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2007

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# The *Cercospora nicotianae* gene encoding dual *O*-methyltransferase and FAD-dependent monooxygenase domains mediates cercosporin toxin biosynthesis

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Received 17 July 2006; accepted 30 August 2006

Available online 30 October 2006

## Abstract

Cercosporin, a photo-activated, non-host-selective phytotoxin produced by many species of the plant pathogenic fungus *Cercospora*, causes peroxidation of plant cell membranes by generating reactive oxygen species and is an important virulence determinant. Here we report a new gene, *CTB3* that is involved in cercosporin biosynthesis in *Cercospora nicotianae*. *CTB3* is adjacent to a previously identified *CTB1* encoding a polyketide synthase which is also required for cercosporin production. *CTB3* contains a putative *O*-methyltransferase domain in the N-terminus and a putative flavin adenine dinucleotide (FAD)-dependent monooxygenase domain in the C-terminus. The N-terminal amino acid sequence also is similar to that of the transcription enhancer AFLS (formerly AFLJ) involved in aflatoxin biosynthesis. Expression of *CTB3* was differentially regulated by light, medium, nitrogen and carbon sources and pH. Disruption of the N- or C-terminus of *CTB3* yielded mutants that failed to accumulate the *CTB3* transcript and cercosporin. The  $\Delta$ ctb3 disruptants produced a yellow pigment that is not toxic to tobacco suspension cells. Production of cercosporin in a  $\Delta$ ctb3 null mutant was fully restored when transformed with a functional *CTB3* clone or when paired with a  $\Delta$ ctb1-null mutant (defective in polyketide synthase) by cross feeding of the biosynthetic intermediates. Pathogenicity assays using detached tobacco leaves revealed that the  $\Delta$ ctb3 disruptants drastically reduced lesion formation.

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**Keywords:** Clustering; Gene replacement; Perylenequinone; Photosensitizer

## 1. Introduction

Cercosporin is a non-host-selective, photo-activated toxin produced by many phytopathogenic *Cercospora* species. *Cercospora* species cause leaf spot and blight diseases on a wide range of plant species, including many economically important crops such as corn, soybean, sugar beet, coffee,

peanut, rice, banana, tobacco as well as ornamental, vegetable and weed species (Daub and Ehrenshaft, 2000). Production of cercosporin has been proven to be essential for full virulence of *Cercospora nicotianae* in the invasion of hosts and for lesion formation on tobacco (Choquer et al., 2005).

Cercosporin belongs to a class of photosensitizing compounds that are activated upon exposure to light and thus is not toxic to cells in the dark. The perylenequinone chromophore of cercosporin absorbs light energy to attain an

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electronically-activated triplet state and produces active oxygen species such as the hydroxyl radical (OH), superoxide ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ) or singlet oxygen ( $^1O_2$ ) upon reaction with oxygen molecules (Foote, 1976; Spikes, 1989). These reactive oxygen species cause damage to various cellular components including lipids, proteins and nucleic acids (Daub and Ehrenshaft, 2000). Cercosporin has been shown to produce both  $^1O_2$  and  $O_2^-$ , causing peroxidation of cell membrane lipids and consequently electrolyte leakage in plants (Daub, 1982a; Daub and Briggs, 1983; Daub and Hangarter, 1983). Due to the production of reactive oxygen species, cercosporin has universal toxicity not only to host plants, but also to fungi, bacteria and mice (Daub, 1982b; Ito, 1981; Yamazaki et al., 1975).

The toxicity of cercosporin to cells and the cellular mechanisms operating in the fungus for cercosporin self-protection have been intensively investigated (reviewed by Daub et al., 2005). By contrast, the biochemical pathway leading to the cercosporin production is largely unknown. It has been known for a long time that light is absolutely required for cercosporin production (Yamazaki et al., 1975). Studies on physiological factors regulating cercosporin production, however, revealed that accumulation of cercosporin in culture also was markedly influenced by temperature, medium composition, developmental stages and other environmental conditions and varied among isolates (Jenns et al., 1989). Studies using pharmacological inhibitors implicated the involvement of calcium and calmodulin signaling in cercosporin production (Chung, 2003). Studies using a precursor feeding also pointed out that cercosporin is synthesized via a polyketide pathway starting at condensation of acetate and malonate units (Okubo et al., 1975). Recently, several genes in *Cercospora* spp. have been cloned and demonstrated to be involved in cercosporin accumulation in culture (Callahan et al., 1999; Chung et al., 1999, 2003a; Shim and Dunkle, 2003). However, none of these genes are directly involved in the biosynthetic steps of cercosporin. To identify genes directly involved in cercosporin biosynthesis, *C. nicotianae* mutants completely deficient in cercosporin production were generated using the restriction enzyme-mediated integration (REMI) mutagenesis (Chung et al., 2003b). A *CTBI* (Cercosporin Toxin Biosynthesis) gene encoding a fungal polyketide synthase was subsequently identified from one of the REMI mutants and shown by gene disruption to be required for cercosporin biosynthesis and for high levels of virulence on tobacco (Choquer et al., 2005).

Gene clusters involved in the biosynthesis of secondary metabolites have been reported in many filamentous fungi (Keller and Hohn, 1997; Walton, 2000). Some examples include the biosynthetic genes for strigimycin and aflatoxin in *Aspergillus* species, fumonisin and trichothecene in *Fusarium* (*Gibberella*) species, sirodesmin in *Leptosphaeria maculans*, compactin in *Penicillium citrinum*, gibberellin in *Gibberella fujikuroi*, alkaloids in *Claviceps purpurea* and *Neotyphodium uncinatum*, AK toxin in *Alternaria alternata* and HC toxin in *Cochliobolus carbonum*

(Abe et al., 2002; Ahn and Walton, 1996; Brown et al., 1996, 1999; Gardiner et al., 2004; Hohn et al., 1993; Proctor et al., 2003; Seo et al., 2001; Spiering et al., 2005; Tanaka and Tsuge, 2000; Tudzynski and Hölter, 1998; Yu et al., 2004). Thus, we hypothesize that genes involved in cercosporin biosynthesis also are clustered in *Cercospora* spp. In this study we describe cloning and characterization of a second gene, named *CTB3*, which is immediately adjacent to the *CTBI* gene, to gain further insight into the molecular basis of cercosporin biosynthesis and regulation. The *CTB3* contains two putative domains: an *O*-methyltransferase domain in the N-terminus and a flavin adenine dinucleotide (FAD)-dependent monooxygenase domain in the C-terminus. Functional analysis indicated that *CTB3* also is required for cercosporin production. The results imply that many, if not all, genes involved in cercosporin biosynthesis and regulation are likely clustered in *C. nicotianae*.

## 2. Materials and methods

### 2.1. Fungal strains, media and cultural conditions

*Cercospora nicotianae* wild-type strain CnA (ATCC18366) and other mutant strains were maintained on complete medium (CM) agar plate at 28 °C (Jenns et al., 1989). For DNA and RNA purification, fungal strains were grown on CM or potato dextrose agar (PDA) (Difco, Detroit, MI) with a layer of cellophane. Fungal isolates were grown in 50 ml of potato dextrose broth supplemented with 100 mM  $Ca(NO_3) \cdot 4H_2O$  in a rotary shaker for 5 days in the dark for protoplast preparation. Screening of cercosporin-deficient mutants was conducted on PDA plates as previously described (Chung et al., 2003b). Assays for photosensitizer sensitivity were conducted on CM medium under continuous light as described by Jenns and Daub (1995). Pure cercosporin and other photosensitizing dyes (eosine Y, methylene blue or toluidine blue) were purchased from Sigma–Aldrich (St. Louis, MO), and dissolved in acetone to make a 10 mM stock solution. The pH of PDA was adjusted with 0.1 M citric acid–0.2 M dibasic sodium phosphate buffer (Ruzin, 1999).

### 2.2. Toxin purification and quantification

Cercosporin and other pigments produced by the wild type and the  $\Delta$ ctb3 disruptants were extracted with 5 N KOH from agar plugs with mycelia as described previously (Choquer et al., 2005; Chung, 2003). Cercosporin in the KOH extracts was quantified by measuring absorbance at 480 nm using a model Genesys 5 spectrophotometer (Spectronic Instruments, Rochester, NY). For TLC and HPLC analyses, cercosporin and putative intermediates were purified from agar plugs with mycelia with ethyl acetate. The organic solvent was evaporated with a Model R110 of Rotavapor (Brinkmann, Büchi, Switzerland) and the

compounds were dissolved in acetone. Thin-layer chromatography (TLC) on plates coated with a 60 F254 fluorescent silica gel (5 × 20 cm, Selecto Scientific Inc., Suwanee, GA) and a solvent system containing ethyl acetate:hexane:methanol:H<sub>2</sub>O (6:4:1.5:1, v/v) was as described by Fore et al. (1988). HPLC was performed in a series 1050 Hewlett Packard autosampler equipped with a Nucleosil 120-5C18 reversed phase column (5 μm, 250 × 4 mm) (Richard Scientific, Novato, CA) and using 5–95% acetonitrile gradient as a mobile phase. Cercosporin and related compounds were detected using a Model 490E detector (Waters-Millipore) at 480 nm.

### 2.3. Cloning and sequencing of the full-length *CTB3* gene

The full-length *CTB3* sequence of *C. nicotianae* was identified from a previously isolated genomic clone, pPKS-8, containing the 5'-end of the *CTB1* gene encoding a fungal polyketide synthase and its upstream sequence (Choquer et al., 2005). DNA sequence analysis was performed in both directions at the Integrated Biotechnology Laboratory, University of Georgia (Athens).

### 2.4. Gene disruption and genetic complementation

All DNA clones were built on a pGEM-T easy vector (Promega, Madison, WI). To disrupt the N-terminus of *CTB3*, two-step PCR was used to create a *CTB3* disruption vector pVG2 (Fig. 1A). A 2.9-kb *CTB3* fragment was amplified with the primers P22 (5'-ggcagcaccgatccacga-3') and OMT-1 (5'-ctctttttgacatcccgcacc-3') by an Expand High Fidelity PCR system (Roche Applied Science, Indianapolis, IN). Primers OMT-1 paired with OMT-2 (5'-gagcggcttgagttgagcatcgc-3'), and OMT-3 (5'-gcgagcactgaaa cctgggg-3') paired with P22 were used to amplify 1.0 and 0.8 kb fragments, respectively, and ligated to a 1.6 kb *Bam*HI fragment containing hygromycin phosphotransferase B gene (*HYG*) cassette from pUCATPH (Lu et al., 1994). A 3.4-kb fragment containing *HYG* flanked by *CTB3* sequence was cloned to produce pVG2. DNA fragments overlapping within the *HYG* region were amplified with primers P22 and *hyg3* (5'-ggatgcctccgctcgaagta-3'), and primers OMT-1 and *hyg4* (5'-cgttgaagaactgcctgaa-3'). PCR fragments were directly transformed into the *C. nicotianae* wild-type strain for gene replacement.

To disrupt the C-terminus of *CTB3*, a 4.2-kb *CTB3* fragment was amplified with primers *CTB3H* (5'-aggagcg gattcgatccctcatg-3') and P40 (5'-cagctacgatgagtcggag c-3') and cloned to yield pCTB3G. A 1.0-kb *Cl*AI and *Age*I fragment of the *CTB3* gene was excised from plasmid pCTB3G and replaced with the end-filled *Eco*RI/*Hind*III phosphinothricin acetyltransferase gene (*BAR*) gene under the *Cochliobolus heterostrophus* promoter 1 (P1) from pBP1T (Straubinger et al., 1992) to result in pCTB3/Bar6 (Fig. 2A). Split *BAR* maker fragments obtained by PCR with the primers P40 and *bar1* (5'-tctgcaccatgctcaaccac-3') and the primers P42 (5'-cctcggctctcacag-3') and *bar2*

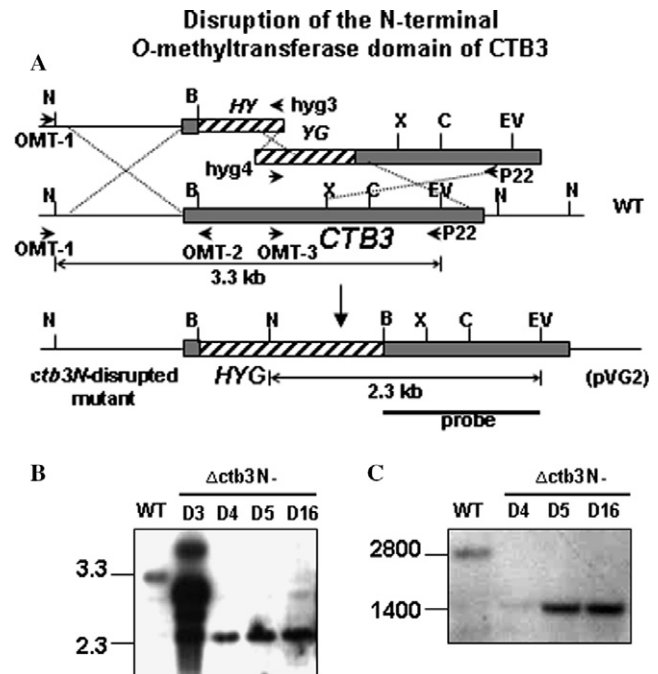


Fig. 1. Disruption of the N-terminal *O*-methyltransferase domain of *CTB3* in *Cercospora nicotianae*. (A) Schematic illustration of a split-hygromycin phosphotransferase B gene (*HYG*) marker used for the 5'-end *CTB3* disruption. Two truncated *CTB3* fragments fused with an overlapping *HYG* were amplified from the disruption construct pVG2 using primers (OMT-1/*hyg3*, P22/*hyg4*) and directly transformed into protoplasts of the *C. nicotianae* wild type (WT). Oligonucleotide primers (OMT-2 and OMT-3) used for cloning are also indicated. Restriction enzyme site abbreviations: B, *Bam*HI; C, *Cl*AI; EV, *Eco*RV; N, *N*coI; X, *Xho*I. Note: drawing is not to scale. (B) Southern-blot analysis of genomic DNA from the wild type and four  $\Delta$ *ctb3N* knockouts (D3, D4, D5, D16). Fungal DNA was digested with *Eco*RV and *N*coI, electrophoresed, blotted onto a nylon membrane, and hybridized to a *CTB3*-specific probe as indicated. The membrane was washed at high stringency. Sizes of hybridizing bands are indicated in kilobase pairs (kb). (C) Northern-blot analysis of total RNA isolated from the wild type and three  $\Delta$ *ctb3N* knockouts (D4, D5, D16). Sizes of hybridizing bands are indicated in nucleotides (nt).

(5'-aaaccacgtcatgccagtt-3') from pCTB3/Bar6 were directly transformed into the *C. nicotianae* wild-type strain.

For genetic complementation, pCTB3G was co-transformed with the plasmid pBARKS1 carrying the *BAR* gene responsible for bialaphos resistance under control of the *Aspergillus nidulans* *trpC* promoter (Pall and Brunelli, 1993) into a  $\Delta$ *ctb3N*-null mutant ( $\Delta$ *ctb3N*-D5). Transformants were selected on medium containing 50 μg/ml bialaphos (gluphosinate ammonium) (Fluka, Milwaukee, WI) and tested for cercosporin production on thin PDA plates as described previously (Chung, 2003). Fungal protoplasts were prepared and transformed using CaCl<sub>2</sub> and polyethylene glycol (PEG) by previously described methods (Chung et al., 2002).

### 2.5. Manipulation of nucleic acids

Fungal DNA was isolated with a DNeasy Plant Mini kit (Qiagen, Valencia, CA). Fungal RNA was extracted with a TRIZOL RNA Isolator kit (Invitrogen). Standard

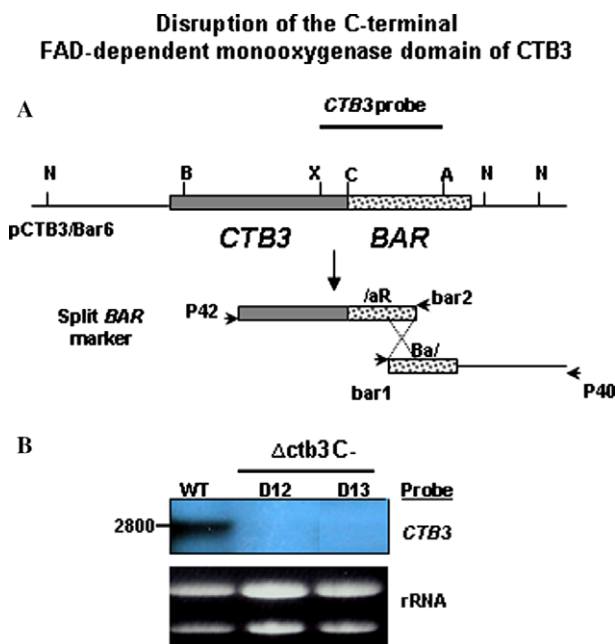


Fig. 2. Disruption of the C terminal FAD-dependent monooxygenase domain of CTB3 in *Cercospora nicotianae*. (A) Schematic illustration of pCTB3/Bar6 plasmid containing a phosphinothricin acetyltransferase gene (*BAR*) marker used for the 3'-end *CTB3* disruption. Two truncated *CTB3* fragments fused with an overlapped *BAR* were amplified from the disruption construct pCTB3/Bar6 using primers (P42/bar-2, bar-1/P40) and directly transformed into protoplasts of the *C. nicotianae* wild type (WT). Restriction enzyme site abbreviations: A, *AgeI*; B, *BamHI*; C, *ClaI*; N, *NcoI*; X, *XhoI*. Note: drawing is not to scale. (B) Northern-blot analysis of total RNA isolated from the wild type and two  $\Delta$ ctb3C knockouts (D12 and D13).

procedures were used for endonuclease digestion of DNA, electrophoresis, and Southern- and Northern-blot hybridizations (Sambrook and Russell, 2001). The hybridization probes were prepared by incorporating digoxigenin-11-dUTP (Roche) into the *CTB3* DNA fragments by PCR with gene-specific primers (Chung et al., 2003c). Probe labeling, hybridization, post-hybridization washing and immunological detection of the probe using a CSPD lumigenic substrate for alkaline phosphatase were conducted following the manufacturer's instructions (Roche). Double-strand cDNA was synthesized from total RNA using a BD SMART cDNA synthesis kit (Clontech, Palo Alto, CA). *CTB3* cDNA was obtained from cDNA pools by nested PCR. Two primers CTB31 (5'-cgtcctggtctcaat gtaacatc-3') and P25 (5'-ttagccaaggcgtcgcatcgact-3') were first used for amplification and the nested primer P22 was paired with CTB31 to amplify a 2.5-kb cDNA fragment comprising the *CTB3* gene with a TITANIUM *Taq* DNA polymerase (Clontech). The resulting fragment was cloned into the pGEM-T easy vector to yield pKAL41 for sequence analysis. All plasmids were propagated in competent *Escherichia coli* DH5 $\alpha$  bacterial cells and purified using a Wizard DNA purification kit (Promega).

## 2.6. Fungal pathogenicity test

Fungal inoculations were performed on detached tobacco (*Nicotiana tabacum*) leaves cultivar Burley 21 as previously described (Choquer et al., 2005). Toxin assays using tobacco cell suspensions were conducted as described by Daub (1982b). Briefly, cercosporin and other pigments extracted with ethyl acetate from agar plugs with mycelia were dried, dissolved in acetone and added to the 6-day-old tobacco cell suspensions ( $10^6$  cells/ml). Cell cultures covered with or without aluminum foil were incubated on a rotary shaker (140 rpm) at 32 °C under fluorescent lights at an intensity of  $3.1 \times 10^3$  J/cm<sup>2</sup>/s. Cell viability was determined with the aid of a microscope after staining with 0.1% Evan's blue (Sigma).

## 3. Results

### 3.1. Sequence analysis and characterization of *C. nicotianae* *CTB3* gene

Sequence analysis of a *CTB1*-containing genomic clone pPKS-8 (Choquer et al., 2005) identified an additional putative gene, hereafter designated as *CTB3*. Coding sequences of *CTB3* and *CTB1* are separated by 846 bp and are divergently transcribed. The promoter sequences of *CTB3* can be retrieved from the GenBank database under GenBank Accession No. AY649543. The *CTB3* open reading frame (ORF) consists of 2613-bp nucleotides, interrupted by two small introns of 48 and 70 bp, and was predicted to encode a polypeptide of 871 amino acids (GenBank Accession No. DQ355149). Analysis of 640 nucleotides upstream from the ATG start codon of *CTB3* identified three CAAT consensus sequences at positions -282, -141 and -37, respectively.

A search of sequence databases suggested that the deduced amino acid sequence of *CTB3* translation product has two putative functional domains related to *O*-methyltransferases and FAD-dependent monooxygenases. The N-terminal amino acids (1-429) of *CTB3* have strong similarity to numerous *S*-adenosyl-methionine (SAM)-dependent *O*-methyltransferases in bacteria and fungi (Table 1 and data not shown). Alignment of the N-terminal amino acids of *CTB3* with AFLO (formerly omtB) of *Aspergillus* is shown in supplementary Figure A. The N-terminal amino acids of *CTB3* also have various degrees of similarity to AFLS (formerly AFLJ), a putative transcription co-regulator involved in aflatoxin biosynthesis in *Aspergillus* (supplementary Figure B). In contrast, the C-terminal amino acids (419-871) of *CTB3* exhibit similarity to many putative FAD-dependent monooxygenases required for energy production in bacteria (supplementary Figure C).

### 3.2. Targeted gene disruption and genetic complementation

To determine if each domain of *CTB3* is required for cercosporin biosynthesis, targeted gene disruption was

Table 1  
Similarity of *Cercospora nicotianae* *CTB3* gene encoding putative dual *O*-methyltransferase (amino acids 1–420) / FAD-dependent monooxygenase/oxidoreductase (amino acids 421–871) domains with homologues to various fungal and bacterial species

Species	Amino acids	Identity (%)	Similarity (%)	<i>E</i> -value	Accession No.	Putative function
<i>Gibberella zeae</i>	439	36	52	6e–59	EAA69866	Unknown
<i>Aspergillus nidulans</i>	459	30	45	4e–39	EAA62575	Unknown
<i>A. parasiticus</i>	386	36	55	9e–25	AAS66016	<i>O</i> -methyltransferase
<i>A. flavus</i>	386	35	51	2e–25	AF159789	<i>O</i> -methyltransferase
<i>Magnaporthe grisea</i>	970	42	56	0	EAA48769	Unknown
<i>Neurospora crassa</i>	528	34	53	8e–32	EAA33703	Unknown
<i>A. flavus</i>	438	26	45	4e–25	AAS90004	AFLJ, transcription enhancer
<i>A. nomius</i>	438	26	44	1e–24	AAS90052	AFLJ
<i>Chromobacterium violaceum</i>	384	29	44	3e–31	AAQ59500	FAD-dependent monooxygenase
<i>Rubrobacter xylophilus</i>	377	32	48	6e–30	EAN36783	FAD-dependent monooxygenase
<i>Bradyrhizobium</i> sp.	409	28	44	2e–25	EAP27500	FAD-dependent monooxygenase
<i>Photobacterium luminescens</i>	411	27	45	3e–20	CAE14529	Unknown

performed separately. A disruption vector pVG2 (Fig. 1A) containing a hygromycin phosphotransferase B gene (*HYG*) cassette flanked with *CTB3* sequence on each side was first constructed to replace the 5' end comprising the N-terminal *O*-methyltransferase/AFLS domain of *CTB3*. In total, 18 of 32 (~56%) transformants recovered were unable to produce cercosporin on a thin PDA plate. The putative *Actb3* knockouts accumulated a yellow pigment.

Southern-blot analysis was performed to validate that the loss of cercosporin production was a consequence of the targeted gene replacement at the 5' end of *CTB3* (Fig. 1B). Genomic DNA from the wild-type strain and four *Actb3* disruptants (*Actb3N*-D3, D4, D5 and D16) was cleaved with restriction enzymes *NcoI* and *EcoRV* and hybridized with a *CTB3*-specific probe. The results indicated that all *Actb3* analyzed displayed a *CTB3* deletion in the *C. nicotianae* genome. As expected, the wild-type strain displayed a 3.3-kb *NcoI*–*EcoRV* hybridizing band from the genomic DNA, whereas the *Actb3N* disruptants contained a 2.3 kb hybridizing band due to the insertion of the *HYG* cassette in *CTB3* and the presence of an additional *NcoI* site in the *HYG* gene cassette. In addition to the 2.3-kb signal, three hybridizing bands of ~5.0, 3.0 and 2.7 kb were also detected in DNA prepared from the *Actb3N*-D3 disruptant (lane 2), likely resulting from the ectopic integrations of split marker fragments in the genome or incomplete digestion of genomic DNA. Northern-blot analysis indicated that hybridization of total RNA from the wild-type strain with a *CTB3*-specific probe identified a 2800-nucleotide (nt) transcript that was completely absent among three *Actb3N* strains (D4, D5, D16) (Fig. 1C). By contrast, a truncated transcript of approximate 1400 nucleotides in size was detected in RNA from the *Actb3N* strains, verifying the disruption of *Actb3N* strains indeed specifically occurred at the *CTB3* locus.

Northern-blot analysis of RNA from the *Actb3N* disruptants identified a truncated *CTB3* transcript that might be able to produce a functional protein with monooxygenase activity. To determine the function of the C-terminal monooxygenase domain of *CTB3* in cercosporin biosynthesis, plasmid vector pCTB3/Bar6 carrying a phosphinothricin acetyltransferase gene (*BAR*) cassette flanked by

the 3' region of *CTB3* was constructed for gene replacement (Fig. 2A). PCR fragments containing the split *BAR* makers fused with *CTB3* sequences were amplified and used for transformation. Two transformants (designated *Actb3C*-D12 and D13) recovered from a medium containing glufosinate ammonium were unable to produce cercosporin. Northern-hybridization analysis indicated that the 2800-nt *CTB3* transcript was completely undetectable in the *Actb3C*-D12 and D13 isolates (Fig. 2B), further validating the disruption occurred at the *CTB3* locus. The *Actb3C* disruptants failed to produce cercosporin but accumulated a yellow/brown pigment similar to the *Actb3N* disruptants. Thus, integration of the *HYG* or *BAR* cassette in *CTB3* has completely disrupted the entire *CTB3* ORF, resulting in *C. nicotianae* mutants with a lack of functional *O*-methyltransferases and FAD-dependent monooxygenase domains.

Genetic complementation was carried out to further verify the essential role of *CTB3* for cercosporin biosynthesis. A DNA fragment containing the full-length *CTB3* gene and its promoter was co-transformed with pBARKS1 plasmid into protoplasts of the *Actb3N*-D5 mutant. In total, 7 of 23 (30%) strains recovered produced large amounts of cercosporin comparable to that of wild type (Table 2).

To determine if mutants disrupted in *CTB1* (encoding a polyketide synthase) or *CTB3* would complement each other for cercosporin biosynthesis by substrate cross feeding, the *Actb1* and *Actb3* knockouts were co-cultured on PDA. Complementation was observed when a *Actb1* disruptant was paired with the *Actb3N* or *Actb3C* disruptants by formation of a distinct red band where the mycelia of the mutant colonies were in contact (Fig. 3A). Formation of cercosporin also was observed when cellophane was placed between two colonies. However, no cercosporin was observed when the *Actb3N* disruptants and the *Actb3C* disruptants were grown together (data not shown).

### 3.3. Detection and quantification of cercosporin

Production of cercosporin (a distinct red pigment) by the wild-type strain, the *Actb3* disruptants and the complemented strains was first assessed by growing the fungal

Table 2

Production of cercosporin by the *Cercospora nicotianae* wild-type strain (CnA), the *ctb3* mutants disrupted in the N-terminus ( $\Delta$ ctb3N), and C-terminus ( $\Delta$ ctb3C), and the *CTB3*-complemented strains (T5C11 and T5C12) under continuous light (LT) or darkness (DK)

Isolates	Treatment	Cercosporin production (nmol/agar plug) <sup>a</sup>
CnA	LT	196.2 ± 58.6
	DK	3.7 ± 0.8
$\Delta$ ctb3N-D3	LT	11.0 ± 4.4
	DK	1.4 ± 0.6
$\Delta$ ctb3N-D4	LT	44.6 ± 4.8
	DK	1.2 ± 0.4
$\Delta$ ctb3N-D5	LT	50.9 ± 14.7
	DK	1.4 ± 0.9
$\Delta$ ctb3N-D16	LT	24.3 ± 11.4
	DK	1.4 ± 0.6
T5C11	LT	229.7 ± 41.3
	DK	6.6 ± 2.9
T5C12	LT	270.3 ± 84.9
	DK	5.2 ± 1.9
$\Delta$ ctb3C-D12	LT	24.6 ± 7.9
	DK	0.6 ± 0.2
$\Delta$ ctb3C-D13	LT	16.6 ± 3.2
	DK	1.0 ± 0.4

<sup>a</sup> Fungal mycelium of *C. nicotianae* strains was grown on thin PDA plates for 7 days. Cercosporin was extracted with 5 N KOH and quantified by measuring absorbance of the extract at 480 nm. Control consisted of five PDA plugs extracted with 5 N KOH. Data are the means of three different experiments with five replicates of each isolate. Data shown ± standard error of the means.

strains on thin PDA under continuous light (Fig. 3B) or in complete darkness. Cercosporin and potential biosynthetic intermediates were then extracted from mycelial plugs with 5 N KOH. As shown in Fig. 3C, the extract from the wild-type strain produced a green color after soaking in KOH solution, whereas the extract from the  $\Delta$ ctb3 disruptants yielded an orange color. The amounts of cercosporin in the extract were quantified by measuring absorbance at 480 nm (Table 2). All extracts of fungal isolates grown in the dark had very low absorbance values, similar to the controls containing agar plugs alone. The wild-type strain produced high levels of cercosporin when grown on PDA under light. Spectrophotometric scanning of the KOH extract from the wild-type strain, the  $\Delta$ ctb3 disruptants, and the complemented strains also revealed a marked difference in the respective absorption spectrum. The KOH extract of wild type had a strong absorbance at 480 nm and two small peaks at 600 and 640 nm, as reported by Solod et al., 1992). By contrast, the KOH extract of the  $\Delta$ ctb3 disruptants had no absorbance at 480 nm.

To further confirm the inability for cercosporin production by the *ctb3* knockouts, fungal cultures were extracted with ethyl acetate and the extracts were analyzed by TLC and HPLC. As shown in Fig. 3D, the wild-type strain and the complemented strains produced a red pigment at  $R_f$  0.7, typical of cercosporin, whereas the  $\Delta$ ctb3-disrupted mutants produced a yellow pigment at  $R_f$  0.9. The yellow pigment gradually converted to a dark brown color on TLC plates (Fig. 3D). HPLC analysis also confirmed that

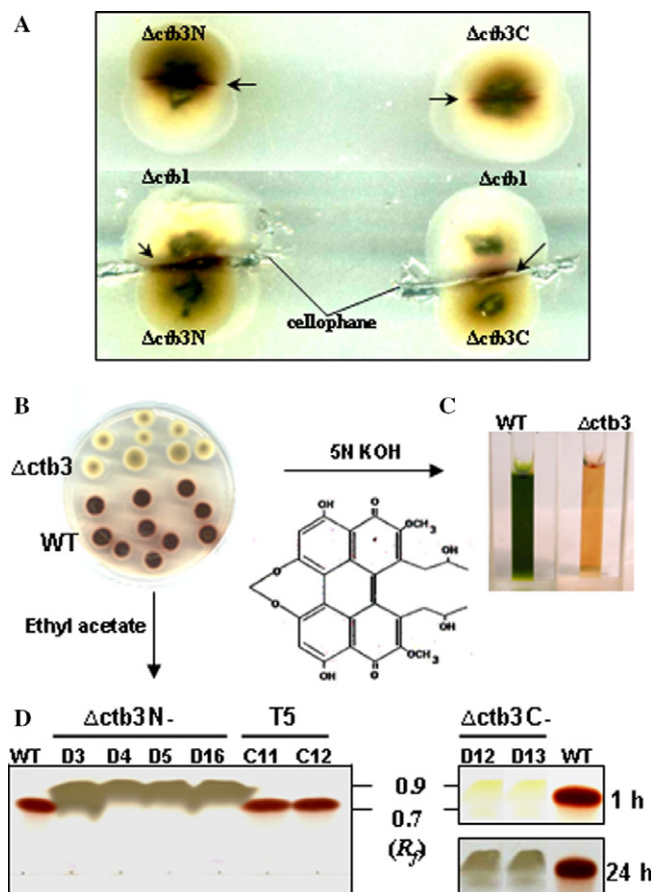


Fig. 3. Characterization of cercosporin produced by the wild type (WT) and a pigment produced by the  $\Delta$ ctb3 disruptant of *Cercospora nicotianae*. (A) Complementation of cercosporin production by pairing the  $\Delta$ ctb1 with  $\Delta$ ctb3 disruptants as indicated on a PDA plate. (B) Production of cercosporin and other pigments by the wild type and the  $\Delta$ ctb3 disruptants of *C. nicotianae* on potato dextrose agar plates. Accumulation of cercosporin is indicated by the red pigment. The chemical structure of cercosporin also is shown. (C) Reaction of cercosporin from the wild type and the pigment from the  $\Delta$ ctb3 disruptants with 5 N KOH. (D) Thin-layer chromatography (TLC) analysis of cercosporin production by the wild-type (WT) strain, the  $\Delta$ ctb3 N terminal disruption mutants ( $\Delta$ ctb3N-D3, D4, D5, D16), the *CTB3*-complemented strains (T5C11, T5C12), and the  $\Delta$ ctb3 C terminal disruption mutants of *C. nicotianae*. Cercosporin extracted from agar plugs was dissolved in acetone, spotted onto a silica gel plate, and separated with ethyl acetate:hexane:methanol:H<sub>2</sub>O (6:4:1.5:1, v/v). Cercosporin produced by the wild type and the complemented strains was detected as a red pigment with mobility at  $R_f$  0.7. By contrast, the  $\Delta$ ctb3-disrupted mutants produced a yellow/brown pigment at  $R_f$  0.9. The pigment produced by the  $\Delta$ ctb3 disruptants was yellowish (right upper panel) and gradually changed to a brown color (right bottom panel). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper).

no measurable cercosporin was produced by the  $\Delta$ ctb3 disruptants (data not shown). The yellow/brown pigment was completely undetectable when the  $\Delta$ ctb3 disruptants were grown in darkness. Collectively, we concluded that the  $\Delta$ ctb3 disruptants are completely defective in cercosporin accumulation.

To determine if the yellow/brown pigment extracted from cultures of the  $\Delta$ ctb3 disruptants is toxic, tobacco cell



suspensions were treated with the extracts from the wild-type strain and  $\Delta$ ctb3 disruptants, and incubated under continuous light or in complete darkness. The results indicated that the yellow pigment extracted from the cultures of the  $\Delta$ ctb3 disruptants was no longer toxic to tobacco cells compared to cercosporin produced by the wild type (Fig. 4).

Marked deficiency in cercosporin production was the only phenotype altered in the  $\Delta$ ctb3 disruptants. Similar to the  $\Delta$ ctb1 knockouts (Choquer et al., 2005), all  $\Delta$ ctb3 disruptants exhibited growth similar to the wild-type strain on the medium containing exogenous cercosporin (10  $\mu$ M) or other photosensitizing compounds (eosine Y, methylene blue or toluidine blue) under continuous light (data not shown), indicating that *CTB3* plays no role in cercosporin or singlet oxygen resistance. The  $\Delta$ ctb3 disruptants exhibited normal growth on various media and maintained normal conidiation on V8 juice agar.

### 3.4. Expression and regulation of *CTB3*

To test if accumulation of the *CTB3* transcript in *C. nicotianae* is coordinately regulated by light and medium composition suited for cercosporin production, the wild-type *C. nicotianae* was grown in either continuous light or continuous darkness on PDA or complete medium (CM). Total RNA purified from these cultures was hybridized to a *CTB3*-specific probe. As shown in Fig. 5A, high levels of the 2800-nt *CTB3* transcript were detected in RNA samples prepared from fungal cultures grown on PDA under continuous light, conducive conditions for cercosporin production and accumulation (Fig. 5B). By contrast, accumulation of *CTB3* transcript and cercosporin were markedly decreased when the fungus was grown on CM under continuous light. The *CTB3* transcript and

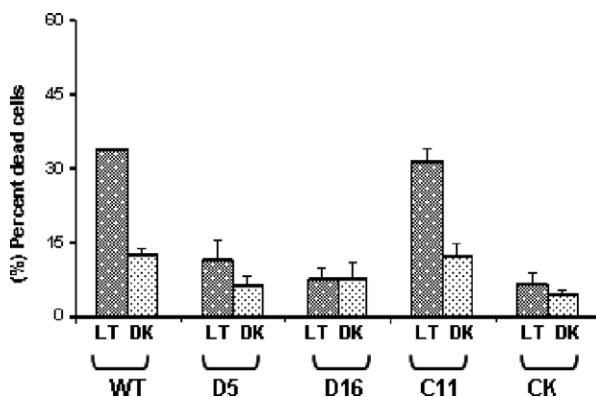


Fig. 4. Viability of tobacco suspension cells treated with pigments produced by the wild type (WT), two *ctb3*-disrupted mutants ( $\Delta$ ctb3N-D5, D16), and a complemented strain (T5C11) of *Cercospora nicotianae*, and the acetone control (CK) under continuous light (LT) or in complete darkness (DK). Tobacco cell suspensions were mixed with the test compounds and incubated at 32 °C for 5 h. Cell viability was evaluated after staining with 0.1% Evan's blue. Data are the means of three replicates of each treatment.

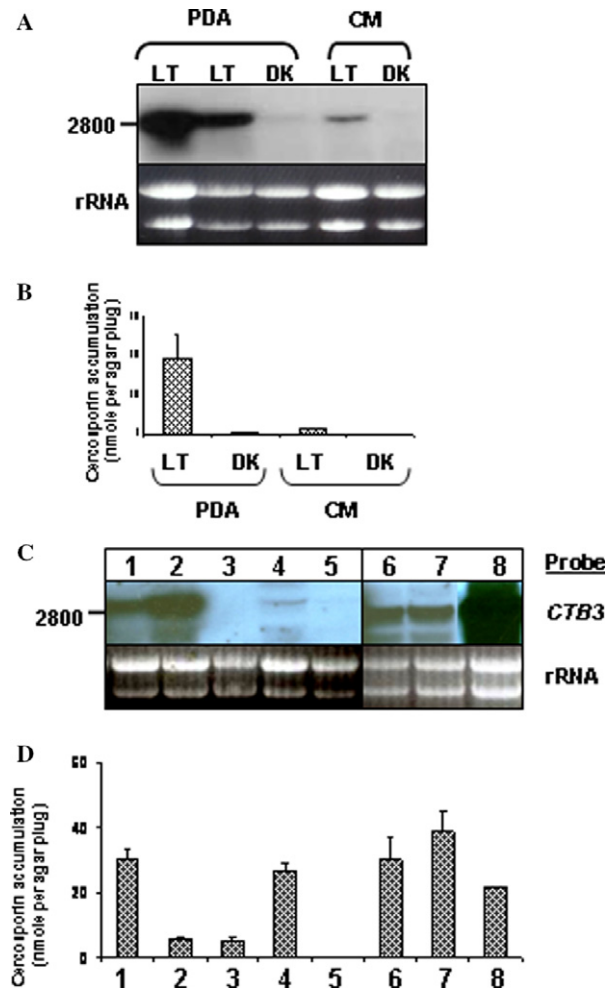


Fig. 5. Differential expression of the *CTB3* gene and accumulation of cercosporin by the *Cercospora nicotianae* wild type. (A) Northern-blot analysis of *CTB3* expression in the *C. nicotianae* wild type grown on potato dextrose agar (PDA) or complete medium (CM) under continuous light (LT) or darkness (DK). Fungal RNA was isolated, electrophoresed in a formaldehyde-containing gel, blotted to a nylon membrane, and hybridized to a *CTB3*-specific probe as indicated in Fig. 1A. Ethidium bromide-stained rRNA is shown for loading of the samples. Sizes of hybridizing bands are indicated in nucleotides. (B) Accumulation of cercosporin by the *C. nicotianae* wild type. The fungus was grown on PDA or CM plates under continuous light (LT) or darkness (DK) for 5 days, and cercosporin was extracted with 5 N KOH and quantified by absorbance at 480 nm. Data shown are the means and standard errors of two different experiments with five replicates of each treatment. (C) Northern-blot analysis of *CTB3* expression in the *C. nicotianae* wild type grown on complete medium (CM) with glucose and calcium nitrate as a sole carbon and nitrogen source, respectively (lane 1), mannitol as a sole carbon source (lane 2), ammonium chloride as a sole nitrogen source (lane 3), no nitrogen added in CM (lane 4), and “shift experiment” (The fungus was grown on PDA for 5 days and shifted to CM) (lane 5), and on PDA at pH 4.0 (lane 6), pH 5.0 (lane 7), and pH 7.0 (lane 8) under continuous light. (D) Accumulation of cercosporin by the *C. nicotianae* wild type grown on media as indicated above. Accumulation of cercosporin was not determined when the fungus was grown on PDA for 5 days and shifted to CM (lane 5).

cercosporin were undetectable or barely detectable when the fungus was grown on either PDA or CM in continuous darkness. Thus, expression of the *CTB3* gene appeared to

coincide well with cercosporin accumulation in response to light and medium as with the *CTB1* transcript (Choquer et al., 2005).

Expression of the *CTB3* gene in culture was further investigated to determine if any ingredients in CM may repress accumulation of the *CTB3* gene transcript. Northern-blot hybridization indicated that substitution of glucose with mannitol as a sole carbon source in CM markedly increased accumulation of the *CTB3* gene transcript (Fig. 5C, lanes 1 and 2), but failed to support increased production of cercosporin (Fig. 5D). Substitution of calcium nitrate with ammonium chloride as a sole nitrogen source in CM, however, completely blocked expression of *CTB3* and production of cercosporin (lane 3). Omission of the nitrogen from CM also decreased expression of the *CTB3* gene (lane 4), but remained normal cercosporin production. The *CTB3* transcript was completely undetected when fungal mycelium grown on PDA for 5 days was shifted to CM (lane 5). Expression of the *CTB3* gene appeared strongest at pH 7.0 when the fungus was grown on the permissive medium (PDA) and occurred to a much lesser extent at pH 4 and 5 (lanes 6–8). However, there was no strong correlation between cercosporin production and accumulation of the *CTB3* transcript on PDA with various pH.

### 3.5. Pathogenicity tests

Previously, we have shown that disruption of the *CTB1* gene resulted in mutants completely defective in cercosporin production and markedly reduced in lesion development on tobacco cv. Burley 21 (Choquer et al., 2005). The  $\Delta$ ctb1 disruptants did not accumulate any pigments. In contrast, disruption of the *CTB3* gene resulted in strains that accumulated a yellow/brown pigment instead of the red cercosporin. Those mutants were inoculated onto detached tobacco leaves cv. Burley 21. Similar to the  $\Delta$ ctb1 disruptants (Choquer et al., 2005), the  $\Delta$ ctb3 disruptants (D5, D8, D16) incited fewer necrotic lesions on tobacco leaves compared to those induced by the wild-type strain, whereas the *CTB3* complemented strain (T5C11) produced necrotic lesions comparable to the wild type (Fig. 6).

## 4. Discussion

In the present study, we identified a *C. nicotianae* *CTB3* gene located immediately adjacent to the *CTB1* gene and provided experimental evidence that *CTB3* encodes an enzyme for cercosporin biosynthesis. Targeted gene replacement and genetic complementation unambiguously confirmed the involvement of *CTB3* in cercosporin biosynthesis in *C. nicotianae*. Furthermore, sequence analysis of the surrounding DNA fragments obtained by chromosome walking outward from the *CTB1* and *CTB3* genes, allowed us to identify several putative ORFs with amino acid similarities to a wide range of *O*-methyltransferases, major facilitator superfamily (MFS) transporters, and Cys<sub>6</sub>N<sub>2</sub>

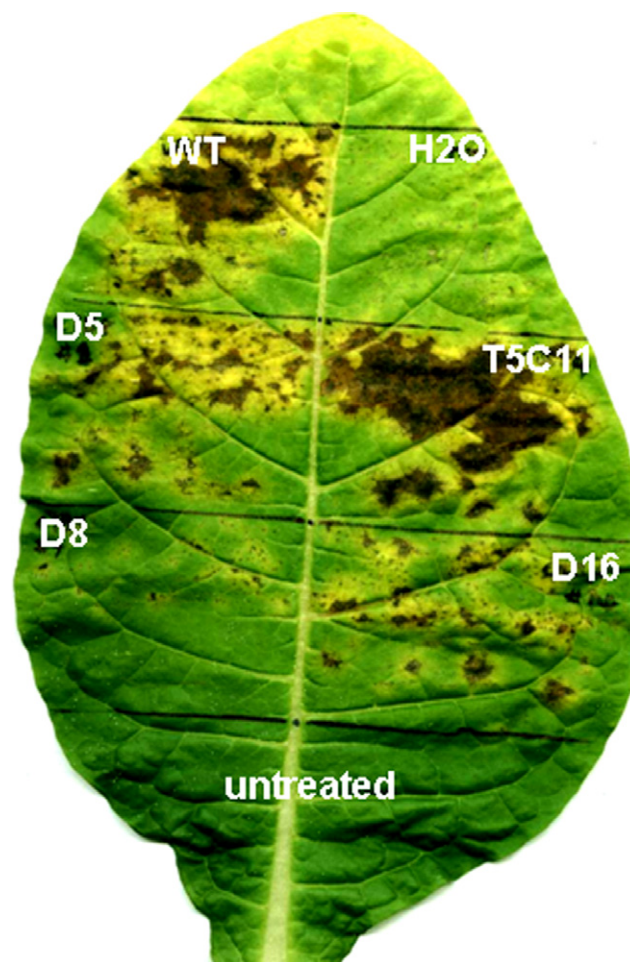


Fig. 6. Pathogenicity assay using detached tobacco leaves cv. Burley 21. The *Cercospora nicotianae* wild type (WT),  $\Delta$ ctb3-knockout mutants ( $\Delta$ ctb3N- D5, D8, D16) and *CTB3*-complemented strain (T5C11) were grown on V8 juice agar for 7–12 days at 20 °C under continuous light. The concentration of conidia was adjusted to  $5 \times 10^4$  conidia/ml and rubbed onto wounded tobacco leaves with cotton swabs. The inoculated leaves were then kept in a moist chamber under fluorescent light.

binuclear cluster motif-containing transcriptional activators in the database, respectively (*unpublished data*). Such proteins are required for the accumulation of many fungal secondary metabolites, including aflatoxin, fumonisin, sterigmatocystin, and trichothecene (Alexander et al., 1999; Brown et al., 1996; Proctor et al., 2003; Yu et al., 2004). Thus, the results suggest that the genes required for cercosporin production likely reside in cluster.

The deduced *CTB3* polypeptide has two putative functional domains with *O*-methyltransferase and FAD-dependent monooxygenase at N and C termini, respectively. The finding that the *CTB3* translation product contains dual functional domains is highly unusual. The functions of *O*-methyltransferase and FAD-dependent monooxygenase are often encoded by separate genes in an organism, with one exception of a hypothetical, uncharacterized protein (GenBank Accession No. EAA48769) from the sequenced genome of *Magnaporthe grisea*. Protein alignments indicated that *CTB3* and the *M. grisea* hypothetical protein have

similar structures, sizes and organizations with dual *O*-methyltransferase and FAD-dependent monooxygenase domains (data not shown). A protein with multiple functional domains also was observed in other fungal species. Fusion of the cytochrome P450 monooxygenase and NADPH-dependent cytochrome reductase functions in a single protein has also been found in the FUM6 involved in fumonisin biosynthesis in *F. verticilloides* (Seo et al., 2001). The EQIS containing a PKS-nonribosomal peptide synthase (NRPS) hybrid was found to be involved in equisetin biosynthesis in *Fusarium heterosporum* (Sims et al., 2005). Similarly, the *alt5* gene encoding a polyketide synthase also contains a *C*-methyltransferase domain required for decaketide alternapyrone biosynthesis in *Alternaria solani* (Fujii et al., 2005).

The backbone of cercosporin is derived from the condensations of acetate and malonate to form polyketomethylene chains, most likely via the polyketide biosynthetic pathway (Okubo et al., 1975). Cercosporin contains two methyl groups at positions C2 and C11, and methylation has also been proposed to be involved in its biosynthesis (Okubo et al., 1975). Thus, the *CTB3* translation product may likely catalyze the addition of one or two methyl groups into the cercosporin backbone (Fig. 3).

The C-terminus (a.a. 419–871) of *CTB3* resembles many putative FAD-dependent monooxygenases from a variety of organisms, particularly in bacteria (supplementary Figure C). Members of this group of enzymes include phenol hydroxylase from *Trichosporon cutaneum* (GenBank Accession nos. 1FOH\_A) and *Ralstonia pickettii* (GenBank Accession No. Q01551), kynurenine 3-monooxygenase for biosynthesis of nicotinic acid (GenBank Accession No. P38169), and putative squalene epoxidase from *Arabidopsis thaliana* (GenBank Accession No. AAC32430). The FAD-dependent monooxygenase domain of *CTB3* likely catalyzes hydroxylation of the cercosporin backbone. Likewise, disruption of the FAD-dependent monooxygenase domain alone in *C. nicotianae* also interfered with cercosporin biosynthesis.

Although the N-terminus of *CTB3* comprises an *O*-methyltransferase domain, database search revealed that the amino acids translated from the same frame also have similarity (26% identity and 48% similarity) to AFLS (formerly AFLJ). AFLS has been shown to be involved in aflatoxin biosynthesis by interacting with a zinc finger transcription factor, AFLR, to activate aflatoxin structural genes in *A. parasiticus* (Chang, 2003). Whether or not the N-terminus of *CTB3* acts as a transcription co-regulator as proposed for AFLS awaits further analysis.

Biosynthesis of secondary metabolites such as aflatoxin, sterigmatocystin, trichothecene, penicillin, and fumonisin in fungi is often regulated by various environmental conditions, developmental processes and cell differentiation (Calvo et al., 2002). Similarly, production of cercosporin by *Cercospora* spp. is primarily induced by light and affected by medium composition such as carbon and nitrogen sources and pH in the culture medium

(Daub and Ehrenshaft, 2000; Ehrenshaft and Upchurch, 1993; Jenks et al., 1989). In general, *C. nicotianae* accumulated high amounts of cercosporin when grown on potato dextrose agar (PDA) under continuous light, but produced no cercosporin when grown in continuous darkness. In a prior study, we have shown that the light- or medium-induced *CTB1* gene transcript accumulation was correlated with light- or medium-induced cercosporin production (Choquer et al., 2005). Northern-hybridization analysis conducted in the present study also revealed that the *CTB3* gene transcript was expressed under conditions highly favorable for cercosporin production by *C. nicotianae*. However, change of the carbon or nitrogen source in CM slightly influenced accumulation of the *CTB3* transcript, but did not enhance cercosporin accumulation. Comparative promoter analysis using TRES (Transcription Regulation Element Search; <http://bioportal.bic.nus.edu.sg/tres>) failed to identify the CREA (carbon regulatory protein) (Dowzer and Kelly, 1991) binding site in the promoter region of *CTB3*. However, an AREA (nitrogen regulatory protein) (Marzluf, 1997) and a PACC (pH regulatory protein) (Espeso et al., 2000) conserved sequence was found in the *CTB3* promoter region (data not shown). Transfer of fungal mycelia from PDA to CM completely abolished accumulation of the *CTB3* transcript and cercosporin, indicating the presence of inhibitors for cercosporin biosynthesis in CM. Further, we observed a significant reduction of cercosporin accumulation when the fungus was grown on PDA buffered with citric acid–phosphate solution. As shown in Fig. 5, the amounts (~40 nmol/plug) of cercosporin accumulated on the PDA buffered with citric acid and phosphate solution were as much as 5-fold lower than those (~200 nmole/plug) accumulated on regular PDA (Difco; pH 5.6).

The  $\Delta$ ctb1 disruptants defective in the polyketide synthase failed to accumulate any detectable pigments in culture (Choquer et al., 2005). In contrast, disruption of *CTB3* with the *HYG* or *BAR* cassette in *C. nicotianae* generated fungal mutants that accumulated a yellow/brown pigment with different mobility from cercosporin on TLC plates when grown on PDA in continuous light. The  $\Delta$ ctb3 disruptants, however, failed to accumulate any pigment when grown in continuous darkness, indicating that the yellow/brown pigment is regulated by light as well. Assays using tobacco suspension cells revealed that the yellow/brown compound extracted from the  $\Delta$ ctb3 disruptants was not toxic to tobacco cells. Whether or not the yellow/brown pigment accumulated by the  $\Delta$ ctb3 knockouts represents an intermediate for cercosporin biosynthesis awaits further structural investigation. Complementation of cercosporin production was achieved when the  $\Delta$ ctb3 disruptants were paired with the  $\Delta$ ctb1 disruptants on PDA even in the presence of cellophane barriers. The results indicated that complementation between paired mutants was the result of cross feeding rather than diploidization, genetic exchange, heterokaryon formation, or

mutant reversion. Judging from all available data, the yellow/brown pigment produced by the  $\Delta$ ctb3 disruptants is likely an intermediate for cercosporin biosynthesis.

Pathogenicity assays using detached tobacco cv. Burley 21 leaves revealed that, as with the  $\Delta$ ctb1 disruptants (Choquer et al., 2005), the  $\Delta$ ctb3-disrupted mutants incited less necrotic lesions compared to those of wild type, further confirming the important role of cercosporin in fungal virulence.

In summary, *CTB3* is tightly linked with *CTB1* and is also essential for cercosporin production. This is the first report describing the possible clustering of genes involved in cercosporin biosynthesis. We have obtained 10 putative ORFs in a span of 34 kb sequences that might be involved in cercosporin biosynthesis and regulation in *C. nicotianae*. Functional determination of the remaining genes that closely link to *CTB1* and *CTB3* is currently in progress.

### Acknowledgments

We thank D.J. Lewandowski for providing tobacco plants and cell cultures, H.Q. Chen for technical assistance and S. Gowda and two anonymous reviewers for helpful comments. This research was supported by the Florida Agricultural Experiment Station.

### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.fgb.2006.08.005](https://doi.org/10.1016/j.fgb.2006.08.005).

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**Supplementary Figure (FGB-06-114R2)**

**(A) O-methyltransferases (a. a. 1-429)**

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Apa omtB -----MTGLDMEIIFAKIKEEYARTDDVGRKQIQGHIRELVGFYSDWDVVMRLSSGPIQLVALTKVGIDLGIIFR 69
Afl omtB -----MTGLDMEIIFAKIKDEYARTDDVGRKQIQGHIRELVGFYSDWDVVMRLSSGPIQLVALAKVADLGIIFR 69
Cna CTB3 MMQFQRDLEASLEAVSANAQELLSKSRKDVQDLNASLPKDPDNCDAQTAARAQLAEAAATRIQLSIR-POEYLEHLQNGYQHLTCFRWLVELNILD 99

Apa omtB SLKESDTPITLAEIVKKTGASPRLLGRILRTQAAGFLIKETGPQEYTSSTAFDVFAN-SDAAGAVVQLFDISGPCTQILPDLAERNYQDITSNKDCVFQ 168
Afl omtB SLKESDTPITLAEIVKKTGASPRLLGRILRTQAAGFLIKETGPQEYTSSTAFDVFAN-SDAAGAVVQLFDISGPCTQILPDLAERNYQDITSNKDCVFQ 168
Cna CTB3 HLPHSCT-ISTYDLARKASVPPMQLRSICRMAICNGFLEEPEANQVRSRISALFARDESYLGWARMWVNSVPAAYKLSL--ATRSWGETVAKDQTAFN 196

Apa omtB KAFGSDLTMFEMWPPHKKHMESLGHMLALERPVSVWDHYVPLEELGGFPAEDKVLVMDIGGGFGQSQKALRAKFPDLPGRILVQDIPOTLANAQPAAG-- 266
Afl omtB KAFGSDLTMFEMWPPHKKHMESLGHMLALERPVSVWDHFFVLEELGKFFPAEDKVLVMDIGGGFGQSQKALRAKFPDLPGRILVQDIPOTLANAQPAAG-- 266
Cna CTB3 LGMDVVPFFDHLRQTPAMKDAFAAYMRNVTSNATWGLQHAVTGFDWASLPRGAKVVDVGGSLGHGSIAIAKEHHTLT--FVIQDLLETVGARKEMAQN 294

Apa omtB -----IEFMEHNFFEPQPIQNAKFFYLRRHVFDWDEQCVLILKQIIPAMGPESQILIDEMVIPSTG----VPWQAFTDLLMNSLGGVVERTR 351
Afl omtB -----IEFMEHNFFEPQPIQNAKFFYLRRHVFDWDEQCVLILKQIIPAMGPESQILIDEMVIPSTG----VPWQAFTDLLMNSLGGVVERTR 351
Cna CTB3 DKIEASVKSRTTFQEHDFGPTVKDADVYFLRMICHWDPNDEAKVILSQIRAAKPGAQIVIMDTILPQPGTISVLQEQQLRIRDLTMEVFNKEREL 394

Apa omtB AEWDDLMEQVGLLEIQSKVYDSKEQAILVAVPKRT 386
Afl omtB AEWDDLMEQAGLEIQSKVYDSKEQAILVAVAKRT 386
Cna CTB3 EDWSSLSMQSAGLEISRVNQPLNSVMGLLTVRSAGQ 429
    
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**(B) AFLS (a. a. 1-438)**

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Afl AFLS --MTLTDLETCAEEIATAARTLVRDGHGTGNS-----AGLPDHLRFVQRTLIANASQVLAASQPADLVRQLALYNQLLACLRLWGEFQVLAC 86
Afu AFLS -MDSITQLEARANEVAAAARRLAECRNARVDGAC---LPAVVPEAPRAIREIQRLLSNVHDHLQILLTEPADLVQRLAVQSLLSCLQWLGEFQVLAC 96
Cna CTB3 MMQFQRDLEASLEAVSANAQELLSKSRKDVQDLNASLPKDPDNCDAQTAARAQLAEAAATRIQLSIR-POEYLEHLQNGYQHLTCFRWLVELNILDH 100

Afl AFLS IELDEPVFFEDVADIAGVPECLRRLLVRLPFTIGFLCEPSGPHVAHSVLSKQVFTVQALLDAILFMSETLAPSASTMTGTQTRRFQ-ASEHAEDSÄWMMVV 185
Afu AFLS LELTDTVSIADLARLSCVPEAQARIIRFTITITIGFLQEPQRGHVAHSVLSLSEVSRPSRLDVMFLADSAAPTALQMASATGRFDTVASEDTYNYNIAF 196
Cna CTB3 LPHSGTISYTDLARKASVPPMQLRSICRMAICNGFLEEPEANQVRSRISALFARDESYLGWARMWVNSVPAAYKLSDATRSWG-ETVAKDQTAFNLGM 199

Afl AFLS GSDSFFAACLQQRPKVKRQLAAYLSYVSSAVDAAVEDTLTRMDWQNLG-MATVVHVGAQSPSLVVALAPQFPPLRFLVQTEAKTESGGHQPCLDNHGISA 284
Afu AFLS DHRDFEYFACEQRPKLQRRWSAYLQHTGGDARDLQVQLSRVDWNLN-NACVVEVLGPQSQSVTMMDLQHPMLHFIVQEARI PNGSTHAALHQD---- 291
Cna CTB3 DVKVPFFDHLRQTPAMKDAFAAYMRNVTSNATWGLQHAVTGFDWASLPRGAKVVDVGGSLGHGSIAIAKEHHTLT-FVQDLLETVGARKEMAQN---- 295

Afl AFLS LKLANIPLHLRARIITWGTRLSTATQVIDAAVYLISIPSPSQSPAMEITTRVAQALKAHVEVLRNNSDARLILTLPMSSATR-SMDAARAASVLSDL 383
Afu AFLS -----VRSVQIRELGGPQRVRDAAYIILNLG---PLPHAILSTSVLTELRAHFSVLAANSCAMLLTAGLLPKPQAVDARVETSVRLHDLS 375
Cna CTB3 ----KIEASVKSRTTFQEHDFGPTVKDADVYFLRMI-----CHWDPNDEAKVILSQIRAAKPG--AQIVIMDTILPQPGTISVLQEQQLRIRDLT 382

Afl AFLS LLQLTNGGSLNMGIEIRDLRSRSD---GLVVMREVRSEPTNAVIAFEIQYRVNDNDNRY---- 438
Afu AFLS LLQLANDRLEMEDELVEVMVHGKDSVGR LAVVNRLHLECTTVALGVRYQAFHGDSKSL 437
Cna CTB3 MMEVFNAKERELEDDSSLSMQSAGL-----EISRVNQPLNSVMGLLTVRSAGQTALSGTNTL 438
    
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**(C) FAD-dependent monooxygenases (a. a. 419-871)**

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Bra protein -----MARQLRIGIIGGGFCGLALAQGLRKAGLDPVVFEKNRARSYVQGFRLRVRRRGLSALLEALLPEALY 67
Rxy protein -----MLGRDVGNRGRSGRTFRVLIVGGGIGGLCLAQGLKKSRIEAVYERDESARFRGQGYRISIKADGGSGALRDLCLPENLF 78
Chv protein -----MKSVIIVGAGIIGGLCLAQGLKAKAGIEFEVWEADAGPQVRGGYRRLIDAMGQRAIRLQCLPPDLF 64
Phl protein -----MHVGIIGAGIGGTCLAHGLRKHGKVTIYERNASAASISLPYGIHINSFGKQALQECLPAENW 63
Cna CTB3 MGLLTVRSAGQTALSGTNTLTPELVAASASTGSADSRFPVLGAGIAGLCLAQALKKAGIDFRVFERDSDHIDARPPQYRLKFEADAASLKNILPDDVY 518

Bra protein QAVLDTAGRAPSETLQFDELLRPLDLRQPD---PAADDALIEMSLSRITLRQVLLTGLDDIIAYGRTFASYTENADGS-VTAHFEDRSSEVFDVLVA 162
Rxy protein DLSVATSIGSATRMVFLDHRINEKFAKPIP----PLAEDFEFCVN-RLTLREIILAGLEGVHFGKTFERFEPVEDGR-VRAYFADGTATGDLVGA 170
Chv protein ELFRASCAVSG-PARFVDPQLNPLPGRPPENWRENADAGEAGGDLSDRQVLRDILSHGLAPQIRWGRRARGFERGGD--IVVHCADGSEHGAAVVAA 161
Phl protein LAFEEASRYIGGQSRFYNERMRLAVHGGIS--FMAGKIISERQLSISRTELKEIENKGLANTIQWNKTFFVRYEHIENG--IKIFFADGSHENVVLVGA 160
Cna CTB3 EAFELSNAVTAGETDFNFNNGNIHRSRTGG--GLSGKKGLYATFFVDRKAFRTQMTGIEDKISFGKEIAYKTDATSTVNAEFKGTAVTGSFLAGT 616

Bra protein DGAGSRVRAQLLESARLIDTGARRFAGKVTFAEAERASLARVLQDYNVNI---RPTAGRTLMVTSHRVDPATFAHGLIG-ANDPSHAGLAGRHFENTAS 258
Rxy protein DGTDSRVALLVPDAGLDELGAFIYKTPITPGALEWVVDALVDSFNRM---GEDGVSVSATCIKRQPLACATARFAP-DLFLTEVQDYLAWMLNAPS 266
Chv protein DGLHSPLRQWLQAEPEAIGALNLYGKTPLDGIDPALLAGPTIVF-----ADGWTLVVEPMRFRAAMSGLAARHAP-DCRLSPDITDYVWAMFGR 252
Phl protein DGSNSKVRKQYLPFFIERFDVGVSMIIGRRLTPALTALLPQNFDRG-----TPNSIVPKSPDWLFIWMWRAPVNIHVEASLAEIDNFVWVYVAAT 251
Cna CTB3 DGLHSVVRKTCVFNHRIVDTGAACIYKVTMTPEFLARFPFERGLRFTMVVSDIAPMLQSCILGDSPTVLLLEPIRFSEASRARYPELPPDYVYWALIGPK 716

Bra protein YVWNVAFWRDEIADATLEALDGRGLLELLLRNANWHPELLKLEITDPSTVAALKVRSSVP-VPPWPTRR-VTLGDAIHTSMYFRALGANSIHDA 356
Rxy protein ALLP-----LAREESRRADGPAHLRLALGMLGEGWHSVRRIVEADAASTFLVRLRSARP-VERWQASN-VTLGDAIHTMSPRGEGANTALRDA 355
Chv protein ACLG-----GSLSGEEDAAGWRRRIEASGGLHPGLSLLRLRTAPEAVMGRVMAAG-VPSWPPGR-LTVLGDATHMSPPAGGANTALRDA 339
Phl protein DSLP-----DNITDFSAEALCDLVQSRMISWDPSSLHTLVQSDMENISPLHLRSMPH-LLPWKSST-VTLGDAIHNMTMPTGSGANTALRDA 337
Cna CTB3 ERFGSQE---VTSMKNFVSLDQAAEQAAKLSLAVTEEWHPSLRALFELQDTKQASLRIVASTIPDIPSWESHNSVTVLGGSIHPMSCGGVGAANTAVDA 813

Bra protein GLLVPAIVQIAAG-APLIETLAAYEAAMTAHGAFAVTD-SLAAMQALGPNSIAA----- 409
Rxy protein ALLRRALVDAVTRVPLYRAKARYETEMLRYGFRVADSRNPPFAPRSGGGSPV----- 410
Chv protein AMLAVCLADGDVD--R--AVARYEADMGRGAERALVATRAGTERLLRL----- 384
Phl protein LLLTQKIASVSGHEELVKAISDYEQMRAYANEIVGISLRSQAQNAVHFSIPPLKQRLHSIRRNKQSOSHQR 411
Cna CTB3 DALAKVLEHGK--PPVNAIEFGAAMTRAKRNIWRSEVSGKRMFGQKLVLDVCSFVF----- 871
    
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Supplementary Fig. (FGB-06-114R2) (A) Alignments of the N-terminal CTB3 of *Cercospora nicotianae* (CnA) with S-adenosyl-methionine (SAM)-dependent O-methyltransferases (omtB) of *A. parasiticus* (Apa; GenBank accession nos. **AAS66016**) and *A. flavus* (Afl; GenBank accession nos. **AF159789**). A putative SAM-binding domain is indicated by dashed underlines. (B) Alignments of the N-terminal CTB3 of *C. nicotianae* (CnA) with the AFLS transcription co-regulator of *A. fumigatus* (Afu; GenBank accession nos. **EAL89340**) and *A. flavus* (Afl; GenBank accession nos. **AAS90004**). (C) Alignments of the C-terminal CTB3 of *C. nicotianae* (CnA) with flavin adenine dinucleotide (FAD)-dependent monooxygenases of *Chromobacterium violaceum* (Chv; GenBank accession nos. **AAQ59500**), *Rubrobacter xylanophilus* (Rxy; GenBank accession nos. **EAN36783**), *Bradyrhizobium* sp. (Bra; GenBank accession nos. **EAP27500**), and *Photobacterium luminescens* subsp. *laumondii* (Phl; GenBank accession nos. **CAE14529**). The putative FAD-binding domains are indicated by dashed underlines.