Functional characterization of three genes encoding putative oxidoreductases required for cercosporin toxin biosynthesis in the fungus Cercospora nicotianae

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Cercosporin is a non-host-selective, photoactivated polyketide toxin produced by many phytopathogenic Cercospora species, which plays a crucial role during pathogenesis on host plants. Upon illumination, cercosporin converts oxygen molecules to toxic superoxide and singlet oxygen that damage various cellular components and induce lipid peroxidation and electrolyte leakage. Three genes (CTB5, CTB6 and CTB7) encoding putative FAD/FMN- or NADPH-dependent oxidoreductases in the cercosporin toxin biosynthetic pathway of C. nicotianae were functionally analysed. Replacement of each gene via double recombination was utilized to create null mutant strains that were completely impaired in cercosporin production as a consequence of specific interruption at the CTB5, CTB6 or CTB7 locus. Expression of CTB1, CTB5, CTB6, CTB7 and CTB8 was drastically reduced or nearly abolished when CTB5, CTB6 or CTB7 was disrupted. Production of cercosporin was revived when a functional gene cassette was introduced into the respective mutants. All ctb5, ctb6 and ctb7 null mutants retained wild-type levels of resistance against toxicity of cercosporin or singlet-oxygen-generating compounds, indicating that none of the genes plays a role in self-protection.

INTRODUCTION

Many phytopathogenic fungi are equipped with various arsenals such as secretion of cell-wall-degradation enzymes and formation of phytotoxins in order to invade their hosts (Schafer, 1994). Host-selective toxins kill plant cells by targeting a specific cellular enzyme or component, and thus are toxic only to a limited range of host cultivars (Walton, 1996). In contrast, non-host-selective toxins, targeting various cellular components, enable the producing pathogens to have wide host ranges. Cercosporin (Fig. 1a) is a non-host-selective perylenequinone toxin produced by many phytopathogenic Cercospora species, which have been reported to affect several hundred plant species, including many major crops such as corn, rice, banana, coffee, sugar beet, soybean, peanut and tobacco (reviewed by Daub & Ehrenshaft, 2000; Daub et al., 2005). Cercosporin is an important virulence determinant in Cercospora species (Callahan et al., 1999; Choquer et al., 2005, 2007; Dekkers et al., 2007; Shim & Dunkle, 2003; Upchurch et al., 1991). Compared to other non-host-selective phytotoxins, cercosporin has several unique features, including light activation for its biosynthesis (Ehrenshaft & Upchurch, 1991), light- and oxygen-dependent cytotoxicity (Yamazaki et al., 1975), and the production of reactive oxygen species such as singlet oxygen and superoxide (Daub & Hangarter, 1983). In the past two decades, intensive research has been focused on elucidation of the genetic mechanisms involved in self-protection from cercosporin and singlet-oxygen-generating compounds (Chung et al., 1999, 2003a; Daub et al., 1992, 2000; Ehrenshaft et al., 1998, 1999; Jenns & Daub, 1995; Solld et al., 1992).

Cercosporin was first isolated from the soybean pathogen Cercospora kikuchii in 1957 (Kuyama & Tamura, 1957) and its structure was chemically determined by Yamazaki & Ogawa (1972). In addition to plants, cercosporin has been
Biosynthesis of cercosporin is also primarily triggered by
been known to be required for cercosporin toxicity.
sequences are also indicated.
Arrows indicate the orientation of transcription. Sizes of the coding
fungal polyketide pathway. However, the genes or enzymes
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et al.
Based on substrate feeding experiments (Okubo

tually cell death (Daub, 1982; Daub & Briggs, 1983).
causing lipid peroxidation, electrolyte leakage and even-
cercosporin has been shown to damage cell membranes by
such as lipids, proteins and nucleic acids, depending on its
shown to be toxic to various cell types, including bacteria
and many fungi and animal cells, due to the production of
reactive oxygen species (Daub et al., 2005). Cercosporin is
capable of breaking down different cellular components
such as lipids, proteins and nucleic acids, depending on its
localization in cells. During pathogenesis in host plants,
cercosporin has been shown to damage cell membranes by
cauing lipid peroxidation, electrolyte leakage and eventually
cell death (Daub, 1982; Daub & Briggs, 1983).

Based on substrate feeding experiments (Okubo et al.,
1975) cercosporin has been proposed to be synthesized by a
fungal polyketide pathway. However, the genes or enzymes
involved in cercosporin production remain largely
unknown. Recent studies with molecular and genetic tools
began to uncover the cercosporin biosynthetic pathway
and regulation network (Daub et al., 2005). Light has long
been known to be required for cercosporin toxicity.
Biosynthesis of cercosporin is also primarily triggered by
light (Jenns et al., 1989). In order to elucidate cercosporin
biosynthesis and regulation at molecular levels, several
cercosporin-deficient mutants were identified via a restric-
tion-enzyme-mediated mutagenesis approach (Chung et al.,
2003b). As a result, two linked genes, CTB1 (encoding a
polyketide synthase) and CTB3 (encoding a dual methyl-
transferase/monoxygenase) that were required for cercos-
porin biosynthesis were identified and characterized from
Cercospora nicotianae (Choquer et al., 2005; Dekkers et al.,
2007). We later obtained nine additional ORFs beyond the
boundaries of CTB1 and CTB3 by combining chromosomal
walking and sequence analysis. Six of them (CTB2, CTB4,
CTB5, CTB6, CTB7 and CTB8) encode polypeptides
proposed to be involved in cercosporin production
(Chen et al., 2007). Expression of eight of the genes was
induced under cercosporin-producing conditions and was
go-ordinately regulated by the Zn(II)Cys6 transcriptional
activator, CTB8 (Chen et al., 2007). We hypothesize that
cercosporin, like many fungal secondary metabolites
(Keller et al., 2005), is synthesized by a cluster of co-
regulated genes, in this case designated the cercosporin
toxin biosynthesis (CTB) gene cluster. The functions of
CTB1, CTB2, CTB3, CTB4 and CTB8 in cercosporin
biosynthesis have been unambiguously demonstrated by
analysing the respective null mutants (Chen et al., 2007;
Choquer et al., 2005, 2007; Dekkers et al., 2007). The
functions of CTB5, CTB6 and CTB7, which encode proteins
similar to numerous FAD/FMN- or NADPH-
dependent oxidoreductases or dehydrogenases, remain to
be elucidated. In the present study, we characterized these
genes by creating loss- and gain-of-function strains; we
present conclusive evidence to demonstrate their roles in
cercosporin biosynthesis, and completely define the core
CTB gene cluster.

Methods

Fungal isolates and culture conditions. Wild-type C. nicotianae
(ATCC 18366) and genetically modified strains were maintained on
complete medium (CM) (Jenns et al., 1989). Cercosporin-production
mutants were screened daily for the lack of red pigment production
on thin potato dextrose (glucose) agar (PDA, Difco, Becton,
Dickinson and Company) plates by a method described previously
(Chung et al., 2003b). We found that a thin PDA plate (less than
15 ml medium in a 100 x 15 mm Petri dish) supported the highest
production of cercosporin under illumination. Assays for sensitivity
to photosensitizing compounds (cercosporin, eosin Y, haematoporph-
phyrin, methylene blue or toluidine blue) were performed by growing
fungai isolates on CM containing 10 or 100 M test compound under
continuous light as described previously (Jenns & Daub, 1995). All
chemicals were purchased from Sigma-Aldrich unless otherwise
stated, and dissolved appropriately in acetone or water to make a
10 or 100 mM stock solution.

Cercosporin analysis. Cercosporin was extracted with 5 M KOH or
with ethyl acetate from agar plugs with mycelia as described
previously (Choquer et al., 2005; Chung, 2003). Cercosporin in
KOH extracts was quantified by measuring A480 using a Genesys 5
spectrophotometer (Spectronic Instruments). Ethyl acetate extracts
were analysed on a TLC plate coated with a 60 F254 fluorescent silica

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**METHODS**

**Fungal isolates and culture conditions.** Wild-type *C. nicotianae* (ATCC 18366) and genetically modified strains were maintained on complete medium (CM) (Jenns et al., 1989). Cercosporin-production mutants were screened daily for the lack of red pigment production on thin potato dextrose (glucose) agar (PDA, Difco, Becton, Dickinson and Company) plates by a method described previously (Chung et al., 2003b). We found that a thin PDA plate (less than 15 ml medium in a 100 x 15 mm Petri dish) supported the highest production of cercosporin under illumination. Assays for sensitivity to photosensitizing compounds (cercosporin, eosin Y, haematoporphyrin, methylene blue or toluidine blue) were performed by growing fungal isolates on CM containing 10 or 100 μM test compound under continuous light as described previously (Jenns & Daub, 1995). All chemicals were purchased from Sigma-Aldrich unless otherwise stated, and dissolved appropriately in acetone or water to make a 10 or 100 mM stock solution.

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CTB5, CTB6 and CTB7 gene disruption. A strategy employing the split-hygromycin phosphotransferase B gene (HYG) marker fused with truncated CTB5, CTB6 or CTB7 fragments was used for targeted gene disruption in C. nicotianae as described previously (Choquer et al., 2005). All DNA clones were built on the backbone of pGEM-T easy vector (Promega). For CTB5 disruption, a 3.3 kb fragment was amplified with primers CTB5R (5'-GCTACTGTCGGAGCAGTCGTT-3') and CTB7I (5'-CTGAGCTCCGAGAAGCTTTG-3') from genomic DNA and cloned to yield pCTB5. A 1.0 kb HindIII–AgeI fragment in pCTB5 was replaced with a 1.6 kb BamHI, end-filled fragment harbouring the HYG cassette from pUCATPH [obtained from the Fungal Genetics Stock Center (FGSC), University of Missouri, Kansas City, MO, USA] to generate the disruption construct, pCTB5. Two truncated HYG and CTB5 fusion fragments overlapping within the HYG region (800 bp) were amplified by PCR from pCTB5, and directly transformed into wild-type C. nicotianae for gene disruption. A 2.3 kb fragment containing 5' CTB5 fused with 3' HYG was amplified with primers CTB7I and Hygsplit2 (5'-CCGACATGCTCCGGATCATGG-3'); a 2.1 kb fragment containing 5' HYG fused with 3' CTB5 was amplified with primers CTB5R and Hygsplit1 (5'-AGGAGGCCGTCGATAATGCTCCGTCCGGG-3').

For CTB6 disruption, a 2 kb fragment was amplified with primers ctb6F (5'-CAGAAGCTGATCACTGCAGCAGT-3') and mfs4 (5'-GCAATATCTCATGGTTATTCCTCCTG-3'), and cloned to form pCTB6. A 0.5 kb BglII–HindIII fragment in pCTB6 was replaced with the HYG cassette to generate pAChb. Two fragments, of 1.7 and 1.9 kb, overlapping within HYG, were obtained from pAChb with primers ctb6F and hygsplit1 and mfs4 and hygsplt2, respectively.

For CTB7 disruption, a 5 kb fragment was amplified with primers tf4 (5'-CCATGAGAATACGCGCATGC-3') and ord3 (5'-CGTATACGGCTAGATCCTCCTG-3'), and cloned to become pCTB7. A 0.9 kb Eco47III fragment was replaced with the HYG cassette to yield the disruption construct, pAChb. Split-HYG marker fragments, of 2.5 and 1.5 kb, were amplified from pAChb with primers tf4 and hygsplt1 and ord3 and hygsplt2, respectively.

Complementation and fungal transformation. The full-length CTB5, CTB6 or CTB7 ORF, including the corresponding endogenous promoter, was independently amplified with gene-specific primers by a high-fidelity DNA polymerase (Roche Applied Science). Genetic complementation was performed by co-transformation of a PCR fragment with the pCB1532 plasmid carrying the Magnaporthe grisea acetalactate synthase gene (SUR) cassette for sulfonylurea resistance (Sweigard et al., 1997, obtained from FGSC) into Δctb5-D8, Δctb6-D18 or Δctb7-D2 null mutants. Transformants were selected on sulfonylurea and screened for cercosporin production. Fungal protoplasts were prepared and transformed using CaCl2 and PEG by methods described previously (Chung et al., 2002). Transformants were selected on medium containing 250 μg hygromycin ml−1 (Roche), or 5 μg chlorimuron ethyl ml−1 (Chem Service) as appropriate and tested for cercosporin production on PDA plates (Chung, 2003).

Sequence analysis. Fungal DNA was isolated with a DNeasy Plant Mini kit (Qiagen). Full-length CTB5, CTB6 or CTB7 was amplified with the respective primers as described above and cloned into pGEM-T easy vector (Promega) for sequence analysis from both directions at Eton Bioscience. PCR primers were synthesized by Integrated DNA Technologies. Searches for sequence similarity and functional domains were performed with web-based software programs as described previously (Chen et al., 2007). Sequence data from this study have been deposited with the EMBL/GenBank Data Libraries under accession nos. DQ991507 (CTB5), DQ991508 (CTB6) and DQ991509 (CTB7).

Molecular techniques. Standard procedures were used for endonuclease digestion of DNA, electrophoresis, and Southern and Northern blot hybridization. The hybridization probes were generated by PCR with gene-specific primers to incorporate DIG-11-dUTPs (Roche) into CTB1, CTB5, CTB6, CTB7 or CTB8 DNA fragments as previously described (Choquer et al., 2005; Chen et al., 2007). The conditions and procedures for probe labelling, hybridization, post-hybridization washing and immunological detection of the probe with a disodium 3-[4-methoxyxypirin]-1,2-dioxetano-3,2’-(5’-chloro)tricyclo[3.3.1.1]decan-4-yl]phenyl phosphate (CSPD) chemiluminescent substrate for alkaline phosphatase were carried out according to the manufacturer’s instructions (Roche).

Pathogenicity assay. Assay for fungal pathogenicity was carried out on detached tobacco leaves (Nicotiana tabacum ‘Burley 21’) with conidia suspensions (5 × 10^4 conidia ml−1) as described previously (Choquer et al., 2005).

RESULTS

Characterization of the CTB5, CTB6 and CTB7 genes

In total, eight CTB genes (CTB1–8) are closely linked in the genome of C. nicotianae and probably involved in biosynthesis of cercosporin (Fig. 1a). In this study we utilized a genetic approach to further define the roles of CTB5, CTB6 and CTB7 in cercosporin biosynthesis. Both CTB5 and CTB7 are located near the left border of the cercosporin biosynthetic gene cluster (Fig. 1b). The coding regions of CTB5 and CTB7 are separated by 867 bp and are transcribed in the same direction. The CTB5 ORF contains 1380 bp with no introns and is predicted to encode a polypeptide of 459 aa. The translation product of CTB5 displays 30–59 % identity and 50–72 % similarity to numerous uncharacterized, conserved, hypothetical proteins from sequenced genomes of fungi (data not shown). The amino acid sequence deduced from the CTB5 ORF also resembles various oxygen- and FAD/FMN-dependent oxidoreductases of various bacteria in the databases. CTB5 has a putative TonB-dependent receptor protein signature, a potential oxygen-interacting site, a FAD-binding site, a tyrosine sulfation site, and a putative NADH-binding site (Fig. 2).

The CTB6 gene is located near the right border of the CTB gene cluster (Fig. 1b), consists of 1074 bp with no introns, and presumably encodes a 357 aa polypeptide. The translation product of CTB6 has 26–40 % identity and 48–61 % similarity to numerous NADPH-dependent reductases, oxidoreductases or dehydrogenases of various micro-organisms. An alcohol dehydrogenase family signature and a motif that probably interacts with NADPH were identified in CTB6 (Fig. 2).

The CTB7 ORF consists of 1401 bp interrupted by a single intron of 48 bp and is predicted to encode a protein of 450 aa. The translation product of CTB7 has 22–33 %
identity and 38–51 % similarity to a wide variety of FAD/FMN-dependent oxidoreductases, hydrolases, or monooxygenases of fungi and bacteria. CTB7 has two FMN/FAD or flavin-containing monooxygenase-binding sites and an amidation site (Fig. 2).

Disruption of the CTB5, CTB6 and CTB7 genes

Targeted gene disruption was performed to evaluate the functions of CTB5, CTB6 and CTB7 associated with cercosporin production in *C. nicotianae*. Transformants were screened for cercosporin production on a thin PDA plate. In total, 53 of 202 (26 %) transformants recovered from CTB5 disruption, 10 of 141 (7.1 %) from CTB6 disruption, and 3 of 16 (19 %) from CTB7 disruption were completely defective in cercosporin accumulation.

Successful disruption of each CTB locus was validated by Southern blot analysis. Hybridization of *Eco*RI/*Hind*III-digested genomic DNA from the wild-type and six putative ctb5 disruptants to a CTB5 gene probe identified a 3.0 kb band in wild-type (Fig. 3a, b). In contrast, all transformants had a 2.4 kb hybridizing band instead, due to the insertion of the split-hygromycin phosphotransferase B gene (*HYG*) marker fused with the truncated CTB5 fragments and used for disruption. Oligonucleotide primers (ctb7I, ctb5R, ctb51, hygsplit 1 and hygsplit 2) used for cloning, probe labelling and amplification of the split markers are also indicated. Restriction enzyme site abbreviations: A, *Age*I; E, *Eco*RI; H3, *Hind*III. Note: drawing is not to scale.

(b) Southern blot hybridization of genomic DNA from the wild-type and six ctb5 knockouts confirms gene-specific replacement at the CTB5 locus. Fungal DNA was digested with *Eco*RI and *Hind*III, electrophoresed, blotted onto a nylon membrane, and hybridized to a CTB5-specific probe as indicated above. The hybridizing bands indicated by arrows in DNA prepared from two disruptants (ctb5-D1 and D9) may be due to the ectopic integrations in the genome.

(c) Northern blot analysis of total RNA isolated from the wild-type and two ctb5 mutants (D8 and D10). Ethidium-bromide-stained rRNA is shown to indicate the relative loading of the samples.

As shown in Fig. 4(a, b), hybridization of *Nco*I/*Xho*I-digested genomic DNA to a CTB6 gene probe yielded a 2.0 kb hybridizing band in wild-type and a 1.8 kb band in three putative Δctb6 disruptants due to the presence of an extra *Nco*I site in the inserted *HYG* fragment. The hybridization patterns confirmed a successful disruption
at the CTB6 locus. Hybridizing bands (>2.0 kb and <1.5 kb) due to the ectopic integration of PCR fragments were detected in DNA prepared from the ctb6-D4 disruptant. Northern blot hybridization of total RNA from two ctb6 disruptants further validated the null mutation at CTB6 (Fig. 4c).

The hybridization profiles shown in Fig. 5(a, b) also confirmed targeted disruption of CTB7. The CTB7 probe hybridized to 2.7 and 3.4 kb BclI fragments in DNA purified from the wild-type and five putative ctb7 disruptants, respectively. Ectopic insertions were also detected in DNA from the Δctb7-D1, D4 and D7 disruptants. Hybridization of total RNA from wild-type and two ctb7 disruptants to a CTB7 probe also confirmed the null mutation at CTB7 (Fig. 5c).

**Functional complementation**

To further evaluate the roles of CTB5, CTB6 and CTB7 in relation to cercosporin biosynthesis, genetic complementation was carried out by co-transforming a functional CTB5, CTB6 or CTB7 gene cassette (under its own promoter) with plasmid pCB1532 into the respective disruptants (ctb5-D8, ctb6-D18 and ctb7-D2). Transformants were selected on media containing sulfonylurea then tested for cercosporin production on thin PDA plates. The results

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**Fig. 4.** Targeted gene disruption of CTB6 in C. nicotianae. (a) Restriction maps of CTB6 in wild-type (WT) and Δctb6-disrupted fungal genomes, and the split-hygromycin phosphotransferase B gene (HYG) marker fused with the truncated CTB6 fragments. Oligonucleotide primers used for cloning, probe labelling and amplification of the split markers are also indicated. (b) Southern blot analysis of genomic DNA prepared from the wild-type and three ctb6 knockouts. Fungal DNA was digested with NcoI and Xhol, electrophoresed, blotted onto a nylon membrane, and hybridized to a CTB6-specific probe. The hybridizing bands indicated by arrows in DNA prepared from ctb6-D4 were probably due to the ectopic integration of genomic DNA. (c) Northern blot analysis of total RNA isolated from the wild-type and two Δctb6 mutants (D8 and D11). Ethidium-bromide-stained rRNA is shown to indicate the relative loading of the samples.

**Fig. 5.** Gene replacement of CTB7 in C. nicotianae. (a) Restriction maps of CTB7 in wild-type (WT) and Δctb7-disrupted fungal genomes, and the split-hygromycin phosphotransferase B gene (HYG) marker fused with the truncated CTB7 fragments. Oligonucleotide primers used for cloning, probe labelling and amplification of the split markers are also indicated. (b) Southern blot analysis of genomic DNA prepared from the wild-type and five putative ctb7 disruptants. Fungal DNA was digested with BclI, electrophoresed, blotted onto a nylon membrane, and hybridized to a CTB7-specific probe. The hybridizing bands indicated by arrows in DNA prepared from three disruptants (ctb6-D1, D4 and D7) were due to the ectopic integration of genomic DNA. (c) Northern blot analysis of total RNA isolated from the wild-type and two Δctb7 mutants (D1 and D2). Ethidium-bromide-stained rRNA is shown to indicate the relative loading of the samples.
indicated that transformation of full-length CTB5, CTB6 or CTB7 genes into the ctb5-D8, ctb6-D18 or ctb7-D2 disruptants, respectively, enabled the mutants to restore cercosporin production to levels comparable to the wild-type (see below).

Cercosporin production

The amounts of cercosporin in KOH extracts were determined, revealing that the absorbance values obtained from the ctb5-, ctb6- or ctb7-disruptants were indistinguishable from the controls (agar plugs only) and were considered to be zero (Table 1). To determine if a trace amount of cercosporin was produced by the disrupted mutants, cercosporin was extracted by ethyl acetate from agar plugs with fungal mycelia and the extracts were analysed by TLC. As shown in Fig. 6, the wild-type and the CTB5-, CTB6- and CTB7-complemented strains produced a red pigment (cercosporin), whereas the ctb5, ctb6 or ctb7 disruptants produced no detectable cercosporin. Thus, disruption of the CTB5, CTB6 or CTB7 gene completely blocked cercosporin biosynthesis, but disruptants accumulated a yellowish or purplish pigment that was barely visible on fluorescent-TLC plates (Fig. 6).

Transcriptional inhibition of the CTB genes in the ctb5-, ctb6- and ctb7-disrupted mutants

Northern blot analysis was performed to determine if disruption of the CTB5, CTB6 or CTB7 gene would affect expression of the other CTB genes in the cluster (Fig. 7). The results indicated that expression of CTB5 was almost undetectable in the ctb6 and ctb7 null mutants (Fig. 7a). Accumulation of the CTB6 transcript was slightly reduced in two ctb5 null mutants, but completely abolished in two ctb7 null mutants (Fig. 7b). Expression of CTB7 was barely detectable in the ctb5 and ctb6 null mutants (Fig. 7c). Similarly, expression of CTB1 and CTB8 was drastically down-regulated in the ctb5 and ctb6 null mutants, and accumulation of the CTB1 but not the CTB8 gene transcript was also reduced in two ctb7 null mutants (Fig. 8).

Sensitivity to cercosporin and other singlet-oxygen-generating photosensitizers

To determine if CTB5, CTB6 or CTB7 play a role in cellular resistance to cercosporin and other singlet-oxygen-generating photosensitizers, wild-type and ctb5, ctb6 and ctb7 disruptants were grown on media containing exogenous photosensitizing compounds (cercosporin, eosin Y, haematoporphyrin, methylene blue or toluidine blue). As compared to the wild-type, ctb5, ctb6 or ctb7 null mutants did not show significant growth retardation in the presence of exogenous cercosporin or other photosensitizers (Table 1). For comparison purpose, strain 205C3, which is deficient in a zinc finger transcriptional factor, CRG1, required for normal cercosporin production and resistance (Chung et al., 2003a), was tested. In agreement with the previous findings (Chung et al., 2003a), the 205C3 mutant

Table 1. Accumulation of cercosporin and radial growth of C. nicotianae isolates in the presence of singlet-oxygen-generating photosensitizers

<table>
<thead>
<tr>
<th>Isolate*</th>
<th>Cercosporin accumulation (nmol)$^\dagger$</th>
<th>Radial growth (mm)$^\ddagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CR 10 μM</td>
<td>10 μM</td>
</tr>
<tr>
<td>WT</td>
<td>107.4±12.7</td>
<td>8.6±0.8</td>
</tr>
<tr>
<td>ctb5-D8</td>
<td>0</td>
<td>9.5±1.5</td>
</tr>
<tr>
<td>ctb5-D11</td>
<td>0</td>
<td>9.8±1.6</td>
</tr>
<tr>
<td>CTB5C1</td>
<td>121.6±42.3</td>
<td>8.6±1.4</td>
</tr>
<tr>
<td>ctb6-D8</td>
<td>0</td>
<td>10.1±1.9</td>
</tr>
<tr>
<td>ctb6-D11</td>
<td>0</td>
<td>9.8±0.6</td>
</tr>
<tr>
<td>CTB6C5</td>
<td>113.1±15.4</td>
<td>9.6±0.8</td>
</tr>
<tr>
<td>ctb7-D2</td>
<td>0</td>
<td>9.7±0.9</td>
</tr>
<tr>
<td>ctb7-D4</td>
<td>0</td>
<td>9.7±1.9</td>
</tr>
<tr>
<td>CTB7C2</td>
<td>119.6±10.4</td>
<td>9.1±1.3</td>
</tr>
<tr>
<td>205C3</td>
<td>45.5</td>
<td>5.1</td>
</tr>
</tbody>
</table>

*Fungal isolates: WT, wild-type; ctb-D, ctb5, ctb6 and ctb7, disruptants; CTB5C1, CTB6C5 and CTB7C2, complementation strains; 205C3, strain deficient in zinc finger transcription factor, CRG1 (Chung et al., 2003a).

$^\dagger$Fungal isolates were grown on thin PDA plates for 5 days. Cercosporin was extracted with 5 M KOH and quantified by measuring $A_{480}$. The blank control contained agar plugs only. Experiments were repeated twice except for 205C3. Data are the mean±SE of two experiments.

$^\ddagger$Fungal isolates were grown on complete medium containing exogenous cercosporin, eosin Y, haematoporphyrin, methylene blue, or toluidine blue under constant fluorescent light at room temperature; radial growth was determined after 7 days. Values are the mean±SE of three experiments with three replicates for each treatment.
exhibited partial sensitivity to cercosporin but not to other photosensitizers, and produced less than 50% of the cercosporin produced by the wild-type (Table 1).

Pathogenicity
As assayed on detached tobacco leaves, the ctb5, ctb6, or ctb7 disruptants caused fewer lesions compared to the wild-type. However, the complementation strains incited necrotic lesions on tobacco, indistinguishable from those induced by the wild-type (data not shown).

DISCUSSION
Cercosporin is a polyketide compound. Biosynthesis of cercosporin has been predicted to start with decarboxylation of acetyl-CoA and malonyl-CoA units to synthesize petaketide, followed by ring closure, oxidation, hydration and methylation to form the polyketomethylene backbone of cercosporin (Okubo et al., 1975). Chromosome walking coupled with sequence analysis led to identification of the cercosporin toxin biosynthesis (CTB) cluster. The core CTB gene cluster in C. nicotianae consists of a transcriptional regulator gene, a potential transporter gene, and six biosynthetic genes (Chen et al., 2007). To fully determine the function of the CTB1–8 genes associated with cercosporin production, we performed genetic and molecular analysis of C. nicotianae strains with loss- and gain-of-function mutations in each CTB gene (Chen et al., 2007; Choquer et al., 2005, 2007; Dekkers et al., 2007). In this present study we analysed three putative oxidoreductase-encoding genes (CTB5, CTB6 and CTB7) that were localized in the CTB cluster, and obtained experimental evidence to support their crucial roles in cercosporin biosynthesis.
CTB5 has amino acid similarity to many oxygen- and FAD/ FMN-dependent oxidoreductases of bacteria and fungi, including vanillyl-alcohol oxidases, D-lactate dehydrogenases, 6-hydroxy-D-nicotine oxidases (accession nos. P56216, P06149, ZP_00522304, and CAA29416), and mitomycin radical oxidases of *Streptomyces* (accession no. P43485). These enzymes catalyse the oxidation of a wide variety of substrates for energy production and conversion (Rule et al., 1985; van den Heuvel et al., 2000). The mitomycin radical oxidase, however, oxidizes the reduced form of mitomycins and is involved in cellular self-defence against mitomycin in *Streptomyces lavendulae* (August et al., 1994). The enzyme encoded by *CTB5* is proposed to utilize FAD and/or FMN as a cofactor and catalyse the oxidation steps in the cercosporin biosynthetic pathway. CTB5 has a putative TonB-dependent receptor signature. The TonB protein is involved in the passive uptake of large and low-affinity substrates by interacting with outer-membrane receptors in *Escherichia coli* (Bell et al., 1990). Without TonB, the receptors bind their substrates but fail to transport them into the periplasmic space. In addition, CTB5 also has a tyrosine sulfation site that is physiologically associated only with proteins or domains that are transported or reside in the Golgi lumen (Huttner, 1988). It will be interesting to determine how those domains contribute to CTB5 function.

The protein encoded by *CTB6* displays considerable similarity to numerous NADPH-dependent reductases, oxidoreductases or dehydrogenases in bacteria, yeasts and plants. Some members include aldehyde reductases, nucleoside-diphosphate sugar epimerases, carbonyl reductases, dihydroflavonol 4-reductases and cinnamyl-alcohol dehydrogenases (accession nos. 1Y1PA, XP_71038, ZP_00592614, and ZP_00528210). A computer search identified an alcohol dehydrogenase (adh) family signature and a NADPH-binding motif in *CTB6*. A *CTB6* homologue in *Saccharomyces cerevisiae* (accession no. NP_011476) was thought to catalyse NADPH-dependent reduction of the bicyclic diketone bicyclo[2,2,2]octane-2,6-dione to the chiral ketoalcohol-6-hydroxybicyclo[2,2,2]octane-2-one (Goffeau et al., 1996). It is tempting to speculate that the *CTB6* enzyme catalyses an NADPH-dependent reduction or hydration step during ring closure of pentaketide in the cercosporin biosynthetic pathway.

Although the biochemical function of *CTB5*, *CTB6* and *CTB7* as oxidoreductases remains to be proven, molecular and genetic analyses clearly indicated that they were required for cercosporin biosynthesis. A prior study revealed that accumulation of *CTB5*, *CTB6* and *CTB7* transcripts was co-ordinately controlled by the *CTB8* transcriptional activator (Chen et al., 2007). In the present study, disruption of *CTB5*, *CTB6* or *CTB7* yielded mutants that were completely impaired in cercosporin production, yet retained the wild-type level of resistance to cercosporin and other singlet-oxygen-generating photosensitizers. We have also demonstrated that disruption of each of the *CTB5*, *CTB6* or *CTB7* genes markedly reduces transcriptional accumulation of the other *CTB* gene transcripts, consistent with the previous finding of the presence of a feedback inhibition mechanism (Chen et al., 2007). However, it appears that such inhibition was not completely stringent since expression of *CTB6* was slightly reduced in two *ctb5* null mutants, but completely undetectable in the *ctb7* null mutants (Fig. 7). Furthermore, expression of *CTB8* was apparently down-regulated in the *ctb5* or *ctb6* null mutants, but was normal in the *ctb7* mutants (Fig. 8).

### Fig. 8. Northern blot analysis of total RNA prepared from the wild-type (WT), and the *ctb5* (D8 and D10), *ctb6* (D8 and D11) and *ctb7* (D1 and D2) disruptants of *C. nicotianae*, indicative of a feedback transcriptional inhibition of the *CTB1* and *CTB8* genes. Total RNA was electrophoresed in formaldehyde-containing gels, blotted onto nylon membranes and hybridized to a *CTB1* or *CTB8* probe as indicated. Ethidium-bromide-stained rRNA is shown to indicate the relative loading of the samples.

The translation product of *CTB7* has an amidation site and two FMN/FAD or flavin-containing monoxygenase-binding sites. Proteins having similarity to the *CTB7* include flavoprotein monoxygenases and pyridine nucleotide-disulfide oxidoreductases of *Pseudomonas* (accession nos. YP_2333205 and YP_272409), and FAD-dependent oxidoreductases of *Xanthomonas* (accession nos. YP_363434 and AAM36534). *CTB7* also has similarity to a *Xanthomonas campestris* pv. *zinniae* oxidoreductase (accession no. AAY86766) which has recently been shown to be involved in cercosporin degradation (Taylor et al., 2006). We propose that the function of *CTB7* in the cercosporin biosynthetic pathway is probably to catalyse a hydration or reduction step during ring closure to form the polypeptide-methylene skeleton of cercosporin prior to the methylation steps.

Biosynthetic gene clusters often include one or more genes for cellular self-protection in some fungi. For example, TRI12 is an efflux pump that is involved in self-protection against trichothecene in *Fusarium sporotrichioides*.
(Alexander et al., 1999), and TOXA is a putative HC-toxin transporter in Cocchiobolus carbonum (Pitkin et al., 1996). Cercosporin is toxic to many cells due to the production of singlet oxygen; however, Cercospora species are very resistant to cercosporin (Daub et al., 2005). The mechanisms involved in cercosporin resistance have been attributed to the ability of Cercospora species to transiently reduce cercosporin, perhaps via membrane reductases (Daub et al., 1992, 2000; Leisman & Daub, 1992; Sollod et al., 1992). Recently, expression of a yeast FAD-dependent reductase in tobacco conferred resistance to cercosporin (Panagiotis et al., 2007). Despite the fact that the CTB5, CTB6 and CTB7 genes encode putative oxidoreductases, targeted gene disruption indicated that none of them is responsible for cercosporin self-resistance. Loss of the ability to synthesize cercosporin was the only phenotypic change caused by mutation of the CTB5, CTB6 or CTB7 genes. Conidiation of ctb5, ctb6 and ctb7 disruptants was not affected (data not shown). Finally, the ctb5, ctb6 and ctb7 disruptants produced fewer lesions compared to the wild-type on tobacco leaves, consistent with previous findings that cercosporin is an important virulence factor (Chen et al., 2007; Choquer et al., 2005, 2007; Dekkers et al., 2007).

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