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The Role of G-Protein Signaling in Pathogenesis in Cercospora zeae-maydis

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The Role of G-Protein Signaling in Pathogenesis in *Cercospora zeae-maydis*

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Plant Pathology

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ABSTRACT

Gray leaf spot, caused by *Cercospora zeae-maydis*, is one of the most destructive foliar diseases of maize worldwide. *C. zeae-maydis* orients hyphal growth towards stomata (stomatal tropism) and forms infectious structures (appressoria) that are necessary for successful infection. Although some genes involved in pathogenesis in *C. zeae-maydis* have been identified, the molecular mechanisms are not well understood. In fungi, heterotrimeric G-proteins consist of three subunits (α, β, and γ) and mediate responses to environmental stimuli. They regulate diverse functions, including nutrient detection, virulence, fungal development, conidiation, secondary metabolism, and pathogenesis in many plant pathogenic fungi. This research explored the role of each G-protein α (Gpα) subunit of *C. zeae-maydis* in pathogenesis. To this end, the three Gpα subunits identified in *C. zeae-maydis* were functionally characterized. All three Gpα genes were required for appressorium formation and pathogenesis. Additionally, all three Gpα genes regulated cercosporin biosynthesis and sporulation. Together, these data demonstrated that the Gpα subunits in *Cercospora zeae-maydis* regulate pathogenesis and suggest that environmental sensing has been impaired. This study links G-protein signaling to infectious development in *C. zeae-maydis* and sets up a study for transcriptomic analysis of genes regulated by the Gpα genes.
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1.1 Introduction

1.1.1 The genus *Cercospora* and the history of gray leaf spot

The genus *Cercospora* contains many diverse plant pathogens that infect economically important crops worldwide. Currently there are over 3,000 named *Cercospora* species, many of which are host specific. *Cercospora* species share several defining morphological characteristics, particularly with respect to the formation of conidiophores and conidia. *Cercospora zeae-maydis* is the causal organism of gray leaf spot, a destructive disease on maize. Conidia from *C. zeae-maydis* are primarily spread by wind and rain. When a conidium germinates on a maize leaf, germ tubes grow towards stomata, which is known as stomatal tropism. Upon encountering a stomate, an appressorium is formed, which allows infectious hyphae to colonize leaf tissues. Conidia are formed on erumpent conidiophores formed in colonized leaf tissue; these are a source of secondary inoculum during the disease cycle. *C. zeae-maydis* overwinters in maize debris until spring, when newly formed conidia re-initiate the disease cycle (Beckman and Payne, 1982).

Broadly, fungal mechanisms of plant infection include biotrophy, necrotrophy, and hemibiotrophy. Biotrophs grow exclusively in or on living tissue, whereas necrotrophs kill host cells in advance of pathogen growth. Hemibiotrophs require living tissue for part of their life cycle before transitioning to a necrotrophic life style. *C. zeae-maydis* transitions
from a biotrophic lifestyle to a necrotrophic lifestyle (Kim et al., 2011 A), and thus is
generally classified as a hemibiotroph.

The process used by Cercospora species to find stomata has been unresolved for
decades (Kim et al., 2011 A). In 1916, Pool and McKay observed that hyphae of C. beticola
consistently took the shortest path from conidia to stomata, which suggested that
Cercospora species sense chemotropic cues (Kim et al., 2011 A; Pool and Mckay, 1916).
Histological studies performed in the 1970’s and 1980’s with C. beticola and C. zeae-maydis
(Rathaiah, 1977; Beckman and Payne, 1982) demonstrated that stomatal tropism was non-
thigmotropic. Currently, a consensus is emerging that stomatal tropism may be directed by
chemoattraction (Kim et al., 2011 A), although the identity of such a cue has not been
resolved.

Gray leaf spot, once considered to have a minor effect on maize production, is now
considered one of the most destructive foliar diseases of maize worldwide (Ward et al.,
1999; Kim et al., 2011 B). In the 1920’s, gray leaf spot occurred at a low rate of incidence,
with symptoms exclusively observed late in the growing season (Tehon and Daniels, 1925).
By the 1980’s, the incidence and severity of gray leaf spot increased from the practice of
reduced tillage and increased use of overhead irrigation (Latterell and Rossi, 1983; Carson
et al., 2002). In 1995, yield losses to gray leaf spot were reported as high as 50% in the U.S.
corn belt (Ward et al., 1999). Similarly, gray leaf spot also became a problem in Africa
where yield losses have been recorded at 60% or greater (Kim et al., 2011 B).

Controlling C. zeae-maydis through breeding has not been fully effective. For over
20 years, breeders have attempted to develop hybrids with an acceptable level of tolerance
to gray leaf spot. Initially, breeding for yield and resistance to other foliar pathogens inadvertently increased susceptibility to *C. zeae-maydis.* (Wang *et al.*, 1998) By the early 2000s, hybrids with an acceptable level of resistance to gray leaf spot were identified. However, there are still problems identifying which hybrids to use due to variability in aggressiveness in *C. zeae-maydis* isolates and other factors that favor disease development (Carson *et al.*, 2002; Wang *et al.*, 1998).

In developing countries, chemical controls for *C. zeae-maydis* are limited and not always widely available. Fungicides from the benzimidazole, the triazole, and the Quinone Outside Inhibitor (QoI) chemical groups have been shown to be effective in controlling grey leaf spot (Ward *et al.*, 1997; Bradley and Pedersen, 2011). However, resistance has developed in response to fungicides for several *Cercospora* species (Kirk *et al.*, 2012; Price III *et al.*, 2014). Due to the likeliness of developing resistance to these chemical controls and the economic constraints of obtaining them in developing countries, additional control strategies are needed for gray leaf spot.

A few genes have been identified to be involved in pathogenesis in *C. zeae-maydis*, such as *CRP1* and *CZK3*. Functional characterization of these genes has implicated distinct phenomena in pathogenesis (Shim *et al.*, 2003; Bluhm and Dunkle, 2008; Kim *et al.*, 2011A). One important aspect is that light is involved in pathogenesis. Despite recent progress in the genetic dissection of pathogenesis, the pathway underlying pathogenesis is not well understood (Figure 1).
1.1.2 The importance of environmental factors for pathogenicity of *C. zeae-maydis*

Environmental factors that play an important role in pathogenicity of *C. zeae-maydis* include temperature, relative humidity, and light. Temperatures around 25-30 °C and prolonged high relative humidity (95% or greater) are favorable for disease development (Beckman and Payne, 1982; Rupe *et al.*, 1982; Paul and Munkvold, 2005). Light is a critical environmental cue that is involved in multiple processes such as cercosporin biosynthesis and conidiation (Beckman and Payne, 1983; Daub and Chung, 2009; Kim *et al.*, 2011 B). In *C. zeae-maydis*, constant light induces the biosynthesis of the phytotoxin cercosporin and inhibits conidiation (Beckman and Payne 1983; Kim *et al.*, 2011 B). Light also affects stomatal tropism and has been shown to regulate pathogenesis (Kim *et al.*, 2011 B). Considering that numerous environmental cues influence pathogenesis in *C. zeae-maydis*, the perception of diverse environmental signals is crucial for survival.

1.1.3 Genes involved in pathogenesis and cercosporin biosynthesis in *C. zeae-maydis*

*CZK3*, a mitogen-activated protein (MAP) kinase kinase kinase homolog in *C. zeae-maydis*, regulates cercosporin biosynthesis, fungal development, and pathogenesis. *CZK3* deletion strains exhibited increased vegetative growth and were impaired in cercosporin biosynthesis and conidiation. *CZK3* mutants were able to penetrate and initiate disease, but were unable to formulate mature lesions. *CZK3* of *C. zeae-maydis* is highly similar to *Wis4* in *Schizosaccharomyces pombe*, a MAP kinase kinase kinase that is involved in various stress responses (Samejima *et al.*, 1997). Due to this and the absence of cercosporin production in *CZK3* deletion strains, cercosporin biosynthesis may be activated by nutrient stress (Shim and Dunkle, 2003). MAP kinase cascades and cyclic adenosine
monophosphate (cAMP) signaling pathways have been implicated in a variety of functions including pathogenesis in many species of filamentous fungi. These cascades regulate environmental sensing through membrane receptors including those involved in G-protein signaling (Talbot, 2003; Liande et al., 2007; Mehrabi et al., 2009; Degani et al., 2015).

The phytotoxin cercosporin, when photoactivated, reacts with oxygen to generate reactive oxygen species such as superoxide (O$_2^-$) and singlet oxygen (¹O$_2$). CRG1, a putative transcription factor in C. nicotianae, is involved in regulating self-resistance to cercosporin, but not to ¹O$_2$-generating photosensitizers (Chung et al., 2003). CRG1 also directly or indirectly regulates the expression of genes in the cercosporin toxin biosynthetic (CTB) gene cluster (Chung et al., 2003; Chen et al., 2007). The CTB gene cluster is associated with cercosporin toxin production in Cercospora species (Chen et al., 2007). Within the CTB gene cluster, CTB1 encodes a polyketide synthase that catalyzes the formation of a precursor molecule required for cercosporin biosynthesis (Choquer et al., 2005; Dorleku, 2014).

CRP1 of C. zeae-maydis, a homolog of WC-1 in Neurospora crassa, is postulated to be a central component of the fungal circadian clock (a biological oscillation that allows coordination of biology with daily environmental changes). Functional characterization of CRP1, which encodes a putative blue-light photoreceptor, implicated the gene in multiple aspects of pathogenesis. Specifically, CRP1 is involved in the regulation of stomatal tropism, appressorium formation, conidiation, and biosynthesis of cercosporin (Kim et al., 2011 A). CRP1 may also be involved in the same signaling pathway as CZK3, as both genes are involved in conidiation and cercosporin biosynthesis. Given that CRP1 encodes a blue-
light photoreceptor that entrains the circadian clock, both blue-light and the circadian clock are implicated in pathogenesis.

Considering that environmental cues regulate numerous important biological functions among fungi, identifying the receptors and downstream genes involved in pathogenesis will provide a greater understanding of molecular mechanisms underlying pathogenesis in *C. zeae-maydis*. Even with the current knowledge of genes involved in pathogenesis and cercosporin biosynthesis in *C. zeae-maydis*, the regulatory circuits involved in pathogenesis are not well understood. Many studies have implicated G-protein signaling in a variety of functions including pathogenesis in fungi (Turner and Borkovich, 1993; Lengeler *et al.*, 2000; Truesdell *et al.*, 2000; Jain *et al.*, 2005; Liande *et al.*, 2007), although no experimental data are available regarding the role of G-protein signaling in pathogenesis in *C. zeae-maydis*.

1.1.4 The function of G-proteins in other systems

Guanine nucleotide binding proteins (G-proteins) transmit external signals through transmembrane receptors. G-protein coupled receptors (GPCRs) go through conformational changes when bound by a ligand. This conformational change exchanges the guanine diphosphate (GDP) in the heterotrimeric G-protein to its active form containing guanine triphosphate (GTP). The exchange of GDP to GTP, catalyzed by guanine nucleotide exchange factors (GEFs – also called GTPases), dissociates the α subunit from the βγ heterodimeric subunit; the βγ and α subunits individually regulate downstream signaling activity. Eventually, the dissociated α subunit becomes inactive when GTP is hydrolyzed to GDP. This process is often accelerated by a class of proteins called regulators
of G-protein signaling (RGS). To complete the cycle, the inactive α subunit binds the βγ dimeric subunit, thus reforming the heterotrimeric complex and repressing downstream signaling. If the GPCR is still stimulated by the ligand, the process could repeat until the receptor itself is inactivated. GPCRs can be inactivated through the binding of β-arrestin facilitated by phosphorylation of the GPCR (Lengeler et al., 2000; Urano et al., 2013) (Figure 2).

**G-protein α subunits (Gpα)** bind and regulate other proteins with assistance from secondary messengers such as cyclic adenosine monophosphate (cAMP) (Lengeler et al., 2000). Additional pathways are regulated through Gpα interactions, such as the N-end rule pathway. **UBL1**, a putative E3 ubiquitin ligase involved in tagging proteins for degradation, is postulated to be regulated by G-protein signaling and is involved in pathogenesis in *Fusarium graminearum* (Ridenour et al., 2014). Among other fungal signaling pathways linked to G-protein signaling, two are broadly conserved in fungi: a MAP kinase cascade which mediates responses to cell fusion, filamentous growth, and pathogenesis (Kronstad et al., 1998; Shim and Dunkle, 2003) and the nutrient sensing Gpα-cAMP-PKA cascade. These cascades regulate carbon sensing, appressorium formation, conidial germination, and pathogenesis in various fungal species (Turner and Borkovich, 1993; Lengeler et al., 2000; Truesdell et al., 2000; Jain et al., 2005; Liande et al., 2007). G-proteins are also broadly categorized into three groups based on sequence alignment. Group I and Group III Gpα subunits are related to mammalian Gpα groups Gαi and Gαs, which inhibit or stimulate the activity of adenylyl cyclase. The Gαs group contains a myristoylation site (MGxxxS) and a Cxxx consensus site that is implicated in Gpα inhibition of adenylyl cyclase. Provisionally, group I contains inhibitors of adenylyl cyclase while group III contains stimulators of
adenylyl cyclase. Group II Gpα subunits are not related to mammalian subunits and are therefore harder to predict functionally. A few members of group II have been characterized, but no distinct phenotype has been observed (Bölker, 1998; Kays and Borkovich, 2004; Yu et al., 2008; Omann and Zeilinger, 2010).

1.1.5 Preliminary study with the G-protein α subunits in Cercospora zeae-maydis

Because G-protein α subunits play crucial roles in development and pathogenesis, an initial study of their function in C. zeae-maydis was performed (Hirsch, 2014) in 2013. Through BLAST analyses, three genes were identified in C. zeae-maydis that were predicted to encode G-protein α subunits (designated as GPA1, GPA2, and GPA3). GPA2 (Group II) appeared to regulate pathogenic development while GPA1 (Group I) and GPA3 (Group III) were initially described as dispensable for pre-penetration infectious development. However, these results were preliminary and thus require more careful experimental validation.

1.2 Justification and Research Objectives

G-protein α subunits are involved in various functions, including pathogenesis, in many fungal species (Coca et al., 1999; Lengeler et al., 2000; Yu JH, 2006; Liande et al., 2007, Jain et al., 2005). BLAST analyses indicated that C. zeae-maydis has three genes encoding G-protein α subunits. Preliminary experiments suggested that GPA1 and GPA3 are involved to some degree in the regulation of pathogenesis, and that GPA2 is required for the formation of appressoria. Studying G-protein α subunits in C. zeae-maydis during
infection will elucidate genes that are involved in pathogenesis and how these genes interact with environmental stimuli. Considering that G-proteins are global regulators of cellular development, it is likely that the G-protein α subunits in *C. zeae-maydis* regulate the expression of novel genes involved in pathogenesis. For this reason, the disruption of the Gpα subunits in *C. zeae-maydis* will identify genes in the pathway underlying pathogenesis. Therefore, the objective of this study is to characterize how each G-protein α subunit of *C. zeae-maydis* is involved in pathogenesis and to identify which novel genes are involved in pathogenesis and are regulated in the G-protein α subunit pathway.

Hypothesis:

G-protein α subunit signaling relays important environmental signals in *Cercospora zeae-maydis* and thus directly or indirectly regulates pathogenesis through important downstream genes or proteins.

Research Plan:

The overarching goal of this project is to functionally characterize the G-protein subunits (*GPA1*, *GPA2*, and *GPA3*) of *C. zeae-maydis*. The specific objectives of this study are to:

1. Structurally characterize the Gpα genes in *C. zeae-maydis*

2. Determine the role of *GPA1*, *GPA2*, and *GPA3* in pathogenesis
1.3 Citations


Dorleku WP (2014) Molecular and Biochemical Mechanisms of Pathogenesis in the Maize Foliar Pathogen *Cercospora zeae-maydis* Available from: [http://library.uark.edu:80/record=b3311206~S1](http://library.uark.edu:80/record=b3311206~S1)


CHAPTER II G-PROTEIN α SUBUNITS ARE INVOLVED IN SPORULATION, CERCOSPORIN BIOSYNTHESIS, APPRESSORIUM FORMATION, AND PATHOGENESIS IN C. ZEAE-MAYDIS

2.1 Abstract

Gray leaf spot, caused by Cercospora zeae-maydis, is a destructive, pervasive disease of corn that requires greater understanding of the mechanisms underlying pathogenesis in order to develop sustainable strategies for control. Studying G-protein signaling in C. zeae-maydis will potentially elucidate novel genes involved in pathogenesis. In other fungi, G-proteins transmit external cues activating a variety of downstream biological responses and functions, including pathogenesis. In this study, three genes were deleted that encode putative G-protein α subunits in C. zeae-maydis (GPA1, GPA2, and GPA3). Deletion of any of the three of the Gpα genes in C. zeae-maydis severely impaired appressorium formation and lesion development. Additionally, all deletion strains were significantly reduced in cercosporin biosynthesis, whereas sporulation was increased. This study strongly implicates G-protein signaling as a regulator of pathogenesis, specifically pre-penetration infectious development, in C. zeae-maydis.
2.2 Introduction

Gray leaf spot (GLS), caused by *Cercospora zeae-maydis*, is one of the most important foliar diseases of maize (*Zea mays*) production worldwide (Ward *et al*., 1999; Kim *et al*., 2011 A). GLS has caused significant losses over the last decade in U.S. maize production, and has become pandemic in Africa and China with losses as high as 60% or greater (Kim *et al*., 2010 B). A greater fundamental understanding of molecular mechanisms underlying pathogenesis in *Cercospora zeae-maydis* is urgently needed as the basis for the development of novel disease management strategies (Figure 1).

G-proteins are crucial for cellular responses to environmental stimuli and have been implicated in signal transduction pathways involved in a variety of functions, including pathogenesis. G-proteins are important for metabolism, cell differentiation, nutrient detection, virulence establishment, infectious development, conidiation, and secondary metabolism (Coca *et al*., 1999; Lengeler *et al*., 2000; Jain *et al*., 2005; Yu JH, 2006; Li and *et al*., 2007). Heterotrimeric G-proteins consist of three subunits (α, β, and γ); upon activation, the α subunit dissociates from the dimeric βγ subunit to initiate downstream regulatory processes. Organisms vary regarding the number of Gα-encoding genes they possess (Gao and Nuss, 1996; Lengeler *et al*., 2000; Yu, 2006; Li and *et al*., 2007). Consistent with observations that Gαs frequently mediate cross-talk between various signaling pathways, many have been demonstrated to possess at least some degree of functional redundancy (Turner and Borkovich, 1993) (Figure 2).
In this study, the involvement of Gpα subunits in pathogenesis in the maize pathogen *C. zeae-maydis* was determined. Through BLAST analyses, *C. zeae-maydis* was determined to possess three Gpα-encoding genes. *C. zeae-maydis* mutants lacking any of the three Gpα-encoding genes (designated *GPA1*, *GPA2*, and *GPA3*) were apathogenic, failed to form appressoria, and were severely reduced in cercosporin biosynthesis. These results indicated that Gpα subunits in *C. zeae-maydis* are likely to function as crucial mediators of environmental cues underlying pathogenesis.

### 2.3 Materials and Methods

#### 2.3.1 Fungal strains and culture conditions

All fungal strains used in this study, including *C. zeae-maydis* strain SCOH1-5 that served as the wild-type strain (Bluhm *et al.*, 2008, Bluhm and Dunkle 2008, Kim *et al.*, 2010 B), are listed in Table 1. All strains were stored as hyphal fragments or spore suspensions in 25% (v/v) glycerol at -80 °C. Working cultures were maintained on V-8 agar medium (180 mL/L V-8 juice, 2 g/L calcium carbonate, 20 g/L agar), grown in constant darkness, and renewed from glycerol every 3-4 months.

#### 2.3.2 Nucleic acid manipulations

Plasmid DNA was isolated with an alkaline lysis midiprep protocol as described by Sambrook and Russell (2001). Genomic DNA was isolated from fungal cultures grown in YEPD medium (5 g/L yeast extract, 10 g/L peptone, and 20 g/L dextrose) with a modified
cetyltrimethylammonium bromide (CTAB) method (Taylor and Natvig 1987; Proctor et al., 1997). For PCR-based screening, fungal DNA was prepared with a modified CTAB method or methods described by Ridenour et al. (2012). Primers were acquired from Integrated DNA Technologies (Coralville, IA, USA) and are listed in Table 2.

2.3.3 Bioinformatics

Sequence data were obtained through web portals at the Joint Genome Institute and the National Center for Biotechnology Information (http://www.jgi.doe.gov and http://www.ncbi.nlm.nih.gov respectively). Predicted functional domains were identified with SMART (Schultz et al., 1998; Letunic et al., 2014). Putative GPA1, GPA2, and GPA3 orthologs were identified with reciprocal protein–protein BLAST analyses (blastp; Altschul et al., 1990; Grigoriev et al., 2011). Multiple sequence alignments were performed with ClustalW (Larkin et al., 2007) and Cobalt (Papadopoulos and Agarwala, 2007). Phylogenetic data was graphed with FigTree (Version 1.4.2, http://www.tree.bio.ed.ac.uk/software/figtree). Pathogenesis data were analyzed with graphing software (KaleidaGraph Version 4.5, http://www.synergy.com/wordpress_650164087). Analyses for ANOVA and Fisher’s least significant difference were performed with statistical software (GraphPad Prism Version 6, http://www.graphpad.com/scientific-software/prism/#1)

2.3.4 Targeted deletion of the Gpα genes

For targeted gene deletion, a split-marker approach was used to replace the endogenous GPA1, GPA2, and GPA3 genes with a hygromycin resistance cassette (Catlett et al., 2003; Yu et al., 2004). To this end, a gene deletion construct was generated as
previously described (Ridenour et al., 2012). Briefly, a region upstream of the \textit{GPA1} open reading frame (ORF) (\textit{GPA1} genomic 5’ flank) and a region downstream of the \textit{GPA1} ORF (\textit{GPA1} genomic 3’ flank) were amplified from \textit{C. zeae-maydis} genomic DNA (strain SCOH1-5) with primer pairs \textit{GPA1\_A1/GPA1\_F2} and \textit{GPA1\_F3/GPA1\_F4}, respectively. Overlapping marker fragments, HY and YG, were amplified from a hygromycin resistance cassette derived from pCB1003 (Carroll et al., 1994) with primer pairs M13F/HY and YG/M13R, respectively. The \textit{GPA1} genomic 5’ flank and the HY marker fragment were joined and amplified by fusion PCR (Yu et al., 2004) with the nested primer pair \textit{GPA1\_F1n/HYn} to generate the 5’ deletion construct. Similarly, the \textit{GPA1} genomic 3’ flank and the YG marker fragment were joined and amplified with the nested primer pair \textit{YGn/GPA1\_F4n} to generate the 3’ deletion construct. A similar approach was used to generate split-marker constructs for targeted deletion of \textit{GPA2} and \textit{GPA3} in \textit{C. zeae-maydis} (Figure 5A).

Preparation and transformation of fungal protoplasts and screening of hygromycin resistant transformants were performed as described previously (Ridenour et al., 2012).

2.3.5 Sporulation and cercosporin biosynthesis assays

The sporulation assay was performed by inoculating each \textit{C. zeae-maydis} strain (1 mL of 1×10^5 per mL conidial suspension) onto V-8 agar plates (180 mL/L V-8 juice, 2 g/L calcium carbonate, 20 g/L agar) that were incubated in constant darkness for seven days. Because sporulation in \textit{C. zeae-maydis} is regulated by blue light (Kim et al., 2010 A; Bluhm and Dunkle, 2008), samples were collected under red light at four, five, six and seven days after inoculation. For each time point and strain, 3 plugs (each plug has a diameter of 1 cm) were collected per plate and placed in a 10 mL tube with 5 mL of sterile water. Each tube
was vortexed for 30 seconds and quantified twice on a hemocytometer and then averaged.
The data were converted and expressed as amount of spores per cm$^2$. For all time points and strains, four replications were assessed.

Cercosporin biosynthesis was assessed with a protocol similar to that previously described (Kim et al., 2010 A; Bluhm and Dunkle, 2008). Briefly, *C. zeae-maydis* strains (1mL of $1.6 \times 10^4$ per mL conidial suspension) were inoculated onto thick (20-30 mL per plate) 0.2× PDA (Difco potato dextrose agar, HiMedia agar) plates. The spore suspension was spread across the plate and allowed to dry. Plates were then incubated at room temperature in constant light (General Electric (GE) F20T12 (daylight) 24 inch bulbs). Cercosporin biosynthesis was quantified seven days after inoculation. The agar medium was collected from each plate, incubated in 20 mL of 5M KOH, and assessed with a Bio-Rad SmartSpec 3000 spectrometer ($\lambda_{480}$ nm). Cercosporin biosynthesis experiments had six replicates and were repeated three times with similar results.

2.3.6 Pathogenicity assays

For assessment of pathogenesis in planta, Silver Queen maize plants (susceptible to infection by *C. zeae-maydis*) were inoculated with spore suspensions ($1 \times 10^5$ per mL) at the V4 stage (approximately 25 days after planting). Plants were then placed into an opaque plastic chamber to facilitate disease progression. Six days after inoculation, leaves were collected to assess appressorium formation. Appressorium formation was analyzed with ImageJ software (Schneider et al., 2012; ImageJ Version 1.49, http://imagej.nih.gov/ij/) and the data were calculated as the percentage of successful interaction with stomata (appressoria formed over stomata/total hyphal interactions with stomata). The
apressorium formation experiments contained 15 replicates per strain and were repeated three times with similar results. To assess lesion development, plants were inoculated with spore suspensions (1×10⁵ per mL) and disease was allowed to progress for 14 days. Leaves were then collected and photographed. The lesion development assay was repeated three times with similar results.

2.4 Results

2.4.1 Bioinformatics and validation of the Gpα subunits in C. zeae-maydis

BLAST analyses were performed to identify and compare the Gpα subunits in C. zeae-maydis. C. zeae-maydis has three G-protein α subunits: GPA1 (Protein ID: 69878; Scaffold 24), GPA2 (Protein ID: 69803; Scaffold 24), and GPA3 (Protein ID: 111563; Scaffold 10). The ORF of GPA1 was not predicted to contain any introns, whereas GPA2 and GPA3 were each predicted to contain three introns (Figure 5A). When the Gpα protein sequences in C. zeae-maydis were compared to putative orthologs from other Dothideomycete fungi the Gpα subunits in C. zeae-maydis separated into distinct clades (Appendix Table 1; Appendix Figure 1).

Analyses with SMART revealed important structural features in G-proteins of C. zeae-maydis (Figure 4). For Gpα subunits, the nucleotide binding domain and P-loop NTPase fold are crucial for function. In C. zeae-maydis, Gpα subunits each have a nucleotide binding domain, which is involved in binding diverse molecules, and a P-loop NTPase fold, which aids in catalyzing the phosphate bond connecting Gpα subunits to the βγ complex.
The G-protein β (Gpβ) subunit in *C. zeae-maydis* contains seven WD-40 repeats that serve as sites for protein interactions, while the G-protein γ (Gpγ) subunit in *C. zeae-maydis* contains a GGL domain that allows binding to the β subunit. In regard to nucleotide binding domains, Gpα subunits have three known consensus motifs that can aid in determining function (Kjeldgaard *et al* 1996; Wittinghofer and Vetter 2011). *GPA1* contains one Σ1 (GxxxxGK(S/T)) motif, two Σ2 (DxxG) motifs, and one Σ3 (NKxD) motif. *GPA2* is different from *GPA1* by having three Σ2 motifs and an extra Σ3 motif. *GPA3* is different from *GPA1* or *GPA2* by having an additional Σ2 motif (Figure 4).

Targeted deletion of the Gpα genes in *C. zeae-maydis* was performed through homologous recombination (Figure 5A). All mutants obtained under hygromycin selection were screened for Gpα deletion and the insertion of the *HPH* gene by PCR (Figure 5B; Table 2). Strains determined to be Gpα deletion mutants were: G1-05 and G1-12 for *GPA1*; G2-11 and G2-24 for *GPA2*; and G3-03 and G3-07 for *GPA3*. During validation of the deletion strains, the *GPA1* deletion strains were noted to grow sparsely when compared to the wild-type strain. There were no observable differences in growth *in vitro* for the other deletion mutants.

2.4.2 Sporulation and cercosporin biosynthesis of the *C. zeae-maydis* strains

Since Gpα subunits are involved in sporulation and the *GPA1* strains grew sparsely, the Gpα subunits were predicted to be involved in sporulation. Spores for each Gpα deletion strain did not have any observable morphological differences from wild-type; however, all of the deletion strains produced significantly more spores at earlier time
points than wild-type. Interestingly, there were no significant differences in sporulation at five, six, or seven days after inoculation. (Figure 6; Table 3).

Because cercosporin biosynthesis is an important biological characteristic of many Cercospora species, a cercosporin biosynthesis assay was performed with the Gpα deletion strains. Although all deletion strains were severely impaired in cercosporin biosynthesis, a low but visible amount of cercosporin biosynthesis was observed in the GPA2 and GPA3 deletion strains. There was no visible amount of cercosporin production in the GPA1 deletion strains. Visual observations were consistent with spectrophotometric quantification (Figure 7; Table 4).

2.4.3 Pathogenesis of the C. zeae-maydis strains

To assess the involvement of the Gpα subunits in infection, the percent of appressoria formed upon stomatal interaction was quantified for each Gpα deletion strain in comparison to the wild-type. Notably, all of the Gpα deletion strains were severely impaired in appressorium formation. The magnitude of impairment was substantial; the wild-type formed appressoria 66% of stomatal encounters, whereas the mutants were impaired by 7-fold or greater levels (Figures 8-11; Table 5).

Although the Gpα deletion strains were impaired in appressorium formation, lesions could theoretically still form if the appressoria were viable. To determine whether the Gpα subunits in C. zeae-maydis were involved in lesion development, disease was allowed to progress on maize leaves for 14 days. From this approach, all of the Gpα deletion strains were determined to be apathogenic when compared to wild-type (Figure 12).
2.5 Discussion

Phenotypic characterization of the Gpα deletion strains indicated that all three Gpα subunits in *C. zeae-maydis* regulate sporulation. All of the deletion mutants produced more conidia per cm² at four days after inoculation (DAI) compared to the wild-type. Interestingly, there was no significant difference in sporulation at later time points among the mutant and wild-type *C. zeae-maydis* strains. This observation could indicate that there is an early difference in spore germination and/or growth rate between the wild-type and Gpα deletion strains. Alternatively, conidiation could be constitutively de-repressed in the Gpα deletion strains. A similar phenomenon was observed during phenotypic characterization of *CRP1* disruption mutants in *C. zeae-maydis*. Disruption of *CRP1*, a putative blue light photoreceptor, led to constitutive expression of both cercosporin and sporulation (Kim *et al.*, 2011 A). The absence of an α subunit could prevent proper sensing of repressive signaling ligands which would lead to de-repressed cellular responses (such as increased sporulation). The increased sporulation observed in the deletion of each Gpα subunit in *C. zeae-maydis* is contrary to what has been observed in other organisms, in which sporulation was reduced or delayed (Liu and Dean, 1997; Jain *et al.*, 2005; García-Rico *et al.*, 2009). This difference is interesting as it indicates that the pathway underlying sporulation in *C. zeae-maydis* could have diverged from other filamentous fungi. The GPA2 deletion strains were significantly increased in sporulation when compared to the other
deletion strains. However, all of the Gpα subunits in *C. zeae-maydis* are implicated in sporulation, which indicates that there may be shared elements between the Gpα subunits.

Cercosporin biosynthesis, an important biological characteristic of many *Cercospora* species, was significantly impaired upon deletion of any G-protein α subunit in *C. zeae-maydis*. Cercosporin is visible by eye as a distinctive purple pigment in conducive culture media (Daub and Chung, 2007; Dorleku, 2014). Interestingly, the *GPA2* and *GPA3* deletion strains produced a faint but visible amount of cercosporin, whereas the *GPA1* deletion strains did not. This suggests that although there is redundancy in function between the Gpα subunits in *C. zeae-maydis*, *GPA1* may serve a distinct regulatory function when compared with *GPA2* or *GPA3*. One likely downstream element is *CZK3*, a MAP kinase kinase kinase that is crucial for cercosporin production (Shim and Dunkle, 2003).

Appressorium formation in *C. zeae-maydis* was significantly impaired in all of the Gpα deletion strains. Before the inception of the current study, *GPA2* was believed to be the only Gpα subunit regulating appressorium formation (Hirsch 2014). However, in the current study, *GPA1*, *GPA2*, and *GPA3* deletion strains were all clearly impaired in appressorium formation.

All of the Gpα deletion strains were also unable to produce necrotic lesions on maize leaves. In theory, if the Gpα subunits specifically regulated appressorium formation, the low number of appressoria formed should still be viable and initiate lesions. The apathogenic nature of the mutants analyzed in this study suggests that the Gpα genes of *C. zeae-maydis* regulate not only pre-penetration infectious development, but also leaf colonization and/or the transition from hemibiotrophy to necrotrophy. A more thorough
histopathological analysis will be required to elucidate exactly which component of pathogenesis is impaired upon disruption of the Gpα genes.

Functional differences in the Gpα subunits in C. zeae-maydis, which appear to have overlapping functions with potentially different downstream elements, might be explained by structural differences. The GTP binding motif differences for each Gpα are extra Σ2 (DxxG) and Σ3 (NKxD) motifs. The Σ2 motif is implicated in the conformational change from GDP to GTP while the Σ3 motif determines the specificity for guanine (Kjeldgaard et al., 1996; Wittinghofer and Vetter, 2011). Most intriguing is the location of the extra Σ3 motif in GPA2. Unlike GPA1 or GPA3, the extra Σ3 motif in GPA2 is located next to the Σ1 (GxxxxGK(S/T)) motif upstream (Figure 3). This is relevant because the Σ1 motif is involved in binding purine nucleoside triphosphates and could mean that GPA2 is even more specific in binding guanine than GPA1 or GPA3. The Gpα subunits in C. zeae-maydis belong to specific groupings for filamentous fungi that could aid in predicting functionality (GPA1 in group 1, GPA2 in group 2, and GPA3 in group 3). These groupings suggest that GPA1 inhibits adenylyl cyclase, GPA3 stimulates adenylyl cyclase, and GPA2 is in an undefined grouping. Additionally, there are no introns in the open reading frame of GPA1, while GPA2 and GPA3 have three introns each (Figure 5A). This suggests that GPA2 and GPA3 could be regulated by alternative splicing or via intronic regulatory elements (Hildebrandt, 1997).

This study indicated that the Gpα genes of C. zeae-maydis regulate similar components of pathogenesis. The three Gpα genes were important for multiple phenotypes (sporulation, cercosporin biosynthesis, appressorium formation, and lesion
development), which suggests that the three genes are not functionally redundant. G-protein coupled receptors are involved in responding to various abiotic and biotic stresses. Thus, the *C. zeae-maydis* Gpα genes could play different roles in mediating various environmental conditions. There may also be important regulatory differences between each of the Gpα subunits. Such differences could be due to variation in receptor and α subunit binding, and subsequent βγ dimer signaling (Hildebrandt, 1997; Lodish *et al.*, 2000; Albert and Robillard, 2002; Gearing *et al.*, 2003). There is also potential for splicing variants in *GPA2* and *GPA3* (Figure 5A). Differences in α subunit receptor binding have been attributed to protein variations arising from splicing events (Hildebrandt, 1997; Albert and Robillard, 2002). The G-protein α subunit splicing variants bind to a suite of G-protein coupled receptors, which are each activated by specific ligands (Lodish *et al.*, 2000; Gearing *et al.*, 2003). Determining the downstream events regulated by the Gpα genes in *C. zeae-maydis*, through gene expression profiling and molecular genetics, would identify candidate genes involved in pathogenesis and help to resolve the exact mechanisms through which the individual Gpα subunits function.
2.6 Citations


Liu S, Dean RA (1997) G protein α subunit genes control growth, development, and pathogenicity of Magnaporthe grisea MPMI 10(9): 1075-1086


In conclusion, this study demonstrates that G-protein signaling is involved in sporulation, cercosporin biosynthesis, appressorium formation, and lesion development in \textit{C. zeae-maydis}. This is the first study linking G-protein signaling to pathogenesis in \textit{C. zeae-maydis}. Overall, the G\textalpha subunits in \textit{C. zeae-maydis} share similar roles in sporulation, cercosporin biosynthesis, and appressorium formation. This was surprising as G\textalpha subunits often have specific functions in filamentous fungi. Even more interesting is that the G\textalpha genes in \textit{C. zeae-maydis} are all independently required even though they regulate similar functions. This suggests that the G\textalpha genes in \textit{C. zeae-maydis} regulate different elements downstream. As G\textalpha subunits are hierarchal, it is expected that the G\textalpha deletion strains of \textit{C. zeae-maydis} could have other phenotypes not detected in this study. Perhaps the G\textalpha genes in \textit{C. zeae-maydis} influence other biological processes through the G\textalpha-cAMP-PKA cascade, the downstream regulation of \textit{CZK3}, or regulation of a \textit{UBL1} ortholog. It is likely that the other genes identified to be involved in pathogenesis and cercosporin biosynthesis (\textit{CRP1}, \textit{CRG1}, and \textit{CZK3}) are also regulated downstream of the G\textalpha subunits in \textit{C. zeae-maydis}. 
### Table 1. Fungal strains used in this study.

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</thead>
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<td>Bluhm et al., 2008</td>
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### Table 2. Primers used in this study

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#### 6PA1-F

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Table 3. Sporulation of *Cercospora zeae-maydis* strains

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<tr>
<th>Strain Name</th>
<th>4 DAI (spores/cm²)</th>
<th>5 DAI (spores/cm²)</th>
<th>6 DAI (spores/cm²)</th>
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<td>SCOH1-5</td>
<td>8.75 ±1.90</td>
<td>37.93±12.78</td>
<td>61.01±13.54</td>
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<td>SCOH1-5 G1-12</td>
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<td>71.09±6.32</td>
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<td>SCOH1-5 G2-11</td>
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<td>77.19±10.34</td>
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Sporulation was quantified as spores/cm² after four, five, six, and seven days after inoculation. Means and standard errors were calculated from one independent experiment. ANOVA and Fisher’s LSD revealed that only four days after inoculation is significantly different.

Table 4. Cerosporin biosynthesis of *Cercospora zeae-maydis* strains

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<td>5.57x10⁻⁶± 3.29x10⁻⁶</td>
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<td>7.53x10⁻⁶± 2.68x10⁻⁶</td>
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<td>1.20x10⁻⁵± 4.99x10⁻⁶</td>
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<td>SCOH1-5 G2-24</td>
<td>9.71x10⁻⁶± 3.27x10⁻⁶</td>
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<td>SCOH1-5 G3-03</td>
<td>4.58x10⁻⁶± 2.19x10⁻⁶</td>
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<td>SCOH1-5 G3-07</td>
<td>6.50x10⁻⁶± 2.45x10⁻⁶</td>
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Cerosporin biosynthesis was quantified in nanomoles at seven days after inoculation. Means and standard errors were calculated from three independent experiments. Plates were grown under constant light. ANOVA and Fisher’s LSD confirmed that these results are significantly different.
Table 5. Appressorium formation of *Cercospora zeae-maydis* strains

<table>
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<th>Strain Name</th>
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<td>SCOH1-5 G3-07</td>
<td>8.46% ± 3.13%</td>
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</table>

<sup>a</sup> 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 three independent experiments. ANOVA and Fisher’s LSD confirmed that these results are significantly different.
Figure 1. A timeline of *C. zeae-maydis*. In 1916, it was discovered that the hyphae of *C. beticola* exhibits stomatal tropism which answered important questions on how *Cercospora* species encounter stomata\(^1\). It wasn’t until 1925 when *C. zeae-maydis* was first reported in maize fields. Due to an insubstantial effect on yield, it was considered a minor problem until the 1980-90s\(^2\). In 1954, Chupp published a culmination of more than 30 years of work in defining distinct morphological features of the *Cercospora* genus\(^3\). Among discovering the phytotoxin cercosporin in 1957\(^4\), a variety research into factors that favor *C. zeae-maydis in vivo* and *in vitro* was conducted around the 1980s\(^5,7\). In 1998, it was discovered that gray leaf spot is caused by two sibling species that are genetically different (Group I – *C. zeina*; Group II – *C. zeae-maydis*)\(^6,8\). In 2000, it was revealed that *C. zeina* is the predominate isolate in Africa\(^9\). Even though hybrids have been developed with an acceptable level of tolerance to *C. zeae-maydis*, controlling gray leaf spot is still a problem\(^10\). Entering the new millennia brought new genetic tools for further research. A few genes were identified to be involved in a variety of functions (pathogenesis, fungal development, sporulation, blue-light responses, metabolism, stomatal tropism, and appressorium formation)\(^11,12,13\). Finally in 2010, isolate SCOH1-5 of *C. zeae-maydis* was sequenced opening the path for genetic research for molecular mechanisms in *C. zeae-maydis*\(^14\).
Figure 2. G-protein signaling overview. The seven transmembrane G-protein coupled receptor (GPCR) goes through a conformational change when an external ligand binds to it. This conformational change exchanges guanine diphosphate (GDP) to an active form containing guanine triphosphate (GTP). The dissociation of the α subunit and the βγ heterodimeric subunit activates downstream cellular activity (such as UBL1, the MAPK or cAMP-PKA cascades). Eventually the GTP on the α subunit is hydrolyzed to GDP reforming the G-protein αβγ heterotrimeric complex. If the GPCR is still stimulated by the ligand, the process can repeat until the receptor itself is inactivated.
Figure 3. Domain organization of the G-protein subunits in *Cercospora zeae-maydis*. This figure illustrates the location of important domains among all the residues for all the *C. zeae-maydis* G-protein subunits. The G-protein α (GPA) subunits contain a general nucleotide binding domain that is used for binding many different molecules and a p-loop NTPase fold which is involved in catalyzing the hydrolysis of the β-γ phosphate bond. The G-protein β (GPB) subunit contains seven WD-40 repeats which serve as sites for protein interactions. The G-protein γ (GPY) subunit contains a GGL domain which allows for binding to the β subunit.
Figure 4. Binding motif organization of the G-protein α subunits in *Cercospora zeae-maydis*. Three major binding motifs are used to fingerprint the binding domain in G-protein α subunits (Kjeldgaard et al. 1996). The GxxxxGK(S/T) motif is found in proteins that bind purine nucleoside triphosphates among which is GTP. The second motif (DxxG) is implicated in the conformational change that takes place when GDP or GTP forms. The third motif (NKxD) determines the specificity for guanine. It is interesting to note that the nucleotide binding domain contains the GxxxxGK(S/T) motif in all three G-protein α subunit proteins; however, unlike the others GPA2 contains an extra NKxD motif in the nucleotide binding domain.
Figure 5. Target deletion and validation of the three G-protein α subunits in Cercospora zeae-maydis. A. Depicted is the deletion strategy using homologous recombination for each alpha gene in C. zeae-maydis. Lines represent where recombination occurred. In the open reading frame, the large gray boxes represent exon coding regions whereas the smaller boxes represent intronic regions. Primers A1 to HSB (Hygromycin Screen B), internally set primers (gpa1PF to gpa1PR), and HSC (Hygromycin Screen C) to A2/A2 primers were used to validate the deletion of Gpa genes by PCR. B. PCR validation of each independent deletion for the alpha genes using 3 sets of primers: the endogenous gene specific primers (wells 1), the 3’ endogenous flank primer to the HSB primer (wells 2), and the HSC to the 5’ endogenous flank primer (wells 3). For all rows, wells 1 demonstrate that wildtype (SCO1H-5) has the endogenous gene that encodes the corresponding G-protein α subunit. Whereas, the endogenous gene has been deleted in all transformants. Wells 2 and 3 demonstrate that the hygromycin cassette was successfully inserted into the endogenous gene locus which could not be amplified in wildtype as the wildtype does not contain a gene encoding for hygromycin resistance. This confirms that the endogenous gene was deleted in the transformants.
Figure 6. Sporulation of *Cercospora zeae-maydis* strains. The amount of spores produced by *C. zeae-maydis* strains were assessed at four (A.), five, six, and seven days after inoculation. Only the most earliest time point was statistically significant. This experiment has 4 replications per strain for each time point. One-way anova and fisher’s least significant difference were used to determine significance ($\alpha=0.05$; letters denote significance). B. Representative micrographs of spores were taken for each *C. zeae-maydis* strain. The bar marks 50uM for all micrographs.
Figure 7. Cercosporin biosynthesis of *Cercospora zeae-maydis* strains. Cercosporin biosynthesis was quantified in wildtype and mutant strains seven days after inoculation through a similar protocol described by Bluhm *et al.*, 2008. Concentration of cercosporin was calculated using a known extinction coefficient for cercosporin. This graph demonstrates that G-protein signaling is involved in cercosporin biosynthesis as can be seen from the severe reduction in cercosporin concentration in the mutant strains. This experiment has six replications per strain and was repeated three times with similar results. One-way anova and fisher’s least significant difference were used to determine significance ($\alpha=0.05$; letters denote significance)
Figure 8. Appressorium formation of the *Cercospora zeae-maydis* strains. Appressorium formation was quantified in wildtype and mutant strains six days after inoculation on V4 maize plants. The amount of appressoria formed over stomata out of all the total stomatal interactions by hyphae was counted. This experiment has 15 replications per strain and was repeated three times with similar results. One-way anova and fisher’s least significant difference were used to determine significance ($\alpha=0.05$; letters denote significance).
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Figure 9. Micrographs of appressorium formation for the *C. zeae-maydis GPA1* deletion strains. Representative micrographs of wildtype and mutant strains six days after inoculation on V4 maize plants. *GPA1* deletion mutants are severely impaired in appressorium formation when compared to wildtype. The bar marks 50uM for all micrographs.
Figure 10. Micrographs of appressorium formation for the *C. zeae-maydis* GPA2 deletion strains. Representative micrographs of wildtype and mutant strains six days after inoculation on V4 maize plants. GPA2 deletion mutants are severely impaired in appressorium formation when compared to wildtype. The bar marks 50uM for all micrographs.
Figure 11. Micrographs of appressorium formation for the *C. zeae-maydis* GPA3 deletion strains. Representative micrographs of wildtype and mutant strains six days after inoculation on V4 maize plants. GPA3 deletion mutants are severely impaired in appressorium formation when compared to wildtype. The bar marks 50uM for all micrographs.
Figure 12. Lesion Development of *Cercospora zeae-maydis* strains. V4 silver queen maize plants were inoculated with either wildtype, a Gpα deletion strain or a mock to assess disease progression after 14 days. When comparing the Gpα deletion strains to the wildtype strain, the Gpα deletion strains are severely impaired in lesion development.
### APPENDIX

**Appendix Table 1. Species in the phylogenetic tree of G-protein α subunits in Dothideomycete fungi**

<table>
<thead>
<tr>
<th>Species Name</th>
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Appendix Table 1. Species in the phylogenetic tree of G-protein α subunits in Dothideomycete fungi (Continued)

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This table contains sequences, obtained through blast analysis, used to generate a phylogenetic tree (Appendix Figure 1). The table contains a total of 268 hits from 90 different Dothideomycete species. Sequences below 40% identify and 40% sequence coverage were not included in the tree. Hits represent a minimum amount of G-protein α genes per species and may not include all genes due to the 40% cut off.
Appendix Figure 1. Protein sequence similarity of G-protein α subunits in Dothideomycete fungi

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C. zeae-maydis GPA1 clade
Appendix Figure 1. Protein sequence similarity of G-protein α subunits in Dothideomycete fungi (Continued)

C. zeae-maydis GPA2 clade
Appendix Figure 1. Protein sequence similarity of G-protein α subunits in Dothideomycete fungi (Continued)

A phylogenetic tree was made comparing *C. zeae-maydis* Gpα protein sequences to other Gpα subunits in Dothideomycete fungi. The Gpα subunits in *C. zeae-maydis* are in red font. Each line points to a specific hit in a specific species. These hits are named by “species accronym|protein ID”. A total of 268 hits among 90 species are included in the tree. These were determined by excluding sequences below 40% identity and 40% sequence coverage. More information on which species are included in the tree is listed in Appendix Table 1.