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Molecular Approaches to Detect and Control Cercospora kikuchii in Soybeans

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MOLECULAR APPROACHES TO DETECT AND CONTROL *CERCOSPORA KIKUCHII* IN SOYBEANS

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DEDICATION

This work is dedicated to my

Dear Mother, **PADMAVATHI**

Dear Father, **MADHAVA RAO**

Sweet Wife, **MALA**

Little Angel, **HAMSINI**

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LIST OF ABBREVIATIONS

2-DE	2-Dimensional electrophoresis
2-DGE	2-Dimensional gel electrophoresis
2-ME	2-Mercaptoethanol
aa	Amino acid
ABC	ATP-binding cassette
ACN	Acetonitrile
AHCY	Adenosylhomocysteinase
ANOVA	Analysis of variance
BiP	Binding innmunoglobulin protein
bp	Base pair
BPMV	*Bean pod mottle virus*
BSMV	*Barley stripe mosaic virus*
CBB	Coomassie brilliant blue
CDD	Conserved domain database
cDNA	Complementary DNA
cDNA-AFLP	cDNA-amplified fragment length polymorphism
CFP	Cercosporin facilitatior protein
CHAPS	3-[(3- cholamidopropyl) dimethylammonio]-1-propane sulfonate
CLB	Cercospora leaf blight
CM	Complete medium
CMC	Carboxymethylcellulose
CTB1	Cercosporin toxin biosynthesis 1

DAP	Days after planting
DD	Differential display
DIGE	Difference gel electrophoresis
DNA	Deoxyribonucleic acid
dpi	Days post inoculation
dsRNA	Double stranded RNA
DSS	Disease severity scores
DTT	Dithiotheritol
Ecp	Extracellular protein
ESI IT MS/MS	Electrospray ionization ion trap tandem mass spectrometry
EST	Expressed sequence tag
ETI	Effector triggered immunity
FAD	Flavin adenine dinucleotide
FCR	Fusarium crown rot
FRET	Fluorescence resonance energy transfer
GUS	β-glucuronidase
HIGS	Host-induced gene silencing
HNR	Hydroxynaphthalene reductase
HYG	Hygromycin
IAA	Iodoacetamide
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
ITS	Internal transcribed spacer

kb	Kilo base
kDa	Kilo dalton
LC-MS/MS	Liquid chromatography tandem mass spectrometry
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MAPKKK	Mitogen activated protein kinase kinase kinase
MFS	Membrane facilitator superfamily
miRNA	Micro RNA
MM	Minimal medium
MP-PCR	Microsatellite-primed PCR
MS	Mass spectrometry
MW	Molecular weight
MWCO	Molecular weight cut off
NCBI	National Center for Biotechnology Information
NIP	Necrosis inducting protein
NL	Nonlinear
NMR	Nuclear magnetic resonance
nt	Nucleotide
NTC	No-template control
ORF	Open reading frame
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
p*I*	Isoelectric point

PKS	Polyketide synthase
PSS	Purple seed stain
PST	*Puccinia striiformis* f. sp. *tritici*
PTGS	Post-transcriptional gene silencing
PTM	Post-translational modification
qPCR	Quantitative polymerase chain reaction
Q-TOF	Quadrupole time-of-flight
RAPD	Random amplified polymorphic DNA
RdRP	RNA dependent RNA polymerase
REMI	Restriction enzyme-mediated integration
RH	Relative humidity
RNA	Ribonucleic acid
RNAi	RNA interference
RNA-Seq	RNA sequencing
rRNA	Ribosomal RNA
RT	Room temperature
RT-PCR	Reverse transcription-polymerase chain reaction
SAGE	Serial analysis of gene expression
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	Short interfering RNA
SIX	Secreted in xylem
sRNA	Small RNA

SSH	Suppression subtractive hybridization
SSR	Simple sequence repeat
TCA	Trichloroacetic acid
TCW	Tomato cell walls
TFA	Trifluoroacetic acid
TM	Transmembrane
TSS	Transcriptional start site
TTSS	Type three secretion system
VCG	Vegetative compatibility group
VIGS	Virus induced gene silencing
WT	Wild type

ABSTRACT

Cercospora leaf blight (CLB) caused by *Cercospora kikuchii*, has become a troublesome disease in the southern United States. *C. kikuchii* produces a non-hostspecific phytotoxin and a pathogenicity factor known as cercosporin during infection of soybean. A quantitative real-time PCR assay was developed for detection and quantification of *C. kikuchii*. The sensitivity of detection is 1 pg of genomic DNA. The assay detected the presence of *C. kikuchii* in soybean leaves long before the appearance of disease symptoms. *C. kikuchii* DNA levels in soybean leaves increased slowly during early soybean development, followed by a quick increase at late reproductive stages. Results from three year field studies of soybean plants with various fungicide treatments showed that multiple fungicide applications beginning from late vegetative stages until late reproductive stages can reduce *C. kikuchii* growth and CLB symptom development. However, different fungicides vary in their effectiveness.

In order to identify genes involved in cercosporin biosynthesis, proteins were isolated from *C. kikuchii* and compared between cultures grown under light (promotes toxin production up to 6 fold) and dark conditions through proteomics. Six proteins were up-regulated and two were down-regulated in *C. kikuchii* grown under light. Two of the up-regulated proteins [hydroxynaphthalene reductase (*HNR*) and adenosylhomocysteinase (*AHCY*)] were further studied through gene disruption. The resulting mutants showed reduced cercosporin production *in vitro* and virulence on soybean leaves.

C. kikuchii secreted proteins from culture were also examined to identify proteins involved in the infection of soybean. Two of them showed high homology to glucan beta 1,3-glucosidase and EAP30 family proteins and identity of several proteins remains unknown. The function of these proteins in infecting soybean remains to be determined.

Two small portions of *AHCY* gene also were inserted into a *Bean Pod Mottle Virus* (BPMV) derived vectors and introduced into soybean to explore the possibility of using host-induced gene silencing (HIGS) in controlling *C. kikuchii* infection of soybean, The target gene expression was reduced by 3.5 to 6.6-fold, and *C. kikuchii* growth was reduced by 16 to18-fold in the HIGS treated soybean compared to vector control plants, indicating a possible new approach to control CLB in soybean.

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CHAPTER: 1. INTRODUCTION

1.1 Justification

Soybean is the second among economic crops grown in the United States with annual production of 261 million tons (FAOSTAT data, 2010). *Cercospora kikuchii* (T. Matsumoto & Tomoy.) M. W. Gardner causes purple seed stain (PSS) and cercospora leaf blight (CLB) in soybeans. In 2006, the estimated yield loss caused by *C. kikuchii* in 16 southern states was estimated to be 2.14 million bushels, out of which Louisiana accounts for 0.43 million bushels (Southern Soybean Disease Workers report, 2006). Purple seed stain was first reported in Korea in 1921 (Suzuki 1921) and later observed in the US in 1924 (Gardner 1926). PSS is now distributed worldwide. Typical symptoms of CLB are observed at the beginning of seed filling (R5) until the end of seed filling (R6) and maturity (R7) (Walters et al. 1980). Varying degrees of resistance to CLB and PSS have been observed in different soybean cultivars, which are highly dependent on favorable weather conditions for disease development, and there is no strong correlation between incidence of CLB and PSS (Orth and Schuh 1994). There has been an increase in number of soybean cultivars susceptible to CLB in field. In 2002, 59 out of 62 cultivars were susceptible to CLB in standardized cultivar trials (Schneider et al. 2003), whereas in 2005, all 285 entries were susceptible (R. W. Schneider, unpublished).

Currently, fungicide application is the only solution to manage CLB under field conditions as most of the soybean cultivars are susceptible to this disease. Different fungicides were evaluated (Schneider and Whitam 2002, Schneider et al. 2005) to manage CLB but none of them were effective when applied alone. When two different fungicides were applied together during reproductive stage, they were slightly effective. Fungicides like pyraclostrobin were only effective when applied more than once during late vegetative stages (Chanda et al. 2009;

Robertson et al. 2011), which is not economically feasible for soybean growers. Earlier studies found that *C. kikuchii* and many other fungi often cause latent infections in soybeans (Cerkauskas and Sinclair 1980; Hartman et al. 1999; Orth and Schuh 1992; Sinclair 1991). Traditional methods involving bioassays, histological and serological assays to detect latent infections in soybeans are often time consuming and not sensitive. Without knowing when infection occurs, producers do not know when to initiate fungicide applications.

Conventional polymerase chain reaction (PCR) has made it possible to detect plant pathogens based on primers designed for a specific part of their genome sequence. However, conventional PCR involves separation of reaction products on an agarose gel and is not suitable for automation when there is a need to screen a large number of samples. The development of high throughput real-time PCR technology, which uses primers and a fluorogenic probe to amplify a specific target sequence, allows monitoring the PCR amplification process in real time and the quantification of target sequence by measuring fluorescence signal without the need for post PCR gel analysis. After the first successful application of real-time PCR for detecting *Phytophthora* strains in their host plants (Böhm et al. 1999), it has been successfully employed in detecting various plant pathogens including fungi (Li et al. 2008; Oliver et al. 2008; Pico et al. 2008), bacteria (De Bellis et al. 2007; Vandroemme et al. 2008), and viruses (Kokkinos and Clark 2006; Stewart et al. 2007). It also has been used to quantify growth of pathogens based on quantification of pathogen DNA (Guo et al. 2006; Qi and Yang 2002). Therefore, there is a strong need to develop an assay for detection and quantification of *C. kikuchii* using real-time PCR primers and probes.

C. kikuchii produces a non-hostspecific phytotoxin known as cercosporin (Kuyama and Tamura 1957). The biosynthesis of cercosporin is induced by light, and cercosporin itself is

photo-activated by blue-wavelength light. Light-activated cercosporin catalyzes the production of singlet oxygen and superoxide ions, which in turn can cause membrane lipid peroxidation resulting in host cell damage (Daub 1987). Cercosporin has been shown to be the crucial pathogenicity factor for *C. kikuchii* (soybean), *C. nicotianae* (tobacco), and *C. zeae-maydis* (maize). Mutants lacking the ability to produce cercosporin did not cause lesions or produced only few lesions when inoculated on respective host plants (Choquer et al. 2005; Shim and Dunkle 2003; Upchurch et al. 1991).

The chemical characteristics of cercosporin are well established, but the biosynthetic pathway in *C. kikuchii* remains uncharacterized. Ehrenshaft and Upchurch (1991) successfully isolated six light enhanced cDNAs of *C. kikuchii* by subtracting the cDNA library of *C. kikuchii* grown under dark from that grown under light. The transcript accumulation of these cDNAs was positively correlated with light, suggesting the role of light in cercosporin production in addition to media composition in some isolates. There are several studies that have shown that the level of gene expression does not necessarily correlate with the protein levels in a cell (Gygi et al. 1999). One approach to identify additional cercosporin biosynthesis pathway genes is to use proteomics. Proteomics is a study of all proteins, including their relative abundance, distribution, post-translational modification, functions and interactions with other macromolecules in a given cell or tissue. The identification of genes involved in the cercosporin biosynthetic pathway may be essential for developing resistance in soybean cultivars and other crops susceptible to diseases caused by cercosporin-producing species of this fungal genus by using modern strategies like host-induced gene silencing (Helber et al. 2011; Nowara et al. 2010; Stärkel 2011; Yin et al. 2011). This approach may also allow the identification of the genes used by *C. kikuchii* to confer self-resistance to cercosporin. Genetic engineering can be used to incorporate these toxin

resistance genes in crops to protect from the damage caused by the phytotoxin cercosporin. We can analyze proteins isolated from *C. kikuchii* using 2-D gel electrophoresis (2-DGE) and compare the resulting protein profile differences between different conditions like cercosporin production favorable (light) and non-favorable conditions (dark) to identify possible proteins involved in cercosporin biosynthesis and/or protein involved in resistance to cercosporin. In addition to proteins involved in cercosporin biosynthesis that can be used to manipulate/enhance host resistance to this pathogen, studying fungal secreted proteins, which often contain virulence factors/effectors to either suppress host defense response or breach host defense mechanism/breach host physical barrier to gain access to host nutrients, is another approach to understand host-pathogen interaction and to enhance soybean resistance to *C. kikuchii* infection in soybean.

1.2 Objectives

1) To develop a real-time PCR assay specific for detection of *Cercospora kikuchii*
2) To quantify *Cercospora kikuchii* directly from naturally infected soybean tissues
3) To compare DNA content of *Cercospora kikuchii* in infected plants subjected to different fungicide treatments to dertermine the efficacy of various treatments
4) To identify differentially expressed proteins from light and dark-grown *Cercospora kikuchii* through proteomics approach
5) To characterize roles of light-induced *HNR* and *AHCY* genes identified through proteomics approach in *Cercospora kikuchii* infection of soybean.
6) To identify secreted proteins from *Cercospora kikuchii*
7) To evaluate whether host-induced gene silencing strategy can be used to control CLB using *AHCY* gene

CHAPTER 2: REVIEW OF LITERATURE

2.1 Soybean

Soybean, *Glycine max* (L.) Merr., was cited as one of the five sacred grains in China as early as 2838 B.C. Soybean was introduced into the United States in 1765 when soybean seed from China was planted by a colonist in the British colony of Georgia (Hartman et al. 1999). In 2010, soybeans were planted on 102 million ha worldwide with a production of 261 million metric tons (FAOSTAT, 2010). Soybean is the second most important crop in the United States next to corn, and is planted on 31 million ha with a total production of 91 million tons in 2010. The top five countries that produced more than 94% of the world soybeans include the United States (35%), Brazil (26%), Argentina (20%), China (9.5%), and India (3.7%) (FAOSTAT, 2010). Soybean seeds are rich in oil (20%) and protein (40%). Soybean meal is mainly used as animal feed and a small proportion is used for human consumption. Soybean oil is mainly used for human consumption (83%) and also for making adhesives, coatings and printing inks, lubricants, plastics and specialty products (United Soybean Board, http://www.unitedsoybean.org/).

2.2 Soybean Growth and Development

Soybeans have two growth habits, determinate and indeterminate growth. The main distinguishing feature is that the indeterminate soybeans continue main stem growth indefinitely after first flowering, whereas determinate soybeans terminate main stem growth shortly after first flowering. Indeterminate varieties generally are classified as early maturing soybeans (maturity group IV or lower), whereas determinate soybeans are generally in the late maturing groups (maturity groups V through VIII) (Louisiana Soybean, 1996, Louisiana Cooperative Extension Service publication). The maturity classification of soybeans is based on the days from emergence to maturity for a specific maturity group in the area of adaptation and when planted at

the optimal planting date. Traditionally late maturing determinate soybeans have been grown in Louisiana and the rest of the southeastern U.S., and early maturing indeterminate varieties are grown in midwestern and northeastern U.S.

Soybean is a quantitative short day plant, which means day-length must be at a critical level or less for rapid profuse flowering to occur. Soybean cultivars require a specific length of dark period to flower (Hartwig 1973), and based on their response to photoperiod, they are divided into 13 maturity groups designated 000, 00, 0 and I to X. Those cultivars with the shortest dark period requirements (group 000) are adapted to highest latitudes, whereas groups IX and X are used primarily in semitropical or tropical production areas. Most cultivars within maturity groups are adapted for full-season growth in a band of latitude no wider than 160 to 240 km (Hartman et al. 1999).

The lifecycle of soybean is divided into two general categories, vegetative period and the reproductive period. The vegetative period extends from emergence until first flowering, whereas the reproductive period lasts from first flowering until maturity. Fehr et al. (1971) developed a well-defined system for describing different stages of soybean development. Vegetative stages are named for the appearance of leaves, and begin with the V1 stage until the beginning of the reproductive stage. Reproductive stages are defined by the letter R. First flowering is R1. Full flowering, pod initiation, pod elongation, the start of seed filling and the end of seed filling are designated as R2, R3, R4, R5, R6, and R7, respectively.

2.3 Yield Loss Caused by *Cercospora kikuchii*

Soybean production can be affected by a variety of pathogens, including fungi, bacteria, viruses and nematodes resulting in significant yield losses. In 2005, 6.9 million tons (9%) of soybeans were lost because of different diseases in the U.S. (Wrather and Kenning 2006) out of

77.3 million tons total production. Among disease caused by fungi, Cercospora leaf blight (CLB) and purple seed stain (PSS) diseases are caused by *Cercospora kikuchii* (T. Matsumoto & Tomoy.) M. W. Gardner (Matsumoto and Tomoyasu 1925; Suzuki 1921; Walters 1980). In 2006, the estimated yield loss caused by *C. kikuchii* in 16 southern states was estimated to be 2.14 million bushels, out of which Louisiana accounts for 0.43 million bushels (Southern Soybean Disease Workers report, 2006).

2.4 *Cercospora kikuchii*

2.4.1 *Cercospora kikuchii* and Latent Infection of Soybeans

Cercospora kikuchii growth on artificial media is uniformly dense, with deep folds radiating from the center. Colonies are white at the edges and light grayish olive toward the centers (Hartman et al. 1999). The medium beneath the colony varies in color but is often dark purple to red in color (Hartman et al. 1999; Murakishi 1951; Roy 1982). Condiophores form in fascicles, and they are medium dark brown, multiseptate and geniculate, unbranched and 45-220 x 4-6 μm (Fig. 1.1A and B). Conidia are hyaline, acicular, indistinctly multiseptate (2-49), straight to curved, base truncate with thickened hilum (Fig. 1.1B) and 50-375 x 2.5-5 μm (Chupp 1954; Hartman et al. 1999). Young hyphae in culture are hyaline, septate, 2-4 μm thick, whereas old hyphae are pale brown, 3-5 μm thick, and closely septate (Hartman et al. 1999). *C. kikuchii* sporulates profusely under high humidity and temperatures of 23-27°C on infected plant tissues and excised seed coats. But, for sporulation on artificial media, addition of senescent soybean leaf powder and incubation under light (Chen 1979; El-Gholl et al. 1981; Vathakos and Walters 1979; Yeh and Sinclair 1980) are essential requirements. *C. kikuchii* can survive on infected seeds and surface debris in the field for extended periods (Jones 1968; Kilpatrick 1956). Cai and Schneider (2005) analyzed the vegetative compatibility groups (VCGs) in *C. kikuchii* and found

that some VCGs are specific to isolates from either leaf or seed. However, randomly amplified DNA polymorphism (RAPD) and microsatellite-primed PCR (MP-PCR) based clustering of these isolates was different from VCG grouping, indicating that VCG is not an indicator of evolutionary lineage and could be possible due to cryptic sexual reproduction (Cai and Schneider 2008).

Purple stained seeds and crop debris on the soil surface may serve as primary inoculum during the growing season (Jones 1968; Kilpatrick 1956; Orth and Schuh 1994). Orth and Schuh (1992) observed latent infection of *C. kikuchii* resulting from active penetration of the host epidermal cell wall followed by subsequent colonization of one to a few cells during soybean growth stage V2-R4. They based their conclusions on a procedure in which they either desiccated asymptomatic leaves for 48 hours or dipped the leaves in 11.64% paraquat solution. Latent infecting hyphae resumed growth and sporulation during the senescent phase, and these conidia may serve as secondary inoculum for sustaining an epidemic. Spore trapping experiments revealed two peaks in aerial spore concentration, one at the beginning of the growing season and the other at the beginning of the seed set. The second sporulation peak, in the absence of visible symptoms or crop residues from the previous season, resulted from latent infections on leaves (Hartman et al. 1999).

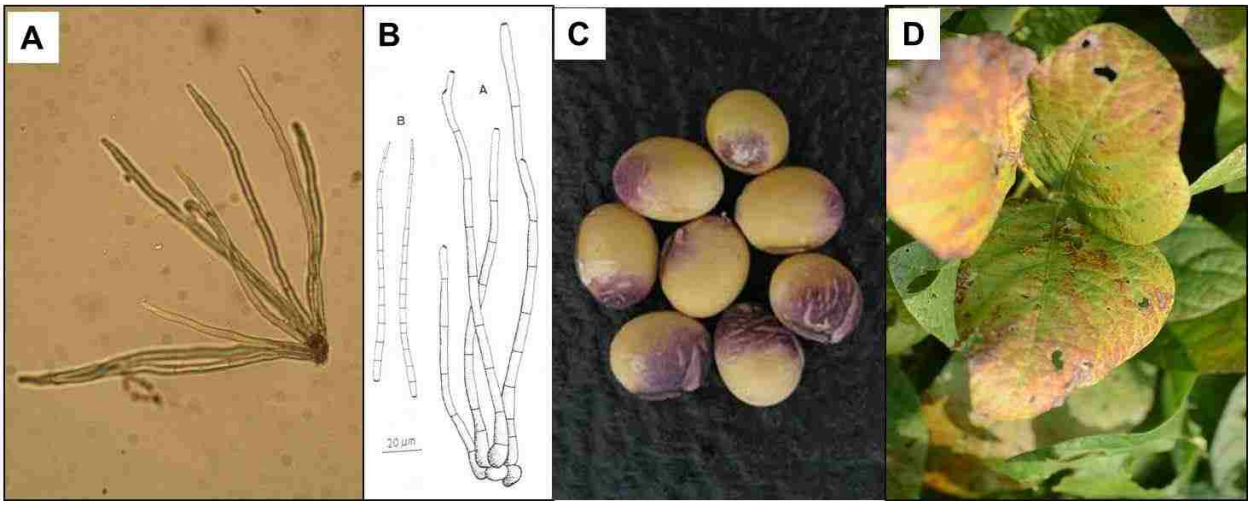


Figure 1.1. *Cercospora kikuchii* characteristics and symptoms on soybean. **A**, *C. kikuchii* conidiophores developed on a soybean leaf showing typical CLB symptoms after incubating in a moist chamber for 48 hours. **B**, Fascicle of conidiophores (A). Conidia (B), Original in: Hsieh, W.H. & Goh, T.K. 1990, Cercospora and similar fungi from Taiwan. Published 1990 by Maw Chang Book Co. in Taipei, Taiwan. Image source: http://www.mycobank.org/MycoTaxo.aspx?Link=T&Rec=252873. **C**, Typical purple seed stain (PSS) symptoms on soybean seeds. (Image source: http://www.omafra.gov.on.ca/english/crops/pub811/14soybean.htm). **D**, Typical cercospora leaf blight (CLB) symptoms on soybean leaves. (Image source: Dr. Zhi-Yuan Chen).

2.4.2 Purple Seed Stain (PSS)

Purple seed stain was first reported in Korea (Suzuki 1921) and later observed in the U.S. (Gardner 1926) and Japan (Kikuchi 1924) in 1924. PSS is now a worldwide disease of soybean. PSS symptoms are characterized by irregular blotches varying from light pink to dark purple and ranging from a tiny spot to the entire area of the seed coat (Murakishi 1951) (Fig. 1.1C) PSS does not affect soybean yield but reduces the quality of soybean seeds. Purple stained seeds have poor germination and produce weak seedlings (Lehman 1950; Murakishi 1951; Pathan and Sinclair 1989; Wilcox and Abney 1973). *Cercospora kikuchii* causes symptoms on hypocotyls, leaves, stems, petioles as well as on pods and seeds (Jones 1968; Kilpatrick 1956; Lehman 1950; Matsumoto and Tomoyasu 1925; Murakishi 1951).

2.4.3 Cercospora Leaf Blight (CLB)

Typical symptoms of CLB are observed at the beginning of seed filling (R5) until the end of seed filling (R6) and maturity (R7). Reddish purple, angular-to-irregular lesions occur on upper leaves exposed to sunlight (Fig. 1.1D). Numerous infections cause rapid chlorosis and necrosis of leaf tissue, resulting in defoliation. The most obvious symptom is the premature blighting of the younger, upper leaves over large areas, even entire fields (Walters et al. 1980). CLB is an economically important disease and can cause substantial yield loss in many countries (Wrather et al. 1997, 2001). In Louisiana, CLB became more severe beginning in 1999 (Moore and Walcott 2000). Originally CLB was limited to the southern United States, but the disease has been slowly moving as far north as Iowa and Ohio in the past few years. Currently, CLB is the top soybean disease in Louisiana causing great concern to soybean growers in the state (Raymond Schneider, personal communication).

2.4.4 Resistance to PSS and CLB

Varying degrees of resistance to leaf blight and purple seed stain have been observed in different soybean cultivars. However, there is no strong correlation between incidence of CLB and PSS and both diseases are highly dependent on favorable weather conditions for development (Orth and Schuh1994). Two studies (Ploper et al. 1992; Roy and Abney 1976) indicated that soybean line PI 80837 is resistant to PSS. Using a F_2 population derived from a cross between cultivars Agripro 350 (AP 350) and PI 80837 and simple sequence repeat (SSR) markers, Jackson et al. (2006, 2008) showed that resistance to PSS in PI 80837 was controlled by a single dominant gene, *Rpss1*, and mapped between Sat_308 (6.6 cM) and Satt594 (11.6 cM) on soybean molecular linkage group G. Walters (1980) screened 10 soybean cultivars for resistance to *C. kikuchii*. Based on greenhouse inoculations, he reported that all cultivars were susceptible

to leaf blight, but cultivars Davis and Lee74 were moderately susceptible, and cultivar Tracy was slightly susceptible. There has been an increase in the number of soybean cultivars susceptible to CLB in the field. In 2002, 59 out of 62 cultivars were susceptible to CLB in standardized cultivar trials (Schneider et al. 2003), whereas in 2005, all 285 entries were susceptible (Raymond Schneider, personal communication). This forced soybean growers in Louisiana to shift to plant early maturing cultivars in part to avoid CLB and other late-season diseases (Moore and Bouquet 2008). Cai et al. (2009) screened 11 varieties under greenhouse and field conditions for resistance to CLB and found only 2 varieties, AG5701 and TV59R85, with a moderate level of resistance to CLB.

2.4.5 Management of Cercospora Leaf Blight

Currently, fungicide application is the only solution to manage CLB under field conditions as most of the soybean cultivars are susceptible to this disease. Different fungicides were evaluated (Schneider and Whitam 2002; Schneider et al. 2005) to manage CLB but none of them were effective when applied alone. However, when two different fungicides were applied together during reproductive stages, they were slightly effective. Fungicides like pyraclostrobin were only effective when applied more than once during late vegetative stages (Chanda et al. 2009, Robertson et al. 2011) but multiple applications are not economically feasible option for soybean growers.

As the symptoms of CLB are not visible until late reproductive stages (R5 and later), it is very difficult to know when the initial infection occurred. The successful use of fungicide application strategy requires early detection of *C. kikuchii* in soybean tissues. Earlier studies found that *C. kikuchii* and many other fungi often cause latent infections in soybeans (Hartman et al. 1999; Orth and Schuh 1992; Sinclair 1991). As a result disease symptoms, such as chlorosis

and necrosis, only become visible long after initial infection and usually at late growth stages when plants are under stress or there is a sudden change in nutritional or environmental conditions (Cerkauskas and Sinclair 1980; Hartman et al. 1999; Orth and Schuh 1994). Fungicide applications at these late growth stages are usually ineffective (Robertson et al. 2011).

2.5 Cercosporin

2.5.1 Cercosporin and Mode of Action

Kuyama and Tamura (1957) isolated a deep red pigment from dried mycelia of *C. kikuchii*. Further study showed that the pigment can be dissolved in aqueous alkali and in reduced state, had a bright yellow color with intense green fluorescence. Because of the presence of a chromophoric system in the pigment that differed remarkably from pigments isolated from other fungi and lichens at that time, they named this toxin as cercosporin. Kuyama (1962) described the nature of aromatic ring of cercosporin. The chemical structure of cercosporin (Fig. 1.2) was elucidated independently by Lousberg et al. (1971) and Yamazaki and Ogawa (1972) to be 1,12-bis(2-hydroxypropyl)-2,11-dimethoxy-6,7-methylenedioxy-4,9-dihydroxyperylene-3,10-quinone ($C_{29}H_{26}O_{10}$). Yamazaki et al. (1975) demonstrated the requirement for both light and oxygen and the photodynamic properties of cercosporin on mice and bacteria. Similar effects also were observed with suspension-cultures of tobacco cells (Daub 1982) and plant tissues (Macri and Vianello 1979), with a linear relationship between cell death and light intensity. The action spectrum of the cell killing response agreed with the absorption spectrum of cercosporin.

Cercosporin is a photosensitizer that absorbs light energy and is converted to an energetically activated triplet state (^3S). ^3S reacts by an electron transfer reaction (type I reaction) through a reducing substrate (R) to generate a reduced sensitizer ($S^{-\cdot}$), which in turn reacts with molecular oxygen to generate superoxide ions ($O_2^{-\cdot}$). Alternatively, ^3S may react directly with

oxygen by an energy transfer process to generate singlet oxygen ($^1O_2$) (type II reaction). Evidence suggests that $^1O_2$ formation is the major mechanism by which cercosporin exerts its toxicity (reviewed in Daub and Ehrenshaft 1997). The production of $^1O_2$ from cercosporin *in*

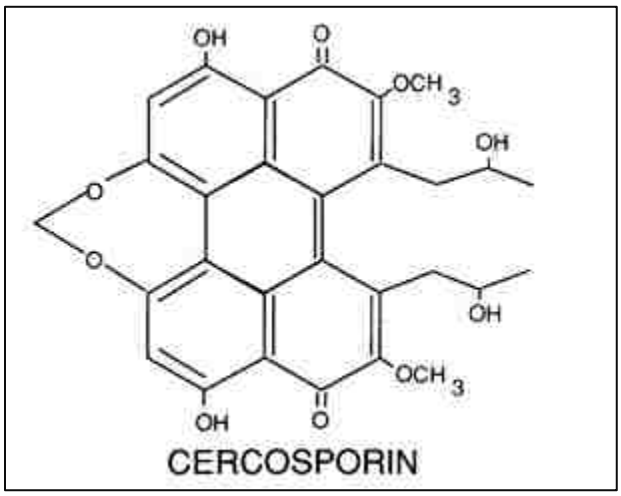


Figure 1.2. Chemical structure of cercosporin. Source: http://www.apsnet.org/publications/apsnetfeatures/Pages/Cercosporin.aspx

vitro has been measured directly (phosphorescence at 1270 nm) and also by chemical reactions (Daub and Hangarter 1983; Dobrowolsky and Foote 1983; Hartman et al. 1988; Leisman and Daub 1992). Cercosporin with photosensitizing activity can cause peroxidation of membrane lipids, leading to membrane breakdown and cell death (Daub and Briggs 1983). Membrane damage may allow leakage of nutrients thus allowing growth and sporulation of intracellularly growing fungal hyphae.

2.5.2 Role of Cercosporin in Plant Diseases

Cercosporin plays a critical role in disease development by *Cercospora* species that synthesize this toxin. Light intensity and day length were strongly correlated with disease severity caused by *Cercospora* species in coffee (reviewed in Daub and Ehrenshaft 1997), banana (Calpouzos and Stalknecht 1966), and sugar beet (Calpouzos and Stalknecht 1967).

Treatment of sugar beet leaves with pure cercosporin reproduced the necrotic symptoms that are both consistent with the known mode of action of cercosporin and similar to the disease symptoms caused by *C. beticola* (Steinkamp et al. 1979, 1981). In addition, cercosporin has been isolated from necrotic lesions of several infected plant hosts (Fajola 1978; Kuyama and Tamura 1957; Venkataramani 1967). Cercosporin has been shown to be the crucial pathogenicity factor for *C. kikuchii* (soybean), *C. nicotianae* (tobacco), and *C. zeae-maydis* (maize). *C. kikuchii* mutants disrupted for the *CFP* transporter gene had reduced ability to produce cercosporin and reduced virulence on soybeans and increased sensitivity to exogenously applied cercosporin (Upchurch et al. 1991). *C. zeae-maydis* mutants disrupted for *CZK3* MAP kinase kinase kinase (MAPKKK) have lost capability to produce cercosporin, and conidia and produced only chlorotic lesions compared to necrotic lesions that are produced by the wild type (Shim and Dunkle 2003). Choquer et al. (2005) disrupted the *CTB1* gene coding for polyketide synthase in *C. nicotianae* and mutants for *CTB1* gene did not produce cercosporin and produced fewer lesions but not necrotic lesions that are typical of wild type *C. nicotianae*.

2.5.3 Cercosporin Biosynthesis

Once the chemical structure and photodynamic characteristics of cercosporin were understood, research focused on understanding cercosporin biosynthesis. Japanese researchers (Okubo et al. 1975; Yamazaki and Ogawa 1972) conducted substrate feeding experiments in which *C. kikuchii* was incubated in the presence of ^{14}C and ^{13}C incorporated acetate and formate compounds. Mass spectrometry (MS) and nuclear magnetic resonance (NMR) data indicated that cercosporin produced from these cultures was synthesized by condensation of acetate and malonate via a polyketide pathway (Fig. 1.3). Ehrenshaft and Upchurch (1991), using subtractive hybridization, found several mRNA transcripts that are accumulated at higher levels in *C.*

kikuchii that was grown in light as compared to dark. Rollins et al. (1993) performed two-dimensional gel electrophoresis (2-DGE) of both extracted proteins and *in vitro* translation products of wild type *C. kikuchii* and found polypeptides and poly (A)+ RNAs that were positively regulated by light, but they did not identify any related proteins. One cDNA, LE6 from Ehrenshaft and Upchurch (1991), was sequenced and identified as cercosporin facilitator protein (*CFP*). CFP has 14 transmembrane (TM) domains with high homology to membrane facilitator superfamily (MFS) drug resistance transporter proteins (Callahan et al. 1999). *C. kikuchii* mutants disrupted for *CFP* produced very low levels of cercosporin, were sensitive to exogenously applied cercosporin and also exhibited greatly reduced virulence to soybeans. Shim and Dunkle (2002) constructed a suppression subtractive hybridization (SSH) library of *C. zeae-maydis* by subtracting poly (A)+ RNA isolated from cercosporin-suppressing medium from poly (A)+ RNA isolated from cercosporin- inducing medium and identified nearly 197 cDNAs. Based on the predicted functions of the proteins encoded by these cDNAs, the genes were grouped into nine categories. Northern blot analysis of seven selected clones showed high expression under cercosporin-inducing conditions and genes were predicted to be involved in fatty acid metabolism (fatty acid synthase, oleate Δ-12 desaturase, and linolate diol synthase), secondary metabolism (cytochrome P450 oxidoreductase, cytochrome P450 monooxygenase, dihydrogeodin phenol oxidase), and coproporphyrinogen oxidase. However, none of these genes was characterized by generating loss-of-function mutants. Further analysis of the cDNA library revealed the presence of a MAPKKK (mitogen-activated protein kinase kinase kinase) gene that plays a key role in many signal transduction pathways (Shim and Dunkle 2003). Targeted disruption of *CZK3* MAPKKK abolished cercosporin production, conidiation, and also mutants were less virulent on maize leaves. Bluhm et al. (2008) constructed an extensive expressed

sequence tag (EST) library of *C. zeae-maydis* and evaluated expression of selected genes during vegetative, infectious, and reproductive growth. They also identified several genes potentially involved in growth regulation, development, and pathogenesis in *C. zeae-maydis*.

Much of the understanding about cercosporin biosynthesis comes from research done on *C. nicotianae*. Chung et al. (2003c) used restriction enzyme-mediated integration (REMI) and generated several *C. nicotianae* mutants that were deficient in cercosporin production. Sequencing one of the rescued plasmids (pCTB1) from one of the toxin deficient mutants revealed the presence of a polyketide synthase (*PKS*) gene which they named *CTB1* (cercosporin toxin biosynthesis 1). Disruption of *CTB1* completely abolished cercosporin production and mutants disrupted for *CTB1* caused only few necrotic lesions compared to wild type *C. nicotianae*. Expression of *CTB1* transcript was also highly up-regulated by light and nutrient media that favor cercosporin production. Dekkers et al. (2007) found another gene, *CTB3*, adjacent to *CTB1*, which encodes two putative domains i.e., an O- methyltransferase domain in the N-terminus and a flavin adenine dinucleotide (FAD)-dependent monooxygenase domain in the C-terminus and demonstrated that *CTB3* is also required for cercosporin biosynthesis and also hypothesized that cercosporin toxin biosynthesis genes in *C. nicotianae* may be arranged as a gene cluster. Genes involved in the biosynthesis of many secondary metabolites are often arranged in clusters, for example, afaltoxin by *Aspergillus* species (Brown et al. 1996, 1999; Yu et al. 2004a), fumonisin and trichothecene in *Fusarium* (*Gibberella*) species (Hohn et al. 1993; Proctor et al. 2003; Seo et al. 2001), sirodesmin in *Leptosphaeria maculans* (Gardiner et al. 2004), compactin in *Penicillium citrinum* (Abe et al. 2002), gibberellin in *Gibberella fujikuroi* (Tudzynski and Hölter 1998), alkaloids in *Claviceps purpurea (*Tudzynski et al. 1999) and *Neotyphodium uncinatum* (Speiring et al. 2005), AK toxin in *Alternaria alternata* (Tanaka and

Tsuge 2000) and HC toxin in *Cochliobolus carbonum* (Ahn and Walton 1996). *CTB3* disruption mutants produced yellow/brown pigment and also were less virulent on tobacco leaves. Sequencing of the 34 kb region identified presence of 10 additional open reading frames (ORFs) that might be part of the gene cluster. Choquer et al. (2007) identified a *CTB4* gene downstream of *CTB1* and *CTB4* has similarity to MFS transporter genes with 12 TM domains. Mutants disrupted for *CTB4* produced less cercosporin and did not secrete cercosporin into the medium, suggesting that *CTB4* is involved in secretion and accumulation of cercosporin. Chen et al. (2007a) reported that *CTB5* and *CTB7* (FMN/FAD-dependent oxidoreductases), and *CTB6* (NADPH-dependent oxidoreductase) genes also were involved in cercosporin biosynthesis. Chen et al. (2007b) characterized a Zinc finger transcription factor encoding gene *CTB8* and putative *CTB2* gene (O-methyltransferase) involvement in cercosporin biosynthesis. Disruption of *CTB8* abolished the accumulation of transcripts *CTB1* through *CTB7* indicating that *CTB8* acts a regulatory switch to control the cercosporin biosynthetic pathway. Based on observations from *CTB1*-*CTB8* genes, Chen et al. (2007b) proposed a possible biosynthetic pathway for cercosporin in *C. nicotianae* (Fig. 1.3). Production of cercosporin is regulated by signaling network interplay between Ca^{2+}/calmodulin and a MAP kinase pathway (Chung 2003; Shim and Dunkle 2003). Chung et al. (2003a and 2003b) also characterized a CRG1 protein containing a Cys6Zn2 binuclear cluster DNA-binding motif with homology to various fungal regulatory proteins, indicating that CRG1 may function as a transcription activator. *CRG1* disruption mutants were partially sensitive to cercosporin and also produced low levels of cercosporin, indicating that CRG1 is involved in the activation of genes associated with cercosporin resistance and production in the fungus *C. nicotianae*. Amnuaykanjanasin and Daub (2009) identified *ATR1* and *CnCFP* genes in *C. nicotianae* from a subtractive library between the wild type (WT) and a *crg1*

mutant. *ATR1* is an ATP-binding cassette (ABC) transporter gene and disruption resulted in lower cercosporin and increased sensitivity to exogenously applied cercosporin. Constitutive expression of *ATR1* in the *crg1* mutant restored cercosporin biosynthesis and moderately increased resistance to cercosporin.

The chemical characteristics of cercosporin are well established, but the biosynthetic pathway in *C. kikuchii* remains uncharacterized. The elucidation of this cercosporin biosynthetic pathway may be essential for developing resistance in soybean cultivars and other crops susceptible to diseases caused by cercosporin-producing species of this fungal genus. One approach to identify additional cercosporin biosynthesis pathway genes is to separate proteins isolated from *C. kikuchii* isolates using two dimensional gel electrophoresis (2-DGE) and to compare the resulting protein profile differences between different conditions such as cercosporin production favoring (light) and suppressive conditions (dark).

2.5.4 Resistance to Cercosporin

Apart from cercosporin biosynthesis, research on *Cercospora* species has also focused on understanding self-resistance to cercosporin, the knowledge of which can be utilized to genetically engineer important crops to protect them from the damage caused by cercosporin. The *CFP* gene in *C. kikuchii* was shown to be involved in resistance to cercosporin (Callahan 1999). Upchurch et al. (2002) transformed the cercosporin sensitive fungus, *Cochliobolus heterostrophus*, with *CFP* and demonstrated enhanced resistance to cercosporin. Certain bacteria such as *Xanthomonas campestris* pv. *zinniae* and *X. campestris* pv. *pruni* can degrade cercosporin (Mitchell et al. 2002) into a non-toxic compound, xanosporic acid. A putative oxidoreductase was involved in this degradation process (Taylor et al. 2006). Studies *C. nicotianae* mutants sensitive to cercosporin identified several genes that confer resistance to

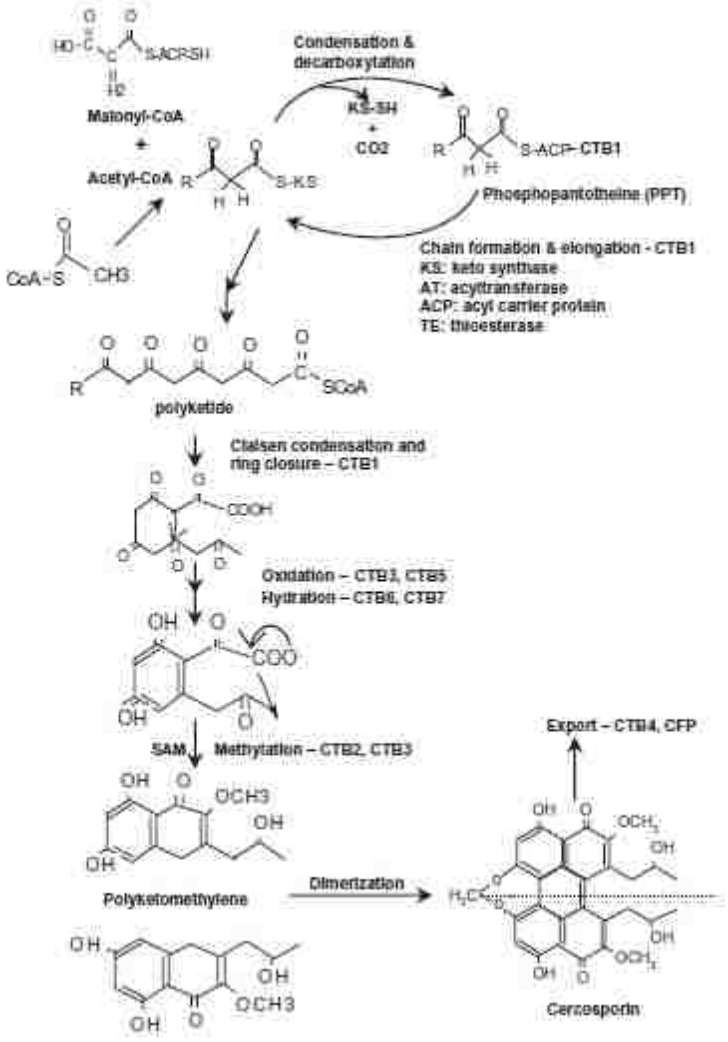


Figure 1.3. Speculated biosynthetic pathway leading to the formation of cercosporin, showing hypothesized functions of the CTB gene products in *Cercospora nicotianae* (Chen et al. 2007b).

cercosporin (reviewed in Daub et al. 2005). In addition, the *PDX1* gene involved in vitamin B_6 biosynthesis also plays an essential role in resistance to cercosporin (Ehrenshaft et al. 1998, 1999). *CRG1* is also involved in the activation of genes associated with cercosporin resistance and production in *C. nicotianae* (Chung et al. 2003b). Reductive detoxification can also provide resistance to cercosporin, in which toxin is maintained in a reduced state in hyphae in

Cercospora species and emits green fluorescence. Upon excretion by fungal cells, this reduced form of cercosporin was oxidized and regained photodynamic activity (Daub et al. 1992).

2.5.5 Factors Affecting Cercosporin Production

Cercosporin biosynthesis is affected by diverse factors such as light, temperature, nutrient conditions, pH, source of carbon and nitrogen, and carbon : nitrogen ratio. Higher cercosporin was accumulated at 20°C compared to 30°C in two media by different *C. kikuchii* isolates. *C. kikuchii* ATCC 86864 produced more cercosporin on malt media while *C. kikuchii* IN (isolated from soybeans in Indiana) produced more on PDA, indicating there is isolate/media specificity for cercosporin production. Peak cercosporin accumulation occurred at C:N ratios of 500:1 in *C. kikuchii* PR (isolated from soybeans in Puerto Rico), 150:1 in *C. kikuchii* IL (isolated from soybeans in Illinois), 50:1 in *C. beticola*, and 10:1 in *C. nicotianae* (Jenns et al. 1989). Depletion of nitrogen in the medium had little effect on cercosporin production whereas depletion of carbon drastically reduced cercosporin production in *C. nicotianae* (You et al. 2008). Unlike the above factors, light is a universally required cue for cercosporin production by all *Cercospora* isolates tested. Light is required for both production and activation of cercosporin. *C. kikuchii* isolates grown in continuous light accumulated 100-fold more cercosporin than dark-grown cultures (Ehrenshaft and Upchurch 1991, Fajola 1978). Lynch and Geoghegan (1979) demonstrated using spectral analysis that cercosporin production was stimulated by wavelengths of 450-490 nm in *C. beticola*.

2.6 Real-Time Polymerase Chain Reaction (Real-time PCR)

2.6.1 Available Real-Time PCR Technologies

Invention of the polymerase chain reaction by Kary Mullis in 1984 (Mullis and Faloona 1987) was a major breakthrough in science and has accelerated scientific discoveries. Soon after

this invention, researchers were interested in quantifying PCR to determine the concentration of nucleic acids for gene quantification and pathogen detection. This has led to development of real-time PCR, in which PCR amplification was monitored from cycle to cycle using detectors, rather than end point detection by electrophoretic gel separation in conventional PCR.

Real-time PCR technology has gained momentum with the use of fluorescent dyes for detection of amplicons. Early detection techniques used intercalating dyes such as SYBR® Green for detecting amplification. SYBR® Green was very useful and can be used with any gene/primer combinations. However, as it can bind to both specific and non-specific amplification products, the reaction needs proper optimization. A SYBR® Green assay should always be accompanied by a melting curve analysis to confirm the amplification of a single target region and also SYBR® Green assays do not allow multiplexing.

The potential problem of detecting non-specific amplicons has led to development of new techniques that involve the use of labeled oligonucleotides that bind specifically to target sequence. This technology uses probes labeled with two different fluorochromes and is based on Fluorescence Resonance Energy Transfer (FRET). This energy transfer only occurs if the two molecules are in close proximity to each other (a few nanometers). Depending on the proximity to the second fluorochrome, the first one may either emit light or transfer its energy to the second, which in turn fluoresces. Thus, bringing the two fluorochromes in close proximity to each other results in the fluorescence quenching of the first one, and fluorescence emission of the second one. As the fluorescence from the emitting fluorochrome increases proportionally with the amount of newly synthesized DNA, both effects can be recorded to follow the amplification of the target DNA. However FRET was not popular in plant sciences owing to the use of four oligonucleotides and was only limited to studies that required very high level of specificity.

FRET was followed by development of the quenching system such as the Taqman® assay which is based on the 5′ → 3′ exonuclease activity of Taq DNA polymerase. In the Taqman® assay, in addition to gene-specific forward and reverse primers, oligonucleotide probes also are included which have a fluorescent reporter dye [e.g. 6-carboxy-fluorescein (FAM)] at the 5′ end and a fluorescent quencher [e.g. 6-carboxy-tetramethylrhodamine (TAMRA)] at the 3′ end with a blocked phosphate group to prevent PCR-driven elongation. Once the PCR is in progress, primers and probe will bind to complementary regions. As the forward primer is extended during the extension step, the 5′ end of the probe is cleaved because of the 5′ → 3′ exonuclease activity of Taq DNA polymerase which releases the fluorescent reporter dye. Once the reporter dye is cleaved and away from the quencher, an increase in fluorescence is detected. As more and more amplicon is produced exponentially during the subsequent cycles, fluorescence will also increase exponentially. By plotting the increase in fluorescence (ΔRn) versus the PCR threshold cycle number (Ct), the system produces plots that provide a more complete picture of the PCR process. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. Finally, the use of an internal positive control (e.g. 18S rRNA or other housekeeping gene) in parallel reactions to the target reactions allows the normalization of DNA/RNA extraction variations between samples (AppliedBiosystems, Foster City, CA). Taqman® probes were followed by the development of Molecular beacons (Tyagi and Kramer 1996) and Scorpion™ probes (Thelwell et al. 2000).

2.6.2 Application of Real-Time PCR to Study Plant Pathogens

Since its first application in the detection of *Phytophthora* strains in their host plants (Böhm et al. 1999), real-time quantitative PCR (qPCR) has proven to be a very simple and

reliable way to detect numerous plant pathogens such as fungi (Filion et al. 2003; Gachon and Saindrenan 2004; Gao et al. 2004; Li et al. 2008; Oliver et al. 2008; Pico et al. 2008), bacteria (De Bellis et al. 2007; Weller et al. 2000; Randhawa et al. 2001; Schaad et al. 1999; Vandroemme et al. 2008; Weller and Stead 2002), viruses (Kokkinos and Clark 2006; Stewart et al. 2007), and nematodes (Cao et al. 2005; Madani et al. 2005; Francois et al. 2007). Compared to other classical diagnostic methods, real-time PCR-based assays are rapid, sensitive, specific and best suited to discriminate between slightly different levels of infection (Winton et al. 2003). Moreover, since their specificity relies on primer/probe sequences, such assays are easy to develop and they can be transposed to virtually every pathosystem. Real-time PCR also has been extensively used in clinical microbiology, food microbiology, and clinical oncology for detection and quantification of various infectious agents (Klein 2002). Real-time PCR is not only useful for detection but also quantification of plant pathogens in host tissues (Guo et al. 2006; Qi and Yang 2002).

Evaluation of host disease resistance was often based on lesion diameter measurement or on the proportion of inoculated leaves exhibiting spreading necrosis (Nandi et al. 2003). Direct assessment of fungal development on inoculated leaves might be an alternative to those techniques and provide more robust results. With the advent of real-time quantitative polymerase chain reaction, researchers possess the unprecedented ability to accurately quantify a specific pathogen within a host plant. By comparing the amplification pattern of an unknown sample against that of known standards, the quantity of target DNA in the unknown sample can be accurately determined (Heid et al. 1996). Strausbaugh et al. (2005) found significant correlations between percent infected root area and *Fusarium* DNA quantities in *F. culmorum*-inoculated plants. Gachon and Saindrenan (2004) developed a real-time PCR-based assay to follow disease

progression on *Arabidopsis* plants infected with the fungi *Alternaria brassicicola* and *Botrytis cinerea*. It was based on the relative quantification of plant and fungal DNA in infected tissues by performing two real-time PCR reactions targeted at fungal and plant sequences on inoculated samples. The study allowed quantitative monitoring of the growth of the fungi *A. brassicicola* and *B. cinerea.* This method was very robust and was able to differentiate between lines displaying slightly different levels of resistance. Similarly, a better and more sensitive real time PCR based method was developed by Hogg et al. (2007) to replace the traditional methods for evaluating Fusarium crown rot (FCR). Disease severity scores (DSS) and *Fusarium* DNA quantities were positively correlated for all three cultivars. The traditional methods involved scoring of mature wheat tillers using a subjective scale that was based on the intensity and distance brown discoloration extended up the tillers. They were labor intensive, time consuming, and often varied greatly from evaluator to evaluator.

Ma and Michailides (2004) designed a real-time PCR assay to detect azoxystrobin-resistant populations of *Alternaria* from pistachio groves in California, based on a single point mutation (G143A) in mitochondrial cytochrome b (*cyt b*) gene in resistant populations. Real-time PCR based pathogen quantification showed a very good correlation to visual methods of disease assessment in many fungal diseases of wheat (Guo et al. 2007; Fraaije 2001).

2.7 Proteomics

2.7.1 Introduction and Advantages

Plant pathogenic fungi cause significant yield losses in crops worldwide and millions of dollars are invested in fungicides in the U.S. every year to control plant diseases. Understanding molecular mechanisms underlying the interaction of fungi with their host plants and virulence mechanisms are essential to devise effective disease management strategies. The availability of

fast and efficient DNA sequencing technologies has led to a genomics era with whole genome sequencing of many plant pathogenic fungi. Once the genome sequences became available, several reverse genetics approaches, such as targeted gene disruption/replacement (knock-out) (Schumacher et al. 2008), gene silencing (knock-down) (Ajiro et al. 2010), insertional mutagenesis (Chi et al. 2009), or targeting induced local lesions in genomes (TILLING) (Lamour et al. 2006) were developed as part of functional genomics research. Transcriptomics, the global analysis of gene expression at the mRNA level has also become an attractive method for analyzing the molecular basis of fungal-plant interactions and pathogenesis (Bhadauria et al. 2007; Oh et al. 2008; Takahara et al. 2009; Wise et al. 2007), and includes differential display (DD) (Venkatesh et al. 2005), cDNA-Amplified Fragment Length Polymorphism (cDNA-AFLP) (Wang et al. 2009b), suppression subtractive hybridization (SSH) (Fekete et al. 2009), serial analysis of gene expression (SAGE) (Venu et al. 2007), expressed sequence tags (ESTs) (Yin et al. 2009), and DNA microarrays (Venu et al. 2007), or RNA-Seq (RNA sequencing) using next generation sequencing technologies (Wang et al. 2009a). However mRNA expression based studies have some limitations: (i) highly expressed mRNAs do not necessarily correlate with protein expression (Gygi et al. 1999, Tsai-Morris et al. 2004) (ii) spliced variants of genes are known to code for different types of proteins (Blencowe and Khanna 2007, Cheah et al. 2007), and (iii) post-translational modifications (PTMs) of a single protein may lead to multiple functions (Davison 2002, de la Cadena et al. 2007). All these limitations can be overcome by a complementary proteomics approach.

The term proteomics was coined by Marc Wilkins to simply refer to “PROTEin complement of a genOME” (Wilkins et al. 1995). In the broadest sense, the proteome can be defined as being the total set of protein species present in a biological unit (organelle, cell, tissue,

organ, individual, species, and ecosystem) at any developmental stage and under specific environmental conditions. Proteomics helps us understand how, where, and when the several hundred thousands of individual protein species are produced in a living organism, and how they interact with one another. Proteomics can include several areas such as (i) descriptive proteomics, including intracellular and subcellular proteomics, (ii) differential expression proteomics, (iii) post-translational modifications, (iv) interactomics, (v) proteinomics (targeted or hypothesis-driven proteomics), and (vi) secretomics (fungal effectors and virulence factors) (González-Fernández et al. 2010).

2.7.2 2-Dimensional Electrophoresis

The original two-dimensional electrophoresis (2-DE) based proteomics technique, developed in the 1970s (O'Farrell 1975), suffered from problems of poor resolution and reproducibility. The development of immobilized pH gradient (IPG) strips, technical advancement in improving quality and reproducibility of 2-DE gels, non-gel based high-throughput separation of proteins using liquid chromatography (LC), and mass spectrometry (MS) have led to accelerated use of proteomics in various scientific disciplines including plant biology (Chen and Harmon 2006; Domon and Aebersold 2006). 2-DE involves separation of proteins in the first dimension according to their isoelectric point (p*I*), by isoelectric focusing, and in the second dimension, according to their molecular weight (MW), by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Görg et al. 2004). 2-DE gels can resolve thousands of protein spots and provide information about MW, p*I*, quantity and post-translational modifications (Görg et al. 2004, Whittmann-Liebold et al. 2006).

2.7.3 Different Staining Techniques

Protein spots separated on 2-DE gels can be visualized by several staining techniques with varying sensitivities that include Coomassie Brilliant Blue (CBB), silver, and fluorescent SYPRO® dyes (Miller et al. 2006). Under acidic conditions, CBB sticks to the amino groups of proteins by electrostatic and hydrophobic interactions (Westermeier and Marouga 2005). CBB R-250 has very low sensitivity requiring 100-500 ng of protein per spot for detection (Candiano et al. 2004). However, by using colloidal Coomassie G-250, the detection limit up to 1 ng per spot can be achieved (Candino et al. 2004) and G-250 staining is also compatible with downstream MS applications. Silver stain is very sensitive with detection limits up to 0.1 ng per spot but it interferes with downstream MS applications (Syrovy and Hodny 1991). Jin et al. (2008) developed a modified silver staining method that is compatible with MS. Fluorescent dyes show very wide dynamic ranges, over four orders of magnitude. Among SYPRO® dyes (Orange, Red, Tangerine and Ruby) SYPRO® Ruby has very high sensitivity with detection limit less than 1 ng per spot (Berggren et al. 2002). Deep Purple™ dye containing fluorophore "epicocconone" can detect a few hundred picograms of proteins (Mackintosh et al. 2003). Fluorescent dyes offer high sensitivity of detection with reliable quantification over a wide linear dynamic range. However, the high cost of dyes and requirement of specialized scanners to document gel images limit the use of fluorescent dyes in many proteomics studies. In addition to staining all the proteins in the gel, glycosylated proteins can be stained specifically with Pro-Q® Emerald dye (Hart et al. 2003) and phosphorylated proteins with Pro-Q® Diamond dye (Steinberg et al. 2003). However, all these staining techniques only allow visualization on the gels, but if the objective is to find differentially up- or down-regulated proteins between different treatments, the gel-to-gel variation of biological and technical repeats poses a problem in

accurate quantification of protein expression. To solve this problem, difference gel electrophoresis (DIGE) was developed by Unlu et al. (1997), which employs the use of size and charge matched cyanine dyes (CyDye^{TM} DIGE flours Cy3, Cy2, and Cy5) with different excitation and emission wavelengths as proteins labels for different samples. DIGE allows multiplexing of samples and use of an internal standard, which is created by using one of the labels (Cy2) for a pooled mixture of all samples in a given experiment (Westermeier and Marouga 2005). Thus, the DIGE method gives highly accurate qualitative and quantitative results, because of elimination of gel-to-gel variation.

2.7.4 Application of Proteomics to Study Plant Pathogenic Fungi

Fernandez-Acero et al. (2006) used two-dimensional electrophoresis coupled with MS to generate a partial proteome map for *Botrytis cinerea* and detected 400 spots in Coomassie-stained 2-DE gels. Out of 60 spots subjected to MS analysis, twenty-two spots were identified using matrix assisted laser desorption/ionization time of flight (MALDI-TOF) or electrospray ionization ion trap tandem mass spectrometry (ESI IT MS/MS), with some proteins corresponding to variants of malate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, and cyclophilin. Comparative proteome analysis of two *B. cinerea* strains with different virulence and toxin production characteristics also revealed the presence of qualitative and quantitative differences in the 2-DE protein profile (Fernandez-Acero et al. 2007). Yajima and Kav (2006) identified 18 secreted proteins and 95 mycelial proteins in *Sclerotinia sclerotiorum* using 2-DE and MS/MS and found many cell wall-degrading enzymes in the secreted proteins that were previously identified as pathogenicity or virulence factors of *S. sclerotiorum*. One-dimensional electrophoresis and 2-DE was also used by Xu et al. (2007) to analyze the protein profile of six isolates of *Curvularia lunata*, a pathogen of maize, and correlation was observed

between the band or spot pattern and virulence. Out of 423 spots resolved by 2-DE, 29 spots were isolate-specific and 39 showed quantitative differences. Sulc et al. (2009) built a mass spectral database with twenty-four *Aspergillus* strains by profiling spores followed by MALDI-TOF MS. These mass fingerprints generated by MS can be used for genotyping and characterizing different *Aspergillus* strains and also finding new biomarkers in host-pathogen interactions. Biotrophic fungi such as downy mildews (Oomycota), powdery mildews (Ascomycota), and rust fungi (Basidiomycota) are some of the most destructive plant pathogens and *in vitro* studies of these biotrophs is difficult as they cannot survive outside of their host. Godfrey et al. (2009) reported a technique to isolate the fungal haustoria from barley plants, infected with *Blumeria graminis* f.sp. *hordei* (*Bgh*), the powdery mildew pathogen, and analyzed haustorial proteins using liquid chromatography tandem mass spectrometry (LC-MS/MS). The resulting MS/MS data were searched against the *Bgh* EST sequence database and the NCBInr fungal protein database. They identified 204 haustorial proteins. Apart from whole cell proteomes, subcellular proteomes such as mitochondrial proteins of *Trichoderma harzianum* were studied by Grinyer et al. (2004). A reference proteome map of the dimorphic maize pathogen, *Ustilago maydis*, was developed by Böhmer et al. (2007) using 2-DE coupled with MALDI-TOF MS and ESI-MS/MS. In its haploid phase, this fungus is unicellular and multiplies vegetatively by budding. It then undergoes a dimorphic transition to infective filamentous growth. They observed 13 protein spots accumulated in high abundance in the filamentous form as compared to the budding form.

Cooper et al. (2007) used multidimensional protein identification technology to survey proteins in germinating asexual urediniospores of the bean rust pathogen, *Uromyces appendiculatus*, and compared the data to proteins discovered in an inactive spore. There were

few changes in amounts of accumulated proteins involved in glycolysis, acetyl Co-A metabolism, citric acid cycle, ATP-coupled proton transport, or gluconeogenesis. Germlings contained a greater amount of proteins involved in mitochondrial ADP:ATP translocation (indicative of increased energy production) and more histone proteins, pointing to the reorganization of the nuclei that occurs after germination prior to appresorium formation. These changes are indicative of metabolic transition from dormancy to germination and are supported by cytological and developmental models of germling growth. Noir et al. (2009) used 2-DE and MALDI TOF MS to generate an annotated proteome map of nongerminated conidiospores of the ascomycete barley powdery mildew pathogen, *Blumeria graminis* f.sp. *hordei*, in which they identified 123 distinct fungal gene products. Most of the identified proteins have a predicted function in carbohydrate, lipid or protein metabolism, indicating that the spore is equipped for the catabolism of storage compounds as well as for protein biosynthesis and folding on germination. Luster et al. (2010) applied 2-DE and MS to identify predominantly soluble proteins present during the germination phase of *Phakopsora pachyrhizi* urediniospores that are involved in early infection and interaction with host legumes. They identified 117 fungal proteins with demonstrated roles in cell biosynthesis, metabolism, regulatory, signaling, stress responses and infection.

2.7.5 Application of Proteomics to Study Secreted Proteins from Plant Pathogenic Fungi

The combination of native secreted proteins and the cell machinery involved in their secretion is defined as the "secretome" (Tjalsma et al. 2000). Many plant pathogenic fungi secrete a large number of degradative enzymes and other proteins, which have diverse functions in colonization, nutrient acquisition, and ecological interactions (de Vries 2003; Freimoser et al. 2003; Walton 1994). Many extracellular enzymes such as polygalacturonase, pectate lyase,

xylanase, and lipase, were shown or proposed to be required for virulence in plant pathogenic fungi (Brito et al. 2006; Deising et al. 1992; Isshiki et al. 2001; Oeser et al. 2002; ten Have et al. 1998; Voigt et al. 2005; Yakoby et al. 2001).

The ability of *Aspergillus flavus* and *A. parasiticus* to degrade the flavonoid, rutin (quercetin 3-*O*-glycoside), secondary metabolite produced by plants in response to invading microorganisms, was studied by Medina et al. (2004). The secreted proteins were analyzed by 2-DE and MALDI-TOF MS and 15 rutin-induced proteins and 7 non-induced proteins and glycosidases were identified. In the second study, the growth media including glucose and potato dextrose broth were used to identify differentially expressed secreted proteins by 1- and 2-DE and MS/MS (Medina et al. 2005). Among identified proteins, ten were unique to rutin-, five to glucose-, and one to potato dextrose-grown *A. flavus* and sixteen proteins were common to all three media. An investigation of the exoproteome of *Fusarium graminearum*, found a greater quantity and diversity of proteins when the fungus was grown on hop cell wall than when grown on glucose (Phalip et al. 2005). Using 1- and 2-DE followed by LC-MS/MS, they identified 84 proteins unique to cell-wall grown fungal exoproteome and most were enzymes involved in carbohydrate metabolism, including cellobiohydrolase, which can act as a sensor of the extra cellular environment. These results indicated that fungal metabolism becomes oriented towards the synthesis and secretion of an arsenal of enzymes able to digest almost the complete plant cell wall.

Lim et al. (2001) extracted and separated 220 cell envelope associated proteins from *Trichoderma reesei* mycelia with great variation in quantities secreted. The most abundant protein identified in both conditions was HEX1, the major woronin body protein, which is unique to fungal mycelia. The extracellular proteome secreted by *T. harzianum* in the presence of

fungal cell walls (*Pythium ultimatum, B. cinerea, R. solani*) and chitin as a nutrient source was studied by Suarez et al. (2005). They found abundant expression of aspartic protease (P6281) under all conditions, which showed homology to polyporopesin from the basidiomycete *Irpex lacteus,* and they speculated upon the putative role of protease P6281 in mycoparasitism. Tseng et al. (2008) studied the biocontrol mechanism of *T. harzianum* in response to *R. solani* by analyzing the secretome. Seven cell-wall degrading enzymes, chitinase, cellulase, xylanase, *β*-1,3-glucanase, *β*-1,6-glucanase, mannanase, and protease, were revealed by activity assay, in-gel activity stain, 2-DE, and LC-MS/MS analysis, and the production of these enzymes increased in response to *R. solani.* Meijer et al. (2006) studied cell wall associated proteins of the oomycete, *Phytophthora ramorum*, the causal agent of sudden oak death. Based on LC-MS/MS, they identified 17 secreted proteins with homology to mucin or mucin-like proteins, putative glycoside hydrolases, transgluataminases, annexin-like protein and kazal-type protease inhibitor. Yajima and Kav (2006) studied the secreted proteins from *Sclerotinia sclerotiorum* and identified 18 secreted proteins by MS/MS, which include mainly cell wall degrading enzymes that were previously identified as pathogenicity or virulence factors of *S. sclerotiorum.* This study also identified an alpha-L-arabinofuranosidase, which was not present in earlier comprehensive EST studies (Li et al. 2004; Sexton et al. 2006). Liang et al. (2010) studied the proteins present in the liquid sclerotial exudates of *S. sclerotiorum* using 1- and 2-DE followed by LC-MS/MS and identified 56 proteins that included five glucanases, GPI anchor protein, glucoamylase, arabinifuranosidase, tyrosinase etc.

Paper et al. (2007) used high-throughput MS/MS to identify secreted proteins of *F. graminearum in vitro* (grown on 13 different media) and *in planta* (infection of wheat heads). A total of 289 proteins (229 *in vitro* and 120 *in planta*) were identified with high statistical

confidence. They identified 49 unique proteins *in planta*, which were absent in *in vitro* conditions. Compared to *in planta* proteins (56%), the majority of *in vitro* proteins had predicted signal peptides. Some of the non-secreted proteins found *in planta* included single-copy housekeeping enzymes such as enolase, triose phosphate isomerase, phosphoglucomutase, calmodulin, aconitase, and malate dehydrogenase, which indicated the occurrence of significant fungal lysis during pathogenesis. Several of the proteins lacking signal peptides that were found *in planta* have been reported to be potent immunogens secreted by animal pathogenic fungi, indicating their importance in interactions between *F. graminearum* and its host plants. A comprehensive proteomic-level comparison of *Pyrenophora tritici-repentis* avirulent race 4 and virulent race 5 by 2-DE followed by MS/MS, identified 29 differentially abundant proteins in the secretome and included the secreted enzymes α-mannosidase and exo-β-1,3-glucanase, heat-shock and BiP (binding imunoglobulin protein) proteins, and various metabolic enzymes (Cao et al. 2009). The proteome-level differences suggested that reduced pathogenic ability in race 4 of *P. tritici-repentis* may reflect its adaptation to a saprophytic habit.

Several studies examined the secretome of *Botrytis cinerea*, an important necrotrophic pathogen of nursery plants, vegetables, ornamental, field, orchard crops, as well as stored and transported agricultural products, causing significant economic losses. Shah et al. (2009a) used 1-DE and LC-MS/MS to identify 89 secreted proteins from *B. cinerea* grown on a solid substrate of a cellophane membrane appressed to the nutrient medium, which was supplemented with an extract from full red tomato, ripened strawberry or *Arabidopsis* leaf extract. Sixty proteins were predicted to contain signal motif indicating extracellular localization. *Botrytis cinerea* increased secreted protein production in the presence of favorable food sources such as full red tomato and ripe strawberry extract compared to Arabidopsis leaf extract. The identified secreted proteins

include transport proteins, proteins involved in carbohydrate metabolism, peptidases, and pathogenicity factors that provide important insight into how *B. cinerea* may use secreted proteins for infection and colonization of hosts. Shah et al. (2009b) also identified 126 proteins secreted by *B. cinerea* by growing the fungus on highly or partially esterified pectin, or on sucrose in liquid culture. They identified 13 proteins related to pectin degradation in both pectin growth conditions indicating that it is unlikely that the activation of *B. cinerea* from the dormant state to active infection is solely dependent on the changes in degree of esterification of the pectin component of the plant cell wall. Fernandez-Acero et al. (2010) studied the secretion of discrete sets of proteins by *B. cinerea* grown on culture media supplemented with different carbon sources [glucose, carboxymethylcellulose (CMC), starch, pectin] and plant-based elicitors [tomato cell walls (TCW)]. A total of 78 spots were identified by MALDI-TOF/TOF MS/MS analysis, corresponding to 56 unique proteins and 45 identified proteins contained secretion signal peptides for both classical and nonclassical secretory pathways. The use of different carbon sources and plant-based elicitors resulted in different degrees of complexity of fungal response, moving from a state of constitutive fungal growth and a simple secretome (by using glucose as a nutrient) toward a more complex and possibly pathogenic secretory behavior (induced by TCW). The corresponding 2-DE profiles showed an increasing number and variety of spots when CMC or TCW were used. Some of the identified proteins are involved in the pathogenicity process (e.g. pectin methylesterases, xylanases, and proteases). Espino et al. (2010) studied the early secreted proteome of *B. cinerea* grown in conditions that resemble the plant environment (a synthetic medium enriched with low molecular weight plant compounds, in a dialysis bag that contained 50% tomato, strawberry, or kiwifruit extracts) by collecting proteins secreted during first 16 hours of conidial germination. Using 2-DE coupled with MALDI TOF

and 1-DE coupled with LC-MS/MS, they identified a total of 121 proteins. One interesting conclusion from this study was that the secreted proteome did not differ significantly during the first 16 h of growth by *B. cinerea* on media with and without plant extracts. Proteasomes dominated the early secretome indicating their role in degradation of plant cell wall proteins to promote fungal hyphal penetration and to suppress plant defense proteins to increase the chances for a successful infection. The first most abundant proteins secreted and detected in all conditions was the BcAP8 (asparatic protease) followed by BcPG1 (endopolygalacturonase), which is also shown to be required for *B. cinerea* virulence (Espino et al. 2010).

CHAPTER 3: LATENT INFECTION BY *CERCOSPORA KIKUCHII*, CAUSAL AGENT OF CERCOSPORA LEAF BLIGHT IN SOYBEAN

3.1 Introduction

Cercospora kikuchii (T. Matsumoto & Tomoy.) M.W. Gardner is the causal agent of Cercospora leaf blight (CLB) and purple seed stain (PSS) of soybean (Orth and Schuh 1994). This pathogen can affect all aerial parts of the plant including leaves, petioles, stems, pods, and seeds (Matsumoto and Tomoyasu 1925; Suzuki 1921; Walters 1980). PSS is widely distributed in all soybean growing regions of the world and primarily affects seed germination leading to reduced stands and poor seed quality (Lehman 1950; Wilcox and Abney 1973). Until recently, CLB was a minor disease in the southern U.S., but it has become a serious concern to soybean producers in recent years (Moore and Wolcott 2000). CLB has been reported in other major soybean producing states such as Iowa. The disease is capable of causing substantial yield losses, and it is associated with a disorder known as green stem in which stems remain green while pods and seeds are mature (Hartman et al. 1999; Leonard et al. 2011; Schneider et al. 2003; Wrather et al. 1997, 2001).

Several attempts have been made to identify soybean cultivars with resistance to CLB. Moderate levels of resistance were found in some soybean lines, but there was no relationship between resistance to CLB and PSS in that soybean cultivars resistant to CLB were susceptible to PSS and *vice versa* (Orth and Schuh 1994; Walters 1980). Development of CLB symptoms depends upon environmental conditions. Warm and humid conditions favor the spread of disease, especially at the end of the growing season, while hot dry conditions at the end of the season lead to severe blight symptoms (Raymond Schneider, Personal communication). Some growers have resorted to early planting to avoid favorable conditions for development of CLB

later in the season because most commercial cultivars lack complete resistance to *C. kikuchii* (Moore and Boquet 2008; Schneider et al. 2003). In addition, the pathogen is extremely diverse, and there are significant location and time interactions with respect to susceptibility (Cai and Schneider 2005, 2008; Cai et al. 2009).

Another viable alternative to the use of resistant cultivars or early planting for CLB management is fungicide applications. However, the successful use of this strategy requires early detection of the pathogen as demonstrated for sheath blight in rice (Sayler and Yang 2007). Earlier studies showed that *C. kikuchii* and many other fungi often cause latent infections in soybeans (Hartman et al. 1999; Sinclair 1991). As a result disease symptoms, such as chlorosis and necrosis, only become visible long after initial infection and usually at late growth stages when plants are under stress or there is a sudden change in nutritional or environmental conditions (Cerkauskas and Sinclair 1980; Hartman et al. 1999). Fungicide applications at these late growth stages are usually ineffective (Robertson et al. 2011). However, traditional methods involving bioassays, histological and serological assays to detect latent infections in soybeans are often time consuming and not sensitive. Without knowing when infection occurs, producers do not know when to initiate fungicide applications.

The advent of traditional polymerase chain reaction (PCR), made it possible to detect plant pathogens based on primers designed for a specific part of their genomic sequence. However, traditional PCR involves separation of reaction products on an agarose gel for visualization and qualitative analysis, which are not suitable for automation when there is a need to screen a large number of samples. The development of high throughput real-time PCR technology, which uses primers and a fluorogenic probe to amplify a specific target sequence, allows monitoring the PCR amplification process in real time and the quantification of target

sequence by measuring fluorescence signal without the need for post PCR gel analysis. After the first successful application of real-time quantitative PCR (qPCR) for detecting *Phytophthora* strains in their host plants, it has been successfully employed in detecting various plant pathogens including fungi (Bohm et al. 1999, Li et al. 2008, Oliver et al. 2008, Pico et al. 2008), bacteria (De Bellis et al. 2007, Vandroemme et al. 2008), and viruses (Kokkinos and Clark 2006, Stewart et al. 2007). It also has been used to quantify progression of pathogens based on absolute quantification of pathogen DNA (Kuhne and Oschmann 2002).

In this study, we report (i) the development of a real-time PCR assay for the detection of DNA of *C. kikuchii*, (ii) quantification of *C. kikuchii* DNA directly from infected soybean leaves during different plant growth stages, and (iii) comparisons of DNA content of *C. kikuchii* in infected plants subjected to different fungicide treatments to determine the efficacy of various fungicide treatements. Preliminary reports of this work were published (Chanda et al. 2009).

3.2 Materials and Methods

3.2.1 *Cercospora* Species

The *Cercospora* species used in this study are listed in Table 3.1. Isolates of *C. kikuchii* were recovered from infected soybean plants collected from fields in different locations in Louisiana in 2001(Cai and Schneider 2005), and *C. sojina* isolate RWS1, causal agent of frog eye leaf spot in soybean, was recovered from a soybean field near Baton Rouge, LA in 2007. All isolates were single-spored and stored on slants of potato dextrose agar at 5°C in the dark. Samples of genomic DNA for C. *sojina* isolate CS223, C. *zeae-maydis*, C. *zeina*, and C. *beticola* were kindly provided by Dr. Burton Bluhm (University of Arkansas, Fayetteville, AR).

Table 3.1. *Cercospora* species tested for the specificity of the real-time PCR primers and probe developed in this study.

***Cercospora* Species**	**Isolate**	**Host**	**Source**	**Detection[z]**
C. kikuchii	MRL 6020-2B[y]	Soybean	Leaf	Yes
C. kikuchii	DLL 6013-1B[y]	Soybean	Leaf	Yes
C. kikuchii	DLL 50908-1B[y]	Soybean	Leaf	Yes
C. kikuchii	MRS 6020-2B[y]	Soybean	Seed	Yes
C. kikuchii	MRS 5012-1A[y]	Soybean	Seed	Yes
C. sojina	RWS1	Soybean	Leaf	No
C. sojina	CS223	Soybean	Leaf	No
C. zeae-maydis	SCOH1-5	Maize	Leaf	No
C. zeina	DYPA	Maize	Leaf	No
C. beticola	S1	Sugarbeet	Leaf	No

[y], See reference Cai and Schneider (2005)
[z], Yes indicates significant amplification in real-time PCR and No indicates no amplification

3.2.2 Development of Specific Real-time PCR Primers and Probe for *C. kikuchii*

Because of limited genome sequence information available for *C. kikuchii*, 14 sets of PCR primers (Table 3.2) specific for eight cercosporin toxin biosynthesis (CTB) pathway genes and four adjacent open reading frames (ORFs) in *C. nicotianae* were designed based upon known sequences (Chen et al. 2007b). These primers were used to amplify their corresponding genes and ORFs from *C. kikuchii*. PCR was performed on genomic DNAs from *C. kikuchii* and *C. sojina.* The CNCTB6 primer pair, which targeted the NADPH-dependent oxidoreductase gene (*CTB6*) from *C. nicotianae* (GenBank accession DQ991508), produced a specific amplification product from *C. kikuchii* but not from *C. sojina*. The PCR fragment amplified from *C. kikuchii* was confirmed to be the *CTB6* gene by direct sequencing (data not shown). The real-time PCR primers (CKCTB6-2F: 5'-CACCATGCTAGATGTGACGACA-3', CKCTB6-2R: 5'-GGTCCTGGAGGCAGCCA-3') and the fluorescent probe (CKCTB6-PRB: 5'-FAM-CTCGTCGCACAGTCCCGCTTCG-TAMRA-3'), which was specific to the *C. kikuchii CTB6* gene, were designed using Primer Express 2.0 (Applied Biosystems, Foster City, CA) based on

the region of *CTB6* DNA sequences of *C. kikuchii* that are different from *C. nicotianae*, and they were custom synthesized by IDT DNA, Inc. (Coralville, IA).

3.2.3 Real-Time PCR Assay and Quantification of DNA of *C. kikuchii* in Field Samples

Real-time PCR was performed with MicroAmp optical 8-tube strips and optical 8-cap strips (Applied Biosystems, Foster City, CA) in the ABI 7000 sequence detection system (Applied Biosystems) under standard conditions. Each reaction contained 7.5 µl 2X TaqMan® Universal PCR Master Mix (Applied Biosystems), 1 µl of 10 µM for each primer (final concentration 666 nM each primer), 0.6 µl of 10 µM (final concentration of 400 nM) probe and 1 µl of 10 ng or 100 ng template DNA. HPLC pure water was added to adjust the final reaction volume to 15 µl. For standard curves, triplicates of 1 µl of serial dilutions of DNA of *C. kikuchii* (1 pg/µl to 1000 pg/µl) in 10 ng/µl of healthy soybean DNA in order to simulate field samples were used as template. DNA from healthy soybean leaves was obtained from greenhouse-grown plants. In addition, each real-time PCR run of field samples also included *C. kikuchii* standards in triplicate as references and DNA from *C. sojina* isolate RWS1 to ensure specificity. DNA concentrations of *C. kikuchii* in each field sample were calculated as pg of *C. kikuchii* DNA/ng soybean DNA. For soybean samples with less than 1 pg of DNA of *C. kikuchii* in 10 ng soybean DNA (Ct value > 37.75), a 100 ng DNA template was used in a second real-time PCR reaction and the resulting Ct value was converted to pg of pathogen DNA/ng soybean DNA. The absolute quantity of DNA of *C. kikuchii* in field samples, which was used to determine the level of infection by *C. kikuchii*, was calculated by using the Ct value of each sample and the regression equation obtained with DNA standards.

Table 3.2. Primer pairs, based upon the CTB gene cluster in *Cercospora nicotianae*, used for amplification in *Cercospora kikuchii*.

Primer name	**Forward sequence (5' - 3')**	**Reverse sequence (5' - 3')**	**Target gene from *C. nicotianae* (GenBank accession number)**
CNCTB1A	CAGCGAGCAACAAGAAGAGAC	AGCGAACGCCATCAAATGC	*CTB1* (AY649543)
CNCTB1B	CAGCGAGCAACAAGAAGAGAC	GACCAGAGGCAGCAAGGATA	*CTB1* (AY649543)
CNCTB1C	GCCCACAAGTAAACAGCCAC	TGAGCAGAACACGACGCTT	*CTB1* (AY649543)
CNCTB1D	CAGACCTTTGCGTCCATCAAT	ATGCTCGCTCTCCACAACT	*CTB1* (AY649543)
CNCTB2	AGCCCGTAGCATCACTGTT	CCGTTGACACCATTAGCCTG	*CTB2* (DQ991505)
CNCTB3	ACAACTGCGATGCTCAAACTC	ATCCTCTTGCTGCCAACCTC	*CTB3* (DQ355149)
CNCTB4	TCGTGACTGCTTGACTGGAG	TTCATCGGGCTCAGCATCA	*CTB4* (DQ991506)
CNCTB5	AGCCTGGGCAAGAACTCAGT	ACCACTCACGCTCCATCTCA	*CTB5* (DQ991507)
CNCTB6	ATACTTCTTCCTACGCCGTCG	TTTGACAACCCGCTCTTGC	*CTB6* (DQ991508)
CNCTB7	CCCAAGCAGTGTGAACATCA	CATCAATCCAGCCCACCAAA	*CTB7* (DQ991509)
CNCTB8	TGCGTGAAGGAACATCGTTTG	TGGGTAGCACATCAGAAGGC	*CTB8* (DQ991510)
CNORF9	CGATAGTCGCCTGGTTGGAA	ACCTTGCTACAATCCACTCCC	*ORF9* (DQ993249)
CNORF10	GAGTGGGCATACATCAGCAG	TTACGCCTACCTCGCTTCTC	*ORF10* (DQ993250)
CNORF11	CCTTGGGTAGAAAGTTGCGAG	TGCTTCGTCCGTCTTTACCA	*ORF11*(DQ993251)
CNORF12	CGATACGAGCGACCTCAGTT	TGCCATTACAGCGTGTCCA	*ORF12* (DQ993252)

3.2.4 Plant Materials, Sampling Details, and Fungicide Sprays

Field studies to determine when soybean plants became infected with *C. kikuchii* using real-time PCR were conducted in 2007, 2009, and 2010. Field experiments in each year were arranged in randomized complete blocks. Each treatment in the experiments described in Table 3.3 consisted of three plots (replications) with each plot comprised of four rows on 76 cm centers by 12 m long. Plants were maintained according to standard practices (Levy et al. 2011). Leaf samples were collected from plants in each plot for each treatment, i.e., treated with fungicide or not treated. If plants were sprayed with fungicides on a sampling date, samples were collected before spraying. Each sample consisted of 30 randomly collected uppermost fully developed leaves from the middle two rows in each plot. Uppermost leaves were used because CLB symptoms first appear on upper leaves that are exposed to sun light (Hartman et al. 1999). Leaf samples were frozen in liquid nitrogen and stored at -80°C until further processing.

3.2.5 DNA Extraction

All 30 leaves from each sample were ground to a fine powder in liquid nitrogen with a pre-chilled mortar and pestle. Three subsamples of 100 mg each were transferred to 2.0 ml microcentrifuge tubes, and DNA was extracted with a GenElute^{TM} Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, St Louis, MO) according to the manufacturer's instructions. DNA of *C. kikuchii* and *C. sojina* was extracted with the same kit using 100 mg of lyophilized ground mycelial powder. Mycelia were collected by filtration from 5-day-old cultures grown in potato dextrose broth on rotary shakers in the dark. Mycelia were washed three times with sterile distilled water before being lyophilized, ground with a mortar and pestle and stored at -80°C until further processing. All DNA samples were diluted to 10 ng/µl based on DNA quantification by measuring absorbance at 260 nm using a NanoDrop ND-1000 Spectrophotometer (Thermo

Table 3.3. Experimental details for field studies related to Cercospora leaf blight of soybean.

Experiment	Year	Cultivar	Planting date	Growth stages at sampling[a]	Sampling date (DAP)[b]	Fungicide application (DAP)	Fungicide treatments (ml/ha ai)[c]
A	2007	CP 5892	22 May	R2 – R6	58, 78, 85, 107	62, 78	pyraclostrobin (322) plus metconazole (548)
B	2007	DP 5634	22 May	R2 – R6	58, 78, 85, 107	62, 78	pyraclostrobin (322) plus metconazole (548)
C	2007	CP 5892	5 June	V8 – R6	44, 64, 71, 93	64	pyraclostrobin (322) plus metconazole (548)
D	2007	DP 5634	5 June	V8 – R6	44, 64, 71, 93	64	pyraclostrobin (322) plus metconazole (548)
E	2007	CP 5892	27 June	V3 – R5	22, 42, 57, 71	42	pyraclostrobin (322) plus metconazole (548)
F	2007	DP 5634	27 June	V3 – R5	22, 42, 57, 71	42	pyraclostrobin (322) plus metconazole (548)
G	2009	DP 5808RR	15 May	V4 – R7	35, 52, 59, 70, 76, 85, 97, 109, 116, 124	48, 55, 74, 90, 103	pyraclostrobin (877)
H	2010	95Y20	26 May	V5 – R5	33, 44, 58, 100	30	flutriafol (512)
I	2010	95Y20	26 May	V5 – R5	33, 44, 58, 100	30, 51, 77	flutriafol (512)
J	2010	95Y20	26 May	V5 – R5	33, 44, 58, 100	30, 51, 77, 99, 113	flutriafol (512)

[a] : See Fehr et al. 1971 for description of different soybean growth stages.
[b] : DAP: days after planting.
[c] : Materials were applied in a spray volume of 187 liters/ha, ai = active ingredient

Fisher Scientific Inc., Wilmington, DE). The uniformity of diluted genomic DNA was verified using real-time PCR assays with primers specific for plant 18S rRNA (Zm18s1F: 5'-GAGAAACGGCTACCACATCCA-3' and Zm18s1R: 5'-ACGCGCCCGGTATTGTTAT-3') and SYBR® Green dye chemistry on eight randomly chosen leaf samples, all of which yielded similar threshold cycle (Ct) values with a mean 14.95 ± 0.2 standard deviations.

3.2.6 Cercospora Leaf Blight Disease Severity Ratings

CLB does not lend itself to quantitative estimates of disease severity, e.g. percent leaf area affected, because symptoms begin as small brick red necrotic lesions on petioles and progress ultimately to the classic leathery purple discoloration of leaves exposed to direct sunlight. Therefore, the following semiquantitative scale was used: 1 = no symptoms; 2 = few symptoms on petioles (up to 10% of petioles that had symptoms); 3 = as above plus up to 5% affected leaves including leaf veins; 4 = as above plus up to 10% affected leaves; 5 = as above with about 50% petioles and up to 30% of leaves with symptoms; 6 = up to 100% of petioles affected but leaves do not have reddish cast and are not chlorotic; 7 = up to 20% of upper leaves becoming reddened or chlorotic; 8 = moderate chlorosis, reddening and necrosis on up to 50% of upper leaves; 9 = as above but symptoms are severe plus defoliation. The 1 to 9 scale was converted to 0 to 100% disease severity for statistical analysis using ANOVA. At least 20 plants were examined in the center two rows, and a composite value was assigned to each plot. Disease incidence was 100%.

3.2.7 Data Analysis

Analysis of variance (ANOVA) using the mixed procedure (PROC MIXED) of SAS (version 9.1, SAS Institute, Cary, NC) was performed on data from the fungicide-sprayed and non-sprayed plants. Means were compared by Tukey's Honestly Significant Difference (HSD)

at $P < 0.05$ with Kramer adjustment for unbalanced design (Kramer 1956).

3.3 Results

3.3.1 Developing Real-Time PCR Primers Specific to *C. kikuchii*

CNCTB6F and CNCTB6R primers (Table 3.2) amplified a 1.14 kb PCR product only in the presence of genomic DNA from *C. kikuchii* but not from *C. sojina* (Fig. 3.1). When field samples were tested, these primers produced a specific product from soybean leaves either with CLB symptoms or from purple stained seeds but not from healthy leaves collected from greenhouse-grown plants (Fig. 3.1). These results indicated that the *CTB6* gene can be used to differentiate between *C. kikuchii* and *C. sojina* and that the primers can detect *C. kikuchii* in soybean leaves. Homology analysis of the sequenced PCR product (GenBank accession JF830016, this study) showed that the *CTB6* gene shared 97% sequence identity between *C. kikuchii* and *C. nicotianae* (data not shown). The DNA region containing the most sequence differences between *C. kikuchii* and *C. nicotianae* was used to design real-time PCR primers and probe (Fig. 3.2). The specificity of real-time PCR primers CKCTB6-2F, CKCTB6-2R and fluorescent probe CKCTB6-PRB was tested by performing real-time PCR with genomic DNA from *C. kikuchii*, *C. sojina*, *C. zeae-maydis*, *C. zeina*, *C. beticola*, greenhouse-grown healthy soybean leaves (negative control), and the no-template control (NTC). Amplification was detected only in the presence of *C. kikuchii* genomic DNA as assessed by changes in fluorescence intensity. No significant fluorescence was detected in real-time PCR reactions containing genomic DNA from the other *Cercospora* species, healthy soybean or NTC.

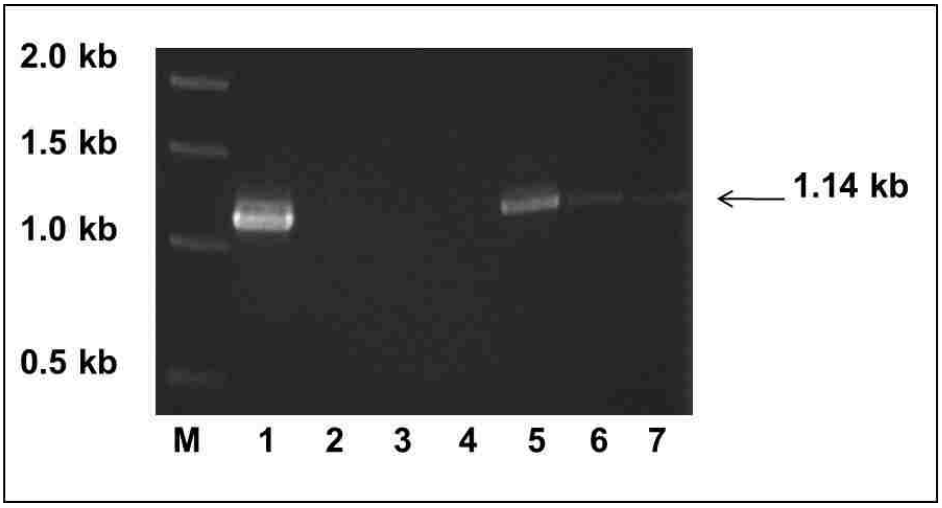


Figure 3.1. CNCTB6F/CNCTB6R primer pair showing specificity to *Cercospora kikuchii* pure culture or *C. kikuchii* infected soybean leaves. Lane 1, *C. kikuchii* isolate MRL 6020-2B; lane 2, *C. sojina* isolate RWS1; lane 3, healthy greenhouse-grown soybean cultivar CP 5892; lane 4, healthy greenhouse-grown soybean cultivar 1001M; lane 5, greenhouse-grown soybean cultivar 1001M grown from purple stained seed; lanes 6 and 7, naturally infected field grown soybean cultivar DP 5634 at R5 stage (71 DAP), showing typical CLB symptoms.

Figure 3.2. Partial DNA sequence of *CTB6* gene from *Cercospora kikuchii* (CK). Open boxes indicate the variable nucleotides between CK and *C. nicotianae* (CN). Arrows indicate location of real-time PCR primers (CKCTB6-2F and CKCTB6-2R) and probe (CKCTB6-PRB) used in real-time PCR detection of *C. kikuchii* in soybean samples.

3.3.2 Standard Curves and Sensitivity of Real-Time PCR Assays

Quantification of serial diluted DNA of *C. kikuchii* showed a linear relationship (Fig. 3.3A) between the amount of genomic DNA (x) and Ct values (y) ($y = -1.434\ln(x) + 37.913$) with a R^2 value of 0.9984 and a slope of 3.3, indicating nearly 100% amplification efficiency (Fig. 3.3B). Mean Ct values for *C. kikuchii* DNA ranged from 27.87 for 1000 pg to 37.75 for 1 pg, which was reliably detected in all samples. Therefore, the lowest detection limit of our assay for DNA of *C. kikuchii* was 1 pg in 10 or 100 ng of soybean DNA. The addition of as much as 1 ng of DNA of *C. sojina* per 10 ng of soybean DNA still produced a negative result ($Ct \geq 40$), which confirmed the high specificity of this assay.

3.3.3 Visual Rating of Disease Severity and Quantification of *C. kikuchii* DNA in Soybean Leaf Samples Using Real-Time PCR

For the first and second plantings in the 2007 (Experiments A, B, C and D, Table 3.3), CLB symptoms were more severe in the non-sprayed DP 5634 plants compared to sprayed plants (Fig. 3.4A), whereas there was no difference in disease severity between fungicide-sprayed and non-sprayed CP 5892 plants. No symptoms were observed for leaf samples collected from the third planting (Experiments E and F, Table 3.3) for either cultivar by the last sampling time (R5 stage).

C. kikuchii DNA was detected in all samples of all three plantings from V3 through R6. In general, the fungal DNA levels were lower in sprayed lines compared to non-sprayed controls. In the first planting, the amount of *C. kikuchii* DNA in soybean leaves collected from non-sprayed plants from R2 to R4 significantly increased from 0.02 to 1.32 pg and 0.11 to 1.86 pg in

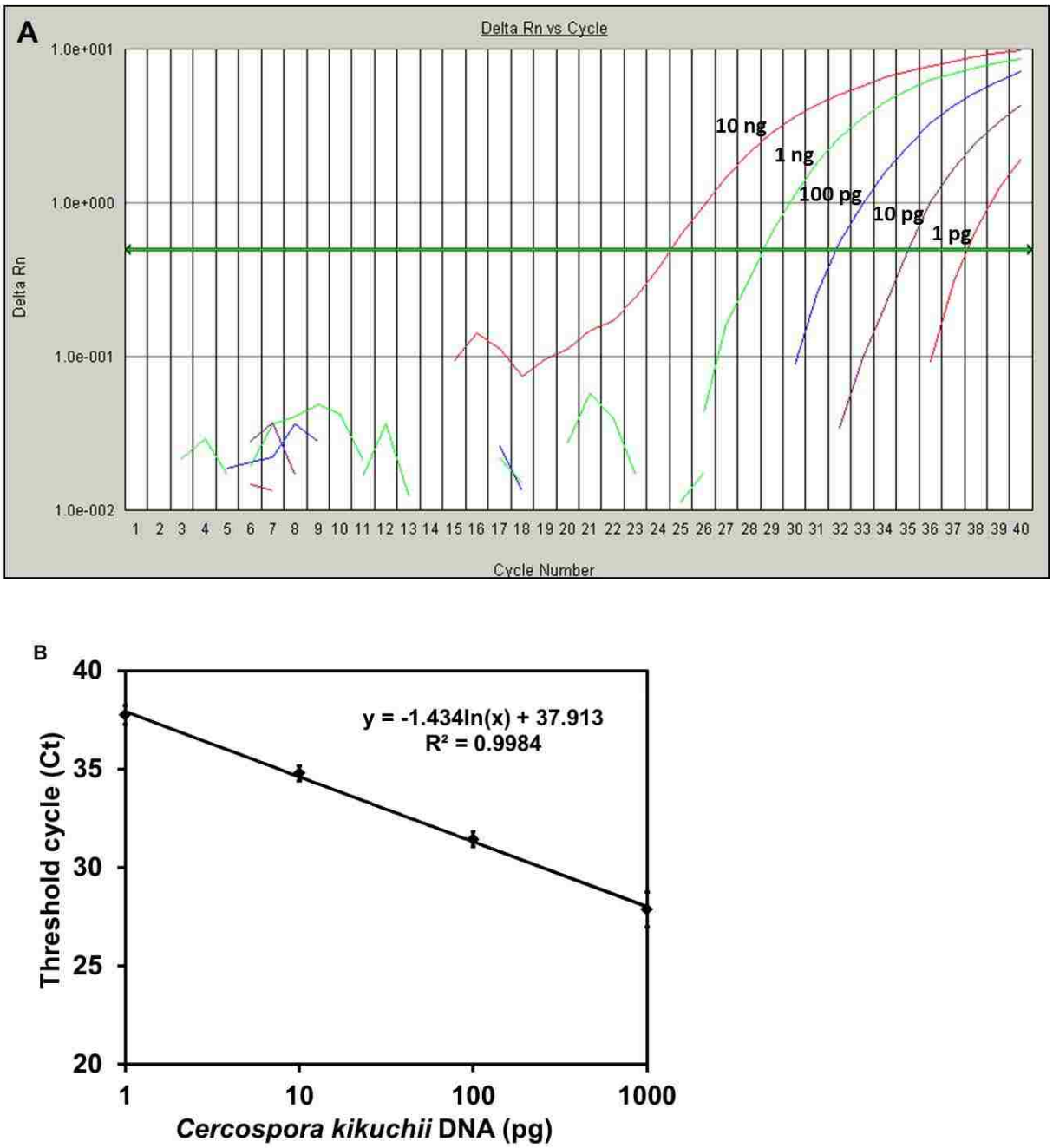


Figure 3.3. Real-time PCR amplification plots and standard curves for serial dilutions of DNA of *Cercospora kikuchii*. **A**, Representative real-time PCR fluorescent amplification curves for *C. kikuchii* DNA concentrations from left to right are 10 ng, 1 ng, 100 pg, 10 pg, and 1 pg. Each standard was spiked with 10 ng of soybean DNA. **B**, Data represent mean cycle threshold (Ct) values of at least six replicates of each dilution and error bars indicate standard deviation. Ct values were plotted against known DNA concentrations of *C. kikuchii* and the linear regression equation for calculating quantity of DNA of *C. kikuchii* in soybean leaf sample is shown inside the graph.

cultivars CP 5892 and DP 5634, respectively; while it remained at low levels in sprayed plants until R4 in both cultivars (Fig. 3.4B). The fungal DNA levels did not change from R4 to R6 in non-sprayed or sprayed plants in both cultivars. Soybean plants receiving two fungicide sprays at 62 DAP (R2) and 78 DAP (R4) had very low levels of fungal DNA as compared to non-sprayed plants (Fig. 3.4B and Table 3.4). In the 2nd planting (Experiments C and D, Table 3.3), the amount of *C. kikuchii* DNA detected in non-sprayed soybean plants from V8-V9 to R6 significantly increased from 0.03 to 1.35 pg in CP 5892 and from 0.1 pg at R2 to 3.12 pg at R6 in DP 5634 (Fig. 3.4C). However, for the fungicide-treated plants, pathogen DNA increased from R5 to R6 in CP 5892 and remained constant in DP 5634. Cultivar DP 5634 had consistently higher levels of fungal DNA as compared to CP 5892 in non-sprayed and sprayed plants at every growth stage (Fig. 3.4C and Table 3.4). For the 3rd planting (Experiments E and F, Table 3.3), DNA of *C. kikuchii* was detected as early as 22 DAP and at V4. Fungal DNA remained low until R3 in both cultivars whether sprayed or non-sprayed but increased from R3 to R5 (Fig. 3.4D and Table 3.4). The levels of *C. kikuchii* detected at R5 were lower than those of soybean plants at R5 in the first and second plantings. For CP 5892, the levels of *C. kikuchii* detected in non-sprayed plants was higher than sprayed plants at R5, but there was no significant difference for DP 5634.

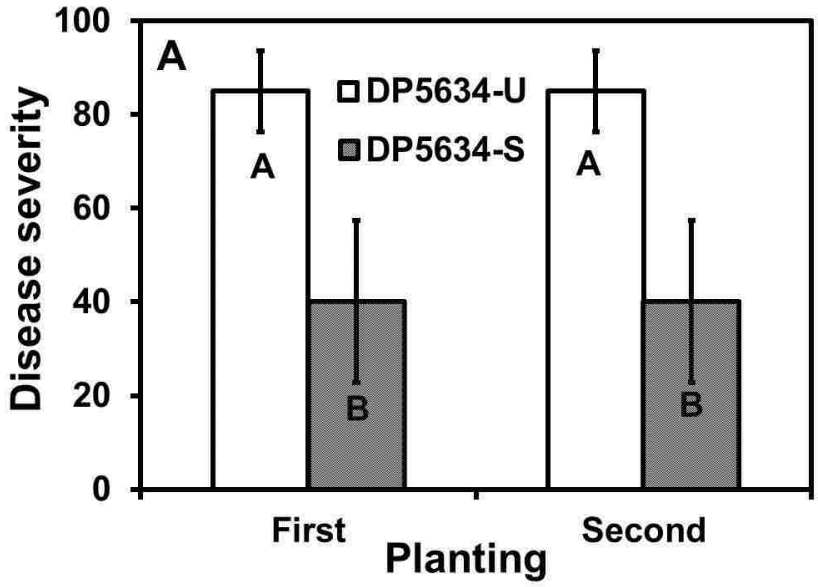
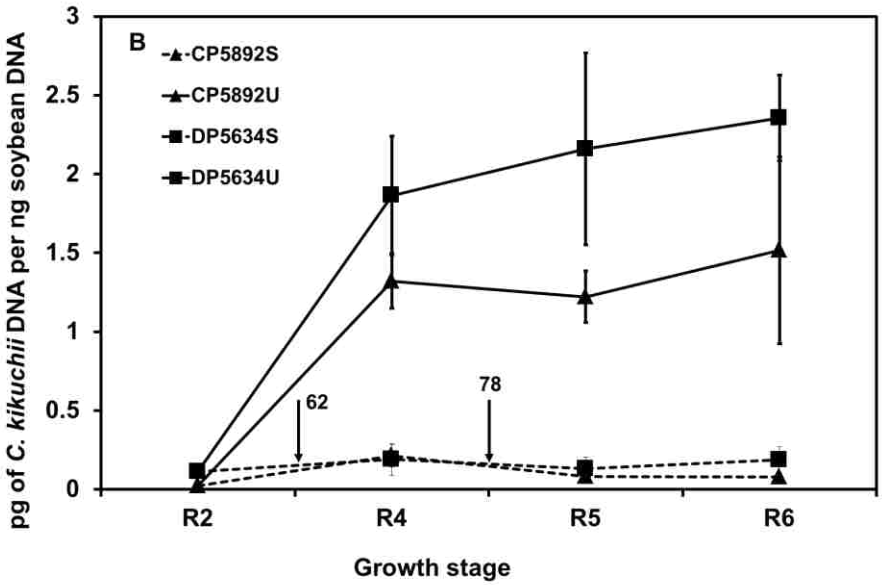


Figure 3.4. Cercospora leaf blight disease severity ratings and quantification of levels of DNA of *Cercospora kikuchii* in leaves of soybean cultivars CP5892 and DP5634 in 2007 in non-sprayed (U) and sprayed (S) plants. Arrows indicate the days after planting (DAP) when fungicides were applied. **A**, Bars represent the mean disease severity of 3 plots within each treatment and the error bars indicate standard deviation. The groups of means indicated with the same letters were not significantly different according to Tukey's honestly significant difference (HSD) test at $P < 0.05$. **B**, First planting. Soybean leaf samples were collected at R2 (58 DAP), R4 (78 DAP), R5 (85 DAP), and R6 (107 DAP) growth stages. In sprayed treatments, a mixture of pyraclostrobin and metconazole was applied at R2 (62 DAP) and R4 (78 DAP) growth stages. Error bars indicate standard deviation. **C**, Second planting. Soybean leaf samples were collected at V8-V9 for CP 5892 and R2 for DP 5634 (44 DAP), R3-R4 (64 DAP), R5 (71 DAP), and R6 (93 DAP) growth stages. In sprayed treatments, a mixture of pyraclostrobin and metconazole was applied at R3-R4 (64 DAP) growth stage. Error bars indicate standard deviation. **D**, Third planting. Soybean leaf samples were collected at V3 for CP 5892 and V3-V4 for DP 5634 (22 DAP), R1-R2 for CP 5892 and R2 for DP 5634 (42 DAP), R2 (49 DAP), R3 (57 DAP), and R5 (71 DAP) growth stages. In sprayed treatments, a mixture of pyraclostrobin and metconazole was applied at R1-R2 (42 DAP) growth stage.

Figure 3.4. continued



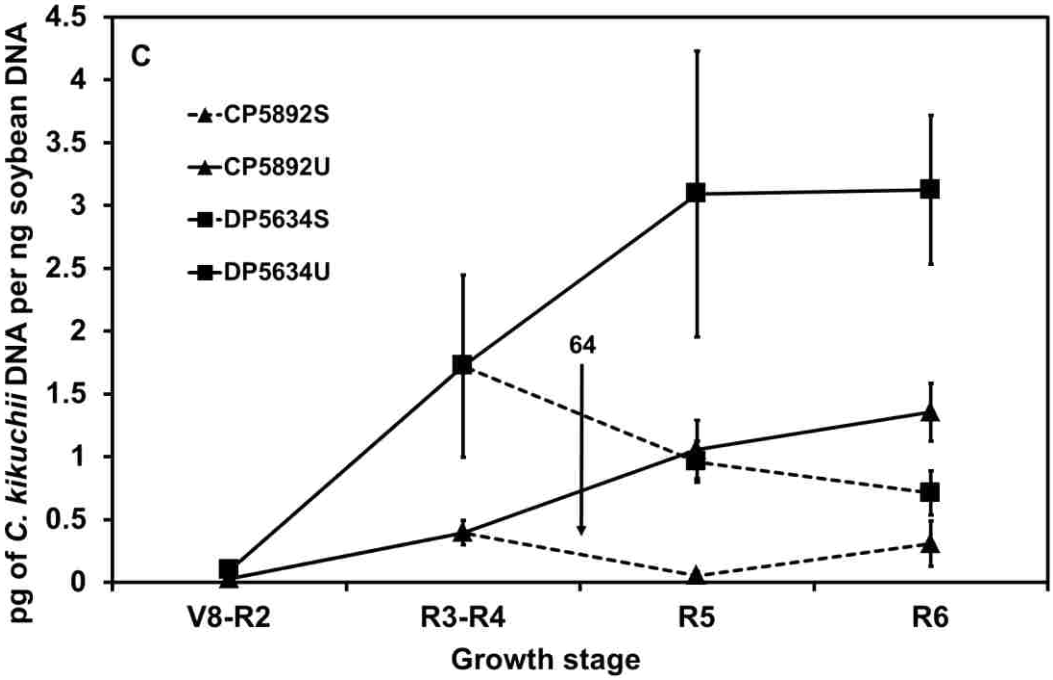


Figure 3.4. continued

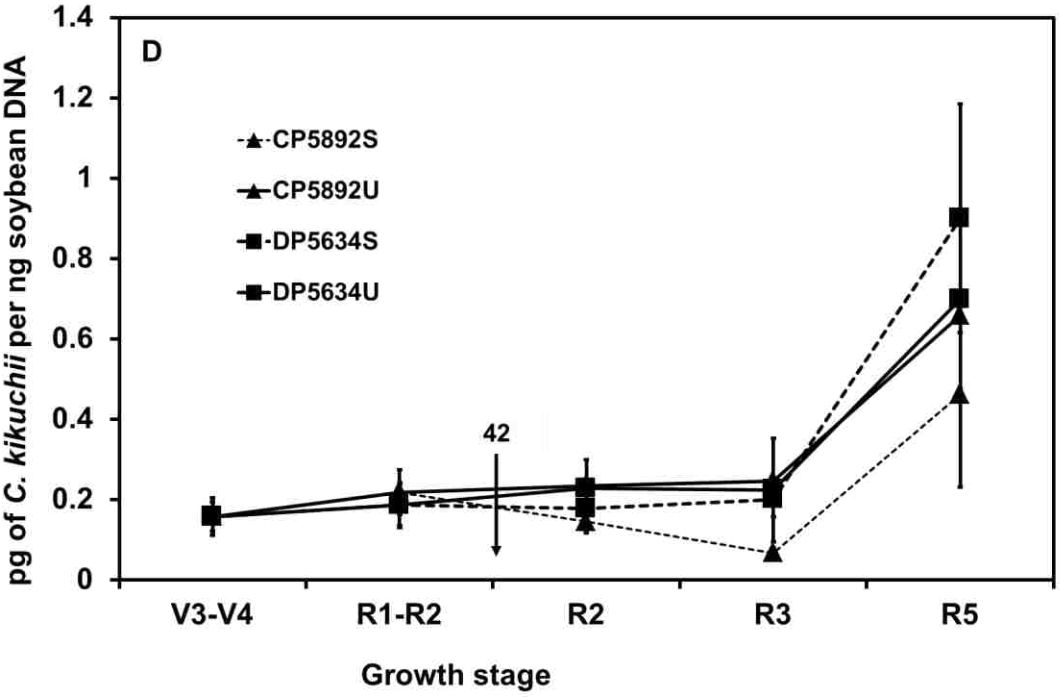


Table 3.4. *Cercospora kikuchii* infection of soybean leaf samples collected at various growth stages in 2007.

Planting	DAP[x]	DNA of *C. kikuchii* (pg/ng soybean DNA)					
		CP 5892			DP 5634		
		Growth Stage	Non-sprayed[y]	Sprayed	Growth Stage	Non-sprayed	Sprayed
First	58	R2	0.02 b	-	R2	0.11 b	-
	78	R4	1.32 a	0.21 b	R4	1.86 a	0.19 b
	85	R5	1.22 a	0.08 b	R5	2.16 a	0.13 b
	107	R6	1.52 a	0.08 b	R6	2.36 a	0.19 b
Second	44	V8-V9	0.03 de	-	R2	0.1 c	-
	64	R3-R4	0.4 c	-	R4	1.72 b	-
	71	R5	1.06 b	0.06 e	R5	3.09 a	0.96 bc
	93	R6	1.35 a	0.31 cd	R6	3.12 a	0.71 c
Third	22	V3	0.16 c	-	V3-V4	0.16 b	-
	42	R1-R2	0.22 bc	-	R2	0.19 b	-
	49	R2	0.23 bc	0.14 bc	R2	0.23 b	0.18 b
	57	R3	0.25 bc	0.07 c	R3	0.22 b	0.2 b
	71	R5	0.66 a	0.46 ab	R5	0.7 a	0.9 a

[x] DAP, days after planting.
[y] Means followed by the same letter in non-sprayed and sprayed treatments in a given cultivar and given planting were not significantly different according to the Tukey's honestly significant difference (HSD) test at $P < 0.05$.

In 2009 (Experiment G, Table 3.3), there was no significant difference in CLB severity between sprayed and non-sprayed plants for DP 5808RR (Fig. 3.5A). There was no significant change in fungal DNA from V4 to R4 in non-sprayed plants and from V4 to R5 in sprayed plants (Fig. 3.5B). However, fungal DNA significantly increased from 1.15 pg at late R4 to 20.4 pg at R7 in non-sprayed plants and significantly increased from 2.41 pg at R6 to 11.67 pg at R7 in sprayed plants. Non-sprayed plants were about 7 to 10 days ahead of sprayed plants with regard to development of *C. kikuchii* at R6 and R7. There was almost a two-fold increase in fungal DNA in non-sprayed plants as compared to sprayed plants at R7 (Fig. 3.5B and Table 3.5).

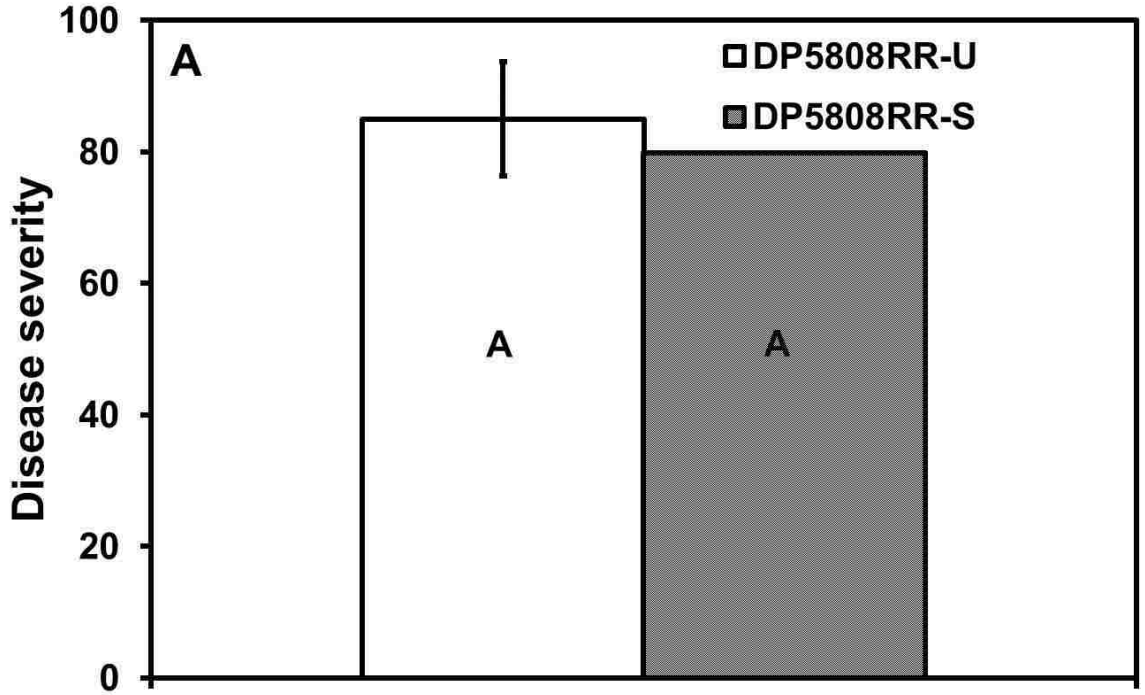


Figure 3.5. Cercospora leaf blight disease severity ratings and quantification of levels DNA of *Cercospora kikuchii* in leaves of soybean cultivar DP 5808RR in 2009. Arrows indicate the days after planting (DAP) when fungicides were applied. **A**, Bars represent the mean disease severity of 3 plots within each treatment and the error bars indicate standard deviation. Letters represent groups of means that were determined according to Tukey's honestly significant difference (HSD) test. Different letters indicate significant differences at $P < 0.05$. **B**, Soybean leaf samples were collected at V4 (35 DAP), Late R1 (52 DAP), R2 (59 DAP), R3 (70 DAP), R4 (76 DAP), Late R4 (85 DAP), R5 (97 DAP), R6 (109 DAP), Late R6 (116 DAP), and R7 (124 DAP) growth stages. In sprayed treatments, pyraclostrobin was applied at R1 (48 DAP), R2 (55 DAP), R3 (74 DAP), R4 (90 DAP), and R5 (103 DAP) growth stages. Error bars indicate standard deviation.

Figure 3.5. continued

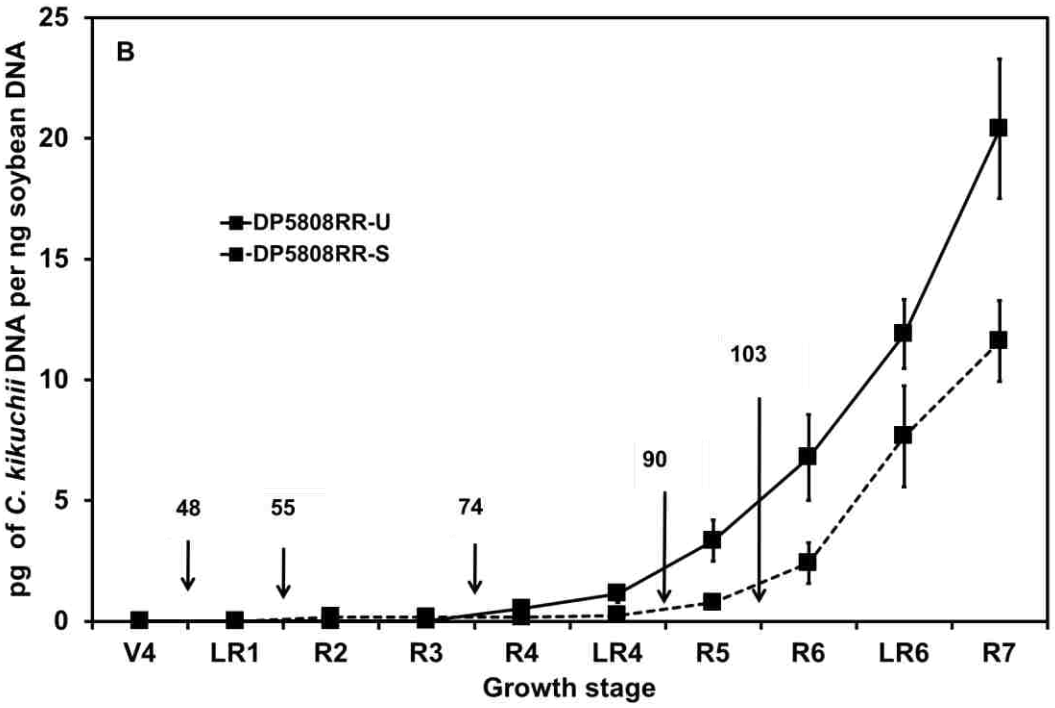


Table 3.5. *Cercospora kikuchii* infection of soybean leaf samples collected from DP 5808RR at various growth stages in 2009.

DAP[x]	Growth Stage	DNA of *C. kikuchii* (pg/ng soybean DNA)	
		Non-sprayed[y]	Sprayed
35	V4	0.015 e	0.014 e
52	Late R1	0.015 e	ND[z]
59	R2	0.017 e	0.184 e
70	R3	0.028 e	0.171 e
76	R4	0.528 e	0.165 e
85	Late R4	1.153 de	0.248 e
97	R5	3.34 d	0.781 e
109	R6	6.79 c	2.414 d
116	Late R6	11.9 b	7.669 c
124	R7	20.4 a	11.607 b

[x] DAP = days after planting,.

[y] Means followed by the same letters in non-sprayed and sprayed treatments were not significantly different according to Tukey's honestly significant difference (HSD) test at $P < 0.05$.

[z] ND, not detected.

In 2010, CLB symptoms were more severe in non-sprayed plants and plants with one spray (1S) as compared to plants with three (3S) and five (5S) sprays (Experiments H, I and J, Table 3.3) (Fig. 3.6A). There was no significant change in fungal DNA from V5 to R2 in any treatment. However, at R5, non-sprayed plants had the highest content of DNA of *C. kikuchii* (4.99 pg), followed by plants with one and three sprays (Fig. 3.6B). The least amount of fungal DNA was detected in plants with five sprays (Fig. 3.6B and Table 3.6). There was a significant difference in the amount of fungal DNA between sprayed and non-sprayed plants at R5. In all sprayed plants, there was more than a ten-fold increase in DNA of *C. kikuchii* from R2 to R5 although the rate of increase was much smaller than in the non-sprayed plants (Fig. 3.6B).

3.4 Discussion

CLB has become a serious concern for soybean producers in the southern U.S. in recent years because most soybean cultivars are susceptible (Moore and Wolcott 2000). Given the lack of disease resistant cultivars, the use of fungicides has become the primary means by which this disease is managed. However, management of CLB with fungicides has been less than satisfactory even though *C. kikuchii* is sensitive to commonly used fungicides in *in vitro* tests (Robertson et al. 2011). We hypothesized that this apparent inconsistency may be attributed to a long latent period during which the pathogen may become established within leaf tissues in sites where fungicide concentration may be too low to suppress subsequent symptom expression. Thus, fungicides would have to be applied during the early stages of latent infection, and there would have to be sufficient residual activity or additional applications to suppress pathogen development during reproductive stages of plant growth. Cerkauskas and Sinclair (1980) first observed the development of *C. kikuchii* lesions on stems and pods of soybean plants

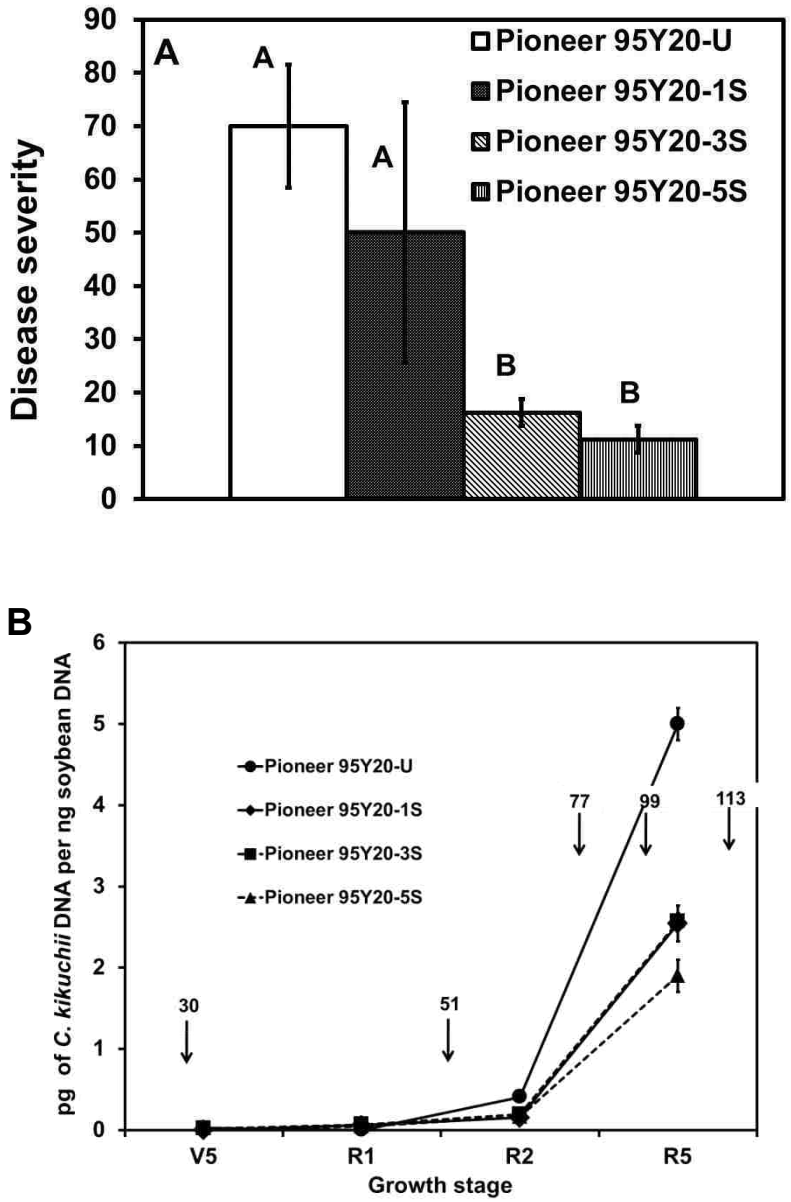


Figure 3.6. Cercospora leaf blight disease severity ratings and quantification of levels of *Cercospora kikuchii* DNA in leaves of soybean cultivar Pioneer 95Y20 in 2010. Arrows indicate the days after planting (DAP) when fungicides were applied. **A**, Bars represent the mean disease severity of 4 plots within each treatment and the error bars indicate standard deviation. Letters represent groups of means that were determined according to Tukey's honestly significant difference (HSD) test. Different letters indicate significant differences at $P < 0.05$. **B**, Soybean leaf samples were collected at V5 (33 DAP), R1 (44 DAP), R2 (58 DAP), and R5 (100 DAP) growth stages. Flutriafol® was sprayed at V5 (30 DAP) growth stage in treatment with one spray (1S); at V5 (30 DAP), R1-R2 (51 DAP), and R3 (77 DAP) in treatment with three sprays (3S); and at V5 (30 DAP), R1-R2 (51 DAP), R3 (77 DAP), R5 (99 DAP), and R6 (113 DAP) growth stages in treatment with five sprays (5S). Error bars indicate standard deviation.

Table 3.6. *Cercospora kikuchii* infection of soybean leaf samples collected from Pioneer 95Y20 at various growth stages in 2010.

Days after planting (DAP)	Growth Stage	DNA of *C. kikuchii* (pg/ng soybean DNA)			
		Non-sprayed[x]	One spray	Three sprays	Five sprays
33	V5	0.023 d	ND	0.015 d	0.021 d
44	R1	ND[y]	0.058 d	0.065 d	0.043 d
58	R2	0.406 d	0.153 d	0.191 d	0.164 d
100	R5	4.995 a	2.543 b	2.563 b	1.898 c

[x] Means followed by the same letters in non-sprayed and sprayed treatments were not significantly different according to Tukey's honestly significant difference (HSD) test at $P < 0.05$.
[y] ND, not detected.

that had been dipped in a solution of the herbicide paraquat 2 weeks before symptoms appeared on non-treated tissues. They suggested that the pathogen exists in a latent state in soybean tissues until the inducement of senescence by the herbicide. Klingelfuss and Yorinori (2001) also observed latent infection of soybeans at the R5 stage and they went on to show that symptoms developed even after three applications of diphenoconazole during the pod filling stage of development (R5.2–R5.5). However, as they did not sample at earlier vegetative or reproductive stages, the leaves may be infected before the R5.2 stage of development. In our study, we documented an extended latent period and showed that fungicides differentially affected pathogen development within the host as a function of time of application. Our findings suggest that it is essential to apply fungicides at the time of infection even though symptoms may not become apparent for several weeks. Results from this study quantified the latent infection process over time using real-time PCR and assessed the effects of fungicide applications on the extent of this process. Furthermore, we showed that cultivar may be a factor in pathogen

development as well. To our knowledge this is the first report of using real-time PCR for detection and quantification of *C. kikuchii* in soybean plants.

The real-time PCR primer set (CKCTB6-2F and CKCTB6-2R) and probe (CKCTB6-PRB), which was based upon the cloned *CTB6* gene sequence from *C. kikuchii*, produced a specific amplicon using genomic DNA from *C. kikuchii* but not from other *Cercospora* species tested in this study. The detection limit was 1 pg of *C. kikuchii* DNA in the presence of 10 to100 ng of soybean DNA. This was equivalent to 34 copies of the genomes, which is based upon an estimate of the genome size of *C. kikuchii* of 28.4 Mb (or equivalent of 0.03 pg) (Hightower et al. 1995). The sensitivity was similar in the absence of soybean DNA. However, in order to simulate soybean leaf samples, the standard curve was constructed with serial dilutions of *C. kikuchii* genomic DNA in the presence of 10 ng of soybean genomic DNA. A similar approach was used in quantifying *Fusarium solani* f. sp. *glycines* in artificially inoculated soybean roots (Li et al. 2008). The real-time PCR protocol developed in this study was quantitative over four orders of magnitude of fungal genomic DNA concentrations, which was similar to results obtained by Guo et al. (2007). The relatively higher detection limit as compared to other studies with femtogram sensitivity may be attributed to the use of internal transcribed spacer (ITS) sequences used in other studies (Pastrik and Maiss 2000) that have far more copies than structural genes, such as the toxin biosynthetic pathway gene (*CTB6*) that we used in this study. Also, using the cercosporin pathway gene was important in differentiating *C. kikuchii* from *C. sojina* because both are capable of infecting soybean, but only *C. kikuchii* is known to produce cercosporin (Goodwin et al. 2001). In addition, a BLAST search done in October 2010 revealed that the ITS1 and ITS2 sequences of *C. kikuchii* share 97 and 100% similarity with *C. sojina*, respectively, and 100% similarity with other species of *Cercospora*, such as *C. beticola*, *C. apii*,

C. capsici, and *C. rodmanii*. These similarities render ITS sequences of *C. kikuchii* of little use for developing species-specific real-time PCR primers.

We showed that *C. kikuchii* was present in soybean leaf samples as early as 22 DAP (V4) in the third planting in 2007, 33 DAP (V5) in 2010, and 35 DAP (V4) in 2009, which were long before the development of symptoms at R6 (107 and 93 DAP in the first and second plantings, respectively, in 2007, 97 DAP in 2009, and 113 DAP in 2010).We documented a general trend of increasing amount of fungal DNA in non-sprayed soybean plants from R2/R3 to R4/R5 during 2007 and 2010 and post R4 during 2009. These findings suggest that latent infection is quiescent until plants begin reproductive growth, at which time there is a steady increase in fungal biomass leading ultimately to symptom development.

Results from this study indicated that latent infection responded differentially to different fungicide combinations. In 2007, the cultivars sprayed with a mixture of pyraclostrobin and metconazole for the first two plantings had significantly lower levels of fungal DNA at later stages of soybean growth as compared to non-sprayed controls. Whereas in 2009, multiple applications of pyraclostrobin alone only delayed the onset of rapid pathogen development by one growth stage as compared to non-sprayed controls. However, in 2010, a single flutriafol application at V5 was enough to limit development of *C. kikuchii* as there was no significant difference in fungal DNA content between the treatment receiving the single V5 spray and the treatment receiving three sprays with the last one being applied at R3. The treatment receiving five sprays, with the last one occurring at R6, had the lowest DNA content at the end of the season. However, three and five fungicide applications are impractical commercial practices for managing CLB. Clearly, fungicides must be applied during early latent stages in order to

suppress pathogen development, and Robertson et al. (2011) demonstrated that application of flutriafol and other fungicides during late vegetative stages provided superior disease control.

It is likely that fungicidal control of other diseases with protracted latent infection periods would benefit from similar studies. For example, Ward et al. (2011) showed that the soybean rust pathogen, *Phakopsora pachyrhizi*, also has an extended latent period and that fungicide applications are most efficacious when applied shortly before the rapid increase in fungal biomass, which occurs about 2 weeks before pustules become evident. Guo et al. (2006) detected *Mycosphaerella graminicola* DNA in wheat two weeks before symptoms were visible.

With the exception of the DP 5634 cultivar in 2007, there was not a clear relationship between DNA content of *C. kikuchii* and disease severity. This may be attributed to the effects of environment on symptom expression. For example, CLB is generally most severe when plants are exposed to hot, dry conditions late in the season. It is possible that the pathogen is induced to produce more cercosporin in the plant, or the plant may be more sensitive to the toxin under these conditions even though fungal biomass may not be affected. These relationships warrant further study.

CHAPTER 4: IDENTIFICATION AND CHARACTERIZATION OF LIGHT-INDUCIBLE PROTEINS FROM *CERCOSPORA KIKUCHII*

4.1 Introduction

Cercospora kikuchii (T. Matsumoto & Tomoy.) M. W. Gardner causes purple seed stain (PSS) and cercospora leaf blight (CLB) in soybeans (Orth and Schuh 1994). *C. kikuchii* and at least 22 other *Cercospora* spp. (Assante et al. 1977) produce a non-hostspecific phytotoxin known as cercosporin. Kuyama and Tamura (1957) isolated this deep red pigment from dried mycelia of *C. kikuchii*. Kuyama (1962) described the nature of aromatic ring of cercosporin. The chemical structure of cercosporin was elucidated to be 1,12-bis(2-hydroxypropyl)-2,11-dimethoxy-6,7-methylenedioxy-4,9-dihydroxyperylene-3,10-quinone ($C_{29}H_{26}O_{10}$) (Lousberg et al. 1971; Yamazaki and Ogawa 1972). Cercosporin is a photosensitizer that absorbs light energy and converts to energetically activated triplet state (^3S), which in turn reacts with molecular oxygen to generate superoxide ions ($O_2^{-\cdot}$) or may react directly with oxygen by an energy transfer process to generate singlet oxygen ($^1O_2$) (Daub and Ehrenshaft 1997). Cercosporin with photosensitizing activity can cause peroxidation of membrane lipids, leading to membrane breakdown and cell death (Daub and Briggs 1983; Macri and Vianello 1979).

Cercosporin production is highly affected by environmental factors such as light, nutrient conditions, temperature, and culture age (Chung 2003; Daub and Ehrenshaft 2000; Ehrenshaft and Upchurch 1991; Jenns et al. 1989). Light is not only essential for cercosporin production but also for activation (Daub 1982). Cercosporin production is stimulated by light of 450-490 nm in wavelengths (Lynch and Geoghegan 1979) and *C. kikuchii* isolates grown in continuous light accumulated more cercosporin than when grown in continuous dark (Ehrenshaft and Upchurch 1991; Fajola 1978; Rollins et al. 1993).

Cercosporin plays a critical role in the disease development by *Cercospora* species that synthesize the toxin. Light intensity and day length were strongly correlated with disease severity caused by *Cercospora* species in coffee (Daub and Ehrenshaft 1997), banana (Calpouzos 1966), and sugar beet (Calpouzos and Stalknecht 1967). Cercosporin has been shown to be the crucial pathogenicity factor for *C. kikuchii* (soybean), *C. nicotianae* (tobacco), and *C. zeae-maydis* (maize) and gene disruption mutants [*CFP* disruption mutants in *C. kikuchii* (Callahan et al. 1999), *CZK3* disruption mutants in *C. zeae-maydis* (Shim and Dunkle 2003), *CTB1* disruption mutants in *C. nicotianae* (Choquer et al. 2005)] showed reduced or abolished cercosporin production and produced fewer or no lesions when inoculated on their respective host plants. Using restriction enzyme mediated insertional mutagenesis (REMI), the cercosporin toxin biosynthesis (CTB) gene cluster harboring 8 genes *CTB1* to *CTB8*, was identified in *C. nicotianae* (Chen et al. 2007b). Suppressive subtractive hybridization (SSH) was used to identify genes involved in cercosporin biosynthesis in *C. kikuchii* (Ehrenshaft and Upchurch 1991) and *C. zeae-maydis* (Shim and Dunkle 2002). However, so far the genes identified are very different in *C. kikuchii*, *C. zeae-maydis*, and *C. nicotianae* indicating that cercosporin biosynthesis is very complex at physiological and genetic levels (You et al. 2008) and also involves calcium signal transduction pathways (Chung 2003)

To aid in developing resistance in plants to *Cercospora* spp. that require cercosporin as a pathogenicity factor, our primary goal is be to identify the genes/proteins that are either directly involved in or regulating cercosporin biosynthesis and conferring resistance to cercosporin. Eventually we can target these genes to suppress the cercosporin production by *Cercospora* spp. in plants by using recent technologies like host-induced gene silencing (HIGS) (Nowara et al. 2010; Tinoco et al. 2010; Yin et al. 2011). In this study, we used 2-dimensional gel

electrophoresis (2-DGE) to identify differentially regulated proteins in *C. kikuchii* grown under continuous light. In this study, we describe the complete cloning and functional characterization of hydroxynaphthalene reductase (*HNR*) and adenosylhomocysteinase (*AHCY*) genes by generating hnr and ahcy disruption mutants, and demonstrated the requirement of *HNR* and *AHCY* genes for fungal pathogenesis of *C. kikuchii* on soybeans.

4.2 Materials and Methods

4.2.1 Fungal Cultures and Cercosporin Determination

Cercospora kikuchii isolate MRL6020-2B was isolated from leaves of naturally infected soybean cultivar SS RT 6299N in Winnsboro, Louisiana (Cai and Schneider 2005). *C. kikuchii* was grown on PDA for 1 week and three 7-mm mycelial plugs (2 mm away from margins) were cut with a cork borer and ground in 2 ml of sterile water in a glass grinder. Two hundred microliters of this mycelial suspension was used to inoculate 250 ml of liquid complete medium [Ingredients per liter were: glucose (10 g); yeast extract (1 g); casein hydrolysate (1 g); $Ca(NO_3)_2.4H_2O$ (1 g); 10 ml of a solution containing 2 g of KH_2PO_4, 2.5 g of $MgSO_4.7H_2O$, and 1.5 g of NaCl in 100 ml of H_2O, adjusted to pH 5.3 with NaOH] (Jenns et al. 1989) in a 500 ml Erlenmeyer flask. Cultures were incubated at 25°C with constant shaking (200 rpm) under light (240 μE $m^{-2}s^{-1}$) or dark (achieved by wrapping flasks with two layers of aluminum foil). Cultures from light and dark were harvested at 4, 6, 8, 10, 12, and 16 days. Entire cultures were blended in Waring blender twice for 20 sec with high speed pulses and 4-ml aliquot of the suspension (mycelium plus medium) was mixed with 4 ml of 5N KOH, incubated in dark for 4 h and cercosporin concentration was determined with spectrophotometer by measuring absorbance of the sample at 480 nm [Molar extinction coefficient of cercosporin in base is 23,300 (Jenns et al. 1989)]. *C. kikuchii* mycelia from the remaining suspension were separated from media by

vacuum filtration. Mycelia were freeze-dried and ground to fine powder using liquid nitrogen and stored at -80°C for further use. The entire experiment was repeated four times.

4.2.2 Protein Extraction

Total proteins were extracted from fungal mycelia using a phenol method followed by methanolic ammonium acetated precipitation (Hurkman and Tanaka 1986). One gram of ground *C. kikuchii* mycelia was transferred to 30 ml oak ridge tube (Nalgene, Rochester, NY) and homogenized with 2.5 ml of Tris pH 8.8 buffered phenol and 2.5 ml of extraction buffer (0.1 M Tris-HCl pH 8.8, 10 mM EDTA, 0.4% 2-mercaptoethanol, 0.9 M sucrose) using a Polytron PT 3100 (Kinematica Inc., Newark, NJ) homogenizer at 10,000 rpm for 1 min. Samples were agitated for 30 min at 4°C and centrifuged at 5000 g for 10 min at 4°C. The phenol layer (top) with proteins was transferred to a second tube. Proteins were re-extracted by adding an equal volume of Tris pH 8.8 buffered phenol to the first tube. After agitation for 30 min and centrifugation at 5000 g for 10 min, the phenol layer (top) was transferred to the second tube. The combined phenol phase was extracted with an equal volume of extraction buffer. The top phenol phase was transferred to a new tube and proteins were precipitated by adding 10 volumes of 0.1 M ammonium acetate in 100% methanol (pre-chilled to -80°C) and tubes were left at -80°C overnight. Proteins were pelleted by centrifugation at 4000 g for 30 min at 4°C and the pellet was washed twice with 0.1 M ammonium acetate in 100% methanol containing 10 mM dithiotheritol (DTT), followed by two washes with ice-cold 80% acetone containing 10 mM DTT. Protein pellets were air dried in a laminar air flow hood and stored at -30°C until further use.

4.2.3 First-Dimension Gel Electrophoresis

Approximately 5 mg protein pellets were solubilized in lysis buffer [8 M urea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS), 40 mM dithiotheritol (DTT), and 2% (w/v) 3-10 NL IPG (Non-linear immobilized pH gradient) buffer (Gorg et al. 1998)] for 30 minutes at room temperature (RT). Samples were centrifuged at 14,000 rpm for 10 min at RT and supernatant was transferred to a fresh 1.5 ml microfuge tube and protein concentration was determined using a protein assay buffer (Bio-Rad) (Bradford 1976). IPG strips (18 cm, pH 3-10 NL, Immobiline^{TM} DryStrip, GE Healthcare) were rehydrated overnight in 340 µl of rehydration buffer (8 M urea, 2% (w/v) CHAPS, 20 mM DTT, 0.002% bromophenol blue, and 0.5% (w/v) 3-10 NL IPG buffer) with 100 µg of protein for silver staining (analytical gels) and 1000 µg of protein for Coomassie Brilliant Blue staining (preparative gels). Strips in the rehydration tray were covered by Immobiline^{TM} DryStrip cover fluid (3 ml per strip) (GE Healthcare, Cat # 17-1335-01), leveled horizontally, and left overnight at RT. Isoelectric focusing (IEF) was done at 20°C using the following conditions: 500 V for 1 h, 1000 V for 1 h and 8000 V for 5 h. The maximum current per strip was limited to 50 µA. The focused IPG strips were equilibrated for 20 min in 7 ml per strip of equilibration buffer I (50 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 20% (w/v) SDS, a trace of bromophenol blue, 1% (w/v) DTT) with gentle shaking, followed by 20 min in 7 ml per strip of equilibration buffer II [50 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 20% (w/v) SDS, a trace of bromophenol blue, 2.5% (w/v) iodoacetamide (IAA)].

4.2.4 Second-Dimension Gel Electrophoresis, Staining, and Gel Analysis

The equilibrated IPG strips were embedded in 1% agarose overlay solution on top of a 12.5% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel [235 mm (width) x 190 mm

(length) x 1.5 mm (thickness)] for the second dimension (Ettan^{TM} DALTtwelve large vertical system, GE Healthcare). Electrophoresis was carried out at 40 V for 1 h and 110 V for 16 h until the bromophenol blue dye reached the bottom of the gel. Proteins were visualized in analytical gels by staining with the Dodeca Silver Stain Kit (Bio-Rad) according to the manufacturer's instructions. Preparative gels were stained with 0.125% (w/v) Coomassie Brilliant Blue (CBB) R-250. All stained gels were scanned using a UMAX PowerLook II scanner (UMAX data systems, Taiwan) and analyzed using the Progenesis SameSpots software (Nonlinear Dynamics, Durham, NC, USA). The 2-DE gels were prepared from four independent biological replicates and only consistent differentially expressed protein spots were processed for identification using LC-MS/MS.

4.2.5 Protein Identification

The selected protein spots were excised from two to three CBB stained gels and were subjected to in-gel trypsin digestion. The digested peptides were subjected to liquid chromatography tandem mass spectrometry (LC-MS/MS) at the Pennington Biomedical Center (Baton Rouge, LA). The gel plugs were destained, reduced, alkylated, and then digested with trypsin at 37ºC overnight using an automated robotic workstation (MassPrep, Waters Corp., Milford, MA). The digested peptide fragments were extracted with 2% (v/v) acetonitrile and 1% (v/v) formic acid and transferred to a 96-well plate for analysis. The peptides from each digested spot were separated by a capillary LC system coupled to a nanospray quadrupole time-of-flight (Q-TOF) tandem mass spectrometer (Waters Corp). Briefly, the peptides were injected onto a 75 µm C_{18} reverse phase capillary column (Dionex) and separated using a gradient of 3 to 40% acetonitrile during a 30 min run. The MS was operated in a data-dependent acquisition mode, in which a full survey of the parental ions was followed by three MS/MS scans using normalized

collision energy. The instrument was operated in positive ion mode, with an electrospray voltage of 3.5 kV, sample cone voltage of 40 V and extraction cone voltage of 1.5 V. The peaklist (pkl) files were generated using ProteinLynx Global Server 2.2.5 (PLGS 2.2.5, Waters Corp.) with default parameters. Tandem mass spectra were searched against the SwissProt database using the PLGS 2.2.5 software (Waters Corp.) with the following settings: one missed tryptic cleavage; precursor-ion mass tolerance, 200 ppm; fragment-ion mass tolerance, 0.1 Da and fixed carbamidomethylation of cysteine residues. Methionine oxidation of proteins was allowed as a variable modification in the database search query in PLGS, and automodification query was selected to identify peptides with further post-translational modifications in PLGS. The top ranking hits (PLGS scores between 8-13) were further evaluated using molecular weight, p*I*, and % sequence coverage to help confirm protein identities. Proteins were identified by searching the non-redundant protein database of the National Center for Biotechnology Information (NCBI), expressed sequence tags (ESTs) from *Cercospora zeae-maydis* with the Mascot search engine (Perkins et al. 1999, http://www.matrixscience.com) and taxonomy limited to fungi. *De novo* sequencing directly from MS/MS data was also done using PEAKS software (Ma et al. 2003) to identify peptides.

4.2.6 Cloning of the *HNR* Gene from *C. kikuchii*

Two degenerate primers were synthesized for spot 57, HNRF (5′-GTIGTIGTBAAYTAYGCIAAY-3′) and HNRR (5′- YTTICCRTTVACCCAYTCICC-3′) [I=Inosine, B=C/G/T, Y= T/C, R=A/G, V=A/C/G], based on peptide sequences VVVNYANS and GEWVNGK, respectively. The resulting 703 bp PCR amplicon was cloned into pCR®2.1-TOPO® TA Cloning vector (Invitrogen, Carlsbad, CA) and sequenced. To clone full length *HNR*, a chromosome library of *C. kikuchii* was constructed using the Universal GenomeWalker™ kit

per the manufacturer's instructions (Clontech Laboratories Inc., Mountain View, CA). To walk upward and downward into unknown genomic regions, primers were synthesized to complement the known regions (HNRL1: 5′- aatgtcaactggctgtccaacacgag-3′; HNRL2: 5′-gaactcacctcatcaacctcgtcatc-3′; HNRR4: 5′-aactcgacatcgtgtgctcaaactct-3′; HNRR3: 5′-gtgctatcaaggagcacggtggagat-3′) and used for two rounds of PCR amplification with adaptor primers (AP1: 5′-gtaatacgactcactatagggc-3′; AP2: 5′- actatagggcacgcgtggt-3′) supplied with the kit. DNA fragments amplified from the library 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 was first performed using the gene-finding software at http://www.softberry.com. The full length *HNR* gene was 862 bp with one intron. Oligonucleotides used for PCR and sequencing were synthesized by Integrated DNA Technologies (IDT, Coralville, IA).

4.2.7 Targeted Disruption of the *HNR* Gene

The *hygromycin* (*HYG*) cassette fused with *HNR* 5′ and 3′ fragments was constructed using three rounds of PCR using a high fidelity DNA polymerase (Roche Applied Science, Indianapolis, IN) according to Yu et al. (2004b). The *HYG* gene encodes a phosphotransferase under the control of the *Aspergillus nidulans* trpC promoter and terminator, conferring hygromycin resistance (pUCATPH vector was kindly provided by Dr. Turgeon, Cornell University). In the first round, *HYG* cassette, 5′ HNR, and 3′ HNR fragments were amplified. The *HYG* cassette (2.5 kb) was amplified from pUCATPH (Lu et al. 1994) with the primers M13R (5′-agcggataacaatttcacacagga-3′) and M13F (5′-cgccagggttttcccagtcacgac-3′). The 5′ HNR fragment (0.8 kb) was amplified by PCR with the primers HNR5F (5′- aacgggcggtgattcttgct-3′) and M13RHNR5R (5′-tcctgtgtgaaattgttatccgctgtccgaatgatacaacgccag-3′) and the 3′ HNR

fragment (1 kb) was amplified with M13FHNR3F (5′-gtccgtgactgggaaaaccctggcgctgttggcggacgaatcatc -3′) and HNR3R (5′- aacggagaagccacacgaa-3′) from the genomic DNA of *C. kikuchii*. The underlined sequence in the primers M13RHNR5R and M13FHNR3F represents the oligonucleotides completely complementary to the sequence of primers M13R and M13F, respectively. In the second round, a fusion PCR was done to combine all three fragments. The long fragment (4.3 kb) was amplified by direct fusion of the 5′ HNR fragment, 3′ HNR fragment, and the *HYG* cassette in equal molar concentration using the end primers HNR5F and HNR3R. In the third round, a nested PCR was done on this using primers HNR5FN2 (5′- gctttcgctctctcgcacat-3′) and HNR3RN2 (5′-agccacacgaataccgcat-3′) to get the final *HNR* gene disruption construct (4.2 kb) (Figure 4.1). Hygromycin resistant colonies were screened by PCR using HYG specific primers HY (5′- ggatgcctccgctcgaagta-3′) and YG (5′-cgttgcaagacctgcctgaa-3′). The primer HNR5F was paired with the primer HY (5′-ggatgcctccgctcgaagta-3′) in PCR to validate site- specific integration. Presence of *HYG* in the middle of the *HNR* gene was confirmed by PCR using primer pairs HNR5F and HNR3R.

4.2.8 Cloning of the *AHCY* Gene from *C. kikuchii*

Two degenerate primers were synthesized for spot 34, AHCYF (5′-TAYAARATGYTNAARAAYAA-3′) and AHCYR (5′-ARYTTNGCRTTNACRTGRTCNA-3′) [Y= T/C, R=A/G, N=A/G/C/T], based on the peptide sequences, YRMLKNK and LEHVNAKL, respectively. The resulting 842 bp PCR amplicon was cloned in to pCR®2.1-TOPO® TA Cloning vector (Invitrogen, Carlsbad, CA) and sequenced. To clone full length *AHCY*, a chromosome library of *C. kikuchii* was constructed as described above. To walk upward and downward into unknown genomic regions, primers were synthesized to complement the known regions (AHCYL1: 5′-gcttgataggatcgatctcagtaacg-3′; AHCYL2: 5′-gcaatcgatctcagtaacgagcacac-3′;

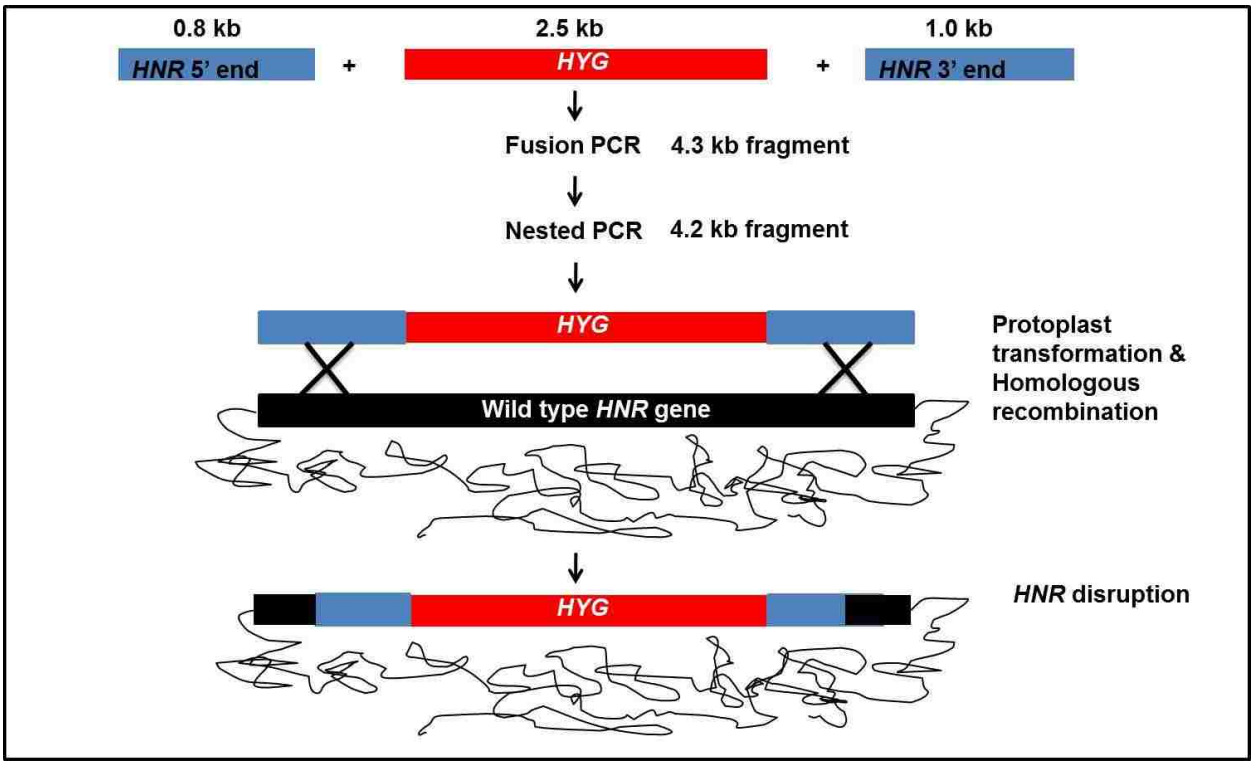


Figure 4.1. Hydroxynaphthalene reductase (*HNR*) disruption strategy in *Cercospora kikuchii*.

AHCYR4: 5′-gc ttactgagatcgatcctatcaacg-3′; AHCYR3: 5′-gcagagcaagtttgacaacctctacg-3′) and used for two rounds of PCR amplification with adaptor primers (AP1: 5′-gtaatacgactcactatagggc-3′; AP2: 5′-actatagggcacgcgtggt-3′) supplied with the kit. DNA fragments amplified from the library 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 was first performed using the gene-finding software at http://www.softberry.com. The full length *AHCY* gene was 1562 bp with two introns. Oligonucleotides used for PCR and sequencing were synthesized by Integrated DNA Technologies.

4.2.9 Targeted Disruption of the *AHCY* Gene

The *HYG* cassette fused with *AHCY* 5′ and 3′ fragments was constructed using three rounds of PCR using a high fidelity DNA polymerase (Roche Applied Science, Indianapolis, IN) according to Yu et al. (2004b). The *HYG* gene encodes a phosphotransferase under the control of the *Aspergillus nidulans* trpC promoter and terminator, conferring hygromycin resistance. In the first round, *HYG* cassette, 5′ AHCY, and 3′ AHCY fragments were amplified. The *HYG* cassette (2.5 kb) was amplified from pUCATPH (Lu et al. 1994) with the primers M13R (5′-agcggataacaatttcacacagga-3′) and M13F (5′-cgccagggttttcccagtcacgac-3′). The 5′ AHCY fragment (1.4 kb) was amplified by PCR with the primers AHCY5F (5′- gcatagcgtgggaggatgtat-3′) and M13RAHCY5R (5′-ttcctgtgtgaaattgttatccgcttcaaacttgctcttggtgacg-3′) and the 3′ AHCY fragment (1.4 kb) was amplified with M13FAHCY3F (5′-gtcgtgactgggaaaaccctggcgccgtgtgctcgttactgaga-3′) and AHCY3R (5′-gttgaagaacggacagcgaa-3′) from the genomic DNA of *C. kikuchii*. The underlined sequence in the primers M13RAHCY5R and M13FAHCY3F represents the oligonucleotides completely complementary to the sequence of primers M13R and M13F, respectively. In the second round, a fusion PCR was performed to combine all three fragments. The long fragment (5.3 kb) was amplified by direct fusion of the 5′ AHCY fragment, 3′ AHCY fragment, and *HYG* cassette in equal molar concentration using the end primers AHCY5F and AHCY3R. In the third round, a nested PCR was done on this using primers AHCY5FN2 (5′-tatgctggtcgttgagagcg-3′) and AHCY3RN2 (5′-gaaacaggcagaggaaacagt-3′) to get the final *AHCY* gene disruption construct (5.1 kb) (Fig. 4.2). Hygromycin resistant colonies were screened by PCR by using HYG specific primers HY (5′- ggatgcctccgctcgaagta-3′) and YG (5′-cgttgcaagacctgcctgaa-3′). The primer AHCY5F was paired with the primer HY (5′-ggatgcctccgctcgaagta-3′) in PCR to validate site-specific integration.

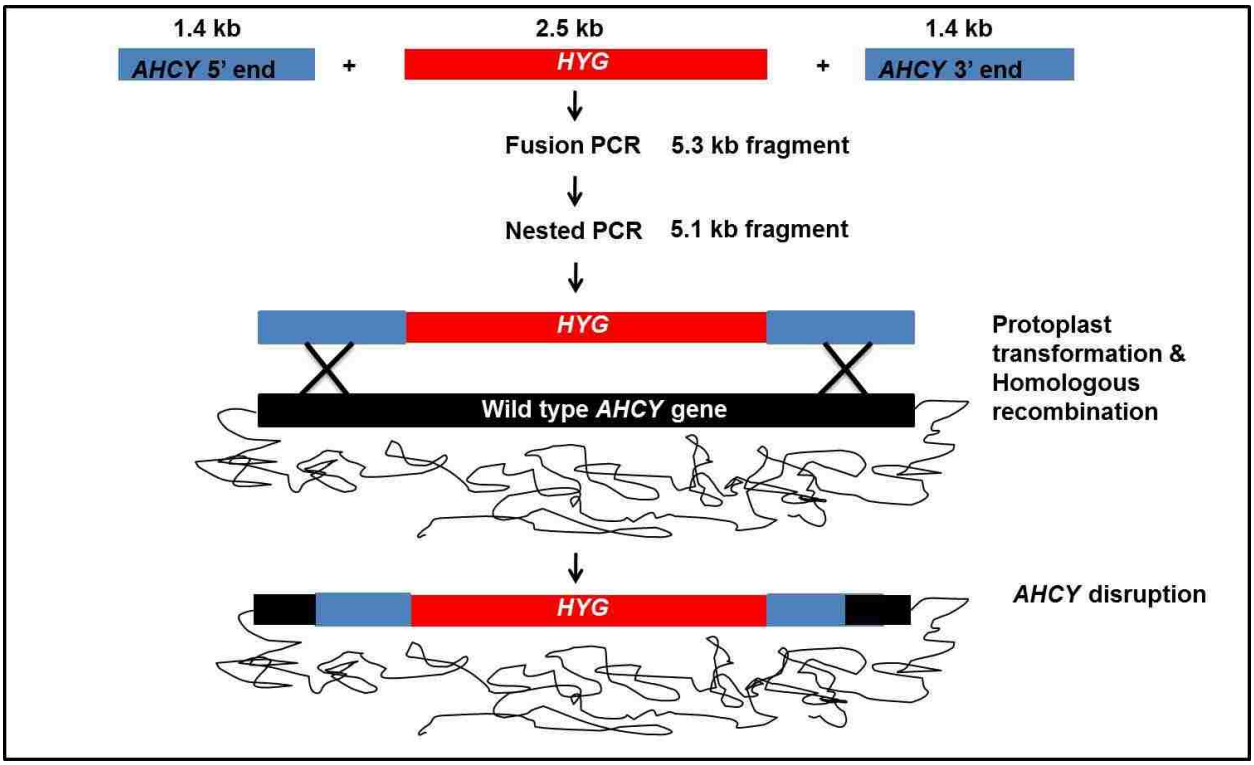


Figure 4.2. Adenosylhomocysteinase (*AHCY*) disruption strategy in *Cercospora kikuchii*.

4.2.10 Preparation of *C. kikuchii* Protoplasts

C. kikuchii protoplasts were prepared as previously described (Chung et al. 2002; Upchurch et al. 1991) with some modifications. Two hundred microliters of mycelial suspension (two 5-mm diameter mycelial agar plugs ground in 2 ml of water) was transferred into 200 ml of complete medium (CM) and grown for 5 days at 25°C with continuous shaking at 180 rpm. Then the culture was blended (two 8-sec cycles) in a Waring blender, and 50 ml of the mycelial slurry was transferred into 200 ml of fresh CM and incubated overnight. Culture flasks were wrapped in two layers of aluminum foil to minimize the production of cercosporin. One gram (fresh wt.) of mycelium was completely resuspended in 40 ml of an enzyme cocktail containing 10 mg/ml Glucanex (Sigma L1412), 5 mg/ml Driselase (Sigma D9515), 1200 U/ml ß-glucuronidase (Sigma G0876), 0.7 M NaCl, 10 mM $CaCl_2$, and 10 mM Na_2HPO_4 (pH 5.8). Digestion was done

at 30°C for 3 h with gentle shaking (90 rpm). 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 0.7 M NaCl followed by two washes with STC buffer (1.2 M sorbitol, 10 mM Tris-HCl pH 7.5, 10 mM $CaCl_2$). Finally protoplasts were adjusted to 10^8 per ml in four parts of STC and one part of 50% PEG (polyethylene glycol 3350, 10 mM Tris-HCl pH 7.5, 10 mM $CaCl_2$), stored as 100 ul aliquots at -80°C.

4.2.11 *C. kikuchii* Transformation

Fungal transformation was performed as described (Turgeon et al. 2010) with modifications. All steps were performed on ice. Approximately $1x10^7$ protoplasts in 100 µl STC/PEG solution were mixed gently with 10 µg of DNA [purified PCR product of *HNR* or *AHCY* gene disruption constructs or pUCATPH plasmid] and incubated on ice for 10 min. Freshly prepared polyethylene glycol (30 g Polyethylene glycol, MW 3,350 (60% w/v), 0.5 ml of 1 M Tris–HCl pH 7.5 (10 mM), 0.37 g $CaCl_2 \cdot 2H_2O$ (50 mM), H_2O to 50 ml) was added in three aliquots of 200, 200, and 800 ul each and mixed well after each addition by rolling. The tubes were incubated for 10 min on ice after the first and second addition and at room temperature after the third addition. Four hundred microliter aliquots were plated in 20 ml molten complete medium (Ingredients per liter: 1 g yeast extract, 1 g casein hydrolysate, 342 g sucrose, and 16 g agar) and incubated overnight in dark at room temperature. The plates were overlaid with 10 ml of 1% agar containing 900 µg/ml Hygromycin B (Sigma H9773). The final concentration of Hygromycin B in the plate was 300 µg/ml. One plate per transformation was kept as no overlay control. Plates were incubated at 25°C and the colonies of transformants that appeared between 6

and 9 days (growing through the Hygromycin B overlay) were selected and transferred to complete media plates containing 300 μg/ml Hygromycin B.

4.2.12 Pathogenicity Assay on Soybeans

For a detached leaf assay, *C. kikuchii* wild type, vector control, hnr and ahcy disruption mutants were grown on CM agar plates for 1 week. Three-millimeter diameter mycelial agar plugs were cut from the plates and placed on the adaxial surface of soybean leaves (collected at the R1 stage from greenhouse grown AG6202 cultivar) with the mycelial side touching the leaf and gently pressed. These leaves were placed on a moist filter paper in a Petri dish and incubated at 25°C with 16 h light and 8 h dark regime and development of chlorotic and necrotic symptoms was observed for 2 weeks and leaves were photographed using a digital camera. The experiment was repeated two times and each time 4 to 5 leaves were used for each control or mutant tested.

For greenhouse inoculations, *C. kikuchii* wild type, vector control, hnr and ahcy disruption mutants were grown on CM agar plates lined with Spectra/Por 1 6-8 kDa MWCO dialysis membrane (VWR labshop, Batavia, IL) placed on top of the medium, for 10 days in dark at 25°C. Mycelia were removed from the top of the dialysis membrane with the help of a forceps. One gram of mycelia (fresh wt.) was blended with 100 ml sterile distilled water in a Waring blender, filtered through three layers of cheese cloth. Tween-20 was added to the filtrate at 0.001% concentration and the filtrate ($1.5x10^4$ CFU/ml) was used to inoculate greenhouse grown soybean variety AG6202 at the R1 stage. Approximately 10 ml filtrate was sprayed on each plant. Immediately after inoculation, the plants were kept in a plastic bag with moist paper towels (to maintain 100 % RH) and kept in dark for 24 h. Later the plants were transferred to greenhouse benches and development of symptoms was observed at 14 days post inoculation

(dpi). The experiment was repeated two times and each time 4 to 5 soybean plants were inoculated for each control or mutant tested.

4.3 Results

4.3.1 Light Enhanced Cercosporin Production in *C. kikuchii*

The *C. kikuchii* isolate MRL 6020-2B used in our study produced high levels of cercosporin in CM broth, whereas it did not produce any detectable levels of cercosporin in potato dextrose broth (PDB) (data not shown). The red color of cercosporin was visible in 4-day-old *C. kikuchii* cultures on CM under continuous light. Spectrophotometric analysis confirmed the production of cercosporin toxin with a characteristic peak of absorbance at 480 nm in 5N KOH (alkali). Dark-grown cultures grown in the dark accumulated significantly less cercosporin compared to those grown in light. On any sampling day after 4 days, cultures in light had up to 6-fold more cercosporin than under dark conditions (Fig. 4.3A). More mycelial growth also was observed in dark-grown cultures compared to light (Fig. 4.3B).

4.3.2 Comparison of Protein Profiles between Light and Dark Grown *C. kikuchii*

2-DE analysis of mycelial proteins extracted at 4, 6, 8, 10, 12, and 16 d from light and dark grown *C. kikuchii* cultures revealed approximately 1100 ± 55 spots from both light and dark samples using silver staining (Fig. 4.4A and 4.4B). Protein profiles from four biological replicates showed reproducible patterns. Protein levels of visible spots were quantified with Progenesis Samespots software after normalization and the average normalized volume of the spots was used for calculating fold change of up- and down-regulation between differentially expressed proteins grown under light and dark during the time-course experiment (Fig. 4.4A, 4.4B, and 4.4C). The up-regulated spots in light include, spot 57: 5-fold at 4 d; spots 34 and 28:

5-fold at 6 d; spot 32: 4-fold at 8 d; spot 129: 4-fold at 4 d; spot 26: >1.5 fold at 4 and 6 d. The down-regulated spots in light include, spot 70: 2.5-fold at 8 d; spot 132: 2-fold at 6 d (Fig 4.4D).

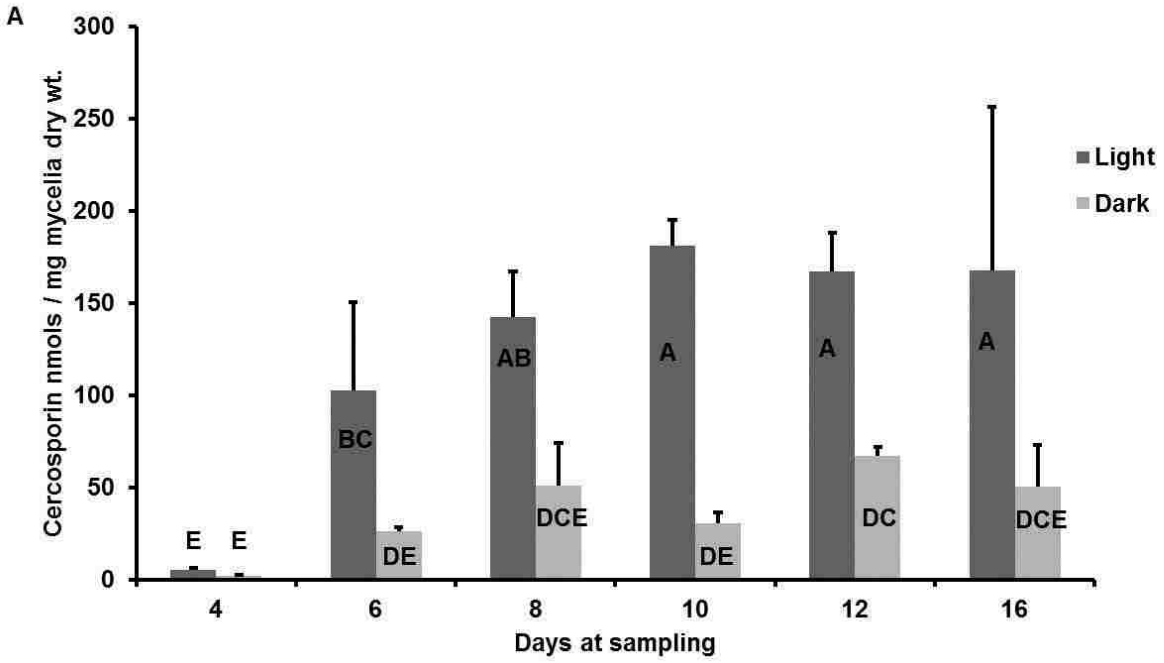
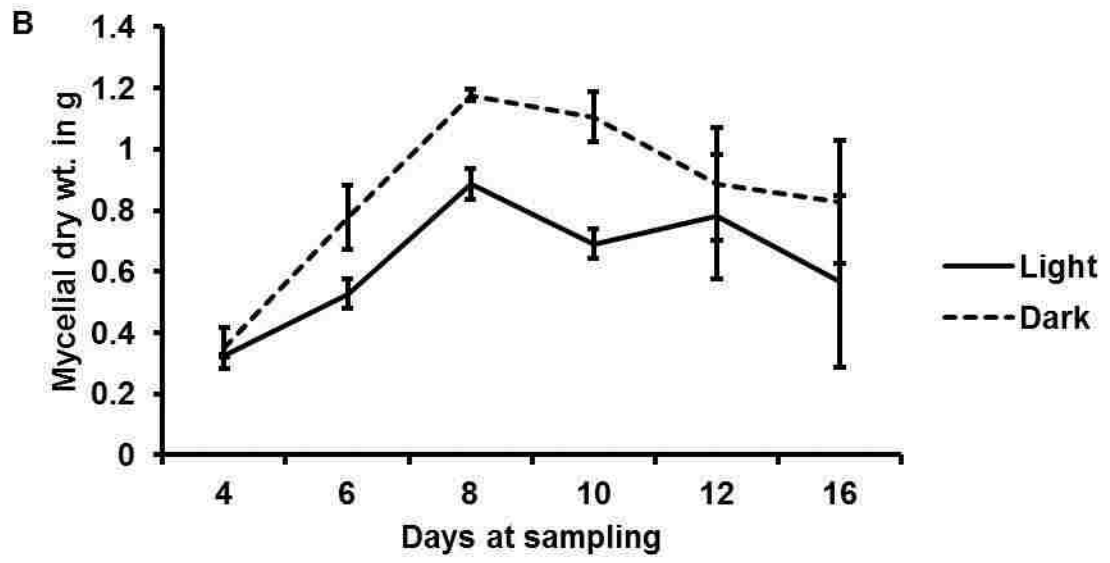


Figure 4.3. Effect of light on Cercosporin production and fungal growth of *Cercospora kikuchii*. **A**, Production of cercosporin toxin by the wild-type *Cercospora kikuchii* isolate MRL 6020-2B. The fungus was grown in CM broth under continuous light or darkness for 4 to 16 days. Cercosporin was extracted with 5 N KOH and quantified by absorbance at 480 nm. Data shown are the means of five biological replications and error bars indicate standard deviation. Means with the same letters were not significantly different at $P < 0.05$ according to Tukey's honestly significant difference (HSD) test. **B**, Fungal growth of *C. kikuchii* under light and dark conditions. Mycelia were separated by vacuum filtration and dry weights were determined after freeze drying. Data shown are the means of five biological replications and error bars indicate standard deviation.

Figure 4.3. continued



4.3.3 Identification of Differentially Expressed Proteins

Six up-regulated and two down-regulated spots visible in Coomassie Brilliant Blue stained gels were recovered and sequenced at Pennington Biomedical Research Center through a fee-based service (Table 4.1). For the hypothetical/predicted proteins, probable function was assigned by considering sequence homology with other species using BLASTP. The presence of highly conserved domains was also taken into consideration using NCBI's Conserved Domain Database (CDD). Peptide sequences of each spot and their sequence homology identified through database searches are summarized in Table 4.1. Peptide sequences of Spot 57 showed high similarity to hydroxynaphthalene reductase (*HNR*) from *Cochliobolus lunatus.* Spot 34 showed homology to adenosylhomocysteinase (*AHCY*) from *Verticillium albo-atrum*. Spots 28 and 26 were identified as S-adenosylmethionine synthetase and superoxide dismutase (mitochondrial precursor), respectively. The peptide sequences from Spots 132 and 70 showed high homology to elongation factor 2 and 60S ribosomal protein L7, respectively.

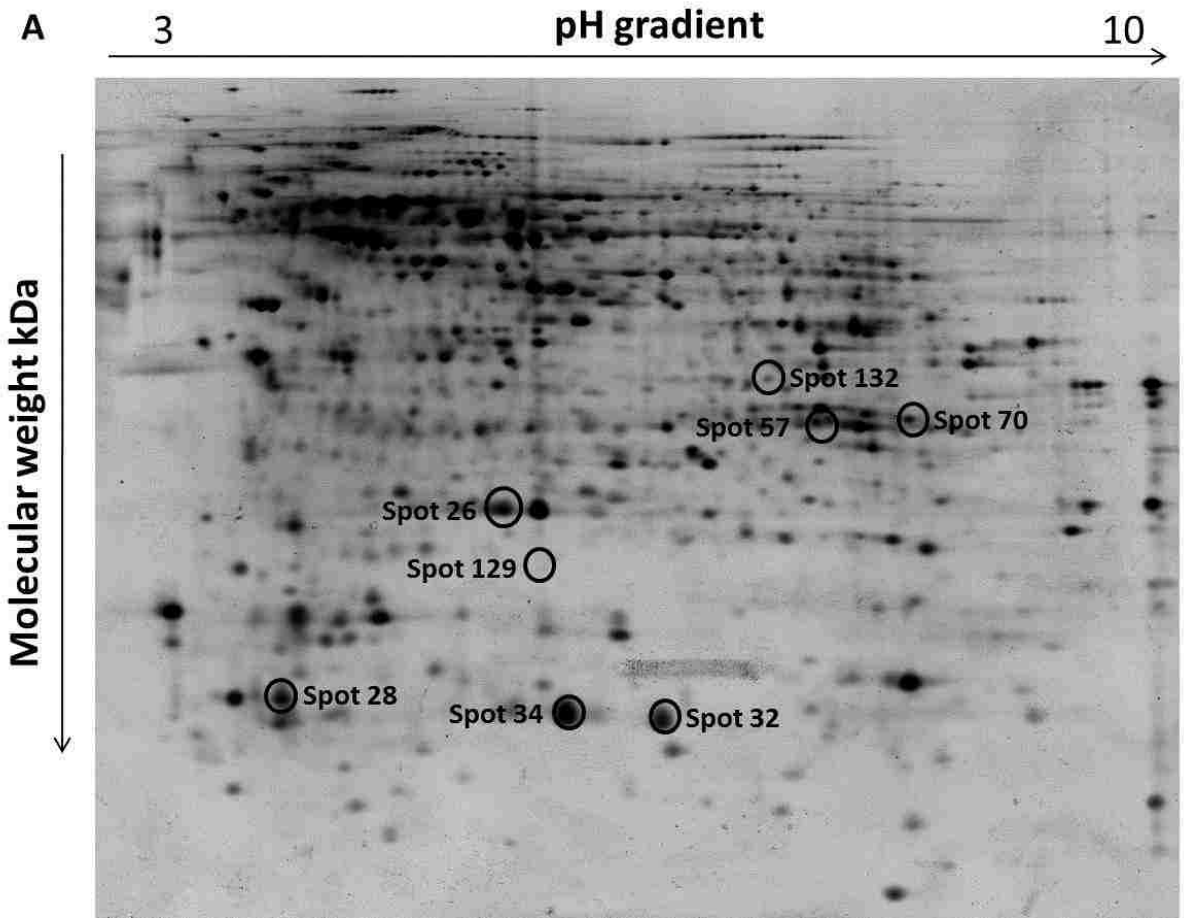


Figure 4.4. Two dimensional gel electrophoresis of proteins extracted from *Cercospora kikuchii* mycelia grown under light and dark conditions. Mycelial protein profiles of *C. kikuchii* grown in continuous light for 6 days (**A**), or under continuous dark for 8 days (**B**). 100 µg mycelia proteins were subjected to IEF on 18 cm 3-10 NL IPG strips followed by second dimension on 12.5% linear SDS-PAGE, and visualization of proteins with silver staining. Differentially expressed protein spots were indicated with open circles and spots were excised from the gels as indicated and subjected to in-gel digestion with trypsin prior to LC-MS/MS analysis. **C**, Gel sub-sections of selective protein spots up- (57 and 34) and down-regulated under light. **D**, Heat map showing fold change up- and down-regulation of protein spots under light.

Figure 4.4 continued

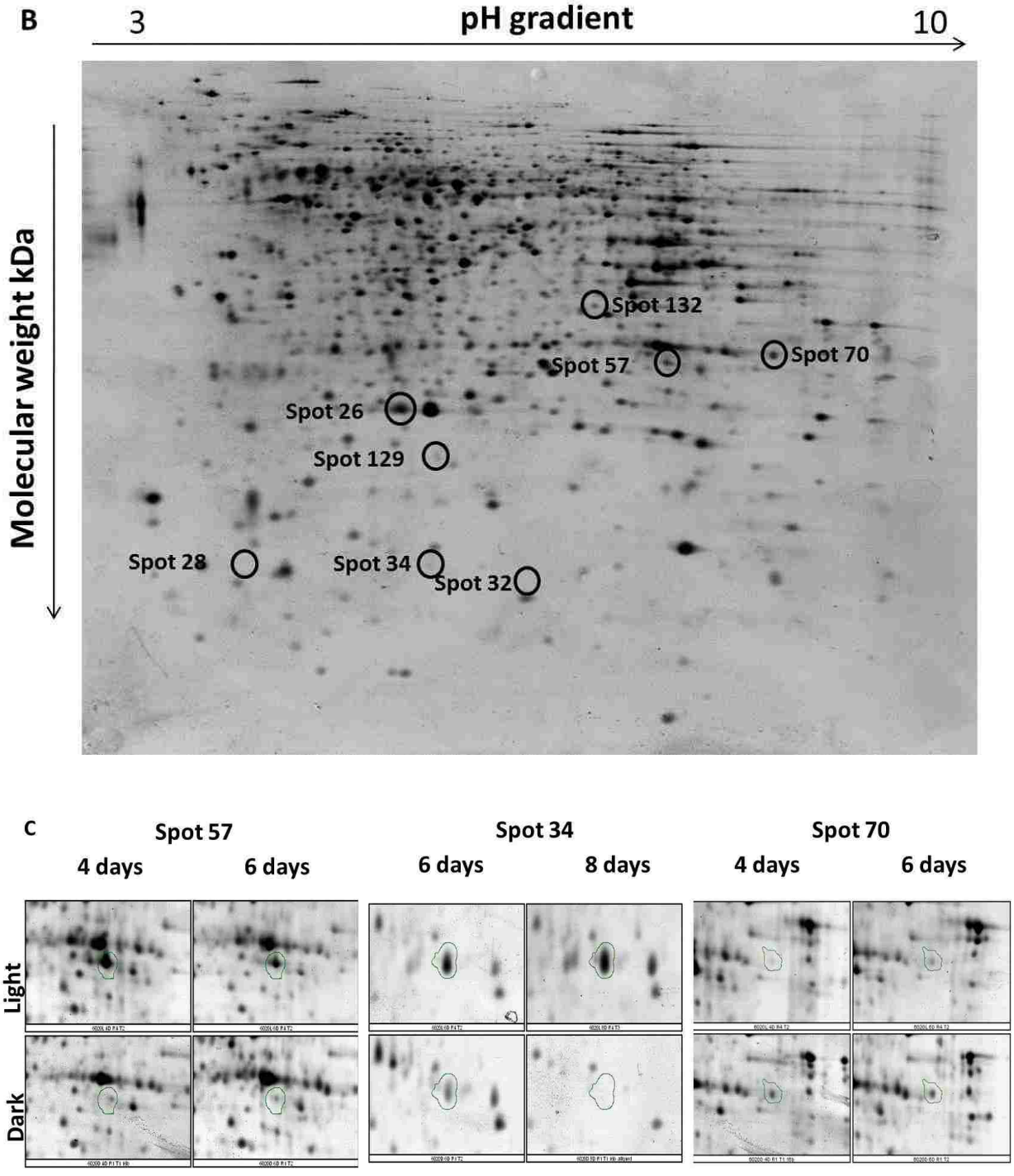
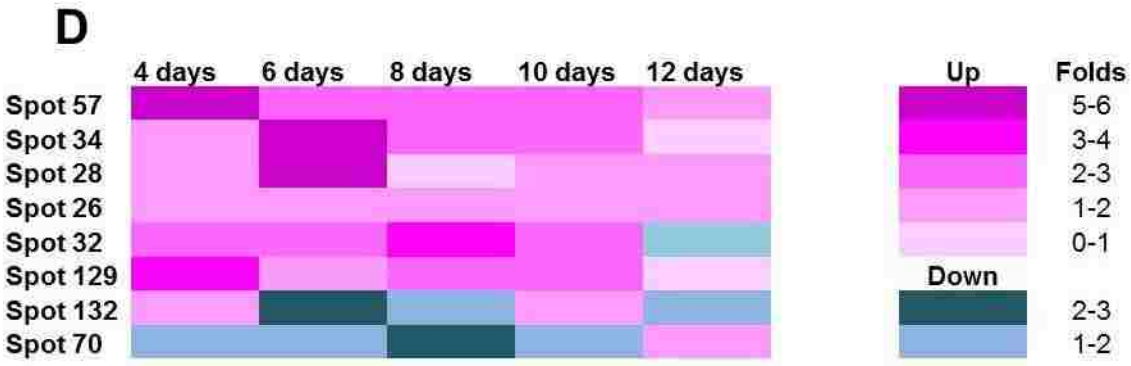


Figure 4.4 continued



4.3.4 Cloning and Characterization of *HNR* and *AHCY* Genes

The 703 bp fragment obtained by PCR using degenerate primers HNRF and HNRR (Fig. 4.5A) showed homology to *Brn1* gene coding for hydroxynaphthalene reductase from *Embellisia hyacinthi* (AB120882.1) and *Cochliobolus lunatus* (AB288866.1). To clone the full length *HNR* gene, a *C. kikuchii* genome library was constructed using the universal GenomeWalker™ kit. Using gene specific primers and adapter primers, 1.8 kb upstream and 2.0 kb downstream fragments were obtained. After sequencing, these 2 fragments were aligned into a 3.2 kb contig. Sequence analysis of this contig revealed the presence of an 862-bp open reading frame with a 52-bp intron (Fig. 4.5B). A transcriptional start site (TSS) was predicted 601 bp upstream of the ATG start codon, and the transcriptional termination site was located 143 bp downstream of the translational stop codon (www.softberry.com). The intron had the characteristic 5′-XX/GT and AG/XX-3′ splicing junctions commonly found in filamentous fungi and also contained an internal lariat consensus sequences CT(A/G) A (T/C) (Radford and Parish 1997). The *HNR* gene was predicted to encode a polypeptide of 269 amino acids (aa) with a predicted molecular mass of 28.6 kDa and a predicted isoelectric point (p*I*) of 5.71. BlastP search (Altschul et al. 1997) of

Table 4.1. Identification of differentially expressed protein spots from *Cercospora kikuchii* grown under continuous light and dark through peptide sequencing using tandem mass spectrometry (MS/MS).

Spot	Top Hit	Organism	NCBI accession number	p*I*$_w$	MW (kDa)[x]	E-value	Peptide Sequence
57	Hydroxynaphthalene reductase	*Cochliobolus lunatus*	AF419330.1	7.0	31	1e-95	VVVNYANS[z] DVTEEFDR[z] LGSDA[z] LVSAEGEWLNGK[z]
34	Adenosylhomocysteinase	*Verticillium albo-atrum*	XP_003009749.1	5.6	17	1e-07	KIAVVAGFGDVGKG[y] KHIILLAEGRL[y] HLLLLAEGR[z]
28	Predicted protein (S-adenosylmethionine synthetase)	*Mycosphaerella graminicola*	EGP86389.1	5.0	17	9e-16	VACETATKTGMVMVFGEITTKS[y] EALKDLGYDDSAL[z]
26	Hypothetical protein (superoxide dismutase, mitochondrial precursor)	*Mycosphaerella graminicola*	EGP88469.1	5.5	24	1e-17	KETMEIHYTKH[y] KHHQTYVNNLNNLIKG[y]
132*	Hypothetical protein (similar to Elongation factor 2)	*Botryotinia fuckeliana*	XP_001551461.1	6.3	33	6e-14	RILADNHGWDVTDARK[y] KAVQYLNEIKD[y] REGPIGEEPMRS[y] REGPIGEEPMRS[y] KDSVVSGFQWATKE[y] KAYLPVNESFGFNGELRQ[y] RKIFAFGPDTNGANLLVDQTKA[y]
70*	Similar to 60S ribosomal protein L7	*Leptosphaeria maculans*	CBY00501.1	8.3	31	5e-13	KHFIEGGDLGNRE[y] KQASNFLWPFKL[y] RLLQINNGVFIRV[y] KIVEPFVAYGYPNLKS[y]

*: Indicates spots that were down-regulated under continuous light; the spots were up-regulated under continuous light

[w]: Experimental pI

[x]: Experimental MW

[y]: Peptides identified using MASCOT

[z]: Peptides identified using PEAKS

the National Center for Biotechnology Information (NCBI) protein database revealed that the predicted 269-aa translation product of *HNR* had high similarity to a hypothetical protein from *Mycosphaerella graminicola* (overall 88% identity, 94% similarity), an ESC reductase from *Elsinoe fawcetti* (overall 84% identity, 92% similarity), and a hydroxynaphthalene reductase from several fungi (maximum overall 78% identity, 87% similarity) (Fig. 4.5C). Analysis of the predicted translational product of the *C. kikuchii HNR* gene revealed the presence of a tetrahydroxynaphthalene/trihydroxynaphthalene reductase-like classical (c) SDR domain, 3-ketoacyl-(acyl-carrier-protein) reductase domain, and a NADP binding site (Rossmann superfamily).

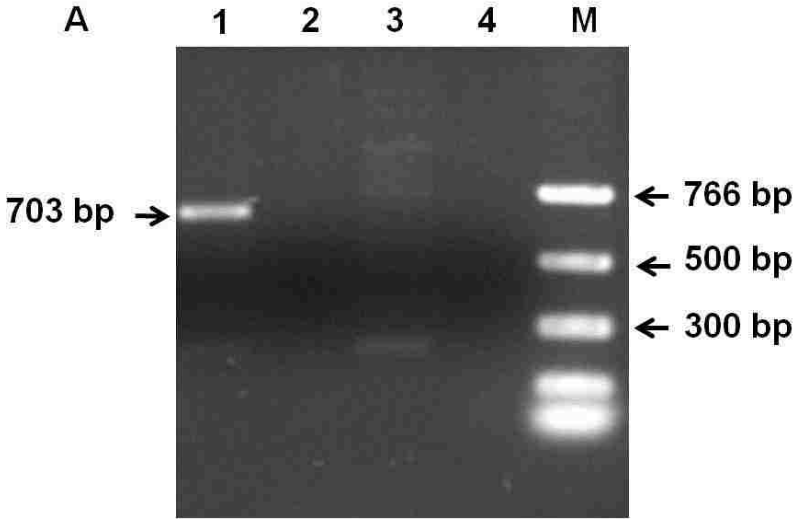
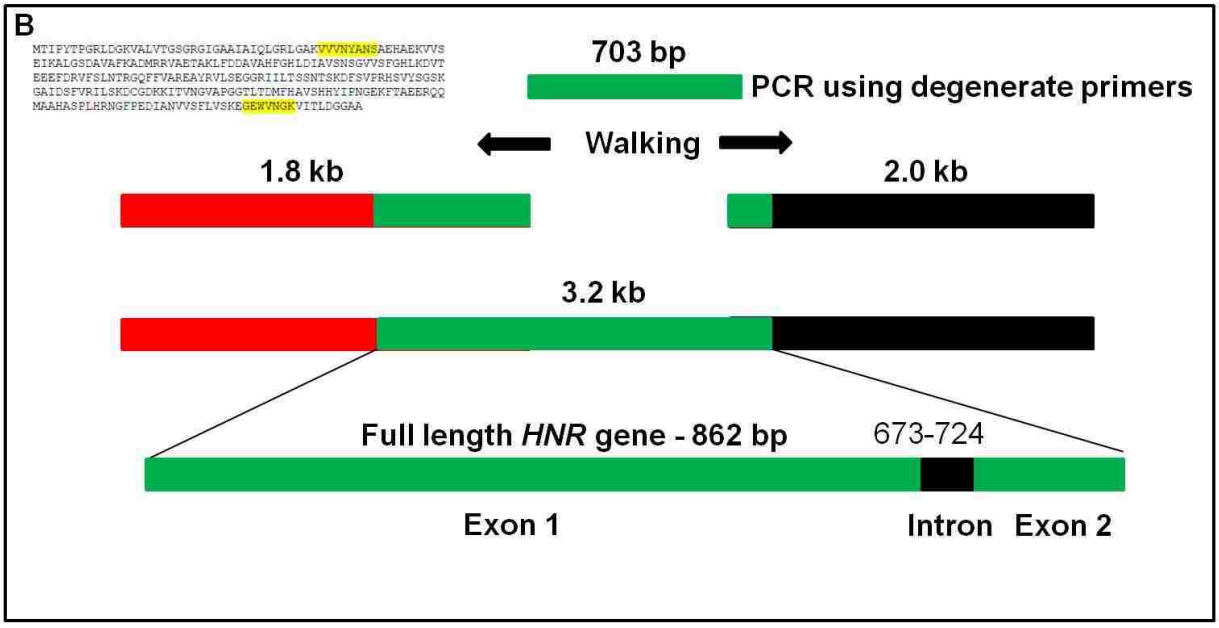
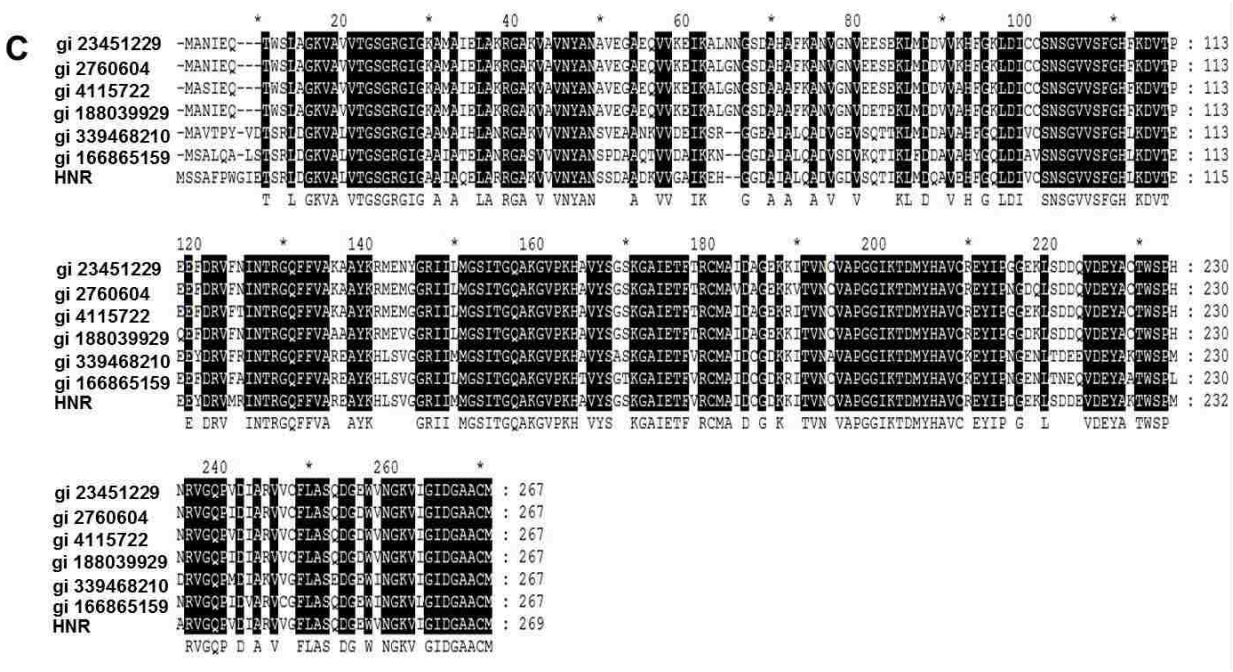


Figure 4.5. Cloning and characterization of the hydroxynaphthalene reductase (*HNR*) gene from *Cercospora kikuchii*. **A**, PCR amplification of *HNR* coding region from *C. kikchii* gDNA using degenerate primers HNRF and HNRR (see Materials and Methods). A specific PCR product was amplified only in the presence of both HNRF and HNRR primers and the template (lane 1). No specific amplifications was visible when PCR with only one primer (HNRF only, lane 2; HNRR only, lane 3) or without template (lane 4). **B**, Schematic diagram of strategy to clone the full length *HNR* gene using genome walking. Note: drawing is not to scale. **C**, Multiple sequence alignment of HNR protein to hydroxynaphthalene reductase from *Cochliobolus lunatus* (gi 23451229), Brn1 from *Cochliobolus heterostrophus* (gi 2760604), 1,3,8-trihydroxynaphthalene reductase from *Alternaria alternata* (gi 4115722), 1,3,8-trihydroxynaphthalene reductase from *Setosphaeria turcica* (gi 188039929), a hypothetical protein from *Mycosphaerella graminicola* (gi 339468210), and an ESC reductase (RDT1) from *Elsinoe fawcettii* (gi 166865159). Conserved amino acid residues across all sequences were shaded in black.

Figure 4.5. continued





An 842 bp PCR fragment was obtained using the degenerate primers AHCYF and AHCYR (Fig. 4.6A), cloned into the pCR2.1 vector and sequenced. BLASTX search of this translated 842 bp DNA sequence against the nonredundant protein database showed that it has a high homology to adenosylhomocysteinase from several fungi [*Paracoccidioides brasiliensis*

(EEH21092.1), *Ajellomyces capsulatus* (EEH07964.1), *Coccidioides posadasii* (XP_003072218.1)]. To clone the full length *AHCY* gene, genome libraries were constructed using the universal GenomeWalker^{TM} kit. Using gene specific primers and adapter primers, a 2.0 kb and a 5.0 kb fragments were amplified for the up- and down-stream of *AHCY*, respectively. After sequencing, these fragments were aligned into a 3.7 kb contig. Sequence analysis of this contig revealed the presence of a 1562 bp ORF with two introns (47 and 69 bp) (Fig. 4.6B). The transcriptional start site (TSS) was predicted 545 bp upstream of the ATG start codon, and the transcriptional termination site was located 18 bp downstream of the translational stop codon (www.softberry.com). The *AHCY* gene was predicted to encode a polypeptide of 481 aa with a predicted molecular mass of 52.2 kDa and a predicted p*I* of 6.12. BlastP search (Altschul et al. 1997) of the NCBI protein database revealed that the predicted 481 aa translation product of *AHCY* had high similarity to a hypothetical protein from *Mycosphaerella graminicola* (overall 79% identity, 85% similarity) and *Pyrenophora teres* f. *teres* (overall 71% identity, 77% similarity), and adenosylhomocysteinase from several fungi (maximum overall 72% identity, 78% similarity) (Fig. 4.6C). Analysis of the predicted protein sequence of the *C. kikuchii AHCY* gene revealed the presence of a S-adenosyl-L-homocysteine hydrolase (AdoHycase) domain, and a NADP binding site (Rossmann superfamily).

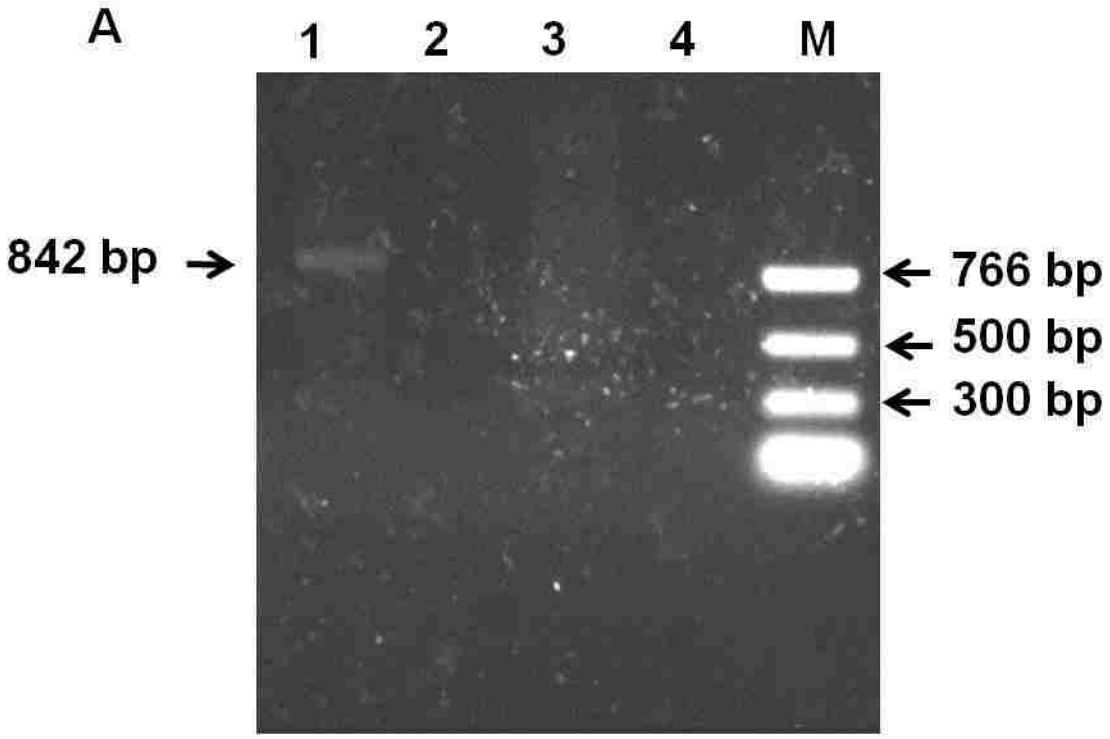
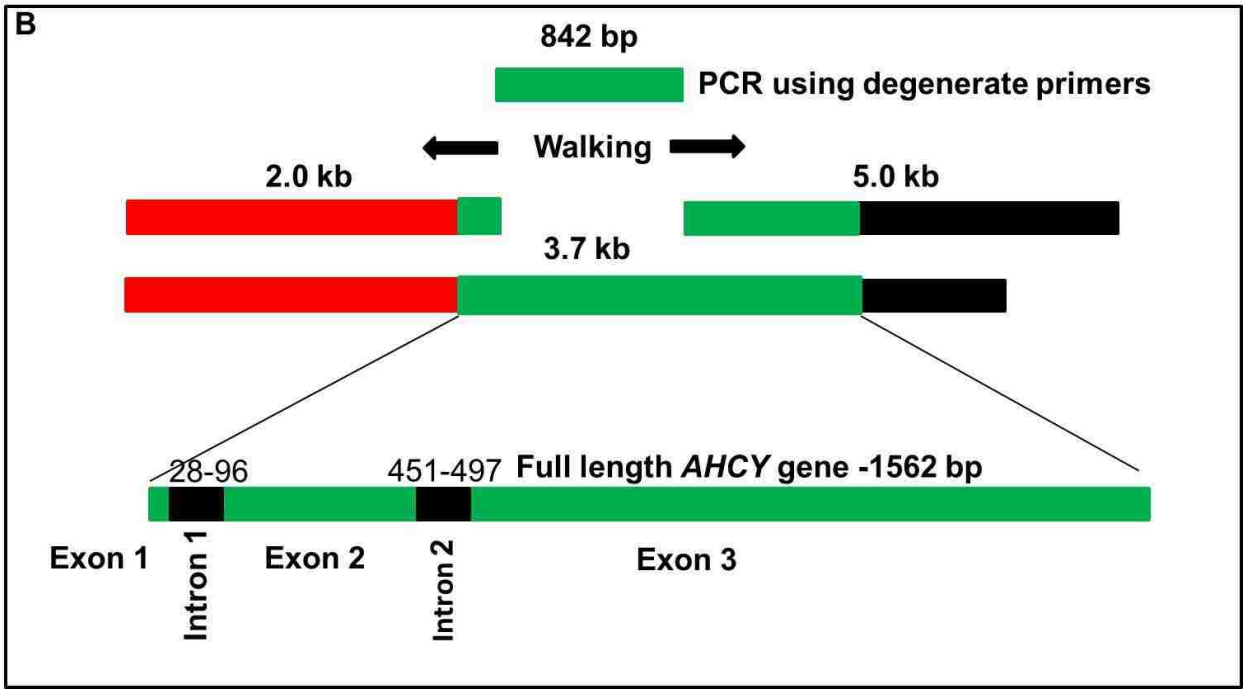
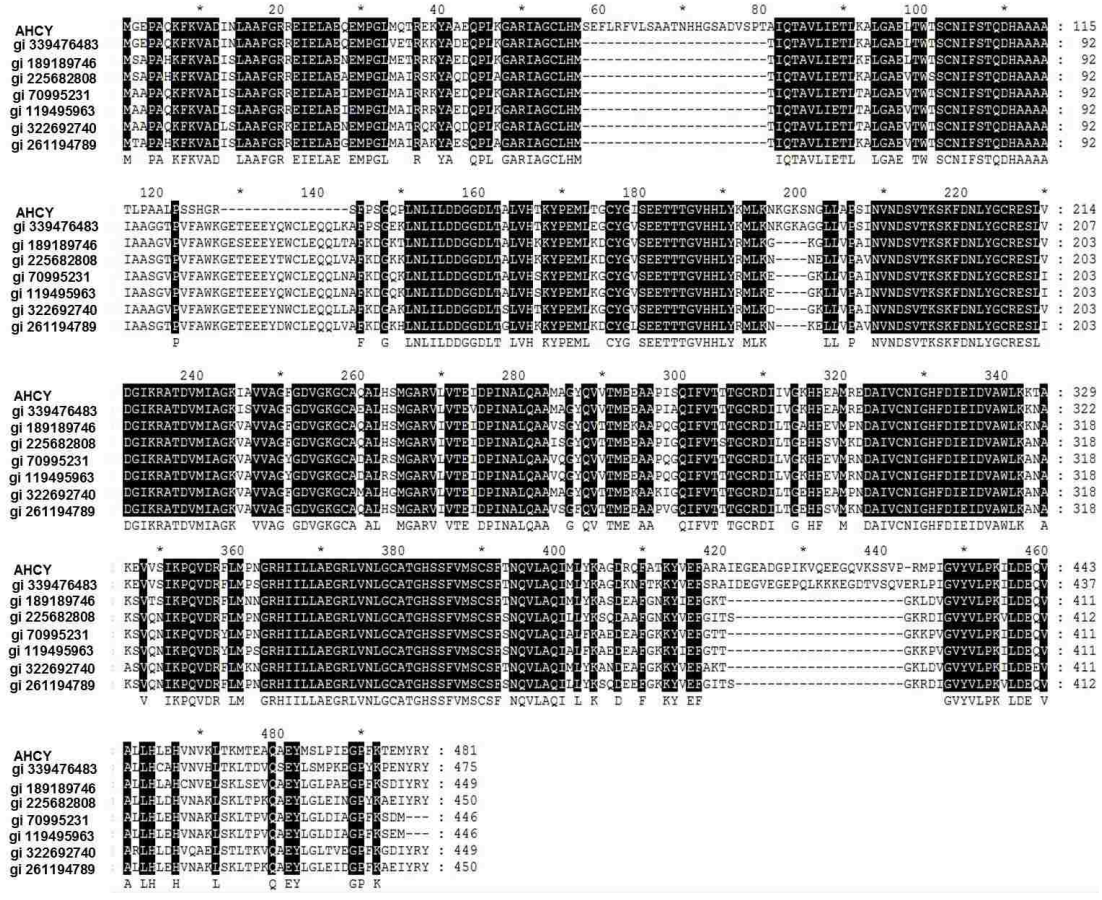


Figure 4.6. Cloning and characterization of the adenosylhomocysteinase (*AHCY*) gene from *Cercospora kikuchii*. **A**, PCR amplification of the *AHCY* coding region from *C. kikchii* gDNA using degenerate primers AHCYF and AHCYR (see Materials and Methods). A specific PCR product was amplified only in the presence of both AHCYF and AHCYR primers and the template (lane 1). No specific amplifications was visible when PCR with only one primer (AHCYF only, lane 2; AHCYR only, lane 3) or without template (lane 4). **B**, Schematic diagram of strategy to clone full length *AHCY* gene using genome walking. Note: drawing is not to scale. **C**, Multiple sequence alignment of AHCY protein to hypothetical protein from *Mycosphaerella graminicola* (gi 339476483), adenosylhomocysteinase from *Pyrenophora tritici-repentis* (gi 189189746), *Paracoccidioides brasiliensis* (gi 225682808), *Aspergillus fumigatus* (gi 70995231), *Neosartorya fischeri* (gi 119495963), *Metarhizium acridum* (gi 322692740), and *Ajellomyces dermatitidis* (gi 261194789). Conserved amino acid residues across all sequences were shaded in black.

Figure 4.6. continued



C



4.3.5 Target Gene Disruption of *HNR* and *AHCY* Genes in *C. kikuchii*

For disruption of *HNR*, the 5′-end of *HNR* (0.8 kb), hygromycin phosphotransferase B gene (*HYG*) cassette (2.5 kb) and 3′-end of *HNR* (1.0 kb) were combined using a fusion PCR to generate a long PCR product of 4.3 kb. A nested PCR using primers HNR5FN2 and HNR3RN2 on this PCR product was employed to obtain the 4.2 kb DNA fragment (Fig. 4.7A). This 4.2 kb product was directly transformed into the wild-type *C. kikuchii* protoplasts. Fungal transformants (20 colonies) were selected on the complete medium (CM) supplemented with hygromycin (300 μg/ml) 7 days after plating the transformed protoplasts and transferred to new CM plates with hygromycin. All 20 colonies (Δhnr1 - Δhnr20) showed the presence of the 466 bp fragment from *HYG* by PCR using *HYG* specific primers HY (5′- ggatgcctccgctcgaagta-3′) and YG (5′-cgttgcaagacctgcctgaa-3′). No amplification was observed from the wild-type *C. kikuchii* (Fig. 4.7B). The primer HNR5F was paired with the primer HY (5′-ggatgcctccgctcgaagta-3′) in PCR to validate site-specific integration of the 4.2 kb DNA fragment used for transformation at HNR locus. Six colonies *(*Δhnr1, Δhnr2, Δhnr11, Δhnr12, Δhnr17 and Δhnr *18*) showed the presence of the expected 2.7 kb fragment and again no amplification was observed from the wild-type *C. kikuchii* (Fig. 4.7C). Finally, the presence of *HYG* in the middle of *HNR* gene was confirmed by PCR using primer pairs HNR5F and HNR3R. Three colonies (Δhnr2, Δhnr12 and Δhnr17) showed the presence of expected 4.3 kb fragment, whereas wild-type *C. kikuchii* and pUCATPH vector controls showed only the 2.0 kb fragment (Fig. 4.7D). From these analyses, colonies Δhnr2, 12, and 17 were considered correct transformants. Quantitative real-time RT-PCR using primers HNR-RT-F1 (5′-atgtcacagaagaggagtacgacc-3′) and HNR-RT-R1 (5′-caggtgcttgtatgcttctcttgc-3′) showed significant reduction in *HNR* transcript levels (Fig. 4.7E)

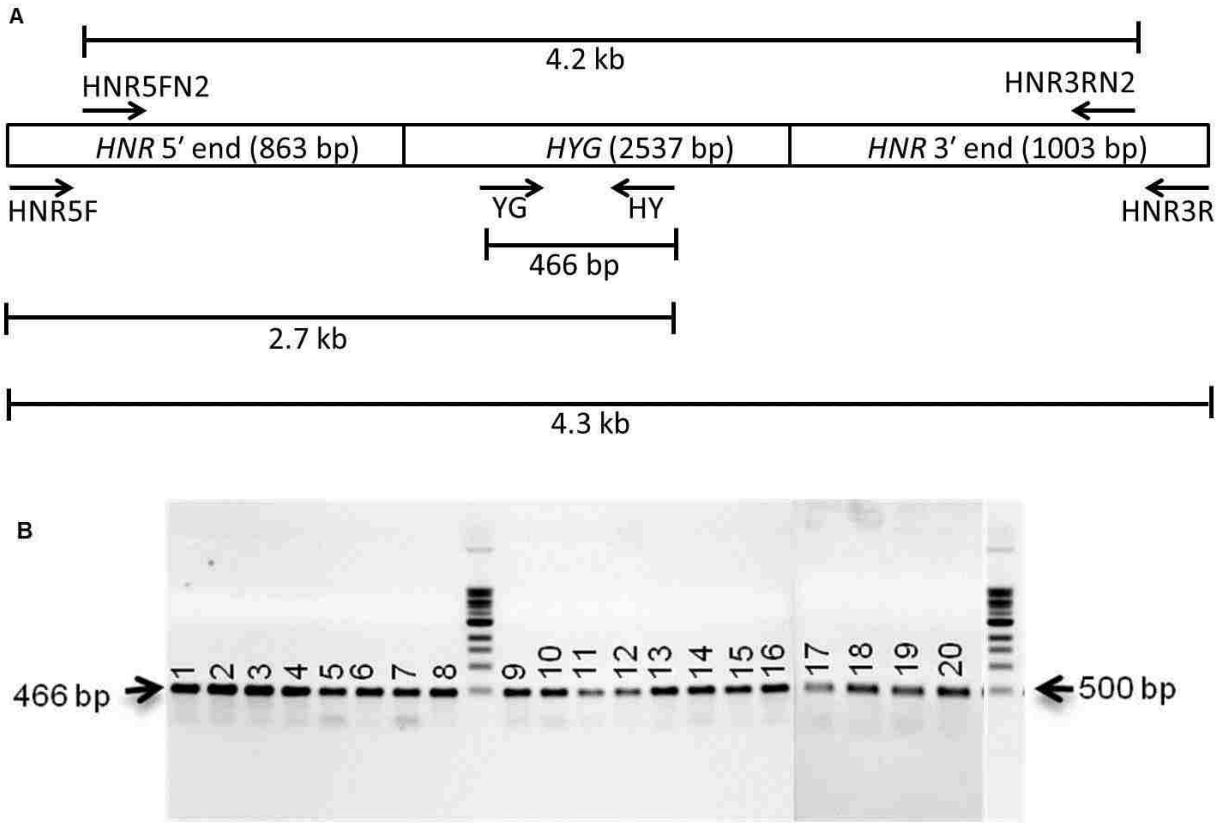
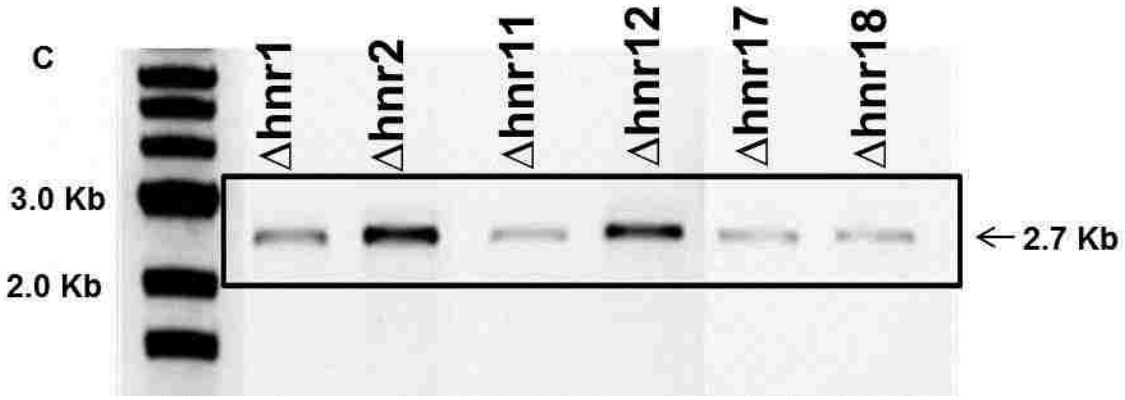
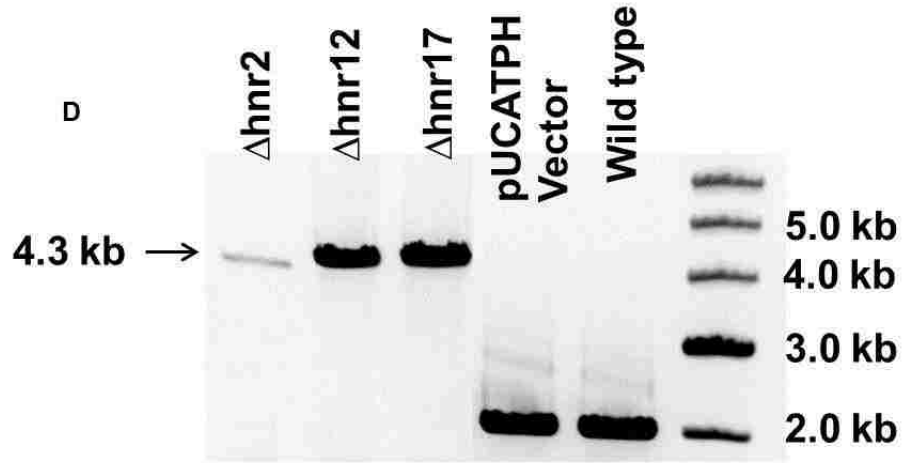
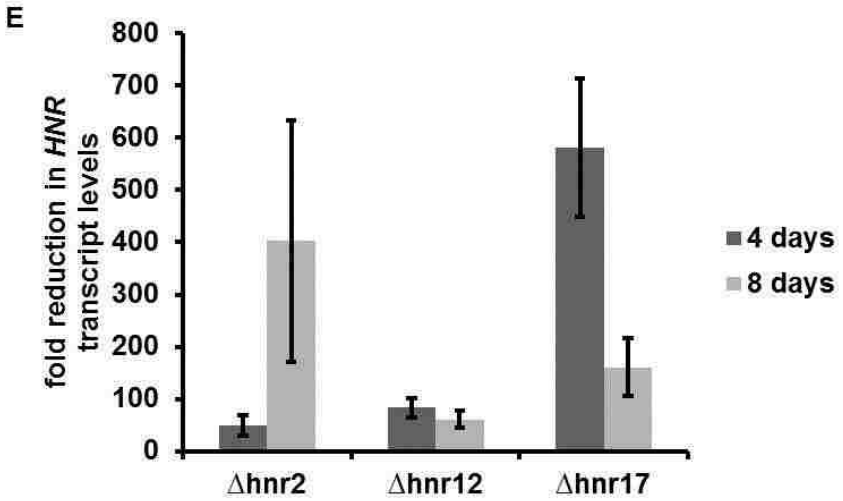


Figure 4.7. Disruption of the hydroxynaphthalene reductase (*HNR*) gene in *Cercospora kikuchii*. **A**, Two truncated HNR fragments (5′ and 3′-end) were joined to hygromycin resistance gene cassette (*HYG*) using polymerase chain reaction (PCR) to generate the 4.3 kb DNA fragment. Nested PCR was done using HNR5FN2 and HNR3RN2 primer pairs and the resultant 4.2 kb fragment was directly transformed into *C. kikuchii* protoplasts. Note: drawing is not to scale. **B**, PCR confirmation of the presence of 466 bp hygromycin fragment in 20 of *HNR* gene disruption mutants (Δhnr1 to Δhnr20)**. C**, Site-specific disruption of the *HNR* gene as confirmed by PCR using primer pairs HNR5F (upstream primer) and HY primer. An expected 2.7 kb PCR product was detected in Δhnr1, Δhnr2, Δhnr11, Δhnr12, Δhnr17, and Δhnr18. **D**, Insertion of *HYG* in the middle of *HNR* gene using primer pairs HNR5F and HNR3R showed presence of a 4.3 kb fragment only in Δhnr2, Δhnr12, and Δhnr17whereas wild type or pUCATPH vector controls showed only the 2.0 kb fragment. **E**, Quantitative real-time RT-PCR data showing fold reduction in *HNR* transcript levels in Δhnr2, Δhnr12, and Δhnr17. The ratio of gene expression in the mutants was normalized to pUCATPH vector control and 18S RNA was used as internal reference. Data shown were means of four replications and error bars represent the standard deviation.

Figure 4.7. continued







For disruption of *AHCY*, the 5′-end of *AHCY* (1.4 kb), *HYG* cassette (2.5 kb) and the 3′-end of *AHCY* (1.4 kb) were combined using a fusion PCR to generate a long DNA fragment of 5.3 kb. A nested PCR using primers AHCY 5FN2 and AHCY3RN2 was performed on this product to obtain the 5.1 kb DNA fragment (Fig. 4.8A). This 5.1 kb fragment was directly transformed into the wild-type *C. kikuchii* protoplasts. Fungal transformants (20 colonies) were selected on the complete medium (CM) supplemented with hygromycin (300 μg/ml) 7 days after plating the transformed protoplasts and transferred to new CM plates with hygromycin. All 20 colonies (Δahcy1 to Δ ahcy20) showed the presence of the 466 bp fragment from *HYG* by PCR using *HYG* specific primers HY (5′-ggatgcctccgctcgaagta-3′) and YG (5′-cgttgcaagacctgcctgaa-3′) (Fig. 4.8B). No amplification was observed from the wild-type *C. kikuchii*. The primer AHCY5F was paired with the primer HY in PCR to validate the site-specific integration of the DNA fragment used for transformation at AHCY locus. Six colonies (Δahcy1, Δahcy3, Δahcy4, Δahcy9, Δahcy10 and Δahcy12) showed the presence of the expected 2.57 kb fragment. Again, no amplification was observed from the wild-type *C. kikuchii* (Fig. 4.8C). The target gene expression in three of these confirmed mutants were examined using quantitative real-time RT-PCR with AHCY-RT-F1 (5′-tgtcatgattgctggcaagatcgc-3′) and AHCY-RT-R1 (5′-atggagtggagagcctgagca-3′), which showed significant reduction in *AHCY* transcript levels in the disruption mutants (Fig. 4.8D).

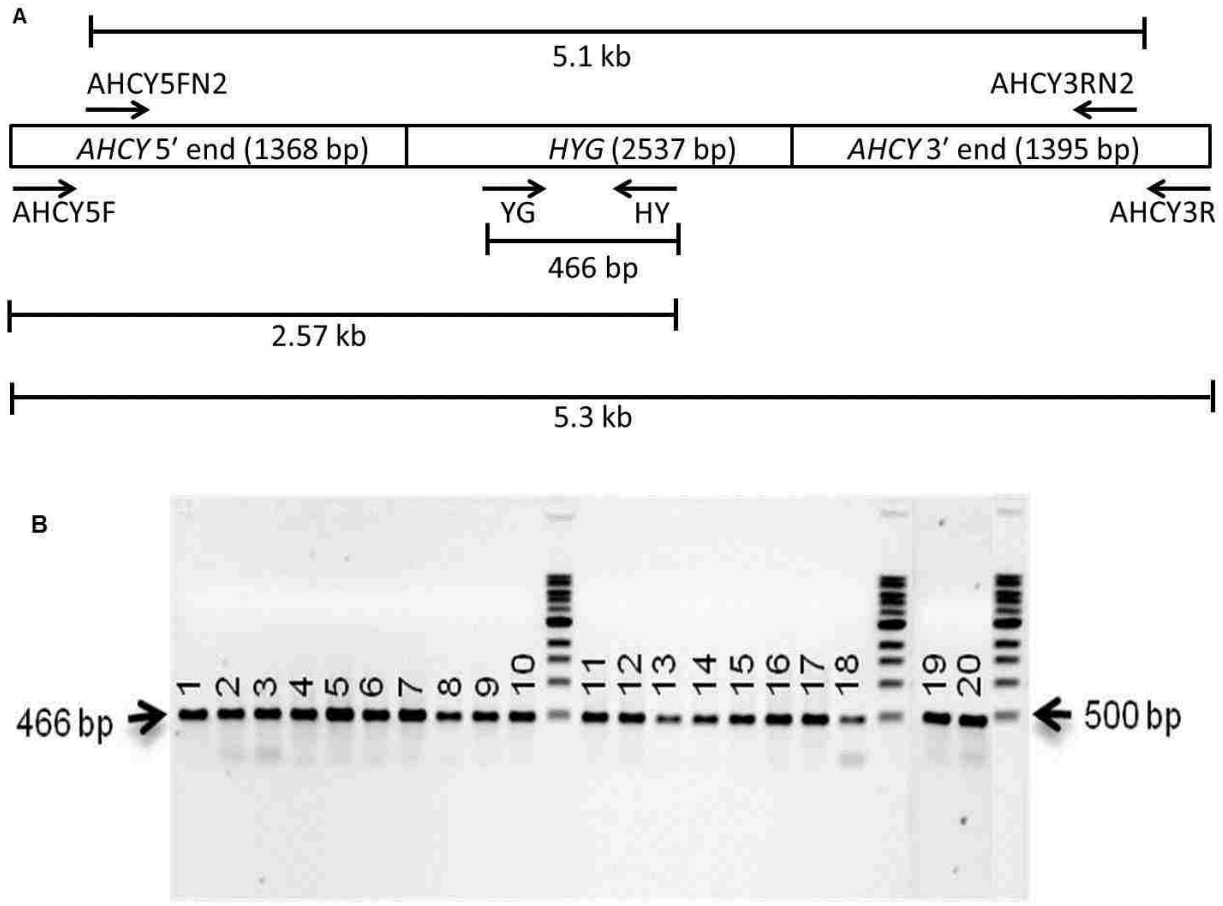
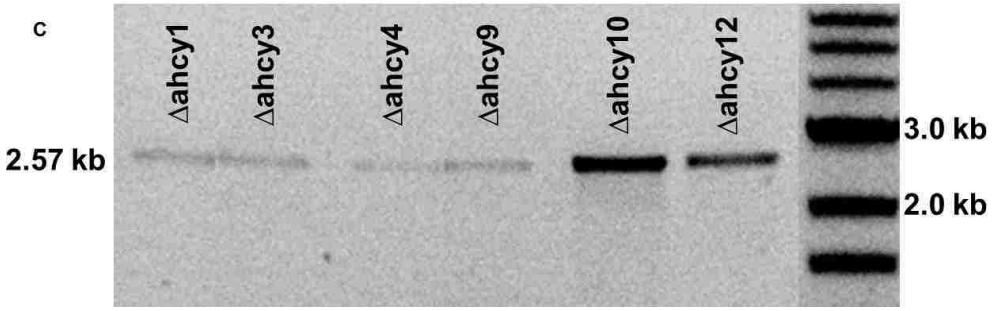
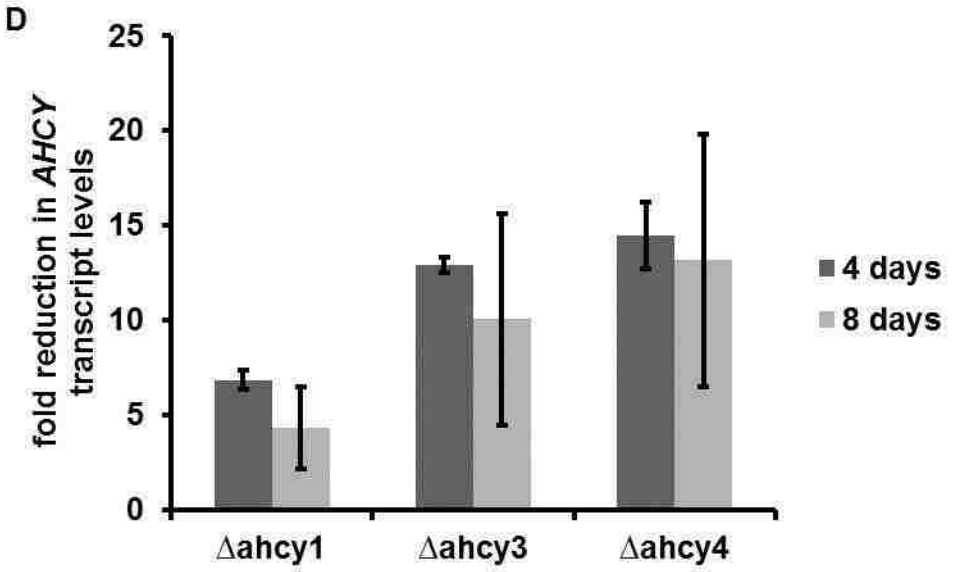


Figure 4.8. Disruption of adenosylhomocysteinase (*AHCY*) gene in *Cercospora kikuchii*. **A**, Two truncated *AHCY* fragments (5′ and 3′-end) were joined to the hygromycin resistance gene cassette (*HYG*) using polymerase chain reaction (PCR) as described in text to generate the 5.3 kb DNA fragment. A nested PCR was conducted using AHCY5FN2 and AHCY3RN2 primer pairs and the resultant 5.1 kb fragment was directly transformed into *C. kikuchii* protoplasts. Note: drawing is not to scale. **B**, PCR confirmation of the presence of 466 bp hygromycin fragment in 20 *AHCY* gene disruption mutants (Δahcy1 to Δahcy20). **C**, Site-specific disruption of the *AHCY* gene as confirmed by PCR using primer pairs AHCY5F (upstream primer) and HY primer, which showed presence of a 2.57 kb fragment in Δahcy1, Δahcy3, Δahcy4, Δahcy9, Δahcy10, and Δahcy12. **D**, Quantitative real-time RT-PCR data showing fold reduction in *AHCY* transcript levels in Δahcy1, Δahcy3, and Δahcy4. The ratio of gene expression in the mutants was normalized to pUCATPH vector control and 18S RNA was used as internal reference. Data shown were means of four replications and error bars represent the standard deviation.

Figure 4.8. continued





4.3.6 Detection and Quantification of Cercosporin

Cercosporin production in the hnr and ahcy disruption mutants was measured and compared to that of pUCATPH vector transformed control by growing the fungal strains on CM plates amended with hygromycin (300 µg/ml). Mycelial plugs were extracted with 5N KOH and the amounts of cercosporin in the extract quantified by measuring the absorbance of the extract at 480 nm (Jenns et al. 1989). Both hnr and ahcy disruption mutants produced significantly lower levels of cercosporin compared to vector transformed control (Fig. 4.9A and B). There was no

significant difference in cercosporin levels among the three hnr or ahcy disruption mutants. The secretion of cercosporin into the media underneath was not affected in hnr or ahcy disruption mutants. Apart from lower cercosporin production, no other phenotypic characteristics were observed among the hnr and ahcy disruption mutants compared to the vector control or the wild-type. All hnr and ahcy disruption mutants exhibited normal growth on CM. In addition, when tested for sensitivity to exogenously applied cercosporin, all mutants retained normal growth similar to the vector control (data not shown).

4.3.7 Pathogenicity Assay on Soybeans

Three hnr disruption mutants (Δhnr2, Δhnr12, and Δhnr17), three ahcy disruption mutants (Δahcy1, Δahcy3, and Δahcy4), and vector-transformed control were compared to wild-type *C. kikuchii* for pathogenicity on soybeans using two methods. In the detached leaf assay, severe necrosis and chlorosis of the entire leaf was observed 14 days after inoculation with wild-type or vector-transformed *C. kikuchii* controls. However, neither hnr nor ahcy disruption mutants were able to cause severe chlorosis and necrosis with the exception of Δhnr17 and Δahcy3, in which mild necrosis was observed at the site of attachment of mycelial plugs (Fig. 4.10). Lactophenol blue staining revealed the mycelia of hnr and ahcy disruption mutants were able to spread throughout the leaf (data not shown) but unable to cause chlorosis and necrosis. In the second method, mycelia from the above fungal strains were directly inoculated on soybean plants at the first flower stage in the greenhouse. Fourteen days after inoculation, small necrotic lesions were

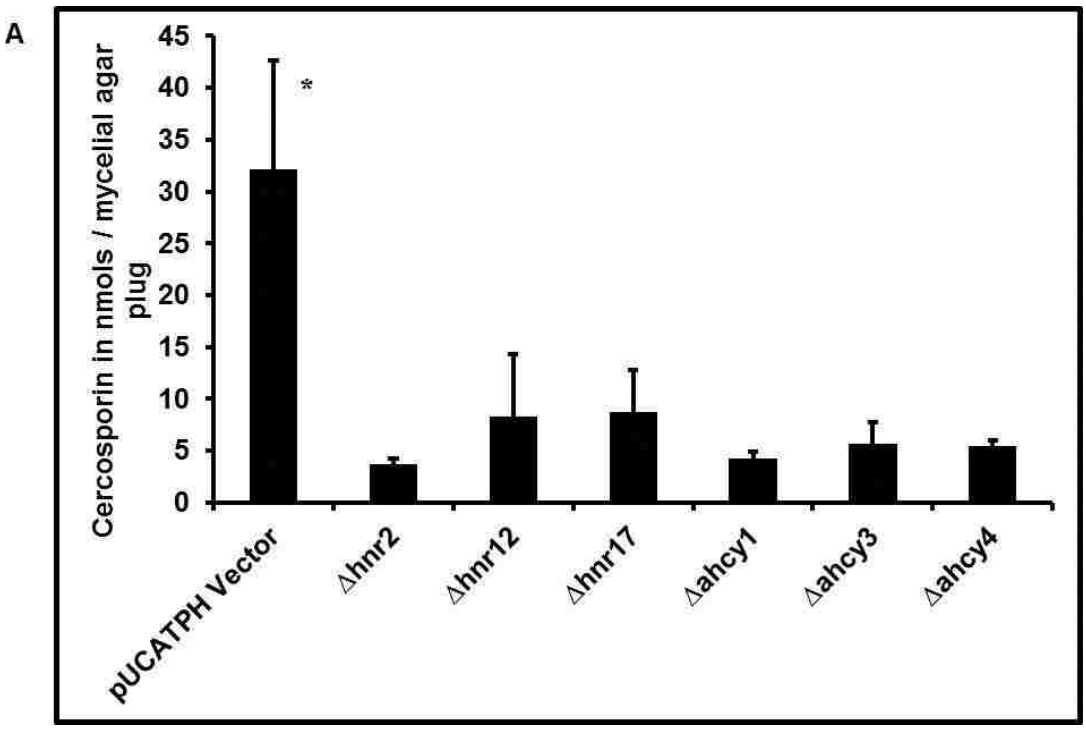
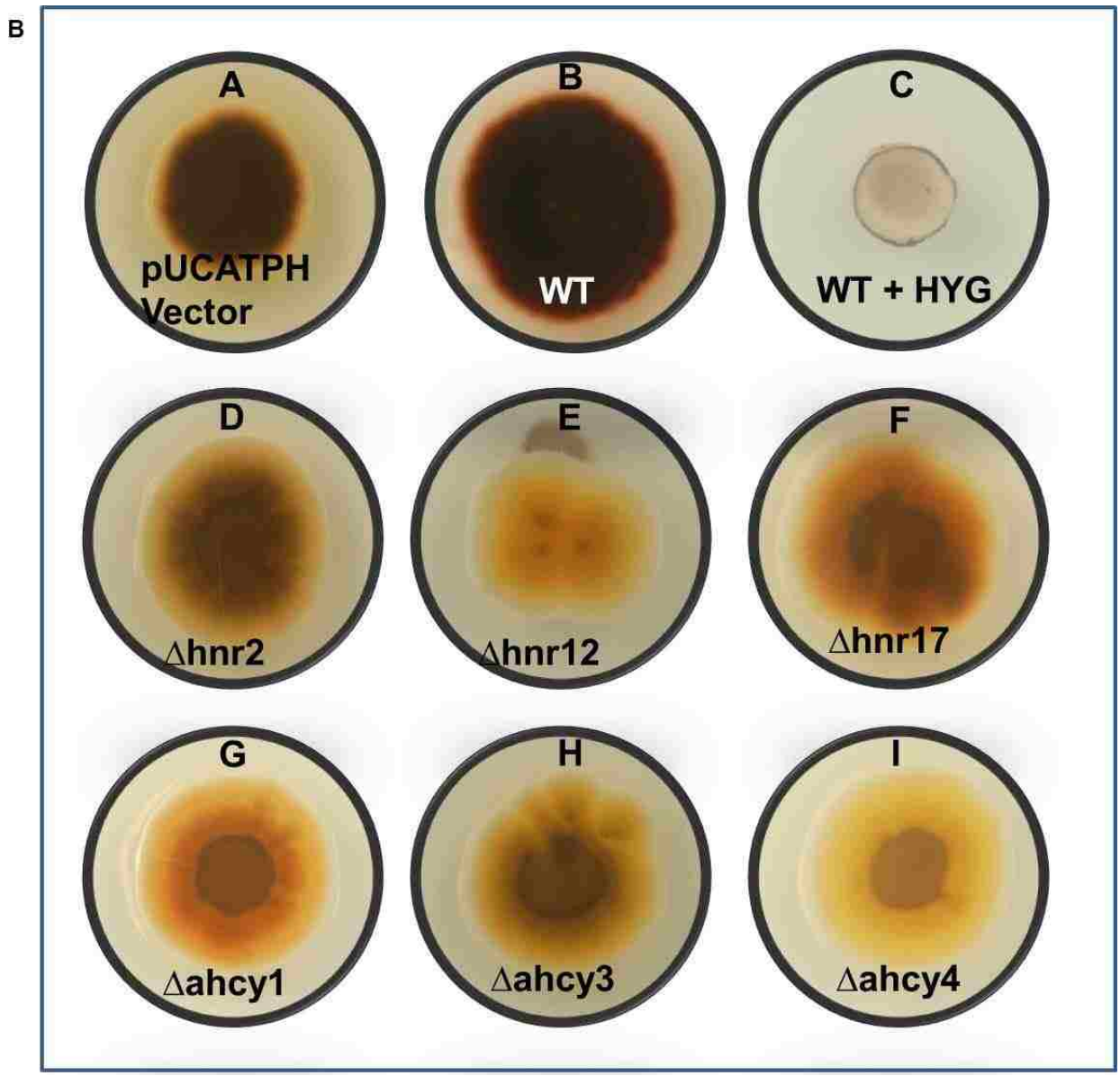


Figure 4.9. Characterization of cercosporin produced by vector-transformed control and hnr and ahcy disruption mutants of *C. kikuchii*. **A**, Vector-transformed control, Δhnr, and Δahcy disruption mutants were grown on CM plates amended with hygromycin (300 μg/ml) in continuous light and cercosporin production was assayed on mycelial agar plugs according to Jenns et al. (1989) and the absorbance at 480 nm was recorded. Error bars represent standard error of four biological replications. Asterisk indicates significant difference at $p < 0.05$ using ANOVA. **B**, Appearance of vector-transformed control (**A**), wild-type with no hygromycin (**B**), wild-type with hygromycin (no growth) (**C**), hnr disruption mutants (**D** through **F**), and ahcy disruption mutants (**G** through **I**) on CM plates viewed from underside of plates (**C** from top side). Reduction in cercosporin production in mutants compared to wild-type and pUCATPH vector control can be observed on underside of plates.

Figure 4.9. continued



observed on soybean leaves inoculated with wild-type or vector-transformed control. Neither hnr nor ahcy disruption mutants caused any visible necrotic lesions (Fig. 4.11). Collectively, disruption of the *HNR* and *AHCY* genes resulted in mutants that produced lower levels of cercosporin and were markedly reduced in virulence. These data support the observation that cercosporin is an important virulence factor in the infection of soybean by *C. kikuchii*.

4.4 Discussion

Cercosporin accumulation by different *Cercospora* species *in vitro* is highly variable and dependent on light, temperature, media type, pH, and C:N ratio of the media and also the complex interaction of the above factors. For the *C. kikuchii* isolate used in this study, CM

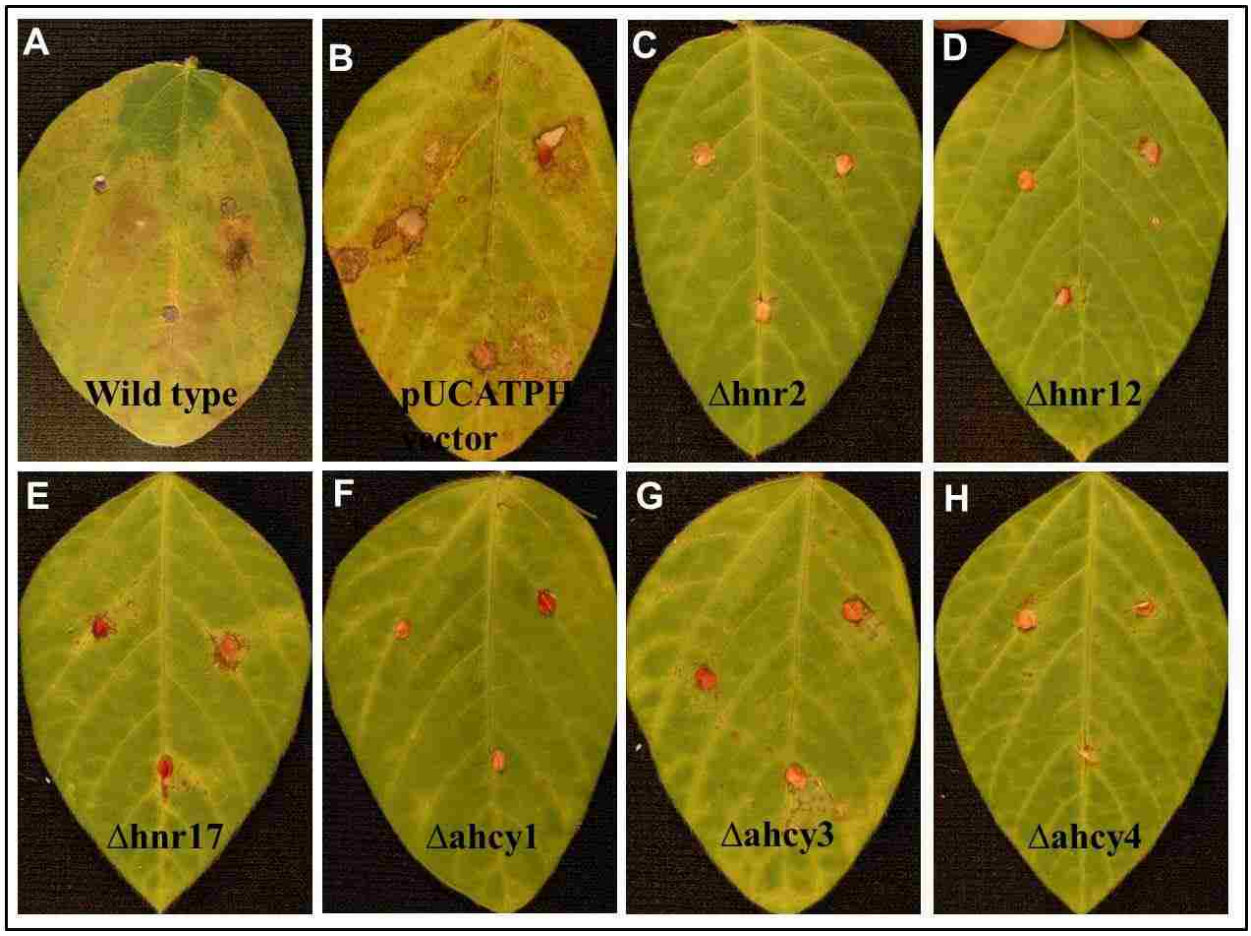


Figure 4.10. Pathogenicity assay of Δhnr and Δahcy mutants on detached soybean leaves harvested at R1 growth stage from cv. ASGROW 6202. Three 3 mm diameter mycelial agar plugs were excised from 7 day-old cultures grown on CM plates and directly placed on the detached soybean leaves with mycelia side touching the adaxial surface of leaves in a Petri plate. Plates were kept under 16 h light and 8 h dark at 25 ºC for 2 weeks and observed for symptoms. Severe necrosis and chlorosis was observed in leaves treated with wild type *C. kikuchii* (**A**) or *C. kikuchii* transformed with pUCATPH vector (**B**) compared to very minimal symptoms in leaves treated with *C. kikuchii* Δ*hnr* (**C** through **E**) or Δ*ahcy* (**F** through **H**) mutants.

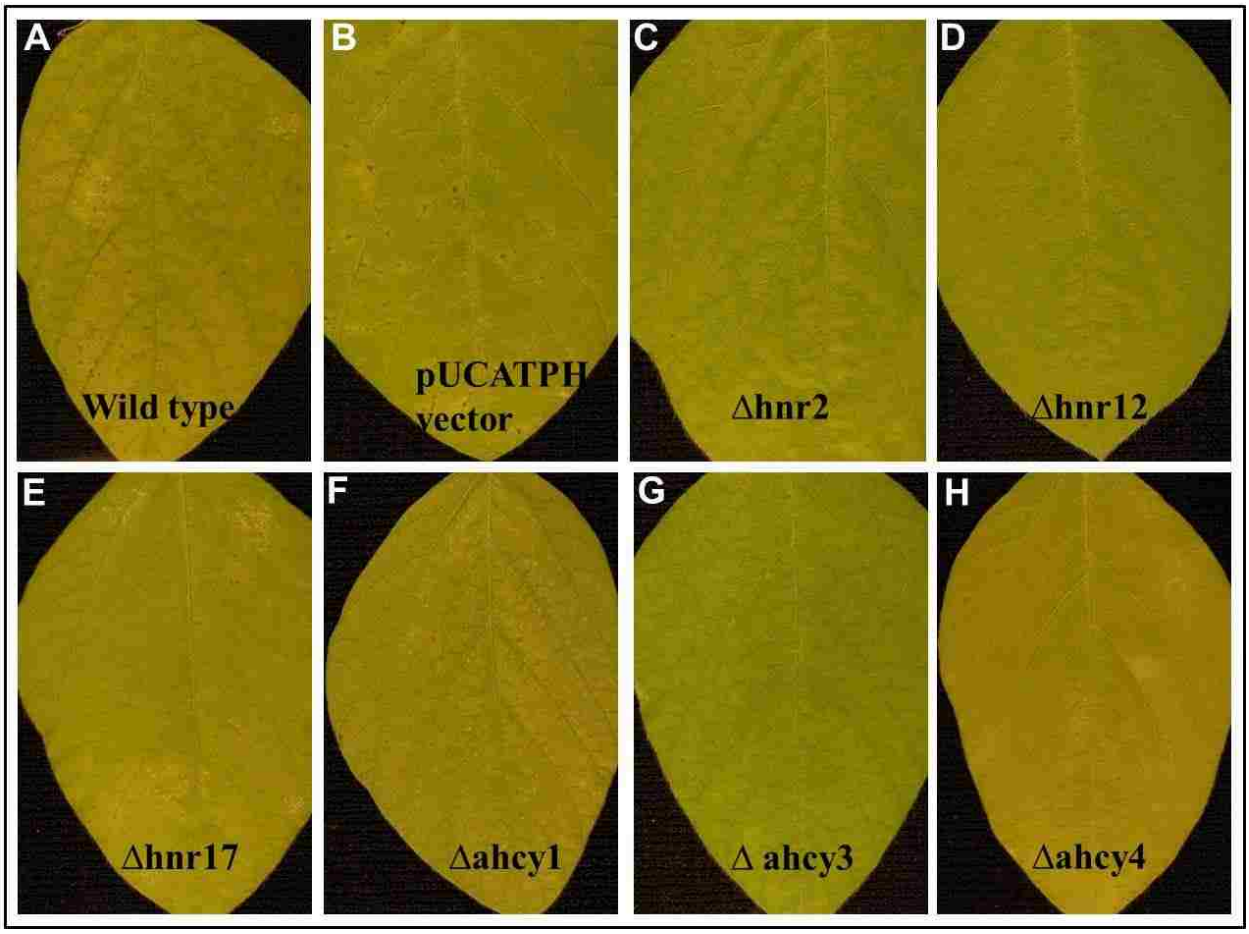


Figure 4.11. Pathogenicity assay of Δ*hnr* and Δ*ahcy* mutants on soybean plants (cv. ASGROW 6202) at R1 growth stage in greenhouse inoculations. All cultures were grown on CM plates lined with Spectra/Por 1 6-8 kDa MWCO dialysis membrane for 10 days in the dark, mycelia were removed, blended in Waring blender at high speed (1 gm fresh wt. mycelia in 100 ml water), and sprayed on soybean plants until runoff. Several necrotic spots (pin head size) surrounded by chlorosis were observed in soybean plants inoculated with wild type (**A**) or pUCATPH vector-transformed *C. kikuchii* (**B**) compared to very tiny necrotic spots (pin tip size) in soybean plants inoculated with Δ*hnr* (**C** through **E**) or Δ*ahcy* (**F** through **H**) mutants 2 weeks after inoculation.

induced cercosporin production, but not PDB, which is different from other studies. In *C. zeae-maydis*, PDB (0.2X) induced cercosporin, but not CM (Shim and Dunkle 2002). Light is not only crucial for cercosporin toxicity, but also is required for the initiation of cercosporin biosynthesis (Ehrenshaft and Upchurch 1991, 1993). *C. kikuchii* cultures incubated in light from 6 to 16 days, accumulated significantly higher cercosporin compared to dark-grown cultures and similar

results were reported in *C. kikuchii*, *C. beticola* and *C. riciniella* (Balis and Payne 1971; Fajola 1978; Lynch and Geoghegan 1979; Rollins et al. 1993).

Before performing 2D experiments, several protein extraction methods, such as the TCA/acetone (Damerval et al. 1986), Tri-HCl (Chen et al. 2006) and phenol-methanol (Hurkman and Tanaka 1986) methods as well as the methods specifically developed for filamentous fungi (Fernandez-Acero et al. 2006; Fragner et al. 2009) were compared for extracting proteins from *C. kikuchii* mycelia. The phenol-methanol method was selected for use as it had greater protein yield and well resolved proteins on 2D gels. This method was also followed to extract proteins from plant samples and shown to be very efficient (Carpentier et al. 2005; Park et al. unpublished data; Saravan and Rose 2004). Phenol extraction followed by methanolic precipitation eliminates nucleic acids, membrane lipids, and cell wall fragments that might interfere with downstream protein solubility and electrophoresis (Saravan and Rose 2004). The 3-10 NL pH gradient IPG strips proteins produced very well resolved profiles of about 1100 ± 55 proteins using silver staining.

Rollins et al. (1993) compared the 2D protein profiles of extracted proteins and *in vitro* translated products (from poly A+ RNAs) from wild-type and mutant *C. kikuchii* isolates and found some proteins up-regulated under light, but they did not sequence any protein spots for further identification. In this study, six protein spots were found up-regulated and two were down-regulated in *C. kikuchii* cultures grown under light compared to dark conditions. These six up-regulated spots (spots 57, 34, 28, 26, 32, and 129) and two down-regulated spots (spots 132 and 70) in light were recovered from Coomassie stained gels, sequenced using LC-MS/MS. Six of them were identified (Table 1) based on peptide sequence homology analysis. Spot 57 showed homology to 1,3,8-trihydroxynaphthalene reductase (3*HNR*) gene from *Cochliobolus lunatus*

(Rižner and Wheeler 2003) and *RDT1* gene from *Elsinoë fawcettii*, (Chung and Liao 2008). *3HNR* codes for a reductase that is involved in 1,8-dihydroxynaphthalene (DHN)-melanin biosynthetic pathway by reducing 1,3,8-trihydroxynaphthalene to vermelone in fungal cells. *RDT1* encodes a putative polypeptide similar to a wide range of reductases, with strong similarity to the 1,3,8-trihydroxynaphthalene reductase (Chung and Liao 2008). It is located upstream of *TSF1* gene, a transcriptional factor that regulates the biosynthesis of elsinochrome phytotoxin in *E. fawcettii* (Chung and Liao 2008), a photosensitizing compound that shares structural similarity to cercosporin (Weiss et al. 1987). Targeted disruption of the *HNR* gene resulted in a significant reduction in cercosporin accumulation by hnr disruption mutants. This suggests that HNR either is involved in the regulation of cercosporin biosynthesis or is one of the key homologs that function in the cercosporin biosynthetic pathway. Presence of *RDT1* upstream of *TSF1* in *E. fawcetti* also suggests that melanin biosynthesis may be related to elsinochrome biosynthesis. Melanin is the key pigment produced by many fungi to protect the spores and mycelia from harmful UV radiation (Bell and Wheeler 1986; Kawamura et al. 1999; Wheeler and Bell 1988) as well as damage caused by reactive oxygen species (ROS), such as singlet oxygen and super oxide radicals (Herrling et al. 2008; Tada et al. 2010). Because the main mode of action of cellular damage caused by cercosporin is through the production of ROS (Daub and Ehrenshaft 1997), *C. kikuchii* might have its own melanin biosynthesis linked to cercosporin biosynthesis. This is supported by the observation that HNR was up-regulated in cultures grown in continuous light, which is favorable for cercosporin production. Melanin is also important for virulence of pathogenic fungi on plants (Chumley and Valent 1990; Howard and Valent 1996; Kawamura et al. 1997; Takano et al. 1995).

Spots 34 and 28 were also up-regulated in light and showed homology to enzymes involved in cysteine and methionine metabolism. Spot 34 showed homology (72%) to adenosylhomocysteinase (*AHCY*) from several fungi. *AHCY* converts S-adenosylhomocysteine to homocysteine, which can be used to regenerate methionine or to form cysteine (Palmer and Abeles 1979) in amino acid metabolism. *AHCY* was also identified in the proteomics studies of extra-radical phase of arbuscular mycorrhiza (Recorbet et al. 2009) and haustoria of the the barley pathogen *Blumeria graminis* f.sp. *hordei* (Godfrey et al. 2009). Spot 28 showed homology (83%) to S-adenosyl methionine synthetase (SAM synthetase). SAM synthetase catalyzes the formation of SAM from methionine, which is involved in transferring methyl groups in many metabolic processes (Peleman et al. 1989). A proposed cercosporin biosynthesis pathway in *C. nicotianae* (Chen et al. 2007b) indicated the involvement of SAM, *CTB2*, and *CTB3* gene products in the formation of pentaketomethylene, which upon dimerization forms cercosporin. SAM synthetase and AHCY proteins that are up-regulated in light might be associated with this step during cercosporin biosynthesis. However, ahcy disruption mutants did not completely abolish cercosporin production indicating that there may be more than one methyl donor involved in that particular step.

Spot 26 showed a high homology to superoxide dismutase, which causes dismutation of superoxide radicals ($O_2\cdot^- + O_2\cdot^- + 2H^+ \rightarrow O_2 + H_2O_2$) (McCord and Fridovich 1969). In the presence of light, cercosporin gets activated, reaches a triplet state that reacts with molecular oxygen, resulting in the formation of singlet oxygen and superoxide radicals collectively known as ROS. ROS cause damage to membrane lipids and also other cellular components ultimately leading to cell death (Daub and Briggs 1983). As *C. kikuchii* grown under light accumulated higher cercosporin compared to dark-grown cultures, it is reasonable to expect that superoxide

dismutase also expressed at higher levels in light to protect fungal cells from the damage caused by ROS. We were unable to identify spots 32 and 129 as the mass spectrometry was not successful. Elongation factor 2 (spot 132) and 60S ribosmal protein L7 (spot 70), which are involved in protein metabolism, were down-regulated under light. This is in accordance with the observation that the *C. kikuchii* grown under dark had more mycelial growth compared to light.

Cercosporin is known to be a crucial pathogenicity factor in *C. kikuchii* (Upchurch et al. 1991), *C. nicotianae* (Choquer et al. 2005), and *C. zeae-maydis* (Shim and Dunkle 2003) and mutants deficient in cercosporin biosynthesis caused reduced or no disease. Both hnr and ahcy gene disruption mutants in *C. kikuchii* resulted in significantly lower production of cercosporin compared to the vector-transformed control and produced minimal or no chlorosis and necrosis on soybean leaves in both detached leaf assay (Fig. 4.10) and greenhouse inoculation experiments (Fig. 4.11). However, growth of the mutants in inoculated leaves as visualized through lactophenol blue staining was comparable to the wild type (data not shown). Slight necrosis at the site of inoculation in the detached leaf assay with disruption mutants Δhnr17 and Δahcy3 might be caused by damage from cercosporin present in the agar block.

Since hnr and ahcy gene disruption mutants were not completely deficient in cercosporin production, the possibility of involvement of these genes in conferring resistance to exogenously applied cercosporin also was tested. In a preliminary experiment, when vector-transformed control and hnr and ahcy gene disruption mutants were grown on CM plates in the presence of 10 μM cercosporin, no visual difference in fungal growth was observed between the mutants and the wild-type, indicating that the mutants were not sensitive to exogenously applied cercosporin (data not shown). One possible explanation for this observation is that the mutants are still

producing lower amounts of cercosporin, and the cercosporin self-protection mechanism is still operational under these conditions.

Various strategies were employed to identify genes involved in or regulating cercosporin biosynthesis in different *Cercospora* species. Ehrenshaft and Upchurch (1991) isolated six light enhanced cDNAs by using suppressive subtractive hybridization (SSH) in *C. kikuchii* and one cDNA was characterized as the *CFP* gene (coding for cercosporin facilitator protein) by Callahan et al. (1999). Shim and Dunkle (2002) also used SSH in *C. zeae-maydis* and identified several genes up-regulated under cercosporin-producing conditions and characterized a MAP kinase kinase kinase (*CZK3*) involved in cercosporin biosynthesis (Shim and Dunkle 2003). Chung et al. (2003c) used restriction enzyme-mediated integration (REMI) mutagenesis in *C. nicotianae* and identified a polyketide synthase gene (*CTB1*) involved in cercosporin biosynthesis and further sequencing of upstream and downstream region of *CTB1* revealed the presence of a gene cluster with *CTB1* through *CTB8* genes that code for various enzymes such as polyketide synthase, oxidoreductases, major facilitator proteins for cercosporin export and also identified four ORFs surrounding the gene clusters for which no putative function could be assigned (Chen et al. 2007a; Chen et al. 2007b; Choquer et al. 2007; Dekkers et al. 2007). The proteomics approach used in this study identified several up- and down-regulated proteins in *C. kikuchii* under light. *HNR* and *AHCY* genes characterized in this study were not identified in earlier studies, indicating that proteomics can be used as a complementary approach to other approaches like SSH and REMI to identify additional genes related to cercosporin biosynthesis.

Further studies indicate that cercosporin biosynthesis is influenced by various factors and is regulated in a very complex manner (Chen et al. 2007b). The general mode of action of cercosporin with the generation of ROS is essential for successful pathogenicity of *Cercospora*

fungi that produce cercosporin. But, some *Cercospora* species, such as *C. sojina* (frog eye leaf spot pathogen of soybeans) do not produce cercosporin and yet it is pathogenic. This suggests the involvement of additional factors in pathogenicity. The *HNR* and *AHCY* genes identified in this study are either involved in regulating cercosporin biosynthesis or part of redundant structural genes involved in cercosporin biosynthesis. Since hnr and ahcy disruption mutants are still producing small amount of cercosporin, further studies are necessary to better understand how *HNR* and *AHCY* genes are interacting with other genes by comparing protein profiles from wild-type, hnr, and ahcy gene disruption mutants and identify differentially expressed proteins.

CHAPTER 5: IDENTIFICATION OF SECRETED PROTEINS FROM *CERCOSPORA KIKCUHII*

5.1 Introduction

Many plant pathogenic fungi secrete a large number of degradative enzymes and other proteins, which have diverse functions in colonization, nutrient acquisition, and ecological interactions (de Vries 2003; Freimoser et al. 2003; Walton 1994). Many extracellular enzymes such as polygalacturonase, pectate lyase, xylanase, and lipase, have been shown or proposed to be required for virulence in plant pathogenic fungi (Brito et al. 2006; Deising et al. 1992; Isshiki et al. 2001; Oeser et al. 2002; ten Have et al. 1998; Voigt et al. 2005; Yakoby et al. 2001). Several proteomics studies have been conducted on plant pathogenic fungi to understand the nature of secreted proteins (Cao et al. 2009; Liang et al. 2010; Lim et al. 2001; Yajima and Kav 2006). The secretome of *Aspergillus flavus* and *A. parasiticus* in response to different growth media containing rutin, glucose and potato dextrose was examined (Medina et al. 2004, 2005). A high number of secreted proteins were identified when *Fusarium graminearum* was grown on hop cell wall (Phalip et al. 2005) indicating that fungal metabolism becomes oriented towards the synthesis and secretion of an arsenal of enzymes able to digest almost the complete plant cell wall. A high-throughput MS/MS (tandem mass spectrometry) to identify secreted proteins of *F. graminearum in vitro* (grown on 13 different media) and *in planta* (infection of wheat heads) identified a total of 289 proteins (229 *in vitro* and 120 *in planta*) with high statistical confidence (Paper et al. 2007). A comprehensive proteomic-level comparison of *Pyrenophora tritici-repentis* avirulence race 4 and virulence race 5, by 2-DE followed by MS/MS, identified 29 differentially abundant proteins in the secretome, including α-mannosidase, exo-β-1,3-glucanase, heat-shock, BiP (binding innmunoglobulin protein) proteins, and various metabolic enzymes

(Cao et al. 2009). The proteome-level differences suggested that reduced pathogenic ability in race 4 of *P. tritici-repentis* may reflect its adaptation to a saprophytic habit. Fernandez-Acero et al. (2010) studied the discrete sets of proteins secreted by *B. cinerea* grown on culture media supplemented with different carbon sources [glucose, carboxymethylcellulose (CMC), starch, pectin] and plant-based elicitors [tomato cell walls (TCW)] and identified 78 spots by MALDI-TOF/TOF MS/MS (matrix-assisted laser desorption/ionization time-of-flight/time-of-flight tandem mass spectrometry) analysis, corresponding to 56 unique proteins and 45 identified proteins contained secretion signal peptides for both classical and nonclassical secretory pathways.

Plant pathogenic bacteria deliver a plethora of proteins, known as effector proteins, into their host plants, using the type III secretion system (TTSS) (Abramovitch et al. 2006). Staskawitz et al. (1984) successfully cloned the first effector protein coding gene *avrA* (*avirulence A*) from *Pseudomonas syringae* pv. g*lycinea* and later several effector proteins were discovered. First fungal effector (*avr9*) was identified in *Cladosporium fulvum* in 1991 (van Kan et al. 1991) and followed by the first oomycete effector (Extracellular Protease Inhibitor 1) in 2004 (Tian et al. 2004). These effector proteins are secreted by the pathogens during the interaction with their corresponding hosts (Catanzariti and Jones 2010). Effector proteins are required for enhanced virulence in the absence of a corresponding R gene in the host (Cui et al. 2009). The pathogen effector and host R gene proteins may interact directly or indirectly (guard model) leading to effector triggered immunity (ETI) (Dangl and Jones 2001). As a result of ETI, pathogens respond by mutating or losing effectors, or by developing novel effectors that can avoid or suppress ETI, whereas plants develop novel R proteins mediating recognition of novel effectors (de Wit 2007; Jones and Dangl 2006). Extracellular fungal pathogens such as

Cladosporium fulvum and *Fusarium oxysporum* f. sp. *lycopersici* (Fol) secrete a large number of effector proteins that are small and rich in cysteine which provides stability in protease-rich environments (reviewed in Stergiopoulos and de Wit 2009). In *C. fulvum*, so far eight effector proteins were identified which include Avr2, Avr4, Avr4E, Avr9, Ecp1, Ecp2, Ecp4, and Ecp5 (Joosten et al. 1994; Laugé et al. 1997; Laugé et al. 2000; Luderer et al. 2002; van den Ackerveken et al. 1992; Westerink et al. 2004). In Fol, seven proteins have been identified in xylem sap during infection known as SIX (Secreted in xylem) proteins (reviewed in Catanzariti and Jones 2010). The fungus *Leptosphaeria maculans*, causal agent of black leg disease in brassicas, secretes three effectors, AvrLm1 (Gout et al. 2006), AvrLm6 (Fudal et al. 2007), and AvrLm4-7 (Parlange et al. 2009). Three small proteins are secreted by *Rhyncosporium secalis*, causal agent of barley leaf scald, known as necrosis inducing proteins (NIP). NIP1 can cause leaf necrosis by affecting the host's plasma membrane H^+-ATPase (Wevelsliep et al. 1993) and also activates ETI in barley cultivars carrying the *Rrs1* resistance gene (Hahn et al. 1993; Rohe et al. 1995). Necrotrophic fungi also secrete small effector proteins that have a functional role as virulence factors (Tan et al. 2010). *Pyrenophora tritici-repentis*, causal agent of tan spot of wheat, secretes PtrToxA and PtrToxB (Ballance et al. 1989; Martinez et al. 2001; Strelkov et al. 1999). The presence of *SnToxA* in *Stagonospora nodorum*, which showed a high homology to *PtrToxA* from *P. tritici-repentis* suggested that the genes coding for effector proteins can be horizontally transferred across different fungal genera (Stukenbrock and McDonald 2007). Biotrophic fungi and oomycetes have evolved haustoria for secretion of particular classes of host-translocated fungal and oomycete effectors (Catanzariti et al. 2006; Dodds et al. 2004; Whisson et al. 2007). Based on the evidence from the above studies, as the effector proteins

produced by fungi are secreted proteins, there is every need to study secreted proteins from *C. kikuchii* to identify potential effector proteins.

The study of fungal secreted proteins, which often contain virulence factors/effectors to either suppress host defense response or breach host defense mechanism/breach host physical barrier to gain access to host nutrients, is another approach to understanding host-pathogen interaction which may be used to enhance resistance to *C. kikuchii* infection in soybean. In this study we isolated secreted proteins form *C. kikuchii* grown under continuous light and dark and protein sequencing was done using liquid chromatography tandem mass spectrometry (LC-MS/MS).

5.2 Materials and Methods

5.2.1 Culture Conditions and Concentrating Culture Supernatant Fraction

Cercospora kikuchii isolate MRL6020-2B was grown on potato dextrose agar (PDA) for 1 week and three 7-mm mycelial plugs (taken 2 mm from the margins) were cut with a cork borer and ground in 2 ml of water in a glass grinder. Two hundred microliters of this mycelial suspension was used to inoculate 200 ml of minimal media [ingredients per liter were: glucose (10.0 g); $Ca(NO_3)_2.4H_2O$ (1.0 g); 10 ml of a solution containing 2.0 g of KH_2PO_4, 2.5 g of $MgSO_4.7H_2O$, and 1.5 g of NaCl in 100 ml of H_2O, adjusted to pH 5.3 with NaOH (Jenns et al. 1989)] in a 500 ml Erlenmeyer flask. Cultures were incubated at 25°C with constant shaking (200 rpm) in light (240 $\mu Em^{-2}s^{-1}$) or dark (achieved by wrapping flasks with two layers of aluminum foil). Cultures from light and dark were harvested at 7 and 14 days. Mycelia were separated from media by vacuum filtration to obtain a culture supernatant. The supernatant was concentrated using Vivaspin 20 centrifugal concentrators (GE Healthcare, Piscataway, NJ) with 5000 Da molecular weight cut off (MWCO) until the final volume was approximately 2 ml (100-

fold concentration). Concentrated protein samples were stored at -30°C until further use. All centrifugations were performed at 4°C.

5.2.2 Protein Extraction

An aliquot of concentrated protein sample was mixed with 3 volumes of pre-chilled 10% (w/v) trichloroacetic acid (TCA) in acetone containing 0.07% 2-mercaptoethanol (2-ME) and kept at -30°C overnight (Damerval et al. 1986; Vincent et al. 2009). Proteins were pelleted by centrifugation at 10,000 g for 30 min at 4°C. The protein pellet was washed three times with 80% acetone containing 0.07% 2-ME. Each time the pellet was resuspended in acetone. After washing, the protein pellet was air dried and stored at -30°C until further use.

5.2.3 2-DE (IEF/SDS-PAGE), Staining, and Scanning

Protein pellets were solubilized in lysis buffer (8 M urea, 4% 3-[(3- cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS), 40 mM dithiotheritol (DTT), and 2% (w/v) 3-10 NL IPG (Non-linear immobilized pH gradient) buffer. IPG strips (7 cm, pH 3-5.6 NL, GE Healthcare) were rehydrated overnight in 125 µl of rehydration solution (8 M urea, 2% (w/v) CHAPS, 20 mM DTT, 0.002% bromophenol blue, and 0.5% (w/v) 3-5.6 NL IPG buffer) containing 51 to 259 µg of proteins. Strips in the re-swelling tray were covered by Immobiline™ DryStrip cover fluid (GE Healthcare, Piscataway, NJ), leveled horizontally, and left overnight at room temperature. Isoelectric focusing (IEF) was performed as follows: (i) step and hold 300 V for 30 min; (ii) gradient to 1000 V for 30 min; (iii) gradient to 5000 V for 90 min; and (iv) step and hold 5000 V for 90 min for a total of 12 kVh. The focused IPG strips were equilibrated for 20 min in 7 ml per strip of equilibration buffer I (50 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 20% (w/v) SDS, a trace of bromophenol blue, 1% (w/v) DTT) with gentle shaking, followed by another 20 min in 7 ml of equilibration buffer II [50 mM Tris-HCl pH 8.8, 6 M urea,

30% (v/v) glycerol, 20% (w/v) SDS, a trace of bromophenol blue, 2.5% (w/v) iodoacetamide (IAA)] per strip. The equilibrated IPG strips were subjected to second dimension on the 12% Mini-PROTEAN® TGX precast gels with IPG comb (Bio-Rad, Cat # 456-1041, Hercules, CA). Electrophoresis was carried out at 20 V for 10 min and 100 V for 90 min until the bromophenol blue dye reached the bottom of the gel. Gels were fixed overnight in fixing solution [10% (v/v) acetic acid and 40% (v/v) methanol] followed by staining with Coomassie Brilliant Blue G-250 dye [(2% (v/v) phosphoric acid, 10% (w/v) $(NH_4)_2SO_4$, 0.1% (w/v) Coomassie Brilliant Blue G-250, and 20% (v/v) methanol] (Candiano et al. 2004; Neuhoff et al. 1985, 1988) for 24 hr at room temperature. The secreted proteins prepared from three biological repeats for each treatment (light and dark) and time point (7 and 14 days) were separated on 2-DE gels. Only consistently expressed protein spots were recovered for identification using LC-MS/MS.

5.2.4 In-Gel Trypsin Digestion

Fifteen protein spots were excised from the 2-DE gels, cut into 1 mm^2 pieces and subjected to in-gel digestion by trypsin (Proteomics grade trypsin, Sigma, Cat # T6567) as previously described (Shevchenko et al. 2007). Briefly, the recovered gel pieces were first destained twice using 100 mM ammonium bicarbonate and acetonitrile (ACN) (1:1 v/v) followed by shrinking by adding 500 µl acetonitrile. On ice, 50 µl of trypsin digestion buffer [20 µg trypsin dissolved in 1.5 ml of 10 mM NH_4HCO_3/10% (v/v) ACN (1:1 v/v)] was added to cover the gel pieces and left for 30 min. Samples were left on ice for another 90 minutes. Samples were incubated at 37°C overnight for trypsin digestion. Peptides were extracted from the gel by adding extraction buffer containing 5% formic acid/acetonitrile (1:2 v/v). The collected supernatants, which contain the peptide mixtures, were dried under vacuum in a Savant SpeedVac (Thermo Scientific) and stored at -30°C. Just before mass spectrometry, peptides were resuspended in 10

μl of 0.1% (v/v) trifluoroacetic acid (TFA). The peptide mixtures were injected into LC-MS/MS to determine peptide mass spectra or *de novo* peptide sequences.

5.2.5 Liquid Chromatography Tandem Mass Spectrometry and Protein Identification

Liquid chromatography tandem mass spectrometry (LC-MS/MS) was used to analyze the peptides extracted from the gel spots. The tryptic peptides dissolved in 10 μl 0.1% (v/v) TFA was injected with a microplate autosampler (Famos, Dionex, Sunnyvale, CA, USA) onto a 0.3 × 1 mm trapping column (PepMap C_{18}, Dionex) on a nano-LC system (Ultimate, Dionex) at a flow rate of 10 μl/min. After sample loading, the trapping column was washed with 0.1% (v/v) formic acid at a flow rate of 5 μl/min for an additional 5 min. The peptides were then eluted onto a 75 μm × 15 cm C_{18} column (Biobasic Vydac, Grace Davison, IL, USA) and separated using a gradient of 5–40% solvent B over 60 min with a flow rate of 200 nl/min. Solvent A was 95% water and 5% acetonitrile containing 0.1% formic acid. Solvent B was 80% acetonitrile and 20% water containing 0.1% formic acid. The effluent was directed to a quadrupole time-of-flight mass spectrometer (QSTAR XL, Applied Biosystems, Foster City, CA, USA) and ionized using a nano-electrospray source at a voltage of 2.5 kV. The mass spectrometer was operated in information-dependent acquisition (IDA) mode. Three collision energies (25, 38 and 50 eV) were selected for fragmentation of the peptides. The MS/MS spectra were subjected to database search using MASCOT (Perkins et al. 1999, http://www.matrixscience.com) and proteins were identified based on homology searches against the NCBI non-redundant (NCBInr) protein database and the SwissProt database with taxonomy limited to fungi. MASCOT individual ions scores of 48 or greater indicate a significant homology to the peptide spectra in the database ($p<0.05$). Ions score is calculated as -10*Log(P), where P is the probability that the observed match is a random event.

5.3 Results

5.3.1 2-D Electrophoresis of the Secreted Proteins

Initial trial 2-D gels in which the first dimension was done with IPG strips spanning a pH range of 3-10, showed most of the protein spots were concentrated in the pH range 3-6 (not shown). Therefore, IPG strips with a pH range of 3–5.6 NL were used for subsequent experiments. For each time point (7 and 14 days) and cultures grown in light and dark, proteins from three biological repeats were separated in three gels. The spot patterns in these gels were very consistent and reproducible. For cultures grown in light, the total number of protein spots was approximately 50, whereas about 60 spots were observed in samples from cultures grown in the dark (Fig. 5.1). Differences in protein profiles between light and dark were observed, such as the presence of high molecular weight proteins in light-grown cultures, but not in dark-grown cultures. In addition, some of the medium and low molecular weight proteins with a p*I* range of 4.5–5.6 were found only in the dark-grown cultures (Fig. 5.1).

5.3.2 Protein Identification

Fifteen protein spots (Fig. 5.1) were excised from 2-D gels. Following in-gel trypsin digestion, peptides were separated by liquid chromatography and subjected to tandem mass spectrometry. Out of 15 spots, only three spots were positively identified based on their MS/MS spectra, corresponding to three different hypothetical proteins (Table 5.1) with only one matching peptide per protein. Spots 4 and 26 showed the presence of possible signal peptides based on predictions with SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP/). Identities of the remaining 12 spots are unknown although their partial peptide sequences have been obtained determined (Table 5.2).

These proteins were annotated according to results of sequence homology search using BLAST against the NCBInr database. Spot 4 showed no significant homology to any proteins in the database. Spot 26 showed homology (63% identity) with glucan endo-1,3-beta-glucosidase eglC from *Aspergillus flavus* (GenBank accession XP_001816626). Spot 45 showed homology to EAP30 family protein Dot2 from *Ajellomyces capsulatus* (GenBank accession EER40012).

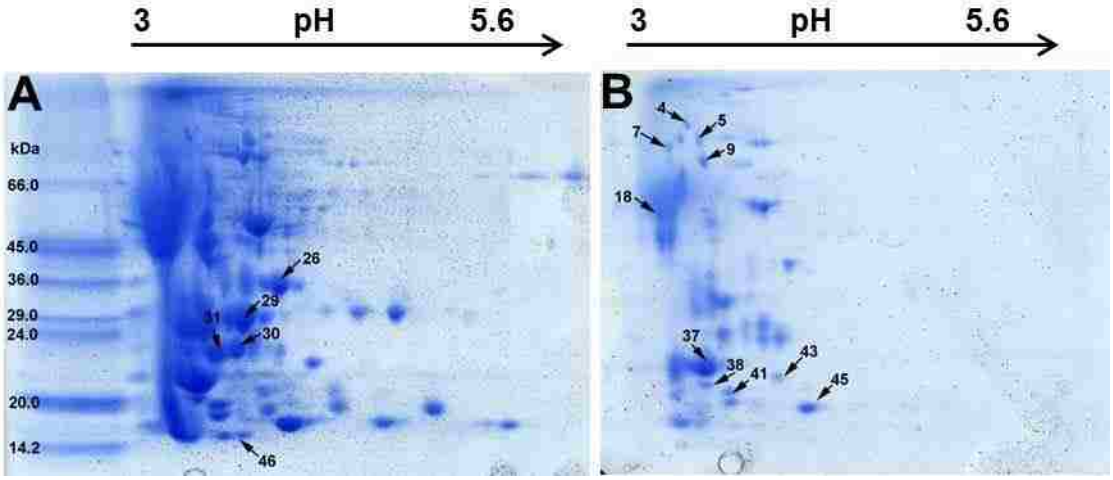


Figure 5.1. Profiles of secreted proteins from *Cercospora kikuchii* grown under dark and light conditions separated on two dimensional polyacrylamide gels. Protein profiles of *C. kikuchii* grown in minimal media broth with constant shaking (200 rpm) for 1 week in continuous dark (**A**, 224 µg) and continuous light (**B**, 51 µg). Culture supernatant was concentrated and secreted proteins were precipitated by acetone precipitation and separated on 3-5.6 NL IPG strip, followed by 12% w/v SDS-PAGE. Arrows indicate the protein spots recovered from the SDS-PAGE gel for LC-MS/MS.

5.4 Discussion

The typical symptoms of CLB only appear during the mid-pod fill stage of development (R5) even though in an earlier study, using real-time PCR primers and probe specific for *C. kikuchii*, we detected *C. kikuchii* in soybean leaf tissues as early as 22 days after planting (V4 growth stage). Such a long latent period prompted us to look into different proteins that may be secreted by *C. kikuchii* during this phase of the disease. Towards this goal, as a first step, we

designed this experiment to gain insight into proteins secreted *by C. kikuchii in vitro* when grown on minimal media. To our knowledge, this is the first report of proteins secreted by *C. kikuchii.*

Out of 15 spots recovered and sequenced, only spots 4, 26, and 45 were positively identified in this study (20% discovery) that show significant homology to other proteins in the database. Spot 4 has no significant homology to any known proteins in the database suggesting this protein may be an uncharacterized protein of *C. kikuchii*. Spot 26 showed homology to glucan beta 1,3-glucosidase (EC 3.2.1.58), which belongs to a class of hydrolytic enzymes that catalyze the cleavage of 1,3-β-D-glucosidic linkages in β-1,3-glucans and are found in many fungi (Espino et al. 2010; Fernandez-Acero et al. 2010; Liang et al. 2010; Vincent et al. 2009) and plants (Doxey et al. 2007). The secretion of beta-1,3-glucanases by the yeast *Pichia membranifaciens* is one of the possible mechanisms related to its antagonism against *Botrytis cinerea* (Masih and Paul 2002). β-glucanases have a role in many developmental processes including fungal cell wall growth and extension (Adams 2004). They are also involved in mobilization of β-glucans in response to conditions of carbon and energy source exhaustion (Piston et al. 1993). They play important nutritional roles in both saprobic and mycoparasitic fungi, and represent primary biochemical offensive weapons for parasitized fungal cell wall degradation (de la Cruz et al. 1995; Pitson et al. 1993). β-glucanases play a crucial role in fungal cell autolysis that is triggered by many factors (White et al. 2002). Given the various roles played by β-glucanases in fungi, it is not surprising to see this protein secreted by *C. kihuchii*.

Table 5.1. List of secreted proteins of *Cercospora kikuchii* identified by LC-MS/MS.

Spot[w]	Top Hit	Organism	NCBI accession number	p*I*[x]	MW (kDa)[y]	MASCOT score[z]	E value	Peptide Sequence
4	Hypothetical protein MYCGRDRAFT_64715	*Mycosphaerella graminicola*	EGP82597.1	4.45	60	88	5.7e-06	AGLTTAGAVYGLDGR
26	Hypothetical protein BC1G_09079	*Botryotinia fuckeliana*	XP_001552608.1	4.34	44	49	0.054	VVGISVGSEDLYR
45	Hypothetical protein MYCGRDRAFT_103841	*Mycosphaerella graminicola*	EGP88932.1	6.1	28	51	0.043	AENGGMIALAEAR

[w]: spot number corresponds to spots shown in Fig. 5.1

[x]: predicted isoelectric point

[y]: predicted Molecular weight

[z]: see materials and methods (5.2.5) for description of scores

Table 5.2. List of peptide sequences of unknown secreted proteins of *Cercospora kikuchii* identified by *de novo* sequecing using Mascot Distiller version 2.4.2.0.

Spot No.	**Peptide sequence**
5	VDP[YT]A[RG] DiPYiqEiYDDi YG[GC]NEVqPR
7	iVD[SGC]NA[RG] NGAGPFVVNEiSFASR RiSSPAMN SSPAT[SG][VA|Gi]R
9	EiSqFiNAAK iqEiYTNTiR iGiDNVAMDDFYR VASEiGiDNVAFDDFYR [VA|Gi]SEiAVE[VN|RG]AFDDFYR Yii[SG]iiYGSqVDAFR YGSqVDAFR YiG[GC]SAGGAqGFK
18	iSSPATRASR iiFWASDEPHR NGAGPFVVNEiSFASR iSSPAMN
29	YiGYiEqiiR PiSGCAEVGTTFEVK RPDiYWVDiVPRR GViiVGFADDAPiFR iDWTEDAVEVPNiDRK AGWGiDSDGGiR PFYDTYG AiTVSGR VGFADDAPFiR AGEiATVAGW
30	WYiENATEEFK iHEPVDGS[NH]A[SG]iYK YVDSGDE[PN]GYiAGGiK NNNYYDGiMK [VD|Ti]GGVPiFEGFiNYYDVAFK
31	iPVTDDEV[VP]TGAGR SP[GC]WPNVVDW[GC]VSAR WTEDAVEFYiDGVK NiiAGR G[GC]ASAEFPSVYAK [NC]ASAEFPSVYAK

Table 5.2. (continued) List of peptide sequences of unknown secreted proteins of *Cercospora kikuchii* identified by *de novo* sequecing using Mascot Distiller version 2.4.2.0.

Spot No.	**Peptide sequence**
37	qEFDqAiTiVK qDFATFAiYSDNR EDFATFANYSDNR FYESiGNR qEAqRAD[NF]R EAEWGiSYAGAiR FAiYSDNR NGADiDi[Pq]ANAK ADVWVNq[VP]NSNTPSPDR TGYSSPDR YiS[GC]PGAiTFDNT[PA]K
38	ATFAiYSDNR GEAEqFiNAAK qGDiiPRVi[GC]R iAASSYN qDFATFAiYSDNR SiDPAVPN EAEWGiSYAGAiR CETiAGEqSiMCD[CC][PA]K
41	GAGPFDqGTNDPK DADqGTNDPK VGEViATADPN NPDATAiVEGVPVR DATAiVEGV[VP]R N[WP]TAiVEGV[VP]R ATAiVEGV [SGC]N[PD|Vi]ATPS[GC]
43	[VD|Ti]ADiiiTSiSR EiV[GCC]iNAAK GNEqFiNAAR FATFAiYSDNR
46	AGPATE iE[NA|Gq]TEEFK MiiRiSSPAT iSSPATGS AqAFNPSTiAN[AA]K WiENATEEFK AqDFCGK NFqqSGqVS[GC]K

Spot 45 showed homology to EAP30 family protein. EAP30 encodes for a 30 kDa protein, which is part of a multiprotein complex of RNA polymerase elongation factor ELL and three ELL-associated proteins (Shilatifard 1998). EAP30 protein shows significant homology to the *Saccharomyces cerevisiae SNF8* gene, which is required for efficient derepression of glucose repressed genes (Schmidt et al. 1999). As *C. kikuchii* was grown on minimal media containing glucose, EAP30 protein might be induced.

MASCOT search uses an algorithm in which MS/MS spectra of peptide ions generated are searched against the available protein information in public databases such as NCBInr and SwissProt. If a particular protein is not present in the database, the chance of having a positive identification is very low. The reason for such a low discovery rate (20%) in this study is the lack of genome sequence information for *C. kikuchii*. The alternative approach to identify the peptides would be to analyze the MS/MS spectra using *de novo* sequencing software such as Mascot Distiller and obtain the peptide sequences by carefully reading the mass spectra. We were not able to identify the proteins based on *de novo* approach. Once genome sequence of *C. kikuchii* is available in future, many secreted proteins can be identified from *C. kikuchii*, and some of the secreted effector proteins can be used as a potential target for silencing using virus induced gene silencing (VIGS), as a means of enhancing resistance to *C. kikuchii* infection in soybeans.

CHAPTER 6: EVALUATION OF HOST-INDUCED GENE SILENCING (HIGS) STRATEGY FOR CONTROL OF *CERCOSPORA KIKUCHII* INFECTION OF SOYBEANS

6.1 Introduction

RNA interference (RNAi) is a conserved gene silencing mechanism in eukaryotes (Baulcombe 2004). RNAi was first described in *Caenorhabditis eleagns* in which double-stranded RNA (dsRNA) induces sequence specific post-transcriptional gene silencing (PTGS) (Fire et al. 1998). RNAi is referred to as quelling in fungi (Romano and Macino 1992) and, co-suppression in plants (Napoli et al. 1990). RNAi is typically achieved by application or expression of dsRNA homologous to a target sequence to silence its expression. Various RNAi pathways utilize small noncoding RNAs (sRNAs) of about 20 to 30 nucleotides (nt) that act as regulators of cellular processes, host defense, transcription and translation (Reviewed in Dang et al. 2011). sRNAs include both short interfering RNAs (siRNAs) and micro RNAs (miRNAs). Both miRNAs and siRNAs are derived from dsRNA precursors that are recognized and processed by Dicer to generate short duplexes (21 to 25 nt). siRNAs are derived from exogenous dsRNA (e.g. viral RNA) or endogenous transcript from repetitive sequences, or from transcripts that can form long hairpins (Carthew and Sontheimer 2009; Ghildiyal and Zamore 2009; Hannon 2002), and siRNA-induced silencing requires RNA-dependent RNA polymerases (RdRPs) (Allen et al. 2005; Cogoni and Macino 1999; Gent et al. 2010) whereas miRNAs are generated from miRNA-encoding genes that generate ssRNA precursor transcripts from hairpin structures. siRNAs trigger RNA cleavage or transcriptional silencing mediated by Argonaute proteins and generally function in genome defense, while miRNAs can target mRNAs that are not fully complementary and cause mRNA degradation and translational repression (Ambros et al. 2003; Bartel 2004). RNAi components (Dicer, Argonaute, and RdRP) have been identified in all major

branches of eukaryotes (Cerutti and Casas-Mollano 2006; Shabalina and Koonin 2008), suggesting that RNAi is an ancient defense and/or regulatory mechanism that existed in the common eukaryotic ancestor.

RNAi has been used commercially to engineer virus resistance in plants by expression of viral sequences as transgenes (Frizzi and Huang 2010). RNAi-based silencing showed reduced development of root knot nematodes as well as *Lepidoptera* and *Coleoptera* insects feeding on transgenic plants carrying RNAi constructs against target genes in these pests (Baum et al. 2007; Huang et al. 2006; Huvenne and Smagghe 2010; Mao et al. 2007;Sindhu et al. 2009; Yadav et al. 2006). Tomilov et al. (2008) reported that β-glucuronidase (GUS) expression was suppressed in *Tryphysaria* spp. by expressing dsRNA of the GUS gene in the host plant, and they demonstrated the application of host-induced gene silencing (HIGS).

Like other eukaryotes, fungi are sensitive to RNAi (Nakayashiki 2005; Nakayashiki and Nguyen 2008; Nakayashiki et al. 2006). Exploration of HIGS to engineer resistance in plants to fungi seems to be a viable alternative based on the outcome from several recent studies. Tinoco et al. (2010) showed that tobacco transformed to express a GUS hairpin-structured dsRNA specifically silenced GUS transcripts in a GUS-expressing strain of *Fusarium verticillioides* during infection. *In planta* expression of fungal dsRNA also triggered HIGS in the biotrophic pathogens *Blumeria graminis* and *Puccinia striiformis* f.sp. *tritici*. Nowara et al. (2010) used virus induced gene silencing (VIGS) in barley using barley stripe mosaic virus (*BSMV*) to produce antisense *GTF1* and *GTF2* transcripts (1,3-β-glucanosyltransferase) that resulted in significant reduction in haustorium formation and the rate of secondary hyphal elongation, respectively. Yin et al. (2011) demonstrated HIGS in wheat targeting *Puccinia striiformis* f.sp. *tritici* (PST) genes in which a highly abundant haustorial PST transcript (PSTha12J12) was

silenced by the expression of dsRNA in VIGS mediated BSMV–wheat transformants. Further examination of other PST genes revealed that only genes highly expressed in haustoria were effectively silenced (Yin et al. 2011).

Bean pod mottle virus (BPMV)-VIGS system has been successfully used to test the candidate genes for their involvement in resistance to *Phakopsora pachyrhizi* in soybeans (Meyer et al. 2009; Pandey et al. 2011). In this study, a *C. kikuchii* gene (*AHCY*) was selected for analysis. *AHCY* codes for adenosylhomocysteinase and in our previous experiments ahcy disruption mutants of *C. kikuchii* produced low levels of cercosporin and also showed reduced virulence on soybeans. The BPMV-VIGS system is used to express dsRNA from *C. kikuchii AHCY* gene in soybean plants to determine whether silencing signals can be delivered to the pathogen and suppress expression of the fungal genes. The broader goals are to develop a host-induced gene-silencing system for functional analysis of *C. kikuchii genes* and for control of *C. kikuchii* infection of soybeans.

6.2 Materials and Methods

6.2.1 Construction of BPMV-Derived Vectors

The *Cercospora kikuchii AHCY* gene was targeted for silencing using BPMV derived vectors (Figure 6.1A; Zhang et al. 2010). The middle and 3′-end fragments of *AHCY* were designated as AHCY #6 and AHCY #7-1, respectively (Figure 6.1B). The AHCY #6 and AHCY #7-1 sequences were used to search public sequence databases and showed no homology to the available soybean genome sequences. AHCY #6 and AHCY #7-1 were amplified by PCR from cDNA of *C. kikuchii* using primer pairs AHCY_F_176/AHCY_R_614 and AHCY_F_491/AHCY_R_883, respectively (Fig. 6.2). The AHCY_F_176/AHCY_R_614 primers contain a *Stu*I restriction site at the 5′-end and a *Sal*I site at the 3′-end allowing insertion

of AHCY #6 in a reverse orientation into the vector pBPMV-IA-V2. The AHCY #6 and #7-1 were subcloned into pCR™2.1-TOPO® TA Cloning vector (Invitrogen, Carlsbad, CA) to obtain pTOPO-AHCY-6 and pTOPO-AHCY-7-1, respectively, and confirmed by DNA sequencing using vector specific primers T7 and M13. The AHCY #6 was released from pTOPO-AHCY-6 by *Stu*I and *Sal*I digestion (Fig. 6.3) and inserted into similarly digested and dephosphorylated pBPMV-IA-V2 (Zhang et al. 2010) to generate pBPMV-AHCY-6. *Stu*I and *Sal*I digestion of pBPMV-AHCY-6 confirmed the release of the insert with the expected size (428 bp) (Fig. 6.3). AHCY #7 was released from pTOPO-AHCY-7-1 by *Sal*I digestion (Fig. 6.3) and inserted into similarly digested and dephosphorylated pBPMV-IA-V2 to generate the pBPMV-AHCY-7. *Sal*I digestion of the pBPMV-AHCY-7 also confirmed the release of the insert with the expected size (241 bp) (Fig. 6.3). 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 and pBPMV-IA-V2 was used as an empty vector control.

6.2.2 Delivery of BPMV-Derived Vectors into Soybean Leaves

For HIGS experiments, pBPMV-IA-R1M (modified RNA1 of BPMV, Zhang et al. 2010) was either mixed with pBPMV-IA-V2 (empty vector control), pBPMV-PDS-3R (positive control), pBPMV-AHCY-6 (AHCY #6), or pBPMV-AHCY-7 (AHCY #7) and biolistically introduced into the primary leaves (vegetative cotyledon stage) of 14-day-old soybean seedlings (cultivar ASGROW 6202) by a single particle bombardment (Please see Appendix for detailed protocol) as described (Sanford et al. 1993) using a Biolistic PDS-1000/He system (Bio-Rad Laboratories, Hercules, CA), 1.0 μm gold particles, and 1,100-psi rupture disks at a distance of

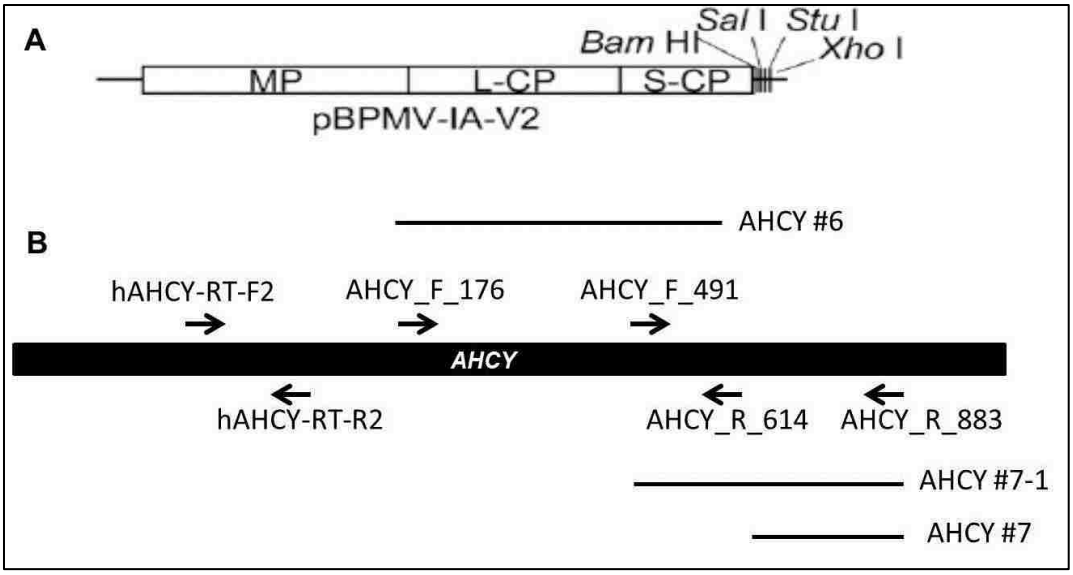


Figure 6.1. **A**, Diagram of the RNA2 molecule of BPMV. The restriction sites at the end are for integration of genes of interests into the vector. **B**, Diagram of *AHCY* gene with arrows indicating location of primers used to amplify the middle and 3′ fragments of the *AHCY* gene in *Cercospora kikuchii*. Note: Map is not to scale.

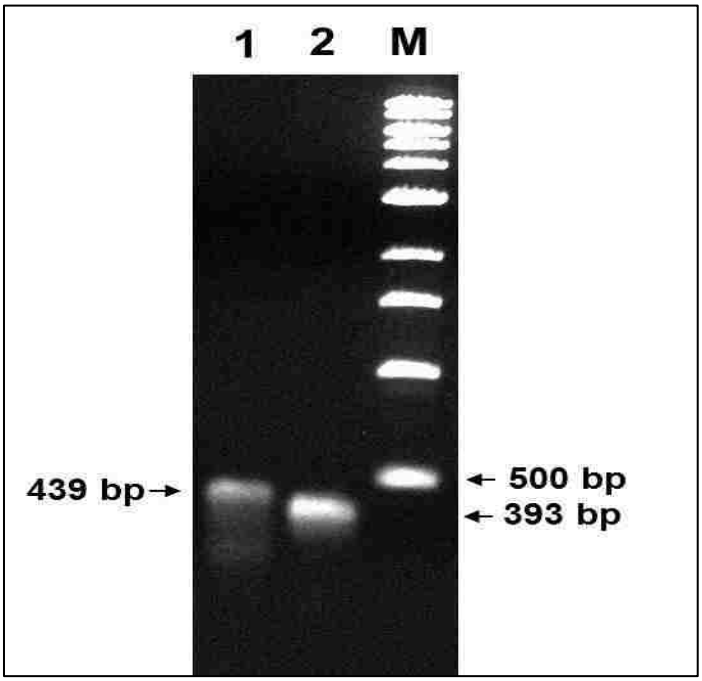


Figure 6.2. PCR amplification of two different regions of *AHCY* gene from *Cercospora kikuchii*. Lane 1, the middle region of *AHCY* (AHCY #6, 439 bp); lane 2, the 3′ end of *AHCY* (AHCY #7-1, 393 bp); and lane M is 1 kb DNA ladder.

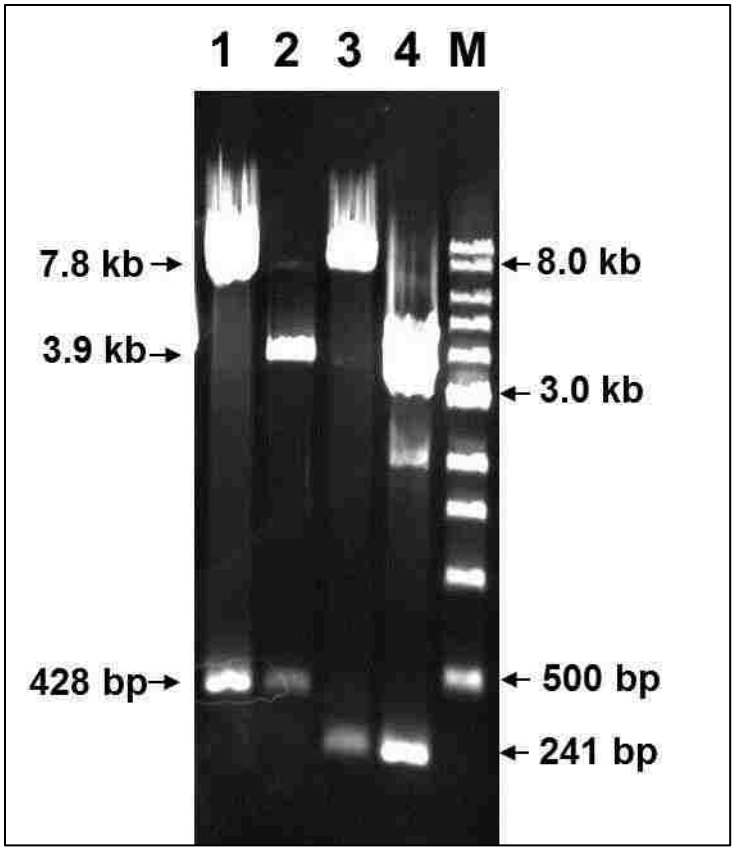


Figure 6.3. Restriction digestion of pBPMV-AHCY-6 (lane 1) and pTOPO-AHCY-6 (lane 2) with *Stu*I and *Sal*I showing release of a 428 bp fragment corresponding to AHCY #6. Restriction digestion of pBPMV-AHCY-7 (lane 3) and pTOPO-AHCY-7-1 (lane 4) with *Sal*I showing release of a 241 bp fragment corresponding to AHCY #7. The linearized pBPMV-IA-V2 (lanes 1 and 3) and pCR™ TOPO®-2.1 (lanes 2 and 4) vector parts are shown as 7.8 kb and 3.9 kb fragments, respectively. Lane M is 1 kb DNA ladder.

6 cm. Following bombardment, plants were lightly misted with water and maintained in the greenhouse at 20°C with a photoperiod of 16 h.

6.2.3 *Cercospora kikuchii* Inoculation of Soybean Plants Carrying BPMV-Derived Vectors

C. kikuchii mycelial suspension (blend of 1g mycelium in 100 ml water with 0.001% Tween-20) was sprayed onto healthy soybean plants and plants (2 weeks after BPMV inoculation by particle bombardment) carrying the empty vector, pBPMV-AHCY-6, or pBPMV-AHCY-7 constructs. Mock inoculated plants were sprayed with 0.001% Tween-20.

Immediately after inoculation, the plants were sealed in a plastic bag with moist paper towels (to maintain 100% RH) and kept in the dark for 24 h. Later the plants were transferred to greenhouse benches and the development of symptoms was observed until 14 days post inoculation (dpi).

6.2.4 Soybean Leaf Tissue Collection

Leaves were collected from mock- and *C. kikuchii*-inoculated healthy (4 plants), empty vector (4 plants), AHCY #6 (4 plants), or AHCY #7 (5 plants) bombarded soybean plants at 0, 7 and 14 dpi. For each treatment, leaves collected from individual plants were considered as one sample. Individual plant is considered as a replicate (minimum of 4 plants). The leaves were immediately frozen in liquid nitrogen and stored at -80°C.

6.2.5 Total RNA Isolation and RT-PCR Analysis

Leaf tissue was ground in liquid nitrogen, and total RNA was extracted using RNeasy Plant Mini Kit (Qiagen) and treated with DNase I to eliminate residual DNA contamination. Total RNA was extracted from the leaves of ASGROW 6202 soybean infected with BPMV carrying the RNAi target regions of *AHCY* of *C. kikuchii*. RNA also was isolated from healthy plants and plants which were infected with the empty pBPMV-IA-V2 vector carrying no fungal sequences. Reverse transcription was conducted with 500 ng of total RNA using Taqman® reverse transcription reagents (Applied Biosystems, Foster City, CA) according to the manufacturer's guidelines. The level of *AHCY* expression in soybean leaves was determined by comparative quantitative real-time polymerase chain reaction (qRT-PCR) using 18S rRNA to normalize RNA amounts. Real-time PCR assays were performed using SYBR® Green PCR Master Mix (Applied Biosystems) in a 25 µl reaction volume with 1 µl of reverse transcribed cDNA, 12.5 µl 2X SYBR® Green Master Mix, and 1 µM of each primer. ABI PRISM 7000 Sequence Detection System was used for real-time PCR under standard conditions. The same protocol was used for 18S rRNA (internal control) primers. Specific primers for *AHCY* gene

were selected from regions outside of those used in RNAi target regions for AHCY #6 and AHCY #7 and were designed using primer design software available at http://www.idtdna.com (Table 6.1). Melting curve analysis was conducted to confirm amplification of a single product and the absence of primer dimers. Relative RNA expression of *AHCY* was determined using the ΔΔCt method ($[Ct_{(18S\ rRNA)} - Ct_{(AHCY)}]_{AHCY\ \#6\ or\ \#7}$ - $[Ct_{(18S\ rRNA)} - Ct_{(AHCY)}]_{empty\ vector}$) (Pfaffl 2001).

6.2.6 DNA Isolation and Quantitative Real-Time PCR Assessment of *C. kikuchii* Accumulation

Leaf tissue was ground in liquid nitrogen, and DNA was extracted using a GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, St Louis, MO*)* according to the manufacturer's instructions. All DNA samples were diluted to 10 ng/µl according to DNA

Table 6.1. List of primers used in this HIGS study.

Primer	Sequence (5′ to 3′)
AHCY_F_176	GAGGCCTCGCACCATCCATC
AHCY_R_614	AACGTCGACGGCGGTCTTCTTCA
AHCY_F_491	GAGGCCGAGACATCATTGTTGG
AHCY_R_883	TGAGTCGACCCTCCTCTTGGA
BPMV-IA-F1	TGTGCTGCTGTTGGCTTGAC
BPMV-IA-R1	TCAGTTTGCCCATAAACCTAT
R2-3195F	CCTCATTGGTACAAGTGTTT
Gm-PDS-3F	CGCGGATCCGCAAGGAATATTATAGCCCAAA
AHCY_R_510	AACAATGATGTCTCGGCAACC
AHCY_R_816	GACATACTTGGTGGCGAACTG
hAHCY-RT-F2	ACTCAAGATCACGCTGCC
hAHCY-RT-R2	CATCTCTGGGTACTTGGTGTG
CKCTB6-2F	CACCATGCTAGATGTGACGACA
CKCTB6-2R	GGTCCTGGAGGCAGCCA

quantification based on absorption at 260 nm using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE). Real-time PCR using primers CKCTB6-2F/CKCTB6-2R and fluorescent probe (CKCTB6-PRB: 5'-FAM-

CTCGTCGCACAGTCCCGCTTCG-TAMRA-3') targeting the *C. kikuchii CTB6* gene, was performed with MicroAmp optical 8-tube strips and optical 8-cap strips (Applied Biosystems, Foster City, CA) in the ABI 7000 sequence detection system (Applied Biosystems) under the standard conditions. Each reaction contained 7.5 µl 2X TaqMan Universal PCR Master Mix (Applied Biosystems), 1 µl of 10 µM each primer (final concentration 666 nM each primer), 0.6 µl of 10 µM (final concentration of 400 nM) probe and 1 µl of 10 ng or 100 ng template DNA. The absolute quantity of DNA of *C. kikuchii* in leaf samples, which was used to determine the level of infection by *C. kikuchii*, was calculated by using the Ct value of each sample and the regression equation obtained with DNA standards of *C. kikuchii* ($y = -1.434\ln(x) + 37.913$), with x being pg of *C. kikuchii* DNA and y being Ct value. Amounts of DNA of *C. kikuchii* are expressed as picograms per nanogram soybean DNA.

6.3 Results

6.3.1 Successful Delivery of VIGS Vectors into Soybeans

The biolistic inoculation method used in this study to deliver the modified BPMV was successful. After approximately 14 days, plants inoculated with recombinant pBPMV-IA-V2 constructs developed typical BPMV symptoms such as crinkled leaves with a mosaic of light and dark green regions and photobleaching was observed in positive control plants bombarded with pBPMV-IA-PDS-3R (Fig 6.4). Reverse transcription (RT)-PCR was performed with pBPMV-IA-V2 vector-specific forward primer R2-3195F and each insert-specific reverse primer, Gm-PDS-3F for *PDS*, AHCY_R_510 for AHCY #6, and AHCY_R_816 for AHCY #7 to examine the integrity of the recombinant BPMV RNA2. A single band with the expected size was observed for each BPMV RNA2 carrying the corresponding *PDS* or *AHCY* insert (Fig. 6.5)

demonstrating that the *PDS* and *AHCY* fragments were stably maintained during the virus infection.

6.3.2 Silencing expression of *Cercospora kikuchii AHCY* Transcript in Soybean using BPMV as a HIGS Vector

In order to determine whether *C. kikuchii* genes can be silenced *in planta*, the RNAi target regions (AHCY #6 and AHCY #7) were chosen to be *C. kikuchii* specific by homology searches to all soybean sequences in the database to avoid nonspecific silencing of plant genes. The level of *AHCY* expression in modified virus inoculated plants was compared with vector control and healthy plants. At 7 dpi, we detected 3.5-fold (ranged from 2.4 to 4.4-fold in 3 plants) and 6.6-fold (ranged from 4.1 to 9.3-fold in 5 plants) reduction in *AHCY* transcript levels in soybean plants expressing HIGS gene constructs AHCY #6 and AHCY #7 respectively, compared to vector control. However, by 14 dpi, *AHCY* transcript levels in both AHCY #6 and ACHY #7 were similar to vector control (data not shown).

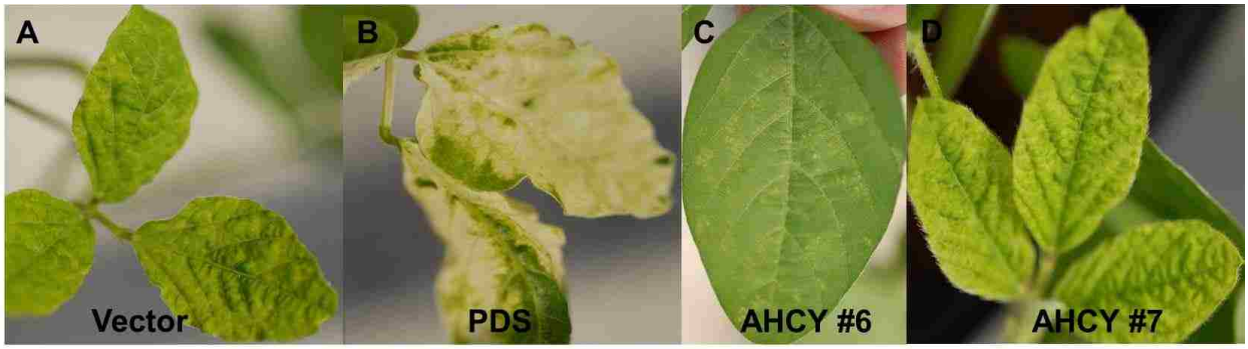


Figure 6.4. Symptoms induced by blank BPMV vector or modified recombinant virus expressing foreign genes on leaves of soybean cultivar ASGROW 6202. pBPMV-IA-R1M was used as the RNA1 for all inoculations. **A**, Vector soybean plants were bombarded with the empty pBPMV-IA-V2. **B**, PDS plants were bombarded with the modified virus containing pBPMV-PDS-3R as RNA2. **C** and **D**, AHCY #6 and AHCY #7 plants that were bombarded with modified virus containing pBPMV-AHCY-6 and pBPMV-AHCY-7 as RNA2, respectively. AHCY #7 induced strong viral symptoms compared to AHCY #6. Plants were photographed at 4 weeks post inoculation.

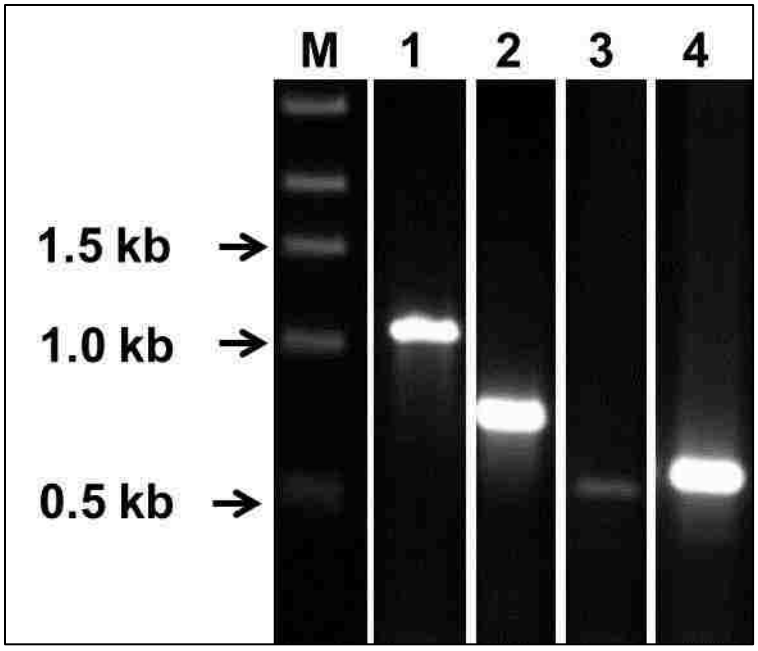


Figure 6.5. Empty vector (pBPMV-IA-V2) amplified with primers BPMV-IA-F1 and BPMV-IA-R1 (lane 1) showing expected 1.1 kb fragment with no insert. RT-PCR analysis using the forward primer R2-3195F and each of the reverse primer corresponding to the PDS, AHCY #6, and AHCY #7 target sequence in each of the HIGS constructs to confirm the PDS (lane 2), AHCY #6 (lane 3) and AHCY #7 (lane 4) fragment insertions. Lane M is 1 kb DNA ladder.

6.3.3 Inoculation of Soybeans Plants Carrying HIGS Constructs with *Cercospora kikuchii*

Soybeans bombarded with two HIGS gene constructs (AHCY #6 and AHCY #7), vector control constructs with no fungal genes, and healthy plants were inoculated with mycelial suspension of *C. kikuchii*. At 7 dpi, healthy and vector control plants developed numerous necrotic lesions, whereas in AHCY #6 and AHCY #7 plants had few necrotic lesions (Fig. 6.7). Real time PCR analysis of DNA samples isolated from *C. kikuchii* inoculated soybean plants that had been pre-bombarded with HIGS constructs found that by 7 dpi, *C. kikuchii* biomass decreased by 16-fold and 18-fold fold in AHCY #6 and AHCY #7 plants respectively (Fig 6.8). We saw good negative correlation between *AHCY* transcript levels and *C. kikuchii* biomass in AHCY #6 (r = -0.81) and AHCY #7 (r = -0.72) plants.

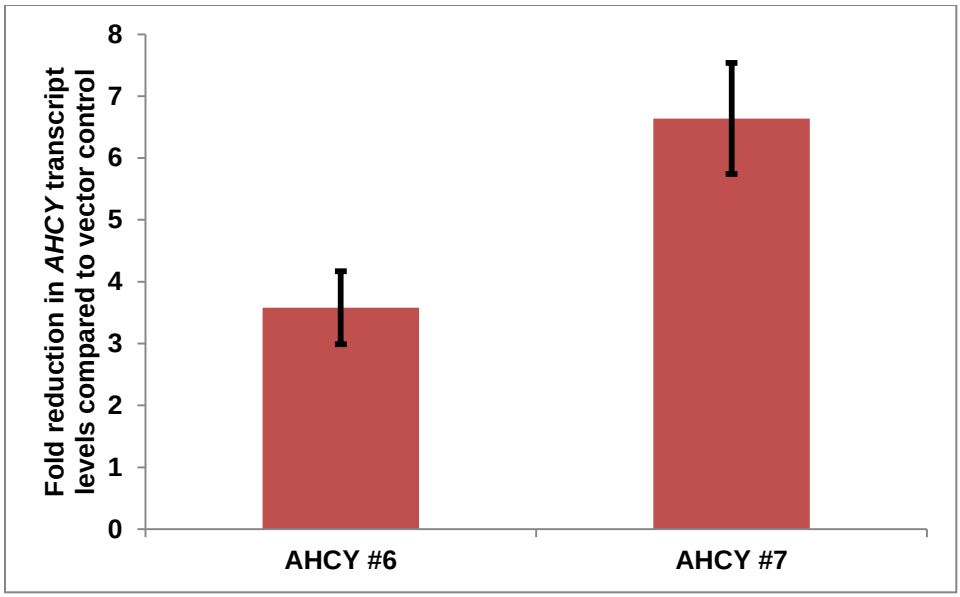


Figure 6.6. RT-PCR analysis showing *C. kikuchii AHCY* transcript levels 7 dpi in soybeans carrying recombinant HIGS vectors. *AHCY* transcript levels in AHCY #6 and AHCY #7 were normalized against empty vector *AHCY* transcript levels and 18S rRNA was used as internal reference. Data is the mean of three or more individual plants per each treatment and error bars indicate standard error of the mean.

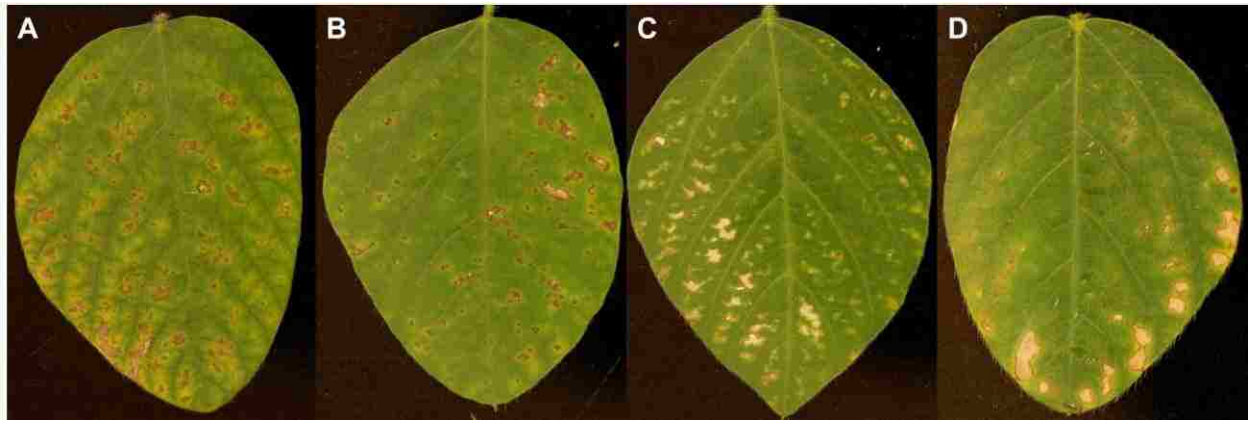


Figure 6.7. Symptoms on soybean plants carrying recombinant HIGS vectors after7 days after *Cercospora kikuchii* inoculation. Healthy (**A**) and Vector control (**B**) leaves showed many necrotic lesions, AHCY #6 plants (**C**), and ACHY #7 (**D**) leaves showed few necrotic lesions.

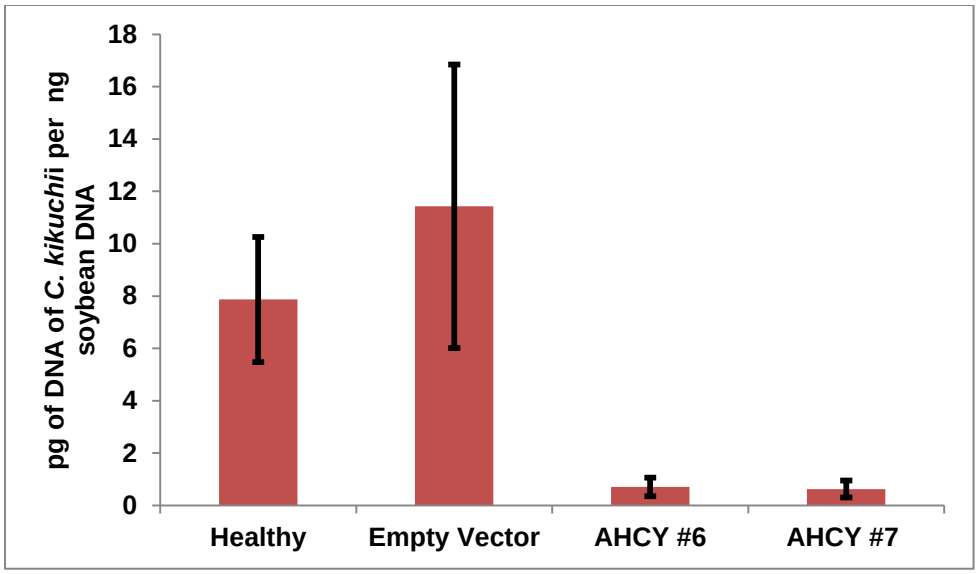


Figure 6.8. Quantitative real-time PCR analysis showing differential accumulation of *C. kikuchii* at 7 dpi in soybeans carrying recombinant HIGS vectors. Leaves collected form individual plant constitute a sample. Data indicate mean for healthy (4 plants), empty vector (3 plants), AHCY #6 (3 plants), and AHCY #7 (3 plants) and error bars indicate standard error of the mean.

6.4 Discussion

HIGS assay with the *AHCY* gene of *C. kikuchii* showed that upon successful inoculation of soybeans with *C. kikuchii*, BPMV carrying parts of the *AHCY* gene could silence the *AHCY* gene of *C. kikuchii in planta*. The *AHCY* transcript levels in AHCY #6 and AHCY #7 plants were reduced compared to empty vector control at 7 dpi. Necrotic spots typical of *C. kikuchii* infection seen in greenhouse soybean inoculations with fungal mycelial suspensions, were evident at 7 dpi. Healthy and vector control plants developed extensive necrotic lesions compared to AHCY #6 and AHCY #7 (Fig. 6.7). In our previous study, ahcy disruption mutants produced significantly less cercosporin compared to wild type and also were less virulent on soybeans. We saw the similar results using this HIGS study by silencing the *AHCY* transcript.

There was a clear difference in *C. kikuchii* biomass accumulation in the vector control, AHCY #6 and AHCY #7 plants at 7 dpi with a rapid increase in *C. kikuchii* biomass in vector

control plants compared to AHCY #6 and AHCY #7 plants. The data are in agreement with reduction in *AHCY* transcript levels in both AHCY #6 and AHCY #7 at 7 dpi. These results are similar to other HIGS studies with fungi. HIGS was promising in the *Blumeria graminis*-barley interaction, in which effector gene *Avra10* was silenced resulting in reduced fungal development in the absence of matching resistance gene *Mla10* (Nowara et al. 2010). Yin et al. (2011) also achieved silencing of highly expressed genes in haustoria of rust fungi belonging to *Puccinia* spp. by using *Barley Stripe Mosaic Virus* (BSMV) as a vector.

The VIGS approach was originally developed for silencing plant genes for functional genomics studies, and now by inserting a piece of pathogen genes in the VIGS vectors, it opens new arenas of research for developing disease resistance in host plants by targeting essential virulence/pathogenicity factors of pathogens. In plants, the mobile RNA silencing signals are transported systemically mainly through phloem to all plant parts (reviewed in Melnyk et al. 2011). In the case of biotrophic pathogens like rusts dsRNA fragments can enter fungal cells through haustoria (Yin et al. 2011). However, the movement of dsRNAs in to the mycelia of *C. kikuchii* in the infected soybean has to be demonstrated. Future studies are needed to demonstrate the presence of gene-specific dsRNA in the fungus, which will be key in determining whether HIGS can be used to control fungal disease in soybean.

CHAPTER 7: GENERAL CONCLUSIONS AND PROSPECTS FOR FUTURE RESEARCH

7.1 General Conclusions

- CLB symptoms are visible at R5 growth stage of soybeans
- PCR primers designed based on *C. nicotianae* CTB gene cluster can amplify specific gene product in *C. kikuchii*, indicating that some CTB genes are conserved across *Cercospora* spp.
- The qPCR assay developed based on *CTB6* gene is highly sensitive for the detection of *C. kikuchii* with a detection limit of 1 pg of genomic DNA
- The qPCR assay can detect *C. kikuchii* as early as 22 DAP in field grown soybeans
- The qPCR also can be used to quantify *C. kikuchii* biomass in soybean leaves at different growth stages
- The *C. kikuchii* biomass showed a slow increase during vegetative stages followed by a quick increase during late reproductive stages
- Multiple fungicide applications beginning from late vegetative stages until reproductive stages reduced the *C. kikuchii* levels and development of CLB symptoms
- Fungicides tested in this study differed in their effectiveness in controlling CLB symptoms
- *C. kikuchii* accumulated up to 6-fold higher cercosporin under light compared to dark
- Differential expression of proteins was detected in *C. kikuchii* grown on CM under light and dark. More proteins were up-regulated under light compared to dark
- HNR protein (Spot 57) up-regulated under light showed homology to RDT1 from *Elsinoë fawcettii* and suggests that melanin biosynthesis in *C. kikuchii* may be tightly linked to cercosporin biosynthesis
- AHCY (Spot 34) and S-adenosyl methionine synthetase (Spot 28) proteins were up-regulated under light, indicating a direct relationship between cysteine and methionine metabolism and cercosporin biosynthesis
- Up-regulation of superoxide dismutase (Spot 26) in general under light indicates a possible role of this protein in protecting *C. kikuchii* from harmful reactive oxygen species generated by photosensitizing cercosporin

- hnr and ahcy disruption mutants showed a significant reduction in cercosporin production *in vitro* suggesting their role in regulating cercosporin biosynthesis

- hnr and ahcy disruption mutants showed a reduced virulence in both detached leaf assay and greenhouse inoculations suggesting their role in pathogenicity on soybeans

- *C. kikuchii* secreted a large number of proteins *in vitro* and three of them have been identified using mass spectrometry. One is a potential glucan endo-1,3-beta-glucosidase based on sequence homology, which is known to play important nutritional and colonization roles in both saprobic and mycoparasitic fungi

- *AHCY gene* of *C. kikuchii* was silenced in *C. kikuchii* infected soybean tissues using the HIG*S* approach resulting in reduced *AHCY* transcript levels and also reduced accumulation of *C. kikuchii* biomass

7.2 Prospects for Future Research

- This qPCR assay can be exploited to screen different soybean varieties for resistance to *C. kikuchii* under field conditions by quantifying the fungal biomass

- This qPCR assay can be used as a testing tool to determine if asymptomatic soybean seeds are infected or infested by *C. kikuchii,* and to determine the efficacy of different fungicide treatments in controlling CLB

- Development of qPCR assay based on ITS sequences unique to *C. kikuchii* may further increase the present detection limit of 1 pg to fg of genomic DNA

- Lack of correlation between *C. kikuchii* biomass and CLB severity in CP 5892 variety suggests that measuring cercosporin levels in leaf tissues may be another assessment

- Since some cercosporin is also produced under dark, proteomics study using cercosporin producing and non-producing conditions might be more useful in identifying key proteins involved in cercosporin biosynthesis

- Comparing protein profiles of *C. kikuchii* wild-type and hnr/ahcy disruption mutants may lead to discovery of other proteins involved in regulation of cercosporin biosynthesis

- Sequencing *C. kikuchii* genome using the currently available next generation sequencing technologies might help in identifying the secreted proteins of *C. kikuchii*

- Verifying the presence of small RNAs in soybean tissues generated by HIGS approach will provide more conclusive evidence as to whether HIGS is functional in soybean

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APPENDIX

A. 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, decent the ethanol
4. Wash three times with 0.5 ml of sterile distilled water (SDW) each time
5. Resuspend gold particles in 500 µl of SDW and store as aliquots of 25 µl in 1.5 ml tubes at -20°C until further use

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

1. To a 25 µl 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 µl of pBPMV-IA-RIM DNA (1 µg/µl) – RNA1
    b. 2.5 µl of pBPMV-IA-V2 DNA (1 µg/µl) – RNA2 or pBPMV-IA-V2 carrying gene of interest
    c. 50 µl of 50% glycerol
    d. 25 µlof 2.5 M $CaCl_2$
    e. 10 µl 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 µl of 70% isopropanol
4. Wash particles with 70 µl of 100% isopropanol
5. Resuspend the particles in 25 µl of 100% isopropanol

6. Wash macrocarrier discs (Bio-Rad, Cat # 165-2335) with 100% isopropanol and air dry
7. Load 6 µl 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

Ashok Kumar Chanda was born in 1979 in Kodad, Andhra Pradesh (State), India. He attended primary schools in Kodad and moved to Vidwan residential college for his Intermediate education. He received a Bachelor's Science degree in Agriculture from ANGR agricultural University, Aswaraopet campus, India. His interest in biotechnology during bachelors made him to pursue a Master of Science in Agricultural Biotechnology in the same university, but in Hyderabad campus. He had the opportunity to complete master's thesis research on peanuts at ICRISAT, Patancheru under the supervision of Dr. Jonathan Crouch. He joined ICRISAT as a Research Technician under supervision of Dr. Tom Hash, where he became familiar with Marker Assisted Breeding and various molecular markers. In the Fall of 2006, Ashok got admitted into the doctoral program in the department of Plant Pathology & Crop Physiology at Louisiana State University under the guidance of Dr. Zhi-Yuan Chen and Dr. Raymond Schneider. During his time at LSU, he acquired the 2-D gel proteomics and fungal molecular biology skills. He became a member of the American Phytopathological Society and attended annual meetings to present his research findings. He served as Treasurer and Vice-President of the PPCP Graduate Student Association and led the Journal club committee. During his PhD program, Ashok met his wife, Mala, and also they were blessed with a baby girl, Hamsini. Mr. Chanda's research efforts were recognized by the Department of Plant Pathology and Crop Physiology by making him the recipient of the 2011 C.W. Edgerton Award. He will receive the degree of Doctor of Philosophy at the Summer 2012 Commencement.