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

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

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

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

Received 17 July 2006; accepted 30 August 2006
Available online 30 October 2006

Abstract

Cercosporin, a photo-activated, non-host-selective phytotoxin produced by many species of the plant pathogenic fungus *Cercospora*, causes peroxidation of plant cell membranes by generating reactive oxygen species and is an important virulence determinant. Here we report a new gene, *CTB3* that is involved in cercosporin biosynthesis in *Cercospora nicotianae*. *CTB3* is adjacent to a previously identified *CTB1* encoding a polyketide synthase which is also required for cercosporin production. CTB3 contains a putative *O*-methyltransferase domain in the N-terminus and a putative flavin adenine dinucleotide (FAD)-dependent monooxygenase domain in the C-terminus. The N-terminal amino acid sequence also is similar to that of the transcription enhancer AFLS (formerly AFLJ) involved in aflatoxin biosynthesis. Expression of *CTB3* was differentially regulated by light, medium, nitrogen and carbon sources and pH. Disruption of the N- or C-terminus of CTB3 yielded mutants that failed to accumulate the *CTB3* transcript and cercosporin. The Δctb3 disruptants produced a yellow pigment that is not toxic to tobacco suspension cells. Production of cercosporin in a Δctb3 null mutant was fully restored when transformed with a functional *CTB3* clone or when paired with a Δctb1-null mutant (defective in polyketide synthase) by cross feeding of the biosynthetic intermediates. Pathogenicity assays using detached tobacco leaves revealed that the Δctb3 disruptants drastically reduced lesion formation.

Keywords: Clustering; Gene replacement; Perylenequinone; Photosensitizer

1. Introduction

Cercosporin is a non-host-selective, photo-activated toxin produced by many phytopathogenic *Cercospora* species. *Cercospora* species cause leaf spot and blight diseases on a wide range of plant species, including many economically important crops such as corn, soybean, sugar beet, coffee, peanut, rice, banana, tobacco as well as ornamental, vegetable and weed species (Daub and Ehrenshaft, 2000). Production of cercosporin has been proven to be essential for full virulence of *Cercospora nicotianae* in the invasion of hosts and for lesion formation on tobacco (Choquer et al., 2005).

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

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

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

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

The toxicity of cercosporin to cells and the cellular mechanisms operating in the fungus for cercosporin self-protection have been intensively investigated (reviewed by Daub et al., 2005). By contrast, the biochemical pathway leading to the cercosporin production is largely unknown. It has been known for a long time that light is absolutely required for cercosporin production (Yamazaki et al., 1975). Studies on physiological factors regulating cercosporin production, however, revealed that accumulation of cercosporin in culture also was markedly influenced by temperature, medium composition, developmental stages and other environmental conditions and varied among isolates (Jenns et al., 1989). Studies using pharmacological inhibitors implicated the involvement of calcium and calmodulin signaling in cercosporin production (Chung, 2003). Studies using a precursor feeding also pointed out that cercosporin is synthesized via a polyketide pathway starting at condensation of acetate and malonate units (Okubo et al., 1975). Recently, several genes in *Cercospora* spp. have been cloned and demonstrated to be involved in cercosporin accumulation in culture (Callahan et al., 1999; Chung et al., 1999, 2003a; Shim and Dunkle, 2003). However, none of these genes are directly involved in the biosynthetic steps of cercosporin. To identify genes directly involved in cercosporin biosynthesis, *C. nicotianae* mutants completely deficient in cercosporin production were generated using the restriction enzyme-mediated integration (REMI) mutagenesis (Chung et al., 2003b). A *CTB1* (Cercosporin Toxin Biosynthesis) gene encoding a fungal polyketide synthase was subsequently identified from one of the REMI mutants and shown by gene disruption to be required for cercosporin biosynthesis and for high levels of virulence on tobacco (Choquer et al., 2005).

Gene clusters involved in the biosynthesis of secondary metabolites have been reported in many filamentous fungi (Keller and Hohn, 1997; Walton, 2000). Some examples include the biosynthetic genes for sterigmatocystin and aflatoxin in *Aspergillus* species, fumonisin and trichothecene in *Fusarium* (*Gibberella*) species, sirodesmin in *Leptosphaeria maculans*, compactin in *Penicillium citrinum*, gibberellin in *Gibberella fujikuroi*, alkaloids in *Claviceps purpurea* and *Neotyphodium uncinatum*, AK toxin in *Alternaria alternata* and HC toxin in *Cochliobolus carbonum* (Abe et al., 2002; Ahn and Walton, 1996; Brown et al., 1996, 1999; Gardiner et al., 2004; Hohn et al., 1993; Proctor et al., 2003; Seo et al., 2001; Spiering et al., 2005; Tanaka and Tsuge, 2000; Tudzynski and Hölter, 1998; Yu et al., 2004). Thus, we hypothesize that genes involved in cercosporin biosynthesis also are clustered in *Cercospora* spp. In this study we describe cloning and characterization of a second gene, named *CTB3*, which is immediately adjacent to the *CTB1* gene, to gain further insight into the molecular basis of cercosporin biosynthesis and regulation. The CTB3 contains two putative domains: an *O*-methyltransferase domain in the N-terminus and a flavin adenine dinucleotide (FAD)-dependent monooxygenase domain in the C-terminus. Functional analysis indicated that *CTB3* also is required for cercosporin production. The results imply that many, if not all, genes involved in cercosporin biosynthesis and regulation are likely clustered in *C. nicotianae*.

2. Materials and methods

2.1. Fungal strains, media and cultural conditions

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

2.2. Toxin purification and quantification

Cercosporin and other pigments produced by the wild type and the Δctb3 disruptants were extracted with 5 N KOH from agar plugs with mycelia as described previously (Choquer et al., 2005; Chung, 2003). Cercosporin in the KOH extracts was quantified by measuring absorbance at 480 nm using a model Genesys 5 spectrophotometer (Spectronic Instruments, Rochester, NY). For TLC and HPLC analyses, cercosporin and putative intermediates were purified from agar plugs with mycelia with ethyl acetate. The organic solvent was evaporated with a Model R110 of Rotavapor (Brinkmann, Büchi, Switzerland) and the