Chapter 12

Plant Mitochondrial Mutations

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Summary

The complex mitochondrial genomes of angiosperms tend to rearrange, leading to rapid structural evolution and to visible mutations. The observed mutations include those affecting growth and morphology, as well as male fertility. The abnormal growth mutations are usually associated with defects in essential mitochondrial genes. In contrast, cytoplasmic male sterility (CMS) usually results from the de novo expression of chimeric open reading

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R. Bock and V. Knoop (eds.), Genomics of Chloroplasts and Mitochondria, Advances in Photosynthesis and Respiration 35, pp. 267–291,
DOI 10.1007/978-94-007-2920-9_12,

frames (ORFs) in rearranged mitochondrial genomes. The expression of the CMS-chimeric ORFs can be modified by nuclear restorer-of-fertility (*Rf*) genes. Most of the *Rf* genes described to date are rapidly evolving members of a class of genes encoding pentatricopeptide repeat (PPR) proteins. Plants may also revert to fertility following mitochondrial DNA (mtDNA) rearrangements that disrupt the sterility-associated region. Alternatively, subgenomes containing a CMS-ORF may be lost or highly suppressed. In many cases, the mtDNA rearrangements that lead to phenotypic changes are mediated by events involving short or microhomologous repeats. In this chapter, we emphasize work on cytoplasmic male sterility, including cytoplasmic reversion to fertility and nuclear restoration of fertility.

I. Introduction

Plant mitochondrial genomes tend to be organizationally complex and diverse, as well as much larger than their animal counterparts (reviewed by Kubo and Newton 2008; Kitazaki and Kubo 2010). Because seed plant mitochondrial genomes are reviewed in depth in another chapter of this volume (Chap. 8), we will only highlight the features here that are most relevant to the topic of mitochondrial mutation.

Most of the DNA in angiosperm mitochondrial genomes is non-coding. These large genomes contain only between 50 and 60 genes, encoding a few components of the electron transfer chain, a few ribosomal proteins, the ribosomal RNAs, and many of the transfer RNAs (Kubo and Newton 2008). There is some variation as to which genes are present in the mitochondria versus the nucleus in different genera, especially those coding for tRNAs and ribosomal proteins. With some notable exceptions, e.g. the plant family Geraniaceae (Parkinson et al. 2005), the protein coding sequences themselves tend to be highly conserved, but the DNA that lies between the genes appears to be different in different genera. Even within a single genus, where large intergenic regions can have high sequence conservation, losses and gains of sequence (presence/absence variation) are commonly seen (Allen et al. 2007). There is also variation among those sequences which can act as promoters (Hazle and Bonen 2007). Indeed, among mitochondrial genotypes of a single species, there can be rearrangements that lead to promoter "swaps" between genes without any apparent deleterious effects.

Although most plant mitochondrial genomes can be mapped as single "master circles", they appear to exist as a set of subgenomes, maintained in a dynamic equilibrium (reviewed in Kubo and Newton 2008). The organizational complexity of plant mitochondrial genomes reflects a propensity to rearrange, resulting from a high level of recombination across repeated sequences (e.g. Palmer and Shields 1984; Allen et al. 2007; reviewed by Hanson and Folkerts 1992; Fauron et al. 1995). Frequent, reversible recombination between pairs of relatively large (>1 kb) repeats can result in alternative molecular forms of these dynamic genomes. Inversions result if repeats are in inverted orientation with respect to one another, and subgenomic circles result if the repeats are in direct orientation. Despite the high levels of recombination and the demonstrable presence of subgenomes, the size and overall

Abbreviations: *atp* – Gene encoding a subunit of ATPase; bp – Base pairs; CMS – Cytoplasmic male sterility; CMS-ORFs – ORFs usually chimeric, associated with CMS; *cox*Gene encoding a subunit of cytochrome oxidase; HR – Homologous recombination; kb – Kilobase; MDL – Maternal distorted leaf; Mmt – Modifier of mitochondrial transcripts; MSC – Paternally transmitted mosaic; mtDNA – Mitochondrial DNA; MSH1 – MutS homolog; *nad* – Gene encoding a subunit of the Complex I NADH dehydrogenase; NCS – Nonchromosomal stripe; ORF – Open reading frame; OSB1 – Organellar single-stranded DNA binding protein; PPR – Pentatricopeptide repeat; *Rf* – Restorer of fertility; RNAi – RNA interference; SSS – Substoichiometric shifting; TCM – Teosinte-cytoplasm miniature; TIRs – Terminal inverted repeats

organization of a specific mitochondrial genome tends to be inherited reproducibly over many generations in "normal" nuclear backgrounds (e.g. Oro et al. 1985). Nuclear genes are responsible for the relative stability of plant mitochondrial genomes (reviewed by Maréchal and Brisson 2010).

Within sequenced mitochondrial genotypes (mitotypes) of a single species, the copy number of individual, conserved genes has been shown to vary from 1 to 4 copies without any apparent deleterious effects of the difference in dosage (see Allen et al. 2007). This suggests that post-transcriptional processes, including assembly of multi-subunit complexes, may be critical for mitochondrial function. Furthermore, a rearrangement between "extra" copies of actual genes and other sequences can lead to the formation of chimeric open reading frames. Chimeric ORFs that include pieces of normal genes are quite common in plant mitochondrial genomes (Marienfeld et al. 1997; Clifton et al. 2004; Allen et al. 2007). The chimeric ORFs associated with CMS are expressed from nearby promoters and have 3′ segments that can stabilize transcripts. Several of the CMS-ORFs have been shown to pre-exist in natural populations.

II. Mitochondrial Rearrangements and Mutations

Although a few plant mitochondrial mutations have been reported to result from base substitutions (e.g. Ducos et al. 2001), most of the mutations studied in angiosperms have been shown to result from rearrangements. Plant mitochondrial mutations tend to be of two types: (1) gain-of-function, such as the acquisition or expression of a CMS-causing chimeric ORF; and (2) loss-of-function, which alters an essential gene. Both types of mutations may be generated via common pathways.

Low-frequency recombination events involving very short (<50 bp) microrepeats may be the first step in generating most plant mitochondrial mutations. Such repeats also appear to be important in generating the types of reorganization inferred to have occurred during the rapid evolution of plant mitochondrial genomes. The microhomology-mediated events are rare and, thus, not usually reversible (reviewed by Fauron et al. 1995). A new subgenome resulting from the rare event can recombine with a subgenome resulting from high-frequency recombination across a pair of large repeats to form a new "master genome". The new genome may carry a deletion of the region between one copy of the microrepeat and one of the large repeats (Small et al. 1989; Fauron et al. 1995). Indeed, data from the abnormal growth mutants are consistent with this model. For example, the NCS3 mutant genome has a short deletion between an actively recombining 11-kb repeat and a 12-bp microrepeat located within the intron of the *rps3* ribosomal protein gene (Hunt and Newton 1991).

Since there are many mitochondrial genomes per cell, a new mutation is not phenotypically detectable until it has accumulated and segregated away ("sorted out") from non-mutant mtDNA. The effects of the mutations are often first detectable as sectors on the plants. Thus, all mitochondrial mutations are expected to exist first at very low ("substoichiometric") levels. Their phenotypic consequences would be seen only after many rounds of replication and sorting out.

When essential mitochondrial genes are mutated, the mutations often remain heteroplasmic; i.e. plants carry both normal and mutant mitochondrial arrangements. Mitochondria carrying only the mutant mtDNA segregate somatically during development to cause sectors of abnormal growth on the plant (Newton and Coe 1986; Newton et al. 1990; Gu et al. 1993; reviewed by Kubo and Newton 2008). This class of mitochondrial mutations has similar effects to those described in yeast and animal systems, and includes the **n**on**c**hromosomal **s**tripe (NCS) mutations of maize (reviewed by Newton et al. 2004, 2009), the MSC (paternally transmitted **m**o**s**ai**c**) mutants of cucumber (Lilly et al. 2001; Bartoszewski et al. 2004) and certain *chm*-derived ("chloroplast mutator")

mutants of Arabidopsis (including **M**aternal **D**istorted **L**eaf, MDL; Sakamoto et al. 1996). Interestingly, in *Nicotiana sylvestris*, tissue-culture-derived deletions for the Complex I gene *nad7* survive as homoplasmic plants, but they grow poorly and are male sterile (Pla et al. 1995; Pineau et al. 2005). In maize, rare plants have been recovered that are homoplasmic for a partial deletion of the Complex I gene *nad4* (Marienfeld and Newton 1994); they are small, uniformly pale, male sterile, and set no seeds (Yamato and Newton 1999). In most cases, the maize NCS mutations cause kernel abortion when the mitochondria of the embryos are homoplasmic or near-homoplasmic (Gu et al. 1994; Baker and Newton 1995); thus, only kernels that contain some normal mitochondria together with the mutant mitochondria, will grow into plants.

Another type of well-studied de novo mutation is the spontaneous reversion to fertility of CMS plants, a phenomenon that has been especially well documented in maize and which depends upon nuclear background (reviewed in Sect. IV). The mutations causing CMS reversions appear to sort out rapidly and homoplasmic plants usually result. In nearly all cases, the maize CMS revertants result from independent rearrangements and do not appear to pre-exist within the maize CMS mitotypes.

Persistent, very low-level alternative arrangements of mitochondrial genomes or "sublimons" can be detected in most mitotypes, including those of maize (Small et al. 1987, 1989). The low copy-number molecules can become suddenly predominant, a process referred to as substoichiometric shifting (SSS). This has been shown to occur spontaneously and reversibly (Janska et al. 1998). Nonetheless, mutant alleles of nuclear genes that affect recombination and differential amplification of mitochondrial genomes can dramatically affect this process (reviewed in Sect. V).

III. Cytoplasmic Male Sterility

The most commonly studied type of mitochondrial rearrangement mutation in higher plants is CMS. CMS is a maternally inherited trait in which plants fail to produce functional pollen (reviewed by Hanson 1991; Chase 2007; Fujii and Toriyama 2008). CMS has been reported in a large number of plant species (Laser and Lersten 1972; Kaul 1988; reviewed by Hanson 1991; Schnable and Wise 1998; Chase 2007). It has long been exploited by plant breeders to produce hybrids that, in most crops, tend to be more vigorous and higher yielding than inbred lines (Havey 2004). In addition to maize (reviewed by Skibbe and Schnable 2005), CMS has also been observed and analyzed in many other crop plants, including *Brassica napus* (Singh and Brown 1993; L'Homme et al. 1997), chili pepper (Kim et al. 2007), common bean (Mackenzie 1991; Chase 1994), onion (Havey and Bark 1994), chives (Engelke and Tatlioglu 2002), sugar beet (Satoh et al. 2004), carrot (Linke et al. 2003), pearl millet (Burton 1977), radish (Iwabuchi et al. 1999), rice (Wang et al. 2006), rye (Tudzynski et al. 1986), sorghum (Bailey-Serres et al. 1986), sunflower (Horn and Friedt 1999), tobacco (Bonnett et al. 1991) and wheat (Song and Hedgcoth 1994a). CMS plants are also found among non-crop species, e.g., petunia (Boeshore et al. 1985), where they may be favored by natural selection (Delph et al. 2007).

Distinct stages in male organ development and pollen formation are affected in different CMS systems. Female fertility is not affected and the morphology of the plants is usually normal, although there may be alterations to flower morphology (reviewed by Zubko 2004; Linke and Börner 2005). Two examples are the petaloid-type of CMS (Kitagawa et al. 1994) and the 'carpeloid' type of CMS (Linke et al. 2003), both in carrot. Abnormal floral development is also observed in some alloplasmic CMS strains (see below). While CMS in these systems alters flower structure, programmed cell death or necrosis within anthers or pollen is associated with CMS in other plants (Warmke and Lee 1977; Balk and Leaver 2001; Wen et al. 2003; reviewed by Chase 2007).

The most extensively studied CMS systems to date are in *Brassica* (L'Homme and Brown 1993; Wang et al. 1995), petunia

(reviewed by Hanson et al. 1999), *Phaseolus* (Chase 1994; Sarria et al. 1998), maize (reviewed by Ward 1995; Gabay-Laughnan et al. 1995), and rice (Fujii et al. 2010). There are two main classes of CMS, one that appears to have arisen naturally in wild populations and a second resulting from intentional manipulation such as interspecific crosses or crosses between different populations of the same species. The latter is termed alloplasmic CMS. This sterility results from nuclear-cytoplasmic incompatibility (Kaul 1988). Alleles of certain nuclear genes, *restorers-of-fertility*, can suppress or override cytoplasmic male sterility (reviewed by Chase 2007). In alloplasmic CMS, restorers existing within a population that mask the existence of CMS may be removed by crossing programs (discussed below).

CMS enables breeders to produce commercial F1 hybrids on a large scale as it eliminates the need for hand emasculation (Schnable and Wise 1998; Havey 2004). In crops such as maize, where the F1 hybrid must be male fertile and produce seed, *restorer-of-fertility genes* can override the CMS. CMS-S and CMS-C maize are presently used but not CMS-T since it was shown to be particularly susceptible to *Bipolaris maydis* (Ward 1995; Schnable and Wise 1998). Whether a breeder prefers CMS-S or CMS-C depends on the stability of the sterility in the environment in which it is grown (Havey 2004) and the CMS-inbred line combination.

A. Naturally Occurring Male-Sterile Cytoplasm

The types of CMS that have arisen naturally are considered to result from a series of recombination events leading to rearrangements in the mtDNA, presumably in a progenitor species. The male sterility is often revealed when outcrossing removes a restorer allele (reviewed by Hanson 1991; Schnable and Wise 1998). A number of naturally occurring male-sterile cytoplasms have been discovered in this manner. For example, a CMS plant was found in a male-fertile line of *Phaseolus vulgaris*. It was later determined that the fertile progenitor line carried a restorer that concealed the male-sterile nature of the cytoplasm (Mackenzie 1991).

Among the best-characterized examples of naturally occurring male-sterile cytoplasms are those of maize (*Zea mays* ssp. *mays*) CMS-S and CMS-T. Five of the mitochondrial genomes of maize were sequenced and compared (Allen et al. 2007). It was determined that the S and T cytoplasms are the most divergent mitotypes. They are distinct from one another and also from the NA and NB male-fertile cytoplasms. These findings are in agreement with theory that the male-sterile S and T cytoplasms had their origin in fertile ancestors of maize. The maize CMS-S cytoplasm is found in some Latin American races of maize (Weissinger et al. 1983). It also appears identical to a cytotype found in some accessions of *Zea mays* ssp. *mexicana* teosinte (Weissinger et al. 1983; Doebley and Sisco 1989), although that strain appears to be male fertile (Allen 2005). The CMS-T cytotype has not been found in any teosinte accession but is seen in several accessions of Latin American maize (Weissinger et al. 1983).

In maize, no case of a spontaneous de novo mutation from male fertile to CMS has been confirmed, despite reports that exact matches to extant CMS-S and CMS-T arose from the fertile NB mitotype (the **n**ormal fertile genome first characterized in inbred line **B**37), each within a single generation (Lemke et al. 1985, 1988). The CMS mtDNAs are structurally very different from NB and each contains some unique DNA (Allen et al. 2007). It is highly improbable that either CMS type could completely replace the fertile mitotype in one generation (Lonsdale 1987; Small et al. 1987). An alternative explanation that substoichiometric shifting would cause the replacement events (Arrieta-Montiel et al. 2009) is also unlikely, because the CMS-ORFs have not been detected in the fertile NB cytoplasm (Liu et al. 2002). The data reported by Lemke et al. (1985, 1988) probably resulted from seed contamination or a sample mix-up.

B. Alloplasmic Male-Sterile Cytoplasm

Alloplasmic CMS may be caused by nuclear-cytoplasmic incompatibility when the nucleus of one species is combined with the cytoplasm of another (Kaul 1988). One of the best examples is found in sunflower (*Helianthus* sp.). The hybrid production of sunflower has always relied heavily on the PET1 system. The PET1 cytoplasm was derived from an interspecific cross between *H. petiolaris* and *H. annuus* (Horn 2002). Nine additional PET1-like CMS cytoplasms have been since been identified (Horn et al. 1996) and it is possible that this CMS exists at subliminally low levels in *H. annuus* (Horn and Friedt 1999). More recently, a new type of sunflower CMS was derived from an interspecific cross of an accession of *H. giganteus* and a cultivar of *H. annuus* (Feng and Jan 2008).

In wheat, cytoplasmic male sterility resulted from the cross of two male-fertile species. The male-sterile phenotype appears when plants carry *Triticum timopheevi* mitochondria in the *T. aestivum* nuclear background (Song and Hedgcoth 1994a, b). A chimeric ORF present in *T. timopheevi* mitochondria with a *T. timopheevi* nucleus either does not synthesize transcripts or the transcripts are unstable. However, in plants with *T. timopheevi* mitochondria and the *T. aestivum* nucleus, this ORF produces a stable protein product (Song and Hedgcoth 1994b).

In some instances, alloplasmic male sterility results in aberrant floral structures (reviewed by Carlsson et al. 2008). For example, pistillody (homeotic transformation of stamens into pistil-like structures) is observed in an alloplasmic line of wheat (Murai et al. 2002), and there is abnormal floral development in alloplasmic lines of male-sterile tobacco (Kofer et al. 1991; Bergman et al. 2000; Farbos et al. 2001) and *Mimulus* (Barr and Fishman 2011). For example, in CMS tobacco that carries the cytoplasm of *Nicotiana repanda* and the nuclear genome of *N. tabacum*, the petals are poorly pigmented, the stamens have shortened filaments, and the anthers are shriveled (Bergman et al. 2000; Farbos et al. 2001). CMS *Brassica napus* plants resulting from somatic hybrids between *Brassica napus* and *Arabidopsis thaliana* also exhibit an aberrant floral phenotype (Leino et al. 2003; Teixeira et al. 2005). Although the phenotype resembles those of homeotic mutants, the cause is the alien Arabidopsis mitotype. The expression of many of the *Brassica napus* nuclear genes, including the homolog of the homeotic *apetela3* gene, is altered, apparently due to aberrant retrograde signaling from the mitochondria (Carlsson et al. 2007).

C. Chimeric Open Reading Frames Associated with CMS

CMS is often associated with the expression of chimeric regions of mtDNA (reviewed by Chase and Gabay-Laughnan 2004; Newton et al. 2004; Hanson and Bentolila 2004; Linke and Börner 2005). These regions usually consist of pieces of known genes along with sequences of unknown function and are thought to be generated via repair of DNA breaks or an illegitimate recombination involving microhomologous repeats (usually ~5–<50 bp; reviewed by Maréchal and Brisson 2010). The chimeric ORFs may be fused to promoter sequences or co-transcribed with genes located upstream (reviewed by Chase and Gabay-Laughnan 2004; Hanson and Bentolila 2004; Newton et al. 2004; Fujii and Toriyama 2008). Chimeric regions associated with CMS have been described in many species including *Brassica* (L'Homme and Brown 1993; L'Homme et al. 1997), maize (Zabala et al. 1997), petunia (reviewed by Hanson et al. 1999), sorghum (Tang et al. 1996), and sunflower (Köhler et al. 1991; Laver et al. 1991; Spassova et al. 1994; Horn and Friedt 1999).

There are often so many rearrangements between a CMS mitochondrial genome and a related fertile genome that it can be a laborious effort to identify CMS-associated regions (reviewed by Hanson and Bentolila 2004). The set of candidate CMS-ORFs can be narrowed down to a very few by sequencing *multiple* fertile and CMS mtDNAs within a species and comparing their chimeric open

reading frames (e.g. Satoh et al. 2004; Allen et al. 2007; Fujii et al. 2010). A chimeric ORF that is unique to one CMS genotype can be considered a candidate CMS gene. Of course, tests must be done to confirm that the candidate gene actually causes the CMS phenotype (reviewed by Hanson and Bentolila 2004). These tests can include loss of the CMS phenotype when there is a mutation that alters or eliminates the candidate CMS-ORF. Another important criterion is whether the expression of the CMS candidate gene is changed in the presence of appropriate nuclear restorer alleles. Strangely, transgenic expression of the candidate CMS-ORF (tethered to a sequence directing protein import into plant mitochondria) from the nuclear genome does not always seem to be a straightforward functional test (Wintz et al. 1995).

One of the first systems in which the CMS chimeric ORF was identified is that in petunia (Boeshore et al. 1985). The CMS-associated gene was designated *pcf* for **p**etunia **C**MS-associated **f**used gene (reviewed by Hanson et al. 1999). It consists of the 5′ portion of the *atp9* gene, segments of the first and second exons of *cox2*, and a large region of unknown origin designated *urfS* (reviewed by Hanson et al. 1999). Multiple CMS-associated chimeric ORFs were identified in *Brassica* species, and were characterized in the *pol* (L'Homme and Brown 1993), *nap* (Dieterich et al. 2003) and *ogu* (Bonhomme et al. 1992) cytoplasms (reviewed by Schnable and Wise 1998; Hanson and Bentolila 2004).

Three common types of CMS have been identified in maize and are designated CMS-C, CMS-T and CMS-S (reviewed by Laughnan and Gabay-Laughnan 1983). The chimeric ORFs associated with CMS-T and CMS-S have been identified. However, no chimeric ORF unique to the CMS-C mitotype has been found even though the genome has been completely sequenced (Allen et al. 2007). In this case, CMS may result from rearrangements that exist 5′ to three essential genes (Dewey et al. 1991). Altered expression of one of them in the tapetal cells during pollen development may cause this type of CMS.

The CMS-T-associated chimeric gene of maize has been designated T-*urf13* (Dewey et al. 1987; Wise et al. 1987a). This ORF contains part of the 3′ flanking region of the mitochondrial 26S ribosomal RNA (*rrn26*) gene, a segment of unknown origin, and a sequence with homology to the coding region of *rrn26* (Dewey et al. 1986). In order for this chimeric gene to have arisen, multiple rearrangements were required (Dewey et al. 1986). Interestingly, this amalgamation of sequences is expressed at high levels, because it lies between a duplicate copy of the *atp6* promoter and the only copy of the essential *atp4* gene in the CMS-T genome (Dewey et al. 1986; Allen et al. 2007). Furthermore, it is translated into a 13-kD membrane protein that is expressed constitutively (Forde et al. 1978; Newton and Walbot 1985). The expression of *T-urf13* has little effect on overall plant growth, but it causes premature mitochondrial degradation in the tapetum during microspore biogenesis, and, therefore, early pollen abortion (Warmke and Lee 1977; reviewed by Levings 1993; Skibbe and Schnable 2005).

The CMS-S type of maize male sterility is correlated with the presence of a sequence, designated R, occurring within a 4,215-bp duplicated region of the mitochondrial genome (reviewed by Newton et al. 2009). This region contains two adjacent chimeric open reading frames, *orf355* and *orf77*. Sequences similar to a portion of the linear R1 plasmid are present in *orf355* along with sequences of unknown origin. *Orf77* contains three segments with similarity to the coding and flanking regions of *atp9*, as well as to sequences normally located 3′ to the *atp4* gene (Zabala et al. 1997; Allen et al. 2007). Two free linear plasmids designated S1 and S2 are also present within CMS-S mitochondria (Pring et al. 1977). 208-bp terminal inverted repeats (TIRs) are present at the ends of each S plasmid (Paillard et al. 1985; reviewed in Handa 2008). Recombination can occur between TIR sequences that precede *orf355/orf77* in the main mitochondrial genome and the TIRs at the ends of each S plasmid (Schardl et al. 1984). Transcription

of a 1.6-kb RNA initiates from the resulting linear ends of mtDNA (Gabay-Laughnan et al. 2009). The CMS phenotype is correlated with high levels of expression of this 1.6-kb transcript (Zabala et al. 1997; Wen and Chase 1999; Gabay-Laughnan et al. 2009).

IV. Cytoplasmic Reversion to Fertility

A. Reversion in CMS Maize

Field-grown CMS-S maize plants have given rise to cytoplasmic revertants on numerous occasions. These revertants first appear as sectors of male fertility on male-sterile tassels or as totally male-fertile tassels in plantings of CMS in inbred or hybrid backgrounds (Newton et al. 2009). The first reported cases arose in plants of the genotype CMS-S in the Wf9 inbred line (Jones 1956). Two exceptional male-fertile plants produced only male-fertile progeny when self-pollinated and crosses of these exceptions as pollen parents yielded only male-sterile progeny. These results indicated that a nuclear restorer gene was not involved. Male-fertile plants arising from male-sterile CMS-S plants in the M825 inbred line were later described (Singh and Laughnan 1972) and, again, nuclear restorer gene action was ruled out. It was proposed that the newly arisen male fertility "involved a change from male-sterile to male-fertile condition in the cytoplasm" (Singh and Laughnan 1972).

We now know that cytoplasmic reversion involves deletions or rearrangements of the CMS-associated sequences of the mtDNA. M825, and to a lesser extent Wf9, are the two most active inbred nuclear backgrounds associated with cytoplasmic fertility reversion of CMS-S in maize (reviewed by Gabay-Laughnan and Newton 2005). In lines that do not show "spontaneous" reversion events in the field, tissue culture may induce mtDNA changes leading to fertility in regenerated plants. Revertant plants regenerated from tissue cultures of CMS-S in the W182BN line (Earle et al. 1987) and also of CMS-T in the Wf9/A188 background (reviewed by Ward 1995) have been observed, but there are no reports of reversion to fertility in field-grown plants of these lines.

Numerous CMS-S cytoplasmic revertants have been isolated and analyzed. The types of events that cause reversion to fertility depend on nuclear background. For example, the S1 and S2 mitochondrial plasmids are always lost from M825, 38–11, H95 and WB182BN cytoplasmic revertants but are retained by all Wf9 revertants (Levings et al. 1980; Kemble and Mans 1983; Escote et al. 1985; Ishige et al. 1985; Schardl et al. 1985; Earle et al. 1987; Escote-Carlson et al. 1988; Small et al. 1988). In addition, various regions of the main mitochondrial genome are rearranged, depending again upon the nuclear background; not all cytoplasmic revertants exhibit the same rearrangements (Small et al. 1988). A comparison of revertants in the M825, 38–11 and W182BN nuclear backgrounds revealed that they differ in the organization of the integrated S1 and S2 sequences. This initially made it difficult to identify the mtDNA region involved with the CMS-S male sterility phenotype.

Comparison of the mtDNA of fertile revertants with that of the progenitor male-sterile strains helped identify the sterility-associated region of the CMS-S mtDNA (Zabala et al. 1997). All the cytoplasmic revertants, regardless of nuclear background, showed alterations in the *orf355-orf77* region of mtDNA; furthermore, the 1.6-kb RNA including *orf355* and *orf77* was missing in all the revertants (Zabala et al. 1997). As was described earlier, this 1.6-kb RNA is transcribed only from linear ends resulting from recombination between TIR sequences preceding *orf355* and the TIRs of the linear S-plasmids (Gabay-Laughnan et al. 2009). Since revertants in most nuclear backgrounds lose the S plasmids, they are unable to produce this transcript. In the case of Wf9 revertants, which retain S plasmids, the *orf355-orf77* region in the main mitochondrial genome is itself rearranged or deleted (Zabala et al. 1997).

Illegitimate recombination between microrepeats can lead to deletion of the CMS-associated regions. In one M825-type

revertant analyzed, the mtDNA sequences that recombined to produce the revertant were shown to contain 19 bp of sequence similarity (16/19 bp matched). One copy of this repeat was located on the S2 plasmid and the event led to the loss of the free S plasmids (Schardl et al. 1985). Without the S plasmids, there are no linear ends preceding the *orf355-orf77* region and no 1.6-kb sterility-associated RNA.

Microhomologies are also involved in the generation of the aberrant-growth NCS mutants of maize, which have deletions in essential mitochondrial genes. Intriguingly, the NCS4 mutation arose during the reversion to fertility of a CMS-S plant in the M825 line. Illegitimate recombination across a near-perfect repeat (15/16 bp) present in the S2 plasmid and the intron of the *rps3* ribosomal protein gene resulted in the loss of both the S plasmids and a portion of the *rps3* gene. Thus, a stunted but male-fertile plant was recovered following the same initiating microrepeat-mediated event (Newton et al. 1996).

In most cases of cytoplasmic reversion of CMS-S maize to male fertility, a unique mitochondrial mutation is associated with each revertant (Schardl et al. 1985; Small et al. 1988; Zabala et al. 1997). However, rare exceptions have been reported in closely related plants. Three sibling Wf9 cytoplasmic revertants were found within one family; two of them were observed as tassel sectors and the third as a totally fertile tassel (Gabay-Laughnan and Laughnan 1983). The possibility of a common origin existed even though sectors usually arise from independent mutations. These three sibling revertants were later determined to have the same mitochondrial mutation and, therefore, probably resulted from the same mutational event (Escote-Carlson et al. 1988). The direct male-sterile progenitor plant must have been heteroplasmic for both CMS-S and revertant mitochondria, which sorted out in the sibling plants.

Recent analyses of another set of three Wf9 cytoplasmic revertants revealed that they have an identical mtDNA rearrangement. In this case, however, the mutation sorted out in three successive generations. It is proposed that the revertant arose in the male-sterile progenitor strain. Subsequently, mutant mitochondria were transmitted to some of the progeny of this heteroplasmic plant, where they amplified to become the predominant mitotype. In all three revertants, the same inversion with a breakpoint between the TIR and *orf355* has been found (Matera et al. 2011). Interestingly, the *orf355-orf77* coding sequences and the TIR sequences remain intact in this inversion, and the free S-plasmids are also present; however, recombination between the displaced TIR and the S-plasmids no longer leads to a linear end 5′ to *orf355-orf77*. Without the TIR-terminating linear end, which contains the transcription start site (Gabay-Laughnan et al. 2009), the CMS-associated 1.6-kb RNA cannot be produced.

In contrast to CMS-S, no reversion event has ever been observed in field-grown maize plants carrying T cytoplasm. As was mentioned above, cytoplasmic reversion of CMS-T has been observed in plants regenerated from tissue cultures. The CMS-T-associated T-*urf13* gene has been deleted in all but one of the tissue-culture-induced revertants studied. In the exceptional revertant, there is a frame shift at codon 74 of the T-*urf13* region. This produces a truncated version of the TURF13 protein (Umbeck and Gengenbach 1983; Wise et al. 1987a, b). One of the "typical" CMS-T revertants was studied in detail, and it was shown that both inter- and intra-molecular recombination events were involved in its generation (Fauron et al. 1990). Some of the resulting subgenomic circles were subsequently eliminated, including the one carrying T-*urf13*.

B. Reversion in Common Bean

The male-sterile CMS-Sprite mitochondrial genome of the common bean, *Phaseolus vulgaris*, is comprised of three inter-recombining, redundant circular molecules, 394, 257 and 210 kb in size (Janska and Mackenzie 1993). The progenitor of this CMS cytoplasm maps as a single circular master chromosome.

This progenitor configuration is retained at substoichiometrically low levels in the CMS genome. Conversely, the three circular molecules characteristic of CMS are present at substoichiometrically low levels in the progenitor (Janska et al. 1998). The mtDNA region that is correlated with Sprite CMS consists of a unique sequence in the mitochondrial DNA designated the *pvs* (for P*haseolus* v*ulgaris* sterility) sequence and is carried on the 210-kb molecule. This region contains at least two novel ORFs, *pvs-orf98* and *pvs-orf239* (Johns et al. 1992; Chase and Ortega 1992; Janska et al. 1998), however, only *pvs-orf239* appears to be translated (Abad et al. 1995). In *P. vulgaris* cytoplasmic revertants, the 210-kb subgenomic circle, carrying *pvs*, is reduced to substoichiometric levels (Mackenzie et al. 1988; Janska and Mackenzie 1993). Since the two remaining circles carry all the essential mitochondrial genes, this reduction is tolerated (Janska and Mackenzie 1993). The progenitor mtDNA configuration, as well as the *pvs-orf239* CMS-associated sequences, are maintained at substoichiometric levels in the cytoplasmic revertants (Janska et al. 1998). Stoichiometric shifting of the levels of mtDNA molecules is proposed to account for both the appearance of sterility and the reversion to fertility of CMS-Sprite (Janska et al. 1998).

C. Reversion in Pearl Millet

Pearl millet [*Pennisetum glaucum* (L.) R. Br.; previously *Pennisetum americanum* (L.) Leek] is a significant food crop in the arid tropics. Cytoplasmic male sterility along with restorers is used commercially to increase productivity. Although there are a number of CMS sources in pearl millet (Delorme et al. 1997), the A1 source of CMS is the most commonly employed. Fertile revertants are observed in CMS A1 at a low frequency and were shown to result from mitochondrial DNA alterations (Smith et al. 1987; Delorme et al. 1997). The region of mtDNA that includes the *cox1* gene appeared to be correlated with A1 CMS (Delorme et al. 1997). Feng et al. (2009) further analyzed this region in the male-sterile A1 and its fertile revertants. They found that three *cox1*-related regions are present in pearl millet; these have been designated *coxI-1*, *coxI-2* and *coxI-3*. The organization of these regions differs in the maintainer, CMS A1, and cytoplasmic fertile revertants. A two-step model involving intermolecular illegitimate recombination across a 7-bp microhomologous repeat followed by intramolecular homologous recombination leading to the novel *cox1* mtDNA organization observed in cytoplasmic revertants is proposed (Feng et al. 2009). The intermolecular recombination involves a substoichiometric molecule and one of the resulting products is stabilized by the subsequent intramolecular recombination.

D. Reversion in Brassica

A unique mitochondrial gene, *orf138*, is responsible for Ogura CMS in *Brassica* plants (Bonhomme et al. 1991, 1992; Grelon et al. 1994) and also for the CMS in Ogura radish (Krishnasamy and Makaroff 1993; Krishnasamy et al. 1994). There exist three different configurations of the *orf138* gene region (Bellaoui et al. 1998). In one form, the *orf138* gene is linked to, and co-transcribed with, the *orfB* gene (now known to be *atp8*; e.g. Heazlewood et al. 2003). In a second form, the *orf138* gene is associated with the *atp1* gene and is not expressed. In the third form, *orf138* is no longer associated with *orfB* or *atp1* but with other sequences. The mtDNA form carrying *orf138* and *orfB* is rearranged upon cytoplasmic reversion to fertility in *Brassica* (Bonhomme et al. 1991). Substoichiometric amounts of mtDNA molecules carrying the different *orf138* configurations are present in CMS plants and also in "unmodified" Ogura cytoplasm. In such cases, one configuration is usually predominant while the others are substoichiometric. Cytoplasmic reversion to fertility of Ogura CMS is associated with changes in the proportions of the different molecules carrying *orf138* (Bonhomme et al. 1991, 1992). A

deleted *orf138* derivative was also detected. The observed changes result from recombination between the different forms (Bellaoui et al. 1998).

E. Reversion in Carrot

A partially male-fertile plant arose spontaneously in a strain of petaloid CMS carrot. It was determined via genetic analyses that the fertility was due to a new nuclear restorer gene. Several generations later, cytoplasmically revertant nonrestoring plants were recovered within a CMS family segregating for this new restorer (Chahal et al. 1998). The mtDNA genome of the CMS line was partially mapped and compared with that of the cytoplasmic revertant. A complex organization, including substoichiometric genomes, was revealed. The mitochondrial genomes of the fertile maintainer and the revertant were similar; however, they can be distinguished by unique restriction enzyme fragments. It was suggested that the mtDNA changes in the revertant could have arisen by the amplification of a substoichiometric genome (Chahal et al. 1998).

V. Nuclear-Cytoplasmic Interactions

Plants represent an excellent model system in which to study the interaction of the nuclear and cytoplasmic genomes. The nuclear-mitochondrial genotype combination can be changed using wide crosses and in vitro manipulations. Variant or defective mitochondrial genes that have easily scored phenotypes, such as growth abnormalities or male sterility, can be used to assay the effects of nuclear genes. Conversely, in plants with mitochondrial dysfunction, retrograde regulation of the expression of nuclear genes can often be seen. Because of its economic importance, one area of active research is the analysis of nuclear genes that control the expression of mitochondrially encoded CMS traits. Nuclear genes also control the organization and stability of mitochondrial genomes and, in plants, their effects are especially striking.

A. Incompatibility Between Nucleus and Cytoplasm

As was discussed above, nuclear-cytoplasmic incompatibility may result in alloplasmic male sterility. Other traits, unrelated to male fertility, may be affected in alloplasmic combinations. Cytoplasms from teosinte relatives have been introduced into maize inbred lines by serial backcrosses to produce alloplasmic cytolines (Allen 2005). When the teosintes were more distantly related, a number of nuclear-cytoplasmic incompatibilities could be documented, including effects on growth and morphology (Allen 2005). For example, a spectrum of effects is seen when the *Zea perennis* teosinte cytoplasm is introduced into certain maize inbred lines. CMS (called CMS-EP) is observed in some lines (Gracen 1972; Gracen and Grogan 1974; reviewed by Laughnan and Gabay-Laughnan 1983), but most inbred lines carry restoring alleles for CMS-EP (Gabay-Laughnan 2001). Plant and seed size is also affected in plants carrying the *Z. perennis* cytoplasm and certain maize nuclear genotypes. This phenotype is termed maize **t**eosinte-**c**ytoplasm-associated **m**iniature (TCM; Allen et al. 1989). Kernels are smaller than normal, and plants grown from these smaller kernels are shorter, paler and slower growing. CMS-EP and TCM are distinct traits, and alleles that suppress these effects are products of different nuclear genes (Allen et al. 1989; Gabay-Laughnan 2001; reviewed by Newton et al. 2004).

Two diverse maize nuclear backgrounds, W23 and A619, carrying *Zea perennis* cytoplasm were examined for the expression of various mitochondrial genes (Cooper et al. 1990). Two major *cox2* transcripts were seen when the inbred background was W23 and three were seen when it was A619. The presence of the additional transcript was associated with a threefold reduction of the Cox2 polypeptide. A single nuclear gene, **m**odifier of ***c**ox2* **t**ranscripts (*Mct*), is responsible for the observed transcript differences (Cooper et al. 1990; Newton and Courtney 1991; Newton et al. 1995). The mitochondrial transcript differences, as well as the nuclear

gene responsible, are not related to the CMS or TCM phenotypes or to their restorers/rectifiers (reviewed by Newton et al. 2004).

Mct is probably a member of a class of genes termed ***m****odifier of* ***m****itochondrial* ***t****ranscripts* (*Mmt*). Restorer alleles for *pol* CMS of *Brassica napus,* CMS-S in maize, Ogura CMS in radish, and sorghum CMS IS1112C have all been correlated with the processing of normal mitochondrial gene transcripts (Makaroff and Palmer 1988; Singh and Brown 1991; Singh et al. 1996; Li et al. 1998; Tang et al. 1998; Wen and Chase 1999; Wen et al. 2003). These restorer loci either encode or regulate *Mmt* activity, or the *Mmt* and *Rf* alleles are closely linked (reviewed by Chase and Gabay-Laughnan 2004).

An ambitious effort to analyze nuclear-cytoplasmic co-adaptation using many accessions of Arabidopsis has revealed more subtle incompatibilities. In particular, germination capacity under challenging conditions can be significantly affected by the cytoplasm donor in F2 progeny (Moison et al. 2010).

B. Nuclear Genes and the Restoration of Fertility

CMS is widely utilized in the production of male-sterile plants for efficient, inexpensive hybrid seed production (Havey 2004). Nuclear restorer genes override CMS and are an important component of hybrid seed production when the F1 crop must be male fertile (Havey 2004). Hence, restorers are under study in the CMS systems of many crop plants, including maize, radish, rice, and sorghum, as well as in the CMS/*Rf* model systems such as *Mimulus,* petunia, and *Phaseolus*. The interesting questions raised by the presence of nuclear restorer genes in plants with normal, fertile cytoplasm have been previously reviewed (Chase and Gabay-Laughnan 2004; Newton et al. 2004).

In many CMS systems, e.g. petunia, radish, rice, and CMS-S maize, one nuclear restorer gene is sufficient to restore fertility. However, in some CMS systems, the coordinate action of two restorer genes is required for fertility restoration, e.g. CMS-T maize and the IS1112C sorghum (reviewed by Chase and Gabay-Laughnan 2004; Newton et al. 2004).

Most, but not all, of the *Rf* genes cloned thus far are members of the pentatricopeptide repeat (PPR) family, a large family of proteins in plants containing tandem arrays of degenerate 35 amino-acid repeats (Small and Peeters 2000; Saha et al. 2007; O'Toole et al. 2008). Most of the PPR proteins in plants are targeted to either mitochondria or chloroplasts, where they play essential roles in post-transcriptional processing events, such as RNA cleavage, splicing, editing and translation (Lurin et al. 2004; Andres et al. 2007; Schmitz-Linneweber and Small 2008). Some of the PPRs targeted to mitochondria have been demonstrated to act as *Rf* alleles for CMS.

The first PPR restorer to be cloned was the petunia *Rf592* gene (Bentolila et al. 2002). It encodes a mitochondrially targeted protein containing 14 tandem copies of a PPR motif, which interacts with transcripts of the CMS-associated locus (Gillman et al. 2007). The *Rfo (Rfk1)* restorer locus for Ogura CMS in radish has also been cloned (Brown et al. 2003; Desloire et al. 2003; Koizuka et al. 2003). This locus contains three PPR genes encoding highly similar proteins, designated PPR-A, PPR-B and PPR-C. PPR-B was genetically determined to be the restorer gene (Desloire et al. 2003). This gene codes for a protein containing 16 repeats of the PPR motif (Brown et al. 2003; Koizuka et al. 2003). PPR-B has a role in the translational regulation of the mRNA of the CMS-associated ORF (Uyttewaal et al. 2008).

The BT (Boro II) type of CMS in rice is an alloplasmic CMS, resulting from the combination of an *indica* cytoplasm and a *japonica* nucleus. The restorer gene *Rf-1* restores fertility to this CMS and is widely used commercially (reviewed by Kato et al. 2007). *Rf-1* encodes a protein containing 18 repeats of a PPR motif (Kazama and Toriyama 2003; Akagi et al. 2004; Komori et al. 2004). Duplicate open reading frames, designated *Rf-1A* and *Rf-1B*, were found in the region of the *Rf-1* gene and it was initially concluded that *Rf-1A* is the restorer gene (Akagi et al.

2004). However, later studies reported that *Rf-1A* and *Rf-1B* are each able to restore BT-type CMS (Wang et al. 2006). A survey of allelic variants of the *Rf-1* locus from a wide variety of *Oryza* species identified six genes (*Rf-1A* through *Rf-1F*) with homology to *Rf-1* all encoding PPR proteins (Kato et al. 2007). Another restorer gene in rice has recently been identified as a potential PPR gene. The WA (wild abortive) type of CMS in rice is the most widely used for the production of hybrid seed. A major *Rf* locus has been mapped to a chromosomal region containing 13 PPR genes. One of these genes is the candidate restorer gene (Ngangkham et al. 2010).

There are several CMS systems in sorghum (Schertz et al. 1989), however, the A1 type of CMS in *Sorghum bicolor* is the one employed almost exclusively for the commercial production of sorghum hybrids. Restoration of this CMS requires two major restorer genes, *Rf1* and *Rf2*. The *Rf1* locus has been mapped and cloned and was shown to encode a PPR protein (Klein et al. 2005). This restorer is not located in the collinear region in the rice genome (Klein et al. 2005). A PPR protein also represents a possible candidate for the sorghum *Rf2* gene (Jordan et al. 2010). This PPR gene is highly similar to the orthologous rice *Rf1*gene.

In several other cases, *Rf* genes have been mapped to PPR-rich regions of genomes. Cytoplasmic male sterility occurs in monkeyflower hybrids with *Mimulus guttatus* cytoplasm and the *M. nasutus* nucleus (Fishman and Willis 2006). The genomic region containing the restorer locus for this CMS has been mapped and characterized as a PPR-gene-rich region (Barr and Fishman 2010). The *Rf3* restorer of CMS-S maize maps to the long arm of chromosome 2 (Laughnan and Gabay-Laughnan 1983; Kamps and Chase 1997), in a region containing several putative PPR genes (Xu et al. 2009).

Although most of the *Rf* genes cloned thus far are members of the PPR family, there are three known exceptions. The maize CMS-T *Rf2* allele was the first restorer to be cloned (Cui et al. 1996). It does not encode a PPR protein but instead encodes a mitochondrially localized aldehyde dehydrogenase (Cui et al. 1996; Liu et al. 2001). The *Rf17* restorer gene for the Chinese wild rice (CW)-type of CMS encodes a 178-aa protein designated **R**etrograde-regulated **M**ale **S**terility (RMS). This protein contains a segment similar to acyl-carrier protein synthase (Fujii and Toriyama 2009). In addition, the *Rf2* gene for Lead Rice-type CMS encodes a 152-aa protein with a glycine-rich domain (Itabashi et al. 2011). Thus, researchers looking for candidate restorers should not limit their searches to PPR genes (Ngangkham et al. 2010).

Maize CMS-S is distinctive in that many independent restorers have arisen by spontaneous mutation. While these alleles do restore viability to pollen grains, many are homozygous lethal (Laughnan and Gabay 1973, 1978; reviewed by Gabay-Laughnan et al. 1995; Chase and Gabay-Laughnan 2004). This kind of newly arisen restorer has been designated *restorer-of-fertility lethal* (Wen et al. 2003). CMS-S pollen aborts relatively late and restoration of function occurs when the restoring allele is present within the individual pollen grain (i.e., restoration is gametophytic). Thus, "lethal" restorers would be expected to reduce the levels of the CMS-associated, *orf355-orf77* transcripts in the maturing pollen, but they might be expected to also affect the expression of one or more essential mitochondrial genes. Indeed, in the case of one lethal restorer, Wen et al. (2003) showed a reduction in transcripts for the alpha subunit of ATPase in addition to the expected reduction in the CMS-S-associated 1.6-kb RNA. Ethanolic fermentation can compensate for respiratory deficiencies in pollen (reviewed by Tadege et al. 1999), but obviously not in the seed or seedling. The products of these lethal-restorer genes are expected to be involved in mitochondrial biogenesis or function, and several may represent mutations in PPR proteins.

C. Nuclear Genes Affecting Mitochondrial Recombination and Substoichiometric Shifting

The organization of mitochondrial genomes and the expression of mitochondrial genes

are controlled by nuclear genes. Some nuclear genotypes are associated with higher rates of mitochondrial rearrangements that lead to abnormal growth or reversion of CMS to fertility. Therefore, nuclear genes are involved in the generation, selection and amplification of mitochondrial mutations. The rate at which NCS mutations arise in maize varies among inbred lines; it is usually extremely low, but it can be as high as 1% in the inbred line Wf9 nuclear background (Duvick 1965; Newton and Coe 1986). In addition, the nuclear background controls the rate at which cytoplasmic reversion of male sterility occurs. For example, reversion is observed in approximately 10% of CMS-S maize plants in the M825 nuclear background (Laughnan et al. 1981). The nuclear background also controls the mtDNA rearrangements observed upon cytoplasmic reversion (reviewed by Gabay-Laughnan et al. 1995). Additionally, cytoplasmic reversion of maize CMS-S in the M825 line is always associated with recombination of S2 sequences with microhomologous sequences elsewhere in the genome, resulting in the loss of the S1 and S2 plasmids. In revertants arising in the Wf9 background, however, the S plasmids are invariably retained, but rearrangements affect the CMS-ORF (Small et al. 1988; reviewed by Newton et al. 2004).

The P2 line of maize, derived from a South American strain of popcorn, exhibits a general increase in mtDNA instability and P2 plants exhibit a variety of maternally transmitted abnormalities such as poor plant growth and leaves with pale sectors (Kuzmin et al. 2005). These phenotypes are associated with destabilized, multiply rearranged mitochondrial genomes. The P2 nuclear genotype appears both to alter the copy number of specific sublimons and to amplify the products of aberrant microhomologous recombination (Kuzmin et al. 2005).

In contrast to the above systems in maize, where no specific causative nuclear allele(s) has been identified, nuclear genes have been shown to affect mitochondrial recombination in some other plant systems. For example, the dominant allele of the *Phaseolus vulgaris Fr* ("fertility restorer") gene is responsible for a reduction in the copy number of the 210-kb mitochondrial subgenome that carries the *pvs-orf239* responsible for CMS-Sprite. This results in a reversion/restoration of the CMS to fertility (Mackenzie and Chase 1990; Janska and Mackenzie 1993; He et al. 1995; Janska et al. 1998). When *Fr* is inactive, the 210-kb subgenome is amplified (Arrieta-Montiel et al. 2001). Thus the *Fr* gene seems to affect mitochondrial substoichiometric shifting.

Recombination within Arabidopsis mitochondrial genomes is influenced by at least three nuclear genes: *MSH1*, *OSB1*, and *REC3A* (reviewed by Maréchal and Brisson 2010). Mutation of *MSH1* (**M**ut**S** **h**omolog; formerly *CHM*) is responsible for the *chm/chm* mutant phenotype (Martinez-Zapater et al. 1992) in Arabidopsis. MSH1 regulates substoichiometric shifting within the mitochondrial genome (Abdelnoor et al. 2003), suppressing recombination at repeat sequences varying in size from 108 to 556 bp. When MSH1 activity is disrupted, over 30 sites within the mitochondrial genome become activated, thus influencing the genome organization (Arrieta-Montiel et al. 2009).

OSB1 (**O**rganellar **S**ingle-stranded DNA-**B**inding protein1) is a member of a plant-specific family of DNA-binding proteins. OSB1 was purified from potato (*Solanum tuberosum*) mitochondria (Vermel et al. 2002) and orthologs of the *OSB1* gene were later found in *Arabidopsis thaliana*, rice and maize (Zaegel et al. 2006). OSB1 is required for the correct transmission of substoichiometric mitochondrial genomes in Arabidopsis (Zaegel et al. 2006). T-DNA insertion mutants accumulate products of homologous recombination and this leads to morphological phenotypes such as leaf variegation and distorted plants. *OSB1* thus controls the stoichiometry of the subgenomes produced by recombination (Zaegel et al. 2006).

Three distinct homologs of the *E. coli recA* gene are found in the Arabidopsis nuclear genome. These map to different chromosomes and are designated *RECA1*, *RECA2*, and *RECA3* (Shedge et al. 2007).

RECA3 is targeted to the mitochondria and mutant alleles result in plants that carry mtDNA rearrangements but which appear to be phenotypically normal. The characterized mtDNA rearrangements in *recA* mutants are similar, but not identical, to those found in *msh1*. Interestingly, loss of both the MSH1 and RECA3 functions simultaneously has extreme effects on the plant via substoichiometric shifting of various subgenomes (Shedge et al. 2007).

A targeted effort to amplify pre-existing mtDNA rearrangements in transgenic tobacco and tomato plants was undertaken, using RNAi constructs to suppress MSH1 (Sandhu et al. 2007). In some of the regenerated plants, aberrant flowers and partial sterility were observed. In subsequent generations, maternally-inherited leaf variegation and increasing degrees of male sterility were seen. Sandhu et al. (2007) were able to correlate amplification of originally low-level mtDNA restriction enzyme fragments with the abnormal plant phenotypes. They suggested that substoichiometric shifting could reveal cryptic CMS-ORFs in the mitochondrial genomes of many crop plants. The types of leaf variegation seen on the tobacco and tomato plants, and the correlated mtDNA changes, are similar to those reported for Arabidoposis *msh1* (*chm*) mutants (Martinez-Zapater et al. 1992). Such changes are also reminiscent of the maternally-inherited defective phenotypes and mtDNA changes generated by the P2 line of maize, which is proposed to have reduced functioning of an MSH-type gene (Kuzmin et al. 2005).

The abnormal growth phenotype MSC in cucumber is correlated with mtDNA rearrangements (Havey et al. 2004). Like maize NCS plants, MSC plants are heteroplasmic for MSC and non-mutant mitochondria. MtDNA is inherited paternally in cucumber, and a single nuclear locus designated *Psm* (for **P**aternal **s**orting of **m**itochondria) controls the sorting of the mtDNA from the paternal parent (Havey et al. 2004; Al-Faifi et al. 2008). Although *Psm* controls the predominance of specific mtDNAs, it is not the cucumber ortholog of *Msh1* (Al-Faifi et al. 2008).

VI. Mitochondrial Repeats and the Induction of Rearrangement Mutations

As is apparent from previous sections, rearrangements in mitochondrial genomes can be "induced" in multiple ways. (1) Certain nuclear backgrounds (e.g., M825 and Wf9 in maize) are associated with elevated rates of mitochondrial rearrangement mutations under normal field-growth conditions. (2) Passage through tissue culture can induce mtDNA rearrangements. It has led to the induction of CMS (e.g., in carrot, *Nicotiana* and *Brassica* species), CMS reversions (e.g., in CMS-S and CMS-T maize), and mutant mosaic plants (e.g., MSC of cucumber). (3) Specific "mitochondrial mutator" genes cause high rates of rearrangement mutations (e.g., the *msh1*, *rec3A* and *osb* alleles described in Arabidopsis, and alleles in the P2 line of maize).

There appear to be multiple mechanisms by which rearrangements arise in plant mitochondrial genomes. A recent review described the mechanisms of homology-dependent and illegitimate recombination operative in plant mitochondria (Maréchal and Brisson 2010). Each of the processes is controlled by nuclear genes, such as those described above, whose normal functioning is vital for maintaining the stability of mitochondrial genomes. The lengths of repeated sequences in the genomes appear to correlate with which process is operative.

Longer mtDNA repeats (>1 kb) recombine via reversible homologous recombination, leading both to inversions (if repeats are in inverted orientation relative to each other) and to subgenomes (if the repeats are in direct orientation). Evidence for HR was originally provided by DNA gel-blot hybridization and by mapping studies (Palmer and Shields 1984; Lonsdale et al. 1984). Although HR itself is reciprocal, and both recombinant products are found, the recombinant products may be present at lower or substoichiometric levels (Small et al. 1987, 1989).

Smaller repeats (usually ~100–500 bp) are associated with the recovery of asymmetric

events, often under the influence of recessive mutant alleles of nuclear genes. For example, when MSH1 is mutant in Arabidopsis, many small repeats have shown greatly enhanced recombination (Arrieta-Montiel et al. 2009), and preferential recovery of one of the recombinants tends to be seen. This outcome could be due either to asymmetry of the events themselves or to selective amplification (discussed in Maréchal and Brisson 2010). Rapid sorting out of the recombinant from the original mitochondrial genome in subsequent cell divisions could explain the phenomenon of "substoichiometric shifting", in which the original predominant organization is replaced by a previously rare recombinant form.

The vast majority of repeats in plant mitochondrial DNAs are very short; e.g., less than 50 bp (Clifton et al. 2004; reviewed by Kubo and Newton 2008). Illegitimate recombination involving these microrepeats (also referred to as 'microhomology-mediated illegitimate recombination') is associated with loss of gene segments as well as with generation of novel, chimeric open reading frames.

Can we delineate more exactly the size ranges for the various types of recombination events? In maize, the mutant alleles in the P2 nuclear background affect small and microrepeat-mediated events, but not ones involving HR. The smallest repeat known to be associated with HR in maize mtDNA is the "0.7-kb" repeat, which is present in two nearly identical copies (714 and 725 bp; Clifton et al. 2004). Reciprocal recombination between the 0.7-kb repeats gives rise to two equally represented recombinant molecules (Lonsdale et al. 1984; Lupold et al. 1999). The amounts detected are in a 1:6 ratio of the recombinant versions relative to the "master circle" copies, suggesting that the frequency of recombination is relatively low for this size of repeat. Alternatively, the recombinant products could be less stable or under-replicated (Lupold et al. 1999). If homologous recombination is responsible for the observed results, this suggests a lower size limit for HR of approximately 700 bp.

Recombination across the 0.7-kb repeat is not affected by the P2 mitochondrial-mutator background, which destabilizes shorter repeats and microrepeats. However, a slightly smaller, 560-bp repeat, is affected by a variant P2 allele (Kuzmin et al. 2005). This repeat normally recombines at low frequency; i.e. substoichiometric amounts of recombinant products between the 560-bp sequences are detectable in mtDNA in a "stabilizing" nuclear background (Kuzmin et al. 2005). In the destabilizing P2 nuclear background, the amounts of one of the recombinant products were shown to be selectively amplified suggesting that the replication of one of the recombinant products was favored (Kuzmin et al. 2005). This asymmetric effect or "substoichiometric shifting" is similar to that described in Arabidopsis and other species. Thus, this would suggest an upper limit for SSS of approximately 550 bp in maize, which is in accordance with the results from studies with Arabidopsis (Arrieta-Montiel et al. 2009).

Aberrant products resulting from illegitimate recombination events also rapidly accumulate in P2 plants. One of the products was studied in detail (Kuzmin et al. 2005). It involved a 15-bp near-identical repeat, one copy of which is found in the *rps13* gene and the other in integrated R1 plasmid sequences. A novel, non-reciprocal R1/*rps13* product was recovered in one set of P2 sibling plants. This arrangement was unique in this P2 family; it could not be shown to pre-exist in other maize mitotypes or in other tested P2 families (Kuzmin et al. 2005). Thus, this novel R1/*rps13* rearrangement was not the result of substoichiometric shifting of a pre-existing sublimon; rather, it appeared to be amplified after a de novo non-reciprocal event. The 15 bp of near-identity appears to be average for the microhomology-mediated events. The repeats involved in generating the maize NCS mutants range in size from 6 bp (Newton et al. 1990) to 31 bp (Lauer et al. 1990).

VII. Conclusions

Plant mitochondrial mutations are widespread in higher plants. Maternally inherited abnormal growth mutants, newly arisen male-sterile plants, and cytoplasmic reversions to male fertility in male-sterile strains are easily recognized. Nuclear factors affect the origin and expression of these mitochondrial mutations. DNA modifications that do not result in obvious phenotypes may also occur in mitochondrial genomes. These neutral mutations are likely to be involved in mitochondrial genome evolution. A number of nuclear restorer-of-fertility alleles have been cloned in recent years and most of them encode PPR proteins that affect the expression of mitochondrially encoded CMS-ORFs. Systematic efforts to generate intra- and interspecific combinations of nucleus and cytoplasm, and to detect subtle phenotypic changes should increase our understanding of nuclear-mitochondrial co-adaptations.

In addition to the de novo visible mutations, there are also alternative arrangements of mitochondrial genomes that pre-exist as sublimons and are suppressed by normal alleles of nuclear genes. Defective alleles of these genes, as well as those controlling mitochondrial DNA repair, replication and recombination, result in the recovery of high levels of rearrangements. Manipulating the expression of these genes to amplify cryptic ORFs could result in new sources of CMS. The application of newer technologies to sequence mitochondrial genomes inexpensively and rapidly will allow researchers to detect low-level mutations and rearrangements. This will not only increase our knowledge of the dynamics of mitochondrial genomic changes, but also should allow for the targeted selection of useful rearrangements within plant mitochondrial genomes.

Acknowledgments

Work from the authors' laboratories was funded by grants from the U.S. National Science Foundation and the U.S. Department of Agriculture. We thank I. Small, H. Millar, J. Whelan and their group members at the Plant Energy Biology Centre in Perth, Australia for helpful discussions with KJN. We thank T. Langewisch, A. Lough, J. Flynn and M. Green for help with manuscript preparation.

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