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Molecular and Biochemical Mechanisms of Pathogenesis in the Maize Foliar Pathogen Cercospora zeae-maydis

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ABSTRACT

GLS is a serious foliar disease of maize, a major staple crop grown commercially in the USA for both human and animal feed production, and increasingly, for ethanol production. The disease is caused by two *Cercospora* species, *C. zeae-maydis* and *C. zeina*, both of which infect maize in the USA and in other parts of the world, with yield losses potentially greater than 50%, depending on local conditions. In culture, *C. zeae-maydis* produces abscisic acid (ABA), for which there is no known pathological or physiological function in the fungus, and cercosporin, a phytotoxic, host non-specific perylenequinone. Experimental evidence indicates that cercosporin, is a virulence factor among some species of *Cercospora*, although evidence from other studies suggests that the phytotoxin may not be universally significant for all species. The overall goal of this project was to determine the biosynthetic regulation and pathological significance of cercosporin and ABA in *C. zeae-maydis.* To this end, the ortholog of *CTB1*, a polyketide synthase gene involved in the biosynthesis of cercosporin, was identified and disrupted. Disruption mutants failed to produce cercosporin but were able to infect maize and induce lesions. Furthermore, upon disruption of the nitrogen responsiveness gene *AREA* and a photoropin-like gene *CzmPhot-24*, cercosporin biosynthesis was significantly reduced. However, these two mutants were still able to infect maize, although the *AREA* mutants were significantly reduced in virulence. AreA is a global regulator of secondary metabolism, thus suggesting that the reduction in virulence may be the result of global down-regulation of other virulence factors. Similarly, deletion of putative ABA biosynthesis pathway genes, which resulted in the loss of ABA biosynthesis, did not affect stomatal infection nor lead to decreased virulence when maize leaves were inoculated with the mutants. Taken together, these findings suggest that cercosporin and ABA individually are not necessary for pathogenesis, but may instead belong to a suite of as-yet unidentified virulence factors produced by *C. zeae-maydis* during infection of maize.

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

Project Overview

Gray leaf spot (GLS) of maize (*Zea mays* L.) is an economically important yield-limiting foliar disease caused by *Cercospora zeae-maydis* Tehon & Daniels and its sibling species *C. zeina* Crous & U. Braun, sp. Nov. The incidence and severity of GLS has been on the increase over the past three decades, and has become a major constraint to maize production worldwide (Ward *et al.*, 1999; Crous *et al*., 2006; Dunkle and Levy, 2000; Goodwin *et al*., 2001). In the USA, GLS is one of the most important foliar diseases of maize, causing both chronic and acute yield losses depending on local severity. Maize is a major staple widely cultivated throughout the world. It is also the most widely grown cereal in the United States, which accounted for 35.53% (313.9 million metric tons) of the world's total production (883.46 million metric tons) in 2011 (FAOSTAT, 2013). Since its initial description in the USA (Tehon and Daniels, 1925), GLS has been reported in the maize growing regions of Asia, Africa and the North and South American continents (Coates and White, 1995; Ward *et al.*, 1999). Management of *C. zeae-maydis* has proven to be difficult, especially because commercial hybrids of maize lack effective resistance to gray leaf spot (Ward *et al.*, 1999), and the fungus can survive between growing seasons in plant debris (de Nazareno *et al.*, 1992). Besides maize, *Cercospora* pathogens affect many other important crops throughout the world, but they have not been studies in detail. A better understanding of the molecular mechanism of interactions between these pathogens and their hosts could potentially help in disease management practices and thus would benefit global agricultural output.

Cercospora spp. exhibit stomatal tropism before infecting host leaves through stomatal pores. On maize plants inoculated with the wild type *C. zeae-maydis* strain SCOH1-5, hyphae frequently reoriented growth in the direction of stomata (Kim *et al*., 2011). Upon encountering stomata, the hyphae differentiate to form appressoria-like multilobed structures over stomata to initiate infection (Bluhm and Dunkle, 2008; Kim *et al*., 2011). The discovery that some species of *Cercospora*, and a limited number of other phytopathogenic fungi, can produce the phytohormone abscisic acid (Assante *et al.*, 1977; Tudzynski and Sharon, 2002), an important

regulator of stomatal aperture and modulator of disease, suggests an important function for fungal ABA in pathogenesis. Plant-produced ABA promotes pre-infection resistance by mediating stomatal closure and callus deposition in response to oomycete and fungal pathogens (Ton *et al.*, 2009). Upon successful penetration, however, ABA interferes with host defense responses mediated by jasmonic acid, salicyclic acid, and ethylene signaling pathways to increase host susceptibility to pathogen invasion (Ton *et al*., 2009). Many plant pathogens induce disease by suppressing inducible plant defenses. For example, in the development of *Cercospora* leaf spot of sugar beet, Schmidt *et al*. (2008) have shown that ABA is involved in suppressing the inducible plant defenses of the host. The observation that ABA levels increased during infection concomitantly with increased fungal biomass in necrotic leaf tissues led them to postulate a contributory role for fungal-produced ABA in the interaction.

Most species of *Cercospora*, including *C. zeae-maydis*, also produce a distinctive perylenequinone phytotoxic polyketide, cercosporin. Cercosporin is a photosensitizing compound that causes membrane lipid peroxidation and ultimately cell death (Daub and Ehrenshaft, 2000). Cercosporin has been implicated as a major virulence factor in the development of *Cercospora* leaf spot diseases, especially among dicot-infecting *Cercospora* species, e.g. in soybean and tobacco (Upchurch *et al.*, 1991; Daub *et al.*,, 2005; Choquer *et al.*, 2005).

The identification of genes involved in the biosynthesis of cercosporin has long been a subject of many studies (Chung *et al*., 2003; Choquer *et al.*, 2005; Chen *et al.*, 2007a; 2007b; Choquer *et al*., 2007; Dekkers *et al.*, 2007). In contrast, the identification of genes involved in ABA biosynthesis has only been investigated in *Botrytis cinerea* (Siewers *et al*., 2006; Gong *et al.*, 2013). Consistent with other secondary metabolite genes, ABA biosynthesis in *B. cinerea* was reported to result from a set of four genes (*BcABA1-4*) organized in a cluster (Siewers *et al*., 2006). In comparison, little is known about the regulation of cercosporin as well as ABA biosynthesis during pathogenesis, and it is not clear whether cercosporin is a critical virulence factor for all species of *Cercospora*.

The ability to produce conidiophores and subsequently conidia, as well as cercosporin biosynthesis, is critical for successful pathogenesis in *C. zeae-maydis* (Payne and Waldron, 1983; Upchurch *et al*., 1991). In fungi, reproductive development and metabolism are often affected by changes in the external environment. Thus, the ability to perceive and integrate changes in environmental signals is an important mechanism for survival in all fungi. Among species of *Cercospora*, nitrogen and light source are two of the most important environmental cues that affect cercosporin biosynthesis (You *et al*., 2008). Similarly, evidence for the regulation of ABA biosynthesis by environmental signals, although limited and inconclusive, points to carbon and nitrogen sources, light, and pH as the most important factors affecting the biosynthesis in species of *Cercospora*. and *B. cinerea* (Bennett *et al*., 1981; Norman *et al.*, 1981; Marumo *et al.*, 1982; Takamaya *et al*., 1983), whereas conidiation is most affected by the quality of light and nutrient availability (Kim *et al*., 2011).

The global nitrogen repressor protein AreA, a GATA-type transcriptional activator conserved among all ascomycetes, mediates the genetic regulation of nitrogen utilization in fungi. AreA regulates the biosynthesis of secondary metabolism in many fungi, including fumonisin B_1 in *Fusarium verticilliodes* (Kim and Woloshuk, 2008) and gibberellin in *F. fujikuroi* (Mihlan *et al.*, 2003). AreA is also required for the full virulence of many fungal pathogens (Bolton and Thomma, 2008). Perception of light, on the other hand, is mediated by three classes of photosensory proteins, the phytochrome red light receptors, the green light receptor rhodopsin and the blue light receptors which include the white collar complex (WCC) and the cryptochromes (CRY) (Rodriguez-Romero *et al*., 2010; Heintzen, 2012). Interestingly, in *Neurospora crassa*, the WC-1 protein also acts as a GATA-type transcription factor to induce gene expression for numerous physiological processes in response to light (Ballario *et al*., 1996). The WC-1 protein interacts with WC-2 to form the WCC, which in addition to phototropic responses, regulates the development of asexual spores, synthesis of carotenoids, and control of the circadian rhythm in *N. crassa* (Idnurm *et al.*, 2010; Rodriguez Romaro *et al*., 2010; Heintzen, 2012).

In *C. zeae-maydis*, cercosporin biosynthesis and vegetative infectious growth occur in blue or white light. Conversely, red light or complete darkness is conducive for asexual development (conidiation) and repression of cercosporin but not ABA biosynthesis (Norman *et al.,* 1981; Kim *et al*., 2011). Unlike *Cercospora* species, light induces asexual sporulation while concurrently down regulating sexual development and secondary metabolism in *A. nidulans* (Bayram *et al.,* 2010). An interaction between orthologs of the WC-1/WC-2 complex, LreA / LreB with the phytochrome red-light receptor, FphA and the velvet protein VeA has been observed in *A. nidulans*. The VeA protein also interacts physically with the well-known pleotropic regulator of secondary metabolism, LeaA, and other velvet-like proteins explaining the phenomena of genetic linkage between the regulation of spore development and secondary metabolism by light (Bayram *et al.,* 2008; Purschwitz *et al*., 2008).

Components of the blue-light photoreceptors Crp1 and Phl1 (orthologs of WC-1 and CRY, respectively) have been identified and characterized in *C. zeae-maydis.* Strains disrupted in *PHL1* (Δ*phl1*) or in *CRP1* (Δ*crp1*) displayed abnormalities in asexual development and secondary metabolism (Bluhm and Dunkle, 2008; Kim *et al*., 2011). The Δ*crp1* deletion strains could make cercosporin in the dark or red light, suggesting that Crp1 acts as a repressor of cercosporin biosynthesis in dark or red light, thus pointing to a possible interaction between blue and red light signaling pathways. Also in blue or white light, the Δ*crp1* strains produced reduced levels of cercosporin, suggesting the existence of additional genes encoding blue-light photoreceptors in *C. zeae-maydis* (Kim *et al*., 2011). Identification and molecular characterization of additional components of blue light sensing, red light sensing, and the role of AreA in secondary metabolism, development, and pathogenesis will form the basis for understanding how *C. zeae-maydis* integrates diverse environmental signals to regulate secondary metabolism in the context of pathogenesis. Thus, the goal of this dissertation was to elucidate the genetic basis of the biosynthesis and regulation of cercosporin and ABA in *C. zeae-maydis* and to determine the relative importance of these metabolites as pathogenesis or virulence factors in grey leaf spot of maize.

Overview of the Genus *Cercospora*

Members of the genus *Cercospora* Fresen are among the most prevalent and destructive group of plant pathogenic fungi that infect numerous crops. The genus belongs to the class Dothideomycetes, one of the largest groups of fungi, which includes numerous economically significant plant pathogens as well as non-pathogenic saprophytes. They affect a wide range of important crops in almost all major families of dicots and most monocots (Pollack, 1987), causing mostly leaf spot diseases. The genus *Cercospora,* described by Fresenius (Fuckel, 1863), has more than 3,000 named species, most of which have a narrow host range (Pollack, 1987) and are thus typically classified according to host association, e.g., *C. beticola* infects sugar beet, *C. oryzae* infects rice, and *C. sorghi* infects sorghum. For most of the 3,000 named species in the genus, there is no known sexual stage, although a *Mycosphaerella* teleomorph has been postulated based on sequence-based molecular phylogenetic analyses (Goodwin *et al*., 2001; Crous *et al*., 2004; Crous *et al*., 2006). However, analysis of mating-type gene distribution in populations of selected *Cercospora* species has discovered that *C. beticola, C. zeae-maydis C. zeina*, and *C. sojina* are heterothallic and are likely reproducing sexually (Groenewald *et al*., 2006; Kim *et al*., 2013). Nearly all species of the genus belong to a highly supported monophyletic group within *Mycosphaerella*, with species infecting cereal crops forming a subgroup within the main *Cercospora* cluster (Goodwin *et al*., 2001).

C. zeae-maydis Tehon & Daniels and its sibling species *C. zeina* Crous & U. Braun, sp. Nov. are two species of *Cercospora* that cause GLS of maize. Both are pathogenic on a wide range of maize hybrids and inbreds, and there are no obvious differences in virulence or infection phenotypes between the two species (Lipps, 1998). Of these two species, *C. zeae-maydis* has been documented in China, North and South America but not in Africa, whereas *C. zeina* has been documented in Africa, Brazil, the Eastern USA and also recently in China (Meisel *et al.*, 2009; Dunkle and Levy 2000; Okori *et al.* 2003, Liu and Xu, 2013). The disease has been reported in maize growing regions of Asia, Africa and the North and South American continents (Coates and White, 1995; Ward *et al.*, 1999).

Gray Leaf Spot Disease of Maize

Gray leaf spot (GLS) is a serious foliar disease of maize, documented throughout maize growing regions of the world in which the growing season is characterized by high humidity and temperatures between 22 and 30°C. The disease is defined by characteristic, rectangular shaped (5 - 70 mm long by 2 to 4 mm wide), brown to tan colored lesions that are constrained within major veins, as the fungus is not able to penetrate sclerenchyma tissue aggressively. Lesion color can differ from shades of brown to tan depending on the maize hybrid, but as lesions mature, they assume a greyish cast due to sporulation of the pathogen. On susceptible hybrids and under favorable conditions, total blighting of leaves can occur, leading to significant reduction in photosynthetic capacity with yield losses exceeding 50%. When this occurs before or during grain filling, affected plants are also predisposed to stalk-rotting pathogens, presumably because carbohydrates in stalks are translocated to meet demands of grain filling (Lipps, 1998).

C. zeae-maydis overwinters in the debris of previously diseased maize plants remaining on the soil surface (de Nazareno *et al*., 1992; de Nazareno *et al*., 1993). Because the pathogen survives between seasons in infected crop debris, the disease tends to be more problematic in regions where maize is cultivated intensively with reduced tillage and successive monoculture, especially in the eastern USA and Africa (Ward *et al*. 1999, Payne and Waldron, 1983). Since it was recognized and described as a "disease on the move" by Latterell & Rossi some 30 years ago (1983), GLS has assumed increased importance with greater incidence and severity in maize growing regions throughout the world. In the USA, significant losses of yield occurred throughout the corn-belt in the mid-1990s, partly due to the widespread use of conservation tillage, planting of susceptible hybrids and weather conditions that favored rapid spread of the disease in some years (Ward & Nowell 1998; Lipps, 1998; Ward *et al*. 1999).

Initiation and Development of GLS by *C. zeae-maydis*

C. zeae-maydis infects host leaves through stomata, similar to most species of *Cercospora* (Beckman and Payne, 1982; Gupta *et al*., 1995; Babu *et al*., 2002). Within 12 – 24 hours after spores land on leaf surfaces, they germinate to form germ tubes provided conditions of high humidity and moderate temperature are met (Beckman and Payne, 1982). In a process that is still being investigated, elongating germ tubes perceive nearby stomata and reorient direction of growth or form lateral branches towards the nearest stomata. Upon reaching stomata, the germ tubes differentiate into amorphous, multilobed infection structures similar to appressoria (2 – 5 days after inoculation), from which infectious hyphae penetrate mesophyll tissues (5 – 8 days after inoculation) to begin a presumably biotrophic growth stage (Beckman and Payne, 1982). After a period of colonization, the fungus switches from a biotrophic to a necrotrophic growth habit, leading to the formation of necrotic lesions that expand and coalesce in severe outbreaks. Under severe disease pressure, entire leaves can be blighted, which significantly reduces the photosynthetic capability of diseased plants, causes severe yield reductions, and potentially leads to the premature death of the host (Ward *et al*. 1999). Reproduction occurs in the colonized tissue through the production of asexual spores (conidia) that infect neighboring plants after dispersal by wind and/or rain splash. Observations that host discoloration was associated with sparse internal hyphae, exclusively intercellular growth, and a halo of discoloration surrounding lesion initials led Beckman and Payne (1982) to postulate that fungal toxins were involved in the development of lesions in GLS. Direct involvement of cercosporin, a phytotoxin in pathogenesis of *Cercospora* leaf spot disease of sugar beet, had earlier been proposed (Steinkamp *et al.*, 1981).

Cercosporin is a photoactive perylenequinone phytotoxin made by species of *Cercospora* and at least one endophytic species of *Mycosphaerella* (Assante *et al.*, 1977; Moreno *et al.*, 2011). Many plant pathogenic fungi utilize a variety of small molecules including secondary metabolites for successful pathogenesis of their hosts. In defined growth media, some species of *Cercospora* produce cercosporin (Assante *et al.*, 1977; Daub and Ehrenshaft 2000), the

phytohormone ABA (Assante *et al.*, 1977; Tudzynski and Sharon, 2002; Schmidt *et al*., 2008), the non-host-specific yellow beticolin toxins (Frandsen, 1955), and presumably many unknown metabolites in response to various environmental cues. These metabolites play a role in species diversification, virulence, and/or host specificity (Panaccione, 1992; Yang *et al.*, 1996; Ohm *et al.*, 2012).

Cercosporin in the development of leaf spot diseases

Accumulated evidence from several studies suggests that cercosporin plays a direct role in the ability of *Cercospora* species to induce lesions. The first studies to implicate cercosporin in pathogenesis compared *C. beticola*-induced lesions to lesions resulting from topical application of cercosporin to leaves and identified membrane damage as the underlying cause of lesions (Steinkamp *et al*., 1981). The first compelling evidence for a role for cercosporin in disease development was provided by Upchurch *et al*. (1991). They obtained spontaneous and UV-induced cercosporin-deficient mutants of *C. kikuchii* and showed that cercosporin-deficient mutants were not pathogenic on their host, whereas pathogenic strains could induce lesions from which cercosporin was extracted. Cercosporin has also been extracted from many infected hosts (Kuyama and Tamura, 1957; Venkataramani, 1967; Fajola, 1978) suggesting biosynthesis in the host during pathogenesis.

Cercosporin is a photosensitizing compound, which is converted to an energetically active triplet state upon absorption of light (Yamazaki *et al*., 1975). This photodynamic property causes the production of reactive oxygen species that cause cell death through membrane lipid peroxidation, thus leading to electrolyte leakage and cell death (Daub, 1982a; Daub and Briggs, 1983) possibly through induction of apoptosis (Dickman *et al*., 2001). Coincidentally, the biosynthesis of cercosporin requires light of the same wavelength as that required for its activity, suggesting an evolutionary mechanism ensuring that the toxin is produced only when it could be activated (Daub 1982b; Daub and Ehrenshaft 2000; Kim *et al*., 2011). These experimental results directly linking cercosporin to pathogenesis are well supported by observational data. For

example, the requirement of light for symptom development in *Cercospora* infection of coffee and sugar beet has long been observed, as symptoms often failed to develop on leaves under canopies (Echandi, 1959; Calpouzos and Stalknecht, 1967).

Development of molecular tools for *Cercospora* pathogens in the last decade has enabled molecular elucidation of the role of cercosporin in pathogenesis. Cercosporin non-producing strains of *C. kikuchii, C. nicotianae* and *C. beticola* have shown significant reductions in virulence (Callahan *et al.,* 1999; Choquer *et al.,* 2005; Staerkel *et al.,* 2013), while a reduction in virulence has not occurred for every mutant of *C. zeae-maydis* altered in the biosynthesis of the toxin. Whereas the reduction in cercosporin production in *C. kikuchii, C. nicotianae* and *C. beticola* resulted from mutation of genes encoding enzymes directly involved in the biosynthesis or export of the toxin, our current knowledge of a role for the toxin in the pathogenesis of gray leaf spot of maize is from mutations in genes with regulatory functions or genes with indirect effects on cercosporin biosynthesis. For example, deletion of *CZK3* or *CRP1*, the respective homologs of a MAP kinase kinase kinase and WC-1 in *C. zeae-maydis*, had pleotropic effects and affected cercosporin biosynthesis, fungal development, and pathogenesis (Shim and Dunkle, 2003; Kim *et al.,* 2011). In contrast, mutation of *PHL1*, a homolog of photolyase/cryptochrome family involved in UV protection and fungal development, caused a reduction in cercosporin biosynthesis but had no effect on pathogenesis or virulence (Bluhm and Dunkle, 2008). Interestingly, *C. zeina*, a sibling species of *C. zeae-maydis* that also cause gray leaf spot of maize, have not been reported to make cercosporin *in vitro*. Additionally, resistance to cercosporin necrosis effect has been observed in leaves, calli and cell suspensions of the annual weed, red rice (Batchvarova *et al*., 1992). This evidence creates an uncertainty of whether cercosporin is a universal virulence factor, especially for species infecting cereal crops.

Role of ABA in pathogenesis

The existence of ABA as an endogenous *Cercospora* metabolite was first identified in *C. rosicola* (Assante *et al.,* 1977) and has since been identified in more species within the genus,

e.g. *C. cruenta, C. pini-densiflorae* and *C. beticola*, as well as related genera (reviewed by Tudzynski and Sharon, 2002; Schmidt *et al*., 2008). ABA is a phytohormone involved in short-term physiological effects (e.g. stomatal closure) as well as long-term developmental processes (e.g. seed maturation) in plants. ABA plays a central role in mediating water uptake-and-loss balance through root growth maintenance and guard cell regulation during water deficits (Webb *et al.,* 2001; Santner *et al*., 2009). In addition to causing stomatal closure, ABA is also involved in preventing blue light-induced stomatal opening (Shimazaki *et al*., 1986).

The involvement of ABA as a first line of defense against pathogen invasion is undoubted due to its role as a regulator of stomatal aperture (Schrode *et al*., 2001). For example, in Arabidopsis, exogenous application of ABA retarded stomatal invasion by the bacterial pathogen *Pseudomonas syringae*, while Arabidopsis mutants deficient in *aba3-1* genes were unable to control stomatal movement and were also susceptible to the bacterial pathogens (Melotto *et al.*, 2006). However, the role of ABA in host defense subsequent to pathogen invasion is unclear and seems to depend partially upon pathosystems and infection stages, although accumulated evidence largely support a negative role of ABA in plant immunity (Asselbergh *et al*. 2008; Mauch-Mani and Mauch 2005; Ton *et al.,* 2009; Cao *et al.,* 2011). For example, in necrotrophic fungal and oomycete pathogens, ABA seems to suppress hypersensitive responses by preventing the characteristic ROS production and by priming the host for rapid deposition of callose-rich cell wall enforcements, all of which play key roles in plant defence in the early-post invasive stage (Ton and Mauch-Mani 2004; Asselbergh *et al*., 2007). Other examples of ABA promoting resistance to necrotrophs such as *Cochliobolus miyabeanus* on rice leaf sheaths following exogenous application of ABA and resistance to *Alternaria brassicicola* and *Pythium irregulare* have been reported (Ton and Mauch-Mani 2004; Adie *et al*., 2007; Vleesschauwer *et al*., 2010).

Accumulated evidence also suggests that ABA negatively regulates the expression of host resistance, through direct and indirect interference with the SA-JA-ET backbone of the plant defense circuitry, thereby increasing susceptibility to invading pathogens (Mauch-Mani and

Mauch, 2005; Hirayama and Shinozaki, 2007; Asselbergh *et al*., 2008; Fan *et al*., 2009). For example, a breakdown in both basal and *R*-gene (*Pish* and *Pi19*)–mediated resistance of rice (*japonica* cv. Nipponbare) to *M. grisea* (races 102.0 and 002.0 respectively) was reported following pre-treatment with ABA (Jiang *et al*., 2010). More recently, Mang *et al*. (2012) demonstrated that ABA deficiency in Arabidopsis promotes *R* gene-mediated defense responses at high temperatures by enhancing the nuclear accumulation and activity of two resistance proteins: *SUPPRESSOR OF npr1-1 CONSTITUTIVE1* (SNC1) and RESISTANCE TO PSEUDOMONAS SYRINGAE4 (RPS4).

The invasion of a plant by pathogens is often characterized by rapid accumulation of ABA at the infection site. In Arabidopsis, invasion by *P. syringae* is characterized by rapid accumulation of ABA in leaves immediately after inoculation (de Torres-Zabala *et al*., 2007). This phenomenon has also been observed in response to *C. beticola* during pathogenesis of sugar beet. The observation that ABA levels increased during infection concomitantly with increased fungal biomass in necrotic leaf tissues led Schmidt *et al*. (2008) to speculate a contributory role for fungal-produced ABA in the development of *Cercospora* leaf spot of sugar beet. ABA has previously been shown to repress inducible plant defenses in sugar beet by down-regulating the activities of defense-related genes such as phenylalanine ammonia lyase (PAL) and cinnamic acid 4-hydroxylase at the enzymatic and transcriptional level in early phases of *Cercospora* infection of sugar beet (Schmidt *et al*., 2004). PAL catalyzes the conversion of L-phenylalanine to *trans*-cinnamic acid, an important intermediate of many secondary metabolites implicated in plant defenses. Recently, Yazawa *et al*., (2012) further demonstrated that reduction of ABA levels or inhibition of ABA signaling in rice during the early phase of *M. oryzae* infection decreases the susceptibility of rice to the fungal pathogen, while changes in the level of ABA or its signaling at later stages of infection does not affect susceptibility. Interestingly, *M. oryzae* also produces ABA (Jiang *et al.,* 2010). It has thus been suggested that infection-induced ABA enables pathogens to tap into the plant's defense signaling circuitry and interfere with host immunity. For example, direct manipulation of ABA biosynthesis and signaling by bacterial

type III effectors as a virulence strategy for *P. syringae* and *X. campestris* pv. *campestris* has been demonstrated (De Torres-Zabala *et al.,* 2007; Goel *et al.,* 2008*;* de Torres Zabala *et al.,* 2009; Ho *et al.,* 2013).

Thus, the ability of some fungal pathogens including *Cercospora* spp., *M. oryzae, B. cinerea* and *Rhizoctonia solani* (Tudzynski and Sharon, 2002) to produce and secret ABA is likely an evolutionary mechanism to tap into and dampen plant immunity to promote the infection process, especially considering there is no evidence supporting a role for ABA in the physiology of the pathogens (Cao *et al*., 2011). The timing of ABA release by the infecting pathogen, the activation of host biosynthesis machinery, and/or manipulation of host ABA signaling mechanisms by the pathogen is therefore likely to be strategically timed to occur at the early post-penetration stage to derive optimum benefit for the infecting pathogen.

Secondary Metabolism in Fungi

The polymer backbones for major groups of secondary metabolites incorporate polyketides, ribosomal and nonribosomal peptides, and terpenoids that are synthesized by polyketide synthases (PKSs), nonribosomal peptide synthases (NPSs), terpene synthases (TSs) or prenyltransferases, respectively. These polymer backbones are converted into a diverse array of metabolites by a plethora of modifying enzymes, including (de)hydratases, oxygenases, hydrolases and methylases among others. In fungi, many biosynthetic genes involved in secondary metabolism are organized in discrete clusters around the backbone polymer biosynthetic synthase gene, often in association with a gene encoding a regulatory protein required to regulate the expression of the whole cluster, as well as proteins required to mediate the transport of the metabolite (Smith, *et al*., 1990, Hoffmeister and Keller, 2007, Brakhage, 2013). With the exception of a few fungal metabolites, such as the meroterpenoids austinol and dehydroaustinol in *A. nidulans* and T-toxin in *Cochliobolus heterostrophus* that have cross-chemistry between two separate gene clusters located on different chromosomes (Baker *et al.,* 2006; Sanchez, *et al.,* 2011; Lo *et al.,* 2012), the entire set of enzymes required to catalyze the

biosynthesis of a secondary metabolite in fungi is generally located within a cluster on a single chromosome, frequently at the subtelomeric region (Perrin, 2007; Farman, 2007; Osbourn, 2010).

On the basis of the characteristic organization of enzymes on the chromosome, algorithms such as antiSMASH – antibiotics and Secondary Metabolite Analysis SHell (Medema *et al.,* 2011) and SMUR – Secondary Metabolite Unique regions Finder (Khaldi *et al.,* 2010) have been used in comparative sequence analysis to predict potential fungal secondary metabolite gene clusters for subsequent experimental verification (Inglis *et al*., 2013). One approach for experimental verification is the deletion of genes with suspected roles in secondary metabolite biosynthesis followed by identification of the specific secondary metabolite profiles of the mutants by metabolite analysis. Other approaches include decreasing or increasing epigenetic regulation through deletion of global transcriptional regulators to increase transcription from a biosynthetic gene cluster at the chromatin level by reducing or inducing modifications of histones (Shwab *et al*., 2007; Bok *et al*., 2009), or the overexpression of predicted transcriptional regulators of secondary metabolite gene clusters (Nakazawa *et al*., 2012) followed by the analysis of secondary metabolite profiles of the resulting strains, which has facilitated the identification of numerous secondary metabolites and the genes responsible for their synthesis. Thus, comparative sequence analysis, coupled with targeted mutations, has been used effectively to discover novel secondary metabolites and identify the genes involved in their synthesis.

Discovery and biosynthesis of cercosporin

The red pigment [1,12-bis(2-hydroxypropyl)-2,11-dimethoxy-6,7- methylenedioxy-4,9-dihydroxyperylene-3, 10-quinone] in cultures of *Cercospora*, named cercosporin in 1957 by Kuyama and Tamura (Fig. 1.1), is a non-host-specific polyketide phytotoxin first noted by Schmidt (1928) in *C. beticola* and later crystallized by Deutschmann (1953) from mycelial cultures of *C. kikuchii,* a soybean pathogen. Kuyama and Tamura (1957) subsequently purified

and determined the physical and chemical fundamentals of its structure. The toxin has since been isolated from a large number of *Cercospora* species and from *Cercospora*-infected plants (Fajola, 1978; Venkataramani, 1967; Upchurch *et al.*, 1991; Daub and Ehrenshaft 2000). The structure and early labeling experiments indicated that cercosporin is produced via a polyketide mode of synthesis (Lousberg *et al*. 1971; Yamazaki and Ogawa, 1972; Okubo *et al.*, 1975).

More than four decades after its structure was first elucidated, the genetic basis of the biosynthesis of cercosporin began to emerge with the identification of the *Cercosporin Toxin Biosynthesis1* (*CTB1*) gene. *CTB1* was identified in a screen of a *C. nicotianae* REMI (restriction enzyme-mediated insertion) library for cercosporin non-producing mutants (Chung *et al*., 2003). *CTB1* is an iterative non-reducing type-1 polyketide synthase-encoding gene, whose

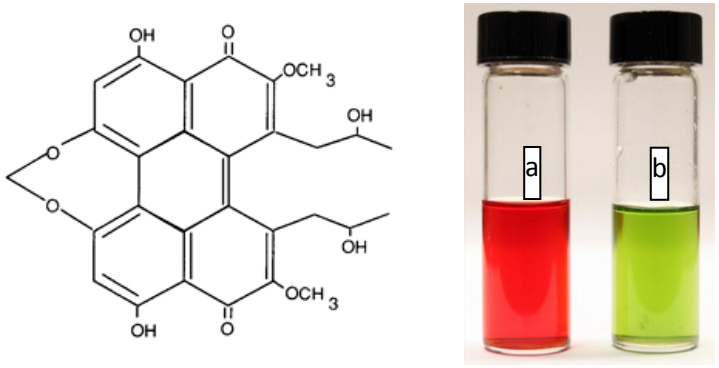

Fig. 1.1: Chemical structure of cercosporin (Yamazaki and Ogawa, 1972). At physiological and acidic pH, cercosporin is red (a) and turns green in alkaline pH (b).

expression was subsequently found to be highly regulated by light and medium composition, and closely correlated with cercosporin production (Choquer *et al.*, 2005). Further analysis with a chromosome walking strategy in *C. nicotianae* led to the identification of eight clustered genes designated *CTB1-8* that were involved in cercosporin biosynthesis. The cluster genes include a pathway-specific Zn-finger transcription factor, *CTB8*, a member of the major facilitator superfamily transporter, *CTB4* (Choquer *et al*., 2007) a methyl transferase, *CTB2* and enzymes

catalyzing redox reactions (Dekkers *et al.*, 2007, Chen *et al.*, 2007a, 2007b). On the basis of the predicted biochemical functions of the Ctb proteins and domain architecture of the polyketide synthase Ctb1, a pathway for the biosynthesis of cercosporin has been predicted. Ctb1 catalyzes the formation of the *nor*-toralactone backbone molecules that are modified by the methyl transferases and oxidoreductases encoded by the cluster genes to dimerize and form the cercosporin (Chen *et al.*, 2007b; Newman *et al.*, 2012).

Discovery and biosynthesis of ABA

The first report of ABA biosynthesis by a fungus appeared in 1969. Rudnicki *et al*., (1969) observed that the phytopathogen *Penicillium italicum*, grown on orange peel substrate, produced ABA but they were unable to conclude definitely whether the source of the ABA was the growth substrate or the fungus. Subsequently, Assante *et al*. (1977) in a screen to identify metabolites produced by the rose pathogen, *C. rosicola*, unequivocally demonstrated the biosynthesis of ABA by the fungal pathogen. The discovery that the fungus accumulated 60 µg ABA/ml after being grown in light for 30 to 40 days on potato dextrose medium was very striking given that *Cercospora* pathogens infect their hosts through stomata, and ABA is known to be an important regulator of stomatal aperture and modulator of disease (Ton *et al*., 2009).

ABA is an isoprenoid phytohormone originally discovered over 50 years ago. It is composed of one chemical compound [*S*-(*Z,E*)]-5-(1-Hydroxy-2,6,6-trimethyl-4-oxo-2-cyclohexen-1-yl)-3-methyl-2,4-pentanedienoic acid. The hormone, previously called Dormin and/or Abscisin II was renamed Abscisic acid [or (+)-2-*cis*-4-*trans*-abscisic acid] in 1968 (Addicott *et al.*, 1968; Addicott and Carns, 1983). Before its chemical properties were fully known, Bennet-Clark and Kefford (1953) identified ABA as a group of impure co-resolving "anti-auxins" that they called Growth Inhibitor-β.

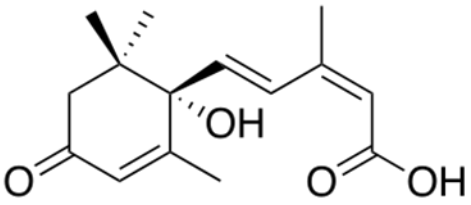

Fig. 1.2: Chemical structure of Abscisic Acid (Ohkuma *et al*. 1965).

The first report of the isolation and chemical characterization of the hormone in its pure form was by Addicott *et al.* in 1963. The group isolated the compound from cotton fruits that abscised prematurely and named it Abscisin II because it was thought to promote abscission (Addicott and Carns, 1983). Two other groups reported the isolation and characterization of ABA at about the same time. One of the groups, led by Philip Wareing, isolated the compound from leaves of *Acer pseudoplatanus* (sycamore), and found levels to correlate with seasonal changes in dormancy of the shoot apexes. Further studies led the group to conclude that the hormone-like substance had a role in seed and bud dormancy and hence named it Dormin. The other group led by van Steveninck was studying compounds thought to be abscission accelerators in flowers and young fruits of *Lupinus luteus* (yellow lupin). ABA's abscission-promoting effect was subsequently determined to be partly an indirect consequence of inducing the biosynthesis of ethylene (Cracker and Abeles, 1969).

ABA biosynthesis in plants occurs via the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway beginning in the plastids where isopentenyl diphosphate is first oxidized to zeaxanthin, and then modified and cleaved by the rate limiting enzyme 9'-*cis*-epoxycarotenoid dioxygenase (NCED) to xanthoxin. Xanthoxin is exported to the cytoplasm where it is converted to ABA by the short-chain dehydrogenase/reductase-like (SDR) enzyme and abscisic aldehyde oxidase (AAO) encoded respectively by the *ABA2* and *ABA3* genes in Arabidopsis. While ABA biosynthesis occurs in plants via the MEP pathway, radioisotopic studies in *B. cinerea* and some species of *Cercospora* point to biosynthesis via the mevalonic acid (MVA) pathway. The evidence suggests that ABA is formed from farnesyl diphosphate, through different oxidative steps, with either 1',4'-dihydroxy-*γ*-ionylidene acetate, 1'-deoxy-ABA, or 1',4'-*trans*-diol-ABA

as intermediates (Nambara and Marion-Poll, 2005). Consistent with the production of many fungal secondary metabolites, ABA biosynthesis in *B. cinerea* was demonstrated to result from the expression of a set of four genes (*BcABA*1-4) organized in a cluster (Siewers *et al*, 2006). These genes included *BcABA3,* a gene of unknown regulatory or catalytic function, a short-chain dehydrogenase/reductase (*BcABA4*) and two copies of cytP$_{450}$ monooxygenases (*BcABA1* and *BcABA*2). The involvement of cytP$_{450}$ in the biosynthesis of ABA in *C. rosicola* has previously been suggested in studies with various inhibitors of terpenoid synthesis (Norman *et al.*, 1986, Al-Nimri and Coolbaugh, 1990). Further evidence of a role for cytP$_{450}$ in ABA biosynthesis was uncovered when targeted disruption of *BcABA1* and *BcABA2* resulted in the accumulation of substances predicted (based on molecular mass) to be α-(γ-)-ionylidene acetic acid and 1′-deoxy-ABA respectively, which are precursors of ABA (Siewers *et al*, 2004; 2006). *BcABA*2 is predicted to be responsible for the final hydroxylation of carbon atom C-1′ of 1′-deoxy-ABA to form ABA. In plants, CytP$_{450}$ (CYP707As) plays the important role of regulating ABA levels by catalyzing its oxidation to phaseic acid. In fungi, however, endogenous ABA levels are most likely regulated by the release of ABA to the external medium. This proposition was evidenced by the observation that mycelial extracts of *C. rosicola* had no detectable levels of ABA after a week of incubation, whereas 5 to 30 µg ABA/ml could be detected from the liquid growth medium (Norman *et al.,* 1981). Further evidence for the proposition of regulation by release into external medium was obtained when conversion to phaseic acid and dihydrophaseic acid was not observed (reviewed in Hartung, 2010). No enzymatic role could be assigned to *BcABA*3 since deletion mutants (Δ*Bcaba*3) did not accumulate any substance that could be correlated to the proposed intermediates of the *B. cinerea* ABA pathway, nor did the deletion affect the expression of other cluster genes, thus ruling out a possible role as a positive pathway regulator (Siewers *et al.*, 2006).

Regulation of Fungal Secondary Metabolism

Fungi perceive and respond to a variety of environmental stimuli such as ambient pH,

temperature, reactive oxygen species/redox status, micronutrient/iron starvation, nitrogen and carbon sources, light, and interspecies communication in order to regulate growth and development, metabolite biosynthesis, and pathogenesis (Brakhage, 2009; Bayram, *et al.*, 2010; Yin and Keller, 2011). In *Cercospora* species, light and nitrogen sources are important environmental stimuli for asexual development, metabolite biosynthesis, and pathogenesis. Regarding metabolite biosynthesis, the expression of cluster genes in response to specific environmental signals is thought to be controlled by complex regulatory networks.

In pathway-specific regulation, the expression of a regulatory gene, often a transcription factor-encoding gene within the cluster, is required for the transcriptional activation of most or all of the cluster genes. For example, in *C. nicotianae*, a functional Zn(II)Cys_6 transcriptional activator, *CTB8*, is required for the expression of the rest of the cercosporin biosynthesis cluster genes (Chen *et al*., 2007b). However, in *A. fumigatus*, deletion of the pathway-specific transcriptional activator of gliotoxin production (*GliZ*) suppressed expression of all but one gene (*GliT*) in the cluster even upon exogenous addition of gliotoxin to the culture medium (Schrettl *et al.* 2010). The transcription of pathway-specific regulatory genes can also be regulated by global-acting regulatory factors or through cross-talk with other regulatory genes located in entirely different clusters (Yu and Keller, 2005; Hoffmeister and Keller, 2007; Bergmann *et al*., 2010). In the biosynthesis of cercosporin, Crg1, a Zn(II)Cys_6 transcription factor, unlinked to the *CTB* gene cluster, acts upstream of *CTB8* to regulate the biosynthesis of cercosporin, at least in part by activating the transcription of *CTB8*, the cluster-specific regulator (Chen *et al*., 2007b). In *A. nidulans*, the activation of a cryptic NPS cluster by overexpression of a putative cluster-specific transcriptional regulator surprisingly resulted in the activation of an additional, physically unrelated, silent asperfuranone gene cluster on another chromosome (Bergmann *et al*., 2010). Some gene clusters do not have pathway specific regulators and are controlled by global acting transcriptional regulators such as the pH responsive factor pacC or the nitrogen responsive factor AreA/Nit2. For example, the *B. cinerea* ABA biosynthesis cluster does not have an identified cluster-specific regulator (Siewers *et al.*, 2006) and may be subject to regulation by

global transcriptional regulators. Globally acting transcriptional regulators are encoded by genes unlinked to the biosynthetic gene clusters and regulate other genes not involved in secondary metabolism.

Regulation of secondary metabolism and pathogenesis

The utilization of secondary nitrogen sources in filamentous fungi is highly regulated and under the transcriptional control of the GATA-type global transcription regulator AreA in *A. nidulans* (Arst and Cove, 1973; Caddick *et al.*, 1986) and Nit2 in *N. crassa* (Stewart and Vollmer, 1986; Fu and Marzluf, 1987). Global nitrogen regulators facilitate the expression of many structural genes encoding enzymes required to metabolize secondary nitrogen sources and ensure that primary sources (ammonia and glutamine) are used preferentially when available. A single ortholog of this nitrogen regulatory gene has been found in the genomes of all filamentous ascomycetes studied to date (Wong *et al.*, 2008).

Disruption of the *AREA* ortholog *AREA-GF* in *Gibberella fujikuroi* led to a 10%–20% reduction of gibberellin production in defined medium (Mihlan *et al*., 2003). In addition, the loss-of-function Δ*areA-GF* strains were insensitive to ammonium-mediated gibberellin repression, supporting the conclusion that *AREA-GF* is directly responsible for nitrogen-induced repression of gibberellin biosynthesis in *Fusarium fujikuroi* (Tudzynski *et al*., 1999; Mihlan *et al*., 2003). The global regulator is also required in the production of fumonisin B_1 in *F. verticillioides* (Kim and Woloshuk, 2008). For *Cercospora*, the presence of the preferred nitrogen source, ammonia, as well as a secondary source of nitrogen, such as nitrates, in cercosporin-inducing medium resulted in significant repression of cercosporin biosynthesis but did not correspond with the accumulation of the *CTB* cluster gene transcripts (You *et al.*, 2008). The involvement of nitrogen in the regulation of ABA biosynthesis in species of *Cercospora* has been amply demonstrated (Norman *et al.*, 1981) but it is yet to be verified how nitrogen exerts its effect on the biosynthesis of these two metabolites at the genetic level.

In pathogenesis, nitrogen limitation has been proposed as one of many signals for

activating the expression of virulence genes in plant pathogens (Coleman *et al.* 1997; Snoeijers *et al.*, 2000; Bolton and Thomma, 2008). Most AreA/Nit2 orthologs studied in plant pathogens thus far have shown that the GATA transcription factor is required for full virulence, suggesting that nitrogen availability and type influence pathogenesis. For example, in the rice blast fungus *M. oryzae*, the pathogenicity gene *Mpg1* is strongly up-regulated under conditions of nitrogen limitation (Talbot *et al.*, 1993) and Nut1, its AreA ortholog, is required for full induction of *Mpg1* expression (Soanes *et al.*, 2002) but not for pathogenicity on rice plants (Froeliger and Carpenter, 1996). Similarly, the avirulence gene *Avr9* of the tomato pathogen *Cladosporium fulvum* is strongly up regulated under nitrogen-limiting conditions (Van denAckerveken *et al.*, 1994). The AreA ortholog Nrf1 partly controlled the expression of *Avr9*, yet *nrf1* mutants produced reduced levels of *Avr9* transcript *in planta* and were fully virulent (Perez-Garcia *et al.*, 2001). Inactivation of *AREA* orthologs in *F. oxysporum* and *Colletotrichum lindemuthianum* decreased virulence to different degrees in the respective mutants (Pellier *et al.*, 2003; Divon *et al.*, 2006). Collectively, these studies suggest that AreA contributes to, but is not essential for, fungal virulence in many pathosystems.

Light in the regulation of secondary metabolism and pathogenesis

Among cercosporin-producing species, light is known to be the most critical factor in cercosporin biosynthesis. In *C. zeae-maydis*, visible light of wavelengths towards blue induce cercosporin biosynthesis, while production is suppressed under complete darkness or red light (Kim *et al*., 2011). Brief exposure to visible light immediately triggers cercosporin biosynthesis in *C. beticola* (Lynch and Geoghegan, 1979; Daub and Chung, 2007). The importance of light has also been demonstrated in ABA biosynthesis (Norman *et al.*, 1981). Among filamentous fungi, changes in external light exposure are frequently detected by several distinct classes of photoreceptor proteins.

Blue-light photosensory genes *CRP1* and *PHL1* (orthologs of *N. crassa WC-1* and *CRY*, respectively) have been identified and characterized in *C. zeae-maydis*. Crp1 regulates the

production of cercosporin and conidiation in *C. zeae-maydis.* Strains disrupted in *PHL1* (Δ*phl1*) or in *CRP1* (Δ*crp1*) displayed abnormalities in development and secondary metabolism (Bluhm and Dunkle, 2008; Kim *et al*., 2011). Deletion of *CRP1* delayed the onset of cercosporin biosynthesis in blue and white light but did not fully eliminate cercosporin biosynthesis; neither did the deletion of *PHL1*. Intriguingly, the *CRP1* deletion mutant could make cercosporin in darkness or red light, suggesting that Crp1 acts as a repressor of cercosporin biosynthesis in these conditions. Contrary to expectations, deletion of *CRP1* in conjunction with *PHL1* restored cercosporin production to wild type levels after seven days of growth (Kim *et al.*, 2011). In contrast, disruption of *CRP1* derepresses conidiation in constant light (Kim *et al*., 2011). These observations indicate that Crp1 mediates the light-dependent repression of conidiation and derepression of cercosporin biosynthesis in *C. zeae-maydis*. During pathogenesis, hyphae of the Δ*crp1* mutants failed to exhibit stomatal tropism, and frequently grew around or across stomata, forming appressoria less frequently than the wild type strain (Kim *et al*., 2011). However, the Δ*phl1* strains were unaffected in their ability to infect maize leaves and cause lesions (Bluhm and Dunkle, 2008).

The LOV Domain Blue-Light Receptors

Most blue-light responses in fungi require the function of the White Collar Complex (WCC), which is a heterodimeric protein complex comprised of white collar-1 (WC-1) and white collar-2 (WC-2), two GATA-type Zn-finger transcription factors. WC-1 is a multi-domain protein that interacts with WC-2 via a PAS (Per-Arnt-Sim) domain to form the WCC which, in addition to phototropic responses, regulates the development of asexual spores, synthesis of carotenoids, and control of the circadian rhythm in *N. crassa* (Idnurm *et al.*, 2010; Rodriguez Romaro *et al*., 2010; Heintzen, 2012; Olmedo *et al*., 2013). WC-1 operates as a photoreceptor through the binding of a flavin chromophore to its LOV domain and a transcription factor that activates gene expression in response to light thorough its Zn-finger DNA binding domain (Ballario *et al.*, 1996).

The LOV domain is a subset of the widely distributed PAS domain superfamily involved in sensing Light, Oxygen, or Voltage (Huala *et al*., 1997; Crosson *et al*., 2003). They were first identified in *Arabidopsis thaliana* phototropins, which control phototropism, chloroplast movement, and stomatal opening (Huala *et al.,* 1997; Briggs and Christie, 2002). The LOV domain proteins have since been identified in different organisms where they regulate the activity of numerous output or effector domains such as kinases, phosphodiesterases, Zn- fingers and stress sigma factors (Crosson *et al.*, 2003;; Losi, 2004). LOV domains adopt a conserved α/β-fold containing a conserved cysteine residue that forms a transient thiol adduct with a flavin cofactor, which is tightly enclosed by five anti-parallel β-sheets and two α-helices upon blue-light excitation. The conformational changes induced in the LOV domain by light dependent adduct formation and dissociation leads to the modulation of protein-protein or protein-DNA interactions that initiate downstream signaling events. Mutation of the conserved cysteine residue to a serine or alanine prevents light-driven cysteinyl adduct formation (Salomon *et al*., 2000). Besides the LOV domains of phototropins in plants, two other groups of LOV proteins are known in plants, one of which is the ADO/FKF1 /LKP/ZTL family of proteins. These proteins closely resemble the fungal VIVID (Schwerdtfeger and Linden, 2003; Shrode *et al.,* 2001) and WC-1 proteins. The LOV domain of these classes of proteins differs from the phototropin LOV domains by having an extended loop in the flavin-binding pocket, which presumably in the case of fungi, is to accommodate the larger FAD molecule in the flavin-binding pocket (Heintzen, 2012). The third class of LOV domain recently identified in Arabidopsis proteins is the PAS/LOV proteins (PLPs) or LOV/LOV proteins (LLP), which are distinguishable from the other LOV domains because they lack the key cysteine residue for light perception in addition to an effector domain (Ogura *et al.,* 2008a; b; Kasahara *et al.,* 2010). Unlike phototropins, LLPs do not contain effector domains, and thus interaction with other binding proteins may serve as effectors. Photochemical properties of LLPs have shown that LLP family proteins have the potential to function in light perception (Kasahara *et al.,* 2010). In a yeast two-hybrid screen, Ogura *et al.* (2008) have shown that *At*LLP interacts with VITAMIN C

DEFECTIVE 2 (VTC2), VTC2-LIKE (VTC2L), and BEL1-LIKE HOMEODOMAIN 10 proteins (BLH10A and BLH10B) as PLP-interacting proteins. The interactions with VTC2L, BLH10A, and BLH10B are specifically diminished by blue light irradiation in yeast cells, suggesting that LLP may function as a photoreceptor.

Red-Light Receptors

Fungal phytochromes are part of a phosphorelay system with a conserved histidine kinase domain and a response regulator domain at their C-terminus. Unlike WC-1/LreA, phytochromes are not transcription factors but instead are presumed to regulate the activity of interacting proteins through a conserved kinase domain (Idnurm and Heitman, 2005). Interestingly, the blue and red light sensory pathways interact both genetically and physically in *A. nidulans*. An interaction between LreA / LreB, orthologs of the WC-1/WC-2 complex, with the phytochrome red-light receptor FphA and the developmental regulator VeA has been observed. VeA also interacts physically with the well-known pleotropic regulator of secondary metabolism, LeaA, and other velvet-like proteins explaining the phenomena of genetic linkage between the regulation of spore development and secondary metabolism by light (Bayram *et al.,* 2008; Purschwitz *et al*., 2008; 2009).

In a genetic analysis, phytochrome deletion mutants of *A. nidulans* produced up to 50% more sterigmatocystin than the wild type. The stimulation of sterigmatocystin synthesis upon phytochrome deletion was even enhanced in combination with the deletion of the blue-light regulators LreA and LreB, while the deletion of LreA and LreB alone resulted in less than WT levels of sterigmatocystin production. These results demonstrate a repressing function for phytochrome and an activating function for the WC orthologs in sterigmatocystin biosynthesis in *A. nidulans* (Purschwitz *et al*., 2008). Unlike *A. nidulans*, strains containing deletions of phytochrome-1 and phytochrome-2, either singly or in tandem, were not compromised in any known photoresponses in *Neurospora* (Fröhlich *et al.,* 2005). Similarly, deletion of the phytochrome gene in *Cryptococcus neoformans* did not exhibit a phenotype (Idnurm and

Heitman, 2005). However, in *N. crassa*, the abundance of phytochrome-1 mRNAs appeared to be under the control of the circadian clock, although the levels were not regulated by light (Fröhlich *et al.*, 2005). A similar pattern of non-light-dependent expression of the phytochrome-like gene was observed in *C. zeae-maydis* (Bluhm *et al*., 2008) leaving the function(s) of phytochromes in fungi unclear.

Regulation of cercosporin biosynthesis

The biosynthesis of cercosporin is affected by a variety of environmental factors. The essential role of light as an inducer of cercosporin biosynthesis and activity is well established (Daub and Ehrenshaft, 2000). Brief exposure of *Cercospora* cultures to light is sufficient to induce cercosporin production during subsequent incubation in darkness, indicating that although initiation of synthesis requires light, the actual synthesis does not (Lynch and Geoghegan, 1979; Daub and Chung, 2007). Hence, the induction of cercosporin biosynthesis by light presumably requires the transcriptional activation of genes by a sensory protein such as a photoreceptor. On the other hand, regulation of cercosporin biosynthesis by other environmental factors, e.g., metal ions, carbon and nitrogen availability, or pH appears to occur by subtle and interconnected mechanisms that do not manifest identically among all *Cercospora* species (You *et al.*, 2008).

A hypothetical model of signal transduction and regulatory control in which light activates cercosporin biosynthesis through Ca/calmodulin and MAPK signaling pathways has been proposed. In this model, environmental signals are perceived by unknown receptors and transmitted to up- or down-regulate *CRG1*, which serves a dual function of increasing biosynthesis by activating the expression of the specific pathway regulator, *CTB8*, and resistance by inducing the efflux pump, Cercosporin Facilitator Protein (CFP). *CTB8*, in turn, induces the entire set of cluster genes (*CTB1-7*) required for the biosynthesis and export of cercosporin in *C. nicotianae* (Daub and Chung, 2007; You *et al.*, 2008). *CRG1* is expressed constitutively in cultures grown in the light and in the dark (Chung *et al.* 1999). However, expression of *CTB8* is differentially regulated by light.

Analysis of the promoter regions of the *CTB* cluster genes in *C. nicotianae* revealed that all (*CTB1– CTB8*) genes have conserved GATA consensus sequences (Chen *et al*., 2007b) which can serve as the binding sites for the WCC regulatory complex (Ballario *et al*., 1996; Linden and Macino 1997) and AreA-type nitrogen regulatory proteins (Arst and Cove, 1973; Caddick *et al.,* 1986).

Regulation of ABA biosynthesis

Unlike in plants where ABA biosynthesis is triggered by known abiotic and biotic stressors, the trigger for its biosynthesis in fungi is unclear. However, light and nutrient availability (e.g. nitrogen/carbon ratio) as well as extracts from various host plant species have been shown to activate biosynthesis in *C. rosicola*, suggesting that host factors may be important triggers for ABA biosynthesis (Norman *et al.,* 1981; Takayama *et al.,* 1983). Carbon and nitrogen utilization play important roles in many aspects of fungal development, secondary metabolite production, and, for some fungi, pathogenesis. Gene expression in response to light, sugars and nitrogenous compounds has been extensively studied (Bahn *et al*., 2007: Palenchar *et al.* 2004; Rolland *et al.,* 2006; Wong *et al*. 2008), although not in the context of ABA biosynthesis. In light of the obvious need to understand the regulation of ABA biosynthesis *in vitro* while still striving to elucidate the biosynthetic pathway(s) in fungi, Gong *et al.* (2013) recently employed large scale cDNA-amplified fragment length polymorphism expression studies to identify candidate ABA biosynthesis and regulatory genes in the ABA overproducing TB-3-H8 strain of *B. cinerea*. Significant among the 31 candidate genes differentially expressed were transcripts of regulatory genes involved in nitrogen - carbon starvation and pH responses. Surprisingly, however, orthologs of pathway genes previously implicated in the biosynthesis of ABA in strain ATCC 58025 (Siewers *et al*, 2006) were not identified or discussed (Gong *et al.,* 2013).

Dissertation Goals and Outline of Thesis

The overall goal of this dissertation is to determine the genetic basis of the regulation of cercosporin biosynthesis in *C. zeae-maydis* by nitrogen and light, to identify the genetic basis of ABA biosynthesis in *C. zeae-maydis* and to determine the relevance of these two secondary metabolites in the pathogenesis of gray leaf spot disease of maize. The experiments and results described in this dissertation provide insight into the relevance of cercosporin and ABA in pathogenesis, the involvement of the *AREA* gene in mediating the regulation of cercosporin biosynthesis by nitrogen, and the involvement of *ABA3* and associated cluster of genes in the biosynthesis of ABA in *C. zeae-maydis*. The results of the experiments conducted are organized into three chapters as outlined below:

Chapter 2 describes the identification of the cercosporin biosynthetic gene cluster in *C. zeae-maydis*, regulation of cercosporin biosynthesis by AreA, the global nitrogen repressor gene, and the relevance of cercosporin as a pathogenicity factor in gray leaf spot disease of maize. The results in this chapter show that cercosporin is dispensable for pathogenesis and may not be a virulence factor in the development of grey leaf spot disease of maize.

Chapter 3 describes the identification of the *ABA3* gene and the putative ABA biosynthesis gene cluster in *C. zeae-maydis*. The results in this chapter suggest that a set of five contiguous genes have a role in the biosynthesis of ABA in *C. zeae-maydis* but ABA is dispensable for pathogenesis.

Chapter 4 describes the identification and partial characterization of a putative blue light receptor gene and the red-light receptor, phytochrome. In the chapter, the molecular functions of the putative light receptor genes in pathogenesis, cercosporin biosynthesis and reproduction were investigated. The results suggest that the putative blue light receptor gene, which regulates cercosporin biosynthesis and asexual reproduction in *C. zeae-maydis*, may be a member of a novel group of blue light receptors in fungi.

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CHAPTER TWO

GENETIC AND ENVIRONMENTAL REGULATION OF CERCOSPORIN BIOSYNTHESIS

ABSTRACT

Gray leaf spot of maize (GLS) is an economically important yield-limiting foliar disease caused by *Cercospora zeae-maydis.* Many species of *Cercospora*, including *C. zeae-maydis,* produce a distinctive perylenequinone phytotoxin, cercosporin. The identification of genes involved in the biosynthesis of cercosporin has long been a subject of many studies. In comparison, little is known about the regulation of cercosporin biosynthesis during pathogenesis, and it is not clear whether cercosporin is a critical virulence factor for all species of *Cercospora*. To this end, we identified a cluster of cercosporin biosynthetic genes based on shared homology and organization with genes in the cercosporin biosynthesis (*CTB*) cluster previously described in *C. nicotianae*. Disruption of *CTB1,* the polyketide synthase gene in the cluster resulted in the total loss of cercosporin production in the mutants, but did not affect fungal growth and development *in vitro*. However, *CTB1* disruption mutants were still capable of infecting maize leaves and forming lesions, which suggests that cercosporin biosynthesis is dispensable for pathogenesis. Additionally, disruption of *AREA* in *C. zeae-maydis*, which encodes a putative global regulator of nitrogen metabolite repression, nearly abolished cercosporin biosynthesis in *C. zeae-maydis*. Interestingly, the *AREA* disruption mutant was able to form necrotic lesions on maize leaves, which provides a second line of evidence that cercosporin may not be essential for pathogenesis in *C. zeae-maydis*. Together, these findings suggest that cercosporin is likely to be a component of a suite of as-yet unidentified virulence factors produced by *C. zeae-maydis* during infection of maize.

INTRODUCTION

Cercosporin is a photosensitizing phytotoxin made by many species of *Cercospora* and at least one endophytic species of *Mycosphaerella* (Moreno *et al.*, 2011). The photosensitizing action of the molecule results in peroxidation of membrane lipids and cell death (Daub, 1982a, b). *Cercospora* species are among the most successful fungal phytopathogens, and the production of cercosporin has been suggested to enhance virulence (Upchurch et al. 1991), but it is not a universal pathogenicity factor as cercosporin is not produced by all species (Assante *et al*., 1977; Goodwin *et al*., 2001; Weiland *et al*., 2010). The genetic basis of cercosporin biosynthesis is well studied in some *Cercospora* species, but little is known about whether cercosporin is a critical virulence factor for all species which produce cercosporin.

The biosynthesis of cercosporin is also affected by numerous factors and its regulation is predicted to occur via complex interplay of network mechanisms (You *et al*., 2008). Cercosporin is produced almost exclusively in the presence of visible light at wavelengths of 400–600 nm, and its production is blocked under complete darkness or in red light (Kim *et al*., 2011). Production of cercosporin is also affected by nutrient conditions, especially nitrogen (Daub and Ehrenshaft 2000), but the genetic basis for the effect of nitrogen on the biosynthesis is not known.

Gray leaf spot (GLS) is a serious foliar disease of maize, a major staple crop grown worldwide. The disease is caused by two *Cercospora* species, *C. zeae-maydis* Tehon & Daniels and *C. zeina* Crous & U. Braun, sp. Nov. in the USA. Both species are pathogenic on maize, and there are no obvious differences in virulence (or infection phenotype) between the two species, although *C. zeina* is not known to produce cercosporin (Lipps, 1998; Goodwin *et al.*, 2001). GLS is increasingly becoming a foliar disease of worldwide concern, especially in regions where management practices are constrained by limited management resources. On susceptible hybrids and under favorable conditions, extensive blighting of leaves can occur, resulting in yield losses exceeding 50% (Lipps, 1998). GLS is difficult to manage, as there are few resistant hybrids. An understanding of the molecular mechanisms underlying pathogenesis has therefore become an

integral part of the efforts to understand and improve the management options. The goals of this dissertation chapter are to identify and characterize the cercosporin biosynthesis cluster in *C. zeae-maydis*, determine its significance with respect to pathogenesis, and determine the genetic basis of regulation by nitrogen.

MATERIALS AND METHODS

Identification of *CTB1*

A cosmid genomic library created from *C. zeae-maydis* strain SCOH1-5 S (Shim and Dunkle, 2003) was kindly provided by Dr. Won-Bo Shim, Texas A&M University. Approximately 5,760 clones from the genomic library were screened by PCR with primers CTB1pF/ CTB1pR and areApF/areApR to identify cosmid clones containing the *CTB1* and *AREA* gene sequences respectively. For initial library screening, bacteria were pooled systematically (96 per pool) and used for colony PCR. In pools where a target sequence was identified, individual strains in the pool were screened separately and cosmid DNA was extracted for a second round of PCR to confirm individual cosmids containing the target sequence.

Nucleic Acid Preparation and Sequencing

DNA for PCR and southern blotting was prepared according to a cetyltrimethyl ammonia bromide (CTAB) method for plant genomic DNA extraction (Rosewich *et al*., 1998). *C. zeae-maydis* was grown in 50 ml YEPD medium (0.5% yeast extract, 1.0% peptone and 3.0% dextrose) for 2-3 days with constant shaking at 100 rpm. Mycelia were harvested by vacuum filtration through Whatman Grade No. 1 filter paper, washed with deionized water, and ground immediately in liquid nitrogen. Genomic DNA was isolated following a standard CTAB DNA preparation protocol (Rosewich *et al*., 1998) optimized for *C. zeae-maydis* as follows. Briefly, 500 µl CTAB DNA-extraction buffer (2.0% CTAB, 100mM Tris-Cl pH 8, 1.4 M NaCl, 20mM EDTA pH 8, 4.0% polyvinylpyrrolidone and 0.5% β-mercaptoethanol) was added to about 50 mg ground mycelium and incubated for 30 minutes at 55°C. Mycelial debris and proteins were pelleted and DNA was precipitated from the aqueous phase with isopropanol following standard molecular biology protocols. Plasmid and cosmid DNA were recovered from *E. coli* cultures following standard protocols described by Sambrook and Russell (2001). All plasmid and cosmid-walking sequencing was performed at the University of Arkansas DNA Resource Center (Fayetteville, AR) with an ABI 3130XL Genetic Analyzer.

Bioinformatics Analysis

Nucleic acid sequences obtained from the sequenced cosmids were manually assembled into contigs with Microsoft Word and Clustal omega to identify consensus regions and overlaps between successive cosmid walks. The assembled sequences were queried with BlastN and BlastX against the *C. zeae-maydis* whole-genome sequence (http://genome.jgi.doe.gov/Cerzm1/Cerzm1. home.html) to retrieve sequences flanking the *CTB1* and *AREA* sequences for analysis. Manual annotation of gene ORFs was performed with Softberry FGENESH (http://linux1.softberry.com/berry.phtml) with *Stagnospora nodorum, Pyrenophora* or *Leptosphaeria* as the training models. Homology searches of nucleotide sequences and database searches were performed with the BLAST service provided by the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov/BLAST). Identification of conserved domains and motifs in the protein sequences was performed with the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/) and the conserved-domain database (CDD) (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) at NCBI. Transcription factor binding motifs in the promoter sequences were predicted with Regulatory Sequence Analysis Tool (RSAT) (http://rsat.ulb.ac.be/) and Suite for Computational identification Of Promoter Elements (SCOPE) Version 2.1.0 (http://genie.dartmouth.edu/scope/).

Construction of Gene Deletion Fragments

A split-marker recombination strategy was used (Fu *et al*., 2006) to disrupt *CTB1* and *AREA*. Two *hygromycin phosphotransferase-B* (*HYG*) gene fragments *HY* and *YG* with a 428 bp sequence overlap in the middle were amplified from pTA-Hyg with primer pairs M13F/HY and M13R/YG, respectively. The *HY* fragment, which was approximately 1.3 kb and contained a *TrpC* promoter and 5′ partial *HYG* sequence, was fused with 550 – 970 bp of the region upstream of each target disruption locus, whereas the *YG* fragment containing ~0.8 kb of the 3’ region of *HYG* was fused at the 3’ end with the 5’ end of the sequence immediately downstream of the

target locus. PCR products corresponding to sequences upstream and downstream of the targeted disruption sites were amplified with primer pairs F1/F2 and F3/F4 respectively, fused with the *HY* and *YG* fragments by overlapping M13 primer regions in a fusion PCR, and amplified with nested primers F1N/HY-N versus F4N/YG-N (Ridenour *et al*., 2012).

For GFP expression strains, protoplasts were transformed with a GFP-Geneticin construct amplified by PCR with primers M13F/M13R from the plasmid pBR0073 (Appendix 2.3).

Protoplasting and Transformation

Freshly germinated *C. zeae-maydis* conidia were harvested by centrifugation and digested overnight in 1.2 M KCl solution of 20% lysing enzyme (Sigma# L-1412) and 0.4% v/v β-glucuronidase (Sigma# G-7017). Protoplast transformation was performed with 60% PEG-8000 in STC (1.2 M sorbitol, 50mM $CaCl_2$ and 10mM Tris pH 8.0). Depending on the selectable marker, transformants were selected on regeneration agar (1.0 M sucrose, 0.02% yeast extract and 1.0% agar) containing 150 µg/ml Hygromycin-B or 2 mg/ml G-418 disulphate (Geneticin) (Research Products Inter. Corp., Mt. Prospect, IL).

Molecular Analysis of Transformants

Mutants recovered from the regeneration agar were initially cultured on V8 juice agar supplemented with 150 µg/ml Hygromycin-B or Geneticin and maintained in continuous darkness to promote conidiation. DNA was extracted and analyzed by PCR to determine the integration of the disruption fragments at the targeted locus. For Southern analysis, 5 µg of genomic DNA was digested overnight with 10 units of restriction enzyme at 37°C. The samples were resolved on a 0.8% agarose gel at 0.6 V/cm for 12 h and transferred to Hybond-N+ (Amersham Pharmacia, Diegem, Belgium) as described by Sambrook and Russell (2001).

Measurement of Conidiation

V8 juice agar plates inoculated with 1 x 10^5 spores of wild type or mutant strains were

grown in the dark for 4 days. Colonized agar plugs of 1 cm diameter were cut from each of three conidiating V8 agar plates with a cork borer. Three plugs cut from each plate were placed into 50 ml tubes containing 3 ml of distilled H_2O containing 0.1% Triton-x and agitated to dislodge the spores into solution. The conidial suspension was appropriately diluted and spores counted with a hemocytometer. For biological replication, the experiment was repeated two times. Results were analyzed with one-way ANOVA in Minitab (v12.23) software.

Measurement of Growth

Radial growth of the mutants was compared to the wild type by measuring colony diameter on 0.2x PDA incubated for 10 days in constant light. A 10 mm diameter plug was extracted from a conidiating colony on V8 and lightly padded on a 0.2x PDA plate. Each strain was padded at a separate spot on each of four replicate plates in each experiment. For each colony, two diameters, measured 10 days after incubation, at right angles to each other were averaged to give the mean diameter for that colony. The experiment was repeated once.

Total biomass was measured by ergosterol concentration. Plates (0.2x PDA with or without NH_4Cl or KNO_3) overlaid with cellophane membranes were inoculated with 1×10^4 spores and incubated under constant light. Two plates were used per strain in each experiment. At 5 and 9 days after inoculation, fungal tissue was collected into a glass vial containing 2 ml chloroform methanol (2:1) and extracted overnight with constant agitation. Ergosterol analysis was performed on an HPLC system with a 4.6 U ODS column (250 x 4.6 mm, Alltech) and a UV detector (Shimadzu) set to measure absorbance at 282 nm as described by Bluhm and Woloshuk (2005). Quantities were determined by comparing peak areas of samples to those of a standard curve generated from HPLC-grade ergosterol (Sigma-Aldrich). The experiments were performed two times. Results were analyzed with one-way ANOVA in Minitab (v12.23) software.

Measurement of Cercosporin Content

For determination of cercosporin, 0.2x PDA plates (pH 5.6) were inoculated with 1 x 10^4 spores of *C. zeae-maydis* and incubated under constant light for 5 to 9 days. Colonized agar plugs (1 cm diameter) were removed into 15 ml tubes and extracted with 5.0 M KOH in darkness for 4 – 5 hours as described by Jenns and Daub (1995). Four plugs were sampled from each of three replicate plates at five, seven and nine days post-inoculation and extracted with 4 ml of 5.0 M KOH. Cercosporin concentrations were quantified by measuring the absorbance of the KOH solution at 480 nm and converted to concentration using the molar extinction coefficient (ε = 23,300 M^{-1} cm^{-1}). The entire experiment was repeated at least three times.

For the *AREA* deletion strains, cercosporin biosynthesis was assessed on 0.2x PDA amended with or without 50mM NH_4Cl or KNO_3. The experimental design and replications were as described above.

Cercosporin biosynthesis in the random insertional mutants recovered from regenetation agar were initiatially assessed by a streak of a colony on 0.2x PDA and incubated in light for 10 days (Appendix 2.3 D). When a mutant was identified as having a significant alteration in the biosynthesis of cercosporin, a plate of 0.2x PDA was inoculated with 1 x 10^4 spores and incubated under constant light for five days as described above. Plates were flooded with 20 ml of 5.0 M KOH and incubated in the dark for 4 hours to extract cercosporin. Cercosporin concentration was determined and expressed as a percentage of wild type production. There were four replications per sample and the experiments were repeated once. Statistical analyses were performed with the Minitab (v12.23) software.

Pathogenesis

Pathogenicity assays on maize hybrids B73, SilverQueen and a commercial DeKalb release (DKC 53-45) were performed with conidia collected from V8-juice agar cultures. To enable comparisons between mutant and wild type strains, leaves of six-week-old maize plants were individually spray-inoculated with each strain (approximately 5 ml of a 1 x 10^5 conidia/ml

suspension) as described by Bluhm and Dunkle (2008). The plants were covered with transparent plastic bags to maintain high relative humidity (>90%) without significantly impeding light transmission and were grown in a 12-12 hour day-night light cycle at 23^{o}C inside a growth chamber. Observations of disease progression were made 5 to 15 days post-inoculation. Leaf samples were collected 5 dai and examined via fluorescent microscopy to document pre-penetration infectious development. To evaluate lesion sizes and intracellular growth, leaves were sampled 14 -15 dai and examined via fluorescent microscopy. Intracellular growth of GFP-expressing strains was examined with a Nikon D-eclipse C1 confocal scanning fluorescent microscope. To measure lesion sizes, representative leaf sections from at least two inoculated plants in each experiment were cut into 8 cm sections and photographed with a Canon EOS REBEL T1i digital camera 14 dai. This experiment was repeated at least two times. In each photograph, a ruler was included in the same focal plane as a scale bar. To allow for precise measurement, the photographs were digitally magnified and the leaf margins cropped out to exclude necrosis resulting from physical damage and normal aging. All discernible lesions were measured with ImageJ (1.47q) image analysis software, and average lesion areas were calculated. Statistical analyses were performed with the Minitab (v12.23) software.

RESULTS

Cloning & Analysis of *CTB* Cluster in *C. zeae-maydis*

The *C. zeae-maydis CTB1* gene was initially identified from an EST sequence (~1.1 kb in length) obtained during differential cDNA screening to identify genes highly expressed during infectious or reproductive development (Bluhm *et al*., 2008). A BLASTx homology search of the EST sequence identified high levels of homology in the thioesterase domain of the *C. nicotianae* Ctb1 protein and other fungal polyketide synthases (Fig 2.1 A) In order to obtain the full sequence of the *C. zeae-maydis CTB1* gene and analyze the flanking genomic regions, primers were designed to screen a genomic library of *C. zeae-maydis* strain SCOH1-5 based on the *CTB1* EST sequence. Two independent sets of cosmid clones with sequence overlaps of at least 500 bp at one or both ends of the EST sequence were selected (Appendix 2.1) and sequenced by the Sanger method (ABI 3130xl analyzer BigDye 3.1) at the University of Arkansas core sequencing facility and assembled into a 15.5 kb contig. The assembled sequence of the *C. zeae-maydis CTB* cluster was confirmed by pairwise alignment with the subsequently released draft whole genome shotgun (WGS) sequence of the wild type strain SCOH1-5 {cluster located on JGI seq. scaffold 15: 626606-650190} with 100% nucleotide sequence identity. Gene ORFs located within 50 kb genomic region of the sequences were predicted with the softberry FGENESH software (http://linux1.softberry.com/berry.phtml) and BLASTx searches in the NCBI database were used to predict gene functions.

The *CTB* Cluster is Conserved in *C. zeae-maydis*

BLASTx homology searches of functions of twelve putative ORFs contiguous within the 50 kb *CTB* cluster revealed numerous genes encoding proteins analogous to various enzymes involved in fungal secondary metabolite biosynthesis. The putative *CTB* genes in *C. zeae-maydis* collectively span 24 kb and consist of eight coding sequences highly similar in structure and organization to the cercosporin biosynthetic genes described in *C. nicotianae* (Chen *et al*., 2007). The eight genes, designated *CTB1-8* to correspond with orthologs in *C. nicotianae*, were

analyzed in addition to two putative ORFs on either side of the core cluster of eight genes (Fig. 2.1. B. and Table 2.1). Relative to *CTB1*, the orientation, location and distribution of predicted genes within the putative cluster were conserved. Downstream of *CTB8*, the putative cluster-specific Zn-finger transcription regulator, are two ORFs encoding phenylalanine ammonia-lyase (PAL), a key enzyme in the biosynthesis of plant defense metabolites, and Cercosporin Facilitator Protein (*CFP*), an MFS type transporter predicted to be involved in cercosporin self-resistance (Callahan *et al*., 1999; Amnuaykanjanasin and Daub, 2009).

Co-regulated expression of genes in a cluster involved in fungal secondary metabolism is a common phenomenon. Because nitrogen and light are two of the most important environmental signals that influence cercosporin biosynthesis, we analyzed the promoter sequences 850 bp upstream from the predicted translation start codon for the presence of GATA binding sites, which are regulated by the global nitrogen repressor AreA, and incidentally, are also recognized by the light responsive regulator, WCC (Wilson and Arst, 1998; Linden and Macino, 1997). The upstream sequences were also analyzed for the presence of other promoter elements potentially overrepresented among cluster genes. In the region upstream of the *CTB1* open reading frame, two TATA binding elements, three CAAT binding elements and two non-symmetric spaced-dyads 5′- CGGN{0,2}GAA-3′ were identified. The GATA binding sites that are potentially recognized by the AreA nitrogen regulator, and/or the light-responsive regulator WCC were most represented. Four binding sites were found within the 850 bp promoter region. Analysis of the other cluster genes identified several consensus sequences in the promoter regions (Table 2.2). The GATA binding element and the non-symmetric spaced dyads 5′-CGGN{0,2}GAA-3′ were the most represented and present in the promoter regions of all the cluster genes. All the genes contain at least two CAAT consensus sequences in their promoter regions, with *CTB5* having seven. The consensus TATA motif was found in the promoters of all genes except *CTB4*. Unlike the GATA consensus sequence, the pH-regulatory pacC-binding sequence (GCCA(A/G)G) (Espeso *et al*., 1997) was only identified in the promoter regions of *CTB1* and *CTB4*, while the binding sequence (G/C)(A/G)GGGG that is recognized by the creA

carbon repressor was found in four (*CTB3*, *CTB5*, *CTB7* and *CTB8*) out of the eight promoters of the cluster genes.

Conceptual translation of the *CTB1* sequence of *C. zeae-maydis* identified a 7,014 bp ORF interrupted by eight introns with 90% identity to *C. nicotianae CTB1* (Table 2.1). Conceptual translation of the sequence resulted in a 2,187 amino acid protein with five conserved domains typical of a type 1 polyketide synthase: a β-ketoacyl synthase (β-KAS), an acyltransferase (AT), a dual-tandem acyl carrier protein (ACP) domain with phosphopantotheine prosthetic (PP) group attachment site, and a thioesterase (TE) domain analogous to that described in *C. nicotianae* (Choquer *et al*., 2005). (Fig.2.1. C).

Functional Disruption of *CTB1*

To characterize the *CTB1* gene and determine its molecular function, we disrupted the gene at the C-terminal end in the thioesterase domain in the wild type strain (SCOH1-5) of *C. zeae-maydis* by insertion of *hygromycin phosphotransferase B* gene cassette (*HYG*) under the control of the trpC promoter and terminator via split-marker triple homologous recombination (Fig. 2.2. A). Wild type strain was transformed with two split but overlapping *HYG* sequences, each flanked on the non-overlapping side with about 550 bp of *CTB1* sequence on either side of the targeted disruption site (Fig. 2.2. A). The resulting hygromycin-B resistant transformants were screened on 0.2x PDA in 24-well plates cultured under constant light for 10 days to identify strains with reduced or total loss of cercosporin biosynthesis; cercosporin accumulates as a visible red pigment in these conditions. This approach led to the identification of five mutant strains with *CTB1* disrupted at the targeted site, all of which failed to produce cercosporin (Fig. 2.2. B, and Appendix 2. 2).

Southern-blot analysis was performed on putative Δ*ctb1* disruption mutants to validate that the loss of cercosporin biosynthesis was a consequence of targeted disruption of *CTB1*. Genomic DNA from the wild type and two mutant strains (Δ*ctb1*-35 and Δ*ctb1*-73) was digested with *Dra*I and *Pvu*II and hybridized with either a *CTB1*-specific probe or *HYG*-specific probe to

confirmed the presence of a single copy of the *CTB1* gene in the wild type or the integration of a single copy of the disruption cassette in the *CTB1* locus in the mutants. As expected, a single 2.5 kb band was obtained for the wild type strain upon hybridization with a *CTB1* specific probe. A 1.6 kb band shift to 4.1 kb from the wild type band position was obtained for the mutants, confirming the insertion of a single *HYG* cassette in the *CTB1* gene (Fig. 2.2. C). No additional bands were present in the mutants, thus confirming lack of ectopic integration of additional copies of the *HYG* cassette . Thus, these results confirmed that the two mutants, Δ*ctb1*-35 and Δ*ctb1*-73, were disrupted only in the *CTB1* gene and the wild type strain, SCOH1-5, contained only a single copy of the wild-type *CTB1* gene. This, to the best of our knowledge, constitutes the first report of successful gene disruption in *C. zeae-maydis* by split marker triple homologous recombination.

Phenotypic Characterization of *CTB1* Disruption Mutants

Effect of *CTB1* disruption on growth and development

Mutants disrupted in the *CTB1* gene and the wild type were inoculated individually onto 0.2x PDA plates at the concentration of 10^2 spores per plate or on V8 juice agar at the rate of 10^6 spores per plate and assessed for colony margin morphology and conidiation, respectively. Colony diameter of a 10 mm diameter padding of spores onto 0.2x PDA plates was measured 10 dai. Mutants were not affected in morphology or radial growth on 0.2x PDA (Fig. 2.3. A-B). On V8 juice agar in constant darkness, the disruption mutants, Δ*ctb1*-35 and Δ*ctb1*-73 appeared to produce fewer conidia compared to the wild type (Fig. 2.3. C). However, lower levels of conidiation in the mutants was not statistically different from the wild type as determined by single factor ANOVA at a 95% confidence interval.

Effect of *CTB1* disruption on cercosporin biosynthesis

We observed that the wild-type strain SCOH1-5 produces cercosporin consistently when inoculated on thicker 0.2x PDA plates and grows throughout a longer incubation period as

opposed to cultures on thinner plates, as recommended for *C. nicotianae* (Daub and Chung, 2007). Thus, thick 0.2x PDA plates were inoculated with 1 x 10^4 spores and incubated under constant fluorescent light illumination for up to nine days. Cercosporin production by the wild type was visible by the fourth day after inoculation. On plates inoculated with the Δ*ctb1*-35 and Δ*ctb1*-73 mutants, cercosporin production was not detected in either strain even up to nine days after incubation (Fig. 2.4. A). Quantitatively, cercosporin accumulation in the wild type increased two fold from about 25 nM on the 5^{th} day to about 50 nM on the 9^{th} day post inoculation, whereas mutants had undetectable accumulation over the same period (Fig. 2.4. B). These data affirm the role of *CTB1* in the biosynthesis of cercosporin in *C. zeae-maydis*.

Effect of *CTB1* disruption on pathogenesis

While the role of *CTB1* in cercosporin biosynthesis has been established, the function of *CTB1* and cercosporin in pathogenesis remains unresolved, especially for species infecting cereal crops. To determine the role and importance of *CTB1* in pathogenesis, we inoculated maize plants at the V6 stage of development with conidia of the wild type isolate SCOH1-5 and disruption mutants Δ*ctb1*-35 and Δ*ctb1*-73. We observed that by 18 days after inoculation, both mutants formed large lesions on leaves that were indistinguishable from those induced by the wild type (Fig. 2.5. A).

To verify whether disruption of *CTB1* affected pre-penetration infectious development, GFP expressing isolates of wild type (WT-GFP) and Δ*ctb1-35* (Δ*ctb1-35*-GFP) were created and inoculated on maize leaves for microscopic evaluation. Wild type or Δ*ctb1-35* were transformed with a linearized vector of a GFP and geneticin (*Neo*) resistant gene ORFs driven by gpdAp constitutive promoter (Appendix 2.3). Geneticin-resistant colonies, which were morphologically similar to the parent strain and showed strong GFP expression, were evaluated for cercosporin biosynthesis phenotype in the WT-GFP and the absence of biosynthesis in the Δ*ctb1-35*-GFP strain. The strains were inoculated onto maize leaves as already described. Representative leaf samples were examined via confocal scanning microscopy at 5 to 14 dai. Stomatal infection,

leaf colonization and conidiation within lesion regions in the disruption mutant (Δ*ctb1-35*-GFP) did not differ from the wild type (WT-GFP) strain. Both the Δ*ctb1-35*-GFP and WT-GFP strains formed appressoria, exhibited extensive intracellular growth, induced large necrotic lesions and produced erumpent conidia from stomata pores within the lesion area (Fig. 2.5. B). To ascertain that the Δ*ctb1* mutant causing lesions was not a wild type revertant from a dikaryon, we prepared sporulating cultures from conidia isolated from lesions on leaves inoculated with Δ*ctb1-35*-GFP and confirmed the persistence of the loss of cercosporin biosynthesis phenotype on 0.2x PDA, disruption of the *CTB1* gene by PCR, and pathogenicity by re-inoculation of maize leaves (Appendix 2.4).

To further examine whether cercosporin is important as a virulence factor, average lesion areas were compared between the wild type and mutants. (Lesion area was calculated from the most representative lesion-bearing sections of leaves inoculated with the Δ*ctb1*strains or wild type and compared with single factor one-way ANOVA (Minitab v12.23). No significant difference was found between the mutant and wild type (Fig. 2.5. C).

Regulation of Cercosporin Biosynthesis in Pathogenesis

Although cercosporin is believed to function as a virulence/ pathogenicity factor in many *Cercospora* species, the dynamics of cercosporin biosynthesis during pathogenesis are largely unknown. Our goal therefore was to determine whether the *CTB1* gene was expressed *in planta* and if so to determine at what stage of pathogenesis. In this respect, a *CTB1* promoter activation reporter strain was constructed and evaluated *in vitro* and *in planta*(Fig. 2.6; Appendix 2.5). The *CTB1* promoter sequence (1 kb) was cloned between *Sal*I and *Bam*HI sites upstream of the eGFP open reading frame in the vector pEGFP (clontech PT3078-5) to drive the conditional expression of eGFP.

Ten transformants confirmed to be capable of producing wild type levels of cercosporin were evaluated for conditional expression of GFP *in vitro* or *in planta*. Surprisingly, in this assay, we were unable to distinctly differentiate between autofluorescence and GFP with

epifluorescence or confocal microscopy (Appendix 2.5). A major resistant mechanism employed by species of *Cercospora* to avoid the toxic effect of cercosporin is the reduction of cercosporin to less toxic hydroxylated derivatives that fluoresce green under blue light excitation, in contrast to the typical red fluorescence observed for cercosporin (Leisman and Daub, 1992). Thus, the autofluorescence observed specifically during cercosporin biosynthesis presumably resulted from fluorescence of reduced cercosporin derivatives.

On inoculated leaves, observations were made under fluorescent microscopy at intervals from 24 hours, to fifteen days post inoculation. No green fluorescence that could be attributed to autofluorescence of cercosporin or expression of GFP was observed in any of the ten reporter strains (Appendix 2.5). Possible explanations are that *CTB* genes are not expressed during pathogenesis, are expressed at low levels, or have a temporal/spatial regulation that obscures detection via the GFP reporter assay.

Genetic Regulation of Cercosporin Biosynthesis by Nitrogenous Salts

Cercosporin biosynthesis is affected by several environmental and physiological factors, among which light and nitrogen are most critical (Bluhm *et al*., 2008; You *et al*., 2008). Analysis of the promoter elements of the cercosporin biosynthesis gene cluster revealed the overrepresentation of binding sites for AreA, the global regulator of nitrogen metabolite repression; the same binding sites are also recognized by the light regulated WCC (Table 2.2). Given the global effect AreA has on secondary metabolism in fungi, the fact that several putative binding elements were present in the promoter regions of the cercosporin biosynthesis genes (Table 2.2) and the observation that ammonium phosphate suppresses biosynthesis in *C. zeae-maydis* (Bluhm *et al*., 2008), we hypothesized that AreA mediates the regulation of cercosporin biosynthesis by nitrogen. To test this hypothesis, we first ascertained the effect of ammonium and nitrate salts on cercosporin biosynthesis *in vitro*. The wild type strain SCOH1-5 was grown on 0.2x PDA with or without added nitrogen salt. . On medium amended with potassium nitrate (KNO_3), cercosporin biosynthesis was decreased by over 80% by 9 dai whiles the addition of

ammonium chloride (NH_4Cl) or ammonium nitrate (NH_4NO_3) to the growth medium resulted in complete suppression of cercosporin biosynthesis (Fig. 2.7).

Sequence analysis of *AREA*

To identify the *AREA* gene in the genome of *C. zeae-maydis* wild type strain SCOH1-5, an EST sequence with high homology to the *A. nidulans AREA* gene was retrieved and PCR primers were designed to screen the SCOH1-5 genomic library as previously described to retrieve cosmid clones for sequencing (Appendix 2.1). The sequence obtained was assembled and compared with subsequently available SCOH1-5 WGS sequence on the JGI web portal {http://genome.jgi.doe.gov/pages/blast.jsf?db=Cerzm1}.

Analysis of the sequence showed an ORF of 3.1 kb interrupted by two introns of 53 and 51 bp. The predicted protein was 898 amino acids long with a conserved 5'-GATA-3' Zn-finger DNA binding domain and a DUF (Fig. 2.8. A-B). In addition to the conserved 5'-GATA-3' Zn-finger DNA binding domain, the *AreA* ortholog contained the two blocks of sequences RMENLTWRMM and WEWLTMSL at the N and C termini, respectively, that are highly conserved among all ascomycetes (Wong *et al*., 2008). The gene was located between a repeat element upstream and the *Xeroderma pigmentosum* group G protein-encoding gene downstream.

Deletion of *AREA* in *C. zeae-maydis*

The *AREA* gene in wild-type strain SCOH1-5 of *C. zeae-maydis* was targeted for deletion in order to determine the genetic basis of the effect nitrogen has on cercosporin biosynthesis *in vitro*. Protoplasts were prepared and transformed with spilt-marker triple homologous recombination as described by Fu *et al*, (2006). Transformants were recovered from regeneration agar containing 150 mg/ml Hygromycin-B. The transformants were initially screened by PCR with primers pF/pR (Fig. 2.9. A) to select putative knockout mutants.

With this approach, seven putative knockouts were identified which were further screened by PCR with primers pairs A1/H1and A1/H2 to verify the integration of the deletion

constructs at targeted sites (Fig. 2.9. B). Southern blotting was performed to identify and eliminate strains with ectopic insertion(s) of the disruption cassette (Fig. 2.9. C). Two of the five independent knockout mutants confirmed by southern blotting, Δ*areA-20* and Δ*areA-34*, showed deletion without multiple insertion of the deletion construct and thus were selected for subsequent characterization.

Phenotypic Characterization of *AREA* Deletion Mutants (Δ*areA*)

Effect of *AREA* deletion on growth and development

To confirm that the deleted gene encoded a nitrogen catabolite repressor protein orthologous in function to AreA, the Δ*areA-20* and Δ*areA-34* mutants were grown on 0.2x PDA supplemented with NH_4Cl or $KClO_3$. As expected, we observed that deletion mutants grew on media supplemented with $KClO_3$, whereas wild type could not sustain growth on the same media (Fig. 2.10 A). Radial growth on 0.2x PDA and conidiation on V8 juice agar were compared in the mutants and the wild type. Conidiation of mutants on V8 media in the dark was significantly reduced compared to wild type. The *AREA* mutants, Δ*areA-20* and Δ*areA-34*, produced only 40% as much conidia as the wild type (Fig. 2.10. B). On 0.2x PDA, radial growth of the mutants appeared to be significantly reduced compared with the wild type (Fig. 2.10. C).

Effect of *AREA* deletion on cercosporin biosynthesis

Levels of cercosporin produced by the *AREA* deletion mutants on 0.2x PDA supplemented with NH_4, a favorable nitrogen source, was indistinguishable from the background absorbance by visible spectrometry. On NO_3 supplemented 0.2x PDA, the *AREA* deletion mutants produced comparable levels of cercosporin as the wild type at all-times albeit in much reduced amounts. On 0.2x PDA without added nitrogen, the wild type made nearly 5 fold the level of cercosporin produced by the mutants on 5 and 7 dai. However, by 9 dai, the production of cercosporin in the wild type had increased to as much as 96 nM/plug compared with 10 nM/plug of the mutants, which represents approximately a 10-fold difference (Fig. 2.11).

As a measure of total fungal biomass, we determined membrane ergosterol concentration at 5 and 9 days after inoculation and compared differences in ergosterol levels between mutants and wild type on different media to levels of cercosporin on the same media. Ergosterol levels were fairly comparable across media between mutant strains, but differed considerably between the mutants and wild type. Total ergosterol was higher in the wild type than the mutants on media supplemented with nitrogen. Conversely, on 0.2x PDA, ergosterol levels of the wild type were somewhat surprisingly lower than the mutants (Fig. 2.11).

When examined as the ratio of cercosporin produced per unit of biomass, there was a drastic reduction in cercosporin production in the mutants compared with the wild type on PDA. On addition of nitrate to the medium, cercosporin biosynthesis in the wild type was repressed while no obvious changes were observed in the levels accumulated by the mutants per unit of biomass (Fig. 2.11. B).

Phenotypic Characterization of *AREA* Deletion Mutants *in planta*

Maize plants at the V6 leaf stage were inoculated with approximately 5 x 10^5 spores of strains Δ*areA*-20, Δ*areA*-34 or the wild type and incubated for 15 days. Interestingly, the *AREA* deletion mutants were able to initiate necrotic lesions on maize leaves, but lesions did not expand to full size (Fig. 2.12. A). To investigate this further, we transformed Δ*areA* with the constitutive GFP expression vector pBR0073 and evaluated the ability of the GFP strain of the deletion mutant to colonize and form lesions during pathogenesis. We observed that the mutants were able to form appressoria and penetrate the stomata but the pervasive intracellular growth associated with the wild type in lesion and surrounding leaf tissues was absent in the *AREA* deletion mutant. Intracellular growth was restricted to the small regions of necrosis.

Identification of Genetic Factors Regulating Cercosporin Biosynthesis in *C. zeae-maydis*

To identify specific signals or genetic factors that affect cercosporin biosynthesis, we adopted a forward genetic approach to generate a collection of 1,300 geneticin-resistant and GFP

expressing random insertional mutants and screened for cercosporin biosynthesis phenotypes. Each of these transformants was evaluated 10 dai for cercosporin biosynthesis and scored as having 1) approximately wild-type levels of cercosporin biosynthesis, 2) reduced levels, or 3) an apparent loss of cercosporin biosynthesis (Appendix 2.3. D). Following this approach, 24 mutants were selected and re-tested for cercosporin biosynthesis. This re-evaluation confirmed that cercosporin production in 22 of the 24 mutants was reduced by at least 25% as compared to wild type (Table 2.3, Appendix 2.6).

DISCUSSION

Cercosporin has long been considered a key virulence factor in diseases caused by *Cercospora* species but it is not clear whether cercosporin is a critical virulence factor for all species of *Cercospora*. In this study, we provide experimental evidence that cercosporin is dispensable for pathogenesis in gray leaf spot disease of maize (GLS) and may not be a critical virulence factor for *C. zeae-maydis*.

The identification of genes involved in the biosynthesis of cercosporin has been a subject of many studies. We identified a polyketide synthase gene (which we named *CTB1*) in *C. zeae-maydis* that has 90% amino acid sequence identity to the Ctb1 protein of *C. nicotianae.* In *C. nicotianae*, the *CTB1* gene is one of eight core cercosporin biosynthesis genes physically located within the *CTB* cluster. Mutation of *CTB1* or other pathway genes has invariably resulted in the loss of cercosporin biosynthesis and virulence (Chen *et al*., 2007, Daub and Chung, 2007). By creating and analyzing *CTB1* disruption strains, we established that Ctb1 in *C. zeae-maydis* is a functional ortholog of *C. nicotianae* Ctb1 and has a clear role in cercosporin biosynthesis. Disruption of the *CTB1* gene in *C. zeae-maydis* resulted in the complete loss of cercosporin production in the mutants in vitro (Fig. 2.4). Subsequent to the disruption of *CTB1* and the availability of full sequence information for the gene, we observed that the disrupted thioesterase domain was located at the N-most terminal of the gene, leaving all the other domains involved in the iterative assembly of the polyketide backbone unaffected at the C-terminal. However, this insertional disruption deleted thioesterase domain of Ctb1, and likely disrupted transcription of the entire locus.

Because secondary metabolite genes in fungi tend to be clustered, and *CTB1* in *C. nicotianae* has been identified in a cluster with other cercosporin biosynthesis pathway and transport genes, we analyzed the genomic regions flanking the *CTB1* gene and identified a conserved cluster of eight genes that are orthologous with the *CTB* cluster described in *C. nicotianae* (Fig. 2.1). Consistent with the coordinated regulation of cluster genes by common transcription factors, binding elements for the global nitrogen regulator AreA and/or the blue-

light regulator WCC were common in the promoter regions of all the cluster genes. This finding together with the loss of cercosporin in the Δ*ctb1* mutant is sufficient evidence to conclude that the cluster of eight *CTB* genes orthologs in *C. zeae-maydis* is conserved in function and is the genetic basis of cercosporin biosynthesis in *C. zeae-maydis*.

To determine the significance of cercosporin in GLS of maize, the cercosporin non-producing mutants were inoculated on maize leaves and observed through the course of disease progression. On maize, the Δ*ctb1* disruption mutants were indistinguishable from the wild type in pathogenicity and virulence. The virulence of the mutants was initially surprising given that cercosporin has long been considered a critical virulence factor among *Cercospora* species. To determine whether *CTB1* is potentially involved in specific aspects of pathogenesis-related development despite the apparent lack of involvement in lesion formation, we performed histological examinations of the developmental stages of pathogenesis on the Δ*ctb1-GFP* and WT-GFP strains inoculated on leaf surfaces. Pathogenesis of *C. zeae-maydis* involves the infection of host leaves through stomata by first sensing and re-orienting growth towards the stomata, formation of multilobed appressoria-like structures over stomata before penetration and colonization of the internal structures after a period of latent intercellular growth (Beckman and Payne, 1982; Bluhm and Dunkle, 2008). No defects were observed in the infection cycle of the mutants; they made appressoria over stomata, exhibited pervasive intracellular hyphal growth and produced erumpent conidia from stomata within lesions. From these findings, we conclude that *CTB1* (and by extension, cercosporin) does not play a significant role in stomatal infection or colonization of host tissue (Fig. 2.5). This hypothesis is consistent with other reports from the literature. Bluhm and Dunkle (2008) had previously shown that disruption of *PHL1* in *C. zeae-maydis* significantly reduces cercosporin biosynthesis but the reduction does not correspondingly affect pathogenesis. Also, other species of *Cercospora,* including *C. zeina*, a sibling species of *C. zeae-maydis* and GLS pathogen of maize, are not known to make cercosporin in culture yet are aggressive pathogens. At the onset of this project, there is no experimental evidence to confirm that *C. zeae-maydis* makes cercosporin during pathogenesis of maize as it does *in vitro*.

To address this concern, we created a CTB1p::GFP reporter strain to determine how *CTB1* is regulated during pathogenesis. Surprisingly, we were unable to distinguish between the green fluorescence of cercosporin and GFP expression *in vitro*. This was probably due to low expression of GFP by the *CTB1* promoter, which has a low activity even under conditions conducive to cercosporin biosynthesis (Bluhm *et al.*, 2008). To allow a distinction to be made between the induced expression of GFP and background cercosporin auto-fluorescence, a threshold minimum expression of the GFP is required which might not have been satisfied by the *CTB1* gene promoter activity.

To explain the regulation of cercosporin biosynthesis by nitrogen and also clarify the basis of discrepancy between the virulence phenotype of the *CTB1* mutants in *C. zeae-maydis* as compared to *C. nicotianae*, we identified and deleted the *AREA* gene in the wild type and evaluated deletion mutants for the ability to produce cercosporin and infect maize. The genome of *C. zeae-maydis* contains a single copy of the *AREA* gene. AreA is a global regulator of nitrogen utilization in ascomycetes and is involved in the regulation of secondary metabolism (Mihlan *et al.*, 2003; Kim and Woloshuk, 2008). Besides the environmental effect nitrogen has on cercosporin biosynthesis, the over representation of AreA-type GATA binding elements in the promoter regions of all the *CTB* cluster genes suggested that AreA mediates the regulation of cercosporin biosynthesis by nitrogen *in vitro*. Presumably, ammonia represses the expression of *AREA* to effect the repression of cercosporin biosynthesis. We therefore anticipated that deletion of the *AREA* gene would mimic the effect of ammonia and result in complete repression of cercosporin biosynthesis in the *AREA* mutants. As expected, no cercosporin biosynthesis occurred when mutants were grown on ammonia containing media. Contrary to expectations, however, *AREA* deletion did not completely abolish cercosporin biosynthesis in the mutants on nitrogen-deficient media or in media with nitrate, a less preferred nitrogen source, as would be expected if *AREA* repression is the sole mechanism by which regulation of cercosporin biosynthesis by ammonia is mediated. It is not obvious how nitrates in the media, which presumably activate *AREA* expression, will cause a reduction in cercosporin biosynthesis while

the absence of a functional *AREA* gene in the mutants was not sufficient to cause complete abolition of cercosporin biosynthesis as it occurs on ammonia. It suggests that the repression of cercosporin biosynthesis in the presence of ammonia cannot be fully explained by the presumed repression of *AREA* gene by preferred nitrogen alone but may involve a complex network of factors including *CRG1* and *CTB8*. It has been noted that the inhibitory effect of NH_4Cl and $NaNO_3$ salts on cercosporin biosynthesis occurs through negative regulation of *CRG1*, *CTB8*, and *CTB1* genes (You *et al*., 2008). *CRG1* is an upstream activator of *CTB8*, which is the master regulator of *CTB (1-7)* and *CFP* genes involved in the biosynthesis and export of cercosporin. (Callahan *et al*., 1999; Chen *et al*., 2007; You *et al*., 2008).

On inoculated leaves, the *AREA* mutants were reduced in virulence and were only able to form necrotic specks that did not expand to form lesions even after 14 days of incubation. The loss of virulence likely results from the global effect of AreA on multiple virulence factors. It is also possible that AreA regulates a single virulence factor other than cercosporin that is not dispensable for virulence in *C. zeae-maydis*. AreA contributes to, but is not directly essential for, fungal virulence in several other pathosystems (Coleman *et al.*, 1997; Snoeijers *et al.*, 2000; Bolton and Thommab, 2008). However, the ability of the *AREA* mutants, which are reduced in cercosporin biosynthesis, to initiate necrosis is further evidence that cercosporin is dispensable for pathogenesis in *C. zeae-maydis*. Thus, it will be interesting to determine the effect of nitrogen and *AREA* deletion on the expression of the *CRG1* and *CTB8* genes and the differential expression of pathogenesis related genes between *CTB1* and *AREA* mutants to predicted factors that contribute to virulence in *C. zeae-maydis*.

Conclusion

CTB1 is a polyketide synthase gene involved in the biosynthesis of cercosporin that physically resides in the cercosporin biosynthesis gene cluster of *C. zeae-maydis.* Disruption of *CTB1* results in the loss of cercosporin biosynthesis in *C. zeae-maydis;* however, the loss of cercosporin biosynthesis does not affect virulence on maize. In contrast, Δ*areA* deletion

mutants, which were significantly reduced in cercosporin biosynthesis, were only able to cause necrotic specks on maize, suggesting that AreA regulates other factors that are more essential for virulence in *C. zeae-maydis* than cercosporin. Taken together, these findings suggest that cercosporin is dispensable for virulence in *C. zeae-maydis* but may belong to a suite of as-yet unidentified virulence factors regulated by AreA that are expressed during infection of maize by *C. zeae-maydis*.

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TABLES

Table 2.1: Characterization of genes in *C. zeae-maydis CTB* cluster

Predicted Gene	Length (bp)	No. of Exons	Amino Acids	Closest BLAST match proteins and species (accession no.)	*E*-value	Identity (%)
CTB1	7,014	9	2,187	Polyketide synthase (AAT69682)	0.00	90
CTB2	1,401	1	467	O-methyltransferase [*Cercospora nicotianae*] (ABK64180.1)	0. 00	91
CTB3	2,762	3	873	Cercosporin toxin biosynthesis protein [*Cercospora nicotianae*] (ABC79591.2)	0. 00	87
CTB4	1,702	4	513	MFS transporter [*Cercospora nicotianae*] (ABK64181.1)	0. 00	91
CTB5	1,410	1	470	Oxidoreductase [*Cercospora nicotianae*] (ABK64182)	0. 00	91
CTB6	1,065	1	355	Reductase [*Cercospora nicotianae*] (ABK64183.1)	0. 00	91
CTB7	1,332	2	411	Oxidoreductase [*Cercospora nicotianae*] (ABK64184.1)	0. 00	73
CTB8	1,251	2	400	Zinc finger transcription factor [*Cercospora nicotianae*](ABK64185.1)	1e-154	69
Orf1	973	4	262	Hypothetical protein [*Cercospora nicotianae*] (ABJ55988.1)	0.00	91
Orf2	1,340	4	360	Hypothetical protein [*Cercospora nicotianae*] (ABJ55989.1)	8e-94	85
CFP	1,942	2	607	Cercosporin facilitator protein [*Cercospora kikuchii*] (AAC78076.1)	0. 00	89
PAL	2,506	6	727	Phenylalanine ammonia-lyase [*Aspergillus fumigatus* Af293] (XP_755245.1)	2e-125	50

Table 2.2: Analysis of promoter sequences upstream of putative translation start codon

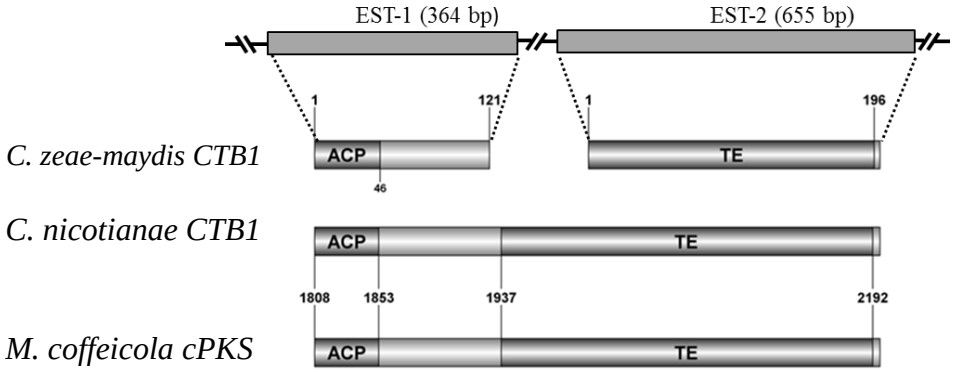
Gene	TATA	CAAT	AreA or WC1/WC2 GATA	creA SYGGGG	pacC GCCARG	Palindrome CGGN{0,2}GAA
CTB1	2	3	4	0	1	2
CTB2	3	3	5	0	0	3
CTB3	1	2	2	1	0	3
CTB4	0	5	2	0	1	4
CTB5	1	7	2	1	0	5
CTB6	2	3	2	0	0	2
CTB7	1	3	2	1	0	1
CTB8	3	2	3	1	0	2

Table 2.3: Phenotypic characterization of the cercosporin biosynthesis deficient mutants

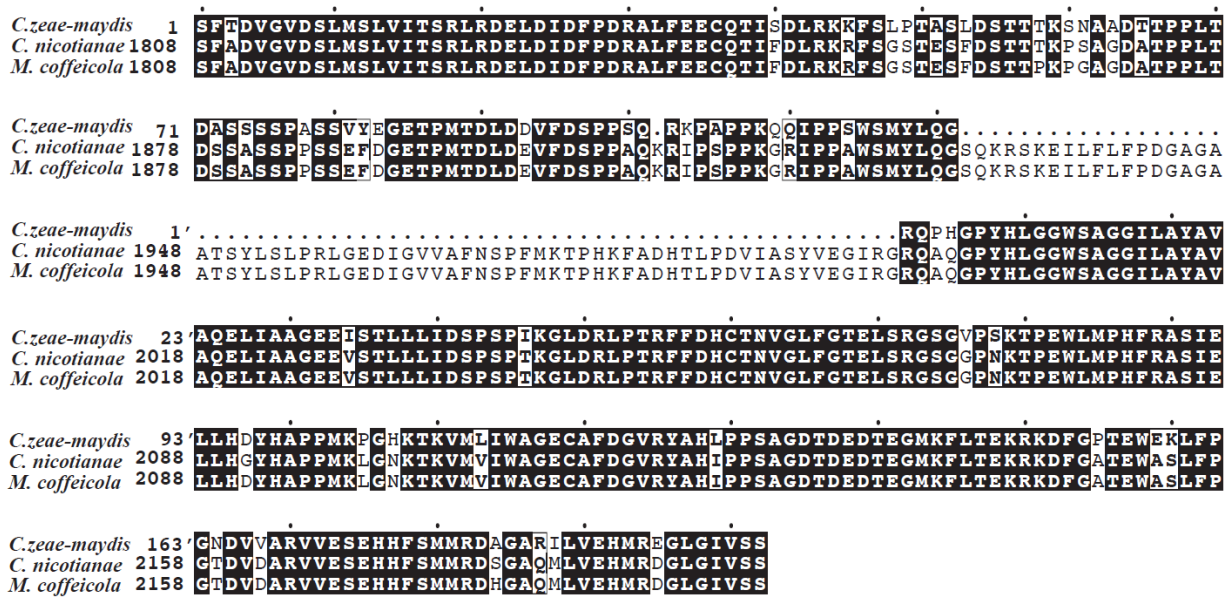
Relative cercosporin production (% of WT production)	No. of mutants	% of mutants screened
$\leq 10\%$	8	0.6
$11\% < x \leq 50\%$	12	0.9
$50\% < x \leq 75\%$	2	0.2

FIGURES AND FIGURE LEGENDS

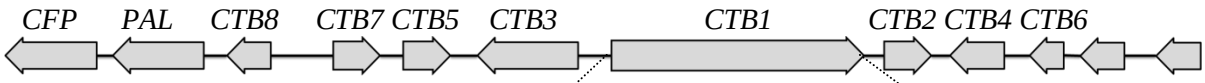
A. (i)



(ii)



B.



C.

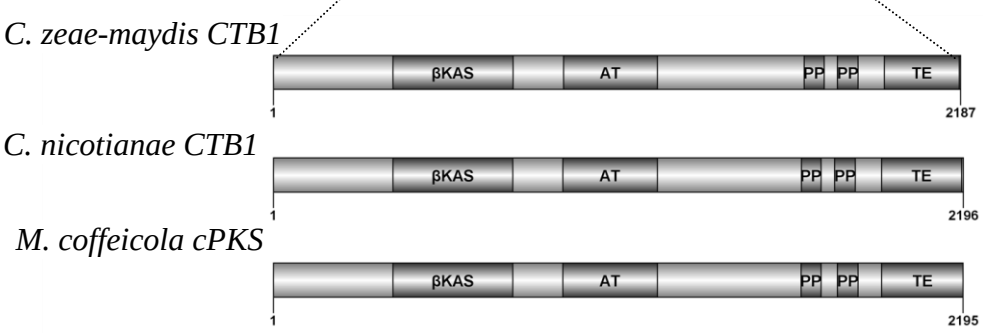
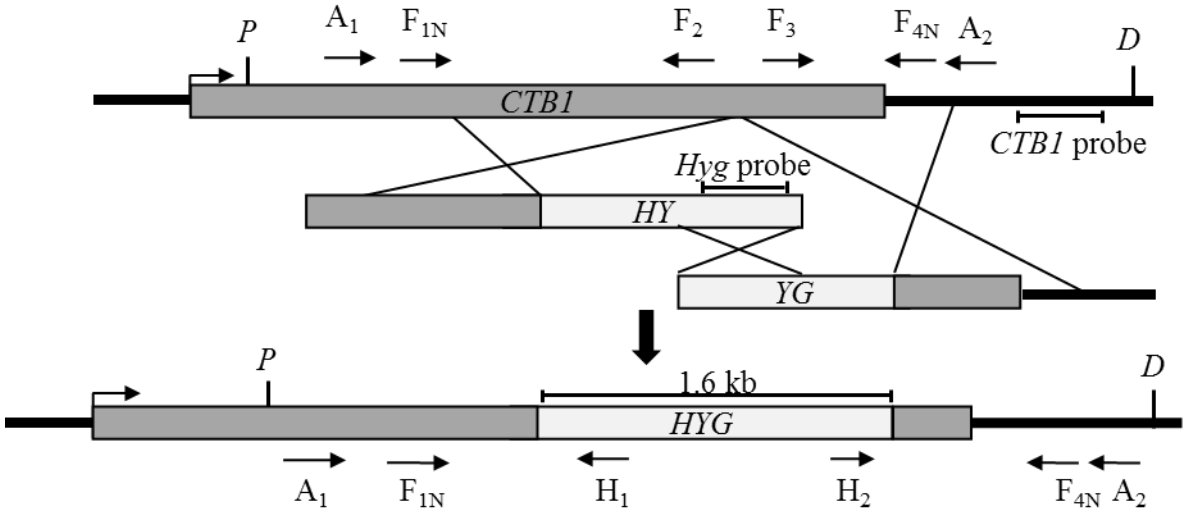
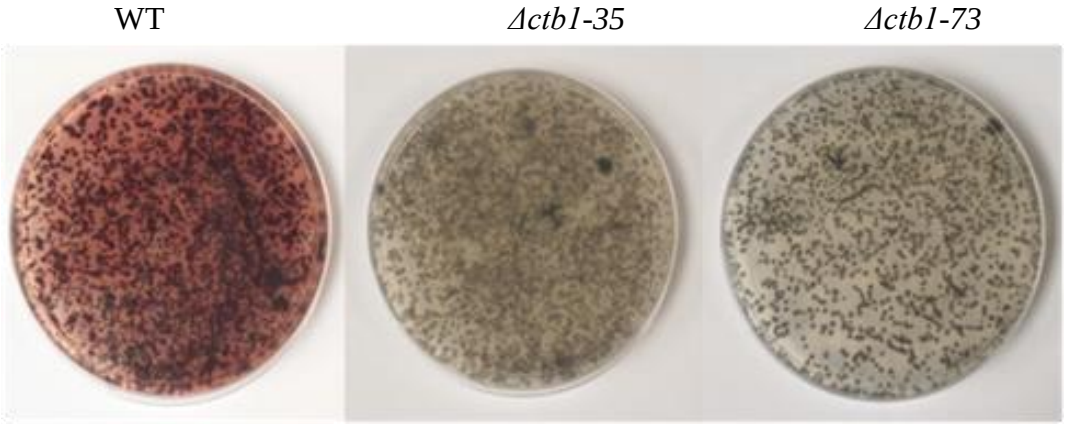


Fig. 2.1

A.


P
A1
F1N
F2
F3
F4N
A2
D
CTB1
CTB1 probe
Hyg probe
HY
YG
P
1.6 kb
D
HYG
A1
F1N
H1
H2
F4N
A2

B.


WT
Δctb1-35
Δctb1-73

C.

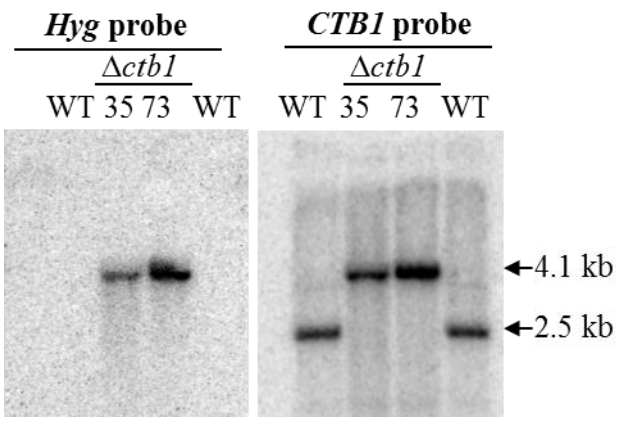

Hyg probe
Δctb1
WT 35 73 WT
CTB1 probe
Δctb1
WT 35 73 WT
4.1 kb
2.5 kb

Fig. 2.2.

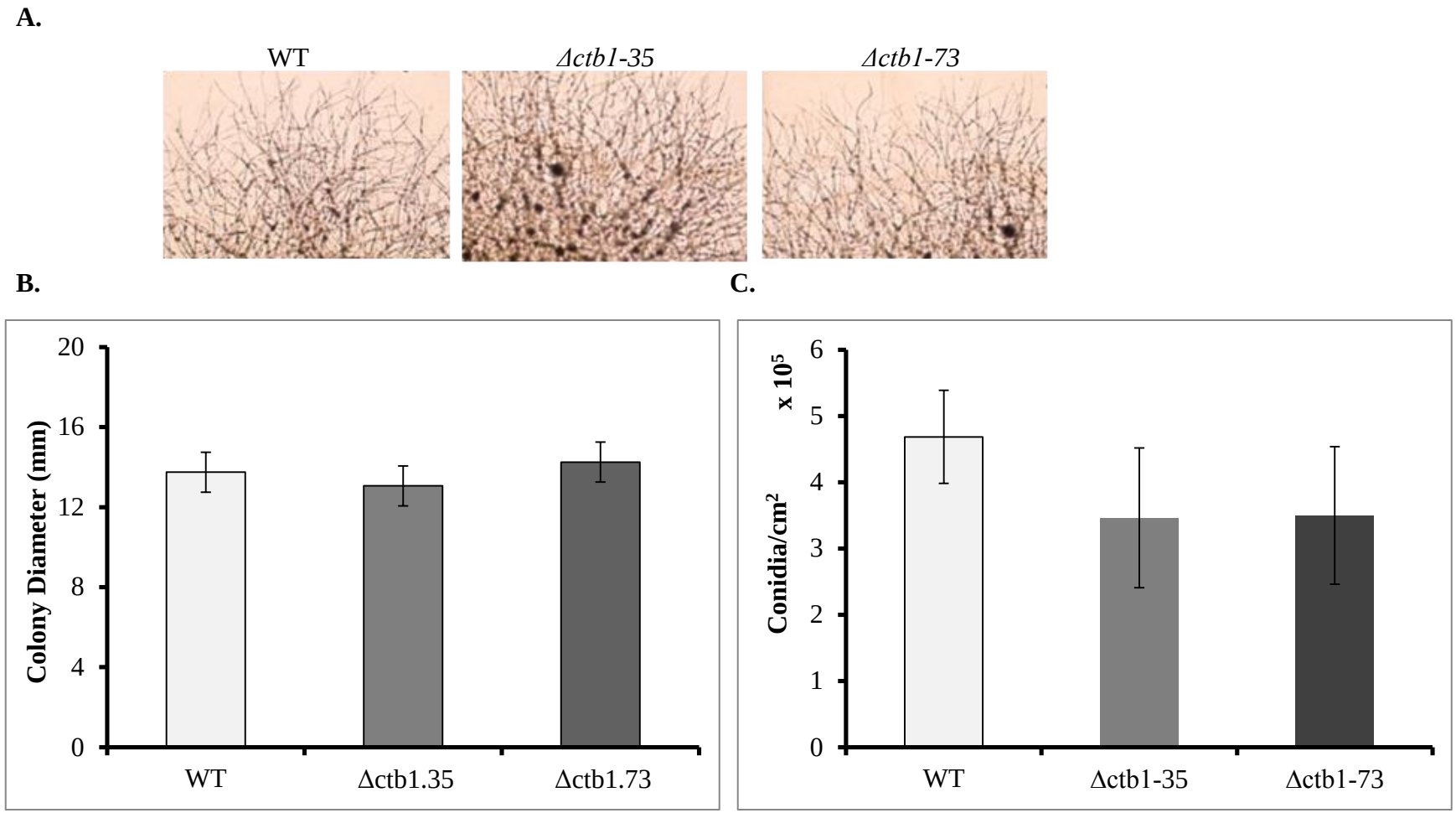

A.
WT
Δctb1-35
Δctb1-73
B.
Colony Diameter (mm)
0
4
8
12
16
20
WT
Δctb1.35
Δctb1.73
C.
Conidia/cm² x 10⁵
0
1
2
3
4
5
6
WT
Δctb1-35
Δctb1-73

Fig. 2.3

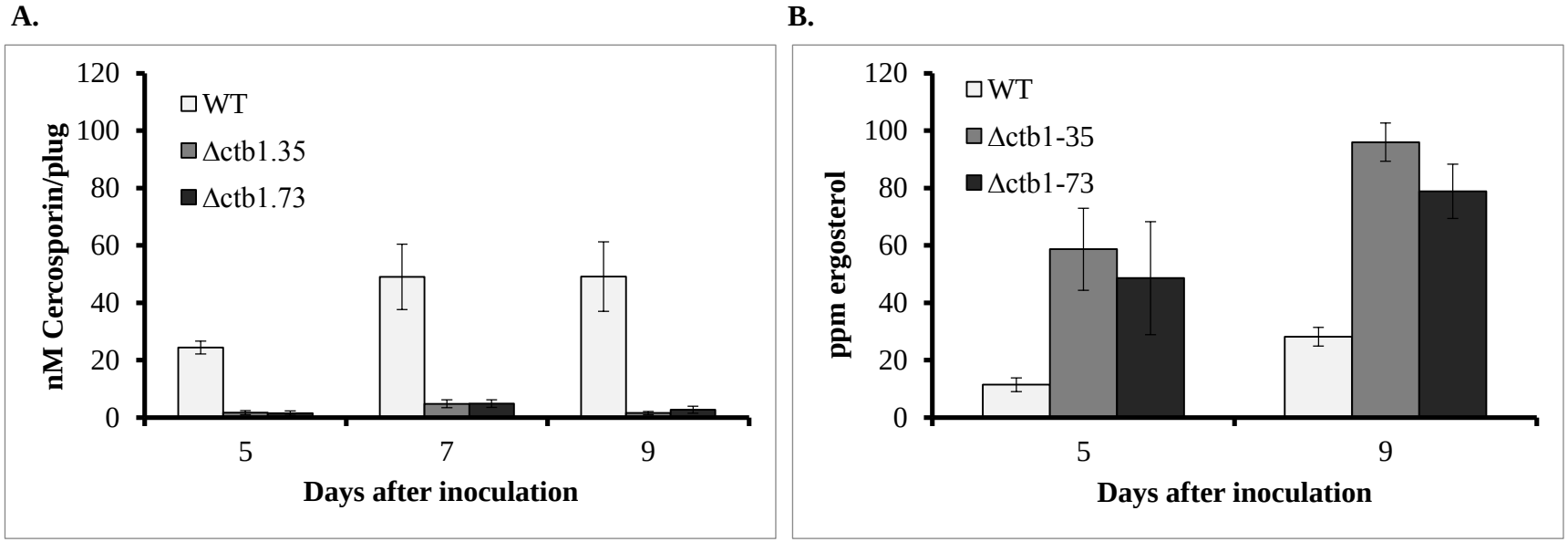
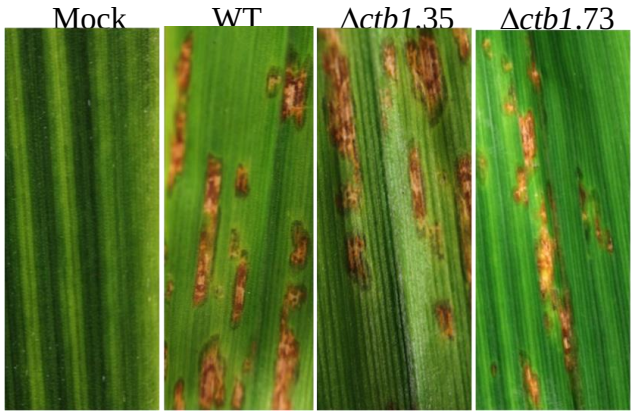
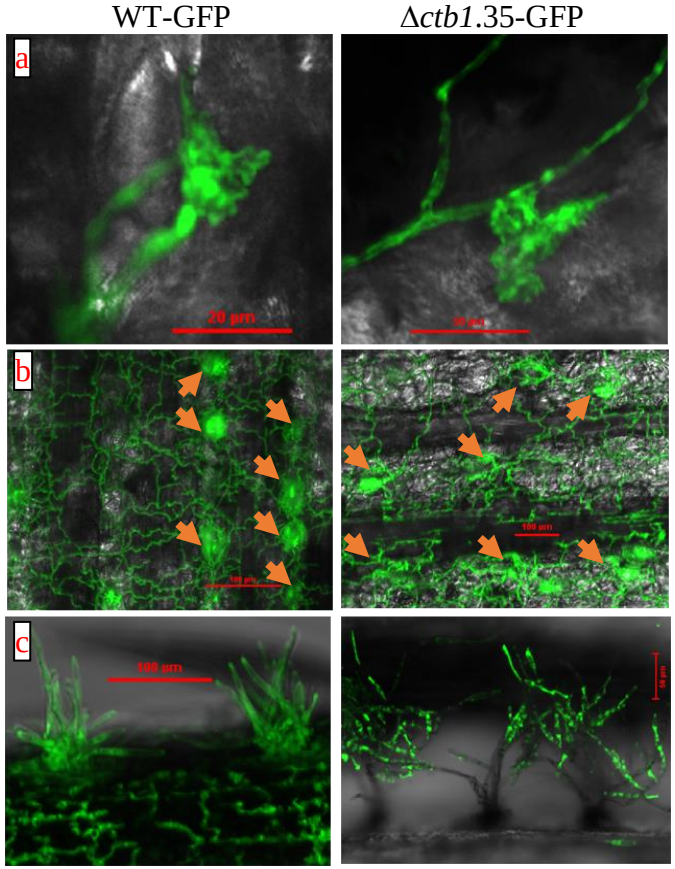

A.
B.
nM Cercosporin/plug
ppm ergosterol
WT
Δctb1.35
Δctb1.73
Δctb1-35
Δctb1-73
Days after inoculation
0
20
40
60
80
100
120
5
7
9

Fig. 2.4

A.


Mock
WT
Δctb1.35
Δctb1.73

B.


WT-GFP
Δctb1.35-GFP
a
b
c

C.

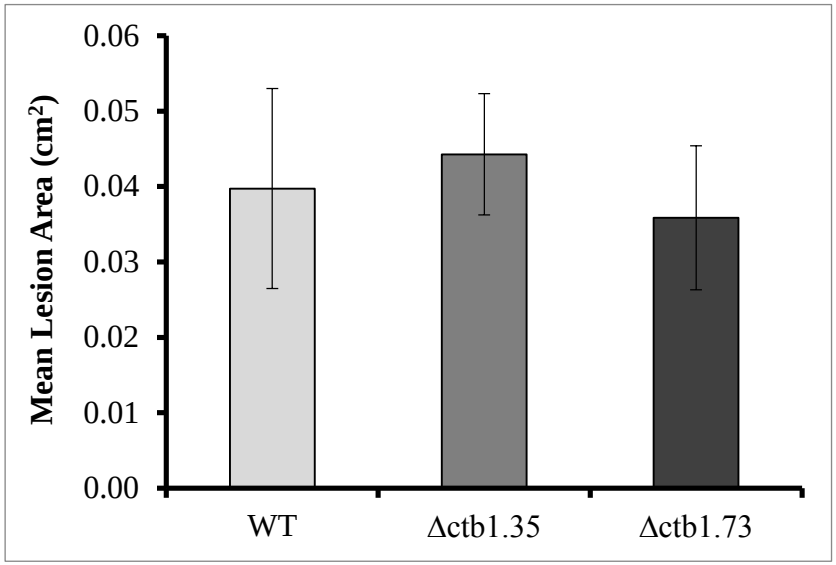
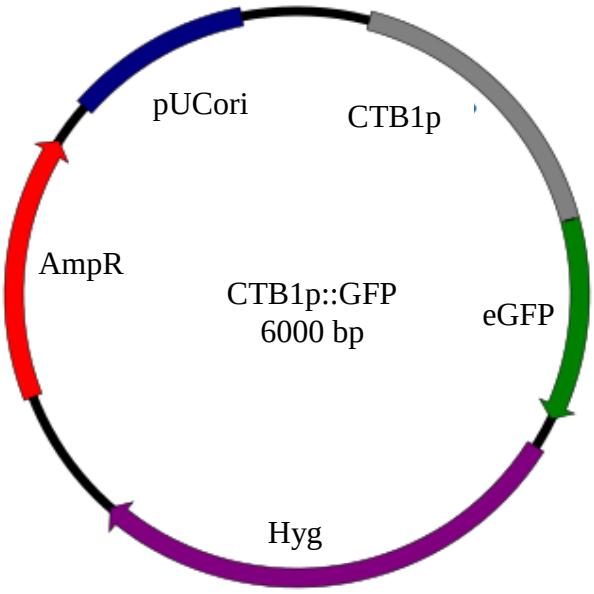

Mean Lesion Area (cm²)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
WT
Δctb1.35
Δctb1.73

Fig. 2.5

A.


pUCori
CTB1p
AmpR
CTB1p::GFP
6000 bp
eGFP
Hyg

B.

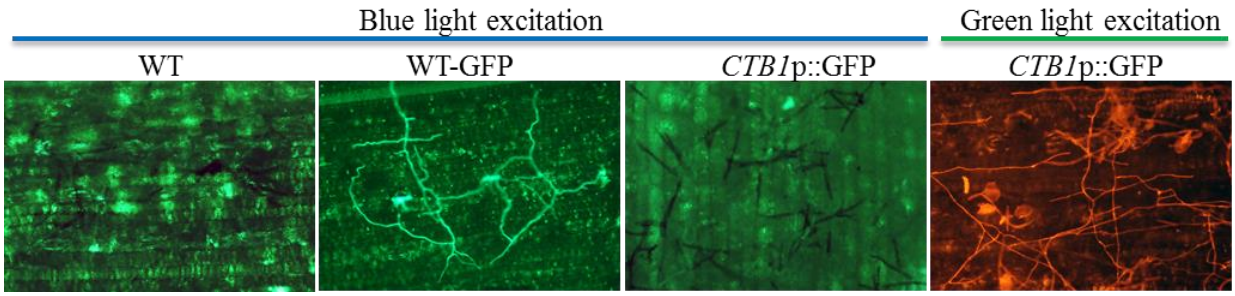

Blue light excitation
Green light excitation
WT
WT-GFP
*CTB1*p::GFP
*CTB1*p::GFP

Fig. 2.6

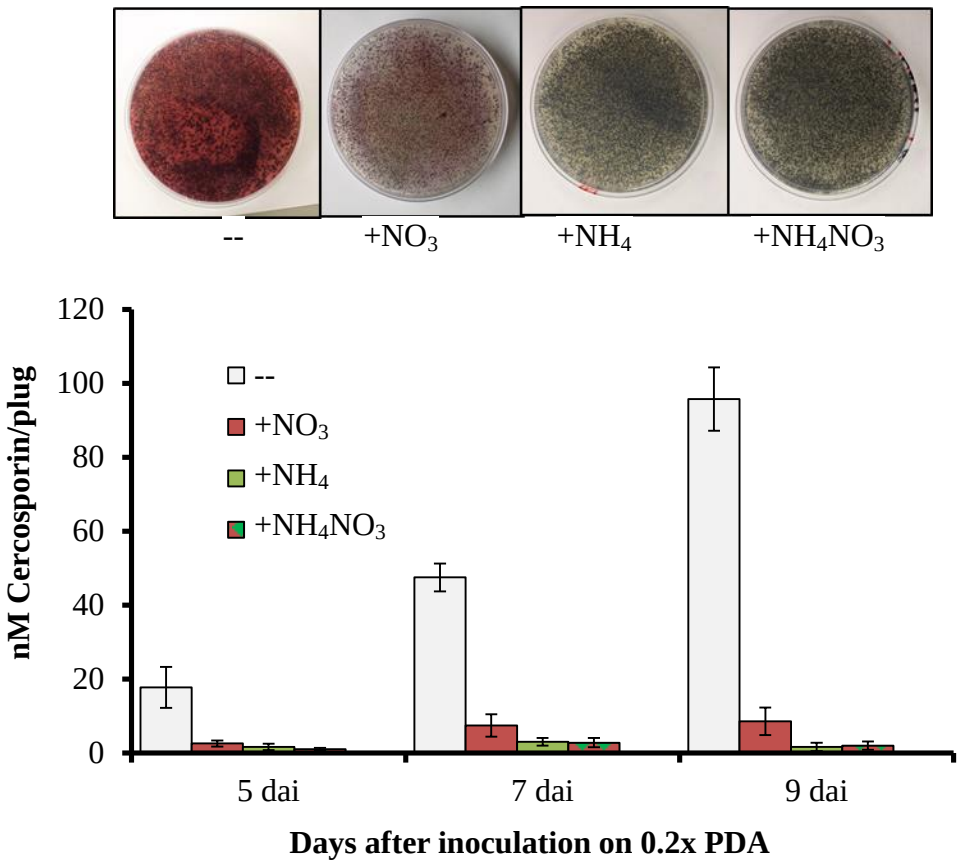

--
+NO3
+NH4
+NH4NO3
120
100
80
60
40
20
0
nM Cercosporin/plug
--
+NO3
+NH4
+NH4NO3
5 dai
7 dai
9 dai
Days after inoculation on 0.2x PDA

Fig. 2.7

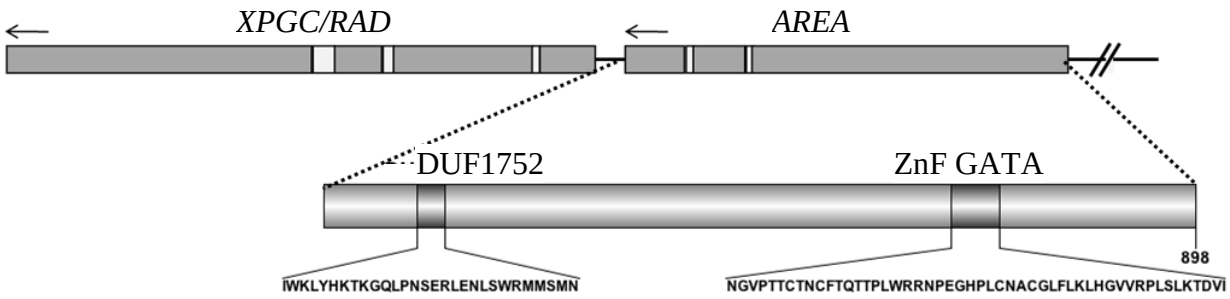
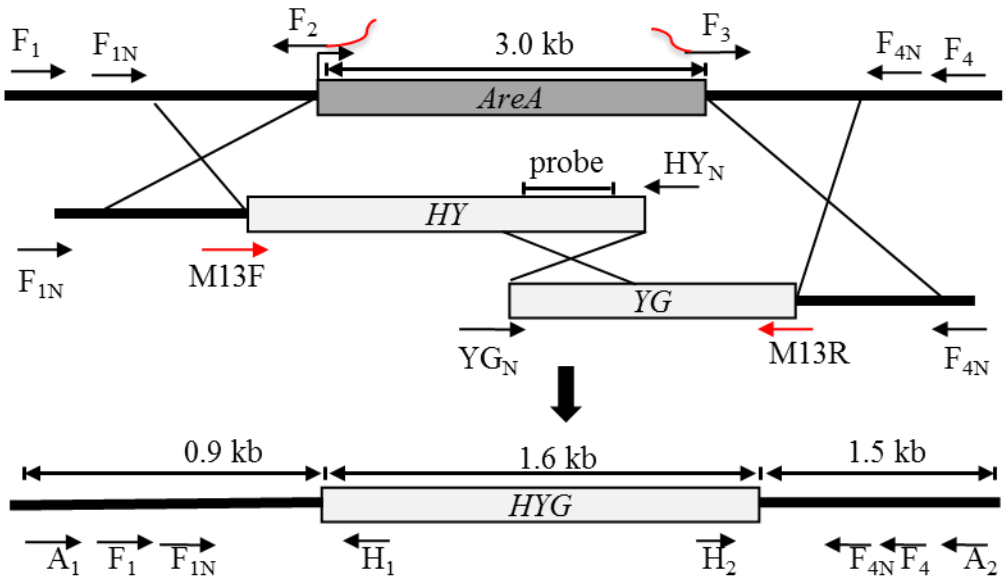
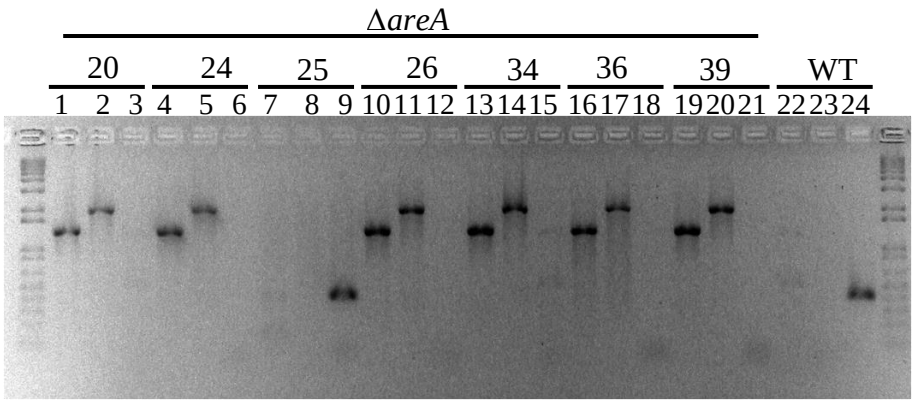

XPGC/RAD
AREA
DUF1752
ZnF GATA
IWKLYHKTKGQLPNSERLENLSWRMMSMN
NGVPTTCTNCFTQTTPLWRRNPEGHPLCNACGLFLKLHGVVRPLSLKTDVI
898

Fig. 2.8

A.


F1
F1N
F2
3.0 kb
F3
F4N
F4
AreA
probe
HYN
HY
F1N
M13F
YG
YGN
M13R
F4N
0.9 kb
1.6 kb
1.5 kb
HYG
A1
F1
F1N
H1
H2
F4N
F4
A2

B.


ΔareA
20
24
25
26
34
36
39
WT
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

C.

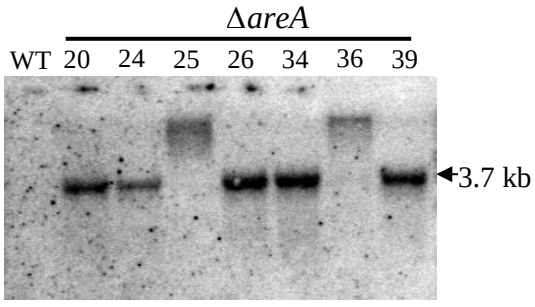
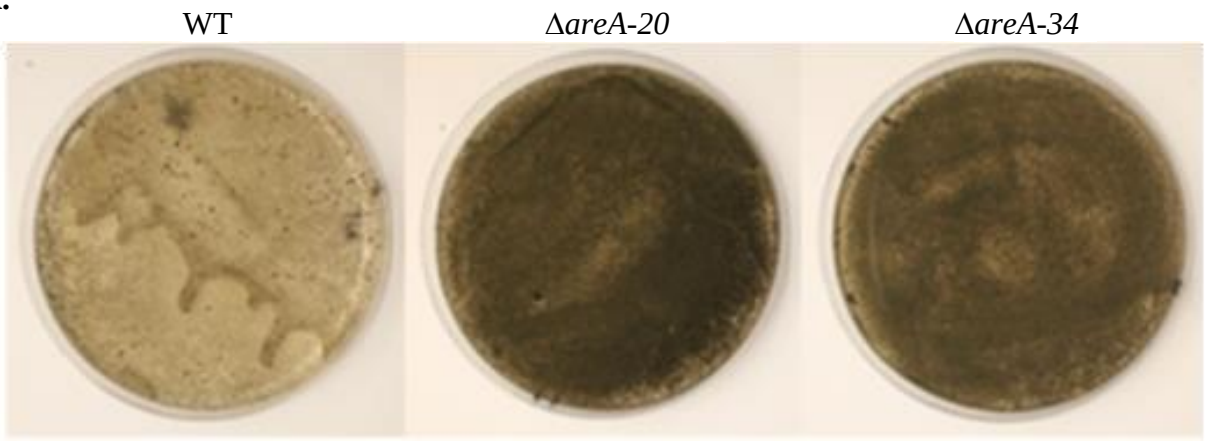

ΔareA
WT
20
24
25
26
34
36
39
3.7 kb

Fig. 2.9

A.


WT
ΔareA-20
ΔareA-34

B.

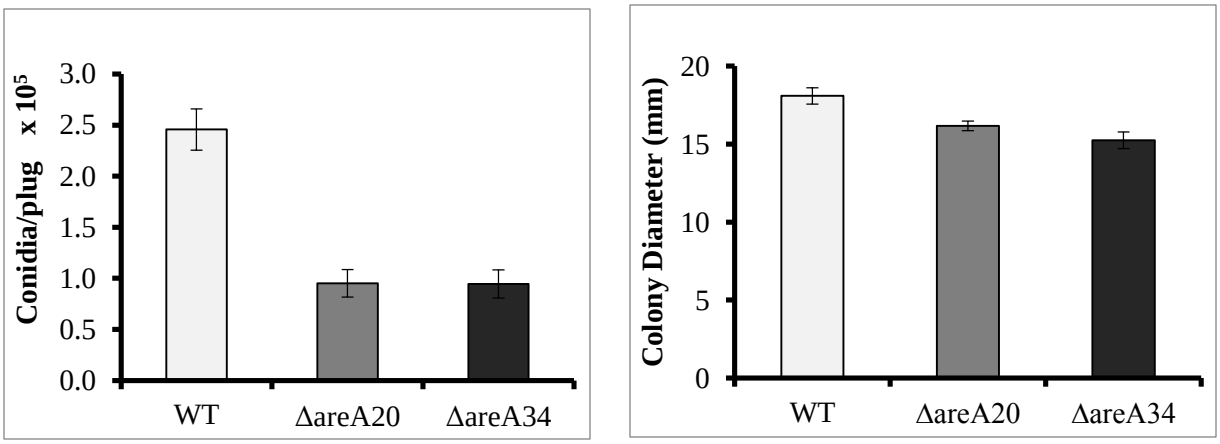
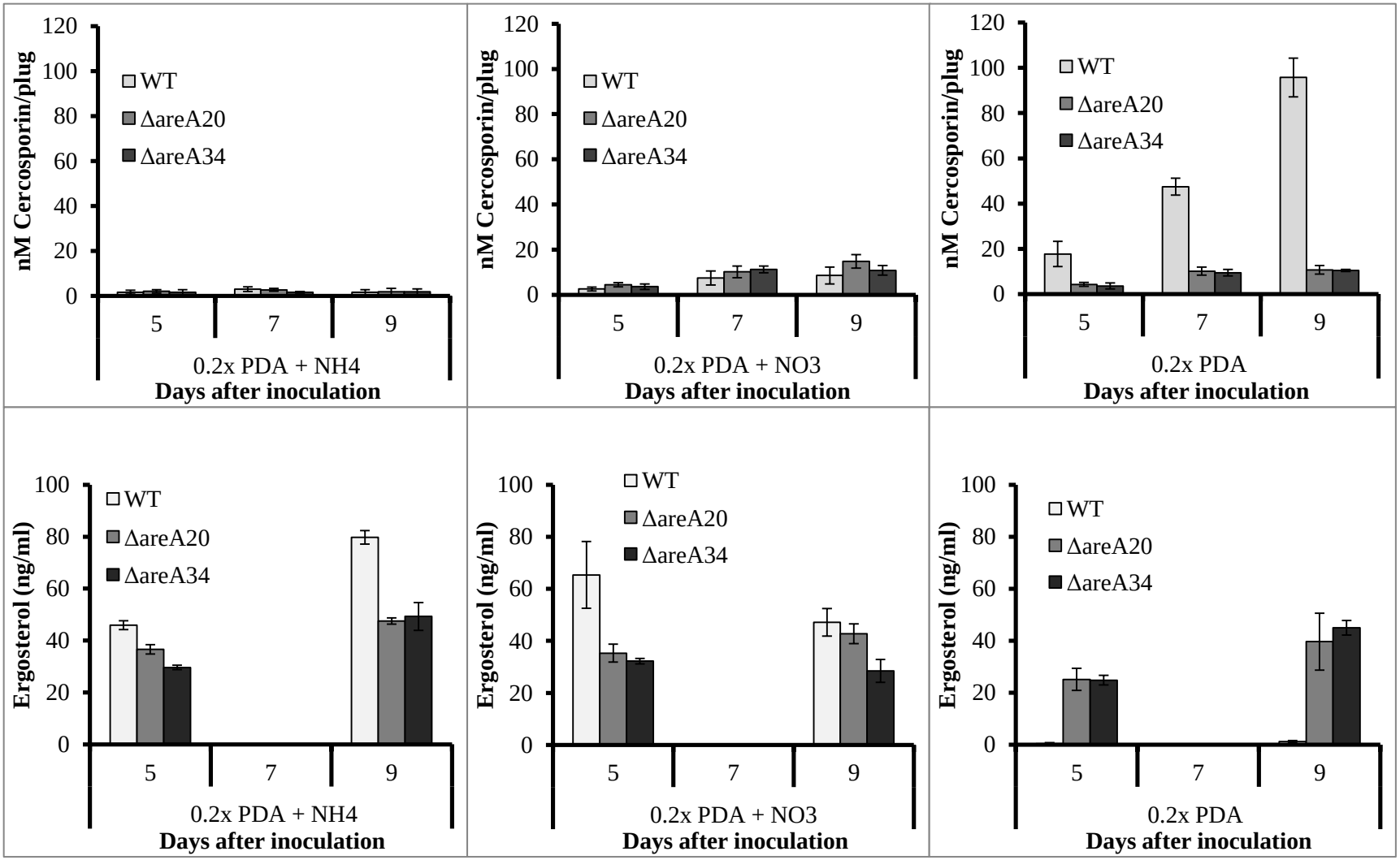

Conidia/plug x 10^5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
WT
ΔareA20
ΔareA34
Colony Diameter (mm)
20
15
10
5
0
WT
ΔareA20
ΔareA34

Fig. 2.10

A.


nM Cercosporin/plug
120
100
80
60
40
20
0
WT
ΔareA20
ΔareA34
5
7
9
0.2x PDA + NH4
Days after inoculation
0.2x PDA + NO3
Days after inoculation
0.2x PDA
Days after inoculation
Ergosterol (ng/ml)

B

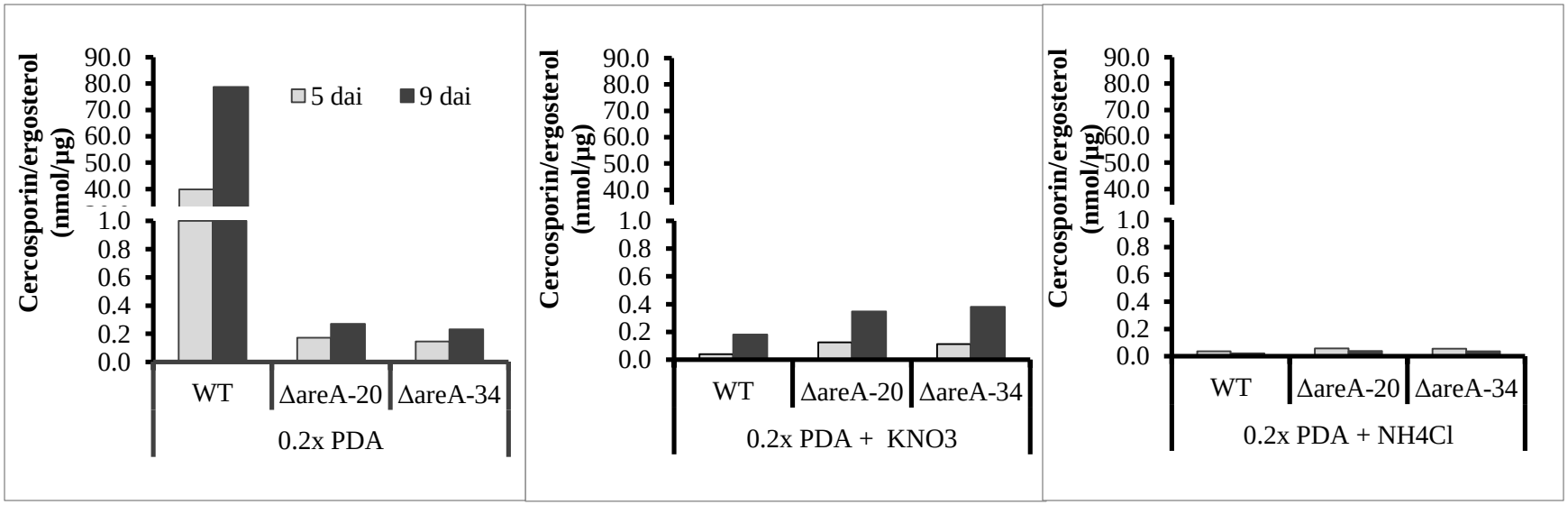
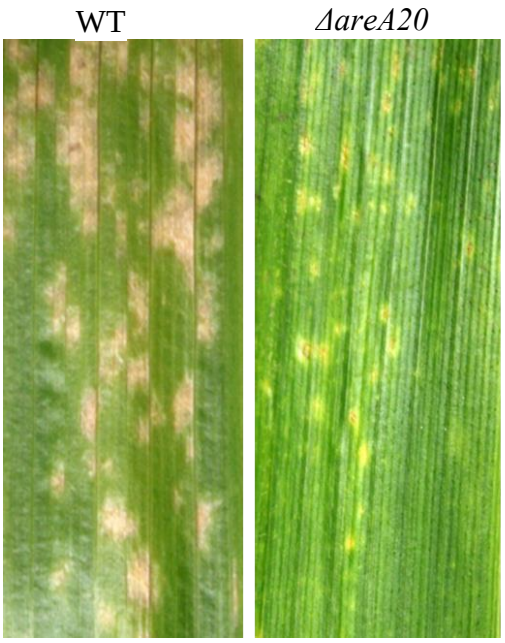

Cercosporin/ergosterol (nmol/µg)
90.0
80.0
70.0
60.0
50.0
40.0
1.0
0.8
0.6
0.4
0.2
0.0
5 dai
9 dai
WT
ΔareA-20
ΔareA-34
0.2x PDA
0.2x PDA + KNO3
0.2x PDA + NH4Cl

Fig. 2.11

A.



B.

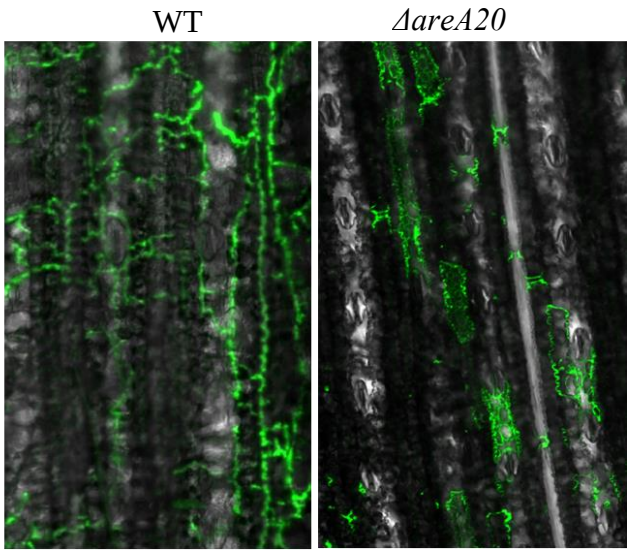


Fig. 2.12

Figure legends

Fig. 2.1

A. Alignment of truncated acyl-carrier protein (ACP) and thioesterase (TE) domains of *C. nicotianae* CTB1 polyketide synthase (accession no. AAT69682) and *Mycosphaerella coffeicola* cercosporin polyketide synthase (accession no. ADO14690) with *C. zeae-maydis* EST sequences. **B.** Diagram of cluster organization showing the order and orientation of genes in the putative *CTB* cluster of *C. zeae-maydis.* Orientation of transcription is indicated by the direction of the arrows. **C**. Full length alignment of conserved domains in *C. zeae-maydis* CTB1 with orthologs in *C. nicotianae* CTB1 and *M. coffeicola*. (β –KAS - β-ketoacyl synthase domain; AT - acyl transferase domain; PP - phosphopantetheine prosthetic group attachment sites of ACP domains; TE - thioesterase domain).

Fig. 2.2

Functional disruption of *CTB1* in *C. zeae-maydis* strain SCOH1-5. **A.** The *CTB1* gene. Schematic illustration of a split *hygromycin phosphotransferase B* gene (*HYG*) maker disruption of the *CTB1* gene in the wild type. Two truncated fragments of *HYG* (*HY* and *YG* with 430 bp overlap region) were amplified from plasmid pTA-Hyg by PCR and were respectively fused to about 550 bp up and downstream sequences flanking the targeted disruption site as described by Fu *et al*., (2006). **B.** Cercosporin production on 0.2x PDA plates in light. Cercosporin accumulates as a visible red pigment in the wild type. Mutants shown (*Δctb1-35* and *Δctb1-73*) did not produce the red pigment. **C.** Southern confirmation of single gene mutation. Southern blot analysis of genomic DNA from the wild type and two mutants: *Δctb1-35* and *Δctb1-73*. Fungal DNA was double digested with *Pvu*II (P) and *Dra*I (D), and hybridized with a *Hyg* gene probe (left panel) and *CTB1*-specific probe (right panel). The membrane was washed at high stringency. Band patterns confirm integration of a single copy of *Hyg* cassette at the targeted disruption site of the *CTB1* gene.

Fig. 2.3

Growth and development of *CTB1* mutants. **A.** Colony margin morphology of wild type strain and mutants on a thin 0.2x PDA plates. **B.** Radial growth on 0.2x PDA. Colonized agar plugs (1 cm diameter) were cut from sporulating V8 agar plates and padded on new 0.2x PDA plates. Radial growth was then measured by measuring colony diameter in mm 10 dai **C.** Conidiation on V8 juice agar 5 dai. Conidiation on V8 juice agar was measured by counting of spores with a haemocytometer. Data plotted are the means from three experiments. Error bars represent confidence intervals at 95%.

Fig. 2.4

Production of cercosporin by the Δ*ctb1* mutants and WT on 0.2x PDA. **A.** Quantitative measure of cercosporin production of wild type compared with mutants. Cercosporin was extracted from colonized 5 mm radii 0.2x PDA agar plugs with 5M KOH for 5 hrs in the dark. The absorbance of the extract was measured at 480 nm and converted to nM cercosporin using the molar extinction coefficient of cercosporin in alkali (23300 L mol^{-1} cm^{-1}) at 480 nm. Data shown are means and 95% confidence intervals of three different experiments with three replication of each treatment. **B.** Biomass was measured by ergosterol concentration. Ergosterol was extracted from mycelia of strains grown on cellophane membrane overlaid 0.2x PDA plates inoculated with conidia and incubated under constant light. Data plotted are the means and 95% confidence intervals as error bars of two experiments with two replications in each experiment.

Fig. 2.5

Pathogenicity assessment of *CTB1* mutants on maize leaves. **A.** Leaves of maize plants at V6 stage were inoculated with about $x10^5$ conidia suspension of the wild type and the Δ*ctb1* disruption mutants (Δ*ctb1-35* and Δ*ctb1-73*) on the abaxial surface. Plants were grown at 23^oC in high relative humidity, and disease development was observed between 5 and 20 dai. Pictures shown are representative leaf sections. **B.** Stages of pathogenesis exhibited by GFP expressing WT and mutant. [a. appressoria over stomata. b. mycelia in a colonized leaf section with aggregation in stomatal regions as shown in orange arrows. c. conidiophores emerging from stomata in lesion regions]. **C**. Virulence was determined by measuring average lesion area on three replicate leaf sections in each experiment. Data plotted are the means and 95% confidence intervals as error bars.

Fig. 2.6

Regulation of *CTB1* during pathogenesis. **A**. *CTB1* promoter fused to a GFP reporter construct to monitor the expression of *CTB1 in vivo*. **B**. Evaluation of *CTB1*p::GFP reporter strains *in planta* for *CTB1* promoter activation.

Fig. 2.7

Effect of nitrogen on cercosporin biosynthesis in *C. zeae-maydis.* The growth medium (0.2x PDA) was supplemented with nitrogenous salts and inoculated with the WT. Cercosporin biosynthesis was determined by extraction with 5.0 M KOH from agar plugs sampled from the plate at five, seven , and nine days after inoculation.

Fig. 2.8

Genomic region of *C. zeae-maydis* coding for the protein AreA and domain structure of the encoded protein. Structure shows conserved Zn-GATA and DUF domains.

Fig. 2.9

Deletion of *AREA* in *C. zeae-maydis*. **A**. Schematic illustration of a split-marker *hygromycin phosphotransferase B* gene (*HYG*) maker deletion of *AREA*. Two truncated fragments of *HYG* (*HY* and *YG*) were amplified from plasmid pTA-Hyg by PCR and fused respectively to 730 and 980 bp up and downstream sequences flanking the targeted *AREA* gene. The two truncated *HYG* deletion fragments, fused with upstream or downstream sequence of the targeted gene, were directly transformed into protoplasts of *C. zeae-maydis* strain SCOH1-5. **B.** PCR screen to select mutants. PCR was performed with primer pairs A1/H1 to verify the integration of HY fusion deletion construct at the targeted site. A 1,363 bp band in lanes 1, 4, 7, 10, 13, 16 and 19 is indicative of correct insertion of the deletion construct upstream of the *AREA* gene translation start codon, while primers A2/H2 amplify a 2,014 bps sequence that was used to verify the integration of deletion construct at the 3' site of insertion. The presence of a band in lanes 2, 5, 8, 11, 14, 17 and 20 is indicative of correct insertion at the downstream targeted site. **C.** Southern-blot analysis of genomic DNA from the WT and putative Δ*areA* knockouts (20, 24, 26, 34, 36, and 39) identified in the PCR screen. Genomic DNA was digested with *Avr*II and *Age*I, resolved on 0.8% agarose gel by electrophoresis and blotted onto nylon membrane. Hybridization was performed with a *Hyg* probe to eliminate mutants with ectopic insertion of the disruption constructs.

Fig. 2.10

A.Growth phenotype on 0.2x PDA supplemented with $KClO_3$ 7 days after inoculation. **B.** Conidiation on V8 juice agar 5 dai. Conidiation on V8 juice agar was measured by counting spores with a haemocytometer **C.** Radial growth on 0.2x PDA. Colonized agar plugs (1 cm diameter) were cut from sporulating V8 agar plates and padded on new 0.2x PDA plates. Radial growth was then measured by measuring colony diameter in mm 10 dai.

Data shown are the means and confidence intervals at 95% (error bars) of all experiments.

Fig. 2.11

Effect of *AREA* deletion on cercosporin biosynthesis. **A**. WT and Δ*areA* mutants were grown on 0.2x PDA with or without added nitrogen salt and incubated under constant light for up to 9 days. Cercosporin was extracted with 5 M KOH. Absorbances was measured at 480 nm and

converted to nM cercosporin using the molar extinction coefficient of absorbance in alkali (23300 L mol^{-1} cm^{-1}). Growth was measured by total ergosterol extracted from mycelia grown on solid media overlaid with cellophane membrane. Data plotted are the means and 95% confidence intervals as error bars of two experiments with two replications in each experiment. **B.** To enable comparison between assay conditions, cercosporin biosynthesis was normalized to fungal growth.

Fig. 2.12

Maize leaves were inoculated with conidial suspensions of the wild type and mutant strains. Inoculated plants were maintained at 23^{o}C in high relative humidity for 18 days for lesions to form. **A**. Necrosis on leaves inoculated with *AREA* deletion mutants. **B**. *AREA* deletion mutants exhibited limited intracellular growth. Intracellular growth was restricted to the small regions of necrosis.

APPENDICES

Appendix 2.1

Table A2.1: List of screening primers and cosmids with target *CTB1* or *AREA* sequences

Primer ID	Primer sequence	Target cosmid		
		6C 4(M-P)	4A 13(I-L)	10H 11(E-H)
CTB1pF	CGTCACGCATCATGCTGAAGTG	x	x	
CTB1pR	TTTCGACACTACTCCTCATCGACTCG	x	x	
areAF1	GGAAAGAACTGGAAAGGCAGGGA			x
AreArtF	ATGGCTGAGGCAGCCCTACATAATT			x

>834_1_CBYB_CBYC_CBYF_CBYG_CCAW_EXTA (*CTB1* EST cluster 834 complement)

GGGATATCACCATGGTTTCATTCGAACGCCGAACTAGAATACTATCACGAAGAAACAATACCTAAACC
TTCTCGCATATGCTCAACGAGTATCCGTGCACCGG**CGTCACGCATCATGCTGAAGTG**GTGCTCGCTC
TCAACAACTCTCGCGACCACATCGTTGCCAGGGAACAGTTTTTCCCACTCCGTTGGCCCGAAATCTTTC
CGCTTCTCCGTCAAGAACTTCATACCTTCAGTGTCTTCGTCGGTATCACCTGCAGATGGAGGCAGATGA
GCGTAGCGCACACCGTCAAACGCACACTCGCCTGCCCATATCAGCATGACTTTCGTCTTGTGGCCAGG
CTTCATGGGAGGAGCGTGATAATCATGCAGCAGCTCAATAGAAGCTCGAAAGTGCGGCATAAGCCATT
CCGGAGTCTTGCTGGGAACACCGCTGCCTCTTGACAACTCGGTTCCAAATAGTCCTACATTTGTGCAAT
GATCAAAGAACCGTGTTGGAAGGCGATCCAGTCCTTTGATAGGCGACGG**CGAGTCGATGAGGAGTA**
GTGTCGAAATCTCCTCGCCGGCGGCGATCAATTCTTGGGCCACGGCGTAAGCCAGAATGCCACCAGC
AGACCAGCCGCCGAGATGATATGGCCCGTGCGGTTGACGGCCTC

>834_0_CBYC1040.b1_CBYB_CBYC_CBYF_CBYG_CCAW_EXTA (*CTB1* EST cluster 834)

GTCCTTCACCGATGTGGGTGTCGACTCGCTGATGTCGCTTGTCATTACGAGTCGCTTGCGCGATGAGTT
GGATATCGACTTTCCAGATCGAGCATTGTTTGAAGAATGCCAGACAATATCCGACCTCCGCAAAAAGT
TCTCTTTGCCAACGGCATCTTTGGACTCCACGACGACCAAGTCCAACGCTGCTGATACGACGCCACCTC
TCACCGATGCCAGCTCATCTTCTCCGGCGTCTTCCGTGTACGAGGGCGAGACCCCAATGACTGATTTGG
ACGACGTCTTCGACAGCCCTCCGAGTCAGAGGAAACCAGCTCCGCCAAAGCAACAGATTCCACCTTCA
TGGTCGATGTATTTGCAAGGC

>3591_0_CBYB879.b1_CBYB_CBYC_CBYF_CBYG_CCAW_EXTA (*AREA* EST)

AGCGCCTGGAGAATCTCTCGTGGCGGATGATGTCCATGAATTTGAAGC**GGAAAGAACTGGAAAGGC**
AGGGACTGGCCGCTCGACCGCTCAATCCTGCGCAGAATGCCCCAAGCGGCATTGCGCAGCTGCGGAA
GTCCTCCGAGCACAGCGGCCCAGCGGCCCAGCAGCAGGCAGCCGCTGATGATCAGATGAACCTTGAC
GAGTTCCTCGAACCGTCATCGATTGGAAGTCCTGCTGGCATCTCGCCCTCGCCCGCTCCCGAGCACTCG
GCGACAGTCAGCGCATCAGCGATTCCGATCAAGCAGCAGCAGCGCCTGCAGAATCACGAGCTATCCGT
AGGGCGCGCCTCAGCACCTTCCGTCCCGCCACTCGAGCAGAGTCGGACCCACCGCGAGTTTTTCTACG
TGCAGCGACACGTGCGCAAGACCAGCATTGATGAGCGAAGGCCCCCAAAACGGCGTGCCGAAGCATC
GCCCCAGGTGCCTCCGGTGCAGAACAGCACTCTCGTAGCGCAAGATCCC**ATGGCTGAGGCAGCCCTA**
CATAATTACACGCTGGACGCTCAGCCGCAAGCACCACACGACGCCCTACCATTCAACCTCGAGACCTT
CAACATTGACAACGATCCCATCCTCAACTCCGCCGGGCCCCTGCAACAGCATTTCTCCTTTTCTCCCGT
TGGGTCTCCGATGATGACC

Screening primer locations are shown in bold and underlined characters.

Table A2.2: List of primers and cosmids sequenced for *CTB* cluster and *AREA*.

Primer ID	Primer sequence	Cosmids sequenced		
		6C 4(M-P)	4A 13(I-L)	10H 11(E-H)
CTB1-F1N	CGCTTGCGCGATGAGTTGG	3’	3’	
CTB1-F4N	ATGCTCAACGAGTATCCGTGCAC	5’	5’	
CTB1W1-3	GTGCACGGATACTCGTTGAGCAT	3’	3’	
CTB1W1-5	CCAACTCATCGCGCAAGCG	5’	5’	
CTB1W2-3	GTTCGCTGCCCACATCCCATTAC	3’	3’	
CTB1W2-5	TCCAGGTCCACTCCCTCGTTC	5’	5’	
CTB1 W3-3	CCTCAATGGACGGGAACGCA		3’	
CTB1 W3-5	CTTCAAGGTCCAGCCATAAGCTG	5’	5’	
CTB1 W4_3	AGTCCTGTGGAAAXAGTCAGCA		3’	
CTB1 W4_5	GGAGGGTCATTCGCTCGCAG	5’	5’	
CTB1 W5-3	TGACGAACGAAGCCATTGCA		3’	
CTB1 W5-5	TCCCGTGCATCTCGCATACAG	5’	5’	
CT1W6-3	GAAGGTCACATAGGGCCGCT		3’	
CTBW6-5	CACGTTCTTGGCAGTGGGATCT	5’	5’	
CTBW7-3	CAGTCTTCCGCGTATCGAACCT		3’	
CTBW7-5	CGTACACTGCGATGCGCTC	5’	5’	
CTBW8-3	CGTGACTCCAGCCATTGTCC		3’	
CTBW8-5	CGGCATCTTTCCTCGCACA	5’	5’	
CTBW9-3	GGAAGCCGATGAAGCCAGTAG		3’	
CTB1W9-5	ATCGTGGGGCGAGACTGT		5’	
areAF1	GGAAAGAACTGGAAAGGCAGGGA			
AreArtF	ATGGCTGAGGCAGCCCTACATAATT			
AreAFW1	CCGTTGGGTCTCCGATGATG			
AreAFW2	TCCCAACAACCTGCACCAAC			
AreARW1	TCCCTGCCTTTCCAGTTCTTTCC			
AreARW2	GCCGCAGCCACAGTATCAAC			

5’ and 3’ in the table indicate the direction of sequencing with the corresponding primer.

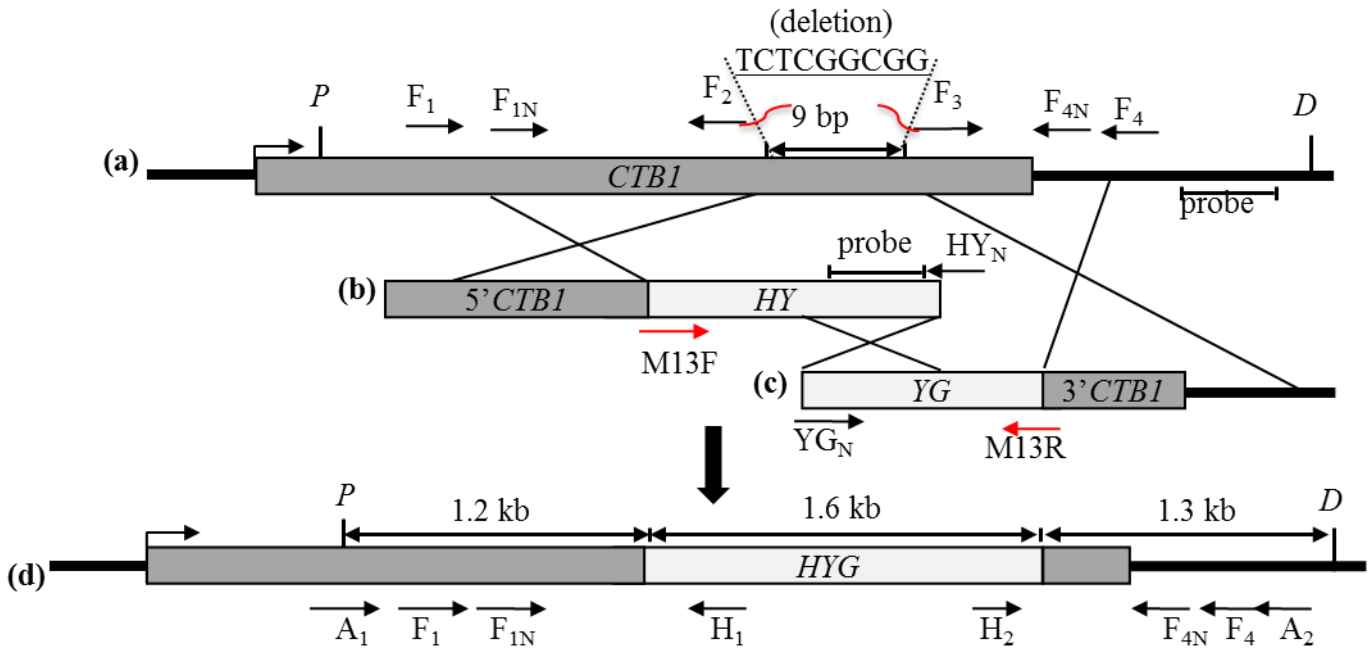
Total *CTB* cluster sequences obtained by cosmid walk = ~15,537 bp.

Total *AREA* sequences obtained cosmid walk = 2,780 bp.

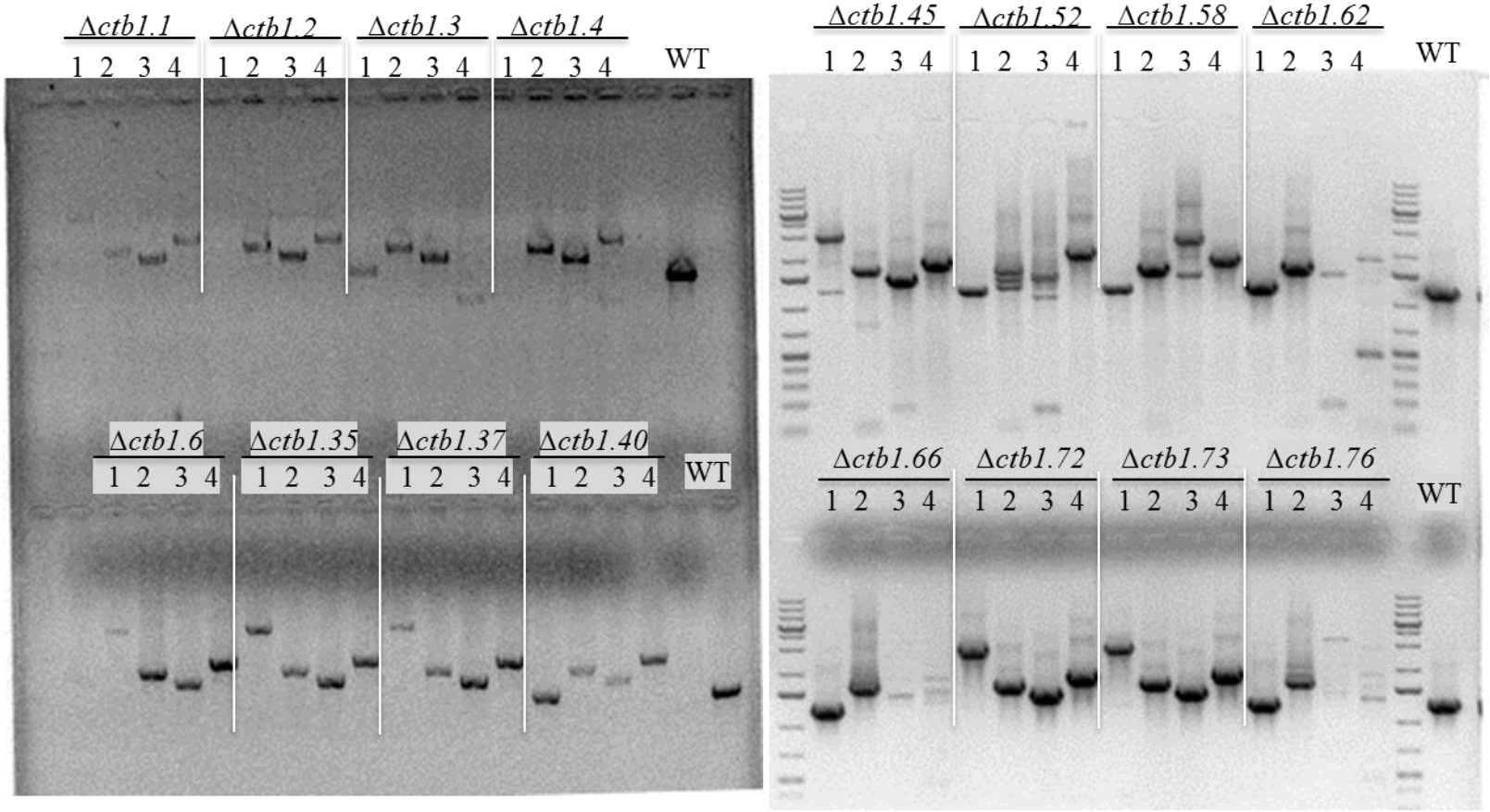
Table A2.3: List of *CTB1* and *AREA* gene disruption and PCR validation primers

Primer Name	Primer sequence
CTB1-A1	GTCCTTCACCGATGTGGGTGTC
CTB1-A2	CTGCGTGATGGGTCTGAGGA
CTB1-A3	GCAAATCCTACGCCCGATGAA
CTB1-F1	GCTGATGTCGCTTGTCATTTACGAGT
CTB1-F1N	CGCTTGCGCGATGAGTTGG
CTB1-F2	**ATTACAATTCACTGGCCGTCGTTTTAC**TGATATGGCCCGTGCGGTTG
CTB1-F3	**CGTAATCATGGTCATAGCTGTTTCCT**CTGGTCTGCTGGTGGCATTCT
CTB1-F4	AACAATACCTAAACCTTCTCGCATATG
CTB1-F4N	ATGCTCAACGAGTATCCGTGCAC
CTB1pF	CGTCACGCATCATGCTGAAGTG
CTB1pR	TTTCGACACTACTCCTCATCGACTCG
ctb1pF1	CGCATAACGCAGGGCAGTAGTC
ctb1pR1	GCTTCTCGCATCGTAGTATCCCT
AreA_A1	CGAAGGCGAGTGTCGAGAGA
AreA_A2	ATCATCGTCGCTGCTCCCTA
AreA_F1	CTCCCCAGCAATCCGAGTC
AreA_F1N	TGTGGTCAGCGTGCTCATCT
AreA_F2	**ATTACAATTCACTGGCCGTCGTTTTAC**GAGGTGCAAAGAGGGTGGTG
AreA_F3	**CGTAATCATGGTCATAGCTGTTTCCT**CTGTCAGTCGCAGGGTTGTG
AreA_F4	CACCCATTACCCTCCCACCT
AreA_F4N	ATTCGCAGCCGACTTCTCAG
M13F	**GTAAAACGACGGCCAGTGAATTGTAAT**
M13R	**CAGGAAACAGCTATGACCATGATTACG**
HY	GGATGCCTCCGCTCGAAGTA
HY-N	TAGCGCGTCTGCTGCTCCATACAAG
YG	CGTTGCAAGACCTGCCTGAA
YG-N	ACCGAACTGCCCGCTGTTTCTC
H1	CTACTGCTACAAGTGGGGCTGA
H2	GTACACAAATCGCCCGCAGAAG

A.

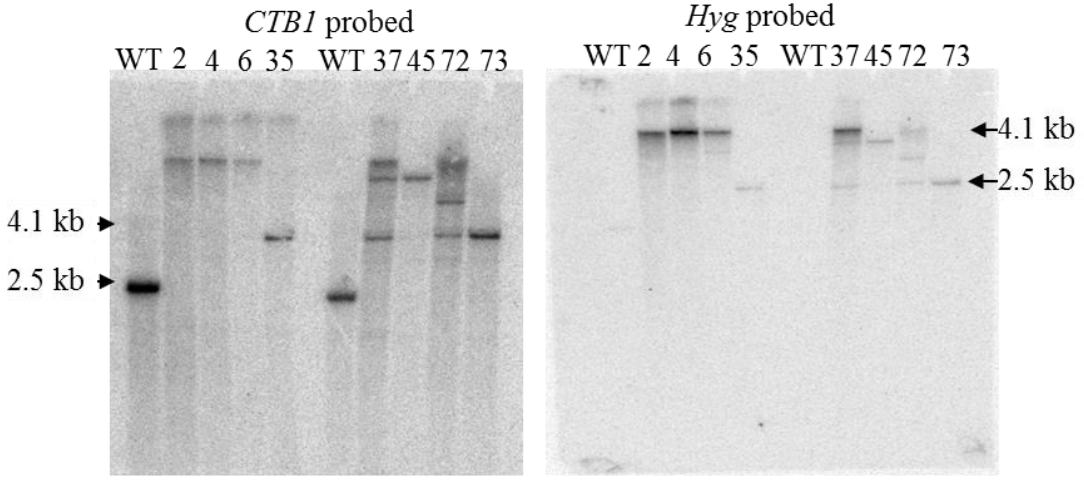

(deletion)
TCTCGGCGG
P
F1
F1N
F2
9 bp
F3
F4N
F4
D
(a)
CTB1
probe
probe
HYN
(b)
5'CTB1
HY
M13F
(c)
YG
3'CTB1
YGN
M13R
P
1.2 kb
1.6 kb
1.3 kb
D
(d)
HYG
A1
F1
F1N
H1
H2
F4N
F4
A2

B.

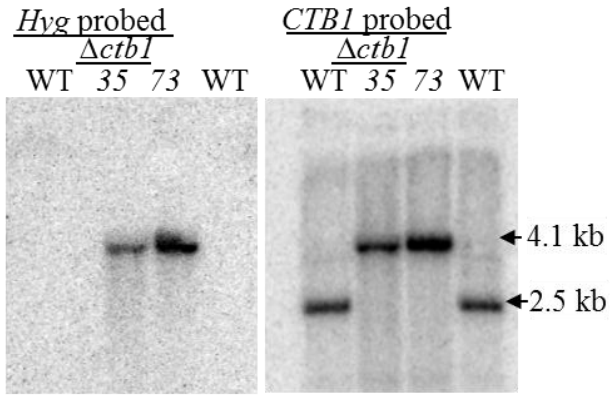


Primers	Lanes	Expected sizes (Δ*ctb1*)	WT
A_1/F_4	1	3.0	1.2
M13F/R	2	1.6	--
F_4/YG_N	3	1.5	--
$A1/HY_N$	4	2.0	--

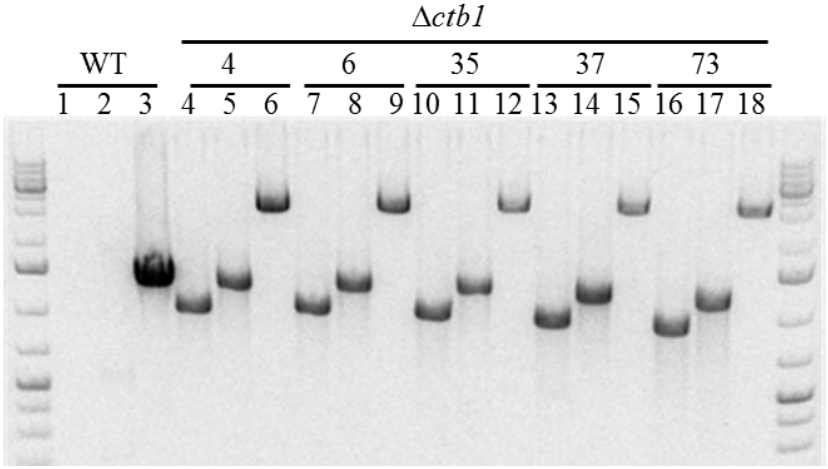
C.



E.

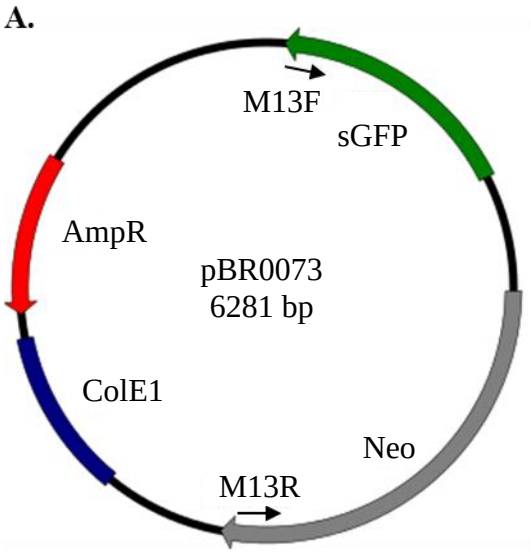


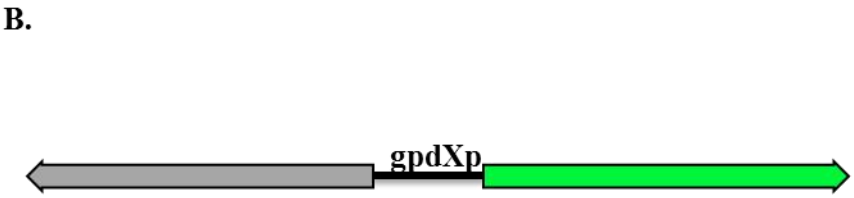
D.



Primers	Expected size (kb)		Lanes
	Δctb1	WT	
A_1/H_1	0.98	--	1, 4, 7, 10, 13, 16
A_2/H_2	1.2	--	2, 5, 8, 11, 14, 17
A_1/A_2	3.1	1.5	3, 6, 9, 12, 15, 18

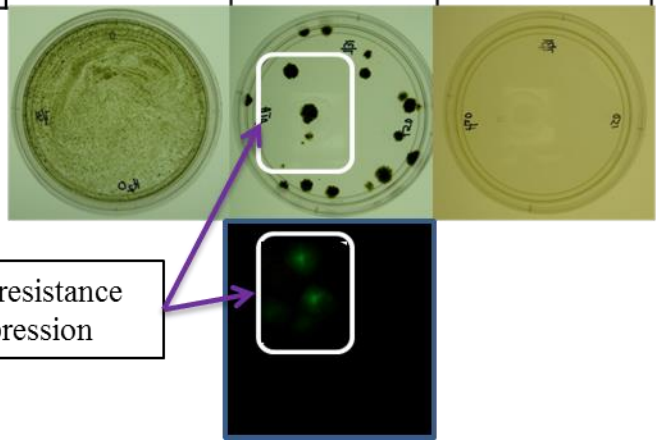
Appendix 2.2



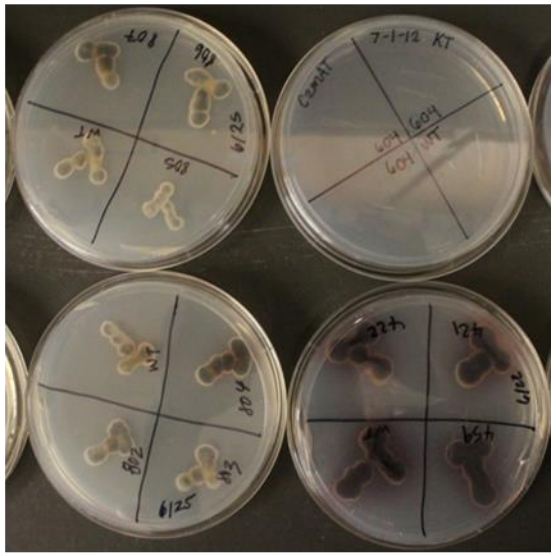


C.

Vector (0.1 µg/µl protoplast)	+	+	-
Geneticin (200µg/ml)	-	+	+

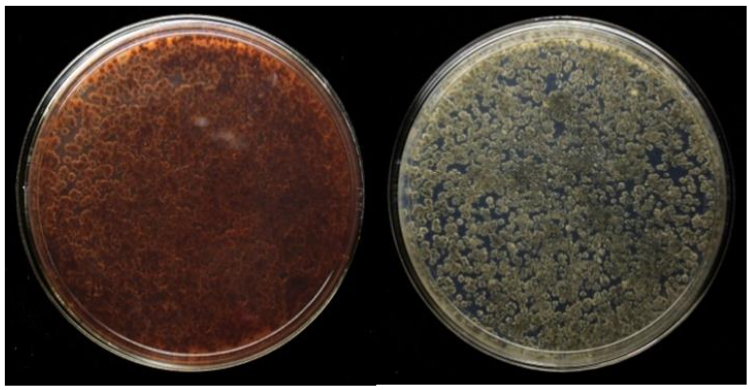


D.

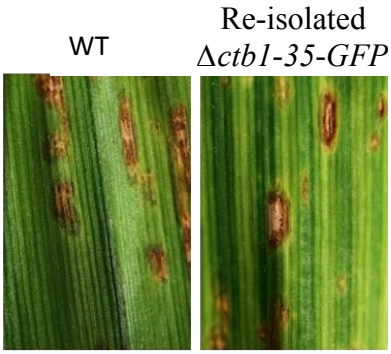


Appendix 2.3

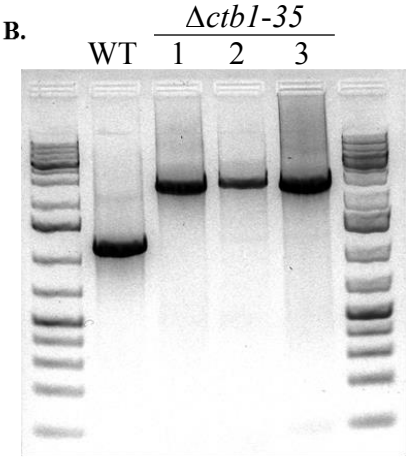
A.



C.



B.



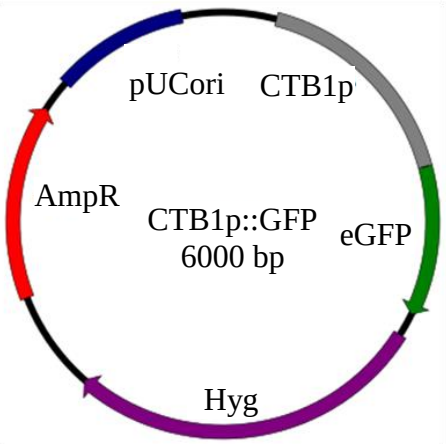
Primers	Expected size (kb) Δ*ctb1*	WT
A_1/A_2	3.1	1.5

Lane

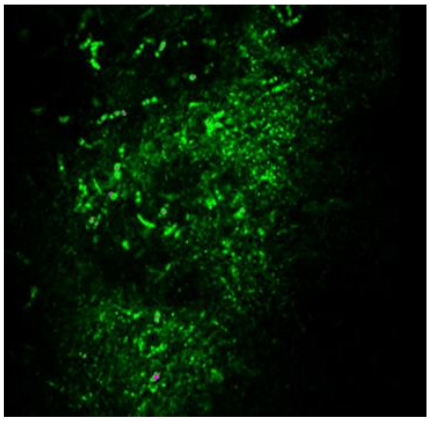
1. -- Δ*ctb1-35*
2. -- Δ*ctb1-35-GFP*
3. -- Δ*ctb1-35-GFP* re-isolated from lesion

Appendix 2.4

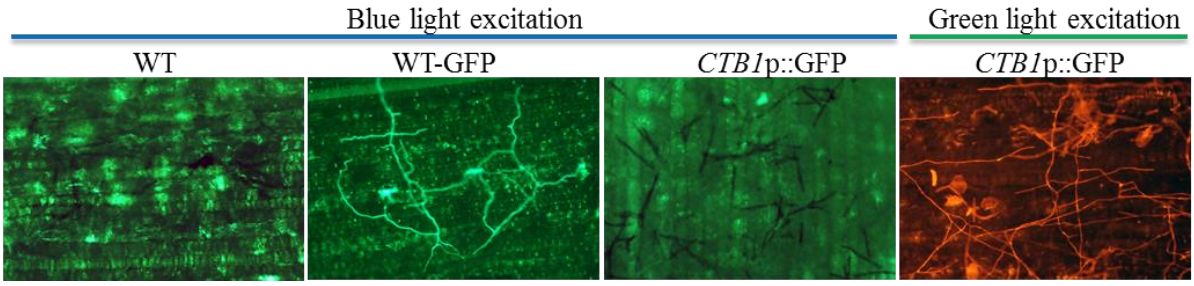
A.



B.



C.



Appendix 2.5

Appendix: 2.6

Table A2.4: List of random insertional mutants and % cercosporin biosynthesis.

	Random mutant Strain	Relative Cercosprin (% of WT)
1	AT1368	3.14
2	AT1108	3.26
3	AT1289	3.76
4	AT773	5.70
5	AT515	7.09
6	AT1164	7.60
7	AT1371	8.04
8	AT1398	8.53
9	AT1159	10.66
10	AT669	11.26
11	AT1193	12.05
12	AT873	12.27
13	AT1200	22.89

	Random mutant	Relative Cercosprin (% of WT)
14	AT1337	25.70
15	AT837	40.37
16	AT665	44.68
17	AT1396	45.29
18	AT1114	46.73
19	AT1374	47.06
20	AT1216	48.49
21	AT1384	55.50
22	AT1370	61.94
23	AT686	77.90
24	AT771	90.60
25	AT1318	x
	WT	100.00

Percentage of biosynthesis was calculated by dividing nM cercosporin of the mutant by nM cercosporin produced by the wild type.

Legends to Figures in Appendices

Appendix 2.2

A. Split-marker gene deletion scheme. The split marker disruption fragments (a, b) were generated by first amplifying the 5' and 3' *CTB1* regions of the target disruption site from the genomic DNA of the wild type with primers F1/F2 and F3/F4 respectively. The *HY* and *YG* pieces were amplified from a plasmid pTA-Hyg with primers HY/M13F and YG/M13R respectively. (The M13 primers are shown as red arrows or squiggle tail on F2 and F3 primers to create a region of homology). The pieces were mixed in equal proportions and used in a fusion PCR and subsequently used as templates in a nested PCR with primers $F1_N/HY_N$ and $YG_N/F4_N$ to generate the disruption fragments (b) and (c) respectively for protoplast transformation. **B.** Transformants were screened by PCR to verify the integration of the disruption fragments (c and d) at the target sites. **C**. Genomic DNA (5µg) of wild type and putative disruptants was double digested in a 100 µl volume with *Dra*I and *Pvu*II overnight. Fifty microliters of the digest was loaded into a well of 0.8% agarose gel and run at 0.6 V/cm overnight. Two gels were loaded for each sample and transferred to a Hybond N^+ nylon membrane. One membrane was hybridized with a probe (495 pb) amplified with primers CTB1pF1/CTB1pR1 from the wild type and the second with a 428 bp *Hyg* resistant gene probe amplified from the plasmid pTA-Hyg with primers HY-N/YG-N. **D.** The strains were single spored and verified for loss of cercosporin biosynthesis and five were rescreen by PCR. The PCR re-verification of Δctb1mutants was performed with primers A1 and A2, which amplified 1.5 kb of the wild type gene or non-disruptants and 3.1 kb in the disruption mutants. Primers A1/H1 and A2/H2 were used to verify the correct insertion of the disruption fragments (b and c) evidenced by the presence of a 0.980 kb and 1.2 kb band in the mutants and none in the wild type. **E**. Southern hybridization was repeated for the two strains, which previously showed single gene mutation (C) for documentation.

Appendix 2.3

Protoplasts of the wild type or mutants (resistant to hygromycin-B) were transformed with a GFP and geneticin resistant cassette (Neo) under constitutive gpdAp bidirectional promoter. The transformation vector (B) was amplified with primers M13F/M13R from the plasmid pBR0073 (A). Geneticin-resistant colonies were selected on regeneration agar (1.0 M sucrose, 0.02% yeast extract and 1.0% agar) amended with 200 µg/ml geneticin (G418). Regenerated colonies were screened under blue fluorescence light to select geneticin-resistant transformants, which showed strong GFP expression (C). Colonies shown inside the white rectangle met the two selection criteria for antibiotic resistance and GFP expression. These transformants were then transferred and maintained on V8 juice agar amended with geneticin (200 µg/ml) for two successive generations and subsequently without the antibiotic selection before phenotypic

evaluation. Except for the random mutagenesis library strains, all transformants, which were morphologically similar to the parent strain, were selected and evaluated for cercosporin biosynthesis, appressoria formation on maize leaves and lesion development. **D**. Rapid screen for cercosporin biosynthesis to select transformants with altered cercosporin phenotype. A colony was streaked on 0.2x PDA with a toothpick and incubated under constant light for 10 days was evaluated for cercosporin biosynthesis, and scored as having 1) approximately wild-type levels of cercosporin biosynthesis, 2) reduced levels, or 3) an apparent loss of cercosporin biosynthesis.

Appendix 2.4

A colony of a single spored conidia isolated from a lesion on leaves inoculated with Δ*ctb1-35*-GFP was prepared. **A**. The persistence of the loss of cercosporin biosynthesis phenotype in the re-isolated Δ*ctb1-35-GFP* was confirmed on 0.2x PDA. **B**. The disruption of the *CTB1* gene was confirmed by PCR with primers A1/A2, which amplified 1.5 kb of the wild type gene and 3.1 kb in the Δ*ctb1-35* or its Δ*ctb1-35-GFP* strains. **C**. Lesions on leaves inoculated with Δ*ctb1-35-GFP* previously isolated from a Δ*ctb1-35-GFP* inoculated leaf lesion.

Appendix 2.5

Regulation of *CTB1* during pathogenesis. **A**. Fusion of *CTB1* promoter to GFP ORF in the vector pEGFP (clontech PT3078-5) to make a CTB1 promoter activation construct to monitor the expression of *CTB1 in vivo*. The promoter sequence (1 kb) of *CTB1* was amplified by PCR and cloned between *Sal*I and *Bam*HI sites upstream of the eGFP open reading frame to drive the conditional expression of eGFP. For antibiotic selection of transformants, a hygromycin resistant gene cassette under *TrpC* promoter and terminator control were amplified from pTA-Hyg and cloned into the *EcoR*I site of the vector downstream of the eGFP reporter gene for positive selection of transformants. Transformants were selected on hygromycin-B selection medium and evaluated for cercosporin biosynthesis. Ten transformants confirmed to be capable of producing wild type levels of cercosporin were selected, grown in conditions that repressed cercosporin production, and observed under epifluorescence microscope to rule out autoactivation of GFP expression in the transformants. The selected transformants were then grown on 0.2X PDA plates under constant light to induce cercosporin biosynthesis and hence expression of the GFP reporter gene. **B**. Autofluorescence of mycelia mass of wild type strain grown on 0.2x PDA under cercosporin inducing conditions. On V8 juice agar under light or 0.2x PDA in dark, mycelia of the wild type did not fluoresce in blue light. **C**. Evaluation of *CTB1*p::GFP reporter strains *in planta* for *CTB1* promoter activation. Observations were made 1 to 14 days after inoculation. Pictures shown are photographs taken 9 days after inoculation.

CHAPTER THREE

IDENTIFICATION AND PARTIAL CHARACTERIZATION OF ABSCISIC ACID BIOSYNTHETIC GENE CLUSTER IN *CERCOSPORA ZEAE-MAYDIS*

ABSTRACT

Many filamentous fungi including Cercospora zeae-maydis make secondary metabolites, some of which have important plant signalling functions. In addition to the distinctive perylenequinone polyketide phytotoxin, cercosporin, C. zeae-maydis makes the phytohormone abscisic acid (ABA) in cultures. Unlike cercosporin, little is known about the regulation and relevance of ABA biosynthesis by fungi, especially in the context of pathogenesis. In the genome of C. zeae-maydis, we identified an ortholog of BcABA3, which has previously been implicated in the biosynthesis of ABA in Botrytis cinerea. Analysis of genes flanking the ABA3 gene ortholog in C. zeae-maydis revealed a cluster of genes containing a polyketide synthase and other genes frequently found in fungal secondary metabolite clusters. We created gene deletion mutants and determined ABA biosynthesis in vitro and pathogenicity on maize leaves. Mutants were reduced in ABA biosynthesis but were not impaired in pre-penetration infectious development. The mutants were also capable of inducing lesions on inoculated leaves with the same degree of virulence as the wild type. This finding suggests that genes involved in ABA biosynthesis in C. zeae-maydis are organised in a cluster, the expression of which may be dispensable for pathogenesis.

INTRODUCTION

The incidence and severity of gray leaf spot (GLS) has been on the increase over the past three decades, and has become a major constraint to maize production worldwide (Lipps, 1998; Ward *et al.*, 1999). GLS is a serious foliar disease of maize, a major staple crop mostly grown for human and animal feed, and recently, for commercial ethanol production. The disease is caused by two *Cercospora* species, *C. zeae-maydis* Tehon & Daniels and *C. zeina* Crous & U. Braun, sp. Nov. On susceptible hybrids and in favorable weather, the pathogen can cause total blighting of leaves resulting in over 50% yield losses (Lipps, 1998). GLS is difficult to manage, as effective genetic resistance is limited among commercial maize hybrids. Besides maize, *Cercospora* pathogens affect many other important crops throughout the world, but are poorly understood. A better understanding of the molecular mechanism of interactions between these pathogens and their hosts would greatly benefit global agricultural output.

Cercospora spp. exhibit stomatal tropism before infecting host leaves through stomatal pores. On maize, *C. zeae-maydis* hyphae frequently reorient growth in the direction of stomata and form multilobed structures similar to appressoria over stomata to initiate infection (Bluhm and Dunkle, 2008; Kim *et al*., 2011). Chemical analysis of fungal metabolites has shown that *Cercospora* species and a limited number of fungi produce the phytohormone abscisic acid (ABA) in addition to the signature phytotoxic metabolite cercosporin (Assante *et al.*, 1977; Tudzynski and Sharon, 2002, Schmidt *et al.*, 2008). This discovery is intriguing given that ABA regulates stomatal aperture in response to biotic and abiotic stresses (Schroed *et al*. 2001; Melotto *et al*., 2006) and stomatal pores are the main portal of host infection by *Cercospora* species (Beckman and Payne, 1982; Gupta *et al*., 1995; Babu *et al*., 2002). ABA has also been cited extensively as an important signalling intermediary in host-pathogen interactions. Although the current knowledge of the role for ABA in pathogenesis is contradictory, the weight of available evidence shows that ABA levels or sensitivity negatively correlate with resistance to both biotrophic and necrotrophic pathogens (Asselbergh *et al*. 2008; Ton *et al*., 2009; Cao *et al*., 2011). Because of the emerging knowledge of its significances in biotic stress responses during

pathogenic invasion of crops, the identification of genes involved in ABA biosynthesis and analysis of its molecular function among producing fungi has begun to attract research interest (Siewers *et al*., 2006; Schmidt *et al*., 2008; Yazawa *et al*., 2012; Gong *et al.*, 2013). The goal of this dissertation chapter is to identify the genetic basis of ABA biosynthesis in *C. zeae-maydis* and determine whether *ABA3* is involved in pathogenesis.

METHODOLOGY

Screening of Genomic Library for *ABA3*

A cosmid genomic library created from *C. zeae-maydis* strain SCOH1-5 (Shim and Dunkle, 2003) was kindly provided by Dr. Won-Bo Shim, Texas A&M University. Approximately 5,760 clones from the genomic library were screened by PCR with primers ABA3pF/ABA3pR and ABA3p2F/ABA3p2R. For initial library screening, bacteria were pooled systematically (96 per pool) and used for colony PCR. In pools where a target sequence was identified, individual strains in the pool were screened separately and cosmid DNA was extracted for a second round of PCR to confirm individual cosmids containing the target sequence (appendix 3.1).

Nucleic Acid Preparation and Sequencing

DNA for PCR and southern blotting was prepared according to a cetyltrimethyl ammonia bromide (CTAB) method for fungal genomic DNA extraction (Rosewich *et al*., 1998). *C. zeae-maydis* was grown in 50 ml YEPD medium (0.5% yeast extract, 1.0% peptone and 3.0% dextrose) for 2-3 days with constant shaking at 100 rpm. Mycelia were harvested by vacuum filtration through Whatman Grade No. 1 filter paper, washed with deionized water, and ground immediately in liquid nitrogen. Genomic DNA was isolated with a standard CTAB DNA preparation protocol (Rosewich *et al*., 1998) optimized for *C. zeae-maydis* as follows. Briefly, 500 µl CTAB DNA-extraction buffer (2.0% CTAB, 100 mM Tris-Cl pH 8, 1.4 M NaCl, 20 mM EDTA pH 8, 4.0% polyvinylpyrrolidone and 0.5% β-mercaptoethanol) was added to approximately 50 mg ground mycelium and incubated for 30 minutes at 55°C. Mycelial debris and proteins were pelleted and DNA was precipitated from the aqueous phase with isopropanol following standard molecular biology protocols. Plasmid and cosmid DNA was recovered from *E. coli* cultures with standard protocols described by Sambrook and Russell (2001). All plasmid and cosmid-walking sequencing was performed at the University of Arkansas DNA Resource Center (Fayetteville, AR) with an ABI 3130XL Genetic Analyzer.

The *C. zeae-maydis* SCOH1-5 genomic DNA sample for Whole Genome Shotgun sequencing was submitted to DOE Joint Genome Institute, while all plasmid and cosmid-walking sequencing were performed at the University of Arkansas DNA Resource Center (Fayetteville, AR) with an ABI 3130XL Genetic Analyzer.

Bioinformatics Analysis

Nucleic acid sequences obtained from the sequenced cosmids were manually assembled into contigs with Microsoft Word processor and ClustalΩ. The assembled sequences were then analyzed with the Basic Local Alignment Search Tool (BLASTN and BLASTX) and queried against the *C. zeae-maydis* whole genome sequence (http://genome.jgi.doe.gov/Cerzm1/Cerzm1.home.html) to identify and retrieve full sequences for analysis. The retrieved sequences were analyzed with the Antibiotics and Secondary Metabolite Analysis Shell (antiSMASH) algorithm (http://antismash.secondarymetabolites.org) to predict the biosynthesis cluster. Manual annotation of gene ORFs in the predicted cluster was performed with Softberry FGENESH (http://linux1.softberry.com/berry.phtml) with *Stagnospora nodorum, Pyrenophora* or *Leptosphaeria* as the training models. Homology searches of nucleotide sequences and database searches were performed with the BLAST service provided by the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov/BLAST). Identification of conserved domains and motifs in the protein sequences was performed with the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/) and the conserved-domain database (CDD) (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) at NCBI. DNA binding motifs in the promoter sequences were predicted with Regulatory Sequence Analysis Tool (RSAT) (http://rsat.ulb.ac.be/) and Suite for Computational identification Of Promoter Elements (SCOPE) Version 2.1.0 (http://genie.dartmouth.edu/scope/).

Construction of Gene Deletion Fragments

For targeted deletion of the cluster genes, a split-marker recombination strategy was

utilized (Fu *et al*., 2006). Two *hygromycin phosphotransferase-B* (*HYG*) gene fragments *HY* and *YG* with a 428 bp sequence overlap in the middle were amplified from pTA-Hyg with primer pairs M13F/HY and M13R/YG, respectively. The *HY* fragments, which were approximately 1.3 Kb and contained a *TrpC* promoter and 5′ *HYG* sequence, was fused with 750 – 1,500 bp of sequence immediately upstream of each target deletion locus. The *YG* fragments contained ~0.8 Kb sequence of the 3' region of *HYG* was and were fused at their 3' ends with the 5' ends of sequences immediately downstream of each target deletion locus. PCR products corresponding to the upstream and downstream sequences of each gene targeted for deletion were generated with target-specific primer pairs F1/F2 and F3/F4 respectively, and fused with the *HY* and *YG* fragments by overlapping M13 primer regions in a fusion PCR. The fusion products were then amplified with nested primers F1N/HY-N versus F4N/YG-N.

Protoplasting and Transformation

Freshly germinated *C. zeae-maydis* conidia were aseptically harvested by centrifugation and digested overnight in 1.2 M KCl containing lysing enzyme (Sigma# L-1412) and β-glucuronidase (Sigma# G-7017). Protoplast transformation was performed with 60% PEG-8000 in STC (1.2 M sorbitol, 50mM $CaCl_2$ and 10mM Tris pH 8.0). Depending on the selectable marker used, transformants were selected on regeneration agar (1.0 M sucrose, 0.02% yeast extract and 1.0% agar) containing 150 µg/ml Hygromycin-B or 2 mg/ml G-418 disulphate (Geneticin) (Research Products Inter. Corp., Mt. Prospect, IL).

For GFP expression strains, protoplasts were transformed with a GFP-Geneticin construct amplified by PCR with primers M13F/M13R from the plasmid pBR0073 (Appendix 2.3).

Molecular Analysis of Transformants

Mutants recovered from the regeneration agar were initially grown and single spored on V8-juice agar plates containing 150 µg/ml Hygromycin-B for two successive generations. DNA was extracted and screened by PCR with primer pair pF/pR to confirm the absence of the

targeted deletion locus, followed by primer pairs A1/H1 and A2/H2 to confirm integration of the 5' and 3' disruption fragments. For Southern analysis, 5 μg of genomic DNA was digested overnight with 10 units of restriction enzyme at 37°C. The DNA samples were resolved on a 0.8% agarose gel at 6 V/cm for 12 h and transferred to Hybond-N+ (Amersham Pharmacia, Diegem, Belgium) as described by Sambrook and Russell (2001). The probes for Southern analysis were a 428 bp sequence of hygromycin resistance gene (*Hyg*) located in the region of the split-marker overlap and for *ABA3* mutants an additional probe of a 456 bp sequence located downstream of the region of homologous recombination was used. The *Hyg* probe was prepared with primers HY-N/YG-N while the *ABA3*-specific gene probe was amplified with ABA3pF1/ABA3pR1 primer pair.

Measurement of Conidiation

V8 juice agar plates inoculated with 1 x 10^5 spores of wild type or mutant strains were grown in the dark for 4 days. Colonized agar plugs of 1 cm diameter were cut from each of three conidiating V8 agar plates with a stainless-steel cork borer of 1 cm inner diameter into 50 ml tubes. Three plugs were cut from each plate into 3 ml of ddH_2O containing 0.1% Triton-x and agitated to dislodge the spores into solution. The conidial suspension was appropriately diluted and spores counted with hemocytometer. The experiment was repeated two times. Statistical analyses (one-way ANOVA) were performed with Minitab v12.23 software.

Measurement of Radial Growth

Radial growth of the mutants on 0.2x PDA incubated for 10 days under light was compared to the wild type by measurement of colony diameter. A 10 mm diameter was extracted from a conidiating colony on V8 and lightly padded once on a 0.2x PDA plate. Each strain was padded at a separate spot on each of four replicate plates in each experiment. For each colony two diameters, measured at right angles to one another, were recorded after 10 days of incubation to give the mean diameter for that colony. The experiment was repeated two times.

Measurement of ABA Content

For determination of ABA, wild type and mutant *C. zeae-maydis* were grown on V8 juice agar plates under constant light for 7 and 14 days. Colonized agar plugs (1 cm diameter) were removed into 50 ml tubes and extracted with 80% methanol containing 0.05% acetic acid at the rate of one plug/ml. A total of 16 plugs were sampled from each plate into 16 ml of the extraction buffer and sonicated twice at 25^{o}C for 3 minutes each. Samples were left on a slow rocker in the cold room in the dark for 12 hours. The contents of the tubes were briefly centrifuged and the supernatant recovered and subjected to ABA determination using Phytodetek reverse competitive ELISA kit (Agdia). There were two replicates of each strain in each experiment. The entire experiment was repeated once.

Measurement of Cercosporin Content

For determination of cercosporin, 0.2x PDA plates (with pH adjusted to 5.6 with NaOH or HCl prior to autoclave sterilization) were inoculated with 1 x 10^4 spores of *C. zeae-maydis* (wild type or mutants) and incubated under constant light for 5 and 9 days. Colonized agar plugs (1 cm diameter) were removed into 15 ml tubes and extracted with 5.0 M KOH in the dark for 4 – 5 hours as described by Jenns and Daub (1995). A total of four plugs were sampled from each of three replicate plates at five, seven and nine days post-inoculation and extracted with 4 ml of 5 M KOH. Cercosporin concentration was quantified by measuring the absorbance of the KOH extraction at 480 nm and converting to concentration using molar extinction coefficient (ε = 23,300 M^{-1} cm^{-1}). The experiment was repeated at least three times.

Pathogenesis

Pathogenicity assays on maize hybrids SilverQueen and Dekalb 53-45, and the inbred line B73, were performed with spores collected from V8-juice plates with 0.1% Tween-x solution. The spore concentration was adjusted to 1.0 x 10^5 spores/ml. To enable comparison between mutants and wild type strains, each leaf of a six-week old maize plant was spray-

inoculated with a single up and down motion to normalize inoculum pressure per leaf area. The plants were covered with transparent plastic bags to maintain high relative humidity (>90%) without significantly impeding light transmission and were grown in a 12-12 hour day-night light cycle at 23^{o}C inside a growth chamber. Observations of disease progression were made 5 to 15 days post-inoculation. Leaf samples were collected 5 dai and examined via fluorescent microscopy to document pre-infection infectious development. For evaluation of lesion sizes and intracellular growth, leaves were sampled 14 -15 dai and examined. Intracellular growth of GFP-expressing strains was examined with a Nikon D-eclipse C1 confocal scanning fluorescent microscope. To measure lesion sizes, representative leaf samples from at least two inoculated plants in each experiment were cut into 8 cm sections and photographed with a Canon EOS REBEL T1i digital camera 14 dai. This experiment was repeated at least two times. In each photograph, a ruler was included in the same focal plane as a scale bar. To allow for precise measurement, the photographs were digitally magnified and the leaf margins cropped out to exclude necrosis resulting from physical damage or normal aging. All discernible lesions were measured with ImageJ (1.47q) image procession and analysis software, and the average lesion area was calculated. Statistical analyses were performed with Minitab software (v12.23).

RESULTS

Identification of the *ABA3* Gene from *C. zeae-maydis*

The *C. zeae-maydis ABA3* gene, an ortholog of *BcABA3* (Acc. # CCD 51887.1) whose function has been elucidated in *Botrytis cinerea* ATCC 58025 (Teleomoph *Botryotinia fuckeliana*), was identified during a differential cDNA screening experiment performed to identify genes differentially expressed under various light conditions (Bluhm *et al*., 2008). The *BcABA3* gene, which encodes a polypeptide of 417 amino acid of unknown enzymatic or regulatory function, is essential for the biosynthesis of ABA in *B. cinerea* (Siewers *et al*., 2006). In order to obtain the full sequence of the predicted *ABA3* ortholog of *C. zeae-maydis*, PCR primers ABA3pF/ABA3pR were designed from the ends of the available 470 bp EST and used to screen 5,760 cosmid colonies of a *C. zeae-maydis* genomic library. This identified one cosmid clone that was sequenced with a cosmid-walking approach. Prior to the whole genome sequence of *C. zeae-maydis* becoming publicly available, an additional screen of the library with primers ABA3p2F/ABA3p2R (located at one end of the first sequenced cosmid) identified two additional cosmid clones that were sequenced to obtain more information about the *ABA3* locus (Appendix 3.1A-B). The sequences which were assembled into a 22 kb contig, was used as a query in a BLAST search of the subsequently released whole genome sequence of the wild type strain (http://genome.jgi.doe.gov/Cerzm1/Cerzm1. home.html) to determine whether there are paralogs of the *ABA3* gene and to retrieve flanking sequences. Open reading frame and intron positions were predicted with the Softberry FGENESH software (http://linux1.softberry.com/berry.phtml). The predicted ORF, which is 1,303 bp long, is located on scaffold 27 from locus 161489 to 162791 consisting of two exons, 338 and 886 bp long, separated by a 79 bp intron. Conceptual translation of the exons resulted in a 408 amino acid long protein with 56% identity to BcAba3 at 95% coverage (Table 3.1). A search for conserved domains in the NCBI CDD (Conserved Domain Database) (Marchler-Bauer A *et al*., 2011), identified a GAT-1 (Type 1 glutamine amidotransferase) superfamily domain with a conserved C-terminal cysteine residue (residue 379) that presumably forms a Cys-His-Glu catalytic triad in the active site to transfer ammonia

from the amide side chain of glutamine to an acceptor substrate. Structural modeling of the protein on the Phyre^2 web server (Kelley and Sternberg, 2009) identified a classic Zn-finger, (C2H2) motif in a beta-beta-alpha Zn-finger fold with 43% identity. A search of the NCBI database of available fungal genomes with the BLASTp tool using the conceptually translated protein as a query identified orthologs among 18 ascomycete fungi. Interestingly, no homologs were found among other fungal phyla (Fig. 3.1.A)

Characterization of the Genomic Region of *ABA3* Gene from *C. zeae-maydis*

Genes encoding enzymes required for biosynthesis of a specific fungal secondary metabolite, as well as related genes such as transporters and transcription factors, are often clustered within a single genetic locus in filamentous fungi (Hoffmeister and Keller, 2007). Comparative analyses based on some of the most widely known genes involved in secondary metabolite biosynthesis (such as NRPS, PKS, TS or prenyltransfease, which are essential for the biosynthesis of non-ribosomal peptides, polyketides, terpenoids/isoprenoids and other metabolites) have been used to identify potential secondary metabolite biosynthetic gene clusters (Inglis *et al*., 2013). Because *C. zeae-maydis* produces ABA and has an ortholog of the *BcABA* gene, which is required for the synthesis of the phytohormone in *B. cinerea* (Siewers *et al*., 2006), we hypothesized that *ABA3* is associated with a cluster of genes involved in ABA biosynthesis in *C. zeae-maydis*. To test this hypothesis, the genome sequence was retrieved from the GJI web portal and analyzed with the antiSMASH (Antibiotics and Secondary Metabolite Analysis Shell) algorithm to identify any association of the *ABA3* gene with a secondary metabolite cluster in *C. zeae-maydis* genome. This analysis identified a total of 33 secondary metabolite biosynthesis clusters distributed among 23 scaffolds in the genome (Appendix 3.2). The *ABA3* gene, located on scaffold 27, was found to be associated with a polyketide synthase gene in a cluster consisting of five predicted ORFs. Further analysis of the polyketide synthase identified the enzyme as a type-1 polyketide synthase made up of the minimal polyketide synthase domains (ketoacyl synthase (KS), acyl transferase (AT), a thioesterase/Claisen-like

cyclase (TE) and acyl carrier protein (ACP)) including ketoreductase (KR), dehydratase (DH), enoylreductase (ER) and SAM dependent methyltransferase domains. The action of KR, DH, and ER activities results in varying degrees of reduction of the polyketide molecule (Table 3.1). In addition to the polyketide synthase and *ABA3* encoding genes, four other ORFs identified in the span of the predicted cluster were further analyzed with the Softberry fgenesh algorithms to annotate intron and exon positions, with function predicted with the NCBI BLASTx tool. A cytochrome P_{450} (Class E, group IV) and acetyltransferase genes, all of which encode structural proteins involved in synthesis of secondary metabolites were found to be contiguous with *ABA3* and a major facilitator superfamily (MFS) transporter in the cluster (Fig. 3.1 B and Table 3.1). The proposed ABA biosynthesis pathway(s) in *Cercospora* involves several oxidation and hydroxylation reactions that are presumably catalyzed by cytochrome P450 monooxygenases (Nambara and Marion-Poll, 2005). Cytochrome P450s catalyze a wide variety of oxidative reactions by introduction of an atom of molecular oxygen into a substrate. Acetyltransferases act by transferring acetyl groups to a variety of substrates including histones, where they might function as specific or global regulators of gene expression. Evidence supporting the role of acetyltransferases in the regulation of secondary metabolism clusters has been found for the sterigmatocystin, penicillin, terrequinone and orsellinic acid gene clusters in *A. nidulans* (Nützmann *et al*., 2011; Soukup *et al*., 2012). The MFS transporter is predicted to be the efflux pump-encoding gene for the release of the ABA into the external environment of the fungus. downstream of the MFS transporter, an ORF predicted to encode a plant homeodomain-like Zn-finger (PHD ZnF) protein of unknown function. We predict that the poorly characterized PHD ZnF protein, which has no fungal homologs in the NCBI genome database, is likely the cluster transcriptional regulator. Based on functional prediction, a degenerate transposable element *Malazy* (AY170475), upstream of *ABA3* gene and an ORF, downstream of PHD ZnF, did not appear to be involved in the metabolic function of the cluster and are predicted to lie outside the cluster.

Analysis of the Promoter Regions for Consensus Sequences

Genes involved in fungal secondary metabolite biosynthesis that are clustered also tend to be co-regulated by common transcription factors. The co-occurrence of particular transcription factor binding sites in the promoter regions of cluster genes can therefore be a useful predictor of co-regulation. Although evidence for the regulation of ABA biosynthesis by environmental cues is limited and inconclusive, nitrogen and carbon sources, light, and pH are the most important environmental signals that have been implicated in the biosynthesis of ABA in *Cercospora* and *B. cinerea* (Bennett *et al*., 1981; Norman *et al.*, 1981; Marumo *et al.*, 1982; Takamaya *et al*., 1983). The promoter regions of the predicted cluster genes were thus analyzed for the presence of binding sites recognized by the global nitrogen repressor AreA (GATA) which, incidentally are also recognized by the light responsive regulator, WCC (Wilson and Arst, 1998; Linden and Macino, 1997), the pH-regulatory gene pacC (GCCARG) (Espeso *et al*., 1997), and the carbon catabolite repressor creA (SYGGGG). The upstream sequences were also analyzed with SCOPE (Suite for Computational identification Of Promoter Elements) for the presence of other overrepresented promoter elements that are common to all cluster genes. In the region upstream of the *ABA3* open reading frame, 16 TATA binding elements, one CAAT binding element, and two non-symmetric spaced-dyads 5′-GTGN{4}TGA-3′ were identified. Analysis of the other cluster genes identified several consensus sequences in the promoter regions (Table 3.3). For example, all of the cluster genes contain at least one CAAT consensus sequence in their promoter regions. The GATA binding sites that are recognized by AreA and/or the WCC were also represented in the promoters of all putative cluster genes. The consensus sequences GATA and ATNRRS, as well as the non-symmetric spaced dyads 5′-GTGN{4}TGA-3′, were the most represented in the promoter regions of all putative cluster genes. On the contrary, the binding elements for pacC and creA were not present on all promoter regions of the cluster.

Disruption of Putative ABA Biosynthesis Pathway Genes

Metabolic profiling is a widely used approach to experimentally validate genes with

suspected roles in secondary metabolism. To characterize the putative ABA biosynthesis cluster and determine the molecular function of genes within the cluster, three genes (*ABA3*, a cytochrome P_{450}, and the polyketide synthase gene) were individually deleted via split-marker triple homologous recombination. The *ABA3* gene was disrupted by the replacement of a 140 bp sequence of the ORF including the predicted translation start codon with the *hygromycin phosphotransferase B* gene cassette. The cytochrome P_{450} and the polyketide synthase were deleted by replacement of the entire ORF including the predicted translation start and stop codons with *hygromycin phosphotransferase B* gene cassette (Fig. 3.2 A). Resulting transformants were recovered from regeneration agar containing 150 mg/ml Hygromycin-B and screened by PCR to verify the absence of the gene targeted for deletion and integration of the *hygromycin phosphotransferase B* gene at the deletion locus (Appendix 3.3 A-C). Southern blotting of the PCR-verified mutants was performed to identify knockout mutants with no ectopic gene insertions (Fig. 3.2. B). The 2.8 kb band on the Δ*aba3* radiograph with the *ABA3* gene-specific probe (Fig. 3.2. B) represents the intact *ABA3* gene of the wild type while the 1.6 kb shift in band to 4.3 kb in the mutants represents the replacement of 140 bp sequence of *ABA3* with the 1.6 kb *HYG* maker gene. The absence of ectopic insertion of the disruption constructs was verified with the *Hyg* probe. Each mutant had a single 4.3 kb band with no additional bands, thus confirming deletion of the targeted region without ectopic insertions. For the Δ*p450* and Δ*pks* mutants, gene deletion without ectopic insertions was verified by hybridization with the *Hyg* probe. Three mutants, Δ*p450-23,* Δ*p450-24* and Δ*p450-28*, each had the expected 4.6 kb band with no additional bands, while mutants Δ*p450-26* and Δ*p450-27* showed bands of larger sizes, possibly due to incomplete digestion of the genomic DNA (Fig. 3.2. B). For the polyketide synthase gene, mutants Δ*pks-18*, Δ*pks-25*, and Δ*pks-33* showed a single expected band (4.98 kb) while Δ*pks-13,* Δ*pks-21* Δ*pks-23* and Δ*pks-38* had more than one band in a lane or bands of unexpected sizes (Fig. 3.2. B). For each gene, two independent deletion mutants were selected for further characterization: Δ*aba3-22*, Δ*aba3-37*, Δ*p450-23,* Δ*p450-24,* Δ*pks-25*, and Δ*pks-33*.

Characterization of *ABA3, P450* and *PKS* Deletion Strains

Growth and development of mutants was compared with the wild type on 0.2x PDA and V8 juice agar. The two independent mutants of each gene and the wild type were inoculated on V8 juice agar at the rate of 1.0 x 10^5 spores per plate and incubated in the dark for four days. The Δ*aba3* mutants produced the least amount of conidia, while the Δ*pks-33* mutant produced the most conidia (Fig. 3.3 A). Both mutants of the *ABA3* gene (Δ*aba3-22 and* (Δ*aba3-37*) recorded 1.4 X 10^5 conidia/plug while Δ*p450-23* and Δ*p450-24* produced 1.8 X 10^5 and 2.1 X 10^5 conidia/plug respectively. Conidia production in Δ*pks-25* and Δ*pks-33* was respectively 2.0 X 10^5 and 2.3 X 10^5. Compared with the 1.7 X 10^5 conidia produced in the wild-type, no significant difference was found between/among wild type and mutants.

Radial growth of the mutants on 0.2x PDA incubated for 10 days was compared to wild type by measurement of colony diameter. A 10 mm diameter was extracted from a conidiating colony on V8 and padded on a 0.2x PDA plate. Radial growth of mutants increased from the initial diameter of 10 mm to an average of 16 mm after 10 days and did not differ from the wild type strain (Fig. 3.3. B).

Metabolite Analysis

The effect of gene deletion on the biosynthesis of cercosporin, which is thought to be a virulence factor (ref. Chapter 2 of this dissertation), was also evaluated. Potato dextrose agar plates (0.2x PDA) were inoculated with 1.0 x 10^4 spores and incubated under constant fluorescent light for up to nine days to stimulate cercosporin biosynthesis. Cercosporin biosynthesis of the Δ*aba3*, Δ*p450*, and Δ*pks* deletion mutants was compared to the wild type from 5 to 9 dai. No significant difference was observed between any of the mutants and the wild type. Also, cercosporin production increased two fold from the fifth to the tenth day of incubation in all strains (Fig. 3.4. A), indicating the distinctness of cercosporin biosynthesis from ABA biosynthesis.

ABA biosynthesis of the strains was quantified by reverse competitive ELISA with the

Phytodek monoclonal antibody ELISA kit (Agdia Inc. USA). Up to 12.55 pM ABA was detected in the wild type samples incubated under constant light on V8 juice agar for 7 days but declined to 7.4 pM ABA after 14 days of continuous incubation under the same conditions. Background detection from an uninoculated agar plate used as a control was unexpectedly as high (8 pM ABA) as the levels recorded for the mutants (Fig. 3.4. B). Because of this high background, the significant reduction in level of ABA detected in the Δ*aba3*, Δ*p450* and Δ*pks* mutants (average 5.4 pM ABA) were effectively considered to be background detection. Thus, the mutants had no detectable levels of ABA. This finding links the *ABA3* gene, the polyketide synthase and cytochrome P450 to ABA biosynthesis in *C. zeae-maydis*.

Characterization of *ABA3, P450* and *PKS* Deletion Mutants *in planta*

Since ABA plays multiple roles in plant pathogen interactions, we evaluated the effect of disrupting fungal ABA biosynthesis on pathogenesis by inoculating three susceptible lines of maize (B73, Silver Queen and Dekalb 53-45) leaves. ABA interferes with defense responses of the host to increase susceptibility to pathogen invasion at the early post-penetration stage of infection (Ton *et al*., 2009; Yazawa *et al*., 2012). We therefore hypothesized that disruption of ABA biosynthesis in the fungus would result in decreased colonization of the host. Maize plants at the V6 leaf stage were inoculated with mutants (approximately 5 ml of 1 x 10^5 spore suspension) and incubated for 15 days together with wild type inoculated plants. Interestingly, all the deletion mutants (Δ*aba3*, Δ*p450* and Δ*pks*) were able to form necrotic lesions on maize leaves (Fig. 3.4. A). To investigate this further, each of the Δ*aba3*, Δ*p450* and Δ*pks* mutants was transformed with a constitutive GFP expression cassette amplified from the vector pBR0073 (Appendix 3.4) for histological examination of the infection process *in planta* The *GFP* and *Neomycin phosphotransferase* genes were both constitutively expressed under the control of *gpdXp* bidirectional promoters. We observed that all the mutants exhibited pervasive intracellular growth similar to the wild type (Fig. 3.5 B) subsequent to forming normal appressoria.

To determine whether ABA biosynthesis in *C. zeae-maydis* is essential as a virulence factor, lesion sizes on inoculated leaf sections were measured and compared with the wild type by single factor one-way ANOVA. Average lesion area for wild type inoculated leaves was 4.2 x 10^2 cm^2 compared with 3.7 x 10^2 cm^2 and 4.3 x 10^2 cm^2 respectively for Δ*aba3-22* and Δ*aba3-37*. Compared with Δ*p450* and Δ*pks* inoculated leaves, lesion size varied from 3.8 X10^2 cm^2 to 5.7 X10^2 cm^2. The observed difference between mutants and the wild type were not statistically significant at the 95% confidence interval (Fig. 3.5 C).

DISCUSSION

The biosynthesis of abscisic acid and its intermediates has been experimentally demonstrated in species of *Cercospora* and the related ascomycete *B. cinerea* to occur mainly via pathways other than the carotenoid biosynthesis pathway (reviewed in Nambara and Marion-Poll, 2005). In this study, we identified and partially characterized a putative ABA biosynthesis gene cluster in *C. zeae-maydis* and performed pathogenicity and virulence assays to show that ABA biosynthesis in *C. zeae-maydis* is apparently dispensable for pathogenesis in gray leaf spot disease of maize.

By performing homology searches, a homolog of the *BcABA3* gene required for the synthesis of ABA in *B. cinerea* was identified in *C. zeae-maydis.* The identity score of 56% at 95% coverage revealed significant homology to the *B. cinerea* ABA3 protein previously implicated in ABA biosynthesis (Siewers *et al*., 2006). Homology was also found with genes in the ABA-producing fungi *F. oxysporum* and *M. oryzae* (Dörffling *et al.,* 1984; Jiang *et al.,* 2010) and related ascomycetes (Fig. 3.1. A), suggesting a role for the *ABA3* gene in ABA biosynthesis in *C. zeae-maydis*. We have shown in this study that ABA biosynthesis occurs in *C. zeae-maydis* (Fig. 3.4). Disruption of the *ABA3* gene resulted in complete loss of ABA biosynthesis in the mutants, confirming a role for the *ABA3* gene in the biosynthesis of ABA in *C. zeae-maydis*.

Consistent with the biosynthesis of fungal secondary metabolites, the biosynthesis of ABA in *C. zeae-maydis* was predicted to occur from a set of genes organized in a cluster. Interestingly however, comparison of the genomic regions flanking the *ABA3* gene in *C. zeae-maydis* to the ABA biosynthesis cluster elucidated in *B. cinerea* (Siewers *et al*., 2006) revealed a lack of significant conservation among flanking genes except for a cytochrome P_{450} gene found next to *ABA3* in both gene clusters. Cytochrome P450s are haem-containing monooxygenases that play a variety of roles in primary, secondary and xenobiotic metabolism. They catalyze a wide variety of reactions, e.g., hydroxylation, epoxidation, dealkylation, dehydrogenation and demethylation, by introducing an atom of molecular oxygen into a substrate (Ortiz de Montellano and De Voss, 2005; Gillam and Hunter, 2007). The proposed ABA biosynthesis

pathway(s) in *Cercospora* involves several oxidation and hydroxylation reactions that are presumably catalyzed by cytochrome P450 monooxygenases (Nambara and Marion-Poll, 2005). The specific function of the cytochrome P450 monooxygenases gene in the cluster can be elucidated when further metabolite profile analysis of mutants is concluded. Additional genes found in the region downstream of the *ABA3* predicted to belong to the putative cluster include an acetyltransferase, a type-1 polyketide synthase and an MFS transporter encoding genes (Fig. 3.1. B), all of which have roles in secondary metabolism. Acetyltransferases act by transferring acetyl groups to a variety of substrate molecules including histones, where they might function as a specific regulator of the cluster through chromatin modification. Evidence supporting the role of acetyltransferases in the regulation of secondary metabolism clusters has been found for the sterigmatocystin, penicillin, terrequinone and orsellinic acid gene clusters in *A. nidulans* (Nützmann *et al*., 2011; Soukup *et al*., 2012). Recently, EsaA, a homologue of the *Saccharomyces cerevisiae* MYST family acetyltransferase Esa1, was reported to play a role in secondary metabolite cluster activation through acetylation of histone 4 lysine-12 (Soukup *et al*., 2012). The MFS transporter is predicted to be the efflux pump-encoding gene for the release of the ABA into the external environment of the fungus. A role for a polyketide synthase in ABA biosynthesis seems surprising given that metabolic studies have suggested a mevalonic acid biosynthesis pathway in fungi. We predicted that the type-1 polyketide synthase in the cluster catalyzes the biosynthesis of the six-membered carbon ring backbone, similar to the synthesis of orselinic or salicyclic acid by 6-methyl salicyclic acid synthase. Unlike the 6-methyl salicyclic acid synthase, however, the ABA polyketide synthase has additional domains (enoylreductase and SAM-dependent methyl transferase) that are predicted to catalyze the desaturation and acylation of the ring to form ABA. To provide experimental evidence for the involvement of the polyketide synthase and the cytochrome P_{450} genes in the biosynthesis of ABA, the two genes were deleted and the effect on ABA biosynthesis determined. Deletion in either of the two genes (polyketide synthase or cytochrome P_{450}) resulted in significant reduction in ABA to levels undistinguishable from the background detection (Fig. 3.4. B). This strongly suggests a role for

the two genes in the biosynthesis of ABA in *C. zeae-maydis*. The lack of conservation between the *C. zeae-maydis* ABA biosynthesis cluster and the cluster elucidated in *B. cinerea* ATCC 58025 may reflect the difference in the intermediates observed amongst different ABA-producing species and an indication of independent acquisition of the *ABA3* gene by the two fungi (Nambara and Marion-Poll, 2005). Whether the cluster is conserved among species of *Cercospora*, among whom different ABA biosynthetic intermediates have been isolated, is yet to be determined.

Since ABA plays multiple roles in plant-pathogen interactions, we evaluated the effect of the disruption of fungal ABA production in the mutants on maize leaves. ABA interferes with host defense responses to increase host susceptibility to pathogen invasion at the early stages of infection (Ton *et al*., 2009; Yazawa *et al*., 2012). We therefore hypothesized that disruption of ABA biosynthesis in the fungus will result in decreased colonization of the host. The observation that mutants in the putative pathway genes could still form appressoria over stomata, colonize leaf tissue and form lesions as well as the wild type isolate suggested that fungal ABA is dispensable for stomatal infection and colonization. If ABA plays a role in the colonization of the leaf tissues, host response to infection characterized by upsurge in ABA levels is sufficient to compensate for the lack of production from the pathogen. Alternatively, it is possible that the hormone plays a role in GLS pathogenesis, but other necrosis-inducing factors may be sufficient to promote lesion formation in the absence of fungal ABA.

Conclusion

The gray leaf spot pathogen of maize, *C. zeae-maydis*, makes ABA and possesses a functional ortholog of the *ABA3* gene, which resides in a cluster. The cluster consists of three enzyme-coding genes ($cytP_{450}$, acetyltransferase and a polyketide synthase), an efflux pump (MFS) and a gene of unknown regulatory or enzymatic function (*ABA3*). Disruption of the $cytP_{450}$, polyketide synthase, or *ABA3* resulted in the loss of ABA biosynthesis but had no discernible effect on pathogenesis.

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Ward, J. M. J., Stromberg, E. L., Nowell, D. C. & Nutter, F. W. (1999) Gray leaf spot - A disease of global importance in maize production. *Plant Disease,* **83:**884-895.

Wilson, R. A. & Arst, H. N., Jr. (1998) Mutational analysis of *AREA,* a transcriptional activator mediating nitrogen metabolite repression in *Aspergillus nidulans* and a member of the "streetwise" GATA family of transcription factors. *Microbiology and Molecular Biology Reviews,* **62:**586-596.

Yazawa, K., Jiang, C.-J., Kojima, M., Sakakibara, H. & Takatsuji, H. (2012) Reduction of abscisic acid levels or inhibition of abscisic acid signaling in rice during the early phase of *Magnaporthe oryzae* infection decreases its susceptibility to the fungus. *Physiological and Molecular Plant Pathology,* **78:**1-7.

TABLES

Table 3.1: ABA biosynthetic genes and their predicted functions in *C. zeae-maydis*

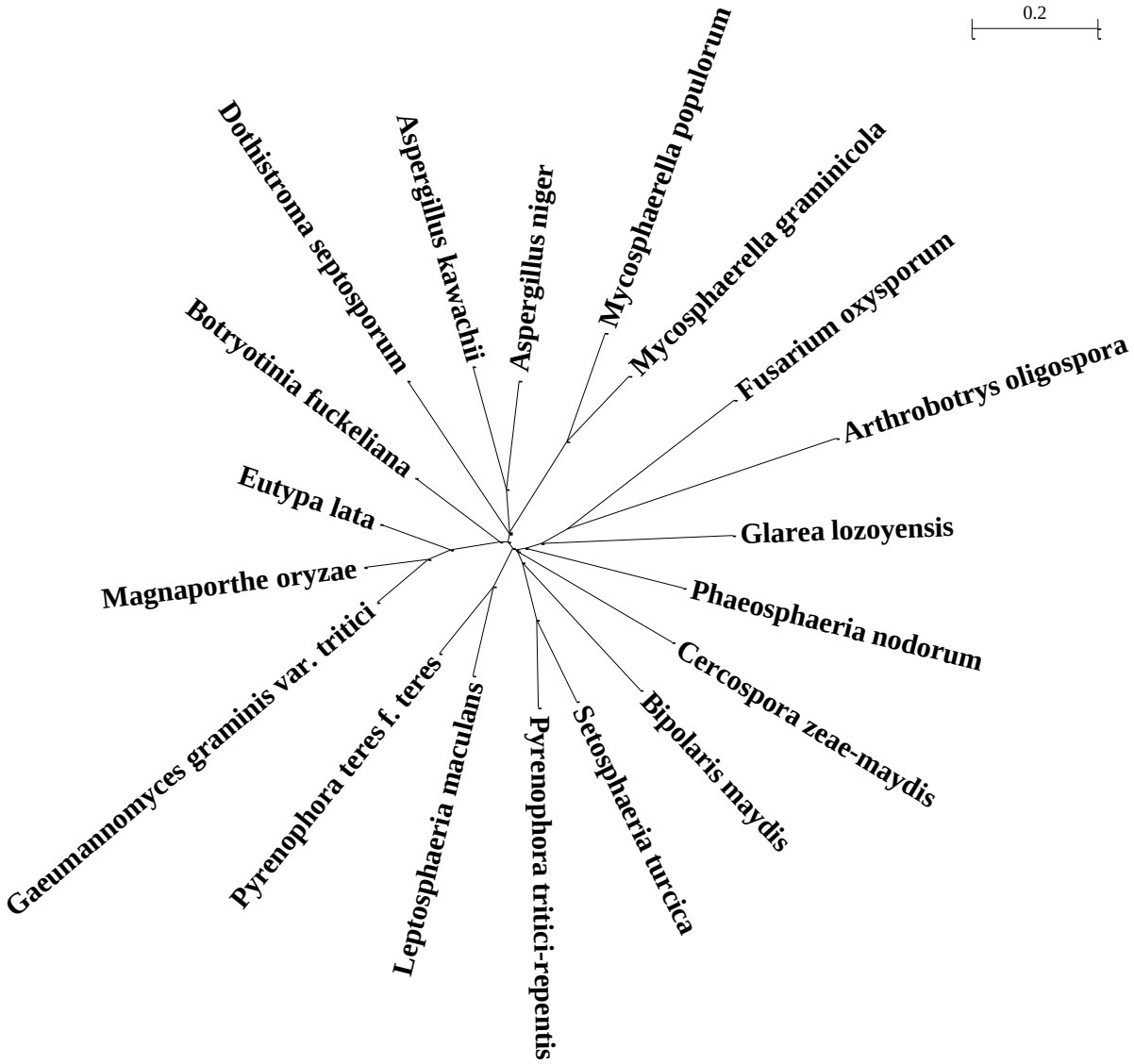
Predicted Gene	Length (bp)	No. of Exons	Amino Acids	Closest BLAST match proteins and species (accession no.)	*E*-value	ID (%)	Domains and motifs
ABA3	1,303	2	408	BcABA3 *Botrytis cinerea* (CCD51887)	$2e^{-144}$	56	GAT-1, (Type 1 glutamine amido transferase)
Cyt P450 monoxygenase	1,729	3	515	Hypothetical protein *Phaeosphaeria nodorum* SN15 (XP 001793167)	0.0	59	Cytochrome P450
Acetyl transferase	1,596	1	532	Hypothetical protein *Phaeosphaeria nodorum* SN15 (XP 001793166)	$8e^{-107}$	44	Transferase
Polyketide synthase	7,513	3	2,289	Hypothetical protein *Phaeosphaeria nodorum* SN15 (XP 001793165)	0.0	51	• β-ketoacyl synthase • Acyl transferase • Dehydratase • SAM-dependent methyl transferase • Enoyl reductase • $NAD(P)^{+}$-dependent ketoreductase • Phosphopantetheine attachment site
MFS Transporter	1,926	4	557	MFS transporter *Aspergillus kawachii* IFO4308 (GAA84669)	$2e^{-79}$	38	Major Facilitator Superfamily
Transcription factor	1,575	6	433	PHD Zn-finger protein *Bombus terrestris* (XP 003398056)	$2e^{-79}$	20	
ORF 1	1,561	4	425	Predicted protein (no match)			
malazy	7,166	6/9	135/ 162	Degenerate transposable element *C. zeae-maydis* (AY170475)	$5e^{-81}$	46	

Table 3.2: Analysis of promoter sequences upstream of putative translation start codon

Predicted Gene	**TATA**	**CAAT**	**AreA or WC1/WC2 (**GATA)	**creA** (SYGGGG)	**pacC** (GCCARG)	(ATNRRS)	GTGN{4}TGA
ABA3	16	1	1	2	0	3	2
Cyt P450 monoxygenase	1	2	1	0	1	6	1
Acetyltransferase	0	1	3	2	0	9	2
Polyketide synthase	0	3	2	1	1	5	2
MFS Transporter	4	4	6	0	1	13	1
Transcription factor	2	6	2	2	0	10	0
ORF1	2	2	3	2	0	10	0

FIGURES AND FIGURE LEGENDS

A.


0.2
Dothistroma septosporum
Aspergillus kawachii
Aspergillus niger
Mycosphaerella populorum
Mycosphaerella graminicola
Fusarium oxysporum
Arthrobotrys oligospora
Botryotinia fuckeliana
Eutypa lata
Glarea lozoyensis
Magnaporthe oryzae
Phaeosphaeria nodorum
Gaeumannomyces graminis var. tritici
Cercospora zeae-maydis
Pyrenophora teres f. teres
Bipolaris maydis
Leptosphaeria maculans
Setosphaeria turcica
Pyrenophora tritici-repentis

B.

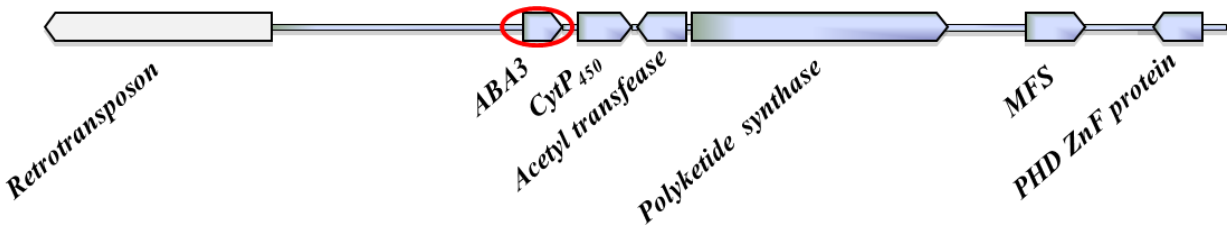
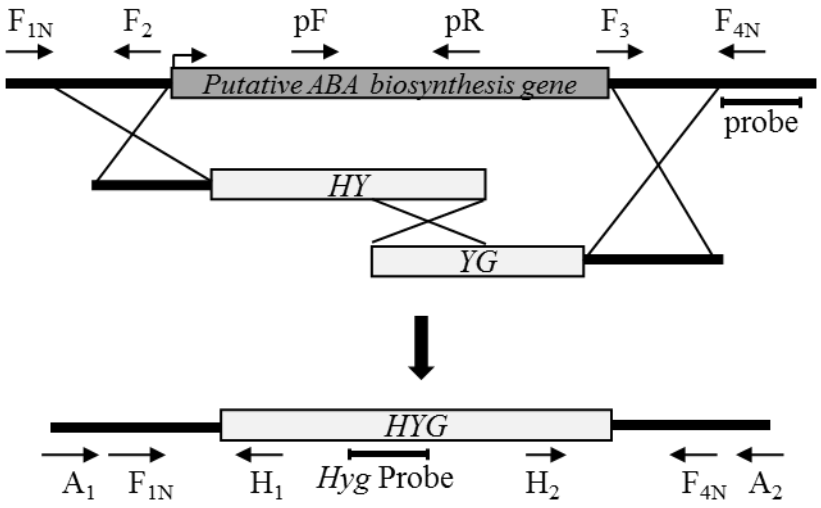

Retrotransposon
ABA3
CytP 450
Acetyl transfease
Polyketide synthase
MFS
PHD ZnF protein

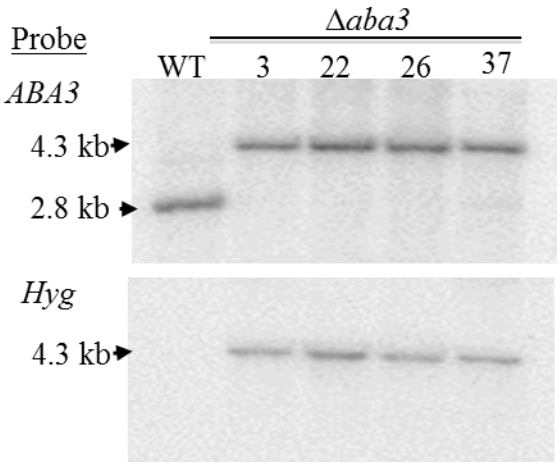
Fig. 3.1

A.

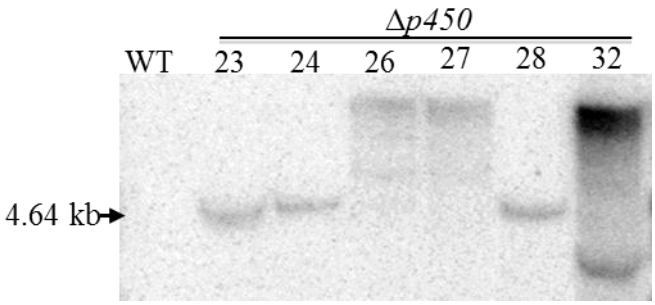


B.

i.



ii.



iii.

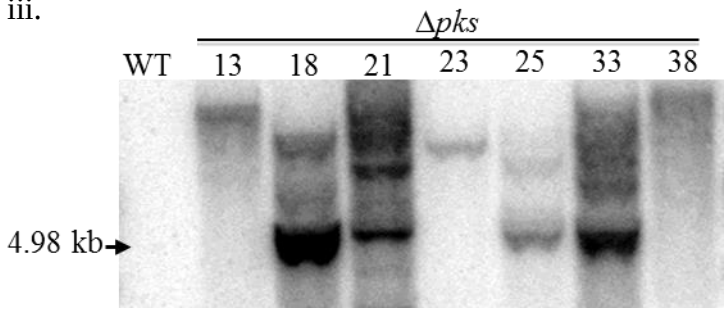
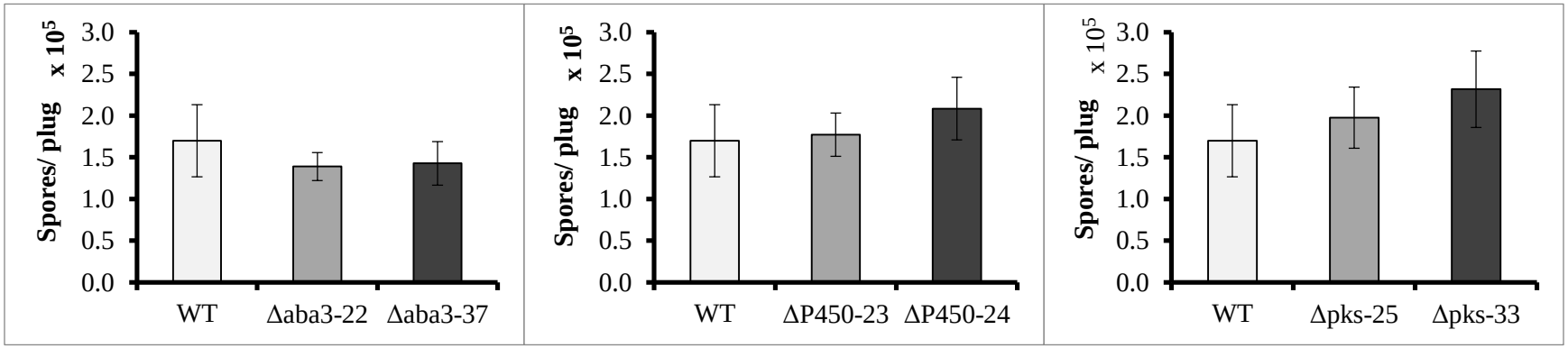


Fig. 3.2

A.


Spores/ plug x 10^5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
WT
Δaba3-22
Δaba3-37
Spores/ plug x 10^5
WT
ΔP450-23
ΔP450-24
Spores/ plug x 10^5
WT
Δpks-25
Δpks-33

B.

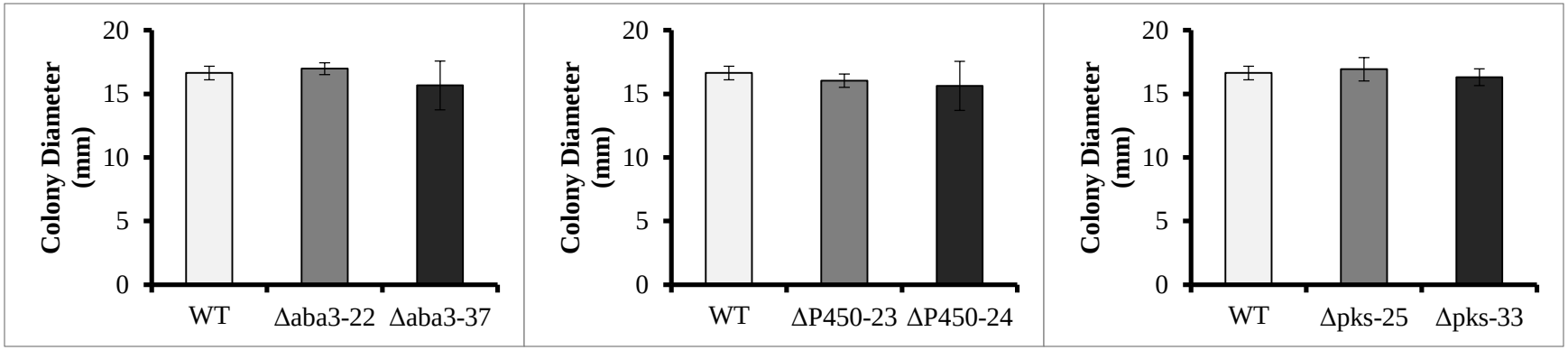
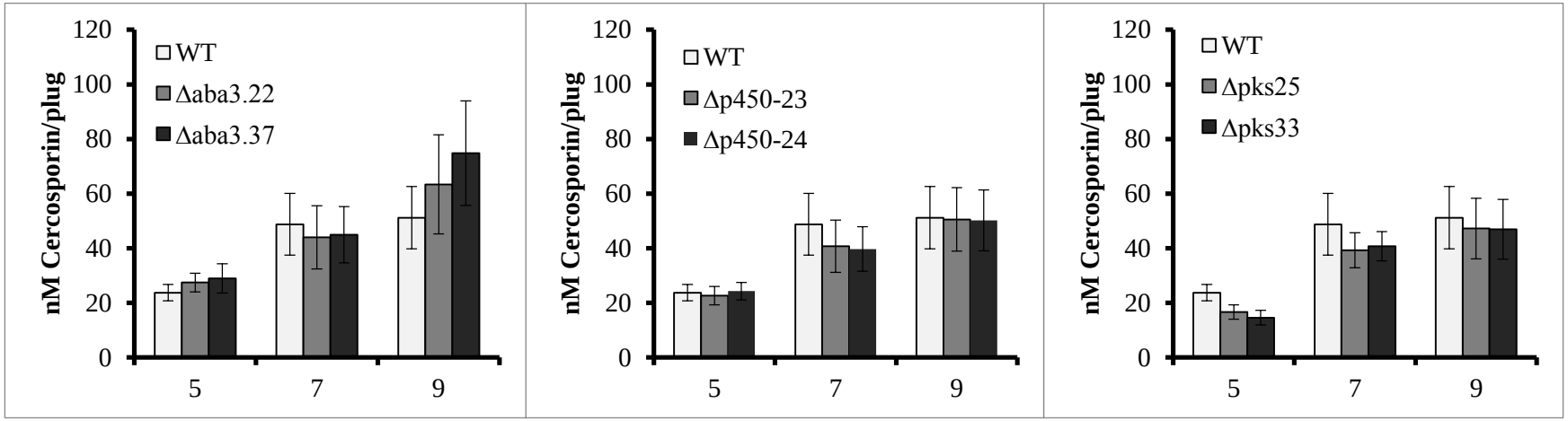

Colony Diameter (mm)
20
15
10
5
0
WT
Δaba3-22
Δaba3-37
Colony Diameter (mm)
WT
ΔP450-23
ΔP450-24
Colony Diameter (mm)
WT
Δpks-25
Δpks-33

Fig. 3.3

A.


nM Cercosporin/plug
120
100
80
60
40
20
0
WT
Δaba3.22
Δaba3.37
5
7
9
nM Cercosporin/plug
WT
Δp450-23
Δp450-24
nM Cercosporin/plug
WT
Δpks25
Δpks33

B.

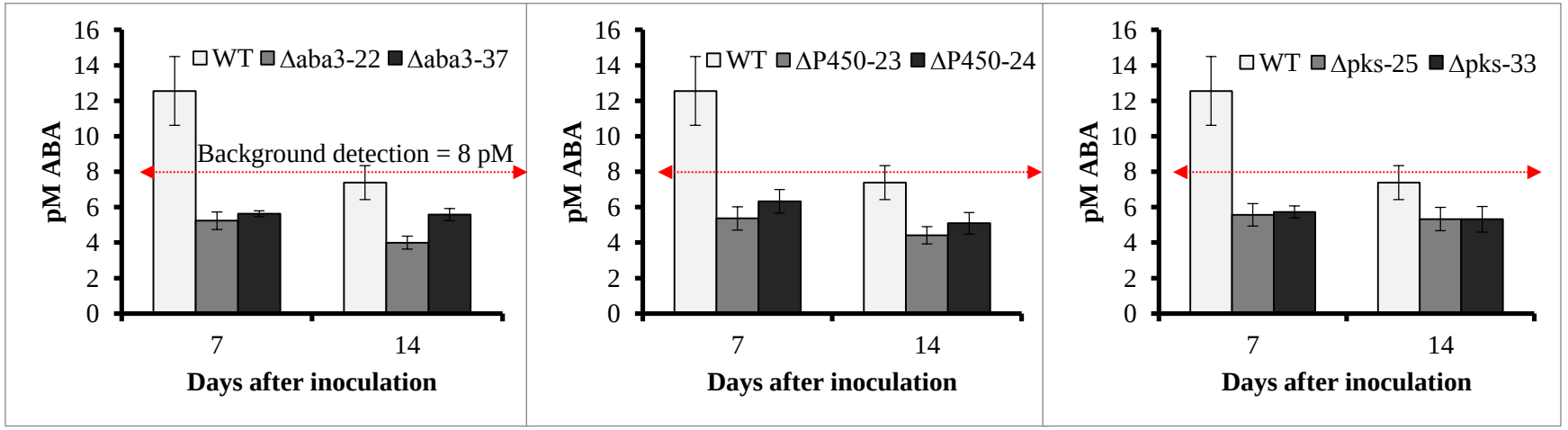
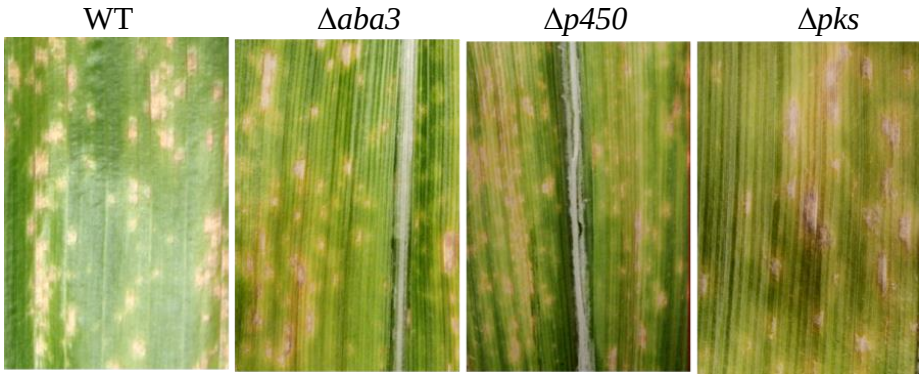
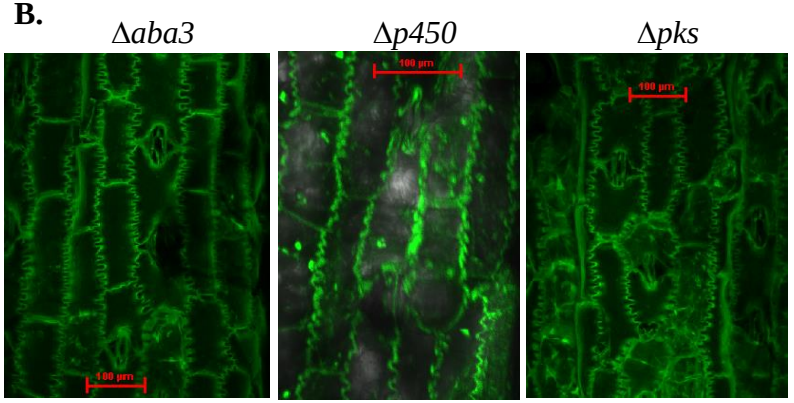

pM ABA
16
14
12
10
8
6
4
2
0
WT
Δaba3-22
Δaba3-37
Background detection = 8 pM
7
14
Days after inoculation
WT
ΔP450-23
ΔP450-24
WT
Δpks-25
Δpks-33

Fig. 3.4

A.


WT
Δaba3
Δp450
Δpks

B.


Δaba3
Δp450
Δpks

C.

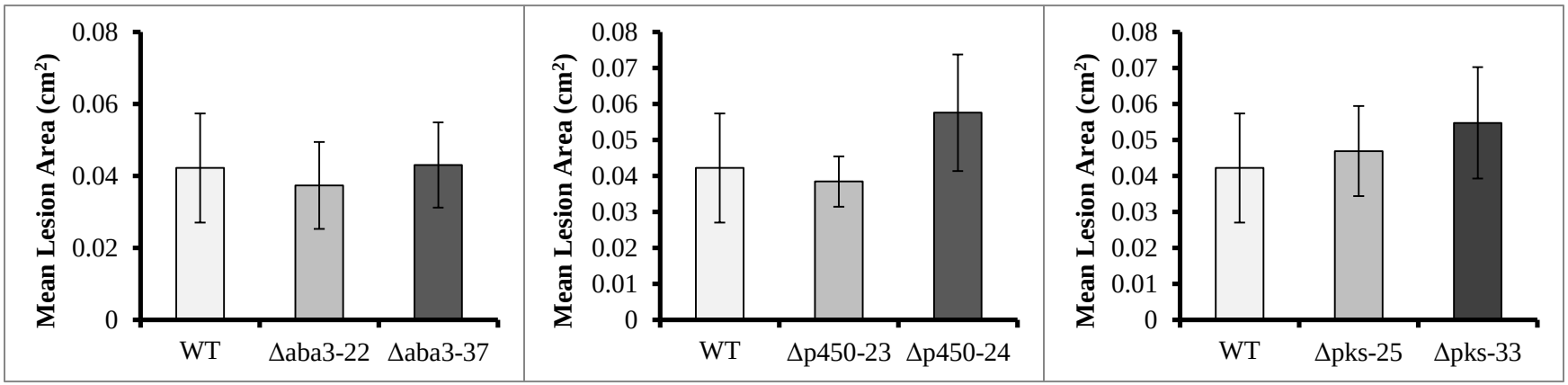

Mean Lesion Area (cm²)
0.08
0.06
0.04
0.02
0
WT
Δaba3-22
Δaba3-37
Mean Lesion Area (cm²)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
WT
Δp450-23
Δp450-24
Mean Lesion Area (cm²)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
WT
Δpks-25
Δpks-33

Fig. 3.5

Figure legends

Fig. 3.1.

A. Phylogenetic analysis of *ABA3* orthologs. The analysis was based on full-length amino acid sequence using the neighbor-joining method. Scale bar indicates number of substitutions per site. **B.** Genomic map of the predicted ABA gene cluster and surrounding genes. Each arrow indicates relative gene size and direction of transcription. On the basis of a set of deletions we created in *C. zeae-maydis* (Δ*aba3*, Δ*p450* and Δ*pks*) and analyzed, the ABA3, CytP450 and PKS proteins have a role in ABA biosynthesis in *C. zeae-maydis*. The *ABA3* gene is circled in red for emphasis.

Fig. 3.2.

Deletion of putative ABA biosynthesis cluster genes in *C. zeae-maydis.* **A.** Schematic illustration of a split *hygromycin phosphotransferase B* gene (*HYG*) maker deletion of targeted genes. Two truncated fragments of *HYG* (*HY* and *YG*) were amplified from plasmid pTA-Hyg by PCR. The *HY* fragment was fused by PCR to sequence immediately upstream of the region targeted for deletion and the *YG* piece was fused to sequence immediately downstream of the targeted gene. Arrows indicate primer locations and directions. **B.** Southern blot analyses of the wild type strain SCOH1-5 and putative mutants were performed with a Hyg gene probe. For *ABA3* knockouts, genomic DNA was digested with *Dra*I and *Bgl*II and hybridized with *HYG* and *ABA3* gene- specific probes. In the case of CytP450 and the polyketide synthase, the DNA was digested with *Xho*1/*Eco*RV and *Psi*1/*Eco*NI respectively. Hybridization was performed with a 428 bp *Hyg* probe located in the region of split-marker homology to identify knockout mutants with ectopic insertion of either of the disruption constructs. In each case, the restriction enzymes were selected to cut only outside the region targeted for deletion and does not cut within the *HYG* gene. For *ABA3* mutants, an additional probe (456 bp long) located downstream of the region of homologous recombination was used.

Fig. 3.3.

Growth and development of Δ*aba3*, Δ*p450* and Δ*pks* mutants of *C. zeae-maydis*. **A.** Conidiation on V8 juice agar 5 dai. Conidiation of Δ*aba3*, Δ*p450* and Δ*pks* mutants and WT on V8 juice agar 5 dai was measured by counting of spores with a haemocytomete. **B.** Radial growth on 0.2x PDA. Colonized agar plugs (1 cm diameter) were cut from colonized V8 agar plates and padded on new 0.2x PDA plates. Radial growth was then measured by measuring colony

diameter in mm at 10 dai. Data plotted are the means from three experiments. Error bars represent confidence intervals at 95%.

Fig. 3.4.

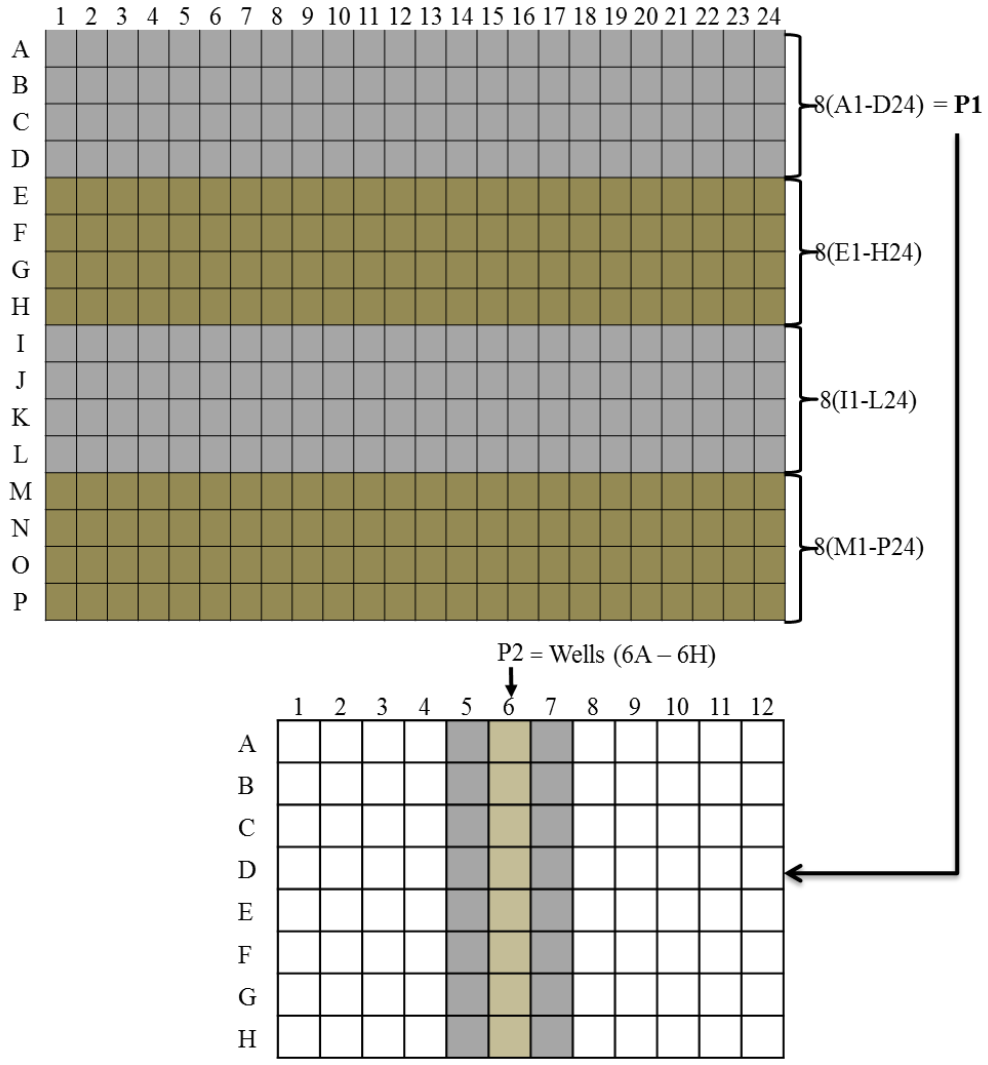
Metabolite analysis. **A.** WT and mutants were grown on 0.2x PDA under constant light between four and ten days and the level of cercosporin accumulation was determined from the absorbance of alkali extracts at 480 nm. **B.** ABA was extracted from colonized V8 agar incubated under constant white light for 7 or 14 days. Amount of ABA in the extracts was estimated by reverse competitive ELISA. Data plotted are the means of all experiments with replicates. Error bars are confidence intervals at 95%.

Fig. 3.5.

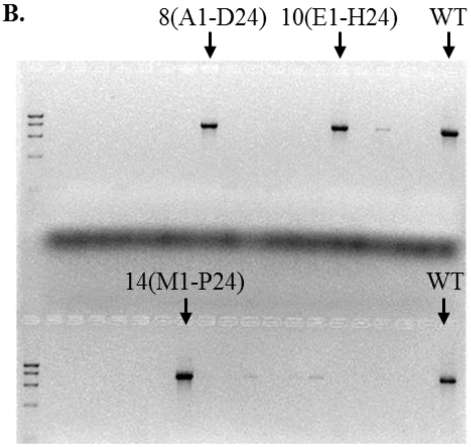
Assessment of pathogenicity of ABA cluster gene mutants on maize leaves. **A.** Leaves of maize plants at the V6 stage were inoculated with conidial suspensions of the wild type and the deletion mutants (Δ*aba3*, Δ*p450* and Δ*pks*) on the abaxial surface. Plants were grown at 23^{o}C in high relative humidity for 15 days. Pictures shown are representative leaf sections bearing lesions. **B.** Intracellular colonized of leaf by mycelia of GFP expressing mutant strains. Pictures were obtained with a Nikon D-eclipse C1 confocal scanning microscope. **C**. Virulence was determined by measuring average lesion areas on three replicate leaf sections in each experiment.

APPENDICES

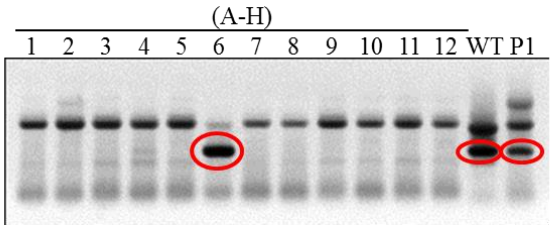
A.



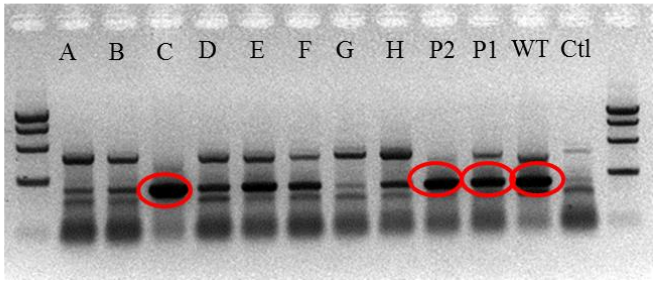
B.



C.



D.



Appendix 3.1 A

Appendix 3.1B

Table A3.1: List of screening primers and cosmids with target *ABA3* sequence

Primer ID	Primer sequence	Cosmid retrieved		
		6C 8(A-D)	8G 10(E-H)	1E 14(M-P)
ABA3pF	CATTCGCGCAAAGTTGATCACGTG	x		
ABA3pR	ATCGCACGTTCCTCCTCATCG	x		
ABA3p2F[1]	CCTGGTCATGGCAAGGACG		x	x
ABA3p2R[2]	GCTGGAAGGAGATGGGTGC		x	x

>pB201 (*ABA3* EST) 966 bp

~~N~~CTATAGAATACTCAAGCTATGCATCCNACGCGTTGGGAGCTCTCCCATATGGTCGACCTGC
AGGCGGCCGCGAATTCACTAGTGATTATCGGTAGCGACGCATGGTCATGTGGATCGGCCCGC
CAAA**CATTCGCGCAAAGTTGATCACGTG**CAGGCGTGCCGCATCGCCACGCCAGGCGACGT
CGAGCGCCCACAGGAGTTGGCGACATTCGGCGTAGACTGCCACGCGTGACGCCGACGGCAC
GTATGCGAACGTACTGCTGATCTCTCCTTCGGCCCGATGCTTGAAGAAGGCGACGGCATCAT
ACATGCAGTCGCACAATTCCGTCAGCACTTCGAGCTGCTCGTCCGAGAACCACGTATCGTCC
GCGTCGTTACACGCCAACGCAGCAGCTATCGTGAAGCGCGCGAGCGCGTCACAGTCGCGCA
TGCGGAACCACTGCTTGGGAGAGGCGACGAGGGCGTTGGCGTATCGCCGAAAGAGCTCGCT
GCTCGTGTTGCTGGACTTCTCGGCCG**CGATGAGGAGGAACGTGCGAT**ACTCTCGTGCCATC
TCGGCGTGAATCGAATTCCCGCGGCCGCCATGGCGGCCGGGAGCATGCGACGTCGGGCCCA
ATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGG
AAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGT
AATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAAT
GGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACC
GCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGT
TCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTT~~N~~

[1] Primer ABA3p2F is in storage with the code "Peck_ABA_CosScreen_F".
[2] Primer ABA3p2F is in storage with the code "Peck-ABA-Walk6".

Table A3.2: List of primers and cosmids sequenced for *ABA* cluster

Primer ID	Primer sequence	Cosmid sequenced		
		6C 8(A-D)	8G 10(E-H)	1E 14(M-P)
ABA3pF	CATTCGCGCAAAGTTGATCACGTG	5’		
ABA3pR	ATCGCACGTTCCTCCTCATCG	3’		
ABA3-seq1	AGCTGTGCTGTGCATGCTCTT	3’		
ABA3-seq2	GCACGTGACTTTAGTGCACACGAT	5’		
PECK ABA3 W3	CGAGGCACTCGCAGAAGTACTG	3’		
ABA Walk4	CAGCATCTTGCGCAGGCTG	3’		
Peck-ABA-Walk5	CATGGGACGACGCTCGACA	3’		
Peck-ABA-Walk6	GCTGGAAGGAGATGGGTGC	3’		
Peck-ABA-Walk7	GCATCGGGTCGTTTTCCTGT	3’	3’	3’
PeckWalk8	CGTGTTCGAGCAGTCTGGTC		3’	3’
PeckWalk9	GACGACCTCACGAGCACAAG		3’	3’
PeckABAWalk10	GGTGCCATGCGCGTATCATTC		3’	3’
ABA3W11	TATCGAGGCCGTGCGCATGG		3’	3’
ABA3 W12	ACAACGCTGGACTCCTTGTTACAG		3’	3’
ABA3 W13	GCTCAAGCAAATCCTTCGCCTTTA		3’	3’
ABA3 W14	TGTCTCATCAGAGGCAGTCGTCG		3’	3’
ABA W15	GAGGGTGCCGCTATTCCCTAC		3’	3’
ABAW16	GAGCGTTACAGACGGAAGCAGA		3’	3’
ABAW17	CGGTGGATTGCGGTCTGTCT		3’	3’
ABAW18	TGGAGAGCAARGTCTCGGGACAG		3’	
ABAW19	GACCCGACCGACAGCACT		3’	
PeckGap1	AGAGCCGAAGGTAGGGCT			
PeckGap2	GACGAAGCTGCCAATCCTGG			
PeckGap3	AGCCCTACCTTCGGCTCT			
PeckGap5	GGTCAGTATCTGCTCCAGCCAC	5’	5’	5’
PeckGap6	GAAGCCTTGCTCGCTGAC	5’		5’
PeckEndExt	GACGACCTCACGAGCACAAG	5'		
PeckEndExt2	CTGCCTAAGAACCGATCCGAAC	5’		
ABA3Ext3	TGCCTAAGAACCGATCCGAACC	5’	5’	5’
ABA3Ext3b	TCGTACTCTCTAGTTTCGCTATAA	5’		
ABA3Ext3c	CCTACCTACTATCTCTACTTCTACT	5’		
ABA3Ext4a	AGCACGAAGGACACAATCCTTAC	5’		
ABA3 Ext4b	GGAAGGTAGAAATCCGGAAGCTG	5’		
ABA3Ext5	TGCCTTTAAAGCCTAAGAGAAGTC			
ABAExt6	AGCCTTTTTAGGCATTTAACCCTC	5’		
ABAExt7	GCCGTAGAGTAGAACCTAAGAGG	5’		
ABAExt8	ATTGCAGCACTAAAGCTAGAAGA	5’		

5’ and 3’ indicate the direction of sequencing.

Total *ABA3* cluster sequences obtained by cosmid walk = 21,982 bp.

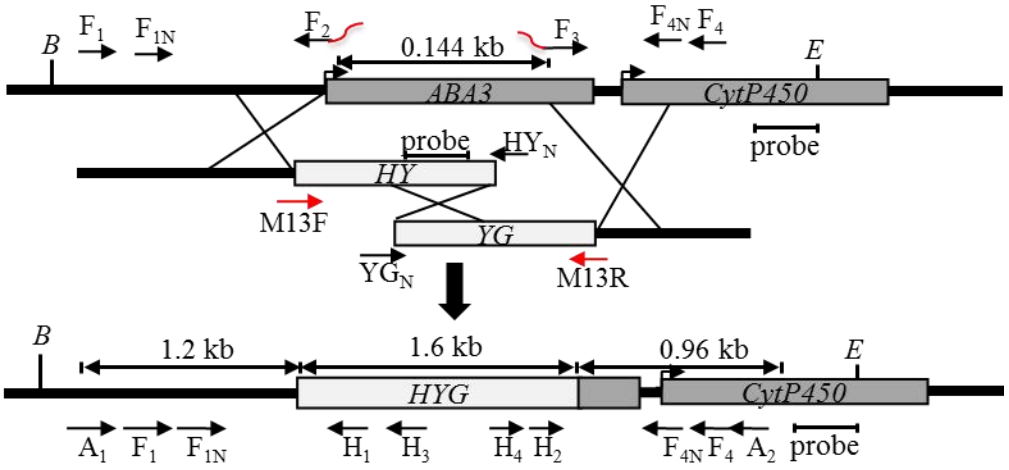
Appendix 3.2

Table A3.3: Distribution of predicted secondary metabolite clusters in *C. zeae-maydis*

Scaffold #	Cluster Type	Scaffold Location	
		From	To
1	Lantipeptide synthase	1505620	1526791
2	NRPS	481538	567539
	NRPS	703376	750171
4	T1PKS	550612	601814
	Other	729924	772540
	NRPS–T1PKS	928706	981251
	Other	984883	1027845
5	T1PKS	991782	1038675
6	T1PKS	225033	272142
	Terpene	470156	494391
9	NRPS	789981	832455
10	Other	331557	375948
	T1PKS	452778	500333
13	T1PKS	143829	190398
15	**T1PKS[3]**	**610624**	**659245**
16	Terpene	544609	566112
17	Other	200240	243972
18	Terpene	732703	760325
19	Other	74483	118467
20	T1PKS	563308	621291
25	NRPS	24892	79603
27	**T1PKS[4]**	**147509**	**195038**
	Terpene	235326	256471
	T1PKS	252449	298192
	NRPS	294899	344790
28	NRPS	429662	474675
29	NRPS–T1PKS	1	50710
36	NRPS	274381	317242
43	NRPS	50511	98286
	Terpene	99150	123215
46	NRPS	235505	296430
56	Other	126864	169832
67	Other	73855	107609

[3] Predicted cercosporin biosynthesis cluster.
[4]Predicted ABA biosynthesis cluster.

A.



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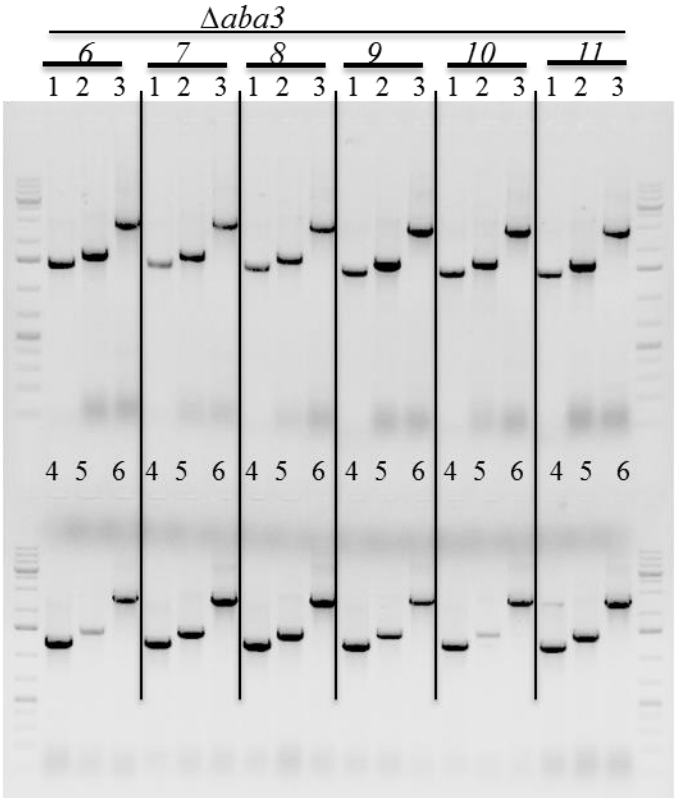
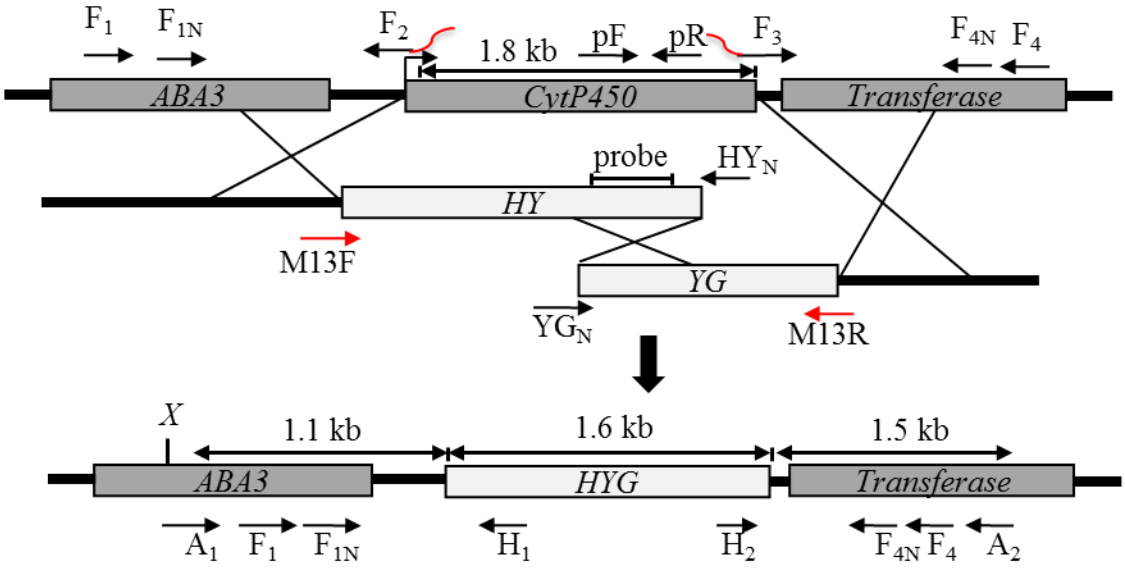
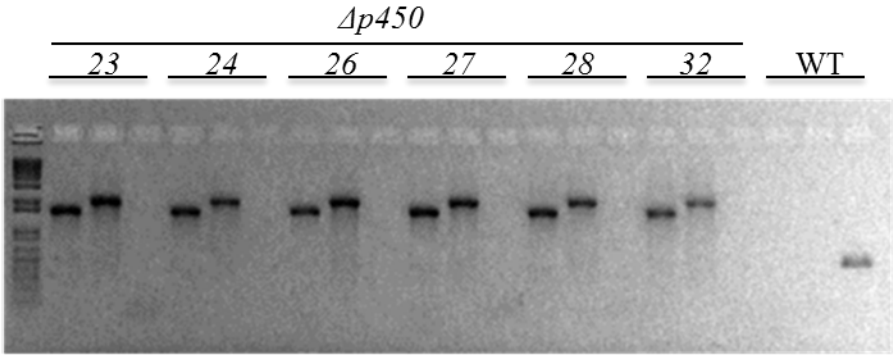


Primers	Lanes
A_1/H_1	1
A_1/H_3	2
A_1/M13R	3
A_2/H_2	4
A_2/H_4	5
A_2/M13F	6

C.

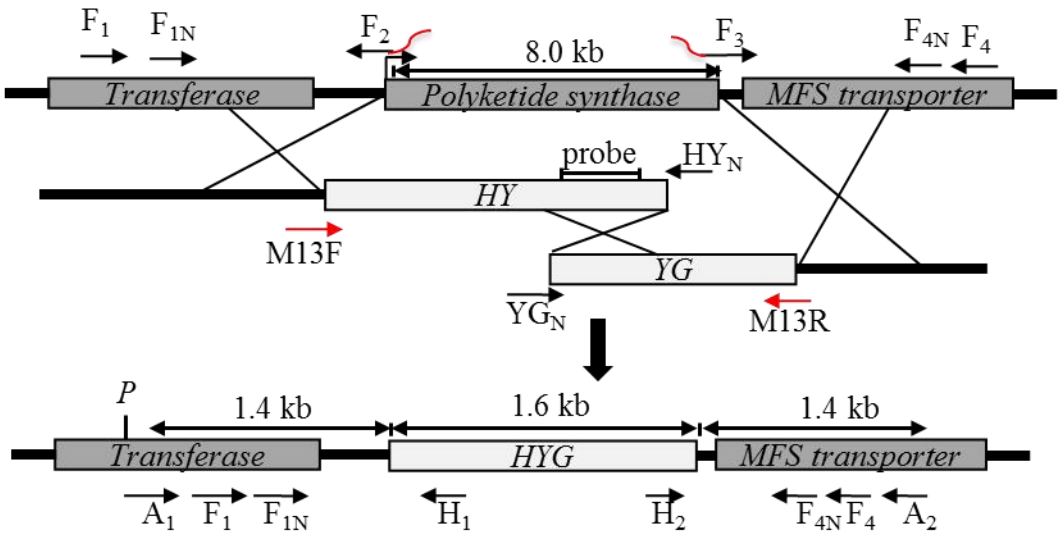


D.

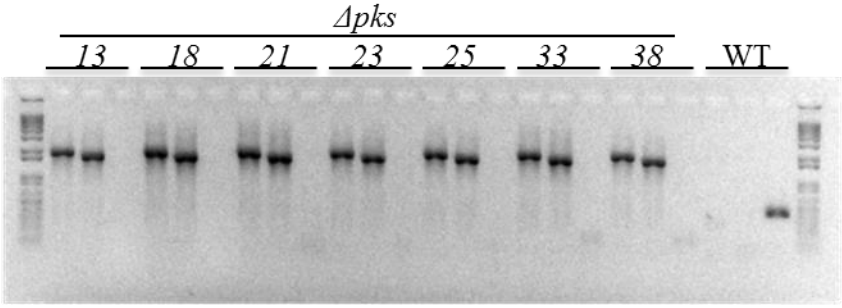


Primers	Size (kb) Δ*p450*	WT
A_1/H_1	1.40	0.00
A_2/H_2	2.00	0.00
pF/pR	0.00	0.46

E.



F.



Primers	Size (kb) Δ*pks*	WT
A_1/H_1	1.7	0.0
A_2/H_2	1.6	0.0
pF/pR	0.0	0.43

Appendix 3.3

Appendix 3.3. G

Table A3.4: List of *ABA3* cluster genes disruption and PCR validation primers

Primer Name	Primer sequence
ABA3-A1	GCCTTATAGCGAAACTAGAGAGTACGAG
ABA3-A2	GAAGTCGGCGAGTCCAGTGAAC
ABA3-F1	GTTAACCTTCTATAACTAATCGTCGGCGTC
ABA3-F1N	CCGTCGATTCTAAGTCGTAATAGGACTAAAG
ABA3-F2	**ATTACAATTCACTGGCCGTCGTTTTAC**CTCTTCCAGCGCTGTGGAT
ABA3-F3	**CGTAATCATGGTCATAGCTGTTTCCTG**TGCATGGGAGTACAGTCG
ABA3-F4	GTTCACGGTTACCGCATCCTC
ABA3-F4N	GTGAGGTCCTCCTCCACGAAG
pskA1	GCATTCTGCCTTCGGTGTAGC
pskA2	AGAACGAAGGGCGTCCATTG
pskF1	CCCACAACCAGCCGATGAG
pskF1N	GAGGAACGCCAAGATACGGC
pskF2	**ATTACAATTCACTGGCCGTCGTTTTAC**ACAGGAAAACGACCCGAT
pskF3	**CGTAATCATGGTCATAGCTGTTTCCTG**CGAGTCCCACAGCGGAGT
pskF4	GACCAATAAACGAACAATCCGACCT
pskF4N	GCGGCTATTGAAGGACCGATA
cypA1	GATGCCGTCGCCTTCTTCA
cypA2	GGAGGCGAAGACCAGGACCA
cypF1	CGCTTCGTGGAGGAGGACC
cypF1N	GAGGATGCGGTAACCGTGAAC
cypF2	**ATTACAATTCACTGGCCGTCGTTTTAC**TGCGGAGAGGCAGGATAG
cypF3	**CGTAATCATGGTCATAGCTGTTTCCTG**TGGGTTGGGGGAGTAGGA
cypF4	TTTGACGAGCATCCCCTGGT
cypF4N	GCCGTATCTTGGCGTTCCTC
M13F	**GTAAAACGACGGCCAGTGAATTGTAAT**
M13R	**CAGGAAACAGCTATGACCATGATTACG**
HY	GGATGCCTCCGCTCGAAGTA
HY-N	TAGCGCGTCTGCTGCTCCATACAAG
YG	CGTTGCAAGACCTGCCTGAA
YG-N	ACCGAACTGCCCGCTGTTTCTC
H1	CTACTGCTACAAGTGGGGCTGA
H2	GTACACAAATCGCCCGCAGAAG
H3	AGGCTTTTTCATTTGGATGCTTGGG
H4	TCTTCTGGAGGCCGTGGTT

Legends to Figures in Appendices

Appendix 3.1 A

Schematic presentation of cosmid library duplication and PCR screen strategy for the identification of bacteria clones with target sequences for cosmid walking. **A.** A replicate of individual bacterial colonies in a 384 well plate were made in four 96 well plates and named according to serial number of the 384 well plate, and the range of wells represented in the replicate plate (e.g. 8(A1-D24)). **B**. All bacterial in the 96 well plate were pooled (P1) for colony PCR with the library screening primers. **C**. When a target sequence is identified in any pool, the bacteria in that 94 well plate were pooled in groups of eight (P2 – e.g. wells 6A to 6H) and screened again with the primers. **D**. Individual bacteria in P2 are screened in a final PCR round to select the bacterial colony e.g. C, bearing the cosmid with the target sequence.

Appendix 3.2

Gene disruption strategy and PCR verification of transformants. **A.** Figure shows the *ABA3* gene locus and targeted deletion by triple homologous recombination approach. **B**. PCR verification of hygromycin resistant colonies to identify mutants with deletion in the *ABA3* gene locus. Primers A1/H1, A1/H3 and A1/M13R were all used to verify the homologous recombination of the HY-fused deletion fragment. Primers A2/H2, A2/H4 and A2/M13F were all used to verify the homologous recombination of the YG-fused deletion fragment. **C.** Figure shows the *cytochrome P450* gene locus and targeted deletion by triple homologous recombination approach. **D**. PCR verification of hygromycin resistant colonies with primers A1/H1 and A2/H2 to identify transformants with the hygromycin resistant cassette insertion at the target locus and with primers pF/pR to confirm deletion of the target cytochrome P450 gene. **E.** Figure shows the polyketide synthase gene locus and targeted deletion by triple homologous recombination approach. **F**. Hygromycin resistant transformants were screen by PCR with primers A1/H1 and A2/H2 to verify insertion of the right and left flank disruption cassettes and primers pF/pR were used to verify the absence of the target gene.

CHAPTER FOUR

ANALYSIS OF PUTATIVE BLUE AND RED LIGHT RESPONSIVE GENES IN *CERCOSPORA ZEAE-MAYDIS*

ABSTRACT

Gray leaf spot of maize is an important yield-limiting foliar disease caused by *Cercospora zeae-maydis*. Diseases caused by *Cercospora* species are of global economic importance because they affect a wide range of food crops and are generally difficult to manage, especially in agricultural settings that rely on minimum tillage. *Cercospora* species respond to several environmental stimuli, including light. Light influences cercosporin biosynthesis, pathogenesis and morphological development in *C. zeae-maydis*, but the molecular factors mediating the effect of light are not completely known. In this study, we describe four novel putative blue light receptors (the *CzmPhot* gene family) and a red light phytochrome homolog in *C. zeae-maydis*. Single deletions in three of the four *CzmPhot* genes and the Phytochrome ortholog had no significant effect on cercosporin biosynthesis and did not affect stomatal infection. However, deletion in one of the four *CzmPhot* genes (*CzmPhot-24*) led to significant reductions in cercosporin biosynthesis and conidiation.

INTRODUCTION

Gray leaf spot (GLS) is a serious foliar disease of maize, a major staple crop grown worldwide. The disease is caused by two *Cercospora* species, *C. zeae-maydis* Tehon & Daniels and *C. zeina* Crous & U. Braun, sp. Nov. in the USA. Both species are pathogenic on maize, and there are no obvious differences in virulence (or infection phenotype) between the two species, although *C. zeina* is not known to produce cercosporin (Lipps, 1998; Godwin *et al.*, 2001). GLS is increasingly becoming a foliar disease of worldwide concern, especially in regions where management practices are constrained by limited management resources. On susceptible hybrids and under favorable conditions, total blighting of leaves can occur, resulting in yield losses exceeding 50% (Lipps, 1998). GLS is difficult to manage, as there are few resistant hybrids, and even those generally express tolerance rather than resistance. The molecular mechanisms underpinning pathogenesis have therefore become an integral part of the efforts to understand and improve management options.

Cercospora species are among the most successful fungal phytopathogens, and the production of cercosporin has been suggested to enhance virulence of species such as *C. beticola*, *C. nicotianae* and *C. kikuchii* (Upchurch et al. 1991; Callahan *et al.*, 1999; Choquer *et al.*, 2005; Staerkel *et al.*, 2013). The biosynthesis of cercosporin is affected by numerous factors and among them light is one of the most critical. Light is required for cercosporin biosynthesis as well as its phytotoxic activity (Daub and Chung, 2009). In *C. beticola*, the absence of light results in almost complete suppression of cercosporin biosynthesis, while a brief exposure of light to cultures grown in darkness is sufficient to induce biosynthesis (Lynch and Geoghegan, 1979). Light also influences morphological development and differentiation such as hyphal melanization, conidiation and pathogenesis in many species of *Cercospora*. For example, in *C. zeae-maydis*, continuous exposure to the full spectrum of light or exclusively to blue light strongly represses conidiation while inducing cercosporin production. Conversely, exposure to complete darkness or red light source results in conidiation and repression of cercosporin biosynthesis (Bluhm *et al.*, 2008; Kim *et al.*, 2011). In *C. kikuchii*, zonate banding of hypha

induced by light entrainment of the circadian clock (Bluhm *et al*., 2010) was used to demonstrate the requirement for light in hyphal development and protection. Interestingly, the blue and red light sensory pathways in *A. nidulans* interact both genetically and physically (Rodriguez-Romero *et al.,* 2010).

Fungi have evolved at least two broad classes of photoreceptors that are able to absorb light in different regions of the spectrum of light: the red light detecting phytochromes and the blue light receptors, cryptochromes (Cry) and white-collar-1 proteins (WC). The phytochromes are red/far-red photochromic biliprotein photoreceptors, which in plants regulate development, but little is known of their function in ascomycete fungi (Blumenstein *et al.,* 2005; Fröhlich *et al.,* 2005; Rodriguez-Romero *et al.,* 2010). On the other hand, *CRY* and the WC-1 protein complex mediate blue light responses. In *C. zeae-maydis,* double mutation of *CRP1* and *PHL1,* the respective orthologs of *WC-1* and *CRY,* did not completely eliminate cercosporin biosynthesis, thus suggesting that other light receptors are involved in the regulation of cercosporin biosynthesis (Kim *et al*., 2011). The goal of this dissertation chapter is to identify and characterize novel components of blue and red light signaling proteins in *C. zeae-maydis*.

MATERIALS AND METHODS

Fungal Isolate and Culture Conditions

C. zeae-maydis SCOH 1-5 was used throughout the experiments as the wild type. Mutant strains were created from the wild type through targeted gene deletions. All fungal cultures were maintained routinely on V8 juice agar at room temperature in the dark to promote conidiation, and transferred every 4 to 5 days to new plates to maintain conidia production for inoculation on maize leaves and for cercosporin analysis. Tissue for genomic DNA was collected from cultures grown in liquid YEPD medium (0.5% yeast extract, 1.0% peptone, and 3% dextrose) or from five- to ten-day-old conidiating cultures. To quantify conidiation, agar cylinders were excised with a cork borer (1 cm diameter) from a four-day-old V8 juice agar culture, were placed in a test tube with 1 ml of sterile water per plug, and were vortexed to dislodge the conidia, which were counted with a haemocytometer. For inoculation, conidia were dislodged from plates flooded with sterile deionized water using an L-shaped colony spreader and filtered through two layers of cheesecloth.

Bioinformatics Analysis

Nucleic acid sequences obtained from sequenced cosmids were manually assembled into contigs with Microsoft Word and Clustal omega to identify consensus regions and overlaps between successive cosmid walks. The assembled sequences were queried with BlastN and BlastX against the *C. zeae-maydis* whole-genome sequence (http://genome.jgi.doe.gov/) to retrieve full-length gene sequences for analysis. Manual annotation of gene ORFs was performed with Softberry FGENESH (Solovyev *et al.,* 2006) with *Stagnospora nodorum, Pyrenophora* or *Leptosphaeria* as the training models as necessary. Homology searches of nucleotide sequences and database searches were performed with the BLAST service provided by the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov/BLAST). Identification of conserved domains and motifs in the protein

sequences was performed with the Simple Modular Architecture Research Tool (SMART) (Schultz *et al.* 1998; Letunic *et al*., 2012) and the conserved-domain database (CDD) (Marchler-Bauer *et al*. 2011), at NCBI and reconstructed with DOG (Ren *et al*., 2009). Secondary structure prediction of domains was performed with Phyr^2 (Kelley and Sternberg, 2009). Multiple sequence alignment of domains was performed with Clustal omega in Seaview v4.4.2 (Gouy *et al.*, 2010) and ESPripts v2.2. (http://espript.ibcp.fr/ESPript/ESPript/). Poorly aligned sections were manually edited and subsequently submitted for phylogenetic tree construction using the distance neighbour-joining method (Saitou and Nei, 1987).

Disruption of Putative Light Receptor Genes

To investigate the function of the *C. zeae-maydis* phytochrome (*Phyto*) and phototropin-like (*CzmPhot*) genes in cercosporin production and pathogenesis, targeted disruption was performed via triple homologous recombination according to the method describe by Fu *et al*. (2006) with modifications. Briefly, freshly germinated conidia were aseptically harvested by centrifugation and digested overnight in 1.2 M KCl solution containing 20 mg/ml lysing enzyme (Sigma# L-1412) and 0.4% v/v β-glucuronidase (Sigma# G-7017) to generate protoplasts. The protoplasts were transformed with split marker gene disruption constructs with 60% PEG-8000 in STC (1.2 M sorbitol, 50mM $CaCl_2$ and 10mM Tris pH 8.0). The disruption fragments were created by fusion PCR as described by Fu *et al*. (2006) and modified by Ridenour *et al*. (2012). Briefly, sequences (0.5 -1.5) kb flanking the gene targeted for deletion were amplified from the wild type strain and fused in a PCR with a split hygromycin resistant gene cassette (Appendix 4.2). Protoplasts were directly transformed with the disruption fragments and hygromycin-resistant colonies were selected on regeneration agar (1.0 M sucrose, 0.02% yeast extract and 1.0% agar) amended with 150 μg/ml Hygromycin-B (Research Products Inter. Corp., Mt. Prospect, IL). The hygromycin-resistant transformants were then maintained on V8 juice agar amended with Hygromycin-B (150 μg/ml) for two successive generations, and subsequently without Hygromycin-B selection, before they were screened by PCR to verify deletion of target

gene. For southern analysis, genomic DNA was probed with a 428 bp region of the hygromycin-resistance gene amplified by PCR with primers HY-N/YG-N from pTA-HYG.

Quantification of Cercosporin Biosynthesis

For analyses of cercosporin biosynthesis, 0.2x PDA plates were inoculated with $1x10^4$ conidia and incubated under appropriate lighting for four to ten days. Colonized agar plugs (1 cm diameter) were removed into 15 ml tubes and extracted with KOH in darkness for 4 – 5 hours as described by Jenns and Daub (1995). Four plugs were sampled from each of three replicate plates at five, seven and nine days post-inoculation and extracted with 4 ml of 5.0 M KOH. Cercosporin concentrations were quantified by measuring the absorbance of the KOH extract at 480 nm and converting to concentration based on the molar extinction coefficient for cercosporin ($\varepsilon = 23{,}300\ M^{-1}\ cm^{-1}$).

Pathogenicity Assays

Pathogenicity assays on maize inbred B73 was performed with conidia collected from inoculated V8-juice agar plates. To enable comparisons between the wild type and mutant strains, leaves of six-week old maize plants were individually spray-inoculated with each strain (approximately 5 ml of a 1×10^5 conidia/ml suspension) as described by Bluhm and Dunkle (2008). The plants were covered with transparent plastic bags to maintain high relative humidity (> 90%) without significantly impeding light transmission and were grown in a 12-12 hour day-night light cycle at 23^oC inside a growth chamber. Observations of disease progression were made periodically from 5 to 15 days post-inoculation. Leaf samples were collected five days after inoculation and examined with a Nikon D-eclipse C1 confocal scanning fluorescent microscope to visualize infectious development.

RESULTS

Light is a critical environmental cue for pathogenesis and cercosporin biosynthesis in *C. zeae-maydis*, the causative pathogen of gray leaf spot of maize. Sequences homologous to photoreceptors from higher eukaryotes, including putative blue-light sensing phototropins and fungal phytochromes, have been identified in EST libraries of *C. zeae-maydis* (Bluhm *et al.*, 2008). In the promoter regions of the cercosporin biosynthesis genes in *C. zeae-maydis*, we identified higher than random occurrences of binding sites for the light-regulated white-collar complex (please refer to Chapter 2, Table 2.2), which are also recognized by the global regulator of nitrogen metabolite repression, AreA. Because of the significance of light to pathogenesis and secondary metabolism, we focused on the identification and characterization of genes putatively involved in photoreception and signaling that potentially mediate the regulation of pathogenesis and cercosporin biosynthesis by light in *C. zeae-maydis*.

Identification of Putative Light Receptor Genes

We sought to identify components of light responsiveness signaling pathways in the genome of *C. zeae-maydis*. For the detection of blue light, three classes of receptor proteins have been described in all kingdoms of life: the cryptochromes, the flavin-binding LOV (light-oxygen-voltage) domain proteins, and the BLUF (blue light-sensing using FAD) domain containing flavoproteins (Losi and Gartner, 2011). LOV domains function as blue light-sensing modules in various photoreceptors in plants, fungi, algae and bacteria. In contrast to blue-light receptors, phytochromes are the only known red/far-red photoreceptors known, which have well-studied roles in plant development (Rodriguez-Romero *et al.*, 2010; Heintzen, 2012). We identified genes encoding a histidine kinase-like phytochrome and an uncharacterized phototropin-like LOV domain protein in *C. zeae-maydis*, both of which were induced by light and had not previously been described in fungi (Bluhm *et al.*, 2008). We named the phototropin-like gene *CzmPhot* on the basis of its initial homology with plant phototropins and hypothesized that the protein was a fungal blue-light receptor involved in the regulation of cercosporin

biosynthesis, pathogenesis, and/or asexual development in *C. zeae-maydis*.

To identify the genes underlying the putative phytochrome and LOV domain-encoding transcripts, primers from each EST identified by Bluhm *et al*. (2008) were designed to screen a cosmid genomic library created from *C. zeae-maydis* strain SCOH1-5 (Appendix 4.1) (Shim and Dunkle, 2003). Cosmids that produced an amplicon were cloned and sequenced with a cosmid-walking approach (Appendix 4.1). The sequences obtained were assembled and queried against the *C. zeae-maydis* genome in BLASTx and BLASTn searches to identify putative paralogs. The search returned one protein-encoding gene for phytochrome and five LOV domain-containing protein-coding genes: *CRP1*, the *C. zeae-maydis* ortholog of *N. crassa WC-1* (Kim *et al.*, 2011) and four other genes, each of which is predicted to encode a phototropin-like LOV domain containing protein. The *CzmPhot* genes were assigned serial number codes to identify each based on the scaffold on which it is located (Table 4.1 and Fig. 4.1. A).

Sequence Analysis of Putative Blue-Light Receptor Genes (*CzmPhot*)

The four *CzmPhot* genes were conceptually translated with the softberry fgenesh webservice (Solovyev *et al*., 2006). BLAST analysis (BLASTx) querying the translated sequence of *CzmPhot-24* against the GenBank nucleotide collection revealed high sequence similarity (E-value of 0.0) with several uncharacterized hypothetical genes in a wide range of fungi. The proteins, which varied in length from 513 to 718 amino acids, contained a single LOV domain and one or more low complexity regions (LCR) near their termini (Fig 4.1. A). Terminal LCR have been hypothesized to play important roles in low-specificity biological events that involve large protein complexes (Coletta *et al*., 2010). In addition, the CzmPhot-24 protein is predicted to contain a coiled-coil (CC) domain in close proximity to the C-terminal end of the LOV domain. Coiled-coil (CC) domains act as protein-protein interaction motifs in transcription factors and receptor kinases (Jakoby *et al*., 2002, Vinson *et al*., 2002, Wang *et al*., 2002).

To explore the phylogenetic relationship among *CzmPhot* genes, a distance

neighborhood-joining phylogenetic tree with 17 proteins from six photosynthetic species and two fungi, *N. crassa* and *C. zeae-maydis*, was inferred from amino acid sequence alignment of their LOV domains (Fig. 4.1. B) with Seaview v4.4.2 (Gouy *et al.*, 2010). The analyses revealed that CzmPhot proteins form a clade within the plant LLP and phototropins and separate from fungal VIVID and WC-1 proteins, which clustered with the ADO/FKF1 /LKP/ZTL family of plant proteins (Fig. 4.1. B). Structural modelling showed that *CzmPhot-6* encoded a protein with a LOV domain that is similar to the LOV1 domain of Arabidopsis phototropin-2, while *CzmPhot-4* encoded a protein that shared structural homology with Arabidopsis PLP LOV domain (Fig. 1. C).

Analysis of the amino acid residues in the putative flavin-binding pocket showed most of the amino acids required for interaction with a flavin chromophore are conserved, except that the key cysteinyl-arginyl residues in the 3_{10}-helix motif (N**CR**FLQ) essential for cysteinyl adduct formation and photoactivation have been replaced by a double serine residue in CzmPhot-44 (Fig. 4.1. D). In addition, residues in the α4 helix (**V/L**AK**IR**) required for flavin binding were less conserved in the CzmPhot proteins.

Sequence Analysis of Putative Phytochrome Red-Light Receptor Gene in *C. zeae-maydis*

Conceptual translation of the *Phyto* gene sequence identified a 4,591 bp ORF interrupted by three introns that is predicted to encoded a 1,416 amino acid phytochrome-like protein kinase with five domains. The predicted protein contains all the conserved features for a functional phytochrome, including an N-terminal photosensory region for bilin binding and a C-terminal output region for signal transmission (Fig. 4.1. E). The N-terminal photosensory region has three domains, the chromophore binding domain (PAS-2), the cGMP-specific phosphodiesterase, adenylyl cyclase and FhlA (GAF) domain, and the phytochrome (PHY) domains. The C-terminus response domains consists of a histidine kinase A (HKA) domain, a histidine kinase-like ATPases (HATP) and signal receiver domain, (REC).

Functional Characterization of Putative Light Receptor Genes

To gain insight into the functions of the putative light receptor genes, the *CzmPhot* genes (*CzmPhot-44, CzmPhot-24, CzmPhot-6 and CzmPhot-4*) and the *Phyto* gene, we performed targeted disruption of the *Phyto* and *CzmPhot* genes via triple homologous recombination (Fu *et al*., 2006) and investigated involvement in cercosporin production and pathogenesis. The gene deletion constructs were constructed by fusion PCR of gene flanks amplified from sequences 0.5 – 1.5 kb up- and downstream of the putative translation start and stop codons. The selectable marker gene (*hygromycin phosphotranferase*) fragments (*HY* and *YG*), to which the gene flanks were fused, were amplified from the plasmid pTA-Hyg (Fig. 4.2. A) and directly transformed into protoplasts of the wild type strain. Hygromycin-resistant transformants were subjected to verification by PCR with primer pairs A1/H1, A2/H2, and pF/pR to probe for the insertion of the up and downstream deletion constructs, and absence of the gene targeted for deletion (Appendix 4.2). At least two independent mutants of each gene were identified by PCR and confirmed to be morphologically indistinguishable during growth on V8 juice agar and 0.2× PDA.

Visual assessment showed that mutants lacking the *CzmPhot-24* gene were significantly different in cercosporin production and colony morphology than the wild type on both 0.2x PDA and V8 agar (Fig. 4.2. B). Six mutants identified by PCR to be lacking *CzmPhot-24* were therefore verified by Southern hybridization to confirm single gene deletion by probing with the *Hyg* gene probe (Fig. 4.2. C). To quantify the difference in cercosporin production, 0.2x PDA plates were inoculated with conidia of two morphologically identical strains of *CzmPhot-24* deletion mutants (Δ*CzmPhot-24-21,* Δ*CzmPhot-24-25*). For comparison, two morphologically identical strains of Δ*CzmPhot-44* (Δ*CzmPhot-44-2,* Δ*CzmPhot-44-3*) and the wild type were included in the experiment. Cercosporin production in Δ*CzmPhot-44* mutants did not differ from substantially from the wild type at five and seven dai, although the Δ*CzmPhot-44-3* strain consistently produced somewhat more cercosporin than the wild type on all three days of sampling (Fig. 4.3. A). On the other hand, mutants of *CzmPhot-24* consistently produced up to 6 fold less cercosporin than the wild type (Fig. 4.3. B).

In contrast, mutants of the *Phyto* gene were not affected in cercosporin biosynthesis compared to the wild type (Fig. 4.4. A). To verify whether deletion of the *Phyto* gene had an effect on red or blue light responses in relation to cercosporin production, specially constructed fungal growth chambers with acrylic filters that exclusively transmitted only blue or red light were utilized. Mutant and wild type strains were grown exclusively in either total darkness or uninterrupted white, blue or red light for nine days. In this assay, the wild type appeared to make more cercosporin under white light that blue, whereas no real difference was detected in the response of mutants to the blue and white light sources (Fig. 4.4. B). In complete darkness or exclusive red light, no difference was observed between the strains and showed that, unlike species described by Daub and Chung (2007), minimal induction of cercosporin still occurred in *C. zeae-maydis* even in complete darkness or in growth under exclusive red light. While this data might give a general view of the role of the *Phyto* gene in cercosporin biosynthesis, it is worth noting that this experiment was not repeated and could benefit from follow-up analyses.

Pre-Infectious Pathogenic Development

Because light is a critical requirement for infection (Kim *et al.*, 2011) we inoculated and incubated plants in high humidity for five days and performed fluorescent microscopic examination on the leaves to determine whether deletion of *Phyto* or *CzmPhot* genes alters pre-penetration infectious development. Leaves sampled from the inoculated plants five days after inoculation were cut into sections and stained in congo red dye to enable fluorescence detection of the fungal mycelia under green light excitation. Similar to the wild type, all mutants (*CzmPhot* and *Phyto*) produced appressoria over stomata and occasionally, (but more frequently than the wild type) the *Phyto* mutants formed appressoria that were not over stomata (Fig. 4.5. A-B). No quantitative data was obtained for this observation.

Conidiation and General Morphology

The morphology of the Δ*CzmPhot-24* strains on V8 and 0.2x PDA prompted us to

examine the strains for the effect of gene deletion on conidiation. On both media, Δ*CzmPhot-24* strains had an elevated colony appearance compared with wild type and the other mutants. On V8, the colonies had a more greenish appearance and did not produce the typical whitish vegetative mycelia of the wild type that is associated with aged cultures (Fig. 4.6. A). Viewed under the stereoscope, the mutants appeared to have increased conidiophore production than the wild type, although fewer conidia appeared to be formed. To verify this observation, production of conidia in the Δ*CzmPhot-24* mutants was quantified in strains grown exclusively in either total darkness or in blue, red, or white light for four uninterrupted days. In complete dark or in red light, the wild type produced about 1.7 fold more conidia/plug than the mutants. In blue or white light, no spores were produced in the mutants, while the wild type produced barely few.

DISCUSSION

Phototropin and phytochrome gene orthologs are present in many fungal species but have no known physiological function. In this study, we demonstrate that *C. zeae-maydis* possesses a putative blue-light receptor encoded by *CzmPhot-24* that has a role in cercosporin biosynthesis and asexual development. Through BLAST homology searches, we observed several fungal species have sequences with homology to the *CzmPhot-24* gene sequence. The wide distribution among fungi implies that the CzmPhot-24 protein may play a broad role in common physiological response of fungi. Comparison with amino acid sequences of phototropins and plant LLPs revealed significant similarities and conservation of residues required for photocyclic function in the LOV domain. LOV domains function as blue light-sensing modules in various photoreceptors in plants, fungi, algae, and bacteria (Kasahara *et al.*, 2010). Thus, this sequence conservation suggests that fungal homologs may be evolutionarily related to plant blue-light receptors and have a biological function in light signaling.

For many proteins that function as signaling molecules, an effector or DNA binding domain is usually necessary for signal output function. In the CzmPhot proteins, no effector or DNA binding domains were identified, but the proteins contain one or more terminal LCRs that are frequently found as homo- and hetero-dimerization motifs in transcription factors and proteins involved in signaling complexes (Iakoucheva *et al.*, 2004). This implies that although the CzmPhot proteins lack components of the typical signaling architecture, the presence of protein-protein interaction motifs and the photosensory domain likely implicates the CzmPhot proteins as components of a signaling complex, possibly similar to the two-component signaling system of bacteria. Incidentally, the Arabidopsis LLP proteins, with which CzmPhot-24 shares significant LOV domain homology, also lack effector domains but exhibit blue light modulated binding affinity towards other proteins in yeast two-hybrid systems (Ogura *et al.*, 2008). Reversible photocyclic binding of the flavin chromophore in Arabidopsis LLP homologs of

tomato and the moss *P. patens* have been demonstrated (Kasahara *et al*., 2010), strongly suggesting that these short sensor proteins function as photoreceptors in plants. A blue light mediated function for *CzmPhot* proteins in *C. zeae-maydis* is therefore feasible. The induction of *CzmPhot-24* expression by light has previously been documented, and the possible existence of multiple blue light receptors besides Phl1 and Crp1 in *C. zeae-maydis* has been predicted (Bluhm *et al*., 2008; Kim *et al*., 2011). We therefore hypothesized that the *CzmPhot-24* gene (and probably its paralogs *CzmPhot-44*, *CzmPhot-4* and *CzmPhot-6*) encode novel blue light signaling proteins in *C. zeae-maydis*.

We performed deletions of the *CzmPhot* genes individually to evaluate changes in cercosporin biosynthesis and stomatal infection on maize leaves. Cercosporin biosynthesis and stomatal infection in *C. zeae-maydis* require the presence of light. Mutants in the *CzmPhot-44* gene had no obvious changes in cercosporin or stomatal infection phenotypes compared with the wild type. This was expected given that the essential cysteinyl-arginyl residues in the LOV domain that are required for light-driven cysteinyl adduct formation and conformational changes were not conserved in *CzmPhot-44* (Fig.4.1 D). The importance of the cysteine residue conservation has been repeatedly demonstrated, whereby non-conserved substitution leads to a loss of protein function both *in vitro* and *in vivo* (Salomon *et al.*, 2000; Swartz *et al*., 2007; Kasahara *et al.*, 2010; Djouani-Tahri el *et al*., 2011). Similarly, deletion of *CzmPhot-6*, which shared very close homology with the LOV1 domain of phototropin-2, did not lead to any obvious gain or loss in phenotype. This was not totally unexpected given that the LOV1 domains of phototropins appear to have a dispensable role in light-regulated functions (Matsuoka and Tokutomi, 2005). It is also possible that the *CzmPhot-44*, *CzmPhot-4* and *CzmPhot-6* genes, which had no stomatal infection or cercosporin biosynthesis phenotype upon deletion, will have a role in oxygen or voltage sensing (Butler, 2013). Deletion of *CzmPhot-24*, however, resulted in significant reduction in cercosporin and conidiation. This suggests a regulatory role for the *CzmPhot-24* gene in cercosporin biosynthesis and asexual reproduction, two phenomena

regulated inversely by blue light in *C. zeae-maydis.*

Whereas three classes of photoreceptors have been described for blue light, phytochromes are the only known red/far-red light receptors to date (Franklin and Quail, 2010; Chen and Chory, 2011) and only a handful of fungal orthologs have been characterized among ascomycetes (Rodriguez-Romero *et al*., 2010). In *C. zeae-maydis*, deletion of the putative ortholog resulted in no obvious cercosporin or pre-infection development phenotypic change. This lack of phenotype is not surprising given that Bluhm *et al*. (2008) had previously reported the absence of significant induction of expression of the phytochrome-like gene under light stimulation. Also, in ascomycetes where the putative orthologs have been characterized, no phenotype has been associated with the gene except in *A. nidulans*, where an ortholog of the gene, *FphA*, has been shown to have a role in reproduction and secondary metabolism (Idnurm and Heitman, 2005; Fröhlich *et al.*, 2005; Purschwitz *et al*., 2008).

To determine whether these putative photoreceptors have a role in pathogenesis in *C. zeae-maydis*, histological examination of pre-infectious development on maize leaves was performed on all mutants. All mutants produced appressoria five days after inoculation on maize leaves. This finding suggests that the *Phyto* and *CzmPhot* genes may not have a role in pre-infection development although they could have a role in virulence of the pathogen. Observations of plants inoculated with Δ*Phyto* or Δ*CzmPhot-24* strains showed wide variability in lesion sizes within the same strain (Fig. 4.7). Unlike Δ*CzmPhot-24* strains (Δ*CzmPhot-24-21 and* Δ*CzmPhot-24-25*) used for this study, ectopic disruption of genes in the Δ*Phyto* strains was not verified. This will therefore be essential to resolve the cause of wide variability in virulence phenotype observed among the Δ*Phyto* strains. The inconsistent and wide range of variability observed in the symptoms made it impossible to determine whether the *Phyto* and *CzmPhot-24* genes have a role in regulation of pathogen virulence. Notwithstanding, the significant reduction in conidiation of the *CzmPhot-24* mutants suggests that the gene has significance in GLS as successful conidiation is a critical part of the disease cycle.

Conclusion

Cercospora zeae-maydis possesses a putative blue-light receptor gene with a LOV domain, orthologous to phototropins, which has not previously been described in fungi. This gene, *CzmPhot-24*, regulates cercosporin biosynthesis and conidiation. The expression of the gene is dispensable for stomatal infection but might have a role in fungal virulence. The putative *Phyto* gene and the *CzmPhot-44*, *CzmPhot-6* and *CzmPhot-4* genes in *C. zeae-maydis* do not appear to have any detectable phenotype in relation to cercosporin biosynthesis and stomatal infection, although further work is required to draw a valid conclusion on their role in virulence.

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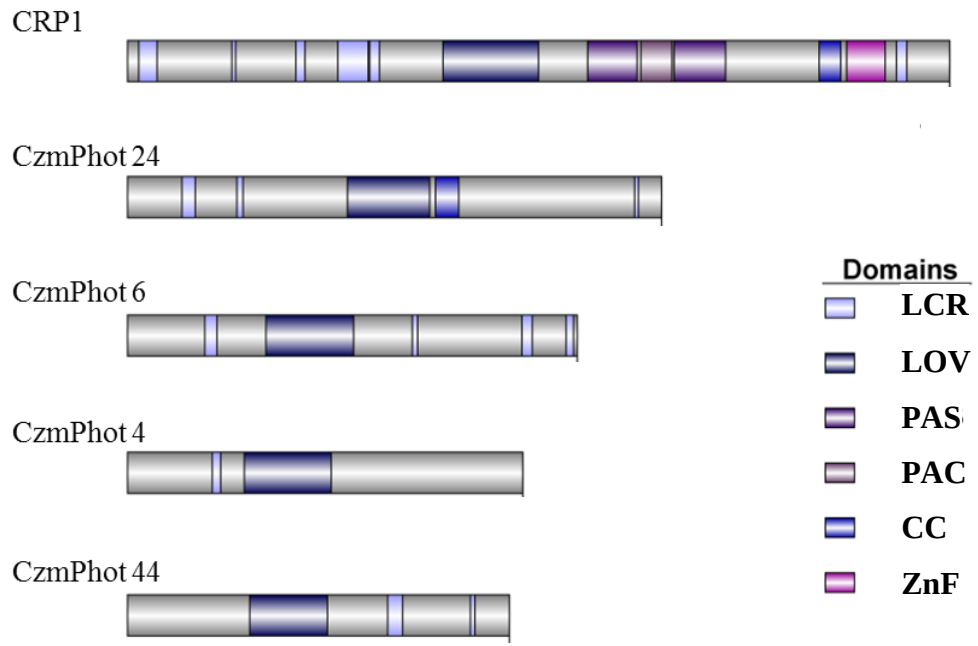
TABLES

Table 4.1. Characteristics of phototropin-like LOV domain-genes in *C. zeae-maydis*

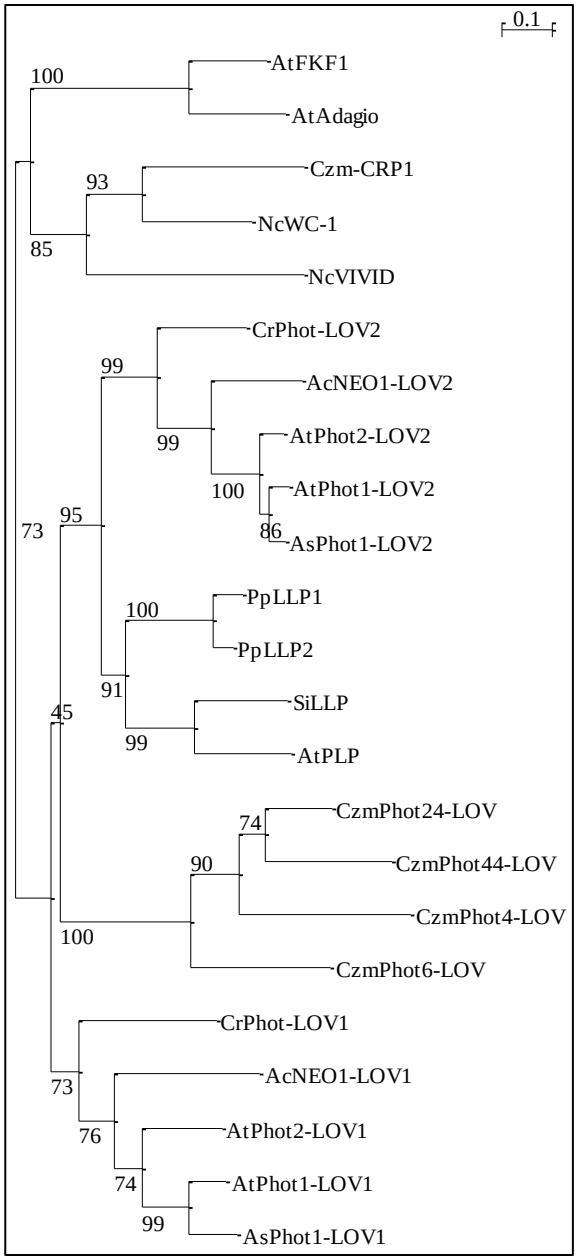
Gene Name	Scaffold #: Locus Identifier	Gene length (bp)	No. of introns	Protein length	Best NCBI Blast Hit (Acc #)	E-value	% ID
czmPhot-44	44 : 99819-101468	1,650	2	513	hypothetical protein *Mycosphaerella populorum* (EMF14092)	0.0	55
czmPhot-24	24: 83370-85581	2,212	1	718	hypothetical protein *Mycosphaerella populorum* (EMF17109)	0.0	79
czmPhot-4	4: 311941-313645	1,705	2	532	hypothetical protein *Mycosphaerella populorum* (EMF14069)	0.0	62
czmPhot-6	6: 504308-506122	1,815	0	605	hypothetical protein *Mycosphaerella fijiensis* (EME89475)	0.0	63

FIGURES AND FIGURE LEGENDS

A.

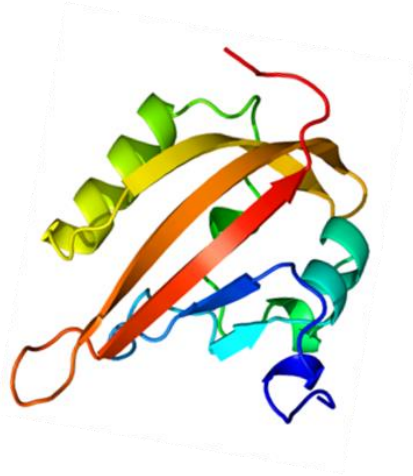

CRP1
CzmPhot 24
CzmPhot 6
CzmPhot 4
CzmPhot 44
Domains
LCR
LOV
PAS
PAC
CC
ZnF

B.


0.1
AtFKF1
AtAdagio
Czm-CRP1
NcWC-1
NcVIVID
CrPhot-LOV2
AcNEO1-LOV2
AtPhot2-LOV2
AtPhot1-LOV2
AsPhot1-LOV2
PpLLP1
PpLLP2
SiLLP
AtPLP
CzmPhot24-LOV
CzmPhot44-LOV
CzmPhot4-LOV
CzmPhot6-LOV
CrPhot-LOV1
AcNEO1-LOV1
AtPhot2-LOV1
AtPhot1-LOV1
AsPhot1-LOV1
100
93
85
99
99
100
86
73
95
100
91
99
45
74
90
100
73
76
74
99

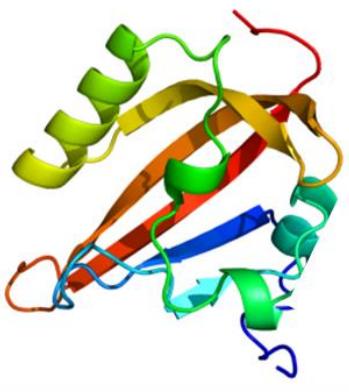
C.

*Czm*Phot-6



*Czm*Phot-4

*Czm*Phot-24



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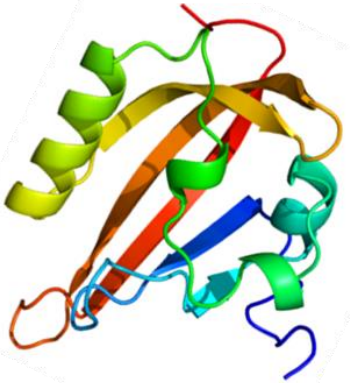
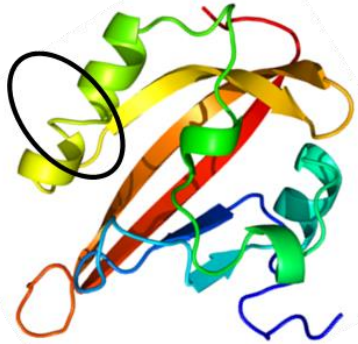


*At*Phot2-LOV1

*At*PLP

*At*Phot2-LOV2



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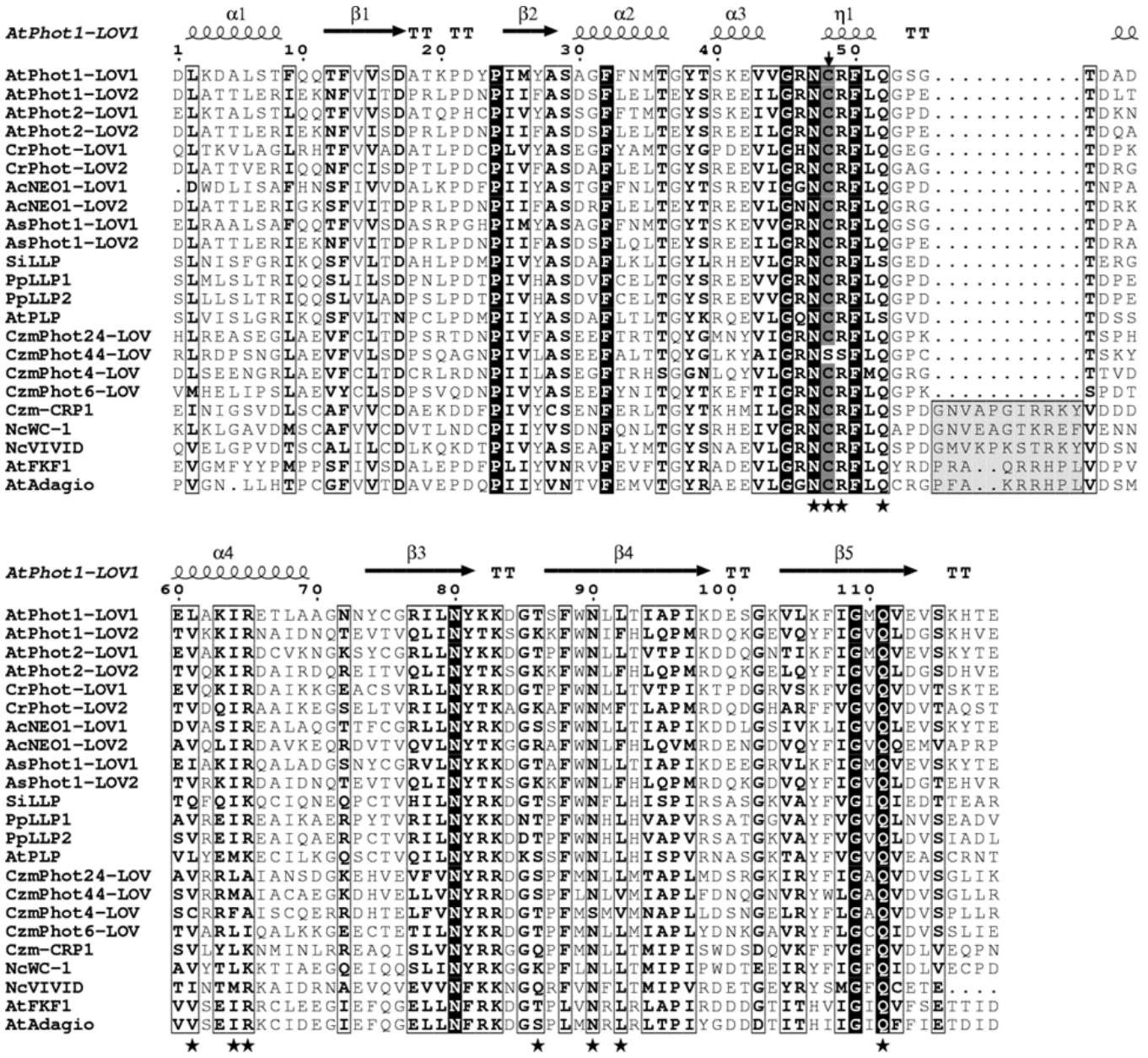
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AtPhot2-LOV1
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CrPhot-LOV1
CrPhot-LOV2
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AcNEO1-LOV2
AsPhot1-LOV1
AsPhot1-LOV2
SiLLP
PpLLP1
PpLLP2
AtPLP
CzmPhot24-LOV
CzmPhot44-LOV
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CzmPhot6-LOV
Czm-CRP1
NcWC-1
NcVIVID
AtFKF1
AtAdagio

E

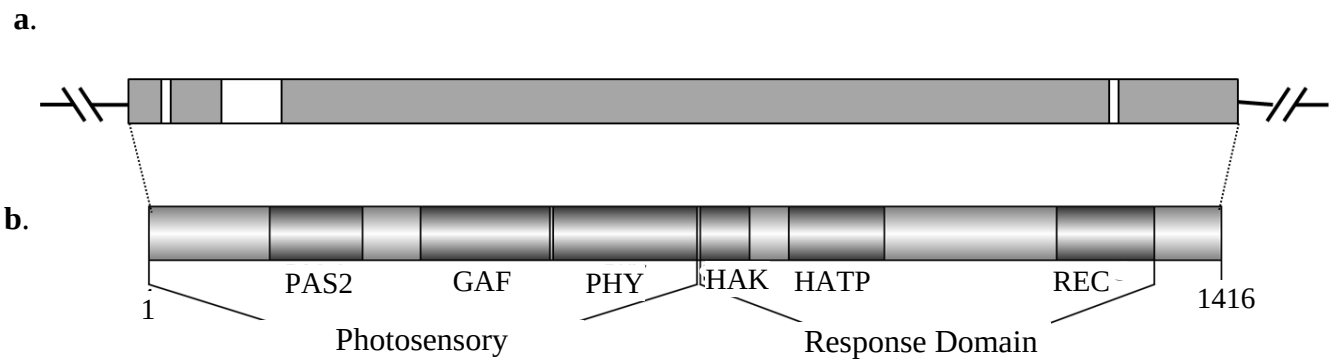
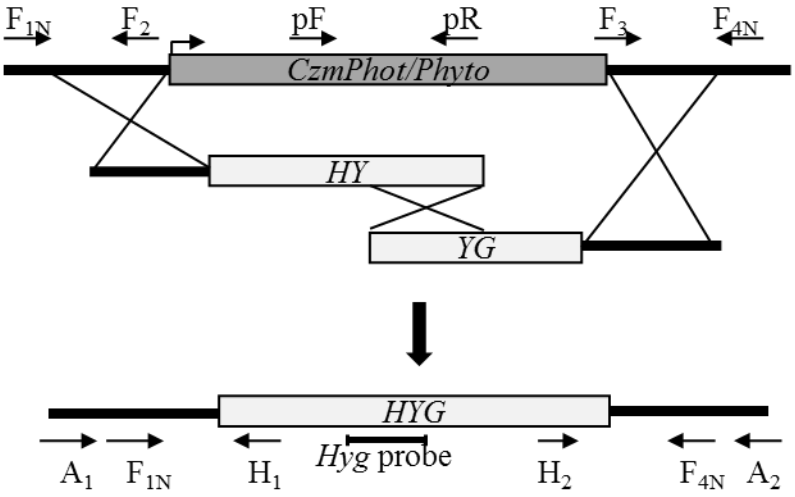
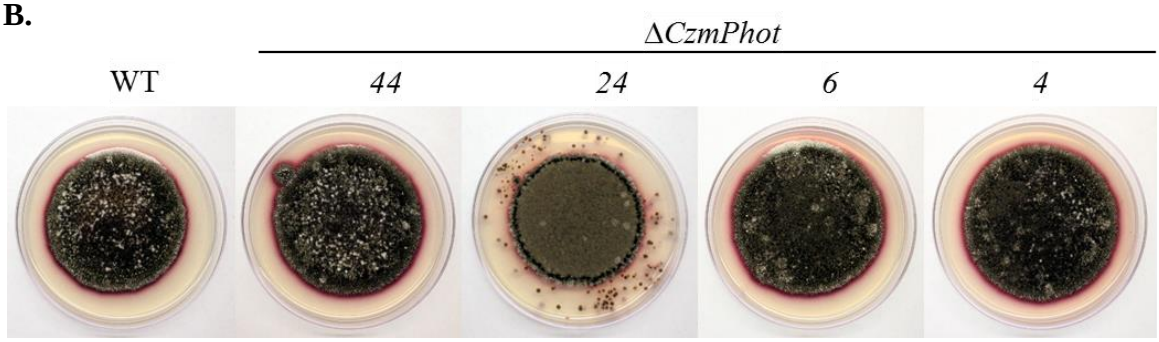

a.
b.
PAS2
GAF
PHY
HAK
HATP
REC
1
1416
Photosensory
Response Domain

Fig. 4.1

A.


F1N
F2
pF
pR
F3
F4N
CzmPhot/Phyto
HY
YG
HYG
Hyg probe
A1
F1N
H1
H2
F4N
A2

B.


ΔCzmPhot
WT
44
24
6
4

C.

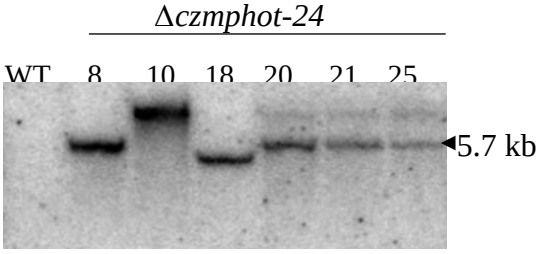
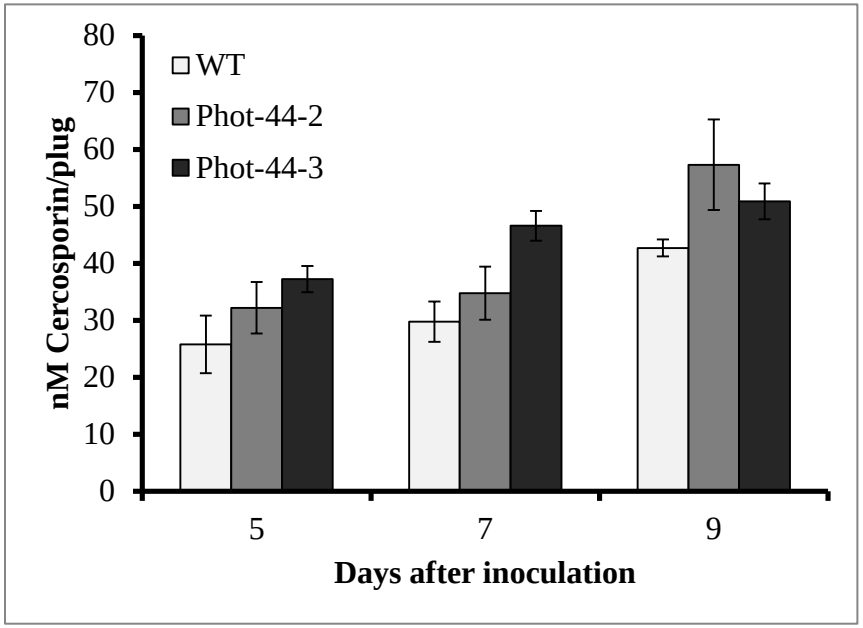

Δczmphot-24
WT
8
10
18
20
21
25
5.7 kb

Fig. 4.2

A.


80
70
60
50
40
30
20
10
0
WT
Phot-44-2
Phot-44-3
nM Cercosporin/plug
5
7
9
Days after inoculation

B.

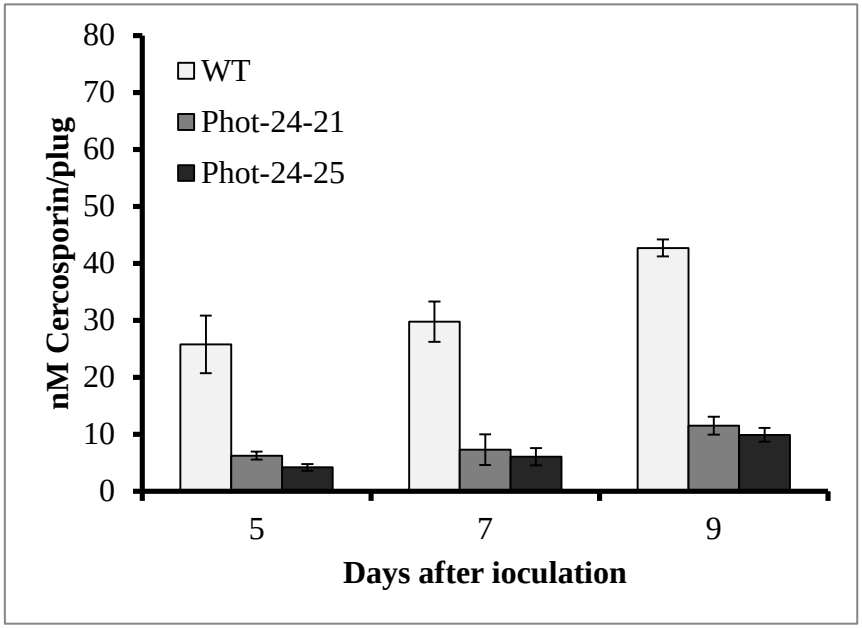
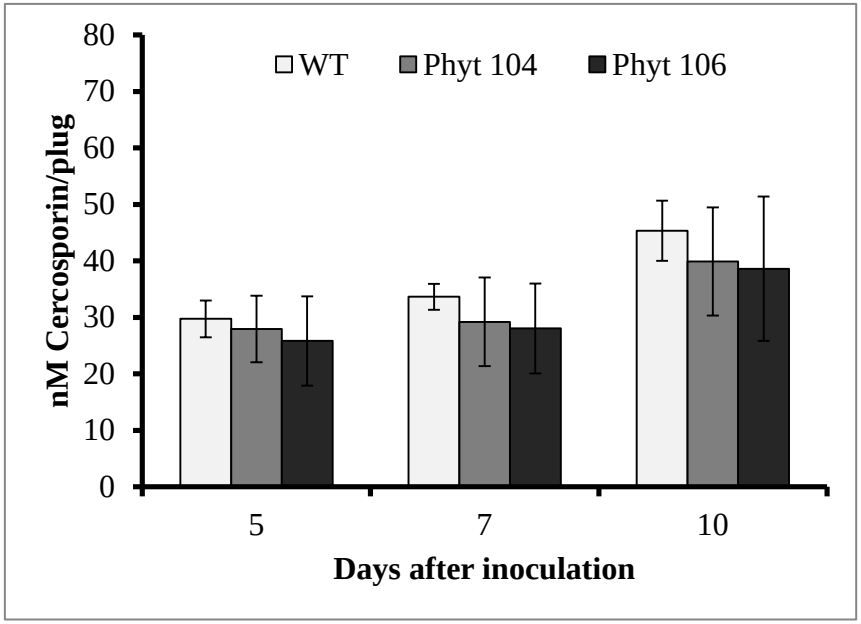

80
70
60
50
40
30
20
10
0
WT
Phot-24-21
Phot-24-25
nM Cercosporin/plug
5
7
9
Days after ioculation

Fig. 4.3

A.


80
70
60
50
40
30
20
10
0
WT
Phyt 104
Phyt 106
nM Cercosporin/plug
5
7
10
Days after inoculation

B.

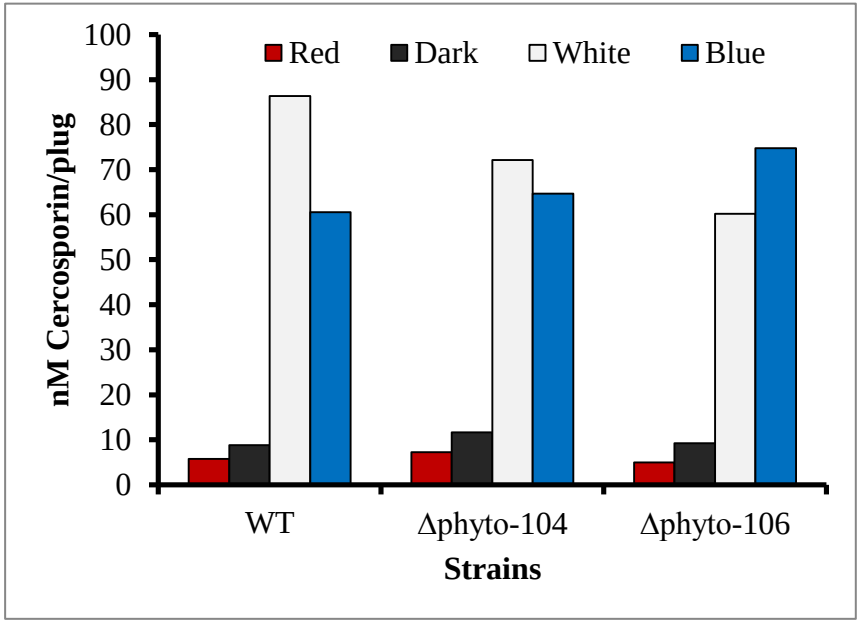
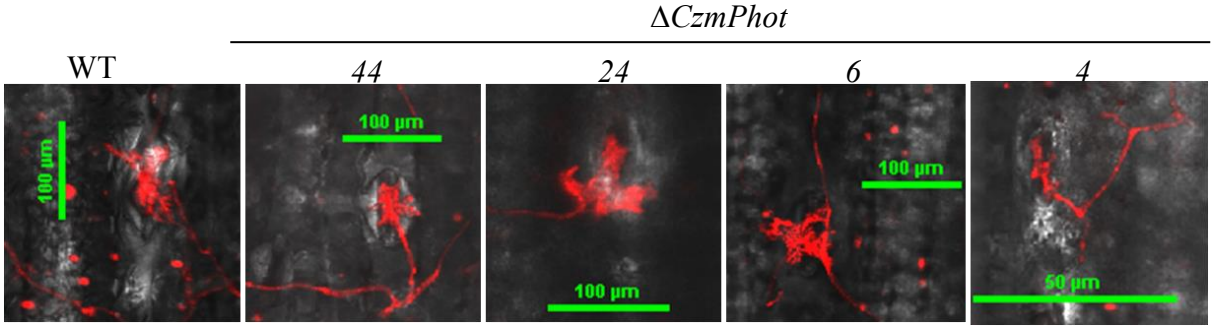

100
90
80
70
60
50
40
30
20
10
0
Red
Dark
White
Blue
nM Cercosporin/plug
WT
Δphyto-104
Δphyto-106
Strains

Fig.4.4

A.


ΔCzmPhot
WT
44
24
6
4
100 µm
100 µm
100 µm
100 µm
50 µm

B.

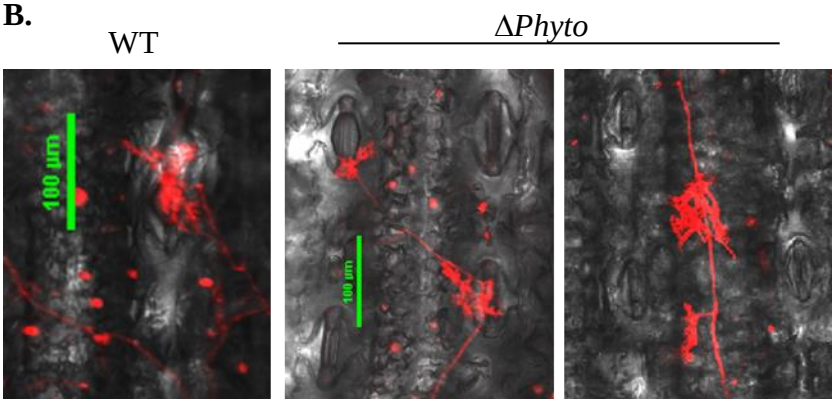
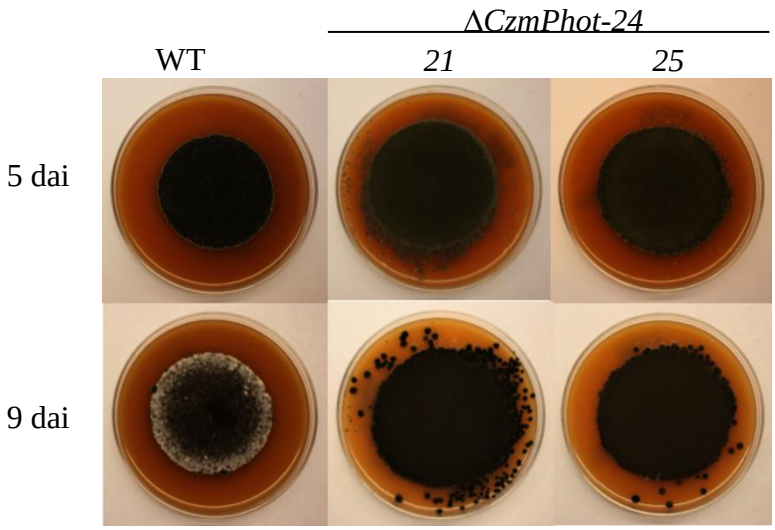

WT
ΔPhyto
100 µm
100 µm

Fig. 4.5

A.


ΔCzmPhot-24
WT
21
25
5 dai
9 dai

B.

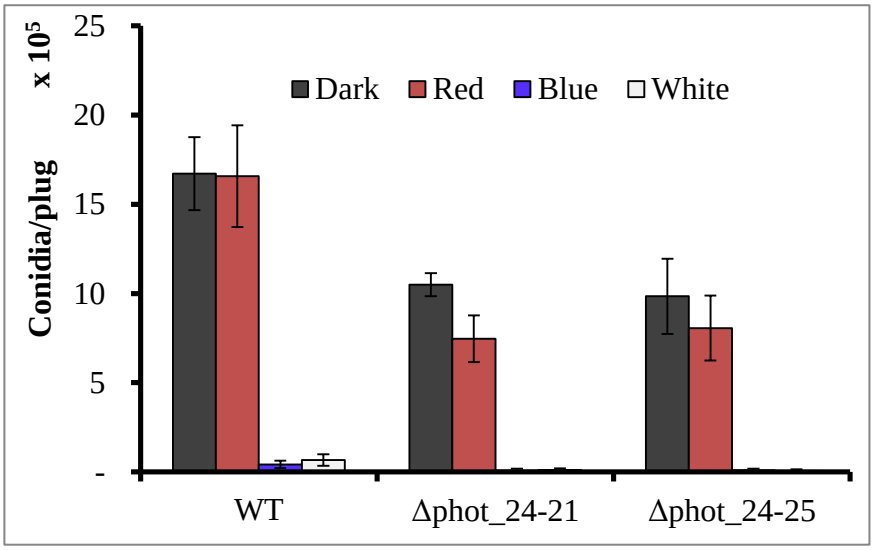

Conidia/plug x 10⁵
25
20
15
10
5
-
Dark
Red
Blue
White
WT
Δphot_24-21
Δphot_24-25

Fig. 4.6

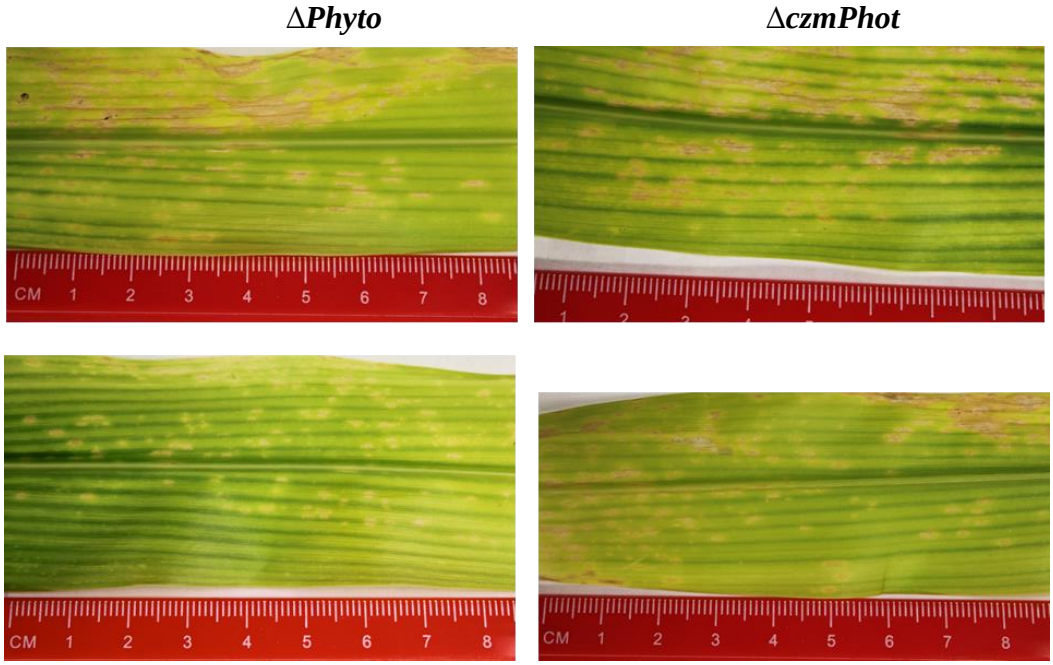


Fig. 4.7

Figure legends

Fig. 4.1.

Analysis of putative light response genes in *C. zeae-maydis* genome. **A**. Domain architectures of conceptually translated putative blue-light receptor proteins reveal the presence of a single LOV domain in the CzmPhot proteins. **B**. Neighborhood-joining phylogenetic tree of LOV domains from selected protein families from *N. crassa* and land and freshwater photosynthetic organisms. {Nc, *N. crassa*, Cr, *Chlamydomonas reinhardtii* At, *Arabidopsis thaliana*; Ac, *Adiantum capillus-veneris*; As, *Avena sativa;* Pp, *Physcomitrella patens;* Sl, *Solanum lycopersicum*. AGI numbers and accession numbers are as follows: NcVIVID, AAK08514; NcWC-1, CAA63964.2; Czm-CRP1, HQ646376 CrPhot, AJ416557; AtPhot1, At3g45780; AtPhot2, At5g58140; AtAdagio1, NP_568855.1; AtFKF1, NP_564919.1; AtPLP, NP_849928.1; AcNEO1, AB012082; AsPhot1, AF033097; PpLLP2, AB576161; PpLLP1, AB576160; SlLLP, AB576162}. **C**. Comparison of LOV domains from *C. zeae-maydis* and Arabidopsis phototropin-2 and PLP. **D**. Alignment of LOV domains encoded by putative blue-light receptor genes in *C. zeae-maydis* and four families of plant and fungal LOV domain proteins (phototropins, FKF/ADO, WC-1, VIVID). Residues that are identical in most LOV domains are shown in bold on white background while residues that are similar across all LOV domains are shown in white on black background. Stars mark the 11 FMN-interacting residues found in the LOV1 domain of Arabidopsis phototropin1. Cysteine residues essential for FMN–cysteinyl adduct formation are identified by an arrow pointing from the top and on a dark gray background. The ADO/FKF1, CRP/WC-1 and VIVID-specific amino acid insertion is represented in a light-gray background between the η1 and α4 helices surrounded by a line frame. For simplicity, the first residue of each LOV domain was numbered as the first residue. Secondary structure elements [α-helices, 3_{10}-helices, β-strands and strict α and β-turns denoted as α, η, β, TTT and TT, respectively] are indicated at the top of the respective residues forming the elements. Multiple sequence alignment was performed with ClustalΩ in Seaview (v4.4.2) and visualized with ESPript 2.2. **E**. Depiction of domain architecture for the putative red-light sensory protein phytochrome in *C. zeae-maydis*. (PAS-2) Per-ARNT-Sim, (GAF) cGMP-specific phosphodiesterases, adenylyl cyclases, and FhlA (PHY) phytochrome, HKA (histidine kinase A), HATP (histidine kinase-like ATPases) and REC, (signal receiver domain).

Fig. 4.2

Gene deletion strategy. A. The *CzmPhot* and *Phyto* genes were deleted by split marker triple homologous recombination to create the mutants. The presence or absence of the targeted genes were verified by PCR with A1/H1, H2/A2 and pF/pR according to the method described by Fu *et al*., (2006). **B**. Cercosporin production phenotype of *CzmPhot* mutants. **C**. Mutants of *CzmPhot-24* verified for gene deletion by PCR were verified by southern hybridization with a *Hyg* probe to determine if there were ectopic mutations. A single 5.7 kb band confirmed single

gene deletion.

Fig. 4.3

Cercosporin analysis. **A.** *C. zeae-maydis* strains were grown on 0.2x PDA under constant light between four and ten days and the level of cercosporin accumulation was measured by the absorbance of alkali extracts at 480 nm. This experiment was repeated two times. Data plotted are the means and error bars represent confidence intervals at 95%.

Fig.4.4

Cercosporin analysis. **A.** *C. zeae-maydis* trains were grown on 0.2x PDA under constant light between four and ten days and the level of cercosporin accumulation was measured by the absorbance of alkali extracts at 480 nm. **B**. Inoculated 0.2x PDA plates were incubated in specially constructed boxes covered with plexiglass acrylic sheets to provide blue or red light exclusively. Samples were incubated for 9 days. Data plotted are the means of two technical plate replication without any repeat.

Fig. 4.5

Representative confocal pictures of pre-infection appressoria development of *CzmPhot* (A) and *Phyto* (B) deletion mutants.

Fig. 4.6

Mutants in the *CzmPhot-24* gene produced fewer conidia and did not produce the whitish mycelia associated with over-aged cultures of the wild type 9 days after inoculation on V8 in the dark.

Fig. 4.7

Representative samples of lesions observed with Δ*CzmPhot-24* and Δ*phyto* inoculated maize leaves. (Δ*phyto* used were not confirm by southern hybridization)

APPENDICES

Appendix 4.1

Tables A4.1: List of cosmids and library screening primers

Primer ID	Primer sequence	Cosmids sequenced for *CzmPhot-24*			
		5A [4(A-D)]	5C [4(A-D)]	5D [4(A-D)]	5E [4(A-D)]
PhotScr	GGATTGAAGAATGCCGGGGA	x	x	x	x
PhotF1	ATTGGCAGAAATTGTCGTTC	x	x	x	x

Primer ID	Primer sequence	Cosmids sequenced for *Phyto*	
		10A [10(E-H)]	1C [12(E-H)]
PhytoF1	GATGTGTCGGCTCGTGGGAGAT	x	x
PhytScr	GCTTCTTTCTGCCGCCACAT	x	x

1. Initial *CzmPhot-24* and *Phyto* sequences were obtained from sequencing of plasmids pDB143 and pDB145 respectively with primer M13R
2. Primers PhotF1 and PhytoF1 were previously available in Laboratory stock.

>Plasmid pDB143 (*CzmPhot-24* sequence length 774 bp)

NNNNNNNNCCGCANGCCGTTCGCAGGTTGGCCNTTGCCNACTCCGATGGCAAAGAACACGTTGAGGT
GTTCGTGAATTATCGCAGAGATGGGAGCCCATTCATGAACCTCTTGATGACTGCGCCCTTGATGGACA
GCCGTGGAAAGATCAGATACTTCATCGGTGCCCAAGTGGACGTTTCTGGTCTGATCAAGGACTGTAGC
GAAATGGAGGGACTTGTACATTTGCTCGAAAGAGAGCGAGCAGAACGCGAAGGCCAAGAACTTGCGG
ATGAGCACAAAGACGAATTCCGAGAACTAAGCGAAATGTTCAATGGCGCCGAGCTAGACACTGTTCG
AAAATATGGCGGGCGCATGCACAAGGAGTATGTGGACGACAGCGACCGCGAGAGCGTACATCGGCCT
CGTTTGTTGCTCAAAGACCCAAGCAATGAGGCGGTGGAAAAGCAGCGCAGCATGTCCAACGACAGCG
CTTCAGCTTTCAAGGAGCGTCTCAACGGCAGACTAGAAGGAGTGTACCAGAATGTGAGTTGGAAGAA
CAAGTCGAATTTGTTCGTGTGCCACAGGCTGACATACCTGACAGTATTTGCTTATCCGGCCGGCACCCT
CATTGCGGATCCTCTTTACATCACCCTCGCTGCGA**GTCCCCGGCATTCTTCAATCC**CCATTCCTGAAT
CACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAG
CTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTANTCNNNNNNNNNNNNNTNNNNNNNNNN

>Plasmid pDB145 (*Phyto* 700 bp)

NNNNNNNNNCGAGCGANTTTCGTACGCCTCGCGGTTGCNAGCACGTAAGTTGATCAACACTGTGCCCA
CACAGAGTAATCCATCTGGCTACATCATCGCATCTTCCGAGGATCTCCTGAAGCTGTTTGACGCCGACT
GTGGCATGTTATCCATCCGCGGAGAAACCAAAATACTTGGTCACCTCGAGCATTCACAAGAGGCCTTG
GCGATGTTGGAATACCTACGCATGCGATCTGTAACGAACGTCATGACCTCGCAGGACATCAGGGAGGA
TTTCCCCGACCTTCGATACGCCCCTGGGTTCAAGGAGATTGCTGGTCTCCTACTTGTGCCTCTGTCTGTT
GGAGGCCAGGACTTTATTGTATTCTTCCGGAAAGGGCACTTGAAAGAAGTCAAATGGGCTGGCAATCC
TTACGAAAAATACGTCAAAGAAGGGACCGAAGGCTACCTCGAACCCCGAAAGAGTTTCCGCACGTGG
AGTGAAATGGTTGTGGGGAAGTGCCGCGAGTGGTCAGAAGAGGAAATCGAGACTGCGGCGGTTCTTT
GCTTAGTCTATGGAAAATTCATCGAG**ATGTGGCGGCAGAAAGAAGC**TGCAATCACTAGTGAATTCGC
GGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATA
GTGTCACCTAANTAGCTTGGCGTAATCNTGNNNNNN

Table A4.2: List of cosmids sequenced and sequencing primers

Primer ID	Primer sequence	Cosmid sequenced				
		5C 4(A-D)	5D 4(A-D)	5E 4(A-D)	10A 10(E-H)	1C 12(E-H)
PhotrW1	CACCTCAACGTGTTCTTT	5’	5’			
PhotfW1	CAGCGAGGGTGATGTAA	3’	3’			
PhotRW2	CGACGCTTGTGTCCAGC	5’	5’			
PhotFW2	TCCTCTTTACATCACCCT	3’	3’			
PhotRW3	CCAGGACGCAAAAGACC	5’				
PhotFW3	CGAAACGGGGCAAAACC	3’	3’			
PhotRW4	GAGCCACTCTTGTGCTGT	5’		5’		
PhotFW4	GGAGGGGTGCGAAGAAC	3’	3’	3’		
Gap1	CTCCTTCGCCAGCCTCGT	5’				
Gap2	TGACAGCACAAGAGTGG			3’		
PhytGap1	TGTCCCGTGTTTCCTGAT					5’
PhytrW1	ATGTGGCGGCAGAAAGA				5’	5’
PhytfW1	CACAGTCGGCGTCAAAC				3’	3’
PhytRW2	GTTCACGGGGTCGTCTTG				5’	5’
PhytFW2	GCTCCGTCTGAAATCCG				3’	3’
PhytRW3	GTTCACGGGGTCGTCTTC				5’	5’
PhytFW3	TCGTCACAGACCCACAA				3’	3’
PhytR4	GCACTGCTGTTCGTGTTC				5’	5’
PhytW4	GATGGCTGGACGTTGCT				3’	3’
PhytGap1	GCGAGCGATCCGATGGC					3’

Sequences assembled.

Total *CzmPhot-24* sequences obtained by cosmid walk = 4,651 bp.

Total *Phyto* sequences obtained by cosmid walk = 6,512 bp.

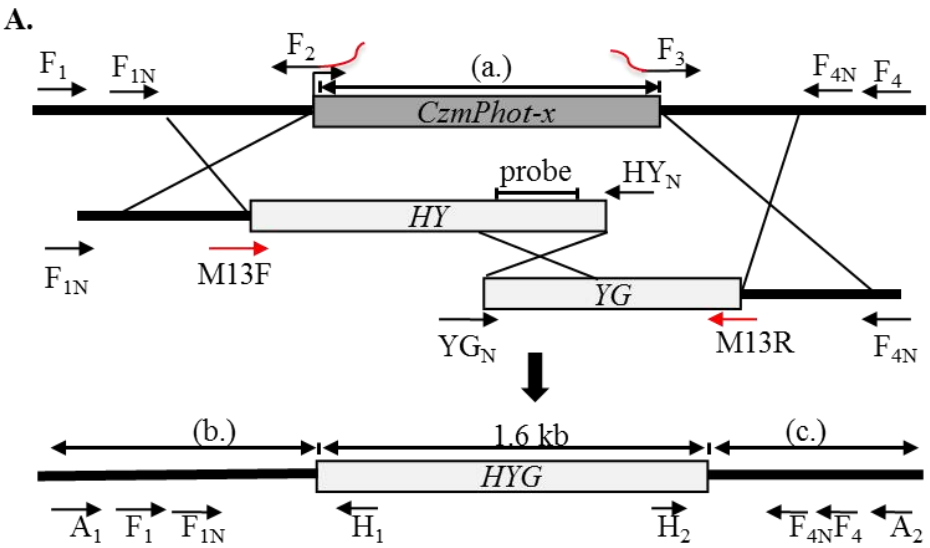
Table A4.3: List of *CzmPhot* and *Phyto* genes disruption and PCR validation primers

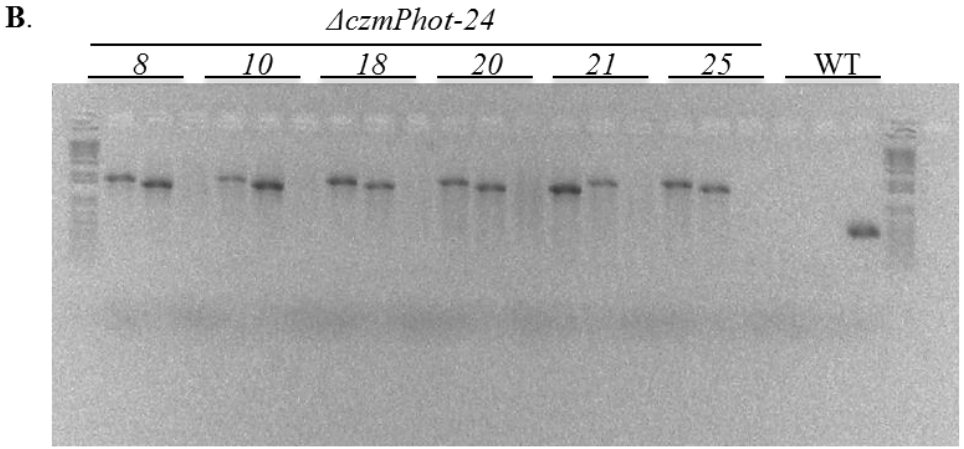
Primer Name	Primer sequence
Phot-S44_A1	GTGAGGAAACGGGGCAGATG
Phot-S44_F1	TGCCTCTTCCACCGCTTACA
Phot-S44_F1N	CACGGGAACTGAGGATGAAGG
Phot-S44_F2	**ATTACAATTCACTGGCCGTCGTTTTA**CGGATGTGGTACGGGTCG
Phot-S44_F3	**CGTAATCATGGTCATAGCTGTTTCCTG**AGGATGGGGTTGACGGA
Phot-S44_F4N	CACGGGACAGGAAAAGCAAAGC
Phot-S44_F4	AGGCAACAGCAGAGAGGGAACA
Phot-S44_A2	ATCCAAGGCTACCACCAAGCAAC
PhotpF-S44	TGAACTACGCACGCTCCATCAT
PhotpR-S44	TGACACAAGCGACCACCATTCT
Phot-S44_A1	GAAACAGACAAACGACATCGCTC
Phot-S4_F1	GTGCGAAGAAAGACGCTGAGAA
Phot-S4_F1N	CGCTCTGACGACTCTCTCCTAC
Phot-S4_F2	**ATTACAATTCACTGGCCGTCGTTTTAC**CTACGGCATCGCTCGAA
Phot-S4_F3	**CGTAATCATGGTCATAGCTGTTTCCTG**AATTCTCGGAAGCGAGG
Phot-S4_F4N	CAGACAGGCGTCCAGTAGTGC
Phot-S4_F4	CAGTATGCTCACACCCTCTGCG
Phot-S4_A2	ATCCACAGAAGACGCTGGGC
PhotpF-S4	GCTGACGAAGACCGAAGTGAGAAT
PhotpR-S4	GCCGTTGCTGGTCACATATCAG
Phot-S6_A1	CATCGCTGGTTCTTGTTTCGTG
Phot-S6_F1	CATTGGCGTCTCTCCACTCG
Phot-S6_F1N	GTTGCGGAATCTCGGACAGACT
Phot-S6_F2	**ATTACAATTCACTGGCCGTCGTTTTAC**AGTGCTGACGAACGGAG
Phot-S6_F3	**CGTAATCATGGTCATAGCTGTTTCCTG**GGCGAAGGCATGGTAAA
Phot-S6_F4N	ATACGATGGCGAAGCACTCAAGAT
Phot-S6_F4	GAAAGCGAAGCCAGCATTGACT
Phot-S6_A2	CATGCTGGCACGTCTACTTCTCAA
PhotpF-S6	CTCACAGAAGAGGAAGCCGAAGTC
PhotpR-S6	GCACTATTCCTCGTTCCATTCGTC
Phot_A1	GGAGGACTGCTGGTCTGGAA
Phot_F1	GCAGTTGGACGCAACAGCAAG
Phot_F1N	GGGCGTTGTGTTGAGAGACC
Phot_F2	**ATTACAATTCACTGGCCGTCGTTTTAC**ATGCTCTTTGGTTGCGTT
Phot_F3	**CGTAATCATGGTCATAGCTGTTTCCTG**ATCGCAGGTGGTTCACG
Phot_F4N	AGCTGCTTGAGGCGAAGTTTCT
Phot_F4	CACAGAGAAACTTCCCGATGGCAAC
Phot_A2	TCCTATGGCAGCGTGTCTCC
PhotpR	GTGCCAGTGATCCTTCGGTGA
PhotpF	GCCGATCCCACGTATTTGGAG

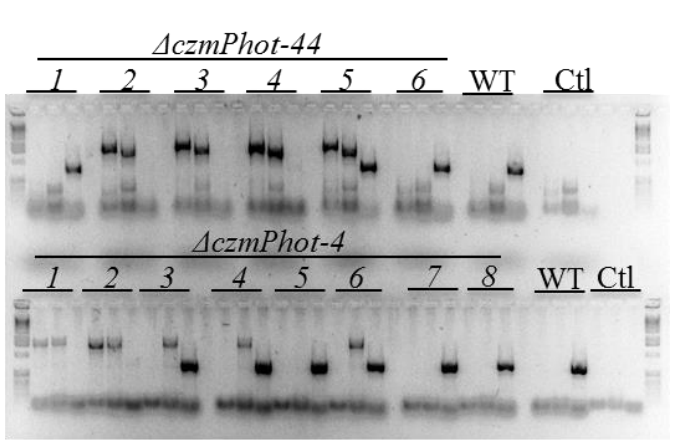
Table A4.3 continued: List of gene disruption and PCR validation primers

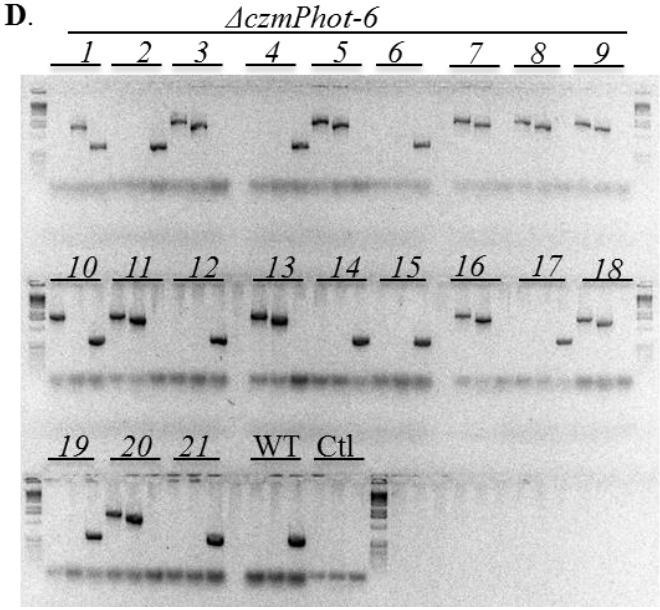
Phyt_A1	TGAGCTGCTTCTTGGTCATTGG
Phyt_F1	GCCTCTTGCTCAATGTCCACTACAC
Phyt_F1N	GATGGAGGCAAAGACGACGTTC
altPhyt_F2	**ATTACAATTCACTGGCCGTCGTTTTAC**GACAGGCGAGGTGGTGAG
Phyt_F3	**CGTAATCATGGTCATAGCTGTTTCCTG**GACGGAGGCTCATGTGGACA
Phyt_F4N	ACTTTCGCTCGACCTTCACTCG
Phyt_F4	ATCTCCGTGTGCTCGTGCTTC
Phyt_A2	AGCCCTCCTCCTTGACAATGC
Phyt_A3	GACCGAGGGGTTGGCGAT
Phyt_A4	CGATGGTCAGTATGAAGACGGCAC
PhytpR	CTTCTTTCTGCCGCCACACCT
PhytpF	AGCTGTTTGACGCCGACTGTG
M13F	**GTAAAACGACGGCCAGTGAATTGTAAT**
M13R	**CAGGAAACAGCTATGACCATGATTACG**
HY	GGATGCCTCCGCTCGAAGTA
HY-N	TAGCGCGTCTGCTGCTCCATACAAG
YG	CGTTGCAAGACCTGCCTGAA
YG-N	ACCGAACTGCCCGCTGTTTCTC
H1	CTACTGCTACAAGTGGGGCTGA
H2	GTACACAAATCGCCCGCAGAAG

All *CzmPhot-24* disruption primers have a Phot prefix while all other *CzmPhot* primers have Phot-Sx-prefix (x – for the scaffold specific CzmPhot) e.g. Phot-S44_F1 is the F1 primer for *CzmPhot-44* gene.


A.
F1
F1N
F2
(a.)
F3
F4N
F4
CzmPhot-x
probe
HYN
HY
F1N
M13F
YG
YGN
M13R
F4N
(b.)
1.6 kb
(c.)
HYG
A1
F1
F1N
H1
H2
F4N
F4
A2


B.
ΔczmPhot-24
8
10
18
20
21
25
WT


C.
ΔczmPhot-44
1
2
3
4
5
6
WT
Ctl
ΔczmPhot-4
1
2
3
4
5
6
7
8
WT
Ctl


D.
ΔczmPhot-6
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
WT
Ctl

Appendix 4.2

Legends to Figures in Appendices

Appendix 4.2

Gene disruption strategy and PCR verification of transformants. **A.** Figure shows a representation of *CzmPhot-24* gene loci and targeted deletion by triple homologous recombination approach. In the gels (**B** — **D**), numbers above each set of three wells represent a transformant and the wells. Each of the two wells above under the transformant number from the left, PCR products for primers A1/H1 and A2/H2 for the verification of the integration of the 5' and 3' split hygromycin resistant cassettes respectively, and pF/pR to verify the absence of the target gene locus. In the gel pictures, bands in the two leftmost wells show the integration of the hygromycin resistant cassette and a third well (rightmost of each set of three wells) represents the target *CzmPhot* gene locus. A blank in the mutants suggests deletion of target. In the WT the leftmost two wells (no bands) indicate the absence of a hygromycin resistant gene and a single band in the third well (rightmost) represent an intact *CzmPhot* target gene locus. **B**. A gel picture of selected *CzmPhot-24* transformants. **C.** Figure shows the gel picture of *CzmPhot-44* (upper panel) and *CzmPhot-4* (lower panel) transformants in a PCR screen to identify target gene deletion. **D**. Figure shows the gel picture of selected *CzmPhot-6* transformants in a PCR screen to identify target gene deletion.

CHAPTER FIVE

DISCUSSION AND CONCLUSIONS

Secondary metabolism in fungi plays a significant role in species diversification, virulence, and host specificity (Panaccione, 1992; Yang *et al.*, 1996; Ohm *et al.*, 2012). Among necrotrophs, small molecules including secondary metabolites are employed to induce necrosis of host cells, from which nutrients are derived for pathogens' physiological needs. *C. zeae-maydis* is a hemi-biotroph, which produces cercosporin and ABA, two secondary metabolites known for their pathological significance in plants. Cercosporin is a membrane-disrupting perylenequinone that is activated by light (Daub and Briggs, 1983). The metabolite, which is produced by many *Cercospora* species, absorbs light to produce reactive oxygen species that cause membrane lipid peroxidation and subsequently electrolyte leakage and cell death (Daub and Ehrenshaft, 2000). Experimental and observational evidence have established that cercosporin is a virulence factor among some species, but it is still uncertain whether cercosporin is a universal virulence factor among all *Cercospora* species, especially among species that colonize cereal crops. Similarly, the biosynthesis of ABA in axenic culture by *Cercospora*, which is also induced by light, has been suggested to occur *in planta* during pathogenesis of *C. beticola* (Assante *et al*., 1977; Schmidt *et al*., 2008). ABA regulates stomatal aperture and has recently been cited extensively in many plant-pathogen interactions, although there is a very limited understanding of the molecular basis of its biosynthesis and pathological significance among the limited range of producing fungi, mostly phytopathogens.

The goal of this dissertation was to determine the regulation and pathological significance of the biosynthesis of cercosporin and ABA in *C. zeae-maydis*, the foliar pathogen of GLS of maize. To this end, chapter two described the identification of a cluster of cercosporin biosynthetic genes based on shared homology with genes in the cercosporin biosynthesis (*CTB*) cluster in *C. nicotianae*. The polyketide synthase-encoding gene in the cluster was disrupted to abolish cercosporin biosynthesis. The mutants were evaluated for stomatal infection (by observation for appressoria formation over stomata), and colonization of leaves to form lesions. The conclusion from this work was that cercosporin biosynthesis is dispensable for pathogenesis and virulence in the development of GLS. Previously, the reduced virulence in cercosporin-

deficient *CZK3* disruption mutants of *C. zeae-maydis* was suggested to be due to the loss of cercosporin biosynthesis, although the pleotropic effects of *CZK3* disruption precluded a conclusive attribution (Shim and Dunkle, 2003). To explore the discrepancy between the virulence of the *CTB1* mutant and the loss of virulence reported for the *CZK3* mutant, we identified and functionally disrupted two putative regulators of cercosporin biosynthesis: *AREA*, a global nitrogen catabolite repressor gene, and a novel fungal gene, *CzmPhot-24*, predicted to be a blue light sensor. The biosynthesis of cercosporin *in vitro* is regulated by light and nitrogen in the growth media. Mutants in either of the two regulatory genes were significantly reduced in cercosporin biosynthesis, thus confirming the prediction that they mediate regulation of cercosporin biosynthesis *in vitro* by nitrogen and light. Regarding pathogenesis, *AREA* mutants were able to form appressoria and colonize maize leaves only to a limited extent, and failed to produce fully necrotic lesions. This reduction in colonization and virulence is most likely due to a global effect of *AREA* deletion on presumably one or multiple factors involved in virulence in addition to cercosporin, a phenomenon similar to the pleotropic effect of the *CZK3* gene disruption. The *CzmPhot-24* mutants, on the other hand, exhibited differing degrees of reduction in virulence, from full lesions similar to wild type to few necrotic specks (Discussed in Chapter 4). Cercosporin, therefore, appears to be one of many virulence factors among the arsenal of weapons employed by *C. zeae-maydis* during pathogenesis on maize.

In line with this prediction, Chapter 3 discusses the identification and partial characterization of genes involved in ABA biosynthesis in *C. zeae-maydis*. ABA was predicted to play a role in stomatal infection and/or suppression of host inducible defenses, thus enabling virulence upon successful infection of the host. Similar to identification of the *CTB* cluster, homology searches identified an ortholog of the *ABA3* gene implicated in ABA biosynthesis in *B. cinerea*. *ABA3* flanking sequence analyses identified a predicted core cluster of six genes that presumably encode enzymes responsible for ABA biosynthesis in *C. zeae-maydis.* We created deletion mutants of three genes in the putative cluster and confirmed the loss of ABA biosynthesis. The mutants, however, were unaffected in pathological development. They

formed appressoria over stomata, colonized leave tissues and induced lesions with no change in virulence compared with the wild type.

Taken together, these findings suggest that cercosporin and ABA are dispensable for pathogenesis and virulence, but could be components of a suite of as-yet unidentified AreA-regulated virulence factors produced by *C. zeae-maydis* during infection of maize. However, if cercosporin or ABA play major roles in virulence, other virulence factors are sufficient for pathogenesis in the absence of either ABA or cercosporin.

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