Toxin-Deficient Mutants From a Toxin-Sensitive Transformant of 
_Cochliobolus heterostrophus_

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ABSTRACT

Tox1 is the only genetic element identified which controls production of T-toxin, a linear polyketide involved in the virulence of _Cochliobolus heterostrophus_ to its host plant, corn. Previous attempts to induce toxin-deficient (Tox1) mutants, using conventional mutagenesis and screening procedures, have been unsuccessful. As a strategy to enrich for Tox1 mutants, we constructed a Tox1+ strain that carried the corn T-urfl3 gene (which confers T-toxin sensitivity) fused to a fungal mitochondrial signal sequence; the fusion was under control of the inducible _Aspergillus nidulans_ pelA promoter which, in both _A. nidulans_ and _C. heterostrophus_, is repressed by glucose and induced by polygalacturonic acid (PGA). We expected that a transformant carrying this construction would be sensitive to its own toxin when the T-urfl3 gene was expressed. Indeed, the strain grew normally on medium containing glucose but was inhibited on medium containing PGA. Conidia of this strain were treated with ethylmethanesulfonate and plated on PGA medium. Among 362 survivors, 9 were defective in T-toxin production. Authenticity of each mutant was established by the presence of the transformation vector, proper mating type, and a restriction fragment length polymorphism tightly linked to the _Tox1_ locus. Progeny of each mutant crossed to a Tox1+ tester segregated 1:1 (for wild type toxin production vs. no or reduced toxin production), indicating a single gene mutation in each case. Progeny of each mutant crossed to a Tox1+ tester segregated 1:1 (for no toxin production vs. no or reduced toxin production) indicating that each mutation mapped at the _Tox1_ locus. Availability of Tox1+ mutants will permit mapping in the _Tox1_ region without interference from a known _Tox1_ linked translocation breakpoint.

_Cochliobolus heterostrophus_, a haploid Ascomycete, exists in nature as either of two races. Race T produces T-toxin, a family of 10–15 linear polyketides which vary in chain length from C_{15} to C_{46} (Kono and Daly 1979; Kono et al. 1981a,b), and is specifically virulent on corn containing Texas male sterile (T) cytoplasm (Yoder 1980). Race O produces none of the members of the T-toxin family and has low virulence on T cytoplasm corn as well as on most other types of corn. When field isolates of race T and race O are crossed, progenies usually segregate 1:1 (Tox+:Tox−), thus defining the single genetic element _Tox1_, which controls production of T-toxin (Bronson et al. 1990; Leach et al. 1982b). Off ratios observed in progeny of certain field isolates (Taga et al. 1985; Yoder 1976; Yoder and Gracen 1975) have been explained by linkage of _Tox1_ to an ascospore abortion factor, possibly a spore killer gene (Bronson et al. 1990). Plant tests of progeny of all crosses reveal that without exception _Tox1_ progeny are highly virulent on T-cytoplasm corn whereas all _Tox1_ progeny are weakly virulent; thus T-toxin is highly associated with enhanced virulence of the fungus to T-cytoplasm corn (Bronson et al. 1990; Yoder 1980).

Sensitivity of T-cytoplasm corn to T-toxin is caused by a 13-kD T-toxin-binding protein found in the inner mitochondrial membrane of the host (Levings and Sadow 1992). The protein is encoded by a mosaic gene (T-urfl3), which is unique to the mitochondrial chromosome of T-cytoplasm corn. When T-urfl3 is expressed in _Escherichia coli_ under control of an _E. coli_ promoter, the cells are sensitive to T-toxin as mitochondrial T-cytoplasm corn (Devey et al. 1988). These T-toxin-sensitive cells have been developed as the basis of an efficient and reliable microbiological assay for T-toxin (Giuffetti et al. 1992). In addition to expression in _E. coli_, T-urfl3 has been shown to function in yeast (Huang et al. 1990), tobacco (Vonallmen et al. 1991) and insects (Korth and Levings 1993). For yeast, a fungal mitochondrial signal sequence fused to T-urfl3 targeted the URFL3 protein to the mitochondria; without the signal neither the cells nor the mitochondria themselves were sensitive to T-toxin. In contrast, when _T-urfl3_ without a mitochondrial signal sequence was expressed in tobacco or insects, both were sensitive to T-toxin.

Successful heterologous expression of _T-urfl3_ suggested that the URFL3 protein might also function to confer T-toxin-sensitivity in _C. heterostrophus_. We predicted that expression in a _Tox1_ strain would be detrimental to the growth of the fungus, since it would be sensitive to its own toxin. This paper describes the successful construction of such a strain and its use to enrich for Tox− mutants in a population of mutagenized conidia. Nine _Tox−_ mutants were collected; all mapped

Materials and methods

strains, media, crosses and transformation: C. heterostrophus strains C4 (Tox1'; MAT-2; ATCC 48331), C5 (Tox1'; MAT-1; ATCC 48332), C9 (Tox1'; MAT-1) are members of an isogenic line generated by backcrossing progeny of field isolates (Leach et al. 1982a). Media, growth conditions, and storage of C. heterostrophus have been described previously (Turgeon et al. 1985) as have mating (Leach et al. 1982a) and transformation (Turgeon et al. 1993) procedures. CMX is CM (complete medium) with xylose instead of glucose (Tseng et al. 1992); CMNS is CM with salts omitted. Polyalgaluronic acid medium (PGA) consisted of 0.1 m sodium phosphate, pH 7.0, 0.5% polygalacturonic acid, 0.5% sorbose, 1% yeast extract and 1% enzymatic cascin hydrolysate. Culture filtrate containing T-toxin was harvested from a seven day old still culture of strain C4 grown in Fries medium (Pringle and Braun 1957). Methomyl (DuPont), which mimics the biological effects of T-toxin (Klein and Koepe 1985), was included in CMNS at a final concentration of 5 mm.

The plasmid carrying the C. heterostrophus restriction fragment length polymorphism (RFLP) marker G264 in pGem2 was supplied by C. Bronson (Tseng et al. 1992). Plasmids pNL9.LORF13, pHPG, and pRD301 were provided by A. Myers (Huang et al. 1990), W. Schäfer (Mönke and Schäfer 1993) and R. Dean and W. Timberlake, respectively. The pelA gene carried by pRD301 has been described (Dean and Timberlake 1989).

Plasmid constructions: Three vectors were made. pHNU5: A 660 bp PstI-EcoRI fragment from the plasmid pNL9.LORF13 containing the T urfl3gene fused to the Neurospora crassa ATP synthase subunit 9 targeting peptide (MSS) was transferred to the same sites in the polylinker of pBluescript SK- (Stratagene). A 0.7 kb BamHI-HindIII fragment, carrying the MSS:T-urfl3sequence, was ligated to a 5.8 kb BamHI-HindIII fragment of pHPG, resulting in plasmid pHNU2 (ca. 6.5 kb). A 1.3 kb SacI fragment of pHNU2 carrying C. heterostrophus promoter1 (Turgeon et al. 1987) was inserted into the vector pHNU2 (MSS::T-urfl3) and the entire cassette was inserted into a vector (pHPG; Mönke and Schäfer 1993) containing the selectable marker hygB and transforming pHNU3 (MSS::T-urfl3) was transformed into C. heterostrophus strain C4. Conidia of one transformant (C4 urfl3-1) was mutagenized with EMS to 99% kill and plated on PGA medium (which induces pelA) to enrich for Tox- mutants. Survivors were screened for ability to produce T-toxin in a microbial bioassay (Cuiffetti et al. 1992).

Results

construction of T-toxin sensitive strains of C. heterostrophus: To determine if T urfl3 could confer T-toxin sensitivity to C. heterostrophus, pHNU3 (promoter1::MSS::T-urfl3) was transformed into C. heterostrophus strain C5 (Tox1'). The recipient strain (C5) was necessarily Tox- since promoter1 is constitutive. Transformants grew normally on CMNS but were inhibited (Figure 2) on the same medium containing either 5 mm methomyl or culture filtrate from strain C4 (Tox1').

To determine if T-urfl3 could cause self-inhibition, pHNU3pelA (pelA::MSS::T-urfl3) was transformed into C. heterostrophus strain C4 (Tox1'). Control transformants carried pHPGpelA (pelA::GUS). Transformants expressing pHNU3pelA grew normally from mycelial inoculum on medium containing glucose (which represses the pelA promoter) but, on PGA medium.
(which induces the pelA promoter), were only half the size after three days and 80% of the size after 1 week, of those grown on glucose as measured by colony diameter. Control transformants carrying pHPGpelA grew normally with either glucose or PGA. When conidial germination and germ tube elongation were monitored on PGA medium, those carrying T-urfl3 lagged behind those carrying GUS. For example, six hours after the start of germination 80% of the GUS conidia had germinated while only 10% of the T-urfl3 conidia had germinated. Within twelve hours however, germination was 95% for conidia of both strains. Similarly, germ tube elongation was initially slower in the T-urfl3 strain but by 12 hr germ tube lengths were the same for both strains. Thus, expression of T-urfl3 in a Toxl+ strain was not lethal, but resulted in inhibition of fungal growth. We then determined whether this level of self inhibition was sufficient to allow enrichment for Tox- mutants.

**Isolation of Tox- mutants:** Conidia of transformant C4urfl3-1 (Toxl+; MAT-2; hygB; T-urfl3) were treated with EMS and plated on PGA medium (Figure 1b). As a control, conidia of strain C4gus-1 (Toxl+; MAT-2; hygB; GUS) were treated similarly. Survivors were tested for T-toxin production by microbial assay. In the first experiment with C4urfl3-1, 7 of 212 survivors were Tox- (defective in T-toxin production) and in a second experiment, two of 150 survivors were Tox-. In the control test with C4gus-1, none of 306 survivors was Tox-. Thus induced expression of T-urfl3 in Toxl+ cells enriches for the Tox- phenotype when cells are plated on PGA medium.

To determine if pHNUN3pelA integrated near the Toxl locus, one of the Tox- mutants, ctm45 (hygR; Tox-, see Table 1) was crossed to tester strain C9 (hygS; Toxl+) and progeny were scored for parental vs recombinant types. A ratio of 26:18 was obtained which is not different from 1:1 (at the 5% level of significance) and indicates that the two genes hygB and Toxl are not closely linked. To verify that the pelA::MSS::T-urfl3 sequence was intact and unrearranged in the genome, blots of DNA from strain C4urfl3-1 (Toxl+; T-urfl3; hygB) were probed with pelA::MSS::T-urfl3. Hybridization of the probe to a band identical in size to that on the transformation vector indicated that this sequence is intact in the genome of strain C4urfl3-1.

**Characterization of Tox- survivors:** T-toxin bioassay: A microbial assay in which the nine Tox- survivors are compared to each other and to ToxP and Toxl- wild type controls is shown in Figure 3. When inocula for the assay were taken from colonies growing on CM, one mutant (ctm45) did not produce a halo; the other eight produced halos of variable size, all clearly smaller than those produced by wild type race T. When inocula were taken from colonies growing on Fries medium, which encourages high production of T-toxin, one strain (ctm45) caused no detectable halo while the other eight showed a range of halo sizes that generally were larger than those on CMX; none was as large as race T control halos. Thus, eight of the mutants appear to be leaky and one of the nine is tight.

**Plant assays:** Each mutant was inoculated on corn plants. Only one of them, ctm45, produced symptoms indistinguishable from those of a race O control strain in repeated assays (Figure 4). The remaining eight candidates showed weak race T type symptoms. These results are consistent with those of the toxin bioassay: the
Assay of C. heterostrophus strains on T-cytoplasm corn. Blocks of agar medium bearing mycelium and conidia were placed in water in the whorls of two week old corn seedlings. Plants were held overnight in a mist chamber, then incubated five days in a growth chamber. For the photograph, three leaves were chosen from sets of plants inoculated with (from left to right): race T (ToxI') strain C4; race O (ToxI') strain C5; 'tox' mutant ctm45; Tox' ascospore progeny (1151-3-1) of a cross of ctm45 × C9. Note that race T caused browning, complete tissue collapse and senescence of the leaves as well as yellow streaks (arrow), associated with T-toxin production in the noncollapsed tissue. The Tox' mutants caused symptoms indistinguishable from those of race O, i.e., small necrotic lesions rather than tissue collapse, and no yellow streaks associated with T-toxin production.

Genetic analyses: To test the heritability of the apparent mutations, each of the nine Tox' survivors was crossed with ToxI' tester strain C9 and the progeny grown on CMX were tested for T-toxin production using the microbial assay. In all cases Tox':Tox' (toxin-defective) segregation was 4:4 in tetrad s and 1:1 among random spores (Table 1). This indicates that each survivor carries a mutation which segregates as a single gene in meiosis.

The relationship between the mutations and the defined ToxI locus was determined by crossing each mutant to ToxI' tester strain C5. In the microbial assay for T-toxin, all tetrad and random spore progeny (grown on CMX) were Tox' (no halos or small halos). The absence of wild-type halos in any progeny indicates that each mutation maps at or near the ToxI locus (Table 2).

Analysis of tight and leaky mutants: Mutant ctm45 has a tight Tox' phenotype. Toxin production by ctm45 tight mutant has a race O phenotype on plants whereas the leaky mutants have weak race T phenotypes.

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of tetrad s</th>
<th>Tox':Tox'</th>
<th>No. of random spores</th>
<th>Tox':Tox'</th>
</tr>
</thead>
<tbody>
<tr>
<td>ctm45</td>
<td>6</td>
<td>4:4</td>
<td>44</td>
<td>22:22</td>
</tr>
<tr>
<td>ctm105</td>
<td>5</td>
<td>4:4</td>
<td>65</td>
<td>52:33</td>
</tr>
<tr>
<td>ctm106</td>
<td>5</td>
<td>4:4</td>
<td>50</td>
<td>27:23</td>
</tr>
<tr>
<td>ctm107</td>
<td>8</td>
<td>4:4</td>
<td>44</td>
<td>21:25</td>
</tr>
<tr>
<td>ctm108</td>
<td>4</td>
<td>4:4</td>
<td>58</td>
<td>29:21</td>
</tr>
<tr>
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<td>4:4</td>
<td>44</td>
<td>24:20</td>
</tr>
<tr>
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<td>4:4</td>
<td>54</td>
<td>24:30</td>
</tr>
<tr>
<td>ctm113</td>
<td>12</td>
<td>4:4</td>
<td>40</td>
<td>18:22</td>
</tr>
<tr>
<td>ctm118</td>
<td>6</td>
<td>4:4</td>
<td>44</td>
<td>21:23</td>
</tr>
</tbody>
</table>

Each Tox' mutant was crossed to tester strain C9 (ToxI'; MAT-1). Progeny were grown on CMX and scored for T-toxin in the microbial assay. In control crosses, ToxI' × ToxI' produced 100% Tox' progeny; ToxI' × ToxI' produced 50% Tox' progeny.

Each strain is a Tox' survivor of EMS mutagenesis (Figure 3).

Tox' = toxin-defective; Tox' progeny of ctm45 produced no halo in the microbial assay for T-toxin; Tox' progeny of the remaining eight mutants produced halos smaller than those of wild-type race T.
was reassessed to determine if its tight phenotype was caused by a defect in toxin secretion. Filtrates and mycelial extracts were prepared from cultures grown in liquid Fries medium. No evidence for T-toxin production by "c" colonies on Fries medium. Ctm106 was chosen to determine genetic control of halo size. It was crossed to produce T-toxin is heritable, as measured in the toxin assay depends on the culture medium used for high.

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of tetrads</th>
<th>No. of random spores</th>
<th>Tox⁻:Tox⁺</th>
<th>Tox⁺:Tox⁻</th>
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</tr>
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<td>0:8</td>
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<td>0:114</td>
</tr>
<tr>
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<td>0:8</td>
<td>82</td>
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</tr>
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<tr>
<td>ctm118</td>
<td>5</td>
<td>0:8</td>
<td>100</td>
<td>0:100</td>
</tr>
</tbody>
</table>

Each Tox⁻ mutant was crossed to tester strain C5 (Tox⁺; MAT-1). Progeny were assayed as in Table 1. In control crosses, Tox⁺ × Tox⁻ produced 50% Tox⁻ progeny. Tox⁺ × Tox⁻ produced 100% Tox⁺ progeny.

DISCUSSION

We have demonstrated that a ToxI⁺ strain of C. heterostrophus carrying the T-urf13 gene from T-cytoplasm corn, which confers sensitivity to T-toxin, is sensitive to its own toxin and have taken advantage of this observation to isolate induced mutants of C. heterostrophus deficient in production of T-toxin. One of the Tox⁺ mutants (ctm45) produces no detectable toxin in culture, either intra- or extracellularly, and causes typical race O symptoms on corn plants. Eight of the Tox⁻ mutants produce detectable amounts of T-toxin in culture (but less than wild type race T strains) and cause weak race T symptoms in plant tests. Tox⁻ progeny of either tight or leaky mutants have the same phenotypes on plants as their Tox⁺ parents. These findings support the hypothesis that T-toxin is required by C. heterostrophus for high...
virulence on T-cytoplasm corn. Previously, the most compelling evidence for this was the cosegregation of T-toxin production and high virulence in progeny of crosses between races T and O (Yoder 1980). Now, an additional line of evidence, analysis of induced mutants, supports the conclusion that T-toxin is a virulence factor. None of the mutants, including ctm45 (which produces no detectable T-toxin), is non pathogenic; thus T-toxin does not appear to be required for pathogenicity. A similar conclusion was also drawn from analyses of segregating progeny from crosses of wild type race T and race O isolates (Yoder 1980).

The mechanism by which T-urfl3 confers T-toxin sensitivity to cells of C. heterostrophus is not known. Although a mitochondrial targeting sequence was fused to the T-urfl3 gene used for transformation, we did not determine whether or not mitochondria of transgenic strains were sensitive to T-toxin. It is clear, however, that expression of T-urfl3 in a Toxl strain of C. heterostrophus under the control of a constitutive promoter, or in a Toxl strain under control of an inducible promoter, caused inhibition of fungal growth in the presence of T-toxin, supplied exogenously in the former case and produced endogenously in the latter case. Expression of T-urfl3 in the presence of T-toxin was not suicidal, but rather caused moderate inhibition of both conidial germination rate and hyphal growth. This modest inhibition was nevertheless sufficient to enrich for Toxl mutants in a population of mutagenized conidia. Nine Toxl mutants among 362 surviving conidia were collected when T-urfl3 was present compared with none among 306 survivors when the GUS gene was substituted for T-urfl3. Presumably, if a transformant were constructed in which T-urfl3 expression was in fact suicidal, selection, rather than enrichment of Toxl mutants would result.

Until now the source of all Toxl strains has been the field collection of race O isolates, all of which differ from Toxl strains by heterozygosity at Toxl (Bronson et al. 1990; Taka et al. 1985; Yoder 1976). The genetic nature of the naturally occurring difference between race O and race T is largely unknown and may be complex. A translocation breakpoint and several different repeated elements have been found, by conventional genetic and RFLP mapping, associated with the Toxl region (Tzeng et al. 1992). Availability of Toxl mutants permits fine structure genetic mapping previously not possible. The existing map of the Toxl region (Tzeng et al. 1992) places Toxl at the breakpoint of the reciprocal translocation and shows several RFLPs closely linked to Toxl (and therefore the breakpoint). The map was made, by necessity, with the progeny of a cross that was heterozygous both for Toxl and for the Toxl-linked translocation breakpoint. Since crossover frequencies can be distorted near translocation breakpoints (Kaper 1974), map distances between markers near the breakpoint may be misleading. The Toxl mutants make it possible to determine map distances using progeny of crosses in which the translocated chromosomes are homozygous, thereby reducing the likelihood of aberrant crossover frequencies. Furthermore, since markers on the two chromosomes involved in a reciprocal translocation appear linked to each other, the Toxl mutants also make it possible to determine whether or not these markers are linked. Toward this end we have analyzed the progeny of a cross between ctm45 (Toxl) and a Toxl strain carrying hygB integrated at an RFLP marker (B88) which maps within one cM of Toxl (Tzeng et al. 1992). Progeny were scored for resistance to hygromycin B and ability to produce T-toxin. Recombinants occurred as frequently as parental types, suggesting that when the breakpoint is homozygous, Toxl and B88 are not closely linked. A similar approach will be used to map additional markers near Toxl.

Crosses between naturally occurring race T and race O isolates usually segregate equal numbers of Toxl and Toxl progeny, consistent with the hypothesis that Toxl is a single gene. While each of the Toxl mutants collected in this study is a single site mutation and each map at Toxl, the fact that most of them are leaky may be relevant to hypotheses for the pathway of T-toxin biosynthesis. Since T-toxin is a large polyketide (predominantly C4) and very large polyketide synthase (PKS) genes, such as the 35-kb eryA gene of Saccharopolyspora erythraea, which produces the C13 polyketide erythromycin (Donadio et al. 1991), and the 65-kb aav gene of Streptomyces avermitilis, which produces the C25 polyketide avermectin (MacNeil et al. 1992), have been described, it is not unreasonable to speculate that the Toxl region encodes a giant PKS gene. Alternatively, the Toxl region may contain several genes, one of which may be a PKS. Our present ability to map the Toxl region without interference from the known translocation breakpoint data should lead to a reliable estimate of the size of Toxl and its eventual cloning.

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LITERATURE CITED


