A 13-Kilodalton Maize Mitochondrial Protein in *E. coli* Confers Sensitivity to *Bipolaris maydis* Toxin

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The Texas male-sterile cytoplasm (*cms-T*) of maize carries the cytoplasmically inherited trait of male sterility. Mitochondria isolated from *cms-T* maize are specifically sensitive to a toxin (BmT-toxin) produced by the fungal pathogen *Bipolaris maydis*, race T, and the carbamate insecticide methomyl. A mitochondrial gene unique to *cms-T* maize, which produces a 13-kilodalton polypeptide associated with cytoplasmic male sterility, was expressed in *Escherichia coli*. After addition of BmT-toxin or methomyl, inhibition of whole cell respiration and swelling of spheroplasts were observed in *Escherichia coli* cultures producing the novel mitochondrial protein; these effects are similar to those observed with isolated *cms-T* mitochondria. The amino-terminal region of the 13-kilodalton polypeptide appears to be essential for proper interaction with the BmT-toxin and methomyl. These results implicate the 13-kilodalton polypeptide in conferring toxin sensitivity to mitochondria of *cms-T* maize.

MAIZE (*Zea mays* L.) PLANTS CARRYING Texas male-sterile cytoplasm (*cms-T*) are particularly susceptible to the fungal pathogen *Bipolaris (Helminthosporium) maydis*, race T, the causative agent of Southern Corn Leaf Blight (*1*). A host-specific pathotoxin (BmT-toxin) isolated from the fungus specifically alters membrane permeabilities of mitochondria from *cms-T* maize. A carbamate insecticide, methomyl, mimics BmT-toxin effects (*2*). In response to BmT-toxin or methomyl, *cms-T* mitochondria exhibit rapid swelling, uncoupling of oxidative phosphorylation, inhibition of malate-driven respiration, and leakage of small molecules such as NAD^+ and Ca^{2+} (*3, 4*). The site of toxin and methomyl action is believed to be at the inner mitochondrial membrane. Two other distinct cytoplasms, S (*cms-S*) and C (*cms-C*), confer cytoplasmically inherited male sterility to maize. Mitochondria from maize plants containing either the S or C male-sterile or normal (male-fertile) cytoplasms are unaffected by the BmT-toxin or methomyl.

A strict correlation exists between susceptibility to the *B. maydis* pathotoxin and the cytoplasmic male-sterility (cms) trait in *cms-T* maize. Both traits are maternally inherited and attempts to separate the two effects have been unsuccessful. Regeneration of *cms-T* maize callus from tissue cultures both with and without BmT-toxin selection has given rise to revertant plants that are not only resistant to the BmT-toxin, but are also male-fertile; no stable revertants have been obtained that are male-sterile and toxin-resistant or male-fertile and toxin sensitive (*5*).

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A possible explanation for the simultaneous reversion of the two traits is that a single locus of extranuclear origin encodes both phenotypes. We have recently isolated and characterized a unique mitochondrial gene from *cms-T* plants, designated *urf13-T*, that encodes a 13-kD protein associated with the cms trait (*6–9*). To determine whether this cms-associated protein is involved with susceptibility to disease pathogenicity, we expressed the *urf13-T* gene in *Escherichia coli* and studied the effects of BmT-toxin and methomyl in this heterologous system.

The entire *urf13-T* gene was cloned into the plasmid vector pATH 3 and expressed in *E. coli* strain JM109 as described (*10*). The pATH 3–*urf13-T* construct, pATH13-T, is transcriptionally regulated by the *trp* promoter of *E. coli* and produces a 13-kD protein that cross-reacts with an antiserum to the *urf13-T* protein and comigrates electrophoretically with the mitochondrial *urf13-T* protein product of *cms-T* maize as determined by protein blot analysis (Fig. 1). Nucleotide sequence analysis confirmed that the *urf13-T* reading frame in pATH13-T was identical with the maize reading frame (*11*). Although identical in nucleotide sequence, a difference in amino acid composition of the pATH13-T and *urf13-T* proteins may exist because of a possible difference in codon usage. In higher plant mitochondria, CGG codons are believed to designate tryptophan rather than arginine as predicted by the universal code (*12*). *Urf13-T* and pATH13-T contain a single CGG codon, corresponding to amino acid position 87 (*6*).

A truncated version of the *urf13-T* gene, pJG 13-T, that produces a form of the 13-kD protein missing amino acids 2 through 11 was also constructed and expressed in *E. coli* by means of the plasmid vector pJG200 (*13*). Construct pJG13-T is under thermoinducible control of the CI857 thermolabile repressor and p_R promoter of bacteriophage λ. *Escherichia coli* cells containing plasmid pJG13-T abundantly expressed the modified form of the 13-kD protein after temperature induction at 42°C (Fig. 1).

We measured the effects of the BmT-toxin and methomyl on respiration in *E. coli* cells containing the pATH13-T and pJG13-T

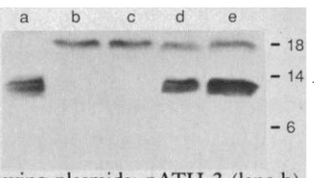

Fig. 1. Expression of the *urf13-T* protein product of *cms-T* mitochondria in *E. coli*. Protein preparations from *cms-T* mitochondria and *E. coli* were separated electrophoretically in 18% (wt/vol) SDS-polyacrylamide gels and blotted to nitrocellulose (*15*). Protein blots were challenged with an antiserum directed against the *urf13-T* gene product as described (*7*). Lane a, total mitochondrial proteins from *cms-T* maize (inbred B73) (*7*). Total *E. coli* proteins were examined from cells containing the following plasmids: pATH 3 (lane b), pATH13-T (lanes c and d), and pJG13-T (lane e). Cultures were induced for maximal plasmid expression for all cells except those in lane c, which were grown in a noninducing medium (*10, 13*). Approximate molecular masses (in kilodaltons) were determined by running molecular weight markers (BRL) in parallel lanes.

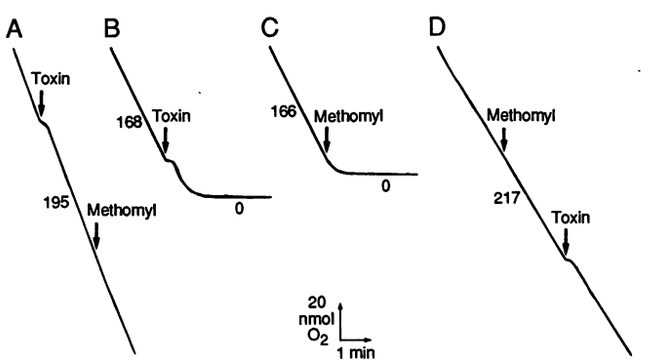

Fig. 2. Effect of BmT-toxin and methomyl on O_2 consumption of *E. coli* cells expressing the *cms-T* 13-kD polypeptide. Additions of BmT-toxin (780 ng/ml) and/or methomyl (4 m*M*) were made to *E. coli* cultures expressing the plasmids pATH 3 (**A**), pATH13-T (**B** and **C**), and pJG13-T (**D**). The reaction medium contained 42 m*M* Na_2HPO_4, 22 m*M* KH_2PO_4, 8.5 m*M* NaCl, 19 m*M* NH_4Cl, 10 m*M* glucose, and from 150 to 200 μg of *E. coli* protein. Oxygen consumption was measured polarographically with a Clark oxygen electrode at 25°C. Respiration rates are expressed as nanomoles of O_2 consumed per minute per milligram of *E. coli* protein.

plasmids. Oxygen consumption was completely inhibited by the addition of BmT-toxin (780 ng/ml) or methomyl (4 m*M*) in cells producing the complete 13-kD protein (Fig. 2, B and C). Respiration was not altered by toxin or methomyl in control cells transformed with the pATH 3 vector containing no insert (Fig. 2A), or in the cells producing the truncated version of the 13 kD protein (Fig. 2D).

The time required to completely inhibit respiration after toxin addition in *E. coli* cells was dependent on the concentration of the toxin. Although O_2 consumption was completely inhibited at toxin concentrations of 7.8 ng/ml, 8 to 9 minutes were required for full inhibition (*11*). In contrast, complete inhibition was achieved after approximately 1 minute of exposure to toxin at 780 ng/ml (Fig. 2B).

In *cms-T* mitochondria, BmT-toxin and methomyl cause a rapid decrease in A_{520} that has been interpreted as mitochondrial swelling (*3*). BmT-toxin and methomyl induced dramatic swelling in spheroplasts from *E. coli* cells that produced the 13-kD polypeptide (Fig. 3, B and C); no effect was seen with spheroplasts containing the pATH 3 control plasmid (Fig. 3A). In accord with the respiration results, *E. coli* spheroplasts producing the truncated 13-kD protein from plasmid pJG13-T showed no stimulation in swelling after the addition of toxin or methomyl (*11*).

To determine the effect of the BmT-toxin on *E. coli* growth, the A_{550} of pATH13-T induced cell cultures was monitored for several hours both in the presence and absence of toxin. No growth was detected in *E. coli* cultures (logarithmically growing) expressing the 13-kD polypeptide during a 6-hour period after addition of BmT-toxin (780 ng/ml), whereas the same cells without toxin exhibited growth rates similar to cells containing the pATH 3 control plasmid (*11*).

Incubation of *cms-T* maize mitochondria with dicyclohexylcarbodiimide (DCCD), a reagent that preferentially binds covalently to carboxyl groups in hydrophobic regions of proteins, confers protection against the effects of BmT-toxin (*14*). Pretreatment of mitochondria with 4 to 15 μ*M* DCCD prevents toxin-induced inhibition of malate-dependent oxidation, dissipation of the membrane potential, and leakage of accumulated calcium. Preincubation with the water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, does not protect *cms-T* mitochondria from toxin action (*14*), suggesting that DCCD modifies a component situated in a hydrophobic environment.

Two major DCCD-binding proteins, approximately 6 and 14 kD in size, were observed when normal and *cms-T* mitochondria were incubated with ^{14}C-labeled DCCD (Fig. 4). The 6-kD protein presumably is subunit 9 of the mitochondrial adenosine triphosphate synthase complex, a polypeptide commonly referred to as the DCCD-binding protein. The identity of the 14-kD polypeptide is unknown. Immunoprecipitation of [^{14}C]DCCD–labeled maize mitochondrial proteins with an antiserum directed against the *urf13-T* protein product revealed that DCCD binds to the 13-kD protein (Fig. 4). In our gel system the DCCD-labeled 13-kD polypeptide comigrated with the [^{14}C]DCCD–labeled 14-kD protein observed in both normal and T cytoplasms. Similar to its characteristics in *cms-T* mitochondria, the 13-kD polypeptide produced in *E. coli* by pATH13-T is also localized in the membrane and binds DCCD (*11*). In the *E. coli* system, we have also been able to confer resistance to the BmT-toxin by preincubating cells with DCCD (*11*).

The evidence indicates that the amino-terminal region of the 13-kD polypeptide is involved in conferring BmT-toxin and methomyl susceptibility to *cms-T* mitochondria or *E. coli*. Hydropathy profiles suggest that the amino-terminal portion of the protein is the region most likely associated with the membrane (*7*), the site where toxin action is believed to occur. In addition, *E. coli* cells that express the truncated form of the 13-kD protein encoded by plasmid pJG13-T, lacking amino acids 2 to 11 from the amino terminus, are not sensitive to BmT-toxin or methomyl. The truncated protein, like the intact 13-kD polypeptide, is localized in the membrane of *E. coli* (*11*). Therefore resistance in these cells cannot be explained simply by a lack of association between the truncated protein and the membrane.

The ability to confer sensitivity to BmT-toxin and methomyl in *E. coli* cells by expressing the pATH13-T plasmid is compelling evidence that an interaction between the toxin (or methomyl) and the 13-kD protein of *cms-T* maize mitochondria is responsible for the deleterious effects of this toxin in *cms-T* mitochondria. Synthesis of the 13-kD protein in *E. coli* may be a convenient system for investigating the interaction between toxin and protein and the mechanism by which respiration is inhibited. The heterologous *E. coli* system may be valuable because mutagenesis studies can easily be conducted to determine the specific amino acids or domains of the 13-kD polypeptide necessary for susceptibility to BmT-toxin and methomyl.

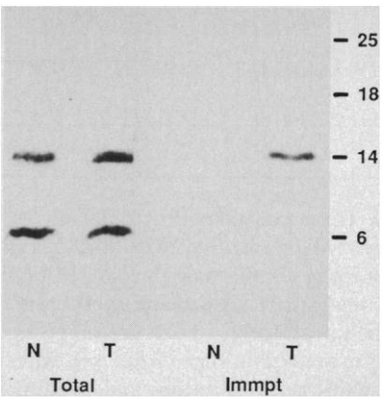

Fig. 4. DCCD binding to mitochondrial proteins from normal (N) and male-sterile (T) cytoplasms of maize. Total mitochondrial proteins were labeled with [^{14}C]DCCD (Amersham), separated on SDS-polyacrylamide gels, and detected by autoradiography as described (*17*). DCCD-labeled mitochondrial protein preparations were immunoprecipitated (Immpt) with an antiserum directed against the protein product of *urf13-T* (*7*). Mitochondria were isolated from etiolated seedlings of the maize inbred B73 (*7*). Approximate molecular masses are indicated in kilodaltons.

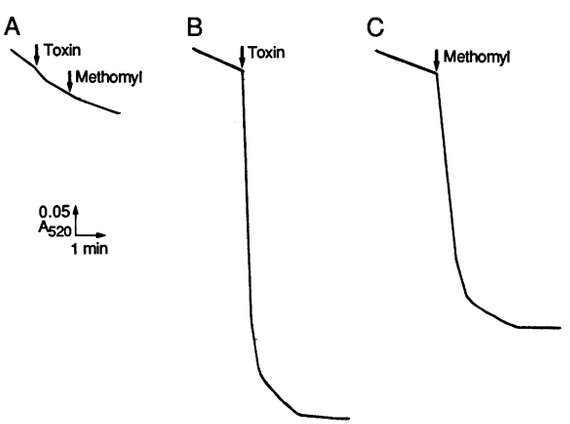

Fig. 3. Effect of BmT-toxin and methomyl on the A_{520} of *E. coli* spheroplasts expressing the 13-kD *cms-T* protein. Decrease in absorbance of *cms-T* mitochondria by BmT-toxin and methomyl has been interpreted as mitochondrial swelling (*3*). *Escherichia coli* cells expressing the plasmids pATH 3 (**A**) and pATH13-T (**B** and **C**) were treated with toxin (780 ng/ml) and methomyl (4 m*M*). *Escherichia coli* spheroplasts were prepared as outlined (*16*). Absorbance measurements were made spectrophotometrically in a medium containing 10 m*M* tris-HCl, *p*H 8.0, 30% sucrose, 10 m*M* EDTA, 10 m*M* glucose, and from 0.45 to 0.6 mg of *E. coli* protein in a final volume of 2.0 ml.

REFERENCES AND NOTES

1. A. L. Hooker, D. R. Smith, S. M. Lim, J. B. Beckett, *Plant Dis. Rep.* **54**, 708 (1970).
2. H. S. Humaydan and E. W. Scott, *Hortic. Sci.* **12**, 312 (1977).

3. R. J. Miller and D. E. Koeppe, *Science* **173**, 67 (1971); D. E. Koeppe, J. K. Cox, C. P. Malone, *ibid.* **201**, 1227 (1978); A. Berville, A. Ghazi, M. Charbonnier, J. F. Bonavent, *Plant Physiol.* **76**, 508 (1984); R. R. Klein and D. E. Koeppe, *ibid.* 77, 912 (1985).
4. M. J. Holden and H. Sze, *Plant Physiol.* **75**, 235 (1984).
5. R. I. S. Brettell, B. V. D. Goddard, D. S. Ingram, *Maydica* **24**, 203 (1979); P. F. Umbeck and B. G. Gengenbach, *Crop Sci.* **23**, 584 (1983).
6. R. E. Dewey, C. S. Levings III, D. H. Timothy, *Cell* **44**, 439 (1986).
7. R. E. Dewey, D. H. Timothy, C. S. Levings III, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5374 (1987).
8. B. G. Forde and C. J. Leaver, *ibid.* 77, 418 (1980).
9. R. P. Wise, D. R. Pring, B. G. Gengenbach, *ibid.* **84**, 2858 (1987); W. H. Rottmann, T. Brears, T. P. Hodge, D. M. Lonsdale, *EMBO J.* **6**, 1541 (1987).
10. The pATH13-T plasmid was constructed by means of the pATH 3 expression vector, the *urf13-T* gene of *cms-T* mitochondria, and two complementary synthetic oligonucleotides. Digestion of the clone containing *urf13-T* with Hind III, followed by a partial digestion with Bcl I gave a fragment that included the entire *urf13-T* gene except for the first two nucleotides of the ATG initiation codon. The pATH 3 plasmid was linearized by cleavage of the Eco RI and Hind III sites located within the polycloning region of the vector. Two complementary oligonucleotides corresponding to the sequences 5′-AATTCTGGAGGAAAAAATTAT-3′ (top strand) and 5′-GATCATAATTTTTTCCTCCAG-3′ (bottom strand) were annealed to yield a fragment with Eco RI and Bcl I sticky ends. The linear pATH 3 vector and the Bcl I–Hind III fragment containing *urf13-T* were then joined by ligation. The oligonucleotides were constructed to provide a prokaryotic ribosome binding site and to reconstitute the initiator ATG codon of *urf13-T*. Cloning procedures were as described [T. Maniatis, E. F. Fritsch, J. Sambrook, in *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982)]. The structure of the pATH 3 expression vector and conditions of transcriptional induction of the *trp* promoter were according to K. R. Spindler, D. S. E. Rosser and A. J. Berk, [*J. Virol.* **49**, 132 (1984)] with modifications described by T. J. Koerner (personal communication). Optimal expression of the pATH13-T vector was obtained in an M9 medium containing casamino acids minus glucose and thiamine. Although the pATH 3 expression vector was designed to enable synthesis of *trp*E fusion proteins, the pATH13-T plasmid was constructed so that the *urf13-T* sequence was out of frame with the *trp*E reading frame allowing production of an unfused 13-kD protein.
11. R. E. Dewey *et al.*, unpublished data.
12. T. D. Fox and C. J. Leaver, *Cell* **26**, 315 (1981).
13. A Bcl I–Bgl II fragment [positions 1248 to 1723 according to Dewey *et al.* (*6*)] containing all of the *urf13-T* reading frame except for the first 11 codons was ligated into the Bam HI site of the expression vector pJG200. The resulting construct (pJG13-T) encoded all of the *urf13-T* reading frame except codons 2 through 11. The pJG200 vector and conditions for induction of transcription are described by J. Germino, J. Charbonneau, T. Vanaman, and D. Bastia [*Proc. Natl. Acad. Sci. U.S.A* **80**, 6848 (1983)] and J. Germino and D. Bastia [*ibid.* **81**, 4692 (1984)].
14. P-Y. Bouthyette, V. Spitsberg, P. Gregory, *J. Exp. Bot.* **36**, 511 (1985); M. J. Holden and H. Sze, in *Plant Mitochondria. Structural, Functional and Physiological Aspects*, A. L. Moore and R. B. Beechey, Eds. (Plenum, New York, 1987), pp. 305–308.
15. U. K. Laemmli, *Nature (London)* **227**, 680 (1970); J. Renart and I. V. Sandoval, *Methods Enzymol.* **104**, 455 (1984).
16. C. Burstein, L. Tiankora, A. Kepes, *Eur. J. Biochem.* **94**, 387 (1979).
17. E. Hack and C. J. Leaver, *Curr. Genet.* **8**, 537 (1984).
18. We are grateful to H. W. Knoche for his generous gift of BmT-toxin, the E. I. DuPont de Nemours & Co. for the methomyl, T. J. Koerner for the pATH 3 expression vector, and D. Bastia for the pJG200 vector. In addition, we thank H. C. Griffin for excellent technical assistance. Supported by grants from Agrigenetics Corporation and the National Science Foundation. R.E.D. is supported by a North Carolina Biotechnology fellowship. Paper No. 11249 of the Journal Series of the North Carolina Agricultural Research Service, Raleigh, NC 27695-7601.

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DNA Amplification for Direct Detection of HIV-1 in DNA of Peripheral Blood Mononuclear Cells

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By means of a selective DNA amplification technique called polymerase chain reaction, proviral sequences of the human immunodeficiency virus (HIV-1) were identified directly in DNA isolated from peripheral blood mononuclear cells (PBMCs) of persons seropositive but not in DNA isolated from PBMCs of persons seronegative for the virus. Primer pairs from multiple regions of the HIV-1 genome were used to achieve maximum sensitivity of provirus detection. HIV-1 sequences were detected in 100% of DNA specimens from seropositive, homosexual men from whom the virus was isolated by coculture, but in none of the DNA specimens from a control group of seronegative, virus culture–negative persons. However, HIV-1 sequences were detected in 64% of DNA specimens from seropositive, virus culture–negative homosexual men. This method of DNA amplification made it possible to obtain results within 3 days, whereas virus isolation takes up to 3 to 4 weeks. The method may therefore be used to complement or replace virus isolation as a routine means of determining HIV-1 infection.

ALTHOUGH SEROLOGIC ASSAYS IDENtify persons with prior exposure to human immunodeficiency virus (HIV-1), they do not specifically determine current infection; this requires isolating the virus from an HIV-1–seropositive person. HIV-1 isolation involves prolonged cocultivation of peripheral blood mononuclear cells (PBMCs) with phytohemagglutinin (PHA)-stimulated lymphocytes from an uninfected donor or with a susceptible uninfected indicator cell line (*1*). The procedure takes up to 3 to 4 weeks and lacks sensitivity in that viruses cannot be consistently isolated from persons with documented infections (*2–4*).

The objective of this study was to directly determine the presence of HIV-1 genetic information in the DNA from PBMCs of a patient and to correlate this with the ability to isolate virus from the same patient. The number of peripheral blood lymphocytes expressing viral RNA, as detected by in situ hybridization (*5*), in an infected person is less than 1 in 10,000 cells; therefore, we used a DNA amplification technique (*6–11*), the polymerase chain reaction (PCR), to amplify specific regions of HIV-1 proviruses present either as the free episomal form or as the integrated form in patients' chromosomal DNA. The amplified viral DNA could be detected by hybridization of a ^{32}P-labeled DNA probe to a portion of the amplified region; a specific restriction endonuclease was then used to cleave the resultant hybrid to yield an HIV-1 diagnostic fragment (*8*). This PCR technique takes less than 3 days to complete.

We tested three groups of patients (Table 1) for the presence of HIV-1 proviral DNA in their PBMCs. Those in group A (n = 11) were randomly selected seropositive and virus culture–positive homosexual men from California (*12*); those in group B (n = 11) were randomly selected seropositive and virus culture–negative homosexual men from California (*12*). Lymphocytes and sera from persons in these two groups were collected between April 1984 and July 1985. Persons in group C (n = 13) were randomly selected negative controls who were seronegative blood donors from the Atlanta area, and their lymphocytes and sera were collected between January 1985 and July 1985.

HIV-1 proviral sequences present in the PBMCs of the persons in groups A through C were amplified by means of primer pairs

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