Characterization of a virulence gene responsive to nitrogen stress in *Fusarium graminearum*

by

Sean Walkowiak

B.Sc. (Carleton University, Ottawa, Canada)

A thesis submitted to the Faculty of Graduate and Postdoctoral Affairs in partial fulfillment of the requirements for the degree of

Master of Science

in

Biology

Carleton University
Ottawa, Ontario

Your file Votre référence
ISBN: 978-0-494-83205-9
Our file Notre référence
ISBN: 978-0-494-83205-9

Abstract

Emerging models indicate that nitrogen availability is an important environmental cue for the induction of virulence in the phytopathogen *Fusarium graminearum*. This was further validated in this study which characterized a putative regulatory gene with three proposed Cys_2His_2 zinc fingers, Accession *FGSG_03881.* High-throughput analyses using promoter::GFP constructs in *F. graminearum* in a 96 well system determined that the promoter activity of *FGSG_03881* increased during nitrogen stress, which was supported by RT-qPCR analyses. In addition, disruption of *FGSG_03881* caused increased virulence in a susceptible variety of wheat in comparison to the wild-type fungal strain, which indicated that *FGSG_03881* is acting as a negative regulator of virulence. Analysis of global gene expression patterns and secreted proteins sheds light on potential targets of *FGSG_03881* involved in virulence.

Acknowledgements

None of this would have been possible without the mentorship of Rajagopal Subramaniam, Owen Rowland, and Winnie Leung. Their continued guidance and friendship have been an asset to both myself personally and the project. I would also like to thank my colleagues, friends, and family who have encouraged my interest in science and research.

I would like to acknowledge the following people, who have also helped guide me, provided me with materials, performed technical tasks, or assisted in data analyses (appearing alphabetically): Sally Buffam, Jacqueline Ching, Anne Johnston, Andre Kalikilio, Cristopher Mogg, Charles Nasmith, Swara Naryanan, Christoph Rampitsch, and Li Wang.

Table of Contents

Abstract..... ii

Acknowledgments..... iii

Table of Contents..... iv

List of Figures..... ix

List of Tables..... xii

List of Abbreviations..... xiv

Chapter 1 - Introduction 1

1.1. *Fusarium graminearum*: A Brief Overview..... 1

1.2. The Economic and Health Implications of *F. graminearum* 2

1.3. *F. graminearum* Pathogenesis..... 4

1.4. Plant Defence and Susceptibility..... 7

1.5. Regulation of Virulence in *F. graminearum* by Environmental Factors 8

1.5.1. Virulence and Nitrogen Stress 9

1.5.2. Virulence and Carbon, pH, Light, and Other Environmental Cues..... 11

1.5.3. Signal Transduction by Environmental Factors..... 13

1.6. The Role of Secreted Proteins in Virulence..... 15

1.7. Mycotoxin Production in *F. graminearum*..... 17

1.7.1. Production and Implications of Fusarin C 19

1.7.2. Production and Implications of Zearelanone (ZON) 19

1.7.3. Production and Implications of Deoxynivalenol 20

1.8. Project Outline..... 22

Chapter 2 - Materials and Methods 23

2.1. Fusarium Strains, Propagation and Storage.....23

2.2. Bioinformatic Analyses of *FGSG_03881*.....23

2.3. Generation of *F. graminearum* Transgenic Strains.....24

2.3.1. Generating the *FGSG_03881* Disruption Strain.....24

2.3.2. Generation of the *FGSG_03881* Complementation,

FGSG_03881 Constitutive Expression, and

pFGSG_03881::*GFP Fusarium* Transgenic Strains.....26

2.4. Pathology Tests and Analyses in *Triticum aestivum* (wheat) 29

2.5. Gene Expression Analyses.....29

2.5.1. Culture Conditions, RNA Isolation, and Quantitative

Real-Time PCR.....29

2.5.2. Global Gene Expression Analyses by Microarrays..... 31

2.5.3. High-Throughput Analyses of *FGSG_03881* Promoter

Activity..... 32

2.6. Analysis of Secreted Proteins..... 34

2.6.1. Isolation of Secreted Proteins Under 15-acetyl-DON Inducing

Conditions.....34

2.6.2. Two-Dimensional Gel Electrophoresis of Secreted Proteins...34

2.6.3. Identification of Secreted Proteins by Mass Spectrometry.....35

2.6.4. Callose Staining of Wheat Leaves Infiltrated with Protein.... 36

2.7. Physiological and Growth Analyses of the Wild-type and Transgenic Strains..... 36

2.7.1. Assessing Growth on Solid Media.....36

2.7.2. Assessing Growth in Liquid Media..... 37

2.8. Mycotoxin Analyses..... 37

2.8.1. Analyses of 15-acetyl-DON Production *in vitro*..... 37

2.8.2. Analyses of DON Production *in vivo* 38

2.9. Diaminobenzidine and Nitroblue Tetrazolium Staining.....38

2.10. Yeast Two-Hybrid Analyses of FGSG_03881 against Trichothecene Regulators..... 39

Chapter 3 - Results.....41

3.1. FGSG_03881 is a Zinc Finger Protein Similar to *F. verticillioides* FVEG_13013.....41

3.2. *Fusarium* Transformations.....44

3.3. The *fgsg_03881Δ* Strain is More Pathogenic than Wild-type at Early Infection Stages in a Susceptible Host.....48

3.4. Expression Analyses of *FGSG_03881*.....50

3.4.1. *FGSG_03881* is Induced in Nitrogen Stress Conditions..... 50

3.4.2. *FGSG_03881* is Co-Regulated with *Tri6* and *Tri10*, but Expression is Independent of DON Production.....56

3.5. Global Gene Expression Patterns of *fgsg_03881Δ* and Wild-type Under Nitrogen-Rich and 15-acetyl-DON Inducing Conditions..... 60

3.6. Secreted Proteins of *F. graminearum*..... 65

3.6.1. Identification and Characterization of the Secreted Proteins.. 65

3.6.2. Callose Deposition was Observed in the Wheat Leaves Infiltrated with Proteins..... 70

3.7. The *fgsg_03881Δ* Strain has Similar Growth, Development, and 15-acetyl-DON Production as Wild-type..... 72

3.8. FGSG_03881 does not Interact with Trichothecene Regulators..... 76

Chapter 4 - Discussion..... 78

4.1. *FGSG_03881* is Associated with Virulence in *F. graminearum*..... 78

4.1.1. Pathological Evidence Links *FGSG_03881* to Virulence..... 78

4.1.2. Genetic Evidence Links *FGSG_03881* to Nutrient Stress and Virulence..... 80

4.2. Identification of Potential Targets of *FGSG_03881* Relating to Virulence..... 82

4.2.1. Potential Virulence Factors Modulated by *FGSG_03881*..... 82

4.2.2. *FGSG_03881* Modulates Genes Involved in Mycotoxin Production..... 85

4.2.3. Disruption of *FGSG_03881* Affects Primary Metabolism..... 87

4.3. An Integrated Model Outlining the Role of *FGSG_03881*..... 91

4.4. Perspectives and Future Studies..... 95

4.4.1. The Possible Identification of a New Class of Regulatory Genes.....95

4.4.2. Additional Insights into the Secretome of *F. graminearum*... 95

References.....97

Appendix.....105

List of Figures

Figure 1: A wheat head infected with 1, 000 *F. graminearum* conidia between the palea and the lemma by point inoculation. 6

Figure 2: The chemical structures of common *Fusarium* mycotoxins..... 18

Figure 3: Generation of the fusion PCR construct used to disrupt *FGSG_03881*..... 25

Figure 4: Cladogram generated by ClustalW2 multiple sequence alignment using a neighbour-joining algorithm..... 42

Figure 5: Multiple sequence alignment by ClustalW2 of the protein sequences for *FGSG_03881*, *FGSG_11025*, *FVEG_13013*, and *FS_Tri15*..... 43

Figure 6: Verification of transformation and integration of the *FGSG_03881* disruption construct into wild-type protoplasts by PCR amplification..... 45

Figure 7: Verification of transformation and integration of the *FGSG_03881* complementation, constitutive expression, and promoter::GFP constructs..... 47

Figure 8: Promoter activity for *FGSG_03881* in pFGSG_03881::*GFP*/wt and pFGSG_03881::*GFP*/*fgsg_03881Δ* strains grown in minimal media supplemented with different compounds as sole carbon and nitrogen sources..... 51

Figure 9: RT-qPCR analyses of the gene expression of *FGSG_03881* in wild-type grown in various nitrogen rich and nitrogen poor conditions.....53

Figure 10: Promoter activity of *FGSG_03881* in pFGSG_03881::*GFP*/wt after growth in minimal media supplemented with different compounds as sole nitrogen sources.....55

Figure 11: RT-qPCR results describing the expression levels of *FGSG_03881*, *Tri6*, and *Tri10* under different nutrient conditions..... 57

Figure 12: RT-qPCR results describing the expression levels of *FGSG_03881* in *tri6*Δ. 58

Figure 13: RT-qPCR results describing the expression levels of *FGSG_03881* in *FGSG_03881/fgsg_03881Δ*..... 59

Figure 14: Pie charts illustrating functional categories of genes differentially expressed in *fgsg_03881Δ*.....64

Figure 15: Two-dimensional separation of *F. graminearum* secreted proteins isolated from 15-acetyl-DON inducing medium.....66

Figure 16: Callose staining of 'Roblin' leaves infiltrated with the Flg22 and proteins secreted from *F. graminearum* grown in 15-acetyl-DON inducing medium.....71

Figure 17: Radial growth measurements of wild-type and *fgsg_03881Δ* grown on solid media..... 73

Figure 18: Growth of wild-type and *fgsg_03881Δ* in liquid media..... 74

Figure 19: Nitroblue tetrazolium and diaminobenzidine staining of wild-type and *fgsg_03881Δ*..... 75

Figure 20: Flow chart illustrating genes involved in purine metabolism..... 89

Figure 21: An integrated model illustrating the role of *FGSG_03881*..... 94

List of Tables

Table 1: Pathology tests of *F. graminearum* in the susceptible variety of wheat 'Roblin'.....49

Table 2: Pathology tests of *F. graminearum* in the resistant variety of wheat 'Sumai 3'..49

Table 3: The top ten genes up-regulated in *fgsg_03881Δ* relative to wild-type grown in GYEP medium.....61

Table 4: The top ten genes down-regulated *fgsg_03881Δ* relative to wild-type grown in GYEP medium.....61

Table 5: The top ten genes up-regulated in *fgsg_03881Δ* relative to wild-type grown in 15-acetyl-DON inducing medium.....62

Table 6: The top ten genes down-regulated in *fgsg_03881Δ* relative to wild-type grown in 15-acetyl-DON inducing medium.....62

Table 7: Proteins secreted by *fgsg_03881Δ* but not by wild-type after 24h of growth in 100mL of 15-acetyl-DON inducing medium. 68

Table 8: Proteins secreted by wild-type but not by *fgsg_03881Δ* after 24h of growth in 100mL of 15-acetyl-DON inducing medium.....69

Table 9: Yeast two-hybrid analyses of FGSG_03881 and trichothecene regulators..... 77

List of Abbreviations

ACN - acetonitrile

BLAST - basic local alignment search tool

CCR - carbon catabolite repression

CWDE - cell wall degrading enzymes

DAB - diaminobenzidine

DON - deoxynivalenol

dpi - days post inoculation

DPI - diphenyliodonium

EST - expressed sequence tag

FA - formic acid

GABA - γ-aminobutyric acid

GFP - green fluorescent protein

MAPK - mitogen activated protein kinase

NBT - nitroblue tetrazolium

NCR - nitrogen catabolite repression

NIV - nivalenol

PAMP - pathogen associated molecular pattern

QTL – quantitative trait loci

ROS - reactive oxygen species

TCA - trichloroacetic acid

ZON - zearalenone

Chapter 1 - Introduction

1.1. Fusarium graminearum: A Brief Overview

Fusarium graminearum, anamorph *Gibberella zeae*, is arguably one of the most intensely studied fungal phytopathogens (Desjardins 2006; Trail 2009). It is in the subphylum Ascomycota and the order Hypocreales (Kendrick 2000). The fungus produces sexual structures called perithecia that contain ascospores located in elongated sacs called asci (Goswami and Kistler 2004). It is homothallic, able to reproduce sexually by itself, so outcrossing is not required for generating ascospores (Trail 2009). In addition to producing sexual spores, it also produces asexual spores called conidia (Trail 2009). *Fusarium* spores infect a number of different cereal crops and are a causal agent of seedling blight, brown foot rot, and head blight; crops of particular economic concern are wheat, barley, rye and corn (Goswami and Kistler 2004; Trail 2009). Fusarium head blight causes substantial agricultural losses in Canada, the United States, Europe, Australia, Asia, and South America (Desjardins 2006). *F. graminearum* is subdivided into subspecies that are often correlated with geographical distribution, as well as mycotoxin and pathological differences (Starkey et al. 2007).

The mycotoxic effects of *F. graminearum* infected crops, first recorded in the 1800's, are attributed to many disease outbreaks and continue to be a serious health risk today (Desjardins 2006; Yazar and Omurtag 2008). *F. graminearum* produces two mycotoxins of major concern, deoxynivalenol (DON) and zearalenone (ZON). DON is a protein synthesis inhibitor that has serious health implications when ingested by either humans or livestock, while ZON is an estrogenic mimicking compound that is of

particular concern with swine (Abramson et al. 1997; Placinta et al. 1998; Desjardins and Proctor 2007). Thermostability of these compounds makes it difficult to decontaminate infected samples. Screening for DON in Canadian grain has become standard practice and the maximal allowance of the toxin is tightly regulated (Egmond and Jonker 2003; Döll and Dänicke 2011). The impact of *F. graminearum*, both economically and biologically, has encouraged governing bodies to concentrate on reducing the effects of this pathogen.

In May 2003, the genome of *F. graminearum* became publicly available, making it easier to study the pathogen and factors that influence its virulence (Goswami and Kistler 2004; Cuomo et al. 2007). The genome has very few repetitive genetic elements and the recent establishment of *Agrobacterium*-mediated transformation of *F. graminearum* makes it an ideal model organism to study fungal pathogenesis (Kimura et al. 2007; Frandsen et al. 2008).

1.2. The Economic and Health Implications of F. graminearum

The mycotoxic effects of *F. graminearum* have been linked to the disease outbreak akakabi-byo, or red mold disease, caused by infected wheat and barley in Asia from the 1930's to 1970's, as well as swine feed refusal and estrogenic syndrome from contaminated corn in the United States from the 1920's to 1980's (Desjardins 2006; Yazar and Omurtag 2008). Both akakabi-byo and swine feed refusal were common reoccurring problems that resulted from the consumption of contaminated grains. High incidence of swine feed refusal in the United States led to the discovery of the mycotoxin

deoxynivalenol or DON in the 1970's (Desjardins 2006). Screening for DON did not start in Canada until the 1980's; the initial survey done on Ontario wheat grain determined that half of the samples studied contained between 0.06μg/g and 8.5μg/g of deoxynivalenol, levels well above today's acceptable limits (Desjardins 2006). Since then, allowances for DON have been set, which vary depending on grain usage, and there have been regular tests for the toxin in Canadian grains (Egmond and Jonker 2003; Döll and Dänicke 2011).

Wheat losses from *F. graminearum* infection in the United States in the early 1900's were recorded to be tens of millions of bushels annually, and increased to over 100 million bushels in 1982. This trend continued through the 1990's when some states lost half of their wheat crop to infection, which resulted in more than $3 billion in primary and secondary losses (McMullen et al. 1997; Windels 2000). Between 1993 and 1998, the losses in Manitoba were estimated to be approximately $300 million (Windels 2000).

Recently, computer models have been employed to predict the amount of *Fusarium* mycotoxins expected in Ontario grain given environmental parameters such as rain (Schaafsma and Hooker 2007). Other models exist for predicting both incidence of head blight disease and mycotoxin accumulation for areas of Europe and North America (Prandini et al. 2009). Because of *F. graminearum's* ubiquity and the challenges associated with controlling the fungus through agronomic practices, there have been increased pressures to develop decontamination techniques. However, these have proven

to be challenging due to the thermostability of both DON and ZON (Trail 2009; Döll and Dänicke 2011).

1.3. F. graminearum Pathogenesis

F. graminearum overwinters in post harvest plant debris as a dikaryon, a state where cells contain two separate nuclei. This is also when the fungus is in its sexual state and produces sexual structures called perithecia (Trail 2009). The ascospores produced in the perithecia are the initial source of inoculum on plants and their release usually coincides with anthesis of host plants in the spring and early summer. The release of the spores is dependent on light and moisture conditions (Trail et al. 2002). There is a strong correlation between years of large epidemics and the timing of ascospore release (Trail et al. 2002; Brown et al. 2010). The ascospores are ejected into the environment by the build-up of turgor pressure in the asci (Trail et al. 2002). Once released, the spores are dispersed by wind, rain or animals, and upon landing on an appropriate host, the spores germinate and infection ensues (Trail 2009).

Unlike most phytopathogenic ascomycete fungi that produce an appressorium, a structure used to penetrate the exterior of plants, *F. graminearum* gains entry to the interior of the plant through stomatal openings and susceptible areas of the inflorescence tissues such as the cracks of anthers (Kikot et al. 2009). The fungus also produces a wide array of enzymes collectively termed extra-cellular cell wall degrading enzymes or CWDE which degrade the protective material on the exterior of the host plant, thereby allowing the fungus to penetrate more easily (Wanjiru et al. 2002; Kikot et al. 2009). In

wheat, the fungus has been observed to enter through the adaxial surface of the glume, lemma and palea of the spikelet (Figure 1). This is facilitated by CWDE that metabolise plant cellulose, pectin and xylan (Pritsch et al. 2000; Wanjiru et al. 2002).

Once inside the host, the developing hyphae gain access to apoplast and cortex of the plant and migrate away from the infected spikelet and towards the rachis (Brown et al. 2010). As the mycelia migrate towards the rachis, the fungus obtains nutrients from the extracellular plant tissues digested by the CWDE and from the dead or dying cells (Wanjiru et al. 2002; Brown et al. 2010). Once the fungus enters the rachis it utilizes the vasculature of the plant to spread and complete its lifecycle (Jansen et al. 2005; Brown et al. 2010). The completed lifecycle often entails the production of conidia (Trail 2009). These spores then colonize nearby plants and plant debris and the cycle is perpetuated (Goswami and Kistler 2004; Trail 2009).

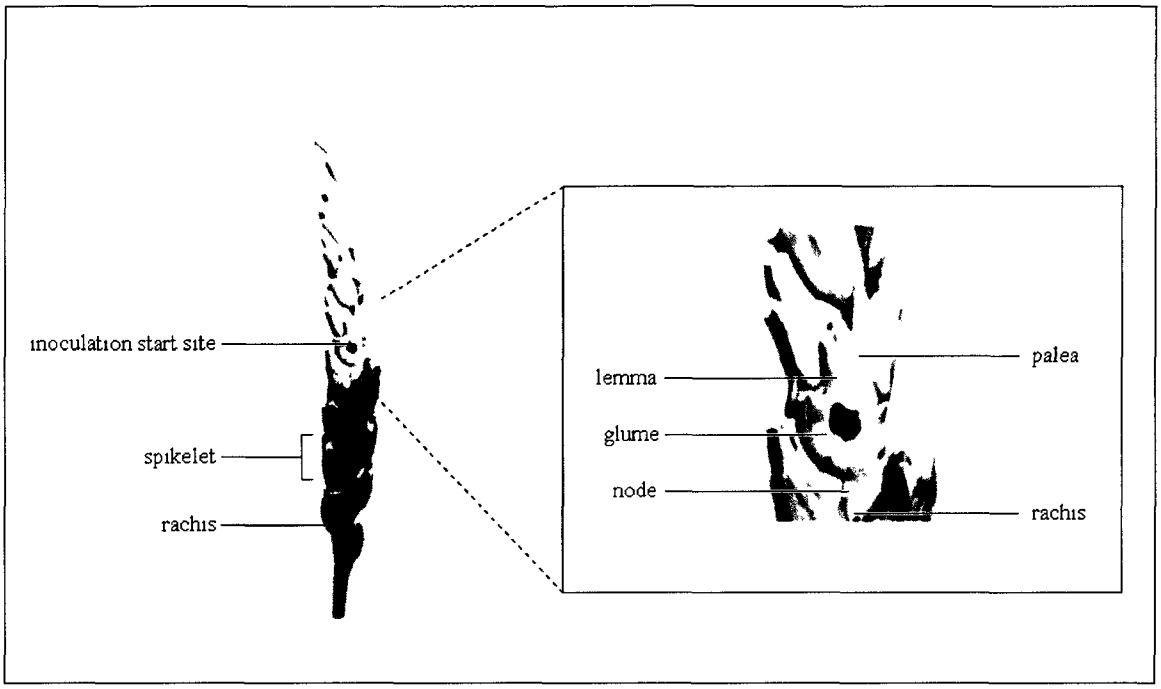


Figure 1: A wheat head infected with 1,000 *F. graminearum* conidia between the palea and the lemma by point inoculation. After 10 days of infection the fungus is able to spread throughout the wheat head and cause an accumulation of mycotoxins in the plant.

1.4. Plant Defence and Susceptibility

Plants depend on their innate immunity to defend themselves against pathogen attacks. The recognition of pathogen associated molecular patterns (PAMPs), such as flagellin in the case of bacterial pathogens, by plant receptors leads to the initiation of a generalized basal defence by the plant (Jones and Dangl 2006). This response by the plant is successful against most pathogens and involves fortification of the cell wall and the mobilization of antimicrobial plant compounds, phytoalexins (Maldonado-Bonilla et al. 2008). For example, in wheat, fortification of the nodes to the rachis has been observed following *Fusarium* infection (Ilgen et al. 2009). In a susceptible interaction, the pathogen produces virulence factors that are able to suppress basal defences in the plant. Using the same example, DON is suspected to suppress the wheat defences at the nodes to the rachis, thereby allowing the fungus to spread to the rest of the plant (Ilgen et al. 2009). Resistant plants on the other hand, have evolved counter mechanisms against these virulence factors (Jones and Dangl 2006). For example, the wheat variety 'Roblin' is susceptible to *Fusarium* infection, whereas the variety 'Sumai 3' has acquired genetic elements that enable it to defend against *Fusarium* infection, making it resistant (Buerstmayr et al. 2002). Breeding for *Fusarium* resistance dates back to the early 1900's and there have been many genetic loci identified to be associated with both susceptibility and resistance to *Fusarium* infection (Rudd et al. 2001).

In wheat, five types of resistance against *Fusarium* have been observed: Type I – resistance to penetration, Type II – resistance to fungal spread, Type III – resistance to kernel infection, Type IV – maintaining yield in the presence of infection, and Type V –

the ability to metabolize mycotoxins (Rudd et al. 2001). An ongoing concern with breeding programs in wheat is that there is cost associated with breeding for resistance, such as reduction in yields. This is partially due to the linkage between genes associated with increased yields, *Rht1* and *Rht2*, and genes associated with susceptibility to *F. graminearum* (Srinivasachary et al. 2008). Despite these challenges, plant breeding has generated many wheat varieties that incorporate a combination of resistance types to *Fusarium* infection (Rudd et al. 2001). Breeding experiments that use resistant varieties such as 'Sumai 3' have identified quantitative loci that provide some Type I and Type II resistance, although the underlying mechanisms to these resistances are still unknown (Buerstmayr et al. 2002). However, other studies have successfully identified genes involved in resistance to *F. graminearum* infection. In *Arabidopsis thaliana* a gene encoding for UDP-glycosyltransferase is capable of detoxifying DON (Poppenberger et al. 2003). Transgenic wheat plants expressing the *Fusarium* gene *Tri101*, coding for trichothecene 3-O-acetyltransferase, are also able to detoxify DON (Okubara et al. 2002). In addition, mutation of the gene *Rpl3*, a target of DON, resulted in a significant reduction of trichothecene activity in the host (Desjardins 2006).

1.5. Regulation of Virulence in F. graminearum by Environmental Factors

Fungal virulence can be modulated by carbon sources, nitrogen sources, pH, salts, fungicides, hydrogen peroxide, light, and various phytochemicals present in the environment (Miller and Greenhalgh 1985; da Silva et al. 2001; Yu and Keller 2005; Desjardins 2006; Li et al. 2007; Gardiner et al. 2009a; Gardiner et al. 2009b; Merhej et al. 2011). It is generally accepted that during the initial stages of infection, pathogenic fungi

are in a state of nutrient starvation and are completely reliant on internal stores (Divon and Fluhr 2007). Nutrient stress conditions that simulate pathology are often used to activate and study virulence factors, including mycotoxins, in many pathogenic fungi (Bolton and Thomma 2008). Understanding how environmental signals are able to modulate virulence could be a pivotal development towards controlling fungal infection and mycotoxin production.

1.5.1. Virulence and Nitrogen Stress

There are many studies that support a link between nitrogen stress and virulence. For example, gene expression patterns during *F. oxysporum* infection are very similar to the patterns observed during nitrogen stress conditions in culture (Divon et al. 2005). Nitrogen-deplete conditions are also able to induce virulence and secondary metabolism in *F. verticillioides*, regulating biosynthesis of the secondary metabolites gibberellin, bikaverin, and carotenoids (Rodriguez-Ortiz et al. 2009). This suggested that fungi may be under nitrogen stress during infection, and that this stress may act as a cue to induce virulence-related genes. In *F. graminearum*, DON biosynthesis genes are induced by non-preferred nitrogen sources such as agmatine and putrescine, and suppressed by preferred nitrogen sources such as glutamine, asparagine, and ammonia (Gardiner et al. 2009a). The suppression of virulence functions by preferred nitrogen sources also occurred during hyphal fusion and root adhesion in *F. oxysporum* (López-Berges et al. 2010). Similarly, preferred nitrogen sources are also able to block *F. graminearum* and *F. oxysporum* penetration of cellophane, an *in vitro* method used to mimic host penetration (López-Berges et al. 2010).

When a preferred nitrogen source such as glutamine or free ammonia is available, pathways involved in the metabolism of non-preferred nitrogen sources are repressed by a mechanism termed nitrogen catabolite repression (NCR), which is mediated by the gene *Nmra* (Divon and Fluhr 2007; Bolton and Thomma 2008; Schonig et al. 2008; Wong et al. 2008). If preferred nitrogen sources are absent, signalling pathways regulated by the *Tor* kinase and *MeaB* suppress the activity of *Nmra* and induce expression of *AreA*, a non-preferred nitrogen catabolism activator (Wong et al. 2008; López-Berges et al. 2010). Evidence indicates that these global regulators have direct implications on virulence. Blocking *Tor* kinase or *MeaB* in *F. oxysporum* derepresses NCR and initiates behaviour associated with virulence such as cellophane penetration, even in the presence of a preferred nitrogen source (López-Berges et al. 2010). In addition, an *AreA* homolog, *Fnr1*, as well as many other genes involved in non-preferred nitrogen acquisition, are induced in early stages of infection for *F. oxysporum* (Divon et al. 2006). Disruption of *Fnr1* results in reduced pathology in tomato as well as reduced ability to metabolize specific nitrogen sources (Divon et al. 2006). Although the association between *Fnr1* and virulence in *F. oxysporum* is clear, the exact mechanism is unknown (López-Berges et al. 2010). Disruption of *AreA* in *F. verticillioides* reduces both virulence and the capacity for the fungus to synthesize the mycotoxins fumonisin and gibberellin, suggesting that *AreA* positively regulates these biosynthetic pathways (Mihlan et al. 2003; Kim and Woloshuk 2008). Evidence also indicates that *AreA* may bind directly to the promoters of genes involved in biosynthesis of the gibberellin in *F. verticillioides* (Mihlan et al. 2003).

Together, the evidence suggests that NCR blocks the developmental transition from vegetative growth to a pathogenic state. Non-preferred nitrogen conditions seem to initiate key signals from *Tor* kinase and *MeaB*, which activate *AreA*. These signals seem to be responsible for inducing many factors associated with virulence in *Fusarium spp.*

1.5.2. Virulence and Carbon, pH, Light, and Other Environmental Cues

In addition to nitrogen stress, carbon stress has also been shown to be important for virulence, affecting the production of both DON and ZON in *F. graminearum* (Miller and Greenhalgh 1985). Glucose is well characterized to be a preferred carbon source for pathogenic fungi (Divon and Fluhr 2007). Similar to nitrogen, biosynthesis of DON is greater in non-preferred sources such as sucrose, 1-kestose and nystose and lower in preferred sources such as glucose (Jiao et al. 2008). ZON biosynthesis follows a similar trend, with biosynthesis being greater in raffinose and D-arabinose in comparison to glucose (Miller and Greenhalgh 1985). It is important to note that cultures that contained both glucose and sucrose had similar production of DON in comparison to cultures that contained only sucrose; this suggested that biosynthesis of DON is responsive to the non-preferred sources as opposed to carbon catabolite repression (CCR) (Jiao et al. 2008). In addition, deletion of a regulatory gene responsive to sucrose in *F. oxysporum* results in reduced activity of CWDE and delayed pathogenicity (Ospina-Giraldo et al. 2003). It is noteworthy to mention that before the genome of *F. graminearum* became available, EST libraries were made under nitrogen and carbon stress conditions to identify genes potentially involved in virulence since these conditions are required for mycotoxin production and virulence in the fungus (Trail et al. 2003).

DON and ZON biosynthesis are also modulated by pH. Although the effects are dependent on the availability of nitrogen and carbon sources; mycotoxin production is lower in alkaline conditions (Miller and Greenhalgh 1985). Studies from *F. verticillioides* suggests that nitrogen availability may be important for initializing the pH drop which in turn elicits secondary metabolism; this may be the case with *F. graminearum* (Kim and Woloshuk 2008). In *F. graminearum*, the transcriptional regulator *PacC* negatively regulates genes involved in DON biosynthesis by responding to pH signals (Merhej et al. 2011). The constitutive expression of *Pac1* (*PacC* homolog) in *F. graminearum* led to a dramatic reduction in both DON biosynthesis and the activity of *Tri5*, the first structural gene leading to the biosynthesis of DON (Merhej et al. 2011).

Light also seems to be an important regulator of secondary metabolism. Photosignals have been shown to modulate the behaviour of a complex composed of the proteins VelB, VeA, and LaeA (Bayram et al. 2008). Manipulation of these genes has direct implications on the production of gibberellic acid, bikaverin, fumonisins and fusarins in *F. verticillioides*, as well as secondary metabolism and nutrient acquisition from host material in *Aspergillus flavus* (Amaike and Keller 2009; Myung et al. 2009; Wiemann et al. 2010). It has been suggested that elements of this regulatory mechanism may also be dependent on nitrogen stress, and act independently of *AreA* in *F. verticilloides* (Wiemann et al. 2010). Light has also been demonstrated to affect production of carotenoids in *F. verticillioides* (Rodriguez-Ortiz et al. 2009).

In addition to carbon, pH, and light, there are many other environmental signals that are able to modulate virulence, although their mechanisms are poorly understood. *F. graminearum* is able to infect wheat differently at 16°C in comparison to 20°C, suggesting a role for temperature in virulence (Brennan et al. 2005). Plant metabolites such as choline and betaine, have also been demonstrated to enhance *F. graminearum* virulence, although the cause of the increased virulence is currently unknown (Strange et al. 1978; Engle et al. 2004). Hydrogen peroxide is able to elevate the expression of DON biosynthesis genes and enhance DON production, whereas magnesium is able to repress DON biosynthesis in *F. graminearum* (Ponts et al. 2007; Pinson-Gadais et al. 2008). Mutation of genes in the high-osmolarity glycerol pathway (HOG), have also been demonstrated to cause reduced virulence in *F. graminearum* (Jiang et al. 2011). Disruption of a HOG-MAPK gene in *F. proliferatum* resulted in increased nitrogen stress sensitivity and induction of fumonisin biosynthesis genes; this suggested a possible link between the HOG-MAPK signalling pathway and nitrogen stress (Kohut et al. 2009).

1.5.3. Signal Transduction by Environmental Factors

Studies from various phytopathogens suggest that G-protein complexes play an important role in integrating a broad range of environmental signals (Yu and Keller 2005). The G-protein consists of α, β, and γ subunits that are part of a large transmembrane receptor complex (Li et al. 2007). After the receptor receives a stimulus, there is dissociation of the α subunit from the β/γ subunit dimer (Li et al. 2007). These then interact with other molecules in the cell to initiate and amplify downstream signals (Li et al. 2007). The large number of genes associated with the G-proteins/receptors in

the *F. graminearum* genome allows for myriad combinations of different subunits and receptors, which enables the fungus to detect different stimuli as well as adjust the downstream responses. For example, disruption of either the α subunit *Gpa1* or the β subunit *Gpb1* in *F. graminearum* results in increased DON and ZON biosynthesis (Yu et al. 2008). Deletion of another α subunit, *Gpa2*, results in decreased pathogenicity and increased chitin accumulation in *F. graminearum* (Yu et al. 2008). Mutation in *Gbb1*, a β subunit in *F. verticilloides*, results in reduced fumonisin biosynthesis (Sagaram and Shim 2007). Similarly, the *Gpa1* homolog *FadA* is negatively associated with aflatoxin and sterigmatocystin biosynthesis in *Aspergillus*, but constitutive expression of the gene in *F. sporotrichioides* results in an increase in trichothecene biosynthesis (Tag et al. 2000). Together, these studies suggest that G-protein complexes are important for perceiving various environmental cues that result in distinct downstream responses involved in virulence. The information perceived by the G-protein complexes is then integrated by mitogen activated protein kinase signalling pathways (MAPKs), which are ubiquitous in all eukaryotes.

In *F. graminearum*, two MAPKs, *Mgv1* and *Gpmk1* are known to be important for virulence functions. Disruption of either *Mgv1* or *Gpmk1* affected virulence and other aspects related to fungal development, such as mating and conidiation (Hou et al. 2002; Jenczmionka et al. 2003; Urban et al. 2003). Both *Mgv1* and *Gpmk1* are also required for fungal protection against defensins, a type of antibiotic protein produced in plants as part of the innate defence response (Thomma et al. 2002; Ramamoorthy et al. 2007). In addition, disruption of *Gpmk1* affected the secretion of proteins involved in lipid,

cellulose, and xylan metabolism thereby affecting pathogenicity in *F. graminearum* (Jenczmionka and Schäfer 2005). In *F. oxysporum*, the MAPK gene *Fmk1* is also required for pathology and has implications on penetration, while dispensable for normal growth and sporulation (Di Pietro et al. 2001; Yu and Keller 2005; López-Berges et al. 2010). Recent evidence suggests that nitrogen signals from *Tor* kinase and *MeaB* also affect penetration and therefore may act through pathways that overlap with *Fmk1* (López-Berges et al. 2010). Phosphorylation events by MAPK have also been demonstrated to play a role in the activation of trichothecene production in *F. graminearum* (Rampitsch et al. 2010). Among the phosphorylated proteins associated with DON biosynthesis were transcription factors that could potentially regulate genes associated with virulence (Rampitsch et al. 2010).

1.6. The Role of Secreted Proteins in Virulence

As with most phytopathogens, *Fusarium spp.* secrete a large number of proteins during interactions with their hosts. In depth mass spectrometry analyses from various laboratories have identified ~120 secreted proteins from infected wheat heads and ~228 proteins from cultures inoculated with *F. graminearum* (Phalip et al. 2005; Paper et al. 2007). The most prevalent among the secreted proteins, were the proteins that belonged to the CWDE family.

In vitro studies have demonstrated that *F. graminearum* is able to modulate the secretion of CWDE depending on the availability of plant nutrients in the environment. For example, *F. graminearum* grown in plant cell wall material secreted more glycosyl

hydrolase proteins in comparison to cultures that contained only sucrose; these proteins were secreted specifically for the degradation of the complex polysaccharides (Paper et al. 2007). This indicates that the fungus has the ability to detect and metabolize plant materials in the environment.

As described before, *F. graminearum* is able to gain access to the host through susceptible areas of the plant such as stomatal openings and cracked anther tissues. In addition, CWDE are used to break down structural barriers to facilitate entry into the host. The first barrier is the cuticle which is composed of cuticular waxes and cutin. It then encounters a cell wall composed of carbohydrates (Kikot et al. 2009). The production and secretion of various CWDE such as cutinases, pectinases, cellulases, xylanases and lipases allows the fungus to break down these barriers and facilitates penetration of the host (Feng et al. 2005; Paper et al. 2007). This is supported by the observation that *F. graminearum* produces a wide array of CWDE during infection in wheat (Phalip et al. 2005; Paper et al. 2007). In addition to the proteins found to be secreted in infected wheat, additional genes encoding for CWDE are up-regulated during infection in barley (Cuomo et al. 2007; Kikot et al. 2009). The large number and diversity of CWDE demonstrates their extensive role in both host penetration and nutrient acquisition (Wanjiru et al. 2002; Phalip et al. 2009).

In addition to CWDE, there are other proteins secreted by *Fusarium spp.* that could have roles in virulence. There are many proteins unrelated to CWDE, such as proteases, that are secreted by *F. graminearum* (Paper et al. 2007). These could interact

with host proteins to suppress host defences, therefore acting as virulence factors. It is also possible that some of these proteins are detected by the host and cause the host to initiate an immune response. Since they contribute to the success of the host, these proteins are called avirulence factors; avirulence factors are recognized by the host in what is called a 'gene-for-gene' interaction, mediated by host resistance genes. Some of these interactions have been identified for *F. oxysporum*; however, little is known about 'gene-for-gene' interactions in other *Fusarium spp.* (Houterman et al. 2009).

1.7. Mycotoxin Production in F. graminearum

Secondary metabolites from fungi are of immeasurable importance; many have desirable antibiotic and pharmaceutical properties, while others are undesirable and can be highly toxic to animals. They may act to increase the virulence of pathogens, defend pathogens against other organisms, or territorially exclude organisms from infected tissues. *F. graminearum* is able to produce many secondary metabolites, such as the trichothecenes nivalenol (NIV) and DON, as well as other metabolites such as ZON, fusarin C, aurofusarin, butenolide, chlamydosporal, culmorin, and cyclonerdosiol (Desjardins 2006). The structures of a subset of mycotoxins can be found in Figure 2.

Many of these metabolites are less studied as they are either not currently attributed to economically important disease symptoms in humans or livestock, or they are not produced at levels that make them economically important (Desjardins 2006). Others, such as the DON, have serious health implications and have been explored with great vigour. Compounds such as DON and ZON are produced simultaneously and their

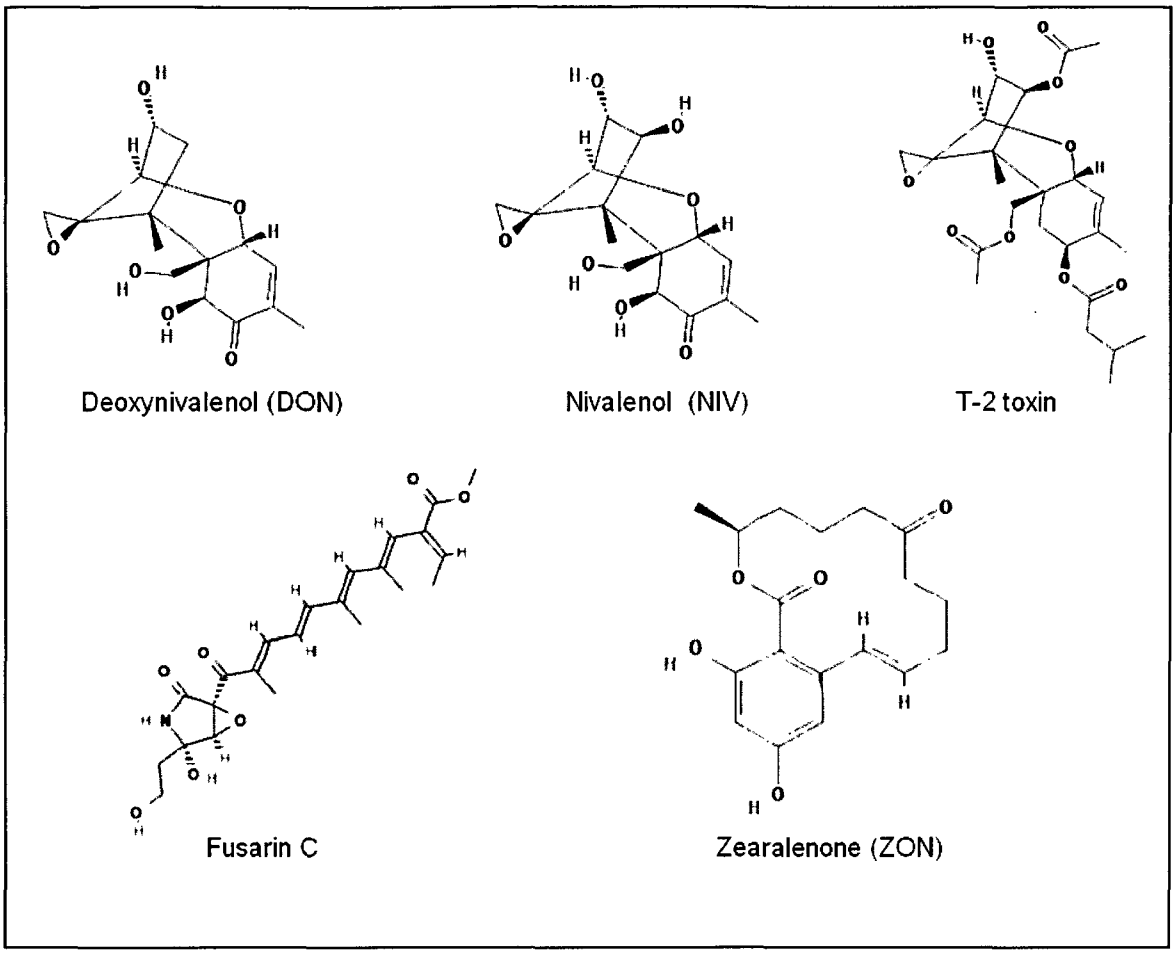


Figure 2: The chemical structures of common *Fusarium* mycotoxins. The secondary metabolites DON, NIV, ZON and fusarin C are produced by *Fusarium graminearum*; T-2 toxin, which differs from DON by three functional groups, is produced by *Fusarium sporotrichioides* (Bolton et al. 2008).

effects are often studied together (Döll and Dänicke 2011). The availability of the genome sequence of *F. graminearum* has placed greater emphasis on the identification of uncharacterized metabolites, as well as the elucidation of genes involved in the production and regulation of known secondary metabolites (Desjardins 2006).

1.7.1. Production and Implications of Fusarin C

Fusarin C is an acyl-tetramic acid produced by many sponges, slime moulds and fungi (Song et al. 2004). It has been identified in food crops infected by *Fusarium spp.*, but there is little known of its biological role *in planta*. Positive tests for fusarin C with liver homogenates have been observed in Ames tests, suggesting that it may have carcinogenic properties (Desjardins 2006). However, since it has not been attributed to any major disease outbreaks, its limits in food have not been set (Desjardins 2006). One of the genes involved in the biosynthesis of fusarin C in *F. graminearum* has been identified, but most of the genes required for its production are unknown (Song et al. 2004; Desjardins and Proctor 2007).

1.7.2. Production and Implications of Zearelanone (ZON)

Zearelanone (ZON) is a resorcyclic acid lactone that predominantly appears in *Fusarium* contaminated corn (Döll and Dänicke 2011). It is an estrogen mimicking compound that can bind to mammalian α and β estrogen receptors, but is not known to be acutely toxic or fatal to humans or livestock (Yazar and Omurtag 2008). It displaces estradiol from a uterine binding protein causing an estrogenic response (Yazar and Omurtag 2008). It is most attributed to causing modifications of the reproductive and

mammary glands of swine, often resulting in infertility (Desjardins 2006). The genes required for the production of this metabolite appear in a gene cluster that contains both biosynthetic genes and gene regulators (Kim et al. 2005; Desjardins and Proctor 2007). Targeted deletion of structural genes involved in the production of ZON does not affect the virulence of the fungus in wheat (Gaffoor et al. 2005; Kim et al. 2005).

1.7.3. Production and Implications of Deoxynivalenol

The impact of *F. graminearum* in disease is mostly attributed to the accumulation of the trichothecene sesquiterpenoid DON, commonly called vomitoxin (Sobrova et al. 2010). It is a ribosomal protein synthesis inhibitor, which acts by binding to the 60S ribosome and inhibiting peptidyltransferase activity (Desjardins 2006; Yazar and Omurtag 2008). This not only blocks the formation of peptides, but induces ribosomal stresses that activate stress-induced pathways, such as MAPK pathways, which impose other secondary effects in the cell (Desjardins 2006). Evidence suggests that *F. graminearum* produces DON at the nodes to the rachis in wheat to help overcome local plant defences (Ilgen et al. 2009). The downstream products would then contain the toxin and would be consumed by humans and livestock. The acute effects of DON consumption in animals are highly dose dependant. Large enough doses have been shown to induce shock-like death; however, a dose of this magnitude would be unlikely from naturally harvested materials. A realistic dose causes nausea, vomiting, dizziness/vertigo, headaches, and hallucinations; it also appears to influence serotonin levels which are suspected to influence appetite, the end result of which is weight loss (Yazar and Omurtag 2008; Sobrova et al. 2010; Döll and Dänicke 2011). Prolonged exposure causes

organ specific abnormalities and diseases such as stomach lesions and spleen haematopoiesis (Sobrova et al. 2010). Since the toxic effects of DON are very severe, a concerted effort is being given to characterize this toxin and its production in the fungus.

Both *Fusarium graminearum* and *Fusarium sporotrichioides* have been used to study the biosynthesis of trichothecenes and currently, many of the biosynthetic genes have been identified (Desjardins 2006). It is important to note that the pathway in *F. sporotrichioides* differs from that of *F. graminearum* and results in the production of T-2 toxin (Alexander et al. 2004). The T-2 toxin differs from DON by four functional groups, as seen in Figure 2 (Abramson et al. 1997). Most of the genes responsible for the production of DON are located in a gene cluster composed of twelve genes that are involved in toxin production, transportation and regulation; the key positive regulators being *Tri6* and *Tri10* (Kimura et al. 2007). There are also four other genes located outside of the cluster that are also associated with DON production, these include the biosynthetic genes *Tri1* and *Tri16*, and the detoxifying genes *Tri101* and *Tri201* (homologue of *Tri101*) (Kimura et al. 2007). DON is produced in one of three acetylated forms in the fungus, either 15-acetyl-DON, 3-acetyl-DON, or 3,15-diacetyl-DON and becomes deacetylated *in planta* (Desjardins 2006; Alexander et al. 2011). The acetylation patterns have recently been attributed to the gene *Tri8* (Desjardins 2006; Alexander et al. 2011).

Disruption of either *Tri6* or *Tri10* results in reduced expression of many genes involved in trichothecene biosynthesis, inhibition of DON production, and renders the

strain non-virulent, suggesting that both are positive regulators of genes in the DON pathway (Proctor et al. 1995; Tag et al. 2001). Alexander et al. (2004) observed that *Tri15*, a gene outside of the cluster that codes for a Cys_2His_2 zinc finger protein, was expressed in a *Tri10* constitutive expression strain in *F. sporotrichioides* (Peplow et al. 2003). Furthermore, addition of T-2 toxin to a *F. sporotrichioides* culture induced *Tri15* expression. This led them to propose that *Tri15* may function as a negative regulator of T-2 toxin production, although further studies would be required to validate this claim (Alexander et al. 2004).

1.8. Project Outline

While investigating the role of *Tri15* in *F. graminearum*, two genes similar to *Tri15* were identified, *FGSG_03881* and *FGSG_11025*. However, disruption of *FGSG_11025* in *F. graminearum* does not affect DON production or virulence in wheat (personal communication, Rajagopal Subramaniam). This project explored the role of *FGSG_03881* in mycotoxin production and virulence in *F. graminearum* using a reverse genetic approach. It also involved the development of a high-throughput system used to monitor expression patterns of *FGSG_03881*. Finally, microarray analyses and mass spectrometry of secreted proteins were performed with *fgsg_03881Δ* to delineate mechanistic roles for *FGSG_03881*.

Chapter 2 - Materials and Methods

2.1. Fusarium Strains, Propagation and Storage

F. graminearum NRRL 29169 (also catalogued as NRRL 38155, GZ3639 and DAOM 233423) was used as the wild-type strain and all transgenic strains were derived from this parental strain. Two additional strains that were also used, *tri6Δ* (DAOM 237993) and *tri10Δ* (DAOM 240167), were also derived from this strain. For storage purposes, conidia were generated using CMC medium according to Cappellini and Peterson (1965), and were resuspended in 15% glycerol and stored at -80°C. This stock was used to propagate fresh conidial stocks that were used as the primary inoculum in subsequent experiments. All of the transgenic strains generated were propagated in the same manner and deposited at the Canadian Collection of Fungal Cultures which is maintained by Agriculture and Agri-Food Canada.

2.2. Bioinformatic Analyses of FGSG_03881

The *FGSG_03881* DNA and protein sequences were imported into Blast (basic local alignment search tool) and analyzed against available genomes for *F. oxysporum* and *F. verticillioides* in the Broad Institute database (www.broadinstitute.org) as well as available sequences in the NCBI database (www.ncbi.nlm.nih.gov). Hits of interest were then aligned to the sequence for *FGSG_03881* to generate a similarity tree using a neighbour-joining algorithm in ClustalW2 (www.ebi.ac.uk/Tools/msa/clustalw2).

2.3. Generation of F. graminearum Transgenic Strains

2.3.1. Generating the FGSG_03881 Disruption Strain

FGSG_03881 was disrupted through homologous recombination between genomic DNA and a fusion PCR construct as described in Schreiber et al. (2011). A summary of the procedure is outlined in Figure 3. Briefly, a fusion PCR construct was obtained by using primers P1 – P6, with *Fusarium* genomic DNA, and the hygromycin resistance gene (Kistler and Benny 1988) as templates. The list of primers can be found in Appendix Table 1. The standard PCR reaction consisted of 0.2 μM forward primer, 0.2 μM reverse primer, 0.2 mM dNTPs, 1.5mM $MgCl_2$, roughly 50 ng of DNA template and Roche Expand Long polymerase and PCR Buffer (Hoffmann-La Roche Limited, Canada) in a 25μL reaction; 35 PCR cycles were performed with a melting temperature of 94°C, annealing temperature of 54°C, extension temperature of 72°C and a final extension for 10 min at 72°C.

The fusion PCR construct, composed of the hygromycin resistance gene flanked by 1kb of the 5' and 3' ends of the gene, was transformed into *F. graminearum* protoplasts. Protoplasts were obtained by digesting 5 x 10^8 germinated spores using an enzyme solution (5% Driselase (Sigma-Aldrich, USA), 1mg Chitinase (Sigma-Aldrich, USA) and 100 mg Lysing enzymes (Sigma-Aldrich, USA)) in 20mL of 1M of NH_4Cl_2 pH 5.0 and centrifuging at 100 RPM at 30°C for 2.5h. After washing with 1M 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 1 x 10^8 protoplasts/mL with storage buffer

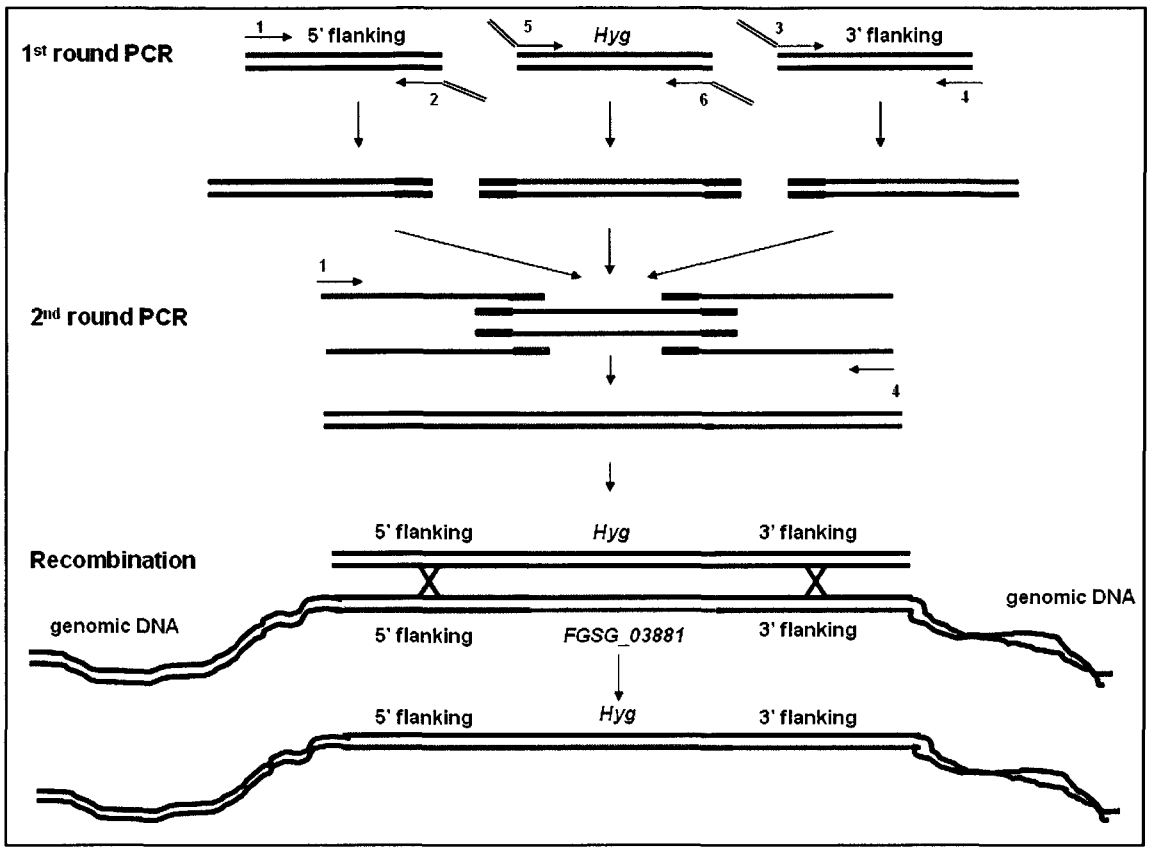


Figure 3: Generation of the fusion PCR construct used to disrupt *FGSG_03881*. The fusion PCR construct was generated using primers P1 - P6 (Appendix Table 1) and standard PCR amplification steps as described in Schreiber et al. (2011). This construct was transformed into *F. graminearum* (DAOM 233423) protoplasts, generating the *fgsg_03881Δ* transgenic strain.

(800µL STC, 200µL of 30% PEG, 10µL of DMSO). Protoplasts were aliquoted into 100µL aliquots and stored in -80°C until use. Transformation was carried out by incubating 100µL protoplasts with 500ng DNA of the fusion PCR construct for 30min at 25°C. After incubation, 2mL of 30% PEG and 4mL of STC buffer were added and the mixture was transferred to 200mL of solidified regeneration medium (0.6g of yeast extract, 0.6g of casein hydrolysate, 9.2g of agar, and 164.3g of sucrose in 600ml of H_2O) and plated. The plates were incubated at 28°C in darkness for 24h prior to the addition of 10mL overlay media (1% agarose and 200µg/mL of Hygromycin B (Calbiochem, USA)). The transformants were selected based on the ability to grow on the hygromycin, generating the *fgsg_03881Δ* transgenic strain. Deletion of the gene was verified by PCR using *Hyg* F / *Hyg* R primers detecting the presence of the hygromycin marker, and *FGSG_03881* F / *FGSG_03881* R primers verifying the absence of the coding region.

2.3.2. Generation of the FGSG_03881 Complementation, FGSG_03881 Constitutive Expression, and pFGSG_03881::*GFP Fusarium Transgenic Strains*

The *FGSG_03881* complementation, *FGSG_03881* constitutive expression, and promoter::GFP transgenic strains were generated using USER (Uracil-Specific Excision Reagent) cloning (New England Biolabs, USA) and *Agrobacterium*-mediated transformation as described in Frandsen et al. (2008). The vector pSW-GU was used to generate the complementation and promoter::GFP strains. This vector was constructed by replacing the hygromycin selection marker with Geneticin resistance encoded by the gene *NptII* (neomycin phosphotransferase). *NptII* was amplified from the vector pII99 (Namiki et al. 2001) using Geneticin USER F / Geneticin USER R primers (Appendix Table 1),

standard PCR conditions and Stratagene PfuTurbo C_x Hotstart polymerase (Stratagene, USA). This construct was then cloned into the pRF-HU2 vector using the USER cloning methods described in Frandsen et al. (2008) and transformed into *E. coli* DH5α. This generated the vector pSW-GU (Appendix Figure 1), which had the same restriction and USER cloning sites as the pRF-HU vector, but contained the Geneticin resistance gene.

Generation of the constitutive expression strains also required the construction of new vectors with Geneticin as a selection marker. To do this, the promoter for glyceraldehyde-3-phosphate dehydrogenase (pGpd) was amplified from the pRF-HUE vector using pGPD USER F / pGPD USER R primers (Appendix Table 1) with the Stratagene PfuTurbo C_x Hotstart polymerase. This construct was cloned into the pSW-GU vector and transformed into *E. coli* DH5α. This generated a vector pSW-GUE (Appendix Figure 2), which has the same restriction and USER cloning sites as the pRF-HUE vector, but the pSW-GU backbone allowed for Geneticin to be used as the selection marker. All of the constructs were verified by sequencing (MWG Operon, USA).

To construct the complementation vector, the promoter, coding region, and terminator of *FGSG_03881* were amplified using the Promoter USER F / Terminator USER R primers (Appendix Table 1) and the Stratagene PfuTurbo C_x Hotstart polymerase. This was then cloned into the pSW-GU vector by the USER cloning method (Frandsen et al. 2008) and transformed into *E. coli* DH5α. To generate the constitutive expression vector, the cDNA sequence of *FGSG_03881* was amplified using *FGSG_03881* USER F / *FGSG_03881* USER R primers using the Stratagene PfuTurbo

C_x Hotstart polymerase as described previously (Appendix Table 1) and was then cloned into the pSW-GUE vector and transformed into *E. coli* DH5α. The fusion PCR method (Section 2.3.1) was used to generate the promoter::GFP vector. The vector consisted of the gene coding for green fluorescent protein (GFP) flanked by the 5' and 3' ends of *FGSG_03881*. The *FGSG_03881* promoter and terminator were amplified using *Fusarium* genomic DNA and Promoter USER F / Promoter R, and Terminator F / Terminator USER R primers (Appendix Table 1). The coding region for the GFP was amplified from the vector pCA42 (Sato et al. 2007) using GFP F / GFP R, standard PCR parameters, and the Stratagene PfuTurbo C_x Hotstart polymerase. This construct was then cloned into pSW-GU by USER cloning and transformed into *E. coli* DH5α as before. All of the constructs were verified by sequencing (MWG Operon, USA).

The complementation vector was transformed into *fgsg_03881Δ* to generate the *FGSG_03881/fgsg_03881Δ* strain. The promoter::GFP vector was transformed into both wild-type and *fgsg_03881Δ* to generate the pFGSG_03881::*GFP*/wt and pFGSG_03881::*GFP/fgsg_03881*Δ strains, respectively. The constitutive expression vector was transformed into both wild-type and *fgsg_03881Δ* to generate pGpd::*FGSG_03881*/wt and pGpd::*FGSG_03881/fgsg_03881Δ*, respectively. All transformations were performed using the *Agrobacterium*-mediated transformation method as described in Frandsen et al. (2008) and selected using Geneticin (Sigma Aldrich, Canada) at 150μg/mL. Verification of the Geneticin resistance gene in the transgenic strains was done by PCR using Gen F2 / Gen R2 (Appendix Table 1). Construct specific primers were used to verify all of the transgenic lines; for the

constitutive expression lines pGpd Int F / *FGSG_03881* R were used, for the complementation line Promoter USER F / *FGSG_03881* R primers were used, and for the promoter::GFP lines Promoter USER F / GFP R were used (Appendix Table 1).

2.4. Pathology Tests and Analyses in Triticum aestivum (wheat)

The susceptible variety of wheat 'Roblin' and a resistant variety of wheat 'Sumai 3' were grown in growth chambers until anthesis was reached as described in Schreiber et al. (2011). At mid-anthesis, the plants were inoculated between the palea and lemma with approximately 1,000 conidia from either wild-type, *fgsg_03881Δ*, or the *FGSG_03881* complement strain. Inoculations were performed over approximately a week, as not all plants reached mid-anthesis simultaneously. For the 'Roblin' plants, the number of inoculated wheat heads were 41, 42, and 42 respectively while for 'Sumai 3' the number of inoculated wheat heads were 30, 32, and 31 respectively. The plants were then transferred into a greenhouse where they experienced regular misting for two days. The infection was scored by counting the number of infected spikelets at 7, 10, 14 and 21 days and an unpaired t-test was performed to assess any differences between the wild-type and transgenic strains.

2.5. Gene Expression Analyses

2.5.1. Culture Conditions, RNA Isolation, and Quantitative Real-Time PCR

GYEP medium (3g NH_4Cl, 2g $MgSO_4 \cdot 7H_2O$, 0.2g $FeSO_4 \cdot 7H_2O$, 2g KH_2PO_4, 2g peptone, 2g yeast extract, 2g malt extract, and 20g glucose in 1L H_2O) was inoculated with either the wild-type or *fgsg_03881Δ* strain at a concentration of approximately 5,000

spores/mL. Four milliliter cultures in 6 well plates were incubated at 28°C and 170 RPM for 18h and 24h. After 24h of growth in GYEP medium, the cultures were transferred to fresh GYEP medium, minimal medium (Leslie and Summerell 2006), minimal medium with 2g/L ammonium chloride as a sole nitrogen source, minimal medium lacking nitrogen, and 15-acetyl-DON inducing medium (1 g $(NH_4)_2HPO_4$, 3g KH_2PO_4, 0.2g $MgSO_4 \cdot 7H_2O$, 5g NaCl, 40g sucrose, and 10g glycerol in 1L H_2O and adjusted to pH 4) and grown for an additional 4h. Mycelia from all conditions were flash frozen in liquid nitrogen and ground using a mortar and pestle prior to RNA isolation. RNA was then isolated using Trizol (Invitrogen, USA) and purified using InviTrap Spin Cell RNA Mini Kit according to the manufacturer's instructions (Invitek, Germany).

For quantitative real-time PCR (RT-qPCR) analyses, RNA was converted into cDNA by the Applied Biosystems cDNA synthesis kit (Applied Biosystems, Canada). All of the RT-qPCR reactions were performed in triplicate using Applied Biosystems Power SYBR Green kit (Applied Biosystem, Canada) and the Applied Biosystems StepOne Plus Real-Time PCR System according to manufacturer's instructions (Applied Biosystems, Canada). A list of RT-qPCR primers can be found in Appendix Table 2. A ten times dilution series was performed for each primer set; *β-tubulin* (*FGSG_09530*) was used as the internal control for relative quantification between samples. The data was imported and analyzed in StepOne 2.1 software (Applied Biosystems, Canada).

2.5.2. Global Gene Expression Analyses by Microarrays

RNA was isolated from mycelia grown for 24h in GYEP media and for 4h in 15-acetyl-DON inducing conditions as in Section 2.5.1. An Agilent 2100 Bioanalyzer (Agilent, Canada) was used to examine the integrity of the RNA. RNA was then converted into cDNA using the Agilent Quick Amp Labeling Kit, and converted back into labeled RNA using T7 RNA polymerase and cyanine 3-labeled CTP or 5-lableled CTP from the Agilent two-color RNA Spike-in kit (Agilent, Canada); each strain had two samples labeled with each dye from each culture condition. The cRNA from the two lines were then hybridized to a custom made *F. graminearum* oligo Chip from Agilent, which contains up to three oligomers for every *F. graminearum* gene, using the Gene Expression Hybridization Kit from Agilent (Agilent, Canada). The hybridized chips were scanned using the GenePix Professional 4200A scanner, and the signals quantified using PenePix Pro 6. The microarray data was then transferred into Acuity 4.0. The data was normalized using Lowess Normalization and data with low intensities were removed. Next, the data was expressed as $\log_2$, corresponding to a two-fold difference in gene expression. In addition, genes with only one oligomer remaining were also removed from the data set. Hybridization values for the oligomers for each gene were averaged between replicates, generating a combined data set. Hybridization values were then averaged between biological replicates and reverse-transformed to generate an average value for each gene. Genes with expression less than two-fold difference or with p-values > 0.05 were removed. Differentially expressed genes were categorized according to annotations in the Munich Information Center for Protein Sequences (MIPS) database (mips.helmholtz-muenchen.de/genre/proj/FGDB/); categories of over represented genes

were identified using MIPS FunCat database (mips.helmholtz-muenchen.de/cgi-bin/proj/funcatDB/search_advanced.pl?gene=2).

2.5.3. High-Throughput Analyses of FGSG_03881 Promoter Activity

Approximately 800 spores from the pFGSG_03881::*GFP*/wt and pFGSG_03881::*GFP*/*fgsg_03881Δ* strains were inoculated into wells of a 96 well plate containing 200μL of minimal medium with either sodium nitrate or ammonium chloride as sole nitrogen sources at 2g /L, and either sucrose or glucose as a sole carbon sources at 30g/L (Leslie and Summerell 2006). All of the cultures were grown in triplicate. The plate was then incubated for over 120h at 28°C in a POLARstar OPTIMA plate reader (BMG LabTech, USA). Samples were shaken for 300s and a 5 X 5 matrix of transmittance measurements were made for each well at 520nm after excitation with 485nm. This was followed by a second measurement taken at 620nm. The data was inputted into FLUOstar OPTIMA 1.32 software (BMG LabTech, USA).

The transmittance values were then represented as a moving average of 10 measurements corrected to pass through the origin. The transmittance values obtained at 620nm, corresponding to growth, were then inverted. The cultures containing sodium nitrate and sucrose as nutrient sources were used as a reference for comparison against the other culture conditions. The GFP transmittance values (T_{GFP}) were expressed as a function of the growth (T_{growth}) in a fourth order polynomial equation for the culture containing sodium nitrate and sucrose:

$T_{GFP\ (predicted)} = \alpha\ T_{growth}^4 + \beta\ T_{growth}^3 - \gamma\ T_{growth}^2 + \delta\ T_{growth} - \varepsilon$; where α, β, γ, δ, and ε are constants

This relationship was then used to predict the T_{GFP} values given T_{growth} for all culture conditions. The relative quantification of the promoter activity was then calculated as:

$$RQ = \frac{T_{GFP\ (actual)}}{T_{GFP\ (predicted)}}$$

The RQ values were then transformed into $\log_2$ and imported into Acuity V 4.0 which was used to generate heat maps.

This was repeated with approximately 800 spores of the pFGSG_03881::*GFP*/wt strain grown in minimal media containing a range of sole nitrogen sources (glutamate, glutamine, aspargine, aspartate, ammonium sulphate, ammonium nitrate, ammonium chloride, arginine, serine, histidine, urea, allantoin, hypoxantine, xanthine, sodium nitrate, uracil, leucine isoleucine, phenylalanine, lysine dichloride, cysteine, or ammonium phosphate dibasic) at 2g/L. The relative quantification was performed using three different nutrient conditions (sodium nitrate, ammonium phosphate dibasic, and glutamate) as references.

2.6. Analysis of Secreted Proteins

2.6.1. Isolation of Secreted Proteins Under 15-acetyl-DON Inducing Conditions

Individual flasks containing 100mL of GYEP medium were inoculated with *fgsg_03881Δ*, *FGSG_03881/fgsg_03881Δ*, *tri6Δ*, *tri10Δ* and wild-type spores at approximately 5,000 spores/mL. The flasks were incubated for 24h at 28°C and 170rpm. The mycelia contents were then washed by filtration using 70mm circular whatman filter paper and transferred to new 250mL flasks containing 100mL 15-acetyl-DON inducing media. Cultures were further incubated for either one or five days. At the end of the five day period, the cultures were filtered and tested for 15-acetyl-DON content as in Section 2.8. Cultures isolated after one day in 15-acetyl-DON inducing conditions were filtered using 70mm circular Whatman filter paper and the supernatant was added to 400mL of acetone with 10% TCA (Trichloroacetic acid) and 0.07% DTT (Dithiothreitol). The supernatant was stored overnight at -20°C for proteins to precipitate. The supernatant was then centrifuged at ~5,000 x g in a high-speed centrifuge in 250mL containers. The pellet was transferred to a microcentrifuge tube and washed six times with 80% acetone containing 0.07% DTT. The final pellet was dried under N_2 gas and stored at -20°C before analyses by either 2-D gel electrophoresis or gel-free mass spectrometry. This process was performed in triplicate for each *Fusarium* strain.

2.6.2. Two-Dimensional Gel Electrophoresis of Secreted Proteins

Seventy-five micrograms of proteins from each *Fusarium* strain were separated by 2-D gel electrophoresis. The proteins were resuspended in rehydration buffer (8M deionzied urea, 2M thiourea, 2% CHAPS, 50mM DTT, 0.2% ampholytes pH 3-10

(BioRad, USA), and 0.0005% bromophenol blue) and quantified using the BioRad Bradford Assay kit (BioRad, USA). Proteins were separated according to their isoelectric point using 7cm IPG strips pH range 3-10 (BioRad, USA). They were then separated according to their molecular weight in a 12% polyacrylamide denaturing gel, and stained using Coomassie R-250 according to manufacturer's instructions (BioRad, USA).

2.6.3. Identification of Secreted Proteins by Mass Spectrometry

The same three biological replicates from each strain were used for mass spectrometry analyses by Christoph Rampitsch (Agriculture and Agri-Food Canada, Winnipeg). Protein pellets were dissolved in 100mM ammonium bicarbonate and digested with trypsin as described by Rampitsch and Bykova (2009). The resulting tryptic peptides were desalted on RP POROS R2 nanocolumns (PerSeptive Biosystems, USA) with 2% acetonitrile (ACN) in 1% formic acid (FA), and 0.5% acetic acid in preparation for LCMS analyses. Peptides were separated by reversed-phase nano-scale liquid chromatography LC: Ultimate 3000 (Dionex, Germany) eluting into a linear ion trap mass spectrometer LTQ XL (Thermo Finnigan, USA). A 10cm C_{18} column (prepared in-house) was used to introduce peptides into the mass spectrometer via nano-spray ionization at 250nl/min using a 4% to 80% ACN gradient in 1% FA, 0.5% acetic acid over 65min. The LTQ XL was programmed to produce a full survey scan (m/z 400-2000). The peptides identified were queried against a database containing translated ORFs from the complete genomic sequence of *F. graminearum* (downloaded June 2010 from www.broadinstitute.org) using the Mascot search engine. The database was expanded by including common mammalian contaminants. Proteins were considered

correctly identified if two or more peptides matched with significant Mascot scores. Secretion signals for positively identified proteins were assessed based on Signal P 3.0 scores (http://www.cbs.dtu.dk/services/SignalP/).

2.6.4. Callose Staining of Wheat Leaves Infiltrated with Protein

Secreted proteins and 100mM Flg22 peptide suspended in 0.5% Triton were infiltrated into the abaxial side of mature wheat leaves ('Roblin') by a needless 1mL syringe. The concentration of proteins from wild-type, *fgsg_03881Δ*, and *FGSG_03881/fgsg_03881Δ* were 120µg/mL, 90µg/ml, and 250µg/ml respectively. The plants were maintained in a growth chamber overnight at 16°C. After 18h, the leaves were transferred to a solution containing a 1:2 solution of (1:1:1:1 phenol : glycerol : lactate : H_2O) : ethanol and incubated at 65°C for 20min. The solution was drained, replaced and incubated at 25°C for approximately 4h. The leaves were washed with 50% ethanol, followed by washing with H_2O, and then stained for 30min with 0.01% aniline blue in 150mM K_2HPO_4 pH 9.5. The samples were mounted in 60% glycerol and observed by epiflourescence illumination (365nm excitation filter; 395nm chromatic beam splitter; 420nm barrier filter) using a Zeiss Axioplan 2 Imager microscope equipped with a Zeiss AxioCam colour camera.

2.7. Physiological and Growth Analyses of the Wild-type and Transgenic Strains

2.7.1. Assessing Growth on Solid Media

Approximately 10,000 spores of *fgsg_03881Δ* and wild-type were inoculated onto the center of sterile disks on 10 PDA and 10 SNA (Leslie and Summerell 2006) agar

plates and incubated at 28°C. Radial growth measurements were taken until the edge of the plates was reached. As the growth was not always perfectly symmetrical, two radial growth measurements were taken at regular intervals; one was taken at the maximum diameter and one at the minimum diameter, and the two measurements were averaged. The standard deviation between the replicates was used to approximate the error.

2.7.2. Assessing Growth in Liquid Media

Approximately 800 spores from either the wild-type or *fgsg_03881*Δ strain were inoculated in triplicate into 96 well plates containing 200μL of the following media: GYEP, minimal medium, CMC medium, SNA medium, 15-acetyl-DON inducing medium, and PDB (Leslie and Summerell 2006). The plate was incubated at 28°C in a POLARstar OPTIMA plate reader (BMG LabTech, USA). Samples were shaken for 300s before a 5 X 5 matrix of A-620nm measurements were made and inputted into FLUOstar OPTIMA V1.32 software (BMG LabTech, USA). The standard deviation of the triplicates was used to approximate the error.

2.8. Mycotoxin Analyses

2.8.1. Analyses of 15-acetyl-DON Production in vitro

Approximately 20,000 spores of *fgsg_03881Δ* and wild-type were grown in 4mL of GYEP in 6 well plates for 24h at 28°C and 170RPM. The cultures were transferred to 4mL of 15-acetyl-DON inducing medium for another 24h. One milliliter of culture was removed and filtered using a 0.2μm Nylon filter (Mandel Scientific, USA). 450μL of the filtrate was combined with 150μL of MeOH and injected into a C_{18} ODS Hypersil

reverse phase column (ThermoFisher Scientific, Canada) connected to an AKTA P-10 purifier (GE Healthcare, Canada). The mycotoxin 15-acetyl-DON was separated by a linear gradient of 85:15 to 60:40 water:methanol in 25min at flow rate of 1mL/min. Under these conditions, a 15-acetyl-DON standard (produced in-house) eluted at a retention time of ~ 11min, detected at 220nM.

2.8.2. Analyses of DON Production in vivo

The susceptible variety of wheat 'Roblin' was grown and point inoculated as in Section 2.3. with the wild-type (n = 47) and *fgsg_03881Δ* (n = 49) strains. The wheat heads were harvested 10 days post inoculation (dpi). The harvested wheat heads were freeze dried at -50°C under vacuum for two days and were ground into a fine powder using a Retsch MM2000 miller/grinder. The amount of DON in 1g of ground samples were then quantified using ELISA methods as described in Sinha et al. (1995). A weighted t-test was then used to compare the mean DON levels for the wild-type and *fgsg_03881Δ* samples.

2.9. Diaminobenzidine and Nitroblue Tetrazolium Staining

Spores from the wild-type and *fgsg_03881Δ* strains were used to inoculate 4mL of PDB in six well plates at approximately 20,000 spores/well. The plates were incubated at 28°C and 170RPM. After 0, 12, and 24h, cultures were harvested and washed with H_2O by centrifugation in preparation for staining.

Prior to staining with nitroblue tetrazolium (NBT), all cultures were treated with 25μM diphenyliodonium (DPI) for 30min. The cultures were washed in water and resuspended in 100μL 0.05% NBT. The cultures with NBT stain were transferred to a syringe and pressure was applied between the tip and the plunger to facilitate the infiltration of NBT stain. The cultures were then incubated at 25°C for 30min. The solution was replaced with 100μL of ethanol and incubated for 1h. The culture was resuspended in 20μL of water and observed under a compound light microscope (Lieca DM 1000). The specimen was scanned for NBT-diformazan, a dark blue precipitate that forms when NBT interacts with superoxide radicals.

For diaminobenzidine (DAB) staining, cultures were resuspended in 100μL of 0.001% DAB and were placed under pressure in a syringe as before and incubated for 30min at 25°C. The DAB was then replaced with 100μL fixing solution (3:1:1 ethanol:lactate:glycerol) and incubated for 1h at 25°C. The culture was then resuspended in 20μL of water and was observed under a compound light microscope (Leica DM 1000). The specimen was scanned for dark brown precipitates formed in a reaction between DAB and hydrogen peroxide.

2.10. Yeast Two-Hybrid Analyses of FGSG_03881 against Trichothecene Regulators

The ProQuest Yeast Two-Hybrid Kit (Invitrogen, USA) was used to test for protein-protein interactions. The cDNA sequence of *FGSG_03881* was amplified from a *Fusarium* cDNA library using standard PCR conditions, the Expand Long PCR system (Hoffmann-La Roche Limited, Canada) and *FGSG_03881* ENTR primers (Appendix

Table 1). The PCR product was cloned into the pENTR D-TOPO vector according to manufacturer's protocol (Invitrogen, USA). The insert was verified by sequencing and cloned into the yeast vectors pDest22 and pDest32 using LR Clonase and grown on selective media according to manufacturer's specifications (Invitrogen, USA). The vectors were transformed into MaV203 (*MATα; leu2-3,112; trp1-901; his3Δ200; ade2-101; cyh2^R; can1^R; gal4Δ; gal80Δ;GAL1::lacZ; HIS3$_{UASGAL1}$::HIS3@LYS2; SPAL10::URA3*) yeast competent cells and their interactions were assessed based on the *Ura3* and β-galactosidase reporter genes according to manufacturer's instructions (Invitrogen, USA).

Chapter 3 - Results

3.1. FGSG_03881 is a Zinc Finger Protein Similar to F. verticillioides FVEG_13013

Computational analyses were performed to characterize *FGSG_03881* and its relationship to other genes based on both nucleotide and protein sequences. The nucleotide blast using the Broad institute and NCBI databases did not find any genes with significant homology to *FGSG_03881*. However, the protein blasts identified many hits in *Fusarium spp.* as well as in other fungal species (Figure 4). Pairwise alignments by ClustalW2 indicate *FGSG_03881* showed little homology to *Tri15* from *F. sporotrichioides* (33% identity and 52% similarity at the protein level and 59% homology at the DNA level), whereas *FGSG_11025* was much more similar (with 82% identity and 88% similarity at the protein level and 84% homology at the nucleotide level). *FGSG_03881* was more related to the *F. verticillioides* gene *FVEG_13013* (65% identity and 80% similarity at the protein level and 67% homology at the nucleotide level) (Figures 4 and 5).

The blast analyses also identified three putative Cys_2His_2 zinc finger motifs in *FGSG_03881*. All three were conserved in *FVEG_13013*, while only two were conserved in *FGSG_11025* and *Tri15* from *F. sporotrichioides* (Figure 5). Despite differences in alignment, all four proteins share some similarity. The similarities are particularly prevalent in the third zinc finger as well as the region between the second and third zinc finger (Figure 5).

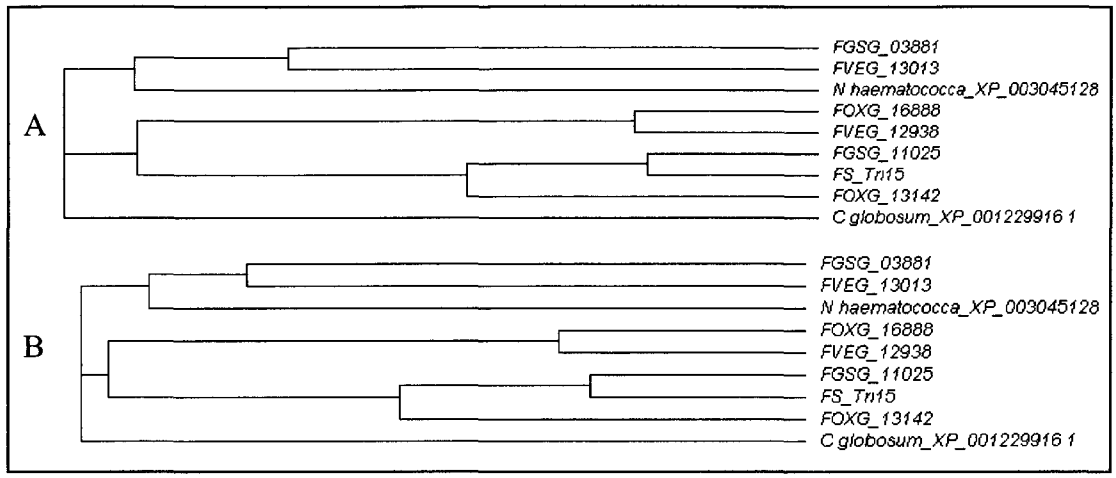


Figure 4: Cladogram generated by ClustalW2 multiple sequence alignment using a neighbour-joining algorithm. The protein (A) and nucleotide (B) similarities between *FGSG_03881* and similar proteins are presented. The top 6 *Fusarium* hits are displayed with *FGSG_11025* originating from *F. graminearum*, *FOXG_16888* and *FOXG_13142* originating from *F. oxysporum*, *FVEG_13013* and *FVEG_12938* originating from *F. verticillioides*, and *FS_Tri15* originating from *F. sporotrichioides*. There are also two genes from other genera represented; they are from the species *N. haematococca* and *C. globosum*.

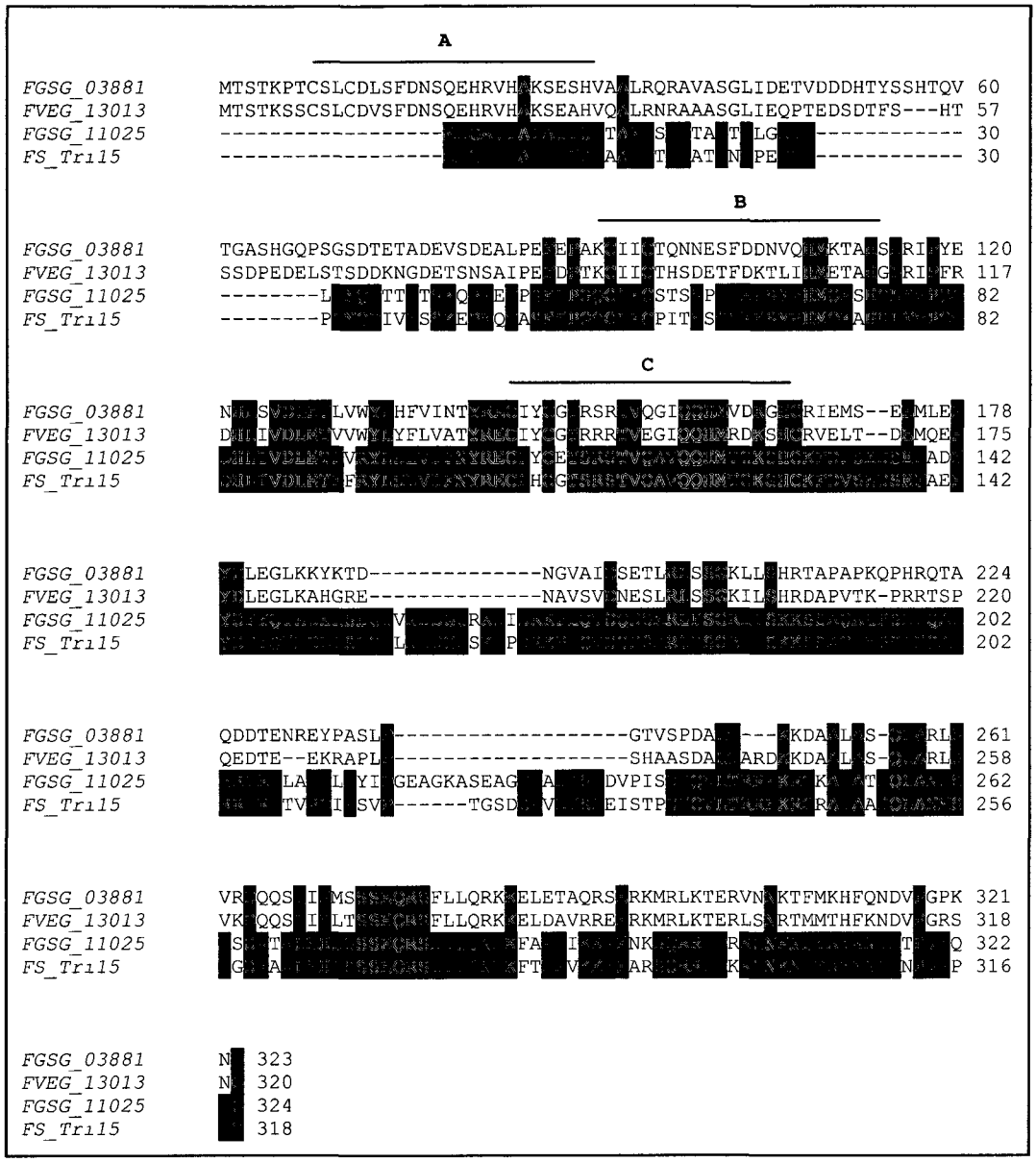


Figure 5: Multiple sequence alignment by ClustalW2 of the protein sequence for *FGSG_03881*, *FGSG_11025*, *FVEG_13013*, and *FS_Tri15*. Identical amino acids between all four are highlighted in red, identities between *FGSG_03881* and *FVEG_13013* are highlighted in yellow and identities between *FGSG_11025* and *F. sporotrichioides Tri15* highlighted in green, and putative zinc fingers are labelled (A, B, and C).

3.2. Fusarium Transformations

The transgenic strains were analyzed based on their ability to grow on selective media and by PCR methods. The *FGSG_03881* disruption, *FGSG_03881* complementation, pFGSG_03881::*GFP* in both the mutant and wild-type, and constitutive expression in wild-type strains were able to grow on selective media. The PCR results for transformation of the disruption construct into wild-type protoplasts confirmed that the hygromycin resistance gene was successfully integrated into the genome thereby disrupting *FGSG_03881* and generating the *fgsg_03881Δ* strain (Figure 6). Similarly, all of the transformations performed using *Agrobacterium*-mediated transformation generated viable colonies with the exception of the pGpd::*FGSG_03881* construct into *fgsg_03881Δ*. This transformation was repeated twice for wild-type generating a single colony, and was repeated three times for the *fgsg_03881Δ* without any success. However, the *Agrobacterium*-mediated transformations were able to generate the *FGSG_03881/fgsg_03881Δ*, pFGSG_03881::*GFP*/wt, pFGSG_03881::*GFP/fgsg_03881Δ*, and pGpd::*FGSG_03881*/wt transgenic lines. The Geneticin resistance gene and the construct specific regions of these strains were also verified by PCR (Figure 7).

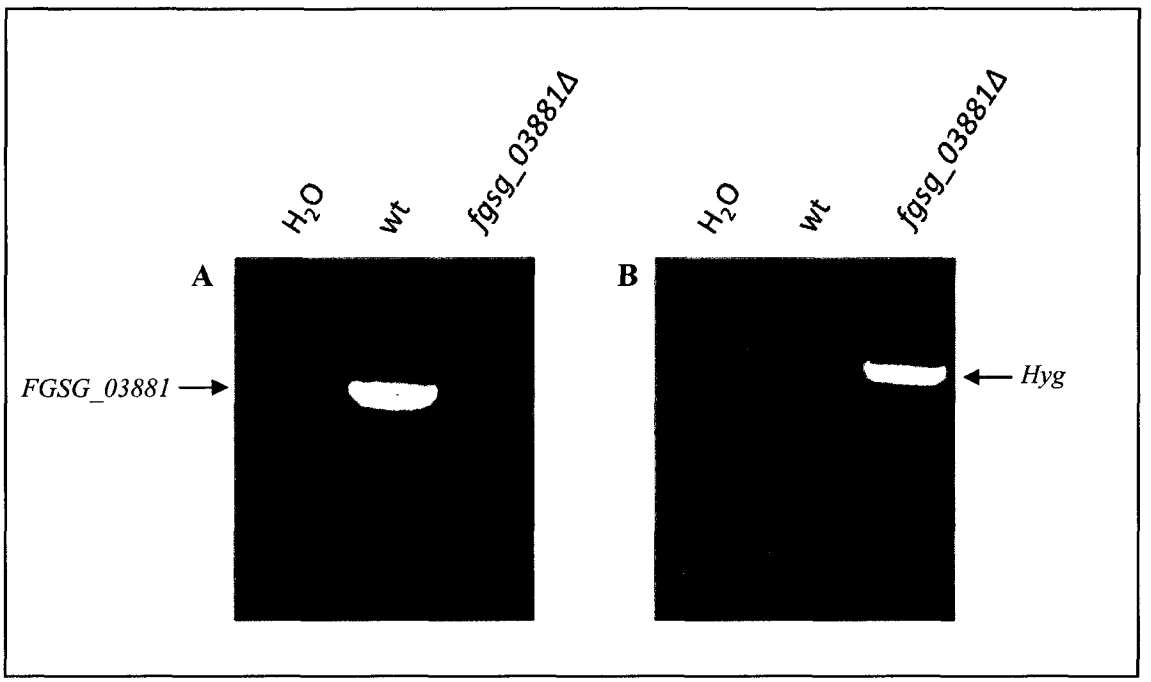
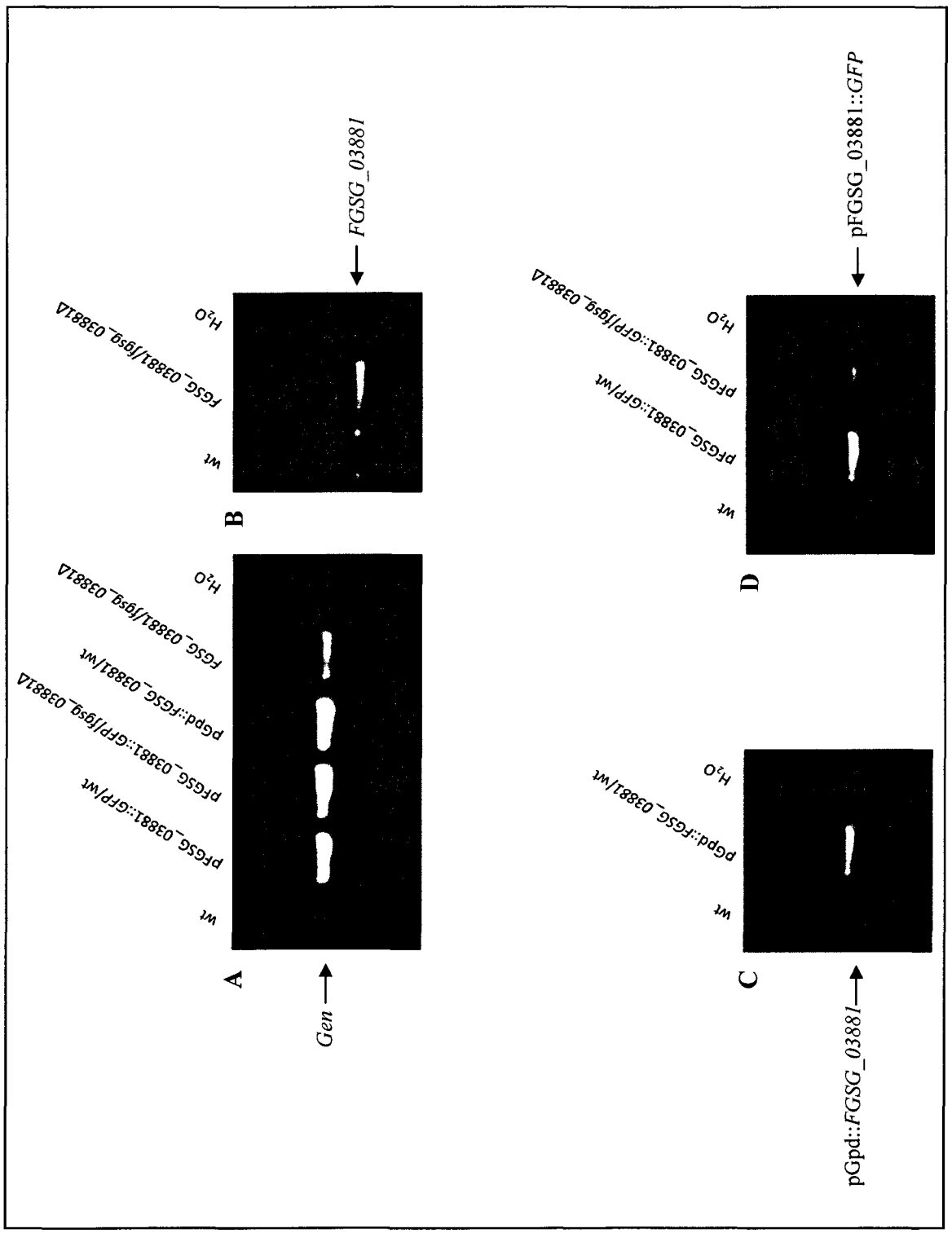


Figure 6: Verification of transformation and integration of the *FGSG_03881* disruption construct into wild-type protoplasts by PCR amplification. **(A)**. *FGSG_03881* was amplified using *FGSG_03881* F / *FGSG_03881* R primers in wild-type (wt), but not the mutant strain. **(B)**. The hygromycin resistance gene (*Hyg*) was amplified using *Hyg* F / *Hyg* R in the mutant strain and failed to amplify in wild-type (wt). The primer sequences can be found in Appendix Table 1.


A
Gen
wt
PFGSG_03881::GFP/wt
PFGSG_03881::GFP/jgsq_03881Δ
PGpd::FGSG_03881/wt
FGSG_03881/jgsq_03881Δ
H2O
B
FGSG_03881
wt
FGSG_03881/jgsq_03881Δ
H2O
C
pGpd::FGSG_03881
wt
PGpd::FGSG_03881/wt
H2O
D
pFGSG_03881::GFP
wt
PFGSG_03881::GFP/wt
PFGSG_03881::GFP/jgsq_03881Δ
H2O

3.3. The fgsg_03881Δ Strain is More Pathogenic than Wild-type at Early Infection Stages in a Susceptible Host

To assess the role of *FGSG_03881* in virulence, spores from the wild-type, *fgsg_03881Δ*, and *FGSG_03881/fgsg_03881Δ* strains were point inoculated into spikelets of 'Roblin' and 'Sumai 3' at mid-anthesis and scored for infection. In 'Roblin', the amount of infection was minimal during the first seven days; however, after the seventh day the infection spread rapidly and colonized the majority of the heads in a short period of time. Overall, the mean number of spikelets infected in 'Roblin' was higher for *fgsg_03881Δ* in comparison to wild-type or *FGSG_03881/fgsg_03881Δ* at 7, 10, and 14 dpi (Table 1). The time point of maximum infection difference was at 14 dpi, where the mean number of infected spikelets for wild-type was 9.5 ± 7.7 and *fgsg_03881Δ* was 12.4 ±7.2 (p-value of 0.04). However, there was no significant difference between the complemented strain, *FGSG_03881/fgsg_03881Δ* (10.7 ± 8.1), and the wild-type at this time point (p-value of 0.25). After 21 dpi, we did not observe any differences between the wild-type and the *fgsg_03881Δ* strains. The p-value comparing *fgsg_03881Δ* to wild-type was 0.30 and the p-value comparing *FGSG_*03881*/fgsg_03881Δ* to wild-type was 0.41 (Table 1). None of the strains caused substantial infection in the more resistant wheat variety 'Sumai 3'. The mean number of infected spikelets was less than two for wild-type, *fgsg_03881Δ*, and *FGSG_03881/ fgsg_03881Δ* after 21 dpi (Table 2).

To quantify the accumulation of DON *in vivo*, an independent point inoculation experiment was performed on 'Roblin' plants and tissues were harvested at 10 dpi. There were observable differences in the amount of DON accumulation between the wild-type

Table 1: Pathology tests of *F. graminearum* in the susceptible variety of wheat 'Roblin'. The wild-type, *fgsg_03881Δ*, and *FGSG_03881/fgsg_03881Δ* strains were point inoculated into the susceptible variety of wheat 'Roblin' and the mean number of infected spikelets was scored after 7, 10, 14, and 21 dpi.

Time / Sample	7 Days		10 Days		14 Days		21 Days	
	Infected Spikelets	p-value*	Infected Spikelets	p-value*	Infected Spikelets	p-value*	Infected Spikelets	p-value*
wild-type (n = 41)**	2 3 ± 3 7	-	6 1 ± 6 0	-	9 5 ± 7 7	-	12 8 ± 6 9	-
fgsg_03881Δ (n = 42)**	3 4 ± 4 2	**0.10**	8 3 ± 6 0	**0.08**	12 4 ± 7 2	**0.04**	13 6 ± 6 6	0 30
FGSG_03881/fgsg_03881Δ (n = 42)**	2 7 ± 3 9	0 34	6 7 ±6 1	0 40	10 7 ± 8 1	0 25	12 5 ± 7 7	0 41

* One-tailed p-value from an unpaired student's t-test in comparison to wild-type. See Appendix Tables 3 to 10 for more details.
** n represents the number of wheat heads inoculated

Table 2: Pathology tests of *F. graminearum* in the resistant variety of wheat 'Sumai 3'. The wild-type, *fgsg_03881Δ*, and *FGSG_03881/fgsg_03881Δ* strains were point inoculated into the susceptible variety of wheat 'Sumai 3' and the mean number of infected spikelets was scored after 7, 14, and 21 dpi.

Time / Sample	7 Days		14 Days		21 Days	
	Infected Spikelets	p-value*	Infected Spikelets	p-value*	Infected Spikelets	p-value*
wild-type (n = 30)**	0 53 ± 0 51	-	0 87 ± 1 1	-	1 2 ± 2 1	-
fgsg_03881Δ (n = 32)**	0 63 ± 0 50	0 24	0 72 ± 0 58	0 25	1 6 ± 2 4	0 23
FGSG_03881/fgsg_03881Δ (n = 31)**	0 61 ± 0 50	0 27	0 97 ± 1 0	0 36	1 4 ± 2 0	0 36

* One-tailed p-value from an unpaired student's t-test in comparison to wild-type. See Appendix Tables 11 to 16 for more details.
** n represents the number of wheat heads inoculated

and mutant strains. Mycotoxin analysis performed on wild-type infected tissues had DON levels of 74 ± 37 ppm and *fgsg_03881Δ* had values of 125 ± 55 ppm (*p*-value of 0.013, Appendix Table 17).

3.4. Expression Analyses of FGSG_03881

The promoter activity and the mRNA levels of *FGSG_03881* were assessed to determine if they correlate with known virulence genes/pathways. The promoter activity was assessed by GFP fluorescence from pFGSG_03881::*GFP*/wt and pFGSG_03881::*GFP/fgsg_03881Δ* grown in various culture conditions. We employed a high-throughput 96-well plate reader method to assess the role of various compounds that could potentially modulate *FGSG_03881* expression. In addition to the GFP fluorescence, we simultaneously monitored the growth of the fungus. This allowed us to normalize the growth and expression for the given culture condition. The RT-qPCR analyses were used to validate the expression of *FGSG_03881* under these conditions.

3.4.1. FGSG_03881 is Induced in Nitrogen Stress Conditions

Promoter::*GFP* analyses were performed to assess *FGSG_03881* expression. The promoter activity of *FGSG_03881*, as measured by GFP fluorescence, decreased in the presence of preferred nitrogen sources such as ammonium chloride and increased in the presence of non-preferred nitrogen sources such as sodium nitrate (Figure 8). To a lesser extent, promoter activity was also greater in sucrose, a non-preferred carbon source, in comparison to glucose, a preferred carbon source. This trend appears to be similar in both the wild-type and the *fgsg_03881Δ* strains (Figure 8). These observations were

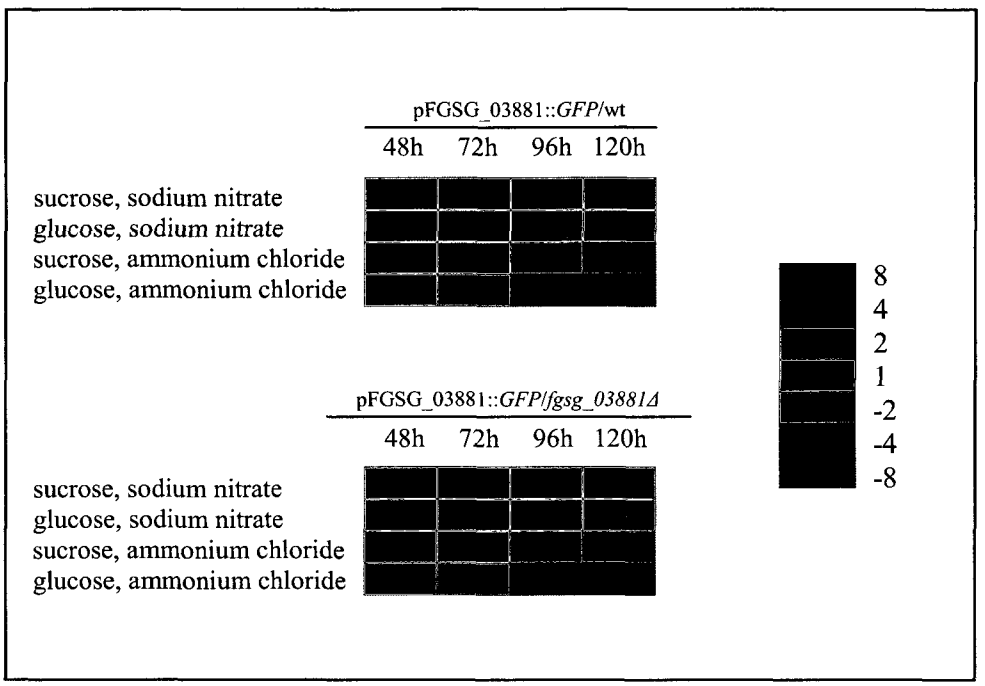


Figure 8: Promoter activity for *FGSG_03881* in pFGSG_03881::*GFP*/wt and pFGSG_03881::*GFP/fgsg_03881Δ* strains grown in minimal media supplemented with different compounds as sole carbon and nitrogen sources. 200μL cultures were grown in triplicate in a 96 well plate. Increased expression of *FGSG_03881* is represented by red and decreased expression is represented in green. Sucrose with sodium nitrate was used as the control for relative comparison. The equation used was: $T_{GFP\ (predicted)} = 0.1 \times 10^{-13}$ $T_{growth}^4 + 1.1 \times 10^{-8}\ T_{growth}^3 - 0.00021\ T_{growth}^2 + 1.585\ T_{growth} - 17.785$.

confirmed by the RT-qPCR analyses, which showed that mRNA levels of *FGSG_03881* is low in the presence of complex nitrogen sources (GYEP medium), increased in conditions containing a sole nitrogen source, and was greatest in conditions where nitrogen was totally absent (Figure 9). In addition, expression was also lower in preferred sources such as ammonium chloride in comparison to non-preferred sources such as sodium nitrate (Figure 9).

To further understand the role of preferred and non-preferred sources of nitrogen which activate *FGSG_03881*, we tested a number of different compounds as sole nitrogen sources in the same plate reader assay. The results indicated that there are three distinct groupings of compounds that affected promoter activity (Figure 10). The compounds that have the lowest activity include preferred nitrogen sources such as glutamine, asparagine, and most ammonium containing compounds. Compounds that exhibited intermediate and high promoter activity are not generally considered preferred sources of nitrogen; these included sodium nitrate, leucine, and lysine. The promoter activity was greatest in cysteine and ammonium phosphate dibasic, which also appeared to affect fungal growth (Figure 10). These observations were validated by multiple reference points. All of the models appear to come to the same conclusion: promoter activity is low in preferred nitrogen sources and is high in non-preferred nitrogen sources.

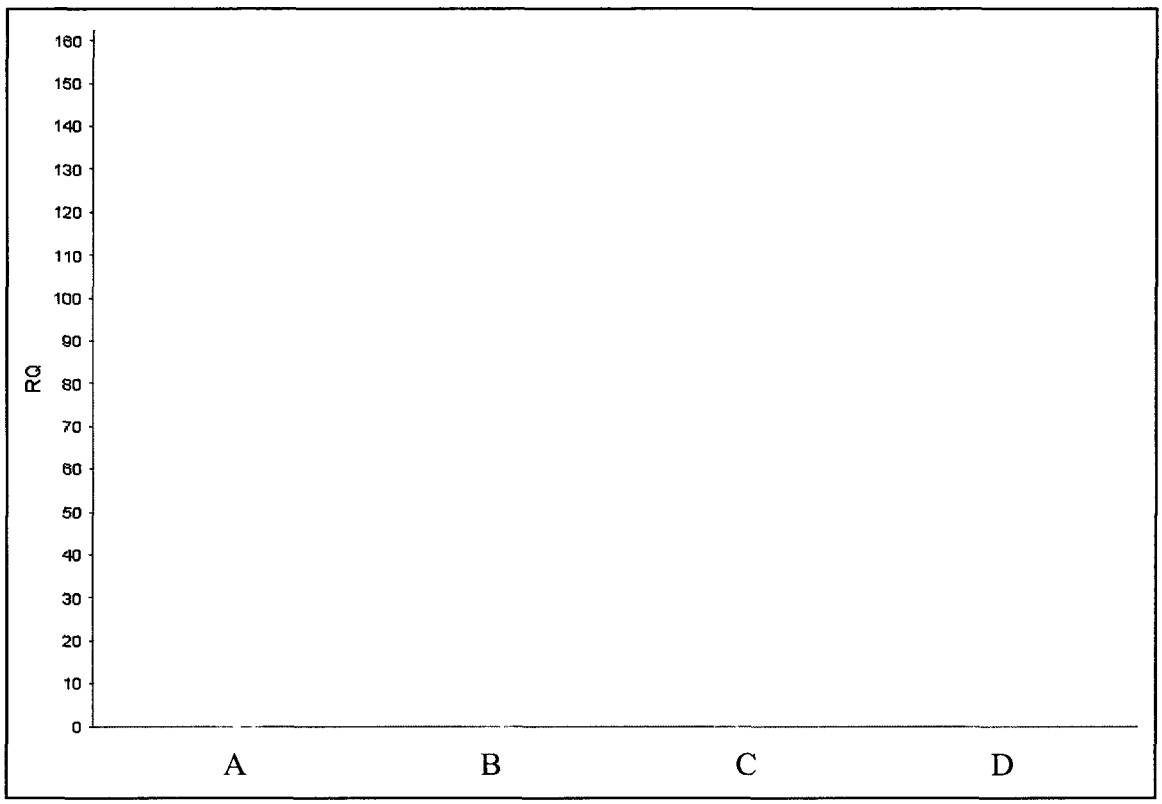
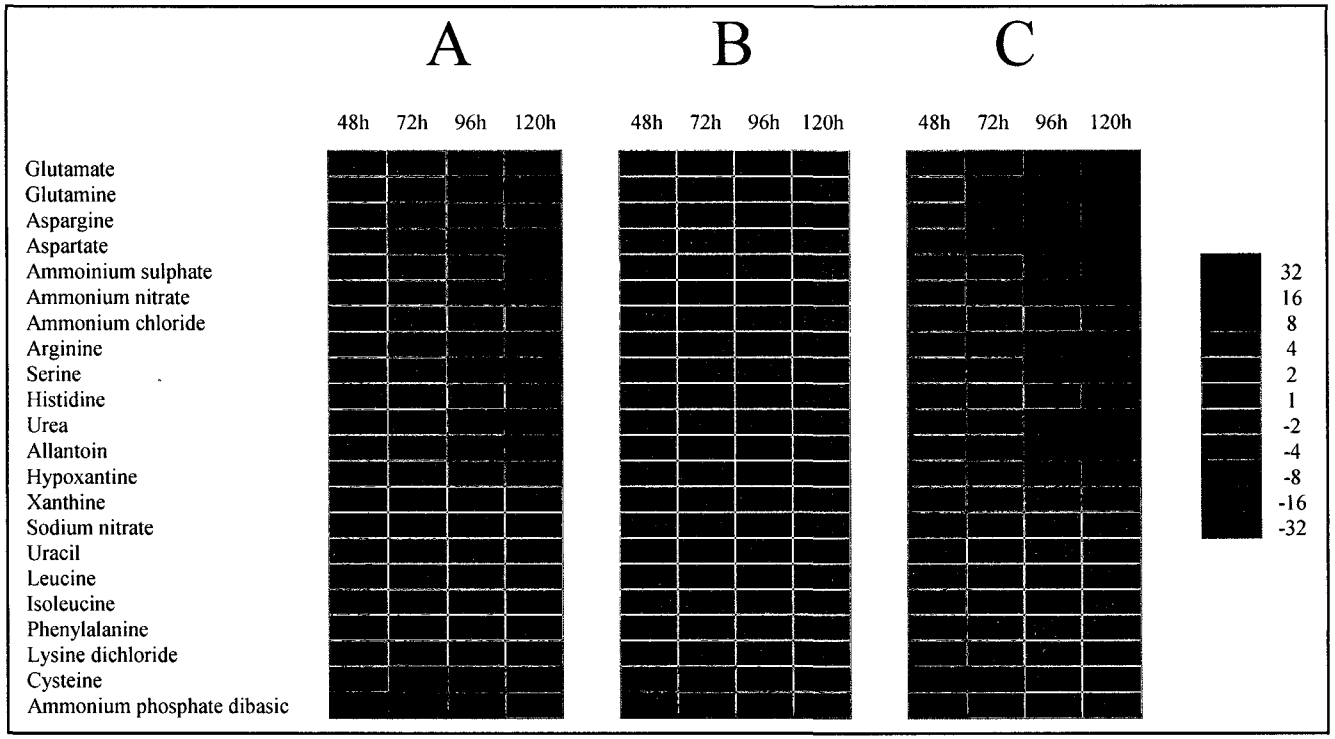


Figure 9: RT-qPCR analyses of the gene expression of *FGSG_03881* in wild-type grown in various nitrogen rich and nitrogen poor conditions. Cultures were grown in GYEP for 24h and transferred to GYEP (A) or minimal media with, ammonium chloride (B), sodium nitrate (C), or water (D) as sole nitrogen sources for 4h. Relative quantification (RQ) of gene expression was determined by RT-qPCR performed in triplicate using primers found in Appendix Table 2. Errors reflect a confidence interval of 0.95.


A
B
C
48h 72h 96h 120h
48h 72h 96h 120h
48h 72h 96h 120h
Glutamate
Glutamine
Aspargine
Aspartate
Ammoinium sulphate
Ammonium nitrate
Ammonium chloride
Arginine
Serine
Histidine
Urea
Allantoin
Hypoxantine
Xanthine
Sodium nitrate
Uracil
Leucine
Isoleucine
Phenylalanine
Lysine dichloride
Cysteine
Ammonium phosphate dibasic
32
16
8
4
2
1
-2
-4
-8
-16
-32

3.4.2. FGSG_03881 is Co-Regulated with Tri6 and Tri10, but Expression is Independent of DON Production

The plate reader assay established that expression of *FGSG_03881* is induced in the presence of ammonium phosphate dibasic, which is also the sole nitrogen source used during 15-acetyl-DON biosynthesis (Figure 10). We wanted to explore the relationship between *FGSG_03881* and the transcription regulators *Tri6* and *Tri10*. As the RT-qPCR analyses indicated, similar to *Tri6* and *Tri10*, the expression of *FGSG_03881* was induced in 15-acetyl-DON inducing conditions (Figure 11). This suggested a positive association between *FGSG_03881* and production of DON, a known virulence factor. Since we observed similar expression of *FGSG_03881* in the *tri6Δ* strain, we suggest that regulation of *FGSG_03881* is independent of *Tri6* (Figures 12). We also observed increased expression of *FGSG_03881* under 15-acetyl-DON inducing conditions in the complemented strain, *FGSG_03881/fgsg_03881Δ*. It is important to note that *FGSG_03881/fgsg_03881Δ* had increased expression relative to wild-type in both GYEP and 15-acetyl-DON inducing media (Figure 13).

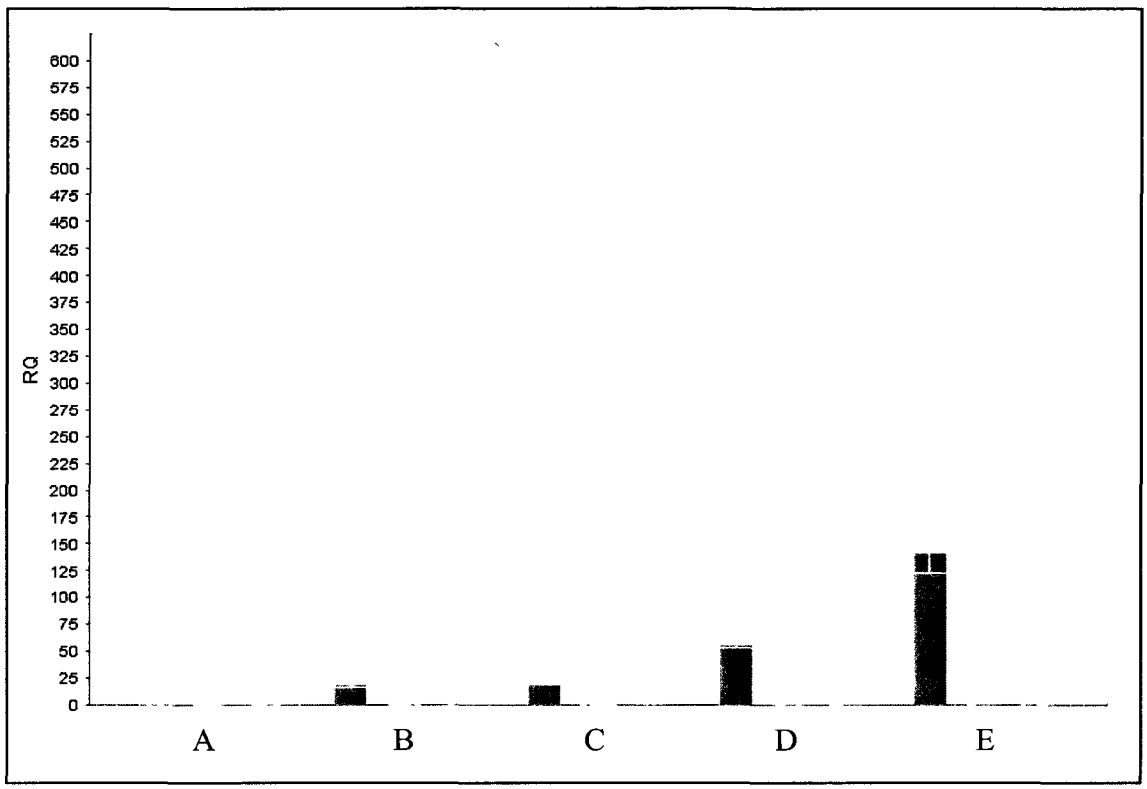


Figure 11: RT-qPCR results describing the expression levels of *FGSG_03881*, *Tri6*, and *Tri10* under different nutrient conditions. Cultures were grown for 24h in GYEP media and then transferred to fresh GYEP medium (A), minimal medium containing ammonium chloride (B), 15-acetyl-DON inducing medium (C), minimal medium containing sodium nitrate (D) and minimal medium containing no nitrogen (E) and grown for an additional 4h. The mRNA levels of *FGSG_03881* (red), *Tri6* (purple) and *Tri10* (beige) were then quantified from each condition. Relative quantification (RQ) of gene expression was determined by RT-qPCR performed in triplicate using primers found in Appendix Table 2. Errors reflect a confidence interval of 0.95.

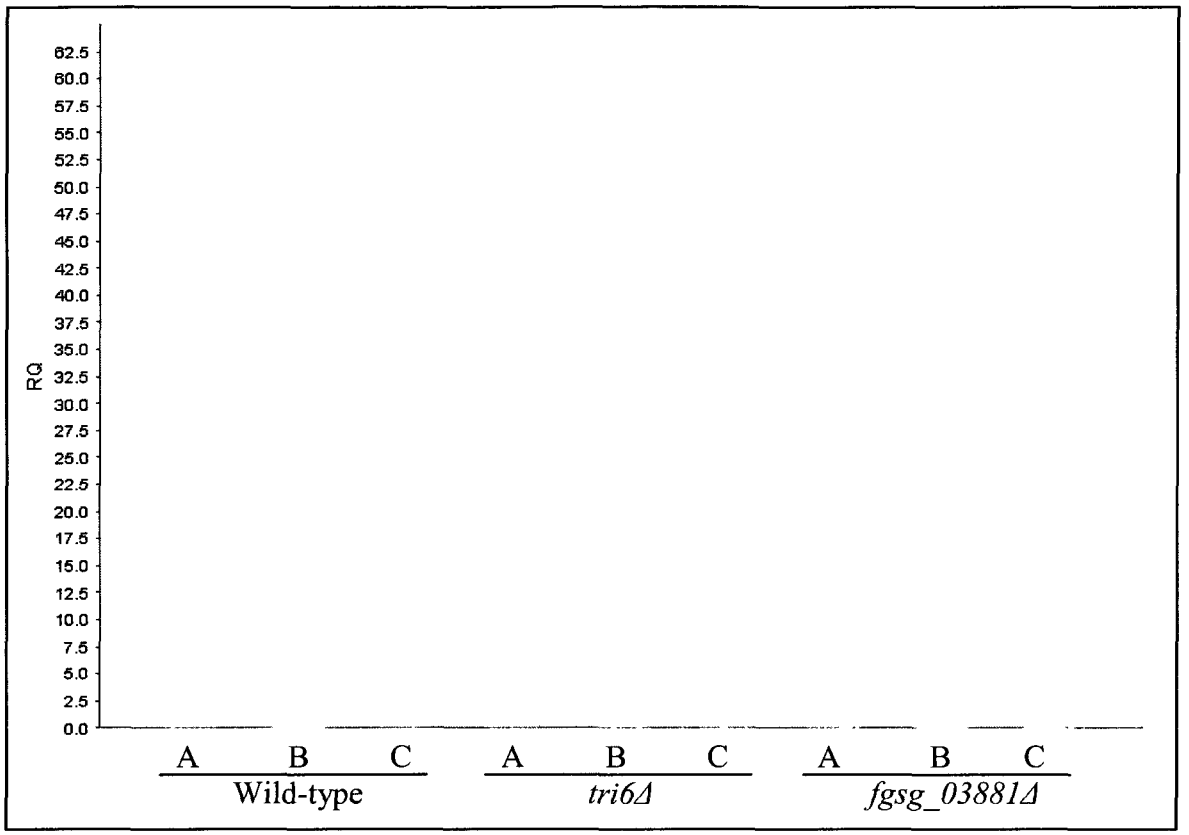


Figure 12: RT-qPCR results describing the expression levels of *FGSG_03881* in *tri6*Δ. The mRNA levels of *FGSG_03881* in wild-type, *tri6Δ*, and *fgsg_03881Δ* were quantified from mycelia grown in nutrient rich medium (GYEP) for 18h (A) and 24h (B). Expression was also quantified from mycelia grown in GYEP medium for 24h and transferred to 15-acetyl-DON inducing medium for 4h (C). Relative quantification (RQ) of gene expression was determined by RT-qPCR performed in triplicate using primers found in Appendix Table 2. Errors reflect a confidence interval of 0.95.

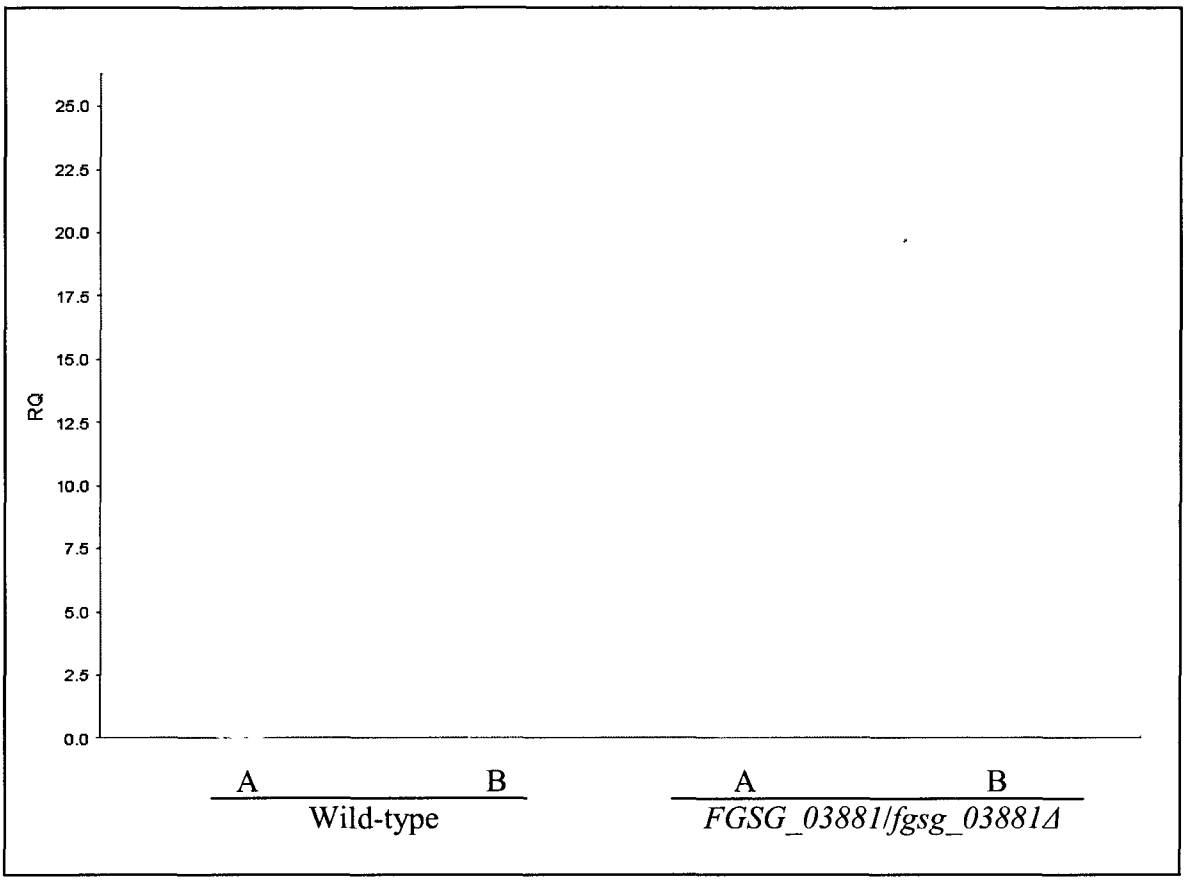


Figure 13: RT-qPCR results describing the expression levels of *FGSG_03881* in *FGSG_03881/fgsg_03881Δ*. The mRNA levels of *FGSG_03881* in wild-type and *FGSG_03881/fgsg_03881Δ* were quantified from mycelia grown in GYEP medium for 24h (A) and mycelia transferred into 15-acetyl-DON inducing medium for 4h after 24h of growth in GYEP medium (B). Relative quantification (RQ) of gene expression was determined by RT-qPCR performed in triplicate using primers found in Appendix Table 2. Errors reflect a confidence interval of 0.95.

3.5. Global Gene Expression Patterns of fgsg_03881Δ and Wild-type Under Nitrogen-Rich and 15-acetyl-DON Inducing Conditions

The increased expression of *FGSG_03881* in 15-acetyl-DON medium and decreased expression in GYEP medium suggested that these would be optimal conditions to analyze the function of *FGSG_03881*. Global gene expression patterns were performed using DNA microarrays to identify genes differentially expressed in *fgsg_03881Δ* in comparison to the wild-type strain. These genes could potentially contribute to the virulence phenotype observed in *fgsg_03881Δ*. A total of 14 genes were up-regulated and 184 genes were down-regulated in *fgsg_03881Δ* grown in GYEP compared to wild-type, while 65 genes are up-regulated and 483 genes are down regulated in 15-acetyl-DON inducing media. The top ten differentially expressed genes from each experiment are listed in Tables 3 – 6; a complete list of genes differentially expressed by two fold or greater can be found in accompanying electronic materials. *FGSG_03881* is not expressed in either the wild-type or the *fgsg_03881Δ* strain in GYEP medium. The gene is expressed in 15-acetyl-DON inducing conditions in the wild-type strain, but there is no detectable expression in the *fgsg_03881Δ* strain.

The differentially expressed genes were organized into functional categories according to their Munich Information Center for Protein Sequences (MIPS) characterization (Figure 14). In total, 39.8% of differentially expressed genes in GYEP and 17.8% of differentially expressed genes in 15-acetyl-DON inducing conditions were involved in metabolism. Among the genes differentially expressed in GYEP media, seven out of 14 (50%) of the up-regulated genes were identified by MIPS to be involved in

Table 3: The top ten genes up-regulated in *fgsg_03881Δ* relative to wild-type grown in GYEP medium

Gene Identifier	Description	Fold Change
FGSG_01561	probable xanthine dehydrogenase	4.693508
FGSG_03520	conserved hypothetical protein	3.781172
FGSG_05323	related to guanine deaminase	3.662512
FGSG_00409	related to cytidine deaminase	2.773421
FGSG_04155	related to uracil phosphoribosyltransferase	2.771499
FGSG_11618	conserved hypothetical protein	2.743625
FGSG_01567	probable AAH1 - adenosine deaminase	2.734607
FGSG_12865	probable molybdenum cofactor sulfurase HxB protein	2.543532
FGSG_11629	conserved hypothetical protein	2.308571
FGSG_02692	conserved hypothetical protein	2.284561

Table 4: The top ten genes down-regulated *fgsg_03881Δ* relative to wild-type grown in GYEP medium

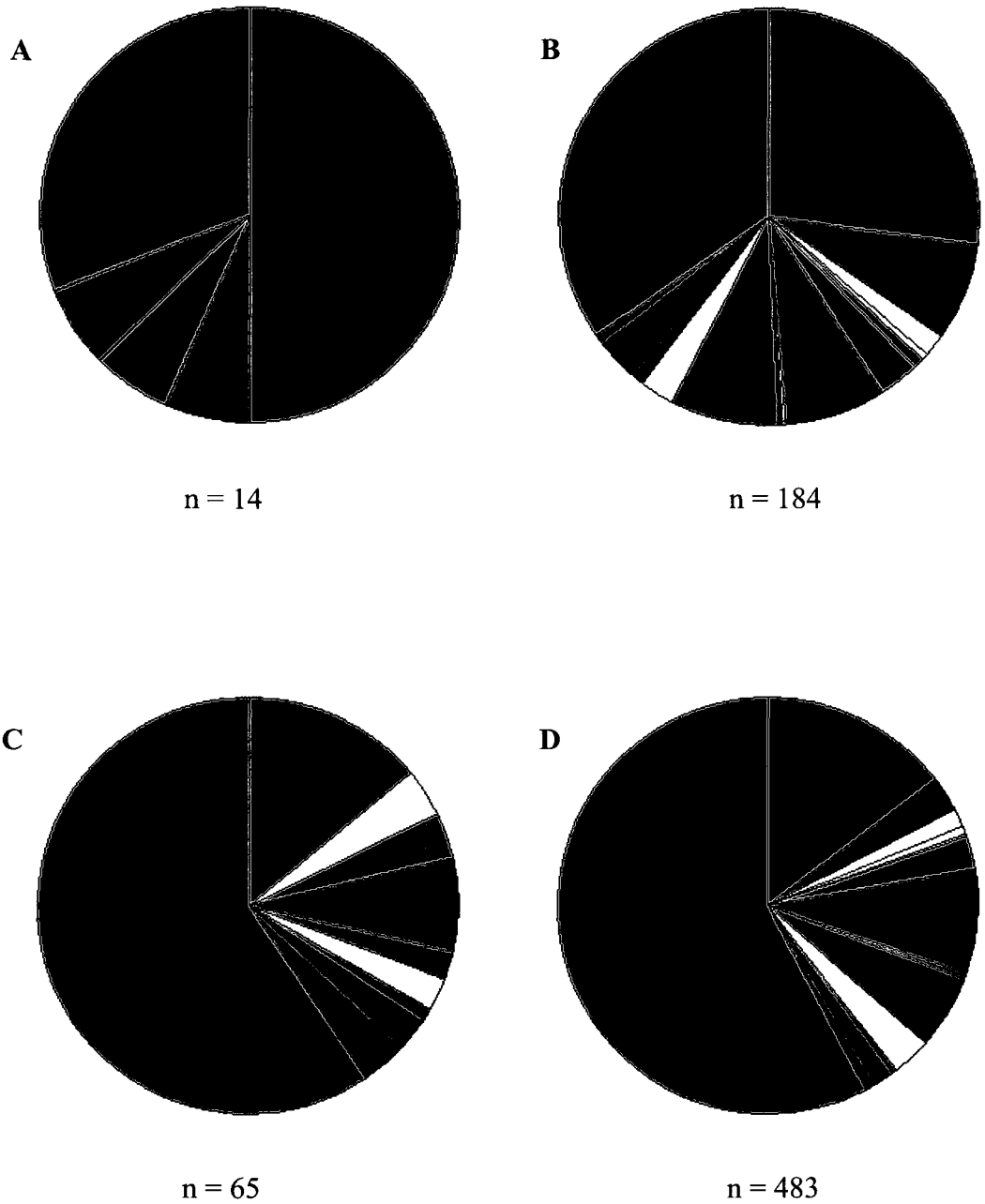
Gene Identifier	Description	Fold Change
FGSG_05828	conserved hypothetical protein	14.6298
FGSG_03882	probable ABC1 transport protein	10.43
FGSG_12565	probable alcohol dehydrogenase I - ADH1	10.3756
FGSG_02291	related to ADH5 - alcohol dehydrogenase V	8.27781
FGSG_03335	conserved hypothetical protein	8.13467
FGSG_08402	probable nitrite reductase	6.7478
FGSG_01417	probable O-acetylhomoserine (thiol)-lyase	6.6919
FGSG_03162	related to formate transport protein	6.55229
FGSG_03422	conserved hypothetical protein	6.28974
FGSG_07697	hypothetical protein	6.17526

Table 5: The top ten genes up-regulated in *fgsg_03881Δ* relative to wild-type grown in 15-acetyl-DON inducing medium

Gene Identifier	Description	Fold Change
FGSG_07836	related to dopamine-responsive protein	17.81783
FGSG_05724	conserved hypothetical protein	13.43493
FGSG_07601	related to integral membrane protein	12.03363
FGSG_08580	conserved hypothetical protein	8.245841
FGSG_02377	conserved hypothetical protein	6.063567
FGSG_07598	conserved hypothetical protein	5.504075
FGSG_11385	related to integral membrane protein PTH11	5.416084
FGSG_11343	related to integral membrane protein	5.375877
FGSG_09098	related to mannosyltransferase	5.30064
FGSG_07597	conserved hypothetical protein	4.722061

Table 6: The top ten genes down-regulated in *fgsg_03881Δ* relative to wild-type grown in 15-acetyl-DON inducing medium

Gene Identifier	Description	Fold Change
FGSG_08212	hypothetical protein	146.745
FGSG_11577	conserved hypothetical protein	69.9499
FGSG_03936	probable UGA2 - succinate semialdehyde dehydrogenase	69.7321
FGSG_09351	related to pisatin demethylase / cytochrome P450 monooxygenase	62.1421
FGSG_12878	related to non-ribosomal peptide synthetase	50.5364
FGSG_12299	related to integral membrane protein	50.3615
FGSG_13202	conserved hypothetical protein	47.6422
FGSG_11578	related to acetylxylan esterase	46.554
FGSG_12150	related to pisatin demethylase / cytochrome P450 monooxygenase	41.4414
FGSG_03881	**related to TRI15 - putative transcription factor**	**40.2209**


A
B
n = 14
n = 184
C
D
n = 65
n = 483
Metabolism
Energy
Cell Cycle
Transcription
Protein Synthesis
Protein Fate
Transport
Regulation of Metabolism and Protein Functi
Communication
Defence and Virulence
Interaction with Environment
Cell Fate
Development
Biogenesis of Cellular Components
Differentiation
Unclassified

nucleotide metabolism. 38.5% of the down-regulated genes in GYEP are involved in metabolism and 22 genes (11.9%) are categorized to be involved in amino acid metabolism: five in glutamate, eight in aspartate, and seven in cysteine metabolism. None of the functional categories were over represented in up-regulated genes in 15-acetyl-DON inducing media. Among the down-regulated genes, 21 (4.3%) were involved in secondary metabolism, and 19 (3.9%) were involved in disease, virulence and defense, and 18 (3.7%) were involved in detoxification.

3.6. Secreted Proteins of F. graminearum

In addition to global gene expression analyses, analyses of the secreted proteins under 15-acetyl-DON inducing were also explored as these conditions simulate virulence and are known to induce *FGSG_03381* expression. Proteins secreted under these conditions could therefore participate in virulence and interactions with host plants. The proteins secreted under 15-acetyl-DON inducing conditions were determined for wild-type, *fgsg_03881Δ*, *FGSG_03881/fgsg_03881Δ*, and the non-pathogenic strains *tri6*Δ and *tri10*Δ, which are also unable to produce DON. The data provided targets potentially involved in virulence that were either common or different amongst the strains.

3.6.1. Identification and Characterization of the Secreted Proteins

The secreted proteins were separated by 2-D electrophoresis (Figure 15). There were observable differences in protein intensities, in addition to proteins that were either present (arrows) or absent (circles) from the secreted proteins of the different strains. Although there were notable differences in the secreted proteins of *fgsg_03881*Δ and

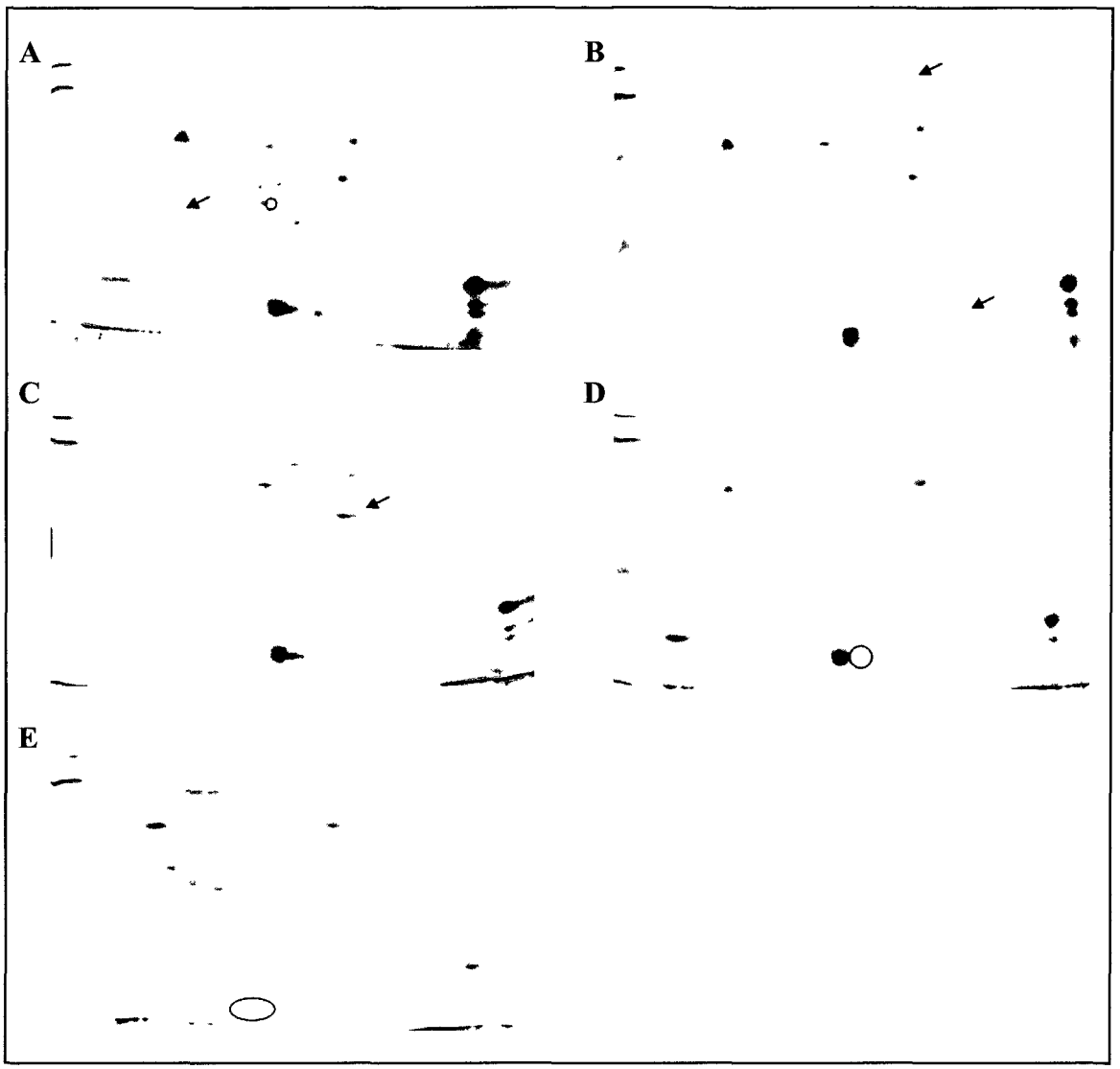


Figure 15: Two-dimensional separation of *F. graminearum* secreted proteins isolated from 15-acetyl-DON inducing medium. 7cm 12% polyacrylamide gels containing 75µg of proteins pH range 3-10 that were secreted by wild-type (A), *fgsg_03881Δ* (B), *FGSG_03881/fgsg_03881Δ* (C), *tri6*Δ (D), and *tri10*Δ (E) grown in 100mL of 15-acetyl-DON inducing medium for 24h are shown. Arrows indicate proteins uniquely identified to the gel and circles represent absent proteins (non-exhaustive).

those of the other strains, the most noticeable difference was that of the secreted proteins in the *tri10*Δ strain in relation to the other strains.

The secreted proteins were also subjected to gel-free mass spectrometry analyses, which identified a total of 162 proteins secreted from all of the strains, 53% of which had secretion signals as their most dominant localization pattern. A complete list of proteins that were identified can be found in the accompanying electronic materials. Of the 162 secreted proteins, 21 were present while 14 were absent in the *fgsg_03881*Δ strain in comparison to wild-type (Tables 7 and 8). Twelve of the 21 present and four of the 14 absent proteins have no known function. Among the proteins that were absent in the *fgsg_03881*Δ strain, two potential CWDE, glucan 1,4-alpha-glucosidase (FGSG_11326) and L-xylulose reductase (FGSG_04826) were identified. Interestingly, only one of the 14 absent proteins (FGSG_02903) was present in the secreted proteins from all of the other strains. Among the 21 proteins that were present only in the *fgsg_03881*Δ strain, five proteins were also absent from all other strains, a CUE domain-containing protein (FGSG_00592), cystathionine gamma-lyase (FGSG_01177), 40S ribosomal protein S2 (FGSG_01509), and two uncharacterized proteins (FGSG_04094 and FGSG_10206). The non-pathogenic strains *tri6*Δ and *tri10*Δ also had noteworthy differences in their secreted proteins. An ATP-citrate synthase subunit (FGSG_06039) was absent only from *tri6*Δ and had good coverage in all other strains and a superoxide dismutase (FGSG_08721) was absent from both the *tri6*Δ and *tri10*Δ strains.

Table 7: Proteins secreted by *fgsg_03881Δ* but not by wild-type after 24h of growth in 100mL of 15-acetyl DON inducing medium.

Gene Identifier	Description	Signal P Score
FGSG_00523	hypothetical protein	0.984
FGSG_00592	**CUE domain-containing protein**	**0.000**
FGSG_00642	hypothetical protein	0.999
FGSG_01080	ATP synthase subunit alpha	0.001
FGSG_01177	**Cystathionine gamma-lyase**	**0.006**
FGSG_01179	hypothetical protein	0.998
FGSG_01509	**40S ribosomal protein S2**	**0.000**
FGSG_02503	hypothetical protein	0.000
FGSG_04094	**hypothetical protein**	**0.998**
FGSG_05175	Uncharacterized protein	0.828
FGSG_05615	hypothetical protein	0.002
FGSG_05633	hypothetical protein	0.999
FGSG_05803	hypothetical protein	0.998
FGSG_06154	Heat shock 70	0.403
FGSG_07291	40S ribosomal protein S22	0.000
FGSG_08238	hypothetical protein	1.000
FGSG_09471	Heat shock 70	0.999
FGSG_09866	60S ribosomal protein	0.000
FGSG_10206	**predicted protein**	**0.996**
FGSG_10235	Allergen Asp f 7	1.000
FGSG_10784	predicted protein	0.999

Note: Bolded items are also not secreted by *FGSG_03881/fgsg_03881Δ*, *tri6Δ*, or *tri10Δ*

Table 8: Proteins secreted by wild-type but not by *fgsg_03881Δ* after 24h of growth in 100mL of 15-acetyl DON inducing medium.

Gene Identifier	Description	Signal P Score
FGSG_02273	Aldehyde dehydrogenase	0.000
FGSG_02903	**hypothetical protein**	**0.997**
FGSG_03894	hypothetical protein	0.999
FGSG_04022	Putative amidase	0.999
FGSG_04826	L-xylulose reductase	0.001
FGSG_05433	40S ribosomal protein S3	0.000
FGSG_06130	hypothetical protein	1.000
FGSG_06932	Adenosine kinase	0.030
FGSG_07401	Elongation factor 1-gamma 2	0.000
FGSG_07468	D-3-phosphoglycerate dehydrogenase 2	0.000
FGSG_07907	Uncharacterized CDP-alcohol phosphatidyltransferase	0.000
FGSG_09119	hypothetical protein	0.667
FGSG_11326	Glucoamylase	0.985
FGSG_11496	Expansin-like protein 5	0.999

Note: Bolded items are also secreted by *FGSG_03881/fgsg_03881Δ*, *tri6Δ*, and *tri10Δ*

3.6.2. Callose Deposition was Observed in the Wheat Leaves Infiltrated with Proteins

Callose deposition is a plant defense response to pathogen exposure. Infiltration of secreted proteins into wheat leaves and staining for callose deposition were performed to assess the possibility that these proteins may induce a defense response in 'Roblin'. As the results showed, infiltration with Flg22 into the 'Roblin' leaves caused observable deposition of callose, compared to the mock control (Figure 16). The leaves infiltrated with secreted proteins generated less of a response than Flg22, but some depositions were observed.

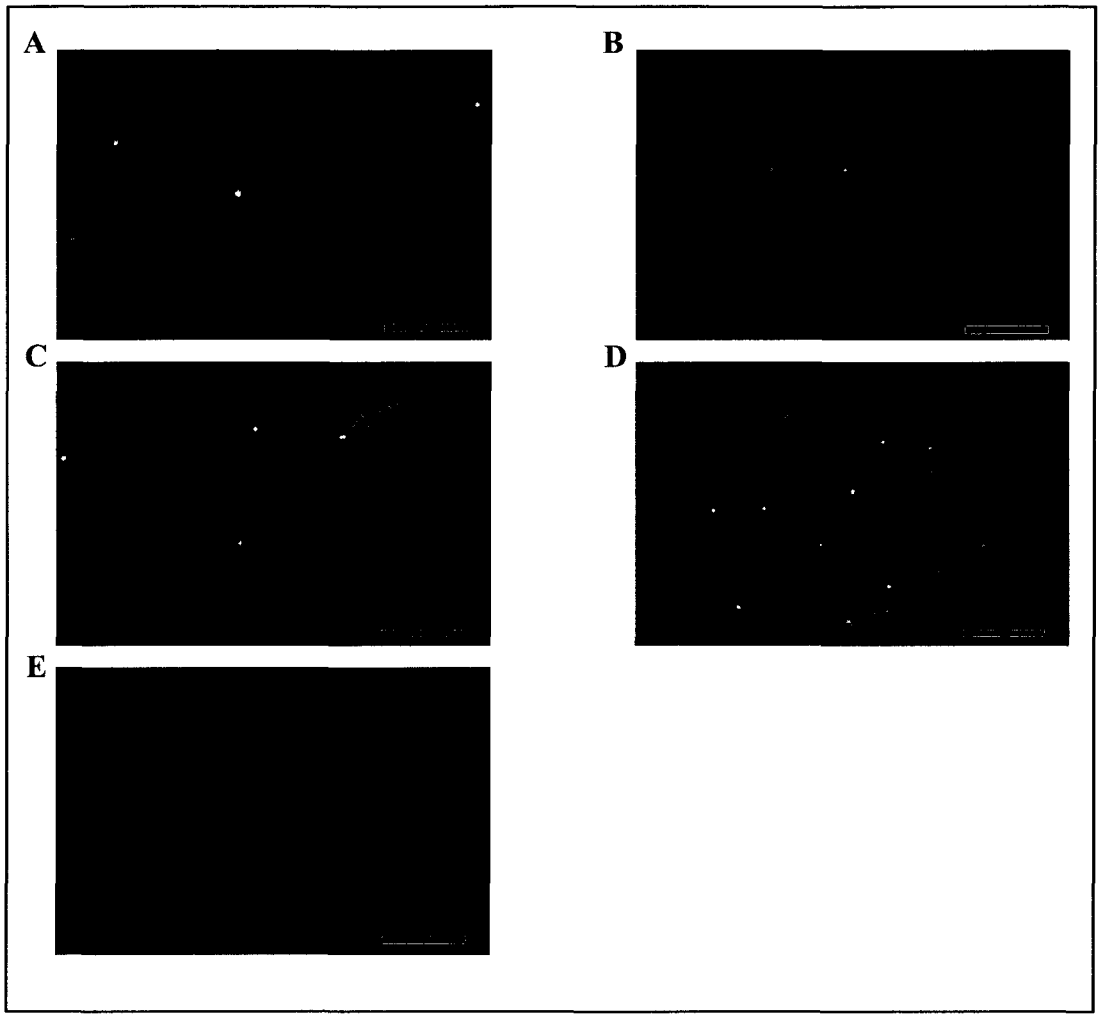


Figure 16: Callose staining of 'Roblin' leaves infiltrated with the Flg22 and proteins secreted from *F. graminearum* grown in 15-acetyl-DON inducing medium. The secreted proteins from wild-type (A), *fgsg_03881Δ* (B), and *FGSG_03881/fgsg_03881Δ* (C) with Flg22 (D) and water (E) infiltrated as controls were viewed using a 365nm excitation filter and a Ziess AxioPlan II Imager. Examples of positively stained callose are indicated by red arrows. The bar in the bottom right corner of each image is corrected to 200μm to indicate scale.

3.7. The fgsg_03881Δ Strain has Similar Growth, Development, and 15-acetyl-DON Production as Wild-type

Wild-type and *fgsg_03881Δ* were assessed for their ability to grow in solid and liquid media, produce reactive oxygen species and hydrogen peroxide, and synthesize 15-acetyl-DON. Both the wild-type and *fgsg_03881Δ* strains had similar growth patterns on plates and in liquid media with different nutrients (Figures 17 and 18). The wild-type, *fgsg_03881Δ*, and *FGSG_03881/fgsg_03881Δ* strains were also able to effectively form conidia in CMC medium. There were no observable differences between the cultures for the production of superoxides, as measured by NBT staining, or for the production of hydrogen peroxide, as measured by DAB staining. Both of the strains produced superoxides, while there was little to no staining by DAB (Figure 19). The HPLC analyses determined that neither strain was able to produce 15-acetyl-DON while grown in GYEP medium; however, both the wild-type and *fgsg_03881Δ* strains were able to produce the toxin with the same capacity after grown in toxin inducing conditions in both flasks (112 Au·min and 80 Au·min, respectively) and 6 well plates (563 Au·min and 449Au·min, respectively). As described in Section 3.3, both wild-type and *fgsg_03881Δ* produce DON during infection *in planta*.

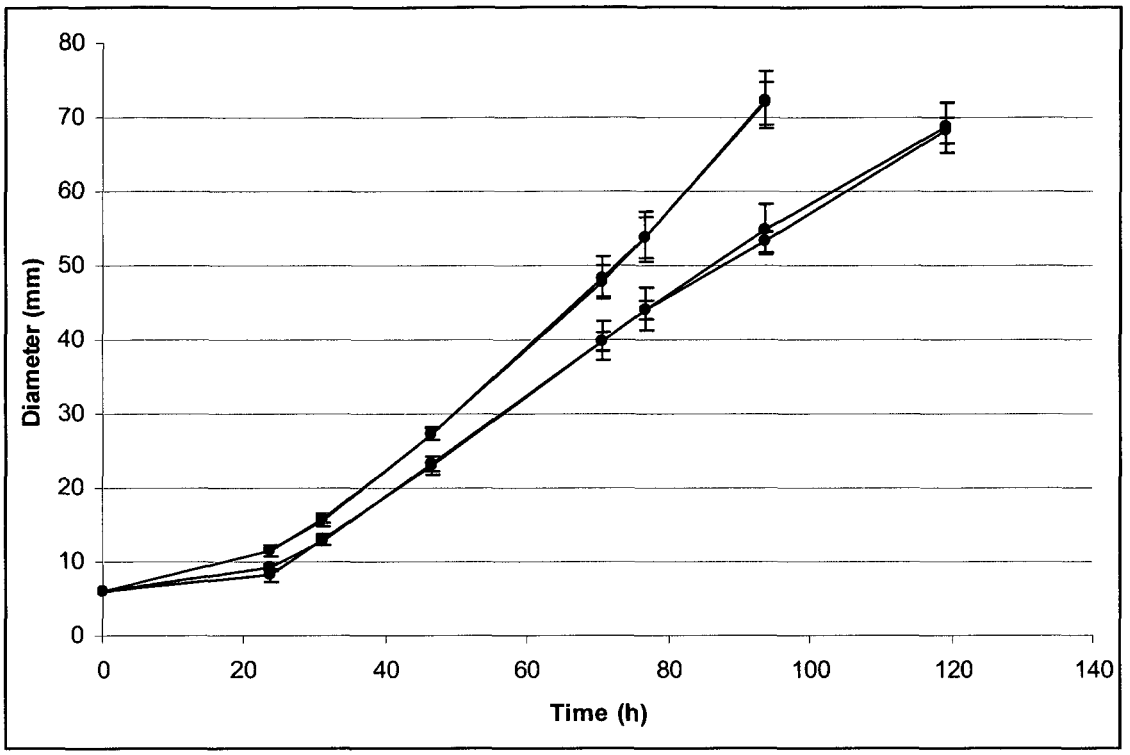


Figure 17: Radial growth measurements of wild-type and *fgsg_03881Δ* grown on solid media. Media was inoculated with approximately 10,000 conidia and grown under nutrient rich conditions (PDA) (blue and black respectively) and in nutrient limiting conditions (SNA) (green and red respectively).

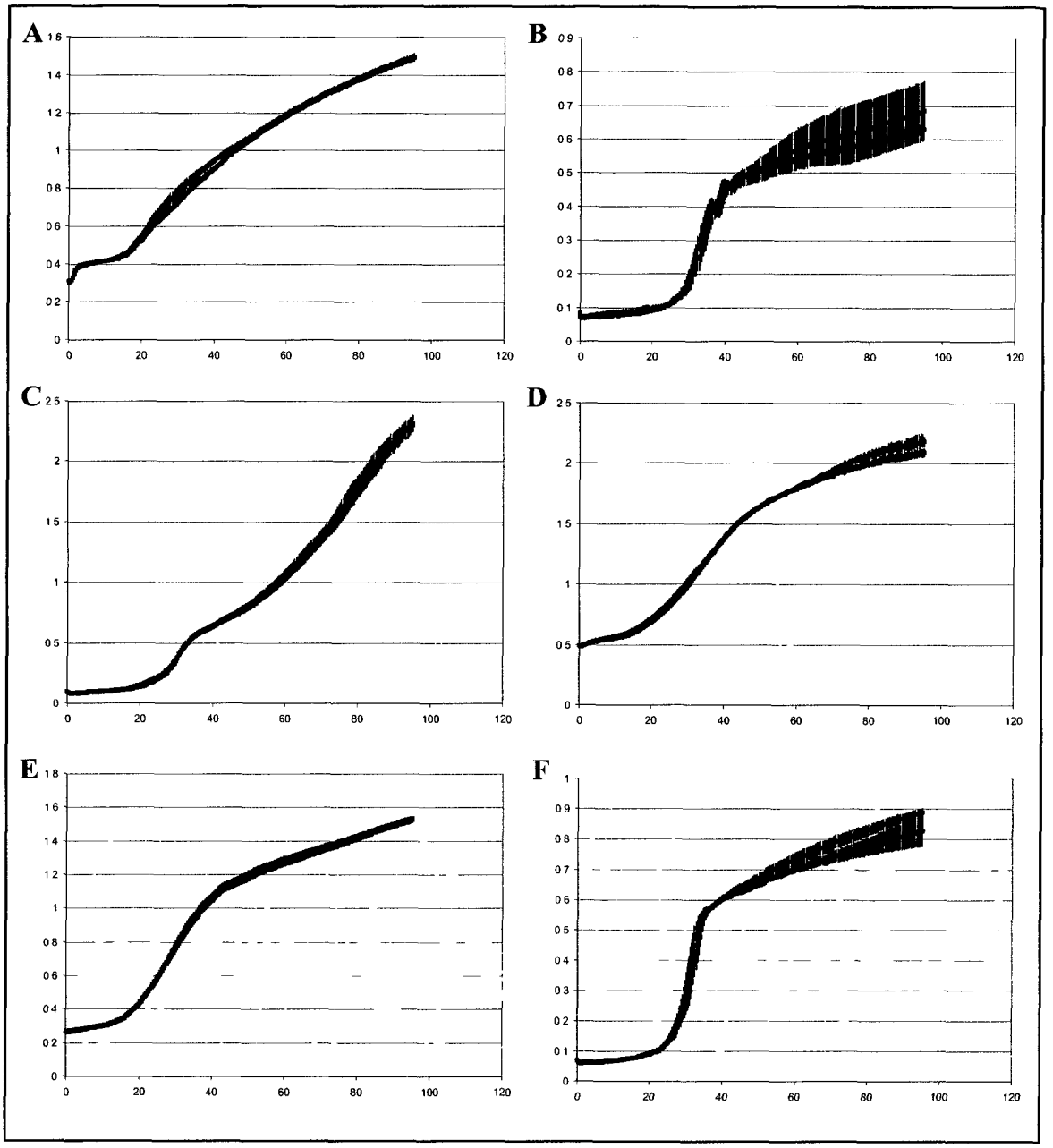


Figure 18: Growth of wild-type and *fgsg_03881Δ* in liquid media. Growth (A-620) was measured over time (hours) for wild-type (blue) and *fgsg_03881Δ* (red) in CMC medium (A), 15-acetyl-DON inducing medium (B), minimal medium (C), GYEP medium (D), PDB (E), and SNA medium (F) grown in 200µL cultures in a 96 well plate.

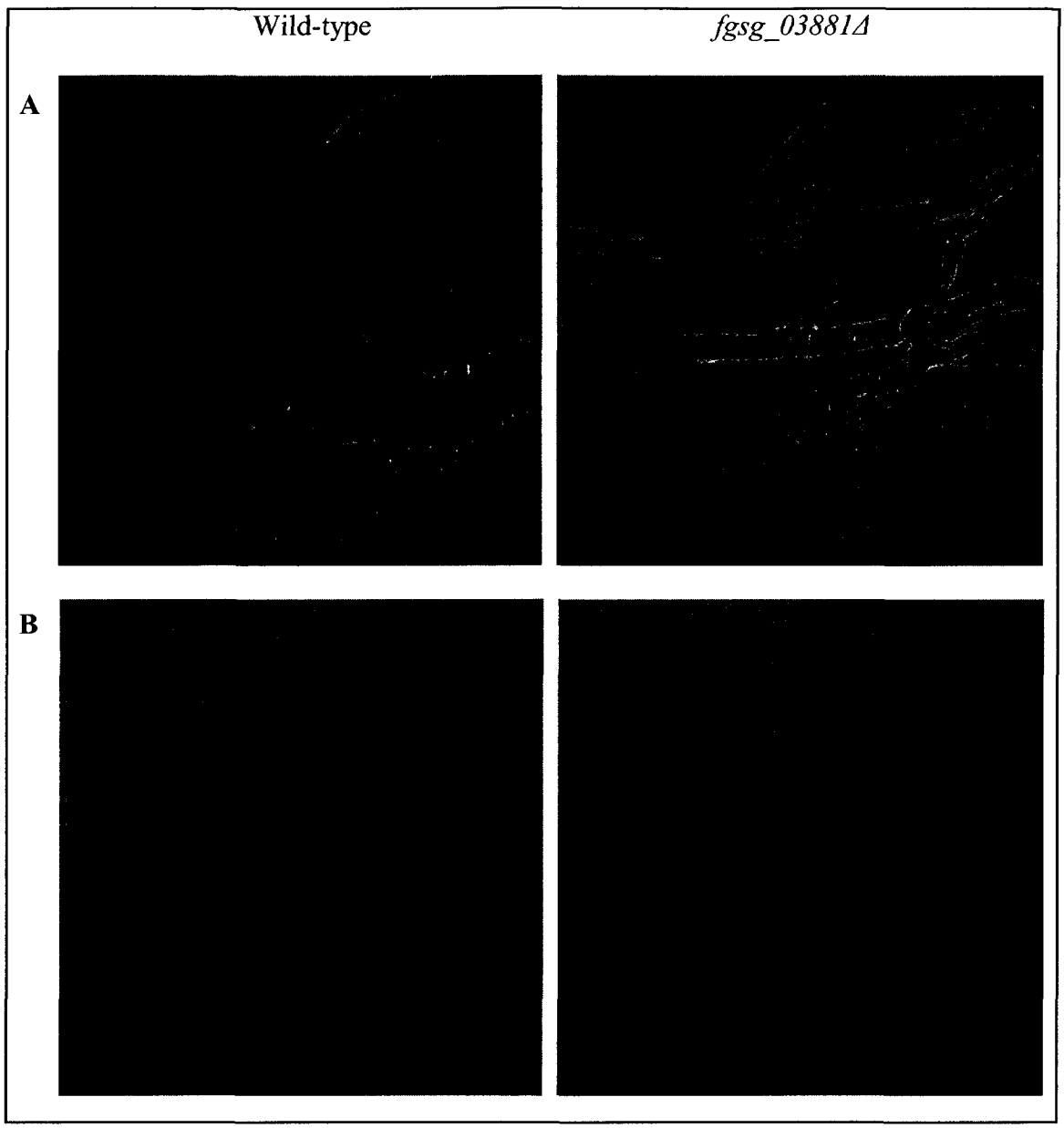


Figure 19: Nitroblue tetrazolium and diaminobenzidine staining of wild-type and *fgsg_03881Δ*. Cultures were stained with NBT (A) and DAB (B) for wild-type and *fgsg_03881Δ* after 24h of growth in a 6 well plate containing 4mL of PDB and viewed under a Leica DM 1000 compound light microscope. Arrows indicate positive staining for superoxides in NBT stained cultures.

3.8. FGSG_03881 does not Interact with Trichothecene Regulators

Since *FGSG_03881* is co-regulated with trichothecene regulators, yeast two-hybrid analyses were performed to determine if FGSG_03881 is able to interact with Tri10, Tri6, or FGSG_11025. The yeast strains were scored based on the expression of the reporter genes that encode for beta-galactosidase and uracil biosynthesis. The observed growth on the uracil deficient media and the beta-galactosidase activity for the various combinations of pDest32 and pDest22 vectors are found in Table 9. The two strains that contained Tri6 fused to the binding domain of *Gal4* auto-activated both reporter genes. The only other combination that activated the reporter genes were RalDGS and Krev1, which are known interactors and were used as a positive control. The destination vectors lacking cloned targets were used as negative controls. The results suggested that FGSG_03881 does not interact with the regulators of trichothecene biosynthesis.

Table 9: Yeast two-hybrid analyses of FGSG_03881 and trichothecene regulators. The expression of yeast two-hybrid reporter genes for yeast MaV203 cultures transformed with various combinations of pDEST32 and pDEST22 vectors containing the coding regions for Tri6, Tri10, and FGSG_11025 were scored against FGSG_03881.

pDest 32	pDest 22	Growth on -Ura Plates	Beta-galactosidase activity
FGSG_03881	FGSG_03881	No	No
FGSG_03881	Tri10	No	No
FGSG_03881	FGSG_11025	No	No
FGSG_03881	Tri6	No	No
FGSG_03881	Empty	No	No
Tri10	FGSG_03881	No	No
FGSG_11025	FGSG_03881	No	No
Tri6	FGSG_03881	**Yes**	**Yes**
Empty	FGSG_03881	No	No
Tri6	Empty	**Yes**	**Yes**
Empty	Tri6	No	No
Tri15	Empty	No	No
Empty (control)	Empty (control)	No	No
Krev1 (control)	RalDGS (control)	**Yes**	**Yes**

Chapter 4 - Discussion

There are many genes and environmental factors that are known to be associated with virulence in *F. graminearum*, but many of the underlying mechanisms that regulate virulence are poorly understood. This thesis project provides some insight into these mechanisms. In the past few years, some virulence functions have been characterized at both the genetic and the molecular level. For example, many of the genes required for DON biosynthesis have been positively associated with virulence in the fungus; some of the environmental factors that regulate the expression of these genes have also been elucidated (Miller and Greenhalgh 1985; Ponts et al. 2007; Jiao et al. 2008; Gardiner et al. 2009b). However, *F. graminearum* has 13,826 genes, of which 69% are currently annotated as unclassified and the vast majority of classified genes have been annotated electronically. The reality is that our current understanding of how *F. graminearum* functions as a pathogen is very limited. The characterization of *FGSG_03881* and its association with virulence complement the current efforts made by many research teams to identify genes involved in virulence.

4.1. FGSG_03881 is Associated with Virulence in F. graminearum

4.1.1. Pathological Evidence Links FGSG_03881 to Virulence

Since *F. graminearum* infection has such devastating consequences, understanding the factors that contribute to infection in the fungus will be important from both a health and an economic perspective. Infection assays indicated that disruption of *FGSG_03881* caused the fungus to become more pathogenic in a susceptible variety of wheat, 'Roblin', during the early stages of infection (7-14 dpi). At 21 dpi, there were no

discernable differences between the number of wheat spikelets infected with *fgsg_03881Δ* or the wild-type strain (p-value = 0.3), indicating that the wild-type strain was eventually able to cause similar amounts of infection as *fgsg_03881Δ* at late infection stages. Together, the results indicated that disruption of *FGSG_03881* allowed the fungus to increase its potential for infection at the earlier stages, and that the presence of *FGSG_03881* delays this potential.

The increased virulence of the *fgsg_03881Δ* strain at early stages is correlated with increased accumulation of DON in the infected wheat heads (Table 1). This observation could be explained by two possibilities: 1) *fgsg_03881Δ* was able to produce more DON than wild-type, or 2) the increased virulence of the *fgsg_03881Δ* strain allowed the fungus to establish more biomass which in turn allowed for more DON to accumulate. Since the wild-type and *fgsg_03881Δ* strains possessed a similar capacity to synthesize 15-acetyl-DON in culture, the likely scenario is that the increased DON levels observed in 'Roblin' infected with *fgsg_03881Δ* is caused by increased biomass accumulation of the strain.

The increased virulence attributed to the disruption of *FGSG_03881* was also tested in the resistant variety of wheat 'Sumai 3'. This variety contains many QTLs (quantitative trait loci) that are associated with resistance, allowing it to be one of the most resistant wheat varieties to *Fusarium* infection (Liu and Anderson 2003). We did not observe any differences in the amount of infection in 'Sumai 3' by wild-type, *fgsg_03881Δ* or *FGSG_03881/fgsg_03881Δ* strains. This suggested that whatever caused

increased infection in 'Roblin' at early stages is insufficient to overcome the resistance present in 'Sumai 3'.

4.1.2. Genetic Evidence Links FGSG_03881 to Nutrient Stress and Virulence

A positive link between virulence and nutrient stress has been established in *F. graminearum* and other *Fusarium spp.* (da Silva et al. 2001; Gardiner et al. 2009a; López-Berges et al. 2010). Previous studies have assessed the role of various nitrogen sources in the activation of genes involved in trichothecene biosynthesis; the results demonstrated that activation is increased in the presence of non-preferred nitrogen sources (Gardiner et al. 2009a). In that study, the authors measured GFP fluorescence every 24h to monitor the expression of *Tri5*, the first gene in the trichothecene biosynthesis pathway. The expression level was estimated by integrating the area under the curve. Since the expression was not corrected for the growth of the fungus, which varies with different nitrogen sources, the results did not represent the actual expression of the *Tri5* (Gardiner et al. 2009a). We developed a high-throughput system that simultaneously monitored both the expression and the growth of the fungus in each nitrogen source. A mathematic equation was derived to normalize the expression of the *FGSG_03881* using the growth of the fungus. Preliminary analyses employed multiple mathematic models to describe the relationship between growth and GFP, which determined that a fourth order polynomial equation had the greatest accuracy. It is important to note that the equation was most accurate when growth was in the mid-log phase, which is why a time course was employed to ensure that sufficient growth was

achieved in all nitrogen conditions. This method was validated by using different nitrogen sources as a baseline in the polynomial equation and by RT-qPCR analyses.

The results indicated that *FGSG_03881* is expressed under nutrient stress conditions, specifically under nitrogen stress. This was in line with previous microarray observations which showed that *FGSG_03881* was induced in nitrogen stress conditions (Güldener et al. 2006). In contrast, *FGSG_03881* expression was suppressed in preferred nitrogen sources, such as glutamate and aspartate. Interestingly, expression appeared to be greatest in ammonium phosphate dibasic, the nitrogen source present in the 15-acetyl-DON inducing media. This expression appears to be independent of *Tri6* and the biosynthesis of 15-acetyl-DON, as the expression level of *FGSG_03881* was comparable between wild-type and *tri6Δ*. In spite of no apparent associations with 15-acetyl-DON, the expression patterns indicate that the gene is positively correlated with the expression of genes that regulate DON biosynthesis, namely *Tri6* and *Tri10*. Taken together, the results indicate that *FGSG_03881* is induced under conditions known to be associated with virulence and is co-expressed with genes that control the biosynthesis of a known virulence factor.

In addition to being expressed under virulence conditions, earlier studies indicated that *FGSG_03881* was also induced during the initial stages of conidial germination (Seong et al. 2008). This suggested a potential role for this gene in initial developmental of the fungus and the infection process. In addition to *FGSG_03881*, genes associated with nitrogen acquisition, specifically nucleotide and amino acid metabolism were also

induced during this developmental stage. Other studies indicated that *FGSG_03881* was also expressed after infection in barley (Güldener et al. 2006) and similar analyses in wheat have corroborated this result (personal communication, Thérèse Ouellet, AAFC, Ottawa). This suggested that *FGSG_03881* may have an important role during the initiation and progression of infection in multiple hosts.

4.2. Identification of Potential Targets of FGSG_03881 Relating to Virulence

Since the disruption of *FGSG_03881* causes the fungus to infect more than wild-type at early infection stages, it is possible that genes regulated by *FGSG_03881* and secreted proteins affected by its mutation might reveal potential targets involved in virulence. Analyses of both microarrays and the secreted proteins (secretome) identified genes and proteins that included CWDE, genes involved in secondary metabolism, and others with known associations with virulence. Other targets included genes encoding proteins involved in central metabolic processes that could also contribute to virulence.

4.2.1. Potential Virulence Factors Modulated by FGSG_03881

Included in the top ten genes up-regulated in the mutant strain grown in 15-acetyl-DON inducing conditions were five proteins that contained secretion signals (*FGSG_07601* (12-fold, accompanying electronic materials), *FGSG_02377* (6-fold), *FGSG_11385* (5-fold), *FGSG_11343* (5-fold), and *FGSG_09098* (5-fold)). The eleventh most up-regulated gene is a predicted secreted serine protease (*FGSG_ 04817* (5-fold)), which could potentially interact with and degrade host proteins. These could be secreted during infection and act as virulence factors, thereby enhancing infection in the mutant.

Among the top ten genes down-regulated in the mutant strain grown in 15-acetyl-DON inducing conditions was a gene predicted to encode an acetylxylan esterase (*FGSG_11578* (47-fold)) and two small secreted proteins (*FGSG_13202* (48-fold) and *FGSG_08212* (147-fold)). Multiple acetylxylan esterases were shown to be induced in *F. graminearum* cultures grown in the presence of cell wall material, as they are able to hydrolyze acetyl groups of xylan, a principal component of the plant cell wall (Hatsch et al. 2006). These could act as avirulence factors and provoke a defence response in the host. The absence of these factors in the mutant could delay this response from the host and render it more susceptible.

A comparison between the gene expression data and the secretome data revealed that 22 of the proteins found in the secretome were differentially expressed in *fgsg_03881Δ* strain. This correlation suggested that *FGSG_03881* may affect the production and secretion of proteins in *F. graminearum*. Since mutation of *FGSG_03881* increases virulence of the fungus, the proteins secreted from the *fgsg_03881Δ* but not by wild-type could offer the mutant a selective advantage. One protein (FGSG_05175) contains a CFEM domain, a fungal domain that is present in some membrane proteins associated with pathology in *M. grisea* (Kulkarni et al. 2003). In addition, two HSP70-like proteins (FGSG_09471, and FGSG_06154) were also secreted. These proteins may be important chaperones for virulence associated molecules in the fungus. Another possibility is that the fungal HSP70s could be interacting with molecules in the host cell and co-opt the function of host HSP70s; it has also been shown that expression of HSP70s in the host increases during *F. graminearum* infection in wheat (Golkari et al.

2007). Many of the other proteins uniquely secreted by the mutant were involved in metabolism or general cellular processes, such as an ATP synthase subunit (FGSG_01080), cystathionine gamma-synthase (FGSG_01177), S-adenosyl-L-homocysteine hydrolase (FGSG_05615), and four ribosomal proteins (FGSG_02503, FGSG_07291, FGSG_01509, and FGSG_09866). There were also ten other proteins that are yet to be characterized.

There were also proteins that were secreted by the wild-type strain, but not by the mutant strain. These proteins could participate in interactions with the host and delay the *Fusarium* infection process. For example, one of the identified proteins was an elongation factor (FGSG_07401) and elongation factors have been previously demonstrated to initiate innate immune responses in plants (Kunze et al. 2004). Another protein FGSG_11496 has a proposed cerato-platanin domain that has been demonstrated to be phytotoxic (Pazzagli et al. 1999). Glucan 1,4-alpha-glucosidase (FGSG_11326), a member of the CWDE family, is also down-regulated in both GYEP (3-fold) and 15-acetyl-DON inducing conditions (3-fold). Mannitol, which is used as a protective mechanism against ROS, is produced by mannitol dehydrogenase (*FGSG_04826*), which is absent from the secretome and down-regulated under GYEP conditions (2-fold) in the *fgsg_03881Δ* strain (Solomon et al. 2007). Another protein absent from the mutant strain is a small secreted protein that is also down-regulated in 15-acetyl-DON inducing conditions (FGSG_06130 (2-fold)). In addition to these, there were many proteins absent from the *fgsg_03881Δ* strain with roles in metabolism or general cellular processes, including aldehyde dehydrogenase (FGSG_02273), amidase (FGSG_04022), adenosine

kinase (FGSG_06932), 40S ribosomal protein (FGSG_05433) and phosphoglycerate dehydrogenase (FGSG_07468). Others had no known functions (FGSG_02903, FGSG_03894, FGSG_07907, FGSG_09119). Since they are down-regulated or absent in the *fgsg_03881Δ* strain, we propose that these proteins may trigger a plant defence response during infection by the wild-type strain.

4.2.2. FGSG_03881 Modulates Genes Involved in Mycotoxin Production

The original goal of the project was to explore a possible connection between trichothecenes and *FGSG_03881*. Since gene expression data suggested that *FGSG_03881* is positively associated with conditions used to induce 15-acetyl-DON production as well as with the trichothecene biosynthesis regulators *Tri6* and *Tri10*, we postulated a link between this pathway and *FGSG_03881*. However, yeast two-hybrid analyses indicated that FGSG_03881 does not interact with FGSG_11025, Tri10 or Tri6. In addition, gene expression analyses suggest that expression of *FGSG_03881* is independent of *Tri6* or trichothecene production. Since none of the essential *Tri* genes are differentially expressed in the GYEP conditions and there is no difference in 15-acetyl-DON production between the wild-type and *fgsg_03881Δ* strains, we suggest that *FGSG_03881* may have a role that is unrelated to DON biosynthesis.

Despite these observations, it is still possible that *FGSG_03881* is involved in modulating some of the genes involved in DON production. For example, we observed the down-regulation of *FGSG_12976* (*Tri201*) in *fgsg_03881Δ* in both GYEP (3-fold) and 15-acetyl-DON inducing conditions (4-fold). *Tri201* is structurally similar to *Tri101*,

an acetyl-transferase that is well established to metabolically detoxify trichothecenes (Kimura et al. 2003). Since the disruption of *Tri101* alone does not completely prevent the fungus from metabolising trichothecenes, *Tri201* may be working synergistically with *Tri101* in the detoxification process (Kimura et al. 2003). In support of this, bacterial cultures expressing *Tri201* were able to detoxify T-2 toxin produced by *F. sporotrichioides* (Kimura et al. 2003). Therefore, it is possible that by down-regulating the detoxifying gene, the mutant is able to increase the potency of the mycotoxin and increase its virulence. Although, *Tri201* was the only *Tri* gene to be down-regulated in both GYEP and 15-acetyl-DON inducing conditions, there were other *Tri* genes that are also down-regulated in the 15-acetyl-DON inducing conditions (*Tri4* (11-fold), *Tri10* (3-fold), and *Tri8* (3-fold)). Despite the differential expression of these genes, the fungus is still able to synthesize 15-acetyl-DON *in vitro* and DON *in vivo*. This suggests that the fungus is able to compensate for these differences in gene expression, or that the differences are insufficient to influence toxin production.

Another secondary metabolic pathway that is affected by *FGSG_03881* disruption is that of aurofusarin and rubrofusarin biosynthesis. In the *fgsg_03881Δ* strain, 7 genes in the pathway are down-regulated in 15-acetyl-DON inducing conditions (between 2 and 22-fold). These genes are involved in regulation, biosynthesis and transport of rubrofusarin and aurofusarin in *F. graminearum* (Frandsen et al. 2006). Rubrofusarin is an intermediate leading to the production of aurofusarin, a pigment molecule that causes red coloration in the cell wall of the fungus (Frandsen et al. 2006). Although disruption of aurofusarin synthesis does not influence pathology directly, it has been associated with

increased levels of zearalenone (Malz et al. 2005). However, zearelanone biosynthesis and regulatory genes, *Zeb1*, *Zeb2*, *Pks4*, and *Pks13* appear to be unaffected in both GYEP and 15-acetyl-DON inducing conditions and are not expressed under either condition. Although these pathways may not be contributing to the pathological differences in the mutant strain, the data does suggest that secondary metabolic pathways are altered in the mutant strain.

Fusarin C biosynthesis is also unaffected by *FGSG_03881* disruption. The fusarin C biosynthesis gene *FusA* is not differentially expressed in either GYEP or 15-acetyl-DON inducing conditions, despite being expressed in the latter. In addition, the biosynthesis of fusarin C is suggested to use intermediates from the citric acid cycle, as well as serine and aspartate metabolites (Song et al. 2004). Based on the gene expression data, *FGSG_03881* is negatively associated with both serine and aspartate, and is therefore less likely to participate in the direct metabolism of these compounds for fusarin C biosynthesis. This suggests that *FGSG_03881* may have a role that is unrelated to fusarin C.

4.2.3. Disruption of FGSG_03881 Affects Primary Metabolism

The gene expression data suggested that many key metabolic processes are differentially regulated in the *fgsg_03881Δ* strain compared to wild-type. In total, metabolic genes accounted for 39.8% and 17.8% of the genes differentially expressed in GYEP and 15-acetyl-DON inducing conditions, respectively. Some of the genes identified are involved in amino acid metabolism and nitrogen acquisition. For example,

one of the genes that are down-regulated in both GYEP (3-fold) and 15-acetyl-DON inducing media (70-fold) is *FGSG_03936*, which encodes for succinate semialdehyde dehydrogenase, an enzyme that converts glutamate into succinate. Another gene, cystathionine gamma-synthase (*FGSG_01417*) is also down-regulated in GYEP (7-fold) and is involved in the production of cysteine, a non-preferred nitrogen source which is able to induce *FGSG_03881* expression. Nitrite reductase (*FGSG_08402*), a gene that facilitates the conversion of nitrogen sources into free ammonia, was also down-regulated in both GYEP (7-fold) and 15-acetyl-DON inducing conditions (8-fold).

In addition to the above mentioned genes, there appears to be an overrepresentation of differentially regulated genes involved in purine metabolism. Seven of the fourteen genes up-regulated in *fgsg_03881Δ* grown in GYEP appear to be involved in purine metabolism (between 2 and 5-fold), and another gene in this pathway (*FGSG_12356*) was down-regulated (4-fold) (Figure 20). However, under 15-acetyl-DON inducing conditions, the pathway is not induced and four genes (*FGSG_05323*, *FGSG_12865*, *FGSG_12356*, and *FGSG_04126*) in the pathway are down-regulated (between 2 and 10-fold). This suggests that this pathway may be sensitive to environmental nitrogen changes, such as the change from preferred nitrogen in GYEP to non-preferred nitrogen in 15-acetyl-DON inducing conditions. This agrees with previously established data that suggested that purine metabolism is regulated by NCR, and is mediated by the genes *AreA* and *UaY* in *Aspergillus sp.* (Marzluf 1997). *UaY* is constitutively expressed and is believed to be independent of *AreA* and responsive to

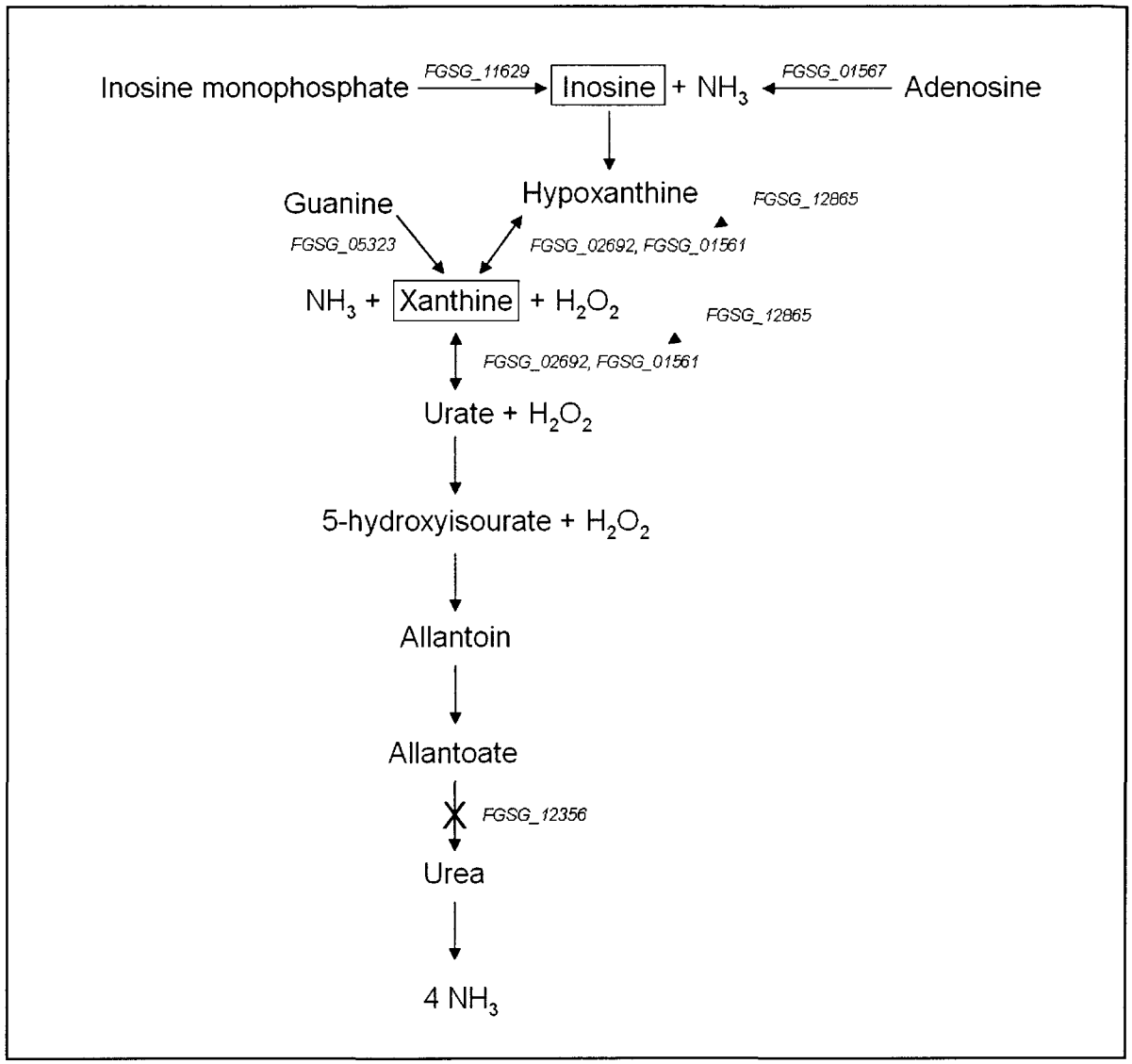


Figure 20: Flow chart illustrating genes involved in purine metabolism. Up-regulated (black) and down-regulated (red) genes in the *fgsg_03881Δ* strain in comparison to wild-type grown in GYEP medium are involved in degrading the purine intermediates into free ammonia. Broken arrows denote proposed protein modifications required for gene activation.

urate (Marzluf 1997). Neither *AreA* (proposed homologue FGSG_08634) nor *UaY* (proposed homologue FGSG_05120) are differentially regulated under either microarray condition. In addition, none of the genes identified to be differentially expressed in the pathway have the proposed *UaY* binding site in their promoters (Suárez et al. 1995). This suggested that the induction of these genes in the mutant strain grown under GYEP conditions may be independent of *AreA* and *UaY*.

It is possible that activation of the purine metabolic pathway enables the fungus to secure free nitrogen in the form of ammonia during plant infection. The *F. oxysporum* strain deleted in *AreA*, the global regulator of NCR, displayed decreased virulence on its host. It was proposed that altered metabolism of non-preferred nitrogen sources, specifically the reduced ability to metabolise the purine intermediates hypoxanthine and urate, contributed to the infection deficiencies (Divon et al. 2006). In addition, it has been suggested that plants produce γ-aminobutyric acid (GABA) and urate during infection, and that these could be used as principal nitrogen sources by some pathogenic fungi (Divon and Fluhr 2007). The induction of purine metabolic genes in the mutant strain independently of *AreA* or *UaY* could allow *fgsg_03881Δ* to acquire nutrients from the host more readily than the wild-type strain, allowing it to infect more spikelets at earlier time points. However, it was also observed that urate oxidase (*FGSG_04126*), an enzyme that metabolizes urate into allantoin, and GABA permease (*FGSG_08221*) are down-regulated (6-fold and 4-fold, respectively) in the mutant strain when grown in the 15-acetyl-DON inducing media. The down-regulation of these genes counters the previous claim that the mutant would be acquiring more nutrients during infection. Although the

data suggests that some nutrient acquisition pathways may be altered in *fgsg_03881Δ*, additional analyses would be required to substantiate an association between these pathways and the pathology phenotype observed in the *fgsg_03881Δ* strain.

The purine metabolic pathway also results in the production of hydrogen peroxide (Figure 20). Hydrogen peroxide is associated with apoptosis in eukaryotic cells, and has been recorded to induce mycotoxin production in *F. graminearum* (Ponts et al. 2007). The lack of differences in the NBT and DAB stained cultures suggests that hydrogen peroxide and superoxide levels may not be associated with *FGSG_03881*. It is possible that the mutant may be able to compensate for any differences that may have developed or that the differences were insufficient to be detected by the procedures used.

The difficulty in obtaining a constitutive expression strain using the pGpd::*FGSG_03881* construct suggested that having high transcript levels may be detrimental to the fitness of the fungus. This could be the case if it is positively regulating metabolic genes that degrade non-preferred nitrogen sources that are essential to cellular function. It is also possible that high transcript levels are causing increased metabolism and the buildup of toxic by-products. Additional analyses would be required to validate these hypotheses.

4.3. An Integrated Model Outlining the Role of FGSG_03881

Several lines of evidence have been obtained that indicate how *FGSG_03881* could be regulated. The expression of *FGSG_03881* increases in nitrogen limiting

conditions and as such it is likely subject to regulation by NCR, a system that is common to many fungal species and is alleviated by the gene *AreA* (Marzluf 1997). *AreA* has been demonstrated to regulate secondary metabolism and virulence functions in other *Fusarium spp.* and would therefore be an ideal candidate for a regulatory mechanism controlling *FGSG_03881* (Mihlan et al. 2003; Kim and Woloshuk 2008; Schonig et al. 2008; López-Berges et al. 2010). Since *AreA* (proposed homologue FGSG_08634) is not differentially expressed in the mutant strain, it is likely that *FGSG_03881* may be a down-stream target of *AreA*.

FGSG_03881 could also be regulated by the structural confines of the DNA. This is supported by the observation that expression of *FGSG_03881* in the complement strain (*FGSG_03881/fgsg_03881Δ*) is over 20 times greater than wild-type. This could be occurring because the complementation construct is not constrained by the epigenetic regulation that the gene may normally encounter in its native position in the genome. Epigenetic regulation is also supported by the observation that the adjacent gene *FGSG_03882* is down-regulated in both GYEP (10-fold) and 15-acetyl-DON inducing conditions (14-fold) in the *fgsg_03881*Δ strain. While the down-regulation of this gene may be a result of genetic clustering and direct regulation by *FGSG_03881*, it may also be the result of the insertion of the disruption construct altering the structure of the chromatin. A recent study has shown that *LaeA* is able to regulate secondary metabolism and virulence in fungi through epigenetics (Bayram et al. 2008; Amaike and Keller 2009; Wiemann et al. 2010). Additionally, LaeA interacts with the velvet-like complex, which is able to regulate secondary metabolism in *F. verticillioides* by responding to nitrogen

stress independently of *AreA* (Wiemann et al. 2010). This suggests that *LaeA* and the velvet-like complex may also be suitable candidates for the regulation of *FGSG_03881*.

Together, the data suggests that *FGSG_03881* may be controlled by global regulators such as *AreA* and *LeaA*. Disruption of the gene enhances the ability for the fungus to infect wheat at early time points. However, culture conditions demonstrate that the mutation has no effect on the fungus' ability to make conidia or grow on various nutrient sources, suggesting that the gene is non-essential to basic cellular functions. Thus, the phenotype of this mutant is similar to other known pathway-specific regulators associated with virulence, as outlined in the hierarchal model (Figure 21).

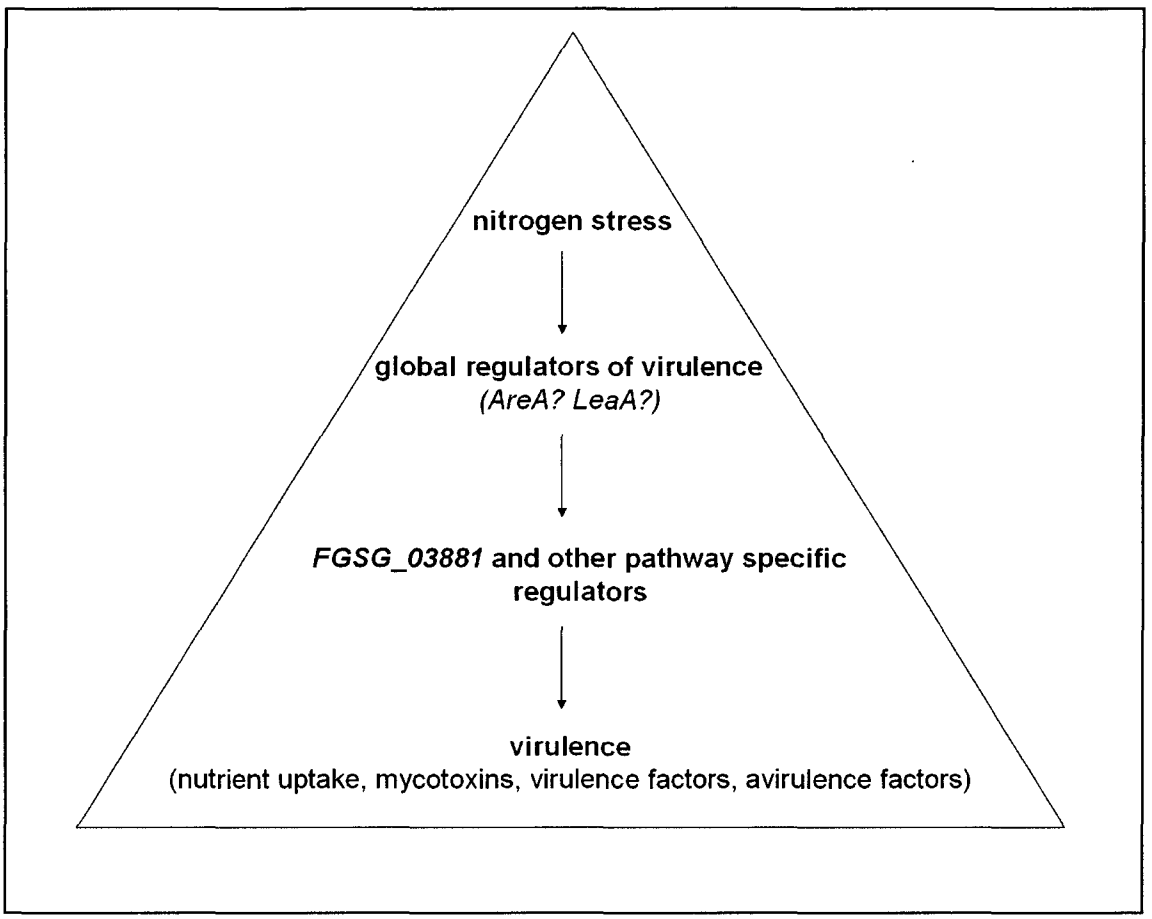


Figure 21: An integrated model illustrating the role of *FGSG_03881*. *FGSG_03881* responds to nitrogen cues and regulates downstream pathways related to pathogen virulence in *F. graminearum*.

4.4. Perspectives and Future Studies

4.4.1. The Possible Identification of a New Class of Regulatory Genes

The identification of *Tri15*-like proteins in both *Fusarium sp.* and other fungi, suggests that there may be other proteins that perform similar roles to *FGSG_03881*. Additional analyses, especially with *FVEG_13013*, may provide insight into whether or not these proteins have similar functions. A bacterial one-hybrid system or chromatin immunoprecipitation combined with next generation sequencing could be used to identify potential targets of *FGSG_03881*. This would also identify the DNA binding site for FGSG_03881, which may overlap with the other *Tri15*-like proteins. Although *FGSG_03881* contains three Cys_2His_2 zinc fingers, which is characteristic of many transcription factors, it is also possible that the role of *FGSG_03881* is better understood through its protein interactions. To explore this hypothesis further, a forward yeast two-hybrid screen would identify potential-protein interactions with FGSG_03881.

4.4.2. Additional Insights into the Secretome of F. graminearum

There are many important aspects to the secreted protein data that are worth closer attention. Firstly, the proteins identified are likely representative of a non-exhaustive list due to limitations of the gel-free mass spectrometry procedure (personal communication with Christoph Rampitsch), suggesting that there are likely many additional proteins that were not identified. With that in mind, many of the proteins that were identified were CWDE, while others had associations with metabolism and stress responses. The identification of many metabolic proteins suggests that there may be contamination from cellular debris. However, it is also possible that these proteins have

roles in virulence that are yet to be characterized. In support, FGSG_06039, a putative ATP citrate synthase subunit, which is uniquely absent from the *tri6Δ*, also has excellent coverage in all of the other samples and has been previously recorded to be associated with virulence and DON production (Son et al. 2011). In addition, a superoxide dismutase (FGSG_08721), which converts oxygen radicals into hydrogen peroxide, is absent from both the *tri6*Δ and *tri10*Δ strains. This suggests an additional link that may be contributing to the reduced pathogenicity of these strains. Additional analyses of these proteins and their associations with virulence are warranted.

The deposition of callose observed in the 'Roblin' leaves infiltrated with Flg22 indicated that this method was successful in inducing basal defense in wheat. It also suggested that this method could be used to determine if the secreted proteins are able to elicit similar responses. The presence of macro deposits of callose after infiltration with the secreted proteins indicated that the secretomes from 15-acetyl-DON inducing cultures may contain proteins that could function as pathogen associated molecular patterns (PAMPs).

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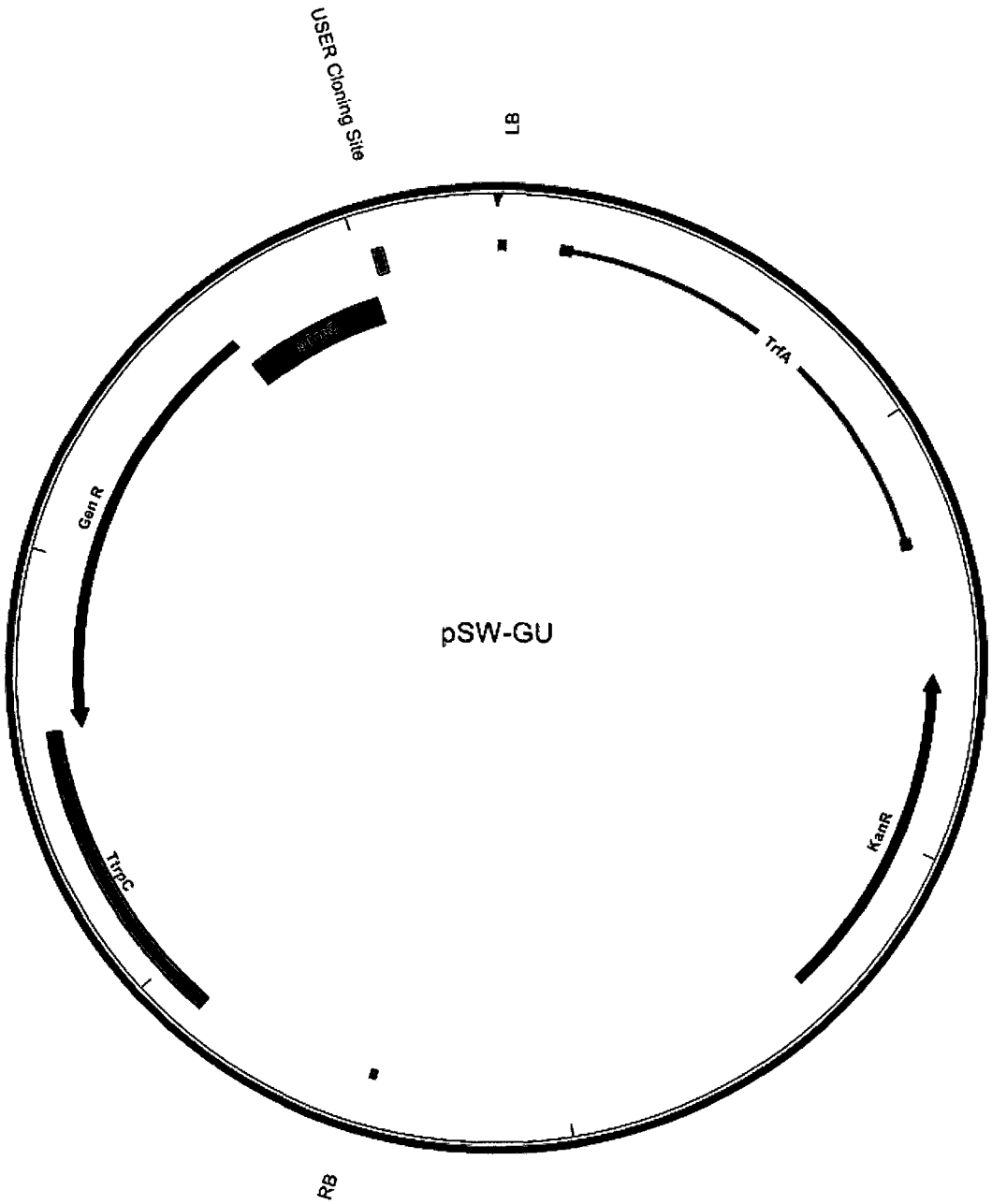
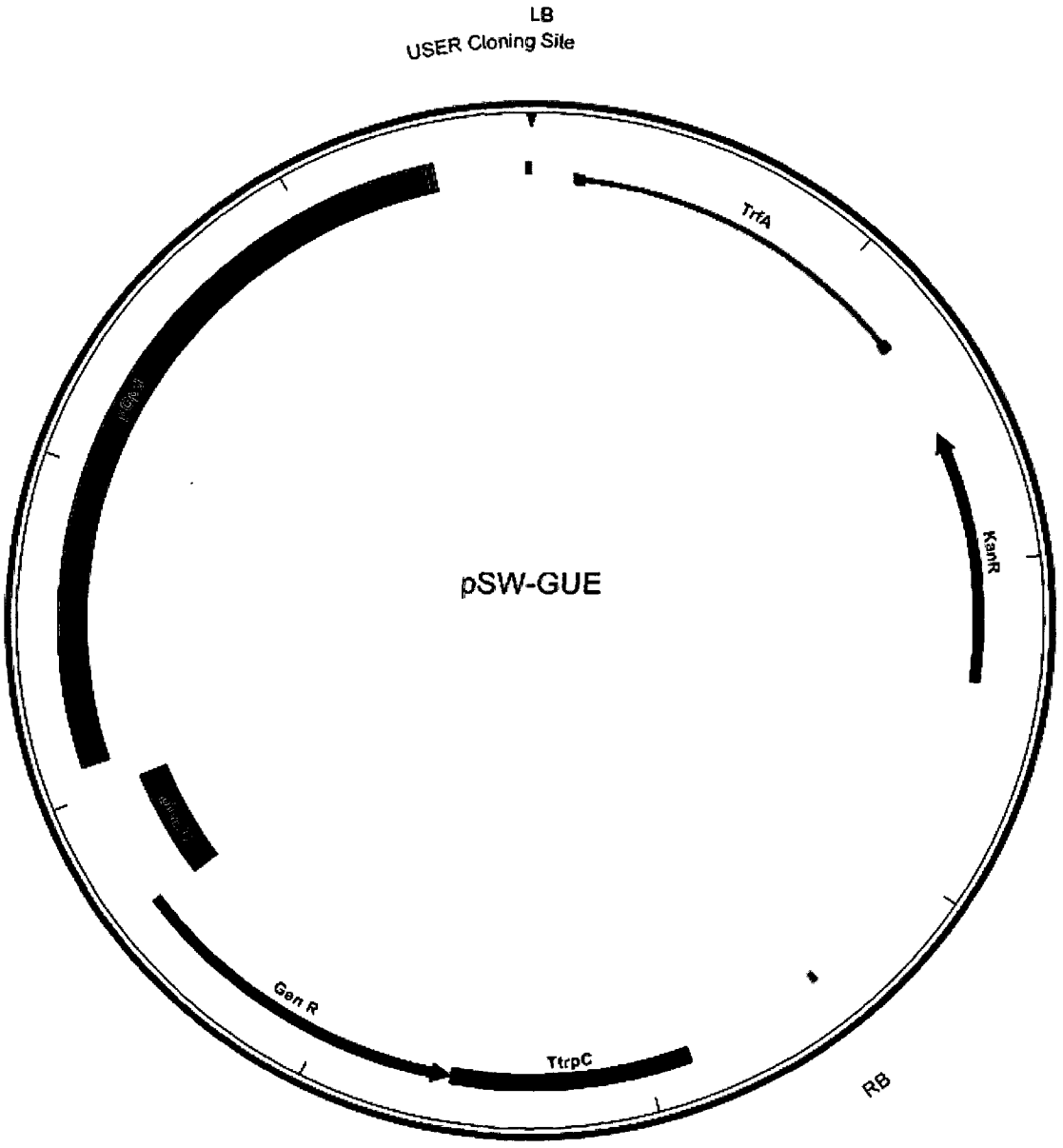
Appendix

Appendix Table 1: Primers used in standard PCR reactions for the formation of transgenic constructs and verification of construct integration

Name	Sequence 5'- 3'
P1	CGACCATTTTGGCTGGTCGTC
P2	GATGTCAGGCCATTTTCACGACAATAGCAATGGATGATG
P3	TGCCGACCGAACAAGAGCTGAAGGGTTTGAGCTACAGAAG
P4	CAACGACGTAGTAATGGTGTC
P5	CATCATCCATTGCTATTGTCGTGAAAATGGCCTGACATC
P6	CTTTCTGTAGCTCAAACCCTTCAGCTCTTGTTCGGTCGGCA
FGSG_03881 F	GATATCGATATCATGACATCCACAAAACCGAC
FGSG_03881 R	AGGCCTAGGCCTCAACCATTCTTCGGACCCG
Hyg F	TGAAAATGGCCTGACATCAT
Hyg R	AGCTCTTGTTCGGTCGGCATCTAC
Geneticin USER F	GGGTTTAAUTAAGTCCTCAGCATGCCAGTTGTTCCCAGTG
Geneticin USER R	GGTCTTAAUAGAGTAAAGAAGAGGAGCATG
pGpd USER F	GGACTTAAUCGTGGCCAGCCCGAATTCCC
pGpd USER R	GGGTTTAAUTAAGTCCTCAGCCCCGGGTGATGTCTGCTCAAG
Promoter USER F	GGACTTAAUGTAAGATCTACTGGGGTGAG
Terminator USER R	GGGTTTAAUGGTGAATTAACTGTCCAAAC
FGSG_03881 USER F	GGACTTAAUATGACATCCACAAAACCGAC
FGSG_03881 USER R	GGGTTTAAUTCAACCATTCTTCGGACCCG
GFP F	GGGAAGCTTGCCACCATGGCCCAGTCCAAGC
GFP R	CCGCTCGAGCCCGGGCTCAGGGCAAGGCG
Promoter R	GGGCCATGGTGGCAAGCTTAGTTGCTGGTGTTTAACAATTG
Terminater F	CTGAGCCCGGGCACGAGCGGAGGGTTTGAGCTACAGAAAG
Gen F2	GAAGCACTTGTCCAGGGAC
Gen R2	GACCGACCTGTCCGGTGCCC
pGpd Int F	CAAATATCGTGCCTCTCCTG
FGSG03881 Int	GGAACAAGAAGCCGCACCGT
ENTR F	CACCATGACATCCACAAAACCGACG
ENTR R	ACCATTCTTCGGACCCGGCA



Appendix Figure 1: Vector map of pSW-GU. The vector was constructed using the vector pRF-HU2 as in Frandsen et al. 2008. The *Agrobacterium* vector contains a USER cloning site consisting of *Pac1* and *Nt.BbvCI* restriction sites which are used in USER cloning as described in the vector pRF-HU in Frandsen et al. (2008). RB and LB indicate the right and left border of the T-DNA.



Appendix Figure 2: Vector map of pSW-GUE. The vector was constructed using the vector pRF-GU. The *Agrobacterium* vector contains a constitutive expression promoter (pGpd) which precedes a USER cloning site consisting of *Pac1* and *Nt.BbvCI* restriction sites which are used in USER cloning as described in the vector pRF-HUE in Frandsen et al. (2008). RB and LB indicate the right and left border of the T-DNA.

Appendix Table 2: Primers used for RT-qPCR analyses

Name	Sequence 5'- 3'
Tri10 F	GAGAGGCTCCCAAATCTTGAAG
Tri10 R	TGACTTTCTGAGGCCATGCA
Tri6 F	CATCGTCGGGACTGTTGGA
Tri6 R	AAGGTGGGAAGGGCGATAA
β-Tubulin F	GTTGATCTCCAAGATCCGTG
β-Tubulin R	CATGCAAATGTCGTAGAGGG
FGSG_03881 F	AGCCTGCTAAATGCATTATTTGC
FGSG_03881 R	TGCTGGACGTTGTCATCGA

Appendix Table 3: Unpaired t-test comparing the number of wild-type and *fgsg_03881Δ* infected spikelets in the susceptible variety of wheat 'Roblin' 7 days after point inoculation with 1,000 spores

	wild-type	*fgsg_03881Δ*
Mean	2.292682927	3.404761905
Variance	13.51219512	18.29558653
Observations	41	42
Pooled Variance	15.93341793	
Hypothesized Mean Difference	0	
df	81	
t Stat	-1.268991417	
P(T<=t) one-tail	0.104039285	
t Critical one-tail	1.663883913	
P(T<=t) two-tail	0.20807857	
t Critical two-tail	1.989686323	

Appendix Table 4: Unpaired t-test comparing the number of wild-type and *FGSG_03881/fgsg_03881Δ* infected spikelets in the susceptible variety of wheat 'Roblin' 7 days after point inoculation with 1,000 spores

	wild-type	*FGSG_03881/fgsg_03881Δ*
Mean	2.292682927	2.642857143
Variance	13.51219512	15.35714286
Observations	41	42
Pooled Variance	14.44605756	
Hypothesized Mean Difference	0	
df	81	
t Stat	-0.419649801	
P(T<=t) one-tail	0.337926136	
t Critical one-tail	1.663883913	
P(T<=t) two-tail	0.675852272	
t Critical two-tail	1.989686323	

Appendix Table 5: Unpaired t-test comparing the number of wild-type and *fgsg_03881Δ* infected spikelets in the susceptible variety of wheat 'Roblin' 10 days after point inoculation with 1,000 spores

	wild-type	*fgsg_03881Δ*
Mean	6.414634146	8.285714286
Variance	36.14878049	36.69686411
Observations	41	42
Pooled Variance	36.42620553	
Hypothesized Mean Difference	0	
df	81	
t Stat	-1.41209186	
P(T<=t) one-tail	0.080876639	
t Critical one-tail	1.663883913	
P(T<=t) two-tail	0.161753277	
t Critical two-tail	1.989686323	

Appendix Table 6: Unpaired t-test comparing the number of wild-type and *FGSG_03881/fgsg_03881Δ* infected spikelets in the susceptible variety of wheat 'Roblin' 10 days after point inoculation with 1,000 spores

	wild-type	*FGSG_03881/fgsg_03881Δ*
Mean	6.414634146	6.761904762
Variance	36.14878049	36.96631823
Observations	41	42
Pooled Variance	36.56259589	
Hypothesized Mean Difference	0	
df	81	
t Stat	-0.261593563	
P(T<=t) one-tail	0.397149423	
t Critical one-tail	1.663883913	
P(T<=t) two-tail	0.794298846	
t Critical two-tail	1.989686323	

Appendix Table 7: Unpaired t-test comparing the number of wild-type and *fgsg_03881Δ* infected spikelets in the susceptible variety of wheat 'Roblin' 14 days after point inoculation with 1,000 spores

	wild-type	*fgsg_03881Δ*
Mean	9.536585366	12.35714286
Variance	59.35487805	51.84494774
Observations	41	42
Pooled Variance	55.5535553	
Hypothesized Mean Difference	0	
df	81	
t Stat	-1.723679652	
P(T<=t) one-tail	0.044290395	
t Critical one-tail	1.663883913	
P(T<=t) two-tail	0.088580791	
t Critical two-tail	1.989686323	

Appendix Table 8: Unpaired t-test comparing the number of wild-type and *FGSG_03881/fgsg_03881Δ* infected spikelets in the susceptible variety of wheat 'Roblin' 14 days after point inoculation with 1,000 spores

	wild-type	*FGSG_03881/fgsg_03881Δ*
Mean	9.536585366	10.71428571
Variance	59.35487805	65.5261324
Observations	41	42
Pooled Variance	62.47859939	
Hypothesized Mean Difference	0	
df	81	
t Stat	-0.678651305	
P(T<=t) one-tail	0.249646625	
t Critical one-tail	1.663883913	
P(T<=t) two-tail	0.499293249	
t Critical two-tail	1.989686323	

Appendix Table 9: Unpaired t-test comparing the number of wild-type and *fgsg_03881Δ* infected spikelets in the susceptible variety of wheat 'Roblin' 21 days after point inoculation with 1,000 spores

	wild-type	*fgsg_03881Δ*
Mean	12.82926829	13.61904762
Variance	47.74512195	43.94889663
Observations	41	42
Hypothesized Mean Difference	0	
df	81	
t Stat	-0.531152825	
P(T<=t) one-tail	0.298383197	
t Critical one-tail	1.663883913	
P(T<=t) two-tail	0.596766395	
t Critical two-tail	1.989686323	

Appendix Table 10: Unpaired t-test comparing the number of wild-type and *FGSG_03881/fgsg_03881Δ* infected spikelets in the susceptible variety of wheat 'Roblin' 21 days after point inoculation with 1,000 spores

	wild-type	*FGSG_03881/fgsg_03881Δ*
Mean	12.82926829	12.45238095
Variance	47.74512195	58.64401858
Observations	41	42
Hypothesized Mean Difference	0	
df	81	
t Stat	0.23551773	
P(T<=t) one-tail	0.407200681	
t Critical one-tail	1.663883913	
P(T<=t) two-tail	0.814401363	
t Critical two-tail	1.989686323	

Appendix Table 11: Unpaired t-test comparing the number of wild-type and *fgsg_03881Δ* infected spikelets in the resistant variety of wheat 'Sumai 3' 7 days after point inoculation with 1,000 spores

	wild-type	*fgsg_03881Δ*
Mean	0.533333	0.625
Variance	0.257471	0.241935
Observations	30	32
Pooled Variance	0.249444	
Hypothesized Mean Difference	0	
df	60	
t Stat	-0.72221	
P(T<=t) one-tail	0.236485	
t Critical one-tail	1.670649	
P(T<=t) two-tail	0.47297	
t Critical two-tail	2.000297	

Appendix Table 12: Unpaired t-test comparing the number of wild-type and *FGSG_03881/fgsg_03881Δ* infected spikelets in the resistant variety of wheat 'Sumai 3' 7 days after point inoculation with 1,000 spores

	wild-type	*FGSG_03881/fgsg_03881Δ*
Mean	0.533333	0.612903
Variance	0.257471	0.245161
Observations	30	31
Pooled Variance	0.251212	
Hypothesized Mean Difference	0	
df	59	
t Stat	-0.61988	
P(T<=t) one-tail	0.268863	
t Critical one-tail	1.671092	
P(T<=t) two-tail	0.537726	
t Critical two-tail	2.000997	

Appendix Table 13: Unpaired t-test comparing the number of wild-type and *fgsg_03881Δ* infected spikelets in the resistant variety of wheat 'Sumai 3' 14 days after point inoculation with 1,000 spores

	wild-type	*fgsg_03881Δ*
Mean	0.866667	0.71875
Variance	1.222989	0.337702
Observations	30	32
Pooled Variance	0.76559	
Hypothesized Mean Difference	0	
df	60	
t Stat	0.66521	
P(T<=t) one-tail	0.254233	
t Critical one-tail	1.670649	
P(T<=t) two-tail	0.508465	
t Critical two-tail	2.000297	

Appendix Table 14: Unpaired t-test comparing the number of wild-type and *FGSG_03881/fgsg_03881Δ* infected spikelets in the resistant variety of wheat 'Sumai 3' 14 days after point inoculation with 1,000 spores

	wild-type	*FGSG_03881/fgsg_03881Δ*
Mean	0.866667	0.967742
Variance	1.222989	1.098925
Observations	30	31
Pooled Variance	1.159905	
Hypothesized Mean Difference	0	
df	59	
t Stat	-0.36645	
P(T<=t) one-tail	0.357672	
t Critical one-tail	1.671092	
P(T<=t) two-tail	0.715343	
t Critical two-tail	2.000997	

Appendix Table 15: Unpaired t-test comparing the number of wild-type and *fgsg_03881Δ* infected spikelets in the resistant variety of wheat 'Sumai 3' 21 days after point inoculation with 1,000 spores

	wild-type	*fgsg_03881Δ*
Mean	1.166667	1.59375
Variance	4.557471	5.797379
Observations	30	32
Pooled Variance	5.19809	
Hypothesized Mean Difference	0	
df	60	
t Stat	-0.73711	
P(T<=t) one-tail	0.231965	
t Critical one-tail	1.670649	
P(T<=t) two-tail	0.46393	
t Critical two-tail	2.000297	

Appendix Table 16: Unpaired t-test comparing the number of wild-type and *FGSG_03881/fgsg_03881Δ* infected spikelets in the resistant variety of wheat 'Sumai 3' 21 days after point inoculation with 1,000 spores

	wild-type	*FGSG_03881/fgsg_03881Δ*
Mean	1.166667	1.354839
Variance	4.557471	4.036559
Observations	30	31
Pooled Variance	4.292601	
Hypothesized Mean Difference	0	
df	59	
t Stat	-0.35463	
P(T<=t) one-tail	0.362067	
t Critical one-tail	1.671092	
P(T<=t) two-tail	0.724134	
t Critical two-tail	2.000997	

Appendix Table 17: Unpaired weighted t-test comparing the amount of DON quantified in wild-type and *fgsg_03881Δ* infected spikelets 10 days after point inoculation with 1,000 spores and quantified by ELISA

	wild-type	*fgsg_03881Δ*
Mean (ppm)	74.29787234	125.4489796
Variance	1423.706142	3085.914341
Observations*	10	10
Hypothesized Mean Difference	0	
df	18	
t Stat	2.408712919	
P(T<=t) one-tail	0.01349	
t Critical one-tail	1.734063062	
P(T<=t) two-tail	0.02698	
t Critical two-tail	2.100923666	

*corresponds to the number of pooled wheat head samples