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Defining the Genetic Regulation of Appressorium Formation in Cercospora zeae-maydis

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Defining the Genetic Regulation of Appressorium Formation in *Cercospora zeae-maydis*

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ABSTRACT

Cercospora zeae-maydis is one of the primary pathogens associated with gray leaf spot, one of the most damaging foliar diseases of maize in the world. Gray leaf spot can be managed to some extent by cultural practices and fungicide applications. To infect maize, *C. zeae-maydis* grows towards stomata and forms infectious structures, termed appressoria, over stomatal pores. Prior research on the pathogen revealed that appressorium formation is crucial for foliar infection. Although several genes involved in pathogenesis have been identified in *C. zeae-maydis*, the molecular regulation of appressorium formation in this pathogen is poorly understood. Specifically, how the fungus senses stomata and induces appressorium formation are unknown. The goal of this research was to elucidate the genetic regulation of pre-penetration infectious development in *C. zeae-maydis*. To identify genes involved in appressorium formation, a collection of 1409 genetically tagged random insertional mutants was generated via *Agrobacterium tumefaciens*-mediated transformation and assayed for defects relating to appressorium formation *in vitro* and *in planta.* Two mutants were identified that were defective in appressorium formation and pathogenicity on maize leaves. Target enrichment sequencing identified two genes that were disrupted in the mutants: a CAZyme gene of the GH76 family, and a mitogen activated kinase kinase kinase (MEKK) gene belonging to the *STE11* family. Targeted deletion mutants of the MEKK-encoding gene failed to form appressoria on maize leaves and were apathogenic, thus confirming a role for the gene in pathogenesis. However, deletion mutants of the GH76 CAZyme formed appressoria and were pathogenic, which suggested that the mutation was not linked to the phenotype of interest. Thus, the key finding of this research implicated a specific MEKK pathway with the regulation of appressorium formation and pathogenesis in *C. zeae-maydis*.

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CHAPTER I: INTRODUCTION

1.1 Introduction

1.1.1 Overview of Genus *Cercospora* and gray leaf spot of maize

The genus *Cercospora* is one of the most diverse and destructive groups of foliar fungal pathogens, containing over 3000 named species that infect a wide range of important crops causing tremendous losses. The species are often classified per host association (e.g. *C. beticola* infects sugar beets, *C. sorghi* infects sorghum, *C. zonata* infects faba beans, *C. zeae-maydis* and *C. zeina* infect maize) (Weiland and Koch, 2004; Okori *et al*., 2004; Kimber 2011; Crous *et al*., 2006). Historically, the species concept and taxonomy of *Cercospora* are based upon morphological criteria, particularly the dimensions and characteristics of the conidia and conidiophores, as well as host range (Chupp, 1954; Ibrahim and Elamin 1974). Recently, new approaches such as molecular phylogenetics have challenged the taxonomic organization of the genus *Cercospora* (Goodwin *et al*., 2001; Tessmann *et al*., 2001; Pretorius *et al*., 2003; Groenewald *et al*., 2010, 2012)

Cercospora zeae-maydis is a causal agent of gray leaf spot, which is one of the most widespread and damaging foliar diseases of maize in the world (Crous *et al*., 2006; Kim *et al*., 2011A). Initially, *C. zeae-maydis* was considered the sole causal agent of gray leaf spot. However, molecular studies later showed that gray leaf spot can be caused by two genetically distinct but morphologically indistinguishable *Cercospora* species. Originally, they were considered 'sibling species' (*C. zeae-maydis* Group I and *C. zeae-maydis* Group II) (Wang *et al*, 1998). In 2006, *C. zeae-maydis* Group II was taxonomically defined as a distinct species, *Cercospora zeina*, while *C. zeae-maydis* Group I remained associated with the name *Cercospora zeae-maydis* (Crous *et al*.,

2006). *C. zeae-maydis* is thought to be predominant in North America, South America and Asia, while *C. zeina* has been found in South America, the Eastern United States and Africa (Meisel *et al*., 2009; Okori *et al*., 2003; Dunkle and Levy, 2000). These assumptions are based on limited studies, some of which are increasingly dated, and therefore, the current geographic distribution of pathogens associated with gray leaf spot is unknown. Visually, both pathogens cause gray leaf spot. However, *C. zeae-maydis* differs from *C. zeina* in that it grows faster in many artificial media, produces the phytotoxin cercosporin in defined culture conditions, typically forms longer conidiophores, and produces broader and longer conidia (Wang *et al*, 1998; Crous *et al*., 2006).

When gray leaf spot was first reported in Illinois in 1924, the disease was determined to minimally affect crop yield as the overall incidence was low and symptoms occurred late in the growing season (Tehon and Daniels, 1925). However, the incidence and severity of the disease drastically increased beginning in the mid 1970's (Ward *et al*., 1999; Beckman and Payne, 1983). This increase coincided with the ascendance of no-till farming, which likely increased the overwintering potential of the pathogen (Payne and Waldron, 1983). Yield losses due to gray leaf spot in 1995 were reported to be as high as 50% in some U.S. localities (Bhatia and Munkvold, 2002). Similarly, the disease became endemic across Africa and South America, with reported losses of 60% or greater (Ward *et al*, 1999).

Currently, gray leaf spot can be managed through a combination of integrated pest management techniques. Crop rotation with non-hosts like soybean or wheat can reduce the amount of primary inoculum available to initiate infections come the spring season. Conventional tillage practices also reduce in-field inoculum and consequently, severity of the disease by burying infected plant tissues and debris. Planting maize hybrids with genetic resistance to gray leaf spot, along with foliar fungicide applications, is another important management strategy. Even though

some resistant germplasm has been identified, resistance has not been broadly incorporated into commercial hybrids (Munkvold *et al*., 2001), partly due to the complex nature of gray leaf spot resistance. Resistance to *C. zeae-maydis* is quantitative (partial resistance) and additive effects are predominant, but dominance is significant as well (Gordon *et al*., 2006). Most resistant genotypes are considered to have two or more factors associated with resistance (Pratt and Gordon, 2010). Genetic mapping experiments have identified quantitative trait loci (QTL) for resistance to *C. zeae-maydis* on all 10 chromosomes of maize (Clements *et al*., 2000; Gordon *et al*., 2004). Commercial hybrids are available that confer partial resistance to gray leaf spot; when infected by the fungus, these hybrids exhibit one or more components of partial resistance such as longer incubation periods, or fewer and smaller lesions (Latterrel and Rossi, 1983). Partially resistant hybrids exhibit fleck-type lesions (Latterrel and Rossi, 1983), while moderately resistant and susceptible hybrids exhibit chlorotic and necrotic lesions, respectively (Paul and Munkvold, 2005). Chemical controls for *C. zeae-maydis* are limited and not always widely available, especially in developing countries. Fungicides currently labeled for use in controlling gray leaf spot contain active ingredients belonging to the quinone outside inhibitor (QOI) and demethylation inhibitor (DMI) classes, the strobilurins and triazoles, either as a single active ingredient or as a premix of the two chemistries (Munkvold *et al*., 2001; Bradley and Pedersen, 2011). However, several *Cercospora* species have developed resistance in response to these fungicides (Kirk *et al*., 2012; Price III *et al*., 2014). Therefore, additional control strategies are needed for gray leaf spot.

1.1.2 Epidemiology of Gray Leaf Spot

C. zeae-maydis survives on infected maize debris, which serves as a source of primary inoculum for the following season's maize crop. Pathogenesis can be divided into four broad, but distinct, stages once the spores encounter the host tissue: germination, infection, colonization, and

sporulation (Kim *et al*., 2011A). The conidia germinate in conditions approaching 100% relative humidity (germination stage) (Thorson and Martinson, 1993). The developing hyphae emerge from conidia and grow across the host leaf surface until they sense stomata and reorient their growth accordingly. The infection stage begins when hyphae differentiate into swollen, bulbous, multi-lobed appressoria upon reaching a stomate (one to five days after germination). Appressoria potentially guide a penetration peg between the guard cells and into the stomatal pore, although the exact function of appressoria has not been determined in this pathogen. After entering the maize leaf mesophyll, *C. zeae-maydis* grows intracellularly and rapidly colonizes host tissue. At an unknown point in the infection stage, the fungus shifts to a necrotrophic growth habit. Lesions are delineated by the major veins of maize leaves, giving gray leaf spot its characteristic rectangular lesions. The sporulation stage occurs after lesions form, typically more than seven days after germination, as erumpent conidiophores emerge through stomata of colonized tissue and produce conidia (Kim *et al*., 2011A). By late spring, conidia are dispersed via wind or rain, and subsequently infect maize leaves at early stages of development (Paul and Munkvold, 2005), initiating secondary infection cycles and subsequently, a polycyclic progression of disease throughout the growing season.

Environmental factors known to impact the infection process of *C. zeae-maydis* are temperature, relative humidity, and light. High relative humidity (95% or greater) and temperatures around 25-30 °C are favorable for disease development (Paul and Munkvold, 2005). Light also affects gray leaf spot development. *C. zeae maydis* produces cercosporin, a phytotoxin produced by many *Cercospora* species (Daub and Chung, 2009). Once activated by light, cercosporin reacts with oxygen to produce reactive oxygen species including singlet oxygen, which induce lipid peroxidation and damage cell membrane, resulting in leakage of cytoplasmic

contents and cell death (Daub and Ehrenshaft, 2000; Daub and Chung, 2009). Light has been shown to be involved in the regulation of cercosporin biosynthesis and conidiation in *C. zeae-maydis* (Kim *et al*., 2011 B; Daub and Chung, 2009). Light also affects stomatal tropism and has been shown to regulate pathogenesis (Shim and Dunkle, 2003; Kim *et al*., 2011 B).

1.1.3 Advancement in functional genomics of *C. zeae-maydis*

The advancement of genomic resources and technical approaches paved the way for the sequencing and annotation of the *C. zeae-maydis* genome in 2011 by the U.S. Department of Energy Joint Genome Institute (https://genome.jgi.doe.gov/pages/projectStatus.jsf?db=Cerzm1). Several genes have since been characterized that provide molecular insight into important biological functions (Shim *et al*, 2003; Bluhm and Dunkle, 2008; Kim *et al*, 2011B). *CZK3*, a mitogen-activated protein kinase kinase kinase, was the first gene to be functionally characterized in *C. zeae-maydis* (Shim and Dunkle, 2003). Targeted disruption of the gene led to the suppressed expression of cercosporin biosynthetic genes and the abolishment of cercosporin production in conducive culture media. Additionally, the disruption mutants grew more quickly compared to the wild-type in culture, failed to produce conidia, and elicited small chlorotic flecks as opposed to necrotic lesions during plant infection, which implicated *CZK3* as a regulator of virulence (Shim and Dunkle, 2003). The characterization of *PHL1*, a cryptochrome/6-4 photolyase-like gene, clarified the genetic regulation of light photoreception and repair of UV-damaged DNA (Bluhm and Dunkle, 2008). Disruption of *PHL1* resulted in abnormalities, including increased levels of conidiation and reduced levels of cercosporin biosynthesis in culture and completely abolished photoreactivation after lethal exposure to UV light. This suggested that *PHL1* encodes either a photolyase, a regulator of photolyase activity, or a protein with both regulatory and DNA-repair activities (Bluhm and Dunkle, 2008). Recently, the Bluhm lab identified and characterized *CRP1*,

a gene encoding a putative blue-light photoreceptor homologous to *White Collar*-1 (*WC*-1) of *Neurospora crassa* (Kim *et al*., 2011B). Disruption of *CRP1* via homologous recombination revealed roles in multiple aspects of pathogenesis, including tropism of hyphae to stomata, the formation of appressoria, conidiation, and the biosynthesis of cercosporin. *CRP1* was also required for photoreactivation after lethal doses of UV exposure (Kim *et al*., 2011B).

1.1.4 Regulation of appressorium-formation and plant penetration

Effective nutrient acquisition is a prerequisite for successful colonization and fungal fitness in host-pathogen interactions (Divon and Fluhr, 2007). Consequently, plant pathogenic fungi have evolved a wide array of strategies to breach the host cell walls to access and colonize underlying tissues, ranging from entry via natural openings, such as stomata, to direct penetration of cuticle (Rodriguez-Moreno *et al*., 2018; Skamnioti and Gurr, 2007). Taxonomically diverse fungi have developed highly specialized infection structures, termed appressoria, to generate focused turgor pressure to breach the host cell wall and gain access to nutrient sources (Ryder and Talbot, 2015). The ability to produce appressoria varies within certain species and depends on specific physical or chemical cues provided by the host plant (Deising *et al*., 2000).

Appressorium development and penetration in *Colletotrichum* species and *Magnaporthe oryzae* is accompanied by a series of precisely and highly regulated events including nuclear division, reorganization and melanization of the cell wall, formation of a penetration hypha, secretion of extracellular enzymes, and generation of turgor pressure to physically penetrate the host surface (Deising *et al*., 2000; Ryder and Talbot, 2015). Appressorium formation in *Colletotrichum* species is regulated by cell-cycle progression. The conidia are elliptical cells containing a single nucleus (Takano *et al*., 2001). Mitosis occurs during conidium germination before the formation of a septum. One nucleus moves into the developing germ tube where a

second mitosis occurs, and a second septum is formed; therefore, only one individual nucleus is in the cell that differentiates into an appressorium. Another mitosis event results in a mature binucleate appressorium. During infection, one of the nuclei moves into the subsequent penetration peg and the following growth produces a multinucleate mycelium in the host cell (Perfect *et al*., 1999).

Like *Colletotrichum* species, appressorium development in *M. oryzae* is regulated by cell-cycle progression (Veneault-Fourrey *et al,* 2006). First, a three-celled conidium lands on the rice leaf surface and adheres to the hydrophobic cuticle. The spore then germinates producing a germ tube, followed by the migration of one nucleus into the developing germ tube. The germ tube then differentiates into an appressorium and subsequently undergoes mitosis, 4-6 hours after germination. During mitosis, an actomyosin ring forms and septation occurs. Following mitosis, a daughter nucleus enters the developing appressorium, while the other returns to the conidium. As the appressorium matures, it becomes melanized and develops substantial turgor. The three nuclei in the conidium are then degraded along with rest of the spore contents, leaving a single nucleus in the mature appressorium. A narrow penetration peg forms at the base of the appressorium, and with the aid of turgor pressure, it punctures the cuticle and enters the rice epidermis (Saunders *et al, 2010*; Talbot and Wilson, 2009).

Unlike *Colletotrichum* species and *M. oryzae*, the process of appressorium formation in *C. zeae-maydis* is poorly defined. Previous research on *C. zeae-maydis* categorized appressorium development into three stages: initiation, proliferation, and maturation (Hirsch, 2014). As the fungus encounters host stomata, hyphae begin to swell and form small lateral growths. These lateral growths then develop into large bulbous growths that spread out from the parental hypha. After several hours, a mature appressorium is formed in close association with the stomata (Hirsch,

2014). However, important questions remain unanswered: how many nuclei are there per appressorium, and how many cells comprise each appressorium? Therefore, more research is required to provide a better descriptive model of appressorium formation and regulation in *C. zeae-maydis.*

1.1.5 Signal transduction during appressorium development

Eukaryotic cells respond to environmental cues and transduce external signals to the cell interior where changes in gene expression are affected, resulting in diverse biochemical responses. Two broadly conserved signal transduction pathways that regulate growth and development in yeast and other filamentous fungi are the cAMP and mitogen activated protein kinase (MAPK) signal transduction pathways (Lengeler *et al*., 2000; Pathak *et al*., 2013; Anjago *et al*., 2018). MAP kinases are activated by MAPK kinases (MEK) which are activated by MEK kinases (MEKK). These MEKK-MEK-MAPK cascades are highly conserved in eukaryotes and have been extensively studied in several organisms. Five MAPK pathways regulate mating, filamentous growth, high osmolarity response, maintenance of cellular integrity, and ascospore formation in the yeast *Saccharomyces cerevisiae* (Chen and Thorner, 2007; Molina *et al*., 2010). The yeast pheromone pathway, arguably the most characterized MAPK pathway, is initiated by the binding of mating pheromones to a receptor and the release of stimulatory Gβ subunits. These Gβ subunits associate with a scaffold protein, *STE5*, and a p21-activated kinase (*PAK*), *STE20*, and are essential for activating the MEKK *STE11*, which in turn activates the MEK *STE7*. Downstream from *STE7, FUS3* and *KSS1* are two partially redundant MAPKs that regulate the mating process (Chen and Thorner, 2007). Several elements of this pheromone response pathway, including kinases *STE20, STE11, STE7*, and *KSS1*, are also involved in filamentous growth in *S. cerevisiae* (Cherkasova *et al*., 2003; Chen and Thorner, 2007).

At the molecular level, appressorium formation is regulated by MAPK and cAMP signal transduction in *Colletotrichum* species, as well as in *M. oryzae* and other fungi. In *M. oryzae*, several genes within the MAPK pathway have been identified that regulate appressorium formation, cellular turgor control, and appressorium function (Anjago *et al*., 2018). The cAMP signaling pathway has been shown to be involved in surface recognition and appressorium turgor generation (Thines *et al*., 2000; Bahn *et al*., 2007). Xu and Hamer (1996) identified *PMK1* (pathogenicity MAP kinase 1), a homolog of yeast *FUS3/KSS1*, and established that it regulates appressorium formation and infectious hyphal growth. Germ tubes of *pmk1* mutants failed to form appressoria but still recognized hydrophobic surfaces or responded to exogenous cAMP and produced subapical swollen bodies (Xu and Hamer, 1996). Zhao *et al* (2005) characterized *MST7* and *MST11* genes in *M. oryzae*, which are homologs of the yeast *STE7* MEK and *STE11* MEKK, respectively. Like the *pmk1* mutant, deletion mutants of *MST7* or *MST11* failed to form appressoria and failed to colonize rice tissues through wounds (Zhao *et al*., 2005).

In the past years, MAPK genes homologous to *PMK1* have been identified in several pathogenic fungi. The *PMK1* orthologs are essential for appressorium formation in several appressorium-forming pathogens including *Colletotrichum lagenarium, Cochliobolus heterostrophus* and *Pyrenophora teres* (Takano *et al*., 2000; Ruiz-Roldan *et al*., 2001). Like *PMK1*, gene replacement mutants of *PTK1* in *P. teres, CMK1* in *C. lagenarium* and *CHK1* in *C. heterostrophus* are apathogenic, failed to form appressoria and failed to colonize healthy or wounded host tissue (Takano *et al*., 2000; Ruiz-Roldan *et al*., 2001).

The infection process of *C. zeae-maydis* differs from *M. oryzae*, the fungal model system for the study of fungus-plant interactions, in terms of stomatal tropism and appressorium formation. However, to date, little is known about the genetic regulation of appressorium

formation in *C. zeae-maydis*. *PMK1* orthologs have been shown to be important for appressorium formation in several filamentous dothidiomycetes. Therefore, an ortholog of this MAPK could be an important regulator of appressorium formation in *C. zeae-maydis*. However, comparisons to the established model systems is insufficient to address important biological questions about appressorium formation regulation in *C. zeae-maydis*. As such, more research is needed to characterize the unique regulation of *C. zeae-maydis* during pathogenesis and identify potential genes required for appressorium formation.

1.2 Project rationale and long-term goals

Appressorium formation is an important phase in the infection process of *C. zeae-maydis*. However, the biological and molecular underpinnings of this process are poorly defined, and many intriguing questions remain unanswered. For example: how does the fungus navigate leaf surfaces, sense, and enter stomata to colonize host tissue? And perhaps most importantly, what regulates appressorium formation in *C. zeae-maydis* at the genetic and molecular levels? This research aims to determine the genetic regulation underlying pre-penetration infectious development by identifying novel genes and regulatory networks in *C. zeae-maydis*.

Objectives

The specific objectives of this research are to:

1. Create a genetic screen to identify mutants defective in appressorial development in *C. zeae-maydis*.
2. Identify and characterize genes in mutants of interest that are impaired in appressoria formation in *C. zeae-maydis*

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CHAPTER II: THE IDENTIFICATION OF MUTANTS DEFECTIVE IN APPRESSORIAL DEVELOPMENT IN *CERCOSPORA ZEAE-MAYDIS*

2.1 Abstract

Despite the global importance of gray leaf spot of maize caused *by Cercospora zeae-maydis*, the molecular mechanisms underlying pathogenesis are poorly defined. Appressorium formation is an important phase in the infection process of *C. zeae-maydis*. However, the biological and molecular regulation of appressorium formation is still largely unknown. Studying the regulation of appressorium formation in *C. zeae-maydis* could potentially elucidate novel genes involved in pathogenesis. In this study, a forward genetic screen utilizing random insertional mutagenesis was used to identify genes involved in pre-penetration infectious development. A collection of 1409 tagged random insertional mutants was created and assayed for defects relating to germination, growth and appressorium formation. Two *C. zeae-maydis* mutants (*Czm* ATMT 3.20 and *Czm* ATMT 3.71) failed to form appressoria on maize leaves and were apathogenic. The identification of these two mutants defective in appressorium formation will lead to the identification of genes regulating appressorium formation in *C. zeae-maydis*.

2.2 Introduction

Gray leaf spot, caused by *Cercospora zeae-maydis,* is one of the most devastating foliar diseases on maize worldwide (Ward *et al*., 1999; Kim *et al*., 2011A). The incidence of the disease significantly increased in the late 1980's due to reduced tillage and overhead irrigation practices (Latterell and Rossi, 1983; Ward *et al*., 1999). The disease continues to be a major problem especially in regions where control methods for the disease are limited mostly due to economic reasons. With the increasing need for alternative methods to control the epidemic, more research on gray leaf spot is needed to better understand the infection process of *C. zeae-maydis*.

Appressorium formation and development is crucial for the infection process and pathogenesis of some plant fungal pathogens. In *Magnaporthe oryzae,* the biotrophic fungal model for the study of plant-microbe interactions (Talbot, 2003; Dean *et al*., 2005; Gilbert *et al*., 2006), germ tubes grow without a specific direction before forming a rigid, melanin-pigmented appressorium (Wang *et al*., 2005; Skamnioti and Gurr 2007). These appressoria generate tremendous intracellular turgor pressure, a consequence of glycerol accumulation in the cell, to penetrate the cuticle and enter mesophyll tissues of rice leaves (Tucker and Talbot, 2001; Talbot, 2003; Liu *et al*., 2007). In *C. zeae-maydis,* conidia germinate in conditions near to 100% relative humidity; the emerging germ tubes sense and reorient their growth towards the stomata (termed stomatal tropism). As they encounter a stomate, the hyphae differentiate into swollen, globular, multi-lobed appressoria, which seem to aid the penetration peg in stomatal entry (Kim *et al*., 2011 A). During stomatal tropism, reorientation happens prior to appressoria formation and when the hyphae encounter stomata, appressoria seem to successfully form approximately 75% of the time (Kim *et al*., 2011B).

Appressorium formation seems to be an important phase in the infection process of *C. zeae-maydis.* However, the biological and molecular basis for this process is poorly defined. There are a few essential questions about the genetic regulation of stomatal penetration in *C. zeae-maydis* that need conclusive answers: what regulates appressorium formation in *C. zeae-maydis*. A better and clear understanding of the genetic regulation of the pre-penetration structure is required to discern how *C. zeae-maydis* encounters and infects leaves through the stomata at the molecular level.

One approach to defining genes important for disease development is to identify mutants defective in pathogenicity, and in the case of *C. zeae-maydis*, mutants defective in appressorium formation. Random insertional mutagenesis approaches have been successfully used with *M. oryzae* to identify several pathogenicity genes (Rho *et al*., 2001; Chen *et al*., 2011). To identify genes important for the ability of the fungus to cause disease, this study chose to saturate the *C. zeae-maydis* genome with random insertions of a defined DNA fragment. Through this approach, any genes of interest can be recovered because they are tagged.

Through *Agrobacterium tumefaciens*- mediated transformation (ATMT), a collection of 1409 tagged random insertional mutants was created and assayed for defects relating to germination, growth and appressorium formation. Of the screened mutants, 41 *C. zeae-maydis* mutants failed to form appressoria *in vitro.* Two of these 41 *C. zeae-maydis* mutants (designated *Czm* ATMT 3.20 and *Czm* ATMT 3.71) were apathogenic and failed to form appressoria on inoculated maize leaves. The identification of these two *C. zeae-maydis* mutants defective in appressorium formation could lead to the identification of genes regulating and involved in appressorium formation in *C. zeae-maydis.*

2.3 Materials and Methods

2.3.1 Fungal strains and culture conditions

All fungal strains used in this study (Table 2.1), including *C. zeae-maydis* strains SCOH1-5 that served as the wild-type (Bluhm *et al*., 2008; Bluhm and Dunkle 2008; Kim *et al*., 2010) and GFP-reporter strain SCOH1-5-GFP (Hirsch 2014), were generated or obtained from the Bluhm lab stocks. The strains were stored as spore suspensions in 25% glycerol at -80°C. Working stocks were maintained on V8 agar medium (180ml/L V-8 juice, 2g/L calcium carbonate, 20g/L agar) grown under constant darkness, and renewed from glycerol stock every 3-4 months.

2.3.2 Nucleic acid manipulation and transformation of tagged *C. zeae-maydis* mutants

A collection of *C. zeae-maydis* tagged random insertional mutants was generated via *Agrobacterium tumefaciens*- mediated transformation, as described by Li *et al*., 2013. The *A. tumefaciens* strain AGL-1 containing the binary vector pBHt2_sGFP (Li *et al*., 2013), a derivative of pBHt2 (Mullins *et al*., 2001) was used in the transformation. AGL-1 was streaked out on liquid LB (Luria-Bertani) medium (Sambrook and Russel 2001) amended with carbenicillin (50µg/ml) and kanamycin (50µg/ml) and incubated two to three days at 28°C until discrete colonies formed. Single colonies of AGL-1 were then suspended in 5ml liquid LB medium (with appropriate antibiotics) and incubated two to three days at 28°C on an orbital shaker (250rpm), until visible opalescent growth was present. A spectrophotometer was used to measure the optical densities (OD) of the AGL-1 cultures, which were then adjusted to $OD_{600} = 0.2$ with liquid inducing minimal medium (IMM; contains per liter: 2.05g K_2HPO_4, 1.45g KH_2PO_4, 0.5g NH_4NO_3, 0.15g NaCl, 0.0025g $FeSO_4$, 0.01g $CaCl_2$, 0.25g $MgSO_4$, 0.9g glucose, 5.33g MES. H_2O (free acid), 5ml glycerol, 20µl Vogel trace elements solution, and 200µM acetosyringone). Shaking was induced

in IMM overnight at 28°C. OD_{600} of the cultures were measured again and adjusted to 0.2 with liquid IMM. This was the induced (virulent) AGL-1 stock suspension. *C. zeae-maydis* SCOH1-5 fungal spores were adjusted to 1×10^6 (colony forming units per ml) concentration in liquid IMM. Equal volumes (500µL) of induced AGL-1 suspension and SCOH1-5 conidial suspension (10^6 conidia/ml) were mixed in a sterile microfuge tube and 200µL aliquots of the mixture were spread on 6cm IMM plates, with appropriate antibiotics, overlaid with 47mm cellophane membranes and co-incubated at room temperature (RT) in the dark for 48-72 hours. After co-incubation, the cellophane membranes were transferred to 0.2x PDA plates (10cm) containing cefotaxime (200 µg/mL) to eliminate *Agrobacterium* and hygromycin B (100µg/ml) to select for fungal transformants by flipping membrane over, transformation-side down onto the surface of the agar and incubated for an additional 48-72 hours at RT in the dark. After transformation, strains expressing GFP were visually selected with the DFP-1 Dual Fluorescent Protein Flashlight and subsequently cultured on V8 medium with hygromycin (100µg/ml). Transformants were purified by sub-culturing single spores three times on selective medium. Transformants were sub-cultured on non-selective medium three times, followed by selective medium PDA amended with hygromycin B (100 µg/ml). 14 separate transformation events produced 1409 individual mutant strains that constitutively expressed GFP (Table 2.2). Because the radial growth of *C. zeae-maydis* is slow in defined culture media (~0.5cm/two weeks) (Hirsh 2014), mutant strains were sub-cultured by transferring conidia to fresh V8 media every four days.

2.3.3 *In vitro* assay of appressorium formation

The effects of relative humidity on germ tube elongation, appressorium formation, and germling survival of *C. zeae-maydis* without the host plant have previously been studied by Thorson and Martison 1993. Following the paper, a modified protocol was developed by the

Bluhm lab to study the *in vitro* appressorium formation by *C. zeae-maydis* fungal strains, using a glass jar humidity chamber (depicted in figure 2.1) and an 8.11% glycerol solution to maintain a 98% relative humidity (RH).

A preliminary *in vitro* screen was performed to identify which membranes available in the Bluhm lab could be used for subsequent *in vitro* appressorium formation assays. The four different membrane discs available that were assessed included membrane discs made of mixed cellular ester (MCE), nylon, nitrocellulose, polyvinylidene difluoride (PVDF) and cellophane (Table 2.3). The MCE, nylon, and nitrocellulose membrane discs are hydrophilic while the PVDF membrane discs are hydrophobic. Appressorium formation was assessed by inoculating the different membrane discs with spore suspensions of SCOH1-5-GFP and observed using a confocal microscope six days after inoculation.

For the *in vitro* assay, to assess appressorium formation, conidia from each mutant strain and wild-type GFP reporter strain (SCOH1-5-GFP) were collected by taking three 5mm plugs of V8 media cultures in 1.5ml microfuge with 1ml sterile dH_2O and vortexing the microfuges for 1 minute to release the conidia from the agar plugs. The conidia suspensions were then quantified by a hemocytometer to a concentration of 10^4 conidia per ml. Sterile MF- Millipore MCE membrane discs (Sigma-Aldrich, catalog number GSWP04700) were inoculated with 200µl of conidial suspensions of SCOH1-5-GFP and tagged random insertional mutant strains (10^4 conidia per ml) and were left to dry in a laminar flow hood for thirty minutes. As a negative control, MCE membrane discs were inoculated with 200µl of sterile dH_2O. The membrane discs were then transferred to a glass jar humidity chamber and ~20ml of an 8.11% glycerol solution was used to maintain a 98% relative humidity in the chamber. The tightly sealed glass jar humidity chamber was then moved to a growth chamber, maintained at 23°C with a 12:12 light: dark photoperiod.

The discs were then removed from the glass jar humidity chamber and assessed using a confocal microscope four days after inoculation to see appressorium formation.

2.3.4 *In planta* assay of appressorium formation and pathogenesis

C. zeae-maydis strains SCOH1-5-GFP and SCOH1-5, as well as the generated tagged random insertional mutants were used to inoculate susceptible maize cultivar Silver Queen at V4 (three weeks after emergence). Conidia were harvested with 10ml sterile water per petri dish and quantified with a hemocytometer. Three leaves per plant per strain were inoculated individually with 10ml of a conidial suspension (10^5 conidia/ml) of strains SCOH1-5-GFP, SCOH1-5, or each mutant strain. Each conidial suspension was amended with 0.01% Tween-20, and plants were inoculated with an atomizer attached to an air compressor until inoculum run-off. Mock-inoculated control plants were sprayed with the same Tween-20 solution but contained no conidia. After inoculation, the maize plants were placed in incubation chambers made of wire mesh wrapped in opaque plastic to maintain free moisture on plants and increase humidity. The incubation chambers were large enough to surround the inoculated plants but not touch the leaves. The chambers containing the inoculated maize plants were placed in a large growth chamber, maintained at 23°C with a 12:12 light: dark photoperiod with a light intensity at leaf level of 300µmol m^{-2} s^{-1}.

To assess appressorium formation on leaf surfaces, infected leaves from each inoculated plant were collected four and seven days after inoculation. Small leaf sections (1.5cm X 3cm) were cut from each leaf for microscopic examination. Appressorium formation was analyzed with a Nikon Eclipse 90i confocal microscope and the data were calculated as the percentage of successful interactions with stomata (appressoria formed over stomata/total hyphal interactions with stomata). Confocal images were edited for color and clarity in Adobe Photoshop or NIS Elements.

The appressorium formation experiments contained three replicates per strain. To assess lesion development, plants were inoculated with spore suspensions (1×10^5 per mL) and disease was allowed to progress for 14 days. Leaves were then collected for routine observations, assessed qualitatively by eye for pathogenesis, and photographed.

2.3.5 Statistical analysis

To determine any significant difference between *in planta* appressorium formation in the WT SCOH1-5-GFP and the tagged mutant strains, two-way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) tests were performed using Excel. Because ANOVA doesn't analyze within group significance, the statistical analysis software (SAS) was used to analyze significance between each mutant strain to each other and to the WT. To run the statistical comparisons of means, the following code was run in the software: proc mixed data=FILE NAME, method=type 3; class run strain; model percent=strain/ddfm=kr; random run; LSmeans strain/diff; run.

2.4 Results

2.4.1 Evaluation of the infection process *in vitro*

A preliminary *in vitro* assay revealed that the MCE, nylon and nitrocellulose membrane discs were better membranes for the *in vitro* appressorium formation assay, compared to the PVDF membrane discs, as shown in figure 2.3. Based on the observations that the MCE membrane discs had less background noise, allowed for better conidia visualization, and appressoria formed on the discs, the MCE discs were selected to be used for the remainder of the *in vitro* assay in this study.

An initial *in vitro* screen of the 1409 tagged random insertional mutant strains, just looking at whether the mutants formed appressoria or not, identified 131 mutant strains impaired in

appressorium formation without the host plant (table 2.4). A re-screen of the 131 mutant strains revealed that 41 of the mutant strains failed to produce appressoria *in vitro* (Table 2.5). Based on qualitative observations comparing appressorium formation in the mutant strains to the wild-type (WT) strain (SCOH1-5 GFP), around 10 mutants had relatively the same appressorium production rate as the WT, five mutants had a higher appressorium production than the WT and 40 mutants had reduced appressorium production (Table 2.5). There were 35 mutants that were designated as unknown for appressorium production; this was mainly because there was some background noise in the membrane discs and some of the samples had lots of hyphal tissue which lead to the uncertainty of whether they formed appressoria.

2.4.2 Evaluation of the infection process *in planta*

To confirm the phenotypes observed in the *in vitro* screen, an *in planta* screen was performed to assess appressorium formation. Notably, two of the mutant strains assayed, *Czm* ATMT 3.20 and *Czm* ATMT 3.71, were completely impaired in appressorium formation *in vitro* and *in planta*. The magnitude of impairment was substantial; the WT formed appressoria 79.39% of stomatal encounters, whereas the two mutants formed appressoria 0% of stomatal encounters, even though, *Czm* ATMT 3.20 encountered 105 stomata and *Czm* ATMT 3.71 encountered 116 stomata (Table 2.6; Figure 2.3). Looking at the micrographs (figure 2.3 A), some of the mutant spores landed on stomata, indicating that some interaction with the stomata took place; however, no appressoria were formed. To evaluate effects on pathogenicity, disease symptoms such as lesion development were assayed 14 days after inoculation. The two mutants, *Czm* ATMT 3.20 and *Czm* ATMT 3.71, were determined to be apathogenic when compared to the wild-type (Figure 2.4).

2.5 Discussion

Several approaches for stable transformation for a wide range of filamentous fungi have been developed. Successful transformations have been performed with $CaCl_2$/polyethylene glycol, electroporation, viral vectors-mediated transformation, liposome-mediated transformation, nanomaterial-mediated, and *Agrobacterium tumefaciens*-mediated transformation (He *et al*., 2016). *Agrobacterium tumefaciens*-mediated transformation (ATMT) is a common and popular approach for the genetic transformation for fungal organisms with several advantages. Firstly, ATMT allows transformation of different types of tissues (such as conidia, mycelium, or fruiting bodies), thereby, avoiding the tedious process of protoplast preparation (Mora-Lugo *et al*., 2014). Secondly, its T-DNA can be randomly inserted as single copy into host genome with more stability of the transgene (Zhang *et al*., 2014; Gong *et al*., 2015). Lastly, the ATMT approach has been proven to increase transformation rate, compared to other techniques (He *et al*., 2016).

Random insertional mutagenesis has been widely utilized in plant pathogenic fungi to generate collections of tagged mutant strains for forward genetic screens (Shim and Woloshuk, 2001; Seong *et al*., 2005). Rho *et al*., generated a mutant library for *Magnaporthe oryzae* using ATMT, which can subsequently be used to screen the mutants that exhibit loss of (or reduced) virulence (Rho *et al*., 2001). Transformation using the binary vector pBHt2 (Mullins *et al*., 2001), carrying the bacterial hygromycin B phosphotransferase gene under the control of the *Aspergillus nidulans trpC* promoter, led to the generation of 500 to >1000 hygromycin B resistant transformants per 1×10^6 conidia of *M. oryzae* (Rho *et al*., 2001). Similarly, Li *et al*., provided the first report of *Phomopsis longicolla* transformation using ATMT as a tool for insertional mutagenesis in an important pathogen of soybean (Li *et al*., 2013). Transformation with the *A. tumefaciens* AGL-1 strain containing the binary vector pBHt2_sGFP, a derivative of pBHt2

(Mullins *et al*., 2001) carrying the hygromycin B phosphotransferase gene driven by the *trpC* promoter and the *sGFP* gene driven by *ToxA* promoter (Lorang *et al*., 2001) yielded 150-250 transformants per 1×10^6 conidia of *P. longicolla,* which were analyzed as the green fluorescent protein (GFP) expression (Li *et al*., 2013). This study took the advantage of ATMT's ability of random insertion during the transformation process. 14 separate transformation events generated 1409 tagged random insertional mutants which were used to identify and characterize mutants impaired in appressorium formation and genes involved in the regulation of appressorium development in *C. zeae-maydis*. The mutants were tagged with GFP and the constitutive expression of GFP was crucial for the genetic screen by allowing the examination of pre-penetration infectious development with epifluorescence microscopy.

Appressorium formation by *Cercospora zeae-maydis* can be studied away from living plants on artificial hydrophobic surfaces by regulating the relative humidity to mimic that of leaf surfaces (Thorson and Martison, 1993). When germlings were maintained at 95 or 100% RH, germ tube elongation continued, and appressoria formed in two to three days. However, germ tube elongation did not occur when discs were subjected to relative humidity environments below 95% (Thorson and Martinson, 1993). Following the paper, a modified protocol was developed to study *in vitro* appressorium formation in *C. zeae-maydis* mutant strains. A 98% relative humidity was maintained using an 8.11% glycerol solution in a glass jar humidity chamber. *In vitro* appressorium screening allows for the analysis of many mutants in membrane discs which reduces the time used in the experiment compared to *in planta* analysis which requires a lot of time allocated towards the experiment. The screen identified 41 mutants that were impaired in appressorium formation *in vitro*, while an *in planta* screen identified two mutants that were impaired in appressorium formation and were apathogenic.

An interesting question arises while looking at data collected from this study. 1409 mutants were generated but only two mutants had the desired phenotype. Therefore, why use this method? This *in vitro* approach still seems to be a much quicker way to screen mutants, compared to directly screening mutants *in planta,* and is more convenient to researchers who are allocated two years to finish their research. Getting less mutants with the desired phenotype might be because of the underlying biology of the *in vitro* screen. Artificial hydrophobic surfaces like membrane discs are different than the host tissue, even though the relative humidity can be maintained using glycerol solution to mimic that of leaf surfaces. Additionally, excessive nutrients, from the agar medium, led to conidia overgrowth on the discs which led to a noisy background environment on the confocal microscope. This contributed to the inconsistency in the data collection process.

2.6 Tables and Figures

Table 2.1: Fungal strains used in this study

Species	***Strain***	***Genotype***	***Reference***
SCOH1-5	*C. zeae-maydis*	Wild-type	Bluhm *et al*., 2008
SCOH1-5-GFP	*C. zeae-maydis*	Wild-type GFP reporter	Hirsch 2014
Czm ATMT 3.20	*C. zeae-maydis*	Insertion mutant	This study
Czm ATMT 3.68	*C. zeae-maydis*	Insertion mutant	This study
Czm ATMT 3.71	*C. zeae-maydis*	Insertion mutant	This study
Czm ATMT 4.176	*C. zeae-maydis*	Insertion mutant	This study
Czm ATMT 9.8	*C. zeae-maydis*	Insertion mutant	This study
Czm ATMT 10.10	*C. zeae-maydis*	Insertion mutant	This study
Czm ATMT 10.16	*C. zeae-maydis*	Insertion mutant	This study
Czm ATMT 10.118	*C. zeae-maydis*	Insertion mutant	This study
Czm ATMT 11.70	*C. zeae-maydis*	Insertion mutant	This study
Czm ATMT 11.147	*C. zeae-maydis*	Insertion mutant	This study
Czm ATMT 12.95	*C. zeae-maydis*	Insertion mutant	This study
Czm ATMT 12.100	*C. zeae-maydis*	Insertion mutant	This study
Czm ATMT 13.16	*C. zeae-maydis*	Insertion mutant	This study
Czm ATMT 13.29	*C. zeae-maydis*	Insertion mutant	This study
Czm ATMT 13.41	*C. zeae-maydis*	Insertion mutant	This study
Czm ATMT 13.70	*C. zeae-maydis*	Insertion mutant	This study
Czm ATMT 13.130	*C. zeae-maydis*	Insertion mutant	This study
Czm ATMT 13.135	*C. zeae-maydis*	Insertion mutant	This study
Czm ATMT 13.147	*C. zeae-maydis*	Insertion mutant	This study
Czm ATMT 13.166	*C. zeae-maydis*	Insertion mutant	This study
Czm ATMT 13.170	*C. zeae-maydis*	Insertion mutant	This study
Czm ATMT 13.174	*C. zeae-maydis*	Insertion mutant	This study
Czm ATMT 13.197	*C. zeae-maydis*	Insertion mutant	This study
Czm ATMT 13.206	*C. zeae-maydis*	Insertion mutant	This study
Czm ATMT 14.54	*C. zeae-maydis*	Insertion mutant	This study
Czm ATMT 14.105	*C. zeae-maydis*	Insertion mutant	This study
Czm ATMT 14.188	*C. zeae-maydis*	Insertion mutant	This study
Czm ATMT 14.297	*C. zeae-maydis*	Insertion mutant	This study

Table 2.2: Transformations performed to create the random mutant collection used in this study

Transformation number	***Subcultured strains***
3	72
4	240
9	63
10	179
11	148
12	136
13	211
14	360
Total	1409

Table 2.3: Membrane discs used in this study

Vendor	***Membrane***	***Catalog Number***	***Pore Size***	
Sigma-Aldrich	Mixed cellular ester	GSWP04700	0.22µm	hydrophilic
Micron	Nylon	NO451P04700	4.5µm	hydrophilic
Whatman	Nitrocellulose	8447800	0.45µm	hydrophilic
Westran	Polyvinylidene difluoride	10413096N	0.2µm	hydrophobic

Table 2.4: *C. zeae-maydis* insertion mutants screened for impaired appressorium formation[a]

Transformation	***Number of mutants***	***No appressoria***
Czm ATMT 3	72	6
Czm ATMT 4	240	19
Czm ATMT 9	63	1
Czm ATMT 10	179	14
Czm ATMT 11	148	8
Czm ATMT 12	136	10
Czm ATMT 13	211	25
Czm ATMT 14	360	48
Total	1409	131

[a] "*Czm* ATMT" denotes *C. zeae-maydis Agrobacterium tumefaciens*-mediated transformation number.

Table 2.5: *In vitro* re-screen of 131 mutants impaired in appressorium formation

Appressorium production	*Number of Mutants*
No appressoria	41
More appressoria production compared to WT	5
Relatively same appressoria production compared to WT	10
Reduction in appressoria production compared to WT	40
Unknown	35

Table 2.6: *C. zeae-maydis* insertion mutants screened for impaired appressorium formation *in planta*

Strain	***Percent of successful appressorium formation[a]***
SCOH1-5-GFP	79.32% ± 3.75%
CZM ATMT 3.20	0.00% ± 1.58 %
CZM ATMT 3.68	67.67% ± 4.33%
CZM ATMT 3.71	0.00% ± 1.98%
CZM ATMT 4.176	64.49% ± 4.43%
CZM ATMT 9.8	73.49% ± 4.09%
CZM ATMT 10.10	91.94% ±2.52%
CZM ATMT 10.16	92.47% ± 2.45%
CZM ATMT 10.118	54.71% ± 4.61%
CZM ATMT 11.70	48.52% ± 4.63%
CZM ATMT 11.147	66.76% ± 4.37%
CZM ATMT 12.95	62.89% ± 4.48%
CZM ATMT 12.100	65.20% ± 4.41%
CZM ATMT 13.16	72.89% ± 4.12%
CZM ATMT 13.29	81.54% ± 3.60%
CZM ATMT 13.41	81.24% ± 3.62%
CZM ATMT 13.70	76.55% ± 3.93%
CZM ATMT 13.130	87.08% ± 3.11%
CZM ATMT 13.135	72.63% ± 4.13%
CZM ATMT 13.147	67.43% ± 4.34%
CZM ATMT 13.166	64.48% ± 4.43%
CZM ATMT 13.170	78.27% ± 3.82%
CZM ATMT 13.174	96.11% ± 2.67%
CZM ATMT 13.197	75.46% ± 3.99%
CZM ATMT 13.206	79.26% ± 3.76%
CZM ATMT 14.54	89.66% ± 2.82%
CZM ATMT 14.105	59.73% ± 4.55%
CZM ATMT 14.188	85.38% ± 3.27%
CZM ATMT 14.297	64.63% ± 4.43%

[a] Appressorium formation was quantified as the amount of successful appressoria formed over stomata over the total amount of stomatal interactions. Means and standard mean errors were calculated from calculated from three runs. Statistical Analysis Software (SAS), two-way ANOVA and Fisher's LSD confirmed these results are significantly different.

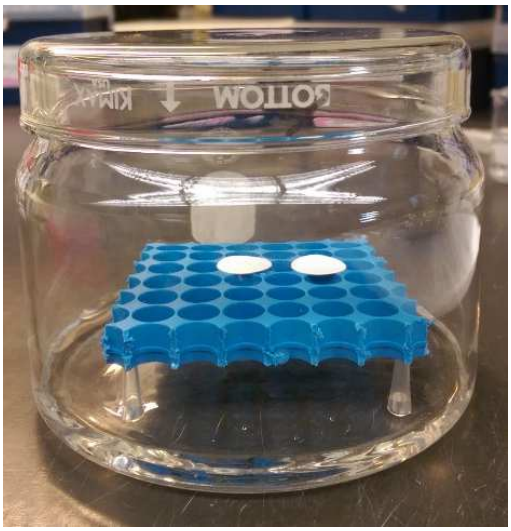


Figure 2.1: *In vitro* appressorium assay set-up. Inoculated mixed cellulose ester membrane discs on a raised apparatus are placed in the glass jar. A relative-humidity of 98% is regulated by the 8.11% glycerol solution. (Modified from Thorson and Martinson, 1993).

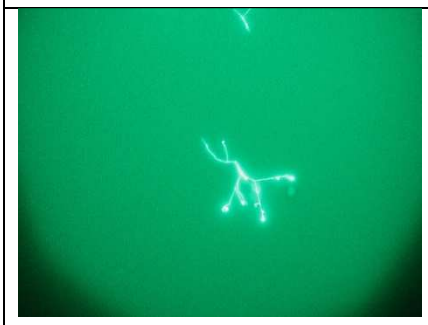
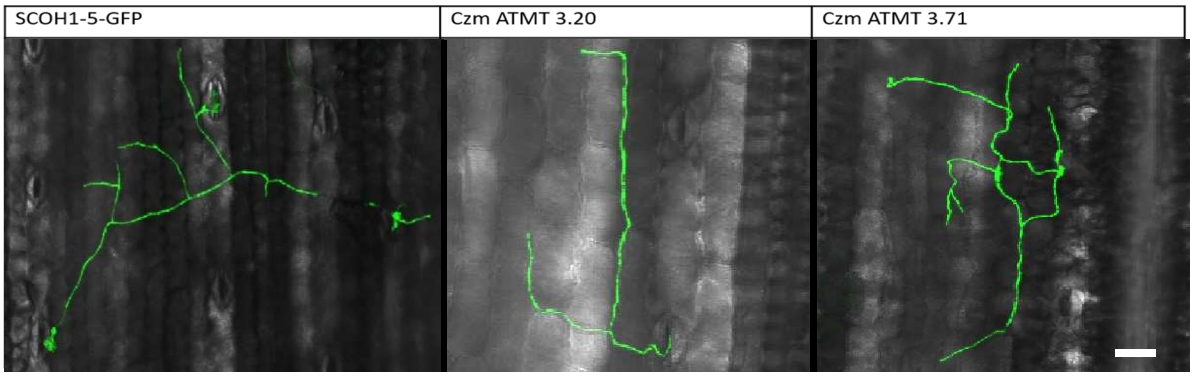
Mixed cellular ester membrane	Nylon membrane
	
Nitrocellulose membrane	Polyvinylidene difluoride membrane (PVDF)
	

Figure 2.2: Appressorium formation on membrane discs. Membrane discs were inoculated with wild-type (SCOH1-5-GFP) to assess *in vitro* appressorium formation. The pictures taken are representative picture of the assay. There is clear appressorium formation in samples assayed in the MCE, Nylon and Nitrocellulose discs compared to those in the PVDF. The bar marks 20µM for all micrographs.

A



B

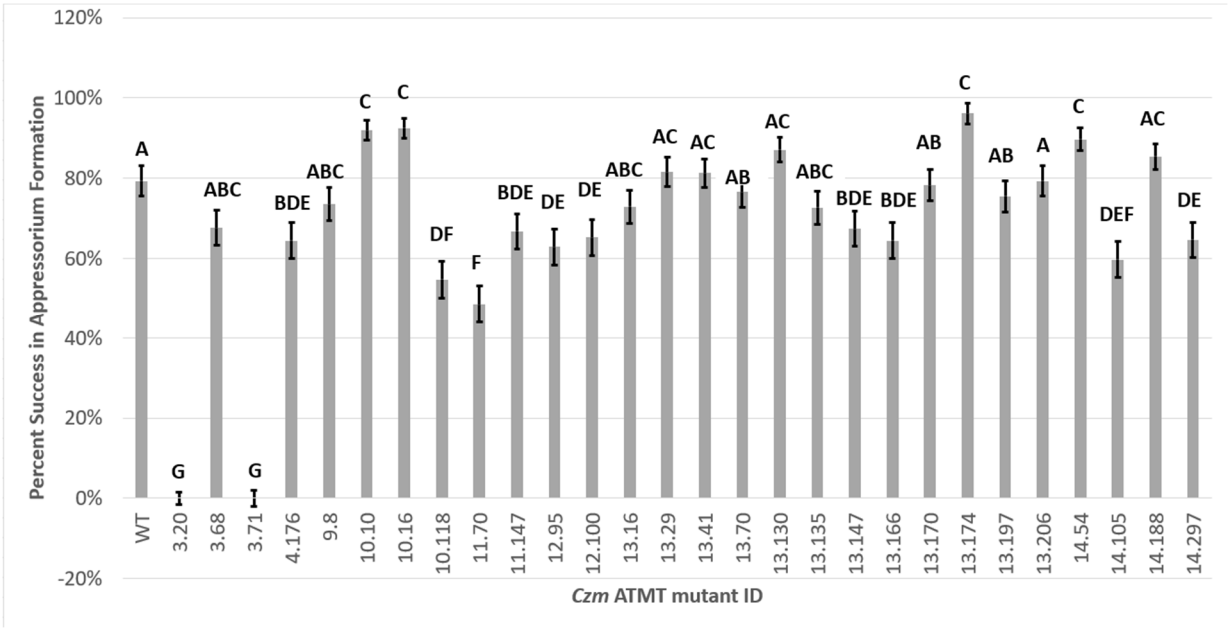


Figure 2.3: In planta appressorium formation. (A) Representative micrographs of wild-type (SCOH1-5-GFP) and mutant strains seven days after inoculation. Following tropic growth toward host stomata, the wild-type strain readily formed appressoria, while the *Czm* ATMT 3.20 and *Czm* ATMT 3.71 mutants failed to form appressoria. The bar marks 20 μM for all micrographs. **(B)** Percent appressorium formation measurements were conducted by counting how many appressoria were formed from 60 germ tubes, with the bars representing standard error. Statistical comparisons of means were performed with Statistical Analysis Software (SAS), two-way ANOVA and Fisher's LSD test (α=0.05; letters denote statistical significance).

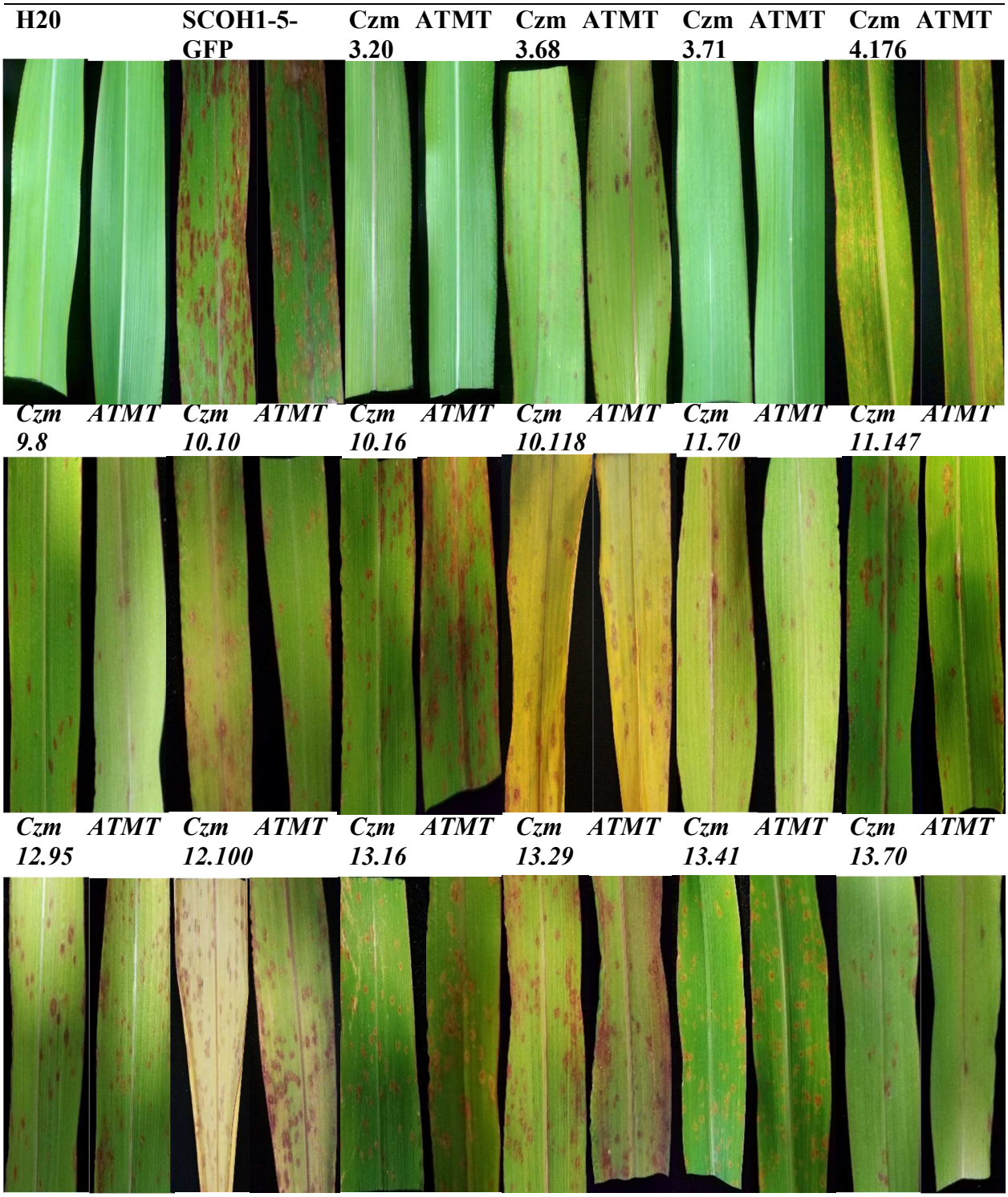


Figure 2.4: Lesion development of *C. zeae-maydis* strains. V4 silver queen maize plants were inoculated with either wild-type (SCOH1-5-GFP), insertion mutants, or a mock to assess disease progression after 14 days. The pictures taken are representative picture of the assay. When compared to wild-type strain, 2 insertion mutants, *Czm* ATMT 3.20 and *Czm* ATMT 3.71, are severely impaired in lesion development.

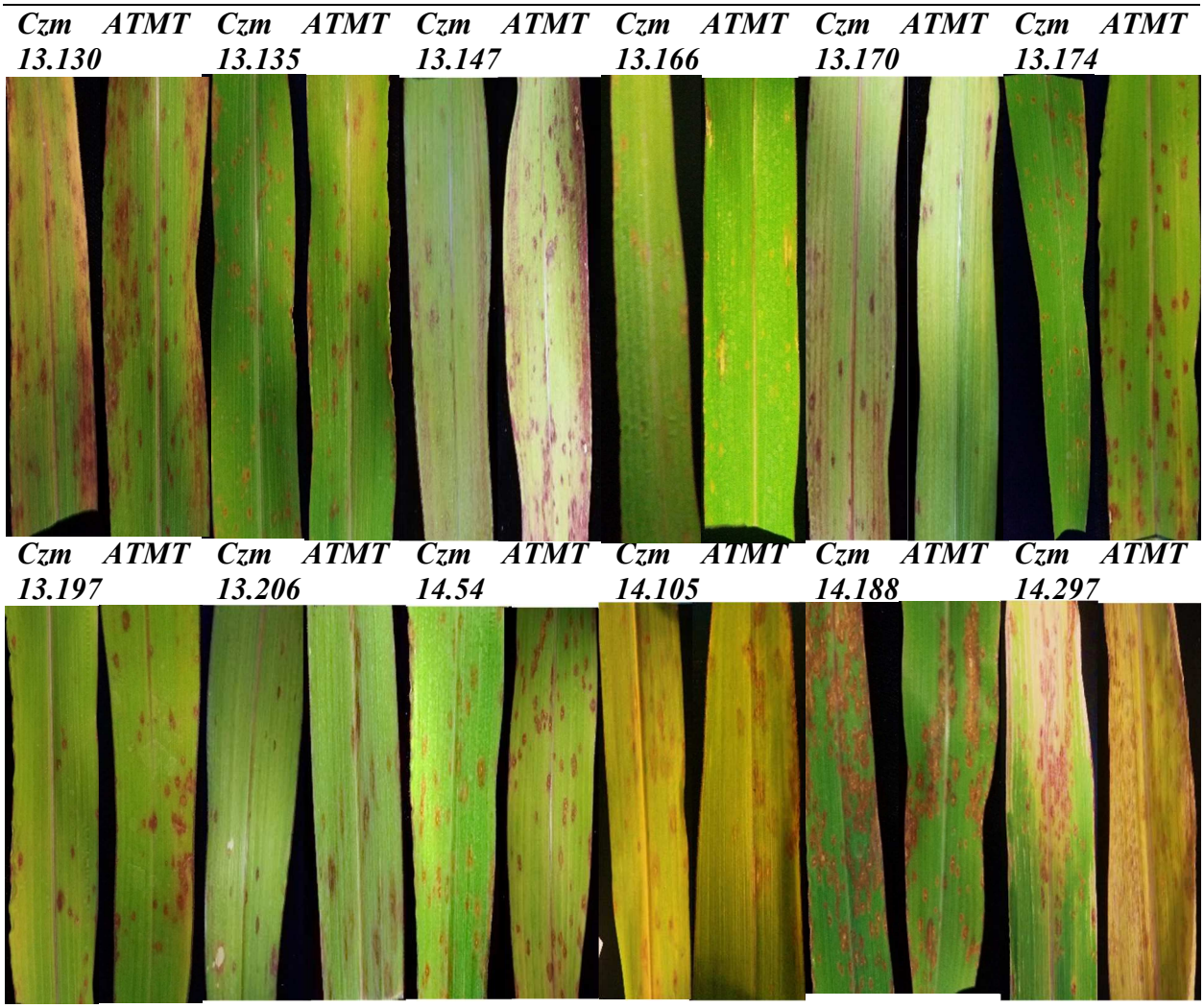


Figure 2.4 Cont. Lesion development of *C. zeae-maydis* strains. V4 silver queen maize plants were inoculated with either wild-type (SCOH1-5-GFP), insertion mutants, or a mock to assess disease progression after 14 days. The pictures taken are representative picture of the assay. When compared to wild-type strain, 2 insertion mutants, *Czm* ATMT 3.20 and *Czm* ATMT 3.71, are severely impaired in lesion development.

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CHAPTER III: THE IDENTIFICATION AND CHARACTERIZATION OF GENES IN MUTANTS OF INTEREST INVOLVED IN APPRESSORIUM FORMATION IN *CERCOSPORA ZEAE-MAYDIS* THROUGH FUNCTIONAL GENOMIC APPROACHES

3.1 Abstract

In this study, forward and reverse genetic approaches were utilized to identify and disrupt genes involved in the pre-infectious phase of *Cercospora zeae-maydis*. Two tagged insertional mutants, previously identified to be defective in appressorium formation and apathogenic, were targeted for functional disruption. Target enrichment sequencing revealed two genes *Czm109919* and *Czm41382 that* were disrupted in the mutants: a CAZyme gene of the GH76 family, and an MEKK gene belonging to the *STE11* family. Targeted deletion mutants of the MEKK-encoding gene failed to form appressoria on maize leaves and were apathogenic, thus confirming a role for the gene in pathogenesis. *STE11* has been shown to be involved in pathogenesis in many plant pathogenic fungi like *Fusarium graminearum*, *Magnaporthe oryzae*, and *Cochliobolus heterostrophus.* However, deletion mutants of the GH76 CAZyme formed appressoria and were pathogenic, indicating that the mutation was not linked to the phenotype of interest. This research implicated a specific MEKK pathway in the regulation of appressorium formation and pathogenesis, providing novel genetic resources to help further discern pathogenesis in *C. zeae-maydis*.

3.2 Introduction

Fungal pathogens have evolved elaborate strategies to infect host tissues. These strategies, however, involve complex regulatory networks that facilitate the disease initiation phase (Deising *et al*., 2000; Ryder and Talbot, 2015). For many pathogens, the correct combination of topographic and chemical cues- surface hardness, hydrophobicity and absence of exogenous nutrients- trigger signal cascades and the formation of mature appressorium (Kamamura *et al*., 2002; Talbot, 2003; Liu *et al*., 2007). The rice blast fungus *Magnaporthe oryzae* produces spores that adhere to the rice surface and germinate shortly after contact (Talbot, 2003; Dean *et al*., 2005; Gilbert *et al*., 2006). As conidia continue to germinate, the rice blast fungus displays major transcriptional reprogramming as appressoria begin to form from the developing germ tubes in response to surface hydrophobicity (Takano *et al*., 2003; Gowda *et al*., 2006; Oh *et al*., 2008). The identification and characterization of genes involved in the crucial development of appressoria during pathogenesis in *M. oryzae* and other filamentous fungi has highlighted the conservation of important signaling networks like mitogen-activated protein kinases (MAPK) and cyclic AMP (Thines *et al*., 2000; Bahn *et al*., 2007; Anjago *et al*., 2018). At the molecular level, appressorium formation has been shown to be regulated by the MAPK and cAMP signal transduction in *Colletotrichum* species, *M. oryzae* and other fungi (Zhao *et al*., 2005). Several MAPK genes essential for appressorium formation have been characterized in several appressorium-forming pathogens including *Colletotrichum lagenarium*, *Cochliobolus heterostrophus* and *Pyrenophora teres* (Lev *et al*., 1999; Takano *et al*., 2000; Ruiz-Roldan *et al*., 2001).

Next-Generation Sequencing (NGS) technology has tremendously changed the field of genetics, enabling largescale, high throughput genetic studies for a variety of research and diagnostic applications (Dapprich *et al*., 2016). Through targeted DNA sequencing of specific

genomic loci, many research and diagnostic applications are best achieved (Dapprich *et al*., 2016). Several methods have been developed for the targeted enrichment of genomic DNA (Mertes *et al*., 2011; Altmuller *et al*., 2014; Turner *et al*., 2009; Ballester *et al*., 2016) and they are typically based upon a multiplexed PCR amplification reaction, DNA hybridization to a capture oligonucleotide (either on an array or in solution) (Gnike *et al*., 2009; Albert *et al*., 2007; Cao *et al*., 2013) or DNA capture via molecular inversion probe circularization (Porreca *et al*., 2007).

In the previous chapter, a collection of tagged random insertional mutants was generated and assayed for phenotypes involved in the pathogenesis of *C. zeae-maydis*. From the mutant collection, two mutants exhibited impaired appressorium formation and impaired pathogenicity. In this study, the two mutants were analyzed through target enrichment sequencing. The sequencing identified two genes *Czm109919* and *Czm41382,* belonging to the CAZyme family (GH76) and *STE11* (MEKK) family, respectively. Concurrently, the two genes were selected for targeted disruption based on similarities to known regulatory genes. Targeted deletion mutants of the MEKK-encoding gene failed to form appressoria on maize leaves and were apathogenic, while deletion mutants of the GH76 CAZyme formed appressoria and were pathogenic. This study identified a specific MEKK pathway involved in the genetic regulation of appressorium formation and pathogenesis in *C. zeae-maydis*, and the strains developed in this study represent a significant increase in molecular resources for future research of this pathogen.

3.3 Materials and Methods

3.3.1 Fungal Strains and culture conditions

As described in the previous chapter, the fungal strains used in this study were obtained from the Bluhm lab stocks. The *C. zeae-maydis* targeted gene-disruption strains generated for this study are described in Table 3.1. All strains were stored as spore suspensions in 25% glycerol at -80°C. Working stocks were maintained on V8 agar medium (180ml/L V-8 juice, 2g/L calcium carbonate, 20g/L agar) grown under constant darkness, subcultured every four days and renewed from glycerol stock every 3-4 months.

3.3.2 Genomic analysis of *C. zeae-maydis* random insertion mutants

Fungal genomic DNA from *Czm* ATMT 3.20 and *Czm* ATMT 3.71 was isolated with the modified CTAB extraction method (Doyle and Doyle, 1990). DNA sequencing libraries were prepared using the NEBNext Fast DNA fragmentation and library prep kit for Ion torrent, following manufacturer's protocol. Each fragment library was from 1 µg of genomic DNA. The DNA fragments were PCR amplified by adding 50µl NEBNext Q5 Hot HiFi PCR master mix, 10µL amplification primer mix, sterile 35µL H_2O and 5µL purified, size-selected library DNA. The reactions were then mixed by pipetting and split into two 0.2-mL PCR tubes, each containing 50µL and PCR was performed. The PCR conditions consisted of one cycle of 98°C for 30 seconds, six cycles of 98°C for 10 seconds, 58°C for 30 seconds, 65°C for 30 seconds, then one cycle of 65°C for 5 minutes. The previously split PCRs were combined into new 1.5-mL LoBind tubes (100 µL total volume). Agencourt AMPure XP beads (0.9X the sample volume) was added to each sample and thoroughly mixed and incubated the mixture for 5 minutes at room temperature. The samples were pulse-spun, and the tubes were placed on a magnetic rack for two to three minutes

until the beads had collected to the side of the tubes and the solution was clear. The supernatants were carefully removed and discarded from the tubes without disturbing the beads. With the tubes still on the magnetic rack, 200µL of freshly prepared 80% ethanol was added to the samples and incubated at room temperature for 30 seconds before carefully removing and discarding the supernatant. The ethanol wash was repeated twice. The samples were then air-dried at room temperature for five minutes. The tubes were removed from the magnetic rack and 25µL of 0.1X TE buffer was added directly to the pellet to disperse the beads. The samples were then mixed thoroughly, pulse-spun, and placed back on a magnetic rack for two minutes until the solutions were clear at room temperature. 20µL of supernatant containing the eluted DNA were transferred to new 1.5-mL LoBind tubes without disturbing the pellets. 2-3µL of the library was diluted in 1:4 0.1X TE buffer. Each purified, pre-capture library was then quantitated using the bioanalyzer, Tapestation. Two barcoded libraries for each mutant, 250 ng each, were then used in the target capture reaction prior to sequencing.

Target enrichment was performed using the next generation protocol from Integrated DNA technologies. The protocol includes the necessary steps for hybridization of xGen lockdown probes with a library prepared from genomic DNA and for target enrichment by PCR before sequencing. Briefly, the xGen lockdown probes and the xGen universal blocking oligos were resuspended in IDTE pH 8.0 to a final concentration of 0.75 pmol/µL and 1rxn/µL (1X final concertation), respectively. The prepared library for *Czm* ATMT3.20 and *Czm* ATMT3.71 was combined with the Cot-1 DNA and the xGen universal blocking oligos. The DNA capture probes were then hybridized to the prepared library by incubating the samples in a thermal cycler at 65°C for four hours. Hybridization at 65°C improves the percentage of on-target capture. The hybridized targets were then bound to Streptavidin beads by placing the samples in a thermal cycler at 65°C for 45

minutes. The streptavidin beads were then washed with preheated wash buffer to remove unbound DNA. A magnetic rack was used to allow beads to fully separate from the supernatant, which contained the unbound DNA. Two washes at 65°C, maintained by a water bath at room temperature were performed. Following the washes, the beads, with bound DNA, were resuspended and a post capture PCR enrichment was performed. The PCR was done using the primer set for the Ion Torrent: 2x KAPA HiFi hot-start ready mix, 10μM Ion Torrent primer and 10μM Ion torrent P1 primer. The post-capture PCR fragments were then purified, following the Agencourt AMPure protocol, and prepared for sequencing via the Ion Torrent. The sequenced data was then compared to the sequenced genome hosted by the Department of Energy Joint Genome Institute (http://genome.jgi-psf.org/Cerzm1/Cerzm1.home.html) with BLASTp search.

3.3.3 Disruption of specific genes in *C. zeae-maydis*

Disruption cassettes were constructed with the split-marker PCR strategy (Table 3.2; Hirsch, 2014; Ridenour *et al*., 2012). DNA fragments corresponding to the 5' upstream and 3' downstream regions flanking the *Czm109919* gene were amplified with primer sets CZM 109919 F1/CZM 109919 F2, and CZM 109919 F3/CZM 109919 F4, respectively (Table 3.2). To create the split selectable marker, 1 kb portions of the 1.4 kb hygromycin B phosphotransferase (HYG^R) gene cassette were amplified from pTA-HYG with primers M13F/HY1 and M13F/YG1. Two fusion products combining the 5' flank to HY and the 3' flank to YG were created using primers CZM 109919 F1n/HY2 and CZM 109919 F4n/YG2. Protoplasts of *C. zeae-maydis* strain SCOH1-5 were transformed as previously reported (Kim *et al*., 2011B). After seven days, transformants were subcultured on V8 medium containing 150 μg/mL hygromycin and sub-cultured every four days to increase colony size. Genomic DNA was extracted following standard protocols (Doyle and Doyle, 1992) and transformants were screened by PCR with primers CZM 109919 A1/ HYG

SCRN B and CZM 109919 A1/ CZM 109919 F2. The isolates that tested positive for a targeted disruption were subjected to a secondary PCR screen with primers CZM 109919 A1/ CZM 109919 F2, CZM 109919 A1/HYG SCRN B, CZM 109919 A2/CZM 109919 F3, and CZM 109919 A2/HYG SCRN C to identify strains disrupted in the *CZM109919* gene. Six isolates that tested positive for disruption of *Czm109919* by PCR were designated *Δczm109919*-8, *Δczm109919*-9, *Δczm109919*-11, *Δczm109919*-13, *Δczm109919*-14, and *Δczm109919*-17.

DNA fragments corresponding to the 5' upstream and 3' downstream region flanking the *CZM41382* gene were amplified with primer sets CZM 41382 F1/CZM 41382 F2, and CZM 41382 F3/CZM 41382 F4, respectively (Table 3.2). 1 kb portions of the 1.4 kb hygromycin B phosphotransferase (HYG^R) gene cassette were amplified from pTA-HYG with primers M13F/HY1 and M13F/YG1 to create the split selectable marker. The two fusion products combining the 5' flank to HY and the 3' flank to YG were created using primers CZM 41382 F1n/HY2 and CZM 41382 F4n/YG2. Protoplasts of *C. zeae-maydis* strain SCOH1-5 were then transformed as previously reported (Kim *et al*., 2011B). After seven days, transformants were subcultured on V8 medium containing 150 µg/mL hygromycin and sub-cultured every four days to increase colony size. Genomic DNA was extracted following standard protocols (Doyle and Doyle, 1990) and transformants were screened by PCR with primers CZM 41382 A1/ HYG SCRN B and CZM 41382 A1/ CZM 41382 F2. The isolates that tested positive for a targeted disruption were subjected to a secondary PCR screen with primers CZM 41382 A1/ CZM 41382 F2, CZM 41382 A1/HYG SCRN B, CZM 41382 A2/CZM 41382 F3, and CZM 41382 A2/HYG SCRN C to identify strains disrupted in the *Czm41382*. Two isolates that tested positive for disruption of *Czm41382* by PCR were designated *Δczm41382*-7 and *Δczm41382*-8.

3.3.4 Evaluation of the infection process

Silver Queen maize plants (three plants per strain) at V4 stage (three weeks after emergence) were inoculated with 10ml of a conidium suspension (10^5 conidia/ml) of strains SCOH1-5-GFP strain, *Czm109919* and *Czm41382* deletion mutant strains (*Δczm109919*-8, *Δczm109919*-9, *Δczm109919*-11, *Δczm109919*-13, *Δczm109919*-14, *Δczm109919*-17, *Δczm41382*-7 and *Δczm41382*-8). The conidial suspensions for all inoculations were amended with 0.01% Tween-20 and plants were inoculated with an atomizer attached to an air compressor until inoculum run-off. Three mock-inoculated control plants were sprayed with the same Tween-20 solution but contained no conidia. After inoculation, the plants were then placed in incubation chambers made of wire mesh wrapped in opaque plastic to maintain free moisture on plants and increase humidity. The incubation chambers were of large enough to surround the inoculated plants but not touch the leaves. The chambers containing inoculated maize plants were placed in a large growth chamber, maintained at 23°C with a 12:12 light: dark photoperiod with a light intensity at leaf level of 300μmol m^{-2} s^{-1}. To assess appressorium formation on leaf surfaces, infected leaves from each inoculated plant were collected four and seven days after inoculation. Small leaf sections (1.5cm x 3cm) were cut from each leaf for microscopic examination as performed previously (chapter II). To assess lesion development, disease was allowed to progress for 14 days.

3.4 Results

3.4.1 Genomic analysis of *C. zeae-maydis* random insertional mutants

The genomic analysis of the two *C. zeae-maydis* random insertional mutants, *Czm* ATMT 3.20 and *Czm* ATMT 3.71, impaired in *in-vitro* appressorium formation and pathogenicity are

shown in table 3.3. The disruption cassette for mutant *Czm* ATMT3.71 inserted in Scaffold 12 (location 569112-572000) and the gene ID is 41382, hereafter referred to as *Czm41382* in this study. The gene contains a 2,892 bp open reading frame with one 243 bp intron and encodes an 883-amino acid sequence. A search in the sequenced genome hosted by the Department of Energy Joint Genome Institute (http://genome.jgi-psf.org/Cerzm1/Cerzm1.home.html) revealed that the gene belongs to STE11 family and is a MAP Kinase Kinase Kinase (MEKK) and is possibly downstream of G protein signaling. A BLASTp analysis of the predicted protein identified homology to *Sphaerilina musiva* (teleomorph *Mycosphaerella populorum*) (Ohm *et al*., 2012). This gene has also been shown to be involved in pathogenesis in many plant pathogenic fungi like *Fusarium graminearum, Magnaporthe oryzae,* and *Cochliobolus heterostrophus* (Gu *et al*., 2014; Zhao *et al*., 2005; Izumitsu *et al*., 2009).

The disruption cassette for mutant *Czm* ATMT3.20 integrated downstream of gene 109919 (referred to as *Czm109919* in this study) in scaffold 5 (location 260121-261826). The gene contains a 1,706 bp open reading frame with four introns (137 bp, 104 bp, 58 bp, and 69 bp) and encodes a 446-amino acid sequence. A search in the JGI showed the gene belongs to Carbohydrate-Active Enzymes (CAZyme) family and is a glycoside hydrolase family 76 (GH76). GH76 are endo-acting α-mannanases and their genes are found within bacteria and fungi. In fungi, the GH76 family of enzymes are speculated to be required for cross linking GPI anchored proteins into cell walls, where they are proposed to act as transglycosylases (Kitagaki *et al*., 2002).

3.4.2 Disruption of *Czm109919* in *C. zeae-maydis*

To elucidate the function of *Czm109919*, the gene belonging to the GH76 CAZyme family, gene-deletion or knock-out mutants for the genes were generated using the split-marker system (Table 3.2; Ridenour *et al*., 2012; Hirsch, 2014). The coding region of the gene was replaced by

the hygromycin B phosphotransferase gene (HYG^R) in the gene deletion mutants as confirmed by PCR analysis. The split-marker approach resulted in the generated 30 gene transformants *Czm109919* (table 3.4). Six putative gene-deletion mutants, *ΔCzm109919*- 8, *ΔCzm109919*-9, *ΔCzm109919*-11, *ΔCzm109919*-13, *ΔCzm109919*-14 and *ΔCzm109919*-17 were obtained from this knock-out experiment (Table 3.5). The deletion mutants were confirmed by PCR analysis with primers CZM 109919 A1/ CZM 109919 F2, CZM 109919 A1/HYG SCRN B, CZM 109919 A2/CZM 109919 F3, and CZM 109919 A2/HYG SCRN C. The expected amplicon appeared only on the deletion mutants but not in the WT (SCOH1-5-GFP), indicating that the gene was successfully replaced by the HYG^R gene (Figure 3.2).

3.4.3 Disruption of *Czm41382* gene in *C. zeae-maydis*

To investigate the function of *Czm41382*, the MEKK gene of the *STE11* family, gene-deletion or knock-out mutants for the genes were generated using the split-marker system (Table 3.2; Ridenour *et al*., 2012; Hirsch, 2014). The coding region of MEKK was replaced by the hygromycin B phosphotransferase gene (HYG^R) in the gene deletion mutants as confirmed by PCR analysis. The split-marker approach resulted in the generation of 25 gene transformants for *Czm41382* (table 3.4). Two putative gene-deletion mutants, *ΔCzm41382*-7 and *ΔCzm41382*-8, were obtained from this knock-out experiment (Table 3.5). The deletion mutants were confirmed by PCR analysis with primers CZM 41382 A1/ CZM 41382 F2, CZM 41382 A1/HYG SCRN B, CZM 41382 A2/CZM 41382 F3, and CZM 41382 A2/HYG SCRN C. The expected amplicon appeared only in the deletion mutants but not in the WT (SCOH1-5-GFP), indicating that the gene was successfully replaced by the HYG^R gene (Figure 3.2).

3.4.4 Evaluation of the infection process

To confirm the phenotype of the gene-deletion mutants generated, an *in-planta* screen was performed assessing appressorium formation. The percent of appressoria formed upon stomatal interaction was quantified for each gene-deletion single-spore isolate in comparison to wild-type. Notably, *Czm41382* gene-deletion single-spore isolates failed to produce appressoria while *Czm109919* gene-deletion single-spore isolates formed appressoria upon stomatal encounter (Table 3.6; Figure 3.3). Two-way ANOVA and Fisher's LSD revealed that the *Czm109919* gene-deletion single-spore isolates were significantly different in appressorium production compared to the wild-type. To see effect on pathogenicity, disease symptoms such as lesion development were assayed 14 days after inoculation. *Czm41382* gene-deletion single-spore isolates were apathogenic while *Czm109919* gene-deletion single-spore isolates were pathogenic on maize plants (Figure 3.4).

3.5 Discussion

The genetic regulation of appressorium formation is poorly understood in *Cercospora zeae-maydis*. The pathogen senses and reorients its growth toward the stomata to form appressoria-like infection structures on stomata in the maize surface (Kim *et al*., 2011A, B). This study characterized and identified a regulatory gene potentially involved in the pre-infectious development prior to stomatal penetration and pathogenesis. The characterizing of this gene regulating appressorium formation adds to the developing narrative of how dothidiomycetes interact with their hosts during infection.

The transduction of a variety of extracellular signals and the regulation of different developmental processes in eukaryotic cells are regulated by a family of serine/threonine protein

kinases known as mitogen-activated protein (MAP) kinases (MAPKs) (Zhao *et al*., 2007). MAP kinases are activated by MAPK kinases (MEK) which are activated by MEK kinases (MEKK). These MEKK-MEK-MAPK cascades are highly conserved in eukaryotes and have been extensively studied in several organisms. There are five MAPK pathways in the yeast *Saccharomyces cerevisiae:* the pheromone *FUS3/KSS1* pathway regulates mating (Dohlman, 2002), the *KSS1* pathway regulates filamentous growth, the *SLT2* pathway regulates cellular integrity and promotes cell wall biosynthesis (Heinsch, 2005), the *HOG1* pathways is required for high osmolarity response (Hohmann, 2002; Saito and Tatebayashi, 2004), and the *SMK1* pathway, a sporulation specific MAPK activated by intracellular signals (Huang *et al*., 2005). The most characterized MAPK pathway is the yeast pheromone response pathway, which is initiated by the binding of mating pheromones to a receptor and the release of stimulatory Gβ subunits. These Gβ associate with a scaffold protein, *STE5*, and a p21-activated kinase (*PAK*), *STE20*, and is essential for activating the MEKK *STE11*, which in turn activates the MEK *STE7*. Downstream from *STE7, FUS3* and *KSS1* are two partially redundant MAPKs that regulate the mating process (Kusari *et al*., 2004). Several elements of this pheromone response pathway, including kinases *STE20, STE11, STE7*, and *KSS1*, also are involved in filamentous growth in *S. cerevisiae* (Cherkasova *et al*., 2003).

At the molecular level, appressorium formation has been shown to be regulated by the MAPK and cAMP signal transduction in *Colletotrichum* species, *M. oryzae* and other fungi. In *M. oryzae*, several genes within the MAPK have been identified that regulate appressorium formation, cellular turgor control and appressorium function (Anjago *et al*., 2018). The cAMP signaling pathway has been shown to be involved in surface recognition and appressorium turgor generation (Thines *et al*., 2000; Bahn *et al*., 2007). Xu and Hamer (1996) identified *PMK1* (pathogenicity

MAP kinase 1), a homolog of yeast *FUS3/KSS1* MAPKs, and found that it regulates the late stages of appressorium formation and infectious hyphal growth. Germ tubes of *pmk1* mutants failed to form appressoria but still recognized hydrophobic surfaces or responded to exogenous cAMP and produce subapical swollen bodies (Xu and Hamer, 1996; Xu, 2000). *PMK1* also is essential for infectious hyphal growth after penetration. In transformants expressing a *PMK1*-green fluorescent protein construct, enhanced green fluorescent protein signals and nuclear localization are observed in appressoria and developing conidia (Bruno *et al*., 2004). The pathway includes three protein kinases, *MST11*, *MST7* and *PMK1*, which appear to be scaffolded by a protein called *MST50*, which interacts with *MST11*, and upon activation and phosphorylation of its components, a phosphor-relay is triggered leading to the detachment of *PMK1* and its transversal to nucleus during appressorium maturation (Zhao *et al*., 2005; Park *et al*., 2006; Wilson and Talbot, 2009). Like the *pmk1* mutants, deletion mutants of *MST7* or *MST11* failed to form appressoria and failed to colonize rice tissues through wounds (Zhao *et al*., 2005).

Homologs of *FUS3/KSS1* MAPKs in several other appressoria forming plant pathogenic fungi have also been characterized, including *Cochliobolus heterostrophus*, *Colletotrichum lagenarium*, and *Pyrenophora teres* (Lev *et al*., 1999; Takano *et al*., 2000; Ruiz-Roldan *et al*., 2001). In all these fungi, *PMK1* homologs are essential for appressorium formation. Like the *pmk1* mutants, the *ptk1* mutants in *P. teres, cmk1* mutants in *C. lagenarium* and *chk1* mutants in *C. heterostrophus* are apathogenic, failed to form appressoria and failed to colonize healthy or wounded host tissue (Takano *et al*., 2000; Ruiz-Roldan *et al*., 2001).

Homologs of *FUS3/KSS1* in several other non-appressoria forming plant pathogenic fungi that have also been characterized. The MEKK *ChSTE11* of *C. heterostrophus* regulates sexual/asexual development, melanization, pathogenicity and adaptation to oxidative stress

(Izumitsu *et al*., 2009). Like the *chk1* mutants, *Δchste11* mutant strains showed defects in conidiation, sexual development, melanization and the formation of appressoria (Izumitsu *et al*., 2009). In *Fusarium graminearum*, the *PMK1* homolog *GPMK1* regulates fungal development and is essential for virulence (Gu *et al*., 2014).

The infection process of *C. zeae-maydis* differs from *M. oryzae* in terms of stomatal tropism and appressorium formation. However, little is known about the genetic regulation of formation of infection structures in *C. zeae-maydis*. *PMK1* orthologs are important for appressorium formation in several filamentous dothidiomycetes. Therefore, this study hypothesized that an ortholog of the MAPK could be an important regulator in *C. zeae-maydis.* Through target enrichment sequencing, this study identified a specific MEKK gene, *Czm41382*, belonging to the *STE11* family that regulates appressorium formation in the insertional mutant *Czm* ATMT 3.71. Gene deletion mutants *Δczm41382-7* and *Δczm41382-8* failed to produce appressorium and were apathogenic in maize plants, confirming that MAPKs are important for appressorium formation.

Unlike the MEKK identified in this study, another MEKK regulates an entirely different process in *C. zeae-maydis*. The MEKK *CZK3* was the first gene to be functionally characterized in *C. zeae-maydis* (Shim and Dunkle, 2003). An analysis of a cDNA subtraction library comprising of genes up-regulated during cercosporin biosynthesis revealed a sequence highly similar to MAP kinases in other fungi. Sequencing and conceptual translation of the full-length genomic sequence indicated that the gene contained a 4,119-bp open reading frame devoid of introns and encoded a 1,373-amino acid sequence that was highly similar to *Wis4*, a MEKK in *S. pombe* (Shim and Dunkle, 2003). Targeted disruption of the gene led to the suppression of the expression of cercosporin biosynthetic genes and the abolishment of cercosporin production. After

plant inoculation, the disrupted *czk3* mutants grew more quickly compared to the wild-type in culture, failed to produce conidia, and elicited small chlorotic flecks as opposed to necrotic lesions during infection, implicating *CZK3* in virulence (Shim and Dunkle, 2003). The MEKK *Czm41382,* characterized in the insertional mutant *Czm* ATMT 3.71, contains a 2,892 bp open reading frame with one 243 bp intron and encodes an 883-amino acid sequence, regulates appressorium formation in *C. zeae-maydis*. A preliminary cercosporin biosynthesis assay on the mutant *Czm* ATMT 3.71 revealed that there was a server reduction in cercosporin production compared to the wild-type, SCOH1-5, (data not presented in this study). However, a more extensive research is needed to discern the extent of the reduction in cercosporin production and whether the gene, *Czm41382,* also regulates cercosporin biosynthesis and virulence in *C. zeae-maydis*.

Target enrichment sequencing also identified a different gene disrupted in the insertional mutant *Czm* ATMT3.20. The gene belongs to the GH76 CAZyme family. Many fungi produce a wide range of carbohydrate activity enzymes (CAZymes) to degrade plant polysaccharides to gain nutrition or cause infection (Zhao *et al*., 2013). At present, CAZymes have been grouped into four functional classes: glycoside hydrolases (GHs), glycosyltransferases (GTs), polysaccharide lyases (PLs), and carbohydrate esterases (CEs) based on their structurally-related catalytic modules or functional domains (Cantarel *et al*., 2009). GHs are the most diverse groups of enzymes used in microbes in the degradation of biomass and over 100 GH families have been classified to date (Cantarel *et al*., 2009). Glycoside hydrolases hydrolyze the glycoside bond between two or more carbohydrate molecules, or between a carbohydrate molecule and another moiety, such as a lipid or protein (Cantarel *et al*., 2009).

A transcriptome analysis of the transcriptional reprograming associated with pre-penetration infectious development on leaf surfaces revealed that glycoside hydrolases were

significantly upregulated during growth on the leaf surface by *C. zeae-maydis* (Hirsch, 2014). Six glycoside hydrolases belonging to families 16, 31, 43, 53, and 54 encode proteins involved in the degradation of β-glycans, pectin, and hemicellulose, which are key structural components of plant cells and therefore represent enzymatic targets for pathogens (Zhao *et al*., 2013). The expression of genes encoding CAZymes by *C. zeae-maydis* prior to host infection and several days before symptoms begin to develop points toward a previously uncharacterized preparatory step of the initiation of host tissue degradation (Hirsch, 2014). This led to the hypothesis that *C. zeae-maydis* might begin to express and synthesize these CAZymes in preparation of the switch to necrotrophy days in advance, cultivating the availability of enzymes that can be quickly secreted to overwhelm plant defenses leading to rapid lesion development (Hirsch, 2014).

The glycoside hydrolase family 76 (GH76) is poorly characterized and is populated exclusively by bacterial and fungal members (Belz, 2016). The only known biochemical function for members of this family has been described for bacterial enzymes, termed α-1,6-mannanases, which hydrolytically cleave α-1,6-mannans, such as those found in the fungal cell wall (Belz, 2016; Maruyama and Nakajima, 2000). Separately, it has been hypothesized that certain fungal members are trans mannosidases, with the ability to cleave an α-mannosidic linkage within the glycosylphosphatidylinositol (GPI) glycan of cell wall proteins, and then catalyze the formation of a new glycoside linkage to the cell wall glycan, resulting in covalent attachment of mannoproteins to the fungal cell wall (Kitagaki *et al*., 2002). However, no biochemical data has been obtained for any fungal GH76 enzyme in support of the so-called anchorage hypothesis. Presently, there are no studies linking the GH76 family in pathogenesis of plant pathogens. Therefore, the identification of the CAZyme gene of the GH76 family in the random insertional mutant *Czm* ATMT 3.20 in this study lead to the hypothesis that a CAZyme gene could potentially

be involved in the regulation of appressorium in *C. zeae-maydis*. However, deletion of this gene resulted in mutants that produced appressoria and were pathogenic on maize leaves. An additional, unlinked mutation that was not detected by the target enrichment sequencing could explain why the random insertional mutant failed to produce appressoria and was apathogenic, but its gene deletion mutant failed to retain the phenotype.

3.6 Tables and Figures

Table 3.1: Fungal strains used in this study

Species	***Strain***	***Genotype***	***Reference***
SCOH1-5	*C. zeae-maydis*	Wild-type	Bluhm *et al*., 2008
SCOH1-5-GFP	*C. zeae-maydis*	Wild-type GFP reporter	Hirsch 2014
Czm ATMT 3.20	*C. zeae-maydis*	Insertion mutant	This study
Czm ATMT 3.71	*C. zeae-maydis*	Insertion mutant	This study
ΔCzm41382	*C. zeae-maydis*	Deletion strain	This study
ΔCzm109919	*C. zeae-maydis*	Deletion strain	This study

Table 3.2: Primers used in this study

Name	***Sequence***
CZM109919_A1	GTAGGTTGTCTCAAGCGACAACG
CZM109919_F1	TATCAACCACTCCCAACAGAGACATG
CZM109919_F1N	CATCAAGGTGCTTGCAGCCT
CZM109919_F2	ATTACAATTCACTGGCCGTCGTTTTACGTAGACTTTGGACTGGCAGAGC
CZM109919_F3	CGTAATCATGGTCATAGCTGTTTCCTGGCTTGATGTTCCAGTCATTCCGTC
CZM109919_F4N	GCAACACGACAAATTCCGATCCAC
CZM109919_F4	GCTTTTGATGAGGAGAAGGAGACC
CZM109919_A2	GTTCACTGCTAACCGCTGAATTGC
CZM109919_PF	GATAACTCGACTTGCGACGGAG
CZM109919_PR	CCATCCACCGGGAGAGATATGC
CZM41382_A1	CCAGTCACAGTGGACAGTAGTG
CZM41382_F1	GTGCCCTACATACTGCTACTGTAC
CZM41382_F1N	GTCGTGCATCTGATCGGCAC
CZM41382_F2	ATTACAATTCACTGGCCGTCGTTTTACGAATAACGCGGCGTCCGAGA
CZM41382_F3	CGTAATCATGGTCATAGCTGTTTCCTGCCTGACACCAACAACAACGAAG
CZM41382_F4N	CACACAGCTTGTGGGCAGTTTAG
CZM41382_F4	TTCCCAAAACACACCATGTGATGA
CZM41382_A2	CTCCCACATCATCAATCACCACG
CZM41382_PF	CGAAGACATCATCCGTGCGAC
CZM41382_PR	CCATAGAAGTCTTTGAGCTTCCTCG

Table 3.3: Genes investigated during this study

Gene name	***Strain name***	***Location (Scaffold: bp-bp)***	***Predicted function***	***Homologous to***	***Citation***
Czm41382	*ΔCzm41382*	12: 569112-572000	Mitogen-activated protein kinase kinase kinase (MEKK)	*Sphaerilina musiva* (teleomorph *Mycosphaerella populorum*)	Ohm *et al*., 2012
				Fusarium graminearum	Gu *et al*., 2014
				Magnaporthe oryzae	Zhao *et al*., 2005
				Cochliobolus heterostrophus	Izumitsu *et al*., 2009
Czm109919	*ΔCzm109919*	5: 260121-261826	Glycoside hydrolase family 76 (GH76)	*S. musiva*	Ohm *et al*., 2012

Table 3.4: Generation of the split-marker constructs and total number of gene transformants

Gene	***Constructs created***				***Total Strains***
	F1/F2	F3/F4	A	B	
Czm41382	Yes	Yes	Yes	Yes	30
Czm109919	Yes	Yes	Yes	Yes	25

Table 3.5: Putative gene deletion mutant strains created for this study and assayed by PCR[a]

Strain name	***5' flank***	***3' flank***
Δczm41382-1	No	No
Δczm41382-2	No	No
Δczm41382-3	Yes	No
Δczm41382-4	No	No
Δczm41382-5	Yes	No
Δczm41382-6	Yes	No
Δczm41382-7	Yes	Yes
Δczm41382-8	Yes	Yes
Δczm41382-9	No	No
Δczm41382-10	No	No
Δczm109919-1	No	No
Δczm109919-2	No	No
Δczm109919-3	No	No
Δczm109919-4	No	No
Δczm109919-5	No	No
Δczm109919-6	No	No
Δczm109919-7	No	No
Δczm109919-8	Yes	Yes
Δczm109919-9	Yes	Yes
Δczm109919-10	No	No
Δczm109919-11	Yes	Yes
Δczm109919-12	No	No
Δczm109919-13	Yes	Yes
Δczm109919-14	Yes	Yes
Δczm109919-15	No	No
Δczm109919-16	No	No
Δczm109919-17	Yes	Yes
Δczm109919-18	No	No

[a] "Yes" denotes a positive PCR result. "No" denotes the absence of expected amplicons during the PCR screens to confirm the integration of the split-marker mutagenesis cassette.

Table 3.6: Appressorium formation of *Cercospora zeae-maydis* gene-deletion mutant strains

Strain Name	***Percent of successful appressorium formation***[a]
SCOH1-5-GFP	79.72% ± 3.76%
Δczm109919-8ss2	63.08% ± 2.02%
Δczm109919-9ss5	64.06% ± 1.95%
Δczm109919-11ss1	73.67% ± 4.69%
Δczm109919-13ss6	63.61% ± 4.40%
Δczm109919-14ss5	61.94% ± 2.38%
Δczm109919-17ss2	67.50% ± 3.18%

[a] Appressorium formation was quantified as the amount of successful appressoria formed over stomata over the total amount of stomatal interactions. Means and standard errors were calculated from calculated from three independent experiments. Two-way ANOVA and Fisher's LSD confirmed that these results are not significantly different.

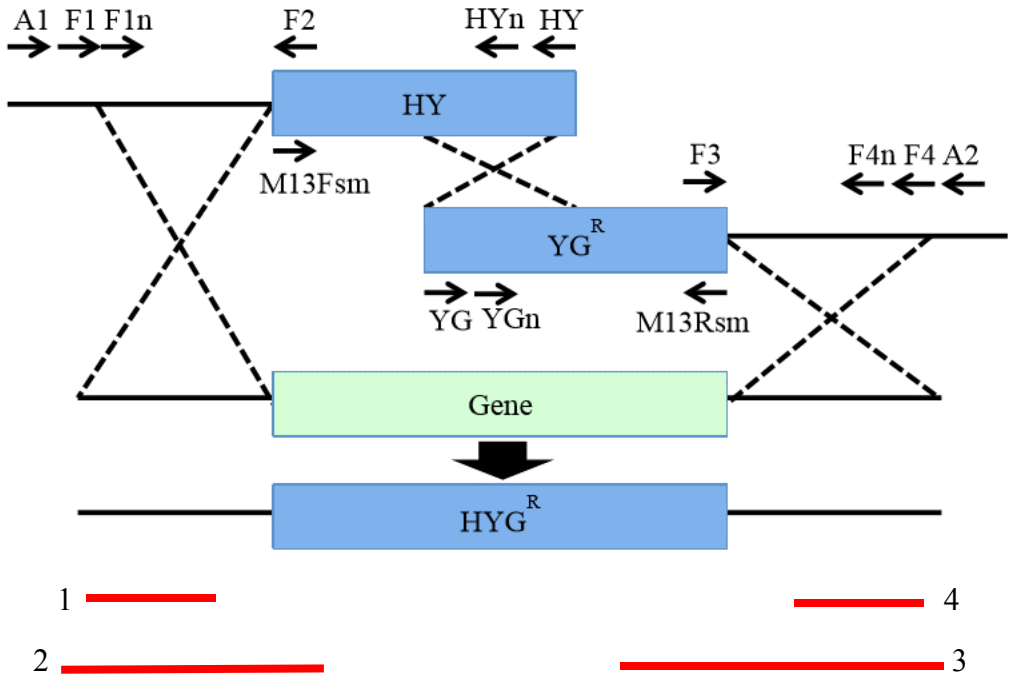

Figure 3.1: Targeted disruption of specific genes in *C. zeae-maydis*. A) Strategy for split-marker gene disruption by amplifying genomic flanks and fusing them to portions of the HYGR gene conferring resistance to the antibiotic hygromycin. Generation of the gene deletion constructs required the amplification of 5' (F1/F2) and 3' (F3/F4) genomic flanking regions and fusing them to fragment HY or YG of the HYG^R cassette, respectively, to form the split-marker constructs A and B. Putative transformants were screened by PCR by amplifying fragments 1 and 4 as positive genomic controls, and fragments 2 and 3 to confirm successful integration of the split-marker fragments on both genomic flanks (Hirsch, 2014).

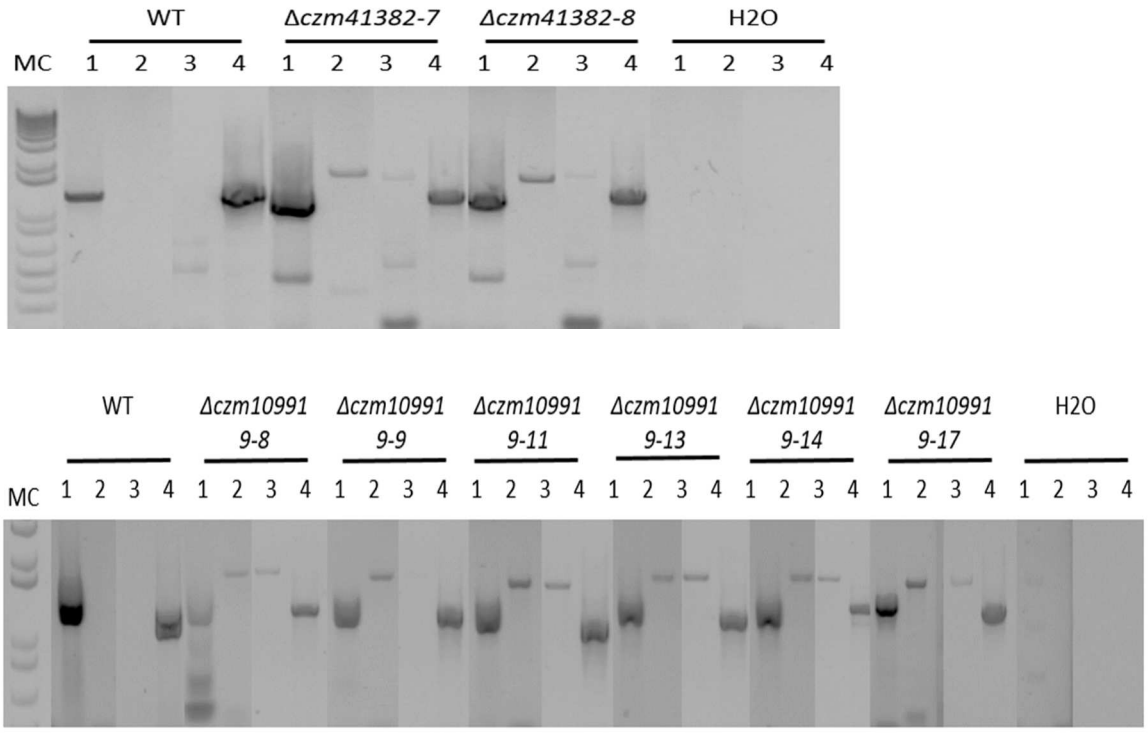

Figure 3.2: PCR validation of putative gene-deletion mutant strains. Independent gene deletion strains for *czm41382* and *czm109919*. Amplicons 1 and 4 (using primers sets A1/F2, and F3/A2, respectively) represent positive controls for DNA quality and quantity, while amplicons 2 and 3 (using primers sets A1/HYG SCRN B, and HYG SCRN C/A2, respectively) were only be expected in the presence of the hygromycin resistance gene integrated into the 5' and 3' genomic flank, respectively. PCR markers represent, in descending order, 2.0 kb, 1.5 kb, 1.0kb and 0.5 kb

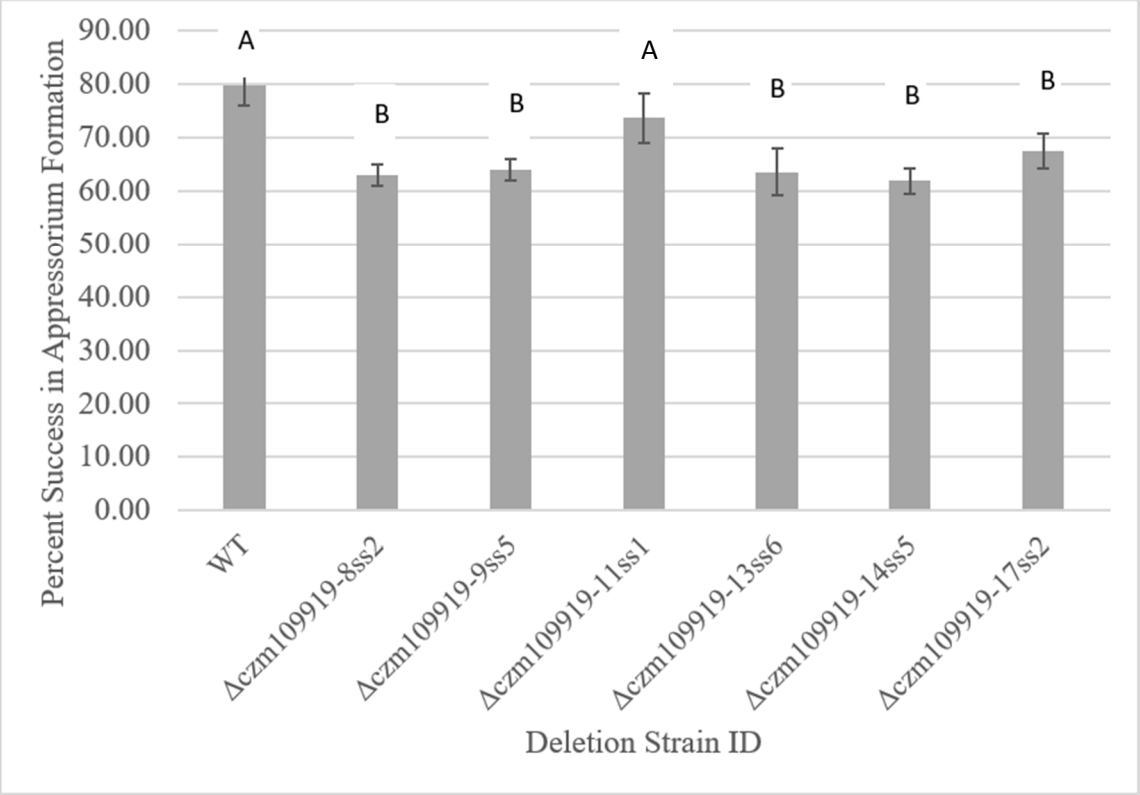

Figure 3.3: Appressorium formation of *Cercospora zeae-maydis* gene-deletion mutant strains. Appressorium formation was quantified in wild-type (SCOH1-5-GFP) and deletion mutant strains seven days after inoculation on V4 maize plants. The amount of appressoria formed over stomatal interactions by conidia was counted. Two-way ANOVA and Fisher's LSD were used to determine significance (α=0.05; letters denote significance).

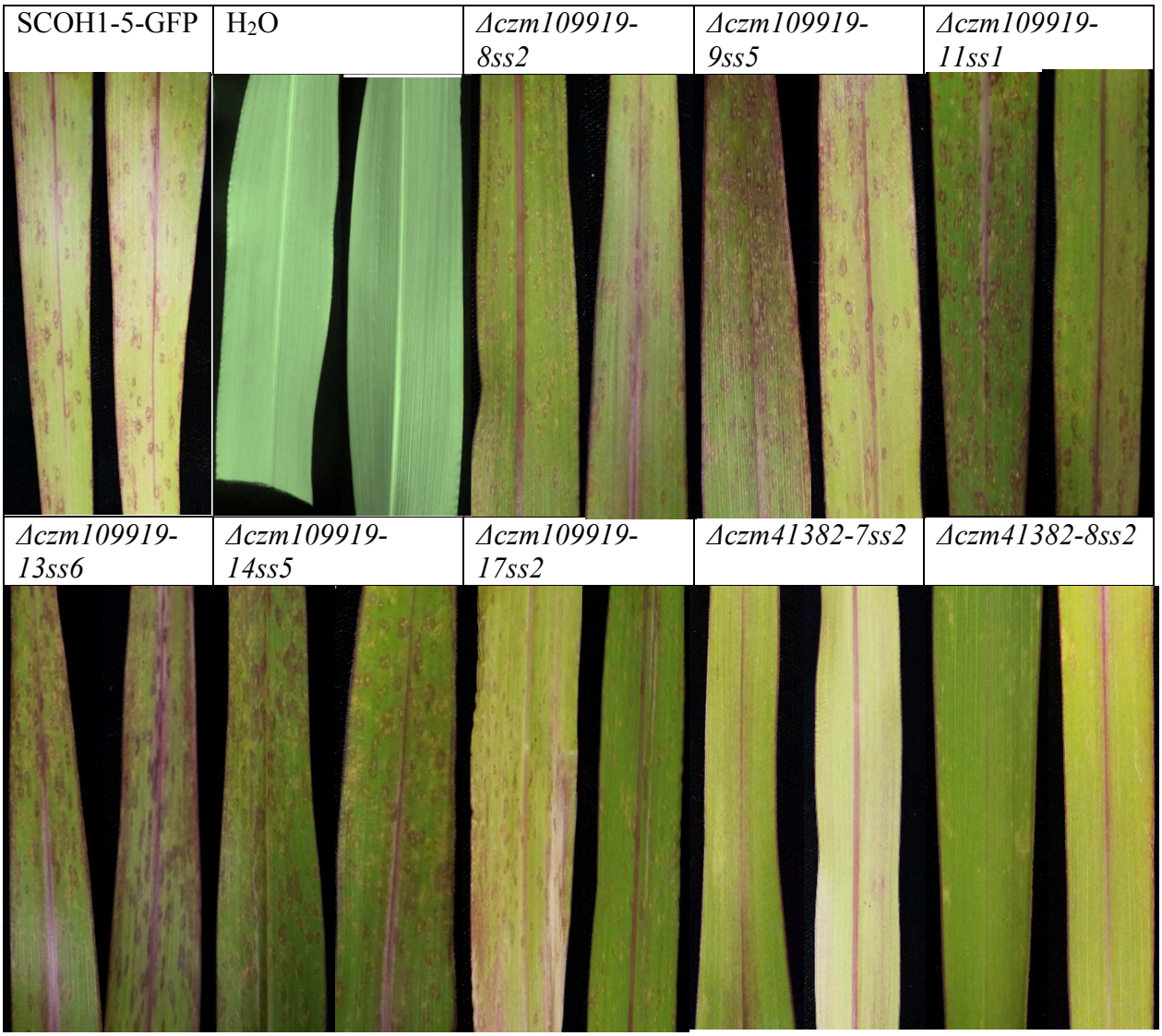

Figure 3.4: Lesion development of *C. zeae-maydis* gene-deletion mutant strains. V4 silver queen maize plants were inoculated with either wild-type (SCOH1-5-GFP), deletion mutants single-spores, or a mock to assess disease progression after 14 days. When compared to wild-type strain, the *Czm41382* gene-deletion mutant strains are apathogenic while the *Czm109919* gene-deletion mutant strains are pathogenic on maize plants.

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CHAPTER IV: CONCLUSIONS

The research presented in this thesis identified novel mechanisms of pathogenesis in *Cercospora zeae-maydis*. The genetic regulation of appressorium formation in *C. zeae-maydis* still remains poorly defined, thereby limiting the ability to dissect specific components associated with appressoria formation prior to stomatal penetration in the pathogen. An important component of this research was the identification and characterization of an MEKK gene, *Czm41382,* potentially involved in the pre-infectious development prior to stomatal penetration and pathogenesis. This study demonstrated that MAPKs are important regulatory components in appressorium formation in *C. zeae-maydis*; thus, confirming what is already known of these kinases in other dothidiomycetes and adding to the developing narrative of how dothidiomycetes interact with their hosts during infection. From the collection of 1409 tagged insertional mutants created and assayed for defects relating to germination, growth and appressorium formation, only two mutants had the phenotype of interest and were consequently sequenced to identify the genes potentially involved with the phenotype. However, the *in vitro* screen additionally revealed five random insertional mutants that had more appressorium production and 40 random insertional mutants that had severe reduction in appressorium formation compared to the wild-type. Therefore, more research into these mutants is required to further discern the genetic regulation of appressorium formation in *C. zeae-maydis*.