Complementation of *CTB7* in the Maize Pathogen *Cercospora zeina* Overcomes the Lack of In Vitro Cercosporin Production

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Gray leaf spot (GLS), caused by the sibling species *Cercospora zeina* or *Cercospora zea-maydis*, is cited as one of the most important diseases threatening global maize production. *C. zeina* fails to produce cercosporin in vitro and, in most cases, causes large coalescing lesions during maize infection, a symptom generally absent from cercosporin-deficient mutants in other *Cercospora* spp. Here, we describe the *C. zeina* cercosporin toxin biosynthetic (*CTB*) gene cluster. The oxidoreductase gene *CTB7* contained several insertions and deletions as compared with the *C. zea-maydis* ortholog. We set out to determine whether complementing the defective *CTB7* gene with the full-length gene from *C. zea-maydis* could confer in vitro cercosporin production. *C. zeina* transformants containing *C. zea-maydis CTB7* were generated by *Agrobacterium tumefaciens*-mediated transformation and were evaluated for in vitro cercosporin production. When grown on nitrogen-limited medium in the light—conditions conducive to cercosporin production in other *Cercospora* spp.—one transformant accumulated a red pigment that was confirmed to be cercosporin by the KOH assay, thin-layer chromatography, and ultra performance liquid chromatography-quadrupole-time-of-flight mass spectrometry. Our results indicated that *C. zeina* has a defective *CTB7*, but all other necessary machinery required for synthesizing cercosporin-like molecules and, thus, *C. zeina* may produce a structural variant of cercosporin during maize infection.

Gray leaf spot (GLS) continues to be a devastating maize foliar disease of global importance that has resulted in extensive yield losses over the past few decades (Crous and Braun 2003; Ward et al. 1999). Previously classified as *Cercospora zea-maydis* Group I and Group II, the causative agents of GLS, *C. zea-maydis* and *Cercospora zeina* are differentiated by both

genetic distance and phenotypic characteristics, such as their ability to produce the phytotoxin cercosporin (Crous et al. 2006; Goodwin et al. 2001). *Cercospora zeina* predominates throughout Africa, while *C. zea-maydis* is most prevalent in the majority of the United States and Mexico (Dunkle and Levy 2000; Goodwin et al. 2001; Meisel et al. 2009; Wang et al. 1998).

The genus *Cercospora* is part of the class Dothideomycetes and consists of more than 600 recognized species of plant pathogens (Crous and Braun 2003). Although *Cercospora* species generally exhibit relatively narrow host ranges, many produce cercosporin, a photosensitizing perylenequinone that functions as a nonspecific toxin that has been identified as a major pathogenicity factor (Daub and Ehrenshaft 2000; Weiland et al. 2010). Cercosporin production has been demonstrated for several *Cercospora* species, with isolates of *C. kikuchii*, *C. beticola*, *C. zea-maydis*, *C. asparagi*, and *C. nicotianae* all shown to accumulate cercosporin in vitro (Jenns et al. 1989). Isolates of *C. zeina*, however, demonstrate a lack of cercosporin production in vitro (Dunkle and Levy 2000; Goodwin et al. 2001; Koshikumo et al. 2014). These species all cause leaf spot diseases that are characterized by severe blighting of leaves (Daub and Ehrenshaft 2000). In contrast to this, isolates of the peanut pathogen *C. arachidicola* fail to produce cercosporin and induce only small chlorotic lesions (Fore et al. 1988). Isolates of *C. arachidicola* have, however, been shown to produce other toxins that may aid in virulence (Fore et al. 1988).

Once activated by visible wavelengths of light, cercosporin functions by producing reactive oxygen species (ROS), such as singlet oxygen and superoxide radicals, that cause oxidative damage to a wide range of macromolecules (Daub and Chung 2009; Daub and Ehrenshaft 2000). Cercosporin is thought to damage plant host cells primarily by inducing oxidative deterioration of lipids within cell membranes; the subsequent leakage of nutrients creates a favorable environment for the growth and sporulation of *Cercospora* spp., which culminates in host cell death (Daub and Chung 2009; Daub and Ehrenshaft 2000). Production of cercosporin during plant colonization has thus been linked to large, coalescing lesions in some *Cercospora* infections, whereas studies have demonstrated the induction of only small, necrotic flecks in cercosporin-deficient isolates (Choquer et al. 2005; Upchurch et al. 1991).

Cercosporin biosynthesis mainly involves a cluster of eight cercosporin toxin biosynthetic (*CTB*) genes, all of which are transcriptionally induced upon exposure to light in the tobacco pathogen *C. nicotianae* (Chen et al. 2007b). Targeted disruption of any *CTB* gene blocked cercosporin production and reduced

Cercospora zeina sequence data is deposited in the DDBJ/EMBL/GenBank database under the accession number KY656140. The raw RNA-seq reads are deposited under the Genome Expression Omnibus accessions GSE90705 and GSE94442.

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virulence in planta (Chen et al. 2007a and b; Choquer et al. 2005, 2007; Dekkers et al. 2007). *CTB8* encodes a Zn(II)Cys₆ transcriptional activator that coordinates expression of the *CTB* genes under appropriate conditions for cercosporin production (Chen et al. 2007b), while *CTB4* encodes a major facilitator superfamily transporter protein involved in cercosporin export (Choquer et al. 2007). The six remaining *CTB* genes (*CTB1*, *CTB2*, *CTB3*, *CTB5*, *CTB6*, and *CTB7*) function directly in cercosporin biosynthesis. Although the biochemical pathway underlying cercosporin biosynthesis has not been fully elucidated, metabolic profiling of individual *C. nicotianae* *CTB* knockout strains has resolved key components of cercosporin biosynthesis (Newman and Townsend 2016).

We hypothesized that there may be an underlying genetic cause for the lack of cercosporin production in *C. zeina*. Here, we present the identification and annotation of the *CTB* gene cluster in *C. zeina* as well as evidence of coding sequence degradation for the *CTB7* gene, as compared with its orthologs in *C. zea-maydis* and *C. nicotianae*. Truncation of the *C. zeina* *CTB7* gene due to the presence of indels was found to be common to *C. zeina* isolates from different African countries and the United States. The *C. zeina* *CTB7* gene region was not transcribed in vitro and exhibited incomplete splicing in planta. *Agrobacterium tumefaciens*-mediated transformation was used to complement *C. zeina* with the *C. zea-maydis* *CTB7* gene copy. Chemical analysis demonstrated cercosporin production under the appropriate in vitro growth conditions in one of the *C. zeina* *CzmCTB7* transformants. Our evidence suggests that *CTB7* is a pseudogene responsible for the lack of cercosporin production in *C. zeina*.

RESULTS

C. zeina fails to produce cercosporin in vitro.

Cercospora zeina CMW25467 from Zambia (Table 1) did not produce the visible red compound cercosporin when

grown in vitro on nitrogen-limiting medium (0.2× potato dextrose agar [PDA]) (Fig. 1A). Similarly, the United States isolate of *C. zeina* (OYPA, USPA-4) was cercosporin negative in vitro, as reported for all African and the United States *C. zeina* isolates tested previously (Dunkle and Levy 2000) (Fig. 1A). This was in contrast to the profuse cercosporin production by *C. zea-maydis* SCOH1-5 (Fig. 1A), a second fungal species that causes gray leaf spot disease of maize (Bluhm et al. 2008).

C. zeina CMW25467 harbors an intact *CTB* gene cluster, except for *CTB7*.

We exploited the availability of the *C. zeina* CMW25467 genome sequence (Muller et al. 2016) to determine whether the *CTB* gene cluster was absent or defective. BLAST analysis using the *C. nicotianae* *CTB* gene sequences as queries revealed a *CTB* gene cluster on a single *C. zeina* contig (with *E* values of 0.0 for *CTB1*, *CTB3*, *CTB5*, and *CTB6*). *CTB* gene models were annotated in silico, using i) gene prediction programs (AUGUSTUS, FGENESH and SNAP) and ii) RNA-seq reads from in vitro-grown *C. zeina* cultures. The predicted *C. zeina* *CTB* genes had best BLASTx matches corresponding to the *C. nicotianae* *CTB* protein sequences (Table 2), and pairwise alignments showed amino acid identities of 85 to 90% for *CTB1* to *CTB6*, 68% identity for *CTB8* (Supplementary File S1), but only 51% identity for *CTB7* (Table 2). In silico annotation of the *C. zeina* *CTB7* gene region in the absence of in vitro transcript sequence data predicted a single intron and a putative *CTB7* polypeptide of 322 amino acids, which was considerably shorter than the *C. nicotianae* 450-amino acid *CTB7* (Chen et al. 2007b). The order and orientation of the predicted *C. zeina* *CTB* genes (Fig. 1B) was the same as the *C. nicotianae* *CTB* cluster reported previously (Chen et al. 2007b). Transcripts were detected for all of the *C. zeina* *CTB* genes except *CTB7*, under seven different in vitro growth conditions (Table 3).

Table 1. *Cercospora* isolates utilized in this study

Species ^a	Isolate ^b	Area, country of isolation	Reference
<i>C. zeina</i>	CMW25467	Mkushi, Zambia	Meisel et al. 2009
<i>C. zeina</i>	2011.GT30	KwaZulu-Natal, South Africa	Muller et al. 2016
<i>C. kikuchii</i>	CMW49223 ^c	KwaZulu-Natal, South Africa	
<i>C. zeina</i>	NLUG 1.23 (B9-P9) ^d	Uganda	
<i>C. zeina</i>	NLUG 1.23 (B9-P11) ^d	Uganda	
<i>C. zeina</i>	NLUG 1.23 (B9-P12) ^d	Uganda	
<i>C. zeina</i>	NLUG 1.23 R (B9-P20) ^d	Uganda	
<i>C. zeina</i>	Zambia (B9-P16) ^d	Zambia	
<i>C. zeina</i>	Zambia 3.2 (B9-P26) ^d	Zambia	
<i>C. zeina</i>	Zambia 3.2 (B9-P31) ^d	Zambia	
<i>C. zeina</i>	Zambia 3.2 (B9-P32) ^d	Zambia	
<i>C. zeina</i>	CMNY C2 (B8-P1) ^d	New York, U.S.A.	
<i>C. zeina</i>	CMNY C2 (B8-P3) ^d	New York, U.S.A.	
<i>C. zeina</i>	CMNY C2 (B8-P2) ^d	New York, U.S.A.	
<i>C. zeina</i>	WOOH-NCR (B8-P25) ^d	Ohio, U.S.A.	
<i>C. zeina</i>	WOOH-NCR (B8-P26) ^d	Ohio, U.S.A.	
<i>C. zeina</i>	WOOH-NCR (B8-P27) ^d	Ohio, U.S.A.	
<i>C. zeina</i>	WOOH-NCR (B8-P28) ^d	Ohio, U.S.A.	
<i>C. zeina</i>	OYPA 16 (B8-P5) ^d	Pennsylvania, U.S.A.	
<i>C. zeina</i>	OYPA 16 (B8-P7) ^d	Pennsylvania, U.S.A.	
<i>C. zeina</i>	OYPA 16 (B8-P9) ^d	Pennsylvania, U.S.A.	
<i>C. zea-maydis</i>	CBS 117757	Wisconsin, U.S.A.	Crous et al. 2006
<i>C. zea-maydis</i>	CBS 117761	Indiana, U.S.A.	Crous et al. 2006
<i>C. zea-maydis</i>	SCOH1-5 ^d	Ohio, U.S.A.	Bluhm et al. 2008

^a Based on diagnostic histone gene polymerase chain reaction analysis (Crous et al. 2006).

^b CMW = Culture collection of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa. CBS = Culture collection of the Centraalbureau voor Schimmelfcultures, Utrecht, The Netherlands.

^c Isolated by V. Coetzee from a soybean leaf with symptoms of *Cercospora* leaf blight.

^d Culture collection of B. H. Bluhm, University of Arkansas, Fayetteville, U.S.A.

The *C. zeina* CTB7 gene region has deletions compared with the corresponding genomic DNA (gDNA) region in *C. zeae-maydis*.

Based on in silico annotation and the lack of in vitro expression, the *C. zeina* CMW25467 CTB7 gene appeared to be defective. Comparison of gDNA sequences between the two GLS pathogens revealed a series of deletions in the *C. zeina* CTB7 region, corresponding to part of exon one and the intron of *C. zeae-maydis* CTB7 (nucleotides 225 to 678) (Fig. 2). There was high sequence identity (86%) for the region corresponding to the first 224 bp of *C. zeae-maydis* exon one as well

as for the second CTB7 exon of *C. zeae-maydis* (87% identity; nucleotides 679 to 1,332) (Fig. 2).

The *C. zeina* CTB7 gene region is transcribed during in planta glasshouse trials and exhibits splicing of an intron at a different position from the in silico prediction.

High nucleotide sequence identity between the *C. zeina* CTB7 gene region and the exons of *C. zeae-maydis* CTB7 (Fig. 2), together with the in silico prediction of a putative 322-amino acid *C. zeina* CTB7 polypeptide (Table 2), led us to seek

Fig. 1. *Cercospora zeina* fails to produce cercosporin in vitro, despite carrying a largely intact cercosporin toxin biosynthetic (CTB) gene cluster. **A**, *Cercospora zeae-maydis* SCOH1-5 produces cercosporin when cultured on 0.2× potato dextrose agar (PDA) (visible as the accumulation of a red pigment in the media). Neither the African nor the United States isolates of *C. zeina*, CMW25467 and OYPA, respectively, produce cercosporin when cultured on 0.2× PDA. **B**, The CTB gene cluster of *C. zeina* CMW25467 is intact except for CTB7. The position, orientation, and structure of the predicted genes are indicated by the arrows. Following automated gene predictions, manual annotations were performed, based on multiple sequence alignments of the encoded amino acid sequences with the CTB amino acid sequences of *C. nicotianae* and *C. zeae-maydis*.

Table 2. The *Cercospora zeina* cercosporin toxin biosynthetic (CTB) gene cluster

<i>C. zeina</i> CTB gene ^a	Length (bp) ^b	Intron number	Amino acids	NCBI BLASTx search ^c		Pairwise alignment ^d		
				Best BLASTx match (accession number)	E value	Identity (%)	Similarity (%)	Gaps (%)
CTB1	6,588	9	2,195	Polyketide synthase (<i>Cercospora nicotianae</i>) (AAT69682.1)	0.0	85.0	91.2	0.9
CTB2	1,359	0	452	O-methyltransferase (<i>Cercospora nicotianae</i>) (ABK64180.1)	0.0	85.9	90.4	4.3
CTB3	2,619	2	872	Cercosporin toxin biosynthesis protein (<i>Cercospora nicotianae</i>) (ABC79591.2)	0.0	87.5	93.1	0.2
CTB4	1,539	3	512	Major facilitator superfamily transporter (<i>Cercospora nicotianae</i>) (ABK64181.1)	0.0	89.9	95.9	0.2
CTB5	1,386	0	461	Oxidoreductase (<i>Cercospora nicotianae</i>) (ABK64182.1)	0.0	90.9	95.9	0.6
CTB6	1,065	0	354	Reductase (<i>Cercospora nicotianae</i>) (ABK64183.1)	0.0	86.6	94.7	0.6
CTB7 gene region ^e	969	1	322	Oxidoreductase (<i>Cercospora nicotianae</i>) (ABK64184.1)	1e-120	51.4	55.6	36.6
CTB8	1,278	1	425	Zinc finger transcription factor (<i>Cercospora nicotianae</i>) (ABK64185.1)	8e-151	68.5	77.1	7.7

^a Annotated nucleotide sequence of the *C. zeina* CMW25467 CTB gene cluster is available on GenBank (accession number KY656140).
^b The length of the predicted coding DNA sequence (introns excluded).
^c Predicted *C. zeina* CTB gene sequences used as queries in a National Center for Biotechnology Information (NCBI) BLASTx search against the nonredundant protein sequences (nr) database (E value indicated).
^d The best BLASTx hits and predicted *C. zeina* CTB amino acid sequences were subjected to a pairwise sequence alignment and the identity, similarity, and gap percentages are indicated.
^e In silico annotation of the CTB7 gene region with incorrect intron position, prior to identification of intron position from in planta expression data (discussed later).

further experimental evidence to annotate the *CTB7* gene model. We hypothesized that, although the gene did not appear to be expressed in vitro, it may be expressed in planta.

We conducted a glasshouse inoculation trial of the susceptible maize inbred B73 with *C. zeina* CMW25467 and extracted RNA at 0, 12, 19, 21, and 25 days postinoculation (dpi). Typical GLS lesions had formed by 19 dpi and had progressed to coalesced lesions by 25 dpi on all replicate plants. *C. zeina* fungal load was quantified by quantitative polymerase chain reaction (qPCR) and was shown to increase over the time course, with a significant difference at 21 and 25 dpi compared with the 0-dpi samples (Supplementary Fig. S1).

RNA from all the time points were pooled and were used for reverse transcription (RT)-PCR analysis with the *CTB7*exon primer pair (Table 4), which flank the in silico-predicted intron in the *C. zeina CTB7* gene region (Fig. 2). Sequence analysis revealed the presence of a 112-bp intron with a canonical GT-AG

donor-accepter pair as well as a consensus branch site (CTAAC) (Reid et al. 2014; Rep et al. 2006) situated 8 bp from the acceptor (Fig. 3A). Importantly, the donor site of the true intron was 10 nucleotides downstream of the in silico-predicted intron (Figs. 2 and 3A). The intron was 12 bp larger than the predicted intron in the *C. zeae-maydis CTB7*, but the acceptor site was at the same position (Fig. 2).

A second glasshouse trial was carried out and RNA was extracted from three biological replicates of *C. zeina*-infected B73 maize plants, at 32 dpi, with typical GLS lesions. All three replicates produced the 122-bp RT-PCR product (Fig. 3B, lanes 4 to 6), confirming expression of the *C. zeina CTB7* gene and indicating removal of the *CTB7* intron. However, there was incomplete splicing of some transcripts, since a 234-bp product was also observed in each sample, which corresponds to the size of the gDNA product (Fig. 3B, lanes 4 to 6). RT-PCR analysis of the *C. zeina elongation factor 1α* gene, the primers

Table 3. Expression of the *Cercospora zeina cercosporin toxin biosynthetic (CTB)* genes under in vitro growth conditions and in planta (field)

Gene	Gene expression (counts) ^a									
	In vitro							In planta ^b		
	V8	PDA+AP	PDA pH8	PDA pH3	Corn agar	CM	YPD	Replicate 1	Replicate 2	Replicate 3
<i>CTB1</i>	835	1,645	1,828	639	1,834	1,930	1,104	149	68	76
<i>CTB2</i>	20	103	45	60	84	84	22	2	1	6
<i>CTB3</i>	492	884	849	579	1,115	1,059	1,906	73	49	85
<i>CTB4</i>	86	135	113	66	128	145	140	35	24	14
<i>CTB5</i>	318	895	669	288	979	1,179	903	61	56	57
<i>CTB6</i>	41	346	65	117	192	250	225	96	81	93
<i>CTB7</i>	0	0	0	0	0	0	0	0	0	0
<i>CTB8</i>	241	416	410	259	489	453	453	18	21	55
<i>40S</i>	31,344	9,852	13,666	10,550	10,857	19,034	9,694	11,368	8,881	13,053
<i>EF1α</i>	179,348	54,366	140,673	85,332	84,579	132,714	243,946	4,192	2,835	4,541

^a Number of RNA sequence reads which mapped to the gene of interest. PDA = potato dextrose agar, AP = ammonium phosphate, CM = complete medium, and YPD = yeast extract peptone dextrose.

^b RNA isolated from field grown B73 maize leaves demonstrating gray leaf spot lesions.

Fig. 2. Pairwise alignment of *Cercospora zeina* CMW25467 and *C. zeae-maydis* SCOHI-5 *CTB7* genomic DNA nucleotide sequences. The three potential translation start sites for the *C. zeina CTB7* gene are underlined and in bold. The positions corresponding to the *CTB7del* and *CTB7exon* primer binding sites are indicated by horizontal arrows. Indels present in the *C. zeina* nucleotide sequence are highlighted in gray. The position of the in silico-predicted intron splice site for *C. zeina CTB7* is indicated by a vertical arrow (used to predict the 322-amino acid *CTB7* polypeptide). The *C. zeae-maydis* intron sequence is underlined. The *C. zeina* intron sequence of the transcripts expressed in planta is shown in bold and underlined (as validated by reverse transcription-polymerase chain reaction and sequencing using the *CTB7exon* primer pair).

of which flank an intron (Table 4), produced only the smaller 99-bp spliced product, indicating that there was no gDNA contamination in the samples (Fig. 3C, lanes 4 to 6).

Additional evidence for expression of *CTB7* in the glasshouse trials was obtained by RT-qPCR, using the *CTB7* primer pair (Table 4) designed to the *C. zeina CTB7* region, with high identity to the C-terminal region of *C. zeae-maydis CTB7*. *C. zeina CTB7* expression could be quantified at all time points from 0 to 25 dpi, although expression was low and there were no significant differences between the time points (Fig. 4C). Similarly, three other *C. zeina CTB* genes (*CTB1*, *CTB2*, and *CTB8*) were analyzed as controls and showed a trend of increased expression over time, although there were no significant differences to the 0-dpi samples (Fig. 4A, B, and D). Amplification of specific products for the *CTB* and normalization control genes was verified by sequencing the RT-qPCR products and melt-curve analysis (Supplementary Fig. S2). This result for *CTB7* was corroborated by RT-PCR analysis of the B73-GLS samples from the second glasshouse trial with the same primers, to show the expected product of 98 bp in all three replicates (Supplementary Fig. S3).

RNA-seq analysis of a field infection of maize B73 with natural isolates of *C. zeina* at a GLS hotspot in South Africa (Greytown, KwaZulu-Natal) revealed the in planta expression of all *CTB* genes except *CTB7* in all three replicate plants (Table 3). The field leaf samples were characterized by individual GLS lesions covering 8% of the leaf surface area, on average, and therefore, had not yet coalesced (Christie et al. 2017), in contrast to the glasshouse samples that were inoculated at high conidial density, resulting in lesions that were coalesced by 21 to 25 dpi.

We suggest that the field samples were at an earlier stage of GLS disease development or the isolates were less aggressive, thus, *CTB7* expression was below the detection threshold. Additionally, for these samples, RT-qPCR (glasshouse samples) may have been more sensitive than RNA-seq (field samples), considering the amount of fungal RNA compared with maize RNA in the field samples with lower fungal load.

The *C. zeina CTB7* gene region does not encode a full-length oxidoreductase protein based on the intron position.

Identification of the intron position in the *C. zeina CTB7* gene region from the in planta transcripts showed that the in silico-predicted intron position was incorrect, and thus, the putative 322-amino acid *CTB7* polypeptide shown in Table 2 was invalid. The correct intron position was used to predict the open reading frames (ORFs) across the gene. None of the three possible ORFs encode a complete *CTB7* protein corresponding to the full-length *CTB7* from *C. zeae-maydis* (Fig. 5A). ORF 1 encodes a 151-amino acid polypeptide with no similarity to the *CTB7* orthologs (Fig. 5A). ORF 2 encodes a 105-amino acid polypeptide that is highly similar to the N-terminus of the *C. nicotianae* (62.9% identity) and *C. zeae-maydis* (59.0% identity) *CTB7* orthologs but ends in a stop codon before the intron (Fig. 5B). ORF 3 encodes a 257-amino acid protein—the first 39 amino acids before the intron show no similarity to the *C. nicotianae* and *C. zeae-maydis CTB7* orthologs. The remaining 218 amino acids of ORF 3 are highly similar to the C-terminal half of the *C. nicotianae* (78.9% identity) and *C. zeae-maydis* (92.6% identity) *CTB7* orthologs and

Table 4. Primer sequences used in this study

Primer name	Sequence (5'–3')	Gene target	Amplicon size (bp)
<i>CTB7</i> gDNA structure analysis			
CTB7del F	AAGAGTGCTTGTGAATGG	<i>Cercospora CTB7</i>	618 (<i>Cercospora zeina</i>)
CTB7del R	GATGCGGGTGAAGTAGAAA	<i>Cercospora CTB7</i>	925 (<i>C. zeae-maydis</i>)
Reverse transcription-polymerase chain reaction (RT-PCR) primers			
CTB7exon F	AAGATGGGCTTGGAGCAG	<i>C. zeina CTB7</i>	234 (gDNA)
CTB7exon R	TGGCGTGTGGAGCTTTC	<i>C. zeina CTB7</i>	122 (cDNA)
CzmCTB7 F	GATTGAGATCATGAGGCGAGAC	<i>C. zeae-maydis CTB7</i>	100
CzmCTB7 R	AAGTTCGTCAACGGTACATCC	<i>C. zeae-maydis CTB7</i>	100
EF1 α F	GTGCTCGACAAGCTGAA	<i>C. zeina elongation factor 1α</i>	154 (gDNA)
EF1 α R	GTGATGACGGTGACATAG	<i>C. zeina elongation factor 1α</i>	99 (cDNA)
Quantitative RT-PCR primers			
CTB1 F	GCCTCCAGATGGCATTAT	<i>C. zeina CTB1</i>	96
CTB1 R	CGTAGAGTGCAGCGTATT	<i>C. zeina CTB1</i>	96
CTB2 F	CCTAGACCAGAAAGGACTTC	<i>C. zeina CTB2</i>	97
CTB2 R	GCTTCTCGCATCGTAGTA	<i>C. zeina CTB2</i>	97
CTB7 F	GCCTCGCCTCATATCAT	<i>C. zeina CTB7</i>	98
CTB7 R	GACTGTGGTAACGCTAACT	<i>C. zeina CTB7</i>	98
CTB8 F	TGTCATCGTGTCAAGTCATC	<i>C. zeina CTB8</i>	95
CTB8 R	AATCTTGGCCTCGACATC	<i>C. zeina CTB8</i>	95
40S F	GGTCTCAAGGTCATTCTC	<i>C. zeina 40S ribosomal protein</i>	102
40S R	TTGACACCCTTTCCAGTC	<i>C. zeina 40S ribosomal protein</i>	102
Cyt III F	GAGCTCTTTATGGTGCTCTA	<i>C. zeina cytochrome c oxidase III</i>	108
Cyt III R	CGTAGGCTCCATCTGATAAA	<i>C. zeina cytochrome c oxidase III</i>	108
Plasmid construction (restriction enzyme sites appear in bold) ^a			
MCS HYG_XhoI F	GGGCTCGAGACTGGTACCGCGG GCCCTAGGCCTTACGTAGAGCTCCACC GCGGTGGCG	Hygromycin-resistance cassette	2,008
HYG_BstEII R	GGCGGTGACCGGGGA TCCACTAGTTCTAGAGCGGCC	Hygromycin-resistance cassette	2,008
GFP_BamHI F	GGCGGATCCGAGAGCTTATACCGAGCTCCC	Green fluorescent protein (GFP) expression cassette	1,814
MCS GFP_BstEII R	GGCGGTGACCTCAAGCTT GCTTAATTAATCCCGGGTTACTT GTACAGCTCGTCCATGCC	GFP expression cassette	1,814
XbaI-CTB7-F	ATATATCTAGAGGTACAGTAGCTCACCACGT	<i>C. zeae-maydis CTB7</i>	3,409
BamHI-CTB7-RC	TATATGGATCCCTGATTGAGAGTAAGCCGC	<i>C. zeae-maydis CTB7</i>	3,409

^a MCS = multiple cloning site, GFP = green fluorescent protein.

include the predicted amidation and FMN/FAD-binding motifs (Fig. 5B). However, the *C. zeina* ORF 3 lacks approximately 220 amino acids at the N-terminus (containing a second FMN/FAD-binding motif) that are present in the CTB7 proteins from the other *Cercospora* species (Fig. 5B). Currently, it is not known if any of these potential CTB7 ORFs are translated into active proteins in *C. zeina*. *C. zea-maydis* CTB7 has a 40–amino acid deletion compared with the *C. nicotianae* CTB7 (Fig. 5B) but, in contrast to *C. zeina*, *C. zea-maydis* still maintains the ability to produce cercosporin (Fig. 1A).

The deletion in the gDNA of the *C. zeina* CTB7 gene region compared with *C. zea-maydis* is conserved in *C. zeina* isolates from Africa and the United States.

The sequence conservation between the *C. zeina* CTB7 gene region and the nucleotides corresponding to the N- and C-termini of *C. zea-maydis* CTB7 was exploited to design a diagnostic PCR assay. The CTB7del primer pair, which flanks the region of deletions and the intron in *C. zeina*, produced 618- and 925-bp amplicons from gDNA of *C. zeina* and *C. zea-maydis*, respectively (Fig. 6A and B). A suite of *C. zeina* isolates from both Africa

(Uganda and Zambia) and the United States (Pennsylvania, Ohio, New York) were screened and all were found to carry the smaller CTB7 amplicon predicted to occur in *C. zeina* CMW25467 (Fig. 6C). The species identity of these isolates was confirmed to be *C. zeina*, using a histone gene diagnostic PCR (Supplementary Fig. S4) (Crous et al. 2006). Sequence analysis of the CTB7 gene region from a United States isolate (OYPA, USPA-4) showed it to be identical to the Zambian isolate CMW25467 and a selection of seven additional isolates from Zambia, Kenya, and South Africa (Supplementary File S2). There was a single nucleotide polymorphism in an isolate from Uganda.

Cercosporin production in *C. zeina* CzmCTB7 transformants.

C. zeina was complemented with the full-length *C. zea-maydis* CTB7 gene using *A. tumefaciens*-mediated transformation (Supplementary Fig. S5). The presence of the *C. zea-maydis* CTB7 gene was confirmed in four transformants, using the CTB7 diagnostic PCR, which showed amplicons for both copies of the CTB7 gene (618 bp from *C. zeina* and the 925 bp from

Fig. 3. The *Cercospora zeina* CTB7 gene region is expressed and an intron is spliced in planta. **A**, Alignment of *C. zeina* CTB7 gDNA and cDNA sequences amplified with CTB7exon primer pair indicates CTB7 expression in *C. zeina*-inoculated B73 maize during glasshouse trial 1 and removal of the 112-bp intron. The consensus GT-AG donor-acceptor and CTAAC branch sites are underlined in the cDNA. The position of the incorrect in silico-predicted intron donor site (used to predict the incorrect 322-amino acid CTB7 polypeptide) is indicated by a vertical arrow and occurs 10 nucleotides upstream of the correct site in *C. zeina* CTB7. **B**, Confirmation of *C. zeina* CTB7 expression and intron splicing in *C. zeina*-inoculated B73 maize during glasshouse trial 2. The CTB7 reverse transcription-polymerase chain reaction (RT-PCR) (CTB7exon primers) generated two products in each inoculated maize replicate (lanes 4 to 6), one corresponding to the expected cDNA amplicon with intron removed (122 bp) and a larger amplicon (234 bp) indicating the presence of nonspliced CTB7 transcripts. Nontemplate/water controls were included in lane 2 and a *C. zeina* gDNA positive control in lane 3. RT-PCR products were separated on a 2% agarose gel stained with ethidium bromide. A size standard (FastRuler low range DNA ladder [ThermoFisher Scientific]) is shown in lane 1. **C**, The *EF1α* RT-PCR (*EF1α* primers) demonstrated no gDNA contamination in the three replicates (lanes 4 to 6). Electrophoresis conditions and equivalent controls were as described for B.

C. zeae-maydis) (Fig. 7A). RT-PCR analysis showed that these transformants also expressed the *C. zeae-maydis* *CTB7* gene when cultured on 0.2× PDA under constant light (Fig. 7B). Furthermore, transformant 3 was found to accumulate a red pigment comparable to an isolate of *C. kikuchii* (Fig. 8), a species known to produce cercosporin (Kuyama and Tamura 1957). As expected, none of the isolates produced the red pigment when grown on 0.2× PDA plus 10 mM ammonium phosphate (Fig. 8), conditions known to suppress cercosporin production (You et al. 2008). Both the KOH assay and thin-layer chromatography (TLC) indicated cercosporin production in transformant 3 (Fig. 9). These results were confirmed by ultra performance liquid chromatography-quadrupole-time-of-flight mass spectrometry (UPLC-QTOF-MS) (Fig. 10).

The high resolution mass spectra (HRMS) for the cercosporin standard and the extract from transformant 3 are shown in Figure 10. Both pure cercosporin and transformant 3 exhibited a peak at a retention time of 6.30 min on the UPLC profile (Fig. 10A and B). The HRMS-electrospray ionization/atmospheric pressure chemical ionization-TOF (m/z) (MH^+) previously calculated for cercosporin ($C_{29}H_{27}O_{10}$) was 535.1604 (Newman and Townsend 2016). We observed an accurate mass of 535.1606 for the cercosporin standard and 535.1605 for transformant 3 (Fig. 10C and D). The values of HRMS main fragments as shown on the MS/MS profiles for cercosporin and transformant 3 (Fig. 10E and F) and *C. kikuchii* (Supplementary Fig. S6C) are provided in Supplementary Table S1. These data are in accordance with that previously published for cercosporin (Yamazaki and Ogawa 1972). Cercosporin was absent from the UPLC profile of the *C. zeina* wild-type extract.

All four transformants retained pathogenicity when reinoculated onto maize plants; however, the presence of a functional *C. zeae-maydis* *CTB7* gene in transformant 3 did not appear to increase pathogenicity under the conditions tested. Koch's postulates were fulfilled for all four reisolated transformants by intergenic transcribed spacer sequencing and PCR of both *CTB7* gene copies (Supplementary File S3).

DISCUSSION

The main finding of this study was that the lack of cercosporin production in isolates of *C. zeina* was due to a non-functional copy of the *CTB7* gene. *CTB7* is predicted to encode a flavin-dependent oxidoreductase that, in *C. nicotianae*, has been shown in two independent studies to be essential for cercosporin biosynthesis (Chen et al. 2007b; Newman and Townsend 2016). The exact role of *CTB7* in the pathway has, however, not yet been proven biochemically.

Several characteristics of the *C. zeina* *CTB7* gene, including the presence of multiple deletions in the gene sequence as compared with its orthologs, fit the definition of a pseudogene. Pseudogenes typically demonstrate evidence of coding sequence degradation (Lafontaine and Dujon 2010), which we observed for *C. zeina* *CTB7*, namely the presence of an in-frame stop codon (*CTB7* ORF 2) (Fig. 2) and truncation of the ORF (*CTB7* ORF 2 and ORF 3 demonstrate a 75 and 37% truncation, respectively, relative to *C. zeae-maydis* *CTB7*). Lack of gene expression has previously been cited as possible evidence for the description of a pseudogene (Gaur et al. 2008); however, in rare cases, pseudogene expression has been reported (Lafontaine and Dujon 2010; Zhang and Gerstein 2004). A study in yeast showed

Fig. 4. *Cercospora zeina* *CTB7* and three other *CTB* genes (*CTB1*, *CTB2*, and *CTB8*) were shown to be expressed in B73 maize inoculated with *C. zeina* CMW25467 during glasshouse trial 1. Reverse transcription quantitative polymerase chain reaction was carried out on RNA extracted from maize at different time points after inoculation and was used to calculate mean calibrated normalized relative quantity (CNRQ) values for **A**, *CTB1*, **B**, *CTB2*, **C**, *CTB7*, and **D**, *CTB8*. The relative expression values were normalized against the stably expressed reference genes *40S* and *Cyt III*. Standard error bars are included on the graphs. Statistical analysis was done using one-way analysis of variance with a Tukey's multiple comparison test. None of the *CTB* genes studied showed a statistically significant ($\alpha = 0.05$) difference in expression compared with 0 days postinoculation (dpi). Only two biological replicates were included in the analysis for 0 dpi.

evidence that 12 of 77 pseudogenes were expressed (Lafontaine and Dujon 2010). *C. zeina* CTB7 was expressed at low levels in planta (Fig. 4C), although not all transcripts were spliced (Fig. 3B). All the other intact CTB genes were expressed both in vitro and in planta (Table 3).

The predicted ORFs for *C. zeina* CTB7 all lack some of the functional groups described in the *C. nicotianae* ortholog and are, thus, unlikely to have full CTB7 activity (Fig. 5). Proteomic analysis of GLS lesions may reveal which, if any, of the *C. zeina* CTB7 ORFs are translated; however, based on the observed low expression level of CTB7 and expected relative high abundance of maize proteins, its presence may be below the detection limit.

If CTB7 represented a nonfunctional gene undergoing pseudogenization, we would expect to observe the accumulation of mutations among different isolates of *C. zeina*. However, sequencing of the CTB7 diagnostic PCR products from the United States isolate OYPA and eight geographically and chronologically separated African *C. zeina* isolates demonstrated remarkable sequence identity, with only one nucleotide difference in a Ugandan isolate. An explanation for this could be a recent geographical separation between *C. zeina* isolates, although this conclusion would require a comprehensive population genetics study.

The accumulated evidence that *C. zeina* CTB7 may be a pseudogene led us to hypothesize that this may explain the lack of cercosporin production in vitro. In a recent study, the metabolite profile of a *C. nicotianae* *ctb7* knockout demonstrated a lack of cercosporin and, furthermore, yielded no major compound (Newman and Townsend 2016), similar to what we observed for the wild-type *C. zeina* UPLC profile. *Cercospora nicotianae* CTB7, a flavin-dependent oxidoreductase, is, thus, essential for cercosporin production, and it has been proposed to be involved in the formation of the dioxepine ring following the dimerization of the two naphthalene moieties (Newman and Townsend 2016).

Considering the importance of CTB7 in other *Cercospora* species and to test our hypothesis that CTB7 is the bottleneck in cercosporin production in *C. zeina*, we set out to complement it with a functional CTB7 from the cercosporin-producing species *C. zeae-maydis* (Bluhm et al. 2008). *A. tumefaciens*-mediated transformation has recently been applied in gene knockout and complementation studies in several Dothideomycetes species, including the northern corn leaf blight pathogen *Setosphaeria turcica* (Xue et al. 2013), the tomato pathogen *Pyrenochaeta lycopersici* (Aragona and Valente 2015), as well as *C. zeae-maydis* (Lu et al. 2017), and thus, it was decided to utilize this approach to complement the defective CTB7 gene in *C. zeina*. We successfully generated four transformants that

Fig. 5. The *Cercospora zeina* CTB7 gene region encodes three potential open reading frames (ORFs), based on the intron position, two of which show similarity to parts of CTB7 in other *Cercospora* spp. **A**, *C. zeina* CTB7 gene region ORF1 encodes a 151-amino acid polypeptide with no sequence similarity to CTB7 orthologs. ORF2 encodes a 105-amino acid polypeptide and shows high similarity to the N-termini of the *C. nicotianae* and *C. zeae-maydis* CTB7 orthologs. ORF3 encodes a 257-amino acid polypeptide, with the last 218 amino acids showing high similarity to the C-terminal region of the *C. nicotianae* and *C. zeae-maydis* CTB7 orthologs. **B**, Alignment of the *C. nicotianae* CTB7, *C. zeae-maydis* CTB7, and potential *C. zeina* ORF2 and ORF3 CTB7 polypeptides. The intron position is indicated by the vertical black line, with the protein motifs described for *C. nicotianae* CTB7 underlined. The gray highlighted region represents a deletion present in *C. zeae-maydis* CTB7 compared with the *C. nicotianae* CTB7.

were shown to carry both copies of the *CTB7* gene (Fig. 7A) and could confirm cercosporin production in one of the transformants (Figs. 8 to 10). The production of cercosporin by the *C. zeina* *CzmCTB7* transformant strongly suggests that the *C. zeina* *CTB7* gene represents a bottleneck in the biosynthesis pathway.

Chemical assays routinely used to study cercosporin production in vitro are largely dependent on the characteristic red color of the cercosporin molecule that is linked to its highly conjugated structure (Kuyama and Tamura 1957; Yamazaki and Ogawa 1972). Our own analyses demonstrated a lack of red metabolite accumulation in the wild-type *C. zeina* but confirmed cercosporin production in the *C. zeina* *CzmCTB7* transformant with the KOH assay, TLC, and UPLC-QTOF-MS (Figs. 8 to 10).

C. zeina is a successful pathogen of maize causing GLS (Meisel et al. 2009; Muller et al. 2016; Wang et al. 1998), despite deletions in the *CTB7* gene observed in a range of diverse isolates in this study (Fig. 6). *CTB7* has been shown to be important for pathogenicity in at least *C. nicotianae*, since *ctb7* knockout mutants show reduced pathogenicity and lack of cercosporin production (Chen et al. 2007a). Mutant studies in several *Cercospora* spp. have shown a congruence between lack of cercosporin production and reduced pathogenicity (Chen et al. 2007a and b; Choquer et al. 2005, 2007; Dekkers et al. 2007; Shim and Dunkle 2003; Staerkel et al. 2013).

The current study has focused on understanding the lack of cercosporin production in vitro by *C. zeina*, and thus, future work will focus on the role of the CTB pathway, if any, in the pathogenicity of this fungus. However, one hypothesis for the

Fig. 6. Screening of *Cercospora zeina* isolates for the presence of *CTB7* genomic DNA deletions. **A**, Predicted *CTB7* gene structures for *C. zeina* and *C. zeae-maydis*, with the position of the CTB7del_F and CTB7del_R primer indicated. The *C. zeae-maydis* *CTB7* gene is indicated with a gray rectangle (exon 1) and a gray arrow (exon 2). The numbers indicate base pair positions from the first nucleotide of the ATG's of the *C. zeae-maydis* *CTB7* and the *C. zeina* ORF1. The *C. zeina* in planta intron is indicated (nucleotides 355 to 467). Regions of greater than 85% nucleotide identity are shaded. **B**, Polymerase chain reaction (PCR) amplification from *C. zeina* and *C. zeae-maydis* isolates with CTB7del_F and CTB7del_R primers. PCR products were separated on a 1.5% agarose gel stained with ethidium bromide. A size standard (GeneRuler 100-bp DNA ladder [ThermoFisher Scientific]) is shown in lane 1. A nontemplate/water control was included in lane 2. *C. zeae-maydis* (CBS 117757 in lane 3 and CBS 115561 in lane 4) demonstrate the larger 925-bp amplicon, while the *C. zeina* isolates (CMW25467 in lane 5 and isolate 2011.GT30 in lane 6) yielded a 618-bp amplicon. **C**, PCR amplification with the same primers was performed on several *C. zeina* isolates from Africa and the United States. PCR products were separated on a 1% agarose gel stained with Gel Red. A size standard (1-kb Plus DNA ladder) is shown in lanes 1 and 22. A nontemplate/water control was included in lane 23. *C. zeae-maydis* SCOH1-5 (lanes 2 and 24) demonstrate the larger 925-bp amplicon, while the *C. zeina* isolates (lanes 3 to 21) yielded a 618-bp amplicon.

success of *C. zeina* despite a nonfunctional CTB7 is that an alternative metabolite is being produced in planta, which maintains a similar function to cercosporin but is not readily detectable visually in vitro.

An alternative hypothesis is that a paralog of *C. zeina* CTB7 is capable of replacing the function of CTB7, leading to cercosporin production in planta. However, BLAST searches of the *C. zeina* genome with its CTB7 gene region failed to reveal a paralog (data not shown), although this does not preclude the possibility of a dissimilar gene encoding a protein with the same function.

Finally, the use of membrane transporter genes associated with cercosporin autoresistance have been highlighted as candidates for engineering resistance to *Cercospora* species that have been demonstrated to produce cercosporin during host plant infection (Beseli et al. 2015). The generation of maize

lines carrying one or more of these transporter genes in order to combat GLS in regions where *C. zeina* is predominant may, however, not be advisable if *C. zeina* isolates are incapable of producing cercosporin.

MATERIALS AND METHODS

All chemicals were purchased from Merck SA, unless otherwise stated.

Biological material and fungal growth conditions.

CMW25467, the wild-type isolate of *Cercospora zeina*, was cultured on V8 agar medium at 25°C in constant darkness to promote conidiation (Meisel et al. 2009). Conidiating cultures were maintained by subculturing on V8 agar. A South African isolate of *C. kikuchii* was isolated from soybean (Table 1). A

Fig. 7. *Cercospora zeina* transformants positive for the presence and expression of the *C. zeae-maydis* CTB7 gene. **A**, Transformants carrying both *Cercospora* species CTB7 gene copies were detected by polymerase chain reaction (PCR) amplification with the CTB7del primers. PCR products were separated on a 1% agarose gel stained with ethidium bromide (EtBr). A size standard (GeneRuler 100-bp DNA ladder [ThermoFisher Scientific]) is shown in lanes 1 and 11. A nontemplate/water control was included in lane 2. Positive controls for both *C. zeae-maydis* (925-bp amplicon, lane 3) and *C. zeina* (618-bp amplicon, lane 4) were included. Four of the transformants were shown to carry both copies of the CTB7 gene (lanes 6 to 9), while two were shown to only carry the smaller *C. zeina* CTB7 gene copy (lanes 5 and 10). **B**, Reverse transcription (RT)-PCR analysis of the transformants grown on 0.2x potato dextrose agar in constant light to determine expression of the *C. zeae-maydis* CTB7 gene (CzmCTB7 primers). RT-PCR products were separated on 2% agarose gels stained with EtBr. A size standard (GeneRuler 100-bp DNA ladder) is shown in lane 1 and a nontemplate/water control is included in lane 2. Lane 3 contained cDNA from *C. zeina* CMW25467, with lanes 4 to 7 containing cDNA from transformants 2 to 5, respectively. The CzmCTB7 100-bp RT-PCR product was detected in all four of the transformants. The reference gene *EF1α* was also included to assess the presence of gDNA contamination.

Fig. 8. Visual assessment of red pigment cercosporin production by *Cercospora zeina* CTB7 transformants complemented with the *C. zeae-maydis* CTB7 gene. Cultures were grown in constant light on cercosporin-conductive conditions (0.2x potato dextrose agar [PDA], top panel) as well as cercosporin-suppressive conditions (0.2x PDA supplemented with 10 mM ammonium phosphate, bottom panel). The positive control *C. kikuchii* culture and transformant 3 produced the red pigment cercosporin on 0.2x PDA, but the *C. zeina* wild-type culture and remaining transformants failed to do so.

vegetative culture was maintained on 0.2× PDA (3 g PDA, 12 g agar, and approximately 1,000 ml of distilled water) and was used as a positive control for the cercosporin chemical analyses. Additional *C. zeina* and *C. zeae-maydis* isolates analyzed in this study are listed in Table 1.

Wild-type *C. zeina* was grown under seven separate in vitro growth conditions to generate material for RNA isolation and in vitro transcriptome sequencing. The seven growth conditions were: i) V8 agar, ii) 0.2× PDA supplemented with 10 mM ammonium phosphate, iii) PDA, pH8 [pH adjusted with $\text{Na}_2\text{CO}_3 + \text{NaHCO}_3$], iv) PDA, pH3 [pH adjusted with citric acid + Na_2HPO_4], v) cornmeal agar, vi) complete medium (10 g of glucose, 1 g of yeast extract, 1 g of casein hydrolysate, 1 g of $\text{Ca}[\text{NO}_3]_2 \cdot 4\text{H}_2\text{O}$, 10 ml of buffer solution [2 g of KH_2PO_4 , 2.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.5 g of NaCl, and approximately 100 ml of distilled water] and approximately 1,000 ml of distilled water), and vii) YPD (0.5 g of peptone, 0.5 g of yeast extract, 5 g of glucose, 18 g of NaCl, and approximately 1,000 ml distilled water). For growth conditions of the first group, the cultures were kept in constant darkness at ambient room temperature for 3 days. For growth conditions for groups ii through vii, the cultures were kept in constant light at 25°C for 7 days.

Fig. 9. Evaluation of cercosporin production by *Cercospora zeina* transformants with the *C. zeae-maydis* CTB7 gene. **A**, Cercosporin production was quantified using the KOH assay. Both *C. kikuchii* and transformant 3 demonstrated significantly higher cercosporin concentrations than the wild-type *C. zeina* (one-way analysis of variance with a Tukey's multiple comparison test). The asterisks (*) indicate concentrations significantly higher than the wild-type *C. zeina* concentrations at $P < 0.05$. **B**, Thin-layer chromatography analysis of *C. zeina* transformant extracts, prepared using ethyl acetate. Compounds were separated using the ethyl acetate/hexane/methanol/ H_2O (6:4:1.5:1, vol/vol) solvent system. A pure cercosporin standard (lane 1) was visible as a red pigment at retention factor 0.45 (arrow). Cercosporin was present in the extract of *C. kikuchii* (lane 2) and transformant 3 (lane 5), but could not be detected in the *C. zeina* wild type (lane 3) and remaining transformants (lane 4, 6, and 7).

Maize inoculations with *C. zeina*.

The first glasshouse inoculation trial was conducted to generate GLS-infected B73 material for RT-qPCR following the methods described by Christie et al. (2017). *C. zeina* conidia were collected from V8 agar cultures and were used to artificially inoculate maize plants at the V12 growth stage, using the brush method (Meisel et al. 2009). The conidial suspension had a concentration of 1×10^6 conidia per milliliter. Inoculated leaf material (two leaves per plant) was harvested at five separate time points from three biological replicates, at 0 dpi, directly following inoculation, at 12 dpi, prior to lesion development, at 19 dpi, when rectangular GLS lesions were visible, at 21 dpi, when the GLS lesions started to coalesce, and at 25 dpi, when the leaves were blighted. The leaf material was flash-frozen in liquid nitrogen and was stored at -80°C prior to DNA and RNA isolation. Subsequently, a second glasshouse trial was conducted in the same manner as described, with inoculated leaf material harvested at 32 dpi when rectangular GLS lesions were visible.

RNA isolation and quality assessment.

For transcriptome sequencing, RNA was isolated from in vitro-grown *C. zeina* cultures, using QIAzol lysis reagent (Qiagen), per the manufacturer's specifications. On-column DNase treatment and RNA purification was performed with the RNeasy mini kit (Qiagen). RNA quality was assessed using the Experion RNA StdSens system (Bio-Rad).

For RT-PCR, biological material was ground in liquid nitrogen and 100 mg was used for RNA isolation with the RNeasy plant mini RNA extraction kit and the RNase-free DNase set for on-column DNA digestion (Qiagen), per the manufacturer's specifications. The High capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, U.S.A.) was used for cDNA synthesis, per the manufacturer's specifications.

Transcriptome sequencing and expression analysis of *C. zeina* CTB genes.

Total RNA samples were submitted to BGI Tech Solutions Co., Ltd. (Beijing Genome Institute) for library construction and sequencing. RNA sequencing of 200-bp short-insert libraries was performed on an Illumina HiSeq 2000 platform (Illumina Inc.), with a 100-bp paired-end module for the seven in vitro *C. zeina* libraries.

Data filtering was done by BGI, which included the removal of adapters, low-quality reads, and reads with more than 5% unknown nucleotides. Read quality was evaluated, using FastQC, and 13 bases were removed from the beginning of each read in the sequencing files with fastx_trimmer from the FASTX Toolkit. Transcripts were assembled using Trinity (Grabherr et al. 2011) and were mapped to the *C. zeina* CMW25467 draft genome (Muller et al. 2016) with TopHat2 (Kim et al. 2013), using the default parameters. The standard deviation for the distribution on inner distances between read pairs was set at 200. The TopHat2 BAM output files were converted to the SAM file format using SAMtools (Li et al. 2009) and read coverage was counted with the htseq-count package (Anders et al. 2015), using the default parameters. Mapped reads were visualized using the Genome View tool (Abeel et al. 2012) and were used to validate the *C. zeina* CTB gene annotations. *C. zeina* in vitro RNA-seq data have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus database (accession number GSE90705).

Maize field infection with *C. zeina* and RNA-seq analysis of CTB genes.

Plants of maize inbred line B73 were subjected to natural infection with *C. zeina* at the Hildesheim Research Station,

PANNAR SEED (Pty) Ltd., Greytown, KwaZulu-Natal. The material for RNA-seq analysis was the same as described by Christie et al. (2017), namely GLS-diseased lower leaves from three biological replicate plants at tasseling stage of development. RNA-seq analysis was conducted as described by Christie et al. (2017), except that the reads were simultaneously mapped to both the maize B73 genome sequence (v5b.60) (Schnable et al. 2009) and the *C. zeina* CMW25467 draft genome (Muller et al. 2016). Reads that mapped to the fungal genome were extracted and were used for read counting in the current study. In planta RNA-seq data have been deposited in the NCBI Gene Expression Omnibus database (accession number: GSE94442).

CTB gene annotation and expression analysis.

The nucleotide sequences of the *Cercospora nicotianae* CTB genes (Chen et al. 2007b) were retrieved from NCBI GenBank and were used to perform a BLASTn search against the draft genome assembly of *C. zeina* (Muller et al. 2016). The relevant contig was subjected to gene prediction using the AUGUSTUS (Stanke and Morgenstern 2005), FGENESH (Solovyev et al. 2006), and SNAP (Korf 2004) web-based gene prediction tools. The gene predictions were manually assessed and annotated using the GenomeView genome browser (Abeel et al. 2012). Manual annotation of the predicted *C. zeina* CTB genes was done based on amino acid sequence

alignments. The *C. nicotianae* CTB amino acid sequences were retrieved from GenBank and were used as query sequences in a BLASTp analysis against the *C. zeae-maydis* filtered model protein database (available from the Joint Genome Institute database) to identify predicted *C. zeae-maydis* CTB amino acid sequences. Pairwise protein sequences alignments were performed using EMBOSS Needle and multiple sequence alignments using MUSCLE 3.8. The annotated *C. zeina* CTB gene cluster has been deposited in GenBank with the accession number KY656140.

DNA isolation and PCR analysis.

Small-scale fungal genomic DNA isolations were performed using a modified version of the CTAB method (Meisel et al. 2009). The ZR fungal/bacterial DNA mini isolation kit (Zymo Research, Irvine, CA, U.S.A.) was used, according to the manufacturer's specifications, for DNA isolation of the *C. zeina* CzmCTB7 transformants. The CTB7del PCR was set up in a total volume of 12.5 µl, which consisted of: 1× KAPA2G Robust HotStart ReadyMix (Kapa Biosystems, Wilmington, MA, U.S.A.), 0.5 µm of each primer, 0.6 µl of dimethyl sulfoxide (DMSO), 25 ng of DNA, and sterile distilled water. The cycling conditions were as follows: 3 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 60°C, and 1 min at 72°C, with a final extension step of 72°C for 10 min.

Fig. 10. Mass spectrometry confirms the presence of cercosporin in the extract from *Cercospora zeina* transformant 3 complemented with the *C. zeae-maydis* CTB7 gene. Samples were processed by ultra performance liquid chromatography-quadrupole-time-of-flight (TOF) mass spectrometry (MS), using a Synapt G2 high-definition MS system (Waters Inc.). The extracted ion chromatogram for m/z 535.1604 (the $[MH]^+$ calculated for cercosporin, $C_{29}H_{27}O_{10}$) showed a peak at 6.30 min for both **A**, the cercosporin standard and **B**, the transformant 3 extract. High resolution mass spectra-electrospray ionization/atmospheric pressure chemical ionization-TOF of these peaks demonstrated an accurate mass-to-charge ratio (m/z) of **C**, 535.1606 for the cercosporin standard and **D**, 535.1605 for the transformant 3 extract, which is consistent with data previously described. The MS/MS spectrum of both **E**, the cercosporin standard peak and **F**, the transformant 3 extract peak demonstrated all of the main fragments previously described for cercosporin.

RT-PCR analysis.

RT-PCR reactions were set up in a total volume of 12.5 µl, which consisted of: 1× KAPA2G Robust HotStart ReadyMix, 0.5 µm of each primer, 0.6 µl DMSO, 1 µl of the cDNA, and sterile distilled water. The cycling conditions were as follows: 3 min at 95°C followed by 30 cycles of 15 s at 95°C, 15 s at 58°C, and 30 s at 72°C, and a final extension step of 72°C for 10 min, with the following exceptions: 35 cycles for the CTB7 exon RT-PCR and an annealing temperature of 62°C for the *EF1α* RT-PCR.

The CTB7exon RT-PCR amplicon was cloned and sequenced using the InsTAclone PCR cloning kit (Thermo Fisher Scientific, Waltham, MA, U.S.A.), as per the manufacturer's specifications. Sequencing reactions were set up using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems), according to the manufacturer's guidelines, and were submitted to the DNA Sequencing Facility of the Natural and Agricultural Sciences Faculty at the University of Pretoria.

Fungal quantification of inoculated maize leaves.

DNA isolated from inoculated maize leaf material was used to quantify the in planta fungal load by means of a real-time PCR method as previously described (Korsman et al. 2010).

RT-qPCR analysis.

RT-qPCR analysis of the *C. zeina* CTB genes (*CTB1*, *CTB2*, *CTB7*, and *CTB8*) was done according to the MIQE guidelines (Bustin et al. 2009), using the Bio-Rad CFX96 touch real-time PCR detection system. Primers were designed using the PrimerQuest Tool (Table 4). Expression was measured in three biological replicates. Reactions were set up in 10-µl volumes, which consisted of 5 µl of Lightcycler 480 SYBR green I master mix (Roche Diagnostics, Basel, Switzerland), 0.5 µM of each of the primers, 1 µl of cDNA template, and sterile distilled water. The cycling conditions used were: 95°C for 10 min, 45 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 10 s. Fluorescence was measured at the end of each elongation step and melt curve analysis was performed. Samples were normalized to the 40S ribosomal protein (*40S*) and cytochrome c oxidase subunit III (*Cyt III*) reference genes. The expression stability of the reference genes were assessed using geNorm (Hellemans et al. 2007; Vandesompele et al. 2002). Relative quantification and normalization was performed using qBASE^{PLUS} v1.0 (Hellemans et al. 2007). Statistical analyses were performed using GraphPad Prism v5.04 (GraphPad Software, Inc., La Jolla, CA, U.S.A.).

Heterologous expression of CTB7

from *C. zea*-*maydis* in *C. zeina*.

Plasmid construction. A 1.8-kb hygromycin-resistance cassette was PCR-amplified from pSilent1 (Nakayashiki et al. 2005), using the primer MCS HYG_*XhoI* F and HYG_*BstEII* R, and was cloned into *XhoI* and *BstEII* restriction sites in the binary vector pCambia-2301 (Cambia, Canberra, Australia). Similarly, a 1.8-kb green fluorescent protein (GFP) expression cassette was PCR-amplified from pBR0073 (Ridenour et al. 2014), using the primer pair GFP_*BamHI* F and MCS GFP_*BstEII* R, and was cloned into *BamHI* and *BstEII* restriction sites in pCambia-2301 modified above. The resulting plasmid was designated pBYR14. Subsequently, *CTB7* of *C. zea*-*maydis* (open reading frame plus 1,473 bp upstream of the predicted start codon and 604 bp downstream of the predicted stop codon) was PCR-amplified from genomic DNA of the reference strain SCOH1-5 (Kim et al. 2011), using the primer pair *XbaI*-CTB7-F and *BamHI*-CTB7-RC, and was cloned into *XbaI* and *BamHI* restriction sites in pBYR14. The resulting plasmid was designated pBEA002.

Transformation of *C. zeina*. *A. tumefaciens* AGL-1 containing plasmid pBEA002 was grown at 28°C with shaking (250 rpm) for 3 days in 5 ml of Luria broth, supplemented with carbenicillin (50 µg/ml) and kanamycin (100 µg/ml). The culture was diluted to an optical density at 600 nm (OD₆₀₀) of 0.2, using *Agrobacterium* induction medium (IAM) (Xue et al. 2013), and was incubated overnight at 28°C with shaking. The cultures were grown and diluted to an OD₆₀₀ of 0.2 using IAM, to produce the induced (virulent) *Agrobacterium* stock. A conidial suspension of *C. zeina* was prepared by flooding V8 agar plates with IAM, dislodging the conidia with a glass spreader, and diluting to a concentration of 2×10^6 CFU/ml. The induced *Agrobacterium* stock and conidial suspension was mixed in a 1:1 (vol/vol) ratio and 200 µl was plated onto a cellophane membrane overlain on IAM agar (18 g/liter) containing hygromycin B (75 µg/ml) and was incubated at 20°C for 3 days. Cellophane membranes were transferred to 0.2× PDA plates containing cefotaxime (50 µg/ml) and hygromycin B (Sigma-Aldrich, St. Louis) (75 µg/ml), to kill off *Agrobacterium* cells and select for transformants, respectively. Plates were incubated at room temperature for 14 days. Single conidia of putative transformants were transferred onto V8 plates containing the same concentrations of cefotaxime and hygromycin B and were subcultured weekly to maintain a sporulating culture.

Cercosporin extraction and chemical characterization. The *C. zeina* CzmCTB7 transformants were cultured for approximately 2 months at ambient room temperature under constant light. Spectrophotometric quantification of cercosporin production was done using the KOH assay (Bluhm and Dunkle 2008; Yamazaki and Ogawa 1972). Absorbance measurements were taken for three plates per mutant and were normalized against the absorbance of the extract from a 0.2× PDA with 10 mM ammonium phosphate–grown culture. For TLC, extracts were prepared using ethyl acetate, as previously described (Dekkers et al. 2007), and an ethyl acetate/hexane/methanol/H₂O (6:4:1.5:1, vol/vol) elution solvent system (Choquer et al. 2005). Pure cercosporin (from *Cercospora hayii* [Sigma-Aldrich]) was dissolved in acetone (1 mg/ml) and was included as a standard.

Compound separation and detection was performed using a Waters Synapt G2 high definition mass spectrometry system (Waters Inc., Milford, MA, U.S.A.). The system is comprised of a Waters Acquity UPLC system hyphenated to a QTOF instrument. The extracts were injected (injection volume of 1 to 5 µl) onto a linear gradient of 5 to 95% solvent B over 15 min at 2.0 ml/min on a Kinetex EVO C18 column (4.6 mm × 50 mm, 17 µm [Phenomenex, Torrance, CA, U.S.A.]). Solvent A was 0.1% formic acid in H₂O and solvent B was acetonitrile. Chromatograms were extracted for *m/z* 535.1604 (Newman and Townsend 2016).

Data access. The sequence of the annotated *C. zeina* CTB gene cluster has been associated with NCBI GenBank BioProject PRJNA355276 and BioSample SAMN06067857 and is deposited in the DDBJ/EMBL/GenBank database under accession number KY656140. The raw in vitro and in planta RNA-seq sequence reads have been uploaded to the NCBI Gene Expression Omnibus database under accession numbers GSE90705 and GSE94442, respectively.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- Cambia website: <http://www.cambia.org/daisy/cambia/585>
- EMBOSS Needle: http://www.ebi.ac.uk/Tools/psa/emboss_needle
- FastQC database: <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>
- FASTX toolkit: http://hannonlab.cshl.edu/fastx_toolkit/index.html
- Joint Genome Institute database: <http://genome.jgi.doe.gov/Cerzm1/Cerzm1.home.html>
- MUSCLE 3.8 database: <http://www.ebi.ac.uk/Tools/msa/muscle>
- PrimerQuest tool: <http://www.idtdna.com/PrimerQuest/Home>