizes the phenotypic characteristics of CMS materials in terms of material and energy metabolism, hormone levels, and other aspects. Simultaneously, it integrates several current hypotheses regarding CMS molecular mechanisms and proposes perspectives on CMS molecular mechanisms based on experimental evidence, aiming to provide assistance for future more in-depth theoretical and experimental research on the basis of summarizing current cytoplasmic male sterility studies.

Full Text

Advances in Cytoplasmic Male Sterility Phenotype and Mechanism Research

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Abstract

Specialized open reading frames (ORFs) within plant mitochondrial genomes can prevent male gamete production or cause abnormal fertilization, a phenomenon known as cytoplasmic male sterility (CMS). The stable male sterility of CMS materials makes them invaluable for commercial hybrid seed production, effectively reducing breeding costs and improving hybrid purity. As research on CMS has progressed, new CMS materials continue to be developed through various approaches, with associated sterility genes being mapped and cloned. This review first summarizes current research on CMS gene evolution and the identification of common CMS materials and their corresponding genes. We then synthesize the phenotypic characteristics of CMS materials regarding metabolic activity and hormone levels. Additionally, we integrate several current hypotheses on CMS molecular mechanisms and propose perspectives on these mechanisms based on experimental evidence. Our aim is to provide a foundation for future theoretical and experimental investigations into cytoplasmic male sterility.

Keywords: cytoplasmic male sterility, mitochondrial genome, reactive oxygen species, open reading frames, CMS molecular mechanism

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Cytoplasmic male sterility (CMS) is widely observed across the plant kingdom, having been documented in over 150 species (Xu et al., 2022). In 1763, German biologist Joseph Gottlieb Kölreuter first recorded anther abortion in intra- and interspecific plant hybrids (Mayr, 1986). In 1931, American botanist Marcus Morton Rhoades discovered that sterility traits followed maternal inheritance in maize (*Zea mays*) (Rhoades, 1931). CMS arises from interactions between specialized ORFs generated through mitochondrial genome recombination and the nuclear genome, resulting in failure to produce male gametes or inability to complete normal fertilization, while female gametes remain functional.

Mitochondria serve not only as cellular energy producers but also play crucial roles in intracellular signal transduction and redox homeostasis regulation through coordination with other cellular structures (Ghifari et al., 2023). According to the endosymbiotic hypothesis, mitochondria originated from α-proteobacteria approximately one billion years ago through a prolonged endosymbiotic process (Gray et al., 1999; Braun & Klusch, 2024). During endosymbiosis, genetic material was continuously transferred among the three genomes (mitochondrial, nuclear, and chloroplast) via intracellular gene transfer (IGT), primarily from organelles to the nucleus (Kleine et al., 2009; Wang et al., 2022). Compared to *Rickettsiale*, the closest extant relatives of the mitochondrial ancestor, modern mitochondrial genomes have shrunk by approximately 98.5% (Roger et al., 2017). This size reduction has diminished mitochondrial protein-coding capacity—of the 1,000–2,000 proteins in plant mitochondria, only

a handful are encoded by the mitochondrial genome itself (Robles et al., 2012; Murcha et al., 2014). For example, the mitochondrial genome of *Paphiopedilum micranthum* contains just 39 protein-coding genes (Yang et al., 2023). Extensive gene transfer and loss have reduced mitochondrial genetic autonomy, placing mitochondrial activity under deep nuclear control. Mitochondria-nucleus regulation operates through two main pathways: first, cells respond to external stimuli by regulating expression of nuclear and mitochondrial genes to synthesize and recruit proteins that modulate mitochondrial activity; second, mitochondria transmit signals to the nucleus in response to stimuli, prompting nuclear responses through mitochondrial stress responses that suppress mitochondrial aberrations and maintain cellular homeostasis—a process known as retrograde regulation (Ryan & Hoogenraad, 2007; Khan et al., 2024).

Nuclear-cytoplasmic interactions profoundly affect plant life activities. CMS genes in the mitochondrial genome control pollen abortion, while nuclear fertility restorer genes (*Rf*) can eliminate or mitigate CMS gene effects to ensure normal pollen development (Chen & Liu, 2014). This controllable male fertility makes CMS materials crucial for crop hybrid seed production. Hybrid offspring often outperform both parents in yield and stress resistance, a phenomenon called heterosis or hybrid vigor (Pei et al., 2022). Utilizing heterosis can increase rice (*Oryza sativa*) yields by 10–20% compared to inbred lines (Luo et al., 2013), while maize shows even stronger heterosis with mid-parent heterosis rates of 35–40% for yield traits (Wei et al., 2022). In hybrid production, CMS maternal lines eliminate the need for manual emasculation, saving labor costs and improving efficiency and seed purity. Unlike chemical emasculation, CMS avoids crop and environmental damage. Researchers have developed the "three-line" system (male-sterile line, restorer line, maintainer line) using CMS and *Rf* genes, enabling both hybrid production and maintenance of the three lines through designed crossing combinations—playing a vital role in large-scale hybrid seed production.

Currently, over half of global rice and maize production uses hybrid varieties, with hybrid vegetables accounting for 80–90% of cultivars in China (Liu et al., 2019). Therefore, investigating CMS mechanisms is essential for harnessing heterosis across more species. This review synthesizes research on plant CMS genes, phenotypes, and mechanisms, discusses the validity of current molecular models in light of cellular metabolism studies, and outlines future research directions to advance CMS molecular mechanism studies and applications in heterosis exploitation and nuclear-cytoplasmic interaction research.

1.1 Mitochondrial Genome and CMS Gene Evolution

Most plant mitochondrial genomes follow maternal inheritance, limiting inter-individual gene exchange (Camus et al., 2022). Mitochondria must therefore maintain highly conserved sequences while eliminating harmful mutations to ensure normal function in progeny. Research shows mitochondria can clear mutated sequences through internal homologous recombination systems (Chevigny

et al., 2020). However, these recombination systems also make mitochondrial genomes prone to rearrangements, creating the paradoxical characteristics of high sequence conservation yet rapid structural change—termed the "mitochondrial evolution paradox" (Chevigny et al., 2020; Christensen, 2021; Kan et al., 2022).

During frequent recombination, different sequence fragments randomly combine to form chimeric sequences, some of which create new ORFs. Certain ORFs can interact with the nuclear genome to cause male gamete abortion, becoming CMS genes. Consequently, CMS gene sequences show clear recombination signatures. Analysis of 28 CMS genes from 13 crops revealed that all are chimeric genes containing fragments of mitochondrial functional genes (Chen & Liu, 2014). Since mitochondrial genetic autonomy is low, sequence recombination is deeply regulated by the nuclear genome. Researchers have identified multiple nuclear genes that actively prevent harmful recombination in organelle genomes, including *MutS homologue 1* (*MSH1*), *RecA-like recombinases* (*RECA*), and two plant-specific single-stranded DNA (ssDNA) binding protein families: *Organellar ssDNA-binding proteins* (*OSBs*) and *Whirlies* (*WHYs*) (Maréchal & Brisson, 2010). *MSH1* is crucial for suppressing ectopic recombination in mitochondrial genomes; its mutation increases recombination activity, sequence mutations, and substoichiometric shifting (SSS) (Wu et al., 2020; Zou et al., 2022; Xu et al., 2022). RNAi silencing of *MSH1* in *Brassica juncea* (Zhao et al., 2016), sorghum (*Sorghum bicolor*), foxtail millet (*Setaria italica*), soybean (*Glycine max*) (Arrieta-Montiel & Mackenzie, 2011), tobacco (*Nicotiana tabacum*), and tomato (*Solanum lycopersicum*) (Sandhu et al., 2007) detected SSS and CMS phenomena in mitochondrial genomes. While silencing *OSB1* and *RECA* also caused rearrangements or SSS, no CMS phenotypes were reported.

CMS gene formation requires multiple rounds of recombination and mutation. Tang et al. (2017) analyzed 808 wild and cultivated rice mitochondrial genomes and proposed a possible evolutionary route for the CMS-WA gene *WA352* [Figure 1: see original paper]. The evolution likely originated from an ancestral *RPL5/COX1/ORF284* structure in *Oryza* ancestors. Through frequent recombination, this structure was repeatedly disrupted and fused with other sequences to form chimeric sequences. Researchers identified multiple *WA352*-like chimeric sequences in ancestral and related species that share fragments with *WA352* but do not cause pollen abortion (Tang et al., 2017). Chimeric sequences also accumulate nucleotide variations during recombination, gradually forming new functional genes through repeated recombination and mutation. Based on this research, scholars proposed a CMS gene evolution model involving multiple recombination events, protogene formation, and functionalization (Tang et al., 2017).

1.2 Overview of CMS Material Development

CMS occurs widely in both wild and cultivated plants. Although CMS genes can form spontaneously, nuclear *Rf* genes typically prevent anther abortion (Chen

& Liu, 2014). CMS material development involves either large-scale screening to identify individuals lacking nuclear *Rf* genes or strategic hybridization to introduce CMS genes into related species followed by backcrossing to eliminate *Rf* genes. For example, between 1964–1965, Academician Yuan Longping screened 140,000 rice panicles and discovered six male-sterile plants in several varieties (Zhu, 2016). Many widely used rice CMS materials, including CMS-WA (wild abortive), CMS-DA (dwarf abortive), and CMS-MX (Ma Xie), were discovered through field screening of wild and cultivated populations. Alternatively, Christov Michail's team obtained 15 CMS materials through interspecific hybridization in sunflower (*Helianthus annuus*) (Michail, 2012).

In summary, large-scale screening and planned interspecific hybridization are the primary means of obtaining CMS materials. Two key factors are essential: maximizing the probability of CMS gene emergence in the screening population, and separating nuclear *Rf* genes after introducing CMS cytoplasm. Continuous backcrossing can stabilize *Rf* gene separation by replacing the nuclear genome, while various methods can increase CMS gene emergence probability. These approaches fall into two categories: (1) expanding survey populations without altering mitochondrial mutation/recombination rates, as used for discovering CMS-WA, CMS-DA, and CMS-MX; or (2) increasing recombination/mutation rates while maintaining relatively stable population sizes.

Advancing technology and deeper CMS mechanism understanding have enabled breeders to develop methods that accelerate mitochondrial genome evolution, improving CMS material acquisition efficiency:

Somatic cell fusion: Used when distant relatives cannot hybridize through pollination, somatic fusion forcibly introduces highly heterogeneous mitochondrial genomes from distant species while potentially causing chromosome elimination and high-frequency mitochondrial genome rearrangements (Liu et al., 2005), simultaneously enabling CMS gene generation and *Rf* gene loss.

Physical/chemical mutagenesis: Mutagenesis using agents like base analogs or radiation induces DNA double-strand breaks and mismatches across all genetic systems. The repair process triggers mitochondrial rearrangements and sequence variations, allowing CMS material selection through subsequent hybridization and screening (Wang et al., 2005).

Nuclear regulation interference: As mitochondrial genome and nuclear-cytoplasmic interaction research advances, breeders can manipulate nuclear gene expression to influence mitochondrial rearrangements and create new CMS materials. For example, Sally's team silenced *MSH1* in *Brassica juncea* to reduce mitochondrial genome stability, successfully developing CMS materials (Zhao et al., 2016).

From costly, time-consuming field screening to various biotechnological applications, CMS material development methods continue to improve as CMS research advances, reducing acquisition difficulty.

1.3 Current Status of CMS Gene Identification and Cloning

CMS has been identified in over 150 plant species, with some crops (e.g., rice, rapeseed) harboring multiple CMS types. CMS gene sequences and chimeric structures determine the sterility mechanism and physiological characteristics, serving as the fundamental basis for classifying different CMS materials . For uncharacterized CMS materials, classification relies on fertility restoration and maintenance relationships (Laughnan & Gabay-Laughnan, 1983). Developers name CMS materials based on their characteristics—for instance, rice CMS-TAA (also CMS-TA) was derived from crossing Philippine indica variety Tadukan with japonica Taichung 65, named after the parental abbreviation TAA/TA (Takatsuka et al., 2022). Carrot (*Daucus carota*) CMS-Carpeloid and CMS-Petaloid types are named according to floral organ abortion features (Linke et al., 2003).

Identifying CMS genes facilitates material classification and deepens understanding of CMS phenomena. Early studies used RNA blotting to identify abnormal transcripts in sterile lines (Luo et al., 2013). With advances in high-throughput sequencing and mitochondrial genome assembly, current approaches primarily use multi-omics comparative analysis. Candidate CMS genes are screened by comparing transcriptomic and proteomic differences between sterile lines and their corresponding maintainer or restorer lines, combined with mitochondrial genome structural variations.

Candidate genes require functional validation through knockout or transformation experiments. Since reliable mitochondrial gene transfer methods were previously unavailable, researchers fused mitochondrial targeting signals (MTS) with candidate gene fragments for nuclear expression and mitochondrial import, observing fertility changes to validate CMS gene function. This strategy successfully analyzed genes including rice CMS-BT *ORF79* (Wang et al., 2006), *Brassica juncea* CMS-Hau *ORF288* (Jing et al., 2012), rice CMS-WA *WA352* (Luo et al., 2013), sugar beet (*Beta vulgaris*) CMS-I-12 *ORF129* (Yamamoto et al., 2008), pepper (*Capsicum annuum*) *ORF456* (Kim et al., 2007), *Brassica ORF220* (Yang et al., 2010), and maize CMS-C *ATP6c* (Yang et al., 2022).

Recently, compact TALENs (cTALENs) technology has enabled mitochondrial genome editing. Combining cTALENs with MTS allows direct knockout of mitochondrial sequences (Beurdeley et al., 2013; Kazama et al., 2019), providing more convincing evidence than nuclear expression with mitochondrial import. Shin-ichi Arimura's team used this technology to validate tomato CMS-P *ORF137* (Kuwabara et al., 2022), rice CMS-RT102 *ORF352* (Omukai et al., 2021), rice CMS-BT *ORF79*, and *Brassica napus* CMS-Kosena *ORF125* (Kazama et al., 2019).

2 Phenotypic and Physiological Characteristics of CMS Materials

During the reproductive growth phase, plants undergo significant changes in morphology, cytology, and physiological-biochemical parameters. All observable traits discussed herein are collectively defined as phenotypes. Reproductive growth is characterized by enhanced metabolic activity, including increased biosynthesis and elevated energy metabolism rates to support floral organ development and seed formation. Numerous studies demonstrate significant phenotypic differences between sterile and fertile lines in stamen morphology, metabolic activity, and hormone levels.

2.1 Abnormal Stamen Morphology and Cytological Behavior

Stamen degeneration and pollen abortion are the most typical CMS traits. For example, cabbage CMS-Ogu sterile lines show slender filaments and shriveled anthers compared to fertile lines (Chen et al., 2023). Male sterility is classified into four types based on stamen abortion: (1) complete stamen degeneration lacking typical anther/filament structures; (2) malformed anthers without contents; (3) normal anther appearance but internally abnormal pollen; and (4) normal pollen morphology but lacking viability (Fan et al., 2016; Mo et al., 2023). Some CMS materials show no obvious stamen phenotypic changes—rice CMS-Tetep sterile lines have normal anthers containing viable pollen throughout most of the growth period, but abnormal anther dehiscence causes CMS (Lee et al., 2022). Beyond stamen phenotype, tapetal cells critical for pollen development show abnormal cytological features in many CMS materials. In *Brassica napus* and wild cabbage (*Brassica oleracea* L.) CMS materials, tapetal cells often exhibit excessive swelling and vacuolation before disintegration, compressing microspores and causing abnormal development and pollen abortion (Xing et al., 2022; Chen et al., 2023).

2.2 Reduced Material and Energy Metabolism

Multiple approaches comparing metabolic intensity between sterile and fertile lines consistently show significantly lower metabolism in sterile lines. Chen et al. (2019) used 3D imaging of tapetal cells during pollen development, revealing increased mitochondrial volume and density, indicating pollen development is highly energy-demanding. ATP content studies show significantly lower anther ATP levels in sterile lines of maize (Yang et al., 2022), rapeseed (Xing et al., 2022), soybean (Bai et al., 2022), and tobacco (Mo et al., 2023). Mo et al. (2023) also found lower pyruvate content in tobacco sterile line anthers. Additionally, activities of respiratory enzymes (e.g., ATP synthase, cytochrome c oxidase) are reduced in sterile lines (Hou et al., 2003). Wang et al. (2023) observed downregulated expression of ATP synthase genes in soybean sterile lines. These findings indicate multi-level suppression of energy metabolism in CMS anthers.

Beyond energy supply, biosynthesis is crucial for pollen development. Studies

of maize anther metabolism revealed that throughout microspore development, starch, soluble sugars, soluble proteins, proline content, and amylase activity were significantly lower in sterile lines (Xia & Liu, 1993). Similar studies in rapeseed and rice showed reduced carbohydrate and protein content and enzyme activity in sterile line anthers (Zhuang & Huang, 1987; Xing et al., 2022). Weakened material metabolism may exacerbate pollen energy deficiency and impede normal development of pollen mother cells and tapetal cells.

2.3 Elevated Reactive Oxygen Species Concentration

During oxidative phosphorylation (OXPHOS), electrons from the mitochondrial electron transfer chain (mETC) can prematurely combine with O_2 to generate superoxide anions ($O_2^{-}\cdot$) or singlet oxygen ($^1O_2$) (Mailloux et al., 2013). Normally, 0.2–2.0% of respiration-derived O_2 is released as reactive oxygen species (ROS) (Balaban et al., 2005). These ROS act as developmental signaling molecules and are rapidly degraded by antioxidant systems including superoxide dismutase (SOD) and peroxidase (POD) after signal transduction (Shadel & Horvath, 2015; Waszczak et al., 2018). However, when respiration slows and ATP synthesis is blocked, ROS production rates increase sharply (Huang et al., 2016). Studies show elevated ROS content in sterile lines with cellular damage indicators.

In rapeseed and maize anthers, sterile lines exhibit significantly higher $O_2^{-}\cdot$ content than restorer or maintainer lines, along with elevated malondialdehyde (MDA) levels indicating membrane damage (Xing et al., 2022; Yang et al., 2022). However, in rapeseed sterile lines, H_2O_2 content is significantly lower than in fertile lines despite higher MDA levels, with both SOD and POD activities reduced compared to maintainer lines (Xing et al., 2022). This suggests abnormal ROS metabolism, where ROS may persist primarily as superoxide anions with impaired conversion to H_2O_2, inadequate antioxidant enzyme activity, and consequent membrane damage. Bai et al. (2022) found differential expression of ROS metabolism-related genes in soybean sterile lines. Overall, CMS anther tissues experience greater ROS stress than fertile tissues, potentially triggering oxidative stress that exacerbates cellular energy deficits.

2.4 Abnormal Hormone Levels

Floral organ development is regulated by multiple plant hormones, and altering hormone content can cause developmental abnormalities. Exogenous cytokinin (CTK) application can modify floral organ sex (Champault, 1973), while Li et al. (2023) altered cotton pollen fertility by manipulating jasmonic acid (JA) content. Mutations in JA synthesis and signaling genes cause sterile phenotypes including short filaments, non-dehiscent anthers, inactive pollen, and poor pollen tube growth—phenotypes reversible by exogenous JA application (Browse, 2009). These findings demonstrate close connections between hormone regulation and stamen development, suggesting hormone level differences may contribute to CMS stamen abortion.

Complex hormone signaling networks interact differently across species and developmental stages, yielding inconsistent conclusions about hormone level differences among the "three lines." For example, abscisic acid (ABA) levels are higher in all developmental stages of *Brassica napus* CMS-Pol, CMS-Nsa, and CMS-Ogu sterile lines compared to fertile lines (Ding et al., 2018), whereas in "Xingao" pear (*Pyrus pyrifolia*), ABA is higher only during early and late uninucleate pollen stages (Li et al., 2006). Moreover, different molecular mechanisms underlie anther abortion in different CMS lines, causing inconsistent hormone differences even within the same species. In *B. napus*, only CMS-Nsa showed significant IAA differences from maintainer lines during early floral bud development, though all three CMS lines had lower IAA than maintainers during later stages, with varying significance levels (Ding et al., 2018).

While hormone level differences are clearly linked to CMS, research on "three-line" hormone variations remains relatively preliminary, particularly regarding hormone regulatory network differences.

3 Hypotheses on CMS Gene Mechanisms

Although numerous CMS genes have been cloned and mapped, in-depth molecular mechanism analysis is limited to a few major crops (rice, maize, etc.). Based on CMS research, four molecular mechanism models have gained acceptance: the cytotoxicity model, energy deficiency model, aberrant programmed cell death model, and retrograde regulation model (Chen & Liu, 2014; Heng et al., 2018).

3.1 Cytotoxicity Model

The cytotoxicity model posits that CMS proteins are toxic, specifically damaging cells involved in stamen development to cause pollen abortion [Figure 2: see original paper] (Levings, 1993; Chen & Liu, 2014). In maize CMS-T, expression of the *URF13* gene in prokaryotes (*E. coli*) and eukaryotes (yeast, *Spodoptera frugiperda* larvae, *Trichoplusia ni* larvae) caused varying degrees of growth inhibition (Dewey et al., 1988; Huang et al., 1990; Korth & Levings, 1993). Similar growth inhibition was observed in *E. coli* expressing sunflower *ORF522* (Nakai et al., 1995), rice CMS-BT *ORF79* (Wang et al., 2006), and *Brassica juncea* CMS-Hau *ORF288* (Jing et al., 2012). However, no studies have successfully detected or isolated "stamen-specific toxins." Another supporting observation is the presence of transmembrane domains in many CMS proteins, a feature common in toxic proteins. Yet studies of rice CMS-WA *WA352* and *Brassica juncea* CMS-Hau proteins found that truncated CMS proteins lacking transmembrane domains still caused pollen abortion (Luo et al., 2013; Heng et al., 2018). Furthermore, fragmenting the *Brassica juncea* CMS-Hau gene revealed that only transmembrane-containing fragments showed cytotoxicity in *E. coli*, but these fragments failed to cause pollen abortion when transformed into *Arabidopsis thaliana*, while non-transmembrane fragments induced sterility (Heng et al., 2018). In rice CMS-Tetep, *ORF312* showed cytotoxicity in *E. coli*

yet the sterile line produced mature pollen (Lee et al., 2022). These findings indicate no necessary correlation between cytotoxicity and pollen viability.

While the cytotoxicity model has some merit, its explanation of CMS mechanisms is relatively one-sided. Direct experimental evidence is lacking; detecting or isolating "stamen-specific toxins" would substantially strengthen the model, which requires further refinement.

3.2 Aberrant Programmed Cell Death Model

Programmed cell death (PCD), or apoptosis, refers to orderly cell dissolution mediated by specific signaling molecules (including ROS, nitric oxide, ethylene) under stress, damage, or during specific life cycle stages (Breusegem & Dat, 2006). The aberrant PCD model proposes that abnormal timing of apoptotic signals (primarily ROS) in stamens causes premature or delayed death of critical pollen development cells, leading to pollen abortion [Figure 2: see original paper].

Tapetal cells are essential for pollen development, regulating developmental progression, protecting microspores, and forming pollen walls. Aberrant tapetal PCD timing causes pollen abortion. Studies show both abnormal tapetal PCD and ROS accumulation in sterile lines. In rice CMS-WA, WA352 protein accumulates in tapetal cells, inhibiting COX2 function, disrupting peroxide metabolism, triggering ROS accumulation, and causing aberrant tapetal PCD and pollen abortion (Luo et al., 2013). Maize CMS-C also shows abnormal ROS accumulation and tapetal PCD (Yang et al., 2022).

The widespread observation of aberrant tapetal PCD across multiple CMS lines strongly supports this model. Although it cannot explain all CMS phenomena, the model effectively links pollen abortion to respiratory dysfunction, providing a clear research direction.

3.3 Energy Deficiency/Metabolic Disorder Model

During pollen development, anther respiration rates and mitochondrial numbers far exceed those in leaf tissues, indicating high ATP consumption (Lee & Warmke, 1979; Chen et al., 2019). The energy deficiency model proposes that CMS proteins impair mitochondrial function, causing energy metabolic disorder and insufficient ATP supply that blocks pollen development [Figure 2: see original paper].

Multiple lines of evidence support this model. Respiratory mutants frequently show pollen abortion phenotypes (Hernould et al., 1993; Heiser et al., 1997). Liu's team transformed tobacco with an RNAi silencing construct targeting ramie (*Boehmeria nivea*) *ATP9*, resulting in ~50% pollen sterility in each transgenic plant (Liu & Yang, 2020). Geisler et al. (2012) used T-DNA insertion to disrupt the nuclear gene encoding the ATP synthase δ subunit, causing partial pollen abortion. Similar phenomena were observed in respiratory mutants of

Arabidopsis (León et al., 2007; Li et al., 2010; Busi et al., 2011), potato (*Solanum tuberosum*) (Heiser et al., 1997), wheat (Hernould et al., 1993), and tobacco (Liu & Yang, 2020), demonstrating that disrupting the respiratory chain and ATP synthesis can induce pollen abortion. Most CMS genes contain fragments of mETC-related genes, suggesting CMS proteins may interact with mETC complexes to affect respiration and ATP synthesis (Chen & Liu, 2014). In rice CMS-HL, the CMS protein ORFH79 interacts with the p61 subunit of mETC Complex III, reducing ATP and NADH content (Wang et al., 2013) and decreasing Complex V quantity and activity (Zhang et al., 2007; Liu et al., 2012). Sunflower CMS protein ORF522 interacts with ATP synthase to reduce its activity and anther ATP synthesis (Sabar et al., 2003). Transmembrane-containing CMS proteins may affect ATP synthesis by altering membrane electrochemical potential—maize CMS-T URF13 (Rhoads et al., 1995) and rapeseed CMS-Ogu ORF138 (Duroc et al., 2005) can form pore structures in the inner mitochondrial membrane, potentially causing proton leakage that reduces the proton motive force and ATP synthesis (Rhoads et al., 1995; Duroc et al., 2009).

Despite substantial supporting evidence, the energy deficiency model has limitations. First, respiratory mutants still produce some fertile pollen, contrasting with complete pollen abortion in CMS (Meyer et al., 2009; Geisler et al., 2012; Touzet & Meyer, 2014). Second, evidence is largely indirect and correlative; causality between energy supply reduction and CMS remains unproven. Demonstrating that exogenous ATP supplementation restores sterile line fertility would strengthen the model considerably.

3.4 Mitochondrial Retrograde Regulation Model

The retrograde regulation model proposes that mitochondria influence pollen development by altering nuclear gene expression, causing CMS [Figure 3: see original paper]. Researchers have identified differentially expressed nuclear genes between sterile and fertile lines.

Theissen and Saedler (2001) proposed the ABCDE model of floral development, where MADS-box transcription factors play crucial roles in different floral structures and stages, with B- and C-class genes specifically involved in stamen development. Linke et al. (2003) found that in carrot CMS-Petaloid and CMS-Carpeloid lines, mitochondrial retrograde signals regulate MADS-box transcription factor expression, affecting B- and C-class genes to cause stamen petaloid or carpeloid conversion [Figure 3: see original paper]. Zhang et al. (2021) identified differentially expressed miRNA169 in soybean CMS-RN sterile lines; since miRNA169 regulates MADS-box genes, it may be involved in the CMS phenotype.

In rice CMS-CW, the *rf17* gene is upregulated by retrograde signals, and increased RF17 protein expression inhibits pollen germination, causing abortion (Fujii & Toriyama, 2009). In restorer lines, the *Rf17* allele has promoter mutations preventing retrograde regulation, allowing normal pollen development

[Figure 3: see original paper]. Additionally, other floral development regulatory genes show differential expression.

Direct evidence for the retrograde regulation model remains limited, and the diverse pathways involved yield inconsistent experimental evidence. Thus, this model provides a general overview of one CMS mechanism class, offering an alternative research direction focused on nuclear gene expression analysis.

3.5 Evaluation of CMS Molecular Mechanism Models

Current CMS molecular mechanism research remains insufficiently deep, with few sterile lines having validated mechanisms. Consequently, existing models have inherent limitations.

Studies show that aberrant tapetal PCD causes maize CMS-C pollen abortion, yet sterile anthers also exhibit decreased ATP content and membrane potential, indicating energy supply reduction (Yang et al., 2022). Since ATP6 protein is a crucial FoF1-ATP synthase component, we hypothesize that transforming *ATP6c* into *E. coli* or yeast may reduce energy supply and increase ROS, inhibiting growth. If confirmed, this would support the cytotoxicity model. These observations demonstrate that current models lack clear delineation criteria, with the same sterile line explainable by multiple mechanisms.

No single model can explain all CMS phenomena. We propose that pollen abortion mechanisms differ substantially among sterile lines, making a universal mechanism unlikely. The cytotoxicity, energy deficiency, and aberrant PCD models show strong connections to respiratory pathways, while the retrograde regulation model focuses on nuclear gene expression differences. Therefore, CMS mechanisms may be broadly categorized as mitochondria-dominant or nucleus-dominant types. Mitochondria-dominant mechanisms primarily interfere with respiratory pathways affecting sporogenous cell-to-pollen development, whereas nucleus-dominant mechanisms regulate nuclear gene expression via mitochondrial retrograde signals to interfere with pollen germination and floral organ development.

4 Summary and Outlook

CMS materials have created enormous economic benefits in hybrid seed production and contributed significantly to food security. Beyond commercial applications, CMS materials are important for studying male gametophyte development and plant fertilization (Chase, 2007). However, challenges remain:

First, CMS gene screening and cloning concentrate on major crops, primarily focusing on Poaceae, Brassicaceae, and Solanaceae, limiting large-scale evolutionary analysis. Among cloned CMS genes, few have deeply resolved mechanisms. Current hypotheses are constrained by limited research foundations. Second, CMS research remains relatively isolated from studies of respiration,

metabolism, floral development, and hormone regulatory networks, hindering detailed physiological characterization and molecular model construction. Third, long-term reliance on one or few CMS cytoplasms creates genetically uniform backgrounds vulnerable to pests and diseases. In 1969, the URF13-encoded protein in maize CMS-T caused hypersensitivity to *Bipolaris maydis* race T, and large-scale monoculture of CMS-T maize in the southern United States led to rapid southern corn leaf blight spread and massive economic losses. Therefore, CMS cytoplasm diversification is essential for increasing genetic complexity and disease resistance.

Emerging technologies are accelerating CMS research. New sequencing and organelle assembly technologies enable precise mitochondrial genome comparisons, while pangenome construction aids traceability of chimeric sequences, reducing unknown origin proportions. Multi-omics approaches (transcriptomics, proteomics) efficiently mine mitochondrial ORFs potentially causing CMS. Mitochondria-targeted TALEN (MitoTALEN) technology enables direct mitochondrial genome editing, allowing more direct knockout validation of candidate CMS genes and expanding research possibilities.

CMS phenomena serve as important models for nuclear-cytoplasmic interactions, and research on mitochondrial replication, recombination, and repair (RRR) genes continues revealing nuclear regulation of mitochondrial sequence changes. Nuclear-cytoplasmic interaction research will not only deepen understanding of mitochondrial recombination mechanisms but also provide theoretical foundations for manipulating CMS gene evolution through nuclear gene regulation, enriching CMS germplasm resources for hybrid production. We anticipate that emerging technologies and mechanisms will provide new insights into plant CMS phenomena, enabling better utilization in production and research.

Note: Figure translations are in progress. See original paper for figures.