2007

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

Katherine L. Dekkers

Bang-Jau You

Vivek S. Gowda

Hui-Ling Liao

Miin-Huey Lee

See next page for additional authors

Available online at www.sciencedirect.com
ScienceDirect
Fungal Genetics and Biology 44 (2007) 444–454
FUNGAL GENETICS AND BIOLOGY
www.elsevier.com/locate/yfgbi

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

Katherine L. Dekkers [a], Bang-Jau You [a], Vivek S. Gowda [a], Hui-Ling Liao [a,b], Miin-Huey Lee [c], Huey-Jiunn Bau [d], Peter P. Ueng [e], Kuang-Ren Chung [a,b,*]

[a] *Citrus Research and Education Center, Institute of Food and Agricultural Sciences (IFAS), University of Florida, 700 Experiment Station Rd., Lake Alfred, FL 33850, USA*
[b] *Department of Plant Pathology, IFAS, University of Florida, Gainesville, FL 32611, USA*
[c] *Department of Plant Pathology, National Chung-Hsing University, Taichung, 402 Taiwan, ROC*
[d] *Department of Biotechnology, Transworld Institute Technology, Douliu, 640 Taiwan, ROC*
[e] *Molecular Plant Pathology Lab., United States Department of Agriculture-Agricultural Research Service, BARC-West, 10300 Baltimore Ave., Beltsville, MD 20705, USA*

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 Δctb3 disruptants produced a yellow pigment that is not toxic to tobacco suspension cells. Production of cercosporin in a Δctb3 null mutant was fully restored when transformed with a functional *CTB3* clone or when paired with a Δctb1-null mutant (defective in polyketide synthase) by cross feeding of the biosynthetic intermediates. Pathogenicity assays using detached tobacco leaves revealed that the Δctb3 disruptants drastically reduced lesion formation.

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

* Corresponding author. Fax: +1 863 956 4631.
E-mail address: krchung@ufl.edu (K.-R. Chung).

1087-1845/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.fgb.2006.08.005

electronically-activated triplet state and produces active oxygen species such as the hydroxyl radical ($OH^{\cdot}$), 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 *CTB1* (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 sterigmatocystin 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 *CTB1* 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 Δctb3 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_2O (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′-ggcagcaccagtatccacga-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′-gagcgg cttgagtttgagcatcgc-3′), and OMT-3 (5′-gcgcagcactgaaa 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′-cgttgcaagaactgcctgaa-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 gattcgatgccctcatg-3′) and P40 (5′-cagctacgatgagtccgagg c-3′) and cloned to yield pCTB3G. A 1.0-kb *Cla*I 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′-tctgcaccatcgtcaaccac-3′) and the primers P42 (5′-cctcggtctcacag-3′) and bar2

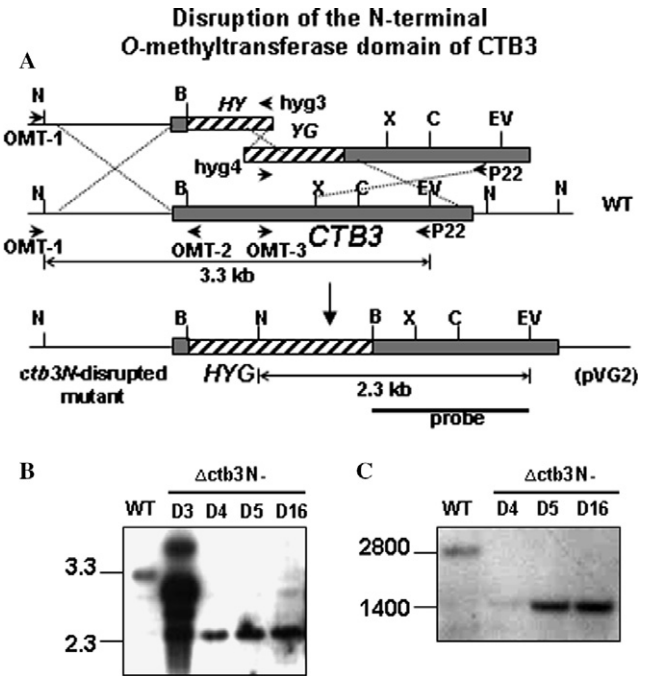

Fig. 1. Disruption of the N-terminal *O*-methyltransferase domain of CTB3 in *Cercospora nicotianae*. (A) Schematic illustration of a split-hygromycin phosphotransferase B gene (*HYG*) marker used for the 5′-end *CTB3* disruption. Two truncated *CTB3* fragments fused with an overlapping *HYG* were amplified 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, *Cla*I; EV, *Eco*RV; N, *Nco*I; X, *Xho*I. Note: drawing is not to scale. (B) Southern-blot analysis of genomic DNA from the wild type and four Δctb3N knockouts (D3, D4, D5, D16). Fungal DNA was digested with *Eco*RV and *Nco*I, 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 Δctb3N knockouts (D4, D5, D16). Sizes of hybridizing bands are indicated in nucleotides (nt).

(5′-aaacccacgtcatgccagtt-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 trp*C promoter (Pall and Brunelli, 1993) into a Δctb3-null mutant (Δctb3N-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 $CaCl_2$ 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

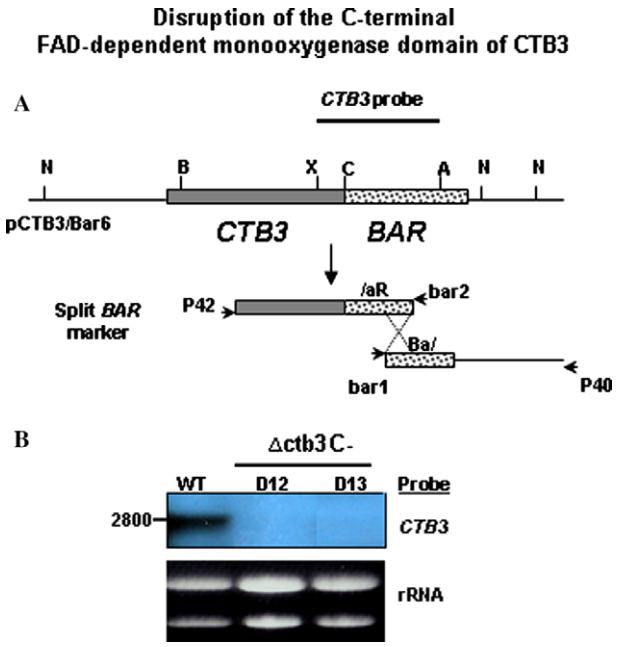

Fig. 2. Disruption of the C terminal FAD-dependent monooxygenase domain of CTB3 in *Cercospora nicotianae*. (A) Schematic illustration of pCTB3Bar6 plasmid containing a phosphinothricin acetyltransferase gene (*BAR*) marker used for the 3′-end *CTB3* disruption. Two truncated *CTB3* fragments fused with 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, *Age*I; B, *Bam*HI; C, *Cla*I; N, *Nco*I; X, *Xho*I. Note: drawing is not to scale. (B) Northern-blot analysis of total RNA isolated from the wild type and two Δctb3C 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 CTB3I (5′-cgtcctggtctcaat gctaacatc-3′) and P25 (5′-ttagccaaggcgtcggcatcgact-3′) were first used for amplification and the nested primer P22 was paired with CTB3I 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* DH5α 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 3.1×10^3 J/cm^2/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 (%)	*E*-value	Accession No.	Putative function
Gibberella zeae	439	36	52	6e−59	EAA69866	Unknown
Aspergillus nidulans	459	30	45	4e−39	EAA62575	Unknown
A. parasiticus	386	36	55	9e−25	AAS66016	*O*-methyltransferase
A. flavus	386	35	51	2e−25	AF159789	*O*-methyltransferase
Magnaporthe grisea	970	42	56	0	EAA48769	Unknown
Neurospora crassa	528	34	53	8e−32	EAA33703	Unknown
A. flavus	438	26	45	4e−25	AAS90004	AFLJ, transcription enhancer
A. nomius	438	26	44	1e−24	AAS90052	AFLJ
Chromobacterium violaceum	384	29	44	3e−31	AAQ59500	FAD-dependent monooxygenase
Rubrobacter xylanophilus	377	32	48	6e−30	EAN36783	FAD-dependent monooxygenase
Bradyrhizobium sp.	409	28	44	2e−25	EAP27500	FAD-dependent monooxygenase
Photorhabdus luminescens	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 Δctb3 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 Δctb3 disruptants (Δctb3N-D3, D4, D5 and D16) was cleaved with restriction enzymes *Nco*I and *Eco*RV and hybridized with a *CTB3*-specific probe. The results indicated that all Δctb3 analyzed displayed a *CTB3* deletion in the *C. nicotianae* genome. As expected, the wild-type strain displayed a 3.3-kb *Nco*I–*Eco*RV hybridizing band from the genomic DNA, whereas the Δctb3N disruptants contained a 2.3 kb hybridizing band due to the insertion of the *HYG* cassette in *CTB3* and the presence of an additional *Nco*I 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 Δctb3N-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 Δctb3N strains (D4, D5, D16) (Fig. 1C). By contrast, a truncated transcript of approximate 1400 nucleotides in size was detected in RNA from the Δctb3N strains, verifying the disruption of Δctb3N strains indeed specifically occurred at the *CTB3* locus.

Northern-blot analysis of RNA from the Δctb3N 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 Δctb3C-D12 and D13) recovered from a medium containing gluphosinate ammonium were unable to produce cercosporin. Northern-hybridization analysis indicated that the 2800-nt *CTB3* transcript was completely undetectable in the Δctb3C- D12 and D13 isolates (Fig. 2B), further validating the disruption occurred at the *CTB3* locus. The Δctb3C disruptants failed to produce cercosporin but accumulated a yellow/brown pigment similar to the Δctb3N 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 monooxygenases 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 Δctb3N-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 Δctb1 and Δctb3 knockouts were co-cultured on PDA. Complementation was observed when a Δctb1 disruptant was paired with the Δctb3N or Δctb3C 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 Δctb3N disruptants and the Δctb3C 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 Δctb3 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 (Δctb3N), and C-terminus (Δctb3C), and the *CTB3*-complemented strains (T5C11 and T5C12) under continuous light (LT) or darkness (DK)

Isolates	Treatment	Cercosporin production (nmol/agar plug)[a]
CnA	LT	196.2 ± 58.6
	DK	3.7 ± 0.8
Δctb3N-D3	LT	11.0 ± 4.4
	DK	1.4 ± 0.6
Δctb3N-D4	LT	44.6 ± 4.8
	DK	1.2 ± 0.4
Δctb3N-D5	LT	50.9 ± 14.7
	DK	1.4 ± 0.9
Δctb3N-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
Δctb3C-D12	LT	24.6 ± 7.9
	DK	0.6 ± 0.2
Δctb3C-D13	LT	16.6 ± 3.2
	DK	1.0 ± 0.4

[a] 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 Δctb3 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 Δctb3 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 Sollod et al., 1992). By contrast, the KOH extract of the Δctb3 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_f0.7, typical of cercosporin, whereas the Δctb3-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

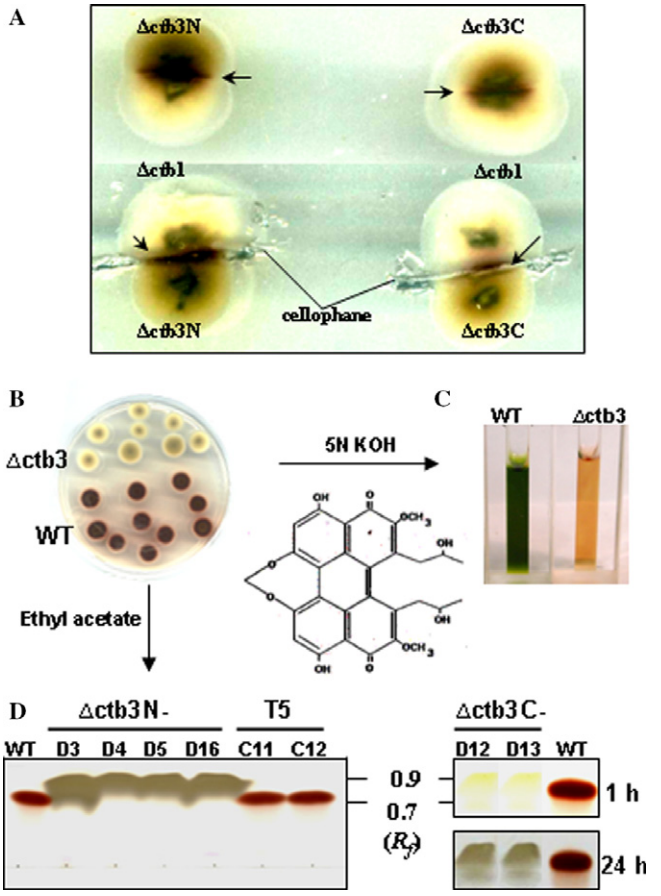

Fig. 3. Characterization of cercosporin produced by the wild type (WT) and a pigment produced by the Δctb3 disruptant of *Cercospora nicotianae*. (A) Complementation of cercosporin production by pairing the Δctb1 with Δctb3 disruptants as indicated on a PDA plate. (B) Production of cercosporin and other pigments by the wild type and the Δctb3 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 Δctb3 disruptants with 5 N KOH. (D) Thin-layer chromatography (TLC) analysis of cercosporin production by the wild-type (WT) strain, the Δctb3 N terminal disruption mutants (Δctb3N-D3, D4, D5, D16), the *CTB3*-complemented strains (T5C11, T5C12), and the Δctb3 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_2O (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_f0.7. By contrast, the Δctb3-disrupted mutants produced a yellow/brown pigment at R_f 0.9. The pigment produced by the Δctb3 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 Δctb3 disruptants (data not shown). The yellow/brown pigment was completely undetectable when the Δctb3 disruptants were grown in darkness. Collectively, we concluded that the Δctb3 disruptants are completely defective in cercosporin accumulation.

To determine if the yellow/brown pigment extracted from cultures of the Δctb3 disruptants is toxic, tobacco cell

suspensions were treated with the extracts from the wild-type strain and Δctb3 disruptants, and incubated under continuous light or in complete darkness. The results indicated that the yellow pigment extracted from the cultures of the Δctb3 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 Δctb3 disruptants. Similar to the Δctb1 knockouts (Choquer et al., 2005), all Δctb3 disruptants exhibited growth similar to the wild-type strain on the medium containing exogenous cercosporin (10 μM) 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 Δctb3 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 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

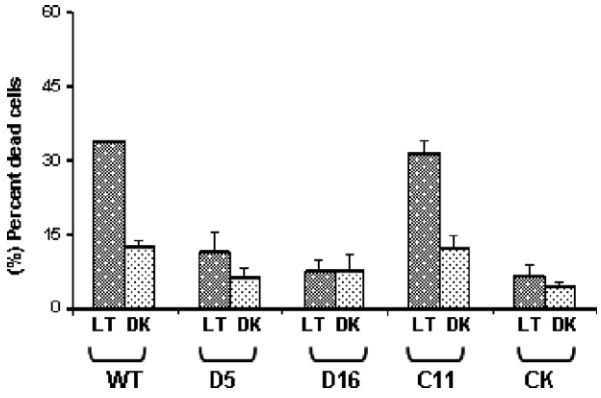

Fig. 4. Viability of tobacco suspension cells treated with pigments produced by the wild type (WT), two ctb3-disrupted mutants (Δctb3N-D5, D16), and a complemented strain (T5C11) of *Cercospora nicotianae*, and the acetone control (CK) under continuous light (LT) or in complete darkness (DK). Tobacco 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.

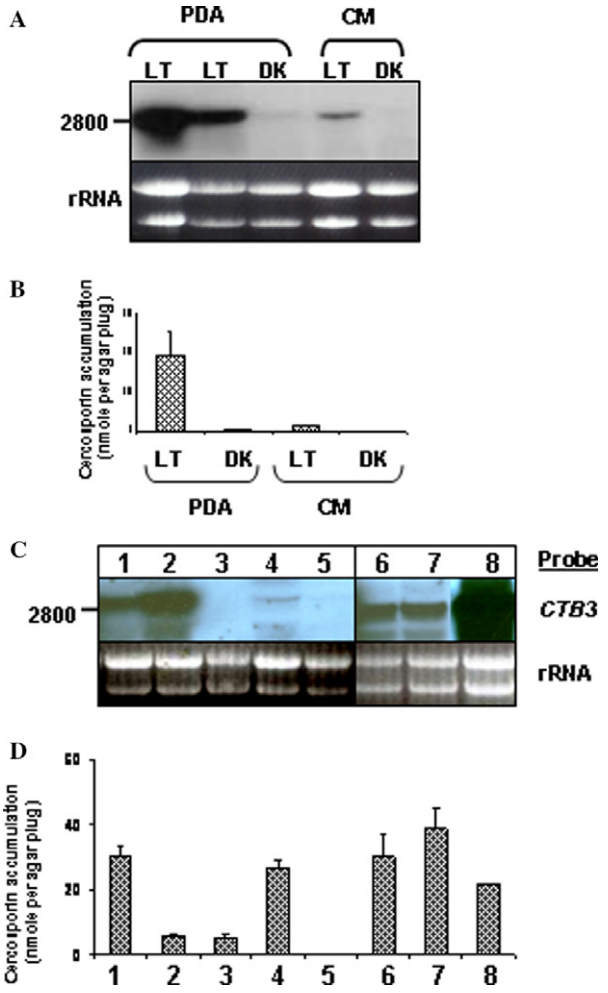

Fig. 5. Differential expression of the *CTB3* gene and accumulation of cercosporin by the *Cercospora nicotianae* wild type. (A) Northern-blot analysis of *CTB3* expression in the *C. nicotianae* wild type grown on potato dextrose agar (PDA) or complete medium (CM) under continuous light (LT) or darkness (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).

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 Δctb1 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 Δctb1 disruptants (Choquer et al., 2005), the Δctb3 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).

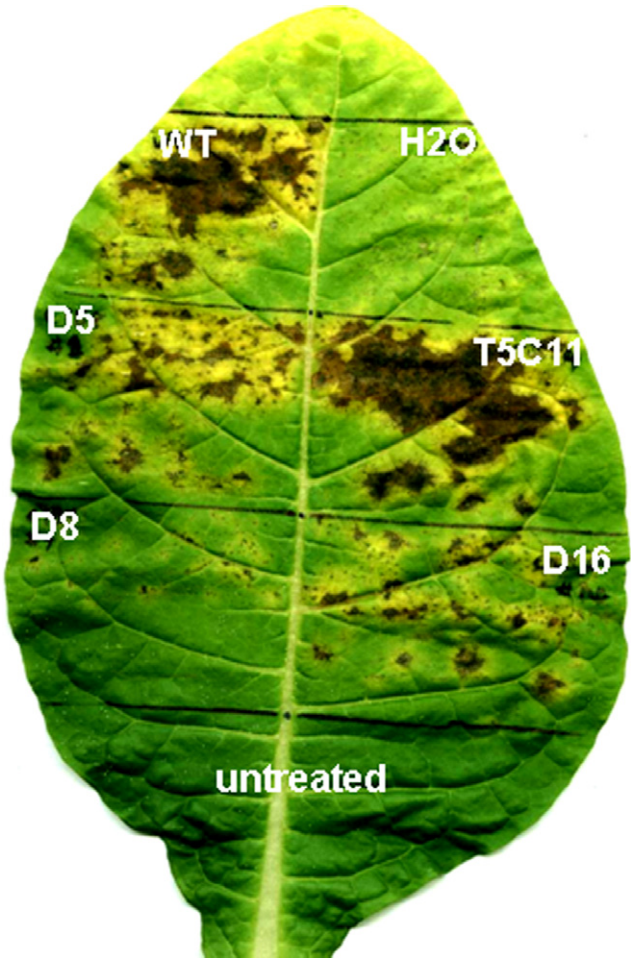

Fig. 6. Pathogenicity assay using detached tobacco leaves cv. Burley 21. The *Cercospora nicotianae* wild type (WT), Δctb3-knockout mutants (Δctb3N- D5, D8, D16) and *CTB3*-complemented strain (T5C11) were grown on V8 juice agar for 7–12 days at 20 °C under continuous light. The concentration of conidia was adjusted to 5×10^4 conidia/ml and rubbed onto wounded tobacco leaves with cotton swabs. The inoculated leaves were then kept in a moist chamber under fluorescent light.

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_6Zn_2 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; Jenns 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 Δctb1 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 Δctb3 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 Δctb3 disruptants was not toxic to tobacco cells. Whether or not the yellow/brown pigment accumulated by the Δctb3 knockouts represents an intermediate for cercosporin biosynthesis awaits further structural investigation. Complementation of cercosporin production was achieved when the Δctb3 disruptants were paired with the Δctb1 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 Δctb3 disruptants is likely an intermediate for cercosporin biosynthesis.

Pathogenicity assays using detached tobacco cv. Burley 21 leaves revealed that, as with the Δctb1 disruptants (Choquer et al., 2005), the Δctb3-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.

References

Abe, Y., Ono, C., Hosobuchi, M., Yoshikawa, H., 2002. Functional analysis of *mlcR*, a regulatory gene for ML-236B (compactin) biosynthesis in *Penicillium citrinum*. Mol. Gen. Genomics 268, 352–361.

Ahn, J.H., Walton, J.D., 1996. Chromosomal organization of *TOX2*, a complex locus controlling host-selective toxin biosynthesis in *Cochliobolus carbonum*. Plant Cell 8, 887–897.

Alexander, N.J., McCormick, S.P., Hohn, T.M., 1999. TRI12, a trichothecene efflux pump from *Fusarium sporotrichioides*: Gene Isolation and Expression in Yeast. Mol. Gen. Genet. 261, 977–984.

Brown, M.P., Brown-Jenco, C.S., Payne, G.A., 1999. Genetic and molecular analysis of aflatoxin biosynthesis. Fungal Genet. Biol. 26, 81–98.

Brown, D.W., Yu, J.H., Keller, H.S., Fernandes, M., Nesbitt, T.C., Keller, N.P., Adams, T.H., Leonard, T.J., 1996. Twenty-five coregulated transcripts define a sterigmatocystin gene cluster in *Aspergillus nidulans*. Proc. Natl. Acad. Sci. USA 93, 1418–1422.

Callahan, T.M., Rose, M.S., Meade, M.J., Ehrenshaft, M., Upchurch, R.G., 1999. *CFP*, the putative cercosporin transporter of *Cercospora kikuchii*, is required for wild type cercosporin production, resistance, and virulence on soybean. Mol. Plant-Microbe Interact. 12, 901–910.

Calvo, A.M., Wilson, R.A., Bok, J.W., Keller, N.P., 2002. Relationship between secondary metabolism and fungal development. Microbiol. Molec. Biol. Rev. 66, 447–459.

Chang, P.K., 2003. The *Aspergillus parasiticus* protein AFLJ interacts with the aflatoxin pathway-specific regulator AFLR. Mol. Gen. Genomics 268, 711–719.

Choquer, M., Dekkers, K.A., Chen, H.Q., Cao, L., Ueng, P.P., Daub, M.E., Chung, K.R., 2005. The *CTB1* gene encoding a fungal polyketide synthase is required for cercosporin biosynthesis and fungal virulence of *Cercospora nicotianae*. Mol. Plant-Microbe Interact. 18, 468–476.

Chung, K.R., 2003. Involvement of calcium/calmodulin signaling in cercosporin toxin biosynthesis by *Cercospora nicotianae*. Appl. Environ. Microbiol. 69, 1187–1196.

Chung, K.R., Jenns, A.E., Ehrenshaft, M., Daub, M.E., 1999. A novel gene required for cercosporin toxin resistance in the fungus, *Cercospora nicotianae*. Mol. Gen. Genet. 262, 382–389.

Chung, K.R., Shilts, T., Li, W., Timmer, L.W., 2002. Engineering a genetic transformation system for *Colletotrichum acutatum*, the causal fungus of lime anthracnose and postbloom fruit drop. FEMS Microbiol. Lett. 213, 33–39.

Chung, K.R., Daub, M.E., Kuchler, K., Schüller, C., 2003a. The *CRG1* gene required for resistance to the singlet oxygen-generating cercosporin toxin in *Cercospora nicotianae* encodes a putative fungal transcription factor. Biochem. Biophys. Res. Comm. 302, 302–310.

Chung, K.R., Ehrenshaft, M., Wetzel, D.K., Daub, M.E., 2003b. Cercosporin toxin deficient mutants by plasmid tagging in the asexual fungus *Cercospora nicotianae*. Mol. Genet. Genomics 270, 103–113.

Chung, K.R., Daub, M.E., Ehrenshaft, M., 2003c. Expression of the cercosporin toxin resistance gene (*CRG1*) as a discistronic mRNA in the filamentous fungus *Cercospora nicotianae*. Curr. Genet. 43, 415–424.

Daub, M.E., 1982a. Peroxidation of tobacco membrane lipids by the photosensitizing toxin, cercosporin. Plant Physiol. 69, 1361–1364.

Daub, M.E., 1982b. Cercosporin, a photosensitizing toxin from *Cercospora* species. Phytopathology 72, 370–374.

Daub, M.E., Briggs, S.P., 1983. Changes in tobacco cell membrane composition and structure caused by the fungal toxin, cercosporin. Plant Physiol. 71, 763–766.

Daub, M.E., Ehrenshaft, M., 2000. The photoactivated *Cercospora* toxin cercosporin: Contributions to plant disease and fundamental biology. Annu. Rev. Phytopathol. 38, 461–490.

Daub, M.E., Hangarter, R.P., 1983. Production of singlet oxygen and superoxide by the fungal toxin, cercosporin. Plant Physiol. 73, 855–857.

Daub, M.E., Herrero, S., Chung, K.R., 2005. Photoactivated perylenequinone toxins in fungal pathogenesis of plants. FEMS Microbiol. Lett. 252, 197–206.

Dowzer, C.D., Kelly, J.M., 1991. Analysis of the *creA* gene, a regulator of carbon catabolite repression in *Aspergillus nidulans*. Mol. Cell. Biol. 11, 5701–5709.

Ehrenshaft, M., Upchurch, R.G., 1993. Host protein(s) induces accumulation of the toxin cercosporin and mRNA in a phytopathogenic strain of *Cercospora kikuchii*. Physiol. Plant Pathol. 79, 157–164.

Espeso, E.A., Arst Jr., H.N., 2000. On the mechanism by which alkaline pH prevents expression of an acid-expressed gene. Mol. Cell. Biol. 20, 3355–3363.

Foote, C.S., 1976. Photosensitized oxidation and singlet oxygen: Consequences in Biological Systems. In: Pryor, W.A. (Ed.), Free Radicals in Biology, II. Academic Press, New York, pp. 85–133.

Fore, S.A., Daub, M.E., Beute, M.K., 1988. Phytotoxic substances produced by some isolates of *Cercospora arachidicola* are not cercosporin. Phytopathology 78, 1082–1086.

Fujii, I., Yoshida, N., Shimomaki, S., Oikawa, H., Ebizuka, Y., 2005. An iterative type I polyketide synthase PKSN catalyzes synthesis of the decaketide alternapyrone with regio-specific octa-methylation. Chem. Biol. 12, 1301–1309.

Gardiner, D.M., Cozijnsen, A.J., Wilson, L.M., Pedras, M.S.C., Howlett, B.J., 2004. The sirodesmin biosynthetic gene cluster of the plant pathogenic fungus *Leptosphaeria maculans*. Mol. Microbiol. 53, 1307–1318.

Hohn, T.M., McCormick, S.P., Desjardins, A.E., 1993. Evidence for a gene cluster involving trichothecene-pathway biosynthetic genes in *Fusarium sporotrichioides*. Curr. Genet. 24, 291–295.

Ito, T., 1981. Dye binding and photodynamic action. Photochem. Photobiol. 33, 947–955.

Jenns, A.E., Daub, M.E., 1995. Characterization of mutants of *Cercospora nicotianae* sensitive to the toxin cercosporin. Phytopathology 85, 906–912.

Jenns, A.E., Daub, M.E., Upchurch, R.G., 1989. Regulation of cercosporin accumulation in culture by medium and temperature manipulation. Phytopathology 79, 213–219.

Keller, N.P., Hohn, T.M., 1997. Metabolic pathway gene clusters in filamentous fungi. Fungal Genet. Biol. 21, 17–29.

Lu, S., Lyngholm, L., Yang, G., Bronson, C., Yoder, O.C., Turgeon, B.G., 1994. Tagged mutations at the *Tox*1 locus of *Cochliobolus heterostrophus* by restriction enzyme-mediated integration. Proc. Natl. Acad. Sci. USA. 91, 12649–12653.

Marzluf, G.A., 1997. Genetic regulation of nitrogen metabolism in the fungi. Microbiol. Mol. Biol. Rev. 61, 17–32.

Okubo, A., Yamazak, S., Fuwa, K., 1975. Biosynthesis of cercosporin. Agric. Biol. Chem. 39, 1173–1175.

Pall, M.L., Brunelli, J.P., 1993. A series of six compact fungal transformation vectors containing polylinkers with multiple unique restriction sites. Fungal Genet. Newslett. 40, 59–62.

Proctor, R.H., Brown, D.W., Plattner, R.D., Desjardins, A.E., 2003. Co-expression of 15 contiguous genes delineates a fumonisin biosynthetic gene cluster in *Gibberella moniliformis*. Fungal Genet. Biol. 38, 237–249.

Ruzin, S.E., 1999. Plant Microtechnique and Microscopy. Oxford University Press, Oxford.

Sambrook, J., Russell, D.W., 2001. Molecular Cloning: A Laboratory Manual, third ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NewYork.

Seo, J.A., Proctor, R.H., Plattner, R.D., 2001. Characterization of four clustered and coregulated genes associated with fumonisin biosynthesis in *Fusarium verticillioides*. Fungal Genet. Biol. 34, 155–165.

Shim, W.B., Dunkle, L.D., 2003. *CZK3*, a MAP kinase kinase kinase homolog in *Cercospora zeae-maydis*, regulates cercosporin biosynthesis, fungal development, and pathogenesis. Mol. Plant-Microbe Interact. 16, 760–768.

Sims, J.W., Fillmore, J.P., Warner, D.D., Schmidt, E.W., 2005. Equisetin biosynthesis in *Fusarium heterosporum*. Chem. Comm. 2, 186–188.

Sollod, C.C., Jenns, A.J., Daub, M.E., 1992. Cell surface redox potential as a mechanism of defense against photosensitizers in fungi. Appl. Environ. Microbiol. 58, 444–449.

Spiering, M.J., Moon, C.D., Wilkinson, H.H., Schardl, C.L., 2005. Gene cluster for insecticidal loline alkaloids in the grass-endophytic fungus *Neotyphodium uncinatum*. Genetics 169, 1403–1414.

Spikes, J.D., 1989. Photosensitization. In: Smith, K.C. (Ed.), The Science of Photobiology, second ed. Plenum Press, New York.

Straubinger, B., Straubinger, E., Wirsel, S., Turgeon, G., Yoder, O.C., 1992. Versatile fungal transformation vectors carrying the selectable *bar* gene of *Streptomyces hygroscopicus*. Fungal Genet. Newslett. 39, 82–83.

Tanaka, A., Tsuge, T., 2000. Structural and functional complexity of the genomic region controlling AK-toxin biosynthesis and pathogenicity in the Japanese pear pathtype of *Alternaria alternate*. Mol. Plant-Microbe Interact. 13, 975–986.

Tudzynski, B., Hölter, K., 1998. Gibberellin biosynthetic pathway in *Gibberella fujikuroi*: Evidence for A Gene Cluster. Fungal Genet. Biol. 25, 157–170.

Walton, J.D., 2000. Horizontal gene transfer and the evolution of secondary metabolite gene clusters in fungi: An Hypothesis. Fungal Genet. Biol. 30, 167–171.

Yamazaki, S., Okube, A., Akiyama, Y., Fuwa, K., 1975. Cercosporin, a novel photodynamic pigment isolated from *Cercospora kikuchii*. Agric. Biol. Chem. 39, 287–288.

Yu, J., Bhatnagar, D., Cleveland, T.E., 2004. Completed sequence of aflatoxin pathway gene cluster in *Aspergillus parasiticus*. FEBS Lett. 564, 126–130.

Supplementary Figure (FGB-06-114R2)

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

```
Apa omtB  ------------------------------MTGLDMEIIFAKIKEEYARTDDVGKRQIQGHIRELQVGFYSDWDVVMRLSSGPLQVALTKVGIDLGIFR  69
Afl omtB  ------------------------------MTGLDMETIFAKIKDEYARTDDVGKRQIQGHIRELQVGFYSDWDVVMRLSSGPLQVALAKVAIDLGIFR  69
CnA CTB3  MMQFQRDLEASLEAVSANAQELLKSLKSRKDVQDLNASLPKDPLDNCDAQTQAARAQLAEAATRILQLSIR-PQEYLEHLQNGYQHLTCFRWLVELNILD  99

Apa omtB   SLKESDTPITLAEIVKKTGASPRLLGRILRTQAAFGLIKETGPQEYTSSAFTDVFAN-SDAAGAVVQLFDISGPCTQILPDFLAERNYQDITSNKDCVFQ 168
Afl omtB   SLKESDTPITLAEFVKKTGASPRLLGRILRTQAAFGLIKETGPQEYTSSAFTDVFAN-SDAAGAVVQLFDISGPCTQILPDFLAERNYQDITSNKDCVFQ 168
CnA CTB3   HLPHSGT-ISYTDLARKASVPPMQLRSICRMAICNGFLEEPEANQVRHSRISALFARDESYLGWARWMVNYSVPAAYKLSD--ATRSWGETVAKDQTAFN 196

Apa omtB   KAFGSDLTMFEWMPQHPKHMESLGHLMALERPVSWVDHYPVLEELGGFPAPDKVLMVDIGGGFGQQSKALRAKFPDLPGRLIVQDIPQTLANAQPAAG-- 266
Afl omtB   KAFGSDLTMFEWMPQHPKHMESLGHLMALERPVSWVDHFPILEELGKFPAPDKVLMVDIGGGFGQQSKALRAKFPNLPGRLIVQDIPQTLANAQPAAG-- 266
CnA CTB3   LGMDVKVPFFDHLRQTPAMKDAFAAYMRNVTSNATWGLQHAVTGFDWASLPRGAKVVDVGGSLGHGSIAIAKEHTHLT--FVIQDLPETVAGARKEMAQN 294

Apa omtB   ----------IEFMEHNFFEPQPIQNAKFYYLRHVFHDWPDEQCVLILKQIIPAMGPESQILIDEMVIPSTG-----VPWQAAFTDLLMMNSLGGVERTR 351
Afl omtB   ----------IEFMEHNFFEPQPIQNAKFYYLRHVFHDWPDEQCVLILKQIIPAMGPESQILIDEMVIPSTG-----VPWQAAFTDLLMMNSLGGVERTR 351
CnA CTB3   DKIEASVKSRITFQEHDFFGPQTVKDADVYFLRMICHDWPDNEAKVILSQIRAALKPGAQIVIMDTILPQPGTISVLQEQQLRIRDLTMMEVFNAKEREL 394
                                  ------------------------------------

Apa omtB  AEWDDLMEQVGLEIIQSKVYDSKEQAILVAVPKRT 386
Afl omtB  AEWDDLMEQAGLEIIQSKVYDSKEQAILVAVAKRT 386
CnA CTB3  EDWSSLMQSAGLEISRVNQPLNSVMGLLTVRSAGQ 429
```

(B) AFLS (a. a. 1-438)

```
Afl AFLS --MTLTDLETCAEEIATAARTLVRDGHTGGNS------------AGLPDHLRPVQRTLIANASQVLALASQPADLVRQLALYNQLLACLRWLGEFQVLAC  86
Afu AFLS -MDSITQLEARANEVAAAARRLAEYCRNARVDGAC---LPAVVPSEAPRAIREIQRLLLSNVDHLQILLTEPADLVQRLAVQSQLLSCLQWLGEFQVLAC  96
CnA CTB3 MMQFQRDLEASLEAVSANAQELLKSLKSRKDVQDLNASLPKDPLDNCDAQTQAARAQLAEAATRILQLSIRPQEYLEHLQNGYQHLTCFRWLVELNILDH 100

Afl AFLS IPLDEPVPFEDVADIAGVPECRLRRLVRPLFTIGFLCEPSPGHVAHSVLSKQFVTQPALLDAILFMSETLAPSASTMGTQTRRFG-ASEHAEDSAWNMVV 185
Afu AFLS LPLTDTVSIADLARLSGVPEAQLARIIRFTITIGFLQEPQRGHVAHSPLSSLFVSRPSLRDAVMFLADSAAPTALQMASATGRFGDTVASETDTAYNIAF 196
CnA CTB3 LPHSGTISYTDLARKASVPPMQLRSICRMAICNGFLEEPEANQVRHSRISALFARDESYLGWARWMVNYSVPAAYKLSDATRSWG-ETVAKDQTAFNLGM 199

Afl AFLS GSDSPFAACLQQRPKVKRQLAAYLSYVSSAVDAAVEDTLTRMDWQNLG-MATVVHVGAQSPSLVVALAPQFPSLRFLVQTEAKTESGGHQPCLDNHGISA 284
Afu AFLS DHRDPFYFACEQRPKLQRRWSAYLQHTGGDARDLTQQVLSRVDWFNLN-NACVVEVLGPQSQSVTMMLDQLHPMLHFIVQEARIPNGSTHAALHQD---- 291
CnA CTB3 DVKVPFFDHLRQTPAMKDAFAAYMRNVTSNATWGLQHAVTGFDWASLPRGAKVVDVGGSLGHGSIAIAKEHTHLTFVIQDLPETVAGARKEMAQND---- 295

Afl AFLS LKLANIPLHLRARITWGTRLSTATQPVIDAAVYLISIPSPSPQSPAMEITTRVAQALKAHVEVLRNNSDARLILTLPMSSATR-SMDAAARAAVSLSDLS 383
Afu AFLS ------------VRSVQIRELGGPQRVRDAAIYILNLG----PLPHAILSTSVLTELRAHFSVLAANSCAMLILTAGLLLPKPGAVDARVETSVRLHDLS 375
CnA CTB3 ----KIEASVKSRITFQEHDFFGPQTVKDADVYFLRMI-------CHDWPDNEAKVILSQIRAALKPG--AQIVIMDTILPQPGTISVLQEQQLRIRDLT 382

Afl AFLS LLQLTNGGSLNMGEIRDLLRSRSD---GLVVMREVRSPTNAVIAFEIQYRVDNDDNRY---- 438
Afu AFLS LLQLANDRLMEEDELVEMVHGVKDSVGRLAVVNRLHLPCTTTVALGVRYQAFGHGDSSAKSL 437
CnA CTB3 MMEVFNAKERELEDWSSLMQSAGL------EISRVNQPLNSVMGLLTVRSAGQTALSGTNTL 438
```

(C) FAD-dependent monooxygenases (a. a. 419-871)

```
Bra protein  ---------------------------------MARQLRIGIIGGGPGGLALAQGLRKAGLDPVVFEKNRARSDYVQGFRLRVRRRGLSALEALLPEALY  67
Rxy protein  ----------------------MLGRDVGNRGRSGRTFRVLIVGGGIGGLCLAQGLKKSRIEVAVYERDESARFRGQGYRISIKADGSGALRDCLPENLF  78
Chv protein  -----------------------------------MKSVIIVGAGIGGLCLAQGLAKAGIEFEVWEADAGPQVRGQGYRLRIDAMGQRALRQCLPPDLF  64
Phl protein  ------------------------------------MHVGIIGAGIGGTCLAHGLRKHGIKVTIYERNSAASSILPGYGIHINSFGKQALQECLPAENW  63
CnA CTB3     MGLLTVRSAGQTALSGTNTLTPELVAAVSASTGSADSRPVLIAGAGIAGLCLAQALKKAGIDFRVFERDSHIDARPQGYRLKFEADAAQSLKNILPDDVY 518
                                                 ---------

Bra protein  QAVLDTAGRAPSETLQFDELLRPLDLRQGPD----PAADDALIEMSLSRITLRQVLLTGLDDIIAYGRTFASYTENADGS-VTAHFEDRSSEVFDVLVGA 162
Rxy protein  DLSVATSIGSATRMVFLDHRLNEKFAKPIP-----PLAEDEFFGVN--RLTLREILLAGLEGVVHFGKTFERFEPVEDGR-VRAYFADGTTATGDLLVGA 170
Chv protein  ELFRASCAVSG-PARFVDPQLNPLPGRRPENWRENADAGEAGGDLSADRQVLRDILSHGLAPQIRWGRRARGFERGGDG--IVVHCADGSEHGAALVVAA 161
Phl protein  LAFEEASRYIGGQSRFYNERMRLLAVHGGIS--PMAGKIISEQRLSISRTELKEILNKGLANTIQWNKTFVRYEHIENGG-IKIFFADGSHENVDVLVGA 160
CnA CTB3     EAFELSNAVTAVGETDFNPFNGNIIHSRTGG--GLSGKKGLYATFTVDRKAFRTQLMTGIEDKISFGKEIAYYKTDDATSTVNAEFKDGTHVTGSFLAGT 616

Bra protein DGAGSRVRAQLLPSARLIDTGARRFAGKVTFAEAERASLARVLQDYNVNI---RPTAGRTLMVTSHRVDPATFAAHGLIG-ANDPSHAGLAGRHFDNTAS 258
Rxy protein DGTDSRVRALLVPDAGLDELGAFIYGKTPITPGALEWVPDALVDSFNRMT---GPDGVSVSVATCIKRQPLACATARFAP-DLFLTEVQDYLAWMLNAPS 266
Chv protein DGLHSPLRRQWLPQAEPEAIGALNLYGKTPLDGIDPALLAGPTIVF--------ADGWTLVVEPMRFRAAMSGLAARHAP-DCRLSPTDDYVYWAMFGRE 252
Phl protein DGSNSKVRKQYLPFIERFDVGVSMIIGRARLTPALTALLPQNFRDG---------TPNSIVPKSPDWLFISMWRAPVNIHVEASLAEIDNFIVWVYVAAT 251
CnA CTB3    DGLHSVVRKTCVPNHRIVDTGAACIYGKTVMTPEFLARFPEKGLRFMTVVSDIAPMLQSCLIGDSPVTLLLEPIRFSEASRARYPELPPDYVYWALIGPK 716

Bra protein YVWWNVAFWRDEIAADATLEALDGRGLLELLLRHNANWHPELLKLIEITDPSTVAALKVRSSVP-VPPWPTRR-VTLLGDAIHSMTYFRALGANSAIHDA 356
Rxy protein ALLP---------LAREESRRADGPALHRLALGMLEGWHPSVRRIVEEADAASTFLVRLRSARP-VERWQASN-VTLLGDAIHTMSPGRGEGANTALRDA 355
Chv protein ACLG-----------GSLSGEEDAAGWRRRIEAASGGLHPGLSDLLRRTAPEAVMGRAVRMAAG-VPSWPPGR-LTVLGDAIHAMSPAGGVGANTALADA 339
Phl protein DSLP-----------DNITDFSAEALCDLVQSRMISWDPSLHTLVQQSDMENISPLHLRSMPH-LLPWKSST-VTLLGDAIHNMTPMTGSGANTALRDA 337
CnA CTB3    ERFGSQE---VTSMKNFVSLDQAAEQAAKLSLAVTEEWHPSLRALFELQDTKQASLIRVASTIPDIPSWESHSNVTVLGGSIHPMSPCGGVGANTAIVDA 813
                                                                                  --------------------------

Bra protein GLLVPALVQIAAG-APLIETLAAYEAAMTAHGFAAVTDS-LAAMQRALGPNSIAA------------------- 409
Rxy protein ALLRRALVDAVTDRVPLYRAKARYETEMLRYGFRAVADSRNNPFAPRSGPGGSPV------------------- 410
Chv protein AMLAVCLADGDVD---R--AVARYEADMRGRAERALVATRAGTERLLRRL------------------------ 384
Phl protein LLLTQKLASVASGHEELVKAISDYEQQMRAYANEIVGISLRSAQNAVIHFSIPPLKQRHLSIRRNKSQSHQHRR 411
CnA CTB3    DALAKVLVEHGTK--PPVNAIAEFGAAMRTRAKRNIWRSEVGSKRMFGQKNLVDCSEFVF-------------- 871
           --------
```

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 *Photorhabdus luminescens* subsp. *laumondii* (Phl; GenBank accession nos. **CAE14529**). The putative FAD-binding domains are indicated by dashed underlines.