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Maize nuclear background regulates the synthesis of a 22-kDa polypeptide in *Zea luxurians* mitochondria

(nuclear–cytoplasmic incompatibility/mitochondrial protein synthesis/teosinte/*Zea mays*)

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ABSTRACT **When cytoplasm of the teosinte *Zea luxurians* is introduced into certain maize inbred nuclear backgrounds, the pattern of protein synthesis in the teosinte mitochondria is altered. Teosinte mitochondria purified from plants possessing a maize A619 nuclear background (*Z.l.*–A619 plants) synthesize a novel 22-kDa polypeptide that is associated exclusively with the membrane fraction of the organelle. Mitochondria from plants possessing a W23 nuclear background do not synthesize this protein. The F_1 hybrids *Z.l.*–A619 × W23 and *Z.l.*–W23 × A619 do not synthesize the protein. However, synthesis of the polypeptide was observed in 14 of 21 individual progeny from the backcross of the F_1 hybrid *Z.l.*–A619 × W23 to the pollen parent A619. These data suggest that a single nuclear gene controls the synthesis of the 22-kDa protein in mitochondria, with the recessive allele of the gene allowing expression of the polypeptide. Mitochondria from the F_1 hybrid *Z.l.*–A619 × Mo17 synthesize the 22-kDa protein, whereas mitochondria from *Z.l.*–A619 × B73 do not. Data from these outcrosses demonstrate that other maize lines also possess nuclear genes capable of regulating the synthesis of the 22-kDa *Zea luxurians* mitochondrial protein.**

Normal plant growth and development depend on properly functioning mitochondria and chloroplasts. The biogenesis and activity of both organelles require cooperativity between the cytoplasmic and nuclear genomes. For example, the enzymes of the inner mitochondrial membrane are composed of subunits encoded by both the mitochondrial and nuclear genomes, and these subunits must be able to interact correctly to give functional holoenzyme complexes. In addition, nuclear gene products may directly influence mitochondrial gene expression by regulating transcription or translation. If two genotypes that have evolved under different sets of selective pressures are combined, as would be the case in an interspecific cross, noncooperating combinations of nuclear and organellar gene products may arise. Such nuclear–cytoplasmic incompatibilities are usually recognized as altered phenotypes, the most common being cytoplasmic male sterility (CMS; ref. 1). CMS, or the failure to shed functional pollen, is generally considered to result from disturbances in nuclear–mitochondrial interactions (2–5).

Kermicle and coworkers (6, 7) have described a constitutive incompatibility between nuclear genes of some maize inbred lines and the cytoplasmic genomes of teosintes from section Luxuriantes. The maize inbred lines W23 and A619 were used as the male parents in recurrent backcrosses with *Zea perennis*, *Zea diploperennis*, or *Zea luxurians* (*Z.l.*), race Guatemala. After several generations of backcrossing, the nuclear genome becomes essentially that of the maize inbred line, while the maternally inherited cytoplasmic genomes remain those from the original teosinte. When the maize inbred line W23 carries the cytoplasmic genomes from any of these three teosintes, small kernels result that give rise to small, late-developing, but male-fertile plants. When the *Z. perennis* or *Z. diploperennis* cytoplasm is present with the maize inbred A619 nuclear genome, normal kernel and plant development occur, but the plants are male-sterile. On the other hand, *Z.l.* cytoplasm in combination with the A619 nuclear genome results in male-fertile plants, although the amount of pollen shed is reduced (K.J.N., unpublished observations). The reciprocal crosses do not result in small kernels or male sterility; the phenotypes are observed only if the cytoplasm is contributed by the teosinte.

We have begun to identify molecular alterations that result from the combination of teosinte cytoplasmic genomes and the nuclear genomes from different maize inbred lines. Here we report that when the cytoplasm from *Z.l.* is present with the nuclear genotype of the maize A619 line, the *Z.l.* mitochondria synthesize a distinctive 22-kDa polypeptide. The synthesis of this polypeptide is not observed when the *Z.l.* mitochondria are present in the maize W23 nuclear background. Maize mitochondria present with their own nuclear genomes also do not synthesize the protein. The synthesis of the polypeptide can be suppressed by outcrosses to other maize inbred lines, and genetic analyses indicate that the synthesis of this mitochondrial polypeptide is controlled by a single recessive nuclear gene.

MATERIALS AND METHODS

Plant Material. Teosinte [*Zea luxurians* (Wilkes collection 51186)] plants were successively backcrossed with maize inbred lines A619 or W23. Plants were analyzed when the teosinte–maize hybrids had been backcrossed at least six to nine generations with the respective maize inbred lines. These materials were generously provided to us by Jerry Kermicle (University of Wisconsin). Other maize inbred lines used for outcrosses were from standard seed stocks maintained and propagated at the University of Missouri. Young ear shoots used for the preparation of mitochondria were removed from field- or greenhouse-grown material when the silks had just emerged from the husk leaves surrounding the ear shoots. Mitrochondria were prepared on the same day the ear shoots were harvested.

Purification of Mitochondria. Mitochondria were prepared from ear shoots surface-sterilized in sodium hypochlorite. Homogenized extracts were centrifuged sequentially at 1000 × *g* (5 min) and 2000 × *g* (10 min). Centrifugation of the 2000 × *g* supernatant at 10,000 × *g* for 15 min yielded a crude mitochondrial pellet (8). The mitochondria were resuspended and purified from the 35–47% interface of a sucrose step gradient prepared as described by Leaver *et al.* (9). The final mitochondrial pellets were used immediately for *in organello* protein synthesis.

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Abbreviation: *Z.l.*, *Zea luxurians*.

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***In Organello* Protein Synthesis and Pulse–chase Analysis.** Mitochondrial proteins were labeled *in organello* with [^{35}S]methionine (>1000 Ci/mmol, NEN; 1 Ci = 37 GBq) for 90 min at 25°C in a sodium succinate/ADP/GTP energy mix (10). The inhibitor cycloheximide, erythromycin, or streptomycin was present at a concentration of 40 μg/ml.

For pulse–chase experiments, mitochondria were labeled as above for 1 hr; then the incubation media were made 1 mM in unlabeled L-methionine and 100 μg/ml in chloramphenicol to prevent further incorporation of radioactivity into protein. The samples were chased for up to 90 min.

Radioactive incorporation into protein was estimated (11), and following the incubation period, mitochondria were pelleted, quick-frozen on dry ice, and stored at −80°C. For electrophoresis, boiling Laemmli sample buffer (12) was added to still-frozen pellets. The pellets were boiled for 5 min and then were sonicated into solution.

Submitochondrial Fractionation. To separate the matrix and membranes of the mitochondria used in pulse–chase experiments, a procedure based upon that of Day *et al.* (13) was employed. Mitochondria were resuspended and sonicated (3 × 30 sec, 75 W, sonicator model W375, Heat Systems/Ultrasonics) in a buffer containing 0.3 M mannitol, 10 mM EDTA, and 10 mM sodium phosphate (pH 7.2). The sonicates were centrifuged 5 min in a microcentrifuge to remove remaining intact organelles, and the supernatants were centrifuged for 30 min at 167,000 × *g* in a Beckman TLA 100.2 rotor. The supernatants were recentrifuged, so that the final supernatants contained membrane-free matrix proteins. The membrane pellets were resuspended in 25 mM Mops/KOH buffer (pH 7.5) and also recentrifuged. The final membrane pellet was solubilized by sonication in hot Laemmli sample buffer. Matrix proteins were concentrated by precipitation with 10% (wt/vol) trichloroacetic acid and then solubilized in Laemmli sample buffer.

Gel Electrophoresis of Proteins. Mitochondrial proteins were separated in $NaDodSO_4$ gels with a linear polyacrylamide gradient of 12–18%, using the buffer system of Laemmli (12). For synthesis studies, equal amounts of trichloroacetic acid-precipitable radioactivity were loaded onto gel lanes. For mitochondrial fractionations, equal amounts of protein were loaded onto lanes of some gels. For other gels, equal amounts of total mitochondrial protein were loaded (usually 20 μg per lane) and an amount of membrane or matrix protein equal to the percentage of total mitochondrial protein in the particular subfraction was loaded onto lanes. Membrane fractions routinely contained about 80% of the total mitochondrial protein, with the remainder in the matrix fraction, regardless of the length of the chase period. Thus 16 μg of mitochondrial membrane protein was loaded in the "membrane" lanes of the gel and 4 μg of matrix protein was loaded in the "matrix" lanes. Protein content was determined by the method of Peterson (14).

Prior to drying, gels were stained with Coomassie blue and impregnated with 2,5-diphenyloxazole (15). Radioactively labeled proteins were detected by placing the gels against Kodak XRP-5 film at −80°C. Estimation of molecular weights of mitochondrial proteins was based on Bio-Rad low molecular weight standards run alongside the samples.

RESULTS

Effect of Maize Nuclear Background on Teosinte Mitochondrial Protein Synthesis. Maize nuclear background had a distinct influence on the pattern of protein synthesis in *Z.l.* mitochondria (Fig. 1). A polypeptide of 22 kDa was synthesized by the mitochondria when the *Z.l.* cytoplasm was in the maize A619 nuclear background (*Z.l.*–A619, Fig. 1, lane 2), but not when the cytoplasm was in the W23 background (*Z.l.*–W23, lane 3). This protein was not synthesized by mitochondria from the maize A619 or W23 lines themselves (lanes 1 and 4).

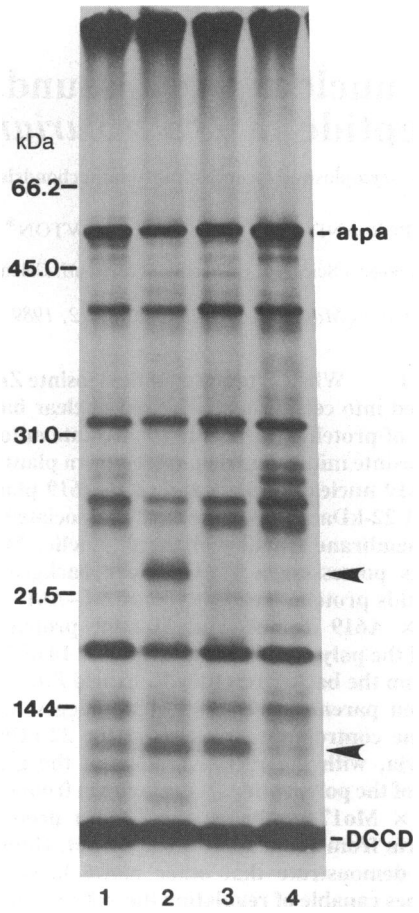

FIG. 1. Effect of maize nuclear background on mitochondrial protein synthesis. Proteins synthesized by purified maize or *Z.l.* mitochondria in the presence of erythromycin were separated in $NaDodSO_4$/polyacrylamide (12–18% linear gradient) gels and visualized by fluorography. Lanes: 1, mitochondria from maize inbred line A619; 2, *Z.l.* mitochondria in a maize inbred A619 nuclear background; 3, *Z.l.* mitochondria in a maize inbred W23 nuclear background; 4, mitochondria from maize inbred line W23. Lanes contained equivalent amounts of radioactivity. Arrowheads indicate 12- and 22-kDa polypeptides synthesized by *Z.l.* mitochondria. Position of size standards are indicated on the left, and those of the ATPase α subunit (atpa) and *N,N'*-dicyclohexylcarbodiimide (DCCD)-binding proteins on the right.

Nuclear background did not generally affect the pattern of *Z.l.* mitochondrial protein synthesis. The overall pattern of synthesis was similar to that of maize. A polypeptide of 12 kDa did exhibit enhanced synthesis in the *Z.l.* mitochondria as compared to maize. But unlike the 22-kDa polypeptide, the synthesis of the 12-kDa polypeptide was not influenced by nuclear background. The 12-kDa protein was synthesized by *Z.l.* mitochondria in the presence of either A619 or W23 nuclear genes (Fig. 1, lanes 2 and 3).

To further test the influence of the maize nuclear genome on the synthesis of the 22-kDa protein in teosinte mitochondria, *Z.l.*–A619 plants were crossed as females with the maize inbred strains W23, B73, and Mo17, and mitochondrial protein synthesis was monitored in the progeny (Fig. 2). Outcrosses to W23 and B73 resulted in the loss of synthesis of the polypeptide in the F_1 progeny plants (Fig. 2, lanes 2 and 3), whereas synthesis of the 22-kDa polypeptide was maintained in progeny from an outcross to Mo17 (lane 4). When *Z.l.* cytoplasm in a W23 background (*Z.l.*–W23, Fig. 1, lane 3) was outcrossed to A619, the synthesis of the polypeptide

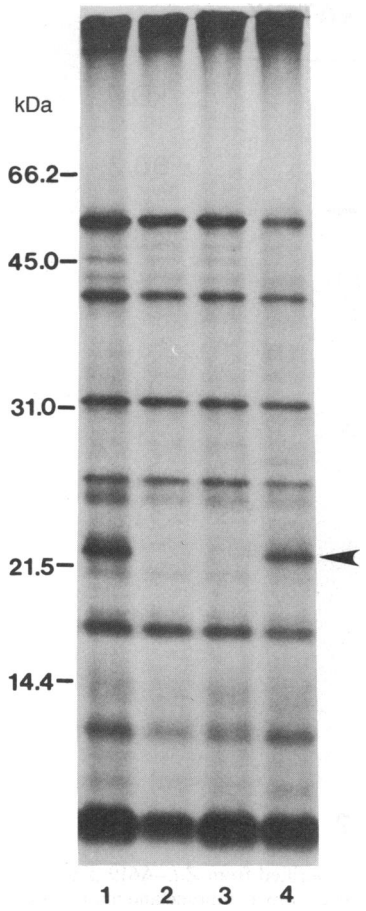

FIG. 2. Effect of outcrossing on the synthesis of the 22-kDa *Z.l.* mitochondrial protein. *In organello* protein synthesis was conducted using mitochondria from *Z.l.* in a maize A619 nuclear background (*Z.l.*–A619; lane 1), *Z.l.*–A619 × W23 (lane 2), *Z.l.*–A619 × B73 (lane 3), or *Z.l.*–A619 × Mo17 (lane 4). The gel was prepared and loaded as in Fig. 1. Positions of size standards are indicated on the left. Arrowhead identifies the 22-kDa protein.

was not observed in the progeny (data not shown). We have also scored for synthesis of the polypeptide in individual progeny from a backcross of the F_1 hybrid *Z.l.*–A619 × W23 to the parent A619. We found that protein synthesis was recovered in 14 and 21 individuals tested.

Submitochondrial Localization of the 22-kDa Polypeptide. To determine the submitochondrial location of the 22-kDa polypeptide, mitochondria from *Z.l.*–A619 plants were separated into their component membrane and matrix fractions after labeling. A pulse–chase labeling protocol was followed to evaluate the stability of the polypeptide relative to other proteins synthesized by the mitochondria.

Like most of the proteins synthesized by the mitochondria, the 22-kDa polypeptide was found in the membrane fraction (Fig. 3). Only two polypeptides, the α subunit of the ATPase and a protein of 40 kDa, were present in significant amounts in the matrix fractions (Fig. 3). Both proteins have previously been identified as "soluble" mitochondrial components (9, 16). The α subunit was partially released from its association with the mitochondrial membrane by the high concentration of EDTA present during sonication. It has been speculated that the 40-kDa protein is analogous to the soluble var1 ribosomal protein of yeast (16). The 22-kDa polypeptide was not detectable in the matrix fractions, even when the x-ray film was heavily overexposed or equivalent amounts of protein were loaded onto all lanes of the gel (data not shown).

The 22-kDa polypeptide was as stable as the other mitochondrially synthesized proteins resolvable by this gel system. The level of the proteins remained largely constant throughout the 90-min chase period (Fig. 3, "total" lanes). There was a gradual decline in the level of one membrane-associated polypeptide of 32 kDa, with the greatest loss occurring in the first 30 min of the chase (Fig. 3, total and membrane lanes 2). Two new polypeptides of 31 and 21 kDa became visible during the chase. Disruption of the mitochondria accelerated their appearance, as they were present at 0 min of chase (membrane lane 1) but only became visible in intact mitochondria after a 30- to 60-min chase (total lanes 2 and 3). They may be degradation products of the 32-kDa protein or of larger mitochondrial proteins that were unable to enter the gel.

DISCUSSION

When *Z.l.* cytoplasm is placed in combination with certain maize nuclear backgrounds, the mitochondria exhibit altered patterns of protein synthesis. When in the maize inbred A619 nuclear background, *Z.l.* mitochondria synthesize a 22-kDa polypeptide, but in the W23 background they do not. The effect is not limited to the Wilkes collection of *Z.l.*, since modulation of the synthesis of the 22-kDa polypeptide by the W23 and A619 backgrounds (see Fig. 1) was also observed when Iltis collection G-36 was the source of cytoplasm (data not shown).

The functional identity of the 22-kDa protein is unknown, although submitochondrial localization of the protein showed it to be associated exclusively with the membrane fraction of the mitochondria. The synthesis of a novel polypeptide by maize plants possessing a particular nuclear–cytoplasmic constitution is reminiscent of the synthesis of the 13-kDa polypeptide by cms-T maize (17, 18). However, unlike cms-T maize, no correlation exists between the presence of the 22-kDa protein and male sterility in these interspecific hybrids. Although *Z.l.*–A619 plants shed less pollen than do *Z.l.*–W23 or inbred A619 plants with their original N cytoplasms, the pollen that is shed is functional.

The absence of the protein in the F_1 hybrids *Z.l.*–A619 × W23 and *Z.l.*–W23 × A619 suggests that a recessive nuclear gene or genes permit the synthesis of the 22-kDa mitochondrial polypeptide. If a single nuclear gene controls the expression of the protein, then in a backcross of the F_1 hybrid to the parent inbred A619, a 1:1 ratio of expression is expected, whereas if two nuclear genes are involved, a 3:1 ratio in favor of the absence of protein synthesis is expected. We found that 14 of 21 individuals from such a backcross synthesized the polypeptide. This ratio is statistically most consistent with the control of the protein's synthesis by only one nuclear gene. For this ratio, we obtained a value of $P(x) = 0.055$. [A binomial exact test was performed on the data: $P(x) = {}_{21}C_{14}\, p^{14} q^{21-14}$, where $p = q = 0.5$.] Thus, at a 0.050 level of significance, the involvement of other factors which skew the ratio in favor of protein synthesis must be considered. One possible explanation is the inviability of some gametes that happen to carry the dominant allele that suppresses 22-kDa protein synthesis.

Because we do not know whether *Z.l.* mitochondria synthesize the 22-kDa protein when present in their own nuclear background, we cannot determine whether the synthesis of the polypeptide represents the compatible or incompatible situation. Nevertheless, the modulation of the synthesis of the polypeptide defines a nuclear factor that controls synthesis of a mitochondrial protein. If this factor acts directly on the mitochondrion rather than influencing yet another nuclear gene, it could act at one of several levels of gene expression. The synthesis of the protein could come about by the fortuitous production of an open reading frame through rearrangement of the mitochondrial genome such as has occurred in cms-T maize (18). However, comparison of the

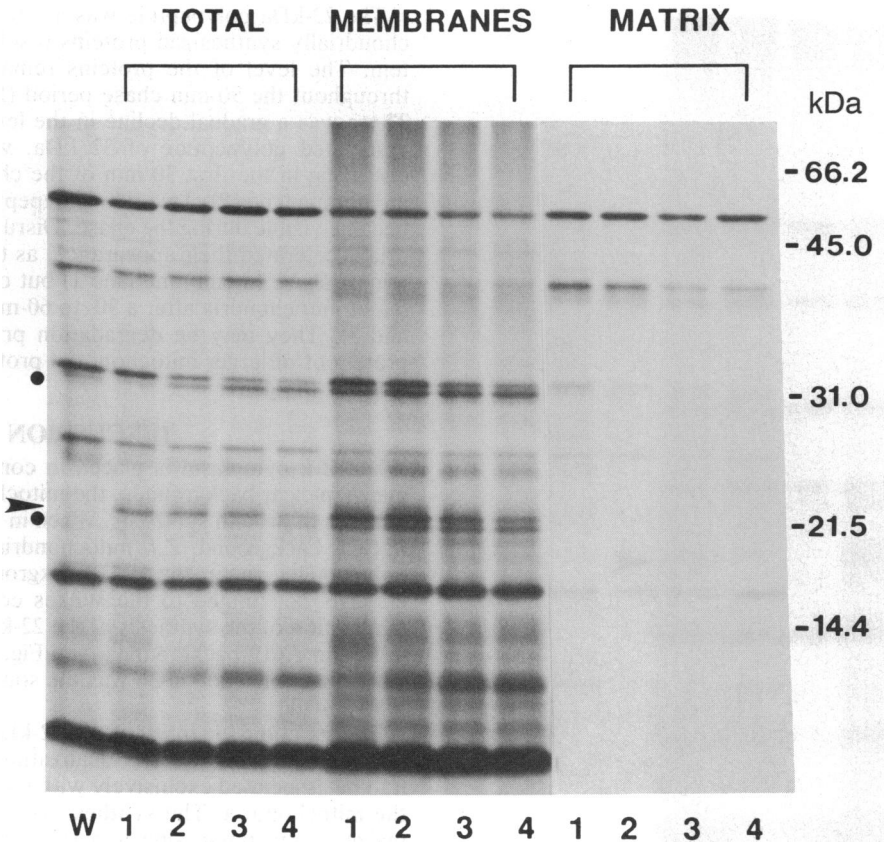

FIG. 3. Intramitochondrial localization of the 22-kDa *Z.l.* polypeptide. Mitochondria were purified from *Z.l.*–A619 plants and incubated with [^{35}S]methionine for 1 hr prior to a chase period of 0–90 min. Mitochondria were then subfractionated into membrane and matrix components. Proteins from the total unfractionated mitochondria, the membrane, and matrix were separated in gels as in Fig. 1, and the newly synthesized proteins were visualized by fluorography. Total mitochondrial protein loaded was 20 μg. Membrane and matrix lanes contained 16 and 4 μg, respectively. Lanes: 1, 0-min chase; 2, 30-min chase; 3, 60-min chase; 4, 90-min chase. Lane W shows total mitochondrial proteins synthesized by *Z.l.* mitochondria when in a maize W23 nuclear background. Positions of size standards are indicated at the right. Arrowhead indicates the 22-kDa protein. •, Proteins that appear during the chase or sonication. The matrix lanes were exposed 3 times as long as the total or membrane lanes.

restriction digestion patterns of mitochondrial DNA revealed no differences between the two nuclear backgrounds. This was true for nine different restriction endonucleases (data not shown). Thus the maize nuclear background appears to have no effect on *Z.l.* mitochondrial genome organization.

The nuclear gene product could be acting at the level of transcription. However, in yeast, transcriptional factors have yet to be identified among the numerous nuclear petite (*pet*) mutants in existence (19). In contrast, several *pet* mutations have been found to define nuclear gene products that regulate the translation of messages encoded by specific mitochondrial genes (20–22). It may be that the nuclear gene product that regulates synthesis of the 22-kDa protein in the teosinte–maize system acts at the level of translation.

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