Fungal Toxins Bind to the URF13 Protein in Maize Mitochondria and Escherichia coli

Carl J. Braun,a,1 James N. Siedow,b and Charles S. Levings IIIa,*

a Department of Genetics, North Carolina State University, Raleigh, North Carolina 27695-7614
b Department of Botany, Duke University, Durham, North Carolina 27706

Expression of the maize mitochondrial T-urf13 gene results in a sensitivity to a family of fungal pathotoxins and to methomyl, a structurally unrelated systemic insecticide. Similar effects of pathotoxins and methomyl are observed when T-urf13 is cloned and expressed in Escherichia coli. An interaction between these compounds and the membrane-bound URFl3 protein permeabilizes the inner mitochondrial and bacterial plasma membranes. To understand the toxin-URF13 effects, we have investigated whether toxin specifically binds to the URFl3 protein. Our studies indicate that toxin binds to the URFl3 protein in maize mitochondria and in E. coli expressing URFl3. Binding analysis in E. coli reveals cooperative toxin binding. A low level of specific toxin binding is also demonstrated in cms-T and cms-T-restored mitochondria; however, binding does not appear to be cooperative in maize mitochondria. Competition and displacement studies in E. coli demonstrate that toxin binding is reversible and that the toxins and methomyl compete for the same, or for overlapping, binding sites. Two toxin-insensitive URFl3 mutants display a diminished capability to bind toxin in E. coli, which identifies residues of URFl3 important in toxin binding. A third toxin-insensitive URFl3 mutant shows considerable toxin binding in E. coli, demonstrating that toxin binding can occur without causing membrane permeabilization. Our results indicate that toxin-mediated membrane permeabilization only occurs when toxin or methomyl is bound to URFl3.

INTRODUCTION

Maize carrying Texas cytoplasm (cms-T) is susceptible to Bipolaris (formerly Helminthosporium) maydis, race T, and Phyllosticta maydis fungal pathogens (Hooker et al., 1970; Miller and Koepepe, 1971; Ullstrup, 1972; Cornstock, Martinson, and Gengenbach, 1973; Yoder, 1973). Colonization of cms-T maize by these pathogenic fungi causes severe leaf blight (Southern corn leaf blight and yellow corn leaf blight, respectively). In contrast, these pathogens cause only small, isolated leaf lesions in other cytoplasmic male-sterile and normal male-fertile maize. B. maydis, race T, and P. maydis produce a related set of pathotoxins, referred to as BmT- and Pm-toxins, respectively, that specifically affect cms-T mitochondria. Pm- and BmT-toxins have identical effects on cms-T mitochondria, including inhibition of malate-supported state-3 respiration, stimulation of NADH-driven state-4 respiration, organelle swelling, leakage of small molecules such as Ca2+ and NAD+, and uncoupling of oxidative phosphorylation (Miller and Koepepe, 1971; Gengenbach et al., 1973; Matthews, Gracen, and Gracen, 1979; Berville et al., 1984; Holden and Sze, 1984; Klein and Koepepe, 1985; Holden and Sze, 1987a).

Pm- and BmT-toxins are structurally similar, respectively containing repeating linear β-oxydioxo and oxy-oxo polyketol structures along a methylene backbone. There are some minor differences: Pm-toxins range from 33 to 35 carbons in length, and BmT-toxins from 35 to 45 carbons (Danko et al., 1984; Kono et al., 1985). Synthetic versions of Pm-toxins, 24 and 16 carbons in length, require 10-fold and 1000-fold higher concentrations, respectively, to show toxicity equivalent to native Pm-toxins (Suzuki et al., 1985). Pm- and BmT-toxins also retain their toxicity toward cms-T mitochondria when their carbonyl groups are reduced with sodium borohydride (Frantzen, Daly, and Knoche, 1987). In this report, we collectively refer to the Pm- and BmT-pathotoxins as T-toxins. Methomyl [S-methyl-N-[(methylcarbamoyl)oxy]thioacetimidate], the active ingredient in the Dupont insecticide Lannate®, is structurally unrelated to the fungal toxins, yet causes equivalent effects on cms-T mitochondria (Koepepe, Cox, and Malone, 1978).

A novel gene, T-urf13, in the cms-T mitochondrial genome is associated with both fungal disease susceptibility and cytoplasmic male sterility (Dewey, Levings, and Tim-
otthy, 1986). A membrane-bound 13-kD polypeptide, designated URF13, is encoded by T-urf13 (Dewey, Timothy, and Levings, 1987; Wise et al., 1987b). Analyses of mitochondrial DNA from revertant cms-T plants, regenerated from embryonic calli challenged with toxin, have demonstrated that T-urf13 is altered. These plants are both male-fertile and disease-resistant (Umbeck and Gengenbach, 1983; Rottman et al., 1987; Wise, Pring, and Gengenbach, 1987a). The correlation of cytoplasmic male sterility and disease susceptibility traits with an intact T-urf13 gene is consistent with the hypothesis that one gene is responsible for both traits.

Expression of URF13 in Escherichia coli imparts toxin sensitivity to the bacterium, demonstrating that T-urf13 is responsible for sensitivity to BrmT-toxin (Dewey et al., 1988). In E. coli, the effects of Pm- and BrmT-toxins and methomyl are analogous to those observed in cms-T mitochondria. These include inhibition of glucose-driven respiration, spheroplast swelling, and ion leakage (Dewey et al., 1988; Braun, Siedow, and Levings, 1989a). Mutagenesis of T-urf13 in E. coli has revealed three regions of the gene required for maintaining toxin sensitivity. Two mutations that delete large portions of the protein, an internal deletion of amino acids 2 through 11, and a deletion of 33 amino acids from the carboxyl end, result in E. coli that are no longer sensitive to T-toxin or methomyl (Dewey et al., 1988; Braun et al., 1989b). Site-directed substitutional mutations at amino acid 39, which is normally an aspartate, define a third region of URF13 capable of eliminating toxin sensitivity. Furthermore, mutations at position 39 abolish one of the two dicyclohexylcarbodiimide (DCCD) binding sites in URF13 (Braun et al., 1989b). Site-directed substitutional mutations at amino acid 39, which is normally an aspartate, define a third region of URF13 capable of eliminating toxin sensitivity. Furthermore, mutations at position 39 abolish one of the two dicyclohexylcarbodiimide (DCCD) binding sites in URF13 (Braun et al., 1989b). Pretreatment with DCCD protects against the effects of T-toxins and methomyl in cms-T mitochondria and in E. coli expressing URF13 (Bouthyette, Spitsberg, and Gregory, 1985; Holden and Sze, 1987b; Braun et al., 1989b). DCCD binding to aspartate-39 is responsible for the protective effect in E. coli expressing URF13. Analysis of numerous URF13 mutants has also indicated that sensitivity to BrmT- and Pm-toxins and methomyl are inseparable, implying that a common binding site exists for these compounds (Braun et al., 1989b).

A binding analysis using chemically reduced, 3H-Pm-toxin showed equivalent toxin binding in normal and cms-T maize mitochondria (Frantzen et al., 1987). Frantzen et al. (1987) pointed out that high levels of nonspecific binding could act to mask a lower level of specific toxin binding; however, they also suggested that there may not be a specific toxin receptor in cms-T mitochondria but rather the mitochondrial membranes from normal maize could contain components that prevent toxin-induced membrane permeabilization.

We have used chemically reduced, 3H-Pm-toxin to investigate toxin binding in E. coli expressing URF13 and in mitochondria of cms-T maize. Our studies indicate that URF13 binds T-toxin. In addition, we have characterized toxin binding by competition and displacement experiments and by binding studies with URF13 mutants.

RESULTS

T-Toxin Binding in E. coli

In the 3H-Pm-toxin used in this study, the carbonyl groups have been chemically reduced to hydroxyl moieties, which are shown in Figure 1A. Activity of 3H-Pm-toxin was assayed by monitoring glucose-driven respiration in E. coli expressing URF13 (Dewey et al., 1988). 3H-Pm-toxin retained its toxicity on E. coli expressing URF13 (data not shown), which corroborates earlier reports of the activity of 3H-Pm-toxin on cms-T mitochondria (Frantzen et al., 1987; Holden and Sze, 1987a).

Figure 2 shows 3H-Pm-toxin binding in E. coli harboring expression vector plasmids which either do (pLC13-T) or do not (pLC236) contain a T-urf13 insert (Remaut, Stansaens, and Fiers, 1981; Braun et al., 1989b). Both cultures were grown under conditions (42°C) that induce transcription from the plasmid's pL promoter. Binding in the control (pLC236) reflects endogenous toxin binding in E. coli, which we refer to as nonspecific binding, whereas E. coli harboring pLC13-T display greater toxin binding due to expression of URF13. Nonspecific toxin binding, fitted to a combination of linear equations, was subtracted from the total binding observed in pLC13-T to calculate specific toxin binding. The difference between total and nonspecific binding was used to calculate specific toxin binding.

![Figure 1. Structure of a Fungal Pathotoxin and Methomyl.](image-url)

(A) Structure of component C of Pm-toxin (redrawn from Frantzen et al., 1987). The 35-carbon methylene backbone is represented by the jagged line. Hydrogens bound to the methylene backbone are not shown for clarity. Arrows indicate carbonyl groups that are the sites of reduction with tritiated sodium borohydride.

(B) Structure of S-methyl-N-[(methylcarbamoyl)oxy]thioacetimide (methomyl).
Fungal Toxins Bind to URF13

**Figure 2.** Total \(^{3}H\)-Pm-Toxin Binding in *E. coli*.

Aliquots of *E. coli* (0.5 mg of protein) were incubated for 30 min with varying concentrations of \(^{3}H\)-Pm-toxin, and the cells were then centrifuged through silicone oil to partition unbound and bound toxin. *E. coli* expressing URF13 (pLC13-T) are indicated by solid boxes (■) and *E. coli* not expressing URF13 (pLC236) by open boxes (□). Bound toxin is expressed as picomoles of \(^{3}H\)-Pm-toxin bound per milligram of *E. coli* protein.

Specific toxin binding displays a sigmoidal-shaped curve, although it is not readily apparent because of the poorly defined inflection point. The sigmoidal binding curve is indicative of cooperative binding. Cooperative binding is better demonstrated when the data are presented in a Scatchard-type plot (Scatchard, 1949) (Figure 3, inset); the resulting nonlinear curve indicates positive cooperativity associated with the binding of toxin to URF13. The Hill coefficient \((n)\), which provides a numerical estimate of cooperativity, was determined from the position of the peak on the Scatchard plot (Segel, 1975). Data in Figure 3 have an \(n\) value of 1.5. An \(n\) value greater than 1.0 indicates positive cooperativity; the larger the \(n\) value, the more cooperative the binding. Oxygen binding by hemoglobin, which has four distinct binding sites, is a classic example of cooperative binding and displays an \(n\) value around 2.7 (Ogata and McConnell, 1971). The apparent dissociation constant \((K_d)\), calculated from the data in Figure 3, is 70 nM. Repetition of the binding experiment has demonstrated that the amount of URF13 expressed in *E. coli* is variable, and this variation affects both the saturating level of toxin binding and the magnitude of cooperativity. Conversely, nonspecific binding in control *E. coli* (pLC236) does not exhibit any appreciable variability. Saturating levels of specific toxin binding are approached asymptotically and generally range between 300 pmol and 400 pmol of toxin per milligram of *E. coli* protein. The observed cooperativity \((n)\) varies between 1.4 and 2.0 (data not shown).

**Competition and Displacement Studies**

Methomyl mimics the effects of Pm- and BmT-toxins in *cms-T* mitochondria and in *E. coli* expressing URF13, although much higher concentrations of methomyl (4 mM) are required to observe a toxic effect (structure of methomyl shown in Figure 1B). Figure 4 shows the results of a binding study in which *E. coli*, either expressing or not expressing URF13, were titrated with \(^{3}H\)-Pm-toxin in the absence or presence of added methomyl. \(^{3}H\)-Pm-toxin binding in control *E. coli* (pLC236), plus or minus 16 mM methomyl, indicates that nonspecific Pm-toxin binding is unaffected by added methomyl. Conversely, in *E. coli* expressing URF13, the presence of even low concentrations (2 mM) of methomyl reduces specific \(^{3}H\)-Pm-toxin binding, shown in Figure 3, estimates specific binding due to URF13 expression. With excess \(^{3}H\)-Pm-toxin, specific toxin binding is linear with respect to *E. coli* protein concentration and requires approximately 20 min of incubation for complete binding (data not shown).

**Figure 3.** Specific \(^{3}H\)-Pm-Toxin Binding in *E. coli* Expressing URF13.

Specific \(^{3}H\)-Pm-toxin binding in *E. coli* was determined by subtracting nonspecific toxin binding to pLC236 from total toxin binding to pLC13-T. The solid line in each plot represents a theoretical curve generated using the following parameters: \(K_d = 70\) nM, maximum binding of 350 pmol/mg of *E. coli* protein, and a Hill coefficient \((n)\) value of 1.5. *Inset.* Scatchard analysis of binding data.
binding. Increasing the methomyl concentration produces a set of binding curves that are shifted along the abscissa toward higher unbound toxin concentrations. If the nonspecific binding is subtracted from the \(^{3}\)H-Pm-toxin plus methomyl binding curves, an apparent dissociation constant \(K_d'\), which is a function of the added methomyl concentration and the methomyl binding constant, can be derived from each titration (Munson and Rodbard, 1980). Based upon the results in Figure 4, the average dissociation constant for methomyl, calculated from each \(K_d'\), is approximately 1.0 mM.

Figure 5 shows a displacement experiment in which aliquots of \(E. coli\), either expressing or not expressing URF13, were incubated for 30 min with 0.2 \(\mu\)M \(^{3}\)H-Pm-toxin. After this incubation, bound \(^{3}\)H-Pm-toxin was displaced during the course of a second 30-min incubation by addition of either 10 \(\mu\)M unlabeled BmT-toxin or 32 mM methomyl. The amount of \(^{3}\)H-Pm-toxin binding in each sample was normalized such that \(E. coli\) expressing URF13 and without added unlabeled toxin or methomyl was defined as 100%. \(^{3}\)H-Pm-toxin binding in control \(E. coli\), not expressing URF13, was only slightly affected by secondary additions. By contrast, a significant amount of \(^{3}\)H-Pm-toxin was displaced in \(E. coli\) expressing URF13 when either unlabeled toxin or methomyl was added. Because specifically bound toxin can be displaced by either unlabeled toxin or methomyl, we conclude that specific toxin binding to URF13 is reversible.

**Toxin Binding in URF13 Mutants**

Mutational analysis in \(E. coli\) has revealed three distinct toxin-insensitive URF13 mutants (Dewey et al., 1988; Braun et al., 1989b). Substitutions replacing the side chain of aspartate-39 define one set of mutants. The other two mutants are deletional mutations: one contains an internal deletion, missing amino acids 2 through 11, and the other a carboxyl-terminal deletion of 33 amino acids, which yields a mutant URF13 of 82 amino acids. Binding studies for each of the three toxin-insensitive URF13 mutants were performed to determine whether toxin insensitivity is correlated with the capacity to bind \(^{3}\)H-Pm-toxin. The results are shown in Figure 6. The substitutional mutation at residue 39 and the carboxyl-deletional mutant each show a marked reduction in specific toxin binding. At higher toxin concentrations (10 \(\mu\)M to 100 \(\mu\)M), these mutants bind a substantial amount of toxin; at these concentrations, the mutants are still insensitive to the effects of toxin (data not shown). In contrast, the internal deletional mutant,
Three toxin-insensitive URF13 mutants, represented by open symbols, were assayed for \(^{3}H\)-Pm-toxin binding as described in the legend to Figure 1: deletional mutant missing amino acid residues 2 through 11 (△), valine substitutional mutant at residue 39 (○), and the 82 amino acid truncated URF13 (■). Specific binding of the mutants is shown by dotted lines; for comparison, the standard URF13-specific \(^{3}H\)-Pm-toxin binding curve (solid line, ■), taken from Figure 2, is shown.

missing residues 2 through 11, specifically binds approximately 300 pmol of toxin, nearly as much as the standard URF13, which binds 350 pmol of toxin. On a Scatchard plot, the 2 to 11 deletional mutant reveals cooperative binding; however, cooperativity, \(n = 1.0 \) to \(1.2\), is lower than with the standard URF13, \(n = 1.5\).

**Toxin Binding in Maize Mitochondria**

The cytoplasmic male sterility phenotype in maize is countermanded by the action of dominant nuclear restorer genes. cms-T mitochondria, isolated from tissue containing the restorer genes, are referred to as T-restored mitochondria. Total toxin binding to isolated, purified cms-T, T-restored, and normal maize mitochondria is shown in Figure 7A. Consistent with the previous report (Frantzen et al., 1987), considerable nonspecific binding is observed in normal mitochondria. In contrast to the previous report, we detect more toxin binding in cms-T than in normal mitochondria, and was later identified as URF13 (Dewey et al., 1987; Wise et al., 1987b). In this report, we provide evidence of a T-toxin-URF13 interaction by demonstrating specific toxin binding in both \(E. coli\) and isolated mitochondria expressing URF13. Previously, differences in toxin binding could not be detected between cms-T and normal mitochondria (Frantzen et al., 1987). We believe that the small, but consistent differences in toxin binding between cms-T, T-restored, and normal mitochondria (Figure 7A) are attributable to a more sensitive binding assay.

**DISCUSSION**

Based on the fungal toxin-mediated ion efflux in \(E. coli\) expressing the T-urfl3 gene, we have suggested that a toxin-URF13 interaction leads to permeabilization of the bacterial plasma membrane (Braun et al., 1989b), analogous to the interaction that occurs in the inner mitochondrial membrane of cms-T mitochondria. Prior to the discovery of T-urf73, Klein and Koepppe (1985) proposed that the effects of T-toxin on cms-T mitochondria could be explained by permeabilization of the inner membrane, and suggested that a 13-kD mitochondrial translation product is involved. The 13-kD protein was shown by Leaver and associates (Forde, Oliver, and Leaver, 1978; Forde and Leaver, 1980) to be unique to T mitochondria, and was later identified as URF13 (Dewey et al., 1987; Wise et al., 1987b). In this report, we provide evidence of a T-toxin-URF13 interaction by demonstrating specific toxin binding in both \(E. coli\) and isolated mitochondria expressing URF13. Previously, differences in toxin binding could not be detected between cms-T and normal mitochondria (Frantzen et al., 1987). We believe that the small, but consistent differences in toxin binding between cms-T, T-restored, and normal mitochondria (Figure 7A) are attributable to a more sensitive binding assay.

Binding of chemically reduced, \(^{3}H\)-Pm-toxin in \(E. coli\) expressing URF13 displays a sigmoidal-shaped curve, indicative of cooperative binding (Figure 3). Analysis of binding data on a Scatchard plot (Figure 3, inset) yields a coefficient (\(n = 1.5\)) whose magnitude indicates positive cooperativity. Such positive cooperative binding could be obtained if there are multiple T-toxin binding sites on each URF13 molecule. Alternatively, if URF13 binds only one toxin molecule per monomer but exists in an oligomeric state in the membrane, positive cooperativity would occur if binding of one toxin molecule to an URF13 oligomer
The Plant Cell

enhances binding of additional toxin molecules. The $n$ value only measures the degree of cooperativity and does not reveal the maximum number of toxin molecules that can bind to an oligomer. For example, an $n$ value of 1.5 could signify either moderate cooperativity with a dimeric URF13 structure or weak cooperativity in a tri- or tetrameric URF13 complex (Wyman, 1963). Preliminary cross-linking studies suggest that URF13 exists as an oligomer in E. coli and cms-T mitochondria (J. N. Siedow and C. S. Levings, unpublished data).

Assuming that each molecule of URF13 binds a single toxin molecule, then 350 pmol of toxin bound per milligram of E. coli protein (Figure 3) is equivalent to 350 pmol of URF13 per milligram of E. coli protein. With the same assumption, we can estimate that cms-T mitochondria contains only 20 pmol of URF13 per milligram of mitochondrial protein. When compared with other membrane-bound components, the abundance of URF13 in cms-T mitochondria is very low. For example, plant mitochondria contain approximately 300 pmol of cytochrome $aa_3$ per milligram of protein (Douce, 1985). These estimates of URF13 are based on the ability to bind T-toxin and may not reflect the actual amount of URF13 present. Comparison of URF13 expression, using anti-URF13 antibody, in E. coli (pLC13-T), cms-T, and T-restored mitochondria do not agree with the estimate from the binding assay. Antibody-decorated protein blots show that the quantity of URF13 in cms-T mitochondria is approximately 30% of the level in E. coli, on a per-milligram-of-protein basis (C. J. Braun and C. S. Levings, unpublished data). URF13 abundance is even less in T-restored mitochondria, where it is substantially reduced when compared with the URF13 present in cms-T mitochondria (Dewey et al., 1987; R. E. Dewey and C. S. Levings, unpublished data). In addition, comparison of in vitro translation products from isolated T restored and cms-T mitochondria show that URF13 expression in T restored is greatly diminished compared with cms-T mitochondria (Forde and Leaver, 1980). It is unclear why antibody detection and toxin binding show such large differences in URF13 abundance in E. coli and cms-T and T-restored mitochondria. Despite this difference in the quantity of URF13, cms-T and T-restored mitochondria are both susceptible to toxin and methomyl. When the inner mitochondrial membrane is permeabilized by the toxin-URF13 interaction, the mitochondrion becomes uncoupled and unable to produce ATP by oxidative phosphorylation. From a functional perspective, permeabilized mitochondria, either by several (e.g., T-restored) or many (e.g., cms-T) toxin-URF13 interactions, are rendered inactive.

Unlike toxin binding in E. coli expressing URF13, toxin binding in cms-T mitochondria does not show cooperativity. Because of the low level of specific binding in cms-T and T-restored mitochondria, experimental error, particularly with lower toxin concentrations, may be large enough to mask the cooperative nature of toxin binding. Alternatively, toxin binding in cms-T mitochondria may not be

---

**Figure 7.** Toxin Binding in Maize Mitochondria.

(A) Total $^3$H-Pm-toxin binding in isolated cms-T, T-restored, and normal mitochondria. 0.5-mg aliquots of sucrose-purified normal (□), T-restored (▲), and cms-T mitochondria (■) were incubated with varying amounts of $^3$H-Pm-toxin as described in Methods.

(B) Specific $^3$H-Pm-toxin binding in T-restored (▲) and cms-T mitochondria (■). Specific $^3$H-Pm-toxin binding was calculated by subtracting total $^3$H-Pm-toxin binding to normal mitochondria from the total $^3$H-Pm-toxin binding from either cms-T or T-restored mitochondria.
cooperative. The lack of cooperativity, however, does not preclude the possibility that URF13 can exist in the cms-T mitochondrial membrane as an oligomer. An example of a trimeric oligomer that does not exhibit allostery is ornithine transcarbamoylase (Kuo, Zambidis, and Caron, 1989). Further research is required to determine how URF13 exists in the inner membrane of cms-T mitochondria.

Although methomyl is structurally unrelated to the fungal T-toxins (Figure 1), it mimics the effects of toxins in cms-T mitochondria and in E. coli expressing URF13. In a series of competition experiments, we bound chemically reduced, $^3$H-Pm-toxin plus increasing amounts of methomyl to E. coli expressing URF13. The resulting toxin binding curves are shifted to progressively higher toxin concentrations with increasing methomyl concentrations (Figure 4). Calculations from these data yield a $K_d$ for methomyl of approximately 1.0 mM. The large difference between the $K_d$ of toxin (about 70 nM) and methomyl (about 1 mM) probably reflects differences in their lipophilicity and, thus, their accessibility to a membrane-localized URF13 binding site. Assays on cms-T mitochondria, using synthetic derivatives of methomyl, have shown that more lipophilic derivatives of methomyl are capable of disrupting mitochondrial function at the same low concentrations as toxin (Aranda, Durlin, and Gauvrit, 1987).

Bound $^3$H-Pm-toxin is displaced by the addition of large amounts of unlabeled BmT-toxin or methomyl (Figure 5). From these displacement and competition experiments, we conclude that BmT-toxin, Pm-toxin, and methomyl compete for the same, or at least for overlapping, binding sites on URF13 and that toxin binding is reversible. The apparent identity of T-toxin and methomyl binding sites on URF13 is supported by the finding that more than 50 site-directed URF13 mutations in E. coli were unable to distinguish sensitivity to Pm- and BmT-toxins from sensitivity to methomyl (Braun et al., 1989b; C. J. Braun and C. S. Levings, unpublished data).

Toxin binding studies in E. coli expressing toxin-insensitive mutant URF13 proteins provide insight into the regions of URF13 required for toxin-URF13-induced membrane permeabilization. Toxin-insensitive URF13 molecules, lacking the carboxyl-terminal 33 amino acids or containing a substitution of the side chain at aspartate-39, display a drastically reduced capacity to bind T-toxin (Figure 6). Because these mutant URF13 molecules bind a small amount of toxin, it is unlikely that the binding site(s) has been eliminated. It is more plausible that these mutations have altered the structure of URF13 in the lipid bilayer and diminished the binding site's affinity for T-toxin. In chloroplasts, for example, atrazine resistance is caused by a mutation in the D1 protein that increases the herbicide's $K_d$ approximately 100-fold (Pfister and Arntzen, 1979; Steinback et al., 1981). In a third toxin-insensitive mutant, missing amino acid residues 2 through 11, the presence of T-toxin binding indicates that toxin insensitivity is possible even with toxin binding. In this mutant, the nature of T-toxin binding is altered; cooperativity is diminished. We speculate that, although amino acids 2 through 11 are unnecessary for toxin binding, they are essential for the T-toxin-URF13 interaction leading to membrane permeabilization.

METHODS

Toxin Isolation and Labeling

BmT- and Pm-toxins were supplied by Dr. J.M. Daly's laboratory, University of Nebraska; the "C" component of Pm-toxin was isolated by Dr. Stephen Danko (Danko et al., 1984). Approximately 10 mg of the C component was reduced with thiolated sodium borohydride by the Amersham Corporation and purified by gel filtration over LH-20-100 Sephadex resin (Frantzen et al., 1987). The final specific activity (11.4 Ci/mmol) of the $^3$H-Pm-toxin was determined by drying a desiccated aliquot, approximately 2.3 mCi, to a constant weight on an analytical CAHN 25 Automatic Electrobalance. The entire sample was then dissolved in DMSO and the total counts per minute determined by liquid scintillation counting. The efficiency of counting was determined with a $^3$H standard.

E. coli: Expression of URF13, Toxin Sensitivity Assay, and Toxin Binding

E. coli containing the c857 temperature-sensitive repressor gene and harboring expression plasmids that either do (pLC13-T) or do not (pLC236) contain the T-urf73 gene were grown under inducing conditions (Braun et al., 1989b). Cells were harvested, and sensitivity to T-toxin was assayed by monitoring the inhibition of glucose-driven respiration when challenged with either T-toxin or methomyl (Dewey et al., 1988). E. coli protein concentration was determined by the method of Lowry et al. (1951). To assay binding of $^3$H-Pm-toxin, 1 mL of M9 salts (42 mM Na$_2$HPO$_4$, 22 mM KH$_2$PO$_4$, 8.6 mM NaCl, 19 mM NH$_4$Cl), and E. coli (0.5 mg of protein) were incubated at room temperature in an Eppendorf tube containing added $^3$H-Pm-toxin. After 30 min, bound and unbound toxin were separated by centrifuging 0.7 mL of the reaction mixture through a 0.5-mL layer of silicone oil (Wacker'silicone AR200) in an Eppendorf microcentrifuge tube. The oil has a greater density than the assay buffer and showed no affinity for chemically reduced $^3$H-Pm-toxin. After centrifugation, the aqueous supernatant and pellet fractions contained unbound and bound toxin, respectively. The total pellet fraction was resuspended in 0.1 mL of distilled water, and the amounts of bound and unbound $^3$H-Pm-toxin were determined using liquid scintillation counting.

Mitochondrial Isolation, Toxin Sensitivity Assays, and Toxin Binding

Normal (N), T-restored, and cms-T maize mitochondria (Zea mays) were isolated and purified from 5-day-old to 7-day-old dark-grown shoots by previously published methods (Siedow and Bickett, 1983). All plants were inbred lines that contained the B37 nuclear background. The genotype of the restorer genes of the cms-T
plants is r1f/r1f and R12/R12; T-restored plants contained each of the dominant T-restorer genes in a homozygous condition (R1f/ R1f and R12/R12). Mitochondrial activity was measured polarographically using a Clark electrode, and toxin sensitivity was assessed as the inhibition of state-3 malate oxidation (Koepp et al., 1978).

Toxin binding studies using mitochondria were similar to those described above for E. coli, except that a 9:1 mixture (v/v) of Wacker AR200 and AR20 silicone oils was used and a mitochondrial reaction buffer (0.4 M mannitol, 1 mM K2HPO4, 10 mM KCl, 5 mM MgCl2, 10 mM Hepes, pH 7.2) was substituted for M9 salts.

**Competition and Displacement Assays**

In the competition experiments, [3H]-Pm-toxin binding titrations were carried out in the absence or presence of a constant concentration of unlabeled methomyl. Both [3H]-Pm-toxin and methomyl were added and mixed before E. coli addition. After a 30-min incubation, bound and unbound toxin were separated by centrifugation through silicone oil and measured by scintillation counting as described above. In the displacement experiments, 0.2 μM (total) [3H]-Pm-toxin was incubated with 0.5 mg of E. coli following the standard procedure. After a 30-min incubation, either 10 μM unlabeled BmT-toxin or 32 mM methomyl was added to the reaction, which was then incubated for a second 30-min period. Bound and unbound toxin were subsequently measured.

**ACKNOWLEDGMENTS**

This is paper no. 12413 of the Journal Series of the North Carolina Agricultural Service (Raleigh, NC 27695-7601). We gratefully acknowledge the technical assistance of Cyril Kaspi. This work was supported by grants from the Agrigenetics Research Corporation (C.S.L.), National Science Foundation (C.S.L.), and U.S. Department of Energy (J.N.S.).

We wish to dedicate this paper to the memory of David E. Koepp, whose research contributed significantly to understanding the nature of the interactions of T-toxin and methomyl with cms-T mitochondria and, as such, laid the experimental foundation for the work presented here.

Received November 22, 1989; revised December 22, 1989.

**REFERENCES**


Fungal Toxins Bind to URF13

161


Fungal toxins bind to the URF13 protein in maize mitochondria and Escherichia coli.
C J Braun, J N Siedow and C S Levings, 3rd
*Plant Cell* 1990;2:153-161
DOI 10.1105/tpc.2.2.153

This information is current as of August 27, 2017