INFLUENCE OF THE NUCLEAR GENOME ON TRANSCRIPTION OF A MAIZE MITOCHONDRIAL REGION ASSOCIATED WITH MALE STERILITY AND TOXIN SENSITIVITY

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1987

PREFACE

"It is hardly an exaggeration to say that Nature tells us, in the most emphatic manner, that she abhors perpetual self-fertilization".

- Final sentence of Charles Darwin's book The Various Contrivances by which Orchids are Fertilized by Insects".

ACKNOWLEDGEMENTS

I thank Daryl Pring for his insightful guidance, constant enthusiasm, and for providing an atmosphere that stimulates productivity as well as creativity. His professionalism is an admirable quality that has hopefully been instilled in me. I would also like to thank the other members of my committee and other faculty, post-doctoral researchers and students that have helped in my research and education. I am particularly grateful to the technical advice given by Al Smith and Roger Wise as well as the help provided by fellow researchers of 1404 Fifield; Al Fliss, Sandy Fogg, Jeff Mullen and Torbert Rocheford. Special thanks go to Robin Roffey for putting up with my "thirst for knowledge" and enabling me to get this part of my life together. Finally, I thank my family for their continued support and encouragement for all my interests and efforts.

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December, 1987

Chairman: Daryl R. Pring
Major Department: Plant Pathology

Maize plants carrying T cytoplasm are male sterile and highly susceptible to the fungal pathogens Cochliobolus heterostrophus and Phyllosticta maydis. Mitochondria isolated from T cytoplasm plants are sensitive to toxins produced by the pathogens. A gene unique to T mtDNA, urf13-T, is associated with the phenotypes of cms and toxin sensitivity. The nuclear genes Rf1 and Rf2 restore plants with T cytoplasm to fertility and decrease the sensitivity of T mitochondria to the toxins; other unidentified nuclear genes, besides the Rf genes, also appear to influence the level of toxin sensitivity.

The urf13-T gene is 5' to the gene ORF25 and adjacent to a 5 kb duplicated region of the maize mitochondrial genome that also is 5' to atp6. Transcripts of atp6, urf13-T and ORF25 from N, T and T-restored cytoplasms were analyzed in five nuclear backgrounds. Sequences within the 5 kb repeated region promote transcription of atp6 and the

cytoplasms were analyzed in five nuclear backgrounds. Sequences within the 5 kb repeated region promote transcription of atp6 and the cotranscribed genes urf13-T and ORF25. The steps in transcript maturation were determined for these genes. Differences between N and T atp6 transcripts were associated with two small DNA insertions that apparently create an RNA processing site in the T mitochondrial genome. The 5' termini of a transcript unique to fertility restored mtRNAs mapped to position +14 of the urf13-T gene and was not a suitable substrate for capping with guanylyl transferase. This transcript is detected in backgrounds containing the dominant gene Rf1 and is associated with a reduction in the urf13-T gene product, a 13 kD protein. The primary role of the RF1 gene may involve aspects of translation rather than an RNA processing event which creates the restorer-specific transcript. Nuclear background, separate from fertlity restoring genes, influenced the synthesis and abundance of specific urf13-T/ORF25 transcripts.

CHAPTER I

INTRODUCTION

Mitochondrial Genomes

Prior to the divergence of the fungal, animal and plant kingdoms, a single endosymbiotic association between free-living aerobic bacteria and primitive eukaryotic cells is one hypothesis for the origin of mitochondria (rev. by Gray and Doolittle, 1982). Despite recognizable differences, mitochondria from all eukaryotic kingdoms appear to have originated from a specific subdivision of the purple eubacterial phylum (Yang et al., 1985) and have maintained their basic structure and function. The fundamental mechanisms of the electron transport system and oxidative phosphorylation have been highly conserved throughout the development and divergence of fungal, animal and plant species (Moore and Rich, 1980). Plant mitochondria, however, have additional routes for oxidizing substrates and a terminal oxidase not found in mammalian mitochodria which may enable plant cells to function under a wide variety of metabolic and/or environmental conditions (Moore and Rich, 1980).

Between 300 to 400 different polypeptides are estimated to be in the protein component of yeast mitochondria. Of these, approximately 10% are synthesized in the mitochondria and the remainder are synthesized in the cytoplasm (Schatz and Mason, 1974). The same basic set of mitochondrial-synthesized proteins are found in mitochondria of all

eukaryotic species yet the mitochondrial genomes which encode these proteins vary considerably with regard to size and organization.

Animal mitochondrial genomes range from 16 to 23 kb (Grivell, 1983; Kessler and Avise, 1985) and exhibit high conservation in gene organization. The complete nucleotide sequence of the human mitochondrial genome (Anderson et al., 1981) identified 13 protein coding regions, two rRNAs and 22 tRNAs (Borst et al., 1984). All metazoan species studied thus far contain the same 13 proteins although their order varies slightly (Anderson et al., 1982; Bibb et al., 1981; Roe et al., 1985). These mitochondrial genomes are very efficient, containing little non-coding regions between genes (Ojala et al., 1981).

Fungal mitochondrial genomes are generally larger than animal mitochondrial genomes, ranging from 19 to 176 kb (Clark-Walker, 1980; Hintz et al., 1985). Besides three additional tRNAs, RNA processing enzymes encoded within an intron of the cytochrome b gene (cob) gene and the F_0-ATPase subunit 9 gene found in some species (e.g. yeast), fungal mitochondrial genomes encode the same set of proteins as animal mitochondrial genomes (Borst et al., 1984). The majority of the increased size of the fungal mitochondrial genomes is accounted for by non-transcribed, A-T rich spacer regions as well as introns (Bernardi, 1982).

The size and organization of higher plant (angiosperms) mitochondrial DNAs (mtDNAs) are quite different than their animal and fungal counterparts. The size of plant mitochondrial genomes range from 208 (Palmer and Herbon, 1987) to 2500 kb (Ward et al., 1981), and can vary greatly within a single plant family. For example, an eight-fold size difference is found within the cucurbit family with sizes of the

watermelon and muskmelon mitochondrial genomes estimated at 330 and 2500 kb, respectively (Ward et al., 1981). The increased size is not the result of repetitive DNA, as less than 10% of the cucurbit mtDNAs are repetitive (Ward et al., 1981), nor is it a result of high A-T spacer regions as is the case in fungal mtDNAs. The base composition of plant mtDNAs examined to date are consistently between 46 to 51% G-C (Pring and Lonsdale, 1985). An explanation for the increased size of plant mitochondrial genomes in comparison to animal and fungal mitochondrial genomes is still unavailable, but there are some unique aspects of higher plant mitochondria that may have contributed to maintaining or expanding their size.

Whereas most animal and fungal mitochondrial genomes are singular circular molecules, all but one (Brassica hirta; Palmer and Herdon, 1987) of the plant mitochondrial genomes studied thus far exist in multipartite circular molecules arising through intra- and intermolecular recombinational events. The complete mtDNA physical maps for two Brassica species and for maize have been reported (Palmer and Shields, 1984; Stern and Palmer, 1986; Lonsdale et al., 1984). The mitochondrial genomes of turnip and spinach exist as three molecules; a complete or "master" circular molecule and two subcircular molecules that arise via recombination through repeated DNA regions. For example, the 218 kb turnip mitochondrial genome contains two direct repeats of about two kb, spaced 135 and 83 kb from each other. Intramolecular recombination through these repeats results in subcircular molecules of 135 and 83 kb (Palmer and Shields, 1984). The maize mitochondrial genome is more complex as it contains at least six repeated regions, five of which are known to participate in recombination (Lonsdale et

al., 1984). The proposed model of recombination predicts a minimum of nine subcircular molecules in addition to the 570 kb master circle. Being typically below 10% of the genome, the repetitive DNA is not significant for enlarging the genome but for conferring the ability for intra- and intermolecular recombination which has played a major role in creating the variability found among plant mitochondrial genomes.

The large size of plant mitochondrial genomes could allow a substantial increase in its coding capacity. In contrast to the 13 protein coding regions in the animal mitochondrial genomes, isolated plant mitochondria synthesize at least 20 to 30 polypeptides (Leaver et al., 1983; Hack and Leaver, 1983). At present, it is known that plant mitochondrial genomes encode at least four genes not found in animal mtDNA. These include the 5S rRNA gene (Bonen and Gray, 1980), a ribosomal binding protein (Bland et al., 1986), alpha-F_1ATPase (atpA; Braun and Levings, 1985), and subunit 9 of F_0-ATPase (atp9; Dewey et al., 1985b). Of these genes, only atp9 has been detected in the mitochondrial genomes of fungal species (Hensgens et al., 1979).

Measurement of the actual coding capacity of the turnip mitochondrial genome has shown that there is approximately 60 kb of abundant, nonoverlapping RNAs (28% of the genome), divided into 24 distinct transcript regions (Makaroff and Palmer, 1987). When less abundant transcripts are included, the percentage of the total genome that is transcriptionally active increases to a level similar to that detected in watermelon (70%; Stern and Newton, 1985) and Brassica napus (100%; Carlson et al., 1986). These data suggest that plant mtDNAs may encode a far greater number of proteins than their animal and fungal counterparts. The synthesis of proteins in isolated mitochondria and

subsequent 2-D gel electrophoresis has detected only 30 polypeptides (Hack and Leaver, 1983), yet this type of analysis may not be capable of detecting less abundant polypeptides or those induced by external factors or by specific plant tissues. It has been reported that mitochondria isolated from different plant organs and the same organ at different developmental stages exhibit qualitative and quantitative differences in the polypeptides they synthesize (Newton and Walbot, 1985). It is also known that certain plant mitochondrial respiratory mechanisms are active only in specific tissues (Moore and Rich, 1980). Although it is not commonly believed that plant mitochondrial genomes encode significantly more proteins than animal and fungal mitochondrial genomes, there is sufficient evidence indicating that there are at least a few more genes in plant mitochondrial genomes and that these genomes are more complex and diversified than the mitochondrial genomes of animals and fungi.

Mitochondrial Transcription

Transcription of the mammalian mitochondrial genome is relatively simple and highly coordinated. Transcription is initiated in the displacement loop region, the site of the heavy strand replication origin. A consensus sequence 5' CANACC(G)CC(A)AAAGAPyA 3' is present on both DNA strands in this region and is regarded as a candidate promoter (Chang and Clayton, 1984). The entire genome is transcribed, from both strands, ensuring stoichiometric transcription of all genes. The 13 protein coding genes are all located on the heavy strand and most are directly adjacent to tRNAs. The tRNAs are posttranscriptionally cleaved from the primary transcript and the intermediate mRNAs are

polyadenylated (Ojala et al., 1981). The relative abundance of the mitochondrial rRNAs is apparently increased by an attenuation process occurring on the heavy strand at the 3' end of the 16S rRNA gene, resulting in the release of transcripts containing both the large and small rRNAs (Clayton, 1984). The punctuation of the structural genes with tRNAs and the process of attenuation both help make mammalian mitochondrial transcription extremely efficient.

Gene order and mode of transcription are much different in fungal mitochondria than in animal mitochondria. In yeast, genes are scattered around the genome without apparent order as different orders are found in different yeast species (Tabak et al., 1983). The genetic information is almost exclusively coded on one strand and 19 sites of transcription initiation have been identified (Christianson and Rabinowitz, 1983). Preceeding the 5' end of yeast primary transcripts (ribosomal and protein coding genes) is the nine nucleotide sequence, 5'$^{T}/_{A}$TATAAGTA 3', representing positions -8 to +1 (Osinga and Tabak, 1982; Christianson and Rabinowitz, 1983). Most of the yeast transcriptional units appear to be monogenic, although a few multigenic transcripts (Christianson and Rabinowitz, 1983) and multiple initiation sites for a single gene (ATPase subunit 9) have been reported (Edwards et al., 1983).

To further characterize the yeast mitochondrial promoter region, in vitro transcription systems have examined the effect of mutations within and adjacent to the nonanucleotide consensus sequence. Mutations within the nonanucleotide sequence were found to either completely inhibit transcription (Biswas and Getz, 1986) or reduce transcriptional efficiency 80 to 90% (Schinkel et al., 1987). Changes at positions +2,

+3, and +4 also affected transcriptional efficiency (Biswas and Getz, 1986; Wettstein-Edwards et al., 1986) whereas changes at +1 had little effect (Schinkel et al., 1987). In general, changes in promoter efficiency could not be correlated to the energy requirements of strand dissociation or to any other obvious mechanisms. Recently, it has been shown that efficient in vitro promoter sequences do not necessarily function well in vivo (Francisci et al., 1987).

The steps involved in posttranscriptional processing of fungal mitochondrial genes have received a great deal of attention. Unlike mitochondrial genes in animals, many fungal mitochondrial genes contain intervening sequences and most genes undergo some sort of posttranscriptional processing. Processing sites for maturation of multigenic transcripts have been determined (Osinga et al., 1984) as well as consensus sequences involved in processing of introns (group I, Waring and Davies, 1983; group II, van der Veen et al., 1986). Fungal mitochondrial genes may also be translationally regulated (rev. by Fox, 1986) or undergo posttranslational modification (Pratje et al., 1983). The genes that control the RNA processing and translational events are encoded in the nucleus or in the mitochondria, and in some cases, within the gene they affect (e.g. maturases; rev. by Grivell and Borst, 1982).

A genetic system analogous to mitochondrial genomes exists in chloroplasts of higher plants. Chloroplast genomes range in size from 120 to 217 kb (Palmer et al., 1987), with most of the size variation being accounted for by differences in a large inverted repeat (rev. by Zurawski and Clegg, 1987). The complete nucleotide sequence of two chloroplast genomes (liverwort, Ohyama et al., 1986; tobacco, Shinozaki et al., 1986) reveals approximately 50 protein-coding genes, about 30

tRNA genes and four rRNA genes. Chloroplast DNA sequences important in gene regulation (e.g. promoters, ribosome binding sites, and transcription termination sites) are similar to those of prokaryotic genomes, supporting the endosymbiotic theory of the origin of chloroplasts. However, expression of many genes involves posttransciptional processing. Furthermore, in the tobacco chloroplast genome, eight of the protein-coding genes and seven tRNAs contain introns, many of which have features similar to eukaryotic introns. This has led to speculation that ancestral photosynthetic prokaryotes had introns in their genomes which have been retained in chloroplasts (Shinozaki et al., 1986).

In vitro expression studies have identified two critical regions in the chloroplast promoter. The first is analogous to the prokaryotic '-35' consensus region and the second to the '-10', or TATA region (Link, 1984). It is important to note that although consensus sequences can be accurately defined, in some prokaryotic systems there appears to be great tolerance for deviation from the consensus sequence (Siebenlist et al., 1980).

The complexities of plant mitochondrial genomes have made gene expression studies difficult and often misleading. Initial transcript analyses found that the majority of maize mitochondrial genes have very complex Northern patterns (rev. by Eckenrode and Levings, 1986). Some of the maize mitochondrial transcript analyses are complicated by the association of certain genes with repeated regions. For example, atpA is internal to a 12 kb repeat in N cytoplasm; cytochrome c oxidase, subunit II (coxII) is immediately 3' to a 2 kb repeat (Dawson et al., 1986) and the 5' flanking region of atp6 is repeated in T cytoplasm

(Dewey et al., 1986). The location of mitochondrial genes in or adjacent to repeated regions also has been found in other plant species. Some of these include the 5S, 18S, and 26S rRNA genes in wheat (Bonen and Gray, 1980; Falconet et al., 1984), atpA in sorghum and Oenothera (Dawson et al., 1986), cytochrome c oxidase subunits I and III (coxI and coxIII) in Oenothera (Heisel et al., 1987), and coxII in soybean (Grabau, 1987). There also is one report of a duplicate gene, not associated with a larger repeat (petunia atp9; Rothenberg and Hanson, 1987). It is clear that many plant mitochondrial genes have complex transcriptional patterns which are unrelated to associations with repeated regions, yet an equal number exhibit simple, one-transcript Northern patterns. For example, five of the ten known genes in the turnip mitochondrial genome have a single major transcript (Makaroff and Palmer, 1987).

At present, there is only one confirmed example of an intron in higher plant mitochondrial genomes. The coxII gene in three monocot species [maize (Fox and Leaver, 1981), rice (Kao et al., 1984) and wheat (Bonen et al., 1984)] contains a single intron which is not detected in dicot species [Oenothera (Heisel and Brennicke, 1983), and pea (Moon et al., 1985)]. An intron also occurs in the gene for complex I of the NADH-ubiquinone oxidoreductase detected in tobacco, maize (Bland et al., 1986) and watermelon (Stern et al., 1986), although it has yet to be determined if this is a functional gene.

There are a few examples of multigenic transcripts in plant mitochondrial genomes, such as atp9 and S13-like genes in tobacco (Bland et al., 1986), urf13-T and ORF25 genes in T cytoplasm maize (Dewey et al., 1986), and coxII and initiator met-tRNA in soybean (Grabau, 1987).

However, the majority of plant mitochondrial genes appear to be independently transcribed. Genetic information is coded on both DNA strands in plant mitochondrial genomes (Dawson et al., 1986) yet without a particular order. In the turnip and maize mitochondrial genomes, genes are located throughout the genome and are not clustered in functional units (e.g. subunits of the ATPase complexes or cytochrome oxidases) either on the master circle or subgenomic circles (Makaroff and Palmer, 1987; Dawson et al., 1986).

Transcriptional initiation sites have not been identified for plant mitochondrial genomes, although there does appear to be a short sequence common to the 5' end of some transcripts (Isaac et al., 1985; Hiesel and Brennicke, 1985; Moon et al., 1985; Young et al., 1986; Rothenberg and Hanson, 1987). However, none of the plant studies distinguished primary transcripts from processed transcripts as has been accomplished in other mitochondrial genomes through the use of a capping enzyme. The capping enzyme, guanylyl transferase, specifically caps transcripts that retain a di- or tri-phosphate 5' terminus and has been used to locate transcript initiation sites in human (Chang and Clayton, 1984) and yeast (Christianson and Rabinowitz, 1983) mitochondrial genomes as well as the chloroplast genome in maize (Mullet et al., 1985).

A transcript termination sequence has been proposed for some plant mitochondrial genes (Schuster et al., 1986). It appears to function in a manner similar to bacterial transcript terminators by the generation of a stem-loop structure. There is no evidence of translational control over the expression of plant mitochondrial genes, but some chloroplast genes appear to be translationally regulated (Rock et al., 1987).

Cytoplasmic Male Sterility

Mutational analysis is a powerful method for understanding the functions of a specific gene product. Many mitochondrial mutations are available for analysis in lower eukaryotes due to the ability to maintain mutants on non-fermenting carbon sources. Unfortunately, most mutations in mitochondrial genes of higher eukaryotes appear to be lethal as few have been documented. A few cytoplasmic traits are known in higher plants; some involve the chloroplast [tentoxin sensitivity (Durbin and Uchytil, 1977); triazine resistance (Pfister et al., 1981)] and others are associated with mitochondria [disease susceptibility (Hooker et al., 1970), non-chromosomal stripe (Shumway and Bauman, 1967; Newton and Coe, 1986) and most types of cytoplasmic male sterility (rev. by Hanson and Conde, 1985)].

Cytoplasmic male sterility (cms) is a cytoplasmically-controlled alteration in the development of the male gametophyte resulting in nonfunctional pollen. This phenomenon is usually specific to microsporogenic and microgametogenic tissues, although abnormalities in megasporogenesis and megagametogenesis are associated with a few cms systems (Grun, 1976). In the T-cms system of maize, all plant tissues are associated with an increased susceptibility to two fungal pathogens (see below). Cytoplasmic male sterility has wide distribution among plant families, being reported in 22 different families and in over 150 species (Edwardson, 1970). The prevalence of cms among plant families suggests that a common mechanism may be involved, however analyses of cms in major crop species have yet to uncover a simple mechanism explaining cytoplasmically controlled pollen abortion. Many mechanisms,

or at least many manifestations of a few mechanisms, appear to be involved.

Cytoplasmic male sterility is an economically desirable trait for the production of hybrid seed. Seeds produced on cms plants are hybrid, and homogeneous populations of male sterile plants are easier to maintain with maternally inherited cms lines than with nuclear male sterile lines. Interspecific crossing has been the most common way of producing defined cms types (Laser and Lersten, 1972). Described as alloplasmic cms, the interspecific crosses are believed to create an incompatibility between the nuclear genome of one species and the cytoplasm of another which is manifested as cms. The idea that cms represents a breakdown in a nuclear-cytoplasmic interaction is further supported by the presence of nuclear genes that "restore" pollen fertility. The process of fertility restoration was first detected in maize when the cms condition was being transfered into standard inbred lines for use in hybrid seed production (Jones et al., 1957). The progeny of cms lines crossed to certain inbred lines were fertile, even though they had maintained a "sterile" cytoplasm. The ability to restore pollen fertility was inherited in a mendelian fashion and usually controlled by one or two genes. Nuclear-controlled fertility restoration was subsequently found in most cms systems [40% of cms types that had arise through interspecific crosses; 80% in intraspecific-derived cms types (Grun, 1976)].

Restoration of pollen fertility can be monogenically or polygenically controlled and fertility restoration genes (Rf) have been found to be recessive or dominant. The Rf genes are classically used to distinguish specific cms cytoplasms within a species. In maize, for

example, there are three distinct types of cms cytoplasms (T, S, and C), each distinguished by different Rf genes; T cytoplasm is restored by Rf1 and Rf2, S cytoplasm by Rf3 and C cytoplasm by Rf4 (Duvick, 1965). Multiple alleles for Rf3 and Rf4 may be present in certain inbred maize lines (Laughnan and Gabay-Laughnan, 1983).

Cytological studies demonstrated that abnormalities associated with cms systems can occur at almost every stage of pollen development and in different anther tissues (rev. by Laser and Lersten, 1972). Some common cytological abnormalities include 1) tapetal abormalities, 2) disfunctioning of the stamen vasculature, and 3) failure of callose dissolution around the developing microspores (rev. by Laser and Lersten, 1972). An example of the variability in the developmental stage at which pollen abortion occurs is observed in the differences between sporophytic and gametophytic restoration. In T-cms and C-cms systems of maize, restoration is sporophytic while in S-cms, restoration is gametophytically controlled. Thus, in Rf3rf3 plants carrying S cytoplasm only 50% of the pollen is functional as restoration is dictated by the genotype of the individual microspore; the presence of Rf3-carrying microspores does not appear to affect abortion of rf3-carrying microspores in the same anther (Buchert, 1961).

The most common defect reported among cms systems is a premature breakdown of the tapetal cells, a layer of nutritive cells that surround the developing microspores. At least four monocot genera and nine dicot genera have cms systems in which the tapetal cells degenerate prematurely (Laser and Lersten, 1972; Horner, 1977; Peters and Jain, 1987). Tapetal abnormalities are not restricted to cms systems, as they also occur in nuclear male-sterile mutants of soybean (rev. by Graybosch

and Palmer, 1984). In the T-cms system of maize, ultrastructural studies have shown that the mitochondria of tapetal cells are frequently the first organelle to degenerate (Warmke and Lee, 1977). The mitochondria in the tapetal cells of both N and T-cms maize plants were found to rapidly increase in number immediately preceding the stage in which T-cms anthers degenerate. The number of mitochondria within an individual tapetal cell increased almost 40-fold during microsporogenesis and microgametogenesis. This observation led to a proposed mechanism of pollen abortion in which the mitochondria of T cytoplasm malfunction under the high stress conditions associated with tapetal cell development (Warmke and Lee, 1978).

Host-selective Toxins and T Cytoplasm of Maize

The T cms system of maize has an additional cytoplasmically inherited trait, that of disease susceptibility. Maize plants having T cytoplasm are highly susceptible to the fungal pathogens Cochliobolus heterostrophus, race T, causal agent of southern corn leaf blight (Hooker et al., 1970), and Phyllosticta maydis Arny and Nelson, causal agent of yellow corn leaf blight (Scheifele et al., 1969). The association of cms with disease susceptibility has never been separated. Whereas cms is solely manifested in anther tissues, T cytoplasm plants are susceptible to the pathogens at all developmental stages, indicating that host factors involved in this host-parasite interaction are constituitively expressed. Understanding the molecular basis for susceptibility of T-cms plants to the pathogens could conceivably provide important clues to the mechanism of cms, and vice versa.

In the late 1960s, T cytoplasm was the primary maize cms cytoplasm used for hybrid seed production leading to a virtual monoculture of plants with T cytoplasm in the U.S. [up to 80% in 1970 (Tatum, 1971)]. This set the stage for the 1970 epiphytotic of the southern corn leaf blight. The imperfect stage of C. heterostrophus [anamorph: Bipolaris maydis (Nisik.) Shoemaker], race T, initially colonized maize plants in Florida and quickly spread throughout the South and parts of the Midwest. The epidemic caused a 50% loss in the corn crop in the corn belt states and almost a complete loss in the southern states, resulting in a total loss of over 1 billion dollars (rev. by Ullstrup, 1972).

Cochliobolus heterostrophus, race T, and Phyllosticta maydis produce toxins that specifically affect T cytoplasm (Bhullar, et al., 1975; Yoder, 1973). A toxinless race of C. heterostrophus (race O) is able to colonize maize plants and incite disease, although the effects are less severe and this race exhibits little or no specificity towards any maize cytoplasm (Hooker et al., 1970). This demonstrates that the toxin from race T is not required for pathogenicity and serves as a virulence factor. Sexual crossing studies between race T and race O have shown that race differences and presence of the toxin are linked and segregate from other traits monogenetically (Lim and Hooker, 1971; Yoder and Gracen, 1975).

There is some controversy over the origin of race T (rev. by Yoder, 1980). It was reportedly isolated from corn leaves in 1955 (Nelson et al., 1970), yet a later report suggested this could be due to contamination in the laboratory (Leonard, 1972). Race T isolates from 1970 were found to be predominately of mating type A, whereas race O isolates were 50% A and 50% a (Leonard, 1973). Several years later,

mating type A and a were equally distributed in both races (Leonard, 1977). This suggests that race T had a recent origin (1969) due to a mutation in a single isolate of race O having mating type A. Additional evidence supporting the recent origin of race T comes from studies that compare its fitness with race O. When race T was equally mixed with race O and inoculated into maize plants with N cytoplasm, race T declined dramatically over a two year period. The TOX 1 locus, or closely linked genes, reduced pathogen fitness of race T compared to race O on N cytoplasm (Klittich and Bronson, 1986).

The host selective toxins from C. heterostrophus, race T (T-toxin), and P. maydis (Pm-toxin) directly interfere with mitochondria isolated from T cytoplasm (Miller and Koeppe, 1971; Comstock et al., 1973). The toxins inhibit electron transport and stimulate NADH oxidation while inhibiting malate oxidation (Miller and Koeppe, 1971; Peterson et al., 1975). The molecular structures of T-toxin and Pm-toxin are very similar and both are active at concentrations of 10^{-8} to 10^{-9} M (Suzuki, et al., 1983). T-toxin is a mixture of several linear polyketols ranging from C-35 to C-45 in length, with C-39 and C-41 comprising 60-90% (Suzuki et al., 1983). Pm-toxin has 10-15 components, the four major being linear C-33 and C-35 compounds with beta-ketol functional groups (Danko et al., 1984). Although their chain length varies, the toxins have nearly identical spacing of the four sets of oxygen functions which may be important for binding to the inner mitochondrial membrane (Danko et al., 1984). The length of T-toxin approximates the length of a lipid bilayer and space filling models demonstrate that the toxin molecules could potentially form a cylinder with a hydrophic core, and could function as an ionophore (Payne et al., 1980). The ionophore

model is further supported by the ability of the T-toxin to form channels in a planar bilayer membrane system, subsequently altering the permeability to Ca^{2+} (Holden et al., 1985). Increased membrane permeablity to Ca^{2+} with the addition of T-toxin was detected in mitochondria isolated from T cytoplasm but not N cytoplasm plants (Holden and Sze, 1984).

Plants with T cytoplasm also are sensitive to the insecticide methomyl (Humaydan and Scott, 1977). Methomyl is a systemic carbamate insecticide that affects T cytoplasm at concentrations of 10^{-3} M (Koeppe et al., 1978). The structure of methomyl bears little resemblance to T-toxin or Pm-toxin, yet has a similar mode of action on T mitochondria. Methomyl has also been proposed to form hydrophillic pores in T mitochondria inner membrane (Klein and Koeppe, 1985).

In an effort to identify binding sites in the inner mitochondrial membrane, tritium-labelled T-toxin and Pm-toxin analogs were prepared (Frantzen et al., 1987). The analogs had high biological activity and specificities identical to the native toxins, but the assay was unable to detect differences in binding characteristics between mitochondria from N (normal) and T cytoplasms. The toxins were found to be lipophillic and bound to mitochondrial membranes in a non-energy dependent manner.

The molecular characterization of the genetic locus (loci) involved with toxin sensitivity and cms associated with T cytoplasm has been approached in various ways. The first approach characterized the mitochondrial genomes of three cms and one normal (N) cytoplasms of maize by restriction enzyme analysis (Pring and Levings, 1978). Restriction fragment length polymorphisms were detected between

cytoplasms yet the extent of these polymorphisms and the apparent size and complexity of the maize mitochondrial genome precluded identification of differences that could easily be correlated to the phenotypic differences between cytoplasms. Analysis of mitochondrial protein-coding gene expression by labelling proteins in isolated mitochondria detect far fewer differences between cytoplasms (Forde et al., 1978; Forde and Leaver, 1980). A protein specific to T cytoplasm was detected, and the abundance of this protein (13 kD) was reduced upon fertility restoration (Forde and Leaver, 1980).

Two different approaches proved to be successful at identifying the DNA region coding for the T-specific 13 kD protein. Dewey and coworkers chose an approach that assumed the differences in protein expression between N and T cytoplasm could be detected as transcript differences (Dewey et al., 1986). They prepared mitochondrial DNA libraries (in the vector pUC8) from N and T cytoplasms and hybridized these clones with total radio-labelled mitochondrial RNA from the two cytoplasms. By sequencing clones that uniquely hybridized to T mitochondrial RNAs they identified a clone that contained two potential open reading frames (ORFs). The first ORF (urf13-T) was found to be unique to T cytoplasm and could potentially encode a protein of approximately 13 kD in size. Evidence supporting this ORFs relation to the T-specific 13kD protein was that its transcripts were affected by restoration, apparently by a differential processing event (Dewey et al., 1986).

The other approach taken to find the urf13-T gene was to analyze tissue-culture derived male fertile, toxin insensitive mutants (Fauron et al., 1987; Rottman et al., 1987; Wise et al., 1987a). There are no reports of natural, heritable revertants of T cytoplasm; however, male

fertile and toxin insensitive mutants have been obtained at relatively high frequencies from plants regenerated from immature embryos in the presence (Gengenbach et al., 1977; Brettell et al., 1980) or absence (Brettell et al., 1980; Umbeck and Gengenbach, 1983) of T-toxin. Restriction enzyme analysis of the fertile, toxin insensitve mutants revealed a common rearrangement relative to the parental T male-sterile, toxin sensitive mtDNA restriction pattern, as well as one mutant that retained the paternal restriction pattern (T-4; Umbeck and Gengenbach, 1983). Sequence comparison of the area of rearrangement in T and T-4 revealed a five bp insertion and a G to A transition located internal to the urf13-T gene in the T-4 mutant (Wise et al., 1987a). The insert places a stop codon in the reading frame which truncates the putative 13 kD protein. Confirmation that the urf13-T gene codes for the T-specific 13 kD protein was made with antibodies raised to synthetic peptides and subsequent immunoprecipitation from polypeptides labelled in isolated T mitochondria (Wise et al., 1987b; Dewey et al., 1987). The 13 kD protein was not detected in N cytoplasm or the T-4 mutant and was reduced in abundance in T cytoplasms restored to fertility. Although both Rf1 and Rf2 are required for fertility restoration of T cytoplasm, Rf1 is solely responsible for the reduction of the 13 kD protein (Dewey et al., 1987). The function of Rf2 in restoration is unclear, as is the specific role the 13 kD protein plays in T-cms and toxin sensitivity.

Nuclear-Mitochondrial Interactions

Pollen fertility restoring genes compensate for nuclear-mitochondrial deficiencies or incompatibilities that are phenotypically expressed during microsporogenesis and

microgametogenesis. In the most thoroughly characterized cms system (T-cytoplasm), one effect of restoration is a decrease in the abundance of a specific mitochondrial gene product (13 kD protein: Forde and Leaver, 1980; Dewey, et al., 1987), apparently by altered RNA processing (Dewey et al., 1986). Fertility restoration also affects the expression of specific mitochondrial proteins in certain cms systems of sorghum (Dixon and Leaver, 1982; Bailey-Serres, et al., 1986). Nuclear gene effects, apart from the effect of Rf genes, on expression of other mitochondrial proteins have also been reported in sorghum (Bailey-Serres, et al., 1986).

The influence of the nuclear genome on mitochondria is also evident in the range of susceptibility of specific maize inbred lines to C. heterostrophus, race T, (Watrud et al., 1975; Hallauer and Martinson, 1975; Payne and Yoder, 1978) and sensitivity to T-toxin (Payne and Yoder, 1978). The Rf genes reduce the sensitivity of T-cms plants (Watrud et al., 1975) and isolated mitochondria (Barrett and Flavell, 1975) to T-toxin. Reductions up to 50%, as measured by the inhibition of malate oxidation in isolated mitochondria, have been detected in some lines (S. J. Danko, J. M. Daly, B. G. Gengenbach, personal communication); however, it is apparent that nuclear genes besides the Rf genes may have an influence on this reaction. An evaluation of a wide range of non-restoring genotypes indictated that both the level of susceptibility to the pathogen and sensitivity to the toxin were influenced by the nuclear genome (Payne and Yoder, 1978), although disease susceptibility and toxin sensitivity did not correlate in every comparison.

Certain maize nuclear genomes have been shown to influence the rate of reversion in the S-cms system of maize as well as the abundance of the S1 and S2 linear DNA molecules (Laughnan et al., 1981). A particular nuclear background, M825, was also shown to affect mitochondrial DNA organization, unrelated to reversion (Escote et al., 1986). Another maize inbred line, WF9, gives rise to a mitochondrial mutation ncs (nonchromosomal stripe) at a frequency around 1% (Coe, 1983). The frequency of reversion to fertility in a cms system of Phaseolus vulgaris is about 15% in one nuclear background and appears to increase dramatically with the introduction of the Fr gene (fertility restorer; Mackenzie and Basset, 1987).

There is a growing list of yeast and Neurospora nuclear genes that regulate some aspect of mitochondrial gene expression. Although no nuclear genes have yet been identified that directly regulate transcipt initiation of mitochondrial genes, there are nuclear genes influencing RNA processing (Dieckman et al., 1984), RNA splicing (Bonitz et al., 1982; McGraw and Tzagoloff, 1983; Faye and Simon, 1983), translation (Fox, 1986) and posttranslational modification (Pratje et al., 1983). As many as six nuclear genes may be specifically involved in the expression of certain yeast mitochondrial genes.

At this early stage of investigation, plant nuclear-mitochondrial interactions involve nuclear influences on mitochondrial RNA processing, genome rearrangement and replication. Considering the size and complexity of plant mitochondrial genomes, it is likely that there are at least an equivalent number of nuclear genes involved in mitochondrial biogenesis and function as there are in other species. The major direction of the research presented in this dissertation evaluates the

influence of the nuclear genome on maize mitochondrial transcription. Specifically, it involves the characterization of transcription and posttranscriptional processing of transcripts from a region of the maize T cytoplasm mitochondrial genome associated with cms and toxin sensitivity.

CHAPTER II

EVALUATION OF MITOCHONDRIAL TRANSCRIPTIONAL DIFFERENCES BETWEEN CYTOPLASMS OF MAIZE

Introduction

There are four major cytoplasms of maize; N (normal) cytoplasm and three male sterile cytoplasms, S (USDA), C (Charrua), and T (Texas). The male sterile cytoplasms are formally distinguished by the genetics of fertility restoration (Laughnan and Gabay-Laughnan, 1983), but also can be characterized by mtDNA restriction patterns (Pring and Levings, 1978) and gel patterns of proteins labelled in isolated mitochondria (Forde et al., 1978; Forde and Leaver, 1980). Additionally, plants having the T-cms cytoplasm are highly susceptible to the fungal pathogens Cochliobolus heterostrophus, race T, and Phyllosticta maydis (Chapter I). Mitochondrial restriction analyses have detected polymorphisms within three of the four types of cytoplasms (N, Levings and Pring, 1977; McNay et al., 1983; C, Pring et al., 1987b; S, Sisco et al., 1985); however, these polymorphisms apparently have little effect on mitochondrially-coded polypeptides within these groups since no heterogeneties have been reported among polypeptide patterns obtained from labelling proteins in isolated mitochondria (Forde et al., 1980).

The polypeptide patterns of proteins labelled in isolated mitochondria have identified a number of polypeptides ranging from 42-88 kD that are unique to S cytoplasm, a 17.5 kD polypeptide unique to C cytoplasm, and a 13 kD polypeptide unique to T cytoplasm (Forde and Leaver, 1980). The S-specific and C-specific polypeptides are

unaffected by fertility restoration, whereas the T-specific 13 kD protein is reduced approximately 67% upon restoration (Forde and Leaver, 1980; Dixon et al., 1982). This reduction is controlled by the nuclear restorer gene Rf1 (Dewey et al., 1987), apparently through differential RNA processing of its transcripts (Dewey et al., 1986). Differences in the abundance of specific polypeptides have been noted in different maize nuclear backgrounds, suggesting the involvement of other nuclear genes on mitochondrial gene expression (Forde et al., 1978). Developmental and tissue specific mitochondrial polypetides have also been reported in maize, indicating that some mitochondrial genes may be differentially regulated (Newton and Walbot, 1985).

If the mtDNA regions coding for the cytoplasm-specific polypeptides are associated with regions of mtDNA polymorphism between cytoplasms, several approaches are available for the isolation of these gene regions. Transcriptional analyses may be the most appropriate method since detection of RNA differences is often a direct way of identifying regions involved in differential gene expression and can at least simplify analysis of DNA heterogenieties by excluding regions of DNA polymorphism occurring in non-coding regions. Most methods employed for the isolation of RNAs unique to a particular developmental stage, tissue or mutation, involve differential hybridization of RNA to either DNA or cDNA libraries. For example, the technique of cascade hybridization developed by Timberlake (1980) for identification of developmentally specific cDNAs of Aspergillus involved several rounds of hybridation of excess RNA from sporulating cultures to cDNAs of vegetative cultures, and vice versa. Nonhybridized cDNAs, unique to either vegetative or sporulating cultures, were separated from RNA:cDNA hybrids by

hydroxyapatite columns. Another techinque, differential plaque hybridization (St. John and Davis, 1979), utilizes multiple nitrocellulose filter replicas of plaque plates produced from lambda libraries as substrates for cDNA probes. Differential hybridization of the probes to the plaques is detected by comparison of the resulting x-ray film exposures.

The relatively small size of the maize mitochondrial genome (approximately 570 kb) simplifies evaluation of mtRNA differences between cytoplasms, permitting the application of simpler approaches. A technique that is a modification of the method used to identify the ribosomal RNA gene regions of the maize chloroplast (Bedbrook and Bogorad, 1976) is described in this chapter. This technique involves the labelling of RNA from different mitochondria and hybridization to nitrocellulose filters containing endonuclease restricted mtDNA. Identification of mtDNA regions associated with differential transcription between N cytoplasm, T-cms cytoplasm and a disease resistant, male fertile mutant of this cytoplasm, as well as differences between cell suspension cultures and coleoptile tissue, are presented.

Materials and Methods

Seed Lines

Maize inbred lines A188(N), A188(T) and tissue culture mutant lines A188(T-4) and A188(T-7) were provided by Dr. Burle Gengenbach, University of Minnesota. The restored line WF9(T) (Rf1Rf1;Rf2Rf2) was provided by Dr. Marc Albertson, Pioneer Hi-Bred International Inc. The Black Mexican inbred line and cell suspension cultures were kindly provided by Dr. Prem Chourey, USDA-ARS, University of Florida.

Preparation of mtRNA and mtDNA

The preparation of mtRNA was performed as described (Wise et al., 1987a). The preparation, restriction endonuclease digestion, electrophoresis and blotting of mtDNA was performed as described (McNay et al., 1983).

End labelling of mtRNA

End labelling involves the transfer of the gamma phosphate of gamma-^{32}P dATP to the 5' hydroxyl of a nucleic acid strand, catalyzed by the enzyme polynucleotide kinase. Hydrolysis of RNA to generate 5' hydroxyl ends was accomplished by mild alkali treatment. Approximately 1 ug of RNA (1 ug/ul) was incubated with glycine hydrolysis buffer (5 mM glycine, 100 uM spermidine, 10 um Na_2EDTA, pH 9.5) in a reaction volume of 10 ul for 10 min at 90°C. Kinase labelling was performed in 15 ul reaction volume containing 50 mM Tris-HCl, pH 9.5, 10 mM $MgCl_2$, 5 mM dithiothreitol, 20 to 30 uCi of gamma-^{32}P dATP (7000 Ci/uM; New England Nuclear) and 4 to 5 units of T4 polynucleotide kinase. The reaction was incubated at 37°C for 30-45 min and run on a Sephadex G-50 column to separate the unincorporated nucleotides from the labelled RNA.

The Southern blots were prehybridized in 4X SSC, with 100 ug/ml yeast RNA and 0.1% SDS at 65°C for 2 to 3 hours. The labelled RNA was heated briefly at 70°C and hybridized at 65°C for 16 hours. The nitrocellulose membranes were washed two times in 2X SSC, 0.1% SDS for 15 minutes at room temperature then two more times at 0.25X SSC, 0.1% SDS for 15 min at room temperature. Membranes were dried and exposed to x-ray film (Kodak).

Results and Discussion

The DNA region coding for the maize chloroplast rRNA genes were initially identified through isolation and end labelling of the rRNAs and subsequent probing of Southern blots containing restricted maize chloroplast DNA (Bedbrook and Bogorad, 1976). In the procedure described here, total mtRNAs from different cytoplasms were end labelled and hybridized to identical Southern blots containing restricted mtDNA from different cytoplasms. All blots contained mtDNAs from the cytoplasms used as probes, to ensure a DNA substrate was available for all RNAs, and these mtDNAs were usually digested with a minimum of three restriction enzymes.

A representative hybridization comparing mtRNA from A188(N) cytoplasm with that of A188(T-7), a fertile, disease resistant mutant that was generated through tissue culture (Umbeck and Gengenbach, 1983) is shown in Figure 2-1. The differences in hybridization patterns obtained with the two RNA species were most easily visualized in the lanes containing N and T mtDNA (Fig. 2-1). Differences in BamHI restriction patterns between N and T mt DNAs are detected in both the ethidium bromide stained lanes (panel A) and in the hybridization patterns (panels B-E). The hybridized DNA fragments that are unique to either the N or T lanes within each panel represent regions of DNA polymorphism between these mitochondrial genomes that are actively transcribed (as detected with N and T-7 mtRNAs).

The differences between N and T-7 mtRNAs are detected by comparing the hybridization pattern of N mtRNA to N and T mtDNA lanes (panel B) with the hybridization pattern of T-7 mtRNA to N and T mtDNAs (panel C). The intensity of hybridization to a 3.0 kb BamHI fragment in T mtDNA

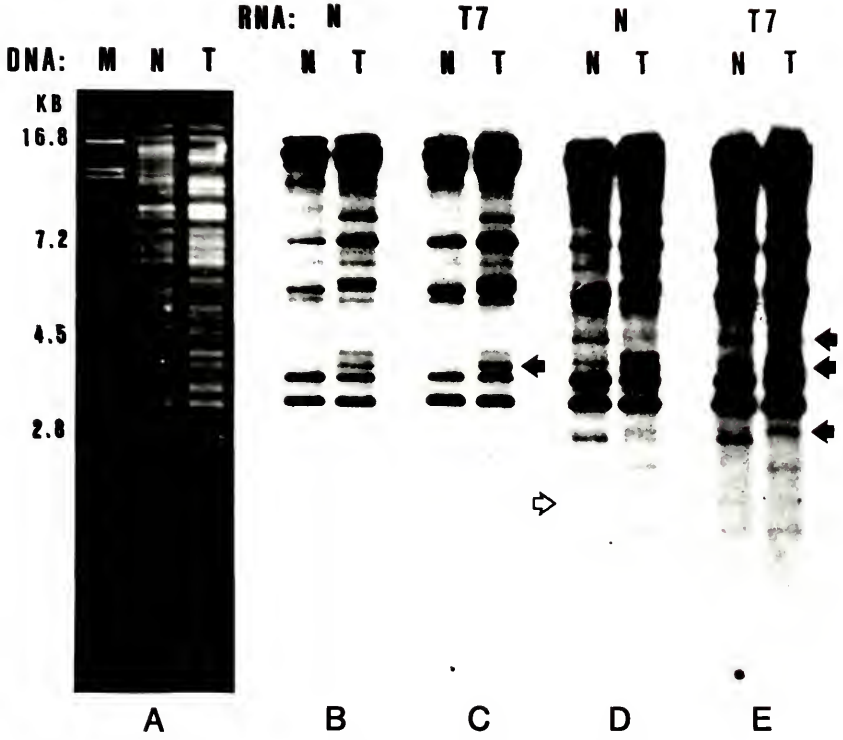


Fig. 2-1. Comparison of mtRNAs from N and T7 mitochondrial genomes. Panel A, ethidium bromide stained agarose gel electrophoresis pattern of BamHI restricted mt DNAs from N and T cytoplasms (lanes N and T, respectively) and lambda DNA separately digested with BamHI and PstI and combined for a marker lane (M). Panels B and C, identical (sandwich) blots of DNAs shown in panel A, hybridized with end-labelled mtRNA from N cytoplasm (panel B) or mtRNA from the fertile, toxin insensitive revertant mutant T7 (panel C). Panels D and E, longer exposures of panels B and C, respectively. Black arrows identify mtDNA bands that show stronger hybridization intensities with T7 mtRNA compared to N mtRNA. Open arrow identifies a mtDNA band detected in both N and T mtDNAs that shows a stronger hybridization intensity with N mtRNA relative to T7 mtRNA. Lambda markers are labelled in kilobases.

lane is greater in panel C (dark arrow) than in panel B, indicating a greater abundance of the RNA fragments that hybridize to this fragment in T-7 mtRNA compared to N mtRNA. A longer exposure also detects at least two other bands (dark arrows) that are more intense with T-7 mtRNA hybridization pattern compared to N mtRNA. All three of these BamH1 fragments appear to be specific to T mtDNA, thus the increased hybridization intensity may represent a DNA polymorphism between N and T mt genomes, resulting in non-identical transcripts which unequally hybridized to this specific DNA fragment, rather than being a reflection of differential transcription between N and T-7 mitochondria. Another fragment displayed greater hybridization intensity when hybridized with N mtRNA compared to T-7 mtRNA (light arrow). This effect was seen in both N and T mtDNA lanes suggesting it may involve a similar fragment. The simplest explanation for this effect is that the transcripts hybridizing to this fragment are more abundant in N mtRNAs and may represent a difference in rate of transcription or in transcript stability between cytoplasms.

This technique merely implicates mtDNA regions that may be differentially transcribed between mt genomes, under these experimental conditions. Subsequent isolation of the DNA fragments and use in Northern hybridizations would be necessary to confirm that transcriptional differences exist. The best application of this technique may be its ability to find or rule out gross transcriptional differences between RNAs from two mt genomes. For example, comparison of mtRNAs isolated from A188(N) and WF9(N) cytoplasms showed that the A188(N) mtRNA hybridized to a 1.3 kb HindIII fragment from T mtDNA, while and WF9(N) did not (data not shown). The absence of hybridization

to this band was later found to be related to a 3.5 kb deletion in the WF9(N) mt genome (see Chapter 3). The technique described in this chapter also was used to compare mtRNAs from T male-sterile cytoplasm with the T-4 male fertile, disease resistant mutant. No differences were detected (data not shown), and further analyses demonstrated that the only difference detected between these mitochondrial genomes was a five bp insertion and a G to A transition in the urf13-T gene, which truncates the polypeptide but does not affect transcription (Wise et al., 1987a; Wise et al., 1987b).

It is also noteworthy that the isolation of the urf13-T gene involved a technique similar to the differential plaque hybridization; Dewey et al. (1986) labelled replica nitrocellulose membranes of pUC8 libraries of N and T mt genomes with N and T mt RNA and examined the clones that were differentially labelled.

Besides evaluating differences between maize cytoplasms, this differential RNA hybridization technique was used to detect differences in mitochondrial transcripts from cell suspension cultures and coleoptile tissue. A representative blot is shown in Figure 2-2. Mitochondrial DNA isolated from cell suspension cultures was digested with BamHI, HindIII, and XhoI restriction enzymes, and identical Southern blots were hybridized with end labelled RNA isolated from log-phase Black Mexican cell suspension or with Black Mexican coleoptile mtRNA. Dramatic differences in hybridization patterns were detected (panel B vs panel C; panels D and E are long exposures of B and C, respectively; panels F1 and F2 are from another experiment that used T-cytoplasm mtDNA). The intense bands that are unique to coleoptile

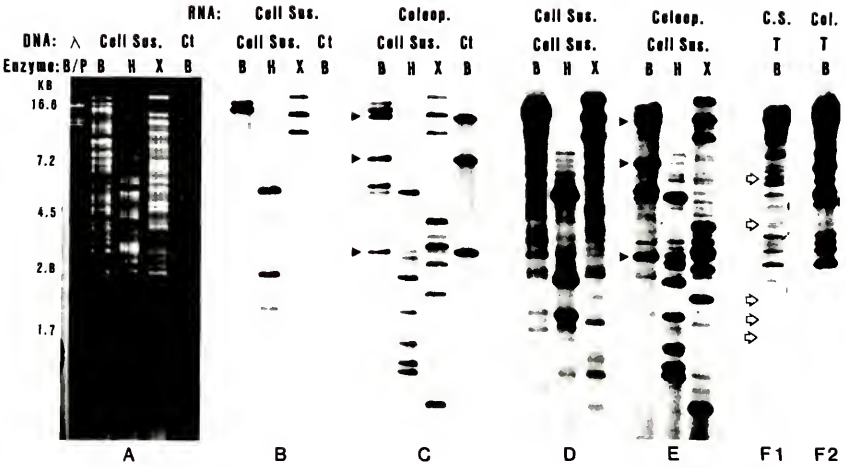


Fig. 2-2. Comparison of mtRNAs from cell suspension cultures and coleoptile tissues. Panel A, ethidium bromide stained agarose gel with electrophoresis pattern of BamHI, HindIII, and XhoI restricted cell suspension mt DNA; BamHI restricted maize chloroplast DNA (ct); and lambda DNA restricted separately with BamHI and PstI and combined. Panels B and C, identical blots of DNAs shown in panel A hybridized with end-labelled mtRNA from log-phase cell suspension cultures (panel B) and 6 day old coleoptile tissue (panel C). Panels D and E, longer exposures of blots shown in panels B and C, respectively. Panel F, BamHI digested mtDNA from T cytoplasm hybridized with cell suspension (F1) or coleoptile (F2) mtRNA. Arrowheads identify chloroplast DNA fragments contaminating the cell suspension mtDNA, hybridizing to chloroplast RNA from the respective mtRNAs. Open arrows distinguish mtDNA fragments that are uniquely or more intensely hybridized with cell suspension mtRNA.

RNA, are primarily from chloroplast contamination. Maize chloroplast DNA, digested with BamHI, is shown in panels A, B, and C, and the BamHI "mitochondrial" DNA fragments that comigrate with the chloroplast hybridized fragments are marked (arrowheads). The cell suspension mtDNA is contaminated with chloroplast DNA (ct DNA), yet the cell suspension mtRNA do not appear to be as contaminated with ctRNA as the coleoptile mtRNA. The most striking result of this analysis is the increase in the number of DNA bands that hybridize to the cell suspension RNA (panels E and F1). Whether this reflects a general increase in transcriptional level of "minor" mitochondrial transcripts or transcripts from regions that are not normally transcribed in coleoptile mitochondria remains to be answered.

CHAPTER III

INFLUENCE OF NUCLEAR BACKGROUND ON TRANSCRIPTION OF A MAIZE MITOCHONDRIAL REGION ASSOCIATED WITH TEXAS MALE STERILE CYTOPLASM

Introduction

The mitochondrial genome of the normal, male-fertile (N) cytoplasm of maize (Zea mays L.) is estimated to be 570 kb (Lonsdale et al., 1984). Approximately 42 kb of the maize mitochondrial genome is represented as six repeated regions that range in size from 1-14 kb; five of these repeats are involved in intramolecular recombination (Lonsdale et al., 1984). Recombination through these repeats generates subgenomic circles from the master circle. The capability for intramolecular recombination apparently has contributed to the diversity of cytoplasms in maize, as mtDNA polymorphisms are observed among (Pring and Levings, 1978) and within (Levings and Pring 1977; McNay et al., 1983; Pring et al., 1987b; Sisco et al., 1985) groups of cytoplasms. Recombination events have resulted in new transcriptional units as inferred from the organization of the gene urf13-T (Dewey et al., 1986; Wise et al., 1987a, 1987b). This 345 bp gene is unique to the T male sterile cytoplasm of maize yet contains 263 bp 87% homologous to a region 3' to the mitochondrial 26S rDNA, and 58 bp of near perfect homology to a region interior to 26S rDNA (Dewey et al., 1986). Another recombination event duplicated a 5 kb DNA region 5' to the gene atp6 resulting in a second configuration located 69 bp 5' to the predicted translational start sequence of urf13-T (Dewey et al., 1986; Wise et al.

1987a). Sequences within this repeated region apparently promote transcription of atp6 and urf13-T, as well as another open reading frame, ORF25, located 80 nucleotides 3' to urf13-T (Dewey et al., 1986).

There are three major sources of cytoplasmic male sterility (cms) in maize (T, C, and S), each distinguished by the ability of specific nuclear genes to restore pollen fertility (Laughnan and Gabay-Laughnan, 1983). Fertility restoration of T cytoplasm plants is conditioned by the dominant nuclear genes Rf1 and Rf2. Maize plants carrying the T cytoplasm are highly susceptible to the fungal pathogens Cochliobolus heterostrophus Drechsler (Bipolaris maydis) race T, the causal agent of Southern corn leaf blight, and Phyllosticta maydis Arny and Nelson, the causal agent of yellow corn leaf blight (Ullstrup, 1972). Both pathogens produce a series of toxic Beta polyketols which are virulence determinants (Suzuki et al., 1983; Danko et al., 1984) and specifically affect T cytoplasm mitochondria (Miller and Koeppe, 1971; Comstock et al., 1973; Yoder, 1973).

The gene urf13-T is strongly implicated in playing a role in the maternally inherited phenotypes of male sterility and toxin sensitivity in T cytoplasm since it is unique to this cytoplasm and is altered or deleted in tissue culture derived male fertile and toxin insensitive mutants (Wise et al., 1987a; Rottman et al., 1987). The gene encodes a polypeptide of approximately 13 kD (Wise et al., 1987b), the synthesis of which is reduced in plants restored to fertility (Forde and Leaver, 1980). Nuclear restorer genes appear to function by differentially processing major urf13-T transcripts (Dewey et al., 1986).

There is substantial evidence of nuclear background effects on reaction of maize lines and isolated mitochondria to the fungal toxins.

Mitochondria isolated from different lines in T cytoplasm exhibit variation in sensitivity to the toxins of C. heterostrophus race T, and mitochondria isolated from restored T cytoplasm plants are less sensitive than mitochondria from nonrestored T plants (Barratt and Flavell, 1975). In another study, sensitivity of malate oxidation to purified toxins was reduced 50% by fertility restoration in many cases, and an effect of nuclear background also was evident (S. J. Danko, J. M. Daly, and B. G. Gengenbach, personal communication). An influence of nuclear background on susceptibility to race T of C. heterostrophus and the toxins was demonstrated among 24 lines and single-cross hybrids (Payne and Yoder, 1978). The lines displayed a range of sensitivity to the pathogen and to toxins, but disease susceptibility and toxin sensitivity rankings were not correlated in every comparison.

This study examined the influence of nuclear background on transcription of urf13-T and RNA processing events associated with fertility restoration. Transcripts of atp6, urf13-T, and ORF25 from N, T, and T-restored cytoplasms in five inbred lines were mapped with a series of contiguous DNA clones. DNA sequence and atp6 transcriptional alterations were detected between N and T cytoplasms. Nuclear background influenced the abundance of urf13-T and ORF25 transcripts, as well as processing of transcripts in a region within ORF25.

Materials and Methods

Seed lines

The maize inbred lines A188(T) (rf1rf1;Rf2Rf2), A188(N) (rf1--;rf2--) and restored WF9(T) (Rf1Rf1;Rf2Rf2) were provided by Dr. B. Gengenbach. Wf9(T) (rf1rf1;rf2rf2) was provided by Pioneer Hi-Bred International, Inc. The lines A632(T) (rf1rf1;----), restored A632(T)

(Rf1--;Rf2--), A632(N) (rf1--;rf2--), W64A(T) (rf1rf1;----), restored W64A(T) (Rf1--;Rf2--), W64A(N) (rf1--;rf2--), C103(T) (rf1rf1;----), restored C103(T) (Rf1rf1;Rf2--) and C103(N) (rf1--;rf2--) were provided by Dr. P. S. Chourey. Other lines used were WF9(N) (rf1--;rf2--) and the single cross hybrid A188(T) X restored WF9(T).

Preparation and analysis of mtDNA and mtRNA

Isolation of mitochondria, purification of nucleic acids, endonuclease digestion, electrophoresis, blotting, and hybridization were performed as previously described (Wise et al., 1987a). The DNA probes used in Northern hybridizations were obtained from pUC8 clones subjected to preparatory digestion. The inserts were recovered from agarose gels with DEAE NA45 paper (Schleicher & Schuell) and ligated overnight prior to nick translation. If nitrocellulose filters were reprobed, the previous probe was washed off with boiling 20 mM Tris, pH 8.0 for 20 min.

DNA sequencing:

The dideoxynucleotide chain termination method of Sanger et al. (1977) was used with the universal primer for clones in the M13 vectors mp18 and mp19 (Yanisch-Perron et al., 1985). The reactions were radiolabeled with S^{35} dATP (New England Nuclear) and separated on 6% denaturing acrylamide wedge gels.

Results

Transcription of atp6

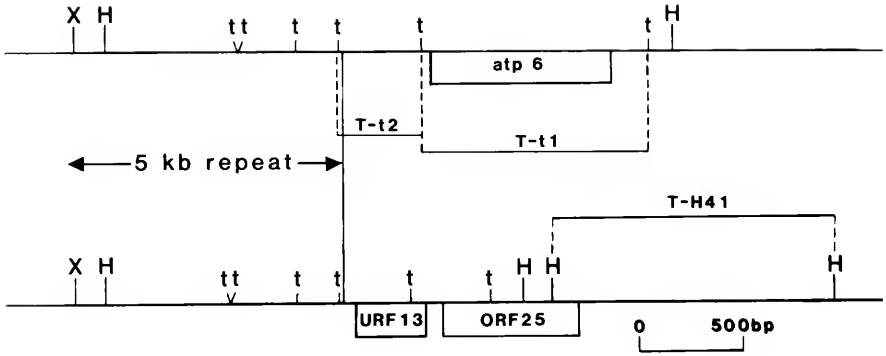
The 5' region of the maize mitochondrial gene atp 6 is repeated in T cytoplasm from the positions -444 relative to the proposed translation initiation site (Dewey et al., 1985a) to over -5000 bp (Wise et al., 1987a; Fig. 3-1). A complex transcript pattern was observed on Northern

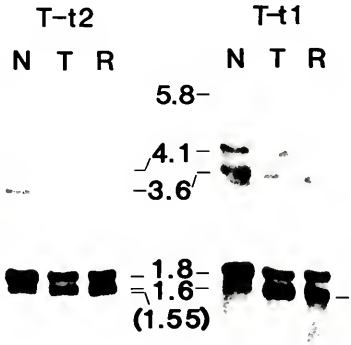
blots of T cytoplasm mtRNA with probes from the repeated region, as they hybridized to transcripts of atp6, and the cotranscribed genes urf13-T and ORF25. The pattern observed with mtRNA from normal (N) cytoplasms was less complex as the probes detected only atp6 transcripts. To distinguish atp6 transcripts from urf13-T/ORF25 transcripts in T cytoplasm, probes containing sequences beginning 22 bp from the 3' edge of the 5 kb repeat and extending through the atp6 coding region (Fig. 3-1; T-t2, T-t1) were hybridized to RNA from N, T, and T cytoplasm plants restored to fertility (R) (Fig. 3-2). Major transcripts of 1.8 and 1.6 kb and minor transcripts of 4.1 and 3.6 kb were detected with the two probes; prolonged exposure also revealed a transcript of 5.8 kb. The exposure level of the N lanes in Fig. 3-2 was longer than the T or R lanes to illustrate a transcript difference between N and T or R mtRNAs, as there appeared to be a transcript migrating at 1.55 kb that was only found in T and R mtRNAs. Since this transcript could not clearly be separated from the 1.6 kb transcript detected in N, T and R mtRNAs, it may reflect a copy number difference of the 1.6 kb transcript rather than a unique 1.55 kb transcript.

Mitochondrial RNA of N, T and R cytoplasms from five different nuclear backgrounds were analyzed with probes T-t2 and T-t1, and the difference between N and T (and R) mtRNAs was consistent among these particular inbreds. The difference may reflect differential transcript initiation or processing events between N and T cytoplasms. Additionally, fertility restoration did not alter atp6 transcription in T cytoplasm.

Fig. 3-1. Restriction map of the mitochondrial region containing the genes atp6, urf13-T and ORF25. Relative postion the genes to the 5 kb repeat in T cytoplasm of maize is shown with solid vertical line representing the 3' edge of the repeat. Dashed lines mark the boundries of three probes used on Northern blots. X, XhoI; H, HindIII; t, TaqI.

Fig. 3-2. Northern blot analysis of atp6 transcripts. Position of the clones (T-t1, T-t2) used as probes is designated in Fig. 3-1. Mitochondrial RNA is from normal (N), Texas male sterile (T), and nuclear restored Texas male sterile (R) cytoplasms. Approximate size of transcripts are in kilobases.


X H
tt
t
t
t
t H
atp 6
T-t2
T-t1
5 kb repeat
T-H41
X H
tt
t
t
t
t
H H
H
URF13
ORF25
0
500bp


T-t2
T-t1
N T R
N T R
5.8-
4.1-
3.6-
1.8-
1.6-
(1.55)

Differences between N and T mtDNA

A difference between N and T mtDNA was detected in the 5 kb repeat at positions -572 to -576 and -587 to -590 relative to the proposed translational start site of atp6. DNA sequence analysis of a TaqI clone (-466 to -689) revealed that N mtDNAs did not contain 9 bp that were present in T mtDNAs; the remaining sequences of the 223 bp clone were identical. The T mtDNA has insertions of 4 and 5 bp in the region shown below:

```
T: (-597) AAAAGGAAGACGGGGCCCTTAGCTTAGGGACAC (-565)
N:        AAAAGGA----GGGGCCCTTA-----GGGACAC
```

The 5 bp insert disrupts an AluI recognition site, thus providing a diagnostic assay for other cytoplasms. Hybridization of the TaqI clone to AluI digests of maize mtDNAs revealed that seven N cytoplasms and C and S cytoplasms do not carry this insert, while it was present in three T cytoplasms tested.

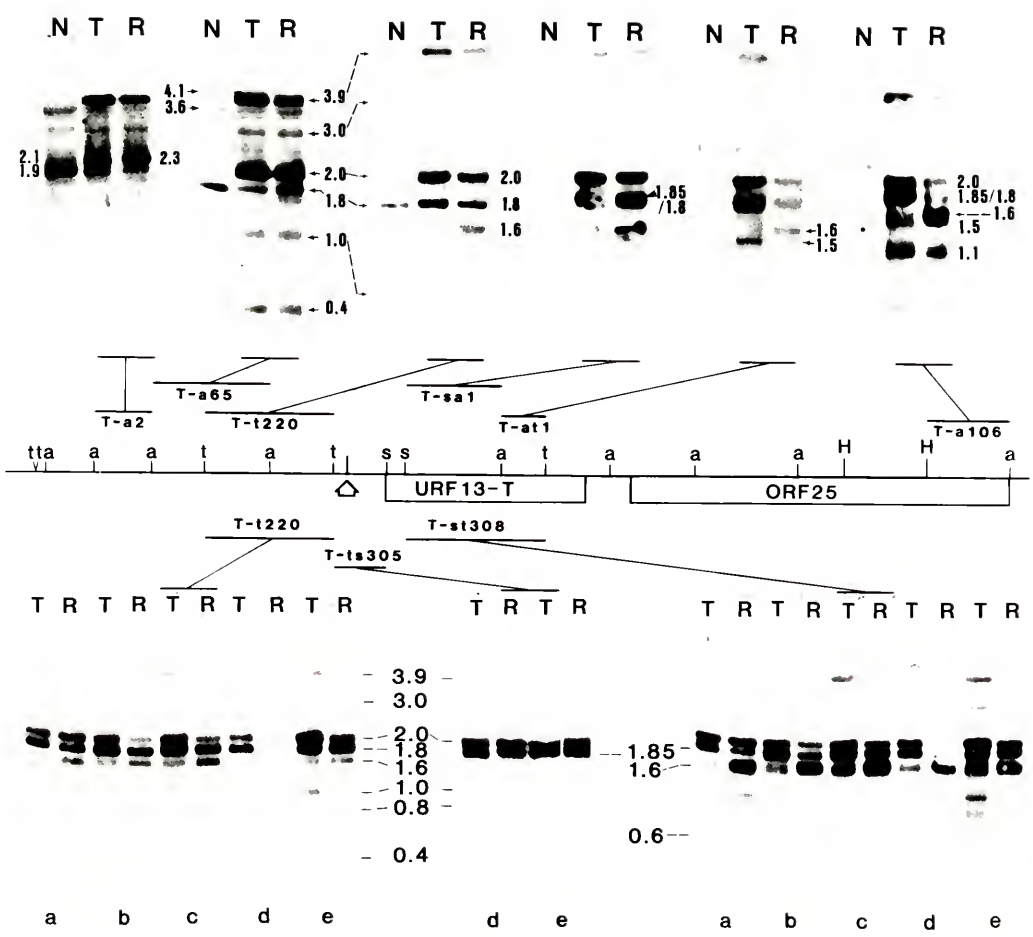
Transcription of urf13-T and ORF25

Transcripts of urf13-T and ORF25 from T and R mitochondria were mapped using 20 DNA probes to "Northern walk" a region 2 kb 5' to urf13-T to over 3 kb 3' to ORF25 (Fig. 3-1). Figure 3-3 shows the location of selected probes and corresponding Northern blots used in characterizing the complex transcriptional pattern found in this area of the T mitochondrial genome. The largest transcript detected was approximately 3.9 kb (detected by all six probes shown in the upper panel of Figure 3-3). This transcript extends from a point within a 700 bp XhoI fragment in the 5 kb repeated region (Wise et al., 1987a) to a point some 200 bp 3' to ORF25 (data not shown). Minor transcripts of

Fig. 3-3. Northern walk through urf13-T and ORF25.

upper: Positions of probes are aligned with a partial restriction map of urf13-T and flanking sequences. Arrowhead marks the 3' edge of the 5 kb repeat; probes 5' of this hybridize to atp6 transcripts as well as urf13-T/ORF25 transcripts. Glyoxylated mtRNA from normal (N), male sterile (T), and nuclear restored male sterile (R) cytoplasms were electrophoresed through 1.0% agarose gels at 100 volts for 12 hr at 4o C. Blots for probes T-t220 (upper), T-sa1 and T-at1 were from 1.5% gels run at 100 volts for 20 hr at 4o C. Arrow in T-sa1 blot distinguishes the 1.85 kb transcript detected 3' to the 5 kb repeat.

lower: Comparison of five nuclear genotypes and the effect of dominant nuclear restorer genes on urf13-T transcripts. Genotypes examined; a=A632, b=W64A, c=C103, d=Wf9, e=A188. All exposures were chosen to best illustrate the major transcripts of urf13-T. H, HindIII; a, AluI; s, Sau3A; t, TaqI. Approximate sizes are in kb.


N T R N T R N T R N T R N T R N T R
4.1
3.6
2.1
1.9
2.3
3.9
3.0
2.0
1.8
1.0
0.4
2.0
1.8
1.6
1.85
/1.8
1.6
1.5
2.0
1.85/1.8
1.6
1.5
1.1
T-a65
T-a2
T-t220
T-sa1
T-at1
T-a106
tta a a t a t s s a t a a a H H a
URF13-T
ORF25
T-t220
T-st308
T-ts305
T R T R T R T R T R T R T R T R T R T R T R T R
3.9
3.0
2.0
1.8
1.6
1.0
0.8
0.4
1.85
1.6
0.6
a b c d e d e a b c d e

3.0 and 2.3 kb also were detected with the 700 bp XhoI probe and downstream probes. The 3.0 kb transcript terminates within the first 200 bp of ORF25 (not shown) and the 2.3 kb transcript terminates within the region represented by the probe T-a2 (positions -505 to -405 relative to the putative translational start of urf13-T). Probe T-a2 also detected transcripts of 2.1 and 1.9 kb in N mtRNAs.

Probes T-a65 and T-t220 (Fig. 3-3; upper panel) hybridized to the major transcripts of atp6 and urf13-T/ORF25. There were two sets of comigrating transcripts (1.8 and 1.6 kb) that only could be distinguished from one another with gene-specific probes that do not contain sequences from the 5 kb repeat. Probe T-a65 (representing positions -404 to -202) hybridized to major transcripts of 1.8 kb in N, T, and R, and 2.0 kb only in T and R mtRNAs. Minor transcripts in N were 4.1 and 3.6 kb, while 3.9 and 3.0 kb transcripts were evident in T and R mtRNAs. The 1.8 kb transcript is an atp6 transcript (Fig. 3-2) and urf13-T-specific probes (Fig. 3-3; T-sa1, T-at1) indicated that the 2.0 kb transcript is an urf13-T/ORF25 transcript and consequently not present in N mtRNAs. It is likely that the transcript initiation site (or the precursor RNA processing site) for the 1.8 kb atp6 transcript also serves to initiate urf13-T/ORF25 transcription. The difference in transcript abundance between the 2.0 and 1.8 kb transcripts also was detected with T-a65.

Probe T-t220 hybridized to major transcripts of 1.8 and 1.6 kb in N mtRNA (the 1.6 kb transcript was detected with longer exposures) and 2.0, 1.8 and 1.6 kb in T and R mtRNAs (Fig. 3-3, upper panel). The atp6-specific probes (Figs. 3-1 and 3-2) distinguished the 1.8 and 1.6 kb transcripts as atp6 transcripts. Probes specific to urf13-T (T-sa1,

T-at1) hybridized to a 1.8 kb transcript in T and R mtRNAs. The shape and migration of this band (arrowhead, T-sa1), in comparison with the 1.8 kb transcript detected with T-t220, suggest it represents two transcripts, of 1.8 and 1.85 kb. If the 1.6 kb atp6 transcript arose by the processing of the 1.8 kb transcript, the same processing event most likely would create a 1.8 kb transcript from the 2.0 kb transcript in T and R mtRNAs. If this scenario is correct, the 1.8 kb transcript detected in T mtRNA with probe T-t220 represent atp6 and urf13-T/ORF25 transcripts.

There was a noticable effect of fertility restoration on the 1.6 kb transcript detected with probe T-t220, resulting in a higher abundance in R mtRNAs than in T mtRNAs. This subtle difference was detected in mtRNAs from five different nuclear backgrounds (Fig 3-3; T-t220, lower panel). The effect of restoration detected with T-t220 was limited to the region representing positions -202 to -92.

A more obvious effect of restoration (Dewey et al., 1986) was detected with probes representing map positions +3 relative to urf13-T and extending through ORF25. Probes T-s1 (+3 to +32; not shown), T-sa1 (+31 to +200; Fig. 3-3) and all downstream probes hybridized to a 1.6 kb transcript in R mtRNA but not T mtRNA. We have designated this transcript as 1.6'. Another transcript of 1.5 kb was detected with probes T-sa1 (with a longer exposure) and T-at1 (Fig. 3-3), and other downstream probes, in both T and R mtRNA. The 1.6' kb transcript was unique to R mtRNA whereas the 1.5 kb was observed in both T and R mtRNAs.

A few weakly hybridizing transcripts were detected in N mtRNA with T-sa1, an urf13-T-specific probe, which may represent transcripts 3' to

the 26S rDNA gene. It should be noted that the region of urf13-T that is homologous to the coding region of 26S rDNA (Dewey et al., 1986) was not used in any of the Northern hybridizations shown in Figure 3-3.

All ORF25-specific probes and those extending some 200 nucleotides 3' to this gene gave the same pattern on Northern blots of T and R mtRNA, with regard to the 2.0, 1.85/1.8, 1.6', and 1.5 kb transcripts (Fig. 3-3; T-a106), as urf13-T-specific probes. Minor transcripts of 1.0 and 0.8 kb (Fig. 3-3; T-st308), which were first detected with T-a65 and T-t220, respectively, do not extend through ORF25, terminating within the first 350 nucleotides of the 663 bp ORF. A minor transcript of 0.6 kb was detected only with urf13-T-specific probes in R mtRNA (Fig. 3-3; T-st308). This transcript was first detected with a probe representing positions of +3 to +32 of urf13-T (not shown) and terminated in approximately the same region as the 1.0 and 0.8 kb transcripts. All restored lines were characterized by the appearance of the 1.6' kb and 0.6 (upon longer exposure) kb transcripts with the probe T-st308. An illustration of these transcripts, with putative start and stop sites, is shown in Figure 3-4. For all transcripts described, except the 1.85 kb transcript, the predicted size was in close agreement with their mapped position on the restriction map.

The relationship between the 1.6 kb transcript detected within the repeat and the 1.6' kb transcript unique to R mtRNA is delineated in the lower panel of Figure 3-3. Probe T-t220 detected the 1.6 kb atp6 transcript in both T and R mtRNAs as well as what appeared to be a more abundant 1.6 kb transcript in R mtRNA. If T-t220 detected a higher copy

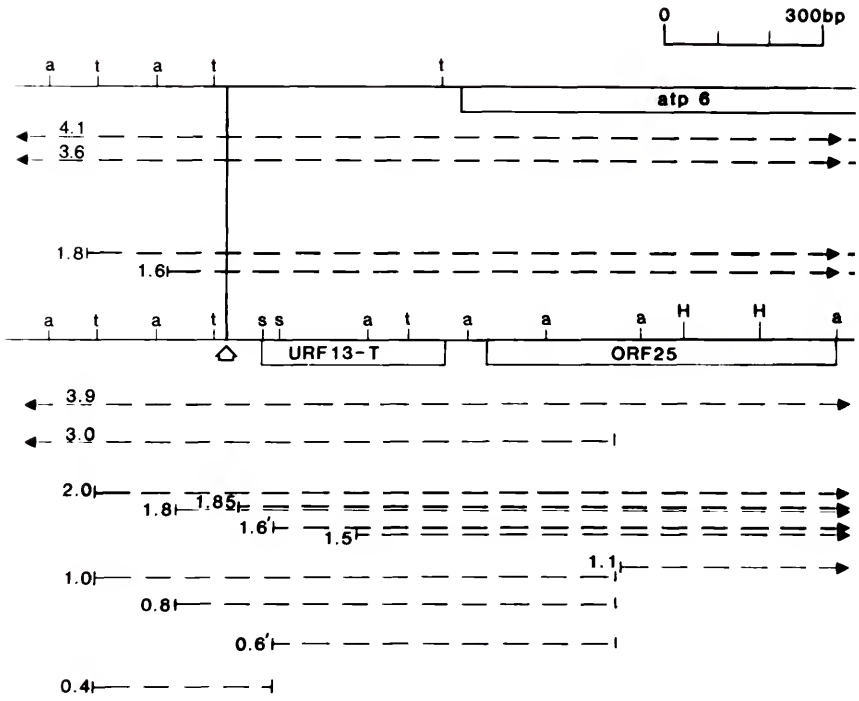


Fig. 3-4. Schematic representation of the major and minor transcripts of the maize mitochondrial genes atp6, urf13-T and ORF25. Start and stops are approximated and marked with vertical lines. Approximate sizes are in kilobases. Transcripts only detected in nuclear-restored T mtRNA are primed.

number of the 1.6 kb atp6 transcript in R mtRNA relative to T mtRNA, the atp6-specific probes shown in Fig. 3-2 should also detect this difference. This was not found in any of the lines analyzed. This suggests that T-t220 may have detected the restorer-specific 1.6' kb transcript. Probe T-ts305 (positions -91 to +2) did not hybridize to the 1.6 kb transcript. Assuming that the 1.6 kb transcript detected with T-t220 is related to the restorer specific 1.6' kb transcript, this observation suggests that T-ts305 may be an intron-specific probe and the 1.6 kb transcript that appears with restoration is the result of an RNA splicing event.

Nuclear effects on urf13-T and ORF25 transcripts

The 2.0, 1.85, and 1.8 kb transcripts appear to be the major mature transcripts of urf13-T due to their abundance and mapped position. The relative abundance of these transcripts was found to be affected by nuclear background with the most noticeable difference detected in the intensity of the 2.0 kb transcript. This transcript was in greater abundance in A188(T) mtRNA (Fig. 3-5 a) than in four other inbreds examined (Fig. 3-5 b). The lower abundance of the 2.0 kb transcript in the latter inbreds was associated with increased abundance of the 1.8/1.85 kb transcripts.

Three different transcript patterns were detected with ORF25-specific probes among N cytoplasms of the five lines surveyed. Figure 3-6 shows a representative of each characteristic pattern obtained with probe T-a106 (Fig. 3-3 defines the probe location). The line Wf9(N) displayed transcripts of 3.5, 1.8, and 1.6 kb (Fig. 3-6, left, a), while A188(N) exhibited transcripts of 3.1, 1.7, and 1.3 kb

Fig. 3-5. Nuclear background effects on urf13-T/ORF25 transcripts. A188(T) mtRNA (a) has a greater abundance of the 2.0 kb transcript relative to the other nuclear genotypes examined [WF9(T) shown, b] as well as decreased abundance of the 1.8 (1.85) kb transcript. Location of probe T-st308 shown in Figure 3-3. Transcript sizes in kb.

Fig. 3-6. Effects of nuclear background and cytoplasm on transciption patterns of ORF25. Left, mitochondrial RNA from Wf9(N) (a), A188(N) (b), and W64A(N) (c). Right, mitochondrial RNA from sterile (T) or restored (R) lines Wf9(T) and A188(T); the restored version of A188(T) is a single cross hybrid of A188(T) X Wf9(T)RF1RF1RF2RF2. Probes are ORF25-specific T-a106 (Fig. 3-3) or T-H41 (Fig. 3-1); the two probes resulted in indistinguishable transcript patterns. Approximate transcript sizes are indicated in kb.

T-st308

a b

T-a106

T-H41

T R T R N N N

a b a b c

(Fig. 3-6, right, b). The pattern detected in W64A(N) mtRNA (Fig. 3-6, right, c) was identical to the patterns found in A632(N) and C103(N) mtRNAs, and had the characteristic Wf9(N) pattern with the addition of a major transcript of 2.2 kb. The difference in the transcript patterns between WF9(N) and A188(N) mtRNAs may be attributed to DNA differences that were detected 3' to ORF25. The WF9(N) mitochondrial genome lacks a region of over 3.5 kb that is located 3' to ORF25 in A188(N), WF9(T) and A188(T) mtDNA genomes. Hybridization of HindIII digests of A188(N), Wf9(N), and A188(T) (Fig. 3-7 a, b, c) mtDNAs with three probes located 3' to ORF25 revealed the extent of the deletion. The probes represented regions of +1340 to +2330 (T-a22), and approximately, +2510 to +2910 (T-H16), and +2911 to +4343 (T-H15), relative to the initiation codon of urf13-T. The probes readily hybridized to their analogous fragments in A188(N) and A188(T), but did not detect these fragments in Wf9(N) mtDNA. Minor homology to all three probes was detected in A188(N) and A188(T) mtDNAs, with a fragment of 3.7 kb evident with T-a22. The next downstream probe contiguous with T-H15 hybridized to WF9(N) mtDNA, delineating the extent of this deletion. Transcripts that are initiated 5' to or within ORF25 are terminated in different regions in WF9(N) and A188(N) cytoplasms which likely explains the difference between A188(N) and WF9(N) transcript size detected with ORF25-specific probes. The abundance of ORF25 transcripts varies greatly among N cytoplasms shown in Figure 3-6, as well as between N and T cytoplasms, perhaps as a consequence of genomic organization.

The detection of a 1.1 kb transcript with ORF25 probes (T-a106 in Fig. 3-6) in T mtRNAs was associated with nuclear background. Of five

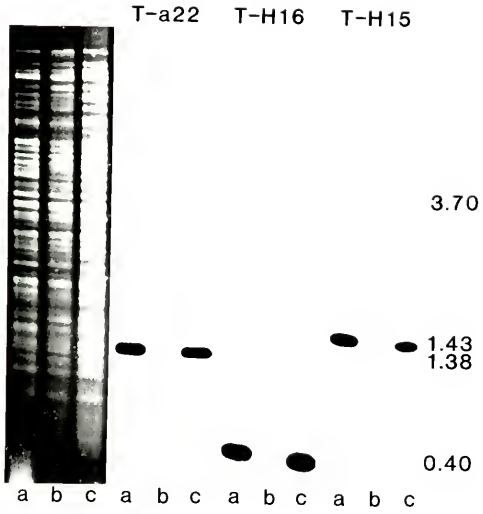


Fig. 3-7. Deletion in the WF9 mt genome. Ethidium bromide stained gel of A188(N) (a), WF9(N) (b), and A188(T) (c) mtDNAs digested with HindIII and three southern blots. Probes are from regions 3' to ORF25 and represent the approximate positions +1340 to +2330 (T-a22), +2510 to +2910 (T-H16), and +2511 to +4343 (T-H15) relative to urf13-T. Size of the hybridizing DNA fragments are approximated in kb.

lines examined, restored and sterile W64A(T) and WF9(T) mtRNAs contained the 1.1 kb transcript (Fig. 3-6, WF9 shown; Fig. 3-3 defines location of probe T-a106), while sterile C103(T), A632(T) and A188(T) (Fig. 3-6, A188 shown) did not. The single cross hybrid A188(T) X restored WF9(T) showed the appearance of the 1.1 kb transcript (Fig. 3-6). The event that gives rise to this transcript apparently is determined by the WF9 nuclear genome, and is distinct from the combined effect of the restorer genes Rf1 and Rf2.

The 1.1 kb transcript was not detected with a probe that contains the first 114 bp of the 663 bp ORF25 gene (not shown), which suggests that it is a processed form of another transcript, or initiated within the gene. The WF9(T) and A188(T) mtDNAs were examined through this region with six restriction enzymes and several probes without evidence of genomic variation which might account for the unusual transcript.

Discussion

The atp6-urf13-T-ORF25 region of T cytoplasm mtDNA represents an interesting complex of two cotranscribed open reading frames (urf13-T-ORF25) that apparently share transcription initiation sequences with atp6 by virtue of the 5 kb repeat. Transcription patterns through this region are therefore complex. Other maize mtDNA genes apparently have complex transcriptional patterns, but are simplified with defined probes. A complex pattern of three major transcripts of 2.5, 3, and 4.5 kb was reported for transcription of atpA when a 2.0 kb probe that spans the coding region was used (Isaac et al., 1985) but a probe specific to the coding region identified a probable major mature transcript of 2.6 kb (Braun and Levings, 1985). Similarly, the complex pattern reported

for atp6 in T cytoplasm (Dewey et al., 1985a) was obtained with a probe that contained part of the 5 kb repeat and consequently detected transcripts of urf13-T and ORF25. As shown here, probes specific to the atp6 coding region clarify the transcript pattern as they identify two major transcripts of 1.8 and 1.6 kb and three minor transcripts, in N and T cytoplasms. Even with gene-specific probes, transcript patterns detected for many higher plant mitochondrial protein encoding genes are not simple and it is not yet known whether this reflects multiple initiation sites, multiple termination sites and/or post-transcriptional processing.

The subtle difference in transcription of atp6 between N and T cytoplasms reflects either a unique transcript in T mtRNA (1.55 kb) or a greater abundance of the same transcript (1.6 kb). Since the abundance of the 1.8 kb atp6 transcript and larger transcripts were equivalent among five inbreds in N and T cytoplasms, it is unlikely that the difference detected in the 1.6 kb range was a consequence of differential processing. This suggests there is a unique transcript (1.55 kb) in T mtRNAs. This subtle difference may be related to nucleotide sequence differences between N and T mtDNAs 5' to the gene, since the 4 and 5 bp inserts found in the T mtDNA genome are located in approximately the same region as the initiation or processing sites of the 1.6/1.55 kb atp6 transcript.

The 5 bp insert, which has indications of a tandem repeat, was not found in N, C, or S cytoplasms, suggesting that the event occurred in T mtDNA. Both copies of the 5 kb repeat in T cytoplasm contain the 4 and 5 bp inserts (Dewey et al., 1986) suggesting the insertion event(s)

occurred prior to the duplication of the 5 kb region or occurred in one copy and was subsequently duplicated by copy correction.

Transcription patterns of atp6 and urf13-T/ORF25 indicate that major transcripts are initiated (and/or processed) at or near the same sequences within the 5 kb repeat. This indicates that the atp6 promoter serves the genes urf13-T and ORF25. The 3.9, 2.0 and 1.85/1.8 kb urf13-T/ORF25 transcripts apparently share the same respective initiation or processing sites within the repeat as the 3.6, 1.8, and 1.6 kb atp6 transcripts. Assuming the 200 nucleotide size difference between atp6 and urf13-T/ORF25 transcripts reflects their termination site in the gene-specific regions, one would expect urf13-T/ORF25 transcripts of 6.0 and 4.3 kb that correspond to the 5.8 and 4.1 kb atp6 transcripts. Transcripts of 6.0 and 4.3 kb were not detected with urf13-T probes. The relative abundance of transcripts from the atp6 and urf13-T/ORF25 gene region also was not equivalent, as the urf13-T transcripts were detected in greater abundance. This may be related to the stability of the RNAs or the extent of processing events that each transcript is subjected to, rather than being a reflection of differential strength of the promoter in the two presumably identical regions.

The effect of the nuclear restorer genes on urf13-T/ORF25 transcripts has been described as a differential RNA processing event that results in the loss of a 1.5 kb transcript and the appearance of 1.6 and 0.6 kb transcripts unique to T cytoplasm lines restored to fertility (Dewey et al., 1986). The basic effect thought to be associated with fertility restoration has been observed in lines carrying the Rf1rf2 genotype (Dewey et al., 1987). Lines carrying

Rf1rf2 and T cytoplasm are male sterile, yet have the 1.6 kb transcript and a reduced abundance of the 13 kD urf13-T gene product. Since these studies were conducted on somatic cells, Rf1 might be considered to be a constitutively expressed gene. We have confirmed the basic effect of restoration and extended the observations in five additional maize inbreds in T cytoplasm. Some anomalies are evident, however, in our transcriptional analyses. Probes from within the 5 kb repeat that include positions -202 to -92 relative to urf13-T, detected an enhanced abundance of a 1.6 kb transcript in R mtDNAs. The abundance of the 1.6 kb atp6 transcript in T mtRNAs was unchanged by fertility restoration, suggesting that the enhanced transcript is related to urf13-T/ORF25. A probe representing positions -91 to +2 (T-ts305) did not hybridize to the 1.6 kb transcript, but probes 3' to this region readily detected the restorer-specific 1.6 kb transcript in R mtDNAs. The possibility of an intervening sequence is raised by these data, but we have been unable to confirm this observation by primer extension or nucleotide protection experiments. The effect of the restorer genes on urf13-T/ORF25 transcripts can, at least, be described as a unique event (whether 5' processing or splicing) leading to the appearance of the 1.6 kb transcript. The 1.5 kb transcript, unaffected by fertility restoration, is unrelated to processes which produce the 1.6 restorer-specific transcript.

The appearance of the unique 1.6 kb transcript characterizes fertility restoration of T cytoplasm in the five lines examined, yet subtle nuclear background effects among these lines are evident. The 1.6 kb transcript was not detected with a 95 bp probe terminating at +2 in urf13-T, suggesting that the 5' terminus of the 1.6 kb transcript may

be within the coding region. This is consistent with the observation that the 13 kD polypeptide is reduced by fertility restoration (Forde and Leaver, 1980). This suggests that fertility restoration effectively reduces the relative amount of the mature message, which appears to be either the 2.0 and/or 1.85/1.8 kb transcript. The abundance of the mature transcripts was found to be affected by nuclear background, as the line A188(T) exhibited a greater abundance of the 2.0 kb transcript relative to the other lines examined. It remains to be determined if the variability in abundance of urf13-T/ORF25 transcripts can be correlated to quantitative differences in the gene product or to the range of toxin sensitivity exhibited among maize inbred lines.

A more striking example of nuclear background effects was evident in the appearance of the 1.1 kb ORF25 transcript in T and R mtRNAs from the lines W64A and WF9. The 1.1 kb transcript was detected in the progeny of a cross between a line not having the 1.1 kb transcript [A188(T)] and restored Wf9(T), indicating nuclear control of its synthesis. The 1.1 kb transcript is not the mature ORF25 transcript, since its 5' terminus is internal to ORF25. This nuclear effect appears to be separate from restoration, and is not associated with any known phenotype.

It is interesting that the posttranscriptional modifications of urf13-T/ORF25 transcripts that are directed by the nuclear genome apparently are gene-specific and reduce, rather than produce, the mature message. Gene specific processing events directed by nuclear genes have been characterized in yeast (Dieckmann et al., 1984; McGraw and Tzagoloff, 1983; Simon and Faye, 1984), although they are involved in the process of maturation, rather than the reduction, of transcripts.

The synthesis of the 1.1 kb transcript directed by the WF9 and W64A nuclear genomes appeared to be specific to T cytoplasm. The ORF25 gene is believed to be a normal constituent of the maize mitochondria and has homology to mtDNA of at least five other diverse plant species (Dewey et al., 1986). The differences in the ORF25 transcript patterns detected among N cytoplasms was not correlated with the presence or absence of the 1.1 kb transcript among the analogous T cytoplasms. Thus the effects of the WF9 or W64A genomes on ORF25 transcripts in T cytoplasm was not exhibited on transcripts of ORF25 in N cytoplasm. The restorer genes have been found to only influence transcripts in the urf13-T region, a region whose organization is unique to T cytoplasm. The processing site recognized by the fertility restoration gene Rf1, however, is in a region that has homology to sequences 3' to the 26S rRNA gene. It is possible that processing of 26S rRNA transcripts may also be affected by Rf1.

For atp6, urf13-T, and ORF25, and perhaps other mitochondrial genes, it is clear that the transcriptional characteristics should be considered with regard to both the particular cytoplasm and nuclear background examined. The recombination events that created the unique organization of the gene urf13-T not only makes its transcript analysis specific to T cytoplasm, but also affects transcription of ORF25. Variation in ORF25 transcriptional patterns among N cytoplasms is likely related to genomic differences 3' to the gene, whereas subtle atp6 transcript differences between N and T cytoplasms may be related to small insertion events in a region 5' to the gene. Finally, nuclear genes have been shown to influence both abundance and synthesis of specific mitochondrial transcripts associated with the urf13-T/ORF25 complex.

CHAPTER IV

INITIATION AND PROCESSING OF atp6, urf13-T AND ORF25 TRANSCRIPTS FROM MITOCHONDRIA OF T CYTOPLASM OF MAIZE

Introduction

Cytoplasmic male sterility (cms) is a cytoplasmically inherited alteration in the development of the male gametophyte resulting in nonfunctional pollen. This phenomenon is widely distributed among higher plant species, having been reported in greater than 22 different families (Edwardson, 1970). In maize, there are three major male-sterile cytoplasms, S, C and T distinguished by the genetics of fertility restoration (Laughnan and Gabay-Laughnan, 1983). Nuclear-encoded fertility restoring genes compensate for nuclear-cytoplasmic incompatibilities or deficiencies that are phenotypically expressed during microsporogenesis and/or microgametogenesis. Plants carrying S and C cytoplasm are restored to fertility by single dominant genes Rf3 and Rf4, respectively, whereas two genes, Rf1 and Rf2, are necessary to restore fertility to T cytoplasm plants.

Plants carrying T cytoplasm are also distinguished from normal (N), S and C cytoplasms by their susceptibility to the fungal pathogens Cochliobolus heterostrophus, race T, and Phyllosticta maydis (rev. by Ullstrup, 1972) and sensitivity to the insecticide Methomyl (Humayden and Scott, 1977). The fungal pathogens produce host-selective toxins that act as virulence determinants and preferentially affect

mitochondria isolated from T cytoplasm plants (Miller and Koeppe, 1971). The association of cms and sensitivity to the toxins has not been separated.

There is abundant evidence associating a region of the T mitochondrial (mt) genome with the traits of cms and toxin sensitivity (rev. by Pring et al., 1987a). The organization of the mitochondrial DNA region encoding the gene urf13-T is unique to T cytoplasm (Dewey et al., 1986) and the urf13-T gene product, a 13 kD polypeptide, is only detected in plants carrying T cytoplasm (Forde et al., 1978; Dewey et al., 1987; Wise et al., 1987b). Synthesis of the 13 kD protein is reduced upon fertility restoration (Forde and Leaver, 1980) which is specifically directed by the nuclear Rf1 gene (Dewey et al., 1987). The primary effect of the Rf1 gene is posttranscriptional, altering the urf13-T transcriptional pattern by functioning as an RNA processing enzyme or additional transcription factor (Dewey et al., 1987). Male fertile, toxin insensitive mutants of T cytoplasm have been obtained through tissue culture regeneration of plants from immature embyros in the presence (Gengenbach et al., 1977; Brettell et al., 1980) or absence (Brettell et al., 1980; Umbeck and Gengenbach, 1983) of the fungal toxins. The majority of the male fertile, toxin insensitive mutants have suffered deletions involving the urf13-T gene (Fauron et al., 1987; Rottman et al., 1987; Wise et al., 1987a), and do not synthesize the 13 kD protein (Dixon et al., 1982). Another mutant, T-4, retains urf13-T sequences but fails to synthesize the 13 kD protein due to a 5 bp insertion in the coding region that places a premature stop codon in the correct reading frame (Wise et al., 1987a, 1987b).

The organization of the urf13-T gene region in the T mitochondrial genome resulted from numerous recombination events involving mitochondrial and perhaps chloroplast DNAs (Dewey et al., 1986). The 345 bp coding region of urf13-T contains 263 bp that has 87% similarity to the 3' flanking region of the 26S rRNA gene and 58 bp of nearly perfect similarity to a region interior to the 26S rRNA gene. Another recombination event duplicated a 5 kb flanking region of the gene coding for subunit 6 of the ATPase complex (atp6). The 3' edge of this repeated region is 444 bp and 69 bp from the predicted translational start codons of atp6 and urf13-T, respectively (Dewey et al., 1986). Sequences within the 5 kb repeat apparently promote transcription of atp6 and urf13-T, as well as another open reading frame, ORF25, that is 80 bp 3' to, and cotranscribed with, urf13-T (Dewey et al., 1986).

The atp6 transcripts were mapped from N and T cytoplasms, and the urf13-T/ORF25 transcripts from several restoring and non-restoring nuclear backgrounds of T cytoplasm (Chapter III). Here we demonstrate that the location of the 5' ends of the major primary and processed transcripts of atp6 and urf13-T/ORF25 are at identical positions within the repeated region. Differences in atp6 transcripts detected between N and T cytoplasms appear to be related to two small DNA insertions that apparently create an RNA processing site. The 5' terminus of a transcript that is unique to mitochondria from fertility restored T cytoplasm plants was not a suitable substrate for capping with guanylyl transferase and mapped to position +14 of urf13-T.

Materials and Methods

Seed lines

The maize inbred lines A188(T) (rf1rf1;Rf2Rf2), A188(N) (rf1--;rf2--) and restored WF9(T) (Rf1Rf1;Rf2Rf2) were provided by Dr. B. Gengenbach. Wf9(T) (rf1rf1;rf2rf2) was provided by Pioneer Hi-Bred International, Inc. The lines A632(T) (rf1rf1;----), restored A632(T) (Rf1--;Rf2--), A632(N) (rf1--;rf2--), W64A(T) (rf1rf1;----), restored W64A(T) (Rf1--;Rf2--), W64A(N) (rf1--;rf2--), C103(T) (rf1rf1;----), restored C103(T) (Rf1rf1;Rf2--) and C103(N) (rf1--;rf2--) were provided by Dr. P. S. Chourey. Other lines used were WF9(N) (rf1--;rf2--) and the single cross hybrid A188(T) X restored WF9(T).

RNA Analysis

Total mitochondrial RNA was isolated using guanidine isothiocyanate and prepared for Northern analysis by denaturation with glyoxal and eletrophoresis in 1.0% agarose gels as described (Wise et al., 1987a). The glyoxal gels used for Northern hybridizations shown in Figure 4-2 were electrophoresed for 12 hr at 100 V at 4°C. The Northern blots in Figure 4-9 were from 2.0% agarose gels run at 80 V for 12 hr at 4°C. Hybridizations were conducted at 50 or 55°C as described (Wise et al., 1987a) and washed according to Thomas (1980).

Primer Extension and RNA Sequencing

Oligonucleotides complementary to the DNA sequence 5'-TTGGCTCAACTCTCCGAG-3' (+132 to +149 of urf13-T) and 5'-GACTAGATGGAGTTCCACTG-3' (-346 to -327 of atp6) were synthesized on an Applied Biosystems 380 1-A DNA synthesizer and purified by recovery from a 15% acrylamide 8 M urea gel. Acrylamide gel slices containing the oligonucleotide primers were crushed in a syringe and eluted overnight

at 37° C with a buffer containing 0.5 M ammonium acetate, 0.01 M magnesium acetate, 0.1% SDS and 0.1 mM Na_2EDTA. The eluted fragments were precipitated by the addition of three volumes of 100% ethanol and 0.1 volume ammonium acetate, followed by a 15 min spin in a microcentrifuge. Approximately 100 pM of the purified oligonucleotides were end-labelled using T4 polynucleotide kinase and gamma-^{32}P dATP (see Chapter II) and 10 to 20 ng were used for primer extension reactions.

The synthetic oligonucleotides were annealed to 15 ug of W64A N, T or restored T mtRNA in a 10 ul volume containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM Dithiothreitol and 3mM $MgCl_2$. The solution was initially heated to 65°C and allowed to cool to 50°C or 42°C over 1 to 2 hrs. The primers were extended in 15 ul volumes with 200 units of M-MLV-reverse transcriptase (or 18 to 20 units of AMV reverse transcriptase) and a mixture of 0.5 mM dATP, dCTP, dGTP and dTTP (dNTP mix) at 42°C for 45 min. The reactions were stopped by adding 5 ul of formamide and boiled for 3-5 min prior to loading on 6% acrylamide 8 M urea wedge gels.

The RNA sequencing reactions were identical to the primer extension reactions except that approximately 100 ng of labelled primer (18mer) was annealed to 30 ug of restored W64A mtRNA and split into 4 reactions containing either 0.5 mM of dideoxy (dd) ATP, ddCTP, ddGTP or ddTTP along with 1 ul of the dNTP mix. Reverse transcriptase was added and the reactions were incubated at 50°C to help eliminate premature termination, as described by Geliebter (1987).

Primary Transcript Capping

Approximately 250 uCi of alpha P^{32} GTP was dried in a vacuum evaporator prior to the addition of 20 ug of total mt RNA from either

W64A N, T or restored T cytoplasm. The reaction included 50 mM Tris pH 7.8, 1.25 mM $MgCl_2$, 6 mM KCl, 2.5 mM DTT, 0.1 mM Na_2EDTA, 10 units RNAsin (Promega Biotech.) and 7 units of guanylyl transferase in a total volume of 20 ul. The reaction was incubated at 37°C for 30 min followed by the addition of another 7 units of guanylyl transferase another 30 min incubation. The reactions were terminated with 50 ul of a solution containing 0.6 M sodium acetate pH 7.0, 50 mM Na_2EDTA, 2% SDS, followed by two extractions with phenol/chloroform and precipitation with 2.5 volumes of ethanol, 0.1 volume of 8 M ammonium acetate.

Specific DNA clones were restricted and subjected to gel electrophoresis and the inserts were recovered by binding and extraction from DEAE paper. In a 20 ul volume, 1 to 3 ug of the DNA inserts were annealed to the capped RNAs in 80% formamide, heated to 85°C for 15 min and cooled to 49°C over a 3 hr period. Single stranded RNAs and DNAs were digested by addition of 250 units of mung bean nuclease in a 300 ul volume containing 50 mM NaCl, 10 mM sodium acetate pH 5.0, 1 mM L-cysteine, 4.5 mM $ZnCl_2$ which was incubated at 37°C for 1 hr. The reaction was stopped by addition of 50 ul 8 M ammonium acetate, 0.1 M Na_2EDTA followed by extraction with phenol/chloroform and ethanol precipitation.

3' Labelling, S-1 Digestion

Restriction fragments having 5' staggered ends were isolated from agarose gels and approximately 1 ug was incubated at 30°C for 30 min in a 10 ul volume containing 7 mM Tris-HCl pH 7.5, 7 mM $MgCl_2$, 50 mM NaCl, 15 uCi of alpha ^{32}P dCTP, 1.5 units of Klenow fragment and 0.5 mM of the particular dNTPs needed to fill in the sticky end. The labelled fragments were purified either by Sephadex G-50 columns or by ethanol

precipitation. The fragments used in determining the 3' end of RNA transcripts were heated to 70°C for 10 min and annealed with 10 ug of total mtRNA by cooling the reaction to 49°C over 3 hrs in a 20 ul volume containing 40 mM PIPES pH 6.4, 1 mM Na_2EDTA pH 8.0, 0.4 M NaCl and 80% formamide. The reaction was brought up to 300 ul in a buffer containing 0.28 M NaCl 0.05 M sodium acetate pH 4.6, 4.5 mM $ZnSO_4$, 300 to 600 units of S-1 nuclease and incubated at 37°C for 45-60 min. The reaction was terminated by extraction with phenol/chloroform followed by ethanol precipitation.

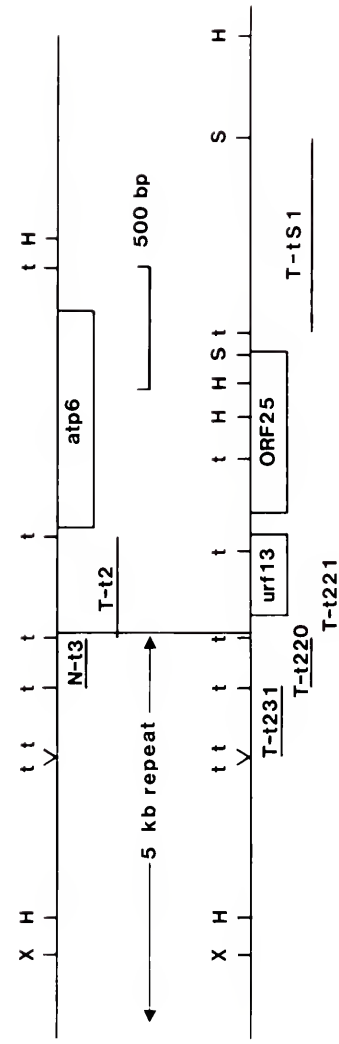
DNA Markers

The markers were either pUC8 (Yannish-Perron et al., 1985) cut with Sau3A or TaqI and 3' labelled as described above or were from DNA sequencing reactions of M13 clones with known sequences. The dideoxy chain termination method (Sanger et al., 1977) was used for sequencing as previously described (Wise et al., 1987a).

Results

The mitochondrial genome regions of T cytoplasm containing the genes atp6, urf13-T and ORF25 are shown in Figure 4-1. A region of approximately 5 kb located 5' to atp6 is duplicated in T cytoplasm and is 5' to urf13-T in its other arrangement. Northern analysis has shown that the transcript patterns for these genes are complex, with atp6 having two major (1.8 and 1.6 kb) and three minor transcripts (Chapter III) and the cotranscribed genes urf13-T and ORF25 having as many as nine distinct transcripts (Dewey et al., 1986; Wise et al., 1987a; Chapter III). The 5' and 3' termini of the atp6 and urf13-T/ ORF25

Fig. 4-1. Restriction map of mitochondrial genes atp6, urf13-T and ORF25 in T cytoplasm showing their location relative to a 5 kb repeat. Clones used in transcriptional analyses are shown. X=XhoI, H=HindIII, t=TaqI.


X H
t t t t t
N-t3
T-t2
atp6
t H
500 bp
5 kb repeat
X H
t t t t
T-t231
urf13
t
T-t220
T-t221
t H H S t
ORF25
S H
T-tS1

transcripts were approximated from Northern analysis and most of the major transcripts appear to originate in the repeated region (Chapter III), suggesting these genes share promoter sequences. To accurately identify the 5' termini of the major transcripts, primer extension analysis was conducted rather than S-1 analysis to clearly distinguish atp6 transcripts from urf13-T/ORF25 transcripts.

Primer Extension

Synthetic primers were prepared, complementary to gene-specific regions of atp6 (-327 to -346 relative to the proposed translational initiation site, Dewey et al., 1985) and urf13-T (+145 to +128, Dewey et al., 1986). The urf13-T primer site is 3' to all major urf13-T/ORF25 transcripts predicted from Northern analysis, in a region that has sequence similarity to a region 3' to 26S rRNA gene (the primer has 83% similarity). The results of primer extension experiments using primers from gene-specific regions of atp6 (20mer) and urf13-T (18mer) are shown in Figure 4-2.

Total mitochondrial RNA from both N and T cytoplasms were analyzed with the atp6-specific 20mer to determine if there is a transcript unique to T cytoplasm. Northern analyses detected a difference in atp6 transcript patterns between N and T cytoplasms (Chapter III). In all nuclear backgrounds examined, T mtRNAs displayed a more abundant (or unique) transcript migrating at approximately 1.55 to 1.6 kb. Major extension bands of 241 and 250-256 nucleotides were detected only in the T mitochondrial RNA lane (Figure 4-2; 20mer-T), indicating a unique transcript(s), designated as the 1.55 kb transcript. These extension bands correspond to positions -568 and -577 to -583 of atp6 and span a five bp insertion that was found to be unique to T mitochondrial DNAs

Fig. 4-2. Primer extension analysis. Primers specific to urf13-T (18) and to atp6 (20) were annealed and extended with mtRNA from T and R, or N and T cytoplasms, respectively. Marker is a from a DNA sequence reaction and indicates distance of primer extension in nucleotides; the corresponding transcript is labeled in kilobases. Unlabelled arrowheads are fragments that do not correspond to known transcripts. Size of fragments are listed in Table 4-1.


18 20
T R N T
2.0
1.8
1.85
399
1.8
302
1.6
1.55
204
1.6
1.5
105

(Chapter III). There are two insertions of four and five bp in the T mitochondrial genome at positions -572 to -576 and -587 to -590 relative to the proposed translational start site of atp6. The insertions affect comparisons between the primer extension fragments from N and T mtRNAs, reflected by a nine nucleotide difference in fragments which are larger than 266 nucleotides. Two other major groups of bands are detected in the T lane at approximately 480/470 and 297/290/279 nucleotides, as well as three minor bands migrating at 391, 195 and 123 nucleotides (arrowheads). The mapped positions of the major bands (-807/-797 and -624/-617/-606, respectively) correlates with the 5' termini of the major atp6 transcripts approximated by Northern analyses. The intensity of these bands and their relation to the 5' ends of the major urf13-T/ORF25 transcripts (see below) support the assumption that these bands represent the 5' termini of the 1.8 and 1.6 kb atp6 transcripts. The DNA sequences at these termini were identical in N and T cytoplasms.

There were numerous primer extension bands obtained with both primers that did not appear to correlate with transcripts detected in Northern analyses. The appearance and intensity of many of these bands were variable under different reaction temperatures and with the reverse transcriptase employed (M-MLV or AMV). Consequently, only bands that were consistently observed were identified in Figure 4-2 (arrowheads). Of these bands, only the -123 nucleotide atp6 band and the -225 nucleotide urf13-T/ORF25 band were associated with the same site within the repeat (Table 4-1). There is no definitive evidence that any of the transcripts associated with atp6, urf13-T, or ORF25 have intervening sequences or that any of the major transcripts have unique termination sites (see below), so a correlation between these minor

Table 4-1. Sequences proximal to primer extension bands.

Band Length 20mer;18mer	Downstream site atp6;urf13-T	Transcript (kb) atp6;urf13/ORF25	Sequence
480; 581+/-5	-807; -432	1.8; 2.0	TATATAAGCGTAGTTAT
470; 571+/-5	-797; -422	1.8; 2.0	
391	-718	?	TATGTTAAGTTCTCT
297; 398+/-3	-624; -249	1.6; 1.85	
290; 391+/-3	-617; -242	1.6; 1.85	CATAGAGAAAGATGTTCTGATTCAGTT
279; 380+/-3	-606; -231	1.6; 1.85	
241; 342	-568; -193	1.55; 1.8	
250; 351	-577; -202	1.55; 1.8	AGACGGGGCCCTTAGCTTAGGGA
252; 353	-579; -204	1.55; 1.8	
254; -	-581; -	1.55; -	
195	-522	?	TGGTAGCCTCTCTGCTG
123; 225	-450; -76	? ; ?	TGTGTGGGTGTTCAGT
; 141	; +10	; 1.6	ATCACTACTTTCTTAAAC
; 136	; +14	; 1.6	
; 107	; +43	; 1.5	TTTGATCAAGGTTTGGTAT

↓ denotes mapped transcript termini
underlined sequences are not in N mtDNAs; mapped termini only detected in T cytoplasm in this region

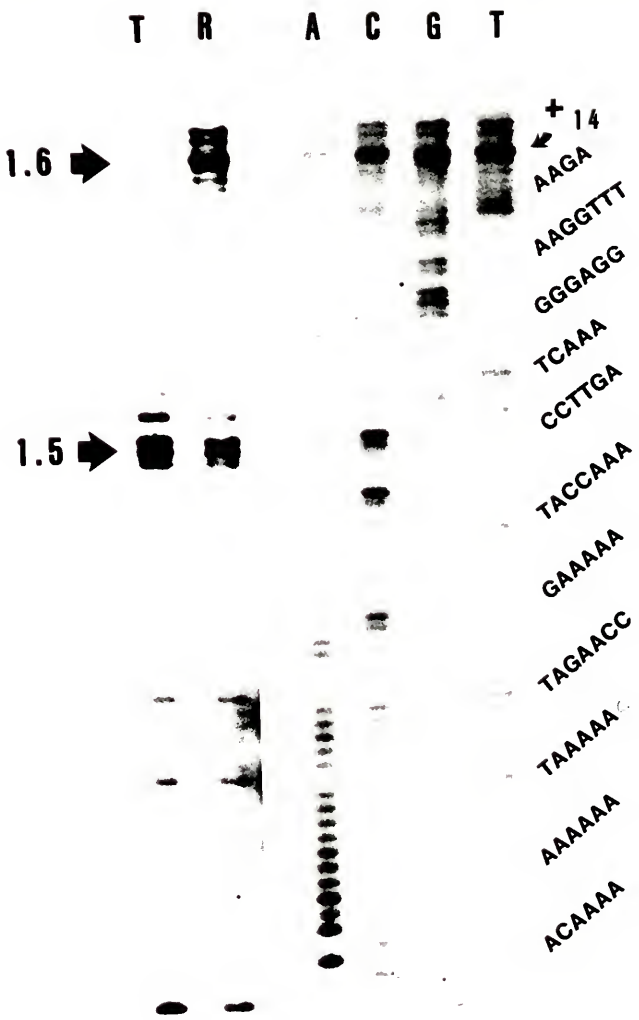


Fig. 4-3. Sequence of the 5' terminus of the 1.6 kb transcript specific to mtRNA from restored cytoplasm. The urf13-T-specific primer was extended in the absence (lanes T and R) or presence of DNA chain terminators (A, C, G and T). All lanes, except the primer extension of T mtRNA, exhibit a band that correspond to position +14 relative to the initiation codon of urf13-T. Sequences continuing beyond this site represent extensions of other major transcripts.

primer extension bands and discrete transcripts detected on Northern hybridizations cannot be made.

Primer extension analysis using the 18mer was conducted with mtRNA from T cytoplasm and T cytoplasm restored to fertility (R). Identical extension band patterns were observed with the exception of a 136 nucleotide band detected only in the R lane (Fig. 4-2). This band corresponds to the restorer-specific 1.6 kb transcript (Dewey et al., 1986; Chapter III). The 5' ends of the major urf13-T/ORF25 transcripts (2.0, 1.85 and 1.8 kb) mapped to approximately the same sequences within the repeat as the major atp6 transcripts. The DNA sequences proximal to all the primer extension bands are listed in Table 4-1. The size of the bands corresponding to the longest urf13-T/ORF25 transcripts was difficult to accurately determine and were estimated with different degrees of accurancy (+/- 3 to 5 nucleotides).

RNA Sequencing

The precise location of the end of the 1.6 kb restorer-specific transcript was identified by primer extension experiment using the 18mer synthetic primer and DNA chain terminators (Figure 4-3). The RNA sequence did not deviate from the DNA sequence (Dewey et al., 1986) and terminated at position +14 of urf13-T, in agreement with the primer extension analysis (Figures 4-2, 4-3). Extension of the RNA sequence beyond the +14 position represents binding to the higher molecular transcripts (e.g. 1.8/1.85, 2.0 or 3.9 kb transcripts). Northern hybridization patterns revealed that the restorer-specific 1.6 kb transcript may participate in an RNA splicing event (Chapter III). The RNA sequencing results presented here appear to rule out this interpretation of the Northern hybridizations.

Primary Transcript Capping

Mitochondrial transcripts originating from initiation, rather than from RNA processing, retain a triphosphate nucleotide at their 5' termini and can be specifically labelled with the G-capping enzyme, guanylyl transferase. Total mitochondrial RNA from N, T and restored T cytoplasms were labelled with guanylyl transferase and subjected to nuclease protection analyses using DNA clones T-t231, T-t220, N-t3, and T-t221 (Fig. 4-1) and mung bean nuclease. Labelled transcripts were only detected when clones T-t220 or the analogous clone from N mitochondrial genome, N-t3, were used as protection fragments (Figure 4-4). Another fragment detected in all lanes most likely represents a highly abundant transcript that is not completely digested with mung bean nuclease. The size difference of the RNA fragments protected by probe T-t220 (or N-t3) is approximately 6 or 7 nucleotides within each lane (lanes 3 and 4) and 9 nucleotides between lanes. The mapped site for these fragments (estimated by DNA sequencing ladders) is -258/-251 for urf13-T or -625/-618 for atp6 (in the T mitochondrial genome), which is within 10 nucleotides of the mapped 5' termini of the 1.85 kb urf13-T/ORF25 and 1.6 kb atp6 transcripts (-249/-242/-231 and -624/-617/-606, respectively). The relative proximity between capped and primer extension sites and the equivalent spacing between specific termini sites (7 nucleotides) implicates this mtDNA region as a putative transcription initiation site. Similar analyses of chloroplast RNAs (Mullet et al., 1985) showed the capped RNAs do not migrate at an equivalent rate as the DNA markers.

When correlated with primer extension and Northern data, the transcript capping experiments identify the 1.85 kb urf13-T/ORF25 and

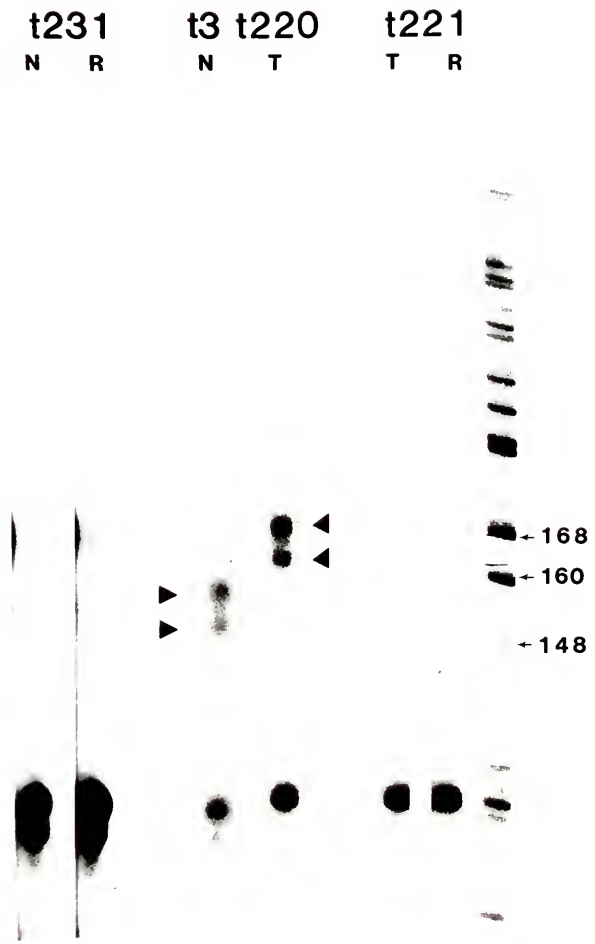


Fig. 4-4. Primary transcript capping with guanylyl transferase. Total mitochondrial RNA from normal (N), male sterile (T), or restored (R) cytoplasms was capped, protected with specific DNA clones, and subjected to nuclease digestion. Protection fragments T-t231 and T-t221 (Fig. 4-1) did not protect any labeled transcripts, whereas clone T-t220 and its equivalent clone from normal cytoplasm (N-t3; Fig. 4-1) protected two transcripts. The size of the protected transcript indicates they correspond to the 1.85 kb urf13-T transcript and the 1.6 kb atp6 transcript. The shift of nine nucleotides is due to a difference between N and T mitochondrial DNA in this region. Marker lane, from a DNA sequencing reaction, is on far right with size of three fragments in nucleotides.

1.6 kb atp6 transcripts as primary transcripts (initiated) whereas the 2.0, 1.8 and 1.5 kb urf13-T/ORF25 transcripts and the 1.8 and 1.55 kb atp6 transcripts are presumably products of RNA processing events. The 1.6 kb restorer-specific transcript was not found to be a suitable substrate for capping under our experimental conditions. The DNA sequences proximal to the atp6/urf13-T/ORF25 transciption initiation site are similar to sequences located at the 5' termini of the largest transcripts from the petunia atp9 gene and maize coxI gene, forming a short consensus sequence (Table 4-2). The similarities between the 5' termini of the shortest transcript of these genes and a processing site described in this report are also included.

The protection clones (T-t231, T-t220, N-t3, and T-t221) were chosen because they span the 5' termini of the major atp6 and urf13-T/ORF25 transcript determined from Northern and primer extension analyses and they were of optimal size for accurate measurement. Consequently, it is possible that there are other transcripts that were capped but escaped our detection (e.g. at or near the TaqI sites common to these adjacent clones).

A schematic representation of location of transcript initiation and major RNA processing sites for atp6 and urf13-T (and ORF25) are shown in Figure 4-5. The distance (in nucleotides) from the 3' edge of the 5 kb repeat to the translation start codon of atp6 and urf13-T, as well as the regions of urf13-T having homology to 26S rDNA regions are also shown. The major events involved in the maturation of atp6 and urf13-T/ORF25 transcripts deduced from the primary transcript capping, primer extension, and Northern analyses are illustrated in Figure 4-6.

Table 4-2. Sequence similarities among 5' termini of plant mitochondrial gene transcripts.

A. Transcript initiation

Petunia atp9[a]	-253	TTTCATA AGATAA GAGAGAT
maize atp6	-635	TTTTCATAGAGAGAAAGAT
maize coxI[b]	-161	TCATA AG TAATC
Young Consensus[a]		AAATYTCNTA AGAGAA T

B. Processing sites

Petunia atp9-1 and 2[c]	-127	TAGCCTATGCTTTGC
maize T-specific atp6	-584	GGCCCTTAGCTTAGGG
maize coxI[b]	-68	GCCCTTCA TTCTTTG
maize atp6-1.8 kb	-817	ATTGACGTATATAAGCGTAGTT
maize urf13-T-1.5 kb	+36	TCAAGGTTTGGTATTTTTCGGT
maize urf13-T-Rf1	+6	CACTACTTTCTTAAACCTTCCT

C. 3' termini

Rothenberg Consensus[c]		ATTTCATCGA
urf13-T/ORF25	+1146	GGGCTTCTTTCATGGTGCCATTCTTTA
maize 26S rRNA[d]	4020	ATGGAACTTTCCAACCTGTTAT

a Young et al., 1986
b Isaac et al., 1985
c Rothenberg and Hanson, 1987
d Dale et al., 1984
↓ denotes mapped transcript termini

Fig. 4-5. Transcription initiation and processing sites 5' to atp6 and urf13-T. The proposed transcript initiation sites (horizontal arrows) and major RNA processing sites (vertical arrows) are shown. Distance from the translational initiation codon (+1) is given in nucleotides. A difference between N and T mitochondrial DNA is distinguished at the top of the figure. The complex organization of urf13-T is shown with sequences beginning 444 nucleotides 5' to atp6 that are found 70 nucleotides 5' to urf13-T, as well as, sequences similar to a region 3' to the 26S ribosomal gene (////) and internal to the 26S gene (XXXX). The processing site directed by the nuclear gene Rf1 (+14) and site of 1.5 kb urf13-T/ORF25 transcript (+43) are indicated. Approximate location of synthetic oligonucleotides are shown. t, TaqI.

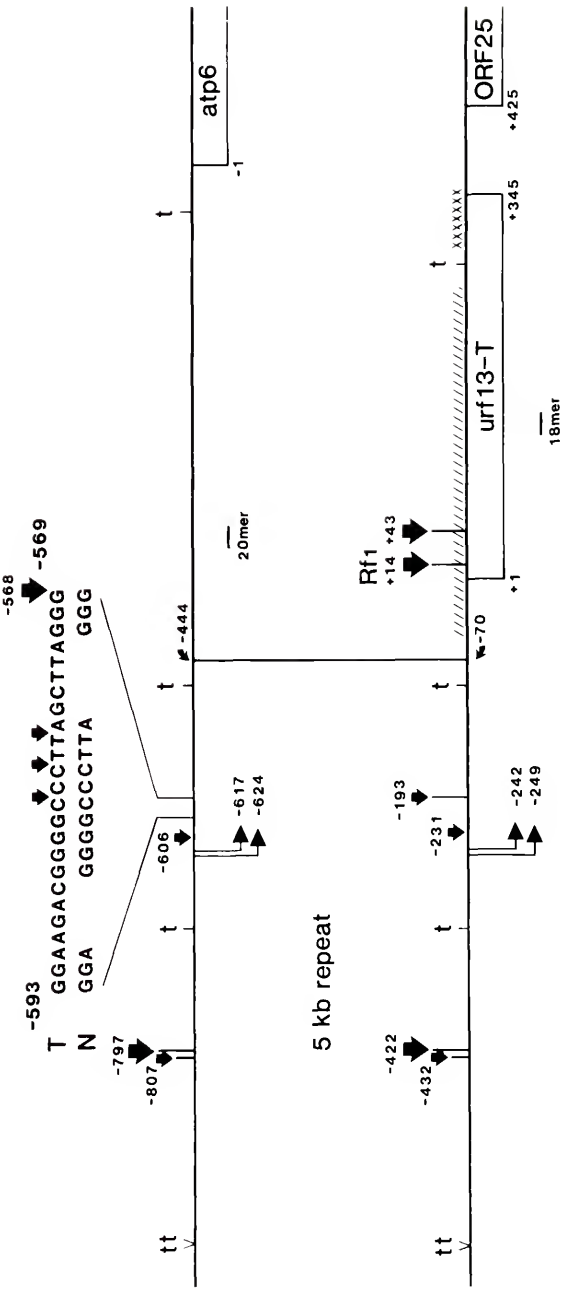
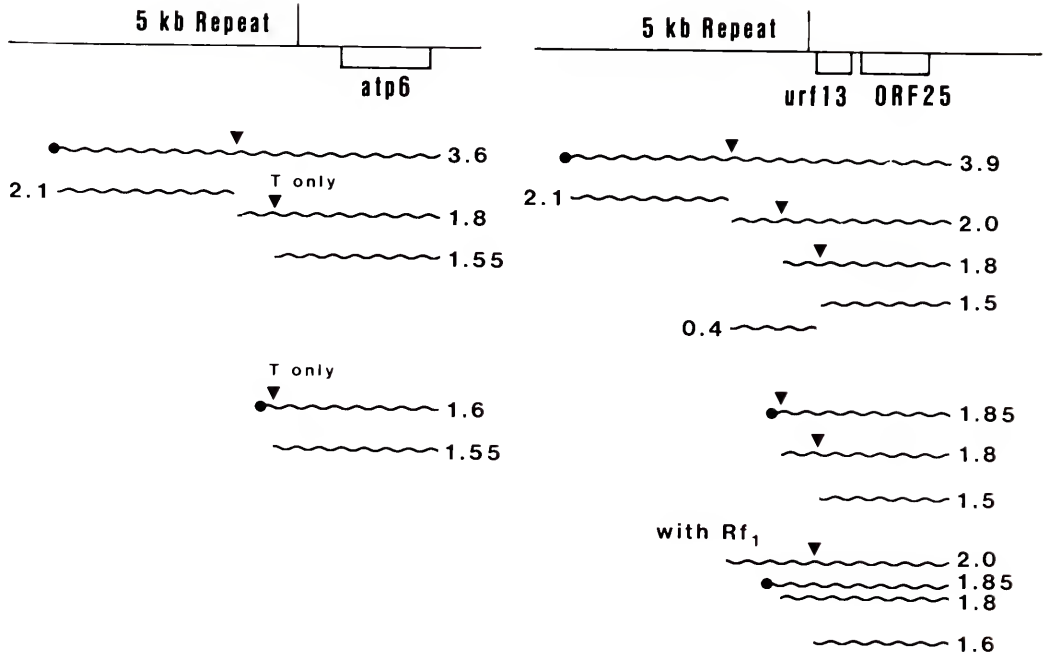

-593
T GGAAGACGGGGCCCTTAGCTTAGGG
N GGA GGGGCCCTTA GGG
-568
-569
-797
-807
-606
-617
-624
-444
20mer
atp6
-1
t
tt
5 kb repeat
-422
-432
-193
-231
-242
-249
Rf1
+14 +43
-70
+1
urf13-T
XXXXXXX
+345
ORF25
+425
18mer

Fig. 4-6. Schematic representation of the atp6 and urf13-T/ORF25 transcript maturation. Initiation (dots) and processing sites (arrowheads) are indicated and the size of transcripts detected by Northern analyses are designated in kilobases. Primary transcripts are marked with a dot (the 3.9 kb and 3.6 kb transcripts have not yet been confirmed as primary transcripts).


5 kb Repeat
atp6
3.6
2.1
T only
1.8
1.55
T only
1.6
1.55
5 kb Repeat
urf13
ORF25
3.9
2.1
2.0
1.8
1.5
0.4
1.85
1.8
1.5
with Rf1
2.0
1.85
1.8
1.6

3' mapping

The 3' termini of the major urf13-T/ORF25 transcripts were located by hybridization of a 3' end-labelled DNA probe representing positions +716 to +1499, relative to the proposed translational start of ORF25 (positions 2356 to 3139 of TURF2H3, Dewey et al., 1986), to total mtRNA from N or T cytoplasms and subsequent digestion with S1 nuclease (Figure 4-8). A protected fragment of approximately 440 nucleotides is detected in the T mtRNA lane, corresponding to position +1155 (2795 of TURF2H3). There appear to be at least four discrete fragments in this size range, suggesting termination does not occur at a specific nucleotide. The urf13-T/ORF25 transcript termination site had been predicted due to the potential for an RNA secondary structure similar to bacterial transcript terminators (Schuster et al., 1986). Comparison of the flanking sequences of this site and a 3' termini consensus sequence are shown in Table 4-2.

The 3' terminus of the 26S rRNA gene was determined for reasons described below. A 1973 bp HindIII (T-ts1; Fig. 4-1) fragment representing positions 3045 to 5018 of the sequence reported by Dale et al. (1984) was 3' end-labelled and protected with total RNA from T cytoplasm (Figure 4-8). A nondiscrete band measuring approximately 972 nucleotides was detected that corresponds to position 4018 (+/- 8 nucleotides) of the DNA sequence (Table 4-2). This site is approximately 20 nucleotides upstream of the stop site predicted by homology to E. coli 23 rDNA (Dale et al., 1984). Other protected fragments of similar size were also detected, but were much less abundant.

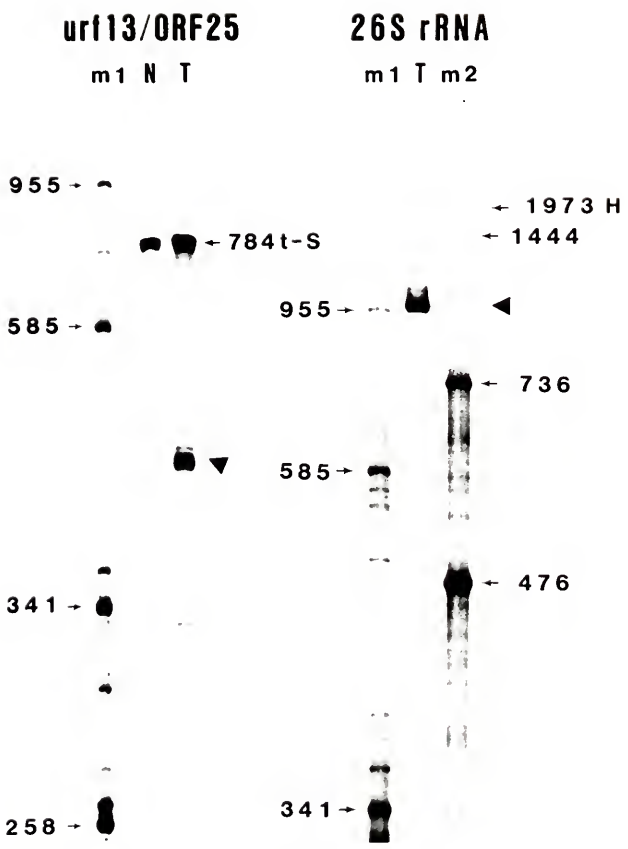
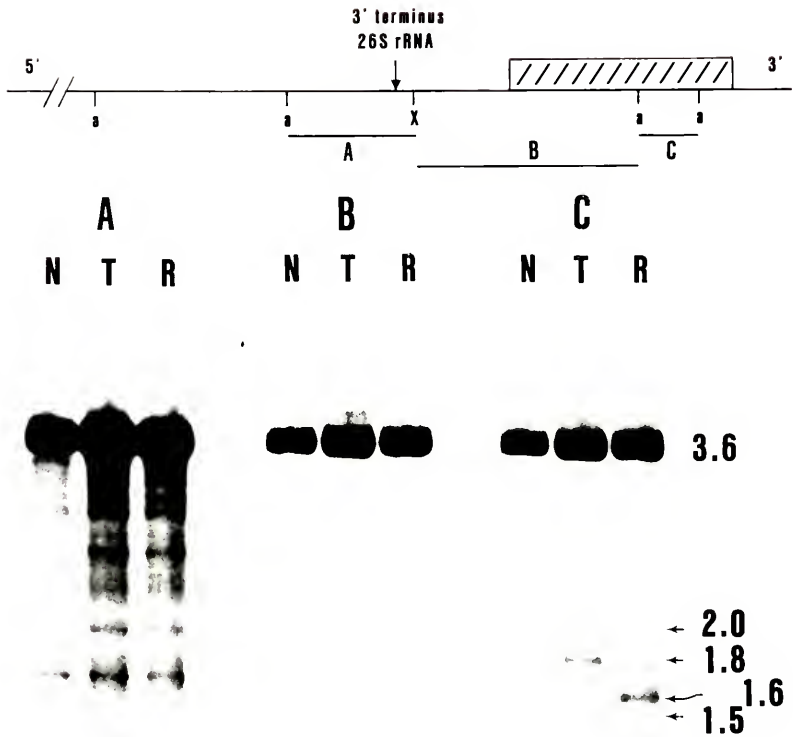


Fig. 4-7. Mapping the 3' termini of urf13-T/ORF25 and 26S rRNA transcripts. A 784 bp TaqI-SmaI fragment (T-ts1, Fig. 4-1) and a 1973 bp HindIII fragment were labelled and hybridized to total mtRNA from N or T cytoplasm followed by S-1 digestion. Arrowheads mark the major protected fragments. Markers are pUC8 digested with Sau3A (m1) or TaqI (m2) and labelled. Size of fragments are in nucleotides.

Does Restoration Affect 26S rRNA Processing?

The proposed RNA processing sites at positions +14 (restorer-specific) and +43 of urf13-T are within the region that has homology to sequences 3' to the 26S rDNA gene. A region of high homology extends 68 nucleotides upstream of the +14 position (90% homology) and 52 nucleotides downstream (98% homology; Dewey et al., 1986). To determine if the +14 or +43 sites are utilized in the processing of the 26S rRNA transcripts, Northern blots of mtRNA from N, T and restored T cytoplasms were probed with DNA clones from the 26S rDNA region of A188(T) genome that represent positions 3881-4049 (A) , 4050-4343 (B), and 4344-4422 (C; Figure 4-9). No differences in transcript patterns between N, T, and restored-T mtRNAs were detected. Probes B and C hybridized to an abundant message of approximately 3.6 kb, comigrating with the 26S rRNA transcript detected with probe A. Although the nitrocellulose membranes probed with B and C were exposed two to three times longer than the membrane probed with A, the transcript detected with B and C could be the mature 26S rRNA, which would indicate that the transcript termination site could extend at least 400 nucleotides downstream from the predicted stop site. The S-1 analysis described above does not support this hypothesis, thus the transcript detected with probes B and C may represent an unprocessed form of the the gene. Probes B and C also detect the major urf13-T/ORF25 transcripts (2.0, 1.85/1.8, 1.6 and 1.5 kb; Fig. 4-9, probe C).

Fig. 4-8. Northern analysis of 3' end of 26S rRNA gene. Probes A, B and C represent the regions shown in the partial restriction map were hybridized to total mtRNAs from N, T and T-restored (R) cytoplasms. Blots B and C were expososed for approximately two to three times longer than blot A. Mapped 3' termini of the major 26S rRNA transcript, predicted in Fig. 4-7, is shown. The 3.6 kb band detected with probe A represents the major 26S rRNA transcript, and a less abundant 3.6 kb transcript detected in B and C may represent a precursor transcript. Hatched region represents region of similarity to urf13-T and probe C detects the major urf13-T/ORF25 transcripts (2.0, 1.85/1.8, 1.6, and 1.5 kb).


3' terminus
26S rRNA
5'
3'
a
a
X
a
a
A
B
C
A
B
C
N T R
N T R
N T R
3.6
2.0
1.8
1.6
1.5

Discussion

The major steps involved in the maturation for the genes atp6, urf13-T, and ORF25 transcripts were elucidated through identification of transcript initiation and processing sites and their relation to the transcript map for these genes developed from Northern hybridizations (Chapter III). Although the events involved in the synthesis of a few minor transcripts, and the authenticity of some primer extension fragments have not been resolved, there are no major inconsistencies between the primer extension/capping data and the hybridization analyses. The size of the major transcripts predicted from the start and stop sites described in Tables 4-1 and 4-2 were all within 40 nucleotides of the size estimated from Northern analyses. Two ambiguous primer extension bands (-450 of atp6; -76 of urf13-T) were detected that corresponded to the same nucleotide sequence (Table 1), but did not correlate with Northern data. It is noteworthy that DNA sequences surrounding this site have great similarity (29 of 30 bp) to a region that is immediately 5' to the 26S rRNA gene (Dewey et al., 1986).

The major atp6 transcripts detected in mtRNA from N cytoplasm (1.8 and 1.6 kb) result from a processing and an initiation event. The processed transcript (1.8 kb) is most likely derived from either the 4.1 or the 3.6 kb transcript, since a 2.1 kb transcript is detected upstream of the processing site (e.g., 1.8 + 2.1=3.9). The relative abundance of the 1.8 kb transcript is three to five times greater than the abundance of the 1.6 initiated transcript (Chapter III), thus the major atp6 transcription initiation site is located approximately 2.5 to 3.0 kb upstream of the predicted translational start. In mtRNA from T cytoplasm, a processing site apparently has been formed by the addition

of two small DNA insertions of 4 and 5 bp. This processing site slightly affects the abundance of both the 1.8 and 1.6 kb transcripts, resulting in a 1.55 kb transcript that is about half as abundant as the 1.8 kb transcript.

The nucleotide sequence of a 3.0 kb region that includes the urf13-T gene (Dewey et al., 1986; Chapter III) reveals as many as six small DNA insertions or deletions, deduced through comparison of sequence similarities to the DNA progenitor regions. Three of these are exhibited as perfect 5 bp tandem direct repeats, two as 4 bp repeats separated by a single bp and one that is a 4 bp insertion with 2 bp in common with the adjacent 4 bp sequence. Most of these insertions or deletions resemble classical proposals for frameshift mutations produced by mechanisms involving misaligned pairing of repeated DNA sequences (Steisinger et al., 1966). The region of urf13-T that has similarities to a sequence 3' to 26S rDNA gene has lost one copy of a tandem 5 bp repeat and suffered a G to A transition when compared to the 26S rDNA region. A tissue culture derived fertile, toxin insensitive mutant (T4; Umbeck and Gengenbach, 1983) was shown to regain the tandem five bp duplication in its urf13-T sequence (Wise et al., 1987a), matching the 26S rDNA sequence in this region. This mutant does not synthesize the 13 kD protein because the 5 bp duplication places a stop codon in frame that truncates the predicted protein to 8.3 kD (Wise et al., 1987a, 1987b). The effect of the small insertions and deletions, presumed to be caused by errors in replication, or failure to correct these errors, is illustrated in two ways within a relatively small DNA region of the T mitochondrial genome; one event apparently creates a new RNA processing site and the other is involved with a frameshift mutation.

The initiation and processing sites for the urf13-T/ORF25 transcripts within the 5 kb repeat are the same as those for atp6 transcripts; however, urf13-T/ORF25 transcripts are subject to as many as three more events outside of 5 kb repeat. Processed transcripts were detected in T mtRNA having 5' termini at +43 (1.5 kb transcript) and in nuclear fertility restoring backgrounds (specifically, those with Rf1; Dewey et al., 1987) at +14 of urf13-T (1.6 kb transcript). Both processing events result in transcripts that do not contain the entire urf13-T gene, yet retain ORF25 sequences. Another event, not yet determined to be the result of processing or initiation, occurs internal to ORF25 resulting in a 1.1 kb transcript. The detection of this transcript, like the 1.6 kb transcript, depends on nuclear background (see Chapter III).

Numerous recombination events were involved in establishing the complex arrangement of the atp6, urf13-T and ORF25 genes in T cytoplasm of maize. An event critical for the transcription of urf13-T was the duplication of the atp6 flanking region which placed DNA sequences involved in transcription initiation 5' to urf13-T and ORF25. If the degree of similarity between sequences that make up the chimeric gene region of urf13-T and their proposed progenitor sequences (e.g. atp6, 26S rDNA) is indicative of the chronological order of the recombination events, the duplication of the 5 kb region would be the most recent event. The regions of urf13-T similar to sequences internal and 3' to the 26S rRNA gene have apparently diverged since their duplication as they are 95% and 85% similar, respectively (Dewey et al., 1986).

All transcripts that are not a suitable substrate for guanylyl transferase are assumed to be the result of RNA processing events,

although they may have alternative origins. Additional evidence for the authenticity of the proposed processing sites that result in the 1.8 kb atp6 and 2.0 kb urf13-T/ORF25 transcripts and the site for the 1.5 kb urf13-T/ORF25 transcript is provided by transcripts 5' to these sites which are of an appropriate size to be products of a processing events of a larger transcript. For example, the 0.4 kb transcript detected immediately upstream of the 1.5 kb urf13-T/ORF25 transcript is most likely a product of the cleavage of the 2.0 kb urf13-T/ORF25 transcript.

The nuclear gene Rf1 has been described to act as an RNA processing enzyme or as an additional transcriptional factor which apparently leads to a reduction of the 13 kD protein by altering or limiting the abundance of the urf13-T transcripts (Dewey et al., 1986, 1987; Chapters III, IV). There are, however, a number of conflicting observations concerning this conclusion. First, no transcripts, specific to fertility restored cytoplasms, are detected 5' of the +14 site of urf13-T. Second, the abundance of the 2.0, 1.85, and 1.8 kb urf13-T/ORF25 transcripts is only slightly reduced, not nearly as dramatic as the 67% reduction detected on protein gels with labelled mitochondrial polypeptides (Dixon et al., 1982) or the even greater reduction apparent on Western blots (estimated from Dewey et al., 1987). Finally, although the urf13-T sequence spanning the +14 site is very similar to sequences 3' to the 26S rRNA gene, fertility restoring genes were not found to have an effect on transcripts in this region. An alternative explanation for the function of the Rf1 gene may be that it affects some aspect of translation, prematurely terminating the translation of some, but not all, transcripts and secondarily creating a 1.6 kb transcript. This would seemingly be in better agreement with the

analyses of the 13 kD and its reduction upon restoration, as well as the observation that fertility restoration does not influence 26S rRNA transcripts. Consequently, although the influence of Rf1 gene effectively reduces the abundance of 13 kD protein, the manner in which it accomplishes this is not completely clear, nor is the role of Rf2 in fertility restoration.

CHAPTER V

DISCUSSION

The following observations implicate the gene urf13-T as having a major role in the phenotypes of cms and toxin sensitivity 1) urf13-T and its gene product, the 13 kD protein, are unique to T cytoplasm, 2) urf13-T transcripts and the abundance of the 13 kD protein are affected by fertility restoration, specifically, the Rf1 gene, and 3) urf13-T is deleted or is truncated in tissue culture derived fertile, toxin insensitive mutants. Despite these facts, much remains to be answered.

The first major question concerns the toxin sensitivity of T cms plants restored to fertility. Although these plants are completely fertile, their sensitivity to the toxins is only slightly moderated, at best 50%. Fertility restoration has been shown to reduce the abundance of the 13 kD protein up to 67% (Dixon et al., 1982), determined by labelling proteins in isolated mitochondria. Western blots, probably a more accurate method of quantitating proteins in vivo, reveal a greater reduction in the abundance of the 13 kD protein upon fertility restoration or with Rf1 alone (estimated from data presented in Dewey et al., 1987). One hypothesis for this apparent separation of the traits of cms and toxin sensitivity in restored plants is that the decreased abundance of the 13 kD protein could be sufficient to overcome cms yet not great enough to affect its ability to serve as a receptor for, or have some other interaction with, the toxin molecules. In this

scenario, only the complete absence of the 13 kD would confer insensitivity to the toxins.

The second enigma involves the role of Rf2 in fertility restoration and toxin sensitivity. The effect of Rf2 on toxin sensitivity can be determined by assaying the response of isolated T cytoplasm mitochondria from the proper nuclear backgrounds. Comparison of the toxin response of T mitochondria from backgrounds having different dominant Rf genes (e.g. rf1rf1;rf2rf2, Rf1--;rf2rf2, rf1rf1;Rf2--, and Rf1--;Rf2--) should discriminate the differences between the individual effects of Rf1 and Rf2. Unfortunately, identifying the influence of Rf2 in fertility restoration will not be as simple.

The primary influence of the Rf1 gene on urf13-T appears to involve an RNA processing event that alters the urf13-T messages (Dewey et al., 1986, 1987; Chapters III and IV). The relative reduction of mature urf13-T messages, determined from Northern analyses (Chapter III), does not appear to be as great as reduction of the 13 kD protein in restoring or Rf1 containing nuclear backgrounds (Dewey et al., 1987). The mature transcripts (assumed to be the 2.0, 1.85 and 1.8 kb urf13-T/ORF25 transcripts) are slightly reduced upon fertility restoration, but they are present in relatively high abundance when compared with atp6 or ORF25 transcripts in N mtRNAs. This inconsistency creates some uncertainty regarding the hypothesis that the Rf1 gene product is directly involved in RNA processing. With the available data, no other obvious mechanisms for Rf1 action are apparent, yet it is conceivable that Rf1 may be related to some aspect of translation and that the restorer-specific 1.6 kb transcript is a secondary effect of an aborted translation. This idea is not without precedent, as nuclear genes that

influence the translation of specific mitochondrial genes have been reported for several genes in yeast (rev. by Fox, 1986).

The loss of the 13 kD protein in the tissue culture derived fertile, toxin insensitive mutants identifies this protein as having an involvement in T-cms. The only mtDNA difference detected between one mutant, T4, and the mtDNA of its progenitor, T cytoplasm, appears to be a G to A transition and a five bp insertion in the urf13-T gene (Wise et al., 1987a). Transcription was unaltered in this mutant and the insertion created a premature stop in the translational reading frame, truncating the predicted gene product to 8.3 kD. If no other DNA regions or polypeptides are affected in this mutant, it would appear that the 13 kD protein is the causal agent of T-cms. However, this mutant was derived from and maintained in the nuclear background A188 that is dominant for Rf2 (rf1rf1;Rf2Rf2). Until it is demonstrated that this mutant, and the other tissue culture derived fertile, toxin insensitive mutants, maintain these characteristics in backgrounds that are recessive for both Rf genes, other factors besides the 13 kD protein may play a role in T-cms.

The novel DNA rearrangements in the urf13-T region of the T mitochondrial genome not only create the urf13-T gene but may have indirect effects on atp6 and ORF25 gene expression. Transcriptional studies (presented in Chapters III and IV) detected differences between atp6 transcripts in mtRNAs isolated from N and T cytoplasms, although these were unrelated to the presence or absence of dominant fertility restoring genes. Major differences were detected between ORF25 transcripts of N and T mtRNAs and among mtRNAs isolated from N cytoplasms from different nuclear backgrounds which appear to relate to

mtDNA sequences 5' and 3' to the ORF25 coding region. ORF25 is cotranscribed with urf13-T in T cytoplasm, whereas in N cytoplasm it is located in another part of the genome. An apparent influence of nuclear background was detected among ORF25 transcripts in mtRNA from T cytoplasm. A 1.1 kb transcript was detected in mtRNAs of two of five nuclear backgrounds examined and there is no evidence to date indicating that there are mtDNA differences between these nuclear backgrounds. The synthesis of this transcript was shown to be under nuclear control as it was detected in the progeny of a cross involving a line [A188-(T)] that did not have the transcript with a line [WF9-(T) restored] that did (Chapter III). The F1 hybrids were backcrossed to the original A188-(T) parental stock in order to follow the segregation of this characteristic. Ten individual plants of this back cross population were surveyed and all contained the 1.1 kb transcript. The failure for this characteristic to segregate within these progeny suggests that the synthesis of the 1.1 kb transcript is controlled by more than one locus. Regardless of the nuclear background, the synthesis of the 1.1 kb transcript was not influenced by the nuclear fertility restoration genes.

Transcriptional studies have yet to uncover any influence of the Rf2 gene on the transcription of atp6, urf13-T, or ORF25, at least within mtRNAs isolated from coleoptiles. If Rf2 were to be expressed only in anther tissue during microsporo- and/or microgametogenesis, its effect on mitochondrial gene expression would escape detection in the transcriptional studies described in this study. Although sensitivity to the fungal toxins occurs in all tissues of T cms plants, this does not rule out the possibility that other, stage- or tissue-specific,

events are involved in cms. There is evidence in a cms system of petunia that mitochondrial genes may be developmentally regulated in anther tissues. A chimeric mitochondrial gene, unique to a cms cytoplasm, was identified that apppears to be transcriptionally more active in anthers than in leaf tissues (Young and Hanson, 1987). However, the single nuclear restoration gene of this cms cytoplasm did not appear to have an effect on transcription of this gene at any developmental stage examined.

The tapetum is the layer of nutritive cells surrounding the developing microspores and is the first cytologically documented site in which abnormalities are detected in T-cms plants. A model has been proposed for T-cms pollen abortion that relates to the stressful conditions associated with tapetal cell development (Warmke and Lee, 1978). Tapetal cells undergo a dramatic increase in the number of mitochondria (increase 40-fold) during microsporogenesis, as well as doubling in ploidy (karyokinesis without cytokinesis). Consideration of these events in light of our current knowledge of the complex arrangement of the urf13-T region in the T mitochondrial genome, leads to several hypotheses concerning the initial event inducing premature tapetal breakdown and manifestation of T cms, and more specifically, the role of Rf2 in compensating for this event.

Increased mitochondrial gene expression during microsporo- and/or microgametogenesis could result in overproduction of the 13 kD protein (or perhaps the ORF25 gene product) that leads to a toxic condition in tapetal cell mitochondria. The effect of Rf2 gene may be similar to that of the Rf1 gene by reducing the amount of the 13 kD protein, or even by, preventing its overproduction. Alternatively, the duplication

of the atp6 transcription initiation regions in T mtDNA could lead to difficulties in adequately increasing the expression of this gene due to competition between repeated initiation sites for components of the transcriptional apparatus. This latter hypothesis could potentially be examined with the T-4 mutant. Since this tissue culture derived fertile, toxin insensitive mutant retains the 5 kb repeat that includes the atp6 transcription initiation sites, yet does not synthesize the 13 kD protein, it represents an ideal control for examination of the effect of the duplicated atp6 promoter.

Other possible roles for Rf2 include; 1) preventing the 13 kD protein from interacting with the inner mitochondrial membrane, 2) preventing an interaction of tapetal-specific gene products from interacting with the 13 kD protein or, 3) serving as a tapetal-specific product (expressed in the recessive state) that interacts with the 13 kD protein leading to mitochondrial dysfunction.

A characteristic feature of plant mitochondrial genomes is the prevalence of recombinational events. These events include intragenomic recombination between large regions that are involved in generating subgenomic circular forms of the master genomic circle as well as the recombination events involved in establishing these and other repeated regions. It is unclear whether plant mitochondrial genomes have a high rate of recombination relative to fungal and animal mitochondrial genomes or, alternatively, plant mitochondria are more capable of maintaining the products of recombinational events.

The existence of plant mitochondrial genomes as a set of subgenomic molecules is contingent on, and may have developed from, the duplication of mtDNA regions. There are no apparent sequence similarities between

mitochondrial repeated regions of unrelated plant species, suggesting that the duplications that created the repeats occurred independently (Stern and Palmer, 1984). The size of the repeated region in the maize mitochondrial genome does not appear to relate to its frequency of intragenomic recombination and not all large repeated regions are involved in intragenomic recombination (e.g. the maize 10 kb repeat; Lonsdale et al., 1984). Another characteristic of the large repeated regions of plant mitochondrial genomes is that they are frequently associated with genes (Stern and Palmer, 1984). This association may relate to the ability for these repeats to be maintained and subsequently become a fixed within plant mitochondrial genomes.

The duplication of the 5 kb region in the maize T mitochondrial genome has all the features of other large repeated mtDNA regions. It is recombinationally active (Wise et al., 1987a), creating a 42 kb subcircular molecule unique to T mtDNA (Fauron et al., 1987), and is associated with at least three mitochondrial genes. The most significant outcome of the duplication of the 5 kb region in the T mitochondrial genome was that it placed sequences involved in transcription initiation (and processing) upstream of two open reading frames (urf13-T and ORF25). This probably led to the expression of the urf13-T gene (and ORF25, although perhaps not for the first time) and presumably to T-cms. Recombination events that locate the promoter region of one gene upstream of the coding region of another have been detected for a number of yeast mitochondrial genes (Costanzo and Fox, 1986). In some cases, this places the chimeric gene under both transcriptional and translational regulation of the gene from which the 5' flanking region originated. There is little information regarding

the regulation of any plant mitochondrial genes, yet it is likely that both urf13-T and ORF25 would be subject to the same transcriptional regulation signals that might influence atp6 gene expression.

The expression of cytoplasmic male sterility in the T-cms system of maize is extremely stable under normal field conditions (no natural revertants have been reported). All the fertile, toxin insensitive mutants of T-cms have arisen from regeneration of immature embryos subjected to tissue culture. The mtDNAs of the fertile mutants that have been analysed all exhibit some rearrangements or alterations of the urf13-T DNA region. Most mutants have lost the urf13-T gene and part or all of the 5 kb repeat (Rottman et al., 1987; Fauron et al., 1987; Wise et al., 1987a).

The genetics of cytoplasmic organelles are quite different from that of nuclear genomes both in regard to inheritance and the affect of mutations. Mitochondria can contain approximately 10-50 copies of the mitochondrial genome and there may be between 50 and 500 mitochondria in an average individual cell, leading to approximately 10^2 to 10^4 mtDNA molecules per cell (Birky, 1983). It is difficult to explain how an individual mitochondrial recombination event that confers no selective advantage can be maintained and established within a cell lineage. The recovery of fertile, toxin-insensitive mutants of T cytoplasm at high frequencies (35 of 60 regenerates in one study; Brettell et al., 1980; rev. by Pring, 1987a) is almost incomprehensible, particularly since many were recovered without the selection pressure of T-toxin.

The events that established the urf13-T region of T cytoplasm in nature are even more difficult to envision. As many as four separate recombination events must have occurred in the urf13-T region (Dewey et

al., 1986 identified seven points of recombination; Pring et al., 1987a identifies one more) and these events appear to be temporally separate (see Discussion of Chapter IV). It is likely that the coding sequences of urf13-T were at one time latent and did not produce a gene product. Consequently, there would be no selective advantage conferred in their replication. The maintenance of such sequences is not uncommon in plant mitochondrial genomes (e.g. non-trancribed regions) and it has recently been demonstrated that alternative genomic constructions may be present in plant mitochondria at sub-stoichiometric levels, relative to the normal gene construction (Small et al., 1987). Although the "ploidy" level of mitochondrial genomes within a cell may permit the maintenance of these molecules, the amplification of these sub-stoichiometric molecules to a level in which they become established as the predominant form is not understood.

Plant mitochondria have the ability to maintain large genomes and cope with frequent recombination events as well as small DNA insertions and deletions apparently created through errors in replication. The process of plant mitochondrial gene expression also appears to be loosely controlled and not very efficient. In maize, the smallest transcript detected for at least five of the protein coding genes is over 400 nucleotides longer than the length of the coding regions. Most of these genes have multiple transcripts; in some cases the largest transcripts is up to eight times as large as the coding region (rev. by Eckenrode and Levings, 1986). The major transcript for the maize atp6 gene appears to be initiated approximately 2.5 to 3.0 kb upstream of the coding region and undergo at least one processing event (Chapter IV). There are at least three processing events associated with urf13-T/ORF25

transcripts, and two more depending on the nuclear background (Rf1 leads to a 1.6 kb transcript and WF9 or W64A backgrounds, *per se*, lead to a 1.1 kb transcript). Both of the nuclear controlled processing events and the event that leads to the 1.5 kb *urf13-T*/ORF25 apparently reduce, rather than produce, the mature transcripts. The role of these RNA processing events in gene expression is unclear as is the raison d'etre of the genes that direct these events. Another aspect of the process of transcript maturation in plant mitochondria is that most of the characterized processing events appear to be quite inefficient. The proposed primary transcripts for *atp6* and *urf13-T*/ORF25 are still detectable in relatively high abundance, even though they have been subjected to as many as five processing events. The processing event associated with the Rf1 gene appears to affect the *urf13-T*/ORF25 transcripts only slightly and the degree of processing is unaffected whether there are one or two dominant Rf1 alleles in the nucleus (no difference between homozygous dominant and heterozygous Rf1 plants; Chapter III). Although the nature of posttranscriptional events affecting the *urf13-T*/ORF25 transcripts may not be analogous to other maize mitochondrial genes, they are indicative of the complexity of transcription and the tolerance of maize mitochondria to what appears to be a very inefficient process of transcript maturation.

According to the endosymbiotic theory of the origin of mitochondria, plant and animal mitochondria developed from the same primitive eukaryotic cells. Comparison of plant and animal mitochondrial genomes and the manner in which mitochondrial genes are expressed reveals a few similarities (e.g. the genes that they encode) but many more differences. Assuming the endosymbiotic theory is

basically correct, plant and animal mitochondrial genomes have diverged in both gene organization and expression with animal mitochondria becoming highly efficient and plant mitochondria expanding in genome size (or remaining large), without an apparent requirement for efficiency.

Although disease susceptiblity confers no selective advantage to the plant, cms leads to outbreeding which increases genetic variability. Male sterility is certainly more important as a mechanism for outbreeding for species that primarily self-pollinate, than it is for plant species that have other mechanisms for outbreeding (such as spatial separation of reproductive parts or self-incompatibility). Although T cytoplasm has only been detected on a few occasions (Duvick, 1965) and subsequently increased by plant breeders, it may have been around for a long time prior to its discovery. The mitochondrial recombination events responsible for the genomic region unique to T mtDNA are an example of how recombination and maintenance of non-essential genomic constructions in the maize mitochondrial genome can conceivably lead to a novel gene and selectable traits.

The organization of the protein coding gene urf13-T, having nucleotide sequence similarity to two or more separate regions of the 26S rDNA mitochondrial gene, has led to the description of urf13-T as a scrap or junk gene. Yet, in the words of the biologist Francios Jacob, nature is an excellent tinkerer, not a divine artificer (Jacob, 1977). Perhaps more appropriate is a metaphor that Charles Darwin (1862) applied to describe the adaptations orchid petals have undergone from being conventional petals to forming tubes that help ensure cross-fertilization:

Although an organ may not have been originally formed for some special purpose, if it now serves this end we are justified in saying that it is specially contrived for it. On the same principle, if a man were to take a machine for some special purpose, but were to use old wheels, springs, and pulleys, only slightly altered, the whole machine, with all its parts, might be said to be specially contrived for that purpose. Thus throughout nature almost every part of each living being has probably served, in a slightly modified condition, for diverse purposes, and has acted in the living machinery of many ancient and distinct specific forms (Gould, 1980, p. 26).

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BIOGRAPHICAL SKETCH

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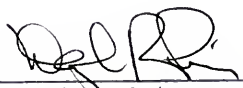

Daryl R. Pring, Chairman
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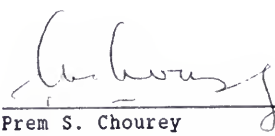

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This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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