Differential Roles of *Tri10* and *Tri6* in *Fusarium graminearum*

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A dissertation submitted to the Faculty of Graduate Studies and Research of Carleton University, in partial fulfillment of the requirements of the degree of Master of Science in Biology

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Abstract

Fusarium graminearum is a globally distributed fungal pathogen that causes disease in cereal crops such as wheat and barley and it also contaminates host plants with a secreted mycotoxin known as deoxynivalenol. Consumption of infected or contaminated cereal products is hazardous to animals, therefore, fungal infection and contamination cause major economic losses in agriculture industries worldwide. My thesis project focused on characterizing two positive regulators, *Tri10* and *Tri6*, of the trichothecene biosynthesis pathway in *F. graminearum*. Gene expression profiling of *Tri10Δ* and *Tri6Δ* mutant strains under DON-inducing conditions provided evidence that the regulatory roles of the two studied genes extend beyond the trichothecene biosynthesis pathway. They are potential regulators of another clustered secondary metabolic pathway, butenolide synthesis pathway, and also genes involved in primary metabolism. In contrast to a prior regulatory model of the trichothecene biosynthesis pathway, RNA transcript analysis demonstrated that regulation of *Tri10* and *Tri6* gene expressions are independent of each other. The distinct gene sets regulated by *Tri10* and *Tri6* indicate different, albeit overlapping, roles in regulating gene expression.

Acknowledgements

First of all, I would like to sincerely thank my supervisor, mentor and friend Dr. Gopal Subramaniam for his guidance and support for the past two years. Not only has he trained me to become a better scientist, to always do things the 'right' way, but has also equipped me with tools and methodology to overcome future obstacles, science-related or not.

I would like to express my gratitude to my co-supervisor Dr. Owen Rowland, for his patience, understanding, and for always taking time out of his busy schedule to answer my questions. I would also like to extend my gratitude to Dr. Therese Ouellet, who has provided me with invaluable input and ideas for the past two years as well.

Without a harmonious working environment, I would have never accomplished what I have for my thesis. For this I must thank my lab members, in alphabetical order, Tina Kirkam, Charles Nasmith, Maria Villota, Sean Walkowiak and Li Wang. Thank you for tolerating my weaknesses, and also for cheering me up on a bad day. This experience would not have been the same without any of you.

Last, but most importantly, I would like to thank my parents, Horace and Josephine Leung, for always being there and providing your support for my decisions. I hope I do you proud. Also, I would like to thank my sister Kitty Ka Yan Leung, whose humour always makes me laugh and brighten my day.

"Courage does not always roar. Sometimes courage is the little voice at the end of the day saying I will try again tomorrow." – Maryanne Radanbacher

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List of Abbreviations

ATA = alimentary toxic aleukia

bp = base pairs

CPM = counts per minute

Cy-3 = cyanine 3

Cy-5 = cyanine 5

DMSO = dimethyl sulfoxide

DON = deoxynivalenol

EST = expressed sequence tags

Fg = *Fusarium graminearum*

FHB = *Fusarium* head blight

Fs = *Fusarium sporotrichioides*

g = gram

HPLC = high performance liquid chromatography or high pressure liquid chromatography

hrs = hours

Hyg = hygromycin

Kb = kilo basepair

mAU = milli-absorbance

Mb = mega base pairs

mg = milligram

mL = millilitre

ng = nanogram

NIV = nivalenol

RPM = revolutions per minute

PCR = polymerase chain reaction

PEG = polyethylene glycol

WT = wild type

ZEA = Zearalenone

4-ANIV= 4-acetylnivalenol

15-ADON = 15-acetyldeoxynivalenol

µg = microgram

^{o}C = degree Celsius

Chapter 1 - Introduction

1.1. Fusarium species: An Overview

1.1.1. Diseases Caused by Fusarium species

Fusarium species are globally distributed fungal pathogens that cause devastating diseases in agriculturally important cereal crops such as wheat and barley (Parry *et al.*, 1995). The most commonly documented disease, *Fusarium* head blight (FHB), also known as scab, is characterized by bleaching, sometimes browning, of infected spikelets of wheat (Parry *et al.*, 1995). In 1995, the five predominant species responsible for FHB around the world were *F. culmonrum, F. avenaceum*, *F. poae*, *F. nivale*, and *F. graminearum.* However, by year 2000, *F. graminearum* had become the primary causal agent of FHB (Parry, 1990; Windels, 2000). Other *Fusarium*-associated diseases include *Fusarium* foot rot, root rot and seedling blight (Parry *et al.*, 1995, Couture *et al.*, 2002; Khan *et al.*, 2006). Symptoms of foot rot are typically described as browning at the stalk or stem base of the infected cereal host plant, and the causal species were identified to be *Fusarium nivale, F. culmorum, F. avenaceum* and *F. graminearum* (Parry, 1990). Although both FHB and foot rot diseases are mainly caused by the same *Fusarium* species, the biological factors and abiotic conditions that determine the establishment of one disease over another are not well understood. Nonetheless, records show that FHB is more likely to occur in early spring during the anthesis stage of cereal host plants, whereas foot rot disease is more frequently observed in autumn on mature plants (Parry *et al.*, 1995; Parry, 1990). Hence, the developmental stage of host plants might be a factor in determining the conquest of each disease. Unlike the wide range of causal species responsible for FHB and foot rot, root rot in crops is most often caused by

Fusarium oxysporum (Sutherland, 1990; Michielse and Rep, 2009). Plants with root rot exhibit symptoms such as stunted growth, leaf discolouration from green to light green or yellow, and narrowed reddish-brown shoots (Sutherland, 1990). Finally, seedling blight occurs when *Fusarium*-infected seeds are sowed, or when seeds are planted in a *Fusarium*-contaminated field (Glynn *et al.*, 2007). Depending on the severity of the disease, infected seeds may fail to germinate. However, if germination was successful, the plant development can be affected manifested by stunted shoot and root growth (Glynn *et al.*, 2007). Many *Fusarium* species cause seedling blight, with the most prevalent species corresponding to the ones mentioned for FHB (Khan *et al.*, 2006).

1.1.2. Fusarium mycotoxins

The capability of *Fusarium spp.* to cause disease is often attributed to their ability to produce and secrete mycotoxins. To date, it is known that these secondary metabolites affect plant or animal biological systems through various means such as interfering with DNA replication or protein synthesis, targeting host immune system and blocking of important metabolic pathways (Pitt, 2000, Desjardins and Proctor, 2007). Since mycotoxins are stable even under sterilizing and food-processing conditions, they also pose serious health hazards to humans and livestocks that have cereal-grain food products in their diets (Yazar and Omurtag, 2008). Based on their frequent occurrence in nature and high toxicity to humans and animals, *Fusarium* mycotoxins have been classified into three major groups: zearalenones, fumonisins, and trichothecenes (De Nijs *et al.*, 1996, Desjardins, 2006). Of the three toxins, zearalenones appear to be the least toxic as they have not been associated with any fatal illnesses after consumption, whereas

trichothecenes and fumonisins were reported to have caused deaths in humans and animals (Desjardins and Proctor, 2007, Yazar and Omurtag, 2008).

The biggest health concern regarding zearalenone is that it functionally mimics the hormone estrogen, and is able to bind and activate human and mammalian α- and β-estrogen receptors (Kuiper-Goodman *et al.*, 1987). Even though this myco-estrogen is not currently linked to any human diseases, numerous swine disease outbreaks revealed that zearalenone is responsible for causing estrogenic syndrome in animals. General symptoms of the estrogenic syndrome in females include enlargement of the mammary gland and reproductive organs such as the uterus and fallopian tubes, decreased number of viable offspring, decreased weight and size of offspring and in more serious cases, ovary deterioration that leads to infertility (Kordic *et al.*, 1992, Desjardins, 2006). Affected males exhibit similar symptoms, including the enlargement of their mammary gland, as well as deterioration of their version of primary reproductive organ, the testes (Kordic *et al.*, 1992, Desjardins, 2006). At present, there are no indications of zearalenones being toxic to plants. Zearalenones (ZEA), even at high concentrations (310μM), did not affect growth of wheat seedlings (Desjardins, 2006). It has been detected in corn and other crops of Australia, Asia, Europe, Africa, South America and North America (Kuiper-Goodman *et al.*, 1987). Several studies have established that ZEA is a heat stable metabolite, as incubation of zearalenone or ZEA-contaminated corn samples at a temperate range of 120°C to 150°C for four to forty-four hours did not reduce the levels of the mycotoxin (Bennett *et al.*, 1980, Kuiper-Goodman *et al.*, 1987). Due to its thermal stability, natural accumulation on crops and harmful effects on farm

animals, 16 countries now limit the amount of zearalenone to less than 1000 μg per kg in corn and other crops (ARO, 2003). Strains of *Fusarium* that consistently produce zearalenone in maize or other crops belong to the species *F. graminearum*, *F. crookwellense*, *F. culmorum, F. equiseti*, and *F. semitectum* (Desjardins, 2006).

All fumonisins share a backbone of 20-carbon aliphatic straight chain, with two ester-linked side-chains at carbon 14 and carbon 15 (Pitt, 2000). The principal fumonisins found naturally in crops are the three homologues in series B, designated FB_1, FB_2 and FB_3 (Yazar and Omurtag, 2008). These three homologues differ by the presence of a hydroxyl group on carbon 5 or carbon 10. *F. verticillioides*, *F. proliferatum* and one strain of *F. oxysporum* have been found to produce fumonisins, with production of each fumonisin homologue being strain-specific as opposed to species-specific (Desjardins, 2006). This group of mycotoxin has been classified by the International Agency for Research on Cancer (IARC) as group 2B, which represents the "probably carcinogenic" group (IARC, 1993). This decision was supported by the unusually high incidence of esophageal cancer in South Africa from 1955 to 1990, which corresponds to the time when collected maize samples, their major food source, were heavily contaminated with FB_1 and FB_2 (Desjardins, 2006). A positive correlation between high levels of fumonisins and esophageal cancer was also observed in rural parts of China. Fumonisins at a concentration ranging from 1 to 50 μM can inhibit the root and shoot growth of maize and tomato seedlings (Desjardins, 2006). Spraying FB_1 at a concentration of up to 200 μg per mL on adult weed and crop plants such as cucumber and soybean plants induced necrosis and wilting, yet there are no visible effects when

FB_1 was sprayed on monocots such as wheat and barley (Abbas and Boyette, 1992). The molecular effects of fumonisins on plants remain obscure. FB_1 was first identified in the 1980s and by 2003 six countries had limited the amount of fumonisins on crops to less than 3000 ug per kg (ARO, 2003). Like zearalenones, fumonisins can be found in all major continents and are stable at temperatures as high as 150°C (Desjardins, 2006, Yazar and Omurtag, 2008).

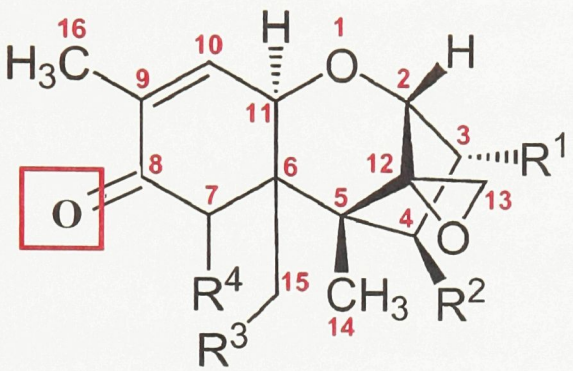
With over 200 trichothecenes discovered, this group of mycotoxin is classified into four types, A to D, according to their chemical structure (Desjardins, 2006). Trichothecenes are a group of sesquiterpenoid compounds with a general backbone of 12, 13-epoxytrichothec-9-enes, with a double bond between carbon 9 and carbon 10, and an epoxide ring linking carbon 12 and carbon 13 (Desjardins, 2006). All *Fusarium*-produced trichothecenes belong to either type A or type B, with T-2 toxin being the most prominent type A trichothecene, and the most frequently encountered type B trichothecenes in crops are nivalenol (NIV) and deoxynivalenol (DON) (Desjardins, 2006, Kimura *et al.*, 2007). Type A trichothecenes are defined by the absence of a ketone group on carbon 8, and within this type, they vary from the side-chains added at carbon 3, 4, 8 and 15 (Kimura *et al.*, 2007). Alternatively, type B trichothecenes are distinguished by the presence of a ketone at carbon 8, allowing for side-chain variation at carbon 3, 4, 7 and 15, see Figure 1 (Kimura *et al.*, 2007).

Figure 1: Chemical structures of type B trichothecenes.

A) General backbone structure of type B trichothecenes, described as 12, 13-epoxytrichothec-9-en-8-one. (Image obtained from Kimura *et al.*, 2007). Carbons 12 and 13 are linked by an epoxide ring whereas carbons 8 and 9 share a double bond. Red square indicates a ketone at carbon 8, a distinguishing feature of type B trichothecenes. Chemotypes of type B trichothecenes differ from each other based on the R-groups attached to carbons 3, 4, 7 and 15.

B) Functional groups specific to DON, NIV, 15-ADON and 3-ADON at carbons 3, 4, 7 and 15. R1 refers to carbon 3, R2 to carbon 4, R3 to carbon 15 and R4 to carbon 7. For example, DON is chemically described as 3, 7, 15-trihydroxy-12, 13-epoxytrichothec-9-en-8-one, illustrating the hydroxyl groups at carbons 3, 7 and 15 in addition to the general type B trichothecene backbone. (Kimura *et al.*, 2007, Desjardins, 2006)

A)



B)

Type B Trichothecene	**R1**	**R2**	**R3**	**R4**
Deoxynivalenol (DON)	-OH	-H	-OH	-OH
Nivalenol (NIV)	-OH	-OH	-OH	-OH
15-acetyl-deoxynivalenol (15-ADON)	-OH	-H	-OAc	-OH
3-Acetyldeoxynivalenol (3-ADON)	-OAc	-H	-OH	-OH

Type C trichothecenes consist of an extra epoxide ring linking carbons 7 and 8, whereas type D trichothecenes are differentiated by the 4, 15-macrocyclic ring (Kimura *et al.*, 2007). Trichothecenes are not only produced by *Fusarium* species, they are also produced by other fungal genera such as *Trichoderma*, *Trichothecium*, *Stachybotrys* and *Myrothecium* (Desjardins, 2006). Nivalenol harbours hydroxyl groups at all four carbons available for side-chains whereas deoxynivalenol only harbours three hydroxyl groups, with carbon 4 unattached (Figure 1). T-2 toxin is produced by *F. sporotrichioides*, *F. poae*, *F. equiseti* and *F. acuminatum* (Yazar and Omurtag, 2008). DON is primarily produced by *F. graminearum* and *F. culmorum*, which are two of the five major species responsible for causing FHB (Yazar and Omurtag, 2008, Windels, 2000). Some isolates of these two *Fusarium* species can also produce NIV, but the main producers of this toxin are *F. cerealis* and *F. poae* (Yazar and Omurtag, 2008). International Agency for Research on Cancer (IARC) classified this group of *Fusarium* mycotoxins as group 3, indicating their carcinogenicity in humans was not classifiable due to insufficient and limited evidence (IARC, 1993). The three representative trichothecenes have demonstrated their high toxicity in humans and animals through previous outbreaks and clinical studies. In humans, T-2 toxin causes a condition known as "alimentary toxic aleukia" (ATA), where affected individuals mainly suffer from a reduction in white blood cells due to damaged bone marrow, which leads to immunosuppression, sometimes resulting in death. DON is sometimes referred to as vomitoxin, because it induces vomiting in animals, including humans, upon consumption of contaminated grain. In 1933, an outbreak of red mold disease, termed akakabi-byo, was associated with DON and nivalenol contamination. Affected patients experienced vomiting, nausea, trembling

and visual hallucinations. There were no reports of deaths. Although the fungus *F. graminearum*, which produces DON and NIV, was consistently isolated from the moldy grains associated with the outbreak, traces of DON and NIV were never reported (Desjardins, 2006). This might be due to the fact that DON was not isolated and characterized until the 1970s. The symptoms of DON were confirmed by feeding experiments of swine, where pigs suffered from vomiting after they were fed with grains contaminated with 0.15 mg per kg of DON (Pestka and Smolinski, 2005). At concentrations as low as 1 μM to 10 μM, trichothecenes can inhibit shoot and root growth in pea and lettuce seedlings. In contrast to zearalenones and fumonisins, trichothecenes have inhibitory effects on monocots such as wheat. T-2 toxin appears to be the most toxic trichothecene, inhibiting 50% of wheat seedling shoot growth at 2 μM, with DON at 3 μM and NIV at 21 μM (Shimada and Otani, 1990, Desjardins, 2006).

1.1.3. Economic Impacts of Fusarium Infection

In spite of the slightly higher phytotoxicity of T-2 toxin, DON is considered the most important trichothecene of the three because of its prevalence in cereal crops and its association with FHB. Incidence of DON was reported worldwide, whereas NIV was hardly detected in Africa, North America and South America and T-2 toxin was never reported in Africa and East Asia (Desjardins, 2006). A study performed in 2003 indicated the occurrence of DON is more frequent than that of NIV and T-2 toxin in Southern Ontario. For instance, 58% of harvested barley samples tested positive for DON, and the average concentration of contaminated samples was 260 ng per g but none tested positive for NIV and T-2 toxin (Lombaert *et al.*, 2003).

Both *Fusarium*-associated disease and *Fusarium* mycotoxin contamination has the potential to reduce crop yield and grain quality. In the 1990s, FHB epidemics in North America cost farmers in Quebec and Ontario up to US $220 million in crops while Manitoba farmers suffered a loss of approximately US $300 million (Windels, 2000). During the same period, the United States lost up to US $2.7 billion in wheat and barley due to *Fusarium* infection (Goswami *et al.,* 2004). According to the Government of Alberta, Canada continues to experience scab epidemics, and suffers from a loss of about $100 million annually in Manitoba alone.

1.2. Fusarium graminearum and Host Range

F. graminearum consists of nine phylogenetically distinct subspecies: *F. austroamericanum*, *F. meridionale*, *F. boothii*, *F. mesoamericanum, F. acaciae-mearnsii*, *F. asiaticum, F. graminearum, F. cortaderiae*, and *F. brasilicum* (O'Donnell *et al.*, 2004). In addition to the molecular markers, morphological features such as conidial width and length, presence or absence of an apical conidial beak, morphological symmetry and the widest position of conidia are also used to distinguish these species (O'Donnell *et al.*, 2004). Global sampling indicated that some of these species are geographically limited, while others are found in more than one continent. Since geography and climate dictate the type of crops grown in the area, these species have evolved and adapted the ability to cause disease in different hosts accordingly. Four of the nine species, *F. cortaderiae*, *F. brasilicum*, *F. austroamericanum*, and *F. meridionale*, belong to the South American clade, which corresponds to their geographic distribution (O'Donnell *et al.*, 2000, O'Donnell *et al.*, 2004). Each of these four species inhabit different hosts, with *F. cortaderiae* isolated from *Cortaderia spp.*, a type of grass native to South America, *F. brasilicum* extracted from oats, and *F. meridionale* from corn (O'Donnell *et al.*, 2000, O'Donnell *et al.*, 2004). Strains of *F. austroamericanum* were recovered from three different hosts, including polypore mushrooms, corn and herbaceous vine, suggesting a diverse host range within the species (O'Donnell *et al.*, 2000, O'Donnell *et al.*, 2004). The Central American-distributed species *F. mesoamericanum* infects fruit crops, as strains of it were isolated from bananas and others from grape ivy. Strains belonging to the species *F. acaciae-mearnsii* were discovered in Australia inhabiting a native legumous tree known as *Acacia mearnsii*

(O'Donnell *et al.*, 2000, O'Donnell *et al.*, 2004). *F. asiaticum* causes disease in barley, wheat and corn, and is restricted to Asia (O'Donnell *et al.*, 2000, O'Donnell *et al.*, 2004). The two species *F. boothi* and *F. graminearum* are perhaps the two most widespread species of all, with *F. boothi* found in Africa, Mexico and Central America, and *F. graminearum* found in all of the major continents (O'Donnell *et al.*, 2000, O'Donnell *et al.*, 2004). However, their hosts remain limited to cereal crops, with *F. boothi* mainly infecting corn and *F. graminearum* infecting wheat, barley and corn (O'Donnell *et al.*, 2000, O'Donnell *et al.*, 2004).

1.3. Life Cycle of Fusarium graminearum

The homothallic ascomycetes, *F. graminearum*, can alternate between its anamorphic state (*F. graminearum* Schwabe) and teleomorphic state (*Gibberella zeae* Schwein) to produce asexual conidia and sexually derived ascospores respectively (Stack, 1989, Trail *et al.*, 2002). While conidia are equally effective in causing disease, ascospores are considered the primary inoculum for FHB because perithecia, fruiting bodies from which ascospores are discharged, are more frequently found on infected cereal crops (Stack, 1989, Guenther and Trail, 2005). In winter, the fungus survives in the form of perithecia on crop debris, even though chlamydospores, a form of conidia, and hyphal fragments can also be found in the soil (Goswami and Kistler, 2004, Champeil *et al.*, 2004). Both perithecia and chlamydospores have thick, multi-layered outer shells, allowing them to endure through the harsh winter months (Mehrotra and Aneja, 1990). Although undamaged, the low temperature, lack of sunlight and drought inhibited the development of these propagules, inducing a dormant phase (Champeil *et al.*, 2004). In spring, an increase in daylight, temperature and rainfall offset previous effects and allowed for maturation of perithecia and chlamydospores to continue (Champeil *et al.*, 2004). Since *F. graminearum* does not produce specialized, penetrating structures such as an appresorium, spore maturation and dispersal must be coordinated with anthesis, when the florets are most susceptible and provide easy access into the host plant. Inside a mature perithecium, ascospores are suspended in sac-like spore-producing cells known as asci, which will individually extend and protrude through the ostiole, a small hole at the top of the perithecium, to discharge ascospores (Mehrotra and Aneja, 1990). This release of ascospores is triggered by a period of drought followed by high

humidity, and the optimal temperature ranges from 13°C to 22°C (Champeil *et al.*, 2004). The mechanism behind ascospore discharge has not been determined, but it is speculated that the presence of water increases osmotic pressure built up by an accumulation of mannitol sugar and an influx of potassium ions inside the vacuole of an ascus, hence leading to a forceful ejaculation (Trail *et al.*, 2002, Trail *et al.*, 2005). Ascospores do not always land on a susceptible part of the host plant, and rely on wind and splash created by rain for aerial dispersal (Goswami and Kistler, 2004, Champeil *et al.*, 2004). Following spore germination, the fungal hyphae enter into the flower, colonizing the anthers and ovaries, and move towards the glumes and the rachis. Colonization of the anthers stimulates hyphae growth, since these male reproductive organs contain two virulence enhancers, choline and betaine. Both compounds contain an amide group and are transported into the fungus, promoting hyphal extension (Robson *et al.*, 1995). As the hyphae progress towards the rachis, they penetrate and degrade hindering cells, until they have successfully colonized the entire wheat grain. In a more detailed study, Guenther and Trail used a *F. graminearum* GFP-tagged strain to examine the process of fungal invasion (Guenther and Trail, 2005). It was discovered that this pathogen takes the form of haploid mycelia when moving down the rachis, but it converts into dikaryotic hyphae when colonizing chlorenchyma cells and stomates (Guenther and Trail, 2005). Dikaryotic hyphae refer to fungal cells that harbour two nuclei, which occurs during sexual reproduction. This change in reproductive state is consistent with the emergence of sexually-developed perithecia within chlorenchyma tissue and stomates, where dikaryotic hyphae are found (Guenther and Trail, 2005). These perithecia will overwinter in crop debris and become the primary inoculum for FHB the subsequent year.

Microarray analysis revealed that ion transporters play an important role in perithecium development (Hallen *et al.*, 2007). In particular, potassium, chloride and calcium ion channels are of interest, because they were activated 72 to 96 hours after the onset of perithecium maturation, corresponding to the developmental stage where asci are formed (Hallen *et al.*, 2007). These ion channels might play a role in ascospore discharge by allowing an influx of ions into the vacuoles of asci, ultimately leading to ascospore discharge.

1.4. The Role of Trichothecenes in Fusarium Pathogenicity

Trichothecenes are typically described as virulence factors that contribute to pathogenicity of *Fusarium spp.* on cereal host plants. This is supported by the observation that a non DON-producing *F. graminearum* mutant strain, designated *Fg Tri6Δ* was able to infect the wheat cultivar Norm, but failed to spread beyond the site of infection (Seong *et al.*, 2009). Independent of the pathogen, pure trichothecenes are also toxic to plants, as discussed in section 1.1.2. Studies have shown that the three trichothecenes, DON, NIV and T-2 toxin, inhibit protein synthesis by targeting the 23S rRNA component of the ribosome that has peptidyl transferase activity (Nissen *et. al.*, 2000). This transferase is responsible for forming peptide links between amino acids and tRNA in the process of translation (Nissen *et. al.*, 2000). Inhibition of an important cellular process such as this generates a wide range of effects on the host plant and among them is the failure to translate genes involved in disease resistance, enabling the fungus to prevail in this host-pathogen battle. Some plants have evolved resistance to *Fusarium* infection, particularly against the effects of trichothecenes. The degree of resistance to FHB varies from cultivar to cultivar in cereal crops, but in general, there are five types of resistance. Type I resistance counteracts initial *Fusarium* infection (Schroeder and Christensen, 1963). Since infection requires the dispersal of ascospores and conidia onto susceptible plant organs, a cultivar with type I resistance possesses physical characteristics such as longer peduncle or stem that prevents spores from landing onto the flowers (Champeil *et al.*, 2004). Also, cultivars with type I resistance decrease susceptibility to *Fusarium* infection by secreting anti-fungal compounds such as chitinases, which are fungal cell-wall degrading enzymes (Champeil *et al.*, 2004). Type

II resistance prevents the spread of the pathogen inside the host plant (Schroeder and Christensen, 1963). Resistant host plants do so by depositing callose and lignin into the periplasmic space to create a defense wall between fungal-colonized tissue and uninfected tissue, blocking further entry of the invading fungus and trichothecenes (Schroeder and Christensen, 1963, Champeil *et al.*, 2004). In order for *F. graminearum* to overcome type II resistance, diffusion of DON into the host plant must precede the establishment of a defense wall, because inhibitory effects of this metabolite on protein synthesis can stop the production of callose and lignin (Champeil *et al.*, 2004). Type III resistance refers to the resistance specifically against kernel infection utilizing similar morphological structures described for type I resistance (Foroud and Eudes, 2009). Host plants with type IV resistance have a modified peptidyl transferase that is not recognized by trichothecenes; therefore, mycotoxins have no affect on the plant's ability to synthesize callose and lignin, thus allowing the host to suppress fungal advancement through type II resistance (Champeil *et al.*, 2004, Foroud and Eudes, 2009). Finally, type V resistance is the resistance against trichothecene accumulation (Champeil *et al.*, 2004, Foroud and Eudes, 2009). Host plants demonstrating this type of resistance are divided into two classes: class 1 refers to plants that can chemically modify trichothecenes such that they render it non-toxic (Boutigny *et al.,* 2008). For instance, certain FHB resistant host plants such as wheat cultivar Frontana have the ability to add a glucose molecule to the hydroxyl group at carbon-3 of type B trichothecenes, which greatly reduces their ability to inhibit protein synthesis (Miller and Arnison, 1986). Using an *in vitro* translation assay with firefly luciferase as a reporter marker, it was determined that 1 μM of unglycosylaed DON inhibited translation by 36.8%, while 20 μM of DON-3-O-

glucoside, glycosylated DON, inhibited luciferase translation by only 8% (Poppenberger *et al.*, 2003). In *Arabidopsis thaliana*, glycosylation of DON is catalyzed by the enzyme deoxynivalenol-glucosyl-transferase (DOGT1), which codes for an UDP-glucoysyltransferase (Poppenberger *et al.*, 2003). However, the gene encoding DON-glucosyltransferase in wheat has not been identified. Alternatively, class 2 host plants reduce trichothecene accumulation by inhibiting trichothecene biosynthesis in the fungal pathogen (Boutigny *et al.,* 2008). It had been reported that flavonoids and furanocoumarins could inhibit T2-toxin production in *F. sporotrichioides* by more than 85% (Desjardins *et al.*, 1988). Flavone was the only flavonoid tested that could inhibit trichothecene production, whereas examples of furanocoumarins that act as inhibitors include pimpinellin, xanthotoxin and xanthotoxol (Desjardins *et al.*, 1988). The first compound formed in the trichothecene biosynthesis pathway, trichodiene, was detected in cultures, indicating these inhibiting compounds block the pathway in subsequent steps.

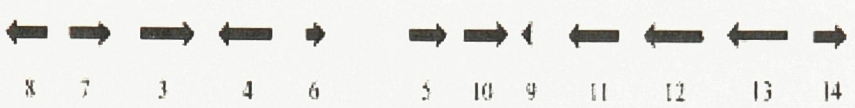
1.5. Trichothecene Biosynthesis Pathway

The genome of *Fusarium graminearum* isolate PH-1 has been sequenced to 99.7% completion and was released to the public by the Broad Institute in 2003 (http://www.broadinstitute.org/annotation/genome/fusarium_graminearum/MultiHome.html). *F. graminearum* contains four chromosomes, with a genome size of 36.1Mb and encodes for approximately 13,937 predicted open reading frames (Goswami and Kistler, 2004, Trail, 2009). Most trichothecene genes involved in the biosynthesis pathway have been identified, with a majority of them found within the 25kb core *Tri* cluster on chromosome 2 (Kimura *et al.*, 2003). Comparison of the *Tri* cluster revealed allelic polymorphisms between chemotype-specific *F. graminearum* strains, and also between type A and type B trichothecene-producing *Fusarium* species (Brown *et al.,* 2002, Kimura *et al.,* 2003, Goswami and Kistler, 2004). Figure 2 illustrates that a functional *Tri7* and *Tri13* are present in the *Tri* cluster of both T-2 toxin-producing *F. sporotrichioides* strains and NIV-producing *F. graminearum* strains, but encodes for non-functional gene products in DON-producing *F. graminearum* strains.

Figure 2: *Tri* gene cluster in T2-toxin-producing *F. sporotrichioides* strain, NIV-producing *F. graminearum* strain and DON-producing *F. graminearum* strain. (Brown *et al.*, 2002)

The twelve *Tri* genes from the *Tri* gene cluster are represented by arrows, which indicate the direction of transcription. The number underneath each arrow identifies the *Tri* gene. *F. sporotrichioides* and NIV-producing *F. graminearum* strains encode functional Tri7 and Tri13 proteins, but DON-producing *F. graminearum* strain does not, as illustrated by the elimination of the corresponding genes.

F. sporotrichioides



F. graminearum (NIV)

F. graminearum (DON)

The presence of *Tri7* and *Tri13* correlates specifically with the production of T2-toxin and NIV. *Tri13* encodes a monooxygenase which hydroxylates carbon 4 of NIV and T-2 toxin while the protein product of *Tri7* acetylates the oxygen atom attached to C4 of T-2 toxin, resulting in an acetyl-ester group in the final product (Lee *et al.*, 2002). The hydroxyl group on C4 of nivalenol remains unaltered in the NIV-producing *F. graminearum* strain, Tri7 participates later on in the pathway to produce the NIV-related trichothecene, 4-acetylnivalenol, in 4-ANIV-producing *F. graminearum* strains (Lee *et al.*, 2002, Brown *et al.*, 2002). Factors regulating the functionality of Tri7 protein in NIV-producing and 4-ANIV-producing strains are not known.

To date, fifteen functional *Tri* genes have been identified in *F. graminearum*. Besides the twelve *Tri* genes in the core *Tri* cluster as indicated in Figure 1, there are also *Tri1* on chromosome 1 (McCormick *et al.*, 2004), *Tri101* on chromosome 4 (Kimura *et al.*, 1998) and *Tri15* on chromosome 3 (Desjardins, 2006). Not all of these genes are involved in catalyzation of substrates; some are involved in regulation of the pathway while others are involved in transport (Kimura *et al.*, 1998, Desjardins, 2006). These genes are functionally categorized into five groups, as shown in Table 1.

Table 1: Functional classification of fifteen *Tri* genes in *Fusarium graminearum*

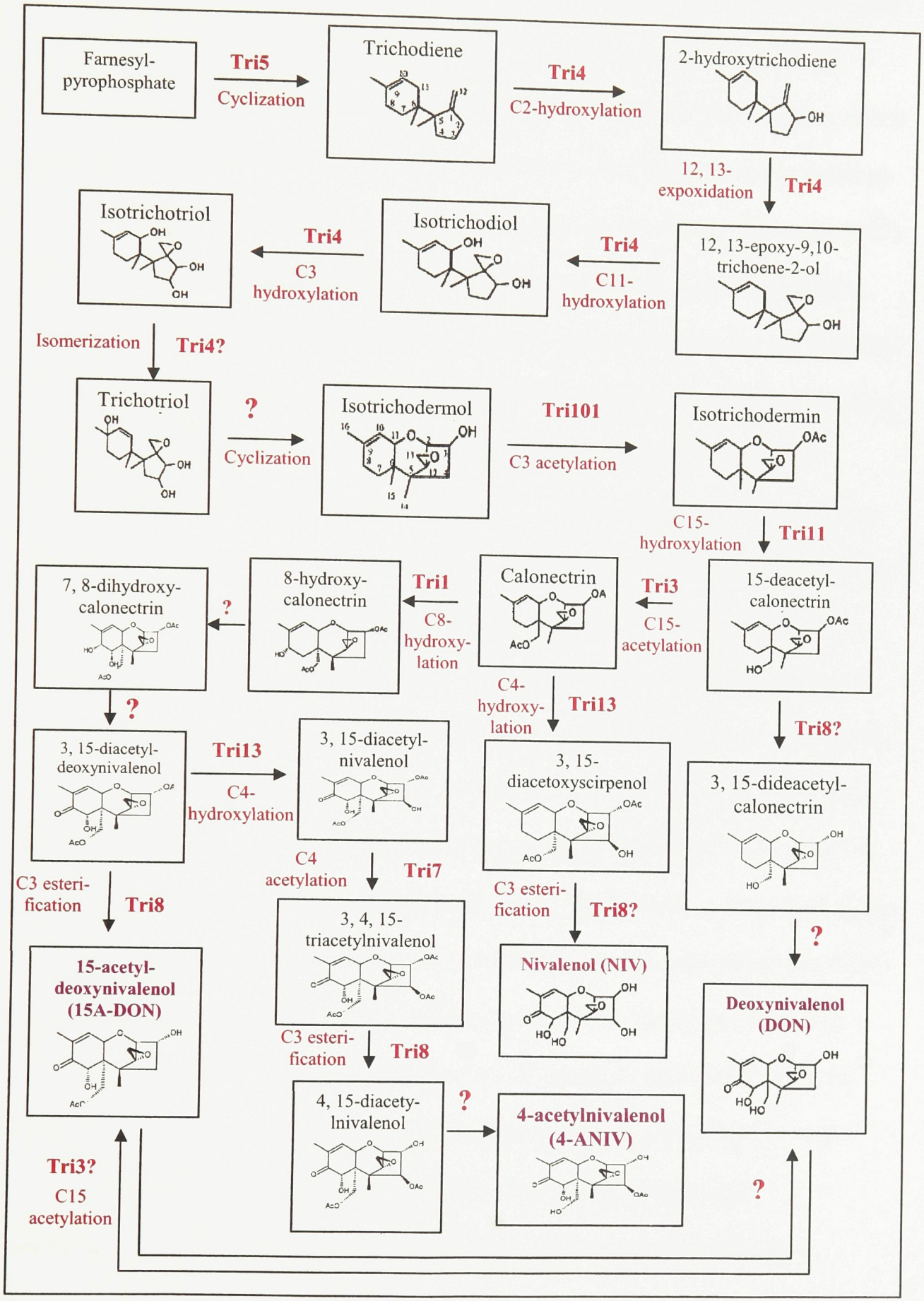
Type of Function	**Gene**	**Specific Role in Trichothecene Biosynthesis Pathway**	**Strains of *F. graminearum***
Sesquiterpene cyclase	*Tri5*	Cyclize farnesyl pyrophosphate (initial substrate) into trichodiene	DON, NIV
Cytochrome P450	*Tri4*	Hydroxylase (multiple carbons)	DON, NIV
	Tri11	C15 hydroxylase	DON, NIV
	Tri1	C8 hydroxylase	DON, NIV
	Tri13	C4 hydroxylase	NIV
Acylation/deacylation (adding or removing an acyl group)	*Tri3*	C15-acetyltransferase	DON, NIV
	Tri101	C3-acetyltransferase	DON, NIV
	Tri8	C3 esterase	DON, NIV
	Tri7	C4-acetyltransferase	NIV
Regulation	*Tri6*	Positive regulator (transcription)	DON, NIV
	Tri10	Positive regulator	DON, NIV
	Tri15	Negative regulator	DON, NIV
Transport	*Tri12*	Transporter involved in trichothecene efflux	DON, NIV
Unknown	*Tri9*	N/A	DON, NIV
	Tri14	N/A	DON, NIV

(Ref: Brown *et al.,* 2004, Desjardins, 2006)

Based on the functions described in Table 1, Foroud and Eudes predicted the trichothecene biosynthesis pathway as illustrated in Figure 3 (Foroud and Eudes, 2009). Some steps in the trichothecene biosynthesis pathway are not catalyzed by any of the known *Tri* genes, and genes encoding these proteins have yet to be identified.

Figure 3: Proposed trichothecene biosynthesis pathway for DON, NIV, 4-ANIV and 15-ADON. Modified from Foroud and Eudes (2009) with information added from Brown *et al.*, 2001 and Brown *et al.,* 2004.

The trichothecene biosynthesis pathway begins with farnesyl pyrophosphate, an intermediate substrate from the mevalonate pathway, being transformed into trichodiene by *Tri5*. Following a series of cyclization, hydroxylation, acetylation and esterification driven by different *Tri* gene products, the appropriate trichothecene is formed.


Farnesyl-pyrophosphate
Tri5
Cyclization
Trichodiene
Tri4
C2-hydroxylation
2-hydroxytrichodiene
12, 13-expoxidation
Tri4
12, 13-epoxy-9,10-trichoene-2-ol
Tri4
C11-hydroxylation
Isotrichodiol
Tri4
C3 hydroxylation
Isotrichotriol
Isomerization
Tri4?
Trichotriol
?
Cyclization
Isotrichodermol
Tri101
C3 acetylation
Isotrichodermin
C15-hydroxylation
Tri11
15-deacetyl-calonectrin
Tri3
C15-acetylation
Calonectrin
Tri1
C8-hydroxy-lation
8-hydroxy-calonectrin
?
7, 8-dihydroxy-calonectrin
?
C4-hydroxy-lation
Tri13
Tri8?
3, 15-dideacetyl-calonectrin
3, 15-diacetoxyscirpenol
Tri13
C4-hydroxylation
3, 15-diacetyl-nivalenol
3, 15-diacetyl-deoxynivalenol
C4 acetylation
Tri7
C3 esteri-fication
Tri8?
Nivalenol (NIV)
C3 esteri-fication
Tri8
3, 4, 15-triacetylnivalenol
?
Deoxynivalenol (DON)
15-acetyl-deoxynivalenol (15A-DON)
C3 esteri-fication
Tri8
4, 15-diacety-lnivalenol
?
4-acetylnivalenol (4-ANIV)
Tri3?
C15 acetylation
?

The trichothecene biosynthesis pathway in *F. graminearum* begins with the cyclization of the initial substrate, farnesyl pyrophosphate, into trichodiene by Tri5. Farnesyl pyrophosphate is an intermediate substrate from the acetate-mevalonate pathway, which produces isoprenoid organic compounds required for a wide range of cellular functions such as cell membrane maintenance and steroid synthesis (Suzuki and Muranaka, 2007). Following a series of hydroxylations, isomerizations, acetylations, and cyclizations, different type B trichothecenes are produced. Four of the known *Tri* genes are identified as cytochrome P450 oxygenases, a superfamily of heme-containing enzymes (Urlacher and Eiben, 2006). They generally function by introducing oxygen into substrate molecules and can catalyze a wide range of reactions, including hydroxylation of fatty acids and aromatic compounds, epoxidation of alkenes (C = C), and cleavage of an acyl-carbon bond (Desjardins, 2006, Urlacher and Eiben, 2006). This explains the re-occurrence of Tri4 to drive several reactions in the early stages of the biosynthesis pathway such as hydroxylation of carbon 2, carbon 11, carbon 3 and also catalyzing the 12, 13-epoxidation reaction. Acetylation, addition of an acetyl group ($-COCH_3$), is the most common form of acylation observed in the pathway. While Tri3, Tri101 and Tri7 catalyze acetylation, Tri8 is the only one in this group that catalyze an esterification. The gene products of *Tri101* and *Tri8* both act on carbon 3, but with opposite effects: Tri101 adds an acetyl group to carbon 3 while Tri8 removes it. There have been numerous studies in the past few years to ascertain the role of regulatory genes *Tri10* and *Tri6*, section 1.6 will summarize these studies. The other regulatory gene *Tri15* has been speculated to act as a negative regulator of the trichothecene pathway in *F. sporotrichioides*, but evidence to support this in *F. graminearum* has not been

forthcoming (Alexander *et al.*, 2004). *Fg Tri12* product and its homologous counterpart in *F. sporotrichioides* is a trichothecene efflux pump involved in self-protection against trichothecenes (Alexander *et al.*, 1999). The functions of *Tri9* and *Tri14* have yet to be elucidated.

1.6. Regulatory Genes of the Trichothecene Biosynthesis Pathway: Tri10 and Tri6

The trichothecene biosynthesis pathway is not constitutively active in *Fusarium spp*. It is activated under nutrient-limiting conditions *in vitro*, especially when carbon and nitrogen sources are insufficient (Pestka *et. al.*, 1985). Possible host plant-derived inducers of DON production have been reported recently. Using a transgenic *F. graminearum* strain that harbours a *Tri5* promoter-GFP construct, Gardiner *et al.* tested the ability of 95 different nitrogen sources to activate the DON biosynthesis pathway (Gardiner *et al.*, 2009). It was discovered that compounds such as agmatine, arginine and guanine can induce DON production to amounts that are equal or greater than the levels of DON detected in infected wheat heads (Gardiner *et al.*, 2009). These compounds are involved in the polyamine biosynthesis pathway of host plants, and they are capable of inducing the T-2 toxin biosynthesis pathway in *F. sporotrichioides* as well (Gardiner *et al.*, 2009). Initially demonstrated in *F. sporotrichioides*, *Tri5* is the first enzyme committed to the trichothecene biosynthesis (see Figure 3) and its expression is tightly controlled by the regulatory genes *Tri10* and *Tri6* (Proctor *et al.*, 1995, Tag *et al.*, 2001).

Initial characterization of *Tri10* and *Tri6* was performed in *F. sporotrichioides*, which sheds light on the roles of their counterparts in *F. graminearum* (Goswami and

Kistler, 2004). *Tri10* is located next to *Tri5* in the *Tri* gene cluster, and encodes a protein of 420 amino acids (Tag *et al.*, 2001). Upon deletion of this gene, *Fs Tri10Δ* did not produce T-2 toxin. Northern analysis indicated a significant decrease in the expression of all *Tri* genes, including the 11 that reside in the *Tri* gene cluster and the three outside of the *Tri* cluster, *Tri1*, *Tri101* and *Tri15* (Tag *et al.*, 2001, Peplow *et al.*, 2003). In the same study, a *Fs Tri6*-deleted mutant also significantly decreased expression of all *Tri* genes with the exception of *Tri10* (Peplow *et al.*, 2003). From this, the authors suggested that while both *Tri10* and *Tri6* regulate the expression of *Tri* genes, *Tri10* functions upstream of *Tri6* (Peplow *et al.*, 2003). Sequence analysis of *Tri10* at the amino acid level does not reveal any conserved motifs (Peplow *et al.*, 2003).

On the other hand, sequence analysis of the 217 amino acid protein encoded by the *Fs Tri6* gene revealed three imperfect repeats of Cys_2His_2 zinc finger-like motifs, each slightly differing from the general consensus of a Cys_2His_2 zinc finger motif, $CX_{(2-4)}CX_3FX_5LX_2HX_{(3-4)}H$ (Proctor *et al.*, 1995, Krizek *et al.*, 1991). The cysteines (C) and histidines (H) in the first zinc finger-like motif in *Fs* Tri6 are conserved but unlike the general consensus sequence where there are only three residues in between the second cysteine (C) and phenylalanine (F), there are five residues (Proctor *et al.*, 1995). Also, the conserved leucine is replaced by another phenylalanine (Proctor *et al.*, 1995). In the second zinc finger-like motif, phenylalanine and leucine are absent but three hydrophobic residues, methionine (M), valine (V) and phenylalanine, are found between the two cysteine and histidine fingers (Proctor *et al.*, 1995). The third zinc finger-like motif is also conserved, including the phenylalanine, but leucine is replaced with a methionine (Proctor *et al.*, 1995). Zinc finger motifs refer to a special structural domain on proteins

which require the binding of a zinc ion for stabilization (Krishna *et al.*, 2003). There are over 20 types of zinc-binding structural domains, for example, zinc ribbon and zinc binding loops, but the most common one is the Cys_2His_2 zinc finger motif (Krishna *et al.*, 2003). This type of zinc finger motifs forms two β-sheets at their N-terminus, which arrange themselves into hairpin, finger-like structure, and also one α-helix at their C-terminus (Krishna *et al.*, 2003, Brayer and Segal, 2008). The side-chains of the two cysteines and two histidines are responsible for binding to the zinc ion, and in proteins with transcription factor properties, the α-helix usually interacts with the major groove of the targeted nucleotide sequence to activate transcription (Iuchi, 2000, Krishna *et al.*, 2003, Brayer and Segal, 2008). Previously, Cys_2His_2 zinc finger motifs have been associated with transcription factors because of their interaction with nucleotides, however, there have been increasing reports of this type of motifs interacting with RNA and also other proteins (Brown, 2005, Brayer and Segal, 2008). In the case of *Fs* Tri6, Cys_2His_2 zinc finger motifs mediated binding to the consensus nucleotide sequence TNAGGCCT on the promoters of *Tri4* and *Tri5* (Hohn *et al.*, 1999). Using gel-shift analysis, the authors demonstrated that *Fs* Tri6 protein was able to bind to all three fragments of the *Tri5* promoter that harbour the consensus sequence, as well as two consensus sequences contained in the promoter of *Tri4* gene (Hohn *et al.*, 1999). The importance of zinc finger motifs was further supported by mutational analysis. For example, *Fs* Tri6 protein with a C187A mutation in its third zinc finger motif failed to bind to the same promoter regions of *Tri5* (Hohn *et al.*, 1999). In order to test the binding of *Fs* Tri6 to DNA *in vivo*, the authors created numerous transgenic *F. sporotrichioides* strains in which the two consensus sequences on the *Tri4* promoter were altered or

deleted, and the open reading frame of *Tri4* was replaced by the reporter gene *β-glucuronidase* (Hohn *et al.*, 1999). The *F. sporotrichioides* mutant strains carrying a *Tri4* promoter with the two intact consensus sequences expressed the same level of GUS as wild-type, however, in strains where one or both of the consensus sequences were missing or altered, their levels of GUS expression were at least 15-fold lower (Hohn *et al.*, 1999). The role of Tri6 as a transcriptional regulator was further documented when it auto-activated GAL4 reporter genes when fused to a GAL4 DNA binding domain in yeast (Proctor *et. al.,* 1995). Together with its DNA binding properties *in vitro*, *Fs Tri6* was hypothesized to be a transcriptional regulator of some *Tri* genes.

Fg Tri10 and *Fs* Tri10 share 88% identity at the amino acid level and *Fg* Tri6 and *Fs* Tri6 proteins also share 86% identity including the three Cys_2His_2 zinc finger-like motifs (Brown *et al.*, 2001). Despite sharing a high degree of identity, regulatory roles of *Tri10* and *Tri6* described in *F. sporotrichioides* remained to be verified in *F. graminearum*. Recently, Seong *et. al.* have confirmed that, similar to *F. sporotrichioides*, both *Fg Tri10* and *Fg Tri6* are required for the production of trichothecene (Seong *et. al.,* 2009). In addition, disruption of *Fg Tri10* resulted in a significantly lowered expression in all of the fifteen *Tri* genes (Seong *et. al.,* 2009). Likewise, disruption of *Fg Tri6* significantly decreased expressions of *Tri* genes, with the exception of *Tri10* (Seong *et. al.,* 2009).

1.7. Thesis Objectives

While it has been established that both *Fg Tri10* and *Fg Tri6* are required for the activation of *Tri* genes, there is increasing evidence suggesting that the two genes function outside of the trichothecene biosynthesis pathway. For example, in *F. sporotrichioides*, four genes, *Ibt1* - *Ibt4*, which code for proteolytic enzymes such as aspartyl protease and cellulase, were down-regulated in both *Fs Tri10Δ* and *Fs Tri6Δ* strains when compared to the wild-type (Peplow *et al.*, 2003). In the same study, expression of the gene *Ibs1*, which codes for a NADH-dependent flavin oxidoreductase, was significantly reduced in *Fs Tri6Δ* strain but was not affected in *Fs Tri10Δ* strain (Peplow *et al.*, 2003), suggesting that the two genes regulate an unique set of genes. This is further supported by a gene expression profiling study on infected wheat heads using both *Tri10Δ* and *Tri6Δ* mutant strains (Seong *et al.*, 2009). My research was undertaken well in advance of the publication of this study, which set out to address the differential roles of the two regulatory genes, *Tri10* and *Tri6*, of the trichothecene biosynthesis pathway in *F. graminearum*. To achieve this goal, the following were performed:

1) Construction of *F. graminearum Tri10*Δ and *Tri6*Δ mutant strains.

2) Expression profile analysis in both disrupted strains under nutrient-rich and nutrient-limiting conditions were performed to identify unique and co-regulated genes.

3) Assign distinct roles for *Tri10* and *Tri6* in *F. graminearum* pathogenicity.

Chapter 2 – Materials and Methods

2.1. Fusarium Strains, Media and Culture Conditions

Fusarium graminearum wild type NRRL 31084 (strain 233423) was used in this study. Fresh spores were grown in 50 mL of CMC liquid media (Cappellini and Peterson, 1965) at 170 RPM, 28°C for 5 days. Spores were harvested by filtering through four layers of sterile cheesecloth (Fisher Healthcare, Houston, TX, USA), washed twice by centrifugation at 4000 RPM, resuspended in sterile water and stored at 4°C until use. *F. graminearum* wild-type, mutant and complemented strains were maintained on potato dextrose agar (PDA) with appropriate antibiotics. Mycelia of both wild-type strains were grown by inoculating 10,000 spores in 4 mL of first stage media (Taylor *et al.*, 2008) for 24 hrs, washed and then transferred to grow in 4 mL of second stage media (Taylor *et al.*, 2008) for 4 hrs or 12 hrs. Mycelia were washed with sterile water, harvested by vacuum filtration using MF-Millipore membrane filters (Bedford, MA, USA), and stored at –80°C until use.

2.2. Gene Disruption Constructs, Protoplast Preparation and Fungal Transformation

Tri10 and *Tri6* disruption constructs consisting of the selection marker, hygromycin, flanked by 1000 bp of each 5'- and 3'- region of the respective genes were created using fusion PCR as described in Figure 4. Primers used to make these constructs are listed in Table A1 and Table A2 of the Appendix. PCR master mix consisted of 0.2 μM forward primer, 0.2 μM reverse primer, 0.2 mM dNTPs, 1.5mM $MgCl_2$, 50 ng of DNA template and 1.8 units of Expand long template PCR system (Hoffmann-La Roche Limited, Mississauga, ON, Canada). Protoplasts were made by digesting 1 – 5 X 10^8 germinated spores using an enzyme solution consisting of 5% Driselase from

Basidiomycetes sp. (Sigma-Aldrich Corporation, St. Louis, MO, USA), 1.0 mg of Chitinase from *Streptomyces sp.* (Sigma-Aldrich Corporation, St. Louis, MO, USA), and 100 mg Lysing enzymes from *Aspergillus sp.* (Sigma-Aldrich Corporation, St. Louis, MO, USA) in 20mL of 1.0M of NH_4Cl_2 pH 5.0 (Wiebe *et al.*, 1997) at 100 RPM at 30°C for 2.5 hrs. After washing with 1.0M NH_4Cl_2 pH 5.0, the protoplasts were resuspended in 300µL of STC buffer (1.2M sorbitol, 10mM Tris pH 8.0, 50mM $CaCl_2$ dihydrate) and diluted to $1X10^8$ protoplasts per mL with storage buffer (800µL STC, 200µL of 30% PEG, 10µL of DMSO). Protoplasts were aliquoted into 100µL aliquots and stored in –80°C until use.

Fungal transformation was carried out by incubating 100µL protoplasts with 500ng DNA of the disruption construct for 30 mins at room temperature containing STC and 30% PEG. After incubation, 2mL of 30% PEG and 4mL of STC were added and subsequently the entire transformation mixture was added to 200mL of Regeneration media (0.6g of yeast extract, 0.6g of casein hydrolysate, 9.2g of agar, 164.3g of sucrose in 600ml of water, autoclaved), mixed and poured into petri dishes. The plates were incubated at 28°C for 24 hours before adding a 10mL overlay consisting of 1% agar and 100 µg/mL of Hygromycin B from *Streptomyces* sp. (Calbiochem, Gibbstown, NJ, USA) or 100 µg/mL of G418 geneticin (Sigma-Aldrich Corporation, St. Louis, MO, USA).

2.3. Complementation of Tri10 and Tri6 Deleted Mutant Strains

To construct the "addback" vector for a disrupted gene mutant, the gene of interest and its native promoter were amplified from *F. graminearum* genomic DNA, digested with *Eco*RI and *Eco*RV restriction enzymes (New England Biolabs Inc.,

Ipswich, MA, USA), and ligated to the *Eco*RI-, *Eco*RV-digested pII99 vector (Namiki *et al.*, 2001). The 5.3 Kb pII99 plasmid is a derivative of pSP72 (Promega Corporation, Madison, WI, USA) that expresses the geneticin gene under the control of the trpC promoter (Namiki *et al.*, 2001). The pII99 vector map is available in Figure A1 in the Appendix. Protoplasts were made from *Tri10Δ* and *Tri6Δ* mutant spores, and 1µg DNA of each addback construct was transformed into protoplasts. The primers used for amplifying *Tri6* promoter–ORF as well as for *Tri10* promoter–ORF are listed in Table A3 in the Appendix.

2.4. DNA Isolation and Analysis

Frozen mycelia samples were ground to a powder using liquid nitrogen and DNA was extracted by using DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). Deleted mutants were confirmed by PCR using hygromycin primers (Hyg P5, Hyg P6), Tri10 ORF primers (Tri10 orf F and Tri10 orf R), and Tri6 ORF primers (T6 Fsp and T6 End R). In addition to the mentioned primers, complemented mutants were also screened for the presence of geneticin, which is the selection marker of the addback vector pII99, using geneticin primers (Geneticin ENTR and Geneticin Down). PCR parameters consisted of a 2-minute 94°C denaturing step, followed by 31 cycles of 30 secs at 94°C, 30 secs at 54°C, and 2 mins at 68°C. The ingredients of the PCR master mix were identical to that discussed in Section 2.2., but 0.6 units of Taq polymerase was used instead of Expand long template PCR system. Primer sequences are available in Table A4 in the Appendix. GeneRuler^{TM} 1 Kb Plus DNA ladder (Fermentas Canada Inc., Burlington, ON, Canada) was used for size confirmation of PCR products.

DNA blot analysis was performed on both *Tri10Δ* or *Tri6Δ* mutant strains. Genomic DNA of wild-type and *Tri10Δ* mutant strain were digested with *Sac*I enzyme (New England Biolabs Inc., Ipswich, MA, USA), while wild-type and *Tri6Δ* genomic DNA were digested with *Not*I enzyme (New England Biolabs Inc., Ipswich, MA, USA). A total of 4 µg of each digested DNA sample was loaded onto the gel and separated at 80 volts for 4 hours. Subsequently, the gel was treated with 0.2M HCl for 15 mins followed by incubation with 1.5M NaCl, 0.5M NaOH for 45 mins with gentle shaking to ensure the denaturation of double stranded DNA. The gel was neutralized with 1M Tris pH 7.4, 1.5M NaCl for 30 mins, and DNA was transferred onto a nitrocellulose membrane (Millipore, Bedford, MA, USA) overnight (Maniatis *et al.*,1989). Following the transfer, the nitrocellulose filter was baked in an 80°C oven for 1 hr and soaked in 20X SSC (3M sodium chloride, 300mM sodium citrate) for hydration prior to storing between Whatman 3MM filter papers (GE Healthcare, Buckinghamshire, UK). For hybridization of hygromycin radioactive probe to membrane, see section 2.6. (*Northern analysis and hybridization of radiolabeled probes).*

2.5. RNA Extraction and Microarray Analysis

RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). Integrity of RNA was measured by Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA). RNA samples were then transcribed into cDNA using a reverse transcriptase provided in the Quick Amp Labeling Kit (Agilent Technologies Inc., Santa Clara, CA, USA) and subsequently converted into cRNA using T7 RNA polymerase and cyanine 3-labeled CTP or cyanine 5-labeled CTP provided by

the Agilent two-color RNA spike-in kit (Santa Clara, CA, USA). Purification of cRNA was carried out using RNeasy mini spin columns from Qiagen (Valencia, CA, USA). Hybridization of purified cRNA to *F. graminearum* Agilent microarray slide was performed using the Gene Expression Hybridization Kit (Agilent Technologies Inc., Santa Clara, CA, USA). Each array consists of approximately 14,666 oligos including 1,417 spike-in and negative controls, and the remaining oligos represent a number of 13,918 expressed sequence tags with three 60mer-oligos representing each gene. Finally, the array slide was washed with Wash Buffer provided by the manufacturer (Agilent Technologies Inc., Santa Clara, CA, USA).

Hybridization signals were scanned using a GenePix Professional 4200A Scanner (Molecular Devices, Sunnyvale, CA, USA) and signal intensities were quantified using the software GenePix Pro 6 (Molecular Devices, Sunnyvale, CA, USA). Microarray data were imported into Acuity 4.0 (Molecular Devices, Sunnyvale, CA, USA) for assessment and analysis. Two-dye microarray data are subjected to non-linear dye bias, where dye intensity does not proportionately correlate to the level of gene expression, therefore rendering unreliable data (Oshlack *et al.*, 2007). Lowess Normalization was applied to raw microarray data to eliminate variations generated from dye bias, so that the only variation between gene expressions would be biological-based. Three biological replicates and two technical replicates for each experiment were compared, and only substances with a p-value < 0.001 were selected. Prior to combining technical and biological replicates, hierarchical clustering was performed on Acuity software to verify that all the same arrays were aligned together. Combined data, expressed as $\log_2$, were

then transferred onto Microsoft Excel to determine genes that were commonly or differentially regulated in *Tri10Δ* and *Tri6Δ*.

Genes with an expression of $\log_2 \geq 1$ (two-fold or more) in wild type, *Tri10Δ* or *Tri6Δ* mutants were considered up-regulated genes in each strain. To identify genes that were specifically up-regulated in WT, genes with an expression of $\log_2 \geq 1$ in wild type but $\log_2 \leq 1$ in *Tri10Δ* and *Tri6Δ* mutants were selected. Similar analysis was performed to identify genes that were specifically up-regulated in *Tri10Δ* or *Tri6Δ*. To identify genes that were up-regulated in both WT and *Tri10Δ* only, genes with an expression of $\log_2 \geq 1$ in WT and *Tri10Δ* but $\log_2 \leq 1$ in *Tri6Δ* were selected. Similar analysis was performed to identify genes that were up-regulated in WT and *Tri6Δ* only, as well as genes that were up-regulated in the two mutants only. Genes with an expression of $\log_2 \geq 1$ in WT, *Tri10Δ* and *Tri6Δ* were regarded as up-regulated genes in all three strains.

Genes with an expression of $\log_2 \leq -1$ in wild type, *Tri10Δ* or *Tri6Δ* were considered down-regulated genes in each strain. To identify genes that were specifically down-regulated in WT, genes with an expression of $\log_2 \leq -1$ in WT but $\log_2 \geq -1$ in *Tri10Δ* and *Tri6Δ* were selected. Similar analysis was performed to identify genes that were specifically down-regulated in *Tri10Δ* or *Tri6Δ*. To identify genes that were down-regulated in both WT and *Tri10Δ* only, genes with an expression of $\log_2 \leq -1$ in WT and *Tri10Δ* but $\log_2 \geq -1$ in *Tri6Δ* were selected. Similar analysis was performed to identify genes that were down-regulated in WT and *Tri6Δ* only, as well as genes that were down-

regulated in the two mutants only. Genes with an expression of $\log_2 \leq -1$ in WT, *Tri10Δ* and *Tri6Δ* were regarded as down-regulated genes in all three strains.

2.6. Northern Analysis and Hybridization of Radiolabeled Probes

Mycelia of *F. graminearum* wild type, *Tri10*Δ and *Tri6*Δ mutant strains were grown in the two-stage media as described in Section 2.1. for microarray and Northern analyses. RNA samples subjected to microarray analysis were extracted from mycelia incubated in 2nd stage media for 4 hours whereas RNA samples subjected to Northern analysis were extracted from mycelia incubated in 2nd stage media for 4 hours and 12 hours. RNA extraction was performed according to section 2.5. Northern analysis was performed on selected genes to confirm expression levels indicated in the microarray data. A total of 10 µg RNA of each sample was loaded and separated on a 1% formaldehyde gel at 65 volts for 2-3 hours (Maniatis *et al.*, 1989). Following electrophoresis, the gel was rinsed in deionized water and incubated in 10X SSC for 45 mins with gentle shaking. RNA was transferred onto an Immobilon-NY+ charged nylon membrane (Millipore, Bedford, MA, USA) by overnight capillary transfer (Maniatis *et al.*, 1989). RNA was immobilized on the nylon membrane by exposure to ultraviolet light in an ultraviolet crosslinker (model CL-1000, Ultra-Violet Products Limited, Science Park, Cambridge, UK). The membrane was stored between two Whatman 3MM filter papers at room temperature until hybridization.

Prior to hybridization of radiolabeled probes, the nitrocellulose filter or nylon membrane was incubated with 10mL of ExpressHyb Hybridization Solution (Clontech,

Mountain View, CA, USA) at 65°C for 1 hr to block non-specific hybridization. Meanwhile, the radioactive probe was prepared in a random priming reaction with 25ng of DNA template and [α^{32}P]-CTP (Perkin Elmer, Boston, MA, USA) using the reagents provided by the NEBlot Kit (New England Biolabs Inc., Ipswich, MA, USA) at 37°C for 1 hr. The hygromycin probe was used in the Southern analysis whereas the probes *Tri10*, *Tri6*, *Tri1* and *Fg00007* were used in Northern analysis. Primers used to amplify non-radiolabeled probe templates are listed in Table A5 in the Appendix. The probes were purified using Illustra NICK columns (GE healthcare, Buckinghamshire, UK). Specific activity of the probe was measured by Cerenkov counting by spotting 1 µL of the probe to a 1.5mm X 1.5mm Whatman 3MM filter paper and subjected to a liquid scintillation analyzer (model 1900 TR, Packard BioScience Company, Meriden, Connecticut, USA). A total of 2.0 X 10^7 CPM of probe was mixed with 200µL salmon sperm DNA, boiled and cooled on ice for 5 mins and then added to the hybridization solution incubated with the membrane at 65°C overnight. After hybridization, membranes were washed with 2X SSC, 0.1% SDS at room temperature for 30 mins and re-washed with the same buffer for 1 hr at 65°C. Membranes were wrapped in plastic wrap and exposed to Kodak BioMax MS film (Carestream Health Inc., Rochester, NY, USA) with a BioMax MS intensifying screen (Eastman Kodak Company, Rochester, NY, USA), and stored in –80°C overnight. The film was developed using a medical film processor (model SRX-101A, Konica Minolta Medical and Graphic Inc., Taiwan) and depending on the band intensity, another film was exposed to the membrane for an alternate amount of time.

2.7. Wheat Infection Assays

Wheat seeds, variety Roblin, were germinated on 1% water agar and transplanted into soil that had been fertilized with 20% nitrogen, 20% phosphorous, and 20% potassium. They were grown in a chamber that provided 16 hours of full spectrum light at a constant temperature of 22°C followed by 8 hours of darkness at a temperature of 15°C for six weeks. During anthesis, different strains of *F. graminearum* were inoculated into the floret at a concentration of 1000 spores per 10µL, as described in Hou *et al.* (2002). Inoculated wheat heads were marked and labelled, then placed in a growth room with 16 hours of full spectrum light at a day temperature of 20°C, followed by 8 hours of darkness at a night temperature of 15°C for 21 days. For the first 48 hours, the infected wheat plants were exposed to water mist every 15 minutes at 30 second intervals, but for the remaining 19 days, the plants were moved to a dry area where disease symptoms were observed. Pictures of infected wheat heads 21 days post inoculation were taken using a Canon PowerShot S50 camera (Canon Canada Inc., Mississauga, ON, Canada).

2.8. Secondary Metabolite Analysis

Mycelia of *F. graminearum* wild type, *Tri10*Δ and *Tri6*Δ mutant strains were grown in first stage media for 24 hours at 28°C, 170 RPM prior to incubation in second stage media under the same conditions for 12 hours. 1mL of filtrate was filtered through 0.2 µm Nylon Syringe filters (Mandel Scientific Company Inc., Guelph, ON, Canada). The sample for analysis was prepared by mixing 450 µL of the filtrate with 150µL of methanol and subjected to HPLC analysis using a 5um Hypersil ODS column (Thermo Fisher Scientific Inc., Waltham, MA, USA) connected to an AKTA P-900 purifier (GE

Healthcare, Buckinghamshire, UK). Trichothecenes were separated by employing a water: methanol gradient of 85: 15 to 60: 40 over 25 minutes at a flow rate of 1 mL/min. It is detected by Monitor UV-900 at 220nm (GE Healthcare, Buckinghamshire, UK). The retention time for 15-ADON was 11.3 mins under these conditions as calibrated by 15-ADON standard (provided by Barbara Blackwell, AAFC, Ottawa).

Chapter 3 – Results

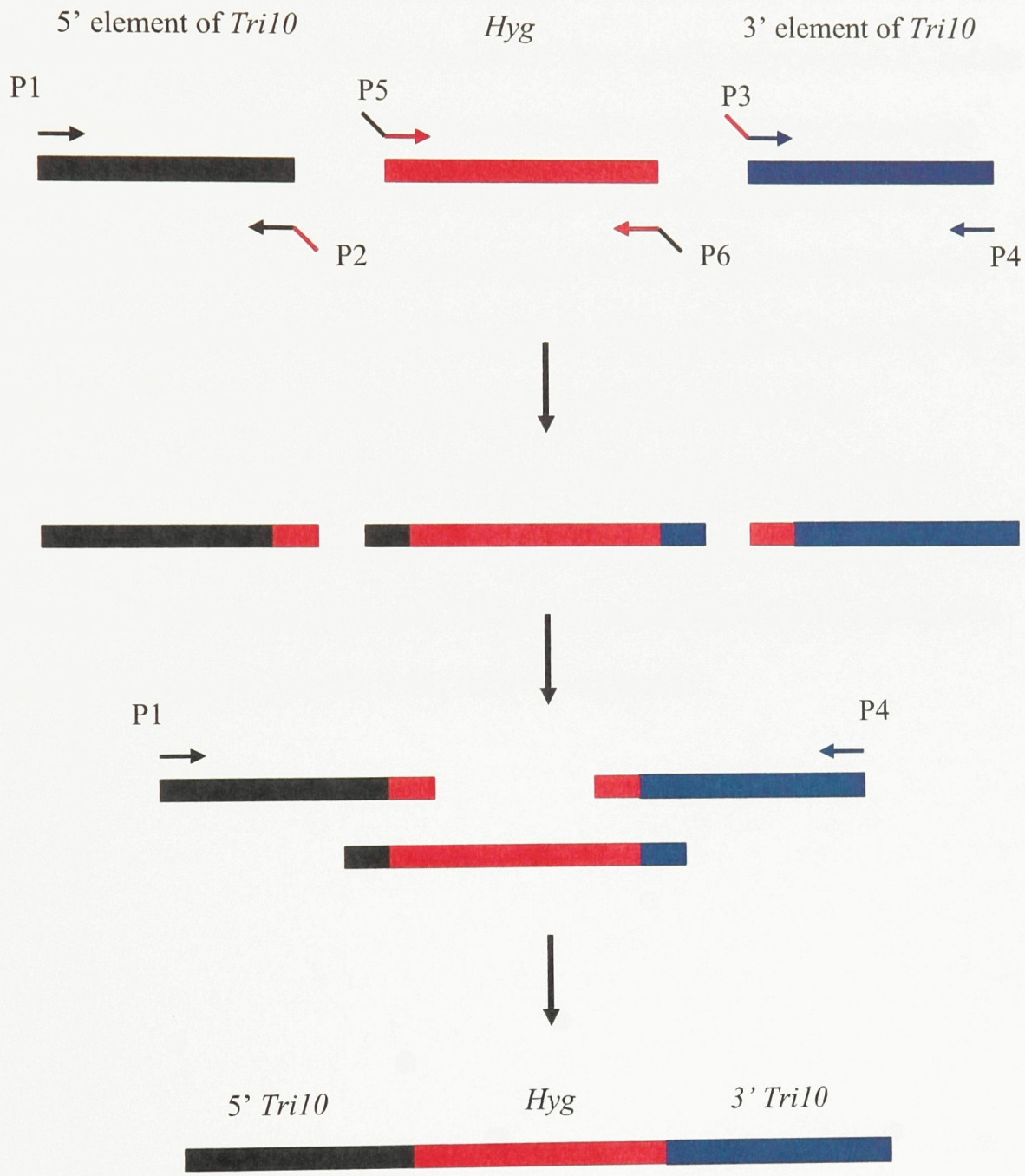
3.1. Fg Tri10 is Involved in Pathogenicity

3.1.1. Construction of F. graminearum Tri10Δ mutant strain

To investigate the effects of *Tri10* in *F. graminearum*, the *Tri10* coding region was replaced with *hygromycin* using homologous recombination. The construct used to replace *Tri10* was made as described in Figure 4. Three polymerase chain reactions (PCRs) were performed to amplify three chimeric gene fusions with overlapping sites of each other. Specifically, the first PCR product consisted of 1 kb of 5' flanking region of *Tri10* with 18 bp of *hygromycin* at its 3' end, the second PCR fragment consisted of 1 kb of 3' flanking region of *Tri10* with 18 bp of *hygromycin* at its 5' end. The third PCR fragment was the hygromycin gene with overhangs homologous to the 3' end of the 5' flanking region and the 5' end of the 3' flanking region of *Tri10*. The deletion construct was achieved with the combination of all three PCR products and amplification with flanking primers.

Figure 4: Generation of *Tri10* disruption construct.

Black solid line represents 5' element of *Tri10*, red solid line represents the *hygromycin* gene and blue solid line represents 3' element of *Tri10*. Using primers P1 and P2, the 5' element of *Tri10* consisting of 1000 bp upstream of the *Tri10* coding region and 18 bp of the 5' end of *hygromycin* gene was amplified. Using primers P5 and P6, the *hygromycin* gene consisting of overlapping regions with the 5' and 3' elements of *Tri10* was amplified. Using primers P3 and P4, the 3' element of *Tri10* consisting of 1000 bp downstream of the *Tri10* coding region and 18 bp of the 3' end of the *hygromycin* gene was amplified. The final disruption construct consisting of 5' element of *Tri10* – *hygromycin* – 3' element of *Tri10* was amplified using the P1 and P4 primers. Sequences of the primers can be found in Table A1 in the Appendix.


5' element of Tri10
Hyg
3' element of Tri10
P1
P5
P3
P2
P6
P4
P1
P4
5' Tri10
Hyg
3' Tri10

Hygromycin-resistant transformants were screened for the absence of the *Tri10* gene, as well as the presence of the *hygromycin* selection marker, and at least one of them verified to have the targeted gene disrupted. Figure 5 represents the PCR confirmation of *Tri10Δ* mutant strain for the absence of *Tri10* using *Tri10* gene-specific primers (lane 2), and the presence of *hygromycin* (lane 6). Southern analysis of the *Tri10Δ* mutant strain using hygromycin as probe indicated that only one copy of the disruption construct was incorporated into the genome (lanes 1 and 2, Figure 6). The *Tri10Δ* mutant strain was then complemented with plasmid (*pTri10*) consisting of the *Tri10* gene and 1 Kb of its promoter, and a second selection marker, *Geneticin*, into the genome. This complemented strain, denoted as *Tri10Δ-AB*, harboured *hygromycin* from the gene replacement (lane 7, Figure 5), *Tri10* gene (lane 3, Figure 5) and *geneticin* (lane 11, Figure 5) from *pTri10* addback plasmid. The primers used in the verification of *Tri10Δ* and *Tri10Δ-AB* strains are listed in Table A4 of the Appendix.

Figure 5: Confirmation of *Tri10* disruption and complementation in *F. graminearum*. DNA was extracted from wild type, *Tri10Δ* and *Tri10Δ-AB* strains and subsequently used as templates for PCR confirmation of *Tri10* disruption and complementation.

Lanes 1-4: WT, *Tri10Δ, Tri10Δ-AB* transformant #9, and water control, respectively, screened with *Tri10* gene specific primers, *Tri10 orf F* and *Tri10 orf R*. The PCR product at ~1200 bp in lanes 1 and 3 represents *Tri10* gene, with an expected size of 1263 bp.

Lanes 5-8: WT, *Tri10Δ, Tri10Δ-AB* transformant #9, and water control, respectively, screened with *hygromycin* primers, *Hyg P5* and *Hyg P6*. The PCR product at ~1800 bp in lanes 6 and 7 represents *hygromycin*, with an expected size of 1822 bp.

Lanes 9-12: WT, *Tri10Δ, Tri10Δ-AB* transformant #9, and water control, respectively, screened with *geneticin* primers, *Geneticin ENTR* and *Geneticin Down*. The PCR product at ~1200 bp in lane 11 represents *geneticin*, with an expected size of 1283 bp.

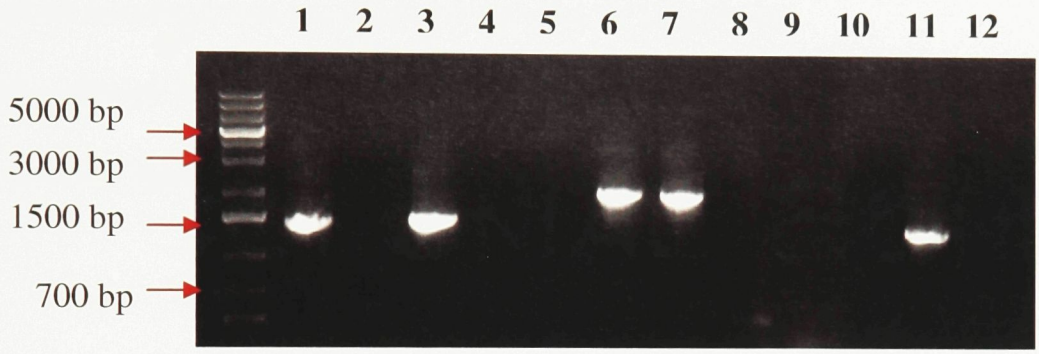

1 2 3 4 5 6 7 8 9 10 11 12
5000 bp
3000 bp
1500 bp
700 bp

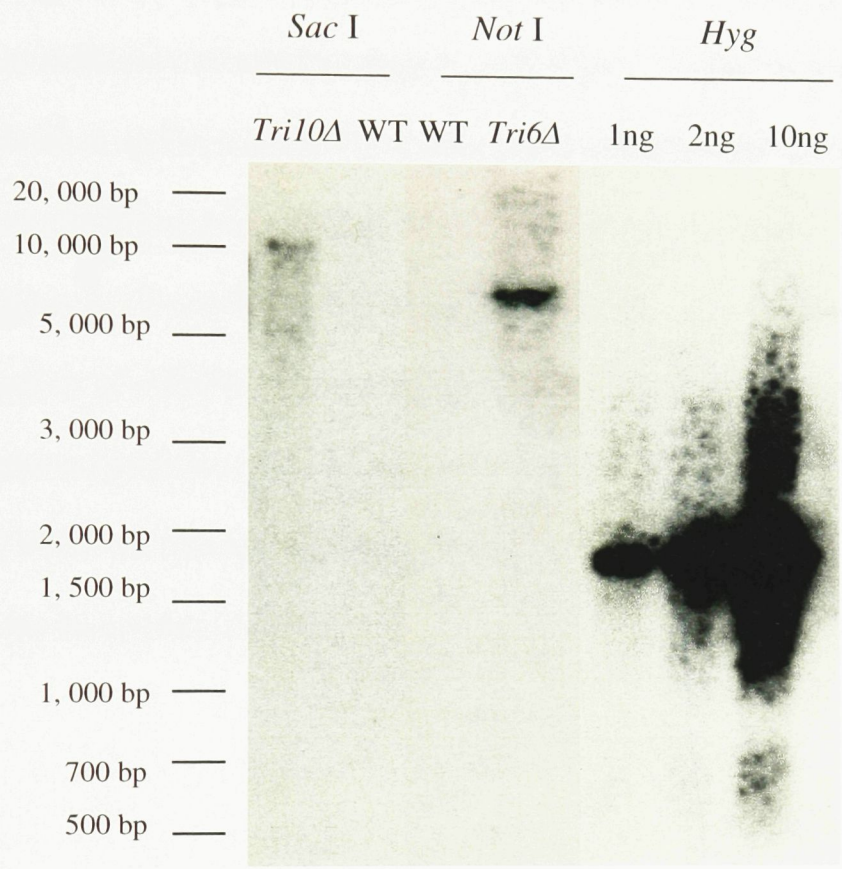
Figure 6: DNA blot analysis of *Tri10*Δ and *Tri6*Δ mutant strains.

To determine the number of disruption construct integrated into the genome, 4 μg of *Sac*I-digested wild type and *Tri10*Δ DNA along with 4 μg of *Not*I-digested wild type and *Tri6Δ* DNA were hybridized with a *hygromycin* probe.

The single band at 10,000 bp present in *Tri10Δ* digested with *Sac*I indicates that only one disruption construct was incorporated into the *Tri10Δ* genome.

The single band at ~8,000 bp present in *Tri6Δ* digested with *Not*I indicates that only one disruption construct was incorporated into the *Tri6Δ* genome.

Loading control for the *hygromycin* probe was monitored by 1 ng, 2 ng, and 10 ng of the PCR product *hygromycin*.


Sac I
Not I
Hyg
Tri10Δ WT WT Tri6Δ 1ng 2ng 10ng
20, 000 bp
10, 000 bp
5, 000 bp
3, 000 bp
2, 000 bp
1, 500 bp
1, 000 bp
700 bp
500 bp

3.1.2. Fg Tri10Δ does not produce trichothecene derivative 15-ADON

A distinctive phenotype of *F. graminearum* strain 233423 is its ability to produce 15-ADON when cultured under nutrient-limiting conditions *in vitro*. To determine the involvement of *Tri10* in the production of trichothecene in *F. graminearum*, the filtrates of *Fg Tri10Δ* and *Fg Tri10Δ-AB* strains cultured under DON-inducing conditions were subjected to secondary metabolite analysis using HPLC. Figure 7b shows that 15-ADON standard elutes at approximately 11.11 mins when added to wild type *F. graminaerum* grown in non DON-inducing media (15-ADON standard provided by Barbara Blackwell, AAFC). Figure 8, 15-ADON was synthesized in the WT strain (panel A), but *Fg Tri10Δ* did not produce 15-ADON, as indicated by the absence of a peak at the retention time indicated above (panel B). The synthesis of 15-ADON from the *Tri10* complemented strain *Fg Tri10Δ-AB* (panel C) indicated that *Tri10* contributes to the activation of the trichothecene biosynthesis pathway.

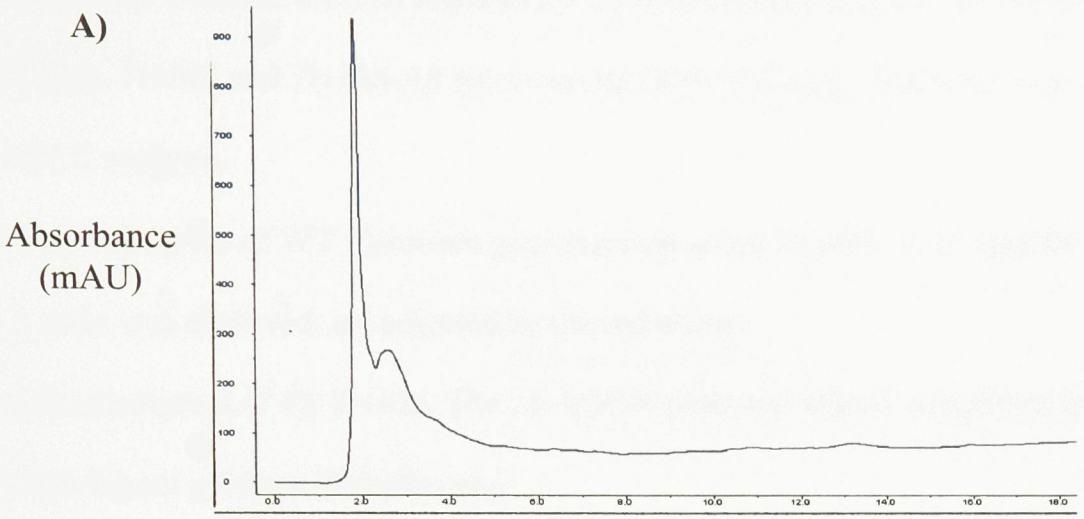
Figure 7: HPLC chromatograms of 15-ADON standard.

In order to identify the elution time of 15-ADON standard, HPLC chromatogram of wild type *F. graminearum* grown in non DON-inducing media was compared to that of wild type *F. graminearum* grown under the same conditions spiked with 15-ADON (provided by Barbara Blackwell, AAFC). The x-axis indicates retention time whereas the y-axis represents absorbance of light transmitted in mAU.

A) Chromatogram of WT *F. graminearum* in non DON-inducing media for 35 hours. A solvent peak was observed at around 2 mins.

B) Chromatogram of WT *F. graminearum* in non DON-inducing media for 35 hours spiked with 150 µg of 15-ADON. In addition to the solvent peak observed at 2 mins, an additional peak was also observed at 11.1 mins, as indicated by the red arrow. This additional peak represents 15-ADON.

A)


Absorbance
(mAU)
900
800
700
600
500
400
300
200
100
0
0.0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
16.0
18.0
Retention Time (mins)

B)

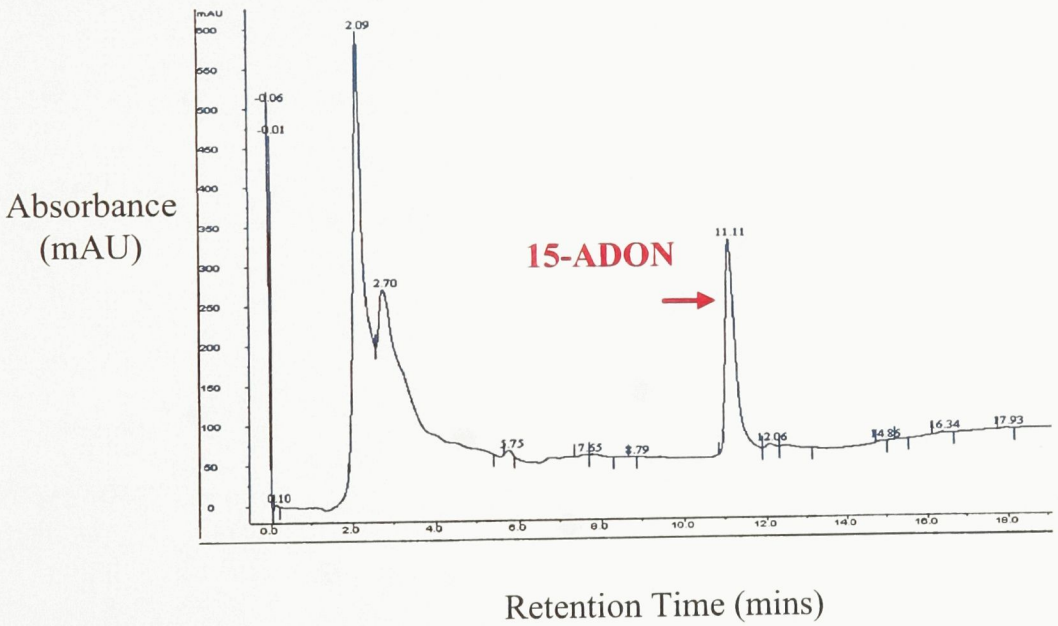

Absorbance
(mAU)
mAU
600
550
500
450
400
350
300
250
200
150
100
50
0
2.09
-0.06
-0.01
2.70
11.11
15-ADON
3.75
7.55
7.79
12.06
14.85
16.34
17.93
0.10
0.0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
16.0
18.0
Retention Time (mins)

Figure 8: HPLC chromatograms of *F. graminearum Tri10Δ* and *Tri10Δ-AB* strains cultured under DON-inducing conditions.

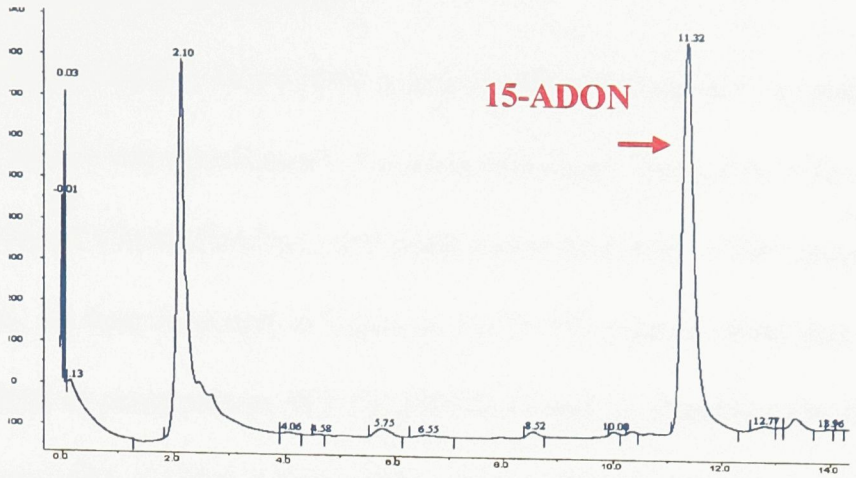
To determine whether *Tri10* is required for trichothecene production, the media filtrate of wild type, *Tri10Δ* and *Tri10Δ-AB* grown under DON-inducing conditions were subjected to HPLC analysis.

A) Chromatogram of WT *Fusarium graminearum* strain 233423. A 15-ADON peak at 11.3 mins was observed, as indicated by the red arrow.

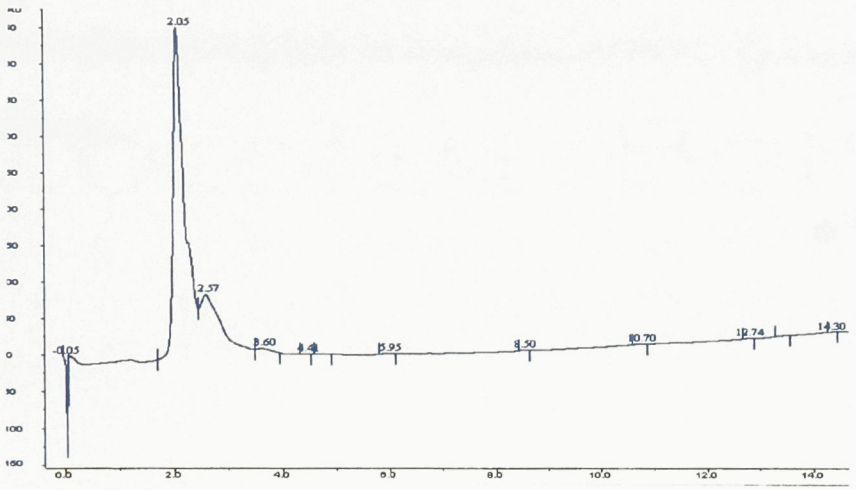
B) Chromatogram of *Fg Tri10Δ*. The 15-ADON peak was absent, suggesting that *Fg Tri10Δ* did not produce trichothecene.

C) Chromatogram of *Fg Tri10Δ-AB*. The 15-ADON peak was present, as indicated by the red arrow, suggesting that the *Tri10* complemented strain was able to produce trichothecene.

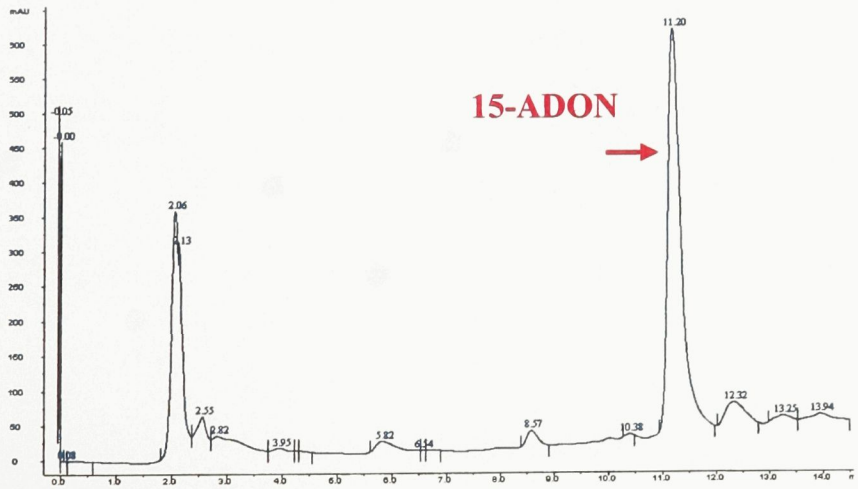
A)


15-ADON
Absorbance (mAU)
Retention Time (mins)

B)


Absorbance (mAU)
Retention Time (mins)

C)


15-ADON
Absorbance (mAU)
Retention Time (mins)

3.1.3. Fg Tri10 contributes to virulence

To determine whether *Tri10* plays a role in pathogenicity of *F. graminearum*, wheat infection assays were performed. For each pathology assay, five wheat heads of a susceptible variety of wheat (Roblin) were point inoculated with ~1000 spores of *Fusarium* strains. As demonstrated in Figure 9, *Fg Tri10Δ* mutant strain was non-virulent (wheat heads 3 and 4) compared to WT *Fg* 233123 (1 and 2). Significantly, the complemented strain *Fg Tri10Δ-AB* was able to rescue *Tri10Δ* phenotype (5 and 6) and cause disease. This, in combination with the HPLC results (Figure 8) conclusively suggested that *Tri10* plays a role in both the biosynthesis of DON, which in turns affects virulence of this fungus.

Figure 9: *Fg Tri10* contributes to virulence in *F.graminearum*

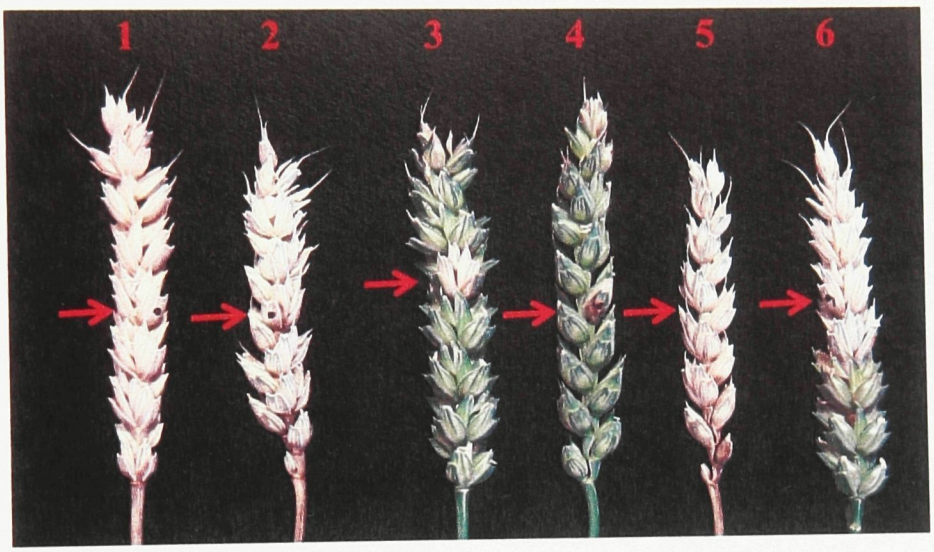
Representative wheat heads (variety Roblin) after infection with WT, *Tri10Δ* and *Tri10Δ-AB* strains.

1, 2: Wheat heads infected with *F. graminearum* WT strain. Twenty-one days subsequent to the initial inoculation, both infected wheat heads were completely bleached.

3, 4: Wheat heads infected with *Fg Tri10Δ* strain. The *Tri10Δ* strain was able to infect wheat heads but could not spread beyond the site of infection, as indicated by the bleaching of the inoculated spikelets only.

5, 6: Wheat heads infected with *Fg Tri10Δ-AB* transformant #9. The *Tri10* complemented strain was able to infect and rescue mutant phenotype to spread beyond the site of infection, as indicated by the bleaching of the entire wheat head.

Picture was taken 21 days post infection. This experiment was repeated three times with similar results. Red arrow indicates site of inoculation.


1
2
3
4
5
6

3.2. Fg Tri6 is Involved in Pathogenicity

3.2.1. Disruption of Fg Tri6 using Homologous Recombination

Fg Tri6Δ strain was constructed by homologous recombination using a similar fusion PCR method used to generate the *Tri10* deletion mutant. However, the structure of the construct was slightly different as shown in Figure 10. Unlike *Tri10Δ* where the entire *Tri10* open reading frame was replaced by *hygromycin*, recombination occurred at 107 bp inside of the ATG start site of *Tri6* and 65 bp from the TGA stop codon; consequently, only 485 bp of the 657bp *Tri6* was replaced by *hygromycin*, as shown in Figure 10a. Figure 10b presents a PCR confirmation of *Fg Tri6Δ* mutant strain, where a 1889 bp product was amplified using *Tri6*-specific primers (lane 2). Since *Tri6*-specific primers, *Tri6 Fsp* and *Tri6 End R*, anneal to regions outside the recombination sites (see Figure 10a), the entire 1822bp *hygromycin* gene and the 67 bp from *Tri6* were amplified as a PCR product with the size of 1889 bp. Using *hygromycin*-specific primers, the 1822bp *hygromycin* was also amplified (lane 6, Figure 10b). Similar to *Tri10Δ*, Southern analysis revealed that a single copy of the disruption construct was integrated into the genome, indicating that *Tri6* was the only gene disrupted during the fungal transformation and recombination process (Figure 6). Complementation of *Fg Tri6Δ* mutant strain was accomplished by re-introducing a *pTri6HA* addback plasmid into the *Tri6* deleted strain. The add back construct with Geneticin as a selection marker, consisted of 1 Kb of the *Tri6* promoter, and the *Tri6* gene fused to a hemagglutinin (HA) epitope tag at the C-terminus. This complemented *Fg Tri6Δ-AB* strain was screened for *Tri6* (lane 3, Figure 10b), *hygromycin* (lane 7, Figure 10b) and *geneticin* (lane 11, Figure 10b) sequences. Notably, two PCR products with a size of 1889 bp and 672 bp were

observed in lane 3 (Figure 10b), with the former representing the hygromycin-disrupted *Tri6* and the latter representing the *Tri6* from the addback plasmid. Primers used in the verification of *Tri6Δ* and *Tri6Δ-AB* strains are listed in Table A4 of the Appendix.

Figure 10: Confirmation of *Tri6* disruption and complementation in *F. graminearum*.

(A) The *Tri6* disruption construct consists of 1000 bp of 5' and 3' element of the *Tri6* coding region. This construct replaces only 485 bp of the *Tri6* coding region with *hygromycin*, leaving 107 bp of the 5' end and 65 bp of the 3' end of the *Tri6* ORF in the genome.

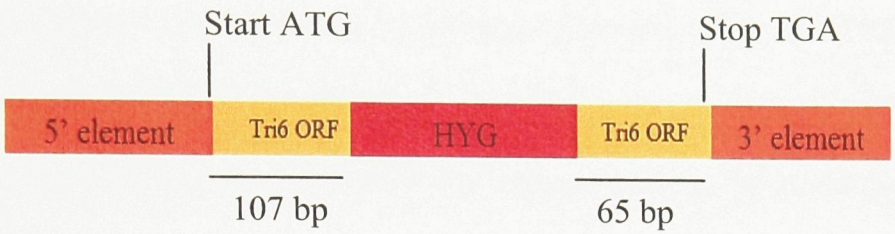
(B) PCR confirmation of *Tri6Δ* and *Tri6Δ-AB* strains.

Lanes 1-4: WT, *Tri6Δ, Tri6Δ-AB* transformant #9, water control respectively, screened with *Tri6* gene specific primers, *Tri6 Fsp* and *Tri6 End R*. The PCR product at ~600 bp in lanes 1 and 3 represents the functional *Tri6* gene, with an expected size is 657 bp. The PCR product at ~1900 bp in lanes 2 and 3 represents a combination of intact *hygromycin* gene and portions of *Tri6*, together with an expected size of 1889 bp.

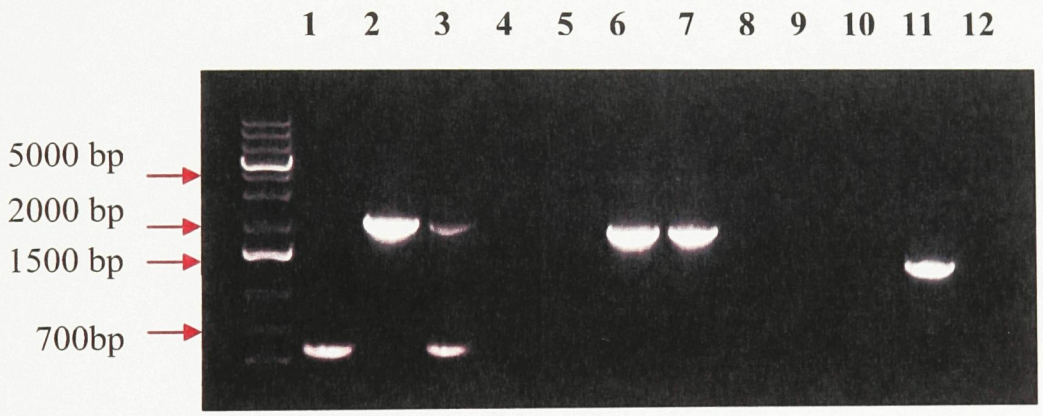
Lanes 5-8: WT, *Tri6Δ, Tri6Δ-AB* transformant #9, water control respectively screened with hygromycin primers, *Hyg P5* and *Hyg P6*. The PCR product at ~1800 bp in lanes 6 and 7 represent *hygromycin* gene, with an expected size of 1822 bp.

Lanes 9-12: WT, *Tri6Δ, Tri6Δ-AB* transformant #9, water control respectively screened with geneticin primers, *Geneticin ENTR* and *Geneticin Down*. The PCR product at ~1200 bp in lane 11 represents *geneticin*, with an expected size of 1283 bp.

A)


Start ATG
Stop TGA
5' element
Tri6 ORF
HYG
Tri6 ORF
3' element
107 bp
65 bp

B)


1 2 3 4 5 6 7 8 9 10 11 12
5000 bp
2000 bp
1500 bp
700bp

3.2.2. Fg Tri6 is required for trichothecene production

To determine the involvement of *Tri6* in the trichothecene biosynthesis pathway of *F. graminearum*, *Tri6Δ* and *Tri6Δ-AB* strains were grown under DON-inducing conditions and their filtrates were subjected to secondary metabolite analysis. As in the case of *Fg Tri10Δ* strain, the WT *F. graminearum* strain was able to synthesize the mycotoxin 15-ADON (panel A, Figure 11) while the *Fg Tri6Δ* strain did not produce 15-ADON, as indicated by the absence of a 15-ADON peak (panel B, Figure 11). Complementation of *Tri6Δ* rescued its ability to produce 15-ADON, as supported by the peak eluting at 11.3 mins (panel C, Figure 11).

Figure 11: HPLC chromatograms of *F. graminearum Tri6Δ* and *Tri6Δ-AB* strains cultured under DON-inducing conditions.

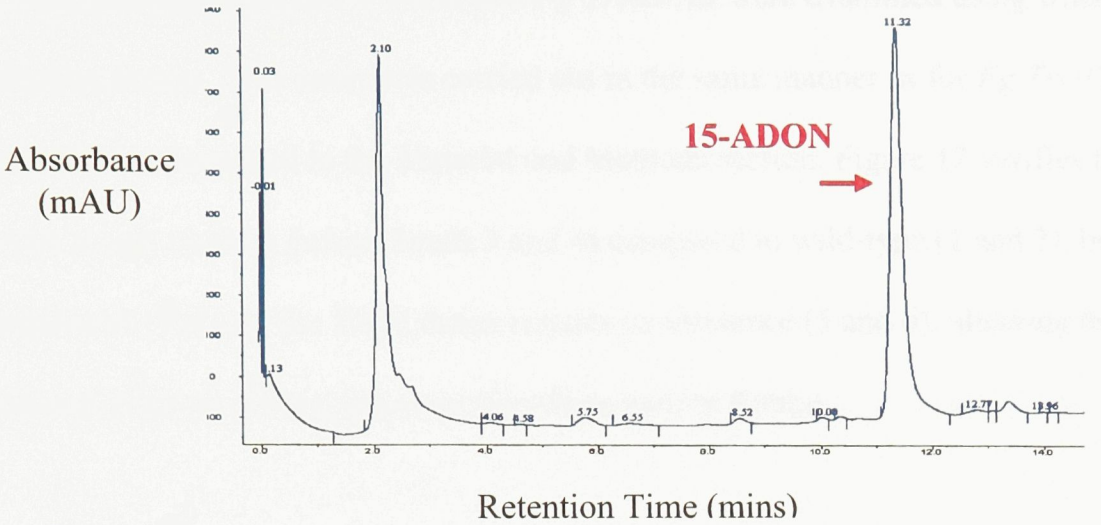
To determine whether *Tri6* is required for trichothecene production, the media filtrate of wild type, *Tri6Δ* and *Tri6Δ-AB* were subjected to HPLC analysis.

A) Chromatogram of WT *F. graminearum* strain 233423 grown under DON-inducing conditions. A 15-ADON peak at 11.3 mins was observed, as indicated by the red arrow.

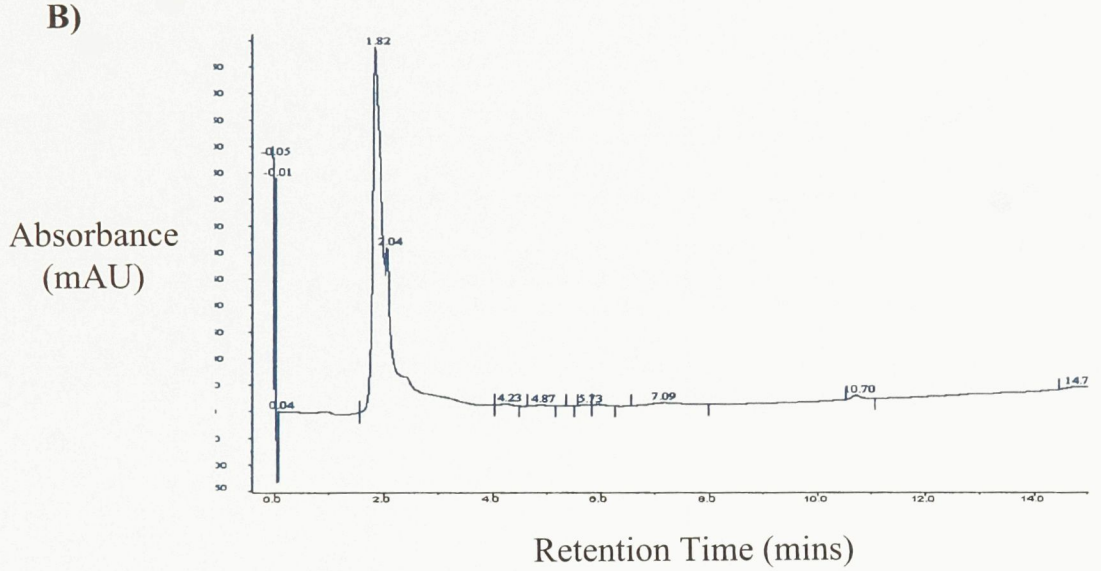
B) Chromatogram of *Fg Tri6Δ* grown under DON-inducing conditions. The 15-ADON peak was absent, suggesting that *Fg Tri6Δ* did not produce trichothecene.

C) Chromatogram of *Fg Tri6Δ-AB* grown under DON-inducing conditions. The 15-ADON peak was present, as indicated by the red arrow, suggesting that the *Tri6* complemented strain was able to produce trichothecene.

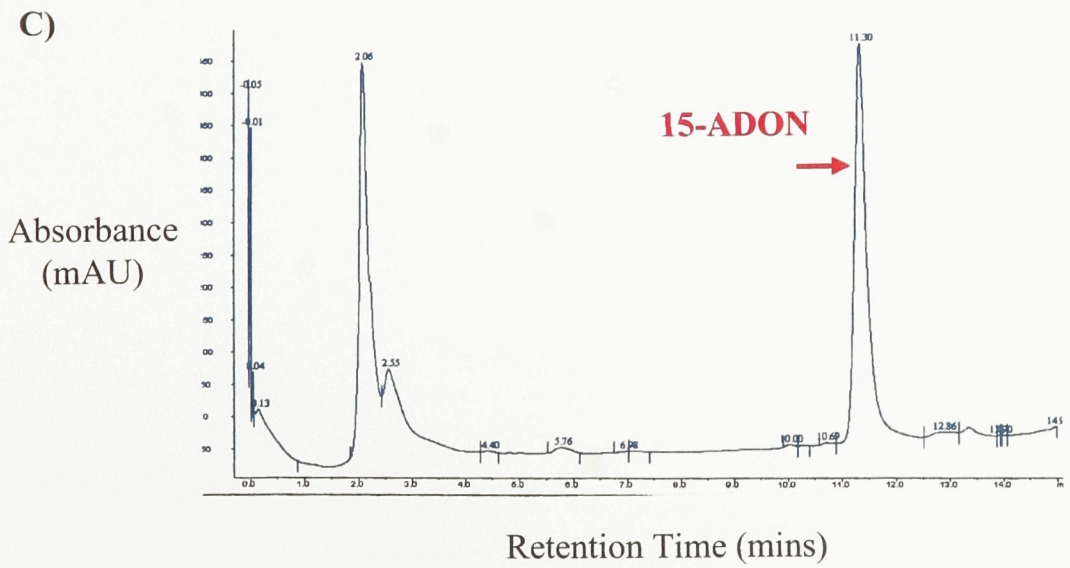
A)


Absorbance
(mAU)
15-ADON
Retention Time (mins)

B)


Absorbance
(mAU)
Retention Time (mins)

C)


Absorbance
(mAU)
15-ADON
Retention Time (mins)

3.2.3. Fg Tri6 contributes to virulence

The pathogenicity of *Fg Tri6Δ* and *Fg Tri6Δ-AB* were examined using wheat infection assays. This assay was carried out in the same manner as for *Fg Tri10Δ*, and the procedure is described in the Material and Methods section. Figure 12 verifies that *Fg Tri6Δ* is non-virulent (wheat heads 3 and 4) compared to wild-type (1 and 2), but the addition of *Tri6* into the *Tri6Δ* strain rescues its virulence (5 and 6), allowing the addback strain to cause disease on the wheat variety Roblin.

Figure 12: *Fg Tri6* contributes to virulence in *F. graminearum*

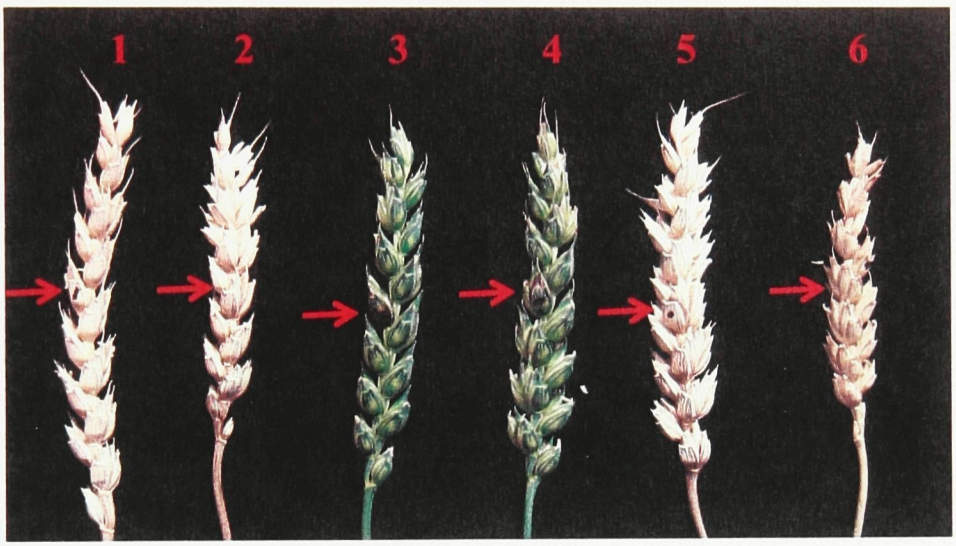
Representative wheat heads (variety Roblin) after infection with WT, *Tri6Δ* and *Tri6Δ-AB* strains.

1, 2: Wheat heads infected with *F. graminearum* WT strain. Twenty-one days subsequent to the initial inoculation, both infected wheat heads were completely bleached.

3, 4: Wheat heads infected with *Fg Tri6Δ* strain. The *Tri6Δ* strain was able to infect wheat heads but could not spread beyond the site of infection, as indicated by the browning of inoculated site only.

5, 6: Wheat heads infected with *Fg Tri6Δ-AB* transformant #9. The *Tri6* complemented strain was able to infect and rescue mutant phenotype to spread beyond the site of infection, as indicated by the bleaching of the entire wheat head.

Picture was taken 21 days post infection. This experiment was repeated three times with similar results. Red arrow indicates site of inoculation.


1
2
3
4
5
6

3.3. Establishment of a Relationship between Tri10 and Tri6 in vitro

From the previous sections, we established that both *Fg Tri10* and *Fg Tri6* play a significant role in the trichothecene biosynthesis pathway because disruption of either gene resulted in failure to produce 15-ADON (Figure 8B and 10B). In order to identify genes that are commonly and differentially regulated by *Tri10* and *Tri6*, we performed whole gene expression profiles on *F. graminearum* wild type, *Tri10*Δ and *Tri6*Δ mutant strains grown under DON-inducing conditions. The following section is the analysis of genes that are commonly or uniquely regulated by *Fg Tri10* and *Fg Tri6*.

3.3.1. Profiling of genes regulated by Tri10Δ and Tri6Δ under DON-inducing conditions in vitro

Gene expression profiles were carried out using an Agilent custom made oligonucleotide microarray that consists of a total of 44, 000 oligos, of which 1,417 are controls and the rest represent 13,918 ESTs, with each gene represented by three 60-mer oligonucleotides (Agilent Technologies Inc., Santa Clara, CA, USA). As a first step, we were interested to know the total number of genes that are differentially expressed in the DON-inducing media. Genes expressed in WT *Fusarium* grown under DON non-inducing conditions were compared to genes expressed in WT *Fusarium* grown under DON-inducing conditions. Next, gene expression profiling was performed in both *Tri10* and *Tri6* mutant strains and compared to the WT *Fusarium*, respectively, grown under DON-inducing conditions. The numbers of genes that are differentially regulated under DON-inducing conditions by these mutant strains are summarized in the Venn diagram (Figure 13).

Figure 13: Venn diagram indicating the number of genes commonly and differentially regulated in WT, *Fg Tri6Δ*, and *Fg Tr10Δ* .

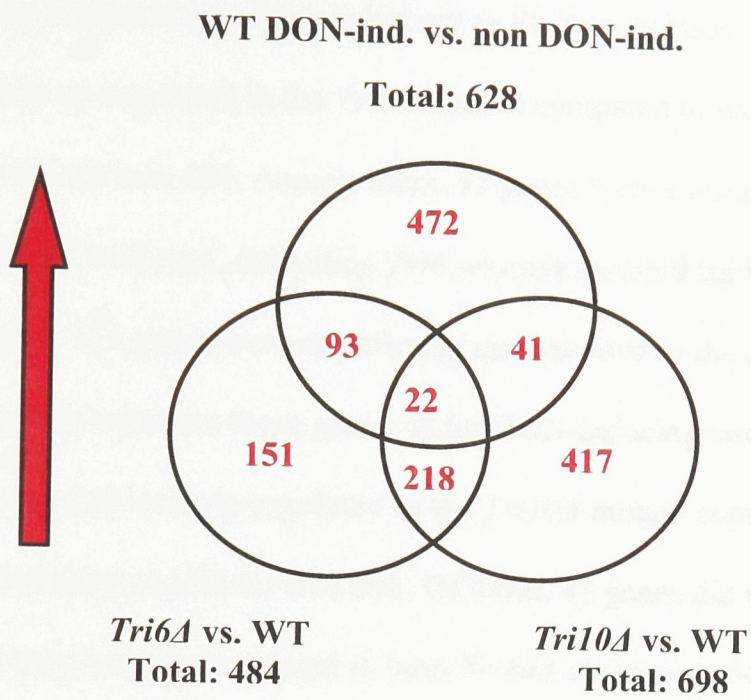
A) Red arrow indicates up-regulated genes in WT, *Tri10*Δ and *Tri6*Δ.

A total number of 628, 698 and 484 genes were up-regulated in wild type, *Tri10Δ*, *Tri6Δ* respectively under DON-inducing conditions. Of those, 472, 417 and 151 genes were unique to the strains. The two mutant strains shared 218 commonly up-regulated genes, with *Tri10Δ* and WT sharing 41 genes, and *Tri6Δ* and WT sharing 93 genes. Together, 22 genes were up-regulated in all three strains.

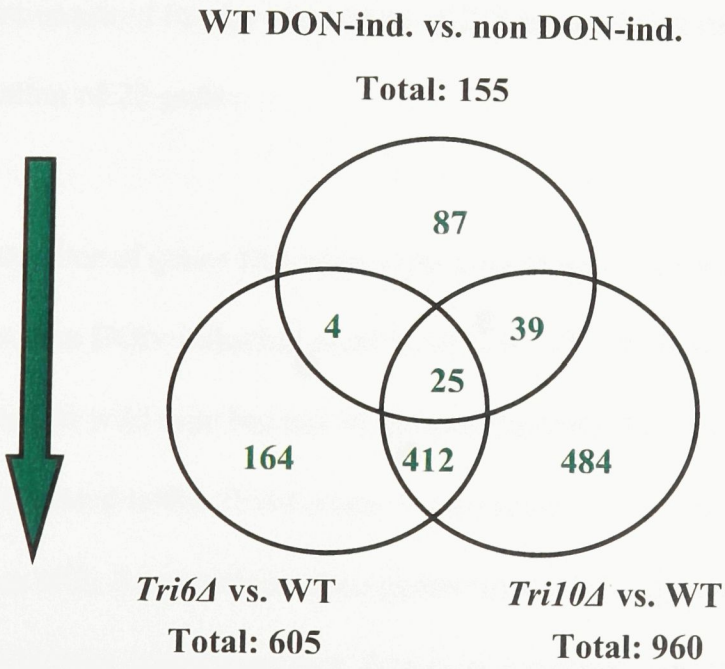
B) Green arrow indicates down-regulated genes in WT, *Tri10*Δ and *Tri6*Δ.

A total number of 960 and 605 genes were down-regulated in *Tri10*Δ and *Tri6*Δ strains compared to wild type under DON-inducing conditions respectively, compared to only 155 down-regulated genes in the wild type under DON-inducing conditions. Of these total numbers of down-regulated genes, 87 genes were uniquely down-regulated in wild type, 484 in *Tri10Δ* strain and 164 in *Tri6Δ* strain. A total number of 412 genes were down-regulated in both *Tri10Δ* and *Tri6Δ* strains, with 39 down-regulated genes shared by *Tri10Δ* and wild type and only four by *Tri6Δ* and wild type. There were 25 genes commonly down-regulated in all three strains.

A)


WT DON-ind. vs. non DON-ind.
Total: 628
472
93
41
22
151
218
417
Tri6Δ vs. WT
Total: 484
Tri10Δ vs. WT
Total: 698

B)


WT DON-ind. vs. non DON-ind.
Total: 155
87
4
39
25
164
412
484
Tri6Δ vs. WT
Total: 605
Tri10Δ vs. WT
Total: 960

The number of genes that were activated in wild type under DON-inducing conditions vs. non DON-inducing conditions was 628. Of those, 472 genes were specifically up regulated in wild type but not in the two mutants. The total number of genes that were up-regulated in the *Tri6Δ* mutant compared to wild type under DON-inducing conditions was 484. Among them, 93 genes were commonly up-regulated in the *Tri6Δ* mutant and wild type, indicating *Tri6* was not required for the activation of these genes. However, 151 genes were specifically up-regulated in the *Tri6Δ* strain, suggesting that *Tri6* normally represses these genes under DON-inducing conditions. The total number of genes that were up-regulated in the *Tri10Δ* mutant compared to wild type under DON-inducing conditions was 698. Of those, 41 genes did not require *Tri10* for activation, as they were up-regulated in both *Tri10Δ* strain and wild type. The majority, 417 genes, were up-regulated in *Tri10Δ* strain only, indicating that these genes were normally repressed in the presence of *Tri10* under DON-inducing conditions. Both *Tri10* and *Tri6* were required for the repression of 218 genes, however, neither was responsible for the activation of 22 genes.

The number of genes that were repressed in wild type under DON-inducing conditions vs. non DON-inducing conditions was 155. Of those, 87 genes were uniquely down-regulated in wild type but not in the two mutants. The total number of genes that were down-regulated in the *Tri6Δ* mutant compared to wild type under DON-inducing conditions was 605. Among them, four genes were commonly down-regulated in *Tri6Δ* mutant and wild type, suggesting that *Tri6* does not repress these genes. However, 164 genes were uniquely down-regulated in the *Tri6Δ* strain, suggesting that Tri6 normally

activates their expression under DON-inducing conditions. The total number of genes that were down-regulated in the *Tri10Δ* mutant compared to WT under DON-inducing conditions were 960. Of those, 39 genes were not repressed by *Tri10* under normal circumstances, as they were down-regulated in both *Tri10Δ* strain and wild type. 484 genes were down-regulated in *Tri10Δ* mutant only, suggesting that these genes were normally activated in the presence of *Tri10* under DON-inducing conditions. Both *Tri10* and *Tri6* were required for the activation of 412 genes, but neither was responsible for the repression of 25 genes.

3.3.2. Fg Tri10 and Fg Tri6 co-regulate genes involved in secondary metabolism

Secondary metabolism refers to the synthesis of metabolites that are not essential to the growth of the organism, but contribute to their overall fitness. These pathways are typically induced under nutrient-limiting conditions or during certain developmental stages to produce and secrete toxic metabolites, hence strengthening the organism's ability to survive by eliminating nearby competitors or weakening a host plant. Biosynthetic genes involved in secondary metabolic pathways often occur in gene clusters in filamentous fungi, for example, the penicillin gene clusters in *P. chrysogenum* and *A. nidulans*, aflatoxin gene clusters in *Aspergillus sp.*, and melanin gene clusters in *A. alternata* (Keller and Hohn, 1997). Within the *Fusarium* genus, some species consist of a cluster of 15 genes involved in the production of fumonisins while others contain another 15 gene-cluster encoding for genes required for the zearalenone biosynthesis pathway (Desjardins, 2006).

Our analysis of the genes up-regulated in wild type under DON-inducing conditions but down-regulated in both *Tri10*Δ and *Tri6*Δ mutant strains revealed regulation of two distinctive secondary metabolite clusters. The first cluster to be identified belonged to the trichothecene biosynthetic pathway leading to the production of DON and the second cluster contained genes involved in the production of butenolide (Harris *et al.*, 2007). It is understood that *Fg Tri10* and *Fg Tri6* regulate the trichothecene biosynthesis pathway (Seong *et al.*, 2009); however, regulation of the butenolide pathway was not previously known. Of the eight genes involved in the butenolide pathway, five were up-regulated in wild type under DON-inducing conditions but down-regulated in both *Tri10Δ* and *Tri6Δ* mutants compared to wild type.

3.3.2.1. Fg Tri10 and Fg Tri6 regulate the Tri gene cluster

As mentioned in the Introduction, there are a total of 15 genes involved in the trichothecene biosynthesis pathway in *F. graminearum*. While 12 genes reside in the *Tri* gene cluster on chromosome 2, three other genes, *Tri1*, *Tri101* and *Tri15*, are located outside the cluster on different chromosomes (McCormick *et al.*, 2004, Kimura *et al.*, 1998, Desjardins, 2006). Expression levels of *Tri* genes in wild type and the two mutant strains can be found in Table 2. Eleven of the *Tri* genes were down-regulated by at least two-fold in both *Tri10Δ* and *Tri6Δ* strain, and they include *Tri1* (*fg00071*), *Tri3* (*fg03534*), *Tri4* (*fg03535*), *Tri5* (*fg03537*), *Tri6* (*fg03536*), *Tri8* (*fg03532*), *Tri9* (*fg03539*), *Tri11* (*fg03540*), *Tri12* (*fg12013*), *Tri14* (*fg03543*) and *Tri101* (*fg07896*). It appears that *Tri3* was the most strongly down-regulated *Tri* gene in both mutant strains, with a 100-fold ($\log_2 = -6.65$) down-regulation in *Tri6Δ* and 247-fold ($\log_2 = -7.95$)

decrease in *Tri10Δ* compared to WT under DON-inducing conditions (Table 2). Although residing outside of the *Tri* gene cluster, *Tri1* was equally down-regulated as other *Tri* genes in both mutant strains, with a 51-fold ($\log_2$ = -5.68) decrease in the *Tri6Δ* mutant and a 82-fold ($\log_2$ = -6.36) decrease in the *Tri10Δ* mutant, compared to WT under DON-inducing conditions. The two *Tri* genes that are differentially regulated in either mutant strains are *Tri10* and *Tri15*. *Tri10* was up-regulated both in wild type by 24-fold ($\log_2$ = 4.59) and in *Tri6Δ* strain by 4.89-fold ($\log_2$ = 2.29). This suggested that activation of *Tri10* in DON-inducing conditions is not regulated by *Tri6*. *Tri15* is commonly down-regulated in wild type, *Tri10Δ* and *Tri6Δ* (Table 2). In wild type grown under DON-inducing conditions, *Tri15* was down-regulated by 2.5-fold ($\log_2$ = -1.33), however in *Tri10Δ* mutant it was significantly down-regulated by 111-fold ($\log_2$ = -6.80) and in *Tri6Δ*, by 126-fold ($\log_2$ = -6.98).

Table 2: Expression levels of *Tri* genes in WT, *Tri10Δ* and *Tri6Δ* under DON-inducing conditions

Fg Number	***Tri* gene**	**Annotated Function/Description**	***Tri6Δ* vs WT (DON-inducing conditions) ***	***Tri10Δ* vs WT (DON-inducing conditions) ***	**WT DON-inducing vs WT non DON inducing conditions ***
fg00071	*Tri1*	cytochrome P450 oxygenase	-5.68 +/- 1.64	-6.36 +/- 1.64	5.28 +/- 0.55
fg03534	*Tri3*	trichothecene 15-O-acetyltransferase	-6.65 +/- 0.53	-7.95 +/- 1.09	6.32 +/- 0.27
fg03535	*Tri4*	trichodiene oxygenase [cytochrome P450]	-2.02 +/- 0.21	-6.45 +/- 0.50	5.26 +/- 0.48
fg03537	*Tri5*	trichodiene synthase [sesquiterpene cyclase]	-5.91 +/- 0.61	-6.40 +/- 1.05	5.40 +/- 0.25
fg03536	*Tri6*	trichothecene biosynthesis positive transcription factor	-3.30 +/- 0.77	-3.95 +/- 0.55	3.64 +/- 0.04
fg03532	*Tri8*	trichothecene 3-O-esterase	-5.06 +/- 0.15	-6.34 +/- 0.52	2.44 +/- 0.14
fg03539	*Tri9*	hypothetical protein	-3.57 +/- 0.11	-5.50 +/- 0.13	2.67 +/- 0.16
fg03538	*Tri10*	regulatory protein	2.29 +/- 0.06	-4.69 +/- 0.50	4.59 +/- 0.05
fg03540	*Tri11*	isotrichodermin C-15 hydroxylase	-4.35 +/- 0.06	-7.10 +/- 0.59	4.86 +/- 0.95
fg12013	*Tri12*	trichothecene efflux pump	-4.26 +/- 0.11	-6.85 +/- 0.56	3.09 +/- 0.09
fg03543	*Tri14*	putative trichothecene biosynthesis gene	-5.63 +/- 0.56	-6.80 +/- 0.54	4.44 +/- 0.12
fg11025	*Tri15*	putative C2H2 zinc finger transcription factor	-6.98 +/- 1.26	-6.80 +/- 0.48	-1.33 +/- 0.14
fg07896	*Tri101*	trichothecene 3-O-acetyltransferase	-5.28 +/- 0.24	-5.14 +/- 0.22	0.05 +/- 0.01

* value represented as $\log_2$.

3.3.2.2. Fg Tri10 and Fg Tri6 regulate the butenolide pathway in vitro

The butenolide gene cluster spans 17 kb on chromosome 2 of *F. graminearum* and codes for eight genes required for the butenolide biosynthesis pathway (Harris *et al.*, 2007). These eight genes, *fg08077 - fg08084*, synthesize a secondary metabolite known as butenolide, which has been associated with the fescue foot syndrome in cattle. Affected cattle experience swelling and lesions in their hooves, weight loss and in severe cases, the loss of feet (Bacon, 1995). Previous secondary metabolite analysis using HPLC indicated the secretion of both 15-ADON and butenolide by *F. graminearum* into the medium under DON-inducing conditions (Harris *et al.*, 2007). Our microarray data suggests that along with the *Tri* gene cluster, *Tri10* and *Tri6* also regulate the butenolide gene cluster in wild type during DON-inducing conditions. According to Table 3, six genes from the butenolide gene cluster, *fg08077* and *fg08079 – fg08083*, were down-regulated in both *Tri10Δ* and *Tri6Δ* mutants. For example, *fg08079* codes for a benzoate 4-monooxygenase cytochrome P450 that was up-regulated in wild type under DON-inducing conditions by 7.31-fold ($\log_2 = 2.87$) and down-regulated in *Tri10Δ* and *Tri6Δ* by 48-fold ($\log_2 = -5.59$) and 16-fold ($\log_2 = -4.02$), respectively. *Fg08078* was not down-regulated in *Tri6Δ* by two-fold, and expression levels of *fg08084* were not available.

Table 3: Expression levels of genes involved in the butenolide biosynthetic pathway under DON-inducing conditions

Fg Number	**Annotated Function/Description**	***Tri6Δ* vs WT (DON-inducing conditions)***	***Tri10Δ* vs WT (DON-inducing conditions)***	**WT DON-inducing vs WT non DON inducing conditions ***
fg08077	related to flavin oxidoreductase	-1.59 +/- 0.15	-2.68 +/- 0.39	0.52 +/- 0.08
fg08078	related to general amidase	-0.90 +/- 0.04	-2.03 +/- 0.07	0.96 +/- 0.03
fg08079	probable benzoate 4-monooxygenase cytochrome P450	-4.02 +/- 0.54	-5.59 +/- 0.91	2.87+/- 0.58
fg08080	conserved hypothetical protein	-1.10 +/- 0.07	-3.54 +/- 0.26	1.93 +/- 0.00
fg08081	related to gibberellin 20-oxidase	-1.64 +/- 0.09	-5.00 +/- 0.83	3.63 +/- 0.56
fg08082	conserved hypothetical protein	-1.68 +/- 0.06	-5.04 +/- 0.17	3.23 +/- 0.15
fg08083	related to glutamic acid decarboxylase	-2.27 +/- 0.12	-4.80 +/- 1.18	2.53 +/- 0.82
fg08084	N/A			

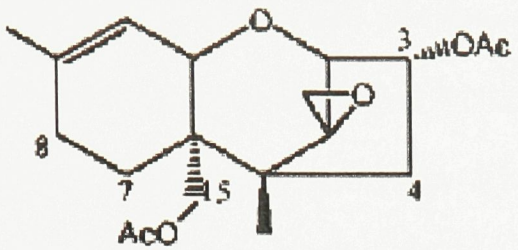
* value represented as $\log_2$.

3.3.3. Fg Tri10 and Fg Tri6 regulate cytochrome P450 oxygenases in vitro

Cytochrome P450 oxygenases are a superfamily of heme-containing enzymes that introduce oxygen into substrate molecules, thus generating a wide range of reactions. In the trichothecene biosynthesis pathway, these enzymes typically hydroxylate a particular carbon on the trichothecene backbone. Four *Tri* genes code for cytochrome P450 oxygenases: *Tri1* hydroxylates carbon 8 in type B trichothecenes to produce 8-hydroxycalonectrin (Figure 14), *Tri4* hydroxylates multiple carbons, while *Tri11* hydroxylates carbon 15, and finally *Tri13* hydroxylates carbon 4 of trichothecene only in the NIV-producing *Fg* strains.

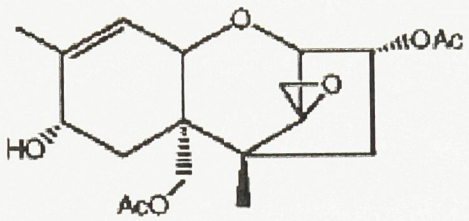
Figure 14: Potential action of a cytochrome P450 oxygenase in the trichothecene biosynthesis pathway. (Image taken from McCormick *et al.,* 2004).

In this selected reaction from the trichothecene biosynthesis pathway, *Tri1* gene product hydroxylates carbon 8 of calonectrin to produce 8-hydroxycalonectrin. However, the enzyme driving the subsequent hydroxylation reaction from 8-hydroxycalonectrin to 7,8-dihydroxylcalonectrin has not been identified.

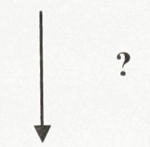


Calonectrin

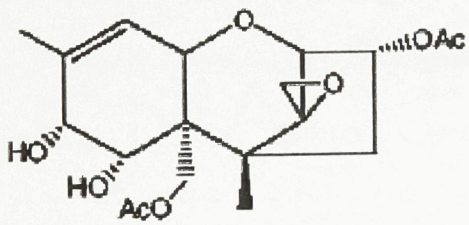
Tri1



8-hydroxycalonectrin



?



7, 8-dihydroxycalonectrin

In addition to regulating the three oxygenase-encoding *Tri* genes (*Tri1*, *Tri4* and *Tri11*) described above, *Tri10* and *Tri6* also regulate three additional cytochrome P450 oxygenases, namely, *fg00007*, *fg08023* and *fgd224-50*. Since not all the enzymes catalyzing the trichothecene biosynthesis pathway have been characterized, these oxygenases could be potential candidates in that pathway. Also, cytochrome P450s have been documented to detoxify phytoalexins to promote fitness of the organism, and it is possible these three cytochrome P450s function to promote fitness of the fungus by way of this.

Of the three oxygenases, the expression level of *fg00007* was the most reduced in *Tri10Δ* and *Tri6Δ*. This gene was down-regulated by 191-fold (log_2= -7.58) in *Tri10Δ* and by 108-fold (log_2= -6.76) in *Tri6Δ* mutant strains (Table 4). The second P450 gene, *fg08023*, was up-regulated by 2-fold (log_2 = 1.03) in WT, but down-regulated by 26.7-fold (log_2 = -4.74) in *Tri10Δ* and by 12.7-fold (log_2 = -3.67) in *Tri6Δ* strains. The third gene, *Fgd224-50*, increased in expression by 2-fold (log_2 = 1.03) in WT grown under DON-inducing conditions, but was decreased in expression by 2.9-fold (log_2 = -1.55) in *Tri10Δ* and by 2.4-fold (log_2 = -1.25) in *Tri6Δ* when compared to WT.

Table 4: Expression levels of genes encoding for cytochrome P450 oxygenases regulated by *Tri10* and *Tri6* under DON-inducing conditions

Fg Number	**Annotated Function/Description**	***Tri6Δ* vs WT (DON-inducing conditions)***	***Tri10Δ* vs WT (DON-inducing conditions)***	**WT DON-inducing vs WT non DON inducing conditions ***
fg00071 (*Tri1*)	cytochrome P450 oxygenase	-5.68 +/- 1.64	-6.36 +/- 1.64	5.28 +/- 0.55
fg03535 (*Tri4*)	trichodiene oxygenase [cytochrome P450]	-2.02 +/- 0.21	-6.45 +/- 0.50	5.26 +/- 0.48
fg03540 (*Tri11*)	isotrichodermin C-15 hydroxylase	-4.35 +/- 0.06	-7.10 +/- 0.59	4.86 +/- 0.95
fg00007	related to O-methylsterigmatocystin oxidoreductase	-6.76 +/- 0.16	-7.58 +/- 0.24	2.71 +/- 0.43
fg08023	related to isotrichodermin C-15 hydroxylase (cytochrome P-450 monooxygenase CYP65A1)	-3.67 +/- 0.14	-4.74 +/- 0.20	1.03 +/- 0.03
fgd224-50	related to benzoate 4-monooxygenase cytochrome P450	-1.25 +/- 0.05	-1.55 +/- 0.06	1.03 +/- 0.00

* value represented as $\log_2$.

3.3.4. Differential regulation of genes by Tri10 and Tri6

Our analysis thus far has focused on genes that are co-regulated by both *Tri10* and *Tri6*. In order to identify genes up-regulated in wild type during DON-inducing conditions that are regulated uniquely by either *Tri10* or *Tri6*, two comparisons were carried out. The first comparison was between the 472 genes uniquely up-regulated in wild type to the 484 down-regulated genes specific to *Tri10*Δ mutant, and the second comparison was between the same 472 up-regulated in wild type and the 164 down-regulated genes specific to the *Tri6*Δ mutant strain. Our analysis revealed that while there were 180 genes uniquely regulated by *Tri10* (Table A21) under DON-inducing conditions *in vitro*, only ten genes could be associated with *Tri6* regulation (Table A22). With the significant difference in the number of affected genes, we were interested to know the functional category of genes that are specifically influenced by *Tri10* and *Tri6*. As displayed in Table 5, among the annotated genes, the major category regulated by *Tri10* or *Tri6* belongs to the genes regulating cellular metabolism, specifically genes involved in carbon and nitrogen metabolism. However, the data also revealed that *Tri10* exerts its influence on genes involved in cellular transport, especially in ion transport, as well as genes involved in defense and virulence. This categorization supports the fact that *Tri10* and *Tri6* play differential roles outside of the trichothecene biosynthesis pathway.

Table 5: MIPS Functional category* of genes potentially activated by *Tri10* or *Tri6* under DON-inducing conditions

Functional Category	Genes potentially activated by *Tri10*	Genes potentially activated by *Tri6*
Metabolism	45	6
Energy	9	0
Cell cycle and DNA processing	3	1
Transcription	1	0
Protein fate (folding, modification, destination)	6	2
Protein with binding function or co-factor requirement	11	1
Cellular transport	18	1
Cell rescue, defense and virulence	17	2
Systemic interaction with environment	1	2
Cell fate and cell type differentiation	4	0
Biogenesis of cellular components	6	1
Regulation of metabolism and protein function	0	1
Cellular communication/ Signal transduction mechanism	0	2
Unclassified	112	4

* MIPS = Munich information center for protein sequences (http://mips.helmholtz-muenchen.de/genre/proj/fusarium)

3.4. Confirmation of Microarray Data by RNA Blot Analysis of Selected Genes

Northern analysis was performed on RNA samples extracted from WT, *Tri10* deleted mutant (*Tri10Δ)*, *Tri10* complemented (*Tri10Δ-AB), Tri6* deleted mutant (*Tri6Δ),* and *Tri6* complemented (*Tri6Δ-AB*) strains of *F. graminearum* harvested after 24 hours incubation in first stage non DON-inducing media and 12 hours in second stage DON-inducing media. Figure 15 shows gene expression of the four genes, *Tri1*, *Fg00007*, *Tri6*, and *Tri10*, grown in non DON-inducing conditions (panel A) and DON-inducing conditions (panel B). While *Tri1*, *Fg00007* and *Tri10* probes span the entire coding region of the genes, the *Tri6* probe only spans 562 bp of the *Tri6* coding region beginning at the 86th bp from the ATG start site.

As demonstrated in Figure 15, the two P450 genes, *Tri1* and *fg00007*, exhibited similar profiles. Both are induced under DON-inducing conditions in the wild type (compare panel A and B, *Tri1* and fg00007, Figure 15) and similarly both are down-regulated in the *Tri10* and *Tri6* mutant strains (lanes 2 and 4, *Tri1* and *fg00007*, panel B, Figure 15). Gene expression of *Tri1* and *fg00007* were recovered to wild type levels in both complemented strains, *Tri10Δ-AB* and *Tri6Δ-AB* (compare lanes 1, 3 and 5, *Tri1* and *fg00007*, panel B, Figure 15).

Northern analysis confirmed that *Tri10* expression was strongly induced under nutrient limiting conditions (lane 1, *Tri10*, panel B, Figure 15). *Tri10* expression was equally induced in the *Tri6Δ* mutant strain. This data, corroborated by our microarray analysis, strongly indicated that activation of *Tri10* was not dependent on the presence of

Tri6 gene. Our analysis of *Tri6* expression was contradictory to the data obtained from our microarray analysis. The microarray analysis (Table 2) suggested that *Tri6* expression was down regulated in the *Tri10Δ* mutant strain, however, Northern analysis indicated that expression of *Tri6* is unaffected by the absence of *Tri10* (lane 4, *Tri6*, panel B, Figure 15).

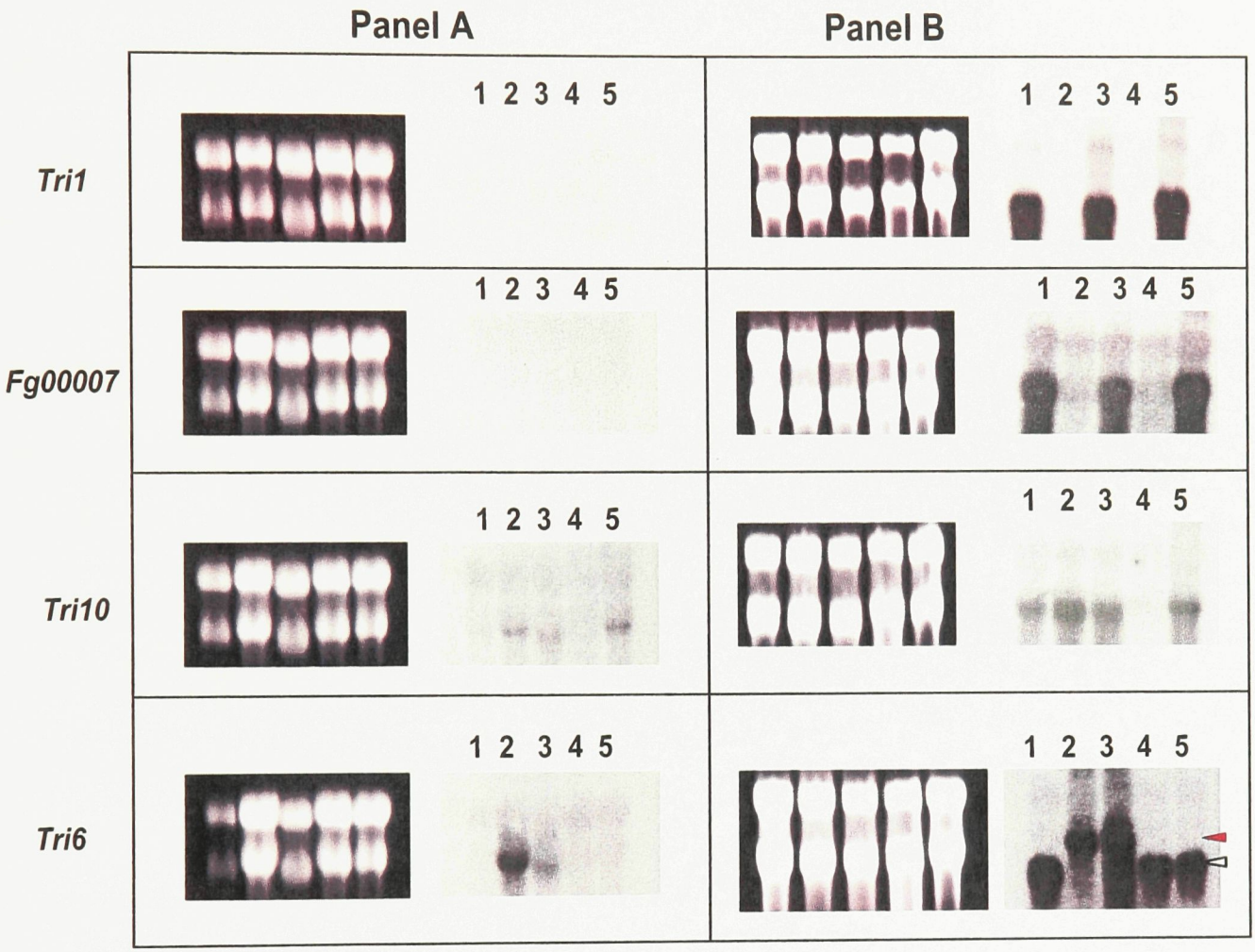
Although Northern analysis showed that *Tri6* expression was significantly up-regulated in nutrient-limiting conditions with RNA corresponding to the correct size (white arrow, lane 1, *Tri6*, panel B, Figure 15), we also observed that in the *Tri6Δ* mutant strain, a new RNA species with a high molecular weight strongly hybridized to the *Tri6* probe (red arrow, lane 2 and 3, *Tri6*, panel B, Figure 15). Since the *Tri6* probe overlaps with the portion of *Tri6* that was not replaced by *Hygromycin*, as indicated by the red line in Figure 10a, we speculate that this is a transcript read through into the *Hygromycin* gene. More significantly, the *Tri6* gene is apparently induced in the *Tri6Δ* mutant strain even in non DON-inducing media (lane 2, *Tri6*, panel A, Figure 15). Therefore, this suggests that the expression of *Tri6* is negatively regulated by its own product. Given the fact that there are two putative binding sites of *Tri6* in its own promoter, this is a likely scenario.

Figure 15: Gene expressions of *Tri1*, *Fg00007, Tri10* and *Tri6* under non DON-inducing conditions and DON-inducing conditions.

Lanes 1-5 represents 10 µg of total RNA extracted from WT, *Tri6*Δ, *Tri6*Δ-*AB*, *Tri10*Δ, *Tri10*Δ-*AB* strains of *F. graminearum*, respectively. Equal loading of RNA was monitored by ethidium bromide straining adjacent to the RNA blot.

Panel A represents expression profiles of *Tri1*, *Fg00007*, *Tri10* and *Tri6* under non DON-inducing conditions. Under these conditions, neither *Tri1* nor *Fg00007* were expressed. Low-level expression of *Tri10* was observed in *Tri6*Δ, *Tri6*Δ-*AB* and *Tri10*Δ-*AB* (lanes 2, 3 and 5). High-level expression of *Tri6* was observed in *Tri6*Δ and *Tri6*Δ-*AB* (lanes 2 and 3).

Panel B represents expression profiles of *Tri1*, *Fg00007*, *Tri10* and *Tri6* under DON-inducing conditions. Under these conditions, both *Tri1* and *Fg00007* were not expressed in *Tri6*Δ and *Tri10*Δ (lanes 2 and 4) but expressed to complementary levels as wild type in *Tri6*Δ-*AB* and *Tri10*Δ-*AB* strains (lanes 1, 3 and 5). Under DON-inducing conditions, *Tri10* was expressed to high levels in WT, *Tri6*Δ and both addback strains (lanes 1, 2, 3 and 5). However, its expression was absent in *Tri10*Δ. Under similar conditions, *Tri6* was highly expressed in WT, *Tri10*Δ and the two addback strains (white arrow, lanes 1, 3, 4 and 5). However, in *Tri6*Δ-*AB* strain, an additional species was observed (red arrow, lane 3). This higher molecular weight RNA species is also observed in *Tri6*Δ strain (lane 2). This likely represents the transcript read-through of the *hygromycin* gene initiating from the *Tri6* ATG start codon.


Panel A
Panel B
Tri1
Fg00007
Tri10
Tri6
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5

Chapter 4 – Discussion

Fusarium graminearum is a remarkably successful fungal pathogen. The pathogen colonizes a wide host range encompassing many habitats. One component of its success could be attributed to its ability to produce a wide range of secondary metabolites, many of whose functions are uncharacterized. One of the well characterized secondary metabolites are the trichothecenes. With inhibitory effects on protein synthesis, it compromises the host's defense system, thus providing the fungus with an advantage in the host –pathogen battle. Although trichothecenes play such an important role in the pathogenicity of *F. graminearum*, their activation and regulation is not fully understood. Many past studies have demonstrated that *Tri10* and *Tri6* act as positive regulators of the mycotoxin production pathway and up-regulate genes involved in the trichothecene pathway under nutrient-limiting conditions (Proctor *et al.*, 1995, Tag *et al.*, 2001, Peplow *et al.*, 2003, Seong *et. al.,* 2009). The existing model suggests that regulation of the trichothecene biosynthesis pathway occurs in a linear fashion, where *Tri10* regulates *Tri6*, which in turn activates other *Tri* genes (Tag *et al.*, 2001). In contrast, this study revealed that the regulation of *Tri10* and *Tri6* is independent of each other. This is also supported by gene expression profiling data performed on infected wheat heads by the same mutants (Seong *et al.*, 2009). Our microarray analysis has provided evidence that the regulatory roles of *Tri10* and *Tri6* in *F. graminearum* extend their control beyond the trichothecene biosynthetic pathway. The analysis showed that another secondary metabolic cluster involved in the production of butenolide is also co-regulated by *Tri10* and *Tri6*. Finally, coordinated expression of genes involved in primary metabolism by *Fg*

Tri10 and *Fg Tri6* identifies a regulatory network that links these primary and secondary metabolic pathways.

4.1. Coordinated and non-coordinated expression of Tri genes by Tri10 and Tri6

Our microarray data indicated that eleven out of the thirteen functional *Tri* genes (*Tri1, 3, 4, 5, 6, 8, 9, 11, 12, 14, 101*) are regulated by both *Tri10* and *Tri6* in DON-producing *F. graminearum*. The two *Tri* genes that are differentially regulated in *Tri10Δ* and *Tri6Δ* mutant strains are *Tri15* and *Tri10*. The expression of *Tri15* was decreased by 2.5-fold ($\log_2$ = -1.33) in wild type under DON-inducing conditions, indicating it might not be required for the production of trichothecenes. In the sister species *F. sporotrichioides*, *Tri15* codes for a Cys_2His_2 zinc finger protein and was described as a negative regulator of the pathway based on the fact that this gene was strongly expressed when the fungal culture was treated with the end product T2-toxin (Alexander *et al.*, 2004). However, the disruption of this gene did not affect trichothecene production (Alexander *et al.*, 2004). Similarly, disruption of this gene in *F. graminearum* did not affect the production of 15-ADON in culture (unpublished results). Our microarray analysis showed that *Fg Tri15* is positively regulated by both *Tri10* and *Tri6*, as its expression was down regulated by 111-fold ($\log_2$ = -6.80) in *Tri10Δ* strain and 125-fold ($\log_2$ = -6.98) in *Tri6Δ* strain. Currently, the role of *Tri15* in the trichothecene pathway is unclear. Since the expression pattern of the two secondary metabolic pathways (trichothecene and butenolide) are co-regulated by *Tri10* and *Tri6*, it would be worthwhile examining the expression of *Tri15* in a *F. graminearum* mutant strain that does not produce butenolide.

In contrast to the expression patterns of most *Tri* genes, *Tri10* expression is not typical. The expression of *Tri10* is induced in the *Tri6Δ* mutant by 4.89-fold ($\log_2$ = 2.29). This was confirmed by the RNA blot analysis, where we showed that *Tri10* was expressed in low amounts in *Tri6Δ*, *Tri6Δ-AB* and *Tri10Δ-AB* strains under non DON-inducing conditions (lanes 2 and 3, *Tri10*, panel A, Figure 15). Under DON-inducing conditions, *Tri10* transcripts accumulated to higher levels in the *Tri6Δ*, suggesting that *Tri6* may act as a negative regulator of *Tri10*. This idea is supported by the data obtained in *F. sporotrichioides*, where deletion of *Tri6* also resulted in the over-expression of *Tri10* (Tag *et al.*, 2001). Since the promoter of *Tri10* does not possess the *Tri6* binding site, the regulation of *Tri10* by *Tri6* is likely indirect.

Microarray analysis also indicated that the expression of *Tri6* was down regulated in the *Tri10*Δ mutant strain, thus implicating the requirement of *Tri10* for *Tri6* activation. This observation corresponds to the regulatory model of trichothecene biosynthesis pathway in *F. sporotrichioides* proposed by Tag *et al.* (2001), where *Tri10* acts upstream of *Tri6*. However, our RNA blot analysis does not agree with this linear regulation of *Tri6* by *Tri10*. *Tri6* transcripts accumulated to comparable amounts in both the wild type strain and *Tri10*Δ mutant strain (lanes 1 and 4, *Tri6*, panel B, Figure 15), implying that activation of *Tri6* expression does not require *Tri10*. The discrepancy between results generated from microarray and Northern analysis suggests a temporal regulation of this gene. The microarray was performed with RNA extracted after four hours under DON-inducing conditions, whereas the Northern analysis was performed with RNA extracted after twelve hours under the same conditions. Recent results from our laboratory

(Subramaniam, personal communications) indicate a complex auto-regulation of *Tri6* expression. This is borne out in the RNA blot analysis of *Tri6* in the non DON-inducing conditions, where expression of *Tri6* is elevated even in the *Tri6*Δ mutant strain (lane 2, *Tri6*, panel A, Figure 15). Therefore, differential expression of *Tri6* in the *Tri10*Δ mutant strain could be attributed to the auto-regulation of *Tri6* gene, rather than any dependence on *Tri10*. In a recent study by Seong *et al.*, who performed expression profile analysis in *Tri10*Δ and *Tri6*Δ mutant strains during wheat infection, concluded that *Tri10* did not regulate *Tri6* since *Tri6* expression was not reduced in the *Tri10*Δ strain during plant infection (Seong *et al.*, 2009). Together, this study and Seong *et al.* (2009) have provided evidence that *Tri10* and *Tri6* are regulated independently of each other *in vitro* and *in planta*.

4.2. Comparison of in vitro and in planta gene expression profiles

In the same study performed by Seong *et al.*, a total of 39 genes were down regulated in the *Tri10*Δ mutant strain whereas a total of 224 genes were down regulated in the *Tri6*Δ mutant strain. Among them, 27 genes were co-regulated by *Tri10* and *Tri6* including ten *Tri* genes, *Tri1*, *3*, *4*, *5*, *8*, *11*, *12*, *14*, *15*, *101* (Seong *et al.*, 2009). Similar to our observations, they also observed that neither *Tri10* nor *Tri6* are regulated by each other. Interestingly, while we observed *Tri9* was equally affected in both *Tri10*Δ and *Tri6*Δ mutants along with the other ten *Tri* genes listed in culture conditions, the expression of this gene was only affected in the *Tri6*Δ mutant strain during wheat infection only. Since Seong *et al.* (2009) did not provide the expression level of this gene,

it is difficult to conclude if the exclusion of *Tri9* in their gene expression profile did not meet the two-fold cut-off criteria or that it is indeed not regulated by *Tri10*.

The genes of the butenolide cluster have been documented to be closely coordinated with the expression of the trichothecene genes (Harris *et al.*, 2007). Our microarray analysis corroborated this observation and indicated that both gene clusters are co-regulated by *Tri10* and *Tri6*. Surprisingly, none of the eight genes in the butenolide cluster was down regulated in *Tri10*Δ and *Tri6*Δ mutant strains during wheat infection. This *in planta* result suggests that the expression of this cluster may be related to the type of host that *F. graminearum* infects. Toxic effects of trichothecene have been documented to be host-specific, for instance, non DON-inducing *F. graminearum Tri5*Δ mutant strain cannot cause disease in wheat but remains virulent in barley, indicating that while DON acts as a virulence factor on wheat, it is not on barley (Maier *et al.*, 2006).

Among the 97 cytochrome P450s expressed in *F. graminearum*, six are regulated by both *Tri10* and *Tri6* in our study, including the three genes (*Tri1*, *4*, *11*) involved in the trichothecene biosynthesis pathway. All three trichothecene-associated P450 genes are also regulated by *Tri10* and *Tri6 in planta*. Of the three other genes coding for P450 monooxygenase regulated in culture, only fg00007 was also down regulated in planta (Seong *et al.*, 2009). As alluded to earlier, host specificity may play a role in the expression of *Fusarium* genes. This is clearly evident when we compare the number of genes that are affected by these mutant strains. Comparison of *in vitro* and *in planta* expression profiles indicated a large discrepancy between the number of genes that are

differentially regulated in culture by the two mutant strains compared to the genes in the infected wheat heads. There are 960 genes that are down regulated in the *Tri10*Δ mutant *in vitro*, compared to only 39 genes that are down regulated *in planta* upon infection with this mutant (Seong *et al.*, 2009). Likewise, the number of down regulated genes in the *Tri6*Δ mutant *in vitro* is 605, compared to the 224 down regulated genes *in planta* (Seong *et al.*, 2009).

4.3. Roles of Tri10 and Tri6 in secondary metabolism

One of the major findings of this research is that the regulatory effects imposed by *Tri10* and *Tri6* extend beyond the control of a single secondary metabolite cluster. In addition to the trichothecene biosynthesis pathway, *Tri10* and *Tri6* also regulate six of the eight genes residing in the butenolide gene cluster on chromosome 2. The seventh gene, *fg08078*, was down regulated by four-fold in *Tri10Δ*, but only by 1.8-fold in *Tri6Δ*, suggesting that its expression is differentially regulated by *Tri10* with respect to our two-fold cut-off criteria. Like trichothecene biosynthesis pathway, butenolide production is activated under nutrient limiting conditions, but at an earlier time point (Harris *et al.*, 2007). Although the expression data suggests a direct relationship between these two pathways, the product of each pathway shows an opposite relationship (Harris *et al.*, 2007). In a *F. graminearum* strain that accumulates 15-ADON to high levels, there is a corresponding decrease in butenolide production (Harris *et al.*, 2007). Deletion analysis of *fg08079*, which codes for a cytochrome P450, resulted in the loss of butenolide production but not trichothecene synthesis (Harris *et al.*, 2007). Wheat infection assays of *fg08079Δ* mutant strain indicated that butenolide production is not required for the infection and spreading of the fungal pathogen in wheat, as the mutant was as virulent as

wild type (Harris *et al.*, 2007). As mentioned, expression of *Tri15* was negatively affected by exogenous addition of T2-toxin in *F. sporotrichioides* (Alexander *et al.*, 2004), however, its role in the trichothecene biosynthesis pathway was not conclusive. Given the inverse relationship between the accumulation of 15-ADON and butenolide, it would be interesting to ascertain a role for *Tri15* in the context of butenolide production.

Antibiotic assays of the two mycotoxins butenolide and T2-toxin showed that butenolide at 200 μg could inhibit the growth of nine bacteria but had no effect on fungi, whereas T2-toxin at 50 μg was toxic to six different fungi but exerted no deleterious effects on bacteria (Burmeister and Hesseltine, 1970). This suggested that *Fusarium spp.* produces different mycotoxins to eliminate different groups of potential competitors in nature, ultimately promoting its own fitness and survival. In addition to eliminating competitors, butenolide was also demonstrated to act synergistically with another mycotoxin, enniatin, to cause lesions on the leaves of a flowering plant 'spotted knapweed' (*Centura maculosa*) (Hershenhorn *et al.*, 1992). *Fusarium avenaceum* produces both butenolide and enniatin to cause necrotic lesions on spotted knapweed, thus promoting its virulence (Hershenhorn *et al.*, 1992). When 5 μg of butenolide and 22 μg of enniatin were applied onto detached leaves of spotted knapweed, necrotic lesions similar to those caused by *F. avenaceum* were observed, however, when individually applied onto the leaves, necrotic symptoms were not observed, suggesting that both mycotoxins act synergistically and promote pathogenicity (Hershenhorn *et al.*, 1992). Although a direct role of butenolide in *F. graminearum* has not been ascertained, it is

possible that it acts synergistically with other toxins, including trichothecenes to increase fitness through the elimination of competitors.

4.4. Other candidates of the trichothecene biosynthesis pathway

As described in Figure 3, a number of genes involved in the biosynthesis of DON have yet to be identified. Our microarray analysis revealed that in addition to regulating the three cytochrome P450s that participate in the trichothecene biosynthesis pathway, *Tri10* and *Tri6* also regulate another three cytochrome P450s, namely, *fg00007*, *fg08023* and *fgd224-50*. In the trichothecene biosynthesis pathway, P450 monooxygenases are involved in various hydroxylation steps. As indicated in Figure 14, the synthesis of 7, 8-dihydroxycalonectrin is speculated to be catalyzed by two P450s. However, only one, namely *Tri1*, has been identified. It is conceivable that any of the remaining P450s identified in our microarray data could participate in this catalyzation step.

In addition to playing a role in the DON biosynthesis pathway, cytochrome P450s also participate in the detoxification of phytoalexins produced by plants during infection. For example, the fungal pathogen *Nectria haematococca* (anamorphic state *Fusarium solani*) produces the enzyme pisatin demethylase, a cytochrome P450 that allows the fungus to demethylate pisatin, a phytoalexin produced by pea plants (Desjardins *et al.*, 1984). The demehtylated pisatin is reduced in toxicity compared to pisatin, therefore allowing the fungus to tolerate and survive through the infection process (Desjardins *et al.*, 1984). Hence, it is worthwhile to investigate the function of these three cytochrome P450s, even if they do not participate in the trichothecene biosynthesis pathway.

4.5. Differential regulation of genes by Tri10 and Tri6

Our microarray analysis showed that the total number of genes differentially regulated by *Tri10* is 1650 compared to 1089 by *Tri6*. We observed that 62% (677) of the genes regulated by *Tri6* are also regulated by *Tri10*. However, a substantial number of genes (60% or 979 genes) are uniquely affected by *Tri10* alone and similarly 40% of the genes are uniquely regulated by *Tri6*. MIPS functional category suggested that while *Tri10* and *Tri6* seem to commonly regulate genes involved in primary metabolism, *Tri10* also appear to exert its influence on genes involved in cellular transport and in defense and virulence. Functional categories of genes assigned by MIPS have overlapping functions and therefore, we cannot conclude that genes regulated by *Tri10* or *Tri6* are limited to distinct category. Experiments with over-expression of *Tri10* in a *Tri6Δ* mutant strain and vice versa might tease out genes that are differentially regulated by *Tri10* and *Tri6*.

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Appendix

Table A1: Primer Sequences used for Creating *Tri10* Disruption Construct

Primer	Sequence 5' → 3'
P1	GACGCTTGCATATTCAGGC
P2	GATGTCAGGCCATTTTCACCGATACCCGAAGTCTTTC
P3	GAAAGACTTCGGGTATCGGTGAAAATGGCCTGACATC
P4	CACTCGTCTAAGCAGATGG
P5	TGAAAATGGCCTGACATCAT
P6	AGCTCTTGTTCGGTCGGCATCTAC

Table A2: Primer Sequences used for Creating *Tri6* Disruption Construct

Primer	Sequence 5' → 3'
P1	GTCCTCAGTATCGCCGTGTCAG
P2	GATGTCAGGCCATTTTCAGAGAGTATGTGGGGAAGTTGTCAAAG
P3	TGCCGACCGAACAAGAGCTGACAGTGCGGTGCCCTTGG
P4	GCCAATGCCAGCAAAAAGTATC
P5	CTTTGACAACTTCCCCACATACTCTCTGAAAATGGCCTGACATC
P6	CCAAGGGCACCGCACTGTCAGCTCTTGTTCGGTCGGCA

Table A3: Primer Sequences used for Creating *Tri6 and Tri10* Addback Constructs

Primer	Sequence 5' → 3'
Tri10 AB F	GACGCTTGCATATTCAGGC
Tri10AB R	CACTCGTCTAAGCAGATGG
Tri6 AB F	GTCCTCAGTATCGCCGTGTCAG
Tri6 AB R	GCCAATGCCAGCAAAAAGTATC

Table A4: Primers used for confirmation *Tri10Δ* and *Tri6Δ* Deleted Mutant Strains

Primer	Sequence 5' → 3'	Gene
Tri10 orf F	GAATTCTAGTCATCATGGATTTCC	*Tri10*
Tri10 orf R	GAATTCATCCCTACCAAAGTAGCACT	*Tri10*
Hyg P5	TGAAAATGGCCTGACATCAT	*Hygromycin*
Hyg P6	AGCTCTTGTTCGGTCGGCATCTAC	*Hygromycin*
Geneticin ENTR	CACCATGCCAGTTGTTCCCAG	*Geneticin*
Geneticin Down	CATATCGATCTTGGGTAGAAT	*Geneticin*
T6 Fsp	CATGCCAAGGACTTTGTCCC	*Tri6*
T6 End R	GTGTATCCGCCTATAGTGAT	*Tri6*

Table A5: Primers used to Amplify Non-Radiolabeled Probe Templates

Primer	Sequence 5' → 3'	Gene
Tri1 RT F	GTATCTGTCCACACCACCACC	*Tri1*
Tri1 RT R	GTCATCCTGTACCAATTCC	*Tri1*
Fg07 RT F	CCCAGAGGTCCTCAAGAAAGCCC	*Fg00007*
Fg07 RT R	CAGTACGACCATATCCTGCTGG	*Fg00007*
T6 Fsp	CATGCCAAGGACTTTGTCCC	*Tri6*
T6 End R	GTGTATCCGCCTATAGTGAT	*Tri6*
Tri10 orf F	GAATTCTAGTCATCATGGATTTCC	*Tri10*
Tri10 orf R	GAATTCATCCCTACCAAAGTAGCACT	*Tri10*
Hyg P5	TGAAAATGGCCTGACATCAT	*Hygromycin*
Hyg P6	AGCTCTTGTTCGGTCGGCATCTAC	*Hygromycin*

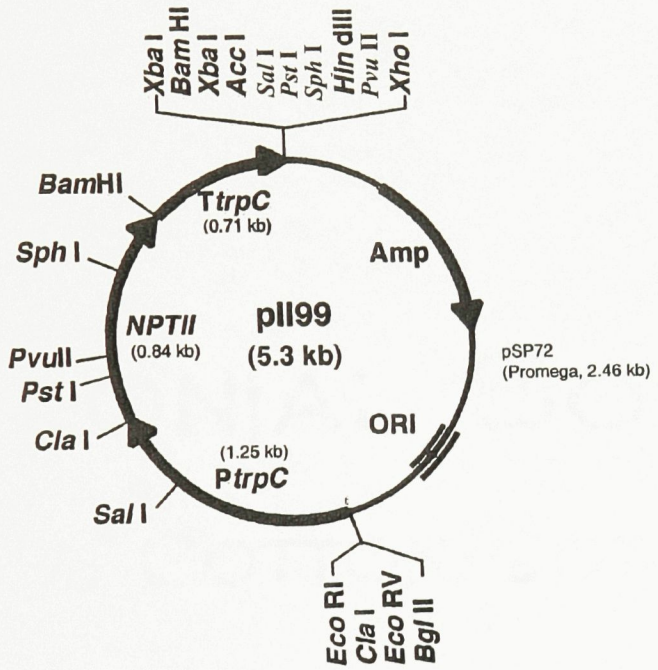

Figure A1: pII99 Vector Map (Namiki *et al.*, 2001)

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Differential roles of TRI10 and TRI6 in Fusarium graminearum

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