A putative branched-chain-amino-acid transaminase gene required for HC-toxin biosynthesis and pathogenicity in Cochliobolus carbonum

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The cyclic tetrapeptide HC-toxin is required for pathogenicity of the filamentous fungus Cochliobolus carbonum on maize. HC-toxin production is controlled by a complex locus, TOX2. The isolation and characterization of a new gene of the TOX2 locus, TOXF, is reported. It is shown that TOXF is specifically required for HC-toxin production and pathogenicity. It is present as two or three copies in all HC-toxin-producing (Tox2+) isolates and is absent in toxin-non-producing strains. The deduced amino acid sequence of TOXF has moderate homology to many known or putative branched-chain-amino-acid transaminases from various species. A strain of C. carbonum with all copies of TOXF disrupted grew normally but lost HC-toxin production and pathogenicity. It is proposed that TOXF has a biosynthetic role in HC-toxin synthesis, perhaps to aminate a precursor of Aeo (2-amino-9,10-epoxi-8-oxodecanoic acid).

Keywords: Cochliobolus (Helminthosporium) carbonum, cyclic peptide, branched-chain-amino-acid transaminase, TOXF, HC-toxin

INTRODUCTION

Host-specific toxins, which are produced exclusively by fungi, are generally low-molecular-mass secondary metabolites with diverse structures. They are critical determinants of virulence or pathogenicity in many plant disease interactions (Walton, 1996). The host-selective toxin made by race 1 isolates of Cochliobolus carbonum, called HC-toxin, selectively affects maize (Zea mays) lines of genotype hm1/hm1. Structurally, HC-toxin is a cyclic tetrapeptide, cyclo(d-Pro-l-Ala-d-Ala-l-Aeo), where Aeo stands for 2-amino-9,10-epoxi-8-oxodecanoic acid.

Previous studies on the Mendelian and molecular genetics of HC-toxin production have shown that it is controlled by a Mendelian locus, TOX2, but that this locus has a complex molecular structure (Nelson & Ullstrup, 1961; Scheffer et al., 1967; Ahn & Walton, 1996). All of the known genes necessary for HC-toxin production are present only in HC-toxin-producing (Tox2+) isolates in multiple functional copies, are dedicated to HC-toxin production and, with one exception, are linked over a ~540 kb region of one chromosome (Ahn & Walton, 1996, 1998). TOX2 genes include HTSI, which encodes a 570 kDa tetrapartite non-ribosomal peptide synthetase called HC-toxin synthetase (HTS) (Panaccione et al., 1992; Scott-Craig et al., 1992); TOXA, encoding a putative HC-toxin transporter of the major facilitator superfamily (Pitkin et al., 1996); and TOXC, encoding a fatty acid synthase β subunit (Ahn & Walton, 1997). TOXE encodes a regulatory protein required for expression of TOXA and TOXC (Ahn & Walton, 1998). Another gene, TOXD, is also Tox2+-unique, linked to the other TOX2 genes and co-regulated by TOXE, but TOXD has no defined role in HC-toxin biosynthesis (Y.-Q. Cheng & J. D. Walton, unpublished results). A homologue of TOXD was recently shown to be necessary for correct processing of the growing polyketide chain by the lovastatin nonaketide synthase in Aspergillus terreus (Kennedy et al., 1999).

The known genes of TOX2 can account for the synthesis and assembly of the components of HC-toxin other than the unusual amino acid Aeo. Biogenetically, Aeo is a
fatty acid or polyketide (Cheng et al., 1999) and the specific requirement of TOXC for toxin production argues that the decanoic acid backbone of Aeo is a fatty acid. Nothing is known about the other steps in Aeo biosynthesis, but at least two oxidations (to produce the 8-carbonyl and the 9,10-epoxide) and an amination (at the 2-position) must also occur.

We describe here a strategy using bacterial artificial chromosomes (BACs) to search for additional genes of TOX2. It is based on the assumption that new TOX2 genes would be physically linked, but not clustered, to chromosomes (BACs) to search for additional genes of the 2-position) must also occur.

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METHODS

Fungal strains. C. carbonum SB111 (ATCC 90305) and SB114 are standard Tox2 and Tox2 laboratory strains, respectively, and 164R10 is a Tox2 progeny of a cross between them (Ahn & Walton, 1997).

BAC library construction. The BAC vector pBACwASHINGTON, a generous gift of the Clemson University Genomics Institute (Zhu et al., 1997), was modified by the insertion of restriction sites for AscI and PmeI. Two oligonucleotides, 5'-AGCTGTTTAAAATCGGCCGCCC-3' and 5'-AGCTGCGCGCCGCGCCTTTAAGC-3', were mixed at equimolar concentration, heated at 95 °C for 5 min and annealed slowly to form a complementary DNA linker. This linker was subcloned in-frame into the unique HindIII site of pBACwashington to make a new vector called pBACocta. Fungal chromosomal DNA was embedded and digested in low-melting agarose as described previously (Ahn & Walton, 1996). Proteinase K was removed from the agarose by washing with 50 mM EDTA, followed by 10 mM Tris/1 mM EDTA (TE), pH 8.0, and then with the appropriate buffer for the particular restriction enzyme. DNA was digested with 10 U AscI per gel block for 24 h at 37 °C, and the gel blocks were then digested with β-agarase I (New England BioLabs) following the manufacturer's instructions. DNA fragments were gently precipitated with ethanol, dried under vacuum and dissolved in TE. Ligation reactions contained 5 µg pBACocta, linearized with AscI and dephosphorylated, and 1 µg of digested DNA in a volume of 20 µl, and were incubated for 15 h at 16 °C. Each aliquot (2 µl) of ligation mixture was transformed into 40 µl ELECTROMAX DH10B Escherichia coli cells (Gibco-BRL) by electroporation (2.5 kV, 25 µF, 100 Ω, 0.1 cm cuvette) using a GenePulser apparatus (Bio-Rad). Cells were transferred immediately to a new tube, diluted with 1 ml SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, and 20 mM glucose, pH 7.0), incubated at 37 °C for 1 h and spread on LB plates with 12.5 µg chloramphenicol ml⁻¹, 0.75 µg X-Gal ml⁻¹ and 100 µg IPTG ml⁻¹. After 30 h growth at 37 °C, white recombinant clones were transferred to 96-well microtitre plates. Each well contained 200 µl LB medium with 12.5 µg chloramphenicol ml⁻¹. Plates were incubated for 24 h at 37 °C, then stored at ~80 °C.

NUCLEIC ACID MANIPULATIONS AND ANALYSIS. Fungal DNA and RNA isolation was as described previously (Puttkamr et al., 1996). DNA and RNA blotting, probe labelling, hybridization, cDNA and genomic library screening, and DNA subcloning were done following standard procedures (Sambrook et al., 1989). Oligonucleotides were synthesized by the Michigan State University Macromolecular Facility.

The method for BAC DNA extraction was adopted from Woo et al. (1994). BACs were analysed by clamped homogeneous electrical field (CHEF) electrophoresis (Bio-Rad) (Ahn & Walton, 1996). One microgram of each BAC was digested with NotI and loaded on a gel of 1% chromosome-grade agarose (Bio-Rad). The gels were run at 130 V voltage for 15 h in 0.5 x TBE buffer (1 x TBE: 0.089 M Tris/borate, 0.089 M boric acid, 2 mM Na,EDTA) at 14 °C with a linearly varied 1-22 s switching interval. Gels were subsequently stained with ethidium bromide for 20 min and destained in water before being photographed. Lambda concatemers (New England BioLabs) were used as molecular size standards.

DNA sequencing was done by automated fluorescence sequencing at the Yale University W. M. Keck Foundation Biotechnology Resource Laboratory and the Michigan State University DNA Sequencing Facility. Sequences were assembled and analysed with the DNAStar software package. Multiple sequence alignments were produced with the CLUSTAL W program (Thompson et al., 1994) and decorated with BOXSHADE (program at http://www.ch.embnet.org/software/boxform.html).

The transcriptional start site of TOXF was determined by 5'-RACE (rapid amplification of cDNA ends) using a kit from Gibco-BRL (Frohman et al., 1988). The primer for reverse transcription (GSP1) was 5'-GGGGTTGAGTCCAAATTTT-GACAAC-3' and the nested primer for PCR amplification (GSP2) was 5'-GACGAAAACAGGGTCTGACACAGTA-3'. Two independent RACE products were sequenced.

Creation of targeted mutants. Fungal protoplast preparation, transformation, selection and single-spore isolation of transformants were done as described previously (Panaccione et al., 1992; Pitkin et al., 1996). To make the disruption vector pTOXFD1 (7.0 kb), the central part of the coding region from a cDNA copy of TOXF (the fragment size was 531 bp; this corresponds to +644 bp to +1236 bp in the genomic sequence, taking into account the presence of one intron in the genomic sequence) was amplified by PCR and subcloned into the SalI/XhoI sites of pAMD72 (Pitkin et al., 1996), which contains the Aspergillus nidulans amdS gene for acetamide utilization (Hynes et al., 1983). pTOXFD1 was linearized with BbrII before transformation. To make the replacement vector pTOXF1, pAATTG1, which contains a genomic copy of TOXF, was trimmed with SmaI and Hpal to delete the 5' upstream region and ligated to make the intermediate construct pAATM3. The 383 bp internal Mscl/BbrII fragment of pAATM3 was then replaced by a hygromycin resistance cassette (composed of the E. coli bph gene encoding hygromycin phosphotransferase driven by the A. nidulans trpC promoter) from plasmid pCB1003 (Carroll et al., 1994) to make the final replacement vector pTOXF1 (6.1 kb). The fragment (3-1 kb) containing the bph cassette plus TOXF DNA was released from pTOXF1 by digestion with BamHI and PstI and used for transformation.

ANALYSIS OF MUTANTS. HC-toxin was extracted and analysed by TLC as described previously (Walton et al., 1982; Meeley & Walton, 1991). Pathogenicity was assayed on maize inbred Pr (genotype bnl1/bnl1) by spraying with conidia (Panaccione et al., 1992; Ahn & Walton, 1997).

RESULTS

BAC library construction and screening

The BAC library was constructed from genomic DNA of C. carbonum cut to completion with AscI. A total of 3880 BAC recombinants were identified by blue/white...
colour selection. Based on a sample of 20, the mean insert size was \( \sim 55 \) kb. The BAC library was screened with a mixture of probes corresponding to the known TOX2 genes (HTSI, TOXA, TOXC, TOXD and TOXE). Four BACs with inserts totalling 220 kb were obtained. These BACs were mapped within the TOX2 region by hybridizing with probes corresponding to the different copies of HTSI, TOXA, TOXC, TOXD and TOXE, which can be distinguished from each other by RFLPs (Ahn & Walton, 1996).

**Analysis of TOXF**

BAC A24C1, which harbours an insert of \( \sim 70 \) kb and contains copy 3 of TOXD (Ahn & Walton, 1996), was used as a probe to screen a \( C. \) carbonum cDNA library. The hybridizing cDNAs were sorted into 12 classes (Fig. 1). One representative of each class was tested by DNA hybridization for presence in fungal isolates SB111 (Tox2\(^+\) indicated by ‘\(+\)’) and SB114 (Tox2\(^-\) indicated by ‘\(-\)’). Genomic DNA of isolate SB111 was digested with BamHI, separated on a 0.9% agarose gel, blotted to a nylon membrane and probed individually. Putative functions based on partial sequencing of each cDNA are indicated.

The sequence of C12 showed moderate similarity to several cytochrome P450 genes from other fungi [GenBank accession numbers X82490 (\( Fusarium oxysporum \)), S09643 (\( N. \) crassa) and Y17243 (\( G. \) fujikuroi)]. The predicted product of C12A contains a conserved cytochrome P450 cysteine haem iron ligand signature. Gene C12 is present in most Tox2\(^+\) isolates, including SB111, and is absent in all tested Tox2\(^-\) isolates. However, C12 is absent in isolate 164R10, which produces HC-toxin (Ahn & Walton, 1997). This fact excludes it from having an essential role in HC-toxin biosynthesis.

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<th>C29 TOXF</th>
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**Sequence analysis of TOXF**

Full-length cDNA (pAATC1) and genomic (pAATG1, containing a 2-9 kb PstI/PstI fragment) copies of TOXF were obtained and sequenced on both strands. The novel Tox2\(^+\)-unique genes C12 and C29 were further investigated.

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The BAC clone was radiolabelled and used to screen a cDNA library of \( C. \) carbonum isolate SB111. Forty-eight positive cDNAs were sorted by cross-hybridization into 12 classes. One representative of each class was tested by DNA hybridization for presence in fungal isolates SB111 (Tox2\(^+\) indicated by ‘\(+\)’) and SB114 (Tox2\(^-\) indicated by ‘\(-\)’). Genomic DNA of isolate SB111 was digested with BamHI, separated on a 0.9% agarose gel, blotted to a nylon membrane and probed individually. Putative functions based on partial sequencing of each cDNA are indicated.


Although initially it appeared to be present in SB111 in only two copies (Fig. 1), a more extensive analysis with additional restriction enzymes (\( AatIII, AervII, BglI, BstXI, KpnI \) or \( PvuI \)) showed that SB111 actually has three copies of C29 (Fig. 2 and data not shown). We have designated the gene corresponding to cDNA C29 as TOXF.
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Fig. 2. Presence of TOXF only in HC-toxin-producing (Tox2+) isolates of C. carbonum. Genomic DNA was digested with BglI and the blot was probed with the full-length TOXF cDNA insert of pAATC1. ‘+’ and ‘−’ indicate whether a particular isolate makes HC-toxin or not. Isolates SB111 and 367-1a are Tox2+. SB114, 243-10, 1368 and 1309 are Tox2−. R5–R16 are the random ascospore progeny of a cross between SB111 and SB114; their full strain designations are 164R5–164R16 (Walton, 1987).

TOXF ORF encodes a predicted 357 amino acid protein with a molecular mass of 39.6 kDa and a pl of 6.60 (Fig. 3). TOXF contains five introns of 58, 93, 52, 126 and 56 bp. All of the introns have the consensus splicing sequence (GT/C...AG). Transcription of TOXF starts 58 bp upstream of the predicted translation start site (Fig. 3). TOXF is predicted to not have a signal peptide and to be located in the cytosol (Nakai & Kanehisa, 1991; Nielsen et al., 1997). A cytosolic location is consistent with the predicted pI of the TOXF product (designated ToxFp), because mitochondrial branched-chain-amino-acid transaminases (BCATs; EC 2.6.1.42) have acidic pIs whereas cytosolic BCATs have acidic pIs (Eden et al., 1995).

BLAST analysis (Altschul et al., 1997) of TOXF indicated a significant similarity between ToxFp and BCATs of bacteria, fungi, nematodes, plants and mammals. The best match was to a putative BCAT of Haemophilus influenzae (52% amino acid identity; GenBank accession no. U32798). Of those sequences corresponding to biochemically defined BCATs, ToxFp was most closely related to the cytosolic BCAT of Saccharomyces cerevisiae (26% identity, U35774; Hutson et al., 1995). ToxFp is 22% identical to the BCAT of E. coli (encoded by ilvE) (Fig. 4). Weaker amino acid similarity was found between ToxFp and bacterial a-amino acid aminotransferases, which are structurally related to BCAT (Tanizawa et al., 1989).

From the crystal structure of E. coli BCAT, nine residues at the active site have been identified (Okada et al., 1997). The single exception is a conservative substitution of Leu for Val at amino acid 144 (corresponding to amino acid 110 in E. coli BCAT). Conserved amino acids in ToxFp include the two critical amino acids that bind pyridoxal phosphate, Glu227 and Lys188 (Glu194 and Lys160, respectively, in E. coli BCAT) (Fig. 4).
ToxFp with BCATs from other organisms. Alignment was performed with CLUSTAL W and decorated with BOXSHADE. Black shading and asterisks underneath the sequence indicate amino acids identical between ToxFp and at least one of the others. Similar amino acids are indicated by dots. The origin of amino acid sequences are: CcTOXF, *C. carbonum* (GenBank accession no. AF157629); EcBCAT, *E. coli* (P00510); and ScBCATc, *S. cerevisiae* (cytosolic form, P47176). The nine conserved residues of all known BCATs are indicated by ‘g’, with ‘(g)’ indicating the one conserved but not identical residue (Okada et al., 1997).

Strains D and 164R10 were transformed with the 3·1 kb fragment from the replacement vector pTOXFR1 (6·1 kb). A double-crossover homologous integration event was predicted to result in the disappearance of one of the 2·9 kb PsI hybridizing bands in 164R10 when probed with the 383 bp internal fragment of TOXF; the blot in (c) was probed with the TOXF cDNA (pAATC1). DNA was cut with PsI in all lanes. PsI does not allow discrimination between the two copies of TOXF (part a and lanes WT in parts b and c).

The conclusions drawn from probing the transformants with the 383 bp internal fragment were confirmed by reprobing with the entire TOXF cDNA (Fig. 5c). In the replacement strain R and the double mutant D/R, a new band of 3·9 kb was visible. This 3·9 kb signal corresponds to the original 2·9 kb PsI fragment plus the 1·4 kb hph gene cassette minus the 383 bp fragment of TOXF that had been deleted (Fig. 5c).

**Phenotypes of TOXF mutants**

The single (D and R) and double (D/R) TOXF mutants showed normal growth and development on V8 agar or

![Fig. 5. Targeted mutation of TOXF.](image-url)
in modified Fries' liquid medium (Walton et al., 1982). Single mutants D and R still made HC-toxin and had TOXF mRNA (data not shown). The D/R strain lacked TOXF mRNA (Fig. 6a) and failed to make detectable HC-toxin in culture (Fig. 6b). Mutation of one or the other copy of TOXF (D or R strains) did not affect pathogenicity of C. carbonum on maize of genotype hm1/hm1 and therefore both copies of TOXF are functional. Mutation of both copies of TOXF completely abolished pathogenicity (data not shown). The disease phenotype of the D/R strain was indistinguishable from null mutants of hts1 (Panaccione et al., 1992), toxC (Ahn & Walton, 1997) or toxE (Ahn & Walton, 1998). These experiments establish that TOXF is not required for normal growth and development but is specifically required for HC-toxin biosynthesis and hence pathogenicity.

**DISCUSSION**

TOX2 is a complex locus consisting of multiple genes each present in multiple functional copies (Ahn & Walton, 1997). Unlike the genes for other secondary metabolite pathways in fungi, such as those for aflatoxin/sterigmatocystin, trichothecenes and gibberellins, those involved in HC-toxin production are spread over more than 550 kb and show only limited clustering (Ahn & Walton, 1996; Brown et al., 1996; Trapp et al., 1998; Tudzynski & Holtzer, 1998; Yu et al., 1995). Therefore, the molecular genetic analysis of HC-toxin biosynthesis cannot rely on sequencing and short-range chromosome walking. The functional redundancy of the HC-toxin biosynthetic genes excludes the use of mutagenesis approaches such as restriction-enzyme-mediated integration (REMI) (Lu et al., 1994; Sweigard et al., 1998). Here, we have described the use of an alternative strategy, based on BACs, to find HC-toxin biosynthetic genes. This approach assumes that new HC-toxin biosynthetic genes will be within 50–150 kb of each other and present only in Tox2+ isolates. The BAC method is being extended by analysis of regions adjacent to other known TOX2 genes (e.g. HTS1, TOXC and TOXE) and by constructing a BAC library with larger inserts (up to 400 kb) (Monaco & Larin, 1994).

The TOXF product, ToxFp, shares moderate homology with a large group of BCATs (Fig. 4). All of the essential amino acid residues necessary for transaminase function are conserved in ToxFp. Considering that three of the four amino acids of HC-toxin (D-Pro, L-Ala and D-Ala) are derived directly from primary metabolism, a role for TOXF in the biosynthesis of Aeo seems most reasonable. A plausible reaction catalysed by ToxFp would be to aminate a precursor of Aeo. Insofar as the decanoic acid backbone of Aeo is biogenically a fatty acid (Ahn & Walton, 1997; Cheng et al., 1999), a transamination reaction seems essential. The amino-acceptor substrate for the reaction catalysed by ToxFp could be 2-oxodecanoic acid, which itself could be produced by oxidation of decanoic acid by an unidentified enzyme. Alternative substrates for ToxFp could be derivatives of 2-oxodecanoic acid that already contained the 8-carbonyl and/or the 9,10-epoxide.

All known BCATs are involved in primary catabolism of branched-chain amino acids. Typically, they catalyse the transfer of an amino group from Leu, Val or Ile to 2-oxoglutaric acid. ToxFp appears to be the first BCAT dedicated to a non-essential secondary metabolic pathway. The failure of TOXF to cross-hybridize with any other genes in C. carbonum at low stringency suggests that TOXF and the sequences of the house-keeping BCAT of C. carbonum are not closely related. It may be significant that BCATs are structurally and functionally related to bacterial d-amino acid aminotransferases and that ToxFp also shows limited sequence similarity to this subfamily of enzymes (Alexander et al., 1994; Mehta et al., 1993; Sugio et al., 1995; Tanizawa et al., 1989). D-Amino acids are common in nonribosomal peptides and HC-toxin contains two d-amino acids. From its similarity to BCATs, ToxFp probably uses a branched-chain amino acid as amino donor, but the possibility that the donor is a d-amino acid should also be considered.

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*Fig. 6. Analyses of TOXF mutants. (a) RNA blot showing the TOXF mRNA in 164R10 (WT) and its disappearance in the double mutant (D/R). Thirty micrograms of total RNA were loaded per lane and the blot was probed with the TOXF cDNA (pAACT1). The blot was subsequently stripped and reprobed with C. carbonum GPD-1 encoding glyceraldehyde-3-phosphate dehydrogenase as loading control. (b) TLC of extracts of culture filtrates of C. carbonum 164R10 (WT) and the double mutant (D/R) showing that the double mutant does not make HC-toxin. HC-toxin was visualized with an epoxide-specific reagent (Meeley & Walton, 1991).*
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Branched-chain-amino-acid transaminase


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