MOLECULAR CHARACTERIZATION OF GENES REGULATING FUMONISIN BIOSYNTHESIS AND DEVELOPMENT IN MAIZE PATHOGEN *FUSARIUM VERTICILLIOIDES*

A Dissertation

by

UMA SHANKAR SAGARAM

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2007

Major Subject: Plant Pathology

MOLECULAR CHARACTERIZATION OF GENES REGULATING FUMONISIN BIOSYNTHESIS AND DEVELOPMENT IN MAIZE PATHOGEN *FUSARIUM VERTICILLIOIDES*

A Dissertation

by

UMA SHANKAR SAGARAM

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Approved by:

Chair of Committee, Won-Bo Shim
Committee Members, Daniel J. Ebbole
Michael V. Kolomiets
Javier F. Betran
Head of Department, Dennis C. Gross

May 2007

Major Subject: Plant Pathology

ABSTRACT

Molecular Characterization of Genes Regulating Fumonisin Biosynthesis and Development in Maize Pathogen *Fusarium verticillioides.* (May 2007)

Uma Shankar Sagaram, B.Sc., Acharya N. G. Ranga Agricultural University;

M.S., West Texas A&M University

Chair of Advisory Committee: Dr. Won-Bo Shim

Fusarium verticillioides (Sacc.) Nirenberg (teleomorph *Gibberella moniliformis* Wineland) is a fungal pathogen of maize that causes ear rots and stalk rots worldwide. In addition, it produces a group of mycotoxins called fumonisins when the fungus colonizes maize and maize-based products. Fumonisin B_1 (FB_1), the predominant form occurring in nature, can cause detrimental health effects in animals and humans. Several efforts were made to study the host and pathogen factors that contribute to the production of fumonisins. Using the available genomic resources, three genes with a potential role in FB_1 regulation and development were identified. The genes are *GBP1*, *GBB1* and *GAP1*. This research describes molecular characterization of these genes with respect to regulation of FB_1 and development in *F. verticillioides*. *GBP1* is a monomeric GTP binding protein with similarity to DRG and Obg sub-classes of G-proteins. *GBB1* encodes heterotrimeric GTP binding protein β subunit. *GAP1* is a GPI (Glycophosphotidylinositol) anchored protein, which belongs to a family of cell wall proteins.

Targeted deletion and complementation studies indicated that *GBP1* is negatively associated with FB_1 biosynthesis but had no effect on conidiation in *F. verticillioides*. *GBB1* plays an important role in regulation of FB_1 biosynthesis, conidiation and hyphal growth, but not virulence. *GAP1* is associated with growth, development and conidiation but not in positive regulation of FB_1 or pathogenicity. The outcome of this study revealed new molecular genetic components that will help scientists better understand signal transduction pathways that regulate FB_1 biosynthesis and conidiation in *F. verticillioides*.

DEDICATION

This dissertation is dedicated with appreciation to my parents, Eswaraiah Sagaram & Laxmi Devi Sagaram and my wife Madhulika Sagaram for their encouragement, untiring patience and moral support.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Won-Bo Shim for his continued guidance and support during the course of my PhD work and preparation of this dissertation. I would also like to thank my committee members Dr. Daniel J. Ebbole, Dr. Michael V. Kolomiets and Dr. Javier F. Betran for their help, insight and review of this dissertation.

My special thanks to Dr. Brian Shaw for his expertise and input in microscopy work. I am grateful to Yoon E Choi and other lab members for sharing scientific knowledge and maintaining a friendly atmosphere in the lab. I also would like to thank my friends and colleagues in the department of Plant Pathology and Microbiology.

TABLE OF CONTENTS

CHAPTER Page

LIST OF FIGURES

FIGURE Page

LIST OF TABLES

CHAPTER I

INTRODUCTION*

Fusarium verticillioides (Sacc.) Nirenberg (teleomorph *Gibberella moniliformis* Wineland; formerly known as *F. moniliforme*) is a fungal pathogen of maize causing ear rots and stalk rots worldwide (Munkvold and Desjardins, 1997). More importantly, *F. verticillioides* produces a group of mycotoxins called fumonisins when the fungus colonizes maize and maize-based products. Fumonisin B_1 (FB_1), the major fumonisin found in nature, can cause detrimental health effects when consumed by animals and humans (Gelderblom et al., 1988; Nelson et al., 1993; Marasas, 2001). Fumonisin contamination of maize has been perceived as a problem in the Midwest, however, recent reports describing the link between fumonisin-contaminated maize and prevalent incidents of neural tube defects (NTD) near the Texas-Mexico border suggest otherwise (Missmer et al., 2006). Since the discovery of fumonisins in 1988, scientists have invested substantial efforts to understand the chemistry, toxicology, and biology of fumonisins as well as the maize-*Fusarium* pathosystem. Several excellent reviews are available on these topics (ApSimson, 2001; Merrill et al., 2001; Munkvold and Desjardins, 1997; Nelson et al., 1993).

This dissertation follows the style and format of *Molecular Plant Pathology*.

* This chapter is reprinted with permission from "Regulation of fumonisin biosynthesis in *Fusarium verticillioides-Maize system*" by Sagaram, U.S., Kolomiets, M. and Shim, W.B. (2006) *Plant Pathol. J.* 22, 230-210.

REGULATION OF FUMONISIN BIOSYNTHESIS IN *FUSARIUM VERTICILLIOIDES*-MAIZE SYSTEM

Our knowledge of fumonisins biosynthesis has greatly increased over the past decade. Fumonisins, a group of polyketide-derived secondary metabolites, that are synthesized by a cluster of genes designated as the *FUM* gene cluster (Proctor et al., 1999; Proctor et al., 2003). To date, the *FUM* gene cluster, which is approximately 42-kb in length, is known to harbor 22 open reading frames (ORFs) – 15 of which are known to be co-regulated. *In silico* analysis suggests that, eleven of the 15 co-regulated genes encode secondary metabolite biosynthesis genes, e.g., mono-oxygenases, dehydrogenases, and fatty acyl-CoA synthetases. The *FUM* gene cluster also contains genes encoding putative transporters and proteins involved in self-protection (Proctor et al., 2003). Molecular genetic characterization of *FUM1*, a polyketide synthase, *FUM6*, a cytochrome P450 monoxygenase, and *FUM8*, an aminotransferase, demonstrated their critical roles in FB_1 biosynthesis; disruption of *FUM1*, *FUM6*, and *FUM8* resulted in significant reduction in FB_1 production (Proctor et al., 1999; Seo et al., 2001). Butchko et al. (2003) provided the first biochemical evidence directly linking a *FUM* gene (*FUM13*) to a specific reaction during fumonisin biosynthesis. The Fum13 protein, which has similarity to short-chain dehydrogenases/reductases, was found to catalyze the reduction of the C-3 carbonyl of the fumonisin backbone to a hydroxyl group. Similarly, *FUM3* encodes a 2-ketoglutarate dependent dioxygenase and catalyzes the hydroxylation of carbon-5 of the fumonisin backbone (Butchko et al., 2003; Ding et al., 2004). Bojja et al. (2004) have also elucidated two of the early steps in fumonisin biosynthesis.

Utilizing disruption mutants, the authors demonstrated that the enzyme encoded by *FUM8* catalyzes the condensation of alanine, which is followed by the oxidation of the backbone at carbons 14 and 15 by the P450 monooxygenase encoded by *FUM6*.

While our knowledge of the fumonisin biosynthesis gene cluster is nearly complete, there is a critical gap in our understanding of the regulatory mechanisms involved in fumonisin biosynthesis. A decade of research has shown that there are multiple factors, environmental as well as genetic, that play a role in this complex biological process. Significantly, the regulation mechanism governing fumonisin biosynthesis in *F. verticillioides* may have several distinct attributes quite different from what is known in other filamentous fungi (Yu and Keller, 2005). Here I discuss the current understanding of how fumonisin biosynthesis is regulated in *F. verticillioides*. I also present the recent functional genomics approaches that are helping scientists to better understand this intriguing regulatory mechanism in *F. verticillioides*.

REGULATION OF FB_1 BIOSYNTHESIS IN *F. VERTICILLIOIDES*

Effect of ambient environmental factors on FB_1 production

A variety of environmental factors, such as host specificity, moisture content, temperature, maize kernel environment, and nutritional condition, are known to significantly influence fumonisin production. A couple of environmental factors known to impact FB_1 production are humidity and temperature. A positive correlation between late season rainfall and *F. verticillioides* infection severity has been reported (Kommedahl and Windels, 1981; Munkvold and Desjardins, 1997). Fumonisin production increased with an increase in water activity (a_W) at all tested temperatures

between 15 – 30 ºC (Samapundo et al., 2005). A marginal effect of temperature on fumonisin was observed at high a_W (optimal for growth), whereas more drastic effect was observed at low a_w. Thus, these studies suggest that relative water activity in maize kernels has more direct effect on fumonisin production whereas the effect of temperature seems to be dependent on a_W (Marín et al., 1999; Samapundo et al., 2005).

Nitrogen limitation, ambient pH, and carbon nutrient specificity are other important factors known to impact FB_1 production in *F. verticillioides* (Bluhm and Woloshuk, 2005; Shim and Woloshuk, 1999). A positive correlation between nitrogen limitation and FB_1 biosynthesis was described in *F. verticillioides* and *F. proliferatum* (Keller et al., 1997; Shim and Woloshuk, 1999). Studies also indicated that carbon and phosphate contents did not have a repressive effect on FB_1 production. It was also determined that the FB_1 production was repressed under higher concentrations of nitrogen (ammonium phosphate, glycine, or glutamate) in the culture media, suggesting that FB_1 biosynthesis is under nitrogen repression (Shim and Woloshuk, 1999). In addition to nitrogen, Keller et al (1997) showed that acidic pH (3.0 – 4.0) under well-aerated conditions enhanced FB_1 biosynthesis in *F. proliferatum*. In a later study, it was determined that acidic pH is critical for FB_1 production which prompted the investigation of *F. verticillioides* pH regulator gene and its role in FB_1 biosynthesis (Shim and Woloshuk, 2001; Flaherty et al., 2003).

Genes associated with fumonisin biosynthesis regulation

Notably, unlike other fungal secondary metabolite gene clusters, the *FUM* cluster does not contain a pathway-specific regulatory gene (Brown et al., 1996; Kennedy et al.,

1999; Proctor et al., 2003; Woloshuk et al., 1994). Rather, fumonisin biosynthesis is regulated by several genes not linked to the *FUM* cluster. To date, the genes that are known to regulate FB_1 biosynthesis are *FCC1*, *FCK1*, *PAC1*, *ZFR1*, and *GBP1*. Mutational analysis of these genes demonstrated that a functional copy of these genes is necessary for proper production of FB_1 in *F. verticillioides*. Detailed information on these genes is presented in Table1.1. Other than *FCC1* and *FCK1*, no definitive epistatic relationship between these regulatory genes has been demonstrated, thus it is likely that these genes operate independently. However, it is clear that additional regulatory genes, yet to be identified, are associated with fumonisin biosynthesis in *F. verticillioides*.

Table 1.1 Genes associated with fumonisin B_1 regulation in *F. verticillioides*.

Gene Name	Gene Family	FB_1 production in mutants	Other Mutant Phenotype	Regulatory mode	Reference
FCC1	C-type Cyclin	No FB_1 when grown on maize kernel. Leaky FB_1 production in acidic defined media	Severe reduction in conidiation	Positive	Shim and Woloshuk, 2001
FCK1	Cyclin-dependent kinase	Significant reduction in FB_1 production	Severe reduction in growth and conidiation	Positive	Bluhm et al., 2006
PAC1	*PACC* group of pH regulatory genes	Higher FB_1 when grown on acidic conditions and on maize kernels	Severely impaired growth at alkaline pH	Negative	Flaherty et al., 2003
ZFR1	Zn(II)2Cys6 binuclear cluster	Severe reduction in FB_1 production	No observable effect on growth and development	Positive	Flaherty and Woloshuk, 2004
AMY1	Amylase	Unable to digest starch. Impaired in FB_1	No observable effect on growth and development	Positive	Bluhm and Woloshuk, 2005

Maize physiology and genomics associated with FB_1 biosynthesis: Maize kernel microenvironment

One of the complicated issues in fumonisin research yet to be understood is the interaction between maize kernel and *F. verticillioides* that ultimately influences fumonisin biosynthesis. It is clear from published reports that maize kernel microenvironment plays a critical role in fumonisins biosynthesis in maize (Bluhm and Woloshuk, 2005; Shim et al., 2003; Warfield and Gilchrist, 1999). Warfield and Gilchrist (1999) observed a developmental stage-dependent relationship between FB_1 production and maize kernels. Their study showed that later developmental stages – dough (R4) and dent (R5) - produced higher levels of FB_1 while the earlier stages produced lower levels indicating that the maize kernel stage influences FB_1 production. The researchers suggested that the effect on toxin production could be due to substrate composition change as well as moisture content. Shim et al. (2003) examined FB_1 production in two components of the corn kernel, namely the germ tissues and the degermed kernel. Growth of *F. verticillioides* was similar in colonized germ tissue and the degermed kernels, but FB_1 production was at least five-times higher in degermed corn kernels than in germ tissue. Expression of the *FUM1*, as measured by a GUS assay and northern blot analysis, followed the same pattern as FB_1 production. Also correlated with FB_1 was a concomitant drop in pH of the colonized degermed kernels. A time course experiment showed that degermed kernels inoculated with *F. verticillioides* became acidified over time (from pH 6.4 to 4.7 after 10 days of incubation), while colonized germ tissue became alkaline over the same period (from pH 6.5 to 8.5). Since

conditions of acidic pH are conducive to FB_1 production and alkaline pH is repressive (Shim and Woloshuk, 2001), the observed correlation between the acidification of degermed kernels and the increase in FB_1 provides one explanation for the observed differences in FB_1 levels.

Recently, studies conducted by Bluhm and Woloshuk (2005) provided further understanding on how the fungal colonization of maize kernels affects FB_1 production. It was demonstrated that kernel development stage, and not the fungal growth, is critical for FB_1 biosynthesis. Subsequently, it was hypothesized that the difference in starch content in the kernels - the dent stage has highest starch content (70%) followed by dough (58%), milk (20%) and blister (12%) - is the key reason for varied FB_1 production at different kernel stages. At ten days after fungal inoculation, high level of FB_1 occurred only in dent stage kernels whereas trace FB_1 level was observed in other stages. In agreement with this set of data, quantitative real-time PCR (qPCR) analysis indicated that kernel development affected transcription of genes involved in fumonisin biosynthesis, nitrogen repression, and starch metabolism. Worth noting is the low expression level of *AREA*, a nitrogen metabolism regulating transcription factor in colonized blister kernels. These data suggest that the blister kernel environment represses *FUM* gene expression that is then derepressed as the kernels mature. Also, expression of *AMY1* (α-amylase) and ATPase (a putative H^+ ATPases) is elevated in the colonized dough and dent kernels, correlating with starch accumulation and the decrease in pH, respectively.

These observations led Bluhm and Woloshuk (2005) to investigate the role of starch in regulation of FB_1 biosynthesis in maize kernels. Four maize mutants with varied starch compositions, i.e., *shrunken-2* (sh2), *sugary-1* (su1), *waxy-1* (*wx1*), and *amylose extender-1* (*ae1*), along with wild-type maize line (5322) were challenged with *F. verticillioides*. One striking discovery from this study was that a very low level of FB_1 was detected in *ae1* (less than 10% of FB1 level observed in 5322) that has low starch content. Significantly, *ae1* was the only maize variety in which the pH increased after *F. verticillioides* colonization. Furthermore, it was demonstrated that α-amylase activity is necessary for growth, FB_1 production, and kernel acidification in degermed-tissue of mature 5322 kernels. Strikingly, cultures provided with amylopectin or dextrin (a product of amylopectin hydrolysis) as sole carbon source produced significantly more FB_1 than ones with other carbon sources, suggesting that these carbon sources induce FB_1 biosynthesis in *F. verticillioides* (Bluhm and Woloshuk, 2005).

FUNCTIONAL GENOMIC APPROACHES TO IDENTIFY PUTATIVE FUMONISIN REGULATORS IN *F. VERTICILLIOIDES*

F. verticillioides cDNA libraries, microarrays, and genome sequence

Genomics have facilitated our efforts to identify genes associated with fumonisin regulation. Shim and Woloshuk (2001) took advantage of subtractive suppressive hybridization (SSH) technique to identify differentially expressed genes in the wild-type and *fcc1* mutant strain. As described in Table 1.1, *fcc1* strain is blocked in FB_1 biosynthesis when grown on maize kernels. The SSH cDNA fragments randomly selected from each strain, which averaged 500 bp in length, were sequenced and putative

functions were designated based on sequence homology. Wild-type SSH cDNAs were of particular interest since these cDNAs are hypothesized as genes positively associated with fumonisin biosynthesis. The fact that cDNAs corresponding to the *FUM* genes, e.g., *FUM1* and *FUM6* through *FUM14,* were identified in the wild-type library strongly support this hypothesis. Significantly, *ZFR1*, positive regulator of FB_1 biosynthesis, was originally identified in the wild-type EST library (Flaherty and Woloshuk, 2004).

Subsequently, Woloshuk and colleagues utilized microarray technology to further verify putative genes associated with fumonisin biosynthesis (Pirtillä et al., 2004). A total of 716 spots of cDNA from the SSH EST libraries described earlier were spotted on the microarray. The sequence data of the SSH cDNAs and microarrays can be accessed at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/). While this microarray represented a small number of genes by comparison to whole-genome microarrays, these cDNAs were obtained from two strains having contrasting FB_1-production phenotypes when grown on corn kernels. The analysis compared genotype (wild type or *fcc1* mutant), pH, and genotype × pH interactions and resulted in isolation of genes that are expressed concomitantly with fumonisin production. The study identified 19 genes displaying expression profile similar to the *FUM* genes (Pirtillä et al. 2004). Thus far, Woloshuk and colleagues have tested expression of 12 of these genes by quantitative real-time PCR (qPCR) during growth on corn kernels and found that their expression was higher in the wild-type strain than the *fcc1* mutant (Woloshuk, unpublished data).

More recently, scientists at the Mycotoxin Research Unit, USDA-ARS (Northern Regional Research Laboratory) reported generating over 87,000 ESTs from *F. verticillioides* (Brown et al., 2005). This collection of ESTs was generated from nine different fungal cDNA libraries and represents over 11,000 unique sequences. Analysis of this extensive collection of ESTs, which is estimated to represent over 80% of the expressed genes in the fungus, revealed candidate genes that are likely to regulate fumonisin biosynthesis and *F. verticillioides*-maize interactions. The EST database is publicly available at The Institute for Genomic Research (TIGR) and can be accessed at TIGR *F. verticillioides* Gene Index website (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=f_verticill). Collaborative research is in progress at USDA-ARS, Purdue University, and Texas A&M University to further characterize the functional role of these putative regulatory genes.

My long-term research goal is to develop fumonisin control strategies that are cost effective and environmentally sustainable. To accomplish this goal, we need better understanding of the molecular and physiological interactions between *F. verticillioides* and maize that triggers fumonisin biosynthesis. The key objective of my research is to isolate and characterize the genes that regulate FB_1 biosynthesis and conidiation in *F. verticillioides*. In an effort to identify additional regulators of fumonisin biosynthesis, I screened *F. verticillioides* EST index (TIGR) and *F. verticillioides* SSH cDNA library (Shim and Woloshuk, 2001). During the screening process, we identified a 400-bp EST encoding a putative GTP-binding protein in *fcc1* SSH library and an 350-bp EST in the wild-type SSH library encoding a protein sequence with high homology to

glycosylphosphatidylinositol (GPI) -anchored β-1, 3-glucanosyltransferases that belong to a class of GPI- anchored glycoproteins. I designated these genes *GBP1* and *GAP1*, respectively. Another group of highly conserved G proteins that I was interested in are heterotrimeric G proteins. Heterotrimeric (αβγ) G proteins are shown to be involved in several developmental and signaling processes in a wide range of organisms including filamentous fungi (Gilman, 1987; Hamm and Gilchrist, 1996). G proteins enable the eukaryotic organisms to recognize and respond to external signals through transmembrane signaling (Gilman, 1987; Hamm and Gilchrist, 1996). Considerable information is available on the role of heterotrimeric G protein signaling in association with secondary metabolism in filamentous fungus *Aspergillus* (reviewed in references Calvo et al., 2002; Yu and Keller, 2005). But very little is known about G protein's involvement in secondary metabolism of other filamentous fungi and especially in relation to fumonisin regulation in *F. verticillioides*. A better understanding of the role of heterotrimeric G proteins in FB_1 regulation would enhance our knowledge in relation to corn and *F. verticillioides* interaction, which in turn enable us to develop improved management strategies. Hence, I selected heterotrimeric G protein β-subunit encoding gene for characterization. A reverse genetic approach was adopted to study the possible involvement of selected genes in FB_1 biosynthesis. The deletion and complementation mutants generated and used in further studies are listed in Table 1.2.

Table 1.2 Fungal strains used in this study.

Strain name	**Description**	**Genotype**
M-3125	*F. verticillioides* 7600 strain	Wild type (WT)
GM40-2 (*Δgbp1*)	Deletion of *GBP1* in M-3125	*Δgbp1::HYG*
GBPC4 (*gbp1-C*)	Complementation of GM40-2	*Δgbp1::HYG, GBP1::GEN*
BM83 (*Δgbb1*)	Deletion of *GBB1* in M-3125	*Δgbb1::HYG*
BC4 (*gbb1C*)	Complementation of BM83	*Δgbb1::HYG, GBB1::GEN*
GAM126	Deletion of *GAP1* in M-3125	*Δgap1::HYG*
GAG8	Deletion of *GAP1* in M-3125	*Δgap1::GEN*
GASC1	Complementation of GAM126	*Δgap1::HYG, GAP1::GEN*
PAC2A	Deletion of *PAC1* in M-3125	*Δpac1::HYG*

CHAPTER II

THE PUTATIVE MONOMERIC G-PROTEIN *GBP1* IS NEGATIVELY ASSOCIATED WITH FUMONISIN B_1 PRODUCTION IN *FUSARIUM VERTICILLIOIDES**

SUMMARY

Fumonisin B_1 (FB_1) is a mycotoxin produced by *Fusarium verticillioides* that contaminates maize. FB_1 has been linked to a number of human and animal mycotoxicoses worldwide. Despite its significance, our understanding of the FB_1 biosynthesis regulatory mechanisms is limited. Here, we describe *F. verticillioides GBP1*, encoding a monomeric G-protein, and its role in FB_1 biosynthesis. *GBP1* was discovered as an expressed sequence tag (EST) up-regulated in the *F. verticillioides fcc1* mutant that showed reduced conidiation and no FB_1 biosynthesis when grown on maize kernels. Sequence analysis showed that *GBP1* encodes a putative 368-amino acid protein with similarity to DRG and Obg sub-classes of G-proteins that are involved in development and stress responses. A *GBP1* knockout mutant (*Δgbp1*) exhibited normal growth, but increased FB_1 production (>58%) compared to the wild type when grown on corn kernels. Complementation of *Δgbp1* with wild-type *GBP1* gene restored FB_1 production level to that of wild type. Our data indicate that *GBP1* is negatively associated with FB_1 biosynthesis but not with conidiation in *F. verticillioides*. The

---

* This chapter is reprinted with permission from "The putative monomeric G-protein *GBP1* is negatively associated with fumonisin B_1 production in *Fusarium verticillioides"* by Sagaram, U.S., Butchko, R.A.E. and Shim, W.B. *Molecular Plant Pathol*. 7, 381-389. Copyright 2006 by Blackwell Publishing LTD.

deletion of *GBP1* led to up-regulation of key FB_1 biosynthetic genes, *FUM1* and *FUM8*, suggesting that the increased FB_1 production in *Δgbp1* is due to over-expression of *FUM* genes.

INTRODUCTION

Fusarium verticillioides (Sacc.) Nirenberg (teleomorph *Gibberella moniliformis* Wineland) is a fungal pathogen of maize causing ear rots and stalk rots worldwide (Munkvold and Desjardins, 1997). More importantly, *F. verticillioides* produces a group of mycotoxins called fumonisins, which can contaminate maize and maize-based products. Fumonisins can cause detrimental health effects when consumed by animals and humans (Marasas, 2001; Nelson *et al*., 1993). A link between fumonisins and human esophageal cancer risks has been reported in South Africa and China (Gelderblom *et al.,* 1988; Yoshizawa *et al.,* 1994). In addition, a link between fumonisins and neural tube defects (NTD) was suggested when laboratory tests showed early somite neurulating mouse embryo exposed to fumonisins resulted in retarded growth and cranial neural tube defects (Sadler *et al*., 2002). More recently, research suggested that the prevalence of NTDs in the US-Mexico border population was attributed to exposure to a diet with high levels of fumonisins (Missmer *et al.,* 2006). Currently over 15 different fumonisin analogs are known in nature, but Fumonisin B_1 (FB_1) is the major, and most toxic, fumonisin identified (Musser and Plattner, 1997). In an effort to minimize public exposure to fumonisins, the US food and Drug Administration has established a guideline for fumonisin levels in food and feeds (Park and Troxell, 2002).

Recent molecular studies have revealed that fumonisins are synthesized via proteins encoded in the fumonisin biosynthesis gene cluster (*FUM* cluster). In *F. verticillioides*, the *FUM* cluster is reported to contain at least 15 genes, including *FUM1* (polyketide synthase gene), *FUM8* (aminotransferase gene), and *FUM12* (cytochrome P450 monooxygenase gene) (Procter *et al.,* 2003; Seo *et al*., 2001). Significantly, the *FUM* cluster does not contain regulatory genes, and studies have shown that the fumonisin biosynthesis regulatory mechanism is complex (Proctor *et al*., 2003; Shim and Woloshuk, 2001). A number of factors, such as available nitrogen, ambient pH, and maize kernel environment, are suggested to play a critical role in the regulation of toxin production. (Shim and Woloshuk, 1999; Shim *et al*., 2003). Also, a few regulatory genes, e.g., *FCC1*, *FCK1*, *ZFR1*, and *PAC1*, that affect fumonisin biosynthesis have been identified and characterized (Bluhm *et al*., 2006; Flaherty *et al*., 2003; Flaherty and Woloshuk, 2004; Shim and Woloshuk, 2001). Among the genes identified, *FCC1*, a gene encoding a C-type cyclin, regulates conidiation and fumonisin production; the *fcc1* mutant shows a significant reduction (> 100-fold) in conidiation and a block in FB_1 biosynthesis when the fungus is grown on maize kernels (Shim and Woloshuk, 2001).

A suppressive subtraction hybridization (SSH) cDNA library and cDNA microarray were constructed from cDNAs from wild-type and *fcc1* mutant strains, and these tools were used for screening differentially-expressed genes during FB_1 biosynthesis (Pirttilä *et al*., 2004; Shim and Woloshuk, 2001). The efforts led to the identification of *ZFR1*, a Gal4-like transcription factor, in the cDNA library enriched with transcripts from the wild-type strain. Molecular characterization determined that

ZFR1 is a *FCC1*-dependent positive regulator of FB_1 biosynthesis in *F. verticillioides*. Recently, Brown and colleagues (2005) reported generation of over 87,000 ESTs from *F. verticillioides* in an effort to identify genes affecting toxin production. Establishment of these molecular and genomic resources is facilitating the efforts to better characterize the complex regulatory mechanisms involved in fumonisin biosynthesis.

In this study we describe identification of a 200-bp EST encoding a putative GTP-binding protein in the *Δfcc1* SSH cDNA library. The gene, designated *GBP1*, encodes a protein that has a high homology to the Obg class of G-proteins and developmentally-regulated GTP-binding proteins (DRGs) in fungi and higher eukaryotes (Li and Trueb, 2000). The fact that these GTPases are known to perform ligand-binding, regulatory roles in eukaryotes, and that we discovered *GBP1* in the *fcc1* SSH cDNA library led us to hypothesize that *GBP1* acts as a negative regulator of FB_1 biosynthesis or conidiation, or both, in *F. verticillioides*. We investigated the transcriptional regulation of *FUM1*, *FUM8*, *GBP1*, and *ZFR1* in culture conditions conducive to FB_1 biosynthesis, and we generated a *F. verticillioides GBP1* deletion (*Δgbp1*) mutant to investigate the functional role of *GBP1*. While the *Δgbp1* mutant showed no defect in conidiation or growth, one key phenotype we observed was increased FB_1 production which was mediated by over-expression of *FUM1* and *FUM8*. The data presented in this study suggests that *GBP1* is a negative regulator of *FUM* genes in *F. verticillioides*.

RESULTS

Transcription profile of EST ft536_1_I17

We isolated a 200-bp EST sequence (ft536_1_I17) from a *F. verticillioides fcc1* SSH cDNA library that shared high similarity to a *Schizosaccharomyces pombe* hypothetical GTP-binding protein (E= 5e-26). We designated the ft536_1_I17 EST as *GBP1* and further investigated its role in fumonisin biosynthesis. The *F. verticillioides* Gene Index was screened for matching cDNA sequence and the 1369-bp TC (tentative consensus) 31258 was identified. A translated BLAST (BLASTx) search revealed that TC 31258 shares high homology (E<1e-100) to a conserved hypothetical GTP-binding protein found in a number of eukaryotes, particularly fungi. We compared the expression profile of TC 31258 with *FUM1*, *FUM8*, and *ZFR1* (Fig. 2.1).

Preliminary analysis showed that TC 31258 exhibited relatively higher expression when the fungus was grown in defined liquid culture than it did in maize kernels. However, little change was observed between the 24 hr and 120 hr sample suggesting that TC 31258 is not expressed concomitantly with FB_1 production. Similar conclusions can be made with the expression study performed in maize kernel samples. We also observed a similar expression pattern in *ZFR1*, a positive regulator of fumonisin biosynthesis in different culture conditions. Flaherty and Woloshuk (2004) previously recognized *ZFR1* as a constitutively expressed gene, and suggested that additional factors could be required for the Zfr1 protein activity.

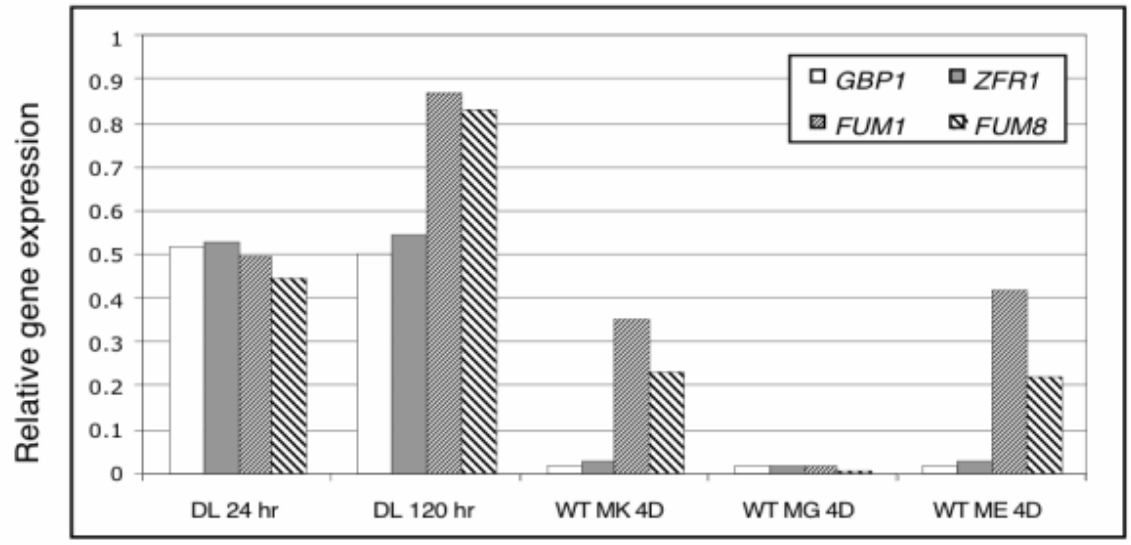

Fig. 2.1 Transcription analysis *GBP1*, *FUM1*, *FUM8* and *ZFR1* from microarray data. Expression of *GBP1*, *FUM1*, *FUM8* and *ZFR1* are presented after growth on defined liquid medium at 24 and 120 hours (DL, see Experimental procedures) and on whole maize kernels (MK), maize germ tissue (MG) and maize endosperm tissue (ME) after a period of 4 days growth. Relative gene expression between experiments is shown as expression relative to Elongation Factor-1-α.

Molecular characterization of *GBP1*

Analysis of genomic DNA sequence containing the *GBP1* EST sequence (ft536_1_I17) and TC 31258 revealed that *GBP1* is a 1,503-bp gene containing 3 putative introns. Analysis of 368-amino acid polypeptide showed the presence of a GTP1-Obg domain at the N-terminus and a TGS (ThrRS, GTPase, and SpoT) domain at the C-terminus. Significantly, all five GTP binding domains that are characteristic of GTP binding proteins are present in Gbp1 (Fig. 2.2) (Ethridge *et al.*, 1999). CLUSTALW analysis indicated that the putative Gbp1 protein shares high homology with predicted protein sequences in filamentous fungi and other higher organisms (Thompson *et al*., 1994). Sequence accession numbers of homologs in other organisms with percentage identity to putative Gbp1 are: *Magnaporthe grisea*, AACU01000565.1

(90 %); *Neurospora crassa* XM_322474.1 (86 %); *Saccharomyces cerevisiae* AY693042.1 (69 %); *Homo sapiens* BC020803.1 (62 %); *Mus musculus* D10715.1 (62 %); *Drosophila melanogaster*, X71866.1 (61 %); and *Arabidopsis thaliana*, AL078620.2 (59 %). The *S. cerevisiae* homolog Rbg1 is known to physically interact with translating ribosomes, however, its biological functions are still unknown. The homologs found in filamentous fungi are designated as hypothetical proteins with no functional characterization.

(B)

	G56	Y79	D102	N234	G326
F. verticillioides	GFPSVGKS	YEFTTL	DLPG	NKID	GKSV
M. grisea	GFPSVGKS	YEFTTL	DLPG	NKID	GKSV
N. crassa	GFPSVGKS	YEFTTL	DLPG	NKID	GKSV
S. cerevaceae	GFPSVGKS	YEFTTL	DLPG	NKID	GSSV
H. sapiens	GFPSVGKS	YEFTTL	DLPG	NKID	GLSV
M. musculus	GFPSVGKS	YEFTTL	DLPG	NKID	GLSV
D. melanogaster	GFPSVGKS	YEFTTL	DLPG	NKID	GSSV
A. thaliana	GFPSVGKS	YEFTTL	DLPG	NKID	GSSA

Fig. 2.2 *F. verticillioides* Gbp1 protein and the five GTP-binding domains. **(A)** Schematic representation of the putative *F. verticillioides* Gbp1 protein and the five GTP-binding domains. Each domain designation denotes its first amino acid and its location in the protein sequence. **(B)** Amino acid alignment of the five GTP-binding motifs in Gbp1 and select homologs.

GBP1 deletion and its impact on FB_1 biosynthesis and conidiation

To investigate the role of *GBP1* in FB_1 biosynthesis, we generated a gene knock-out strain (*Δgbp1*) by double homologous recombination strategy (Fig. 2.3). After

screening 29 transformants, we identified one transformant where *GBP1* was replaced with the *HYG* marker. Southern blot showing ^{32}P-labeled 1.3-kb DNA hybridizing to a 3.8-kb band in wild type and 1.8-kb band in *Δgbp1* confirmed targeted gene deletion (Fig. 2.4A).

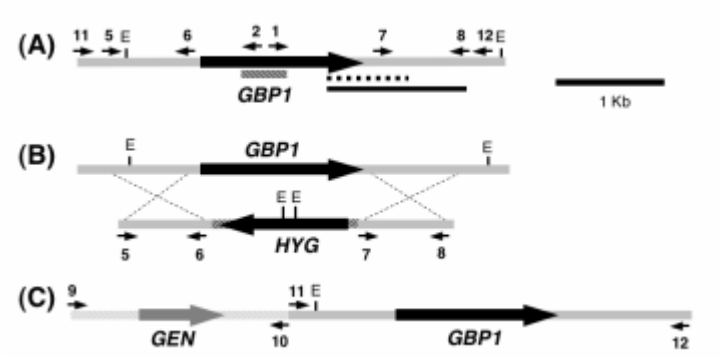

Fig. 2.3 Description of *F. verticillioides GBP1* locus: disruption and complementation strategies. **(A)** Schematic representation of sequenced 3.9-kb *GBP1* locus in wild-type genome. The shaded box represents the 400-bp *GBP1* EST, which was used to design the primers for Genome Walker® PCR. Solid line and dashed line represent the genomic regions used as probes for Southern and northern blots, respectively. Primer positions are indicated with small arrows. Primers 1, 2, 3, and 4 were used to sequence the *GBP1* genomic DNA. Primers 3 and 4 are not shown as they are present on the Genome Walker® linkers. Primer pairs 5-6 and 7-8 were used to amplify 5′ flanking and 3′ flanking DNA, respectively, for the disruption vector pFVGDV. Primer pair 11-12 was used to amplify the *GBP1* genomic DNA (3,700 bps) for the complementation construct. E: *Eag*I restriction site. **(B)** Schematic representation of *GBP1* deletion vector and double homologous event. The homologous recombination resulted in replacement of *GBP1* with hygromycin-resistant gene (*HYG*). **(C)** The complementation construct was generated by fusing geneticin resistant gene (*GEN*) to *GBP1* gene by single joint PCR. Primer pair 9-10 was used to amplify *GEN*.

The decrease in band size is due to the presence of *Eag*I restriction site in *HYG*. Subsequent northern analysis showed that *GBP1* expression is completely abolished in *Δgbp1* (Fig. 2.4B). When wild-type and *Δgbp1* strains were grown in a variety of media, including PDA, V8 agar, KCl agar, and maize kernels, no morphological differences,

such as conidiation, mycelial growth, and pigmentation, were observed (data not shown). Also, *Δgbp1* grew normally on a variety of minimal media, particularly with different carbon and nitrogen sources, suggesting no physiological defect was caused by the mutation. Wild-type and mutant strains were inoculated on cracked corn medium to analyze FB_1 production. The wild-type strain produced 84 μg of FB_1 per g of cracked corn after 10 days incubation. In contrast, *Δgbp1* produced 133 μg of FB_1 per g of cracked corn, which is significantly greater (>58% increase) than that of wild type ($P<0.05$) (Fig. 2.5).

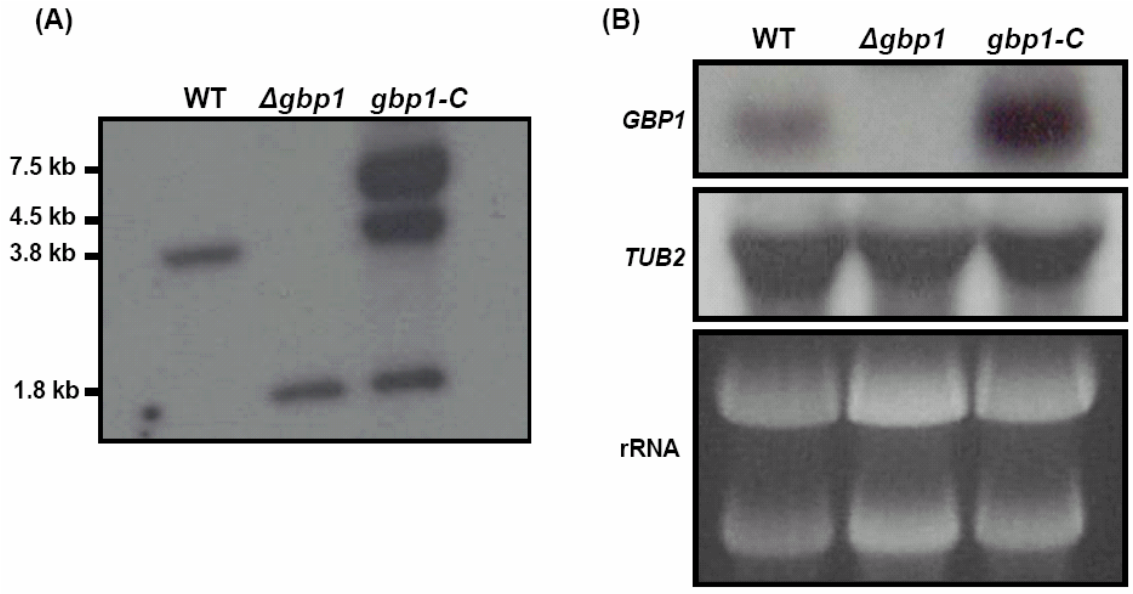

Fig. 2.4 Southern and northern analyses of wild type (WT), deletion mutant (*Δgbp1*), and complement strain *gbp1-comp* (*gbp1-C*). **(A)** To confirm targeted disruption and complementation of *GBP1,* Southern analysis of WT, *Δgbp1, and gbp1-comp* was performed. Genomic DNA (12 μg) was digested with *Eag*I and the blot was hybridized to 1.3-kb ^{32}P-labeled probe (shown in Fig. 2.3A). Molecular size markers are indicated on the left. **(B)** Northern analysis of *GBP1* expression in WT, *Δgbp1,* and *gbp1-comp* strains. Total RNA samples were isolated from fungal cultures grown in defined liquid medium (pH 6.0) for 7 days and the blots were hybridized with ^{32}P-labeled *GBP1* and *TUB2* probes, independently. Ribosomal RNA stained with ethidium bromide is shown as equal loading control.

GBP1 gene complementation

To confirm that increased FB_1 production is due to *GBP1* deletion, the *Δgbp1* mutant was complemented with a wild-type copy of *GBP1* fused to the geneticin-resistance gene (*GEN*) (Fig. 2.3C). The complemented strain (*gbp1-comp*) was isolated from transformants growing on 0.2 × PDA with hygromycin and geneticin.

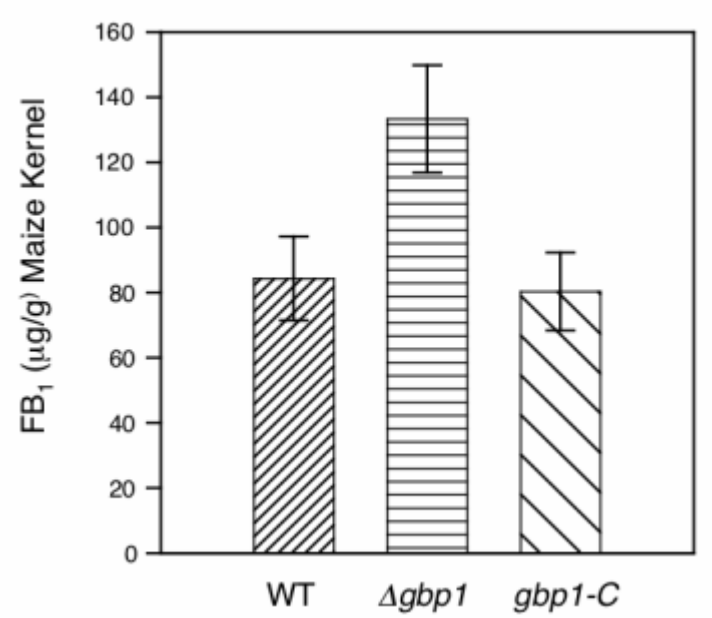

Fig. 2.5 Analysis of fumonisin B_1 (FB_1) in wild type (WT), *Δgbp1,* and *gbp1-comp* (*gbp1-C*). Sterile cracked corn (2 g) was inoculated with 10^6 spores of WT and mutant strains. After 10 days of incubation at room temperature, FB_1 was extracted with 10 mL of 50% acetonitrile in water, purified through SPE C18 columns and eluted in 2 mL 70% acetonitrile in water and quantified by HPLC. All values represent means of 3 replications with standard deviations shown as error bars. The data suggest that FB_1 production in *Δgbp1* was 58% greater than WT ($P<0.05$ least significant difference), and that FB_1 production in WT and *gbp1-comp* were not statistically different.

The presence of complete *GBP1* ORF was verified by Southern analysis (Fig. 2.4A). In addition to the 1.8-kb band, *gbp1-comp* produced a 4.5-kb and 7.5-kb bands indicating the insertion of an intact complementation construct into *Δgbp1* genome (Fig.

2.4A). Expression of *GBP1* in *gbp1-comp* was confirmed by northern hybridization. Wild type and *gbp1-comp* produced a 2.3-kb transcript while *Δgbp1* did not produce any detectable transcript (Fig. 2.4B). The higher expression levels of *GBP1* in *gbp1-comp* can be attributed to the ectopic insertion of two independent copies of *GBP1* in the *gbp1-comp* (Fig. 2.4A). The FB_1 levels in *gbp1-comp* (80 μg per g corn) were not statistically different from wild-type levels ($P<0.05$) (Fig. 2.5). Interestingly, elevated expression of *GBP1* in *gbp1-comp* did not result in suppression of FB_1 levels.

Influence of *Δgbp1* on FB_1 biosynthetic and regulatory genes

The role of *GBP1* in the fumonisin biosynthetic pathway was investigated by examining the expression levels of *FUM1* and *FUM8* (AF155773) in wild-type, *Δgbp1,* and *gbp1-comp* strains. Deletion of *GBP1* results in up-regulation of *FUM1* (> 3-fold) and *FUM8* (>2.5-fold) (Table 2.1).

Table 2.1 Expression of *FUM1* and *FUM8* genes in wild-type, *Δgbp1,* and *gbp1-comp* strains [a, c].

Fungal Strains	Wild type	*Δgbp1*	*gbp1-comp*
FUM1	1.0 (0.8-1.2)[b]	3.2 (2.3-4.4)	0.4 (0.3-0.5)
FUM8	1.0 (0.8-1.2)	2.5 (2.0-3.2)	0.25 (0.2-0.3)
ZFR1	1.0 (0.5-1.5)	0.9 (0.8-1.0)	0.8 (0.7-1.0)

[a] Total RNA samples were prepared from fungal strains grown on cracked corn medium for 11 days. Quantitative real-time (QRT)-PCR analysis of gene expression was performed with SYBR-Green® as the fluorescent reporter. The expression of each gene was normalized to endogenous β-tubulin (*TUB2*) (GenBank U27303) gene expression.

[b] The gene expression was calibrated using $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). Data represent the fold differences in gene expression. The range of expression was calibrated using $2^{-\Delta\Delta Ct-s}$ - $2^{-\Delta\Delta Ct+s}$ where s is the standard deviation of ΔCt value. Ct=Threshold Cycle.

[c] Each value is the mean of three technical replicates from one biological experiment. The experiment was repeated with similar results.

When the *Δgbp1* strain was complemented with the wild-type *GBP1* gene, *FUM1* and *FUM8* expression levels were restored to wild-type levels. The Ct values of *FUM1* and *FUM8* are higher in *gbp1-comp* than wild type, which may suggest that the *FUM* genes are repressed in the complemented strain, however, the Ct values were not different from those of the wild type ($P<0.05$) (data not shown). Significantly, the *FUM* gene expression levels in wild-type, *Δgbp1,* and *gbp1-comp* strains is consistent with the FB_1 levels produced in these strains (Table 2.1 and Fig. 2.5). Subsequently, we hypothesized that *GBP1* may negatively regulate FB_1 biosynthesis in a *ZFR1*-dependent manner, and that the up-regulation of *ZFR1* led to the transcriptional activation of *FUM1* and *FUM8* in *Δgbp1* strain. However, our data showed no significant difference in the Ct value of *ZFR1* in wild type, *Δgbp1*, and *gbp1-comp* indicating that *GBP1* regulates FB_1 in a *ZFR*- independent manner (data not shown).

DISCUSSION

GTP-binding proteins (G-proteins) are a large group of highly conserved proteins. G-proteins are categorized into several super-families and sub-classes but can be structurally categorized as either monomeric or heterotrimeric G-proteins. G-proteins act as 'on' (GTP binding) and 'off' (GTP hydrolysis) switches in several cellular processes such as protein translation, vesicle transport, intracellular transport, organization of cytoskeleton and signal transduction (Bourne, 1990; 1991). Heterotrimeric G-proteins consist of α, β and γ subunits, which are activated by dissociation into G(α) and G(βγ) subunits. Heterotrimeric G-proteins have been shown to be involved in conidiation, virulence mating in several filamentous fungi (Degani *et*

al., 2004; Delgado-Jarana *et al*., 2005; Gao and Nuss, 1996; Ganem *et al*., 2004; Krystofova and Borkovich, 2005). It was shown in *A. nidulans* that inactivation of α subunit (*FadA*) signal transduction pathway is required for asexual spore production and sterigmatocystin production, suggesting that G-proteins play an important role in fungal development and secondary metabolism (Hicks *et al*., 1997). Monomeric G-proteins such as *CLPT1* and *RAS2* are involved in pathogenesis in *Colletotrichum lindemuthianum* (Siriputthaiwan *et al.*, 2005) and *Ustilago maydis* (Lee and Kronstad, 2002) respectively. Developmentally-regulated GTP binding proteins (DRGs) (Devitt *et al*., 1999; Sazuka *et al*., 1992) and Obg-G proteins (Buglino *et al*., 2002) are sub-classes of G-proteins that contain conserved motifs characteristic of GTP binding proteins. Members of DRGs have been extensively studied in mouse and humans, yet, very little is known about the function of fungal DRGs. DRGs from animals, plants and fungi encode similar proteins, however, they seem to perform different functions (Devitt *et al*., 1999). DRG genes show a variety of expression patterns under different conditions in *Xenopus* (Kumar *et al*., 1993), mouse (Sazuka *et al*., 1992), humans (Schenker *et al*., 1994) and plants (Devitt *et al*., 1999). In contrast, our QRT-PCR data indicate that *GBP1* is expressed at a consistent level in different culture media (cracked corn, defined liquid medium, and potato dextrose broth) and at different time points (4, 6, 8 days after inoculation) (data not shown).

Not surprisingly, we are discovering that regulation of fumonisin biosynthesis, like other fungal secondary metabolism regulations, is achieved via complex controlling mechanisms in which multiple genetic components are involved. One of the better

understood mechanisms is the G-protein mediated aflatoxin (AF)/sterigmatocystin (ST) biosynthesis regulation in *A. flavus*/*A. nidulans*. A recent review article by Yu and Keller (2005) provides an excellent description of this complexity. Studies have shown that heterotrimeric G-protein complex in *A. nidulans* serves as a primary mediator of vegetative growth, asexual development, and ST biosynthesis. However, to date the complexity of the genetic components interacting with the heterotrimeric G-protein complex, in both positive and negative manners, have not been fully deciphered to allow clear understanding of the mechanisms involved in fungal development and secondary metabolite production.

Recent characterization of *A. nidulans* RasA (a monomeric GTP-binding protein) and its role in ST biosynthesis suggested that small G-proteins serve a role in fungal secondary metabolism (Shimizu *et al*., 2003). The study showed that RasA is negatively associated with ST biosynthesis and that over-expression of *RasA* repressed *aflR* expression, a key transcriptional activator of ST biosynthetic genes. We hypothesized that *GBP1* may serve a similar role in fumonisin biosynthesis in *F. verticillioides*. A significant increase (> 58%) of FB_1 production in *Δgbp1* was consistently observed, but no difference in spore production, vegetative growth, and perithecia development of the mutant was observed. The data suggest that *GBP1* is involved in regulating FB_1 biosynthesis but not conidiation in *F. verticillioides*. We also demonstrated that the deletion of *GBP1* is the direct cause of increased FB_1 production by complementing *Δgbp1* mutant with wild-type *GBP1* gene. Re-introduction of the wild-type copy of *GBP1* with its native promoter restored the FB_1 levels that are comparable to wild-type

levels (Fig. 2.5). Southern and northern analyses showed that the complemented strain *gbp1-comp* contained two copies of the complementation construct (Fig. 2.4A) and therefore resulted in over-expression of *GBP1* in the complemented strain (Fig. 2.4B). If our hypothesis that *GBP1* is a negative regulator of FB_1 biosynthesis is correct, over-expression of *GBP1* should have resulted in repression of FB_1 production. However, the FB_1 production and *FUM* gene expression analyses in wild type, *Δgbp1,* and *gbp1-comp* suggested that while deletion of *GBP1* is necessary for increased FB_1 production in *F. verticillioides*, over-expression of *GBP1*, at least at the level observed here, does not suppress FB_1 biosynthesis.

In conclusion, we identified and characterized a putative monomeric GTP-binding protein that negatively regulates *FUM* genes in *F. verticillioides*. Our data showed that *GBP1* is fumonisin-specific regulator; deletion of the gene did not cause developmental or morphological impairments, but resulted in de-repression of *FUM1* and *FUM8* that led to over-production of FB_1. Interestingly, over-expression of *GBP1* did not result in repression or inhibition of FB_1 biosynthesis. Such mechanisms, where regulatory genes function in a unilateral manner, can be found in other signaling pathways. One such example, coincidentally, is the *ZFR1*-mediated regulation of FB_1 biosynthesis in *F. verticillioides* (Flaherty and Woloshuk, 2004). *ZFR1* is a zinc binuclear cluster-type gene that is a positive regulator of FB_1 biosynthesis. Deletion of *ZFR1* resulted in a significant reduction of FB_1 production, and repression of *FUM1* and *FUM8* gene expression. However, over-expression of *ZFR1* did not lead to increased production of FB_1 but only restored the FB_1 level and *FUM* gene expression levels to

that of wild type. One possible explanation for such regulatory mechanism in *ZFR1* and *GBP1* is that additional, unidentified, factors other than *ZFR1* or *GBP1* are involved in transcriptional regulation of FB_1 biosynthesis. Also, a possibility exists that over-expression of *GBP1* transcript does not necessarily lead to the production of increased functional enzyme, potentially due to limiting post-translational modifications. Alternatively, wild-type levels of transcript may already be at saturating levels for this signal transduction.

Lastly, we can also hypothesize that *GBP1* controls FB_1-biosynthetic feedback mechanism in *F. verticillioides*. Such feedback mechanism may exist in *F. verticillioides* to protect itself from toxic secondary metabolites, as observed in other toxin-producing fungi, e. g., *Cercospora* species (Callahan *et al*., 1999; Chung *et al*., 1999). Functional genomics analyses, e. g., microarray experiments or proteome analysis, may provide further insight into the *GBP1*-mediated FB_1 regulation. Availability of the *F. verticillioides* genome sequence will facilitate these genomics approaches and lead to a better understanding of *GBP1*-mediated FB_1 regulation in *F. verticillioides*.

EXPERIMENTAL PROCEDURES

Fungal strain and culture media

The wild-type *F. verticillioides* strain 7600 (M3125; Fungal Genetics Stock Center, Kansas City, KS) was stored in 30 % glycerol at -80 ºC. The fungus was grown on V8 agar (200 mL of V8 juice per liter, 3 g of $CaCO_3$ per liter, and 20 g agar per liter) for inoculum. For genomic DNA extraction, the fungus was grown in YEPD medium (3 g yeast extract per liter, 10 g peptone per liter, 20 g dextrose per liter) on a rotary shaker

(150 rpm) at 24 ºC. For total RNA isolation, the fungus was grown in defined liquid medium (Shim and Woloshuk, 1999), cracked corn medium (Shim and Woloshuk, 2001), or potato dextrose broth (Difco Laboratories, Sparks, Maryland).

Transcription Profile Analysis

Oligonucleotide microarrays designed from the *F. verticillioides* EST library (Brown *et al.*, 2005) were created by NimbleGen Systems, Inc. (Madison, WI) (Butchko *et al*., manuscript in preparation). The NimbleGen arrays contain 12 24-nucleotide probes for each unique EST from the library**.** Samples of mRNA used for analysis were shipped on dry ice to NimbleGen for labeling, hybridization and relative quantification across individual chips. Sample mRNA was generated from five culture conditions of wild-type 7600: 1) defined liquid medium (GYAM) (Proctor *et al*., 2002) for 24 hours and 2) 120 hours, 3) whole maize kernels, 4) dissected maize germ tissue and 5) dissected maize endosperm tissue. Maize tissue was autoclaved prior to inoculation. Two biological replicates of each sample were analyzed. Expression profile data was returned from NimbleGen after being subjected to robust multi-array analysis (RMA) (Bolstad *et al*., 2003). To compare expression of ESTs from different chips, the average expression levels from the two replicates are reported relative to the expression of elongation factor-1-a (TC29728, Brown *et al.*, 2005). EF-1- α is constitutively expressed under all conditions we have investigated with the NimbleGen microarrays.

Nucleic acid isolation and analysis

Bacterial plasmid DNA was isolated with Wizard miniprep DNA purification system (Promega, Madison, WI). All primers used in this study are listed in Table 2.2.

Fungal genomic DNA was extracted using an OmniPrep Genomic DNA Extraction kit (G Biosciences, St. Louis, MO). Total RNA for northern analysis and QRT-PCR was prepared with Trizol reagent (Invitrogen, Carlsbad, CA) or with RNeasy plant mini kit (Qiagen, Valencia, CA) as per the manufacturers' protocols. For Southern analysis, genomic DNA samples (12 μg) were digested with *Eag*I, subjected to electrophoresis on a 1% agarose gel, and transferred to Nytran membrane (Amersham, Arlington Heights, IL) by standard procedures (Sambrook and Russell, 2001). For northern analysis, total RNA samples (15 μg) were subjected to electrophoresis on 1.2% denaturing agarose gel and transferred to Nytran membrane. Gels were stained with ethidium bromide to confirm the uniformity of RNA sample loading. The probes used in all hybridization experiments were ^{32}P-labeled with Prime-It random primer labeling kit (Stratagene, La Jolla, CA). DNA sequencing was performed at Gene Technologies Lab, Texas A&M University. BLAST algorithms were used to perform database searches (Altschul *et al*., 1997).

Isolation and sequencing of *GBP1*

A 200-bp EST (ft536_1_I17) encoding a putative GTP-binding protein was identified in the SSH cDNA library that was enriched for transcripts of *F. verticillioides fcc1* mutant (Shim and Woloshuk, 2001). A *F. verticillioides* genomic DNA library, constructed with Universal Genome Walker Kit (BD Biosciences, Palo Alto, CA), was used as a template to amplify DNA fragments containing a 1.7-kb downstream and a 3.0-kb upstream of *GBP1* EST. The outward primers FvGBP-DL and FvGBP-UL were designed based in the 200-bp *GBP1* EST. Primers AP1 and AP2 were provided in the

Kit. A 1.7-kb downstream DNA fragment from conserved region was obtained by primary and secondary PCR amplifications of a *Stu*I genome walker library. The DNA fragment was cloned into pGEMT-Easy vector (Promega, Valencia, CA) and the resulting plasmid was designated as pFVG202.

Table 2.2 Primers used in chapter II.

Number	Name	Primer sequence (5′ – 3′)
1	FvGBP-DL	CCGTCAGAGGCTTGTTGACATCCAGCACA
2	FvGBP-UL	GGGTGGCCTCAATATCACCAGCACTGTACC
3	AP1	GTAATACGACTCACTATAGGGC
4	AP2	ACTATAGGGCACGCGTGGT
5	FvGBPkpn1R	TTCCTCATGAGCATTGGTACCGGATCGGA[a]
6	FvGBPHind3F	GGCCATCTGGTTACAAAGCTTCATGTCAGAG
7	FvGBPEcoR1R	ATCCCAGTCTCAGTCGAATTCTGATCCGTT
8	FvGBPSac1F	GGTCAAGCCTTTGTGAGCTCGCATTTCAG
9	M13-F	TTGTAAAACGACGGCCAGTGA
10	M13-R	CAGGAAACAGCTATGACCATG
11	FvGBP-U10t	AAT*CATGGTCATAGCTGTTTCCTG*TTATGATCATGGGTGGCGCAGAGA[b]
12	FvGBP-D5	TGCTTCTGCTCGTTGACATC

[a] Underlined sequences represent the restriction sites used to clone the flanking regions for disruption vector construction.
[b] Italicized sequence of primer FvGBP-U10t represent complimentary sequence of M13-R primer used to fuse *GBP1* to geneticin-resistant marker.

Similarly, a 3.0-kb upstream fragment was obtained from a *Dra*I genome walker library and cloned into pCR® 2.1-TOPO® vector (Invitrogen, Carlsbad, CA). The resulting plasmid was designated as pFVG201. The middle 350-bp region between upstream and downstream regions was amplified from genomic DNA and cloned into

pGEMT-Easy vector. The resultant plasmid was named as pFVG203. Subsequently, a 3.9-kb genomic DNA, which contained *GBP1*, was amplified and sequenced.

Molecular characterization of *GBP1*

The *GBP1* disruption vector (pFVGDV) was created by insertion of 890-bp 5' and 870-bp 3' flanking regions of *GBP1* into pBP15 vector, which contains a hygromycin phosphotransferase (*HYG*) gene as selectable marker (Fig. 2.3B). The primers FvGBPkpn1R, FvGBPHind3F, FvGBPEcoR1R, and FvGBPSac1F were used to amplify the 5' and 3' flanking regions (Fig. 2.3A). Prior to transformation of *F. verticillioides*, the disruption vector was linearized with *Sac*I. Protoplasts of *F. verticillioides* were obtained and transformed as described previously (Shim and Woloshuk, 2001). Transformants were selected on regeneration medium (343 g sucrose per liter, 0.2 g yeast extract per liter, 10 g agar per liter) containing 150 μg per milliliter of hygromycin B (Calbiochem, La Jolla, CA). Hygromycin-resistant colonies were selected and screened for *GBP1* deletion by PCR (not shown) and Southern analysis (Fig. 2.4A).

The *GBP1* deletion mutant was complemented with wild-type *GBP1* gene that was fused to the geneticin resistant gene (*GEN*) (Flaherty *et al*., 2003). The complementation construct was made via single-joint PCR strategy (Yu *et al*., 2004). First, *GEN* was amplified with the primers M13-F and M13-R, and the full-length *GBP1* (including 960-bp 5' UTR and 1200-bp 3' UTR) was amplified with FvGBP-U10t and FvGBP-D5 (Fig. 2.3C). Subsequently, the two amplicons were mixed in a single tube and were joined by PCR without using any primers: the primer FvGBPU10t contains

complimentary sequence of M13-R primer at the 5' end of the *GBP1* fragment (Table 2.2). The joined PCR product was then used as template to generate fused complementation construct. The final complementation construct was amplified using Expand Long Polymerase (Roche, Indianapolis, IN). The *Δgbp1* protoplasts were generated and transformed with the complementation construct, and the transformants were selected by growth on regeneration medium containing 150 μg per milliliter of G-418 (Cellgro, Herndon, VA). The complemented strain (*gbp1-comp*) was identified by PCR and confirmed by Southern analysis (Fig. 2.4A). The expression level of *GBP1* in *gbp1-comp* was analyzed by northern hybridization (Fig. 2.4B).

PCR and Quantitative Real-Time (QRT)-PCR

All PCR reactions were performed in GeneAmp PCR system 9700 thermocycler (PE Applied Biosystems, Norwalk, Connecticut). PCR amplification of DNA was performed in 25 μL or 50 μL total volumes with *Taq* DNA polymerase (Promega). The PCR conditions were a 2 min of pre-denaturation at 94 ºC followed by 30 cycles of 30 s denaturation at 94 ºC, 30 s annealing at 55-57 ºC and 1-2 min extension at 72 ºC unless specified. All the QRT-PCR reactions were performed in Cepheid Smart Cycler System (Cepheid, Sunnyvale, CA) with QuantiTect SYBR Green RT-PCR kit (Qiagen). QRT-PCR reactions were carried out with 30 min of reverse transcription at 50 ºC followed by 15 min of pre-denaturation at 95 ºC and 35 cycles of 15 s of denaturation at 95 ºC, 30 s of annealing at 55 ºC and 30 s of extension at 72 ºC. The β-tubulin gene (*TUB2*) (GenBank U27303) expression was used as a reference.

Fumonisin B_1 analysis

F. verticillioides strains were grown in whole kernel cracked-corn medium (B73 line) for 10 days for FB_1 analysis. FB_1 was extracted overnight in 50% acetonitrile in water and the extracts were filtered through C18 solid phase extraction columns (J&W Scientific, Folsom, CA) prior to high performance liquid chromatography (HPLC) analysis (Shim and Woloshuk 1999). The HPLC analyses were performed at Purdue University (West Lafayette, IN) as described previously (Shim and Woloshuk, 1999: Flaherty and Woloshuk, 2004). Data were analyzed using analysis of variance (ANOVA) and the significant means were separated by least significant difference (LSD) method.

Nucleotide sequence accession number

The nucleotide sequence of *GBP1* and the predicted amino acid sequence were submitted to GenBank (Accession Number AY862193).

CHAPTER III

FUSARIUM VERTICILLIOIDES GBB1, A GENE ENCODING HETEROTRIMERIC G PROTEIN β SUBUNIT, IS ASSOCIATED WITH FUMONISIN B_1 BIOSYNTHESIS AND HYPHAL DEVELOPMENT BUT NOT WITH FUNGAL VIRULENCE*

SUMMARY

Fusarium verticillioides (Sacc.) Nirenberg (teleomorph *Gibberella moniliformis* Wineland) is a maize pathogen that causes ear rots and stalk rots. Also, the fungus produces a group of mycotoxins, fumonisins, on infected ears, which cause considerable health and economic concerns for humans and animals worldwide. To date, our understanding of the molecular mechanisms associated with fungal virulence and fumonisin biosynthesis in *F. verticillioides* is limited. In this study, *GBB1*, a gene encoding a putative β subunit of a heterotrimeric G protein, was disrupted and the effects on fumonisin biosynthesis and virulence were evaluated. A *GBB1* deletion mutant (*Δgbb1*) showed no significant differences in radial growth and mycelial mass but produced significantly less fumonisin B_1 (FB_1) than its wild-type progenitor. HPLC analysis showed that *Δgbb1* produced less than 10 ppm FB_1 while the wild type produced over 140 ppm when strains were grown on cracked corn kernels. Reduced

---

* This chapter is reprinted with permission from "*Fusarium verticillioides GBB1*, a gene encoding heterotrimeric G protein β subunit, is associated with fumonisin B_1 biosynthesis and hyphal development but not with fungal virulence" by Sagaram, U.S. and Shim, W.B. (2006) *Molecular Plant Pathol.* (Accepted).

expression of the key FB_1 biosynthetic genes, *FUM1* and *FUM8*, in *Δgbb1* provides further evidence that *GBB1* is involved in FB_1 regulation. Stalk rot virulence, as measured by mean lesion length and by area, was not significantly different in *Δgbb1* compared to the wild type, suggesting that *GBB1* does not regulate virulence in *F. verticillioides*. Developmentally, hyphae of *Δgbb1* do not deviate from the original axis of polarity established upon germ tube emergence in contrast to wild type hyphae that meander on and off axis as they grow. Complementation of *Δgbb1* with *GBB1* restored FB_1 production and hyphal growth to wild-type. The results of this study demonstrate that heterotrimeric G protein β subunit plays an important role in regulation of FB_1 biosynthesis and hyphal growth, but not virulence in *F. verticillioides*.

INTRODUCTION

Fusarium verticillioides (Sacc.) Nirenberg (teleomorph *Gibberella moniliformis* Wineland) is a maize pathogen responsible for ear rots and stalk rots worldwide (Munkvold and Desjardins, 1997). Fusarium stalk rot of maize is known to be prevalent in the warmer, drier regions (White, 1999). Losses due to stalk rot come in several different forms including stalk breakage, lodging, premature death of the plant, and the interruption of the normal grain filling process. It is generally perceived that when maize experiences abiotic stress, particularly at the end of the growing season, pathogens take advantage of the weak host and colonize the vulnerable stalk tissues (Dodd, 1980; Michaelson, 1957; White, 1999). Significantly, *F. verticillioides* can exist as an asymptomatic, intercellular endophyte (Bacon and Hinton, 1996). Fusarium ear rot can be found wherever maize is grown and was considered to be of minor importance until

the disease was linked to fumonisins, a group of mycotoxins with cancer-promoting activities.

Fumonisins are polyketide-derived mycotoxins that are structurally similar to sphinganine (a natural precursor of sphingolipids), and can inhibit the activity of sphingosine N-acyltransferase thereby disrupting the sphingolipid biosynthetic pathway (Merrill et al., 2001; Wang et al., 1991). Most recently, reports describing the link between fumonisin-contaminated maize and prevalent neural tube defect (NTD) incidents on the Texas-Mexico border demonstrate that fumonisins still pose a threat to food safety (Missmer et al., 2006). Due to its significance, the US Food and Drug Administration has established guidelines for maximum allowable fumonisin content in food and feed (Park and Troxell, 2002). Fumonisins are synthesized by a cluster of 15 co-regulated genes, designated as the *FUM* cluster (Proctor et al., 2003). Among the genes in the *FUM* cluster are *FUM1*, encoding a polyketide synthase, *FUM6*, encoding a cytochrome P450 monoxygenase, and *FUM8*, encoding an aminotransferase. Each is critical for fumonisin biosynthesis (Proctor et al., 1999; Seo et al., 2001). Interestingly, the *FUM* cluster, unlike other fungal secondary metabolite gene clusters, does not contain a pathway-specific regulatory gene (Brown et al., 1996, Kennedy et al., 1999, Proctor et al., 2003). Rather, fumonisin biosynthesis is regulated by several environmental factors, such as available nitrogen, ambient pH and maize kernel constitution environment (Bluhm and Woloshuk, 2005; Keller et al., 1997; Shim and Woloshuk, 1999; Shim et al., 2003). FB_1 biosynthesis has also been linked to several regulatory genes not physically linked to the *FUM* cluster (Bluhm and Woloshuk, 2005;

Flaherty et. al 2003; Flaherty and Woloshuk, 2004; Shim and Woloshuk, 2001). Since this diversity of regulatory genes and environmental factors appear to exert their effects independently, additional factors or genes associated with fumonisin regulation probably remain to be identified (Flaherty and Woloshuk, 2004; Shim and Woloshuk, 2001).

To expand our understanding of the molecular mechanisms associated with fungal virulence and regulation of fumonisin biosynthesis in *F. verticillioides*, we targeted β-subunit gene of heterotrimeric G protein complex for molecular characterization. Heterotrimeric ($\alpha\beta\gamma$) G proteins are highly conserved and enable eukaryotes to recognize and respond to external signals (Gilman, 1987; Hamm and Gilchrist, 1996; Morris and Malbon, 1999). G proteins transduce signal from cell surface receptors to cytoplasmic effector proteins, which lead to the activation of regulatory cascades with subsequent changes in gene expression and cellular function (Gilman, 1987). In filamentous fungi, G protein complexes are associated with virulence and secondary metabolism (Bölker, 1998; Lengeler et al., 2000, Yu and Keller, 2005). Heterotrimeric G protein signaling pathways associated with secondary metabolism in fungi have been extensively studied in *Aspergillus* species (Calvo et al., 2002; Yu, 2006; Yu and Keller, 2005). *Aspergillus nidulans* and *A. flavus* produce sterigmatocystin (ST) and aflatoxin (AF), respectively, and the gene clusters responsible for ST and AF biosynthesis are well characterized. A pathway-specific transcription factor, *aflR*, is present in the ST and AF biosynthetic clusters, and it has been shown that *aflR* controls the regulation of ST and AF biosynthetic genes (Brown et al., 1996; Woloshuk et al., 1994). Significantly, the heterotrimeric G protein subunits and their

interacting proteins in *A. nidulans* are involved in eliciting ST biosynthesis and asexual/sexual development (Chang et al., 2004; Hicks et al., 1997; Rosen et al., 1999; Seo et al., 2003; Seo et al., 2005; Yu, 2006). In particular, Gβ and Gγ subunits (SfaD and GpgA) have been shown to be essential for ST biosynthesis and vegetative growth (Seo and Yu, 2006). In contrast, it has been demonstrated that Gα subunit (FadA) negatively regulates *aflR*, ultimately blocking ST biosynthesis, but promoting hyphal growth (Hicks et al., 1997). It is important to note, however, that heterotrimeric G proteins in fungi play diverse roles, including mating, pathogenicity, and development, by regulating a variety of downstream signaling pathways (Bölker, 1998; Choi et al., 1995; Delgado-Jarana et al., 2005; Nishimura et al., 2003). Surprisingly, any role of heterotrimeric G protein signaling in *F. verticillioides* remains largely unknown to date.

Our objective in this study was to test the hypothesis that *GBB1*, a gene encoding a heterotrimeric G protein β subunit in *F. verticillioides*, has a role in fumonisin biosynthesis and *F. verticillioides* development. We used a homologous recombination strategy to inactivate *GBB1* in a wild-type strain and showed that gene deletion results in severely impaired fumonisin production and aberrant hyphal growth.

RESULTS

Molecular characterization of *GBB1*

To identify the *F. verticillioides* G β subunit, we selected heterotrimeric G protein β subunit protein sequences from *Fusarium oxysporum* (Fgb1) and *A. nidulans* (SfaD), and performed a comparison against the *F. verticillioides* 7600 strain genome sequence database (*Fusarium verticillioides* Sequencing Project. Broad Institute of

Harvard and MIT [http://www.broad.mit.edu]) using the tblastN algorithm (Altschul et al., 1997). The best match (E value = e-174) was identified on supercontig 3.14 (Locus: FVEG_10291.3). The nucleotide sequence was obtained and the gene was named *GBB1*. Analysis of the sequence determined that *GBB1* gene is 1,412-bp long with four putative introns and encodes a putative protein (Gbb1) of 359 amino acids. The nucleotide sequence (DQ457053) and the conceptually translated protein sequence (ABE67098) were submitted to GenBank. Gbb1 is highly similar to the protein sequences of heterotrimeric G protein β subunits of several filamentous fungi: *F. oxysporum f. sp. lycopersici* (AAO91808, 99% identity), *Magnaporthe grisea* (BAC01165, 95% identity), *Cryphonectria parasitica* (AAC49838, 93% identity), *F. graminearum* (EAA72306, 91% identity), and *Emericella nidulans* (AAC33436, 83% identity). Conserved domain searches revealed that the protein contains a WD-40 repeat structure, characteristic of β subunits of heterotrimeric G proteins (Gilman, 1987).

Role of *GBB1* in FB_1 biosynthesis and *FUM* gene regulation

We generated a *F. verticillioides GBB1* knock-out mutant strain (*Δgbb1*) by using a double homologous recombination strategy (Fig. 3.1B). Eighty-six hygromycin-resistant transformants were selected and screened for homologous recombination insertion at the native *GBB1* locus event by PCR (data not shown) and subsequently verified by Southern analysis (Fig. 3.1D). The wild-type strain produced a 7-kb band whereas the deletion mutant produced a 4-kb band, indicating that *GBB1* gene was replaced with the disruption construct (Fig. 3.1D). Northern analysis showed that *GBB1*

expression was completely abolished in *Δgbb1*, while the wild-type produced a 2.2-kb transcript (Fig. 3.1E).

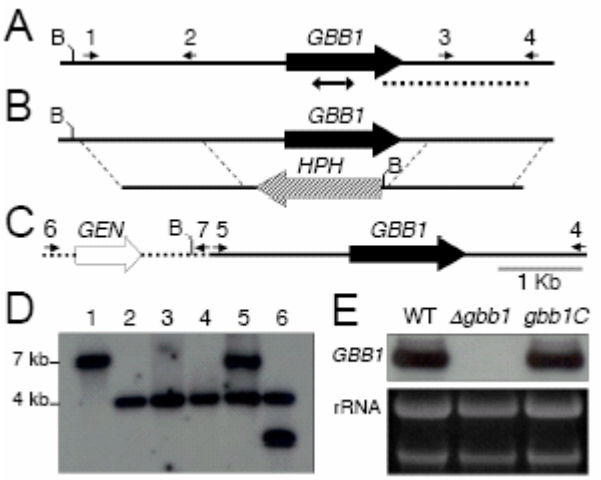

Fig. 3.1 Description of *F. verticillioides GBB1* locus: deletion and complementation strategies. **(A)** Schematic representation of the 5.8-kb *GBB1* locus in the wild-type genome. The dashed line and double arrow represent the genomic regions used as probes for Southern and northern blots, respectively. Primer pairs 1-2 and 3-4 were used to amplify 5′ flanking and 3′ flanking DNA, respectively, for the disruption construct. B: *BamH*I restriction site. **(B)** Targeted replacement of *GBB1* with hygromycin phosphotransferase gene (*HPH*) via double homologous integration event. **(C)** The complementation construct was generated by fusing the geneticin-resistant gene (*GEN*) to *GBB1* gene by single joint PCR. Primer pair 4-5 was used to amplify the *GBB1* genomic DNA (4.8-kb) for the complementation construct. Primer pair 6-7 was used to amplify *GEN*. **(D)** Southern analysis of transformants. Genomic DNA (10 μg) was digested with *BamHI*, subjected to electrophoresis on a 1% agarose gel. The DNA was transferred on to nylon membrane and probed with ^{32}P-labeled DNA fragment amplified from genomic DNA with primers FvGBB-F4+FvGBB-R8 (Table 3.1). Molecular sizes are indicated on the left. 1=WT, 2=*Δgbb1*, 3-6=Complemented strains (5=*gbb1C*). **(E)** Northern analysis of *GBB1* expression in wild type (WT), deletion mutant (*Δgbb1*) and complement (*gbb1C*) strains. Total RNA (12 μg) extracted from fungal cultures grown in defined liquid medium pH 4.5 for 7 days was subjected to electrophoresis on a 1.2% denaturing agarose gel, transferred on to a nylon membrane and probed with a ^{32}P-labeled 520-bp *GBB1* genomic DNA fragment amplified from genomic DNA with primers FvGBB-F8+FvGBB-R4 (Table 3.1). WT and *gbb1C* produced a 2.2-kb transcript while *Δgbb1* did not produce any detectable transcript. Ribosomal RNA stained with ethidium bromide is shown as equal loading control.

No significant differences in radial growth and mycelial mass (dry weight) were observed between the wild-type and *Δgbb1* strains when grown on agar plates (PDA and V8 juice agar) and YEPD broth, respectively (data not shown). Wild-type and *Δgbb1* were grown on cracked corn kernels to determine the effect of the mutation on FB_1 biosynthesis. *Δgbb1* grew similarly to its wild-type progenitor on cracked maize kernels (B73 line) (data not shown) but the TLC (Fig. 3.2A) and HPLC (Fig. 3.2B) analyses showed significantly lower levels (10 ppm) of FB_1 when compared to the wild-type strain, which consistently produced high levels (140 ppm) of FB_1. The expression levels of *FUM1* and *FUM8* are concomitant to FB_1 biosynthesis in *F. verticillioides* (Flaherty et al., 2003; Flaherty and Woloshuk, 2004; Shim and Woloshuk, 2001). Northern blot analysis indicated that expression of both *FUM1* and *FUM8* was non-detectable in *Δgbb1* strain, suggesting that functional *GBB1* is required for proper transcription of *FUM* genes in *F. verticillioides* (Fig. 3.2C).

To verify that the reduced-FB_1 phenotype in *Δgbb1* strain is due to the targeted deletion of *GBB1*, we complemented the mutant with a wild-type copy of *GBB1* (Fig. 3.1C). Geneticin-resistant transformants were analyzed for intact *GBB1* gene by PCR (data not shown) and by Southern analysis (Fig. 3.1D). The complemented strain *gbb1C* contains a 7-kb *BamH*I fragment, indicative of an ectopic insertion of the complementation construct, in addition to the 4-kb fragment containing the deletion cassette. The expression level of *GBB1* in *gbb1C* assessed by northern blot was equal to that of wild type, showing that gene expression was fully restored in *gbb1C* (Fig. 3.1E). In addition, FB_1 analysis showed that FB_1 levels were restored in the complemented

strain (Fig. 3.2A and 3.2B). Reversion of expression levels of *FUM1* and *FUM8* in *gbb1C* provides further evidence of *GBB1* involvement in transcriptional regulation *FUM* cluster genes (Fig. 3.2C).

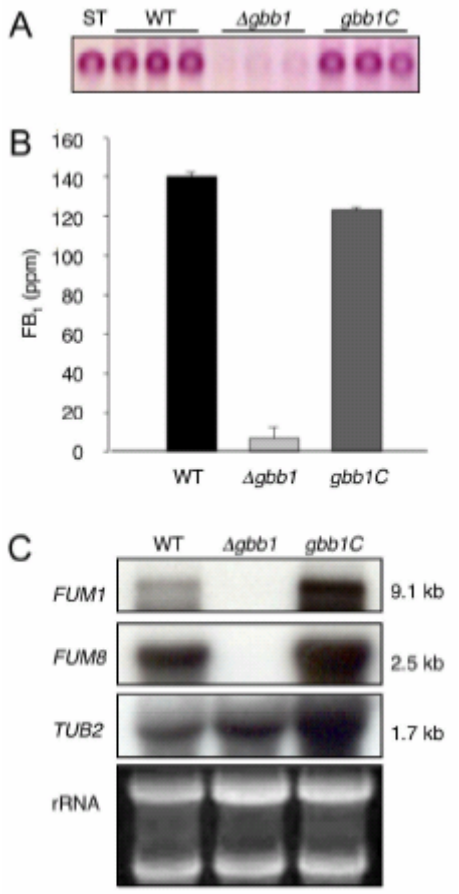

Fig. 3.2 Fumonisin (FB_1) analysis and expression of *FUM* genes in wild type (WT), deletion mutant (*Δgbb1*), and complement strain (*gbb1C*). **(A)** A thin layer chromatography plate (TLC) showing the FB_1 production in wild-type, *Δgbb1*, and *gbb1C* strains. After development, the TLC plate was sprayed with 0.05% para-anisaldehyde solution and charred. Presence of FB_1 is indicated by purple color reaction. Three replicates of each strain are shown. The experiment was repeated two times with similar results. ST = FB_1 standard (50 ppm). **(B)** HPLC quantification of fumonisin production in wild-type, *Δgbb1*, and *gbb1C* strains. Results are means of three replicates **(C)** Northern analysis of *FUM1* and *FUM8* gene expression in wild-type (WT), deletion mutant (*Δgbb1*) and complement (*gbb1C*) strains. Total RNA (12 μg) extracted from fungal cultures grown in bovine serum albumin liquid (BSAL) pH 6.0 for 7 days was subjected to electrophoresis on a 1.2% denaturing agarose gel, transferred on to a nylon membrane and hybridized with ^{32}P-labeled gene-specific probes. Target genes and transcript sizes are indicated on the left and right, respectively. Ribosomal RNA stained with ethidium bromide is shown to verify equal loading control.

Impact of *GBB1* on microconidia production, pathogenicity and sexual development

The deletion mutant produced significantly ($p>0.05$) fewer microconidia than wild type on both V8 agar medium (wild type $119\pm11\times10^6$ to *Δgbb1* $64\pm6\times10^6$ per mL) and CLA (wild type $58\pm6\times10^5$ to *Δgbb1* $24\pm2\times10^5$ per mL). Complementation of the deletion mutant with the wild type copy of *GBB1* restored conidia production in *Δgbb1* to that of wild type in both V8 agar ($123\pm10\times10^6$ per mL) and CLA ($54\pm10\times10^5$ per mL).

In maize stalk rot assays, both the wild type and *gbb1* strains successfully colonized and rotted the tissue. While the *Δgbb1* strain showed slightly reduced fungal colonization and stalk rotting when compared to its wild-type progenitor, the difference was not statistically significant ($p>0.05$) (Fig. 3.3). The complemented strain *gbb1C* showed comparable virulence to wild type and *Δgbb1* strains (not shown). The mean lesion length of *Δgbb1* (3.5±0.3 cm) is not significantly different ($p>0.05$) from wild type (3.7±0.7 cm) indicating that *GBB1* is not a key genetic factor associated with *F. verticillioides* stalk rot virulence.

We also demonstrated that the *GBB1* mutation had no impact on sexual development of *F. verticillioides*. Wild-type, *Δgbb1*, and *gbb1C* were crossed to the opposite mating type wild-type strain 7598, and after 7 days of incubation at 25°C with a 14-h light and 10-h dark cycle we observed the development of perithecia (Fig. 3.4). After 14 days of incubation, we observed ascospore development in mature perithecia verifying that *Δgbb1* is fertile (not shown).

Fig. 3.3 Comparison of stalk rot virulence in wild type (WT), deletion mutant (*Δgbb1*). Eight-week-old maize stalks were inoculated with 10^6 spores of wild-type (WT) and deletion mutant *(Δgbb1*) strains at inter-nodal region and incubated in a growth chamber for 14 days at 25 °C. Subsequently, the maize stalks were split longitudinally to assay the severity of stalk rot. Four independent biological repetitions were performed, and two biological repetitions were selected and shown in the figure. Maize stalk inoculated with sterile water is shown as the negative control.

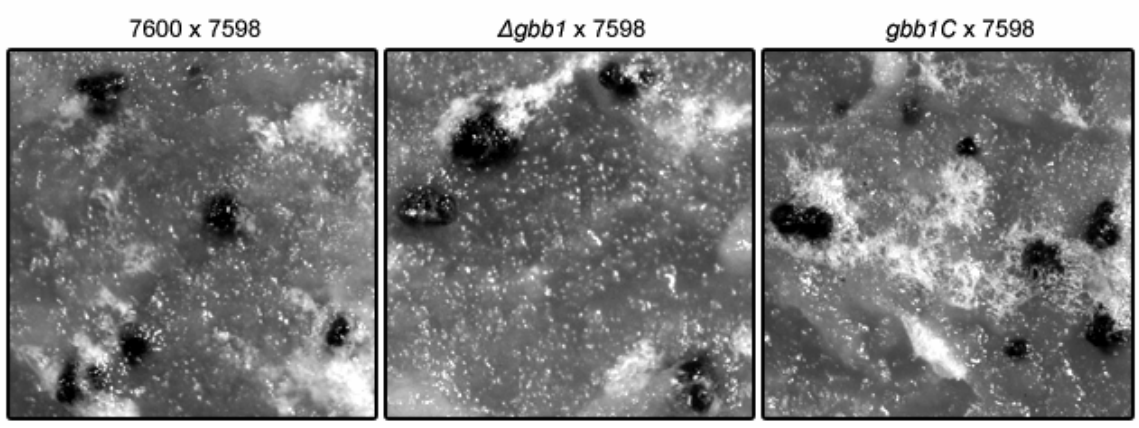

Fig. 3.4 Effect of *GBB1* deletion on sexual development of *F. verticillioides***.** Sexual crosses were performed on carrot agar as explained in experimental procedures. The Petri plates were photographed 14 days after incubation. The mycelium from the surface of the plates was scraped to visualize the perithecia embedded into agar.

GBB1 and *F. verticillioides* hyphae development

Δgbb1 cultures produced germ tubes and hyphae with an undeviating, straight axis of polarity relative to the point of germ tube or branch emergence (Fig. 3.5, 3.6). Establishment of hyphal polarity via germ tube emergence was similar for both wild type

and *Δgbb1* (Fig. 3.5), but by 60 min after emergence the wild type germ tube consistently deviated noticeably from the original axis of growth while *Δgbb1* consistently continued growth with minimal deviation from its axis of polarity. Growth patterns in *gbb1C* were indistinguishable from wild type (Fig. 3.6). Older germlings and mature hyphae of wild type and *gbb1C* strains continued this pattern of development (Fig. 3.6). Lateral branches of *Δgbb1* established growth off axis from their parent hypha but nonetheless continued their undeviated straight growth once they emerged (Fig. 3.6C). This hyphal growth morphology was observed in *Δgbb1* cultures grown in both solid and liquid medium (Fig. 3.6).

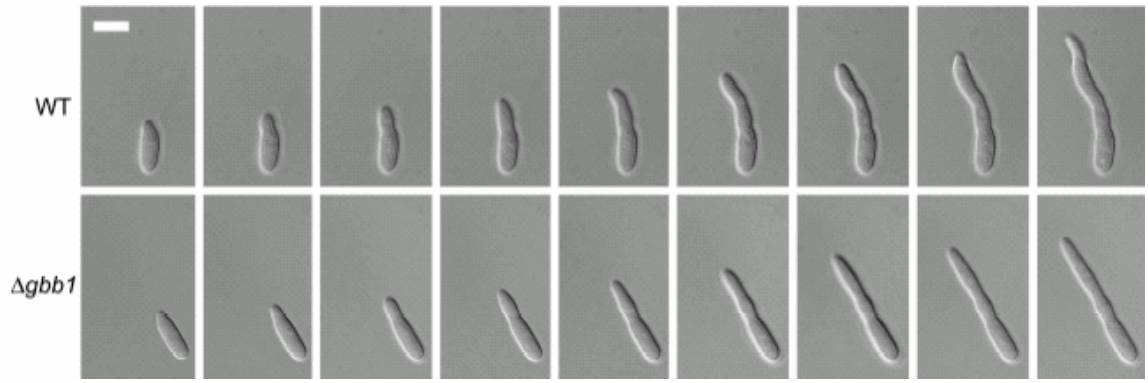

Fig. 3.5 Time-lapsed conidium germination of wild-type and *Δgbb1* strains. The first panel demonstrates the first detectable emergence of the germ tube. Each subsequent panel represents exactly 20 minutes after the panel to its left. Upper panel = wild type. Lower panel = *Δgbb1*. Scale bar = 10 μm

DISCUSSION

Heterotrimeric G protein signaling complexes typically consist of α, β, and γ subunits, G-protein-coupled receptors, and respond to a variety of effectors (Gilman,

1987; Hamm and Gilchrist, 1996). In filamentous fungi, these elements are involved in the regulation of a variety of important cellular functions, including vegetative growth, sexual development, pathogenicity, and secondary metabolite production by transducing an external signal into changes in gene expression (Bolker, 1998; Jain et al., 2005; Liu and Dean, 1997, Tag et al., 2000).

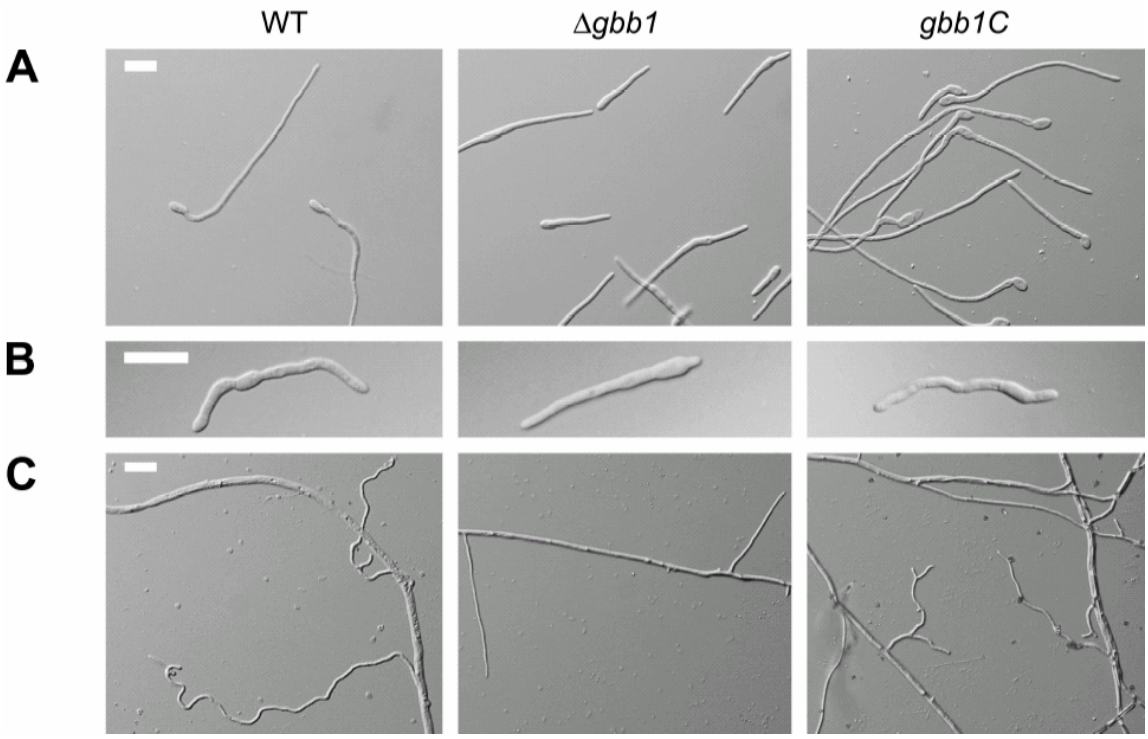

Fig. 3.6 Effect of *GBB1* mutation on colony hyphal development. (A) Microconidia of the designated strains were allowed to germinate in 0.5× potato dextrose broth for 15 hours at 23°C. Scale bar = 20 µm. (B) A single conidial germling of each strain is shown. Scale bar = 20 µm. (C) Fungal strains were spot inoculated on an agar plug and a Riddell mount was observed. Pictures of hyphal development were obtained 4 days after inoculation. Scale bar = 20 µm. In all panels it should be noted that the *Δgbb1* strain produces hyphae that maintain undeviating straight hyphal growth. Both the wild type and *gbb1C* strains exhibit a more random, meandering growth habit, as if the cells are responding to or searching for an external signal.

We selected *GBB1,* which encodes a Gβ subunit, for molecular genetic characterization to investigate the role of the heterotrimeric G protein complex in *F. verticillioides*, particularly in fumonisin biosynthesis. Our data suggest that *GBB1* positively regulates transcription of *FUM* genes and is necessary for biosynthesis of FB_1 but not for fungal virulence. The finding that *GBB1* influences FB_1 regulation in *F. verticillioides* is similar to ST production in *A. nidulans* where Gβ (SfaD) plays an essential role (Seo and Yu, 2006). SfaD also influences *aflR* expression and interacts with other components of the heterotrimeric G protein complex to control the complicated regulatory mechanism associated with ST production (Seo and Yu, 2006).

Virulence is another phenotype that is influenced by Gβ subunit genes. Studies conducted in several plant pathogenic fungi demonstrated that Gβ deletion mutants were severely impaired in virulence compared to their wild-type progenitors (Delgado-Jarana et al., 2005; Ganem et al., 2004; Jain et al., 2003). In *C. heterostrophus* and *M. grisea,* deletion of Gβ subunit encoding genes resulted in lack of visible lesions when inoculated on corn and rice leaves, respectively (Ganem et al., 2004; Nishimura et al., 2003). The mutants were defective in producing functional appressoria (fungal infection structures), which explains the reason for loss of virulence in these mutants. In *F. oxysporum*, deletion of *FGB1*, a Gβ subunit gene, resulted in reduced wilting of cucumber host plants (Jain et al., 2003). The authors suggested that the reduced pathogenicity observed in this mutant might be due to defective penetration or colonization. Surprisingly and in contrast to our hypothesis, the *Δgbb1* mutant of *F. verticillioides* did not show significant difference in stalk rot virulence when compared to its wild-type progenitor.

From studies performed in *C. heterostrophus* and *M. grisea*, one can argue that Gβ subunit gene has a role in development of infection structures and thus impacts fungal virulence (Ganem et al., 2004; Nishimura et al., 2003). Perhaps that is why *F. verticillioides*, which does not produce a specialized infection structure, is not affected in virulence. However, this proposal falls short of the explanation why we observe contradictory virulence assay results in *F. verticillioides* and *F. oxysporum*, which also does not produce a specialized infection structure. But we can also reason that the molecular and physiological mechanisms of virulence in the two *Fusarium* species, one a rot pathogen and the other a wilt pathogen, are quite different, leading to divergent G β functions in the two *Fusarium* species. Further characterization of fungal colonization in combination with cytology of the infection process in *Fusarium* species is needed to test this hypothesis.

Our results also indicated that *GBB1* regulates conidiation and hyphal development in *F. verticillioides*. The Gβ subunit has been shown to be a positive regulator of conidiation in several filamentous fungi. Targeted disruption of the Gβ subunit encoding gene caused suppression of conidia production in *F. oxysporum* (Jain et al., 2005), *C. parasitica* (Kashahara and Nuss, 1997), *Neurospora crassa* (Krystofova and Borkovich, 2005; Yang et al., 2002), *M. grisea* (Nishimura et al., 2003), and *Cochliobolus heterostrophus* (Ganem et al., 2004). In contrast, deletion of the Gβ subunit-encoding gene resulted in hyperactive sporulation in *A. nidulans* (Rosen et al., 1999). In *F. verticillioides,* we observed a significant reduction in microconidia production when grown on V8 agar and CLA. Particularly, we observed fewer

microconidial chains produced in *Δgbb1* mutants when grown on CLA (data not shown) and KCL agar (Fig. 3.7). Therefore, we hypothesize that *GBB1* is associated with conidiophore development and may ultimately influence conidia production. One developmental phenotype associated with a functional Gβ subunit is the preponderance of straight hyphal growth along the axis of polarity of the hyphal cell in Gβ deletion strains (Fig. 3.6).

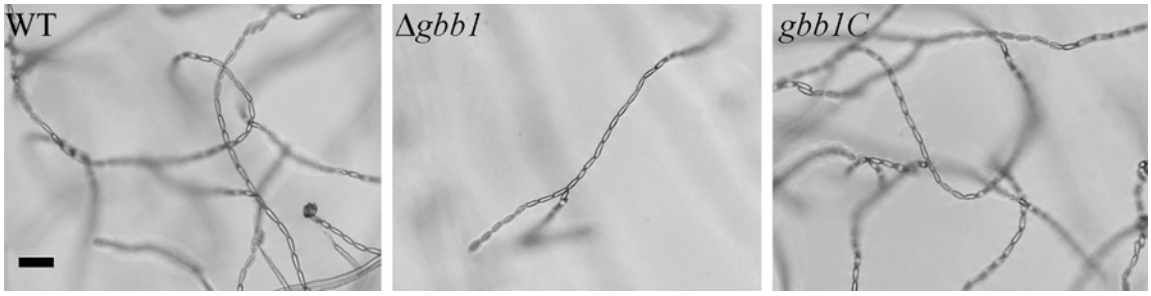

Fig. 3.7 Microconidial chains of wild-type and mutant strains. Fungal strains were spot inoculated on KCL medium and allowed to grow for 8 days. The Δ*gbb1* strain produces significantly fewer microconidia than does wild type or *gbb1C*. WT = wild-type, Δ*gbb1* = deletion mutant, and *gbb1C* = complement strain. Scale bar = 10 μm.

Similar observations have been made previously in Gβ disruption mutants of other filamentous fungi including: *F. oxysporum* (Delgado-Jarana et al., 2005), *C. heterostrophus* (Ganem et al., 2004), and *C. parasitica* (Kasahara and Nuss, 1997). Our study further demonstrates that the straight hyphal phenotype starts from germination and continues during hyphal branching and growth in liquid as well as solid media. Delgado-Jarana et. al. (2005) suggested that the α and β subunits may inhibit apical growth and cell elongation by triggering cAMP-PKA pathway, which results in induction of cell differentiation and conidiation. We agree with Ganem et. al. (2004)

who asserts that these cells are still following their normal growth pattern of polarized hyphal extension and therefore this should not be considered a cell polarization phenotype *per se*. Instead, it appears that the wild type cells are either searching for or responding to an external stimulus that causes the cell to make minor adjustments to its axis of polarity while growing. In the Gβ deletion strain the cells are not able to sense this stimulus and therefore are not triggered to make these minor adjustments to their growth direction. The mechanism of this phenotype remains to be elucidated, but it is clear that Gβ protein plays some role in modulating the main axis of polarity in hyphae.

A complex but intriguing aspect of G protein signaling in filamentous fungi is that it modulates secondary metabolism in close association with development, e.g. *A nidulans* and *F. graminearum* (Calvo et al., 2002; Yu and Keller, 2005). Our study re-affirms the existence of a link between secondary metabolism and conidiation in filamentous fungi. However, it is important to note that G proteins are not the sole, direct regulators of either secondary metabolism or conidiation. For example, deletion of *GBB1* did not lead to complete elimination of FB_1 production (Fig. 3.2B) and *FUM1* and *FUM8* gene expression when grown in DL medium pH 6.0 (data not shown). Furthermore, deletion of the Gβ subunit-encoding gene in *F. verticillioides* and Gβ subunit, and also an α subunit, in *F. oxysporum* did not lead to complete abolition of conidiation (Jain et al., 2002; Jain et al., 2003). Even though G proteins play an important role in the regulation of secondary metabolism and asexual/sexual development, the conservation of the regulatory function in filamentous fungi is far from certain. Based on our sexual cross studies, it is clear that *GBB1* is not essential for

perithecia development and ascospore formation in *F. verticillioides*. This is in contrast to the role *sfaD* plays in *A. nidulans* sexual development (Rosen at al., 1999; Seo and Yu, 2006). Our comparative protein sequence analysis via BLASTP and CLUSTALW (Thompson et al., 1994) showed that Gbb1 shares high similarity to Gβ subunits in several filamentous fungi, however the similarity in protein sequences does not always imply similarity in function. For example, in *A. nidulans* and *F. verticillioides*, the Gβ subunits both positively regulate production of polyketide-derived mycotoxins (ST and FB_1), but have contradictory effects on conidiation (Rosen et al., 1999; Seo and Yu, 2006, this study). Similar regulation of toxin might be due to the analogous nature of downstream components to Gβ in the two fungi. In contrast, the downstream components of conidiation may function in a dissimilar fashion which may explain why we observe opposing phenotypes. Functional variation of Gβ-interacting proteins is also critical for downstream gene signaling. For instance, the Gα gene FadA, which serves as a negative regulator of mycotoxin (ST) production in *A. nidulans* and *A. parasiticus*, was introduced into *F. sporotrichioides* and worked as a positive regulator of trichothecene biosynthesis (Tag at al., 2000). In addition, FadA regulates ST and penicillin (PN) production in an opposite manner (Hicks et al., 1997). These examples suggest multiple ways in which G protein complexes can influence critical biological functions.

Even though the characterization of the Gβ subunit encoding gene in *F. verticillioides* provided new insight into FB_1 regulation, our complete understanding of G protein signaling in FB_1 regulation is far from complete. In *F. verticillioides*, the G

protein complex and the signaling pathways are less clearly defined. Components of the heterotrimeric G protein complex have not been characterized and no putative transcription factor similar to *A. nidulans* AflR that may regulate *FUM* genes in *F. verticillioides* has been identified. The significant information available about G protein involvement in mycotoxin production in *A. nidulans* can not be applied in parallel to *F. verticillioides* since the signaling mechanism may not be conserved. Our Southern analysis showed that a single copy of *GBB1* is present in the *F. verticillioides* genome and this was confirmed via a search of genome database (http://www.broad.mit.edu). We used the protein and nucleotide sequences of Gα and Gγ subunits from *F. graminearum* and *A. nidulans* to query *F. verticillioides* genome and identified three putative Gα genes and a Gγ gene (data not shown). One of the putative Gα genes in *F. verticillioides* is orthologous to *A. nidulans fadA* and *M. grisea MAGB*. The second Gα gene is orthologous to *A. nidulans ganB*, but the last Gα have no functionally characterized orthologs in other fungi. The Gγ gene is similar to *A. nidulans gpgA* (Seo et al., 2005). As we have discussed earlier, these G protein subunits play important roles in secondary metabolism and fungal development in *A. nidulans* (Chang et al., 2004; Hicks et al., 1997; Seo et al., 2005). But once again, sequence similarity does not always imply similarity in function. For example, FadA, a negative regulator of ST production in *A. nidulans*, operates in an opposite manner when regulating penicillin and trichothecene production (Hicks et al., 1997; Tag et al., 2000). Therefore, molecular characterization of individual G protein subunits and other associated factors like RGS

genes is critical for better understanding the role of heterotrimeric G protein complex in FB_1 biosynthesis, conidiation and hyphal development in *F. verticillioides*.

EXPERIMENTAL PROCEDURES

Fungal strains and media

The wild-type *F. verticillioides* strain 7600 (Fungal Genetics Stock Center, University of Missouri-Kansas City, Kansas City, MO) was stored in 30 % glycerol at -80 ºC. For inoculum, the fungus was grown on V8 juice agar (200 mL of V8 juice, 3 g of $CaCO_3$, and 20 g agar per liter). For genomic DNA extraction, the fungus was grown in YEPD medium (3 g of yeast extract, 10 g of peptone, and 20 g of dextrose per liter) on a rotary shaker (150 rpm) at 25 °C. For RNA isolation, the fungus was grown in defined liquid (DL) medium, pH 4.5 or 6.0 (1 g $NH_4H_2PO_4$, 40 g sucrose, 3 g KH_2PO_4, 2 g $MgSO_4 \cdot 7H_2O$, and 5 g NaCl per liter) or bovine serum albumin liquid (BSAL) pH 6.0 (1 g BSA, 40 g sucrose, 3 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, and 5 g NaCl per liter) (Shim and Woloshuk, 2001) under shaking conditions (150 rpm) at 25 °C. To obtain hyphal growth photos, the fungal strains were grown using the Riddell mount protocol (Riddell, 1950). For spore germination studies, incubation was in 0.5× potato dextrose broth (PDB) (Difco, Sparks, MD) without agitation. For microconidia counts, 10^4 microconidia were inoculated at the center of V8 agar or carnation leaf agar (CLA) plate (Nelson et al., 1983) and allowed to grow for 9 and 14 days, respectively. The spores were harvested in 0.1% triton and counted using a hemacytometer. The spore suspension from V8 agar plates were passed through 8 layers of cheese cloth to eliminate mycelia which might obstruct spore counting.

Nucleic acid manipulation

Bacterial plasmid DNA was isolated with the Wizard miniprep DNA purification system (Promega, Madison, WI). Fungal Genomic DNA was extracted as described previously (Shim and Woloshuk, 2001). Total RNA was extracted with the RNeasy plant mini kit (Qiagen, Valencia, CA) following the manufacturer's protocol. Southern and northern analyses were performed following standard procedures (Sambrook and Russell, 2001). The probes used in all hybridization experiments were ^{32}P-labeled with Prime-It random primer labeling kit (Stratagene, La Jolla, CA). The primers used in this study are listed in Table 3.1.

Table 3.1 Primers used in chapter III.

Number [a]	Name	Primer sequence (5′ ------ 3′)
1	FvGBB-F5	TCGTGAATCGTGATAGCCGTGTGT
2	FvGBB-R6t	TCACTGGCCGTCGTTTTACAAATCACCTTCACTGCCCGTAATCCA [b]
3	FvGBB-F6t	CATGGTCATAGCTGTTTCCTGACTGGCTGATGCGATACCTCCTTT [c]
4	FvGBB-R7	ACAGTAACACCTTCATTGCCGTGG
5	FvGBB-F8t	CATGGTCATAGCTGTTTCCTGAGTGGGCGCAATCTGGGTAGTAAT [c]
6	M13-F	TTGTAAAACGACGGCCAGTGA
7	M13-R	CAGGAAACAGCTATGACCATG
8	FvGBB-F4	TACATCTGTATCGGGTCGCCTTCT
9	FvGBB-R8	TCTCAACCTTCGCCAGACTGGATT
10	FvGBB-F8	TGAAAGCCAAGCGAACACTGAAGG
11	FvGBB-R4	TTTCCAGCGCGAATATCCCAAAGC

[a] Primers 1-7 are shown in Fig. 3.1A and C.
[b] Underlined sequence represent complimentary sequence of M13-F primer used in double joint PCR.
[c] Underlined sequence represent complimentary sequence of M13-R primer used in double joint PCR and single joint PCR.

Polymerase chain reaction

All PCR reactions were performed in a GeneAmp PCR system 9700 thermocycler (PE Applied Biosystems, Norwalk, CT). DNA amplification was performed in a 25-μL or a 50-μL mixture with either *Taq* DNA polymerase (Promega) or Expand Long Polymerase (Roche, Indianapolis, IN). The PCR conditions included: 2 min of predenaturation at 94 °C followed by 30 cycles of 30 s denaturation at 94 °C, 30 s annealing at 55-57 °C (based on primer pair), and 1-5 min (based on length of the amplicon) of elongation at 72 °C (68 °C for Expand Long Polymerase amplifications). A 10-min post-elongation was carried out after 30 cycles.

***GBB1* deletion and complementation constructs**

The *GBB1* disruption cassette was constructed by using a previously described double-joint PCR strategy (Yu et al., 2004). DNA fragments corresponding to regions 5' (1.5 kb) and 3' (1.5 kb) of the *GBB1* gene were amplified from the *F. verticillioides* genomic DNA with primer pairs FvGBB-F5 + FvGBB-R6t and FvGBB-F6t + FvGBB-R7, respectively. A hygromycin phosphotransferase (*HPH*) gene cloned into pBluescript II (Stratagene) was amplified with the primers M13-F + M13-R. Primers FvGBB-F5 + FvGBB-R7 were used to amplify the 4.4-kb amplicon carrying the *HPH* marker fused to the *GBB1* flanking regions.

The *GBB1* deletion mutant (*Δgbb1*) was complemented with wild-type *GBB1* gene that was fused to a geneticin resistant gene (*GEN*). *GEN*, which had been cloned into pBluescript II (Stratagene), was amplified with primers M13-F + M13-R, and *GBB1* (including 1650-bp 5' UTR and 1450-bp 3' UTR) was amplified with FvGBB-F8t +

FvGBB-R7. *GEN* and *GBB1* were fused by single-joint PCR strategy to generate the complementation construct (Shim et al., 2006).

Fungal transformation

Fungal protoplasts were prepared and transformed as described previously (Shim and Woloshuk, 2001), except that Mureinase (2 mg per mL) was replaced by Driselase (5 mg per mL) (Sigma, St. Louis, MO). Transformants were selected on regeneration medium (Shim and Woloshuk, 2001) containing 150 μg per mL of hygromycin B (Calbiochem, La Jolla, CA) or G-418 (Cellgro, Herndon, VA). Hygromycin-resistant and geneticin-resistant transformants were screened for homologous recombination and ectopic insertion events, respectively, by PCR and further verified by Southern analysis.

Fumonisin B_1 (FB_1) analysis

FB_1 was analyzed as described previously with modifications (Shim and Woloshuk, 1999). Briefly, fungal strains were grown on cracked corn (B73 line; 1 g dry weight) medium in a 20mL glass vial (VWR, West Chester, PA) for 14 days at room temperature (22-23 °C). FB_1 was extracted with acetonitrile: water (1:1, v/v) for 48 h. The crude extracts were passed over equilibrated PrepSep SPE C18 columns (Fisher Scientific, Pittsburgh, PA) prior to thin layer chromatography (TLC) analysis (Shim and Woloshuk, 2001). Following the TLC experiment, FB_1 concentration of samples was analyzed on a Shimatzu LC-20AT HPLC system (Shimatzu Scientific Instruments, Inc. Kyoto, Japan) equipped with an analytical Zorbax ODS column (4.6 × 150 mm) (Agilent Technologies, Santa Clara, CA) and a Shimatzu SPD-20A Prominence UV/VIS detector (335nm). The HPLC system was operated following the protocol described by Shim and

Woloshuk (1999). FB_1 was quantified by comparing HPLC peak areas with FB_1 standards (Sigma, St. Louis, MO). The experiment was repeated twice with three technical replications within each experiment.

Maize stalk rot assay

Stalk rot severity was assayed on 8-week-old B73 maize plants as previously described (Shim et al., 2006). Inter-nodal regions of the stalk were punctured approximately 2 mm deep with a sterile needle and fungal conidia suspension (10^5 spores/1 μL water) was inoculated into the wound. Plants were incubated for 14 days in a growth chamber at 25°C, 70% RH with a 14 h light/10 h dark light cycle. Stalks were split longitudinally to quantify the extent of rot. The lesions were assayed by measuring lesion length from four biological replications. Disease severity was quantified by measuring the length of longitudinal lesion that developed on either side from the inoculation point (Jardine and Leslie, 1999). The experiment was performed twice and with four independent biological replications.

***F. verticillioides* sexual cross experiments**

Sexual crosses were performed as explained in Shim et al. (2006) with modifications. Fresh carrots (400 g) were washed, diced and boiled for 20 min in 400 ml of distilled water. The carrots were macerated and the mixture was passed through cheesecloth. The filtrate was mixed with 20 g of agar and the final volume was brought to 1 liter with distilled water. The carrot agar medium was autoclaved for 20 min and poured into 100 mm diameter Petri plates. Both mating types were grown on V8 agar prior to mating. *F. verticillioides* strain 7600, *Δgbb1* and *gbb1C* strains (all with

genotype *MAT1-1*) were crossed with *F. verticillioides* strain 7598 (genotype *MAT1-2*) (Fungal Genetics Stock Center) on carrot agar plates. The growth conditions were maintained at 25°C with a 14-h light and 10-h dark cycle.

Microscopy

Microscopic observations were made using an Olympus BX51 microscope (Olympus America Inc., Melville, NY, USA). A detailed description of features used for imaging from this microscope has been described earlier (Shaw and Upadhyay, 2005). Images of hyphal growth phenotypes were obtained with an Olympus DP70 camera using DP70-BSW software (version 01.01). For time lapsed imaging of germ tube growth, a Q-imaging MicroPublisher RTV 5.0 camera (Q-Imaging, Burnaby, B.C. Canada) controlled by Simple PCI software (Version 5.3.1.081004) (Compix Inc., Imaging Systems, Cranberry Township, PA) was used. Images were acquired at one minute intervals while the light source was shuttered using a software controlled Prior shutter placed in the light path to limit exposure time to the cells. For time-lapse live-cell imaging, cells were grown on coverslips in 0.5× PDB at 23°C in an aluminum growth chamber as previously described (Kuo and Hoch, 1996). This chamber provided a sealed environment in which the cells can develop over long periods of time with minimal disruption from handling and imaging. Coverslips were pretreated with 0.01% poly-L-lysine solution (Sigma) to maintain the attachment of the cells to the glass surface. Germlings grown in the absence of poly-L-lysine had a similar phenotype but had a tendency to grow away from the plane of focus (data not shown). Adobe

Photoshop version 7.0.1 (Adobe, Mountain View, CA) was used to prepare images for publication.

Nucleotide sequence accession number

The nucleotide sequence of *GBB1* (DQ457053) and the predicted amino acid sequence (ABE67098) were submitted to GenBank.

Statistical analysis

FB_1, conidia, and stalk rot data were analyzed using analysis of variance (ANOVA) and the significant means were separated by least significant difference (LSD) method.

CHAPTER IV

FUSARIUM VERTICILLIOIDES GAP1, A GENE ENCODING A PUTATIVE GLYCOLIPID ANCHORED SURFACE PROTEIN, PARTICIPATES IN CONIDIATION AND CELL WALL STRUCTURE BUT NOT VIRULENCE

SUMMARY

Fusarium verticillioides is an important pathogen of maize that causes ear rot and produces the mycotoxins fumonisins. To date, our knowledge about the pathogenicity and the regulation of fumonisin biosynthesis in *F. verticillioides* is limited. Here, we present the molecular characterization of *GAP1*, a gene encoding a putative 540-amino acid protein that belongs to a glycolipid anchored surface protein (GASP) family. *F. verticillioides GAP1* was identified as an expressed sequence tag (EST) up-regulated in a culture condition conducive to conidiation and fumonisin B_1 (FB_1) production. *GAP1* null mutants GAM126 (*Δgap1::HYG*) and GAG8 (*Δgap1::GEN*) exhibited restricted growth with more aerial hyphae compared to their wild-type progenitor on solid media. No defect in mycelial mass or filamentous growth was observed when the GAM126 and GAG8 strains were grown in liquid media under shaking conditions. When grown in suspended conditions, GAM126 and GAG8 strains produced significantly fewer conidia and produced comparatively thin and densely branched hyphae. Concanavalin A staining indicated that the *GAP1* deletion altered the cell wall carbohydrate composition/deposition process. Deletion of *GAP1* did not affect FB_1 production level or *F. verticillioides* virulence on maize seedlings and stalks. Complementation of GAM126 with the wild-type *GAP1* gene restored growth, conidiation and cell wall

abnormality phenotypes. Our results suggest that *GAP1* is associated with growth, development and conidiation in *F. verticillioides* but not in positive regulation of FB_1 or pathogenicity.

INTRODUCTION

Fusarium verticillioides (Sacc.) Nirenberg (teleomorph: *Gibberella moniliformis* Wineland) causes rots of maize (*Zea mays)* stalks and ears (Munkvold and Desjardins, 1997). Stalk rot of maize is widespread in dry, warm climates (White, 1999). Stalk rots can incur losses in several different forms such as stalk breakage, lodging, interruption of the normal grain filling process and sometimes premature death of the plant (Dodd, 1980; Michaelson, 1957; White, 1999). The fungus also produces polyketide-derived carcinogenic mycotoxins called fumonisins (Nelson et al., 1993; Marasas, 2001). Since the discovery of fumonisins, several efforts were made to understand the regulation of fumonisin biosynthesis in *F. verticillioides*-maize system. (reviewed in Sagaram et al., 2006). Briefly, fumonisins are synthesized by a cluster of 22 *FUM* genes, of which 15 are co-regulated (Proctor at al., 1999; Proctor et al., 2003). The *FUM* cluster is not known to contain any regulatory genes. Regulation is controlled by several environmental factors, including available nitrogen, ambient pH and maize kernel constitution environment and regulatory genes not physically linked to the *FUM* cluster (reviewed in Sagaram et al., 2006).

The first regulatory gene identified by restriction enzyme mediated integration (REMI) strategy is *FCC1*, which encodes a C-type cyclin (Shim and Woloshuk, 2001). Mutation in *FCC1* led to severe reduction in conidiation and fumonisin production.

Nitrogen source and pH heavily influenced the conidiation and expression of the fumonisin biosynthetic gene (*FUM1*) in a *Δfcc1* strain, indicating a regulatory role of *FCC1* in fumonisin biosynthesis and fungal development (Shim and Woloshuk, 2001). Subsequent genomic approaches such as construction of cDNA subtraction libraries using a wild type strain and *Δfcc1* strains (Shim and Woloshuk, 2001) and microarray analysis (Prittilä et al., 2004) were made to identify additional regulatory genes of fumonisin biosynthesis. As a result, several genes, e. g., *FCK1*, a C-type cyclin-dependent kinase, *PAC1*, a pH regulatory gene, *ZFR1*, a Zn(II)2Cys6 gene and *AMY1*, a gene encoding a putative α-amylase (reviewed in Sagaram et al., 2006), have been identified and characterized.

During the screening of *F. verticillioides* EST index (TIGR) and *F. verticillioides* SSH cDNA libraries (Shim and Woloshuk, 2001), a 350-bp EST was identified in the wild-type SSH library that encodes a protein sequence with high homology to glycosylphosphatidylinositol (GPI) -anchored β-1, 3-glucanosyltransferases that belong to a class of GPI- anchored glycoproteins. We designated this gene *GAP1* (GPI-anchored protein 1). GPI-anchored cell wall proteins (GPI-CWPs) are among the most common proteins in eukaryotic cell walls (De Groot et al., 2005) that are covalently linked to β-1,6-glucan, which can be further linked to β-1,3-glucan or chitin resulting in a strong anchorage of these proteins to cell walls (Kapteyn et al., 1996, 1997; Kollar et al., 1997). The β-1, 3-glucanosyltransferases are involved in the elongation of β-1,3-glucan side chains of the cell wall (Mouyna et al., 2000). These enzymes split the β-1,3-glucan molecule and transfer the newly produced reducing end to the non-reducing end

of another β-1,3-glucan molecule (Mouyna et al., 2000). GPI-anchored β-1, 3-glucanosyltransferases have been shown to play a critical role in fungal cell wall biogenesis (Mouyna et al., 2000), morphogenesis and virulence in opportunistic fungal pathogens of humans (Muhlschlegel and Fonzi, 1997; DeBernardis et al., 1998; Ghannoum et al., 1995; Mouyna et al., 2005). For example, *GEL1* and *GEL2* are GPI-anchored β-1, 3-glucanosyltransferase encoding genes characterized in *Aspergillus fumigatus* (Mouyna et al., 2000; Mouyna et al., 2006). Disruption of *GEL1* did not result in a phenotype while disruption of *GEL2* and disruption of both *GEL1 and GEL2* resulted in altered cell wall composition, slower growth, unusual conidiogenesis and reduced invasive aspergillosis in a murine model (Mouyna et al., 2005). Significantly, a β-1, 3-glucanosyltransferase encoding gene, *gas1* is also involved in virulence in a plant pathogenic fungus *F. oxysporum* (Caracuel et al., 2005), suggesting that *GAP1* may be associated with fungal virulence in *F. verticillioides*. Caracuel et al. (2005) also reported that *F. oxysproum GAS1* expression is pH-independent which is in contrast to *C. albicans PHR1* and *PHR2* (Muhlschlegel and Fonzi, 1997).

The fact that the *GAP1* EST was isolated in the *F. verticillioides* wild-type SSH cDNA library (Shim and Woloshuk, 2001) prompted us to initially hypothesize that *GAP1* is primarily associated with fumonisin biosynthesis. However, we recognized that *GAP1* may alternately be associated with fungal development and conidiation (Shim and Woloshuk, 2001), which in turn may affect fungal virulence. Thus, we formulated a hypothesis that *GAP1* regulates fungal development and virulence in *F. verticillioides*. To test this hypothesis, we generated *GAP1* deletion mutants of *F. verticillioides* and

investigated growth, conidiation, and cell wall biogenesis. In addition, we analyzed fungal virulence on maize and fumonisin production. In this study, we demonstrate that *GAP1* is required for proper growth and conidiation on solid synthetic media and conidiation in liquid media. Our study revealed that *GAP1* influences cell wall structure by organizing the carbohydrate deposition in the cell walls when the fungus is growing on solid synthetic media. We also show that *GAP1* is not involved in *F. verticillioides* virulence on maize and does not positively regulate fumonisin biosynthesis.

RESULTS

Molecular characterization of *GAP1*

We isolated a 350-bp EST sequence (wt_0_G15) from *F. verticillioides* SSH cDNA library (Shim and Woloshuk, 2001) that shared high similarity to *Schizosaccharomyces cerevisiae* glycolipid-anchored surface protein (E=1e-33) and *Candida albicans* pH responsive protein (E= 3e-33). The complete *GAP1* sequence was obtained from the *F. verticillioides* 7600 strain genome sequence (Broad Institute of Harvard and MIT [http://www.broad.mit.edu]) GPI-anchored surface proteins are known to be differentially expressed with changes in ambient pH (Saporitoirwin et al., 1995; Muhlschlegel and Fonzi, 1997). In *F. verticillioides*, *GAP1* expression was repressed at pH 8.5 and was induced proportionately at acidic pH (Fig. 4.1). The *GAP1* gene is 1,669-bp long with single putative intron (49 bp) and encodes a putative protein of 540 amino acids. The nucleotide sequence and the conceptually translated protein sequence were submitted to the GenBank (DQ458798). A database search for conserved domains in the Conserved Domain Database (CDD, NCBI) revealed the presence of characteristic

glycolipid anchored surface protein (GAS1) domain at the N-terminal region of the protein (Marchler-Bauer et al., 2005). Amino acid comparison using BlastP analysis indicated that Gap1 shares high homology with predicted protein sequences of other filamentous fungi and yeast (Altschul et al., 1997). Sequence accession numbers of homologues in other fungi with percentage identity to putative Gap1 are; *Fusarium oxysporum* AAX78216 (94%); *F graminearum* EAA69544 (81%); *Magnaporthe grisea* XP_362679 (53%); *Neurospora crassa* CAD70754 (51%); *Aspergillus nidulans* XP_661334 (50%) and *A. fumigatus* EAL84955 (49%).

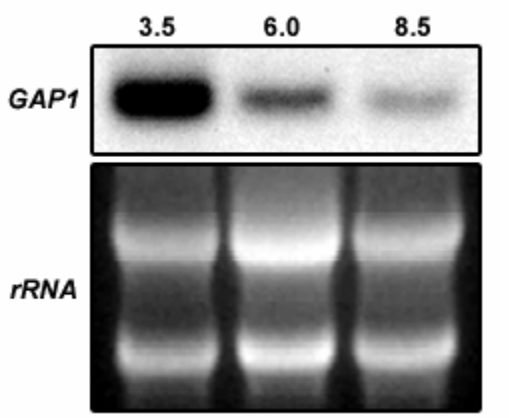

Fig. 4.1 Northern analysis of *GAP1* expression. For RNA isolation, 10^6 spores were inoculated into BSAL (pHs 3.5, 6.0 and 8.5) medium and allowed to grow for 7 days at room temperature (22-23 ºC) at 150 rpm. Total RNA (15 μg) was subjected to electrophoresis on a 1.2% denaturing agarose gel, transferred on to a nylon membrane and probed with a ^{32}P-labeled 1-kb *GAP1* genomic DNA fragment amplified with primers FvGAS-F1+FvGAS-R5 (Table 4.2). Ribosomal RNA stained with ethidium bromide is shown as equal loading control.

Generation of *GAP1* deletion mutant

To test our central hypothesis, we generated *F. verticillioides GAP1* deletion mutant strains; GAM126 (*Δgap1::HYG*) and GAG8 (*Δgap1::GEN*) using a double

homologous recombination strategy. After protoplast transformation, hygromycin-resistant colonies were selected and screened for gene deletion by PCR (data not shown). The homologous recombination event was verified by Southern analysis (Fig. 4.2D). The wild-type strain produced a 5-kb band whereas the deletion mutant, GAM126 produced a 3-kb band, indicating that the *GAP1* gene was replaced with the disruption construct GAS1DV2-H (Fig. 4.2B, D). The deletion of *GAP1* in GAG8 was also confirmed by Southern analysis (not shown). Northern analysis showed that the *GAP1* expression was completely abolished in both GAM126 and GAG8 strains where as the wild-type strain produced a 2.7-kb transcript (Fig. 4.3).

Mutation of *GAP1* influences cell wall composition and architecture

We noted a brighter calcofluor staining pattern for the *GAP1* deletion strains GAM126 and GAG8 as compared to wild type and the complemented strain GASC1 (data not shown). To better visualize this difference, we assayed the staining pattern of all four strains incubated in Concanavalin A (ConA) conjugated to the fluorophore FITC. ConA is a lectin that selectively binds to glucan and mannan moieties (Shaw et al., 1999). The GAM126 and GAG8 deletion strains each exhibited a significantly altered and brighter staining pattern compared to that of wild type and GASC1 when observed after identical staining protocols using camera settings (Fig. 4.4). Deletion strains each exhibited brightly stained cell walls punctuated by spaced discrete binding foci. Wild type and the GASC1 strain each lacked significant ConA binding.

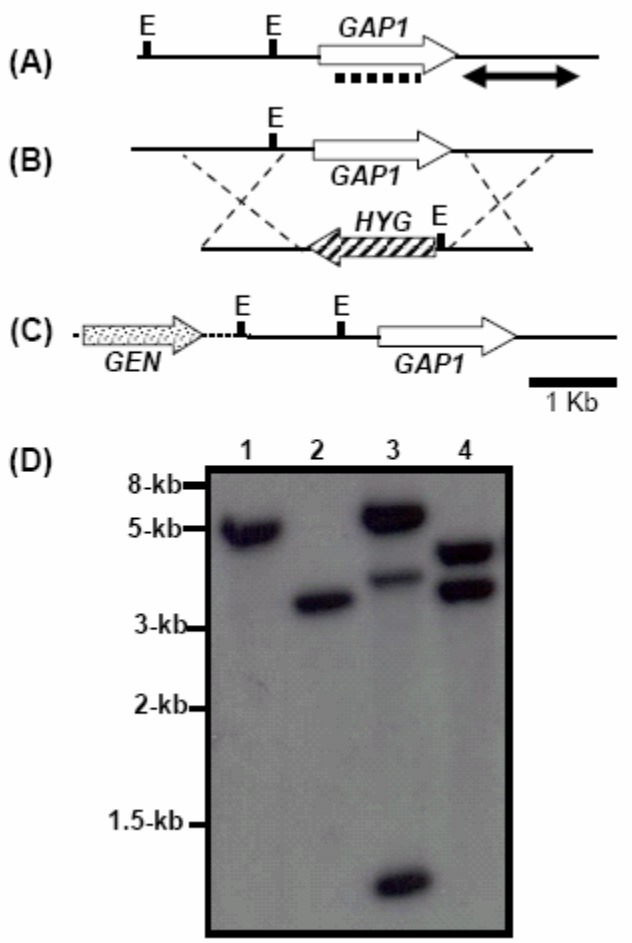

Fig. 4.2 Schematic representation of *GAP1* locus: deletion and complementation strategies. **(A).** Schematic representation of a 5.5-kb *F. verticillioides GAP1* locus. The dashed line and double arrow represent the genomic regions used as probes for northern and Southern blots, respectively. E: EcoRI restriction site. **(B)** Targeted replacement of *GAP1* with hygromycin phosphotransferase gene (*HYG*) and geneticin resistant marker (*GEN*), respectively, via double homologous integration event. **(C)** The complementation construct (GAS1CV1) used to complement GAM126 with *GAP1* wild type copy. GAS1CV1 was generated by fusing the geneticin-resistant gene (*GEN*) to *GAP1* gene by single joint PCR. **(D)** Southern analysis of transformants. Genomic DNA (10 µg) was digested with EcoRI, subjected to electrophoresis on a 1% agarose gel. The DNA was transferred on to nylon membrane and probed with ^{32}P-labeled DNA fragment amplified from genomic DNA with primers FvGAS-F4t+FvGAS-R3 (Table 4.2). Molecular sizes are indicated on the left. 1=Wild type, 2=GAM126, 3=Ectopic integration, 4=GASC1.

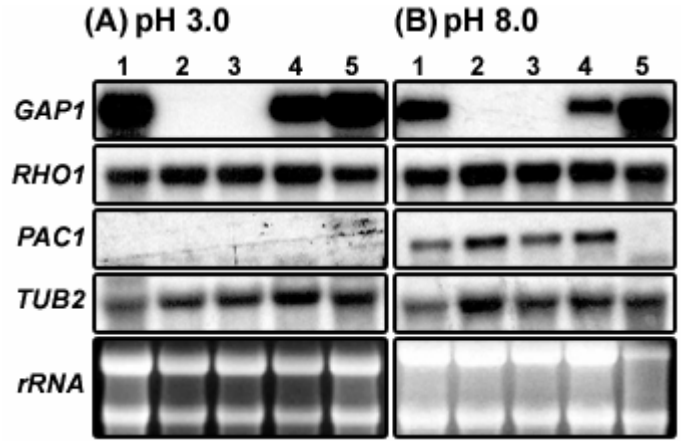

Fig. 4.3 Northern analysis to study the gene expression in wild type, GAM126, GAG8 and GASC1. For RNA isolation, 10^7 spores were inoculated into DL (Initial pHs 3.0 and 8.0) medium and allowed to grew for 7 days at room temperature (22-23 ºC) at 125 rpm. Total RNA was subjected to electrophoresis on a 1.2% denaturing agarose gel, transferred on to a nylon membrane and hybridized with ^{32}P-labeled gene-specific probes. Target genes are indicated on the left. Ribosomal RNA stained with ethidium bromide is shown to verify equal loading control. Fungal strains: 1=Wild type; 2= GAM126, 3= GAG8, 4=*GASC1* and 5=*PAC2A* (*PAC1* deletion strain).

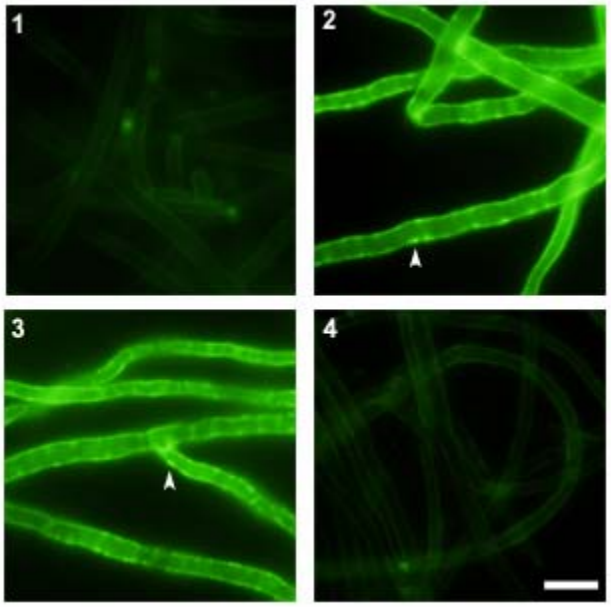

Fig. 4.4 Effect of *GAP1* mutation on cell walls as visualized by Concanavalin A staining. Fungal strains: 1=Wild type; 2= GAM126, 3= GAG8, 4=*GASC1*. Scale = 10 µm. It should be noted that GAM126 and GAG8 strains exhibited a significantly brighter staining pattern separated by regularly spaced discrete binding foci (arrow heads) compared to that of wild type and GASC1. Identical staining protocols, exposure time (1/55 sec) and camera settings were used.

Since, the cell walls of the deletion strains showed an unusual patterning, we decided to study the expression pattern of *RHO1*, which encodes a small monomeric G protein that is an important regulatory component of cell wall biogenesis (Bickle et al., 1998; Madaule et al., 1987). In *F. oxysporum*, deletion of *gas1* resulted in increased expression of *rho1* gene, which is orthologous gene to *RHO1* (Caracuel et al., 2005). *RHO1* of *F. verticillioides* was identified by using the nucleotide sequence of *rho1* of *F. oxysporum* (AY884607) as a query in BlastN analysis of the *F. verticillioides* genome sequence (http://www.broad.mit.edu).

Conceptually translated *RHO1* of *F. verticillioides* is 98% identical to *rho1* of *F. oxysporum*. A 500-bp DNA fragment that spans the *RHO1* gene was amplified from *F. verticillioides* genomic DNA using the primers RhoGase-D1+RhoGase-U1 and used as a probe for northern analysis. Surprisingly, *RHO1* transcript levels in GAM126 and GAG8 mutants are similar to wild type under both pH conditions tested (Fig. 4.3). These results indicate that the deletion of *GAP1* might alter the composition of cell wall through a mechanism not involving the *RHO1* unlike *F. oxysporum*, or *Rho1* involvement in cell wall biogenesis in *F. verticillioides* may be post transcriptional.

Deletion of *GAP1* affects growth and microconidia production

Deletion of *GAP1* affected growth on synthetic solid media and in suspension in liquid medium (Table 4.1 and Fig 4.5). When grown on agar medium, both the GAM126 and GAG8 strains growth was restricted when compared to wild-type. The deletion mutants produced a compact mass of mycelium with restricted edges that tended to grow into the agar medium while their wild-type progenitor produced thin and spreading

borders on the surface of the medium (Fig. 4.5A). Microscopic examination of spore germination revealed that deletion strains produced short and highly branched hyphae where as the wild-type strain produced long and less branched hyphae that extended on the surface of agar (Fig. 4.5B). However, we observed no defect in deletion strains with regard to mycelial mass production (Table 4.1) or filamentous growth (Fig. 4.5C) when grown in liquid media under shaking conditions. In YEPD suspension, GAM126 and GAG8 produced thin and densely branched hyphae that tended to remain submerged when compared to their wild-type progenitor (not shown). In PDB suspension, GAM126 and GAG8 produced densely branched hyphae but hyphal diameter was not noticeably different from wild type (not shown). Since ambient pH plays a critical role in the expression of *GAP1* and *GAP1* plays an important role in growth, we decided to test if the pH variation affects the growth of *GAP1* deletion strains. Unlike the *C. albicans* deletion strains (Muhlschlegel and Fronzi, 1997; Saporitoirwin et al., 1995), differences in pH (4.5 and 7.5) did not affect the growth (dry mass production) of the GAM126 and GAG8 strains in either solid or liquid media (not shown).

GAP1 deletion strains produced significantly ($p>0.05$) fewer microconidia than wild type when grown on ½ PDA and V8 agar (Table 4.1). We speculated that the production of fewer conidia on agar medium might be due to the restricted growth of deletion strains. Hence, we tested the microconidia production in a liquid medium (PDB). Under this condition GAM126 and GAG8 produced approximately half the microconidia produced by their wild-type progenitor (Table 4.1).

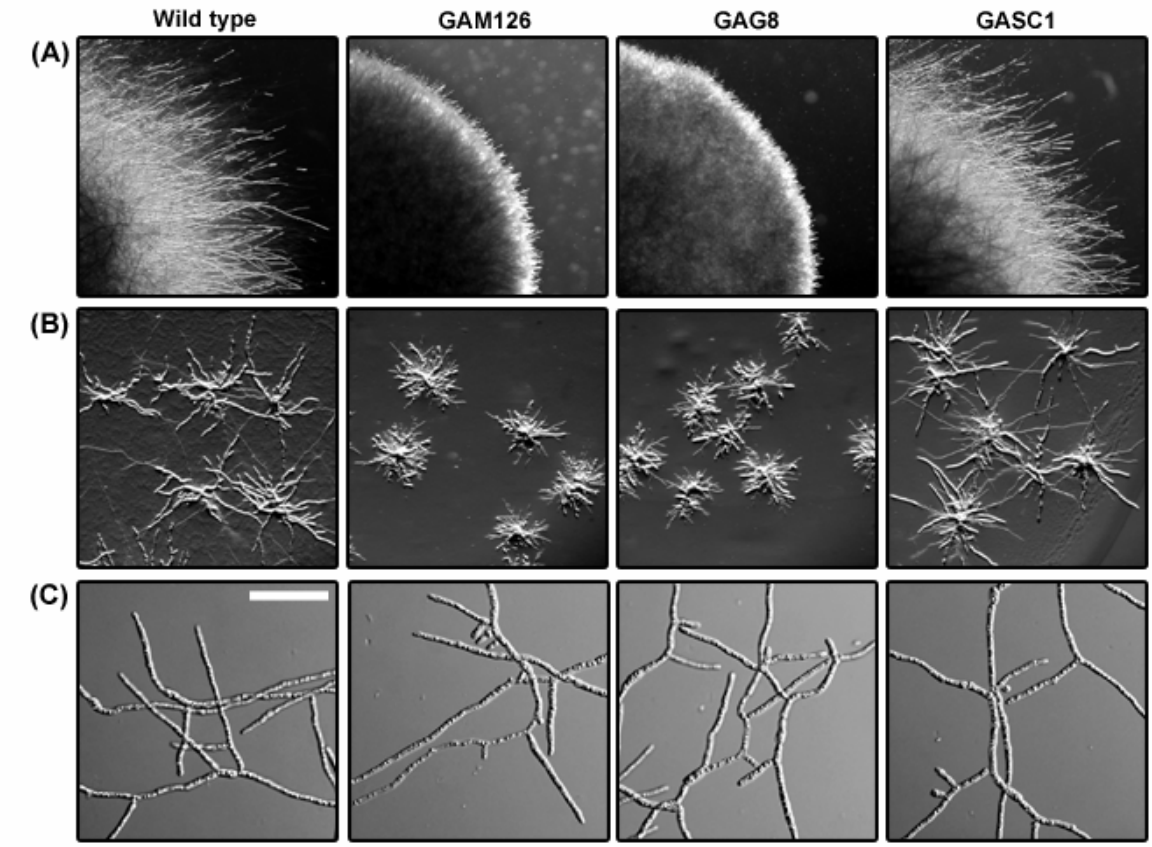

Fig. 4.5 Effect of *GAP1* mutation on growth and hyphal development. **(A)** Colony edges of fungal strains grown on 0.5× PDA (potato dextrose agar). **(B)** 10^3 spores in 10 µL volume were spot inoculated on agar 0.5× PDA. Pictures of individual colonies were photographed 4 days after inoculation. **(C)** Growth of *GAP1* deletion mutants and complement in 0.5× PDB (potato dextrose broth) in comparison with wild-type strain. In (A) and (B) it should be noted that the deletion strain produces short and hyper-branching hyphae that results in compact and restricted growth on solid media. Panels (A) and (B) pictures were photographed at 20× magnification and Panel (C) pictures were photographed at 10× magnification. Scale = 50 µm.

Deletion of *GAP1* did not influence pathogenicity and FB_1 production

Previous reports showing that GAS proteins in filamentous fungi are involved in virulence (Caracuel et al., 2005; Martinez-Lopez et al., 2004; Mouyna et al., 2005) prompted us to examine the involvement of *GAP1* in virulence. To achieve this, we performed maize stalk and seedling rot assays. In the maize stalk rot assay, we tested the

ability of deletion strains to infect and invade stalk tissue. As observed 21 days after infection, the deletion strains successfully colonized and rotted the tissue similar to the wild type (Fig. 4.6A). In the maize seedling assay, the deletion strains were as efficient as wild type in infecting and colonizing seedlings (Fig. 4.6B). After eight days, all the strains colonized the kernels completely and invaded the radical and plumule regions of the seedlings. Similar results were observed in both the maize lines tested (not shown). Based on these results, we concluded that *GAP1* is not involved in virulence in *F. verticillioides*.

Table 4.1. Comparison of growth and micro-conidial production in wild-type, GAM126, GAG8 and GASC1 strains.

Strains[m]	**Growth**[n]			**Microconidia**[o]		
	½ PDA (mm)	**V8 agar (mm)**	**½ PDB (mg)**	**½ PDA**[p] **10^5**	**V8 agar**[p] **10^6**	**½ PDB 10^4**
Wild type (WT)	52±1	66±1	58±1	131±18 a	70±7 a	112±7 a
GAM126	11±1	20±1	58±1	33±2 b	12±1 c	53±6 b
GAG8	11±1	19±1	60±1	32±6 b	14±1 c	47±3 b
GASC1	48±1	60±0	58±1	146±14 a	57±12 b	109±5 a

[m] Fungal strains were spot inoculated with 10^4 spores (agar plate) or 10^6 spores (in 100 mL stagnant broth) and allowed to grow for 7 days at room temperature (22 ºC).
[n] The growth was measured as diameter (mm) for agar plates and dry mass (mg) for broth. Values represent the mean of three biological replicates ± standard deviation.
[o]Mean number of conidia per milliliter ± standard deviation.
[p] Agar plates were flooded with 10mL of 0.1% triton, passed through 8 layers of cheese cloth (VWR) to eliminate mycelium and microconidia were counted using hemacytometer.
Significantly different means for microconidial number (the same column) are represented with a, b and c ($p<0.05$, least significant difference).
PDA = Potato dextrose agar; PDB = Potato dextrose broth.
All experiments were repeated twice with similar results.

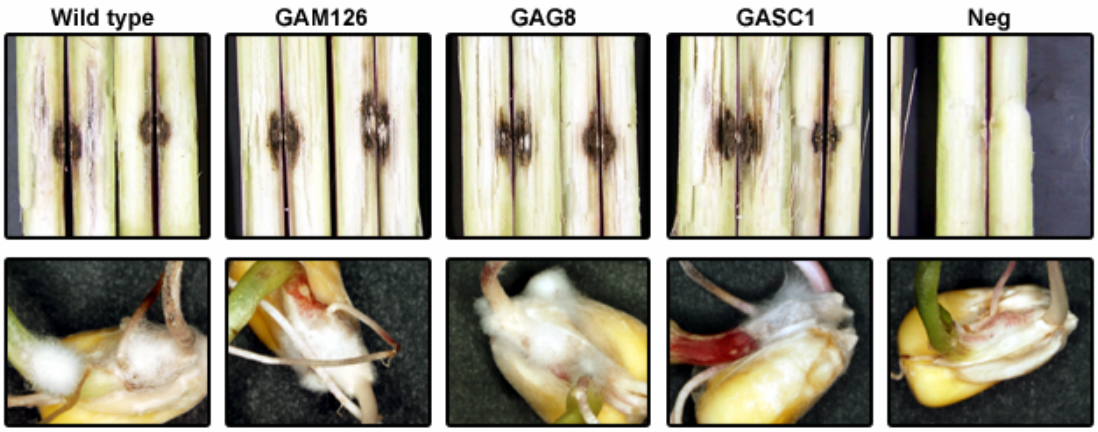

Fig. 4.6 Maize stalk rot and seedling rot assays of wild type, GAM126, GAG8 and GASC1. **(A).** Eight-week-old maize stalks were inoculated with 10^4 spores of fungal strains at inter-nodal region and incubated in a growth chamber for 21 days at 25 °C. Subsequently, the maize stalks were split longitudinally to assay the severity of stalk rot. Four independent biological repetitions were performed, and two biological repetitions were selected and shown in the figure. Maize stalk inoculated with sterile water is shown as the negative control. **(B).** Germinated corn seedlings were inoculated with 10^4 spores of fungal strains at embryo region. Eight seedlings were infected with each strain. Seedlings were periodically observed and photographed 8 days after infection. A close up picture of a single seedling per each strain showing the fungal colonization is shown in the figure.

To determine the effect of the *GAP1* mutation on FB_1 biosynthesis, we compared the FB_1 production ability of *GAP1* deletion strains with that of the wild-type strain. Observations made at 25 days post inoculation revealed that, GAM126 and GAG8 strains grew similarly but TLC analysis showed higher FB_1 levels when compared to the wild-type strain (not shown). However, further HPLC analysis indicated that only the GAG8 strain produced significantly ($p>0.05$) higher levels of FB_1 (47.63 ± 2.87 ppm) than its wild-type progenitor (29.69 ± 6.87 ppm). The FB_1 levels produced by GAM126 (37.38 ± 4.79 ppm) and GASC1 (26.25 ± 6.36 ppm) were not significantly different from the wild type strain. Contradictory to our hypothesis, the data clearly indicates that *GAP1* is not a positive regulator of FB_1 biosynthesis in *F. verticillioides*.

Gene complementation

To reconfirm that restricted growth and the reduced conidiation phenotype in GAM126 and GAG8 strains is due to the deletion of *GAP1*, we complemented the GAM126 with a wild-type copy of *GAP1*. Geneticin-resistant transformants were analyzed for functional *GAP1* gene by PCR (data not shown) and by Southern analysis (Fig. 4.2C and D). The complemented strain, GASC1 contains a 4-kb fragment, indicative of complementation construct insertion at an ectopic location, in addition to 3-kb fragment resulting from GAS1DV2-H. While we observed that the restoration of *GAP1* expression in GASC1 was not to that of wild type (lane 4, Fig. 4.3A and B), all the phenotypes observed in GAM126 and GAG8 strains such as conidiation, growth, cell wall phenotypes were completely restored to wild-type levels (Figs. 4.4 and 4.5) providing evidence that *GAP1* deletion is directly associated with these mutant phenotypes.

DISCUSSION

We regard *FCC1* as a key regulatory gene that impacts two important biological features - fumonisin biosynthesis and conidiation - in *F. verticillioides* (Shim and Woloshuk, 2001). The *F. verticillioides GAP1* gene was isolated from the wild-type SSH cDNA library, suggesting that *GAP1* is downstream of *FCC1* in the signaling pathway that regulates fumonisin biosynthesis or conidiation, or both. *In silico* analysis showed that *GAP1* encodes a putative protein that shares high structural similarity to a family of GPI-anchored glycoproteins that are known to be involved in cell development in filamentous fungi (Chabane et al., 2006; Mouyna et al., 2000; Mouyna et al., 2005).

Therefore we hypothesized that *GAP1* is primarily associated with conidiation and fungal development. Mutational analyses of GPI-anchored proteins in other fungi resulted in a variety of cell wall development defects (Chabane et al., 2006; Davydenko et al., 2005; Kottom et al., 2001; Mouyna et al., 2005), and therefore one of the key biological functions of *GAP1* we were interested in was its involvement in cell wall biogenesis. In this study, we observed that GAM126 and GAG 8 strains are not sensitive to the cell wall binding agent calcofluor (50 μg/mL) (Fluorescence brightener, Sigma) (not shown). This result is in agreement with *F. oxysporum gas1* deletion mutant phenotype but in contrast to *S cerevisiae gas1* mutants which were sensitive to calcofluor (Caracuel et al., 2005; Popolo et al., 1993). Surprisingly, the *F. verticillioides* wild-type strain is sensitive to 0.1% sodium dodecyl sulfate (not shown). While previous studies showed that osmotic stabilization with 1.2M sorbitol solution partially remediated the restricted colony phenotype in *gas1* mutant (Caracuel et al., 2005), the restricted colony growth phenotypes in *F. verticillioides GAP1* mutant strains were not remediated upon addition of osmotic stabilizers including 1.2M KCl or 1.2 M sorbitol solutions (not shown).

When mycelia of wild type, GAM126, and GAG8 were stained with calcofluor, we noticed that cell walls of GAM126 and GAG8 strains were more brightly stained compared to wild type, indicating that the mutant strains either produced more chitin and β-glucans in their cell walls, or that these moieties were more readily stained by calcofluor in the mutant (not shown). When stained with ConA-FITC, GAM126 and GAG8 exhibited uniform binding of the lectin to cell walls punctuated by discrete sites

of greater binding (Fig. 4.4). In contrast neither wild type nor GASC1 revealed any significant ConA binding sites. ConA is routinely used to assay the presence of glucan and mannan moieties in fungal cell walls (Shaw et al. 1999). It is generally believed to bind to terminal α-mannan and α-glucan (Smith and Goldstein 1967) One might have hypothesized that the *GAP1* mutants would exhibited fewer ConA binding sites than wild type, since the *GAP1* protein is thought to function in the linking of β-linked glucan groups. Our result is not surprising however since the alteration of the cell wall by lack of *GAP1* function may lead to a more amorphous cell wall that is less cross-linked, therefore leading to exposure of more ConA binding sites. While it is difficult to unambiguously explain this ConA staining pattern, it is clear from our results that the *GAP1* mutant cell wall is significantly altered from wild type.

The expression of pH responsive genes in filamentous fungi is regulated by key transcriptional regulators such as *pacC in A. nidulans* (Triburan et al., 1995) and *PRR1 in C. albicans* (Porta et al., 1999). For example, the expression of *PHR1* and *PHR2*, the β-(1,3)-glucanotransferase encoding genes in *C. albicans* is regulated by changes in the external pH via *PRR1* (Muhlschlegel and Fronzi, 1997; Porta et al., 1999; Saporitoirwin et al., 1995). In contrast, *GEL1* and *GEL2*, the two characterized β-(1,3)-glucanotransferase encoding genes in *A. fumigatus* are constitutively expressed over a range of growth conditions (Mouyna et al., 2000). Interestingly, the expression of *gas1* in *F. oxysporum* is not controlled by ambient pH, or by the responsive regulator PacC (Caracuel et al., 2005). In contrast to *F. oxysporum* studies, expression of *GAP1* in *F. verticillioides* is induced under acidic conditions (Fig. 4.1) and seems to be dependent

upon the pH responsive transcription factor *PAC1* (Fig 4.3). Our northern analysis revealed that *GAS1* expression is upregulated under alkaline conditions in a *PAC1*-deletion mutant compared to the wild type (Fig. 4.3B, lane 5). We propose that under alkaline conditions, induction of the *PAC1* suppresses the expression of *GAP1* and vice versa. However, further studies are needed to confirm the regulation of *GAP1* by *PAC1*.

In addition to investigating the developmental phenotypes of GAM126 and GAG8, we studied the impact of *Δgap1* on key biological features that are linked to the *FCC1* in *F. verticillioides*. In our study we observed a decrease in production of conidia due to the deletion of *GAP1* (Table 4.1). Flaherty et al., (2003) showed that *PAC1* is critical to maintaining proper growth and conidiation under alkaline pH conditions. However, the ambient pH did not have any impact on growth in GAM126 and GAG8 strains. Hence, we speculate that the reduction in microconidia production in our deletion mutants is due to a developmental defect similar to the abnormal conidiophores observed in *A. fumigatus* Δgel2 and Δgel2 Δgel1 mutants (Mouyna et al., 2005) rather than molecular regulation of conidiation genes. Over all, very little information is available regarding GPI-anchored proteins in relation to conidiation. Identification and characterization of developmental-related regulatory genes in *F. verticillioides* are necessary to clarify this relationship. In contrast to the conidiation data, we were intrigued that *GAP1* deletion did not have a negative impact on fumonisin production. Rather, we observed an increased level of FB_1 production in GAG8 strain. This data provides indirect evidence that the primary functional role of *GAP1* is to regulate proper hyphal development and conidiation in *F. verticillioides*. Discovery of additional genes

in the *FCC1*-mediated signaling pathways will allow us to investigate epistatic relationship between the genes and clarify the pathway-phenotype association.

In this report we also show that *GAP1* in *F. verticillioides* is not essential for invasion and colonization of maize stalks and seedlings. In a way it is interesting that GAM126 and GAG8 that are severely impaired in hyphal development on synthetic solid surfaces can grow as effectively as wild type in maize stalks and on seedlings which in theory can be considered solid surfaces. Based on our observations it is unlikely that *GAP1* plays a signalling role in interaction between maize and *F. verticillioides*. Our finding is significant based on the fact that supposedly orthologous proteins (94% identical) performed dissimilar functions (Caracuel et al., 2005, this study) although both fungi might have different modes of infection. However, it is not surprising since the fungus might have a compensatory mechanism to over come the loss of *GAP1*. Similar results were observed in other filamentous fungi. For example, deletion of *GEL1* in *A. fumigatus* did not have an impact on virulence but deletion of its homolog *GEL2* affected morphogenesis and virulence (Mouyna et al., 2005).

Surprisingly, *GEL1* and *GEL2* of *A. fumigatus* both complement *GAS1* deletion strain of *Saccharomyces cerevisiae* indicating that both proteins can act as functional homologous in yeast (Mouyna et al., 2005). In addition, under *in vitro* conditions Gel2p exhibited the same enzymatic activity as Gel1p reconfirming the redundancy in function (Mouyna et al., 2005). The regulation of expression of structurally similar but functionally different genes can be under the control of external signals such as pH, nutrient source etc. On the other hand, *PHR1* and *PHR2*, are GPI anchored proteins in *C.*

albicans that exhibit structurally and functionally inverse relationship between pH and morphology. *PHR1* and *PHR2* mutants are morphologically abnormal at alkaline and acidic pHs respectively (Muhlschlegel and Fronzi, 1997; Saporitoirwin et al., 1995). The fact that deletion of *GAP1* had no effect on pathogenicity where as its structural homolog in *F. oxysporum* has a significant impact on virulence (Caracuel et al., 2005) further indicates that the function of GPI proteins is specific to each organism. Analyses of publicly available fungal genomes revealed that more than one GAS family gene member is present in filamentous fungi, and as expected two additional GPI-anchored glycoprotein-encoding genes were identified in the *F. verticillioides* genome. This might indicate the possibility that β-(1,3)-glucanotransferases might be functionally homologous or might perform a different function. It is evident that β-1,3-glucanotransferases share a common function of involvement in cell wall biogenesis. In spite of their homology, GPI proteins of fungi perform different cellular functions probably to meet the specific requirements of an organism.

EXPERIMENTAL PROCEDURES

Fungal strains, media and growth conditions

The wild-type *F. verticillioides* strain 7600 (M3125; Fungal Genetics Stock Center, Kansas City, KS) and mutant strains generated for this study were stored in 30% glycerol at -80ºC. For genomic DNA extraction, the fungus was grown in YEPD medium (3 g yeast extract, 10 g peptone, and 20 g dextrose per liter). For production of initial inoculum (for RNA isolation, growth measurements, spore counts, virulence assays and FB_1 analysis) the fungal strains were grown on V8 juice agar plates (200 mL

V8 juice, 3 g $CaCO_3$, 20 g agar per liter) for 7-10 days. The spores were harvested in sterile water and passed through cheese cloth and quantified using a hemacytometer. The fungal strains were grown in bovine serum albumin liquid (BSAL) (1 g $NH_4H_2PO_4$, 40 g sucrose, 3 g KH_2PO_4, 2 g $MgSO_4.7H_2O$, 5 g NaCl per liter) or defined liquid (DL) (1 g $NH_4H_2PO_4$, 40 g sucrose, 3 g KH_2PO_4, 2 g $MgSO_4 \cdot 7H_2O$, and 5 g NaCl per liter) medium for RNA extraction.

Nucleic acid manipulation

Bacterial plasmid DNA was isolated with the Wizard miniprep DNA purification system (Promega, Madison, WI.). Fungal Genomic DNA was extracted by methods described earlier (Shim and Woloshuk, 2001). Total RNA was extracted with RNeasy plant mini kit (Qiagen, Valencia, CA) or Trizol reagent (Invitrogen, Carlsbad, CA) as per the manufacturer's protocols. Southern and northern blotting were performed following standard procedures (Sambrook and Russell, 2001). The probes used in all hybridization experiments were ^{32}P-labeled with Prime-It random primer labeling kit (Stratagene, La Jolla, CA).

PCR

All PCR reactions were performed in GeneAmp PCR system 9700 thermocycler (PE Applied Biosystems, Norwalk, CT). The primers used in this study are listed in Table 4.2. PCR amplification of DNA was performed in 25 µL or 50 µL total volumes with *Taq* DNA polymerase (Promega, Madison, WI) or Expand Long Polymerase (Roche, Indianapolis, IN). The PCR conditions were a 2 min of pre-denaturation at 94 ºC followed by 30 cycles of 30 s of denaturation at 94 ºC, 30 s of annealing at 55-57 ºC and

1-2 min of extension at 72 ºC for *Taq* DNA polymerase and 68 ºC for Expand Long Polymerase.

Table 4.2 Primers used in Chapter IV.

Number[c]	Name	Primer sequence (5′ ------ 3′)
1	FvGAS-F5	TTCGAGTTGTCGTCGTCTGAACCA
2	FvGAS-R2t	TCACTGGCCGTCGTTTTACAATAAAGCAGTCTAGCTACGGGACCA[a]
3	FvGAS-F4t	CATGGTCATAGCTGTTTCCTGATGCCATCTTGCGCGATTGCTATG[b]
4	FvGAS-R3	TTGAGCGTGTCTAATGGAGCTGGT
5	FvGAS-R4	TTCCAAGTCAGGTTCGCAACAACG
6	FvGAS-R5	ACCAAAGTTCTCGTCATCCTCGCT
7	FvGAS-F7t	CATGGTCATAGCTGTTTCCTGTTCGAGTTGTCGTCGTCTGAACCA[b]
8	M13-F	TTGTAAAACGACGGCCAGTGA
9	M13-R	CAGGAAACAGCTATGACCATG
10	FvGaseRho-D1	AAACAGCCTTATCATGCAACGGGC
11	FvGaseRho-U1	AGAGCTTGATTTGGCGCTTGTTGG

[a] and [b] Underlined sequences represent complimentary sequence of M13-F and M13-R (respectively) primers used in double joint and single joint PCR techniques.

Deletion and complementation vector construction

Two deletion constructs were generated by double joint PCR strategy (Yu et al., 2004) to replace *GAP1* open reading frame in *F. verticillioides* strain 7600. First, 1150-bp 5’ and 1050-bp 3’ flanking regions of *GAP1* gene were amplified from *F. verticillioides* genomic DNA using *Taq* DNA polymerase. The primers FvGAS-F2 and FvGAS-R2t were used to amplify 5’ flanking region and primers FvGAS-F4t and FvGAS-R4 were used to amplify 3’ flanking region. Simultaneously, hygromycin phosphotransferase (*HYG*) encoding gene and Geneticin (G418)-resistant gene (*GEN*)

were amplified from plasmid vectors pBP15 and pBS-G respectively using the primers M13-F and M13-R. The drug-resistant markers were amplified using Expand Long Polymerase which has proof reading activity. Subsequently, the three amplicons were mixed in a single tube in a 1:3:1 (5' fragment: Marker: 3' fragment) ratio and joined by PCR without using any primers. Finally, nested primers FvGAS-F5 + FvGAS-R4 were used to amplify the 3.9-kb amplicon carrying the *HPH* or *GEN* marker fused to the *GAP1* flanking regions. This fused product named GAS1DV2-H and GAS1DV2-G that harbor HYG and GEN respectively, were used as *GAP1*disruption constructs.

The *GAP1* deletion mutant strain GAM126 (*Δgap1::HYG*) was complemented with a wild-type *GAP1* gene that was fused to *GEN*. *GEN* was amplified with the primers M13-F and M13-R from pBS-G and *GAP1* (including 1530-bp 5' UTR and 1200-bp 3' UTR) was amplified from genomic DNA with FvGAS-F7t and FvGAS-R3 using Expand Long Polymerase. *GEN* and *GAP1* were fused by single-joint PCR strategy to generate the complementation construct (Shim et al., 2006; Yu et al., 2004). The joined-PCR product, GAS1CV1 was amplified with the primers M13-F and FvGAS-R4 using Expand Long Polymerase and used for transformation to complement GAM126.

Fungal transformation

Fungal protoplasts were prepared and transformed as described previously (Shim and Woloshuk, 2001), except that Mureinase (2 mg per mL) was replaced by Driselase (5 mg per mL) (Sigma, St. Louis, MO) and 1 g of wet mycelium was digested in 20 mL enzyme solution (10 mM NaH2PO4 (pH 5.8), 20 mM CaCl2, β-glucuronidase (1,343 U

per ml) (Sigma, St. Louis, Mo.), Driselase (5 mg per ml) (Sigma, St. Louis, Mo.) and 1.2 M KCl. Transformants were selected on regeneration medium (343 g L^{-1} sucrose, 0.2 g L^{-1} yeast extract, 10 g L^{-1} agar) containing 150 μg mL^{-1} of hygromycin B (Calbiochem, La Jolla, CA) or G-418 (Cellgro, Herndon, VA).

Mycelial staining and microscopy

For mycelial staining**,** 10^5 spores were inoculated on PDA and allowed to grow. After two days, the mycelia were scraped and collected in a 1.5 mL tube. Concanavalin A Type VI conjugated to FITC (Sigma, Cat # C7642) was used at a final concentration of 100 μg/ml using previously published conditions (Shaw et al., 1999). Concanavalin A in 100 μL volume was added to fungal mycelia and incubated for 45 minutes. Later, samples were washed twice with 100 μL of water by successive centrifugation and resuspensions. Finally, the samples were resuspended in 100 μL of water before observation under microscope. Microscopic imaging seed an Olympus BX51 microscope (Olympus America, Melville, NY) outfitted with Uplanapo objectives and an Olympus DP70 cooled CCD digital camera. Further details on this imaging system were recently described (Upadhyay and Shaw, 2006). Images were acquired DP70-BSW software (version 01.01) and prepared for publication with Adobe Photoshop. For FITC fluorescence visualization an Olympus U-MNIBA2 (Olympus) filter cube was used with excitation wavelengths from 470-480nm, emission wavelengths from 510-550nm and a dichroic mirror at 505nm FITC.

Stalk rot and seedling rot assay

Stalk rot assays were performed on 8 week old plants of the B-73 corn line. Inter-nodal regions of the stalk were punctured (2mm deep) with a sterile needle and 10^4 spores were inoculated into the punctured hole. Plants were incubated in a growth chamber with 25 ºC temperature and 40% humidity. Stalks were split open longitudinally after 21 days and the disease symptoms were photographed. For seedling rot assays, B73 corn line and Golden Cross Bantam hybrid sweet corn (Ed Hume seeds, Inc. Puyallup, WA) were surface sterilized with 100% bleach for 15 min, washed twice with sterile water and soaked in sterile water for 1 hr. The seeds were placed in between two layers of moist filter paper in a sterile glass saucer (30 cm × 15 cm) and allowed to germinate. After 7 days, the top filter paper is removed and the ungerminated seeds were discarded. Seeds were inoculated at the embryo region with 10^4 spores in 5 µL volume. Eight seeds were inoculated per each strain. The glass saucer was covered with Saran® wrap and incubated at room temperature (22-23 ºC) for 8 days. The experiments were repeated twice with similar results.

Fumonisin B_1 (FB_1) analysis

FB1 analysis was performed as explained earlier with some modifications (Shim and Woloshuk, 1999). Fungal strains were grown on cracked corn (B73 line; 1 g dry weight) medium for 15 days and 25 days at room temperature (22-23 ºC). FB_1 was extracted with acetonitrile: water (1:1, v/v) for 48 h. The crude extracts were passed over equilibrated PrepSep SPE C18 columns (Fisher Scientific, Pittsburgh, PA) to eliminate unwanted metabolites coming from corn and fungus. FB_1 concentration of samples was

analyzed by high performance liquid chromatography (HPLC) analysis using the Shimatzu LC-20AT HPLC system (Shimatzu Scientific Instruments, Inc. Kyoto, Japan) equipped with an analytical Zorbax ODS column (4.6 × 150 mm) (Agilent Technologies, Santa Clara, CA) and a Shimatzu SPD-20A Prominence UV/VIS detector (335nm). The HPLC system was operated following the protocol described by Shim and Woloshuk (1999). FB_1 was quantified by comparing HPLC peak areas with FB_1 standards (Sigma, St. Louis, MO). Four biological replicates were included in the experiment and the experiment was repeated twice.

Statistical analysis

Data were analyzed using analysis of variance (ANOVA) and the significant means were separated by least significant difference (LSD) method.

CHAPTER V

CONCLUSION AND FUTURE PROSPECTS

In this study, three putative FB_1 regulators in *F. verticillioides* were isolated and functionally characterized. The results demonstrated that *GBP1* and *GBB1* are involved in regulation of FB_1 production by controlling the expression of *FUM* genes. *GAP1*, however, was not directly associated with FB_1 production but rather hyphal development. In addition to FB_1 production, the role of these candidate genes in virulence and development, especially morphology and conidiation, was investigated in this study. Interestingly, the three genes characterized in this study all regulated FB_1 production in independent fashion, suggesting a lack of epistatic relationship with previously characterized FB_1 regulatory genes. Accumulating evidence suggests that regulatory mechanism involved in fumonisin biosynthesis is complex.

A model of FB_1 regulatory pathways in *F. verticillioides* is presented in Fig. 5.1. It has been well documented that multiple environmental and genetic factors play a role in the regulation, and what is more intriguing is that recent data demonstrate the interaction between maize kernel and *F. verticillioides* is also a key factor influencing the toxin biosynthesis. *FCC1* is the first FB_1 regulatory gene identified (Shim and Woloshuk, 2001). Fck1, a cyclin dependant kinase, physically interacts with Fcc1 (Bluhm and Woloshuk, 2006) and controls either unidentified downstream factors or directly regulates *FUM* gene expression, ultimately affecting FB_1 production. Fcc1/Fck1 complex directly or indirectly controls expression of Zfr1 (Flaherty and Woloshuk, 2004). Zfr1 positively regulates *FUM* genes and FB_1 production (Flaherty and

Woloshuk, 2004). FB_1 production and conidiation are each negatively regulated by Pac1 (Flaherty et al., 2003). Amy1, which is responsible for digestion of starch in corn kernels positively, affects FB_1 production (Bluhm and Woloshuk, 2005). My research has shown that Gbp1 is negatively associated with *FUM* gene expression and FB_1 production (Chapter II), and that Gbb1, a β subunit of heterotrimeric G protein positively governs FB_1 production, conidiation and hyphal development. (Chapter III). Gene expression studies revealed that Gbp1 and Gbb1 do not regulate the transcription of FB_1 regulatory genes identified to date (not shown). Gap1 is not involved in FB_1 regulation but positively controls conidiation in *F. verticillioides* (Chapter IV). It is also important to note that a number of regulatory genes and environmental factors that have been characterized to date operate in independent fashion (Fig 5.1). Therefore, I anticipate that additional genes associated with fumonisin regulation await discovery, and perhaps some of these genes will serve as anchors that will link signaling pathways that regulate fumonisin biosynthesis and cellular development in *F. verticillioides*.

The future challenge is to identify additional physiological and molecular factors that are associated with fumonisin regulation and to ultimately incorporate these factors into the current model (Fig. 5.1) to better understand the regulatory network. For instance, α subunit gene (named as *GBA1*) and γ subunit genes (named as *GBG1*) are prime target for further molecular characterization. The heterotrimeric G-protein complex has been well-characterized in other filamentous fungi, and it is reasonable to anticipate that α and γ subunits may perform distinct roles in FB_1 regulation in *F. verticillioides*.

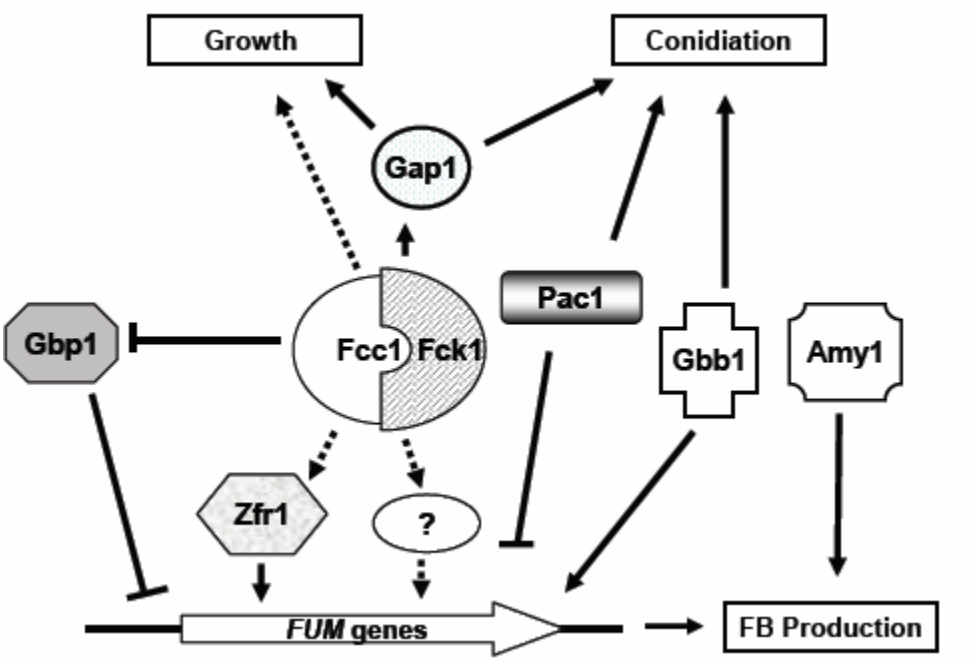

Fig. 5.1 Proposed model for regulation of fumonisin (FB_1) biosynthesis in *F. verticillioides*. Solid lines indicate a confirmed direct/indirect regulation. The dotted lines indicate putative relation that has not been characterized yet

Technical advancements, particularly genomics and proteomics technologies, are expected to facilitate the discovery of key genes and regulators in *F. verticillioides* – maize interaction. These new discoveries will lead to a better understanding of the molecular mechanisms that govern fumonisin biosynthesis in *F. verticillioides* as it grows on maize kernels, thus enhancing the prospects for control. Efforts are currently being made to identify new regulatory genes using the available genetic resources like cDNA SSH library, *F. verticillioides* Gene Index (www.tigr.org), and *F. verticillioides* genome (www.broad.mit.edu). Further studies will elucidate the interaction between these putative regulatory genes which will help us define fumonisin regulatory signaling pathway in *F. verticillioides*. While *in silico* analysis and transcriptional profiling are

more frequently selected genomics strategies for mining genes involved in certain biological processes, it is important not to overlook its limitations. The change in mRNA level of a certain gene can be transient while influencing down-stream gene regulation and ultimately cellular function. On the other hand, mRNA abundance may not be indicative of a gene's regulatory or metabolic potential. In some instances, pathway regulations occur at the post-transcriptional level, and therefore activity of the final gene product, the protein, may provide better evaluation of a gene's functional role. With the advancements in protein mass spectrometry technology that can perform high-throughput analyses of protein spots isolated from 2D protein gels, researchers are utilizing proteomics to investigate fumonisin regulatory pathways in *F. verticillioides*. As discussed earlier in this dissertation, nitrogen limitation is one of the important factors in triggering fumonisin biosynthesis in *F. verticillioides*. For example, proteomic technology can be used to obtain a differentially expressed proteome under varied nitrogen conditions that can be further analyzed to identify and isolate possible candidates of fumonisin regulation. The differentially expressed protein spots can be excised and sequenced, and subsequently this information can be used to compare the correlation between gene transcription and translation. Furthermore, characterization of genes that encode the respective proteins can provide valuable information that can be used to elucidate fumonisin regulatory signaling pathway.

REFERENCES

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389-3402.

ApSimon, J.W. (2001) Structure, synthesis, and biosynthesis of fumonisin B1 and related compounds. *Environ. Health Perspect.* **109**, 245-249.

Bacon, C.W. and Hinton, D.M. (1996) Symptomless endophytic colonization of maize by *Fusarium moniliforme*. *Can. J. Bot.* **74**, 1195-1202.

Bickle, M., Delley, P. A., Schmidt, A. and Hall, M. N. (1998) Cell wall integrity modulates RHO1 activity via the exchange factor ROM2. *EMBO J.* **17**, 2235-2245.

Bluhm, B.H. and Woloshuk, C.P. (2005) Amylopectin induces fumonisin B1 production by *Fusarium verticillioides* during colonization of maize kernels. *Mol. Plant-Microbe. Interact.* **18**, 1333-1339.

Bojja, R. S., Cerny, R.L., Proctor, R.H. and Du, L.C. (2004) Determining the biosynthetic sequence in the early steps the fumonisin pathway by use of three gene-disruption mutants of *Fusarium verticillioides*. *J. Agri. Food Chem.* **52**, 2855-2860.

Bölker, M. (1998) Sex and crime: heterotrimeric G proteins in fungal mating and pathogenesis. *Fungal Genet. Biol.* **25**, 143-156.

Bolstad, B., Irizarry, R., Astrand, M. and Speed, T. (2003) A comparison of normalization methods for high density oligonucleotide array data based on bias and variance. *Bioinformatics* **19**, 185-193.

Bourne, H.R., Sanders, D.A. and McCormick, F. (1990) The GTPase superfamily: a conserved switch for diverse cell functions. *Nature* **348**, 125-132.

Bourne, H.R., Sanders, D.A. and McCormick, F. (1991) The GTPase superfamily: conserved structure and molecular mechanism. *Nature* **349**, 117-127.

Brown, D.W., Cheung, F., Proctor, R.H., Butchko, R.A.E., Zheng, L., Lee, Y., Utterback, T., Smith, S., Feldblyum, T., Glenn, A.E., Plattner, R.D., Kendra, D.F., Town, C.D. and Whitelaw, C.A. (2005) Comparative analysis of 87,000 expressed sequence tags from the fumonisin-producing fungus *Fusarium verticillioides*. *Fungal Genet. Biol.* **42**, 848-861.

Brown, D.W., Yu, J.H., Kelkar, H.S., Fernandes, M., Nesbitt, T.C., Keller, N.P., Adams, T.H. and Leonard, T.J. (1996) Twenty-five coregulated transcripts define a sterigmatocystin gene cluster in *Aspergillus nidulans*. *Proc. Natl. Acad. Sci.* USA, **93**, 1418-1422.

Butchko, R.A.E., Plattner, R.D. and Proctor, R.H. (2003) FUM13 encodes a short chain dehydrogenase/reductase required for C-3 carbonyl reduction during fumonisin biosynthesis in *Gibberella moniliformis*. *J. Agri. Food Chem.* **51**, 3000-3006.

Buglino, J., Shen, V., Hakimian, P. and Lima, C.D. (2002) Structural and biochemical analysis of the Obg GTP binding protein. *Structure* **10**, 1581-1592.

Callahan, T.M., Rose, M.S., Meade, M.J., Ehrenshaft, M. and Upchurch, R.G. (1999) *CFP*, the putative cercosporin transporter of *Cercospora kikuchii*, is required for wild type cercosporin production, resistance, and virulence on soybean. *Mol. Plant-Microbe Int.* **12**, 901-910.

Calvo, A.M., Wilson, R.A., Bok, J.W. and Keller, N.P. (2002) Relationship between Secondary Metabolism and Fungal Development. *Microbiol. Mol. Biol. Rev.* **66**, 447-459.

Caracuel, Z., Martinez-Rocha, A. L., Di Pietro, A., Madrid, M. P. and Roncero, M. I. (2005) *Fusarium oxysporum* gas1 encodes a putative beta-1,3-glucanosyltransferase required for virulence on tomato plants. *Mol. Plant Microbe Interact.* **18**, 1140-1147.

Chabane, S., Sarfati, J., Ibrahim-Granet, O., Du, C., Schmidt, C., Mouyna, I., Prevost, M. C., Calderone, R. and Latge J. P. (2006) Glycosylphosphatidylinositol-anchored Ecm33p influences conidial cell wall biosynthesis in *Aspergillus fumigatus*. *Appl. Environ. Microbiol.* **72**, 3259-3267.

Chang, M.H., Chae, K.S., Han, D.M. and Jahng, K.Y. (2004) The GanB G α-protein negatively regulates asexual sporulation and plays a positive role in conidial germination in *Aspergillus nidulans*. *Genetics,* **167**, 1305-1315.

Choi, G.H., Chen, B. and Nuss, D.L. (1995) Virus-mediated or transgenic suppression of a G-protein α subunit and attenuation of fungal virulence. *Proc. Natl. Acad. Sci.* USA, **92**, 305-309.

Chung, K.R., Jenns, A.E., Ehrenshaft, M. and Daub, M.E. (1999) A novel gene required for cercosporin toxin resistance in the fungus, *Cercospora nicotianae*. *Mol. Gen. Genet.* **262**, 382-389.

Davydenko, S. G., Feng, D., Jantti, J. and Keranen. S. (2005) Characterization of GPI14/YJR013w mutation that induces the cell wall integrity signalling pathway and

results in increased protein production in *Saccharomyces cerevisiae*. *Yeast* **22**, 993-1009.

De Bernardis, F., Muhlschlegel, F. A., Cassone, A. and Fonzi, W. A. (1998) The pH of the host niche controls gene expression in and virulence of *Candida albicans*. *Infect. Immun.* **66**, 3317-3325.

Degani, O., Maor, R., Hadar, R., Sharon, A. and Horwitz, B.A. (2004) Host physiology and pathogenic variation of *Cochliobolus heterostrophus* strains with mutations in the G protein α subunit, CGA1. *Appl. Environ. Microbiol.* **70**, 5005-5009.

De Groot, P. W. J., Ram, A. F. and Klis, F. M. (2005) Features and functions of covalently linked proteins in fungal cell walls. *Fungal Genet. Biol.* **42**, 657-675.

Delgado-Jarana, J., Martinez-Rocha, A.L., Roldan-Rodriguez, R., Roncero, G., and Pietro, A.D. (2005) *Fusarium oxysporum* G-protein β subunit Fgb1 regulates hyphal growth, development, and virulence through multiple signalling pathways. *Fungal Genet. Biol.* **42**, 61-72.

Devitt, M.L., Maas, K. J. and Stafstrom, J. P. (1999) Characterization of DRGs, developmentally regulated GTP-binding proteins, from pea and Arabidopsis. *Plant Mol. Biol.* **39**, 75-82.

Ding, Y., Bojja, R.S. and Du, L.C. (2004) Fum3p, a 2-ketoglutarate-dependent dioxygenase required for C-5 hydroxylation of fumonisins in *Fusarium verticillioides*. *Appl. Envir. Microbiol.* **70**, 1931-1934.

Dodd, J.L. (1980) The role of plant stresses in development of corn stalk rots. *Plant Dis.* **64**, 533-537.

Etheridge, N., Trusov, Y., Verbelen, J. and Botella, J.R. (1999) Characterization of ATDRG1, a member of a new class of GTP-binding proteins in plants. *Plant Mol. Biol.* **39**, 1113-1126.

Flaherty, J.E., Pirttila, A.M., Bluhm, B.H. and Woloshuk, C.P. (2003) *PAC1*, a pH-regulatory gene from *Fusarium verticillioides*. *Appl. Environ. Microbiol.* **69**, 5222-5227.

Flaherty, J.E. and Woloshuk, C.P. (2004) Regulation of fumonisin biosynthesis in *Fusarium verticillioides* by a zinc binuclear cluster-type gene, *ZFR1*. *Appl. Environ. Microbiol.* **70**, 2653-2659.

Ganem, S., Lu, S.W., Lee, B.N., Chou, D.Y.T. Hadar, R., Turgeon, B.G. and Horwitz, B.A. (2004) G-Protein β Subunit of *Cochliobolus heterostrophus* involved in virulence, asexual and sexual reproductive ability, and morphogenesis. *Eukaryot. Cell,* **3**, 1653-1663.

Gao, S.J. and Nuss, D.L. (1996) Distinct roles for two G protein α subunits in fungal virulence, morphology, and reproduction revealed by targeted gene disruption. *Proc. Natl. Acad. Sci. USA* **93**, 14122-14127.

Gelderblom, W.C.A., Jaskiewicz, K., Marasas, W.F.O., Thiel, P.G., Horak, R.M., Vleggaar, R. and Kriek, N.P.J. (1988) Fumonisins - novel mycotoxins with cancer-promoting activity produced by *Fusarium moniliforme*. *Appl. Environ. Microbiol.* **54**, 1806-1811.

Ghannoum, M. A., Spellberg, B., Saporito-Irwin, S. M. and Fonzi, W. A. (1995) Reduced virulence of *Candida albicans* PHR1 mutants. *Infect. Immun.* **63**, 4528-4530.

Gilman, A.G. (1987) G proteins - Transducers of receptor-generated signals. *Annu. Rev. Biochem.* **56,** 615-649.

Hamm, H.E. and Gilchrist, A. (1996) Heterotrimeric G proteins. *Curr. Opin. Cell. Biol.* **8**, 189-196.

Hicks, J.K., Yu, J.H., Keller, N.P., and Adams, T.H. (1997) *Aspergillus* sporulation and mycotoxin production both require inactivation of the FadA, Gα protein-dependent signaling pathway. *EMBO J.* **16**, 4916-4923.

Jain, S., Akiyama, K., Kan, T., Ohguchi, T., and Takata, R. (2003) The G protein β subunit *FGB1* regulates development and pathogenicity in *Fusarium oxysporum*. *Curr. Genet.* **43**, 79-86.

Jain, S., Akiyama, K., Kan, T., Ohguchi, T., and Takata, R. (2002) Targeted disruption of a G protein α subunit gene results in reduced pathogenicity in *Fusarium oxysporum*. *Curr. Genet.* **41**, 407-413.

Jain, S., Akiyama, K., Takata, R., and Ohguchi, T. (2005) Signaling via the G protein α subunit *FGA2* is necessary for pathogenesis in *Fusarium oxysporum*. *FEMS Microbiol. Lett.* **243**, 165-172.

Jardine, D.J. and Leslie, J.F. (1999) Aggressiveness to mature maize plants of *Fusarium* differing in ability to produce fumonisin. *Plant Dis.* **83**, 690-693.

Kapteyn, J. C., Montijn, R. C., Vink, E., De la Cruz, J.,. Llobell, A., Douwes J. E. et al. (1996) Retention of *Saccharomyces cerevisiae* cell wall proteins through a phosphodiester-linked β-1,3-/β-1,6-glucan heteropolymer. *Glycobiol.* **6**, 337–345.

Kapteyn, J. C., Ram, A. F., Groos, E. M., Kollar, R., Montijn, R. C., Van den Ende H. et al. (1997) Altered extent of cross-linking of β1,6-glucosylated mannoproteins to chitin in *Saccharomyces cerevisiae* mutants with reduced cell wall β1,3-glucan content, *J. Bacteriol.* **179**, 6279–6284.

Kasahara, S. and Nuss, D.L. (1997) Targeted disruption of a fungal G-protein β subunit gene results in increased vegetative growth but reduced virulence. *Mol. Plant-Micr. Interact.* **10**, 984-993.

Keller, S.E., Sullivan, T.M. and Chirtel, **S.** (1997) Factors affecting the growth of *Fusarium proliferatum* and the production of fumonisin B1: oxygen and pH. J. *Indust. Microbiol. Biotechnol.* **19**, 305-309.

Kennedy, J., Auclair, K., Kendrew, S.G., Park, C., Vederas, J.C. and Hutchinson, C.R. (1999) Modulation of polyketide synthase activity by accessory proteins during lovastatin biosynthesis. *Science,* **284**, 1368-1372.

Kollar, R., Reinhold, B. B., Petrakova, E., Yeh, H. J., Ashwell, G., Drgonova J. et al. (1997) Architecture of the yeast cell wall. β(1 → 6)-glucan interconnects mannoprotein, β(1 → 3)-glucan, and chitin. *J. Biol. Chem.* **272**, 17762–17775.

Kottom, T. J., Thomas, Jr. C. F. and Limper, A. H. (2001) Characterization of *Pneumocystis carinii* PHR1, a pH-Regulated Gene Important for Cell Wall Integrity. *J. Bacteriol.* **183**, 6740-6745.

Krystofova, S. and Borkovich, K. A. (2005) The Heterotrimeric G-Protein subunits GNG-1 and GNB-1 form a G βγ dimer required for normal female fertility, asexual development, and Gα protein levels in *Neurospora crassa*. *Eukaryot. Cell,* **4,** 365-378.

Kumar, S., Iwao, M., Yamagishi, T., Noda, M. and Asashima, M. (1993) Expression of GTP-binding protein gene *drg* during *Xenopus laevis* development. *Int. J. Dev. Biol.* **37**, 539-546.

Kuo, K.C. and Hoch, H.C. (1996) Germination of *Phyllosticta ampelicida* pycnidiospores: prerequisite of adhesion to the substratum and the relationship of substratum wettability. *Fungal Genet. Biol.* **20,** 18-29.

Lee, N. and Kronstad, J. W. (2002) *ras2* controls morphogenesis, pheromone response, and pathogenicity in the fungal pathogen *Ustilago maydis*. *Eukaryot. Cell* **1**, 954-966.

Lengeler, K.B., Davidson, R.C., D'Souza, C., Harashima, T., Shen, W.C., Wang, P., Pan, X.W., Waugh, M. and Heitman, J. (2000) Signal transduction cascades regulating fungal development and virulence. *Microbiol. Mol. Biol. Rev.* **64**, 746-785.

Li, B. and Trueb, B. (2000) DRG represents a family of two closely related GTP-binding proteins. *Biochem. Biophys. Acta,* **1491**, 196-204.

Liu, S. and Dean, R.A. (1997) G protein α subunit genes control growth, development, and pathogenicity of *Magnaporthe grisea*. *Mol. Plant-Micr. Int.* **10**, 1075-1086.

Livak, K. J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}{}_T$ Method. *Methods* **25**, 402-408.

Madaule, P., Axel, R. and Myers, A. M. (1987) Characterization of two members of the *rho* gene family from the yeast *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U S A* **84**, 779-783

Marasas, W.F.O. (2001) Discovery and occurrence of the fumonisins: A historical perspective. *Environ. Health Perspect.* **109**, 239-243.

Marchler-Bauer, A., Anderson J. B., Cherukuri, P. F., DeWeese-Scott, C., Geer, L. Y., Gwadz, M., He, S., Hurwitz, D, I., Jackson, J. D., Ke, Z. et al. (2005) CDD: a Conserved Domain Database for protein classification. *Nucleic Acids Res.* **33**, D192-196

Martinez-Lopez, R., Monteoliva, L., Ez-Orejas, R., Nombela, C. and Gil, C. (2004) The GPI-anchored protein CaEcm33p is required for cell wall integrity, morphogenesis and virulence in *Candida albicans*. *Microbiol.* **150**, 3341-3354.

Merrill, A.H., Sullards, M.C., Wang, E., Voss, K.A. and Riley, R.T. (2001) Sphingolipid metabolism: roles in signal transduction and disruption by fumonisins. *Environ. Health Perspect.* **109**, 283-289.

Michaelson, M.E. (1957) Factors affecting development of stalk rots of corn caused by *Diplodia zeae* and *Gibberella zeae*. *Phytopathol.* **47**, 499-503.

Missmer, S.A., Suarez, L., Felkner, M., Wang, E., Merrill Jr., A.H., Rothman, K.J. and Hendricks, K.A. (2006) Exposure to fumonisins and the occurrence of neural tube defects along the Texas-Mexico Border. *Environ. Health Perspect.* **114**, 237-

241.

Mouyna, I., Fontaine, T., Vai, M., Monod, M., Fonzi, W. A., Diaquin, M., Popolo, L., Hartland, R. P. and Latge, J. P. (2000) Glycosylphosphatidylinositol-anchored glucanosyltransferases play an active role in the biosynthesis of the fungal cell wall. *J. Biol. Chem.* **275**, 14882-14889.

Mouyna, I., Morelle, W., Vai, M., Monod, M., Lechenne, B., Fontaine, T., Beauvais, A., Sarfati, J., Prevost, M. C., Henry, C. and Latge, J. P. (2005) Deletion of GEL2 encoding for a beta(1-3)glucanosyltransferase affects morphogenesis and virulence in *Aspergillus fumigatus*. *Mol. Microbiol.* **56**, 1675-1688.

Morris, A. J. and Malbon, C.C (1999) Physiological regulation of G protein-linked signaling. *Phys. Rev.* **79**, 1373-1430.

Muhlschlegel, F. A. and Fonzi, W. A. (1997) PHR2 of *Candida albicans* encodes a functional homolog of the pH-regulated gene *PHR1* with an inverted pattern of pH-dependent expression. *Mol. Cell. Biol.* **17**, 5960-5967.

Munkvold, G.P. and Desjardins, A.E. (1997) Fumonisins in maize - Can we reduce their occurrence? *Plant Dis.* **81**, 556-565.

Nelson, P.E., Desjardins, A.E. and Plattner, R.D. (1993) Fumonisins: Mycotoxins produced by Fusarium species: Biology, Chemistry, and Significance. *Annu. Rev. Phytopathol.* **31**, 233-252.

Nishimura, M., Park, G. and Xu J.R. (2003) The G-beta subunit MGB1 is involved in regulating multiple steps of infection-related morphogenesis in *Magnaporthe grisea*. *Mol. Microbiol.* **50**, 231-243.

Park, D.L. and Troxell, T.C. (2002) US perspective on mycotoxin regulatory issues. *Adv. Exp. Med. Biol.* **504**, 277-285.

Pirttilä, A.M., McIntyre, L.M., Payne, G.A. and Woloshuk, C.P. (2004) Expression profile analysis of wild-type and *fcc1* mutant strains of *Fusarium verticillioides* during fumonisin biosynthesis. *Fungal Genet. Biol.* **41**, 647-56.

Popolo, L., Vai, M. Gatti, E., Porello, S., Bonfante, P., Balestrini, R. and L. Alberghina. (1993) Physiological analysis of mutants indicates involvement of the *Saccharomyces cerevisiae* GPI-anchored protein gp115 in morphogenesis and cell separation. *J. Bacteriol.* **175**, 1879-1885.

Porta, A., Ramon, A. M. and Fonzi, W. A. (1999) PRR1, a homolog of *Aspergillus nidulans* palF, controls pH-dependent gene expression and filamentation in *Candida albicans*. *J. Bacteriol.* **181**, 7516-7523.

Proctor, R.H., Brown, D.W., Plattner, R.D. and Desjardins, A.E. (2003) Co-expression of 15 contiguous genes delineates a fumonisin biosynthetic gene cluster in *Gibberella moniliformis*. *Fungal Genet. Biol.* **38**, 237-249.

Proctor, R.H., Desjardins, A.E., Plattner, R.D. and Hohn, T.M. (1999) A polyketide synthase gene required for biosynthesis of fumonisin mycotoxins in *Gibberella fujikuroi* mating population A. *Fungal Genet. Biol.* **27**, 100-112.

Riddell, R.W. (1950) Permanent stained mycological preparations obtained by slide culture. *Mycologia,* **42**, 265-270.

Rosen, S., Yu, J.H. and Adams, T.H. (1999) The Aspergillus nidulans *sfaD* gene encodes a G protein β subunit that is required for normal growth and repression of sporulation. *EMBO J.* **18**, 5592-5600.

Sadler, T.W., Merrill Jr., A.H., Stevens, V.L., Sullard, M.C., Wang, E. and Wang, P. (2002) Prevention of fumonisin B1-induced neural tube defects by folic acid. *Teratol.* ***66,*** 169-176.

Sagaram, U.S., Kolomiets, M. and Shim, W.B. (2006) Regulation of Fumonisin Biosynthesis in *Fusarium verticillioides*-Maize system. *Plant. Path. J.* **22**, 203-210.

Sambrook, J. and Russell, D.W. (2001) *Molecular cloning*: *a laboratory manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Saporitoirwin, S. M., Birse, C. E., Sypherd, P. S. and Fonzi, W. A. (1995) Phr1, a pH-regulated gene of *Candida albicans*, is required for morphogenesis. *Mol. Cell. Biol.* **15**, 601-613.

Sazuka, T., Tomooka, Y., Ikawa, Y., Noda, M. and Kumar, S. (1992) DRG: a novel developmentally regulated GTP-binding protein. *Biochem. Biophys. Res. Commun.* **189**, 363-370.

Schenker, T., Lach, C., Kessler, B., Calderara, S. and Trueb, B. (1994) A novel GTP-binding protein which is selectively repressed in SV40 transformed fibroblasts. *J. Biol. Chem.* **269**, 25447-25453.

Seo, J.A., Proctor, R.H., and Plattner, R.D. (2001) Characterization of four clustered and coregulated genes associated with fumonisin biosynthesis in *Fusarium verticillioides*. *Fungal Genet. Biol.* **34**, 155-165.

Seo, J.A., Guan, Y. and Yu, J.H. (2003) Suppressor mutations bypass the requirement of fluG for asexual sporulation and sterigmatocystin production in *Aspergillus nidulans*. *Genet.* **165**, 1083-1093.

Seo, J.A., Han, K.H. and Yu, J.H. (2005) Multiple roles of a heterotrimeric G-protein γ-subunit in governing growth and development of *Aspergillus nidulans*. *Genet,* **171**, 81-89.

Seo, J.A. and Yu, J.H. (2006) The phosducin-like protein PhnA is required for Gβγ-mediated signaling for vegetative growth, developmental control, and toxin biosynthesis in *Aspergillus nidulans*. *Eukaryot. Cell,* **5**, 400-410.

Shaw, B. D. and Hoch, H. C. (1999) The pycnidiospore of *Phyllosticta ampelicida*: surface properties involved in substratum attachment and germination. *Mycol. Res.* **103**, 915-924.

Shaw, B.D. and Upadhyay, S. (2005) *Aspergillus nidulans swoK* encodes an RNA binding protein that is important for cell polarity. *Fungal Genet. Biol.* **42**, 862-872.

Shim, W.B., Flaherty, J.E. and Woloshuk, C.P. (2003) Comparison of fumonisin B-1 biosynthesis in maize germ and degermed kernels by *Fusarium verticillioides*. *J. Food Prot.* **66**, 2116-2122.

Shim, W.B., Sagaram, U.S., Choi, Y.E., So, J., Wilkinson, H.H. and Lee, Y.W. (2006) *FSR1* is essential for virulence and female fertility in *Fusarium verticillioides* and *F. graminearum*. *Mol. Plant-Microbe Interact.* **19**, 725-733.

Shim, W.B. and Woloshuk, C.P. (1999) Nitrogen repression of fumonisin B1 biosynthesis in *Gibberella fujikuroi*. *FEMS Microbiol. Lett.* **177**, 109-116.

Shim, W.B. and Woloshuk, C.P. (2001) Regulation of fumonisin B1 biosynthesis and conidiation in *Fusarium verticillioides* by a cyclin-like (C-type) gene, *FCC1*. *Appl. Environ. Microbiol.* **67**, 1607-1612.

Shimizu, K., Hicks, J.K., Huang, T.P. and Keller, N.P. (2003) Pka, Ras and RGS protein interactions regulate activity of AflR, a Zn(II)2Cys6 transcription factor in *Aspergillus nidulans*. *Genet.* **165**, 1095-1104.

Siriputthaiwan, P., Jauneau, A., Herbert, C., Garcin, D. and Dumas, B. (2005) Functional analysis of CLPT1, a Rab/GTPase required for protein secretion and pathogenesis in the plant fungal pathogen *Colletotrichum lindemuthianum*. *J. Cell Sci.* **118**, 323-329.

Smith, E. and Goldstein, I. (1967) Protein-Carbohydrate Interaction. V. Further inhibition studies directed toward defining the stererochemical requirements of the reactive sites of concanavalin A. *Arch. Biochem. Biophys.* **121**, 88.

Tag, A., Hicks, J., Garifullina, G., Ake, C., Phillips, T.D., Beremand, M. and Keller, N.P. (2000) G-protein signalling mediates differential production of toxic secondary metabolites. *Mol. Microbiol.* **38**, 658-665.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673-4680.

Tilburn, J., Sarkar, S., Widdick, D. A., Espeso, E. A., Orejas, M., Mungroo, J., Penalva, M. A. and Arst, H. **N.** (1995) The Aspergillus PacC zinc-finger

transcription factor mediates regulation of both acid-expressed and alkaline-expressed genes by ambient pH. *Embo. J.* **14**, 779-790.

Upadhyay, S., and Shaw B. D. (2006) A phosphoglucose isomerase mutant in *Aspergillus nidulans* is defective in hyphal polarity and conidiation. *Fungal Genet. Biol.* **43**, 739-751.

Wang, E., Norred, W.P., Bacon, C.W., Riley, R.T. and Merrill, Jr. A.H. (1991) Inhibition of sphingolipid biosynthesis by fumonisins. Implications for diseases associated with *Fusarium moniliforme*. *J. Biol. Chem.* **266**, 14486-14490.

White, D. G. (1999) *Compendium of Corn Disease*. 3rd ed. APS Press, St. Paul, MN, USA.

Woloshuk, C.P., Foutz, K.R., Brewer, J.F., Bhatnagar, D., Cleveland, T.E and Payne, G.A. (1994) Molecular characterization of *aflR*, a regulatory locus for aflatoxin biosynthesis. *Appl. Environ. Microbiol.* **60**, 2408-2414.

Yang, Q., Poole, S.I., and Borkovich K.A. (2002) A G-Protein β subunit required for sexual and vegetative development and maintenance of normal G α protein levels in *Neurospora crassa*. *Eukaryot. Cell,* **1**, 378-390.

Yoshizawa, T., Yamashita, A. and Luo, Y. (1994) Fumonisin occurrence in corn from high-risk and low-risk areas for human esophageal cancer in China. *Appl. Environ. Microbiol.* **60**, 1626-1629.

Yu, J.H. (2006) Heterotrimeric G protein signaling and RGSs in *Aspergillus nidulans*. *J. Microbiol.* **44**, 145-154.

Yu, J.H., Hamari, Z., Han, K.H., Seo, J.A., Reyes-Dominguez, Y. and Scazzocchio, C. (2004) Double-joint PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi. *Fungal Genet. Biol.* **41**, 973-981.

Yu, J.H. and Keller, N.P. (2005) Regulation of Secondary Metabolism in filamentous fungi. *Annu. Rev. Phytopathol.* **43**, 437-458.

VITA
Uma Shankar Sagaram

Education

Ph. D. in Plant Pathology (2007)
- Texas A&M University, College Station, TX
- Advisor: Won-Bo Shim
- Dissertation: "Molecular Characterization of Genes Regulating Fumonisin Biosynthesis and Development in Maize Pathogen *Fusarium verticillioides*".

M.S. in Plant, Soil and Environmental Science (2002)
- West Texas A&M University, Canyon, TX
- Advisors: Greta L. Schuster and Terry A. Wheeler
- Thesis: "Identification of Critical Factors that Influence the Screening of Cotton Cultivars for Bacterial Blight Resistance".

B.Sc. in Agriculture (1999)
- Acharya N.G. Ranga Agricultural University, Hyderabad, A.P., India

Selected Publications

1. Sagaram U.S., Shaw, B.D and Shim, W.B. 2007. *Fusarium verticillioides GAP1*, a gene encoding a putative glycolipid-anchored surface protein participates in conidiation and cell wall structure but not virulence. Microbiology (Submitted for publication).
2. Sagaram U.S. and Shim, W.B. 2007. *Fusarium verticillioides GBB1*, a gene encoding heterotrimeric G protein β subunit, is associated with fumonisin B_1 biosynthesis and hyphal development but not with fungal virulence. Molecular Plant Pathology (Accepted for publication).
3. Sagaram U.S., Kolomiets, M. and Shim, W.B. 2006. Regulation of fumonisin biosynthesis in *Fusarium verticillioides*-Maize system. The Plant Pathology Journal 22(3): 203-210.
4. Sagaram U.S. and Shim, W.B. 2006. The putative monomeric G-protein *GBP1* is negatively associated with fumonisin B_1 production in *Fusarium verticillioides.* Molecular Plant Pathology 7(5):381-389.
5. Shim, W.B., Sagaram, U.S., Choi, Y.E., So, J., Wilkinson, H.H. and Lee, Y.W. 2006. *FSR1* is essential for both virulence and female fertility in *Fusarium verticillioides* and *F. graminearum*. Molecular Plant Microbe Interactions 19(7):725-733.

Professional Organization Membership

- American Phytopathological Society. *Student Member*. 2001-present.
- Gamma Sigma Delta. The Honor Society of Agriculture. 2004-2006.