Reduction of the mycotoxin deoxynivalenol in barley ethanol co-products using trichothecene 3-*O*-acetyltransferases

Piyum A. Khatibi

Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Plant Pathology, Physiology, and Weed Science

David G. Schmale III
Carl A. Griffey
John M. McDowell
Dorothea Tholl

July 12, 2011
Blacksburg, Virginia

Keywords: *Fusarium graminearum*, Fusarium head blight, deoxynivalenol, barley, *Hordeum vulgare*, ethanol, dried distillers grains with solubles, *TRI101*, *TRI201*

Reduction of the mycotoxin deoxynivalenol in barley ethanol co-products using trichothecene 3-*O*-acetyltransferases

Piyum A. Khatibi

ABSTRACT

The fungal plant pathogen *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae*) produces a dangerous trichothecene mycotoxin called deoxynivalenol (DON) and causes a devastating disease of barley (*Hordeum vulgare* L.) called Fusarium head blight (FHB). Food and feed products derived from barley, such as dried distillers grains with solubles (DDGS), may be contaminated with DON and pose a threat to the health of humans and domestic animals. New methods to mitigate the threat of DON in barley need to be developed and implemented. TRI101 and TRI201 are trichothecene 3-*O*-acetyltransferases that modify DON and reduce its toxicity. The first objective of this research was to isolate unique TRI101 and TRI201 enzymes that modify DON efficiently. We hypothesized that TRI101/TRI201 enzymes from different species of *Fusarium* would have varying rates and abilities to modify DON. Using degenerate primers, an internal portion of *TRI101* or *TRI201* was identified in 54 strains of *Fusarium*. Full-length sequences of seven *TRI101* or *TRI201* genes were cloned and expressed in yeast. All seven genes acetylated DON, but at different rates. The second objective of this research was to utilize transformed yeast expressing TRI101 or TRI201 to reduce DON levels in barley mashes and ultimately in DDGS. We hypothesized that DON levels would be reduced in DDGS derived from mashes prepared with transformed yeast. Five different barley genotypes were used to prepare the fermentation mashes and DON levels were reduced in all DDGS samples derived from mashes prepared with transformed yeast. The third objective of this study was to characterize barley genotypes developed at Virginia Tech for resistance to FHB and DON. We hypothesized that significant differences in resistance would be observed among barley

genotypes and FHB resistance would be associated with reduced DON accumulation. From 2006 to 2010, FHB resistance was assessed in hulled (22 to 37) and hulless (13 to 32) barley genotypes by measuring incidence and index, and DON resistance was determined by quantifying DON levels in ground grain using gas chromatography-mass spectrometry. Our study showed that FHB and DON resistance is significantly determined by genotype. The final objective of this study was to develop a robust tissue culture system necessary for future development of transformed barley plants with FHB resistance gene(s). We hypothesized that callus production would vary among barley genotypes. In our analysis of 47 Virginia barley genotypes, 76% (36/47) of the genotypes produced callus tissue and there were significant differences in callus size. Our work sets the stage for identifying and characterizing DON detoxification genes in the future. The development of commercial barley lines that do not accumulate DON and that are resistant to FHB will directly impact growers and producers of small grains in the eastern U.S.

ACKNOWLEDGEMENTS

Throughout my time in graduate school I am grateful for the support and encouragement from numerous colleagues, friends, and family members. I would first like to thank my advisor Dr. David Schmale for his guidance, advice, and encouragement over the five years of my project. I am forever in debt to my wife Abby whose support, patience, and positivity outside of the lab, allowed me to continue during the most difficult times in my research and academic obligations. I would also like to thank members of my committee, Drs. Carl Griffey, Dorothea Tholl, and John McDowell for their time dedicated towards committee meetings and advice provided throughout my graduate career. I am also thankful to colleagues and friends whose involvement in outside-of-the-lab activities, discussions (scientific or not), experimental advice, or experimental assistance over the years. These people include (in no particular order), Ryan Anderson, Peter Bowerman, Stephen Filingeri, Chris Clarke, Kevin Crosby, Jonathan Watkinson, Troy Hoff, Melissa Ramirez, Sarah Durant, Nicole Juba, Sherry Hildreth, Will Slade, Melissa Keller, John Cianchetti, Niki McMaster, AJ Prussin, BinBin Lin, Diane Reaver, Greg Berger, and all those part of the molecular plant sciences community at Virginia Tech. I am also thankful to the PPWS staff members including Donna Ford, Patsy Niece, and Judy Fielder for all their help in organizing and completing graduate school forms, all financial matters, and all school related trips. Last but not least, I am forever grateful to my parents and sister for their encouragement, advice, and support.

Table of Contents

List of Tables

Chapter II Tables

Chapter III Tables

Chapter IV Tables

Chapter V Tables

List of Figures

Chapter I Figures

Chapter II Figures

Chapter III Figures

Chapter V Figures

CHAPTER I

Literature Review

BARLEY

Barley History and Domestication

Barley (*Hordeum vulgare* L.) is currently the fourth most important cereal crop in the world and is the fifth most produced cereal crop in the United States (according to FAO in 2009; http://www.faostat.fao.org). Barley is one of the oldest domesticated crops dating back to about 10,000 years before present (BP) in the Fertile Crescent of the Middle East (Pourkheirandish and Komatsuda, 2007; Zohary and Hopf, 2000). The wild relative of *Hordeum vulgare* is *Hordeum spontaneum* C. Koch, and these two species differ in part due to selection pressures in the wild versus man-made environments (Zohary, 2004). *Hordeum vulgare* has broader leaves, shorter stems, shorter awns, a tough ear rachis, shorter and thicker spikes, and larger grains than its wild relative (Zohary, 1969).

Barley spikes are composed of a central rachis and two lateral spikelets. In six-rowed barley cultivars all spikelets are fully fertile and develop into grain in contrast to two-rowed barley in which only the central spikelets develop into grain (Komatsuda et al., 2007). The earliest domesticated barley plants were two-rowed, with six-rowed barley showing up around 8,800 years BP (Zohary and Hopf, 2000). Six-rowed barley was likely derived from the ancestral two-rowed genotype and developed via a mutation during domestication (Komatsuda et al., 2007; Pourkheirandish and Komatsuda, 2007). In cultivated barley, a non-brittle rachis, very important in early domestication, allowed the spikes to remain longer on the plant resulting in higher levels of grain to be harvested (Pourkheirandish and Komatsuda, 2007). Selection for

larger grains together with six-rowed barley genotypes resulted in increased yields for the earliest farmers (Pourkheirandish and Komatsuda, 2007).

Archaeological evidence shows that barley originated in the Near Eastern Neolithic agriculture together with wheat (Zohary and Hopf, 2000). From the Middle East, barley emerged in Greece about 8,000 to 7,000 years BP, in the Mediterranean basin 7,000 to 5,000 years BP, and east into China about 4,000 years BP (Zohary and Hopf, 2000). Throughout history, barley has been primarily used as food for humans; however, when barley finally reached North America in 1492 it was primarily used for malting and animal feed (Newman and Newman, 2006). This still holds true today where most barley is designated for feed, malting and brewing (Baik and Ullrich, 2008). However, areas in Asia and northern Africa still maintain barley as a major food source (Newman and Newman, 2006).

Barley as Food and Feed

Food products of barley include porridges, breakfast cereals, bread, and other baked goods (Andersson and Aman, 2008). In the United States, barley is not a major food source for humans and has been replaced by other grains such as wheat and rice (Baik and Ullrich, 2008). However, there are many nutritional and health benefits from the fiber and phytochemicals found in barley (Griffey et al., 2010). Consumption of fiber is important for reducing the risk of diseases such as heart disease, hypertension, diabetes, obesity, and certain gastrointestinal disorders (Anderson et al., 2009). There are also a variety of health benefitting phytochemicals such as tocols, sterols, phenolic acids, alkylresorcinols, and the vitamin folate (Andersson et al., 2008). Foods that are enriched in β-glucans may elicit a low glycemic response (Wood, 2007) which may help in the prevention and control of diabetes (Rizkalla et al., 2002). Seventy-five

percent of the barley endosperm cell walls are composed of β-D-glucans (Fincher, 1975), while total mean concentrations in the kernel can range from 4.0% to 4.2% (Griffey et al., 2010).

Although barley has a long history as a human food, a large portion is used in feed for domestic animals. In 2009, 27% of barley grown in the U.S was destined for feed (U.S. Grains Council, www.grains.org). Barley with high protein and high starch content is preferred over barley types with high fiber (Griffey et al., 2010). Barley also has a nutritional advantage over corn as feed, having a higher lysine content than that of corn (Griffey et al., 2010). Many domestic animals are unable to synthesize lysine, one of eight essential amino acids (Renneberg, 2008). Additionally, barley is a high energy source, with starch concentrations reported to be as high as 61% (Huth et al., 2000).

Winter Barley

Winter barley is a perennial plant that requires a period of cold exposure to make the physiological switch from vegetative to reproductive growth (Sasani et al., 2009). This vernalization requirement prevents flowering before the winter season and establishes a life cycle in which flowering only occurs during the warmer spring weather (Schmitz and Amasino, 2007). Most strains of wild barley (*Hordeum spontaneum*) are of the winter type (*sgh2*) (Takahashi et al., 1968; Takahashi et al., 1963) leading to the hypothesis that spring barley (*Sgh2*) arose from a mutation in the *sgh2* locus, allowing barley to expand to milder climates (Bothmer et al., 2003; Pourkheirandish and Komatsuda, 2007). In germinating barley seedlings, the *Sgh2* gene (*HvVRN1)* (Cockram et al., 2007), has increased expression levels during the vernalization period (Sasani et al., 2009).

Flower timing in plants such as *Arabidopsis thaliana* are regulated by both length of day and temperature. *Arabidopsis* is a long day plant requiring a period of vernalization to induce flowering. These environmental factors influence the epigenetics of genes involved in flower timing. In the absence of cold temperatures, the repressor *FLOWERING LOCUS C* (*FLC*), a MADS domain transcription factor (Michaels and Amasino, 1999), is in the active conformation due to the acetylation of lysine residues (Schmitz and Amasino, 2007). FLC expression results in the repression of *FLOWERING LOCUS T* (*FT)* and ultimately prevents the switch from the vegetative to flowering state (Helliwell et al., 2006; Michaels and Amasino, 1999). In winter barley, a period of 4 to 9 weeks at 2±1 ^{o}C is sufficient to promote heading within 60 days (Sasani et al., 2009). Barley vernalization creates an open chromatin state (loss of trimethylation at histone 3 lysine 27 and gain of trimethylation at histone 3 lysine 4) and induction of the floral promoter *VERNALIZATION1* (*HvVRN1*) (Oliver et al., 2009). Unlike *Arabidopsis,* where flowering is dictated by expression of the repressor *FLC* (expression results in no flowering), barley flowering is controlled by the floral repressor *VERNALIZATION2* (*HvVRN2*). *HvVRN2* (zinc finger transcription factor) is down-regulated by *HvVRN1* (a MADS box transcription factor), which leads to the activation of *FT* (a polyethanolamine binding protein which regulates meristem identity) and the switch from vegetative to flowering growth (Trevaskis et al., 2006).

In addition to spring and winter genotypes, there are also hulled and hulless genotypes. In hulled barley, the hull is tightly affixed to the seed epidermis; however, due to a mutation event that led to the deletion of the *Nud* gene, hulless barley lacks this phenotype (Taketa et al., 2008). This hulless barley phenotype was selected by early farmers and ultimately led to its distribution around the world. The *Nud* gene regulates the deposition of lipids on the pericarp epidermis to allow adhesion of the pericarp to the hull (Taketa et al., 2008). Since the hull is

separate from the seed coat there is no need for pearling and the resulting kernel has more starch per volume (Griffey et al., 2010). The increased starch density of hulless barley renders it a viable alternative to corn as a fuel ethanol source.

'Doyce' is a winter hulless barley genotype, which was developed by the Virginia Agricultural Experimental Station (Brooks et al., 2005). Through a progression of crosses detailed in Brooks et al. (2005), Doyce was created in 1994 and released in 2003 (Brooks et al., 2005). Hulless barley cultivars are a promising choice for use in ethanol production and as a feed product for domestic animals because of their high starch and low fiber composition (Brooks et al., 2005; Griffey et al., 2010).

Barley for Fuel Ethanol Production

Many important agricultural crops (e.g., soybean, corn, and sugarcane) have been proposed as important sources of alternative fuels. Biodiesel, for example, is primarily produced from soybeans; biodiesel reduces carbon dioxide emissions, particulate matter, and sulfur oxides compared to traditional diesel (Manuel, 2007). Ethanol, produced primarily from corn and sugarcane, offers many advantages over traditional gasoline including a higher-octane value, higher theoretical efficiency for engines, and far less particulates and NO_x emissions (Balat et al., 2008). During the fuel shortage crisis of the 1970s (Dias De Oliveira et al., 2005), a major policy shift by Brazil led to ethanol production using sugarcane (Madson and Monceaux, 1995). In Brazil, ethanol produced from sugarcane provides about 40% of Brazil's fuel needs (Balat et al., 2008). The U.S., however, only produces enough ethanol to replace at most 4% of the gasoline market (Harari, 2007). American ethanol production requires more fossil fuel and is

less cost effective than sugarcane ethanol produced in Brazil (Harari, 2007). The U.S. has a long way to go before it can significantly reduce the demand for foreign oil (Manuel, 2007).

Currently, 95% of the ethanol in the U.S is produced from corn (Drapcho et al., 2008) but many parts of the country including the eastern, western, and northwestern states do not have good growing climates for corn (Hicks et al., 2005). Barley can be grown in these regions as a winter crop (Hicks et al., 2005), providing both ground cover and preventing nutrient loss from the soil (Chesapeake-Bay-Commission, 2007). Furthermore, growing winter barley allows a two-year three crop rotation that includes winter barley, soybean, and corn.

The average corn kernel is composed of 72% starch (Sohn et al., 2007) and hulled genotypes of barley can have starch values between 50% and 55% (Sohn et al., 2007); however, new hulless cultivars such as Doyce can have levels up to 64% (Griffey et al., 2010). In 2007, the U.S used approximately 60% of total corn destined for food and industrial applications for fuel ethanol (Drapcho et al., 2008). This statistic underscores the need to produce fuel ethanol from non-food crops. The USDA Agricultural Research Service predicts the U.S will be able to produce up to 2 billion gallons of ethanol per year from barley (Drapcho et al., 2008).

A significant challenge to utilizing barley as a fuel ethanol source is the presence of β-D-glucans, which can represent 3.31% to 5.76% of total weight (Griffey et al., 2010). These non-starch molecules are polymers composed of D-glucose units with fermentation potential. The Enhanced Dry Grind Enzymatic (EDGE) process was developed by researchers at the USDA Eastern Regional Research center (ERRC), with the goal of hydrolyzing the β-D-glucans, to resolve viscosity issues and to increase ethanol yields by increasing available glucose (Nghiem et al., 2010). Mash viscosity issues are a concern with β-glucans as they are water-soluble and consequently increase mash thickness and hinder complete integration of the mash components

(i.e. yeast and nutrients) (Nghiem et al., 2010). During the EDGE process, a preparation of β-glucanases are added to reduce the viscosity by hydrolyzing the β-glucans to oligomeric fractions (Nghiem et al., 2010). To release the glucose in the starch granules of the kernel endosperm, α-amylase is added to the mash during liquefaction (Nghiem et al., 2010). β-glucosidases are included in the mash to cleave the β-glucan monomers into glucose units at the saccharification and fermentation stage (Nghiem et al., 2010).

Virginia Tech plant breeders and scientists at the USDA ERRC have conducted collaborative research promoting the use of barley as an ethanol feedstock and to incorporate fermentation methods such as the EDGE process, which optimizes ethanol yields (Schill, 2008). However, the success and future of barley as a feedstock for ethanol production is going to heavily rely on getting growers to grow barley to provide a sufficient supply for ethanol production.

FUSARIUM

Overview

Fungi in the genus *Fusarium* cause devastating diseases in both plants (Nelson et al., 1994) and animals (Evans et al., 2004; Nucci and Anaissie, 2007). There are over 70 biological species of *Fusarium*, some with the ability to produce secondary metabolites such as mycotoxins (Leslie and Summerell, 2006). *Fusarium* species most common in animal infections include *F. solani, F. oxysporum, F. verticillioides,* and *F. fujikuroi* (Nucci and Anaissie, 2007). For example, *F. solani* was the causal agent of brain inflammation called meningoencephalitis in a dog (Evans et al., 2004). *F. solani and F. oxysporum* isolates were responsible for a multistate outbreak of *Fusarium* keratitis due to the use of contaminated contact lens solutions (Chang et al.,

2006). Other animal infections caused by *Fusarium* species include nail, skin, bone, and intranasal infections (Nelson et al., 1994).

Fusarium infections in humans are problematic, however, they generally occur in immunocompromised individuals (Nucci and Anaissie, 2007). On the other hand, *Fusarium* causes substantial damage to agricultural crops, resulting in several billion dollars worth in losses for wheat and barley farmers (Windels, 2000). In the United States, *Fusarium graminearum* causes the disease Fusarum head blight (FHB). Other species such as *F. pseudograminearum*, *F. culmorum*, and *F. sporotrichoides* also contribute to FHB around the world. *Fusarium oxysporum* and *F. fujikuroi* have been occasionally isolated from barley in the Midwest (Salas et al., 1999); however, these pathogens primarily cause disease in tomato and rice, respectively (Lagopodi et al., 2002; Sunder, 1998). Morphological and physiological differences vary considerably among *Fusarium* species, enabling these fungi to occupy diverse ecological niches in many geographic areas (Nelson et al., 1994). Except for *F. graminearum*, most *Fusarium* species have two mating types (they are heterothallic) and outcross (Cuomo et al., 2007; Goswami and Kistler, 2004). *F. graminearum* and species in the *F. graminearum* species complex have four chromosomes, while others in the genus such as *F. verticillioides, F. oxysporum,* and *F. solani* have chromosome numbers ranging from 9 to more than 17 (Cuomo et al., 2007). There are approximately 11,640 genes in the *Fusarium graminearum* genome (Goswami and Kistler, 2004) and the most variable DNA regions are in areas associated with plant infection, which likely help the organism cause disease (Cuomo et al., 2007).

Fusarium species may produce four types of asexual spores called macroconidia (large), mesoconidia (medium), microconidia (small), and chlamydospores (Leslie and Summerell, 2006). Chlamydospoores are thick-walled cells, which can survive extreme weather conditions. All

four types of spores are haploid and germinate to form germ tubes, which differentiate and grow into hyphae. A hypha is made up of one or more cells, which may be divided by internal walls called setpa. Hyphae can form structures called conidiophores, which give rise to the asexual spores. The sexual stage (teleomorph) of *Fusarium* (anamorph) is called *Gibberella* (Desjardins, 2003). The sexual stage is characterized by the formation of fruiting bodies called perithecia that produce ascospores (Desjardins, 2003). For the sexual stage to take place, a conidium or hyphal fragment from one mating type transfers a nucleus through a specialized structure called the trichogyne to the opposite mating type (Leslie and Summerell, 2006). Mating type is determined by the mating type locus (*MAT*), which may either be *MAT1 or MAT2* (Turgeon, 1998). After fertilization, perithecia and ascospores develop (Leslie and Summerell, 2006).

In a study with *Giberella zeae* there was no difference between disease levels of conidia and ascosopores (Stack, 1989). In experiments to examine the levels of ascospores and conidia in a wheat field, ascospores were found at twice the levels as conidia (Markell and Francl, 2003). Similarily, in 1995, densities of ascospores were ten times greater than macroconidia over a seven day time period in a wheat field (Fernando et al., 2000). The relative abundance of these spore types suggests that ascospores play a greater role in field infections. Unlike conidia, ascospores are ejected with great force and acceleration from perithicia (Trail et al., 2002), potentially resulting in a greater contribution to field infections.

Fusarium head blight

Fusarium head blight (FHB) of wheat and barley resulted in economic losses of $2.7 billion from 1998 to 2000 (Nganje et al., 2001). FHB causes considerable reductions in yield and grain quality (Kang and Buchenauer, 2000; Parry et al., 1995). The causal agent, *Fusarium*

graminearum, overwinters in decaying crop residue as a saprophyte (Parry et al., 1995) and during the spring and summer months, perithecia eject ascospores into the environment leading to infection of emerging wheat and barley heads (Goswami and Kistler, 2004).

Presently, there are conflicting claims on whether *F. graminearum* invades floral tissue as a necrotroph or hemibiotroph (Brown et al., 2010). Jansen et al. (2005) harvested and inoculated seed from different barley lines and could not identify a biotrophic phase. Furthermore, a look at the *F. graminearum* interaction with *Arabidopsis thaliana* showed that jasmonic acid/ethylene pathways were more enhanced than the salicylic acid pathway, typical of a necrotrophic pathogen (Chen et al., 2006). On the other hand, Brown et al. (2010) concluded that there is both a biotrophic phase and necrotrophic phase of colonization. Brown and colleagues report that *Fusarium* initially feeds off the extracellular exudates in the apoplast and is followed by necrotrophic intracellular colonization (Brown et al., 2010). They tracked the infection pathways and hyphal networks of *F. graminearum* from the original inoculated floret, into the rachis nodes, and the movement to adjacent spikelets (Brown et al., 2010).

Upon inoculation of a wheat spikelet, there was evidence of fungal colonization of the palea 2 days post infection (dpi) (Brown et al., 2010). By 5 days post infection (dpi) intercellular hyphae colonized the cortex cells of the rachilla and eventually entered the rachilla vasculature (Brown et al., 2010). In order to spread to adjacent spikelets, the hyphae progressed both inter- and intracellularly into the rachis node and moved into the cortex and vasculature of the rachis where the fungus could spread vertically to additional spikelets (Brown et al., 2010). Alternatively, hyphae could move laterally by intracellular colonization from the rachis node to rachis (Brown et al., 2010). Behind the advancing front of infection, the cytoplasm of cortex cells became granulated and plasmolysed (Brown et al., 2010). Without hyphal contact, the

chlorenchyma cells were the only cells that died ahead of infection (Brown et al., 2010); however, since deoxynivalenol (DON) (**Figure 1A**) was not tracked in this study it is difficult to determine whether this mycotoxin had any role in causing cell death. Towards the late infection state, older colonized cortex and phloem collapsed and was devoid of cell contents, while the advancing hyphae grew through the epidermis onto the surface to produce aerial mycelium (Brown et al., 2010). Brown et al. (2010) provide a detailed description of *F. graminearum* movement from spikelet to spikelet in wheat; in spite of this, they do not report infection of the grains. Jansen et al. (2005) show that in isolated kernels, the traveling hyphae move along the epicarp, subsequently penetrating into the pericarp and progress into the endosperm. Hyphae attacking the epicarp cells displayed appressoria-like structures before entering into the cytosol of individual cells and causing plasmolysis and cell death, indicating a nectrophic only phase of infection (Jansen et al., 2005).

Fusarium graminearum produces the mycotoxin DON to promote hyphal spread in wheat (Proctor et al., 1995). DON has been demonstrated to contribute to aggressiveness of *F. graminearum* (Mesterhazy, 2002). Higher aggressiveness was correlated with higher FHB severity, higher Fusarium damaged kernels (FDK), and higher yield loss in wheat (Mesterhazy, 2002). However, cultivar resistance is the final determinant on the isolate's aggressiveness during infection (Mesterhazy, 2002). For example, resistant wheat cultivars were shown to accumulate low levels of mycotoxin, while the same isolate produced high levels of mycotoxins in susceptible cultivars (Mesterhazy, 2002).

Previous work has shown that in several fungi their respective mycotoxins are present within or on the surface of the spores (Land et al., 1993; Sorenson et al., 1987; Wicklow and Shotwell, 1983). Conversely, Evan et al. (2000) showed that there was no detectable DON in or

on macroconidia and then further investigated the timing of DON biosynthesis upon infection of barley spikelets. DON was detected at 36 h post-inoculation in barley spikelets and spikelet chlorosis and necrosis was visible at 54 and 72 h post-inoculation, respectively (Evans et al., 2000). Deoxynivalenol has been demonstrated to be a virulence factor in wheat, but not in barley (Jansen et al., 2005). In barley, hyphae of both wild-type and DON synthesis knockout mutant (*tri5*), are unable to spread to adjacent spikelets and are inhibited at the rachis node and rachilla (Jansen et al., 2005). Instead infection to neighboring florets must proceed via the phloem and the surface of the rachis (Jansen et al., 2005). Upon barley infection by *F. graminearum* and release of DON, a variety of genes are differentially expressed in the plant. Boddu et al. (2007) reported that 63 different transcripts were induced during trichothecene release into barley, including trichothecene detoxification and transport proteins, ubiquitination-related proteins, programmed cell death-related proteins, transcription factors, and cytochrome P450s (Boddu et al., 2007). Uridine diphosphate (UDP) glucosyltransferases are expressed during trichothcene accumulation in barley (Boddu et al., 2007) and have been shown to detoxify deoxynivalenol in *Arabidopsis thaliana* (Poppenberger et al., 2003). Moreover in the absence of DON (*tri5*), 414 genes were expressed and likely represents the basal defense response (Boddu et al., 2007). Basal defense genes expressed include pathogenesis-related proteins, oxidative burst enzymes, glutathione-S-transferases, proteases, tryptophan biosynthetic genes, shikimate pathway genes, and phenylpropanoid pathway genes (Boddu et al., 2007).

FUSARIUM MYCOTOXINS

Trichothecenes

Trichothecenes are sesquiterpene epoxides (Desjardins et al., 1993) and were named after the trichothecin producing fungus *Trichothecium roseum* (Desjardins, 2006). *Trichothecium roseum* is an endophyte (Zhang et al., 2010) which infects cereals such as corn, barley, and wheat (Bamburg et al., 1969; Ishii et al., 1986; Magan, 1988). Like other trichothecenes, trichothecin is a protein synthesis inhibitor and has recently been implicated in inhibiting mitochondrial translation (McLaughlin et al., 2009). In 1961, the first *Fusarium* trichothecene isolated was diacetoxyscirpenol from *F. scirpi (Desjardins, 2006)*. In 1968, nivalenol was isolated from *F. nivale* from contaminated grain in Japan (Desjardins, 2006). From moldy maize in France, T-2 toxin was isolated from *F. tricinctum* (*F. sporotrichioides*) (Desjardins, 2006). The relative toxicity of trichothecene mycotoxins varies between plants and animals (Desjardins et al., 2007). To determine the toxicity in plants, assays were conducted with *Arabidopsis thaliana* leaves (Desjardins et al., 2007). From the smallest to the largest concentration of trichothecene mycotoxin that resulted in 50% (LD_{50}) of the leaves to die, were T-2 toxin, 4,15-diacetoxyscirpenol, deoxynivalenol, and nivalenol (Desjardins et al., 2007). Similarly, experiments with wheat demonstrated that T-2 toxin is the most inhibitive to growth followed by deoxynivalenol, and nivalenol which was seven times less toxic than DON (Desjardins, 2006). In animal studies, the more oxygenated trichothecenes tend to be the most dangerous (Desjardins, 2006). Animal toxicity experiments have demonstrated that T-2 toxin is the most toxic, followed by nivalenol and then deoxynivlenol (Desjardins, 2006).

Zearalenones

Zearalenones are trichothecenes that are produced by a range of *Fusarium* species such as *F. graminearum*, *F. culmorum*, and *F. crookwellense* (Desjardins, 2006). This group of

mycotoxins, despite their nonsteroidal structure, mimic the steroidal hormone estrogen in animals by binding to estrogen receptors (Desjardins, 2006). Intake of zearalenones is known to cause problematic reproductive issues (Desjardins, 2006). In nonpregnant female pigs, zearalenone resulted in pseudopregancies, while during pregnancy there was a reduced weight of the uterus, placental membrane, and fetus (Etienne and Jemmali, 1982). In plants, zearalenone does not appear to be phytotoxic. In fact, varying concentrations of zearalenone (up to 310 μM) did not inhibit the germination or growth of wheat seedlings (Eudes et al., 2000).

Fumonisins

Fumonisins are inhibitors of sphingolipid metabolism in animals and plants (Desjardins, 2006). Specifically, fumonisins strongly inhibit the third enzyme in sphingolipid biosynthesis and cause cell apoptosis (Desjardins, 2006). Sphingolipids make up the plasma membrane in cells and are important in cell recognition and signaling. There are 6 analogues of fumonisins (FA_1, FA_2, FB_1, FB_2, FB_3, FB_4) (Marasas, 2001) that differ by structure and biological activity (Desjardins, 2006). FB_1 is the most prevalent and naturally occurring toxin in corn and corn products and is the most studied of the fumonisins (Marasas, 1995). FB_2 and FB_3 are less oxygenated than the FB_1 analogue and were less toxic in detached tomato leaf assays (Desjardins, 2006). The fumonisin A analogues are the N-acetyl derivatives of the FB type (Marasas, 1995) and were less toxic than the deoxygenated derivatives (FB_2, FB_3) in detached tomato leaves (Desjardins, 2006).

The toxicity of fumonisins to plants is dependent on plant species and may even vary between cultivars of the same species (Desjardins, 2006). The first reports of phytotoxicity were demonstrated in growth inhibition assays with maize callus cultures (Desjardins, 2006), where a

concentration of 13 μM fumonisin B_1 caused 50% reduction in callus growth (Van Asch et al., 1992).

In humans, risks of exposure to fumonisins are high due to the high consumption of corn products, especially in the U.S. The effects of fumonisins on humans are unknown; however, there are numerous studies in animal systems. For example, fumonisins (FB_1) have been shown to cause brain lesions in horses (leukoencephalomalcia) and cancer in laboratory rodents (Desjardins, 2006). *F. verticillioides* is the primary agent that infects corn and produces fumonisins (Marasas, 2001). The federal drug administration (FDA) has set guidance levels for fumonisins in formulated animal feed that range from 5 ppm (horses) to 50 ppm (chickens designated for slaughter) (Voss et al., 2007).

DEOXNIVALENOL

Overview

DON is a sesquiterpene epoxide and secondary metabolite (Desjardins et al., 1993). DON has been identified in the cytosol, mitochondria, vacuoles, and the cell wall of hyphae and in the ribosomes, chloroplasts, cell membrane, cell walls, and vacuoles in wheat (Jansen et al., 2005). DON is an inhibitor of protein synthesis, causes necrosis, and interferes with the expression of defense-related genes, thereby increasing susceptibility to plant diseases (Mitterbauer et al., 2004). For animals, DON is toxic in very small amounts, causing vomiting, immune system suppression, reproductive problems, feed refusal, and even death (Pestka and Smolinski, 2005). DON is likely not a volatile molecule and requires derivatization to increase volatility for gas chromatography mass spectrometry (GC/MS); however others have speculated that feed refusal in domestic animals is caused by detecting the mycotoxin through smell,

although little evidence for this is available (Snijders, 1990). The U.S. Food and Drug Administration has set limits for DON in food and feed products. Food for human consumption such as bran, flour, and germ has a limit of 1 ppm DON (Pestka and Smolinski, 2005). There is a 5 ppm DON limit in grains and grain products intended for swine, a 10 ppm DON limit for grain feed, and 30 ppm limit for distillers grains destined for cattle (www.fda.gov/food).

DON Biosynthesis

Trichothecenes such as deoxynivalenol, nivalenol, and T-2 toxin are sesquiterpenes derived from the mevalonic acid pathway (MVA) (Kimura et al., 2007). The MVA pathway is responsible for the production of secondary metabolites and in the synthesis of terpenes (Lange et al., 2000). Pyruvate produced from glycolysis is shuttled into the MVA pathway to produce isopentenyl diphosphate (IPP), which is then converted to farnesyl diphosphate (Lange et al., 2000), the first substrate in DON biosynthesis (Foroud and Eudes, 2009). In the first step, farnesyl diphosphate is cyclized into trichodiene by trichodiene synthase (TRI5) (**Figure 2)** (Foroud and Eudes, 2009). The next three steps include a hydroxylation, epoxidation, and another hydroxylation, catalyzed by a P450 monooxygenase (TRI4), to produce isotrichodiol (**Figure 2)** (Kimura et al., 2007). An additional hydroxylation by TRI4 produces isotrichotriol (**Figure 2)** (Kimura et al., 2007). Two non-enzymatic isomerization steps produce the first trichothecene in the pathway called isotrichodermol (**Figure 2)** (Foroud and Eudes, 2009). A trichothecene 3-*O*-acetylatransferase called TRI101 acetylates isotrichodermol at C-3 to produce isotrichodermin, which is then hydoxylated at C-15 by TRI11, a C-15 monooxygenase, to form 15-deacetylcalonectrin (Foroud and Eudes, 2009). Subsequently, TRI3 (15-*O*-acetyltransferase) produces calonectrin, followed by 7,8 dihdroxycalonectrin catalyzed by TRI1 (C-7

monooxygenase) (McCormick et al., 2011). The next product is 3,15-dideacetylcalonectrin, which is converted to 3-acetyldeoxynivalenol (3ADON) (**Figure 1B)** by TRI8 (C-3 deacetylase) **(**McCormick et al., 2011)**.** In the final step, 3ADON is converted to DON by TRI101 **(Figure 2)** (McCormick et al., 2011).

Trichothecene 3-*O*-acetyltransferases

TRI101 is a trichothecene 3-*O*-acetyltransferase that converts isotrichodermol to isotrichodermin in the trichothecene biosynthetic pathway and is required for mycotoxin biosynthesis (McCormick et al., 1999). This enzyme has been implicated as a self-protection mechanism utilized by *Fusarium* by attaching an acetyl to the C-3 hydroxyl moiety of DON to form 3-acetyldeoxynivalenol (3ADON) (Garvey et al., 2008). DON is a known protein synthesis inhibitor that targets the L3 subunit of the ribosome (Mitterbauer et al., 2004), indicating that the acetyl group may render DON less toxic by restricting access to the ribosomal binding site (Alexander et al., 1999). Kimura et al (1998) demonstrated that *TRI101* provided resistance to *F. graminearum* (DON producing) from exogenous T-2 toxin and therefore may have a role in self-protection (Kimura et al., 1998a). There are numerous examples of trichothecene 3-*O*-acetyltransferases reducing toxicity by converting DON to 3ADON. In *Saccharomyces pombe* TRI101 decreased the inhibitory effects of T-2 toxin on cell growth (Kimura et al., 1998a). Similar effects were also observed in *Chlamydomonas reinnardtii* and *Arabidopsis thaliana* (Alexander et al., 1999; Desjardins et al., 2007). *In vitro* assays have demonstrated DON to be more inhibitive than 3ADON on protein translation using rabbit reticulocytes (Kimura et al., 1998a), DNA synthesis using mouse 3T3 fibroblasts (Eriksen et al., 2004), and proliferation of murine lymphocytes (Bondy et al., 1991). However, DON was only 1.4 times less toxic than

3ADON based on 50% lethal dose (LD_{50}) values in mice, which was assessed through intraperitoneal injections (Peska, 2007; Thompson and Wannemacher, 1986). Although the differences in the LD_{50} concentrations between DON and 3ADON was small, the relative toxicity of DON and 3ADON in mammals requires further investigation, as toxicology data is limited at this time (Scientific Committee on Food, 1999).

TRI101 transfers an acetyl group from acetyl coenzyme A (acetyl-CoA) to the C-3 hydroxyl moiety of deoxynivalenol **(Figure 3)** (Garvey et al., 2008). The proposed mechanism of acetylation begins with TRI101 from *F. graminearum* (FgTRI101) binding DON and acetyl-CoA **(Figure 3)** (Garvey et al., 2008). Then the catalytic histidine, $FgHis^{156}$, of FgTRI101 extracts a proton from the C-3 hydroxyl group of DON **(Figure 3)** (Garvey et al., 2008). The deprotonated oxygen, now having a negative charge, nucleophilically attacks acetyl-CoA ultimately forming 3ADON **(Figure 3)** (Garvey et al., 2008). There are kinetic differences among TRI101 enzymes from different species (Khatibi et al., 2011). For example, structural differences between FgTRI101 and the *F. sporotrichioides* TRI101 (FsTRI101) have shown that FgTRI101 is better able to accommodate DON (Garvey et al., 2008). Kinetic experiments showed that 85 times more DON substrate is required for FsTRI101 to reach the half-maximal rate than for FgTRI101 (Garvey et al., 2008).

TRI101 is located between the phosphate permease gene (*PHO5*) and the UTP-ammonia ligase gene (*URA7*) in *F. graminearum*. Kimura et al. (2003) looked at the same genomic regions in *F. oxysporum* and *F. fujikuroi*, trichothecene non-producers, and found *FoTRI101* and *FfTRI101* genes that had inactivating mutations. However, trichothecene 3-*O*-acetltransferase activity was still detected in fungal cultures of *F. oxysporum* and *F. fujikuroi* and was determined to be encoded by a gene called *TRI201* (Kimura et al., 2003). Tokai et al. (2005) also looked for

TRI201 in other trichothecene non-producers and found that some non-producers such as *F. acuminatum* and *F. avenaceum* lack *TRI201*. *TRI201* is believed to be the result of gene duplication with a different evolutionary history from that of other trichothecene biosynthesis genes (Tokai et al., 2005). *TRI101* and *TRI201* are not linked to the trichothecene biosynthetic gene cluster (Kimura et al., 1998b; Tokai et al., 2005), and likely existed in the genome before the divergence of trichothecene producers from non-producers (Kimura et al., 2003). Functional trichothecene 3-*O*-acetyltransferases have been found in other fungal genera including *Albonectria*, *Neocosmospora,* (Tokai et al., 2005) and *Saccharomyces* (Alexander et al., 2002). These enzymes may be antibiotic resistance genes broadly dispersed among other fungal species (Tokai et al., 2005).

FHB AND DON MANAGEMENT STRATEGIES

Management of FHB should involve an integrated strategy that includes the following (Wegulo et al., 2008): (i) plant fungicide treated seeds to prevent seedling blight, (ii) maintain crop rotations with broad leaf crop such as soybean, (iii) plant resistance cultivars, (iv) plant diverse cultivars with different flower timing, (v) utilize FHB prediction tools, and (vi) apply fungicides early during flowering.

Seedling blight of wheat and barley, caused by *F. graminearum* and *F. culmorum*, can cause significant damage to growing seedlings (Parry et al., 1995). Seedling blight is characterized by decay of the seed and radicle, including reduced emergence, shorter shoot and root length, and lower dry weight of seedlings (Munkvold and O'Mara, 2002). Although treatment can reduce seedling blight, it does not protect against head blight (McMullen et al.,

1997). Difenoconazole is effective against seed decay and seed weight and fludioxonil helped improve emergence (Munkvold and O'Mara, 2002).

FHB mycelium and chlamydospores overwinter in crop residue (Parry et al., 1995), therefore rotations with non-host crops such as soybeans may reduce FHB levels in wheat or barley (McMullen et al., 1997). Dill-Macky and Jones (2000) reported that in a wheat-soybean rotation, there was 25% and 50% less DON than a wheat-wheat rotation and a wheat-corn rotation, respectively. In addition to crop rotations, tillage can be an effective way to reduce the impact of overwintering *Fusarium* spores (Dill-Macky and Jones, 2000). Tillage reduces the availability of contaminated crop debris by mixing the top layer of soil (Edwards, 2004); however, many soil conservation practices include reducing tillage (Bai and Shaner, 2004).

Planting resistant cultivars of wheat and barley can be one of the most effective ways to reduce FHB and DON. Mesterhazy et al. (1999) demonstrated that resistant wheat cultivars showed lower FHB symptoms and lower accumulation of DON than susceptible genotypes. Further studies have indicated that resistant cultivars reduce the occurrence of severe FHB epidemics (Hongxiang et al., 2008). Tall genotypes and awnless types tend to be more resistant than dwarfs and awn-having types (Mesterhazy, 1987; Mesterhazy, 1995) and should be considered when choosing cultivars. Barley has natural resistance to spread (Jansen et al., 2005) bus not initial infection (Type I resistance). Barley breeding programs in the U.S should be geared towards increasing type I resistance (Foroud and Eudes, 2009). However, farmers tend to choose barley cultivars that have good agronomic traits which often are not the most resistant to FHB (Foroud and Eudes, 2009).

Cereal grains are most susceptible to head blight during head emergence and anthesis (Bai and Shaner, 2004). Planting cultivars having different flower timing, may reduce the chance that the entire crop will be diseased (De Wolf, 2003).

Use of FHB prediction tools may help growers prepare for heavy disease years by employing appropriate strategies, as mentioned above. In a 2009 survey, 77% of FHB prediction tool users applied the information to their farm practices and 92% considered the information valuable (De Wolf et al., 2009). In a 2009 study involving the Dutch winter wheat, a model was developed to predict DON levels (Franz et al., 2009). The model was based on agronomic and climatic variables and was found to be highly predictive, with an R^2 of 0.59 between observed and predicted values (Franz et al., 2009).

Lastly, the use of fungicides to control FHB is an effective, yet costly method to reduce disease (Bai and Shaner, 2004). Jones (2000) conducted a series of field experiments between 1994 and 1997 to test the effectiveness of select fungicides. For example, the fungicides benomyl and tebuconazole reduced head blight incidence, FHB severity, scabby kernels in harvested wheat kernels, and DON levels (Jones, 2000). In barley, fludioxonil reduced FHB incidence, severity, and DON concentration (Jones, 2000). Use of fungicides in wheat and barley also increased yields (Jones, 2000). Although these fungicides appear effective, one must weigh the cost of the applied fungicide versus the dangers of the mycotoxin. Also, some fungicides, such as azoxystrobin, may actually increase mycotoxin production in *Fusarium* (D'Mello et al., 2001).

Post-harvest strategies can also be employed to reduce mycotoxin contamination in grain. For example, maintaining a dry environment in storage may prevent additional fungal growth and mycotoxin production (Kabak et al., 2006). Other examples to reduce the effects of

mycotoxins in grain include blending clean grain or dried distillers grains with solubles (DDGS) with contaminated material (Binder, 2007). Stepanik et al., (2007) demonstrated the potential for electron beam irradiation to reduce DON in contaminated grain (Stepanik et al., 2007). Others have studied the use of adsorbants to bind mycotoxins (Diaz-Llano and Smith, 2006), washing contaminated grain with water (Trenholm et al., 1992), or the use of fungistats to prevent fungal growth in storage (Magan and Aldred, 2007). Many of these strategies are likely to incur high equipment and labor costs. New cost-effective and commercially viable methods to reduce mycotoxin contamination in DDGS need to be developed and implemented.

RESEARCH OBJECTIVES

My graduate research has been interdisciplinary, ranging from molecular biology to plant pathology to fuel ethanol production, and has included collaborations with the USDA and the University of Wisconsin. This research was centered on Fusarium head blight (FHB) caused by the fungal plant pathogen *Fusarium graminearum*. In particular, I worked on solutions to mitigate a dangerous mycotoxin released by this fungus known as deoxynivalenol (DON), which contaminates wheat and barley. A barley derived co-product, remaining after fuel ethanol production, called dried distillers grains with solubles (DDGS) is increasingly being used as a feed source for domestic animals. My project was designed to solve this contamination problem (**Figure 4)** and was addressed in the following objectives:

1. **Investigate the detoxification efficiency of seven different variants of resistance genes, isolated from seven different *Fusarium* species, which encode trichothecene 3-*O*-acetyltransferases.** A series of DON feeding assays with yeast and *in vitro* assays were conducted to determine the steady-state kinetic profiles.

2. **Reduce DON during barley ethanol fermentation**. Transformed yeast expressing two different trichothecene 3-*O*-acetyltransferases (FgTRI101 and FfTRI201) were used in a series of small-scale ethanol fermentations.

3. **Determine the natural DON and FHB resistance of Virginia hulled and hulless barley lines/cultivars.** Genotypes were identified that are resistant to DON and FHB

(Severity, Incidence, and Index), and correlations between DON levels and FHB were determined.

4. **Towards the development of transgenic hulless barley lines expressing a trichothecene 3-*O*-acetyltranserase**. Forty-seven barley genotypes were evaluated for their abilities to develop callus. Transformed barley plants harboring *TRI101* were not identified.

FIGURES

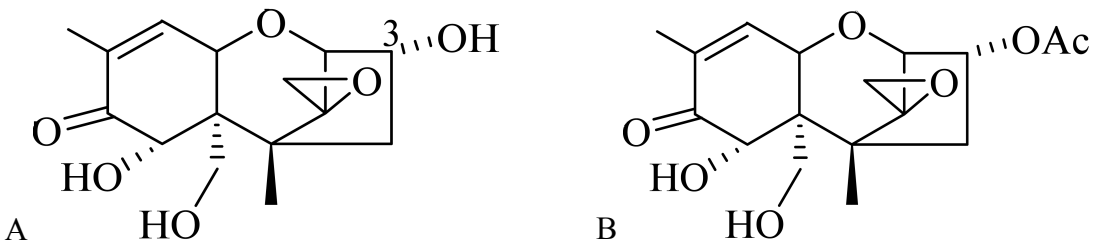

Figure 1. Structure of (A) deoxynivalenol and (B) 3-acetyl deoxynivalenol

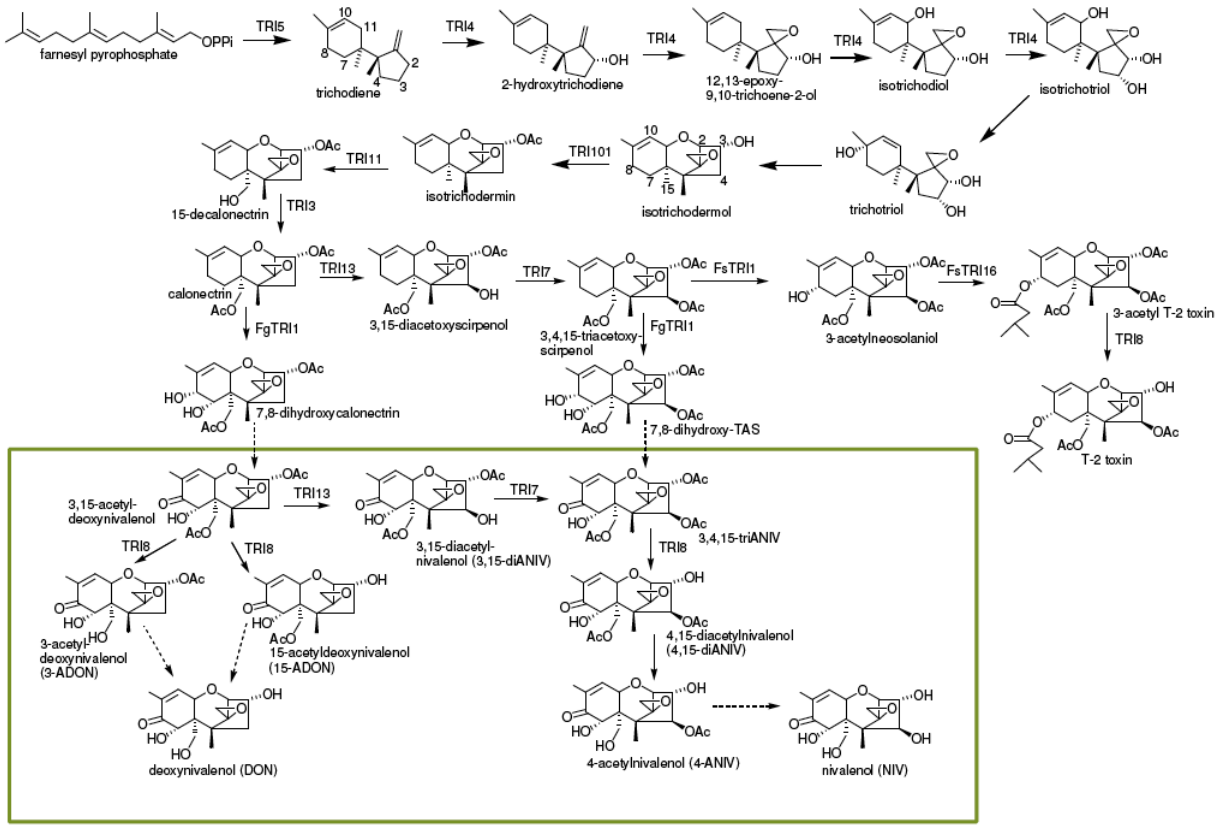

Figure 2. Proposed deoxynivalenol (DON) biosynthetic pathway. The box represents Type B trichothecenes. Figure taken from McCormick, Stanley, Stover, and Alexander (2011).

Permission to use this figure can be found at the following website:
http://www.mdpi.com/about/openaccess/

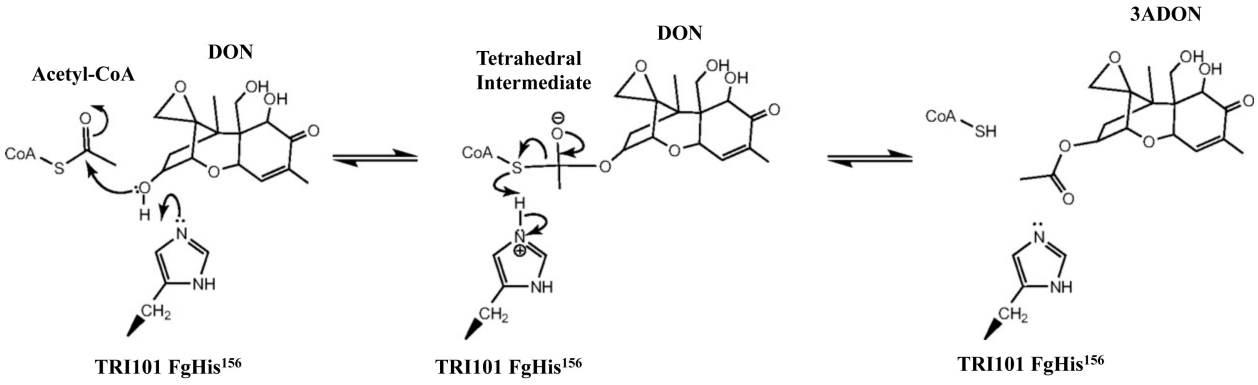

Figure 3. Proposed mechanism of TRI101 mediated DON acetylation. Acetylation of deoxynivalenol (DON) to 3-acetyl-deoxynivalenol (3-ADON). TRI101 binds both DON and the acetyl group of acetyl-CoA. The catalytic histidine of TRI101, $FgHis^{156}$, removes a proton from the C3 hydroxyl of DON. Deprotonated DON then nucleophilically attacks acetyl-CoA. This results in the transfer of the acetyl group to the C3 hydroxyl moiety of deoxynivalenol to form 3-ADON. Figure adapted from Garvey, McCormick, and Rayment (2008).

Permission to use this figure can be found at the following website:
http://www.jbc.org/site/misc/Copyright_Permission.xhtml

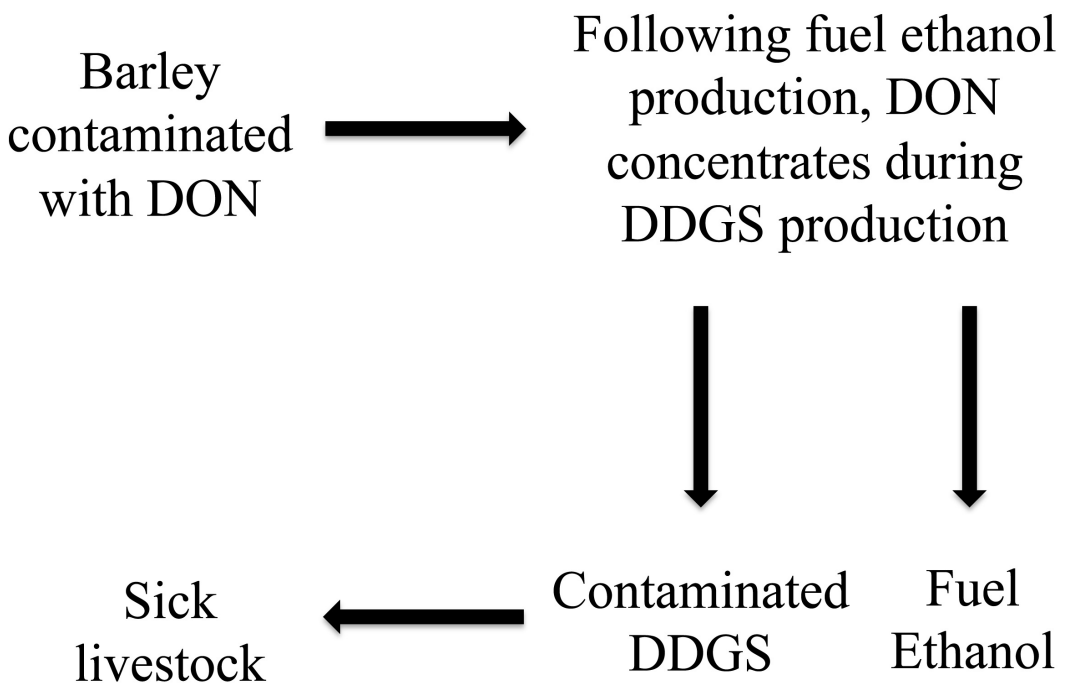

Figure 4. Flow chart of problem this project aims to solve. Mycotoxin contaminated fuel ethanol co-products (DDGS) fed to domestic animals may result in severe illnesses i.e. feed refusal and vomiting.

LITERATURE CITED

Alexander N.J., McCormick S.P., Hohn T.M. (2002) The identification of the Saccharomyces cerevisiae gene AYT1(ORF-YLL063c) encoding an acetyltransferase. Yeast 19:1425-30.

Alexander N.J., McCormick S.P., Ziegenhorn S.L. (1999) Phytotoxicity of Selected Trichothecenes using *Chlamydomonas reinhardtii* as a Model System. Natural Toxins:265-269.

Anderson J.W., Baird P., Davis R.H., Jr., Ferreri S., Knudtson M., Koraym A., Waters V., Williams C.L. (2009) Health benefits of dietary fiber. Nutr Rev 67:188-205.

Andersson A.A., Aman P. (2008) Functional barley products Woodhead Publishing, Cambridge, U.K.

Andersson A.A., Lampi A.M., Nystrom L., Piironen V., Li L., Ward J.L., Gebruers K., Courtin C.M., Delcour J.A., Boros D., Fras A., Dynkowska W., Rakszegi M., Bedo Z., Shewry P.R., Aman P. (2008) Phytochemical and dietary fiber components in barley varieties in the HEALTHGRAIN Diversity Screen. J Agric Food Chem 56:9767-76.

Bai G., Shaner G. (2004) Management and resistance in wheat and barley to fusarium head blight. Annu Rev Phytopathol 42:135-61.

Baik B.-K., Ullrich S.E. (2008) Barley for food: Characterstics, improvements, and renewed interest. Journal of Cereal Science 48:233-242.

Balat M., Balat H., Oz C. (2008) Progress in Bioethanol Processing. Progress in Energy and Combustion Science:551-573.

Bamburg J.R., Strong F.M., Smalley E.B. (1969) Toxins from Moldy Cereals. Journal of Agriculture and Food Chemistry 17.

Binder E.M. (2007) Managing the risk of mycotoxins inmodern feed production. Animal feed science and technology 133:149-166.

Boddu J., Cho S., Muehlbauer G.J. (2007) Transcriptome analysis of trichothecene-induced gene expression in barley. Mol Plant Microbe Interact 20:1364-75.

Bondy G.S., McCormick S.P., Beremand M.N., Pestka J.J. (1991) Murine lymphocyte proliferation impaired by substituted neosolaniols and calonectrins--Fusarium metabolites associated with trichothecene biosynthesis. Toxicon : official journal of the International Society on Toxinology 29:1107-13.

Bothmer R., Hintum T., Knupffer H., Sato K. (2003) Diversity in barley Elsevier Science, Amsterdam, The Netherlands.

Brooks W., Vaughn M.E., Griffey C., Price A.M., Pridgen T.H., Rohrer W.L., Brann D.E., Rucker E.G., Behl H.D., Sisson W.L., Corbin R.A., Kenner J.C., Dunaway D.W., Pitman R.M., Premakumar R., Livingston D.P., Vivar H.E., Paris R.L. (2005) Registration of 'Doyce' Hulless Barley. Crop Science 45:792-293.

Brown N.A., Urban M., van de Meene A.M., Hammond-Kosack K.E. (2010) The infection biology of Fusarium graminearum: defining the pathways of spikelet to spikelet colonisation in wheat ears. Fungal Biol 114:555-71.

Chang D.C., Grant G.B., O'Donnell K., Wannemuehler K.A., Noble-Wang J., Rao C.Y., Jacobson L.M., Crowell C.S., Sneed R.S., Lewis F.M., Schaffzin J.K., Kainer M.A., Genese C.A., Alfonso E.C., Jones D.B., Srinivasan A., Fridkin S.K., Park B.J. (2006) Multistate outbreak of Fusarium keratitis associated with use of a contact lens solution. JAMA 296:953-63.

Chen X., Steed A., Harden C., Nicholson P. (2006) Characterization of Arabidopsis thaliana-Fusarium interactions and identification of variation in resistance among ecotypes. Molecular Plant Pathology 7:391-403.

Chesapeake-Bay-Commission. (2007) Biofuels and the Bay: Getting it Right to Benefit Farms, Forests, and the Chesapeake.

Cockram J., Chiapparino E., Taylor S.A., Stamati K., Donini P., Laurie D.A., O'Sullivan D M. (2007) Haplotype analysis of vernalization loci in European barley germplasm reveals novel VRN-H1 alleles and a predominant winter VRN-H1/VRN-H2 multi-locus haplotype. Theor Appl Genet 115:993-1001.

Cuomo C.A., Guldener U., Xu J.R., Trail F., Turgeon B.G., Di Pietro A., Walton J.D., Ma L.J., Baker S.E., Rep M., Adam G., Antoniw J., Baldwin T., Calvo S., Chang Y.L., Decaprio D., Gale L.R., Gnerre S., Goswami R.S., Hammond-Kosack K., Harris L.J., Hilburn K., Kennell J.C., Kroken S., Magnuson J.K., Mannhaupt G., Mauceli E., Mewes H.W., Mitterbauer R., Muehlbauer G., Munsterkotter M., Nelson D., O'Donnell K., Ouellet T.,

Qi W., Quesneville H., Roncero M.I., Seong K.Y., Tetko I.V., Urban M., Waalwijk C., Ward T.J., Yao J., Birren B.W., Kistler H.C. (2007) The Fusarium graminearum genome reveals a link between localized polymorphism and pathogen specialization. Science 317:1400-2.

D'Mello J.P.F., Macdonald A.M.C., Rinna R. (2001) Effects of azoxystrobin on mycotoxin production in carbendazim-resistant strain of *Fusarium sporotrichioides*. Phytoparasitica 29:431-440.

De Wolf E. (2003) Fusarium Head Blight. Penn State Agricultural Research and Cooperative Extension.

De Wolf E., Knight P., Miller D., Paul P., Madden L. (2009) Evaluating the Use and Potential Impacts of Fusarium Head Blight Prediction Models in the U.S, Proceedings of the 2009 National Fusarium Head Blight Forum, Orlando, Fl.

Desjardins A.E. (2003) Gibberella from A (venaceae) to Z (eae). Annu Rev Phytopathol 41:177-98.

Desjardins A.E. (2006) *Fusarium* Mycotoxins: Chemistry, Genetics, and Biology American Phytopathological Society Press, St. Paul, Minnesota.

Desjardins A.E., Hohn T.M., McCormick S.P. (1993) Trichothecene biosynthesis in Fusarium species: chemistry, genetics, and significance. Microbiol Rev 57:595-604.

Desjardins A.E., McCormick S.P., Appell M. (2007) Structure-Activity Relationships of Trichothecene Toxins in an *Arabidopsis thaliana* Leaf Assay. Journal of Agriculture and Food Chemistry 55:6487-6492.

Dias De Oliveira M.E., Vaughan B.E., Rykiel E.J. (2005) Ethanol as Fuel: Energy, Carbon Dioxide Balances, and Ecological Footprint. BioScience 55.

Diaz-Llano G., Smith T.K. (2006) Effects of feeding grains naturally contaminated with Fusarium mycotoxins with and without a polymeric glucomannan mycotoxin adsorbent on reproductive performance and serum chemistry of pregnant gilts. J Anim Sci 84:2361-6.

Dill-Macky R., Jones R.K. (2000) The Effect of Previous Crop Residues and Tillage on Fusarium Head Blight of Wheat. Plant Disease 84:71-76.

Drapcho C.M., Nghiem N.P., Walker T.H. (2008) Biofuels engineering process technology McGraw-Hill.

Edwards S.G. (2004) Influence of agricultural practices on fusarium infection of cereals and subsequent contamination of grain by trichothecene mycotoxins. Toxicol Lett 153:29-35.

Eriksen G.S., Pettersson H., Lundh T. (2004) Comparatie cytotoxicity of deoxynivalenol, nivalenol, their acetylated derivatives and de-epoxy metabolites. Food and Chemical Toxicology 42:619-624.

Etienne M., Jemmali M. (1982) Effects of zearalenone (F2) on estrous activity and reproduction in gilts. J Anim Sci 55:1-10.

Eudes F., Comeau A., Rioux S., Colllin J. (2000) Phytotoxicite de huit mycotoxines associees a la fusariose de l'epi chez le ble. Canadian Journal of Plant Pathology 23:318-322.

Evans C.K., Xie W., Dill-Macky R., Mirocha C.J. (2000) Biosynthesis of Deoxynivalenol in Spikelets of Barley Inoculated with Macroconidia of Fusarium graminearum. Plant Disease 84.

Evans J., Levesque D., de Lahunta A., Jensen H.E. (2004) Intracranial fusariosis: a novel cause of fungal meningoencephalitis in a dog. Vet Pathol 41:510-4.

Fernando W.G.D., Miller J.D., Seaman W.L., Seifert K., Paulitz T.C. (2000) Daily and seasonal dynamics of airborne spores of *Fusarium graminearum* and other *Fusarium* species sampled over wheat plots. Canadian Journal of Botany 78:497-505.

Fincher G.B. (1975) Morphology and chemical composition of barley endosperm cell walls. Journal of the Institute of Brewing 81:116-122.

Foroud N.A., Eudes F. (2009) Trichothecenes in cereal grains. Int J Mol Sci 10:147-73.

Franz E., Booij K., van der Fels-Klerx I. (2009) Prediction of deoxynivalenol content in Dutch winter wheat. J Food Prot 72:2170-7.

Garvey G.S., McCormick S.P., Rayment I. (2008) Structural and functional characterization of the TRI101 trichothecene 3-O-acetyltransferase from Fusarium sporotrichioides and Fusarium graminearum: kinetic insights to combating Fusarium head blight. J Biol Chem 283:1660-9.

Goswami R.S., Kistler H.C. (2004) Heading for disaster: *Fusarium graminearum* on cereal crops. Molecular Plant Pathology 5:515-525.

Griffey C., Brooks W., Kurantz M., Thomason W., Taylor F., Obert D., Moreau R., Flores R., Sohn M., Hicks K. (2010) Grain Composition of Virginia winter barley and implications for use in feed, food, and biofuels production. Journal of Cereal Science 51:41-49.

Harari E.B. (2007) Bioethanol and Technological Innovation Systems. A comparative analysis between U.S. and Brazil. Science, Technology, and Innovation Policy:1-3.

Helliwell C.A., Wood C.C., Robertson M., James Peacock W., Dennis E.S. (2006) The Arabidopsis FLC protein interacts directly in vivo with SOC1 and FT chromatin and is part of a high-molecular-weight protein complex. Plant J 46:183-92.

Hicks K.B., Flores R.A., Taylor F., McAloon A.J., Moreau R., Johnston D., Senske G., Brooks W., Griffey C. (2005) Hulless Barley-A New Feedstock for Fuel Ethanol?, 15th EPAC Meeting.

Hongxiang M., Yao J., Zhou M., Zhang X., Ren L., Yu G., Lu W. (2008) Molecular breeding for wheat fusarium head blight resistance in China. Cereal Resistance 36:1-730.

Huth M., Dongowski G., Gebhardt E., Flamme W. (2000) Functional Properties of Dietary Fibre Enriched Extrudates from Barley. Journal of Cereal Science 32:115-128.

Ishii K., Kobayashi J., Ueno Y., Ichinoe M. (1986) Occurrence of Trichothecin in Wheat. Appl Environ Microbiol 52:331-333.

Jansen C., von Wettstein D., Schafer W., Kogel K.H., Felk A., Maier F.J. (2005) Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted Fusarium graminearum. Proc Natl Acad Sci U S A 102:16892-7.

Jones R.K. (2000) Assessments of Fusarium Head Blight of Wheat and Barley in Response to Fungicide Treatment. Plant Disease 84:1021-1030.

Kabak B., Dobson A.D., Var I. (2006) Strategies to prevent mycotoxin contamination of food and animal feed: a review. Crit Rev Food Sci Nutr 46:593-619.

Kang Z., Buchenauer H. (2000) Ultrastructural and Cytochemical Studies on Cellulose, Xylan and Pectin Degradation in Wheat Spikes Infected by *Fusarium culmorum*. J. Phytopathology 148:263-275.

Khatibi P.A., Newmister S.A., Rayment I., McCormick S.P., Alexander N.J., Schmale D.G., 3rd. (2011) Bioprospecting for trichothecene 3-o-acetyltransferases in the fungal genus fusarium yields functional enzymes with different abilities to modify the mycotoxin deoxynivalenol. Appl Environ Microbiol 77:1162-70.

Kimura M., Kaneko I., Komiyama M., Takatsuki A., Koshino H., Yoneyama K., Yamaguchi I. (1998a) Trichothecene 3-O-acetyltransferase protects both the producing organism and transformed yeast from related mycotoxins. Cloning and characterization of Tri101. J Biol Chem 273:1654-61.

Kimura M., Matsumoto G., Shingu Y., Yoneyama K., Yamaguchi I. (1998b) The mystery of the trichothecene 3-O-acetyltransferase gene. Analysis of the region around Tri101 and characterization of its homologue from Fusarium sporotrichioides. FEBS Lett 435:163-8.

Kimura M., Tokai T., Matsumoto G., Fujimura M., Hamamoto H., Yoneyama K., Shibata T., Yamaguchi I. (2003) Trichothecene nonproducer Gibberella species have both functional and nonfunctional 3-O-acetyltransferase genes. Genetics 163:677-84.

Kimura M., Tokai T., Takahashi-Ando N., Ohsato S., Fujimura M. (2007) Molecular and genetic studies of fusarium trichothecene biosynthesis: pathways, genes, and evolution. Biosci Biotechnol Biochem 71:2105-23.

Komatsuda T., Pourkheirandish M., He C., Azhaguvel P., Kanamori H., Perovic D., Stein N., Graner A., Wicker T., Tagiri A., Lundqvist U., Fujimura T., Matsuoka M., Matsumoto T., Yano M. (2007) Six-rowed barley originated from a mutation in a homeodomain-leucine zipper I-class homeobox gene. Proc Natl Acad Sci U S A 104:1424-9.

Lagopodi A.L., Ram A.F., Lamers G.E., Punt P.J., Van den Hondel C.A., Lugtenberg B.J., Bloemberg G.V. (2002) Novel aspects of tomato root colonization and infection by Fusarium oxysporum f. sp. radicis-lycopersici revealed by confocal laser scanning microscopic analysis using the green fluorescent protein as a marker. Mol Plant Microbe Interact 15:172-9.

Land C.J., Lundstrom H., Werner S. (1993) Production of tremorgenic mycotoxins by isolates of Aspergillus fumigatus from sawmills in Sweden. Mycopathologia 124:87-93.

Lange B.M., Rujan T., Martin W., Croteau R. (2000) Isoprenoid biosynthesis: the evolution of two ancient and distinct pathways across genomes. Proc Natl Acad Sci U S A 97:13172-7.

Leslie J.F., Summerell B.A. (2006) The Fusarium Laboratory Manual Blackwell Publishing, Ames, Iowa.

Madson P.W., Monceaux D.A. (1995) Fuel ethanol production KATZEN International Inc, Cincinnati, Ohio.

Magan N. (1988) Fungal Colonization and Decomposition of Cereal Straw. International Biodeterioration 24:435-443.

Magan N., Aldred D. (2007) Post-harvest control strategies: minimizing mycotoxins in the food chain. Int J Food Microbiol 119:131-9.

Manuel J. (2007) Battle of the Biofuels. Environmental Health Perspectives 115.

Marasas W.F. (1995) Fumonisins: Their Implications for Human and Animal Health. Natural Toxins 3:193-198.

Marasas W.F. (2001) Discovery and occurrence of the fumonisins: a historical perspective. Environ Health Perspect 109 Suppl 2:239-43.

Markell S.G., Francl L.J. (2003) Fusarium Head Blight Inoculum: Species Prevalence and *Gibberella zeae* Spore Type. Plant Disease 87.

McCormick S.P., Alexander N.J., Trapp S.E., Hohn T.M. (1999) Disruption of TRI101, the gene encoding trichothecene 3-O-acetyltransferase, from Fusarium sporotrichioides. Appl Environ Microbiol 65:5252-6.

McCormick S.P., Stanley A.M., Stover N.A., Alexander N.J. (2011) Trichothecene: From simple to complex mycotoxins. Toxins 3:802-814.

McLaughlin J.E., Bin-Umer M.A., Tortora A., Mendez N., McCormick S., Tumer N.E. (2009) A genome-wide screen in Saccharomyces cerevisiae reveals a critical role for the mitochondria in the toxicity of a trichothecene mycotoxin. Proc Natl Acad Sci U S A 106:21883-8.

McMullen M., Jones R., Gallenberg D. (1997) Scab of Wheat and Barley: A Re-emerging Disease of Devastating Impact. Plant Disease 81.

Mesterhazy A. (1987) Selection of head blight resistant wheat through improved seedling resistance. Plant Breeding 98:25-36.

Mesterhazy A. (1995) Types and components of resistance against *Fusarium* head blight of wheat. Plant Breeding 114:377-386.

Mesterhazy A. (2002) Role of deoxynivalenol in aggressiveness of *Fusarium graminearum* and *F. culmorum* and in resistance to *Fusarium* head blight. European Journal of Plant Pathology 108:675-684.

Michaels S.D., Amasino R.M. (1999) FLOWERING LOCUS C encodes a novel MADS domain protein that acts as a repressor of flowering. Plant Cell 11:949-56.

Mitterbauer R., Poppenberger B., Raditschnig A., Lucyshyn D., Lemmens M., Glossl J., Adam G. (2004) Toxin-dependent utilization of engineered ribosomal protein L3 limits trichothecene resistance in transgenic plants. Plant Biotechnol J 2:329-40.

Munkvold G.P., O'Mara J.K. (2002) Laboratory and Growth Chamber Evaluation of Fungicidal Seed Treatments for Maize Seedling Blight Caused by *Fusarium* Species. Plant Disease 86:143-150.

Nelson P.E., Dignani M.C., Anaissie E.J. (1994) Taxonomy, biology, and clinical aspects of Fusarium species. Clin Microbiol Rev 7:479-504.

Newman C.W., Newman R.K. (2006) A Brief History of Barley Foods. Cereal Foods World 51.

Nganje W.E., Johnson D.D., Wilson W.W., Leistritz F.L., Bangsund D.A., Tiapo N.M. (2001) Economic Impacts of Fusarium Head Blight in Wheat and Barley: 1998-2000 North Dakota State University, Fargo, ND.

Nghiem N., Hicks K., Johnston D., Senske G., Kurantz M., Li M., Shetty J., Konieczny-Janda G. (2010) Production of ethanol from winter barley by the EDGE (enhanced dry grind enzymatic) process. Biotechnol Biofuels 3:8.

Nucci M., Anaissie E. (2007) Fusarium infections in immunocompromised patients. Clin Microbiol Rev 20:695-704.

Oliver S.N., Finnegan E.J., Dennis E.S., Peacock W.J., Trevaskis B. (2009) Vernalization-induced flowering in cereals is associated with changes in histone methylation at the VERNALIZATION1 gene. Proc Natl Acad Sci U S A 106:8386-91.

Parry D., Jenkinson P., McLeod L. (1995) Fusarium ear blight (scab) in small grain cereals-a review. Plant Pathology 44:207-238.

Peska J.J. (2007) Deoxynivalenol: toxicity, mechanisms and animal health risks. Animal feed science and technology 137:283-298.

Pestka J.J., Smolinski A.T. (2005) Deoxynivalenol: toxicology and potential effects on humans. J Toxicol Environ Health B Crit Rev 8:39-69.

Poppenberger B., Berthiller F., Lucyshyn D., Sieberer T., Schuhmacher R., Krska R., Kuchler K., Glossl J., Luschnig C., Adam G. (2003) Detoxification of the Fusarium mycotoxin deoxynivalenol by a UDP-glucosyltransferase from Arabidopsis thaliana. J Biol Chem 278:47905-14.

Pourkheirandish M., Komatsuda T. (2007) The importance of barley genetics and domestication in a global perspective. Ann Bot 100:999-1008.

Proctor R.H., Hohn T.M., McCormick S.P. (1995) Reduced virulence of Gibberella zeae caused by disruption of a trichothecene toxin biosynthetic gene. Mol Plant Microbe Interact 8:593-601.

Renneberg R. (2008) Biotechnology for Beginners Elsevier, Munich, GA.

Rizkalla S.W., Bellisle F., Slama G. (2002) Health benefits of low glycaemic index foods, such as pulses, in diabetic patients and healthy individuals. Br J Nutr 88 Suppl 3:S255-62.

Salas B., Steffenson B.J., Casper H.H., Tacke B., Prom L.K., Fetch Jr. T.G., Schwarz P.B. (1999) *Fusarium* Species Pathogenic to Barley and Their Associated Mycotoxins. Plant Disease 83.

Sasani S., Hemming M.N., Oliver S.N., Greenup A., Tavakkol-Afshari R., Mahfoozi S., Poustini K., Sharifi H.R., Dennis E.S., Peacock W.J., Trevaskis B. (2009) The influence of vernalization and daylength on expression of flowering-time genes in the shoot apex and leaves of barley (Hordeum vulgare). J Exp Bot 60:2169-78.

Schill S.R. (2008) Beefing Up Barley, Ethanol Producer Magazine.

Schmitz R.J., Amasino R.M. (2007) Vernalization: a model for investigating epigenetics and eukaryotic gene regulation in plants. Biochim Biophys Acta 1769:269-75.

Scientific Committee on Food. (1999) Opinion on *Fusarium* toxins. Part 1: Deoxynivalenol (DON). European Commission: Health & Consumer Protection Directorate-General.

Snijders C.H.A. (1990) Fusarium head blight and mycotoxin contamination of wheat, a review. Netherlands Journal of Plant Pathology 96:187-198.

Sohn M., Himmelsbach D.S., Barton F.E., 2nd, Griffey C.A., Brooks W., Hicks K.B. (2007) Near-infrared analysis of ground barley for use as a feedstock for fuel ethanol production. Appl Spectrosc 61:1178-83.

Sorenson W.G., Frazer D.G., Jarvis B.B., Simpson J., Robinson V.A. (1987) Trichothecene mycotoxins in aerosolized conidia of Stachybotrys atra. Appl Environ Microbiol 53:1370-5.

Stack R.W. (1989) A comparison of the inoculum potential of ascospores and conidia of *Gibberella zeae*. Canadian Journal of Plant Pathology 11:137-142.

Stepanik T., Kost D., Nowicki T., Gaba D. (2007) Effects of electron beam irradiation on deoxynivalenol levels in distillers dried grain and solubles and in production intermediates. Food Addit Contam 24:1001-6.

Sunder S. (1998) Vegetative compatibility, biosynthesis of GA3 and virulence of *Fusarium moniliforme* isolates from bakanae disease of rice. Plant Pathology 47:767-772.

Takahashi R., Hayashi S., Hirua U., Yasuda S. (1968) A study of cultivated barleys from Nepal, Himalaya and North India with special reference to their phylogenetic differentiation. Bericht des Ohara Instituts fur Landwirtschaftliche Biologie 14:85-122.

Takahashi R., Hayashi S., Yasuda S., Hiura U. (1963) Chatacteristics of the wild and cultivated barleys from Afghanistan and its neighbouring regions. Bericht des Ohara Instituts fur Landwirtschaftliche Biologie 12:1-23.

Taketa S., Amano S., Tsujino Y., Sato T., Saisho D., Kakeda K., Nomura M., Suzuki T., Matsumoto T., Sato K., Kanamori H., Kawasaki S., Takeda K. (2008) Barley grain with adhering hulls is controlled by an ERF family transcription factor gene regulating a lipid biosynthesis pathway. Proc Natl Acad Sci U S A 105:4062-7.

Thompson W.L., Wannemacher R.W., Jr. (1986) Structure-function relationships of 12,13-epoxytrichothecene mycotoxins in cell culture: comparison to whole animal lethality. Toxicon : official journal of the International Society on Toxinology 24:985-94.

Tokai T., Fujimura M., Inoue H., Aoki T., Ohta K., Shibata T., Yamaguchi I., Kimura M. (2005) Concordant evolution of trichothecene 3-O-acetyltransferase and an rDNA species phylogeny of trichothecene-producing and non-producing fusaria and other ascomycetous fungi. Microbiology 151:509-19.

Trail F., Xu H., Loranger R., Gadoury D. (2002) Physiological and environmental aspects of ascospore discharge in Gibberella zeae (anamorph Fusarium graminearum). Mycologia 94:181-9.

Trenholm H.L., Charmley L.L., Prelusky D.B., Warner R.M. (1992) Washing Procedures Using Water or Sodium Carbonate Solutions for the Decontamination of Three Cereals Contaminated with Deoxynivalenol and Zearalenone. Journal of Agriculture and Food Chemistry 40:2147-51.

Trevaskis B., Hemming M.N., Peacock W.J., Dennis E.S. (2006) HvVRN2 responds to daylength, whereas HvVRN1 is regulated by vernalization and developmental status. Plant Physiol 140:1397-405.

Turgeon B.G. (1998) Application of mating type gene technology to problems in fungal biology. Annu Rev Phytopathol 36:115-37.

Van Asch M.A.J., Rijkenberg F.H.J., Coutinho T.A. (1992) Phytotoxicity of fumonisin B1, moniliformin, and T-2 toxin to corn callus cultures. Phytopathology 82:1330-1332.

Voss K.A., Smith G.W., Haschek W.M. (2007) Fumonisins: Toxicokinetics, mechanism of action and toxicity. Animal feed science and technology 137:299-325.

Wegulo S.N., Baenziger P.S., Nelson L.A., Nopsa J.H., Millhouse J.C., Mengistu N., Breathnach-Stevens J. (2008) The 2008 Fusarium Head Blight Epidemic in Nebraska, United States Wheat and Barley Scab Initiative, Indianapolis, Indiana.

Wicklow D.T., Shotwell O.L. (1983) Intrafungal distribution of aflatoxins among conidia and sclerotia of Aspergillus flavus and Aspergillus parasiticus. Can J Microbiol 29:1-5.

Windels C.E. (2000) Economic and social impacts of fusarium head blight: changing farms and rural communities in the northern great plains. Phytopathology 90:17-21.

Wood P.J. (2007) Cereal β-glucans in diet and health. Journal of Cereal Science 46:230-238.

Zhang X., Li G., Ma J., Zeng Y., Ma W., Zhao P. (2010) Endophytic fungus Trichothecium roseum LZ93 antagonizing pathogenic fungi in vitro and its secondary metabolites. J Microbiol 48:784-90.

Zohary D. (1969) The domestication and exploitation of plants and animals Gerald Duckworth & Co., London.

Zohary D. (2004) Unconscious Selection and the Evolution of Domesticated Plants. Economic Botany 58:5-10.

Zohary D., Hopf M. (2000) Domestication of Plants in the Old World. Third ed. Oxford University Press Inc., Oxford, New York.

CHAPTER II

Bioprospecting for Trichothecene 3-*O*-Acetyltransferases in the Fungal Genus *Fusarium* Yields Functional Enzymes that Vary in their Ability to Modify the Mycotoxin Deoxynivalenol

Piyum A. Khatibi[1], Sean A. Newmister[2], Ivan Rayment[2], Susan P. McCormick[3], Nancy J. Alexander[3], and David G. Schmale III[1*]

Virginia Polytechnic Institute and State University, Department of Plant Pathology, Physiology, and Weed Science, Blacksburg, VA 24061[1], University of Wisconsin, Department of Biochemistry, Madison, Wisconsin 53706[2], Bacterial Foodborne Pathogen and Mycology Unit, USDA, ARS, National Center for Agricultural Utilization Research, Peoria, IL 61604[3]

[*]Corresponding author. Mailing address: Virginia Polytechnic Institute and State University, Department of Plant Pathology, Physiology, and Weed Science, 403 Latham Hall, Blacksburg, VA 24061. Phone: (540) 231-6943. Fax: (540) 231-7477. E-mail: dschmale@vt.edu.

Note: The following chapter was published in Applied and Environmental Microbiology (AEM) in February 2011. Copyright release information is located at the following web address: http://journals.asm.org/misc/ASM_Author_Statement.dtl

ATTRIBUTION

Piyum Khatibi was the lead writer on this manuscript and performed all work except for the enzyme kinetic work and thermostability measurements which was conducted by Sean A. Newmister. Sean contributed in both writing and experiments. He was responsible for the steady-state kinetics and thermostability measurements of the TRI101/TRI201 enzymes described in this manuscript. Ivan Rayment and David G. Schmale provided the laboratory and resources to perform all the experiments described in this manuscript. Susan P. McCormick and Nancy J. Alexander both contributed to the writing and improvement of the manuscript.

We thank J. Golin at the Catholic University of America for providing RW2802 for our transformations. We thank J. Leslie at Kansas State University for providing holotype strains of *Fusarium* from a teaching collection to screen for Tri101. We thank R. Anderson, M. White, and S. Yu for their technical expertise in support of this work.

This work was supported in part by the U.S. Department of Agriculture under agreement no. 59-0790-7-078 (D.G.S.) and 59-0790-6-066 (I.R.). This is a cooperative project with the U.S. Wheat & Barley Scab Initiative. Additional support was provided by grants through the Virginia Small Grains Board (grant 448226 to D.G.S.) and through the NIH (grant GM086351 to I.R.).

Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the view of the U.S. Department of Agriculture.

ABSTRACT

The trichothecene mycotoxin deoxynivalenol (DON) is a common contaminant of small grains, such as wheat and barley, in the United States. New strategies to mitigate the threat of DON need to be developed and implemented. TRI101 and TRI201 are trichothecene 3-*O*-acetyltransferases that are able to modify DON and reduce its toxicity. Recent work has highlighted differences in the activities of TRI101 from two different species of *Fusarium* (*F. graminearum* and *F. sporotrichioides*), but little is known about the relative activities of TRI101/TRI201 enzymes produced by other species of *Fusarium*. We cloned *TRI101* or *TRI201* genes from seven different species of *Fusarium* and found genetic identity between sequences ranging from 66% to 98%. *In vitro* feeding studies using transformed yeast showed that all of the TRI101/TRI201 enzymes tested were able to acetylate DON; conversion of DON to 3-acetyl-deoxynivalenol (3ADON) ranged from 50.5% to 100.0%, depending on the *Fusarium* species from which the gene originated. A time course assay showed that the rate of acetylation varied from species to species, with the gene from *F. sporotrichioides* having the lowest rate. Steady-state kinetic assays using seven purified enzymes produced catalytic efficiencies for DON acetylation ranging from from 6.8×10^4 $M^{-1} \cdot s^{-1}$ to 4.7×10^6 $M^{-1} \cdot s^{-1}$. Thermostability measurements for the seven orthologs ranged from 37.1°C to 43.2°C. Extended sequence analysis of portions of *TRI101/TRI201* from 31 species of *Fusarium* (including known trichothecene producers and nonproducers) suggested that other members of the genus may contain functional *TRI101/TRI201* genes, some with the potential to outperform those evaluated in the present study.

INTRODUCTION

Fusarium head blight (FHB), caused by the plant pathogen *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae*), is a detrimental disease to grains such as wheat and barley (Goswami and Kistler, 2004). The fungus is a major producer of deoxynivalenol (DON), a trichothecene mycotoxin that contaminates cereal crops and compromises the health of humans and domestic animals (Desjardins, 2006). DON is a known eukaryotic protein synthesis inhibitor (McLaughlin et al., 1977) and can cause vomiting, reproductive problems, feed refusal, and even death in mammals (Pestka and Smolinski, 2005). Its acetylated derivative 3-acetyl-deoxynivalenol (3ADON) has been shown to be equivalent to or lower than DON in toxicity based on 50% lethal dose (LD_{50}) values in mice (Peska, 2007) and 50% inhibitory concentrations (IC_{50}s) on rabbit reticulocytes (Kimura et al., 1998a).

Fusarium graminearum contains a gene called *TRI101* that encodes a trichothecene 3-*O*-acetyltransferase (Kimura et al., 1998a) that transfers an acetyl group from acetyl coenzyme A (acetyl-CoA) to the C-3 hydroxyl moiety of the trichothecene molecule (Garvey et al., 2008). This acetyltransferase reaction has been demonstrated to decrease the inhibitory effects of trichothecenes on the growth of *Saccharomyces pombe* (Kimura et al., 1998a), as well as that of *Chlamydomonas reinnardtii* and *Arabidopsis thaliana* (Alexander et al., 1999b; Desjardins et al., 2007). Disruption of *TRI101* in *F. sporotrichioides* (*FsTRI101*) blocked the production of T-2 toxin, a trichothecene-derived metabolite, and led to the accumulation of an intermediate, isotrichodermol, suggesting that *TRI101* is necessary for the production of trichothecenes (McCormick et al., 1999). Interestingly, in trichothecene-producing *Fusarium* spp., *TRI101* is usually not located near the trichothecene biosynthetic gene cluster (Kimura et al., 1998b; Proctor et al., 2009; Tokai et al., 2005). *TRI101* is located between the phosphate permease gene

(*PHO5)* and the UTP-ammonia ligase gene (*URA7*) in *F. graminearum* (Kimura et al., 1998b). In several trichothecene non-producing *Fusarium* species that have nonfunctioning TRI101s, C-3 acetyltransferase activity is still detected and is encoded by a gene named *TRI201* (Kimura et al., 2003). *TRI201* is believed to be the result of gene duplication (Tokai et al., 2005), with a different evolutionary history from that of other trichothecene biosynthesis genes.

Structural differences between *F. graminearum* TRI101 (FgTRI101) and FsTRI101 active sites demonstrated that FgTRI101 is better able to accommodate DON than is FsTRI101 (Garvey et al., 2008). In addition to structural differences, there are large kinetic differences between the two enzymes. The work by Garvey et al. (2008) showed that 85 times more DON substrate is required for FsTRI101 to reach the half-maximal rate than for FgTRI101. Likewise, the catalytic efficiency of FgTRI101 toward DON is considerably higher than that of FsTRI101 (Garvey et al., 2008).

TRI12, another important gene involved in trichothecene production, encodes a trichothecene efflux pump, an integral membrane protein responsible for exporting trichothecenes from hyphal cells (Alexander et al., 1999a). Alexander et al. (1999) suggested that the expression of an efflux pump in yeast could amplify trichothecene flux into yeast cells; consequently, we predicted that we would observe increased acetylation of DON in our expression studies. Our work confirmed the interaction of TRI12 with trichothecenes when the coexpression of *TRI101/TRI201* and *TRI12* increased the acetylation of DON in the yeast *Saccharomyces cerevisiae*.

F. sporotrichioides TRI101 has previously been transformed into several plant systems (Alexander, 2008). Experiments using tobacco showed that FsTRI101 reduced the phytotoxic effects of trichothecenes (Muhitch et al., 2000). Okubara et al. (2002) obtained four transgenic

wheat lines that accumulated *FsTRI101* transcripts; however, out of the four lines, only one showed partial resistance against the spread of *F. graminearum* in greenhouse assays. Ohsato et al. (2007) were the first to demonstrate DON acetylation in a cereal plant expressing TRI101 by determining DON resistance in rice root growth assays. Rice roots subjected to DON showed reduced growth, while roots expressing TRI101 were not inhibited and grew similarly to unexposed wild-type roots. Barley transformed with *FsTRI101* had reduced FHB in greenhouse assays, although field tests showed that transgenic plants were as susceptible to FHB as the controls (Manoharan et al., 2006). These noted works all illustrate the great potential of utilizing trichothecene acetyltransferase genes, such as *TRI101*, to detoxify DON. However, protection of cereal crops against DON may require the employment of a more effective and stable trichothecene acetyltransferase.

Our goal is to discover novel trichothecene acetyltransferases to facilitate the development of FHB-resistant cereal crops with reduced potential for DON contamination. As shown by Garvey et al. (2008), the large disparity in the ability of TRI101 enzymes from two different species of *Fusarium* to bind DON may be explained in part by large differences in kinetic profiles. We hypothesized (i) that both producers and nonproducers of DON in the fungal genus *Fusarium* contain functional *TRI101/TRI201* genes and (ii) that resulting TRI101/TRI201 enzymes vary in their ability to modify DON. Bioprospecting for functional trichothecene acetyltransferases in *Fusarium* species may yield more effective and efficient enzymes that may be used to enhance disease resistance in cereal crops and reduce the threat of DON contamination. Here, we demonstrate the use of yeast feeding assays to indicate the potential functionality of trichothecene 3-*O*-acetyltransferases from different *Fusarium* species and followed this work with enzyme kinetics.

METHODS AND MATERIALS

Yeast Strain. *S. cerevisiae* strain RW2802 (PDR5 *leu2 ura3-52 met5*) was used for all of the experiments (Meyers et al., 1992) and was kindly provided by Dr. J. Golin, The Catholic University, Washington, D.C..

Isolation of *TRI101*, *TRI201*, and *FsTRI12*. Since *TRI101* and *TRI201* do not contain introns, each *TRI101/TRI201* gene was amplified from the genomic DNA of the appropriate *Fusarium* species using primers specific for each species (**Table 1**). Entire genes were obtained from the Schmale (David Schmale, Virginia Polytechnic Institute and State University) and Leslie (John Leslie, Kansas State University) collections, which are designated with a 1 and 2, respectively in gene and protein designations hereinafter. Complete *TRI101* gene sequences were acquired from *F. crookwellense* strain 11451 (*Fcr2TRI101*), *F. culmorum* strain 11427 (*Fcu2TRI101*), *F. pseudograminearum* strain 11435 (*Fps2TRI101*), *F. graminearum* strain Z3639 *(Fg2TRI101), F. graminearum* strain T1S1 (*Fg1TRI101*), *F. sporotrichioides* strain 11552 (*Fs2TRI101*), and *F. sporotrichioides* strain F4n23 (*Fs1TRI101*). Complete *TRI201* sequences were acquired from *F. fujikuroi* strain C1994 (*Ff2TRI201*), *F. fujikuroi* strain F4n17 (*Ff1TRI201*), *F. oxysporum* strain 11390 (*Fo2TRI201*), and *F. oxysporum* strain F4n26 (*Fo1TRI201*). Pairwise comparisons of nucleotide identity and divergence between full-length *TRI101/TRI201* orthologs were conducted using MegAlign (Lasergene v8.1.1; DNAStar, Madison, WI). *Fusarium* genomic DNA was isolated by using the DNeasy Plant Mini Kit following the manufacturer's protocols (Qiagen, Germantown, MD). PCR amplifications were carried out in a 50-μl reaction volumes using Easy-A high-fidelity PCR cloning enzyme (Stratagene, La Jolla, CA) under the following

conditions: one cycle at 95°C for 2 min; 30 cycles of 95°C for 40 s, 54°C for 30 s, and 72°C for 1.5 min; and finally one cycle at 72°C for 7 min. *FsTRI12*, from *F. sporotrichioides* NRRL 3299, was amplified from the YIplac128 vector containing the *FsTRI12* gene. *FsTRI12* amplifications were carried out in 50-μl reaction volumes using Easy-A high-fidelity PCR cloning enzyme (Stratagene, La Jolla, CA) as described above, with an annealing temperature of 57°C and an extension time of 2 min.

Bioprospecting for trichothecene acetyltransferases in *Fusarium* species. *TRI101 or TRI201* sequences from 17 species of *Fusarium* were downloaded from GenBank and aligned using SeqMan Pro version 8.1 (Lasergene version 8.1.1; DNAstar, Madison, WI) (see Table S1 in the supplemental material). Degenerate primers were designed based on regions of homology among available partial *TRI101* and *TRI201* cDNA and known full-length cDNA of the *Fusarium* species listed in Table S1 in the supplemental material. Strains of *Fusarium* from the Leslie collection (see Table S2 in the supplemental material) were screened for the presence of an internal portion of *TRI101/TRI201* (893/890 bp) (Table 1, primers 11 and 12).

RW2802 Transformations. *TRI101/TRI201* genes isolated from both the Leslie and Schmale collections were transformed into *Saccharomyces cerevisiae* strain RW2802 (see Table S3 in the supplemental material). *TRI101/TRI201* genes were cloned into Invitrogen's yeast expression vector pYES2.1 (Invitrogen, Carlsbad, CA) **(Table 1**). The *F. sporotrichioides TRI12* gene (*FsTRI12*) was amplified from its vector (Alexander et al., 1999a) and cut with restriction enzymes BamHI and SalHI obtained from New England Biolabs (NEB, Ipswich, MA), and ligated into Stratagene's pESC-LEU vector. Digestions were performed at 37°C for 1 h, and

ligations were performed at 16°C for 2 h using NEB T4 DNA ligase (NEB, Ipswich, MA). Both vectors contain the GAL1 promoter and the CYC termination sequence, allowing induced expression by the addition of galactose to the liquid medium. All vector-gene combinations were first transformed into TOP10 chemically competent *Escherichia coli* cells by following Invitrogen's One Shot TOP10 chemical transformation protocol. All yeast transformations were conducted by following Invitrogen's small-scale yeast transformation protocol. Yeast transformations were performed for each *TRI101/TRI201* gene, and one colony was chosen from each transformation reaction mixture for the yeast assay. Double yeast transformants carrying both *TRI101/TRI201* and *TRI12* were made by simultaneous transformation of RW2802 using both plasmids, pTRI101YES and pTRI12ESC, and plating onto the appropriate selective medium lacking both uracil and leucine.

Yeast media. Yeast extract-peptone-dextrose (YPD) medium [1% yeast extract, 2% peptone, and 2% dextrose (D-glucose)] was used to grow untransformed *S. cerevisiae* strain RW2802. Transformed *S. cerevisiae* lines were grown on synthetic drop-out medium containing yeast nitrogen base without amino acids (0.67%), 2% glucose, and supplemented with amino acids. The drop-out medium without leucine contained all standard amino acids (76 mg/L final) except leucine (Sigma, St. Louis, MO). The drop-out medium without uracil contained all standard amino acids (76 mg/L final) plus leucine (380mg/L final) and lacked uracil (Sigma, St. Louis, MO). The drop-out medium without uracil and leucine contained all standard amino acids (76 mg/L final) except leucine and uracil (Sigma, St. Louis, MO). The induction medium was similar to synthetic drop-out medium except that glucose was supplemented with filter-sterilized galactose (final concentration of 2%) and raffinose (final concentration 1%). Glucose and

raffinose were filter-sterilized using the Millex GP 0.22-μm filter unit (SLGP033RS; Millipore, Billerica, MA) and galactose was filter sterilized using a 0.45-μm filter unit.

Acetylation assays with TRI101/TRI201 constructs. Liquid drop-out medium, containing the appropriate selective amino acids, was inoculated with transformed yeast cells from a plate and grown at 28°C overnight until an optical density at 600 nm (OD_{600}) greater than 3.0 was reached. Optical density was measured using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA). A volume of culture was spun down and resuspended in induction medium at an OD_{600} 3.0. Cultures were transferred to a culture tube containing an amount of dried DON that, upon resuspension, equaled 10 ppm. Cultures were placed on a rotary shaker at 200 rpm and 28°C for 30 h or 1 day, depending on the experiment. After the selected time had passed, 500 μl of yeast culture was removed and added to 3.5 ml of acetonitrile. Cultures were placed on a shaker at 200 rpm for 1 h at room temperature. The yeast-acetonitrile mixture was then passed through an alumina:C_{18} column for cleanup. A 2-ml aliquot of eluant was transferred to a glass test tube and evaporated to dryness using a nitrogen evaporator set at 55°C. One hundred microliters of the derivatization agent *N*-trimethylsilylimidazole (TMSI) was then added to the dried down samples. After 30 min, 500 μl of isooctane was added to each tube, followed by 500 μl of water to quench the reaction. Samples were exposed to 10 s of vortexing, and 150 μl of the isooctane supernatant was removed and transferred to chromatography vials for gas chromatography-mass spectrometry (GC-MS) analysis. Conversion of DON to 3ADON was determined by calculating a percent based on the amount of 3ADON produced in relation to total toxin (DON plus 3ADON).

GC-MS Analysis. GC-MS analysis was conducted using an Agilent 6890/5975 system operating in selected ion monitoring (SIM) mode. DON, 3ADON, and mirex eluted off the column (HP-5MS, 30.0 m, 250 μm, 0.25 μm) at 6.23 6.70, and 6.89 min, respectively. Mirex was used as an internal control at 0.5 ppm. SIM mode detected DON and 3ADON target ions at a mass/charge ratio of 512 and 392, respectively, with reference ions at 422 and 497 for DON and a reference ion at 467 for 3ADON. SIM mode detected mirex target ions at mass/charge ratio of 272 with reference ions at 276 and 237. The initial column temperature was held at 150°C for 1 min, and increased to 280°C at a rate of 30°C/min, and held constant for 5 min. The injection temperature was set at 300°C, and the flow rate of the column was 1 ml/min. DON and 3ADON were quantified in the samples using a quadratic regression model using pure DON and 3ADON standards (Biopure, Austria) at concentrations of 0.5 ppm, 1.0 ppm, 5 ppm, 10 ppm and 30 ppm.

Cloning and expression of TRI101/TRI201 enzymes in *E. coli.* *TRI101/TRI201* genes were amplified by PCR from pYES2.1 plasmids bearing the appropriate insert by using *Pfu* Herculase(r) II fusion DNA polymerase (Stratagene). Amplified products were digested with NheI and BlpI restriction enzymes (New England Biolabs) and ligated into a modified pET31b (Novagen, Darmstadt, Germany) vector containing an N-terminal His_6 tag followed by a TEV protease cleavage site (Rocco et al., 2008). The TRI101/TRI201 orthologs were overexpressed in *E. coli* strain BL21 Codon Plus (DE3). Starter cultures from a single colony were grown overnight in Luria broth (LB) supplemented with 100 μg/ml ampicillin and 30 μg/ml chloramphenicol. Fifteen milliliters of the starter culture was used to inoculate 1 liter of LB medium including ampicillin and chloramphenicol. Cultures were grown to mid-log phase (OD_{600} ~ 0.8) at 37°C, cooled to 16°C, and induced with 1 mM β-D-thiogalactopyanoside (IPTG,

Sigma Chemical Co.). Cells were harvested after 20 h by centrifugation at 3000 x *g*, washed with a buffer containing 10 mM HEPES and 100 mM NaCl at pH 7.6, and flash-frozen in liquid nitrogen. Cells were stored at -80°C.

Purification of TRI101/TRI201 proteins. All purification steps were carried out on ice or at 4°C. Seven grams of transformed *E. coli* cells were resuspended in 50 ml lysis buffer [20 mM HEPES, 50 mM NaCl, 20 mM imidazole, 0.2 mM tris(2-carboxyethyl)phosphine (TCEP), 1 mM phenylmethanesulfonyl fluoride (PMSF), and 25 mg lysozyme at pH 7.6). Cells were lysed with 5 pulses of 20 to 40 s using a Misonix XL2015 sonicator. The NaCl concentration was brought to 300 mM by the addition of 4 M NaCl and the lysate was clarified by centrifugation at 40,000 rpm in a Beckman 45 Ti rotor for 30 min. The supernatant was loaded onto a 4-ml column of nickel-nitrilotriacetic acid-agarose (Ni-NTA) equilibrated with buffer A (50 mM potassium phosphate, 300 mM NaCl, 20 mM imidazole, 0.2 mM TCEP at pH 7.8). After washing with 80 ml buffer A and 40 ml buffer A containing 40 mM imidazole, the protein was eluted in a linear gradient of 40 to 300 mM imidazole in buffer A. Fractions containing the TRI101/TRI201 protein were identified by SDS-PAGE and Coomassie blue staining. Tobacco etch virus (TEV) protease, purified by following the protocol of Blommel and Fox (Blommel and Fox, 2007), was added at approximately a 1:40 molar ratio to remove the His_6 tag, and the fractions were dialyzed overnight against buffer B (10 mM HEPES, 100 mM NaCl, 0.2 mM TCEP). The NaCl and imidazole concentrations were brought to 300 mM and 20 mM, respectively, and the solution was passed over a 2.5-ml NiNTA column equilibrated with buffer A. The flowthrough was collected; the column removed the TEV protease and undigested TRI101/TRI201 enzyme. The resultant protein was concentrated using a stirred-cell pressure concentrator (Amicon) fit with a

30,000 molecular-weight-cutoff membrane (Millipore) and dialyzed against storage buffer (10 mM HEPES, 100 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol [DTT] at pH 7.6). Protein was drop-frozen in liquid nitrogen and stored at -80°C.

TRI101/TRI201 enzymatic assay. Enzymatic assays were performed as described in Garvey et al. (2008). Briefly, the acetyltransferase reaction was monitored by following the production of CoA in a 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) coupled continuous assay (Ellman, 1959). Reactions were carried out at 25°C in a cuvette containing 100 mM potassium phosphate at pH 8.0, 1.5 mM acetyl-CoA (Sigma), 0.6 mM DTNB (Sigma), 200 μg/mL bovine serum albumin (BSA), 34 ng/ml recombinant TRI101/TRI201 enzyme, and various concentrations of DON in 4.5% dimethyl sulfoxide (DMSO). The change in absorbance at 412 nm was followed, and data were fit by nonlinear regression to the Michaelis-Menton equation.

Circular dichroism. Thermal melting curves of the purified, recombinant TRI101/TRI201 proteins were generated using circular dichroism spectroscopy. The TRI101/TRI201 proteins were diluted to 0.2 mg/ml in a degassed buffer containing 100 mM potassium phosphate at pH 8, and spectra were recorded from 190 to 260 nm in a model 202SF circular dichroism spectrophotometer (Aviv Biomedical). The temperature was increased in 2.5-degree increments from 10°C to 60°C. The melting point (T_m) was determined from the change in molar ellipticity at 222 nm, which resulted in a sigmoidal curve that was fit to a modified version of the van't Hoff equation (Ramsay and Eftink, 1994).

TRI101/TRI201 Western blot analysis. Protein extraction for each yeast transformant was conducted based on the method of Kushnirov (2000). Yeast was grown in 5 ml of liquid drop-out medium containing the appropriate amino acids for selection. The liquid medium was inoculated with transformed yeast cells from a plate and grown at 28°C overnight in a shaking incubator until an optical density (OD_{600}) greater than 2.5 was reached. A volume of liquid culture necessary to achieve an OD_{600} of 2.5 in a 5-ml volume was removed and spun down in a 1.5-ml centrifuge tube. The pellet was resuspended in 100 µl of distilled H_2O, and then 100 µl of 0.2 M NaOH was added to the reaction mixture and held at room temperature for 5 min. Following room temperature incubation, the yeast cells were centrifuged and resuspended in 100 µl of SDS sample buffer (Kushnirov, 2000) and boiled for 3 min. Ten microliters of supernatant was loaded onto a 12% acrylamide-SDS-PAGE gel and run at 150V for 1 h. Precision plus protein dual color (Bio-Rad, Hercules, CA) standard was used to determine protein size. Following separation, protein transfer to a nitrocellulose membrane (Bio-Rad) was conducted in a transfer chamber at 55 mA for 1 h at room temperature. The transfer buffer was composed of 25 mM Tris, 190 mM glycine, 2% SDS, and 20% liquid chromatography-mass spectrometry-grade methanol. The membrane was then blocked in 7% nonfat dry milk in TBST (10 mM Tris, pH 8, 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature. The membrane was incubated with rabbit anti-FsTri101 primary antibody for 1 h in 7% milk TBST (1:5,000). After incubation with the primary antibody, the membrane was washed with 7% milk TBST three times for 15 min each time. The membrane was incubated with secondary antibody (alkaline phosphatase-conjugated anti-rabbit) for 1 h in 7% milk TBST solution (1:10,000). The membrane was washed in TBST three times for 15 min each time and then washed in TBS (no tween 20) once for 15 min. The membrane was incubated with Lumi-Phos WB substrate (Fisher

Scientific, Pittsburgh, PA) at a volume of 0.125 ml for every square centimeter of membrane for 3 min. X-ray film was exposed to the membrane for 1 min and developed. Protein levels were quantified using the freeware software program ImageJ version 1.43u. The band intensities for known concentrations of FsTR101 and FfTRI201 purified from *E. coli* were scored in ImageJ and used to generate standard curves to calculate protein levels for the unknown TRI101 and TRI201 proteins from seven *Fusarium* spp. A correlation analysis was conducted using PROC CORR in SAS (version 9.2; SAS Institute, Cary, NC) to determine relationships between (i) predicted protein concentrations based on standard curves generated using FsTRI101 or FfTRI101 and (ii) percent conversion of DON to 3ADON at 8 or 24 h and predicted protein concentrations based on standard curves generated using FsTRI101 or FfTRI101.

RESULTS

Presence of *TRI101/TRI201* in at least 31 species of *Fusarium*. An internal region of *TRI101/TRI201* was amplified from 33 *Fusarium* strains from the Leslie collection (see Table S2 in the supplemental material). Together with the sequence from an additional four strains from the Schmale collection and 17 sequences from GenBank (see Table S1 in the supplemental material), a phylogenetic tree was constructed in MegAlign using ClustalW (Lasergene version 8.1.1; DNAStar, Madison, WI) (**Figure 1**), based on a total of 688 bp of sequence internal to the 3' end of *TRI101/TRI201*. The tree shows two large clusters that consist mostly of trichothecene producers (top cluster) and nonproducers (bottom cluster) (**Figure 1**). The large top cluster (*F. graminearum*, *F. culmorum*, etc.) contains portions of characterized and uncharacterized *TRI101* genes, and the large bottom cluster (*F. oxysporum*, *F. fujikuroi*, etc.) contains portions of characterized and uncharacterized *TRI201* genes.

TRI12 enhances the conversion of DON to 3ADON. In the absence of FsTRI12, our transformed yeasts expressing FgTRI101 alone were extremely limited in their ability to acetylate DON, converting only 4.7% ± 0.5% (mean ± standard deviation) of DON to 3ADON in 24 h. Yeast transformed with *FsTRI12* alone converted 7.4% ± 0.7% of DON to 3ADON in 24 h, which was greater than both the untransformed control and *Fg1TRI101*-transformed yeast, most likely due to the existence of the endogenous 3-*O*-acetyltranferase encoded by *S. cerevisiae AYT1* (4). In transformed yeast carrying both *Fg1TRI101* and *FsTRI12*, the conversion of DON to 3ADON was 66% in 24 h.

Comparisons of sequence identity. Eleven full-length *TRI101/TRI201* sequences from seven species of *Fusarium* from the Leslie and Schmale collections were compared for sequence identity (**Table 2**). The seven selected species were placed into two groups: trichothecene producers (*F. graminearum, F. sporotrichioides, F. crookwellense, F. culmorum,* and *F. pseudograminarum*) and trichothecene nonproducers (*F. fujikuroi* and *F. oxysporum*) as described by Desjardins (2006). Pairwise comparisons of genetic identity between full-length *TRI101/TRI201* DNA sequences from the seven *Fusarium* species ranged from 66% to 98% **(Table 2)**. The trichothecene nonproducers had sequence similarity to each other but lower levels of identity to trichothecene producers (**Table 2**).

Time course yeast feeding assays. A time course assay was conducted using seven *TRI101/TRI201* genes from the Schmale and Leslie collections (see Table S3 in the supplemental material**)** expressed in yeast to illustrate rate differences of DON acetylation across a 30-h period

(Table 3). Trichothecene extractions were conducted over six different time points: 0 h, 4 h, 8 h, 16 h, 24 h and 30 h. At a concentration of 10 ppm, DON was acetylated at varying rates by each protein encoded by a *TRI101/TRI201* gene. Ff1TRI201 reached 50% and 90% DON acetylation the earliest at 10.3 h and 15.1 h, respectively. During the first 8 h of our assay, Ff1TRI201 had the highest level of acetylation, converting an average of 30.8 ± 2.2% of DON to 3ADON at a rate of 3.6% per hour. At 16 h, Fcu2TRI101 and Ff1TRI201 had relatively the same conversion percentage, at 97.4% ± 0.7% and 97.5% ± 1.1%, respectively; however, Fcu2TRI101 had the highest conversion rate from 8 to 16 h, converting 9.3% DON per hour. Within 24 h Ff1TRI201, Fcr2TRI101, and Fcu2TRI101 reached complete conversion of DON to 3ADON. Fo1TRI201 was the fourth best converter of DON to 3ADON, reaching complete conversion in 30 h. During the time tested, Fs1TRI101 converted the smallest amount of DON overall, converting only 50.5%. Unexpectedly, Fg1TRI101 and Fps2TRI101 showed only intermediate abilities at acetylating DON, converting 92.6% and 81.5% in 30 h, respectively. Pairwise comparisons using the Tukey-Kramer honestly significant difference test between TRI101/TRI201 of each species showed that FfTRI201, Fcu2TRI101, Fcr2TRI101, and Fo1FTRI201were not significantly different at the 30-h time point ($P = 0.05$).

Steady-state kinetic analysis. Steady-state kinetic analyses were conducted in order to compare *in vitro* enzymatic differences among seven TRI101/TRI201 proteins (**Table 4**). A chemically coupled assay was used to follow the rate of CoA production generated by acetyl transfer to the DON substrate. TRI101 from *F. pseudograminearum, F. graminearum, F. crookwellense,* and *F. culmorum* (all are trichothecene producers) had similar catalytic efficiencies for DON acetylation. Fps2TRI101 had the largest turnover number, 195 s^{-1}. TRI201 from *F. fujikuroi* (a trichothecene

non-producer) had an intermediate catalytic efficiency of 4.8×10^5 $M^{-1} \cdot s^{-1}$, with a lower turnover number and higher K_m. TRI201 from *F. oxysporum* (a trichothecene non-producer) had both the lowest k_{cat}/K_m value (6.8×10^4 $M^{-1} \cdot s^{-1}$) and the lowest turnover number (34 s^{-1}) for DON acetylation of the seven orthologs tested. TRI101 from *F. sporotrichioides* (a T-2 toxin trichothecene producer) had a K_m of 550 ± 70, suggesting a low affinity for DON.

Thermostability measurements. Thermal melting curves were generated by circular dichroism spectroscopy to assess differences in thermostability among the TRI101/TRI201 proteins (**Table 4**). Fs1TRI101 had the highest T_m, 43.3°C, while Ff1TRI201 had the lowest T_m, 37.1°C. Fps2TRI101, which had the highest catalytic efficiency for DON acetylation, had a T_m of 37.8°C, indicating that the differences in T_m did not correlate with measured kinetic parameters.

TRI101/TRI201 Western blot analysis. A western blot assay was performed to detect and quantify the TRI101/TRI201 protein produced by each yeast transformant at 24 h (**Figure 2**) and at 12 hours (data not shown) to resolve whether acetylation rates may have been influenced by the amount of protein produced. The predicted protein concentrations based on a standard curve using Fs1TRI101 were the following: 16.5 ng/µl of Fps2TRI101, 21.2 ng/µl of Fcr2TRI101, 26.9 ng/µl of Fg1TRI101, 27.4 ng/µl of Fo1TRI201, 34.4 ng/µl of Fcu2TRI101, 45.1 ng/µl of Fs1TRI101, and 65.3 ng/µl of Ff1TRI201. Predicted protein concentrations based on a standard curve using Ff1TRI201 were the following: 18.8 ng/µl of Fps2TRI101, 24.1 ng/µl of Fcr2TRI101, 30.6 ng/µl of Fg1TRI101, 31.2 ng/µl of Fo1TRI201, 39.2 ng/µl of Fcu2TRI101, 51.3 ng/µl of Fs1TRI101, and 74.3 ng/µl of Ff1TRI201. The relative band intensities did not vary between the 12- and 24-h time points (data not shown). Yeast strain RW2802 produced

Ff1TRI201 at the highest levels, and this enzyme also had the highest rate of acetylation (**Figure 2 and Table 3)**. The Fps2TRI101 enzyme was produced in the smallest amount and had the second lowest rate of DON acetylation in our yeast assay (**Figure 2 and Table 3)**. A Western blot analysis of four dilutions (1:1, 1:2, 1:4, and 1:16) of Fg1TRI101 and Ff1TRI201 did not reveal variations in the binding affinity of the rabbit anti-FsTri101 primary antibody (data not shown**)**. There was perfect correlation between predicted protein concentrations based on standard curves generated using Fs1TRI101 and Ff1TRI201 (r^2=1.0, $P < 0.0001$). The percent conversion of DON to 3ADON at 8 h and predicted protein concentrations based on the standard curves generated using Fs1TRI101 and Ff1TRI201 were weakly correlated (r^2=0.63, $P = 0.13$ for both Fs and Ff). The percent conversion of DON to 3ADON at 24 h and predicted protein concentrations based on standard curves generated using Fs1TRI101 and Ff1TRI201 were not correlated (r^2=0.02, $P = 0.96$ for both Fs and Ff).

DISCUSSION

In principle, there are two ways in which a trichothecene 3-*O*-acetyltransferase might be improved to facilitate detoxification of trichothecenes *in planta*. One way would be to genetically engineer the enzyme to enhance its kinetic properties and bioavailability. Given the structural and functional knowledge for TRI101, this is a reasonable approach, but current methods for predicting functional changes induced by site-directed mutagenesis are still not entirely reliable. Furthermore, given the time and effort needed for each new version of the enzyme, it is not possible to create all combinations that might yield a superior enzyme. Bioprospecting offers an alternative approach to isolating better enzymes that have evolved through natural selection. In our study, *TRI101/TRI201* sequence differences were observed

within and among different species of *Fusarium*, illustrating the potential for utilizing natural variation to find an improved enzyme (**Table 2**). Years of selection and a wide range of environmental conditions are expected to yield a number of TRI101/TRI201 isoforms, some of which might be expected to be better than either FgTRI101 or FsTRI101. The main challenge with this approach is to select the most likely candidates for detailed study. Here, we have used the known structures and kinetic characteristics of FgTRI101 and FsTRI101 to identify alternative promising isoforms with the future goal of determining the parameters that control the specificity and stability of the enzyme.

Our study confirmed that TRI101/TRI201 enzymes from fusaria classified as producers and nonproducers of trichothecenes were able to acetylate DON; all seven enzymes tested, two of which were isolated from trichothecene nonproducers, converted DON to 3ADON. Since TRI101 from *F. graminearum* has been shown to have a high relative affinity for DON (Garvey et al., 2008), we hypothesized (i) that TRI101 from *F. sporotrichioides* (a producer of T-2 toxin) would have an intermediate ability to acetylate DON and (ii) that TRI201 from *F. oxysporum* and *F. fujikuroi* (nonproducers of DON) would have lesser abilities (if any) to acetylate DON. We found that FsTRI101 acetylated DON more slowly than FgTRI101; however, we were surprised to find that the TRI201s from the two trichothecene-nonproducing species were highly capable of acetylating DON. This lead to the comparison of DNA sequences among the tested strains. Pairwise comparisons of TRI101/TRI201 DNA sequence between trichothecene nonproducers (*F. fujikuroi* and *F. oxysporum)* and producers *(F. graminearum)* showed identities of about 67%. In contrast, sequence identities between trichothecene producers (e.g. *F. graminearum* and *F. sporotrichioides*) were 80% and higher.

C-3 acetyltransferase activity can be detected in trichothecene-nonproducing species and is encoded by *TRI201* (Kimura et al., 2003). The trichothecene-nonproducing strains examined in this study have fully functional TRI201s, suggesting that certain nonproducing strains have maintained trichothecene acetyltransferase function. The fact that TRI201 from the trichothecene nonproducer *F. fujikuroi* was demonstrated to have higher acetylation efficiency than TRI101 from the trichothecene producers was unexpected. The demonstration of TRI201 functional activity in our assays begs the question as to why nonproducers have a nonfunctional TRI101 yet an active TRI201. *TRI201* is believed to be the result of gene duplication (Tokai et al., 2005). There may be selective pressures (Finley et al., 1987; Thatcher et al., 1998) yet to be studied that act to maintain a functional *TRI201* in trichothecene nonproducers. Perhaps TRI201 functions as an antibiotic resistance gene (Tokai et al., 2005) or plays an ecological role in fungi that coexist with trichothecene producers. It is also interesting to note that when *TRI101* is inactivated in thrichothecene producers by gene disruption, there is no C-3 acetyltransferase activity (McCormick et al., 1999), suggesting that there is no active *TRI201* gene in trichothecene producers.

TRI101/TRI201 amino acid sequence identity in all seven species examined is considerably higher in the cofactor and substrate binding regions than in other regions, with all seven species maintaining the conserved HXXXMDXG and DFGXG structural motifs important for catalytic activity (Garvey et al., 2008). There is also divergence in the sequences from the nonproducers and producers occurring in the flexible surface loop that is between $FgPro^{215}$ and $FgPro^{226}$ in FgTRI101 (see Figure S1 in the supplemental material). The influence of these residues on enzyme activity is uncertain, but the trichothecene nonproducers FoTRI201 and

FfTRI201 contain four proline residues in this region. Interestingly, these enzymes show the lowest catalytic efficiency of the orthologs tested.

The difficulty lies in explaining why the products of *TRI201* genes isolated from the trichothecene nonproducers *F. oxysporum* and *F. fujikuroi* converted DON to 3ADON at levels similar to the levels of conversion of the producers of *TRI101* genes from the trichothecene producers, such as *F. culmorum*, by the end of our 30-h assay but at different rates. Western blot analysis showed that TRI101/TRI201 enzyme levels might play a role in affecting levels of DON conversion to 3ADON. The percent conversion of DON to 3ADON in our yeast assay at 8 h was weakly correlated with predicted protein concentrations for all of the TRI101/TRI201 enzymes tested. Thus, small variations (less than 4-fold differences in concentrations) in enzyme levels may explain some of the observed variation in acetylation activity at early time points (< 12 h). However, at 24 h there was no correlation between the percent conversion of DON to 3ADON and the predicted protein concentrations based on standard curves, perhaps because most of the reactions were close to completion. Our kinetic measurements indicated a lowered catalytic efficiency for TRI201 enzymes and for TRI101 from *F. sporotrichioides*. This might explain in part the low of conversion of DON to 3ADON for TRI101 from *F. sporotrichioides*, but it does not resolve the ability of TRI201 from *F. fujikuroi* to have one of the highest conversion rates of DON to 3ADON in 24 h. Together, our data suggests that both enzyme levels and kinetics may contribute to conversion rates of DON to 3ADON, at least during the time course experiments conducted as part of this work.

The exploitation of TRI101/TRI201 activity may be helpful in the production of fuel ethanol coproducts with reduced levels of DON. At present, corn is the predominant grain used in the United States for the fermentation of starch to fuel ethanol by yeast. The dried residue

remaining is a nutrient-rich feed for domestic animals, called dried distillers grains with solubles (DDGS) (Ham et al., 1994). If the demand for fuel ethanol increases, wheat and barley may be used as a fermentation source (Griffey et al., 2010). Any DON-contaminated grains may also be toxic to yeast used for the bioconversion process to ethanol. However, *S. cerevisiae* is known to carry the gene *AYT1*, which can acetylate the C-3 position of trichothecenes (Alexander et al., 2002). The addition of appropriate *TRI101/TRI201* genes to fermentation yeast might increase the acetylation activity and allow greater ethanol conversion in contaminated grains. The engineering of either yeast or grains to contain more efficient TRI101/TRI201s may also lower the amount of residual DON in the DDGS.

This work has important implications for the use of TRI101/TRI201 to combat FHB. For example, a transgenic plant expressing *FsTRI101*, which has a lower rate of activity against DON, might not be able to reduce DON levels faster than the invading fungus is releasing the mycotoxin, thus causing greater disease. Inefficient enzymes would likely, in time, convert most DON to 3ADON; however, given that the C-3 acetyl in plants may be unstable, a high rate of acetylation would be preferred *in planta* (Zamir et al., 1996). Putting into plants an engineered *TRI101*/*TRI201* that is kinetically geared toward DON acetylation so that rapid modification occurs as the fungus invades the plant tissue might be successful in limiting FHB. An unknown in all this, however, is the effects of plant or *Fusarium* esterases that may remove the C-3 acetyl group as soon as a TRI101/TRI201 acetylates the trichothecene.

Our work confirmed the value of FsTRI12 to enhance the conversion of DON. If the influx of mycotoxin into yeast cells is through passive diffusion, then the expression of an efflux pump could raise trichothecene flux into yeast cells, thereby increasing the acetylation of DON (Alexander et al., 1999a). In our feeding studies, in the absence of *FsTRI12*, transformed yeasts

with *FgTRI101* alone demonstrated little to no ability to acetylate DON. In yeast transformed with *FsTRI12* alone, the acetylation activity was greater than in both the untransformed control and FgTRI101-transformed yeast. This is likely due to the FsTRI12 enzyme working together with the native 3-*O*-acetyltransferase AYT1 that is present in yeast (Alexander et al., 2002). The added benefit of a trichothecene efflux pump should also be factored into the design of engineering wheat and barley to resist FHB in the future.

Most phylogenetic studies, including this one, compare gene sequences of a specific gene in order to deduce a tree that suggests the evolutionary relationships among species. Rarely do they examine the sequence for continuity of the reading frame. When Kimura et al. (2003) examined the sequence between *PHO5* and *URA7* in *F. oxysporum*, they found a pseudo-*TRI101* whose coding region was interrupted by 2 stop codons. Although the sequence of the pseudo-*TRI101* was similar to that of *FgTRI101*, the FoTRI101 was non-functional. In the same study, the authors found an open reading frame (ORF) for *TRI101* from *Fusarium moniliforme* (later assigned to *F. fujikuroi*), yet no transcriptional product was found, suggesting that no enzyme activity was present. This shows the importance of examining the sequence for an entire ORF for a gene, as well as performing functional studies, because the isolation of a full-length ORF does not indicate the translated product will be functional. The results from our phylogenetic analysis are consistent with those of Proctor et al. (2009). Our tree identified two large clusters that separate many known trichothecene producers (top cluster) from nonproducers (bottom cluster). Future functional work with TRI101 and TRI201 from fusaria that have not yet been characterized for trichothecene production and acetyltransferase activity may help to clarify the relationships among members within each of these clusters.

Our work demonstrates the tremendous potential for bioprospecting for additional trichothecene acetyltransferase genes in members of *Fusarium* and other fungal genera in the near future. There are other fungi that carry trichothecene acetyltransferases, but it is unknown whether these genes are active. Perhaps a fungus, which has to compete in the environment with Fusarium, has a method to combat trichothecene toxicity. Also, nature may be able to provide clues to better engineer more stable and efficient trichothecene acetyltransferase enzymes in the future or even deliver an improved TRI101/TRI201 to directly combat FHB through the implementation of transgenic plants. However, obtaining full-length sequences of *TRI101/TRI201* genes from new and/or understudied *Fusarium* species may be limited by challenges associated with the location and orientation of these genes in the genomes of these species (Proctor et al., 2009). Isolating and screening additional orthologs of *TRI101/TRI201* will undoubtedly play an important role in finding more stable and efficient enzymes to modify DON.

SUPPLEMENTAL TABLES & FIGURES

Table S1. *TRI101/TRI201* sequences from the following *Fusarium* species were from GenBank. Partial or full-length *TRI101* sequence obtained from GenBank were used to create degenerate primers for the purpose of bioprospecting for more genes.

Species	TRI101/TRI201	GenBank Accession Number
F. acaciae-mearnsi	TRI101	DQ452408.1
F. asiaticum	TRI101	AF212600.1
F. austroamericanum	TRI101	AF212586.1
F. boothii	TRI101	AF212593.1
F. brasilicum	TRI101	AY452814.1
F. cerealis	TRI101	AF212614.1
F. cortaderiae	TRI101	AY452812.1
F. culmorum	TRI101	AF212610.1
F. meridionale	TRI101	AF212584.1
F. mesoamericanum	TRI101	AF212589.1
F. nygamai	TRI101	AB193099
F. pseudograminearum	TRI101	AF212618.1
F. solani	TRI101	AB181464
F. graminearum	TRI101	AB011417.1
F. sporotrichioides	TRI101	AF127176.1
F. fujikuroi	TRI201	AB181462.2
F. oxysporum	TRI201	AB083516.1

Table S2. Thirty-one species of *Fusarium* (33 strains) contain tentative orthologs of *TRI101/TRI201*, based on amplification of an internal portion of *TRI101/TRI201* (893/890 bp) using degenerate primers.

Species	Strain ID	GenBank accession ID
F. acutatum	10769	HQ149770
F. andiyazi	4647	HQ149745
F. armeniacum	20970	HQ149746
F. babinda	11478	HQ149747
F. chlamydosporum	11397	HQ149748
F. compactum	20974	HQ149749
F. crookwellense	11451	HQ149735
F. culmorum	11427	HQ149734
F. dlamini	5009	HQ149750
F. equiseti	20978	HQ149751
F. fujikuroi	C1993	HQ149752
F. fujikuroi	C1994	HQ149741
F. globosum	11554	HQ149753
F. graminearum	Z3639	HQ149737
F. lactis	10757	HQ149754
F. langsethiae	19084	HQ149755
F. longipes	20983	HQ149756
F. nelsonii	11564	HQ149757
F. oxysporum	11390	HQ149743
F. polyphialidicum	11413	HQ149758
F. proliferatum	D4853	HQ149760
F. proliferatum	D4854	HQ149759
F. pseudograminearum	11435	HQ149736
F. ramigenum	10760	HQ149761
F. redolens	18979	HQ149762
F. sambucinum	20481	HQ149763
F. scirpi	20986	HQ149764
F. solani	11420	HQ149765
F. sporotrichioides	11552	HQ149740
F. sterilihyphosum	11783	HQ149766
F. succisae	3832	HQ149767
F. thapsinum	F4094	HQ149768
F. venenatum	18982	HQ149769

Table S3. Gene combinations in yeast for DON detoxification assays. Species designations are as follows: *FsTR101 (Fusarium sporotrichioides)*, *FgTRI101 (Fusarium graminearum)*, *FoTRI201 (Fusarium oxysporum), FfTRI201 (Fusarium fujikuroi), FcrTRI101 (F. crookwellense), FcuTRI101 (F. culmorum),* and *F. pseudograminearum (FpsTRI101).* The detoxification assay was performed using *Saccharomyces cerevisiae* strain RW2802 (PDR5 *leu2 ura3-52 met5)* to express *TRI101/TRI201*. The *TRI101/TRI201* and *TRI12* genes were cloned into the pYES2.1 (Invitrogen) vector and pESC-LEU (Stratagene) vector respectively. Both contain the *GAL1* promoter for constitutive expression and are terminated by *CYC1*.

Gene combination	Source	Vector	Yeast strain	Media
None	J. Golin	None	RW2802	YPD
Fg1TRI101	D. Schmale	pYes2.1	RW2802	SC -uracil
FsTRI12	N. Alexander	pESC-LEU	RW2802	SC -leucine
Fs1TRI101/FsTRI12	D. Schmale	pYes2.1/pESC-LEU	RW2802	SC -uracil/leucine
Fg1TRI101/FsTRI12	D. Schmale	pYes2.1/pESC-LEU	RW2802	SC -uracil/leucine
Ff1TRI201/FsTRI12	D. Schmale	pYes2.1/pESC-LEU	RW2802	SC -uracil/leucine
Fo1TRI201/FsTRI12	D. Schmale	pYes2.1/pESC-LEU	RW2802	SC -uracil/leucine
Fs2TRI101/FsTRI12	J. Leslie	pYes2.1/pESC-LEU	RW2802	SC -uracil/leucine
Fg2TRI101/FsTRI12	J. Leslie	pYes2.1/pESC-LEU	RW2802	SC -uracil/leucine
Ff2TRI201/FsTRI12	J. Leslie	pYes2.1/pESC-LEU	RW2802	SC -uracil/leucine
Fo2TRI201/FsTRI12	J. Leslie	pYes2.1/pESC-LEU	RW2802	SC -uracil/leucine
Fcr2TRI101/FsTRI12	J. Leslie	pYes2.1/pESC-LEU	RW2802	SC -uracil/leucine
Fcu2TRI101/FsTRI12	J. Leslie	pYes2.1/pESC-LEU	RW2802	SC -uracil/leucine
Fps2TRI101/FsTRI12	J. Leslie	pYes2.1/pESC-LEU	RW2802	SC -uracil/leucine


Fcr2TRI101 M---------AFKIQLDTLGQLPGLLSIYTQISLLYPVSDPSQYPTIVSTFEQGLKRFSE 51
Fg1TRI101 M---------AFKIQLDTLGQLPGLLSIYTQISLLYPVSDSSQYPTIVSTFEQGLKRFSE 51
Fcu2TRI101 M---------AFKIQLDTLGQLPGLLSIYTQISLLYPVSDPSQYPTIVSTLEQGLKRFSE 51
Fs1TRI101 MAATSSTSSQSFDIELDIIGQQPPLLSIYTQISLVYPVSDPSQYPTIVSTLEEGLKRLSQ 60
Fps2TRI101 M---------AFKIELDTLGQLPGLLSIYTQISLLYPVSDPSQHPTIVSTLEQGLKRFSE 51
Ff1TRI201 MTALNTTNMDELDIELDIIGQQPFMVKIYTQISFCFPITDPSTHPAITATLKTGLQRLSQ 60
Fo1TRI201 MTALNVTNMVDLDIELDIIGQQPFMVKIYTQVSFCFPITDPSTHPAITATIKNGLQRLSQ 60
Fcr2TRI101 AVPWVAGQVKAEGISEGNTGTSFIVPFEDVPRVVVKDLRDDPSAPTIEGMRKAGYPMAMF 111
Fg1TRI101 AVPWVAGQVKAEGISEGNTGTSFIVPFEDVPRVVVKDLRDDPSAPTIEGMRKAGYPMAMF 111
Fcu2TRI101 AFPWVAGQVKAEGISEGNTGTSFIVPFEDVPRVIVKDLRDDPSAPTIEGMRKAGYPMAMF 111
Fs1TRI101 TFPWVAGQVKTEGISEGNTGTSKIIPYEETPRLVVKDLRDDSSAPTIEGLRKAGFPLEMF 120
Fps2TRI101 AFPWVAGQVKAEGISEGNTGTSFIIPFEDVPRLVVKDLRDDPSAPTIEGMRKAGYPMAMF 111
Ff1TRI201 SFPWVAGQVKDDG-----TGVFKIKPFEETPPLVVKDLRDDPSAPTMEGLRKAEFPMSMF 115
Fo1TRI201 SFPWVAGQVKDDG-----TGVFKIKPFEATPRLVVKDLRDDPSAPTMEGLRKAEFPMSMF 115
Fcr2TRI101 DENIIAPRKTLPIGPGTGPNDPKPVILLQLNFIKGGLILTVNGHHGAMDMVGQDAVIRLL 171
Fg1TRI101 DENIIAPRKTLPIGPGTGPDDPKPVILLQLNFIKGGLILTVNGQHGAMDMVGQDAVIRLL 171
Fcu2TRI101 DENIIAPRKTLPIGPGTGPNDPKPVIMLQLNFIKGGLILTVNGQHGAMDMVGQDAVIRLL 171
Fs1TRI101 DENVVAPRKTLAIGPGNGPNDPKPVLLLQLNFIKGGLILTVNGQHGAMDMTGQDAIIRLL 180
Fps2TRI101 DENIIAPRKTLPIGPGTGPNDPKPVILLQLNFIEGGLIFTVNGQHGAMDMVGQDAMIRLL 171
Ff1TRI201 DEKKIASKKTLPFGPDYSPDDPSPVLMFQLNFIEGGLIFTVNGQHGCMDMTGQDELIRLL 175
Fo1TRI201 DENKIAPKKTLPIGPDYSPDDPIPVLIFQLNFIEGGLILTVNGQHGCMDMTGQDELIRLL 175
Fcr2TRI101 SKACRNDPFTEEEKTAMNLDRKTIVPYLENYTIGPEVDHQIVKPDVAGGDAVLTPVSASW 231
Fg1TRI101 SKACRNDPFTEEEMTAMNLDRKTIVPYLENYTIGPEVDHQIVKPDVAGGDAVLTPVSASW 231
Fcu2TRI101 SKACRNDPFTEEEKTAMNLDRKTIVPYLENYTIGPEVDHQIVKPDVAGGDAVLTPVSASW 231
Fs1TRI101 SKACRNESFTEEEISAMNLDRKTVVPLLENYKVGPELDHQIAKPAPA-GDAPPAPAKASW 239
Fps2TRI101 SKACRNDPFTEEEKTAMNLDRTTVVPFLENYKLGPEVDHQIVKPDVAGGDAVLTPVSASW 231
Ff1TRI201 SKACRDEAFSEQEISTMNLDRKTIVPLLENYELGPELDHQIIKPPPT-TETPPTPPKASW 234
Fo1TRI201 SKACRDEAFTQEEISTMNLERKTIVPPLKNYELGPELDHQIIKPPPT-TETPPAPPKASW 234
Fcr2TRI101 AFFTFSPKAMSELKDAATKTLDASTKFVSTDDALSAFIWKSASRVRLERIDGSAPTEFCR 291
Fg1TRI101 AFFTFSPKAMSELKDAATKTLDASTKFVSTDDALSAFIWKSASRVRLERIDGSAPTEFCR 291
Fcu2TRI101 AFFTFSPKAMSELKDAATKTLDASTKFVSTDDALSAFIWKSASRVRLERIDGSAPTEFCR 291
Fs1TRI101 AFFSFTPKALSELKDAATKTLDASSKFVSTDDALSAFIWQSTSRVRLARLDASTPTEFCR 299
Fps2TRI101 AFFTFSPKAMSELKDAATKTLDTSTKFVSTDDALSAFIWKSASRVRLERIDGSAPTEFCR 291
Ff1TRI201 AFFSFSSEALCELKDKATQSLDGQTKFISTDDALSAFIWQSVSRARLPRLDDSTSTQFCR 294
Fo1TRI201 AFFSFSPQALSDLKDKATQTLDAGTKFVSTDDALSAFIWQSVSRARRARLDDSTSTQFCR 294
Fcr2TRI101 AVDARPAMGVSNNYPGLLQNMTYHNSTVGEIANESLGATASRLRSELDPASMRQRTRGLA 351
Fg1TRI101 AVDARPAMGVSNNYPGLLQNMTYHNSTIGEIANESLGATASRLRSELDPASMRQRTRGLA 351
Fcu2TRI101 AVDARPAMGVSNNYPGLLQNMTYHNSTVGEIANEPLGATASRLRSELDPASMRQRTRGLA 351
Fs1TRI101 AVDMRGPMGVSSTYPGLLQNMTYHDSTVAEIANEPLGATASRLRSELNSDRLRRRTQALA 359
Fps2TRI101 AVDARPPMGVSNNYPGLLQNMTYHNSTVGEIANEPLGATASRLRSELDPARMRQRTRGLA 351
Ff1TRI201 AVDVRTQLDVPKAYPGILQNMTYSVSNLSQIANEPLGIVASRLRSQLGRDDLRRRTQAIV 354
Fo1TRI201 AVDVRTQLDVPKNYPGILQNMTYSVSKLSQIANEPLGIVASRLRSELGRDDLRRRTQALV 354
Fcr2TRI101 TYLHNNPDKSNVSLTADADPSTSVMLSSWAKVGLWDYDFGFGLGKPETVRRPIFEPVESL 411
Fg1TRI101 TYLHNNPDKSNVSLTADADPSTSVMLSSWAKVGLWDYDFGLGLGKPETVRRPIFEPVESL 411
Fcu2TRI101 TYLHNNPDKSSVSLTADADPSTSVMLSSWAKVGLWEYDFGFGLGKPETVRRPIFEPVESL 411
Fs1TRI101 TYMHGLPDKSSVSLTADANPSSSIMLSSWAKVGCWEYDFGFGLGKPESVRRPRFEPFESL 419
Fps2TRI101 TYLHNNPDKSNVSLTADADPSTSIMLSSWAKVGCWEYDFGFGLGKPETVRRPIFEPVESL 411
Ff1TRI201 TYLQDQTNRANVSVTADANSSIDIMLSSWAKLKCWEYDFGLGLGNPESVRRPLFEPFESL 414
Fo1TRI201 TYLHDQTNRASVSVTADANPSTDIMLSSWAKLKCWDYDFGLGLGKPESVRRPLFEPFESL 414
Fcr2TRI101 MYFMPKKPDGEFCAALSLRDEDMDRLKADKEWTKYAQYVG 451
Fg1TRI101 MYFMPKKPDGEFCAALSLRDEDMDRLKADKEWTKYAQYVG 451
Fcu2TRI101 MYFMPKKPDGEFCAALSLRDEDMDRLKADKEWTKYAQYVG 451
Fs1TRI101 MYFMPKKPDGEFTASISLRDEDMERLKADEEWTKYAKYIG 459
Fps2TRI101 MYFMPKKPDGEFCAALSLRDEDMDRLKADKEWTKYAQYVG 451
Ff1TRI201 MYLMPKRPDGEITAAISLRDEDMERLKSDEEWKKYGKFIG 454
Fo1TRI201 MYLMPKRPDGEITAALSLRDEDMEILKQDEKWKKYGQFIG 454

Figure S1. Alignment of TRI101/TRI201 protein sequences. Substrate contacts with DON (light gray) and CoA (dark gray) are shaded. The conserved HXXXMDXG and DFGXG motifs are surrounded by boxes. The flexible surface loop is shown in boldface. Fs1TRI101, Ff1TRI101, and Fo1TRI201 show relatively high divergence in the flexible surface loop.

TABLES

Table 1. Sequences of primers used to amplify all or portions of *TRI101/TRI201* and *FsTRI12*. Underlined sequence represents restriction enzyme cut sites. Also shown are oligonucleotides for *F. graminearum URA7* and *PHO5* used to isolate the full-length *TRI101* sequence of *F. pseudograminearum, F. culmorum,* and *F. crookwellense.* After sequence analysis, we developed species-specific primers to amplify the coding region of each *TRI101*.

Primer number/Name	Primer sequence	Application/Gene
1) FsTRI101_FWD	5'-ATGGCCGCAACAAGCA-3'	Entire *TRI101*
2) FsTRI101_REV	5'-CTACCCAATATACTTTGCGTACTTTGT-3'	
3) FgTRI101_FWD	5'-ATGGCTTTCAAGATACAGCTCG-3'	Entire *TRI101*
4) FgTRI101_REV	5'-CTAACCAACGTACTGCGCATACT-3'	
5) FoTRI201_FWD	5'-ATGACTGCACTAAACGTTACAAACA-3'	Entire *TRI201*
6) FoTRI201_REV	5'-CTAGCCAATGAATTGCCCATAC-3'	
7) FfTRI201_FWD	5'-ATGACAGCACTAAACACCACAAAC-3'	Entire *TRI201*
8) FfTRI201_REV	5'-CTAGCCAATGAACTTCCCATACTTT-3'	
9) FsTRI12_FWD_BAMHI	5'-GACTGGATCCATGACTGTCGTAGTTCCAGAGG-3'	Cloning into pESC-
10) FsTRI12_REV_SALI	5'-GACTGTCGACTCATTCTTTATCAGCCATCGAA-3'	LEU
11) *TRI101*_Conserved_FWD	5'-MTVTTKCAGCTCAAYTTYAT-3'	Screening for *TRI101*
12) *TRI101*_Conserved_REV	5'-TYCRTATCCTCATCBCTCA-3'	Screening for *TRI101*
13) *Fgraminearum_URA7*_FWD	5'-CAAGCGTCATCTTTCTCAGCGC-3'	Entire *TRI101*, *F.ps,*
14) *Fgraminearum_PHO5*_REV	5'-GGATGGAACGCTTCGACCAC-3'	*F.cu,* & *F.cr*
15) *Fpseudograminearum*_FWD	5'-ATGGCTTTCAAGATAGAGCTCG-3'	Entire *TRI101*
16) *Fpseudograminearum*_REV	5'-CTAACCAACGTACTGCGCG-3'	
17) *Fculmorum*_FWD	5'-ATGGCTTTCAAGATACAGCTCG-3'	Entire *TRI101*
18) *Fculmorum*_REV	5'-CTAACCAACGTACTGCGCG-3'	
19) *Fcrookwellense*_FWD	5'-ATGGCTTTCAAGATACAGCTCG-3'	Entire *TRI101*
20) *Fcrookwellense*_REV	5'-CTAACCAACATACTGCGCATACTTG-3'	

Table 2. Pairwise comparisons between full-length *TRI101* and *TRI201* DNA sequences evaluated in the present study. Percent identity is above the diagonal, and percent divergence is below the diagonal. Strain abbreviations: 1, strain from Schmale collection; 2, strain from Leslie collection; Fg, *F. graminearum*; Fcr, *F. crookwellense*; Fcu, *F. culmorum*; Fps, *F. pseudograminearum*; Fs, *F. sporotrichioides*; Ff, *F. fujikuroi*; Fo, *F. oxysporum*; +, trichothecene producer; -, trichothecene nonproducer.

	+	+	+	+	+	+	+	-	-	-	-
	Fg1	**Fg2**	**Fcr2**	**Fcu2**	**Fps2**	**Fs1**	**Fs2**	**Ff1**	**Ff2**	**Fo1**	**Fo2**
Fg1		99.9	97.7	97.0	95.9	80.2	80.3	65.6	65.8	67.6	67.3
Fg2	0.1		97.6	96.9	95.8	80.1	80.3	65.5	65.7	67.5	67.2
Fcr2	2.3	2.4		97.7	96.5	80.4	80.6	65.5	65.6	67.5	67.3
Fcu2	3.1	3.2	2.3		96.7	80.7	81.7	65.9	67.0	67.6	67.3
Fps2	4.3	4.3	3.6	3.4		81.7	81.7	67.0	67.0	68.1	67.8
Fs1	23.3	23.4	23.0	22.6	21.2		99.6	69.6	69.7	69.7	69.3
Fs2	23.1	23.2	22.8	22.6	21.2	0.4		69.5	69.6	69.6	69.4
Ff1	46.8	47.0	47.2	46.3	44.2	39.8	39.9		99.3	86.2	86.0
Ff2	46.5	46.7	46.9	45.9	44.1	39.6	39.8	0.7		86.1	85.8
Fo1	46.8	43.1	43.2	42.9	42.1	39.8	39.7	15.5	15.6		98.8
Fo2	43.5	43.6	43.5	43.3	42.5	40.1	40.0	15.6	15.9	1.2	

Table 3. Percentage of DON converted to 3ADON for each time point for each combination of *TRI101* or *TRI201* with *TRI12* and respective time to reach 50% and 90% DON conversion. The study was conducted for 30 h, and cultures were exposed to 10 ppm DON at the time of inoculation. Results are based on four replications. TRI101 from *F. graminearum* (Fg), *F. sporotrichioides* (Fs), *F. crookwellense* (Fcr), *F. culmorum* (Fcu), and *F. pseudograminearum* (Fps) and TRI201 from *F. fujikuroi* (Ff) and *F. oxysporum* (Fo) were analyzed in this study; 1 and 2 indicate strains from the Schmale or Leslie collection, respectively. ND, not determined.

	% DON converted to 3ADON (mean ± SD) at indicated time point (h)						Time (h) to reach indicated % convesion	
Gene combination	**0**	**4**	**8**	**16**	**24**	**30**	**50%**	**90%**
FfTRI101/ FsTRI12	1.7± 0.19	7.2± 0.75	30.8± 2.19	97.5± 1.05	100.0± 0.0	99.9± 0.17	10.3	15.1
FcuTRI101/ FsTRI12	1.6± 0.14	3.7± 0.41	22.7± 0.71	97.4± 0.7	99.9± 0.17	99.8± 0.3	10.9	15.2
FcrTRI101/ FsTRI12	2.3± 0.28	3.8± 0.77	19.7± 1.72	88.2± 3.57	99.0± 0.62	99.7± 0.4	11.5	17.3
FoTRI101/ FsTRI12	2.7± 0.91	2.8± 0.63	9.3± 1.09	70.7± 4.41	97.0± 0.79	99.2± 0.5	13.3	21.9
FgTRI101/ FsTRI12	1.5± 0.31	2.2± 0.05	9.1± 0.58	70.2± 2.67	89.8± 3.27	92.6± 3.11	13.4	24.4
FpsTRI101/ FsTRI12	1.9± 0.29	2.1± 0.3	3.7± 0.8	34.2± 3.05	69.1± 1.19	81.5± 0.41	19.6	ND
FsTRI101/ FsTRI12	1.5± 0.16	2.9± 0.52	6.5± 0.62	21.0± 1.01	39.0± 1.42	50.5± 1.01	29.7	ND
RW2802 Untransformed	1.7± 0.23	2.4± 0.59	1.9± 0.35	1.1± 0.22	1.7± 0.56	2.4± 0.70	ND	ND

Table 4. Kinetic constant K_m and k_{cat} values and T_m values for TRI101/TRI201 enzymes[a].

TRI101/TRI201 variant	K_m (μM) ±SD	k_{cat} (s^{-1}) ±SD	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	T_m (°C)
Fps2TRI101	41 ± 2	195 ± 5	4.7×10^6	37.8
Fg1TRI101	24 ± 2	106 ± 3	4.4×10^6	39.2
Fcr2TRI101	37 ± 2	153 ± 4	4.2×10^6	38.8
Fcu2TRI101	36 ± 2	140 ± 4	3.9×10^6	41.9
Ff1TRI201	170 ± 15	84 ± 3	4.8×10^5	37.1
Fs1TRI101	550 ± 70	73 ± 4	1.3×10^5	43.2
Fo1TRI201	510 ± 90	34 ± 3	6.8×10^4	38.2

[a]Results are listed in order of decreasing k_{cat}/K_m ratio. Species abbreviations: Fs, *F. sporotrichioides*; Fg, *F. graminearum*; Fo, *F. oxysporum*; Ff, *F. fujikuoi*; Fcr, *F. crookwellense*; Fcu, *F. culmorum*; Fps, *F. pseudograminearum*; 1, obtained from the Schmale collection; 2, obtained from the Leslie Collection.

FIGURES

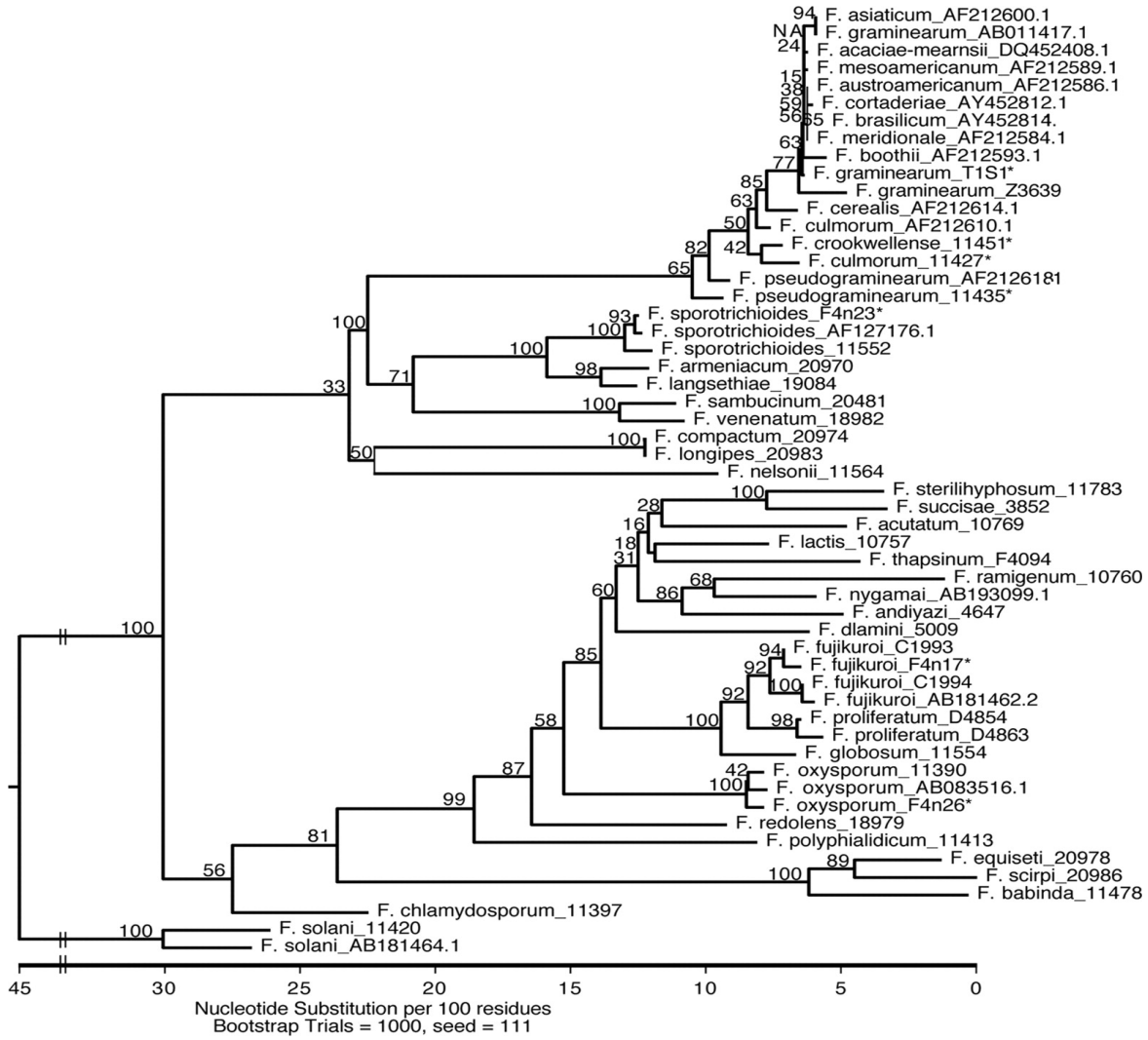

Figure 1. Phylogenetic tree based on a total of 688 bp of sequence internal to the 3' end of *TRI101/TRI201*. Sequences included in the tree are from a total of 54 strains of *Fusarium*: 33 strains from the Leslie collection (see Table S2 in the supplemental material), four strains from the Schmale collection, and 17 strains from GenBank (see Table S1 in the supplemental material). Strain numbers or GenBank accession numbers follow the underscores. *, species whose full-length *TRI101/TRI201* gene products were analyzed in this study for their ability to detoxify DON. Numbers at branch points represent bootstrap values.

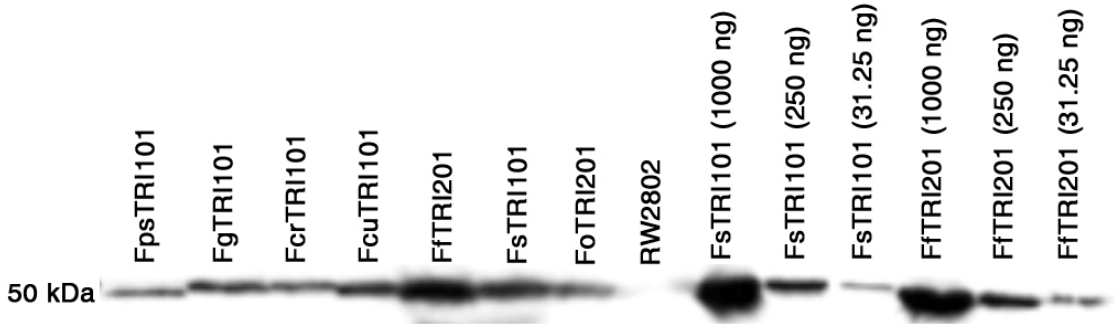

Figure 2. Western blot of TRI101/TRI201 enzymes from *F. pseudograminearum* (Fps2TRI101), *F. graminearum* (Fg1TRI101), *F. crookwellense* (Fcr2TRI101), *F. culmorum* (Fcu2TRI101), *F. fujikuroi* (Ff1TRI201), *F. sporotrichioides* (Fs1TRI101), and *F. oxysporum* (Fo1TRI201) (1 and 2 indicate the strain is from the Schmale or Leslie collection, respectively). Protein extracts from yeast cultures were collected following 24 h of expression. Untransformed yeast strain RW2802 represents the negative control. Ten microliters of each protein extract was loaded onto a 12% acrylamide-SDS-PAGE gel and run at 150 V for 1 h. Precision plus protein dual color standard was used to determine protein size. Ten microliters of TRI101 from *F. sporotrichoides* and TRI201 from *F. fujikuroi*, both purified from *E. coli*, were loaded as positive controls at known amounts of 1,000 ng, 250 ng, and 31.25 ng. The Western blot was probed with rabbit anti-FsTRI101 primary antibody and detected with alkaline phosphatase-conjugated anti-rabbit antibody.

LITERATURE CITED

Alexander N.J. (2008) The TRI101 story: engineering wheat and barley to resist fusarium head blight. World Mycotoxin Journal 1:31-37.

Alexander N.J., McCormick S.P., Hohn T.M. (1999a) TRI12, a trichothecene efflux pump from Fusarium sporotrichioides: gene isolation and expression in yeast. Mol Gen Genet 261:977-84.

Alexander N.J., McCormick S.P., Hohn T.M. (2002) The identification of the Saccharomyces cerevisiae gene AYT1(ORF-YLL063c) encoding an acetyltransferase. Yeast 19:1425-30.

Alexander N.J., McCormick S.P., Ziegenhorn S.L. (1999b) Phytotoxicity of Selected Trichothecenes using *Chlamydomonas reinhardtii* as a Model System. Natural Toxins:265-269.

Blommel P.G., Fox B.G. (2007) A combined approach to improving large-scale production of tobacco etch virus protease. Protein Expr Purif 55:53-68.

Desjardins A.E. (2006) *Fusarium* Mycotoxins: Chemistry, Genetics, and Biology American Phytopathological Society Press, St. Paul, Minnesota.

Desjardins A.E., McCormick S.P., Appell M. (2007) Structure-Activity Relationships of Trichothecene Toxins in an *Arabidopsis thaliana* Leaf Assay. Journal of Agriculture and Food Chemistry 55:6487-6492.

Ellman G.L. (1959) Tissue sulfhydryl groups. Arch Biochem Biophys 82:70-7.

Finley D., Ozkaynak E., Varshavsky A. (1987) The yeast polyubiquitin gene is essential for resistance to high temperatures, starvation, and other stresses. Cell 48:1035-46.

Garvey G.S., McCormick S.P., Rayment I. (2008) Structural and functional characterization of the TRI101 trichothecene 3-O-acetyltransferase from Fusarium sporotrichioides and Fusarium graminearum: kinetic insights to combating Fusarium head blight. J Biol Chem 283:1660-9.

Goswami R.S., Kistler H.C. (2004) Heading for disaster: *Fusarium graminearum* on cereal crops. Molecular Plant Pathology 5:515-525.

Griffey C., Brooks W., Kurantz M., Thomason W., Taylor F., Obert D., Moreau R., Flores R., Sohn M., Hicks K. (2010) Grain Composition of Virginia winter barley and implications for use in feed, food, and biofuels production. Journal of Cereal Science 51:41-49.

Ham G.A., Stock R.A., Klopfenstein T.J., Larson E.M., Shain D.H., Huffman R.P. (1994) Wet corn distillers byproducts compared with dried corn distillers grains with solubles as a source of protein and energy for ruminants. J Anim Sci 72:3246-57.

Kimura M., Kaneko I., Komiyama M., Takatsuki A., Koshino H., Yoneyama K., Yamaguchi I. (1998a) Trichothecene 3-O-acetyltransferase protects both the producing organism and transformed yeast from related mycotoxins. Cloning and characterization of Tri101. J Biol Chem 273:1654-61.

Kimura M., Matsumoto G., Shingu Y., Yoneyama K., Yamaguchi I. (1998b) The mystery of the trichothecene 3-O-acetyltransferase gene. Analysis of the region around Tri101 and characterization of its homologue from Fusarium sporotrichioides. FEBS Lett 435:163-8.

Kimura M., Tokai T., Matsumoto G., Fujimura M., Hamamoto H., Yoneyama K., Shibata T., Yamaguchi I. (2003) Trichothecene nonproducer Gibberella species have both functional and nonfunctional 3-O-acetyltransferase genes. Genetics 163:677-84.

Kushnirov V.V. (2000) Rapid and reliable protein extraction from yeast. Yeast 16:857-60.

Manoharan M., Dahleen L.S., Hohn T.M., Neate S.M., Yu X.-H., Alexander N.J., McCormick S.P., Bregitzer P., Schwarz P.B., Horsley R.D. (2006) Expression of 3-OH trichothecene acetyltransferase in barley (*Hordeum vulgare* L.) and effects on deoxynivalenol. Plant Science:699-706.

McCormick S.P., Alexander N.J., Trapp S.E., Hohn T.M. (1999) Disruption of TRI101, the gene encoding trichothecene 3-O-acetyltransferase, from Fusarium sporotrichioides. Appl Environ Microbiol 65:5252-6.

McLaughlin C.S., Vaughn M.H., Campbell J.M., Wei C.M., Stafford M.E., Hansin B.S. (1977) Inhibition of protein synthesis by trichothecenes. Mycotoxins in Human and Animal Health 19:263-273.

Meyers S., Schauer W., Balzi E., Wagner M., Goffeau A., Golin J. (1992) Interaction of the yeast pleiotropic drug resistance genes PDR1 and PDR5. Curr Genet 21:431-6.

Muhitch M.J., McCormick S.P., Alexander N.J., Hohn T.M. (2000) Transgenic expression of the TRI101 or PDR5 gene increases resistance of tobacco to the phytotoxic effects of the trichothecene 4,15-diacetoxyscirpenol. Plant Sci 157:201-207.

Peska J.J. (2007) Deoxynivalenol: toxicity, mechanisms and animal health risks. Animal feed science and technology 137:283-298.

Pestka J.J., Smolinski A.T. (2005) Deoxynivalenol: toxicology and potential effects on humans. J Toxicol Environ Health B Crit Rev 8:39-69.

Proctor R.H., McCormick S.P., Alexander N.J., Desjardins A.E. (2009) Evidence that a secondary metabolic biosynthetic gene cluster has grown by gene relocation during evolution of the filamentous fungus Fusarium. Mol Microbiol 74:1128-42.

Ramsay G.D., Eftink M.R. (1994) Analysis of multidimensional spectroscopic data to monitor unfolding of proteins. Methods Enzymol 240:615-45.

Rocco C.J., Dennison K.L., Klenchin V.A., Rayment I., Escalante-Semerena J.C. (2008) Construction and use of new cloning vectors for the rapid isolation of recombinant proteins from Escherichia coli. Plasmid 59:231-7.

Thatcher J.W., Shaw J.M., Dickinson W.J. (1998) Marginal fitness contributions of nonessential genes in yeast. Proc Natl Acad Sci U S A 95:253-7.

Tokai T., Fujimura M., Inoue H., Aoki T., Ohta K., Shibata T., Yamaguchi I., Kimura M. (2005) Concordant evolution of trichothecene 3-O-acetyltransferase and an rDNA species phylogeny of trichothecene-producing and non-producing fusaria and other ascomycetous fungi. Microbiology 151:509-19.

Zamir L.O., Nikolakakis A., Devor K.A., Sauriol F. (1996) Biosynthesis of the trichothecene 3-acetyldeoxynivalenol. Is isotrichodermin a biosynthetic precursor? J Biol Chem 271:27353-9.

CHAPTER III

Conversion of deoxynivalenol to 3-acetyldeoxynivalenol in barley derived fuel ethanol co-products with yeast expressing trichothecene 3-*O*-acetyltransferases

Piyum A. Khatibi[1], Justin Montanti[3], Nhuan P. Nghiem[3], Kevin B. Hicks[3], Greg Berger[2], Wynse S. Brooks[2], Carl A. Griffey[2], and David G. Schmale III[1*]

[1]*Virginia Tech, Department of Plant Pathology, Physiology, and Weed Science, Blacksburg, VA 24061, Virginia Tech*

[2]*Department of Crop and Soil Environmental Sciences, Blacksburg, VA 24061*

[3]*Sustainable Biofuels and Co-Products Research Unit, USDA, ARS, Eastern Regional Research Center, Wyndmoor, PA 19038*

[*]Correspondence: dschmale@vt.edu, Phone: (540) 231-6943. Fax: (540) 231-7477

Note: The following chapter was recently submitted to the journal Biotechnology for Biofuels.

ABSTRACT

Background: The trichothecene mycotoxin deoxynivalenol (DON) may be concentrated in dried distillers grains with solubles (DDGS), a co-product of fuel ethanol fermentation, when grain containing DON is used to produce fuel ethanol. Even low levels of DON (≤ 5ppm) in DDGS sold as feed pose a significant threat to the health of monogastric animals. New and improved strategies to reduce DON in DDGS need to be developed and implemented to address this problem. Enzymes known as trichothecene 3-*O*-acetyltransferases convert DON to 3-acetyldeoxynivalenol (3ADON) and reduce its toxicity in plants and animals.

Results: Two *Fusarium* trichothecene 3-*O*-acetyltransferases (FgTRI101 and FfTRI201) were cloned and expressed in yeast (*Saccharomyces cerevisiae*) during a series of small-scale barley (*Hordeum vulgare*) ethanol fermentations. DON was concentrated 1.6 to 8.2 times in DDGS compared to the starting ground grain. During the fermentation process, FgTRI101 converted 9.2% to 55.3% of DON to 3ADON, resulting in DDGS with reductions in DON and increases in 3ADON when Virginia winter barley cultivars Eve, Thoroughbred, and Price and experimental line VA06H-25 were used. Barley mashes from the barley line VA04B-125 showed that yeast expressing FfTRI201 were more effective at acetylating DON than FgTRI101; DON conversion for FfTRI201 ranged from 26.1% to 28.3%, while FgTRI101 ranged from 18.3% to 21.8% in VA04B-125 mashes. Ethanol yields were highest with the industrial yeast strain Ethanol Red ®, which also consumed galactose when present in the mash.

Conclusions: This study demonstrates the potential of using yeast expressing a trichothecene 3-*O*-acetyltransferase to modify DON during commercial fuel ethanol fermentation.

BACKGROUND

As the U.S. attempts to decrease its reliance on fossil fuels, alternative fuel sources are in high demand. Barley is an emerging alternative to corn as an important source for fuel ethanol (Hicks et al., 2005). Winter barley may be grown during the winter months, supplying an additional crop on land that would otherwise be fallow (Chesapeake-Bay-Commission, 2007). This would provide additional income and an ethanol feedstock that does not compete with feed and food markets. In Virginia, new cultivars of barley are being developed with high starch content to support fuel ethanol production (Thomason et al., 2005). The USDA Agricultural Research Service predicts that North America will be able to produce up to 2 billion gallons of ethanol per year from barley alone (Drapcho et al., 2008; Schill, 2008).

A valuable co-product of fuel ethanol production known as dried distillers grains with solubles (DDGS) is increasingly being used as a feed source for domestic animals (McChesney, 2006). DDGS contain high levels of protein, fiber, minerals, and vitamins (Ingledew, 1999; Stone, 1998). An increase in the supply and demand for DDGS (Wu and Munkvold, 2008) is expected to coincide with the increased production of fuel ethanol in commercial plants (Mielenz, 2001), which rely on the sale of DDGS to turn a profit (Madson and Monceaux, 1995).

One of the challenges facing the fuel ethanol industry is the management of mycotoxins such as deoxynivalenol (DON) in DDGS. Barley can become contaminated with DON in the field following infection with the fungal plant pathogen *Fusarium graminearum (Gibberella zeae)*. Barley contaminated with high levels of DON are excluded from feeds and foods (McMullen et al., 1997). DON is a potent inhibitor of protein synthesis (McLaughlin et al., 1977), and animals ingesting DON may demonstrate symptoms of vomiting, feed refusal, and even death (Pestka and Smolinski, 2005).

Levels of DON in DDGS following ethanol production may be concentrated up to three times compared to the starting material (Schaafsma et al., 2009b). DDGS contaminated with mycotoxins are estimated to contribute to losses in swine (*Sus scrofa domesticus*) production in excess of $147 million annually (Wu and Munkvold, 2008). Current methods to reduce the effects of mycotoxins in grain include blending clean grain with contaminated material or washing grain with water (Binder, 2007). Washing DDGS with water is not practical as many nutritional components are water soluble (Kim et al., 2008). Stepanik and co-workers demonstrated the potential use of electron beam irradiation to reduce DON in contaminated grain (Stepanik et al., 2007). Others have studied the use of adsorbants to bind mycotoxins (Diaz-Llano and Smith, 2006) or the use of fungistats to prevent fungal growth in storage (Magan and Aldred, 2007). Many of these strategies are likely to incur high equipment and labor costs. New cost-effective and commercially viable methods to reduce mycotoxin contamination in DDGS need to be developed and implemented.

Enzymes known as trichothecene 3-*O*-acetyltransferases have the ability to modify DON by converting it to an acetylated derivative (Garvey et al., 2008). These enzymes are produced by fungi in the genus *Fusarium* and are encoded by genes known as *TRI101* or *TRI201* (Khatibi et al., 2011; Kimura et al., 2003). The enzymatic modification involves the attachment of an acetyl group to the C-3 hydroxyl moiety of the trichothecene molecule (Garvey et al., 2008), forming the derivative 3-acetyldeoxynivalenol (3ADON) (Kimura et al., 1998). The expression of TRI101 has reduced the phytotoxic effects of trichothecenes in tobacco and rice (Muhitch et al., 2000; Ohsato et al., 2007), and has also decreased inhibitory effects of trichothecenes on the growth of *Saccharomyces pombe* (Kimura et al., 1998) *and Chlamydomonas reinnardtii* (Alexander et al., 1999). *In vitro* assays have demonstrated DON to be more inhibitive than

3ADON on protein translation using rabbit reticulocytes (Kimura et al., 1998), DNA synthesis using mouse 3T3 fibroblasts (Eriksen et al., 2004), and proliferation of murine lymphocytes (Bondy et al., 1991). However, DON was only 1.4 times less toxic than 3ADON based on 50% lethal dose (LD_{50}) values in mice (Peska, 2007; Thompson and Wannemacher, 1986). Although the differences in toxicity between DON and 3ADON *in vivo* is small, the relative toxicity of DON and 3ADON in mammals requires further investigation, as toxicology data is limited (Scientific Committee on Food, 1999). The increased toxicity of 3ADON compared to *in vitro* studies may be due to the removal of the acetyl group during metabolism by esterases and the prevalence of these esterases in animals is unknown.

Recently, seven trichothecene 3-*O*-acetyltransferases were evaluated for their ability to modify the mycotoxin DON (Khatibi et al., 2011). In this study, we tested the hypothesis that two of these enzymes (FgTRI101 and FfTRI201) would reduce DON in DDGS following a series of small-scale barley ethanol fermentations. To our knowledge, this is the first detailed report of yeast expressing a DON modification enzyme during barley ethanol fermentation and sets the stage for evaluating alternative enzymes such as DON de-epoxide hydrolases to reduce and detoxify DON in DDGS (Bala and Chimni, 2010; Eriksen et al., 2004).

METHODS AND MATERIALS

Yeast strains. Ethanol Red ® dry alcohol yeast (Fermentis, Marcq-en-Baroeul, France) was used as a representative strain for industrial ethanol production. *S. cerevisiae* strain RW2802 (PDR5 *leu2 ura3-52 met5*) was kindly provided by Dr. J. Golin, The Catholic University, Washington, D.C. Media for culturing yeast strain RW2802 and transformed RW2802 have been described in Khatibi *et al.* (Khatibi et al., 2011).

Acetyltransferases. Gene isolation**,** cloning, and expression of *FgTRI101*, *FfTRI201*, and *FsTRI12* (a trichothecene efflux pump) were conducted as described in Khatibi *et al.* (Khatibi et al., 2011). The two vectors transformed into yeast strain RW2802 for fermentation assays are shown in **Figure 1** and were created using SeqBuilder (Lasergene version 8.1.1; DNAStar, Madison, WI). Plasmid pTRI101YES contained either the *FgTRI101* or *FfTRI201* gene (**Figure 1)**. The pTRI12ESC vector was derived from pESC-LEU and contained the *F. sporotrichioides TRI12* gene (FsTRI12) (**Figure 1)**.

Barley grain production and source. Winter hulled barley cultivars Price and Thoroughbred, experimental hulled line VA04B-125, hulless cultivar Eve and hulless line VA06H-25 used in this study were planted in a randomized complete block in mist-irrigated nurseries at Mt. Holly, VA. Plots having dimensions of 1.5 m x 13.4 m were used to produce sufficient material for analysis of DON concentrations in barley grain, barley mash, and in DDGS. *Fusarium graminearum* colonized corn (*Zea mays*) kernels were applied to plots at the boot stage to encourage infection and DON contamination in the harvested grain. Grain was harvested in summer 2010 using a small plot research combine.

Preparation of Eve, Price, Thoroughbred, and VA06H-25 mashes. The following method for making the mash was based on the enhanced dry grind enzymatic (EDGE) process developed by Nghiem *et al.* (Nghiem et al., 2010). Small lots of barley kernels (2000 g) were cleaned using a dockage-tester (Carter Day International, Minneapolis, MN) and ground to a particle size of 1 mm in a Model 1 Wiley mill (Thomas Scientific, Swedesboro, NJ). Two 1250 g mashes were

prepared, each containing 20% (w/w) dry solids. Deionized (DI) water (mash 1) or 10% (w/w) galactose solution (mash 2) was added to the ground grain to reach a final mass of 1250 g and the pH was adjusted to 5.2 with 5 M sulfuric acid. Two enzymes, OPTIMASH BG, a β-glucanase, and SPEZYME XTRA, a α-amylase (Genencor, Palo Alto, CA) were then added at 29.6 µl (0.13 kg/ton dry solids) and 68.2 µl (0.30 kg/ton dry solids), respectively. Liquefaction was carried out at 90 ˚C for 2 h in an oil bath with mechanical stirring. During liquefaction, small volumes of DI water were added to compensate for water loss due to evaporation. After 2 h, the mash was cooled in an ice-water bath to a temperature of 32 ˚C. Once cooled, the mass of the mash was adjusted with DI water back to 1250 g. The pH was then adjusted to 4.5 with 5 M sulfuric acid. FERMENZYME L-400, a glucoamylase/protease mix (Genencor, Palo Alto, CA) and a developmental β-glucosidase (Genencor, Palo Alto, CA) were added at 147.7 µl (0.65 kg/ton dry solids) and 138.6 µl (0.61 kg/ton dry solids), respectively. To provide a nitrogen source, 0.5g of urea was added to achieve a final concentration of 400 mg/l.

Fermentation was then carried out in 250 ml shake flasks containing 100 g each of the appropriate mash. Of the nine flasks, three were designated for each strain of yeast: Dry Ethanol Red, RW2802, and RW2802 transformed with FgTRI101/FsTRI12. Each flask was inoculated with the appropriate yeast strain and placed in a shaking incubator set at a speed of 200 rpm and a temperature of 30 ˚C for 66 h. Dry Ethanol Red yeast was rehydrated in DI water at 5% w/w and 0.75 ml of this slurry was added to each designated flask. For untransformed and transformed yeast strain RW2802 inocula, 100 ml liquid cultures were grown for two days at 30 ˚C in a shaking incubator set at 200 rpm. Cultures of RW2802 and the transformed yeast cells were then centrifuged at 1500 x g for five minutes. The supernatants were discarded and the cell

pellets were resuspended in 2 ml DI water (final optical density at 600 nm (OD_{600}) ~15.2). A 1.0 ml aliquot of appropriate liquid culture was added to each designated flask.

VA04B-125 hulled barley mashes were prepared using the same procedure described above for Price, Thoroughbred, Eve, and VA06H-25 mashes, except for the following modifications. Two 1500 g mashes were prepared. β-glucanase and α-amylase were added at 35.5 µl (0.13 kg/ton dry solids) and 81.8 µl (0.30 kg/ton dry solids), respectively. Glucoamylase/protease mix and β-glucosidase were added at 177.4 µl (0.65 kg/ton dry solids) and 166.4 µl (0.61 kg/ton dry solids), respectively. Urea (0.6 g) was added. Twelve small-scale fermentations were performed, of which three were designated for each of the four yeast strains. These included Dry Ethanol Red, untransformed RW2802, RW2802 transformed with FgTRI101/FsTRI12, and RW2802 transformed with FfTRI201/FsTRI12. Fermentations were carried out for 71 h. Cultures of untransformed and transformed yeast strain RW2802 were centrifuged. The supernatants were discarded and the cell pellets were resuspended in 3 ml DI water (OD_{600} ~7.0). A 1.0 ml aliquot of liquid culture was added to each designated flask. A summary of the experiments described in this section is provided in **Table 1**.

Extraction of trichothecene mycotoxins from ground barley grain, barley mash and DDGS. Grains from barley lines VA06H-25, VA04B-125, and cultivars Thoroughbred, Price, and Eve were ground in a Wiley mill and mycotoxin extractions were performed on 1 g subsamples. Each subsample was combined with 8 ml of extraction solvent (86 % (v/v) acetonitrile in DI water) in a capped polypropylene tube and placed on a shaker at 200 rpm for 1 hour at room temperature (~ 25 ˚C). DON was detected and quantified using GC/MS (see below).

For the fermentation mashes, subsamples of 1 mL (weighing about 1 g) were taken at 0 h, 20 h, 44 h, and 66 h, except in the case of VA04B-125 where samples were taken at 0 h, 23 h, 47 h, and 71 h. Each mash subsample was added to 7 ml of extraction solvent (described previously). The mash sample-solvent mixtures were placed on a shaker at 200 rpm for 1 h at room temperature.

At the end of fermentation, the entire contents of the experimental flasks were transferred into aluminum weighing pans and dried in an oven at 55 ˚C for four days. The collected DDGS were ground in a coffee grinder (Hamilton Beach, Model 80365, Southern Pines, NC) positioned at the expresso and twelve cup setting. The ground DDGS samples weighing 1 g were added to 8 ml of extraction solvent and shaken at 200 rpm for 1 h at room temperature. The mass of DON and 3ADON in each DDGS sample was determined by multiplying the concentration [ppm] with the mass of each corresponding DDGS sample.

Extraction solvents from the mash and DDGS containing trichothecene mycotoxins were passed through an Alumina:C18 column for clean-up. A 2 ml aliquot of eluent was transferred to a glass test tube and evaporated to dryness using a nitrogen evaporator set at 55°C. One-hundred microliters of the derivatization agent n-trimethylsilylimidazole (TMSI) (Sigma-Aldrich, St. Louis, MO) was then added to the dried samples. After 30 minutes, 500 µl of isooctane containing 5-ppm mirex, was added to each tube followed by 500 µl of water to quench the reaction. Samples were vortexed for 10 seconds and 150 µl of the isooctane-mirex supernatant was removed and transferred to chromatography vials for GC/MS analysis. The GC/MS detected and quantified DON and 3ADON in parts per million (ppm). Percent DON conversion (DON to 3ADON) was determined by calculating the percentage of 3ADON concentration in

relation to total toxin in the subsample (DON + 3ADON). The concentration of DON and 3ADON, and the percent conversion values are reported ± standard error of the mean (SEM).

GC/MS analysis. GC/MS analysis was conducted using an Agilent 6890/5975 system operating in selected ion monitoring (SIM) mode as described by Khatibi *et al.* (Khatibi et al., 2011). Mirex (Sigma-Aldrich, St. Louis, MO) was used as an internal control at 0.5 ppm. SIM mode detected DON and 3ADON target ions at a mass:charge ratio of 512 and 392 respectively, with reference ions at 422 and 497 for DON and a reference ion at 467 for 3ADON. SIM mode detected mirex target ions at mass:charge ratio of 272 with reference ions at 276 and 237. DON and 3ADON were quantified in the samples using a quadratic regression model using pure DON and 3ADON standards (Biopure, Austria) at concentrations of 0.5 ppm, 1.0 ppm, 5 ppm, 10 ppm and 30 ppm.

Protein extraction from VA04B-125 mashes. Protein extraction for barley mash subsamples were conducted based on the method of Kushnirov (2000) for Western blot analysis. A subsample of 1 g was taken at the end of fermentation (71 h) from mashes containing transformed RW2802 expressing FgTRI101, mashes with transformed RW2802 expressing FfTRI201, and mashes containing untransformed RW2802. Subsamples were centrifuged at 1500 x g for 5 min. The supernatant was removed and the mash pellet was resuspended in a mixture of 500 µl DI water and 500 µl of 0.2 M NaOH and held at room temperature for 5 min. Following room temperature incubation, the yeast cells were recovered by centrifugation and resuspended in 250 µl of SDS sample buffer (Kushnirov, 2000) and boiled for 3 min. Four microliters of supernatant was loaded onto a 12% acrylamide-SDS-PAGE gel and run at 150V

for 1 h. Precision plus protein dual color (Bio-Rad, Hercules, CA) standard was used to determine protein size. Following separation, protein transfer to a nitrocellulose membrane (Bio-Rad) was conducted in a transfer chamber at 34 mA for 1 h at room temperature. The transfer buffer was composed of 25 mM Tris, 190 mM glycine, 2% SDS, and 20% liquid chromatography-mass spectrometry-grade methanol. The membrane was then blocked in 7% nonfat dry milk in TBST (10 mM Tris, pH 8, 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature. The membrane was incubated with rabbit anti-FsTri101 primary antibody for 1 h in 7% milk TBST (1:5,000). After incubation with the primary antibody, the membrane was washed with 7% milk TBST three times for 15 min each time. The membrane was incubated with secondary antibody (alkaline phosphatase-conjugated anti-rabbit) for 1 h in 7% milk TBST solution (1:10,000). The membrane was washed in TBST three times for 15 min each time and then washed in TBS (without Tween 20) once for 15 min. The membrane was incubated with Lumi-Phos WB substrate (Fisher Scientific, Pittsburgh, PA) at a volume of 0.125 ml for every square centimeter of membrane for 3 min. X-ray film was exposed to the membrane for 5 min and developed. FgTRI101 and FfTRI201 were purified from *E. coli* as described in Khatibi *et al.*(Khatibi et al., 2011) and were used as reference controls in the western blot.

Ethanol and sugar quantification. For each subsample of mash taken during the time course study, the concentrations of ethanol, glucose, and galactose were measured using HPLC. An additional 1ml mash sample was removed and centrifuged at 12,000 rpm for 5 minutes. The supernatants were passed through a TITAN 0.2 μm filter (Fisher Scientific, Pittsburg, PA) and stored in the freezer until HPLC analysis. The HPLC used was an Agilent 1200 Series equipped

with a refractive index detector and a Bio-Rad Aminex HPX-87H column with a guard column operating at 65˚C. The mobile phase was 5mM H_2SO_4 pumped at a flow rate of 0.6 ml/min.

Theoretical ethanol yields based on the total starch plus b-glucans were calculated according to the procedure described by Nghiem *et al.* (Nghiem et al., 2010). In experiments using Dry Ethanol Red yeast and 10 % (w/w) galactose solution for mashing, galactose was also included in the total available fermentable substrates since the yeast strain used was also capable of metabolizing this sugar.

DDGS composition. Compositional analysis of DDGS samples were conducted as described in Nghiem *et al.* (Nghiem et al., 2010).

Statistical analyses. All comparisons were performed using the statistical program JMP 9.0.0 (Cary, NC). To measure significant differences, analyses of variance (ANOVA) were performed. If an ANOVA showed a difference ($P<0.05$), then Tukey-Kramer's honestly significant difference (HSD) post-hoc test was performed.

RESULTS

Data were analyzed from 96 small-scale barley fermentation mashes. Five barley lines/cultivars were used to prepare the mashes, which included VA06H-25, VA04B-125, Thoroughbred, Price, and Eve. Mean DON levels ± SEM in the ground grain were 129.5±14.0 ppm, 118.3±10.4, 26.7±1.3 ppm, 17.7±0.5 ppm, and 2.8±0.3 ppm, respectively (**Figure 2**). Two mashes, one with galactose and one without galactose, were prepared for each barley line/cultivars using ground grain **(Table 1)**.

DON/3ADON concentrations in barley mashes with galactose. Addition of galactose induced FgTRI101/FsTRI12 or FfTRI201/FsTRI12 expression in the transformed yeast strain RW2802. Upon preparation of the mash (0 h), DON levels in the dry grain were diluted with the addition of 10% galactose solution **(Figure 2)**. At 0 h of fermentation (no yeast), DON levels were 52.1±1.5 ppm (VA06H-25), 28.5±1.4 ppm (VA04B-125), 17.7±2.3 ppm (Thoroughbred), 12.5±0.5 ppm (Price), and 2.3±0.1 ppm (Eve) **(Figure 2)**. At the end of the fermentation, DON concentrations were reduced in all mashes containing transformed yeast, but were only significantly reduced in mashes with barley line VA06H-25 ($P < 0.01$) **(Figure 3)**. The concentration of DON in VA06H-25 at 66 h of fermentation was 15.3±1.6 ppm (transformed RW2802), 56.8±1.3 ppm (untransformed RW2802), and 47.8±1.0 ppm (Ethanol Red) **(Figure 3)**. The concentration of 3ADON in VA06H-25 at 66 h of fermentation was 18.8±0.7 ppm, 2.8±0.1 ppm, and 2.5±0.0 ppm for transformed RW2802, untransformed RW2802, and Ethanol Red, respectively **(Figure 3)**.

DON percent conversion in Eve, Price, Thoroughbred and VA06H-25 mashes. Mash subsamples were collected over a period of 66 h from all mashes containing galactose. Subsamples were taken at 0 h, 20 h, 44 h, and 66 h. In 20 h, mean percent conversion levels ranged from 4.7%±0.4 (Thoroughbred) to 28.9%±1.0 (VA06H-25). At 44 h, the mean percent conversion ranged from 8.0%±0.5 (Eve) to 55.0%±1.2 (VA06H-25) for mashes with transformed yeast. The end of fermentation with transformed yeast yielded mean percent conversions that ranged from 9.2%±0.7 (Eve) to 55.3%±1.8 (VA06H-25). For each time point after 0 h, mashes with transformed yeast had significantly higher conversion values than untransformed yeast strains ($P<0.05$ for pairwise comparisons conducted within each barley line/cultivar) **(Figure 4).**

For untransformed yeast strains, the highest percent conversion at the end of the assay was 5.7%±0.1% (Price 66 h) **(Figure 4)**.

DON percent conversion in VA04B-125 mashes. In a separate experiment, the acetylation levels of two different acetyltransferases (FgTRI101 and FfTRI201) were compared using ground grain from hulled barley line VA04B-125. Subsamples were collected at 0 h, 23 h, 47 h, and 71 h. In VA04B-125 mashes, FfTRI201 demonstrated a greater reduction of DON and an increased conversion of DON to 3ADON compared to FgTRI101 **(Figure 4)**. DON conversion levels at the end of the assay for mashes with Ethanol Red and untransformed RW2802 were 10.6%±0.9% and 9.6%±0.4%, respectively, while RW2802 expressing FgTRI101 or FfTRI201 had levels of 19.9%±1.0% and 26.9%±0.7%, respectively **(Figure 4)**.

Protein extractions were conducted on mashes to reveal levels of FgTRI101 and FfTRI201. Western blot analysis demonstrated the presence of FfTRI201 protein in all three mashes with transformed RW2802 containing *FfTRI201* (**Figure 5**). No protein was detected in untransformed RW2802 mashes (**Figure 5**). Protein extractions conducted on FgTRI101 mashes did not yield FgTRI101 in our Western blot, and were likely below detection levels (data not shown).

DON/3ADON in Eve, Price, Thoroughbred and VA06H-25 DDGS. The mean concentration of DON was concentrated 1.6 to 8.2 times in DDGS compared to the original ground grain used to prepare the mashes (**Table 2)**. The mass of DON in DDGS samples was lower for all mashes with transformed yeast compared to untransformed yeast strains RW2802 and Ethanol Red **(Table 3)**. In recovered DDGS samples, the mass of DON in untransformed RW2802 DDGS

ranged from 178.4±8.2 µg (Eve) to 2496.2±47.6 µg (VA06H-25) and the mass of DON in transformed RW2802 DDGS ranged from 157.2±7.2 µg (Eve) to 1098.7±39.4 µg (VA06H-25) **(Table 3)**. This reduction was significant for barley cultivars Price and Thoroughbred ($P < 0.05$) **(Table 3)**. The DON reduction in hulless line VA06H-25 DDGS was significantly lower than both untransformed RW2802 and Ethanol Red DDGS ($P < 0.05$) **(Table 3)**. The mass of 3ADON in DDGS samples was significantly higher in all transformed RW2802 DDGS samples compared to both untransformed yeast strains' DDGS **(Table 3)**. The largest DON reduction was observed in VA06H-25 DDGS, with a DON to 3ADON ratio of 0.4±0.0 **(Table 3)**. DDGS from barley cultivar Thoroughbred had the smallest DON to 3ADON ratio of 2.9±0.8 **(Table 3)**.

DON/3ADON in VA04B-125 DDGS. In barley hulled line VA04B-125 DDGS samples, the mean concentration of DON was concentrated 1.8 times compared to starting ground grain used to prepare the mashes (**Table 2**). In a comparison between FgTRI101 and FfTRI201 in DDGS derived from VA04B-125 mashes, DON was reduced the most by FfTRI201**.** Average DON levels were 1775.1±36.1 µg (FfTRI201), 1845.7±55.5 µg (FgTRI101), 1951.8±34.4 µg (untransformed RW2802), and 2206.2±8.1 µg (Ethanol Red) for the VA04B-125 DDGS (**Table 3)**. Transformed yeast DDGS had DON levels that were significantly lower than Ethanol Red and untransformed RW2802 DDGS ($P<0.05$) (**Table 3)**. The 3ADON mean mass for transformed yeast were significantly higher than both Ethanol Red and untransformed RW2802 derived DDGS ($P<0.05$) (**Table 3)**.

Sugar consumption and ethanol yields. Unlike yeast strain RW2802, Ethanol Red yeast consumed galactose (**Table 4)**, leading to significantly higher ethanol concentrations compared

to no galactose mashes *(P* < 0.05) (**Table 4**). In mashes without galactose, ethanol yields ranged from 67.45% (VA06H-25 with untransformed RW2802) to 91.41% (VA04B-125 with Ethanol Red) (**Table 4**). In mashes with galactose, ethanol yields ranged from 36.06% (Thoroughbred with untransformed RW2802) to 94.74% (VA04B-125 with Ethanol Red) (**Table 4**). In a comparison between mashes without galactose, yeast strain RW2802 produced significantly less ethanol than Ethanol Red *(P* < 0.01) (**Table 4**). Since transgene expression only occurred by galactose induction, we examined whether ethanol yields were different between transformed and untransformed RW2802, not accounting for barley line/cultivar. An ANOVA showed that ethanol yields were not significantly different between untransformed RW2802 and yeast expressing FgTRI101/FsTRI12 (*P* = 0.23).

DDGS composition. In a preliminary analysis of DDGS composition, DDGS samples produced by transformed RW2802 yeast were found to be similar to DDGS produced by commercial Ethanol Red yeast (data not shown). Differences in composition only occurred with mashes amended with galactose, where galactose and residual sugars in fermentations did not finish to completion. Yeast strain RW2802 did not consume galactose during fermentations causing components of the DDGS to be diluted compared to Ethanol Red DDGS. For example, the DDGS from barley hulless line VA06H-25 fermented with Ethanol Red in the presence of galactose had the following composition: protein, 24.43%; NDF (neutral detergent fiber), 31.27%; starch, 1.50%; β-glucan, 0.22%; crude fat, 5.06%; whereas the DDGS produced from the transformed yeast on the same barley line in the presence of galactose had the following composition: protein, 13.33%; NDF, 14.32%; starch, 11.23%; β-glucan, 0.14%; crude fat, 2.41% (data not shown).

DISCUSSION

The fungal plant pathogen *Fusarium graminearum* produces trichothecene mycotoxins that may contaminate DDGS following fuel ethanol production (Goswami and Kistler, 2004). New cost-effective and commercially viable methods to reduce mycotoxin contamination in DDGS need to be developed and implemented. Preparation of the barley mash dilutes mycotoxin levels from the ground grain due to the addition of DI water or 10% galactose solution (**Figure 2**). Mycotoxin levels are then concentrated during the formation of DDGS (**Table 2**). DON is soluble in water (Hazel and Patel, 2004) and therefore we would expect a mycotoxin dilution of approximately four-times in the mash compared to dry grain (all mashes in this study were 20% solids). However, not all DON may dissolve in water (Trenholm et al., 1992), and therefore increases in ground grain taken from the mash during subsampling may explain smaller dilutions when the concentration of dry ground grain is compared to levels in the mash at the start of fermentation (**Figure 2)**.

Large reductions in DON via conversion (52.4% to 58.1%) during fermentation were observed in hulless barley line VA06H-25, which contained the highest levels of DON in the starting ground grain **(Figure 4)**. This alone demonstrates the tremendous potential for commercial ethanol yeasts to be engineered to express mycotoxin modification enzymes such as trichothecene 3-*O*-acetyltransferases during fermentation. Recent work has demonstrated the abilities of seven different trichothecene 3-*O*-acetyltransferases in the yeast strain RW2802 to modify DON into 3ADON during a series of feeding assays (Khatibi et al., 2011). In these assays, conversion levels ranged from 50.5% to 100%, depending on the source of the acetyltransferase (Khatibi et al., 2011). With FgTRI101, we observed a 55.3% mean conversion of DON for barley line VA06H-25, but previous feeding assays with the same enzyme showed a

reduction of 92.6% in yeast cultures (Khatibi et al., 2011). The difficulty lies in explaining why we observed different levels of conversion in the barley ethanol fermentations compared to previously published feeding assays. Perhaps 'pure' yeast cultures allow higher acetylation rates due to the high accessibility to DON by the acetyltransferases. The complex matrix of proteins and sugars in barley mashes (Vietor and Voragen, 1993) might impede the ability of the acetyltransferases to interact with DON. The starting concentration of yeast might also play a role in determining DON acetylation rates; the OD_{600} of yeast inoculum for hulled line VA04B-125 was approximately two times less than hulless line VA06H-25, and might have contributed to differences in acetylation rates during fermentation between these two lines.

The acetylation levels of two different acetyltransferases (FgTRI101 and FfTRI201) were compared during fermentation using ground grain from hulled barley line VA04B-125. Previous work has shown that the enzyme FgTRI101 has a 9.2 times greater catalytic efficiency towards DON than FfTRI201; however, FfTRI201 resulted in higher DON conversion levels than FgTRI101 because of higher protein expression (Khatibi et al., 2011). During fermentation FfTRI201 converted more DON to 3ADON than FgTRI101 (**Figure 4**) and this was confirmed in the corresponding DDGS (data not shown). Western blot analyses with mashes containing barley line VA04B-125 detected FfTRI201 in all three mashes tested; however, FgTRI101 was not detected. Previous reports have demonstrated that FfTRI201 is expressed at higher levels than FgTRI101 in yeast (Khatibi et al., 2011), and this might help explain why the FgTRI101 levels in the VA04B-125 mashes were below our limit of detection in our Western blot.

In our fermentation assays, glucose (repression) and galactose (induction) were likely competing for control of the GAL1 promoter (**Figure 1**), responsible for FgTRI101 and FfTRI201 expression, and therefore the expression of the acetyltransferases may not have been

optimal in the fermentations. Alternative methods to induce protein expression (for example, using inducers other than galactose) may yield larger reductions in DON, especially in grain containing reduced amounts of the substrate (DON). Future studies could use promoters such as *CUP1* (Macreadie et al., 1989) induced by copper (100 μM Cu^{2+}) (Mascorro-Gallardo et al., 1996; Mizutani et al., 2011). The effect of copper on fermentation and DDGS production is unknown; however, addition of copper (30 mg/kg dry mass) to animal feed has been reported to suppress bacterial infections in the gut of swine (Nicholson et al., 1999). Alternatively, for constitutive expression, the phosphoglycerate kinase promoter (*PGK1*) can be employed and requires no additional components (Lilly et al., 2000).

Previous reports have indicated a three-fold concentration of DON in DDGS (Schaafsma et al., 2009a). In our studies, mycotoxin levels were concentrated in the DDGS 1.6 times to 8.2 times (ground grain vs. Ethanol Red DDGS) (**Table 2**). Ground grain from resistant genotypes (Eve), containing a low DON concentration, resulted in corresponding DDGS with DON levels that were concentrated more than DDGS derived from ground grain with high DON (**Table 2**). Perhaps resistant genotypes harbor more masked DON (DON-glucosides), through expression of a UDP-glucosyltransferase, (Berthiller et al., 2005; Poppenberger et al., 2003) than susceptible genotypes (accumulate high levels of DON), which are subsequently hydrolyzed by the yeast, causing DON to be released during fermentation (Young et al., 1984). This may help explain the results in Table 2 regarding DON concentrating in DDGS relative to the ground grain, but this was not investigated in the present study and a proper mass balance could not be calculated to compare the masses of DON due to the subsampling of mashes during fermentation.

The reduction of total solid mass during fermentation, where glucose is converted to ethanol and carbon dioxide, together with the loss of moisture during the DDGS drying,

increases the concentration of mycotoxins in DDGS. Since the laboratory yeast strain RW2802 did not consume galactose, the components (including DON) of corresponding DDGS were diluted. Mycotoxin dilutions caused by galactose and other residuals (i.e. unreacted starch, oligosaccharides, maltose, and glucose) remaining due to incomplete fermentation, made calculating the concentration of mycotoxins in the DDGS unreliable. Fermentations containing yeast transformed with FgTRI101 or FfTRI201 reduced the mass of DON and increased the mass of 3ADON in all DDGS samples (**Table 3**). These enzymes are likely inactive in the DDGS since the thermostability of these enzymes (Khatibi et al., 2011) are approximately 15 ˚C lower than the temperature in which the DDGS are prepared. Reduction of DON in fuel ethanol co-products such as DDGS destined for feed may save at least $147 million annually (Wu and Munkvold, 2008).

Ethanol yields were greatest in mashes containing Ethanol Red and galactose. This industrial yeast strain was developed for fuel ethanol production and appears to have the unique ability to utilize galactose as well as glucose. In most yeast strains, galactose utilization is about three-fold lower than glucose metabolism (Ostergaard et al., 2000). The model (laboratory) yeast strain RW2802 did not demonstrate an ability to utilize galactose efficiently in our experiments. Ethanol yields for RW2802 were significantly lower in the presence of galactose, perhaps due to the energy cost on the yeast cells to synthesize enzymes in the Leloir pathway, which make up approximately 5% of all total cellular enzymes (Bhat, 2008). DON is a known protein synthesis inhibitor (McLaughlin et al., 1977), but ethanol yields were not impacted by DON in our fermentations.

Another approach to reduce DON in DDGS might be to add an exogenous trichothecene 3-*O*-acetyltransferase preparation to the mash at the start of fermentation. However, the amount

of enzyme needed for this approach to be successful is presently unknown. Moreover, enzyme stability may limit the effectiveness of this strategy (Garvey et al., 2008) and such preparation is not commercially available. Washing grain (Trenholm et al., 1992) before fermentation can be implemented to reduce DON levels before mash preparation in addition to detoxification during fermentation. Reduction of mycotoxins in fermentation mashes does not have to be limited to barley. This strategy could also be applied to other fuel ethanol crops such as corn, wheat, and sugarcane. For example, in addition to deoxynivalenol, the mycotoxin zearalenone is another common contaminant of corn ethanol co-products (Schaafsma et al., 2009b) and a lactonohydrolase was shown to decrease levels of zearalenone in spiked cultures of *Schizosaccharomyces pombe* and *Escherichia coli* (Takahashi-Ando et al., 2005).

The EDGE process was developed as a new method for increasing ethanol yields from barley in a commercial setting to advance biofuels made from non-food feedstocks (Nghiem et al., 2010). Employing yeast to express mycotoxin detoxification genes represents a potential strategy to reduce mycotoxin levels in fuel ethanol co-products. However, a number of issues must be addressed before this process is commercialized. First, integrating a detoxification transgene into the yeast genome would be preferred over maintaining the gene on a plasmid (which generally requires selective conditions for plasmid propagation). Second, the composition of DDGS in future work using transformed yeast would need to be evaluated. Analysis of DDGS composition in this study revealed that DDGS produced by transformed yeast were similar to DDGS produced by commercial yeast, except for the change in composition due to added galactose and residual sugars that did not finish to completion. Third, the use of a transgenic yeast strain for fuel ethanol production will need to be accepted by policy makers and ethanol production facilities in order to be implemented on a commercial scale.

CONCLUSIONS

When using transformed yeast expressing a trichothecene 3-*O*-acetyltransferase in small-scale barley fermentations, DON contaminating the ground grain was converted to 3ADON, and thereby reduced in DDGS. FfTRI201 resulted in higher acetylation levels than FgTRI101 during fermentations in VA04B-125 mashes. DON levels in ground grain compared to DDGS derived from Ethanol Red mashes, were concentrated 1.6 to 8.2 times depending on the barley line/cultivar used in the mash, but were reduced when transformed yeast expressing either FgTRI101 or FfTRI201 was used. Mashes with Ethanol Red yeast had higher ethanol yields than mashes with yeast strain RW2802. In mashes with galactose, Ethanol Red was able to utilize this sugar for conversion into ethanol while the laboratory strain RW2802 had reduced ethanol yields.

ABBREVIATIONS

ANOVA: analysis of variance; 3ADON: 3-acetyldeoxynivalenol; DDGS: dried distillers grains with solubles; DI: deionized DON; deoxynivalenol; EDGE: enhanced dry grind enzymatic; FHB: fusarium head blight; GC/MS: gas chromatography mass spectrometry; HPLC: high performance liquid chromatography; OD: optical density; PPM: parts per million; RPM: rotations per minute; SEM: standard error of the mean; SIM: select ion monitoring; TMSI: trimethylsilylimidazole.

AUTHOR DETAILS

[1]*Virginia Tech, Department of Plant Pathology, Physiology, and Weed Science, Blacksburg, VA 24061, Virginia Tech.*

[2]*Department of Crop and Soil Environmental Sciences, Blacksburg, VA 24061.*

[3]*Sustainable Biofuels and Co-Products Research Unit, USDA, ARS, Eastern Regional Research Center, Wyndmoor, PA 19038.*

AUTHORS' CONTRIBUTIONS

PAK was involved in the design of the experimental work, performed experiments and analytical work involving transgenic yeast, and was the lead writer on the manuscript. JM performed experiments and analytical work on ethanol and sugar quantification and assisted in editing and writing the manuscript. NPN and KBH contributed to the design, helped coordinate experimental work, and assisted in editing and writing the manuscript. GB, WSB, and CAG were involved in development of the barley lines/cultivars used in this study and performed all field work necessary to grow barley, harvest barley, and provide grain. DGS secured funding for the project and helped design and coordinate the experiments and resulting data analyses.

ACKNOWLEDGEMENTS

This work was supported primarily by a grant to DGS, CAG, and KBH from the Biodesign and Bioprocessing Research Center at Virginia Tech (project #208-11-110A-012-331-1). Grants to DGS by the Maryland Grains Producers Utilization Board (proposal #10121612), the Virginia Agricultural Council (proposal #10183402), the Virginia Small Grains Board (proposal #10278306) and the United States Wheat and Barley Scab Initiative (proposal #07185403) also provided support for the work. The conclusions presented here are those of the authors and do not necessarily reflect the views of the United States Department of Agriculture, the Maryland Grain Producers Board, the Virginia Agricultural Council, and the Virginia Small Grains Board. Mention of trade names or commercial products in this article is solely for the purpose of

providing specific information and does not imply recommendation or endorsement by the USDA, the Maryland Grain Producers Board, the Virginia Agricultural Council, and the Virginia Small Grains Board. USDA is an equal opportunity provider and employer.

TABLES

Table 1: Combination of Virginia barley line/cultivar, yeast strain, and treatment for each mash prepared for DON modification. For each barley genotype, three replicate mashes were prepared for each yeast strain for a total of 96 mashes. Mashes were prepared using either DI water or 10 % (w/w) galactose solution.

Barley Line/Cultivar	Yeast Strain	Flasks	Treatment
VA06H-25[1]	Dry Ethanol Red	3	-Galactose
	RW2802	3	-Galactose
	RW2802 FgTRI101/FsTRI12	3	-Galactose
VA06H-25[1]	Dry Ethanol Red	3	+Galactose
	RW2802	3	+Galactose
	RW2802 FgTRI101/FsTRI12	3	+Galactose
VA04B-125[2]	Dry Ethanol Red	3	-Galactose
	RW2802	3	-Galactose
	RW2802 FgTRI101/FsTRI12	3	-Galactose
	RW2802 FfTRI201/FsTRI12	3	-Galactose
VA04B-125[2]	Dry Ethanol Red	3	+Galactose
	RW2802	3	+Galactose
	RW2802 FgTRI101/FsTRI12	3	+Galactose
	RW2802 FfTRI201/FsTRI12	3	+Galactose
Thoroughbred[2]	Dry Ethanol Red	3	-Galactose
	RW2802	3	-Galactose
	RW2802 FgTRI101/FsTRI12	3	-Galactose
Thoroughbred[2]	Dry Ethanol Red	3	+Galactose
	RW2802	3	+Galactose
	RW2802 FgTRI101/FsTRI12	3	+Galactose
Price[2]	Dry Ethanol Red	3	-Galactose
	RW2802	3	-Galactose
	RW2802 FgTRI101/FsTRI12	3	-Galactose
Price[2]	Dry Ethanol Red	3	+Galactose
	RW2802	3	+Galactose
	RW2802 FgTRI101/FsTRI12	3	+Galactose
Eve[1]	Dry Ethanol Red	3	-Galactose
	RW2802	3	-Galactose
	RW2802 FgTRI101/FsTRI12	3	-Galactose
Eve[1]	Dry Ethanol Red	3	+Galactose
	RW2802	3	+Galactose
	RW2802 FgTRI101/FsTRI12	3	+Galactose

[1]Hulless Genotype

[2]Hulled Genotype

Table 2: **Comparison of DON concentration in ground grain versus Ethanol Red DDGS.**

DON was concentrated in the DDGS 1.6 to 8.2 times compared to the original starting grain.

Barley Line/Cultivar	Ground Grain (Mean ppm± SEM)	Ethanol Red DDGS (no galactose) (Mean ppm± SEM)	Times DON Concentrated in DDGS
VA06H-25[1]	129.5±14.0	212.9±3.0	1.6
VA04B-125[2]	118.3±10.4	207.1±3.0	1.8
Thoroughbred[2]	26.7±1.3	130.9±2.4	4.9
Price[2]	17.7±0.5	99.4±3.5	5.6
Eve[1]	2.8±0.3	23.0±0.3	8.2

[1]Hulless Genotype

[2]Hulled Genotype

Table 3: **DON and 3ADON in DDGS derived from mashes amended with galactose.** A mycotoxin extraction was performed on 1 g subsamples of DDGS and analyzed on the GC/MS.

Barley Line/Cultivar	Mycotoxin	Ethanol Red DDGS (Mean µg±SEM)*	Untransformed RW2802 DDGS (Mean µg±SEM)*	Transformed RW2802 DDGS (Mean µg±SEM)*
VA06H-25[1]	DON	1854.6±55.8[a]	2496.2±47.6[b]	1098.7±39.4[c]
	3ADON	689.2±12.4[a]	573.8±27.5[a]	2500.2±40.7[b]
	Ratio	2.7±0.1[a]	4.4±0.1[b]	0.4±0.0[c]
VA04B-125[2]	DON	2206.2±8.1[a]	1951.8±34.4[b]	1775.1±36.1[†,c]
	3ADON	233.8±3.1[a]	229.1±6.1[a]	560.1±10.7[†,b]
	Ratio	9.4±0.1[a]	8.5±0.1[b]	3.2±0.1[†,c]
Thoroughbred[2]	DON	1100.0±4.2[a,b]	1165.7±55.6[a]	1001.5±33.2[b]
	3ADON	183.4±1.5[a]	130.2±7.5[b]	369.0±7.5[c]
	Ratio	6.0±0.1[a]	9.0±0.1[b]	2.9±0.5[c]
Price[2]	DON	974.4±8.1[a,b]	1090.4±35.4[a]	785.2±86.7[b]
	3ADON	123.6±1.6[a]	113.0±5.2[a]	391.0±123.3[b]
	Ratio	7.9±0.1[a]	9.7±0.1[b]	2.5±0.8[c]
Eve[1]	DON	195.0±3.7[a]	178.4±8.2[a,b]	157.2±7.2[b]
	3ADON	36.2±2.0[a]	22.6±4.0[b]	54.2±2.1[c]
	Ratio	5.4±0.4[a]	8.7±2.3[a]	2.9±0.3[b]

*Mass values not connected by the same letter, across rows, are significantly different ($P < 0.05$)

† DON and 3ADON mass from transformed yeast expressing FfTRI201.

[1]Hulless Genotype

[2]Hulled Genotype

Table 4: Final average ethanol (%v/v) for mashes with ethanol red, untransformed RW2802, and transformed RW2802. Ethanol yields are also shown for mashes with and without galactose.

		No Galactose	10% Galactose		Ethanol Yields	
Barley	**Yeast Strain**	**Final Ethanol (%v/v)**	**Final Ethanol (%v/v)**	**Final Galactose (% v/v)**	**w/o Galactose**	**w/Galactose**
VA06H-25[1]	Ethanol Red	9.84	14.43*	0.14	88.49%	90.93%
	Untransformed RW2802	7.50	7.40	8.37	67.45%	46.63%
	Transformed (FgTRI101)	7.51	7.57	8.03	67.54%	47.70%
VA04B-125[2]	Ethanol Red	7.75	12.79*	0.44	91.41%	94.74%
	Untransformed RW2802	6.72	6.24	8.78	79.26%	46.22%
	Transformed (FgTRI101)	7.63	7.68	8.84	90.00%	56.89%
	Transformed (FfTRI201)	7.74	7.59	8.73	91.30%	56.22%
Thoroughbred[2]	Ethanol Red	9.43	14.26*	0.33	83.53%	88.96%
	Untransformed RW2802	8.48	5.78	8.90	75.11%	36.06%
	Transformed (FgTRI101)	8.25	6.93	8.77	73.07%	43.23%
Price[2]	Ethanol Red	8.27	13.24*	0.39	87.42%	92.01%
	Untransformed RW2802	6.47	7.37	8.57	68.39%	51.22%
	Transformed (FgTRI101)	7.93	7.07	8.83	83.83%	49.13%
Eve[1]	Ethanol Red	9.87	13.58*	1.72	85.60%	83.62%
	Untransformed RW2802	9.49	6.68	9.11	82.31%	41.13%
	Transformed (FgTRI101)	9.24	6.19	8.94	80.14%	38.12%

*Dry Ethanol Red yeast in mashes with galactose had significantly higher percent ethanol than the same mash without galactose ($P < 0.05$).

[1]Hulless Genotype

[2]Hulled Genotype

FIGURES

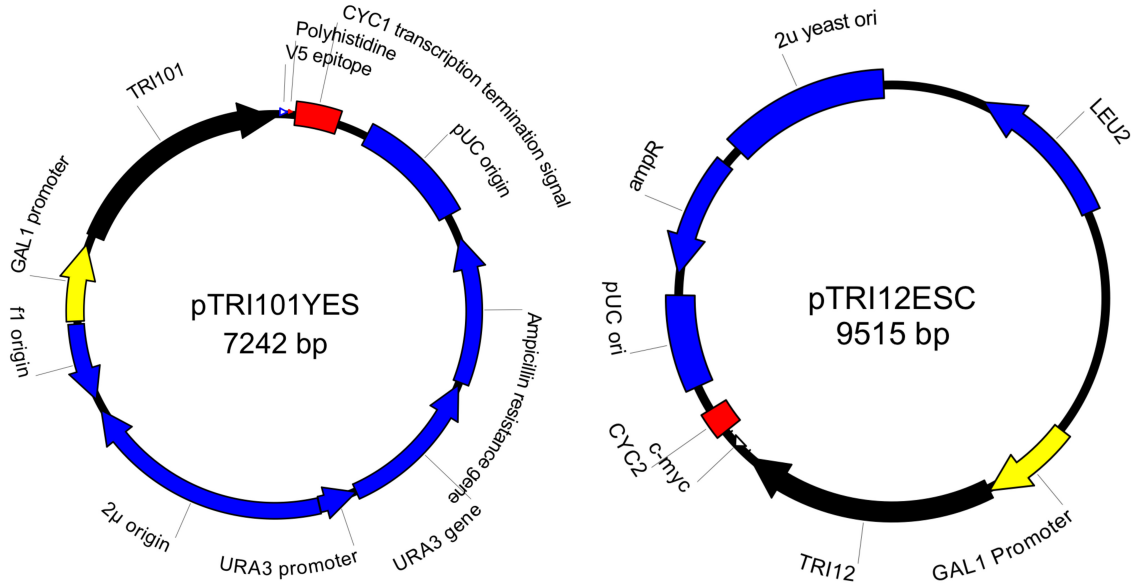

Figure 1. Two vectors transformed into yeast strain RW2802 for barley fermentation assays. Plasmid pTRI101YES (left) contains either the *FgTRI101 or FfTRI201* gene. The pTRI12ESC vector (right) is derived from pESC-LEU and contains the *F. sporotrichioides TRI12* gene.

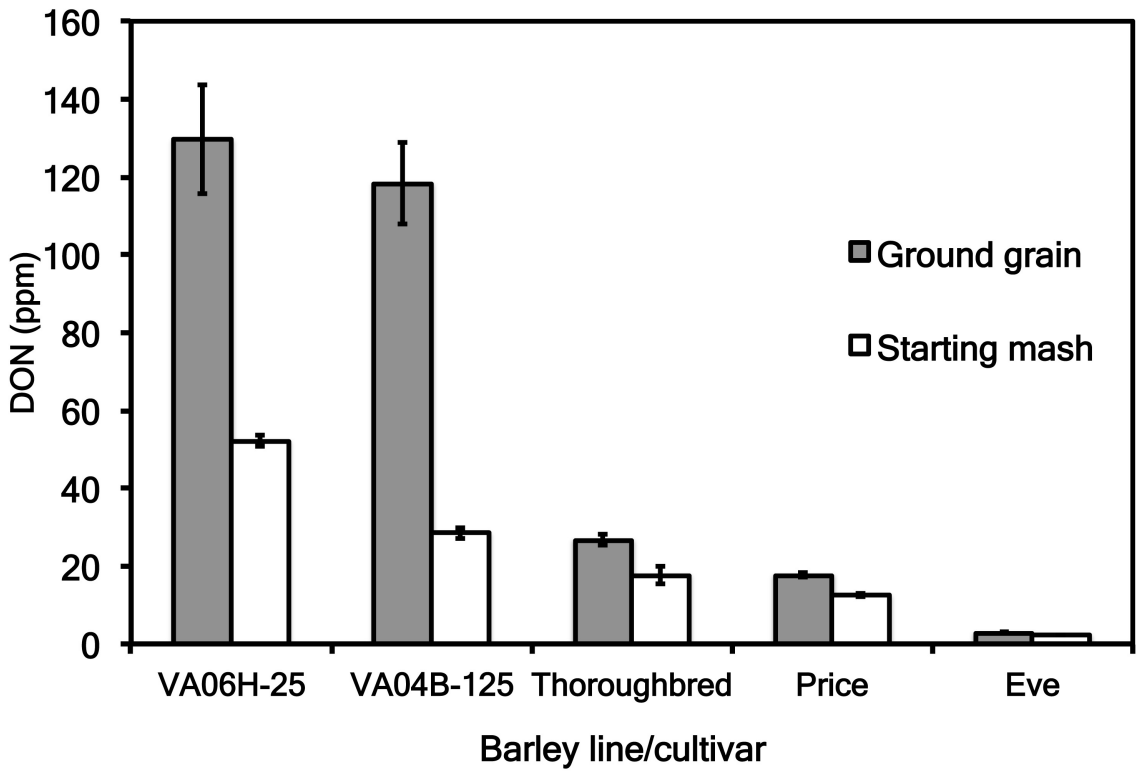

Figure 2. DON concentrations in ground grain and starting (0h) mash (with galactose). DON was diluted upon creation of the mash 1.2 to 4.2x.

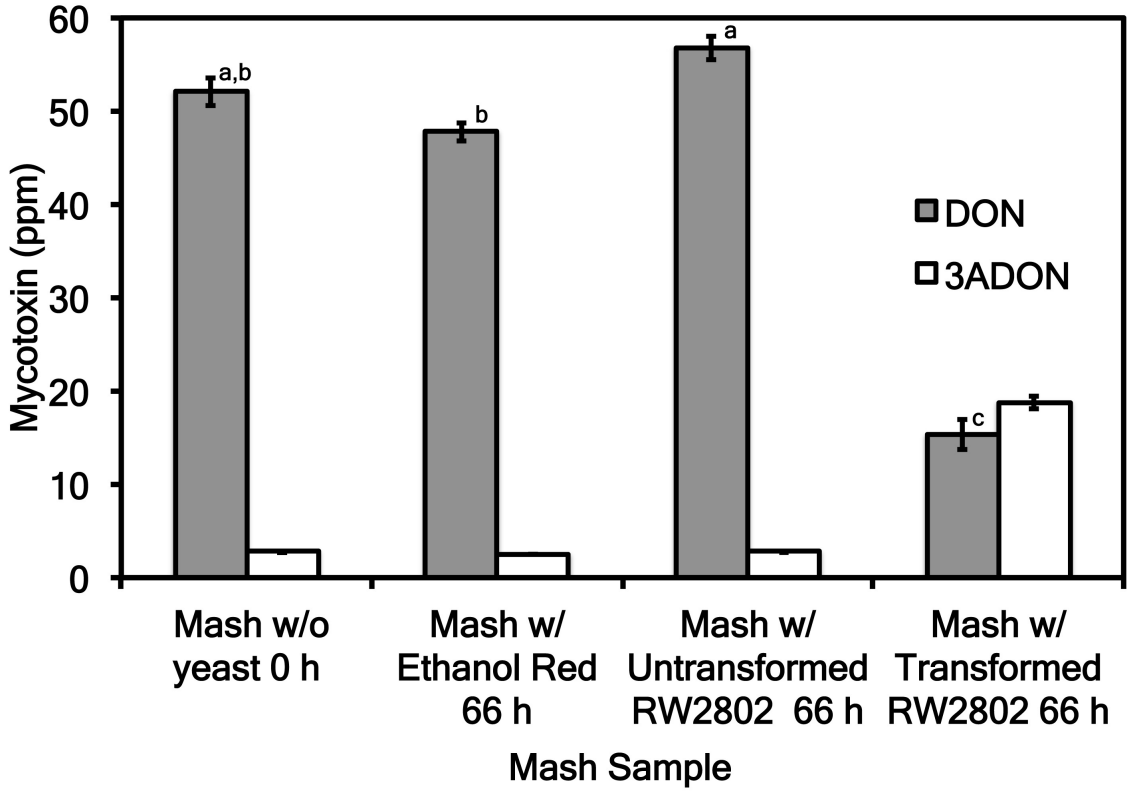

Figure 3. DON and 3ADON concentrations for VA06H-25 hulless barley fermentations containing galactose. Bars not connected by the same letter are significantly different. The concentration of DON in the mash with transformed RW2802 was significantly lower than both the starting mash without yeast and mashes containing Ethanol Red and untransformed RW2802 yeast ($P<0.05$).

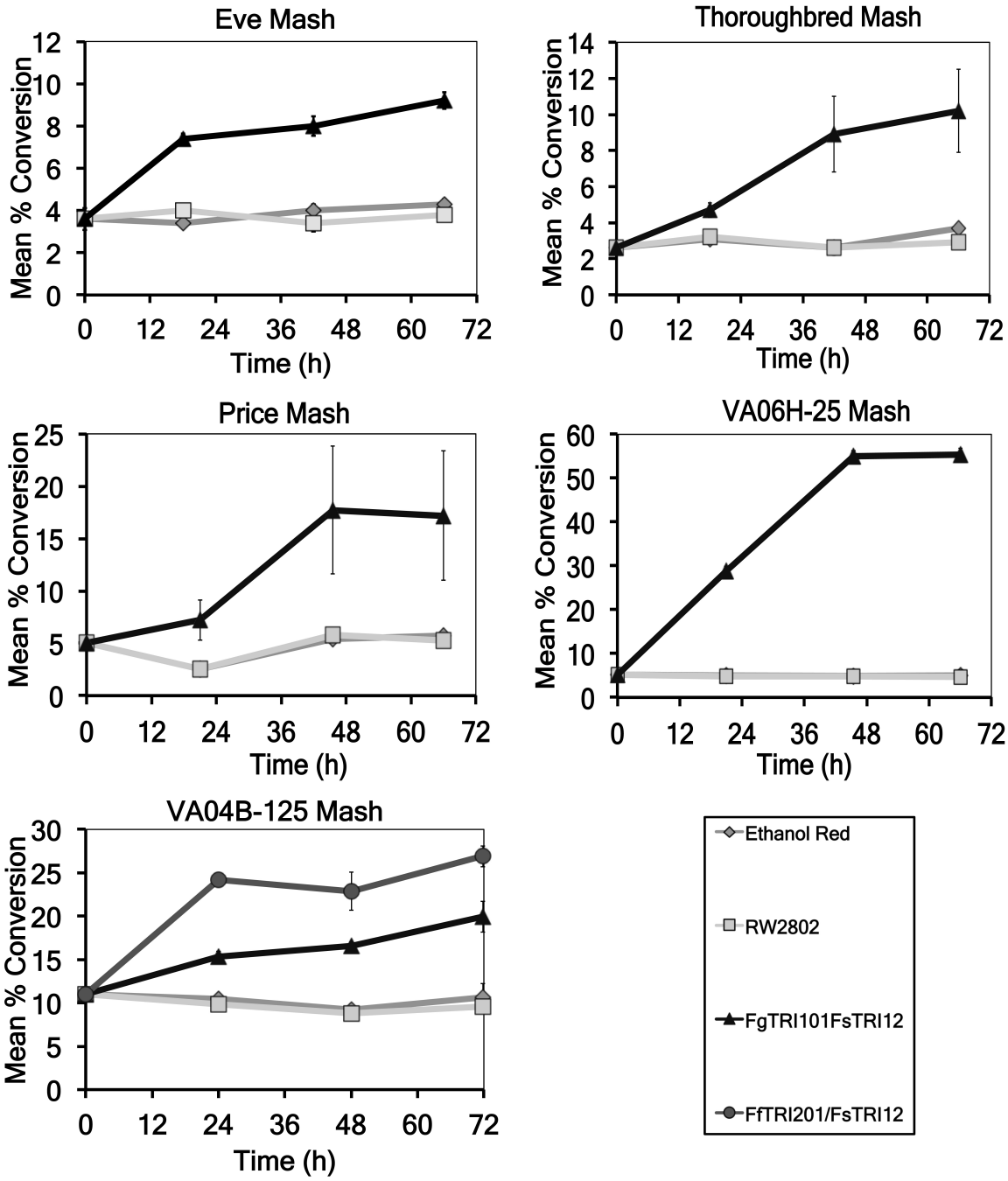

Figure 4. Mean percentage of DON converted to 3ADON for barley fermentations containing galactose. For each time point, the ± SEM is based on three replications.

Note that the Y-axes have been scaled for each of the barley genotypes. Study was

conducted for 66 hours and subsamples were taken at 0 h, 20 h, 44 h, and 66 h. In a comparison between two trichothecene 3-*O*-acetyltransferases, VA04B-125 mash samples were taken at 0 h, 23 h, 47 h, and 71 h. Mycotoxin extractions were performed on all subsamples and analyzed on the GC-MS.

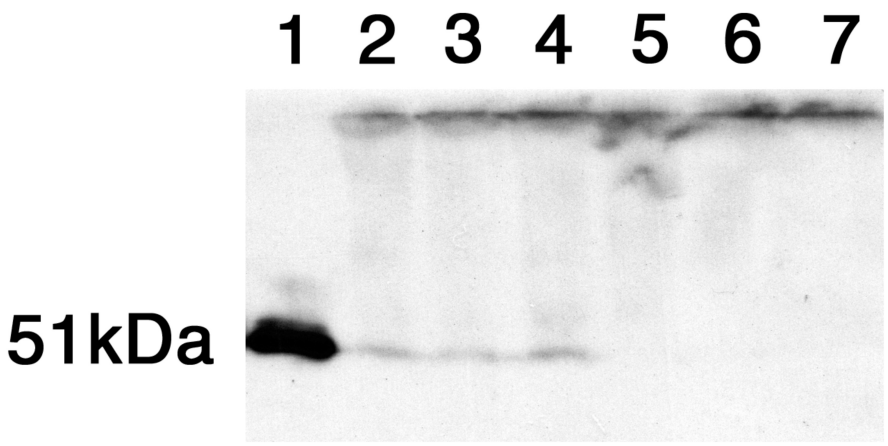

Figure 5. Western blot of TRI201 enzyme from *F. fujikuroi* (FfTRI201) extracted from VA04B-125 mashes. Mash samples weighing 1 g were collected at the end of fermentation (71 h) and protein extractions were performed. Mashes containing untransformed RW2802 were used as a negative control. Four microliters of each extract was loaded onto a 12% acrylamide-SDS-PAGE gel and run at 150V for 1 h. Precision plus protein dual color standard was used to determine protein size. The Western blot was probed with rabbit anti-FsTRI101 primary antibody and detected with alkaline phosphatase-conjugated anti-rabbit antibody. Lane designations are the following: (1) purified FfTRI201 from *E. coli*, (2) FfTRI201 from mash 1 containing transformed yeast, (3) FfTRI201 from mash 2 containing transformed yeast, (4) FfTRI201 from mash 3 containing transformed yeast, (5) mash 1 containing untransformed RW2802, (6) mash 2 containing untransformed RW2802, and (7) mash 3 containing untransformed RW2802.

LITERATURE CITED

Alexander N.J., McCormick S.P., Ziegenhorn S.L. (1999) Phytotoxicity of Selected Trichothecenes using *Chlamydomonas reinhardtii* as a Model System. Natural Toxins:265-269.

Bala N., Chimni S.S. (2010) Recent developments in the asymmetric hydrolytic ring opening of epoxides catalysed by microbial epoxide hydrolase. Tetrahedron: *Asymmetry* 21:2879-2898.

Berthiller F., Dall'Asta C., Schuhmacher R., Lemmens M., Adam G., Krska R. (2005) Masked mycotoxins: determination of a deoxynivalenol glucoside in artificially and naturally contaminated wheat by liquid chromatography-tandem mass spectrometry. Journal of agricultural and food chemistry 53:3421-5.

Bhat P.J. (2008) Galactose Regulon of Yeast Springer, Berlin, Germany.

Binder E.M. (2007) Managing the risk of mycotoxins in modern feed production. Animal feed science and technology 133:149-166.

Bondy G.S., McCormick S.P., Beremand M.N., Pestka J.J. (1991) Murine lymphocyte proliferation impaired by substituted neosolaniols and calonectrins--Fusarium metabolites associated with trichothecene biosynthesis. Toxicon : official journal of the International Society on Toxinology 29:1107-13.

Chesapeake-Bay-Commission. (2007) Biofuels and the Bay: Getting it Right to Benefit Farms, Forests, and the Chesapeake.

Diaz-Llano G., Smith T.K. (2006) Effects of feeding grains naturally contaminated with Fusarium mycotoxins with and without a polymeric glucomannan mycotoxin adsorbent on reproductive performance and serum chemistry of pregnant gilts. J Anim Sci 84:2361-6.

Drapcho C.M., Nghiem N.P., Walker T.H. (2008) Biofuels engineering process technology McGraw-Hill.

Eriksen G.S., Pettersson H., Lundh T. (2004) Comparatie cytotoxicity of deoxynivalenol, nivalenol, their acetylated derivatives and de-epoxy metabolites. Food and Chemical Toxicology 42:619-624.

Garvey G.S., McCormick S.P., Rayment I. (2008) Structural and functional characterization of the TRI101 trichothecene 3-O-acetyltransferase from Fusarium sporotrichioides and Fusarium graminearum: kinetic insights to combating Fusarium head blight. J Biol Chem 283:1660-9.

Goswami R.S., Kistler H.C. (2004) Heading for disaster: *Fusarium graminearum* on cereal crops. Molecular Plant Pathology 5:515-525.

Hazel C.M., Patel S. (2004) Influence of processing on trichothecene levels. Toxicol Lett 153:51-9.

Hicks K.B., Flores R.A., Taylor F., McAloon A.J., Moreau R.A., Johnston D.B. (2005) Hulless Barley-A New Feedstock for Fuel Ethanol?, 15th EPAC Meeting.

Ingledew W.M. (1999) Yeast--Could you base a business on this bug?, in: T. P. Lyons and K. A. Jacques (Eds.), Biotechnology in the Feed Industry, Nottingham University Press, Nottingham, UK. pp. 27-47.

Khatibi P.A., Newmister S.A., Rayment I., McCormick S.P., Alexander N.J., Schmale D.G., 3rd. (2011) Bioprospecting for trichothecene 3-o-acetyltransferases in the fungal genus fusarium yields functional enzymes with different abilities to modify the mycotoxin deoxynivalenol. Appl Environ Microbiol 77:1162-70.

Kim Y., Mosier N.S., Hendrickson R., Ezeji T., Blaschek H., Dien B., Cotta M., Dale B., Ladisch M.R. (2008) Composition of corn dry-grind ethanol by-products: DDGS, wet cake, and thin stillage. Bioresource technology 99:5165-76.

Kimura M., Kaneko I., Komiyama M., Takatsuki A., Koshino H., Yoneyama K., Yamaguchi I. (1998) Trichothecene 3-O-acetyltransferase protects both the producing organism and transformed yeast from related mycotoxins. Cloning and characterization of Tri101. J Biol Chem 273:1654-61.

Kimura M., Tokai T., Matsumoto G., Fujimura M., Hamamoto H., Yoneyama K., Shibata T., Yamaguchi I. (2003) Trichothecene nonproducer Gibberella species have both functional and nonfunctional 3-O-acetyltransferase genes. Genetics 163:677-84.

Kushnirov V.V. (2000) Rapid and reliable protein extraction from yeast. Yeast 16:857-60.

Lilly M., Lambrechts M.G., Pretorius I.S. (2000) Effect of increased yeast alcohol acetyltransferase activity on flavor profiles of wine and distillates. Applied and environmental microbiology 66:744-53.

Macreadie I.G., Jagadish M.N., Azad A.A., Vaughan P.R. (1989) Versatile cassettes designed for the copper inducible expression of proteins in yeast. Plasmid 21:147-50.

Madson P.W., Monceaux D.A. (1995) Fuel Ethanol Production. The Alcohol Textbook:227-238.

Magan N., Aldred D. (2007) Post-harvest control strategies: minimizing mycotoxins in the food chain. Int J Food Microbiol 119:131-9.

Mascorro-Gallardo J.O., Covarrubias A.A., Gaxiola R. (1996) Construction of a CUP1 promoter-based vector to modulate gene expression in Saccharomyces cerevisiae. Gene 172:169-70.

McChesney D. (2006) Nationwide Survey of Distillers Grains for Aflatoxins. U.S Food and Drug Administration.

McLaughlin C.S., Vaughn M.H., Campbell J.M., Wei C.M., Stafford M.E., Hansin B.S. (1977) Inhibition of protein synthesis by trichothecenes. Mycotoxins in Human and Animal Health:263-273.

McMullen M., Jones R., Gallenberg D. (1997) Scab of Wheat and Barley: A Re-emerging Disease of Devastating Impact. Plant Disease 81.

Mielenz J.R. (2001) Ethanol production from biomass: technology and commercialization status. Curr Opin Microbiol 4:324-9.

Mizutani K., Yoshioka S., Mizutani Y., Iwata S., Mikami B. (2011) High-throughput construction of expression system using yeast Pichia pastoris, and its application to membrane proteins. Protein expression and purification 77:1-8.

Muhitch M.J., McCormick S.P., Alexander N.J., Hohn T.M. (2000) Transgenic expression of the TRI101 or PDR5 gene increases resistance of tobacco to the phytotoxic effects of the trichothecene 4,15-diacetoxyscirpenol. Plant Sci 157:201-207.

Nghiem N., Hicks K., Johnston D., Senske G., Kurantz M., Li M., Shetty J., Konieczny-Janda G. (2010) Production of ethanol from winter barley by the EDGE (enhanced dry grind enzymatic) process. Biotechnol Biofuels 3:8.

Nicholson F.A., Chambers B.J., Williams J.R., Unwin R.J. (1999) Heavy metal contents of livestock feeds and animal manures in England and Wales. Bioresour Technol 70:23-31.

Ohsato S., Ochiai-Fukuda T., Nishiuchi T., Takahashi-Ando N., Koizumi S., Hamamoto H., Kudo T., Yamaguchi I., Kimura M. (2007) Transgenic rice plants expressing trichothecene 3-O-acetyltransferase show resistance to the Fusarium phytotoxin deoxynivalenol. Plant Cell Rep 26:531-8.

Ostergaard S., Roca C., Ronnow B., Nielsen J., Olsson L. (2000) Physiological studies in aerobic batch cultivations of Saccharomyces cerevisiae strains harboring the MEL1 gene. Biotechnol Bioeng 68:252-9.

Peska J.J. (2007) Deoxynivalenol: toxicity, mechanisms and animal health risks. Animal feed science and technology 137:283-298.

Pestka J.J., Smolinski A.T. (2005) Deoxynivalenol: toxicology and potential effects on humans. J Toxicol Environ Health B Crit Rev 8:39-69.

Poppenberger B., Berthiller F., Lucyshyn D., Sieberer T., Schuhmacher R., Krska R., Kuchler K., Glossl J., Luschnig C., Adam G. (2003) Detoxification of the Fusarium mycotoxin deoxynivalenol by a UDP-glucosyltransferase from Arabidopsis thaliana. J Biol Chem 278:47905-14.

Schaafsma A.W., Limay-Rios V., Paul D.E., Miller J.D. (2009a) Mycotoxins in fuel ethanol co-products derived from maize: a mass balance for deoxynivalenol. Journal of The Science of Food and Agriculture 89:1574-80.

Schaafsma A.W., Limay-Rios V., Paul D.E., Miller J.D. (2009b) Mycotoxins in fuel ethanol co-products derived from maize: a mass balance for deoxynivalenol. Science of Food and Agriculture 89:1574-80.

Schill S.R. (2008) Beefing Up Barley., Ethanol Producer Magazine. pp. 78-84.

Scientific Committee on Food. (1999) Opinion on *Fusarium* toxins. Part 1: Deoxynivalenol (DON). European Commission: Health & Consumer Protection Directorate-General.

Stepanik T., Kost D., Nowicki T., Gaba D. (2007) Effects of electron beam irradiation on deoxynivalenol levels in distillers dried grain and solubles and in production intermediates. Food Addit Contam 24:1001-6.

Stone C.W. (1998) Yeast Products in the Feed Industry: A Practical Guide for Feed Professionals. Diamond V Mills Inc, Cedar Rapids, IA.

Takahashi-Ando N., Tokai T., Hamamoto H., Yamaguchi I., Kimura M. (2005) Efficient decontamination of zearalenone, the mycotoxin of cereal pathogen, by transgenic yeasts through the expression of a synthetic lactonohydrolase gene. Appl Microbiol Biotechnol 67:838-44.

Thomason W.E., Griffey C.A., Alley M.M., Phillips S.B., Hagood E.S., Herbert D.A. (2005) Growing Hulless Barley in the Mid-Atlantic. Virginia Cooperative Extension.

Thompson W.L., Wannemacher R.W., Jr. (1986) Structure-function relationships of 12,13-epoxytrichothecene mycotoxins in cell culture: comparison to whole animal lethality. Toxicon : official journal of the International Society on Toxinology 24:985-94.

Trenholm H.L., Charmley L.L., Prelusky D.B., Warner R.M. (1992) Washing Procedures Using Water or Sodium Carbonate Solutions for the Decontamination of Three Cereals Contaminated with Deoxynivalenol and Zearalenone. Journal of Agriculture and Food Chemistry 40:2147-51.

Vietor R.J., Voragen G.J. (1993) Composition of non-starch polysaccharides in wort and spent grain from brewing trials with malt from a good malting quality barley and feed barley. The Journal of The Institute of Brewing 99:243-248.

Wu F., Munkvold G.P. (2008) Mycotoxins in ethanol co-products: modeling economic impacts on the livestock industry and management strategies. J Agric Food Chem 56:3900-11.

Young J.C., Fulcher R.G., Hayhoe H.J., Scott M.P., Dexter E.J. (1984) Effect of milling and baking deoxynivalenol (vomitoxin) content of eastern Canadian wheats. Journal of Agriculture and Food Chemistry 32:659-664.

CHAPTER IV

Resistance to Fusarium head blight and deoxynivalenol accumulation in Virginia barley

Piyum A. Khatibi*, Department of Plant Pathology, Physiology, and Weed Science, Virginia Tech, Blacksburg, VA 24061-0390; **Greg Berger*,** Department of Crop and Soil Environmental Sciences, Virginia Tech, 24061-0404; **Shuyu Liu,** Texas AgriLife Research, Texas A & M University, Amarillo, TX 79106; **Wynse S. Brooks**, Department of Crop and Soil Environmental Sciences, Virginia Tech, 24061-0404; **Carl A. Griffey,** Department of Crop and Soil Environmental Sciences, Virginia Tech, 24061-0404; **David G. Schmale III,** Department of Plant Pathology, Physiology, and Weed Science, Virginia Tech, Blacksburg, VA 24061-0390

*These authors contributed equally to this work and should be considered co-first authors

Corresponding author: D.G. Schmale III

Email: dschmale@vt.edu; Phone: (540) 231-6943; Fax: (540) 231-7477.

Note: The following chapter was formatted to facilitate publication in Plant Disease.

ATTRIBUTION

Piyum Khatibi was the lead writer on the manuscript and performed all DON extractions, DON quantification, and DON analyses and aided in the statistical analyses. Greg Berger performed the statistical work presented in this manuscript. Shuyu Liu performed ratings of FHB incidence and FHB severity. Wynse S. Brooks was involved in the development of the lines and cultivars used in this study. Carl A. Griffey and David G. Schmale III contributed to the design, helped coordinate experimental work, assisted in editing and writing the manuscript, and secured funding for the project.

For correspondence about the specific genotypes in this work, please contact CAG. For correspondence about the DON testing in this work, please contact DGS. This work was supported in part by grants to DGS from the Virginia Small Grains Board (proposal #10278306) and the U.S. Wheat and Barley Scab Initiative (proposal #07185403), and to CAG from the USWBSI (59-0790-4-102). The conclusions presented here are those of the authors and do not necessarily reflect the views of the United States Department of Agriculture or the Virginia Small Grains Board.

ABSTRACT

Khatibi, P.A., Berger, G., Liu, S., Brooks, W.S., Griffey, C.A., and Schmale, D.G., III. 2011. Resistance to Fusarium head blight and deoxynivalenol accumulation in Virginia barley. Plant Dis. XX: XXX-XXX.

Fusarium head blight (FHB), caused by the fungal plant pathogen *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae*), is a devastating disease of barley (*Hordeum vulgare* L.) in the United States. Recent epidemics of FHB in the mid-Atlantic region have underscored the need to develop new commercial varieties of barley that are resistant to FHB and that restrict accumulation of the mycotoxin deoxynivalenol (DON). FHB incidence, FHB index, and deoxynivalenol (DON) levels of Virginia hulled and hulless barley genotypes were evaluated over five years (2006-2010). FHB incidence ranged from 22.5% (2010) to 80.1% (2008), and mean DON levels ranged from 0.5±0.4 (2008) to 2.4±2.1 ppm (2010). Barley genotype played a significant role in determining FHB resistance in 2006-2009. DON levels were significantly different among barley genotypes in 2007, 2008, and 2009. FHB incidence was positively correlated with FHB index in all five years studied. In 2006 and 2010, FHB incidence and index were positively correlated with DON. Early spike emergence resulted in higher FHB incidence and index in 2007, 2008, and 2010. This preliminary work has identified some promising hulled and hulless barley genotypes for targeted breeding and commercialization efforts in the future; Eve (hulless) and Thoroughbred (hulled) ranked among the most FHB resistant genotypes.

INTRODUCTION

One of the most important diseases of barley (*Hordeum vulgare* L.) in the U.S. is Fusarium head blight (FHB), caused primarily by the fungal plant pathogen *Fusarium graminearum* Schwabe (teleomorph Gibberella *zeae*) (Goswami and Kistler, 2004). FHB can cause considerable reductions in yield and grain quality when the invading fungus releases cell wall degrading enzymes and mycotoxins (Kang and Buchenauer, 2000; Parry et al., 1995). *F. graminearum* produces a dangerous mycotoxin called deoxynivalenol (DON) that is released during infection (Mirocha et al., 1994). DON is a sesquiterpene epoxide that inhibits protein synthesis in eukaryotes (McLaughlin et al., 1977) and may cause vomiting and feed refusal in domestic animals (Pestka and Smolinski, 2005). From 1998 to 2000, barley contaminated with DON resulted in an economic loss of an estimated $136 million (Tiapo, 2001).

Since the 1990s, FHB screening programs have been set up to evaluate disease resistance and DON levels in barley (Steffenson, 1998). Disease levels are assessed by measuring FHB severity (average percentage of diseased spikelets per diseased spike), FHB incidence (the percentage of diseased heads out of all heads sampled), and FHB index (average percentage of diseased spikelets out of all heads sampled) (Paul et al., 2005). There are two primary types of resistance to FHB and DON in barley: Type I; resistance to initial infection, measured by incidence (Schroeder and Christensen, 1963), Type II; resistance to the movement and spread of the fungus (Schroeder and Christensen, 1963). Barley has natural type II resistance where disease spreading to adjacent spikelets is prevented by the rachis node and rachilla, although movement can still proceed through the phloem and rachis surface (Jansen et al., 2005).

Type I resistance in barley may be determined by measuring FHB incidence and FHB index and is critical to reducing FHB; however, FHB resistance is controlled by multiple genes

(Bai and Shaner, 2004), and coupling disease resistance with suitable agronomic and quality traits is a challenge (Dahleen et al., 2001). For example, in a cross between barley cultivars Chevron and M69, quantitative trait loci (QTLs) linked to FHB resistance were identified across seven chromosomes (de la Pena et al., 1999). Ten QTLs were associated with FHB resistance, 11 QTLs were linked to low DON accumulation, and 4 QTLs were coupled to kernel discoloration (de la Pena et al., 1999). In particular, the chromosome 2 (2H) bin10 region has been labeled as a major QTL for FHB resistance (Boyd et al., 2007). Chromosome 7H (de la Pena et al., 1999) and chromosome 6H (Canci et al., 2003) have been associated with low DON accumulation and low kernel discoloration, respectively (Bai and Shaner, 2004). There is evidence that FHB severity and DON levels in cereals are negatively correlated with plant height, days to heading, spike angle, and spike density (Urrea et al., 2002).

Epidemics of FHB in the mid-Atlantic (McMullen et al., 1997) and southeast (Cowger and Sutton, 2005) regions of the U.S. have underscored the need to develop new commercial varieties of barley that are resistant to FHB and accumulate low levels of DON. An increased knowledge of these traits will directly benefit growers and producers of barley in the eastern U.S. Here, we present the results of a five-year research effort focused on identifying promising barley genotypes that are resistant to FHB and accumulate low levels of DON. We hypothesized that: (1) FHB resistance is associated with reduced DON accumulation in barley and (2) specific barley genotypes show consistent year-to-year FHB resistance and reduced DON accumulation. The specific objective of our work is to identify genotypes of barley in Virginia that are resistant to FHB and reduced in their ability to accumulate DON. These genotypes could be used for targeted breeding and commercialization efforts in the future.

METHODS AND MATERIALS

DON and FHB assessments. Thirteen (2006) to 32 (2008) hulless and 22 (2009) to 37 (2010) hulled genotypes were planted in a randomized complete block with two replications in a mist-irrigated nursery at Blacksburg, VA over five years (2006-2010). Barley plants in the scab nursery were grown in 2-row plots with dimensions of 1.22 m long and 0.31 m between each row and were inoculated with aqueous suspensions of *Fusarium graminearum* macroconidia. Barley heads were spray inoculated with macroconidia (50,000 spores/ml) at approximately 50% anthesis (flowering). Plots were rated for FHB incidence (percentage of infected heads among 10 random heads per plot) and FHB severity (number of infected spikelets divided by the total number of spikelets for 10 diseased heads per plot). FHB severity data was used in the calculation of FHB index (FHB incidence x FHB severity/100).

Quantification of DON from barley samples. Extraction and quantification of DON from barley samples was conducted following standard protocols (Goswami and Kistler, 2005; Lagana et al., 2003; Tacke and Casper, 1996). Kernel samples of 100 g were run through a grinding mill and then passed through a number 20 sieve to maintain a uniform particle size. Sieved samples were weighed to 1 g and added to 4 ml solvent (86% acetonitrile/ 14% water) and placed on a shaker at 200 RPM for 1 hour. The solvent containing DON was then passed through an Alumina: C18 column (Alltech) for clean-up, and 1 ml of eluent was transferred to a glass test tube and evaporated to dryness using a nitrogen evaporator set at 55°C. Dried samples were then silyated with 50 μl of TMSI. After 15 minutes, 250 μl of isooctane was added to each tube followed by 250 μl of water to quench the reaction. Samples were vortexed for 30 seconds and

the supernatant was removed and transferred to chromatography vials for quantification using GC/MS.

In 2007, 2008, and 2009, DON was quantified from two replicates of each barley genotype. In 2006 and 2010, DON was quantified from the composite of two replicates. DON quantification was performed using an Agilent 6890/5975 system operating in selected ion-monitoring mode (SIM). This mode detected the DON target ion at a mass:charge ratio of 235, with reference ions at 259 and 422. The initial column temperature was held at 150°C for 1 min, and increased to 280°C at a rate of 30°C/min and held constant for 5 min. The injection temperature was set at 300°C, and the flow rate of the column was 1 ml/min. DON was quantified using a linear regression model for appropriate trichothecene standards (Biopure, Austria) of 0.01 ppm, 0.1 ppm, 1 ppm and 10 ppm.

Statistical analyses. PROC GLIMMIX in SAS (version 9.2; SAS Institute, Cary, NC) was used to test for significant differences across years in flowering date (FD), FHB incidence (INC), FHB index (IND), and DON concentration. Tukey-Kramer honestly significant difference (HSD) was used to test for all pairwise comparisons between factors. PROC CORR in SAS (version 9.2; SAS Institute, Cary, NC) was used to test for correlations between FHB incidence, FHB index, DON, and flowering date.

A subset of 10 (7 hulled and 3 hulless) genotypes were evaluated during all 5 years of the study and were analyzed to determine the stability of genotypes for FHB resistance across years. PROC GLIMMIX in SAS was used to test significant differences across years in flowering date (FD), FHB incidence (INC), FHB index (IND), and DON concentration, when controlling for year, genotype, and the interaction between genotype × year.

RESULTS

Analysis of Virginia winter barley genotypes. From 2006 to 2010, mean DON levels in Virginia barley genotypes ranged from 0.5 ppm to 2.4 ppm and mean flowering date ranged from 114.8 to 124.8 days **(Table 1)**. Mean FHB incidence and index were highest in 2008 at 80.1% and 38.0%, respectively, but had the lowest mean DON concentration at 0.5 ppm **(Table 1).** The lowest FHB incidence occurred in 2010 with an average rating of 22.5% **(Table 1)**. Mean FHB index was the lowest in 2006 at 4.8% **(Table 1)**.

To determine significant differences within each year for flowering date, incidence, index, and DON, the mean square errors were calculated from an ANOVA and significant differences were determined **(Table 2)**. Each year from 2006 to 2009, there were significant differences among genotypes for flowering date ($P \leq 0.01$), FHB incidence ($P \leq 0.01$), and FHB index ($P \leq 0.05$) **(Table 2)**. In 2010, significant differences among genotypes were observed for flowering date ($P \leq 0.001$) and FHB incidence ($P \leq 0.001$), but not FHB index ($P > 0.05$) **(Table 2)**. DON levels from 2007 to 2009 showed significant differences among barley genotypes ($P \leq 0.05$) **(Table 2)**. Significant differences were observed among replicates in 2007 (incidence and index), 2008 (DON), and 2009 (incidence and index) **(Table 2)**.

Correlations between FHB Incidence, FHB Index, and DON. Correlations were performed to investigate whether there was an association between FHB and DON levels in grain **(Table 3)**. For every year during the five-year study, there was a significant positive correlation between FHB incidence and index **(Table 3**). FHB incidence and index were positively correlated with DON levels in 2006 ($r = 0.44$, $P \leq 0.01$ and $r = 0.67$, $P \leq 0.001$, respectively) and in 2010 ($r = 0.82$ $P \leq 0.001$ and $r = 0.91$ $P \leq 0.001$, respectively) **(Table 3)**. However, from 2007 to 2009,

DON levels were not correlated with FHB incidence or index **(Table 3)**. In years 2007, 2008, and 2010, there was a significant negative association between flowering date with FHB incidence and index, with correlation coefficients ranging from -0.43 to -0.83 ($P \leq 0.01$) **(Table 3)**. In 2006 and 2009 there was no association between flowering date with FHB incidence and index **(Table 3)**.

Analysis of individual genotypes across all five years. A subset of 10 (7 hulled and 3 hulless) genotypes were analyzed to determine stability of genotypes for FHB resistance across years. Significant differences ($P \leq 0.001$) for year, genotypes, and genotype × year interactions were observed for flowering date, FHB incidence, and FHB index (**Table 4**). Significant differences ($P \leq 0.05$) for genotypes and genotype × year interaction were observed for DON concentration.

The hulless barley cultivar Eve was ranked in the top three for lowest FHB incidence (ranging from 7.5% in 2010 to 45% in 2007) (**Table 5**). Eve was ranked in the top four for lowest FHB index in 2006, 2008, 2009, and 2010 and had the lowest DON accumulation levels in years 2006, 2009 and 2010 (**Table 5**). The hulled barley cultivar Thoroughbred was consistently resistant to FHB from 2006 to 2009 with rankings in the top four for lowest FHB incidence (ranging from 7.5% in 2009 to 65% in 2008) and index (ranging from 3.5% in 2006 to 12.3% in 2008) (**Table 5**). Hulled barley cultivar Callao was consistently susceptible to FHB, with incidence ratings that were steadily in the bottom four each year (ranging from 20% in 2009 to 95% in 2008) (**Table 5**). Callao also demonstrated consistent high FHB index ratings (**Table 5**). Callao accumulated low levels of DON in 2008 and 2010, however, in the other three years DON levels were high with concentrations ranging from 1.40 ppm (ranked 7^{th}) to 2.28 ppm (ranked 10^{th}) (**Table 5**).

DISCUSSION

This study reported resistance to FHB and DON accumulation in Virginia barley across five years of field experiments (2006 to 2010). Susceptibility and resistance of Virginia barley genotypes to FHB and DON were determined by evaluating FHB incidence, FHB index, and DON concentrations. We also determined the effect of flowering date on these parameters. Barley genotypes played a significant role in determining the level of disease and DON accumulation in all years of the study **(Table 2)**, illustrating the impact individual barley lines/cultivars and their respective genetics and morphology have on the level of disease. In previous reports, resistant cultivars of wheat (*Triticum aestivum* L.) were shown to have lower levels of disease and DON compared to susceptible genotypes (Mesterhazy et al., 1999). Barley has natural type II resistance to FHB (Jansen et al., 2005), and differences in FHB disease between genotypes is therefore likely due to differences in type I resistance.

Significant differences among replicates in FHB incidence or FHB index may be due to differences in plot location of replicate genotypes within the field. For example, if one replicate of a particular barley genotype is close in proximity to a plot with high disease, movement of inoculum from adjacent plots may explain differences in disease ratings between replicates (Keller et al., 2010). Furthermore, orientation of the misting system and background inocula naturally present in the field (and different from the artificially applied inoculum in this study) may help explain some of the differences among replicates. Aggressiveness of *Fusarium* spp. can vary strain to strain and may depend on the mycotoxin potential of the strains used for inoculation (Mesterhazy, 2002); therefore, plots of susceptible barley genotypes may be exposed to different background inoculum sources (in addition to the artificial inoculum applied to the plots) and could demonstrate variable disease ratings and DON levels.

Mean DON concentration was lowest in 2008, while FHB incidence and index were highest in the same year **(Table 1)**. The three years with the lowest DON levels (2007-2009) did not correlate with FHB incidence or index **(Table 3)**. Differences in levels of resistance between genotypes of wheat have previously been shown to impact DON accumulation (Mesterhazy, 2002). Mesterhazy (2002) showed that susceptible wheat cultivars with low FHB contained equal DON levels to those of resistant cultivars with high FHB ratings. Associations between FHB and DON may not be evident when susceptible and moderately susceptible cultivars are analyzed for correlations in contrast to analyses that incorporate a range of susceptible and resistant genotypes (Bai and Shaner, 2004; Bai et al., 2001). Therefore, the population of barley genotypes within each year may play an important role in determining the association between DON concentration and FHB.

FHB and DON are thought to be positively correlated in wheat (Bai and Shaner, 2004). For instance, 116 cultivars and breeding lines in greenhouse tests and 33 cultivars in field tests were evaluated in 1997 for FHB resistance, and a positive correlation was found between FHB and DON (Bai et al., 2001). In a meta-analysis of 163 studies involving FHB and DON accumulation in wheat, correlation coefficients ranged from -0.58 to 0.99, and more than 65% of correlation coefficients were greater than 0.50 (Paul et al., 2005). This study revealed that FHB and DON accumulation are generally correlated, yet there are examples of negative associations (Paul et al., 2005). Cases of negative associations may be explained by the unintentional removal of small and light weight kernels from FHB infected spikes during threshing (Bai and Shaner, 2004). These badly damaged kernels, possibly containing high levels of DON, would not be included in DON measurements, thereby impacting associations with high ratings of FHB incidence and FHB index (Bai and Shaner, 2004). In our analysis, weak negative associations

between FHB and DON were seen from 2007 to 2009, however they were not significant **(Table 3)**. Significant negative correlations were observed between flowering date and FHB incidence and index **(Table 3)**. This is consistent with previous studies reporting negative correlations between heading date and FHB (Choo et al., 2004). However, since spray inoculation was used in this study, weather conditions (Xu, 2003) likely played a primary role in determining differences between flowering date and FHB than the timing of head emergence.

Efforts within the Virginia Tech winter barley breeding program are currently focused on further characterizing hulled and hulless genotypes for FHB resistance. Significant differences in FHB and DON resistance were observed among genotypes selected to evaluate resistance stability across years (**Table 4**). Fluctuations of FHB resistance and DON accumulation across years for individual genotypes may be contributed to factors such as variable weather conditions and plot locations within the field as described previously in this paper; however, stable genotypes were identified. Barley cultivar Thoroughbred (hulled) was identified as consistently being resistant to FHB while barley cultivar Eve (hulless) was identified as consistently resistant to both FHB and DON accumulation (**Table 5**). Barley cultivar Callao (hulled) was identified in this study as consistently being susceptible to FHB and accumulating high levels of DON (**Table 5**). This study sets the stage for further work and characterization of resistance in Virginia hulled and hulless barley. Genotypes with indications of resistance and susceptibility from this data will be further evaluated during the following growing seasons. Future efforts will be focused on the development of populations to map QTLs contributing to FHB and DON resistance in both hulled and hulless barley genotypes and develop hulled and hulless cultivars with improved FHB resistance.

TABLES

Table 1. Grand means ± standard error of the means for FHB incidence, index, DON concentration (ppm), and flowering date (FD), for all Virginia barley genotypes studied from 2006-2010.

	2006	**2007**	**2008**	**2009**	**2010**
No. Genotypes	39	49	64	52	63
FHB Incidence (%)	59.4±1.7	55.3±2.3	80.1±2.6	25.1±2.3	22.5±2.4
FHB Index (%)	4.8±0.3	11.9±0.9	38.0±2.8	5.6±0.8	5.3±0.8
DON (ppm)	2.1±0.4	1.3±0.1	0.5±0.1	1.5±0.2	2.4±0.3
FD (days from Jan 1st)	114.8±0.5	124.8±0.6	117.3±0.4	115.0±0.2	117.8±0.5

Table 2. Mean square errors (MSE) from analysis of variance for lines/cultivars within years and the respective degrees of freedom (DF).

Effect	DF	FD[a,b]	Incidence[b]	Index[b]	DON[b,c]
2006					
Rep	1	0.63	370.65	3.06	-
Genotype	38	17.00***	221.65***	8.85*	-
Residual	38	1.79	123.14	4.56	-
2007					
Rep	1	0.66	2158.43**	340.04**	1.74
Genotype	48	30.22**	521.42**	78.40**	0.86*
Residual	48	10.95	242.52	46.39	0.53
2008					
Rep	1	9.02	0.00	64.05	0.79*
Genotype	63	23.21***	832.46***	999.18***	0.33***
Residual	63	8.76	232.53	256.20	0.12
2009					
Rep	1	0.16	1144.53***	272.20***	1.39
Genotype	51	2.49***	535.46***	68.92***	3.43**
Residual	51	0.33	100.84	18.33	1.74
2010					
Rep	1	0.02	5.07	0.55	-
Genotype	62	26.63***	709.35***	79.32	-
Residual	62	2.50	253.34	55.47	-

[a] FD denotes flowering date (Days from Jan 1st)
[b] *,**,*** Significant at P≤0.05, 0.01, and 0.001
[c] DON for 2006 and 2010 was omitted from the analysis due to lack of replication.

Table 3. Correlation coefficients for flowering date (FD), FHB incidence, FHB index, and DON concentration from 2006 to 2010.

	Incidence[a]	Index[a]	DON[a]
	2006		
FD[b]	0.21	0.26	0.71***
Incidence		0.78***	0.44**
Index			0.67***
	2007		
FD	-0.43**	-0.54***	0.12
Incidence		0.74***	-0.12
Index			-0.04
	2008		
FD	-0.60***	-0.63***	0.05
Incidence		0.83***	-0.10
Index			-0.18
	2009		
FD	-0.10	-0.03	0.08
Incidence		0.94***	0.10
Index			0.07
	2010		
FD	-0.83***	-0.69***	-0.68***
Incidence		0.89***	0.82***
Index			0.91***

[a] *,**,*** Significant at P≤0.05, 0.01, and 0.001
[b] FD denotes flowering date (days from Jan 1st)

Table 4. Mean square errors (MSE) from analysis of variance for years, genotypes and genotype × year interaction for 10 selected barley genotypes (7 hulled and 3 hulless) across included in all five years of the study.

Effect	DF[a]	FD[b,c]	Incidence[c]	Index[c]	DF[c]	DON[c,d]
Year	4	317.79***	655.28***	315.28***	2	1.42
Year (Rep)	5	3.55	6.24	6.60	3	0.52
Genotype	9	46.93***	1298.74***	470.62***	9	2.70*
Genotype × Year	36	14.59***	701.03***	435.27***	18	3.54**
Residual	45	3.55	130.79	52.76	27	1.18

[a] DF denotes degrees of freedom
[b]FD denotes flowering date (Days from Jan 1st)
[c] *,**,*** Significant at P≤0.05, 0.01, and 0.001
[d] DON for 2006 and 2010 was omitted from the analysis due to lack of replication.

Table 5. Means and rank, separated by year, for 10 selected barley genotypes (7 hulled and 3 hulless) included in all five years of the study.

	2006	Rank	2007	Rank	2008	Rank	2009	Rank	2010	Rank
Line[a]	**Flowering Date (Days from Jan 1st)**									
BARSOY	110.0	1	118.0	1	112.5	2	114.0	3	119.0	4
EVE*	110.0	2	126.5	7	120.5	7	113.0	1	119.0	3
CALLAO	113.0	3	123.0	4	111.5	1	114.0	2	112.0	1
NOMINI	113.0	5	119.0	2	117.0	4	115.0	4	119.0	5
DOYCE*	113.0	4	128.5	9	117.0	5	115.5	7	119.0	7
VA92-42-46	114.0	6	125.0	5	115.5	3	117.0	9	119.0	9
PRICE	116.0	7	131.0	10	120.5	8	115.0	5	119.0	6
WYSOR	117.0	8	127.5	8	119.5	6	116.0	8	119.0	8
DAN*	118.0	9	121.0	3	123.5	9	115.0	6	121.0	10
THOROUGHBRED	119.0	10	125.0	6	124.0	10	117.0	10	118.0	2
Std error	1.0		3.3		2.2		0.5		1.0	
	FHB Incidence (%)									
EVE*	40.0	1	45.0	1	35.0	2	10.0	3	7.5	3
BARSOY	45.0	2	65.0	6	95.0	6	27.5	8	5.0	2
THOROUGHBRED	50.0	4	45.0	2	65.0	4	7.5	1	17.5	6
NOMINI	50.0	3	75.0	8	100.0	9	15.0	5	30.0	9
DAN*	55.0	5	65.0	7	30.0	1	20.0	6	1.0	1
WYSOR	60.0	7	50.0	3	100.0	8	7.5	2	17.5	7
VA92-42-46	60.0	6	85.0	10	100.0	10	10.0	4	30.0	8
PRICE	65.0	9	55.0	5	90.0	5	45.0	9	15.0	5
CALLAO	65.0	8	75.0	9	95.0	7	20.0	7	60.0	10
DOYCE*	70.0	10	50.0	4	55.0	3	92.5	10	11.0	4
Std. error	10.0		11.9		14.3		7.3		11.4	
	FHB Index (%)									
DAN*	2.7	1	13.2	5	14.8	3	4.5	7	0.1	1
EVE*	3.0	2	13.4	6	5.0	1	1.3	3	1.1	4
THOROUGHBRED	3.5	3	5.2	1	12.3	2	1.0	2	3.5	7
VA92-42-46	4.0	4	21.2	9	90.5	10	2.3	4	4.0	8
WYSOR	4.2	5	5.3	2	78.0	9	0.9	1	2.4	6
NOMINI	4.2	6	24.2	10	72.0	8	3.8	5	6.0	9
CALLAO	4.3	7	16.6	7	55.0	7	4.0	6	10.5	10
BARSOY	4.9	8	21.1	8	50.0	6	4.8	8	1.0	3
PRICE	5.5	9	12.9	4	43.3	5	11.5	9	1.8	5
DOYCE*	9.4	10	8.7	3	15.5	4	35.3	10	0.6	2
Std. Error	2.4		6.6		13.3		4.4		2.6	
	DON (ppm)[b]									
EVE*	0.3	1	1.1	6	0.8	9	0.5	1	0.7	2
BARSOY	0.5	2	0.5	1	0.2	4	2.1	6	0.8	4
NOMINI	0.7	3	0.7	3	0.6	8	2.5	7	1.0	7
DAN*	0.9	4	1.6	9	0.0	1	0.6	4	0.5	1
VA92-42-46	1.0	5	0.5	2	0.1	3	1.4	5	0.7	3
DOYCE*	1.2	6	2.4	10	1.7	10	0.6	2	0.9	5
CALLAO	1.4	7	1.5	8	0.1	2	0.6	3	2.3	10
WYSOR	2.4	8	0.8	4	0.2	5	2.5	8	0.9	6
PRICE	3.1	9	1.0	5	0.3	7	5.8	9	2.0	9
THOROUGHBRED	4.4	10	1.2	7	0.3	6	6.0	10	1.9	8

Std. Error	-	0.5	0.3	1.8	-

[a] Barely cultivars or lines followed by an * indicate a hulless genotype; those without a star indicate a hulled genotype

[b] Standard error for DON in 2006 and 2010 was not reported due to lack of replication.

LITERATURE CITED

Bai G., Shaner G. (2004) Management and resistance in wheat and barley to fusarium head blight. Annu Rev Phytopathol 42:135-61.

Bai G.H., Plattner R., Desjardins A.E., Kolb F. (2001) Resistance to Fusarium head blight and deoxynivalenol accumulation in wheat. Plant Breeding 120:1-6.

Boyd C., Horsley R., Kleinhofs A. (2007) Barley chromosome 2(2H) Bin 10 fusarium head blight resistance QTL: Mapping and Development of isolines., United States Wheat and Barley Scab Initiative, Kansas City, Missouri.

Canci P., Nduulu L., Dillmacky R., Muehlbauer G.J., Rasmusson D.C. (2003) Genetic relationships between kernel discoloration and grain protein concentration in barley. Crop Science 43:1671-1679.

Choo T.M., Martin R.A., Ho K.M., Shen Q.Q., Fedak G., Savard M., Voldeng H., Falk D.E., Etienne M., Sparry E. (2004) Fusarium Head Blight and Deoxynivalenol Accumulation of Barley in Eastern Canada: Cultivar Response and Correlation Analysis. Plant Disease 88:837-844.

Cowger C., Sutton A.L. (2005) The Southeastern U.S. Fusarium Head Blight Epidemic of 2003. Plant Health Progress.

Dahleen L.S., Okubara P.A., Blechl A.E. (2001) Transgenic Approaches to Combat Fusarium Head Blight in Wheat and Barley. Crop Science 41:621-637.

de la Pena R.C., Smith K.P., Capettini F., Muehlbauer G.J., Gallo-Meagher M., Dill-Macky R., Somers D.A., Rasmusson D.C. (1999) Quantitative trait loci associated with resistance to Fusarium head blight and kernel discoloration in barley. Theoretical and Applied Genetics 99:561-569.

Goswami R.S., Kistler H.C. (2004) Heading for disaster: *Fusarium graminearum* on cereal crops. Molecular Plant Pathology 5:515-525.

Goswami R.S., Kistler H.C. (2005) Pathogenicity and In Planta Mycotoxin Accumulation Among Members of the Fusarium graminearum Species Complex on Wheat and Rice. Phytopathology 95:1397-404.

Jansen C., von Wettstein D., Schafer W., Kogel K.H., Felk A., Maier F.J. (2005) Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted Fusarium graminearum. Proc Natl Acad Sci U S A 102:16892-7.

Kang Z., Buchenauer H. (2000) Ultrastructural and Cytochemical Studies on Cellulose, Xylan and Pectin Degradation in Wheat Spikes Infected by *Fusarium culmorum*. J. Phytopathology 148:263-275.

Keller M.D., Waxman K.D., Bergstrom G.C., Schmale III D.G. (2010) Local Distance of Wheat Spike Infection by Released Clones of *Gibberella zeae* Disseminated from Infested Corn Residue. Plant Disease 94:1151-1155.

Lagana A., Curini R., D'Ascenzo G., De Leva I., Faberi A., Pastorini E. (2003) Liquid chromatography/tandem mass spectrometry for the identification and determination of trichothecenes in maize. Rapid Commun Mass Spectrom 17:1037-43.

McLaughlin C.S., Vaughn M.H., Campbell J.M., Wei C.M., Stafford M.E., Hansin B.S. (1977) Inhibition of protein synthesis by trichothecenes. Mycotoxins in Human and Animal Health:263-273.

McMullen M., Jones R., Gallenberg D. (1997) Scab of Wheat and Barley: A Re-emerging Disease of Devastating Impact. Plant Disease 81.

Mesterhazy A. (2002) Role of deoxynivalenol in aggressiveness of *Fusarium graminearum* and *F. culmorum* and in resistance to *Fusarium* head blight. European Journal of Plant Pathology 108:675-684.

Mesterhazy A., Bartok T., Mirocha C.G., Komoroczy R. (1999) Nature of wheat resistance to *Fusarium* head blight and the role of deoxynivalenol for breeding. Plant Breeding 118:97-110.

Mirocha C.J., Xie W., Xu Y., Wilcoxson R.D., Woodward R.P., Etebarian R.H., Behele G. (1994) Production of trichothecene mycotoxins by Fusarium graminearum and Fusarium culmorum on barley and wheat. Mycopathologia 128:19-23.

Parry D., Jenkinson P., McLeod L. (1995) Fusarium ear blight (scab) in small grain cereals-a review. Plant Pathology 44:207-238.

Paul P.A., Lipps P.E., Madden L.V. (2005) Relationship Between Visual Estimates of Fusairum Head Blight Intensity and Deoxynivalenol Accumulation in Harvested Wheat Grain: A Meta-Analysis. Ecology and Epidemiology 95:1225-1236.

Pestka J.J., Smolinski A.T. (2005) Deoxynivalenol: toxicology and potential effects on humans. J Toxicol Environ Health B Crit Rev 8:39-69.

Schroeder H.W., Christensen J.J. (1963) Factors affecting resistance of wheat to scab by *Gibberella zeae*. Pytopathology 53:831-838.

Steffenson B. (1998) *Fusarium* head blight of barley: epidemics, impact, and breeding for resistance. *Tech* 35:177-184.

Tacke B.K., Casper H.H. (1996) Determination of deoxynivalenol in wheat, barley, and malt by column cleanup and gas chromatography with electron capture detection. J AOAC Int 79:472-5.

Tiapo N.M. (2001) Economic Impacts of Fusarium Head Blight in Wheat and Barley: 1998-2000.

Urrea C., Horsley R., Steffenson B., Schwarz P. (2002) Heritability of Fusarium Head Blight Resistance and Deoxynivalenol Accumulation from Barley Accession CIho 4196. Crop Science 42.

Xu X. (2003) Effects of environmental conditions on the development of *Fusarium* ear blight. European Journal of Plant Pathology 109:683-689.

CHAPTER V

Callus production and transformation methods of Virginia winter barley

Piyum A. Khatibi[1]

[1]*Virginia Polytechnic Institute and State University, Department of Plant Pathology, Physiology, and Weed Science, Blacksburg, VA 24061*

ABSTRACT

Barley (*Hordeum vulgare* L.) contaminated with the mycotoxin deoxynivalenol (DON) is a threat to the health of humans and domestic animals and transgenic barley expressing a detoxification gene may mitigate the threat of DON. We determined the tissue culture potential of 47 Virginia hulled and hulless barley genotypes. Over 2,350 immature barley embryos from these genotypes were assessed for their ability to develop callus on callus induction medium over a period of 21 days. The percent callus formation across all of the barley genotypes ranged from 1.8% (VA04B-180) to 97.9% (Eve). The FHB susceptible Virginia barley cultivar Doyce produced callus from 49.1% of plated immature embryos and was chosen for transformation with a trichothecene 3-*O*-acetyltransferase gene called *TRI101*. The trichothecene mycotoxin deoxynivalenol (DON) is a dangerous mycotoxin that contaminates feed and food barley products. TRI101 modifies DON to a less toxic product (3ADON), and was isolated from the fungal plant pathogen *Fusarium graminearum* (*FgTRI101*). *FgTRI101* was gateway cloned into the binary vector pBract214, and five hundred immature embryos were inoculated with *Agrobacterium* strain AGL1 harboring *FgTRI101*. Of these five hundred, only two hundred immature embryos developed into callus tissue and thirteen successfully formed roots and shoots and grew into plants. Attempts to verify transgenic barley plants via PCR in the first generation (T_o) were unsuccessful, and 904 T_1 kernels were susceptible to hygromycin. Though we failed to generate transgenic barley plants expressing TRI101, we (1) demonstrated that different barley genotypes have varying abilities to produce callus tissue and (2) established a system for barley transformation that could lead to transgenic plants in the future.

INTRODUCTION

Fusarium head blight (FHB) is a major disease of wheat (*Triticum aestivum* L.) and barley that has caused economic losses of billions of dollars in the United States (Nganje et al., 2001). *Fusarium graminearum*, a fungal plant pathogen, is the etiological agent of FHB, which produces a dangerous mycotoxin called deoxynivalenol (DON) and reduces grain yield and quality (Mirocha et al., 1994; Parry et al., 1995). Many agricultural research programs in the U.S are focused on reducing the threat of FHB by breeding resistance or by genetic modification. For example, the chromosome 2 (2H) bin10 region has been identified as a strong resistance quantitative trait loci (QTL) for FHB resistance (Boyd et al., 2007). Alternatively, there are many gene candidates that may be introduced into the genomes of wheat and barley that may confer FHB resistance (Dahleen et al., 2001). Fungal cell wall-degrading enzymes such as endo- and exochitinases as well as β-1,3-glucanases offer antifungal activity. Additionally, small proteins such as thionins and thaumatin-like proteins have been demonstrated to disrupt fungal membranes (Dahleen et al., 2001). DON is a potent protein synthesis inhibitor (McLaughlin et al., 1977) that has been demonstrated to increase aggressiveness of fungal pathogens of wheat and barley (Hestbjerg et al., 2002; Mesterhazy, 2002). Expression of a modified ribosomal protein (RPL3), a target of DON, conferred trichothecene resistance in tobacco (Mitterbauer et al., 2004). However, these noted resistance genes do not directly reduce or detoxify any released DON.

Trichothecene 3-*O*-acetyltransferases called TRI101 and TRI201, convert DON into an acetylated derivative called 3ADON (3-acetyl deoxynivalenol) (Garvey et al., 2008). This conversion reduces DON levels and produces a derivative (3ADON), which is less toxic to plants and animals (Alexander et al., 1999; Kimura et al., 1998; Peska, 2007).

TRI101 has been transformed into numerous plant systems (Alexander, 2008). For example, TRI101 from *F. sporotrichoides* (FsTRI101) reduced the phytotoxic effects of trichothecenes in tobacco (Muhitch et al., 2000) and in rice (Ohsato et al., 2007). FsTRI101 has also been transformed into wheat and barley; however, only partial resistance was shown in wheat (Okubara et al., 2002) and barley resistance was only demonstrated in greenhouse assays but not in field trials (Manoharan et al., 2006). A potential reason why FsTRI101 has not been effective in wheat and barley may be due to the low affinity of the enzyme towards DON (Garvey et al., 2008). In contrast, TRI101 from *F. graminearum* (FgTRI101) is better able to bind DON because of structural differences between FsTRI101 and FgTRI101 (Garvey et al., 2008). In fact, FgTRI101 requires 85 times less DON to reach the half-maximal rate (K_m) (Garvey et al., 2008). FgTRI101 and other trichothecene 3-*O*-acetyltransferases may yield better enzymes destined for plant transformation (Khatibi et al., 2011).

Particle bombardment and *Agrobacterium*-mediated transformation are two common methods that may be employed to genetically engineer barley (Travella et al., 2005). *Agrobacterium*-mediated transformation has several important advantages over particle bombardment. For instance, particle bombardment tends to result in higher silencing rates due to the integration of multiple transgene copies (Travella et al., 2005). For example, work by Travella et al., (2005) reported that 100% of their *Agrobacterium*-derived lines integrated between one and three copies of the transgene whereas 60% of lines from particle bombardment incorporated more than eight copies. Travella et al., (2005) also reported that Mendelian segregation patterns were only present in three of the nine barley lines transformed via particle bombardment while Mendelian inheritance and an absence of transgene silencing was shown in the majority of *Agrobacterium*-derived lines. Furthermore, transformation efficiencies with

Agrobacterium were double that of particle bombardment (Travella et al., 2005). For both transformation techniques, the location of insertion cannot be dictated and has important effects on gene stability (Salvo-Garrido et al., 2004). Linked transgenes may be expressed at different levels even when driven under the same promoter due to the epigenetic state of the flanking DNA regions (Halpin, 2005). Salvo-Garrido et al., (2004) used fluorescence *in situ* hybridization (FISH) to physically map transgene insertion sites. This was followed by genetic mapping, showing that integration was nonrandom and was primarily in gene-rich regions. Integration patterns are also important when introducing multiple genes into a plant genome. When multiple genes are introduced into plants, they tend to co-integrate at the same chromosomal position (Halpin, 2005). Introduction of multiple genes may be necessary to achieve the desired effect. For example, Golden rice was developed to increase the production of provitamin A for malnourished populations and was created by the co-transformation of two T-DNA's (Ye et al., 2000). Co-transformation of multiple transgenes can be performed by co-inoculation of plant tissue with two *Agrobacterium* cultures containing different T-DNAs (Halpin, 2005).

Plants have a unique ability to initiate cell division from almost any tissue and are considered totipotent (Slater et al., 2003). This ability allows plants, such as monocots, to generate transformed plants from callus (Slater et al., 2003). Cultures are generated from pieces of a whole plant, called explants (Slater et al., 2003). Scutellar cells of immature embryo explants produce callus and are the targets for barley transformation (Dahleen et al., 2001). Since the advent of the first genetically modified dicotyledonous species reported in 1987, which provided tobacco with insecticidal resistance (Vaeck et al., 1987), plant transformation has been used extensively to improve agricultural crops. Monocots, such as barley, constitute a large

portion of agricultural crops and therefore agricultural researchers work on improving this commodity through genetic engineering (Shrawat et al., 2007).

Immature embryos have been determined to be the best explant for barley (Tingay et al., 1997). Since the establishment of utilizing immature embryos as explants for callus development for the transformation of barley by Tingay et al., (1997), co-cultivation of immature embryos with *Agrobacterium* has become a major strategy used for transformation. Callus development from immature embryos may vary based on genotype or cultivar of barley chosen for transformation (Cheng et al., 2004).

The spring cultivar Golden Promise has been used in the majority of barley transformation experiments (Cheng et al., 2004). Few studies have involved alternative elite cultivars but have included Australian cultivars Schooner, Sloop, and Chebec, which generated transformed callus at a frequency of 47 to 76% (Murray et al., 2004). Callus tissue was also generated from the four Nordic cultivars Anni, Elo, Kymppi and Teele (Tiidema and Truve, 2004). When working with a large number of barley genotypes, determining which genotypes are efficient callus producers is necessary before transformation can proceed. In fact, unsuccessful transformants may not be the failure of *Agrobacterium* but rather the inability to generate callus, thereby demonstrating the importance of barley genotype in the development of transgenic barley (Shrawat et al., 2007).

Here, we demonstrate the ability of different Virginia barley lines and cultivars to produce callus necessary for generating transgenic barley plants. This work sets the stage for the development of the barley cultivar Doyce expressing a trichothecene 3-*O*-acetyltransferase isolated from *Fusarium graminearum* (FgTRI101) to enhance resistance against FHB and DON.

METHODS AND MATERIALS

Tissue culture media used in this study. All media used in this study is based on the protocol from Bartlett et al., (2008). Callus Induction Medium (CI) contained 4.3gl^{-1} Murashige & Skoog plant salt base with vitamins and glycine (Caissonlabs), 30 g/L Maltose, 1.0 g/L Casein hydrolysate, 350 mg/L Myo-inositol, 690 mg/L Proline, 1.0 mg/L Thiamine HCl, 2.5 mg/L Dicamba, 0.5 ml Plant Preservative Mixture (PPM) (Plant Cell Technology), pH 5.8, 3.5 g/L Phytagel. Shoot Initiation/transition Medium: 2.7g/L Murashige & Skoog modified plant salt base (without NH_4NO_3), 20g/L Maltose, 165 mg/L NH_4NO_3, 1.25 mg/L $CuSO_4*5H_2O$, 750mg/L Glutamine, 100 mg/L Myo-inositol, 0.4 mg/L Thiamine HCl, 2.5 mg/l 2,4-D, 0.1 mg/L BAP, pH 5.8, 3.5 g/L Phytagel. Regeneration Medium: 2.7g/L Murashige & Skoog modified plant salt base (without NH_4NO_3), 20g/L Maltose, 165 mg/L NH_4NO_3, 750mg/L Glutamine, 100 mg/L Myo-inositol, 0.4 mg/L Thiamine HCl, pH 5.8, 3.5 g/L Phytagel. Rooting Medium: 4.3 g/L Murashige & Skoog plant salt base, 30 g/L Maltose, 1.0 g/L Casein hydrolysate, 350 mg/L Myo-inositol, 690 mg/L Proline, 1.0 mg/L Thiamine HCl, pH 5.8, 3.5 g/L Phytagel.

Collection of plant material for tissue culture assays. Barley plants were grown at Virginia Tech's Kentland farm in Blacksburg, VA. Winter barley kernels were sown in October 2006 and began heading in April 2007. The 19 hulless genotypes and 28 hulled genotypes studied are noted in **Table 1**. Five spikes from each genotype were removed from primary tillers 16 to 21 days post-anthesis. For each spike, 15 spikelets were chosen randomly. Hulls were removed by hand and naked kernels were placed in a sterile 15 ml conical tube.

Plating of embryos for callus formation. Kernels were surface disinfested with 70% ethanol for 1 minute and 20% bleach for 20 minutes. Kernels were then rinsed 5 times with sterilized deionized water. At the fifth rinsing cycle, the water was kept in the conical tube until the moment of embryo excision. Following rinsing, ten embryos were excised per spike with fine tipped forceps and placed on CI medium. Embryos were incubated at 24°C in the dark for 21 days.

Callus development and measurement. Immediately following placement of the immature embryo on callus induction medium, the length of each of the embryos was measured in millimeters. A stereomicroscope and a transparent ruler were used to measure callus development from each of the embryos every 7 days for 21 days. The size of the callus at its longest length was measured and recorded at each of the time points.

Statistical analyses. Analysis of variance (ANOVA) on the mean percent callus production and the callus length for each barley genotype was performed using the statistical program JMP 9.0.0 (Cary, NC).

Isolation of immature barley embryos for barley transformation. Kernels from Virginia hulless barley cultivar 'Doyce' were surface disinfested with 70% ethanol for 1 minute and 20% bleach for 5 minutes in sterile 50ml conical tubes. Kernels were then rinsed 5 times with sterilized deionized water. At the fifth rinsing cycle, the water was kept in the conical tube until the moment of embryo excision. The embryonic axis was removed from all embryos and

discarded. Embryos were then temporarily plated on a plate containing 2% water agar until *Agrobacterium* inoculation.

***FgTRI101* cloning into pBract214.** *FgTRI101*, an intronless gene, was directly amplified from the genomic DNA of *F. graminearum* strain T1S1 using primers FgTRI101_FWD (5'-ATGGCTTTCAAGATACAGCTCG-3') and FgTRI101_REV (5'-CTAACCAACGTACTGCGCATACT-3'). *Fusarium* genomic DNA was isolated using the DNeasy Plant Mini Kit following manufacturer's protocols (Qiagen, Germantown, MD). PCR amplifications were carried out in a 50 μl reaction volume using Easy-A High-Fidelity PCR Cloning Enzyme (Stratagene, La Jolla, CA) as described by Khatibi et al., (2011). *FgTRI101* was TOPO cloned into entry vector pCR8/GW/TOPO (Invitrogen, Carlsbad, CA), following Invitrogen's pCR8/GW/TOPO Cloning Kit's recommended protocol. Successful ligations were transformed into TOP10 *E.coli* cells for propagation of pCR8/GW/TOPO containing *FgTRI101*. The plasmid pBract214 (**Figure 1**) (John Innes Centre, Norwich Research Park, Norwich, UK) was propagated in ccdB resistant *E.* coli DB3.1 cells. FgTRI101 was then gateway cloned into pBract214 using Invitrogen's LR Clonase II, following the manufacturer's recommended protocol. PCR using primers FgTRI101_FWD and FgTRI101_REV, as described above, checked insertion of FgTRI101 into the pBract214 plasmid. Correct orientation was verified by sequencing (Virginia Bioinformatics Institute core laboratory facility (VBI) Blacksburg, VA) from the ubiquitin promoter of the pBract214 plasmid using the primer pBract214_UBI_FWD (5'-GGATGATGGCATATGCAGCA-3'). Reactions were then transformed into DH5α *E.coli* cells (Invitrogen, Carlsbad, CA).

Transformation of *Agrobacterium* with *FgTRI101*. The protocol for *Agrobacterium* transformation was based on the Cold Spring Harbor (2006) method, which was adapted from Shen and Ford (1989) and Mersereau et al., (1990). *FgTRI101* in pBract214, were electroporated into *Agrobacterium tumefaciens* strain AGL1 together with the helper plasmid pSoup (John Innes Centre, Norwich Research Park, Norwich, UK), which is required by the pBract plasmids for replication. A 5 ml culture of *Agrobacterium* was started and grown overnight. The following day, a microcentrifuge was used to pellet the cells at 5000 revolutions per minute (RPM) for 10 minutes at room temperature. After centrifugation, the pellet was washed with sterile 1x TE buffer. Cells were resuspended in 500 µl of Luria-Bertani (LB) medium and distributed in 50 µl aliquots into microcentrifuge tubes. Cells were stored at -80°C until electroporation. To begin transformation, cells were thawed on ice and 500 µg of pBract vector and 500 µg pSoup were added to the cells and mixed together on ice. The mixture was transferred to a pre-chilled electroporation cuvette. The electroporator (BioRad MicroPulser, Hercules, CA) was set with a voltage of 2.4 kV and a pulse length of 5 mili-seconds. Before electroporating it was important to make sure there was no moisture on the outside of the cuvette. Immediately following electroporation, 1 ml of LB medium was added to the cuvette and this mixture was transferred to a 15 ml conical tube and incubated for 4 h at 28°C at 100 RPM. Cells were collected by centrifugation and plated on LB agar plate containing kanamycin (50 µg/ul) and rifampcin (50 µg/ul) for 2 to 3 days at 28°C.

Embryo-*Agrobacterium* inoculations. Using a pipette, full strength suspensions of transformed *Agrobacterium* strain AGL1 containing either *FgTRI101* were drip inoculated onto each embryo. To remove as much excess *Agrobacterium* culture as possible from each embryo, embryos were

dragged across the 2% water agar medium and then transferred to callus induction medium. Embryos were incubated in the dark at 24°C and co-cultivated for three days. Following three days of cultivation, embryos were transferred to fresh callus induction medium containing 150 mg/L Timentin and 50 mg/L Hygromycin B, and returned to dark incubation at 24°C. Calli were subcultured every 14 days to fresh callus induction medium containing 150 mg/L Timentin and 50 mg/L Hygromycin B for 6 to 12 weeks.

Generation of plantlets from callus. Shoot initiation, regeneration, and rooting media were prepared as described by Bartlett et al., (2008). Following 6 to 12 weeks of callus development, barley calli were transferred to shoot initiation/transition medium (**Figure 2**). Once shoots were established, plant material was plated onto regeneration medium in which shoots further developed (**Figure 2**). After sufficient shoot development, barley plantlets were placed on rooting medium (**Figure 2**). Following plentiful root development, plantlets were transferred to a sterile environment inside a magenta box containing soil (Sun-Gro Metro-Mix 360) (**Figure 2**). Plantlets were grown at 24°C with 16 h light and 8 h dark at 80 $\mu m^2 s^{-1}$ of light. Once plantlets became more developed, they were transferred to a larger incubator and covered in plastic wrap to acclimate the plants to drier conditions (**Figure 2**). Plants grew at the red light spectrum, by having the incandescent lamps turned on and having the fluorescent lamps turned off, to simulate dawn and dusk. To simulate daytime conditions, both lamps were on for 12.5 h with up to 420 $\mu m^2 s^{-1}$ of light. Plants were in complete darkness for 5 h. Every few days, holes were punctured in the plastic wrap to allow for more airflow. The plastic wrap was completely removed after 2 weeks and barley plants grew to heading following six weeks of vernalization at 4°C.

Selection of tentative transgenic plants. Genomic DNA was extracted from small portions of leaf tissue that were taken from different areas of T_o plants (**Figure 3**) using the DNeasy Plant Mini Kit following the manufacturer's protocols (Qiagen, Germantown, MD). PCR on genomic DNA was conducted using MyTaq DNA polymerase (Bioline, Tauton, MA) and FgTRI101 forward and reverse primers under the following conditions: one cycle at 95°C for 1 min; 35 cycles of 95°C for 15 s, 47°C for 15 s, and 72°C for 20 s. PCR on genomic DNA was also conducted using Phire Plant Direct PCR kit (Finnzymes, Vantaa, Finland) under the following conditions: one cycle at 98°C for 5 min; 40 cycles of 98°C for 5 s, 47°C for 5 s, and 72°C for 25 s, and one cycle of final extension at 72°C for 1 minute.

To screen for T_1 transformants, T_1 kernels were plated on rooting media containing 50 mg/L hygromycin to test for successful transformants and propagation of transgene into reproductive tissue. These kernels were taken from heads grown from T_0 barley plants that were derived from callus developed from *Agrobacterium*-inoculated immature embryos.

RESULTS

Virginia barley callus formation. In our 21-day study, we examined the abilities of immature embryos from 28 hulled and 19 hulless Virginia barley genotypes to produce callus. Of the 47 barley genotypes, eleven did not form callus, and of those that were able to produce callus, the percent formation ranged from 1.8% (VA04B-180) to 97.9% (Eve) (**Table 1**). In an analysis between hulled and hulless genotypes, there was no difference in the means for percent callus formation ($P = 0.15$). Mean callus length ranged from 1.0 mm ('Barsoy') to 7.8 mm (VA96-44-

304) (**Table 1**). There were significant differences between barley genotypes in the length of callus after three weeks ($P < 0.0001$).

***Agrobacterium* embryo inoculations, callus development, and differentiation.** Five hundred immature embryos from the Virginia barley cultivar Doyce were excised out of kernels and inoculated with *Agrobacterium* strain AGL1 containing *FgTRI101* in the plasmid pBract214. Of these five hundred, only two hundred immature embryos developed into callus tissue and thirteen successfully formed roots and shoots and grew into plants.

Screening T_o and T_1 for *FgTRI101*. From each of the 13 tentative transgenic plants, leaves from different areas were tested by PCR for the presence or absence of *FgTRI101* (**Figure 3**). Preliminary PCR data suggested evidence of *FgTRI101* within the barley genome (data not shown); however, successive DNA extractions from corresponding plants and leaves were unable to repeat the positive results. In spite of the inconsistent data through repetitive DNA extractions and PCR reactions, T_1 kernels from four heads of each plant, totaling 904 kernels, were plated on selection medium containing hygromycin B. Plants susceptible to hygromycin displayed a phenotype of slowed aerial growth, reduced central root length, and root hair growth compared to seedlings grown on non-selective medium (**Figure 4**). Unfortunately, no hygromycin resistant plants were identified following this screen likely due to the failure to develop transformed plants expressing the hygromycin resistance gene (*hpt*).

DISCUSSION

Development of callus is important for generation of transgenic barley, and our study demonstrated that different barley genotypes vary in their ability to generate undifferentiated tissue. Barley genotype was the most important factor in determining the generation of friable and regenerable callus in an analysis of 15 different barley genotypes (Bregitzer, 1992). Friable and compact calli are favored during production of callus cultures (Tiidema and Truve, 2004). In another study involving 24 spring barley genotypes, callus production ranged from 88 to 100% depending on genotype (Halamkova et al., 2004). We used a media and transformation protocol that was developed using the barley cultivar Golden Promise (Bartlett et al., 2008), and only 6.5% of the calli in this study regenerated into plants. Media components may also influence the ability of different genotypes to develop callus and regenerate into a plant (Dahleen and Bregitzer, 2002). Changes in the concentrations of copper (electron-transfer reactions) (Dahleen, 1995), ammonium nitrate (nitrogen source for protein production) (Nuutila et al., 2000), 2,4-dichlorophenoxy acetic acid (root development) (Bregitzer et al., 1998), benzylaminopurine (shoot development) (Cho et al., 1998), boric acid (plant growth and development), and iron (II) sulfate (electron-transfer) may impact regeneration abilities (Dahleen and Bregitzer, 2002; Slater et al., 2003).

We were unsuccessful in transforming the barley cultivar Doyce with FgTRI101. Plant genotype may largely determine how amenable a barley line or cultivar is to genetic transformation (Cheng et al., 2004). Other factors include explant type, the *Agrobacterium* strain used, and the binary vector (Cheng et al., 2004). The majority of reports on successful generation of transgenic barley are based on the highly regenerable spring barley cultivar Golden Promise (Dahleen and Bregitzer, 2002; Tiidema and Truve, 2004), and many barley lines and

cultivars are recalcitrant to genetic modification (Cho et al., 1998; Koprek et al., 1996). Perhaps a barley genotype other than Doyce should be considered as a future candidate for transformation with FgTRI101.

A preliminary round of PCRs was performed to verify *FgTRI101* in the barley cultivar Doyce and amplicons were generated (data not shown). However, PCR amplifications were inconsistent and most PCR reactions did not yield amplicons. These conflicting results may have been a result of chimeric plants. Plant transformation involving callus culturing may produce chimeric plants (Harwood et al., 2000; Khachatourians, 2002) which develop from transformed and untransformed callus cells coexisting on selection media (Hiei et al., 1994). Although less common during somatic embryogenesis than organogenesis, having one or more cells with differing genotypes contribute to a shoot primordium is possible, and may lead to the production of chimeric plants (Khachatourians, 2002).

There are many potential modifications to the protocol used in this study, which may yield more success. Antinecrotic treatments on explants may improve explant viability by reducing the effects of any oxidative burst events that occur from co-culture with *A. tumefaciens* (Cheng et al., 2004). Antinecrotic treatments have increased the recovery of transformed callus cells in rice (Enriquez-Obregon et al., 1999) and increased stable transformation frequency in maize (Frame et al., 2002). Osmotic treatments on explant tissue have been shown to enhance stable transformation of particle bombardment mediated transformation (Vain et al., 1993) and have also been used to enhance *Agrobacterium* mediated transformation of maize and rice (Hiei et al., 1994; Zhao et al., 2001). Desiccation of plant tissue following *Agrobacterium* inoculation is another potential protocol modification which has been shown to enhance T-DNA delivery in wheat (Cheng et al., 2003). Desiccation inhibits the growth of *A. tumefaciens*, which allowed

better cell recovery and higher transformation efficiency (Cheng et al., 2003). Surfactants may also be employed to increase T-DNA delivery, with the notion of increasing the probability of *Agrobacterium* attachment to the explant (Cheng et al., 2004). Incorporating the surfactants Silwet L77 and pluronic acid F68 in inoculation medium improved transformation efficiency in immature wheat embryos (Cheng et al., 1997). Amending co-culture medium with acetosyringone, a chemical released following plant wounding, may increase infection and T-DNA delivery. Transformation in this study, based on the methods of Bartlett et al., (2008), did not find acetosyringone necessary for high transformation efficiencies in their transformation of spring barley cv. Golden Promise; however, numerous studies have reported the benefits of using acetosyringone during co-cultivation (Hensel et al., 2008; Hiei et al., 1994; Shrawat et al., 2007; Zhao et al., 2000) and should be considered in future transformation experiments.

Alternative transformation methods are available and include floral dipping into *Agrobacterium* liquid culture (Zale et al., 2009) and infecting *in vitro* cultured ovules with *Agrobacterium* (Holme et al., 2006). In 2009, transformation via floral dip was reported in wheat with a transformation efficiency of 0.44%. The target tissue for wheat transformation was not determined in this study but was likely the ovule, as is the target in *Arabidopsis* (Desfeux et al., 2000). Transformation was conducted during the early boot stage (uninucleate microspore stage) when the unemerged spike is 6 to 7 cm in length (Zale et al., 2009). Holme et al., (2006) reported the transformation of cultured ovules of the barley cultivar Golden Promise. Ovaries were removed following pollination, dissected to expose the ovule, and then placed onto co-cultivation medium (Holme et al., 2006). The transformation efficiency for this method was 3.1% (Holme et al., 2006). Tissue culturing is a time consuming process and these two methods

may help circumvent the numerous tissue culturing difficulties experienced and described in this project.

Though we failed to generate transgenic barley plants expressing TRI101, we (1) demonstrated that different barley genotypes have varying abilities to produce callus tissue and (2) established a preliminary system for barley transformation that upon optimization could lead to transgenic plants in the future.

TABLES

Table 1. Percent callus formation and the mean callus length at the end of our 21 day study for 28 hulled and 19 hulless Virginia barley genotypes. There was no difference between the means for percent callus formation between hulled and hulless barley ($P = 0.15$). Callus formation ranged from 1.8% (VA04B-180) to 97.9% (Eve).

Barley Line/Cultivar	Hull Phenotype	# of Calli	No. of Immature Embryos Plated	% Callus Formation	21 d Mean±SD Callus Length (mm)
EVE	Hulless	46	47	97.9%	6.7±2.5
VA03B-171	Hulled	40	42	95.2%	5.8±1.1
VA04H-25	Hulless	46	51	90.2%	5.3±1.4
VA04H-114	Hulless	48	54	88.9%	5.3±1.5
VA05H-59	Hulless	46	55	83.6%	6.0±1.6
VA04H-53	Hulless	42	55	76.4%	4.0±1.2
VA04B-127	Hulled	40	53	75.5%	5.1±1.2
H-585	Hulless	30	42	71.4%	6.7±2.2
Thoroughbred	Hulled	37	55	67.3%	5.4±1.4
VA05H-162	Hulless	34	52	65.4%	4.4±1.1
VA04B-8	Hulled	35	54	64.8%	6.4±1.5
VA03B-25	Hulled	32	55	58.2%	4.5±1.0
VA04B-178	Hulled	28	50	56.0%	6.8±3.0
Doyce	Hulless	27	55	49.1%	3.8±1.1
VA92-42-46	Hulled	25	51	49.0%	5.2±1.0
Callao	Hulled	15	31	48.4%	5.0±2.4
VA04B-93	Hulled	24	51	47.1%	5.4±1.6
VA04H-113	Hulless	21	55	38.2%	5.5±1.5
VA03H-64	Hulless	19	51	37.3%	4.3±1.1
VA05B-141	Hulled	20	54	37.0%	4.8±1.8
VA04B-7	Hulled	19	55	34.5%	4.5±1.5
VA04B-62	Hulled	18	57	31.6%	3.8±2.1
Nomini	Hulled	16	54	29.6%	4.9±2.0
VA05H-161	Hulless	16	55	29.1%	5.4±1.3
VA05B-64	Hulled	14	53	26.4%	3.9±0.9
DAN	Hulless	13	55	23.6%	5.0±1.6
VA03B-58	Hulled	8	48	16.7%	5.5±1.9
VA01H-125	Hulless	8	53	15.1%	3.7±0.7
Wysor	Hulled	8	55	14.5%	4.4±1.8
VA05H-159	Hulless	7	51	13.7%	5.2±1.0

VA96-44-304	Hulled	5	48	10.4%	7.8±2.1
VA03H-100	Hulless	4	53	7.5%	5.0±1.8
VA04H-111	Hulless	2	49	4.1%	2.8±0.4
Barsoy	Hulled	1	41	2.4%	1.0±0.0
VA03B-176	Hulled	1	52	1.9%	3.0±0.0
VA04B-180	Hulled	1	55	1.8%	7.0±0.0
VA04B-29	Hulled	0	20	0.0%	0.0
VA04B-125	Hulled	0	29	0.0%	0.0
VA05B-98	Hulled	0	54	0.0%	0.0
VA04B-54	Hulled	0	55	0.0%	0.0
Price	Hulled	0	57	0.0%	0.0
VA04B-95	Hulled	0	28	0.0%	0.0
VA04B-120	Hulled	0	59	0.0%	0.0
VA05B-97	Hulled	0	54	0.0%	0.0
VA05H-147	Hulless	0	44	0.0%	0.0
VA05H-120	Hulless	0	52	0.0%	0.0
VA05H-158	Hulless	0	54	0.0%	0.0

FIGURES

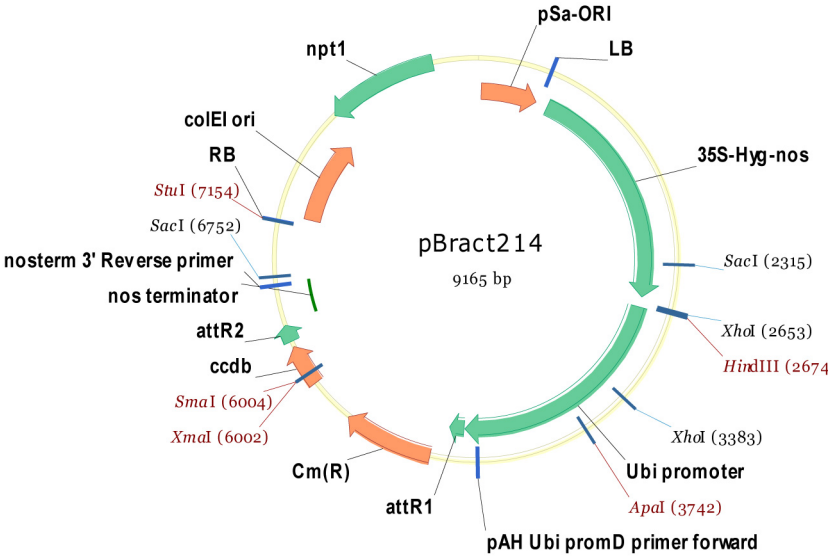

Figure 1. Binary vector pBract214. The pBract214 vector contains the hygromycin phosphotransferase (*hpt)* gene driven by the 35S promoter for hygromycin resistance for selection of successfully transformed plants. *FgTRI101* was gateway cloned into the vector and is driven by the maize ubiquitin promoter.

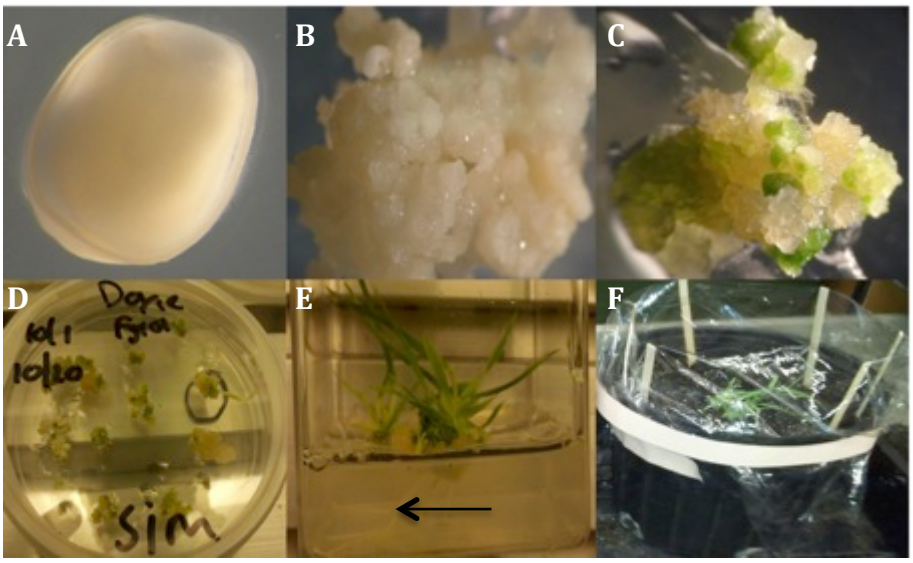

Figure 2. Different stages of barley transformation and differentiation of callus to vegetative tissue. First, an (**A**) immature barley embryo is plated on callus induction medium and inoculated with a small drop of *Agrobacterium* suspension culture and co-cultivated for 3 days at 24°C. (**B**) Calli developed for 6 to 12 weeks. After this time, (**C, D**) calli were transferred to shoot initiation/transition medium to initiate shoot differentiation, then regeneration medium for shoot development, and once shoots were established, plant material was plated onto regeneration medium in which shoots further developed. After sufficient shoot development, (**E)** barley plantlets were placed on rooting medium. Following plentiful root development (arrow), (**F**) plantlets were transferred to a larger incubator and covered in plastic wrap in which holes were created every few days to acclimate the plants to drier conditions.

Photographs taken by Piyum Khatibi

Figure 3. Small portions of leaf tissue were taken from different areas of T_o plants (as shown with black arrows). DNA was extracted from these plant pieces, and PCR was performed to check for the presence of *FgTRI101*.

Photograph taken by Piyum Khatibi

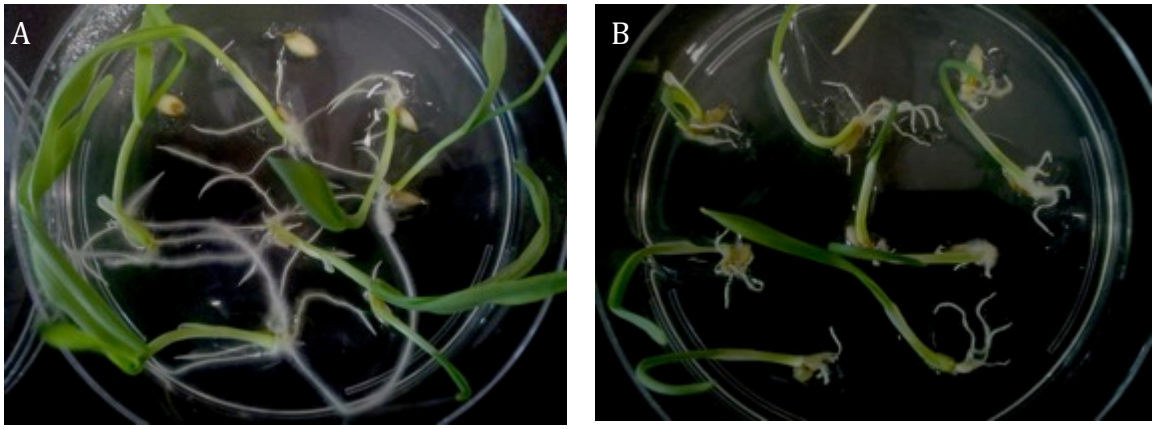

Figure 4. Representative picture showing the effects of hygromycin on wild-type Doyce seedlings. (**A)**, wild-type Doyce on non-selective MS rooting medium and **(B)**, wild-type Doyce on MS rooting medium containing 50mg/L hygromycin. Susceptible seedlings on selective medium displayed reduced aerial growth and reduced central root length and root hair growth compared to seedlings grown on non-selective medium. These demonstrated phenotypes are important in selection of successful transformants containing the hygromycin resistance gene, *hpt*.

Photograph taken by Piyum Khatibi

LITERATURE CITED

Alexander N.J. (2008) The TRI101 story: engineering wheat and barley to resist Fusarium head blight. World Mycotoxin Journal 1:31-37.

Alexander N.J., McCormick S.P., Ziegenhorn S.L. (1999) Phytotoxicity of Selected Trichothecenes using *Chlamydomonas reinhardtii* as a Model System. Natural Toxins:265-269.

Bartlett J.G., Alves S.C., Smedley M., Harwood W.A. (2008) High-throughput Agrobacterium-mediated barley transformation. Plant Methods 4:1-12.

Boyd C., Horsley R., Kleinhofs A. (2007) Barley chromosome 2(2H) Bin 10 fusarium head blight resistance QTL: Mapping and Development of isolines., United States Wheat and Barley Scab Initiative, Kansas City, Missouri.

Bregitzer P. (1992) Plant Regeneration and Callus Type in Barley: Effects of Genotype and Culture Medium. Crop Science 32:1108-1112.

Bregitzer P., Dahleen L.S., Campbell R.D. (1998) Enhancement of plant regeneration from embryogenic callus of commercial barley cultivars. Plant Cell Rep. 17:941-945.

Cheng M., Fry J.E., Pang S., Zhou H., Hironaka C.M., Duncan D.R., Conner T.W., Wan Y. (1997) Genetic Transformation of Wheat Mediated by Agrobacterium tumefaciens. Plant Physiology 115:971-980.

Cheng M., Hu T., Layton J., Liu C.-N., Fry J. (2003) Desiccation of plant tissues post-*Agrobacterium* infection enhances T-DNA delivery and increases stable transformation efficiency in wheat. In Vitro Cellular & Developmental Biology - Plant 39:595-604.

Cheng M., Lowe B.A., Spencer M., Ye X., Armstrong C.L. (2004) Factors Influencing *Agrobacterium*-Mediated Transformation of Monocotyledonous Species. In Vitro Cellular & Developmental Biology - Plant 40:31-45.

Cho M.-J., Jiang W., Laemaux P.G. (1998) Transformation of recalcitrant barley cultivars through improvement of regenerability and decreased albinism. Plant Science 138:229-244.

Dahleen L.S. (1995) Improved plant regeneration from barley callus cultures by increased copper levels. Plant Cell, Tissue and Organ Culture 43:267-269.

Dahleen L.S., Bregitzer P. (2002) An Improved Media System for High Regeneration Rates from Barley Immature Embryo-Derived Callus Cultures of Commericial Cultivars. Crop Science 42:934-938.

Dahleen L.S., Okubara P.A., Blechl A.E. (2001) Transgenic Approaches to Combat Fusarium Head Blight in Wheat and Barley. Crop Science 41:621-637.

Desfeux C., Clough S.J., Bent A.F. (2000) Female reproductive tissues are the primary target of Agrobacterium-mediated transformation by the Arabidopsis floral-dip method. Plant Physiology 123:895-904.

Enriquez-Obregon G.A., Prieto-Samsonov D.L., de la Riva G.A., Perez M., Selman-Housein G., Vazquez-Padron R.I. (1999) *Agrobacterium*-mediated Japonica rice transformation: a procedure assisted by an antinecrotic treatment. Plant Cell Tissue and Organ Culture 59:159-168.

Frame B.R., Shou H., Chikwamaba R.K., Zhang Z., Xiang C., Fonger T.M., Pegg S.E.K., Li B., Nettleton D.S., Pei D., Wang K. (2002) *Agrobacterium tumefaciens*-Mediated Transformation of Maize Embryos Using a Standard Binary Vector System. Plant Physiology 129:13-22.

Garvey G.S., McCormick S.P., Rayment I. (2008) Structural and functional characterization of the TRI101 trichothecene 3-O-acetyltransferase from Fusarium sporotrichioides and Fusarium graminearum: kinetic insights to combating Fusarium head blight. J Biol Chem 283:1660-9.

Halamkova E., Vagera J., Ohnoutkova L. (2004) Regeneration capactiy of calli derived from immature embryos in spring barley cultivars. Biologia Plantarium 48:313-316.

Halpin C. (2005) Gene stacking in transgenic plants--the challenge for 21st century plant biotechnology. Plant biotechnology journal 3:141-55.

Harwood W.A., Ross S.M., Cilento P., Snape J.W. (2000) The effect of DNA/gold particle preparation technique, and particle bombardment device, on the transformation of barley (*Hordeum vulgare*). Euphytica 111:67-76.

Hensel G., Valkov V., Middlefell-Williams J., Kumlehn J. (2008) Efficient generation of transgenic barley: the way forward to modulate plant-microbe interactions. Journal of plant physiology 165:71-82.

Hestbjerg H., Felding G., Elmholt S. (2002) *Fusarium culmorum* Infection of Barley Seedlings: Correlation between Aggressiveness and Deoxynivalenol Content. Phytopathology 150:308-312.

Hiei Y., Ohta S., Komari T., Kumashiro T. (1994) Efficient transformation of rice (Oryza sativa L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. The Plant journal : for cell and molecular biology 6:271-82.

Holme I.B., Brinch-Pedersen H., Lange M., Holm P.B. (2006) Transformation of barley (Hordeum vulgare L.) by Agrobacterium tumefaciens infection of in vitro cultured ovules. Plant cell reports 25:1325-35.

Khachatourians G.G. (2002) Transgenic plants and crops CRC Press, Honolulu, USA.

Khatibi P.A., Newmister S.A., Rayment I., McCormick S.P., Alexander N.J., Schmale D.G., 3rd. (2011) Bioprospecting for trichothecene 3-o-acetyltransferases in the fungal genus fusarium yields functional enzymes with different abilities to modify the mycotoxin deoxynivalenol. Appl Environ Microbiol 77:1162-70.

Kimura M., Kaneko I., Komiyama M., Takatsuki A., Koshino H., Yoneyama K., Yamaguchi I. (1998) Trichothecene 3-O-acetyltransferase protects both the producing organism and transformed yeast from related mycotoxins. Cloning and characterization of Tri101. J Biol Chem 273:1654-61.

Koprek T., Hansch R., Nerlich A., Mendel R.R., Schulze J. (1996) Fertile transgenic barley of different cultivars obtained by adjustment of bombardement conditions to tissue response. Plant Science 119:79-91.

Manoharan M., Dahleen L.S., Hohn T.M., Neate S.M., Yu X.-H., Alexander N.J., McCormick S.P., Bregitzer P., Schwarz P.B., Horsley R.D. (2006) Expression of 3-OH trichothecene acetyltransferase in barley (*Hordeum vulgare* L.) and effects on deoxynivalenol. Plant Science:699-706.

McLaughlin C.S., Vaughn M.H., Campbell J.M., Wei C.M., Stafford M.E., Hansin B.S. (1977) Inhibition of protein synthesis by trichothecenes. Mycotoxins in Human and Animal Health 19:263-273.

Mesterhazy A. (2002) Role of deoxynivalenol in aggressiveness of *Fusarium graminearum* and *F. culmorum* and in resistance to *Fusarium* head blight. European Journal of Plant Pathology 108:675-684.

Mirocha C.J., Xie W., Xu Y., Wilcoxson R.D., Woodward R.P., Etebarian R.H., Behele G. (1994) Production of trichothecene mycotoxins by Fusarium graminearum and Fusarium culmorum on barley and wheat. Mycopathologia 128:19-23.

Mitterbauer R., Poppenberger B., Raditschnig A., Lucyshyn D., Lemmens M., Glossl J., Adam G. (2004) Toxin-dependent utilization of engineered ribosomal protein L3 limits trichothecene resistance in transgenic plants. Plant Biotechnol J 2:329-40.

Muhitch M.J., McCormick S.P., Alexander N.J., Hohn T.M. (2000) Transgenic expression of the TRI101 or PDR5 gene increases resistance of tobacco to the phytotoxic effects of the trichothecene 4,15-diacetoxyscirpenol. Plant Sci 157:201-207.

Murray F., Brettell R., Matthews P., Bishop D., Jacobsen J. (2004) Comparison of Agrobacterium-mediated transformation of four barley cultivars using the GFP and GUS reporter genes. Plant cell reports 22:397-402.

Nganje W.E., Johnson D.D., Wilson W.W., Leistritz F.L., Bangsund D.A., Tiapo N.M. (2001) Economic Impacts of Fusarium Head Blight in Wheat and Barley: 1998-2000 North Dakota State University, Fargo, ND.

Nuutila A.M., Hamalainen J., Mannonen L. (2000) Optimization of media nitrogen and copper concentrations for regeneration of green plants from polyembroygenic cultures of barley (*Hordeum vulgare* L.). . Plant Sci 151:85-92.

Ohsato S., Ochiai-Fukuda T., Nishiuchi T., Takahashi-Ando N., Koizumi S., Hamamoto H., Kudo T., Yamaguchi I., Kimura M. (2007) Transgenic rice plants expressing trichothecene 3-O-acetyltransferase show resistance to the Fusarium phytotoxin deoxynivalenol. Plant Cell Rep 26:531-8.

Okubara P.A., Blechl A.E., McCormick S.P., Alexander N.J., Dill-Macky R., Hohn T.M. (2002) Engineering deoxynivalenol metabolism in wheat through the expression of a fungal trichothecene acetyltransferase gene. Theor Appl Genet 106:74-83.

Parry D., Jenkinson P., McLeod L. (1995) Fusarium ear blight (scab) in small grain cereals-a review. Plant Pathology 44:207-238.

Peska J.J. (2007) Deoxynivalenol: toxicity, mechanisms and animal health risks. Animal feed science and technology 137:283-298.

Salvo-Garrido H., Travella S., Bilham L.J., Harwood W.A., Snape J.W. (2004) The distribution of transgene insertion sites in barley determined by physical and genetic mapping. Genetics 167:1371-9.

Shrawat A.K., Becker D., Lorz H. (2007) *Agrobacterium tumefaciens*-mediated genetic transformation of barley (*Hordeum vulgare* L.). Plant Science 172:281-290.

Slater A., Scott N.W., Fowler M.R. (2003) Plant biotechnology: the genetic manipulation of plants, Leicester, UK.

Tiidema A., Truve E. (2004) Efficient regeneration of fertile barley plants from callus cultures of several Nordic cultivars. Hereditas 140:171-176.

Tingay S., McElroy D., Kalla R., Fieg S., Wang M., Thornton S., Brettell R. (1997) Agrobacterium tumefaciens-mediated barley transformation. The Plant Journal 11.

Travella S., Ross S.M., Harden J., Everett C., Snape J.W., Harwood W.A. (2005) A comparison of transgenic barley lines produced by particle bombardment and Agrobacterium-mediated techniques. Plant Cell Rep 23:780-9.

Vaeck M., Reynaerts A., Hofte H., Jansens S., De Beuckeleer M., Dean C., Zabeau M., Van Montagu M., Leemans J. (1987) Transgenic plants protected from insect attack. Nature 328.

Vain P., McMullen M.D., Finer J.J. (1993) Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize. Plant Cell Rep. 12:84-88.

Ye X., Al-Babili S., Kloti A., Zhang J., Lucca P., Beyer P., Potrykus I. (2000) Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. Science 287:303-5.

Zale J.M., Agarwal S., Loar S., Steber C.M. (2009) Evidence for stable transformation of wheat by floral dip in Agrobacterium tumefaciens. Plant cell reports 28:903-13.

Zhao Z.-y., Gu W., Cai T., Tagliani L., Hondred D., Bond D., Schroeder S., Rudert M., Pierce D. (2001) High throughput genetic transformation mediated by *Agrobacterium tumefaciens* in maize. High through genetic transformation mediated by *Agrobacterium tumefaciens* in maize 8:323-333.

Zhao Z.Y., Cai T., Tagliani L., Miller M., Wang N., Pang H., Rudert M., Schroeder S., Hondred D., Seltzer J., Pierce D. (2000) Agrobacterium-mediated sorghum transformation. Plant molecular biology 44:789-98.

CHAPTER VI

Future Directions

Research opportunities that would continue to reduce deoxynivalenol (DON) contamination in barley and distillers grains should be geared toward: (1) finding and testing new trichothecene acetyltransferases, (2) identifying different enzymatic methods of detoxification, (3) developing industrial fermentation yeast with the ability to produce high ethanol yields and express detoxification enzymes constitutively (4) investigating new *TRI12* genes, their activity, and their impact on DON modification and detoxification *in planta* and (5) identify, validate, and map fusarium head blight (FHB) resistance in winter barley and to utilize marker-assisted breeding to enhance resistance.

Our research has characterized seven trichothecene 3-*O*-acetyltransferases (TRI101/TRI201) from seven different *Fusarium* species for their abilities to acetylate DON (Khatibi et al., 2011). Future experiments may include isolating additional 3-*O*-acetyltransferases that offer a promising ability to acetylate DON at higher levels than that reported for other enzymes (Khatibi et al., 2011). Predicted abilities could be determined by looking at gene sequence and protein similarities to enzymes already tested (Khatibi et al., 2011). Enzymes with varying steady-state kinetics towards DON could be compared structurally through crystallization, together with a comparison of amino acid sequences, to help identify areas that could be targeted for site-directed mutagenesis to help generate a more effective enzyme. In this same regard, enzyme kinetic work and structural interactions with alternative TRI101/TRI201 substrates such as isotrichodermol could also be performed to help determine other targets for site-directed mutagenesis.

Preliminary evidence suggests that enzymes such as TRI101/TRI201 may be produced by different groups of fungi (Tokai et al., 2005). Bioprospecting for better TRI101/TRI201 enzymes does not have to be limited to *Fusarium*, but may include other fungal genera such as *Saccharomyces* (*AYT1* gene) (Alexander et al., 2002), *Aspergillus* and *Neurospora* (Tokai et al., 2005). Bioprospecting in other fungal genera may yield trichothecene acetyltransferases with sought after characteristics such as superior steady-state kinetics towards DON and enhanced temperature stability; however, prospective work should also focus on the search for enzymes that break the trichothecene epoxide ring, which is the major toxicity structure within this metabolite (Binder, 2007). If successful, varying levels of de-epoxide DON in feed products will need to be evaluated for toxicity to domestic animals. *In vitro* assays assessing DNA synthesis have demonstrated de-epoxy DON to be 54 times less toxic than DON (Eriksen et al., 2004).

Further work would also investigate the kinetic profiles of trichothecene 3-*O*-acetyltransferases from different isolates of the same species, especially from trichothecene nonproducers such as *F. fujikuroi* and *F. oxysporum* (TRI201). *TRI201* is believed to be a product of a duplication event and an inactivation event of *TRI101* in the evolutionary history of *F. fujikuroi* and *F. oxysporum* (Tokai et al., 2005). Preliminary evidence has shown differences at the flexible loop region within different isolates of *F. fujikuroi* (Khatibi, Newmister, and Schmale unpublished observations). The flexible loop region influences the catalytic efficiency of the TRI101/TRI201 enzymes (Garvey et al., 2008) and therefore it would be interesting to determine whether there are significant kinetic differences within species and if so, to investigate why such differences exist.

To verify *in vitro* activity of DON detoxification enzymes, transient expression assays in plant cells could be performed. These experiments would show enzyme activity in plant cells

and could also be used to demonstrate the ability of TRI12 to function *in planta*. TRI12 is a trichothecene efflux pump that has been demonstrated to increase acetylation rates in transformed yeast (Alexander et al., 1999). To date, stable transformation of TRI101 from *F. sporotrichioides* has increased DON resistance in greenhouse assays (Manoharan et al., 2006); however, the potential of TRI12 to increase acetylation in plants has not yet been demonstrated. Barley seedlings could be cocultivated with an *Agrobacterium* strain containing a binary vector harboring either *TRI101* or *TRI12* (Li and Nebenfuhr, 2010). A detached leaf assay could then be conducted in a solution containing DON, and resistance and acetylation could be measured (Desjardins et al., 2007).

The utilization of TRI101/TRI201 in the reduction of DON in DDGS was demonstrated in barley fermentation mashes conducted at the USDA. FgTRI101 and FfTRI201 were both expressed after induction by the addition of galactose; however, this may not be the optimum method of expression for this application. During fermentation, glucose (repression) and galactose (induction) are likely competing for control of the GAL1 promoter, and therefore expression of the acetyltransferase is probably not optimized during the course of fermentation. Alternative methods to induce protein expression (other than galactose) may yield larger reductions, especially in grain containing lower concentrations of DON. For instance, promoters such as *CUP1* are inducible via small amounts of copper (Macreadie et al., 1989) and could be an alternative to galactose induction, although the effect of copper on fermentations is unknown. On the other hand, stable gene integration into the yeast genome under the control of a constitutive promoter, would eliminate the need for plasmid selection and induction components. Ultimately, transformed industrial yeast strains will need to be studied to determine whether transgene expression alters ethanol production and DDGS composition.

Another approach to reduce DON in DDGS might be to add an exogenous trichothecene 3-*O*-acetyltransferase preparation to the mash at the start of fermentation. However, the amount of enzyme needed is unknown, enzyme stability may be a limiting factor in this strategy (Garvey et al., 2008), and no such preparation is currently commercially available. Detoxification of mycotoxins in fermentation mashes does not have to be limited to barley. This strategy could also be applied to other fuel ethanol crops such as corn. For example, in addition to deoxynivalenol, the mycotoxin zearalenone is another common contaminant of corn ethanol co-products (Schaafsma et al., 2009) and a lactonohydrolase was shown to decrease levels of zearalenone in spiked cultures of *Schizosaccharomyces pombe*, *Escherichia coli*, and genetically modified corn (Igawa et al., 2007; Takahashi-Ando et al., 2005). In addition to zearalenone, aflatoxin is another common contaminant of corn. Aflatoxin is a dangerous mycotoxin and carcinogen that is often produced by the fungus *Aspergillus flavus* (Wu et al., 2008). Recent work has identified a family of nine enzymes, encoding $F_{420}H_2$ dependent reductases, from *Mycobacterium smegmatis* that decreases the stability of aflatoxin, causing spontaneous hydrolysis and detoxification (Taylor et al., 2010). Future experiments could include yeast detoxification assays and looking at whether TRI12 increases detoxification as it does for TRI101/TRI201. However, an apparent limitation in this aflatoxin detoxification strategy is that the cofactor $F_{420}H_2$ must be present (Taylor et al., 2010).

Important in our DON conversion experiments was a gene called *TRI12* that is located within the trichothecene biosynthetic cluster in *F. sporotrichioides* and is likely not involved in trichothecene biosynthesis (Alexander et al., 1999). TRI12 is a trichothecene efflux pump that is predicted to be an integral membrane protein (Alexander et al., 1999) implicated in exporting trichothecenes such as 4,15-DAS, 3,4,15-TAS, isotrichodermin, 15-decalonectrin, 3,15-

didecalonectrin, and 8-hydroxyisotrichodermin out of the cell (McCormick et al., 1999). The transport of trichothecenes out of the fungal cell is important for the necrotrophic phase (Jansen et al., 2005) of *Fusarium* and may also be a self-protection mechanism (Alexander et al., 1999). Previous work has demonstrated that the hyphal growth of *tri12* mutants on trichothecene-containing media is inhibited (Alexander et al., 1999). Determining the role of TRI12 in preventing autotoxicity could be investigated in future research. For example, one could examine the individual contribution of TRI101 and TRI12 to self-protection in a double mutant.

Future experiments could involve determining the role and mechanism of TRI12 in the transport of DON and 3ADON out of the fungal cell. Feeding wild type yeast and transformed yeast expressing TRI12 with labeled substrates may help elucidate DON and 3ADON movement. This could also be applied to *Fusarium graminearum tri5* (trichothecene biosynthesis mutant) and *tri12* mutants.

When TRI12 from *F. sporotrichoides* (FsTRI12) is coexpressed with a trichothecene 3-*O*-acetyltransferase (TRI101/TRI201) in yeast, acetyltration rates increase relative to yeast transformed with TRI101 or TRI201 alone (Khatibi et al., 2011). It is currently unknown whether other *TRI12* genes, from other trichothecene producing *Fusarium* species, may be better exporters of trichothecenes resulting in larger acetylation rates above that reported of FsTRI12. For example, the *F. graminearum* TRI101 has been shown to have 85 times more affinity for DON (Garvey et al., 2008), leading to the possibility that FgTRI12 may have a larger DON efflux capacity, which could be investigated in future work.

Efforts within the Virginia Tech winter barley breeding program are currently focused on further characterizing hulled and hulless genotypes for FHB resistance. Significant differences in FHB and DON resistance were observed among genotypes selected to evaluate resistance

stability across years. Hulled barley cultivars Thoroughbred and Nomini and hulless cultivars Eve and Doyce should be utilized in future work involving the characterization of resistance in Virginia hulled and hulless barley. Genotypes with indications of resistance and susceptibility should be further evaluated during successive growing seasons. Future efforts should be focused on the development of populations to map QTLs contributing to FHB and DON resistance in both hulled and hulless barley genotypes, the generation of hulled and hulless cultivars with improved FHB resistance, and the potential of hull removal strategies to reduce DON in barley food and feed.

Susceptible lines containing favorable agronomic traits could be enhanced with a DON detoxification gene in future work. The method for stable transformation and insertion of *FgTRI101* was conducted for the Virginia hulless barley cultivar Doyce. Although transformation was unsuccessful, future work should consider adjustments to the method (described in Chapter V) or investigate other avenues of transformation such as the floral dip method (Zale et al., 2009). Future transformants would need to undergo PCR to demonstrate the presence of *FgTRI101* (or other detoxification gene), real-time PCR to verify gene copy number, and western blot analysis to determine enzyme levels. Following these verification methods, transformed plants would be inoculated with *Fusarium graminearum* and tested for resistance to DON compared to wild-type plants. Future work should also include assessing whether the reduction of DON in grain increases susceptibility to other pathogens. Since DON is an antimicrobial metabolite, conversion of DON to a less toxic product may alter population levels of other pathogens. Evaluation of transformed plants could first be done in growth chambers, followed by greenhouse studies, and eventually field trials. Field trials with transgenic plants will require permits from the Biotechnology Regulatory Service Unit of the USDA-APHIS.

During this process, there will be a determination of environmental risks due to genetically modified barley plants expressing a detoxification gene.

LITERATURE CITED

Alexander N.J., McCormick S.P., Hohn T.M. (1999) TRI12, a trichothecene efflux pump from Fusarium sporotrichioides: gene isolation and expression in yeast. Mol Gen Genet 261:977-84.

Alexander N.J., McCormick S.P., Hohn T.M. (2002) The identification of the Saccharomyces cerevisiae gene AYT1(ORF-YLL063c) encoding an acetyltransferase. Yeast 19:1425-30.

Binder E.M. (2007) Managing the risk of mycotoxins inmodern feed production. Animal feed science and technology 133:149-166.

Desjardins A.E., McCormick S.P., Appell M. (2007) Structure-Activity Relationships of Trichothecene Toxins in an *Arabidopsis thaliana* Leaf Assay. Journal of Agriculture and Food Chemistry 55:6487-6492.

Eriksen G.S., Pettersson H., Lundh T. (2004) Comparatie cytotoxicity of deoxynivalenol, nivalenol, their acetylated derivatives and de-epoxy metabolites. Food and Chemical Toxicology 42:619-624.

Garvey G.S., McCormick S.P., Rayment I. (2008) Structural and functional characterization of the TRI101 trichothecene 3-O-acetyltransferase from Fusarium sporotrichioides and Fusarium graminearum: kinetic insights to combating Fusarium head blight. J Biol Chem 283:1660-9.

Igawa T., Takahashi-Ando N., Ochiai N., Ohsato S., Shimizu T., Kudo T., Yamaguchi I., Kimura M. (2007) Reduced contamination by the Fusarium mycotoxin zearalenone in maize kernels through genetic modification with a detoxification gene. Appl Environ Microbiol 73:1622-9.

Jansen C., von Wettstein D., Schafer W., Kogel K.H., Felk A., Maier F.J. (2005) Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted Fusarium graminearum. Proc Natl Acad Sci U S A 102:16892-7.

Khatibi P.A., Newmister S.A., Rayment I., McCormick S.P., Alexander N.J., Schmale D.G., 3rd. (2011) Bioprospecting for trichothecene 3-o-acetyltransferases in the fungal genus fusarium yields functional enzymes with different abilities to modify the mycotoxin deoxynivalenol. Appl Environ Microbiol 77:1162-70.

Li J.F., Nebenfuhr A. (2010) FAST technique for Agrobacterium-mediated transient gene expression in seedlings of Arabidopsis and other plant species. Cold Spring Harb Protoc 2010:pdb prot5428.

Macreadie I.G., Jagadish M.N., Azad A.A., Vaughan P.R. (1989) Versatile cassettes designed for the copper inducible expression of proteins in yeast. Plasmid 21:147-50.

Manoharan M., Dahleen L.S., Hohn T.M., Neate S.M., Yu X.-H., Alexander N.J., McCormick S.P., Bregitzer P., Schwarz P.B., Horsley R.D. (2006) Expression of 3-OH trichothecene acetyltransferase in barley (*Hordeum vulgare* L.) and effects on deoxynivalenol. Plant Science:699-706.

McCormick S.P., Alexander N.J., Trapp S.E., Hohn T.M. (1999) Disruption of TRI101, the gene encoding trichothecene 3-O-acetyltransferase, from Fusarium sporotrichioides. Appl Environ Microbiol 65:5252-6.

Schaafsma A.W., Limay-Rios V., Paul D.E., Miller J.D. (2009) Mycotoxins in fuel ethanol co-products derived from maize: a mass balance for deoxynivalenol. Journal of The Science of Food and Agriculture 89:1574-80.

Takahashi-Ando N., Tokai T., Hamamoto H., Yamaguchi I., Kimura M. (2005) Efficient decontamination of zearalenone, the mycotoxin of cereal pathogen, by transgenic yeasts through the expression of a synthetic lactonohydrolase gene. Appl Microbiol Biotechnol 67:838-44.

Taylor M.C., Jackson C.J., Tattersall D.B., French N., Peat T.S., Newman J., Briggs L.J., Lapalikar G.V., Campbell P.M., Scott C., Russell R.J., Oakeshott J.G. (2010) Identification and characterization of two families of F420 H2-dependent reductases from Mycobacteria that catalyse aflatoxin degradation. Mol Microbiol 78:561-75.

Tokai T., Fujimura M., Inoue H., Aoki T., Ohta K., Shibata T., Yamaguchi I., Kimura M. (2005) Concordant evolution of trichothecene 3-O-acetyltransferase and an rDNA species phylogeny of trichothecene-producing and non-producing fusaria and other ascomycetous fungi. Microbiology 151:509-19.

Wu F., Liu Y., Bhatnagar D. (2008) Cost-Effectiveness of Aflatoxin Control Methods: Economic Incentives. Toxin Reviews 27:203-225.

Zale J.M., Agarwal S., Loar S., Steber C.M. (2009) Evidence for stable transformation of wheat by floral dip in Agrobacterium tumefaciens. Plant cell reports 28:903-13.