

Molecular analysis of the cercosporin biosynthetic gene cluster in *Cercospora nicotianae*

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Summary

We describe a core gene cluster, comprised of eight genes (designated *CTB1–8*), and associated with cercosporin toxin production in *Cercospora nicotianae*. Sequence analysis identified 10 putative open reading frames (ORFs) flanking the previously characterized *CTB1* and *CTB3* genes that encode, respectively, the polyketide synthase and a dual methyltransferase/monooxygenase required for cercosporin production. Expression of eight of the genes was co-ordinately induced under cercosporin-producing conditions and was regulated by the Zn(II)Cys₆ transcriptional activator, CTB8. Expression of the genes, affected by nitrogen and carbon sources and pH, was also controlled by another transcription activator, CRG1, previously shown to regulate cercosporin production and resistance. Disruption of the *CTB2* gene encoding a methyltransferase or the *CTB8* gene yielded mutants that were completely defective in cercosporin production and inhibitory expression of the other *CTB* cluster genes. Similar ‘feedback’ transcriptional inhibition was observed when the *CTB1*, or *CTB3* but not *CTB4* gene was inactivated. Expression of four ORFs located on the two distal ends of the cluster did not correlate with cercosporin biosynthesis and did not show regulation by CTB8, suggesting that the biosynthetic cluster was limited to *CTB1–8*. A biosynthetic pathway and a regulatory network leading to cercosporin formation are proposed.

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Introduction

Many phytopathogenic *Cercospora* species produce a host non-selective, photoactivated phytotoxin, called cercosporin that is required for high levels of virulence by these fungi on plants (Daub, 1982a; Daub *et al.*, 2005). Cercosporin is a perylenequinone that absorbs light and reacts with oxygen molecules to generate reactive oxygen species, including singlet oxygen (¹O₂) and superoxide radicals (O₂⁻) (Daub and Hangarter, 1983). The reactive oxygen species cause peroxidation of cell membranes and electrolyte leakage in host plants (Daub, 1982b; Daub and Briggs, 1983; Daub and Ehrenshaft, 2000). In addition, cercosporin is able to damage major cellular components including nucleic acids, proteins and lipids, and has also been shown to exert broad toxicity to bacteria, many fungi and mice (Yamazaki *et al.*, 1975; Ito, 1981; Daub, 1982a). Extensive investigations have focused on understanding the chemistry and mode of action of cercosporin as well as on the mechanisms involved in fungal self-defence against singlet oxygen and related photosensitizing compounds (Daub *et al.*, 1992; Sollod *et al.*, 1992; 2000; Jenns and Daub, 1995; Ehrenshaft *et al.*, 1998; 1999; Chung *et al.*, 1999; 2003a). Cercosporin has recently been demonstrated to play a crucial role in pathogenicity of *C. nicotianae* and lesion formation on tobacco (Choquer *et al.*, 2005; 2007; Dekkers *et al.*, 2007).

In contrast to the large body of information on cercosporin toxicity and resistance, considerably less is known about the cercosporin biosynthetic pathway and its regulation. Accumulation of cercosporin in culture is affected by environmental and developmental factors (Jenns *et al.*, 1989; Daub and Ehrenshaft, 2000). Light has been shown to be the most critical factor, not only for toxicity, but also for cercosporin production. Cercosporin is only produced in the light, and its production is completely suppressed under complete darkness (Ehrenshaft and Upchurch, 1991); brief exposure to light immediately triggers its biosynthesis. A Zn(II)Cys₆ transcription factor, CRG1, has been identified with a role in regulating both cercosporin resistance and production (Chung *et al.*, 1999; 2003a). The mechanism of CRG1 regulation and its relation to signalling pathways and environmental signals is not yet understood.

A substrate-feeding study conducted by Okubo *et al.* (1975) suggested that cercosporin is synthesized via the

polyketide pathway using acetate and malonate subunits. The first gene putatively identified in the pathway was *CFP* (cercosporin facilitator protein) encoding a major facilitator superfamily (MFS) transporter required for exportation of cercosporin out of the mycelium (Callahan *et al.*, 1999). In order to identify genes involved in cercosporin biosynthesis, we identified several classes of cercosporin deficiency mutants in *C. nicotianae* through a restriction enzyme-mediated integration (REMI) mutagenesis approach (Chung *et al.*, 2003b). A fungal polyketide synthase encoding gene, named *CTB1* (Cercosporin Toxin Biosynthesis), was subsequently recovered from one of the REMI mutants and was demonstrated to be required for cercosporin production through genetic and molecular analysis (Choquer *et al.*, 2005). Further study revealed that another gene (*CTB3*), immediately adjacent to the *CTB1*, was also required for cercosporin biosynthesis (Dekkers *et al.*, 2007), suggesting that the biosynthetic pathway genes are clustered.

A number of fungal secondary metabolites, including aflatoxins, compactin, ergot alkaloids, fumonisins, gibberellins, gliotoxin, HC toxin, indole-diterpenes, loline alkaloids, penicillin, sterigmatocystin, sirodesmin and trichothecenes, are synthesized by genes found in clusters in the genome (Hohn *et al.*, 1993; Brown *et al.*, 1996; 2004; Brakhaage, 1998; Young *et al.*, 2001; 2006; Abe *et al.*, 2002; Ahn *et al.*, 2002; Proctor *et al.*, 2003; Gardiner *et al.*, 2004; 2005; Yu *et al.*, 2004; Haarmann *et al.*, 2005; Spiering *et al.*, 2005; Tudzynski, 2005). We thus sequenced the regions flanking *CTB1* and *CTB3* as a means to identify the putative biosynthetic genes. Here we report on the identity of a core cluster of six genes in addition to *CTB1* and *CTB3* that were co-ordinately regulated and highly induced under cercosporin-producing conditions. In addition, we confirm the function of two of these genes, *CTB2* and *CTB8*, in cercosporin biosynthesis using targeted gene disruption and genetic complementation. This work provides a genetic framework for better understanding the metabolic synthesis and regulation of cercosporin.

Results

Chromosomal walking, sequence analysis and identification of putative open reading frames

We previously characterized the *CTB1* gene encoding a fungal polyketide synthase (Choquer *et al.*, 2005) and the *CTB3* gene encoding a dual *O*-methyltransferase and FAD-dependent monooxygenase (Dekkers *et al.*, 2007) from *C. nicotianae* to be responsible for cercosporin biosynthesis. Sequences flanking the *CTB1* and *CTB3* genes were obtained by polymerase chain reaction (PCR) using a chromosomal walking strategy with *CTB1* and

CTB3-specific primers. New primers were designed based on the end sequences of compiled sequences and used for further rounds of PCR. Overlapping sequences were carefully assembled. The *CTB1*, *CTB2*, *CTB3*, *CTB4* and *CTB8* open reading frames (ORFs) were determined by sequencing cDNA clones, whereas other ORFs were predicted using the computer software.

In addition to *CTB1* and *CTB3*, 10 putative ORFs were identified in a span of approximately 36 kb analysed (Table 1; Fig. 1A). BLASTX searches in the NCBI database were used to identify function for the predicted polypeptide. The conceptually translated *CTB2* protein is similar to many methyltransferases (see below for details). The *CTB4* protein is highly similar to many hypothetical proteins and MFS transporters of fungi and bacteria, with greatest similarity to an *Aspergillus fumigatus* membrane transporter. The *CTB5* protein has homology to numerous hypothetical proteins and to oxygen, FAD/FMN-dependent oxidoreductases of fungi and bacteria including *A. fumigatus*, *Neurospora crassa* and *Thermobifida fusca*. *CTB6* encodes a putative protein with similarity to a wide range of NADPH-dependent dehydrogenases or oxidoreductases such as D-lactaldehyde dehydrogenase of *Cryptococcus neoformans* and ketoreductase of *A. fumigatus*. The *CTB7* protein is similar to many FAD/FMN-dependent oxidoreductases of bacteria, such as *Xanthomonas axonopodis* pv. *citri*, *Streptomyces coelicolor* and *Pseudomonas syringae*. The *CTB8* protein has similarity to many fungal transcription factors containing zinc finger DNA-binding domains (see below for details).

Two ORFs (9 and 10) downstream of *CTB4* did not appear to encode proteins involved in metabolic functions. The predicted ORF9 protein contains a DUF850 domain and has amino acid similarity to many conserved eukaryotic proteins of unknown function. The ORF10 protein has similarity to many hypothetical proteins of fungi and proteins containing a GTP-binding motif that are presumably involved in vegetative compatibility in *Podospora anserina*. Two small ORFs (11 and 12) showing similarity to GAL4-like Zn(II)Cys₆ transcription regulators were found upstream of *CTB8*. ORF11 appears to encode a truncated zinc binding protein due to missing two cysteine residues in the first zinc cluster (xxxxxxxxC----CxxCxxxxxC). ORF12 displays amino acid similarity to *CTB8* and ORF11, but completely lacks the zinc binding domain.

In the present study *CTB2* and *CTB8* were characterized in detail. Comparisons of the *CTB2* genomic and cDNA sequences revealed that the *CTB2* gene coding region contains 1389 nucleotides with a 53 bp intron. The predicted translation product (462 amino acids) of *CTB2* displayed a strong similarity to numerous fungal hypothetical proteins derived from genome sequencing projects and to *O*-methyltransferases of *Aspergillus*

Table 1. The cercosporin biosynthetic gene cluster in *Cercospora nicotianae*.

Gene ^a (accession number)	Length (bp)	Intron number	Predicted function	Amino acids	Closest blast match (accession number)	Identity (%)	E-value
<i>CTB1</i> (AY649543)	7036	8	Polyketide synthase	2196	Polyketide synthase PksP (XP_756095)	37	0
<i>CTB2</i> (DQ991505)	1439	1	O-methyltransferase	462	O-methyltransferase (XP_748078) (AAS90087) (AAS90065) (AAS66017) (BAE71329)	26	3e-31 2e-19 1e-19
<i>CTB3</i> (DQ355149)	2731	2	O-methyltransferase/ FAD-dependent monooxygenase	871	O-methyltransferase (XP_748078); FAD-dependent monooxygenase (NP_901496)	37	1e-26 4e-32
<i>CTB4</i> (DQ991506)	1816	3	Major Facilitator Superfamily (MFS) Transporter	512	Membrane transporter (XP_756023)	30	3e-112
<i>CTB5</i> (DQ991507)	1380	0	Oxygen, FAD/FMN-dependent oxidoreductase	459	Oxidoreductase, FAD-binding (XP_755780) (XP_961030) (YP_288121)	48	2e-69
<i>CTB6</i> (DQ991508)	1074	0	NADPH-dependent oxidoreductase	357	D-lactaldehyde dehydrogenase (XP_570921) (XP_755361)	29	1e-26
<i>CTB7</i> (DQ991509)	1401	1	FAD/FMN-dependent oxidoreductase	450	Oxidoreductase (AAM36534) (NP_624584) (NP_790149)	33	1e-45
<i>CTB8</i> (DQ991510)	1245	1	Zinc finger transcription factor	397	AFLR_EMENI Sterigmatocystin biosynthesis regulatory protein (XP_681089) (XP_411957) (AAM02988) (AAM46650) (AAM02989) (AAW32181)	25	4e-11
ORF9 (DQ993249)	976	3	Conserved eukaryotic protein of unknown function	271	Hypothetical protein with DUF850 domain (XP_747337)	60	9e-63
ORF10 (DQ993250)	1385	3	Hypothetical protein	395	Unnamed protein product (BAE64725) (Q00808)	40	2e-50
ORF11 (DQ993251)	999	0	Truncated transcription factor	332	AFLR (AAM02976)	36	0.001
ORF12 (DQ993252)	960	0	Truncated transcription factor	319	Hypothetical protein (EAT83777)	33	1e-09

a. Genes with putative roles in the cercosporin pathway are designated *CTB* (Cercosporin Toxin Biosynthesis), whereas other flanking genes with no predicted function in cercosporin production are tentatively designated ORFs 9, 10, 11 and 12.

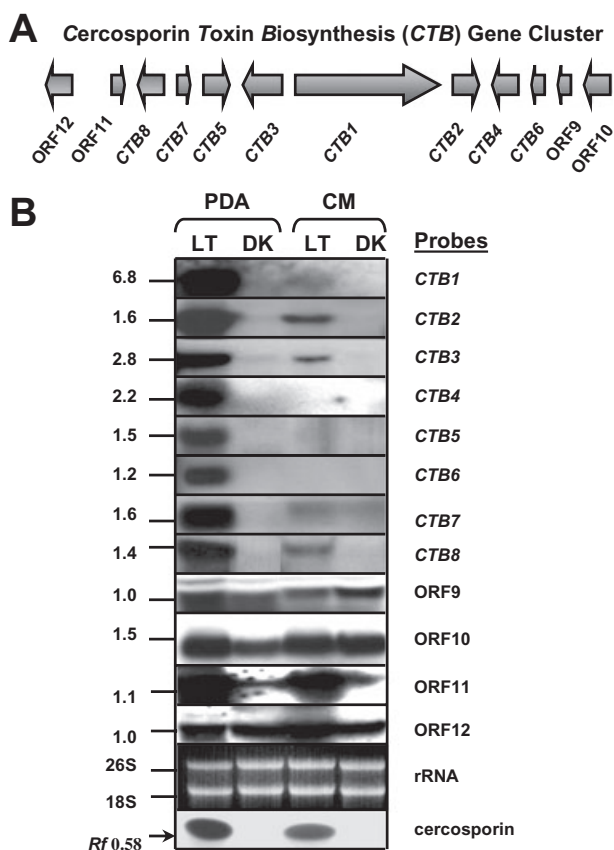


Fig. 1. The cercosporin toxin biosynthesis (CTB) gene cluster in *Cercospora nicotianae*.

A. Transcription map of the CTB cluster genes and the adjacent open reading frames (ORFs). Arrows indicate the orientation of transcription based on sequence analysis and/or deduced from comparisons of the related genes in the databases.

B. Expression of the CTB cluster genes assessed by Northern-blot analysis and production of cercosporin by the wild-type *C. nicotianae*. Total RNA was purified from fungal mycelia grown on potato dextrose agar (PDA) or complete medium (CM) under continuous light (LT) or darkness (DK), electrophoresed in formaldehyde-containing gels, blotted to nylon membranes and hybridized to a specific probe as indicated. Sizes of hybridizing bands are indicated in kilobase pairs (kb). Cercosporin was extracted with ethyl-acetate and analysed by thin-layer chromatography (TLC). Cercosporin (R_f 0.58) was indicated by an arrow.

flavus, *Aspergillus nomius*, *Aspergillus parasiticus* and *Aspergillus oryzae*. CTB2 contains a conserved S-adenosyl methionine (SAM)-binding domain similar to a wide range of O-methyltransferases. CTB2 amino acids also display similarity to the N-terminal methyltransferase domain of CTB3.

Comparisons of the CTB8 genomic and cDNA sequences identified a 1185 bp ORF and the presence of a 51 bp intron. The translated 397 amino acids of CTB8 contained a GAL4-like Zn(II)Cys₆ binuclear cluster DNA-binding domain (data not shown), and exhibited similarity to the sterigmatocystin biosynthesis regulatory proteins in

Emericella nidulans, and the aflatoxin biosynthesis AFLR transcriptional activators in *Aspergillus* species. These results suggested that CTB8 may function as a transcriptional regulator involved in cercosporin biosynthesis.

Coexpression of the CTB1–8 genes and cercosporin accumulation

Expression of the CTB1–CTB8 genes and four putative ORFs was performed by Northern-blot hybridization of total RNA of the wild-type *C. nicotianae* grown on PDA or CM in the light or under darkness to determine if they were co-ordinately regulated under conditions highly favourable (PDA and light), moderately favourable (CM and light) or unfavourable (dark) for cercosporin accumulation (Fig. 1B). Accumulation of transcripts for CTB1, CTB2, CTB3, CTB4, CTB5, CTB6, CTB7 and CTB8 was observed when the fungus was grown on PDA in the light, whereas their expression was completely inhibited when grown under complete darkness. Accumulation of the eight CTB1–CTB8 transcripts was significantly downregulated when grown on CM, compared with PDA, in the light and was almost not detected under darkness. Production of cercosporin was well co-ordinated with the patterns of gene expression of the eight CTB1–CTB8 genes (Fig. 1B). Large amounts of cercosporin were detected when the fungus was grown on PDA, with markedly reduced levels in cultures grown on CM in the light. Cercosporin was nearly undetectable when grown under complete darkness in either medium. In contrast, the ORF9, ORF10 and ORF12 transcripts were constitutively expressed and did not appear to respond to light or medium. Expression of ORF11 was slightly regulated by light, but did not show the pronounced medium-regulation of the CTB1–CTB8 genes.

Promoter analysis of the CTB1–8 genes

Because the CTB clustered genes were co-ordinately regulated, the promoters of the CTB1–CTB8 genes and their adjacent ORFs were analysed for potential common regulatory elements. Analysis of the 650 bp sequences upstream of the putative ATG translational start codon revealed that the consensus sequence CAAT was found in all the promoter regions except for ORF10 (Table 2). The consensus TATA sequence was found in the promoters of CTB1, CTB2, CTB4, CTB5, CTB7 and ORF11 but not in CTB3, CTB6, ORF9 and ORF12. All the promoter regions have one or multiple putative GATA motifs found in the AreA (nitrogen regulatory protein) gene of *E. nidulans* (Wilson and Arst, 1998), whereas none of the CTB clustered gene promoters has the CreA (carbon regulatory protein) binding site (Panozzo *et al.*, 1998). All but CTB1, CTB2, CTB5 and ORF10 have a putative pH

Table 2. Promoter analysis of genes in the cercosporin biosynthetic gene cluster of *Cercospora nicotianae*.

Gene	Translation initiation site CA(C/A)(A/C)ATGGC ^a	TATA ^b	CAAT ^b	pH regulatory GCCA(A/G)G	AreA or WC1/WC2:GATA ^b	Palindrome TCG(N ₃₋₆)CGA
<i>CTB1</i>	CACCATGGA	1	3	0	2	TCG (N ₃)CGA
<i>CTB2</i>	AAAAATGGT	1	5	0	1	TCG (N ₆)CGA
<i>CTB3</i>	CACACATGAT	0	3	1	1	TCG (N ₆)CGA
<i>CTB4</i>	CCACAATGGC	1	5	1	2	TCG (N ₃)CGA
<i>CTB5</i>	AATCCATGGG	1	7	0	3	TCG (N ₃)CGA
<i>CTB6</i>	AGTTATGGC	0	3	1	2	TCG (N ₅)CGA
<i>CTB7</i>	AGACATGGC	1	1	1	1	TCG (N ₆)CGA
<i>CTB8</i>	AACCATGGC	1	2	1	3	TCG (N ₆)CGA
ORF9	AACCATGGC	0	2	1	1	–
ORF10	AGTGATGCG	1	0	0	1	TCG (N ₃)CGA
ORF11	GCTGATGAG	1	2	1	2	–
ORF12	GTCCATGCG	0	4	1	1	–

a. The translation initiation site is underlined.

b. Numbers of the consensus sequences with 100% matches as indicated.

regulatory protein PacC binding motif (Espeso and Arst, 2000). A palindrome sequence 5'-TCG(N₃₋₆)CGA-3' was identified in all the *CTB1-8* and ORF10 promoters, but was absent in the promoters of ORF9, ORF11 and ORF12 (Table 2).

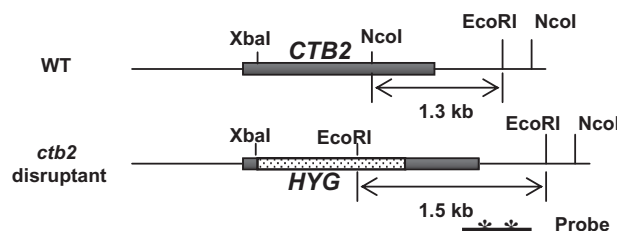
Functional characterization of *CTB2* and *CTB8* involved in cercosporin biosynthesis

To determine the involvement of *CTB2* and *CTB8* in cercosporin formation, disruption plasmids, p Δ ctb2 and p Δ ctb8, were constructed to replace the entire coding regions of *CTB2* and *CTB8*, respectively, via homologous integration. Because cercosporin is a visible red pigment, all transformants recovered were visually screened for cercosporin production on solid potato dextrose agar medium. After transforming with split marker fragments obtained from p Δ ctb2 (Fig. 2A), four of 52 transformants (7.7%) failed to produce visible cercosporin. Southern-blot analysis of the EcoRI/NcoI-digested genomic DNA from wild type and two putative *ctb2* knockouts (Δ ctb2-D5 and D18) confirmed that gene disruption specifically occurred at the *CTB2* locus (Fig. 2B). The two *ctb2* mutants did not produce any visible cercosporin, but accumulated a yellow pigment (Fig. 2C). Transformation of a full-length *CTB2* gene cassette into a Δ ctb2 null mutant restored cercosporin production.

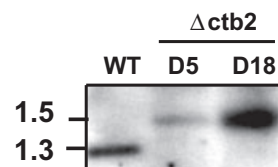
To disrupt *CTB8*, split marker fragments amplified from the disruption construct, p Δ ctb8 (Fig. 3A), were transformed into the wild-type *C. nicotianae* strain, resulting in six cercosporin-defective mutants out of 55 transformants screened (11%). Targeted gene disruption of the *CTB8* gene in *C. nicotianae* was verified by Southern-blot analysis of the XhoI/PvuII-digested genomic DNA from wild type and five mutants using a *CTB8*-specific probe (Fig. 3B). TLC analysis of culture extracts indicated that production of cercosporin was completely abolished in the

A

Targeted Disruption of *CTB2* gene



B



C

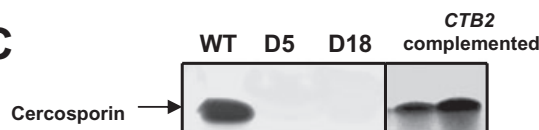


Fig. 2. Targeted disruption of the *CTB2* gene encoding a putative *O*-methyltransferase in *Cercospora nicotianae*.

A. Physical map of *CTB2* in wild type (WT) and in a Δ ctb2 disruptant carrying a hygromycin phosphotransferase B gene (*HYG*) marker integrated within *CTB2* via homologous recombination. Sizes of hybridizing fragments from wild type and the Δ ctb2 disruptant are also indicated.

B. Southern-blot analysis of genomic DNA from wild type and two Δ ctb2 disruptants (D5 and D18). Fungal DNA was digested with NcoI and EcoRI, electrophoresed, blotted and hybridized to a *CTB2*-specific probe. Band patterns show a successful disruption of the *CTB2* gene.

C. TLC analysis of cercosporin produced by wild type, two Δ ctb2 disruptants (D5 and D18), and two complemented strains. Cercosporin and putative intermediates were extracted with ethyl acetate and analysed by TLC.

Targeted Disruption of *CTB8* gene

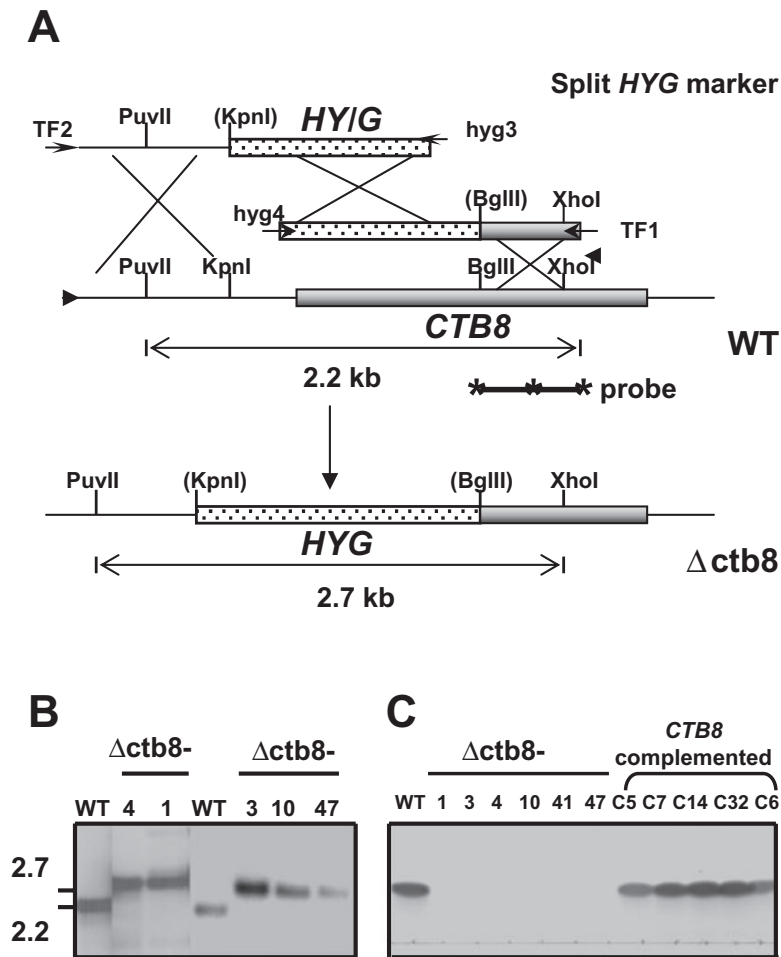


Fig. 3. Targeted gene disruption of *CTB8* encoding a putative Zn(II)Cys₂ transcription regulator using a split-marker strategy in *Cercospora nicotianae*.

A. Restriction maps of the *CTB8* gene in the genome of wild type (WT) and in the Δ ctb8-disrupted mutant. Two truncated *CTB8* fragments fused with an overlapped *HYIG* (hygromycin resistance gene cassette) were amplified by PCR with oligonucleotide primers (TF1 paired with hyg4; TF2 paired with hyg3) as indicated. Note: drawing is not to scale.

B. Southern-blot analysis of genomic DNA from wild type (WT) and five Δ ctb8 disrupted mutants (Δ ctb8-D1, 3, 4, 10 and 47). Fungal DNA was digested with PuvII and XhoI, electrophoresed, blotted onto a nylon membrane and hybridized with a *CTB8*-specific probe. Hybridizing patterns indicate disruption of the *CTB8* gene.

C. TLC analysis of cercosporin produced by wild type, six Δ ctb8 disruptants (D1, D3, D4, D10, D41 and D47), and five complemented strains (C5, C6, C7, C14 and C32).

Δ ctb8 knockouts (Fig. 3C). Transformation of a full-length *CTB8* gene and its promoter region into a Δ ctb8 mutant restored cercosporin production (Fig. 3C).

The Δ ctb2 or Δ ctb8 disruptants grew slightly faster than wild type, but retained the wild-type level of resistance to exogenous cercosporin or other photosensitizers such as eosin Y, haematoporphyrin, methylene blue and toluidine blue (data not shown). Pathogenicity assays using detached tobacco leaves also revealed that the Δ ctb2 and Δ ctb8 knockouts incited fewer lesions compared with wild type (data not shown), consistent with the crucial role of cercosporin in fungal virulence and lesion formation.

Gene regulation in cercosporin biosynthesis

As stated above, the *CTB1-CTB8* genes were regulated by light and medium corresponding to the conditions conducive to cercosporin accumulation, indicating that *CTB1-CTB8* may represent the core cluster for cercosporin biosynthesis. Northern-blot analysis of total RNA from wild

type and two Δ ctb8 null mutants grown on PDA in the light showed that expression of the *CTB1-CTB7* genes was nearly abolished in two Δ ctb8 null mutants (Fig. 4), indicating that the *CTB8* transcriptional activator controls cercosporin production by controlling gene transcript levels. In contrast, levels of the ORF9-ORF12 transcripts were not reduced in the Δ ctb8 mutants as compared with wild type. Regulation of the *CTB1*, *CTB3*, *CTB7* and *CTB8* genes was also examined in a mutant (205C3) disrupted for the *CRG1* transcription regulator; this mutant produces approximately 50% of the cercosporin produced by wild type and exhibits partial sensitivity to exogenous cercosporin (Chung *et al.*, 2003a). Northern-blot hybridization showed a drastic reduction of transcripts of the *CTB1*, *CTB3*, *CTB7* and *CTB8* genes in the 205C3 mutant (Fig. 5A). Accumulation of the ORF10 transcript was slightly reduced in the 205C3 mutant compared with wild type. However, there was no significant difference in expression of the *CRG1* gene between the Δ ctb8 null mutants and wild type (Fig. 5B).

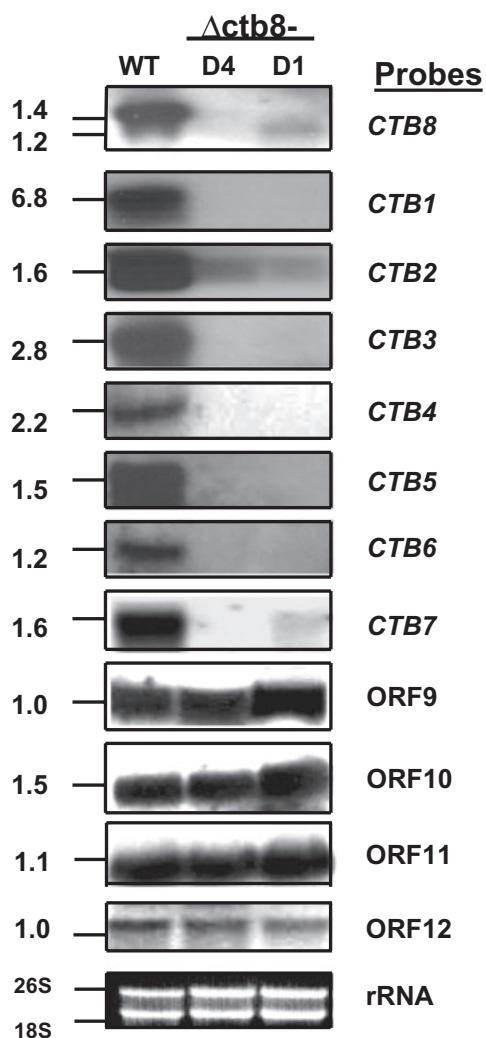


Fig. 4. Northern-blot hybridization indicates cosuppression of transcripts of the *CTB* cluster genes but not the adjacent ORFs in the *ctb8* disruptants of *Cercospora nicotianae*. Total RNA purified from wild type (WT) and two Δ ctb8 disruptants (D1 and D4) was electrophoresed in formaldehyde-containing gels, blotted onto nylon membranes and hybridized to the probes at 65°C as indicated. The membranes were washed at high stringency, and sizes of hybridizing bands are indicated in kilobase pairs (kb).

To further determine coregulation of the *CTB* genes in the cluster, accumulation of the *CTB* gene transcripts also was examined in the Δ ctb2 null mutants as well as in the Δ ctb1 and Δ ctb3 null mutants identified in the previous studies (Fig. 6). The results showed that accumulation of the *CTB3* and *CTB8* gene transcripts in the Δ ctb1 knockouts was drastically reduced in four knockouts tested (Fig. 6A). In contrast, regulation of expression of *CTB2* and *CTB4* in the Δ ctb1 knockouts was apparently variable because two Δ ctb1-D1 and D11 knockouts had the wild-type levels of expression of *CTB2* and *CTB4*, whereas expression of the two genes was nearly undetectable in the Δ ctb1-D6 and D7 knock-

outs. Analysis of two Δ ctb2 null mutants also revealed that transcripts of the *CTB1*, *CTB2*, *CTB3*, *CTB4* and *CTB8* genes were completely undetectable when *CTB2* was disrupted (Fig. 6B). A small, but truncated transcript (less than 1.2 kb) was detected by the *CTB2* probe (Fig. 6B) in the *ctb2* knockout (D5), likely resulting from expression driven by the *trpC* promoter in the *HYG* cassette. When the *CTB3* coding region was disrupted by a hygromycin gene cassette, expression of the *CTB1* gene was markedly reduced and expression of the *CTB2*, *CTB4* and *CTB8* genes was nearly abolished (Fig. 6C).

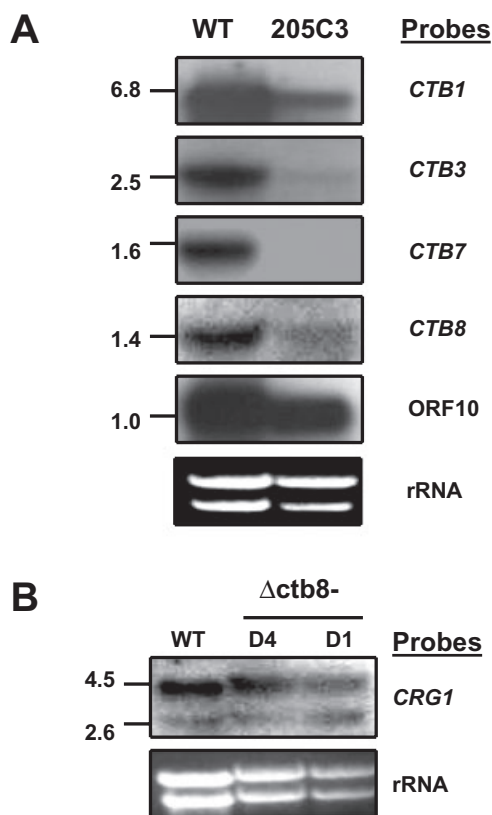


Fig. 5. Epistatic analysis between the *CTB8* and *CRG1* transcriptional regulators in *Cercospora nicotianae*. **A.** Northern-blot analysis of expression of the *CTB* genes in wild type (WT) and a previously generated 205C3 strain that is defective in a putative Zn(II)Cys₆ transcriptional regulator, *CRG1*, and is required for normal cercosporin production and resistance (Chung *et al.*, 1999; 2003a). **B.** Gene expression of the *CRG1* gene in wild type (WT) and two Δ ctb8 disruptants of *C. nicotianae*. In addition to the *CRG1* transcript (2.6 kb), a 4.5 kb transcript is produced due to production of a dicistronic mRNA of *CRG1* and an adjacent putative uracil transcript gene (*PUT1*) (Chung *et al.*, 2003c). Ethidium bromide-stained rRNA indicating the relative loading of the samples is also shown. Total RNA was isolated from fungal strains grown on potato dextrose agar under continuous light for 5 days, electrophoresed in formaldehyde-containing gels, blotted onto nylon membranes and hybridized to the probes at 65°C as indicated.

Feedback Inhibition

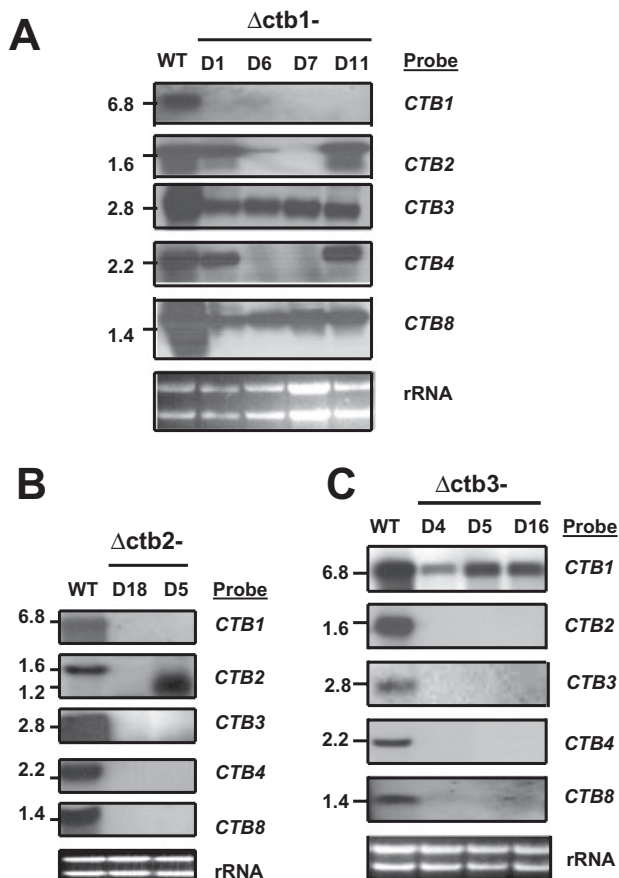


Fig. 6. Northern-blot analysis indicates a feedback inhibition of the *CTB* clustering genes in *Cercospora nicotianae*.

A. Accumulation of transcripts of the *CTB* genes in wild type (WT) and four Δ *ctb1* disruptants (D1, D6, D7 and D11).

B. Expression of the *CTB* genes in wild type and two Δ *ctb2* disruptants (D5 and D18). A truncated transcript (small than 1.2 kb) was detected by the *CTB2* probe in the Δ *ctb2*-D5 mutant, probably resulting from expression driven by the *trpC* promoter in *HYG*.

C. Expression of the *CTB* genes in wild type and three Δ *ctb3* disruptants (D4, D5 and D16). Total RNA was isolated from fungal strains grown on potato dextrose agar under continuous light for 5 days, electrophoresed in formaldehyde-containing gels, blotted onto nylon membranes and hybridized to the probes as indicated. Ethidium bromide-stained rRNA is shown to indicate the relative loading of the samples.

Differential expression of the *CTB1*–*8* genes

A prior study indicated that expression of the *CTB3* gene was affected by changing the carbon and/or nitrogen sources or pH in the medium (Dekkers *et al.*, 2007). Northern-blot analyses were performed to further determine if expression of the *CTB1*, *2*, *4*, *5*, *6*, *7* and *8* genes also was influenced by the carbon and nitrogen sources and pH in culture (Fig. 7A). Except for *CTB4*, accumulation of the *CTB1*, *2*, *5*, *6*, *7* and *8* gene transcripts was markedly elevated when the fungus was cultured on a

synthetic medium containing mannitol, instead of glucose, as the sole carbon source (lanes 1 and 2). Substitution of nitrate with ammonium as the sole nitrogen source or depletion of nitrogen in the medium caused a drastic reduction of the *CTB1*, *2*, *4*, *5*, *6*, *7* and *8* gene transcripts (lanes 3 and 4). All the *CTB* genes tested, except for *CTB4*, were expressed abundantly at pH 4, pH 7, and non-buffered PDA (pH 5.6). The *CTB4* gene transcript was not detected when the fungus was grown on CM under light or in acidic conditions. The amounts of cercosporin accumulated in the medium with different carbon

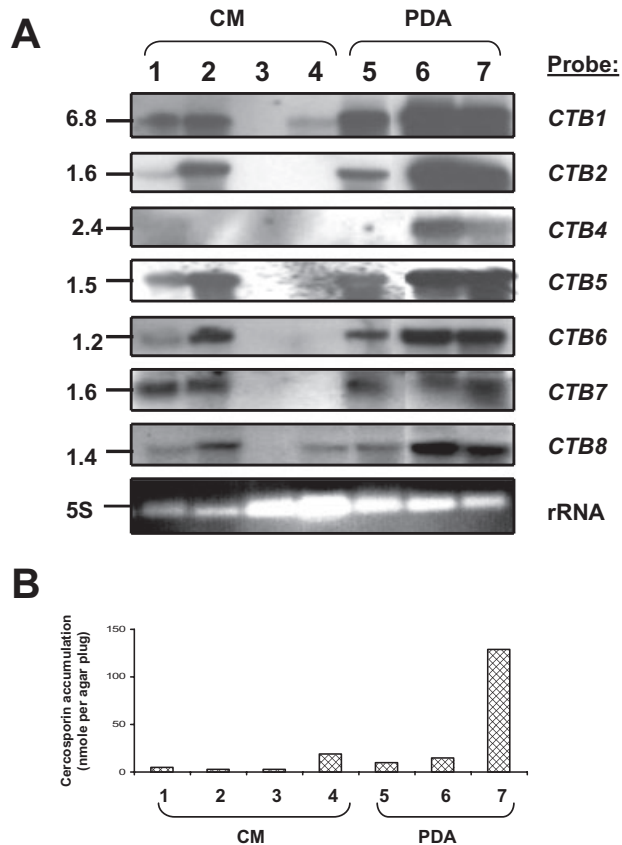


Fig. 7. Differential expression of the *CTB* genes in *Cercospora nicotianae*.

A. Northern-blot analysis of expression of the *CTB* genes in the *C. nicotianae* wild type grown on complete medium (CM with glucose and calcium nitrate as the carbon and nitrogen sources respectively; pH 5.3) (lane 1), mannitol as the sole carbon source (lane 2), ammonium chloride as the sole nitrogen source (lane 3), no nitrogen source (lane 4), and on PDA buffered with citrate-phosphate solution to pH 4 (lane 5), or pH 7 (lane 6), and non-buffered PDA (pH 5.6) (lane 7) under continuous light. Total RNA was isolated, electrophoresed in formaldehyde-containing gels, blotted onto nylon membranes and hybridized to the probes as indicated. Sizes of hybridizing bands are indicated in kb. Ethidium bromide-stained rRNA is shown to indicate the relative loading of the samples.

B. Accumulation of cercosporin by the wild-type *C. nicotianae* grown in the conditions as described above. Cercosporin was purified with 5 N KOH and quantified by a spectrophotometer at 480 nm.

and nitrogen sources and pH did not correlate with the levels of the *CTB* gene transcripts (Fig. 7B). Noticeably, all the *CTB* genes were highly expressed as the fungus was grown on PDA under light, whereas accumulation of cercosporin was drastically reduced on PDA buffered with a citrate-phosphate solution. Compared with the amounts of cercosporin produced on non-buffered PDA (pH 5.6; > 120 nmole cercosporin per agar plug), accumulation of cercosporin on CM (pH 5.3; < 10 nmole per agar plug) was much lower, and did not show significant differences between treatments. Depletion of the nitrogen source from CM slightly enhanced the production of cercosporin, but failed to support high levels of expression of the *CTB* genes.

Discussion

Previous characterization of the *CTB1* gene encoding a polyketide synthase and the *CTB3* gene encoding dual *O*-methyltransferase and FAD-dependent monooxygenase domains revealed the requirement of both genes in cercosporin biosynthesis and fungal pathogenesis (Choquer *et al.*, 2005; Dekkers *et al.*, 2007). The genes involved in secondary metabolite pathways in filamentous fungi are often organized in a cluster (Keller and Hohn, 1997; Keller *et al.*, 2005). Thus, we hypothesized that similar clustering organization of the genes for cercosporin biosynthesis may also occur in *Cercospora* spp. Clustering of the biosynthetic genes may provide a great advantage in coordinated gene regulation at transcriptional levels during cercosporin biosynthesis. Analysis of genomic sequence flanking the *CTB1* and *CTB3* genes identified 10 additional ORFs. All the ORFs were expressed as examined by Northern-blot analysis. However, only accumulation of transcripts of the *CTB1-CTB8* genes correlated with conditions conducive for cercosporin production. We propose that these genes represent the core cercosporin biosynthesis gene cluster in *C. nicotianae*. The core gene cluster for cercosporin biosynthesis includes genes encoding a polyketide synthase (*CTB1*), two *O*-methyltransferases (*CTB2* and *CTB3* N terminus), a monooxygenase (*CTB3* C terminus), an MFS transporter (*CTB4*), three oxidoreductases (*CTB5*, 6 and 7) and a Zn(II)Cys₆ transcription regulator (*CTB8*). When the *CTB8* gene was disrupted, expression of all of the core cercosporin cluster genes was eliminated, or significantly reduced, suggesting that *CTB8* did play a major role in regulation of the pathway.

Cercosporin is a polyketide compound. Although the biochemical functions of the *CTB* proteins remain to be determined, we propose a pathway for cercosporin biosynthesis in a route that resembles fatty acid synthesis based on putative functions deduced from sequence similarities and studies of the biosynthesis of fungal

polyketides (Rawlings *et al.*, 1989; Watanabe and Ebizuka, 2004). The proposed pathway shall provide a framework for further investigation of cercosporin biosynthesis. As depicted in Fig. 8, the early steps for biosynthesis of cercosporin have been predicted to begin with condensation of acetyl-CoA (starter) and malonyl-CoA (extender) subunits (Okubo *et al.*, 1975) by the function of the polyketide synthase encoded by *CTB1*. *CTB1* contains a set of active site domains including a keto synthase (KS), an acyltransferase (AT), a thioesterase (TE) and two acyl carrier protein (ACP) domains (Choquer *et al.*, 2005) that act cooperatively to form the polyketomethylene backbone of cercosporin. The malonyl-CoA subunit is assumed to attach to the ACP domains of *CTB1* by formation of phosphopantotheine (PPT) (Rawlings *et al.*, 1989; Watanabe and Ebizuka, 2004). The AT domain is in turn responsible for transferring the acetate unit from acetyl-CoA to the PPT of the ACP domain. The KS domain of *CTB1* functions to condense the malonyl- and acetyl-CoAs by decarboxylation. After each cycle of condensation, the malonyl keto group is reduced. The putative polyketide synthase encoded by *CTB1* iteratively catalyses the synthesis by incorporating two carbons in each cycle to form a linear polyketide that is released by the function of TE. Unlike fatty acid synthesis, biosynthesis of cercosporin may not involve β -ketoreduction, dehydration and enoyl reduction because *CTB1* lacks β -ketoreductase (KR), dehydratase (DH) and enoylreductase (ER) domains (Choquer *et al.*, 2005).

The polyketide generated by the function of *CTB1* must undergo successive ring closure, oxidation, hydration and methylation reactions to form the polyketomethylene backbone of cercosporin. Cyclization of the aromatic ring of cercosporin is likely accomplished via the Claisen condensation as proposed for other polyketide compounds (Birch, 1967), and would be mainly catalysed by the function of TE domain in the *CTB1*. The N-terminal conserved sequence (LFGDQ) and a conserved histidine residue in the C terminus of *CTB1* may also involve cyclization as shown for the WA protein involved in naphthopyrone production (Fujii *et al.*, 2001). The polypeptides encoded by *CTB3* and *CTB5* show similarities to FAD/FMN-dependent monooxygenases/oxidoreductases that are likely involved in the polyketide oxidations. The *CTB6* and *CTB7* products are similar to oxidoreductases/hydrogenases and are likely responsible for hydration. Methylation at C2 and C11 of cercosporin is presumably completed by the translational products of *CTB2* and/or *CTB3*. 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 translated products of the *CTB2* and *CTB3* genes may likely catalyse the addition of one or two methyl groups into the cercosporin backbone. Substrate

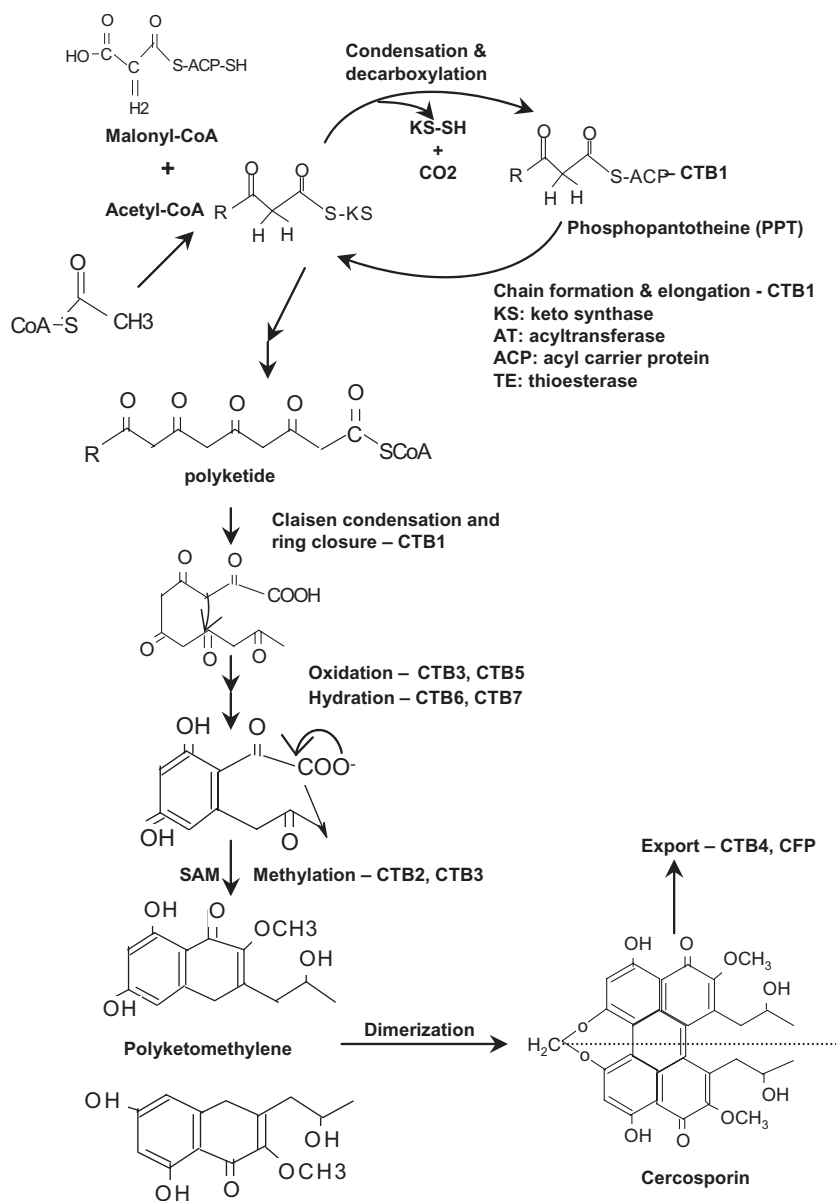


Fig. 8. Summary of a probable biosynthetic pathway led to the formation of cercosporin, showing hypothesized functions of the *CTB* gene products in *Cercospora nicotianae*. The keto synthase (KS) domain and acyl carrier protein (ACP) of the CTB1 likely involved in chain elongation are also indicated. More details are discussed in the text.

feeding analysis by coculturing the *ctb2* and *ctb3* disruptants failed to form any cercosporin (data not shown). However, cercosporin was observed when a *ctb1* disruptant was paired with a *ctb2* or *ctb3* disruptant by forming a distinct red band where the mycelia of the mutant colonies were in contact (Dekkers *et al.*, 2007 and unpubl. data). Thus, the functions of CTB2 and CTB3 as an *O*-methyltransferase were not redundant and both were required for cercosporin biosynthesis.

Cercosporin has a bilateral symmetrical structure (Fig. 8). Thus, formation of the mature cercosporin is likely mediated by dimerization of two identical polyketomethylene units. The enzymatic reaction directly contributing to dimerization is currently unknown. It is also possible that the *CTB3*, 5, 6, and/or 7 translational products may be

involved in dimerization. Alternatively, dimerization of polyketomethylene units may proceed non-enzymatically as noted for trichothecene biosynthesis in *Fusarium* (McCormick *et al.*, 1990).

We propose that exportation of cercosporin outside fungal cells is mediated by a putative 12-transmembrane MFS transporter encoded by *CTB4* (Choquer *et al.*, 2007). Previous studies have argued that CFP, a putative 14-transmembrane MFS transporter, is the major transporter responsible for cercosporin secretion in *Cercospora kikuchii* (Callahan *et al.*, 1999). Interestingly, comparative analysis showed that CFP has no similarity to CTB4, and the *CFP* gene is not linked with the *CTB* gene cluster. The respective roles of these two transporters are not known at this time. Finally, the *CTB* core gene

cluster was apparently coregulated through *CTB8*, which encodes a Zn(II)Cys₆ transcriptional activator, as expression of the *CTB1-CTB7* genes was completely or nearly abolished in two Δ ctb8 disruptants.

Unlike the *CTB1-CTB8* genes, the ORF9 and ORF10 transcripts were present in all conditions tested, thus representing the end of the *CTB* cluster on the right. ORF11, immediately adjacent to *CTB8*, is located in the opposite end of the cluster. Expression of ORF11 appeared to be light regulated, but was unaffected by medium composition. The deduced polypeptide of ORF11 showing low similarity to Zn(II)Cys₆ transcriptional regulators has only one zinc-binding motif, suggesting that ORF11 may encode a non-functional protein. Attempts to disrupt ORF11 using similar split marker approach failed to identify any cercosporin deficiency mutants out of 385 transformants screened, suggesting that ORF11 likely plays no role in cercosporin biosynthesis. The conceptually translated ORF12 also has low similarity to GAL4-like, Zn(II)Cys₆ transcriptional regulators, but completely lacks the zinc-binding domain. *CTB8* did not regulate expression of ORFs 9–12 as accumulation of these gene transcripts in two Δ ctb8 disruptants had no significant difference from wild type. The results support *CTB1-CTB8* as the core biosynthetic cluster for cercosporin biosynthesis.

Many microorganisms including *Cercospora* spp. that produce biologically toxic secondary metabolites have mechanisms for self-protection to avoid suicide (Daub *et al.*, 2005). In some cases, the genes contributing to self-defence are also situated in the biosynthetic gene cluster. For example, genes for longevity assurance factors and an ABC transporter have been identified in the fumonisin biosynthetic gene cluster in *Gibberella moniliformis* (Proctor *et al.*, 2003). *TRI12*, responsible for toxin transport and self-protection in *Fusarium sporotrichioides* clusters with the trichothecene biosynthetic genes (Alexander *et al.*, 1999). *SIRA* in *Leptosphaeria maculans*, located in the sirodesmin biosynthetic cluster, is not required for production, but is involved in self-protection (Gardiner *et al.*, 2005). In *Cercospora*, genes have been shown to have a dual role in cercosporin resistance and biosynthesis. For example, inactivation of the *CFP* gene encoding a putative cercosporin transporter in *C. kikuchii* yielded a mutant deficient in cercosporin production as well as in resistance (Callahan *et al.*, 1999). Further, disruption mutants for *CRG1*, encoding a putative Zn(II)Cys₆ transcriptional activator, resulted in a parallel reduction in both cercosporin production and the ability to tolerate cercosporin toxicity (Chung *et al.*, 1999; 2003a). To date, however, we have no evidence that the *CTB* biosynthetic cluster contains genes involved in resistance. Disruption of the *CTB8* gene resulted in a mutant that failed to express the related genes in the cluster, but retained normal resistance to exogenous cercosporin and to other

singlet oxygen-generating photosensitizers (data not shown). As auto-resistance has frequently been associated with membrane transporters, we specifically tested a disruption of *CTB4* encoding a putative MFS transporter. Disruption of *CTB4* yielded a mutant that was partially defective in cercosporin production but was unchanged in cercosporin sensitivity (Choquer *et al.*, 2007), and ruled out its role in self-protection against cercosporin.

Production of cercosporin is regulated by a wide range of factors such as light, medium composition, cultural age, growth stage and developmental stage (Jenks *et al.*, 1989; Ehrenshaft and Upchurch, 1991; Daub and Ehrenshaft, 2000), and also appears to be regulated by a signalling network interplay between Ca²⁺/calmodulin and a MAP kinase pathway (Chung, 2003; Shim and Dunkle, 2003). Further, there is a regulatory relationship between cercosporin production and resistance, as evidenced by the dual phenotype of *crg1* disrupted mutants (Chung *et al.*, 1999; 2003a). These findings suggest a complex and intertwined regulatory network, where *CTB8* is a pathway-specific regulator and *CRG1* has broader regulatory ability to activate transcript levels of the genes responsible for both biosynthesis and resistance. Figure 9 shows a proposed model for regulation. The fungus receives signal cues, particularly from light, through membrane receptors that subsequently activate the Ca²⁺/calmodulin and/or the MAP kinase signalling pathways. *CRG1*, which is unlinked to the *CTB* gene cluster, appears to exert its regulation of cercosporin biosynthesis, at least in part, by activating the transcription of *CTB8*; disruption of the *CRG1* gene in the 205C3 mutant caused a marked reduction in the *CTB1*, *CTB3*, *CTB7* and *CTB8* gene transcripts (Fig. 5), indicating that *CRG1* may directly and indirectly trigger expression of the *CTB8* transcript. *CTB8* is specifically responsible for activating expression of the other *CTB* (1–7) genes, which eventually leads to cercosporin production. Biosynthesis of secondary metabolites in fungi is often regulated by various environmental factors (Yu and Keller, 2005). In addition to *CRG1*, many positive- or negative-acting global regulatory factors such as AreA (nitrogen regulatory protein; Marzluf, 1997), PacC (pH regulatory protein; Espeso and Arst, 2000), WC (light regulatory proteins; Iwasaki and Dunlap, 2000), and/or AP-1 (oxidative stress-responsive transcription activator; Toone and Jones, 1999) might be involved in cercosporin biosynthesis and/or self-defence as well. Recently, a novel transcriptional regulator, LaeA, has been shown to be involved in a global regulation of the gene clusters for a wide range of secondary metabolites including gliotoxin, lovastatin, penicillin and sterigmatocystin in *Aspergillus* spp. (Bok and Keller, 2004). Whether or not those transcription orthologues are involved in the regulation of cercosporin biosynthesis in *Cercospora* spp. awaits further investigation by disrupting the corresponding genes.

of the *CTB1–8* genes, substitution of glucose with mannitol as the sole carbon source increased accumulation of the *CTB1–8* gene transcripts, yet did not enhance cercosporin production. Expression of the *CTB1–8* genes and production of cercosporin could also be affected by pH. However, this study revealed that accumulation of the *CTB1–8* gene transcripts and cercosporin were more likely affected by the citrate-phosphate buffer rather than by the pH values (Fig. 7). Although several *CTB1–8* gene promoters have putative PacC binding motifs (Table 2), there were no direct correlations between cercosporin production and accumulation of the *CTB1–8* gene transcripts in different pH. Accumulation of the *CTB1–8* gene transcripts and cercosporin was, however, co-ordinately regulated by light. All *CTB1–CTB8* gene promoters also have CAAT consensus sequences that could be recognized by many other transcriptional regulators. These results imply a regulatory complexity in the biosynthetic pathway to form cercosporin.

Our studies are continuing to further define the pathway for cercosporin biosynthesis. Characterization of intermediates accumulated in various mutants will likely provide more definitive data on the pathway. Disruption of *CTB8* blocked cercosporin production but did not lead to the accumulation of obvious intermediates, most likely because Δ ctb8 mutants were downregulated for the entire pathway. Similarly, disruption of *CTB1* encoding the polyketide synthase failed to detect any pigments (Choquer *et al.*, 2005), as this gene encodes the first step in the pathway. By contrast, disruption of *CTB2* or *CTB3* resulted in accumulation of a yellow pigment (Fig. 2 and Dekkers *et al.*, 2007), whereas disruption of *CTB4* yielded a brown pigment (Choquer *et al.*, 2007). Preliminary analysis of the yellow-brown pigments that were accumulated by the *CTB2*, *CTB3* or *CTB4* disruptants failed to identify any distinct peaks by spectrophotometry or HPLC (data not shown). It is presently unknown if those pigments are the intermediates for cercosporin biosynthesis. Continued characterization of the *CTB1–8* genes and pathway intermediates will lead to a fuller understanding of the biosynthetic pathway for this important polyketide toxin and for pathway regulation.

Experimental procedures

Biological materials and cultural conditions

The wild-type (ATCC18366) *Cercospora nicotianae* (Ellis & Everh.) and genetically modified strains were maintained on a complete medium (CM) (Jenns *et al.*, 1989). The Δ ctb1 (D1, D6, D7 and D11) and Δ ctb3 (D4, D5 and D16) disruptants were created in previous studies (Choquer *et al.*, 2005; Dekkers *et al.*, 2007). Fungal mutants defective in cercosporin biosynthesis were screened and identified by the lack of red pigmentation (due to cercosporin production)

when grown on potato dextrose agar (PDA, Difco) plates by the method described (Chung *et al.*, 2003b). Assays for sensitivity to photosensitizing compounds (cercosporin, eosin Y, haematoporphyrin, methylene blue or toluidine blue) were performed by growing fungal isolates on CM medium containing 10 or 100 μ M of the test compound under continuous light as described by Jenns and Daub (1995). Cercosporin and photosensitizing compounds were purchased from Sigma-Aldrich (St. Louis, MO), and dissolved in acetone or water to make a 10 or 100 mM stock solution as appropriate. The pH of medium was adjusted appropriately with 0.1 M citric acid and 0.2 M dibasic sodium phosphate buffer.

Purification and quantification of cercosporin

Cercosporin and the biosynthetic intermediates were extracted with 5 N KOH or with ethyl acetate from agar plugs cut from mycelial cultures as described previously (Chung, 2003; Choquer *et al.*, 2005). Cercosporin and the biosynthetic intermediates in the KOH extracts were quantified by measuring absorbance at 480 nm using a model Genesys 5 spectrophotometer (Spectronic Instruments, Rochester, NY). The ethyl acetate extracts were separated on thin-layer chromatographic (TLC) plates coated with a 60 F254 fluorescent silica gel using ethyl acetate: hexane: methanol: H₂O (6:4:1.5:1, v/v) mixture as the solvent.

Chromosomal walking and sequence analysis

Genomic DNA of *C. nicotianae* was isolated using a DNeasy Plant Mini kit (Qiagen, Valencia, CA). A DNA library of *C. nicotianae* was constructed from genomic DNA digested with DraI, EcoRI, PvuI and StuI, and ligated to adaptors using a Universal GenomeWalker kit (BD Biosciences, Palo Alto, CA) according to the manufacturer's instructions. To obtain unknown genomic regions, primers were designed to complement sequenced regions and used for two rounds of PCR amplification with adaptor primers using a Titanium or Advantage 2 DNA polymerase (BD Biosciences). The amplified DNA fragments were purified with a DNA purification kit (Mo Bio Laboratories, Carlsbad, CA) and either directly sequenced or cloned into pGEM-T easy vector (Promega, Madison, WI) for sequence analysis from both directions at Eton Bioscience, (San Diego, CA). PCR primers were synthesized by Integrated DNA Technologies (Coralville, IA) and Allele Biotechnology and Pharmaceuticals, (San Diego, CA). BLAST similarity searches (Altschul *et al.*, 1997) were performed at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). ORF and exon/intron positions were deduced from comparisons of genomic and cDNA sequences and/or predicted using the FGENESH gene-finding software at <http://www.softberry.com>. A search for functional domains was conducted using the PROSITE database in the Expasy Molecular Biology Server (<http://us.expasy.org>) and Motif/ProDom and Block programs (<http://motif.genome.jp/>). Analysis of the *CTB* promoter regions was conducted using regulatory sequence analysis tools (van Helden, 2003) at <http://rsat.ulb.ac.be/rsat/> and the output threshold was set at 1 (no mismatches were allowed). Palindrome searches were performed at <http://bioweb.pasteur.fr/seqanal/interfaces/palindrome.html>.

Disruption of CTB2 and CTB8 genes

Fungal protoplasts were prepared and transformed using CaCl_2 and polyethylene glycol by previously described methods (Chung *et al.*, 2002). To disrupt *CTB8*, a 2.4 kb DNA fragment encompassing the entire *CTB8* ORF and its 5'- and 3' untranslated regions was amplified using a high fidelity DNA polymerase (Roche Applied Science) with primers TF1 (5'-ctggatcaccgcagaggaagc-3') and TF2 (5'-gctgagcgcaccactgagta-3'), and cloned into the pGEM-T easy vector to yield pCTB8. A 1.1 kb KpnI–BglII fragment in pCTB8 was replaced with a 1.6 kb BamHI end-filled fragment harbouring the hygromycin phosphotransferase B gene (*HYG*) cassette from pUCATPH (Lu *et al.*, 1994) to generate a disruption construct named p Δ ctb8 (Fig. 3). PCR products containing truncated *HYG* and *CTB8* fusion fragments were amplified from p Δ ctb8 for gene disruption. A 1.5 kb fragment containing 5'-*CTB8* fused with 3' *HYG* was amplified with primers TF2 and hyg3 (5'-ggatgcctccgctcgaagta-3'). A 2.0 kb fragment containing 5' *HYG* fused with 3' *CTB8* was amplified with primers TF1 and hyg4 (5'-cggtgcaagaactgcctgaa-3'). PCR fragments, overlapping within the *HYG* region, were purified with a PCR clean-up kit (Mo Bio Laboratories), mixed, and directly transformed into the *C. nicotianae* wild-type strain for gene disruption.

To disrupt *CTB2*, a 2.7 kb fragment containing the full-length *CTB2* gene was amplified from *C. nicotianae* genomic DNA by PCR with primers mts1 (5'-ggattcccgatcttggcgtaag-3') and mts2 (5'-catgagcagatggttggggattgt-3') and cloned into pGEM-T easy vector to form pCTB2 (Fig. 2). A 1.1 kb fragment in pCTB2 was replaced with the *HYG* cassette to generate the disruption construct p Δ ctb2. The *CTB2* fragments fused with the *HYG* marker were amplified with primers mts2 and hyg4 (5'-cggtgcaagaactgcctgaa-3') and primers mts1 and hyg3 (5'-ggatgcctccgctcgaagta-3'), respectively, and used for gene disruption.

Genetic complementation

Genetic complementation was conducted by cotransformation of a PCR fragment containing the full-length *CTB2* or *CTB8* ORF and its endogenous promoter with plasmid pBARKS1 carrying the *BAR* gene responsible for Ignite/basta (bialaphos) resistance under control of the *Aspergillus nidulans* *trpC* promoter [Pall and Brunelli, 1993; obtained from the Fungal Genetics Stock Center (FGSC)] or with the pCB1532 plasmid carrying the *Magnaporthe grisea* acetolactate synthase gene (*SUR*) cassette for sulfonylurea resistance (Sweigard *et al.*, 1997; obtained from the FGSC) into the Δ ctb8-D4 or Δ ctb2-D3 null mutant. Putative transformants were selected on medium containing 250 $\mu\text{g ml}^{-1}$ hygromycin (Roche Applied Science), 5 $\mu\text{g ml}^{-1}$ sulfonylurea (chlorimuron ethyl; Chem Service, West Chester, PA) or 50 $\mu\text{g ml}^{-1}$ bialaphos (Phytotechnology Laboratory, Lenexa, KS) as appropriate and assessed for cercosporin production on PDA plates as described previously (Chung, 2003).

Reverse transcriptase (RT)-PCR and cDNA isolation

Fungal RNA was isolated with TRIZOL reagent (Invitrogen). The poly (A⁺) mRNA was purified with an Oligotex kit (Qiagen). Double strand cDNA of *C. nicotianae* was gener-

ated with the BD SMART PCR cDNA synthesis kit (BD Biosciences) according to the manufacturer's instructions. Each cDNA fragment encompassing the entire ORF and its partial 5'- and 3' regions was subsequently amplified from the cDNA pools with a TITANIUM Taq polymerase (BD Biosciences) and the respective gene primers, and the fragments were purified and directly subjected to sequence analysis.

Manipulation of nucleic acids

All plasmids were propagated in *Escherichia coli* DH5 α bacterial cells and purified using a Wizard DNA purification kit (Promega). Standard procedures were used for endonuclease digestion of DNA, electrophoresis, and Southern-blot hybridizations (Sambrook and Russell, 2001). For Northern-blot hybridizations, total RNA was denatured in a formaldehyde-containing agarose and buffer solution as described previously (Chung *et al.*, 2003c), blotted onto a positively charged nylon membrane, and hybridized to a PCR-generated DNA probe as appropriate. The hybridization probes were generated by PCR with gene-specific primers to insert a digoxigenin (DIG)-11-dUTP into the *CTB1–8* DNA fragments. The conditions and procedures used for probe labelling, hybridization, post-hybridization washing and immunological detection of the probe using a CSPD chemofluorescent substrate for alkaline phosphatase were carried out according to the manufacturer's recommendations (Roche Applied Science).

Nucleotide sequences

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession nos. AY649543 (*CTB1*), DQ991505 (*CTB2*), DQ355149 (*CTB3*), DQ991506 (*CTB4*), DQ991507 (*CTB5*), DQ991508 (*CTB6*), DQ991509 (*CTB7*), DQ991510 (*CTB8*), DQ993249 (ORF9), DQ993250 (ORF10), DQ993251 (ORF11) and DQ993252 (ORF12).

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