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Functional and structural analysis of FST1 in Fusarium verticillioides

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For my parents and grandparents

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ABSTRACT

Niu, Chenxing. Ph.D., Purdue University, May, 2016. Functional and Structural Analysis of Fst1 in *Fusarium Verticillioides*. Major Professor: Charles Woloshuk.

Fusarium verticillioides causes an important seed disease on maize and produces fumonisin B1 (FB1), a mycotoxin that is detrimental to human and animal health. Previous studies discovered that expression of *FST1* is required for FB1 production and wild-type level of virulence on maize seeds. *FST1* encodes a putative protein with 12 transmembrane domains with sequence similarity to hexose transporters. However, those studies have failed to prove its ability to transport glucose, fructose or mannose. I identified another three phenotypes associated with the lack of a functional *FST1*, which includes reduced hydrophobicity of hyphae, reduced macroconidia production, and increased sensitivity to hydrogen peroxide. My research compared the transcriptome of the wild type and strain Δfst1 when grown on autoclaved maize kernels. The 17 % of transcriptome (2677 genes) were differentially expressed. Examination of these genes indicated that the disruption of FST1 function affected genes involved in secondary metabolism, cell structure, conidiogenesis, virulence, and resistance to reactive oxygen species. Additionally, I used a *Saccharomyces cerevisiae* strain (Δitr1) lacking a

functional inositol transporter gene (*ITR1*) to study the function of *FST1*. This yeast mutant grows poorly in myo-inositol medium and is not inhibited by FB1. I found that expression of *FST1* in strain Δitr1 restored growth on myo-inositol medium and sensitivity to FB1 to levels observed in the wild-type yeast strain. The results indicate that FST1 can function as an inositol transporter and suggests it can transport FB1 into fungal cells. Finally, the functional importance of amino acids in FST1 was examined by creating targeted mutations in the central loop and C-terminus regions of the protein. Expression of these engineered *FST1* genes in stain Δitr1 of *S. cerevisiae* and strain Δfst1 of *F. verticillioides* indicated that both the central loop and C-terminus are critical for FST1 functionality. Overall this research has established the first characterized inositol transporter in filamentous fungi and has advanced our knowledge about the global regulatory functions of *FST1*.

CHAPTER 1. INTRODUCTION

1.1 *Fusarium verticillioides* and Fusarium ear rot

Fusarium verticillioides (Sacc.) Nirenburg (teleomorph: *Gibberella moniliformis* Wineland) is the ubiquitous causal agent of seedling, stalk, ear, and kernel diseases in maize (*Zea mays*), the most economically significant of which is Fusarium ear rot. *F. verticillioides* is a necrotroph (Oren *et al.*, 2003) that can be found in plant residues in almost every maize field at harvest, with symptoms ranging from asymptomatic infection to severe rotting of all plant tissues. This fungus has the ability to colonize at any maize development stages, which results in seedling blight, root rot, stalk rot and ear rot (Purdue extension https://extension.purdue.edu/extmedia/ BP/BP-86-W.pdf). Stressed plants usually show more severe disease synptoms (Wu *et al.*, 1995). Environmental conditions, water availability, and the genetic background of both the plant host and the pathogen may all be important factors in disease development.

Infection of maize by *F. verticillioides* can occur via several routes. The most commonly reported method of kernel infection is through airborne conidia that infect the silks. After infection through the silk, the fungus moves to the kernels, though only a small percentage of infected kernels may actually become symptomatic. The infected

kernels show the characteristic “starburst” symptom wherein the fungus grows under the pericarp and light-colored streaks radiate from the top of kernels where the silks were attached (Figure 1.1). Insect wounds can also facilitate fungal infection. Another infection pathway is systemically through the seed. Systemic infection starts from fungal conidia or mycelia that are already present inside the infected seeds or on the seed surface (Limber, 1920). The fungus develops inside the young plant, moving from the roots to the stalk and finally to the cob and the kernels. Rarely, the fungus is able to enter from the butt of the cob (Koehler, 1942).

F. verticillioides is the primary causal agent for Fusarium ear rot in Indiana, though *F. proliferatum* and *F. subglutnans* can also cause Fusarium ear rot (Purdue Extension). Although the disease symptom caused by these three species are similar, *F. verticillioides* and *F. proliferatum* can be differentiated from *F. subglutnans* since they form microconidal chains. Additionally, *F. proliferatum* has polyphialides whereas *F. verticillioides* does not (Nirenberg and O’Donnell, 1998). In addition to the yield loss caused by these Fusarium species, *F. verticillioides* and *F. prolifeatum* also produce fumonisin (Purdue extension).

1.2 Discovery of Fumonisins and mode of action

Fumonisins are potentially harmful for humans and domesticated animals. Both *F. verticillioides* and *F. prolifeatum* can produce four different families of fumonisins, categorized as A, B, C and P-series fumonisins (Lazzaro et al., 2012). B series of fumonisins (Figure 1.2) consist of a 20-carbon-long backbone with an amine function, one to four hydroxyl functions, and two propane-1,2,3-tricarboxylate esters at various

positions along the backbone. Though a few *Fusarium* species produce the C series of fumonisin as their major toxins (Seo *et al.*, 1996), the most important fumonisin related to human health is fumonisin B1 (FB1). FB1 has been shown to be mutagenic to *Salmonella typhimurium* (Gelderblom *et al.*, 1983), while ingestion has been implicated as a cause of leukoencephalo-malacia in horses (Kriek *et al.*, 1981), porcine pulmonary edema in swine (Haschek *et al.*, 2001), liver cancer in rats (Marasas *et al.*, 1984a), and esophageal cancer and birth defects in humans (Nelson *et al.*, 1993). The amount of FB1 that is cytotoxic to baby hamster kidney cells is 1 ug/ul (Cecile *et al.*, 1990). The chemical natures of fumonisins were not elucidated until 1988 (Bezuidenhout *et al.*, 1988) (Figure 1.2). FB1 is structurally similar to sphingosine and can block the biosynthesis of complex sphingolipids by inhibiting ceramide synthesis, causing accumulation of sphinganine and sphingosine. These sphingoid bases are the primary cause of the toxicity of fumonisin B (Merrill *et al.*, 2001; Seiferlein *et al.*, 2007). Sphingolipids are important for membrane and lipoprotein structure and as second messengers for growth factors, differentiation factors and cytokines in cell regulation. The amounts of bioactive sphingolipids are highly regulated, with the regulation of lipid biosynthesis in *S. cerevisiae* by FB1 was examined by Wu *et al.* (1995).

Since the production of fumonisin by *F. verticillioides* during colonization of maize kernels is associated with both endophytic and pathogenic host-pathogen relationships (Seo *et al.*, 2001), control of fumonisin contamination in maize has become a priority area in food safety research and is strictly regulated. Tables 1.1 and 1.2 show the maximum levels for fumonisins in human foods and animal feeds as set by FDA.

Although FB1 is harmful to both animals and humans, it is not required for *F.verticillioides* to infected and rot maize ears (Desjardins, Munkvold et al. 2002).

1.3 Fumonisin biosynthesis

F. verticilioides has an estimated genome size of 41.7 Mb (Ma *et al*., 2010). There are 12 chromosomes that have been detected by contour-clamped homogeneous electric field gels (CHEF) (Xu and Leslie, 1996), but only 11 chromosomes were mapped to the assembled genome, which lacks the smallest chromosome (600kb or less) (Ma *et al*., 2010). Fumonisin biosynthesis genes are clustered in chromosome 1 (Desjardins *et al*, 1996; Proctor *et al*., 1999). In this cluster, FUM1 was identified as a fumonisin biosynthesis gene from the fumonisin-nonproducing mutant GfA2364 (Desjardins *et al.*, 2002). FUM1 encodes a polyketide synthase and was found to be one of the 15 FUM genes clustered on chromosome 1 (Proctor *et al.*, 1999). Other genes in the FUM cluster are FUM6 and FUM12 (similar to cytochrome P450), FUM7 and FUM13 (dehydrogenases), FUM9 (dioxygenase), FUM10 (fatty acyl-coenzyme A synthetase), FUM11 (tricarboxylate transporter), FUM14 (peptide synthetase), FUM17 and FUM18 (longevity assurance factors), and FUM19 (ABC transporter).

FB1 biosynthesis is influenced by the contents of the maize kernel, which can vary depending on environmental conditions (Shim *et al.*, 2003). For example, immature kernels lacking starch do not facilitate FB1 biosynthesis, but mature kernels that have accumulated starch support high levels of FB1 production (Bluhm and Woloshuk, 2005). Nitrogen also affects fumonisin biosynthesis. Expression of the global nitrogen regulatory gene AREA is required for FB1 production (Kim and Woloshuk, 2008). High

ammonium salt media can repress AREA expression and result in no FB1 production, while strains constitutively expressing AREA produce FB1 at levels similar to wild type under nonrepressive conditions. Light is another factor that influences fumonisin production. FB1 production in *F. proliferatum* increased about 40-fold under red light, 35-fold under blue, 10-fold under green, 5-fold under yellow, and 3-fold under white light compared to dark conditions (Fanelli *et al.*, 2012). Carbon source was also found to play an important role for FB1 production. The mature kernel is primarily composed of endosperm and germ. The endosperm, representing the largest part of the kernel, provides about 83 percent of the kernel weight (Wolf *et al.*, 1969). Endosperm tissue is rich in starch compared to germ tissue, and the amount of FB1 produced in the endosperm is 10- to 20-fold higher than in germ tissue (Shim *et al.*, 2003). ZFR1 was identified in a study of gene expression during FB1 biosynthesis as a candidate regulatory gene predicted to encode a Zn (II) 2Cys6 zinc finger transcription factor. A ZFR1 deletion strain (Δzfr1) exhibited greatly reduced levels of FB1, indicating ZFR1 is necessary for FB1 production during kernel growth (Bluhm *et al.*, 2008). The growth of Δzfr1 was approximately 2.5-fold less than the wild-type strain in the endosperm tissue but was conversely over 2-fold greater than wild-type in the germ tissue. However, α-amylase activity and expression of starch saccharification genes were not hindered in the Δzfr1 strain, indicating that the observed reduction in growth of the Δzfr1strain was not due to inhibition of amylolytic enzymes (Bluhm *et al.*, 2008). This suggests that the role of ZFR1 is to regulate genes involved in sugar sensing or transportation.

1.4 FST1

The *F. verticillioides* putative sugar transporter gene FST1 was identified by a comparative microarray analysis of RNA isolated from colonized maize germ and endosperm tissue. Of the 50 microarray probes specific for putative sugar transporter genes, FST1 is one of six genes that was more highly expressed during growth of *F. verticillioides* in maize endosperm tissue compared with germ tissue and was also shown to be negatively affected by disruption of ZFR1 (Bluhm *et al.*, 2008). FST1 is located on chromosome 10 of the *F. verticillioides* genome (FVEG_08441.3), encoding a 574-amino-acid protein with 12 putative transmembrane domains (Kim and Woloshuk, 2011) (Figure 1.3) and belonging to the major facilitator superfamily (MFS, Broad Institute Fusarium Comparative Database).

Kim and Woloshuk (2011) compared the structure of FST1 to other known and putative sugar sensors and transporters. The two sugar sensors in *Saccharomyces cerevisiae* (Snf3p and Rgt2p) have long cytoplasmic C-termini that function in signal transduction. The C-termini of RCO3 of *N. crassa* and FST1 are considerably shorter than those in yeast proteins. Topological analysis also indicated that for many of the sugar sensors the large central loop extend into the cytoplasm (Kim and Woloshuk, 2011) (Table 1.3). Single amino acids change can also affect the function of the whole gene. For example, GLUT1 has an extracellular glycosylation site on the first outside loop (Stein and Litman, 2014). Since FST1 encodes 12 putative transmembrane domains with the N before transmembrane domain 1 (TM1) and C termini after TM12 (74 residues and 64 residues) as well as a long central loop (70 residues) extending into the cytoplasm, it may also function as a sugar sensor. Although FST1 is structurally similar to other sugar

sensors and transporters, expression of this protein failed to complement a yeast strain lacking functional hexose transporters. Its ability to transport other sugar types (such as di- or trisaccharides) or function as a sugar sensor is still unknown (Kim and Woloshuk, 2011). Recent updates in the *F. verticillioides* genome in the Fusarium Comparative Database at the Broad Institute changed the annotation of FST1 from "hypothetical protein" to "myo-inositol transporter" without explanation. To date, no myo-inositol transporters have been functionally described in filamentous fungi. In this research, whether FST1 has inositol transport activity will be tested.

1.5 Functions of inositol in cells

Myo-inositol is a hexose that plays an important role as the structural basis for a number of secondary messengers in eukaryotic cells. In addition, inositol serves as the precursor of phosphatidylinositol (PI), the structural lipids in cellular structure and important component in intracellular signal regulation. The various phosphates of PI, the phosphatidylinositol phosphate (PIP) lipids, are essential in membrane trafficking, membrane identity, nuclear export, chromatin remodeling, and transcription (De Camilli *et al.*, 1996; Dubois, Evelyne *et al.*, 2002; Jesch and Henry, 2005; Lemmon, 2003; Seeds and York, 2007; Steger *et al.*, 2003; York *et al.*, 2005). Fungal cells acquire inositol by a multiple-step internal inositol synthesis from glucose-6-phosphate to inositol-3-phosphate, followed by dephosphorylation (Chen and Charalampous, 1964; Culbertson *et al.*, 1976; Majerus, Philip W., 2009; Reynolds, 2009) or by uptake from the environment through inositol transporters, such as ITR1 in *S. cerevisiae* (Lai and McGraw, 1994; Nikawa, J., 1995). When inositol levels are higher than 50 uM, inositol biosynthesis is

repressed and inositol transport from artificial media supports normal growth (Bisson *et al.*, 1993; White *et al.*, 1991).

1.6 Sugar transporters and inositol transporters

As a membrane protein, FST1 is categorized into the major facilitator superfamily (MFS) in the Fusarium database (Broad Institute). There are two major types of membrane proteins. Extrinsic proteins (also known as peripheral proteins) are lightly attached and can be removed by treatment with EDTA (ethylenediaminetetraacetic acid)-containing solutions with low ionic strength. Many extrinsic proteins are bound to the membrane via interaction with the intrinsic proteins (Stein W. D. and Litman T., 2014). Intrinsic proteins, also known as integral proteins, are firmly embedded in the phospholipids bilayer and can be removed only by treatment with strong detergents. These proteins span the membrane, sometimes multiple times. It is estimated that 20-30% of all genes in most genomes encode membrane proteins. Due to difficulties with crystallization, however, there are only around 500 of membrane proteins are recorded in the Protein Data Bank (PDB), which contains the structures of over 80,000 proteins (Stein W. D. and Litman T., 2014).

Transporter proteins play a crucial role in cell survival by taking up nutrients from the environment and disposing toxic compounds. Five transmembrane transporter families have been identified: (1) Channel proteins (e.g. major intrinsic proteins, voltage-sensitive ion channel, chloride channels); (2) Secondary active transporters (e.g. major facilitator superfamily (MFS) transporters, gluconate: H^+ symporters, lactate permeases, sodium: solute symporters, citrate: Na^+ symporters); (3) Primary active transporters (e.g.

ATP-binding cassette (ABC) transporters, H^+ or Na^+ translocating F-type transporters, V-type and A-type ATPases); (4) Group translocators (e.g. PTS glucose-glucoside transporters; PTS fructose-mannitol transporters; PTS galactitol transporters); (5) Unclassified (e.g. polysaccharide transporters, K^+ uptake permeases, ferrous iron uptake transporters) (Pao *et al.*, 1998). Among these five families, the ATP-binding cassette superfamily and the major facilitator superfamily have been found in all major groups of living organisms (Pao *et al.*, 1998). MFS transporters are single polypeptide secondary carriers that transport small solutes in response to chemiosmotic ion gradients, whereas ABC transporters can transport both small molecules and macromolecules in response to ATP hydrolysis, which are usually multicomponent primary active transporters.

Pao *et al.* (1998) categorized MFS transporters into 12 subfamilies. Although the sequence homology is low, MFS proteins share remarkable structural conservation (Wisedchaisri *et al.*, 2014). MFS proteins are usually formed by N- and C-terminal domains, with each domain containing a bundle of six or seven transmembrane helices. Using a rocker-switch mode, MFS members can open and close the two domains to load and release substrates (Wisedchaisri *et al.*, 2014). Sugar porters, one of the subfamilies, transports monosaccharides (hexoses, pentoses), disaccharides, quinate, inositols etc., and are usually composed of 404-818 amino acids forming 12 transmembrane domains. The sugar porter subfamily is the largest MFS subfamily, with 133 members out of 374 total MFS proteins (35.6% of the MFS family) (Pao *et al.*, 1998). XyIE of *E.coli* is the representative of this subfamily.

Because *F. verticillioides* has different infection mechanisms including airborne conidia entering through silks or insect-caused wounds or systematic infection from

infected seeds, it needs to be able to sense and respond to the changing host environment as well as to stimulatory and inhibitory compounds within the cells. How *F. verticillioides* navigates through the complex environment of silk, seed, or stem tissues while obtaining nutrition from these different tissues, at different seed maturity stages is unknown. The role FST1 plays in this plant-fungi interaction is a mystery.

1.7 Research justification and objectives

Mycotoxins are toxic chemicals produced by fungi during crop infection. Ingestion of fumonisin B1 (FB1) can result in serious health concerns for livestock and humans. Furthermore, economic losses associated with fumonisin contamination in maize production is enormous. Though environmental factors such as nitrogen source, pH, carbon source, and host tissue type can all affect the interaction between *F. verticilliodes* and maize kernels. However, the biological regulation of FB1 synthesis is still unclear. The *F. verticillioides* gene FST1, a putative sugar transporter, was found to repress fumonisin production. The purpose of this research is to study the impact of FST1 on fumonisin production during colonization of maize kernels by *F. verticillioides* and to investigate the function of FST1 in *F. verticillioides*. The following three objectives will be addressed in this dissertation:

Objective 1: Evaluate the impact of the Δfst1 mutation on the *F. verticillioides* transcriptome during kernel colonization. This objective is covered in Chapter 2.

Objective 2: Determine if FST1 has inositol transport activity. This objective is covered in Chapters 3.

Objective 3: Analyze of the importance of the central loop and C-terminus on the function of FST1. This objective is addressed in Chapter 3.

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Table 1.1. The recommended maximum levels for fumonisins in human foods.

Products	Total Fumonisins (FB1+FB2+FB3) ppm (parts per million)
Degermed dry milled corn products	2 ppm
Whole or partially degermed dry milled corn products	4 ppm
Dry milled corn bran	4 ppm
Cleaned corn intended for masa production	4 ppm
Cleaned corn intended for popcorn	3 ppm

http://www.fda.gov/food/guidanceregulation/guidancedocumentsregulatoryinformation/ucm109231.htm

Table 1.2. The recommended maximum levels for fumonisins in animal feed.

Corn and corn by-products intended for:	Total Fumonisins (FB1+FB2+FB3)
Equids and rabbits	5 ppm (no more than 20% of diet)
Swine and catfish	20 ppm (no more than 50% of diet)
Breeding ruminants, breeding poultry and breeding mink	30 ppm (no more than 50% of diet)
Ruminants > 3 months old being raised for slaughter and mink being raised for pelt production	60 ppm (no more than 50% of diet)
Poultry being raised for slaughter	100 ppm (no more than 50% of diet)
All other species or classes of livestock and pet animals	100 ppm (no more than 50% of diet)

http://www.fda.gov/food/guidanceregulation/guidancedocumentsregulatoryinformation/ucm109231.htm

Table 1.3. Central loop orientation of sugar sensors and transporters in fungi (Kim and Woloshuk, 2011)

Species	Protein	Central loop orientation
S. cerevisiae	Rgt2p	Cytoplasmic
S. cerevisiae	Snf3p	Cytoplasmic
S. cerevisiae	Hxt1p	External
N. crassa	RCO3	Cytoplasmic
Botrytis cinerea	FRT1	External
Uromyces fabae	HXT1	Cytoplasmic
F. verticilliodes	FST1	Cytoplasmic
F. verticillioides	FST13	External

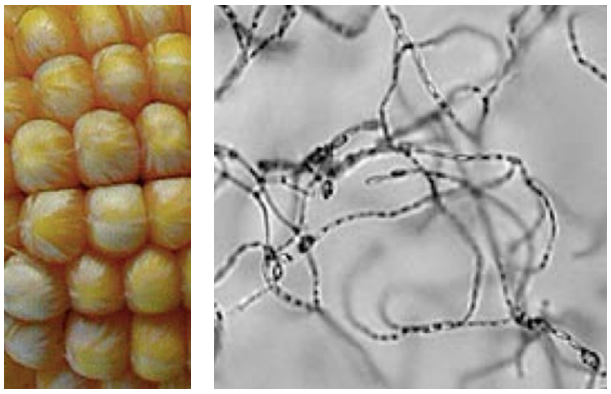

Figure 1.1. Infected kernels are scattered in the ears and show “starburst” pattern (A), and conidia chains of *F. verticillioides* (B) (Marasas *et al*., 1984b).

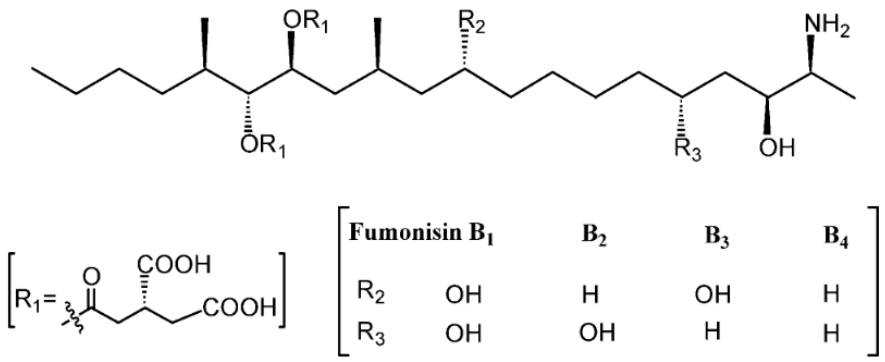

Figure 1.2. Structure of the B series of fumonisin (Bojja *et al*, 2004).

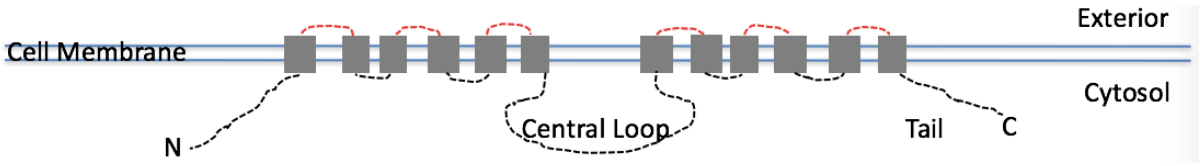

Figure 1.3. Twelve putative transmembrane domains of FST1 across the membrane (Kim and Woloshuk, 2011).

CHAPTER 2. TRANSCRIPTOME CHANGES IN *FUSARIUM VERTICILLIOIDES* CAUSED BY MUTATION IN THE TRANSPORTER-LIKE GENE *FST1*

This chapter contains both text and figures from a published manuscript: Niu, C., Payne, G.A. and Woloshuk, C.P. (2015) Transcriptome changes in *Fusarium verticillioides* caused by mutation in the transporter-like gene *FST1*. BMC Microbiology 15, doi: 10. 1186/s12866-015-0427-3.

2.1 Abstract

Fusarium verticillioides causes an important seed disease on maize and produces the fumonisin group of mycotoxins, which are toxic to humans and livestock. A previous study discovered that a gene (*FST1*) in the pathogen affects fumonisin production and virulence. Although the predicted amino acid sequence of FST1 is similar to hexose transporters, previous experimental evidence failed to prove function. Three new phenotypes were identified that are associated with the *FST1* mutant of *F. verticillioides* (delta-fst1), namely reduction in macroconidia production, increased sensitivity to hydrogen peroxide, and reduced mycelial hydrophobicity. A transcriptome comparison of the wild type and strain delta-fst1 grown on autoclaved maize kernels for six days identified 2677 genes that were differentially expressed. Through gene ontology analysis, 961 genes were assigned to one of 13 molecular function categories. Sets of

down-regulated genes in strain Δfst1 were identified that could account for each of the mutant phenotypes. The study provides evidence that disruption of *FST1* causes several metabolic and developmental defects in *F. verticillioides*. *FST1* appears to connect the expression of several gene metworks, including those involved in sencondary metabolism, cell structure, conidiogenesis, virulence, and resistance to reactive oxygen species. The results support our hypothesis that *FST1* functions within the framework of environmental sensing.

2.2 Introduction

Fusarium verticillioides (telemorph, *Gibberella moniliformis*), which is present in most maize fields, can be an asymptomatic endophyte or the causal agent of seedling, stalk, ear, and kernel diseases (Oren *et al*., 2003). The pathogen produces fumonisins, a group of structurally related polyketide mycotoxins, during colonization of maize kernels. Ingestion of fumonisin B1 (FB1), the most predominant fumonisin analog, can result in leukoencephalomalacia in horses and pulmonary edema in swine. The mycotoxin also has been implicated in human diseases, including cancer and birth defects (Hendricks, 1999). Guidelines for maximum fumonisin levels in human food and animal feeds have been established worldwide (Oren *et al*., 2003). Furthermore, economic losses associated with fumonisin contamination in maize exports by the three major maize-exporting nations (US, China and Argentina) was estimated at $100 million annually (Wu, 2006) with the US losses alone at nearly $40 million annually (Wu, 2004).

Recent publications describe the complexity of genes that influence regulation of fumonisin biosynthesis. Pathway-specific activator *FUM21* (FVEG_14633), which

controls transcription of the cluster of *FUM* genes (Brown *et al*., 2007), was shown to increase when *F. verticillioides* was treated with the histone deacetylase inhibitor chostatin A (Visentin *et al*., 2012). These results support evidence that histone modification plays an important role in the epigenetic regulation of fumonisin production (Liu *et al.*, 2012; Woloshuk and Shim, 2013). There are several intriguing reports indicating that environmental conditions (nutrients and pH) also affect the transcription of *FUM* genes and FB1 production. Expression of the nitrogen utilization gene *AREA* (FVEG_02033) was found to be responsible for repression of FB1 production by ammonium (Kim and Woloshuk, 2008). Under repression conditions, AREA is hypothesized to bind to GATA sequences in the promoters of the *FUM* genes. Generally, acidic conditions favor FB1 production (Shim and Woloshuk, 2001). Experimental evidence indicates that *PACC* (FVEG_05393), which has homology to the alkaline-activator gene *PACC* in *A. nidulans* (Tilburn *et al*., 2010), inhibits FB1 production and *FUM1* (FVEG_00316) transcription at pH 8 (Flaherty *et al*., 2003). Finally, carbon source and availability, especially amylopectin, greatly affect FB1 biosynthesis (Bluhm and Woloshuk, 2005). Studies on carbon utilization have led to the identification of two genes, *HXK1* (FVEG_00957) and *FST1* (FVEG_08441). HXK1, a putative hexose kinase was shown to be required for fructose metabolism (Kim *et al*., 2011). Strains without a functional HXK1 also produced less FB1 and were less virulent on maize kernel than the wild type (WT). The function of *FST1* is the focus of the current study.

FST1 was identified through a comparative analysis of genes expressed in colonized maize germ and endosperm tissues (Bluhm *et al*., 2008). Of 50 putative sugar transporter genes represented on a microarray, *FST1* was one of six genes identified as

highly expressed during fungal growth in endosperm tissue compared to germ tissue (Bluhm *et al*., 2008). Expression of *FST1* was also reduced in a *F. verticillioides* strain with a disrupted *ZFR1* gene, a putative Zn_2Cys_6 transcription factor (Bluhm *et al*., 2008). *FST1* encodes a 574-amino-acid protein with 12 putative transmembrane domains. Heterologous expression of *FST1* in yeast system failed to show hexose transporter activity (Kim and Woloshuk, 2011). Disruption of *FST1* in *F. verticillioides* resulted in reduced virulence and FB1 production (Bluhm *et al*., 2008; Kim and Woloshuk, 2011). The reduced virulence phenotype in inoculated kernels was manifested as slower growth and rot symptoms when compared to the WT (Kim and Woloshuk, 2011). When inoculated onto autoclaved kernels or synthetic media, mutant growth was the same as WT (Bluhm *et al*., 2008). In contrast, the mutant failed to produce FB1 on either living or dead kernels.

In the current study, we describe three new phenotypes attributed to a non-functional *FST1*. Furthermore, we describe the effects of *FST1* on whole genome expression by comparing the transcriptomes of the WT and Δfst1 strains of *F. verticillioides* grown on autoclaved maize kernels. The results support our hypothesis that *FST1* has a regulatory function that globally impacts gene expression.

2.3 Materials and methods

2.3.1 Fungal strains and culture conditions

Fusarium verticillioides strain 7600 (wild type, WT) is deposited in the Fungal Genetics Stock Center, University of Kansas Medical School, Kansas City, KS, USA. The mutant strain Δfst1 and corresponding complemented stain fst1-comp were

previously described by Bluhm *et al*. (2008). Cultures were stored long-term in 50% glycerol at -80°C and maintained as working stock on PDA medium (B&D, Sparks, MD).

2.3.2 Phenotype assessment

To assess conidiation, strains were inoculated onto Petri dishes containing 1.5% water agar with six to eight gamma-irradiated carnation leaves (average size 18 mm^2) on the agar surface (Fisher *et al*., 1982). For each fungal strain, nine carnation leaves were sampled after 7 days of incubation. Individual carnation leaves were placed into 1.5 ml microcentrifuge tubes containing 0.3 ml of water and vortexed briefly. Conidial number was determined with a hemacytometer (Aberkane *et al*., 2002). Macroconidia and microconidia were recorded as the number of conidia per carnation leaf.

Resistance to hydrogen peroxide was measured as described by Lessing (2007) and Ridenour (2014) with some modifications. Conidia (1 ml of 1×10^6 conidia) were mixed with 20 ml of molten PDA and poured into a Petri plate. After incubation for 24 hours at room temperature, a well was cut into the center of the plate with a cork borer (1 cm). To each well, 200 µl of 15 % H_2O_2 (v/v) was added. Plates were incubated for another 24 hours at room temperature in the dark. Inhibition of growth appeared as a clear zone around the well. The area of the inhibition zone was determined. Test on each fungal strain was replicated three times.

Mycelial hydrophobicity was tested by placing droplets (10 µl) of water or a detergent solution (0.2% SDS, 50 mM EDTA) on the colony surface of strains grown on PDA medium for 10 days in the dark at room temperature. After 30 minutes, we

determined whether or not the droplets maintained their spherical shape on the surface of the mycelium (Ridenour and Bluhm, 2011; Yan *et al*., 2011).

2.3.3 Transcriptome analysis

Next-generation sequencing methods were used to obtain transcriptome data from the WT and strain Δfst1 grown on autoclaved maize kernels. Kernels of maize inbred B73 were submerged in deionized water and autoclaved for 15 min. Afterwards, the kernels were crushed slightly to disrupt the pericarp, and approximately 7 g of kernels (10-12 kernels) were placed in glass vials (20 ml) and autoclaved for 30 min. Four replicate vials of the WT and Δfst1 were inoculated with 100 µl of 10^6 conidia/ml. Vials were incubated at 28°C for 6 days, then flash frozen in liquid nitrogen and stored at -80°C.

Total RNA was isolated from the content of each vial as described by Bluhm *et al.* (2008) and purified with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Further purification was achieved by treatment with the DNA-Free RNA kit (Zymo Research, Irvine, CA, USA). The Purdue Genetic Core Facility conducted quality assessment, processing, and sequencing of the RNA. The RNA samples had a RIN (RNA Integrity Number) over 7.0 as determined with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Paired-end sequences were obtained with an Illumina HiSeq 2500 sequencer (Illumina, San Diego, CA, USA). Sequence data were trimmed of adapters and filtered to remove low quality sequence and reads less than 30 nt.

RNA sequence data from each sample were mapped to the reference genome of *Gibberella moniliformis*, which was downloaded (June 2014) from the Broad Institute

Fusarium Comparative Database (http://www.broadinstitute.org). Sequence data were mapped to the reference genome was done with CLC Genomics Workbench (version 7.0.4, CLC Bio, Boston), and gene expression was quantified as reads per kilobase per million mapped reads (RPKM) (Mortazavi *et al*., 2008). Statistical analysis (pairwise t-testing) was also conducted with the CLC Genomics software. Differentially expressed genes between WT and Δfst1 were sorted to identify those with absolute fold change values of > 2.0 and P value < 0.01. Genes expressed uniquely in each fungal strain were identified also. The selected genes from the differentially expressed and those in the uniquely expressed groups were analyzed for gene ontology (GO). For each gene, the translated sequence was analyzed with Blast2GO (version 2.7.2, Blast2Go.com). Results were sorted with respect to molecular function of the top BLAST descriptors.

2.3.4 Quantitative real time-PCR

Quantitative PCR (qPCR) analysis was conducted on RNA isolated from both autoclaved and living maize kernels. For autoclaved kernels, equal amounts of purified RNA were pooled from the four biological replicates of WT and strain Δfst1 used in the RNAseq anlaysis. To obtain living kernels, maize B73 was greenhouse-grown and ears were inoculated with the *F. verticillioides* strains as described by Kim and Woloshuk (2011). Six days after inoculation, infected kernels were collected from three ears (biological replicates) and total RNA was isolated. As with the autoclaved kernels, purified RNA were pooled from the three biological replicates of WT and strain Δfst1.

cDNA was synthesized as described by Reese *et al*. (2011). Gene-specific PCR primers were designed with PrimerQuest Design Tool (Integrated DNA Technologies, Inc.). Quantitative PCR (qPCR) was conducted a described by Bluhm *et al*. (2008) and

reactions were replicated three times for each gene. Each reaction contained 1.5 µl of each primer pair (10 µM), 10 µl of iTaq Universal SYBR Green Supermix (Bio-rad, Hercules, CA), 5 µl of cDNA template, 2 µl of nuclease-free water. Reaction conditions were one cycle of 3 min at 95°C, 40 cycles of 5 s at 95°C and 30 s at 57°C. Expression of *TUB1* gene (FVEG_04081) was used to assure efficiencies of the target and reference reactions were approximately equal. The ΔΔCt method (Livak ans Schmittgen, 2001) was used to calculate expression level with *TUB1* as the internal normalizer.

2.4 Results

2.4.1 Macroconidia production and sensitivity to H_2O_2

Wild type *F. verticillioides* produces primarily microconidia and very few macroconidia. When grown on carnation leaf agar (CLA) medium, higher numbers of macroconidia are produced on the leaves. We found that strain Δfst1 produced only 12 % as many macroconidia as the WT (Table 2.1). In the complemented strain fst1-comp, macroconidia production approached WT levels (82%). There were no measurable differences between Δfst1 and WT in the production or morphology of microconidia, conidiophores, or microconidal chains.

To determine if the reduced growth phenotype of Δfst1 mutants grown on living kernels was associated with increased sensitivity to reactive oxygen species, we evaluated growth of Δfst1 mutants on agar plates amended with hydrogen peroxide. Δfst1 was found to be more sensitive than WT, and the differences were most pronounced at 15 % H_2O_2 (v/v) (Figure 2.1). The zone of the inhibition for strain Δfst1 was 2.4 and 3.6 times larger than that of the WT and strain fst1-comp, respectively.

2.4.2 Analysis of transcriptome

RNA isolated from four biological replicates of Δfst1 and WT were sequenced, which resulted in a total of over 836 million reads (Table 2.2). Approximately 752 million (90%) of the total reads uniquely mapped to the reference genome of *F. verticillioides*. Results from the mapping indicated that of the 15,869 annotated genes of *F. verticillioides*, 14,769 and 14,893 genes were expressed (RPKM > 0) in Δfst1 and WT, respectively. To identify differentially expressed genes, a pairwise t-test was made between the expression data of WT and strain Δfst1. The expression of 2,677 genes was found to be significantly different (P value < 0.01) with an absolute fold difference greater than two. Of these, 1,081 (40.4%) genes were up-regulated in Δfst1 and 1,596 (59.6%) genes were down-regulated. Also, we identified 373 and 249 genes that were uniquely expressed in WT and Δfst1, respectively. Expression of four putative tubulin and three putative elongation factor genes was similar in both strains and not statistically different (Table 2.3), indicating that the mutation in strain Δfst1 did not impact expression of these house-keeping genes. The differentially expressed genes were functionally categorized based on gene ontology (GO) annotation and placed into one of 13 groups (Table 2.4). Two-thirds of the genes were classified as encoding hypothetical proteins.

2.4.3 FUM gene cluster

One of the functional categories included the genes involved in fumonisin biosynthesis (Table 2.4). Expression of all 15 *FUM* genes was measurable in both the WT and strain Δfst1 (Table 2.5). Statistical testing indicated that 12 genes had significantly different (P value < 0.01, absolute fold change > 2) expression between the

two strains. *FUM* 11, 16 and 21 with P values of less than 0.02 did not meet the criteria for statistical significance. All *FUM* genes were down-regulated in strain Δfst1, with at least 4-fold reduction in expression compared to WT. The greatest difference was in the expression of *FUM1* (polyketide synthase gene), which was reduced more than 37-fold in the mutant. Analysis of expression by qPCR verified that both *FUM*1 and *FUM*21 expression was less in Δfst1 compared to WT (Table 2.6).

2.4.4 Hydrophobin genes

Eight hydrophobin genes have been identified in *F. verticillioides, HYD1-8* (Fuchs *et al*., 2004; Ridenour and Bluhm, 2014). Hydrophobins are a group of small, cysteine-rich proteins expressed in filamentous fungi, which form a hydrophobic/hydrophilic interface on the surface of hyphae and conidia. RNAseq analysis revealed significant differences in the expression of *HYD3*, *HYD*4, *HYD*5 and *HYD*7, with a 49.5-fold, 4.4-fold, 6.3-fold reduction and 54-fold increase, respectively, in strain Δfst1 (Table 2.7). The differences in expression of *HYD3* and *HYD7* were verified by qPCR (Table 2.6). The expression of *HYD1*, *HYD2*, *HYD6* and *HYG8* was not significantly different. To test for defects in hydrophobicity, droplets of water or a detergent solution were placed on fungal mycelium of WT, Δfst1, and the complemented strain fst1-comp. For all three strains, droplets of water maintained a spherical shape for more than 30 min. Droplets of detergent solution on the WT and strain fst1-comp also remained intact (Figure 2.2). However, on strain Δfst1, the droplet spread out over the surface of the mycelium, indicating a defect in hydrophobicity.

2.4.5 Transcription factors

Ma *et al*. (2010) predicted 683 putative transcription factor (TF) genes in *F. verticillioides* and Wiemann *et al*. (2013) predicted 640. Of these predicted TF, our analysis identified 115 differentially expressed (Table 2.4). Transcription factors in fungi have been classified into 61 families (Park *et al*., 2008), and we found that 108 of the differentially expressed TF genes were in 12 of the 61 families. Most (80%) of the TFs were C_2H_2 zinc finger (16 genes) and Zn_2Cys_6 (76 genes). *FUM21* is classified in the Zn_2Cys_6 family and its expression in Δfst1 was 4.6-fold less compared to that of the WT (Table 2.5). However, its P-value (0.012) was just outside the threshold we selected for statistical testing.

2.4.6 Transporters

A total of 191 differentially expressed genes and 35 genes in the uniquely expressed category were classified as transporters (Table 2.4). We separated the 191 differentially expressed transporter genes into seven categories: ABC transporter, amino acid related transporter, ammonium related transporter, mineral/ion related transporter, major facilitator superfamily, sugar transporter, and uncategorized (Table 2.8). In the categories for sugar and ammonium transporters, considerably more genes were up-regulated in strain Δfst1. In contrast, most of the differentially expressed genes in the ABC and ion transporter categories were down-regulated. Expression of one putative inositol transporter (*ITR1*, FVEG_06504) was decreased by 19-fold in strain Δfst1 compared to WT, which was verified by qPCR analysis (34-fold) (Table 2.6).

2.4.7 Oxidases

A total of 189 of the differentially expressed genes were categorized with putative oxidase functions (Table 2.4). Compared to the WT, two-thirds of these genes exhibited reduced expression in strain Δfst1 and the other third were expressed at higher levels. Additionally, the expression of 21 oxidase genes was only measured in the WT and seven only in Δfst1. A word-search of the *F. verticillioides* genome database identified 30 putative peroxidase and seven catalase genes, and ten of these genes were differentially expressed. The peroxidase genes (*POD1*, FVEG_10866; *POD3* FVEG_12884; *POD4*, FVEG_12465; FVEG_04790) were all down-regulated as much as 100-fold in strain Δfst1 compared to WT. Four catalase genes (*CAT1*, FVEG_05529; FVEG_05976; FVEG_03348; FVEG_05591) also were down-regulated in Δfst1. Expression of the putative catalases *CAT2* (FVEG_12611) and *CAT3* (FVEG_11955) was up-regulated 4-fold and 2-fold, respectively, in strain Δfst1. We used qPCR analysis to measure the expression of peroxidases and catalases in both autoclaved kernels and infected living kernels. In autoclaved kernels, expression of three peroxidase genes (*POD1, POD3* and *POD4*) and three catalase genes (*CAT1, CAT2* and *CAT3*) were found to be similar to expression indicated by the RNAseq results (Table 6). qPCR analysis of the inoculated living kernels indicated similar effects on expression of the peroxidases and catalases (Table 2.6).

2.4.8 Secretome

The Fungal Secretome Database (http://fsd.riceblast.snu.ac.kr) lists 1412 genes in *F. verticillioides* that encode putative secreted proteins, and a comparison with the updated reference genome at the Broad Institute matched 1402 of these genes. Our

RNAseq analysis indicated that 1310 and 1330 of the genes were expressed (RPKM > 0) in Δfst1 and WT, respectively, and significant differences were found in the expression of 367 genes. Of these, 147 (40.0%) genes were up-regulated in strain Δfst1 and 220 (60.0%) genes were down-regulated. In addition, we identified 39 and 19 genes that were uniquely expressed in WT and Δfst1, respectively. A previous study indicated that *FST1* is preferentially expressed in endosperm tissue relative to expression in germ (Bluhm *et al*., 2008); therefore, we examined genes that encode secreted enzymes for starch and cell wall degradation, many that were previously described by Ravalason *et al.* (2012). Thirty-four differentially expressed genes were separated into five enzyme groups, cellulose-degrading, xylan-degrading, pectin-degrading, xylan/pectin-degrading and starch-degrading enzymes (Table 2.9). All groups contained genes that were affected (up- and down-regulated) by the mutation in strain Δfst1. Two genes with putative functions in starch degradation were expressed at reduced levels in strain Δfst1. We measured the expression of one of these, *AGD1* (FVEG_14136) by qPCR and verified its reduction (Table 2.6).

2.5 Discussion

Previous studies indicated that deletion of *FST1* in *F. verticillioides* results in reduced fumonisin production and virulence (Bluhm *et al*., 2008; Kim and Woloshuk, 2011). Here we have linked the mutation to increased sensitivity to H_2O_2, reduced macroconidia production and reduced hydrophobicity. Considering these diverse phenotypes, the goal of this research was to characterize the effects of *FST1* on genome-wide expression during colonization of maize kernels. Autoclaved kernels were chosen

to eliminate the effects associated with reduced biomass and fungal development caused by the slower growth of the *FST1* mutant when inoculated to living kernels. Even without a living host environment, significant changes in transcription were found in the mutant, many of which may contribute to the observed phenotypes.

For our comparison of the transcriptomes of WT and Δfst1, we relied on the *F. verticillioides* reference genome at the Broad Institute. Recent updates in the annotation of the genome created changes in gene reference numbers and gene identifications. Two changes were important to our study. First, the *FUM8* gene (originally: FVEG_00318, GenBank Accession No AAG27130) was separated into two genes: FVEG_14634 and 14635. In the original annotations, *FUM8* contained a 2532-bp open reading frame encoding a 839 amino acid protein described as the aminotransferase responsible for the condensation of alanine to the polyketide backbone of B-series fumonisins (Seo *et al*., 2001). The disruption of *FUM8* in *F. verticillioides*, which blocks fumonisin production and mycotoxin production, was recovered in the mutant by complementation with the WT *FUM8* gene (Seo *et al*., 2001). In the latest annotation of the genome, the sequence encoding the first 279 amino acids of FVEG_00318 plus 11 additional amino acids was designated as FVEG_14635, and the sequence encoding the last 554 amino acids of FVEG_00318, which contains aminotransferase domain, was designated as FVEG_14634. Regardless of this particular annotation error, expression of *FUM8* is significantly reduced in strain Δfst1 along with most of the other *FUM* genes, confirming the role of *FST1* in fumonisin production.

The second peculiar annotation change in the reference genome was that for *FST1* (FVEG_08441). Originally listed as a “hypothetical protein”, with similarity to hexose

transporters, the gene is now listed as a “myo-inositol transporter”. Inositol is a polyol that functions as an essential constituent of cell membranes as derivatives of phosphatidylinositol and as important cell signaling molecules of inositol phosphates (Barker *et al*., 2009). Two myo-inositol transporter genes have been described in *S. cerevisiae* by complementation of a strain defective in myo-inositol uptake (Nikawa *et al*., 1991). A BLAST analysis of the *F. verticillioides* genome with the yeast ITR1p sequence identified eight genes with high sequence similarity (FVEG_01519, FVEG_01638, FVEG_02081, FVEG_03992, FVEG_06504, FVEG_07757, FVEG_11293, and FVEG_12687). The sequence of *FST1* was not identified by the search. Among the eight identified genes, expression was significantly down-regulated in Δfst1 for FVEG_06504 (named *ITR1*) (19-fold) and FVEG_03992 (5-fold), while the expression of FVEG_12687 was significantly up-regulated (12-fold). We measured the expression of *ITR1* by qPCR and verified that its expression was significantly reduced (Table 2.6). In light of these observations, the assignment of the functional role of myo-inositol transporter to *FST1* is premature.

Kim and Woloshuk (2011) described the phenotype of Δfst1 as having slower growth and symptom development, and thus reduced virulence, compared to WT on wound-inoculated maize kernels. This growth inhibition was not observed on autoclaved kernels (Bluhm *et al*., 2008). We hypothesized that the reduced virulence of Δfst1 resulted from an increased sensitivity to the effects of reactive oxygen species (ROS), which includes H_2O_2 produced by the living kernel (Torres *et al*., 2006; Heller and Tudzynski, 2011). The greater inhibition of the growth of strain Δfst1 by H_2O_2 compared to the WT and fst1-comp strains supports this hypothesis.

During pathogenesis, *F. verticillioides* could encounter ROS produced in maize kernels through several independent pathways. Kim and Woloshuk (2011) inoculated the crown of maize kernels at the R4 (dough) stage of development, a period when the endosperm tissue is undergoing program cell death (PCD) (Young and Gallie, 2000). ROS molecules, including H_2O_2, are produced during PCD in plants (Van Breusegem and Dat, 2006) and likely during endosperm development (Sabelli, 2012). ROS production is also a characterized response of plants to pathogen invasion and plays a major role in host defense (Torres, 2010). Most pathogens respond to ROS by the production of peroxidases and catalases (Torres, 2010). Our RNAseq analysis of *F. verticillioides* grown on autoclaved kernels identified several putative catalases and peroxidases whose expression was changed in Δfst1 mutants. Four putative peroxidase genes were down-regulated in Δfst1, as were four of the six putative catalases. We also found that these oxidases were similarly affected in living kernels infected with the *F. verticillioides* strains.

To gain greater insight into a possible function of the catalases and how they may affect virulence, we examined their function in other plant pathogens. Catalases have been separated by phylogenetic analysis into four clades: peroxisomal, cytoplasmic, spore-specific, and secreted (Giles *et al*., 2006). We found sequence similarity in the five differentially expressed catalases from our study when compared to the catalases assigned to the four clades in Giles (2006). FVEG_11955 was most similar to XP324526 in *Neurospora crassa* and FG02881 in *Gibberella zeae*, both of which belong to the peroxisomal catalase (clade P). FVEG_05976 was similar to FG05695 in *G. zeae*, which belongs to the cytoplasmic catalase (clade C). FVEG_05591 was similar to AAK15808 in

N. crassa and FG06554 in *G. zeae*, which belong to the spore-specific catalase (clade A). Sequence analysis of the N-termini of the five predicted catalase proteins indicated that none are secreted.

As mentioned, catalases also have an important role in fungal development, including conidiogenesis. The sequences of the five differentially expressed, putative catalases in *F. verticillioides* are highly similar to *CATB* in *Magnaporthe grisea*, *CATA* and *CATB* in *A. nidulans*, *CAT1* and *CAT3* in *N. crassa*, and *CATB* in *Blumeria graminis*. In *M. grisea*, *CATB* is up regulated during infection of rice (Skamnioti *et al*., 2007). A strain disrupted in *CATB* was reduced in virulence with increased sensitivity to hydrogen peroxide, and was severely affected in conidia production. In addition, *CATA* mutants in *A. nidulans* exhibited reduction in conidiation and increased sensitivity to hydrogen peroxide (Navarro *et al*., 1996). The vast majority of conidial produced by *F. verticillioides* are microconidia. Although the number of macroconidia produced by the WT used in our study comprised only about 7% of the total conidia population, the reduction of macroconidia was consistently observed in strain Δfst1. From our study, it is not possible to determine if the altered expression of the five catalases in strain Δfst1 is responsible for the reduced production of macroconidia.

Aside from the role of catalases in conidial development, transcription factors are known to impact conidiation in fungi, and the expression of several putative TF genes were down-regulated in strain Δfst1. These genes include FVEG_16516 similar to *REN1* of *Fusarium oxysporum*, FVEG_09661 and FVEG_00646 similar to *BRLA* and *ABAA* of *A. nidulans*, respectively, FVEG_12826 similar to *FL* (*fluffy*) in *N. crassa*, and FVEG_06118 similar to FGSG_06160 in *F. graminearum.* Mutants of *REN1* and *ABAA*

fail to produce normal conidia because of developmental malfunctions associated with phialides, the conidiogenous cells (Sewall *et al.*, 1990; Ohara *et al*., 2004). Mutants of *BRLA* fail to produce conidiophores (Adam *et al*., 1988) and *FL* mutants fail to produce conidia in chains (Bailey and Ebbole, 1998). Furthermore, expression of the conidiation-specific gene *CON-10* is not induced in *FL* mutants of *N. crassa*. In strain Δfst1, a gene (FVEG_00227) with high sequence identity to *CON-10* was down-regulated 14-fold compared to the WT. In *F. graminearum*, Son *et al*. (2011) reported that deletion of FGSG_06160 results in a reduction in conidia production but no effect on virulence. We measured the expression of *FL*-like gene (*FLF1*, FVEG_12826) by qPCR (Table 2.6). The expression was 2.9-fold of WT, which is near the 2.2- fold reduction obtained from the RNAseq analysis. These results indicate that reduced expression of one or more of these TFs may impact production of macroconidia but not microconidia.

Hydrophobins are another family of proteins that are associated with conidiogenesis as well as aerial hypha formation and have been shown to be involved in virulence (Wosten *et al*., 1993; Talbot *et al*., 1996; Wosten, 2001; Fuchs *et al*., 2004). Hydrophobins are separated into two classes based on spacing of cysteine residues and physical characteristics. Class I hydrophobins are highly insoluble proteins that form rodlets, and class II are more soluble and do not form rodlets. Fuchs *et al*. (2004) predicted that hydrophobin genes *in F. verticillioides* encode three class I proteins (*HYD1* FVEG_03689, *HYD2* FVEG_03685 and *HYD3* FVEG_06538) and two class II proteins (*HYD4* FVEG_01575 and *HYD5* FVEG_07695). Examination of the protein sequences derived from *HYD6* (FVEG_01573) and *HYD8* (FVEG_10008) suggests they are class II and class I hydrophobins, respectively. We could not discern the class of *HYD7*

(FVEG_09843) based on sequence alignments. Mutants of *F. verticillioides* with deletions of *HYD1* or *HYD2* are not defective in radial growth, conidial numbers, or corn seedling infection. However, these mutants fail to form microconidial chains (Fuchs *et al*., 2004). Expression of these two genes was unaffected in Δfst1 and the strain produced normal microconidial chains. We observed the spreading of droplets of detergent solution placed on the surface of strain Δfst1, suggesting a deficiency in the more soluble class II hydrophobins (Wosten *et al*., 1993). The down-regulated expression of *HYD4*, *HYD5* and *HYD6* in strain Δfst1 is likely associated with this phenotype.

Previous studies have shown that fumonisin production and *FST1* expression are higher in the endosperm than in germ tissues (Bluhm *et al*., 2008; Kim and Woloshuk, 2011). These observations suggest that components within the endosperm provide an environment conducive for the pathogen. Strain Δfst1 grows as well as the WT on autoclaved maize, implying that it produces the secreted enzymes needed to breakdown macromolecules in the kernel and transporters to move nutrients into growing hyphae. However, our transcriptome results indicate that the mutation in *FST1* greatly impacts the expression of several genes that encode secreted enzymes. We found that the expression of genes encoding enzymes that degrade complex carbohydrate polymers, which make up host cell walls, was altered in strain Δfst1, but not uniformly. The lack of a growth phenotype in the mutant when grown on autoclaved maize and culture media may reflect functional redundancy in these large gene families (Wagner, 2005). For example, the expression of the alpha-amylase gene FVEG_12957 (Bluhm *et al*., 2008) was not affected in strain Δfst1. Expression of this gene would likely mask the potential effects

caused by the down regulation of the two starch degradation genes (FVEG_12681 and FVEG_14136).

In this study, we described three new phenotypes associated with a mutation in *FST1* that may contribute to the reduced virulence phenotype, namely the increased sensitivity to hydrogen peroxide, reduction of macroconidia production, and changes in mycelial hydrophobicity associated with Δfst1 mutants. We propose that reduced resistance to H_2O_2 in Δfst1 may impede the strain's ability to respond to ROS encountered during pathogenesis. Our analysis of the transcriptomes of WT and Δfst1 indicated that the mutation of *FST1* affects the expression of 17% of the genes in *F. verticillioides*. Among the genes affected were many that impact mycotoxin biosynthesis, virulence, resistance to H_2O_2, and conidiogenesis. Our study supports the hypothesis that *FST1* has a role other than sugar transport. Other researchers have described putative sugar transporters that appear to have broader functions. Mutants of *RCO-3* in *N. crassa* displayed altered responses to increasing glucose concentrations in culture media (Madi *et al*., 1997). The authors suggested that RCO-3 functions as a sugar sensor and a regulator of conidia production. In *Magnaporthe oryzae*, mutations affecting *MOST1* result in reduced conidiation and production of the secondary metabolite melanin (Saitoh *et al*., 2014). The authors were not able to complement the defects by expression of other sugar transporter genes. Further studies are needed to determine how these genes (including *FST1*) regulate the function of multiple cell processes. (Availability of supporting data: Supporting sequence data are available in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE66044. http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE66044)

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Table 2.1. Effect of Δfst1 on conidiation.

Strain	Macroconidia [a]	Microconidia
WT	90 ± 7	1,252 ± 118
Δfst1	9 ± 3*	1,052 ± 123
Fst1-comp	75 ± 7	1,603 ± 163

[a] Macroconidia and microconidia values are mean number of conidia per carnation leaf from nine replicates at 7dpi +/- standard error.
* Indicates significant difference from other values in column. (α=0.05)

Table 2.2. Summary of RNAseq data from Illumina sequencing[a]

Sample Name	Total Reads	Percent Mapped
Fst1-1	89,159,452	91
Fst1-2	90,508,852	91
Fst1-3	120,993,332	90
Fst1-4	122,219,758	89
WT1	111,712,848	89
WT2	96,499,202	90
WT3	106,782,286	90
WT4	98,144,940	90

[a] Paired-end data were trimmed to remove low quality sequence, and reads less than 30 nt were filtered out of the final data sets. These data were mapped to the *F. verticillioides* reference genome with CLC Genomic Workbench 7.0.4 software.

Table 2.3. Expression of tubulin and elongation factor (EF) genes during colonization of autoclaved maize kernels by strains Δfst1 and WT [a]

FVEG number	Gene name	WT	Δfst1
00855	Tubulin alpha chain	198 [b]	172
00557	Tubulin alpha chain	195	220
05512	Tubulin beta chain	162	183
02785	Tubulin gamma chain	23	21
02381	EF 1-alpha	2345	2360
04016	EF 1-alpha	25	23
09131	EF 2	9	8

[a] Data were collected from cultures grown for 6 days on autoclaved maize kernels
[b] Data are the mean RPKM values of four biological replicates

Table 2.4. Molecular function ontology of differentially expressed genes in WT and Δfst1 during colonization of autoclaved maize kernels [a].

Molecular Function[b]	Up in Δfst1	Down in Δfst1	Not expressed in WT	Not expressed in Δ*fst1*
Hydrophobins	1	3	0	0
Fumonisin biosynthesis	0	12	0	0
Decarboxylases	6	9	2	0
Reductases	20	18	1	3
Kinases	18	32	3	3
Peptidases and Proteases	13	39	1	5
Integral Membrane Proteins	40	22	3	7
Hydrolases	42	64	5	12
Transcription Factors	49	66	5	3
Dehydrogenases	48	78	6	7
Oxidases	66	123	7	21
Transporters	97	94	16	19
Hypothetical Proteins	681	1035	200	293
Total	1081	1596	249	373

[a] Data were collected from cultures grown for 6 days on autoclaved maize kernels
[b] Ontology assignments based on top BLAST from Blast2GO analysis

Table 2.5. Comparison of expression of FUM genes in wild type (WT) and Δfst1[a].

Gene name	FVEG ID[b]	P value[c]	Log_2 fold change[d]
FUM1	00316	0.004*	-5.21
FUM2	00323	0.008*	-3.15
FUM3	00320	0.003*	-3.41
FUM6	00317	0.003*	-3.63
FUM7	00319	0.002*	-2.96
FUM8	14634	0.005*	-3.54
FUM10	00321	0.004*	-3.60
FUM11	00322	0.012	-3.06
FUM13	00324	0.002*	-3.59
FUM14	00325	0.006*	-3.20
FUM16	00326	0.019	-2.74
FUM17	00327	0.007*	-3.03
FUM18	00328	0.008*	-2.76
FUM19	00329	0.003*	-2.90
FUM21	14633	0.012	-2.20

[a] Data were collected from cultures grown for 6 days on autoclaved maize kernels.
[b] Fusarium Comparative Database (Broadinstitute.org)
[c] P value from pairwise t-test of mean RPKM of Δfst1 and WT. Values with * meet the criteria of $P < 0.01$.
[d] Values derived from the mean RPKM of Δfst1/WT.

Table 2.6. Expression of selected genes in strain Δfst1 relative to expression in wild type (WT) of *F. verticillioides*.

Gene Name	FVEG Number	Relative Expression[a]	
		Autoclaved Kernels	Living Kernels
FUM1	00316	-33.3 (-25.8, - 48.5)	
FUM21	14633	-9.4 (-7.6, - 11.7)	
POD1	10866	-3.9 (-3.7, - 4.1)	-2.0 (-2.0, - 2.0)
POD3	12884	-86.7 (-73.3, - 102.6)	-102.1 (-71.0, -147.0)
POD4	12465	-119.1 (-115.8, – 122.5)	-4.1 (-4.1, - 4.2)
CAT1	05529	-12.0 (-10.1, – 14.2)	-32.4 (-21.8, - 47.1)
CAT2	12611	3.7 (3.3, 4.0)	2.2 (1.9, 2.4)
CAT3	11955	1.5 (1.5, 1.6)	4.9 (4.8, 4.9)
HYD3	06538	-76.0 (-73.3, – 78.7)	
HYD7	09843	33.7 (31.8, 35.7)	
ITR1	06504	-34.4 (-23.5, - 50.4)	
FLF1	12826	-2.9 (-2.8, - 3.0)	
TFS1	06118	-2.6 (-2.4, - 2.8)	
AGD1	14136	-22.1 (-20.5, - 24.0)	

[a] Expression was measured by quantitative reverse-transcriptase polymerase chain reaction (qPCR). RNA from biological replicate samples of WT or Δfst1 was pooled for cDNA synthesis, and three technical replicates were analyzed for each gene. Expression of *TUB1* (FVEG_04081) was used to normalize data. For each gene, values represent fold differences in Δfst1 with WT expression set at a value of 1. Expression of each gene was calculated as $2^{\Delta\Delta Ct}$. Range of expression is in parentheses equals $2^{\Delta\Delta Ct-s}$, $2^{\Delta\Delta Ct+s}$, where s equals the standard deviation of the $2^{\Delta\Delta Ct}$ value.

Table 2.7. Comparison of hydrophobin (*HYD*) genes during colonization of autoclaved maize kernels by strains Δfst1 and WT [a].

Gene name	FVEG ID[b]	P value[c]	Log_2 fold change[d]
HYD1	03689	-[e]	-
HYD2	03685	-	-
HYD3	06538	0.0001	-5.63
HYD4	01575	0.0019	-2.13
HYD5	07695	0.0083	-2.65
HYD6	01573	-	-
HYD7	09843	0.0036	5.76
HYD8	10008	-	-

[a] Data were collected from cultures grown for 6 days
[b] Fusarium Comparative Database (Broadinstitute.org)
[c] P value from pairwise t-test of mean RPKM of Δfst1 and WT
[d] Values derived from the mean RPKM of Δfst1/WT
[e] Data not significant (P value > 0.01)

Table 2.8. Classification of putative transporter genes differentially expressed during colonization of autoclaved maize kernels by wild type (WT) and strain Δfst1[a].

Transporter Type[b]	Up in Δfst1	Down in Δfst1
Amino acid related	14	10
ABC transporter	1	9
Ammonium related	4	0
Mineral/ion related	9	19
Sugar transporter	30	7
Major facilitator superfamily	23	28
Uncategorized transporter	16	21

[a] Data were collected from cultures grown for 6 days on autoclaved maize kernels.

[b] Ontology assignments based on top BLAST from Blast2GO analysis.

Table 2.9. Differences in expression of putative, secreted, cell wall-degradation genes during colonization of autoclaved maize kernels by wild type (WT) and strain Δfst1.

FVEG ID[b]	Molecular function	P value[c]	Log_2 fold change[d]
	Cellulose-degrading		
05521	Glucosidase	0.0021	1.30
08733	Glycosidase	0.0010	1.35
09772	Glucosidase	0.0027	2.22
12965	Glycosidase	0.0004	1.93
13391	Glucosidase	0.0002	2.53
07232	Glucanase	0.0008	-1.32
01870	Glucosidase	0.0024	-1.80
11944	Glucanase	0.0009	-3.47
12142	Glucanase	0.0023	-1.74
13055	Glucosidase	0.0005	-1.31
12840	Glucanase	-	Δfst1[e]
10897	Glucanase	-	Δfst1
12594	Glucosidase	-	WT
	Xylan-degrading		
08344	Xylanase	0.0042	1.09
10098	Xylanase	0.0006	3.58
10625	Xylosidase	0.0006	1.69
12502	Xylanase	0.0006	1.19
13062	Xylanase	0.0055	1.18

Table 2.9. continued.

13426	Xylosidase	0.0006	1.88
13578	Xylanase	0.0031	1.64
07261	Xylanase	0.0009	-1.42
13553	Xylanase	0.0068	-1.08
	Pectin-degrading		
04421	Galactosidase	0.0005	2.47
12299	Galactanase	8.3 E-06	2.02
08734	Pectic Lyase	0.0017	-2.76
11228	Pectinesterase	0.0095	-1.10
13516	Polygalacturonase	0.0023	-2.01
	Xylan/pectin-degrading		
05689	Arabinofuranosidase	0.0007	1.41
13426	Arabinofuranosidase	0.0006	1.88
07490	Glycosidase	2.2 E-06	-2.50
08421	arabinase	0.0004	-1.16
16349	Glycosidase	0.0002	-1.26
	Starch-degrading		
12681	Dextranase	0.0001	-1.62
14136	Glucosidase	9.4 E-05	-3.94

[a] Data were collected from cultures grown for 6 days.
[b] Fusarium Comparative Database (Broadinstitute.org), secretion prediction based on SignalP algorithm (Livak and Schmittgen, 2001).
[c] P value from pairwise t-test of mean RPKM of Δfst1 and WT.

[d] Values derived from the mean RPKM of Δfst1/WT.
[e] Transcript only detected in WT or Δfst1 as designated.

Table 2.10. PCR primers used in this study.

Primer Name	FVEG Number[a]	Primer Sequence (5'-3')
FST1-F	08441	CTT CTG ATG CTC TTC TCT TCC TCG C
FST1-R		TCT GGT ATA TCT CAC CAA TGA ACG CGA T
FUM1-F	00316	ACA CCA AAG CCT CTA CAA GTG A
FUM1-R		AGG TAT CGG GCA CCG CT
FUM21-F	14633	TTG CGA GGA TCT GTT CTT CTA TC
FUM21-R		TAT TAC CGA GCT TGC GCT ATA C
POD1-F	10866	TCA TTG ACC GTG CTC AAC TCC TCA
POD1-R		TGT CGA GTT GAC GAA GAA GT
POD3-F	12884	TCC TGG AAC AAC TGG AAT GG
POD3-R		CAA TCA AGA CAG ACA GGA GAG G
POD4-F	12465	GGC TAG CTA CAT CCA AGA AGA C
POD4-R		GTA CCA TCA GCC ATG ATC TCA A
CAT1-F	05529	GAT CTT CTG GAC CAA CCT CAA T
CAT1-R		CCT GAA CTT GGG CTC CTT ATA C
CAT2-F	12611	AGA AGA AGG CTG GTG CTA ATG
CAT2-R		GGC TCC ATG ACC TGA ACA TAC
CAT3-F	11955	GAG CGA CAC GCA AAC CAT TGA AGT
CAT3-R		ACC ACC AAC AGT CGA GAT TCG TGT
TUB1-F	04081	TGC TCA TTT CCA AGA TCC GCG

Table 2.10. continued

TUB1-R		GCG CAT GCA GAT ATC GTA GAG G
HYD3-F	06538	TTG CTC CAC CAA CTC TTA CTG
HYD3-R		GCG TTG ATG TTG ATG AGA GCA
HYD7-F	09843	AGC TCT CCG CCA TCT TCT A
HYD7-R		GCT CAA TGT CTC TCT CCT CAA C
ITR1-F	06504	GTC TCT CCC GTT CAT GAT TCT C
ITR1-R		GGG TTG ACT TGG GTG GTA TT
FLF1-F	12826	AGC GAT GCT TCT TGT CCT TAC
FLF1-R		AAC CAA GCT CAC GAC CTA TTT
TFS1-F	06118	GGG ACC TGT TGC CAT TAA GA
TFS1-R		TCA TCC TCC GGC ATT TCA TAG
AGD1-F	14136	CGT ATG GCA GAG TGG GTA AAT
AGD1-R		CAT CAG GAT TCG GAC GGT ATA TG

[a] Fusarium Comparative Database (Broadinstitute.org)

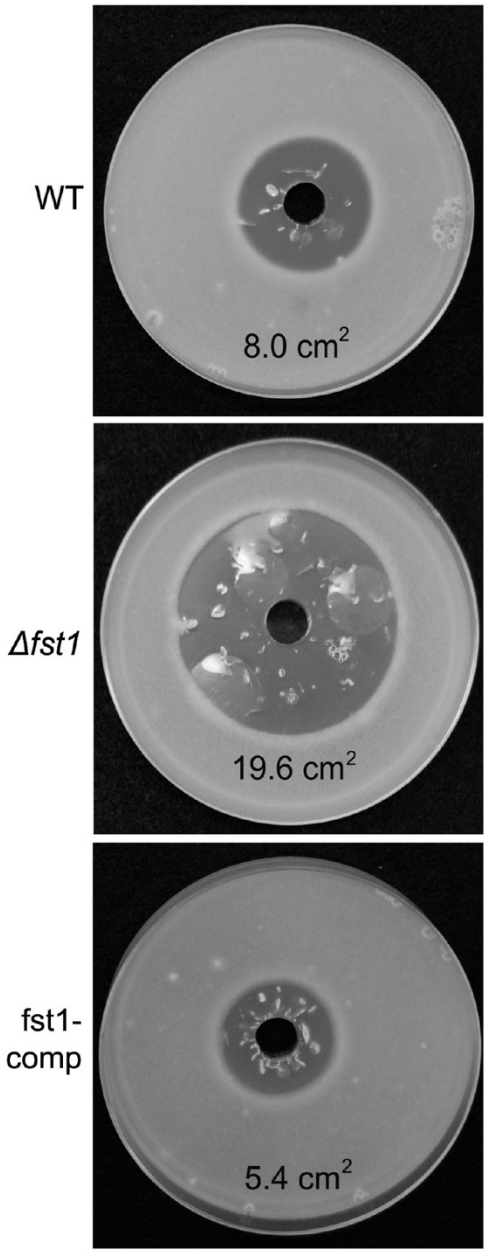

Figure 2.1. Resistance to hydrogen peroxide assay. Conidia of wild type, Δfst1, and fst1-comp were suspended into molten PDA medium. After 24 hours, 15% hydrogen peroxide solution was added to a well cut into each culture. Photograph was taken after two days of incubation. Each plate is labeled with the mean area of inhibition (clear zone) for three replicates. The standard errors were 0.01 cm^2 or less.

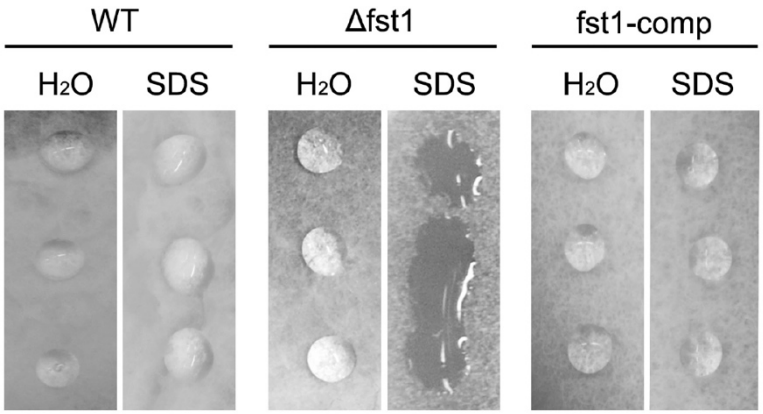

Figure 2.2. Mycelial hydrophobicity assay. Cultures of wild type and Δfst1 were grown for 10 days on PDA medium. Photograph was taken 30 min after placement of droplets (10µl) of water and SDS solution on the colony surface.

CHAPTER 3. INVOLVEMENT OF FST1 FROM *FUSARIUM VERTICILLIOIDES* IN VIRULENCE AND TRANSPORT OF INOSITOL

This chapter contains both text and figures that have been submitted to Molecular Plant Pathology: Niu, C., Payne, G.A. and Woloshuk, C.P. Involvement of FST1 from *Fusarium verticillioides* in virulence and transport of inositol. April 2016.

3.1 Abstract

Fumonisin B1 (FB1), a polyketide mycotoxin produced by *Fusarium verticillioides* during the colonization of maize kernels, is detrimental to human and animal health. The *FST1* gene is highly expressed in the endosperm of maize kernels, which is the carbohydrate-rich storage tissue, compared levels of expression in germ tissues. *FST1*, a gene of unknown function, encodes a putative protein with 12 transmembrane domains, however its function remains unknown. Previous research has shown that *FST1* of *F. verticillioides* affects FB1 production, virulence, hydrogen peroxide resistance, hydrophobicity and macroconidia production. Here we examine the phylogeny of FST1, its expression in a *Saccharomyces cerevisiae* strain lacking a functional myo-inositol transporter (ITR1), and the effect of amino acids changes in the central loop and C-terminus regions of FST1 to functionality. The results indicate that expression of *FST1* in a *ITR1* mutant strain can restore growth on myo-inositol medium

to wild-type levels as well as restore the inhibitory effects of FB1, suggesting that FST1 can transport both myo-inositol and FB1 into yeast cells. Our results with engineered FST1 also indicated that amino acids in the central loop and C-terminus regions are important for FST1 functionality in both S. *cerevisiae* and *F. verticillioides*. Overall this research has established the first characterized inositol transporter in filamentous fungi and advanced our knowledge about the global regulatory functions of *FST1*.

3.2 Introduction

Mycotoxins are toxic chemicals produced by fungi, often associated with crop diseases. For example, *Aspergillus flavus* and *Fusarium verticillioides* cause ear and kernel rots of maize, and produce aflatoxins and fumonisins, respectively. In this study, we focus on fumonisin B1 (FB1), which causes leukoencephalomalacia in horses, pulmonary edema in swine, and birth defects and cancer in human (Hendricks, 1999). Our goal is to better understand the function of genes and the impact of environmental factors on fumonisin biosynthesis. Several genes responding to environmental factors such as pH and nitrogen and carbon sources have been reported to affect FB1 production. Ammonium and glutamine were shown to repress FB1 production by the expression of *AREA*, a global regulator involved in nitrogen metabolism (Kim & Woloshuk, 2008). Flaherty *et al*. (2003) demonstrated that the pH regulatory gene *PAC1* was associated with repression of fumonisin production under alkaline conditions. Previous studies also have revealed that starch, especially amylopectin, is an excellent inducer of FB1 production (Bluhm & Woloshuk, 2005) and that amylopectin-rich endosperm of maize

seeds supports higher FB1 production than germ tissue even though growth is similar in both tissues (Bluhm *et al.*, 2008, Shim *et al.*, 2003).

The sequencing and assembly of the *F. verticillioides* genome (Ma *et al.*, 2010) and the advent of microarray analysis facilitated a comparison of gene expression during growth on endosperm and germ to discover genes involved in sugar transport. Bluhm *et al*. (2008) identified six putative sugar transporter genes whose expression was higher in endosperm tissues than in germ. Subsequent experiments indicated that disruption of one of these genes (*FST1*) resulted in a loss of FB1 production (Bluhm *et al*., 2008, Kim & Woloshuk, 2011). *FST1* (GenBank number EU152990.1, FVEG_08441) encodes a 574-amino-acid protein with 12 putative transmembrane domains. Further examination of the mutated strain (Δfst1) revealed reduced virulence (colonization rate) on living kernels, reduced macroconidia production, increased sensitivity to hydrogen peroxide and reduced mycelial hydrophobicity (Bluhm *et al*., 2008, Kim & Woloshuk, 2011, Niu *et al.*, 2015). Previous efforts failed to demonstrate hexose transporter activity in a strain of *Saccharomyces cerevisiae* lacking of 17 hexose transporters (Kim & Woloshuk, 2011).

In this study, we established a phylogenetic history of FST1 by comparing its sequence with 64 other FST1-like and ITR1-like proteins in other fungi. We also examined two important questions concerning the function of FST1: (1) Does the protein have myo-inositol transporter activity, and (2) are the central loop and C-terminus regions important for the function of the protein? We addressed these questions using a *S. cerevisiae* strain lacking a functional myo-inositol transporter gene (*ITR1*) and also insensitive to FB1. Our results show that FST1 transports myo-inositol and possibly

FB1. Manipulations of the central loop and C-terminus also demonstrated the importance of these regions in FST1 function in both *S. cerevisiae* and *F. verticillioides*.

3.3 Materials and Methods

3.3.1 Fungal strains and culture conditions

Fusarium verticillioides 7600 (M3125; Fungal Genetics Stock Center, University of Kansas Medical School, Kansas City, KS, USA) was used as the wild-type (FvWT) strain. Cultures were stored long-term in 50% glycerol at -80°C and maintained as working stock on PDA medium (B&D, Sparks, MD).

Saccharomyces cerevisiae strains BY4741 (ScWT; MATa, his3Δ, leu2Δ, met15Δ, ura3Δ) and Δitr1 (MATa, his3Δ, Δitr1, leu2Δ, met15Δ, ura3Δ; GE Dharmacon, Lafayette, CO) were grown in Yeast Extract Peptone Dextrose (YEPD) medium at 30°C. In yeast growth assays, ScWT, ScΔitr1 and other strains were grown on YNB medium modified from Culbertson *et al*. (1976): 6.7 g yeast nitrogen base without amino acids (BD Bionutrients, Franklin Lakes, NJ), 20 mg lysine, 10 mg arginine, 10 mg methionine, 60 mg threonine, 10 mg tryptophan, 10 mg leucine, 10 mg histidine, 10 mg adenine and 10 mg uracil per liter supplemented with myo-inositol, sucrose or galactose and raffinose at appropriate concentrations.

3.3.2 Generation of Δfst1 strain

The *FST1* in *F. verticillioides* was deleted by the split-marker method described by Ridenour *et al.* (2012). Upstream and downstream fragments of FST1 were amplified by PCR with primer pairs FST1_F1/FST1_F2 and FST1_F3/FST1_F4, respectively. The split marker PCR fragments (hy and yg) of the hygromycin phosphotransferase (HYG)

gene were amplified with PCR primer pairs M13F/HY1 and YG1/M13R (Ridenour & Bluhm, 2014). Fragment hy was fused to the upstream fragment by PCR with primers FST1_F1N and HYN, and fragment yg was fused to the downstream fragment with primers YGN and FST1_F4N. PCR conditions for the split marker fusion were: 94°C for 1 min, 15 cycles of 94°C for 20 s, 58°C for 4 min and 65°C for 7 min, and one cycle of 65°C for 10 min. This was followed by 94°C for 1 min, 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 140 s, and one cycle of 72°C for 5 min. The resulting split marker products were concentrated by ethanol precipitation and used to transform protoplasts of FvWT as described by Ridenour *et al*. (2012). Hygromycin-resistant transformants were screened by PCR with primers FST1_A1/HYGSCRN_B. To complement the fst1-deletion strain (FvΔfst1), the *FST1* gene was PCR amplified with the primer pair FST1LP-ApaI / FST1LP-14c. The resulting product was fused to the vector pFL1 (linearized with *Xho*I/*Apa*I; kindly provided by Dr. Jin-Rong Xu, Purdue University, IN, USA) in *Saccharomyces cerevisiae* strain XK1-25 (Wang *et al.*, 2011) as described Zhou *et al.* (2011). The sequence of the resulting fusion construct pFL1-FST1comp was verified and transformed into protoplast of FvΔfst1. Geneticin-resistant transformants were confirmed by sequencing analysis.

3.3.3 Phylogenetic analysis

BLASTp analysis with the amino acid sequence of ITR1 from *S. cerevisiae* and FST1 was conducted in the Fungal Genomics Resource JGI MycoCosm database (http://genome.jgi.doe.gov/programs/fungi/index.jsf, Grigoriev *et al*, 2014). Three *ITR1*-like and *FST1*-like genes in the *Pezizomycotina* and *Saccharomycotina* classes with the highest scores were selected (Table 3.1). *ITR1*-like and *FST1*-like genes in *F.*

verticillioides were also identified by BLASTp. Proteins identified with NCBI total score larger than 190 were confirmed in genomic database: Broad Institute for *Fusarium* (http://www.broadinstitute.org/annotation/genome/fusarium_group /MultiHome.html). The alignment of amino acid sequences was generated by clustalX 2.1 with default gap penalties. Maximum likelihood reconstruction of the phylogenetic history was generated by methods of Miller *et al.* (2010) with RAxML-HPC BlackBox 8.2.3 using maximum likelihood/rapid bootstrapping on XSEDE based on the alignment of amino acid sequences of targeted genes (http://www.phylo.org/sub_sections/portal/).

3.3.4 Engineered *FST1*

Conserved and divergent regions of the FST1 central loop and C-terminus were identified arbitrarily from amino acid sequence alignments to other fungal FST1-like proteins by clustalX 2.1 with default gap penalties (Larkin *et al.*, 2007). Mutations (three amino acid deletions and single amino acid changes) in the FST1 were made with overlapping PCR products containing the specific mutations and subsequently fused together into the pFL1 vector in *S. cereviseae* strain XK1-25 (Zhou *et al*., 2011). For deletion in the conserved region of the central loop (IRG), divergent region of central loop (VVS), conserved C-terminus region (EEI) and divergent C-terminus region (HLL), primer pairs FST1-ApaI/FST1LP-Dc12 and FST1LP-Dc13/FST1-14c, FST1-ApaI /FST1LP-Dd12 and FST1LP-Dd13/FST1-14c, FST1-ApaI/FST1TL-Dc12 and FST1TL-Dc13/FST1-14c, FST1-ApaI/FST1TL-Dd12 and FST1TL-Dd13/FST1-14c were used to amplify the two fragments, respectively. For the single amino acid changes in the conserved region of central loop IRG to IGG, IRG to IKG, and the divergent C-terminus region (HLL to HDL), primer pairs FST1-ApaI/FST1LPRG-R and FST1LPRG-F/FST1-

14c, FST1-ApaI/FST1LPRK-R and FST1LPRK-F/FST1-14c, FST1-ApaI/FST1TLLD-R and FST1TLLD-F/FST1-14c were used to amplify the two fragments, respectively. The sequences of the mutation vectors were verified. Mutation vectors were transformed into protoplasts of FvΔfst1, and geneticin-resistant transformants were confirmed by sequencing analysis. Three transformants for each engineered construct were selected for the phenotype assessment.

3.3.5 Phenotype assessment of *F. verticillioides*

Maize B73 was greenhouse-grown and ears were inoculated with the *F. verticillioides* strains as described by Bluhm *et al*. (2008). Six days-post-inoculation (dpi), virulence was assessed by determining fungal growth (ergosterol content) as described by Kim and Woloshuk (2011). FB1 was extracted from autoclaved kernels and analyzed by methods described by Kim and Woloshuk (2008). For conidiation assays, strains were inoculated onto carnation leaf agar medium as previously described (Niu *et al*., 2015). The size of carnation leaves is around 18 mm^2 (4 ×4.5 mm). Resistance to hydrogen peroxide was measured as described by Niu *et al*. (2015). Mycelial hydrophobicity was tested by placing a 10-µl droplet of water or a detergent solution (0.2% SDS, 50 mM EDTA) on the colony surface of strains as described by Niu *et al*. (2015). The ability of the mycelia to maintain a spherical droplet was assessed after 30 min. All experiments were repeated three times.

3.3.6 RNA extraction, cDNA synthesis and quantitative real time-PCR

Six days after inoculation of B73 maize, infected kernels were collected from three ears (biological replicates) and total RNA was isolated as described by Bluhm *et al*. (2008). Extraction of RNA from yeast was conducted following the protocol described

by Schmitt *et al.* (1990). Purified RNA was pooled from the three biological replicates and cDNA was synthesized as described by Reese *et al.* (2011). Gene specific PCR primers were designed with PrimerQuest Design Tool (Integrated DNA Technologies, Inc.). Quantitative PCR (qPCR) was conducted as described by Bluhm *et al*. (2008) and reactions were replicated three times for each gene. Each reaction contained 1.5 µl of each primer pair (10 µM), 10 µl of iTaq Universal SYBR Green Supermix (Bio-rad, Hercules, CA), 5 µl of cDNA template, and 2 µl of nuclease-free water. Reaction conditions were one cycle of 3 min at 95°C, 40 cycles of 5 s at 95°C and 30 s at 57°C. Expression of the *TUB1* gene (FVEG_04081) and *ACT1* (YFL039C) was used to assure that efficiencies of the target and reference reactions were approximately equal in fungal RNA and qPCR, respectively. The ΔΔCt method (Livak & Schmittgen, 2001) was used to calculate expression level with *TUB1/ACT1* as the internal normalizer.

3.3.7 Complementation of Δitr1

The ITR1 ORF was amplified with PCR primers ITR1yes_F/ ITR1yes_R and cloned into the yeast expression vector pYES2 (BamHI and XhoI digested) (Liu, Zhou, Li, Xu 2011; Invitrogen, Grand Island, NY, USA), kindly provided by Dr. Jin-Rong Xu, Purdue University, IN, USA. Expression of genes in this vector was induced with galactose. The resulting construct was transformed into ScΔitr1 competent cells with the alkali-cation yeast transformation kit (MP Biomedicals, Solon, OH). $Ura3^+$ colonies were screened with PCR primers ITR1ScF/ITR1ScR. The FST1 ORF and other mutated FST1 ORFs were PCR amplified from corresponding cDNA with primers FST1yes_F/ FST1yes_R and cloned into *Eco*RI and *Xho*I -digested pYES2 vector. $Ura3^+$ colonies were screened with PCR primers FST1-500-1/FST1-500-2 and FST1-ns5/FST1-ns3. The

FST13 ORF was amplified with primers FST13yes_F/ FST13yes_R from a construct containing FST13 ORF (pFST13-cDNA; Kim and Woloshuk, 2011) and cloned into *Hind*III and *Eco*RI digested pYES2 vector. Ura3^{+} colonies were screened with PCR primers FST13Sc1F/FST13Sc1R and FST13Sc3F /FST13Sc3R. Transformed cells were grown at 30°C in YNB medium with 2% glucose as the only carbon source (Trenkamp *et al.*, 2004). Two transformants for each engineered construct were selected for the yeast growth assay.

3.3.8 Yeast growth assay

A single colony of each yeast transformant was transferred to YNB medium with 100 µM sucrose and grown overnight with shaking at 30°C. Expression of heterologous proteins was induced by transferring overnight cultured cells to 50 ml of YNB medium containing 2% galactose and 1% raffinose (YNB + GARA medium) as described (pYES2 user's manual, Invitrogen, Grand Island, NY, USA) to obtain an OD_{600} of 0.4. After 2 hr of shaking at 30°C, 1 ml of the culture was used for RNA extraction to verify expression of heterologous genes by qPCR. Expression of the entire gene sequence was also confirmed by PCR. One ml of culture was used to wash the cells and 1 ml of YNB media was used to suspend the cells at final wash. Washed cells (in 20 µl) of each strain were transferred to 96-well culture plates (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) containing 200 µl YNB, YNB+sucrose (100 µM) or YNB+myo-inositol (100 µM) medium for testing the growth effect of different carbon sources. Another 20 µl of washed cells from each strain was transferred into a 96-well culture plate containing 200 µl of YNB + GARA medium with 0, 150, 450 or 900 µM FB1. Culture plates were

shaken at 30°C, and growth was measured as OD_{600} at various time points. These experiments were repeated three times and the results showed similar growth effects.

3.4 Results

3.4.1 ITR-like and FST1-like families in *F. verticillioides* and other fungi

Recent updates in the annotation of the *F. verticillioides* genome in the Fusarium Comparative Database at the Broad Institute assigned a function of myo-inositol transporter to gene FVEG_08441, which we designate *FST1*. The only characterized fungal myo-inositol transporters are *ITR1* and *ITR2* in *S. cerevisiae* and ITRs in *Crytococcus neoformans* (Xue *et. al*, 2010; Nikawas *et. al*, 1991). To address the hypothesis that FST1 is a myo-inositol transporter, we first examined the phylogeny of FST1 and ITR1. Our objective was to determine if these proteins co-evolved. By a BLASTp search of fungal genomes, we identified families of ITR1-like and FST1-like proteins. Three ITR1-like and FST1-like proteins, with the highest similarity scores, were selected from the Saccharomycotina and from each of the eight classes of the Pezizomycotina (Table 3.1). A BLASTp search of the *F. verticillioides* genome identified genes that encode five putative ITR1-like proteins and two FST1-like proteins with high similarity scores (Table 3.1). To better understand the relationship between these proteins, a maximum likelihood phylogenetic analysis was made and the phylogenetic tree was generated (Figure 3.1). The tree was rooted with FST1-like and ITR1-like proteins from the Mucoromycotina species *Phycomyces blakesleeanus*. The resulting tree revealed the separation of FST-1-like and ITR1-like proteins into two distinct groups (Figure 3.1). Group 1 contains all the IRT1-like proteins and Group 2

contains all the FST1-like proteins except two (protein ID: 7135 and 2157), which clustered within the outgroup protein 136576 of Group 2.

3.4.2 Growth on myo-inositol

No differences were observed in the growth of *F. verticillioides* strains FvWT, FvΔfst1 and FvΔfst1comp when grown on minimal medium containing myo-inositol as a carbon source (data not shown). In contrast, the ITR1-mutant strain ScΔitr1 of *S. cerevisiae* grew poorly in YNB + myo-inositol medium (Figure 3.2B) compared to the wild type (ScWT) and its growth in YNB + sucrose medium (Figures 3.2A). Expression of the wild-type *ITR1* in the mutant (ScΔitr1/ITR1comp) restored growth on myo-inositol to wild-type levels as well as to levels observed on medium containing sucrose (Figure 3.2C). Expression of *FST1* in ScΔitr1 (ScΔitr1/FST1comp) also complemented growth (Figure 3.2D). Growth was not complemented by expression of *FST13*, which encodes a hexose transporter in *F. verticillioides* (Figure 3.2E).

3.4.3 Growth inhibition by FB1

Growth of wild-type and FvΔfst1 strains of *F. verticillioides* were not inhibited by FB1 concentrations as high as 900 µM (data not shown). However, the growth of wild-type strain (ScWT) of *S. cerevisiae* displayed a concentration-dependent inhibition by FB1 (Figure 3.3A). In contrast, the ITR1-mutant strain ScΔitr1 strain was not affected by the mycotoxin (Figure 3.3B). Expression of the wild-type *ITR1* (ScΔitr1/ITR1comp) and *FST1* (ScΔitr1/FST1comp) restored FB1 sensitivity (Figures 3.3C and D). Strains expressing *FST13* (ScΔitr1/FST13comp) remained insensitive to FB1 (Figure 3.3E).

3.4.4 Central loop and C-terminus amino acids of FST1

Topological analysis of FST1 indicated that the protein has an N-terminus (74 amino acids), a central loop (70 amino acids) and a C-terminus (64 amino acids) that are cytoplasmic (Figure 3.4). To test the hypothesis that the central loop and the C-terminus are important for FST1 function, we aligned the amino acid sequences from these two regions with those of FST1-like proteins from *F. graminearum* (FGSG_03168), *Nectria haematococca* (Necha2_73052), *A. nidulans* (AN2794) *and M. oryzae* (MGG_01373) and arbitrarily selected conserved (IRG and EEI) and non-conserved (VVS and HLL) sets of three amino acids (Figures 3.4A and B). We prepared four *FST1* constructs, each containing specific deletions of these conserved and divergent amino acids. Within the central loop region, we also changed the arginine residue in the conserved IRG to either glycine (G) or lysine (K) (Figure 3.4C), and in the C-terminus, we changed the middle leucine (L) in non-conserved HLL to aspartic acid (D) (Figure 3.4D). These engineered genes were transformed into *F. verticillioides* strain FvΔfst1 and in the yeast strain ScΔitr1, and we confirmed the expression of the engineered genes in the resulting transformants (Table 3.3).

When grown on autoclaved maize, FB1 production is not measurable for strain FvΔfst1 (Table 3.2). In contrast, the wild-type (FvWT) and the complemented strain FvΔfst1comp produced 42 ± 9 and 36 ± 8 µg FB1/g of maize, respectively. Of the engineered *FST1* constructs only ΔHLL and HDL restored measurable fumonisin B1 (Table 3.2). When grown on carnation leaf agar medium, the FvWT produced 92 ± 6 macroconidia on an 18 mm^2 piece of carnation leaf. The FST1 mutant FvΔfst1 produced 9 ± 3 macroconidia and strain FvΔfst1comp produced 78 ± 7 per carnation leaf. Only

strain FvHDL restored macroconidia production (Table 3.2). When assayed for H_2O_2 resistance, the inhibition zone for strain FvΔfst1 was about four times larger than for strains FvWT and FvΔfst1comp. Only strains FvΔVVS and FvΔHLL exhibited the same sensitivity to H_2O_2 as the strain FvΔfst1. Expression of the other constructs resulted in resistance that was not significantly different ($P > 0.05$) from the complemented strain FvΔfst1comp (Table 3.2). In the hydrophobicity assay, a drop of detergent (SDS) solution places on the colony surface of FvWT did not spread at 30 min after application. In contrast, the droplet spread when placed onto the mutant strain FvΔfst1. The hydrophobicity phenotype was only restored by expression of the central loop constructs ΔVVS and ΔIKG in strain FvΔfst1 (Table 3.2). Virulence was measured by the fungal growth (ergosterol content) in inoculated kernels. After six days, the ergosterol content in the kernels inoculated with strain FvΔfst1 was 2.5 times less that in kernels inoculated with strains FvWT and FvΔfst1comp (Table 3.2). Constructs ΔVVS and ΔHLL failed to complement this virulence phenotype whereas expression of the other constructs resulted in ergosterol content values that were not significantly different ($P > 0.05$) from the complemented strain FvΔfst1comp (Table 3.2).

Yeast strains expressing each of the three amino acid-deletion constructs failed to complement myo-inositol transport and fumonisin sensitivity in ScΔitr1 (Figures 3.5A, D, E, F and 3.6A, D, E, F). The strains with the single amino acid-mutated *FST1* R to G (Figure 3.5B) and R to K (Figure 3.5C) both restored the myo-inositol transport and sensitivity to FB1, while the strain with the L to D change (Figure 3.5G) did not complement myo-inositol transport but sensitivity to FB1 was partially restored (Figures

3.6G). This strain (ScHDL) was not inhibited by 150 µM FB1 and 450µM was more inhibitory than 900µM (Figure 3.6G).

3.5 Discussion

Our original BLAST analysis suggested that FST1 is similar to putative sugar transporters in other fungi, such as a hypothetical fructose symporter in *F. oxysporum* (FOXG_10063.2, 98%), a hypothetical fructose transporter in *F. graminearum* (FGSG_03168.3, 90%), a hypothetical glucose transporter in *A. nidulans* (AN2794.1, 80%) and a hypothetical arabinose-proton symporter in *M. grisea* (MGG_01373.6, 73%) (Kim and Woloshuk, 2011). However, experiments failed to prove hexose transporter activity (Kim and Woloshuk, 2011). The current annotation for FST1 is myo-inositol transporter in the *F. verticillioides* reference genome at the Broad Institute. To date, no myo-inositol transporter has been functionally described in filamentous fungi. A BLASTp analysis revealed little sequence similarity (89% coverage, 26% identity, BLAST score 156) between the myo-inositol transporter in yeast (ITR1) and FST1. In *S. cerevisiae*, inositol is required for viability (Nikawa *et al*., 1991). It can be synthesized by a de novo pathway in which inositol-1-phosphate synthase, encoded by *INO1*, has a critical role (Culbertson *et al.*, 1976; Klig & Henry, 1984). Alternatively, inositol can be obtained from the environment and two genes (*ITR1* and *ITR2*) encode myo-inositol transporters required for its uptake. Nikawa *et al*. (1982, 1991) demonstrated that *ITR1* encodes the major myo-inositol transporter and that strains without a functional *ITR1* grow poorly on medium with myo-inositol as the carbon source. In our study we utilized

the *ITR1* mutant strain ScΔitr1 to establish that FST1 can function as a myo-inositol transporter.

We examined the phylogeny of FST1-like proteins and ITR1-like proteins from nine classes of the Ascomycota. Based on the Mucoromycotina root in the phylogenetic tree, these proteins likely diverged from each other prior to the formation of the Ascomycota. Several duplications also occurred after the formation of these two groups. Within the FST1 group, duplication occurred that separated the Saccharomycotina from the Pezizomycetes and two of the Orbiliomycetes species. Two of the *F. verticillioides* FST1-like proteins were also in this subgroup. The Pezizomycetes and Orbiliomycetes are considered the earliest to evolve away from the other Ascomycota (Pfister, 2015). These fungi produce apothecia and include species associated with lichens and nematode-trapping fungi (Pfister, 2015). The subgroup containing FST1 separated from the Saccharomycotina in a subsequent duplication. In the ITR1 branch, duplication also separated the Saccharomycotina. The low bootstrap value does not strongly support the separation of ITR1 from the other fungi in this subgroup, which includes two *F. verticillioides* proteins. These results provide evidence of the divergent evolution of FST1 and ITR1. However, we agree with Gerlt and Babbit (2000) that caution is needed when assigning functional annotation based on sequence similarities in other genome databases.

In all organisms examined, sphingolipids reside in cell membranes and function in cell regulation, and FB1 has been shown to inhibit those functions (Merrill *et al.*, 1996, Merrill *et al.*, 2001). Wu *et al*. (1995) showed that FB1 inhibits the growth of *S. cerevisiae* by inhibiting the synthesis of inositol-containing phospholipids. We found

that the growth of strain ScΔitr1 was insensitive to FB1 in contrast to the wild type ScWT, which was inhibited by increasing concentrations of FB1. Expression of *ITR1* or *FST1* in the mutant strain restored the sensitivity to FB1. These results suggest that FB1 is transported into the yeast cell by the myo-inositol transporter. There is precedence for mycotoxin transporters in fungi. *TRI12* in *F. sporotrichioides* encodes a trichothecene efflux pump that was shown to be functional when expressed in yeast (Alexander *et al.*, 1999). Trichothecenes are *Fusarium* mycotoxins that inhibit protein synthesis in mammals (Richard, 2007, Woloshuk & Shim, 2013). The annotation of *FST1* in the *F. verticillioides* reference genome indicates the presence of a trichothecene efflux pump domain, which spans (200 amino acids) the entire central loop and trans-membrane domains 4, 5, 6 and 7. Our results indicate that, unlike the efflux activity of TRI12, FST1 facilitates the influx of fumonisin into the yeast cells.

At the onset of these studies, we hypothesized that the central loop and C-terminus regions are important for functional activity of FST1. Precedence is established for the sugar transporters RGT2/SNF3 in *S. cerevisiae* in which the C-terminus is essential for its signaling transduction function (Moriya & Johnston, 2004, Ozcan *et al.*, 1998). The cytoplasmic C-terminus of the mammalian glucose transporter GLUT1 activates ERK protein kinase (Bandyopadhyay *et al.*, 2000), and the internal cytoplasmic loop of the mammalian glucose transporter GLUT2 also functions in transducing the glucose signal from the plasma membrane (Guillemain *et al.*, 2000). In this current study, we found that removing sets of three amino acids in conserved and divergent regions in the central loop and C-terminus abolished the myo-inositol transport and FB1 sensitivity in *S. cerevisiae*. In *F. verticillioides*, the amino acid deletions affected in

different ways for the five phenotypes (FB1 production, macroconidia production, sensitivity to hydrogen peroxide, virulence and mycelial hydrophobicity) (Table 3.2). Removal of the conserved amino acids IRG in the central loop did not restore hydrophobicity, FB1 and macroconidia production. Changing the arginine to glycine also rendered the protein inactive, whereas changing the arginine to lysine restored hydrophobicity to strain FvΔfst1. Interestingly, these changes did not affect FST1 function in the yeast mutant ScΔitr1. Amino acids in the divergent region (VVS) in the central loop appear to be essential in *F. verticillioides* except for hydrophobicity, which was unaffected by the deletion. The *F. verticillioides* strain containing the deletion of these amino acids was as sensitive to hydrogen peroxide as FvΔfst1. Deletion of the conserved amino acids (EEI) in the C-terminus demonstrated that they are not essential for resistant to H_2O_2 and virulence, but are required for FB1 production, macroconidia production, and hydrophobicity. Our results suggest that the three amino acids (HLL) in the divergent region of the C-terminus are not involved in FB1 production. We can also conclude that the middle leucine of the HLL triplet is essential for the myo-inositol transport function in ScΔitr1, but changing the uncharged middle leucine to a negatively charged aspartic acid partially restores FB1 sensitivity.

In conclusion, this study has provided experimental results establishing that FST1 has myo-inositol and FB1 transport activities in *S. cerevisiae*. These results are consistent with the annotations in the *F. verticillioides* reference genome. However, in *F. verticillioides*, the loss of *FST1* leads to multiple mutant phenotypes (Niu *et al*., 2015), suggesting direct or indirect alternative functions for FST1 in *F. verticillioides*. We also

demonstrated the functional importance of amino acids in FST1. The results indicate that both the central loop and C-terminus are critical for FST1 functionality.

3.6 List of References

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Table 3.1. List of ITR1-like and FST1-like sequences used for phylogeny analysis[a].

	ITR1-like			FST1-like		
	Protein ID	Organism	Score	**Protein ID**	Organism	Score
Pezizomycetes		*Tuber melanosporum*			*Choiromyces venosus*	
(PEZI)	**7104**	(Tubme)	973	**1791185**	(Chove)	633
					Ascodesmis nigricans	
	481912	*Morchella conica* (Morco)	950	**379391**	(Ascni)	623
	410036	*Wilcoxina mikolae* (Wilmi)	925	**836083**	*Terfezia boudieri* (Terbo)	619
Orbiliomycetes		*Monacrosporium*			*Monacrosporium*	
(ORBI)	**9902**	*haptotylum* (Monha)	839	**853**	*haptotylum* (Monha)	570
		Arthrobotrys oligospora			*Monacrosporium*	
	941	(Artol)	832	**1179**	*haptotylum* (Monha)	562
		Arthrobotrys oligospora			*Arthrobotrys oligospora*	
	2157	(Artol)	621	**3692**	(Artol)	548

Table 3.1. continued

Eurotiomycetes (EURO)	**381105**	*Penicillium brevicompactum* (Penbr)	1057	**436483**	*Aspergillus ochraceoroseus* (Aspoch)	2150
	412244	*Penicillium bilaiae* (Penbi)	1044	**40258**	*Aspergillus versicolor* (Aspve)	2136
	8102	*Aspergillus oryzae* (Aspor)	1025	**26353**	*Penicillium brevicompactum* (Penbr)	1685
Dothideomycetes (DOTH)	**60649**	*Aureobasidium pullulans var. melanogenum* (Aurpu)	1065	**231244**	*Myriangium duriaei* (Myrdu)	1978
	14488	*Venturia inaequalis* (Venin)	1060	**135800**	*Acidomyces richmondensis* (Aciri)	1974
	289437	*Ophiobolus disseminans* (Ophdi)	1038	**373921**	*Microthyrium microscopicum* (Micmi)	1949
Lecanoromycetes (LECA)	**1581329**	*Xanthoria parietina* (Xanpa)	928	**3658**	*Cladonia grayi* (Clagr)	1730
	5624	*Cladonia grayi* (Clagr)	908	**1606405**	*Xanthoria parietina* (Xanpa)	582
	7135	*Cladonia grayi* (Clagr)	629	**1645041**	*Xanthoria parietina* (Xanpa)	578

Table 3.1. continued

Leotiomycetes		*Meliniomyces variabilis*			*Oidiodendron maius*	
(LEOT)	**531442**	(Melva)	1017	**132835**	(Oidma)	1777
		Rhizoscyphus ericae				
	663153	(Rhier)	1013	**620699**	*Coccomyces strobi* (Cocst)	1688
		Leptodontium sp.				
	302520	(Leptod)	1012	**675934**	*Rutstroemia firma* (Rutfi)	1687
Sordariomycetes		*Glomerella acutata*			*Nectria haematococca*	
(SORD)	**1357310**	(Gloac)	1032	**73052**	(Necha)	2447
	637000	*Thozetella sp.* (ThoPMI)	1031	**368948**	*Ilyonectria sp.* (Ilysp)	2236
	1739233	*Ilyonectria sp.* (Ilysp)	1027	**499171**	*Clonostachys rosea* (Cloro)	2157
		Fusarium verticillioides			*Fusarium verticillioides*	
	FVEG_02081*	(Fusve)	395	**FVEG_10089***	(Fusve)	216
		Fusarium verticillioides			*Fusarium verticillioides*	
	FVEG_01519*	(Fusve)	389	**FVEG_01545***	(Fusve)	205
		Fusarium verticillioides				
	FVEG_07757*	(Fusve)	362			

Table 3.1. continued

	FVEG_11293*	*Fusarium verticillioides* (Fusve)	343			
	FVEG_06504*	*Fusarium verticillioides* (Fusve)	306			
Xylonomycetes (XYLO)	**1075393**	*Trinosporium guianense* (Trigu)	1014	**773082**	*Symbiotaphrina kochii* (Symko)	1717
	977828	*Symbiotaphrina kochii* (Symko)	998	**252413**	*Xylona heveae* (Xylhe)	1694
	227483	*Xylona heveae* (Xylhe)	981	**1057322**	*Trinosporium guianense* (Trigu)	1687
Saccharomycetes (SACC)	**2783**	*Torulaspora delbrueckii* (Torde)	2185	**79581**	*Lipomyces starkeyi* (Lipst)	1579
	2442	*Kazachstania Africana* (Kazaf)	2112	**4126**	*Blastobotrys (Arxula) adeninivorans* (Arxad)	1541
	1653	*Kluyveromyces lactis* (Klula)	2010	**143966**	*Trichomonascus petasosporus* (Tripe)	1535
Mucoromycotina (MURO)	**79609**	*Phycomyces blakesleeanus* (Phybl)	787	**136576**	*Phycomyces blakesleeanus* (Phybl)	562

[a] Results of the highest sequence similarity obtained from BLASTp analysis in Fungal Genomics Resource JGI MycoCosm database.
* Gene ID from species genome databases: Broad Institute Fusarium Comparative Database for *F. verticillioides*. Genes with BLAST score higher than 190 were recorded.

Table 3.2. Complementation of defects in FvΔfst1 by various engineered FST1 genes.

Fungal strains	FB1 [a]	Macroconidia [b]	H_2O_2 inhibition [c]	Detergent Hydrophobicity [d]	Virulence [e]
FvWT	42 ± 9	92 ± 6	517 ± 9	+	320.4 ± 0.2
FvΔfst1	nd	9 ± 3	2042 ± 79	-	117.5 ± 0.1
FvΔfst1comp	36 ± 8	78 ± 7	478 ± 35	+	302.2 ± 0.1
FvΔIRG	nd	13 ± 4	1046 ± 7	-	293.3 ± 12.8
FvΔVVS	nd	11 ± 2	2261 ± 35	+	271.6 ± 7.9
FvΔEEI	nd	20 ± 6	544 ± 9	-	257.7 ± 23.9
FvΔHLL	4 ± 1	9 ± 1	1907 ± 26	-	131.7 ± 0.1
FvIGG	nd	24 ± 5	1277 ± 9	-	342.3 ± 13.5
FvIKG	nd	16 ± 1	1096 ± 9	+	122.9 ± 15.3
FvHDL	21 ± 5	75 ± 17	1298 ± 9	-	229.9 ± 5.8

[a] Values are the mean of three replicates in µg/g of maize ± standard error (n=3). (nd) = not detected.
[b] Macroconidia produced per 18 mm^2 carnation leaf (n=3).
[c] Inhibition zones for each strain were compared to strain FvΔfst1comp (n=3).

[d] Hydrophobicity was tested by placing droplets (10ul) of water or detergent solution (0.2% SDS, 50 mM EDTA) on the colony surface of strains grown on PDA medium for six days (n=3). Results were determined after 30 min. (+) = complemented to WT and (-) = not complemented.
[e] Virulence was measured as ergsterol content (6 dpi) of kernels that inoculated with different *F. verticillioides* strains (n=2).

Table 3.3. Expression of engineered FST1 genes in *F. verticillioides* and *S. cerevisiaea*.

Construct	*F. verticillioides*	*S. cerevisiae*
FST1	3.68 (2.69, 5.03)	1.00 (0.81, 1.23)
ΔIRG	4.83 (4.34, 5.87)	1.63 (1.50, 1.78)
ΔVVS	1.55 (1.50, 1.59)	1.43 (1.27, 1.60)
ΔEEI	1.34 (1.32, 1.37)	1.35 (1.19, 1.53)
ΔHLL	3.22 (3.03, 3.43)	1.54 (1.47, 1.62)
IGG	4.98 (4.60, 5.39)	0.76 (0.75, 0.77)
IKG	0.97 (0.92, 1.02)	1.45 (1.43, 1.46)
HDL	2.02 (1.87, 2.18)	1.45 (1.33, 1.58)

Total RNA was extracted from 6 dpi of B73 kernels and yeast cells cultured 2 hours in YNB-GARA medium. Equal amounts of RNA were pooled from three biological samples. Three technical replicates were analyzed for each gene. Expression was measured by quantitative reverse-transcriptase polymerase chain reaction (qPCR). Expression of *TUB1* (FVEG_04081) was used to normalize *F. verticillioides* data and *ACT1* (YFL039C) was used to normalize *S. cerevisiae* data. For each gene construct, values represent fold differences compared to the expression in wild-type strain FvWT, which was set at a value of 1.00 (0.86, 1.17). For *S. cerevisiae* values represent fold differences compared to that in ScΔitr1/FST1comp, which was set at a value of 1. Expression of each gene was calculated as 2ΔΔCt. Range of expression is in parentheses equals 2 ΔΔCt-s, 2 ΔΔCt+s, where s equals the standard deviation of the ΔΔCt value.

Table 3.4. PCR primers used in this research.

Primer name	Sequence 5'-3'
FST1_A1	GCAATTGGACTGGGCACGTTG
FST1_F1	ACATGAGATCGGCTGGGTAGG
FST1_F1N	TCTCACAGTGAGGCATCATCATGT
	ATTACAATTCACTGGCCGTCGTTTTAGTTGTCAGTGACACCTGTT
FST1_F2	GGTAG
	CGTAATCATGGTCATAGCTGTTTCCTGCCTCATGGAGAGATTCGG
FST1_F3	TCGA
FST1_F4N	CAGGCGGAAAGGGTGATCTCTATC
FST1_F4	TGGAATCAGGAGGGATACTCGGA
FST1LP-ApaI	TCGGACACACACGGGGCCGCCAAAGTGTGGGTAGGCCTAGGGTA
	GCTTTCT
FST1LP-Dc12	GCGGTCCTCAATGCGCTTCCAGACCTTGTA
FST1LP-Dc13	AAGGTCTGGAAGCGCATTGAGGACCGCGAATCT
FST1LP-14c	CACCACCCCGGTGAACAGCTCCTCGCCCTTGCTCACGATGAGGG
	GGAGGAAAGCTAA
FST1LP-Dd12	AGCATTCTCCTCAGAGGCACTCATGACGTA
FST1LP-Dd13	ATGAGTGCCTCTGAGGAGAATGCTGTTGCT
FST1TL-Dc12	AAGAACAAGACTCTGGATGTTGTCTTCTCTCGA
FST1TL-Dc13	AGAGAAGACAACATCCAGAGTCTTGTTCTTGGT
FST1TL-Dd12	ATGTCCAGTGAGGGTAGTCTCCTTAACACC
FST1TL-Dd13	AAGGAGACTACCCTCACTGGACATTGGCAC

Table 3.4. continued.

FST1LPRG-F	AAGGTCTGGAAGCGCATTGGTGGTATTGAGGACCGCGAATCT
FST1LPRG-R	GCGGTCCTCAATACCACCAATGCGCTTCCAGACCTTGTA
FST1LPRK-F	AAGGTCTGGAAGCGCATTAAAGGTATTGAGGACCGCGAATCT
FST1LPRK-R	GCGGTCCTCAATACCTTTAATGCGCTTCCAGACCTTGTA
FST1TLLD-F	AAGGAGACTACCCATGATCTTCTCACTGGACATTGGCAC
FST1TLLD-R	ATGTCCAGTGAGAAGATCATGGGTAGTCTCCTTAACACC
FST1-500-1	GATTGTCTCATCGCGTGTCATTCTCGG
FST1-500-2	GATAGCGTTGACACCAGTAAGTTGACC
FST1-ns5	TGCTCTTCTCTTCCTCGCCTCTTT
FST1-ns3	AACACCAGCCCAATTCTCCCTAAC
FST1yes_F	TAAGAATTCATGGGCAAGAGTCGA
FST1yes_R	CTACTCGAGCTAAGCATGAGAGAC
ITR1yes_F	TAAGGATCCATGGGAATACACATACCATAT
ITR1yes_R	CTAGAGCTCCTATATATCCTCTATAATCTCTTGAGT
ITR1ScF	CACTGACACTTCGGAGGAAAT
ITR1ScR	TGAACCAGCCCAGTTTGTAG
FST13yes_F	TAAAAGCTTATGAACGCTATTCGAAGGGCATCC
FST13yes_R	CTACTCGAGCCGACAGCAGAACTTC
FST13Sc1F	GGTGGTGTTCTCTACGGTTATG
FST13Sc1R	CTCATCTCATGGTCGTGGTTAG
FST13Sc3F	GCCGTCTCTTTCGTCTGTATC

Table 3.4.continued

FST13Sc3R	CTCAGCAACCCAAACAACATC
HY1	GGATGCCTCCGCTCGAAGTA
YG1	CGTTGCAAGACCTGCCTGAA
M13F	GTAAAACGACGGCCAGTGAATTGTAA
M13R	CAGGAAACAGCTATGACCATGATTAC
HYN	TAGCGCGTCTGCTGCTCCATACAAG
YGN	ACCGAACTGCCCGCTGTTCTC
HYGSCRN_B	AGGCTTTTTCATTTGGATGCTTGGG

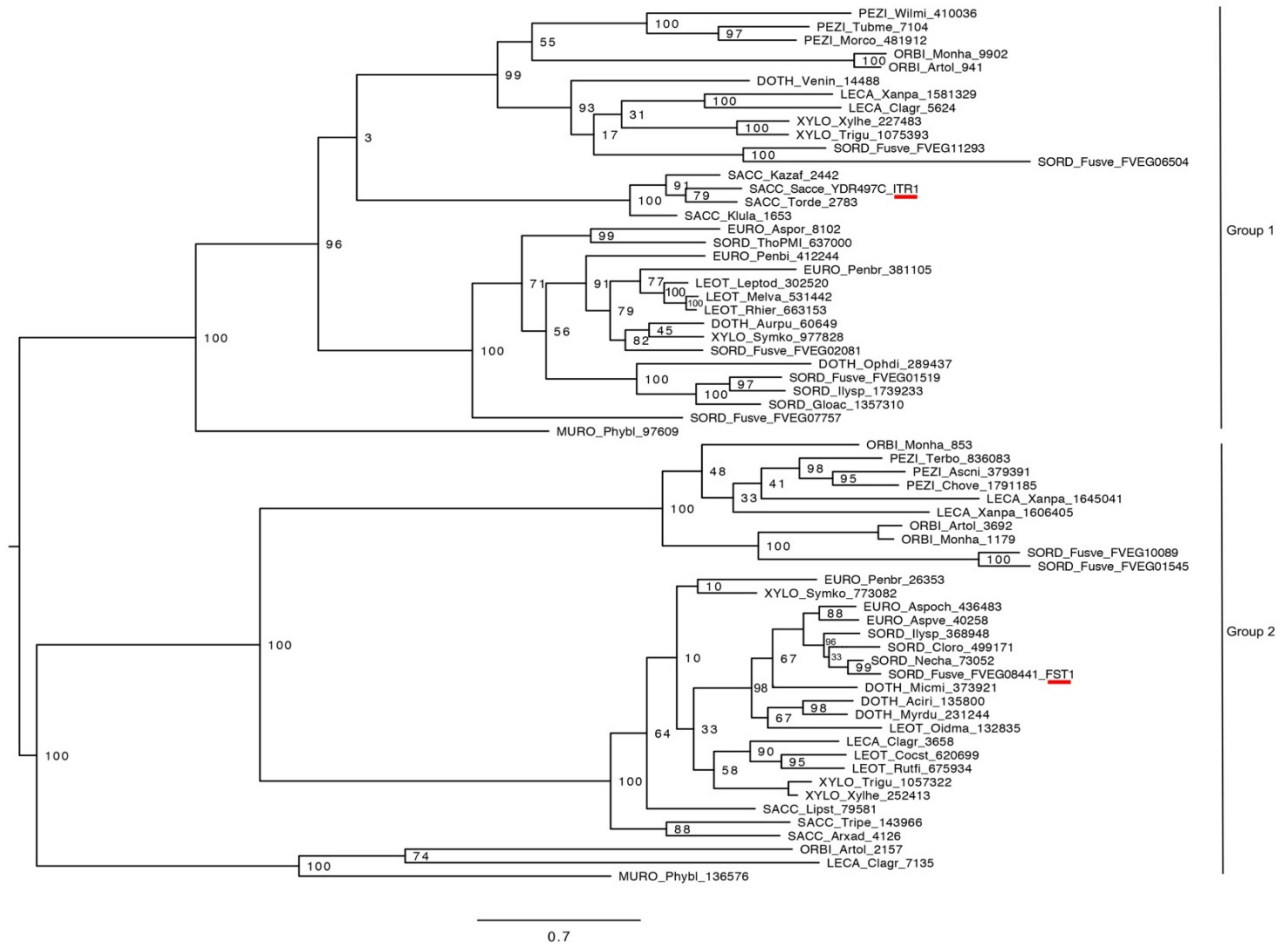

Figure 3.1. Maximum likelihood reconstruction of the phylogenetic history of FST1-like and ITR1-like proteins. Proteins with highest sequence identity to ITR1 and FST1 from Saccharomycotina and from each of the eight classes of the Pezizomycotina were selected

for comparison (PEZI, Pezizomycetes; ORBI, Orbiliomycetes; EURO, Eurotiomycetes; DOTH, Dothideomycetes; LECA, Lecanoromycetes; LEOT, Leotiomycetes; SORD, Sordariomycetes; XYLO, Xylonomycetes). Proteins were also selected from Mucoromycotina (MURO) as the outgroup. The amino acid sequence alignment was generated by clustalX 2.1 and the rooted tree was generated by CIPRES science gateway (Miller *et al*., 2010) with RAxML-HPC BlackBox (version 8.2.3, phylogenetic tree inference using maximum likelihood/rapid bootstrapping on XSEDE). Numbers on nodes are the bootstrap values.

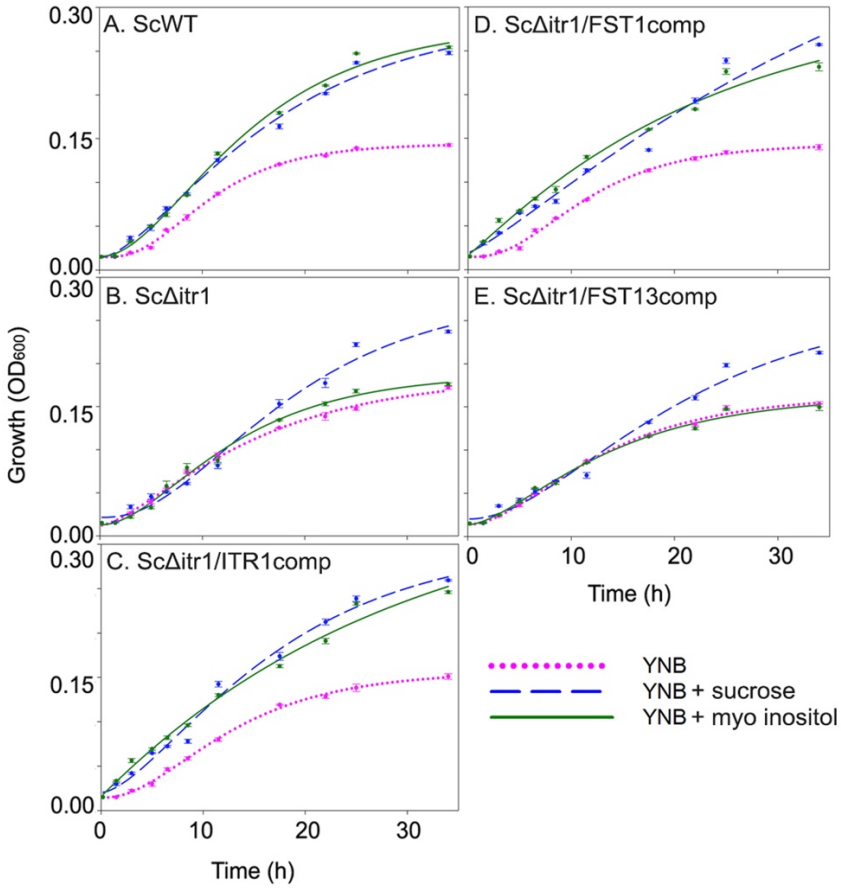

Figure 3.2. Growth of wild type ScWT, ITR1 mutant of *S. cerevisiae* (ScΔitr1) and ScΔitr1 complemented with ITR1, FST1, or FST13 in YNB, YNB + sucrose, and YNB + myo-inositol media. Growth was measured as absorbance at 600 nm (OD_{600}) (n=3). Error bars represent the standard error of the mean.

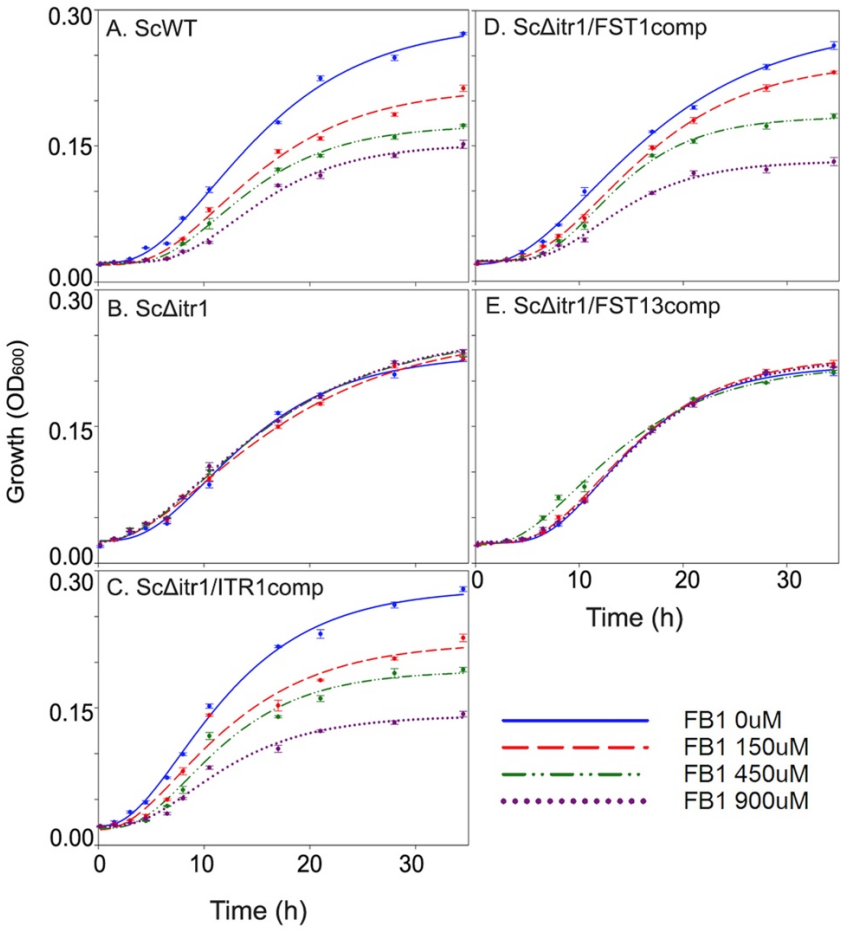

Figure 3.3. Growth of wild type ScWT, an ITR1 mutant of *S. cerevisiae* (ScΔitr1) and ScΔitr1 complemented with ITR1, FST1, or FST13 on medium amended with four concentrations of FB1. Growth was measured absorbance at 600 nm (OD_{600}) (n=3). Error bars represent the standard error of the mean.

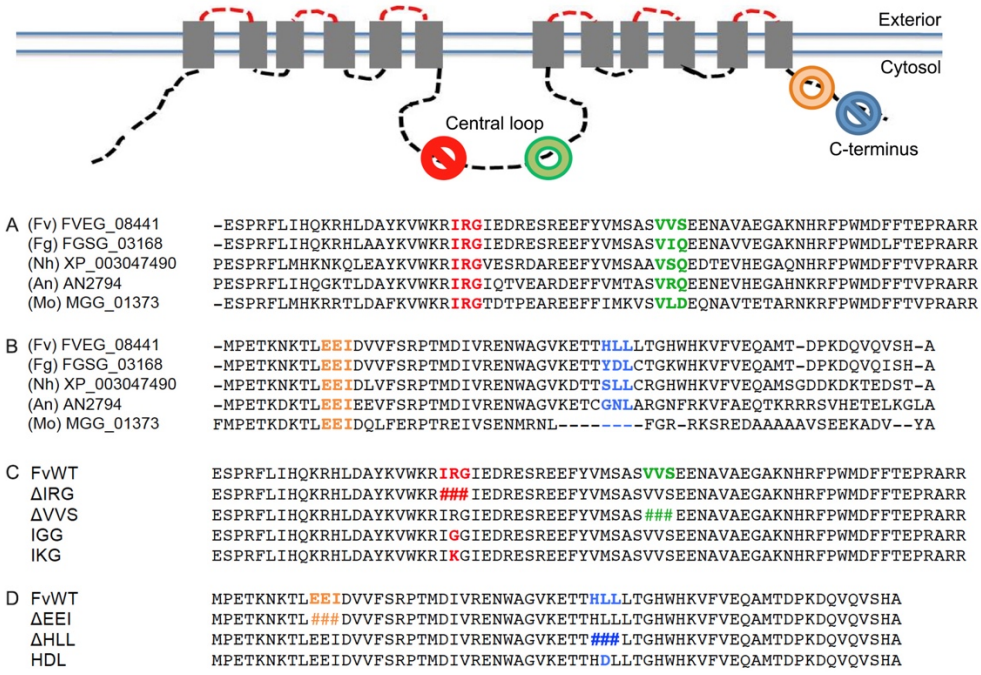

Figure 3.4. Amino acid sequences of FST1-like proteins from various fungi (A and B) and engineered amino acid deletions and substitutions made in FST1 from *F. verticillioides* (C and D). Sequences of the central loop region (A and C) show the selected conserved amino acids in red and divergent in green, and C-terminus (B and D) show the selected conserved amino acids in orange and divergent in blue. Deleted amino acids are represented as (###), substituted amino acids are bold letters and gaps in the alignments are represented as (-). Fungal genes represented are from *Fusarium verticillioides* (Fv), *Fusarium graminearum* (Fg), *Nectria haematococca* (Nh), *Aspergillus nidulans* (An), and *Magnaporthe oryzae* (Mo).

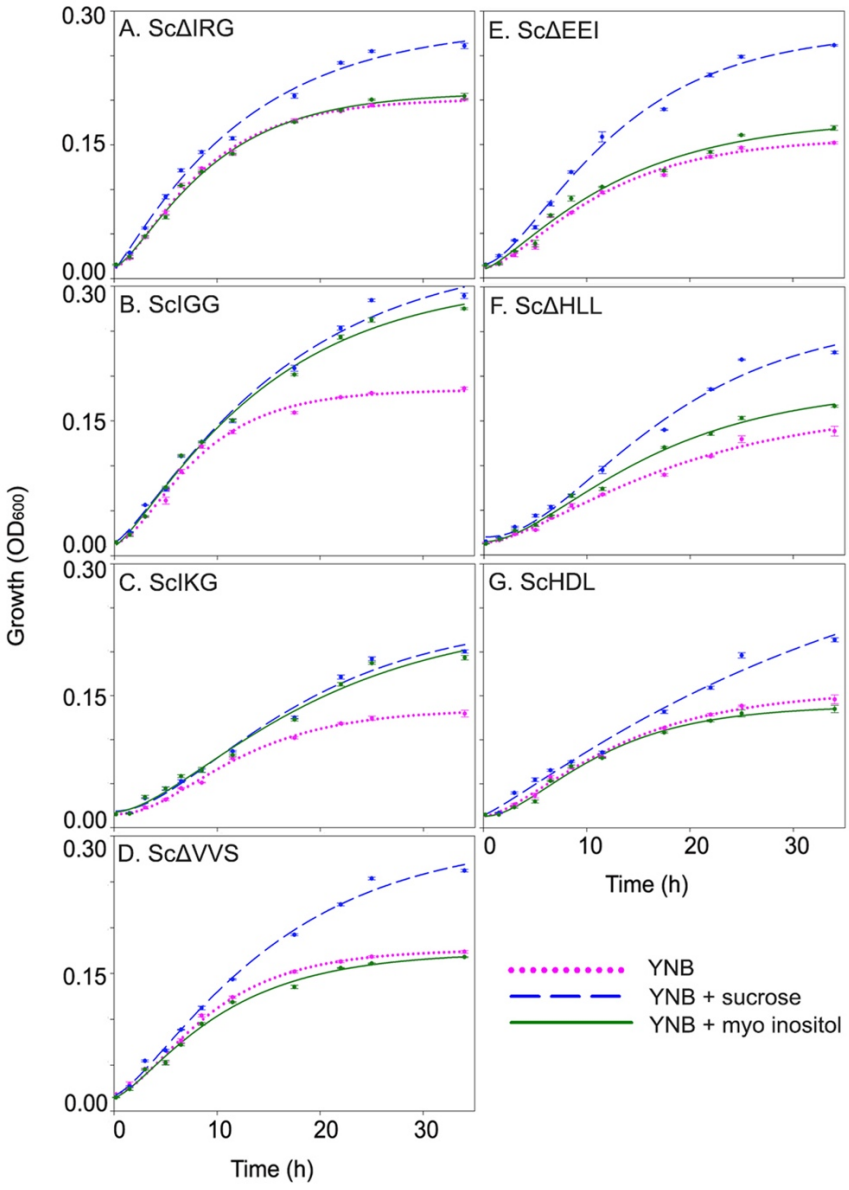

Figure 3.5. Growth of yeast strain ScΔitr1 with engineered FST1 genes in YNB, YNB + sucrose, and YNB + myo-inositol media. Growth was measured as absorbance at 600 nm (OD_{600}) (n=3) in different media. Error bars represent the standard error of the mean.

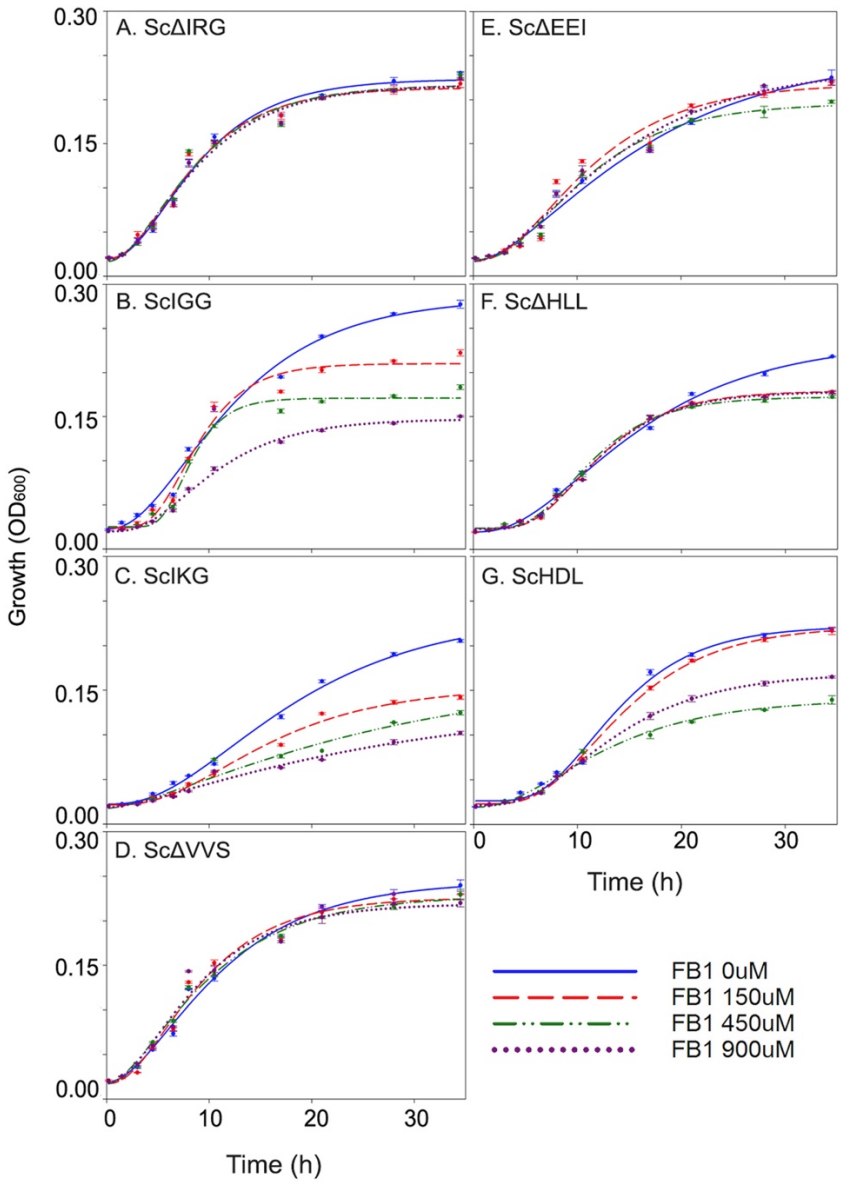

Figure 3.6. Effect of FB1 on the growth of various yeast strains expressing engineered FST1 genes. Growth was measured absorbance at 600 nm (OD_{600}) (n=3). Error bars represent the standard error of the mean.

CHAPTER 4. CONCLUSION AND FUTURE WORK

In this study, we showed that the FST1 gene, encoding a putative sugar transporter, is critical for macroconidia production, reactions to H_2O_2 and hydrophobicity of *F. verticillioides* (Niu *et al.*, 2015), in addition to its previously reported functions in FB1 production and virulence (Kim and Woloshuk, 2011). By comparing the transcriptomes of the WT and Δfst1 strains of *F. verticillioides* grown on autoclaved maize kernels, we examined the effects of FST1 on whole genome expression. Results indicate that FST1 has regulatory functions that globally impacts gene expression. The annotation change of FST1 in the *F. verticillioides* database (Broad Institute) to that of a myo-inositol transporter led us to study the inositol transporting function of FST1. Phylogenetic analysis demonstrated that FST1-like proteins and ITR1-like proteins diverged from each other prior to the formation of Ascomycota. The complementation of the Δitr1 mutant yeast strain by FST1 to WT growth levels indicates FST1 does have inositol transport activity. Since FST1 also restored the FB1 sensitivity of Δitr1 mutant yeast strain, FST1 may also facilitates the transport of FB1 in yeast strain. The central loop and C-terminus regions are important for the functionality of FST1. We identified which amino acid residues important for the functions of FST1. Additional study of sugar transporters in fungal pathogens may help to develop a control strategies for

pathogenic fungi, and understanding the relationship of FB1 with inositol transport in yeast may shed light on certain gene regulation and networks in mammals.

Fungal secondary metabolites have the ability to do us both good and harm. They can be applied in health, medicine, and agriculture as beneficial compounds, such as antibiotics and stains, or they can be severely detrimental to human and animal health as mycotoxins (e.g. aflatoxin, fumnisin, tricothecenes). There are three main groups of fungal secondary metabolites: 1) polyketides and fatty acid-derived compounds (e.g. aflatoxin/sterigmatocystin/dothistromin, ochratoxin, spore pigments, aurofusarin, miscellaneous aromatic polyketides, lovastatin, squalestatin, T-toxin, fumonisin), 2) non-ribosomal peptides and amino acid-derived compounds (e.g. penicillin, cyclosporine, ergot alkaloids, HC-toxin, destruxin, peramine) and 3) terpenes (e.g. gibberellin, trichothecenes, carotenoids) (Hoffmeister and Keller, 2006). Without knowledge of how these metabolites are arranged in the fungal cells, what mechanisms mediate the export of these secondary metabolites, and why this export occurs, we cannot increase the production of beneficial secondary metabolites while limiting mycotoxin production.

The compartmentalization of penicillin has been studied for decades. Penicillin biosynthesis is not derived from the cytosol since the vacuole is the only cellular compartment that has been found to contain amino acid precursors for penicillin synthesis (Lendenfeld *et al.*, 1993). The important penicillin biosynthesis enzyme (L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS) was reported to be loosely bound to the vacuole membrane (Lendenfeld *et al.*, 1993) and later localized in the cytoplasm (Van Der Lende *et al.*, 2002). Other penicillin biosynthesis enzymes such as isopenicillin N synthase (IPNS) and acyl-coenzyme A:isopenicillin N acyltransferase (IAT) are

located in cytosol and peroxisomes, respectively (Van De Kamp *et al.*, 1999; Van Der Lende *et al.*, 2002; Muller *et al.*, 1991).

Recent publications on aflatoxins produced by *Aspergillus parasiticus* have shown that aflatoxin biosynthesis occurs in specialized vesicles known as aflatoxisomes (Chanda *et al.*, 2009; Chanda *et al.*, 2010). The subcellular localization of the early pathway enzyme Nor-1, the middle pathway enzymes Ver-1 and Vbs, and the late pathway enzyme OmtA have been studied using the concentric circle colony fractionation technique, which detects immunogold labeling of the antibodies against these enzymes or the EGFP reporter system. Nor-1, Ver-1 and OmtA were detected in the cytosol at 24-48 h of growth and localized in vesicles and vacuoles later (Chiou *et al.*, 2004; Hong and Linz, 2008; Lee *et al.*, 2004). The final enzyme in the biosynthesis pathway, OrdA, was localized in the vacuole (Lee *et al.*, 2004). During aflatoxin biosynthesis, an increase in the number of vesicles was observed on aflatoxin-inducing media (Chanda *et al.*, 2009). By blocking vesicle-vacuole fusion using the vb1 strain (lacking of a GTPase known to regulate vesicle fusion in *Aspergillus nidulans*) or using Sortin3 to block the function of Vps16 (a protein in class C tethering complex) to affect protein trafficking to vacuoles, the involvement of vesicles involved in aflatoxin biosynthesis was confirmed (Chanda *et al.*, 2009).

Difficulties in extracting intact vacuoles have been reported as a hindrance in the study of compartmentalization of fungal secondary metabolites (Keller and Mattel, 1985; Lendenfeld *et al.*, 1993; Vaughn and Davis, 1981). Though plasma membrane and cytosol fractions can be extracted by shearing with glass beads, the strong shear forces destroy intracellular organelles. To preserve the integrity of the organelles, cells can be

converted to protoplasts/spheroplasts using enzymes and then lysed under isosmotic or hypoosmotic conditions with moderated shear force to release the subcellular fractions (Rieder and Emr, 2001). Shimaoka *et al.* (2004) conducted sucrose density gradient and chromatographic separation to isolate intact purified *Arabidopsis thaliana* vacuoles. Robert *et al.* (2007) treated osmotically and thermally lysed Arabidopsis protoplasts with one-step Ficoll gradient fractionation to release the vacuole. Methods for extracting fungal vacuoles and vesicles have also been studied and reported. Chanda *et al.* (2009) isolated vesicle-vacuole fractions using a high-density sucrose cushion method to solve the problem of different sizes and densities of vesicles and vacuoles from fungal active growth to stationary phase. Different methods to extract different subcellular fractions such as vacuoles, nuclei, mitochondria, endoplasmic reticulum, or peroxisomes have been reported (Rieder and Emr, 2001).

Chanda *et al.* (2010) proposed three possible ways that vesicles mediate mycotoxin export (Figure 4.1): shuttle (intact vesicles that containing mycotoxin pass through the cytoplast membrane and shuttle the mycotoxin out to the environment), pump (intact vesicles transfers the mycotoxin to the cytoplast membrane and fuse with the cytoplast membrane, with mycotoxin efflux pumps on the cytoplast membrane facilitating the export of mycotoxin) and blast (vesicles that contain mycotoxin burst after they fuse with the cytoplast membrane). The detection of aflatoxin antibodies on the fungal mycelial surface and craters of the vesicle-blast-product supported aflatoxin export being done by either the blast mechanism or a combination of pump and blast mechanisms (Chanda *et al.,* 2010).

It is still unclear where and how fumonisin is orchestrated within the cell. We hypothesize that fumonisin biosynthesis occurs in a similar vesicle structures. Future studies will analyze the localization of several enzymes involved in fumonisin biosynthesis by developing strains containing GFP-tagged pathway proteins. We will use confocal microscopy to observe localization of the proteins. We anticipate determining whether these proteins are localized to secretion vesicles similar to those described in *A. parasiticus*.

In addition to the localization of fumonisin, we want to discover the localization of FST1, since it has regulatory function in fumonisin biosynthesis. Our hypothesis is that FST1 is localized to the plasma membrane, with turnover occurring in vacuole-like structures. This hypothesis was derived from the work published by Kim and Woloshuk (Kim and Woloshuk, 2011) (but has yet to be explored) as well as the FB1 transport activity found in yeast strain in this dissertation. Using a strain containing a functional FST1 with a mCherry-FP reporter attached, we will address questions about the inducers of the protein, the hyphae that express the FST1 protein, and the specific location of the protein over a time course. We will use confocal microscopy to conduct observations of the fusion protein to obtain more detailed localization information, including any subcellular transport.

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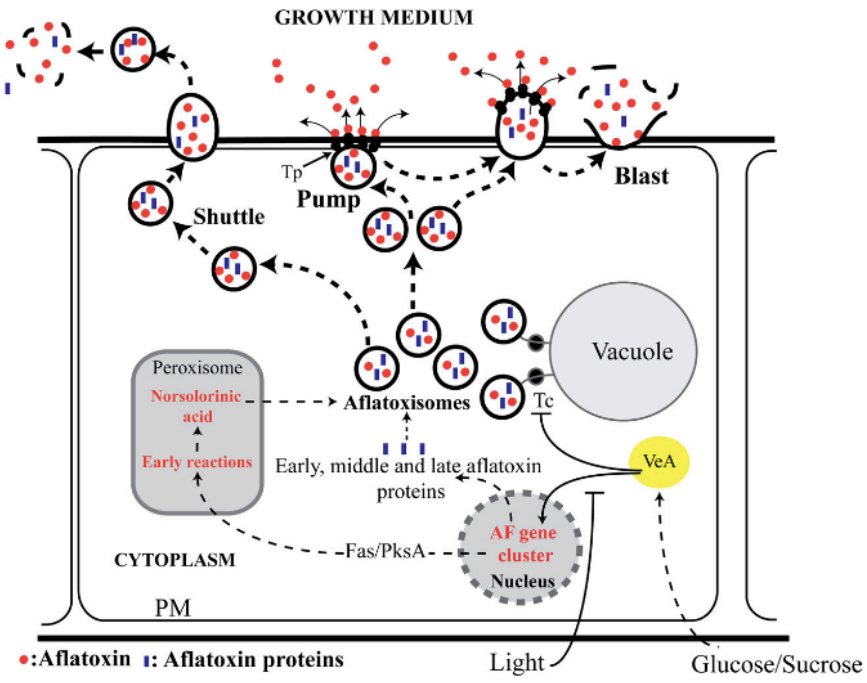

Figure 4.1. Shuttle, pump and blast models for vesicle-mediated export (Chanda *et al*., 2010).

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04/2012	Organizer of BPP graduate student organization for PU Spring Fest

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