Chapter 9
Molecular Basis of Cytoplasmic Male Sterility

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Abstract Cytoplasmic male sterility (CMS), a maternally inherited trait failing to produce functional pollens, is an alternative approach for utilization of hybrid vigor and hybrid seed production in Crucifer crops. CMS and its fertility restoration lines are also good system to explore the interaction between nuclear and mitochondrial genomes. Several cytoplasmic male sterility-associated genes, namely mitochondrial novel open reading frames (*ORFs*), had been identified from different sources of CMS, some of which were confirmed to be associated with CMS phenotypes by transgenic experiments. There were many molecular events occurring at transcriptional and/or editing levels pertaining to energy-related mitochondrial genes. Here, we reviewed the current advances in the research of genetic and molecular basis of CMS in crucifer crops. Thus, the understandings of CMS could help us to recognize the vital role of mitochondria and the manipulation of organellar genetics in practical breeding programs.

Keywords Crucifer crop • Cytoplasmic male sterility • Fertility restoration • Mitochondrial rearrangement • Nuclear-cytoplasmic communication

9.1 Background

Cytoplasmic male sterility (CMS), as a maternally inherited trait that prevents the production of functional pollens, was first termed by Rhoades (Rhoades 1933), and is widely applied in hybrid breeding and currently, is observed in >150 plant species (Laser and Lersten 1972). It was firstly documented that mitochondrial genes

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S.K. Gupta (ed.), *Biotechnology of Crucifers*, DOI 10.1007/978-1-4614-7795-2_9,

contributed to CMS in terms of their molecular aspect in maize (Dewey et al. 1986). Generally, mutations in mitochondrial genes might induce severe defects in respiration and be lethal. Another kind of mitochondrial gene mutants, chimeric plants, containing wild-type mitochondria (Karpova et al. 2002 and refs therein), usually lead to CMS. In most cases, the CMS systems were developed by intra-specific, inter-specific or inter-generic crosses with alien cytoplasm from other species or genera or by natural mutation (Schnable and Wise 1998; Budar and Pelletier 2001). Most researches considered that the alloplasmic CMS usually affected reproductive development but not other developmental events, however, an increasing number of evidence proofed that some vegetative developments were also impacted in CMS (Leino et al. 2003; Liu et al. 2012; Yang et al. 2012).

To date, CMS has been associated with expressions of mitochondrial novel open reading frames (*ORFs*) that arise from rearrangements of mitochondrial genomes. Since the first case of mitochondrial CMS-associated *ORF, urf13*, was identified from T-maize (Dewey et al. 1987), many CMS-associated *ORFs* have been identified from CMS crops. Such *ORFs* are often located adjacent to genes encoding components of the ATPase complex, and co-transcribed with these genes (Hanson and Bentolila 2004 and refs therein). Fertility of CMS can be recovered by restorer genes encoded by nuclear genome (Schnable and Wise 1998). Coupled with the identification of restorer genes, the mechanism of fertility recovery and incongruity between nucleus and mitochondria has been revealed in CMS systems (Hanson and Bentolila 2004). Here, we reviewed some major progresses in the genetic and molecular basis of cytoplasmic male sterility in crucifer crops, mainly in *Brassica* and *Raphanns* of crucifer crops.

9.2 Identification of Mitochondrial Novel *ORFs* from CMS Crucifer Crops

In crucifer crops, CMS has been paid more attention in important economic crops, like *Brassica* and *Raphanns*. Several types of CMS lines were defined by different sources of sterile cytoplasm with distinct genetic features. These types of CMS include *pol* (Singh and Brown 1991) and *nap* (Brown and Mona 1998) CMS in *Brassica napus*, *orf220* (Zhang et al. 2003), *hau* (Wan et al. 2008), *berthautii* (Bhat et al. 2008), *moricandia* (Prakash et al. 1998), *erucoides* (Bhat et al. 2006), *siifolia* (Rao et al. 1994), *erucastrum* (Prakash et al. 2001), *oxyrrhina* (Prakash and Chopra 1990) and *tournefortii* (Pradhan et al. 1991) CMS in *Brassica juncea* and *Ogura* CMS in *Raphanns sativus* (Ogura 1968).

9.2.1 Cloning of Novel Mitochondrial CMS-Associated ORFs

Since the first case of mitochondrial CMS-associated *orf*, *urf13*, was identified from T-maize (Dewey et al. 1986, 1987), many reports were focused on the isolation of

Table 9.1 Examples of novel *ORFs* and mitochondrial genes involved in CMS crucifer crops

Plant species	*ORFs*	Mitochondrial genes	References
Raphanns sativus	*orf138*	*orf158*	Bonhomme et al. (1992)
Brassica nap	*orf222*	*atp8*, *nad5c*, *orf139*	L'Homme et al. (1997)
Brassica pol	*orf224*	*atp6*, *rps3*	Singh and Brown (1991), Handa and Nakajima (1992)
Brassica juncea	*orf263*	*atp6*	Landgren et al. (1996)
Brassica juncea	*orf220*	*atpA, nad2, atp9*	Zhang et al. (2003), Yang et al. (2009a, b, 2010)
Brassica juncea	*orf193*	*atp9*	Dieterich et al. (2003)
Brassica juncea	*orf288*	*atp6*	Wan et al. (2008)
Brassica juncea	*orf108*	*atpA*	Ashutosh et al. (2008), Kumar et al. (2012)

CMS-associated mitochondrial genes through comparisons of CMS and its maintainer fertile (MF) lines. In crucifer crops, several *ORFs* were isolated from CMS. These include *orf138* from CMS *Raphanns sativus* (Bonhomme et al. 1992), *orf222* from *nap* CMS of *Brassica napus* (L'Homme et al. 1997), *orf224* from *pol* CMS of *Brassica napus* (Singh and Brown 1991), *orf263* from *tournefortii Brassica juncea*, *orf220* from *orf220*-type *Brassica juncea* (Zhang et al. 2003), *orf193* from *tournefortii-Stiewe Brassica juncea*, *orf288* from *hau Brassica juncea* (Wan et al. 2008) and *orf108* from *moricandia Brassica juncea* (Ashutosh et al. 2008). Examples of such novel mitochondrial *ORFs* associated with CMS crucifer crops are listed in Table 9.1. Usually, such *ORFs* are located at the flanking ends of mitochondrial encoding genes and are co-transcribed with these genes (Hanson and Bentolila 2004). Furthermore, comprehensive *ORFs* and genomic structure of mitochondria are identified in CMS based on mitochondrial genome sequencing, which could be quite helpful for cloning more candidate CMS-associated *ORFs* (Chen et al. 2011).

9.2.2 *Functional Analysis of Mitochondrial CMS-Associated ORFs*

Common strategies to discover CMS-associated mitochondrial factors through comparative research have not been absolutely powerful. Sometimes, the differences between two cytoplasms might arise from evolutionary divergence of different mitochondrial genomes in alloplasmic cytoplasm. Likewise, it should be emphasized that some chimeric mitochondrial *ORFs* clearly do not exhibit CMS phenotypes in *Arabidopsis* (Marienfeld et al. 1997). Usually, there are two routine ways to prove the relationship between specific mitochondrial genes and CMS. One approach is to study the expression patterns of those genes under the

control of the restorer genes to compare their characterizations in CMS, maintainer fertile and restored lines (Desloire et al. 2003; Koizuka et al. 2003). Another approach is to directly validate their functions by transgenic engineering, although plant mitochondria could not be easily genetically manipulated. Only some CMS-associated *ORFs* were confirmed to functionally cause male sterility in crucifer crops, for instances, *ORF220* (Yang et al. 2010), *ORF108* (Kumar et al. 2012) and *ORF288* (Jing et al. 2012) in *Brassica juncea*. In those successful cases, mitochondrial targeted pre-sequence is often needed to be fused into forepart of *ORFs*, of which CMS-associated *ORFs* could be guided into mitochondria and cause CMS phenotype when expressed in nuclear genome (Yang et al. 2010; Kumar et al. 2012; Jing et al. 2012). Nevertheless, mitochondrial-targeted expression of *ORFs* failed to induce male sterility in some cases (Chaumont et al. 1995; Duroc et al. 2006; Wintz et al. 1995). The cause is probably due to the failure of sub-mitochondrial location (Duroc et al. 2006), the expression period of *ORFs* (Wintz et al. 1995) or the substoichiometric levels of *ORFs* (Chaumont et al. 1995) in transgenic plants. Thus, precise locations of alien CMS-associated *ORFs* and their adequate dose of gene expressions seem to be quite vital to pinpoint their functions.

9.2.3 Occurrence and Origination of Mitochondrial CMS-Associated ORFs

In many cases, CMS-associated genes were caused by rearrangements of mitochondrial genomes resulting in the birth of new *ORFs* composed of fragments of other mitochondrial respiratory-related genes or non-coding sequences (Schnable and Wise 1998; Hanson and Bentolila 2004). However, the exact mechanism of the origination of CMS-associated *ORFs* is still largely unknown in CMS. In plants, an unusual nature of mitochondrial genome undergoes genomic recombination (Mackenzie and Mclntosh 1999; Mackenzie 2005). This type of mitochondrial recombination appears to play a key role in plant mitochondrial genome evolution, generating novel mitotypes (Small et al. 1989), and also serves as a possible mechanism for fertility reversion (Fauron et al. 1995). Dramatic changes of mitochondrial DNA molecule stoichiomtries, a phenomenon termed substoichiometric shifting (SSS), often accompanies recombination in mitochondrial genome (Janska et al. 1998). Experimental evidence indicates that SSS of mitochondrial genome leads to male sterility and spontaneous reversion to fertility (Janska et al. 1998; Feng et al. 2009). One of the nuclear genes, *MSH1* (*MutS* Homolog 1), controls the mitochondrial genome recombination (Shedge et al. 2007). When *MSH1* gene is suppressed, it dramatically alters mitochondrial and plastid properties and plant response to environment, meanwhile, triggers developmental reprogramming (Xu et al. 2011, 2012).

9.3 Transcriptional Regulation of Mitochondrial Genes in CMS

Usually, chimeric *ORFs* are located at the flanking end of genes encoding subunits of mitochondrial complexes. Consequently, the expressions of those genes are altered in the CMS systems. Moreover, RNA editings of mitochondrial genes were observed to be changed in CMS compared with its MF line (Hanson and Bentolila 2004).

9.3.1 Mitochondrial Genes are Co-Transcribed with ORFs

So far, numerous mitochondrial rearrangement regions associated with the CMS phenotype have been identified indicating the striking manner and frequency of recombination events in mitochondrial genome. Most rearrangements on mitochondrial loci were focused on subunit genes of ATP synthesis such as ATP synthesis subunit 4, 6, 8 and 9 genes in CMS-associated loci (Schnable and Wise 1998; Hanson and Bentolila 2004). Some other subunit genes of the mitochondrial respiratory complexes were also displayed to be associated with CMS including NDAH complex in some CMS species. In crucifer crops, several co-transcribed CMS-associated *ORFs* and mitochondrial genes were observed from CMS. These include *orf138* co-transcription with *orf158* in CMS *Raphanns sativus* (Bonhomme et al. 1992), *orf222* co-transcription with *nad5c* in *nap* CMS *Brassica* napus (L'Homme et al. 1997), *orf224* co-transcription with *atp6* in *pol* CMS *Brassica napus* (Singh and Brown 1991), *orf263* co-transcription with *atp6* in *tournefortii* CMS *Brassica juncea* (Landgren et al. 1996), *orfB* co-transcription with *nad2* in *orf220*-type *Brassica juncea* (Yang et al. 2009a), *orf193* co-transcription with *atp9* in *tournefortii-Stiewe Brassica juncea* (Dieterich et al. 2003), *orf288* co-transcription with *atp6* in *hau* CMS *Brassica juncea* (Wan et al. 2008) and *orf108* co-transcription with *atpA* in CMS *Brassica juncea* (Ashutosh et al. 2008). Examples of co-transcribed CMS-associated *ORFs* and mitochondrial genes in crucifer crops are listed in Table 9.1. Actually, more evidences are needed to clarify whether the event of *ORFs* co-transcription with mitochondrial genes is causal or phenomenal factors in CMS.

9.3.2 RNA Editings of Mitochondrial Genes Are Altered in CMS

RNA editing, as a crucial post-transcriptional step for RNA processing in higher plant organelle, regulates most mitochondrial and chloroplast gene expression in plant (Maier et al. 1996). Through comparative study, alterations on RNA editing sites were observed in CMS crucifer crops. Three silent RNA editing sites were reported for mitochondrial *nad3* transcripts in the CMS line of carrot (Rurek 2001).

Temporal and spatial characteristics of RNA editing for *atp9* gene were found in *orf220*-type CMS *Brassica juncea* (Yang et al. 2007). Different RNA editing patterns of mitochondrial *nad3*/*rps12* gene were identified in CMS *Brassica oleracea* (Wang et al. 2007). When RNA editing is specifically altered in some types of CMS, several attempts were made to correlate RNA editings with the occurrence of CMS. Expression of an unedited mitochondrial *atp9* gene in a fertile line caused male sterile in *Arabidopsis*, of which unedited mitochondrial *atp9* gene led to mitochondrial dysfunction (Gomez-Casati et al. 2002). Obviously, CMS trait could be induced at the post-transcriptional level through RNA editings and this result points to an alternative approach for generating engineered male sterile plants.

9.4 Molecular Control of Nuclear-Cytoplasmic Communication

In plant cells, mitochondria and chloroplast are semi-autonomous organelles that encode partial genetic information, with the majority being derived and imported from the nucleus (Unseld et al. 1997). Wide inter-organellar communications among the three organelles, in which signals from nucleus to mitochondria and chloroplast is termed anterograde regulation and signals from mitochondria and chloroplast to nucleus is termed retrograde regulation correspondingly (Woodson and Chory 2008). Because of the nature of nuclear-cytoplasmic interaction, it is a good model of CMS to study the anterograde and retrograde regulation among the organelles. To date, two types of nuclear-cytoplasmic regulation pathway have been found in CMS including restorer gene in mediation of CMS-associated *ORFs* and mitochondrial retrograde regulation of nuclear genes involved in reproductive development.

9.4.1 Identification of Restorer Gene in Mediation of Nuclear Regulation of Mitochondria

Recovery of fertility mediated by nuclear restorer gene has been well described in terms of nuclear restorer genes which suppress the function of CMS-associated *ORFs* in the fertile restored line. The majority of nuclear restorer genes operate at post-transcriptional level, such as RNA editing, processing, and polyadenylation, acting by controlling copy numbers at the DNA level, post-translational modification of CMS-associated proteins and compensation of mitochondrial dysfunction at metabolic level (Hanson and Bentolila 2004). In crucifer crops, several restorer genes or loci had already been identified or mapped, including *Rfo* gene from *Ogura* CMS *Raphanus sativus* (Koizuka et al. 2003; Yasumoto et al. 2009), *Rfd1* loci from *DCGMS* CMS *Raphanus sativus* (Cho et al. 2012), *Rfp* loci from *pol* CMS *Brassica napus* (Formanová et al. 2010; Liu et al. 2012), *Rfk1* loci from *Ogu*-INRA *Brassica rapa* (Niemelä et al. 2012), *Rf* loci from *Moricandia* CMS *Brassica juncea*

Table 9.2 Examples of mapping or identification of restorer genes in CMS crucifer crops

Restorer gene	Encoded protein/ function	CMS type	References
Rfp	mapping	*pol Brassica napus*	Formanová et al. (2010), Liu et al. (2012)
Rfk1	mapping	*Ogu*-INRA *Brassica rapa*	Niemelä et al. (2012)
Rf	mapping	*Moricandia Brassica juncea*	Ashutosh et al. (2007)
Rfd1	mapping	*DCGMS Raphanus sativus*	Cho et al. (2012)
Rfo	PPR protein/RNA processing	*Ogura Raphanus sativus*	Koizuka et al. (2003), Yasumoto et al. (2009)

(Ashutosh et al. 2007). Examples of mapping or identification of restorer genes in CMS crucifer crops are listed in Table 9.2. The *Rfo* gene from *Ogura* CMS *Raphanus sativus* encodes a pentatricopeptide repeat (PPR) protein like most restorer genes from other CMS crops (Koizuka et al. 2003; Yasumoto et al. 2009; Hanson and Bentolila 2004).

PPR proteins constitute a large family, more than 400 members in plants, of which about 60 % were predicted to be targeted in mitochondria and involved in post-transcriptional processes (Lurin et al. 2004). PPR proteins had been suggested to function as sequence-specific adaptors for a variety of other RNA-associated proteins (Lurin et al. 2004), which were primarily and definitively supported by some experimental evidence (Wang et al. 2006b). Hence, PPR proteins were considered as probable candidates for molecules of nuclear-mitochondrial interactions with essential effectors in CMS systems.

9.4.2 *Identification of Candidate Nuclear Targeted Gene in Mitochondrial Retrograde Regulation*

Until now, there has been no evidence supporting the idea that mitochondrial genes are directly involved in floral organ development, microsporogenesis, or other reproductive development. We thought that all types of abnormal phenotypes in CMS should originate from alterations in the expression of nuclear gene signals regulated by mitochondria, which would lead indirectly, but specifically to male sterility. Indeed, two groups of nuclear genes had been reported to be potential target genes: genes involved in programmed cell death (PCD) and transcriptional factor genes needed for development of floral organs as well as pollen.

In tapetal cell degeneration inducing the male sterile type of CMS, PCD of tapetal cells were subsequently extended to other tissues of anthers and were shown to be activated by the partial release of cytochrome c from the mitochondria into the above cells (Balk and Leaver 2001). Studies on the homeotic-type of CMS clearly demonstrated the probable pathway for the effect of mitochondria on the expression of

specific nuclear homeotic genes for floral organ development. In higher plants, floral organ development has been intensively researched in dicotyledonary plants, especially in *Arabidopsis* and *Antirrhinum*, which are mainly controlled by the homeotic genes (Theissen 2001). One classical genetic model, the ABC model, in developmental biology, can explain and predict flower organ families based on three classes of nuclear homeotic genes, termed A, B, and C. Any alterations of transcription or mutation in these genes could lead to global variations in four whorl structures of a flower, of which a certain type of flower organ would be replaced by the another (Coen and Meyerowitz 1991). Interestingly, such dramatic variations in flower organ were observed in alloplasmic cytoplasmic male sterile (CMS) tobacco (Kofer et al. 1991), carrot (Linke et al. 1999), wheat (Murai and Tsunewaki 1993), *Brassica juncea* (Yang et al. 2005) and *Brassica napus* (Teixeira et al. 2005), which, in most cases, exhibited a complete conversion of stamens into other floral organs. In recent research, nuclear MADS-box transcriptional factor (TF) genes, *AGAMOUS* (*AG*), *APETALA3* (*AP3*), *PISTILLATA* (*PI*), *GLOBOSA*- and *DEFICIENS*-like genes, were found to be transcriptionally down-regulated in CMS carpelloid tobacco flowers (Zubko et al. 2001), CMS pistillody carrot (Linke et al. 2003), CMS *Brassica napus* (Teixeira et al. 2005), CMS wheat (Murai et al. 2002; Hama et al. 2004) and CMS *Brassica juncea* (Yang et al. 2008a). Insightful studies of microsporogensis in *Arabidopsis* reveals *SPOROCYTELESS* (*SPL*, also known as *NOZZLE*, *NZZ*) gene, encoding a novel nuclear protein related to MADS-box transcription factor, was required to promote the differentiation of the primary sporogenous cells and cells of the anther wall (Schiefthaler et al. 1999; Yang et al. 1999). The research about putative target genes of *AG* showed that the homeotic protein *AG* controlled microsporogenesis by regulation of the *SPL* gene in *Arabidopsis* (Ito et al. 2004). In cytoplasmic male sterile plants, pollen development was halted at a very early or late developmental stage depending on the CMS system (Hanson and Bentolila 2004). In CMS *Brassica juncea* with failure of microsporogenesis, the absent expression of *SPL* gene was considered as the failure of pollen development (Yang et al. 2008b).

In addition, other candidate nuclear genes manipulated by mitochondrial retrograde regulation were identified, including retrograde regulating of *CTR1* (a negative regulator of ethylene signaling pathway) gene in ethylene response and retrograde regulating of *RCE1* (Related to ubiquitin1-conjugating enzyme) gene in auxin response (Liu et al. 2012; Yang et al. 2012). All the above down-regulated nuclear TF or other genes in CMS abnormal reproductive and vegetative development allow us to hypothesize the pathway of the molecular mechanism of mitochondrial retrograde regulation in CMS.

9.5 Discussion and Perspectives

CMS provides a path to explore the role of mitochondria in vegetative and reproductive development and interactions between the mitochondria and nucleus, apart from its agronomic importance in hybrid production. Indeed, so many mitochondrial

CMS-associated causal factors have been identified to date. Likewise, some restorer genes in mediating of CMS-associated *ORFs* expression and potential nuclear targeted genes regulated by mitochondria have also been studied in some CMS types. Hence, mitochondrial genes could not directly operate on nuclear targeted genes, and thus there must be a signal pathway from mitochondria to nucleus inducing male sterility and affecting other traits. However, our understanding of CMS remains limited, in part because many of the genes involved is still not known including the genes of controlling mitochondrial recombination, and how their functions are controlled by nucleus/organelle and how to place anterograde or retrograde signaling. Moreover, from some breeders' personal communications, we were puzzled that they were unable to observe heterosis in some CMS crops, especially in vegetative growth. Thus, in certain CMS sources applied to crops with vegetative organs as economic trait, CMS could probably only contribute to the seed hybrid production, but not heterosis vigor in hybrids.

Indeed, when *MSH1* gene that controls organellar genomic recombination is suppressed, some research groups observed extremely similar phenotypes to CMS, including male sterility, alterations on phytohormone metabolism and others (Sandhu et al. 2007; Xu et al. 2011, 2012). However, most of the previous researches were mainly focused on genes dysfunction from mitochondria and their reversions by restorer genes in nucleus. The striking findings of the CMS-inducing function of *MSH1* gene provide us with a new window and shed light on further clarifying how nuclear genes cause mitochondrial recombination in anterograde regulation, and vice versa, how mitochondrial genes affect responsive nuclear genes expressions in retrograde regulation, ultimately leading to CMS occurrence.

Acknowledgements This work was supported by serial grants from the National Natural Science Foundation of China (30971994).

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