Exploiting sterility and fertility variation in cytoplasmic male sterile vegetable crops

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Abstract

Cytoplasmic male sterility (CMS) has long been used to economically produce hybrids that harness growth vigor through heterosis. Yet, how CMS systems operate within commercially viable seed production strategies in various economically important vegetable crops, and their underlying molecular mechanisms, are often overlooked details that could expand the utility of CMS as a cost-effective and stable system. We provide here an update on the nature of cytoplasmic–nuclear interplay for pollen sterility and fertility transitions in vegetable crops, based on the discovery of components of nuclear fertility restoration and reversion determinants. Within plant CMS systems, pollen fertility can be rescued by the introduction of nuclear fertility restorer genes (Rfs), which operate by varied mechanisms to countermand the sterility phenotype. By understanding these systems, it is now becoming feasible to achieve fertility restoration with Rfs designed for programmable CMS-associated open reading frames (ORFs). Likewise, new opportunities exist for targeted disruption of CMS-associated ORFs by mito-TALENs in crops where natural Rfs have not been readily identified, providing an alternative approach to recovering fertility of cytoplasmic male sterile lines in crops. Recent findings show that facultative gynodioecy, as a reproductive strategy, can coordinate the sterility and fertility transition in response to environmental cues and/or metabolic signals that reflect ecological conditions of reproductive isolation. This information is important to devising future systems that are more inherently stable.

An overview

Cytoplasmic male sterility (CMS) is a maternally inherited phenomenon that prevents the production of functional pollen by virtue of mitochondrial dysfunction. The phenomenon has been documented in at least 150 plant species [1–3], comprising one of the very few systems of nuclear–mitochondrial interaction amenable to detailed study in plants. CMS systems have served as models in the study of male gamete development, fertilization, and cytoplasmic effects on inheritance. The CMS system, a three-component assembly of male-sterile mutant, fertility restorer line, and sterility maintainer line, has had important practical significance for agriculture by facilitating the economical exploitation of heterosis as a breeding advancement in several important crops [4–6].

CMS is associated with the expression of dominant mitochondrial mutations that arise by recombination-mediated rearrangement of mitochondrial genomic sequences to produce novel ORFs [1, 7, 8]. Mitochondrial expression of these CMS-associated ORFs induces pollen disruption, revealing the biological function of this set of unique mitochondrial proteins while informing alternative strategies to create new CMS germplasm [1, 10]. The mitochondrial ORFs are thought to form and evolve via a “multi-recombination/protoplast formation/function-alization” mechanism that involves gradual variation in the structure, sequence, copy number, and function [11]. In most cases, nuclear-encoded pentatricopeptide repeat (PPR) proteins, comprising an enormous family of plant proteins with RNA and protein binding affinities [12], can act as fertility restorer (Rf) genes to alter or suppress the expression of the CMS-associated ORFs at post-transcriptional or post-translational levels [1, 7].

In many CMS systems, spontaneous fertility reversion can occur, generally at a relatively low frequency (ranging from 1% in common bean to less than 0.0051% in mustard greens) that is influenced by genetic background. Reversion has been reported in carrot [13], common bean [14], mustard greens [15] as well as in monocot crops, such as rice [16], maize [17], and pearl millet [18]. The progress of dissecting the molecular mechanism of CMS has similarly informed a model
for spontaneous reversion in the system. In plants, mitochondria and chloroplasts act as semi-autonomous organelles, encoding limited genetic information and, in the case of the mitochondrial genome, ectopic and aberrant recombination is unusually prevalent as a consequence of incoordinate nuclear-mitochondrial properties [19]. Evidence suggests that differential mitochondrial recombination and replication activity, influencing plant male sterility and fertility transition, may be under nuclear control [17, 20, 21]. For example, the nuclear gene MutS HOMOLOG 1 (MSH1) is a suppressor of mitochondrial ectopic recombination, influencing the frequency of mitochondrial genome substoichiometric shifting activity that can induce male sterility in crops [15, 22, 23]. Altering expression of MSH1, or nuclear background more generally, in a male sterile line can produce conditions for fertility reversion [18, 24, 25].

The ability to self-transition from male sterility to pollen fertility, allowing for both obligate outcrossing and hermaphroditic reproductive strategies, represents an evolutionarily important process of plant adaptation to dynamic environmental changes that accompany seed/spore dispersal [26, 27]. We review recent advances in research on the molecular basis of cytoplasmic male sterility in crops, with particular focus on vegetable crops, and the various sterility and fertility transition mechanisms. We also consider future directions to target nuclear-encoded Rf systems, exploit the versatile protein MSH1, and incorporate direct editing of CMS-associated mitochondrial ORFs.

Utilization of CMS-derived hybrid heterosis in vegetable crops

Operationally, CMS-based hybrid seed technology generally relies on a three-component system, requiring a stable CMS line, a sterility maintainer line that shares isogenic nuclear background with the CMS line, and a fertility restorer line that contains the Rf gene(s) on a genetic background that is heterotic to that of the CMS line (Fig. 1a). The CMS line is distinguished by a male sterile cytoplasm conditioned by a mitochondrially encoded, CMS-associated ORF that is expressed in the absence of a nuclear fertility restorer gene. The maintainer line is isogenic to the CMS line but with normal fertile cytoplasm to permit its use as a pollen donor to the CMS line without impacting sterility expression. The restorer line contains a dominant functional Rf nuclear genetic factor within a genetic background that is selected for heterotic influence on F1 progeny. In derived F1 plants, the Rf gene restores male fertility, and the combination of CMS and restorer line nuclear genotypes gives rise to hybrid vigor. In most vegetable crops that take advantage of vegetative organs as their economic yield, the F1 hybrid
plant can derive from crossing a CMS line and a heterotic maintainer (non-restorer) line, without the need for seed production or a restorer system. Consequently, only a few fertility restorer lines have been developed for the hybrid breeding pipeline in these crops (Fig. 1a).

CMS hybrid seed production systems are employed effectively for a number of vegetable crop species, including radish [28], cabbage [29], mustard greens [30, 31], chili pepper [32], onion [33], carrot [34], and others (Table 1), with some plant families remarkably tractable for cytoplasmic-nuclear breeding manipulations. The family Brassicaceae contains a large number of vegetable crops adapted to CMS induction, with the Ogura CMS cytoplasm the first identification from Japanese radish in 1968 [35]. The system was not well utilized until its introduction into Brassica napus through intergeneric hybridization and recurrent backcrossing [36]. Plants with the Ogura cytoplasm can exhibit undesirable chlorotic leaves at low temperatures [37] so, to overcome the chlorosis, protoplast fusion was used to introduce normal chloroplast function to B. napus [38, 39] and Brassica juncea [40, 41] with Ogura-derived CMS. An improved, stable Ogura cytoplasm was later introduced into other Brassica vegetable crops like Brassica oleracea and Brassica rapa by intergeneric hybridization and recurrent backcrossing [36, 42] and is now used worldwide in hybrid production. In radish, two additional CMS types, Kosena and DCGMS, were identified [43, 44] and in the vegetable B. juncea, the three CMS types ORF220, Oxa, and Hau are used successfully for hybrid production [30, 31]. As additional evidence of the cytoplasmic-nuclear plasticity of this family, cytoplasms from other Brassicaceae crops have also been successfully transferred into B. rapa, B. juncea, and B. oleracea to induce alloplasmic CMS plants [36].

Mitochondrial-encoded CMS-associated ORFs cause male sterility

CMS mitochondrial mutations can arise de novo by spontaneous aberrant recombination events in the mitochondrial genome or by hybridization strategies designed to create nuclear-mitochondrial incompatibility, including recurrent sexual interspecific or intergeneric backcrosses and somatic cell fusions (Fig. 1a).

Numerous studies have shown an association of the CMS trait with novel chimeric ORFs that are generated from rearrangement and intragenic recombination activity in the mitochondrial genome [1, 7, 10]. The CMS-associated ORFs generally contain portions of known mitochondrial genes and are either fused with a mitochondrial promoter directly or located upstream or downstream of a functional mitochondrial gene. Several reported CMS DNA sequences encode small transmembrane proteins, such as ORF138 from radish, that are suspected to be cytotoxic [51, 71]. These CMS proteins may interact with cognate mitochondrial protein complexes, such as ATP8, causing dysfunction by impairing respiration and ATP production [70]. Most CMS proteins are identified by a detailed comparison of mitochondrial transcriptome or proteome data from CMS and corresponding maintainer or fertility-restored lines, including ORF125 and ORF463 in Kosena and DCGMS radish [46, 48], ORF220 and ORF288 in B. juncea [30, 72], ORF239 in common bean [53], ORF456 and ORF507 from pepper [36, 55], ORFB from carrot [34], ORF132 and ORF128 in eggplant [58], and ORF752 in onion [64] (Table 1).

Verifying CMS gene candidates requires testing their effect on pollen development. With the aid of restorer lines, differential expression of CMS-associated ORFs and their functional relevance to the male sterility phenotype can be ascertained. Alternatively, a recombinant

Table 1. Examples of CMS types, associated ORFs and corresponding Rfs in vegetable crops

| Species                        | CMS type         | Associated ORFs | Rfs  | Refs |
|--------------------------------|------------------|-----------------|------|------|
| Radish (Raphanus sativus)      | Ogura<sup>a</sup> | ORF138          | Rfo  | [28, 45] |
|                                | Kosena           | ORF125          | Rfk1 | [46, 47] |
|                                | DCGMS            | ORF463          | Rfd  | [44, 48, 49] |
| Mustard (Brassica juncea)      | ORF220           | -               | -    | -    |
|                                | Oxa              | -               | -    | [31] |
|                                | Hau              | ORF288          | Rfh  | [51, 52] |
| Common bean (Phaseolus vulgaris) | Sprite          | ORF259          | -    | [10, 53] |
| Faba bean (Vicia faba)         | CMS199/CMS297    | -               | -    | [54] |
| Pepper (Capsicum annum)        | Peterson<sup>a</sup> | ORF456/ORS07    | CaPR6 | [32, 55–57] |
| Carrot (Daucus carota)         | Petaloid<sup>a</sup> | ORFB            | -    | [34] |
| Eggplant (Solanum melongena)   | Anther indesincent | ORF132          | -    | [59] |
|                                | Non-pollen       | ORF218          | -    | -    |
| Sugar beet (Beta vulgaris)     | Owenn            | preSatp6        | Rf1, Rf2 | [59–61] |
|                                | 1-2CMS (R)       | ORF129          | -    | [60] |
|                                | G                | ox2             | RfG1 | [62, 63] |
| Onion (Allium cepa)            | S<sup>a</sup>    | ORF725          | Ms   | [64–67] |
|                                | T                | -               | -    | [66] |
| Welsh onion (Allium fistulosum) | S                | atpA            | -    | [67] |
| Chives (Allium schoenoprasum)  | S                | ORF501          | -    | [68, 69] |

CMS: cytoplasmic male sterility; ORF: open reading frame; Rf: restoration of fertility gene. *Used widely and commercially.
construct of the CMS-associated ORF fused with a mitochondrial targeting presequence can be tested for the ability to induce male sterility, verifying CMS function of an ORF in common bean [10], Brassica juncea [9], and other crops [1].

Natural generation of new mitochondrial CMS-associated ORFs in plants can facilitate population outcrossing and enhance fitness, but the induction and origin of mitochondrial CMS-associated ORFs remain largely unknown. In WA rice, mitochondrial genomic recombinant structures were associated with the formation of ORF352 [11]. ORF352 appears to have been generated by recombination of ORF284 from Oryza rufipogon and unknown sequences from another Oryza species. These sequence variations may have experienced purifying selection during evolution, leading to the expression of the recombinant forms in patterns that influence male sterility specifically. The case of ORF220 formation in B. juncea, involving a truncated mitochondrial functional gene and fragments of mitochondrial genomic sequences that recombined to create a new open reading frame, represents what appears to be the most common CMS ORF formation paradigm (Fig. 2a). In general, new CMS genes are assumed to have formed within a lineage via mitochondrial genomic DNA duplication, multirecombination, formation of protogenes, and further functionalization via gradual diversification of structure, sequence, and copy number [11]. Because mitochondrial genomes are multi-partite in structure [73], variation in subgenomic stoichiometries within the genome permits the suppressed retention of mitochondrial genetic variation at substoichiometric levels [74].

The mechanism by which CMS-associated ORFs cause male sterility is not fully understood but varies in different systems. In some, it appears that the sterility-associated protein is only allowed to accumulate in reproductive tissues, but this is not always the case [7]. It is possible in some cases that the sterility-associated protein has little influence on mitochondria until mitochondrial biogenesis and respiration activity are enhanced during pollen development [75, 76]. As multi-partite, partially redundant, and recombinogenic structures, plant mitochondrial genomes are dynamic, with frequent variations detected in structure and copy number of mitochondrial DNA molecules within a species [73]. Spontaneous reversion to fertility occurs at variable frequencies, involving substoichiometric shifting to alter the relative copy number of the CMS-associated ORF; this behavior
Proteins have been identified [88], while 552 PPR domain-containing proteins have been predicted in the chili pepper (Capsicum annuum) genome [45, 87]. In CMS of radish [45, 87], the CMS-associated ORFs can also induce male sterility in fertile plants.

In CMS radish, a PPR-encoding allele was identified by its linkage to the RsRf3 fertility restorer gene. However, there exist three different alleles at this locus, RsRf3-5, which encodes a putative fertility restoring protein, and RsRf3-6 and RsRf3-7 as non-restoring alleles. Intriguingly, RsRf3-6 encodes the same protein as RsRf3-5 but possesses a different promoter region, and RsRf3-7 shares the identical promoter to RsRf3-5 but differs in the gene coding region. This observation points to a potential role of intragenic recombination in the creation of novel fertility restorer genes [90].

A more detailed study of the PPR-B restorer system in radish has permitted the modeling of a putative fertility restoration mechanism. Suppression of male sterility by the PPR-B (Rf0) fertility restorer appears to occur through the specific inhibition of mitochondrial ORF138 translation. This inhibition is thought to involve PPR-B protein binding to the ORF138 coding sequence, acting as a ribosome blocker to specifically impede translation elongation along the orf138 mRNA [91].

Numerous Rf searches have been focused on high-throughput sequencing. Candidate genes associated with fertility restoration of cytoplasmic male-sterility in onion (Allium cepa L.) were identified by a combination of bulked segregant analysis and RNA-seq. The gene PMS1, involved in DNA mismatch repair, was considered to be the best candidate for fertility restoration from this approach [92]. From transcriptome profiling of differentially expressed genes in the cytoplasmic male-sterile and fertility restored lines of pigeon pea, 34 fertility restorer candidate genes were identified, involving carbon metabolism, the tricarboxylic acid cycle, oxidative phosphorylation, and elimination of reactive oxygen species [93].

The variation in potential fertility restorer mechanisms in plants remains unclear. Yet, while each mitochondrial CMS-associated ORF appears unique and each Rf system may appear to act distinctly, what emerges from these studies is the integral role of mitochondrial ectopic recombination and the evolution of nuclear-encoded mitochondrial suppressor gene families like PPRs as fundamental to understanding the evolutionary strategies for facultative gynodioecy in plants. Although in most vegetable species derived from introgression, stable restorer lines have not yet been identified, the information that has emerged from the most detailed studies provides valuable insight into where to look and how to benefit from well-assembled genomic information.

### Fertility reversion and the nuclear-encoded MutS HOMOLOG1

Fertility reversion is a common natural phenomenon in some CMS systems, and a reversion event often produces

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**Figure 3.** Fertility restoration and fertility reversion mediated by Rfs and MSH1. (a) Fertility restoration by Rfs that target to the mRNAs or proteins of CMS-associated ORFs in the CMS line. (b) Fertility reversion by MSH1-mediating substoichiometric shifting of CMS-associated ORFs in the CMS line. Conversely, MSH1-mediating substoichiometric shifting of CMS-associated ORFs can also induce male sterility in fertile plants.

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Pollination by nuclear restorer of fertility

Cytoplasmic male sterility can be restored by nuclear restorer of fertility (Rf) genes. These Rf proteins are encoded in the nucleus and post-translationally imported to mitochondria to suppress the effects of the mitochondrial CMS mutation (Fig. 3a). The majority of Rf genes identified to date encode RNA-binding PPR proteins [81]. Rf genes can suppress gene products from CMS ORFs at the transcript level by blocking the CMS-associated transcript [82] or post-transcriptionally by RNA editing [83], mRNA splicing [84], or protein degradation [85]. Additional types of Rf genes have also been identified, such as in sugar beet (Beta vulgaris), where a post-translational interaction occurs between preSATP6, a non-PPR Rf, and BoORF20, a sterility-inducing ORF, to alter the higher-order structure of preSATP6 [86].

A number of fertility restorer systems have been intensively bred to accommodate vegetable crop production (Table 1). The Ogura cytoplasm was introduced into European radish and B. napus because no Rf genes were observed in the Japanese radish cultivar [36]. Rfo (also known as Rfk1), was later identified to encode a PPR protein (PPR-B) that binds to and suppresses the translation of ORF138 mRNA in CMS-Ogu of Brassica and CMS-Kos of radish [45, 87]. In B. napus, a total of 53 PPR proteins have been identified [88], while 552 PPR domain-containing proteins have been predicted in the chili pepper (Capsicum annuum) genome [89]. Because PPR gene families are large in plants, the mapping of all putative Rf candidates can be complicated, but the one-to-one relationship between an Rf and its target CMS ORF is feasible to demonstrate.

In CMS radish, a PPR-encoding allele was identified by its linkage to the RsRf3 fertility restorer gene. However, there exist three different alleles at this locus, RsRf3-5, which encodes a putative fertility restoring protein, and RsRf3-6 and RsRf3-7 as non-restoring alleles. Intriguingly, RsRf3-6 encodes the same protein as RsRf3-5 but possesses a different promoter region, and RsRf3-7 shares the identical promoter to RsRf3-5 but differs in the gene coding region. This observation points to a potential role of intragenic recombination in the creation of novel fertility restorer genes [90].

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a mixture of male sterile and fertile progeny. This mixed outcome reflects the timing of the reversion event relative to gametogenesis, such that an early event can influence both female and male gametes, while delayed timing influences pollen only. Fertile plants arising through reversion do not restore fertility in crosses to the CMS line, distinguishing this process from nuclear fertility restoration [15, 25, 79].

The reversion phenomenon has been characterized by de novo recombination and subgenomic copy number changes of the plant mitochondrial genome by substoichiometric shifting [94]. The process involves rapid and dramatic changes in the relative copy number of sub-portions of the mitochondrial genome, effectively suppressing the sterility-associated sequence in a single generation. Reversion frequency is influenced by nuclear genetic background [13, 17, 20, 21]. In vitro culture and specific somatic cell hybridization processes can also lead to fertility reversion in petunia and maize [17, 95–97]. In CMS B. juncea, low-frequency substoichiometric shifting of the CMS-associated mitochondrial gene ORF220 has been associated with spontaneous fertility reversion and the occurrence of male sterility [15].

Nuclear genes regulating mitochondrial genomic rearrangement have been identified by genetic studies in various species. In common bean, a single nuclear gene designated Fr was reported to influence substoichiometric shifting of the mitochondrial configuration encoding CMS-associated pos-orf239 by a process that appears very similar to spontaneous reversion [78]. In Arabidopsis, the nuclear gene CHLOROPLAST MUTATOR (CHM, aka MSH1) was shown to encode a protein that is targeted to both mitochondria and plastids and represents a plant counterpart to the yeast mitochondrial MutS homolog designated MSH1 [98, 99]. In plants, mutation or suppression of MSH1 gives rise to mitochondrial illegitimate recombination activity [95] as well as evidence of male sterility [20]. The MSH1 protein is highly conserved within the plant kingdom, with a GIY-YIG endonuclease domain [91, 20] and has been shown to participate in recombination surveillance to suppress mitochondrial ectopic recombination in tobacco [95]. Transgenic suppression of MSH1 has led to evidence of male sterility in tomato [21]. In CMS B. juncea, transgenic MSH1 suppression in a male fertile line mediates the copy number amplification of ORF220 and leads to male sterility, while RNAi suppression of MSH1 in a CMS line induces reversion to fertility and ORF220 copy number suppression [15, 24]. This process, while offering new insight into the dynamic nature of plant mitochondrial genomes, also may represent a potential method for CMS induction.

Other nuclear genes participating in mitochondrial recombination surveillance have been identified, including OSB1 (organellar single-strand DNA binding protein 1) and RECA3 (RECA HOMOLOG 3) [22, 100]. Similar to MSH1, OSB1 also encodes a protein targeted to mitochondria and plastids, but the gene is part of a family of plant-specific DNA binding proteins. Disruption of OSB1 leads to a change in mitochondrial genomic stoichiometry in Arabidopsis [100] but has not been investigated for its influence on CMS behavior. The plant gene RecA3 encodes a mitochondrial recombinase that participates in homologous recombination. RecA3 disruption leads to similar but not identical mitochondrial rearrangements to those in msh1 mutants, but the two genes appear to act in distinct but overlapping pathways [22].

In nature, spontaneous fertility reversion represents an alternative strategy to nuclear fertility restorer loci for counteracting mitochondrially encoded fertility [25, 79]. A male-sterile plant located in isolation will, during flowering, encounter no opportunity for cross-pollination. This non-pollination state leads to altered plant growth and MSH1 expression behavior, triggering spontaneous fertility reversion [22]. One hypothesis for MSH1 behavior during flowering is that the gene responds to local source-sink carbon flow, leading to MSH1 suppression in non-pollination conditions, to create conditions conducive for mitochondrial genomic change and fertility reversion, versus MSH1 amplified expression during successful pollination [22]. Evidence in CMS B. juncea and Arabidopsis suggest that MSH1 transcript levels do, in fact, decline under non-pollination and elevate in response to sucrose availability [24, 101]. These findings, if found to be supported generally, suggest that facultative gynodioecy as a reproductive strategy can incorporate environmentally responsive genes like MSH1 as an “on–off” switch for sterility and fertility transition under changing ecological conditions [24].

Conclusions and perspective

CMS represents an important genetic component of an economical hybrid seed production system, while simul-

![Diagram](https://academic.oup.com/hr/article-lookup/10.1093/hr/uhab039/6510196)
taneously offering important insight into the interaction and co-evolution of nucleus and mitochondrion. Whereas much of the impetus of early CMS research came from the value of heterosis to the agricultural enterprise, what has emerged has also provided insight into the natural workings of facultative gynodioecy as a reproductive strategy in plants.

The sterility and fertility transition and its stable regulation is vitally important to establishing a CMS breeding pipeline. Three strategies have been proposed here to engineer “on–off” switches for the sterility and fertility transition (Fig. 4). Recent attempts have been made to develop restorer lines using wide hybridization [102] and genetic transformation [103]. With the success of artificial PPR scaffold design for programmed RNA recognition [104, 105], it is now possible to contemplate RF design for targeting the mRNA or protein of CMS-associated ORFs.

With growing understanding of nuclear gene influence on recombination activity of the mitochondrial genome, this system may also present opportunities for novel CMS engineering. Three strategies have been proposed here to target the mRNA or protein of CMS-associated ORFs via mitoTALEN-mediated mitochondrial genome editing [106, 107] could similarly lead to the design and engineering of novel CMS-causing lesions.

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Author contributions
J.Y. conceived the outline of the review. J.Y., F.X., X.Y., N.Z., and Z.H. drafted the review. S.M. and M.F. provided revisions.

Data availability
The raw data that support the study are available from the corresponding author upon reasonable request.

Conflict of interest statement
The authors declared no conflict of interest.

Supplementary data
Supplementary data is available at Horticulture Research Journal online.

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