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The roles of AVR4 in fungal virulence, cercosporin biosynthesis and its potential use in host induced gene silencing for controlling cercospora leaf blight disease of soybeans

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THE ROLES OF AVR4 IN FUNGAL VIRULENCE, CERCOSPORIN BIOSYNTHESIS AND
ITS POTENTIAL USE IN HOST INDUCED GENE SILENCING FOR CONTROLLING
CERCOSPORA LEAF BLIGHT DISEASE OF SOYBEANS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Plant Pathology and Crop Physiology

by

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B.S., Federal University of Uberlandia, 2008
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May 2017

This work is dedicated to my

Lovely Mother, Vera

Lovely Father, Jovanio

And all my ancestors

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ABSTRACT

The AVR4 effector, secreted by *Cladosporium fulvum*, has been demonstrated to be involved in pathogen virulence. Recent studies further demonstrated that Avr4 is highly conserved among several *Cercospora* species, indicating a potential important role of this gene in fungal virulence. Therefore, investigation to determine whether this fungal effector gene is present in *Cercospora* cf. *flagellaris* (previously known as *C. kikuchii*), the causal agent of soybean cercospora leaf blight (CLB) disease, and whether it plays any role in CLB disease development, is of great interest. In the present study, the Avr4 gene from *C. cf. flagellaris* was cloned and mutants lacking the expression of Avr4 were created by homologous recombination to investigate its role in fungal virulence, cercosporin production as well as CLB disease development. The Δ avr4 mutants produced little or no cercosporin *in vitro* and the mutants also had significantly reduced cercosporin toxin biosynthesis genes expression. Furthermore, Δ avr4 mutants grew faster and were more sensitive to chitinase *in vitro* than the wild type. Surprisingly, cercosporin could also directly suppress chitinolytic activity *in vitro*. When inoculated onto detached soybean leaves, these mutants exhibited reduced virulence compared to the wild type and no cercosporin was detected in mutant inoculated soybean leaves. Taken all together, the results suggest that AVR4 may contribute to the virulence of *C. cf. flagellaris* on soybean through protecting fungal hyphae and regulating cercosporin biosynthesis. Considering the importance of AVR4 in *C. cf. flagellaris* virulence, we selected a region of this gene for targeted gene suppression through host induced gene silencing (HIGS) to determine whether this can reduce CLB disease. It was found that HIGS plants carrying the BPMV-Avr4 construct showed less disease symptoms compared to control plants, and the reduction of symptoms was positively

correlated with 80% reduction in Avr4 transcript levels and more than 50% reduction in fungal growth. To the best of our knowledge, this is the first study demonstrating the involvement of AVR4 in CLB disease development possibly through regulating cercosporin production as well as protecting fungal hyphae. In addition, this study also showed the potential of using HIGS as a tool to control this important disease of soybeans.

CHAPTER 1: INTRODUCTION

1.1. Justification

The AVR4 effector, secreted by *Cladosporium fulvum*, has been demonstrated to be involved in pathogen virulence. Silencing of the Avr4 gene in *C. fulvum* clearly decreases its growth and virulence on tomato plants (van Esse et al., 2007). Moreover, the virulence function of AVR4 is related to its primary biological function as a chitin binding protein which protects the fungal cell wall against plant chitinases (van den Burg et al., 2006). Interestingly, Avr4 functional homologs have been found in phylogenetically closely related species of Dothideomycete fungi including several *Cercospora* species (Stergiopoulos et al., 2010).

Thus, AVR4 was suggested to be a core effector with a conserved biological function which contributes to fungal virulence of several fungal plant pathogens on distantly related plant species (Stergiopoulos et al., 2012; Stergiopoulos et al., 2010). However, there is no report on whether an Avr4 homologue is present in *Cercospora cf. flagellaris* (previously known as *C. kikuchii*). Therefore, considering the importance of this effector in other fungi, we hypothesized that if this effector exists in *Cercospora cf. flagellaris* it may play an important role in fungal virulence and cercospora leaf blight (CLB) disease development.

CLB is an economically important disease which occurs on soybeans, one of the most important global crops. This disease is responsible for substantial yield losses in important soybean crop regions in the United States and worldwide. The damage caused by CLB has increased continuously, especially in the Mid-Southern United States and it is now responsible for substantial yield losses in the southern U.S. as well (Cai et al., 2009; Moore and Wolcott, 2000; Schneider et al., 2003). Yet, there is no resistant soybean variety currently available and no

effective fungicide to control CLB. Certainly, further investigation of the AVR4 effector in *Cercospora cf. flagellaris* can provide important information in understanding host-pathogen interaction, which may be used to enhance resistance to *C. cf. flagellaris* infection in soybean.

Host Induced Gene Silencing (HIGS) is a biotechnological method that has been exploited as a tool to engineer resistance against plant pathogens and predators (Lilley et al., 2012; Nunes and Dean, 2012; Zhang et al., 2013). This can be achieved by expressing a dsRNA construct against specific genes endogenous to the pathogen in the host plant. When the pathogen attacks the host expressing a HIGS construct, the gene of interest can be down-regulated in the pathogen to slow down or suppress its invasion.

HIGS is based on the RNA interference (RNAi) mechanism conserved in eukaryotes, and plants naturally utilize RNAi to protect against invading viruses (Baulcombe, 2004; Csorba et al., 2009). Indeed, HIGS has been shown to be effective against several plant pathogens, including biotrophic and necrotrophic fungi as well as oomycetes (Andrade et al., 2015; Chen et al., 2016; Koch et al., 2013; Nowara et al., 2010; Nunes and Dean, 2012; Panwar et al., 2013; Song and Thomma, 2016; Tinoco et al., 2010; Yin et al., 2015; Zhou et al., 2016). Therefore, HIGS could be another approach to be exploited to enhance soybean resistance to CLB.

1.2. Objectives

1. Determine the presence of the Avr4 gene in *Cercospora cf. flagellaris*.
2. Clone and sequence the full length Avr4 gene from *C. cf. flagellaris*.

3. Create Δ avr4 disruption mutants and perform *in vitro* and *in vivo* studies to determine the importance of this gene in *C. cf. flagellaris* virulence and CLB development.
4. Verify if Avr4 from *C. cf. flagellaris* is also involved in fungal protection against chitinases.
5. Evaluate the potential control of CLB through HIGS targeting the Avr4 gene.

CHAPTER 2: LITERATURE REVIEW

2.1. Soybean Crop

Soybean (*Glycine max* (L.) Merr.) is one of the most important global crops, which plays an important role in the food production for human and animal consumption. Soybean was introduced into the U.S. during the 1700s, but it was not used as a grain crop until the 1920s to 1930s (Hymowitz, 1990). Although there is contradictory evidence regarding the origin of domestication, it is believed that soybean was domesticated from its wild progenitor *Glycine soja* Sieb. et Zucc. in Asia (Kim et al., 2010). Soybean belongs to the Fabaceae/Leguminosae family which includes important legumes such as alfalfa (*Medicago sativa*), pea (*Pisum sativum*), common bean (*Phaseolus vulgaris*), peanut (*Arachis hypogaea*), and lentil (*Lens culinaris*). *G. max*, a self-pollinated diploid, has 20 chromosomes ($2n = 40$) and it is among the plant species whose genome has been sequenced. Its size is about 1.1 GB with 46,430 protein-coding genes predicted (Schmutz et al., 2010).

Soybean development can be separated into two phases, vegetative (V) and reproductive (R), and both are subdivided into a number of growth stages. The vegetative phase starts with the emergence of the plant from the soil designated as VE (emergence) followed by VC (cotyledon stage) and then each V stage is designated numerically as the number of nodes on the main stem with a fully developed leaf. The unifoliolate (simple) leaf node is the first node to be counted and all following leaves are trifoliates (compound) growing on alternate sides of the stem. The reproductive phase begins with plant flowering (R1 and R2), followed by pod development (R3 and R4), then seed development (R5 and R6), and finally plant maturation (R7 and R8). Although environmental stresses at any stage of soybean development can result in yield losses,

stresses occurring between R1 and R6 stages cause the greatest yield reductions as the plant is developing pods and seeds (Carlson and Lersten, 2004; Lersten and Carlson, 2004).

The duration of the soybean growth stages is controlled by genetics, temperature, and day length. In the United States two types of soybeans with different morphological growth habits are planted. Indeterminate soybean plants continue to develop after flowering begins, whereas determinate plants cease vegetative growth at the time of flowering (R1). Soybeans are also divided into different maturity groups (MG) according to their time of maturation and roman numerals are used to indicate the adaptability of a soybean variety to a given region. Thus, MG number 0 is recommended for the northernmost regions of the U.S., which include northern Minnesota and North Dakota; whereas MG number VIII is intended for the southernmost regions including Florida and the southern parts of the Gulf Coast states (Carlson and Lersten, 2004; Lersten and Carlson, 2004). In Louisiana, MG IV-V give the best seed quality and yield per acre (Ronald et al., 2016).

The global soybean production for 2015/16 was projected at 330.4 million metric tons (MMT) and the forecast for the U.S. was at 110.5 MMT and Brazil at 103 MMT (USDA, 2016b). Currently, the U.S. produces more than 30 percent of all soybeans grown worldwide, which positions the U.S. as the largest global producer and exporter of soybean, followed by Brazil and Argentina. Within the U.S., the Western and Northern Corn Belt regions (Iowa, Kansas, Minnesota, Missouri, Nebraska, North Dakota, South Dakota, Illinois, Indiana, Michigan, Ohio and Wisconsin) are responsible for more than 80 percent of all soybeans grown in the country (Miller-Garvin and Naeve, 2015). In 2016, soybean yields were expected to reach a record-high mark of 48.9 bushels per acre in some states, such as Illinois, Iowa, Missouri,

Nebraska, and Wisconsin (USDA, 2016a). The Mid-south region (Arkansas, Kentucky, Louisiana, Mississippi, Oklahoma, Tennessee, and Texas) is another important area that is responsible for more than 12% of soybean production in the U.S. Within the Mid-south, Louisiana is the third largest producer with 1.6 MMT produced in 2015 (Miller-Garvin and Naeve, 2015). In fact, soybean is a very important Louisiana commodity with a state average yield of 57 bushels per acre in 2014, which is leading the nation in yield according to the U.S. Department of Agriculture.

2.2. Soybean diseases

More than 300 diseases occur in soybean plants (Hartman et al., 1999). The increasing number of diseases is a result of the expansion of acreage cultivated with this crop worldwide, accumulation of pathogen inocula in areas where soybeans are constantly grown, and global warming (Eastburn et al., 2010; Hartman et al., 2011; Rosenzweig et al., 2001). Attack by plant pathogens can drastically reduce soybean yield. In fact, this crop is susceptible to diseases caused by fungi, oomycetes, bacteria, viruses, and nematodes. Among them, several fungal species are considered economically important soybean pathogens.

The genus *Cercospora* (Mycosphaerellaceae, Ascomycota), which was first established by Fresenius (1854) with more than 3,000 species, contains some of the most important plant pathogens occurring on a wide range of hosts (Pollack, 1987). Two species of *Cercospora* are known to infect soybeans, *C. sojina* Hara and *C. kikuchii* (Mat and Tom [Gard]), which is currently reclassified as *Cercospora cf. flagellaris* according to Soares et al. (2015) and Albu et al. (2016). *Cercospora cf. flagellaris* is used instead of its old name *C. kikuchii* throughout this dissertation whenever appropriate.

Cercospora sojina causes frogeye leaf spot (FLS) disease on soybean and the resulting foliage lesions can lead to premature defoliation and yield loss (Hartman et al., 1999).

Defoliation and yield losses are also observed when *C. cf. flagellaris* infects soybean plants causing reddish purple foliage lesions which are a typical symptom of cercospora leaf blight (CLB), a significant disease of soybean in the Southern United States.

2.3. Cercospora leaf blight

Cercospora leaf blight (CLB), caused by the fungus *Cercospora cf. flagellaris*, is among the most devastating fungal diseases of soybean in many countries. CLB symptoms are normally first observed at the late R5 and early R6 soybean growth stages on upper leaves exposed to sunlight (Walters, 1980). The symptomatic leaves show reddish purple, angular-to-irregular lesions and leathery appearance. Moreover, heavily infected leaves lead to premature defoliation causing as high as 15 to 30% yield losses (Hartman et al., 1999; Schneider et al., 2003; Wrather et al., 2001; Wrather et al., 1997).

In the United States, CLB is an economically important soybean disease and yield losses caused by CLB from 1996 to 2012 were estimated to be 5.2 million bushels (Wrather and Koenning, 2013). Since 1999, the damage caused by CLB has increased continuously, especially in the Mid-Southern United States (Cai et al., 2009; Moore and Wolcott, 2000; Schneider et al., 2003) and it is now responsible for substantial yield losses in the southern U.S. as well. Yet, there is no resistant soybean variety currently available and no effective fungicide to control CLB.

2.4. The cercosporin toxin and its role in plant disease

Several *Cercospora* species produce cercosporin, a deep red color toxin. Cercosporin was first isolated in 1957 from *C. kikuchii* and since it has been isolated from many other *Cercospora* species and infected host plants (Assante et al., 1977; Balis and Payne, 1971; Daub and Briggs, 1983a; Fajola, 1978; Gunasinghe et al., 2016; Kuyama and Tamura, 1957; Lynch and Geoghegan, 1977; Mumma et al., 1973; Stergiopoulos et al., 2013; Venkataramani, 1967). Cercosporin belongs to the perylenequinone family, which is a major class of photo-activated polyketide non-host specific toxins (Mulrooney et al., 2010). This toxin was the first perylenequinone isolated from fungi and like all perylenequinone metabolites it functions as a photosensitizing agent (Daub, 1982; Daub and Briggs, 1983a). Since then, several perylenequinones from various fungi have been characterized, including alteichin, altertoxins, and alterlosins, produced by *Elsinoë*, *Cladosporium* and *Alternaria* species, respectively (Daub et al., 2005; Glaeser et al., 2011; Stack et al., 1986).

When exposed to visible light, cercosporin facilitates the transfer of light energy to O₂, resulting in the production of the potent reactive oxygen species (ROS) singlet oxygen (¹O₂) and superoxide radical (O₂^{•-}) (Dobrowolski and Foote, 1983). As a result, it has been shown that cercosporin exerts a broad toxicity to plants, bacteria, fungi, and mice because ROS damages a variety of cellular targets including cell membranes, nucleic acids, proteins, and lipids (Daub, 1982; Daub and Briggs, 1983a; Daub and Ehrenshaft, 2000; Fajola, 1978; Gunasinghe et al., 2016; Staerkel et al., 2013; Steinkamp et al., 1981; Steinkamp et al., 1979; Stergiopoulos et al., 2013; Yamazaki et al., 1975). Several studies show that cercosporin causes peroxidation of membrane lipids after binding to the cell membrane, resulting in ion leakage from the host

organism (Daub, 1982; Daub and Briggs, 1983a). Thus, peroxidation of the cell membrane is the primary mode of toxicity as a direct cellular target of cercosporin has not been found yet.

Cercosporin production is influenced by several environmental conditions. Indeed, light is the most critical factor, whereas temperature, pH, medium nutrient composition, and C:N ratios, have all been demonstrated to influence cercosporin biosynthesis (Choquer et al., 2005; Jenks et al., 1989). Cercosporin production is suppressed in the absence of light, whereas introduction of light elicits its biosynthesis (Ehrenshaft and Upchurch, 1991). The production can be altered by simply alternating light and dark periods and *C. kikuchii* cultures grown under dark conditions were shown to produce 100-fold less toxin than cultures grown under light (Rollins et al., 1993). The composition and particularly the carbon and nitrogen ratio in the medium of lab-grown *Cercospora* cultures was shown to highly influence cercosporin production. In *C. nicotianae*, when ammonium was used as the sole nitrogen source cercosporin production was inhibited, whereas calcium promoted its production (You et al., 2008). Moreover, differences in cercosporin production can be observed among fungal strains and even among different isolates of the same *Cercospora* species, which often synthesize different amounts of the toxin under the same set of growth conditions (Goodwin et al., 2001).

The important role of cercosporin in fungal pathogenicity was demonstrated for different *Cercospora* species, including *C. kikuchii*, *C. nicotianae*, and *C. beticola* (Choquer et al., 2005; Staerkel et al., 2013; Upchurch et al., 1991). Upchurch et al. (1991) showed that *C. kikuchii* mutants producing less than 2% of the cercosporin synthesized by the wild type isolate, caused few small lesions on inoculated soybean leaves. Thus, production of cercosporin by *Cercospora kikuchii* is crucial to the fungus's virulence and has been associated with lesion formation on

soybean leaves. Similarly, in *C. nicotianae* and *C. beticola*, cercosporin deficient knock-out mutants triggered fewer disease lesions on tobacco and sugar beets, respectively, as compared to the wild type fungus (Choquer et al., 2005; Staerkel et al., 2013). Recently, cercosporin was also shown to play a critical role in white leaf spot development of brassicas caused by the fungus *Pseudocercospora capsellae* (Gunasinghe et al., 2016).

Another evidence of the role of cercosporin in plant diseases, is the importance of light in symptom expression and the correlation of it with the mode of toxicity of cercosporin. Light intensity and amount of light exposure has been shown to be positively correlated with severity of disease symptoms caused by *Cercospora* species. For example, when coffee plants were grown close together causing shading of the leaves, the symptoms caused by *C. coffeicola* were less severe (Daub and Ehrenshaft, 2000). Disease severity was also reduced on leaves of sugar beet infected with *C. beticola* when plants were grown under low light conditions (Calpouzos and Stalknecht, 1967). In addition, low light intensities caused a delay in disease symptoms on leaves of sugar beet infected with *C. beticola*. Banana plants grown at low light intensities infected with *Cercospora musae* also showed either no disease development or delayed symptoms (Calpouzos, 1966; Thorold, 1940). Furthermore, cercosporin concentration and light intensity directly affect the rate of electrolyte leakage from tobacco leaf disks. Low light intensity and decreasing cercosporin concentration delayed the electrolyte leakage. In contrast, tobacco leaf discs treated with cercosporin and exposed to high light conditions showed increased electrolyte leakage (Daub, 1982).

2.5. The cercosporin biosynthetic pathway

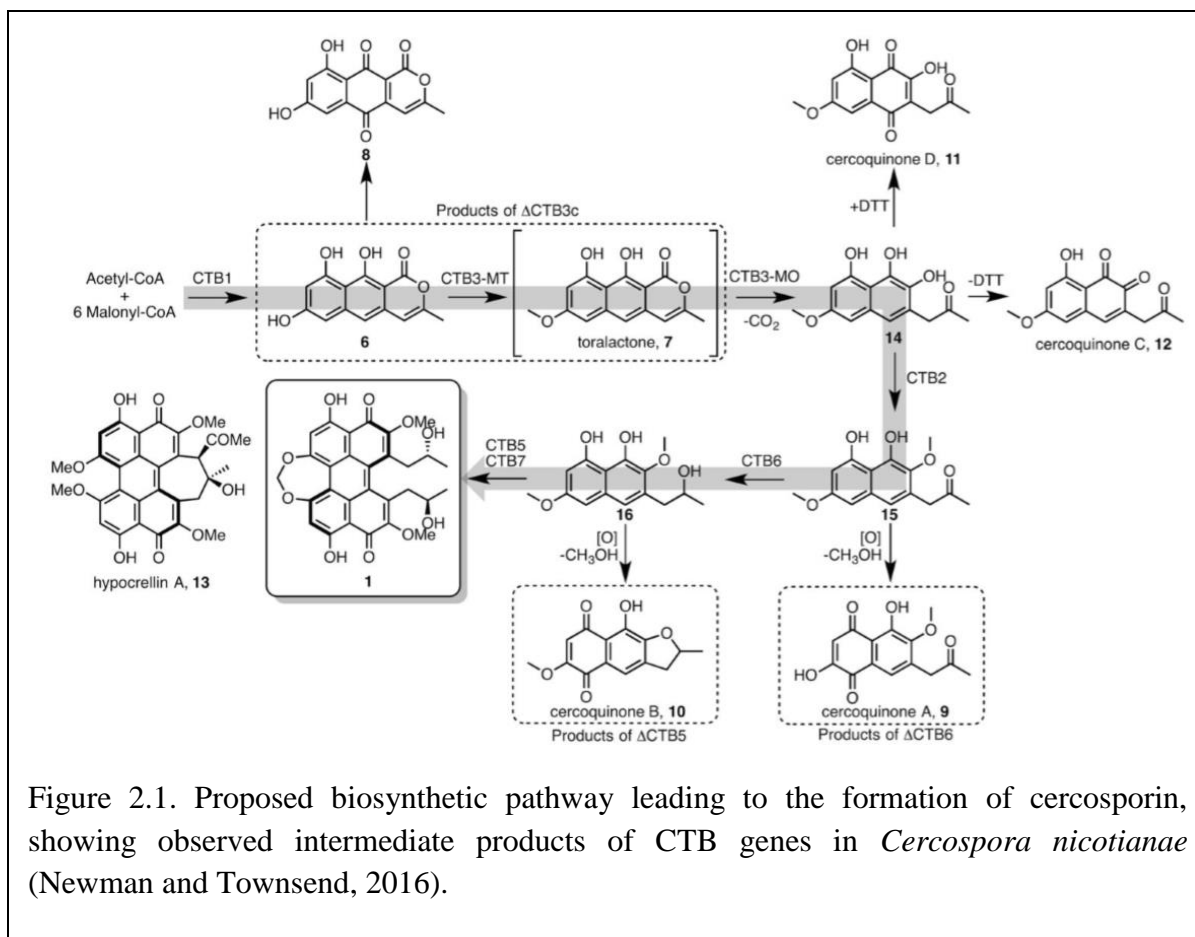
The cercosporin biosynthesis gene cluster was identified in *C. nicotianae* and consists of eight genes, CTB1-to-CTB8 (Chen et al., 2007b). CTB1 (cercosporin toxin biosynthesis 1) was the first gene of the cluster identified and this gene encodes a fungal polyketide synthase (PKS), a crucial enzyme for cercosporin biosynthesis. Choquer et al. (2005) created Δ ctb1 knock-out mutants and showed that mutants were unable to produce cercosporin and elicited fewer and smaller necrotic spots compared with the wild type fungus. In addition, the expression of CTB1 was shown to be correlated with light conditions. CTB2 and CTB3 genes encode an O-methyltransferase and an O-methyltransferase (N-terminus)/FAD-dependent monooxygenase (C-terminus), respectively. Both genes are required for the biosynthesis of cercosporin and virulence of the fungus (Chen et al., 2007a; Dekkers et al., 2007).

Another gene of the cluster, CTB4, encodes a major facilitator superfamily (MFS) transporter that is involved in the secretion of the toxin (Choquer et al., 2007). Disruption of the CTB4 gene significantly reduced cercosporin production and resulted in fewer lesions on tobacco leaves. Interestingly, cercosporin was detected predominantly from fungal hyphae of Δ ctb4 disruptants but not from the medium, suggesting that the mutants were defective in both cercosporin biosynthesis and secretion. CTB5, CTB6, and CTB7 encode FAD/FMN- or NADPH-dependent oxidoreductases in the cercosporin toxin biosynthetic pathway and disruption of these genes in null mutant strains resulted in altered cercosporin production and fewer lesions on the host plant compared with the wild type fungus (Chen et al., 2007b).

The last gene found in the gene cluster was CTB8, which encodes a Zn(II)Cys(6) transcription factor (Chen et al., 2007b). Cercosporin production was completely abolished in

Δ ctb8 knock-out mutants, which were less virulent than the wild type. Furthermore, the disruption of CTB8 gene inhibited the expression of all CTB genes of the cluster and, as expected, CTB8 expression was induced under light exposure. Prior to CTB8 identification, Chung et al. (2003) reported the existence of another Zn(II)Cys₆ transcription factor, CRG1, which is associated with both cercosporin production and resistance (Chung et al., 2003a; Chung et al., 1999). CTB8 appears to act downstream of CRG1, as CTB8 and all CTB genes are down-regulated in a Δ crg1 disrupted mutant, whereas CRG1 expression is not affected in a Δ ctb8 mutant (Chen et al., 2007b).

Although several genes involved in cercosporin production have been identified, the cercosporin biosynthetic pathway still has not been completely solved. Chen et al. (2007) proposed a pathway in which dimerization of two identical aromatic intermediates would lead to the perylenequinone core and these aromatic intermediates would be derived from a carboxylic acid produced by CTB1. However, recently Newman and Townsend (2016) demonstrated that the cercosporin pathway previously proposed is incorrect and they reported that naphthopyrone nor-toralactone is an essential intermediate of the cercosporin pathway and is the product of the CTB1 gene. Thus, a revised biosynthetic scheme for cercosporin (Figure 2.1) has been proposed based on metabolites identified from *C. nicotianae* gene knockout strains (Newman and Townsend, 2016). Furthermore, they characterized the *in vitro* activity of CTB3 showing its role in toralactone formation and in an oxidative aromatic cleavage.



Nevertheless, very recently de Jonge et al. (2017) claimed that the pathway proposed by Newman and Townsend (2016) must be revised. In their new study using an evolutionary comparative genomics approach, de Jonge et al. (2017) reported the existence of additional genes in the CTB cluster of *C. beticola*. This expanded CTB cluster to include one gene that contributes to cercosporin auto-resistance (CbCFP) and at least three novel CTB genes (CbCTB9, CbCTB10, CbCTB11) that are involved in the toxin biosynthesis. The new CTB genes encode the extracellular proteins fasciclin and laccase required for cercosporin production and a final pathway enzyme able to assemble the cercosporin methylenedioxy bridge. Cercosporin production was completely abolished when CbCTB10 and CbCTB11 were

disrupted, while disruption of CbCTB9 resulted in the accumulation of a new metabolite. Moreover, the new CTB genes were up-regulated under cercosporin-inducing conditions. Thus, these new findings suggested that the CTB cluster is larger than previously described.

2.6. Plant pathogen effectors and their role in causing plant diseases

In nature, plants are often attacked by various pathogens, such as fungi, bacteria, viruses, and nematodes. However, only some of these pathogens can successfully overcome plant's defense system and establish a compatible plant pathogen interaction, thus resulting in disease. The plant defense system involves basal defense responses and resistance (R) gene mediated defense (Gururani et al., 2012). These two forms of plant defense are also known as pathogen associated molecular patterns (PAMPs)-triggered immunity (PTI) and effector triggered immunity (ETI), respectively (Jones and Dangl, 2006). The basal defense relies on recognition of conserved PAMPs, including chitins, flagellins, lipopolysaccharides, and glucans (Zipfel and Felix, 2005), which are recognized by several plant transmembrane pattern recognition receptors (PRRs) leading to PTI (Zipfel, 2008).

However, pathogens evolved to produce molecules encoded by Avr (avirulence) genes, named effectors to overcome the recognition by PRRs and thus the basal defense (Giraldo and Valent, 2013), forcing plants to develop a new defense mechanism based on effector recognition by resistance (R) proteins. The R gene mediated defense, also called gene-for-gene resistance (Flor, 1942), relies on recognition of an AVR effector from the pathogen by a corresponding plant resistance (R) protein from the host leading to host defense responses, including hypersensitive response (HR) (Giraldo and Valent, 2013). The pathogen effector and the host R proteins may interact directly or indirectly leading to the immune response called effector

triggered immunity (ETI) (Jones and Dangl, 2006), which suppress PTI and facilitates infection in the absence of the R gene (Koeck et al., 2011). To avoid recognition by host R gene products or to suppress ETI, pathogens mutate, lose or generate novel effectors, and consequently plants develop novel R proteins able to identify these novel effectors, thus continuing the escalating arms-race between plants and pathogens (Jones and Dangl, 2006).

Staskawicz et al. (1984) cloned the first bacterial Avr gene, *avrA* (avirulence A) from *Pseudomonas syringae* pv. *glycinea*. Following that, van Kan et al. (1991) successfully cloned the first fungal Avr gene (Avr9) from *Cladosporium fulvum* and afterward Shan et al. (2004) reported the cloning of the first oomycete Avr gene (Extracellular Protease Inhibitor 1). Several fungal Avr genes and corresponding host resistance genes have now been identified and cloned (Gururani et al., 2012; Stergiopoulos and de Wit, 2009). The cysteine-rich secreted protein, named AVR9, which elicits HR in tomato containing the Cf-9 resistance gene, was the first well-characterized fungal effector (van Kan et al., 1991). However, as for many effectors, the biological function of AVR9 is still unknown.

Cladosporium fulvum (Cf) is considered a model pathogen as its interaction with its host tomato is governed by a gene-for-gene relationship (Thomma et al., 2005). Up to now, five Avr effector genes have been cloned from *C. fulvum* including Avr9, Avr4, Avr4E, Avr2 and Avr5, whose recognition in tomato plants is mediated by the cognate Cf proteins Cf-9, Cf-4, Cf-4E, Cf-2 and Cf-5, respectively (reviewed in de Wit 2016). All of the Avr genes in *C. fulvum* encode small cysteine rich proteins and their primary function is known only for AVR2 and AVR4. Other than inducing HR in Cf-2 tomato, AVR2 is a virulence factor that inhibits several cysteine proteases, including Rcr3, required for plant basal defense (van Esse et al., 2008). AVR4 is also

a virulence factor and it had been demonstrated to contain a functional chitin-binding domain that protects fungal cell walls against hydrolysis by plant chitinases (van den Burg et al., 2006; van Esse et al., 2007).

Unlike PAMPs, effectors appear as a fungal counter-defense later in the pathogen evolution to facilitate infection on a particular plant host. Thus, most fungal effectors were assumed to be species-specific (Jones and Dangl, 2006; Stergiopoulos and de Wit, 2009). In fact, Ecp6 effector from the tomato pathogen *Cladosporium fulvum* was one of the few examples of effectors with orthologs in several other pathogenic and nonpathogenic fungal species (Bolton et al., 2008a). However, later Stergiopoulos et al. (2012; 2010) showed that homologs of the Avr4 and Ecp2 effector genes from *C. fulvum* are also present in several Dothideomycetes fungal species. To date, functional homologs of Avr4, Ecp2, and Ecp6 have been found in phylogenetically closely related species of Dothideomycete fungi and beyond (Bolton et al., 2008b; Stergiopoulos et al., 2010). Several functional Cf-Avr4 orthologues have been identified from diverse plant fungal pathogens, including the banana pathogen *Mycosphaerella fijiensis* (Mf-Avr4), the pine tree pathogen *Dothistroma septosporum* (Ds-Avr4), the poplar pathogen *Septoria musiva* (Sm-Avr4), and Ca-Avr4 from *Cercospora apii*, Cb-Avr4 from *C. beticola* and Cn-Avr4 from *C. nicotianae* (the leaf spot pathogens of celery, sugar beet and tobacco, respectively) (de Wit et al., 2012; Stergiopoulos et al., 2010). Interestingly, most of the Cf-Avr4 orthologues can be recognized by Cf-4 (R) protein and elicit HR despite their low sequence similarity (de Wit et al., 2012; Stergiopoulos et al., 2010).

The broad distribution of effectors like Avr4 and *Ecp2* among different fungal species raised the question of whether these chitin binding lectins are core effectors conserved during

fungal evolution to contribute to fungal pathogenicity on a wide range of hosts. In addition, their preservation across fungal species may be explained by their roles in pathogen virulence and also the conservation of their virulence targets in different hosts. Thus, AVR4 was suggested to be a core effector with a conserved biological function which contributes to fungal virulence of several plant fungal pathogens on distantly related plant species (Stergiopoulos et al., 2010).

2.7. AVR4 effector in Dothideomycetes

Using apoplastic fluid from a compatible interaction between race 5 of *C. fulvum* and tomato genotype Cf-5, Joosten et al. (1994) cloned Avr4 gene. The AVR4 protein is secreted into the apoplastic space of tomato as a preprotein of 135 amino acids which is processed in planta at both the N and C termini by fungal and plant proteases resulting in a mature protein of 86 aa with eight cysteine residues (Joosten et al., 1997; Joosten et al., 1994). *C. fulvum* races circumventing Cf-4-mediated resistance were found to contain nucleotide changes in Avr4, which result in amino acid substitutions mostly in cysteine residues in AVR4 and cause protein instability in the plant apoplast. This can culminate in protein degradation to prevent AVR4 from being recognized and result in virulence on Cf-4 tomato plants by fungal strains that produce these isoforms (Joosten et al., 1997; van den Burg et al., 2003).

Based on its disulfide-bond pattern and protein folding, AVR4 shares structural homology with invertebrate proteins containing the chitin binding domain of carbohydrate-binding module family 14 (CBM14) (Chang and Stergiopoulos, 2015; van den Burg et al., 2004; van den Burg et al., 2003). In fact, AVR4 is a chitin binding protein which protects the fungal cell wall against plant chitinases most likely interfering with substrate accessibility rather than directly inhibiting chitinase activity (van den Burg et al., 2006). *In vitro* experiments showed that

AVR4 binds specifically to chitin and does not interact with other plant cell wall polysaccharides, such as cellulose and xylan. It was also demonstrated that AVR4 binds to cell walls of *C. fulvum* during infection of tomato and it can protect other fungi including *Trichoderma viride* and *Fusarium solani* against plant chitinases and β -1,3-glucanases (van den Burg et al., 2006). Moreover, accumulation of AVR4 was observed on hyphal tips of *C. fulvum* during growth in the tomato apoplast suggesting that the main function of AVR4 is to protect the fungus at positions where chitin is exposed to the surface (van den Burg et al., 2003). However, the tolerance of *C. fulvum* to cell-wall-degrading enzymes does not depend on the production of AVR4 indicating that other components are involved on the protection of the fungus against these deleterious hydrolases (Joosten et al., 1995; van den Burg et al., 2006).

Although AVR4 is recognized by tomato R (Cf) proteins, Avr4 remains present in *C. fulvum* fungal population. Moreover, it had been shown that Avr4 gene is not or hardly expressed *in vitro* but it is highly expressed *in planta* during fungal colonization when the fungus is exposed to chitinases (de Wit, 1992; Joosten et al., 1997; Joosten et al., 1994). In addition, *C. fulvum* natural strains are able to circumvent Cf-4-mediated resistance by producing AVR4 isoforms which preserve their ability to bind chitin, but are unrecognized by corresponding R protein (van den Burg et al., 2003). Overall, those observations suggest that AVR4 plays an important role in fitness and might be involved in pathogen virulence.

The later studies by van de Burg et al. (2006) and van Esse et al. (2007) demonstrated that although its primary function is a chitin binding protein, AVR4 has the ability to modulate its virulence function. This was the first report of a fungal avirulence protein whose primary biological function contributes to pathogen virulence. Studies to silence Avr4 in *C. fulvum* and to

express AVR4 in *Arabidopsis* further confirmed the importance of AVR4 in virulence of chitin-containing fungal pathogens. Silencing of *Avr4* gene in *C. fulvum* clearly decreases its virulence on tomato plants. Virulence assays showed reduced fungal growth and disease symptoms on tomato leaves inoculated with *Avr4*-silenced *C. fulvum* transformants compared to the wild type fungus (van Esse et al., 2007). Moreover, transgenic AVR4-producing *Arabidopsis* plants showed increased disease symptoms compared with the control plants when inoculated with the necrotrophic fungal pathogens *Botrytis cinerea* and *Plectosphaerella cucumerina*. A similar increase in disease susceptibility was observed for AVR4-producing tomato plants when inoculated with *Fusarium oxysporum* f. sp. *lycopersici*. However, the virulence of non-chitin producing oomycete *Phytophthora brassicae* and the bacteria *Pseudomonas syringae* pv. *tomato* remained unaltered when they were inoculated onto *Avr4*-producing *Arabidopsis* plants. Therefore, those results demonstrated that AVR4 increases virulence of chitin containing fungal pathogens to their hosts (van Esse et al., 2007).

In the past years, several Cf-Avr4 orthologues have been identified and the chitin binding ability is a common feature for all of them. Although all bind chitin, some orthologues cannot be recognized by the cognate R protein and thus, they do not elicit HR in Cf-4 containing plants (Stergiopoulos et al., 2010). Ds-Avr4 from *Dothistroma septosporum* and Mf-Avr4 from *Mycosphaerella fijiensis* showed that they conserved the chitin binding ability and they also triggered an HR in *Nicotiana benthamiana* and tomato plants carrying the Cf-4 R gene. On the other hand, the Cf-4 orthologues identified in *Cercospora* species do not trigger a CF-4-mediated HR, although all of them had been shown to bind chitin (de Wit et al., 2012; Stergiopoulos et al., 2010).

Several recent studies may have found answers to why these Cf-4 orthologues behave differently in different hosts. Mesarich et al. (2016) demonstrated that a conserved proline residue located within the Cys6–Cys7 region of AVR4 is required to elicit a Cf-4-dependent HR. In addition, the authors also showed that like wild type fungus, mutants carrying mutations on different cysteine residues were still able to bind chitin. In another study, the AVR4 from the tomato pathogen *Pseudocercospora fuligena* (Pf-Avr4), a functional ortholog of Cf-Avr4 with the same chitin-binding ability, was functionally and structurally characterized (Kohler et al., 2016). Besides fungal protection against plant chitinases, Pf-AVR4 contributes to virulence of *P. fuligena* on a susceptible tomato host, as well as protects *P. fuligena* against microbial-derived chitinases, suggesting that core effectors like AVR4 may not only be involved in plant infection, but also play a role in interaction with other microorganisms (Kohler et al., 2016; Stergiopoulos et al., 2012). Furthermore, Kohler et al. (2016) showed that residues in Pf-AVR4 which are important for chitin binding are not required for Cf-4 mediated recognition as point mutations on these residues did not eliminate Pf-AVR4 recognition by Cf-4. These findings suggested that the chitin binding ability and recognition of Pf-AVR4 by CF-4 protein are structurally separated.

Lately, a possible new function of AVR4 has been reported, which was discovered to promote the association of the tomato Cf-4 immune receptor with RLK BAK1/SERK3 as part of the induced Cf-4 signaling which results in receptor BAK1/SERK3-dependent endocytosis and plant resistance (Postma et al., 2016). The activation of immune signaling mediated by the well-known cell surface receptor FLS2 involves the association with the receptor like kinase BAK1/SERK3 (Heese et al., 2007; Roux et al., 2011). FLS2 is a member of receptor-like kinases (RLKs), whereas CF receptors are members of receptor-like proteins (RLPs). Based on these findings, CF immune receptors are part of a signaling pathway that can be activated by fungal

effectors and function similarly to bacterial flagellin receptor FLS2. Thus, besides binding to chitin to protect fungal cell wall and increasing virulence of fungal pathogens, it is possible AVR4 is also involved in R-gene mediated host-pathogen interaction and activation of host immune responses.

2.8. Host Induced Gene Silence (HIGS) as an RNAi tool to control plant diseases

RNA interference (RNAi) is a conserved gene silencing mechanism in eukaryotes (Baulcombe, 2004). Other than regulation of gene expression employing small RNAs (sRNAs), plants naturally utilize RNAi to protect against invading viruses (Csorba et al., 2009). RNAi was first demonstrated in *Caenorhabditis elegans* in which double stranded RNA (dsRNA) induces sequence specific post-transcriptional gene silencing (PTGS), and studies in *Neurospora crassa* revealed the existence of the RNA silencing machinery which is referred to as quelling in fungi and, co-suppression in plants (Fire et al., 1998; Napoli et al., 1990; Romano and Macino, 1992).

In fact, RNAi was first observed in petunia plants when Napoli et al. (1990) tried to over-express the enzyme chalcone synthase (CHS) to generate flowers with a more intense purple color. However, plants with white or variegated flowers were obtained when CHS gene was overexpressed, showing that an unknown mechanism silenced the introduced transgene as well as the plant's endogenous CHS gene responsible for producing the purple-color. Nevertheless, the mechanism causing these effects was not understood until the research work by Fire et al. (1998) on *C. elegans* was published. They discovered that injecting double stranded ribonucleic acids (dsRNA) into the worm triggered the silencing of genes matching the sequences of the dsRNA.

RNAi mechanism involves several components and is triggered by a precursor double-stranded (ds) RNA which is cleaved into short 21–25 nucleotide small-interfering RNA (siRNA) duplexes by an enzyme called Dicer. The resulted siRNA duplexes are incorporated into an RNA-induced silencing complex (RISC) which is able to unwind the siRNA strands and the antisense strand is used as a guide to target messenger RNAs (mRNA) in a sequence-specific manner. Subsequently, mRNAs are cleaved by RISC resulting in inhibition of target protein biosynthesis (Baulcombe, 2004). Thus, this mechanism can be used for different purposes, such as discovering or characterizing gene functions, engineering virus resistant plants, altering accumulation of specific metabolites and the nutritional values of food crops, and controlling plant pathogens (Koch and Kogel, 2014; Koch et al., 2013; Miyamoto et al., 2008; Qu et al., 2007).

Host Induced Gene Silencing (HIGS) is based on Virus Induced Gene Silencing (VIGS), which is a RNAi mechanism used by plants to protect against invasion by viruses (Lu et al., 2003). This biotechnological method has been exploited as a tool to control plant pathogens and predators (Lilley et al., 2012; Nunes and Dean, 2012; Zhang et al., 2013). This can be achieved by expressing a dsRNA construct against specific genes endogenous to the pathogen in the host plant. When the pathogen attacks the host expressing a HIGS construct, the gene of interest can be downregulated in the pathogen. HIGS was first found effective against nematodes based on earlier *in vitro* feeding studies. For example, RNA silencing was used to target nematode parasitism genes resulting in disease resistance against four major *Meloidogyne* spp (Huang et al., 2006). Moreover, dsRNA delivered by tobacco rattle virus mediates gene silencing in a plant parasitic root-knot nematode (Dubreuil et al., 2009).

RNAi also occurs in fungi and studies have shown the potential of HIGS in controlling fungal diseases in different crops. The first study showing that HIGS could silence genes in fungi was conducted by Tinoco et al. (2010) using tobacco plants expressing a GUS gene hairpin-structured dsRNA to silence GUS transcripts in a GUS-expressing strain of *Fusarium verticillioides* during plant colonization. HIGS has also been demonstrated in cereals infected with biotrophic pathogens *Blumeria graminis* and *Puccinia striiformis* f.sp. *tritici*. Nowara et al. (2010) showed reduction in the number of *B. graminis* spores able to develop haustoria in barley plants transiently transformed with fungal dsRNA silencing constructs. Moreover, hypha elongation was also reduced and HIGS plants inoculated with *B. graminis* exhibited reduced symptoms compared with control plants (Nowara et al., 2010). Yin et al. (2011) used VIGS mediated BSMV to deliver gene fragments from the rust fungi *Puccinia striiformis* f. sp. *tritici* and to express dsRNA from the fungus in wheat plants, which resulted in silencing of fungal haustorial genes.

HIGS was successfully used against another wheat pathogen, *P. triticina*, showing reduction in fungal biomass and disease symptoms at 10 days post-infection in wheat leaves transiently expressing dsRNA constructs targeting *P. triticina* pathogenicity genes (Panwar et al., 2013). Furthermore, targeting the cytochrome P450 lanosterol C-14 α -demethylase (CYP51) gene family using HIGS, was shown to be a highly efficient strategy for controlling *Fusarium graminearum*, which resulted in inhibition of fungal growth and plant infection (Koch et al., 2013). Recently, Ghag et al (2014b) showed that transgenic banana producing small interfering RNAs (siRNAs) targeting vital fungal genes increased its resistance against *Fusarium oxysporum* f.sp. *cubense*. Jahan et al. (2015) demonstrated successful control of *Phytophthora infestans* in potato using the same strategy. Recently, HIGS was also used to target

genes involved in aflatoxin production in *Aspergillus flavus*. Arias et al. (2015) demonstrated the potential of HIGS in reducing accumulation of the mycotoxin produced by *Aspergillus* in peanut. Significant aflatoxin reduction (60% to 100%) was observed in the transgenic peanuts compared to the control, suggesting that RNAi silencing of aflatoxin genes in peanut can contribute to reduction or elimination of mycotoxins in food crops.

Most recently, HIGS was also demonstrated to be an efficient tool to control necrotrophic fungal pathogens, such as *Sclerotinia sclerotiorum*. Andrade et al. (2015) transformed tobacco plants with RNA constructs targeting the fungal chitin synthase (chs) gene. Tobacco plants carrying the HIGS constructs showed significant reduction in disease severity compared with the control plants and the disease resistance was positively correlated with the presence of detectable fungal chs gene siRNA in the HIGS plants. In another recent study, host induced gene silencing was also found to be a promising strategy for improving plant resistance against Verticillium wilt, an economically important and notoriously difficult to control disease that affects a wide range of host plants. Song et al. (2016) were able to suppress Verticillium wilt disease in tomato and Arabidopsis plants by silencing previously identified virulence genes of *V. dahliae* through HIGS. Taken together, these studies convincingly demonstrated that trafficking small RNAs between plants and fungal pathogens provides a new powerful tool to control plant diseases.

CHAPTER 3: THE ROLE OF AVR4 IN *Cercospora cf. flagellaris* VIRULENCE, CERCOSPORIN PRODUCTION, AND INFECTION OF SOYBEAN

3.1. Introduction

Soybean (*Glycine max* L.) is one of the most important agricultural crops, cultivated in about 120.8 million ha in 2015/2016 worldwide with a production projected at 330.4 million metric tons (USDA, 2016a). It plays an important role in the food chain production for human and animal consumption. Currently, the USA produces more than 30 percent of all soybeans grown worldwide which positions U.S. as the largest global producer and exporter of soybean, followed by Brazil and Argentina. However, soybeans are susceptible to attack by various pathogens and pests throughout their growing season. In the southern U.S., one of the most devastating fungal diseases of soybean is Cercospora leaf blight (CLB) caused by *Cercospora cf. flagellaris* (previously known as *C. kikuchii*).

In the U.S., CLB was first reported in 1978 (Walters, 1980). The first symptoms of CLB are usually observed in the late R5 and early R6 soybean growth stages on upper leaves exposed to sunlight as reddish purple lesions, which become leathery and dark purplish-red with bronze highlights as the disease progresses (Walters, 1980). At late soybean growth stages, angular lesions form on infected leaf surfaces, and eventually lead to premature defoliation.

In recent years, the damage caused by CLB has been a serious concern (Moore and Wolcott, 2000) because CLB has spread from southern states to as far north as Iowa (R. W Schneider, personal communication) and has caused as high as 15 to 30% yield losses (Hartman et al., 1999; Wrather et al., 2001; Wrather et al., 1997). CLB was found in 45% (2008), 77% (2009), and 70% (2010) of the soybean fields surveyed in Alabama from 2008-2010 (Sikora et

al., 2011). The yield loss caused by CLB in the U.S. was estimated to be 12.8 and 7.1 million bushels in 2009 and 2010, respectively. It is now the most destructive foliar disease of soybean in the South. There is no effective management to control CLB in soybean due to lack of host resistance, effective fungicides and long latent disease period.

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

The genes involved in cercosporin biosynthesis has also been identified in *C. nicotianae*, and consists of at least 8 cercosporin toxin biosynthesis (CTB) genes with possible 4 additional open reading frames (ORFs) (ORF9-12) that are organized in a cluster (Chen et al., 2007b). The importance of some of these genes has also 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., 2007b). In addition to CTB8, cercosporin biosynthesis is also regulated by CRG1, a transcription factor involved in cercosporin resistance (Chung et al., 2003b) 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., 2003a; Daub et al., 2005; Shim and Dunkle, 2003). A biosynthetic pathway for cercosporin was also proposed (Chen et al., 2007b). However, recent studies presented alternative biosynthetic pathways obtained by a comparative genomic approach and characterization of metabolites from a series of biosynthetic pathway gene knockouts (de Jonge et al., 2017; Newman and Townsend, 2016).

Other than cercosporin production, there is little information on *Cercospora cf. flagellaris* pathogenesis. One interesting observation made by Stergiopoulos et al. (2010) is that several *Cercospora* spp, such as *C. beticola*, *C. apii*, *C. nicotianae*, and *C. zeina*, contain a homolog 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 wall and shield it from 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 sequence 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 or in interaction with other microbes in a pathogen's environment (Kohler et al., 2016).

AVR4 from *C. cf. flagellaris* (Cfla-Avr4) may also contribute to its virulence on soybean. Therefore, the present study cloned Cfla-Avr4, created a disruption mutant and compared the changes in CLB disease development, CTB gene expression, and cercosporin production both *in*

in vitro and *in vivo* to determine the possible roles played by Cfla-AVR4 in *C. cf. flagellaris*. The Δ avr4 disruption mutants produced little cercosporin on potato dextrose agar (PDA, Difco, Detroit, MI, USA) or complete medium, and had lower levels of Ctb gene expression under light conditions. Cercosporin was also found to inhibit chitinase activity *in vitro*. In addition, the mutants were found to grow slower compared to wild type *C. cf. flagellaris*, and elicited less disease symptoms on detached soybean leaves. The above results suggest Cfla-AVR4 regulates the expression of cercosporin pathway genes and the toxin production, in addition to the previously reported function of suppressing host defense through protecting fungal hyphae from degradation by host hydrolytic enzymes, such as chitinases. The knowledge gained from the present study can lead to development of more efficient strategies to control CLB disease in soybean.

3.2. Materials and Methods

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

C. cf. flagellaris isolate MRL 6020-2B obtained from Dr. Schneider's lab was grown for 5 days in potato dextrose broth (PDB, Difco, Detroit, MI, USA) with constant shaking (200 rpm) at 25°C under continuous light. Fungal mycelia were harvested and dried before being ground to a fine powder in liquid nitrogen for DNA extraction with a GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, St Louis, MO) following the manufacturer's instructions. First, PCR primers were designed based on the Avr4 gene sequences available in GenBank (GU574324; GU574325; GU574326; GU574327) to confirm its presence in *C. cf. flagellaris*. The primers Avr4F and Avr4R2 (See Table 3.1 for all primer sequences) were designed using Primer Express 2.0 (Applied Biosystems, Foster City, CA) and synthesized by Integrated

DNA Technologies (Coralville, IA). The amplified Avr4 fragment was cloned into pCR[®]2.1-TOPO[®] TA Cloning vector (Invitrogen, Carlsbad, CA) and sequenced at Louisiana State University GeneLab (Baton Rouge, Louisiana).

Table 3.1: List of primers used in Chapter 3

Primer name	Oligonucleotide sequence (5'→3')
Avr4F	AAGGATCCATGTACGGCCTCTTCCACCTC
Avr4R2	AAGGTACCTTGGTGCAGGTGCAGGTGCTGA
AP1	GTAATACGACTCACTATAGGGC
AP2	ACTATAGGGCACGCGTGGT
GSP1F	AGACATGAAACGAACACACTGTATGG
GSP1R	AGTGCGTAGAGGCAGTCGAATGGAC
GSP2F	ACTACCAGGGAAGTGCCTCGGATACAT
GSP2R	CCATACAGTGTGTTTCGTTTCATGTCT
M13R	AGCGGATAACAATTCACACAGGA
M13F	CGCCAGGGTTTTCCAGTCACGAC
FP1-1	CCCGATGCTTGTCCGCAATA
RP1-1	<u>TCCTGTGTGAAATTGTTATCCGCTTCTTGTTGTTGAGGCAGTGC</u>
FP2-2	<u>GTCGTGACTGGGAAAACCCCTGGCGTGCTGGTCAATGGTGGAACG</u>
RP2-2	CACAGTCTAACGCCTTCCGT
NLC37	GGATGCCTCCGCTCGAAGTA
NLC38	CGTTGCAAGAACTGCCTGAA
Avr4F1	GCATTGCCCTAACAGACCATC
Avr4R1	AGAATAGTGGGCGTTGCGT
CTB12F	ACCTTGCCTCAACTGTCTTAC
CTB12R	TGAAGCGACGAACGGATTT
CTB81F	GACAGCAGGTTATCTTCCAGAG
CTB81R	GTACTTATGCATCCACCACCA
Avr4WT-F	CCGGTATCGCGTATGAAAGG
Avr4WT-R	GAGAAGAAGTACTGCGACACGGT
Avr4WT-PRB	FAM-ATGCCGTGCTGGTCAATGGTGGA-TAMRA
CKctb6-2F	CACCATGCTAGATGTGACGACA
CKctb6-2R	GGTCCTGGAGGCAGCCA
CKctb6-PRB	FAM-CTCGTCGCACAGTCCCGCTTCG-TAMRA

After confirming the presence of Avr4 in *C. cf. flagellaris*, a chromosome library of *C. cf. flagellaris* was constructed using the Universal GenomeWalker™ kit to clone the full length Avr4 gene according to the manufacturer's guideline (Clontech Laboratories Inc., Mountain View, CA). *Cercospora cf. flagellaris* genomic DNA was digested with four different restriction enzymes. Libraries 1, 2, 3 and 4 were created using *Dra I*, *EcoR V*, *Pvu II* and *Stu I*, respectively. *Pvu II* digestion of human genomic DNA was used as a positive control (provided in the kit). For each reaction the following was combined in a separate 1.5-ml tube: 25 µl of genomic DNA (0.1 µg/µl), 8 µl of restriction enzyme (10 U/µl), 10 µl of restriction enzyme buffer (10X) and 57 µl of deionized H₂O. From each library construction 4 µl of digested, purified DNA were transferred to a fresh 0.5-ml tube and the following was added: 1.9 µl of GenomeWalker Adaptor (25 µM), 1.6 µl of 10X ligation buffer and 0.5 µl of T4 DNA ligase (6 U/µl). To walk upward and downward into unknown genomic regions, internal walking primers were designed based on the partial Avr4 sequence obtained previously. The gene specific primers GSP1F and GSP1R and the adaptor primer AP1, were used in the first round of PCR. In the second round of PCR the following primers were used, GSP2F, GSP2R and AP2. For primary PCR, 1 µl of each library was used and 1 µl of a 50X dilution of the primary PCR product was used as the template for the secondary PCR. Subsequently, all DNA fragments amplified by PCR were cloned into pCR®2.1-TOPO® TA Cloning vector (Invitrogen, Carlsbad, CA) for sequencing at Louisiana State University GeneLab (Baton Rouge, Louisiana). Prediction of ORFs and exon/intron junctions were performed using the gene-finding software at <http://www.softberry.com>.

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

Avr4 was disrupted by double homologous recombination using fusion PCR to generate two constructs consisting of Avr4 5' and 3' fragments fused to the *hygromycin (HYG)* cassette

according to Yu et al. (2004). The *HYG* cassette (2.5 kb) was amplified from pUCATPH (Lu et al., 1994) with the primers M13R and M13F. *C. cf. flagellaris* genomic DNA was used to amplify the 5' Avr4 fragment (0.4 kb) with the primers FP1-1 and RP1-1, and the 3' Avr4 fragment (0.5 kb) was amplified with the primers 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), and equal molar concentrations of both fragments were mixed and directly transformed into the *C. cf. flagellaris* wild type protoplasts for gene disruption.

Protoplasts were prepared as previously described (Chung et al., 2002; Upchurch et al., 1991). Briefly, one gram of mycelium was digested in 40 ml of an enzyme cocktail containing 10 mg/ml Glucanex (Sigma L1412), 5 mg/ml Driselase (Sigma D9515), 1200 U/ml β -glucuronidase (Sigma G0876), 0.7 M NaCl, 10 mM CaCl₂, and 10 mM Na₂HPO₄ (pH 5.8). After digestion, the solution was passed, in succession, through cheesecloth, glass wool and Miracloth (EMD Millipore). Fungal protoplasts were harvested by centrifugation at 4000 g for 5 min at 4°C. Protoplasts were washed once with 10 ml 0.7M NaCl followed by two washes with STC buffer (1.2 M sorbitol, 10 mM Tris-HCl pH 7.5, 10 mM CaCl₂). Finally, protoplasts were adjusted to 10⁸ per ml in four parts of STC and one part of 50% PEG. Fungal transformation was carried out using polyethylene glycol as described by Turgeon et al., (2010). Approximately 1x10⁷ protoplasts in 100 μ l of STC/PEG solution were mixed gently with 10 μ g of each DNA fragment, incubated on ice and then mixed with PEG. Protoplasts were spread on molten complete medium

plates overlaid with 1% agar containing 300 µg/ml Hygromycin B (Sigma H9773). The colonies of transformants that appeared between 5 and 8 days were selected and transferred to complete media plates containing 300 µg/ml Hygromycin B. 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.

3.2.3 Fungal isolates, growth conditions and growth measurements

Cercospora cf. flagellaris wild type isolate MRL 6020-2B (Cai and Schneider, 2005) and mutants (Δ avr4M1, Δ avr4M2, Δ avr4M3) were maintained on complete medium (CM, see Appendix) (Jenns et al., 1989) and CM amended with 300 µg/ml Hygromycin B (Roche Applied Science, Indianapolis, IN), respectively. Fungal growth was assessed by placing a 5-mm-diameter mycelial plug in the center of a PDA plate and colony radial growth measured every two days to determine the colony size increase over a period of 13 days. For liquid culture, *C. cf. flagellaris* wild type and mutants were grown on solid CM for 1 week and three 7-mm mycelial plugs were cut with a cork borer and ground in 2 ml of sterile water using a sterile mortar and pestle. This mycelial suspension was used to inoculate 100 ml of liquid complete medium or potato dextrose broth (PDB, Difco, Detroit, MI, USA). Cultures were incubated at 25°C with constant shaking (200 rpm) under light (240 µE m⁻²s⁻¹) or dark (achieved by wrapping flasks with two layers of aluminum foil) for five days.

3.2.4 Cercosporin extraction and quantification

Cercosporin was extracted from solid media using mycelial cultures grown on PDA plates for 14 days. Three 7-mm mycelial plugs were soaked in 5N KOH, incubated in dark for 4 h and cercosporin was quantified with spectrophotometer by measuring absorbance at 480 nm as described in Jenns et al. (1989). Cercosporin was extract from liquid culture using 25 ml of

culture filtrates from *C. cf. flagellaris* wild type or mutants grown in CM or PDB for five days as described above. Cercosporin was extracted by adding 10 ml of ethyl acetate (EtoAc) to 25 ml of fungal culture filtrate for 4 hours, and then adding an additional 10 ml of EtoAc for 4 more hours. During these 8 hours, tubes were shaken vigorously every hour of extraction. Finally, one hundred microliters of the ethyl acetate extract was removed and analyzed by HPLC. Extraction of cercosporin from soybean leaves infected with *C. cf. flagellaris* was performed following the methodology described by Gunasinghe et al. (2016). Briefly, soybean leaf samples (0.2 g) showing typical CLB symptoms were ground in 2 ml of ethyl acetate and kept overnight at 4°C. Following centrifugation, the supernatant was collected and cercosporin analyzed by HPLC.

3.2.5 High Performance Liquid Chromatography (HPLC) analyses

Cercosporin identification was based on the comparison of retention time of the peaks from HPLC between the pigment extracted from infected leaves or fungal cultures and the cercosporin standard (Sigma C6696). The calibration curve for cercosporin was generated from peak area versus the amount of standard cercosporin injected (See Appendix for cercosporin standard curve). HPLC analysis of cercosporin was performed using a Waters (Milford, MA) 2695 Separations Module with a Waters Atlantis C18 column (150 mm x 4.6 mm with 5 µm pore size) and detected using a Waters 2475 Fluorescence Detector as described by Gunasinghe et al. (2016). In brief, a gradient mobile phase consisting of eluent A (acetonitrile : acetic acid 95:5) and eluent B (Milli-Q water : acetic acid 95:5), at a flow rate of 1.5 ml min⁻¹ was used with an initial linear gradient from 50 to 70% eluent A over the first 8 min followed by 70% eluent A for 1 min. Next, the mobile phase of 100% eluent A was maintained for 6 min, and then changed to 50% eluent A for 10 min to allow the re-equilibration of the column.

3.2.6 RNA isolation and real time PCR analysis of Ctb1 and Ctb8 gene expressions

Fungal cultures were grown for 5 days in potato dextrose broth (PDB, Difco, Detroit, MI, USA) 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 aluminum 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) according to the manufacturer's recommendations. RNase-Free DNase Set (Qiagen) was used to eliminate possible residual DNA contamination.

Gene specific primers were designed within exon regions of Ctb1 (CTB12F and CTB12R) and Ctb8 (CTB81F and CTB81R) genes using Primer Express 2.0. Real-time PCR experiments were performed using SYBR® Green PCR Master Mix (Applied Biosystems) and the ABI PRISM 7000 Sequence Detection System under standard conditions. RT-PCR 25 µl reaction volume was composed of 1 µl of reverse transcribed cDNA, 12.5 µl 2X SYBR® Green Master Mix, and 1 µM of each primer. The quantification of the relative transcript levels of Ctb1 and Ctb8 genes were normalized to the β-tubulin gene and relative expression was calculated using the $2^{-\Delta\Delta C_t}$ method (Schmittgen and Livak, 2008). Three biological replicates and three technical replicates within each biological replicate, were used for each sample and a negative (template free) control.

3.2.7 Fungal pathogenicity assay on soybean

Soybean detached leaf assays were performed to check the differences in fungal pathogenicity among the *C. cf. flagellaris* wild type and $\Delta avr4$ mutants. Soybean plants cv. Syngenta 02JR423003 were grown in the greenhouse until R1 stage when leaves were collected

and placed inside the transparent plastic boxes containing moist paper towels. Three-millimeter diameter mycelial agar plugs containing 2 week-old *C. cf. flagellaris* wild type or Δ avr4 mutants grown PDA, were placed on the adaxial surface of soybean leaves with the mycelial side touching the leaf and gently pressed. The inoculated leaves were incubated under fluorescent light at room temperature and disease severity was assessed 14 days post-infection (dpi).

Disease severity and fungal growth were determined using a 1-5 rating scale representing less to more growth or severe symptoms. Three boxes containing eight leaves each 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.

3.2.8 DNA isolation and quantitative real-time PCR

Fungal growth or biomass in inoculated soybean leaves was also quantified using real-time PCR. Soybean leaves infected with *C. cf. flagellaris* wild type or Δ avr4 mutants were ground in liquid nitrogen and DNA was extracted with a GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, St Louis, MO) according to the manufacturer's instructions. Quantitative real-time PCR targeting the *C. cf. flagellaris* Ctb6 gene was performed using primers CKCTB6-2F/CKCTB6-2R and fluorescent probe CKCTB6-PRB in the ABI 7000 sequence detection system (Applied Biosystems) under standard conditions. Each reaction contained 7.5 μ l 2X TaqMan Universal PCR Master Mix (Applied Biosystems), 1 μ l of 10 μ M each primer (final concentration 666 nM each primer), 0.6 μ l of 10 μ M (final concentration of 400 nM) probe and 1 μ l of 10 ng template DNA. The fungal biomass of *C. cf. flagellaris* in leaf samples was determined according to Chanda et al. (2014).

3.2.9 Growth of *C. cf. flagellaris* wild type and Δ avr4 mutants in the presence of chitinase and agarose plate assay for chitinolytic 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, C8241) was dissolved in 0.1M phosphate buffer. CM plates were inoculated with *C. cf. flagellaris* wild type or Δ avr4 mutants using 5-mm-diameter mycelial plugs. Plates were kept at 25°C, and 14 days later a cork borer was used to cut 2-mm-diameter wells in the agar 1 mm away from the edge of the fungal colony. The individual wells were filled with 0.1M phosphate buffer containing 1 unit of chitinase and wells filled with 0.1M phosphate buffer only was used as a control. Plates were sealed and kept at 25°C. Fungal growth was visually assessed two days later and pictures were taken to determine whether chitinase affects mycelial development around the well containing the enzyme.

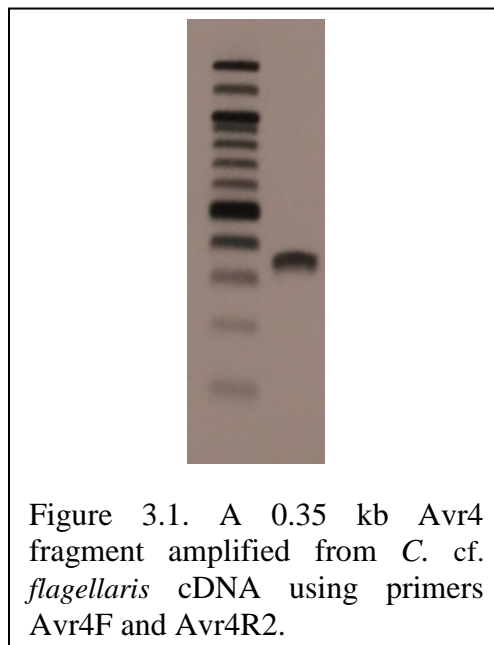
To verify if chitinolytic activity is influenced by cercosporin, a chitinase cup-plate assay was performed. Glycol chitin substrate was synthesized according to Roberts et al. (1988) from shrimp-shell chitin (Sigma C9752). Briefly, 2 g of chitin was dissolved in 35 ml of cold concentrated HCl and placed at 4°C for 24 h. Then, the mixture was filtered through cheesecloth into 200 ml of ethanol at - 20°C with rapid stirring. Next, the chitin suspension was centrifuged at 10000 g for 20 min and finally chitin pellets were washed repeatedly with distilled water until the pH was neutral. To prepare chitin plates, agarose was dissolved (1.6% [w/v]) in 0.05M phosphate buffer in a microwave oven. The dissolved agarose/buffer solution was cooled to 50–60°C and 0.0165 g of glycol chitin and 30 μ l of cercosporin (10 mM) were added to 20 ml of agarose/buffer solution. Plates containing no cercosporin were used as controls. A cork borer was used to cut 4-mm-diameter wells in the gel, with a volume per well of approximately 10 μ L. Samples of 10 μ L of 0.05M phosphate buffer only or containing 0.25 or 0.5 unit of chitinase

enzyme were pipetted into individual wells in the agarose plates, which were sealed with parafilm. The plates were incubated at 25°C and chitin hydrolysis, visualized as clear zones around the wells, was assessed after 48, 72, and 120 hours.

3.3. Results

3.3.1 *C. cf. flagellaris* Avr4 gene molecular cloning and sequence analysis

A 0.35 kb Avr4 fragment was amplified from *C. cf. flagellaris* gDNA using primers designed based on conserved sequences of Avr4 homologs from other *Cercospora* species (Figure 3.1). Sequence analysis showed high identity (97% - 99%) at the deduced amino acid level to AVR4 of *C. beticola*, *C. nicotianae*, *C. zeina*, and *C. apii*, (Figure 3.2). The full-length Avr4 gene was obtained by PCR of a *C. cf. flagellaris* genomic library previously constructed by Chanda 2012 using primers designed to walk upward and downward into unknown genomic regions (Figure 3.2). In total, six overlapping fragments were amplified (Figure 3.3), sequenced, and assembled into a 1.6 kb DNA fragment (Figure 3.4). This Cfla-Avr4 gene has an open



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1      ATGTACGGCCTCTTCCACCTCGCCGTCCTTTCGGCGACAGCGCTCTCCGCATCTGCACTG
      TACATGCCGGAGAAGGTGGAGCGGCAGGAAAAGCCGCTGTCGCGAGAGGCGTAGACGTGAC
      M Y G L F H L A V L S A T A L S A S A L

61     CCTCAACAACAAGAGAAGCCTGGCGTCAACAAAGATTTTCATCTGCCACCCGAAGACATG
      GGAGTTGTTGTTCTCTTCGGACCGCAGTTGTTTCTAAAGTAGACGGGTGGGCTTCTGTAC
      P Q Q Q E K P G V N K D F I C P P E D M

121    AAACGAACACACTGTATGGGTCCATTTCGACTGCCTCTACGCCTACTACCAGGGAAGTGCCTC
      TTTGCTTGTGTGACATAACCAGGTAAGCTGACGGAGATGCGTGAATGGTCCCTTGACGGAG
      K R T H C M G P F D C L Y A L P G N C L

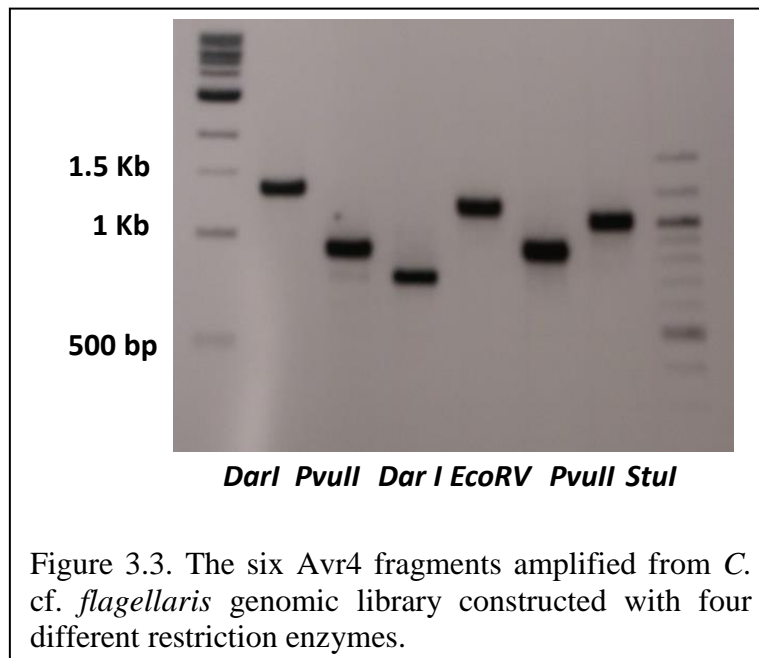
181    GGATACATCCAATGCCAGCCCAAGGACACGACATATGAGACCGGTATCGCGTATGAAAGG
      CCTATGTAGGTTACGGTTCGGGTTCTGTGCTGTATACTCTGGCCATAGCGCATACTTTCC
      G Y I Q C Q P K D T T Y E T G I A Y E R

241    CCATGCCGTGCTGGTCAATGGTGGAAACGACGAGAAGAAGTACTGCGACACGGTGGAGCCT
      GGTACGGCACGACCAGTTACCACCTTGCTGCTCTTCTTCATGACGCTGTGCCACCTCGGA
      P C R A G Q W W N D E K K Y C D T V E P

301    CCAACATGCAAAGCCCCAGAGAATCCTGCGTCAGCACCTGCACCTGCACCAAGGTACCTT
      GGTGTACGTTTCGGGGTCTCTTAGGACGCAGTCGTGGACGTGGACGTGGTTCCATGGAA
      P T C K A P E N P A S A P A P A P R Y L

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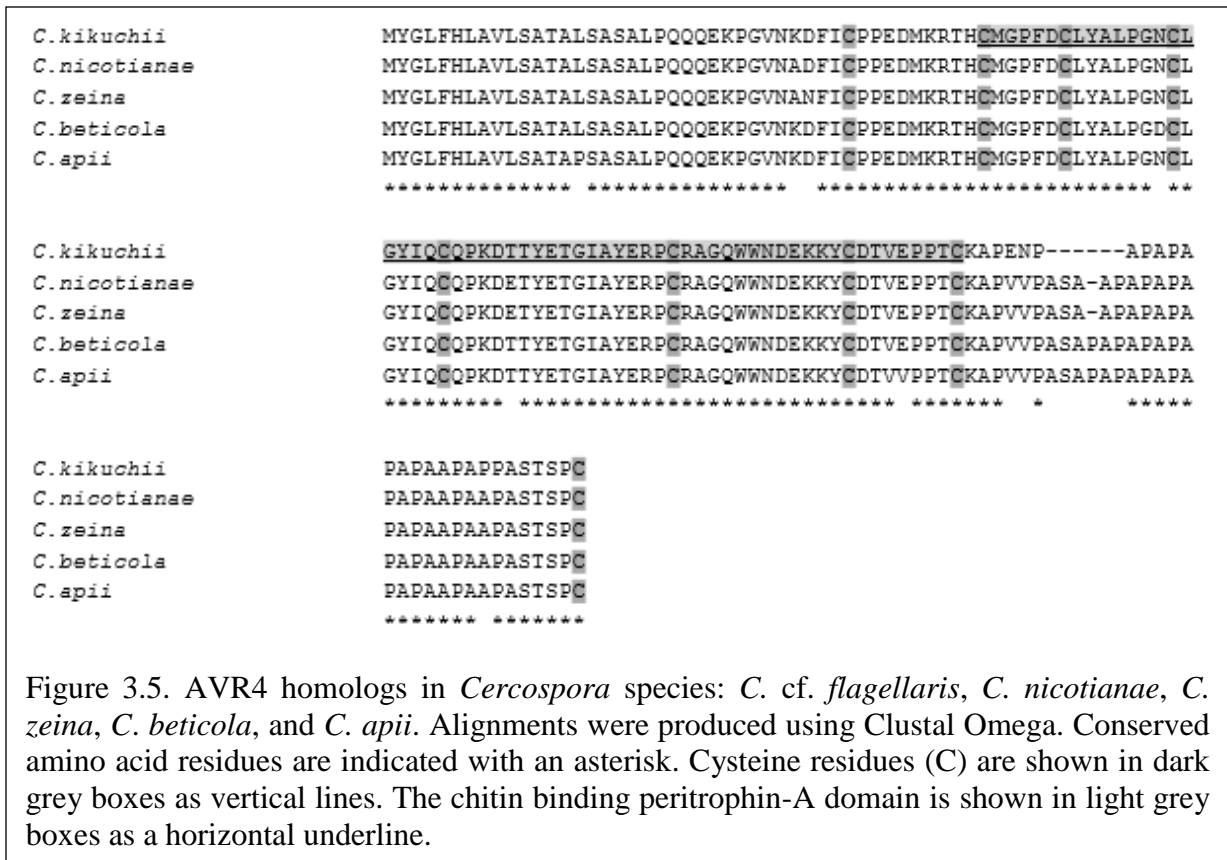
Figure 3.2. *Cercospora cf. flagellaris* Avr4 gene partial sequence showing gene specific primers designed to walk upward and downward into unknown genomic regions. Dark gray boxes represent primers GSP1F and GSP1R. Pink boxes represent primers GSP2F and GSP2R.



1 ACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGTCTGCAAGGCAAACGCTGGAAGGAC
 61 AACGTCGCTGAGTCATCAGTCCGAAGTGCATTGCCCTAACAGACCATCGAGGGCGACTAC
 121 AGTGTGTCATCTGCATACGCGGATTCAAGGAATATAAGCTCAAGGTCCTCTCCGGGCTCT
 181 CCTGCCAGTAAGCTGCCTCAAATCGGCTGCCAAAAGGTTGCAAACGCCCAAAAATCGAGA
 241 GATACCTTGCTACCCGCTCCCCGTATGCAGGTGACACACTGTACAGCACAAAACCTCCGCC
 301 TGGAGACAACGAAGATCATGACGCTGTGCCCGACGGACACACGACCAGTGATCACCCATT
 361 CCGGGTTTCTGCAGCCCCGATGCTTGTCCGCAATAGGCTGCGCGGAAGATGTTCAAATCA
 421 AACCATGGTGTGGTAATTTGCCAGATCCTTGCTAGGCACAAGGACAAAAGTTGACCAGGGT
 481 TCACCAAGACGGCAATGCGAGATGAGGAGGACGTCGCAGTCAGTTCGAACGACGCACCGC
 541 CGCACTTTCACCTTCCACCACCCTCTCTTTCCAAAAGCGACTAGAAAAGACGCACATCG
 601 TCAACCACGAGAGCCTCTGTTGCAGGACAACACCAAGCGGCAGTACACTCGTTTATATCA
 661 TTCGCCACTT**C**CAACTCATCACACCATG**TACGGCCTCTTCCACCTCGCCGTCCTTTCCGGC**
 721 GACAGCGCTCTCCGCATCTGCACTGCCTCAACAACAAGAGAAGCCTGGCGTCAACAAAAGA
 T A L S A S A L P Q Q Q E K P G V N K D
 781 TTTTACTGCCCCACCCGAAGACATGAAACGAACACACTGTATGGGTCCATTGACTGCCT
 F I C P P E D M K R T H C M G P F D C L
 841 CTACGCACTACCAGGGAAGTGCCTCGGATACATCCAATGCCAGCCCAAGGACACGACATA
 Y A L P G N C L G Y I Q C Q P K D T T Y
 901 TGAGACCGGTATCGCGTATGAAAGGCCATGCCGTGCTGGTCAATGGTGGAACGACGAGAA
 E T G I A Y E R P C R A G Q W W N D E K
 961 GAAGTACTGCGACACGGTGGAGCCTCCAACATGCAAAGCCCCAGAGAATCCTGCGCCAGC
 K Y C D T V E P P T C K A P E N P A P A
 1021 ACCAGCACCAGCACCAGCAGCTCCAGCGCCGCCAGTACGTCTCCCTGCTGATCAGG
 P A P A P A A P A P P A S T S P C
 1081 CTTCTGTTGGGCGTTGCTGCGTAAGGGGAAGACGTGGTGTACTGATTTGCACTTGTGCAGG
 1141 CGCTGCCGGTCTGCCTCATACTAGACATTAGATCAAGGAAAAGATAATGCGCTGTTT
 1201 GGAGAACATGACATGGGAAAAGAGGGATTGGTCCCTATACAGAGGACAAAAAGGGAAAAC
 1261 CACATCATCATACTATCCCAGGCTGAGCCAGCTACGATCGGCATATTCTTTCTTGGTAT
 1321 TTTATCATATTTTCTTTTATATTGTATATATTCGCTCGCGCCAGACGGAAAGCGTTAGAC
 1381 TGTGTATGCTCGAAGCAAGTAGATGCATGCCGAGGTTGGCAGGAACTACATGTGTCAGA
 1441 CGTTCGAACATTCGCCACCGTAGTCTACTCTCGACAATATCTAAAACGCAACGCCCACT
 1501 ATTCTCTATCAGCAATCCGCTCATCCAATAAAAAGCCTCAACCATGGACCCAGAAGTGAAC
 1561 TCAAGATCGAAAAGTCTGGTG

Figure 3.4. *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.

reading frame from 686-1073 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 (Figure 3.4). National Center for Biotechnology Information (NCBI) BLAST searches revealed the presence of a putative chitin binding peritrophin-A domain (NCBI accession: pfam01607) conserved in all five *Cercospora* spp. (Figure 3.5). Furthermore, the nine Cys residues reported in *C. beticola*, *C. nicotianae*, *C. zeina*, and *C. apii* (Stergiopoulos et al., 2010) were also present in *C. cf. flagellaris* Avr4 gene (Figure 3.5).



3.3.2 Target gene disruption and confirmation of Δ avr4 mutants

To elucidate the functions of Avr4 in *C. cf. flagellaris* virulence and fitness, Δ avr4 disruption mutants of *C. cf. flagellaris* were created using the strategies outlined in Figure 3.6A. Two DNA fragments containing 5' Avr4 fused with 3' HYG and 3' Avr4 fused with 5' HYG were obtained by fusion PCR, and transformed directly into protoplasts of wild type isolate. In total, 17 out of 57 hygromycin resistant colonies were selected and transferred to new hygromycin selective CM plates. All selected fungal transformants showed the presence of the 466 bp fragment from HYG by PCR using HYG (hygromycin) specific primers NLC37 and NLC38 (Figure 3.6B). 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 transformants were confirmed to contain the expected extra 2.4 kb insertion of HYG compared to wild type strain (1.6 kb) when its full-length was amplified with primers Avr4F1 and Avr4R1 (Figure 3.6C). Three Δ avr4 mutants (Δ avr4M1, Δ avr4M2, Δ avr4M3) were selected for the following experiments.

3.3.3 Δ avr4 mutants grew faster, produced little or no cercosporin in vitro and had reduced expression of Ctb genes

The Avr4 insertion mutants grew faster on PDA and CM than the wild type (Figure 3.7). The difference in mycelium mat diameter became clear and significant 9 days after inoculation on PDA and it was about 50% larger in diameter than the wild type 13 days after fungal inoculation (Figure 3.7).

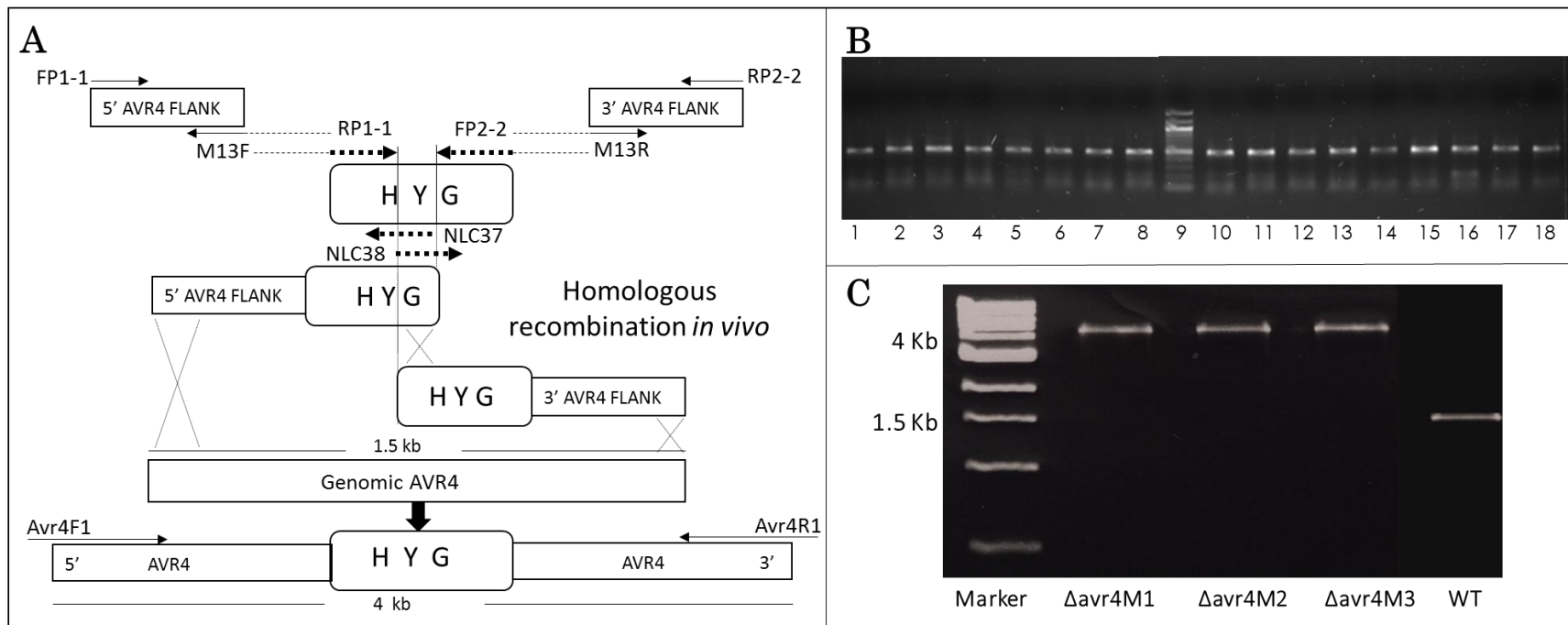


Figure 3.6. Targeted disruption of Avr4 gene in *Cercospora cf. flagellaris*. A) The 5' and 3' fragments of Avr4 were amplified separately with primer pairs FP1-1/RP1-1 and FP2-2/RP2-2 and fused with the hygromycin cassette (HYG) by overlapping regions using primers M13F and M13R, followed by a nested PCR using primer pairs FP1-1/NLC37 and NLC38/RP2-2 to amplify 5' Avr4 fused with 3'HYG and 3' Avr4 fused with 5' HYG, respectively. The resulting two PCR fragments were directly transformed into *C. cf. flagellaris* protoplasts. Note: drawing is not to scale. B) Sixteen selected Δ avr4 mutants (lines 1 to 7 and 10 to 18) showing the presence of the 466 bp fragment from HYG amplified by PCR using HYG specific primer pair NLC37/NLC38. Line 8 and 9 shows the 466 bp HYG fragment amplified from pUCATPH vector and the 1 kb DNA marker, respectively. C) Confirmative PCR using external primers Avr4F1/Avr4R1 showing the 4 kb fragment containing Avr4 fused to HYG in Δ avr4 mutants (Δ avr4M1, Δ avr4M2, Δ avr4M3) and the 1.6 kb Avr4 gene in *C. cf. flagellaris* wild type (WT).

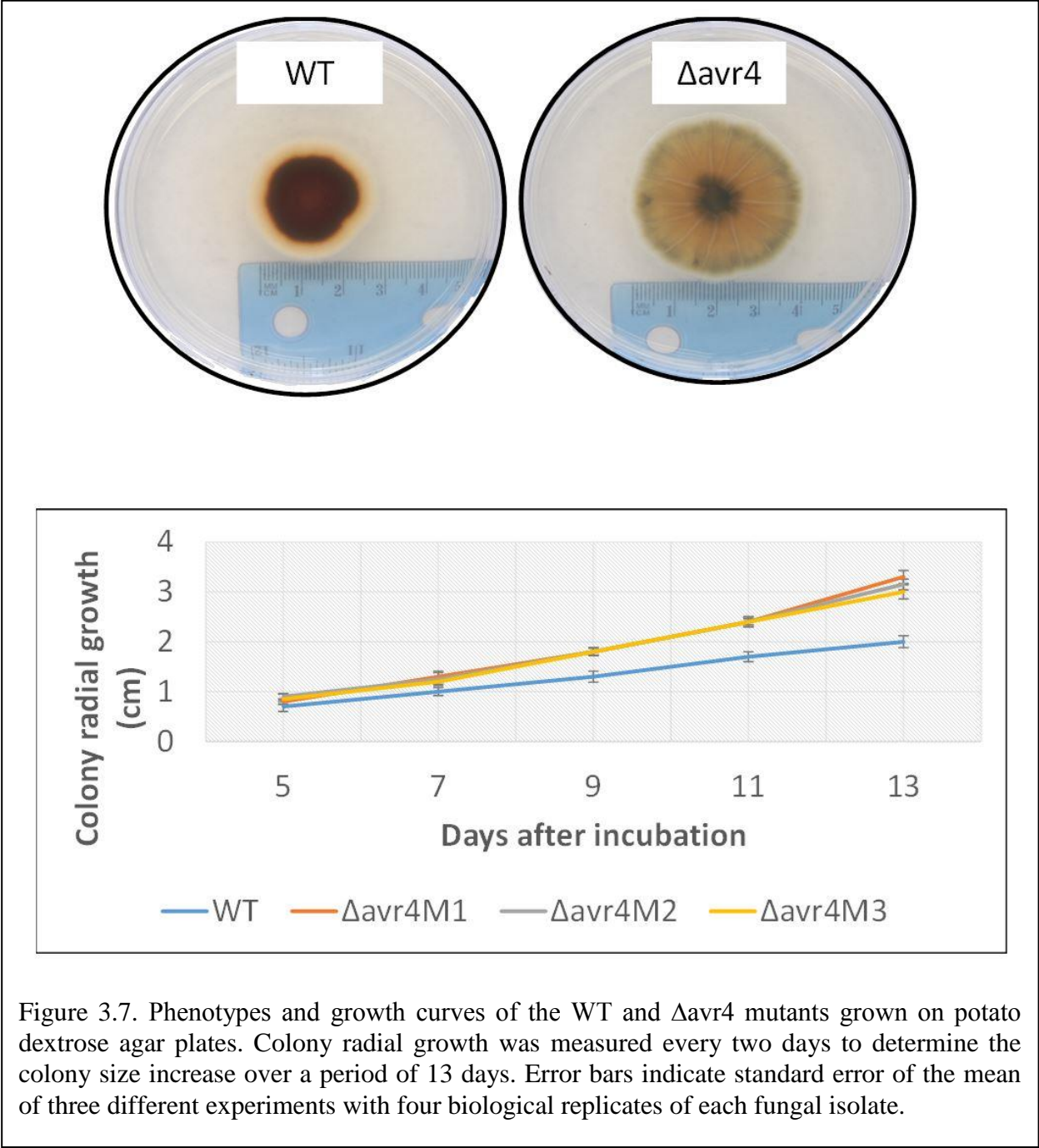
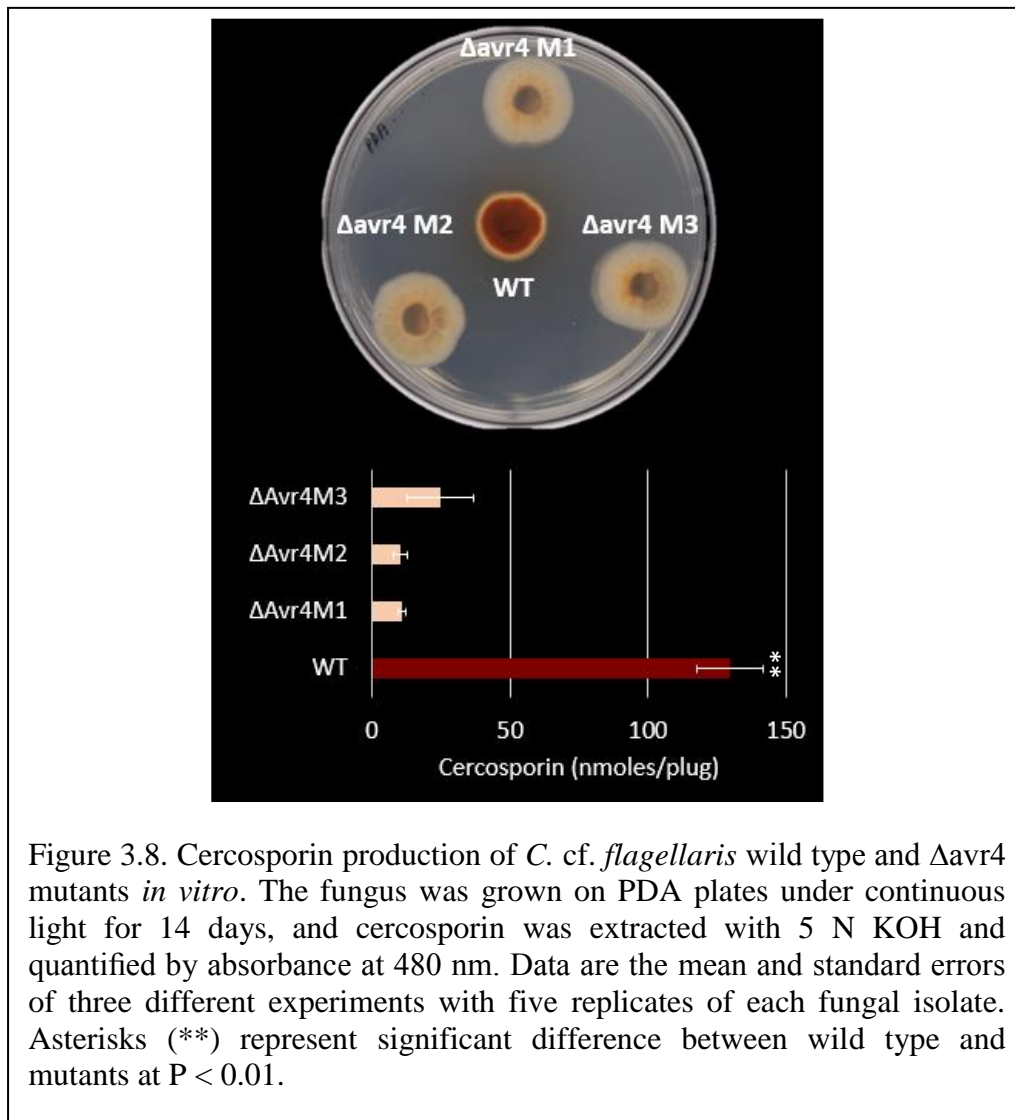
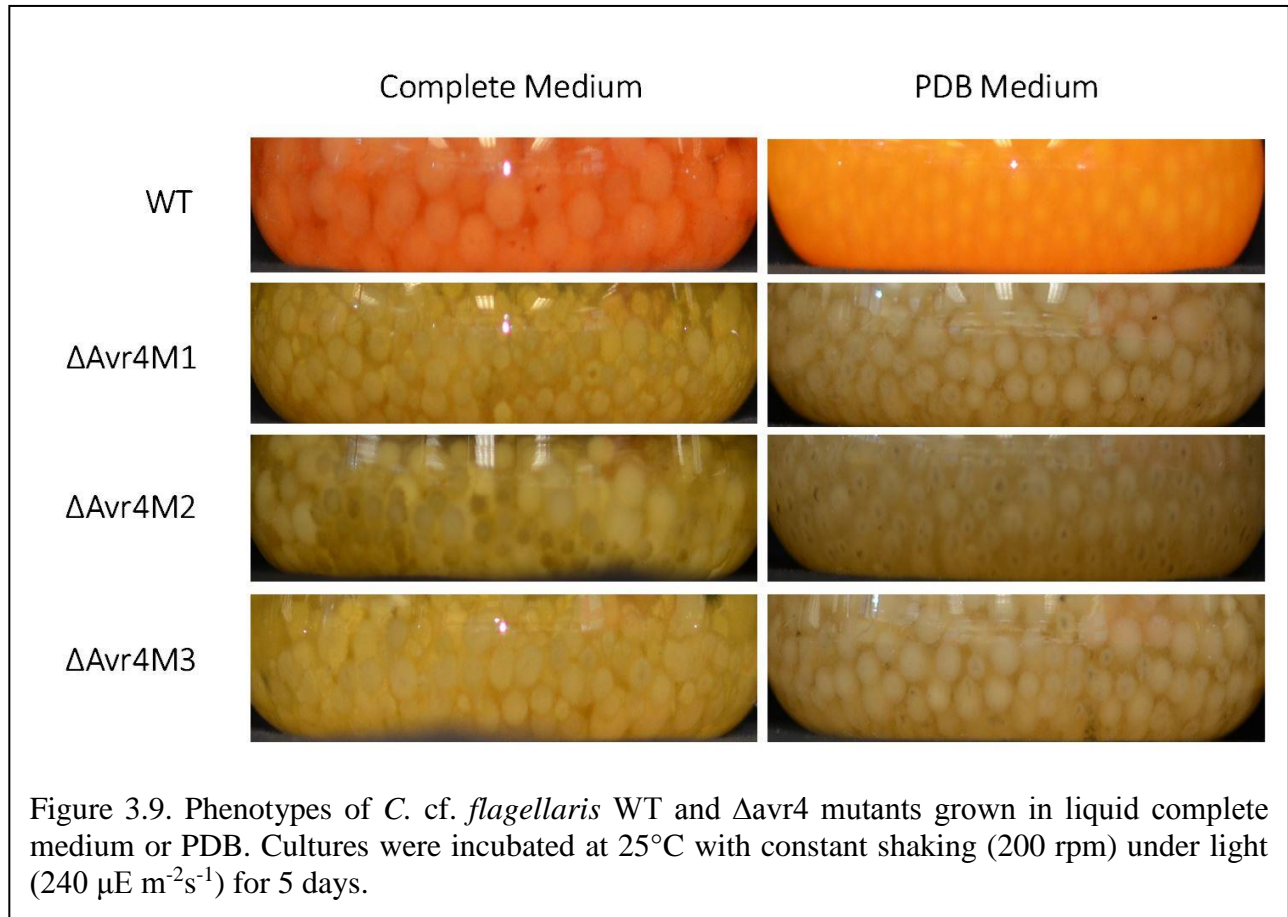


Figure 3.7. Phenotypes and growth curves of the WT and Δ avr4 mutants grown on potato dextrose agar plates. Colony radial growth was measured every two days to determine the colony size increase over a period of 13 days. Error bars indicate standard error of the mean of three different experiments with four biological replicates of each fungal isolate.

In addition, the Avr4 mutants produced very little to no dark red/purple pigment under the fungal colony, a typical color of condensed cercosporin, on either solid complete medium or PDA (Figure 3.7 and 3.8 top), which is strikingly different from that of the wild type. In contrast to the wild type, Δ avr4 mutants produced a light yellow pigment under the center of the fungal colony (Figure 3.7 and 3.8 top). Cercosporin production of Δ avr4 mutants on PDA plates was approximately 10% of the amount of cercosporin produced by the wild type (Figure 3.8). The difference in cercosporin production among the three Δ avr4 mutants was not significant.



When grown in liquid PDB or CM the same phenotype could be observed in which the wild type fungal culture became dark-red in color 5 days after incubation at room temperature, whereas the mutant cultures exhibited a light yellow color (Figure 3.9).



Cercosporin was not detected in either liquid CM or PDB inoculated with Δ avr4 mutants using HPLC. In contrast, the wild type fungus consistently produced greater amounts of cercosporin in both liquid media under light conditions than under darkness. HPLC analysis of culture filtrates showed that cercosporin accumulation in liquid CM inoculated wild type fungus were $3.81 \mu\text{g/ml}$ and $0.97 \mu\text{g/ml}$ under light and dark growth conditions, respectively.

Cercosporin accumulation was drastically reduced when the wild type fungus was grown in PDB medium under light conditions (approx. 1 µg/ml).

Considering that the mutants produced only 10% of the cercosporin produced by the wild type in PDA and also considering that no cercosporin was detected in liquid media inoculated with Δ Avr4 mutants, the expression of cercosporin biosynthesis pathway genes was examined using real time RT-PCR (Figure 3.10). It was found that disruption of Avr4 gene significantly reduced the expression of both Ctb1 and Ctb8 genes under light conditions (about 2-3 fold) (Figure 3.10).

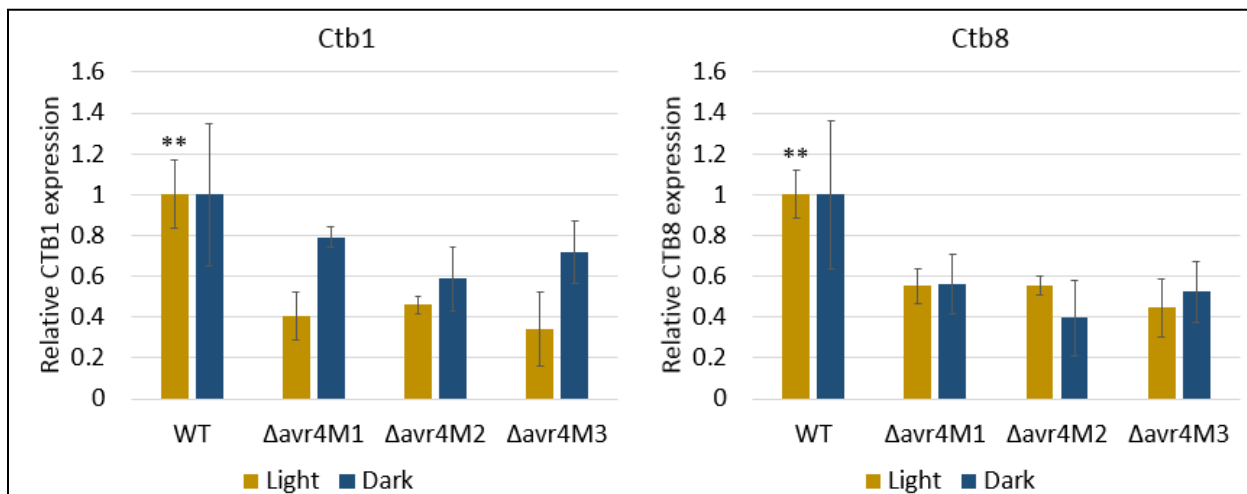
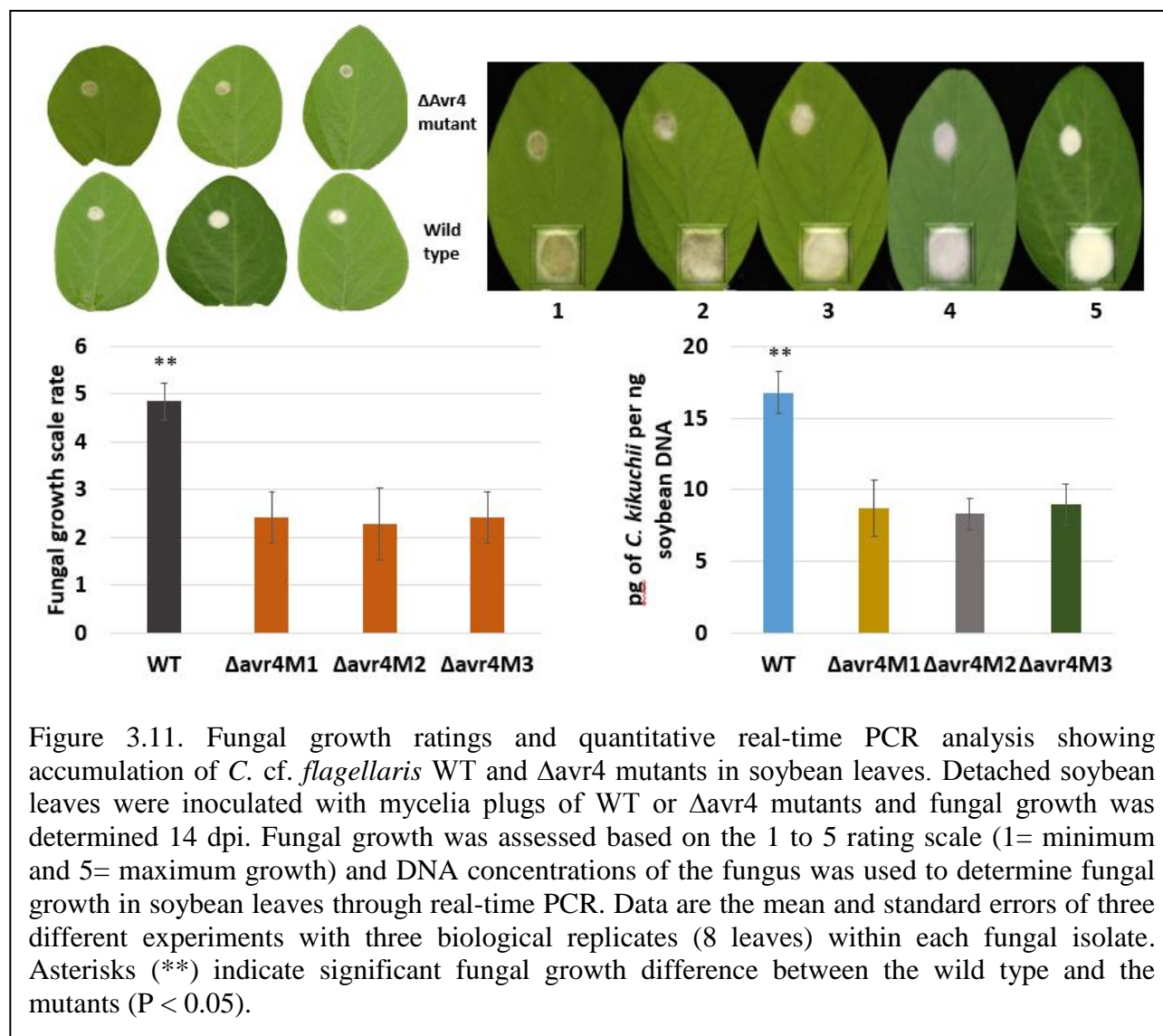


Figure 3.10. Real Time PCR analysis of Ctb1 and Ctb8 expression in *C. cf. flagellaris* WT and Δ avr4 mutants grown on PDB under continuous light or darkness for 5 days. Fungal RNA was isolated and 500 ng of total RNA was used for cDNA synthesis. The quantification of the relative transcript levels of CTB1 and CTB8 genes were normalized to the control gene β -tubulin and relative expression was calculated using the $2^{-\Delta\Delta C_t}$ method. Data are the mean and standard errors of three different experiments with three biological replicates of each fungal isolate and three technical replicates of RT-PCR reactions. Asterisks (**) indicate significant difference between the wild type and the mutants when grown under light ($P < 0.05$).

3.3.4 *C. 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* to soybean, the mycelia mattes of wild type isolate and Δ avr4 mutants from PDA were inoculated onto detached soybean leaves. Mutants grew much slower on the detached soybean leaves, about half of that of the wild type based on visual growth rating (Figure 3.11), which is the opposite of what was observed when they were grown on PDA plates. The abundant fungal growth observed on soybean leaves inoculated with *C. cf. flagellaris* wild type was also confirmed quantitatively using real-time PCR. Soybean leaves inoculated with Δ avr4 mutants showed a statistically less fungal growth than the leaves inoculated wild type pathogen (Figure 3.11).

In addition, the mutants incited fewer necrotic and chlorotic lesions compared to the wild type (Figure 3.12), indicating a clear reduction in fungal virulence. The similar necrotic and chlorotic symptom was reproduced on detached soybean leaves when inoculated with agar plugs without fungal mycelia matte from the wild type, but not from the mutants (Figure 3.12 bottom right), confirming that cercosporin, secreted by the wild type fungus in the PDA plates, contributes to the development of necrotic and chlorotic lesions.



In addition, the development of minor necrotic and chlorotic lesions on soybean leaves inoculated with mutants suggested that Avr4 mutants may produce small amount of cercosporin during infection of soybean leaves. To verify this, cercosporin was extracted from detached soybean leaves inoculated with *C. cf. flagellaris* wild type and mutants and analyzed through HPLC. Interestingly, no cercosporin was detected in leaves inoculated with Δ avr4 mutants.

Cercosporin accumulation in soybean leaves inoculated with wild type fungus was 3.9 $\mu\text{g/g}$ of leaf tissue.

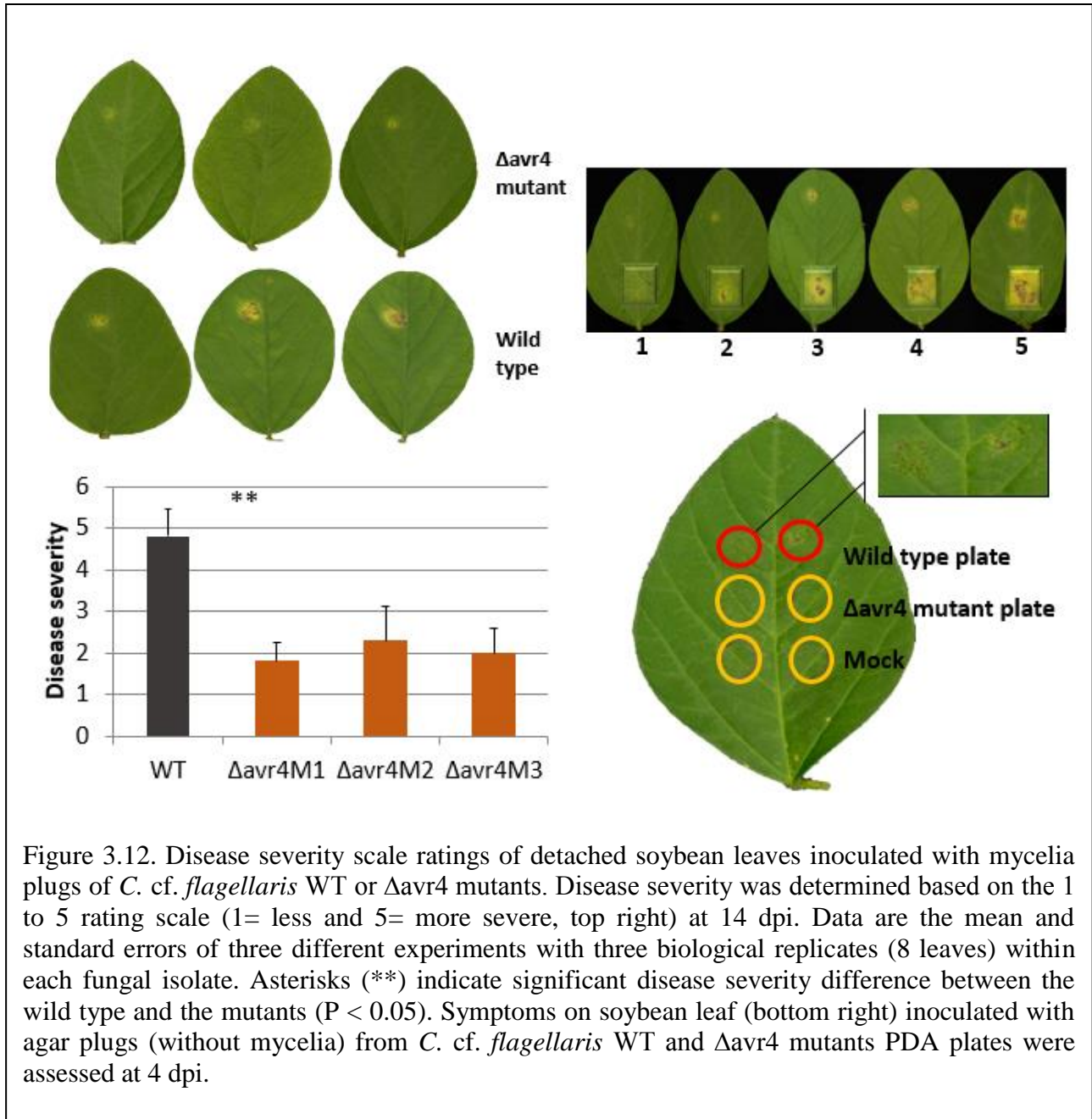
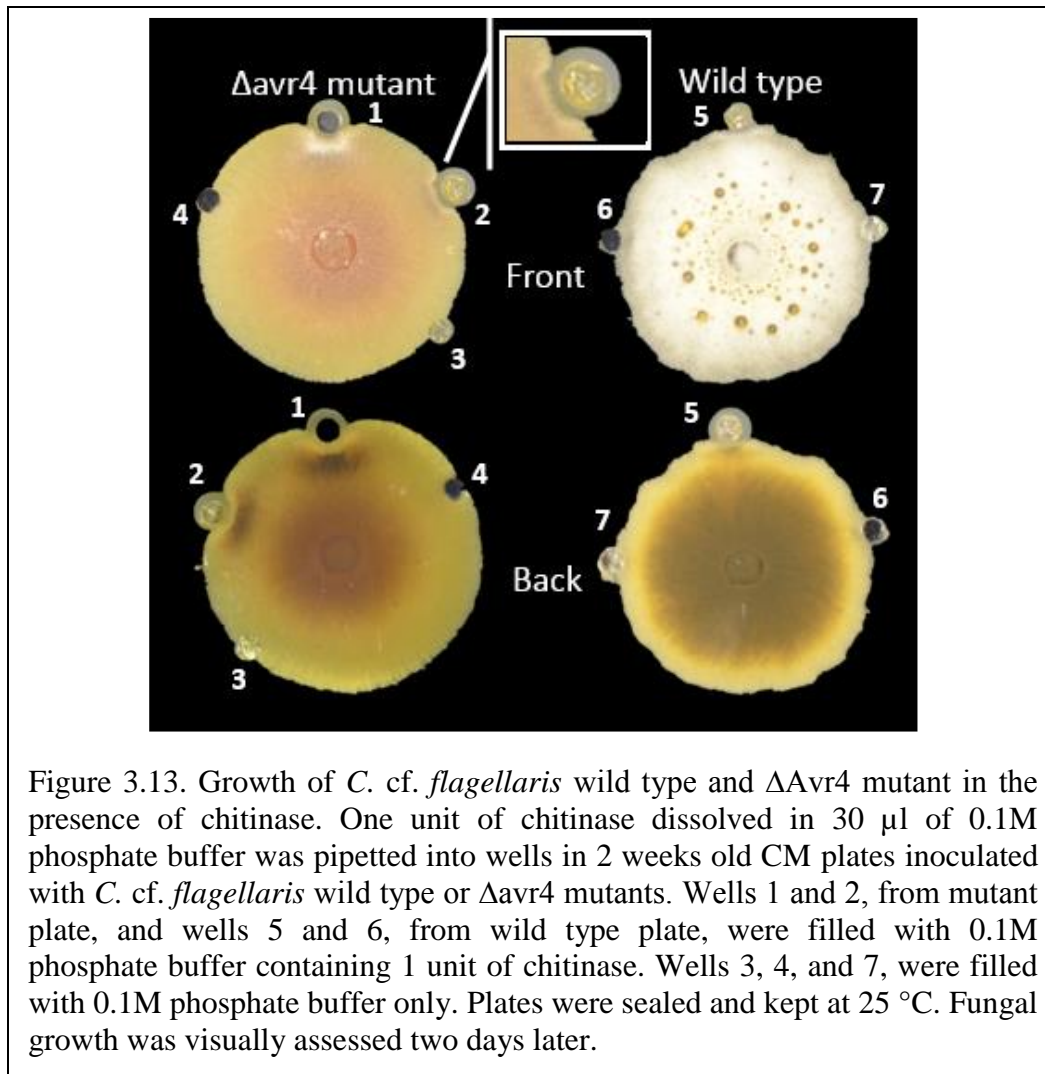


Figure 3.12. Disease severity scale ratings of detached soybean leaves inoculated with mycelia plugs of *C. cf. flagellaris* WT or Δavr4 mutants. Disease severity was determined based on the 1 to 5 rating scale (1= less and 5= more severe, top right) at 14 dpi. Data are the mean and standard errors of three different experiments with three biological replicates (8 leaves) within each fungal isolate. Asterisks (**) indicate significant disease severity difference between the wild type and the mutants ($P < 0.05$). Symptoms on soybean leaf (bottom right) inoculated with agar plugs (without mycelia) from *C. cf. flagellaris* WT and Δavr4 mutants PDA plates were assessed at 4 dpi.

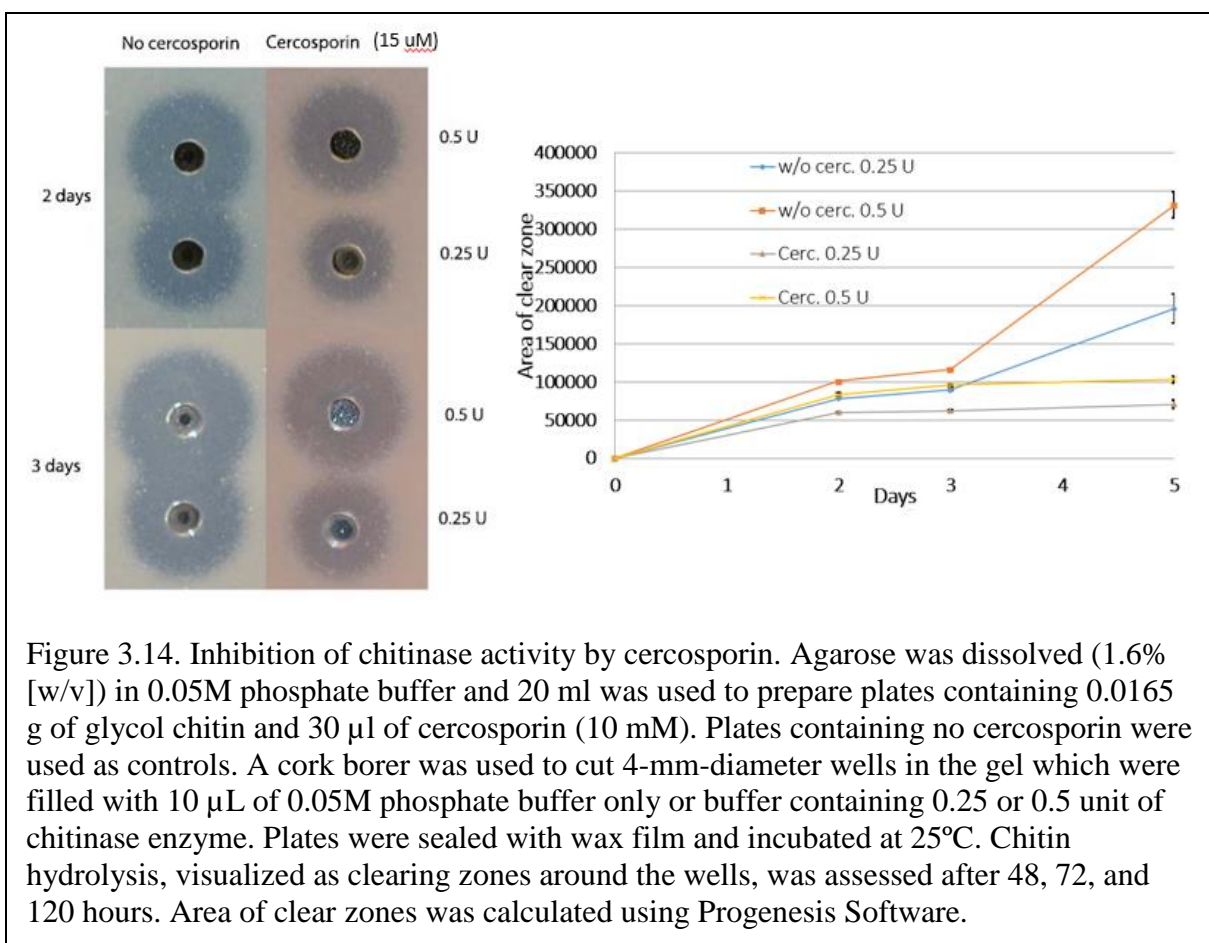
3.3.5 Chitinase reduced the growth of Δ avr4 mutants and chitinolytic activity is inhibited by cercosporin *in vitro*

In order to better understand the function of Avr4, which has been previously reported to act as a chitin binding protein to protect the fungal cell wall from degradation by plant chitinases (van den Burg et al., 2006), we also examined the effect of chitinases on the growth of Avr4 disruption mutants compared to the wild type fungus. The fungal growth was reduced in the mutants when growing next to wells containing 1 unit of chitinase on CM agar plates (Figure 3.13 left). Such suppression of growth by chitinase was not observed in the wild type (Figure 3.13 right), demonstrating that Avr4 does provide the fungus with some level of protection against chitinase.

In addition, the presence of chitinase stimulated production of some dark pigment under the mycelia next to the wells containing the enzyme by the Δ avr4 mutants (Figure 3.13 bottom left). Based on the above observation of increased pigmentation in mycelium near chitinase and considering that the pigment produced could be cercosporin, a cup plate assay was also performed to determine whether cercosporin has any direct effect on chitinolytic activities. A 19% and 30% reduction in chitinase activity was observed after 2 and 3 days when the wells contained 0.25 U of chitinase, respectively (Figure 3.14).



A slightly less reduction in chitinolytic activity (12% and 16%, respectively) was also observed after 2 and 3 days when 0.5 U of chitinase was used. The chitinase lost its activity after 3 days in the presence of 15 μ M of cercosporin whereas the chitinase remained active in the control even after 5 days (Figure 3.14 right). The difference among the agarose plates with and without cercosporin became very clear after 5 days, when 60% and 70% enzyme activity reduction were observed using 0.25 U and 0.5 U of chitinase, respectively (Figure 3.14 right).



3.4. Discussion

The interest in studying this particular effector was raised from the study by Stergiopoulos et al. (2010), which showed the presence of homologs 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 (van Esse et al., 2007).

In our study, Avr4 gene was found present in all 150 isolates of *C. cf. flagellaris* collected from several locations throughout Louisiana (data not shown). It is possible that *Cercospora* species conserve AVR4 to target a diverse host range (Crous and Braun, 2003; Groenewald et al., 2013). Certainly, AVR4 as a chitin binding protein can be very useful in assisting the fungus to enter and colonize different host plants that use chitinases as a defense mechanism to combat fungal invasion. Considering all the information described above, we hypothesized the presence of this effector in *C. cf. flagellaris* plays an important role in the virulence of this pathogen on soybean.

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 between its coding region and those sequences of Avr4 from other Dothideomycetes fungi (*Cercospora beticola*, *C. nicotianae*, *C. apii*, *C. zeina*, *Mycosphaerella fijiensis*, and *Cladosporium fulvum*). A *C. cf. flagellaris* isolate (MRL 6020 2B), well known for producing cercosporin *in vitro* and *in planta*, was used to create Avr4 disruption mutants for elucidating the role of AVR4 in *C. cf. flagellaris* fitness and virulence. The first noticeable change in phenotype of the Δ avr4 transformants was the reduced production of the dark purple pigmentation on PDA and CM, a characteristic of cercosporin.

It is well known that cercosporin is a virulence factor in *C. cf. flagellaris*, as mutants deficient in cercosporin production are compromised in virulence when inoculated into soybean plants (Upchurch et al., 1991). Moreover, the reduced virulence of several *Cercospora* toxin-deficient mutants indicates that cercosporin plays a critical role in many plant diseases (Choquer et al., 2005; Gunasinghe et al., 2016; Staerkel et al., 2013; Weiland et al., 2010). Therefore, *C.*

cf. flagellaris Δ avr4 mutants with reduced cercosporin production were expected to be less virulent on soybean than the wild type *C. cf. flagellaris*. In fact, our detached soybean leaf assay showed that wild type fungus elicited more necrotic and chlorotic lesions compared to those caused by the Δ avr4 mutants. Thus, this result is consistent with those obtained using mutants of other *Cercospora* spp. with altered cercosporin production, showing a positive correlation between fungal virulence and toxin production (Upchurch et al., 1991). Further investigation revealed that reduced cercosporin production was associated with downregulation of Ctb1 and Ctb8 gene expression in Δ avr4 mutants (Figure 3.10). This result agreed with earlier studies showing that expression of Ctb (Cercosporin Toxin Biosynthesis) genes was correlated to toxin production (Chen et al., 2007b).

Apart from the alteration in toxin production, fungal growth was also different between *C. cf. flagellaris* Δ avr4 mutants and the wild type. Comparison of the radial growth measurements *in vitro* demonstrated that mutant mycelia grew more rapidly compared to the wild type. This finding agrees with previous studies showing a negative correlation between fungal growth and cercosporin production (Jenks et al., 1989; Upchurch et al., 1991). However, wild type strain grew more abundantly on the surface and inside of soybean leaves compared to Δ avr4 mutants based on our microscopic assessment (data not shown) and real time PCR quantification (Figure 3.11). 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 (Figure 3.12). Interestingly, cercosporin was not detected from disease lesions caused by mutants based on our HPLC analysis, indicating that *C. cf. flagellaris* is able to cause disease without relying on cercosporin production alone. Therefore, the reduction in disease

development caused by the mutants could be the result of both the altered cercosporin production and a lack of protection against host chitinases without a functional AVR4.

Previous studies showed that AVR4, a small conserved cysteine-rich protein, binds chitin and protects *Cladosporium fulvum* against plant chitinases during infection of tomato and 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 *Cercospora 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 this same function, a chitinase assay was performed using CM agar plates with wells containing 1 unit of chitinase (Sigma C8241). As expected, Δ avr4 mutants were found more sensitive to chitinase than wild type *C. cf. flagellaris* (Figure 3.13).

In addition, a noticeable increase in production of a dark cercosporin-like pigment in mycelium next to wells containing chitinase was observed. This induced biosynthesis of the dark pigment in the presence of chitinase suggests that this compound might function as an inhibitor of chitinase. 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 (Figure 3.14). Therefore, the observed reduced colonization of the Δ avr4 mutants on soybean leaves could be the combined results of lacking a functional AVR4 effector, which can bind chitin and protect fungal mycelium, and the reduced production of cercosporin, which not only can cause peroxidation of membrane lipids leading to membrane breakdown and cell death (Daub and Briggs, 1983b), but also can directly inhibit chitinolytic activity.

Taken together, our studies suggest that this fungal effector protein suppresses host defense during infection of soybean by regulating the expression of cercosporin pathway genes and the toxin production as well as the previously reported function as a chitin binding protein. To the best of our knowledge, this is the first report concerning the roles of an effector homolog in *Cercospora cf. flagellaris* and the first report in linking Avr4 in cercosporin production. Further studies will be needed to understand how Avr4 regulates cercosporin production in *C. cf. flagellaris*.

CHAPTER 4: HOST INDUCED GENE SILENCING TARGETING AVR4 GENE FOR CONTROL OF CERCOSPORA LEAF BLIGHT OF SOYBEAN

4.1. Introduction

RNA interference (RNAi) is an RNA-dependent gene silencing process which affects gene expression in eukaryotic organisms. RNAi is triggered by a double-stranded RNA (dsRNA) which is processed into double stranded small (~20–25 bp nucleotide) interfering RNAs (siRNAs) by an RNase III enzyme called Dicer. Each siRNA is composed of two strands, a sense and an antisense strand (also called guide strand), which is incorporated into a RNA-induced silencing complex (RISC). Complementary mRNA target sequences base-pair with the guide strand resulting in cleavage by the Argonaute protein, thus preventing the translation of the target transcript (Baulcombe, 2004).

One of the most important and well-known functions of plant's siRNA-mediated RNAi is to protect itself against infection by viruses (Lu et al., 2003). This natural mechanism has recently been applied to help determining host gene function in functional genomics (Kamthan et al., 2015), such as virus induced gene silencing (VIGS). VIGS requires the construction of a recombinant virus carrying a fragment of a host target gene that will be down-regulated by the RNAi machinery of the plant host (Robertson, 2004). The viral vector can be delivered into plants by various techniques such as *Agrobacterium*-mediated infiltration, mechanical inoculation of *in-vitro* transcribed RNA, or biolistic delivery of infectious plasmid DNA (Burch-Smith et al., 2004).

One of the VIGS vectors that have been widely used to determine the function of unknown genes in soybean was developed based on the bean pod mottle virus (BPMV). BPMV

was first discovered in common bean and it was later shown to infect different legume species including soybeans (Giesler et al., 2002; Zaumeyer and Thomas, 1948). BPMV is a positive-strand RNA virus of the family Comoviridae and has a bipartite genome composed of two RNA molecules RNA1 (~6 kb) and RNA2 (~3.6 kb) that are encapsulated in separate particles (Giesler et al., 2002). Bean pod mottle virus (BPMV)-VIGS system has been successfully used to test the candidate genes for their involvement in resistance to fungal pathogens in soybeans (Meyer et al., 2009; Pandey et al., 2011) The earlier BPMV based VIGS vectors produced severe virus symptoms on soybean leaves, which can often interfere with phenotypes produced by silencing of target genes due to the pathogenicity component that determines foliar symptom severity is present in the RNA1 molecule (Gu and Ghabrial, 2005). In order to address this limitation, a set of new VIGS vectors based on a mild BPMV isolate (IA-Di1) from Iowa were developed by Zhang et al. (2010).

Besides manipulating host genes to understand their functions, RNAi technology has also been used to target plant pathogens and pests to improve plant disease resistance (Sharma et al., 2013). Eukaryotes that live within or develop intimate contact with a host, such as fungi, are sensitive to RNAi. Several recent studies indicate that small interfering RNAs appear to be able to travel from host to pathogen or *vice versa* and cause gene silencing (Weiberg et al., 2015; Weiberg et al., 2013). This phenomenon is also referred to as cross-kingdom gene silencing (Gardiner et al., 2013; Weiberg and Jin, 2015). Over recent years, this bidirectional cross-kingdom gene silencing has been proposed as a natural mechanism to confer plant protection (Chaloner et al., 2016; Wang et al., 2016; Weiberg et al., 2013). Moreover, several examples of

exchange of small RNAs between host plants and invading pathogens have been described and contributed to better understand plant-pathogen interactions. Recently, this mechanism has been explored to silence genes in plant pathogens by interfering RNAs derived from engineered plant genomes, a strategy referred to as host-induced gene silencing (HIGS).

HIGS relies on the natural RNAi mechanism present in eukaryotes and the unique feature of HIGS is to express an RNAi construct of a pathogen gene in its host plant, which results in suppression of the target gene and consequently reduces pathogen infection or enhances plant resistance. So far, the widely explored targets in recently published HIGS studies have been to suppress the expression of crucial fungal pathogen genes that are required for disease progression in the host. The success of these studies also demonstrates that an *in planta* RNAi approach, such as HIGS may offer a promising strategy to develop crop resistance to different fungal pathogens. The first successes of HIGS against filamentous pathogens were described for *Fusarium verticillioides* and the barley powdery mildew fungus *Blumeria graminis* (Nowara et al., 2010; Tinoco et al., 2010). Over the past years, several studies demonstrated the efficacy of HIGS in suppressing diseases caused by other fungi, such as *Puccinia* spp. (Panwar et al., 2013; Yin et al., 2015; Yin et al., 2011; Zhang et al., 2012b), *Fusarium* spp. (Chen et al., 2016; Cheng et al., 2015; Ghag et al., 2014a; Hu et al., 2015; Koch et al., 2013), *Sclerotinia sclerotiorum* (Andrade et al., 2015), *Rhizoctonia solani* (Zhou et al., 2016), *Verticillium dahliae* (Song and Thomma, 2016), as well as by the oomycete pathogens *Phytophthora infestans* (Jahan et al., 2015; Sanju et al., 2015), and *Bremia lactucae* (Govindarajulu et al., 2015).

Since HIGS was evolved from Virus Induced Gene Silencing (VIGS), many previously developed VIGS vectors, such as the bean pod mottle virus (BPMV) based vectors which have

been widely used to determine the function of unknown genes in soybean (*Glycine max*) (Cooper et al., 2013; Meyer et al., 2009; Pandey et al., 2011; Zhang et al., 2012a), can also be used to silence pathogen genes through HIGS.

Our earlier studies demonstrated that Δ Avr4 disruption mutants produced significantly less cercosporin compared to wild type and also were less virulent on soybeans. In this study, a BPMV based VIGS vector was used to express dsRNA from *C. cf. flagellaris* (previously known as *C. kikuchii*) Avr4 gene in soybean plants to determine whether this novel approach can be used to suppress cercospora leaf blight (CLB) disease by silencing the *C. cf. flagellaris* Avr4 gene through HIGS.

4.2. Material and Methods

4.2.1 Construction of BPMV-derived vectors

The BPMV derived vector (pBPMV-PDS-3R), described in Zhang et al. (2010), was kindly provided by C. Zhang (Figure 4.1A). A 477 bp sequence within Avr4 gene coding region was selected as it showed no homology to the available soybean genome sequences (Figure 4.1B).

The Avr4 fragment was amplified by PCR from cDNA of *C. cf. flagellaris* using the primer pair Avr4HIGS4B F and Avr4HIGS4B R (See Table 4.1 for all primer sequences). These primers contain a BamHI restriction site at the 5'-end and at the 3'-end. The pCRTM2.1- TOPO[®] TA Cloning vector (Invitrogen, Carlsbad, CA) was used to subclone the Avr4 PCR fragment and the product was confirmed by DNA sequencing using vector specific primers T7 and M13.

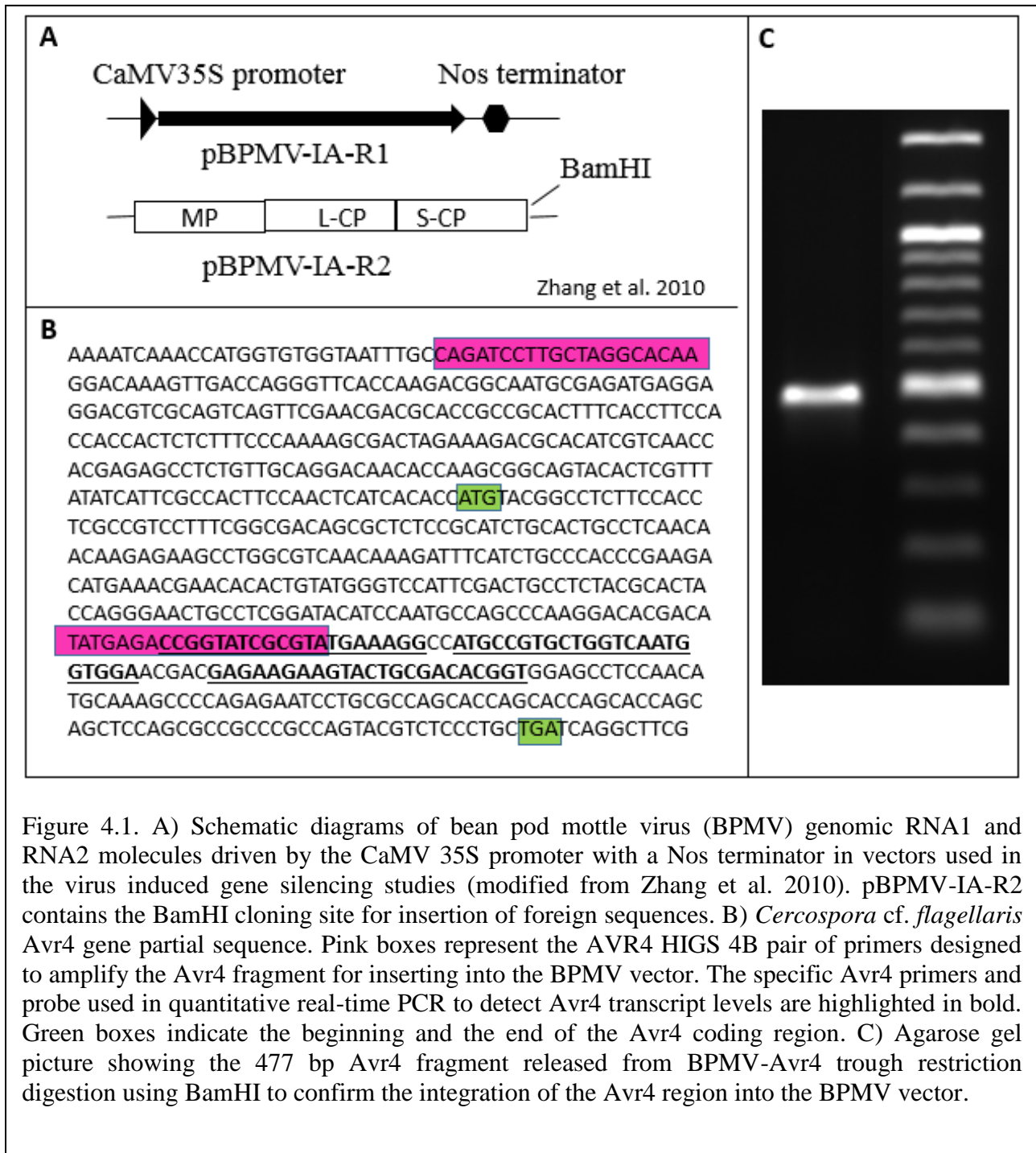


Figure 4.1. A) Schematic diagrams of bean pod mottle virus (BPMV) genomic RNA1 and RNA2 molecules driven by the CaMV 35S promoter with a Nos terminator in vectors used in the virus induced gene silencing studies (modified from Zhang et al. 2010). pBPMV-IA-R2 contains the BamHI cloning site for insertion of foreign sequences. B) *Cercospora cf. flagellaris* Avr4 gene partial sequence. Pink boxes represent the AVR4 HIGS 4B pair of primers designed to amplify the Avr4 fragment for inserting into the BPMV vector. The specific Avr4 primers and probe used in quantitative real-time PCR to detect Avr4 transcript levels are highlighted in bold. Green boxes indicate the beginning and the end of the Avr4 coding region. C) Agarose gel picture showing the 477 bp Avr4 fragment released from BPMV-Avr4 through restriction digestion using BamHI to confirm the integration of the Avr4 region into the BPMV vector.

Table 4.1: List of primers used in Chapter 4.

Primer name	Oligonucleotide sequence (5'→3')
Avr4 HIGS 4B F	CGGATCCTTGCTAGGCACAA
Avr4 HIGS 4B R	TACGGGATCCCGGTCTCATA
Avr4qPCRWT F	CCGGTATCGCGTATGAAAGG
Avr4qPCRWT R	GAGAAGAAGTACTGCGACACGGT
Avr4qPCRWT P	FAM-ATGCCGTGCTGGTCAATGGTGGA-TAMRA
CKCTB6-2F	CACCATGCTAGATGTGACGACA
CKCTB6-2R	GGTCCTGGAGGCAGCCA
CKCTB6-PRB	FAM-CTCGTCGCACAGTCCCGCTTCG-TAMRA
BPMV F2	ACCTTCTCGGTGGAGGAA
BPMV R2	TAATCATCGCAAGACCGGCA
CKBtub F	TCCGGCAACAAGTATGTCCC
CKBtub R	GCCGAAGACGAAGTTGTCTG

The Avr4 fragment was released from TOPO vector by BamHI digestion and subsequently inserted into similarly digested and dephosphorylated pBPMV-PDS-3R (to remove the PDS gene) (Zhang et al., 2010) to generate pBPMV-Avr4. Restriction digestion using BamHI was performed to confirm the release of the insert with the expected size, 477 bp, from pBPMV-Avr4 (Figure 4.1C). The vector pBPMV-PDS-3R (Zhang et al., 2010) was used as a positive control in which silencing of the phytoene desaturase (PDS) gene shows photo-bleaching of soybean leaves, while pBPMV-IA-V2 was used as an empty vector control.

4.2.2 Delivery of BPMV-derived vectors into soybean leaves

Two weeks old soybean plants (cultivar Pioneer 94Y82) were placed in the dark 24 h prior to plant transformation. Using a Biolistic PDS-1000/He system (Bio-Rad Laboratories, Hercules, CA), a DNA-plasmid mix containing pBPMV-IA-R1M (modified RNA1 of BPMV) with pBPMV-IA-V2, pBPMV-PDS-3R, or pBPMV-Avr4, was biolistically introduced into the soybean primary leaves (vegetative cotyledon stage) (See Appendix for complete protocol). Plants were lightly misted with water after bombardment and kept in a moist chamber overnight. Subsequently, plants were maintained in the greenhouse at 20°C with a photoperiod of 16 h. In general, virus symptoms were observed in successfully inoculated plants at three weeks after bombardment.

Soybean leaves showing typical virus symptoms were collected and ground in a mortar with 50 mM potassium phosphate buffer, pH 7.0 (=mock buffer) to make leaf sap for secondary inoculations. Fourteen days old plants were placed in the dark 24 h prior to inoculation, and were mechanically inoculated by rubbing the upper surface of soybean primary leaves (vegetative cotyledon stage) with the leaf sap, using cheesecloth and carborundum (Sigma-Aldrich, St Louis, MO) as an abrasive. Plants were lightly misted with water after inoculation and maintained in the greenhouse at 20°C with a 16 h photoperiod.

4.2.3 *Cercospora cf. flagellaris* inoculation of soybean plants carrying BPMV-derived vectors

Soybean leaves showing typical virus symptoms at 20 dpi, were collected and inoculated with *C. cf. flagellaris*. *C. cf. flagellaris* was grown on PDA plates for 3 weeks and 7 mm mycelial plugs were used to inoculate healthy soybean leaves (No BPMV) and leaves carrying the empty vector, or pBPMV-Avr4 construct. PDA plugs were used to mock inoculate leaves.

Leaves were incubated inside of transparent plastic boxes containing moist paper towels under light. Three boxes containing six leaves each were used for each of the BPMV constructs. The development of disease symptoms was observed 14 days post inoculation (dpi), leaves were photographed and immediately frozen in liquid nitrogen and stored at -80°C. Three boxes containing 6 leaves each were used for each treatment. Thus, leaves from individual boxes were considered as one sample and individual boxes were considered as replicates. The experiment was repeated twice.

4.2.4 Total RNA extraction and quantitative real-time PCR

Leaf tissue was ground in liquid nitrogen, and total RNA was extracted using RNeasy Plant Mini Kit (Qiagen) according to the manufacture instructions. Reverse transcription was conducted with 500 ng of total RNA using Taqman® reverse transcription reagents and guideline (Applied Biosystems, Foster City, CA). Reverse transcription (RT)-PCR was performed with pBPMV IA-V2 vector-specific pair of primers BPMV F2 and BPMV R2 to examine the integrity of the recombinant BPMV RNA2 on the leaves showing viral symptoms. The level of Avr4 expression in soybean leaves was determined by comparative quantitative real-time polymerase chain reaction (qRT-PCR) using *C. cf. flagellaris* β -tubulin specific primers, CKBtub F and CKBtub R, to normalize RNA amounts. Real-time PCR assays were performed using 7.5 μ l 2X TaqMan Universal PCR Master Mix (Applied Biosystems), 1 μ l of 10 μ M each primer (final concentration 666 nM each primer), 0.6 μ l of 10 μ M (final concentration of 400 nM) probe and 1 μ l of cDNA template. An ABI PRISM 7000 Sequence Detection System was used for real-time PCR under standard conditions. The primer pair Avr4qPCRWT F / Avr4qPCRWT R and the gene specific probe Avr4qPCRWT P (Table 4.1) used to amplify Avr4 were designed to anneal outside the region targeted for silencing. Relative Avr4 expression was determined using the 2⁻

$\Delta\Delta C_t$ method ($[Ct (Avr4) - Ct (\beta\text{-tubulin})]_{\text{treatment}} - [Ct (Avr4) - Ct (\beta\text{-tubulin})]_{\text{empty vector}}$) (Pfaffl, 2001).

4.2.5 DNA isolation and quantitative real-time PCR assessment of *C. cf. flagellaris* accumulation

Soybean leaves were ground in liquid nitrogen and DNA was extracted with a GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, St Louis, MO) according to the manufacturer's instructions. Quantitative real-time PCR targeting the *C. cf. flagellaris* Ctb6 gene was performed using primers CKCTB6-2F/CKCTB6-2R and fluorescent probe CKCTB6-PRB in the ABI 7000 sequence detection system (Applied Biosystems) under the standard conditions. Each reaction contained 7.5 μ l 2X TaqMan Universal PCR Master Mix (Applied Biosystems), 1 μ l of 10 μ M each primer (final concentration 666 nM each primer), 0.6 μ l of 10 μ M (final concentration of 400 nM) probe and 1 μ l of 10 ng template DNA. The DNA quantification of *C. cf. flagellaris* in leaf samples was determined as previously described by Chanda et al. (2014).

4.3. Results

4.3.1 Delivery of BPMV vectors into soybeans

In order to investigate whether HIGS can be accomplished against *Cercospora cf. flagellaris* targeting Avr4 gene, we explored a BPMV-based VIGS vector to produce dsRNAs that are targeting the fungus Avr4 transcripts. Particle bombardment was used to deliver the modified BPMV vector into two week old soybean plants. After approximately 20 days, plants inoculated with recombinant pBPMV-IA-V2 mixed with pBPMV-IA-R1M constructs, developed typical BPMV symptoms such as crinkled leaves with a mosaic of light and dark green regions (Figure 4.2A). As expected, photobleaching was observed in positive control plants bombarded with pBPMV-IA-PDS-3R along with pBPMV-IA-R1M (Figure 4.2B).

Considering that the spread of the virus can differ among plants when using biolistic inoculation, a second inoculation was performed by rubbing the upper surface of 2 weeks old soybean leaves with the leaf sap containing the BPMV constructs from the leaves collected from the bombardment experiment in order to obtain plants with consistent virus infection. As expected, more consistent virus symptoms were observed approximately 20 days post inoculation (dpi).

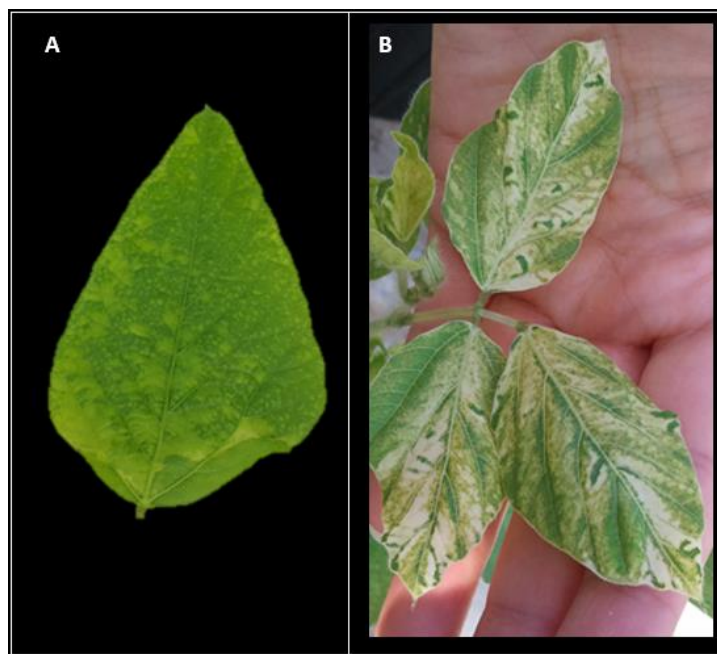


Figure 4.2. A) Soybean leaf inoculated with empty BPMV constructs (pBPMV-IA-V2 mixed to pBPMV-IA-R1M) showing typical viral symptoms, such as crinkled leaves with a mosaic of light and dark green regions as a negative control. B) Soybean leaf inoculated with pBPMV-IA-PDS-3R mixed to pBPMV-IA-R1M showing photo-bleaching as a positive control for successful viral expression. Pictures were taken 20 days after bombardment inoculation.

4.3.2 Inoculation of soybean plants carrying HIGS constructs with *Cercospora cf. flagellaris*

To verify if soybean plants carrying the BPMV-Avr4 construct respond differently to *C. cf. flagellaris* infection, plants carrying the Avr4 gene construct, vector control construct with no fungal genes, and healthy plants were challenged with *C. cf. flagellaris* at 20 days after BPMV treatment. Plants were inspected for CLB disease symptoms two weeks post inoculation. At 14 days post inoculation (dpi), healthy (No BPMV) and vector control plants developed more severe disease symptoms compared to plants carrying the pBPMV-Avr4 construct which showed fewer necrotic and chlorotic lesions (Figure 4.3A). This result demonstrated that successful delivery of BPMV derived VIGS vector carrying the Avr4 sequence, contribute to reduction of CLB disease on soybean leaves.

4.3.3 Expression of Avr4 transcripts and *Cercospora cf. flagellaris* accumulation in soybean leaves carrying the BPMV HIGS vectors

In order to determine whether *C. cf. flagellaris* Avr4 was silenced in plants inoculated with soybean leaf sap containing pBPMV-Avr4 vector, the level of Avr4 expression in virus inoculated plants was compared with empty vector control and healthy plants carrying no BPMV construct. At 14 dpi, we detected a significant lower level of Avr4 transcript in soybean plants carrying the Avr4 gene HIGS construct compared to vector control and healthy plants (Figure 4.3B). To confirm whether the reduced cercospora leaf blight symptoms were positively correlated with reduced fungal growth, quantitative real time PCR analysis was performed using the pair of primers and probe described by Chanda et al. (2014) and DNA samples isolated from *C. cf. flagellaris* inoculated soybean plants carrying HIGS constructs. At 14 dpi, *C. cf. flagellaris* biomass also significantly decreased in BPMV-Avr4 plants compared to empty vector control and healthy plants (Figure 4.3C).

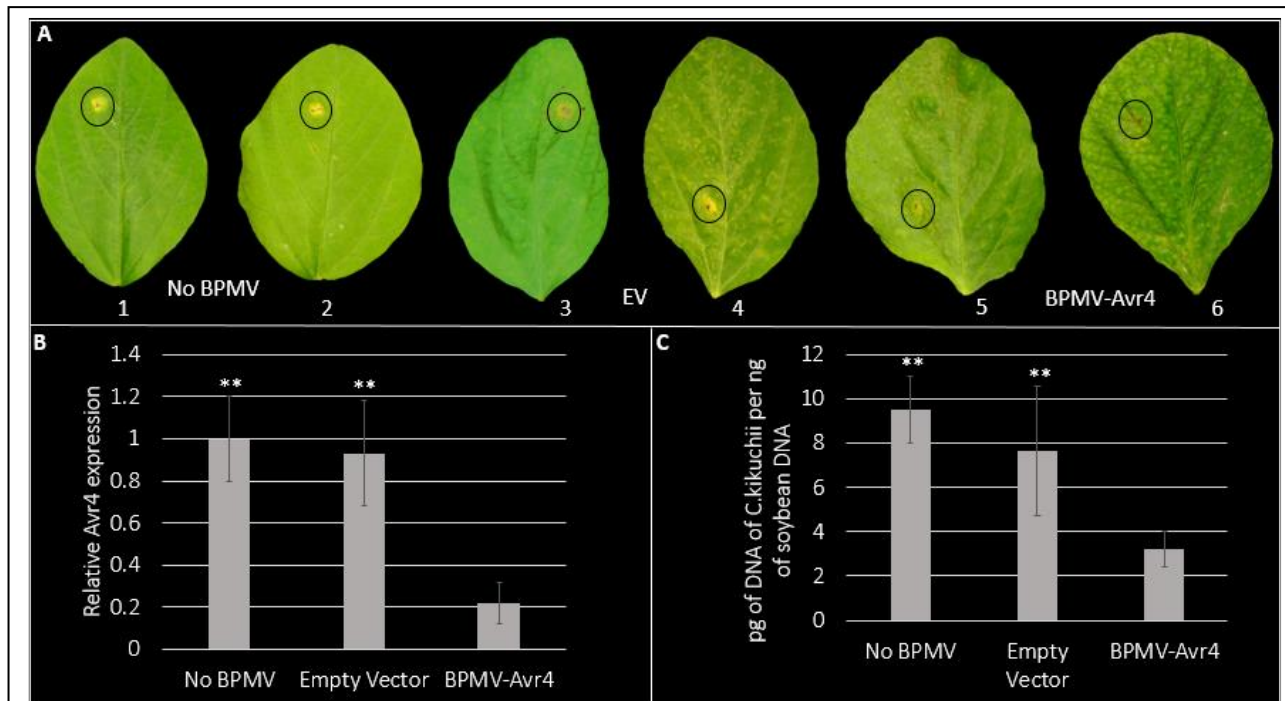


Figure 4.3: A) Symptoms of soybean leaves carrying recombinant HIGS vectors 14 days after *Cercospora cf. flagellaris* inoculation. No BPMV (1 and 2) and empty vector control (3 and 4) leaves showed more necrotic and chlorotic lesions compared to BPMV-Avr4 treated leaves (5 and 6) which showed fewer necrotic lesions. B) Quantitative real-time PCR analysis showing *C. cf. flagellaris* Avr4 transcript levels 14 dpi in soybean leaves carrying recombinant HIGS vectors. The quantification of the relative transcript levels of Avr4 gene was normalized to the control gene β -tubulin and relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. C) Quantitative real-time PCR analysis showing differential accumulation of *C. cf. flagellaris* at 14 dpi in soybeans carrying recombinant HIGS vectors. *C. cf. flagellaris* accumulation on soybean leaves was determined according to Chanda et al. (2014). Data are the means of 18 leaves (3 replicates containing 6 leaves each) from each treatment and error bars indicate standard error of the mean. Asterisks (**) indicate significant difference between control plants (No BPMV and Empty Vector) and the leaves containing the BPMV-Avr4 construct ($P < 0.05$).

To ensure that systemic leaves with viral symptoms still contained the BPMV RNA2 carrying the Avr4 insert, reverse transcription (RT)-PCR was performed with pBPMVIA-V2 vector-specific pair of primers BPMV F2 and BPMV R2 to examine the integrity of the recombinant BPMV RNA2. A product size of 758 bp was obtained from leaves of plants inoculated with BPMV empty vector, corresponding to the size of PCR products between the

two primers in the absence of insert. A larger PCR product of 1.2 Kb was amplified in samples of BPMV-Avr4 inoculated plants, thus, confirming the presence of the 477 bp Avr4 insert within BPMV RNA2 in the soybean leaves.

4.4. Discussion

In our previous study (Chapter 3), Δ avr4 disruption mutants produced significantly less cercosporin compared to wild type and also were less virulent on soybeans. Based on this as well as the importance of Avr4 in host-pathogen interactions from earlier studies, Avr4 gene was selected in the present study as a potential target for silencing through HIGS to determine whether this novel approach can be used to control soybean CLB disease.

In this study, protection of soybean against *C. cf. flagellaris* by HIGS was demonstrated using a transient Avr4 gene silencing in soybeans through a BPMV-based vector. Upon successful inoculation of soybeans with *C. cf. flagellaris*, BPMV constructs carrying Avr4 gene target sequence reduced the expression of Avr4 gene from *C. cf. flagellaris in planta* by 80% in pBPMV-Avr4 plants compared to empty vector control and plants carrying no BPMV construct at 14 dpi (Figure 4.3B). In addition, these HIGS plants carrying the pBPMV-Avr4 construct showed less disease symptoms than control plants carrying the empty vector construct at 14 days after inoculation with *C. cf. flagellaris*. In contrast, healthy and vector control plants developed more necrotic and chlorotic lesions compared to pBPMV-Avr4 plants (Figure 4.3A). In addition, the reduction of CLB symptoms was positively correlated with reduction in fungal growth in soybean plants carrying the pBPMV-Avr4 construct compared to the control plants (Figure 4.3C).

Evidences from the present study and other HIGS studies in plant disease control, reveal that HIGS is not restricted to controlling biotrophic pathogens which have close interactions with host, but can also be used as an important tool to control hemibiotrophic or necrotrophic pathogens (Andrade et al., 2015; Chen et al., 2016; Cheng et al., 2015; Ghag et al., 2014a; Hu et al., 2015; Koch et al., 2013; Nowara et al., 2010; Nunes and Dean, 2012; Panwar et al., 2013; Pliego et al., 2013; Song and Thomma, 2016; Tinoco et al., 2010; Yin et al., 2015; Yin et al., 2011; Zhang et al., 2012b; Zhou et al., 2016). The genes targeted in these studies include not only pathogenicity genes, but also genes encoding biosynthetic enzymes of chitin and ergosterol, or enzymes involved in developmental regulations as well as secondary metabolism of plant pathogens.

Although the present study demonstrated that a virus vector based HIGS approach has the great potential in controlling plant fungal diseases and that suppression of Avr4 significantly reduced the target gene expression in this hemibiotrophic pathogen and resulted in reducing CLB disease symptom development in soybean plants, the virus vector based siRNA delivery system is transient and not inheritable in nature. In fact, the HIGS construct containing plants still show visible CLB symptom. Future efforts may need to focus on developing stable transgenic plants to express the siRNA constructs to achieve the long-lasting suppression effects. In addition, other genes from *C. cf. flagellaris*, such as genes involved in the biosynthesis of cercosporin, a known virulence factor of the pathogen, and combinations of them should be examined to achieve a better suppression of the CLB disease development in soybean.

CHAPTER 5: GENERAL CONCLUSIONS AND PROSPECTS FOR FUTURE RESEARCH

The results presented in this study suggest that AVR4 contributes to the virulence of *C. cf. flagellaris* (previously known as *C. kikuchii*) on soybean through protecting fungal hyphae and regulating cercosporin biosynthesis. Growth of the fungus in the presence of chitinase *in vitro*, showed that chitinase inhibited mycelial growth of Δ avr4 mutants whereas the wild type fungus was not affected by the enzyme. Moreover, growth of Δ avr4 mutants was clearly compromised in infecting soybean leaves suggesting that mutants were more vulnerable to the deleterious effects of soybean host chitinases. Taken together, these results indicate that AVR4 in *C. cf. flagellaris* may have the same biological function as the AVR4 present in *Cladosporium fulvum* in which this effector is a chitin binding protein involved in fungal protection against host chitinolytic enzymes.

Cercosporin had been shown to be an important virulence factor in several *Cercospora* plant pathogens. Cercosporin production was drastically reduced in Δ avr4 mutants compared to the wild type fungus in our *in vitro* and *in planta* assays. However, there is no literature reporting a direct involvement of Avr4 in cercosporin biosynthesis even though the genes and the enzymes involved in cercosporin biosynthesis have been extensively studied. According to the data presented in our study, Avr4 seems to play an important role in regulating the toxin biosynthesis. Therefore, future studies to elucidate how Avr4 is involved in cercosporin production can be extremely helpful to reveal the complete toxin pathway which can contribute to development of new methods to control this soybean pathogen.

In the present study, *C. cf. flagellaris* virulence was also compromised when Avr4 expression was reduced through host induced gene silencing (HIGS). The successful delivery of a BPMV based VIGS vector carrying a selected region of the Avr4 gene into soybean plants, followed by inoculation with *C. cf. flagellaris* resulted in significant reduction of the target gene expression, fungal growth and CLB disease development. Although our study demonstrated that the HIGS approach has the great potential in controlling this important disease of soybeans, future efforts may need to focus on developing a stable transgenic plant targeting fungal Avr4 and other important fungal genes, such as genes involved in the biosynthesis of cercosporin to achieve a better disease suppression. Other than HIGS, direct application of *in vitro* synthesized double strand RNAs targeting Avr4 may also offer a more effective way of managing this disease based on recent success in direct dsRNA applications to control *Fusarium graminearum* and other fungal pathogens in barley (Koch et al., 2016).

Taken together, our results suggest that Avr4 is an important gene in *C. cf. flagellaris* as it is involved in production of cercosporin, one of the most conserved virulence factors among *Cercospora* species. Therefore, studies accessing the roles played by AVR4 in *C. cf. flagellaris* can reveal important aspects related to fungal virulence and also host pathogen interactions which may lead to the development of new approaches to control cercospora leaf blight in soybeans.

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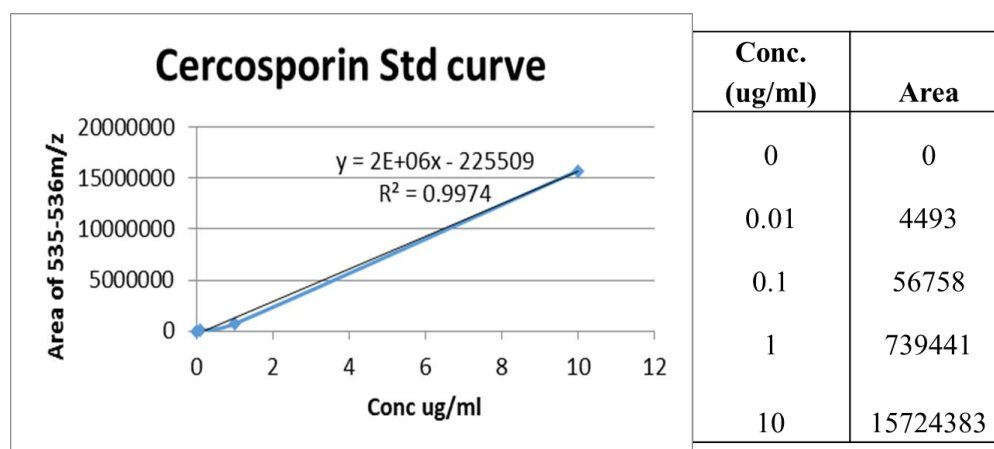
APPENDIX: LIQUID COMPLETE MEDIA, CERCOSPORIN STANDARD CURVE FOR HPLC ANALYSIS, AND DELIVERY OF BPMV CONSTRUCTS TO SOYBEANS

A. Liquid Complete Media (Jenns et al. 1989)

Ingredients per liter:

- 10 g of glucose;
- 1 g of yeast extract;
- 1 g of casein hydrolysate;
- 1 g of $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$;
- 10 ml of a solution containing: 2 g of KH_2PO_4 , 2.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 1.5 g of NaCl in 100 ml of H_2O ,
- Adjust to pH 5.3 in a 500 ml Erlenmeyer flask.

B. Cercosporin standard curve for HPLC analysis



C. Delivery of BPMV constructs to soybeans

C1. Preparation of gold particles

1. Transfer 30 mg gold particles (Bio-Rad Cat # 165-2263) to a 1.5 ml low retention microcentrifuge tube (Phenix Research Products Cat # MAX-815S), and vortex vigorously in 0.5 ml of 100% (v/v) ethanol

2. Incubate at room temperature for 10 min
3. Pellet the gold particle by centrifugation @ 15,000 RPM for 2 min, decant the ethanol
4. Wash three times with 0.5 ml of sterile distilled water (SDW) each time

Resuspend gold particles in 500 μ l of SDW and store as aliquots of 25 μ l in 1.5 ml tubes at -20°C until further use.

C2. Coating of gold particles with Plasmid DNA and particle bombardment

1. To a 25 μ l aliquot of prepared gold particles in 1.5 ml tube, add the following while vigorously vortexing after adding each component (very important to ensure uniform coating)
 - a. 2.5 μ l of pBPMV-IA-RIM DNA (1 μ g/ μ l) – RNA1
 - b. 2.5 μ l of pBPMV-IA-V2 DNA (1 μ g/ μ l) – RNA2 or pBPMV-IA-V2 carrying gene of interest
 - c. 50 μ l of 50% glycerol
 - d. 25 μ l of 2.5 M CaCl₂
 - e. 10 μ l of 0.1 M freshly prepared spermidine (Sigma, Cat # S-0266)
2. Centrifuge at 14,000 RPM and discard the supernatant
3. Wash particles with 70 μ l of 70% isopropanol
4. Wash particles with 70 μ l of 100% isopropanol
5. Resuspend the particles in 25 μ l of 100% isopropanol
6. Wash macrocarrier discs (Bio-Rad, Cat # 165-2335) with 100% isopropanol and air dry
7. Load 6 μ l of prepared gold particles onto center of the macrocarrier and spread uniformly using a yellow pipet tip
8. Load rupture disc (Bio-Rad Cat # 165-2329), prepared macrocarrier, and stopping screen (Bio-Rad Cat # 165-2336) into sample holder

9. Place 14 days old soybean seedlings in the bottom chamber with primary leaves spread out directly under the macrocarrier
10. Press the vacuum switch
11. Press the fire switch and hold it until you hear the shooting sound
12. Remove the plants from chamber, lightly mist with water
13. Transfer the plant to a growth chamber that maintains 22°C and 16 h of light period
14. After 1 week, transfer the plants to big pots in the greenhouse and add fertilizer
15. Observe the development of viral symptoms in 2 weeks on newly developing trifoliates

(This protocol is adapted from Dr. Chunquan Zhang, Alcorn State University)

VITA

Josielle Santos Rezende was born in Uberlândia, Minas Gerais (State), Brazil. She received a Bachelor's Science degree in Agronomy Engineer from Federal University of Uberlândia in 2008. She earned a M.S. in Plant Science in the same university. She joined the Department of Plant Pathology and Crop Physiology at Louisiana State University as a research assistant in 2010. In the spring of 2011, Josielle got admitted into the doctoral program in the same department under supervision of Dr. Zhi Yuan Chen. She anticipates graduating with her Ph.D. degree in May 2017.