Mutations in the maize mitochondrial *T-urf13* gene eliminate sensitivity to a fungal pathotoxin

(site-directed mutagenesis/disease susceptibility/dicyclohexylcarbodiimide binding/cytoplasmic male sterility/*Bipolaris maydis* race T)

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ABSTRACT URF13, the product of the mitochondrial *T-urf13* gene, confers on Texas cytoplasmic male-sterile maize (*Zea mays* L.) a unique susceptibility to a fungal pathogen (*Bipolaris maydis* race T) and sensitivity to its pathotoxin. Expression of URF13 in *Escherichia coli* imparts pathotoxin sensitivity to the bacterium. We show by ion uptake studies in *E. coli* that a pathotoxin–URF13 interaction causes membrane permeability. Similarly, mitochondrial dysfunction caused by membrane permeabilization probably accounts for increased colonization of maize carrying the Texas cytoplasm by toxin-producing pathogens. Site-directed mutagenesis studies show that approximately one-quarter of the amino acids at the carboxyl end of URF13 can be eliminated without affecting toxin sensitivity. We have identified two dicyclohexylcarbodiimide (DCCD) binding sites in the URF13 protein and show that one of the sites is involved in conferring DCCD protection against the pathotoxin. Substitutional mutations at this DCCD binding site also eliminate toxin sensitivity.

The Texas cytoplasm (*cms-T*) confers cytoplasmic male sterility and disease susceptibility to maize plants (1, 2). *cms-T* plants are uniquely susceptible to the fungal pathogens *Bipolaris* (formerly *Helminthosporium*) *maydis* race T (Southern corn leaf blight) and *Phyllosticta maydis* (yellow leaf blight). Both fungi produce a set of structurally related pathotoxins, collectively referred to as T-toxin in this report, that specifically affect mitochondria from the Texas cytoplasm (T mitochondria) (3–5). The T-toxin effects include inhibition of malate-driven electron transport, stimulation of succinate and NADH-driven state-4 respiration, uncoupling of oxidative phosphorylation, mitochondrial swelling, and massive leakage of small molecules such as Ca^{2+} and NAD^+ (6–11). Klein and Koeppe (9) speculate that an interaction between T-toxin and a 13-kDa protein unique to T mitochondria causes permeabilization of the inner mitochondrial membrane. *S*-Methyl *N*-[(methylcarbamoyl)oxy]thioacetimidate (methomyl), the active ingredient in Dupont's systemic insecticide Lannate, is structurally unrelated to T-toxins yet mimics their effects on *cms-T* mitochondria (12).

Disease susceptibility and cytoplasmic male sterility in *cms-T* maize plants are correlated with a mitochondrial gene, *T-urf13*. *T-urf13* contains 115 codons with significant sequence similarity to mtDNA located within and 3' to the 26S rRNA mitochondrial gene (13). *T-urf13* encodes a 13-kDa polypeptide, designated URF13, that is membrane bound and binds dicyclohexylcarbodiimide (DCCD) (14–16). Preincubation with DCCD, which preferentially and covalently binds to acidic amino acid residues located in hydrophobic domains (17), protects T mitochondria from the effects of T-toxin and methomyl (18, 19). A frameshift mutation of *T-urf13* in the tissue culture-derived revertant T-4 shortens the *T-urf13*

reading frame to 74 codons (20). T-4 plants are disease resistant, male fertile, and insensitive to T-toxin.

When *T-urf13* is expressed in *Escherichia coli*, T-toxin and methomyl cause spheroplast swelling and inhibit bacterial respiration. These results show that URF13 is responsible for toxin sensitivity in *E. coli* and *cms-T* maize (15). Previously, we have shown that two mutations of *T-urf13* are no longer sensitive to T-toxin. An internal *T-urf13* mutation, missing codons 2–11 (15), and a frameshift mutation of *T-urf13*, similar to the mutant in the T-4 maize revertant, do not confer toxin sensitivity to *E. coli* (21). The observed effects of T-toxin in *cms-T* mitochondria and *E. coli*, shown with mutant and standard versions of *T-urf13*, indicate that the T-toxin–URF13 response is similar in both systems (15, 21).

We have used site-directed mutagenesis to define additional regions of URF13 important for toxin sensitivity. Two DCCD binding sites have been identified in URF13 and the relationship between DCCD binding and protection against T-toxin has been determined. Finally, we have shown by ion uptake experiments that the T-toxin–URF13 interaction results in rapid permeabilization of the bacterial membrane.

MATERIALS AND METHODS

Oligonucleotide Synthesis, Site-Directed Mutagenesis, and DNA Sequence Analysis. Oligonucleotides were produced with an Applied Biosystems 380A DNA synthesizer and purified according to manufacturer's instructions. Mutagenesis followed the procedure described by Kunkel (22) and used a *T-urf13* template inserted into bacteriophage M13mp-19. Mutant clones were screened by DNA sequencing (23); DNA synthesis was primed by various *T-urf13* oligonucleotides or by the M13 universal primer.

Inducible Prokaryotic Expression of URF13 Using λP_L Promoter. Mutant *T-urf13* DNAs containing prokaryotic translation signals were isolated from the replicative form of M13 and inserted into the pLC236 expression plasmid 3' of the thermal-inducible λP_L promoter (24). Recombinant plasmids were transformed into an *E. coli* host carrying the *cI857* gene that produces a temperature-sensitive repressor protein. URF13 was produced in *E. coli* by diluting a 28°C stationary phase culture 1:50 into Luria–Bertani broth (25) supplemented with ampicillin (100 $\mu\text{g}/\text{ml}$) and incubating for 2 hr at 42°C. Cells were harvested by centrifugation at $5000 \times g$ for 10 min at 4°C, washed once with M9 salts (42 mM $\text{Na}_2\text{HPO}_4/22$ mM $\text{KH}_2\text{PO}_4/8.5$ mM $\text{NaCl}/19$ mM NH_4Cl), and resuspended in 1/100th of the initial volume with M9 salts. Protein concentration was determined by the Lowry assay (26).

Ion Uptake in *E. coli*. Ion uptake experiments followed methods described (21, 27) using $^{86}\text{RbCl}$ (Amersham) at a specific activity of 7.4 MBq/mmol. In these experiments, URF13 was produced in the PATH expression vector; the

insertion of T-*urf13* into the pATH vector has been described (15).

Antibody Production, Purification, and Immunoblot Analysis. Preparation of polyclonal antisera, directed against a synthetic 15-amino acid residue immunogen identical to amino acids 92–106 of URF13, was described (14). The 15-amino acid oligopeptide was coupled to cyanogen bromide-activated Sepharose (Pharmacia) and the crude antiserum was affinity-purified according to the manufacturer's instructions. Mutant and standard versions of URF13 expressed in *E. coli* were electrophoretically resolved on 18% (wt/vol) NaDodSO₄/polyacrylamide gels using a modified Laemmli system (28). Modifications included a resolving gel that contained 0.75 M Tris-HCl (pH 8.8), an acrylamide/bisacrylamide ratio of 150:1, and 2 M urea. Proteins were electroblotted and URF13 was detected by techniques described previously (29, 30).

Membrane Isolation and DCCD Binding. *E. coli* cells were disrupted by sonication and centrifuged for 10 min at 12,000 × *g* to eliminate cell debris. The membrane fraction was isolated by centrifugation (100,000 × *g*) for 1 hr at 4°C. Pellets were resuspended in M9 salts. Each 100 μl of DCCD labeling reaction mixture contained 250 μg of membrane protein and 0.02 μmol of [¹⁴C]DCCD (31). Labeled [¹⁴C]DCCD (2.1 Gbq/mmol; Amersham) was dried prior to use and resuspended at a concentration of 4 mM with 100% ethanol. DCCD-labeled proteins were visualized by fluorography. Unlabeled DCCD (Sigma) at 0.5 mM was used to protect *E. coli* cells expressing sensitive versions of URF13 from the effects of T-toxin. *E. coli* cells (400 μg of protein) were shaken slowly in the presence of DCCD for 1 hr at room temperature.

RESULTS

Expression and Analysis of T-*urf13* in *E. coli*. In previous T-*urf13* experiments in *E. coli*, we used the pATH plasmid expression vector (15, 21). The pATH expression vector was also used in ion uptake experiments described below; however, in other experiments URF13 was expressed with the pLC236 plasmid expression vector, designated pLC13-T when it contains the T-*urf13* gene. We switched to the pLC236 vector because it contains a stronger promoter (AP_L in pLC236 versus *trp* in pATH) and allows for better regulation of URF13 expression [temperature-sensitive repressor protein (*cI857*) in pLC236 versus tryptophan concentration in pATH] (24). In the pLC236 vector, a small DNA cassette containing an *Eco*RI restriction site and a ribosomal binding site was placed between the promoter and the initiator methionine of T-*urf13*. Although the same DNA cassette was used to insert T-*urf13* into pATH and pLC236 (15), the position of T-*urf13* with respect to the promoters is different; T-*urf13* is contiguous with the P_L promoter in the pLC236 vector but 3' to the *trp* promoter and *trpE* gene in the pATH vector. In both vectors, codon 87 (CGG) of T-*urf13* was unaltered and thus encodes arginine in all *E. coli* experiments. In higher plant mitochondria, CGG may be an exception to the universal code and specify tryptophan rather than arginine (32).

URF13 mutants were expressed in *E. coli* and assayed for sensitivity to the *B. maydis* race T toxin, *P. maydis* toxin, and methomyl by measuring the rate of O₂ consumption. Sensitivity to these compounds was also determined by monitoring swelling-associated absorbance changes using spheroplasts (15). We created both toxin-sensitive and toxin-insensitive versions of URF13 (Tables 1 and 2); in every instance, mutations were either sensitive to both toxin and methomyl or insensitive to both compounds. Immunoblot analysis, using affinity-purified anti-URF13 antibody, demonstrated the production of mutant URF13 (Fig. 1). Since terminal-deletional mutants were not recognized by the antibody (see Fig. 2 for the location of the immunogen), the production of

Table 1. Site-directed T-*urf13* deletional mutations in *E. coli*

Original URF13	Mutant URF13	Toxin sensitivity	URF13 length
72W-73L-74R	72S-73G-74 stop ¹	—	73
83L	83 stop ²	—	82
84P	84 stop ¹	+	83
85I	85 stop ²	+	84
86Q	86 stop ²	+	85
88N	88 stop ²	+	87
93R	93 stop ¹	+	92
102K	102 stop ³	+	101

E. coli respiration, measured by O₂ consumption, was used to assay sensitivity to T-toxin and methomyl. Each carboxyl-terminal mutation truncates the open reading frame by either inserting a stop codon or by creating a frameshift. Original T-*urf13* residues are listed by position and single-letter amino acid designation followed by position and identification of mutated residues. Stop codons are distinguished by superscripts: 1, UGA (opal); 2, UAA (ochre); 3, UAG (amber). For each mutation, toxin and methomyl sensitivity is designated by +; insensitive mutants are designated by —. The length in amino acid residues is listed for URF13 mutants. All of these URF13 mutants bind DCCD.

these URF13 mutants was confirmed by [¹⁴C]DCCD labeling (Fig. 3; data not shown).

URF13 Deletional Mutations. Previously we reported a site-directed frameshift mutation of T-*urf13* in *E. coli* that creates a toxin-insensitive URF13 of 73 amino acids (21). This mutant is similar to a T-*urf13* mutation, T-4, recovered from toxin-insensitive maize plants regenerated from tissue culture (20). Deletional mutants were constructed between amino acid 73 and the carboxyl terminus of URF13 to determine the minimum length of URF13 required for toxin sensitivity (Table 1). T-toxin sensitivity was assayed by the methods described above. Three deletional mutants were created by placing termination codons within the T-*urf13* open reading frame. These shortened URF13 mutants were 82, 92, and 101 amino acids, respectively (Table 1). The 82-amino acid mutant was insensitive to T-toxin and methomyl; the longer mutants were sensitive to both compounds. Additional deletional mutants, ranging between 83 and 92 amino acids, were constructed and assayed for toxin sensi-

Table 2. Internal deletional and substitutional T-*urf13* mutants in *E. coli*

Original URF13	Mutant URF13	Toxin sensitivity	DCCD binding
12D	12A, R, G, E, H, or V	+	+
39D	39E, H, or V	—	+
40D	40H or Y	+	+
48E	48Q or K	+	+
56E	56K*	—	—
56E	56Q	+	+
67E	67K†	+	+
67E	67Q	+	+
83L	Codon deleted†	+	+
12D-39D	12V-39A	—	—
12D-39D-40D	12G-39A-40H	—	—
12D-40D	12G-40H	+	+
12D-48E	12R-48Q	+	+
12D-67E	12R-67Q	+	+
39D-40D	39A-40H	—	+

E. coli respiration, measured by O₂ consumption, was used to assay sensitivity to T-toxin and methomyl. Abbreviations are the same as in Table 1. The internal deletion at position 83 creates a 114-codon reading frame. All other mutants described in this table have 115 codons. DCCD binding is indicated by a +; no binding is indicated by a —.

*A mutation that does not produce detectable URF13.

†A reduced amount of URF13.

FIG. 1. Immunoblot of standard and mutant URF13 proteins expressed in *E. coli*. Substitutional mutants are designated by the original amino acid (single-letter code) and its location, followed by the amino acid change. Lanes from left to right are the standard 115-amino acid URF13; molecular mass markers; control, pLC13-T *E. coli* grown under noninducing conditions; 2-11, amino acids 2-11 deleted; D39A; molecular mass markers; D12V-D39A; D12R; and D40H. URF13 was detected by using affinity-purified antibody, followed by 125 I-labeled protein A. Except for the molecular mass markers (Bethesda Research Laboratories), each lane contained 75 μ g of *E. coli* protein. Molecular mass markers are expressed in kDa.

tivity (Table 1). All of these mutants were sensitive to T-toxin and methomyl, demonstrating that URF13 must contain at least the first 83 amino acids to be toxin sensitive. An internal deletion that only eliminated the leucine residue at position 83 was sensitive to T-toxin; the abundance of URF13 produced by this deletional mutant was reduced compared with other URF13 mutants (data not shown). This suggests that a leucine

FIG. 3. Fluorogram of [14 C]DCCD-labeled membrane proteins from *E. coli* expressing standard and mutant versions of URF13. Lanes from left to right are standard URF13; D12V-D39A, double substitutional mutation; D39A; D12R; molecular mass markers; 85 aa, deletional mutant containing the first 85 amino acids of URF13; 101 aa, deletional mutant containing the first 101 amino acids. Mutants are designated as described in Fig. 1. 14 C-labeled molecular mass markers (Amersham) are expressed in kDa.

at position 83 is not required for toxin sensitivity but contributes to the stability of URF13 in *E. coli*.

Membrane Permeability Assays. We used an ion uptake assay to determine the effect of T-toxin on bacterial mem-

FIG. 2. (A) Hydropathy profile of URF13. Predicted hydrophobic regions according to Kyte and Doolittle (33) are indicated by shaded areas above the single-letter amino acid sequence and hydrophilic regions are indicated by open areas below the sequence. The arrows show the location of regions where mutations cause loss of T-toxin sensitivity. Arrow 1, internal deletion of amino acids 2-11 described by Dewey *et al.* (15); arrow 2, Asp-39. Substitutional mutants at this position eliminate toxin sensitivity. Arrow 3, a junction between toxin insensitivity and sensitivity. Deletional mutants having the first 82 amino acids or fewer are toxin insensitive. Any deletional mutant containing the first standard 83 amino acids or more is toxin sensitive. Box above amino acids 92-106 indicates the immunogen used to generate the anti-URF13 antibody. (B) Schematic representation of the origin of DNA in the T-*urf13* open reading frame. The first 88 codons are from mtDNA located 3' to the 26S rRNA gene. The DNA composing the next 9 codons is of unknown origin. The final portion of the open reading frame contains 18 codons from the transcribed portion of the mitochondrial 26S rRNA gene.

brane permeability. A preliminary account of these results has been reported (21). *E. coli* expressing standard and mutant URF13 were allowed to accumulate labeled ^{86}Rb (27); after 30 min of uptake, T-toxin was added. Cells expressing the standard URF13 lost the accumulated label when exposed to T-toxin (Fig. 4). The loss of label was immediate and nearly complete and suggested that a dramatic membrane permeabilization had occurred. Control cells, without the T-*urf13* insert, retained the labeled Rb upon T-toxin addition. Sensitive and insensitive deletional URF13 mutants were tested for the effect of T-toxin on ion uptake. Ion loss did not occur in the insensitive 82-amino acid URF13 mutant. In contrast, the sensitive 92-amino acid URF13 lost the accumulated Rb when T-toxin was added.

DCCD Protection and Binding. The effect of T-toxin on mitochondria can be eliminated by pretreatment of mitochondria with 5–15 μM DCCD (18, 19). We found a similar protective effect of DCCD in *E. coli* expressing T-*urf13*; however, the concentration of DCCD required for protection in *E. coli* was 100 times greater than in mitochondria. The rate of *E. coli* respiration, measured by O_2 consumption, was not inhibited by methomyl and T-toxin when *E. coli* cells expressing T-*urf13* were pretreated with 0.5–1.0 mM DCCD (Fig. 5; data not shown).

DCCD binds covalently and preferentially to acidic amino acids localized in hydrophobic environments (17). URF13 contains 9 acidic residues—5 aspartates and 4 glutamates. To determine the DCCD binding sites in URF13, substitutional mutations were made for each of the first 6 acidic residues—positions 12, 39, 40, 48, 56, and 67. Several deletional mutants described above eliminated the three acidic residues at positions 89, 96, and 110 (Table 2). Binding studies with [^{14}C]DCCD showed that each of the deletional and single substitutional mutants has a DCCD binding site (Fig. 3 and Table 2). In addition to binding to URF13, DCCD binds to an *E. coli* protein with an approximate molecular mass of 6 kDa. The 6-kDa polypeptide is not recognized by the URF13 antibody and is present in *E. coli* not carrying a T-*urf13* insert (data not shown). This protein may be subunit c of the F_0

FIG. 4. ^{86}Rb uptake in *E. coli*. Data presented are from several experiments and are normalized such that 100% is defined as uptake after 30 min. Assay conditions have been described (21). T-toxin (final concentration, 1 μM) was added at 30 min. *E. coli* cells tested carried the following expression plasmids: URF13, standard 115-amino acid protein; control, expression plasmid without a T-*urf13* insert; 82 aa, deletional mutant containing the first 82 amino acids of URF13; 92 aa, deletional mutant carrying the first 92 amino acids of URF13.

FIG. 5. Protection effect of DCCD in *E. coli* cells expressing T-*urf13*. Sensitivity of respiration-stimulated O_2 uptake to methomyl was measured with a Clark electrode. (A) *E. coli* pretreated with 0.5 mM DCCD for 1 hr. (B) Untreated *E. coli*. Reactions were run at 25°C and contained *E. coli* cells in M9 salts and glucose (10 mM). Methomyl (8 mM) was added as indicated.

component of the ATP synthase complex, a polypeptide known to bind DCCD (34). To test for multiple DCCD binding sites, double and triple substitutional mutants were created at various acidic residues and assayed for DCCD binding (Fig. 3 and Table 2). A double mutation at positions 12 and 39 eliminated DCCD binding, clearly demonstrating the presence of two DCCD binding sites (Table 2 and Fig. 3). Although individual substitutional mutants at either residue reduced DCCD labeling, this analysis showed that aspartate at position 39 binds DCCD to a greater extent than aspartate at position 12 (Fig. 3).

URF13 mutations that substitute for the aspartate at position 39 no longer conferred toxin sensitivity (Table 2). This was true even for the conservative replacement of the aspartate with glutamate. Mutations that eliminated the DCCD binding site at position 12 were sensitive to T-toxin and were also protected from the effects of T-toxin by pretreatment with DCCD. Thus, it is the binding of DCCD at position 39 that confers protection against T-toxin and methomyl.

Substitutional mutants that replaced the glutamate residues ($\text{pK}_a = 4.25$) at positions 56 or 67 with a lysine ($\text{pK}_a = 10.53$) eliminated or reduced the detectable level of URF13. In contrast, glutamine substitutions at positions 56 and 67 remained sensitive to T-toxin (Table 2). Mutant URF13 with a lysine substitution at position 56 could not be detected by immunoblot analysis; these cells were also insensitive to T-toxin and methomyl. Lysine substitution at position 67 drastically reduced the detectable quantity of mutant URF13. The respiration rate of *E. coli* carrying this URF13 mutant was reduced but not eliminated by T-toxin and methomyl. Similar substitutional mutations at acidic residues, positions 12 and 48, did not affect the production of mutant URF13, as detected by immunoblot analysis. These data suggest that the introduction of positively charged amino acids between residues 55 and 68 destabilized URF13.

DISCUSSION

Regulation of molecular traffic through membranes is particularly important in the mitochondrion, where electron transfer across the inner membrane creates a proton motive force that drives oxidative phosphorylation, producing cellular energy. Our data show that *E. coli* cells expressing

URF13 undergo massive ionic leakage when exposed to T-toxin (Fig. 4). We have concluded that ionic leakage from plasma membrane permeabilization is caused by the T-toxin-URF13 interaction. Perturbation of mitochondrial inner membrane integrity by a similar T-toxin-URF13 interaction probably accounts for the toxic effect on *cms-T* mitochondria. Mitochondrial dysfunction (6–11), manifested by the inability to produce ATP by oxidative phosphorylation, may explain the extensive colonization of *cms-T* plants by these toxin-producing pathogens.

Membrane-bound proteins have stretches of hydrophobic amino acid residues that may associate with the lipid bilayer (35). URF13 contains several hydrophobic domains (Fig. 2A). Amino acids 14–33 are predicted by the algorithm of Rao and Argos (36) to traverse the membrane as an α -helix. We have identified membrane-bound portions of URF13 experimentally by specifically modifying acidic amino acid residues with radiolabeled [¹⁴C]DCCD, a compound that stably binds only in nonaqueous environments (17). Site-directed mutations demonstrate that DCCD binds to URF13 at two aspartate residues, positions 12 and 39. DCCD binding to aspartate at position 12 is expected because it is located between the two largest hydrophobic domains (Fig. 2A), and the residues adjacent to the aspartate at position 12 are similar to those found in other DCCD binding sites (37). Conversely, DCCD binding to the aspartate at position 39 is unexpected because this residue is located in an area predicted to be hydrophilic and where three of the adjacent six residues contain charged side chains. Taken together, these data suggest amino acids 12 and 39 are membrane bound. In addition, aspartate at position 39 is required for the functional T-toxin-URF13 interaction; this is indicated because substitutional mutations at this aspartate residue eliminate sensitivity to toxin.

Pretreatment with DCCD protects against the effects of T-toxin in *cms-T* mitochondria (18, 19) and *E. coli* expressing URF13. Even though DCCD binds at two sites on URF13, only binding at position 39 confers protection against T-toxin. DCCD may protect against T-toxin by interfering directly with T-toxin binding or alternatively by modifying the tertiary or quaternary structure of URF13 in the membrane. Other inner mitochondrial membrane activities are affected by DCCD. DCCD is known to bind to the proteolipid component of the F₁F₀-ATPase complex (32) and to block the movement of protons through the complex.

Mutations that cause insensitivity to T-toxin are located within the first 82 codons of the *T-urf13* gene (Fig. 2A, arrows). The first insensitive URF13 mutant is an internal deletion, missing amino acids 2–11, near the amino terminus (Fig. 2A, arrow 1). This mutant is missing one of the large hydrophobic domains yet is membrane bound (15). Arrow 2 in Fig. 2A denotes aspartate at position 39, where substitutional mutations eliminate toxin sensitivity and one of the two DCCD binding sites. A series of deletional mutants near the carboxyl end of URF13 defines the third group of insensitive mutations. Arrow 3 is between amino acids 82 and 83 and marks a bifurcation between T-toxin insensitivity and sensitivity. Truncated versions of URF13 containing 82 amino acids or less are toxin insensitive; URF13 mutants 83 amino acids or longer are toxin sensitive. Therefore, the DNA encoding toxin sensitivity is located in an area with sequence similarity to mtDNA normally found 3' to the 26S rRNA gene (Fig. 2B). In normal maize mitochondria, this DNA is not transcribed (13); yet in T mitochondria, recombinational events have positioned this DNA such that it is transcribed. Although the last quarter of URF13 is unnecessary for conferring toxin sensitivity, it may be important in cytoplas-

mic male sterility. Little is known about the role *T-urf13* plays in the T type of cytoplasmic male sterility.

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