THE GENETICS, PATHOLOGY, AND MOLECULAR BIOLOGY OF T-CYTOPLASM MALE STERILITY IN MAIZE*

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*Product names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

I. INTRODUCTION

Texas cytoplasmic male sterility (cms-T or T cytoplasm)[†] in maize has been studied intensely since its discovery in the 1940s. It is the most environmentally stable system for producing hybrid seed in maize and, as such, dominated the maize seed industry in the United States and elsewhere throughout the 1960s. In 1969, however, T-cytoplasm lines exhibited a high degree of susceptibility to two fungi, *Mycosphaerella zeae-maydis* and race T of *Cochliobolus heterostrophus,* which induce severe disease by the production of β-polyketol toxins. In 1970, an epidemic caused by *C. heterostrophus* effectively ended the use of cms-T for commercial maize production. The epidemic emphasized to breeders and others the real and potential dangers of genetic homogeneity in modern cropping systems.

In contrast, the discovery that T-cytoplasm lines are acutely susceptible to the fungi and their toxins has proven a boon to research on mechanisms of male sterility in maize. Research stimulated by the epidemic has shown that male sterility is controlled by the same mitochondrial gene that is responsible for sensitivity to the fungal toxins. In fact, identification and cloning of the gene was facilitated by the use of toxin sensitivity, rather than male sterility, as an assay for the presence of a functional gene. This gene, T-*urf13*, encodes an oligomeric protein, URF13, assembled in the inner mitochondrial membrane. Binding of the fungal toxins to URF13 causes membrane leakage and disruption of mitochondrial function; thus, the toxins were crucial in demonstrating that the URF13 protein may have the capacity to form a pore spanning the inner mitochondrial membrane. The toxins may also provide insight into how T-*urf13* causes male sterility. In cms-T plants, male sterility is associated with premature breakdown of the mitochondria-rich, tapetal cell layer of the anther; this layer is crucial to pollen production because it supplies nutrients to the developing microspores. The ability of the toxins to cause mitochondrial dysfunction suggests that male sterility may be due to an endogenous, toxin-like compound in maize that interacts with URF13 to cause mitochondrial dysfunction and subsequent death of the tapetal cells. Because of the wealth of molecular information available, cms-T now serves as a model for studies of male sterility and fertility restoration in plants.

This review complements previous reviews on cms-T (Duvick, 1965; Edwardson, 1970; Ullstrup, 1972; Levings and Pring, 1979; Laughnan and Gabay-Laughnan, 1983; Kaul, 1988; Pring and Lonsdale, 1989; Levings, 1990, 1993; Levings

[†]Nomenclature: According to the present maize nomenclature, loci and recessive alleles are designated by lowercase symbols; for example, the *rf1* allele of the *rf1* locus is a recessive mutant. Dominant alleles are designated by uppercase symbols; for example, the *Rf1* allele of the *rf1* locus is wild type. Lines that carry T cytoplasm (sterile or fertile) are referred to as T-cytoplasm lines. Male-sterile lines that carry T cytoplasm are designated cms-T. Restored T cytoplasm designates lines restored to fertility via the presence of nuclear restorer genes. N-cytoplasm lines are male fertile.

and Seidow, 1992; Williams and Levings, 1992; Dewey and Korth, 1994; Ward, 1995) by providing, in addition to the directly pertinent information regarding the mechanisms of toxin sensitivity and male sterility, information about the production of hybrids, the cytology of male sterility, the mechanisms of pathogenesis in *C. heterostrophus* and *M. zeae-maydis,* and recent breakthroughs in understanding fertility restoration in cms-T. This review should be useful, therefore, to students new to cms-T research, as well as to advanced investigators. At the end of the review are commentaries by prominent researchers that provide the reader with insights gained from years of work with cms-T.

II. CYTOPLASMIC MALE STERILITY SYSTEMS

Heterosis plays a key role in applied plant breeding programs. F_1 progeny resulting from a cross between two (usually inbred) lines often exhibit enhanced expression of one or more positive characters relative to both parents. This improved condition is termed hybrid vigor or heterosis. Because selected maize hybrids display significant amounts of heterosis, they are widely used for agricultural production.

To produce hybrids, pollen must be transferred from one parent (the male donor) and used to fertilize another (the female recipient). In addition, the female parent must not produce pollen that can compete with that provided by the male parent. Self-pollination of the female parent can be avoided by removing the male floral organs (emasculation), disrupting the development of these structures, or otherwise preventing the female parent from producing functional pollen. For example, the pollen-bearing tassel of maize can be removed from the female parent using machine or hand emasculation (Fig. 1, see color plate). However, these procedures can reduce the yield of F_1 seed from the female parent and they are expensive.

Alternatively, the female parent can be provided with a genetic constitution that ensures that it will be male sterile. Male sterility results when plants do not form complete anthers; or when the internal male cells of the anther (microspore mother cells, microspores, or pollen) become nonfunctional during some stage of development; or when the anthers fail to release pollen. Mutations that cause these effects have been described in nuclear and mitochondrial genes from many plant species (Laser and Lersten, 1972; Palmer *et al.,* 1992), including a large number of crops, such as maize (Duvick, 1965). Although methods have been proposed to use nuclear male-sterile mutants to facilitate the production of hybrid seed (Jones *et al.,* 1957), none have proved practical, at least in maize. However, it has been possible to develop efficient male sterility systems in maize by using nuclear genes (restorers) that complement cytoplasmic mutations inherited solely through the maternal line.
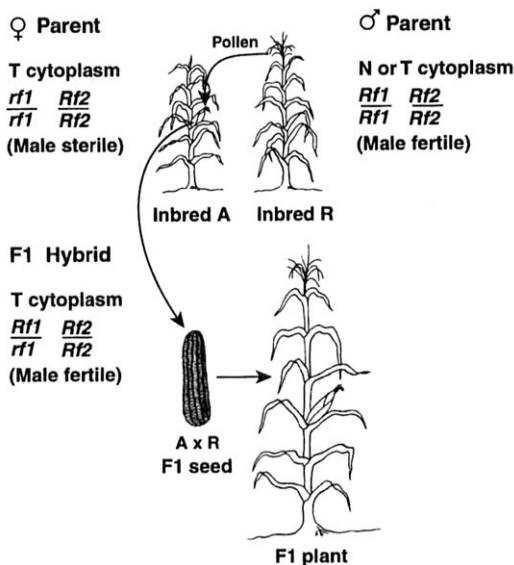
Figure 2 Production of F_1 hybrid seed from two inbred lines using the cms-T maize system. An A line that is male sterile because it carries T cytoplasm, but not all of the nuclear restorers required for fertility restoration, is used as the female parent. An R line that is male fertile and carries the necessary restorers serves as the male parent. Because the A and R lines are planted in isolation from other maize, the only pollen available to fertilize the A line is derived from the R line. Hence, all seed set on the A line is hybrid. Although plants produced from this F_1 seed will carry the maternally transmitted T cytoplasm, they will be male fertile due to the paternal contribution of nuclear restorers (Rf).

Commercial maize hybrid seed production requires the use of three lines: an A line (the female parent of the F_1) that is cms; a B line (the maintainer of the A line) that is isogenic relative to the A line, but that carries a normal (N) cytoplasm and is therefore male fertile; and an R line that carries the necessary restorer of fertility genes (designated Rf) and serves as the male parent of the F_1 (Fig. 2). The cms-A line is formed by backcrossing a male-fertile line to another line that carries a cms cytoplasm. One or both of these parents must lack a necessary nuclear Rf gene. This creates a new pair of A and B lines. An R line is formed by backcrossing Rf genes into the desired male parent of the F_1 hybrid. On a commercial scale, F_1 seed is produced by planting alternating rows (4–6) of the cms-T, male-sterile, female-fertile A line with one or two rows of the male-fertile R line. The F_1 hybrid seed is harvested from the A line.

Three major groups of male-sterile cytoplasms have restorer capabilities and, hence, the potential to be used for the production of hybrid seed, S (USDA), C (Charrau), and T (Texas). These cytoplasms have different nuclear genes that suppress their associated male-sterile phenotype and restore normal pollen development (Duvick, 1965; Beckett, 1971; Gracen and Grogan, 1974; Laughnan and

Gabay-Laughnan, 1983). They can also be distinguished from each other by mitochondrial DNA (mtDNA) restriction endonuclease profiles (Levings and Pring, 1976; Pring and Levings, 1978; Borck and Walbot, 1982) and by characteristic polypeptide patterns resulting from [^{35}S]methionine incorporation by isolated mitochondria (Forde *et al.,* 1978; Forde and Leaver, 1980).

The male-sterile C, S, and T cytoplasms produce fertile plants only in nuclear backgrounds carrying the appropriate restorer genes. These nuclear-encoded fertility-restoring genes compensate for cytoplasmic dysfunctions that are phenotypically expressed during microsporogenesis and/or microgametogenesis. Plants carrying S and C cytoplasms are restored to fertility by single dominant alleles of the *rf3* and *rf4* genes, respectively (Laughnan and Gabay, 1978; Kheyr-Pour *et al.,* 1981; Laughnan and Gabay-Laughnan, 1983). In contrast, T cytoplasm is restored to fertility by the combined action of dominant alleles of the *rf1* and *rf2* genes (Duvick, 1956, 1965; Laughnan and Gabay-Laughnan, 1983). Of the three types of cms found in maize, the cms-T type is the most stable under all environmental conditions and was the primary type used in maize hybrid seed production through 1970.

III. CMS-T CAUSES PREMATURE DEGENERATION OF THE TAPETUM

In normal, male-fertile plants, pollen production is the result of a precisely timed sequence of events known as microsporogenesis and microgametogenesis (Mascarenhas, 1988, 1990; Koltunow *et al.,* 1990; Goldberg *et al.,* 1993). Disruption or incorrect timing of these events may lead to the failure of pollen development or the prevention of pollen release (Beals and Goldberg, 1997). It is now clear that male sterility in cms-T is caused by premature degeneration of the tapetum, a metabolically active cell layer of the anther.

The processes of pollen formation in male-fertile maize and abortive development in cms-T maize are diagrammed in Fig. 3. Each anther consists of four microsporangia (sacs) held together by connective tissue (Palmer *et al.,* 1992; Goldberg *et al.,* 1995; Horner and Palmer, 1995). The earliest stage at which all of the anther tissues are distinguishable is the sporogenous mass stage. During this stage, the sporogenous cells undergo DNA synthesis, secrete callose walls around themselves, and become somewhat rounded. Meiosis occurs during the meiocyte, dyad, and tetrad stages. Following meiosis I at the dyad stage, a partitioning callose wall forms and separates the two resulting nuclei. Following meiosis II at the tetrad stage, two more callose walls form that separate the four resulting nuclei and their cytoplasms. Individual tetrads, each of which now consists of four microspores, are encased in callose. A thin microspore wall forms interior to the callose around each microspore, and within each thin wall a single pore is delimited.
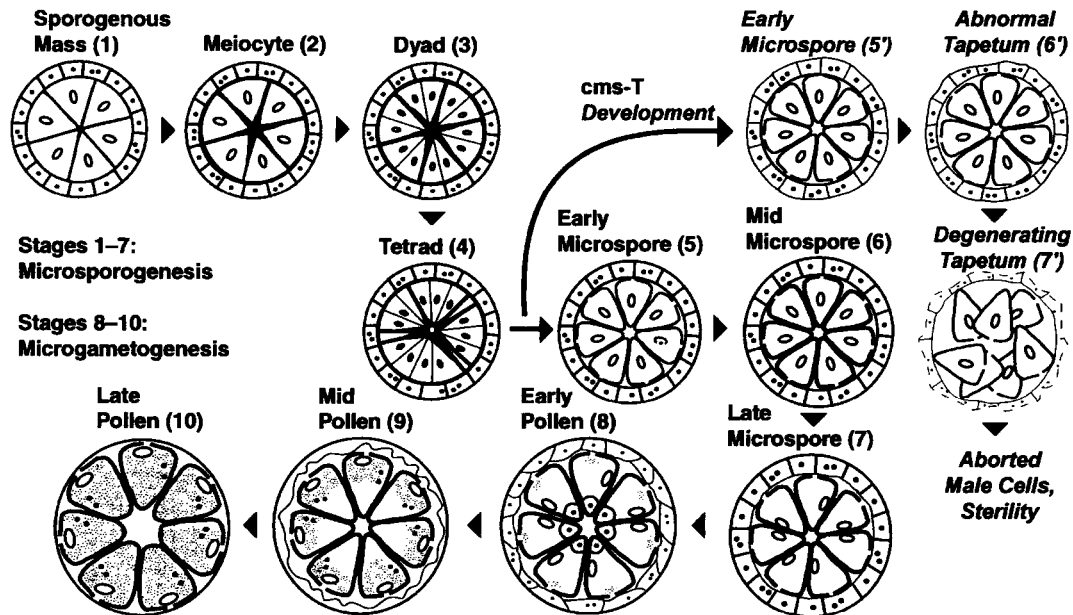
Figure 3 Scheme showing the stages of microsporogenesis (1–7) and microgametogenesis (8–10) in fertile anthers of maize and stages that lead to tapetal degeneration and male-cell abortion in anthers of cms-T maize (5′–7′). Each stage depicts only one of four locules in an anther and includes only affected cells, namely male cells and the surrounding tapetum. cms-T tapetal cells show initial signs of degeneration at about the early microspore stage, and by the mid to late microspore stages the tapetum and male cells appear in various states of degeneration. In most circumstances, no pollen is produced by cms-T plants.

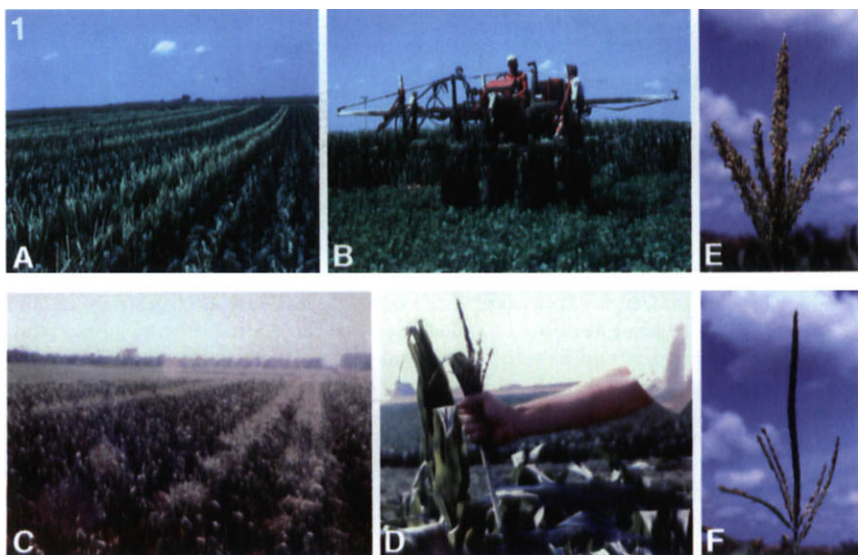**Figure 1** Photographs of emasculated maize fields and individual tassels of fertile and cms-T plants. (A) Machine-emasculated field. (B) A tractor with boom carries modified lawn mowers separated at distances to emasculate rows that serve as female parents. (C) Hand-emasculated field. (D) Hand removal of tassel (emasculation). (E) Tassel of male-fertile plant. (F) Tassel of cms-T plant (male sterile).
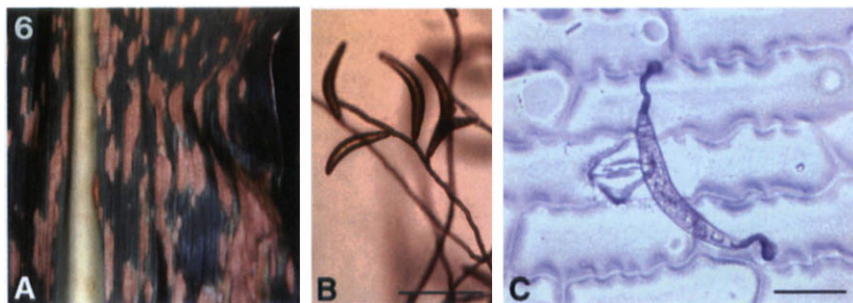
Figure 6 Aspects of southern corn leaf blight. (A) Lesions caused by race T of *Cochliobolus heterostrophus* on leaves of T-cytoplasm maize. Lesions, which may extend up to 27 mm long and 12 mm wide, are spindle-shaped due to their expansion beyond the leaf veins. In contrast, lesions of race T on N-cytoplasm maize, or race O on either N- or T-cytoplasm maize are small, generally less than 15 mm long and 1 to 3 mm wide; lesions are parallel sided due to their restriction by the leaf veins. (B) Conidia of *C. heterostrophus*. Conidia, which measure 30 to 115 μm in length and 10 to 17 μm in diameter, are produced on the surface of infected maize leaves or infested debris under moist conditions. (Photograph by E. J. Braun, Iowa State University.) (C) Infection of maize by *C. heterostrophus*. In presence of free moisture, conidia germinate from both ends to form germ tubes which grow along the leaf surface until they reach depressions formed at the junctions between epidermal cells. At the ends of germ tubes, swellings, known as appressoria, form. Penetration of corn leaves occurs below appressoria. Conidia are shown on a plastic replica of a corn leaf. (Photograph by E. J. Braun, Iowa State University.)
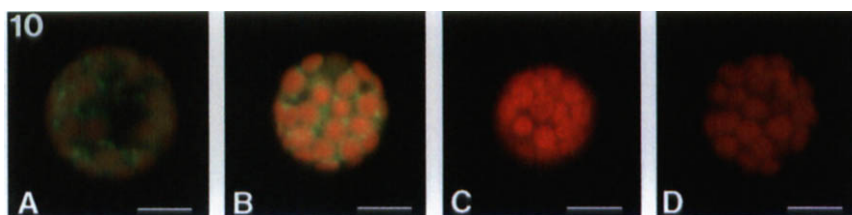
Figure 10 Isolated N-cytoplasm and T-cytoplasm maize leaf protoplasts, untreated or treated with the cationic potential-sensitive fluorescence dye 3,3′-dihexyloxacarbocyanine iodide to indicate metabolically active and inactive mitochondria when treated with T-toxin and respiratory inhibitors. (A) Untreated N-cytoplasm protoplast showing active mitochondria (bright dots). (B) Untreated T-cytoplasm protoplast showing active mitochondria (bright dots). (C) T-cytoplasm protoplast treated with carbonyl cyanide *m*-chlorophenylhydrazone, a respiratory inhibitor; no mitochondria fluoresce. (D) T-cytoplasm protoplast treated with T-toxin; no mitochondria fluoresce. Bars = 17 μm. N-cytoplasm protoplast mitochondria do not fluoresce when treated with the respiratory inhibitor, but they do when treated with T-toxin (not shown).

Each pore is oriented toward the tapetum, the cell layer immediately surrounding the microspores. This layer, which consists of a mixture of uni- and bi-nucleate cells (Horner *et al.*, 1993), functions in secretion, providing an array of substances needed by the developing male cells (Pacini *et al.*, 1985; Pacini, 1997). The tapetum is thus considered critical for normal pollen development (Laser and Lersten, 1972). The very high metabolic activity of these cells is reflected in the number of mitochondria present. Tapetal cells have about twice the number of mitochondria as sporogenous cells and about 40 times the number of mitochondria as most maize somatic tissues (Lee and Warmke, 1979). The tapetal layers of N and cms-T lines are similar; however, cms-T lines have a consistently greater frequency of cells that are bi-nucleate versus uni-nucleate and, uni-nucleate tapetal nuclei of cms-T lines contain a higher level of DNA than their fertile counterparts (Figs. 4A and 4B; Horner *et al.*, 1993). The significance of these differences is not clear.

Following the tetrad stage, the callose around the tetrads is digested, releasing individual micropsores. All microspores remain pressed to the tapetum and, as the microspores round up and enlarge, the single nucleus moves to the microspore wall. Each microspore wall continues to thicken. These last stages are referred to as the early, mid, and late microspore stages. By the latter stage, the nucleus undergoes one mitotic division to form a pollen grain containing a generative cell and a tube cell. Interior to the microspore wall (exine), an inner wall (intine) is initiated, thus creating the pollen wall. At about this time, nutrient reserves form in each pollen grain as the tapetum degenerates. During the next two pollen stages, reserves continue to build up in each grain and the generative cell divides to form
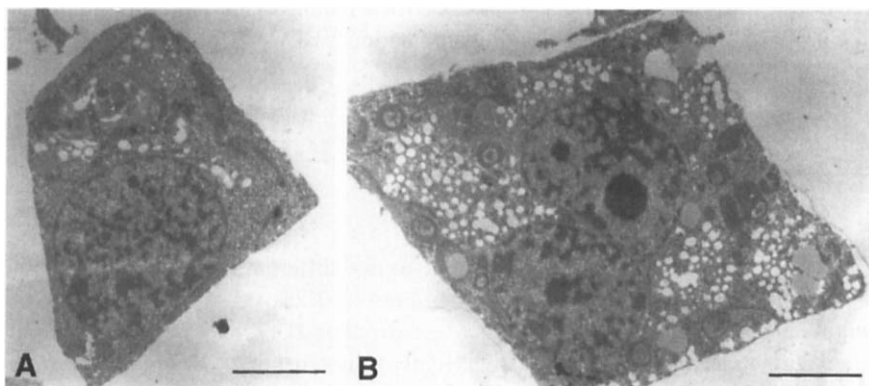
Figure 4   Electron micrographs of flow cytometrically isolated fertile uni- and bi-nucleate tapetal protoplasts from developing maize anthers. The ratio of bi-nucleate to uni-nucleate tapetal cells is greater in cms-T anthers than in fertile anthers, from sporogenous mass stage through microspore stage. (A) Uni-nucleate tapetal protoplast. (B) Bi-nucleate tapetal protoplast. Bars = 5 μm. Published with permission from Springer-Verlag, Austria from Horner *et al.* (1993).

two sperm cells. Each pollen grain (three-celled, male gametophyte) is engorged with reserves at the time the pollen is shed for wind pollination (Fig. 1E, see color plate).

In cms-T anthers, degeneration of the tapetum occurs prematurely, beginning at the dyad (Yang, 1989) to the early microspore stages (Warmke and Lee, 1977). Mitochondria in the tapetal cells and middle wall layer show signs of structural disorganization, becoming enlarged with a less dense matrix and more diffuse cristae (Gengenbach *et al.*, 1973; Watrud *et al.*, 1975b; Gregory *et al.*, 1977, 1978; Warmke and Lee, 1977; Yang, 1989). Dysfunction of the mitochondria precedes premature degeneration of the tapetum and abortion of the microspores (male cells) before they reach the pollen stage (Fig. 3). Thus, pollen does not form and the anthers do not exsert (Fig. 1F, see color plate).

The cause of premature tapetum degeneration in cms-T anthers is not known. However, given the very high metabolic activity of these cells, any mutation that disrupts mitochondrial function has the potential to result in premature cell death and male sterility (Warmke and Lee, 1978; Lee and Warmke, 1979; Levings, 1993). Mitochondria of cms-T tapetal cells differ from those of N-cytoplasm tapetal cells in the presence of the URF13 protein in the inner mitochondrial membrane. URF13 thus has the potential to interfere with normal mitochondrial function. cms-T mitochondria are also sensitive to the fungal toxins that interact with URF13. Based on these observations, a model has been suggested for tapetum degeneration in which an unknown "Factor X," acting similarly to the fungal toxins, disrupts mitochondrial function in the tapetal cells (Fig. 5; Flavell, 1974). Either Factor X is tapetal specific, or tapetal cells are especially sensitive to it, since the mitochondria of all cells in cms-T lines express URF13, but only the tapetum degenerates. Intriguing preliminary evidence for a factor in maize with these toxin-like properties is described by Levings in Section VIID.

IV. SOUTHERN CORN LEAF BLIGHT EPIDEMIC OF 1970

T-cytoplasm maize is highly susceptible to two different fungi, *Mycosphaerella zeae-maydis* (asexual stage: *Phyllosticta maydis*), causal agent of yellow leaf blight, and race T of *Cochliobolus heterostrophus* (Drechs.) Drechs. [asexual stage: *Bipolaris maydis* (Nisikado & Miyake) Shoemaker = *Helminthosporium maydis* (Nisikado & Miyake)], causal agent of southern corn leaf blight. Both fungi produce toxins that induce susceptibility in male-sterile and fertility-restored T-cytoplasm lines by disrupting mitochondrial function. The toxin from *M. zeae-maydis* is known as PM-toxin; the toxin from *C. heterostrophus* is known as T-toxin.
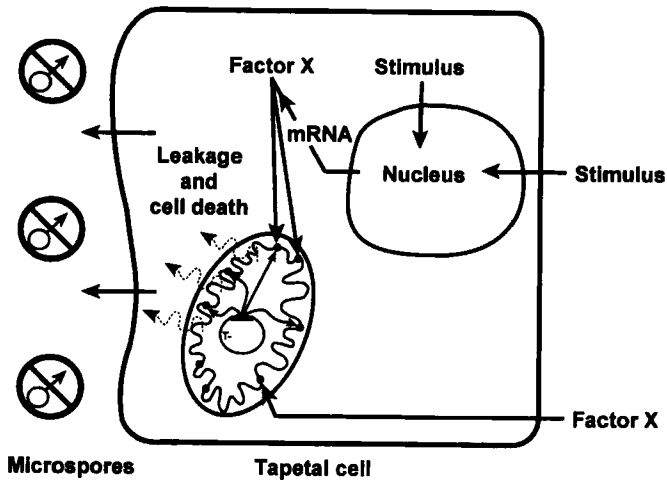
Figure 5 Model showing how cms-T tapetal cells might be triggered to break down prematurely, resulting in male-cell abortion and sterility. The model depicts just one of the two possible nuclei and just one of the many mitochondria in a tapetal cell. The URF13 protein is located in the inner mitochondrial membrane. An as yet unidentified, toxin-like "Factor X" interacts with URF13 to cause membrane leakage (Flavell, 1974). Factor X may be either imported from outside of the tapetal cell or produced within the tapetal cell as a result of gene expression following an unidentified stimulus from either within or outside of the tapetal cell.

Of the two fungi, the best known is *C. heterostrophus* because it caused a worldwide pandemic in 1970. The epidemic was most severe in the United States. The history of the epidemic has been reviewed previously (Tatum, 1971; Hooker, 1972; Ullstrup, 1972); however, it is worth repeating here because of its impact on both the utility of T cytoplasm as a source of male sterility and research into mechanisms of male sterility.

Prior to 1970, *C. heterostrophus* was considered a moderate leaf-spotting pathogen on maize and teosinte in tropical and subtropical regions (Drechsler, 1925). The disease favors warm, wet environments and is generally of little concern to growers in temperate climates. In the United States, it is restricted primarily to the southeastern states (Leonard, 1987). Only a single race of *C. heterostrophus,* which we now call race O, was known prior to the epidemic. Race O normally infects only leaf tissues, where it causes small, elongated, parallel-sided lesions up to 15 mm in length and 1–3 mm in width. The lesions are generally limited to a single interveinal region (Drechsler, 1925).

Perhaps because the disease was not considered a threat to maize in temperate climates, the first published report that T-cytoplasm lines might be highly suscep-

tible to *C. heterostrophus* went generally unnoticed. In 1961, Mercado and Lanti-
can reported that T-cytoplasm lines, which had been introduced into the Philip-
pines by 1957, were highly susceptible to *C. heterostrophus* (Mercado and Lanti-
can, 1961). On T-cytoplasm lines, *C. heterostrophus* caused irregularly shaped
lesions that coalesced, killing entire leaves and causing the plants to die prema-
turely. Efforts to backcross male sterility from these lines into resistant Philippine
inbred lines resulted in the conversion of the Philippine lines to susceptibility and
demonstrated that the high susceptibility was cytoplasmically inherited along with
male sterility (Mercado and Lantican, 1961; Villareal and Lantican, 1965). The
presence of restorer genes did not alter susceptibility (Villareal and Lantican,
1965).

Despite these early indications that T-cytoplasm maize might be highly suscep-
tible to *C. heterostrophus,* no signs of unusual susceptibility were noticed in oth-
er parts of the world and use of T-cytoplasm lines in temperate regions increased
during the 1960s. By 1970, 85% of the hybrid maize in the United States was T
cytoplasm (Ullstrup, 1972). In 1969, severe symptoms due to *C. heterostrophus*
were reported in the United States in a number of midwestern and central states
(Ullstrup, 1972; Scheifele *et al.,* 1970; Leonard, 1987). The symptoms, which
were attributed to a new race of the fungus called race T (Hooker *et al.,* 1970b;
Smith *et al.,* 1970), included foliar lesions up to 27 mm in length and 12 mm in
width. The lesions expanded beyond the leaf veins, giving them a spindle-shaped
appearance (Fig. 6A, see color plate). The high, specific virulence of race T was
attributed to its production of a toxin known as T-toxin, which specifically affects
maize lines with T cytoplasm (Hooker *et al.,* 1970a; Lim and Hooker, 1971, 1972).

Warm, wet conditions combined with inoculum of race T carried over from 1969
set the stage for a severe epidemic in the United States in 1970. The first reports
of serious damage were in Florida in January 1970 (Moore, 1970). In addition to
leaf infections race T attacked leaf sheaths, stalks, husks, shanks, ears, and cobs.
Lodging due to secondary infections was also common (Hooker, 1972). The dis-
ease spread rapidly from the southern states into the midwestern states, northern
states, and finally into southern Canada. Losses in the United States were estimat-
ed at 710 million bushels (Ullstrup, 1972). Losses were reported in other countries
as well. Severe damage due to race T was noted in West Africa in 1970 (Craig,
1971). Fortunately less favorable weather conditions and reduced planting of T-
cytoplasm lines resulted in only moderate damage in Brazil, thus reducing interna-
tional losses (Ullstrup, 1972).

In the United States, race T overwintered from 1970 to 1971 in corn debris on
the soil surface and in refuse piles, resulting in localized outbreaks in 1971. How-
ever, drier, cooler weather combined with the reduced planting of T-cytoplasm
lines in the spring of 1971 resulted in less severe disease and near record harvests.
By the winter of 1971, sufficient seed of N-cytoplasm lines was available to meet
demands for spring planting in 1972 (Ullstrup, 1972).

A. The Rise and Fall of Race T

The origin of race T is not known. It is clear that widespread planting of T-cytoplasm lines in the United States and elsewhere in the 1960s created a situation favorable for the rapid increase of race T. However, its source remains a mystery. Race T was not endemic, or at least not widespread, in most countries prior to 1970. The first known occurrence of race T in the United States was on seed stored in 1968 in Iowa (Foley and Knaphus, 1971), with subsequent occurrences in several midwestern and central states in 1969 (Leonard, 1987). In West Africa, race T was observed for the first time in 1970, even though T-cytoplasm lines had been grown there since 1963 (Craig, 1971). Race T was detected in Australia for the first time in 1972 (Alcorn, 1975). If race T had been endemic in these countries prior to 1970, it should have been detected earlier, shortly after T-cytoplasm lines were introduced.

Epidemiological studies have provided further evidence that race T was not endemic in the United States. Iowa, the site of the first known occurrence of race T in the United States, is well outside the normal endemic range of *C. heterostrophus*. The subsequent occurrences in 1969 in several midwestern and central states were also outside of the normal range. If race T had been endemic in the United States, or had arisen by mutation from the race O population in the United States, its first appearance would have been more likely in the southeastern states because of their prevalent race O population and more favorable environmental conditions. In addition, when race T first appeared in the United States, it was exclusively mating type 1, whereas the endemic race 0 population had equal frequencies of both mating types (Leonard, 1971). If race T had been in the United States for more than a few years before 1968, it should have included both mating types due to intermating with race O. Race T was also exclusively mating type 1 when it first appeared in Australia in 1972 (Alcorn, 1975).

In contrast, race T appears to have been in the Philippines when T-cytoplasm maize was first introduced there in 1957 (Mercado and Lantican, 1961). The disease symptoms reported on T-cytoplasm lines were typical of those we now associate with race T, whereas N-cytoplasm lines showed normal symptoms, indicating that the high virulence was not simply a function of the favorable climatic conditions in the Philippines. In addition, the unusual susceptibility was inherited cytoplasmically. The fact that T-cytoplasm lines were not grown on a widespread basis before the susceptibility was observed suggests that race T may have been present in the Philippines, at least at low levels, at the time of the introduction of T cytoplasm.

Given the available information, the simplest hypothesis is that race T was introduced into the United States from the Philippines or elsewhere in the mid-1960s and concurrently or subsequently spread to other countries. In fact, Leonard (1987) proposed that the epidemic in the United States arose from a single isolate of race T that became established in the Midwest during the mid-1960s. This hypothesis

is consistent with both the pattern of spread of race T and the distribution of mating types. Leonard further postulated that race T spread to Florida, where the epidemic began in January 1970, on contaminated seed from the Midwest planted in winter breeding nurseries (Leonard, 1987).

The frequency of race T in the United States declined rapidly after the epidemic and the withdrawal of T-cytoplasm lines from widespread use (Leonard, 1974, 1977a, 1987). If race T is still present, it is at very low levels. For example, race T symptoms have not been observed in experimental fields planted each year in Iowa from 1988 to 1997, even though these fields have contained up to 100,000 T-cytoplasm plants each year (R. P. Wise and P. S. Schnable, unpublished results). The decline of race T has been attributed to its reduced virulence relative to race O on N-cytoplasm maize; race T produces lesions that are 10 to 38% shorter than those produced by race O on the same genotypes (Leonard, 1977b; Klittich and Bronson, 1986). Race T also grows more slowly as a saprophyte than does race O; the reduction in growth measured as dry mass accumulation in 1 week is about 9% (Bronson, 1998). Both of these differences have been shown to be tightly linked to T-toxin production, suggesting that T-toxin production, or genes tightly linked to T-toxin production, reduce fitness when the fungus grows on substrates other than T-cytoplasm maize. A possible cause of the reduced fitness is the metabolic drain imposed by the synthesis of the toxin; T-toxin has been calculated to constitute 2% of the mycelial dry mass of race T (Tegtmeier *et al.,* 1982).

The low fitness of race T has raised questions about the timing of its evolution. If race T is as unfit everywhere as it is in the United States, it must have evolved shortly after T-cytoplasm lines became available as a host, otherwise, it would have never survived. Such a coincidental evolution at the time of widespread planting of T cytoplasm is possible, but unlikely. An alternative hypothesis is that race T can survive, at least at low levels, in some as yet undiscovered niche, for example, on a wild plant that is susceptible to β-polyketol toxins. Since race T was presumably present in the Philippines at the time of the introduction of T-cytoplasm lines, and since *C. heterostrophus* occurs predominately in the tropics and subtropics, regions such as the Philippines might be reasonable places to search for the origin of race T.

B. The Exceptional Virulence of Race T

The 1970 epidemic resulted in a variety of cytological, genetic, and molecular studies into how *C. heterostrophus* causes disease. This research has provided insight into the strategies used by races T and O to attack maize, and, more specifically, the mechanism by which race T causes severe disease on T-cytoplasm lines.

As far as is known, races T and O behave identically prior to penetration into maize tissues. The primary infectious propagules of *C. heterostrophus* are asexu-

ally produced spores known as conidia (Fig. 6B, see color plate). The conidia, which are wind-dispersed, are released from lesions on infected tissue, usually leaves, and from colonies in debris on the soil surface. Conidia germinate within about 20 min after hydration by rain or dew. The first visible sign of germination is the production of a two-layered mucilaginous matrix on the spore tips (Evans *et al.*, 1982). The inner layer is believed to be responsible for adhering the fungus to surfaces and thus preventing it from being washed off (Braun and Howard, 1994). The outer layer is thought to be necessary for lesion development once the fungus is inside the plant (Zhu *et al.*, 1998). Germ tubes, surrounded by the two matrix layers, emerge a few minutes later from both ends of the conidia. About 2 h after hydration, swellings, known as appressoria, form at the ends of the germ tubes. Most of the appressoria form over the depressions on leaf surfaces at the junctions between epidermal cells. This preference seems to be a response to the physical shape of the surface, rather than to any difference in chemical composition; the fungus shows a similar preference for depressions whether the surface is a maize leaf or a plastic replica of a maize leaf (Fig. 6C, see color plate). Penetration generally occurs within 3–4 h by the production of a penetration peg below the appressorium. Penetration is believed to be enzymatic because of the relatively undifferentiated appressoria and the degraded appearance of cell walls at the sites of penetration. Hyphae are generally observed within mesophyll tissues 6 h after the hydration (E. J. Braun, personal communication).

Little is known about the activities of *C. heterostrophus* after it enters maize tissues. Hyphae of both races T and O ramify through tissues, killing host cells and producing the characteristic necrotic lesions. The factor or factors responsible for host cell death or the ability of the fungus to resist host defense responses are unknown. The only gene currently proven to be needed by race O to produce lesions is *CPS1*, which encodes a cyclic peptide synthetase (Yoder, 1998). The substrate(s) of the peptide synthetase is not known.

Race T presumably has all the genes needed for normal lesion development as does race O. However, in addition, it has genes that confer an added level of virulence to maize lines with T cytoplasm. The high virulence of race T to T-cytoplasm maize has been shown to be due to its production of a host-selective toxin known as T-toxin (Yoder, 1976, 1980; Yoder *et al.*, 1977). T-toxin is a family of long-chain, linear β-polyketols with a chain length of C_{35} to C_{45} (Kono and Daly, 1979; Kono *et al.*, 1980, 1981; Daly and Knoche, 1982; Suzuki *et al.*, 1982, 1983, 1984) (Fig. 7). Because T-toxin specifically affects T-cytoplasm lines, whether male sterile or restored, it was of interest as a tool for plant breeding immediately after its discovery (Gracen *et al.*, 1971; Comstock and Scheffer, 1972) and was used extensively between 1970–1971 to detect T cytoplasm in seed lots (Lim *et al.*, 1971).

Early attempts to determine the genetic control of T-toxin production indicated a single genetic locus difference between race T and race O (Lim and Hooker, 1971; Tegtmeier *et al.*, 1982; Bronson, 1991); this locus was named *Tox1*. A few
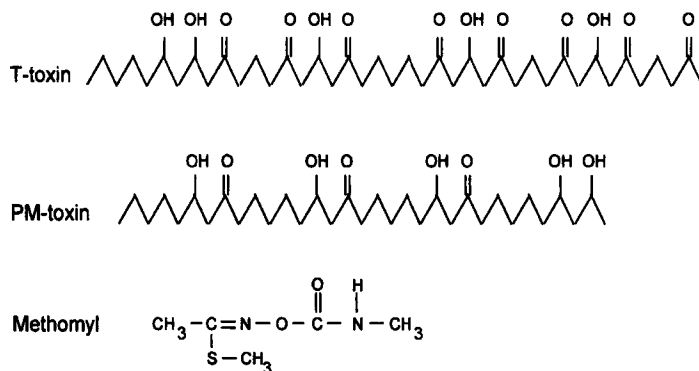
Figure 7 Structures of T-toxin, PM-toxin, and methomyl. Both T-toxin and PM-toxin are families of linear β-polyketols varying in chain length. Shown is one major component of each toxin: band 1 of T-toxin and band B of PM-toxin. Drawings of T-toxin and PM-toxin structures are adapted from Kono *et al.* (1985).

crosses produced segregation ratios suggestive of additional loci (Yoder and Gracen, 1975; Yoder, 1976); however, these ratios were later shown to be artifacts of a factor linked to *Tox1* that caused nonrandom abortion of progeny and the illusion of multiple-locus segregation (Bronson *et al.*, 1990). It is now clear that two distinct loci, *Tox1A* and *Tox1B*, are required for production of T-toxin (Turgeon *et al.*, 1995) and that these two loci absolutely cosegregate in crosses among naturally occurring strains due to their tight genetic linkage to the breakpoint of a reciprocal translocation (Bronson, 1988; Tzeng *et al.*, 1992; Chang and Bronson, 1996). Race T and race O differ by a reciprocal translocation involving chromosomes 6 and *12*; the breakpoint of the translocation is at or very near *Tox1*. Race T also contains, relative to race O, one or more insertions totaling about 1.2 Mb; this extra DNA maps to the translocation breakpoint (Fig. 8; Chang and Bronson, 1996).

Two genes involved in T-toxin synthesis have been cloned: *ChPKS1*, which maps to the *Tox1A* locus on chromosome *12;6*, and *DEC1*, which maps to the *Tox1B* locus on chromosome *6;12*. *ChPKS1* was cloned by tagging using restriction enzyme-mediated integration (REMI) of nonhomologous DNA (Lu *et al.*, 1994). *ChPKS1* contains a 7.6-kb open reading frame encoding a polyketide synthase containing six catalytic domains (Table I; Fig. 9; Yang *et al.*, 1996). The gene is flanked on both sides by A+T-rich, repeated, noncoding DNA. The other gene, *DEC1*, encodes a decarboxylase required for the modification of T-toxin to its biologically active form (Rose *et al.*, 1996; Yoder, 1998). Disruption of either *ChPKS1* or *DEC1* results in the simultaneous loss of ability to make active T-toxin and loss of high virulence to T-cytoplasm lines. Sequences corresponding to *ChPKS1* and *DEC1* are absent in race O and in other *Cochliobolus* species tested,
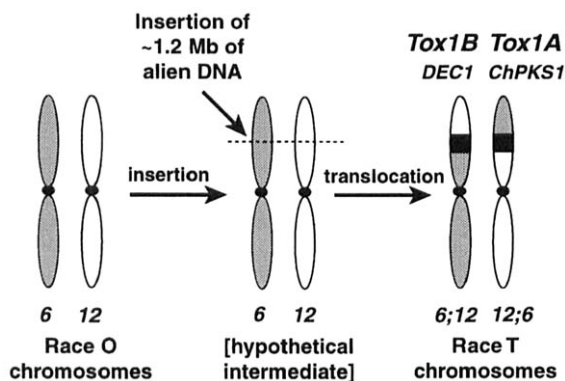
Figure 8   Model for the evolution of race T. Race T is hypothesized to have evolved from race O by the insertion of a ~1.2-Mb segment of alien DNA containing genes for T-toxin production. Concurrently or subsequently, a translocation event occurred that placed the genes on two different chromosomes. It is not known whether the segment initially inserted into chromosome *6* or chromosome *12*. Chromosomes in this diagram are not drawn to scale. Adapted from an unpublished drawing by B. G. Turgeon and O. C. Yoder, Cornell University.

suggesting that these genes may have been acquired by horizontal transfer from another genus (Turgeon and Berbee, 1998; Yang *et al.,* 1996; Yoder, 1998).

The tight genetic linkage of *ChPKS1* and *DEC1* to a chromosome rearrangement has led to a model for the evolution of race T, shown in Fig. 8 (Yang *et al.,* 1996; Yoder, 1998). Given the nature of the chromosomal rearrangement, the simplest hypothesis is that race T evolved from race O, and that it did so by an insertion into either chromosome *6* or *12* of a ~1.2-Mb segment of alien DNA containing the genes for T-toxin production. Either simultaneously or subsequently, a translocation occurred between chromosomes *6* and *12* that placed *Tox1A* and *Tox1B* on two different chromosomes. The source of the alien DNA is not known.

Evidence for additional genes involved in T-toxin production has been obtained recently by mutational analysis (Lu *et al.,* 1995; Yoder, 1998) and by the analysis of quantitative genetic variation among naturally occurring strains (Bronson, 1998). These studies have identified genes unlinked to *Tox1* that control the amount of toxin produced by race T strains. These genes may be involved in the production of precursors, or the transport, regulation, or metabolism of T-toxin.

As far as is known, T-toxin is the only host-specific toxin produced by *C. heterostrophus.* However, one impact of the 1970 epidemic was a lingering fear that *C. heterostrophus* might evolve the ability to produce additional toxins specific for other maize cytoplasms (Hooker, 1972). Species of the genus *Cochliobolus* produce at least three different host-specific toxins. In addition to T-toxin, there is HC-toxin, which allows *C. carbonum* to attack certain maize genotypes, and HV-

Table I

Genbank Accession Numbers for cms-T Related Gene Sequences

Definition	Maize (cms-T) mitochondrial TURF 2H3 sequence containing 2 ORFs
Genbank Acc. No.	M12582
Reference	Dewey *et al.* (1986)
Comment	Complete sequence of T-*urf13* and the co-transcribed *orf221*
Definition	*Zea mays* mitochondrial T mutant ORF13 DNA
Genbank Acc. No.	M16366
Reference	Wise *et al.* (1987a)
Definition	*Zea mays* mitochondrial mutant T-4 ORF8.3 DNA
Genbank Acc. No.	M16268
Reference	Wise *et al.* (1987a)
Comment	T-4 mutant has TCTCA tandem duplication in T-*urf13* reading frame
Definition	CMS T maize mitochondrial DNA for T-URF25 N-terminus
Genbank Acc. No.	X05446
Reference	Rottmann *et al.* (1987)
Definition	CMS T fertile revertant mit-DNA for T-URF25 N-terminus
Genbank Acc. No.	X05447
Reference	Rottmann *et al.* (1987)
Comment	V3 mutant has lost T-*urf13* via homologous recombination
Definition	Maize B73 cms-T mitochondrion mRNA
Genbank Acc. No.	X60239
Reference	Ward and Levings (1991)
Comment	*orf221* but not T-*urf13* is subject to RNA editing
Definition	Maize B73 X MO17 mitochondrial mRNA
Genbank Acc. No.	X60238
Reference	Ward and Levings (1991)
Comment	*orf221* but not T-*urf13* is subject to RNA editing
Definition	*Zea mays* T-cytoplasm male-sterility restorer factor 2 (*rf2*) mRNA
Genbank Acc. No.	U43082
Reference	Cui *et al.* (1996)
Comment	Significant similarity to mitochondrial ALDHs
Definition	*Cochliobolus heterostrophus* polyketide synthase (*PKS1*) gene
Genbank Acc. No.	U68040
Reference	Yang *et al.* (1996)
Comment	Currently designated *ChPKS1*

toxin, which gives *C. victoriae* the ability to cause disease on certain genotypes of oats (Yoder *et al.,* 1997). Thus, a healthy fear of this fungus is not unfounded. In fact, in 1988, researchers in China startled the corn-breeding community by announcing the discovery of a race of *C. heterostrophus* highly virulent to C cytoplasm (Wei *et al.,* 1988). They reported that this new race, which they called "race C," produced a toxin specific for C cytoplasm. Fortunately, these claims have not been confirmed by other laboratories. In fact, in experiments using the same fun-
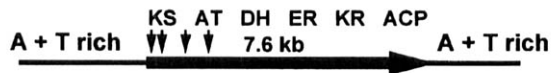
ChPKS1

MzPKS1

Figure 9 Polyketide synthase genes from *C. heterostrophus* and *M. zeae-maydis*. *ChPKS1*, a gene encoding a polyketide synthase involved in T-toxin production in *C. heterostrophus* race T. *MzPKS1*, a gene encoding a polyketide synthase involved in the production of PM-toxin in *M. zeae-maydis*. KS, β-ketoacyl synthase; AT, acyltransferase; DH, dehydratase; ER, enoyl reductase; KR, β-ketoacyl reductase; ACP, acyl carrier protein. *MzRED1* and *MzRED2* are reductases. *Mzt1* through *Mzt5* are transposons. Vertical arrows indicate the location of introns. Adapted from an unpublished drawing by B.G. Turgeon and O. C. Yoder, Cornell University.

gal isolates as used by Wei *et al.*, no differences in either virulence or toxin production were detected between "race C" and authentic race O isolates (O. C. Yoder, personal communication). Thus, to date, there is no evidence that *C. heterostrophus* has evolved the ability to attack other maize cytoplasms.

C. YELLOW LEAF BLIGHT AND THE INSECTICIDE METHOMYL

The popularity of T cytoplasm in the 1960s resulted in the selection of another fungus to which T-cytoplasm hybrids were susceptible. The fungus, *Mycosphaerella zeae-maydis,* was unknown as a pathogen on maize in the United States until 1968, when severe outbreaks of yellow leaf blight were reported in the northern maize-growing states and Ontario, Canada (Scheifele and Nelson, 1969; Scheifele *et al.*, 1969; Ayers *et al.*, 1970). Susceptibility was shown to be cytoplasmically inherited. Maize with N cytoplasm or forms of male sterility other than cms-T are resistant.

The virulence of *M. zeae-maydis* is due to its production of a host-specific toxin, known as PM-toxin (after *Phyllosticta maydis,* the name applied to the asexual stage of the fungus) (Yoder, 1973). The fungus has no known races and all known naturally occurring isolates of *M. zeae-maydis* produce the toxin. Toxin-deficient mutants have been created by REMI mutagenesis and shown to be nonpathogenic on maize (Yun *et al.*, 1998). PM-toxin is strikingly similar to T-toxin, in that it is a family of linear β-polyketols ranging in length from C_{33} to C_{35} (Fig. 7; Kono

et al., 1983, 1985; Danko *et al.,* 1984; Suzuki *et al.,* 1988). Conformational studies suggest that PM-toxin assumes a helical or hairpin form in its native state (Suzuki *et al.,* 1988).

Three genes have been shown to be required for both the production of PM-toxin and virulence to T-cytoplasm maize (Yun *et al.,* 1997; Yoder, 1998; O. C. Yoder, personal communication). *MzPKS1* encodes a polyketide synthase and is similar to *ChPKS1* from *C. heterostrophus* in having six enzymatic domains and four introns (Fig. 9). However, *MzPKS1* lacks the A+T-rich flanking regions and has only 59% nucleotide identity to *ChPKS1*. In addition, immediately 5' of *MzPKS1* are *MzRED1* and *MzRED2*. *MzRED1* and *MzRED2* have high similarity to the ketoreductases associated with plant and bacterial polyketide synthases. Disruption of *MzPKS1,* *MzRED1,* or *MzRED2* results in loss of PM-toxin production. The differences in the organization and nucleotide sequences of genes involved in β-polyketol synthesis between *M. zeae-maydis* and *C. heterostrophus* suggest that the ability to make PM-toxin and T-toxin evolved separately. Thus, it was chance that two, unrelated fungi produced such similar compounds (Yun *et al.,* 1997; Yoder, 1998).

T-cytoplasm maize is also susceptible to damage from *S*-methyl-*N*-[(methyl-carbamoyl)oxy]thioacetimidate (methomyl), the active ingredient in Lannate insecticide (Fig. 7). Susceptibility was discovered accidentally in 1974 when a field containing T-cytoplasm lines was sprayed with Lannate. Methomyl caused necrotic bands on leaf tips and margins and, in severe cases, complete leaf necrosis. Because all other cytoplasms of maize are unaffected, methomyl treatment is a rapid and convenient method for identifying T-cytoplasm plants (Humaydan and Scott, 1977).

D. T-TOXIN AND PM-TOXIN DISRUPT MITOCHONDRIAL FUNCTION

Consistent with the maternal inheritance of disease susceptibility in T-cytoplasm lines, mitochondria of T-cytoplasm lines are highly sensitive to T-toxin and PM-toxin. The toxins have a wide range of effects on tissues of T-cytoplasm plants, including promotion of ion leakage from cells, induction of stomatal closure, and inhibition of root growth, transpiration, photosynthesis, and dark CO_2 fixation (Arntzen *et al.,* 1973a,b; Bhullar *et al.,* 1975). However, the most direct effects are on mitochondrial function. Effects of the toxins on mitochondria include uncoupling of oxidative phosphorylation, inhibition of malate oxidation, stimulation of NADH and succinate oxidation, swelling and loss of matrix density (Miller and Koeppe, 1971; Gengenbach *et al.,* 1973; Peterson *et al.,* 1975; Aldrich *et al.,* 1977; Hack *et al.,* 1991). All of these effects are consequences of increases in mitochondrial membrane permeability, as evidenced by leakage of NAD+, Ca^{2+}, and H^+ (Bervillé *et al.,* 1984; Holden and Sze, 1984, 1987). The effect of the toxins on

membrane permeability can be detected visually with the florescent dye $DiOC_6$, which is sensitive to mitochondrial cationic potential (Figs. 10A–D, see color plate; Yang, 1989). Methomyl, although structurally unrelated to T-toxin and PM-toxin (Fig. 7), induces the same physiological responses in T-cytoplasm mitochondria. The damage to mitochondrial function induced by the fungal toxins and methomyl is believed to be the cause of cell death.

The presence of restorer genes in the nuclear genome modifies the response of maize, but not enough to eliminate disease susceptibility. Leaves, roots, and isolated mitochondria from male-sterile plants are highly sensitive to T-toxin; those from plants restored to fertility show an intermediate reaction; while those from N-cytoplasm plants are insensitive (Watrud et al., 1975a; Barratt and Flavell, 1975). The intermediate response of lines restored to fertility is clearly insufficient to confer resistance to C. heterostrophus and M. zeae-maydis, however, since the 1970 epidemic occurred on restored maize (Ullstrup, 1972).

E. The Hazards of Genetic Homogeneity

Although it has been over 25 years since the southern corn leaf blight epidemic, its repercussions are still being felt. The susceptibility of T-cytoplasm lines to race T of C. heterostrophus has severely limited the use of cms-T as a source of male sterility for commercial maize production. The vast majority of hybrid seed is now produced at considerable expense by hand or machine emasculation.

The most significant impact of the epidemic, however, has been the increased public, political, and scientific awareness of the potentially devastating consequences of genetic homogeneity. The southern corn leaf blight epidemic was highly publicized in both print and electronic media (Ullstrup, 1972); most well-informed individuals in the United States in 1970 would have been aware of its occurrence and the effects it was having, either directly or indirectly, on their lives. Fear of similar pandemics in the future motivated sober appraisals of the extent of genetic homogeneity in crop plants and a wide range of studies on ways to increase genetic diversity in modern agricultural systems (Day, 1977; Mundt and Browning, 1985; Wolfe, 1985).

V. DISEASE SUSCEPTIBILITY AND MALE STERILITY

In many species, cms is associated with the expression of novel open reading frames in the mitochondrial genome (Schnable and Wise, 1998). Although each open reading frame is unique, the feature that these open reading frames have in common are one or more predicted hydrophobic domains, consistent with mem-
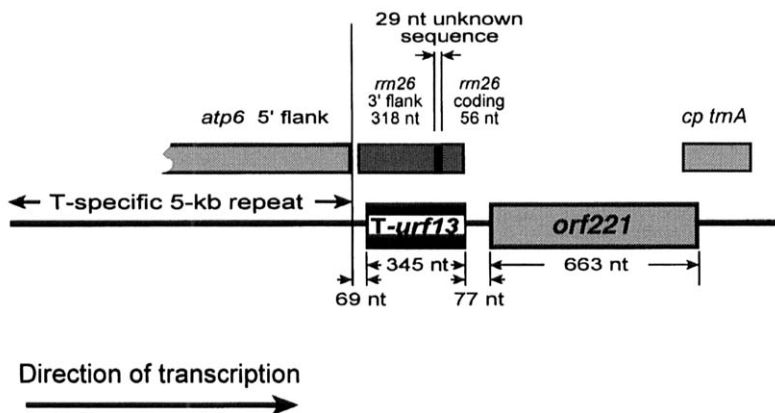
Figure 11 Structure of T-*urf13* region in the mitochondrial genome of T-cytoplasm maize. Shown above the chimeric T-*urf13/orf221* transcriptional unit are the presumed progenitor sequences for these open reading frames. A 5-kb T-specific repeated region contains promoter sequences 5' to *atp6*. There are 15 nucleotides of unknown origin between the 5' *atp6* flanking region and the start of the 3' *rrn26* flanking region. Sixty-nine nucleotides 3' to the T-specific, 5-kb repeat junction is the T-*urf13* ATG start codon. The co-transcribed T-*urf13* and *orf221* reading frames most likely utilize a duplication of the *atp6* promoter within the 5-kb repeat region.

brane-bound proteins. In cms-T, both male sterility and toxin sensitivity are attributed to T-*urf13*, a 345-bp reading frame that encodes a membrane-bound 13-kDa protein found only in T-cytoplasm maize mitochondria (Fig. 11; Levings, 1993).

One of the first indications that male sterility and toxin sensitivity are controlled by the same gene came from attempts to select for toxin-insensitive plants from tissue cultures of toxin-sensitive, cms-T maize (Gengenbach and Green, 1975; Gengenbach *et al.,* 1977; Brettell *et al.,* 1979). Cultures were formed from immature embryos of the T-cytoplasm line, A188T. Tissue cultures from several cell lines were treated with a sublethal dose of T-toxin, and then passed through several more cycles with higher concentrations of the toxin. The survivors of these treatments were regenerated to yield T-toxin-resistant plants. Interestingly, all of the resulting toxin-resistant mutants were also male fertile and both of these traits were maternally inherited. Toxin-resistant mutants were also obtained from tissue cultures without T-toxin selection. All of these plants were also male fertile (Brettell *et al.,* 1980; Umbeck and Gengenbach, 1983). Because all of the tissue-culture derived mutants resulted in the coordinate change in phenotype from toxin sensitivity to insensitivity and male sterility to male fertility, it was suggested that these two traits were controlled by the same or tightly linked mitochondrial genes (Gengenbach *et al.,* 1981; Kemble *et al.,* 1982; Umbeck and Gengenbach, 1983).

The gene controlling both traits, T-*urf13*, was identified by two complementary methods. One approach took advantage of differential expression patterns of mtDNA regions between the N and T cytoplasms. A mtDNA region consisting of three contiguous *Hind*III restriction fragments, designated TURF 2H3, revealed an abundant family of transcripts that was T specific. Sequence analysis of the 3547-bp TURF 2H3 revealed two open reading frames, T-*urf13* and the co-transcribed *orf221* (Table I; Dewey *et al.*, 1986). An alteration in the accumulation of T-*urf13*-specific transcripts was shown to occur in plants restored to fertility; subsequent genetic analysis identified the *Rf1* nuclear restorer gene as responsible for these alterations (Dewey *et al.*, 1987; Kennell *et al.*, 1987; Kennell and Pring, 1989; Wise *et al.*, 1996). Concurrently, a map-based approach took advantage of the male-fertile, toxin-insensitive mutants described above. In 19 of 20 such mutants, a 6.7-kb *Xho*I mitochondrial DNA restriction fragment was altered due to a partial deletion (Gengenbach *et al.*, 1981; Kemble *et al.*, 1982; Umbeck and Gengenbach, 1983; Fauron *et al.*, 1987). The remaining male-fertile mutant, T-4, retained the 6.7-kb *Xho*I fragment. Comparative restriction and sequence analysis of the 6.7-kb *Xho*I fragment from cms-T and T-4 revealed a tandem TCTCA repeat in the T-*urf13* coding region of the T-4 mutant. This tandem 5-bp repeat generates a frameshift, truncating the reading frame at nucleotide 222 (Wise *et al.*, 1987a). In the deletion mutants examined, the T-*urf13* reading frame was excised via recombination through a 127-bp repeat (Rottmann *et al.*, 1987; Fauron *et al.*, 1990). The 127-bp repeat carries a 55-bp conserved core with 85% similar flanking regions and begins 6 bp 3' to the T-*urf13* TGA stop codon. Although the 127-bp repeat extends 56 bp into the co-transcribed *orf221*, this reading frame is unaltered in the deletion (Rottmann *et al.*, 1987; Fauron *et al.*, 1990) and T-4 frameshift mutants (Rocheford *et al.*, 1992), providing rigorous genetic evidence that it is the T-*urf13* gene that is responsible for both toxin sensitivity and male sterility.

The physical organization of the T-*urf13/orf221* complex appears to be the result of numerous recombination events among the 5' flanking region of *atp6* (contained within the T-specific 5-kb repeat), the coding and 3' flanking region of *rrn26*, the cholorplast *trnA* gene, and several unidentified sequences (Fig. 11; Dewey *et al.*, 1986; Wise *et al.*, 1987a). Interestingly, the insertion of the tandem TCTCA repeat in the T-*urf13* coding region in the T-4 mutant creates 86 nucleotides of perfect identity with the 3' flanking region of the *rrn26* sequence (Dale *et al.*, 1984), consistent with the hypothesis that T-4 also arose by homologous recombination or gene conversion with similar sequences in the mitochondrial genome (Wise *et al.*, 1987a; Pring *et al.*, 1988).

T-*urf13* encodes a 13-kDa mitochondrial protein (URF13), located in the mitochondrial membrane (Dewey *et al.*, 1987). Initially identified by Forde *et al.* (1978), the identity of this protein was confirmed by immunoprecipitation and Western blot analysis with antiserum prepared against synthetic peptides derived from the predicted amino acid sequence of the T-*urf13* reading frame (Dewey *et*
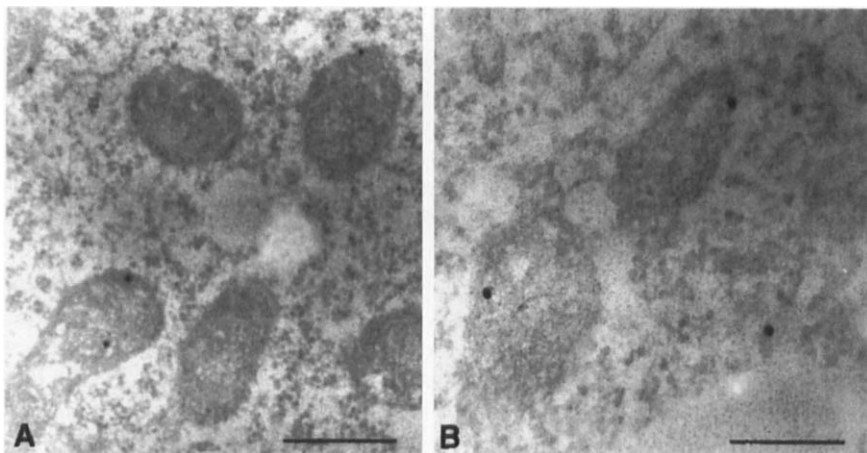
Figure 12 Immunocytochemical labeling with 20-nm gold particles (black dots) of URF13 protein associated with inner membrane of mitochondria in all cells of cms-T maize plants. (A) Portion of root-tip cell. (B) Portion of tapetal cell at early microspore stage. Bars = 0.6 μm (A) and 0.3 μm (B). There is no labeling of N-cytoplasm mitochondria (not shown).

al., 1987; Wise et al., 1987b). This protein is not synthesized by deletion mutants (Dixon et al., 1982), and is truncated to approximately 8 kDa in the T-4 frameshift mutant (Wise et al., 1987b). Immunogold localization further confirmed that URF13 is localized primarily in the mitochondrial inner membrane of mitochondria isolated from etiolated shoots, roots, and tapetal cells from the meiocyte to microspore stages (Figs. 12A and 12B; Yang, 1989; Hack et al., 1991).

A. How Fungal Toxins Disrupt Mitochondrial Function

In addition to the genetic evidence described above, biochemical evidence that T-urf13 is responsible for toxin and methomyl sensitivity comes from expression of the URF13 protein in heterologous systems, Escherichia coli, yeast, and tobacco. When T-urf13 is transformed into E. coli in an inducible expression vector, cells that express URF13 are sensitive to toxin and to methomyl. The E. coli cells not expressing URF13 or expressing a truncated version of URF13 are not sensitive to these compounds (Dewey et al., 1988). Expressed URF13 is associated with the E. coli plasma membrane, similar to its association with the mitochondrial membrane in T-cytoplasm maize. Likewise, the effects of toxin and methomyl treatment on T-urf13–E. coli are similar to those previously observed from isolated T-cytoplasm mitochondria, including inhibition of whole cell respiration, in-

duction of massive ion leakage, and swelling of spheroplasts (Miller and Koeppe, 1971; Barratt and Flavell, 1975; Matthews *et al.,* 1979; Payne *et al.,* 1980; Holden and Sze, 1987; Dewey *et al.,* 1988). The availability of *E. coli* expressing URF13 has resulted in the development of a rapid microbiological assay for T-toxin and PM-toxin, thus facilitating research on toxin production by *C. heterostrophus* and *M. zeae-maydis* (Ciuffetti *et al.,* 1992).

Extensive site-directed mutagenesis experiments of the T-*urf13* sequence have provided insight into the structure–function relationships of URF13 and toxin sensitivity (Braun *et al.,* 1989). These studies demonstrated that approximately one-quarter of the amino acids from the carboxyl end of the URF13 protein are not essential for toxin sensitivity. In maize, URF13 is truncated from 113 to 74 amino acids in the T-4 toxin-insensitive, frameshift mutant (Wise *et al.,* 1987b). Similarly, *E. coli* that has been transformed with T-*urf13* mutations that result in truncated URF13 proteins of 73 or 82 amino acids in length are toxin insensitive, whereas *E. coli* with truncated URF13 proteins of 83 amino acids or longer are toxin sensitive.

N,N'-Dicychlohexylcarbodiimide (DCCD) protection studies have revealed specific amino acid residues that are important in the URF13/T-toxin interaction (Braun *et al.,* 1989). DCCD binds covalently to acidic amino acids localized in hydrophobic environments. Hence, T-cytoplasm mitochondria or *E. coli* cells expressing URF13 preincubated with DCCD are protected against T-toxin. Use of site-directed substitutional mutations demonstrated that DCCD binds to the URF13 protein at two aspartate residues at positions 12 and 39. However, it is the aspartate at position 39 that is required for a functional URF13/T-toxin interaction because substitutional mutations at this position eliminate toxin sensitivity.

[3H]PM-toxin binds to wild-type URF13 in *E. coli,* but not to the 82-amino-acid truncated URF13 (Braun *et al.,* 1990). This is consistent with the toxin sensitivity phenotypes in cms-T maize and insensitivity in the T-4 mutant. Additionally [3H]PM-toxin does not bind to the *E. coli* toxin-sensitive URF13 valine substitutional mutant at position 39. However, in the *E. coli* toxin-insensitive mutant missing amino acids 2 through 11, the presence of significant toxin binding indicates that it is possible to be toxin insensitive even in the presence of toxin binding. Based on this finding, it was proposed that amino acids 2 through 11 are unnecessary for toxin binding, but essential for the toxin–URF13 interaction that leads to membrane permeabilization. Hence, the binding to URF13 in *E. coli* cells is cooperative, suggesting that URF13 exists as oligomers in *E. coli* membranes (Braun *et al.,* 1990). Based on the additional studies of Korth *et al.* (1991) and Rhoades *et al.* (1994), it has now been postulated that URF13 monomers containing three *trans*-membrane α-helices interact with T-toxin (or methomyl) and are assembled as tetrameric pore-forming structures spanning the inner mitochondrial membrane. This pore-forming model is consistent with early observations on mitochondrial electrolyte leakage, uncoupling of oxidative phosphorylation, and chan-

nel formation in the presence of T- and PM-toxins (Matthews *et al.*, 1979; Bervillé *et al.*, 1984; Holden *et al.*, 1985).

Corroborating results were obtained when T-*urf13* was transformed into the yeast *Saccharomyces cerevisiae* and targeted to the mitochondrial membrane utilizing a *Neurospora* ATP synthase subunit 9 leader sequence (Huang *et al.*, 1990; Glab *et al.*, 1990, 1993). Yeast cells expressing T-*urf13* were sensitive to both T-toxin and PM-toxin, in addition to methomyl. These compounds inhibited growth of yeast cells and stimulated respiration by isolated mitochondria with NADH as a substrate, similar to the effects seen on isolated maize mitochondria. Yeast cells expressing URF13 without a targeting peptide do not exhibit sensitivity to the toxins or methomyl, demonstrating that localization of URF13 to mitochondria is essential for sensitivity in yeast.

Similar effects are observed when the URF13 protein is expressed in tobacco. For example, it is essential that URF13 be targeted to mitochondria to cause toxin or methomyl sensitivity. Tobacco cells expressing URF13 without a targeting peptide do not exhibit sensitivity to the toxins or methomyl (von Allmen *et al.*, 1991; Chaumont *et al.*, 1995). However, none of the toxin-sensitive, transgenic tobacco plants produced to date has been male sterile. The failure to obtain male-sterile plants suggests that correct tissue-specific expression and subcellular localization is required for URF13 to cause male sterility.

B. How URF13 Causes Sterility Remains a Mystery

Although the interaction of pathotoxins or methomyl with URF13 causes significant damage to URF13-containing membranes, the mechanism by which URF13 causes tapetal degeneration and male sterility in the absence of pathotoxins and methomyl is still not clear. In particular, the selective nature of this degeneration is paradoxical, because URF13 is expressed in many, if not all, maize tissues (Hack *et al.*, 1991). Two explanations for this paradox have been summarized by Levings (1993). One possibility (Flavell, 1974) is that there is a tapetum-specific, toxin-like compound (Factor X) that is a necessary prerequisite for URF13-induced toxicity (Fig. 5). Consistent with this hypothesis, it is well established that mitochondrial proteins can be regulated in tissue-specific ways (Bedinger, 1992; Conley and Hanson, 1994). Alternatively, Wallace (1989) has proposed that tissue-specific degeneration could occur if tissues differ in their requirements for mitochondrial function. Microsporogenesis seems to be an energy-intensive process; the number of mitochondria undergoes a 40-fold increase in the tapetal cells (Lee and Warmke, 1979). In addition, antisense repression of the mitochondrial tricarboxylic acid (TCA) cycle enzyme, citrate synthase, results in the degeneration of ovary tissues of potato, apparently without affecting other organs (Landschütze *et al.*, 1995). Hence, reproductive tissues seem to be especially sensitive to perturbations in mitochondrial function. Consistent with the hypothesis that URF13 can cause

subtle perturbations in mitochondrial function even in the absence of pathotoxins and methomyl, there is some evidence that URF13 has a slight (but significant) deleterious effect on maize cells (Duvick, 1965; Pring *et al.,* 1988) (see also discussion by Duvick in Section VIIa). Hence, for the Wallace hypothesis to be correct, it would only be necessary that these perturbations in mitochondrial function be particularly deleterious to tapetal cells.

VI. NUCLEAR–CYTOPLASMIC INTERACTIONS AND RESTORATION OF CMS-T

T-cytoplasm lines can be restored to male fertility by the action of dominant alleles of the *rf1* and *rf2* nuclear genes. These genes are thought to suppress or compensate for cytoplasmic dysfunctions that are phenotypically expressed during pollen development. The mode of fertility restoration of cms-T is sporophytic; the genetic constitution of the diploid, sporophytic anther tissue, rather than the haploid, gametophytic pollen, determines pollen development. Therefore, a cms-T plant that is heterozygous for both restorer gene loci (*Rf1/rf1, Rf2/rf2*), will produce all fertile pollen even though only one-fourth of the pollen grains carry both *Rf1* and *Rf2* (Laughnan and Gabay-Laughnan, 1983). *Rf8* and *Rf** are newly described restorers that can each (at least partially) substitute for *Rf1* (Dill *et al.,* 1997). Such overlapping functions are either a consequence of duplications of gene function or an indication that multiple mechanisms can induce restoration.

The allelic frequency of restorers in the maize gene pool can provide clues as to the origins and functions of these restorers. For example, the *Rf1, Rf8,* and *Rf** alleles are all rare among maize lines (Table II). This suggests that they are probably neomorphic alleles and do not have essential functions other than their fortuitous ability to influence restoration of cms-T. In contrast, the RF2 protein probably has a physiological function independent of its role as a nuclear restorer of cms-T (Schnable and Wise, 1994). The argument for this is that most maize lines have never been exposed to cms-T, but carry a functional *Rf2* allele. This finding indicates that there has existed selective pressure for the RF2 protein that is independent of cms-T. Based on this reasoning it has been proposed that the RF2 protein was recruited in T-cytoplasm maize to ameliorate the mitochondrial lesion associated with T-*urf13* expression (Schnable and Wise, 1994).

A. *Rf1*, BUT NOT *Rf2*, ALTERS THE EXPRESSION OF T-*URF13*

In many species, restoration of fertility is associated with the processing and possible post-transcriptional editing of cms-associated mitochondrial transcripts. Typical examples of this are observed in oilseed rape, *Petunia,* common bean,

Table II

Restorer Genotypes in T-Cytoplasm Maize Lines

Line	Restorer genotypes[a]			
A188	*rf1*	*Rf2*	*rf8*	*rf**
A632	*rf1*	ND[c]	*rf8*	*rf**
B37	*rf1*	*Rf2*	*rf8*	*rf**
B73	*rf1*	*Rf2*	*rf8*	*rf**
C103	*rf1*	ND	*rf8*	*rf**
33-16	*rf1*	*Rf2*	*rf8*	*Rf**
38-11	*rf1*	*Rf2*	ND	ND
8703[b]	*rf1*	*Rf2*	*Rf8*[b]	*rf**
Ky21	*Rf1*	*Rf2*	*rf8*	*rf**
Line C	*rf1*	*Rf2*	*rf8*	*rf**
Mo17	*rf1*	*Rf2*	*rf8*	*rf**
Mo17 × B73	*rf1*	*Rf2*	*rf8*	*rf**
N6	*rf1*	*Rf2*	ND	ND
R213	*Rf1*	*rf2*	*rf8*	*rf**
W23	*rf1*	*Rf2*	ND	ND
W64A	*rf1*	*Rf2*	*rf8*	*rf**
Wf9	*rf1*	*rf2*	*rf8*	*rf**
Wf9-BG	*Rf1*	*Rf2*	*rf8*	*rf**
wx1-m8[b]	*rf1*	*Rf2*	*Rf8*[b]	*rf**

[a]Data derived from Dewey *et al.* (1987), Kennell *et al.* (1987), Kennell and Pring (1989), Rocheford *et al.* (1992), Gabay-Laughnan and Laughnan (1994), Wise *et al.* (1996), Dill *et al.* (1997), and P. S. Schnable and R. P. Wise (unpublished results). Unless otherwise indicated all lines are homozyous for the indicated allele.

[b]These stocks are not inbred lines, they segregate at many loci, including *rf8.*

[c]ND, no data.

rice, and sorghum, as well as cms-S and -T maize (reviewed by Schnable and Wise, 1998). In cms-T maize, seven major transcripts of T-*urf13* and the co-transcribed *orf221* range in size between 1.0 and 3.9 kb (Dewey *et al.,* 1986; Kennell *et al.,* 1987; Kennell and Pring, 1989; Wise *et al.,* 1996; Dill *et al.,* 1997). Many of these transcripts are presumably products of a series of processing events stemming from the 3.9-kb transcript. However, transcript capping experiments with guany-lyl transferase identified the 1.85-kb transcript as a primary (initiated) transcript; hence, the 1.85-kb transcript is not the result of RNA processing (Kennell and Pring, 1989).

Plants segregating for the *Rf1* restorer accumulate additional 1.6- and 0.6-kb T-*urf13* transcripts as shown in Fig. 13 and Table III (Dewey *et al.,* 1986, 1987; Kennell *et al.,* 1987; Wise *et al.,* 1996). Mitochondrial RNA gel blot analyses have
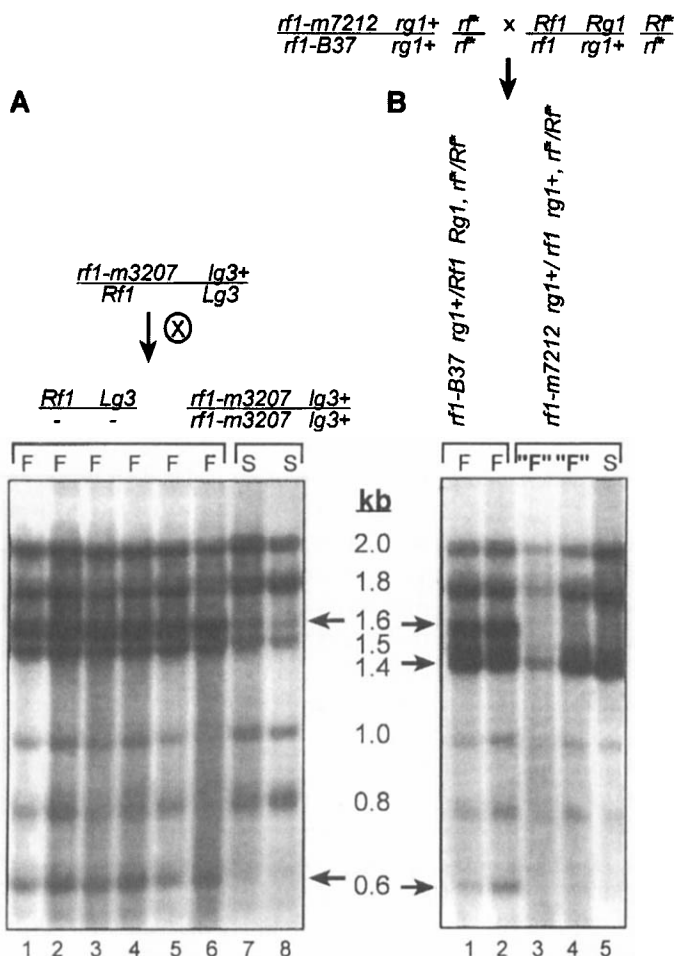
Figure 13 Effect of different restorer genotypes on the accumulation of T-*urf13* mitochondrial transcripts. F, "F", and S denote male-fertile, partially fertile, and male-sterile plants, respectively. (A) Lanes 1–8 contain RNA from selfed progeny of plants carrying the mutant allele, *rf1-m3207*. The RNA in lanes 1–6 originated from plants that carried a dominant *Rf1* allele, which is tightly linked to the morphological marker *Lg3*. *Rf1* mediates the processing of the larger 2.0-, 1.8-, and 1.0-kb T-*urf13* transcripts, resulting in the accumulation of 1.6- and 0.6-kb transcripts. As can be seen in lanes 7 and 8, RNA from plants homozygous for the *rf1-m3207* mutant allele are deficient in these processing products. (B) RNA was isolated from progeny of a cross involving the *rf1-m7212* allele. Plants that carry the dominant *Rf1* allele accumulate 1.6- and 0.6-kb transcripts, whereas those homozygous for the *rf1-m7212* allele do not. However, all of the plants in this family carry the unlinked *Rf** allele, which also mediates the processing of the larger 2.0-, 1.8-, and 1.0-kb transcripts, but results in the accumulation of 1.4- and 0.4-kb transcripts. Plants homozygous for *rf1-m7212* and that accumulate the 1.4-kb transcript are partially fertile or sterile (lanes 3–5) because of the weakly penetrant nature of the *Rf** restorer (Dill *et al.*, 1997). Transcripts were detected with the T-*urf13* specific probe T-st308. Modified and published with permission from the Genetics Society of America, from Wise *et al.* (1996).

Table III

Effect of Nuclear Restorers on T-*urf13*-Associated Mitochondrial Transcripts

Restorer gene	Proposed mechanism	Additional accumulation of *Rf*-associated T-*urf13* transcripts	Concurrent effect on novel proteins
Rf1	Transcript processing	+1.6, 0.6	Reduction of 13-kDa URF13 protein
Rf8	Transcript processing	+1.42, 0.42	Reduction of 13-kDa URF13 protein
Rf*	Transcript processing	+1.4, 0.4	Unknown

indicated that four independent mutant alleles of *rf1* isolated by Wise *et al.* (1996) condition reduced steady-state accumulations of the 1.6- and 0.6-kb T-*urf13* transcripts. This result demonstrates that these transcripts are *Rf1* dependent. Results from the guanylyl transferase capping experiments described above indicate that the *Rf1*-dependent T-*urf13* transcripts do not arise via novel initiation sites, but instead arise via processing from the transcripts synthesized and processed in all cms-T lines (Kennell and Pring, 1989). Even in the presence of *Rf1*, however, the steady-state accumulation of the larger T-*urf13* transcripts is not measurably reduced (Fig. 13). Similarly, novel 1.42- and 0.42-kb T-*urf13* transcripts accumulate in plants segregating for the partial fertility restorer, *Rf8*, and 1.4- and 0.4-kb T-*urf13* transcripts accumulate in plants segregating for *Rf**. Extensive mapping of these transcripts via primer extension and Northern blot analyses indicates that the *Rf1*-dependent 1.6-kb, the *Rf8*-dependent 1.42-kb, and the *Rf**-dependent 1.4-kb transcripts are all derivatives of the 1.8-,1.85-, or 2.0-kb transcripts. Likewise, the smaller 0.6-, 0.42-, and 0.4-kb transcripts are likely derivatives of the 1.0-kb T-*urf13* transcript (Fig. 14; Kennell *et al.*, 1987; Kennell and Pring, 1989; Rocheford *et al.*, 1992; Wise *et al.*, 1996; Dill *et al.*, 1997).

Like the majority of the T-*urf13* and *orf221* transcripts, *atp6* transcripts are most likely initiated from identical promoter sequences within the T-specific 5-kb repeat (Fig. 11). However, differential processing of *atp6*-specific transcripts does not seem to occur in the presence of *Rf1*, *Rf8*, or *Rf** (Kennell *et al.*, 1989; Dill *et al.*, 1997). As described in the next section, this may indicate sequence specificity in the processing events mediated by these restorer genes.

Post-transcriptional RNA editing may also play a role in fertility restoration in some cms systems (Iwabuchi *et al.*, 1993). Possibly, editing might shorten predicted cms-associated ORFs by creating UAA, UAG, and UGA stop codons, because the most prevalent example of editing in plant mitochondrial sequences is C-to-U (Hanson *et al.*, 1996). Although the co-transcribed *orf221* is edited post-transcriptionally in cDNAs from cms-T mitochondria, T-*urf13* transcripts are not. Additionally, no differences in editing are observed between the *orf221* mRNA isolated from T-cytoplasm mitochondria and mRNA isolated from mitochondria
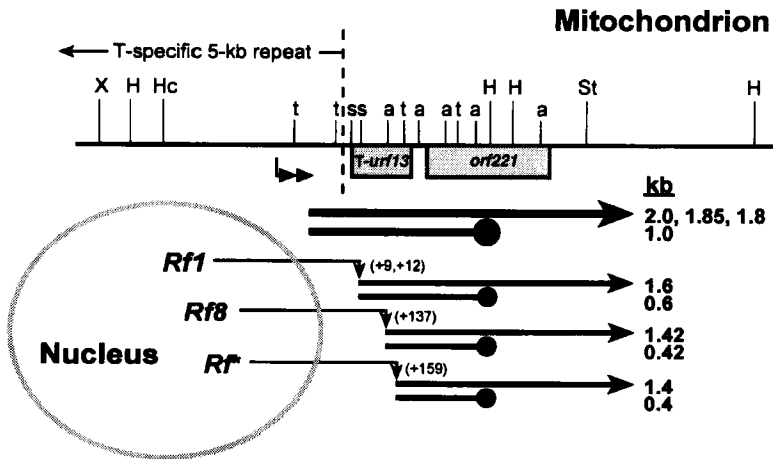
Figure 14 Effect of nuclear *Rf* genes on T-*urf13* transcript processing. *Rf1*, *Rf8*, and *Rf** each mediate processing of T-*urf13* transcripts at specific sites within T-*urf13* reading frame. Diagrammed (horizontal lines terminated by arrowheads or filled circles) are novel T-*urf13* transcripts that accumulate in the presence of the three nuclear restorers. Numbers in parentheses indicate 5' termini (designated by the number of nucleotides 3' of the start of the T-*urf13* reading frame) of each transcript as determined by primer extension experiments (Dill *et al.*, 1997). To the right of each group of transcripts are their respective molecular sizes as determined by Northern analyses. a, *Alu*I; H, *Hind*III; Hc, *Hinc*II; s, *Sau*3a; St, *Sst*II; t, *Taq*I; X, *Xho*I.

of plants restored by *Rf1*, thus, apparently ruling out the influence of RNA editing on restoration of cms-T maize (Table I; Ward and Levings, 1991).

1. *Rf*-Mediated T-*urf13* Transcript Processing Sites Share a Conserved Motif

Comparison of the sequences encompassing the 5' termini of the *Rf1*-, *Rf8*-, and *Rf**-dependent T-*urf13* transcripts and the *Rf3*-associated *orf107* transcript from the A3 cytoplasm of cms sorghum has revealed a small conserved motif associated with fertility restoration and transcript processing (Fig. 15). The conserved motif 5'-CNACNNU-3' overlaps a 5'-terminal U of each of these restorer-dependent transcripts. Notably, overlapping the 5'-terminal U of the *Rf**-associated transcripts and the *Rf3*-associated *orf107* transcript from cms sorghum is the highly conserved sequence 5'-AC(U/C)ACAAUA-3', revealing striking similarities among restorer-associated processing sites in these two grasses (Tang *et al.*, 1996; Dill *et al.*, 1997). This mitochondrial sequence may represent a recognition site for proteins regulated or expressed by the nuclear restorer genes. Thus, it appears that the *Rf1*, *Rf8*, and *Rf** restorers may encode functionally similar gene products that mediate the specific modification of T-*urf13* transcripts synthesized in all T-cytoplasm lines (Fig. 16). Motifs have been described in other cms systems, such as
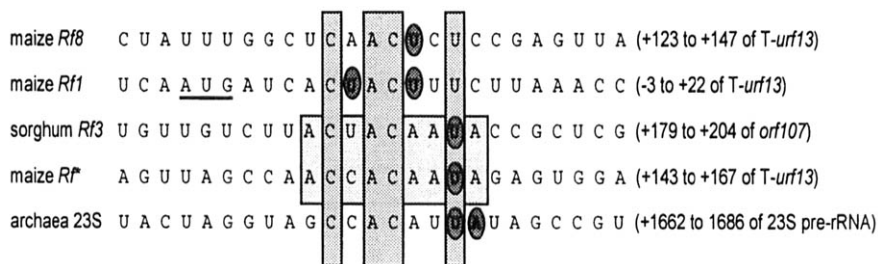
```
maize Rf8      C U A U U U G G C U |C A|A C|  |C U|C C G A G U U A  (+123 to +147 of T-urf13)

maize Rf1      U C A A U G A U C A |C  |A C|  |U U|C U U A A A C C  (-3 to +22 of T-urf13)

sorghum Rf3    U G U U G U C U U |A C|U A C|A A  |A|C C G C U C G  (+179 to +204 of orf107)

maize Rf*      A G U U A G C C A |A C|C A C|A A  |A|G A G U G G A  (+143 to +167 of T-urf13)

archaea 23S    U A C U A G G U A G |C|C|A C|A U    |U A G C C G U  (+1662 to 1686 of 23S pre-rRNA)
```

Figure 15   A conserved motif associated with fertility restoration and transcript processing. The sequence 5'-CNACNNU-3' overlaps the 5' termini of the 1.6- and 0.6-kb (*Rf1*-associated), 1.42- and 0.42-kb (*Rf8*-associated), 1.4- and 0.4-kb (*Rf**-associated), and the 380-nucleotide (*Rf3*-associated) transcripts from maize and sorghum. Letters highlighted by dark gray filled circles represent 5' termini of mtRNA transcripts associated with fertility restoration as determined by primer extension experiments (Tang *et al.*, 1996; Dill *et al.*, 1997). Conservation between *Rf3*- and *Rf**-associated processing sites [AC(U/C)ACAAUA] is highlighted in the light-gray horizontal boxed area. The translation initiation codon for T-*urf13* is underlined. The line designated archaea 23S is a partial sequence from the pre-rRNA transcript of the 23S precursor processing stem from *Sulfolobus acidocaldarius* (Potter *et al.*, 1995); the encircled U and A have been shown to be *in vivo* processing sites. Modified and published with permission of the Genetics Society of America, from Dill *et al.* (1997).
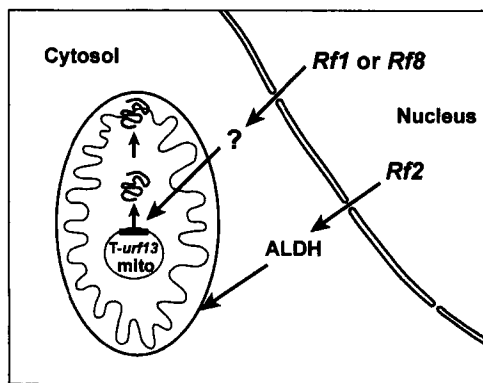

Figure 16   Molecular mechanisms by which restoration occurs are not well understood. Although the nuclear *Rf1* or *Rf8* restorers are known to affect the accumulation of novel T-*urf13* transcripts in mitochondria, the mechanisms by which this occurs have not been established. The *Rf2* restorer does not affect the accumulation of T-*urf13* transcripts. Although essential for restoration of cms-T, the molecular role of *Rf2* in fertility restoration is not known. However, the recent discovery that this gene exhibits a high degree of sequence similarity to mammalian mitochondrial aldehyde dehydrogenases has yielded a number of testable hypotheses. Adapted from an unpublished figure prepared by Xiangqin Cui, Department of Zoology & Genetics, Iowa State University.

the putative recognition sequence, 5'-UUGUUG-3' within the *orf224/atp6* transcriptional unit, downstream of the 5'-terminus of the *Rfp1*-polima-restorer-associated transcripts in *Brassica napus* (Singh *et al.*, 1996). Yet, how or why rare nuclear (*Rf*) genes evolved to mediate recognition of distinct sites within specific transcripts is an unresolved question.

2. *Rf*-Mediated Processing of T-*urf13* Transcripts Is Accompanied by a Reduction in URF13

Rf-mediated processing of cms-associated mitochondrial transcripts is often concurrent with a reduction in the accumulation of the cms-associated mitochondrial proteins. Although *Rf2* has no detectable effect on transcript processing or URF13 accumulation in maize (Dewey *et al.*, 1987; R. P. Wise, C. L. Dill, and P. S. Schnable, unpublished results), the abundance of URF13 is reduced by approximately 80% in plants that possess the *Rf1* restorer (Forde and Leaver, 1980; Dewey *et al.*, 1987). A reduction in URF13 can also be mediated by *Rf8*, however, the effect is not as pronounced as that mediated by *Rf1*, and may even be tissue dependent. For example, the difference in URF13 accumulation among plants segregating for *Rf8* is much more evident in tassels as compared to ears. In contrast, plants that carry *Rf1* exhibit a marked decrease of URF13 accumulation in both tassels and ears (Fig. 17; Dill *et al.*, 1997). *Rf** has not yet been tested for this capacity.

The disparity that surrounds these observations is that there is a yet unknown mechanism for *Rf*-mediated, URF13 reduction. As shown in Fig. 13, mature T-*urf13* transcripts still seem to be available for translation in the various *Rf* genotypes and an individual plant can accumulate an abundance of *Rf1*-, *Rf8*-, or *Rf**-associated transcripts, with no obvious decrease in the steady-state accumulation of the mature 2.0-, 1.8-, or 1.0-kb transcripts (Wise *et al.*, 1996; Dill *et al.*, 1997). Therefore, the end result of *Rf1* and *Rf8* function is likely post-transcriptional, that is, inhibition of translation or protein degradation, and not regulation of transcript accumulation. An excess of these processed transcripts might compete with the unprocessed transcript for translation, thereby reducing the accumulation of URF13.

B. CLONING OF NUCLEAR RESTORER GENES

As a step toward elucidating the molecular mechanisms by which restoration occurs, experiments were initiated to clone the *rf1* and *rf2* genes via a transposon tagging strategy (Figs. 18A and 18B). To assist in the generation of stocks necessary to transposon tag these restorer genes, the *rf1* locus on chromosome *3* (Duvick *et al.*, 1961) and the *rf2* locus on chromosome *9* (Snyder and Duvick, 1969) were positioned in reference to closely linked RFLP and visible markers (Fig. 19; Wise and Schnable, 1994). Subsequently, four *rf1-m* and seven *rf2-m* alleles were
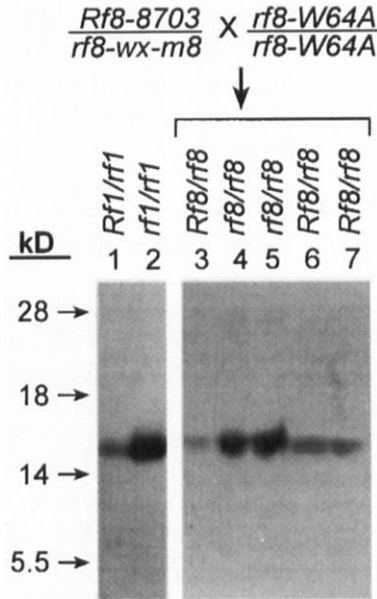
Figure 17   Effect of *Rf1* and *Rf8* on the accumulation of URF13 mitochondrial protein. Plants from families segregating for *Rf1* display a decrease in URF13 as compared to *rf1*-containing plants, although variability has been observed among genotypes. Similarly, within families segregating for *Rf8*, three plants (lanes 3, 6, and 7) that carried the *Rf8* allele accumulated less URF13 protein than two siblings that did not carry this restorer (lanes 4 and 5) (Dill *et al.*, 1997). All plants, regardless of *rf8* genotype, accumulated less URF13 in tassels than in ears (data not shown). In addition, the difference in URF13 accumulation among plants segregating for *Rf8* was much more evident in tassels than in ears. In contrast, plants that carried *Rf1* exhibited a marked decrease of URF13 accumulation in both tassels and ears. Detection of URF13 was performed with an URF13 monoclonal antibody (a gift from C. S. Levings III, North Carolina State University) using goat anti-mouse IgG (H + L) alkaline phosphatase detection. Modified and published with permission from the Genetics Society of America, from Dill *et al.* (1997).

isolated from populations of 123,500 and 178,300 transposon-bearing plants, respectively (Schnable and Wise, 1994; Wise *et al.*, 1996).

   To identify DNA fragments containing *rf1* and *rf2* sequences, DNA gel-blot cosegregation analyses were performed. *Mu1*-hybridizing *Eco*RI restriction fragments were identified that segregated absolutely with male sterility in large families segregating for the transposon-induced *rf1-m3207* and *rf1-m3310* alleles, suggesting that they contained *Mu* transposon insertions in the *Rf1* gene (Wise *et al.*, 1996). These candidate *rf1* DNA fragments were cloned and corresponding cDNAs isolated (R. P. Wise, K. S. Gobelman-Werner, D. Pei, C. L. Dill, P. S. Schnable, unpublished results). However, further experimentation is necessary to establish whether these sequences are part of the *rf1* gene.

   Concurrently, a 3.4-kb *Mu1*-hybridizing *Eco*RI–*Hin*dIII restriction fragment

Transposon tagging of *Rf* genes

Functional gene = Fertile tassel

Mu1

Insertion mutant = Sterile tassel

(A)

(B)

Figure 18 Transposon tagging of *Rf* genes. (A) An insertional mutagenesis strategy employed to generate transposon-tagged mutants of the *rf1* and *rf2* genes. The following two crosses were used to generate large populations of plants that were screened for their inability to restore pollen fertility to cms-T maize (Schnable and Wise, 1994; Wise *et al.*, 1996). Cross 1: Screen for *rf1-m* alleles (123,500 plants): T *Rf1/Rf1, Rf2/Rf2 (Mutator)* × N *rf1/rf1, Rf2/Rf2* (inbred B37). Cross 2: Screen for *rf2-m* alleles (178,300 plants): T *Rf1/Rf1, rf2/rf2* (inbred R213) × N *rf1/rf1, Rf2/Rf2 (Mutator, Cy, Spm)*. These screenings resulted in the isolation of four heritable *rf1-m* alleles and seven heritable *rf2-m* alleles. Subsequently, a cloned *Mu1* transposon was used as a probe to recover the candidate *rf-m* DNA sequences. (B) A male-sterile *rf1-m/rf1-B37* plant identified by detasselling all male-fertile plants resulting from Cross 1.
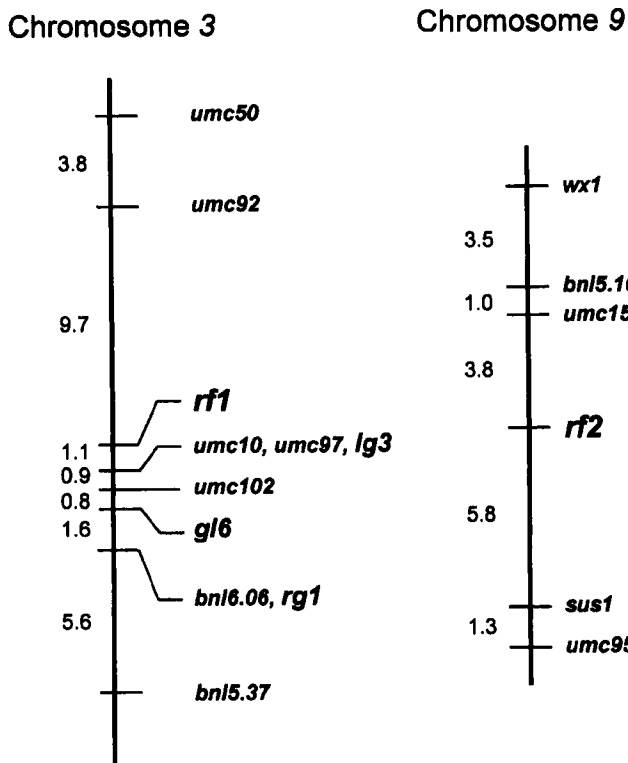
Chromosome 3   Chromosome 9

Figure 19 Consensus genetic maps of the *rf1* and *rf2* regions of chromosomes *3* and *9*, respectively (Wise and Schnable, 1994; Wise *et al.*, 1996). The *rf1* locus and/or linked visible markers were mapped in seven populations consisting of 729 individuals; the *rf2* locus was mapped in three populations consisting of 304 individuals. Data from individual populations were joined with the aid of Join-Map software. The resulting consensus maps place *rf1* between *umc97* and *umc92* on chromosome *3* and *rf2* between *umc153* and *sus1* on chromosome *9*. Numbers to the left of the linkage lines indicate distances in cM. Modified and published with permission from the Genetics Society of America, from Schnable and Wise (1994), and Wise *et al.* (1996).

that cosegragated with male sterility in a family segregating for the *rf2-m8122 Mutator*-induced allele was cloned and shown to contain a portion of the *rf2* gene via allelic cross-referencing experiments. This fragment was used to isolate several *rf2* cDNA clones. One of these clones seemed to be full length because its size corresponds well with that of the *rf2* transcript (ca. 2.2 kb), its translated sequence contains in-frame stop codons 5' of two methionine residues, and it encodes a putative mitochondrial targeting signal (Table I). Computer-based sequence similarity searches of various genome databases revealed that the predicted RF2 protein exhibits 60% identity and 75% similarity to Class II mammalian mitochondrial aldehyde dehydrogenases (ALDH) (Fig. 20; Cui *et al.*, 1996).
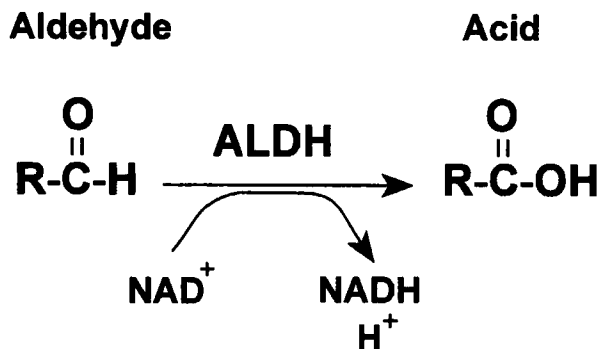
Figure 20 The protein encoded by the *Rf2* nuclear restorer exhibits a high degree of sequence similarity to mammalian mitochondrial aldehyde dehydrogenases (ALDH, EC 1.2.1.3). These enzymes catalyze the oxidation of aldehydes to the corresponding acids; in so doing, they generate NADH.

The diverse array of potential substrates for an ALDH complicates the effort to establish the precise physiological role of the RF2 protein. ALDHs are capable of oxidizing a wide spectrum of aldehydes to their corresponding acids (Fig. 20; Lindahl, 1992). Aldehydes are highly reactive molecules due to the electrophilic nature of their carbonyl groups. Hence, one presumed physiological role for ALDHs in mammals is the detoxification of aldehydes produced as intermediates in various endogenous biochemical pathways. For example, aldehydes are produced during amino acid, carbohydrate, and lipid metabolism. They can also be produced as the result of membrane peroxidation such as may occur during oxidative stress when reactive oxygen species are produced.

An additional complexity to assigning a physiological function for the RF2 protein in restoration relates to the fact that, as discussed above, it probably has a physiological function independent of its role as a nuclear restorer of cms-T. Hence, the physiological functions of the RF2 protein in normal maize and during fertility restoration of cms-T may differ. The difficulty in determining the physiological role of the RF2 protein in fertility restoration is exacerbated by the fact that only limited analyses have been conducted on plant ALDHs (Asker and Davies, 1985; op den Camp and Kuhlemeier, 1997).

C. HOW DOES THE *RF2*-ENCODED ALDH MEDIATE FERTILITY RESTORATION?

Two hypotheses have been articulated for the mechanism by which an ALDH could participate in fertility restoration (Cui *et al.*, 1996). These hypotheses are referred to as the "metabolic hypothesis" and the "interaction hypothesis." Central to both of these hypotheses, is the finding that URF13 seems to alter cell viabili-

ty, even in the absence of toxin (Duvick, 1965; Pring *et al.,* 1988; Korth and Lev-ings, 1993).

According to the metabolic hypothesis, these perturbations in mitochondrial function create either a greater need for ALDH activity or the need for a novel ALDH activity. For example, if URF13 reduces the efficiency of mitochondrial function, the α-oxidation of lipids may become critical as a source of energy dur-ing microsporogenesis. In these circumstances, the role of the RF2 protein in fer-tility restoration would be clear, because α-oxidation requires ALDH activity.

Alternatively, the RF2 protein might function to limit the concentration of ac-etaldehyde, an intermediate produced during ethanolic fermentation and ethanol metabolism (Fig. 21). Indeed, one of the major functions of Class II ALDHs in yeast, *Drosophila,* and mammals, is the oxidation of acetaldehyde produced dur-ing ethanol metabolism. Alcohol dehydrogenase (ADH) and ALDH function in se-ries to metabolize ethanol to acetate. ADH oxidizes ethanol to form acetaldehyde, which is then oxidized to acetate. Both of these oxidation reactions produce NADH. Indeed, it is this NADH-producing pathway that allows these organisms to survive (at least marginally) under aerobic conditions on diets in which ethanol compris-es the sole carbon and energy source.

Although plants are not normally faced with the need to metabolize exogenous ethanol, they do produce ethanol during anaerobic stress. The ATP molecules that play a central role in cellular metabolism are generated both directly by glycoly-
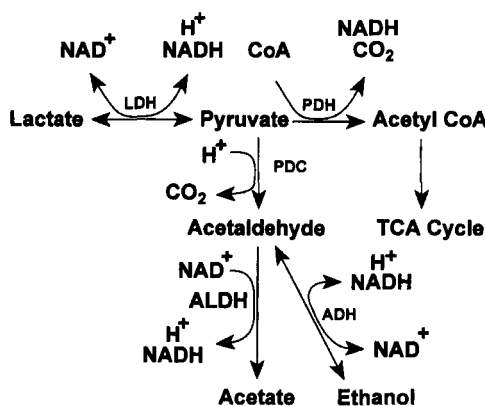

Figure 21   RF2 exhibits a high degree of sequence similarity to mammalian mitochondrial alde-hyde dehydrogenases (ALDH, EC 1.2.1.3). According to the "metabolic hypothesis" T-*urf13*-mediat-ed perturbations in mitochondrial function create either a greater need for ALDH activity or a need for a novel ALDH activity. For example, if these perturbations affect partitioning of pyruvate between the pyruvate dehydrogenase complex (PDH, EC 1.1.1.27) and pyruvate decarboxylase (PDC, EC 4.1.1.1), the RF2 protein may function in the detoxification of acetaldehyde. ADH (EC 1.1.1.1), alcohol dehy-drogenase.

sis and by the oxidative phosphorylation of NADH that is produced by the TCA cycle. In the presence of oxygen, pyruvate generated via glycolysis is imported into the mitochondria by the action of pyruvate dehydrogenase (PDH), which oxidatively decarboxylates pyruvate to form acetyl-CoA (Fig. 21). Acetyl-CoA enters the TCA cycle, where additional NADH is produced during oxidation. The continued functioning of glycolysis and the TCA cycle requires that the NADH molecules generated by these pathways be reoxidized to NAD^+. Under aerobic conditions, this occurs via oxidative phosphorylation, which generates a tremendous amount of ATP. However, under anaerobic conditions, oxidative phosphorylation is not possible. In this situation, pyruvate is shunted to lactate dehydrogenase (LDH) and pyruvate decarboxylase (PDC). LDH can regenerate a single NAD^+ molecule for each molecule of pyruvate reduced to lactate. PDC generates acetaldehyde, which is subsequently reduced by ADH to ethanol, a process that also regenerates an NAD^+ molecule. Hence, under anaerobic conditions, LDH and ADH function to regenerate NAD^+ so the glycolysis can be sustained and thereby continue to produce some quantity of ATP, even in the absence of oxidative phosphorylation.

Therefore, ethanolic fermentation allows plants to survive brief periods of anaerobic stress, such as might occur during transient flooding. However, ethanol is itself toxic. Hence, one possible role for ALDH in plants is in the second step of the oxidation of ethanol (through acetaldehyde) following the return to aerobic conditions after an anaerobic period. Of course, it is not likely that anthers experience anaerobic stress. However ethanolic fermentation can also occur under aerobic conditions. For example, it has been shown that PDC and ADH are expressed (and ethanol accumulates) during tobacco pollen development (Bucher *et al.,* 1994, 1995; Tadege and Kuhlemeier, 1997).

The activity of PDH determines the fate of pyruvate. Acetyl-CoA and NADH both inhibit PDH, while pyruvate and ADP activate it. Hence, although pyruvate preferentially enters the TCA cycle, when respiration is inhibited by some mechanism such as anoxia, or the presence of respiratory inhibitors (Bucher *et al.,* 1994), this balance can be shifted in favor of ethanolic fermentation. It is therefore possible that URF13-mediated perturbations in mitochondrial function could result in additional flux through the ethanolic fermentation pathway during microsporogenesis. Under these conditions, an absence of ALDH activity would result in the accumulation of acetaldehyde and/or ethanol. Because both of these compounds are toxic, such an accumulation could result in tapetal death and male sterility. Hence, one interpretation of the metabolic hypothesis would be that RF2 functions to correct an URF13-mediated metabolic disturbance by preventing the ethanol and/or acetaldehyde poisoning of the tapetum. This interpretation is currently being tested by the analysis of single and double mutants of genes involved in ethanolic fermentation. However, because, as indicated above, there are many physiological pathways that generate aldehydes, ethanol metabolism is only one of many pathways that may be involved in RF2-mediated fertility restoration.

Alternatively, it is also possible that the RF2 protein interacts indirectly with URF13 and thereby prevents the mitochondrial disturbance from occurring at all. This possibility is covered by the interaction hypothesis. Specifically, the RF2 protein could oxidize either an aldehyde component of the inner mitochondrial membrane or Factor X (assuming it is an aldehyde). These oxidation reactions could alter the binding of URF13 to the inner mitochondrial membrane or inactivate Factor X, respectively. Either of these events could reduce the toxicity associated with the accumulation of URF13. Hence, the cloning of the *rf2* gene has stimulated the formulation of several testable hypotheses regarding the specific molecular functions of the RF2 protein in fertility restoration.

VII. PERSPECTIVES BY CMS-T RESEARCHERS

a. Donald N. Duvick, Department of Agronomy, Iowa State University

For more than three decades I have wondered why maize hybrids in T cytoplasm are slightly less vigorous than their isogenic equivalents in normal cytoplasm. When isogenic hybrids in normal and in T cytoplasm were compared, plants in.T cytoplasm were 2 to 3% shorter during all growth stages, they had about 2% fewer leaves at all growth stages, and their grain yield was 2 to 3% lower (see Duvick, 1965, pages 20–26). These differences were statistically significant, and repeatable. They were independent of and different from the effect that pollen sterility per se can have on plant height and grain yield. Pollen-sterile plants are much shorter and yield more grain than pollen-fertile plants, particularly when plants are subjected to stress at the time of flowering, and particularly with genotypes that are highly susceptible to stress. These differences are only an indirect consequence of the changes induced by T cytoplasm. The yield gain probably is due directly to the energy-sparing effect of pollen sterility per se, and is expressed in any cytoplasmic male-sterile system (or even by detasselled plants in normal cytoplasm).

I speculated that the size and yield reduction caused by T cytoplasm is due to a defective cytoplasmic gene or genes, independent of the genetic defect that causes cytoplasmic pollen sterility. Alternatively, fertility restoration might confer only partial correction of the basic defect in T cytoplasm, enough to restore pollen fertility to a nearly normal condition, but not enough to bring general background metabolism up to completely normal levels. But I was unable to test these hypotheses, 30 years ago. Perhaps today's technology will enable design and execution of experiments to test these conjectures.

The question needing an answer is: Why are pollen-fertile plants (plants with restorer genes) in T cytoplasm less vigorous than their nuclear isogenic equivalents in normal cytoplasm?

b. Olen C. Yoder and B. Gillian Turgeon, Department of Plant Pathology, Cornell University

The uniqueness of the *C. heterostrophus Tox* genes to the genome of race T suggests that they were acquired horizontally rather than vertically. The source of these genes, however, remains a mystery and information gained from scrutiny of the gene structures is inconclusive. On one hand, these genes have introns, suggesting eukaryotic origin; on the other, they have highest sequence similarity to bacterial polyketide synthases, opening the possibility of a prokaryotic source. Of particular interest is the fact that *M. zeae-maydis* produces a polyketide toxin similar to T-toxin and has a functional homolog of the *C. heterostrophus PKS1* gene. In both fungi, multiple genes are required for toxin biosynthesis; in *M. zeae-maydis* these clearly constitute a gene cluster, whereas in *C. heterostrophus* it is likely that *PKS1* and *DEC1* were initially linked but are now on two different chromosomes as the result of their linkage to the breakpoint of a reciprocal translocation. Gene clusters are commonly found to control secondary metabolite biosynthesis in fungi. An immediate goal in the continuing analysis of *C. heterostrophus* and *M. zeae-maydis* is to define the limits of the cluster in each fungus and to determine the function of each gene contained within the cluster. A long-term goal is to identify additional genes, not involved in polyketide production, whose products are essential for pathogenesis. Toward that end, we have generated several mutants of *C. heterostrophus* defective in pathogenesis, using the REMI procedure to induce tagged mutations. Analysis of one such mutation site has revealed a gene cluster containing an ORF encoding a cyclic peptide synthetase (*CPS1*) and several other ORFs whose putative products are known in other organisms to be involved in cyclic peptide biosynthesis. Disruption of *CPS1* results in loss of pathogenic capability. In contrast to the genes for T-toxin biosynthesis, which are unique to race T, *CPS1* is found in fungi generally and disruption of its homolog in *C. victoriae,* a pathogen of oats, leads to a drastic reduction in virulence. These results suggest the existence of a cyclic peptide required for general fungal virulence; host specificity of various pathogens is determined by unique factors such as T-toxin, PM-toxin, and in the case of *C. victoriae,* victorin.

c. Daryl R. Pring, Crop Genetic & Environmental Research Unit, USDA-ARS and Plant Pathology Department, University of Florida

The historic question concerning cms-T is the nature of the fertility-restoring genes and their recessive alleles. Since maize *Rf2* may represent an aldehyde dehydrogenase, an assumed normal gene, determination of the nature of the *rf2* allele would be a major advance in our understanding of abnormal processes resulting in male sterility. What, then, might be the "normal" functions of *Rf1, Rf8,* or *Rf*?* A key may be the site(s) of action of these genes. Each of the three 5'-

termini associated with *Rf1, Rf8,* and *Rf** occur within T-*urf13,* in a region high-
ly similar to the progenitor sequences located 3′ to *rrn26.* Analyses of *rrn26*
transcripts revealed three species (Maloney *et al.,* 1989). The largest transcript
represents the initiated species, and a processing event generates the 5′ mature
species. The third transcript probably represents a precursor to 3′ processing,
generating the mature *rrn26.* Sequences 3′ to the mapped 3′-terminus of mature
rrn26 (Kennell and Pring, 1989), which may include the terminus of the *rrn26*
progenitor, are represented within the 317 bp shared by T-*urf13* and *rrn26*
(Dewey *et al.,* 1986). These T-*urf13* sequences may then represent a template
for processes associated with *rrn26* maturation. The assignment of a 3′-termi-
nus to the immature *rrn26* does not necessarily reflect transcript termination se-
quences, but rather, a probable intermediate, stable terminus. The complexity of
3′ transcript processing in chloroplasts, recently invoked for mitochondria, raises
the distinct possibility that endo- and exonucleases, and regulatory factors, may
be involved. Since such a scenario may invoke multiple mechanisms, *Rf1* and
its analogs may represent components involved in *rrn26* maturation. Thus, it is
conceivable that mechanisms operative in the putative 3′ transcription termina-
tion and processing of *rrn26* are also operative in cleavage within T-*urf13.* The
consensus sequence derived for 5′-termini associated with *Rf1, Rf8,* and *Rf** are
retained in the progenitor sequence 3′ to *rrn26,* albeit two sites exhibit 1-nt
changes. The development of *in vitro* transcript processing assays, using ex-
tracts from lines differing in the candidate alleles, may allow the pursuit of these
points. How the recessive alleles of the three *Rf* genes may fit a possible
rrn26/T-*urf13* processing model may emerge from such studies.

Still, a more profound question remains: Why does the presence of truncated
transcripts in *Rf1, Rf8,* or *Rf** lines, which have abundant, whole-length T-*urf13*
transcripts, apparently interfere with accumulation of URF13? The develop-
ment of *in vitro* translation systems programmed with appropriate templates
may allow the effect of the truncated transcripts on T-*urf13* translation to be ad-
dressed.

d. Charles S. Levings III, Department of Genetics, North Carolina State Uni-
versity

Despite substantial correlative evidence linking T-*urf13* and its polypeptide
product URF13 with cytoplasmic male sterility (cms), the precise relationship
between T-*urf13* and cms remains unresolved. Especially supportive of a cause-
and-effect relationship are the studies of *cms*-T revertants in which a mutation
of deletion of T-*urf13* that knocks out URF13 synthesis results in the phenotypic
reversion from male sterility and disease susceptibility to male fertility and dis-
ease resistance (Rottmann *et al.,* 1987; Wise *et al.,* 1987a; Fauron *et al.,* 1990).
Other data also suggest the importance of URF13 in causing cms, for example,
the effect of *Rf1* on the transcriptional profile of T-*urf13* (Dewey *et al.,* 1987;
Kennell *et al.,* 1987; Kennell and Pring, 1989).

Firmly established is the cause-and-effect association between URF13 and disease susceptibility (toxin sensitivity). In this instance, pathotoxins (called T-toxins) produced by *Bipolaris maydis* race T or *Phyllosticta maydis* interact with URF13 to permeabilize the inner mitochondrial membrane, thereby dissipating the membrane potential, terminating oxidative phosphorylation, and causing the loss of mitochondrial function and cell death (Levings and Siedow, 1992). Expression of T-*urf13* in *Escherichia coli* (Dewey *et al.,* 1988; Braun *et al.,* 1989), tobacco (von Allmen *et al.,* 1991; Chaumont *et al.,* 1995), yeast (Glab *et al.,* 1990; Huang *et al.,* 1990), and insects (Korth and Levings, 1993) also confers T-toxin and methomyl sensitivity, confirming the capacity of URF13 to interact with toxin to permeabilize membranes in a variety of organisms.

An attractive explanation for cms is that the mechanism for toxin sensitivity and cms are the same. In cms-T maize, URF13 accumulates in the inner mitochondrial membrane. If an endogenous compound with toxin-like properties exists, it could interact with URF13 to permeabilize the inner mitochondrial membrane and cause cell death. When this compound occurs uniquely or predominately in the anther tissue, it could interact with URF13 to terminate pollen development and explain the cms phenomenon. Because URF13 accumulates in all cms-T plant tissues, the endogenous compound must exhibit limited expression in non-anther tissues to avoid a lethal plant effect. In cms-T plants there is evidence that the T cytoplasm alters leaf number, internode length, and grain yield (Duvick, 1965). Limited expression of a toxin-like compound may account for these unique effects in plants carrying the T cytoplasm.

Indeed, preliminary studies support the occurrence of an endogenous compound with toxin-like properties that interact with URF13 to cause cms (D. Cho and C. S. Levings III, unpublished data). Treatment of cms-T mitochondria with an aqueous extract prepared from anther tissue results in the dissipation of membrane potential ($\Delta\Psi$) and the stimulation of respiration in the presence of NADH. Treatment of mitochondria from normal maize with the extract has no effect on membrane potential or respiration. These results, which are similar to those observed with cms-T and normal mitochondria treated with T-toxin or methomyl, suggest that a compound with toxin-like properties occurs in the anther tissue. Thus far, the specific identity of this compound remains unresolved. These initial studies support the view that the capacity of URF13 to interact with toxin or an endogenous compound accounts for disease susceptibility and cms, respectively.

VIII. FUTURE DIRECTIONS

Substantial progress has been made in understanding the cms-T system of maize. Early studies revealed that both male sterility and disease susceptibility are maternally inherited, and that susceptibility is due to the sensitivity of T-cytoplasm

mitochondria to β-polyketol fungal toxins. These studies provided a foundation for further research that resulted in the cloning of the T-*urf13* and *Rf2* genes from maize and the *ChPKS1* gene from *C. heterostrophus,* and the generation of models for the topology of URF13 in the inner mitochondrial membrane, *Rf1*-mediated processing of T-*urf13* transcripts, and the evolution of toxin biosynthesis in *C. heterostrophus* and *M. zeae-maydis.* Although these advances have provided significant insight into this interesting nuclear–mitochondrial and host–pathogen interaction, much remains to be learned. For example:

What is the molecular basis of male sterility? It is well established that fungal pathotoxins interact with URF13 to cause mitochondrial disruption leading to cell death. However, it is still unclear how URF13 causes mitochondrial dysfunction leading to male sterility; this dysfunction occurs in the absence of the fungal toxins. Preliminary studies by D. Cho and C. S. Levings (Section VIId) support the existence of a tassel-specific, toxin-like compound (termed Factor X by Flavell, 1974) that could interact with URF13 to permeabilize the mitochondrial membrane and cause cell death. If confirmed, this finding would suggest that the mechanism of male sterility is similar to the mechanism of disease susceptibility. Although this hypothesis is intriguing, it is also possible that T-cytoplasm mitochondria are less efficient than N mitochondria and are therefore unable to meet the exceptional energy demands of microsporogenesis (Warmke and Lee, 1978; Lee and Warmke, 1979), thereby causing premature degeneration of the tapetum and hence male sterility. In support of this hypothesis is the observation by D. N. Duvick (Section VIIa; Duvick, 1965) that T-cytoplasm lines grow less vigorously than N-cytoplasm lines, even in the absence of disease. Of course, it is also possible that Factor X works in concert with the high metabolic rate of tapetal cells to cause sterility.

How do *Rf1* and *Rf2* mediate fertility restoration? Although both *Rf1* and *Rf2* are required to restore fertility to cms-T, they have very different functions. The molecular phenotypes mediated by *Rf1* and two similar restorers, *Rf8* and *Rf**, indicate that they are involved in the differential processing of T-*urf13* transcripts and the concurrent reduction of the URF13 protein. These processing events result in novel transcripts, but do not significantly decrease the levels of the original T-*urf13* transcripts. Hence, the mechanism by which T-*urf13* transcript alteration influences the reduction of URF13 is not clear. The *Rf1*/T-*urf13* interaction has the potential to serve as a model for restorer-mediated processing of mitochondrial transcripts because this process occurs in many cms systems (e.g., sorghum, rice, petunia, and rapeseed). In addition, the analysis of this interaction may contribute to our understanding of fundamental processes of RNA processing in plants.The sequence of *rf2* indicates that it encodes a mitochondrial ALDH. Identification of the physiologically significant substrate(s) of this enzyme may provide support for one of the several hy-

potheses that have been proposed to explain the role of an ALDH in fertility restoration.

Why are restored T-cytoplasm lines acutely susceptible to *C. heterostrophus* and *M. zeae-maydis*? There is evidence that restoration can reduce the sensitivity of T-cytoplasm mitochondria to T-toxin (Watrud *et al.,* 1975a; Barratt and Flavell, 1975). This is likely due to the ability of *Rf1* to reduce URF13 levels in the mitochondria and thus reduce the amount of substrate available for toxin binding (Braun *et al.,* 1990; Hack *et al.,* 1991). However, this reduction in mitochondrial sensitivity to the toxins does not provide significant resistance to the fungi (as evidenced by the 1970 epidemic on restored T-cytoplasm maize). Would further suppression of URF13 levels result in resistance? As the molecular mechanisms associated with *Rf1-* and *Rf2*-mediated restoration are elucidated, it may become possible to decrease (or even eliminate) disease susceptibility without the loss of the valuable characteristic of male sterility.

Alternatively, if it is not possible to generate T-cytoplasm lines that are toxin resistant, are there ways to confer disease resistance to toxin-sensitive lines? Perhaps research on the mechanisms of pathogenesis in the fungi will identify ways to modify maize to better defend itself from fungal infection, such as by interfering with the function of fungal genes involved in disease induction, such as *CPS1*. Current efforts to identify and clone genes responsible for pathogenesis in these fungi may provide answers.

Although it is clear that our understanding of cms-T and its associated diseases has advanced considerably, many significant questions remain to be answered. The challenge now is to build on the existing cytological, physiological, genetic, and molecular foundation to answer the remaining questions and assist in maize improvement.

ACKNOWLEDGMENTS

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