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The *CRG1* gene required for resistance to the singlet oxygen-generating cercosporin toxin in *Cercospora nicotianae* encodes a putative fungal transcription factor

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Abstract

The *Cercospora nicotianae* *CRG1* gene is involved in cellular resistance to the perylenequinone toxin, cercosporin, that generates highly toxic singlet oxygen upon exposure to light. The entire open reading frame (ORF) of *CRG1* was isolated and sequenced. The gene contains an ORF of 1950 bp including a 65-bp intron. The predicted 650 amino acid CRG1 protein contains a Cys₆Zn₂ binuclear cluster DNA-binding motif with homology to various fungal regulatory proteins, indicating that CRG1 may act functionally as a transcription activator. Targeted gene disruption of *CRG1* resulted in mutants that are partially sensitive to cercosporin and reduced in cercosporin production. Genetic complementation revealed that *CRG1* fully restored cercosporin resistance, but only slightly restored cercosporin production in a UV-derived mutant (CS10) containing a single nucleotide substitution in *crg1*. Complementation of a *crg1*-null mutant, however, yielded strains that are similar to the wild-type in both phenotypes. These results indicate that the transcription regulator CRG1 is involved in the activation of genes associated with cercosporin resistance and production in the fungus *Cercospora nicotianae*.

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The genus *Cercospora* contains a large group of fungal plant pathogens and causes diseases on a wide range of plants including corn, rice, sugar beet, tobacco, coffee, soybean, banana, and many ornamental and weed species [9]. Many *Cercospora* species synthesize a light-activated perylenequinone phytotoxin, cercosporin. Cercosporin toxin causes fatty acid peroxidation of plasma membrane lipids, and is considered important for fungal pathogenicity and symptom development in diseases caused by *Cercospora* species [10,15,42].

Cercosporin is a photosensitizing compound [15]. When activated by light, cercosporin absorbs light energy, converting it to an electronically activated (triplet) state, which reacts with oxygen molecules via either

electron or energy transfer to generate reactive oxygen species such as superoxide (O₂⁻) and singlet oxygen (¹O₂). Cercosporin has been shown to produce both ¹O₂ and O₂⁻ in vitro, but the major toxicity of cercosporin to the cells is dependent on the production of ¹O₂ [14] that destructively reacts with lipids, proteins, and DNA. Thus, cercosporin is not only highly toxic to plants, but is also toxic to bacteria, many fungi, and cultured human tumor cells [9].

The cellular defense mechanisms against radical and reduced reactive oxygen species (such as O₂⁻ and OH[•]) are well known in biological systems, but resistance mechanisms to nonradical ¹O₂ are poorly understood. *Cercospora* species and other fungi producing similar phytotoxins are highly resistant to cercosporin and to other ¹O₂-generating compounds, providing a good model to elucidate cellular resistance mechanisms to ¹O₂

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[11]. Studies in *Cercospora nicotianae* have tested and ruled out many mechanisms including antioxidant enzymes (SOD, peroxidase, and catalase) and internal levels of $^1\text{O}_2$ -quenching or reducing agents (ascorbate, thiols, and carotenoid) [13,20]. A potential mechanism associated with cercosporin and $^1\text{O}_2$ resistance is a transient and reversible reduction of the cercosporin molecule by *Cercospora* fungi [12,32,38]. Reduced forms of cercosporin synthesized in vitro were shown to generate less $^1\text{O}_2$ and to be less toxic to cells, and fungi sensitive to cercosporin were unable to reduce cercosporin [26,32]. Thus *Cercospora* fungi have the ability to maintain cercosporin in a reduced state and protect themselves from the toxicity of $^1\text{O}_2$ [12]. It is likely that *Cercospora* species may contain unique reductases or may have the ability to grow at a redox potential sufficient to maintain cercosporin in a reduced state. Studies in yeast also showed that over-expression of a FAD-dependent pyridine nucleotide reductase gene (*CPD1*) and a multidrug ABC transporter gene (*Snq2p*) in a sensitive strain conferred resistance to cercosporin and other $^1\text{O}_2$ -generating photosensitizing compounds [43]. Interestingly, over-expression of a facilitator transporter protein (*CFP*) gene in a sensitive fungus, likely involving in toxin efflux, also increased cercosporin resistance [41].

Previously, functional complementation of two groups of cercosporin-sensitive mutants of *C. nicotianae* [25,26] resulted in the cloning of three genes (*PDX1*, *PDX2*, and *CRG1*) required for cercosporin and singlet oxygen resistance [7,16–19]. *PDX1* and *PDX2* were shown to be involved in a novel pathway of pyridoxine (Vitamin B6) biosynthesis and to be required for both cercosporin and $^1\text{O}_2$ resistance. Further studies indicated that pyridoxine is able to efficiently quench $^1\text{O}_2$ [17]. In contrast, *CRG1* gene was shown to be essential specifically for cercosporin toxin resistance and not for $^1\text{O}_2$ in resistance in *C. nicotianae* [7]. The biochemical function of *CRG1* remained unknown due to lack of obvious functional domains or co-factor binding sites.

Since the initially identified ATG codon lacked obvious eukaryotic consensus sequences [28], we hypothesize that the previously identified *CRG1* sequences only contained a partial open reading frame (ORF). Here we report the full-length ORF of *CRG1* determined using 5'-RACE to identify the 5'-end cDNA sequences. Database searches uncovered that the *CRG1* protein contains a Cys_6Zn_2 binuclear cluster DNA-binding motif similar to transcription factors of various fungi. Targeted gene disruption of *CRG1* and genetic complementation were also performed, and demonstrate that *CRG1* is involved in both cercosporin toxin resistance and in toxin production in the fungus *C. nicotianae*. Understanding the function of *CRG1* will help elucidate the mechanisms that act to protect cells against cercosporin in *C. nicotianae*.

Materials and methods

Fungal strains and culture conditions. *Cercospora nicotianae* (ATCC 18366), a *C. nicotianae* mutant strain (CS10) partially sensitive to cercosporin [25,26], and *erg1*-disruption mutants ($\Delta 41\text{C1}$, $\Delta 50\text{C2}$, $\Delta 114\text{C2}$, and $\Delta 205\text{C3}$) were maintained routinely on malt medium [27]. Mycelia for protoplast preparation, and DNA and RNA isolation were cultured in complete medium (CM) as described [27]. Quantification of cercosporin was performed by extracting plugs cut from mycelial cultures grown on potato dextrose agar (PDA) with 5 N KOH as described [4,25]. Cercosporin used for sensitivity assays was purified with ethyl acetate. Briefly, air-dried cultures grown on PDA were chopped into small pieces and extracted with ethyl acetate (1:10, w/v) at 4°C for 16 h. The ethyl acetate extract was evaporated and the residue containing cercosporin was dissolved in acetone. Assays for resistance to cercosporin were conducted on CM medium supplemented with 10 μM cercosporin as described [9,25]. Fungal mycelium was transferred onto test plates using sterile toothpicks. All culture tests were conducted under constant fluorescent light (20 $\mu\text{E m}^{-2} \text{s}^{-1}$) for 6 days. All treatments were performed at least three times with five replicates each.

RNA isolation, RT-PCR, and 5'-RACE. Fungal total RNA was extracted with a TRIZOL RNA Isolator kit (Invitrogen Life Technologies, Carlsbad, CA). The poly(A⁺) mRNA was purified using an Oligotex kit (Qiagen, Valencia, CA). 5'-RACE was conducted as described by Frohman et al. [21] with modifications. The first strand of cDNA was synthesized from poly(A⁺) mRNA using M-MLV reverse transcriptase (Promega, Madison, WI) and *CRG1*-specific primer *crr9* (5'-GCAACGTCGCGATAGAAAGA-3'). The first-strand cDNA was further tailed with oligo dATP using terminal deoxynucleotidyl transferase (Invitrogen Life Technologies) and then subjected to PCR amplification using d(T)₁₇-adaptor primer (5'-GACTCGAGTCGACATCGAT₁₇-3') containing *XhoI*, *Sall*, and *Clal* recognition sites, and *CRG1*-specific primer *crr14* (5'-CGCTCTCATTTCCGGGCAACG-3'). As shown in Fig. 1, the 5'-RACE products were digested with *Sall* and *Eco47III*, and then cloned into the pMECA plasmid vector [39]. The positive clones identified by colony hybridization using a *CRG1*-specific probe were subjected to sequencing analysis. Oligonucleotide primers used for this study were synthesized by Genosys Biotechnologies (Woodlands, TX) and their positions are shown in Fig. 1.

Gene disruption and genetic complementation. The 5' terminus of *CRG1* is located at the end of a cosmid clone 30H2 [7]. A 6.8-kb fragment containing both up- and down-stream of *CRG1* was amplified from the cosmid using a high fidelity DNA polymerase (Roche), and primers *se5* (5'-CAGCTAACACCGTAGTGATA-3') and *ber5* (5'-TCTGCCACATAACCATCCCG-3') (Fig. 1). The amplified fragment was first cloned into pGEM-T easy vector (Promega) to yield the plasmid pKRC201. To create the disruption plasmid pKRC205, a 2.1-kb *BamHI/Eco47III* fragment of the *CRG1* gene was removed from pKRC201 and replaced with an end-filled phosphinothricin acetyltransferase (*Bar*) gene cassette [37] (Fig. 4). Two C-terminal disruption plasmids (pKRC42C1 and pKRC42C2) were generated in a previous study [7].

Plasmid pKRC209 was created to complement the *erg1*-null mutant $\Delta 205\text{C3}$. A 3.4-kb *EcoRV/SmaI* fragment from pKRC33 [7] containing the whole *CRG1* gene was cloned into pCB1532, which contains the acetolactate synthase (*Sur*) gene cassette for sulfonylurea (chlorimuron ethyl) resistance [6] to yield pKRC209. The UV-derived mutant CS10 was complemented with plasmid pKRC01. Fungal protoplast isolation and transformation were performed as described previously [6]. Transformants with *Bar*-carrying plasmids were selected in 50 $\mu\text{g/ml}$ of glufosinate ammonium (Fluka, Milwaukee, WI), whereas transformants with *Sur*-carrying plasmids were selected in 200 ng/ml of sulfonylurea (Chem Service, West Chester, PA).

Southern blot analysis. Fungal DNA was purified using a DNeasy Plant Mini kit (Qiagen). Standard procedures were used for endonuclease digestion of DNA, electrophoresis, and Southern blotting. The

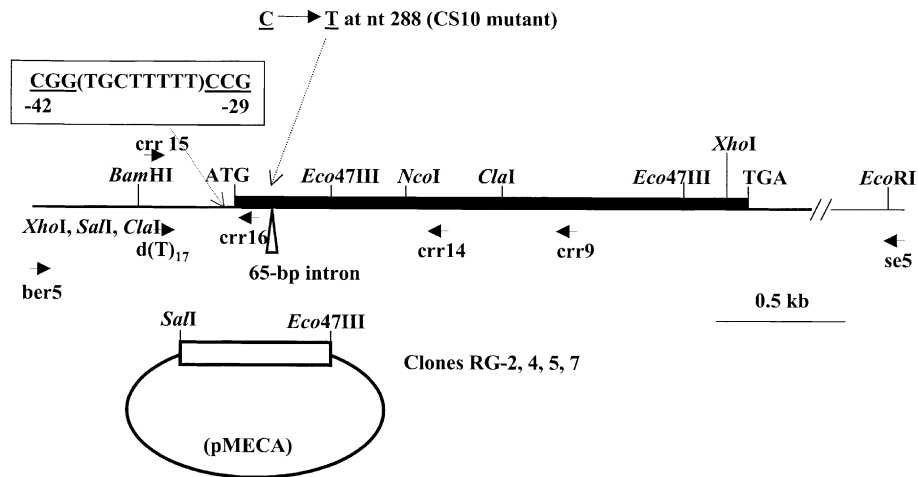


Fig. 1. Schematic illustration of the *CRG1* gene identified in the fungus *Cercospora nicotianae*. The *CRG1* open reading frame (ORF) is indicated by an initiation codon (ATG) and a stop codon (TGA). Four cDNA clones (RG-2, 3, 5, 7) derived from RT-PCR were digested with *Eco47III* and *SalI*, and cloned into pMECA vector for sequencing analysis. The open arrowhead indicates the intron sequences in the ORF of *CRG1*. A putative recognition site (CGGTGCTTTTCCG) for Cys₆Zn₂-containing transcription factors is boxed. The mutated nucleotide (C → T) at 288 in the *CRG1* ORF of the CS10 mutant and oligonucleotide primers used for RT-PCR amplification are also shown.

hybridization probes used for Southern blot analysis were labeled with digoxigenin-11-dUTP (Roche Molecular Biochemicals, Indianapolis, IN) using polymerase chain reaction and *CRG1*-specific primers. The DNA probe was amplified and labeled using primers crr15 (5'-CCAGCGGGAGTATTTGACAT-3') and crr16 (5'-TGTTCAATCGCACGCATCTC-3') as previously described [8]. After hybridization, membranes were washed in 0.1 × SSC and 0.1% SDS at 65 °C for 1 h. Immunological detection of labeled probe using CSPD ready-to-use lumigenic substrate for alkaline phosphatase was conducted according to the manufacturer's recommendations (Roche Molecular Biochemicals).

DNA sequencing and data analysis. Sequencing analysis was performed at the Molecular Genetics Instrumental Facility, University of Georgia (Athens, GA). Database searches and comparisons were conducted using the BLAST network service at NCBI and the ExpASY Molecular Biology servers [1]. Hydropathy plot analysis was performed as described by Kyte and Doolittle [30].

Results and discussion

Previously, we isolated a cercosporin-resistance gene (*CRG1*) from the phytopathogenic fungus *C. nicotianae* by genetic complementation of a cercosporin-sensitive mutant CS10 [7]. Computer prediction based on the genomic sequences identified one putative ORF of 1650 bp. A search of all available databases failed to identify significant sequence identity or similarity between *CRG1* and any known proteins. Further, the lack of obvious eukaryotic consensus sequences (GCC)GCC (A/G)CCATGG [28] in the *CRG1* fragment indicated that this sequence represented a partial ORF. We were thus unable to elucidate the biochemical function of *CRG1*.

In this study we used 5'-RACE with poly(A)⁺ RNA from a *C. nicotianae* wild-type strain to obtain the 5' end of *CRG1*. Four cDNA clones (RG-2, RG-4, RG-5, and

RG-7) were obtained from 5'-RACE. Sequencing analysis revealed that a 65-bp sequence located in the 5' end of *CRG1* was missing in all four cDNA clones (Fig. 1), and *CRG1* has 5' untranslated region of 120 bp. Sequence comparisons of genomic and cDNA clones revealed that *CRG1* gene contained a single ORF of 1950 bp plus a 65-bp intron, encoding a predicted protein of 650 amino acids (Fig. 2) with a calculated molecular weight of 71.8 kDa and a predicted pI of 5.59. The *C. nicotianae* genome contains a single copy of *CRG1* based on restriction enzyme digestion and followed by Southern blot analysis [7].

Sequence database searches revealed several motifs in the deduced protein. The N-terminus contains a Cys₆Zn₂ binuclear cluster DNA-binding motif located from amino acid residues 12–48 (Fig. 2). Hydropathy analysis identified five putative membrane-spanning helical regions (Fig. 2A). As indicated in Fig. 2B, *CRG1* contains one putative PEST region (a.a. 113–139) that is likely responsible for short lifetimes of proteins in eukaryotes [36]. Similar to other fungal transcription factors in the Cys₆Zn₂ family, *CRG1* also contains several potential phosphorylation sites (Fig. 2B). Phosphorylation of DNA-binding transcription factors is critical for DNA-binding ability, transactivation, and protein localization [23,24]. Whether any of these consensus sites are phosphorylated and related to activation of *CRG1* requires further investigation. One plausible mitochondrial localization signal with the residue pattern of IR-CSLG (0.5 certainty on a scale of 1) and a nuclear localization signal with residue pattern of amino acids KKRR (0.3 certainty) as predicted by PSORT were identified in *CRG1* (Fig. 2), suggesting that *CRG1* may be involved in gene activation in both compartments.

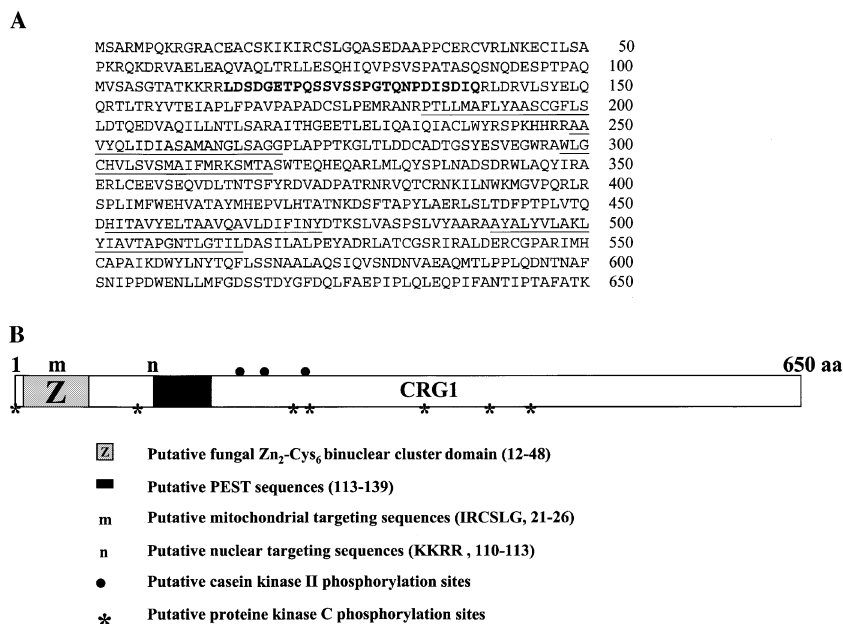


Fig. 2. (A) The amino acid sequences of CRG1 deduced from the cDNA sequences (GenBank Accession No. AF121137). A putative PEST motif is shown in bold. The putative helical membrane-spanning regions are underlined. (B) Physical map of CRG1 protein with the Cys₆Zn₂ DNA-binding and PEST motifs, and various conserved consensus sequences for modification by protein kinases.

Localization studies of CRG1 using a GFP fusion protein, however, only identified very faint, scattered points of fluorescence in the cytoplasm of *C. nicotianae*, but no solid conclusions could be made due to instability of the fusion protein [5].

The CRG1 N-terminus zinc finger motif shares homology to the zinc finger domains of various regulatory proteins such as CTF- α and CTF- β of *Fusarium solani* f. sp. *pisi*; CHAP4, LEU3p, ARO80p, GAL4, UGA3, and PPR1 of *Saccharomyces cerevisiae*; PI067 of *Schizosaccharomyces pombe*; ACU-15 of *Neurospora crassa*; AMYR, PRNA, SU-1, FACB, and AFLR of *Aspergillus* species; PRIB of *Lentinula edodes*; and FCR1p of *Candida albicans* (Fig. 3). Functionally, many of those transcription factors involve in various metabolic processes including carbon utilization (GAL4, AMYR), amino acid biosynthesis (LEU3p, PUT3p), acetate metabolism (ACU-15 and FACB), or pleiotropic drug resistance (FCR1p, PDR1p, and PDR3p) [2,22,31,40]. CTF- α and CTF- β are transcription activators that are required for the regulation of cutinase genes [33,34]. AFLR is a transcription factor involved in gene regulation for aflatoxin [44]. CRG1 shares no significant homology to other regions of those proteins. However, the C-terminal region of CRG1 excluding the zinc finger domain shares low similarity to a novel yeast transcription factor, WAR1p (YMH6_YEAST in Swiss-Prot), that is involved in weak acid response in *S. cerevisiae* [29]. Among the predicted 583 Amino acids, CRG1 shares 17% identity and 40% similarity to WAR1p (Schüller et al., unpublished data).

Many of the Cys₆Zn₂-containing transcription factors recognize a palindrome DNA sequence with two inverted repeats of CGG elements separated by a spacer with various nucleotides [5'-CGG(n_{4-11})CCG-3'] [35]. The CTF- α and CTF- β transcription activators, however, bind to a palindrome with oppositely orientated sequences [5'-GCC(n_2)GGC-3'] [33,34]. Interestingly, examination of the 5' untranslated region (5'-UTR) of *CRG1* also identified the CGG-CCG inverted sequence with a spacer of eight nucleotides (nt -29 to -42) (Fig. 1), suggesting that *CRG1* is likely self-regulated.

The *CRG1* gene was originally isolated by complementation of a UV-derived mutant (CS10), which is partially sensitive to cercosporin. Examination of the *CRG1* ORF and its flanking sequences in the CS10 mutant revealed there is one nucleotide substitution (C \rightarrow T) at position 288 (Fig. 1). The substitution results in a change from a glutamine (CAG) to a stop codon (TAG) and would yield a truncated polypeptide but with a complete Cys₆Zn₂ DNA-binding domain. In previous work, disruption of the 3'-end sequences of *CRG1* (plasmids pKRC42C1 and pKRC42C2) (Fig. 4) resulted in mutants (Δ 41C1, Δ 50C2, and Δ 114C2) that had the same cercosporin-sensitive phenotype as CS10 mutant [7], suggesting that the truncated CRG1 with an intact Cys₆Zn₂ binuclear cluster DNA-binding motif may partially retain its function in DNA-binding and gene activation.

To further elucidate the function of *CRG1*, a disruption vector (pKRC205) was developed and transformed into the wild-type strain to replace the 5' end of

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CRG1      RACEACSKIKIRCSLGOASEDAAPPCEKCVRLNKECTIL
FCR1p     KACDSRIKKTCD-GKK-----PCNRCTLDNKICVF
PRNA      RACDGCRRVKEKCE-GGV-----PCRRCTRYRQCVF
ARO80p    QACISCRSRKVKCDLGPVDNPHDPPCARCKRELKKCIF
CHA4p     LACQNCRRRRRKCN-----MEK--PCSNCIKFRTECVF
PRIB      LACQNCRRRRRKCN-----MEK--PCSNCIKFRTECVF
GAL4      QACDICRLKCLKCS-----KEK-PKCAKCLKNWECRI
LEU3p     FACVECRQQKSKCDA---HERAPEPCTKCAKKNVPCIL
PPR1      TACKRCRLKKIKCD-----QEF-PSCKRCAKLEVPVCS
PI067     RACAKCQKDNKKC-----DDARPCQRCIKAKTDCID
ACU-15    QACDRCRSCKIRCD----GIR--PCCSQCANVGFECT
FACB      LACDRCRSCKIRCD----GVR--PCCTQCANVGFECT
SU-1      RACDGC SLRKTCS----GGQ--P-CQPCAQSGFECYSY
CTF-α     RACETCHARKVRC-----AASLGVPCTNCVAFQIECRI
CTF-β     RACVSCRARKVRC-----VVE-GAPCGNCRWDNVECVV
AMYR      QACDNCRRRKIKCS-----RELP-CDKCRLLLSCSY
UGA3      HGCITCKIRKKCS-----EDKPVCRDCRRLSFFPCIY
AFLR      DSCTSCASSKVRCT-----KEKPACARCIERGLACQY
          * * * * *

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Fig. 3. Alignment of the Cys₆Zn₂ binuclear cluster motif of CRG1 from *Cercospora nicotianae* with the zinc finger regions of transcription factors identified in other fungi. CTF-α and CTF-β are transcription activators for cutinase genes in *Fusarium solani* f. sp. *pisi*. CHAP4, LEU3p, ARO80p, GAL4, UGA3, and PPR1 identified from *Saccharomyces cerevisiae*, PI067 from *Schizosaccharomyces pombe*, ACU-15 from *Neurospora crassa*, AMYR, PRNA, SU-1, and FACB from *Aspergillus* species, and PRIB from *Lentinula edodes* are transcription activators required for nutrition metabolism. AFLR is involved in gene regulation of aflatoxin biosynthesis in *A. flavus* and FCR1p is involved in drug resistance in *Candida albicans*. The conserved cysteine residues (asterisks) and proline residues (dots) are also indicated. The amino acids identical to those of the CRG1 are shaded.

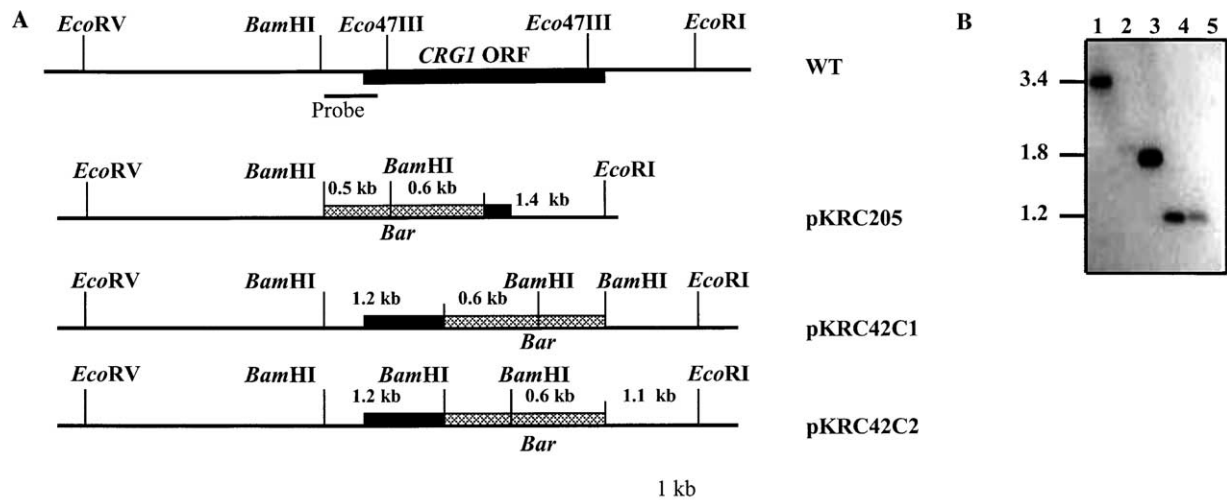


Fig. 4. Targeted gene disruption of *CRG1* in *Cercospora nicotianae*. (A) Restriction maps of the *CRG1* gene (filled box) in the wild-type (WT) fungal genome, and three disruption plasmids (pKRC42C1, C2, and pKRC205) with portions of *CRG1* replaced by the *Bar* gene cassette (hatched box). *Bam*HI and *Eco*47III sites (pKRC205), or *Nco*I and *Xho*I (plasmid pKRC42C1 and pKRC42C2), were blunt-ended and ligated with a filled-in *Bar* gene cassette. The DNA probe generated with PCR amplification is also indicated. (B) Southern blot analysis of genomic DNA from *C. nicotianae* wild-type (WT) (lane 1), and *crg1*-disruption mutants Δ205C3 (lane 2), Δ41C1 (lane 3), Δ50C2 (lane 4), and Δ114C2 (lane 5). Fungal DNA was digested with *Eco*RI and *Bam*HI, electrophoresed, blotted onto a nylon membrane, hybridized with probe, and washed at high stringency. The hybridizing bands are indicated in kilobase pairs (kb).

CRG1, including the Cys₆Zn₂ binuclear cluster DNA-binding motif with the selectable *Bar* marker (Fig. 4A). The plasmid was transformed into the wild-type strain, and transformants were screened for cercosporin sensitivity on the medium containing 10 μM cercosporin. One putative disruption mutant (Δ205C3) out of 300 trans-

formants derived from pKRC205C exhibited partial sensitivity to cercosporin. With all disruption mutants (Δ205C3, Δ41C1, Δ50C2, and Δ114C2), sensitivity to cercosporin was equivalent to that expressed by mutant CS10 (Table 1), indicating that *CRG1* is only partially involved in cercosporin resistance. Gene disruption was

Table 1

Growth of *C. nicotianae* wild-type (WT), the CS10 mutant, *crg1*-disruption mutants ($\Delta 41C1$, $\Delta 50C2$, $\Delta 114C2$, and $\Delta 205C3$), and *CRG1*-complementation strains (CS10/*CRG1* and 205C3/*CRG1*) of CS10 and $\Delta 205C3$ mutants in complete medium (CM), and CM containing 10 μ M cercosporin (CR), 10 μ M eosin Y (EY), or 100 μ M toluidine blue (TB)

Strain	Colony diameter (mm)			
	CM	CM + CR	CM + EY	CM + TB
WT	13.1 \pm 1.0	12.9 \pm 0.6	11.9 \pm 0.2	13.1 \pm 1.6
CS10	12.4 \pm 1.0	6.8 \pm 0.3	11.4 \pm 0.4	11.2 \pm 1.3
$\Delta 205C3$	11.5 \pm 1.3	6.9 \pm 0.2	9.9 \pm 1.3	10.8 \pm 1.5
$\Delta 41C1$	12.2 \pm 0.3	6.6 \pm 0.5	10.2 \pm 0.3	11.2 \pm 1.2
$\Delta 50C2$	12.1 \pm 0.6	6.4 \pm 0.5	10.3 \pm 0.3	11.6 \pm 1.2
$\Delta 114C2$	12.6 \pm 1.5	6.9 \pm 1.0	10.8 \pm 0.8	11.8 \pm 1.4
CS10/ <i>CRG1</i>	12.7 \pm 0.8	11.6 \pm 0.6	11.3 \pm 0.5	11.8 \pm 1.4
205C3/ <i>CRG1</i>	12.0 \pm 0.2	11.7 \pm 0.5	10.8 \pm 0.2	11.2 \pm 0.2

Fungal cultures were grown under constant fluorescent light at room temperature and radial growth (mm) was measured after 6 days. Numbers are means of at least three different experiments with five replicates each and standard error of mean.

confirmed by Southern blot analysis (Fig. 4B). Fungal genomic DNA was digested with *Bam*HI and *Eco*RI, and hybridized to a 0.7-kb PCR probe. The probe hybridized to a 3.4-kb fragment in the wild-type strain (Fig. 4B, lane 1), but not in any of the disruption mutants. The $\Delta 205C3$ disruptant contained no hybridizing band to the probe (lane 2), indicating that the 5' end of *CRG1* was successfully replaced with the marker gene. Hybridization of DNA from the *crg1*-mutant strains derived from pKRC42C1($\Delta 41C1$) and pKRC42C2 ($\Delta 50C2$, and $\Delta 114C2$) indicated that $\Delta 41C1$ disruptant contained a 1.8-kb hybridizing fragment (lane 3), whereas $\Delta 50C2$ (lane 4) and $\Delta 114C2$ (lane 5) contained 1.2-kb fragments. Northern-blot analysis using a *CRG1* probe failed to detect the *CRG1* transcript in the *crg1*-disrupted mutants ($\Delta 41C1$, $\Delta 50C2$, and $\Delta 114C2$), indi-

cating that these transformants were *crg1*-null mutants (data not shown).

The CS10 and *crg1*-null mutants exhibited radial growth similar to the wild-type in complete medium (CM), but were significantly reduced in growth (by $\sim 50\%$) in CM with 10 μ M cercosporin (Table 1). Genetic complementation was conducted to further verify the role of *CRG1* in cercosporin toxin resistance. The CS10 and $\Delta 205C3$ mutants were transformed with plasmids pKRC01 and pKRC205C3, respectively. A total of 25/56 transformants of CS10 and 41/54 transformants of $\Delta 205C3$ exhibited wild-type levels of resistance to cercosporin after transformation with *CRG1* (Figs. 5A and B), confirming the involvement of *CRG1* in cercosporin toxin resistance. Southern hybridization to a *CRG1* gene-specific probe indicated that the

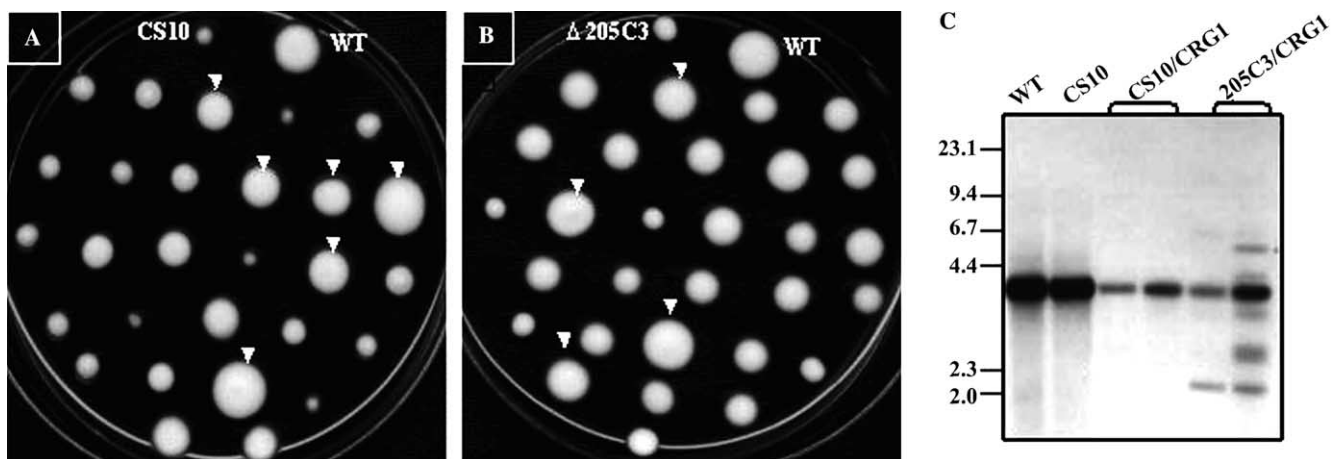


Fig. 5. Genetic complementation of *Cercospora nicotianae* mutants CS10 (A) and $\Delta 205C3$ (B) with *CRG1*. The transformants of CS10 and $\Delta 205C3$ were grown in complete medium amending with 10 μ M cercosporin and incubated under constant fluorescent light for 6 days. Complementation was evident by strains (indicated by arrowheads) that were restored to wild-type levels of growth in the presence of cercosporin toxin. Wild-type (WT) and CS10 and $\Delta 205C3$ non-transformed controls are spotted at the top of the plates as indicated. (C) Southern blot analysis of genomic DNA from *C. nicotianae* wild-type (WT), CS10, and *CRG1* complementation strains (CS10/*CRG1* and 205C3/*CRG1*). Fungal DNA was digested with *Eco*RI and *Bam*HI, and hybridized with a *CRG1* probe (as indicated in Fig. 4A). The position of size markers (DNA from bacteriophage lambda cleaved with *Hind*III) is indicated in kb.

complementation strains contained a single copy or multiple copies of *CRG1* in the genome (Fig. 5C).

The CS10 mutant is partially sensitive to cercosporin, but is unaffected in its resistance to other $^1\text{O}_2$ -generating photosensitizers [25,26]. The disruption mutants were therefore tested for resistance to other $^1\text{O}_2$ -generating photosensitizers (eosin Y and toluidine blue). As compared to the wild-type, growth of CS10 and *crg1*-disruption mutants was unaffected or decreased only slightly in the presence of these compounds (Table 1), indicating that *CRG1* plays little or no role in general $^1\text{O}_2$ resistance. The partial sensitivity to cercosporin of these mutants indicates that resistance to cercosporin and $^1\text{O}_2$ in *C. nicotianae* is likely regulated by multiple transcription activators. Also, *Cercospora* fungi appear to have developed multiple mechanisms to defend

themselves against cercosporin and the toxic $^1\text{O}_2$ molecule.

In addition to sensitivity to cercosporin, the CS10 mutant has reduced cercosporin toxin production, producing only 15–20% of the cercosporin produced by wild-type [27]. As CS10 is a UV-derived mutant, it was not known if the toxin down-regulation phenotype was due to the mutation in *CRG1*. Cercosporin production by the *crg1* disruption mutants was quantified (Fig. 6). All *crg1* disruption mutants had significantly reduced cercosporin production, producing only 40–55% of the cercosporin produced by wild-type (Fig. 6A), although they all produced more cercosporin than CS10. Fungal strains derived from complementation of CS10 and $\Delta 205\text{C3}$ mutants were also tested for cercosporin production. The data indicated that transformation with a

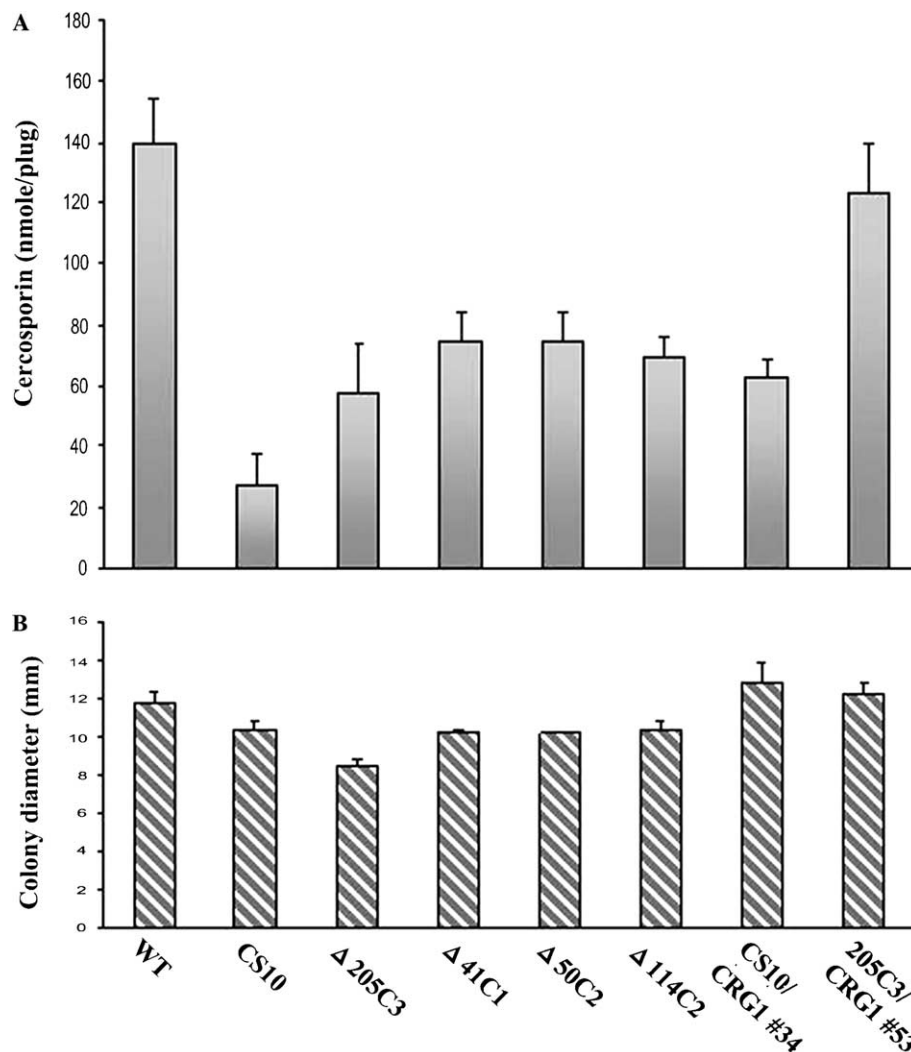


Fig. 6. (A) Cercosporin toxin production, and (B) fungal growth by wild-type (WT), the UV-derived CS10 mutant, the *crg1*-disruption mutants ($\Delta 205\text{C3}$, $\Delta 41\text{C1}$, $\Delta 50\text{C2}$, and $\Delta 114\text{C2}$), and strains recovered from genetic complementation (CS10/*CRG1* #34 and 205C3/*CRG1* #53) on PDA medium. Fungal cultures were grown on potato dextrose agar medium under continuous light for 6 days. Cercosporin toxin was extracted using 5 N KOH and quantified using a spectrophotometer at a wavelength of 480 nm. The data shown are means of at least three different experiments with five replicates for each treatment.

CRG1 clone increased cercosporin production in each of the two mutants by more than 2-fold, bringing production to 89% of wild-type in the null mutant $\Delta 205C3$, but to only 45% of wild-type in CS10 (Fig. 6A). Production of cercosporin is affected by many environmental cues and developmental stages [9,27]. It is likely that the UV-derived CS10 mutant also contains an additional mutation(s) that affects cercosporin production. Furthermore, CS 10 and *crG1*-null mutants slightly reduced growth on PDA under constant light (Fig. 6B), likely due to their sensitivity to cercosporin produced in the medium. Complementation of CS10 and $\Delta 205C3$ mutants with *CRG1* recreated strains, like wild-type, that exhibited normal growth on PDA.

In this study we have shown that *CRG1* contains a zinc finger DNA-binding domain similar to many fungal transcription activators, and have demonstrated its role in the regulation of both cercosporin resistance and production using targeted gene disruption and genetic complementation. Interestingly, disruption of the *CFP* gene, which encodes a putative protein with homology to the family of membrane facilitators in a closely related species *C. kikuchii*, also resulted in mutants that were partially defective in cercosporin sensitivity and toxin production [3]. We speculate that regulation of cercosporin resistance and production is likely operated by the simultaneous interplay of multiple counteracting processes in *Cercospora* fungi. Although disruption of *CRG1* only resulted in partial sensitivity to cercosporin and its production, *CRG1* transcription activator may play a profound role in the regulation of both phenotypes since the original UV mutant CS10 was the only mutant identified after screening 11,835 protoplast-derived colonies for reduced growth on cercosporin-containing medium [26]. Identification of *CRG1* as a fungal transcription factor will open an avenue to the isolation of the genes associated with toxin resistance and biosynthesis in *C. nicotianae*.

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