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Thoughts on Cytoplasmic Male Sterility in *cms-T* Maize

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INTRODUCTION

Cytoplasmic male sterility (CMS) is a maternally inherited trait that suppresses the production of viable pollen grains. The Texas, or T, cytoplasm (*cms-T*) of maize, which was first described in the Golden June line in Texas (Rogers and Edwardson, 1952), carries the CMS trait. The Texas cytoplasm was an important discovery to geneticists and plant breeders because it eliminated the costly detasseling procedure used in maize hybrid seed production. Its commercial value has prompted many applied and basic studies of *cms-T* aimed at understanding CMS and its relationship with mitochondrial and nuclear genes. Although CMS has been observed in more than 150 plant species (Laser and Lersten, 1972), the mechanism by which it interrupts normal pollen development is not well understood. In this review, the basis of CMS in *cms-T* maize is considered.

In *cms-T*, male sterility is characterized by the failure of anther exertion and pollen abortion. Female fertility is not affected by CMS, so male-sterile plants can set seed if viable pollen is provided. Comparisons of plants carrying the T and normal cytoplasms reveal very slight differences in several other morphological characters (see below; Duvick, 1965). Initially, the three male-sterile cytoplasms of maize—*cms-T*, *cms-C*, and *cms-S*—were distinguished by specific nuclear genes, termed restorers of fertility (*Rf*), that suppress the male-sterile effect of the various cytoplasms and allow viable pollen production. For example, two genes, *Rf1* and *Rf2*, acting jointly, restore pollen fertility to *cms-T*. *Rf1* and *Rf2* do not restore pollen fertility to *cms-C* or *cms-S*; instead, different restorer genes are necessary to restore pollen fertility to these cytoplasms. Other characteristics also distinguish male-sterile and normal maize cytoplasms; they include mitochondrial DNA (mtDNA) restriction fragment length polymorphisms, variations in mitochondrial RNA (mtRNA), and differences in mitochondrial translational products (Pring and Levings, 1978; Leaver and Gray, 1982; Newton, 1988). Early on, these distinctions indicated that the various CMS types are based on different mechanisms and hereditary factors.

The T cytoplasm is best known for the part it played in the U.S. epidemic of Southern corn leaf blight of 1969 and 1970 (Williams and Levings, 1992). In the two decades before the epidemic, *cms-T* had replaced detasseling as the chief method of pollen control in hybrid corn production (Wych, 1988), and by 1970, 85% of the hybrid corn grown in the United States carried the T cytoplasm. After it was determined that *cms-T* was specifically susceptible to *Bipolaris maydis* race T, the organism responsible for the blight, its use by the hybrid seed corn industry was largely terminated (Ullstrup, 1972). *Phyllosticta maydis*, another fungal pathogen, is also specifically virulent on *cms-T*.

Normally, *B. maydis* race T is a serious pathogen only on maize containing the T cytoplasm. Other male-sterile and normal maize cytoplasms support only limited colonization by the pathogen, and the lesions on the leaves remain small and isolated. By contrast, *B. maydis* race T can quickly and completely colonize *cms-T* maize plants, causing extensive plant damage and sometimes death. Susceptibility of *cms-T* to *B. maydis* race T is caused by mitochondrial sensitivity to a host-specific pathotoxin, designated BmT toxin, produced by the pathogen. *P. maydis* also produces a pathotoxin that is structurally similar to the BmT toxin of *B. maydis*, to which *cms-T* mitochondria are sensitive. Accordingly, susceptibility of *cms-T* maize to these fungal pathogens is due to mitochondrial sensitivity to the pathotoxins, whereas disease-resistant maize types have mitochondria that are insensitive to the pathotoxins.

THE T-*urf13* GENE OF *cms-T* MAIZE

Compelling evidence indicates that a mitochondrial gene designated T-*urf13* is responsible for the CMS and disease susceptibility traits borne by *cms-T* maize (Dewey et al., 1986, 1987). T-*urf13* encodes a 13-kD polypeptide (URF13) that is a component of the inner mitochondrial membrane (Dewey et al., 1987; Wise et al., 1987a) and is uniquely associated with the *cms-T* type of CMS (Forde et al., 1978).

The effect of restorer genes on T-*urf13* expression indicates an association between CMS and T-*urf13* (Dewey et al., 1986). The dominant nuclear genes *Rf1* and *Rf2* jointly suppress pollen sterility in *cms-T* maize. Specifically, the dominant allele *Rf1* affects the transcriptional profile of T-*urf13* and decreases the abundance of URF13 protein by about 80% (Dewey et al., 1987; Kennell et al., 1987; Kennell and Pring, 1989). The reduction in the abundance of URF13 caused by *Rf1* is essential for restoration of pollen fertility; however, it is not sufficient to restore fertility without the activity of the *Rf2* gene. It is uncertain

how *Rf2* participates in pollen restoration, although there is no evidence that it alters the expression of T-*urf13*, as *Rf1* does. It is also unclear how *Rf1* reduces T-*urf13* expression; the gene could affect either translational events or RNA processing.

Studies of *cms-T* revertants have provided the best genetic evidence that T-*urf13* is responsible for the CMS and disease susceptibility phenotypes. Revertant plants that are male fertile and disease resistant have arisen in cell culture experiments with *cms-T* maize. This was unexpected because spontaneous reversion of *cms-T* plants to male-fertile and disease-resistant types has never been reported. When *cms-T* maize calli are grown on a medium containing BmT toxin, toxin-resistant calli can be selected that give rise to male-fertile plants (Gengenbach et al., 1977; Umbeck and Gengenbach, 1983). Normally, *cms-T* calli are sensitive to toxin and unable to survive on toxin-containing medium. Surprisingly, when these same experiments were conducted without toxin selection, similar male-fertile and disease-resistant revertants were occasionally obtained. This unexpected outcome—that is, the obtaining of revertants even without toxin selection—suggests that the T-*urf13* gene confers a selective disadvantage to callus cells that is independent of toxin sensitivity (Pring et al., 1988).

Approximately 200 plants have been regenerated from toxin-resistant calli; these plants or their progeny have all been male fertile (Gengenbach and Green, 1975; Gengenbach et al., 1977; Brettell et al., 1979, 1980; Gengenbach et al., 1981; Umbeck and Gengenbach, 1983; Kuehnle and Earle, 1989). Approximately a dozen revertants have been analyzed by restriction enzyme mapping and nucleotide sequencing of their mtDNAs. Most commonly, the T-*urf13* gene had been deleted from the mitochondrial genome by recombination (Rottmann et al., 1987; Wise et al., 1987b; Fauron et al., 1990). A more detailed analysis of several revertants has shown that the T-*urf13* gene was deleted by a complex series of intra- and intermolecular recombinations followed by the selective elimination of some of the resultant subgenomic circles (Fauron et al., 1990, 1992). One unusual revertant, designated T4, contains a change in the T-*urf13* gene that causes a frameshift mutation and a premature stop codon, resulting in a truncated URF13 of only 74 amino acids (Wise et al., 1987b). Because the simultaneous loss of CMS and disease susceptibility is linked to the deletion or mutation of the T-*urf13* genes in the revertants, T-*urf13* is believed to be responsible for both traits. The revertant studies also suggest that CMS and disease susceptibility are inseparable; however, this has not been demonstrated rigorously.

It is well established that T-*urf13* is responsible for sensitivity to the fungal pathotoxins produced by *B. maydis* race T and *P. maydis* as well as the insecticide methomyl. This was first shown by pathotoxin studies performed in *Escherichia coli* expressing the T-*urf13* mitochondrial gene (Dewey et al., 1988). *E. coli* not expressing URF13 are insensitive to both pathotoxins and methomyl. In contrast, *E. coli* expressing URF13 are sensitive to these compounds, which cause massive ion leakage, spheroplast swelling, and inhibition of glucose-driven respiration and growth in URF13-expressing cells. These toxic effects are similar to the adverse effects the pathotoxins and methomyl have on mitochondria from *cms-T* maize (for reviews, Levings, 1990; Levings and Siedow, 1992). Furthermore, URF13 expressed in *E. coli* is associated with the plasma membrane fraction (Dewey et al., 1988), which is analogous to its location in the inner mitochondrial membrane of *cms-T* maize. Ion uptake experiments in *E. coli* have shown that the toxin–URF13 interaction permeabilizes the plasma membrane (Braun et al., 1989). A parallel response to pathotoxin and URF13 takes place in the mitochondria of *cms-T* maize (Holden and Sze, 1987). These results demonstrate that the toxic response is due to the capacity of URF13 to form membrane pores in the presence of toxin. In addition, T-*urf13* has also been shown to confer toxin sensitivity to yeast mitochondria (Glab et al., 1990; Huang et al., 1990), transgenic tobacco (von Allmen et al., 1991), and insect cells and larvae (Korth and Levings, 1993).

HOW URF13 CAUSES CMS

The puzzling question is, how can mitochondrial dysfunction interfere specifically with pollen development? Plant mitochondrial genes encode polypeptides that are components of the electron transport system (Complex I, III, IV), the F_0F_1 ATP synthase, and the mitochondrial protein translational system (Eckenrode and Levings, 1986; Newton, 1988). They also code for structural RNAs, such as the 26S, 18S, and 5S rRNAs, and for tRNAs. Maturase and reverse transcriptase genes may also be coded by the mitochondrial genome; however, proteins encoded by these genes have not been demonstrated. Mutations among this restricted array of essential genes are predicted to directly affect the electron transfer system, ATP formation, or the translation of mitochondrial messengers. It is difficult, therefore, to understand how mutations that affect these particular genes could block pollen formation specifically.

Because electron transfer, ATP formation, and mitochondrial translation are essential functions in every phase of plant growth and development, mitochondrial gene mutations that interfere with these activities are likely to be extremely deleterious or even lethal. The various nonchromosomal stripe (NCS) mutants that carry lesions in essential mitochondrial genes are an example of how such mutations profoundly disturb the normal phenotype (Newton, 1988; Newton et al., 1990). These mutant plants contain sectors of reduced and normal growth in the leaves. To survive, the NCS mutants apparently must be maintained as chimeras containing mixtures of defective and functional mitochondria. In contrast, most CMS plants have phenotypes that are nearly normal except for pollen fertility. This is particularly underscored by the fact that CMS has been exploited in crop plants, in which anything other than normal growth, development, and productivity is unacceptable. This means that CMS is unlikely to result from a major lesion in an essential mitochondrial gene.

It is unclear whether any essential mitochondrial gene mutations are responsible for CMS. In *cms-T* maize, the T-*urf13* gene is a unique chimeric sequence that encodes a nonessential polypeptide. The usual complement of essential mitochondrial genes appears to be normal in expression and

function in *cms-T* maize. The same situation seems to be true for the *pcf* gene, which is associated with CMS in petunia (Young and Hanson, 1987), and which is also a chimeric sequence that encodes a nonessential polypeptide. There are other CMS types for which preliminary studies have suggested that mutations of essential genes are responsible. In many of these CMS types, a correlation between an altered gene and/or its expression and CMS is the only evidence suggesting that the two are linked mechanistically. Without stronger data, it may be premature to implicate mutations of essential mitochondrial genes in CMS.

Almost any mitochondrial gene or its product could be involved in CMS if there is something unusual or different about the way mitochondrial genes are expressed or about the way they function in the anther during pollen development. Some mitochondrial gene mutations could have little or no effect on mitochondrial function in most plant cells but could have profoundly deleterious effects in anther cells. In fact, a human disease associated with mitochondrial dysfunction provides evidence that mutant mitochondrial genes may have different effects in different tissues. Leber's hereditary optic neuropathy, which causes optic nerve degeneration and cardiac dysrhythmia, has been attributed to a point mutation in subunit 4 of complex I (NADH dehydrogenase subunit 4; Wallace et al., 1988). This mitochondrial mutation lessens but does not eliminate the respiratory function of this subunit. Importantly, these studies have indicated that mitochondrial mutations may not have effects in all tissues, even though the mutant gene product is expressed in all cell types. That is, a minor impairment of mitochondrial function may result in the degeneration of only certain cells and tissues. Furthermore, Wallace (1989) has suggested that selective degeneration of certain tissues observed for a human mitochondrial defect is caused by differences in the relative importance of mitochondrial function in different cell types. An analogous situation may exist in plants, in which a CMS gene manifests its adverse effect only in certain cells involved in pollen development, whereas other cell types are relatively unaffected.

A possible explanation for the specific effects of CMS mutations on pollen development is that demands on the mitochondria of anther cells are greatest at the time of pollen development. There is a 40-fold increase in mitochondria per cell in the tapetal cell layer of the maize anther and a 20-fold increase in the sporogenous cells (Warmke and Lee, 1977, 1978; Lee and Warmke, 1979). These mitochondrial amplification events suggest an increased demand for energy during pollen formation. Male gamete abortion in *cms-T* occurs soon after the rapid mitochondrial divisions. In *cms-T* maize, the first signs of sterility occur when the tapetal and adjacent middle-cell layers become disorganized soon after meiosis, resulting in precocious tapetal vacuolation and degeneration. Rapid increases in mitochondrial numbers such as those observed in the tapetum have not been seen in other maize cell types, including cells of the developing ear. A mutant mitochondrial gene product could be a serious impairment to development under conditions in which unusually heavy demands for energy exist. Under these conditions, proteins such as URF13 that are located in the inner mitochondrial membrane could interfere with the rapid amplification of mitochondria or, perhaps, with the electron transfer or phosphorylation processes. Anther mitochondria may thus be uniquely sensitive to a mutant mitochondrial gene product—a sensitivity that does not occur in other plant cells.

Alternatively, the same mechanism that is responsible for pathotoxin sensitivity in *cms-T* maize could also cause CMS. Even before the discovery of URF13 and its capacity to interact with toxin to form membrane pores, Flavell (1974) proposed the existence of an anther-specific substance that interacts with the Texas (T) cytoplasm to cause CMS. In our extension of this model to include T-*urf13*, the anther-specific substance is expected to interact with the URF13 protein to permeabilize the inner mitochondrial membrane, resulting in mitochondrial dysfunction and cell death. The proposed anther-specific substance should have properties similar to T-toxins or methomyl; however, it is especially important that its expression be limited to the anther because URF13 is present in other plant organs. The precocious death of anther cells, particularly the tapetal cell layer, which plays an essential role in pollen development (see Goldberg et al., 1993, this issue), is expected to interfere with normal pollen development and lead to sterility. The existence of such an anther-specific compound is feasible because it is known that anthers carry out specialized functions and that unique compounds are synthesized in anthers (e.g., sporopollenin; Bedinger, 1992). This model is also attractive because it is already known that URF13 has the capacity to destroy mitochondrial activity and cause cell death—events that could lead to pollen abortion if they occurred in the anther.

We have made several attempts to find a compound unique to the anther that can interact with the URF13 protein to permeabilize membranes (R.E. Dewey, C.J. Braun, and M.E. Williams, unpublished data). One approach was to use an *E. coli* bioassay to detect anther compounds with properties analogous to T-toxins or methomyl. Anther extracts were placed on paper disks on top of bacterial lawns expressing URF13 to test the capacity of the extract to diffuse out and kill the surrounding bacteria in a fashion similar to methomyl or T-toxin. Although this assay is sensitive enough to detect small amounts of methomyl, it was unable to detect any anther-specific compound capable of interacting with URF13 to kill bacterial cells. Our negative experimental results make the notion that an anther-specific compound is involved in causing CMS less attractive, but they do not eliminate this possibility.

URF13 TOXICITY

URF13, which is confined to the inner mitochondrial membrane, is expressed in roots, shoots, ears, and leaves as well as in tassels. Although URF13 is not toxic to maize cells other than those of the anther, it is likely that URF13 has some effects on plant growth and development independent of its effects on pollen fertility. Duvick (1965) has reported differences in morphological traits between T and normal cytoplasms in

common inbred backgrounds. These studies showed small (1 to 3%) but statistically significant reductions in plant height, leaf number, and grain yield due to the T cytoplasm. Separately, cell culture experiments have suggested that mutant *cms-T* calli that no longer express URF13 have a selective advantage over calli expressing URF13 (see above). Together, these observations indicate that URF13 can adversely affect other cell types besides those of the anther.

There is ample evidence from transgenic experiments that URF13 is toxic to cell viability. In *E. coli*, T-*urf13* has been introduced into several plasmid vectors behind strong, inducible promoters (R.E. Dewey, C.J. Braun, and M.E. Williams, unpublished data). After induction, URF13 builds up to levels that are lethal to cell growth. In fact, it is necessary to clone T-*urf13* behind inducible rather than constitutive promoters to maintain the cloned sequence. Induced bacterial cells expressing URF13 fail to divide properly, so that "snakelike" structures are observed under the microscope that are due to the failure of cytokinesis. When T-*urf13* is cloned behind a leaky promoter (e.g., the *tac* promoter in certain bacterial strains) (G.C. Ward and M.E. Williams, unpublished data), low levels of URF13 are expressed that affect bacterial growth. Colonies with leaky expression are evident because they grow slowly and appear translucent. Thus, it is clear that URF13 affects bacterial viability, heavy expression leading to cell death and leaky expression to slow, impaired growth. These effects on cell viability are independent of toxin sensitivity because bacterial cells expressing toxin-insensitive mutant forms of URF13 also show a loss of viability.

We have expressed URF13 in insect cell cultures and larvae using the baculovirus expression system (Korth and Levings, 1993). Although these experiments were initially conducted to produce large amounts of the URF13 protein for other studies, they have also provided us with important insights into the toxicity of URF13 in insect cells. *Spodoptera frugiperda* (fall armyworm) cells (Sf9) infected with baculovirus expressing *urf13* produced URF13 that is localized in the membranes as multimeric structures. When Sf9 cells expressing URF13 were treated with methomyl or T-toxin, their plasma membranes were permeabilized. In contrast, neither cells expressing the toxin-insensitive mutant URF13 (39/40) nor control baculovirus-infected cells were affected by the addition of toxins. (The URF 13 39/40 mutant contains amino acid changes at positions 39 and 40 that render it toxin insensitive in *E. coli.*) Thus, toxin sensitivity can be conferred on Sf9 cells by expressing URF13 via the baculovirus.

As it is to bacterial cells, URF13 is also toxic to insect cells grown in culture without T-toxin or methomyl. Even the T-toxin–insensitive mutant form of URF13 (URF13 39/40) is lethal to Sf9 cells; it is as lethal as the standard toxin-sensitive form of URF13. The toxic nature of URF13 has also been demonstrated in *Trichoplusia ni* (cabbage looper) by injecting baculovirus expressing URF13 into larvae (Korth and Levings, 1993). Larvae injected with baculovirus expressing either URF13 or the toxin-insensitive mutant URF13 (39/40) die ~50 hr after injection, whereas larvae injected with control baculovirus die ~100 hr after injection. These results indicate that the URF13 protein is lethal to insects independent of T-toxin or methomyl effects.

MECHANISMS FOR CMS

It is evident from the various transgenic studies that URF13 is toxic to a wide variety of organisms. How URF13 adversely affects viability has not yet been determined in either bacterial or insect cells. It is possible that compounds indigenous to these organisms interact with URF13 to permeabilize the cell or organellar membranes in a fashion analogous to the T-toxin–URF13 response. Alternatively, URF13 may affect viability by a mechanism that does not depend on the formation of membrane pores. This is suggested by the finding that the toxin-insensitive mutant URF13 (39/40) is as detrimental to insect cell culture and larvae viability and to bacterial growth as the standard URF13 protein. Because the URF13 (39/40) mutant protein is unable to interact with T-toxin or methomyl to permeabilize membranes, its toxic effect must occur by different means. It may be that the accumulation—or perhaps the overaccumulation—of URF13 leads to mitochondrial dysfunction. Although it is unclear how this could occur, URF13 has been found to be associated with each of the individual electron transfer complexes from *cms-T* maize mitochondria, presumably as a result of nonspecific interactions (S. Ferguson-Miller, W.E. Peiffer, and K.L. Korth, personal communication). Thus, binding of URF13 to mitochondrial membrane proteins might disrupt normal mitochondrial function.

Topological studies of URF13 in the membrane have been useful in understanding how membrane pores are formed by the toxin–URF13 interaction (Korth et al., 1991). URF13 crosses the inner mitochondrial membrane three times, and the carboxyl terminus of URF13 is oriented toward the mitochondrial matrix in maize (Korth et al., 1991). Furthermore, cross-linking studies show that URF13 exists as monomers, dimers, trimers, and other oligomeric structures in the membrane. A relationship between CMS and the structure of URF13 in the membrane, however, remains unclear.

PARALLELS WITH CMS IN PETUNIA

Petunia CMS and the mitochondrial locus that probably encodes it, *pcf*, have also been studied thoroughly. These investigations have revealed many parallels between the petunia and maize *cms-T* type of CMS (Hanson, 1991). *pcf*, like T-*urf13*, is a fusion gene; *pcf* contains part of the coding region of *atp9*, part of each exon of *coxII*, and an unidentified reading frame called *urf-S* (Young and Hanson, 1987). *urf225*, an open reading frame that encodes a mitochondrial membrane polypeptide of unknown function, is 77 nucleotides downstream from the maize T-*urf13* gene (Prioli et al., 1993). *urf225* is probably a standard mitochondrial gene because it is present in other plant mitochondrial genomes, including

tobacco (Stamper et al., 1987) and petunia (Folkerts and Hanson, 1989). Two mitochondrial genes, *nad3* and *rps12*, are located closely downstream of the petunia *pcf* gene. It is possible that the downstream proximity of these nearby mitochondrial genes to the abnormal chimeric genes could disrupt normal expression and cause sterility in maize and petunia. This proposal is considered unlikely because there is no evidence to support it in either maize or petunia CMS.

In petunia, antibodies prepared from synthetic peptides have identified a 25-kD protein associated with the CMS-associated gene *pcf*. This 25-kD protein is completely absent in fertile lines and is much reduced in fertility-restored petunia lines (Nivison and Hanson, 1989). Thus, in *cms-T* maize and petunia, nuclear restorer genes reduce the abundance of the unique polypeptides associated with the CMS type. These results suggest that *pcf* and T-*urf13* produce toxic protein products and that their nuclear restorer genes function by affecting the abundance of these toxic products.

Consistent differences among CMS, fertile, and fertile-restored petunia lines were found in the partitioning of electron transport through the cytochrome oxidase and alternative oxidase pathways in suspension culture and immature anthers (Connett and Hanson, 1990). Reduced alternative oxidase activity was consistently associated with CMS petunia lines, and alternative oxidase activity was restored in fertility-restored petunia lines (Hanson, 1991). Currently, the interesting relationship between CMS in petunia and alternative oxidase activity and how it might cause pollen sterility remains a puzzle. In contrast, differences in alternative oxidase activity have not been detected when *cms-T*, *cms-T*–restored, and fertile maize were compared (C.S. Levings and J.N. Siedow, unpublished data).

EPILOGUE

The toxic nature of URF13 furnishes an attractive explanation for the *cms-T* type of pollen sterility. The toxic URF13 protein could interfere with pollen development by impairing or killing anther cells essential to pollen formation. Why anther cells, such as the tapetal cell layer, should be more sensitive to URF13 than are other cell types is puzzling. One possibility is that URF13 may be overexpressed in anther cells, resulting in highly toxic levels that cause cellular dysfunction, death, and the cessation of pollen development. Overexpression of URF13 could be related to the increased levels of mitochondrial biogenesis observed in the tapetum during pollen formation. Alternatively, anther cells, especially the tapetum, may be more sensitive to URF13 than are other cell types. Because unique functions pertinent to pollen formation occur in these cells, it is possible that these cells with their special functions are more vulnerable to the toxicity of URF13 than are other cell types. Although there are still many unanswered questions, exciting leads are emerging that should soon bring about an understanding of the CMS mechanism.

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