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Maize mitochondria synthesize organ-specific polypeptides

(differentiation/mitochondrial protein synthesis/cytoplasmic male sterility)

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ABSTRACT **We detected both quantitative and qualitative organ-specific differences in the total protein composition of mitochondria of maize. Labeling of isolated mitochondria from each organ demonstrated that a few protein differences are due to changes in the polypeptides synthesized by the organelle. The synthesis of developmental stage-specific mitochondrial polypeptides was found in the scutella of developing and germinating kernels. The approximately 13-kDa polypeptide synthesized by mitochondria from seedlings of the Texas (T) male-sterile cytoplasm was shown to be constitutively expressed in all organs of line B37T tested. Methomyl, an insecticide known to inhibit the growth of T sterile plants, was shown to be an effective inhibitor of protein synthesis in mitochondria from T plants.**

Mitochondria are composed primarily of proteins synthesized in the cytoplasm and encoded by nuclear genes, but the mitochondria of animals, fungi, and plants all contain the genes for some components of the electron transport and ATP synthetase complexes (1). Although the coding capacity of mitochondria is quite limited in fungi and animals, the mitochondrial genomes of higher plants are considerably larger, ranging from 218 kilobases (kb) in *Brassica* (2) to 2500 kb in muskmelon (3), with the maize genome estimated to be approximately 570 kb (4). This larger genome of plants does encode at least one additional protein, the α subunit of the F_1 ATPase (5, 6), which is encoded in the nucleus of fungi and animals (1).

Additional coding functions in plant mitochondria could be required for differentiation of the organelle. Mitochondria vary considerably in morphology in various tissues of plants (7, 8) and clearly have a variety of physiological roles in green and nongreen plant cells (9). But, in studies to date, differences in mitochondrial morphology and function have been ascribed to changes in nuclearly coded gene products. By comparing the *in organello* synthesis products of maize mitochondria purified from various organs, we have found several examples of organ-specific and developmental stage-specific patterns of polypeptide synthesis.

Male sterility resulting from a failure to produce viable pollen is widespread among higher plants; in many cases the defect is maternally inherited, and the syndrome is classified as cytoplasmic male sterility (CMS) (10). Mitochondrial malfunction has been suggested as a general cause of CMS in plants (1). In maize there are three types of male-sterile cytoplasms—T, C, and S (11)—each distinguished from the fertile (N) plants by a unique digestion pattern of the mitochondrial DNA (12, 13). Although the precise cause of sterility is unknown, mitochondrial degeneration occurs in the tapetal cells of the anther during T pollen development (14) and in individual S pollen grains (15). In C and S there is no whole plant phenotype associated with CMS; however, T plants are highly susceptible to toxins produced by the fungal pathogen *Bipolaris maydis* (formerly *Helminthosporium maydis*) (16–18) and to methomyl, a carbamate insecticide (19). Furthermore, mitochondria from seedlings of the T plants synthesize a major low molecular weight polypeptide not found in the other cytoplasmic types (20). To explore the possibility that the detrimental whole plant phenotypes exhibited by T male-sterile plants are correlated with this polypeptide, we have determined the level of expression of the T polypeptide during plant development.

MATERIALS AND METHODS

Plant Material. Seed stocks of the inbred B37N and B37T maize lines were originally supplied as a gift from Pioneer Hi-Bred International and were propagated at Stanford. Kernels were germinated in the dark on vermiculite at 25°C for 5–7 days and the shoots and scutella were excised. Young cobs were removed from field-grown plants just as the silks emerged from the leaves surrounding the ear shoots. Tassels were removed from the interior of the stalk when meiosis was nearing completion in the anthers. Developing endosperm and scutellum samples were dissected from immature kernels.

Purification of Mitochondria. Mitochondria were prepared from plant tissue surface-sterilized in sodium hypochlorite and the initial steps of mitochondrial purification were as described by Kemble *et al.* (21). The pellet from the initial 1000 × *g* (5 min) centrifugation contains most of the chloroplasts and nuclei and was used as a crude plastid preparation for protein synthesis studies. Our subsequent procedures to purify mitochondria from the 1000 × *g* supernatant were similar to those of Leaver *et al.* (22), employing sucrose gradient purification of the mitochondria. The final mitochondrial pellets were used immediately for *in organello* protein synthesis.

***In Organello* Protein Labeling and Gel Electrophoresis.** Mitochondrial proteins were labeled *in organello* with [^{35}S]-methionine (>600 Ci/mmol, Amersham; 1 Ci = 37 GBq) for up to 60 min at 25°C in a sodium succinate/ADP/GTP energy mix (22). At the end of the labeling period, radioactive incorporation was measured (22) and the organelles were frozen at −80°C. The 1000 × *g* first pellets mentioned in the isolation procedure were also labeled by the same method. Various inhibitors were added to the reaction mix, as described in the text. All chemicals were obtained from Sigma with the exception of methomyl, a gift from DuPont.

For gel analyses, the mitochondria and first pellets were thawed and sonicated on ice in 50 μl of cold sodium carbonate/dithiothreitol (0.1 M each), and the proteins were precipitated with 10 vol of cold acetone on ice. Subsequent treatment of the protein pellets followed the procedures of Piccioni *et al.* (23). After the proteins had been pelleted in a Microfuge (10 min, 4°C), the pellets were dispersed by

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Abbreviations: CMS, cytoplasmic male sterility; kb, kilobase(s).

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sonication in 20–50 μl of the carbonate/dithiothreitol solution and then treated by one of two procedures. (*i*) Proteins were solubilized by heating for 1 min at 95°C with ⅓ vol of sucrose/$NaDodSO_4$/bromophenol blue (30%/5%/0.1%) solution and subjected to electrophoresis on 12–18% gradient polyacrylamide gels containing 8 M urea in the running gel (23). (*ii*) Alternatively, proteins were solubilized in the sample buffer of Laemmli and electrophoresed on 12–18% gradient polyacrylamide gels with no added urea (24). Subsequently, both types of gel were stained with Coomassie blue, fluorographed (25), and exposed to preflashed Kodak XAR-5 film at −80°C.

To test for reproducibility, at least three separate mitochondrial preparations from each of the different tissues were labeled and analyzed by gel electrophoresis and fluorography. Because the final mitochondrial pellet was sufficient for 4–6 reactions, not all inhibitor treatments were performed each time with each mitochondrial preparation. In a typical experiment, mitochondrial aliquots from each organ were labeled without an energy source and in the presence of cycloheximide, chloramphenicol, and at least one additional inhibitor and then analyzed on a single gel. In the inhibitor studies, equal amounts of the mitochondrial preparations were loaded in the gel lanes. When mitochondrial protein synthesis from different tissues was to be compared, the volumes loaded in each gel lane were adjusted to yield approximately equal amounts of radioactivity. Protein standards from Bio-Rad (phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme) were included as molecular weight markers on each gel. The positions of these markers are indicated in each figure.

RESULTS

Detection of Plastid Contamination and Inhibitor Studies. To discount differential contamination by plastids as contributing to the patterns of *in organello* mitochondrial protein synthesis, two types of controls were used: comparison of protein synthesis patterns in the plastid-enriched first pellet of the organelle purification protocol to those of purified mitochondria, and use of inhibitors in an attempt to specifically depress either plastid or mitochondrial protein synthesis. Lane 1 in Fig. 1 illustrates the protein synthesis pattern of the plastid-enriched first pellet isolated from young cob tissue without an added energy source. If incubated in the light, chloroplasts will incorporate label without an exogenous energy source. Some nongreen plastids, such as etioplasts, can synthesize proteins even in the absence of an exogenous energy source (26). However, highly purified mitochondria incorporate little [^{35}S]methionine in the absence of an energy-generating system (Table 1; Fig. 5, lane 1) but show a 10- to 30-fold increase in incorporation when an energy source is added. Consequently, the pattern of lane 1 represents only plastid protein synthesis. In lane 2, ADP and GTP were added to the labeling mixture for the first pellet, and additional polypeptides are synthesized that comigrate with authentic mitochondrial translation products. Therefore, if an energy source is added, mitochondria contaminating the first pellet will synthesize proteins. Because etioplasts incorporate more label per microgram of organelle protein than do mitochondria, even a low level of plastid contamination of certain mitochondrial preparations is detectable in a control without an energy source (Fig. 1, lane 5). All plastid profiles show two similarities that can be used to distinguish them from mitochondrial translation products: (*i*) high molecular weight proteins are synthesized and (*ii*) there is intense labeling of a band of protein of approximately 50 kDa.

To further distinguish plastid and mitochondrial translation products, various inhibitors of oxidative phosphorylation and electron transport were surveyed for their effects on [^{35}S]-methionine incorporation. Lane 7 in Fig. 1 shows the standard pattern of labeled polypeptides for mitochondria from cob tissue with cycloheximide added to inhibit synthesis on cytosolic ribosomes. Incorporation of label was not inhibited in the presence of 100 μM rotenone (lane 6). A mixture of carboxyatractyloside, mersalyl, and malonate (lane 3), or carboxyatractyloside alone (lane 4) reduced label incorporation somewhat at the concentrations used, and quantitative effects on the protein synthesis profiles were seen. Antimycin A (400 μM) was found to be as effective an inhibitor of mitochondrial protein synthesis as chloramphenicol was (Table 1). Although erythromycin (100–200 μM) is a potent inhibitor of plastid translation, it causes only a small reduction in the label incorporation into mitochondria (Table 1) and has little effect on the pattern of protein synthesis (Fig. 5, lane 2). This result confirms similar findings in tobacco (27).

Specific effects on the pattern of mitochondrial protein synthesis have been found in yeast treated with nalidixic acid, including a selective reduction in the synthesis of cytochrome oxidase subunit 1 and the var 1 ribosomal protein (28). In maize mitochondria, we also find that 400 μM nalidixic acid depresses [^{35}S]methionine incorporation and that certain polypeptides are more strongly inhibited than others (Fig. 1, lane 8).

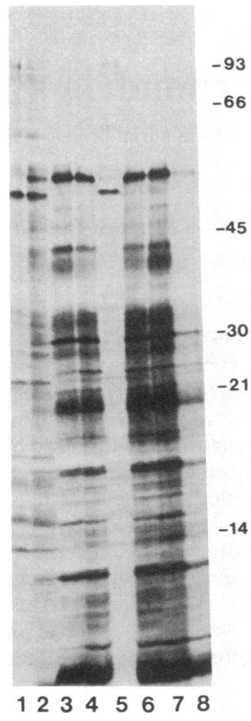

FIG. 1. Fluorograph showing controls for plastid protein synthesis and the effects of inhibitors on mitochondrial protein synthesis. All samples are from the same preparation of B37N cob tissue, with the exception of lane 5, which is from shoots. Approximately equal amounts of protein were loaded in each lane. Lane 1, proteins synthesized by plastid-enriched fraction (initial 1000 × *g* pellet) without exogenous ADP and GTP. Lane 2, same as 1 with the energy mix and 100 μM cycloheximide added. Lane 3, mitochondrial translation products in the presence of carboxyatractyloside, mersalyl, and malonate (100 μM each). Lane 4, mitochondria with 100 μM carboxyatractyloside only. Lane 5, shoot mitochondrial preparation contaminated with etioplasts, with no added ADP and GTP, to show plastid translation products. Lanes 6–8, mitochondrial translation products in the presence of inhibitors: 100 μM rotenone (lane 6), 100 μM cycloheximide (lane 7), or 400 μM nalidixic acid (lane 8). The 12–18% gradient polyacrylamide gel contained 8 M urea. The migrations of protein standards of known molecular mass are shown in kDa on the right side of the figure.

Table 1. Effects of inhibitors on [^{35}S]methionine incorporation into normal and cms-T mitochondria

	Incorporation, cpm × 10^{-5}						
	N shoots		T shoots		N cobs		
Inhibitor	No. 1	No. 2	No. 1	No. 2	No. 1	No. 2	No. 3
Minus energy	2.1	0.43	0.53	0.35	0.23	0.77	2.04
Cycloheximide	16.3	10.11	16.90	9.88	10.33	12.51	28.21
Methomyl	8.8	5.63	2.49	1.60	—	—	—
Chloramphenicol	2.7	1.50	2.32	—	—	1.80	—
Antimycin A	—	—	2.78	—	0.89	—	—
Erythromycin	—	—	—	6.49	—	—	20.29
Streptomycin	—	—	—	—	—	—	27.52

The concentrations of inhibitors used are given in the text. Incorporation was measured in 250-μl reaction mixtures. Numbers refer to individual mitochondrial preparations, from differing amounts of starting material.

These investigations of the properties of the *in organello* translation assay of maize mitochondrial protein synthesis emphasize the requirement for appropriate controls. An assay for contaminating plastid ribosomes active in protein synthesis was included in our surveys of mitochondria from various organs by testing the preparations for energy-independent protein synthesis.

Differences in Protein Staining and Protein Synthesis Patterns. Variation in the protein composition of mitochondrial preparations from different tissues can be seen after Coomassie blue staining of electrophoretically separated polypeptides. In Fig. 2*A*, mitochondrial polypeptides from dark-grown seedling shoots of B37N are compared with those from unfertilized cobs from the same inbred line. In Fig. 2*B* mitochondrial polypeptides from unfertilized cobs, developing endosperm, and scutellum are compared. There are striking differences in the protein profiles of mitochondria from different tissues. These differences may reflect both alterations in the nucleus-encoded components of mitochondria of different tissue types and contamination of the mitochondrial preparations with different subcellular components that purify with the mitochondria. The mitochondrially synthesized polypeptides are in low abundance and are not easily detectable by protein staining with Coomassie blue.

In Fig. 3*A*, the mitochondrial translation products of shoots and cobs are compared. Unlike the major stained polypeptides in Fig. 2*A*, the labeled polypeptides from shoots and cobs are quite similar. There is one 28-kDa polypeptide made in shoots that is not present at discernible levels in unfertil-

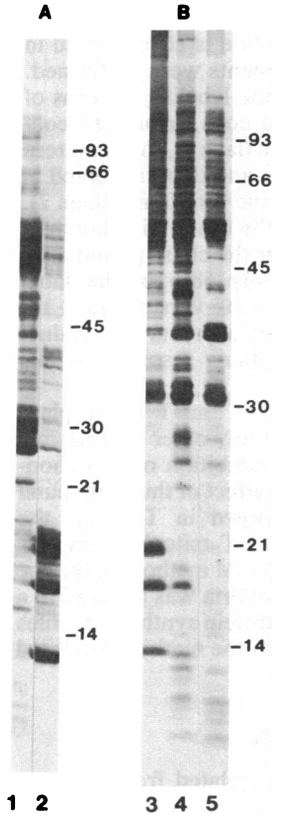

FIG. 2. Proteins present in mitochondrial pellets from different organs. Coomassie blue stained polypeptides separated on 12–18% gradient Laemmli polyacrylamide gels that either included 8 M urea in the resolving gel (*A*) or lacked urea (*B*). Lane 1, mitochondria isolated from B37N seedling shoots. Lane 2, mitochondria from B37N unfertilized cob. Lane 3, mitochondria from B37T unfertilized cob. Lane 4, mitochondria from B37T milky-stage endosperm (17 days after pollination). Lane 5, mitochondria from B37T developing kernel scutella, 23 days after pollination.

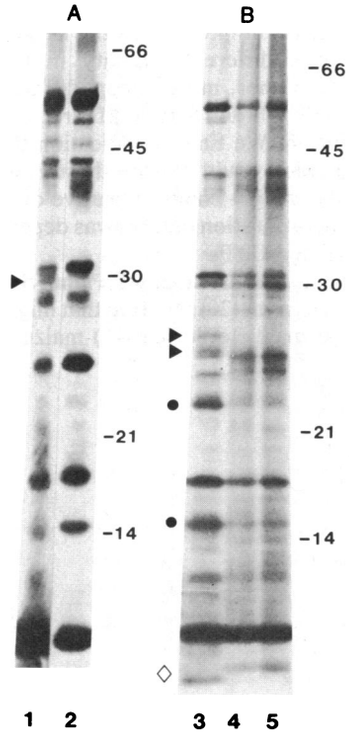

FIG. 3. Protein synthesis in mitochondria isolated from different organs: Fluorographs of [^{35}S]methionine-labeled polypeptides separated on 12–18% gradient PAGE in the absence of urea. Lanes 1 and 2 were from one gel (*A*) and lanes 3–5 were from a second gel (*B*). Lane 1, from N seedling shoots; lane 2, from N unfertilized cobs; lane 3, from N developing kernel scutella, approximately 26 days after pollination (field-grown plants); lanes 4 and 5, N tassels prior to emergence from the stalk. All samples were labeled in the presence of cycloheximide to inhibit synthesis from cytosolic ribosomes except for lane 5, where 100 μM erythromycin was substituted. The arrowheads indicate major qualitative differences and the circles, strong quantitative differences in the protein profiles. The open diamond indicates a band difference in the very low molecular weight region.

ized cobs. When other tissues are compared, additional differences in mitochondrial translation products are apparent. In Fig. 3*B*, developing kernel scutella (lane 3) have additional polypeptides relative to tassel (lanes 4 and 5). These qualitative differences are indicated by arrowheads. Large quantitative differences are also observed and are indicated by circles. A band difference in the lower portion of the gel is indicated by an open diamond.

Differences in Mitochondrial Translation Products in One Organ at Different Stages of Development. To test whether or not mitochondria from a single organ can synthesize some stage-specific polypeptides when assayed at different developmental stages, we compared mitochondrial translation products from scutella of maturing kernels to those from scutella of germinated seedlings. Scutella were excised from developing ears of greenhouse-grown plants at 22–23 days after pollination (at the end of the milky endosperm stage) and from maturing kernels at 24–25 days after pollination. Analysis of the *in organello* labeling patterns by gel electrophoresis (Fig. 4) showed both quantitative and qualitative differences. A heavily labeled 22-kDa mitochondrial polypeptide (arrowhead in Fig. 4) in the maturing kernel scutellar preparations is not detectable as a labeled band in the sample from germinated scutella. A 24-kDa polypeptide (upper dot) is only faintly detectable in the scutella 22–23 days after pollination (lane 1) but is much more apparent in the 24- to 25-day sample (lane 2) and represents a major translation product from the germinated scutella mitochondria (lane 3). A polypeptide in the low molecular weight region of the gel (lower dot) decreases in intensity as the kernel matures and appears to have a different mobility in the germinated scutellum sample. Other, more minor, differences, particularly in the 21- to 30-kDa and 8- to 10-kDa regions, can be seen by inspecting Fig. 4. We find no indication that differential plastid contamination could explain these results: there are no high molecular weight bands indicative of plastid protein synthesis, and incorporation of label was dependent upon the addition of ADP and GTP.

All Organs Examined from cms-T Plants Synthesize the T Polypeptide. Forde *et al.* (20) reported that mitochondria from shoots of T-type male-sterile (cms-T) maize seedlings synthesize a major polypeptide of approximately 13 kDa; this polypeptide is not made by mitochondria from normal or other kinds of male-sterile plants. We have found that it is also synthesized at high levels in cms-T mitochondria from tassels, cobs, milky-stage endosperm, and maturing kernel scutella. Our results with unfertilized cobs are shown in Fig. 5. Thus, the T-polypeptide appears to be synthesized constitutively in cms-T plants. In our gel system, the T polypeptide migrates as an approximately 15-kDa polypeptide. This discrepancy from the molecular mass originally reported (20) reflects the use of different standards for sizing the polypeptides. No other protein in this size range distinguishes T mitochondria from N, C, and S (20).

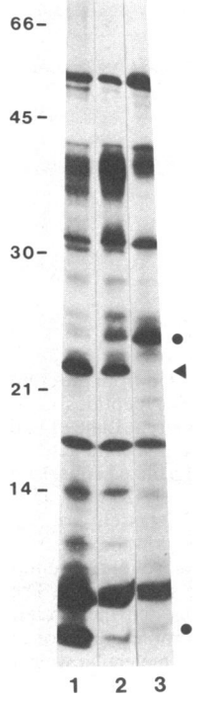

FIG. 4. Protein synthesis in scutellar mitochondria from different developmental stages in B37N: Fluorograph of labeled polypeptides separated on 12–18% PAGE without urea. Lane 1, mitochondria from maturing kernel scutella, 22–23 days after pollination (greenhouse grown). Lane 2, mitochondria from maturing kernel scutella, 24–25 days after pollination. Lane 3, mitochondria from scutella of 5-day germinated seedlings. The arrowhead indicates a major qualitative change and the solid circles, strong quantitative changes.

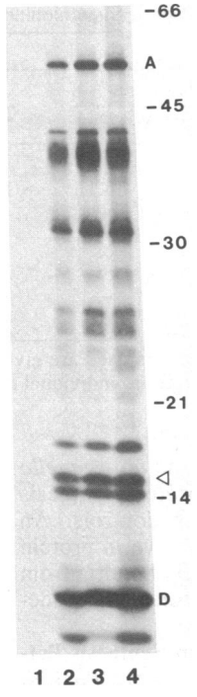

FIG. 5. Synthesis of the T polypeptide by mitochondria isolated from B37T cobs: Fluorograph of labeled polypeptides separated by 12–18% PAGE with no urea. The migration of the T polypeptide is marked by an open arrowhead. All samples were from the same mitochondrial preparation. Lane 1, no exogenous energy source (ADP and GTP were lacking). Lane 2, 30 min of labeling with [^{35}S]methionine followed by a 30-min chase with unlabeled methionine in the presence of cycloheximide. Lane 3, 1-hr labeling with [^{35}S]methionine in the presence of erythromycin. Lane 4, 1-hr labeling with [^{35}S]methionine in the presence of cycloheximide. Matching these patterns to the published gels of Hack and Leaver (6), we have indicated the α subunit of the F_1 ATPase (A) and the dicyclohexylcarbodiimide-binding protein (D).

To demonstrate that the T polypeptide is accumulated in each tissue type, pulse–chase experiments were performed. For example, in Fig. 5 we compare the labeling patterns of mitochondria from B37T unfertilized cobs, incubated continuously in [^{35}S]methionine for 60 min (lane 4), to the pattern from mitochondria labeled for 30 min and then incubated in an excess of nonradioactive methionine for 30 min (lane 2). The T polypeptide, as well as all of the other mitochondrial translational products, is present after the chase period. The pulse–chase sample incorporated only 58% of the label compared to the control sample (1.8×10^6 versus 3.15×10^6 cpm). Thus, there is no evidence for a precursor–product relationship for any of the polypeptides resolved in this experiment.

To determine whether the killing of cms-T maize plant tissue by methomyl, but not tissue of any other cytoplasmic type, could result from differential inhibition of mitochondrial protein synthesis, we tested the effect of this compound on *in organello* translation. As shown in Table 1, the incorporation of [^{35}S]methionine into T mitochondria was strongly inhibited in the presence of 3 mM methomyl, but the incorporation of label into N mitochondria was affected to a much lesser extent. In addition, the protein synthesis profiles of the N mitochondria were similar in the methomyl-treated and control samples (not shown).

DISCUSSION

We found that maize mitochondria isolated from different organs and from a single organ at three stages of development

exhibit both qualitative and quantitative differences in the patterns of polypeptides they synthesize. We see no evidence that differential plastid contamination could account for the organ-to-organ variation in mitochondrial translation products. Pulse–chase experiments suggest that processing intermediates are not detectable as strongly labeled bands in maize mitochondria. Thus, we propose, although it is not yet proven, that the differentially synthesized polypeptides do represent different translation products.

The role and origin of the differentially expressed mitochondrial translation products are as yet unknown. There are several possibilities: (*i*) Plant mitochondria might have more genes than do mitochondria from other organisms. Some of these genes might be transcribed and translated differentially during development, or in response to altered cellular milieu or environmental stimuli. (*ii*) Plant mitochondrial genes might be differentially regulated at the level of RNA initiation, termination, or processing, such that related polypeptides of different sizes are synthesized in mitochondria of different cell types. (*iii*) There may be multiple molecular forms of mitochondrially synthesized proteins, tissue- or stage-specific isozymes, with differing gel electrophoretic mobilities. Alternatively, processing or modification in a tissue- or stage-specific manner of the same gene product could give rise to differentially migrating polypeptides. Whether or not any of the differentially expressed mitochondrial polypeptides are related to each other requires the use of tools not yet available, such as individual antisera to all mitochondrially synthesized polypeptides from a single plant tissue. Any processing step could ultimately be a reflection of the differential activity of nuclear genes, because it is probable that the state of differentiation of the organelle is regulated by nuclear gene products.

The T polypeptide, approximately 15 kDa by our estimates, is an enigma. The mutant phenotype of T-type CMS is associated with the *production* of this polypeptide, not with the absence of a normal mitochondrial polypeptide. The T polypeptide appears to be expressed constitutively at high levels, and yet its origin and its relationship to other mitochondrially synthesized polypeptides is unknown. One hypothesis is that it represents a prematurely terminated translation product (1). However, in our analyses, the rest of the mitochondrial protein synthesis profile is essentially unaffected. Perhaps the T polypeptide is a truly neomorphic character, arising through rearrangements that created a new open reading frame and/or that placed a strong promoter near an open reading frame. Nuclear genes that restore fertility to cms-T plants suppress the synthesis of the T polypeptide, but its production is not completely inhibited (29). Further investigations will be required to establish the role of the T polypeptide in pollen development and to determine whether this protein conditions the sensitivity of cms-T mitochondria to methomyl and *B. maydis* toxin.

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