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The AVR4 effector is involved in cercosporin biosynthesis and likely affects the virulence of *Cercospora* cf. *flagellaris* on soybean

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SUMMARY

One of the most devastating fungal diseases of soybean in the southern USA is Cercospora leaf blight (CLB), which is caused mainly by *Cercospora* cf. *flagellaris*. Recent studies found that the fungal effector AVR4, originally identified in *Cladosporium fulvum* as a chitin-binding protein, is highly conserved among other *Cercospora* species. We wanted to determine whether it is present in *C.* cf. *flagellaris* and, if so, whether it plays a role in the pathogen infection of soybean. We cloned the *Avr4* gene and created *C.* cf. *flagellaris* Δ*avr4* mutants, which produced little cercosporin and significantly reduced expression of cercosporin biosynthesis genes. The Δ*avr4* mutants were also more sensitive to chitinase and showed reduced virulence on soybean compared to the wild-type. The observed reduced virulence of *C.* cf. *flagellaris* Δ*avr4* mutants on detached soybean leaves is likely due to reduced cercosporin biosynthesis. The phenotypes of reduced cercosporin production and cercosporin pathway gene expression, similar to those of the Δ*avr4* mutants, were reproduced when wild-type *C.* cf. *flagellaris* was treated with double-stranded RNA targeting *Avr4 in vitro*. These two independent approaches demonstrated for the first time the direct involvement of AVR4 in the biosynthesis of cercosporin.

Keywords: *C. kikuchii*, Cercospora leaf blight, cercosporin biosynthesis, double-stranded RNA, fungal virulence, gene disruption, gene silencing.

INTRODUCTION

Soybean (*Glycine max*) is one of the most important agricultural crops and plays an important role in human and animal consumption. However, soybean is susceptible to various pathogens and pests throughout the growing season. Among fungal diseases, Cercospora leaf blight (CLB) and purple seed stain (PSS) are mainly caused by *Cercospora kikuchii*, *C.* cf. *sigesbeckiae* and *C.* cf. *flagellaris* (Albu *et al.*, 2016; Matsumoto and Tomoyasu, 1925; Suzuki, 1921; Walters, 1980), with *C.* cf. *flagellaris* being the dominant causal agent of CLB in the southern USA (Albu *et al.*, 2016). In the USA, CLB was first reported in 1978 (Walters, 1980). The first symptoms of CLB are usually observed in the late R5 (beginning of seed filling) and early R6 (end of seed filling) soybean growth stages on upper soybean leaves exposed to sunlight as reddish purple lesions, which become leathery, darken and gain bronze highlights as the disease progresses (Walters, 1980). At late soybean growth stages, angular-to-irregular lesions on infected leaf surfaces coalesce and eventually lead to premature defoliation.

In recent years, CLB was found in up to 70% of soybean fields surveyed in Alabama (Sikora *et al.*, 2011). The damage caused by CLB has been a serious concern (Moore and Wolcott, 2000) because the disease has spread from the southern states to as far north as Iowa and has caused as high as 15–30% yield losses (Hartman *et al.*, 1999; Wrather *et al.*, 1997, 2001). The yield losses caused by CLB in Louisiana and Mississippi were estimated to be 2.38 and 2.07 million bushels in 2016, respectively (Allen *et al.*, 2017). CLB is now among the top five most important diseases of soybean in the South. There is no effective management for CLB in soybean due to the long latent disease period, the lack of host resistance and effective fungicides.

During infection of soybean, *C.* cf. *flagellaris* produces a reddish-purple pigment called cercosporin. It was first extracted from dry mycelia of *C. kikuchii* in 1957 and was identified as a perylenequinone (Kuyama and Tamura, 1957). It can absorb light energy and be converted to an energetically activated triplet state, which can react with oxygen and result in the generation of toxic reactive oxygen species, such as singlet oxygen and superoxide (Daub and Chung, 2007). The photosensitized cercosporin can cause peroxidation of membrane lipids, leading to membrane breakdown and cell death (Daub and Briggs, 1983), which contributes to leakage of nutrients and promotes growth and spread of fungal hyphae intracellularly (Daub, 1982; Daub and Ehrenshaft, 2000). Cercosporin is therefore considered a virulence factor and has been associated with lesion formation on soybean leaves (Upchurch *et al.*, 1991a).

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The genes involved in cercosporin biosynthesis have been identified in *Cercospora nicotianae* and consist of at least eight cercosporin toxin biosynthesis (CTB) genes with a possible four additional open reading frames (ORFs) (ORF9–12) that are organized in a cluster (Chen *et al*., 2007). The importance of some of these genes has been further investigated through gene disruption studies, which found that the expression of the eight CTB genes was coordinated and regulated by CTB8, a zinc finger transcription factor (Chen *et al*., 2007). In addition to CTB8, cercosporin biosynthesis is also regulated by CRG1, a transcription factor involved in cercosporin resistance (Chung *et al*., 2003) and by a MAP kinase kinase kinase (CZK3) (Shim and Dunkle, 2003). Several studies further demonstrated the importance of a cercosporin facilitator protein (CFP) and an ABC transporter (ATR1) in the biosynthesis or efflux of cercosporin and in fungal pathogenesis (Amnuaykanjanasin and Daub, 2009; Callahan *et al*., 1999; Choquer *et al*., 2005; Chung *et al*., 2003; Daub *et al*., 2005). A biosynthetic pathway for cercosporin was also proposed (Chen *et al*., 2007). However, recent studies presented alternative biosynthetic pathways obtained by the characterization of metabolites from a series of biosynthetic pathway gene knockouts, as well as the discovery of several additional genes required for cercosporin biosynthesis through a comparative genomic and conserved gene cluster analysis (de Jonge *et al*., 2017, 2018; Newman and Townsend, 2016; Shim and Dunkle, 2003).

Other than cercosporin production, there is little information on *C.* cf. *flagellaris* pathogenesis. One interesting observation made by Stergiopoulos *et al.* (2010) was that several *Cercospora* species, such as *C. beticola*, *C. apii*, *C. nicotianae* and *C. zeina*, contain a homologue of AVR4, a well-studied fungal effector and a virulence factor of *Cladosporium fulvum* (Cf), on its host tomato (van Esse *et al*., 2007). Cf-AVR4 was demonstrated to bind chitin on the fungal cell wall and shield it from digestion by host-derived chitinases during infection (Joosten *et al*., 1994; van den Burg *et al*., 2006). The exact function of AVR4 is still unclear. However, the broadly conserved nature of AVR4 sequences across fungi with diverse lifestyles indicates that this effector may have conserved virulence functions that deregulate host immunity and facilitate infection on a wide range of hosts (Kohler *et al*., 2016).

If present, AVR4 from *C.* cf. *flagellaris* (Cfla-AVR4) also may contribute to its virulence on soybean. Therefore, the current study aimed to clone the Cfla-AVR4, disrupt it or suppress its expression through double-stranded RNA (dsRNA) treatment, and then compare the changes in CLB disease development, expression of the CTB genes and cercosporin production both *in vitro* and *in vivo* to determine the possible roles played by AVR4 in *C.* cf. *flagellaris*. Cercosporin production was drastically reduced in Δ*avr4* mutants compared to the wild-type fungus in our *in vitro* and *in planta* assays. In addition, the Δ*avr4* disruption mutants had lower expression levels of the CTB genes under light and dark conditions. Chitinase inhibited mycelial growth of Δ*avr4* mutants *in vitro*, whereas the wild-type fungus was not affected by the enzyme. Moreover, the growth and virulence of Δ*avr4* mutants were clearly compromised on soybean leaves, suggesting that the mutants were more vulnerable to the deleterious effects of the host chitinases. Interestingly, cercosporin was also found to inhibit chitinase activity *in vitro*. These results suggest that AVR4 contributes to the virulence of *C.* cf. *flagellaris* on soybean through protecting fungal hyphae and affecting cercosporin biosynthesis. Wild-type *C.* cf. *flagellaris* also exhibited reduced cercosporin production and reduced cercosporin biosynthesis gene expression when it was grown in liquid culture in the presence of *Avr4*-specific dsRNA. The knowledge gained from the present study can lead to the development of more efficient strategies to manage CLB in soybean.

RESULTS

Cloning and sequence analysis of *C.* cf. *flagellaris Avr4* gene

A 0.35 kb *Avr4* fragment was amplified from *C.* cf. *flagellaris* gDNA using primers designed based on conserved sequences of *Avr4* homologues from other *Cercospora* species. The full-length *Avr4* gene was obtained by polymerase chain reaction (PCR) of previously constructed *C.* cf. *flagellaris* genomic libraries using primers designed to walk upward and downward into unknown genomic regions. In total, six overlapping fragments were amplified, sequenced and assembled into a 1.6 kb DNA fragment (Fig. S1, Supporting information), which has been deposited into GenBank under accession number MH673051. This *Cfla-Avr4* gene has an ORF from 686 to 1075 bp with no introns and encodes a protein of 129 amino acids with a putative transcriptional start site predicted at −15 bp upstream of the ATG start codon (Fig. S1). Sequence analysis showed high identity (97–99%) at the deduced amino acid level to AVR4 from *C. beticola*, *C. nicotianae*, *C. zeina* and *C. apii*, (Fig. S2). A BLAST search of NCBI databases revealed the presence of a putative chitin-binding peritrophin-A domain (NCBI accession: pfam01607) that is conserved in all five *Cercospora* species (Fig. S2). Furthermore, the nine cysteine residues reported in *C. beticola*, *C. nicotianae*, *C. zeina* and *C. apii* (Stergiopoulos *et al*., 2010) were also present in the *C.* cf. *flagellaris Avr4* gene (Fig. S2).

Target gene disruption and confirmation of Δ*avr4* mutants

To elucidate the role of AVR4 in *C.* cf. *flagellaris* virulence and fitness, Δ*avr4* disruption mutants were created using the strategies outlined in Fig. 1A. Two DNA fragments containing 5′ *Avr4* fused with 3′ of hygromycin phosphotransferase B gene (*HYG*) and 3′ *Avr4* fused with 5′ *HYG* were obtained by fusion PCR and transformed directly into protoplasts of the wild-type isolate. All 17 selected hygromycin-resistant fungal transformants showed the

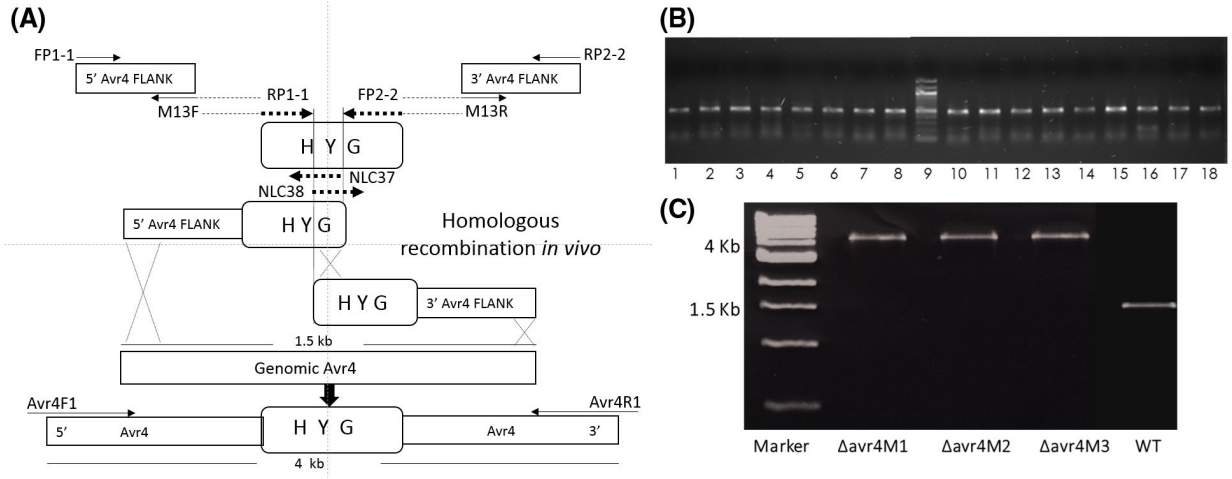

Fig. 1 Targeted disruption of the *Avr4* gene in *C.* cf. *flagellaris*. (A) The 5′ and 3′ fragments of *Avr4* were amplified separately with primer pairs indicated and fused with the hygromycin cassette (*HYG*) as described in Experimental procedures. The resulting two PCR fragments were used to transform *Cercospora* cf. *flagellaris* protoplasts. Note: drawing is not to scale. (B) Sixteen selected Δ*avr4* mutants (lines 1 to 7 and 10 to 18) contained the 466 bp *HYG* fragment when amplified by PCR using primer pair NLC37/NLC38. Lane 8 shows the 466 bp *HYG* fragment amplified from pUCATPH vector. Lane 9 shows the DNA size marker. (C) The presence of 4 kb PCR fragment using external primers Avr4F1/Avr4R1 in Δ*avr4* mutants compared to the 1.6 kb *Avr4* gene in *C.* cf. *flagellaris* wild-type (WT) confirming the *HYG* insertion.

presence of the 466 bp fragment from *HYG* by PCR using *HYG*-specific primers NLC37 and NLC38 (Fig. 1B). Site-specific integration of the split marker DNA fragments into the *Avr4* gene locus was also verified through PCR with an upstream and downstream pair of primers. Selected mutants were confirmed to contain the expected extra 2.4 kb insertion of *HYG* compared to the wild-type (1.6 kb) when its full length was amplified with primers Avr4F1 and Avr4R1(Fig. 1C). Three mutants (Δ*avr4M1*, Δ*avr4M2* and Δ*avr4M3*) were selected for further studies.

Δ*avr4* mutants grew faster on solid media, produced little cercosporin and had reduced expression of CTB genes *in vitro*

The Δ*avr4* mutants grew faster on solid media than the wild-type (Fig. S3). The difference in mycelium mat diameter became clear and significant 9 days after inoculation and it was about 50% larger in diameter than the wild-type 13 days after inoculation (Fig. S3). In addition, the Δ*avr4* mutants produced very little dark red pigment (a typical colour of concentrated cercosporin) under the fungal colony, which was strikingly different from the colour under the wild-type colony (Figs S3 and 2). Cercosporin production of Δ*avr4* mutants on potato dextrose agar (PDA) plates was approximately 10% of the amount of cercosporin produced by the wild-type (Fig. 2). The difference in cercosporin production among the three Δ*avr4* mutants was not significant.

When grown in liquid potato dextrose broth (PDB) or complete medium (CM) the same phenotype could be observed in which the wild-type fungal culture became dark red in colour after 5 days of incubation at room temperature, whereas the mutant cultures exhibited a light yellow colour (Fig. S4). Δ*avr4* mutants produced only 3–7% of the level of cercosporin compared to the wild-type when grown in either liquid CM or PDB medium based on HPLC analysis (Fig. S5). Cercosporin was confirmed to be the main constituent in the red pigment produced by *C.* cf. *flagellaris* wild-type in liquid media based on mass spectrometry analysis of the mass/charge (*m/z*) ratio of the dark red pigment and the cercosporin standard (Fig. S6).

Considering the reduction in cercosporin production by the Δ*avr4* mutants in liquid and solid media compared to the wild-type, the expression of cercosporin biosynthesis pathway genes was examined using real-time reverse transcription (RT)-PCR. It was found that disruption of *Avr4* significantly reduced the expression of both *CTB1* and *CTB8* genes relative to *β-tubulin* under light and dark conditions (about two- to three-fold) (Fig. 3).

Chitinase reduced the growth of Δ*avr4* mutants and chitinolytic activity is inhibited by cercosporin *in vitro*

AVR4 from *C. fulvum* was reported to protect the fungal cell wall from degradation by host chitinase during infection of tomato (van den Burg *et al.*, 2006), therefore the effect of chitinase on the growth of Δ*avr4* mutants compared to the wild-type fungus was also examined. Inhibition of hyphal growth was observed when the mutants were grown next to wells containing 1 unit (U) of chitinase on CM agar plates (Fig. 4). Such suppression of growth by chitinase was not observed in the wild-type (Fig. 4), demonstrating that AVR4 from *C.* cf. *flagellaris* plays a similar role in protecting the pathogen against host chitinases.

A cup-plate assay was also performed to determine whether cercosporin has a direct effect on chitinolytic activity. Reductions of 19%

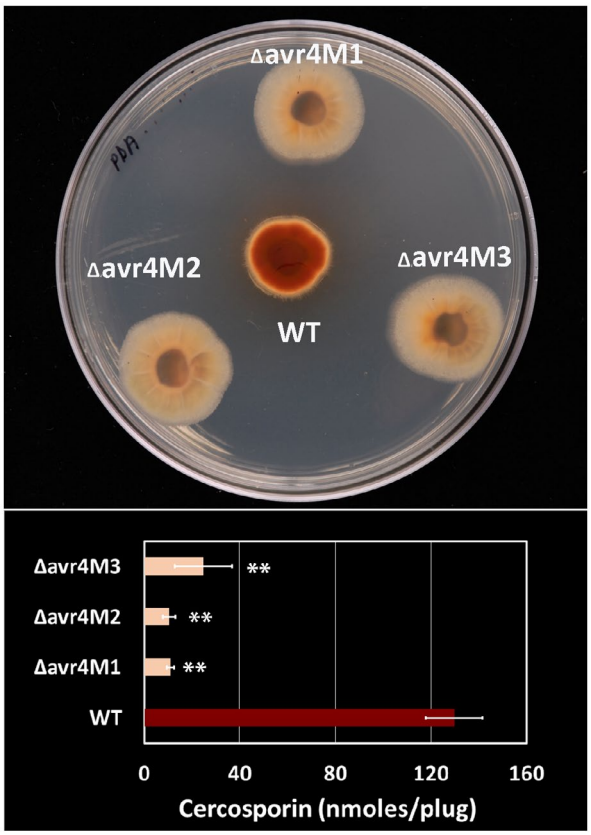

Fig. 2 Cercosporin production of *Cercospora* cf. *flagellaris* wild-type (WT) and Δ*avr4* mutants *in vitro*. The fungus was grown on potato dextrose agar plates under light for 5 days (top) and cercosporin was extracted with 5 M KOH and quantified by absorbance at 480 nm (bottom). Data are the mean and standard errors of three different experiments with five replicates of each fungal isolate. Asterisks (**) represent significant difference between WT and the mutants at $P < 0.01$.

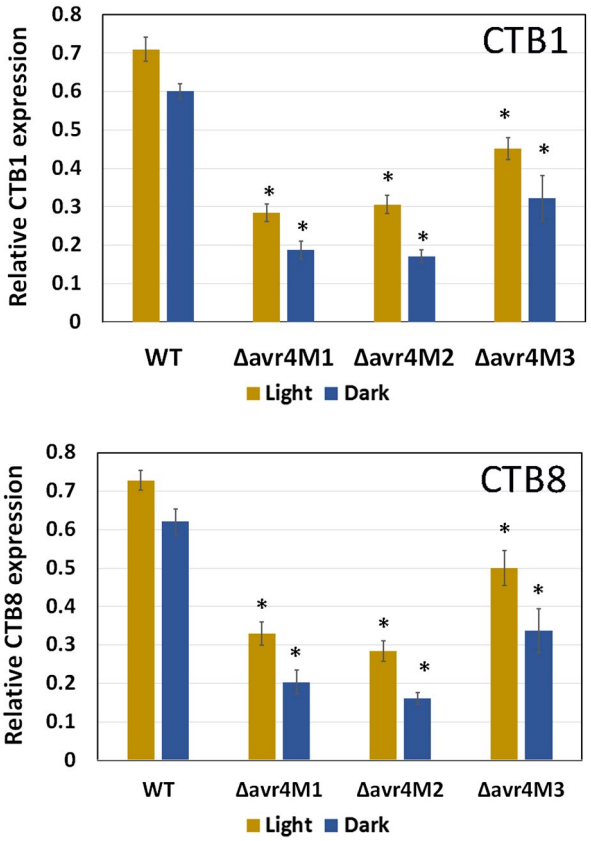

Fig. 3 The expression of *CTB1* and *CTB8* relative to *β-tubulin* in *Cercospora* cf. *flagellaris* wild-type (WT) and Δ*avr4* mutants. Fungal RNA was isolated from cultures grown in potato dextrose broth under light or darkness for 5 days. The expressions of *CTB1* and *CTB8* genes were normalized to that of *β-tubulin* from the same sample. Data are the mean and standard errors of three different experiments with three biological replicates of each fungal isolate and three technical replicates of real-time reverse transcription PCRs. Asterisks (*) indicate significant difference between the wild-type and the mutants when grown under light or dark conditions ($P < 0.05$).

and 30% in chitinase activity were observed in the presence of 15 μM of cercosporin in the plate after 2 and 3 days, respectively, when the wells contained 0.25 U of chitinase (Fig. 5A,B). A slightly lower reduction in chitinolytic activity (12% and 16%, respectively) was observed after 2 and 3 days when 0.5 U of chitinase was used under the same conditions (Fig. 5A,B). The difference among the agarose plates with and without cercosporin became very clear after 5 days, when 60% and 70% reductions in enzyme activity were observed using 0.25 and 0.5 U of chitinase, respectively (Fig. 5B). However, the same concentration of cercosporin did not significantly inhibit the enzyme activity of the α-amylase from *Aspergillus oryzae* on starch agarose cup plates compared to the control plates (Fig. 5C), suggesting that the inhibition of chitinase activity by cercosporin is enzyme-specific.

Cercospora cf. *flagellaris* Δ*avr4* mutants showed reduced virulence and growth on soybean leaves

To determine whether the loss of AVR4 alters the virulence of *C.* cf. *flagellaris* on soybean, the mycelial mats of the wild-type isolate and Δ*avr4* mutants from PDA were inoculated onto detached soybean leaves. The mutants grew twice as slowly as the wild-type on the detached soybean leaves based on visual assessment, which is the opposite of what was observed when they were grown on PDA plates (Fig. S3). In addition, the mutants incited smaller necrotic and chlorotic lesions compared to the wild-type at the site of mycelial inoculation (Fig. 6A,B), indicating a clear reduction in fungal virulence. This reduced virulence of Δ*avr4* mutants on soybean leaves was confirmed by a 50% reduction in fungal growth inside the leaves inoculated with the mutants compared to those inoculated with the wild-type pathogen (Fig. 6C).

The development of minor necrotic and chlorotic lesions on soybean leaves inoculated with the mutants indicates that Δ*avr4* mutants still produce a small amount of cercosporin during infection of soybean leaves. This was confirmed through HPLC analysis, which detected low levels of cercosporin ranging from

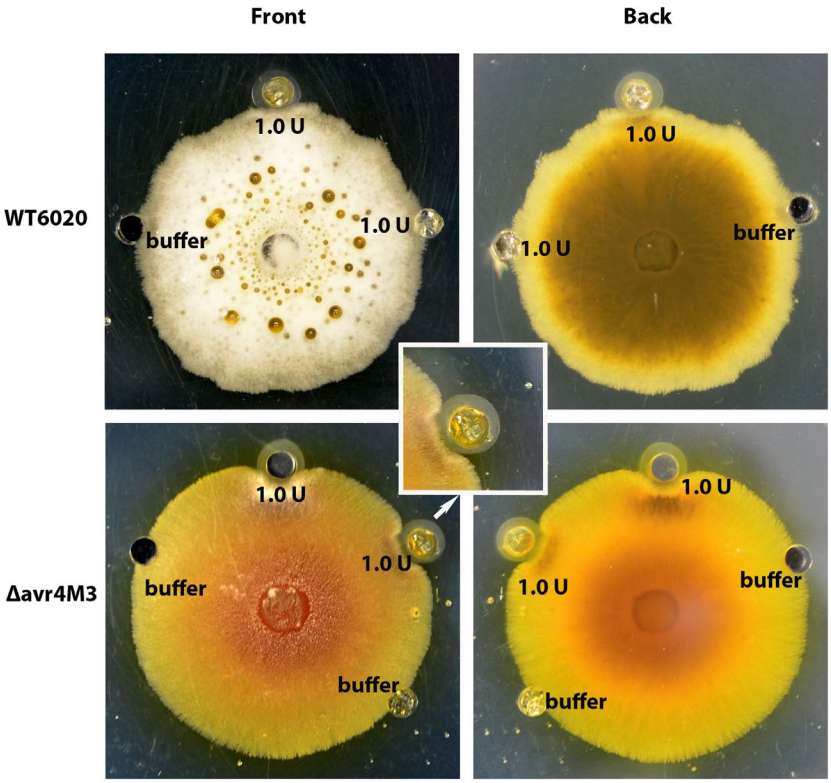

Fig. 4 Growth of *Cercospora* cf. *flagellaris* wild-type (WT) and Δ*avr4* mutant in the presence of chitinase. One unit (U) of chitinase dissolved in 30 μL of 0.1 M potassium phosphate buffer (pH 6.5) was pipetted into wells of 1-week-old complete medium plates inoculated with *C.* cf. *flagellaris* WT or Δ*avr4* mutants. Wells filled with 0.1 M phosphate buffer only were used as controls. Plates were sealed and kept at 25 °C. Fungal growth was visually assessed 2 days later.

0.31 to 0.55 μg/g of leaf tissue in soybean leaves inoculated with *C.* cf. *flagellaris* mutants compared to 3.9 μg/g in leaves inoculated with the wild-type fungus (Fig. S5).

Droplet digital PCR detected the presence of additional copies of *HYG* cassette in the mutants

To address the concern that ectopic integration of the *HYG* cassette could have contributed to any of the phenotypes observed above, genomic DNA from the wild-type and the three mutants was analysed through droplet digital PCR using primers and probe for *HYG* as a target and the known single-copy aminoadipate reductase gene (*LYS2*) as a reference. The results indicated that all three mutants have more than one copy of *HYG* in their genome (Table S1), which suggests that the observed phenotypes of the mutants could be due to knockout of unknown gene(s) at non-target location(s). An independent study of suppressing *Avr4* expression of the wild-type *C.* cf. *flagellaris* in culture using dsRNA targeting the *Avr4* gene was therefore conducted.

Avr4 dsRNA suppressed cercosporin production of the wild-type *C.* cf. *flagellaris* grown in CM

Wild-type *C.* cf. *flagellaris* produced 6.0 and 6.1 μg/mL cercosporin in half-strength CM after 4 days in the absence of any dsRNA, or in the presence of the lysate prepared from *Escherichia coli* HT115 cells with L4440 plasmid only (EV), respectively (Fig. 7A,B). However, the cercosporin production in the wild-type was reduced by 88% to 0.7 μg/mL when the culture was treated with *Avr4* dsRNA prepared from bacterial cells containing the L4440-*Avr4* construct (Fig. 7A,B). This is consistent with the visual colour differences among the cercosporin extracts following different treatments (Fig. 7C). After 4 days of culturing in the presence of *Avr4*-specific dsRNA, the expression of *Avr4* was suppressed by 78% compared to the EV control (Fig. 7D). In addition, the expression of cercosporin biosynthesis pathway genes *CTB1* and *CTB8* was also significantly reduced (75% and 58%, respectively) in the mycelia that had been exposed to the extract containing *Avr4*-specific dsRNA compared to the control that was treated with dsRNA prepared from *E. coli* cells that contained only the EV (Fig. 7D). This *Avr4* dsRNA treatment study independently confirmed the involvement of *Avr4* in cercosporin biosynthesis in *C.* cf. *flagellaris*, possibly through affecting the expression of cercosporin biosynthesis pathway genes such as *CTB1* and *CTB8*.

DISCUSSION

Cercospora cf. *flagellaris* is the causal agent of CLB disease in Louisiana and the interest in studying the AVR4 effector arose

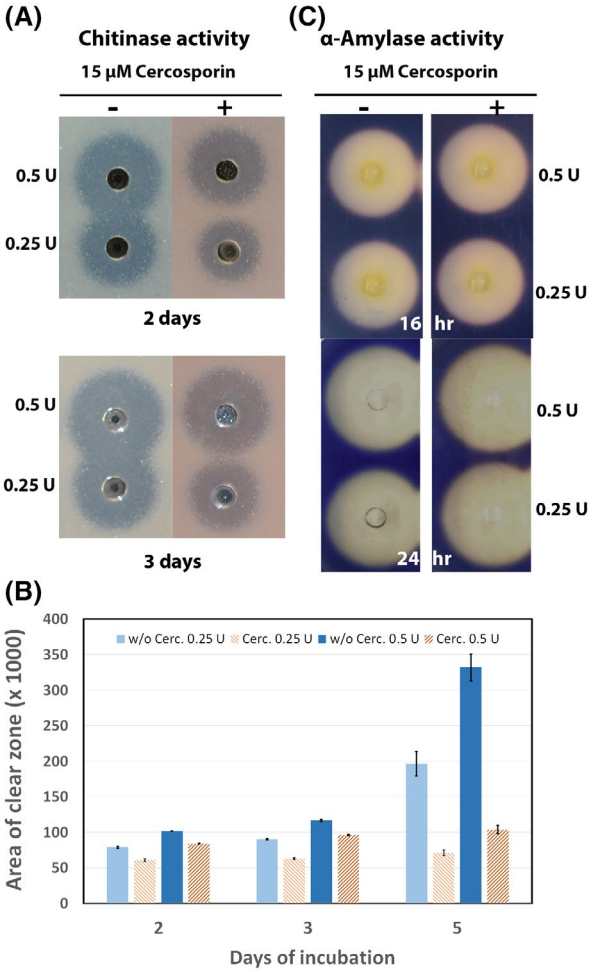

Fig. 5 Chitinase and α-amylase activity in the presence of cercosporin. (A) Agarose plates containing chitin and 15 µM or no cercosporin were used to assess chitin hydrolysis visualized as clearing zones around the wells containing 0.5 or 0.25 units (U) of chitinase after 48 and 72 h of incubation at 25 °C. (B) Area of clear zone assessed after 48, 72 and 120 h using Progenesis Software. (C) Enzyme activity of the α-amylase from *Aspergillus oryzae* on 0.25% soluble starch agarose plates containing 15 µM or no cercosporin after 16 and 24 h of incubation at 25 °C.

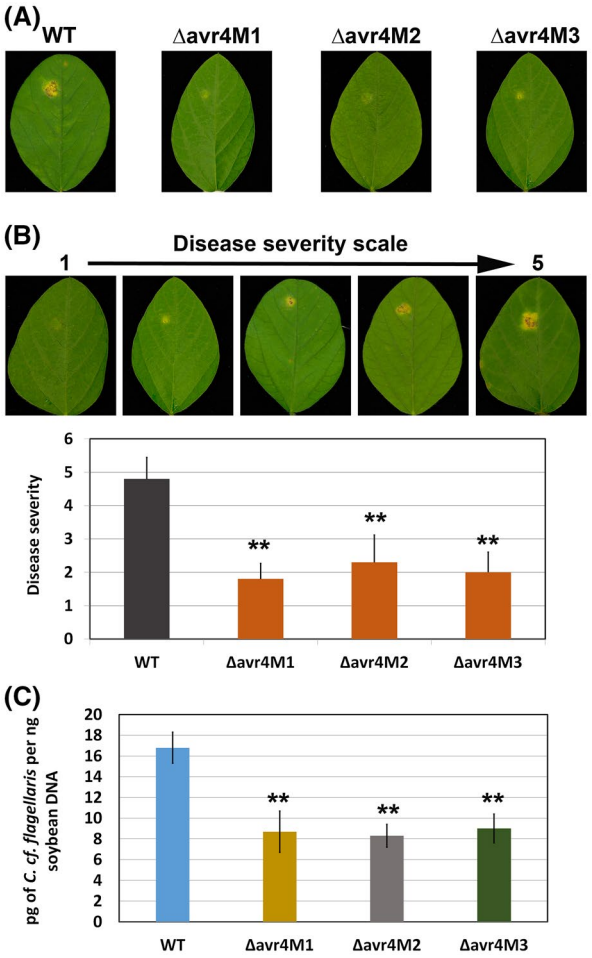

Fig. 6 Virulence of *Cercospora* cf. *flagellaris* wild-type (WT) and Δ*avr4* mutants on detached soybean leaves. (A) Detached soybean leaves were inoculated with mycelia plugs of the WT or Δ*avr4* mutants and disease severity was determined at 14 days after inoculation. (B) Disease severity was determined based on the 1–5 rating scale (1 = less and 5 = more severe). Data are the mean and standard errors of three different experiments with three biological replicates (eight leaves) within each fungal isolate. Asterisks (**) indicate significant disease severity difference between the wild-type and the mutants ($P < 0.01$). (C) Fungal growth was quantitatively assessed by determining the fungal DNA levels in soybean leaves through real-time PCR. Data are the mean and standard errors of three different experiments with three biological replicates (eight leaves) for each wild-type or mutant.

from the study by Stergiopoulos *et al.* (2010), which showed the presence of homologues of *Avr4* in different species of the Dothideomycetes, including *Cercospora* spp., indicating its possible function as a virulence factor on distantly related host plants. Silencing of this gene in *C. fulvum* resulted in reduction of disease symptoms and fungal growth on tomato leaves (van Esse *et al.*, 2007). AVR4 was found to be a chitin-binding protein (van den Burg *et al.*, 2006) and the presence of AVR4 in the apoplast of *Arabidopsis* and tomato plants has also been shown to enhance the susceptibility of these plants to several fungal pathogens (Cai *et al.*, 2009; van Esse *et al.*, 2007).

In the present study, the 1.6 kb fragment cloned from *C.* cf. *flagellaris* was identified as *Avr4* based on its nucleotide and deduced amino acid sequence homologies with the *Avr4* sequences from other Dothideomycete fungi (*C. beticola*, *C. nicotianae*, *C. apii*, *C. zeina*, *Mycosphaerella fijiensis* and *C. fulvum*). In addition, the *Avr4* gene was detected in 150 isolates of *C.* cf. *flagellaris* collected from several locations throughout Louisiana. It is possible that *Cercospora* species maintain the conserved *Avr4* to target a diverse range of hosts (Crous and Braun, 2003; Groenewald *et al.*, 2013). Certainly, AVR4 as a chitin-binding protein can be very useful in assisting

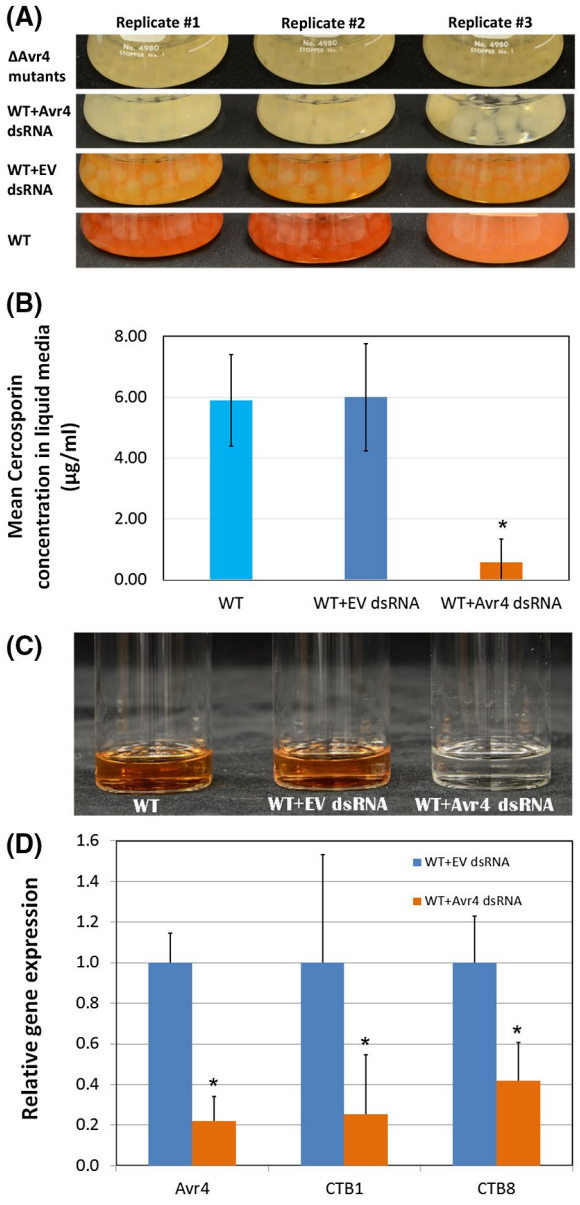

Fig. 7 The effect of *Avr4* dsRNA on cercosporin production and target gene expression. (A) Visual phenotype changes of the wild-type (WT) *Cercospora* cf. *flagellaris* grown in half-strength complete medium (CM) alone or treated with total nucleic acid extracted from *Escherichia coli* containing L4440 vector (WT + EV dsRNA) or containing *Avr4* gene-specific dsRNA (WT + Avr4 dsRNA) for 4 days in comparison to that of Δ*avr4* mutant. The final concentration in the culture was 16 µg/mL for either EV dsRNA or *Avr4* dsRNA. (B) Bar graph of cercosporin production in non-treated WT *C.* cf. *flagellaris* grown in half-strength CM or treated with total nucleic acid extract containing L4440 vector (EV) or containing *Avr4* gene-specific dsRNA (Avr4 dsRNA). (C) Visual comparison of cercosporin extracted from the WT *C.* cf. *flagellaris* under different treatments for 4 days. (D) Relative expressions of *Avr4*, *CTB1* and *CTB8* genes in the WT *C.* cf. *flagellaris* after different treatments.

fungi to enter and colonize different host plants that use chitinases as a defence mechanism to combat fungal invasion. Considering all the information above, we hypothesized that the presence of this effector in *C.* cf. *flagellaris* plays an important role in the virulence of this pathogen on soybean.

Avr4 disruption mutants were created from *C.* cf. *flagellaris* wild-type isolate MRL 6020 2B, a well-known cercosporin-producing isolate, to elucidate the role of AVR4 in *C.* cf. *flagellaris* fitness and virulence. The first noticeable change in the phenotype of the Δ*avr4* mutants was the reduced production of the dark-red pigmentation on solid and liquid media, a characteristic of cercosporin that was verified through mass spectrometry (Fig. S6). Further investigation revealed that reduced cercosporin production was associated with the down-regulation of *CTB1* and *CTB8* gene expression in the Δ*avr4* mutants (Fig. 3). This result agreed with earlier studies showing that expression of CTB genes was correlated with toxin production (Chen *et al.*, 2007).

The Δ*avr4* mutants were found to contain more than one copy of the *HYG* cassette, indicating the presence of possible ectopic integrations besides the intended insertion in the *Avr4* target gene (as determined through PCR). However, all three mutants shared the same phenotype and it is unlikely that random, unintended gene knockouts in three separately generated mutants contributed to those phenotypes without causing any other noticeable differences among them. Moreover, the above phenotypes, such as reduction in cercosporin production (Fig. 2) and significant suppression of cercosporin pathway genes (Fig. 3), were independently confirmed by *in vitro* dsRNA-mediated suppression of *Avr4* in the wild-type (Fig. 7). These combined data conclusively demonstrated a direct role of AVR4 in affecting cercosporin biosynthetic pathway gene expression and cercosporin production. It is reasonable to speculate that the reduced virulence of the Δ*avr4* mutants was most likely due to the reduced cercosporin production, since cercosporin was repeatedly reported as a virulence factor in *Cercospora* species, as well as a critical factor in the development of many plant diseases (Choquer *et al.*, 2005; Gunasinghe *et al.*, 2016; Staerkel *et al.*, 2013; Upchurch *et al.*, 1991a; Weiland *et al.*, 2010). One possible explanation as to why different mutants with multiple copies of a *HYG* insertion exhibited the same phenotype is that the integrations may not be ectopic. Instead, they might have been integrated into the genome of the mutants as a concatemer at the intended target location (*Avr4*), which has been reported to occur (Kamisugi *et al.*, 2006).

Apart from the altered cercosporin production, fungal growth was also different between *C.* cf. *flagellaris* Δ*avr4* mutants and the wild-type. Mutants were found to grow more rapidly on solid media compared to the wild-type based on radial growth measurements (Fig. S3). This finding agrees with previous studies showing a negative correlation between fungal growth and cercosporin production (Jenns *et al.*, 1989; Upchurch *et al.*, 1991a). Compared to Δ*avr4* mutants, the wild-type strain grew more, not only saprophytically on the surface of inoculated soybean leaves based on our visual assessment,

but also more abundantly inside the soybean leaves based on real-time PCR quantification of fungal biomass (Fig. 6C), which clearly reflects the differences in virulence between the wild-type pathogen and the mutants. Moreover, fungal growth was positively correlated with disease severity on soybean leaves, as wild-type fungus caused more severe symptoms than the Δ*avr4* mutants (Fig. 6).

Previous studies showed that the small conserved cysteine-rich AVR4 protein binds chitin and protects *C. fulvum* against plant chitinases during infection of tomato and it also protects *Trichoderma viride* and *Fusarium solani* f. sp. *phaseoli* in the presence of chitinase and β-1,3-glucanase *in vitro* (van den Burg *et al*., 2006). This kind of protection against plant chitinases is essential for fungal development and colonization of its host. Recently, AVR4 from *C. apii* and *C. beticola* has been shown to bind chitin as well (Mesarich *et al*., 2016). In order to verify whether AVR4 from *C.* cf. *flagellaris* has the same protective function, a chitinase assay was also performed. As expected, Δ*avr4* mutants were found to be more sensitive to chitinase than wild-type *C.* cf. *flagellaris* (Fig. 4).

In order to verify if cercosporin could inhibit chitinase activity, a cup-plate assay was performed and a clear reduction of the chitinolytic activity was observed in the presence of cercosporin, suggesting that this inhibition by cercosporin is protein-specific, since the activity of an α-amylase was not inhibited in the presence of cercosporin (Fig. 5). Therefore, the observed reduced colonization of soybean leaves by Δ*avr4* mutants could be the combined result of lacking a functional AVR4 effector and the reduced production of cercosporin, which can not only cause peroxidation of membrane lipids leading to membrane breakdown and cell death (Daub and Chung, 2007; Daub and Hangarter, 1983), but can also directly inhibit chitinolytic activity.

Taken together, our findings suggest that this fungal effector protein suppresses host defence by affecting the expression of cercosporin pathway genes and toxin production. To the best of our knowledge, this is the first report concerning the roles of an AVR4 effector homologue in *C.* cf. *flagellaris* and the first report in linking AVR4 to cercosporin production. Further studies will be needed to understand how AVR4 affects cercosporin production in *C.* cf. *flagellaris*.

EXPERIMENTAL PROCEDURES

Cloning of *Avr4* from *C.* cf. *flagellaris*

Cercospora cf. *flagellaris* wild-type isolate MRL 6020-2B, a well-known cercosporin-producing isolate (Cai and Schneider, 2008), was grown for 5 days in PDB (Difco Laboratories Inc., Detroit, MI, USA) with constant shaking (200 rpm) at 25 °C under continuous light. Fungal dried mycelia were ground in liquid nitrogen and DNA was extracted with a GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, St Louis, MO, USA). Based on the *Avr4* sequences available in GenBank (GU574324, GU574325, GU574326 and GU574327), Avr4F and Avr4R2 primers (see Table 1 for all primer sequences) were designed to confirm the presence of *Avr4* in *C.* cf. *flagellaris*. The amplified fragment was cloned into pCR2.1-TOPO TA Cloning vector (Invitrogen, Carlsbad, CA, USA) and sequenced. Genomic libraries of *C.* cf. *flagellaris* previously constructed using the Universal GenomeWalker kit (Clontech Laboratories Inc., Mountain View, CA, USA) by Chanda (2012) were used to clone the full-length *Avr4* gene according to the manufacturer's guidelines. Adapter primers (AP1 and AP2) and gene-specific primers (GSP1F and GSP1R, GSP2F and GSP2R) were used in two rounds of PCR to walk upwards and downwards into unknown genomic regions. The resulting DNA fragments were cloned into pCR2.1-TOPO and sequenced.

Disruption of *Avr4* in *C.* cf. *flagellaris*

Avr4 was disrupted by double homologous recombination using fusion PCR according to Yu *et al.* (2004). The hygromycin (*HYG*) cassette (2.5 kb) was amplified from pUCATPH (Lu *et al*., 1994) with the primers M13R and M13F. *Cercospora* cf. *flagellaris* genomic DNA was used to amplify the 5′ *Avr4* fragment (0.4 kb) with FP1-1 and RP1-1, and the 3′ *Avr4* fragment (0.5 kb) with FP2-2 and RP2-2. The underlined sequence in the primers RP1-1 and FP2-2 is complementary to the sequence of primers M13R and M13F, respectively. A second round of PCR was performed and a 1.5 kb fragment containing 5′ *Avr4* fused with 3′ *HYG* was amplified using primers FP1-1 and NLC37. A 2.5 kb fragment containing 5′ *HYG* fused with 3′ *Avr4* was amplified with primers RP2-2 and NLC38. The two PCR fragments, overlapping within the *HYG* region, were purified using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). These purified DNA fragments were used to transform the protoplasts of the *C.* cf. *flagellaris* wild-type isolate as described below.

Protoplasts were prepared as previously described (Chung *et al*., 2002; Upchurch *et al*., 1991a). Fungal transformation was carried out as described by Turgeon *et al.* (2010). The colonies that appeared between 5 and 8 days were selected and transferred to CM plates containing 300 μg/mL hygromycin B (Roche Diagnostics, Indianaplis, IN, USA). Hygromycin-resistant colonies were screened by PCR by using *HYG*-specific primers NLC37 and NLC38. The primers Avr4F1 and Avr4R1 were used to validate site-specific integration.

Fungal isolates, growth conditions and growth measurements

Cercospora cf. *flagellaris* MRL 6020-2B (Cai and Schneider, 2005) and mutants (Δ*avr4M1*, Δ*avr4M2* and Δ*avr4M3*) were maintained on CM (Jenns *et al*., 1989) and the CM amended with 300 μg/mL

Table 1 List of primers used in this study

Primer name	Oligonucleotide sequence (5′→3′)
Avr4F	AAGGATCCATGTACGGCCTCTTCCACCTC
Avr4R2	AAGGTACCTTGGTGCAGGTGCAGGTGCTGA
AP1	GTAATACGACTCACTATAGGGC
AP2	ACTATAGGGCACGCGTGGT
GSP1F	AGACATGAAACGAACACACTGTATGG
GSP1R	AGTGCGTAGAGGCAGTCGAATGGAC
GSP2F	ACTACCAGGGAACTGCCTCGGATACAT
GSP2R	CCATACAGTGTGTTCGTTTCATGTCT
M13R	AGCGGATAACAATTTCACACAGGA
M13F	CGCCAGGGTTTTCCCAGTCACGAC
FP1-1	CCCGATGCTTGTCCGCAATA
RP1-1*	TCCTGTGTGAAATTGTTATCCGCTTCTTGTT GTTGAGGCAGTGC
FP2-2*	GTCGTGACTGGGAAAACCCTGGCGTGCTGGTCA ATGGTGGAACG
RP2-2	CACAGTCTAACGCCTTCCGT
NLC37	GGATGCCTCCGCTCGAAGTA
NLC38	CGTTGCAAGAACTGCCTGAA
Avr4F1	GCATTGCCCTAACAGACCATC
Avr4R1	AGAATAGTGGGCGTTGCGT
Avra4sh_F*	GAATTAATACGACTCACTATAGGGAGACCATGT ACGGCCTCTTCC
Avra4sh_R*	GAATTAATACGACTCACTATAGGGAGAGCATG TTGGAGGCTCCAC
CTB1 2F	ACCTTGCCTCAACTGTCTTAC
CTB1 2R	TGAAGCGACGAACGGATTT
CTB8 1F	GACAGCAGGTTATCTTCCAGAG
CTB8 1R	GTACTTATGCATCCACCACCA
Avr4WT-F	CCGGTATCGCGTATGAAAGG
Avr4WT-R	GAGAAGAAGTACTGCGACACGGT
Avr4WT-PRB$	FAM-ATGCCGTGCTGGTCAATGGTGGA-TAMRA
CKCTB6-2F	CACCATGCTAGATGTGACGACA
CKCTB6-2R	GGTCCTGGAGGCAGCCA
CKCTB6-PRB$	FAM-CTCGTCGCACAGTCCCGCTTCG-TAMRA
Hyg B qPCR F1	GCT TTC AGC TTC GAT GTA GGA
Hyg B Prb 1$	5HEX/TAG CTG CGC /ZEN/CGA TGG TTT CTA CAA /3IABkFQ
Hyg B qPCR R1	CGA TGC AAA GTG CCG ATA AAC
Lys2 qPCR Fl	GGT CAG TCG AAA TGG GTA TCT G
Lys2 qPCR Prb 1$	6-FAM/TTG TTC GTT /ZEN/CCG GCT ACG TGA TGG /3IABkFQ
Lys2 qPCR Rl	TTG CTC ACG CCA GTC TTT

*The underlined are M13Foward and M13Reverse primer sequences (RP1-1 and FP2-2) or T7 promoter sequences needed for dsRNA production (Avr4sh_F and Avr4sh_R).$FAM and HEX are two different fluorescent dyes used to label the probes; TAMERA, ZEN and 3IABkFQ are different fluorescent quenchers.

hygromycin B, respectively. Fungal growth was assessed by placing a 7-mm diameter mycelial plug in the centre of a PDA (IBI Scientific, Peosta, IA, USA) plate and colony radial growth measured every 2 days to determine the colony size increase over a period of 13 days. For liquid culture, the mycelial suspension prepared by grinding three 7-mm mycelial plugs of 1-week-old fungal colony on solid CM in 2 mL of sterile water was used to inoculate 100 mL of liquid CM. Cultures were incubated at 25 °C with constant shaking (200 rpm) under light (240 μmol/m^2/s) or dark (achieved by wrapping the flasks with two layers of aluminium foil) for 5 days.

Cercosporin extraction and quantification

From solid media, cercosporin was extracted from three 7-mm mycelial plugs of 5-day-old fungal colony on PDA by soaking in 4 mL of 5 M KOH in the dark for 4 h and cercosporin was quantified using a spectrophotometer by measuring absorbance at 480 nm (Jenns *et al.*, 1989). From liquid culture, cercosporin was extracted from 25 mL of 5-day-old fungal cultures grown in CM with 20 mL of ethyl acetate for 8 h as described by Gunasinghe *et al.* (2016). Extraction of cercosporin from soybean leaves infected with *C.* cf. *flagellaris* was also performed according to Gunasinghe *et al.* (2016). Briefly, soybean leaves (0.2 g) showing typical CLB symptoms were ground in 2 mL of ethyl acetate and the mixtures were kept overnight at 4 °C. Following centrifugation, the supernatant was collected and cercosporin was identified and quantified by high-performance liquid chromatography (HPLC) as described below.

HPLC and mass spectrometry analyses of cercosporin

Cercosporin identification and quantification were achieved through comparing the retention time and area under the target peak of the pigment extracted from infected leaves or fungal cultures to the cercosporin standard (Sigma-Aldrich). HPLC analysis of cercosporin was performed using a Waters (Milford, MA, USA) 2695 Separations Module with a Waters Atlantis C18 column (150 mm × 4.6 mm with 5 μm pore size) and detected using a Waters 2475 fluorescence detector with the mobile phases as described by Gunasinghe *et al.* (2016). In addition, cercosporin standard and the red pigment extracted from fungal cultures as well as *C.* cf. *flagellaris* inoculated soybean leaves were analysed by LC-mass spectrometry through a fee-based service at LSU Chemistry Department.

RNA isolation and real-time PCR analysis of CTB1 and CTB8 gene expressions

Fungal cultures were grown for 5 days in CM with constant shaking (200 rpm). Cultures were incubated at 25 °C under either continuous white fluorescent light or in darkness, attained by wrapping flasks with two layers of aluminium foil. Dried fungal

mycelia were ground to a fine powder in liquid nitrogen and RNA was isolated using RNeasy Plant Mini Kit (Qiagen). Subsequently, cDNA was synthesized from 500 ng of total RNA using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations. RNase-free DNase (Qiagen) was used to remove possible residual DNA contamination. Real-time PCR experiments were performed using SYBR Green Master Mix (Applied Biosystems), CTB1 (2F and 2R) and CTB8 (1F and 1R) gene-specific primers, and the ABI PRISM 7000 Sequence Detection System (Applied Biosystems) under standard conditions. The expression of CTB genes was normalized to *β-tubulin* in the same culture to correct for template variations from one sample to another using the ΔC_t method. The data presented here are a relative expression of target genes to *β-tubulin*. Three biological replicates and three technical replicates within each biological replicate were used for each sample and a negative (template-free) control.

Growth of *C.* cf. *flagellaris* wild-type and Δ*avr4* mutants in the presence of chitinase and agarose plate assay for chitinase and α-amylase activity in the presence of cercosporin

A plate assay was performed to verify if growth of *C.* cf. *flagellaris* wild-type and Δ*avr4* mutants were affected by chitinase. Chitinase (Sigma-Aldrich, #C8241-25UN, ≥600 units/g) was dissolved in 0.1 M phosphate buffer. CM plates were inoculated with *C.* cf. *flagellaris* wild-type or Δ*avr4* mutants using 7-mm diameter mycelial plugs and kept at 25 °C. After 14 days 4-mm diameter wells were cut with a cork borer in the agar 1 mm away from the edge of the fungal colony and filled with 30 μL of 0.1 M phosphate buffer containing 1 U of chitinase or with 0.1 M phosphate buffer only as a control. Plates were sealed and kept at 25 °C for two additional days before fungal growth was visually assessed to determine whether mycelial development was affected around the wells or filter-paper discs containing chitinase.

To verify if chitinolytic activity is influenced by cercosporin, a chitinase cup-plate assay was performed. Glycol chitin substrate was synthesized according to Roberts and Selitrennikoff (1988) from shrimp-shell chitin (Sigma-Aldrich). To prepare chitin plates, agarose was dissolved (1.6% [w/v]) in 50 mM potassium phosphate buffer (pH 6.5). The dissolved agarose/buffer solution (20 mL) was cooled to 50–60 °C and 16.5 mg of glycol chitin and 30 μL of cercosporin (10 mM in acetonitrile) were added. Plates containing 30 μL acetonitrile only were used as controls. Four 7-mm diameter wells were cut in the plates with a cork borer and 10 μL of 50 mM phosphate buffer only or buffer containing 0.25 or 0.5 U of chitinase enzyme were added to individual wells in the agarose plates. The plates were incubated at 25 °C and chitin hydrolysis, visualized as clear zones around the wells, was assessed after 48, 72 and 120 h. In order to determine whether the inhibition of activity by cercosporin is enzyme specific, 0.25 and 0.5 units of an α-amylase from *Aspergillus oryzae* (Sigma, cat log #10065-10G; 30 units/mg) in 10 μL of 50 mM potassium phosphate buffer (pH 6.5) was added to duplicated wells in 1% agarose plates containing 0.25% soluble starch in the above phosphate buffer with or without cercosporin. Four sets of plates (one control plate plus one cercosporin plate comprise a set) were used and one set of plates was stopped by adding 5 mM KI-I_2 solution at 16, 24, 48 and 72 h.

Fungal virulence assay on soybean

Soybean detached leaf assays were performed to examine the differences in fungal growth and virulence among the *C.* cf. *flagellaris* wild-type and Δ*avr4* mutants. Soybean plants cv. Syngenta 02JR423003 were grown in the greenhouse until R1 (beginning bloom) stage when leaves were collected and placed inside transparent plastic boxes containing moist paper towels. Mycelial agar plugs (7-mm diameter) containing 2-week-old *C.* cf. *flagellaris* wild-type or Δ*avr4* mutants grown on PDA were placed on the adaxial surface of soybean leaves with the mycelial side touching the leaf and gently pressed. Mycelial plugs or macerated mycelia instead of spores are often used to study *C. kikuchii* infection on soybean due to the poor sporulation of the pathogen *in vitro*, a common issue for many *Cercospora* species (Almeida *et al.*, 2005; Callahan *et al.*, 1999; Upchurch *et al.*, 1991b). The inoculated leaves were incubated under fluorescent light at room temperature. Disease severity was assessed 14 days post-inoculation (dpi) using a 1–5 rating scale. The photos for the rating scale were selected from a large pool of plug-inoculated soybean leaf photographs 2 weeks after inoculation, and assigned a number of 1 for least and 5 for most severe based on size and severity of lesion. Three boxes each containing eight trifoliate leaves were used for each treatment. Leaves from individual boxes were considered as one sample and individual boxes was considered as a replicate. The experiment was repeated three times.

Quantification of fungal growth using real-time PCR

Fourteen days post-inoculation, soybean leaves infected with *C. cf. flagellaris* wild-type or Δ*avr4* mutants were washed with sterile distilled water to remove agar plugs and fungal mycelia on the leaf surfaces before 22-mm leaf discs were cut with a cork borer from inoculated leaves and ground in liquid nitrogen. Leaf genomic DNA was extracted as described above. Fungal biomass of *C.* cf. *flagellaris* in inoculated soybean leaves was quantified using real-time PCR with primers CKCTB6-2F/CKCTB6-2R and fluorescent probe CKCTB6-PRB in the ABI 7000 equipment (Applied Biosystems) according to Chanda *et al.* (2014).

Droplet digital PCR to determine the number of integrations of the *Avr4* deletion cassette in the mutants

To determine whether there is any ectopic integration of the *HYG* cassette in non-targeted areas in the mutants, a phenomenon

that appears to occur frequently in *C. fulvum* even when using *Agrobacterium tumefaciens*-mediated transformation (Ökmen *et al*., 2014), droplet digital PCR was performed at the Core Research Facility, at the Interdisciplinary Center for Biotechnology Research, University of Florida, Gainesville, FL, USA. The *HYG* gene copy number (target) was quantified using genomic DNA samples extracted from the wild-type and mutants as templates, with a known single-copy gene encoding aminoadipate reductase (*LYS2*) (An *et al*., 2002) as a reference. The primer and probe sequences used for droplet digital PCR are presented in Table 1. The accuracy of using droplet digital PCR to determine target gene copy number in comparison to Southern blot analysis has been well documented in earlier studies (Collier *et al*., 2017; Głowacka *et al*., 2016).

Avr4 dsRNA production in *E. coli*

To independently verify the role of AVR4 on cercosporin production, a 309 bp fragment of the *Avr4* gene was amplified with primer pair Avr4sh_F and Avr4sh_R (Table 1), cloned into pCR2.1-TOPO, and then into pL4440 between *Xho*I and *Sac*I sites to form L4440-Avr4 for dsRNA production in *E. coli* HT115 (DE3) cells according to Tenllado *et al.* (2003). dsRNA synthesis was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.4 mM) for 5 h before the bacterial cells were harvested, washed twice with distilled water, resuspended in RNase-free water to 1/20 of original culture volume and lysed by passing through a French press twice at 1000–1250 psi. After centrifugation for 10 min at 4 °C and 10 000*g*, the supernatant of the lysate was saved as crude dsRNA extract. To further purify the dsRNA, the crude extract was vigorously mixed with an equal volume of chloroform and pelleted by centrifugation at 10 000*g* for 10 min at 4 °C. The aqueous phase was transferred to a new 50 mL centrifuge tube and the dsRNA was ethanol precipitated and finally resuspended in 50 mL RNase-free water for the *in vitro* culture study below.

Culturing *C.* cf. *flagellaris* in the presence of dsRNA

Freshly macerated 2-week-old *C.* cf. *flagellaris* wild-type mycelia (0.5 mL) was added to 50 mL flasks containing 20 mL of half-strength CM only (control) or 20 mL of half-strength CM with dsRNA (at a final concentration of 16 μg/mL of culture) prepared from bacterial cells containing L4440-Avr4 construct or half-strength CM with cell lysate prepared from HT115 bacterial culture with the empty L4440 vector only (at a final concentration of 16 μg dsRNA per millilitre of culture) to serve as an additional control. Twenty millilitres of half-strength CM inoculated with ground mycelia of 2-week-old *C.* cf. *flagellaris Avr4* mutant was included as a positive control. After 4 days of culturing at 25 °C with constant light (240 μmol/m^2/s), cercosporin production in liquid media was measured as described above using HPLC. Each treatment had five replicates and this experiment was repeated three times.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web site:

Fig. S1 *Cercospora* cf. *flagellaris Avr4* gene sequence. The putative transcription start site at –15 relative to the putative start codon ATG (highlighted and italicized) is highlighted in bold and larger font size on top of the amino acid sequences.

Fig. S2 Alignment of AVR4 homologues from several *Cercospora* species. Alignment of AVR4 from *C.* cf. *flagellaris*, *C. nicotianae*, *C. zeina*, *C. beticola* and *C. apii*. was produced using Clustal Omega. Conserved amino acid residues are indicated with an asterisk. Cysteine residues (C) are shown in dark grey boxes as vertical lines. The chitin-binding peritrophin-A domain is shown in light grey boxes as a horizontal underline.

Fig. S3 Differences in phenotype and growth of *Cercospora* cf. *flagellaris* wild-type (WT) and Δ*avr4* mutants. Colony radial growth of fungal cultures on potato dextrose agar plates was measured every 2 days to determine the colony size increase over a period of 13 days. Error bars indicate standard error of the mean of three different experiments with four biological replicates for each fungal isolate.

Fig. S4 Phenotypes of *Cercospora* cf. *flagellaris* wild-type (WT) and Δ*avr4* mutants grown in liquid complete medium and potato dextrose broth. Cultures were incubated at 25 °C with constant shaking (200 rpm) under light (240 μmol/m^2/s) for 5 days.

Fig. S5 HPLC analysis of red pigments extracted from *Cercospora* cf. *flagellaris* wild-type (W)T and Δ*avr4* mutants grown in liquid complete medium, potato dextrose broth and soybean leaves that had been inoculated with the pathogen. The peak height and retention time of the cercosporin standard (10 μg/mL) and the putative cercosporin in the extracts are indicated in the chromatograph for each of the samples.

Fig. S6 Mass spectrometry analysis of cercosporin standard and the red pigment extracted from *Cercospora* cf. *flagellaris* wild-type (WT) grown in liquid complete medium. The major peak in cercosporin standard (A) has a measured mass to charge ratio (*m*/*z*) of 535.1603 and that of the major peak in the red pigment (B) is 535.1517. The measured *m*/*z* of both are very close to the calculated *m*/*z* of cercosporin standard (535.1599), confirming the red pigment is indeed composed mainly of cercosporin.

Table S1 Gene copy number analysis through droplet digital PCR of genomic DNA from the wild-type *Cercospora* cf. *flagellaris* (MRL 6020-2B) and the Δ*avr4* mutants.

