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Molecular studies of cytoplasmic male sterility in maize

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A few extra genes that are not found in the mitochondria of other organisms are encoded by plant mitochondrial genomes. Current evidence suggests that the cytoplasmic male sterility (CMS) trait of maize is due to mitochondrial gene mutations. In the sterile maize (CMS-T) a unique mitochondrial gene, designated *urf*13-T, appears to cause CMS and susceptibility to the fungal pathogen *Helminthosporium maydis* race T, and its pathotoxin, T-toxin. The *urf*13-T gene encodes a 13 kDa polypeptide that is located in the mitochondrial membrane. In CMS-T two nuclear restorer genes, *Rf*1 and *Rf*2, countermand the CMS trait and restore viable pollen production. The *Rf*1 locus appears to contribute to pollen restoration by reducing the expression of the 13 kDa protein. The function of the *Rf*2 gene is unknown.

T-toxin and the insecticide methomyl inhibit respiration of mitochondria from CMS-T but not from other maize cytoplasms. When the *urf*13-T gene is transformed into *E. coli* cells and expressed, bacterial respiration is inhibited by both T-toxin and methomyl. Respiration is not inhibited by these compounds in the absence of the 13 kDa protein or with a truncated version of the protein. These studies indicate that the 13 kDa protein is responsible for conferring sensitivity to T-toxin and methomyl.

The male-sterile cytoplasm, CMS-C, contains mutations of the mitochondrial genes *atp*9, *atp*6 and *cox*II. These mutations have resulted from rearrangements involving portions of mitochondrial genes and chloroplast DNA. One of these gene mutations may be responsible for CMS; however, we currently have no evidence confirming this possibility. Nevertheless, it is clear that different factors cause male sterility in CMS-T and CMS-C because the *urf*13-T gene is only found in CMS-T.

Introduction

Even though the mitochondrial genome of maize (*ca.* 570 kilobases (kb)) (Lonsdale *et al.* 1984) is 30 times larger than that of man (*ca.* 16 kb), it encodes only a few extra genes not found in the mitochondrial genome of man. Among them are the genes for a 5*S* rRNA and the F_1-ATPase-α subunit (Leaver & Gray 1982; Chao *et al.* 1984; Braun and Levings 1985; Isaac *et al.* 1985). The mitochondrial 5*S* rRNA is not encountered in insects, fungi or mammals and is apparently unique to plant mitochondria. The ATPase α subunit is common to all mitochondria but in most organisms it is encoded by a nuclear gene and is imported into the mitochondria. In plants the α subunit is a mitochondrial gene product. A few mitochondrial genes common to fungi and mammals have not yet been identified in plant mitochondrial genomes (Eckenrode & Levings 1986, 1987). This includes genes for some tRNAs, a 9*S* RNA, ribosomal proteins, subunits of the NADH–Q reductase and maturases.

Other mitochondrial genes are suggested by the discovery of unidentified open reading frames (URFs) in the maize mitochondrial genome and in plasmid-like DNA associated with

the mitochondria. The plasmid-like DNAs carry several URFs that are thought to encode genes important to their replication and persistence (Levings & Sederoff 1983; Paillard *et al.* 1985). In the S2 plasmid-like DNA a gene product for *urf*I has been demonstrated by immunological techniques (Manson *et al.* 1986); however, the function of the URFI polypeptide is unknown.

Cytoplasmic male sterility (CMS) is common among higher plants, where it has been described in more than 140 different plant species (Laser & Lersten 1972). The CMS trait causes pollen abortion, is inherited in a non-Mendelian fashion and does not usually affect female fertility. The specific mechanism responsible for CMS differs among species. In tobacco, for example, one CMS type (Gerstel 1980) is caused by the stamens becoming petaloid-like instead of developing into normal stamens. In contrast, pollen abortion in the sterile maize, CMS-S, occurs late in development when nearly mature pollen grains suddenly abort (Lee *et al.* 1980). The wide variation in mechanisms causing CMS indicates that different factors are responsible for the trait. It has been proposed that CMS may be due to mitochondrial, chloroplast or viral factors; however, in maize, abundant evidence indicates that mutant mitochondrial genes are responsible for the CMS trait. Thus CMS constitutes one of the rare opportunities for investigating mitochondrial gene mutations in higher plants.

Three major CMS types have been identified in maize: CMS-C, CMS-S and CMS-T (Laughnan & Gabay-Laughnan 1983). These male-sterile cytoplasms are distinguished on the basis of specific nuclear genes (*Rf*) that restore pollen fertility (table 1). Normal (fertile) and the three male-sterile maize cytoplasms can also be differentiated by other characteristics. The cytoplasms can be distinguished by restriction-fragment length polymorphisms, by variations in their mtRNAs with Northern blot analysis and by differences in their mitochondrial translation products (see review by Eckenrode & Levings (1987)). In a few cases, the cytoplasms differ in disease resistance, toxin and insecticide resistance, histological differences, reversion to fertility, and mitochondrial DNA and RNA plasmids.

TABLE 1. CHARACTERISTICS OF THE MAIZE CMS CYTOPLASMS AND THEIR RESTORER GENES

male-sterile cytoplasm	nuclear restorer genes	chromosomal location
T	*Rf*1 and *Rf*2	chr. 3, 9
C	*Rf*4	chr. 2 or 8
S	*Rf*3	chr. 2

CMS-T

We have discovered several mutant mitochondrial genes by investigating mtRNAs from normal and male-sterile cytoplasms of maize. In these studies, Northern blot analyses have revealed variations in mtRNA profiles (steady-state) among the various cytoplasms when hybridized with certain mitochondrial gene probes. A mtDNA sequence, TURF2B, that is abundantly transcribed (steady-state) in the mitochondria of CMS-T plants has been identified in this fashion (Dewey *et al.* 1986). Low levels of transcripts with homology to TURF2B have been detected in other fertile and sterile cytoplasms; however, these are attributable to the unusual makeup of the TURF2B sequence and are explained later. Transcription of TURF2B is confined to three adjacent *Hin*dIII DNA fragments containing a

total of 3547 nucleotides. The 3547 nucleotide sequence is called TURF2H3 and has been characterized.

The TURF2H3 sequence contains two long open reading frames that could encode polypeptides of 12961 Da (URF13) and 24675 Da (URF25). URF13 and URF25 sequences are separated by an intergenic region of 77 nucleotides. TURF2H3 is unusual in that it is composed of sequences with nucleotide homology to other mitochondrial genes and a chloroplast gene. Specifically, TURF2H3 contains sequences homologous with the 5′ flanking region of the *atp*6 gene, the 3′ flanking region of the 26*S* rRNA gene, a part of the coding region of the 26*S* rRNA gene and a chloroplast $tRNA^{Arg}$ gene. These analyses suggest that the chimeric sequence, TURF2H3, has arisen by rearrangements involving both intra- and intermolecular recombinational events. In fact, at least seven recombinational sites are evident in the TURF2H3 sequence. Finally, it should be pointed out that the homologous sequences in TURF2H3 are duplications (repeats) of the regions of the *atp*6 and 26*S* rRNA genes and that the *atp*6 and 26*S* rRNA genes are complete and functional in CMS-T.

The *urf*13 reading frame is unique to the mitochondrial genome of CMS-T plants. We have not found it in the mitochondrial genomes of normal or other male-sterile cytoplasms of maize, and a limited survey has not detected it in the mitochondria of other plant species. Indeed, because of its peculiar origin, it seems unlikely that a sequence similar to *urf*13 would be found in other plant mitochondria.

The *urf*13 gene is abundantly transcribed (steady-state) in CMS-T. The sequence appears to be constitutively expressed because transcripts are observed in coleoptiles, roots, leaves, ear shoots and tassels. Major RNA species of 3900, 2000, 1800 and 1500 nucleotides are readily identified by Northern blot analysis. The 3900 nucleotide transcript is the largest observed and may be the primary transcript. The identity of the URF13 messenger is not established. S1 protection and primer extension studies show that the 2000 and 1800 nucleotide transcripts contain the entire coding region of *urf*13, and thus they could be *urf*13 messengers. In contrast, the 1500 nucleotide RNA species lacks some of the 5′ coding region of *urf*13, and therefore, cannot be the messenger.

We and others (Wise *et al.* 1987) have shown that a 13 kDa polypeptide (115 amino acids) is encoded by the *urf*13 sequence. The gene product has been demonstrated with an antiserum prepared in rabbits against a chemically synthesized oligopeptide based on the predicted amino acid sequence of URF13 (Dewey *et al.* 1987). The specific oligopeptide sequence (15 amino acids) is located near the C-terminus of URF13 in a hydrophilic region. Western blot analyses have clearly shown that the 13 kDa protein is present in the mitochondria of CMS-T plants, but that it is not detectable in mitochondria from normal (fertile) and other sterile maize cytoplasms, CMS-C and CMS-S. We have identified the 13 kDa protein in every organ type of CMS-T plants assayed to date, i.e. shoots, leaves, roots and ears. These results suggest that the 13 kDa protein is constitutively expressed. The *urf*13 gene may be regulated and expressed in a manner similar to the *atp*6 gene; this is predicted because the *urf*13 and *atp*6 genes both have similar 5′ flanking sequences.

Additional studies have verified that the 13 kDa protein is mitochondrially encoded. This has been established by the immunoprecipitation of a translational product from an *in vitro* protein synthesis with isolated mitochondria. A labelled 13 kDa polypeptide is immunoprecipitated by the URF13-specific antiserum from the translational products of CMS-T mitochondria. With normal (fertile) mitochondria similar experiments do not precipitate a

labelled 13 kDa polypeptide. Earlier, Forde *et al.* (1978) used the *in vitro* protein synthesizing system to show that a unique 13 kDa protein is synthesized in CMS-T mitochondria but not in normal (fertile) ones. Because of the similarity in size and other characteristics, we believe that their 13 kDa polypeptide is the one encoded by the *urf*13 gene. Finally, we have designated the symbol *urf*13-T to indicate the mitochondrial gene encoding the 13 kDa polypeptide of CMS-T.

The 13 kDa polypeptide is prominently identified in the mitochondrial membranes by Western blot analysis with the *urf*13-T specific antiserum. In these studies, soluble and membrane fractions of CMS-T mitochondria have been isolated and compared. Large amounts of the 13 kDa protein are detected in the membrane fraction. In contrast, the soluble fraction contains only a trace amount, which is probably attributable to cross-contamination between fractions.

Two nuclear restorer genes, *Rf*1 and *Rf*2, suppress pollen abortion in CMS-T maize (Duvick 1965). In the past, these genes have been used to restore pollen fertility in hybrid maize seed produced with the T cytoplasm. Forde & Leaver (1980) have studied the effect of restorer genes on *in organello* protein synthesis. They have shown that the abundance of 13 kDa protein is substantially reduced in CMS-T restored (pollen-fertile) plants as compared with CMS-T male-sterile plants.

We have determined the effect of various combinations of the restorer genes, *Rf*1 and *Rf*2, on the expression of the *urf*13-T gene. Four major *urf*13-T transcripts (steady-state) are evident (3900, 2000, 1800 and 1500 nucleotides) in CMS-T plants with the nuclear genotypes *rf*1 *rf*1, *rf*2 *rf*2 and *rf*1 *rf*1, *Rf*2 ___. In contrast, five *urf*13-T transcripts are observed in CMS-T plants carrying the nuclear genotypes *Rf*1 ___, *rf*2 *rf*2 and *Rf*1 ___, *Rf*2 ___. A 1600 nucleotide RNA species is observed in those genotypes with the dominant allele of *Rf*1 that is not detected in CMS-T plants homozygous recessive for *Rf*1. Thus, the *Rf*1 allele is able to alter the expression of the *urf*13-T gene in CMS-T, but the *Rf*2 gene does not affect *urf*13-T expression. Alterations in the *urf*13-T transcripts could be caused by additional RNA processing or transcriptional initiation events; however, as yet we do not understand the mechanism by which the *Rf*1 gene modifies the expression of *urf*13-T. In addition, we have contrasted the DNA sequence of *urf*13-T in restored (pollen-fertile) and non-restored (pollen-sterile) genotypes, because DNA sequence rearrangements have been implicated in causing changes in expression in both yeast (Klar *et al.* 1981) and *Salmonella* (Zieg *et al.* 1978). These comparisons have not detected variations in the *urf*13-T gene sequence between restored and non-restored types (Stamper *et al.* 1987).

We have also characterized the effect of different combinations of restorer genes on the expression of the 13 kDa polypeptide of *urf*13-T. In CMS-T plants with the recessive alleles of *Rf*1, namely *rf*1 *rf*1, *rf*2 *rf*2 or *rf*1 *rf*1, *Rf*2 ___, the 13 kDa protein is fully expressed. The quantity of the protein, however, is drastically diminished in CMS-T plants carrying the dominant allele of *Rf*1, namely *Rf*1 ___, *rf*2 *rf*2 or *Rf*1 ___, *Rf*2 ___. Thus, it is evident that the dominant allele of *Rf*1 accounts for the reduced levels of the 13 kDa protein. Although the precise means is obscure, it is clear that the effect of the nuclear *Rf*1 gene on the expression of the mitochondrial gene *urf*13-T is related to the abundance of 13 kDa protein.

Tissue culture experiments with CMS-T maize have derived plants, called revertants, that are pollen-fertile and disease-resistant. Examination of these revertants has shown that they

have lost the *urf*13-T sequence or that a significant mutation has occurred in the *urf*13-T sequence (see papers by Lonsdale and Pring (this symposium) for details and references). These results implicate the *urf*13-T gene with the CMS trait.

Other characteristics of the 13 kDa polypeptide

CMS-T maize plants are susceptible to the fungal pathogen *Helminthosporium maydis* (*Bipolaris maydis*), race T, and its pathotoxin, T-toxin; by contrast, fertile and other male-sterile cytoplasms are not susceptible. Mitochondria isolated from CMS-T maize preferentially bind the T-toxin (Miller & Koeppe 1971). The T-toxin increases the permeability of the inner mitochondrial membrane to NAD^+ and Ca^{2+} (Holden & Sze 1984), inhibits respiration dependent on NAD^+-linked substrates (Matthews *et al.* 1979), uncouples oxidative phosphorylation (Bednarski *et al.* 1977) and causes mitochondrial swelling (Miller & Koeppe 1971). Conversely, the toxin does not affect mitochondria from normal maize, other male-sterile maize, CMS-T revertants or other plant species. It has also been shown that the insecticide methomyl causes effects similar to those of the T-toxin on mitochondria from CMS-T maize (Klein & Koeppe 1985). Disease susceptibility and pollen sterility appear to be inseparable; however, it is not known if these traits are due to a single locus or to closely linked loci.

We have devised an approach for investigating the relation between the T-toxin and the 13 kDa polypeptide (Dewey *et al.* 1988). The *urf*13-T gene has been inserted into several *E. coli* plasmid expression vectors; these include the pUC18 vector with the *lac* promoter (Messing 1983) and the pATH vector with the *trp*E promoter (T. J. Koerner, personal communication). These plasmid constructs have been transformed into *E. coli* where the inducible expression of the 13 kDa protein has been verified by means of Western blots with the 13 kDa-specific antiserum. To measure respiration in *E. coli* cells, we have monitored oxygen consumption by polarographic techniques. In these experiments we have compared the effect of T-toxin on bacterial respiration with and without the expression of the 13 kDa protein. The addition of T-toxin to cells in which the 13 kDa protein is not present results in no change in the respiration rate. In contrast, the addition of T-toxin to *E. coli* cells in which the 13 kDa protein is expressed causes a sharp reduction in the respiration rate. Methomyl has been substituted for T-toxin in a similar set of experiments; it behaves like the T-toxin. In the presence of the 13 kDa protein, methomyl inhibits respiration of the cells. When the 13 kDa protein is absent from the cells, methomyl does not alter respiration rates.

In another study, we have removed the nucleotides of the *urf*13-T gene that encode amino acid residues 2–10 of the 13 kDa protein. The truncated *urf*13-T gene has been transformed into *E. coli* cells, where it is inducibly expressed. In similar studies, the addition of T-toxin or methomyl does not change the respiration rate. These studies indicate that *E. coli* cells containing the truncated 13 kDa protein are not sensitive to T-toxin or methomyl. Furthermore, they suggest that a region very near the N-terminus of the polypeptide is important in conferring sensitivity.

These investigations indicate that the 13 kDa protein is involved in sensitivity to T-toxin and methomyl. In these studies, we have tested a single gene, *urf*13-T, from CMS-T mitochondria, and found that its gene product is able to confer toxin-sensitivity to *E. coli* cells. These results

strongly support the notion that the 13 kDa protein encoded by *urf*13-T is responsible for sensitivity to the *H. maydis* T-toxin. Our findings also suggest the importance of the N-terminal end of the 13 kDa polypeptide to toxin sensitivity, perhaps as a binding site for the toxin.

Several mitochondrial polypeptides are able to bind covalently to the lipophilic compound dicyclohexylcarbodiimide (DCCD). The proteolipid, subunit 9 of the F_0-ATPase, is a well-known example of a plant mitochondrial polypeptide that binds DCCD (Hack & Leaver 1984; Dewey *et al.* 1985*a*). We have investigated the binding of DCCD to the 13 kDa protein because a characterization of its amino acid sequence has suggested that it might be capable of binding DCCD. The N-terminal region of the 13 kDa protein contains an aspartic acid residue situated in a hydrophobic domain.

We have studied the binding of ^{14}C-labelled DCCD to mitochondrial proteins from normal and CMS-T maize by SDS–gel electrophoresis (Dewey *et al.* 1988). The labelled DCCD binds strongly to the proteolipid, the ATP9 subunit, from normal and CMS-T mitochondria. In addition, it binds to protein(s) from normal and CMS-T mitochondria with molecular mass of *ca.* 13 kDa. To determine whether DCCD binds to the 13 kDa protein encoded by *urf*13-T, we have immunoprecipitated the mitochondrial proteins after the binding reaction with an antiserum specific to the 13 kDa protein. Fractionation by gel electrophoresis has revealed a 13 kDa DCCD-labelled protein from CMS-T mitochondria but not from normal ones. These results demonstrate that the 13 kDa protein binds DCCD. Interestingly, Holden & Sze (1987) have shown that the binding of DCCD to CMS-T mitochondria reduces the sensitivity of the mitochondria to the T-toxin. These preliminary findings suggest that the T-toxin and DCCD may compete for a similar site on the 13 kDa protein.

CMS-C

To investigate the cause of cytoplasmic male sterility in CMS-C maize, we have looked for mitochondrial mutations affecting the size and/or the abundance of their gene transcripts. Northern blot analyses with total mtRNA have been used to compare the transcriptional profiles (steady-state) of mitochondrial genes from normal (fertile) and CMS-C (sterile) maize. Most of the genes examined have similar transcriptional patterns; however, three genes (*atp*9, *atp*6 and *cox*II) show distinct variations in the size and number of their transcripts when normal and CMS-C cytoplasms are contrasted. To learn why these genes produce variable transcripts, we have isolated, cloned and characterized the genes from the mitochondria of CMS-C maize. Interestingly, our studies indicate that these unusual genes are the only copies in CMS-C mitochondria available to encode the important mitochondrial polypeptides ATP9, ATP6 and COXII.

The *atp*9 gene encodes a 74 amino acid polypeptide that is a subunit of the F_0-ATPase located in the inner mitochondrial membrane (Dewey *et al.* 1985*a*). The nucleotide sequence of the *atp*9 coding region is identical in normal and CMS-C maize (figure 1). The 5′ flanking regions are similar from the translational initiation site to nucleotide position 119. At this point, the sequences diverge abruptly; this divergence indicates that a rearrangement has taken place. This rearrangement in the 5′ flanking region accounts for the variation in *atp*9 transcripts between normal and CMS-C maize. Presumably, the change has provided the *atp*9 gene of CMS-C maize with a different promoter region from that associated with the *atp*9 gene of normal maize. This substitution does not appear to have significantly affected the abundance of *atp*9 transcript in CMS-C maize, based on Northern blot comparisons.

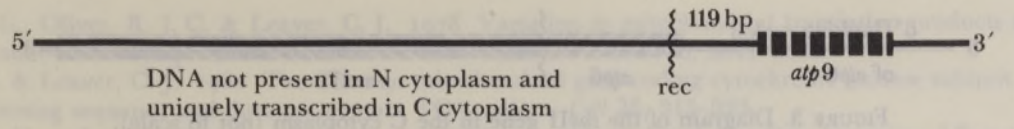

FIGURE 1. Diagram of the *atp*9 gene in the C cytoplasm (not to scale). The point of recombination (rec) indicates the site in the 5′ flanking region of the *atp*9 gene where the difference between N and C cytoplasm begins.

We have also compared the mutant mitochondrial *atp*6 gene of CMS-C maize with the *atp*6 gene from CMS-T maize (Dewey *et al.* 1985*b*). The *atp*6 gene encodes a subunit of the F_0-ATPase that is located in the inner mitochondrial membrane. The *atp*6 gene from CMS-C, designated *atp*6-C, is a complex sequence containing rearrangements involving chloroplast DNA and a portion of another mitochondrial gene, *atp*9 (figure 2). The 5′ flanking region of

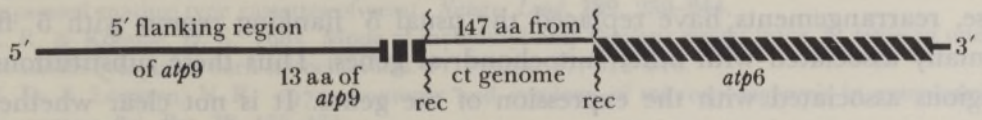

FIGURE 2. Diagram of the *atp*6 gene in the C cytoplasm (not to scale). Regions of homology with the *atp*9 gene and the chloroplast (ct) genome are indicated.

the *atp*6-C gene begins with the 5′ flanking sequence and the nucleotides encoding the first 13 amino acids of the *atp*9 gene. Because the nucleotides of the *atp*9 gene are in frame, they presumably encode the first 13 amino acids of the ATP9 subunit. At this point, sequence homology with the *atp*9 gene ends abruptly and an open reading frame of 441 nucleotides occurs, which has significant homology with the chloroplast genome. Homology with the chloroplast DNA ends after 441 nucleotides and the *atp*6 gene begins. The fusion of the chloroplast DNA with the *atp*6 gene occurs at the nucleotide position encoding amino acid 24 of the *atp*6 gene in CMS-T maize according to Dewey *et al.* (1985*b*). The remainder of the *atp*6-C gene is homologous with the *atp*6 gene from *CMS-T* maize. Because this entire rearranged nucleotide sequence is in frame, a chimeric polypeptide is predicted for the *atp*6-C gene. If we assume that translation begins with the methionine (Met) codon of *atp*9, then the gene encodes, beginning at the N terminus, 13 amino acids of ATP9, 147 amino acids of a chloroplast sequence and the last 268 amino acids of ATP6. Northern blot analysis has indicated that the DNA sequence is transcribed. In the CMS-C maize, sequence analysis has suggested that the promoter region usually associated with *atp*9 has replaced the promoter region ordinarily linked with *atp*6 in CMS-T and normal maize. As yet we have not identified the translational products of the *atp*6-C gene to confirm that a chimeric polypeptide is produced.

Next, we have analysed the *cox*II gene of CMS-C maize. This gene encodes a subunit of complex IV (cytochrome oxidase), a component of the inner mitochondrial membrane (Fox & Leaver 1981). The *cox*II gene of CMS-C maize appears to be a fusion between an *atp*6 and a *cox*II gene (figure 3). The *cox*II gene of CMS-C maize, designated *cox*II-C, begins with the 5′ flanking region of the *atp*6 gene and continues into its coding region. At this point, the *atp*6 sequence ends and the *cox*II sequence begins. Because the sequence is in frame, the predicted amino acid sequence of COXII-C contains amino acids of ATP6 fused to the amino acid sequence of COXII. We have sequenced the *cox*II-C gene through its termination codon; the remainder of the sequence is intact. Because the *cox*II-C gene contains the 5′ flanking sequence

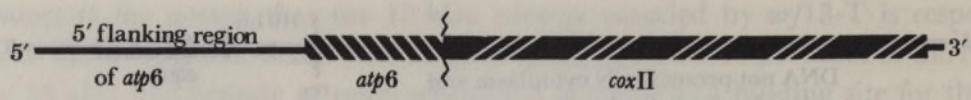

FIGURE 3. Diagram of the *cox*II gene in the C cytoplasm (not to scale). The region of homology with the *atp*6 gene is indicated.

of *atp*6, it is probable that a promoter region similar to that of a normal *atp*6 gene serves this function in the *cox*II-C gene.

We have also looked for the 5′ flanking region usually associated with the *cox*II gene in normal cytoplasm. This region is present in the mtDNA of CMS-C maize, but it does not appear to be adjacent to another gene-coding region. In fact, the region downstream from the *cox*II 5′ flanking region does not contain any large open reading frames.

In CMS-C maize, the mutant genes *atp*9, *atp*6 and *cox*II all have one variation in common. In each case, rearrangements have replaced the usual 5′ flanking region with 5′ flanking regions normally associated with other mitochondrial genes. Thus these substitutions could alter the regions associated with the expression of the genes. It is not clear whether these changes have quantitatively affected the level of transcription and translation of these genes.

These mutant genes of CMS-C maize could be responsible for the C-type of male sterility; however, we currently have no evidence to support this possibility. These mutant genes may be useful in characterizing mitochondrial transcriptional and translational signals. Finally, because the *urf*13-T gene of CMS-T mitochondria is not found in the CMS-C mitochondrial genome, it is clear that male sterility in CMS-T maize is caused by a different factor than in CMS-C. This finding is important because it shows that different mitochondrial gene mutations can cause the CMS trait in plants.

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