The *CTB1* Gene Encoding a Fungal Polyketide Synthase Is Required for Cercosporin Biosynthesis and Fungal Virulence of *Cercospora nicotianae*

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Cercosporin is a light-activated, non-host-selective toxin produced by many *Cercospora* fungal species. In this study, a polyketide synthase gene (*CTB1*) was functionally identified and molecularly characterized to play a key role in cercosporin biosynthesis by *Cercospora nicotianae*. We also provide conclusive evidence to confirm the crucial role of cercosporin in fungal pathogenesis. *CTB1* encoded a polypeptide with a deduced length of 2,196 amino acids containing a keto synthase (KS), an acyltransferase (AT), a thioesterase/claisen cyclase (TE/CYC), and two acyl carrier protein (ACP) domains, and had high levels of similarity to many fungal type I polyketide synthases. Expression of a 6.8-kb *CTB1* transcript was highly regulated by light and medium composition, consistent with the conditions required for cercosporin biosynthesis in cultures. Targeted disruption of *CTB1* resulted in the loss of both *CTB1* transcript and cercosporin biosynthesis in *C. nicotianae*. The *ctb1*-null mutants incited fewer necrotic lesions on inoculated tobacco leaves compared with the wild type. Complementation of *ctb1*-null mutants with a full-length *CTB1* clone restored wild-type levels of cercosporin production as well as the ability to induce lesions on tobacco. Thus, we have demonstrated conclusively that cercosporin is synthesized via a polyketide pathway, and cercosporin is an important virulence factor in *C. nicotianae*. The results also suggest that strategies that avoid the toxicity of cercosporin will be useful in reduction of disease incidence caused by *Cercospora* spp.

Additional keywords: gene disruption, pathogenicity, perylenequinone.

Cercospora spp. are a highly successful group of fungal pathogens causing disease in more than 100 plant species, such as soybean, sugar beet, coffee, rice, corn, peanut, and tobacco (Daub and Ehrenshaft 2000). *Cercospora* spp. cause major economic problems because of their worldwide distribution, wide host range, and low levels of resistance in many host plants. Many *Cercospora* spp. produce a non-host-specific toxin, cercosporin (1,12-bis-(2-hydroxypropyl)-2,11-dimethoxy-6,7-methylenedioxy-4,9-dihydroxyperylene-3,10-quinone), which

has been considered to be an important virulence factor (Upchurch et al. 1991). Cercosporin is a light-activated photosensitizing toxin that, upon illumination, interacts with molecular oxygen to produce both superoxide radicals and singlet oxygen (Daub and Ehrenshaft 2000; Daub and Hangarter 1983). These activated oxygen species cause peroxidation of cell membrane lipids and breakdown of the host plasma membrane, resulting in electrolytic leakage, a decrease in membrane permeability, and, eventually, cell death (Daub 1982a). Nutrients leaking from host cells support pathogen growth and sporulation in the host (Daub and Ehrenshaft 2000).

Cercosporin first was isolated from a soybean pathogen, *Cercospora kikuchii* (Kuyama and Tamura 1957), and its toxicity via photosensitizing activity to mice, bacteria, several fungi, and host plants has been established to be light and oxygen dependent (Daub 1982b; Macri and Vianello 1979; Yamazaki et al. 1975). Despite the universal toxicity of cercosporin, *Cercospora* spp. apparently have evolved sophisticated mechanisms associated with transient reduction of cercosporin, singlet oxygen quenching, toxin export, and other unknown functions that allow them to tolerate cercosporin toxicity (Callahan et al. 1999; Chung et al. 2003b; Daub et al. 1992, 2000; Ehrenshaft et al. 1998, 1999). Production of cercosporin is highly affected by environmental factors (e.g., nutrient conditions, light, temperature, and cultural age) and signal transduction involving calcium or calmodulin and MAP kinase pathways (Chung 2003; Daub and Ehrenshaft 2000; Ehrenshaft and Upchurch 1991, 1993; Jenks et al. 1989; Shim and Dunkle 2003). However, little progress has been made toward understanding the biosynthetic pathway of cercosporin. Early studies using nuclear magnetic resonance and mass-spectrometric analyses of products derived from fungal culture fed with ¹⁴C-labeled acetate suggested that cercosporin is produced via a polyketide pathway by the condensation of acetate and malonate subunits (Okubo et al. 1975). No chemical intermediates or enzymes have ever been identified for the cercosporin biosynthetic pathway, however.

Cercosporin has been suggested to play a crucial role in virulence of *Cercospora* spp. for many years. Light intensity is correlated with both disease severity and toxin action (Calpouzos and Stalknecht 1967; Daub 1982b). Cercosporin has been isolated from affected tissues, and application of toxin alone can produce similar symptoms on host plants (Balis and Payne 1971; Fajola 1978; Upchurch et al. 1991). Non-toxin-producing mutants of *C. kikuchii* derived from UV mutagenesis

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sis failed to cause disease symptoms in soybean plants (Upchurch et al. 1991). Furthermore, disruption of a *CFP* gene encoding a facilitator protein in *C. kikuchii* (Callahan et al. 1999) and a *CZK3* gene encoding a MAP kinase kinase kinase homolog in *C. zea-maydis* (Shim and Dunkle 2003) resulted in mutants that were defective in cercosporin biosynthesis and that also exhibited lower virulence on soybean and maize, respectively. Disruption of *CFP* and *CZK3* genes resulted in pleiotrophic phenotypes; therefore, the pathological roles of cercosporin in symptom development and fungal pathogenesis remain undefined at molecular levels.

In previous studies using the restriction enzyme-mediated integration (REMI) approach, we identified several groups of mutants of *C. nicotianae* that are altered in cercosporin production (Chung et al. 2003c). Sequence analysis of a flanking DNA fragment in the rescued plasmid clone (pCTB1) recovered from one of the cercosporin-deficient mutants revealed a sequence with strong amino acid similarities to many fungal polyketide synthases (PKSs) in the databases. We partially characterized the clone as *CTB1* (for cercosporin toxin biosynthesis) and proposed that it encoded a fungal PKS responsible for cercosporin production (Chung et al. 2003c). In this article, we describe the complete cloning, regulated expression, and functional characterization of the *CTB1* gene and, using *ctb1*-disrupted mutants, unambiguously demonstrate its involvement in cercosporin biosynthesis and requirement for fungal pathogenesis in *C. nicotianae*.

RESULTS

Molecular cloning and characterization of *CTB1* gene encoding a fungal PKS.

Partial *CTB1* sequences showing amino acid similarities to various fungal PKSs were identified first from a 1.6-kb flanking region rescued from a cercosporin-deficient REMI mutant (*ctb1*) (Chung et al. 2003c). The full-length *CTB1* gene subsequently was obtained by polymerase chain reaction (PCR) with two inverse primers using strategies outlined in Figure 1. The primers (pks5/pks6 and 1209L/1209R) were designed based on the sequences in the 1.6-kb flanking DNA and were used for PCR amplification from *EcoRV*- and *NcoI*-digested and self-ligated genomic DNA pools. Two overlapping fragments were amplified and cloned into the pGEM-T easy vector, resulting in plasmids pKS-8 and pCNA1, respectively (Fig. 1). The cDNA fragments of the *CTB1* were cloned collectively and sequenced as four overlapping clones, pXOC2, pXOC7, pXOC10, and pTAR3 (Fig. 1). Analysis of the combined sequences derived from genomic clones and the cDNA identified a 6,591-bp open reading frame (ORF) and the presence of eight small introns ranging from 48 to 74 bp in size. Seven of the eight introns (except intron VI) had characteristic 5'-XX/GT and AG/XX-3' splicing junctions commonly found in filamentous fungi. Six of them also contain an internal lariat consensus sequences CT(A/G)A (T/C) (Radford and Parish 1997). Sequence analysis of 520 nucleotides upstream of the ATG start codon revealed that the promoter region of *CTB1* contains one putative TATA box and three putative CAAT elements (data not shown). The predicted start codon and flanking sequences of *CTB1* also have the eukaryotic translation initiation consensus sequence [CA(C/A)(A/C)ATGTC] (Kozak 1984). Using 5' and 3' RACE, putative transcriptional initiation sites were mapped to 101 and 172 bp upstream of the predicted ATG translational start codon, and the transcriptional termination site was localized 57 bp downstream of the translational stop codon. The *CTB1* sequence was deposited in the GenBank database under accession number AY649543.

The *CTB1* gene was predicted to encode a polypeptide of 2,196 amino acids (aa) rich in alanine (11.2% overall). The CTB1 protein had a predicted molecular mass of 236.7 kDa and a predicted pI of 5.6. BlastP search (Altschul et al. 1997) of the National Center for Biotechnology Information (NCBI) protein database revealed that the predicted 2,196-aa translation product of *CTB1* has 35 to 40% identity and 50 to 60% similarity to several type I fungal PKSs. Analysis of the predicted translational product of the *C. nicotianae CTB1* gene revealed the presence of a keto synthase (KS), an acyltransferase (AT), a thioesterase/claisen cyclase (TE/CYC), and two acyl carrier protein (ACP, phosphopantothene attachment site) domains (Fig. 2A). Comparisons of the individual domains within various fungal PKSs are shown in Figure 2B. In addition to the KS, AT, TE/CYC, and ACP domains, comparison of the N-terminal sequences revealed that CTB1 has a conserved AFGDQT sequences similar to those of fungal type-I PKSs. No conserved amino acid sequences similar to ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) domains were found in CTB1.

Gene disruption and genetic complementation.

The function of the *CTB1* in *C. nicotianae* was determined using a gene disruption strategy. A disruption vector pCTB115 containing a hygromycin-resistance gene (*HYG*) cassette was developed and used for *CTB1* gene replacement. To facilitate

Fig. 1. Restriction map of the *Cercospora nicotianae CTB1* gene and schematic describing the strategy to clone the 5' and 3' regions. Arrowheads indicate relative positions of primers (pks5/pks6 and 1209L/1209R) used for polymerase chain reaction (PCR) amplification. DNA fragments amplified from *EcoRV*- and *NcoI*-digested, self-ligated genomic pools were cloned into a pGEM-T vector to yield pKS-8 and pCNA1, respectively. Four overlapping cDNA fragments encompassing the entire *CTB1* gene as well as the 5' and 3' nontranslation regions were obtained from reverse-transcriptase PCR, and cloned to result in pXOC10, pXOC7, pXOC2, and pTAR3 for sequencing analysis. Restriction enzymes used were: E = *EcoRI*, EV = *EcoRV*, N = *NcoI*, and X = *XhoI*. Note: drawing is not to scale. Details on cloning are indicated in the methods section.

Fig. 2. A, Physical map of CTB1 protein (GenBank accession number AY649543) with a keto synthase (KS), an acyltransferase (AT), a thioesterase/claisen cyclase (TE/CYC), and two acyl carrier protein (ACP, phosphopantotheine attachment site) domains in *Cercospora nicotianae*. **B**, Alignments of CTB1 with conserved active sites of other fungal polyketide synthases (PKSs). Amino acid sequences shown are as follows: *Exophiala dermatitidis* WDPKS1 (accession number AAD31436), *Emericella nidulans* WA (Q03149), *Aspergillus parasiticus* PKSL (Q12053), *E. nidulans* STCA (Q12397), *Botryotinia fuckeliana* BfPKS13 (AAR90249), and *A. fumigatus* ALB1 (AAC39471). Amino acid identities to those of CTB1 in *C. nicotianae* are shaded. Conserved amino acids for all peptides are shown under each alignment, and the active amino acids are underlined.

gene replacement, DNA fragments containing a split hygromycin phosphotransferase B gene (*HYG*) marker (5' *CTB1* fused with 3' *HYG* fragment and 5' *HYG* fused with 3' *CTB1* fragment) were amplified from pCTB115 (Fig. 3A), and the PCR products were directly transformed into the wild-type strain. Because cercosporin is a red pigment, toxin-deficient mutants can be detected easily by screening for lack of red pigmentation in the medium. Fungal transformants were first selected on a medium supplemented with hygromycin and then tested for cercosporin production by plating on thin potato dextrose agar (PDA) plates. The wild-type isolate displayed dark red color due to secretion and accumulation of cercosporin around hyphae. Transformants that lacked red pigmentation were identified as cercosporin-deficient mutants. In all, 16 of 41 transformants displayed complete lack of visible cercosporin production.

Southern blot analysis of genomic DNA from three putative disruption mutants (Δ ctb1-T1, T11, and T18) showed that they all had an integrated fragment at the *CTB1* site in the genome (Fig. 3B). Fungal genomic DNA was digested with *NcoI* and *EcoRV* and hybridized to a *CTB1* gene probe generated using

PCR. Both the wild-type strain and the putative *ctb1*-disruptants contained a 1.4-kb *NcoI* hybridizing band in the genomic DNA (Fig. 3B, lanes 1 to 4). The probe also hybridized to a 4.1-kb *NcoI*-*EcoRV* fragment in the wild-type strain (lane 1). Because an *NcoI* restriction enzyme site is present in the *HYG* gene cassette, hybridization of the genomic DNA from the *ctb1*-disruptants failed to detect the 4.1-kb hybridizing band, but detected a 2.1-kb *NcoI*-hybridizing band (lanes 2 and 3). The 2.0-kb hybridizing band in the Δ ctb1-T18 mutant (lane 4) was smaller than expected, likely due to a small deletion caused by the double crossover recombination. Nevertheless, these results indicate that *CTB1* was replaced successfully with the marker gene. Northern blot analysis of total RNA from four *ctb1*-disruptants (Δ ctb1-T1, T11, T6, and T7) using a *CTB1* gene probe failed to detect the 6.8-kb transcript of *CTB1* (Fig. 3C), strongly indicating that these transformants are *ctb1*-null mutants. Due to the orientation of the disruption construct, a truncated transcript (less than 2 kb) was detected in the Δ ctb1-T7 mutant (Fig. 3C, lane 5).

Genetic complementation was performed to further verify the role of *CTB1* in cercosporin biosynthesis. Protoplasts prepared from two *ctb1*-null mutants (Δ ctb1-T1 and T19) were transformed with plasmid pCTB117 containing the full-length *CTB1* gene as well as its upstream and downstream flanking sequences. A total of 25 Δ ctb1-T1 and of 34 Δ ctb1-T19 transformants exhibited wild-type levels of cercosporin production after complementation with *CTB1* (Table 1), confirming the involvement of *CTB1* in cercosporin biosynthesis.

Cercosporin production by the *ctb1* disruptants and disruption mutants complemented with *CTB1* was measured and compared with that of the wild type. Mycelial plugs were extracted with 5 N KOH and the amounts of cercosporin in the extract quantified by measuring absorbance of the extract at 480 nm (Jenns et al. 1989) (Table 1). Agar plugs soaked with 5 N KOH resulted in light yellow pigment, producing absorbance values of approximately 3 to 6 nmoles/plug. The wild-type and the complemented disruption mutants produced large amounts of cercosporin (122 to 136 nmoles/plug). Very low absorbance values,

Fig. 3. Targeted gene disruption of *CTB1* using a split-marker strategy in *Cercospora nicotianae*. **A**, Restriction maps of the *CTB1* gene in the wild type (WT) and *ctb1*-disrupted fungal genome. Two truncated *CTB1* fragments fused with an overlapped *HYG* (*HYG* = hygromycin resistance gene cassette) were obtained by polymerase chain reaction (PCR) amplification as described in the text. Oligonucleotide primers (0315L/hyg3, 0315R/hyg4) used for PCR amplification also are shown. Note: drawing is not to scale. **B**, Southern blot analysis of genomic DNA from the wild type (lane 1) and three *ctb1* disruption mutants: Δ ctb1-T1 (lane 2), Δ ctb1-T11 (lane 3), and Δ ctb1-T18 (lane 4). Fungal DNA was digested with *NcoI* and *EcoRV*, electrophoresed, blotted onto a nylon membrane, and hybridized with a *CTB1*-specific probe. The membrane was washed at high stringency. Sizes of hybridizing bands are indicated in kilobase pairs (kb). Band patterns indicate disruption of the *CTB1* gene, as described in the text. **C**, Northern blot analysis of total RNA from the wild type (lane 1) and four *ctb1* disruption mutants: Δ ctb1-T1 (lane 2), Δ ctb1-T11 (lane 3), Δ ctb1-T6 (lane 4), and Δ ctb1-T7 (lane 5).

Table 1. Production of cercosporin and necrotic lesions on tobacco by the *Cercospora nicotianae* wild-type strain (CnA), the *crg1*-null mutant (205C3), the *ctb1*-null mutants (Δ ctb1-T1), and the *CTB1*-complemented disruption strains (T1R18 and T1R95)

Isolates	Cercosporin production (nmole/agar plug) ^a	Numbers of lesions per leaf ^b
CnA	128.6 ± 28.5	663 ± 56
205C3	58.4 ± 13.7	487 ± 28
Δ ctb1-T1	4.5 ± 0.8	64 ± 31
Δ ctb1-T6	4.3 ± 0.5	57 ± 18
Δ ctb1-T7	5.8 ± 1.2	16 ± 8
Δ ctb1-T11	4.9 ± 1.5	24 ± 9
Δ ctb1-T18	6.8 ± 2.1	78 ± 21
Δ ctb1-T19	4.6 ± 0.4	15 ± 7
Δ ctb1-T26	4.4 ± 0.2	63 ± 16
Δ ctb1-T31	5.1 ± 1.1	4 ± 2
Δ ctb1-T32	3.6 ± 0.8	30 ± 11
T1R18	136.1 ± 12.7	528 ± 72
T1R95	122.9 ± 23.6	617 ± 54
Control	4.7 ± 1.8	...

^a Fungal mycelium of *C. nicotianae* strains was grown on thin potato dextrose agar (PDA) plates. 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 mean of two different experiments with five replicates of each isolate. Data shown ± standard error of the mean.

^b Fungal conidia were sprayed onto leaves of 6- to 8-week-old tobacco plants (cv. Burley 21) and necrotic lesions were scored 21 days after inoculation. Values are the mean of at least three different experiments with three leaves per isolate. Data shown ± standard error of the mean.

equivalent to those of the controls, were obtained for all *ctb1*-disruption mutants. To determine whether the low calculated amounts were due to the presence of cercosporin or to small amounts of interfering pigments in the crude extract, fungal mycelium also was extracted with ethyl acetate and the extract separated by thin-layer chromatography (TLC). No cercosporin was identified by TLC from any of the eight *ctb1*-null mutants, indicating that disruption of the *CTB1* gene completely blocks cercosporin biosynthesis (Fig. 4). As an additional control for these experiments, a mutant (205C3) deficient in a zinc cluster transcription factor, CRG1, which is required for normal cercosporin resistance and production, was tested. Cercosporin production by this mutant was approximately half that of the wild type (Table 1), consistent with previous findings with this mutant (Chung et al. 2003b).

Beyond abolishing cercosporin production, no other phenotypic characteristics were observed among the *ctb1*-null mutants. All *ctb1*-null mutants exhibited normal conidiation on V8 juice agar. In addition, when tested for cercosporin resistance by growth, in the presence of exogenous cercosporin, they retained normal growth compared with the wild-type strain (data not shown).

Expression of the *CTB1* gene.

The production of cercosporin by *Cercospora* spp. in vitro is strongly influenced by medium composition and environmental factors (Jenks et al. 1989). To determine whether *CTB1* transcript accumulation in *C. nicotianae* is correlated with cercosporin production and regulated by light and medium composition, wild-type *C. nicotianae* cultures were grown in either continuous light or continuous darkness on PDA or complete medium (CM). Total RNA extracted from these cultures was hybridized to a *CTB1*-specific probe. Northern blot analysis indicated that the 6.8-kb *CTB1* gene transcript accumulates to high levels when the fungus was grown in the PDA medium under continuous light (Fig. 5A), a condition also supporting high amounts of cercosporin accumulation (Fig. 5B). In contrast, *CTB1* transcript was undetectable and little cercosporin accumulated when the fungus was grown in continuous darkness. The accumulation of *CTB1* transcript was markedly reduced when the fungus was grown in the CM and cercosporin biosynthesis was suppressed in culture grown under continuous light (Fig. 5).

Cercosporin as a virulence factor.

To test whether production of cercosporin is required for fungal virulence and symptom development, the wild type, two *CTB1*-complemented strains (T1R18 and T1R5), and nine *ctb1*-null mutants (Δ ctb-T1, T6, T7, T11, T18, T19, T26, T31, and T32) were inoculated onto tobacco leaves. Inocula-

Fig. 4. Thin-layer chromatography analysis of cercosporin production by the wild type (WT), *ctb1*-null mutants (T), and *CTB1*-complemented (R) strains of *Cercospora nicotianae*. Cercosporin was extracted from fungal mycelium with ethyl acetate, spotted onto a silica gel plate, and separated with ethyl acetate:hexane:methanol:H₂O (6:4:1.5:1, vol/vol). Cercosporin (indicated by an arrowhead) was easily detected as a red pigment at *R*_f0.55.

tion of tobacco leaves with the wild type and complemented strains resulted in numerous necrotic lesions (Table 1; Fig. 6A). The necrotic lesions and the surrounding yellow halos caused by the wild-type strain and complemented strains enlarged and often coalesced to form blight regions on the leaf. In comparison, *ctb1*-null mutants produced significantly fewer lesions (Table 1; Fig. 6A). Lesions produced by *ctb1*-null mutants generally remained small and did not coalesce. The *crg1* transcription regulator-disruption mutant (205C3), which produced approximately half of the cercosporin produced by the wild type, resulted in a disease level intermediate between wild type and the *ctb1*-disruption mutants (Table 1). Inoculation with the 205C3 strain resulted in 20 to 25% fewer lesions, and the size of lesions induced by 205C3 was much smaller compared with those of the wild type (Fig. 6A).

Infection assessed in a detached leaf assay indicated that the *ctb1*-null mutants exhibit lower virulence on both wounded and unwounded tobacco leaves (Fig. 6B). In general, chlorotic symptoms developed earlier on wounded leaves inoculated with either the wild type or the *ctb1*-null mutants. However, significant differences in the progression of lesions between the wild

Fig. 5. Expression of the *CTB1* gene and production of cercosporin toxin by the wild-type *Cercospora nicotianae*. **A**, Northern blot analysis of *CTB1* expression in *C. nicotianae* wild type grown on potato dextrose agar (PDA) or complete medium (CM) under continuous light (LT) or darkness (DK). Total fungal RNA was purified, electrophoresed in a formaldehyde-containing gel, blotted to membrane, and hybridized with a *CTB1*-specific probe. Ethidium bromide-stained rRNA is shown to ensure equal loading of the samples. **B**, Production of cercosporin toxin by the wild-type *C. nicotianae*. 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 three different experiments with five replicates of each treatment.

type and the *ctb1*-null mutants were noticed. The wild-type strain produced coalesced necrotic lesions with large yellow halos 2 weeks after inoculation on wounded leaves. In contrast, the *ctb1*-null mutants incited fewer and smaller necrotic spots compared with those of the wild type on either unwounded or wounded leaves, and the lesions progressed slowly. Collectively, disruption of the *CTB1* gene resulted in mutants that are completely devoid of cercosporin production and are markedly reduced in virulence. Complementation of *ctb1*-null mutants with the full-length *CTB1* clone restored both cercosporin biosynthesis and full virulence to levels equivalent to those of the wild type. These data clearly indicate that cercosporin is an important virulence factor in *C. nicotianae*.

DISCUSSION

Based on structure and limited ^{14}C -labeling experiments, the biosynthesis of the perylenequinone cercosporin has long been proposed to be via a polyketide pathway (Okubo et al. 1975). However, experimental documentation of this hypothesis has never been obtained. In this study, we isolated a fungal PKS

gene, *CTB1*, and determined its role in cercosporin biosynthesis in *C. nicotianae*. Disruption of the *CTB1* gene with a hygromycin-resistance cassette yielded mutants that were defective in cercosporin production. Further, transformation of the *CTB1* gene into *ctb1*-null mutants restored cercosporin production. Thus, our results provide conclusive molecular evidence to support the hypothesis that cercosporin is biosynthesized via a polyketide pathway.

The predicted amino acid sequences of the *CTB1* translation product showed strong similarity to many fungal type I PKSs. The *CTB1* exhibited strong similarities to the *Aspergillus fumigatus* ALB1 protein (accession number AAC39471) involved in conidial pigment biosynthesis, the *Emericella nidulans* STCA-EMENI protein (accession number Q12397) involved in sterigmatocystin biosynthesis, and the *E. nidulans* WA protein (accession number Q03149) involved in conidial yellow pigment biosynthesis (Brown et al. 1996; Mayorga and Timberlake 1992; Tsai et al. 1998). *CTB1* also displayed strong similarity to *A. parasiticus* PKS1 protein (accession number Q12053) involved in aflatoxin biosynthesis (Feng and Leonard 1995).

Fig. 6. Requirement of *CTB1* for disease severity on tobacco leaves infected with *Cercospora nicotianae*. **A**, Pathogenicity assay using the whole tobacco plants. The wild type (WT), *ctb1*-null mutant ($\Delta\text{ctb1-T19}$), *CTB1*-complemented strain (T19R-5), and *crg1*-null mutant (205C3) were grown on V8 juice agar for 7 days at 20°C under continuous light. Conidia were harvested and sprayed onto leaves of 6- to 8-week-old tobacco cv. Burley 21. Disease severity, evident by lesions with necrotic centers and yellow halos, was documented 14 to 21 days post inoculation. **B**, Pathogenicity assay on detached tobacco leaves. Conidial suspensions prepared from the wild type (WT) and *ctb1*-null mutants ($\Delta\text{ctb1-T1}$, T6, T7, and T11) of *C. nicotianae* were rubbed onto wounded and unwounded (separated by midrib) tobacco leaves with cotton swabs. The inoculated leaves then were incubated in a moist chamber under fluorescent light for 2 to 3 weeks.

Given that cercosporin is a polyketide, the *CTB1* product likely functions as do fatty acid synthases, catalyzing the condensation of two-carbon units in each biosynthetic cycle using acetate and malonate to form polyketomethylene backbones (Okubo et al. 1975). *CTB1* consisted of five catalytic domains, including a KS, an AT, a TE/CYC, and two ACP domains (KS-AT-ACP-ACP-TE) required for elongation and condensation of cyclic cercosporin. The five catalytic domains were distributed at comparable distances along the protein with similar length to other fungal polyketides (data not shown). *CTB1* contained a conserved cysteine residue in the KS domain and conserved serine residues in the AT, ACP, and TE domains similar to many fungal PKSs (Yang et al. 1996). The two ACP domains likely are involved in carbon chain elongation by serving as binding sites for phosphopantotheine (Katz and Donadio 1993). It is uncertain why *CTB1* contains two ACP motifs. However, studies by using site-directed mutagenesis of the active serine residue in two ACP motifs of the *E. nidulans* WA protein revealed that either of them is sufficient to allow PKS function (Fujii et al. 2001). The AT domain likely is required for transferring the acetate unit from acetyl-CoA to the pantotheine arm of the ACP motif, whereas the KS domain is involved in condensation of the malonate unit with acetate by decarboxylation (Katz and Donadio 1993). The chain elongation and condensation steps are repeated until the chain reaches full length and the mature chain is released by the action of TE, followed by methylation. The β -keto group in the polyketide chain presumably remains unchanged or reduced to a hydroxyl because *CTB1* lacks KR, DH, and ER domains.

The catalytic serine residue in the TE domain of *CTB1* may likely be involved in cyclization of the aromatic ring of cercosporin as demonstrated for the WA protein that catalyzes naphthopyrone biosynthesis (Fujii et al. 2001). Deletion and site-directed mutagenesis of WA also revealed the important role in cyclization of the histidine residue in the C-terminus of WA. *CTB1* also contained a conserved histidine residue in the C-terminus, suggestive of a similar function for ring closure. Furthermore, *CTB1* shares with many fungal type-I PKSs a conserved sequence (LFGDQ) in the N-terminus (data not shown). Deletion of these five amino acids in the N-terminus of WA eliminates the polyketide synthase activity in *E. nidulans* (Fujii et al. 2001).

Cercosporin contains a bilateral symmetrical structure; therefore, cercosporin likely is formed from fusing two identical polyketomethylene chains in the final step (Okubo et al. 1975). Except for *CTB1*, proteins that catalyze subsequent reactions involving decarboxylation, methylation, and hydroxylation also must be required for cercosporin biosynthesis. As has been shown with many fungal secondary metabolites (Keller and Hohn 1997), genes involved in the biosynthetic pathway of cercosporin may be clustered in *Cercospora* spp. Recently, sequence analysis of the surrounding DNA fragments of the *CTB1* gene identified several putative ORFs with amino acid similarities to *O*-methyltransferases, oxidoreductases, hydroxylases, major facilitator superfamily transporters, and transcriptional activators (K.-R. Chung, K. L. Dekkers, and M. Choquer, unpublished data). Whether or not these genes that cluster with *CTB1* are also involved in cercosporin biosynthesis awaits further analysis.

Light is not only crucial for cercosporin toxicity, but also is required for the initiation of cercosporin biosynthesis (Ehrenshaft and Upchurch 1991, 1993). Production of cercosporin is also markedly affected by medium composition. For example, PDA medium supports high amounts of cercosporin accumulation in culture, whereas CM significantly reduced its biosynthesis (Jenns et al. 1989). Expression of the *CTB1* gene, as assessed by Northern blot hybridization, revealed that the

CTB1 gene transcript is highly inducible by light and in mycelium grown on PDA. The light- or medium-induced cercosporin production was correlated with light- or medium-enhanced *CTB1* gene transcript accumulation, suggesting that *Cercospora* spp. synthesize cercosporin toxin in response to light or other environmental cues by activation of *CTB1* gene expression. *CTB1* gene activation by environmental cues likely may involve calcium or calmodulin signaling because cercosporin production is significantly reduced by various calcium or calmodulin inhibitors in *C. nicotianae* (Chung 2003). A preliminary study also indicated that the *CTB1* gene transcript was markedly reduced when the fungus was grown in media containing calcium or calmodulin inhibitors (K. L. Dekkers, personal communication).

One of the most significant findings of this study is the confirmation that cercosporin is required for full virulence in *C. nicotianae*. For many years, cercosporin has been suggested to be crucial for fungal pathogenesis by *Cercospora* spp. (Daub and Ehrenshaft 2000). The earliest studies correlated toxin-deficient UV-generated mutants of *C. kikuchii* with reduced virulence on soybean (Upchurch et al. 1991). Subsequently, Upchurch and co-workers isolated from *C. kikuchii* a gene for a cercosporin facilitator protein that is hypothesized to be the cercosporin transporter (Callahan et al. 1999). Disruption of the *CFP* gene severely attenuated disease symptoms on soybean and reduced, but did not eliminate, cercosporin production. Mutants of *C. zeae-maydis* disrupted in the MAP kinase gene *CZK3*, which is upregulated during cercosporin production, were completely defective in cercosporin production and also significantly reduced in virulence; however, these mutants also were defective in conidiation, preventing definitive conclusions for a role of cercosporin in *Cercospora* pathogenesis (Shim and Dunkle 2003). In our present study, we were able to recover mutants completely deficient in cercosporin production by specifically disrupting the initial biosynthetic gene in the pathway. All *ctb1* disruption mutants were significantly reduced in virulence when inoculated on tobacco. Moreover, genetic complementation of *ctb1*-null mutants with the full-length *CTB1* gene fully restored normal production of cercosporin in culture as well as normal disease symptoms. Taken together, these results conclusively demonstrated that cercosporin is a crucial factor in pathogenesis of tobacco by *C. nicotianae*. The study suggests that strategies that interfere with the toxicity of cercosporin will be useful in reduction of disease incidence caused by *Cercospora* spp.

MATERIALS AND METHODS

Fungal strains, media, cultural conditions, and toxin quantification.

C. nicotianae wild-type strain ATCC18366, the cercosporin toxin biosynthesis mutant (*ctb1*), and other strains were maintained on CM (Jenns et al. 1989). For protoplast preparation, fungal isolates were grown in 50 ml of potato dextrose broth (PDB; Difco Laboratories, Detroit) with shaking for 4 to 5 days in the dark. Fungal mycelium was ground in a sterile blender, mixed with 200 ml of fresh medium, and grown for an additional 16 h at 28°C in the dark. For DNA and RNA purification, fungal strains were grown on CM or PDA with a layer of cellophane (cellulose; Paper Mart, Los Angeles). Fungal mycelium can be separated easily from cellophane without contamination of agar. Screening of cercosporin-deficient mutants was conducted on thin PDA (Difco Laboratories) plates as described previously (Chung et al. 2003c). Quantitative assays for cercosporin production were conducted by extracting agar plugs cut from mycelial cultures with 5 N KOH or with

ethyl acetate. Spectrophotometric quantification and TLC were conducted as described (Chung 2003; Fore et al. 1988). KOH extracts were quantified by measuring absorbance at 480 nm. TLC utilized TLC plates with a 60 F254 fluorescent silica gel (5 by 20 cm; Selecto Scientific Inc., Suwanee, GA) and a solvent system containing ethyl acetate/hexane/methanol/H₂O (6:4:1.5:1, vol/vol). Assays for cercosporin sensitivity were performed on CM amended with 10 μ M cercosporin as described by Jenns and Daub (1995). Pure cercosporin was purchased from Sigma-Aldrich (St. Louis) and dissolved in acetone to make a 10 mM stock solution.

Cloning

of the full-length cercosporin biosynthesis (*CTBI*) gene.

The full-length *CTBI* gene encoding a fungal PKS was obtained with two inverse primers derived from the rescued plasmid pCTB1 (Fig. 1) (Chung et al. 2003c). Briefly, *C. nicotianae* genomic DNA was digested with *EcoRV* and self-ligated with a T4 DNA ligase. An 8.0-kb fragment containing the 5' end *CTBI* was amplified with primers pks-5 (5'-ttgac taggaggacggcag-3') and pks-6 (5'-ttgatggacgcaaaggtctg-3') with Elongase enzyme mix (Invitrogen, Carlsbad, CA) and cloned into the pGEM-T easy vector (Promega Corp., Madison, WI, U.S.A.) to yield pPKS-8 for sequence analysis (Fig. 1). A 3.7-kb fragment containing the 3' *CTBI* terminus then was amplified with inverse primers 1209R (5'-gtgtgtcggcactg atagcc-3') and 1209L (5'-ctgacctgcttcgcttcat-3') from *NcoI*-digested and self-ligated genomic DNA pools, and cloned into the pGEM-T vector to yield pCNA1 for sequence analysis (Fig. 1). DNA sequence analysis with the ABI Prism BigDye primer cyler sequencing kit (Applied Biosystems, Foster City, CA, U.S.A.) was conducted at the Integrated Biotechnology Laboratory, University of Georgia (Athens). Database searches and comparisons were performed at the National Center of Biotechnology Information using the BLAST network service (Altschul et al. 1997) and ExPASy Molecular Biology servers.

Fungal transformation, gene disruption, and genetic complementation.

Fungal protoplasts were prepared and transformed as described previously (Chung et al. 2002). To create a *CTBI* disruption vector, a 7-kb fragment containing the whole *CTBI* gene was amplified from genomic DNA of *C. nicotianae* with two primers, P1 (5'-gacatccgcaccatcgaag-3') and P12 (5'-gctgcgaatgcgagcgtag-3'), by an Expand High Fidelity PCR system (Roche Applied Science, Indianapolis, IN, U.S.A.), and cloned into pGEM-T vector to result in pCTB112. A 2.9-kb *KpnI/EcoRV* fragment in pCTB112 then was replaced with a 1.6-kb hygromycin phosphotransferase B gene (*HYG*) cassette from pUCATPH (Lu et al. 1994) to yield pCTB115. To facilitate *CTBI* gene replacement, a split-marker recombination procedure (Catlett et al. 2003; Fairhead et al. 1996) was adapted for *C. nicotianae*. Split-*HYG* marker fused with truncated *CTBI* was generated by PCR with two sets of primers from pCTB115 (Fig. 3A). A 2.6-kb fragment containing 5' *CTBI* fused with 3' *HYG* was amplified with primers 0315L (5'-ggcagtcacagctcttgag-3') and *hyg3* (5'-ggatgcctccgctcgaagta-3'). A 2.4-kb fragment containing 5' *HYG* fused with 3' *CTBI* was amplified with primers 0315R (5'-ccgggtaagaggtgcagtttcg-3') and *hyg4* (5'-cgttgcaa gaactgcctgaa-3'). PCR fragments, overlapping 450 bp at the *HYG* region, were purified with an Ultraclean PCR clean-up kit (Mo Bio Laboratories, Carlsbad, CA, U.S.A.) and directly transformed into the *C. nicotianae* wild-type strain. For genetic complementation, an 8.2-kb fragment was amplified with primers *ctb1-NotI* (5'-gttgtgttccgcggcgtgtacgc-3') and

ctb1-SpeI (5'-gtcatgataactagtcggcca-3') containing restriction enzyme sites *NotI* and *SpeI*, respectively. After digestion with *NotI* and *SpeI*, the fragment was cloned into pCB1532 containing a sulfonyleurea-resistance gene cassette (Sweigard et al. 1997) to yield pCTB117. The pCTB117 was used to complement the *ctb1*-null mutants Δ ctb-T1 and T19. Transformants were selected in medium containing hygromycin (Roche Applied Science) at 250 μ g/ml or sulfonyleurea (chlormuron ethyl; Chem Service, West Chester, PA, U.S.A.) at 5 μ g/ml as appropriate and tested for cercosporin production on thin PDA plates as described previously (Chung 2003).

Southern and Northern blot analyses.

Total fungal DNA was isolated with a DNeasy Plant Mini kit (Qiagen, Valencia, CA, U.S.A.). Total fungal RNA was extracted with a TRIZOL RNA Isolator kit (Invitrogen). Standard procedures were used for endonuclease digestion of DNA, electrophoresis, and Southern and Northern blotting (Sambrook and Russell 2001). The hybridization probes were labeled with digoxigenin-11-dUTP (Roche Applied Science) by PCR as described previously (Chung et al. 2003a). Posthybridization washing and immunological detection of the probe using a CSPD lumigenic substrate for alkaline phosphatase were conducted according to the manufacturer's recommendations (Roche Applied Science). Oligonucleotide primers for PCR were synthesized by Integrated DNA Technologies (Coralville, IA, U.S.A.).

Reverse-transcriptase PCR, and cDNA isolation.

The poly (A⁺) mRNA was isolated with an Oligotex kit (Qiagen). The first strand cDNA was synthesized from poly (A⁺) mRNA with a Sensiscript reverse transcriptase (Qiagen) and oligo dT₃₀ primer. Four overlapping cDNA fragments were subsequently amplified with an AccuPrime *Taq* DNA polymerase (Invitrogen) and *CTBI* gene-specific primers, and the fragments were cloned into the pGEM-T easy vector (Promega Corp.) for sequence analysis (Fig. 1).

Fungal pathogenicity test.

Fungal inoculations initially were performed in greenhouse tests on 6- to 8-week-old tobacco plants, cv. Burley 21. Cultures of fungal strains were induced to produce conidia by culturing them on V8-juice-agar medium as described by Jenns and Daub (1995). Conidia were harvested after 7 days of growth at 20°C under continuous light. The concentration of conidia was adjusted to approximately 5×10^4 conidia/ml with the aid of a hemacytometer, and then sprayed onto tobacco leaves with a SpraTool sprayer (Crown, Woodstock, IL, U.S.A.). Inoculated plants were covered individually with plastic bags, kept shaded for 4 days, and then transferred to greenhouse benches. The number and size of lesions from at least three leaves of each plant were determined 14 to 21 days after inoculation. To minimize a potential release of genetically modified fungi, pathogenicity assays also were conducted on detached tobacco leaves. Briefly, conidial suspensions were rubbed onto the detached leaves with a cotton swab, and the inoculated leaves were kept in a moist chamber under fluorescent light at room temperature for 2 to 3 weeks. Leaves were wounded with sterile needles prior to inoculation. The experiments were repeated three times with at least three replicates.

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