Male Sterility and Fertility Restoration in Crops

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Keywords

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Abstract

In plants, male sterility can be caused either by mitochondrial genes with coupled nuclear genes or by nuclear genes alone; the resulting conditions are known as cytoplasmic male sterility (CMS) and genic male sterility (GMS), respectively. CMS and GMS facilitate hybrid seed production for many crops and thus allow breeders to harness yield gains associated with hybrid vigor (heterosis). In CMS, layers of interaction between mitochondrial and nuclear genes control its male specificity, occurrence, and restoration of fertility. Environment-sensitive GMS (EGMS) mutants may involve epigenetic control by noncoding RNAs and can revert to fertility under different growth conditions, making them useful breeding materials in the hybrid seed industry. Here, we review recent research on CMS and EGMS systems in crops, summarize general models of male sterility and fertility restoration, and discuss the evolutionary significance of these reproductive systems.

Contents

INTRODUCTION

Plant male sterility, in its broadest sense, refers to the failure to produce dehiscent anthers, functional pollen, and viable male gametes. First observed by the German botanist Joseph Gottlieb Kölreuter in 1763 (98), male sterility has been reported in more than 610 plant species (63); it

includes cytoplasmic male sterility (CMS), which is caused by mitochondrial genes with coupled nuclear genes, and genic male sterility (GMS), which is caused by nuclear genes alone (132). Male-sterility mutants can cause abnormal development of either the sporophytic or gametophytic anther tissues. Most sporophytic male-sterility mutants affect primarily tapeta and meiocytes (cells undergoing meiosis), leading to pollen abortion or pollenless sterility (41). By contrast, gametophytic male-sterility mutants affect mainly the development of microspores or pollen grains.

Environment-sensitive genic male sterility (EGMS): male sterility that is reversible in response to environmental conditions such as day length and temperature

Male-sterility plants provide crucial breeding tools to harness hybrid vigor, or heterosis, in hybrid crops and also provide important materials to study stamen and pollen development and cytoplasmic-nuclear genomic interactions. Therefore, scientists have long been interested in the genetic and molecular mechanisms of male sterility and fertility restoration (reviewed in 11, 41, 46, 63, 122). Dozens of CMS and environment-sensitive GMS (EGMS) systems have been studied at the genetic and molecular levels. Here, we review the advances in research on CMS and EGMS and discuss emerging insights on cytoplasmic-nuclear genomic interactions in plants. CMS nomenclature varies in the literature, including different formats such as CMS-*XX* or *XX*-CMS, where *XX* indicates a specific CMS type; in this article, we use the format CMS-*XX*. Other kinds of GMS, including general male-sterility mutants caused by defective essential anther genes and hybrid sterility caused by divergence of speciation genes, have been reviewed recently (41, 109, 110) and thus are not described here.

THE IMPORTANCE OF MALE STERILITY AND FERTILITY RESTORATION

Cytoplasmic and Genic Male Sterility: Important Genetic Resources to Harness Heterosis in Crop Production

Hybrid vigor, or heterosis, refers to the phenomenon in which the progeny derived from a cross of two inbred lines outperform the parent lines. For example, hybrid crops can produce 15–50% higher yields than inbred varieties (130). The utilization of heterosis has produced tremendous economic benefits in worldwide crop production. More than half of the production of major crops such as maize, rice, sorghum, rapeseed, and sunflower comes from hybrid varieties (84). Thus, hybrid breeding contributes significantly to the food supply in the world.

Producing hybrid seeds of self-pollinating plants requires emasculation—the removal of functional pollen grains—to prevent self-pollination. Before the mid-twentieth century, emasculation in hybrid seed production involved manual labor, machines, or chemical treatments and thus was costly, inefficient, and even damaging to the environment. CMS and EGMS lines do not require emasculation and therefore are ideal female lines for hybrid seed production. In the 1950s, the maize CMS-T (Texas) system was first used for hybrid corn, greatly increasing the efficiency of hybrid seed production and improving maize yields. Later, CMS-based hybrid technology was developed in many other crops, including rice. Commercial hybrid rice, which increases the grain yield by over 20%, was first released in 1976 in China, and it has accounted for approximately 55% of the total rice planting area in China since the late 1980s (15).

CMS-based hybrid seed technology uses a three-line system, which requires three different breeding lines: the CMS line, the maintainer line, and the restorer line (**Figure 1*a***). The CMS line has male-sterile cytoplasm with a CMS-causing gene (hereafter termed a CMS gene) and lacks a functional nuclear *restorer of fertility* (*Rf*, or restorer) gene or genes (122), and is used as the female parent. The maintainer line has normal fertile cytoplasm but contains the same nuclear genome as the CMS line, and thus serves as the male parent in crosses for the propagation of the CMS line. The restorer line possesses a functional *Rf* gene or genes, and thus serves as the male

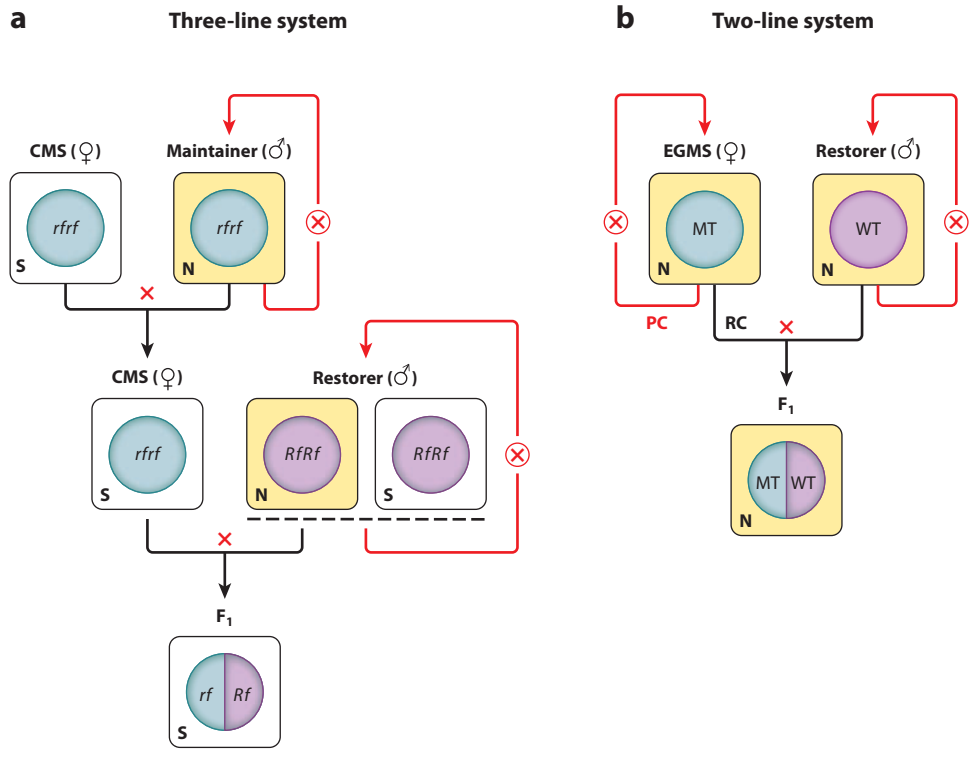


Figure 1

Application of cytoplasmic male sterility (CMS) and environment-sensitive genic male sterility (EGMS) for hybrid seed production in a three-line system and a two-line system. (*a*) The three-line system requires a CMS line, containing sterile cytoplasm (S) and a nonfunctional (recessive) restorer (*rf*) gene or genes; a maintainer line, containing normal cytoplasm (N) and a nuclear genome identical to that of the CMS line; and a restorer line, with normal (N) or sterile (S) cytoplasm and a functional (dominant) restorer (*Rf*) gene or genes. The CMS line is propagated by crossing with the maintainer line; the maintainer and restorer lines can produce seeds by self-pollination. The CMS line is crossed with the restorer line to produce male-fertile hybrids. (*b*) In the two-line system, an EGMS [photoperiod-sensitive GMS (PGMS), reverse PGMS, or temperature-sensitive GMS (TGMS)] mutant (MT) line is propagated by self-pollination when grown under permissive conditions (PC) (short-day conditions for PGMS, long-day conditions for reverse PGMS, or low-temperature conditions for TGMS). The EGMS line is male sterile under restrictive conditions (RC) (long-day conditions for PGMS, short-day conditions for reverse PGMS, or high-temperature conditions for TGMS) and thus serves as the female parent for crossing with a wild-type (WT) line to produce hybrid seeds.

parent to cross with the CMS line to produce F_1 hybrid seeds. In the F_1 plants, the *Rf* gene restores male fertility, and the combination of nuclear genomes from the CMS line and the restorer line produces hybrid vigor.

Photoperiod-sensitive genic male sterility (PGMS): male sterility that is determined by day length

In contrast to CMS, most GMS mutants are not suitable for hybrid seed production because their male-sterility traits cannot be efficiently maintained. However, the discovery of EGMS mutants has enabled some GMS traits to be used for hybrid crop breeding (134). The pollen fertility of EGMS lines changes in response to environmental cues such as day length and temperature. The first photoperiod-sensitive GMS (PGMS) mutant in rice, Nongken 58S (NK58S), was discovered in *japonica* rice (*Oryza sativa* ssp. *japonica*) in 1973. NK58S is completely male sterile when grown under long-day conditions but male fertile when grown under short-day conditions (123). A

temperature-sensitive GMS (TGMS) mutant, Annong S-1, was found in *indica* rice (*O. sativa* ssp. *indica*) in 1988. Annong S-1 is completely male sterile when grown at high temperatures but male fertile at low temperatures (21).

The reversibility of male fertility in PGMS and TGMS enables hybrid seed production with a two-line system. A PGMS or TGMS line grown under restrictive conditions (long-day or high-temperature conditions) serves as the male-sterile female parent. This same line can be propagated under permissive conditions (short-day or low-temperature conditions) (**Figure 1*b***). The two-line system thus eliminates the requirement for crossing to propagate the male-sterility line. Moreover, all normal varieties possess the wild-type fertility gene alleles that can restore male fertility, and thus they can be used as the male parents for hybridization. Therefore, a two-line system simplifies hybrid seed production and reduces costs. Recently, the production of two-line hybrid rice based on PGMS or TGMS has occupied approximately 20% of the total hybrid rice planting area in China (84).

Temperature-sensitive genic male sterility (TGMS): male sterility that is determined by temperature

Pentatricopeptide repeat (PPR): a 35-amino acid protein motif; most PPR-containing proteins in plants target to mitochondria or plastids for RNA processing

Cytoplasmic Male Sterility/Restorer Systems: Models to Study Mitochondrial-Nuclear Coevolution and Interaction

Nuclear genomes play important roles in regulating gene expression in response to developmental and environmental cues. Plant mitochondria and plastids are semiautonomous organelles; their genomes contain only part of the genetic information required for normal function. Mitochondria are important organelles in the tricarboxylic acid cycle, the respiratory electron transfer chain, and ATP synthesis (89). Plant mitochondrial genomes have only approximately 60 known genes for the electron transfer chain, ribosomal proteins, transfer RNAs, and ribosomal RNAs (76). However, proteomics analysis showed that plant mitochondria contain more than 1,000 proteins, most of which are encoded by the nuclear genome (75). For example, *Arabidopsis* and rice have approximately 450 and 650 nucleus-encoded pentatricopeptide repeat (PPR)–containing proteins, respectively, and most PPR proteins target to the mitochondria or plastids for their functions (119). In anterograde regulation, nuclear genes, including *Rf* genes, affect the functions of mitochondrial or chloroplast (plastid) genes. In retrograde regulation, the functions of some mitochondrial or chloroplast (plastid) genes, like CMS genes, may regulate the expression of certain nuclear genes (16, 138). Several plant mitochondrial genomes, including those of maize, rice, wheat, rapeseed, and sugar beet, have been sequenced (17, 45, 77, 105, 107). Sequence analysis of the mitochondrial genomes of CMS and maintainer lines may identify CMS candidate genes. Therefore, extensive studies on CMS/*Rf* systems can reveal the molecular basis of the interactions, including conflicts between the mitochondrial and nuclear genomes, and improve our understanding of the origin of novel mitochondrial genes and their evolutionary significance for the fitness of a species.

CURRENT PROGRESS ON CYTOPLASMIC MALE STERILITY AND RESTORER GENES IN MAJOR CROPS

Identification and Functional Analysis of Cytoplasmic Male Sterility Genes

Some wild plants contain CMS cytoplasm but are male fertile because of the broad presence of *Rf* genes in their nuclear genomes. Therefore, CMS cytoplasms are usually discovered by genetic crossing or somatic hybridization (protoplast fusion) that separates the CMS cytoplasm from its nuclear *Rf* gene(s). Crosses between the potential CMS cytoplasm-containing lines (as female parents) and non-*Rf* lines (i.e., lines containing recessive *rf* alleles) of the same or different species can be followed by selection of male-sterile progeny.

ORF: open reading frame

Mitochondrial electron transport chain (mtETC): a series of inner-membrane-bound metalloproteins of the mitochondria that pass electrons from the reducing products to the oxidizing dioxygen

SUO: sequence of unknown origin

CMS candidate genes can be identified by several strategies. The most common is to search for differences in mitochondrial gene organization and/or differences in the mitochondrial transcriptome or proteome in CMS cytoplasm lines with and without the *Rf* gene(s). For example, to clone the gene for rice CMS-WA—the male-abortive wild rice (*Oryza rufipogon*)–derived CMS system that is most widely used for hybrid rice breeding—Liu et al. (88) used 43 probe sequences covering the whole rice mitochondrial genome to perform RNA-blot analysis of the CMS-WA line, maintainer line, and fertility-restored lines (with the restorer gene *Rf3* or *Rf4*). A transcript was found to be specific to the CMS-WA line, and the abundance of this transcript was reduced in the *Rf4*-restored lines. Sequence analysis identified a novel chimeric open reading frame (ORF), named *WA352*, as the CMS-WA candidate gene (88, 91). In other CMS systems, several CMS-causing proteins, such as URF13 of maize CMS-T (32) and the truncated COX2 of sugar beet CMS-G (28), were detected by comparing the proteomes of CMS and fertile lines. In a few cases, such as radish CMS-Ogu (7) and wheat alloplasmic CMS-AP (115), the CMS candidate genes were identified by analysis of the mitochondrial DNAs of segregating somatic hybrids (cybrids) derived from protoplast fusion between CMS-carrying lines and normal fertile lines. However, for most crops this approach is not effective because of the great difficulty in obtaining cybrids and the uncertainty of recombination events between the mitochondrial DNAs of the fusion lines.

Verifying CMS gene candidates requires testing their effect on male sterility. One of the main technical barriers for functionally testing CMS genes is the lack of a successful method to transform plant mitochondrial genomes. As an alternative strategy, He et al. (47) prepared a recombinant construct of the CMS candidate gene *orf239* in common bean fused with a 5′ mitochondrial targeting signal sequence, and transferring this construct into the nuclei of tobacco plants caused male sterility, thus verifying the CMS function of *orf239*. This strategy has succeeded in functional analysis of other CMS genes, such as *orf79*, *orfH79*, and *WA352* in rice (51, 91, 113, 136); *orf129* in sugar beet (140); *orf288* in rapeseed (59); *orf220* in mustard (141); and *orf456* in pepper (68) (**Table 1**). The use of the mitochondrial targeting signal is critical for this method; in our experience, the mitochondrial targeting signal-encoding sequence (+1 ~ +105 base pairs) from the restorer *Rf1b* (136) works in CMS-transgenic rice and *Arabidopsis*.

Sequence Characteristics of Cytoplasmic Male Sterility Genes

Many CMS genes result from rearrangements of the mitochondrial genome. **Table 1** summarizes 28 types of CMS from 13 crop species. At least 10 essential mitochondrial genes, most belonging to the mitochondrial electron transfer chain (mtETC) pathways, have been found to be involved in the formation of CMS genes. Among them, *cox1*, *atp8*, and *atp6* are frequently involved in the origination of CMS genes. In addition, most CMS genes encode transmembrane proteins (**Table 1**). In rice, *orf79* for CMS-BT (derived from the *indica* rice variety Boro II) and its variant *orfH79* for CMS-HL (derived from the wild rice accession Hong-Lian) encode small proteins with an N terminus similar to COX1 and the remaining SUO (sequence of unknown origin) portion (136) (**Figure 2**). In sorghum CMS-A3, the chimeric *orf107* encodes a protein with a segment of ATP9 at the N terminus and a remaining portion that is similar to ORF79 (129). In wheat, the CMS-AP line, containing the nuclear genome of *Triticum aestivum* and the cytoplasm of *Triticum timopheevii*, is associated with *orf256* (115). The 5′ flanking region of *orf256* and the first 11-amino-acid coding sequence are identical to *cox1*. The *atp8* sequences form parts of the CMS genes in dicot species. The *Brassica* CMS genes *orf138* and *orf125*, which originated from radish, encode *atp8*-like proteins. The *Brassica* CMS-Pol and CMS-Nap genes *orf224* and *orf222*, encoding membrane proteins with 79% sequence similarity, contain an *atp8*-derived sequence

Table 1 Characterized cytoplasmic male sterility (CMS)/restorer (*Rf*) gene systems in major crops

Crop species	CMS type[a]	Associated ORF[b]	Protein property[c]	*Rf* locus[d]	Protein property[c]	Reference(s)
Maize (*Zea mays*)	CMS-T (S)	*urf13-atp4*	13-kDa toxic membrane protein	*Rf1* (R)	UK	18, 23, 25
				Rf2 (M)	Aldehyde dehydrogenase	
	CMS-S (G)	*orf355-orf77*	UK	*Rf3* (R)	UK	144
	CMS-C (S)	*atp6-C*	UK	*Rf4*	UK	24
Rice (*Oryza sativa*)	CMS-BT (G)	B-*atp6-orf79* (T)	Membrane protein	*Rf1a* (R), *Rf1b* (R)	PPR protein	2, 66, 72, 136
	CMS-HL (G)	*atp6-orfH79* (T)	Membrane protein	*Rf5* (*Rf1a*) (R)	PPR protein	51, 135
	CMS-LD (G)	L-*atp6-orf79*[e]	UK	*Rf2* (P)	Glycine-rich protein	53, 54
	CMS-CW (G)	*orf307*	UK	*Rf17* (P)	Acyl-carrier protein synthase	34–36
	CMS-WA (S)	*rpl5-WA352* (T)	Membrane protein	*Rf3* (P), *Rf4* (R)	UK	91, 145, 149
	CMS-RT120	*rpl5-orf352*	Membrane protein	*Rf102*	UK	108
	CMS-RT98	*orf113-atp4-cox3*	Membrane protein	UK	UK	52
Sunflower (*Helianthus annuus*)	CMS-PET1 (G)	*atp1-orf522*	15-kDa toxic membrane protein	*Rf1* (R)	UK	49, 70, 79
Brassica (*B. napus*)	CMS-Ogu (S)	*orf138-atp8*	30-kDa ATP8-like toxic membrane protein	*Rfo* (P)	PPR protein	10, 131
	CMS-Pol (S)	*orf224-atp6*	ATP8-like protein	*Rfp* (R)	UK	99, 125
	CMS-Nap (S)	*orf222-nad5c-orf139*	ATP8-like protein	*Rfn* (R)	UK	9, 82
Brassica (*B. juncea*)	CMS-Hau (S)	*atp6-orf288* (T)	Toxic protein	UK	UK	59
	CMS-*orf220*	*orf220* (T)	26-kDa membrane protein	UK	UK	141, 148
Brassica (*B. tournefortii*)	CMS-Tour (S)	*atp6-orf263*	32-kDa membrane protein (deduced 29 kDa)	UK (P)	UK	78
Radish (*Raphanus sativus*)	CMS-Kos (S)	*orf125-atp8*	ATP8-like membrane protein	*Rfk1* (P)	PPR protein	55, 71
	CMS-Don (S)	*orf463*	Membrane protein	*Rfd1* (P)	UK	112
Sorghum (*Sorghum bicolor*)	CMS-A3 (G)	*orf107*	UK	*Rf3* (R)	UK	129
	CMS-A1 (G)	UK	UK	*Rf1*, *Rf2*	PPR protein	61, 69

(*Continued*)

Table 1 (*Continued*)

Crop species	CMS type[a]	Associated ORF[b]	Protein property[c]	*Rf* locus[d]	Protein property[c]	Reference(s)
Wheat (*Triticum aestivum*)	CMS-AP	*orf256*	7-kDa membrane protein	UK	UK	127
Common bean (*Proteus vulgaris*)	CMS-Sprite (S)	*atp1-orf98-orf239* (T)	27-kDa protein	*Fr* (G), *Fr2* (P)	UK	1, 47, 95
Pepper (*Capsicum annuum*)	CMS-Peterson	*cox2-orf456* (T)	17-kDa protein	UK	UK	39, 58, 68
		cox2-orf507	19.5-kDa protein			
Carrot (*Daucus carota*)	CMS-Petaloid	*orfB*	ATP8-like membrane protein	UK (R)	UK	104
Sugar beet (*Beta vulgaris*)	CMS-Owen	*preSatp6*	35-kDa membrane protein	*Rf1* (P)	Peptidase	97, 139
	I-12CMS(3)	*orf129* (T)	12-kDa matrix protein	UK (P)	UK	140
	CMS-G	*cox2*	31-kDa truncated COX2 protein	*RfG1*, *RfG2*	UK	28

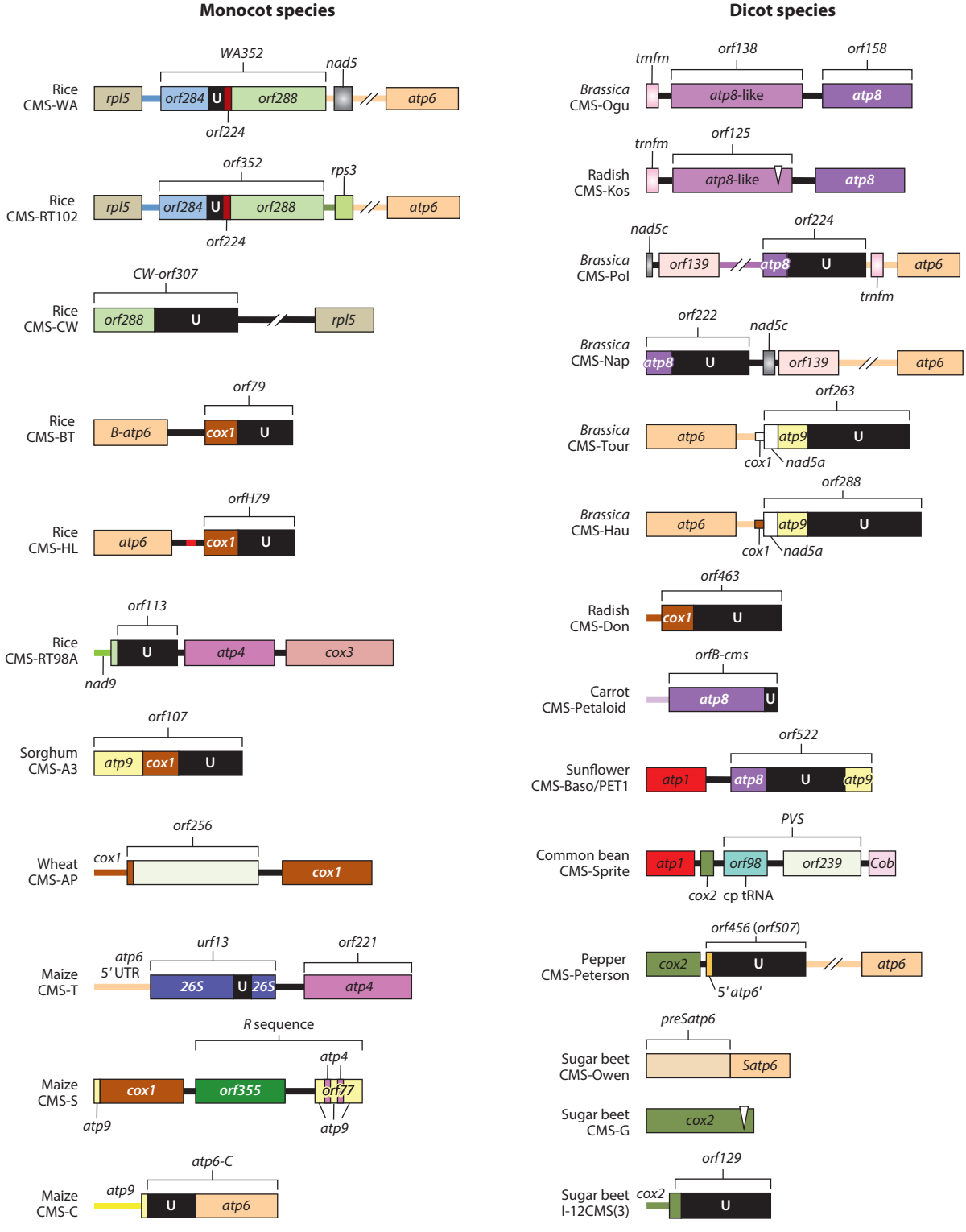
[a]Letters in parentheses indicate the generation where the CMS acts: S, sporophytic; G, gametophytic.
[b]Underlined cotranscripts indicate the CMS-causing open reading frame (ORF); a letter T in parentheses indicates that the biological function as a CMS gene has been validated in transgenic plants.
[c]PPR, pentatricopeptide repeat; UK, gene or product is unknown.
[d]Letters in parentheses indicate the level at which male fertility is restored: G, genomic level; R, RNA (posttranscriptional) level; P, protein (translational or posttranslational) level; M, metabolic level.
[e]K. Toriyama, personal communication.

and a SUO (125). The *atp8* sequences are also present in *orf522* in sunflower CMS-PET1 and *orfB-cms* in carrot CMS-Petaloid, with an additional SUO (70, 79, 104). Many other CMS genes contain *atp6* sequences of different lengths, including *atp6-C* in maize CMS-C (24), *orf456* in pepper CMS-Peterson (68), and *preSatp6* in sugar beet CMS-Owen (139).

Distinct from most CMS genes, which contain sequences of the known functional mitochondrial essential genes, several recently identified rice CMS-associated ORFs consist of sequences of putative mitochondrial ORFs. For instance, the CMS-WA gene *WA352* and its variant *orf352* comprise three segments derived from the putative mitochondrial ORFs *orf284*, *orf224*, and *orf288* and a short SUO (91, 108) (**Figure 2**). *CW-orf307* for CMS-CW contains the 5′ sequence of *orf288* and a SUO (34). Therefore, sequences of the known functional mitochondrial genes are not necessarily the components of some functional CMS genes. In addition, several identified CMS genes (such as *orf125* in radish CMS-Kos and its variant *orf138* in *Brassica* CMS-Ogu) and the mutated *cox2* in sugar beet CMS-G are nonchimeric genes that contain sequences from single sources (8, 28, 55).

Figure 2

Genomic structures of cytoplasmic male sterility (CMS)–associated genes in various crop species. The boxes represent coding sequences, and the horizontal lines indicate flanking regions of the open reading frames (ORFs). The sequences with similarity to the same genes (including flanking and coding sequences) are shown in the same colors. Black boxes represent sequences of unknown origin (U). Diagrams not to scale. Abbreviation: cp, chloroplast; UTR, untranslated region.


Monocot species
Dicot species
Rice CMS-WA
WA352
nad5
rpl5
orf284
U
orf288
atp6
orf224
Rice CMS-RT102
orf352
rps3
rpl5
orf284
U
orf288
atp6
orf224
Rice CMS-CW
CW-orf307
orf288
U
rpl5
Rice CMS-BT
orf79
B-atp6
cox1
U
Rice CMS-HL
orfH79
atp6
cox1
U
Rice CMS-RT98A
orf113
U
atp4
cox3
nad9
Sorghum CMS-A3
orf107
atp9
cox1
U
Wheat CMS-AP
orf256
cox1
cox1
Maize CMS-T
urf13
orf221
atp6
5′ UTR
26S
U
26S
atp4
Maize CMS-S
R sequence
atp4
cox1
orf355
orf77
atp9
atp9
Maize CMS-C
atp6-C
atp9
U
atp6
Brassica CMS-Ogu
trnfm
orf138
orf158
atp8-like
atp8
Radish CMS-Kos
trnfm
orf125
atp8-like
atp8
Brassica CMS-Pol
nad5c
orf224
orf139
atp8
U
atp6
trnfm
Brassica CMS-Nap
orf222
nad5c
atp8
U
orf139
atp6
Brassica CMS-Tour
orf263
atp6
atp9
U
cox1
nad5a
Brassica CMS-Hau
orf288
atp6
atp9
U
cox1
nad5a
Radish CMS-Don
orf463
cox1
U
Carrot CMS-Petaloid
orfB-cms
atp8
U
Sunflower CMS-Baso/PET1
orf522
atp1
atp8
U
atp9
Common bean CMS-Sprite
PVS
atp1
orf98
orf239
Cob
cox2
cp tRNA
Pepper CMS-Peterson
orf456 (orf507)
cox2
U
atp6
5′ atp6′
Sugar beet CMS-Owen
preSatp6
Satp6
Sugar beet CMS-G
cox2
Sugar beet I-12CMS(3)
orf129
cox2
U

Identification and Sequence Characteristics of Restorer Genes

Nine *Rf* genes have been isolated in seven plant species: *Rf2* (maize) (18, 86), *Rf-PPR592* (*Petunia*) (6), *Rfo* (*Rfk1*) (radish, *Brassica*) (10, 22, 71), *Rf1a* (*Rf5*) (rice) (2, 51, 72, 136), *Rf1b* (rice) (136), *Rf2* (rice) (53), *Rf17* (rice) (36), *Rf1* (sorghum) (69), and *Rf1* (*bvORF20*) (sugar beet) (43, 97) (**Table 1**). *Rf-PPR592*, *Rfo*, *Rf1a*, *Rf1b*, and sorghum *Rf1* encode PPR proteins. PPRs are a group of RNA-binding proteins, and most act in organellar posttranscriptional mRNA processing, such as editing, splicing, cleavage, degradation, and translation (19, 121). *Rf2* in maize CMS-T, the first isolated plant restorer gene, encodes an aldehyde dehydrogenase (18). *Rf17* in CMS-CW encodes a 178-amino-acid mitochondrial sorting protein containing an acyl-carrier protein synthase-like domain (36). *Rf2* in CMS-LD encodes a mitochondrial glycine-rich protein (53). Recently, *bvORF20*, encoding a putative peptidase of the M48 family, has been shown to be a strong candidate for *Rf1* in sugar beet CMS-Owen (43, 97). Therefore, except for the conserved PPR *Rf* genes, *Rf* genes are highly multifarious.

CURRENT PROGRESS ON ENVIRONMENT-SENSITIVE GENIC MALE STERILITY IN MAJOR CROPS

Several EGMS mutants have been reported in crops (84), but few EGMS genes have been functionally studied, and most research has been on rice (**Table 2**). The *japonica* rice PGMS mutant line NK58S is mainly photoperiod sensitive, but when this trait is crossed into the *indica* genetic background, the resulting lines—such as PeiAi 64S (PA64S)—become mainly temperature sensitive. The NK58S PGMS locus (*pms3*) and the same locus for TGMS in PA64S (*p/tms12-1*) were mapped to chromosome 12 (90, 151). Recently, a long noncoding RNA (lncRNA) gene was identified at the *pms3* (*p/tms12-1*) locus, and in the male-sterility lines, this lncRNA gene has a single nucleotide mutation (C-to-G) (26, 151). The expressed lncRNA is processed into a 21-nucleotide small RNA (osa-smR5864), and the mutation is located in this small RNA (151). Therefore, this mutation mediates the PGMS trait in *japonica* rice and the TGMS trait in *indica* rice, suggesting that the potential target gene or genes of the lncRNA and osa-smR5864 may respond to day length and temperature, respectively, in the *japonica* and *indica* genetic backgrounds

Table 2 Functionally studied environment-sensitive genic male sterility (EGMS) genes in crops

Crop species	EGMS line	GMS type[a]	EGMS gene	Protein and function	Reference(s)
Rice (*Oryza sativa*)	NK58S	PGMS ≤13 h (F), ≥13.75 h (S)	*pms3*	Noncoding RNA	26, 100
	PA64S	TGMS ≤23.5°C (F), ≥27°C (S)	*tms12-1*	Noncoding RNA/small RNA	151
	CSA	rPGMS ≥13.5 h (F), ≤12.5 (S)	*csa*	MYB transcript regulator	147
	Ugp1	TGMS ≤21°C (F), ≥28°C (S)	*Ugp1*	UDP-glucose pyrophosphorylase	14
Wheat (*Triticum timopheevii*)	Norin26 (D^2-type cytoplasm)	PCMS ≤14.5 h (F), ≥15 h (S)	*orf25/Rfd1*	Unknown	101

[a]Abbreviations: PGMS, photoperiod-sensitive genic male sterility; TGMS, temperature-sensitive genic male sterility; rPGMS, reverse photoperiod-sensitive genic male sterility; PCMS, photoperiod-sensitive cytoplasmic male sterility. A letter F in parentheses indicates male fertility; a letter S in parentheses indicates male sterility.

(151). Expression of the lncRNA is affected by differential DNA methylation at its promoter region in NK58S and wild-type rice, and this methylation is mediated by a small interfering RNA that targets the promoter region (27). Although the potential target gene or genes of the lncRNA and osa-smR5864 have not been identified, these studies have revealed a mechanism by which environmental factors interact with the genetic and epigenetic elements that regulate male fertility (152).

The rice TGMS gene *Ugp1* encodes a UDP-glucose pyrophosphorylase (UGPase). *Ugp1* is expressed in developing pollen and is required for callose deposition; *Ugp1*-silenced plants are male sterile. *Ugp1*-overexpressing transgenic rice plants also show male sterility caused by cosuppression of the endogenous *Ugp1* in which the primary mRNA is not spliced (14). However, these cosuppressed plants revert to being male fertile when grown at low temperatures ($\leq$21°C) owing to the highly efficient splicing of the primary *Ugp1* mRNA into mature mRNA (**Table 2**). Therefore, male sterility caused by *Ugp1* cosuppression represents another type of TGMS.

Rice CARBON STARVED ANTHER (CSA) is an R2R3-MYB transcription factor that regulates the expression of *OsMST8*, encoding a monosaccharide transporter family member that functions in sugar partitioning from vegetative tissues to anthers for pollen maturation (146). Interestingly, *csa* mutant plants are male sterile under short-day conditions and male fertile under long-day conditions, a reverse PGMS trait (147) (**Table 2**). The *csa* mutant phenotype can be recovered under long-day conditions, probably because one or more similar pathways compensate for the defective *CSA-OsMST8* pathway under long-day conditions. Introduction of the *csa* locus from the original *japonica* mutant into an *indica* background maintains the reverse PGMS character. F_1 hybrid plants made by crossing the *indica csa* line with an *indica* fertile line exhibited heterosis and higher yield, suggesting the potential for using this reverse PGMS mutant for hybrid rice breeding (147).

A special alloplasmic wheat containing the nuclear genome of *T. aestivum* cv. Norin 26 and the D^2-type cytoplasm of *Aegilops juvenalis* or *Aegilops vavilovii* exhibits pistil-like stamens (pistillody), and shows complete male sterility under long-day conditions ($\geq$15 h) and high male fertility under short-day conditions ($\leq$14.5 h) (102) (**Table 2**). This photoperiod-sensitive CMS (PCMS) may be caused by unprocessed mitochondrial *orf25* through alterations in the expression of nuclear class-B MADS-box genes, and it can be restored by *Rfd1* on chromosome 7B (44, 101, 106). PCMS may provide promising breeding material for hybrid wheat production in a two-line system.

CURRENT MODELS FOR CYTOPLASMIC MALE STERILITY MECHANISMS

Expression of Cytoplasmic Male Sterility Genes and Mechanisms Determining Male Specificity

One interesting and long-standing question is how CMS genes affect only male development. This male specificity may result from one of three possible types of expression patterns of CMS genes: male organ (anther)–specific mRNA expression and protein accumulation, constitutive mRNA expression and protein accumulation throughout plant tissues, or constitutive mRNA expression but specific or preferential protein accumulation in the male organs (anthers). Anther-specific expression provides a straightforward explanation of the male specificity of the trait, but, to our knowledge, no CMS system shows anther-specific expression. The I-12CMS(3) cytoplasm derived from wild beet shows constitutive *orf129* mRNA expression and protein accumulation; the CMS protein ORF129 accumulates in flowers and also in roots and leaves (140), but the mechanism restricting

its function in the male organ is unknown. One possible explanation is that normal development of anthers requires much more energy than vegetative organ development (see The Energy Deficiency Model, below); thus, impaired mitochondrial function may affect only male development (137).

For most CMS systems, the expression patterns belong to the third type, constitutive mRNA expression but tissue-specific protein accumulation. In CMS-Sprite common bean, the CMS gene *orf239* is transcribed ubiquitously, but the ORF239 protein is present only in the reproductive organs (1). A nucleus-encoded mitochondrial protease, LON, appears to be responsible for the turnover of ORF239 in vegetative tissues (120). In CMS-WA rice, *WA352* is transcribed constitutively, but the production of WA352 protein is highly spatiotemporally specific; it accumulates mainly in the tapetal cells at the microspore mother cell stage and not in the anther cells before or after this stage (91). In CMS-BT rice, ORF79 accumulates preferentially in the microspores to cause gametophytic male sterility (136). Therefore, for most CMS systems, the spatial and temporal regulation of CMS protein production determines the male specificity of the trait.

Emerging research on CMS systems of diverse crops supports four models for the mechanisms that cause CMS (**Figure 3**): the cytotoxicity model, the energy deficiency model, the aberrant

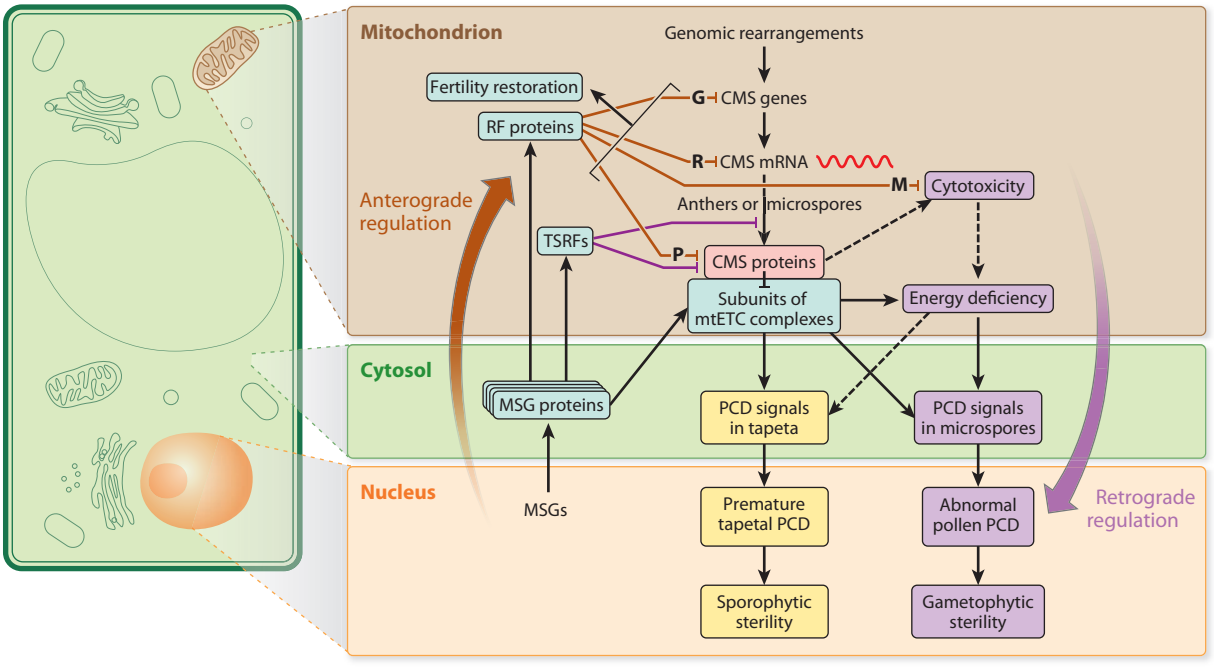


Figure 3

A general model for different layers of mitochondrial-nuclear gene interactions in cytoplasmic male sterility (CMS)/restorer (*Rf*) systems. The nucleus-encoded mitochondrial-sorting gene (MSG) products—including RF proteins and tissue-specific regulatory factors (TSRFs) and subunits of the mitochondrial electron transfer chain (mtETC) complexes—target to the mitochondria for anterograde regulation. TSRFs may regulate, at the translational or posttranslational level, the male organ (anther)–specific accumulation of CMS proteins for male specificity. The CMS proteins may interact with the mtETC subunits to affect their functions or redox status or ATP biogenesis, leading to production of retrograde signals (such as reactive oxygen species and cytochrome *c* release) that trigger aberrant programmed cell death (PCD) in tapeta or microspores. CMS restoration by RF proteins can be achieved at the genomic level (G), mRNA level (R), protein level (P), or metabolic level (M).

programmed cell death (PCD) model, and the retrograde regulation model. These models are discussed in the sections below.

The Cytotoxicity Model

In the cytotoxicity model, the CMS protein directly kills the cells. The first discovered CMS protein, URF13 for maize CMS-T, is toxic to *Escherichia coli* (23, 73) and to many eukaryotic cells (74, 81). Since the discovery of URF13, several CMS proteins—including ORF522 for sunflower CMS-PET1 (103), ORF138 for radish CMS-Ogu (29), ORF288 for *Brassica* CMS-Hau (59), ORF79 for rice CMS-BT (91, 136), and WA352 for rice CMS-WA (H. Xu & Y.-G. Liu, unpublished data)—have also been shown to be toxic to *E. coli* (**Table 1**). Most CMS proteins are 10–35-kDa transmembrane proteins and have a hydrophobic region (**Table 1**), which are typical features of cytotoxic proteins. Therefore, a straightforward model for CMS in these systems is that the CMS proteins cause mitochondrial dysfunction in the sporophytic or gametophytic cells of the anthers, leading to male abortion (81).

However, our information on the cytotoxicity of CMS proteins is derived from the transgenic expression of CMS genes in prokaryotic or eukaryotic cultured cell systems. Direct biochemical evidence for the cytotoxicity of the CMS proteins in plant anther cells is currently lacking. A mechanistic relationship between the structures of the CMS-causing toxic proteins and CMS occurrence remains unclear. The toxicity of the CMS-WA protein WA352 to *E. coli* is dependent on the specific transmembrane structure (H. Xu & Y.-G. Liu, unpublished data). However, transgenic plants expressing truncated WA352 proteins that contain no transmembrane segments still showed male sterility (91), indicating that CMS induction by WA352 is not dependent on the transmembrane domains.

The Energy Deficiency Model

In the energy deficiency model, the CMS protein causes mitochondrial deficiencies, and the cells fail to meet the substantial energy requirements of male reproductive development. Mitochondria are vital for producing biological energy (ATP) via mtETC of the respiratory complexes. The biogenesis of the sporophytic and gametophytic cells of plant anthers is thought to demand more cellular energy than other organs. Cells may produce more ATP either by increasing the number of mitochondria or by increasing the metabolic activity of mitochondria. Lee & Warmke (80) observed rapid division of mitochondria during anther development of maize CMS-T, and therefore proposed that the CMS gene causes mitochondrial dysfunction and the mitochondria fail to provide enough ATP for male development. According to the chemiosmotic theory of ATP production, primary proton pumps such as complexes I (NADH dehydrogenase), II (succinate dehydrogenase), III (ubiquinol-cytochrome *c* reductase), and IV (cytochrome *c* oxidase) produce a high proton gradient and a downhill proton flow, which allow complex V (F_1F_o-ATPase) to generate ATP (89). Therefore, an intact mitochondrial membrane is essential for mtETC function and energy production in mitochondria.

The sequences and structural features of many CMS proteins have provided a possible molecular basis for the energy deficiency model. First, many CMS proteins—including URF13 for maize CMS-T, ORF138 for *Brassica* CMS-Ogu, ORF79 for rice CMS-BT, ORFH79 for rice CMS-HL, and preSatp6 for sugar beet CMS-Owen—are mitochondrial transmembrane proteins (**Table 1**). These proteins may integrate into the inner mitochondrial membrane, destroying the proton gradient and affecting ATP synthesis (117).

Reactive oxygen species (ROS): molecules that act as secondary messengers or retrograde signals

Second, as chimeric ORFs, many (but not all) CMS genes consist of portions of some essential mitochondrial genes involved in respiration pathways, including *nad3*, *nad5*, and *nad7* for complex I; *cox1* and *cox2* for complex IV; and *atp1* (*atpA*), *atp4*, *atp6*, *atp8*, and *atp9* for complex V (**Figure 2**). The CMS proteins may competitively interact with the mtETC complexes. These features of CMS genes strongly indicate a link between respiratory pathways and CMS.

Direct evidence supporting the energy deficiency model comes from the sugar beet CMS-G system (28). Molecular study of CMS-G identified two mutated proteins in male-sterile plants: an extended NAD9 (the core of complex I) and a truncated COX2 (a subunit of complex IV). The CMS phenotype is correlated with this deletion form of *cox2*, which diminishes the activity of normal COX2, providing a molecular link between the mtETC defect and CMS. Another example comes from pepper CMS-Peterson, in which two analogs of the CMS gene, *orf456* and *orf507*, are cotranscribed with *cox2*, and *orf507* impairs cytochrome *c* oxidase activity in the mitochondria in the CMS line (58). Similarly, CMS-HL rice shows reduced ATP and NADH levels in anthers expressing ORFH79, which is a homolog of ORF79 for CMS-BT (113). ORFH79 interacts with the nucleus-encoded subunit P61 of mtETC complex III in the mitochondria, impairing the activity of complex III and resulting in decreased ATP production (135).

In mammalian and yeast cells, *atp8* encodes a core subunit for the assembly of F_1F_o-ATPase. In plants, the mitochondrial *orfB* is an ortholog of *atp8*, despite their limited sequence similarity, and the protein functions as a subunit of plant F_1F_o-ATPase (118). In sunflower CMS-PET1, the chimeric *orf522* gene encodes a novel protein containing a small portion (19 amino acids) identical to the N terminus of ORFB. The ATPase activity of CMS-PET1 plants is significantly lower than that of fertile plants, suggesting that ORF522 may compromise F_1F_o-ATPase activity (118). In CMS pearl millet, the activities of F_1F_o-ATPase and cytochrome *c* oxidase are impaired (62). Other CMS proteins containing sequences similar to ORFB, including ORF138 and ORF125 in CMS radish, ORF224 and ORF222 in *Brassica*, and ORFB-cms in carrot, may share similar mechanisms. In fact, ORF138 forms part of a large complex in the mitochondrial inner membrane (30).

Together, the lines of molecular evidence support the hypothesis that some CMS may be related to energy deficiency in developing anthers, which require more energy. CMS proteins may affect mitochondrial membrane integrity, leading to proton leakage and inadequate ATP production. Alternatively, CMS proteins may serve as dysfunctional homologs competing with native components of the mtETC or F_1F_o-ATPase complexes, forming inactive or less efficient mtETC or F_1F_o-ATPase complexes for ATP production.

The Aberrant Programmed Cell Death Model

PCD in plants is an apoptosis-like cellular process that includes fragmentation of nuclear DNA and is controlled by mitochondrion-driven signals. Plant PCD functions in developmental processes such as senescence, seed germination, organ development, root tip elongation, xylem and aerenchyma formation, and disease resistance (116). The release of cytochrome *c* from mitochondria into the cytosol and overproduction of reactive oxygen species (ROS) function as the major retrograde signals triggering animal apoptosis and plant PCD (38, 87, 142).

The development of plant male gametophytes in anthers requires cooperative interactions between sporophytic (anther wall) and gametophytic (microspore) cells and requires proper PCD-controlled cellular degeneration of the tapetum, the innermost cell layer of the anther wall tissue (92). Therefore, normal tapetum function requires the properly timed initiation and progression of PCD; premature or delayed tapetal PCD leads to male sterility (57, 64, 111). For example, the gametophytic CMS-PET1 cytoplasm in sunflower causes premature PCD of the tapetal cells,

which is associated with early release of cytochrome *c* from the mitochondria to the cytosol (4). ORF522 is proposed to affect the activities of F_1F_o-ATPase and cytochrome *c* oxidase (4, 118); however, the mechanism by which ORF522 induces premature tapetal PCD remains unclear.

COX11 is a nucleus-encoded assembly factor for cytochrome *c* oxidase, which is highly conserved in eukaryotic organisms (5). Yeast COX11 (ScCOX11) and rice COX11 (OsCOX11) have another role in hydrogen peroxide degradation and may act as negative regulators of PCD (91, 133). In rice CMS-WA lines, the CMS protein WA352 accumulates preferentially in the tapetal cells at the microspore mother cell stage and physically interacts with OsCOX11. This detrimental interaction inhibits OsCOX11's function in peroxide metabolism, thus causing an early cytochrome *c* release and ROS burst, which then triggers premature tapetal PCD at the meiotic prophase I stage—much earlier than normal tapetal PCD, which occurs at the dyad stage in male-fertile rice plants (91). These abnormal molecular events lead to rapid cellular degeneration of the tapeta and consequent pollen abortion. Abnormal PCD was also observed in anthers of CMS-HL rice at the tetrad and early-uninucleate stages, associated with excess ROS production (83). However, because CMS-HL is gametophytic, whether the aberrant PCD occurs in the microspores remains to be clarified by in situ assays.

The Retrograde Regulation Model

In CMS-CW rice, derived from *O. rufipogon*, a mitochondrial chimeric ORF, *CW-orf307*, was identified as a CMS gene candidate (34). The restorer gene *Rf17* in CMS-CW encodes the mitochondrial protein RETROGRADE-REGULATED MALE STERILITY (RMS), which has an acyl-carrier protein synthase-like domain. *Rf17* and *rf17* alleles do not alter *CW-orf307* transcription. However, the CMS-CW cytoplasm upregulates, through one or more unknown retrograde signals, the expression of *rf17*, whereas *Rf17* is not upregulated by the CMS cytoplasm, owing to the presence of a mutation in the *Rf17* promoter regulatory region. Increased *RMS* expression suppresses pollen germination, thus leading to gametophytic sterility (36).

MADS-box transcription factors have major roles in flower development (96). Carpeloid CMS and petaloid CMS refer to CMS systems in which the stamens are replaced by carpels and petals, respectively, in the florets. These CMS phenotypes morphologically resemble homeotic mutants. Indeed, expression of two MADS-box genes controlling whorls 2 and 3 in carrot flowers is suppressed in the carpeloid CMS lines. This strongly indicates that retrograde signaling from mitochondria regulates the expression of these nuclear MADS-box genes, determining the organ conversion in carpeloid CMS (85).

In general, we speculate that the various CMS systems involve complex processes; for example, energy deficiency and premature PCD may be parts of the same mechanisms underlying CMS. Regardless of the mechanisms causing CMS, the sporophytic cells (mainly the tapetum) or the gametes eventually die because of the abnormalities in redox processes and energy production in the mitochondria, which may be the primary causes of the retrograde signaling for aberrant PCD in tapeta or microspores (**Figure 3**).

MECHANISMS FOR CYTOPLASMIC MALE STERILITY RESTORATION

As described above, the deleterious effects of CMS proteins cause CMS phenotypes. In this scenario, any mechanisms that suppress the expression of CMS genes or counteract their negative effects will restore male fertility. Thus, the restoration of fertility in CMS/*Rf* systems (referred to as CMS restoration) may be achieved by various mechanisms at different molecular levels (**Figure 3**).

Cytoplasmic Male Sterility Restoration at the Genomic Level

Mitochondrial genomes are highly dynamic, with frequent variations in the structure and copy number of mitochondrial DNA molecules (95). Spontaneous reversion to fertility occurs occasionally in some CMS plants and involves substoichiometric shifting in the relative copy numbers of certain subgenomic molecules containing CMS genes. Common bean CMS-Sprite is caused by the mitochondrial *PVS* sequence located downstream of *atp1* (12, 60). The 25-kb *PVS*-associated mitochondrial genomic sequence is altered by specific excision in the presence of a dominant nuclear gene, *Fr*. As a result, the majority of the CMS mitochondrial DNA molecules convert to normal ones, and thus the progeny of the following generations are male fertile regardless of whether *Fr* is present (1, 56, 94). This system provides the first example of an *Rf* gene that recovers CMS at the genomic level through substoichiometric shifting. Another case of substoichiometric control of a CMS gene occurs in pearl millet CMS-A1. CMS-A1 plants are prone to spontaneous reversion to fertility, in which a subgenomic molecule containing the *cox1-1-2* junction region is amplified 10-fold in fertility-reversed plants (31).

Cytoplasmic Male Sterility Restoration at the Posttranscriptional Level

In plant mitochondria and chloroplasts, transcripts usually undergo modifications such as editing, splicing, and cleavage. The RNA editing changes cytidine (C) residues to uridine (U) at certain sites of the RNA sequences in plant organelles, especially in mitochondria (124). RNA exo- or endonucleolytic cleavage may occur in the coding regions and/or the intercistronic (spacer) sequences of multicistronic transcripts. Expression and sequencing analyses have indicated that most CMS-associated (co)transcripts in various crops are processed by different posttranscriptional mechanisms, such as editing, polyadenylation, cleavage, and degradation mediated by *Rf* gene products (**Table 1**). For example, in sorghum, the CMS-A3-associated *orf107* transcript has four C-to-U editing sites that are processed with different levels of efficiency. In the sterile plants, site 1 is edited frequently and site 2 is edited infrequently. The effective editing of sites 3 and 4, with rates of approximately 80% and 60%, respectively, requires the action of *Rf3*. The edited *orf107* transcript is degraded rapidly in the *Rf3*-restored plants (114, 128). In CMS-PET1 sunflower, *orf522* is cotranscribed with *atp1*. The *atp1-orf522* transcript is edited at two sites in *orf522* and one site in *atp1*. The *atp1-orf522* mRNAs in the florets of the *Rf1*-restored plants are polyadenylated at the 3′ untranslated region and then degraded by two distinct ribonuclease activities (37, 100). Therefore, *Rf1* may function in the specific polyadenylation of the transcript, which is unstable and rapidly degraded.

Multiple CMS transcripts in various CMS crops are cleaved or degraded by the corresponding Rf proteins. In CMS-T maize, which is restored by *Rf1* and *Rf2*, the *urf13-orf221* dicistronic transcript is processed in an *Rf1*-specific manner, causing reduced abundance of the cleaved *urf13* RNA fragment (67). In CMS-Pol *Brassica* plants, the *orf224-atp6* dicistronic transcript is cleaved by the action of *Rfp*, and the resulting *orf224* RNA fragment is degraded rapidly in the reproductive organ (99). ORF222 in CMS-Nap has a 79% sequence similarity to ORF224. The cotranscripts of *orf222-nad5c-orf139* undergo processing similar to that of *orf224-atp6* (82). The specificity of the processing events conditioned by *Rfn* and *Rfp* may depend on RNA structural differences of the CMS cotranscripts (9).

In CMS-BT rice, RF1A cleaves the *B-atp6-orf79* dicistronic transcripts at the intercistronic region and the 5′ untranslated region of *B-atp6*, but RF1B promotes rapid degradation of the *B-atp6-orf79* transcripts (136). When *Rf1a* and *Rf1b* are both present in the restored plants, RF1A has an epistatic effect on the cleavage of the transcripts (136). The cleaved *orf79* RNA fragment loses its ribosome-binding site and is not translated (65, 136). In CMS-HL rice, *atp6-orfH79*

is a homolog of *B-atp6-orf79* (143). In the presence of *Rf5*—the same gene as *Rf1a* for CMS-BT—*atp6-orfH79* transcripts are cleaved similarly to the cleavage pattern of *B-atp6-orf79* in *Rf1a*-restored CMS-BT plants. However, in contrast to the direct binding of RF1A with *B-atp6-orf79* mRNA (65), RF5 alone cannot bind with *atp6-orfH79* mRNA; it requires the glycine-rich protein GRP162 as an adaptor in a restoration-of-fertility complex to bind to the *atp6-orfH79* mRNA for cleavage (51). These different mRNA binding modes may be due to the divergence of their binding sequences in the intercistronic regions of *B-atp6-orf79* and *atp6-orfH79*. In CMS-WA rice, *Rf4* acts to degrade the dicistronic *rpl5-WA352* and monocistronic *WA352* transcripts; the abundance of these transcripts is reduced to approximately 20% in the *Rf4*-restored plants (91).

These findings indicate that most PPR RF proteins suppress CMS gene expression through posttranscriptional mechanisms such as editing, cleavage, and degradation of the target mRNAs. However, because PPR proteins contain no nucleolytic domains, they may function with other cofactors for RNA processing (19).

Cytoplasmic Male Sterility Restoration at the Translational or Posttranslational Level

In some CMS systems, the amounts and sizes of CMS-associated transcripts do not change in CMS and fertility-restored plants. In these cases, fertility restoration may be controlled by translational or posttranslational mechanisms (**Table 1**). For example, in maize CMS-C, *Rf4* does not affect the steady-state level of *atp6-C* mRNA, suggesting that restoration may act at the protein level (24). In common bean CMS-Sprite, *Fr2* does not affect *PVS* transcript levels but does suppress the accumulation of ORF239 protein (120). In fertility-restored CMS-Tour *Brassica* plants, the level of CMS-associated *orf263* mRNA is unchanged but the ORF263 protein is absent (78). In *Brassica* and radish, CMS-Ogu is restored by *Rfo*, which encodes the PPR protein PPR-B (also named ORF687). The amount of *orf138* mRNA in the anthers is not altered, but the accumulation of ORF138 is suppressed (131). PPR-B binds the *orf138* mRNA, thus probably blocking ORF138 translation (131). In CMS-WA rice, *Rf3* does not affect *WA352* mRNA levels, but WA352 protein does not accumulate in the anthers of the plants restored by *Rf3* alone, suggesting that *Rf3* may function at the translational or posttranslational level (91). *Rf2* in CMS-LD rice encodes a glycine-rich protein. Unlike the CMS-HL RF5-interacting protein GRP162, which contains an RNA-binding domain, RF2 has no RNA-binding domain, suggesting that RF2 may restore CMS-LD at the protein level (53).

Cytoplasmic Male Sterility Restoration at the Metabolic Level

Rf2 in maize CMS-T encodes an aldehyde dehydrogenase enzyme (18, 86). Aldehyde dehydrogenase enzymes play roles in the metabolism of fatty acids and amino acids and detoxify alcohols and toxins by altering aldehyde damage to cells and tissues. The RF2 protein oxidizes at least three aldehydes (86). Given that neither the *urf13-orf221* transcripts nor the URF13 protein is changed in the presence of *Rf2*, RF2 may restore CMS-T by eliminating harmful molecules caused by URF13 (86).

EMERGING INSIGHTS INTO MALE STERILITY AND RESTORATION OF FERTILITY

The Origins and Coevolution of Cytoplasmic Male Sterility Genes and Restorer Genes

The structures and sizes of plant mitochondrial genomes are highly variable. Plant mitochondrial genomes possess many recombinantly active sequences; recombination among them can divide

the genome into subgenomic molecules or join different molecules into new ones. These recombination processes may create new molecular structures and novel ORFs, including CMS genes (95). In addition, plant mitochondrial genomes are subject to frequent invasions of DNA sequences from chloroplast and nuclear genomes as well as SUOs (46). In fact, most identified CMS genes contain SUOs, in addition to those from mitochondrial genes of known functions or putative ORFs (**Figure 2**). For example, in the CMS-WA mitochondrial genome, a 15.7-kb rearranged region related to the origin of *WA352* contains a chromosomal sequence, four mitochondrial sequences, and two SUOs; the new ORF *WA352* consists of three mitochondrial sequences and one SUO (91) (**Figure 2**). Notably, the major part of *WA352*, encoding the COX11-interacting domains, was derived from *orf288*, which is not expressed in anthers (91). Therefore, DNA sequences that originally do not function in anthers can be the major donor sequences for the formation of new active CMS genes.

The creation and establishment of active CMS genes during evolution involve several steps. First, DNA fragments of some mitochondrial genes and other resources integrate into the mitochondrial genomic sites through multiple recombination events. Second, the newly created ORFs may possess promoter-5′ ORF sequences derived from other mitochondrial genes, or may land next to local sequences with promoter activity or locate downstream of preexisting active genes. Consequently, the new genes may be expressed independently, be cotranscribed with upstream genes, or both. For instance, *WA352* is located downstream of *rpl5* and contains a promoter-5′ ORF sequence derived from the putative mitochondrial gene *orf284*. *WA352* is transcribed as three transcripts: one that is cotranscribed with *rpl5* and two that are independently expressed (91). However, the initially created CMS potential genes may be present in low copy numbers, in a substoichiometric manner, and thus have only low expression levels, preventing expression of CMS phenotypes even in the absence of *Rf* genes (46). This may prevent the rapid elimination of these genes by negative selection. During evolution, when substoichiometric shifting occurs that increases the copy numbers of the recombinant mitochondrial DNA molecules, the CMS potential genes are normally expressed, finally becoming truly active CMS genes. This mechanism of CMS gene origination occurs in the common bean CMS-Sprite-associated *PVS* region; the *PVS*-containing sequence is present at substoichiometric levels (low copy numbers) in most (~90%) undomesticated *Phaseolus vulgaris* lines but is present at high copy numbers in other lines (3). Similar substoichiometric variations have also been observed in the *WA352*-related sequences in wild rice (H. Tang & Y.-G. Liu, unpublished data).

More than half of the identified *Rf* genes encode PPR proteins. The PPR gene family has expanded in land plant species, and the members tend to exist as tandem gene clusters (19, 121). Chromosome 10 in rice has a cluster of nine PPR genes and includes at least three *Rf* genes: *Rf1a* (*Rf5*) and *Rf1b* for CMS-BT (*Rf5* for CMS-HL) and likely *Rf4* for CMS-WA (2, 33, 51, 72, 136, 149). The *Rfo* locus for CMS-Ogu also has three highly similar PPR-encoding genes (10, 22). In some PPR gene clusters, such as that containing *Rf1a* and that containing *Rfo*, more copies of the PPR genes are present in restorer lines than in nonrestorer lines, suggesting that some *Rf* genes originated recently through duplication events (48, 136). The generation of PPR and diverse other types of *Rf* genes demonstrates that plants have evolved complex pathways to counteract the effects of CMS. The evolved CMS/*Rf*-based gynodioecy, a gender dimorphic genetic system, promotes outcrossing, increases genetic diversity, and provides adaptive flexibility of plant populations to increase evolutionary fitness in changing environments (20, 93).

Some *Rf* genes may have functions in addition to their roles in fertility restoration. For example, rice RF1A promotes the editing of *atp6* mRNA (136), and maize RF2 has an aldehyde dehydrogenase activity required for male fertility in plants with normal cytoplasm (86). In *Brassica*, the *Rf* genes *Rfp*, *Mmt*, and *Rfn* for CMS-Pol and CMS-Nap are divergent alleles or haplotypes of the

same locus (9). The *Rfp*-encoded product, RFP, modifies the CMS-Pol-related *orf224-atp6* (99) and processes CMS-independent mitochondrial transcripts such as those of *nad4* and *ccl1*, strongly suggesting that the endonucleolytic cleavage activity on these transcripts is the original function of RFP, apart from its role as a restorer (9, 126). Therefore, the generation of *Rf* genes is thought to result from coevolution with CMS through origination of new genes or functional divergence of existing genes. This situation is similar to the coevolution of avirulence genes in pathogens and resistance genes in plant immune systems (13).

Whether some CMS genes have additional biological functions apart from CMS or some of them just have negative side effects is also an interesting question. For example, URF13 serves as a receptor for the T toxin of fungal pathogens, and the interaction between URF13 and this toxin causes the formation of hydrophilic pores in the mitochondrial inner membrane and disrupts the proton gradient, leading to cell death and pathogenesis in CMS-T maize (81). ORFH79 also affects root growth in CMS-HL rice seedlings (113).

Mitochondrion-sorting genes (MSGs): nuclear genes encoding proteins containing a mitochondrion-sorting signal that target to mitochondria for biological functions

Cytoplasmic Male Sterility/Restorer Systems Involve Multiple Layers of Mitochondrial-Nuclear Gene Interaction

CMS phenotypes generally result from incompatible interactions between the mitochondrial and nuclear genomes in divergent plant populations. The layers of interaction between the mitochondrial and nuclear genes involve the spatial and temporal regulation of CMS proteins, CMS occurrence, and CMS restoration (**Figure 3**). For example, nuclear mitochondrion-sorting gene (MSG)–encoded proteins target to mitochondria and interact with the CMS proteins to regulate the anther-specific accumulation of most, if not all, CMS proteins. However, so far, only one nucleus-encoded mitochondrial protease, LON, has been identified as involved in the anther-specific accumulation of a CMS protein (120).

Whether CMS induction, like CMS restoration, involves direct interaction between CMS genes and nucleus-encoded factors was unclear for a long time (40). Examinations of the interaction between WA352 and COX11 in CMS-WA (91) and the interaction between ORFH79 and P61 in CMS-HL (135) revealed that this layer of mitochondrial-nuclear gene interaction comprises the key processes for CMS occurrence, which reflects the incompatibility between the cytoplasmic and nuclear genomes. The subunits of the mtETC complexes are critical CMS protein interactors in mediating the release of retrograde signals such as ROS and cytochrome *c* to trigger abnormal PCD in tapetal cells or microspores, leading to sporophytic or gametophytic CMS (**Figure 3**).

Another layer of mitochondrial-nuclear gene interaction includes processes for CMS restoration. In these processes, RF proteins are targeted to mitochondria and interact with CMS genes to restore fertility by suppressing the expression of CMS genes or eliminating the detrimental effect of the CMS proteins via different mechanisms at the genomic DNA, RNA, protein, or metabolic level (**Figure 3**).

In addition to the retrograde signaling that induces abnormal tapetal and microspore PCD, recent studies have uncovered distinct retrograde regulation of CMS occurrence and restoration, such as in the carrot CMS-Petaloid and rice CMS-CW systems (35, 36, 85). Little is known about mitochondrial retrograde regulation in plants, and the question of whether ROS and certain unknown mitochondrial noncoding RNAs may serve as retrograde signals, as implicated by those identified in plastids (50), remains to be investigated.

Noncoding RNAs Emerge as Players in Plant Male Sterility

Noncoding RNAs represent a group of widely distributed transcripts that are not translated into proteins but serve as epigenetic regulatory factors at the DNA or RNA level to mediate several

biological processes (42). Identification of the gene responsible for PGMS and TGMS in rice suggests that lncRNAs or small RNAs may be important players in regulating male development in response to environmental cues (26, 151).

Challenges and Opportunities in the Study and Application of Male Sterility and Fertility Restoration

Many CMS-associated ORFs and some of the corresponding *Rf* genes have been identified and characterized, and these studies have provided some molecular insights into plant mitochondrial signaling pathways. However, for most CMS systems, the interactors of CMS proteins and RF proteins and the mitochondrial retrograde signals have not been identified. In addition, the mechanisms underlying the regulation of the male-specific accumulation of CMS proteins and induction of CMS remain unclear.

Recent development of new technologies may help resolve current challenges in the study of CMS/*Rf* systems. High-throughput next-generation sequencing enables whole-genome sequencing in more crops with large and complicated genomes, such as sorghum, wheat, maize, and soybean, which will accelerate the identification of *Rf* genes in crop CMS systems. Determining the complete mitochondrial genome sequences of CMS lines through high-throughput sequencing also provides an effective way to search for novel CMS candidate ORFs, as exemplified in rice and radish (34, 52, 112). When more genomes are sequenced, the black box of the SUOs may be exposed, thus adding valuable information to our understanding of the evolution of CMS/*Rf* systems. In addition, recent implementation of transcription activator-like effector nuclease (TALEN) technology in plants (150) may provide a powerful tool to knock out target mitochondrial ORFs, offering a new strategy to validate the functions of CMS-associated ORFs and to restore CMS at the genomic level.

Isolation of genes in PGMS and TGMS genetic resources provides important clues for understanding EGMS traits regulated by novel mechanisms such as noncoding RNA or alternative splicing. Further identification of the target genes of the noncoding RNA regulators and the study of their interaction with environmental factors as well as their biological functions will uncover the molecular mechanisms underlying the control of EGMS.

CONCLUDING REMARKS

Although our understanding of the molecular mechanisms of CMS/*Rf* systems in rice and *Brassica* has advanced tremendously, CMS/*Rf* research in some major cereal crops, such as wheat, sorghum, and corn, has remained stagnant in recent years. More CMS systems in staple crops need to be studied and applied in agriculture to avoid genetic vulnerability in hybrid crop production that relies on a few CMS cytoplasms, such as CMS-WA in rice, CMS-Owen in sugar beet, and CMS-Peterson in pepper. Scientists and breeders need to pay more attention to research on EGMS because of its great potential and advantages in hybrid seed production to meet the increasing demand for food.

SUMMARY POINTS

1. Most cytoplasmic male sterility (CMS)–associated genes are chimeric, consisting of multiple fragments with different origins, and most of them encode membrane proteins.
2. CMS genes are transcribed constitutively, but most CMS proteins are produced mainly in male organs.

3. CMS proteins may interact with nucleus-encoded mitochondrial factors to induce abnormal programmed cell death in tapeta and microspores, leading to male sterility.
4. CMS restoration can be achieved at the genomic, posttranscriptional, posttranslational, or metabolic level.
5. Many restorer genes encode pentatricopeptide repeat (PPR) proteins, which target to the mitochondria and suppress the expression of CMS genes through cleavage or degradation of the CMS transcripts.
6. Noncoding RNAs and epigenetic control are involved in environment-sensitive genic male sterility (EGMS) traits.

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