MOLECULAR CHARACTERIZATION OF THE CYSTEINE$_2$–HISTIDINE$_2$ TRANSCRIPTION FACTOR *ACE1* IN *FUSARIUM VERTICILLIOIDES*

A Senior Scholars Thesis

by

JACQUELYN CAMPBELL

Submitted to Honors and Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

May 2012

Major: Bioenvironmental Sciences

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Approved by:

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Associate Director, Honors and Undergraduate Research: Duncan MacKenzie

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ABSTRACT

Molecular Characterization of the Cysteine$_2$-Histidine$_2$ Transcription Factor *Ace1* in *Fusarium verticillioides.*(May 2012)

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Fusarium verticillioides is a pathogen of corn that causes yield losses and food safety issues due to mycotoxin production. This research project focuses on a cysteine2-histidine2 transcription factor, named FvAce1, which is predicted to play a critical role in regulating fungal colonization of corn kernels and subsequent mycotoxin production. In particular, earlier studies have shown that FvAce1 is critical for utilization of sorbitol as carbon source in *F. verticillioides*. The objective of this study was to test the hypothesis that *F. verticillioides* FvAce1 shares conserved function with *Trichoderma reesei* Ace1. To test this hypothesis, we created a construct harboring *T. reesei* Ace1 gene, and complemented *F. verticillioides* FvAce1 null mutant. If the function of the two genes is conserved, we would anticipate restoration of wild-type phenotypes in the complemented *F. verticillioides* strain. The result showed that the complemented strains were able to grow on sorbitol as the sole carbon source, which suggested *T. reesei* Ace1 can restore the loss of function caused by FvAce1 mutation. Better understanding of how F. verticillioides FvAce1 regulate diseases on corn and mycotoxin production can

lead to improve disease and mycotoxin management.

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CHAPTER I

INTRODUCTION

In the United States, corn accounts for 90% of the total volume of feed grain produced, and occupies nearly 80 million acres of land. Corn is utilized primarily for the consumption of livestock, and it also serves as a direct source of food for humans. Corn is a major component in common household food items, such as sweeteners, corn-oil, beverages, and industrial alcohol. Recently, it has also become a prominent source of alternative fuel (www.ers.usda.gov). *Fusarium* species has a broad host range, where it has been reported to infect over 10,000 different plant species, including sugar, rice, wheat and asparagus (1). *F. verticillioides* is the key pathogen of corn, causing ear rot and stalk rot, and produces mycotoxin fumonisins in infested corn. In humans, ingestion of contaminated corn with fumonisins, namely FB1 - the most common fumonisin found in nature - has been linked to birth defects and esophageal cancer (2). *F. verticillioides* has also been found to be allergenic to humans and capable of systemically infecting immuno-compromised patients. This fungus has also been associated with corneal ulcers keratitis and the ability to produce skin lesions (3). Multiple environmental and genetic factors play an essential role in fumonisin biosynthesis, and to date, there is no complete understanding of the mechanisms that regulate its production.

This thesis follows the style of The Journal of Biological Chemistry.

Multiple environmental and genetic factors play an essential role in fumonisin biosynthesis, and to date, there is no complete understanding of the mechanisms that regulate its production. Previous studies showed that fungal cell regulation is controlled by a variety of extracellular stimuli that results in the activation of signal transduction pathways (4). These pathways are involved in the regulation of fungal growth, development, pathogenicity, and synthesis of secondary metabolites. In order to further understand how signal transduction pathways regulate these processes, there is a need to characterize the downstream genetic elements, namely transcription factors (TFs), which are involved in gene expression. TFs possess the ability to activate or repress expression of genes by stabilizing or blocking the recruitment of RNA polymerase (5). While the role of TFs has been shown to be essential in the eukaryotic transcription regulation, a limited number of TFs has been characterized to be involved in fumonisns regulation in *F. verticillioides*.

We have created mutants of *F. verticillioides* by knocking out the gene *Fvace1* and replacing it by incorporating the TF *ace1,* out of *Trichoderma reesei,* into the genetic sequence. This mutant is referred to as Δ9. The phenotypes of the mutants exhibit poor growth on mediums such as glycerol, manitol, and sorbitol (Malapi, unpublished data). When compared to the wild type phenotype of *F. verticillioides* the mutants produce FB1 levels that are up to three times as high in concentration. The phenotypes of the mutants show no difference, when compared to the wild type phenotype, in growth and FB1 production in the presence of cellulose or xylan. The mutant phenotypes do exhibit

a decrease in conidia production. The TF *ace1* was also found to deactivate the expression of genes that are able to utilize carbon and polyols in the mutant phenotypes. The TF *ace1* has also been found to activate genes regulating conidiation in the mutant phenotypes. FB1 biosynthesis has been repressed in the mutant phenotype by *ace1*.

Trichoderma reesei is an alternate species of fungi that has been well characterized by previous studies. *T. reesei* is well known for its ability to produce celluloltic enzymes and to hydrolyze cellulose into glucose (6). The absence of the TF *ace1* in these fungi resulted in the retardation of *T. reesei* on a medium containing cellulose. *Ace1* in *T. reesei* has also been proven to be a repressor of cellulase and xylanase expression (7). These parameters gave us a baseline to experiment with in order to determine whether or not *ace1* in *T. reesei* is an orthologue to *Fvace1*. The intent of our study is to prove that *ace1* and *Fvace1* are orthologues by creating and indentifying the mutant, and then complementing that mutant back to it's original wild type phenotype by interchanging these two genes. In this way we are able to prove that *ace1* and *Fvace1* are the only genes involved in these phenotypic changes. By characterizing these orthologues we hope to further characterize genetic sequences responsible for the functions of this pathogen, and eventually determine which genes are responsible for the biosynthesis of the mycotoxins produced by *F.verticillioides*. Only then can we take steps towards determining proper control and mitigation for this pathogen.

CHAPTER II

METHODS

Strains and media

Wild-type *F. verticillioides* strain 7600 (Fungal Genetics Stock Center, University of Missouri-Kansas City, Kansas City, MO) and mutant stains generated previously were stored in 30% glycerol at -80 °C. These strains were routinely maintained and grown on V8 juice agar (200 mL of V8 juice, 3 g of $CaCO_3$ and 20 g agar per liter), potato dextrose agar (PDA; Difco, Detroit, MI), or YEPD medium (3 g of yeast extract, 10 g of peptone, and 20 g of dextrose per L) for general propagation, growth measurement or genomic DNA extraction. For micro conidia counts, 10^4 microconidia were inoculated at the centre of V8 agar or KCl agar (8) and allowed to grow for 7 days. The spores were harvested in sterile water and were counted using a haemocytometer.

Validation of complementation by biomass assay

In order to validate the complementation of the mutant phenotypes with the introduction of the native gene, four flasks containing 100 ml of Modified Myro Medium (1.0 g/L of ammonium phosphate, 3.0 g/ L of potassium dihydrogen phosphate, 2.0 g/ L of magnesium sulfate, and 5.0 g/ L of sodium chloride) amended with 2% sorbitol will be inoculated with 10^7 spores for each mutant isolate along with the wild-type. The inoculated flasks will then be shaken for six days at 100rpm in

room temperature. Subsequently, mycelia will be harvested by filtering the culture through a filter paper embedded in a Buchner funnel. These filter papers, which harbor fungal mycelia, will then be placed in an oven for one day at 100°C in order to remove moisture. The following day the filter papers will be weighed to determine the differences in biomass (dry weight) between the complemented mutants and the two controls (wild-type and Δ 9). This process will then be repeated, but rather than adding sorbitol to the Modified Myro medium, 2% glucose will be added.

Validation of complementation by fumonisin B1 (FB1) assay

In order to compare the amount of FB1 production in corn between the mutant and wild-type strains, 1 g of cracked corn was transferred to 10 ml vials, deionized water was added to the vials and left overnight. The vials were then autoclaved and inoculated with 10^6 conidia and incubated for ten days in room temperature without agitation. In order to extract FB1, acetonitrile/water (50/50, v/v, 10 mL) was added to each vial. The vials were vortexed vigorously and incubated at room temperature for a minimum of 12 hours to a maximum of 48 hours. Supernatants (2 mL) from each sample were then processed through a standard cleanup process described by Shim and Woloshuk (1999) before HPLC analysis (8). The samples were derivatized with o-phthaldehyde (OPA) (Sigma Chemical Co., St. Louis, MO, USA) prior to HPLC injection. The column eluate (0.1 ml) was added to a vial that held 0.1 ml of borate buffer (0.05 M boric acid/0.05 M sodium borate (50/50, v/v), pH 8.5) and 0.1 ml OPA (0.1 mg ml^{-1} in acetonitrile with 0.5% β-mercaptoethanol). After 10 minutes the

reaction will be stopped and 0.5 ml of acetonitrile/0.01 M boric acid (40/60, v/v) will be added. The FB1 sample was then analyzed on an HPLC. A linear gradient was utilized (solvent A: acetonitrile/0.1 M sodium phosphate (40/60), pH 3.3; solvent B: acetonitrile/0.1 M sodium phosphate (60/40), pH 3.3) and the gradient program was as follows: 100% A to 100% B in 10 min, 100% B for 5 min. FB1 was quantified by comparing peak areas with FB1 standards (Sigma Chemical Co., St. Louis, MO, USA).

CHAPTER III

SUMMARY

Molecular characterization of FvSDA

The *SDA* gene found in *F. verticillioides* is predicted to encode a polypeptide with the presence of a three C_2H_2 zinc finger domain. The BLAST program was run to find a fungal protein similar to that of *FvSDA*. The search resulted in the *Trichoderma reesei* zinc finger TF *Ace1*. To analyze the similarities between the amino acid sequences between *F. verticillioides* SDA and *T. reesei* Ace1, amino acid alignment was run utilizing the ClustalW program. The results showed that *SDA* shares 65% identity, and 76 % similarity with Ace1 (Fig. 1).

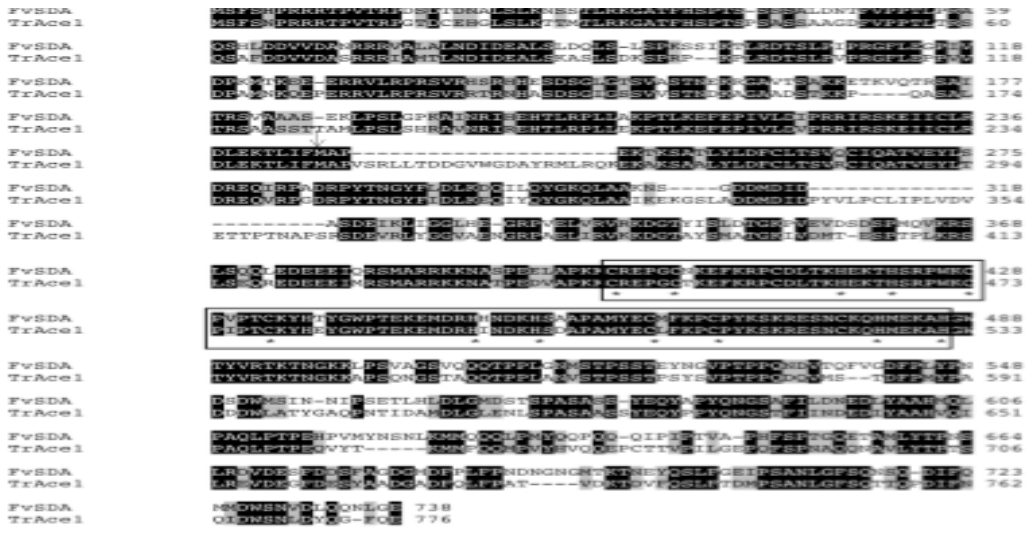

Figure 1. Amino acid alignment of F. verticillioides SDA and T. reesei. This figure displays the results of the amino acid alignment of *F. verticillioides SDA* and *T. reesei* Ace1 using the ClustalW program. The results show that SDA shares 65% identity, and 76 % similarity with Ace1. The sequences that were identical were black shaded and the regions corresponding to the three zinc fingers are indicated by the boxes.

Creation of FvAc1 knock out mutant

The SDA gene was knocked out by utilizing the split-marker recombination strategy, which was established in the Shim lab (Figure 2(A)). Southern analysis was run on the mutant genome and showed successful gene knock out as the wild type SDA band was present at 7kb and the two mutant knock out bands were both present at 3.5 kb (Fig. 2). Of the two mutants, designated fvSDA-7 and fvSDA-9, we chose fvSDA-7 to study phenotypic traits and further characterize the mutant strain. Complementation of ΔSDA was achieved by transforming F. verticillioides ΔSDA gene knock out strain with a construct containing the wild type SDA coding region, promoter and terminator (created earlier in the Shim lab Figure 2). Transformants were then grown in fusarium regeneration agar (FRA) containing hygromycin and geneticin (Fig 3), and PCR screened for complemented phenotypes (Fig 4).

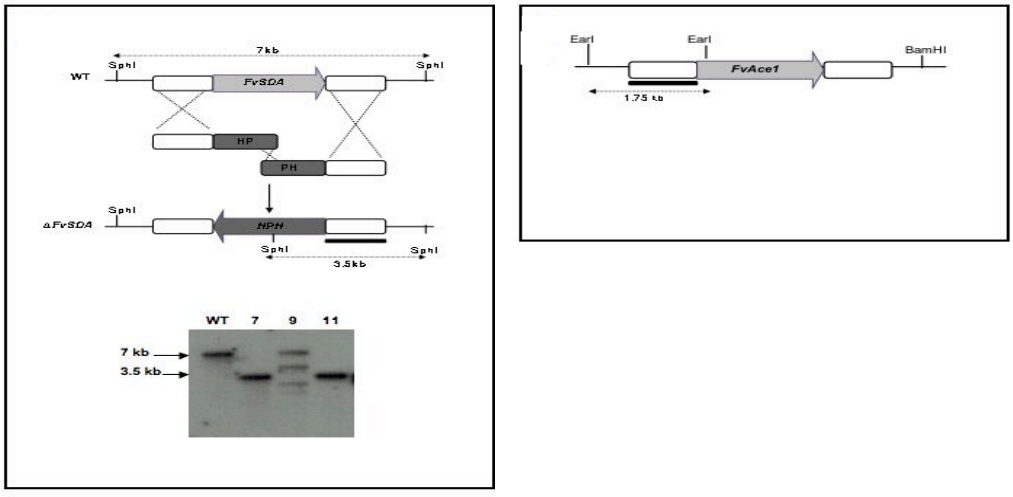

Figure 2. Targeted gene knockout of *SDA* from the genome of *Fusarium verticillioides*. In this representation the solid bar indicates DNA fragment used as the probe for Southern hybridization. Southern analysis of wild-type and two mutants (7 and 11) and the designated probe labelled with ^{32}P. The complementation constructs used to complement ΔSDA strain with a TF9 wild-type copy with its native promoter and terminator.

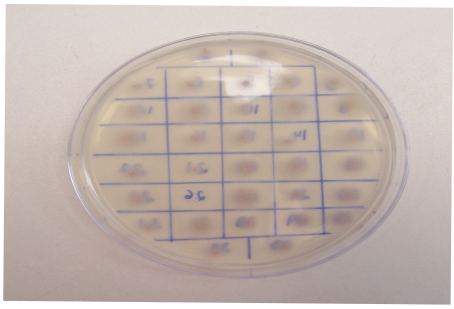

Figure 3:. **Growth of transformants.** Photograph of a fusarium regeneration agar plate containing hygromycin and geneticin on which transformed protoplast have been grown.

Complemented mutants growth on alternate carbon sources

The complemented strains TF9-1, TF9-9, TF9-13, TF9-32, and TF9-17, along with the wild type and ΔSDA, were inoculated into 100ml flasks of defined liquid (DL) media with 2% sorbitol with shaking at 100 rpm for six days. The mycelia was then harvested, dried, and weighed in order to compare the dry biomass of the strains. F. verticillioides wild type can utilize sorbitol as carbon source by oxidizing it into fructose by the enzyme sorbitol dehydrogenase, through the polyol pathway. On the other hand, SDA deletion mutants show complete impaired growth in the presence of sorbitol as the only carbon source When grown on sorbitol, the complemented mutants TF9-13, TF9-32, and TF9-9 exhibited a recovered phenotype, close to wild-type levels (Fig 4).

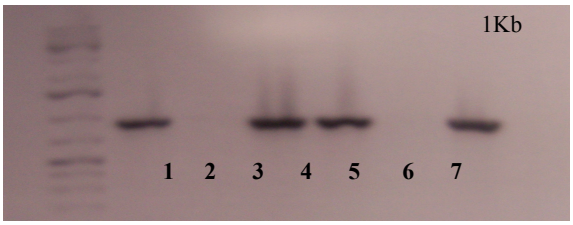

Figure 4: PCR analysis of transformants. Molecular size is indicated on the left. Lanes: 1, 1 kb DNA ladder; 2, *F. verticillioides* WT; 3 *F. verticillioides* ΔSDA; 4,5 7 complemented strains.

As control, we inoculated the strains in DL media + 2% glucose, where we did not observe a significant growth difference among them (Fig. 5)

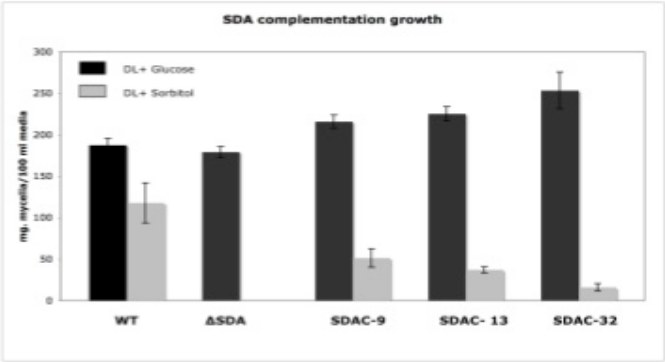

Figure 5: SDA complementation growth. Biomass quantification of the wild-type and complemented strains when supplied with glucose and sorbitol as the sole carbon sources. When grown on sorbitol, complemented strains TF9-9, TF9-13 and TF9-32, exhibited recovered phenotype, close to wild-type levels.

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