IDENTIFICATION AND ANALYSIS OF *FUSARIUM VERTICILLIOIDES* GENES DIFFERENTIALLY EXPRESSED DURING SPORULATION

by

ALISHA MICHELE BURNHAM

(Under the Direction of ANTHONY GLENN)

ABSTRACT

Fusarium verticillioides is the causal agent of diseases such as ear rot, resulting in economic losses in maize production. This fungus can also cause diseases in animals due to mycotoxin-contaminated feed. A major aspect of *F. verticillioides* survival and dispersal is the production of microconidial chains from phialide tips. Upon mating two previously characterized conidia-producing strains, spontaneous mutations occurred resulting in progeny unable to produce conidia. These mutants produced germ tube-like growths from the tips of phialides instead of normal enteroblastic conidia. Based on microarray data comparing a wild-type strain and an aconidial mutant, thirteen candidate genes were chosen for further analysis. One of the thirteen was targeted for gene deletion because of its high fold-change, lack of homology to previously characterized proteins, and similarity only to other filamentous fungi in BLAST searches. Further analysis of these genes may identify novel characteristics of sporulation in *F. verticillioides*.

INDEX WORDS: *Fusarium verticillioides*, corn, ear rot, mycotoxin, conidia, phialide, microarray, sporulation

IDENTIFICATION AND ANALYSIS OF *FUSARIUM VERTICILLIOIDES* GENES DIFFERENTIALLY EXPRESSED DURING SPORULATION

by

ALISHA MICHELE BURNHAM

BA, COKER COLLEGE, 2009

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2011

IDENTIFICATION AND ANALYSIS OF *FUSARIUM VERTICILLIOIDES* GENES

DIFFERENTIALLY EXPRESSED DURING SPORULATION

by

ALISHA MICHELE BURNHAM

Major Professor:	ANTHONY GLENN
Committee:	SCOTT GOLD
	MICHELLE MOMANY

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
August 2011

DEDICATION

This I dedicate to my support system, my parents Lisa and Edward Roddey, grandparents John and Julia Cooper, and brother Michael Elton Burnham, III.

ACKNOWLEDGEMENTS

First I would like to thank my advisor Dr. Anthony Glenn for his support and helpful guidance. His knowledge and advice have helped me to keep on track and work at a smooth pace. His constant faith in me helped to build my confidence as a researcher and a person. My gratitude goes out as well to the other members of my Master's thesis committee, Dr. Scott Gold and Dr. Michelle Momany for their continuous help throughout the research and writing processes. I am forever indebted to my laboratory technician and friend Mrs. Britton Ormiston for always being there and understanding, even when our communication was a little off. She served multiple roles in my life and I will always remember the unconditional support and love she had for me. I would like to thank Dr. Daren Brown of USDA-ARS in Peoria, IL for his collaboration. To everyone in the USDA-ARS Toxicology and Mycotoxin Research Unit at the Richard B. Russell Research Center in Athens, GA, I want to say thanks for their help in various aspects of my research and learning experience. I am indebted to my school colleagues who have helped make my learning an enjoyable and stimulating experience. I am grateful for the UGA Fungal Group for allowing me to present my research and for providing input, advice and feedback. Lastly I wish to thank my family and my many close friends, whose enthusiasm, interest and support in this project have given me the motivation to realize this achievement.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

The fungus *Fusarium verticillioides* is the causal agent of corn diseases such as ear rot, seedling blight, and stalk rot, resulting in major economic losses in corn production. This fungus can also cause diseases in certain animals due to consumption of feed contaminated with fumonisin mycotoxins. Mycotoxins are toxic secondary metabolites produced by fungi that cause animal diseases. In the case of fumonisins, the mycotoxins may also aid colonization of the crop. Once the fungus enters the plant, mycotoxin levels elevate and these toxic chemicals are released. Along with toxins, another step in the colonization of the crop is dissemination of spores. *F. verticillioides* produces non-motile, asexual spores called microconidia. These microconidia form in chains from the tip of a phialide, which is a specialized cell producing spores through a reiterative mitotic process. Microconidia are essential for infection of the plant and the survival and dispersal of the fungus. Few genes involved in the process of sporulation in *F. verticillioides* have been studied. To expand our understanding of the genetics of sporulation, a collection of previously characterized conidiation mutants (Glenn *et al.*2004) were examined. Upon mating the two strains MRC 826 and NRRL 25029, both of which are capable of producing conidia, spontaneous mutations occurred resulting in half of the progeny being unable to produce conidia. These results suggest post-meiotic mutations that may have resulted in deletion of one or more genes from a chromosome. These conidiation mutants produced germ tube-like growths from the tips of phialides instead of normal conidial development. The

mutants lack a wall building zone at the phialide tip needed for forming a conidium wall. The first hypothesis of this project was that microarray analyses utilizing the available conidiation mutation will provide a dataset of sporulation associated genes. An initial microarray experiment was conducted comparing MRC 826 (wild-type strain) and AEG 3-A3-5 (aconidial mutant), and from this thirteen candidate genes having at least 9-fold change in expression in the wild-type strain were chosen for further analysis. One of the thirteen, FVEG_10983, has been targeted for gene deletion because of its 72-fold change. FVEG_10983 lacks homology to any previously characterized proteins and does not have any conserved protein domains to suggest its function. BLASTP searches showed significant similarity (1e-04 or less) only to a few accessions from phialide-producing filamentous fungi of the phylum Ascomycota thus suggesting that FVEG_10983 may be important in the proper production of conidia by *F. verticillioides*. In order to more thoroughly characterize these sporulation-related genes, phenotypic assessment of FVEG_10983 deletion mutants were performed. These efforts contribute to detailed molecular genetic studies of a key biological feature of this economically important fungus.

LITERATURE REVIEW

Disease cycle

Fusarium verticillioides is pathogenic at any stage of maize development (Foley 1962). Infection of maize by this fungus can result from different routes including soil, air, and vector transmission, each affecting different parts of the plant including the seeds, silks, roots, leaves, and ears. Germinating seed are a most common target due to the presence of the fungus in the soil by overwintering in the plant debris or new introduction of the fungus via spores (Alessandra *et al.* 2010). In the soil, *F. verticillioides* may penetrate the plant through the roots and

mesocotyl. Airborne spores (conidia) arising from fungal growth on plant debris or current growth on silks or leaves may cause infection. The prolific production of these spores, microconidia, allows successful plant infection in addition to survival and dispersal of the fungus. A postulated pathway for airborne conidia begins with the landing of the spores on corn silks followed by a downward growth towards the developing kernels. This strategy is believed to be the main pathway for kernel infection (Munkvold *et al.* 1997b). Ear rot and stalk infections also occur via wounding caused by insects, mainly the European corn borer (ECB), *Ostrinia nubilalis* (Munkvold *et al.* 1997a). The ECB larvae carry the fungus into corn stalks and ears, resulting in infection.

Symptoms of infection are exceptionally variable, ranging from asymptomatic with no signs of infection to severely diseased resulting in complete rotting or wilting. Because the plant can remain asymptomatic throughout the entire disease cycle, the infection can go unnoticed (Brown *et al.* 2007). *F. verticillioides* is an endophytic fungus that grows systemically within the plant (Bacon and Hinton 1996). Previously, it was unknown if or how the plant benefited from symptomless endophytic relationships with the fungus until 1997 when Yates *et al*. identified possible advantages for this relationship. They found in asymptomatic infections, the hyphae grow intercellularly throughout the plant. This intercellular growth was found in roots and shoots of infected plants but growth in the leaves had a limited distribution. Root dry weight of maize seedlings inoculated with *F. moniliforme* (*F. verticillioides*) surpassed that of non-infected seedlings, thus suggesting a possible advantage for the plant in the endophytic relationship. Williams *et al*. 2007 showed how low doses of fumonisins (mainly FB1) stimulated root growth, thus suggesting a possible advantage of the endophytic relationship.

Normal Development

F. verticillioides (teleomorph *Gibberella moniliformis*), is a filamentous fungus that produces two types of conidia – macroconidia and microconidia. Macroconidia are the larger of the two types and have a distinct crescent shape. Microconidia are single-celled and much smaller in size. The fungus produces a copious amount of single-celled microconidia and a less plentiful amount of septate macroconidia (Li *et al.* 2006). Microconidia are thought to serve as the primary inoculum for plant infection. These microconidia form in chains from the tip of a phialide, which is a specialized cell producing spores through a reiterative mitotic process called enteroblastic conidiation (Leslie *et al.* 2006, Glass *et al.* 2000, Glenn 2006). The production of long conidial chains is a unique characteristic for only a few fungi including *F. verticillioides*. In normal conidium development, a specific zone at the phialide apex called the wall building zone (WBZ) is present to initiate and aid in the development of the conidium (Glenn *et al.* 2004). The WBZ synthesizes the new conidium wall. The phialide nucleus undergoes mitosis and a daughter nucleus migrates through the apex to the developing conidium. A thickened inner wall layer at the phialide apex contains the WBZ. Each of these aspects is important for conidium development and consequently important for survival, dispersal, and possibly virulence of *F. verticillioides*.

Several genes are noted to affect sporulation of *F. verticillioides*. Li *et al*, reported that as of 2006, the only genes known to have an effect on the morphology of *F. verticillioides* were *fph1*, *hyd1*, *hyd2*, and *fcc1* (Glenn 2004, Fuchs *et al.*2004, and Shim and Woloshuk, 2001). The *fph1* mutant was unable to produce conidia and produced a germ tube-like outgrowth instead of normal enteroblastic conidiation (Glenn 2004). The *hyd1* and *hyd2* mutants produce microconidia in heads (collection of conidia in a spherical manner from the apex of the phialide)

instead of chains (Fuchs *et al.*2004). Lastly, the *fcc1* mutant showed reduced production of conidia when the pH was changed; there was reduced conidiation at pH 6 and conidiation was restored at pH 3 (Shim and Woloshuk, 2001). Since then, four other genes have been reported to be involved in conidiation, including *fvve1*, *fpk1*, *fvmk1*, and *gap1* (Li *et al.*2006, Zhao *et al*. 2010, Zhang *et al.* 2011, and Sagaram *et al.* 2007). Of these, *fpk1* and *fvmk1* have also been found to be involved in pathogenesis and *fvmk1* involved in fumonisin production.

Of particular note is *fvve1*, a homolog of *veA* from *Aspergillus nidulans*, which is important for cell wall integrity, cell surface hydrophobicity and conidiation pattern (Li *et al.* 2006). To determine the function of *fvve1*, several phenotypic tests were performed. When plated on V8 agar, the *fvve1* mutant produced slimy colonies without aerial hyphae, instead of the fluffy colonies with abundant aerial hyphae as in the wild-type. When comparing micro- and macroconidia in the wild-type versus mutant, there was an increase in the production of macroconidia. The wild-type ratio of macroconidia to microconidia was restored when the mutants were grown on V8 agar medium supplemented with osmotic stabilizers. This suggests the mutants had a weakened cell wall (because the cell walls of macroconidia were thicker than the cell walls of microconidia) thus resulting in the increased production of macroconidia over microconidia.

In the *fpk1* study, an important observation was that hyphal growth of the mutant was reduced in both solid and liquid media (Zhao *et al.* 2010). Shorter aerial hyphae were produced from the mutant and the colonies formed were sparse, more spread out than the wild type. Therefore, this gene is involved in hyphal development. They also found *fpk1* to be involved in spore germination and plant infection.

Glenn and co–workers discovered conidiation mutants as the result of mating two strains wild type for conidia production, MRC826 and NRRL 25059 (Glenn *et al*. 2004). MRC826, isolated from corn in South Africa, is wild type for conidia production and contains the fumonisin (FUM) gene cluster, whereas NRRL 25059, isolated from banana in Honduras, is also wild type for conidia production but lacks the FUM gene cluster (Figure 1). When the two strains were mated, a new strain, AEG 1–1–57 was created. Although the parental strains are from different geographic locations and were isolated from different hosts, the fact that they were able to mate indicates a close relationship between the two. The progeny AEG 1-1-57 was also wild type for conidial production but, like NRRL 25059, does not contain the FUM gene cluster. AEG 1-1-57 was back-crossed with the wild type parent, MRC 826 resulting in the formation of an octad of meiotically related progeny. The octad, labeled AEG 3–A3–1 through AEG 3-A3-8, consisted of four sets of twins, half of which contained the FUM gene cluster and half that did not. Half of the octad lost the ability to produce conidia and they no longer produced the wall building zone needed for conidium formation from the phialide apex. The loss of conidial production from the mating of two conidia–producing parents invites many questions. These aconidial mutants could be helpful in identifying mechanism(s) of sporulation in *F. verticillioides*.

Mycotoxins

Mycotoxins are secondary metabolites released by and specific to fungal species. Once a phytopathogenic fungus is established in the plant, it can release mycotoxins to damage the host and contribute to an infestation (Bennett and Klich 2003). These mycotoxins may be harmful to humans, plants, and animals. Some of the major groups of mycotoxins are aflatoxin, ochratoxin, citrinin, ergot alkaloids, patulin, and *Fusarium* toxins (Gelderblom *et al.* 1988). For some

Fusarium species, several mycotoxins such as trichothecenes, zearalenone, and fumonisin are produced (Logrieco *et al.* 2002). These mycotoxins released from *Fusarium* species normally affect maize, wheat, and other grain crops. Crops like maize are in high demand for humans and animals, therefore concern exists for mycotoxin-associated diseases, including cancer (Marasas 1996).

Fumonisin Production

Fumonisin mycotoxins, produced by *F. verticillioides* and other species, are polyketide-derived secondary metabolites (Sagaram *et al*. 2007, Brown *et al.* 2008). These mycotoxins are known to be harmful to humans, animals, and plants and have been noted to be carcinogenic. Upon consumption of fumonisin-contaminated crops such as maize, animals and humans may develop several diseases, such as esophageal cancer and neural tube defects in humans (Marassas *et al*. 2004). The production of fumonisins can also cause damage to plants, such as seedling disease (Glenn *et al.* 2008).

The structure of these compounds is similar to the sphingolipid precursor sphinganine (Brown *et al.* 2007 and 2008). Sphingolipids serve many roles in eukaryotes; they are found in cell membranes and are involved in signaling pathways (Glenn *et al.* 2008). Due to its structural similarity to sphinganine, fumonisins can inhibit the ceramide synthase enzyme involved in sphingolipid metabolism (Wang *et al.* 1991, Kim and Woloshuk 2011). Fumonisin B1 is most notably responsible for this mechanism of action. Fumonisin derivatives B1, B2, and B3 are the most studied due to their involvement in virulence of the fungus (Glenn and Bacon 2009, Glenn *et al.*2002).

Genes encoding fumonisin biosynthesis are part of a cluster of genes called the FUM gene cluster (Desjardins *et al.* 1996, Glenn *et al.* 2008 and Brown *et al.* 2008). One of the genes

in the FUM gene cluster, *FUM1*, has been further studied. In 2002, Desjardins *et al.* identified *FUM1* as encoding a polyketide synthase required for fumonisin biosynthesis. This study also showed *FUM1* is not involved in maize ear rot or ear infection. Genes *FUM2* and *FUM3* are closely linked to *FUM1* (Desjardins *et al.* 1996) and collectively, the cluster of these three genes constitute a fumonisin biosynthetic gene cluster. Another well studied gene from the FUM gene cluster is *FUM21* (Brown *et al.* 2007). *FUM21* is located adjacent to *FUM1* and is postulated to be involved in transcriptional regulation due to the presence of a Zn2Cys6 DNA-binding domain. To date, there are 22 genes located in this FUM gene cluster (Brown *et al*. 2007).

LITERATURE CITED

1. Alessandra L, Luca P, Adriano M. 2010. Differential gene expression in kernels and silks of maize lines with contrasting levels of ear rot resistance after Fusarium verticillioides infection. *Journal of Plant Physiology* 167: 1398-1406.

2. Bacon CW and Hinton DM. 1996. Symptomless endophytic colonization of maize by *Fusarium moniliforme*. *Canadian Journal of Botany* 74: 1195-1202.

3. Bennett JW and Klich M. 2003. Mycotoxins. *Clinical Microbiology Reviews* 16(3): 497-516.

4. Brown DW, Butchko RAE, Busman M, and Proctor RH. 2007. The *Fusarium verticillioides FUM* gene cluster encodes a Zn(II)2Cys6 protein that affects *FUM* gene expression and fumonisin production. *Eukaryotic Cell* 6(7): 1210-1218.

5. Brown DW, Butchko RAE, and Proctor RH. 2008. Genomic analysis of *Fusarium verticillioides*. *Food Additives and Contaminations* 25(9): 1158-1165.

6. Desjardins AE, Munkvold GP, Plattner RD, and Proctor RH. 2002. *FUM1*—A gene required for fumonisin biosynthesis but not for maize ear rot and ear infection by *Gibberella moniliformis* in field tests. *Molecular Plant-Microbe Interactions* 15(11): 1157-1164.

7. Desjardins AE, Plattner RD, and Proctor RH. 1996. Linkage among genes responsible for fumonisin biosynthesis in *Gibberella fujikuroi* mating population A. *Applied and Environmental Microbiology* 62(7): 2571-2576.

8. Foley DC. 1962. Systemic infection of corn by *Fusarium moniliforme*. *Phytopathology* 52: 870-872.

9. Fuchs U, Czymmek KJ, and Sweigard JA. 2004. Five hydrophobin genes in Fusarium verticillioides include two required for microconidial chain formation. *Fungal Genetics and Biology* 41: 852–864.

10. Gelderblom WC, Jaskiewicz K, Marasas WF, Thiel PG, Horak RM, Vleggaar R, and Kriek NP. 1988. Fumonisins – novel mycotoxins with cancer-promoting activity produced by *Fusarium moniliforme*. *Applied and Environmental Microbiology* 54(7): 1806-1811.

11. Glass NL, Jacobson DJ, and Shiu PKT. 2000. The genetics of hyphal fusion and vegetative incompatibility in filamentous ascomycete fungi. *Annual Review of Genetics* 34: 165-186.

12. Glenn AE. 2006. Natural variation of ascospore and conidial germination by *Fusarium verticillioides* and other *Fusarium* species. *Mycological Research* 110: 211-219.

13. Glenn AE and Bacon CW. 2009. FDB2 encodes a member of the arylamine N-acetyltransferase family and is necessary for biotransformation of benzoxazolinones by *Fusarium verticillioides*. *Journal of Applied Microbiology* 107: 657-671.

14. Glenn AE, Gold SE, and Bacon CW. 2002. *Fdb1* and *Fdb2*, *Fusarium verticillioides* loci necessary for detoxification of performed antimicrobials from corn. *Molecular Plant-Microbe Interactions* 15(2): 91-101.

15. Glenn AE, Richardson EA, and Bacon CW. 2004. Genetic and morphological characterization of a *Fusarium verticillioides* conidiation mutant. *Mycologia* 96(5): 968-980.

16. Glenn AE, Zitomer NC, Zimeri AM, Williams LD, Riley RT, and Procter RH. 2008. Transformation-mediated complementation of a FUM gene cluster deletion in *Fusarium verticillioides* restores both fumonisin production and pathogenicity on maize seedlings. *Molecular Plant Microbe Interactions* 21(1): 87-97.

17. Hagen I, Ecker M, Lagorce A, Francois JM, Sestak S, Rachel R, Grossman G, Hauser NC, Hoheisel JD, Tanner W, and Strahl S. 2004. Sed1p and Srl1p are required to compensate for cell wall instability in *Saccharomyces cerevisiae* mutants defective in multiple GPI-anchored mannoproteins. *Molecular Microbiology* 52(5): 1413-1425.

18. Kim H and Woloshuk CP. 2011. Functional characterization of *fst1*in *Fusarium verticillioides* during colonization of maize kernels. *Molecular Plant-Microbe Interactions* 24(1):18-24

19. Larson TM, Kendra DF, Busman M, and Brown DW. 2011. Fusarium verticillioides chitin synthases CHS5 and CHS7 are required for normal growth and pathogenicity. *Current Genetics* 57: 177-189.

20. Leslie JF and Summerell BA. 2006. The *Fusarium* laboratory manual. APS Press.

21. Li S, Myung K, Guse D, Donkin B, Proctor RH, Grayburn WS, and Calvo AM. 2006. *FvVE1* regulates filamentous growth, the ratio of microconidia to macroconidia and cell wall formation in *Fusarium verticillioides. Molecular Microbiology* 62(5): 1418-1432.

22. Logrieco A, Mulé G, Moretti A, and Bottalico A. 2002. Toxigenic *Fusarium* species and mycotoxins associated with maize ear rot in Europe. *European Journal of Plant Pathology*. 108: 597-609.

23. Marasas WFO. 1996. Fumonisins: history, world-wide occurrence, and impact. *Advanced Experimental Medical Biology* 392: 1-17.

24. Marasas WFO, Riley RT, Hendricks KA, Stevens VL, Sadler TW, Gelineau-van Waes J, Missmer SA, Cabrera J, Torres O, Gelderblom WCA, Allegood J, Martı´nez C, Maddox J, Miller JD, Starr L, Sullards MC, Roman AV, Voss KA, Wang E, and Merrill, Jr AH. 2004. Fumonisins disrupt sphingolipid metabolism, folate transport, and neural tube development in embryo culture and in vivo: a potential risk factor for human neural tube defects among populations consuming fumonisin-contaminated maize. *The Journal of Nutrition* 134(4): 711-716.

25. Munkvold GP, Hellmich RL, and Showers WB. 1997a. Reduced fusarium ear rot and symptomless infection in kernels of maize genetically engineered for European Corn Borer resistance. *Disease Control and Pest Management* 87(10): 1071-1077.

26. Munkvold GP, McGee DC, and Carlton WM. 1997b. Importance of different pathways for Maize kernel infection by *Fusarium moniliforme*, *Ecology and Epidemiology* 87(2): 209-217.

27. Ohara T and Tsuge T. 2004. *FoSTUA*, Encoding a basic helix-loop-helix protein, differentially regulates development of three kinds of asexual spores, macroconidia, microconidia, and chlamydospores, in the fungal plant pathogen *Fusarium oxysporum.* 3(6): 1412-1422.

28. Ohara T, Inoue I, Namiki F, Kunoh H and Tsuge T. 2004. *REN1* is required for development of microconidia and macroconidia, but not of chlamydospores, in the plant pathogenic fungus *Fusarium oxysporum*. *Genetics* 166: 113-124.

29. Rittenour WR, and Harris SD. 2008. Characterization of Fusarium graminearum Mes1 reveals roles in cell-surface organization and virulence. *Fungal Genetics and Biology* 45: 922-946.

30. Ruiz-Rolda´n MC, Ko¨hli M, Roncero MIG, Philippsen P, Di Pietro A, and Espeso EA. 2010. Nuclear dynamics during germination, conidiation, and hyphal fusion of *Fusarium oxysporum. Eukaryotic Cell* 9(8): 1216-1224.

31. Sagaram US, Shaw BD, and Shim WB. 2007. *Fusarium verticillioides* GAP1, a gene encoding a putative glycolipid-anchored surface protein, participates in conidiation and cell wall structure but not virulence. *Microbiology* 153: 2850-2861.

32. Shim WB and Woloshuk CP. 2001. Regulation of fumonisin B(1) biosynthesis and conidiation in Fusarium verticillioides by a cyclin-like (C-type) gene FCC1. *Applied Environmental Microbiology* 67: 1607–1612.

33. Shonig B, Brown DW, Oeser, and Tudzynski B. 2008. Cross-species hybridization with *Fusarium verticillioides* microarrays reveals new insights into *Fusarium fujikuroi* nitrogen regulation and the role of AreA and NMR. *Eukaryotic Cell* 7(10): 1831-1846.

34. Sutton JC. 1982. Epidemiology of wheat head blight and maize ear rot caused by *Fusarium graminearum*. *Canadian Journal of Plant Pathology* 4: 195-209.

35. Vincent P, Goubet F, Carapito R, and Jeltsch J-M. Plant cell wall degradation with a powerful *Fusarium graminearum* enzymatic arsenal. *Journal of Microbiological Biotechnology* 19(6): 573-581.

36. Wang E, Norred WP, Bacon CW, Riley RT and Merrill, Jr. AH. 1991. Inhibition of sphingolipid biosynthesis by fumonisins implications for diseases associated with *Fusarium moniliforme*. *Journal of Biological Chemistry* 266(22): 14486-14490.

37. Williams LD, Glenn AE, Zimeri AM, Bacon CW, Smith MA, Riley RT. 2007. Fumonisin disruption of ceramide biosynthesis in maize roots and the effects on plant development and *Fusarium verticillioides*-induced seedling disease. *Journal of Agricultural and Food Chemistry* 55: 2937-2946.

38. Wiemann P, Brown DW, Kleigrewe K, Bok JW, Keller NP, Humpf H-U and Tudzynski B. 2010. FfVel1 and FfLae1, components of a velvet-like complex in *Fusarium fujikuroi*, affect differentiation, secondary metabolism and virulence. *Molecular Microbiology* 77(4): 972-994.

39. Yates IE, Bacon CW, and Hinton DM. 1997. Effects of endophytic infection by *Fusarium moniliforme* on corn growth and cellular morphology. *Plant Disease* 81(7): 723-728.

40. Zhang Y, Choi YE, Zou X, and Xu JR. 2011. The FvMK1 mitogen-activated protein kinase gene regulates conidiation, pathogenesis, and fumonisin production in *Fusarium verticillioides*. *Fungal Genetics and Biology* 48: 71-79.

41. Zhao PB, Ren AZ, Xu HJ, and Li DC. 2010. The gene *fpk1*, encoding a cAMP-dependent protein kinase catalytic subunit homolog, is required for hyphal growth, spore germination, and plant infection in *Fusarium verticillioides*. *Journal of Microbiology and Biotechnology* 20(1): 208-216.

CHAPTER 2

IDENTIFICATION AND ANALYSIS OF *FUSARIUM VERTICILLIOIDES* GENES DIFFERENTIALLY EXPRESSED DURING SPORULATION

INTRODUCTION

The ubiquitous endophytic fungus *Fusarium verticillioides* (teleomorph *Gibberella moniliformis*) is among the most important phytopathogenic and mycotoxigenic fungi (Li *et al.* 2010). It has a relatively narrow host range, typically limited to cereals. *F. verticillioides* is most commonly seen as a major pathogen of maize (*Zea mays*). It causes root rot, stalk rot, seedling blight, and most notably maize ear rot (Leslie and Summerell 2006, Alessandra *et al.*2010, Cotton and Munkvold 1998). These diseases result in major economic losses in maize production. Because the United States is the leading producer and exporter of maize worldwide, *F. verticillioides* is a threat to the industry.

This fungus can cause diseases in animals due to consumption of feed contaminated with fumonisin mycotoxins, toxic secondary metabolites that cause severe species-specific diseases (Glenn 2007). *Fusarium* species collectively release several mycotoxins such as trichothecenes, zearalenone, and fumonisins. The economic losses and reduced quality of maize contaminated with fumonisins are major concerns with *Fusarium* mycotoxins. The presence of FB1 in contaminated feed is linked to equine leukoencephalomalacia and porcine pulmonary oedema syndrome when toxic material is consumed (Gelderblom *et al.* 1988). Scientists are also studying the correlation between FB1 and human esophageal cancer and neural tube defects

(Marasas *et al.* 2004). These are just a few examples of how *F. verticillioides* can be so detrimental to the maize industry.

A major facet in the colonization of maize is dissemination of spores. In the case of *F. verticillioides*, the perfect stage is rarely seen as compared to its close relative *Gibberella zeae* (Li *et al.* 2010). *F. verticillioides* produces conidia, non-motile asexual spores, which are disseminated via wind, rain, or insects. Conidia are thought to play a key role in the survival of the fungus and are therefore, cardinal in the infection of maize plants. There are two types of conidia produced by *F. verticillioides* – macroconidia and microconidia (Leslie and Summerell 2006). Macroconidia are hyaline, 3-5 septate and have tapered ends. These conidia are the larger of the two types and have a distinct crescent shape. Microconidia are single celled and much smaller in size. The prolific production of microconidia allows successful plant infection in addition to survival and dispersal of the fungus. These microconidia form in chains from the tip of a phialide, which is a specialized cell producing spores through a reiterative mitotic process (Glenn *et al.* 2004).

Although the disease cycle is well understood, the molecular mechanisms that drive these pathways have yet to be completely defined. To expand the understanding of the genetics involved in sporulation, examinations of previously characterized conidiation mutants were initiated. As described by Glenn *et al* (2004), upon mating the two strains MRC 826 and AEG 1-1-57, both of which are capable of producing conidia, spontaneous mutations occurred resulting in half of the progeny being unable to produce conidia (Figure 1). These results suggest a meiotically-induced genetic mutation that may have resulted in deletion of one or more genes involved in conidiation. The aconidial mutants produced germ tube-like outgrowths from the tips

of phialides instead of normal conidial development. The mutants also lack a wall building zone at the phialide tip needed for forming a conidium wall.

The overall objective of this project was to characterize genes involved in sporulation, specifically the production of spores from phialides. This study aims to analyze sporulation-associated genes by using mutants from the previous genetic crosses discussed above. A primary objective was to determine if the absence of a single gene or multiple genes is responsible for the mutant phenotype previously characterized. Targeted deletions were performed on select candidate genes also identified from the microarray data. This research will allow further characterization of possible conidiation-associated genes based on morphology, pathogenicity, and mycotoxin production analyses.

MATERIALS AND METHODS

Fungal strains and culture conditions

Many of the fungal strains used in this study (MRC 826, NRRL 25059, AEG 1-1-57, and the octad of strains AEG 3-A3-1 to AEG 3-A3-8) were described previously (Glenn *et al*. 2004). Other strains were created during this study. For routine culturing, strains were grown in potato dextrose broth (PDB), on potato dextrose agar (PDA), or on V8 agar. Cultures were incubated at 27°C in the dark, in addition to shaking (180 rpm) for PDB cultures. Conidiating strains typically were grown for three days in PDB and non-conidiating strains for 7 days. All strains were kept frozen in 10% glycerol at -80°C for long term storage.

Microarray experiment

MRC826 and AEG 3-A3-5 cultures were grown in PDB at 27°C in the dark, shaking at 180 rpm for three and 7 days, respectively. A 1ml sample of each culture was transferred to a new PDB flask to initiate fresh growth. Cultures were grown at two different time-points (24h and 48h) for

each strain. Fungal material was harvested via vacuum filtration and stored at -80°C until ready to use. RNA was extracted using the Qiagen Total RNA Prep Kit (Qiagen Sciences, Maryland, USA), including the DNase step. The RNA samples were sent to Roche Nimblegen (Roche NimbleGen Inc., Madison, WI, USA) for processing (Brown *et al.*2005).

Southern blot analysis of Chromosome 11

Microarray data revealed a possible deletion of the entire Chromosome 11. Three probes, *CRH1* (Congo Red Hypersensitivity 1) forward primer CTGATGATGACAATTGGGAGGG and reverse primer GAACAGTCATGGTGTAAGGACC, *DCW1* (Defect in Cell Wall 1) forward primer CATAAATATCCTGACCCGCCTG and reverse primer CACAGTCTTTATGTCC TCCAGC, and 3.21.1 (lysophospholipase) forward primer GCTACATACGACCGTGTTGTTT and reverse primer ATTCGTCTTTCCATCCCAGCAG were used to target different regions of Chromosome 11. These gene-specific probes were generated using the digoxigenin (DIG) PCR labeling kit according to manufacturer's protocol (Roche). PCR cycling parameters were as follows: initial denaturation at 95°C for 5m, followed by 40 cycles of 95°C for 10s, 60°C for 10s, and 72°C for 40s. A final extension was included at 72°C for 7m. The template DNA used for probe PCR was from MRC 826. Genomic DNA from MRC826, NRRL 25059, AEG 1-1-57, and the AEG 3-A3 octad was extracted using the DNeasy Plant Mini Kit (Qiagen Sciences, Maryland, USA). DNA (2µg) was digested with *EcoRI*. A 1% agarose gel using 1X TAE buffer, verified the digestion. A Southern blot was performed based on standard techniques and procedures followed by detection with DIG labeling (Roche) (Glenn *et al.* 2008). Detection (50 minutes) was performed using the Alpha Innotech FluorChem 8000 digital imaging system (San Leandro, CA, USA). Probing and detection were repeated using the same stripped membrane for all probes.

Sequence and statistical analysis

Gene and protein sequence analyses were performed using *F. verticillioides* genomic information available from the BROAD Institute via the *Fusarium* Comparative Database (www.broadinstitute.org). Protein sequences provided by the BROAD Institute were submitted to the web-based peptide prediction databases, Protein Prowler and SoftBerry ProtComp. Primers and probes were identified using Sequencher (Version 4.10.1, Gene Codes Corp., Ann Arbor, MI) and Integrated DNA Technologies, Inc. (IDT). Sequence similarity searches were performed using BLASTP and BLASTX searches of the National Center for Biotechnology (NCBI) database. Statistical analyses were performed using the Analyse-It Standard Edition software (Analyse-It Software, Ltd., Microsoft Corporation).

Gene deletion

Generation of gene deletion constructs was performed using the DelsGate method with hygromycin as a selectable marker for fungal transformation (Garcia-Pedrajas *et al.* 2008). Briefly, an 865-bp DNA fragment (5' flank) and an 864-bp DNA fragment (3' flank) were amplified via PCR. Primer #1 and #2 amplified the 5' flank, while #3 and #4 amplified the 3' flank (Table 1). The fragments were collectively cloned into a previously designed pDONR-A-Hyg deletion plasmid vector via the Invitrogen BP Clonase system (Invitrogen Corporation, Carlsbad, CA, USA). This reaction generated a linear molecule of the vector and flanking DNA, which was transformed into One Shot TOP10 Electrocompetent *E. coli* cells (Invitrogen Corporation, Carlsbad, CA, USA) using electroporation (BioRad micropulser, Ec1 setting, 1.8kV, 3.6ms), according to manufacturer's protocol. *E. coli* transformants were screened to isolate proper constructs. Transformants were grown in LB + kanamycin (50µg/ml) overnight at 37°C with shaking at 250rpm. Plasmid preps were performed using the Qiagen Spin Miniprep

Kit (Qiagen Sciences, Maryland, USA). Plasmids were screened via PCR using primer sets "I-*SceI* F" and "Donr F," then "I-*SceI* R" and "Donr R" (Table 1). PCR product was viewed on a 1% agarose gel using TAE buffer. After confirmation, the plasmid was digested with I-*SceI* to linearize the deletion construct for fungal transformation. Digestion was incubated overnight at 37°C. Reaction clean-up was performed using Qiagen PCR Purification Kit (Qiagen Sciences, Maryland, USA).

Fungal transformation

Fungal transformation was performed using *F. verticillioides* strain MRC 826 protoplasts according to established protocol (Glenn *et al.* 2008). Briefly, the linear deletion construct was added to 100µl of STC (1.2M Sorbitol, 50mM $CaCl_2$-$2H_2O$, 10mM Tris-HCl) solution followed by the addition of 20µl of protoplasts and 50µl 30% PEG (30% PEG 8000, 10mM Tris-HCl, 50mM $CaCl_2$) solution. Cells clumped together and more STC (2ml) was added. The transformation mixture was poured into a previously prepared molten overlay medium (Glenn *et al.* 2008). The transformation/overlay mixture was added over solidified 2x regeneration medium plates. The next morning, 1% water agar containing hygromycin B was added to each plate (final concentration of 150µg/ml hygromycin B in the total agar). Hygromycin-resistant isolates were recovered after 3-7 days of incubation at 27°C. Transformants were screened by PCR to identify knockouts. The first round of PCR used gene-specific primers (Table 3). Primers used for secondary PCR on putative knockouts were "5'outer" and "HygF DG" and "KanR DG" and "3'outer" (Table 1). Southern blot was performed as described above on potential knockouts using *XbaI*. The probe used for this Southern blot was amplified using FVEG_10983 primers "10983 So. F" (TGGCGACGTAGACGAGTCATT) and "10983 So. R" (AAACCAGAGCCAACCACCA CAA).

Inoculation of maize seed

Five treatments were used to test the virulence of the FVEG_10983 mutants versus wild type; one wild type strain MRC 826, one ectopic strain Transformant #21, two knockouts #37 and #46, and a water control. Kernels of sweet corn (Silver Queen) were externally sterilized using 100% bleach and rinsed with distilled water followed by an imbibation period of 1 hour. Seed were then heat-treated by placing the containers into a 60°C water bath for 5 minutes. Fresh sterile water was added to the containers after draining. Forty seed were added to the appropriately labeled petri dish. A fungal spore suspension (10^6 conidia/ml) was prepared from PDB cultures. The spore suspension (10ml) was added to the seed. Sterile water was applied to the un-inoculated control seed. Seed were incubated 16-18 hours at 27°C.

Planting of inoculated seed

Three replicate pots were prepared for each treatment. Soil was sterilized and added to each pot and gently compacted. Ten seed were placed in each pot and then covered with a final layer of sterilized soil. The pots were each watered from below in saucers and placed in the growth chamber with day/night temperature cycles of 30°C/20°C, respectively. Plants were watered on days 2, 4, 6, and then watered thereafter based on need.

Virulence analysis

After 16 days, virulence was assessed based on the presence of lesions on leaves. Only plants with necrotic lesions were labeled as diseased. Disease was quantified per pot and per treatment. Disease incidence per pot was calculated by the number of diseased plants in each pot compared to the total number of plants per pot multiplied by 100. Disease incidence per treatment was calculated by averaging the percent disease incidence per pot for each of the three replicates. Aerial growth (shoots) was stored at -80°C immediately after harvesting. The aerial growth

samples were lyophilized using a VirTis Benchtop K system (Warminster, PA, USA) for 48 hours, and biomass measurements (grams dry weight) were taken with an analytical scale.

Endophytic growth analysis

To test the ability of the mutant strains to endophytically grow within the maize plants, a section (1-2cm) of each stalk was removed for sampling. These stalk sections were externally sterilized with 70% ethanol followed by 10% bleach then sterile water, each with a duration of 1 minute. Leaf sections were then placed on PDA plates and incubated for 5-7 days at 27°C. The number of stalk sections with fungi growing from the cuticles was recorded. Fungi growing from the ends of knockout-inoculated stalk sections were then transferred to PDA supplemented with hygromycin B (150µg/mL) to determine if the knockout strain was retrieved. Because the deletion construct contains hygromycin resistance, only the knockout strains should grow. If there was growth after 3-7 days, the true knockout strain was considered retrieved from the plant; therefore validating the ability to grow endophytically.

Soil colonization analysis

Next, the ability of the strains to colonize soil was tested. The soil, including the root system, from each pot was placed in a sterile plastic bag. Fresh weight (1g) of soil was placed into 40 ml of sterile water. Serial dilutions were made and 100µl of a 1000-fold soil dilution was inoculated onto PDA plates supplemented with streptomycin and ampicillin (50µg/ml each). Plates were incubated at 27°C for 3-5 days. Five plates were prepared for each treatment. Individual fungal colonies were counted per plate using an Alpha Innotech FluorChem 8000 digital imaging system (San Leandro, CA, USA). Ten single colonies from each knockout treatment were grown on PDA supplemented with hygromycin B (150µg/mL) to determine if the knockout strain was retrieved.

Fumonisin quantification

Fumonisin quantification was performed as previously described (Zitomer *et al.* 2008). The corn used was Pioneer 3223. Cracked corn (5g) was placed in 20ml scintillation vials and distilled water (2.5mL) was added to each vial. Foam plugs were used to close the vials, and corn was imbibed for four hours at RT. Vials were autoclaved for 30 mins and left to cool. Sterile water (10mL) was added to a 7 day old culture of fungal strain grown on PDA. Mycelium was rubbed with a glass rod to create a fungal spore suspension. The fungal spore suspension (250µl) was added to each vial, three replicates were used for each strain. Vials were incubated in the dark for two weeks at 27°C. At the end of the incubation period, 10ml of acetonitrile:water (1:1) containing 5% formic acid was added to each vial. The vials were capped tightly using the original caps with Teflon inserts placed inside. The vials were shaken vigorously to completely disrupt the fungal cells. Vials were placed on a rocker shaker for three hours. HPLC and mass spectrometry analyses were conducted as previously described (Zitomer *et al.* 2008) using a Finigan Micro AS Autosampler coupled to a Surveyor MS pump (Thermo-Fisher, Woodstock, GA, USA). Separation was accomplished using a Metachem Inertsil 5µm ODS-3 column, 150x3mm (Metachem Technologies, Inc. Torrance, CA, USA).

Conidial counts

To quantify the production of conidia, agar plugs (6mm diameter) were taken from the outer margin of growth on PDA plates, three days for wild type (MRC 826) and ectopic (transformant #21) and 6 days for knockout strains #37 and #46. Plugs were transferred to 1ml of sterile water. After vortexing, dilutions were made (10x and 100x), and conidia were counted using a hemacytometer. Next, a comparison of the number of phialides was made. Again, 10x and 100x

dilutions were used but from PDB cultures. The number of phialides per field of view and the number of phialides per single hyphal strand were the basis for measurement.

Light microscopy and fluorescent staining

Observations were made using a Leica DM600B microscope (Leica Microsystems, Germany) equipped with a Hamamatsu ORCA ER digital camera (Hamamatsu Photonics K.K., Japan). The software used was LAS-AF6000 (Leica Application Suite Advanced Fluorescence 6000, Leica Microsystems, Germany). For light microscopy, the strains were grown routinely as stated above. For staining, strains MRC826, ectopic (transformant #21), and two knockouts (#37 and #46) were each grown for 7 days. To allow manipulation of the fungi during staining, the strains were grown on Spectra/POR dialysis membrane (Spectrum Medical Industries, Inc., Los Angeles, CA, USA) cut into 70mm circles, washed in sterile water, and autoclaved for 30mins. After cooling, sterile forceps were used to place each membrane on a previously prepared PDA plate. Frozen fungal strains were directly inoculated onto the membrane surface using a sterile loop. Plates were incubated at 27°C in the dark for 7 days. The fixer (3.2% formaldehyde, 50mM K_2HPO_4, and 0.2% Triton X-100), Hoechst/Calcofluor Stain (Hoechst 33342 – 1mg/ml, Calcofluor – 1 mg/ml, and sterile water), and mounting solution (100mM K_2HPO_4, 50% Glycerol, and 0.1% n-Propyl-gallate) were prepared according to a previous protocol (Glenn *et al.* 2004). Using a sterile razor blade, small square sections (~0.25cm^2) were cut from the margin of growth where the newest growth appears. Sections were thoroughly saturated by the fixer for 30 minutes. Hyphal mats were removed from sections at this point. Hyphal mats were transferred to sterile water and rinsed briefly. The Hoechst/Calcofluor stain solution (450µl) was transferred to a sheet of parafilm and the hyphal mats were submerged into the stain solution and incubated for 10 minutes. During this time, mounting medium (15µl) was placed on a slide.

Hyphal mats were transferred to the mounting medium and a coverslip was applied. Slides were covered in foil to protect from light and left to dry for 30 minutes. The cover slip was sealed with nail polish and the slides were stored at 4°C until ready to use. Observations were made using DAPI and GFP fluorescent filters.

RESULTS

Microarray analyses identify large chromosomal deletion

A Roche NimbleGen microarray experiment was performed to measure changes in gene expression levels, specifically genes expressed in the conidiating wild-type strains MRC826 and not in the aconidial mutant AEG 3-A3-5. The purpose was to narrow the search for genes that may be involved in the conidiation mutation generated previously from a series of crosses (Figure 1). Both strains were grown at two different time-points, 24 hours and 48 hours, in liquid media (PDB) with two biological repetitions for each strain at each time-point. Dr. Daren Brown, a collaborator from United States Department of Agriculture, Agriculture Research Service in Peoria, IL provided assistance in the analysis and interpretation of the microarray data.

The data revealed a possible deletion of an entire chromosome, Chromosome 11. The genomic sequence data for Chromosome 11 from BROAD Institute is split between two supercontigs; Supercontig 15 containing over 1.3 million base pairs and Supercontig 21 with almost 700,000 base pairs. The microarray analysis showed a total of 834 genes expressed in wild type but not in the mutant at both the 24 and 48 hour time points. Of those, 472 (56.6%) are located on Chromosome 11 and 362 (43.4%) are located on other chromosomes. The fact that over half of the differentially expressed genes are located on Chromosome 11 suggested the conidiation mutation described in Glenn *et al.* (2004) (see Figure 1) may be the result of a large chromosomal deletion. Southern blot experiments were performed using probes designed from

loci located along Chromosome 11. Three probes, targeting the genes *CRH1* (Congo Red Hypersensitivity 1; FVEG_10527), *DCW1* (Defect in Cell Wall 1; FVEG_10899), and 3.21 (lysophospholipase; FVEG_12994), were previously designed. *CRH1* and *DCW1* are two loci on Supercontig 15, and 3.21 is located on Supercontig 21. All three probes revealed the same hybridization pattern; the genes were present for all strains except AEG 3-A3-5 and AEG 3-A3-6 (Figure 2). These Southern blot results suggested Chromosome 11 (or major parts of it) was deleted from one set of mutant twins, AEG 3-A3-5 and AEG 3-A3-6. However, the three probes hybridized to all other strains, including the other aconidial strains (i.e., AEG 3-A3-2 and AEG 3-A3-8; see Figure 1), thus indicating the apparent Chromosome 11 deletion was not linked to the conidiation mutation.

Identification of candidate genes

From the microarray analysis, the remaining 362 genes not expressed in AEG 3-A3-5 and not located on Chromosome 11 were sorted based on fold change, mainly focusing on the 24 hour time-point to simplify data analysis. From this list, 13 genes had a 9-fold or higher difference in expression in the wild type as compared to the mutant (Table 2). In particular, FVEG_10983 had a 72-fold difference and FVEG_14159 was 38-fold different.

To further narrow the gene list to candidate genes potentially involved in the AEG 3-A3-5 conidiation mutation, a co-segregation PCR analysis was performed. This involved designing gene-specific primers (Table 3) for each of the 13 genes previously described and utilizing PCR to determine if the gene is present in the conidia-producing strains and absent in the aconidial mutant strains. As a positive PCR control, an elongation factor (EF1) primer set was used. Figure 3 shows the results of the co-segregation PCR experiments. FVEG_14159 was the only gene found to be present in the conidia-producing strains and absent in the mutant strains.

Because of these results, FVEG_14159 was targeted for knockout. NCBI BLAST searches indicated FVEG_14159 as a putative C6 transcription factor, containing a Zn2Cys6 fungal-type DNA-binding domain.

Although shown to be present in aconidial mutant strains AEG 3-A3-2 & AEG 3-A3-8, FVEG_10983 also was targeted for knockout. This gene is unique in the fact that it has a 72-fold difference in expression between wild-type and mutant. FVEG_10983 was also found to lack homology to any other characterized protein, possesses no conserved protein domains to suggest its function, and NCBI BLASTP searches showed significant similarity (1e-04 or less) only to a few accessions of predicted proteins from phialide-producing filamentous fungi of the phylum Ascomycota. Web-based databases were consulted to give further information on the location, function, or interaction of this protein. The predicted protein sequence from the BROAD Institute was submitted to the signal peptide prediction programs Protein Prowler (Boden and Hawkins, 2005) and SoftBerry ProtComp (SoftBerry, Inc., NY, USA). Both revealed that FVEG_10983 is neither secreted nor located in the mitochondria but could be nuclear localized. The Protein Prowler database uses TargetP 1.1 software (Emanuelsson *et al.* 2000) to determine the localization of protein sequences. When interpreting the data, the lower the reliability class (RC) number, the more accurate the results. The RC classes rank from 1 (most accurate) to 5 (least accurate). The RC result for FVEG_10983 was class 1, thus suggesting the prediction is correct. The other signal peptide prediction program SoftBerry ProtComp uses neural networks and integral prediction systems to determine the localization of protein sequences.

Deletion of FVEG_10983

The selected candidate genes, FVEG_10983 and FVEG_14159, were targeted for deletion using the DelsGate method (García-Pedrajas *et al.* 2008). Several attempts to delete

FVEG_14159 were unsuccessful. While able to generate the DelsGate construct, 70 transformants were screened by PCR and none were determined to be knockouts. However, gene deletion of FVEG_10983 was successful, generating five potential knockouts. Table 1 lists the primers used for the DelsGate deletion experiments for FVEG_14159 and FVEG_10983.

PCR and Southern blot analyses were used to verify true knockout and ectopic strains. Figure 4 shows the anticipated results. For the Southern blot, wild type strain MRC826 hybridized as expected, with a ~3.8-kb fragment (*XbaI* digestion). Aconidial mutant strains AEG 3-A3-2 and AEG 3-A3-5 were tested to serve as positive and negative controls, respectively. FVEG_10983, proven by microarray and co-segregation PCR (previously described), is not expressed in strain AEG 3-A3-5 and does not amplify with gene-specific primers. The Southern blot, using an FVEG_10983 probe, showed hybridization for AEG 3-A3-2 (~3.8-kb band similar to wild type) and not AEG 3-A3-5. Transformant #21 was determined to be an ectopic strain because it possesses the native FVEG_10983 allele plus an insertion of the knockout construct giving an *XbaI* fragment of approximately 6.4-kb. Transformants #37 and #46 were true knockouts having hybridization to a single fragment of the expected size (6.6-kb) for the DelsGate deletion allele.

***Phenotypes of* ΔFVEG_10983**

The most obvious observation made about the *Δfveg_10983* mutants was the delay in growth. In liquid media, wild type MRC826 takes 2-3 days to reach confluent growth; however, the *Δfveg_10983* mutants require 6-7 days. This observation is similar to the conidiation mutants (e.g., AEG 3-A3-5), where it also takes 6-7 days. As shown in Figure 5, when grown in PDB liquid media, the wild type and ectopic strains produced a pinkish, cloudy growth in the flask (~3 days), whereas the *Δfveg_10983* mutants produced very little growth; the medium appeared

unchanged. After 6-7 days, the *Δfveg_10983* mutants began to grow but they produced a darker pink color without the cloudy appearance in the media. Microscopically, wild type and ectopic strains produced long hyphae with abundant conidia; however, the *Δfveg_10983* mutants produced hyphal fragments with significantly less conidia.

The same basic observations were made when the strains were grown in solid media (V8 agar). Figure 6 shows the *Δfveg_10983* mutants lack the "fluffy" aerial hyphae which the wild type possesses. To see if this reduction in aerial hyphae is also reflected in radial growth, measurements were taken (Figure 7). The *Δfveg_10983* mutant strains (as well as the ectopic strain) had slightly reduced radial growth compared to wild type. When comparing the wild type strain and the *Δfveg_10983* mutant strains, the data suggested FVEG_10983 is involved in development and growth of hyphae. Yet, when the ectopic results are calculated in, it appears that the transformation process itself may have an effect on the radial growth of these strains. Although the radial growth was only slightly reduced when comparing *Δfveg_10983* mutant strains to the wild type, the major *in vitro* difference between the strains was the loss of the production of aerial hyphae on agar media.

Microscopic analysis of ΔFVEG_10983

Deletion of FVEG_10983 resulted in reduced conidiation (Figure 5). There was much less growth in liquid media compared to agar, therefore conidia production was quantified from both media environments. To count conidial production on agar, a cork borer 6mm in diameter was used to collect a sample of the fungal colony from the margin of growth. Spores from these plugs were suspended in water and quantified. Wild-type produced an average of $1.4x10^8$ conidia/ml and the ectopic strain #21 produced an average of $1.1x10^8$ conidia/ml, while the *Δfveg_10983* mutant strains #37 and #46 produced an average of $5.8x10^7$ conidia/ml and

$4.2x10^7$ conidia/ml, respectively. Microscopic analyses of the *Δfveg_10983* mutants showed the possible loss of a unique characteristic for *F. verticillioides* which is the production of conidia in long chains from the tip of the phialide. Instead, the *Δfveg_10983* mutant strains were not observed to produce these chains and had few conidia at phialide tips. This suggests FVEG_10983 may be involved in the proper development of conidia. A time lapse experiment was attempted to observe the production of conidia from the tips of phialides over a 24 hour period. After numerous attempts, this experiment was unsuccessful.

The ratio of conidia per phialide was quantified from PDB liquid cultures over the span of 7 days based on number per field of view (FOV), resulting in a significant difference between the strains. On average per FOV, the wild type and ectopic strains produced 4.6 conidia/phialide and 4.5 conidia/phialide, respectively. On the other hand, the *Δfveg_10983* mutant strains #37 and #46 produced 1.8 conidia/phialide and 2.2 conidia/phialide on average per FOV, respectively. This suggests the *Δfveg_10983* mutant strains are reduced in their ability to produce multiple conidia from a single phialide.

Given that FVEG_10983 may encode a nuclear localized protein based on signal peptide predictions (previously discussed), wild-type MRC826, ectopic (transformant #21), and the 2 *Δfveg_10983* mutant strains (transformant #37 and #46) were stained with Hoechst 33342 and Calcofluor White to observe any changes in nuclear positioning or number. Figure 8 shows the strains after staining. MRC826 displays one nucleus per hyphal compartment, as does the ectopic strain. The *Δfveg_10983* mutant strains seem to possess several nuclei per hyphal compartment. This characteristic, however, is not seen throughout each hypha but it is seen commonly enough to suggest the deletion of FVEG_10983 resulted in abnormal compartmentalization of nuclei.

Maize seedling and virulence assays

Because *F. verticillioides* is known to be a major maize pathogen, *in planta* assays were performed in order to test virulence of the *Δfveg_10983* mutant strains. MRC826 was the positive control for this test and sterile water was used in place of inoculum to serve as the negative control. Three transformant strains, *Δfveg_10983* mutant strains #37 and #46, and ectopic strain #21 were tested. Both *Δfveg_10983* mutant strains were reduced in virulence, showing an increase in plant vigor as compare to the wild type and ectopic-inoculated plants (Figure 9). Lesions were present on the seedlings treated with the mutant strains but not as frequently as the wild type treated plants (Figure 10). Statistical analyses were performed on the dataset using the Analysis of Variance (ANOVA) test, which showed significant differences between trials and treatments. To ensure consistency, the two trials were not combined throughout the remainder of the experiments. Consistent with the virulence data, dry weight measurements of the aerial tissue showed less leaf and stem biomass for wild-type and ectopic strains compared to the *Δfveg_10983* strains (Figure 11). Seedlings survived at similar rates across transformants (Figure 12). ANOVA results showed no significant difference between trials and treatments; however, the data are not combined for consistency. Thus, the pathogenicity results are not based on increased or decreased survival of seedlings.

Endophytic growth and soil colonization

There was much variation in the endophytic growth results among trials (Figure 13). The main objective of this experiment was to determine if the fungal mutant strains can grow endophytically. This experiment showed that the *Δfveg_10983* strains are capable of growing endophytically throughout the plant. Only the stems were tested for endophytic growth. These results directly correlate with the growth pattern seen for the ectopic strain throughout this study.

Figure 14 shows the data from the soil colonization experiments. The *Δfveg_10983* strains showed reduced presence in the soil as compared to wild type. Statistical analyses showed no significant effect between trials for each treatment. The ectopic strain, though, also seems to have a reduced ability to colonize the soil when compared to wild type, similar to the observations of radial growth.

Chemical analysis of fumonisin production

Production of FB1 mycotoxin was assessed with the help of USDA ARS Toxicology and Mycotoxin Research Unit scientists, Dr. Ron Riley and Dr. Nik Zitomer. The *Δfveg_10983* mutant strains produced significantly less FB1 compared to the wild type (Figure 15). MRC826 produced 6.8 and 5.3 times more FB1 than did *Δfveg_10983* mutant #37 and #46, respectively. The ectopic strain produced nearly half as much FB1 as did MRC826; again indicating the transformation process itself may be impacting proper growth and physiological activity.

DISCUSSION

The main objective of this study was to identify and characterize genes differentially expressed during sporulation. While the developmental pattern of sporulation for *F. verticillioides* is well understood, the genetic mechanisms behind this event remain obscure. Previously, conidiation mutants were identified as spontaneous mutations from genetic crosses between conidiating strains (Glenn *et al.* 2004). Glenn and co-workers found these mutants produced an elongated germ tube-like outgrowth from the tip of phialides instead of the normal enteroblastic production of conidia. After further investigation, it was found that the mutants lost the ability to form a wall building zone needed to help build the conidium wall. To date, this is the first record of this type of mutation in *F. verticillioides* or other phialidic ascomycetes. In order to expand on this previous work and identify genes involved in conidiation, a microarray

analysis was performed using a conidiating strain (MRC826) and an aconidial mutant strain (AEG 3-A3-5).

The microarray revealed 834 genes expressed in the wild type but not in the mutant. Of these, over half (56.6%) were located on Chromosome 11. In *F. verticillioides*, there are 11 stable chromosomes and a dispensable 12^{th} chromosome. The fact that 472 genes were not expressed in the mutant and are located on Chromosome 11 led to the hypothesis that the conidiation mutation was caused by the deletion of a large portion of Chromosome 11. Our efforts have disproved this hypothesis by performing Southern blot experiments. One set of non-conidiating strains (AEG 3-A3-2 and AEG 3-A3-8) hybridized to three probes designed from loci spaced along Chromosome 11, while the other set of aconidial mutant strains (AEG 3-A3-5 and AEG 3-A3-6) did not. Because the probes were found to be present in one set of mutants, the conidiation mutation is not the result of the deletion of Chromosome 11; however, a large set of genes from Chromosome 11 are still proven to be deleted from AEG 3-A3-5. Using microarray technology to identify large chromosomal deletions is a novel result for this study. Also, the apparent loss of an entire chromosome (~2Mb) is notable since Chromosome 11 is not considered a dispensable chromosome such as observed in *Nectria haematococca* MP VI (Covert 1998).

The other 362 genes not expressed in the mutant but in the wild type were sorted based on fold change difference between the two strains (Table 1). Genes with at least a 10-fold difference in expression between the mutant and the wild type were targeted for co-segregation PCR. From these results, we eliminated all possible genes but one (FVEG_14159) because of the co-segregation pattern, present in the conidiating strains and not in the aconidial strains. NCBI BLASTP searches identified FVEG_14159 as a Zn2Cys6 transcription factor; thus making

it an even more attractive target for gene knockout because these proteins are strictly fungal proteins, predominately in Ascomycete fungi (McPhearson *et al,* 2006). This same type of Zn2Cys6 DNA-binding domain is present in the gene *FUM21* from the fumonisin gene cluster; which is involved in transcriptional regulation of fumonisin production (Brown *et al.*2007). Another of the top 13 genes, FVEG_00366, was also characterized as a second Zn2Cys6 transcription factor. This gene had only a 9-fold difference in expression and was eliminated from further consideration due to its presence in conidiating and non-conidiating strains as displayed by the co-segregation PCR experiment (Figure 3). Given that FVEG_14159 may be a transcriptional regulator, it was targeted for gene knockout, although unsuccessfully. The DelsGate construct was generated but no knockouts have been found to date.

Another gene with at least a 10-fold difference in expression was FVEG_10983. This gene did not have the co-segregation pattern we were looking for (it was present in aconidial strains AEG 3-A3-2 and AEG 3-A3-8) but it had an extremely high 72-fold change difference in expression. FVEG_10983 is an uncharacterized protein and found to be limited to only phialide producing filamentous fungi from the phylum Ascomycota. Because of its anonymity and high fold change, this gene was also targeted for knockout and characterization.

Few genes involved in conidiation, growth and development, virulence, and fumonisin production in *F. verticillioides* have been characterized by gene deletions. Some examples include *GAP1*, *FvMK1*, *CPK1*, and *FAC1* (Sagaram *et al.* 2007; Zhang *et al.* 2011; Choi *et al.* 2010). In contrast to the *Δfveg_10983* mutant strains generated in this study, *GAP1* deletion mutants did not affect virulence or fumonisin production. However, growth and hyphal development were affected in the *GAP1* mutants; hyphae were compacted and grew into the medium. Similar to this finding, the *Δfveg_10983* mutants resulted in restricted hyphal growth,

but instead of hyphal compaction and growth into the medium, as with the *GAP1* mutants, *Δfveg_10983* mutants did not produce aerial hyphae (Sagaram *et al* 2007). More similar to the *Δfveg_10983* mutant strains generated in this study are mutants in the *FvMK1* gene (Zhang *et al* 2011). Aerial hyphae and the production of microconidia in chains were reduced in the *FvMK1* mutants. The reduction of microconidia resulting from the deletion of *FvMK1* triggered an increase in the production of macroconidia. In the case of virulence, *FvMK1* deletion mutants showed no symptoms of disease and the production of FB1 was reduced, whereas the wild type and complement strains resulted in severe infection. *FvMK1* was found (by GFP fusion) to be nuclear localized, which was my hypothesis for FVEG_10983.

The first observation made about the Δ*fveg_10983* mutants was the delay in growth in liquid culture (PDB). It took the Δ*fveg_10983* mutants 6-7 days to grow to turbidity whereas it only took the wild type and ectopic strains three days to grow. This observation was similar to that made for the aconidial mutants such as AEG 3-A3-5. The Δ*fveg_10983* mutants did produce conidia but less than the wild type and ectopic strains. Because of these observations, we know that FVEG_10983 is not solely involved in the conidiation mutation but must play a part in the normal production of conidia. While the Δ*fveg_10983* mutants were still able to produce conidia, the question remained whether the mutants were producing only a single conidium or a limited number of conidia per phialide. To address this question, the number of conidia compared to the number of phialides was calculated. The results showed a reduction in conidial production per phialide for the knockout mutants compared to the wild type strain.

Because *F. verticillioides* is a major maize pathogen, *in planta* virulence assays were performed using the Δ*fveg_10983* mutant strains. The Δ*fveg_10983* mutants were less virulent on the maize plants compared to the wild type and ectopic strains. Virulence, though, is not only

measured by the presence of *F. verticillioides* in the plant but also the production of fumonisin mycotoxins (Glenn *et al*. 2008, Williams *et al.*2007, Zitomer *et al.* 2008, 2010). FB1 is the most abundant of the fumonisin mycotoxins released by *F. verticillioides in planta*. In this study, we show that the virulence of the Δ*fveg_10983* mutants on the maize plants is directly correlated with the amount of FB1 produced by the strain. The wild type and ectopic strains both were highly virulent and released high amounts of FB1 per gram of corn. On the other hand, the Δ*fveg_10983* mutant strains were less virulent and produced less FB1. Consistent with previous results (Glenn *et al.* 2008), FB1 released by the fungal strain is directly related to the amount of maize seedling disease. To verify the actual inoculated Δ*fveg_10983* mutant strain is present in the plant and causing disease, endophytic tests were performed. *F. verticillioides* can live endophytically within the plant without causing disease (Bacon and Hinton 1996); therefore, to be sure the inoculated strain is causing the observed symptoms, the strains were tested for their ability to endophytically grow in the maize plant. Our efforts show the strains identified from the stems were the Δ*fveg_10983* mutant strains. These results show that the Δ*fveg_10983* mutants are still capable of growing endophytically within the plant. Also, biomass of the aerial plant growth was measured and correlated with the virulence results.

Another characteristic of *F. verticillioides* is the ability to colonize soil. *F. verticillioides* can survive in the soil for years (Cotten and Munkvold, 1998). The soil colonization was measured by plating soil dilutions on PDA plates supplemented with antibiotics (streptomycin and ampicillin). The Δ*fveg_10983* mutants were less capable of colonizing the soil when compared to wild type; so was the ectopic strain. The ectopic strain had similar virulence and fumonisin production to that of the wild type but its growth pattern is not as similar. When radial growth measurements were taken, the ectopic strain grew at the same rate as the

Δ*fveg_10983* mutant strains. This is the same for the soil colonization. Even though the ectopic growth rate is more similar to the Δ*fveg_10983* mutants than the wild type, the Southern blot proved it to be a true ectopic. This suggests the transformation procedure itself played a role in negatively impacting the growth of the transformant strains. This coincides with the fact that the pigmentation of the transformant strains in PDA was all an orange color rather than the purple color of the wild type culture.

While this study did not fully characterize a gene involved in sporulation of *F. verticillioides*, it identified genes differentially expressed between wild type and aconidial mutants, used DelsGate to generate knockout mutants of FVEG_10983, and showed that FVEG_10983 plays a role in conidiation. FVEG_10983 was found to be involved in the virulence of *F. verticillioides* both due to the decrease in the production of conidia as well as the reduction of FB1 production. This gene also may be involved in the endophytic growth of the fungus as well as the ability to colonize soil. A main observation made of these Δ*fveg_10983* mutants was the reduction of aerial hyphae. Although the Δ*fveg_10983* mutants still produced conidia, though less abundantly, the growth and development of aerial hyphae was stunted. This was not seen in the aconidial mutants, they were still capable of producing aerial hyphae even though no conidia were produced.

One unique characteristic found in the Δ*fveg_10983* mutant strains is the presence of multiple nuclei per hyphal compartment in some areas. This suggests FVEG_10983 may be involved in cell cycle regulation in terms of proper nuclear division and septum formation. This was a phenotype noted by Glenn *et al.* (2004) in the aconidial mutants such as AEG 3-A3-5. More research needs to be done to ensure accurate characterization of the FVEG_10983 gene. One major next step would be labeling the FVEG_10983 protein with green fluorescent protein

(GFP) to visualize its localization. This will help to identify whether FVEG_10983 gene is indeed localized to nuclei as suggested by the signal peptide predictions.

The significance of this study as it relates to plant pathology is apparent. The mechanisms and genes involved in the sporulation pathway of *F. verticillioides* are still unknown, and identifying aconidial mutants and comparing them to conidia-producing strains can lead to the identification of the genes responsible for sporulation. When the sporulation pathway is characterized fully, then scientists will have a better understanding as to how the fungus is dispersed and survives and may, therefore develop more specific management and control efforts. The results of this study have led to the characterization of the gene FVEG_10983 and set-up a procedure for testing other candidate genes thought to play a role in the sporulation pathway. Ultimately fungicides or inhibiters could be targeted for particular aspects of sporulation. For example, given the limited taxonomic distribution of FVEG_10983 and its potential involvement in growth and reproduction, targeted inhibitors of the protein could prove to be effective treatments for disease and mycotoxin management.

LITERATURE CITED

1. Alessandra L, Luca P, Adriano M. 2010. Differential gene expression in kernels and silks of maize lines with contrasting levels of ear rot resistance after Fusarium verticillioides infection. *Journal of Plant Physiology* 167: 1398-1406.

2. Bacon CW and Hinton DM. 1996. Symptomless endophytic colonization of maize by *Fusarium moniliforme*. *Canadian Journal of Botany* 74: 1195-1202.

3. Baldwin TK, Gaffoor I, Antoniw J, Andries C, Guenther J, Urban M, Hallen-Adams HE, Pitkin J, Hammond-Kosack KE, and Trail F. 2010. A partial chromosomal deletion caused by random plasmid integration resulted in a reduced virulence phenotype in *Fusarium graminearum. Molecular Plant-Microbe Interactions* 23(8): 1083-1096.

4. Bennett JW and Klich M. 2003. Mycotoxins. *Clinical Microbiology Reviews* 16(3): 497-516.

5. Brown DW, Butchko RAE, Busman M, and Proctor RH. 2007. The *Fusarium verticillioides FUM* gene cluster encodes a Zn(II)2Cys6 protein that affects *FUM* gene expression and fumonisin production. *Eukaryotic Cell* 6(7): 1210-1218.

6. Brown DW, Butchko RAE, and Proctor RH. 2008. Genomic analysis of *Fusarium verticillioides*. *Food Additives and Contaminations* 25(9): 1158-1165.

7. Choi YE and Xu JR. 2010. The cAMP signaling pathway in *Fusarium verticillioides* is important for conidiation, plant infection, and stress responses but not fumonisin production. *Molecular Plant-Microbe Interactions* 23(4): 522-533.

8. Cotton TK and Munkvold GP. 1998. Survival of *Fusarium moniliforme*, *F. proliferatum* and *F. subglutinans* in maize stalk residues. *Phytopathology* 88:550-555.

9. Covert S. 1998. Supernumerary chromosomes in filamentous fungi. *Current Genetics* 33: 311-319.

10. Emanuelsson, Nielsen H, Brunak S, and von Heijne G. 2000. Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *Journal of Molecular Biology* 300: 1005-1016.

11. Espagne E, Balhade`re P, Penin M-L, Barreau C, and Turcq B. 2002. HET-E and HET-D belong to a new subfamily of WD40 proteins involved in vegetative incompatibility specificity in the fungus *Podospora anserine. Genetics* 161: 71-81.

12. García-Pedrajas MD, Nadal M, Kapa LB, Perlin MH, Andrews DL, and Gold SE. 2008. DelsGate, a robust and rapid gene deletion construction method. *Fungal Genetics and Biology* 45(4): 379-388.

13. Gelderblom WC, Jaskiewicz K, Marasas WF, Thiel PG, Horak RM, Vleggaar R, and Kriek NP. 1988. Fumonisins – novel mycotoxins with cancer-promoting activity produced by *Fusarium moniliforme*. *Applied and Environmental Microbiology* 54(7): 1806-1811.

14. Glass NL, Jacobson DJ, and Shiu PKT. 2000. The genetics of hyphal fusion and vegetative incompatibility in filamentous ascomycete fungi. *Annual Review of Genetics* 34: 165-186.

15. Glenn AE. 2006. Natural variation of ascospore and conidial germination by *Fusarium verticillioides* and other *Fusarium* species. *Mycological Research* 110: 211-219.

16. Glenn AE and Bacon CW. 2009. FDB2 encodes a member of the arylamine N-acetyltransferase family and is necessary for biotransformation of benzoxazolinones by *Fusarium verticillioides*. *Journal of Applied Microbiology* 107: 657-671.

17. Glenn AE, Gold SE, and Bacon CW. 2002. *Fdb1* and *Fdb2*, *Fusarium verticillioides* loci necessary for detoxification of performed antimicrobials from corn. *Molecular Plant-Microbe Interactions* 15(2): 91-101.

18. Glenn AE, Richardson EA, and Bacon CW. 2004. Genetic and morphological characterization of a *Fusarium verticillioides* conidiation mutant. *Mycologia* 96(5): 968-980.

19. Glenn AE, Zitomer NC, Zimeri AM, Williams LD, Riley RT, and Procter RH. 2008. Transformation-mediated complementation of a FUM gene cluster deletion in *Fusarium verticillioides* restores both fumonisin production and pathogenicity on maize seedlings. *Molecular Plant Microbe Interactions* 21(1): 87-97.

20. Hagen I, Ecker M, Lagorce A, Francois JM, Sestak S, Rachel R, Grossman G, Hauser NC, Hoheisel JD, Tanner W, and Strahl S. 2004. Sed1p and Srl1p are required to compensate for cell wall instability in *Saccharomyces cerevisiae* mutants defective in multiple GPI-anchored mannoproteins. *Molecular Microbiology* 52(5): 1413-1425.

21. Kim H and Woloshuk CP. 2011. Functional characterization of *fst1*in *Fusarium verticillioides* during colonization of maize kernels. *Molecular Plant-Microbe Interactions* 24(1):18-24

22. Larson TM, Kendra DF, Busman M, and Brown DW. 2011. Fusarium verticillioides chitin synthases CHS5 and CHS7 are required for normal growth and pathogenicity. *Current Genetics* 57: 177-189.

23. Leslie JF and Summerell BA. 2006. The *Fusarium* laboratory manual. APS Press.

24. Li-Jun M, H. van der Does C, Borkovich KA, Coleman JJ, Daboussi M-J, Pietro AD, Dufresne M, Freitag M, Grabherr M, Henrissat B, Houterman PM, Kang S, Shim W-B, Woloshuk C, Xie X, Xu J-R, Antoniw J, Baker SE, Bluhm BH, Breakspear A, Brown DW, Butchko RAE, Chapman S, Coulson R, Coutinho PM, Danchin EGJ, Diener A, Gale LR, Gardiner DM, Goff S, Hammond-Kosack KE, Hilburn K, Hua-Van A, Jonkers W, Kazan K, Kodira CD, Koehrsen M,Kumar L, Lee Y-H, Li L, Manners JM, Miranda-Saavedra D, Mukherjee M, Park G, Park J, Park SY, Proctor RH, Regev A, Ruiz-Roldan MC, Sain D, Sakthikumar S, Sykes S, Schwartz DC, Turgeon BG, Wapinski I, Yoder O, Young S, Zeng Q, Zhou S, Galagan J, Cuomo CA, Kistler HC, and Rep M. 2010. Comparative genomics reveals mobile pathogenicity chromosomes in *Fusarium*. *Nature* 464: 367-373.

25. Li X, Yu H-Y, Lin Y-F, Teng H-M, Du L, and Ma G-G. 2010. Morphological changes of *Fusarium oxysporum* induced by CF66I, an antifungal compound from *Burkholderia cepacia*. *Biotechnology Letters* 32(10): 1487-1495.

26. Marasas WFO, Riley RT, Hendricks KA, Stevens VL, Sadler TW, Gelineau-van Waes J, Missmer SA, Cabrera J, Torres O, Gelderblom WCA, Allegood J, Martı´nez C, Maddox J, Miller JD, Starr L, Sullards MC, Roman AV, Voss KA, Wang E, and Merrill, Jr AH. 2004. Fumonisins disrupt sphingolipid metabolism, folate transport, and neural tube development in embryo culture and in vivo: a potential risk factor for human neural tube defects among populations consuming fumonisin-contaminated maize. *The Journal of Nutrition* 134(4): 711-716.

27. McPhearson S, Larochelle M, and Turcotte B. 2006. A fungal family of transcriptional regulators: the zinc cluster proteins. *Microbiology and Molecular Biology Reviews* 70(3): 583-604.

28. Ohara T and Tsuge T. 2004. *FoSTUA*, Encoding a basic helix-loop-helix protein, differentially regulates development of three kinds of asexual spores, macroconidia, microconidia, and chlamydospores, in the fungal plant pathogen *Fusarium oxysporum.* 3(6): 1412-1422.

29. Ohara T, Inoue I, Namiki F, Kunoh H and Tsuge T. 2004. *REN1* is required for development of microconidia and macroconidia, but not of chlamydospores, in the plant pathogenic fungus *Fusarium oxysporum*. *Genetics* 166: 113-124.

30. Rittenour WR, and Harris SD. 2008. Characterization of *Fusarium graminearum* Mes1 reveals roles in cell-surface organization and virulence. *Fungal Genetics and Biology* 45: 922-946.

31. Ruiz-Rolda´n MC, Ko¨hli M, Roncero MIG, Philippsen P, Di Pietro A, and Espeso EA. 2010. Nuclear dynamics during germination, conidiation, and hyphal fusion of *Fusarium oxysporum. Eukaryotic Cell* 9(8): 1216-1224.

32. Sagaram US, Shaw BD, and Shim WB. 2007. *Fusarium verticillioides* GAP1, a gene encoding a putative glycolipid-anchored surface protein, participates in conidiation and cell wall structure but not virulence. *Microbiology* 153: 2850-2861.

33. Shonig B, Brown DW, Oeser, and Tudzynski B. 2008. Cross-species hybridization with *Fusarium verticillioides* microarrays reveals new insights into *Fusarium fujikuroi* nitrogen regulation and the role of AreA and NMR. *Eukaryotic Cell* 7(10): 1831-1846.

34. Sutton JC. 1982. Epidemiology of wheat head blight and maize ear rot caused by *Fusarium graminearum*. *Canadian Journal of Plant Pathology* 4: 195-209.

35. Vincent P, Goubet F, Carapito R, and Jeltsch J-M. Plant cell wall degradation with a powerful *Fusarium graminearum* enzymatic arsenal. *Journal of Microbiological Biotechnology* 19(6): 573-581.

36. Wiemann P, Brown DW, Kleigrewe K, Bok JW, Keller NP, Humpf H-U and Tudzynski B. 2010. FfVel1 and FfLae1, components of a velvet-like complex in *Fusarium fujikuroi*, affect differentiation, secondary metabolism and virulence. *Molecular Microbiology* 77(4): 972-994.

37. Williams LD, Glenn AE, Zimeri AM, Bacon CW, Smith MA, Riley RT. 2007. Fumonisin disruption of ceramide biosynthesis in maize roots and the effects on plant development and *Fusarium verticillioides*-induced seedling disease. *Journal of Agricultural and Food Chemistry* 55: 2937-2946.

38. Woo PCY, Ngan AHY, Chui H-K, Lau SKP, Yuen K-Y. 2010. Agar block smear preparation: a novel method of slide preparation for preservation of native fungal structures for microscopic examination and long-term storage. *Journal of Clinical Microbiology* 48(9): 3053-3061.

39. Zhang Y, Choi YE, Zou X, and Xu JR. 2011. The FvMK1 mitogen-activated protein kinase gene regulates conidiation, pathogenesis, and fumonisin production in *Fusarium verticillioides*. *Fungal Genetics and Biology* 48: 71-79.

40. Zitomer NC, Glenn AE, Bacon CW, and Riley RT. 2008. A single extraction method for the analysis by liquid chromatography/tandem mass spectrometry of fumonisins and biomarkers of disrupted sphingolipid metabolism in tissues of maize seedlings. *Annual Bioanalytical Chemistry* 391: 2257-2263.

41. Zitomer NC, Jones S, Bacon C, Glenn AE, Baldwin T, and Riley RT. 2010. Translocation of sphingoid bases and their 1-phosphates, but not fumonisins, from roots to aerial tissues of maize seedlings watered with fumonisins. *Journal of Agriculture and Food Chemistry* 58(12): 7476-7481.

Table 1. DelsGate primers used to generate deletion constructs for FVEG_14159 and FVEG_10983. The underlined text in primers #1 and #4 represents the I-*Sce*I sequence in the forward and reverse orientation, respectively. Bold text in primers #2 and #3 represents the *attB1* and *attB2* sequences, respectively.

Primer	Primer Target	Primer Sequence
#1 (5' flank)	FVEG_14159	TAGGGATAACAGGGTAATAAACAGACCAGTCCGCAAAGAG
#2 (5' flank)	FVEG_14159	**GGGGACAAGTTTGTACAAAAAAGCAGGCTAT**ACCCTCAATCAGTAGCCACCAA
#3 (3' flank)	FVEG_14159	**GGGGACCACTTTGTACAAGAAAGCTGGGTA**TTTATGTGTGGCGGTTCCTGGT
#4 (3' flank)	FVEG_14159	ATTACCCTGTTATCCCTATGAACACCACACTAGCCTGACA
I-*SceI*-F	DelsGate	TAGGGATAACAGGGTAAT
I-*SceI*-R	DelsGate	ATTACCCTGTTATCCCTA
Donr F	DelsGate	ATCAGTTAACGCTAGCATGGATCTC
Donr R	DelsGate	GTAACATCAGAGATTTTGAGACAC
HygF DG	DelsGate	ATCGCGGCCTCGACGTTTCC
KanR DG	DelsGate	TTATCGTGCACCAAGCAGCA
#1 (5' flank)	FVEG_10983	TAGGGATAACAGGGTAATTTTGCTACGCTCGTTGACCTCT
#2 (5' flank)	FVEG_10983	**GGGGACAAGTTTGTACAAAAAAGCAGGCTAT**TGTGCTCTTCAACTGCTTGCGT
#3 (3' flank)	FVEG_10983	**GGGGACCACTTTGTACAAGAAAGCTGGGTA**TCTTGGCAAAGGCCCAAACA
#4 (3' flank)	FVEG_10983	ATTACCCTGTTATCCCTAATGTGACTGCATTGTCCACGCT
5’ Outer	FVEG_10983	ACCTGCTTGCGATAAGAGGTAA
3’ Outer	FVEG_10983	ACAGGAGAAAGGTAAGCGCCAT

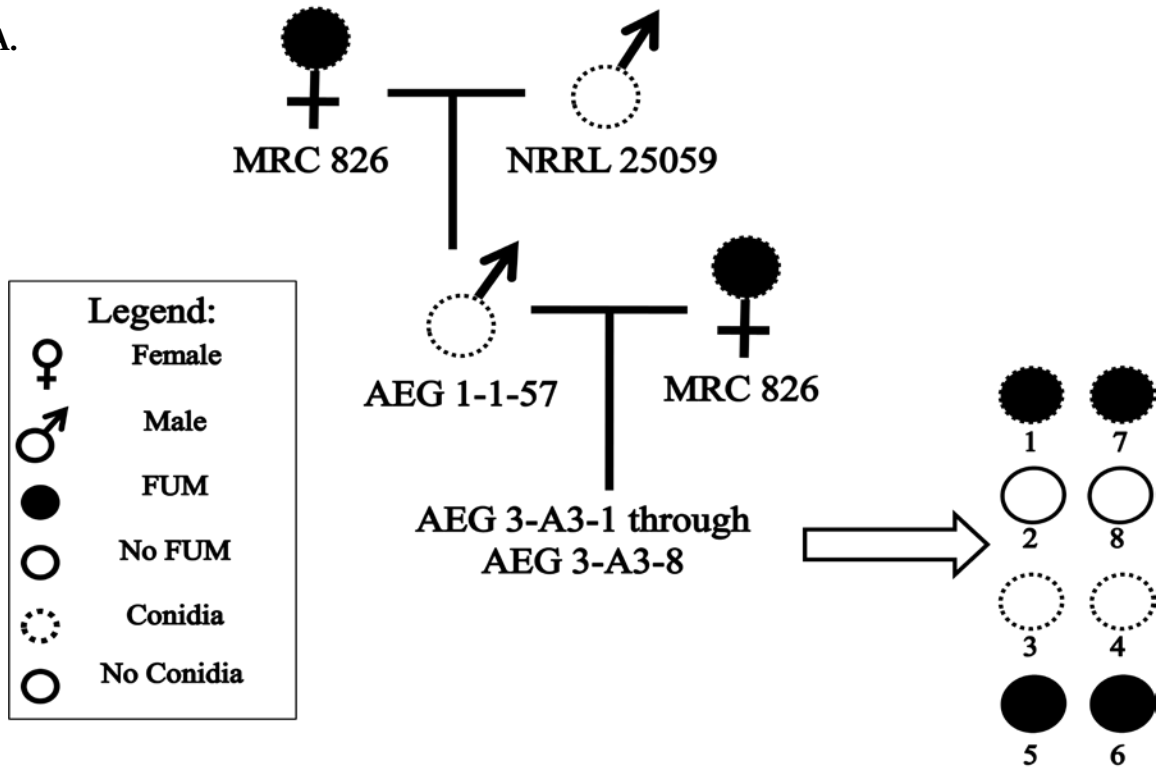
Table 2. List of candidate genes generated from microarray analyses. The candidate genes are labeled in the first column and their putative function in the second. Chromosome number (Ch.) on which the gene is present is listed as well as the accession number and E value from BLASTP search of the NCBI database. The last column gives the fold changes of gene expression in wild type (MRC826) compared to mutant (AEG 3-A3-5) from microarray analyses.

Gene	Putative Function	Ch	Accession	E value	Fold Change
FVEG_10983	Uncharacterized Protein	9	XP_381950.1	1e-137	72
FVEG_14159	Zn2Cys6 Transcription Factor	12	XP_002149432.1	0	38
FVEG_12429	HET Domain Protein	4	XP_383122.1	4e-82	25
FVEG_11785	NACHT Domain Protein	7	XP_002149694.1	2e-74	23
FVEG_00107	Uncharacterized Protein	1	XP_388436.1	2e-126	19
FVEG_12438	Ankyrin Domain Protein	4	AAV66110.1	0	17
FVEG_01538	Uncharacterized Protein	6	XP_391463.1	4e-78	12
FVEG_08499	Kinesin Light Chain	10	XP_384557.1	2e-59	12
FVEG_08500	Kinesin Light Chain	10	XP_001225993.1	2e-104	11
FVEG_08403	Secretory Aspartyl Proteinase	10	XP_384557.1	2e-59	10
FVEG_08383	HET Domain Protein	10	EEU48939.1	5e-130	10
FVEG_00158	Uncharacterized Protein	1	EEU35946.1	5e-06	10
FVEG_08366	Zn2Cys6 Transcription Factor	10	XP_383314.1	0	9

Table 3. Co-segregation PCR primers. For each gene, the primer sequence on top indicates forward primer and the primer sequence on bottom indicates reverse primer. FVEG_02381 is the gene ID designation for the Translation Elongation Factor 1α, used as a positive control with primers EF1 (forward) and EF2 (reverse).

Gene	Putative Function	Primer Sequence
FVEG_10983	Uncharacterized Protein	CACGATTTGGAAGATGGAGGCA TAGTATTGCAGGCTGGCCTGTT
FVEG_14159	Zn2Cys6 Transcription Factor	ATTGCGAGGGCAATGAACCT TGTTGTGTTCCAAAGGCGGT
FVEG_12429	HET Domain Protein	AGCAAGCATCCATCTGCGTTGT TGGATAGGCTAAGGCTGTGCTT
FVEG_11785	NACHT Domain Protein	AGGTTTCAGAAGCTCAACCACG TTTGTCGGAACGAATGAGCTGC
FVEG_00107	Uncharacterized Protein	AGTGCTCTCGACTGCATCATCA CAAGGATCATTGCGACACCAGT
FVEG_12438	Ankyrin Domain Protein	TCCAATGGCTCGGACAAGACAA TTGAGCAGGCAACTTGCGGAAA
FVEG_01538	Uncharacterized Protein	AAAGTCCTTCCCGAACCAAGCA AAAGGTGTCGCCTGCAAAGA
FVEG_08499	Kinesin Light Chain	TCTTTGGAACTCGTCAAGGCCA AAGCCAGAATGGCTGTTTCGCA
FVEG_08500	Kinesin Light Chain	TTGTCAGCCAAAGCGTTGTCCA AAACAACCCAATGGCGCAGT
FVEG_08403	Secretory Aspartyl Proteinase	AAGTGCACTGACAGGTTCTCCT TGCGCAGGAAAGGATCACCAAA
FVEG_08383	HET Domain Protein	AGCCAGAGAGTGCGTTCAGAAA TCCCAACAGTCAAGCACACCAA
FVEG_00158	Uncharacterized Protein	TGTGCCAGACTATAGCAGGAGT AAATAGCTGTTGCGGCGAACCT
FVEG_08366	Zn2Cys6 Transcription Factor	AATCTTCGGTCCACTCGCTTCT AAACTCTCCGCCGCACTTCATT
FVEG_02381	Translation Elongation Factor 1α	ATGGGTAAGGARGACAAGAC GGARGTACCAGTSATCATGTT

A.



B.

Strain	MRC 826	NRRL 25059	AEG 1-1-57	AEG 3-A3-1	AEG 3-A3-2	AEG 3-A3-3	AEG 3-A3-4	AEG 3-A3-5	AEG 3-A3-6	AEG 3-A3-7	AEG 3-A3-8
Conidia	+	+	+	+	-	+	+	-	-	+	-
FUM	+	-	-	+	-	-	-	+	+	+	-

Figure 1. Schematic of mating, backcrossing, and resulting octad originally reported in Glenn *et al.*(2004). **A.** Female symbol indicates female strain, male symbol indicates male strain, filled circle indicated presence of FUM gene cluster, open circle indicates absence of FUM gene cluster, dashed circle outline indicates ability to produce conidia, solid circle outline indicates loss of conidia production. Progeny labeled AEG 3-A3-1 (1)**,** AEG 3-A3-2 (2), AEG 3-A3-3 (3), AEG 3-A3-4 (4), AEG 3-A3-5 (5), AEG 3-A3-6 (6), AEG 3-A3-7 (7), and AEG 3-S3-8 (8). **B.** Table organizing strain characteristics. First row is the strain name, second row is the ability to produce conidia, and the third row is presence of FUM gene cluster. "+" indicates the strain produces conidia or possesses the FUM gene cluster, respectively. "-" indicates the strain does not produce conidia or possess the FUM gene cluster, respectively.

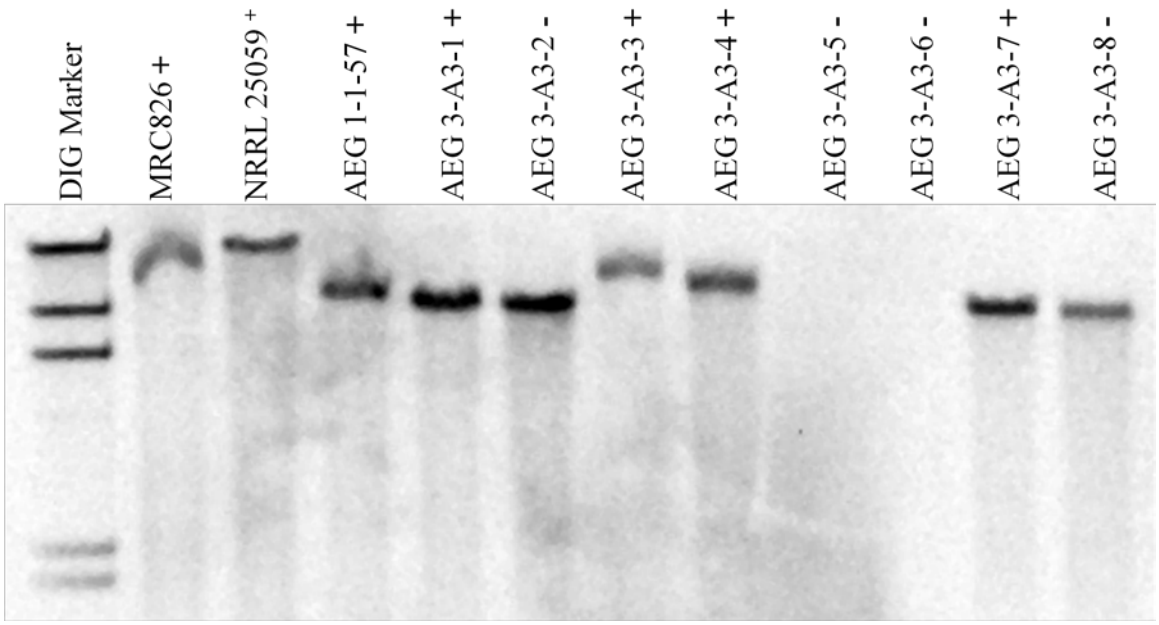


Figure 2. Chromosome 11 Southern blot experiments. All strains, including parental and progeny were used to test for presence or absence of Chromosome 11. Southern results shown are representative of three separate experiments using probes CRH1, DCW1 and 3.21.1. Each experiment showed the same results. "+" indicates the strain's ability to produce conidia, "-" indicates lack of conidia production.

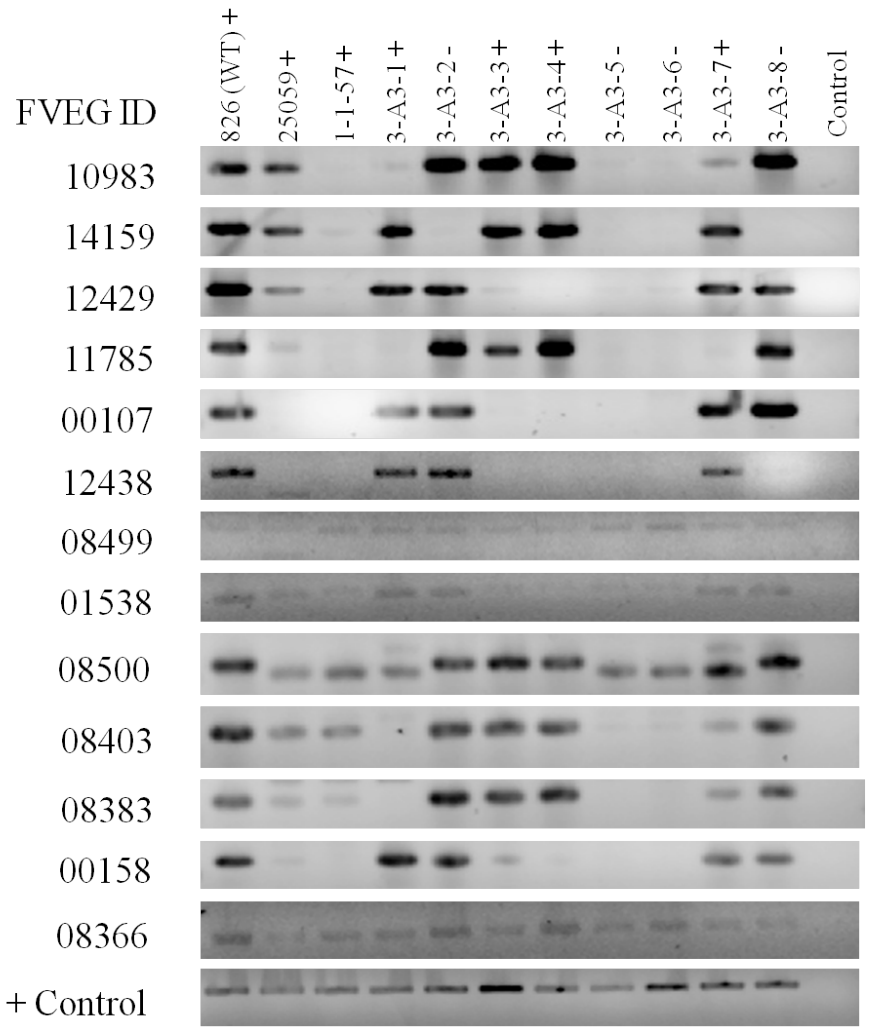
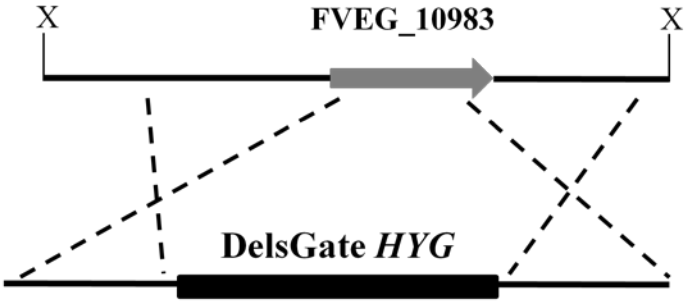


Figure 3. Co-segregation analysis of conidia production and PCR detection of genes identified from the microarray experiment. Across the top are the strains. Along the side are the genes. Positive control represents PCR results using elongation factor primers specific for *Fusarium* species. Presence of a band indicates presence of the gene in the strain. "+" indicates the strain produces conidia; "-" indicates the strain does not produce conidia.

A.


X
Probe
FVEG_10983
X

B.


X
FVEG_10983
X
DelsGate HYG

C.


X
Probe
DelsGate HYG
X

D.

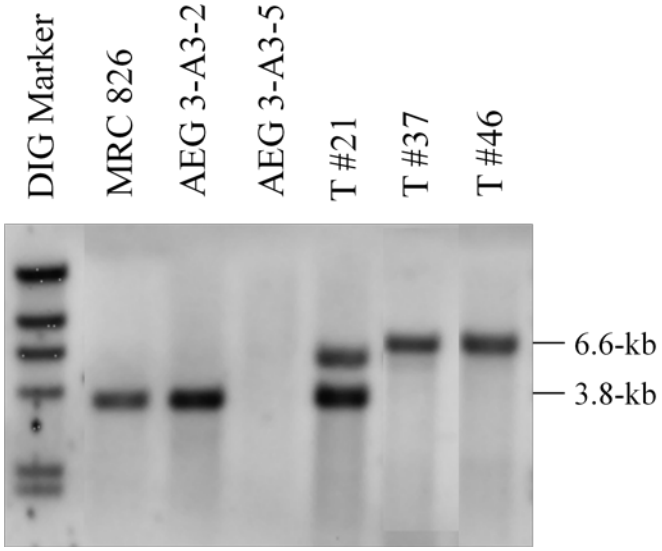

DIG Marker
MRC 826
AEG 3-A3-2
AEG 3-A3-5
T #21
T #37
T #46
6.6-kb
3.8-kb

Figure 4. Deletion construct and Southern blot to confirm knockouts. A.) FVEG_10983 gene (1424-bp) located on a 3.8-kb *Xba1* fragment. The 488-bp probe used in the Southern analyses was generated via PCR using primers "10983 So. F" and "10983 So. R." B.) An 865-bp portion of FVEG_10983 was targeted for deletion via homologous recombination with a DelsGate construct containing the hygromycin resistance gene cassette (not drawn to scale). C.) The 6.6-kb *Δfveg_10983* allele resulting from homologous recombination. D.) Southern hybridizations of wild-type MRC826 and transformants (T) #21, #37, and #46. T #21 is an ectopic strain possessing the wild-type 3.8-kb fragment. T#37 and T#46 are true knockouts having the 6.6-kb fragment.

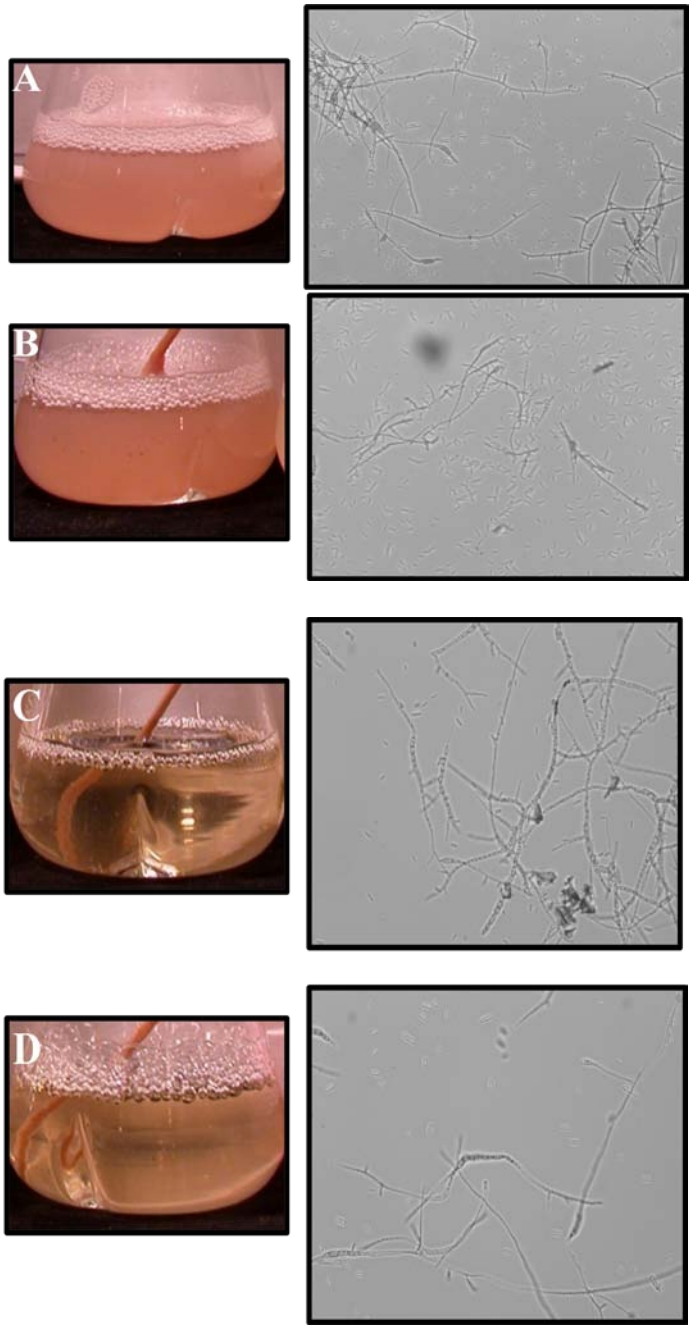

A
B
C
D

Figure 5. Comparison of wild type, ectopic, and two knockout strains for gene FVEG_10983 in PDB culture. A) Wild-type MRC826, B) Ectopic strain transformant #21, C) knockout strain #37, D) knockout strain #46. For each strain, the picture on the left shows characteristic growth in PDB after 5 days, and the picture on the right shows microscopic characteristics at 100x magnification. Strands seen in microscopy pictures are hyphal fragments, circular structures are microconidia.

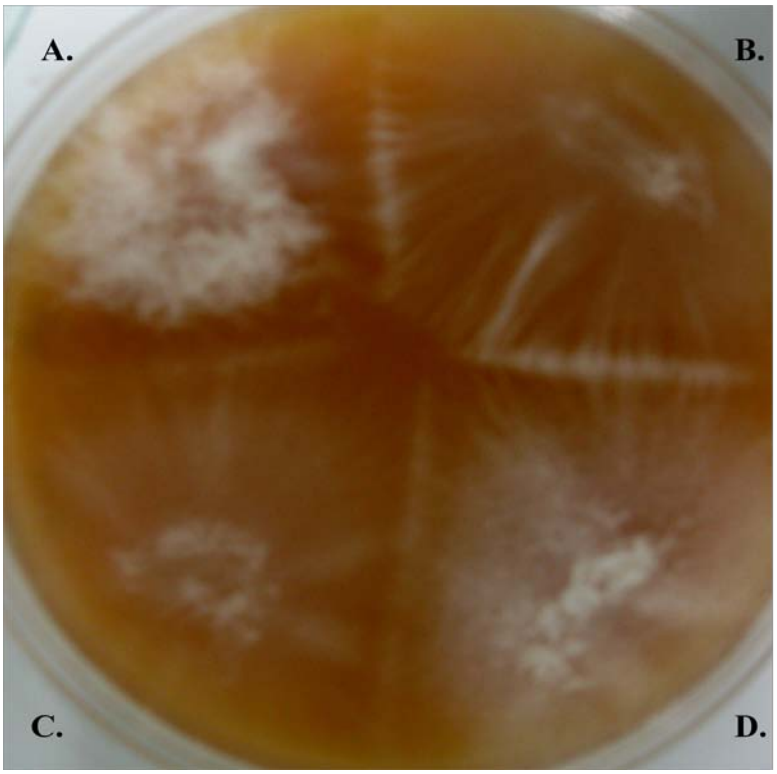


Figure 6. Aerial hyphae phenotype of strains. **A.** Wild type strain MRC826. **B.** *Δfveg_10983* mutant strain #37. **C.** *Δfveg_10983* mutant strain #46. **D.** Ectopic strain transformant #21. Cultures were grown on V8 for 7 days at 27°C in the dark.

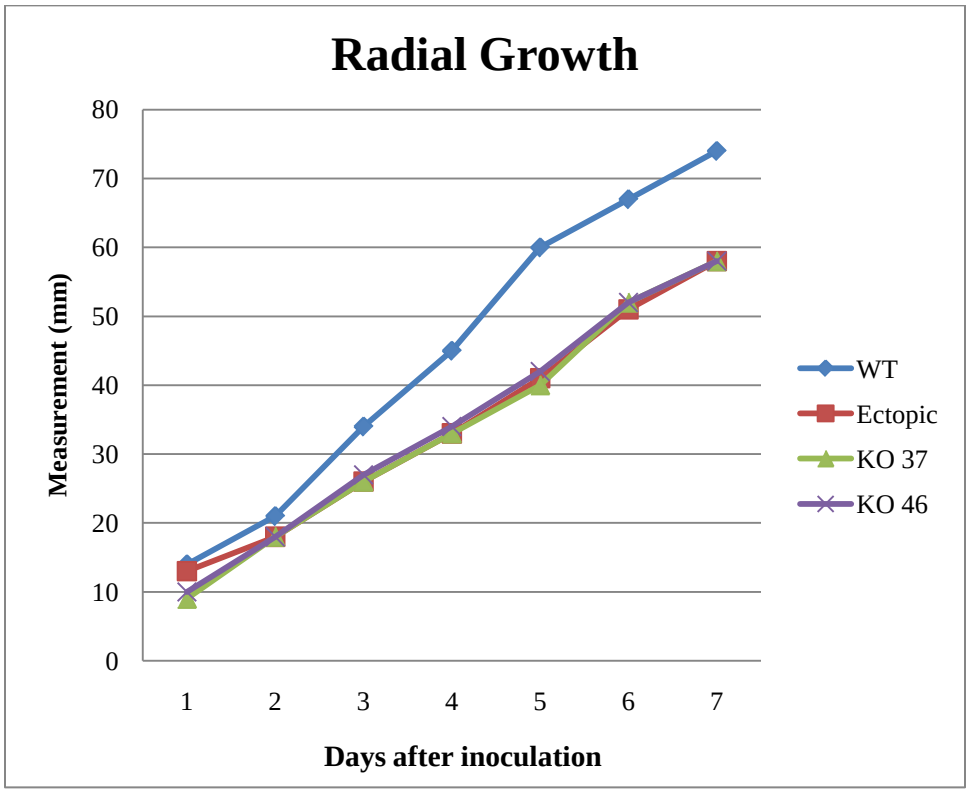


Figure 7. Radial growth measurements of wild-type MRC826 compared to ectopic transformant (strain #21) and the knockout strains #37 and #46. Cultures were grown on PDA plates for 3-7 days, depending on the strain. A 6mm plug was taken from the margin of each strain and placed on a new PDA plate. Radial growth was measured every day for 7 days.

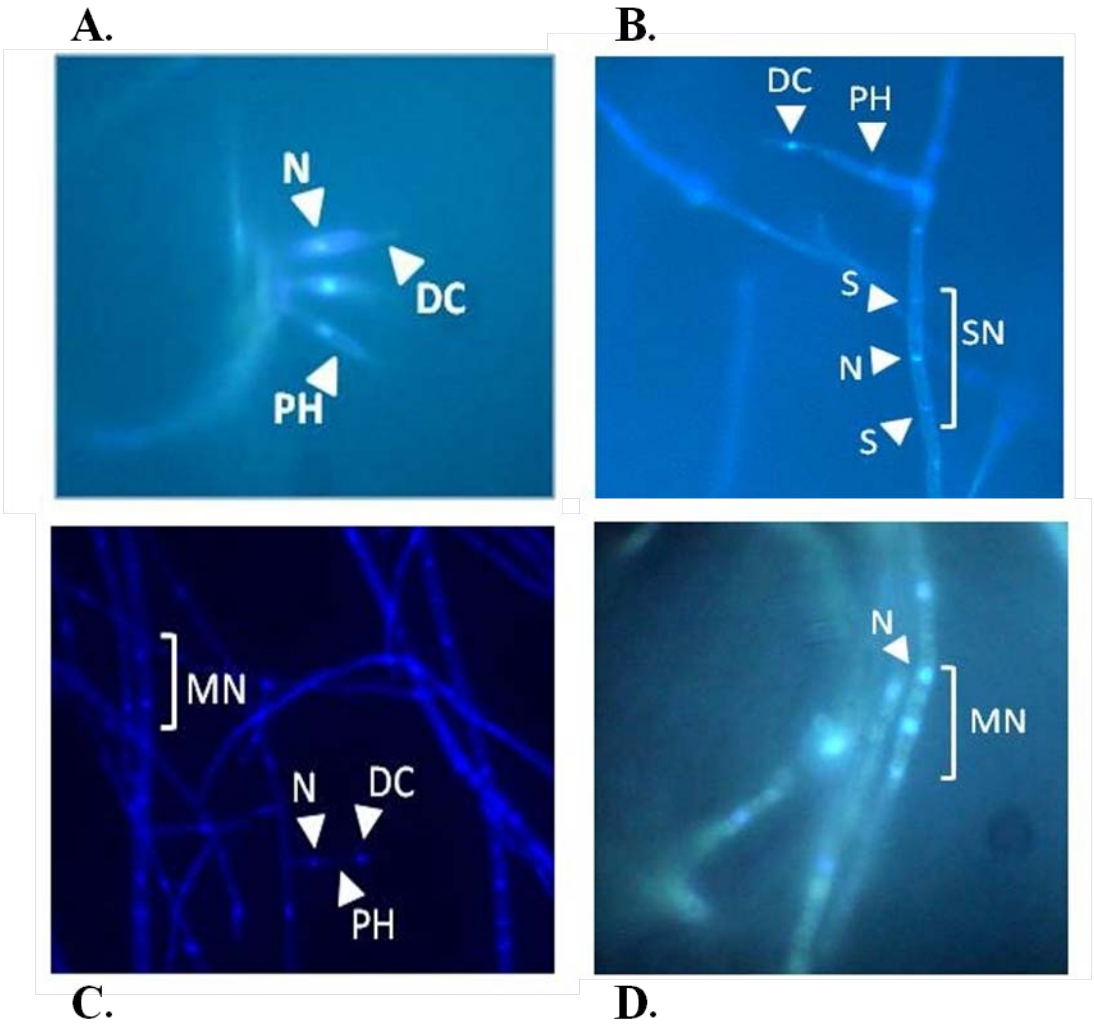


Figure 8. Hoechst and Calcofluor staining of nuclei and cell walls, respectively, of wild-type and knockout strains. A. Wild-type MRC826 displaying three consecutive phialides (PH) each containing a single nucleus (N) and a developing conidium (DC) from the apex. B. Wild-type MRC826 displaying a single nucleus (SN) per hyphal compartment separated by septa (S). C. Knockout strain #37 with a single phialide, nucleus and developing conidium. Multiple nuclei (MN) per hyphal compartment is seen here as well. D. Knockout strain #46 presenting the same characteristic staining of multiple nuclei per hyphal compartment. A Leica DM600B microscope was used with DAPI filter. Pictures taken 30 minutes after staining 7 day-old cultures.

Figure 9. Maize seedling virulence assays. Inoculated seed were planted in sterile soil (mock inoculation for the water control treatment). See the materials and methods for details. Plants were assessed 16 days after planting the seed. Three replicates of each treatment were used to report disease incidence.

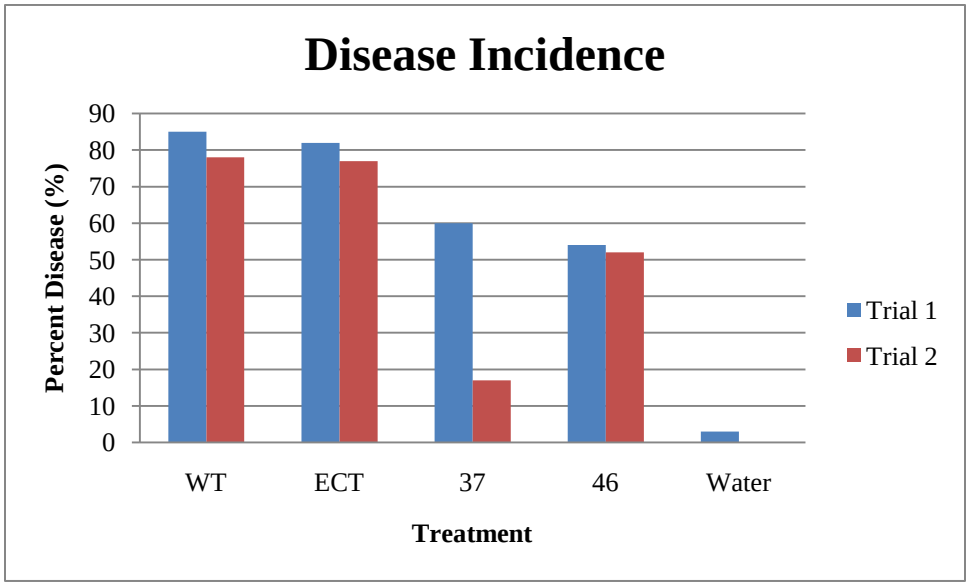


Figure 10. Percent disease incidence 16 days after planting. Disease incidence was calculated as the percentage of diseased plants per pot. Disease incidence per treatment was calculated by averaging the percent disease incidence for each of the three replicates. Only plants with distinct lesions were counted as diseased. Two rounds of the experiment were performed.

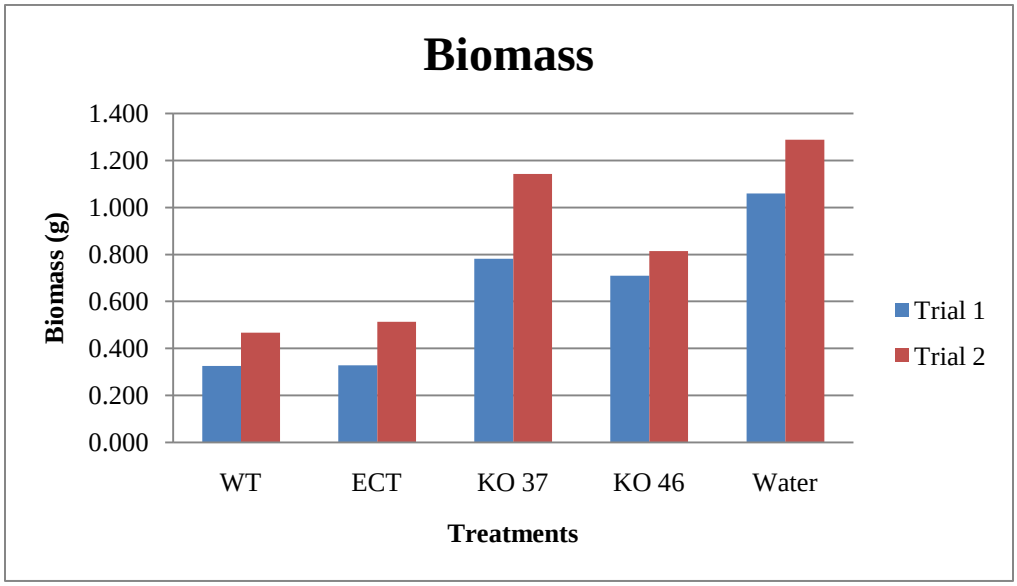


Figure 11. Measurement of shoot biomass (g dry wt.) 16 days after planting. Three replicates were assessed for each treatment. Two rounds of the experiment were performed.

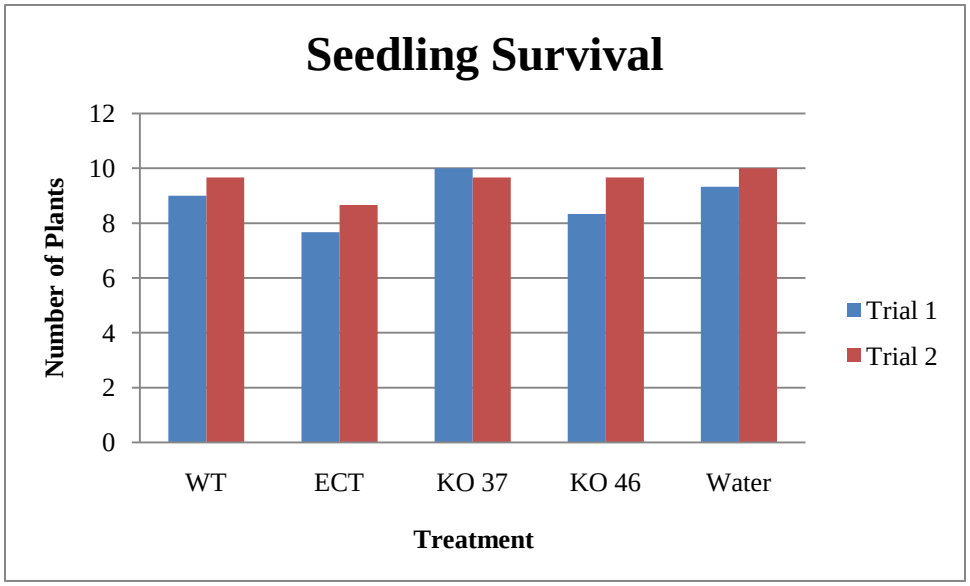


Figure 12. Measurement of seedling survival. Ten seeds were planted for each pot. Three replicates were assessed for each treatment. Two rounds of the experiment were performed. The number of surviving seedlings 16 days after planting was assessed for each treatment.

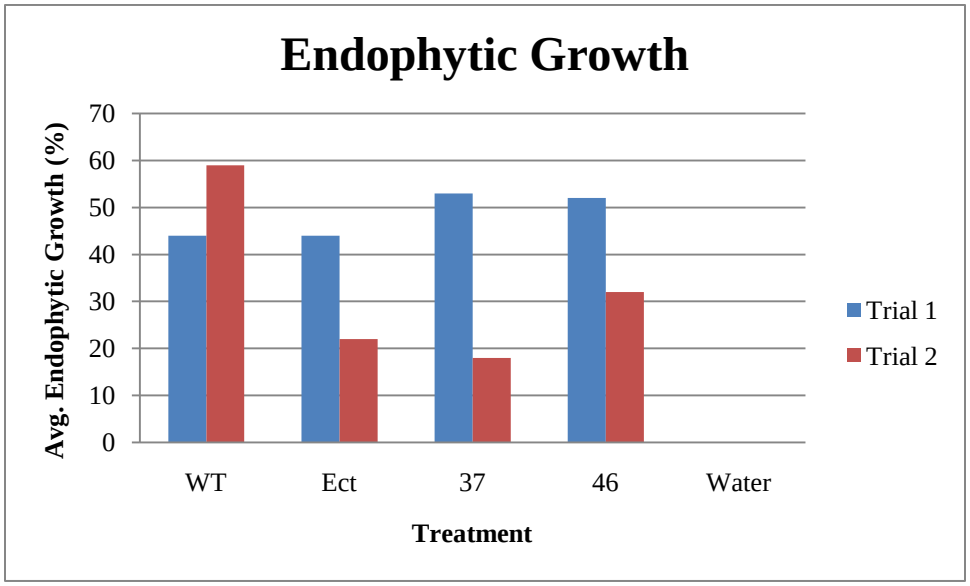


Figure 13. Average percent endophytic growth 16 days after planting. To test for endophytic growth, 1-2cm sections were cut from each stalk, surface sterilized, and grown on PDA supplemented with streptomycin and ampicillin (50µg/ml) for 3-5 days at 27°C.

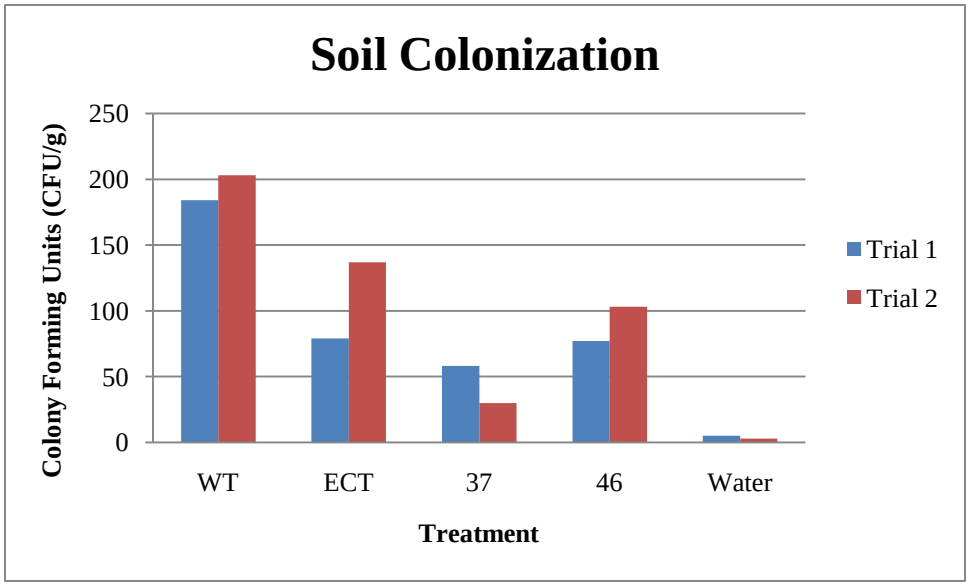


Figure 14. Soil colonization by each fungal strain. Soil colonization was determined for two trials of experiments. From each replicate, a 10,000-fold soil dilution was prepared and plated on five PDA + ampicillin/streptomycin plates. Numbers of colonies were counted after incubating the plates at 27°C for 3 days.

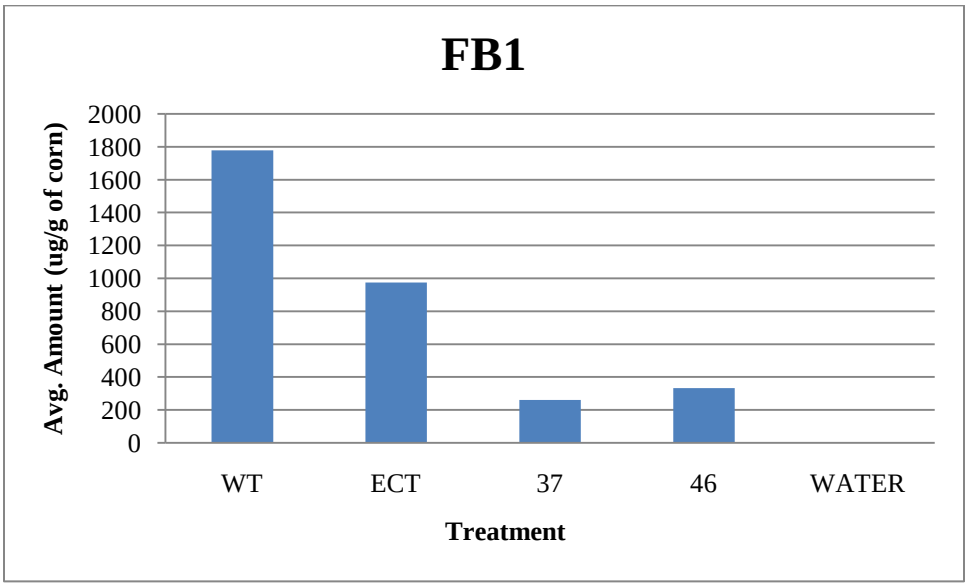


Figure 15. Amount of fumonisin B1 (FB1) per gram of corn for each treatment. Strains were grown for two weeks on cracked corn prepared in scintillation vials. Fumonisin production was analyzed using a Finigan Micro AS Autosampler coupled to a Surveyor MS pump (Thermo-Fisher, Woodstock, GA, USA). Separation was accomplished using a Metachem Inertsil 5µm ODS-3 column, 150x3mm (Metachem Technologies, Inc. Torrance, CA, USA). Two trials were performed with three replicates for each strain per run.

CHAPTER 3

CONCLUSIONS

The research conducted for this thesis involved the identification of genes differentially expressed during sporulation in the pathogenic fungus *Fusarium verticillioides*. Microarray analyses indentified 834 genes differentially expressed during sporulation between a wild type conidia producing strain and an aconidial mutant strain. These genes were assessed based on fold change differences between the two strains. The two genes with the highest fold change, FVEG_10983 and FVEG_14159, were targeted for gene deletion. FVEG_10983 was successfully deleted; FVEG_14159 was not. FVEG_14159 should still be targeted for deletion because the progeny show a 100% correlation of FVEG_14159 presence and absence with sporulation.

The *Δfveg_10983* mutant strains were assessed microscopically for phenotypic changes. The *Δfveg_10983* mutant strains produced less conidia than the wild type strain as well as were reduced in virulence, fumonisin (FB1) production, aerial hyphae production, radial growth, endophytic growth, and soil colonization. The *Δfveg_10983* mutant strains also possessed multiple nuclei per hyphal compartment when stained with Hoechst and Calcofluor stains. Further investigation is needed to determine the exact involvement of FVEG_10983 in each of these aspects. Further studies should also address the function of FVEG_14159 in sporulation.