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 zeae-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 gene cluster. The oxidoreductase gene *CTB7* contained several insertions and deletions as compared to the *C. zeae-maydis* ortholog. We set out to determine whether complementing the defective *CTB7* gene with the full-length gene from *C. zeae-maydis* could confer *in vitro* cercosporin production. *C. zeina* transformants containing *C. zeae-maydis CTB7* were generated by *Agrobacterium tumefaciens*-mediated transformation and 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 which was confirmed to be cercosporin by the KOH assay, thin-layer chromatography and UPLC-QTOF-MS. Our results indicated that *C. zeina* has a defective *CTB7* but all the 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 continues to be a devastating maize foliar disease of global importance that has resulted in extensive yield losses over the past few decades (Ward et al. 1999; Crous and Braun 2003). Previously classified as *Cercospora zeae-maydis* Group I and Group II, the causative agents of GLS, *C. zeae-maydis* and *Cercospora zeina* are differentiated by both genetic distance and phenotypic characteristics, such as their ability to produce the phytotoxin cercosporin (Goodwin et al. 2001; Crous et al. 2006). *Cercospora zeina* predominates throughout Africa, while *C. zeae-maydis* is most prevalent in the majority of the USA and Mexico (Wang et al. 1998; Dunkle and Levy 2000; Goodwin et al. 2001; Meisel et al. 2009).

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 non-specific 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 *Cercospora kikuchii*, *Cercospora beticola*, *C. zeae-maydis*, *Cercospora asparagi* and *Cercospora 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 characterised by severe blighting of leaves (Daub and Ehrenshaft 2000). In contrast to this, isolates of the peanut pathogen *Cercospora 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 which 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, which cause oxidative damage to a wide range of macromolecules (Daub and Ehrenshaft 2000; Daub and Chung 2009). 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 favourable environment for the growth and sporulation of *Cercospora* spp., which culminates in host cell death (Daub and Ehrenshaft 2000; Daub and Chung 2009). 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 (Upchurch et al. 1991; Choquer et al. 2005).

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 virulence *in planta* (Choquer et al. 2005; Chen et al. 2007a; Chen et al. 2007b; Choquer et al. 2007; Dekkers et al. 2007). *CTB8* encodes a $Zn(II)Cys_6$ transcriptional activator which co-ordinates expression of the *CTB* genes under appropriate conditions for cercosporin production (Chen et al. 2007b), while *CTB4* encodes a major facilitator superfamily (MFS) transporter protein involved in cercosporin export (Choquer et al. 2007). The six remaining *CTB* genes (*CTB1, 2, 3, 5, 6* and *7*), 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 to its orthologs in *C. zeae-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 USA. 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. zeae-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

Cercospora 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 (0.2x Potato Dextrose Agar (PDA)) (Fig. 1A). Similarly, the USA isolate of *C. zeina* (OYPA = USPA-4) was cercosporin negative *in vitro*, as reported for all African and USA *C. zeina* isolates tested previously (Dunkle and Levy 2000) (Fig.1A). This was in contrast to the profuse cercosporin production by *C. zeae-maydis* SCOH1-5 (Fig. 1A), a second fungal species that causes gray leaf spot disease of maize (Bluhm et al. 2008).

Cercospora zeina* CMW25467 harbours 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*, *3*, *5* and *6*). *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).

The *C. zeina CTB7* gene region has deletions compared to the corresponding 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-

678; Fig. 2). There was high sequence identity (86%) for the region corresponding to the first 224 bp of the *C. zeae-maydis* exon one, as well as for the second *CTB7* exon of *C. zeae-maydis* (87% identity; nucleotides 679-1332; 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 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 post-inoculation (dpi). Typical GLS lesions had formed by 19 dpi and progressed to coalesced lesions by 25 dpi on all replicate plants (Supplementary Fig. S1A). *Cercospora zeina* fungal load was quantified by qPCR and was shown to increase over the time course with a significant difference at 21 and 25 dpi compared to the post-inoculation 0 dpi samples (Supplementary Fig. S1B).

RNA from all of the time points were pooled and used for RT-PCR analysis with the CTB7exon 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) (Rep et al. 2006; Reid et al. 2014), 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 (Fig. 3A;

Fig. 2). 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 (Supplementary Fig. S1C). All three replicates produced the 122 bp RT-PCR product (Fig.3B, lanes 4-6), confirming expression of the *C. zeina CTB7* gene and indicating removal of the *CTB7* intron. However, there was incomplete splicing of some transcripts since the 234 bp product was also observed in each sample, which corresponds to the size of the gDNA product (Fig. 3B, lanes 4-6). RT-PCR analysis of the *C. zeina elongation factor 1α* gene, the primers of which flank an intron (Table 4), produced only the smaller 99bp spliced product, indicating that there was no gDNA contamination in the samples (Fig. 3C, lanes 4-6).

Additional evidence for expression of *CTB7* in the glasshouse trials was obtained by reverse transcriptase quantitative PCR (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*. *Cercospora zeina CTB7* expression could be quantified at all of the time points from 0 dpi 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 analysed as controls and showed a trend of increased expression over time, although there were no significant differences to the post-inoculation 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 [image is shown in Methods S1 File of (Christie et al. 2017)], in contrast to the glasshouse samples that were inoculated at high conidial density resulting in lesions that were coalesced by 21-25 dpi (Supplementary Fig. S1A). 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 RNAseq (field samples), considering the amount of fungal RNA compared to 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 including the predicted amidation and FMN/FAD-binding motifs (Fig. 5B). However, the *C. zeina* ORF 3 lacks ~220 amino acids at the *N*-terminus (containing a second FMN/FAD-binding motif) which 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. zeae-maydis CTB7* has a 40 amino acid deletion compared to the *C. nicotianae CTB7* (Fig. 5B), but in contrast to *C. zeina*, *C. zeae-maydis* still maintains the ability to produce cercosporin (Fig. 1A).

The deletion in the gDNA of the *C. zeina CTB7* gene region compared to *C. zeae-maydis* is conserved in *C. zeina* isolates from Africa and USA.

The sequence conservation between the *C. zeina CTB7* gene region and the nucleotides corresponding to the N- and C-termini of *C. zeae-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 bp and 925 bp amplicons from gDNA of *C. zeina* and *C. zeae-maydis*, respectively (Fig. 6A and B). A suite of *C. zeina* isolates both from Africa (Uganda and Zambia) and the USA (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 USA 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 (Supplementary File S2).

Cercosporin production in *C. zeina CzmCTB7* transformants

Cercospora zeina was complemented with the full-length *C. zeae-maydis CTB7* gene using *Agrobacterium tumefaciens*-mediated transformation (Supplementary Fig. S5). The presence of the *C. zeae-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 *C. zeae-maydis*) (Fig. 7A). RT-PCR analysis showed that these transformants also expressed the *C. zeae-maydis CTB7* gene when cultured on 0.2x PDA under constant light (Fig. 7B). Furthermore, transformant-3 was found to accumulate a red pigment comparable to an isolate of *Cercospora 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 0.2x PDA + 10mM ammonium phosphate, conditions known to suppress cercosporin production (You et al. 2008) (Fig. 8). Both the KOH assay and TLC indicated cercosporin production in transformant-3 (Fig. 9). These results were confirmed by UPLC-QTOF-MS (Fig.10).

The high resolution mass spectra (HRMS) for the cercosporin standard and the extract from transformant-3 are shown in Fig. 10. Both pure cercosporin and transformant-3 exhibited a peak at an RT of 6.30 min on the UPLC profile (Fig. 10A and B). The HRMS-ESI/ACPI-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 (Supplementary Fig. S6D).

All four transformants retained pathogenicity when re-inoculated 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 (Supplementary File S3A). Koch's postulates were fulfilled for all four re-isolated transformants by ITS sequencing and PCR of both CTB7 gene copies (Supplementary File S3B & C).

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, which in *C. nicotianae* has been shown to be essential for cercosporin biosynthesis in two independent studies (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 to its orthologs, fit the definition of a pseudogene. Pseudogenes typically demonstrate evidence of coding sequence degradation (Lafontaine and Dujon 2010), which we observed for the *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 relative to the *C. zeae-maydis* CTB7, respectively). 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 (Zhang and Gerstein 2004; Lafontaine and Dujon 2010). A study in yeast showed evidence that 12 out of 77 pseudogenes were expressed (Lafontaine and Dujon 2010). *Cercospora 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 non-functional gene undergoing pseudogenisation, we would expect to observe the accumulation of mutations amongst different isolates of *C. zeina*. However, sequencing of the CTB7 diagnostic PCR products from the USA 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 (Supplementary File S2). 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 (Supplementary Fig. S6). *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). *Agrobacterium tumefaciens*-mediated transformation has recently been applied in gene knockout and complementation studies in several Dothideomycetes 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 utilise this approach to complement the defective *CTB7* gene in *C. zeina*. We successfully generated four transformants, which were shown to carry both copies of the *CTB7* gene (Fig. 7A), and could confirm cercosporin production in one of the transformants (Fig. 8 - 10). The production of cercosporin by the *CTB7* over-expression 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 colour 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 *CTB7* over-expression transformant with the KOH assay, TLC and UPLC-QTOF-MS (Fig. 8 - 10).

Cercospora zeina is a successful pathogen of maize causing GLS (Wang et al. 1998; Meisel et al. 2009; Muller et al. 2016), 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* 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 (Shim and Dunkle 2003; Choquer et al. 2005; Chen et al. 2007a; Chen et al. 2007b; Choquer et al. 2007; Dekkers et al. 2007; 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 success of *C. zeina* despite a non-functional 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 paralogue 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 paralogue (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, which 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

The wild-type isolate of *Cercospora zeina*, CMW25467, was cultured on V8 agar medium at 25°C in constant darkness to promote conidiation (Meisel et al. 2009). Conidiating cultures were maintained by sub-culturing on V8 agar. A South African isolate of *C. kikuchii* was isolated from soybean (Table 1). A vegetative culture was maintained on 0.2x PDA (3 g PDA, 12 g Agar and ~1000 ml of distilled water) and used as a positive control for the cercosporin chemical analyses. Additional *C. zeina* and *C. zeae-maydis* isolates analysed 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 as follows: (i) V8 agar, (ii) 0.2x PDA supplemented with 10mM Ammonium phosphate, (iii) PDA pH8 [pH adjusted with Na_2CO_3+$NaHCO_3$], (iv) PDA pH3 [pH adjusted with citric acid + Na_2HPO_4], (v) Cornmeal agar, (vi) Complete medium (10g glucose, 1g yeast extract, 1g casein hydrolysate, 1g $Ca(NO_3)_2.4H_2O$, 10ml buffer solution [2g KH_2PO_4, 2.5g $MgSO_4.7H_2O$, 1.5g NaCl and ~100ml distilled water] and ~1000ml distilled water) and (vii) YPD (0.5g peptone, 0.5g yeast extract, 5g glucose, 18g NaCl and ~1000ml distilled water). For growth condition (i), the cultures were kept in constant darkness at ambient room temperature for 3 days. For growth conditions (ii) to (vii), the cultures were kept in constant light at 25°C for 7 days.

Maize inoculations with *C. zeina*

The first glasshouse inoculation trial was conducted to generate GLS-infected B73 material for quantitative RT-PCR following the methods described in (Christie et al. 2017). *Cercospora zeina* conidia were collected from V8 agar cultures and 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 x 10^6 conidia/ml. Inoculated leaf material (two leaves per plant) was harvested at five separated 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 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, California, USA) as 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, California, USA).

For RT-PCR, biological material was ground in liquid nitrogen and 100mg used for RNA isolation with the RNeasy Plant Mini RNA Extraction Kit and the RNase-free DNase set for on-column DNA digestion (Qiagen) as per the manufacturer's specifications. The High

Capacity RNA-to-cDNA Kit (Applied Biosystems) was used for cDNA synthesis as 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; Hong Kong) for library construction and sequencing. RNA sequencing of 200 bp short-insert libraries was performed on Illumina HiSeq^{TM} 2000 platform (Illumina Inc., San Diego, USA) 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 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and 13 bases were removed from the beginning of each read in the sequencing files with fastx_trimmer from the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/index.html). Transcripts were assembled using Trinity (Grabherr et al. 2011) and 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 formant using SAMtools (Li et al. 2009) and read coverage counted with the htseq-count package (Anders et al. 2015), using the default parameters. Mapped reads were visualised using the Genome View tool (Abeel et al. 2012) and used to validate the *C. zeina CTB* gene annotations. *C zeina in vitro* RNA-seq data have been deposited in the NCBI Gene Expression Omnibus (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 in (Christie et al. 2017), namely GLS-diseased lower leaves from three biological replicate plants at VT stage of development. RNA-seq analysis was conducted as described in (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 used for read counting in the current study. *In planta* RNA-seq data have been deposited in the NCBI Gene Expression Omnibus (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 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 used as query sequences in a BLASTp analysis against the *C. zeae-maydis* filtered model protein database [available on the Joint Genome Institute (JGI) (http://genome.jgi.doe.gov/Cerzm1/Cerzm1.home.html)] to identify predicted *C. zeae-maydis* CTB amino acid sequences. Pairwise protein sequences alignments were performed using

EMBOSS Needle (http://www.ebi.ac.uk/Tools/psa/emboss_needle/) and multiple sequence alignments using MUSCLE 3.8 (http://www.ebi.ac.uk/Tools/msa/muscle/). 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, California, USA) was used according to the manufacturer's specifications for DNA isolation of the *CzmCTB7* transformants. The CTB7del PCR was set up in a total volume of 12.5 µl, which consisted of: 1 x KAPA2G Robust HotStart ReadyMix (KapaBiosystems, Wilmington, Massachusetts), 0.5 µm of each primer, 0.6 µl DMSO, 25 ng of DNA and sterile distilled water. The cycling conditions were as follows: 3 min at 95°C followed by 30 x (30s at 95°C, 30s at 60°C, 1 min at 72°C) with a final extension step of 72°C for 10 min.

RT-PCR analysis

RT-PCR reactions were set up in a total volume of 12.5 µl, which consisted of: 1 x KAPA2G Robust HotStart ReadyMix, 0.5 µm of each primer, 0.6 µl DMSO, 1ul of the cDNA and sterile distilled water. The cycling conditions were as follows: 3 min at 95°C followed by 30 x (15s at 95°C, 15s at 58°C, 30s at 72°C) with 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) as per the manufacturer's specifications. Sequencing reactions were setup using the BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, CA, USA) according to the manufacturer's guidelines and 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 Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al. 2009) using the Bio-Rad CFX96 Touch^{TM} Real-Time PCR Detection System. Primers were designed using the PrimerQuest Tool (https://eu.idtdna.com/PrimerQuest/Home) (Table 4). Expression was measured in three biological replicates. Reactions were set up in 10 µl volumes, which consisted of: 5 µl Lightcycler® 480 SYBR Green I Master Mix (Roche Diagnostics, Basel, Switzerland), 0.5 µM of each of the primers, 1 µl cDNA template and sterile distilled water. The cycling conditions used were: 95°C for 10 minutes, 45 cycles of 95°C for 10 seconds, 60°C for 15 seconds and 72°C for 10 second. 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 (Vandesompele et al. 2002; Hellemans et al. 2007). 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., CA, USA).

Heterologous expression of *CTB7* from *C. zeae-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 pair MCS HYG_XhoI F and HYG_BstEII R and cloned into XhoI and BstEII restriction sites in the binary vector pCAMBIA-2301 (www.cambia.org). Similarly, a 1.8-kb 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 cloned into BamHI and BstEII restriction sites in pCAMBIA-2301 modified above. The resulting plasmid was designated pBYR14. Subsequently, *CTB7* of *C. zeae-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 cloned into XbaI and BamHI restriction sites in pBYR14. The resulting plasmid was designated pBEA002 (Supplementary Fig. S5A).

Transformation of *C. zeina*

Agrobacterium tumefaciens AGL-1 containing plasmid pBEA002, was grown at 28°C with shaking (250rpm) for 3 days in 5 ml Luria broth (LB), supplemented with carbenicillin (50 µg/ml) and kanamycin (100 µg/ml). The culture was diluted to an optical density of 0.2 at 600nm (OD_{600}), using *Agrobacterium* Induction Medium (IAM) (Xue et al. 2013) and incubated overnight at 28°C with shaking. The cultures were grown and diluted to an OD_{600} 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 x 10^6 CFU/ml. The induced *Agrobacterium* stock and conidial suspension was mixed in a 1:1 (vol/vol) ratio and 200 µl plated onto a cellophane membrane overlain on IAM agar (18 g/litre) containing hygromycin B (75 µg/ml) and incubated at 20°C for 3 days. Cellophane membranes were transferred to 0.2x PDA plates containing cefotaxime (50 µg/ml) and hygromycin B (Sigma-Aldrich, St. Louis, USA) (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 sub-cultured weekly to maintain a sporulating culture.

Cercosporin extraction and chemical characterisation

The *C. zeina CzmCTB7* transformants were cultured for approximately two months at ambient room temperature under constant light. Spectrophotometric quantification of cercosporin production was done using the KOH assay (Yamazaki and Ogawa 1972; Bluhm and Dunkle 2008). Absorbance measurements were taken for three plates per mutant and normalised against the absorbance of the extract from a 0.2x PDA with 10mM 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/H_2O (6:4:1.5:1, v/v) elution solvent system (Choquer et al. 2005). Pure cercosporin (from *Cercospora hayii*, Sigma-Aldrich) was dissolved in acetone (1 mg/ml) and included as a standard.

Compound separation and detection was performed using a Waters® Synapt G2 high definition mass spectrometry (HDMS) system (Waters Inc., Milford, Massachusetts, USA). The system comprises of a Waters Acquity Ultra Performance Liquid Chromatography (UPLC®) system hyphenated to a quadrupole-time-of-flight (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 x 50 mm, 17 µm, Phenomenex, Torrence, CA). Solvent A was 0.1% formic acid in H_2O 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* cercosporin biosynthesis (*CTB*) gene cluster has been associated with the NCBI GenBank BioProject PRJNA355276 and the BioSample SAMN06067857, and is deposited in the DDBJ/EMBL/GenBank database under the accession number KY656140. The raw *in vitro* and *in planta* RNA-seq sequence reads have been uploaded to the NCBI Gene Expression Omnibus under the accession numbers GSE90705 and GSE94442, respectively.

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LITERATURE CITED

Abeel, T., Van Parys, T., Saeys, Y., Galagan, J., and Van de Peer, Y. 2012. GenomeView: a next-generation genome browser. Nucleic Acids Research 40:e12-e12.

Anders, S., Pyl, P.T., and Huber, W. 2015. HTSeq—a Python framework to work with high-throughput sequencing data. Bioinformatics 31:166-169.

Aragona, M., and Valente, M.T. 2015. Genetic transformation of the tomato pathogen *Pyrenochaeta lycopersici* allowed gene knockout using a split-marker approach. Current Genetics 61:211-220.

Beseli, A., Amnuaykanjanasin, A., Herrero, S., Thomas, E., and Daub, M. 2015. Membrane transporters in self resistance of *Cercospora nicotianae* to the photoactivated toxin cercosporin. Current Genetics:1-20.

Bluhm, B., Dhillon, B., Lindquist, E., Kema, G., Goodwin, S., and Dunkle, L. 2008. Analyses of expressed sequence tags from the maize foliar pathogen *Cercospora zeae-maydis* identify novel genes expressed during vegetative, infectious, and reproductive growth. BMC Genomics 9:523.

Bluhm, B.H., and Dunkle, L.D. 2008. *PHL1* of *Cercospora zeae-maydis* encodes a member of the photolyase/cryptochrome family involved in UV protection and fungal development. Fungal Genetics and Biology 45:1364-1372.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., and Wittwer, C.T. 2009. The MIQE Guidelines: Minimum information for publication of quantitative Real-Time PCR experiments. Clinical Chemistry 55:611-622.

Chen, H.-Q., Lee, M.-H., and Chung, K.-R. 2007a. Functional characterization of three genes encoding putative oxidoreductases required for cercosporin toxin biosynthesis in the fungus *Cercospora nicotianae*. Microbiology 153:2781-2790.

Chen, H., Lee, M.-H., Daub, M.E., and Chung, K.-R. 2007b. Molecular analysis of the cercosporin biosynthetic gene cluster in *Cercospora nicotianae*. Molecular Microbiology 64:755-770.

Choquer, M., Lee, M.-H., Bau, H.-J., and Chung, K.-R. 2007. Deletion of a MFS transporter-like gene in *Cercospora nicotianae* reduces cercosporin toxin accumulation and fungal virulence. FEBS Letters 581:489-494.

Choquer, M., Dekkers, K.L., Chen, H.-Q., Cao, L., Ueng, P.P., Daub, M.E., and Chung, K.-R. 2005. The *CTB1* gene encoding a fungal polyketide synthase is required for cercosporin biosynthesis and fungal virulence of *Cercospora nicotianae*. Molecular Plant-Microbe Interactions 18:468-476.

Christie, N., Myburg, A.A., Joubert, F., Murray, S.L., Carstens, M., Lin, Y.-C., Meyer, J., Crampton, B.G., Christensen, S.A., Ntuli, J.F., Wighard, S.S., Van de Peer, Y., and Berger, D.K. 2017. Systems genetics reveals a transcriptional network associated with susceptibility in the maize-gray leaf spot pathosystem. The Plant Journal 89:746–763.

Crous, P.W., and Braun, U. 2003. *Mycosphaerella* and its anamorphs: 1. Names published in *Cercospora* and *Passalora*. Pages 1-571 in: CBS Biodiversity Series.

Crous, P.W., Groenewald, J.Z., Groenewald, M., Caldwell, P., Braun, U., and Harrington, T.C. 2006. Species of *Cercospora* associated with grey leaf spot of maize. Studies in Mycology 55:189-197.

Daub, M.E., and Ehrenshaft, M. 2000. The photoactivated *Cercospora* toxin cercosporin: Contributions to plant disease and fundamental biology. Annu. Rev. Phytopathol. 38:461-490.

Daub, M.E., and Chung, K.-R. 2009. Photoactivated perylenequinone toxins in plant pathogenesis. Pages 201-219 in: The Mycota, H.B. Deising, ed. Springer Berlin Heidelberg.

Dekkers, K.L., You, B.-J., Gowda, V.S., Liao, H.-L., Lee, M.-H., Bau, H.-J., Ueng, P.P., and Chung, K.-R. 2007. The *Cercospora nicotianae* gene encoding dual O-methyltransferase and FAD-dependent monooxygenase domains mediates cercosporin toxin biosynthesis. Fungal Genetics and Biology 44:444-454.

Dunkle, L.D., and Levy, M. 2000. Genetic relatedness of African and United States populations of *Cercospora zeae-maydis*. Phytopathology 90:486-490.

Fore, S.A., Daub, M.E., and Beute, M.K. 1988. Phytotoxic substances produced by some isolates of *Cercospora arachidicola* are not cercosporin. Phytopathology 78:1082-1086.

Gaur, M., Puri, N., Manoharlal, R., Rai, V., Mukhopadhayay, G., Choudhury, D., and Prasad, R. 2008. MFS transportome of the human pathogenic yeast *Candida albicans*. BMC Genomics 9:1-12.

Goodwin, S.B., Dunkle, L.D., and Zismann, V.L. 2001. Phylogenetic analysis of *Cercospora* and *Mycosphaerella* based on the Internal Transcribed Spacer Region of ribosomal DNA. Phytopathology 91:648-658.

Grabherr, M.G., Haas, B.J., Yassour, M., Levin, J.Z., Thompson, D.A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q.D., Chen, Z.H., Mauceli, E., Hacohen, N., Gnirke, A., Rhind, N., di Palma, F., Birren, B.W., Nusbaum, C., Lindblad-Toh, K., Friedman, N., and Regev, A. 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotechnol 29:644-U130.

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., and Vandesompele, J. 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol 8:R19.

Jenns, A.E., Daub, M.E., and Upchurch, R.G. 1989. Regulation of cercosporin accumulation in culture by medium and temperature manipulation. Phytopathology 79:213-219.

Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., and Salzberg, S.L. 2013. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol 14:R36-R36.

Kim, H., Ridenour, J.B., Dunkle, L.D., and Bluhm, B.H. 2011. Regulation of stomatal tropism and infection by light in *Cercospora zeae-maydis*: Evidence for coordinated host/pathogen responses to photoperiod? PLoS Pathog 7:e1002113.

Korf, I. 2004. Gene finding in novel genomes. Bmc Bioinformatics 5:59.

Korsman, J., Meisel, B., Kloppers, F., Crampton, B., and Berger, D. 2010. Quantitative phenotyping of grey leaf spot disease in maize using real-time PCR. European Journal of Plant Pathology 18:183-194.

Koshikumo, E.S.M., Alves, E., and Von Pinho, R.G. 2014. Induction and detection of toxin in *Cercospora zeina* and *Cercospora sorghi f. sp. maydis*. Brazilian Journal of Agricultural Sciences 9:31-35.

Kuyama, S., and Tamura, T. 1957. Cercosporin. A pigment of *Cercosporina kikuchii Matsumoto et Tomoyasu*. I. Cultivation of fungus, isolation and purification of pigment. Journal of the American Chemical Society 79:5725-5726.

Lafontaine, I., and Dujon, B. 2010. Origin and fate of pseudogenes in Hemiascomycetes: a comparative analysis. BMC Genomics 11:1-15.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R., and Genome Project Data Processing, S. 2009. The sequence alignment/map format and SAMtools. Bioinformatics 25:2078-2079.

Lu, Y., Xiao, S., Wang, F., Sun, J., Zhao, L., Yan, L., and Xue, C. 2017. *Agrobacterium tumefaciens*-mediated transformation as an efficient tool for insertional mutagenesis of *Cercospora zeae-maydis*. Journal of Microbiological Methods 133:8-13.

Meisel, B., Korsman, J., Kloppers, F.J., and Berger, D.K. 2009. *Cercospora zeina* is the causal agent of grey leaf spot disease of maize in southern Africa. European Journal of Plant Pathology 124:577-583.

Muller, M.F., Barnes, I., Kunene, N.T., Crampton, B.G., Bluhm, B., Phillips, S., Olivier, N.A., and Berger, D.K. 2016. *Cercospora zeina* from maize in South Africa exhibits high genetic diversity and lack of regional population differentiation. Phytopathology 106:1194-1205.

Nakayashiki, H., Hanada, S., Quoc, N.B., Kadotani, N., Tosa, Y., and Mayama, S. 2005. RNA silencing as a tool for exploring gene function in ascomycete fungi. Fungal Genetics and Biology 42:275-283.

Newman, A.G., and Townsend, C.A. 2016. Molecular characterization of the cercosporin biosynthetic pathway in the fungal plant pathogen *Cercospora nicotianae*. Journal of the American Chemical Society 138:4219-4228.

Reid, I., O'Toole, N., Zabaneh, O., Nourzadeh, R., Dahdouli, M., Abdellateef, M., Gordon, P.M., Soh, J., Butler, G., Sensen, C.W., and Tsang, A. 2014. SnowyOwl: accurate prediction of fungal genes by using RNA-Seq and homology information to select among *ab initio* models. Bmc Bioinformatics 15:1-16.

Rep, M., Duyvesteijn, R.G.E., Gale, L., Usgaard, T., Cornelissen, B.J.C., Ma, L.-J., and Ward, T.J. 2006. The presence of GC-AG introns in *Neurospora crassa* and other euascomycetes determined from analyses of complete genomes: implications for automated gene prediction. Genomics 87:338-347.

Ridenour, J.B., Smith, J.E., Hirsch, R.L., Horevaj, P., Kim, H., Sharma, S., and Bluhm, B.H. 2014. *UBL1* of *Fusarium verticillioides* links the N-end rule pathway to extracellular sensing and plant pathogenesis. Environmental Microbiology 16:2004-2022.

Schnable, P.S., Ware, D., Fulton, R.S., Stein, J.C., Wei, F., Pasternak, S., Liang, C., Zhang, J., Fulton, L., Graves, T.A., Minx, P., Reily, A.D., Courtney, L., Kruchowski, S.S., Tomlinson, C., Strong, C., Delehaunty, K., Fronick, C., Courtney, B., Rock, S.M., Belter, E., Du, F., Kim, K., Abbott, R.M., Cotton, M., Levy, A., Marchetto, P., Ochoa, K., Jackson, S.M., Gillam, B., Chen, W., Yan, L., Higginbotham, J., Cardenas, M., Waligorski, J., Applebaum, E., Phelps, L., Falcone, J., Kanchi, K., Thane, T., Scimone, A., Thane, N., Henke, J., Wang, T., Ruppert, J., Shah, N., Rotter, K., Hodges, J., Ingenthron, E., Cordes, M., Kohlberg, S., Sgro, J., Delgado, B., Mead, K., Chinwalla, A., Leonard, S., Crouse, K., Collura, K., Kudrna, D., Currie, J., He, R., Angelova, A., Rajasekar, S., Mueller, T., Lomeli, R., Scara, G., Ko, A., Delaney, K., Wissotski, M.,

Lopez, G., Campos, D., Braidotti, M., Ashley, E., Golser, W., Kim, H., Lee, S., Lin, J., Dujmic, Z., Kim, W., Talag, J., Zuccolo, A., Fan, C., Sebastian, A., Kramer, M., Spiegel, L., Nascimento, L., Zutavern, T., Miller, B., Ambroise, C., Muller, S., Spooner, W., Narechania, A., Ren, L., Wei, S., Kumari, S., Faga, B., Levy, M.J., McMahan, L., Van Buren, P., Vaughn, M.W., Ying, K., Yeh, C.-T., Emrich, S.J., Jia, Y., Kalyanaraman, A., Hsia, A.-P., Barbazuk, W.B., Baucom, R.S., Brutnell, T.P., Carpita, N.C., Chaparro, C., Chia, J.-M., Deragon, J.-M., Estill, J.C., Fu, Y., Jeddeloh, J.A., Han, Y., Lee, H., Li, P., Lisch, D.R., Liu, S., Liu, Z., Nagel, D.H., McCann, M.C., SanMiguel, P., Myers, A.M., Nettleton, D., Nguyen, J., Penning, B.W., Ponnala, L., Schneider, K.L., Schwartz, D.C., Sharma, A., Soderlund, C., Springer, N.M., Sun, Q., Wang, H., Waterman, M., Westerman, R., Wolfgruber, T.K., Yang, L., Yu, Y., Zhang, L., Zhou, S., Zhu, Q., Bennetzen, J.L., Dawe, R.K., Jiang, J., Jiang, N., Presting, G.G., Wessler, S.R., Aluru, S., Martienssen, R.A., Clifton, S.W., McCombie, W.R., Wing, R.A., and Wilson, R.K. 2009. The B73 maize genome: Complexity, diversity and dynamics. Science 326:1112-1115.

Shim, W.B., and Dunkle, L.D. 2003. *CZK3*, a MAP kinase kinase kinase homolog in *Cercospora zeae-maydis*, regulates cercosporin biosynthesis, fungal development, and pathogenesis. Mol Plant Microbe Interact 16:760-768.

Solovyev, V., Kosarev, P., Seledsov, I., and Vorobyev, D. 2006. Automatic annotation of eukaryotic genes, pseudogenes and promoters. Genome Biol 7.

Staerkel, C., Boenisch, M., Kroger, C., Bormann, J., Schafer, W., and Stahl, D. 2013. *CbCTB2*, an *O*-methyltransferase is essential for biosynthesis of the phytotoxin cercosporin and infection of sugar beet by *Cercospora beticola*. BMC Plant Biology 13:50.

Stanke, M., and Morgenstern, B. 2005. AUGUSTUS: a web server for gene prediction in eukaryotes that allows user-defined constraints. Nucleic Acids Research 33:W465-W467.

Upchurch, R.G., Walker, D.C., Rollins, J.A., Ehrenshaft, M., and Daub, M.E. 1991. Mutants of *Cercospora kikuchii* altered in cercosporin synthesis and pathogenicity. Applied and Environmental Microbiology 57:2940-2945.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., and Speleman, F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:research0034.0031 - research0034.0011.

Wang, J., Levy, M., and Dunkle, L.D. 1998. Sibling species of *Cercospora* associated with gray leaf spot of maize. Phytopathology 88:1269-1275.

Ward, J.M.J., Stromberg, E.L., and Nutter, F.W. 1999. Grey Leaf Spot: A disease of global importance in maize production. Plant Disease 83:884-895.

Weiland, J.J., Chung, K.R., and Suttle, J.C. 2010. The role of cercosporin in the virulence of *Cercospora* spp. to plant hosts. Pages 109-117 in: *Cercospora* Leaf Spot of Sugar Beet and Related Species, L.T. Lartey, J.J. Weiland, L. Panella, P.W. Crous, and C.E. Windels, eds. The American Phytopathological Society, Minnesota U.S.A.

Xue, C., Wu, D., Condon, B.J., Bi, Q., Wang, W., and Turgeon, B.G. 2013. Efficient gene knockout in the maize pathogen *Setosphaeria turcica* using *Agrobacterium tumefaciens*-mediated transformation. Phytopathology 103:641-647.

Yamazaki, S., and Ogawa, T. 1972. The chemistry and stereochemistry of cercosporin. Agricultural and Biological Chemistry 36:1707-1718.

You, B.-J., Lee, M.-H., and Chung, K.-R. 2008. Production of cercosporin toxin by the phytopathogenic *Cercospora* fungi is affected by diverse environmental signals. Canadian Journal of Microbiology 54:259-269.

Zhang, Z., and Gerstein, M. 2004. Large-scale analysis of pseudogenes in the human genome. Current Opinion in Genetics & Development 14:328-335.

Table 1. *Cercospora* isolates utilised in this study.

Species[a]	Isolate[b]	Area, Country of isolation	Reference
C. zeina	CMW25467	Mkushi, Zambia	(Meisel et al. 2009)
C. zeina	2011.GT30	KwaZulu-Natal, South Africa	(Muller et al. 2016)
C. kikuchii	CMW49223[c]	KwaZulu-Natal, South Africa	
C. zeina	NLUG 1.23 (B9-P9)[d]	Uganda	
C. zeina	NLUG 1.23 (B9-P11)[d]	Uganda	
C. zeina	NLUG 1.23 (B9-P12)[d]	Uganda	
C. zeina	NLUG 1.23 R (B9-P20)[d]	Uganda	
C. zeina	Zambia (B9-P16)[d]	Zambia	
C. zeina	Zambia 3.2 (B9-P26)[d]	Zambia	
C. zeina	Zambia 3.2 (B9-P31)[d]	Zambia	
C. zeina	Zambia 3.2 (B9-P32)[d]	Zambia	
C. zeina	CMNY C2 (B8-P1)[d]	New York, USA	
C. zeina	CMNY C2 (B8-P3)[d]	New York, USA	

C. zeina	CMNY C2 (B8-P2)[d]	New York, USA	
C. zeina	WOOH-NCR (B8-P25)[d]	Ohio, USA	
C. zeina	WOOH-NCR (B8-P26)[d]	Ohio, USA	
C. zeina	WOOH-NCR (B8-P27)[d]	Ohio, USA	
C. zeina	WOOH-NCR (B8-P28)[d]	Ohio, USA	
C. zeina	OYPA 16 (B8-P5)[d]	Pennsylvania, USA	
C. zeina	OYPA 16 (B8-P7)[d]	Pennsylvania, USA	
C. zeina	OYPA 16 (B8-P9)[d]	Pennsylvania, USA	
C. zeae-maydis	CBS 117757	Wisconsin, USA	(Crous et al. 2006)
C. zeae-maydis	CBS 117761	Indiana, USA	(Crous et al. 2006)
C. zeae-maydis	SCOH1-5[d]	Ohio, USA	(Bluhm et al. 2008)

[a] Based on diagnostic histone gene PCR analysis (Crous et al. 2006).

[b] CMW: Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. CBS: Culture collection of the Centraalbureau voor Schimmelcultures, 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.

Table 2. The *C. zeina CTB* gene cluster.

Cercospora zeina 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	6588	9	2195	polyketide synthase [*Cercospora nicotianae*] (AAT69682.1)	0.0	85.0	91.2	0.9
CTB2	1359	0	452	O-methyltransferase [*Cercospora nicotianae*] (ABK64180.1)	0.0	85.9	90.4	4.3
CTB3	2619	2	872	cercosporin toxin biosynthesis protein [*Cercospora nicotianae*] (ABC79591.2)	0.0	87.5	93.1	0.2
CTB4	1539	3	512	MFS transporter [*Cercospora nicotianae*]	0.0	89.9	95.9	0.2

				(ABK64181.1)				
CTB5	1386	0	461	oxidoreductase [*Cercospora nicotianae*] (ABK64182.1)	0.0	90.9	95.9	0.6
CTB6	1065	0	354	reductase [*Cercospora nicotianae*] (ABK64183.1)	0.0	86.6	94.7	0.6
CTB7 gene region*	969	1	322	oxidoreductase [*Cercospora nicotianae*] (ABK64184.1)	1e-120	51.4	55.6	36.6
CTB8	1278	1	425	zinc finger transcription factor [*Cercospora nicotianae*] (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 an NCBI BLASTx search against the non-redundant 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.

**In silico* annotation of *CTB7* gene region with incorrect intron position, prior to identification of intron position from *in planta* expression data (see later).

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

	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**
CTB1	835	1645	1828	639	1834	1930	1104	149	68	76
CTB2	20	103	45	60	84	84	22	2	1	6
CTB3	492	884	849	579	1115	1059	1906	73	49	85
CTB4	86	135	113	66	128	145	140	35	24	14
CTB5	318	895	669	288	979	1179	903	61	56	57
CTB6	41	346	65	117	192	250	225	96	81	93
CTB7	0	0	0	0	0	0	0	0	0	0
CTB8	241	416	410	259	489	453	453	18	21	55

40S	31344	9852	13666	10550	10857	19034	9694	11368	8881	13053
EF1α	179348	54366	140673	85332	84579	132714	243946	4192	2835	4541

[a] Number of RNA sequence reads which mapped to the gene of interest

[b] RNA isolated from field grown B73 maize leaves demonstrating GLS lesions

Table 4. Primer sequences used in this study.

Primer name	Sequence (5' - 3')	Gene target	Amplicon size (bp)
***CTB7* gDNA structure analysis**			
CTB7del F	AAGAGTGCTTGTGAATGG	*Cercospora CTB7*	618 (*C. zeina*)
CTB7del R	GATGCGGGTGAAGTAGAAA		925 (*C. zeae-maydis*)
RT-PCR primers			
CTB7exon F	AAGATGGGCTTGGAGCAG	*C. zeina CTB7*	234 (gDNA)
CTB7exon R	TGGCGTGTTGGAGCTTTC		122 (cDNA)
CzmCTB7 F	GATTGAGATCATGAGGCGAGAC	*C. zeae-maydis CTB7*	100
CzmCTB7 R	AAGTTCGTCAACGGTACATCC		
EF1α F	GTGCTCGACAAGCTGAA	*C. zeina Elongation factor 1α*	154 (gDNA)
EF1α R	GTCGATGACGGTGACATAG		99 (cDNA)
Quantitative RT-PCR primers			
CTB1 F	GCCTCCAGATGGCATTAT	*C. zeina CTB1*	96
CTB1 R	CGTAGAGTGCAGCGTATT		

CTB2 F	CCTAGACCAGAAAGGACTTC	*C. zeina CTB2*	97
CTB2 R	GCTTCTCGCATCGTAGTA		
CTB7 F	GCCTTCGCCTCATATCAT	*C. zeina CTB7*	98
CTB7 R	GACTGTGGTAACGCTAACT		
CTB8 F	TGTCATCGTGTCAGTCATC	*C. zeina CTB8*	95
CTB8 R	AATCTTGGCCTCGACATC		
40S F	GGTCCTCAAGGTCATTCTC	*C. zeina 40S* ribosomal protein	102
40S R	TTGACACCCTTTCCAGTC		
Cyt III F	GAGCTCTTTATGGTGCTCTA	*C. zeina* cytochrome c oxidase III	108
Cyt III R	CGTAGGCTCCATCTGATAAA		

Plasmid construction (restriction enzymes sites shown in bold)

MCS HYG_XhoI F	GGG**CTCGAG**ACT**GGTACC**GC**G GGCCC**T**AGGCCTTACGTA**GGA GCTCCACCGCGGTGGCG	Hygromycin resistance cassette	2008
HYG_BstEII R	GGC**GGTGACC**GGGGATCCACT AGTTCTAGAGCGGCC	Hygromycin resistance cassette	
GFP_BamHI F	GGC**GGATCC**GGAGAGCTTATA CCGAGCTCCC	GFP expression cassette	1814

MCS	GGC**GGTGACC**TC**AAGCTT**GC**TT**		
GFP_BstEII R	**AATTAA**TCCCGGGTTACTT GTACAGCTCGTCCATGCC	GFP expression cassette	
XbaI-CTB7-F	ATATA**TCTAGA**GGTACAGTAGC TCACCACGT	*C. zeae-maydis CTB7*	3409
BamHI-CTB7-RC	TATAT**GGATCC**TGATTGAGAGT AAGCCGC	*C. zeae-maydis CTB7*	

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 0.2x PDA (visible as the accumulation of a red pigment in the media). Neither the African nor the USA isolate of *C. zeina,* CMW25467 and OYPA respectively, produce cercosporin when cultured on 0.2x 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*.

Fig. 2. Pairwise alignment of the *C. zeina* CMW25467 and the *C. zeae-maydis* SCOH1-5 *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 shown in Table 2). 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 RT-PCR and sequencing using the CTB7exon primer pair, Fig 3A).

Fig. 3. *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 AA CTB7 polypeptide shown in Table 2), 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* RT-PCR (CTB7exon primers) generated two products in each inoculated maize replicate (lanes 4 – 6), one corresponding to the expected cDNA amplicon with intron removed (122 bp) and a larger amplicon (234 bp) indicating the presence of non-spliced *CTB7* transcripts. Non-template/water controls was 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 EtBr. 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 (lane 4 – 6). Electrophoresis conditions and equivalent controls were as described for B.

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 transcriptase qPCR was carried out on RNA extracted from maize at different time points after inoculation and used to calculate mean

calibrated normalised 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 ANOVA analysis with a Tukey's Multiple Comparison test. None of the *CTB* genes studied showed a statistically significant (α=0.05) difference in expression compared to 0 dpi. Only two biological replicates were included in the analysis for 0 dpi.

Fig. 5. The *C. 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 acids 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 to the *C. nicotianae* CTB7.

Fig. 6. Screening of *C. 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 355-467). Regions of greater than 85% nucleotide identity are shaded. **B**, PCR amplification from *C. zeina* and *C. zeae-maydis* isolates with CTB7del_F and CTB7del_R primers (Table 4). PCR products were separated on a 1.5% agarose gel stained with EtBr. A size standard (GeneRuler 100bp DNA ladder, ThermoFisher Scientific) is shown in lane 1. A non-template/water control was included in lane 2. *Cercospora 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 USA (Table 1). PCR products were separated on a 1% agarose gel stained with Gel Red. A size standard (1Kb Plus DNA ladder) is shown in lane 1 and 22. A non-template/water control was included in lane 23. *Cercospora zeae-maydis* SCOH1-5 (lane 2 and 24) demonstrate the larger 925 bp amplicon while the *C. zeina* isolates (lane 3 - 21) yielded a 618 bp amplicon.

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 PCR amplification with the CTB7del primers. PCR products were separated on a 1% agarose gel stained with EtBr. A size standard (GeneRuler 100bp DNA ladder, ThermoFisher Scientific) is shown in lane 1 and 11. A non-template/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 (lane 6 – 9), while two were shown to only carry the smaller *C. zeina CTB7* gene copy (lane 5 and 10). **B**, RT-PCR analysis of the transformants grown on 0.2x PDA in constant light to determine expression of the *C. zeae-maydis CTB7* gene (CzmCTB7 primers, Table 4). RT-PCR products were separated on a 2% agarose gels stained with EtBr. A size standard (GeneRuler 100bp DNA ladder) is shown in lane 1 and a non-template/water control included in lane 2. Lane 3 contained cDNA from *C. zeina* CMW25467, with lane 4-7 containing cDNA from transformants 2-5, 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 *C. zeina CTB7* transformants complemented with the *C. zeae-maydis CTB7* gene. Cultures were grown in constant light on cercosporin conducive conditions (0.2x PDA; top panel) as well as cercosporin suppressive conditions (0.2x PDA supplemented with 10mM 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.

Fig. 9. Evaluation of cercosporin production by *C. 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 ANOVA with a Tukey's Multiple Comparison Test). The stars (*) indicate concentrations significantly higher than the wild type *C. zeina* concentrations at $P < 0.05$. **B**, Thin-layer chromatography analysis of *C. zeina* transformants extracts, prepared using ethyl acetate. Compounds were separated using the ethyl acetate/hexane/methanol/H_2O (6:4:1.5:1, v/v) solvent system. A pure cercosporin standard (lane 1) was visible as a red pigment at R_f 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).

Fig. 10. Mass spectrometry confirms the presence of cercosporin in the extract from *C. zeina* transformant-3 complemented with the *C. zeae-maydis CTB7* gene. Samples were processed by UPLC-QTOF-MS using a Synapt G2 high definition mass spectrometry (HDMS) 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. HRMS-ESI/ACPI-TOF of these peaks demonstrated an accurate mass (*m*/*z*) of 535.1606 for **C**, the cercosporin standard and 535.1605 for **D**, 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 (Supplementary Table S1).

Supplementary File S1. Multiple sequence alignment of the *Cercospora nicotianae*, *Cercospora zeina* and *Cercospora zeae-maydis* CTB amino acid sequences.

Supplementary File S2. Sequencing of *Cercospora zeina CTB7* fragment in geographically and chronologically separated isolates.

Supplementary File S3. Pathogenicity of *Cercospora zeina CzmCTB7* transformants.

Supplementary Fig. S1. Maize line B73 developed GLS symptoms after glasshouse inoculation with *C. zeina* CMW25467. **A**, Glasshouse trial 1: GLS susceptible B73 maize plants were inoculated with a conidial suspension of *C. zeina* and leaf samples were harvested in triplicate at 0, 12 (images not shown), 19, 21 and 25 dpi. Maize leaf images show GLS lesions at 19 dpi, lesions coalescing at 21 dpi and blighting of the leaves at 25 dpi. Control mock-inoculated plants did not show lesions. **B**, *Cercospora zeina* fungal genomic DNA content (a proxy for fungal biomass) increased significantly in B73 maize leaves after inoculation with *C. zeina* CMW25467 in glasshouse trial 1. Fungal quantities are presented as µg of *C. zeina* DNA per ng of *Z. mays* gDNA measured by qPCR of the *CPR1* gene (Korsman et al. 2010). Standard error bars are included on the graphs. Statistical analysis was done using one-way ANOVA analysis with a Tukey's Multiple Comparison test. The fungal load at 21 and 25 dpi was

significantly higher as compared to 0 dpi ($p \leq 0.05$). Only two biological replicates were included in the analysis for 0 dpi. **C**, B73 maize leaves harvested at 32 dpi for RNA isolation following a second glasshouse inoculation trial.

Supplementary Fig. S2. Quality control for *in planta* expression analysis of selected *CTB* genes during glasshouse trial #1. **A**, Selected RT-qPCR products were separated on a 1% agarose gel stained with EtBr. A size standard (GeneRuler 100bp DNA ladder, ThermoFisher Scientific) is shown in lane 1, 6 and 13. The *CTB* target genes are shown in lane 2 (*CTB1*), lane 3 (*CTB2*), lane 4 (*CTB7*) and lane 5 (*CTB8*) and are 96 bp, 97 bp, 98 bp and 95 bp in length, respectively. The RT-qPCR products of a suite of putative reference genes are shown in lane 7 – 12. *40S* (lane 10) and *Cyt III* (lane 11) showed single amplicons of 102 bp and 108 bp, respectively. Putative reference genes *GAPDH* (lane 7), *EF1α* (lane 8), *β-TUB* (lane 9) and *Cyt b* (lane 12), were not included in the expression analysis study due to poor stability values. **B**, Sequencing of the RT-PCR amplicons of target genes *CTB1, CTB2, CTB7* and *CTB8* as well for the reference genes *40S* and *Cyt III*. RT-PCR amplicons were cloned into the pJET vector and sequenced at the DNA sequencing Facility of the Natural and Agricultural faculty at the University of Pretoria. The trace files were analysed and the sequences aligned to the predicted *C. zeina* gene amplicons using the CLC Main Workbench 6.0 (CLC Bio, Denmark). **C**, Melt curve analysis of target genes, *CTB1, CTB2, CTB7* and *CTB8,* as well for the reference genes, *40S* and *Cyt III*. Melt peaks were plotted as the negative rate of change in the relative fluorescent units [-d(RFU)] against the change in temperature [dT]. Single melt peaks indicate specific amplification and the absence of

primer dimers. Non-template controls generated no melt peaks, indicating that no contamination was present. Melting points for *CTB1, CTB2, CTB7, CTB8, 40S* and *Cyt III* were 81.5°C, 83.5°C, 82°C, 88°C, 83.5°C and 74.5°C, respectively.

Supplementary Fig. S3. *Cercospora zeina CTB7* expression demonstrated by RT-PCR analysis of B73-GLS samples from glasshouse trial #2. RT-PCR products were separated on a 2% agarose gel stained with EtBr. A size standard (FastRuler Low Range DNA ladder, ThermoFisher Scientific) is shown in lane 1 and 7. A non-template/water control was included in lane 2 and a *C. zeina* gDNA positive control in lane 3. The *CTB7* RT-PCR demonstrated the expected 98bp amplicon in all the three B73-*C. zeina* replicates (lanes 4 – 6).

Supplementary Fig. S4. The histone diagnostic PCR with the CzeinaHIST and CylH3R primers (Crous et al. 2006), was performed on the *C. zeina* isolates (Table 1). PCR products were separated on a 1% agarose gel stained with Gel Red. A size standard (1Kb Plus DNA ladder) is shown in lane 1 and 22. A non-template/water control was included in lane 23. *Cercospora zeae-maydis* SCOH1-5 (lane 2 and 24) demonstrate no amplification, while the *C. zeina* isolates (lane 3 - 21) yielded a 284 bp amplicon (Crous et al. 2006).

Supplementary Fig. S5. *Agrobacterium tumefaciens*-mediated transformation of *Cercospora zeina*. **A,** Map of the pBEA002 binary vector for *Agrobacterium tumefaciens*-mediated transformation. The *C. zeae-maydis* FAD-oxidoreductase *CTB7*

gene with its native promoter is flanked by a 1.8-kb hygromycin resistance cassette (Nakayashiki et al. 2005) and a 1.8-kb GFP expression cassette (Ridenour et al. 2014), respectively. **B,** *Agrobacterium tumefaciens* transformed *C. zeina* colonies on 0.2x PDA plates supplemented with hygromycin, under normal and UV light conditions. Six colonies were obtained, which grew on media supplemented with hygromycin and demonstrated emission of green fluorescence under UV light. Non-transformed *C. zeina* colonies demonstrated no fluorescence, given their lack of the *Agrobacterium* construct carrying the *GFP* gene.

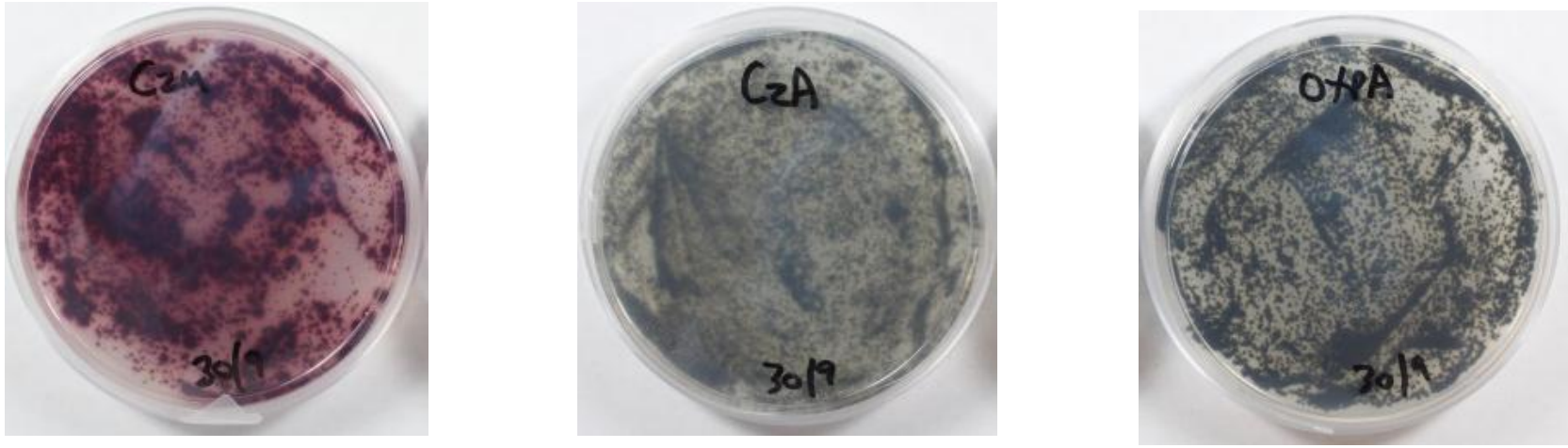
Supplementary Fig. S6. High resolution mass spectra for the *C. kikuchii* CMW49223 and *C. zeina* CMW25467 wild-type extracts. **A**, Extracted ion chromatogram for *m*/*z* 535.1604, **B**, MS and **C**, MS/MS for positive control *C. kikuchii*. **D**, The UPLC profile of the wild-type *C. zeina* extract showed no peaks at 6.30 min, confirming a lack of cercosporin production.

A

C. zeae-maydis SCOH1-5

C. zeina CMW25467

C. zeina OYPA



B

Fig.1 Swart et al. MPMI

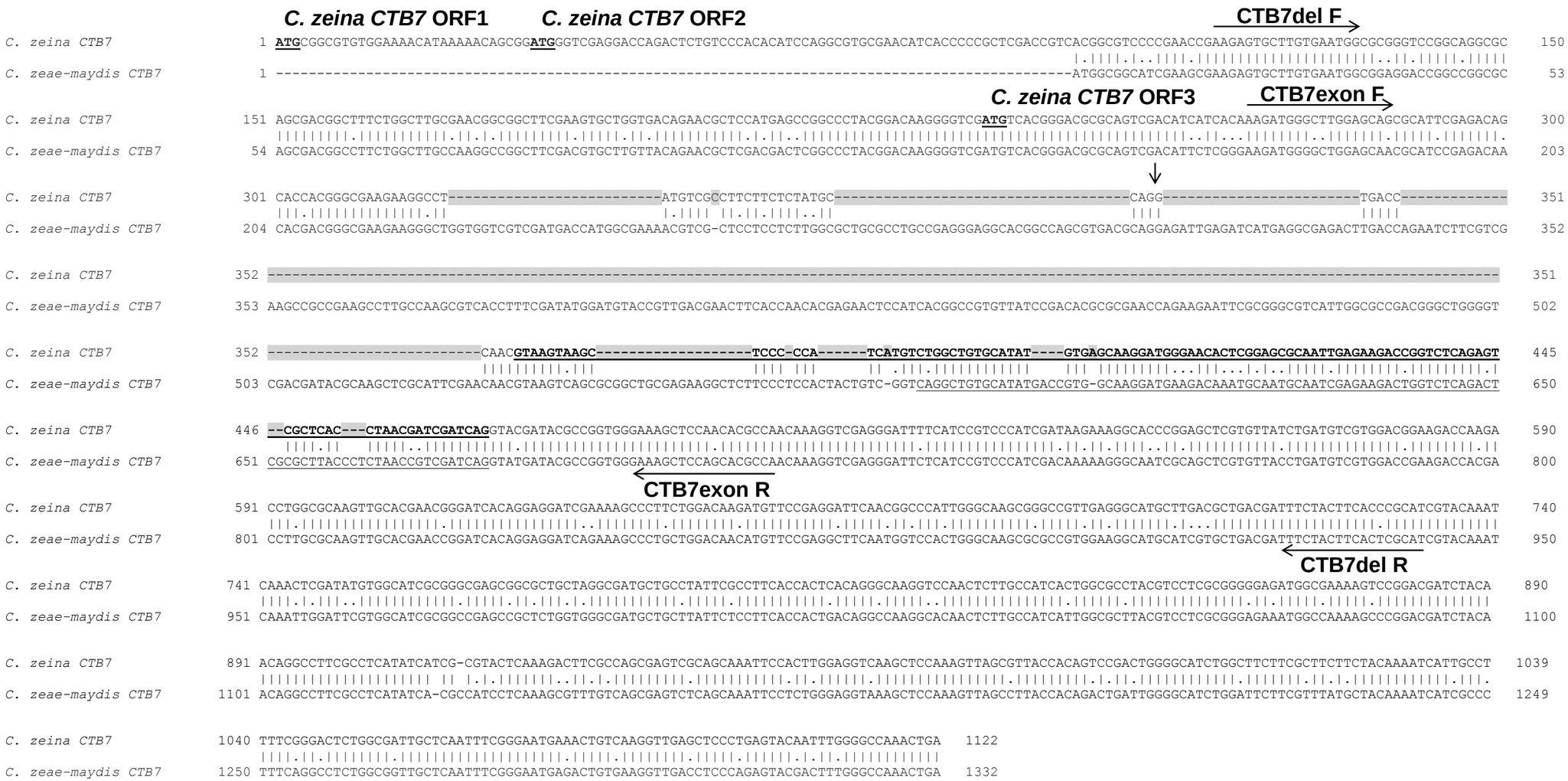


Fig. 2. Pairwise alignment of the *C. zeina* CMW25467 and the *C. zeae-maydis* SCOH1-5 *CTB7* genomic DNA nucleotide sequences. The three potential translation start sites for the *C. zeina CTB7* gene are underlined and in bold. The positions corresponding to the CTB7del and CTB7exon primer binding sites 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 shown in Table 2). 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 RT-PCR and sequencing using the CTB7exon primer pair, Fig 3A).

A

```
CTB7 gDNA  AAGATGGGCTTGGAGCAGCGCATTCGAGACAGCACCACGGGCGAA 45
CTB7 cDNA  AAGATGGGCTTGGAGCAGCGCATTCGAGACAGCACCACGGGCGAA 45

                                                  ↓
CTB7 gDNA  GAAGGCCTATGTCGCCTTCTTCTCTATGCCAGGTGACCCAACGTA 90
CTB7 cDNA  GAAGGCCTATGTCGCCTTCTTCTCTATGCCAGGTGACCCAAC--- 87

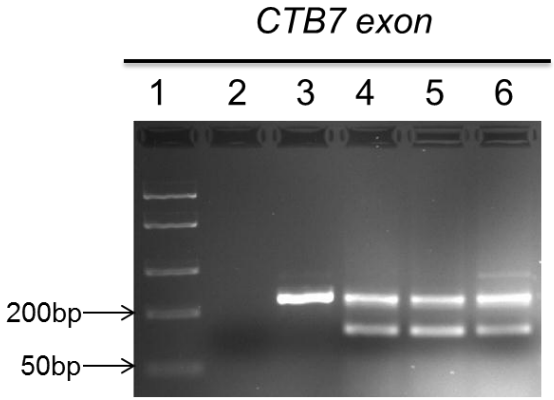
CTB7 gDNA  AGTAAGCTCCCCCATCATGTCTGGCTGTGCATATGTGAGCAAGGA 135
CTB7 cDNA  ----------------------------------------------- 87

CTB7 gDNA  TGGGAACACTCGGAGCGCAATTGAGAAGACCGGTCTCAGAGTCGC 180
CTB7 cDNA  ----------------------------------------------- 87

CTB7 gDNA  TCACCTAACGATCGATCAGGTACGATACGCCGGTGGGAAAGCTCC 225
CTB7 cDNA  ----------------------GTACGATACGCCGGTGGGAAAGCTCC 113

CTB7 gDNA  AACACGCCA 234
CTB7 cDNA  AACACGCCA 122
```

B



C

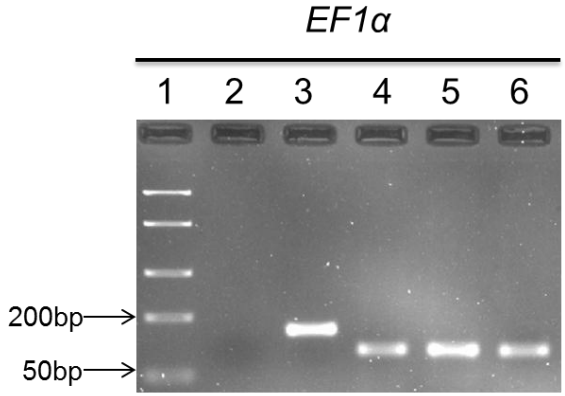


Fig.3 Swart et al. MPMI

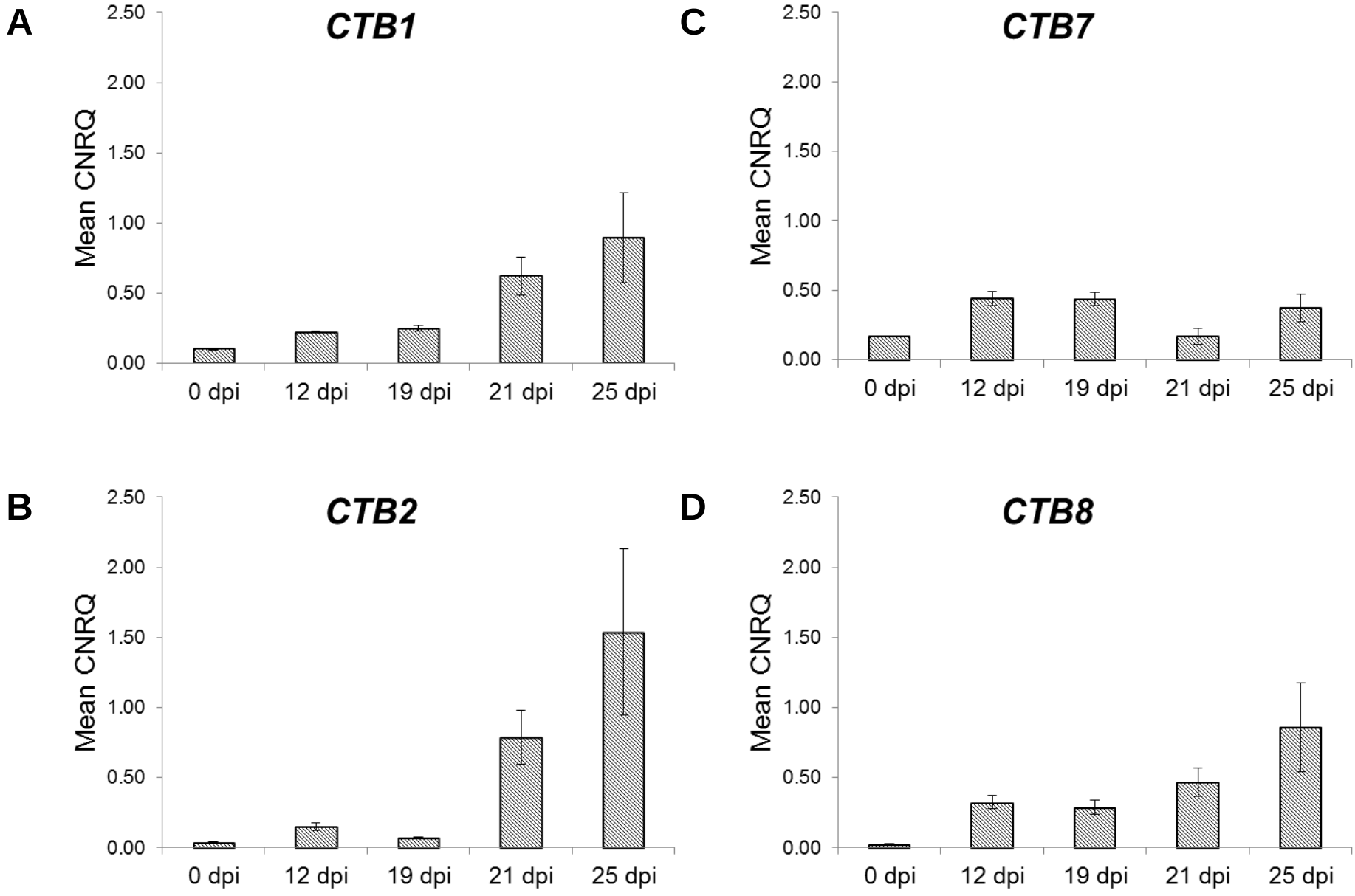
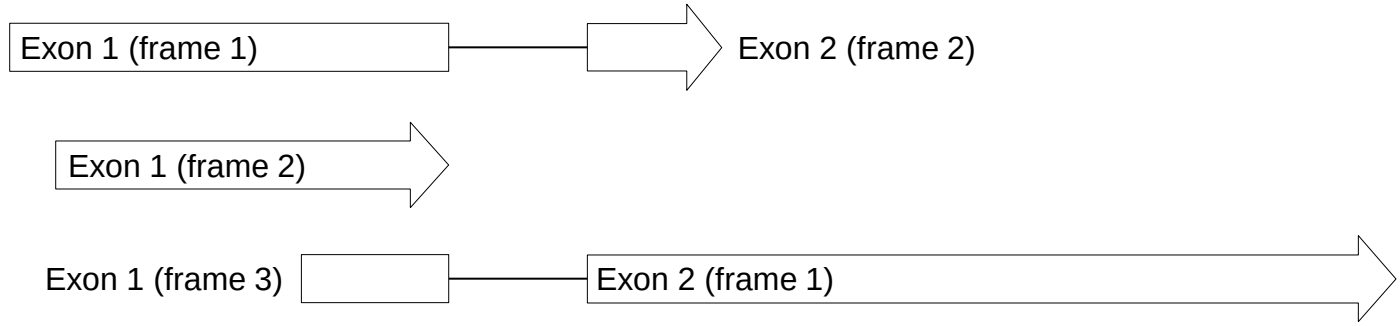


Fig.4 Swart et al. MPMI

A



B

FMN/FAD-binding site

```
Cercospora nicotianae CTB7      ----------------------MASSNRRVLVNGGGPAGAVTAFWLAKGGFEVVVTERSMSRPYGQGVDVMGRAVDIIKKMGLEQ
Cercospora zeae-maydis CTB7     ----------------------MAASKRRVLVNGGGPAGAATAFWLAKAGFDVLVTERSTTRPYGQGVDVTGRAVDILGKMGLEQ
Cercospora zeina CTB7 ORF2      MGRGPDSVPHIQACEHHPRSTVTASPNRRVLVNGAGPAGAATAFWLANGGFEVLVTERSMSRPYGQGVDVTGRAVDIITKMGLEQ

Cercospora nicotianae CTB7      RIRDSTTGEAGLTVVDDQGEDVAPPLGTAPIEGGTASVTQEIEIMRRDLTKIFVDAAEALPNVTFRYGCTVDEVQQHEKSITAVL
Cercospora zeae-maydis CTB7     RIRDNTTGEEGLVVVDDHGENVAPPLGAAPAEGGTASVTQEIEIMRRDLTRIFVEAAEALPSVTFRYGCTVDELHQHENSITAVL
Cercospora zeina CTB7 ORF2      RIRDSTTGEEGLCRLLLYAR*
Cercospora zeina CTB7 ORF3                                                      MSRD--------AQSTSSQRWAWSSAFETAPRAKKAYVAFF

Cercospora nicotianae CTB7      SDTGDPEDFTAIIGADGLGSAIRKLTFDPEINRRSVSPTNTYVAFFSIPGDPKYVSSAARRLSPAPSLCPRSELCDSEGGHDANA
Cercospora zeae-maydis CTB7     SDTREPEEFAGVIGADGLGSTIRKLAFEQRKSARLREGSSLHYCR----------------------------------------
Cercospora zeina CTB7 ORF3      SMPGDPT-------------------------------------------------------------------------------
```

Amidation site

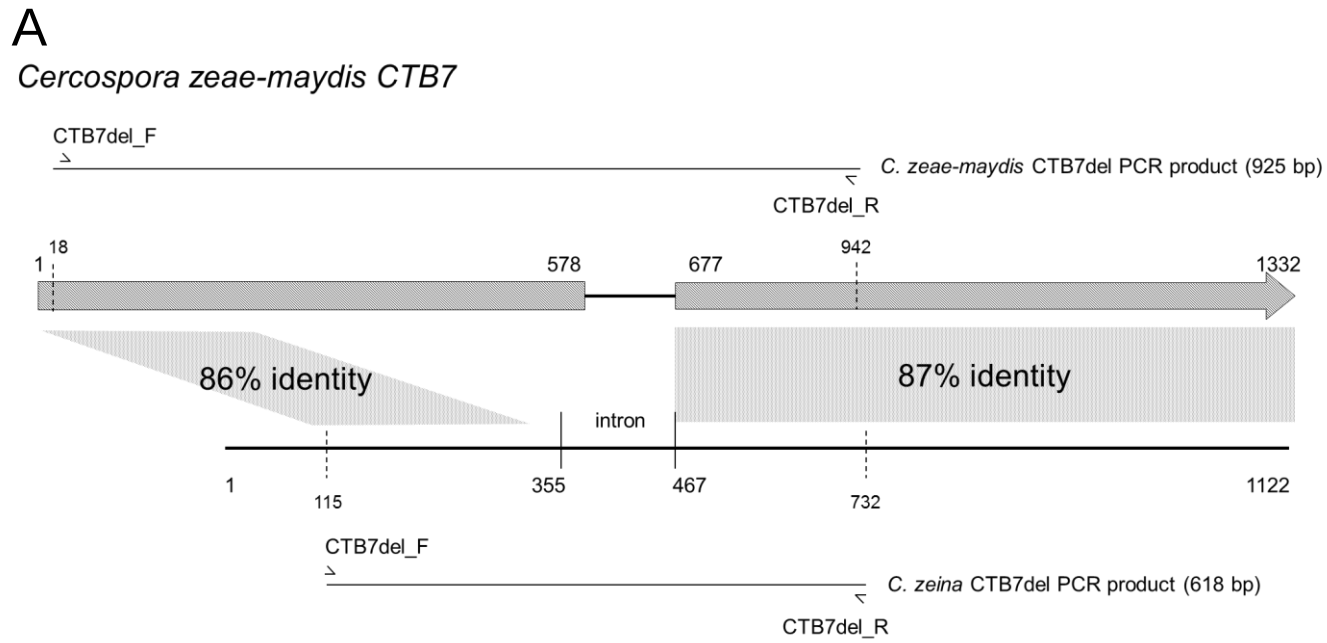
```
Cercospora nicotianae CTB7      YDTPVGKLQHANKGRGILVRPIDKKGTQRSCYLMSQSDSQELAQVARTGSQEDQKALLDNRFREFTGPLGKRAVEGMHSADDFYF
Cercospora zeae-maydis CTB7     YDTPVGKLQHANKGRGILIRPIDKKGNRSSCYLMSWTEDHDLAQVARTGSQEDQKALLDNMFRGFNGPLGKRAVEGMHRADDFYF
Cercospora zeina CTB7 ORF3      YDTPVGKLQHANKGRGIFIRPIDKKGTRSSCYLMSWTEDQDLAQVARTGSQEDRKALLDKMFRGFNGPLGKRAVEGMLDADDFYF
```

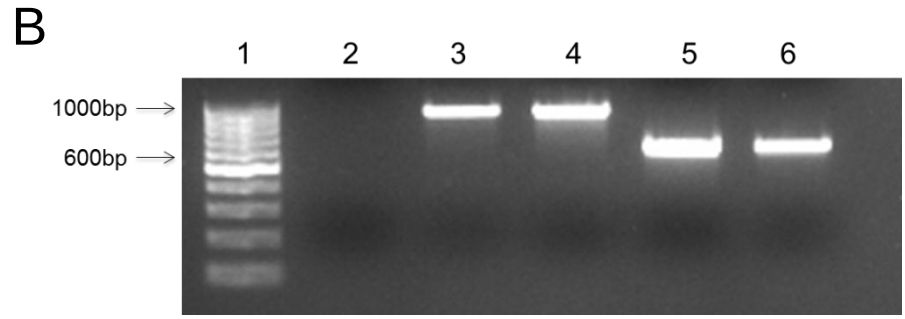
FMN/FAD-binding site

```
Cercospora nicotianae CTB7      TRIVQIKLDSWHSGRAALVGDAGYSPSPLTGQGTTLAIIGAYVLAGEMAKSPDDLERAFTSYYDILNKFVSESQEIPFGGQAPKL
Cercospora zeae-maydis CTB7     TRIVQIKLDSWHRGRAALVGDAAYSPSPLTGQGTTLAIIGAYVLAGEMAKSPDDLQQAFASYHAILKAFVSESQQIPLGGKAPKL
Cercospora zeina CTB7 ORF3      TRIVQIKLDMWHRGRAALLGDAAYSPSPLTGQGPTLAITGAYVLAGEMAKSPDDLQQAFASYHRVLKDFASESQQIPLGGQAPKL

Cercospora nicotianae CTB7      ILPQSDWGIWLLRTFYKIISWTGIWRLLNFGNETVKIE-PEYDFGGLD*
Cercospora zeae-maydis CTB7     ALPQTDWGIWILRLCYKIIALSGLWRLLNFGNETVKVDLPEYDFGPN-*
Cercospora zeina CTB7 ORF3      ALPQSDWGIWLLRFFYKIIAFSGLWRLLNFGNETVKVELPEYNLGPN-*
```

Fig.5 Swart et al. MPMI





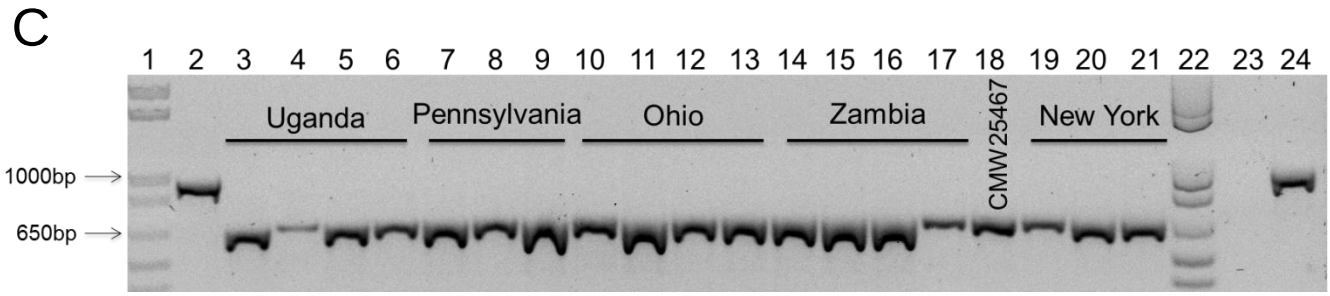


Fig.6 Swart et al. MPMI

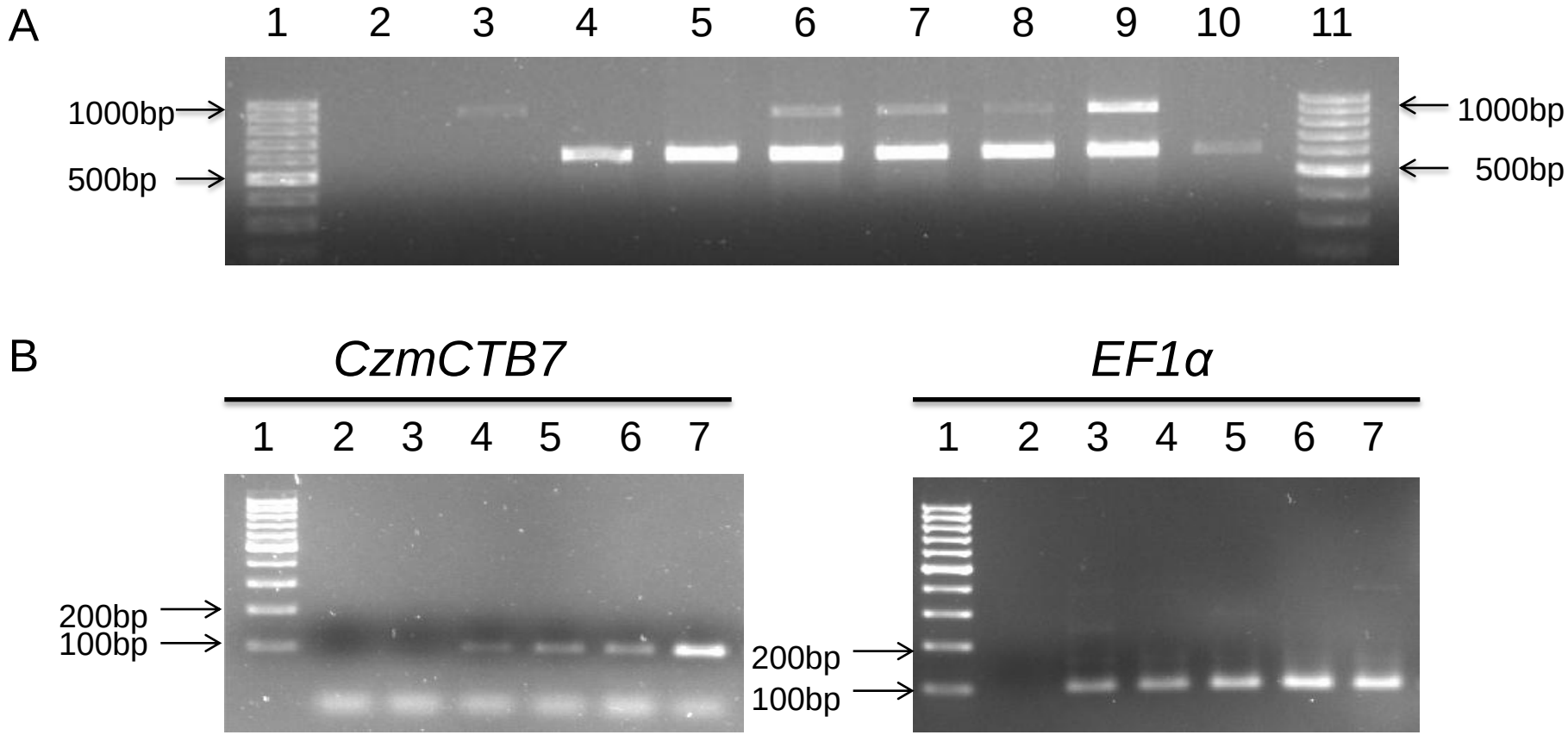


Fig.7 Swart et al. MPMI

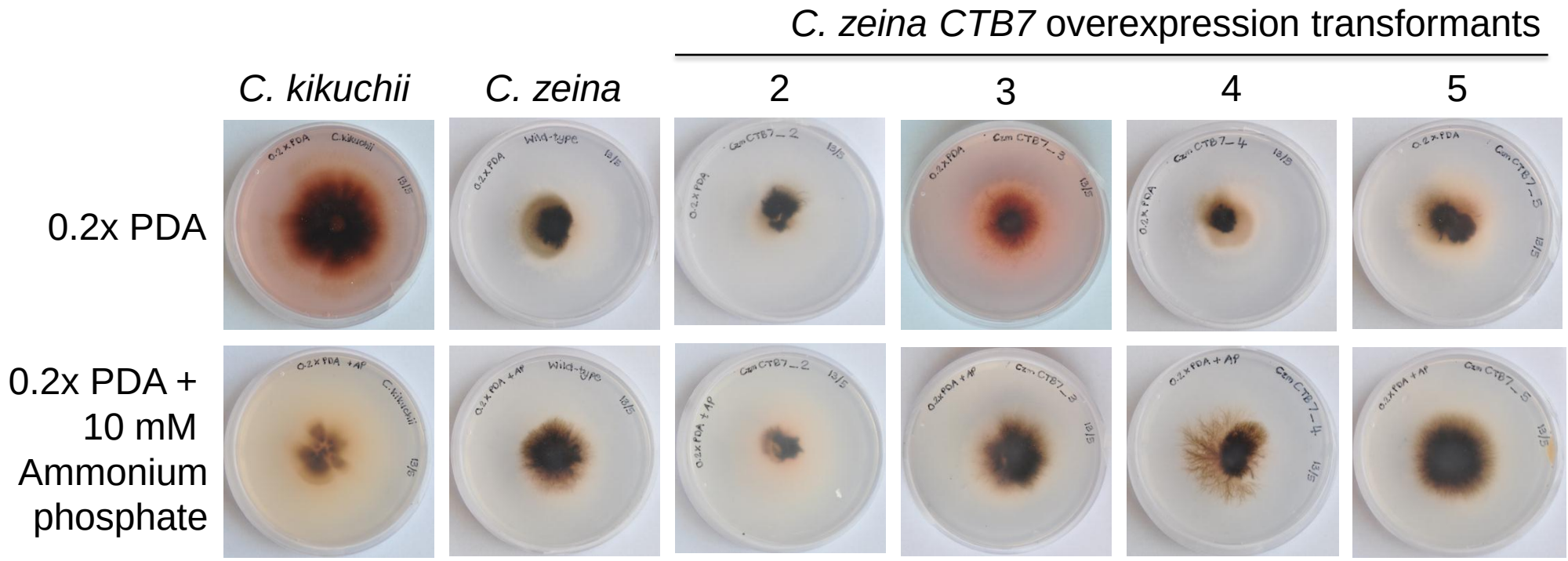
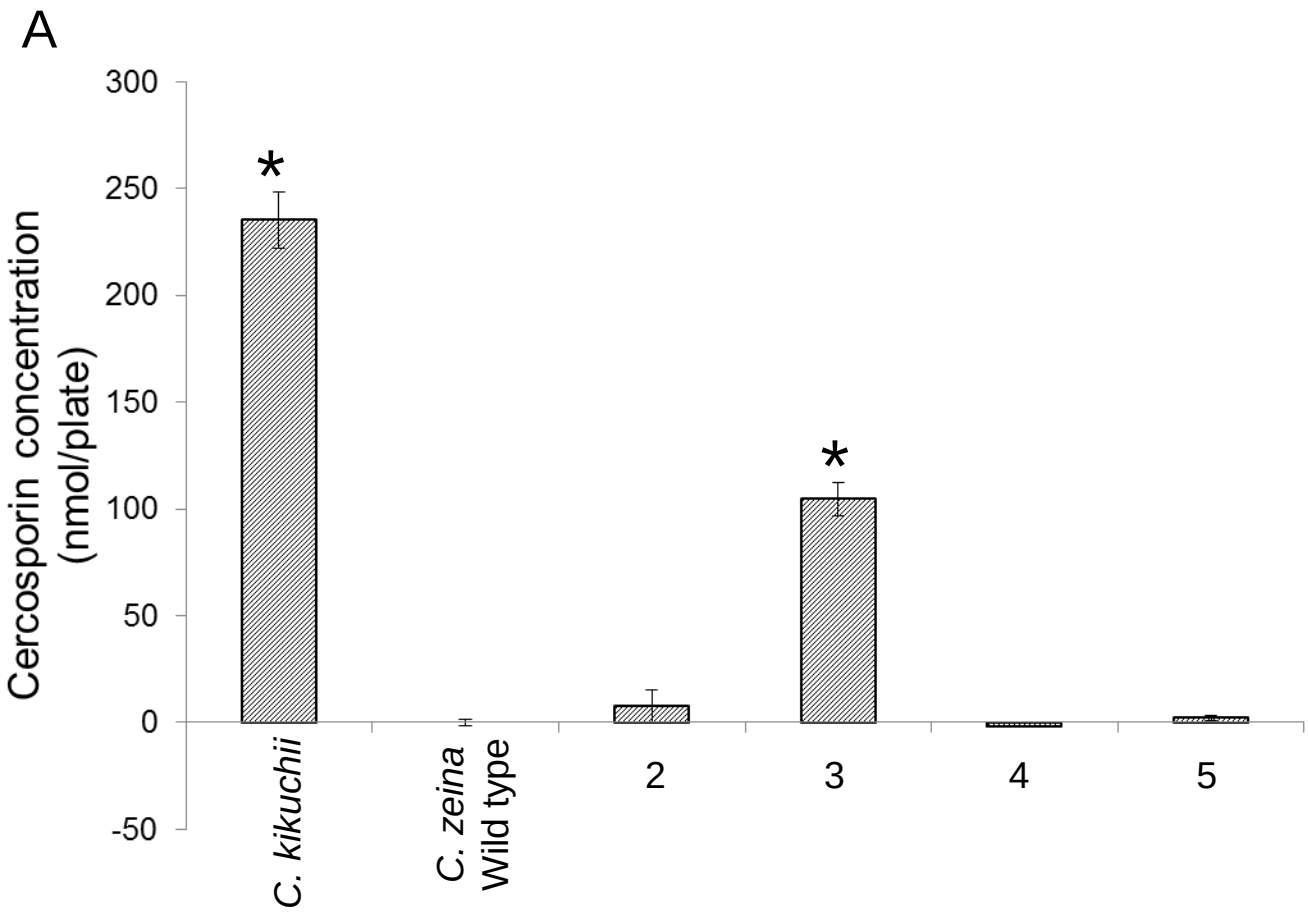


Fig.8 Swart et al. MPMI



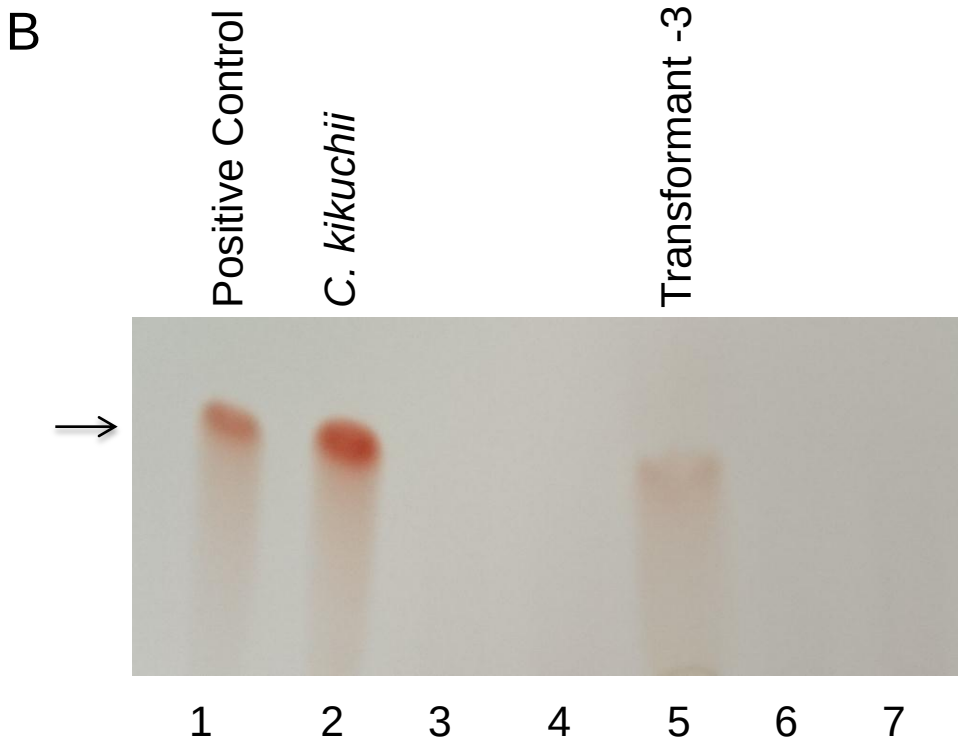


Fig.9 Swart et al. MPMI

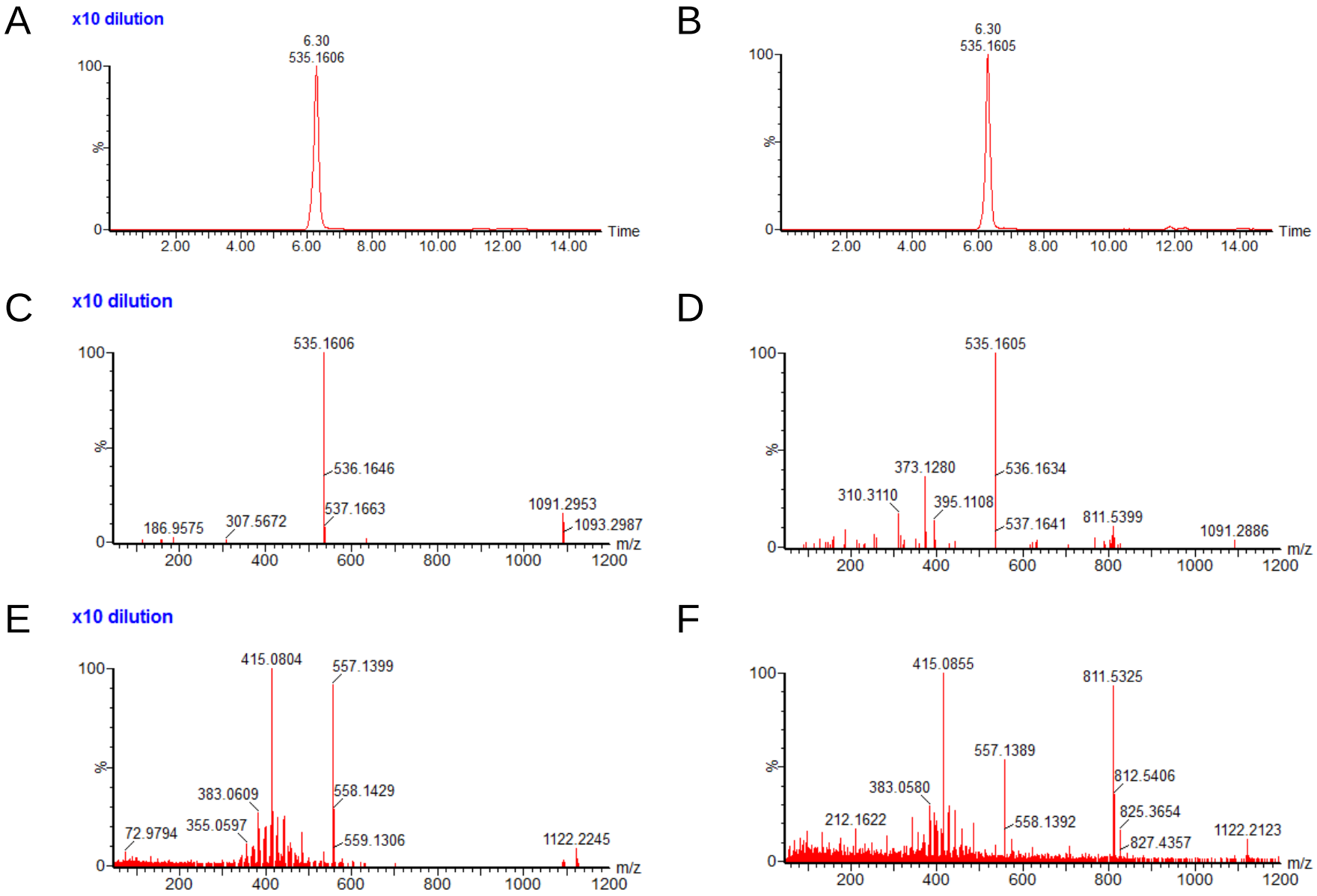


Fig.10 Swart et al. MPMI

Supplementary File S1.

Multiple sequence alignment of the *Cercospora nicotianae*, *Cercospora zeina* and *Cercospora zeae-maydis* CTB amino acid sequences.

File S1 Table 1. Percentage identity and similarity of the *C. nicotianae, C. zeina* and *C. zeae-maydis* CTB1 amino acid sequences.

	C. nicotianae	***C. zeina***	***C. zeae-maydis***
C. nicotianae	-	85.0 % identity	84.9 % identity
C. zeina	91.2 % similarity	-	90.9 % identity
C. zeae-maydis	91.5 % similarity	94.8 % similarity	-

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Cercospora nicotianae CTB1          MEDGAQMRVVAFGDQTYDCSEAVSQLLRVRDDAIVVDFLERAPAVLKAELARLSSEQQEE
Cercospora zeina CTB1               MGDDTQMRVLAFGDQTYDCSEAVSQLLRVRDDAVVVDFLERSCAVLKSELARLSSEQQRE
Cercospora zeae-maydis CTB1         MSDDMQMRVWAFGDQTYDCSEALSQLLRVRDDAIVVDFLERSCAVLKSELAGLSSEQQQE
                                    * *. **** ************:**********:********: ****:*** ****** *

Cercospora nicotianae CTB1          TPRFATLAELVPRYRAGTLNPAVSQALTCIAQLGLFIRQHSSGQEAYPTAHDSCITGVCT
Cercospora zeina CTB1               NPRFAILAELVPPYRAGTLNPALSQALSCIAQLGLFIRQHSSGQAAYPTARDSCLTGVCT
Cercospora zeae-maydis CTB1         NPRVATLAELMPAYRSGTLNPALSQALTCITQLGLFIQQHSSGQAAYPTAQDSCLTGVCT
                                    .**.* ****:* **:******:****:**:******.****** *****.***:*****

Cercospora nicotianae CTB1          GALTAVAVGSASSVTALVPLALHTVAVAVRLGARAWEIGSCLADARRGANGRYASWTSAV
Cercospora zeina CTB1               GLLSAVAVGCASSVTALVPLALHTVAVAVRLGARAWEMGRCLADVRRDAQSRYASWTAAV
Cercospora zeae-maydis CTB1         GVLSAVAVGCASSVTALVPLALHAVAVAVRLGARAWEMGRCLADVRRDAQGRYASWTAAV
                                    * *:******.*************:*************:* ****.**.*:.******:**

Cercospora nicotianae CTB1          GGISPQDLQDRISAYTAEQALASVSVPYLSAAVGPGQSSVSAAPVILDAFLSTLLRPLTT
Cercospora zeina CTB1               GGVSPQDLRERIAGYAKEQALSPISVPFVSARVGPSSGSVSAPPAILDGFLSTLPGPLTG
Cercospora zeae-maydis CTB1         GGAGLQELQERIAVYAAEKALPPLSIPFVSARVGPSSGSVSAPPVILDAFLSTLLRPLTT
                                    ** . *:*.:**: *: *:**..:*:*::** ***...****.*.***.*****  ***

Cercospora nicotianae CTB1          TRLPITAPYHAPHLFTAKDVQHVTDCLPPSEAWPTVRIPIISFSRDEAVSRGASFPAAMS
Cercospora zeina CTB1               TRLPITAPYHASHLFTSDDVQHVTDCLPRSESWPAVQVPLISFSRDEVALPGASFPAAMN
Cercospora zeae-maydis CTB1         TRLPITAPYHAPHLFTSDDVQHITDCLPRSESWPAVQIPIVSFSRDEVASHGAAFPAAMN
                                    ***********.****:.****:***** **:**:*.:*::******..  **:*****.

Cercospora nicotianae CTB1          EAVRDCLIRPIALDRMAVSITNHARDLGKDSVLPSPIALSFSDKLGPQVNSHLPGAKAPT
Cercospora zeina CTB1               EAVRDCLIRPIALDRMAVSIADYARSIGKDHVLPVPFALSFSDKLGPQVNSHLPGAKAPT
Cercospora zeae-maydis CTB1         EAVRDCLIRPIALDRMAASIAAHARSMGKDHVLPVPIALSFSDKLAPQVNSHLPGARAPT
                                    *****************.**: :**.:*** *** *:********.**********.***

Cercospora nicotianae CTB1          PELTSKSIPSAIGAEQQPMAKSPIAILAASGRFPQSSSMDQFWDVLINGVDTHELVPPTR
Cercospora zeina CTB1               LQAASTTIPPSVAAGQEPLSKSPIAILAASGRFPQSSSMDQFWDVLINGIDTHELVPPSR
Cercospora zeae-maydis CTB1         PEATSTTIPPSVAAGQEPMAKSPIAILAASGRFPQSSSMDQFWDVLINGIDTHELVPPSR
                                     : :*.:**.:::.* *:*::*******************************:********:*

Cercospora nicotianae CTB1          WNAATHVSEDPKAKNVSGTGFGCWLHEAGEFDAAYFNMSPREAPQVDPAQRLALLTATEA
Cercospora zeina CTB1               WDAAAHVSEHPKAKNVSGTGFGCWLHEAGQFDAAYFNMSPREAPQVDPAQRLALLTATEA
Cercospora zeae-maydis CTB1         WNAATHVSADPTAKNVSGTGFGCWLHEAGQFDAAYFNMSPREAPQVDPAQRLALLTATEA
                                    *:**:***  *.*****************:******************************
```

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Cercospora nicotianae CTB1          LEQAGVVPNRTSSTQKNRVGVWYGATSNDWMETNSAQNVDTYFIPGGNRAFIPGRVNYFH
Cercospora zeina CTB1               LEQAGIVPDRTSSTQKNRVGVWYGATSNDWMETNSAQNIDTYFIPGGNRAFIPGRVNFHF
Cercospora zeae-maydis CTB1         LEQAGIVPDRTSSTQRKRVGVWYGATSNDWMEVNSAQNIDTYFIPGGNRAFIPGRVNFHF
                                    *****:**:******.:***************.*****:******************:..

Cercospora nicotianae CTB1          KFSGPSYTIDTACSSSLAALHMACNALWRGEVDTAIVGGTNVLTNPDMTAGLDAGHFLSR
Cercospora zeina CTB1               KFSGPSYTIDTACSSSLAALHMACNALWRGEVDMAIVGGTNVLCNPDMTAGLDRGHFLSR
Cercospora zeae-maydis CTB1         KFSGPSYTIDTACSSSLAALHMACSALWRGEVDTAIVGGTNVLCNSDMTAGLDRGHFLSR
                                    ************************.******** ********* *.******* ******

Cercospora nicotianae CTB1          SGNCKTFDDEADGYCRGEAVVTLILKRLPDAQADKDPIQASILGIATNHSAEAASITRPH
Cercospora zeina CTB1               TGNCKTFDDEADGYCRGEAVVTLILKRLPDAQSDKDPIQAVIRGIATNHSAEADSITRPH
Cercospora zeae-maydis CTB1         TGNCKTFDDEADGYCRGEAVVTLVLKRLPDAQSDKDPIQAVIRGIATNHSAEAASITRPH
                                    :***********************:********:******* * ********** ******

Cercospora nicotianae CTB1          AGAQQDLFQQVLTETGLTANDISVCEMHGTGTQAGDSGETTSVVETLAPLNRSGSAVRTT
Cercospora zeina CTB1               AGAQQNLFQQVLTETGISANDISVCEMHGTGTQAGDNGETTSVVETLAPLNRSGCAVRPS
Cercospora zeae-maydis CTB1         PEAQQSLFQQVLAETGISANDISVCEMHGTGTQAGDNGETTSVVETLAPLNRSGSAVRPS
                                    . ***.******:***::******************.*****************.***.:

Cercospora nicotianae CTB1          --PLYIGAVKSNVGHAESAAGVSSLAKILLMLKHSKIPPHVGIKTKLNHRLPDLAARNTH
Cercospora zeina CTB1               DKKLYIGSAKSNVGHGESAAGVTSLAKVLLMLKHSKIPPHIGIKTKLNHRLPDIAARNTH
Cercospora zeae-maydis CTB1         DKKLYIGSAKANVGHGESAAGVTSLAKVLLMLKHSKIPPHIGIKTKLNHRLPDIAARNTH
                                       ****:.*:****.******:****:************:************:******

Cercospora nicotianae CTB1          IARSEVPWPRPKNGKRRVLLNNFSAAGGNTCLVLEDAPEPEDSQEVDPREHHIVALSAKT
Cercospora zeina CTB1               IPRAEVAWPRPENGKRRVLLNNFSAAGGNTCLVLEDAPEVEQFQELDPRLHHIVTLSAKT
Cercospora zeae-maydis CTB1         IPLTEVAWPRPENGKRRVLLNNFSAAGGNTCLVLEDAPELVDSQEPDPRTHHIITLSAKT
                                    *. :**.****:***************************  : ** *** ***::*****

Cercospora nicotianae CTB1          PDSMVNNLTNMITWIDKHSGDSLATLPQLSYTTTARRVHHRHRAVATGTDLLQIRSSLQE
Cercospora zeina CTB1               ADSMASNLMNMITWIDQNSGDSKNTLPRLSYTTTARRMHHKHRAVAVGTDLLQIRTSLQE
Cercospora zeae-maydis CTB1         AESMASNLMNMISWIDKNSGDSKTTLPRLSYTTTARRMHHRHRAVATGSDLSQIRKSLQE
                                    .:**..** ***:***::****  ***.*********:**.*****.*:** ***.****

Cercospora nicotianae CTB1          QLDRRVSGERSIPHPPNGPSFVLAFTGQGSAFAGMGVDLYKRFASFRSDIARYDQICEGM
Cercospora zeina CTB1               QLDRRMAGEKSVPHPPKGPSFVFAFTGQGSAFAAMGADLYQHFATFRSDIARYDQICERM
Cercospora zeae-maydis CTB1         QLDRRMAGEKSVPHPPKGPSFVFAFTGQGSAFAGMGADLYQRFATFRSDIARYDQICERM
                                    *****::**.*:****:*****:**********.**.***:.**:************** *

Cercospora nicotianae CTB1          SLPSIKAMFEDEKVFSTASPTLQQLTHVCFQMALYRLWKSLGVQAKAVVGHSLGEYAALY
Cercospora zeina CTB1               SLPSIKAMFEDDQAFLTASPTVQQLTHVCLQMALYRLWKSFGIQAKAVVGHSLGEYAALY
Cercospora zeae-maydis CTB1         TLPSIKAMFEDDQAFLTASPTVQQLAHVCFQMGLYRLWKSFGIQAKAVVGHSLGEYTALY
                                    :**********::.* *****:***:***:**.*******:*:*************:***

Cercospora nicotianae CTB1          AAGVLSQSDTLYLVGRRAQLMEKHLSQGTHAMLAVRAKEEAIVAAIDGPPGEAYEFSCRN
Cercospora zeina CTB1               AAGVLSQSDVLYLVGRRAQLMEQHLSQGTHAMLAVRAKEEAIVAAIAGPPGDAYEFSCRN
Cercospora zeae-maydis CTB1         AAGVLSQSDVLYLVGRRAQLMEQHLSQGTHAMLAVRAKEEAIVAAIAGPPGDTYEFSCRN
                                    *********.************:********************** ****::*******

Cercospora nicotianae CTB1          GEQRNVLGGTVAQIQAAKAALEAKKIRCQYLDTPMAFHTGQVDPILPELLQVAAACSIQD
Cercospora zeina CTB1               GEQRNVLGGTVDQINAAKSALETKKIRCQYLDTPMAFHTAQVDPILPELLQVAAACSIQE
Cercospora zeae-maydis CTB1         GEQRNVLGGTVEQIHAAKAALENKKIRCQYLDTPMAFHTAQVDPILPELLKVAAACSIQE
                                    *********** **:***:*** ***************.**********:********:

Cercospora nicotianae CTB1          PQIPVISPAYGKVIRSAKDFQPEYFTHHCRSSVNMVDALQSAVEEGLLDKNVIGLEIGPG
Cercospora zeina CTB1               PQIPVISPTYGRVIR--NDIQPEYFTHHCRRPVNMVDALESAIEEGLLDKNTIGLELGPA
Cercospora zeae-maydis CTB1         PQIPVISPTYGRVIR--KDMQPDYFTHHCRRPVNMVDALQSAVEEGLLDKTTIGLEVGPA
                                    ********:**.***  :*:**:******* .*******:**:*******..****:**.

Cercospora nicotianae CTB1          PVVTQFVKEAVGTTMQTFASINKDKDTWQLMTQALAKFYLAGASVEWSRYHEDFPGAQKV
Cercospora zeina CTB1               AVVTQLIKEAVGTSMQAFASINKDKDTWQLMTHALARMYLAGAGVEWSRYHEDFSGAQKV
Cercospora zeae-maydis CTB1         PVVTQLIKEAIGTTMQTFASISKDKDTWQLMTLALARIYLAGANVEWSRYHEDFPGAQKV
                                    .****::***:**:**:****.********** ***.:*****.**********.*****

Cercospora nicotianae CTB1          LELPAYGWALKNYWLQYVNDWSLRKGDPAVVVAASNLELSSSIHKVITNTITANSDGELV
Cercospora zeina CTB1               LELPAYGWTLKNYWLQYVNDWSLRKGDPPTIVTASNLELSPSIHKVVTNTINPSSDGELV
Cercospora zeae-maydis CTB1         LELPAYGWTLKNYWLQYVHDWCLRKGDPPIIVAASNLELSSSVHKVITNTINPSSDGELI
                                    ********:*********:**.******. :*:********.*:***:****...*****:
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```
Cercospora nicotianae CTB1          VDADLSREDLHPMVQGHQVYGVPLCTPSVYADIALTLGEYIRQVIKPGEVAQTSVEVAEM
Cercospora zeina CTB1               VDADLSREDLHPMVQGHQVYGVPLCTPSVYADIAMTLGEYIRKIIKPGQIAQTAVEVAEM
Cercospora zeae-maydis CTB1         VDADLSREDLHPMVQGHQVYGVPLCTPSVYADIAMTLGEYIRQIIKPGQIAQTAVEVAEM
                                    ************************************:********::****::***:******

Cercospora nicotianae CTB1          NIQSALVANNTGRVQLLRTCAKFDPKAQVASCTFSSI-------VEQHANCKIRFGSLEK
Cercospora zeina CTB1               NIQSALVANSTGKVQLLRTCAKFDPKAQIASCTFSTVKEDGNSVIEQHANCQIRFFDLEN
Cercospora zeae-maydis CTB1         NIQSALVANSTGKVQLLRTCAKFDPKAQVASCTFSTI---------KHANCQIRFVNLEN
                                    *********.**.***************:******::         :****:*** .**:

Cercospora nicotianae CTB1          EKTALKSAALAAQASMAALKTQVGQDDNTYRFSKGMIYKMIGQLADFDEKYRGLCAITLD
Cercospora zeina CTB1               EKRGLRDAAIAAQARMGALKAQIGQDDNTYRFSKGMIYKMIGQLADFDEKYRGLCAITLD
Cercospora zeae-maydis CTB1         EKRGLRNAAIAAQARMAALKAQIGQDDHTYRFSKGMIYKMIGQLADFDEKYRGLCAITLD
                                    ** .*..**:**** *.***:*:****:*********************************

Cercospora nicotianae CTB1          NDAMEASGKVSFKGIPNEGKFHSSPAYLDALSQLGGFVMNANEGVDLEKEVFVNHGWGSM
Cercospora zeina CTB1               NDQMEASGTVSFKGIPNDGKFHTSPAYLDALSQLGGFVMNGNEGLDLEKEVFVNHGWGSM
Cercospora zeae-maydis CTB1         NDQMEASGTVSFKGIPNEGKFHTSPAYLDALSQLGGFVMNGNEGVDLEKEVFVNHGWGSM
                                    ** *****.********:****:*****************.***:***************

Cercospora nicotianae CTB1          RFFAALDPAMTYYTHVKMTQGKDKLWTGDVLIFDDKQALIGIVGGVALQGVPKRLMHYIV
Cercospora zeina CTB1               CFFAALDPAMTYYTHVKMTQGKDKLWTGDVLIFDGKQALIGIVRGVALQGVPKRLMHYIV
Cercospora zeae-maydis CTB1         CFFAALDPRMTYYTHVKMTQGKDKLWTGDVLIFDEKQALIGIVRGVALQGVPKRLMHYIV
                                     ******* ************************ ******** ****************

Cercospora nicotianae CTB1          TAANKKASGPPTEKKTSSPPVEKKASAPVAPTRPAIQRKNASIPPPATQVTPQNKTIKTP
Cercospora zeina CTB1               TAANKKASG-------GTAPAEKKVSTPVAPTRPAIQRKNASIPPPSTQSTVQTKTNNTP
Cercospora zeae-maydis CTB1         TAANKKACG-------GKAPAEKSASVPVAPTRPAIQRKNASTPPPSIQSTVRTKINDTP
                                    *******.*       ...*.**..*.*************** ***: * * ..*  .**

Cercospora nicotianae CTB1          SVSALIAPALEIVSEEIRMPIDELKDDIDFTDAGLDSLLSLVISSRMRDQLGIEFESAQF
Cercospora zeina CTB1               SVSALIAPALEIVSEEIGMPIDELKDDIDFTDAGLDSLLSLVISSRMRDQLGIEFESAQF
Cercospora zeae-maydis CTB1         SVSALIAPALEIVSEEIGMPVDELKDDIDFTDAGLDSLLSLVISSRMRDQLGIEFESAQF
                                    ***************** **:*************************************

Cercospora nicotianae CTB1          MEIGSIGGLKEFLTRLSPPVAVAVATAVEIVKEEALTSLEELTDPSPNEIGTVWRDALKI
Cercospora zeina CTB1               MEIGSIGGLKQFLTKLSPPVAVAVATAVEVVKEEALAALEELASPTSDEIGAVWRDALKI
Cercospora zeae-maydis CTB1         MEIGSIGGLKQFLTKLSPPVAVAVATAVEVVKEEALTSLEELANPTPDEIGAVWRDALKI
                                    **********:***.**************:******::****:.*:.:***:********

Cercospora nicotianae CTB1          LSEESGLTDEELTDDTSFADVGVDSLMSLVITSRLRDELDIDFPDRALFEECQTIFDLRK
Cercospora zeina CTB1               LTEESGLTNDELTDDVSFTDVGVDSLMSLVITSRLRDELDIDFPDRALFEECQTISDLRK
Cercospora zeae-maydis CTB1         LSEESGLTGEELTDDVSFTDVGVDSLMSLVITSRLRDELDIDFPDRALFEECQTISDLRK
                                    *:*******.:******.**:************************************ ****

Cercospora nicotianae CTB1          RFSGSTESFDSTTTKPSAGDATPPLTDSSASSPPSSEFDGETPMTDLDEVFDSPPAQKRI
Cercospora zeina CTB1               KFSLPTEYLDSTSTEANAGHTTPQLTDSSSSSPSSSVYEGETPMTDLDEVFDSPPAQKK-
Cercospora zeae-maydis CTB1         KFSLPTASLDSTTTKSNAADTTPPLTDASSSSPASSVYEGETPMTDLDDVFDSPPSQRK-
                                    .** .*  :***:*:..*. :** ***:*:***.** ::*********:******:*..

Cercospora nicotianae CTB1          PSPPKGRIPPAWSMYLQGSQKRSKEILFLFPDGAGAATSYLSLPRLGEDIGVVAFNSPFM
Cercospora zeina CTB1               PGPPKQQIPPAWSMYLQGSQKRSKEILFLFPDGAGAATSYLSLPRLSPDIGVVAFNSPFM
Cercospora zeae-maydis CTB1         PAPPKQQIPPAWSMYLQGSQKRSKEILFLFPDGAGAATSYLSLPRLSPDIGVVAFNSPFM
                                    *.*** .************************************. ************

Cercospora nicotianae CTB1          KTPHKFADHTLPDVIASYVEGIRGRQAQGPYHLGGWSAGGILAYAVAQELIAAGEEVSTL
Cercospora zeina CTB1               KTPHKFADHTLPEVIASYIEGIRGRQPQGPYHLGGWSAGGILAYAVAQELISAGEEISTL
Cercospora zeae-maydis CTB1         KTPHKFADHTLPEVIASYIEGIRGRQPHGPYHLGGWSAGGILAYAVAQELIAAGEEISTL
                                    ************:*****:*******.:************************:****:***

Cercospora nicotianae CTB1          LLIDSPSPTKGLDRLPTRFFDHCTNVGLFGTELSRGSGGPNKTPEWLMPHFRASIELLHG
Cercospora zeina CTB1               LLIDSPSPIKGLDRLPTRFFDHCTNVGLFGTELSRGSGGSKTPPEWLMPHFRASIELLHD
Cercospora zeae-maydis CTB1         LLIDSPSPIKGLDRLPTRFFDHCTNVGLFGTELSRGSGVPSKTPEWLMPHFRASIELLHD
                                    ******** ***************************** ....****************.

Cercospora nicotianae CTB1          YHAPPMKLGNKTKVMVIWAGECAFDGVRYAHIPPSAGDTDEDTEGMKFLTEKRKDFGATE
Cercospora zeina CTB1               YHAPPMKPGNKTKVMLIWAGECAFDGVRYAHLPPSSGDTDEDTEGMKFLTEKRKDFGATE
Cercospora zeae-maydis CTB1         YHAPPMKPGHKTKVMLIWAGECAFDGVRYAHLPPSAGDTDEDTEGMKFLTEKRKDFGPTE
                                    ******* *:*****:***************:***:**********************.**
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Cercospora nicotianae CTB1          WASLFPGTDVDARVVESEHHFSMMRDSGAQMLVEHMRDGLGIVSS
Cercospora zeina CTB1               WSGLFPGNDVDARVVESEHHFSMMRDGGAQTLVEHMRDGLGIVAS
Cercospora zeae-maydis CTB1         WEKLFPGNDVVARVVESEHHFSMMRDAGARILVEHMREGLGIVSS
                                    *  ****.** **************.**. ******:******:*
```

File S1 Fig. 1. Multiple sequence alignment of the *C. nicotianae, C. zeina* and *C. zeae-maydis* CTB1 amino acid sequences.

File S1 Table 2. Percentage identity and similarity of the *C. nicotianae, C. zeina* and *C. zeae-maydis* CTB2 amino acid sequences.

	C. nicotianae	***C. zeina***	***C. zeae-maydis***
C. nicotianae	-	85.9 % identity	89.5 % identity
C. zeina	90.4 % similarity	-	91.0 % identity
C. zeae-maydis	92.7 % similarity	94.0 % similarity	-

```
Cercospora nicotianae CTB2          MVKRIEADNLFELTAELVSASSKLHKFLDQKNLPQPSFDAPAPSVALNSANKPYYDARSA
Cercospora zeina CTB2               MASRIEADGLFELTAELVSASSKLNKFLDQKGLPQPSFDAPAPSVALNTENKPYYDARSA
Cercospora zeae-maydis CTB2         MSNRIEADGLFELTAELVSASSKLHKFLHQKGLPQPSFDAPAPSVALNTANKPYYDARSA
                                    * .******.***************:*** **.****************: **********

Cercospora nicotianae CTB2          IVEAAEQLIRLVRGPRDTLLALSFEHCATASMQVVFKYKFANHIPLHGSTTYSKIAEAVG
Cercospora zeina CTB2               IVEAAEQIIRLVRGPRDTLLALSFEHCATASMQVIFRYKFASHIPLHGSTTYSKIAAAVG
Cercospora zeae-maydis CTB2         IVEAAEQIIRLVRGPRDTLLALSFEHCATASMQVVFKYKFAAHIPLHGSTTYSKIAEAVG
                                    ********:***************************:*.**** ************** ***

Cercospora nicotianae CTB2          DGVTTALVERTIQHCASFGLFETIPGAMLLQ-CYLVLLVTDPDLEAWMYLSAVIAYPAGA
Cercospora zeina CTB2               EGVTAALVERTIQHCASFGLFETIPGGYVTHNATSSLLVTDPDLEAWMYLSAVIAYPAGA
Cercospora zeae-maydis CTB2         QGVTTPLVERTIQHCASFGLFETIPGGYVTHNATSALLVTDPDLEAWMYLSAVIAYPAGA
                                    :***:.*********************. : : .   ************************

Cercospora nicotianae CTB2          AIPKAVEQYGVSHEADESGYGASIGRKIAQFQRFREPDGKKDHEMFARAMRGIAAGGAYD
Cercospora zeina CTB2               AIPKAVEQYGVSMEADEAGYGASIGRKIAQFQRFREPDGKKDHEMFARAMRGIAAGGAYD
Cercospora zeae-maydis CTB2         AIPKAVEQYGVSMEADEAGYGASIGRKIAQFQRFREPDGKKDHEMFARAMRGIAAGGAYD
                                    ************ ****:****************************************

Cercospora nicotianae CTB2          FRHAVDGGYPWHLLAEGAGHLVVDVGGGPGHVAMALAEKYPSLRFQVQDLPETVQVGAKN
Cercospora zeina CTB2               FRHAVDGGYPWHLLAKGAGHLVVDVGGGPGHVAMALAEKYPTLRFEVQDLPETVQVGAKN
Cercospora zeae-maydis CTB2         FRHAVDGGYPWHLLAEGAGHLVVDVGGGPGHVAMALAEKYPSLRFEVQDLPETVQVGAKN
                                    ***************:************************:***:**************

Cercospora nicotianae CTB2          CPEHLKSRVSFQSHDFFTSQPAHEVQDGEGIVYFARFILHDWSDKYATKIVQQLATGLRP
Cercospora zeina CTB2               CPGHLQKRVAFRAHDFMTLQPAHEVQGDEGIAYFARFILHDWSDKYATKIVQQLASGLRP
Cercospora zeae-maydis CTB2         CPEHLQKRVAFRAHDFMTPQPAHEVKGDEGIAYFARFILHDWSDKYATKIVQQLASGLRP
                                    ** **:.**:*.:***:* ******:..***.***********************:****

Cercospora nicotianae CTB2          QDRIILNEVVVPEAGQVGRETERRMHDRDLLMLMNLNGRERTQSAFEAIFASVTPKLRLQ
Cercospora zeina CTB2               QDRIILNEVVVPESGQVGRETQRRMHDRDLLMLMNLNGRERTQSAFEAIFASVTPKLRLQ
Cercospora zeae-maydis CTB2         QDRIILNEVVVPESGQVGRETERRMHDRDLLMLMNLNGRERTQSAFEAIFASVTPKLRLQ
                                    *************:*******:************************************

Cercospora nicotianae CTB2          RVIHPEQGELSLIEVTLDGVELPAQANGVNG----HANGTNGVNGH
Cercospora zeina CTB2               RVHYPEQGELSLIEVTLDGVELPGHGDA--------------VKWH
Cercospora zeae-maydis CTB2         RIHHPEQGELSLIEVTLDGVELPSHSQAVNGTNGVHGNGTNGVNGH
                                    *: :*******************.:.:.             *: *
```

File S1 Fig. 2. Multiple sequence alignment of the *C. nicotianae, C. zeina* and *C. zeae-maydis* CTB2 amino acid sequences.

File S1 Table 3. Percentage identity and similarity of the *Cercospora nicotianae, C. zeina* and *C. zeae-maydis* CTB3 amino acid sequences.

	C. nicotianae	***C. zeina***	***C. zeae-maydis***
C. nicotianae	-	87.5 % identity	87.2 % identity
C. zeina	93.1 % similarity	-	90.6 % identity
C. zeae-maydis	92.4 % similarity	94.5 % similarity	-

```
Cercospora nicotianae CTB3          MMQFQRDLEASLEAVSANAQELLKSLKSRKDVQDLNASLPKDPLDNCDAQTQAARAQLAE
Cercospora zeina CTB3               MMQFQRDLEASLEAVSTNAQKLLAYLESCKDVQNLNTSLPKDPLDDCDAPTQAARAQLAE
Cercospora zeae-maydis CTB3         MMQFERDLEASLEAVSTNAQKLLAYLKSGKNVQSLDTALPKDPLDNCDAQTQAARGQLAE
                                    ****:***********:***:**  *:* *:**.*:::*******:*** *****.****

Cercospora nicotianae CTB3          AATRILQLSIRPQEYLEHLQNGYQHLTCFRWLVELNILDHLPHSGTISYTDLARKASVPP
Cercospora zeina CTB3               AATRVLELSIRPQDYLEHLQNGYQNLTCIRWLVELNILDHVPHSGTISYSDLASKASVPP
Cercospora zeae-maydis CTB3         AATRILELSTRPQEYLEHLQNGYQNLTCIRWLVELNILDHVPHSGTISYSDLASKASVPP
                                    ****:*:** ***:**********:***:***********:********:*** ******

Cercospora nicotianae CTB3          MQLRSICRMAICNGFLEEPEANQVRHSRISALFARDESYLGWARWMVNYSVPAAYKLSDA
Cercospora zeina CTB3               MQLRSVCRMAICNGFLQEPEANQVCHSRISALFARDESYLGWARWMVNYSVPSAYKLSDA
Cercospora zeae-maydis CTB3         MQLRSICRMAICNGFLREPQLNQVGHSRISALFARDESYLAWARWMVNYSVPSAYKLSDA
                                    *****:********** **: *** ***************.***********:*******

Cercospora nicotianae CTB3          TRSWGETVAKDQTAFNLGMDVKVPFFDHLRQTPAMKDAFAAYMRNVTSNATWGLQHAVTG
Cercospora zeina CTB3               TRSWGETVAKDQTAFNLGMDVKVPFFDHLRQTPEMKDAFAAYMRNVTSNETWGLQHAVSG
Cercospora zeae-maydis CTB3         TRSWGETVAKDQTAFNLGMDVKVPFFDHLRQTPEMKDAFAAYMRNVTSNETWGLQHAVSG
                                    ********************************** ************** ********:*

Cercospora nicotianae CTB3          FDWASLPRGAKVVDVGGSLGHGSIAIAKEHTHLTFVIQDLPETVAGARKEMAQNDKIEAS
Cercospora zeina CTB3               FDWASLRPGAKVVDVGGSLGHGSIAIAKQHTHLNFIVQDLPETIAGARKEIAQDSKIDDS
Cercospora zeae-maydis CTB3         FDWASLPPGAKVVDVGGSLGHGSIAIAKQHPHLSFIVQDLPETIAGARKGMAEDGKIDDS
                                    ******  *********************:*.**.*::******:***** :*::.**: *

Cercospora nicotianae CTB3          VKSRITFQEHDFFGPQTVKDADVYFLRMICHDWPDNEAKVILSQIRAALKPGAQIVIMDT
Cercospora zeina CTB3               VKSRIQYMEHDFFGEQPVKDADVYFLRMICHDWPDNEAKIILSQIRAAMKPGAQIVIMDT
Cercospora zeae-maydis CTB3         VKSRIQYMEHDFFGEQPVKDADVYFLRMICHDWPDNEAKVILSQIRAAMKPGAQIVIMDT
                                    ***** : ****** *.**********************:********:***********

Cercospora nicotianae CTB3          ILPQPGTISVLQEQQLRIRDLTMMEVFNAKERELEDWSSLMQSAGLEISRVNQPLNSVMG
Cercospora zeina CTB3               ILPQPGTISVLQEQQLRIRDLTMMEVFNAKEREYEDWSALMQSAGLEISHVNQPLNSVMG
Cercospora zeae-maydis CTB3         ILPQPGTISVLQEQQLRIRDLTMMEVFNAKEREFEDWSSLMQSAGLEISHVNQPLNSVMG
                                    ********************************** ****:**********.**********

Cercospora nicotianae CTB3          LLTVRSAGQTALSGTNTLTPELVAAVSASTGSADSRPVLIAGAGIAGLCLAQALKKAGID
Cercospora zeina CTB3               LLTVRSADKPALPSAGPVARELSLAVPTSGRSEIAKPVLITGAGIAGLCLAQALKKAGID
Cercospora zeae-maydis CTB3         LLTVRSVGQSALPNAETSAPALSAAVSTSRDSALTKPVLIVGAGVAGLCLAQALKKAGID
                                    ******..:.**..: . :  *  **.:*  *  :.****.***:***************

Cercospora nicotianae CTB3          FRVFERDSHIDARPQGYRLKFEADAAQSLKNILPDDVYEAFELSNAVTAVGETDFNPFNG
Cercospora zeina CTB3               FRVFERDPHIDARPQGYRLKFEADAAQSLKNILPDRVHEAFELSNAITAVGETDFNPLNG
Cercospora zeae-maydis CTB3         FRVFERDAHIDARPQGYRLKFEADAAQSLKNILPDSVYEAFELSNAITAVGETDFNPFNG
                                    *******.************************** *:********:**********:**

Cercospora nicotianae CTB3          NIIHSRTGGGLSGKKGLYATFTVDRKAFRTQLMTGIEDKISFGKEIAYYKTDDATSTVNA
Cercospora zeina CTB3               TIIHSRTGGGLSGKQGLYATFTVDRKAFRTQLMTGIEDKISFGKELAYYKTDEATSTVTA
Cercospora zeae-maydis CTB3         TIIHSRTGGGLSGTQGLYATYTVDRTAFRTQLLTGIEDKISFGKELAYYKTDDSTSTVTA
                                    .************.:*****:****.******:************:******::****.*
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Cercospora nicotianae CTB3          EFKDGTHVTGSFLAGTDGLHSVVRKTCVPNHRIVDTGAACIYGKTVMTPEFLARFPEKGL
Cercospora zeina CTB3               EFKDGTHFTGSFLAGTDGLHSVVRKTCVPNHRIVDTGAACIYGKTVMTPEFLARFPEKGL
Cercospora zeae-maydis CTB3         EFKDGTHFTGSFLAGADGLHSAVRKRRVPNHRVVDTGAACIYGKTVMTPEFLARFPEKGL
                                    *******.*******:*****.***  *****:**************************

Cercospora nicotianae CTB3          RFMTVVSDIAPMLQSCLIGDSPVTLLLEPIRFSEASRARYPELPPDYVYWALIGPKERFG
Cercospora zeina CTB3               RFMTVCSDVAPMLQSCLIGDSPVTLLLEPIRFSEASRARHSELPPDYVYWALIGPTERFG
Cercospora zeae-maydis CTB3         RFMTVCSDVAPMLQSCLIGDSPVTLLLEPIRFSEASRARHPELPPDYVYWALIGPKERFG
                                    ***** **:*******************************:.**************.****

Cercospora nicotianae CTB3          SQEVTSMKNFVSLDQAAEQAAKLSLAVTEEWHPSLRALFELQDTKQASLIRVASTIPDIP
Cercospora zeina CTB3               SPEVTAMKNFVSLEQAAQQAAQLSLAVTEEWHPSIRALFELQDTKQASLIRVASTIPDVP
Cercospora zeae-maydis CTB3         SPEVTAMKNFVSLEQAAHQAAKLSLAVTEEWHHSLRALFELQDIQQASLIRVASTIPDVP
                                    * ***:*******:*** ***:********** *:******** :**************:*

Cercospora nicotianae CTB3          SWESHSNVTVLGGSIHPMSPCGGVGANTAIVDADALAKVLVEHGTKPPVNAIAEFGAAMR
Cercospora zeina CTB3               SWEPHSNVTLLGDSIHPMSPCGGVGANTAIVDADALAQVLVEHGTKPSVKAIAEFEAAMR
Cercospora zeae-maydis CTB3         SWEPHSNLTVLGDSIHPMSPCGGVGANTAIVDADALAKVLVEHGTKPPVHAIAAFEADMR
                                    ***.***:*:**.************************:*********.*:*** * * **

Cercospora nicotianae CTB3          TRAKRNIWRSEVGSKRMFGQKNLVDCSEFVF-
Cercospora zeina CTB3               MRAKKNICRSEIGSKRMFGQKDLVDCADFGFQ
Cercospora zeae-maydis CTB3         ARAKKNICRSEIGSKRMFGQKDLVDCDDFGF-
                                     ***.** ***:*********:**** :* *
```

File S1 Fig. 3. Multiple sequence alignment of the *C. nicotianae, C. zeina* and *C. zeae-maydis* CTB3 amino acid sequences.

File S1 Table 4. Percentage identity and similarity of the *C. nicotianae, C. zeina* and *C. zeae-maydis* CTB4 amino acid sequences.

	C. nicotianae	***C. zeina***	***C. zeae-maydis***
C. nicotianae	-	89.9 % identity	90.4 % identity
C. zeina	95.9 % similarity	-	91.6 % identity
C. zeae-maydis	94.7 % similarity	95.5 % similarity	-

```
Cercospora nicotianae CTB4          MAPPITDDDLDGLKQPYVTFSSGSASPPRSTAEAMDFEEQILEAIKSDAFLVDWIGEDDK
Cercospora zeae-maydis CTB4         MALSIADDDLDGLKRPYVTFSSGSASPPRSTNEAMEFEEQILAAIKSDAFLVDWVGEDDK
Cercospora zeina CTB4               MAPSIADDDLDGLKRPHVTFSSGAASPPQSTAEAMEFEEQILETIKSEAYLVDWVGDNDK
                                    ** .*:********.*:******:****.** ***:****** :***:*:****:*::**

Cercospora nicotianae CTB4          GNPQNLPYWRKWVITMSLALYALSTTFSSSVFGAATHVLAEEFALPAETVVLGCTSLFMV
Cercospora zeae-maydis CTB4         GNPQNLPYWRKWVITMSLALYALSTTFSSSVFGAATHVLAKEFTLPAETVVLGCTSLFMV
Cercospora zeina CTB4               GDPQNLPYWRKWVITMSLALYALSTTFSSSVFGAATHVLAKEFALPAETVVLGCTSLFMV
                                    *:**************************************:**:****************

Cercospora nicotianae CTB4          GFATGPIFWGPFSEAFGRTRPLLAGYLGFAVLQLPIADARSLTSICILRFLGGFFGAAPS
Cercospora zeae-maydis CTB4         GFAGGPILWGPLSEAFGRTRPLIVGYLLFAILQLPIADARSPTTIFGLRLLGGFFAAAPS
Cercospora zeina CTB4               GFATGPVFWGPFSEAFGRTRPLIAGYLAFAILQLPIADAQSPTTIFCLRYLQGLTGAAPS
                                    *** **::***:**********:.*** **:********.* *:*  ** * *: .****

Cercospora nicotianae CTB4          SILSGILADIWSPRERGFAMPTVGAFLTIGPILGPLIGSVLVQSVLGWRWIANVVAIASF
Cercospora zeae-maydis CTB4         SILSGTLADIWSPRERGFAMPTVGAFLTIGPILGPLIGSVLVQSVLGWRWIANVVAMASF
Cercospora zeina CTB4               SILSGTLADIWSPRERGFAMPTVGAFLTIGPILGPLIGSVLVQSALGWRWIANVVAIASF
                                    ***** ************************************.**********:***

Cercospora nicotianae CTB4          LIALSTFPFLPETYTPLLLARRAERMRHMTRNWAYRSKSEEAQSSIGDFAERYLLRPARM
Cercospora zeae-maydis CTB4         VIAICTFPFMPETYPPLLLARRAERMRHMTRNWAYRSKSEEAQSSLGDFAERYLLRPARM
Cercospora zeina CTB4               LIAICTFPFMPETYSPLLLARRAERMRHMTRNWAYRSKSEEARSSLGDFAERYLLRPARM
                                    :**:.****:****.**************************.**:**************

Cercospora nicotianae CTB4          LALEPILLMMTLYVSVSFGLLYNFFLAYPTSFIQERGWDQTTASLPLISILVGAIIAGAL
Cercospora zeae-maydis CTB4         LALEPILLMMTLYVSVSFGLLYNFFLAYPTSFIQERGWDQISASLPLISILVGVIIAGAL
Cercospora zeina CTB4               LALEPILLMMTMYVSVSFGLLYNFFLAYPTSFIQERGWDQISASLPLISILVGVIMAGAL
                                    ***********:*************************** :***********.*:****

Cercospora nicotianae CTB4          LSFSTNSRWAPNAKEGRPQETRLLLMMVGAVSLPAGMFLFAWTSSATMNPWPQILSGIPT
Cercospora zeae-maydis CTB4         LSFTTNSRWAPNVAKGRPQETRLLLMMAGAVSLPAGMFCFAWTSAATMSPWPQILSGIPT
Cercospora zeina CTB4               LSFTTNSRWAPNANEGRPQETRLLLMMVGAVSLPAGMFCFAWTSSATMNPWPQILSGVPT
                                    ***:********. :************.********** *****:***.********:**

Cercospora nicotianae CTB4          GFGIHLINMQGMNYIIDSYKIYANSAIAANTFLRSLFAAGFPILATSMYAAIGVKWGTTI
Cercospora zeae-maydis CTB4         GFGIHLINMQGLNYIIDSYKIYANSAVAANTFLRSLFAAGFPILATSMYAAIGVKWGTTI
Cercospora zeina CTB4               GFGIHLINMQGLNYIIDSYKIYANSAVAANTFLRSLFAAGFPILATSMYATIGVKWGTTI
                                    ***********:**************:************************:*********

Cercospora nicotianae CTB4          LALLAVAMIPIPILFYYFGAKIRAKSKWQPPL
Cercospora zeae-maydis CTB4         LALLAVTMIPIPILFYYFGAQIRAKSRWQPPM
Cercospora zeina CTB4               LALLAVTMIPIPILFYYFGANIRAKSKWQPPM
                                    ******:*************:*****.****:
```

File S1 Fig. 4. Multiple sequence alignment of the *C. nicotianae, C. zeina* and *C. zeae-maydis* CTB4 amino acid sequences.

File S1 Table 5. Percentage identity and similarity of the *C. nicotianae, C. zeina* and *C. zeae-maydis* CTB5 amino acid sequences.

	C. nicotianae	***C. zeina***	***C. zeae-maydis***
C. nicotianae	-	90.9 % identity	88.9 % identity
C. zeina	95.9 % similarity	-	91.9 % identity
C. zeae-maydis	94.0 % similarity	95.3 % similarity	-

```
Cercospora nicotianae CTB5          MGSYFSLKNSDLHPSCIALPRSAEEVSKAVRTLSLGAHKWEGQCQFGVRGGGHTPFKGAA
Cercospora zeina CTB5               MGSYFSLKNSDLHPSCIALPRSAEDVSKAVQTLSLGAHKWEGKCQFGVRGGGHTPFKGAA
Cercospora zeae-maydis CTB5         MGSYFSLINSDLHPSCIALPRSAEDVSKAVQTLSLGAHKWEGQCQFGVRGGGHTPFKGAA
                                    ******* ****************:*****.***********:*****************

Cercospora nicotianae CTB5          STDNGIVLDLLHMPSAGISPDYETITVSPSTTWDLVYEVLDAHNRSTLGTKVAGIGVGGA
Cercospora zeina CTB5               STDNGIVLDLLHMPSAGISPDYETITVSPSTTWDLVYEVLDAHNRSTLGTKVAGIGVGGA
Cercospora zeae-maydis CTB5         SIDKGIVLDLLHMPSAGISPDYDTITVSPSTTWDLVYEVLDAHNRSTLGTKVAGIGVGGA
                                    * *:******************:************************************

Cercospora nicotianae CTB5          STSCGVSYFSPRYGYICDMVENWEVVLATGDIVNANANENADLWKALRGGINNFGIVTAV
Cercospora zeina CTB5               STSCGVSYFSPRYGYICDMVENWEVVLATGDIVNANAQENADLWKALRGGINNFGVVTAV
Cercospora zeae-maydis CTB5         STSCGVSYFSPRYGYICDMVENWEVVLATGDIVNANAHENADLWKALRGGVNNFGIVTAV
                                    ************************************:************:****:****

Cercospora nicotianae CTB5          TLKAFEQGPFWGGQTFHSIETRQEHFKNHAKLASAHPYDPYAHYINTLVLAN--GGHWFI
Cercospora zeina CTB5               TLKTFEQGAFWGGQTFHSIDTRKEHFQNHAELASAPSYDPYAHYINTLVLANMTGGHWFI
Cercospora zeae-maydis CTB5         TLKTFEQGVFWGGQTFHSIDTREEHFQNLAELASAPSYDTYAHYINTLVLANMTGGHWFI
                                    ***:**** **********:**:***:* *:**** .**.************  ******

Cercospora nicotianae CTB5          GNSIQYTKSDPPVAEPEVFKPFLKTERTPIFPGLPEDTLRVDNVTSFSREYAANTLYPQR
Cercospora zeina CTB5               GNSIQYTKSDPPVAEPEVFKPFLQTKRTPIFPGAPEDTLRVDNVTSFSREYAANTLYPQR
Cercospora zeae-maydis CTB5         GNSIQYTKSDPPVAEPEVFKPFLKTKRTPIFPGAPEDSLRVDNVTSFSREYAANTLYPQR
                                    ************************:*:******* ***:**********************

Cercospora nicotianae CTB5          WQFACISFAPDADFMETFFQMANDAMQQYVKLPGFKLILNYQPAPTVQLERNGAVDSLGP
Cercospora zeina CTB5               WQFACISFAPDADFMETFFQLADAAMRQYVSLPGFKLILNYQPAPTIQLERNRAIDSLGP
Cercospora zeae-maydis CTB5         WQFACISFAPDADFMETFFQMADTAMREYVRLPGFKLILNYQPAPTVQLERNNAVDSLGP
                                    ********************:*: **.:** ***************:***** *:*****

Cercospora nicotianae CTB5          IQTEGNVVFVHWAVSYDESEAQFDDAITKSVQDLFHAANTKAKELGIYRHFIQPTYADSW
Cercospora zeina CTB5               IQTEGNIVFVHWAVSYDESEAHTDDAITTSVQQLFHAANAKAKELGVYRHYIQPTYADSW
Cercospora zeae-maydis CTB5         IQTEGNIVFVHWAVSYDESEAHMDDAITKSVQKLFHAANAKAKELGVYRHYVQPTYADSW
                                    ******:**************: *****.***.******:******:***::********

Cercospora nicotianae CTB5          QSPFDYRSKSTIEELVATSKKYDPLQVFQKQVPGGFKLPQI--------
Cercospora zeina CTB5               QNPFEFRSKSTVEELVATSKKYDPLQVFQNQVPGGFKLPTV--------
Cercospora zeae-maydis CTB5         QNPFEFRSKSTIEELVATSKKYDPLQIFQNQVPGGFKLPKIRGGESAGA
                                    *.**::*****:**************:**:********* :
```

File S1 Fig. 5. Multiple sequence alignment of the *C. nicotianae, C. zeina* and *C. zeae-maydis* CTB5 amino acid sequences.

File S1 Table 6. Percentage identity and similarity of the *C. nicotianae, C. zeina* and *C. zeae-maydis* CTB6 amino acid sequences.

	C. nicotianae	***C. zeina***	***C. zeae-maydis***
C. nicotianae	-	86.6 % identity	89.6 % identity
C. zeina	94.7 % similarity	-	91.3 % identity
C. zeae-maydis	95.8 % similarity	95.5 % similarity	-

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Cercospora nicotianae CTB6          MADSLVLLTGATGFIGFRILVELLRQGYSVRAVIRSAAKGQWLESRLTAVMKGSDYKDRF
Cercospora zeina CTB6               MADSRVLLTGATGFIGFRILVELLHQGYNVRAVVRSPTKGRWLESRLAAVTKGANWKAGF
Cercospora zeae-maydis CTB6         MTNSLVLLTGATGFIGFRILVELLHQGYNVRAVIRSLAKGQWLESRLAAVMKGANWRDRF
                                    *::* ******************.***.****:** :**.******:** **::::.  *

Cercospora nicotianae CTB6          QTTIVADFVTDGAFDQAAENTSYIIHVASPIVSSDNPDDWEHDFKRVAVKGSIGVLEAAK
Cercospora zeina CTB6               QTTIVTDFVTEGAFDQAAENTSYIIHVASPIVSSDNPEDWEHDFKRVAVRGSIGILEAAK
Cercospora zeae-maydis CTB6         QTTIVTDFVTEGAFDQAAENTSYIIHVASPIVSSDNPEDWEHDFKRVAVKGSIGILEAAK
                                    *****:****:**************************:***********.****:*****

Cercospora nicotianae CTB6          RSGTVRRVVITSSMVGLFSPKALFAEPSEVPLNAESRIPEMEPPYAHKMLAYQAGKIASI
Cercospora zeina CTB6               RSATVRRIVITSSMVALFTPKAIFAEPSKVPLDAESRIPEMEPPYAHKMMAYQAGKIASL
Cercospora zeae-maydis CTB6         RSGTVRRVVITSSMVALFTPKAIFAEPSEVPLSAEQRIPEMEPPYAHKMMAYQAGKIASL
                                    **.****:*******.**:***:*****:***.**.*************:*********:

Cercospora nicotianae CTB6          NSAEAWIKHEKPAFDLIHMHPSFVTGRDDLATTREDLRKFSSNWHSMQIVLGHKNPIGKP
Cercospora zeina CTB6               NSAEAWIRREQPAFDLIHMHPSFVTGRDDLATTREDLRKFSSNWHSMQIVLGHKNPVGKP
Cercospora zeae-maydis CTB6         NSAEAWIKHEKPAFDLIHMHPSFVTGRDDLATTREDMRKFSSNWHSMQIVLGHKNPIGKP
                                    *******..*:**************************:********************:***

Cercospora nicotianae CTB6          ILTCHNDDVARCHVSALDPKVAGNQSFLISCSPEDGSEWDNVKKIVQREFPEAVAQGVLP
Cercospora zeina CTB6               LLTCHNDDVARCHVSALNPKIAGNQSFLISCSPEDGSEWDDVKEFVQRDYPEAVAEGVLP
Cercospora zeae-maydis CTB6         LLTCHNDDVARCHVSALDPKIVGNQSFLISCSPEDGSEWDDVKKFVQRDYPEAVEQGVLP
                                    :*****************:**:.*****************:**::***::**** :****

Cercospora nicotianae CTB6          NDGHMPTVNKGVRFDVRKTEETFGFKHIPYEAQVLDVVKQYLELPEKDEGVEISTTA
Cercospora zeina CTB6               NDGHMPTVNKGVRFDVRKTEETFEFKHTPYEAQVLDVVKQYLELPEKDEGVEVV---
Cercospora zeae-maydis CTB6         NDGHMPTVNKGVRFDTRKTEETFGFKHIPYEAQVLDVVRQYLELPEKDEGVEVV---
                                    ***************.******* *** **********.*************:
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File S1 Fig. 6. Multiple sequence alignment of the *C. nicotianae, C. zeina* and *C. zeae-maydis* CTB6 amino acid sequences.

File S1 Table 7. Percentage identity and similarity of the *C. nicotianae, C. zeina* and *C. zeae-maydis* CTB8 amino acid sequences.

	C. nicotianae	***C. zeina***	***C. zeae-maydis***
C. nicotianae	-	68.5 % identity	70.0 % identity
C. zeina	77.1 % similarity	-	79.6 % identity
C. zeae-maydis	80.6 % similarity	85.2 % similarity	-

```
Cercospora nicotianae CTB8       MAKGSAGDAPNTRDTSFKRPKIRESCTHCSSQKIRCTKERPACARCVNKGLLCQYNISRR
Cercospora zeina CTB8            MARGGASDMPNTRDTSFKRPKIRESCTHCSSQKIRCTKERPACARCVNKGLLCQYNISRR
Cercospora zeae-maydis CTB8      MARGGASDAPNTRDASFKRPKIRESCTHCSSQKIRCTKERPACARCVNKGLLCQYNISRR
                                 **.*.*.* *****:*********************************************

Cercospora nicotianae CTB8       TGTRRHSVRATPEPETTISNAPTSSVPPDSVKIDGKQSPAMSDFALLDGLETFNNSLWHQ
Cercospora zeina CTB8            TGTRRPSIRATPEPDTLKPMAPTSSASADSIVIHGRLSPSLSDLAMLDGLDAFDDSMWAQ
Cercospora zeae-maydis CTB8      TGTRRQSIRATPEPDTLIPTAPTSSASAESIAIDARLSPTLSDLAMLDGLDILDDTMWAQ
                                 ***** *:******:*  . *****...:*: * .. **::**:*:****: :::::* *

Cercospora nicotianae CTB8       PITTDIQDIDMQYFDFFDPGGYQAEPEPINSFDIDSTLLCGTSTA-------GYLPELDA
Cercospora zeina CTB8            PVVTNVEDMDMQYFDFFSAGGFEAEPEAKGLSGVDSTPLSPTEVAKLFGISTGGLSKVGM
Cercospora zeae-maydis CTB8      PTVTSVEDMEVPYLDFFSAGAFQAEPEAKGWNGVESTPLSQNKMAKLFGGSTGSLPEVDR
                                 * .*.::*::: *:***..*.::****. .  .::** *. .. *       * *.::.

Cercospora nicotianae CTB8       EASTRPSSSSSPPSQRSDGGRATTHGGGGCISTALQIFSELHVSSSACPIAAGAPSHNIR
Cercospora zeina CTB8            EASTRPSSSASPRSQRGGGSTGHGGGGGGCISTALQIFSELHVSGSACPIAAGSADQDVR
Cercospora zeae-maydis CTB8      EASTRPSSSSSPHSQRGGGTAGH--GGGGCISTALQIFSQLHVSSAACPIAAGSAGEDVR
                                 *********:** ***..*  .   **************:****.:*******:.. ::*

Cercospora nicotianae CTB8       EFDHVLDSNRAALEKLSSILDCPPCCHDQEVLTALFLAVQKALSWYSAALDVAGDGEP--
Cercospora zeina CTB8            DFDHVLDSNRTALERLSSILDCRPCCRNHEVLTAAFLAIHKALSWYSAALDVASDDEPSS
Cercospora zeae-maydis CTB8      EFDHVLDSNRTALERLSSILDCPPCCRNHEVLTASFLAIHKALSWYSAALDVESDDEP--
                                 :*********:***.******* ***.::***** ***::************* .*.**

Cercospora nicotianae CTB8       TSPSSRVKSPPAFLGSYALGAQAQTLARAYVVMAQLQQHFQPLLAKLQRK----------
Cercospora zeina CTB8            TSSSSRVTSPPAFLGSYALGTQAQALARAYVVTAQLQQHFQPLMAKLQRISWSSPPPPSS
Cercospora zeae-maydis CTB8      ----SRVTSPPAFLGSYALGTQAQTLARAYVVMAQLQQHFQPLMAKLRRI----------
                                     ***.************:***:******* **********:***.*

Cercospora nicotianae CTB8       ---------SSLSALGARSSSTTSLSSVSS-LQSSTSGSAVIECQKRALQEALEDVVAKI
Cercospora zeina CTB8            SSSSSSSASSASSSPGACSPSTASLSSVSS-CQSSASGSAVVECQQRALQEALEDVEAKI
Cercospora zeae-maydis CTB8      ---------SSSSGVRSPSAASSSLSSVSSMCQSSASGSAVVECQQRALQDALDDVVAKI
                                          *: *.  : *.:::*******  ***:*****:***:****:**:** ***

Cercospora nicotianae CTB8       EGIKRG
Cercospora zeina CTB8            DGIKRA
Cercospora zeae-maydis CTB8      EGIKRA
                                 :****.
```

File S1 Fig. 7. Multiple sequence alignment of the *C. nicotianae, C. zeina* and *C. zeae-maydis* CTB8 amino acid sequences.

Supplementary File S2.

Sequencing of *Cercospora zeina CTB7* fragment in geographically and chronologically separated isolates.

METHODS

DNA samples from isolates of *C. zeina* are described in Supplementary File S2A (Table). The CTB7del PCR was set up as described under the Material and Methods section. Sequencing reactions were setup using the BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, CA, USA) according to the manufacturer's guidelines. The sequencing products were purified using the Sephadex G-50 clean-up protocol (Sigma-Aldrich, St. Louis, USA) according to the manufacturer's specifications, and sequenced in both the forward and reverse directions using the CTB7del primers at the DNA sequencing Facility of the Faculty of Natural and Agricultural Sciences, University of Pretoria. The trace files were analysed and edited in CLC Main Workbench 6.0 (CLC Bio, Denmark).

RESULTS

The CTB7 diagnostic PCR yielded 618 bp amplicons for each of the *C. zeina* isolates tested (Supplementary File S2B), which were subsequently sequenced. These sequences were aligned to the corresponding region from CMW 25467 (African isolate) and OYPA (USA isolate) and showed 100% sequence identity (File S2C) for all isolates except 2016.UG.LR.089. This isolate demonstrated a single nucleotide difference (cytosine instead of a thymine) as compared to the remaining isolates (Supplementary File S2C).

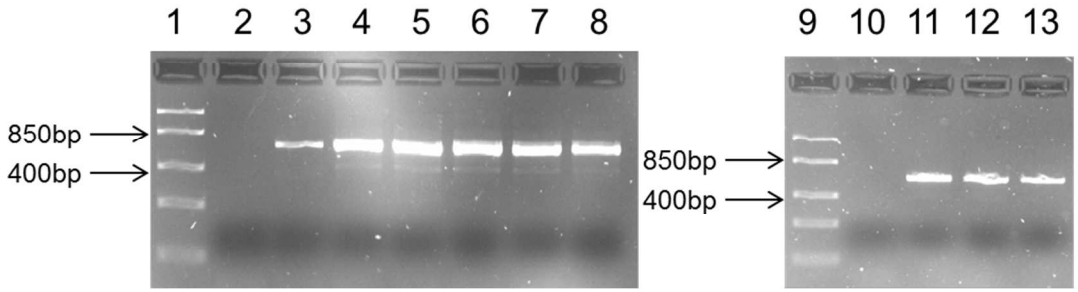
Supplementary File S2A (Table). *Cercospora zeina* isolates.

Isolate	Area, Country of isolation	Year of isolation	Reference
OYPA (= USPA-4)	Ohio, USA	Prior to 2000	(DUNKLE and LEVY 2000)
CMW25467	Mkushi, Zambia	2007	(MEISEL *et al.* 2009)
CBS 118820[a]	KwaZulu-Natal, South Africa	Prior to 2006	(CROUS *et al.* 2006)
2011.ZA.GT.05	Greytown, KwaZulu-Natal, South Africa	2011	(Muller et al. 2016)
2013.ZA.GT.04[b]	Greytown, KwaZulu-Natal, South Africa	2013	(Christie et al. 2017)
2012.ZA.NW.18	Hoogkraal, North West, South Africa	2012	(MULLER *et al.* 2016)
2015.ZA.BZ.002[c]	Bizana, Eastern Cape, South Africa	2015	
2015.ZM.CH.037[c]	Chisamba, Zambia	2015	
2016.KE.VH.094[c]	Vihiga,Kenya	2016	
2016.UG.LR.089[c]	Lira, Uganda	2016	

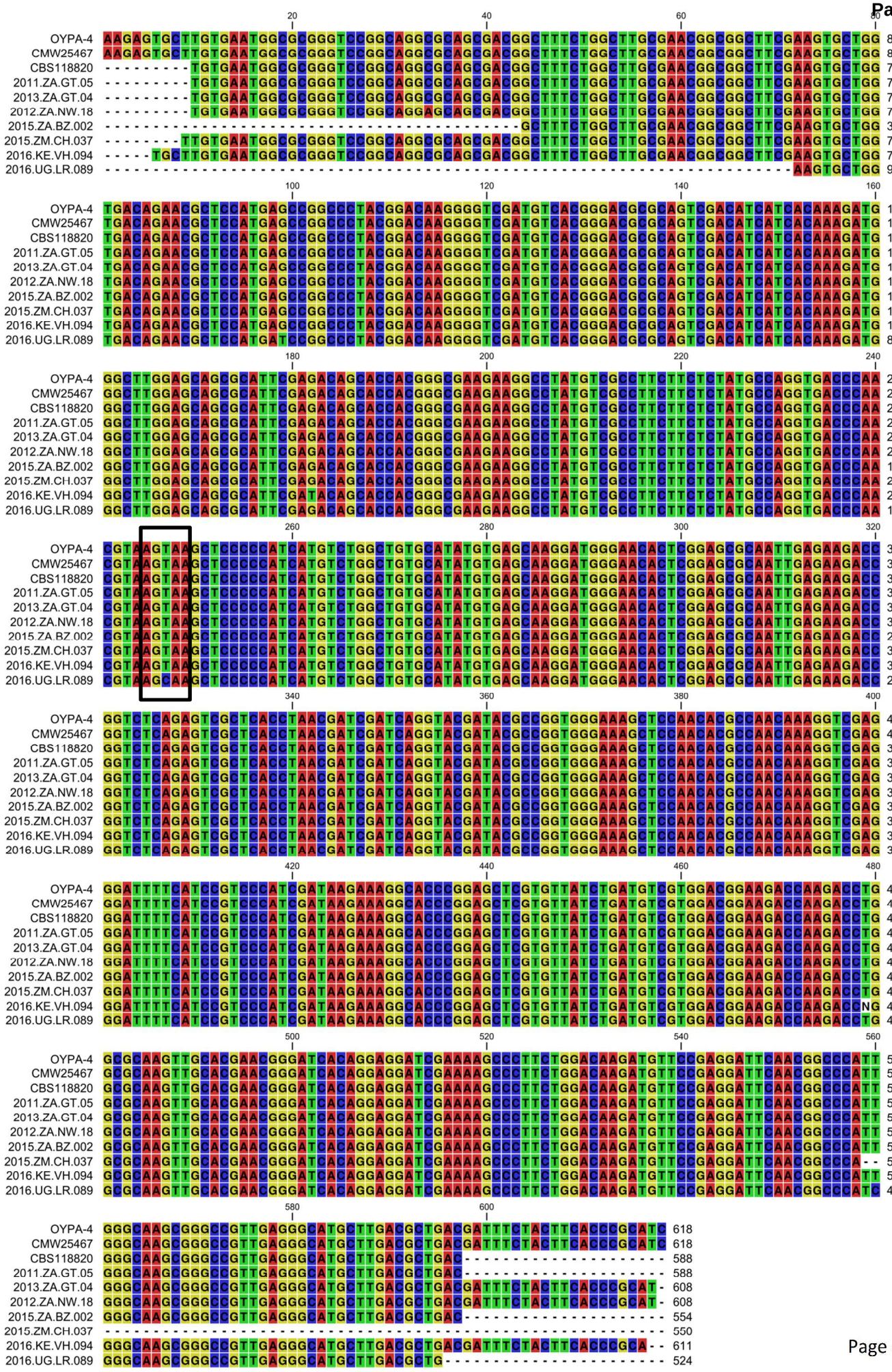
[a] *C. zeina* ex-type cultures. CBS: Culture collection of the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

[b] PCR amplified and sequenced directly from GLS lesions of sample named “B73 (lower leaf) (*C. zeina* infected) Rep 1” from Methods S1 File of (Christie et al. 2017).

[c] DNA of isolates received from David. L. Nsibo (University of Pretoria).



Supplementary File S2B. *CTB7* diagnostic PCR of African isolates of *C. zeina*. PCR amplification with CTB7del_F and CTB7del_R primers (Table 4) from *C. zeina* isolates: CBS 118820 (lane 4), 2011.ZA.GT.05 (lane 5), 2015.ZA.BZ.002 (lane 6), 2015.ZM.CH.037 (lane 7), 2013.ZA.GT.04 (lane 8), 2016.UG.LR.089 (lane 11), 2016.KE.VH.094 (lane 12) and 2012.ZA.NW.18 (lane 13). All of the isolates yielded a 618 bp amplicon as expected for *C. zeina*. PCR products were separated on a 1.5% agarose gel stained with EtBr. A size standard (FastRuler Low Range DNA ladder, ThermoFisher Scientific) is shown in lane 1 and 9. A non-template/water control was included in lane 2 and 10. A positive control, CMW25467 is included in lane 3.


OYPA-4
CMW25467
CBS118820
2011.ZA.GT.05
2013.ZA.GT.04
2012.ZA.NW.18
2015.ZA.BZ.002
2015.ZM.CH.037
2016.KE.VH.094
2016.UG.LR.089

Supplementary File S2C. Sequencing of the CTB7 diagnostic PCR products generated from *C. zeina* isolates. The sequences were found to be identical over the region analysed for all of the isolates, except 2016.UG.LR.089, which showed a single nucleotide difference as compare to the remaining isolates (position indicated by the black block). The differences in the length of the sequences are due to extent of the Sanger sequence data generated and do not indicate deletions among the isolates.

LITERATURE CITED

Christie, N., Myburg, A.A., Joubert, F., Murray, S.L., Carstens, M., Lin, Y.-C., Meyer, J., Crampton, B.G., Christensen, S.A., Ntuli, J.F., Wighard, S.S., Van de Peer, Y., and Berger, D.K. 2017. Systems genetics reveals a transcriptional network associated with susceptibility in the maize-gray leaf spot pathosystem. The Plant Journal 89:746–763.

Crous, P.W., Groenewald, J.Z., Groenewald, M., Caldwell, P., Braun, U., and Harrington, T.C. 2006. Species of *Cercospora* associated with grey leaf spot of maize. Studies in Mycology 55:189-197.

Dunkle, L.D., and Levy, M. 2000. Genetic relatedness of African and United States populations of *Cercospora zeae-maydis*. Phytopathology 90:486-490.

Meisel, B., Korsman, J., Kloppers, F.J., and Berger, D.K. 2009. *Cercospora zeina* is the causal agent of grey leaf spot disease of maize in southern Africa. European Journal of Plant Pathology 124:577-583.

Muller, M.F., Barnes, I., Kunene, N.T., Crampton, B.G., Bluhm, B., Phillips, S., Olivier, N.A., and Berger, D.K. 2016. *Cercospora zeina* from maize in South Africa exhibits high genetic diversity and lack of regional population differentiation. Phytopathology 106:1194-1205.

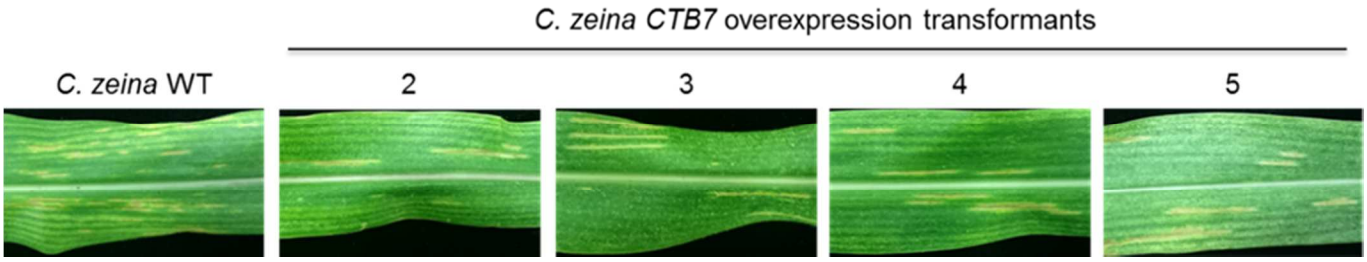
Supplementary File S3.

Pathogenicity of *Cercospora zeina CzmCTB7* transformants.

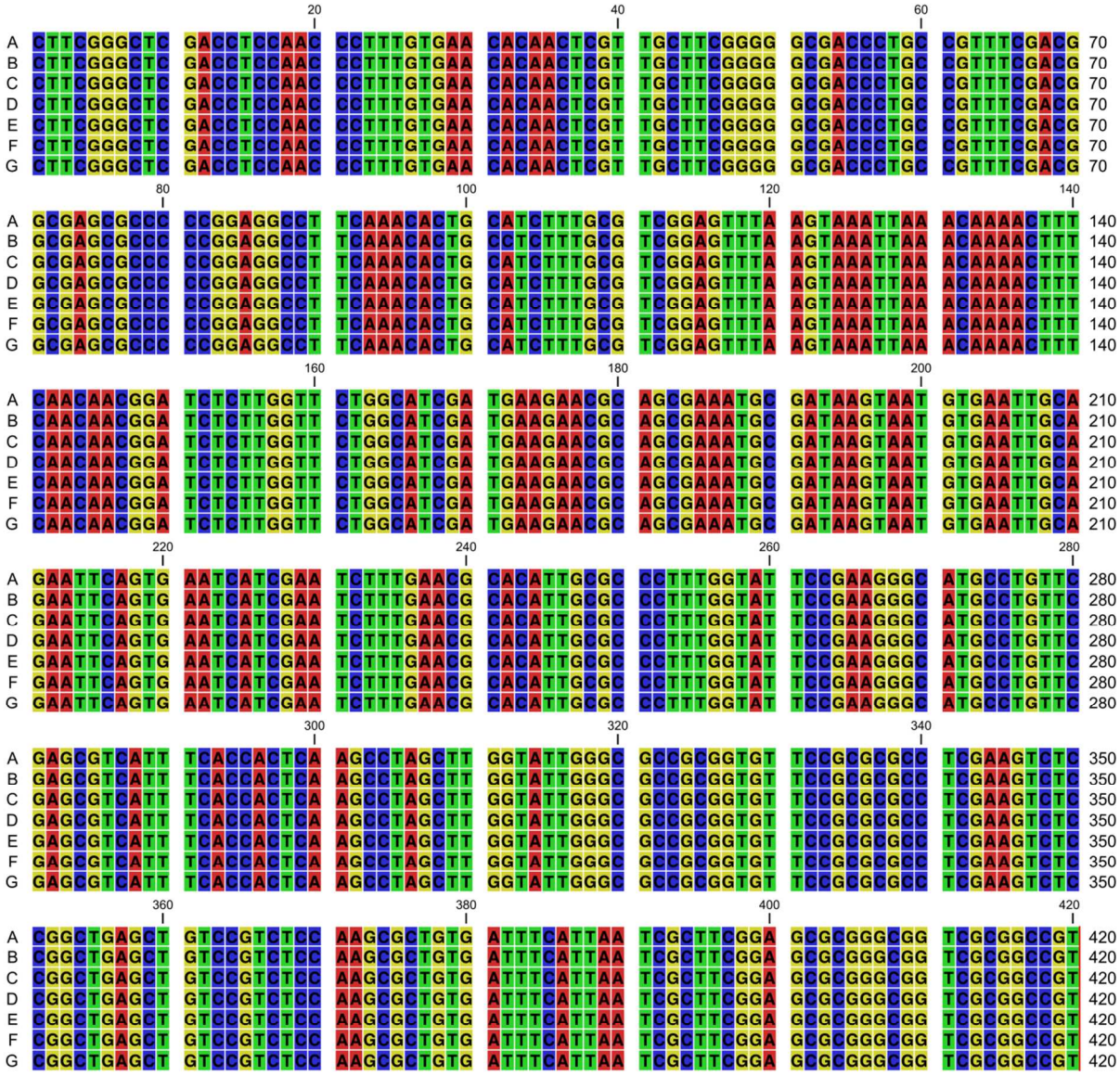
METHODS AND RESULTS

A phytotron inoculation trial was conducted at the University of Pretoria, to confirm that the *C. zeina CzmCTB7* transformants maintained their pathogenicity. The Plant Sciences Complex phytotron is registered as a Type I containment unit with the Department of Agriculture, Forestry and Fisheries of South Africa (registration number: 39.2/University of Pretoria-12/091). The phytotron conditions were set to a 18 hour light/6 hour dark cycle, a temperature of 25°C and 100% relative humidity. High humidity levels were maintained throughout the trial by covering the plants with a wire cage and clear plastic. Eight week old seedlings of a GLS moderately-susceptible, early-maturing maize hybrid, PAN6126 (PANNAR SEED Pty Ltd, Greytown, KwaZulu-Natal), were inoculated using the paint brush method with a conidial suspension collected from V8 agar cultures, as described in (Meisel *et al.* 2009).

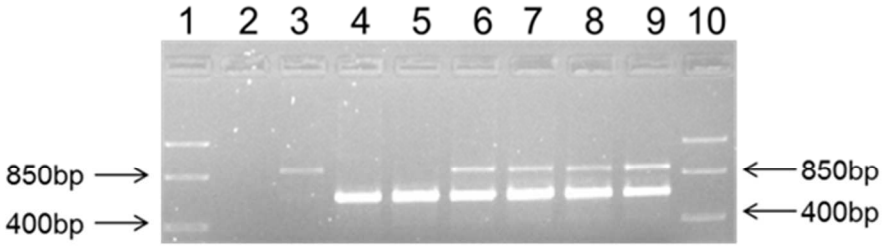
Infection progressed similarly for both the wild-type as well as the *C. zeina* transformants with rectangular GLS lesions observed at 27 dpi (File S3A). For all of the transformants, single conidia were re-isolated from the mature GLS lesions and sub-cultured on V8 agar to obtain sufficient fungal material for DNA isolations using methods described in (MULLER *et al.* 2016). Koch's postulates was demonstrated by conducting ITS sequencing on the isolates, which confirmed that the GLS lesions observed on the maize were due to *C. zeina* infection (File S3B). Furthermore, to confirm that GLS lesions observed were due to infection by the *C. zeina* transformants, the CTB7del screening PCR was performed. DNA obtained from conidia re-isolated from lesions of the maize leaves inoculated with the *C. zeina CzmCTB7* transformants, yielded two amplicons corresponding to both the *C. zeina* and *C. zeae-maydis CTB7* gene copies (File S3C). These findings confirm that the *C. zeina CzmCTB7* transformants were still capable of maize infection.



File S3A. Maize leaves demonstrating GLS disease symptoms. Maize plants of the PAN6126 were inoculated with a conidial suspension of *C. zeina* WT and the *C. zeina CzmCTB7* transformants 2-5, respectively. Images show the GLS symptoms at 27 dpi.



File S3B. ITS sequencing of the single-spore isolates obtained from *C. zeina* wild-type and *CzmCTB7* transformant inoculated maize plants. (A) *C. zeina* CBS 118820. ITS sequence obtained from NCBI [accession number: NR_111205] (CROUS *et al.* 2006). (B) *C. zeina* CMW 25467. ITS sequence obtained from NCBI [accession number: EU569227.1] (MEISEL *et al.* 2009). (C) *C. zeina* CMW 25467 wild-type single-spore isolate. (D) *C. zeina CzmCTB7* transformant-2 single-spore isolate. (E) *C. zeina CzmCTB7* transformant-3 single-spore isolate. (F) *C. zeina CzmCTB7* transformant-4 single-spore isolate. (G) *C. zeina CzmCTB7* transformant-5 single-spore isolate. ITS sequencing was carried out as described in Meisel et al (2009).



File S3C. Screening of the re-isolated *C. zeina-CTB7* transformants for the presence of the *C. zeae-maydis CTB7* gene copy. Transformants carrying both *Cercospora* species' *CTB7* gene copies were detected by PCR amplification with the CTB7del primers. Positive controls for both *C. zeae-maydis* (925 bp amplicon, lane 3) and *C. zeina* (618 bp amplicon, lane 4) were included. DNA from the single-spore isolate culture from the maize plants inoculated with the *C. zeina* CMW25467 WT conidial suspension showed only a single band corresponding to the *C. zeina CTB7* (lane 5). DNA from the single-spore isolates cultured from maize plants inoculated with the *C. zeina* CzmCTB7 transformants 2-5 were shown to carry both copies of *CTB7* (lanes 6 – 9, respectively). PCR products were separated on a 1% agarose gel stained with EtBr. A size standard (FastRuler Low Range DNA ladder, ThermoFisher Scientific) is shown in lane 1 and 10. A non-template/water control was included in lane 2.

LITERATURE CITED

Crous, P.W., Groenewald, J.Z., Groenewald, M., Caldwell, P., Braun, U., and Harrington, T.C. 2006. Species of *Cercospora* associated with grey leaf spot of maize. Studies in Mycology 55:189-197.

Meisel, B., Korsman, J., Kloppers, F.J., and Berger, D.K. 2009. *Cercospora zeina* is the causal agent of grey leaf spot disease of maize in southern Africa. European Journal of Plant Pathology 124:577-583.

Muller, M.F., Barnes, I., Kunene, N.T., Crampton, B.G., Bluhm, B., Phillips, S., Olivier, N.A., and Berger, D.K. 2016. *Cercospora zeina* from maize in South Africa exhibits high genetic diversity and lack of regional population differentiation. Phytopathology 106:1194-1205.

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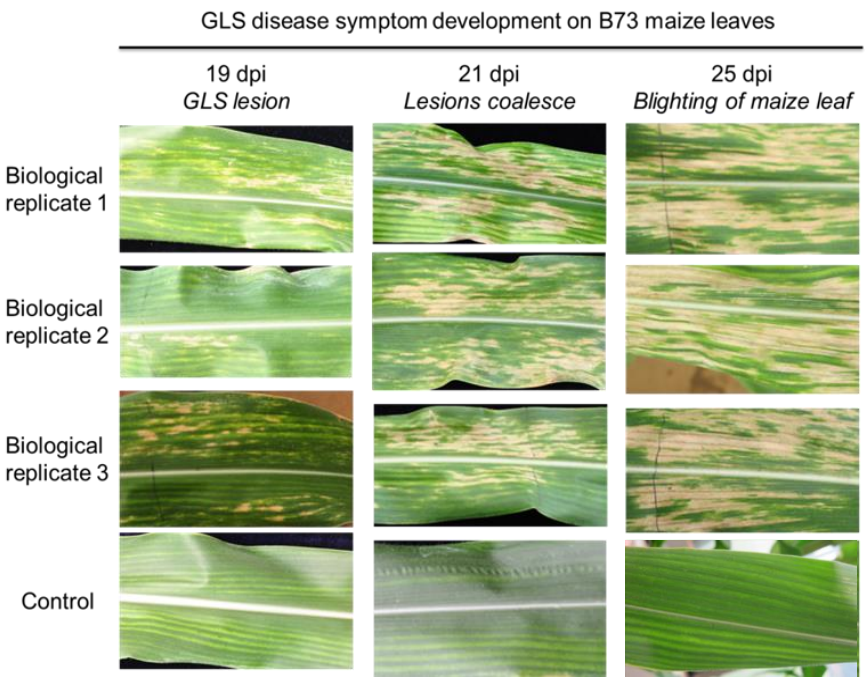
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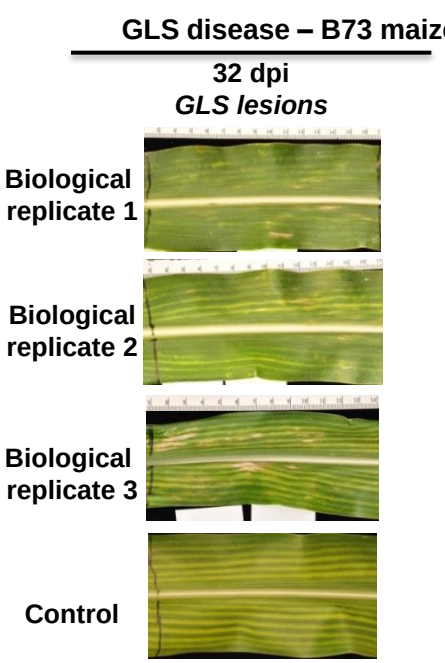
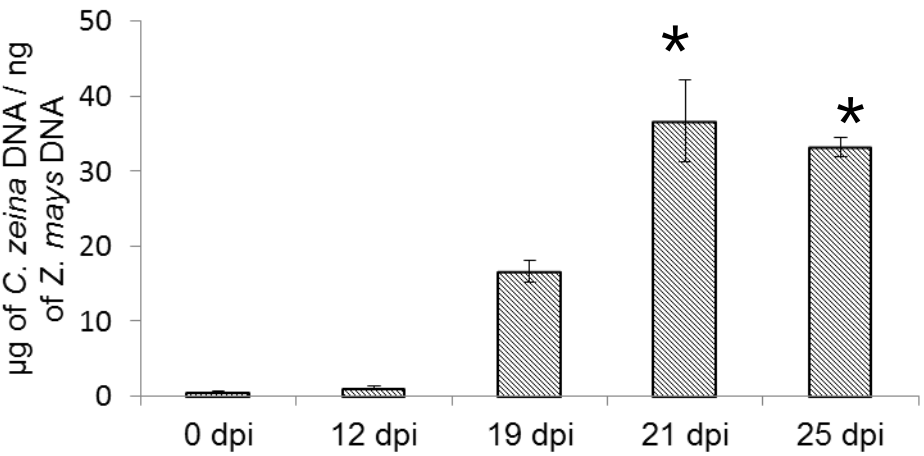
GLS lesion
21 dpi
Lesions coalesce
25 dpi
Blighting of maize leaf
Biological replicate 1
Biological replicate 2
Biological replicate 3
Control

C


GLS disease – B73 maize
32 dpi
GLS lesions
Biological replicate 1
Biological replicate 2
Biological replicate 3
Control

B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50
40
30
20
10
0
*
*
0 dpi
12 dpi
19 dpi
21 dpi
25 dpi

Supplementary Fig. S1. Maize line B73 developed GLS symptoms after glasshouse inoculation with *C. zeina* CMW25467. **A**, Glasshouse trial 1: GLS susceptible B73 maize plants were inoculated with a conidial suspension of *C. zeina* and leaf samples were harvested in triplicate at 0, 12 (images not shown), 19, 21 and 25 dpi. Maize leaf images show GLS lesions at 19 dpi, lesions coalescing at 21 dpi and blighting of the leaves at 25 dpi. Control mock-inoculated plants did not show lesions. **B**, *Cercospora zeina* fungal genomic DNA content (a proxy for fungal biomass) increased significantly in B73 maize leaves after inoculation with *C. zeina* CMW25467 in glasshouse trial 1. Fungal quantities are presented as µg of *C. zeina* DNA per ng of *Z. mays* gDNA measured by qPCR of the *CPR1* gene (Korsman et al. 2010). Standard error bars are included on the graphs. Statistical analysis was done using one-way ANOVA analysis with a Tukey's Multiple Comparison test. The fungal load at 21 and 25 dpi was significantly higher as compared to 0 dpi ($p \leq 0.05$). Only two biological replicates were included in the analysis for 0 dpi. **C**, B73 maize leaves harvested at 32 dpi for RNA isolation following a second glasshouse inoculation trial.

LITERATURE CITED

Korsman, J., Meisel, B., Kloppers, F., Crampton, B., and Berger, D. 2010. Quantitative phenotyping of grey leaf spot disease in maize using real-time PCR. European Journal of Plant Pathology 18:183-194.

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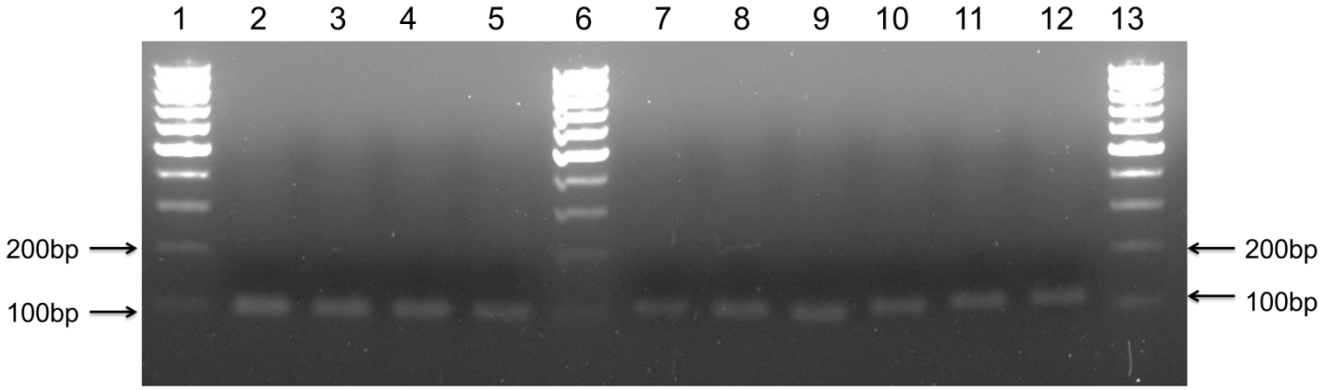
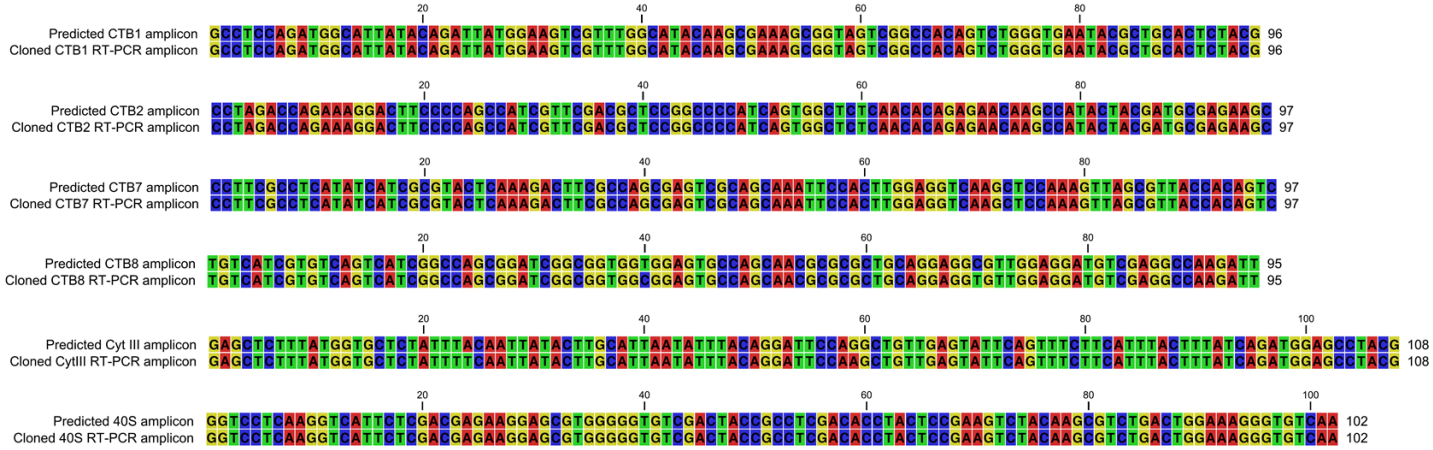

1 2 3 4 5 6 7 8 9 10 11 12 13
200bp
100bp
200bp
100bp

B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Predicted CTB2 amplicon CCTAGACCAGAAAGGACTTCCCCAGCCATCGTTCGACGCTCCGGCCCCATCAGTGGCTCTCAACACAGAGAACAAGCCATACTACGATGCGAGAAGC 97
Cloned CTB2 RT-PCR amplicon CCTAGACCAGAAAGGACTTCCCCAGCCATCGTTCGACGCTCCGGCCCCATCAGTGGCTCTCAACACAGAGAACAAGCCATACTACGATGCGAGAAGC 97
Predicted CTB7 amplicon CCTTCGCCTCATATCATCGCGTACTCAAAGACTTCGCCAGCGAGTCGCAGCAAATTCCACTTGGAGGTCAAGCTCCAAAGTTAGCGTTACCACAGTC 97
Cloned CTB7 RT-PCR amplicon CCTTCGCCTCATATCATCGCGTACTCAAAGACTTCGCCAGCGAGTCGCAGCAAATTCCACTTGGAGGTCAAGCTCCAAAGTTAGCGTTACCACAGTC 97
Predicted CTB8 amplicon TGTCATCGTGTCAGTCATCGGCCAGCGGATCGGCGGTGGTGGAGTGCCAGCAACGCGCGCTGCAGGAGGCGTTGGAGGATGTCGAGGCCAAGATT 95
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Predicted 40S amplicon GGTCCTCAAGGTCATTCTCGACGAGAAGGAGCGTGGGGGTGTCGACTACCGCCTCGACACCTACTCCGAAGTCTACAAGCGTCTGACTGGAAAGGGTGTCAA 102
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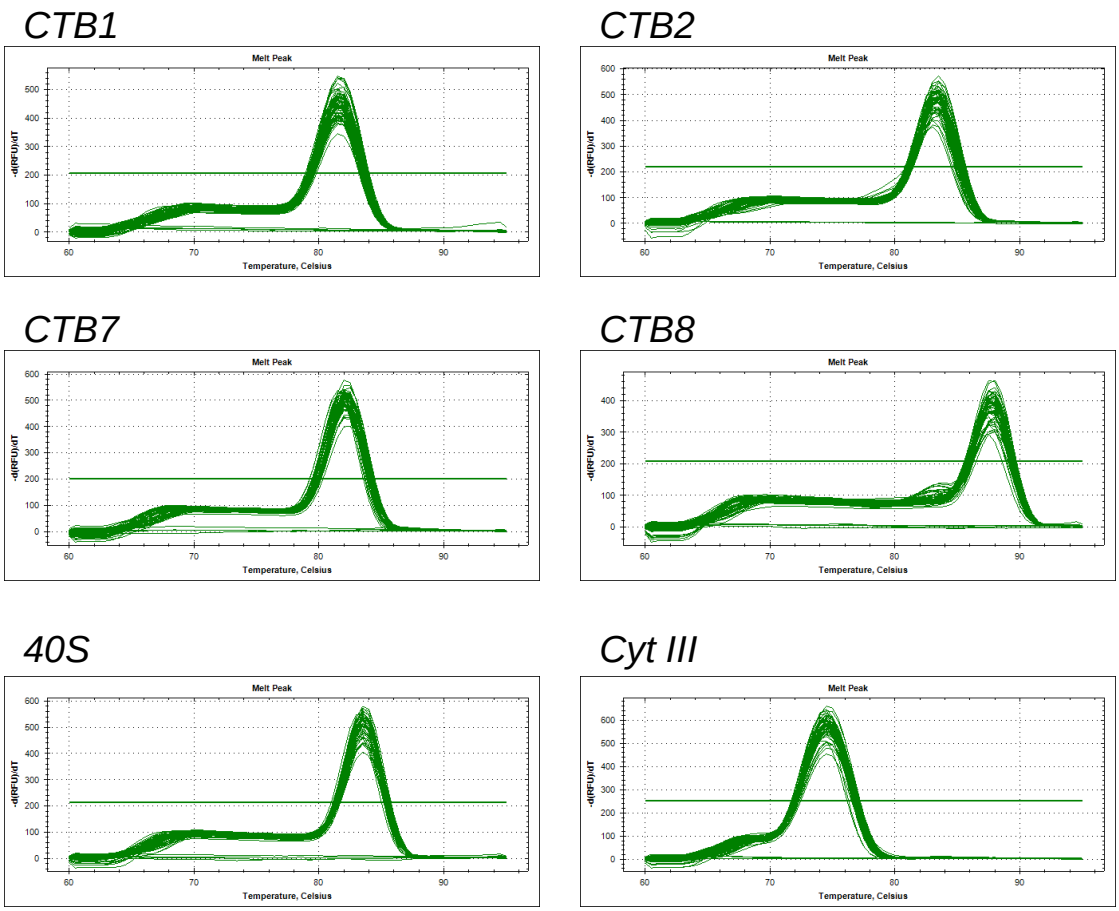
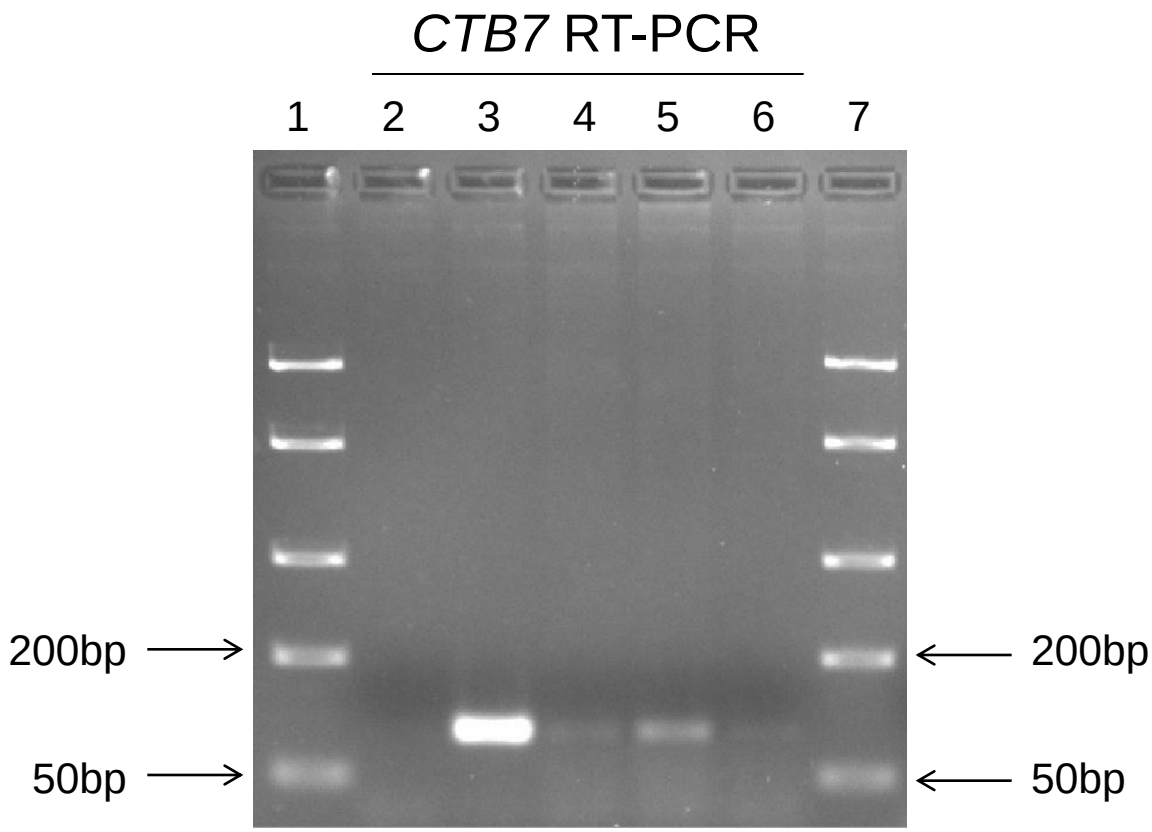
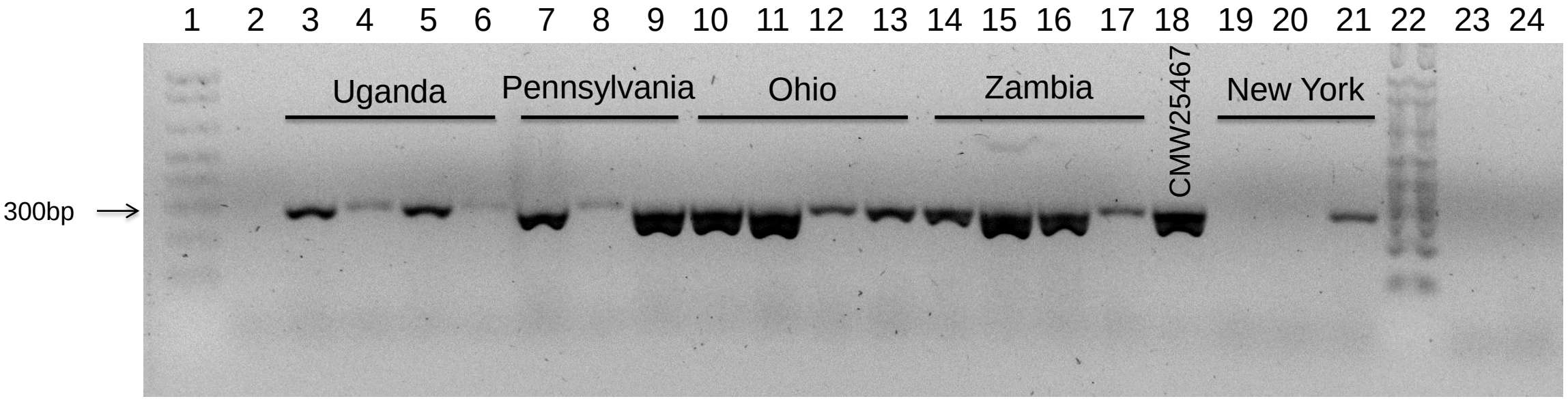
C


CTB1
CTB2
CTB7
CTB8
40S
Cyt III
Melt Peak
-d(RFU)/dT
Temperature, Celsius

Supplementary Fig. S2. Quality control for *in planta* expression analysis of selected *CTB* genes during glasshouse trial #1. **A**, Selected RT-qPCR products were separated on a 1% agarose gel stained with EtBr. A size standard (GeneRuler 100bp DNA ladder, ThermoFisher Scientific) is shown in lane 1, 6 and 13. The *CTB* target genes are shown in lane 2 (*CTB1*), lane 3 (*CTB2*), lane 4 (*CTB7*) and lane 5 (*CTB8*) and are 96 bp, 97 bp, 98 bp and 95 bp in length, respectively. The RT-qPCR products of a suite of putative reference genes are shown in lane 7 – 12. *40S* (lane 10) and *Cyt III* (lane 11) showed single amplicons of 102 bp and 108 bp, respectively. Putative reference genes *GAPDH* (lane 7), *EF1α* (lane 8), *β-TUB* (lane 9) and *Cyt b* (lane 12), were not included in the expression analysis study due to poor stability values. **B**, Sequencing of the RT-PCR amplicons of target genes *CTB1, CTB2, CTB7* and *CTB8* as well for the reference genes *40S* and *Cyt III*. RT-PCR amplicons were cloned into the pJET vector and sequenced at the DNA sequencing Facility of the Natural and Agricultural faculty at the University of Pretoria. The trace files were analysed and the sequences aligned to the predicted *C. zeina* gene amplicons using the CLC Main Workbench 6.0 (CLC Bio, Denmark). **C**, Melt curve analysis of target genes, *CTB1, CTB2, CTB7* and *CTB8,* as well for the reference genes, *40S* and *Cyt III*. Melt peaks were plotted as the negative rate of change in the relative fluorescent units [-d(RFU)] against the change in temperature [dT]. Single melt peaks indicate specific amplification and the absence of primer dimers. Non-template controls generated no melt peaks, indicating that no contamination was present. Melting points for *CTB1, CTB2, CTB7, CTB8, 40S* and *Cyt III* were 81.5°C, 83.5°C, 82°C, 88°C, 83.5°C and 74.5°C, respectively.



Supplementary Fig. S3. *Cercospora zeina CTB7* expression demonstrated by RT-PCR analysis of B73-GLS samples from glasshouse trial #2. RT-PCR products were separated on a 2% agarose gel stained with EtBr. A size standard (FastRuler Low Range DNA ladder, ThermoFisher Scientific) is shown in lane 1 and 7. A non-template/water control was included in lane 2 and a *C. zeina* gDNA positive control in lane 3. The *CTB7* RT-PCR demonstrated the expected 98bp amplicon in all the three B73-*C. zeina* replicates (lanes 4 – 6).



Supplementary Fig. S4. The histone diagnostic PCR with the CzeinaHIST and CylH3R primers (Crous et al. 2006), was performed on the *C. zeina* isolates (Table 1). PCR products were separated on a 1% agarose gel stained with Gel Red. A size standard (1Kb Plus DNA ladder) is shown in lane 1 and 22. A non-template/water control was included in lane 23. *Cercospora zeae-maydis* SCOH1-5 (c; lane 2 and 24) demonstrate no amplification, while the *C. zeina* isolates (lane 3 - 21) yielded a 284 bp amplicon (Crous et al. 2006).

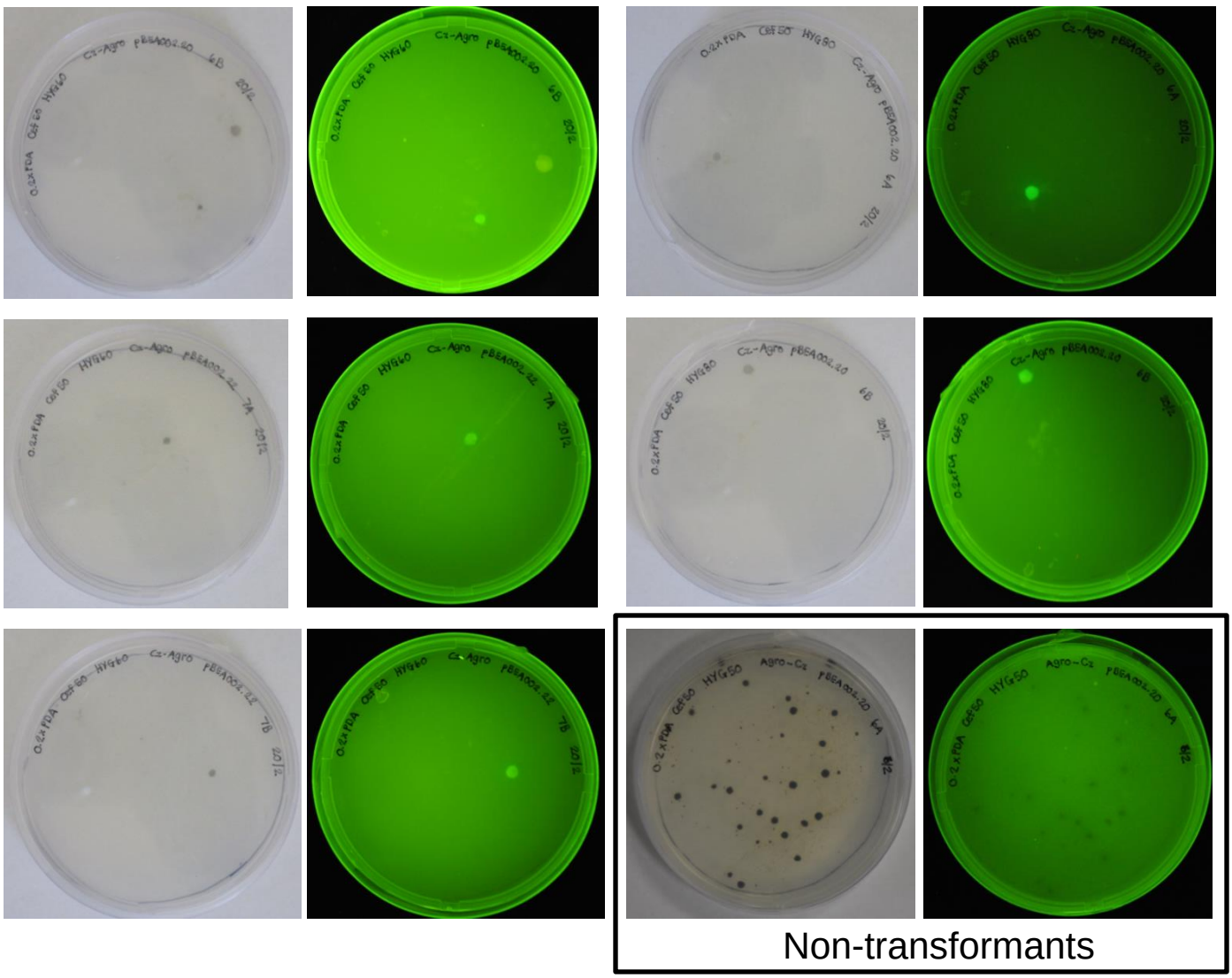
LITERATURE CITED

Crous, P.W., Groenewald, J.Z., Groenewald, M., Caldwell, P., Braun, U., and Harrington, T.C. 2006. Species of *Cercospora* associated with grey leaf spot of maize. Studies in Mycology 55:189-197.

A


14041 BstEII (1)
14033 HindIII (1)
14023 PacI (1)
sgfp 13297..14016
NOS polyA 19..271
RB 309..334
gpdAp 12234..13296
12729 MfeI (1)
12227 BamHI (1)
11962 NruI (1)
pVS1 sta 1375..2375
3286 BsiWI (1)
pVS1 ori 2968..3968
pBEA002
14047 bp
10813 BstBI (1)
XbaI-CzmCTB7-BamHI 8818..12226
9920 Eco47III (1)
pBR322 bom 4378..4638
pBR322 ori 4778..5058
8812 XbaI (1)
5375 SspI (1)
TrpC ter 8318..8803
5448 EcoRV (1)
HygR 7273..8298
Kan R 5349..6143
7633 SgfI (1)
6197 PsiI (1)
TrpC pro 6906..7272
LB 6568..6593
6854 Acc65I (1)
6854 KpnI (1)
6862 ApaI (1)
6862 Bsp120I (1)
6909 HpaI (1)

B

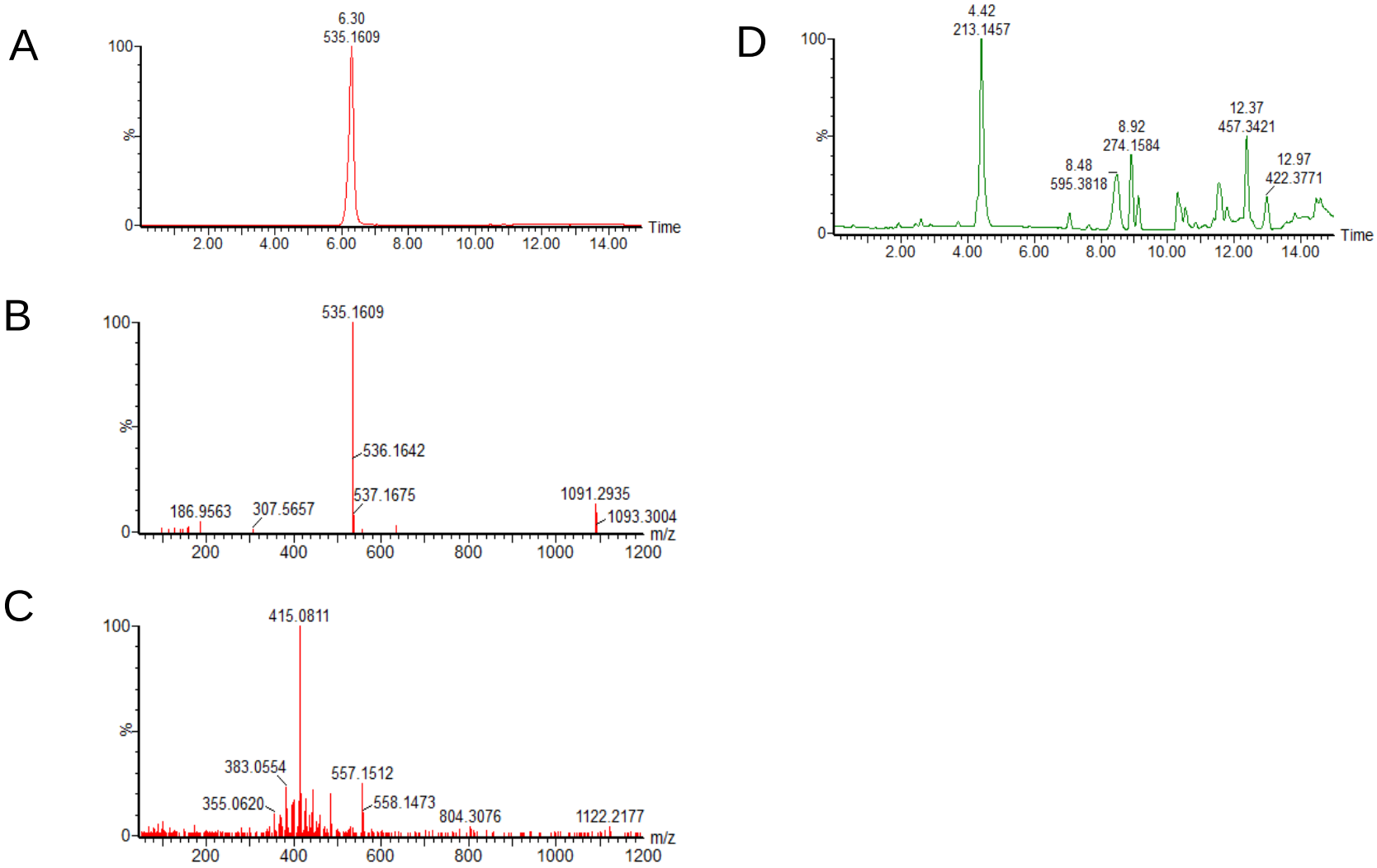

Non-transformants

Supplementary Fig. S5. *Agrobacterium tumefaciens*-mediated transformation of *Cercospora zeina*. **A,** Map of the pBEA002 binary vector for *Agrobacterium tumefaciens*-mediated transformation. The *C. zeae-maydis* FAD-oxidoreductase *CTB*7 gene with its native promoter is flanked by a 1.8-kb hygromycin resistance cassette(Nakayashiki et al. 2005) and a 1.8-kb GFP expression cassette (Ridenour et al. 2014), respectively. **B,** *Agrobacterium tumefaciens* transformed *C. zeina* colonies on 0.2x PDA plates supplemented with hygromycin, under normal and UV light conditions. Six colonies were obtained, which grew on media supplemented with hygromycin and demonstrated emission of green fluorescence under UV light. Non-transformed *C. zeina* colonies demonstrated no fluorescence, given their lack of the *Agrobacterium* construct carrying the *GFP* gene.

LITERATURE CITED

Nakayashiki, H., Hanada, S., Quoc, N.B., Kadotani, N., Tosa, Y., and Mayama, S. 2005. RNA silencing as a tool for exploring gene function in ascomycete fungi. Fungal Genetics and Biology 42:275-283.

Ridenour, J.B., Smith, J.E., Hirsch, R.L., Horevaj, P., Kim, H., Sharma, S., and Bluhm, B.H. 2014. *UBL1* of *Fusarium verticillioides* links the N-end rule pathway to extracellular sensing and plant pathogenesis. Environmental Microbiology 16:2004-2022.



Supplementary Fig. S6. High resolution mass spectra for the *C. kikuchii* CMW49223 and *C. zeina* CMW25467 wild-type extracts. **A**, Extracted ion chromatogram for *m*/*z* 535.1604, **B**, MS and **C**, MS/MS for positive control *C. kikuchii*. **D**, The UPLC profile of the wild-type *C. zeina* extract showed no peaks at 6.30 min, confirming a lack of cercosporin production.

Supplementary Table S1.

High resolution mass spectrum main fragments for cercosporin.

	Cercosporin standard	*C. kikuchii*	Transformant-3
M – 130	401.0673	401.0659	401.0722
M – 119	415.0804	415.0805	415.0855
M – 103	431.1124	431.0755	431.0980
M – 91	443.0878	443.0899	443.0957
M – 76	458.0999	458.0869	458.1486
M – 44	491.0743	490.0828	490.3422
M – 31	503.0930	503.1000	503.2107
M – 18	516.0049	515.9921	516.1905
M + 1	535.1578	535.1492	534.3112
M + 23 (Sodium adduct)	557.1398	557.1380	557.1389

These data are in agreement with those previously obtained for cercosporin (Yamazaki and Ogawa 1972).

LITERATURE CITED

Yamazaki, S., and Ogawa, T. 1972. The chemistry and stereochemistry of cercosporin. Agricultural and Biological Chemistry 36:1707-1718.