SECRETED PROTEINS FROM THE PLANT PATHOGENIC FUNGUS *FUSARIUM GRAMINEARUM*: IDENTIFICATION, ROLE IN DISEASE, AND BIOTECHNOLOGICAL APPLICATIONS

By

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ABSTRACT

SECRETED PROTEINS FROM THE PLANT PATHOGENIC FUNGUS *FUSARIUM GRAMINEARUM*: IDENTIFICATION, ROLE IN DISEASE, AND BIOTECHNOLOGICAL APPLICATIONS

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Fusarium graminearum, the causal agent of wheat head blight, is a necrotrophic pathogen of cereal crops worldwide, including wheat, maize and barley. Like other fungi, it acquires nutrients by secreting a large number of degradative enzymes into its environment. During pathogenesis, these proteins represent one of the first contacts between the pathogen and its host. In this dissertation, the secreted proteins of *F. graminearium* were identified and studied for their role in pathogenesis.

The proteins secreted by *F. graminearum* during growth *in vitro* on 13 media containing different carbon sources and *in planta* during the infection of wheat heads were identified using mass spectrometry based proteomics. Out of a total of 256 fungal proteins identified, forty-nine were found in *in planta* growth but in none of the *in vitro* conditions. These proteins included secreted glycosyl hydrolases, unknown proteins, and a surprisingly large number of housekeeping proteins. The role of 38 of the *in planta*-specific proteins in pathogenesis was investigated by creating gene replacement mutants. None of the mutants had a strong virulence phenotype, probably due to genetic redundancy. One mutant in a gene encoding a putative alpha-arabinofuranosidase showed a small but statistically significant decrease in virulence.

The secreted proteins were also investigated for their possible activity as elicitors, i.e., triggers of defense responses in *Arabidopsis*. The same culture filtrates used for proteomics analysis were assayed on *Arabidopsis* tissue for initiation of classic defense responses such as stunting of growth and induction of reactive oxygen species (ROS). The culture filtrates did inhibit the growth of Arabidopsis seedlings but did not induce ROS.

One of the proteins that *F. graminearum* secretes *in vitro* on various carbon sources, including corn cell walls, is a putative α-fucosidase, but this functional assignment had never been proven biochemically. The gene was expressed in *Pichia pastoris* and demonstrated to have activity on fucosylated pea xyloglucan but not on the model substrate p-nitrophenyl-fucoside. An α- fucosidase from *F. oxysporum* that was previously biochemically identified but whose gene had never been cloned was purified and its gene cloned. This protein has activity on pNP-fucoside but not pea xyloglucan. The *F. graminearum* and *F. oxysporum* fucosidases are the first fungal fucosidases to be completely characterized from activity to protein to gene. They have possible applications in the conversion of biomass to fermentable sugars.

Dedicated to my teachers, especially my parents, who have greatly influenced me.

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CHAPTER 1: LITERATURE REVIEW

A primary characteristic of the Kingdom Mycota is their secretion of hydrolytic enzymes into the extracellular environment to break down complex substrates into simple metabolites that are then absorbed for nutrition. For this reason, fungi are important in all ecosystems as major decomposers of organic material. They are also economically important as plant pathogens, plant symbionts, fermentation agents, and for supplying many enzymes, antibiotics and other chemicals like citric acid and amino acids (Deacon, 2005). There is also significant interest in fungal secreted hydrolytic enzymes to break down lignocellulosic material to release fermentable sugars suitable for conversion into biofuels (Banerjee et al., 2010d; Gibson et al., 2011; King et al., 2011).

During plant pathogenesis, fungi secrete many proteins into the host. These proteins have been studied as virulence factors and also as agents of recognition by plants to induce defenses. Here, I will review how secreted and non-secreted proteins from fungi and other organisms are involved in plant pathogenesis, and will examine how secreted fungal proteins may also be used in the enzymatic digestion of plant cell walls for biofuel production.

The zig-zag model of plant immunity

Plants have developed an innate immune system that allows them to detect and respond to pathogens. The first component of this system involves recognizing the pathogen through cell surface receptors specific to a conserved molecular signature essential to the pathogen itself, called a PAMP, for pathogen associated molecular pattern, or MAMP, for microbe associated molecular pattern (Boller and He, 2009; Chisholm et al., 2006; Hematy et al., 2009; Jones and Dangl, 2006; Zipfel, 2008; Zipfel and Felix, 2005). These receptors, commonly referred to PRR's (pattern recognition receptors) usually include a leucine-rich repeat (LRR) receptor on the outside of the cell and a transmembrane domain (Boller and Felix, 2009; Dodds and Rathjen, 2010). They may or may not be attached to intracellular receptor-like kinase (RLK) domains. Those receptors without RLK domains are referred to as receptor-like proteins (RLPs) (Zipfel, 2008). Binding of a MAMP or PAMP to its cognate receptor triggers a defense response that ultimately results in inhibition of the growth of the pathogen (Ausubel, 2005). This initial response is called PAMP-triggered immunity (PTI) (Chisholm et al., 2006; Jones and Dangl, 2006). Pathogens have evolved proteins, called effectors, to suppress this initial response. When these effectors successfully suppress host responses, the plant becomes susceptible, in what is referred to as effector-triggered susceptibility (ETS) (Chisholm et al., 2006; Jones and Dangl, 2006). Plants have also evolved receptors specific for effectors, resulting in effector-triggered immunity (ETI) that in turn allows the plant to be resistant to the pathogen. In what has been coined an arms race between pathogen effectors and plant receptors, plant receptors and

pathogen effectors co-evolve to evade or maintain pathogenesis (Boller and Felix, 2009; Boller and He, 2009; Chisholm et al., 2006; Jones and Dangl, 2006).

A zig – zag model has been used to describe this process with the initial PTI and the secondary ETI responses represented as a line pointing up towards immunity and the introduction of effectors forcing the line back down towards susceptibility (Jones and Dangl, 2006). Although it has been proposed that the strength of the plant immune response increases from PTI to ETI (i.e., the hypersensitive response resulting in localized cell death occurs after ETI), there are exceptions to this rule. That is, strength of the response is dependent on individual plant species and pathogens (Bailey et al., 1990; Ron and Avni, 2004; Rotblat et al., 2002; Zipfel, 2008).

PAMP/MAMP triggered immunity (PTI)

Historically, molecules that initiate a defense response at low concentrations have been defined as elicitors (Boller and Felix, 2009). Defense responses include stunted seedling growth, a burst of reactive oxygen species (ROS), alkalinization across the cell membrane, synthesis of ethylene, and the accumulation of pathogenesis-related proteins (PRs). These elicitors are now more commonly referred to as PAMPs or MAMPs. There are many different types of elicitors. I will review several well-characterized examples of MAMPs of bacteria, fungi, and oomycetes.

Bacterial MAMP-triggered immunity

Two of the best understood elicitors are bacterial flagellin and elongation factor tu (EF-tu). A peptide fragment from the bacterial flagellin protein (flg22) is perceived by the receptor FLS2, an LRR-RLK. FLS2 has been characterized in Arabidopsis and tomato. Binding of flg22 to FLS2 initiates a defense signal cascade (Chinchilla et al., 2006; Gomez-Gomez et al., 1999; Robatzek et al., 2007; Zipfel et al., 2004). Those plants or, in the case of Arabidopsis, ecotypes, that lack a functional receptor cannot perceive flagellin (Bauer et al., 2001). EF-tu is another characterized MAMP. A conserved 18-26 amino acid peptide of EF-tu, called elf18, binds to a specific receptor in Arabidopsis, called ERF, which is also an LRR-RLK, to initiate PTI (Kunze et al., 2004; Shiu et al., 2004; Zipfel et al., 2006).

Once flg22 and elf18 bind to FLS and ERF, respectively, both of these receptors interact with BAK1 (BRI1-associated receptor kinase 1), which is a positive regulator of PTI (Chinchilla et al., 2007; Heese et al., 2007; Shan et al., 2008). One of the earliest known responses to BAK1/FLS2 dimerization is the opening of anion channels in the plasma membrane. Dimerization of BAK1 with FLS2 is dependent on an influx of calcium (Jeworutzki et al., 2010). It is hypothesized that the dimerization of BAK1 with aPRR induces phosphorylation of both proteins resulting in the start of a MAPK (mitogen activated protein kinase) and/or CADK (calcium-dependent protein kinase) signal cascade(s) (Cardinale et al., 2000; Dodds and Rathjen, 2010; Nuhse et al., 2000).

Oomycete MAMP triggered immunity

Cell walls from the oomycete *Phytophthora* species contain an abundant glycoprotein that can trigger PTI. Pep-13 is a highly conserved sequence in a cell wall-associated transglutamase common to all members of the genus. It triggers a defense-related response in parsley, *Arabidopsis* and potato, which represent two non-host and one host plant, respectively (Brunner et al., 2002; Fellbrich et al., 2002; Hahlbrock et al., 1995; Nurnberger et al., 1994). Thus, MAMP triggering occurs in Oomycetes as well as bacteria.

Fungal MAMP triggered immunity

Many fungal molecules, such as chitin and ergosterol, are elicitors of plant defense responses. The receptor for chitin (CeBiP) has been identified in rice. Unlike ERF and FLS2, this receptor consists of an extracellular domain containing a lysine motif (Lys-M domain), a transmembrane region, and a short cytoplasmic tail (Kaku et al., 2006). Mutation in one of the Lys M-containing genes of Arabidopsis (CERK1) results in the loss of chitin-induced PTI (Miya et al., 2007; Wan et al., 2008).

Another well-characterized fungal MAMP is a xylanase from *Trichoderma viride*. This protein induces ethylene production, which is a hallmark of plant defense (Furman-Matarasso et al., 1999). Xylanase activity is not required for the response, but a peptide fragment containing five amino acids located on an exposed strand of the protein is sufficient (Furman-Matarasso et al., 1999; Rotblat et al., 2002). The receptor for this peptide has been identified in tomato (LeEIX2) and is an RLP consisting of a LRR

extracellular domain, a transmembrane domain, and a cytoplasmic tail containing an endocytosis signal (Ron and Avni, 2004). This receptor may interact with a protein that induces endocytosis of the receptor, thereby initiating PTI (Bar and Avni, 2009). This ultimately leads to a PTI response that includes HR (Furman-Matarasso et al., 1999).

DAMP triggered immunity

Molecules from the host can also act as elicitors to trigger defense responses. These molecules are released from host macromolecules as a result of damage and now are known as DAMPs (damage-associated molecular patterns) (Boller and Felix, 2009). Some of these elicitors, including oligogalacturonides and cutin monomers, are released from plant cell walls by the action of pathogen enzymes (Darvill and Albersheim, 1984; Kauss et al., 1999).

Several endogenous peptides are also recognized as DAMPs (Boller and Felix, 2009). A peptide fragment from the protein systemin triggers PTI in tomato plants. Although the precursor to systemin is a cytoplasmic protein, it is thought to be released from the cell by injury (Lotze et al., 2007). Other endogenous peptides include *A.t.*Pep1, a 23-amino acid peptide from a protein precursor encoded by a gene induced upon wounding, and peptides that come from secreted proteins, and RALF (for rapid alkalinization inducing factor) a peptide originally isolated from tobacco but apparently ubiquitous in plants (Huffaker et al., 2006; Pearce et al., 2001a, b). These peptides initiate common hallmarks of PTI, including stunted growth, alkalinization, and MAPK

signaling (Huffaker et al., 2006; Pearce et al., 2001a, b). The receptors for these proteins are still unknown.

There are over 600 RLKs or RLPs in Arabidopis and an equally large number in rice. Few of these genes have been characterized, indicating that there may be significantly more MAMP/PAMP/DAMP receptors than are currently known (Shiu et al., 2004). If indeed the idea of an evolutionary arms race is correct, there should be many more molecules from other organisms that also trigger PTI (Boller and He, 2009). In particular, relatively little is known about possible MAMP/DAMPs from fungi, so there may still be many to be discovered from this group of important pathogens. A wide variety of secreted fungal proteins could function as MAMPs, either directly or by releasing DAMPs from plant macromolecules such as cell wall polysaccharides.

Effector Triggered Susceptibility and Immunity (ETS and ETI)

In order for a microbe to become pathogenic on a host, it must either not trigger PTI or it must suppress it. Since most triggers of PTI are highly conserved parts of essential cellular components, pathogens have often taken the strategy of suppressing PTI (Boller and He, 2009; Chisholm et al., 2006; Jones and Dangl, 2006). Historically, the gene-for-gene theory of pathogenicity defined effectors as avirulence factors (encoded by avr genes). This terminology was used because these effectors were initially identified by their genetic interaction with corresponding resistance (R) genes in the host. The result of recognition is to cause the pathogen to be avirulent as a result of

ETI. When the effector is not recognized by the host (because there is no R gene present) the plant is susceptible as a result of ETS (Boller and He, 2009; Chisholm et al., 2006; Dodds and Rathjen, 2010; Jones and Dangl, 2006). Here I will review what is known about a few of these effectors from different organisms.

Bacterial effectors

Effectors from bacterial pathogens are secreted into the host cytoplasm through a needle-like structure called the type III secretion system (T3SS). In one genome there can be as many as 20 -30 effectors with sometimes a high level of redundancy. One of the best characterized bacterial pathogens employing T3SS is *Pseudomonas syringae Pto* DC3000. Effectors from *P. syringae* have targeting motifs to direct them to the T3SS. Two effectors in *P. syringae*, AvrPto and AvrPtoB, suppress PTI by targeting the perception of the PAMP itself. These effectors directly interact with FLS2, thereby preventing the kinase from transferring the signal. Other *P. syringae* effectors target the PTI signal cascade downstream of the receptor. HopA1 is a phosphothreonine lyase that is responsible for dephosphorylating MAPKs within the signal cascades (Boller and He, 2009; Zhang et al., 2007). Several effectors (AvrB, AvrRPM1, AvrRpt2 and HopF2) target the plant protein RIN4, although it is still unclear exactly how RIN4 regulates PTI (Mudgett, 2005) (Dodds and Rathjen, 2010). Not all effectors target PTI. In *Xanthomonas*, the TAL (transcription activator-like) effectors target gene promoters to induce expression of specific genes (Boch et al., 2009; Moscou and Bogdanove, 2009).

Oomycete effectors

There are two main types of effectors from oomycete pathogens (Kamoun, 2007). The RXLR effectors, including ATR1, ATR13, AVR3a and AVR1b, contain a secretion signal and a defined N-terminal motif (Birch et al., 2009; Birch et al., 2006; Kamoun, 2006). The RxLR motif is responsible for entry of the effector (comprised of the C- terminal portion of the protein) into the host cell (Bos et al., 2006). Hundreds of these effectors have been identified bioinformatically in the genomes of several haustorium-forming species, including *Phytophthora* and *Hylanoperonospora*. They accumulate in the haustorium before delivery into the plant cell (Kamoun, 2006, 2007; Tyler et al., 2006). Although the suppression function of these elicitors is not clear, AVR3a is thought to bind to an E3 ligase required for initiation of programmed cell death (PCD), thus preventing signaling of ETI (Gilroy et al., 2011). Those plants expressing the corresponding potato R-gene (R3a) are not susceptible to attack (Armstrong et al., 2005; Bos et al., 2006; Whisson et al., 2007).

The second group of effectors in oomycete pathogens includes the Crinkler (CRN) proteins (Kamoun, 2007). Like RxLR effectors, CRNs are modular proteins with an N-terminal secretion signal and a conserved motif (LxLFLAK) that mediates import into the host cell. Another conserved motif is found at the end of the N-terminal domain and represents the space where the C-terminal effector is located (Haas et al., 2009; Torto et al., 2003). These effectors are also highly conserved and are even found in non-haustorium forming species, indicating that the mechanism of effector transportation evolved early (Schornack et al., 2010). The effector proteins of CRNs are

targeted to the nucleus. One, CRN8, accumulates in the nucleus, where it inhibits nuclear reactions to pathogen attack (Schornack et al., 2010; Torto et al., 2003).

Fungal effectors

Effector proteins of filamentous fungi are not as well described as those from bacteria and oomycetes, but recent advances have improved our understanding. Like oomycete effectors, these proteins are secreted and must either act in the apoplast or be transported into the cell. Fungal pathogens have many different lifestyles and many different pathogenesis strategies. Biotrophs parasitize their hosts without killing until late in infection, whereas necrotrophs are those that kill their hosts relatively quickly (Deacon, 2005). Hemibiotrophs are in between – initially they grow within the tissue without causing cell damage or killing, but cell death occurs later in infection.

Effectors of biotrophic fungi

Biotrophs, like oomycetes, form haustoria that form an interface between the pathogen and plant, allowing the pathogen to acquire nutrients from the host cell. The haustorium is also the location where effector molecules are expressed and transferred into the host cell (Deacon, 2005). *Blumeria graminis* and *Melampspora lini* are two fungal species that form haustoria. Effector molecules of both organisms are small proteins. Those from *M. lini* contain secretion signals for transport, whereas those from *B. graminis* do not. Effectors from both organisms act within the host cell as evidenced by a direct interaction with host cytoplasmic R proteins (Dodds et al., 2004; Dodds et al., 2009; Duplessis et al., 2011; Lawrence et al., 2007; Ridout et al., 2006).

Although hemibiotrophs, such as *Magnaporthe oryzae*, the causative agent of rice blast, do not form haustoria, they develop structures that form a close interface with their host cells (Dodds et al., 2009; Valent and Khang, 2010). *M. oryzae* uses an appressorium to penetrate rice cell walls and then grows throughthe tissue, invaginating the plasma membrane (Valent and Khang, 2010). There is a great diversity of R-genes and effectors within both rice and *M. oryzae* respectively. This diversity has apparently resulted in multiple strategies for effector targeting (Ballini et al., 2008; Liu et al., 2010; Wang and Valent, 2009). After appressorial penetration, the first elongating hypha forms a specific structure as an interface between the fungus and the host's plasma membrane. Effectors such as AVR-Pita, PWL1 and PWL2 accumulate in the biotrophic interfacial complex (BIC) (Liu et al., 2010; Valent and Khang, 2010). Using GFP tags of these proteins, it was shown that the secretion signal of the effectors were sufficient for effector transport into the cytoplasm (Khang et al., 2010; Mosquera et al., 2009).

Another effector (ACE1), recognized by the R-gene Pi33, is a cytoplasmic non-ribosomal peptide synthetase/polyketide synthase. It is active only while the apressorium is penetrating the cuticle (Collemare et al., 2008a; Collemare et al., 2008b). It is hypothesized that once inside the host cell, this large protein synthesizes the actual effector molecule (Böhnert et al., 2004). The *M. oryzae* genome contains the largest number of hybrid PKS-NRPS genes of all sequenced fungi, leading to the intriguing thought that there may be many more PKS-NRPS effectors (Collemare et al., 2008a; Collemare et al., 2008b; Valent and Khang, 2010).

Effectors of necrotrophic fungi

Necrotrophic fungi have previously been thought to cause disease through the secretion of toxins and cell wall degrading enzymes without the involvement of effectors that interact with the plant immune system (Hammond-Kosack and Rudd, 2008; van Kan, 2006). Recently, the identification of host targets for toxins and other effector molecules it is becoming clear that the interactions of necrotrophs with their hosts is more subtle that previously thought (Hammond-Kosack and Rudd, 2008; van der Does and Rep, 2007). For example, *Fusarium oxysporum* is a diverse species containing non- virulent and virulent host-specific strains. It causes vascular wilt disease on a broad range of hosts (Michielse and Rep, 2009). These strains are described by the term *formae specialis*, (f.sp.) indicating the host from which the strain was isolated.

F. oxysporum has been shown to make a number of specific effectors. Avr1, Avr2 and Avr3, were identified as proteins extracted from infected tomato xylem (Houterman et al., 2008; Houterman et al., 2009; Houterman et al., 2007). Avr3 is genetically recognized by the corresponding tomato R-gene, I-3, with this interaction being required for resistance (Rep et al., 2004). Other small secreted proteins were also identified in the xylem tissue; the corresponding genes are referred to as SIX genes (for Secreted In Xylem) (Houterman et al., 2007).

Fungal and oomycete effector-targeting and import

Biotrophic and necrotrophic fungal effectors do not have a motif like the RxLR domain of oomycete effectors (Kale et al., 2010). The N-terminal region of several fungal effectors is sufficient to import GFP fusions and Avr1b from *Phytophthora infestans* into plant cells. By investigating the RxLR motif in oomycetes further to determine what amino acids properties are essential to function, the motif for five fungal effectors could be confirmed (Kale et al., 2010). It has been suggested that fungal effectors possess a "loose" targeting motif compared to Oomycetes (Kale et al., 2010).

Although the RxLR motif of oomycete effectors is well defined, and recent work has identified the targeting motif in some fungal effectors, the actual mechanism of import into the host cell is still unknown. In preliminary experiments, Kale et al. (2010) showed that the both oomycete and fungal effectors can bind to phosphatidylinostitol 3-phosphate (PI3P) on the surface of animal cells as well as plant cells (Kale et al., 2010). Upon binding to the ligand, PI3P mediates the uptake of the effector through endocytosis. This work hints at a possible conserved mechanism for effector entry in animals and plants.

Cell wall degrading enzymes secreted by fungi

Several secreted hydrolytic enzymes of pathogenic fungi are important virulence factors, including specific xylanases of *Botrytis cinerea* and a polygalacturonase in *Claviceps purpurea* (Brito et al., 2006; Oeser et al., 2002). Secreted lipases are also

virulence factors for *F. graminearum*, *Alternaria brassicicola*, and *B. cinerea* (Berto et al., 1999; Commenil et al., 1995; Voigt et al., 2005). There is contradictory evidence whether cutinases have a role in initiating the onset of disease (Belbahri et al., 2008; Rocha et al., 2008; Skamnioti and Gurr, 2008).

Although proteomic studies of secreted proteins from pathogens have identified a diversity of hydrolytic enzymes, specific secreted hydrolytic enzymes are virulence factors in some pathogen/host interactions but not all (Nagendran et al., 2009; Paper et al., 2007; Phalip et al., 2005; Phalip et al., 2009; Taylor et al., 2008; Wang et al., 2005). For example, polygalacturonase is a virulence factor in *C. purpurea*, but not in *Cochliobolus carbonum* (Oeser et al., 2002; Scott-Craig et al., 1998; Scott-Craig et al., 1990). Hydrolytic enzymes may also have a role in activating host defense responses. Some products of hydrolytic enzymes derived from plant cell walls can act as DAMPs to activate PTI (Boller and Felix, 2009; Kauss et al., 1999).

The role of fungal enzymes in lignocellulosic biofuel production

In order to reduce the use of oil and other non-renewable fuels, interest in the production of fuels from lignocellulosic material has risen (Langridge, 2011). Cell walls consist of cellulose microfibrils intertwined with hemicelluloses and lignin. Multiple enzymes are needed to break the diverse bonds in this complex substrate to release sugars that can be fermented into fuels such as ethanol (Banerjee et al., 2010d; McCann and Carpita, 2008; Pauly and Keegstra, 2010).

Commercial enzyme mixtures available today for cellulose degradation are expensive and not optimized for diverse lignocellulosic substrates. The effectiveness and cost of these mixtures might be improved by optimizing enzyme loading and adding accessory enzymes to hydrolyze specific polymers. An optimized enzyme mixture would contain the correct amount of each enzyme needed for degradation of any particular biomass (Banerjee et al., 2010d; Lynd et al., 2008). Optimization has begun by robotically performing thousands of digestions with different combinations of enzymes (Banerjee et al., 2010a; Banerjee et al., 2010b; Banerjee et al., 2010c). Because the same enzymes that pathogenic and saprophytic fungi secrete to degrade cell wall polymers for nutrients can also be used to degrade cell walls into fermentable sugars for biofuel production, fungal proteins are being studied as sources of enzymes for biofuel applications.

Fungal pathogens secrete a diversity of hydrolytic enzymes to promote infection and to support saprophytic growth in the absence of the host. *F. graminearum*, *B. cinera*, and *M. grisea* all have a large and diverse set of genes encoding cellulases and hemicellulases (Gibson et al., 2011; King et al., 2011). The enzymes from *F. graminearum* may be particularly promising because this fungus causes disease in a broad range of cereals, which are the major feedstocks for lignocellulosic ethanol (Gibson et al., 2011; King et al., 2011; Phalip et al., 2009). Insight can be gained to what types and in some cases concentrations of enzymes are needed by investigating their range of secreted proteins, known as the secretome (Gibson et al., 2011; King et al., 2011). Proteomic investigation of fungal pathogens has already identified many glycosyl hydrolases secreted on different media and *in planta* (Paper et al., 2007; Phalip

et al., 2005; Phalip et al., 2009). These data are being used to identify the different types and, it is hoped, ultimately more efficient enzymes for cellulose degradation. Since there is great diversity in the fungal kingdom and each fungus can secrete hundreds of proteins, there is a high potential for finding novel and better enzymes.

Fusarium graminearum

Economic Importance

Fusarium graminearum (telomorph *Gibberella zeae)*, is the causal agent of Fusarium head blight (FHB) or scab. It infects wheat, barley, oats and corn in all parts of the world (Goswami and Kistler, 2004; McMullen et al., 1997; Windels, 2000). Within the last twenty years *F. graminearum* has become a major wheat pathogen in Southern Canada and the North Central United States (Windels, 2000). The infection, which occurs mostly in temperate areas during warm periods, can reduce crop yields by causing plant bleaching and reduced grain yield, but its major economic impact is due to its production of mycotoxins (Goswami and Kistler, 2004; McMullen et al., 1997; Windels, 2000). Wheat that contains more than 10 ppm or 1 ppm is unusable for animal or human consumption respectively. From 1998 – 2000 Fusarium head blight resulted in an estimated $3 billion loss in central North America alone (Nganje et al., 2004). Since outbreaks depend on appropriate weather conditions, many years may

pass between severe disease outbreaks. Recent outbreaks in other parts of the world make it a limiting factor of wheat production world-wide (Goswami and Kistler, 2004).

Life cycle of F. graminearum

The disease is spread through saprophytic hyphae and perithecial initials (sexual structures for the development of ascospores) that have overwintered on dead plant tissue. During warmer weather, perithecia and conidia (asexual spores) are forcibly discharged or released, respectively (Guenther and Trail, 2005; Leonard and Bushnell, 2003; Markell and Francl, 2003; Trail, 2009). Disease development usually coincides with the development of flowers on the cereal hosts. Conidia are dispersed by wind, insects and rain (Trail et al., 2002). After germination of the spores, the fungus enters the host through stomata, anthers, or other sites in the inflorescence. *F. graminearum* also infects other tissue such as the foot of cereal plants and stalks of corn (Chakraborty et al., 2010; Mudge et al.).

Disease Progression

After germination, the hyphae most commonly colonize the floret. The disease then spreads through the rachis to neighboring florets. Symptoms include chlorosis of the infected floret. Under laboratory conditions it takes at least 4-5 d to see visible signs of infection, with symptoms being bleaching and sometimes browning of the spikelet. The awn attached to the spikelet also starts to distend (Guenther and Trail, 2005). At this time hyphae are generally growing down the rachis to the next spikelet.

Occasionally one can see thin fungal hyphae protruding from the floret. As the days progress, hyphal growth and infection of cells are visible microscopically, but macroscopic symptoms lag behind the infection front (Brown et al., 2010; Hallen et al.).

Twelve to 14 d after inoculation of susceptible wheat cultivars, the infection front grows down through the pith cavity and vessels into the stem, following which radial hyphae branch from the vertical hyphae and grow through the pith into the xylem cells and then grow cell-to-cell through direct penetration or pit fields (Brown et al., 2010; Guenther and Trail, 2005). Perethecia begin to develop when the hyphae emerge from the stem in substomatal cavities (Guenther and Trail, 2005). There is some question whether *F. graminearum* should be classified as necrotrophic (i.e., killing cells before digestion of their contents) or hemibiotrophic (i.e., able to live biotrophically in the cell for a period of time before causing death). Brown et al. (2010) conclude that *F. graminearum* is actually both, depending on the infection stage. Near the infection front it infects living cells, but the tissue becomes necrotic at later stages (Brown et al., 2010).

Genomic resources

F. graminearum provides a good model to study secreted fungal proteins and their interactions with their hosts. A high quality genome sequence is available and annotated (Cuomo et al., 2007; Guldener et al., 2006a; Wong et al., 2011). Furthermore, the sequencing of two other *Fusarium* species allows gene comparison studies (Ma et al., 2010; Rep and Kistler, 2010). *F. graminearum* is amenable to transformation and high levels of homologous recombination make targeted gene replacements possible. These facts, along with the availability of an Affymetrix GeneChip for genome-wide expression analysis, the data from several expression

studies, and inclusion in host/pathogen databases, have made it a valuable model in the study of necrotrophic fungal pathogens (Baldwin et al., 2006; Guldener et al., 2006a; Guldener et al., 2006b; Trail, 2009; Trail et al., 2003; Wong et al., 2011).

Known virulence determinants

The first virulence factor to be identified in *F. graminearum* was the trichothecene mycotoxin deoxynivalenol (DON). In head blight of wheat, DON or nivalenol are required for infection, but they are not required for crown root (Bai et al., 2002; Desjardins et al., 1996; Proctor et al., 1995). DON acts by preventing the host from strengthening the cell wall at infection (Jansen et al., 2005). Disruption of the gene, Tri5, that controls the first step in the biosynthetic pathway of tricothecenes halts production of DON and thereby reduces the virulence of the fungus on wheat by preventing the spread of the disease to the rachis (Bai et al., 2002; Desjardins et al., 1996; Proctor et al., 1995, 1997). In population genetic studies, different *F. graminearum* isolates were found to produce different chemotypes of tricothecenes (nivalenol, 3-acetyldeoxynivalenol, or 15-acetyldeoxynivalenol). These chemotypes appear to be maintained in wild populations, indicating a selective advantage to containing multiple forms of toxins (Starkey et al., 2007).

Other virulence factors have also been identified in *F. graminearum*. Voigt and colleagues (2005) showed that a mutant in a secreted lipase (FGL2) could not spread from the initial infected spikelet to the rachis and to other spikelets. They hypothesized that the lipase may function directly in cell wall degradation. The delay in pathogenesis

by the mutant may allow enough time for the plant to mount a defense response of forming a barrier at the rachis. Voigt et al. (2005) also proposed that the lipase could function indirectly, by releasing a messenger molecule to up-regulate the production of other cell wall degrading enzymes. For example, diacylglycerol released from the degradation of lipids could serve as this messenger (Voigt et al., 2005).

A topoisomerase I (TOP1) has also been suggested to be involved in virulence and to be necessary for perithecium development (Baldwin et al., 2010b). *In vitro* growth as well as conidium formation was equal or slightly reduced under some conditions. Baldwin et al. (2010 b) hypothesized that the reason for reduced virulence of the mutant was slowed growth of the fungus caused by a slowed gene expression response, giving the plant time to mount a stronger defense (Baldwin et al., 2010b).

Studies of sexual development have also shed light on other factors necessary for virulence of *F. graminearum*. Since forcibly discharged ascospores serve as the primary inoculum for the disease, understanding the regulation of sexual development could lead to the development of ways to break the disease cycle. Gene expression analysis of sexual development identified a subset of genes unique to perithecial development (Hallen et al., 2007). Lipid accumulation in overwintering hyphae is also required for sexual development and thus inoculum production (Guenther et al., 2009)

Gene expression analysis, large scale mutant screening, and proteomic analysis have also been used to better understand the pathogenicity of *F. graminearum*. Both gene expression and proteomic studies have both been done on the fungus grown in a variety of environments such as nutrient-limiting, mycotoxin-producing, and *in planta*

(Guldener et al., 2006b; Hallen et al., 2007; Hallen et al.; Paper et al., 2007; Phalip et al., 2005; Seong et al., 2008; Taylor et al., 2008). These data have allowed and will continue to allow comparison of transcription and translation information for particular environmental conditions. Metabolite profiling can distinguish between several wild type strains expressing different chemotypes of DON and mutants strains (Lowe et al., 2010). Large scale screening of mutants generated by random insertional mutagenesis has also identified regions of the genome that are involved in virulence (Baldwin et al., 2010a; Seong et al., 2005).

Genomic organization of *F. graminearum* and *F. oxysporum*

The sequences of three *Fusarium* species are complete and annotated, allowing valuable information to be gathered by comparison (Cuomo et al., 2007; Ma et al., 2010). This sequence information has revealed that *F. graminearum* has a lower number of repetitive sequences, transposable elements and duplicated genes than other sequenced fungal species (Cuomo et al., 2007). *F. graminearum* only has four chromosomes, whereas the other, more distantly related, *Fusarium* species contain eight to nine. It has been hypothesized that chromosomes from *F. graminearum* have fused, creating regions within each chromosome that undergo high rates of recombination (derived from telomeric regions of the ancestor unfused chromosomes) (Cuomo et al., 2007).

By comparing *F. oxysporum* to other *Fusarium* species (*F. graminearum and F. verticillioides*), lineage-specific regions were identified, including four entire chromosomes (Ma et al., 2010). The genes of the SIX effectors as well as other similar genes are located on chromosome 14. This chromosome is the basis of virulence on tomato. Furthermore, it has been demonstrated that this chromosome as well as another lineage-specific chromosome can transfer to non-pathogenic isolates of *F. oxysporum*, which then acquire the ability to infect tomato. It is hypothesized that this horizontal gene transfer is the basis for new pathogenic lines (Ma et al., 2010).

Many effector or virulence genes in Fusarium species like those described above are clustered in chromosomal regions with more repetitive DNA or near telomeres where there is higher probability of genomic reorganization (Cuomo et al., 2007; Fudal et al., 2007; Gout et al., 2007; Ma et al., 2010; Rep and Kistler, 2010). Genes with secretion signals, implicated in virulence, those expressed *in planta*, and genes encoding proteins found *in planta* were all present at a higher rate in these regions than elsewhere on chromosomes, indicating that genomic reorganization is important for pathogens (Cuomo et al., 2007; Ma et al., 2010; Paper et al., 2007; Rep and Kistler, 2010).

Chapter 2: Comparative proteomics of extracellular proteins *in vitro* and *in planta* from the pathogenic fungus *Fusarium graminearum**

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** This paper is dedicated to the memory of Hans Kende (1937-2006), esteemed colleague and friend.*

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Abstract

High-throughput MS/MS was used to identify proteins secreted by *Fusarium graminearum* (*Gibberella zeae*) during growth on 13 media *in vitro* and *in planta* during infection of wheat heads. *In vitro* secreted proteins were collected from the culture filtrates, and *in planta* proteins were collected by vacuum infiltration. A total of 289 proteins (229 *in vitro* and 120 *in planta*) were identified with high statistical confidence. Forty-nine of the *in planta* proteins were not found in any of the *in vitro* conditions. The majority (91–100%) of the *in vitro* proteins had predicted signal peptides, but only 56% of the *in planta* proteins. At least 13 of the non secreted proteins found only *in planta* were single-copy housekeeping enzymes, including enolase, triose phosphate isomerase, phosphoglucomutase, calmodulin, aconitase, and malate dehydrogenase. The presence of these proteins in the *in planta* but not *in vitro* secretome might indicate that significant fungal lysis occurs during pathogenesis. On the other hand, several of the proteins lacking signal peptides that were found *in planta* have been reported to be potent immunogens secreted by animal pathogenic fungi, and therefore could be important in the interaction between *F. graminearum* and its host plants.

Introduction

A defining characteristic of filamentous fungi is the secretion of a large number of degradative enzymes and other proteins, which have diverse functions in nutrient

acquisition, substrate colonization, and ecological interactions (de Vries, 2003) (Freimoser et al., 2003) (Walton, 1994). Several extracellular fungal enzymes, such as polygalacturonase, pectate lyase, xylanase, and lipase, have been shown or postulated to be required for virulence in at least one host/pathogen interaction (Brito et al., 2006; Deising et al., 1992; Isshiki et al., 2001; Oeser et al., 2002; ten Have et al., 1998; Voigt et al., 2005; Yakoby et al., 2001). In addition, some extracellular proteins, including certain polygalacturonases and xylanases, are elicitors of plant defense responses (Federici et al., 2006; Poinssot et al., 2003; Ron and Avni, 2004). Fungi also secrete many small, often cysteine-rich proteins with no known enzymatic activity, and some of these are also important in some host/pathogen interactions as phytotoxins or elicitors of plant defense responses(Keates et al., 2003; Lauge and De Wit, 1998; Manning and Ciuffetti, 2005; Rep, 2005; Rep et al., 2004; Rohe et al., 1995).

The recent availability of the complete genome sequences of several pathogenic fungi has opened new avenues to the identification of which among their ,14 000 genes have a role in pathogenicity (Xu et al., 2006). Subtracted cDNA libraries and microarrays have identified fungal genes expressed during growth *in planta* in comparison to different *in vitro* growth conditions (Goswami et al., 2006; Guldener et al., 2006a). Proteomics approaches complement and extend RNA-based methods. MS/MS on unfractionated proteins, in which predicted peptide masses and peptide fragmentation patterns are used to identify proteins, as opposed to direct *de novo* sequencing of individual proteins, permits high-throughput analysis of even complex protein mixtures with little or no prior fractionation(Yates, 2004). This method is particularly well suited to the study of two intermixed genomes, such as an infected

plant, because the proteins of the undesired partner are invisible to the computation software. Furthermore, with proteomics it is possible to use differential extraction methods to isolate the proteins that reside in a particular cellular compartment, for example, the secreted proteins (known as the secretome or the exoproteome) (Oh et al., 2005; Phalip et al., 2005).

Fusarium graminearum is a filamentous fungal pathogen of wheat, maize, and other grains. It is currently a serious agricultural and public health problem in several parts of the world, especially because it produces mycotoxins that contaminate harvested grain. Infection starts during early flower development upon infection with ascospores or conidia. The fungus spreads through the spikelet causing bleaching and shriveled grain(Goswami et al., 2006). The genome of *F. graminearum* has been sequenced and the resulting assembly displays high quality and contiguity (www.broad.mit.edu/annotation/genome/ fusarium_graminearum/Home.html). The quality of the genome sequence combined with significant manual reannotation of the gene models, curated through the *Fusarium graminearum* Genome Database (FGDB; http://mips.gsf.de/ genre/proj/fusarium/) makes *F. graminearum* particularly well suited for proteomics studies.

Proteomics has previously been applied to plant and animal pathogenic bacteria and fungi grown *in vitro*, and to plants in response to infection(Chivasa et al., 2006; Colditz et al., 2004; Oh et al., 2005; Phalip et al., 2005; Wang et al., 2005; Zhou et al., 2005). Previous proteomic analyses of *Fusarium*-infected wheat have focused on the host proteins. Wang *et al. (Wang et al., 2005)* and Zhou *et al.(Zhou et al., 2005)* identified 30 and 15 host proteins, respectively, that were upregulated by infection. After

the work, presented in this paper, was completed, Zhou *et al. (Zhou et al., 2006)* published the identification of 41 proteins that were differentially regulated during infection. Eight of these were fungal proteins, although identification of two cannot be unambiguously identified because they were based on obsolete gene models. The major limitation of previous proteomics studies of *F. graminearum* is their reliance on 2-DE to fractionate proteins. In infected plant tissue, where the fungal biomass is a small portion of the total, pathogen proteins will be minor component of the total, and gels cannot distinguish between the two. Here, we demonstrate that it is possible to identify fungal proteins in infected tissues without any prior fractionation. The proteins identified *in planta* differ significantly from those found when *F. graminearum* is grown *in vitro*.

Materials and methods

Growth of F. graminearum

For most of the *in vitro* experiments, *F. graminearum* PH-1 was grown on a base of HMT medium (also known as modified Fries'), which contains 5 g/L ammonium tartrate, 1 g/L NH_4NO_3, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4*7H_2O$, 0.13 g/L $CaCl_2*2H_2O$, 1 g/L NaCl, and 0.1% yeast extract. The medium was dispensed at 125 mL *per* 1-L Erlenmeyer flask, autoclaved, and inoculated with 1 mL of conidial suspension (106 spores). The flasks were incubated at room temperature (207C) for 1 wk without shaking. All medium supplements were added at 10 g/L. Pectin (citrus, P-9135), birchwood xylan (X-0502), and collagen (bovine Type 1, C-9879) were purchased from

Sigma. Cell walls were prepared from maize leaves grown in a standard greenhouse for 6 wk or from carrot roots purchased at a local organic grocery store. The maize leaves or carrots were lyophilized, ground in liquid N_2, and extracted sequentially with water, chloroform, and methanol before drying(Sposato et al., 1995). Maize bran was purchased from a local organic grocery store. Maize stover (leaves and stems) and dried distillers' grains (DDG) was obtained from the National Renewable Energy Lab and treated by the ammonia fiber expansion (AFEX) process as described(Teymouri et al., 2005). For comparison to previous gene expression studies, *F. graminearum* was also grown on complete medium, minimal media lacking carbon, or minimal medium lacking nitrogen (Guldener et al., 2006b).

For production of conidia, *F. graminearum* was grown on 1.5% carboxymethylcellulose (sodium salt, low viscosity, Sigma C-5678), 1% NH_4NO_3, 1% KH_2PO_4, 0.5% $MgSO_4*7H_2O$, and 1% yeast extract (Difco). Flasks (250-mL Erlenmeyer each containing 100 mL medium) were grown for 3 days at room temperature (217C) with shaking at 145 rpm. Conidia were collected by filtering through Miracloth (Calbiochem), and centrifugation at 15006X*g*. The conidia were resususpended in 0.01% Tween-20 to a concentrationof 106 spores/mL.

Wheat growth and inoculation

Wheat (*Triticum aestivum*) cultivar Norm (susceptible to *F. graminearum*) was sown in 4-inch clay pots in Baccto high porosity professional planting mix (Michigan Peat, Houston, TX) and grown in a greenhouse. Plants were inoculated at 8– 10 wk when the anthers began to extrude. In the first five experiments, the plants were

inoculated by injecting 10 mL of conidia (104 conidia) between the lemma and palea of as many florets on the flower head as possible, maximizing contact of the conidia with the anthers. In the other nine experiments, the heads were inoculated by aerosol spraying of the whole head and the heads then covered with plastic bags. All plants were placed in a mist chamber for 3 days, after which the bags were removed and the plants moved to a bench in the greenhouse until harvest 1–11 days later.

Protein extraction: In vitro proteins

After 7 days growth, culture filtrates were collected by filtering through cheesecloth and then lyophilized, redissolved in 3 mL of water, passed through a PD-10 (BioRad) desalting column (5 kDa molecular weight cutoff; (MWCO)), and lyophilized to 1 mL. A portion (100 mL) of each sample was precipitated by the addition of 0.11 volume of 100% w/v TCA. After centrifugation (15 000x*g*, 10 min), the protein pellet was washed three times with 80% v/v ethanol and redissolved in 50 mL of 50 mM ammonium bicarbonate.

Protein extraction: In planta proteins

Infected heads were cut from the plants, the awns trimmed, and the heads fully submerged in deionized water. Each sample comprised six to ten heads. The submerged heads were placed in a vacuum chamber (100 mm Hg) for 5 min. Three to four heads were then placed in a centrifuge tube with 1 inch of 3 mm glass beads and centrifuged at 1500x*g* for 10 min. The liquid at the bottom of the tube was collected,

lyophilized, redissolved in 3 mL of water, desalted, and lyophilized again to 1 mL. A portion (100 mL) of each sample was precipitated by the addition of 0.11 volume of 100% v/v TCA. After centrifugation (15 000x*g*, 10 min), the protein pellet was washed three times with 80% v/v ethanol and redissolved in 50 mL of 50 mM ammonium bicarbonate.

Protease digestion and MS

Each bulk sample was alkylated with iodoacetamide and digested with sequencing-grade modified porcine trypsin (Promega). One sample was fractionated on a BioRad 4–20% gradient SDS-PAGE gel. The gel was divided into 18 pieces and each was individually alkylated and digested in the gel. Samples were then analyzed by one of two methods. In the first, the digested peptides were automatically injected using a ThermoElectron Micro-Autosampler onto an Agilent Zorbax 300 SB-C18 560.3 mm peptide trap column and desalted for 10 min. The bound peptides were then eluted onto a 10 cm675 mm New Objectives Picofrit column packed with MicromMagic C18 AQ and eluted over 120 min with a gradient of 5–50% B in 75 min, then 50–90% B from 75 to 79 min using a ThemoElectron Surveyor high-pressure liquid chromatograph. Buffer A was 99.9% water 1 0.1% formic acid, and buffer B was 99.9% ACN 1 0.1% formic acid. The peptides were eluted into a ThermoElectron LTQ/FT mass spectrometer at a flow rate of 250 nL/min. Survey scans were taken at a resolution of 100 000 and the top ten ions in each survey scan were subjected to automatic low energy CID in the linear trap (LTQ).

In the second method, the extracted peptides were automatically injected by a Michrom Paradigm Endurance Bio-Cool Autosampler onto a Paradigm Platinum Peptide Nanotrap column (C18, 0.15650 mm) and desalted for 5 min. The bound peptides were

eluted onto a10 cm675 mm New Objectives Picofrit column packed with MicromMagic C18 AQ and eluted over 120 min with a gradient of 5–50% B, with constant 10% C, in 80 min, followed by 50–90% B, with constant 10% C, from 80 to 100 min using a Michrom Paradigm MD liquid chromatograph. Buffer A was 100% water, buffer B was 100% ACN, and buffer C was 1% formic acid. The peptides were eluted into a ThermoElectron LTQ mass spectrometer with a flow rate of 250 nL/min. The top five ions in each survey scan were subjected to data-dependent zoom scans followed by low energy CID. In both cases, the same dynamic exclusion parameters were used. The repeat count was 2, the duration 30 s, the list size 500, and the exclude duration 60 s. All resulting MS/MS spectra were converted to peak lists using BioWorks Browser v3.2 and saved in the MASCOT Generic Format (*.mgf).

MS files were searched against the FGDB as of February, 2006, using X!TANDEM (http://www.thegpm.org) (Craig and Beavis, 2004; Craig et al., 2004). The database contains 13 600 proteins. Search parameters consisted of a fragment mass error of 0.8 Da and parent mass error of 6 200 ppm. Complete and partial residue modifications were allowed as well as point mutations and two missed cleavage sites. Protein identifications were considered reliable with an *e* value of, 23.0 and at least two peptides. A simultaneous search against the peptide database in reverse sequence was run to evaluate the false positive rate. No reverse peptide hits achieved the cutoff of *e*, 23.0 and two peptides, indicating that the false positive rate was low to zero. As another control for false positives due to wheat proteins, proteins from mock-inoculated wheat plants were analyzed. Only three wheat proteins “matched” proteins in the *F. graminearum* database, and all three were below the significance threshold.

All proteins were reannotated by comparison to the latest models in the FGDB (http://mips.gsf.de/genre/proj/fusarium/). Because the FGDB is being continuously updated, sometimes gene models had been revised between the time of the experimental work and the analysis. When there was a discrepancy between the original analysis and a newer gene model that resulted in the splitting of one gene into two, the MS results were manually reanalyzed to determine which protein had been detected. All proteins were also reassessed for signal peptides using SignalP (Bendtsen et al., 2004), and by comparison to proteins of known function in the nonredundant GenBank database. This sometimes resulted in differences in assignment of probable function compared to the FGDB annotation.

Chromosome mapping

The locations along each chromosomal axis of the genes encoding all secreted proteins were determined by ordering and orienting supercontigs based on the genetic map (Gale et al., 2005). This anchored 99.8% of the *Fusarium* assembly to chromosomes. A default gap size of 10 000 bp between supercontigs was used to calculate overall chromosome position for each gene. Gene frequencies were calculated per 100 kb.

Results

In vitro proteome

In order to capture as much of the secretome as possible, *F. graminearum* was grown on ten carbon supplements in a common background medium. The fungus was also grown on the complete, carbon-limiting, and the nitrogen-limiting media used by Güldener *et al. (Guldener et al., 2006b)* for microarray transcription profiling. All experiments were done in at least two biological replicates. Uninoculated media yielded no significant protein identifications. Proteins were analyzed in bulk, *i.e.*, with no prior fractionation at the protein level except removal of molecules smaller than 5 kDa. A summary of the results is shown in Table 1.

A total of 228 proteins were found at high reliability *in vitro* (Table 1; Table S1 of Supporting Information). Phalip *et al. (Phalip et al., 2005)* reported 103 proteins in the secretome of *F. graminearum* grown on glucose or cell walls of hop (*Humulus lupus*). We found all of these except ten (FG01603 (now reannotated as FG13207), FG01671, FG02720, FG03875, FG03909, FG04678, FG04738, FG05906, FG07912 (now split into FG12384 and FG12385), and FG09142). The fact that some proteins were found by us and not by the earlier study (Phalip et al., 2005), and *vice versa*, probably indicates that neither of the experiments were saturating in regard to defining the entire secretome. Of the *in vitro* proteins, between 91 and 100% have predicted signal peptides (Table 1). Of those that lack predicted signal peptides, a few are most likely cytoplasmic houskeeping proteins (*e.g.*, FG00777, FG05615, FG05972, FG10942, and FG10677), some are probably misannotated (*e.g.*, fgd463-80, FG12104), and others are orthologous to

proteins known to be secreted by other fungi (*e.g.*, FG08946) (Table S1 of Supporting Information).

In 13 *in vitro* analyses, 56 proteins (25%) were found in only a single growth medium and 47 (21%) were found in eight or more media. Six proteins were found in all experiments: FG03662 (unknown), FG11164 (trypsin), FG11205 (SnodProt), FG11249 (unknown), FG11472 (serine protease), and FG12070 (subtilisin). Sucrose and collagen induced the smallest percentage of glycosyl hydrolases (GHs), and plant cell walls (maize, carrot, AFEX stover, or AFEX DDG) induced the highest percentages of GHs (Table 1). This is probably due to the higher degree of complexity of the polysaccharides in these substrates compared to the others, and because most of the GHs are regulated by catabolite repression and/or substrate availability. Sucrose induced the highest percentage of unknown proteins, which is also probably related to the fact that this medium suppresses the expression of GHs, many of whose functions are known. In the absence of any source of carbon (other than yeast extract), *F. graminearum* grew very poorly yet still secreted many proteins, predominantly GHs and proteases (Table 2.1).

Three-way comparisons of the proteins secreted during growth on different media revealed several trends (Fig. 2.1). Comparison of pectin, xylan, and collagen indicated that pectin and xylan, both of which are polysaccharides, had more proteins in common than either did with collagen. Carrot and maize cell walls had more proteins in common with each other than either did with sucrose, indicative of a similarity between dicot and cereal cell walls as inducers of catabolite-repressed genes (Fig. 2.1). In contrast to Phalip *et al.* (Phalip et al., 2005), we found a large overlap between the

proteins secreted on cell walls *vs.* sugar (Fig. 2.1). Whereas they found only four proteins in common between hop and glucose, we found 50 in common between maize cell walls and sucrose and 51 in common between sucrose and carrot cell walls (Fig. 2.1). In our experiments, 46 proteins could be considered to be constitutively expressed, defined as those found in six or more of the eight HMT-based media (Table S1 of Supporting Information). The difference in the results might be because of differences in the media used (glucose *vs.* sucrose, and maize and carrot *vs.* hop cell walls), or because Phalip *et al. (Phalip et al., 2005)* analyzed glucose proteins from 1-D gels but hop proteins from 2-D gels.

In planta proteome

A total of 14 *in planta* proteomics experiments were performed. For 13 of them, infected wheat heads were extracted by vacuum infiltration at 3–14 days after inoculation and the proteins digested and analyzed en masse. The number of proteins identified in different experiments ranged from 15 to 63. Many of the proteins appeared in multiple samples bothers were found only once. Included in the 14 samples were two time courses, in which the infected wheat heads were extracted over a period of 3–10 days after inoculation in one experiment and 3–14 days in the other. There were no discernible, repeatable time-dependent trends in the numbers or types of proteins found, perhaps because of inevitable small differences in inoculation protocols, variable growth of the pathogen and the host, and technical differences in the extraction and processing of the proteins. Analysis of proteins extracted from mock-inoculated wheat plants gave zero *F. graminearum* proteins with a score above the threshold of significance.

In one experiment, vacuum-extracted proteins were first separated by 1-D SDS-PAGE. The gel was cut into 18 sections and each section individually digested with trypsin and analyzed. This identified eight new proteins, and four of the ones identified in the bulk experiments were identified at higher statistical significance. On the other hand, six proteins found in the bulk experiments were not found in the gel slices.

From all of the experiments combined, a total of 120 fungal proteins were identified at high reliability *in planta* (Table 2.2). All of the proteins were rechecked for the presence of predicted signal peptides, for the presence of orthologs and paralogs, and for function based on sequence similarity to proteins of known or probable biochemical function. This resulted in the revision of some gene models and annotations, which have been submitted to FGDB (http://mips.gsf.de/genre/proj/fusarium/). Proteins that could not be assigned a probable function based on a high sequence similarity to proteins of biochemically established function are listed simply as “unknown” in the tables.

Of the *in planta* proteins whose function could be reliably determined, the two largest classes are enzymes that act on plant cell walls, and proteases. The first group (27 total) includes GHs of 15 different families(Coutinho and Henrissat, 1999), one lyase, and two esterases. All of these have signal peptides. Cytoplasmic FG04826 (xylulose reductase) and secreted FG11032 (galactose oxidase) can also be postulated to have a role in extracting nutrition from plant cell polymers. Of the 13 proteases, 7 have signal peptides and are probably involved in extracellular protein scavenging.

Eleven of the *in planta* proteins are smaller than 17 kDa. Five of these have signal peptides, and the majority has no known enzymatic function. Because the protein samples were desalted on a column with a 5-kDa MWCO, we cannot exclude that there are proteins smaller than 5 kDa in the secretome of *F. graminearum*.

Six of the *in planta* proteins are not found in other organisms (defined as a best BLASTP E value of > 10^{-10} against the GenBank nr database). These are FG00060, FG02560, FG02897, FG03211, FG04213, and FG07647, all of which are classified as unknown function. In addition, FG07741 is orthologous only to volvatoxin from *Volvariella* (Agaricales) (Lin et al., 2004); FG07822 is orthologous only to a single protein from *Chaetomium globosum*; and the only significant orthologs of FG04746 are proteins in two species of *Aspergillus*. The taxonomic distribution of orthologs of FG07558 (fungal lectin) is restricted to *Arthrobotrys*, *Podospora*, and several species in the Agaricales.

Many of the *in planta* proteins have paralogs. A total of 32 of the proteins identified *in planta* have paralogs that are also expressed *in planta* (Table 2.3). Thus, there is a high degree of genetic redundancy in the proteins expressed *in planta*, which might reflect their importance to the fungus and will make it difficult to test their function by gene disruption. A high number (60 or 50%) of the proteins found *in planta* were not found in any of the *in vitro* experiments. Furthermore, of the 120 *in planta* proteins, only 57% have signal peptides. Two proteins without signal peptides, FG07558 (fungal lectin) and FG08721 (superoxide dismutase), are orthologous to proteins that have been experimentally determined to be secreted in other fungi despite lacking canonical

signal peptides (Moore et al., 2002; Oguri et al., 1996). Both of these observations can be explained by the presence of many proteins with probable housekeeping functions, defined as having strong sequence identity to proteins that are widely distributed in many organisms and having a known biochemical function in primary metabolism. We identified enolase, elongation factor eEF1, triose phosphate isomerase, phosphoglyceromutase, aconitase calmodulin, methionine synthase, malate dehydrogenase, and others (Table 2.1). As expected for enzymes involved in primary metabolism, none of them have predicted signal peptides, with the possible exception of FG00346 (saccharopine reductase involved in lysine biosynthesis). It is likely that some of the other identified proteins, such as some of the nonsecreted proteases, cyclophilin, aldose epimerase, *etc.*, are also housekeeping enzymes.

By definition, housekeeping proteins are made under all growth conditions. Therefore, if the presence of these proteins in the *in planta* proteome is due to active secretion and not fungal lysis, then it is not clear why they should be absent from the *in vitro* secretome. Possibly they are secreted only in response to the plant milieu. Alternatively, the absence of housekeeping proteins in the *in vitro* secretome might be due to different extraction methods. The *in vitro* proteomes were obtained from culture filtrates, whereas the *in planta* proteins were extracted by vacuum infiltration; the latter method would tend to extract proteins that are within the mycelia walls and also might cause more cell breakage. To test whether vacuum infiltration might account for the greater number of housekeeping proteins seen in the *in planta* secretome, we extracted proteins from mats grown *in vitro* using the same vacuum infiltration conditions used for infected plants. We thereby identified 47 proteins, all of which had previously been

found in one or more of the *in vitro* culture filtrates. More than 94% of these proteins had predicted signal peptides. This result indicates that the *in planta* results are probably not due to the extraction method used.

Of the eight *F. graminearum* proteins reported by Zhou *et al. (Zhou et al., 2006) in planta* using 2-D gels, seven lack predicted signal peptides (the eighth, FG01246, has since been remodeled into two proteins, FG12291 and FG12918. One is predicted to be secreted and the other not, and it is not possible from the available evidence to know which protein was detected *in planta*). At least two of these proteins are almost certainly single-copy housekeeping proteins: aldolase (FG02770) and glyceraldehyde-3-phosphate dehydrogenase (FG06257) (Zhou et al., 2006). Thus, an independent study also found a high percentage of nonsecreted proteins *in planta* compared to *in vitro (Phalip et al., 2005)*.

Comparison of just the secreted proteins in the *in planta* and the *in vitro* experiments indicates that there are more proteins in common between *in planta* and maize cell walls than between *in planta* and sucrose (Fig. 2.1). This is consistent with the *in planta* milieu being a catabolite-derepressing environment.

The *in planta* proteomic results were compared with the mRNA expression results obtained with the *F. graminearum* Affymetrix gene chip(Guldener et al., 2006b). In order to optimize the comparison, *F. graminearum* was grown on the same media (Guldener et al., 2006b). Only three new proteins were found on these media compared to the HMT-based media (FG08048, FG10941, FG11190) (Table 2.1; Table S1 of Supporting Information). The genes for all but 6 out of the 120 *in planta* proteins in

Table 2 are among the, 7000 *F. graminearum* probe sets whose mRNAs were detected at one or more time-points during infection. In regard to *in planta* specific genes and proteins, Güldener *et al. (Guldener et al., 2006b)* found 431 genes expressed *in planta* but not during growth *in vitro* on complete media or minimal media lacking carbon or nitrogen. Of these genes, 367 (85%) of the corresponding proteins were not found either the *in planta* or *in vitro* proteome by us or by Phalip *et al. (Phalip et al., 2005)*. Twenty-two (5%) were found in both the *in vitro* and the *in planta* proteomes, and 34 (8%) were identified in the *in vitro* but not the *in planta* proteome. The existence of these two groups of proteins suggests that proteomics might, at least for some proteins under some conditions, have sensitivity that is superior to microarrays. Eight (2%) of the *in planta*-specific genes of Güldener *et al.* (Guldener et al., 2006b) were also found by us in the set of *in planta*-specific proteins. These are FG00028 (peptidase), FG00060 (KP4 killer toxin), FG02897 (unknown), FG03483 (pectate lyase), FG03632 (endoglucanase), FG10675 (gluconolactonase), FG11348 (unknown), and FG11399 (FAD-oxidoreductase). All eight of these have predicted signal peptides, which makes sense because many, perhaps most, of the nonsecreted proteins found by us *in planta* have housekeeping functions and are therefore also expressed *in vitro*.

If one hypothesizes that the *in planta* secretome is biased toward proteins involved in pathogenesis, and if pathogenesis genes are clustered into “pathogenicity islands” (Temporini and VanEtten, 2004), then the genes for the *in planta* proteins might show genomic clustering. The genes for the 120 proteins identified *in planta* are over-represented in two regions, corresponding to original FG numbers 3003-4074 (chromosome 2) and 10999-11487 (chromosome 3). The first region contains 20 *in*

planta proteins, all of which are secreted, and the second has 18, all but one of which are secreted (Table 2.1; Table S1 of Supporting Information). This clustering extends to all of the secreted proteins, both *in vitro* and *in planta* (Fig. 2.2). The 60 *in planta*-specific proteins do not show any genomic clustering, only four of them being in these two regions (Table 2.2). The two regions thus represent clusters of proteins that are secreted both *in vitro* and *in planta*, and therefore they might be more appropriately called “saprobic” islands rather than pathogenicity islands.

Discussion

The results presented here and in earlier studies demonstrate that MS-based proteomics is an effective method to survey the proteins secreted by a filamentous fungus under different growth conditions (Phalip et al., 2005; Zhou et al., 2006). In particular, our results demonstrate that it is possible to identify many fungal proteins inside the infected host, despite a low ratio of fungal to plant biomass (Guenther and Trail, 2005; Jansen et al., 2005).

In our experiments, fungal proteins could be detected against a high background of plant proteins both in bulk (unfractionated) preparations directly after extraction by vacuum infiltration as well as in proteins fractionated by 1-D SDS-PAGE. Prefractionation yielded more proteins than any one bulk analysis, but was roughly equivalent to the combined bulk analyses. Both the combined bulk analyses and the gel-fractionated samples had proteins that the other did not. The most striking difference between the two was that SDS-PAGE prefractionation gave higher statistical reliability

for the proteins it did identify. For example, one or two peptides from FG08723 were found in three bulk experiments, with a best log (e) score of –11.1, but the same protein yielded 11 peptides and a log (e) score of –77.3 in one of the gel slices. This indicates that prefractionation can increase the probability of finding proteins in complex mixtures, such as pathogen proteins in infected tissues, but it is not essential for obtaining significant results.

The proteomics approach complements and extends gene expression studies on the interaction between *F. graminearum* and its hosts (maize, barley, and wheat) (Goswami et al., 2006; Guldener et al., 2006a; Trail et al., 2003). Arrays based on the whole genome should have virtually every gene represented, whereas proteomics analyses will always miss some proteins due to such factors as interference from plant proteins, PTMs, lack of trypsin cleavage sites, and poor ionizability of some peptides. For example, we did not detect FG05906, encoding a secreted lipase, which is expressed *in planta* (Guldener et al., 2006b; Voigt et al., 2005). Advantages of proteomics over gene expression studies include an ability to enrich for the proteins in particular compartments, such as the apoplast, and the fact that the proteome represents the ultimate readout of the regulated processes of transcription, mRNA stability, translation, secretion, and protein stability.

In planta proteins that we identified include degradative enzymes (hydrolases, oxidoreductases, esterases, and proteases), small nonenzymatic proteins, housekeeping proteins, and proteins of unknown or only general predicted function. Many of the proteins identified *in planta* are of classes known from other experimental studies to be present in infected tissues, in particular cell wall-degrading enzymes and

proteases. Unexpectedly, of the 120 proteins found *in planta*, only 50% are predicted to have signal peptides, compared to 90% of the proteins identified in culture filtrates of *F. graminearum* grown *in vitro* (Table 2.1)(Phalip et al., 2005; Zhou et al., 2006). The subgroup of *in planta*-specific proteins shows a skewed functional distribution compared to the total *in planta* proteins or the total *in vitro* proteins. Only four are hydrolases, nine are proteases, and seven are oxidoreductases. At least 13 of the proteins found only *in planta* are predicted to be cytoplasmic "housekeeping" proteins. Of the eight proteins found by Zhou *et al. (Zhou et al., 2006) in planta* using 2-D gels, seven lack a signal peptide (one is ambiguous) and three of these are almost certainly housekeeping proteins. Between our results and those of Zhou *et al. (Zhou et al., 2006)*, six out of the ten glycolytic enzymes of *F. graminearum* have been detected *in planta*. All of these proteins lack signal peptides.

One possible explanation for the high percentage of proteins without signal peptides and the high percentage of apparent housekeeping proteins is that the extraction procedure causes breakage of the fungal cells. However, this explanation seems unlikely because fungal cells are stronger than plant cells (Carpita, 1985), and the low level of green pigment in the samples indicated minimal rupture of plant cells. Also, vacuum infiltration of *in vitro* fungal mats did not extract cytoplasmic proteins. Another possible explanation is that fungal hyphae undergo some degree of lysis *in planta*, perhaps due to plant defense proteins with fungal degradative activity, such as b-glucanase and chitinase, resulting in the leakage of fungal cytoplasmic proteins into the plant apoplast. Arguing against this hypothesis is that many abundant cytoplasmic

proteins, such as actin, tubulin, histones, and most of the ribosomal proteins, were not found in the *in planta* secretome (Table 2.2).

Another plausible explanation is that these fungal proteins lacking canonical signal peptides are, in fact, secreted. Some fungal proteins, such as b-xylosidase, lectin, and superoxide dismutase, are known to be true secreted proteins despite lacking typical signal peptides (Moore et al., 2002; Oguri et al., 1996; Rolke et al., 2004; Wegener et al., 1999). Furthermore, there are a growing number of documented cases of housekeeping enzymes that are secreted. Such proteins, which have known cytoplasmic functions and no known extracellular functions, have been found to exist naturally in the cell wall or on the surface of pathogenic and saprobic bacteria and fungi (Pancholi and Chhatwal, 2003). Enolase (FG01346), triose phosphate isomerase (FG05843), aconitase (FG07953), methionine synthase (FG10825), and phosphoglyceromutase (FG06055), all of which were found in our *in planta* experiments, have been reported to be on the surface of cells and to be allergens in bacteria or fungi (Pardo et al., 2000; Smalheiser, 1996). Enolase in particular has been reported in the cell wall and extracellular medium of many fungi and is a major fungal allergen for humans (Achatz et al., 1995; Angiolella et al., 1996; Breitenbach et al., 1997; Sundstrom and Aliaga, 1994).

Whether or not the housekeeping and other proteins without signal peptides are actively secreted *in planta* or are in the apoplast because of fungal lysis, in either case they would be in a position to interact with host cells and receptors and could therefore influence the course of the host/ pathogen interaction. One housekeeping protein that has been shown to be important in pathogenesis is bacterial EFTu. EF-Tu is an

abundant cytoplasmic protein and is also found on the surface of some animal pathogenic bacteria. In *Arabidopsis* it triggers the plant innate immune system by binding to a specific receptor (Kunze et al., 2004). Thus, it is conceivable that plants, like the mammalian immune system, recognize one or more fungal housekeeping proteins.

Because secreted proteins constitute the first contact between a pathogen and its host, and because many secreted proteins are virulence or avirulence effectors in various fungal diseases including head blight, the *in planta* proteins identified in this study are promising candidates to have a role in pathogenesis, either as virulence or as avirulence effectors. This includes not just proteins with predicted signal peptides and a plausible role in disease, such as cell wall degrading enzymes, toxic proteins, lipases, proteases, *etc.*, but also apparent housekeeping proteins such as enolase.

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Table 2.1. Summary of extracellular proteins identified in *Fusarium graminearum* grown *in vitro* on different media and *in planta*. The numbers in the table proper are percentages of the total proteins detected. Numbers in parentheses in the left column are our conservative estimates of the numbers of each class of protein in the predicted secretome of *F. graminearum*. The numbers in parentheses in the top row are the total number of proteins from each growth condition that were detected experimentally. HMT: HMT medium (see Materials and Methods); C: carbon; N: nitrogen; AFEX: ammonia fiber expansion; DDG, dried distillers' grain.

Growth conditions:	HMT no carbon (55)	HMT+ sucrose (92)	HMT + pectin (101)	HMT+ maize cell walls (126)	HMT+ carrot cell walls (120)	HMT + maize bran (154)	HMT + xylan (89)	HMT+ collagen (43)	HMT+ AFEX stover (46)	HMT+ AFEX DDG (37)	complete (54)	N-limited (38)	C-limited (44)	in planta (120)
glycosyl hydrolases (136)	20	25	27	40	39	34	37	16	43	54	37	24	27	21
lyases (20)	0	1	7	6	7	3	3	5	2	3	6	5	5	1
esterases (80)	7	9	12	13	13	13	11	12	15	14	13	16	18	4
proteases (75)	29	11	11	11	8	13	11	16	11	8	15	18	16	11

Table 2.1 cont'd

Growth conditions:	HMT no carbon (55)	HMT+ sucrose (92)	HMT + pectin (101)	HMT+ maize cell walls (126)	HMT+ carrot cell walls (120)	HMT + maize bran (154)	HMT + xylan (89)	HMT+ collagen (43)	HMT+ AFEX stover (46)	HMT+ AFEX DDG (37)	complete (54)	N-limited (38)	C-limited (44)	in planta (120)
oxidoreductases (64)	16	13	11	8	6	10	11	14	4	8	7	8	5	10
nucleases (5)	0	2	2	0	0	1	1	0	0	0	0	0	2	1
non-enzymatic or unknown	25	39	31	22	28	26	25	30	24	14	22	26	23	29
housekeeping	2	0	0	0	1	0	0	7	0	0	0	3	5	23
secretion signal	91	98	97	97	98	96	97	91	96	100	96	97	93	56

Table 2.2. *Fusarium graminearum* proteins identified at high reliability in infected wheat heads.

Gene designation[a]	Function[b]	Classification[c]	Frequency[d]	Best log(e)[e]	Best # of peptides[f]	Signal peptide[g]
FG00028*	metallopeptidase MEP1	P	1	-6.8	2	yes
FG00060*	KP4 killer toxin	S	3	-5.6	2	yes
FG00150*	NADP-dependent oxidoreductase (COG2130)		4	-9.7	4	no
FG00192	peptidase S8 (pfam00082)	P	4	-7.3	2	yes
FG00237*	O-acyltransferase (pfam02458)		1	-7.6	2	no
FG00346*	saccharopine reductase	H (no paralogs)	1	-20.4	4	yes
FG00496*	inorganic pyrophosphatase		5	-18.2	4	no
FG00571	cellobiohydrolase (GH7+CBD)		4	-40.7	7	yes
FG00609*	purine regulatory protein (cd02198.2)	S	3	-5.7	2	no
FG00777	cyclophilin (cd01926)		5	-99.1	14	no

Table 2.2 cont'd

Gene designation[a]	Function[b]	Classification[c]	Frequency[d]	Best log(e)[e]	Best # of peptides[f]	Signal peptide[g]
FG00832*	dienelactone hydrolase (pfam01738.12)		2	-17.1	3	no
FG00979*	aldehyde dehydrogenase		2	-11.7	4	no
FG01017*	dipeptidyl peptidase (M49)		2	-35	7	no
FG01346*	enolase	H (no paralogs)	7	-66.3	10	no
FG01485*	aldose-1-epimerase (COG0676.2)		1	-8.5	2	no
FG01604*	peptidase M1	P	4	-48	8	no
FG01621	cellobiohydrolase (GH5, no CBD)	C	2	-6.3	2	yes
FG01818	peptidase M28		3	-46.2	6	yes
FG01826*	aldehyde dehydrogenase		1	-19.6	4	no
FG01891*	calmodulin	H (5235, 7404)	1	-13.0	3	no
FG01956	ubiquitin/ribosomal protein fusion		3	-9.5	2	no
FG02059	α-galactosidase (GH27)	C	3	-29.8	6	yes

Table 2.2 cont'd

Gene designation[a]	Function[b]	Classification[c]	Frequency[d]	Best log(e)[e]	Best # of peptides[f]	Signal peptide[g]
FG02433*	NAD-disulphide oxidoreductase (pfam00070.12)		1	-8.7	2	no
FG02461*	malate dehydrogenase	H (2504)	4	-35.6	7	no
FG02560	unknown		2	-9.2	2	yes
FG02897*	unknown		4	-10.7	3	yes
FG02974*	catalase/peroxidase		3	-9.9	4	no
FG03003	β-xylosidase (GH43)	C	1	-9.5	3	yes
FG03027	peptidase M28	P	3	-7.8	2	yes
FG03143	glucuronyl hydrolase (GH88)	C	2	-3.9	2	yes
FG03211	unknown		1	-6.7	2	yes
FG03315	peptidase S8 (pfam00082)	P	2	-21.3	4	yes
FG03379	ribonuclease T2		2	-5.3	2	yes
FG03467	metalloproteinase M36		4	-16.9	4	yes
FG03483*	pectate lyase	C	7	-13.6	3	yes

Table 2.2 cont'd

Gene designation[a]	Function[b]	Classification[c]	Frequency[d]	Best log(e)[e]	Best # of peptides[f]	Signal peptide[g]
FG03598	exo-arabinanase (GH93)	C	3	-7.1	2	yes
FG03624	β-xylanase (GH11)	C	4	-18.5	4	yes
FG03628	cellulase (GH6+CBD)	C	1	-11.6	3	yes
FG03632*	endoglucanase (GH61)	C	2	-5.6	2	yes
FG03662	unknown, related to PhiA		4	-22.9	4	yes
FG03695	endoglucanase (GH61)	C	3	-16.5	3	yes
FG03795	cellobiohydrolase (GH5 + CBD)	C	2	-14.7	3	yes
FG03842	α-amylase (GH13)	C	7	-43.3	7	yes
FG03905	unknown		2	-4.5	2	yes
FG03986	endonuclease/phosphatase (pfam03372.11)		2	-11.0	2	yes
FG04074	unknown; related to PhiA		8	-26.2	7	yes
FG04196*	aldehyde dehydrogenase		3	-7.7	2	no
FG04213	unknown		1	-7.2	2	yes
FG04223*	aldo/keto reductase (pfam00248)		1	-4.1	2	no

Table 2.2 cont'd

Gene designation[a]	Function[b]	Classification[c]	Frequency[d]	Best log(e)[e]	Best # of peptides[f]	Signal peptide[g]
FG04741	unknown		6	-27.7	4	yes
FG04746*	unknown		1	-4.2	2	no
FG04826	L-xylulose reductase		8	-36.5	6	no
FG04848	rhamnogalacturonan acetylesterase	C	3	-5.3	2	yes
FG05236*	metalloproteinase peptidase M3	P	1	-74.4	13	no
FG05554*	aminobutyrate aminotransferase (pfam00202.12)		1	-7.3	2	no
FG05615	S-adenosylhomocysteine hydrolase (pfam05221.6)		1	-6.8	2	no
FG05843*	phosphoglucose isomerase	H (no paralogs)	2	-60.0	10	no
FG05972	nucleoside-diphosphate kinase		8	-22.4	4	no
FG06055*	phosphoglyceromutase	H (no paralogs)	3	-28.9	5	no
FG06127*	2-hydroxycarboxylate dehydrogenase (pfam02826.12)		2	-7.0	2	no
FG06397	endoglucanase (GH61)	C	2	-28.4	5	yes

Table 2.2 cont'd

Gene designation[a]	Function[b]	Classification[c]	Frequency[d]	Best log(e)[e]	Best # of peptides[f]	Signal peptide[g]
FG06445	β-xylanase (GH10)	C	3	-26.8	5	yes
FG06452	polysaccharide deacetylase	C	2	-4.3	2	yes
FG06496*	prolidase (cd01087.2)	P	1	-5.5	2	no
FG06527	peptidase M28 (pfam04389.6)	P	1	-3.7	2	yes
FG06616	β1,3-glucanase (GH55)	C	1	-14.4	3	yes
FG06655*	aminopeptidase M18 (pfam02127.12)	P	1	-5.1	2	no
FG06702*	triose phosphate isomerase	H (no paralogs)	3	-15.2	3	no
FG06744*	epoxide hydrolase (pfam00561.12)		1	-6.0	2	no
FG06932*	adenosine kinase (cd01168.2)		2	-5.8	2	no
FG07401*	elongation factor eEF1	H (12102)	1	-6.9	2	no
FG07439*	cyclophilin (cd01926)		2	-18.5	3	yes
FG07558*	fungal lectin (pfam07367.2)		5	-26.5	5	no[i]

Table 2.2 cont'd

Gene designation[a]	Function[b]	Classification[c]	Frequency[d]	Best log(e)[e]	Best # of peptides[f]	Signal peptide[g]
FG07647*	unknown		1	-29.8	4	no
FG07671	lysophospholipase (pfam00561)		4	-18.2	4	yes
FG07741*	volvatoxin (pfam01338.12)		1	-13.4	3	no
FG07822	unknown		12	-127.9	16	no
FG07953*	aconitase	H (9589, 10198, 10949, fgd460-1170, fgd461-10)	1	-3.8	2	no
FG07988	unknown; related to PhiA		5	-28.2	5	yes
FG08037	intradiol dioxygenase (cd03457.1)		2	-18.3	4	yes
FG08078*	acetamidase (pfam01425)		1	-32.6	6	no
FG08677*	AhpC/TSA family (pfam00578.12)		1	-11.6	3	no
FG08721	Cu-Zn superoxide dismutase		13	-19.8	7	no[i]
FG08723*	transaldolase	H (no paralogs)	4	-77.3	11	no
FG08964*	signal recognition particle SRP54	H (8785)	1	-3.4	2	no

Table 2.2 cont'd

Gene designation[a]	Function[b]	Classification[c]	Frequency[d]	Best log(e)[e]	Best # of peptides[f]	Signal peptide[g]
FG08979*	glutamine amidotransferase (cd03141.1)		1	-8.2	2	no
FG09366	β-1,3-glucosidase (GH17)	C	3	-7.3	2	yes
FG09373*	fumarate reductase		1	-9.1	2	yes
FG09844*	dihydrolipoamide dehydrogenase (COG1249.2)		1	-8.7	2	no
FG09998*	transketolase	H (4065)	4	-28.0	5	no
FG10212	SnodProt		3	-8.5	2	yes
FG10229*	glycine/D-amino acid oxidase (COG0665.2)		1	-16.5	4	no
FG10249*	spermidine synthase (pfam01564.12)		2	-7.6	2	no
FG10675*	gluconolactonase (COG3386.2)		2	-21.5	5	yes
FG10782*	aspartyl protease (pfam00026.12)	P	1	-25.0	5	yes
FG10825*	methionine synthase	H (no paralogs)	4	-27.0	5	no

Table 2.2 cont'd

Gene designation[a]	Function[b]	Classification[c]	Frequency[d]	Best log(e)[e]	Best # of peptides[f]	Signal peptide[g]
FG10999	β-xylanase (GH11)	C	1	-7.5	2	yes
FG11036	esterase (COG3509.2)		3	-17.4	3	yes
FG11037	endoglucanase (GH12)	C	1	-4.9	2	yes
FG11164	trypsin	P	14	-71.9	9	yes
FG11169	α-galactosidase (GH27)	C	2	-19.8	3	yes
FG11176*	unknown		1	-3.4	2	yes
FG11184	mixed-linked glucanase (GH5)	C	2	-7.1	2	yes
FG11205	SnodProt		1	-14.5	3	yes
FG11208	xyloglucanase (GH74, no CBD)	C	1	-4.2	2	yes
FG11249	Zn carboxypeptidase (pfam00246.12)	P	2	-36.5	5	yes
FG11304	β-xylanase (GH10)	C	1	-19.4	4	yes
FG11348*	unknown		1	-4.1	2	yes

Table 2.2 cont'd

Gene designation[a]	Function[b]	Classification[c]	Frequency[d]	Best log(e)[e]	Best # of peptides[f]	Signal peptide[g]
FG11366	galactan 1,3-beta-galactosidase (GH43)	C	3	-12.2	3	yes
FG11399*	FAD-dependent oxidoreductase (pfam01565.12)		1	-9.6	3	yes
FG11487	β-xylanase (GH10)	C	1	-10.1	2	yes
FG12070	peptidase S8 (pfam00082)	P	14	-48.6	6	yes
FG12269	glucoamylase (GH15 + SBD)	C	4	-15.5	4	yes
FG12305*	unknown		1	-27.0	5	no
FG12973	FAD-dependent oxidoreductase (pfam01565.12)		1	-18.3	4	yes[h]
FG13053*	peptidylprolyl isomerase (pfam00254.12)		5	-32.5	5	no
FG13077*	serine-pyruvate aminotransferase (COG0075.2)		2	-53.6	8	no
FG13094	galactose oxidase (cd02851.1)		2	-20.3	4	yes

Table 2.2 cont'd

Gene designation[a]	Function[b]	Classification[c]	Frequency[d]	Best log(e)[e]	Best # of peptides[f]	Signal peptide[g]
FG13095	unknown		7	-15.7	5	yes
fgd112-390*	FAD-dependent oxidoreductase (pfam01565.12)		1	-35.5	5	yes
fgd166-400	catalase		1	-24.5	6	yes

[a]Asterisk indicates that the protein was not been found in the *in vitro* secretome (Voigt et al., 2005) and our results.

[b]For proteins of only general prediction, pfam, Conserved Domain (CD), or COG references are given. GH, glycosyl hydrolase family [39]; CBD, cellulose binding domain; SBD, starch binding domain.

[c]Category classification: C, cell-wall-degrading; P, protease; H, housekeeping (with FG numbers of paralogs given in parentheses). Housekeeping proteins are defined in the text.

[d]Number of experiments in which the protein was found.

[e]Best X! TANDEM score in any experiment.

[f]Highest number of peptides found in any experiment.

[g]Presence of signal peptide as predicted by SignalP (Bendtsen et al., 2004)

[h]Presence of signal peptide based on our revision of the gene model.

[i]Lacks a predicted signal peptide but is orthologous to a secreted protein in another fungus.

Table 2.3: *In planta* proteins that have at least one paralog also found *in planta*. SP = predicted signal peptide. "Paralogs not found *in planta*" includes all predicted proteins in the entire genome.

Function	**Paralogous proteins identified *in planta*** **(FG or fgd numbers)**	**Paralogs not found *in planta***
FAD-dependent oxidoreductase (6-hydroxynicotine oxidase)	11399 (SP), 12973 (SP), 112-390 (SP)	17 others
peptidase S8 (subtilisin)	192 (SP), 3315 (SP), 12070 (SP)	10 others
peptidase M28	3027 (SP), 6527 (SP)	4936, 4246, 6545, 11411
Snodprot	10212 (SP), 11205 (SP)	none
unknown; related to PhiA	3662 (SP), 4074 (SP), 7988 (SP)	8122
α-galactosidase	2059 (SP), 11169 (SP)	none
β-xylanase (GH10)	6445 (SP), 11304 (SP), 11487 (SP)	4856, 10411
β-xylanase (GH11)	3624 (SP), 10999 (SP)	fgd108-10
catalase	2974 (no SP), 166-400 (SP)	1245, 10606
aldehyde dehydrogenase	979 (no SP), 1826 (no SP), 4196 (no SP)	27 others
cellobiohydrolase (GH5)	1621 (SP), 3795 (SP)	none
cellulase (GH61)	3632 (SP), 3695 (SP), 6397 (SP)	8 others
cyclophilin	777 (no SP), 7439 (SP)	6 others

Fig. 2.1. Selected three-way comparisons of secreted proteins on different media *in vitro*.

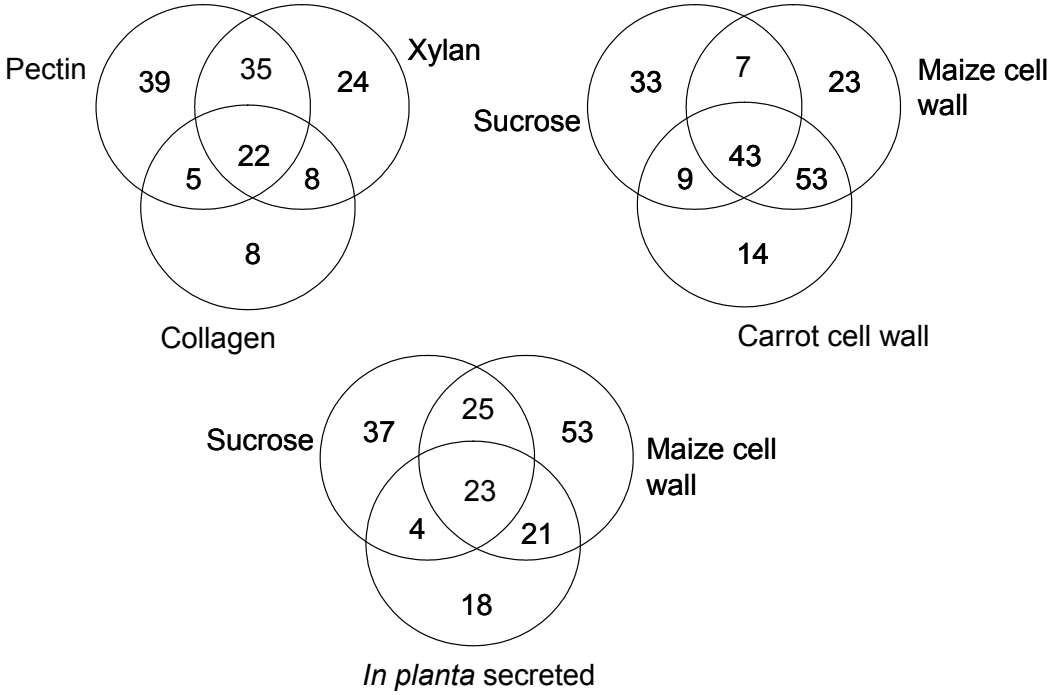
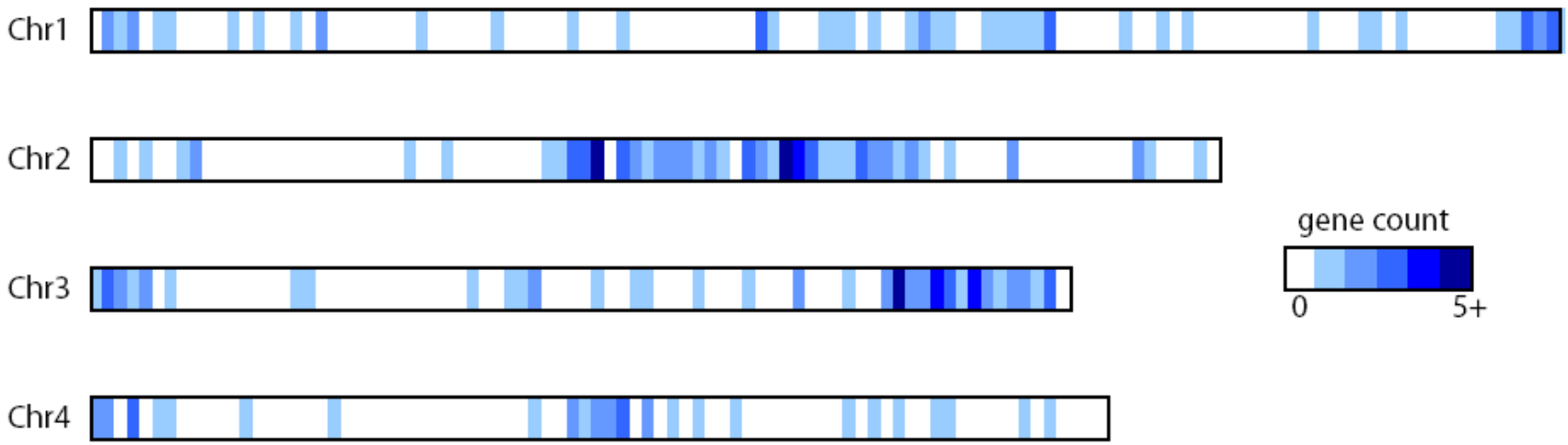


Fig.2. 2. Chromosome distribution of genes for secreted proteins of *F. graminearum* identified *in vitro* or *in planta*. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.



Chapter 3: Characterization of genes for secreted α-fucosidase from Fusarium graminearum and Fusarium oxysprorum

Introduction

In recent years, the practicality of using lignocellulosic material as a feedstock for biofuel production has been heavily studied (McCann and Carpita, 2008; Pauly and Keegstra, 2010). A major area of research in this field is to define the enzymes needed to break biomass down to fermentable sugars, based on the structure of the polymers in the feedstock (Banerjee et al., 2010a; Banerjee et al., 2010b; Banerjee et al., 2010c; Farinas et al., 2010; Hess et al., 2011; Pauly and Keegstra, 2010). Plant cell walls are inherently hard to break down because they provide the strength and support for the plant. A further complication is the fact that there is an enormous diversity in cell wall composition from species to species and among the walls of different cell types within a species (Hayashi and Kaida, 2011; Pauly and Keegstra, 2010). Different walls contain diverse forms and amounts of polysaccharides (i.e., cellulose, hemicelluloses, and pectin). In order to efficiently break down these polymers into individual sugars, different enzymes specific to each bond in the polymer must be used.

Commercial enzyme mixtures available today have many components but are expensive and may not be optimized specifically for the biomass being used (Banerjee et al., 2010b; Nagendran et al., 2009). The amount of enzymes used and thus their cost could be significantly reduced if these mixtures were optimized for the polymers present in the biomass (Lynd et al., 2008).

Nature can offer clues to the development of better ways to break down plant cell wall polymers for biofuels. In particular, fungi are a promising source of new and better enzymes because they gain their nutrition by secreting enzymes to break down polysaccharides and other macromolecules (Yip and Withers, 2004). We can therefore

identify the types of enzymes they secrete and use these for biofuel production. Fungi also secrete slightly different forms of the same enzymes, some of which may be more suited for industrial applications. Since there is an enormous diversity in fungi, they provide a good source of enzymes. Hydrolytic enzymes from fungal plant pathogens have been studied with respect to their role in causing disease, and genomic and proteomic data suggest that pathogens secrete a diverse array of enzymes to promote infection (Cantu et al., 2008; Gibson et al., 2011; King et al., 2011; Paper et al., 2007; Phalip et al., 2005; Phalip et al., 2009). Studies on the secreted proteins of saprophytic fungi when grown on different substrates are one source of information about how these organisms efficiently break down plant cell wall polymers (Nagendran et al., 2009).

One of the hemicelluloses prevalent in cell walls of both monocotyledons and dicototyledons is xyloglucan, which consists of a β-1,4 linked glucose backbone substituted with α-1,6 linked xylose molecules. In dicots, galactosyl and fucosyl – galactosyl residues can also be attached to the xylosyl residues (Hayashi and Kaida, 2011). Because of the different monomers and linkages that are present in xyloglucans, they are challenging to industrially degrade, but due to its glucose backbone, it is an important molecule to target to increase glucose yield from lignocellosic biomass (Pauly and Keegstra, 2010). Although not a large part of the secondary cell wall structure in most monocots, the degradation of xyloglucan may become more important if diverse feedstocks containing dicots are used for biomass. Biomass from restored prairies or old fields, which contain mixed grasses and forbs (herbaceous dicots) has been proposed as an environmentally preferable source of biomass feedstock compared to

monocultures of corn or switchgrass (Adler et al., 2009; Brennan and Harris, 2011; Sykes et al., 2009; Tilman et al., 2006; Vogel, 2008).

Since enzymes needed for degradation of plant cell polymers are specific to the sugar residue and the type of bond, these polysaccharides may be more efficiently degraded if terminal residues were cleaved first. Fucose is the terminal sugar on xyloglucan (attached by an alpha-1, 2 linkage) in nonsolanaceous dicots (Fig. 3.1). For efficient degradation of XG, α-fucosidase would have to act first. Without removal of the fucose residue, β-galactoside, α-xylosidase, and β1,4-glucanase cannot degrade XG all the way to free monomeric, fermentable sugars.

Alpha-fucosidases are widespread in prokaryotic and eukaryotic organisms. They are included in three of the CAZy (Carbohydrate Active Enzymes database) (http://www.cazy.org/) database families (GH1, 29, and 95) (Cantarel et al., 2009). There are many fucosylated substrates in cells and many genes encoding active fucosidases have been characterized in a diversity of organisms (Augur et al., 1995; Eneyskaya et al., 2001; Ishimizu et al., 2007; Yoshida et al., 2011). FUCA1 of humans and other mammals is involved in protein glycosylation in the Golgi. A mutation of this gene causes the disease fucosidosis (Barker et al., 1988; Di Matteo et al., 1976; Kondagari et al., 2011; Zielke et al., 1972). Recently, specific fucosidase activity has been tested as an early marker for some liver diseases and pancreatic cancer (Szajda et al., 2011). Several plant fucosidases are implicated in the modification of cell wall structure during growth, including AtFXG1, AtFUC1 (which belong to CAZy family 29) and AtFUC95A (family 95) (de La Torre et al., 2002; Leonard et al., 2008).

A fucosidase was identified in a proteomic study that identified the secreted proteins of *F. graminearum* grown on various carbon sources and *in planta*. FGSG_11254, annotated as a hypothetical protein with a fucosidase domain as predicted by Pfam, was found to be secreted on various carbon substrates in vitro (Finn et al., 2010; Paper et al., 2007). Another fucosidase, from the plant pathogen *F. oxysporum,* is active on pNP-fucoside, an artificial substrate used to test fucosidase activity, but the gene was not identified (Yamamoto et al., 1986). Although many fungal genes are annotated as predicted fucosidases, these predictions are based solely on gene homology to characterized fucosidases of other organisms. To our knowledge, no fungal fucosidase gene has been definitively characterized. In this study, the gene FGSG_11254 from *F. graminearum* strain PH-1 was shown to encode a bonafide fucosidase, and the gene for the characterized fucosidase of *F. oxysporum* was identified.

Materials and Methods

Fungal growth

F. graminearum strain PH-1 was grown as previously described (Paper et al., 2007). *F. oxysporum* strain 0685 was obtained from the Penn State Fusarium Research Center and grown on potato dextrose agar (PDA) (Difco, 213400). To induce conidial formation, a small piece of inoculated agar was transferred to a 250-mL flask containing 50 mL of carboxymethylcellulose (CMC) medium (Sigma, C-503) and grown shaking at 250 rpm for 2 d. For DNA and RNA, *F. oxysporum* was grown in multiple 1-L

flasks containing 100 mL of potato dextrose broth (PDB) (Difco, 254920) for one week. For induction of fucosidase and isolation of fucosidase-induced RNA, 1% fucose was added (Sigma, F2252). The fungal mats were rinsed with sterile water before being frozen in liquid nitrogen.

Purification of native fucosidase from F. oxysporum

After the *F. oxysporum* mat had been harvested for RNA, the culture filtrate was concentrated by adding 51.6 grams of ammonium sulfate (80% saturation), and the pellet was resuspended in 10 mL of 25 mM sodium acetate, pH 5.0. The filtrate was then desalted/ buffer-exchanged with 25 mM sodium acetate, pH 5.0, using a size exclusion column with a 6000 Da MW cutoff (BioRad 732-2010).

Anion exchange chromatography was performed with a buffer system consisting of 25 mM Tris pH 8.0 (Buffer A) and Buffer A containing 0.6 M NaCl (Buffer B). Four mL of culture filtrate was added to 1 mL 5X buffer A and the entire 5 mL sample was loaded onto a TOSOH Bioscience (part #08803) 8.0 mm X 7.5 cm TSK gel SP-5PW with a 10 µm particle size. The gradient consisted of 0 – 5 min 100% A, 5 - 45 min to 100% B, 45 - 50 min 100% B, with a flow rate of 1 mL/min. Two-mL fractions were collected during the course of the run and desalted as described above. Enzyme assays were done to determine the active fractions, which were then pooled.

Hydrophobic interaction chromatography was performed with a 7.5 mm ID, 7.5 cm TSK gel Phenyl-5PW column (Tosoh Bioscience). The starting buffer (Buffer A) was 1.7M $(NH_4)_2SO_4$ + 100 mM KH_2PO_4 pH 7.0, and buffer B was water. The gradient

consisted of 0-5 min 100% A, 5-35 min to 100% B, and 35-45 min at 100% B, at a flow rate of 1 mL/min. One-mL fractions were collected.

SDS-PAGE and proteomic analysis

All active fractions were analyzed by SDS-PAGE using a gel containing 4-20% acrylamide (BioRad, 161-1159). The most prominent band in the HIC fractions was cut from the gel for proteomic analysis. Mass spectrometry based proteomics was done as described in Nagendran et. al. (2009).(Nagendran et al., 2009). The *Fusarium oxysporum* strain 4287 genome sequenced by The Broad Institute of MIT and Harvard for the Fusarium Comparative Project was used as the initial database for proteomic identifications (http://www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome.html). After the characterization of the gene in *F. oxysporum* 0685, the corrected amino acid sequence was added to the original database for reanalysis.

pNP-fucoside activity assays

4-nitrophenyl-alpha-L-fucopyranoside (pNp-fucoside) (Sigma, 3846) was used as a substrate for enzyme assays. Assays were done in 1 mL total volume by mixing of enzyme with 100 µL of 100 mM sodium acetate, pH 5.0, and 100 µL of 10 mM pNP-fucoside in a total volume of 300 µL. Assays were performed at 37°C, usually for 30 min, after which the reaction was stopped by addition of 600 µL 1 M sodium carbonate. Absorbance at 400 nm was then recorded for all samples with the blank consisting of a

reaction that was immediately stopped with the addition of sodium carbonate after the addition of the enzyme.

DNA isolation

DNA was purified using the Qiagen Puregene Core Kit A following the mouse tail protocol.

RNA isolation

F. graminearum RNA was isolated by 48 h after induction of sexual development (Hallen et al., 2007). RNA from *F. oxysporum* grown on PDB and 1% fucose medium was isolated using a method slightly modified from Hallen et. al. (Hallen et al., 2007). The sample was frozen and lyophilized until completely dry and then ground to a fine powder with a baked mortar and pestle. Trizol® was added in 1 mL increments until a pipetable slurry resulted. The slurry was transferred into three 1.5-mL Eppendorf tubes and allowed to remain at room temperature for 5 min. Chloroform (200 µL) was added to each tube. After 3 min at room temperature, the tubes were spun using a microfuge set at 15,000 rpm for 15 min. The aqueous portion was transferred to new tubes. One volume of Pine Tree CTAB (2% hexadecyl trimethyl ammonium bromide (CTAB); 2% PVP K30; 100 mM Tris-HCl,pH 8.0; 25 mM EDTA; 2 M NaCl; 0.5 mg/ml spermidine) was added to each tube and samples were placed at 65°C for 25 min. One volume of 24:1 chloroform: isoamyl alcohol was added and the samples were centrifuged at 15,000 rpm for 10 min. The aqueous layer was collected and 1 volume of chloroform

added. The centrifugation and aqueous collection was repeated. One-quarter volume of 3M sodium acetate was added with one volume of isopropanol. Samples were put on ice for at least 20 min and then centrifuged for 10 min at 15,000 rpm. The supernatant was discarded and the pellet washed twice with 70% ethanol . The pellet was redissolved in 100 µL of RNase-free water. The samples were placed at 65C for approximately 30 min to evaporate the residual ethanol. They were then treated with DNase (Roche, #776785) and purified with the clean-up protocol and columns in the RNA Easy kit (Qiagen, 74904).

Sequencing F. oxysporum strain 0685 fucosidase

The genomic and predicted amino acid sequences of the α-fucosidase gene from *F. oxysporum* were obtained by a combination of 5' and 3' RACE and genomic sequencing. RACE was done with Clontech SMARTer^{TM} RACE cDNA Amplification Kit (634932) according to the provided protocol. Primers were originally designed based on the sequence from *F. oxysporum* strain 4287 available at the Fusarium Comparative Project at The Broad Institute. Once specific sequence data from strain 0685 were available, primers were designed based on the new sequence. For a list of primers used, see Appendix.

Cloning and expression of F. graminearum and F. oxysporum FCO1 genes

The *F. graminearum* and *F. oxysporum FCO1* genes, named *FgFCO1* and *FoFCO1*, were cloned using standard molecular techniques. After sequence

confirmation, both genes including secretion signals were inserted into PPicZA and PPiCZAα expression vectors with the Myc/6XHis tags in and out of frame (Invitrogen, K1740-01). Specific primers, restriction sites, and vector versions can be found in Appendix A. Confirmation of transformation was done by Zeocin® selection and colony PCR. Expression was done in 50 mL cultures according to previously described method (Banerjee et al., 2010a). FgFCO1 was induced for 3 d while *FoFCO1* was induced for 2 d. Cells were harvested by centrifuging at 8,000xg for 30 min. The supernatants were collected and concentrated using a tangential flow filtration system equipped with a 10 kDa-cutoff membrane (Vivaflow, Sartorious). Buffer exchange was performed during the concentration step with five volumes of 25 mM sodium acetate buffer, pH 5.0. Western analysis of tagged proteins was done using a 1:20,000 dilution of 6xHis antibody/HRP conjugate from Clontech (631210) following the manufacturer's protocol. Chemiluminescent detection of the conjugate was done with Pierce® ECL Western Blotting Substrate.

Anion exchange purification of FoFCO1

Further purification of FoFCO1 was done by anion exchange chromatography as described for purification of native fucosidase (above).

Preparation of fucosylated xyloglucan from etiolated peas

Xyloglucan was isolated by the method of (Zablackis et al., 1995). Pea seeds were soaked in H_2O for 24 hr prior to being sown on top of vermiculite, and placed in the dark for 5-7 d. The seedlings were lyophilized and ground with a mortar and pestle in liquid nitrogen into a fine powder. To isolate cell wall material, approximately 10 gm was suspended in 100 mL 100 mM potassium phosphate, pH 7.0, in a 250 mL centrifuge bottle and and vortexed for 1min. The bottle was then centrifuged at 8,000xg for 10 min. The pellet was washed three times with 100 mM potassium phosphate, pH 7.0, and then resuspended in 0.5% SDS and stirred at 4C overnight. The pellet was collected by centrifugation as described above and washed three times with 500 mM potassium phosphate and three times with sterile water. It was then washed three times with 30 mL of 1:1 chlorform: methanol. The pellet was finally washed with acetone and allowed to air dry.

In order to remove pectic material, the dried pellet was incubated at room temperature overnight in 50 mL of 50 mM trans-1,2-cyclohexanediaminetetraacetic acid (CDTA), pH 7.5, containing 0.05% sodium azide. The pellet was then collected by centrifugation.

The final step in xyloglucan isolation was treating the pellet from the last step with 30 mL of 4M KOH at room temperature overnight with stirring. The sample was centrifuged as above, and the supernatant collected. The supernatant was neutralized with acetic acid to pH 7.0 and dialyzed 6 times against 4L of water for approximately 12 hr each time. It was then lyophilized and stored at -20C.

Enzyme assays with pea xyloglucan

Lyophilized xyloglucan material was dissolved in 25 mM sodium acetate, pH 5.0, at a concentration of 1 mg/mL. The undissolved material was pelleted with a microfuge and discarded. The dissolved xyloglucan was digested with 2 units/mL of β-1,4 endoglucanase (Megazyme, E-CLTR) (E.C. 3.2.1.46) for approximately 8 hr. The resulting oligosaccharides were used for enzyme assays. Each assay was run with 125 µL xyloglucan oligosaccharides, 25 µL of enzyme (or water for control), and 50 µL of 50 mM sodium acetate, pH 5.0. The assays were performed at 37C for 12 hours. The samples were desalted, filtered with Spin-X® centrifuge tube filters (Costar®, 8169) and analyzed on a Shimadzu Axima MALDI-TOF in reflectron mode according to the method of Lerouxel et al.(2002).

Results

Purification and identification of native F. oxysporum fucosidase

After growth of *F. oxysporum* strain 0685 on fucose, activity on pNP-fucoside was present in the culture filtrate (Fig. 3.2A). When analyzed by SDS-PAGE, the culture filtrates contained a single major band of about 70 kDa. The fucosidase activity was purified by anion exchange and hydrophobic interaction chromatography. After anion exchange, the activity was present in two fractions corresponding to a major peak of UV absorbance (Fig. 3.1). A major band at approximately 70kDa was again

observed in the fraction corresponding to this peak (Fig 3.2). These fractions were combined and fractionated by HIC. Fraction 39, containing the peak of activity, was analyzed by SDS – PAGE and the single major band was analyzed by proteomics (Fig. 3.3). The protein was identified as the product of gene FOXG_15218 (Fig. 3.4). This gene, annotated as a hypothetical protein, contains a Pfam predicted alpha-L-fucosidase domain and was renamed *FoFCO1* (Finn et al., 2010).

In order to confirm the annotation, sequence, and gene structure of FoFCO1 in strain 0685, a combination of 5' and 3' RACE, as well as genomic sequencing was done. The sequence of a cDNA indicated a possible misannotated intron in the original F*. oxysporum* strain 4287 sequence resulting in the addition of 66 nucleotides in the coding sequence (22 amino acids in the protein sequence). There were also numerous single nucleotide base pair differences between the two strains resulting in an additional 37 amino acid changes (Fig 3.5). Re-analysis of the original mass spectrometry data from the proteomics experiment with the correct sequence added to the database resulted in a better match and 44% coverage instead of 21% (Fig. 3.6).

Expression of FoFCO1

Both His-tagged and untagged versions of *F.o*. FCO1 were successfully expressed in *P. pastoris*, but only culture filtrates of the untagged versions displayed enzyme activity (Fig. 3.7) on pNP-fucoside. SDS-PAGE analysis of culture filtrates at 24 hr indicated multiple faint protein bands. Western blotting of the same gel did not detect any tagged protein (Fig. 3.8). Although activity in the culture filtrate increased after induction for 48 hr, there were still only faint bands visible on SDS-PAGE (Fig. 3.7 and 3.9). After 72 hr of induction the activity was only slightly higher than at 48 hr.

After the concentrated culture filtrate was fractionated by anion exchange chromatography, a faint, diffuse band between 75 and 100 kDa was visible (Fig. 3.11B). This band is present in the fractions corresponding to a UV peak at approximately 20 min and to pNP-fucosidase activity (Fig. 3.10 and 3.11).

Identification and expression of F. g. FCO1

BLASTP results against the other *Fusarium* sequences available at the Fusarium Comparative Project http://www.broadinstitute.org/annotation/genome/fusarium_graminearum/ identified one *F. graminearum* hypothetical protein (FGSG_11254) with weak similarity to FoFCO1 (expect = $1.05E^{-23}$). This protein is predicted to be 68 kDa and also has a Pfam alpha-L-fucosidase domain and is predicted to belong to CAZy family GH29 (Cantarel et al., 2009; Finn et al., 2010). Although no introns were predicted in the sequence, a cDNA of this gene was sequenced to confirm the database annotation. Both tagged and untagged versions of this protein were cloned and expressed in *P. pastoris*. The culture filtrates from neither the tagged nor the untagged isolates contained activity on pNP-fucoside. Presence of the protein was confirmed by SDS-PAGE. Both pPICZ A and pPICZ Aα vectors expressed proteins of the same size (Fig. 3.12). Western blotting confirmed that the expressed proteins had the His tag (Fig. 3.12).

Activity of F.o. and F.g. FCO1 on pea xyloglucan

Fucosylated xyloglucan was extracted from etiolated peas and the presence of fucosyl - galactosyl oligosaccharides in the β – 1,4 glucosidase-digested xyloglucan was confirmed by mass spectrometry (Fig. 3.13). For reference structures and nomenclature for XG oligosaccharides refer to Fig. 3.1 (Fry et al., 1993). Note that XLXG and XXLG have the same mass and cannot be distinguished in the spectra. When compared to tamarind xyloglucan (which contains no fucosyl residues), pea XG has a peak at mass/charge 1394 which corresponds to XXFG. This peak is not present in the tamarind xyloglucan (Fig. 3.13). When pea XG was digested with FoFCO1 and FgFCO1, the peak corresponding to XXFG disappeared only when digested with FgFCO1 and not when digested with FoFCO1. Also, the ratio of the peak height between XXXG and XXLG/ XLXG shifted so that there was more XXLG/XLXG than XXXG (Fig. 3.14). When digested with FoFCO1, the XXFG peak remained unchanged, as did the ratio between XXXG and XXLG/ XLXG (Fig. 3.15).

Discussion

We purified a protein with fucosidase activity from *Fusarium oxysporum* and determined that it is the product of gene FOXG_15218 from the genome reference strain 4892. According to the annotated genome, the gene is predicted to have 2096 nucleotides and 5 introns (Fusarium Comparative Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org/). However, by sequencing genomic

DNA and cDNA copies of the same gene from *F. oxysporum* strain 0685, we determined that the third intron is mis annotated and the coding sequence around this intron is actually 20 bp longer at the 5' end and 46 bp longer at 3' end. The shortened intron results in the addition of 66 nucleotides encoding 22 amino acids. There are also multiple single nucleotide polymorphisms between the two strains, which result in 37 amino acid substitutions (Fig. 3.5).

F. oxysporum is known to be a genetically diverse species (Ma et al., 2010; Rep and Kistler, 2010). The sequenced strain of *F. oxysporum* (strain 4287) is a pathogen of tomato, whereas the strain used in this study was isolated from cabbage. Given the genomic diversity and the fact that we did not confirm the gene structure in the sequenced strain it is possible that the intron could be different in the two strains. However, it is more likely that the genome reference strain sequence is incorrect, because when we synthesized and expressed this gene in *Pichia*, it did not give an active fucosidase protein, whereas the corrected version from strain 0682 did.

Expression of the gene in *Pichia* confirmed that we had identified the correct protein and gene. There was no activity or protein in the tagged versions which may indicate that the Myc/His tag (21 amino acids) interfered with protein biosynthesis or stability (Fig. 3.7 and 3.8). Although there was activity in the culture filtrates from *Pichia* expressing the protein (Fig. 3.7), the actual protein was not reliably observed by SDS-PAGE (Fig 3.8). FoFCO1 could only be reliably visualized by SDS-PAGE after purification by anion exchange HPLC, and then it ran as a diffuse band, perhaps due to abnormal glycosylation (Fig 3.11).

The native FoFCO1 protein purified from *F. oxysporum* culture filtrates was present on SDS-PAGE as a distinct band at ~70 kDa, which correlates well with the predicted size of 69 kDa (fig 3.2). However, FoFCO1 expressed in *Pichia* was visible as a diffuse band between 100 and 75 kDa (fig 3.11). This size difference could indicate differences in glycosylation patterns between the two organisms. The protein is predicted to contain three glycosylation sites by the ExPASy FindMod tool (Gasteiger et al., 2005; Wilkins et al., 1999).

To date, specific activities of the native and expressed proteins have not been calculated. Further work purifying the proteins is necessary so that an accurate protein concentration can be obtained and the activities of the native and expressed version of this protein can be compared.

Culture filtrates of other fungi including *Cochliobolus carbonum, Trichoderma reesei, F. graminearum,* and *Phanerochaete chrysosporium* grown on fucose were also assayed for fucosidase activity using pNP-fucoside. Although activity was not detected, the genome of *F. graminearum* has a predicted protein that is weakly similar (FGSG_11254). This protein is expressed and secreted *in vitro* (Paper et al., 2007). FgFCO1 proteins were clearly visible as discrete bands at ~70 kDa on SDS-PAGE which corresponds to the predicted size and confirmed by Western, but none of the versions had activity on pNP-fucoside. Other fucosidases have also been shown to lack activity on pNP-fucoside (de La Torre et al., 2002; Leonard et al., 2008).

In contrast to FgFCO1, FoFCO1 was active on pNP-fucoside but not pea xyloglucan oligosaccharides. The natural substrate of FoFCO1 might be a fucosylated

substrate other than xyloglucan. There are other fucosylated substrates that are present in plant cells, for example in N-glycans covalently attached to proteins (Lerouge et al., 1998). α-Linked fucose is also present in rhamnogalacturonan I and II in plant cell walls (Zablackis et al., 1995). Fucose is also present in arabinogalactan proteins in plant cell walls (Wu et al., 2010). Sequence analysis of FoFCO1 and FgFCO1 as well as other annotated hypothetical fungal fucosidase proteins with homology to CAZy family GH29 shows two distinct families, one containing FgFCO1 and the other containing FoFCO1 (Fig. 3.16). It is reasonable to hypothesize that the two families may be active on different natural substrates, possibly the family containing FgFCO1 on α 1-2 linked fucose in xyloglucan and the family containing FoFCO1 on another substrate. The family containing FoFCO1 is present in many more organisms than that containing FgFCO1, possibly implying that the ability to remove fucose from xyloglucan is unique to a few organisms.

Future work could study the activity of both FCO1s on other substrates. The native FoFCO1 should also be assayed on these oligosaccharides. Possibly, the protein expressed in *Pichia* has lost its ability to act on substrates other than pNp-fucose.

My studies have several implications for how potential enzymes are assessed for their utility in improved or new activities. The idea of bioprospecting for more efficient enzymes is popular due to the large number of unstudied microbial and fungal enzymes (Li et al., 2009). para-Nitrophenyl linked substrates are commonly utilized because of their convenience. However, enzymes selected for high activity on artificial substrates may not be as efficient or even active on natural polymers. Alpha-fucosidases may also

play a role in degrading xyloglucan from dicots. Since fucose represents the terminal sugar on this substrate, its removal may be necessary before the rest of the molecule is degraded.

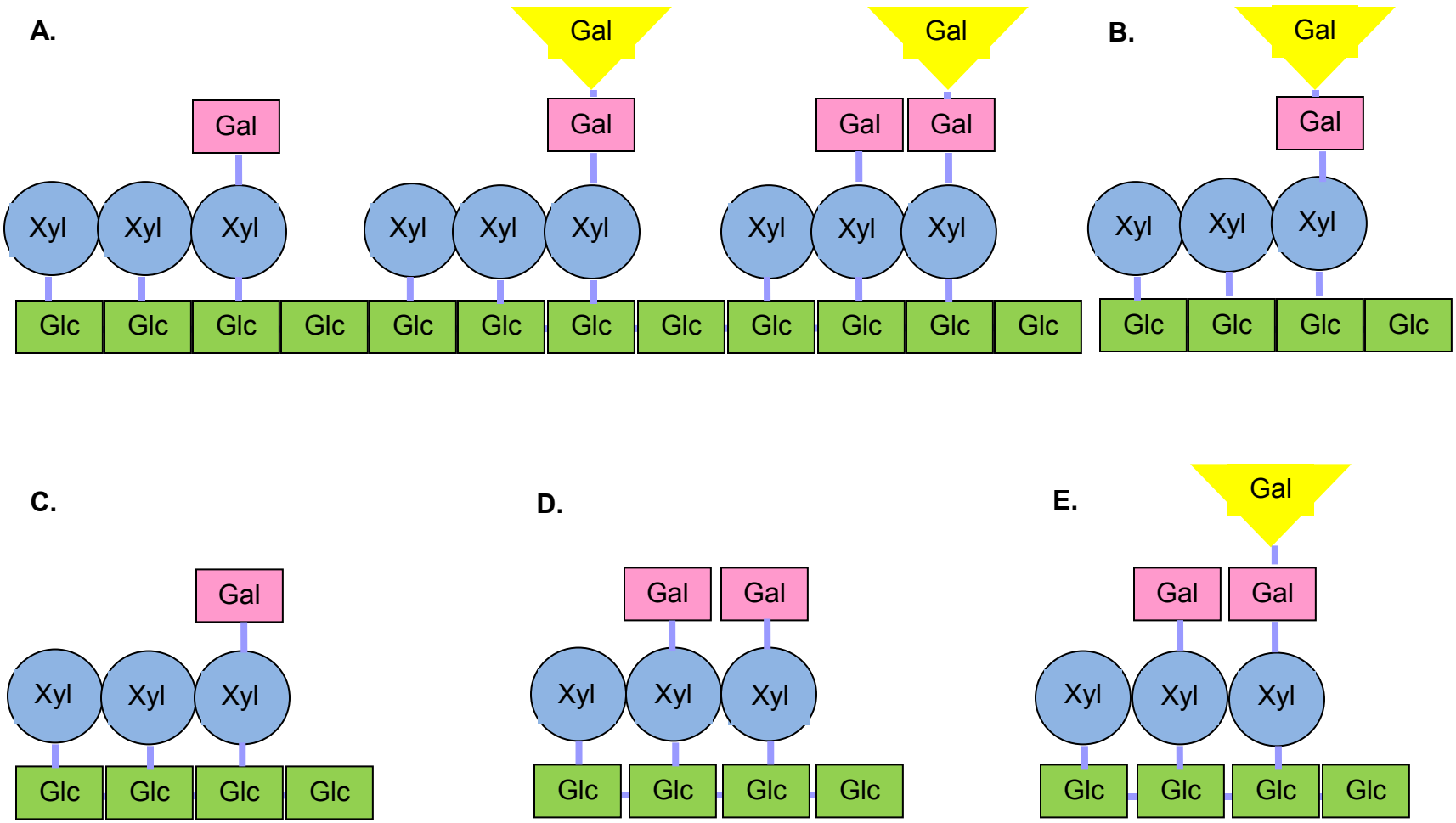


Figure 3.1 A. The structure of xyloglucan in non-solanaceous dicot cell walls (Pauly and Keegstra, 2010). (B) XXFG , (C) XXLG , (D) XLLG, and (E) XLFG according to the nomenclature of (Fry et al., 1993).

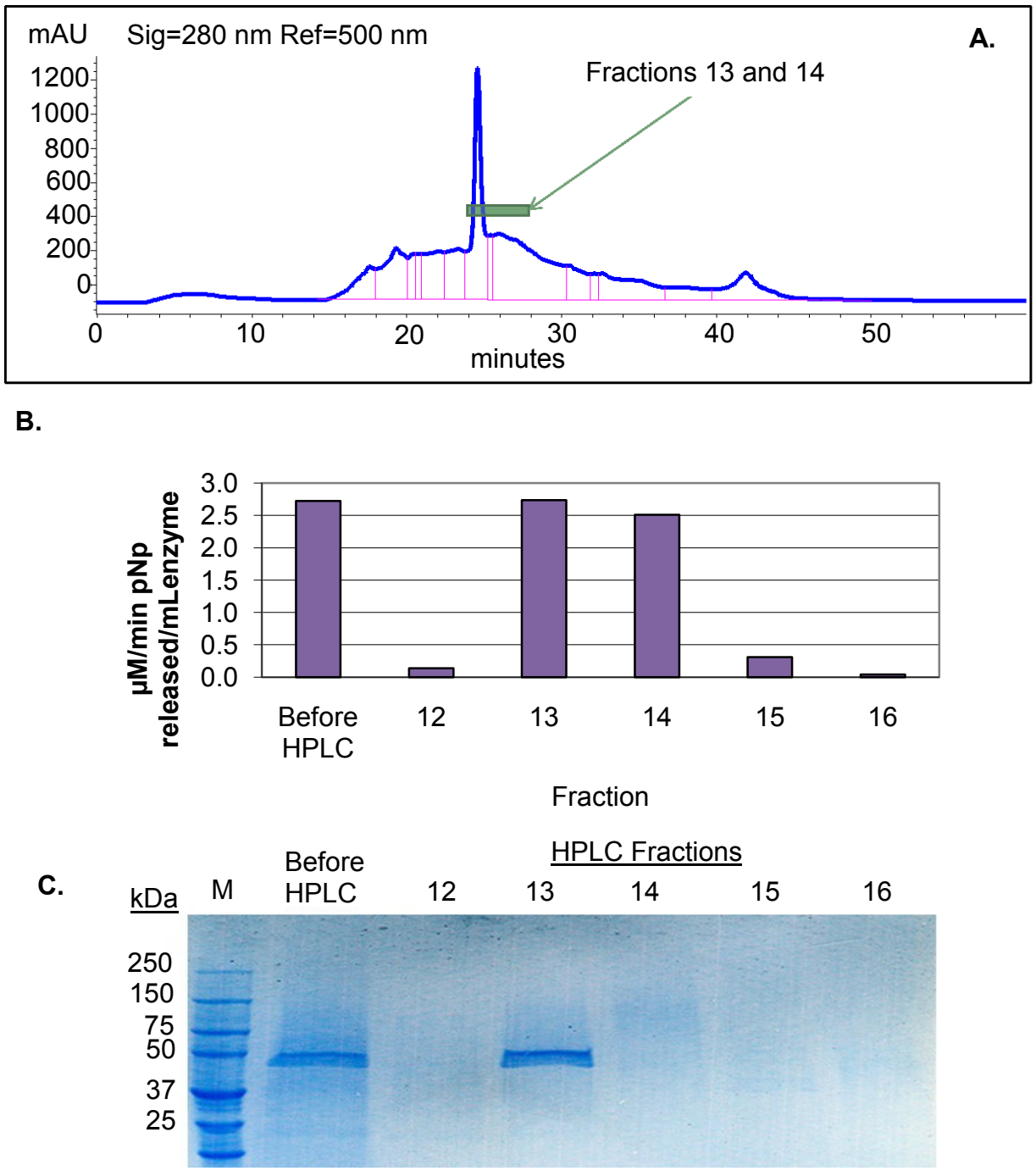


Figure 3.2

A. Anion exchange chromatogram of culture filtrate from *F. oxysporum* grown in 1% fucose. Highlighted green bar represents fractions 13 and 14 which were combined for HIC. **B.** Fucosidase activity before and after anion exchange fractionation. Activity of 10 µL concentrated culture filtrate before chromatography is also shown. **C.** SDS PAGE of culture filtrate and HPLC fractions from anion exchange. Fraction 13 contained the fucosidase activity.

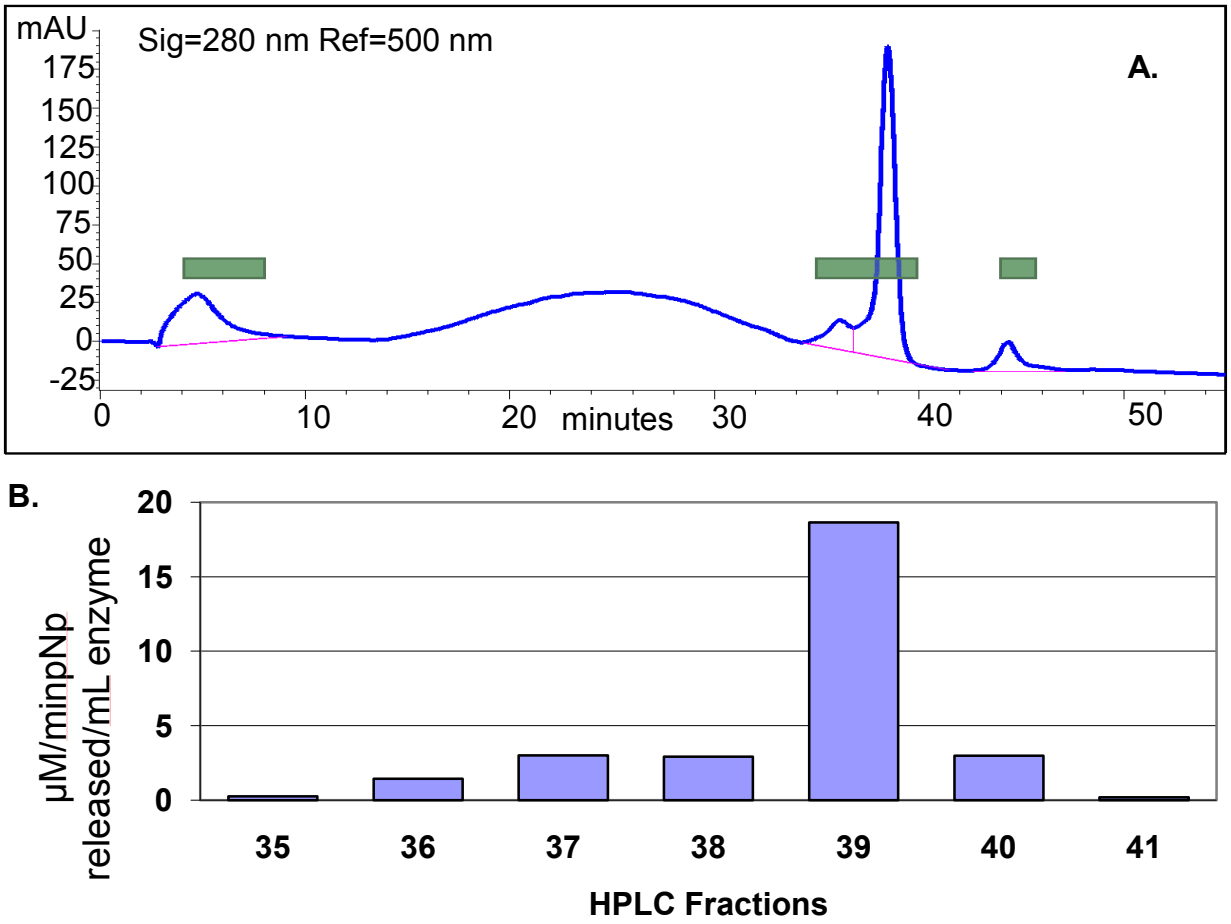


Figure 3.3

A. UV absorbance profile of fractionation of fucosidase by hydrophobic interaction chromatography of fractions 13 and 14 from anion exchange (Fig. 2.2). Activity was assayed in the fractions marked by green bars. **B.** Fucosidase activity in HIC fractions **C.** SDS-PAGE of HIC fractions; A – sample before HIC. The circled band was cut from the gel for proteomics analysis.

Figure 3.4

Identification of FCO1 (FOXG_15218) as the fucosidase purified from *F. oxysporum* strain 0685. The identified peptides are shown in yellow.

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MHIRLLSQLGTVVCLGTASVGALSLKHDKRATSPASLKIGSPVLTSKWLE
MHIRFLSHLGTGLCLGTASVGALSLKHDKRATSPASIVIGNPVLTSKWLE

GSDYEQIVEFFITNSDGKNPLTWTDQLHVIVESSSLETTTPGTLLRLGPK
GSDYEQVVEFFITNSDANNPLTWADQLQVTVESSSLETTTPGTLLRLGPN

QSAVVQVGVKNKAGVKAGTRCDATAVVTWGPKQDPKKSSKAFSGQCGIGD
QSAVVQVGVKNKAGVKAGTQCGATAVLTWGPKENPKKSSKDFSGQCGIGD

YEASESSLEHHWNPDWFHEIKYGIFIHWGLYSVPAFGNRPGPKQDYAEWY
YDASASSLEHHWNPDWFHEIKYGIFIHWGLYSVPAFGNRPGPNQDYAEWY

GYRMTQPDFPSQTYQHHRDTYGENFNYDDFVSNFTGANFDAEDWMNLVAD
GYRMTQPDFPSQTYQYHRATYGENFNYDDFVSNFTGASFDAEDWMNLVAD

AGAH----------------------KRSTVHYGPKRDFVKELLDVAKAK
AGAQYVVPVTKHHDGWALFDFPESVSKRSTVHYGPKRDFVKELLDVAKAK

HPEIRRGTYFSMPEWFNPAYVKYAWDQHYKEIYWGRPPTNPYTNKSIEYT
HPEIRRGTYFSMPEWFNPAYAKYYWDQHYKEIYWGRPPTNPYTNKSIEYT

GYVEVDDFINDIQNPQIEALFYDYDIEMLWCDIGGPNKAPDVLAPWLNWA
GYVEVNDFINDIQNPQMEALFYDYFIEMLWCDIGGPNKAPDVLAPWLNWA

RDQGRQVTFNDRCGAAGDYSTPEYSGISFNPKKFESNRGLDPFSFGYNYL
RDQGRQVTFNDRCGAAGDYSTPEYAGISFNPRKFESNRGLDPFSYGYNYL

TTDDEYLSGEEIVKTLVDNVVNNGNFLLNMGPKGDGTIPKQQQLNLLDAG
TTDDEYLSGEEIVKTLVDNVVNNGNFLLNMGPKGDGTIPKQQQLNLLDAG

EWIKDHGEGIFGTRYWPTAQTSGSLRFAMKPDAFYIHHVGQPSSPLVINE
EWIKSHGEGIFGTRYWPTAQTSGSLRFAMKPDAFYIHHVGQPSSPLVISQ

PVPWVEGDEVTAVGGSAHGTVLQVARNDGSFSVQLPDNVVQGDKYIWTIKIAYSTGK
PVPWVEGDEVTAVGGSAHGTVLQVARNGGSFSVQLPDNVVQGDKYIWTIKIAYSTGK
```

Figure 3.5

Sequence alignment of the predicted amino acid sequence of FoFCO1 from *F. oxysporum* strain 2872 (genome reference strain)(top)and the predicted sequence of *F. oxysporum* strain 0685 (bottom) determined from a corresponding cDNA (work in this thesis).

Figure 3.6

Re-analysis of mass spectrometry data with the corrected *F.oxysporum* FCO1 gene product from strain 0685 added to the proteome database.

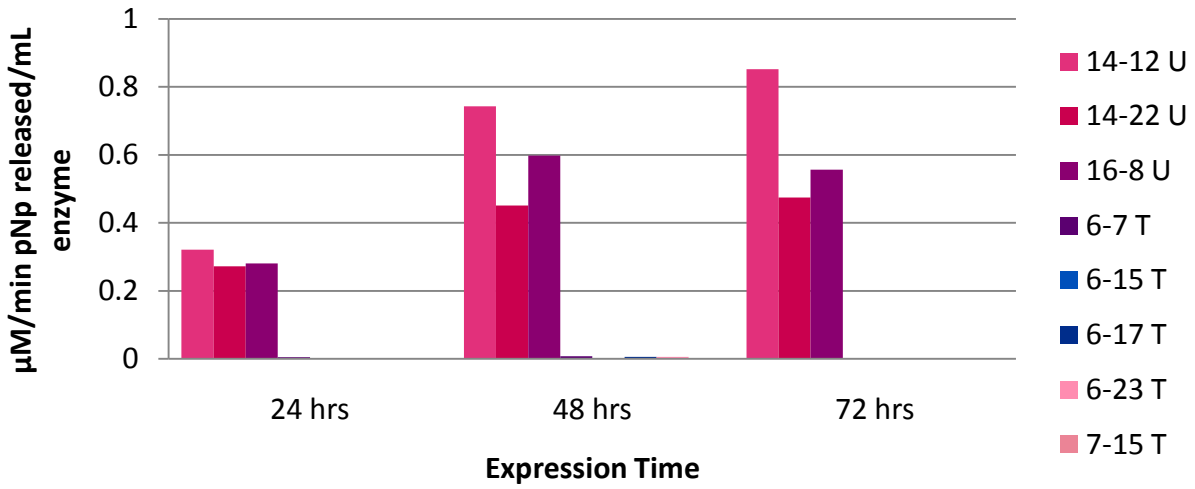

Figure 3.7

Enzyme activity of culture filtrates from *Pichia pastoris* expressing tagged (T) and untagged (U) versions of FoFCO1. Times are hr after methanol induction. Tagged versions did not contain detectable activity.

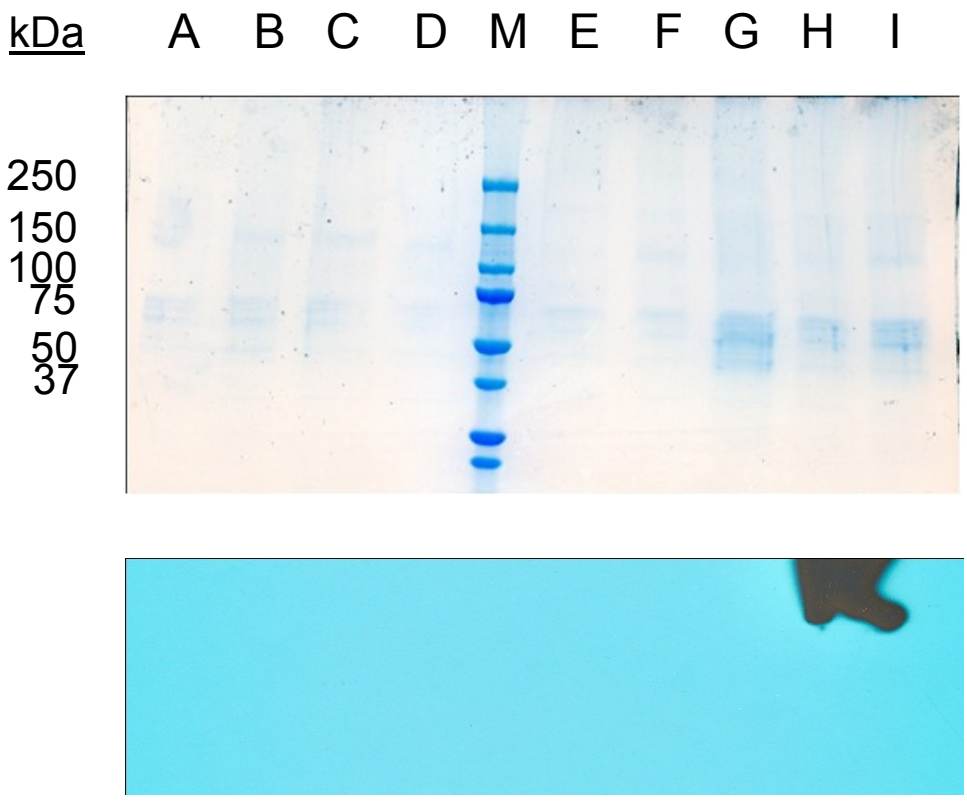

Figure 3.8

A. SDS-PAGE of *P. pastoris* transformants expressing untagged and tagged versions of FoFCO1. **B.** Western analysis of the same gel indicating no detectable tagged protein. Blob on the right is from the luminescent marker used for orientation.

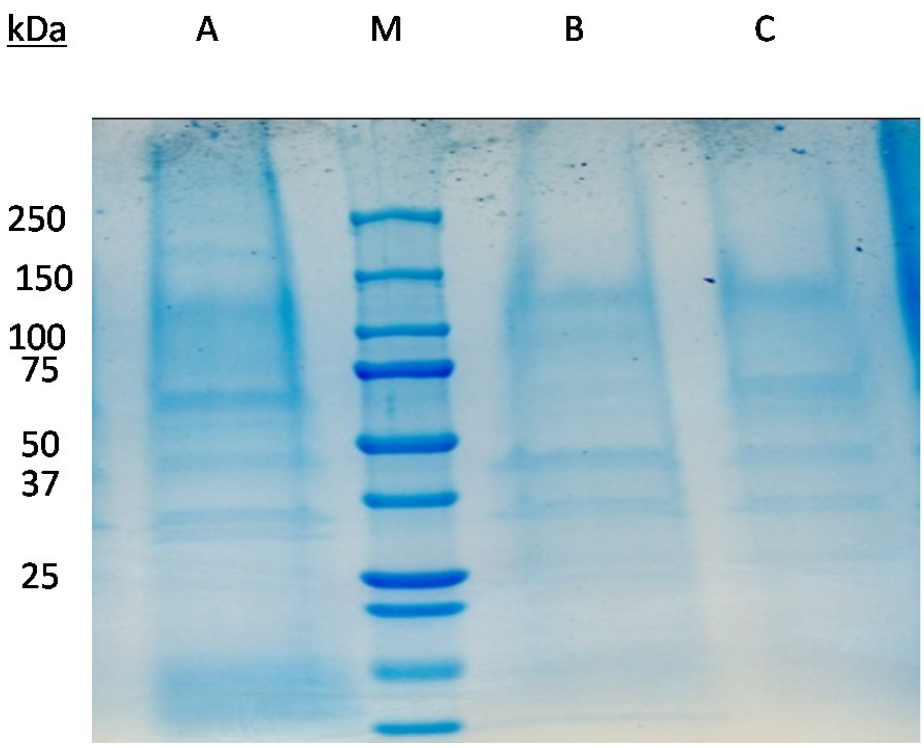

Figure 3.9

SDS-PAGE of three *P. pastoris* transformants (lanes A, B, and C) hr expressing an untagged version of FoFCO1.

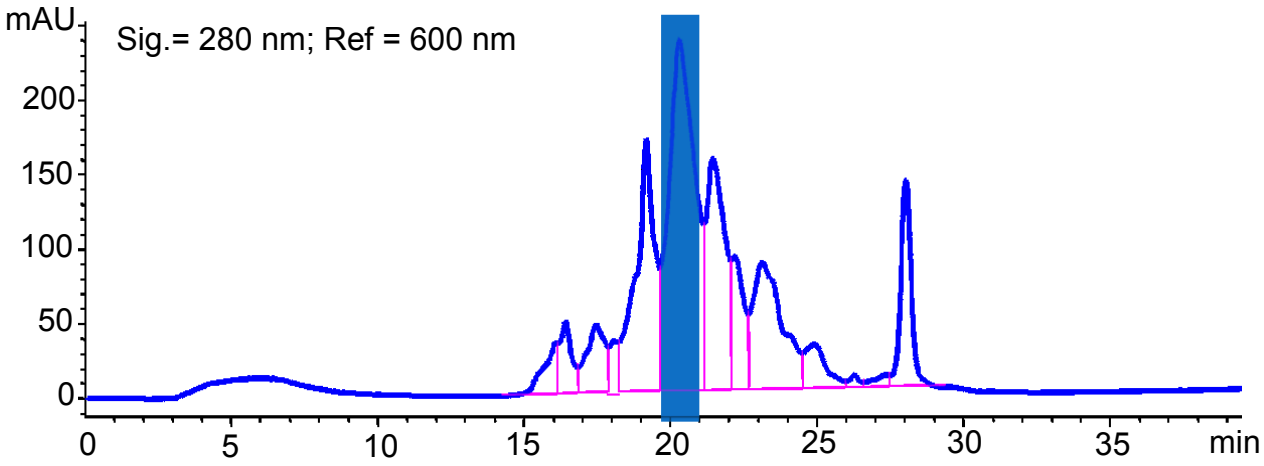

Figure 3.10

Anion exchange chromatography of *P. pastoris* culture filtrate expressing FoFCO1. . Vertical blue bar indicates FCO1 activity on p-NP-fucoside.

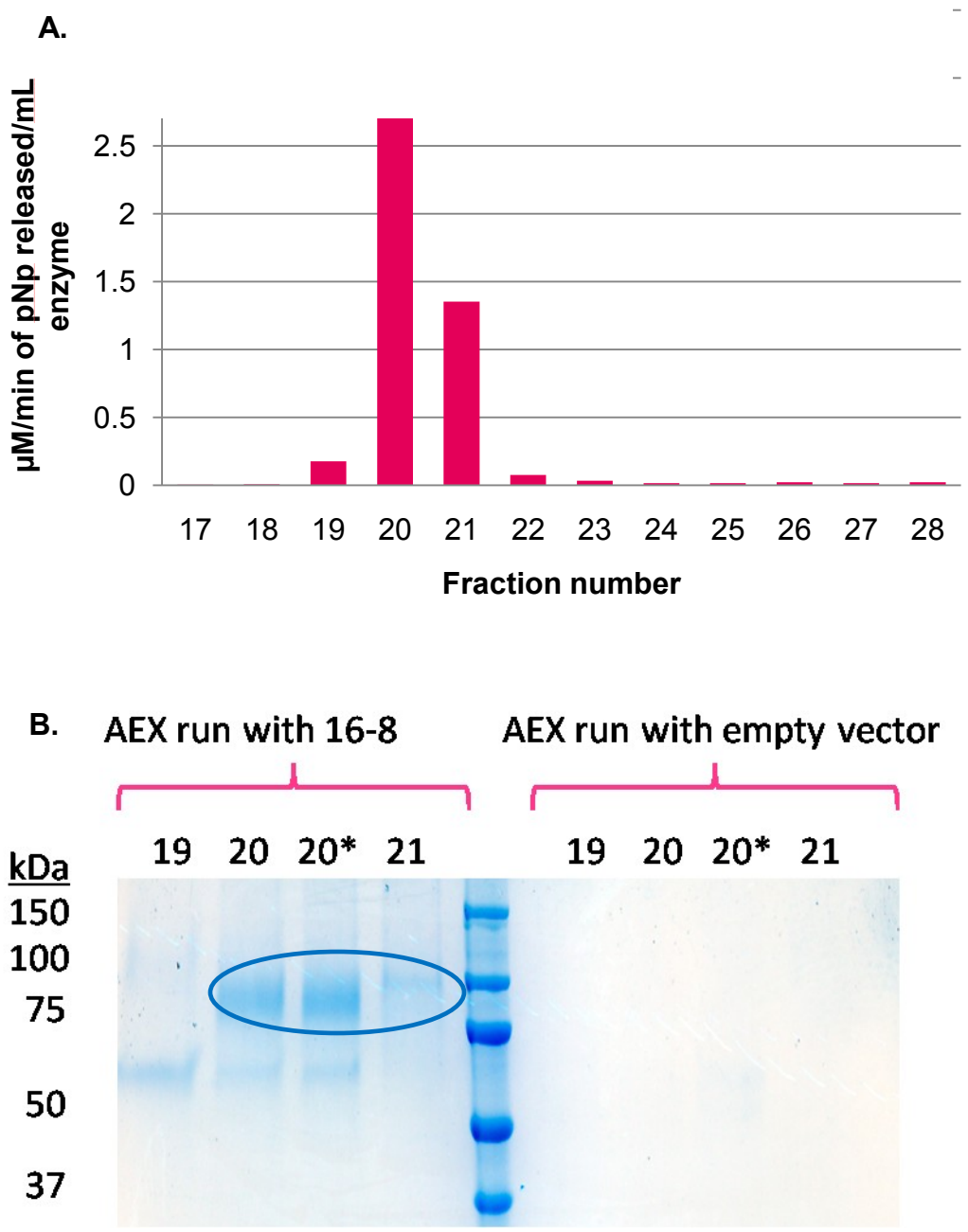

Figure 3.11

A. Activity of HPLC fractions from anion exchange (AEX) fractionation of Pichia transformant 16-8 expressing FoFCO1. **B.** SDS PAGE of active fractions of transformant 16-8 and those corresponding in the empty vector. * indicates that the sample was concentrated 1.5 times.

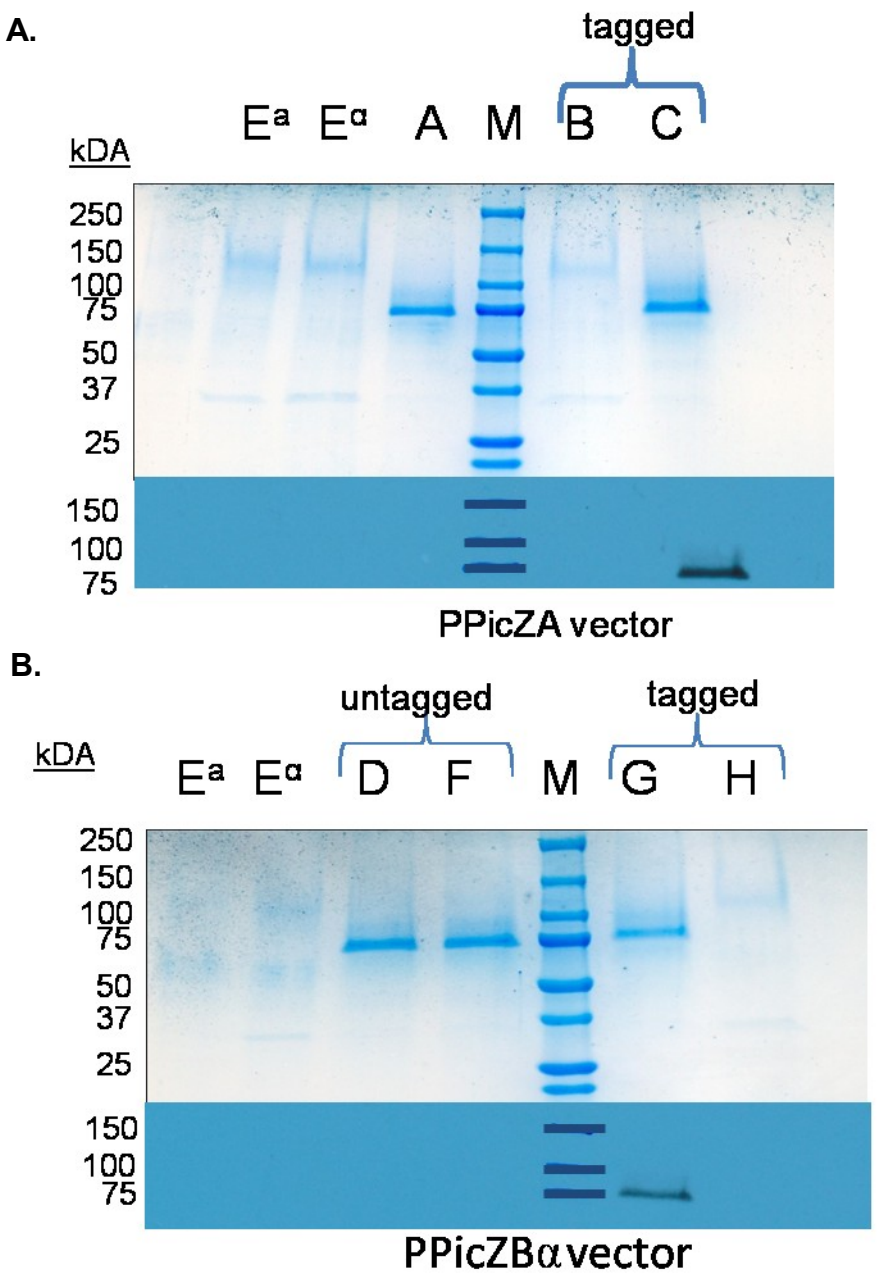

Figure 3.12

A. *P. pastoris* expression of tagged and untagged versions of FgFCO1. E= Empty vector A or Aα; A= Untagged 1, B= Tagged 1, C= Tagged 2. Below, Western analysis of tagged proteins confirming tagged FgFCO1 at just above 75 kDa. **B.** *P. pastoris* expression of tagged and untagged F.g. FgFCO1 in pPICZAα vector. E= Empty vector A or Aα, D= Untagged 2, F = Untagged 3, G = Tagged 3, H = Tagged 4. Below, Western analysis showing the presence of the tagged version of FgFCO1 at 78 kDa. Blue lines on the western blot indicate the location of protein standards.

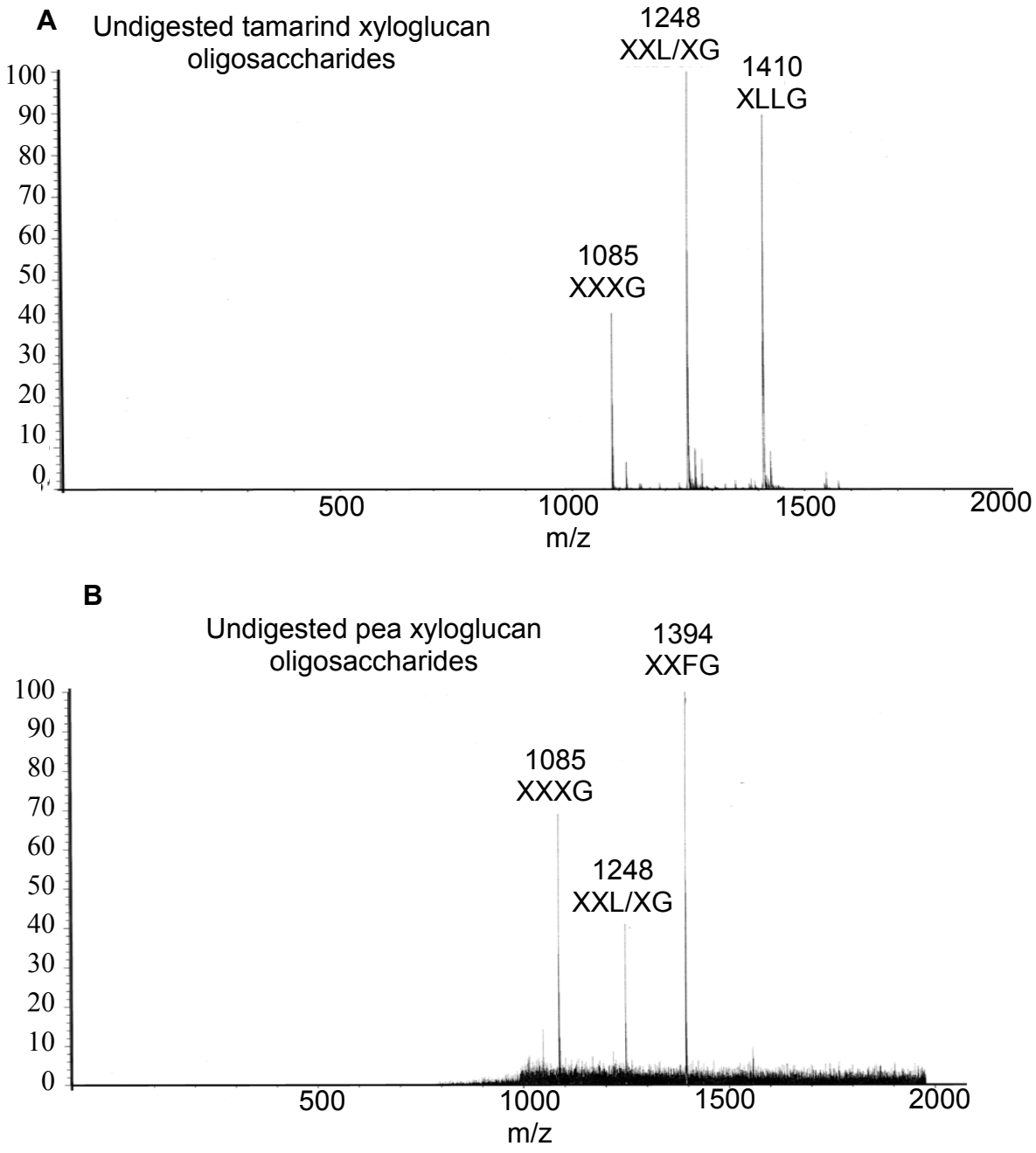

Figure 3.13 A. Mass spectrum of tamarind seed xyloglucan oligosaccharides. Peaks at 1086, 1248, and 1410 correspond to XXXG, XXLG/ XLXG, and XLLG respectively. (XXLG and XLXG cannot be distinguished on the basis of mass). B. Mass spectrum of pea xyloglucan oligosaccharides. Peaks at 1086, 1248 and 1394 m/z correspond to XXXG, XXLG /XLXG, and XXFG respectively.

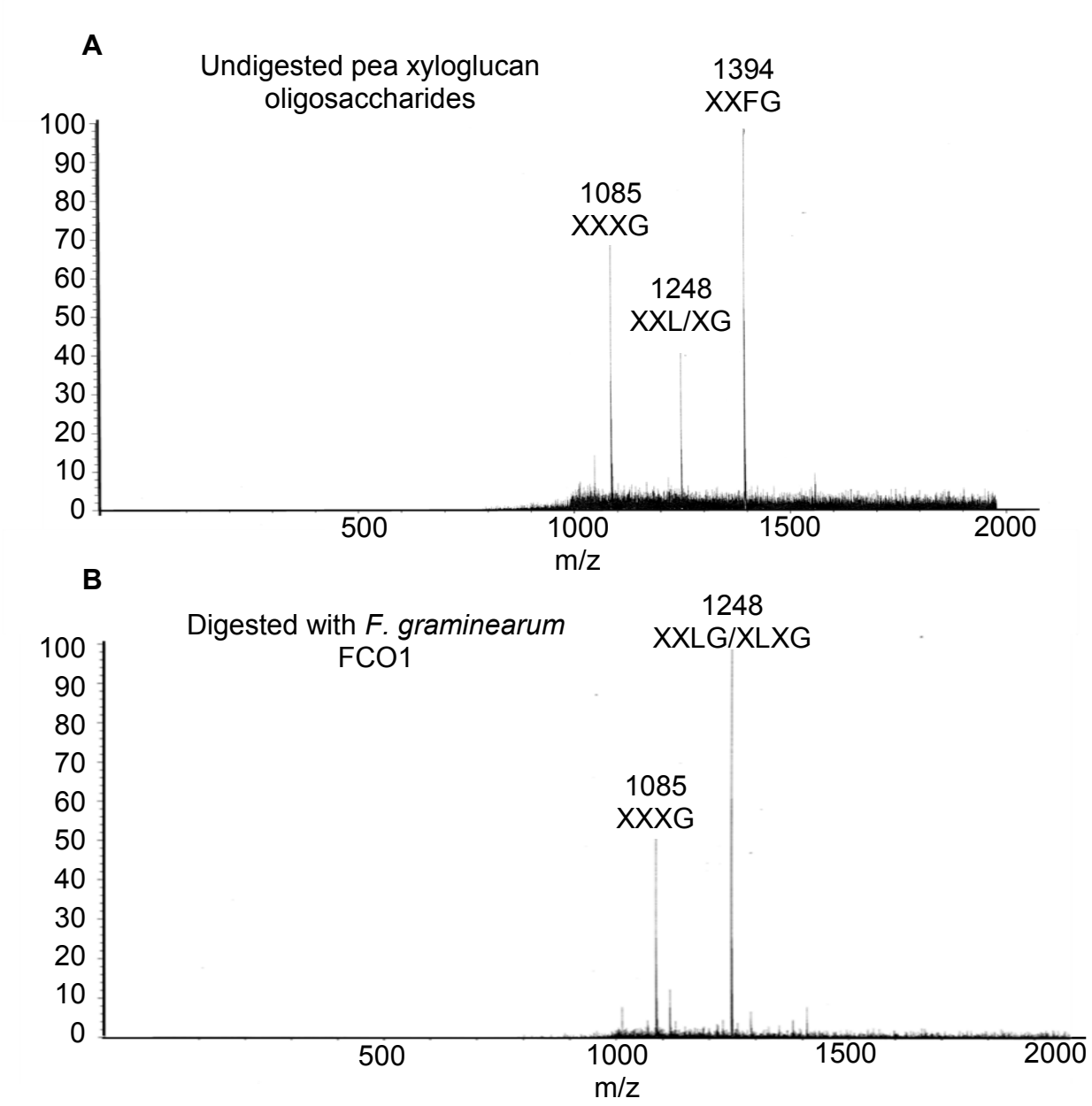

Figure 3.14

A. Mass spectrum of pea xyloglucan oligosaccharides showing the presence of fucosylation. The peaks at 1086, 1248 and 1394 correspond to XXXG, XXLG/XLXG, and XXFG respectively. B. Mass spectrum of pea xyloglucan oligosaccharides digested with culture filtrate of *P. pastoris* expressing FgFCO1. The peak at 1086 is XXXG and the peak at 1248 is either XXLG or XLXG.

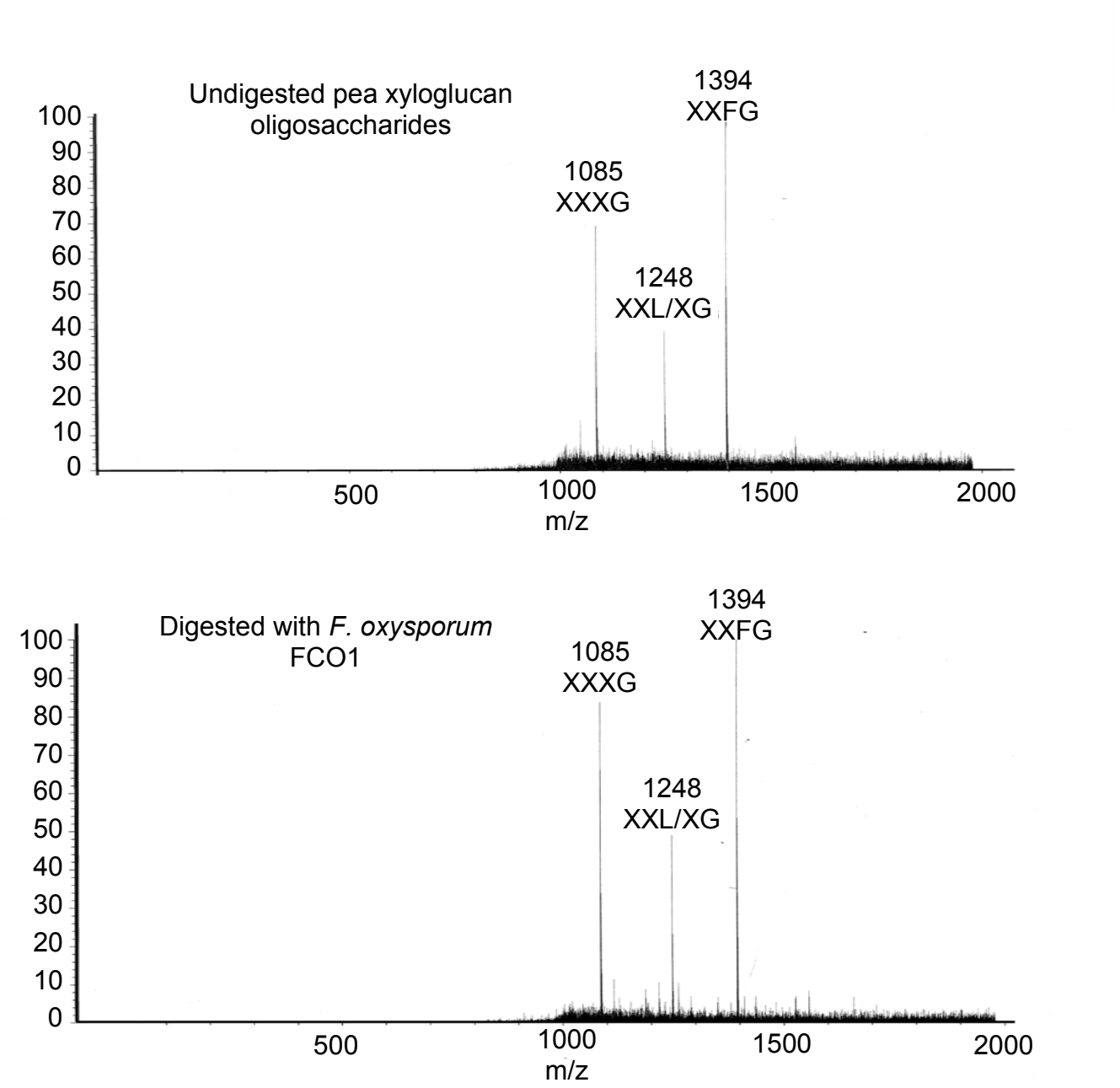

Figure 3.15

A. Mass spectrum of pea xyloglucan oligosaccharides. Peaks at 1086, 1248 and 1394 correspond to XXXG, XXLG or XLXG, and XXFG respectively. B. Mass spectrum of pea xyloglucan oligosaccharides digested with culture filtrate of *P. pastoris* expressing FoFCO1. Peaks at 1086,1248, and 1394 m/z represent XXXG, XXLG or XLXG, and XXFG, respectively.

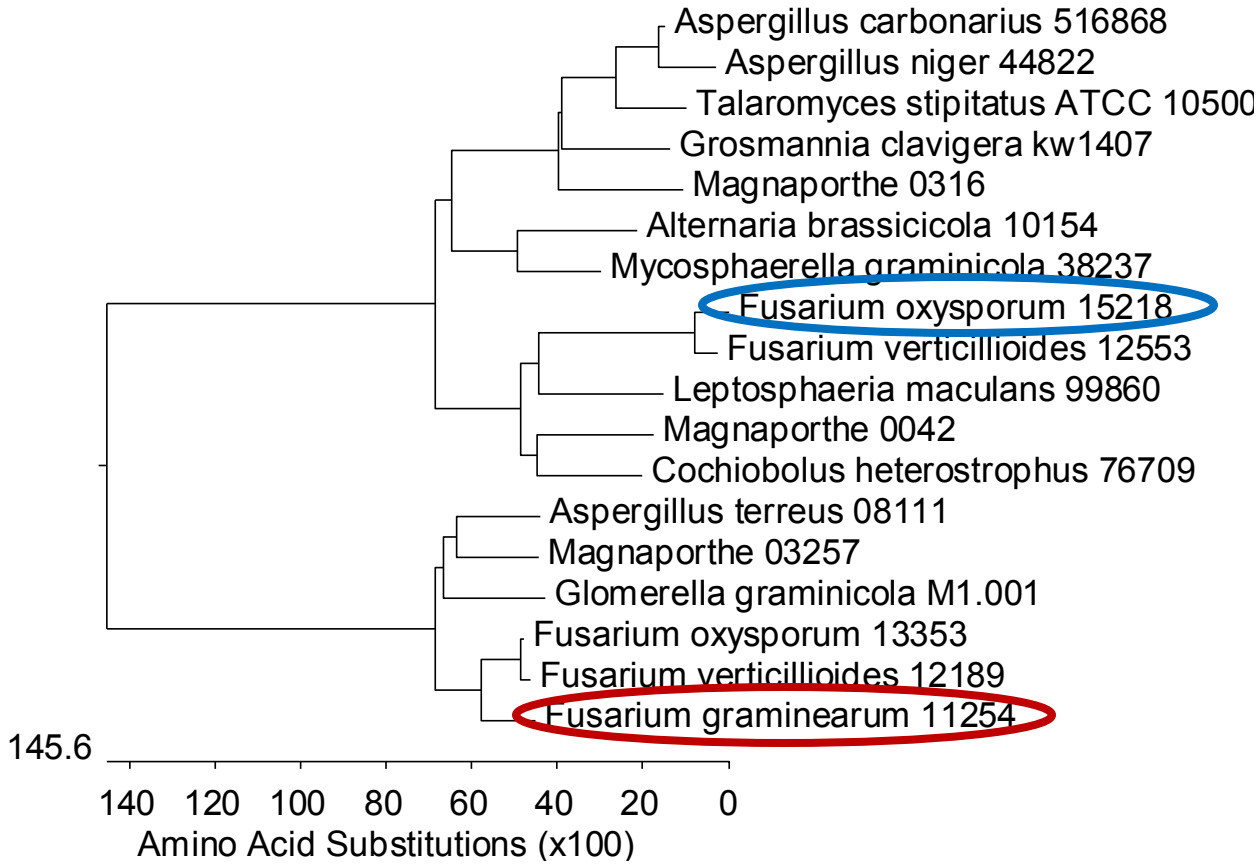

Figure 3.16

A phylogenetic tree of fungal fucosidase sequences classified in CAZy GH family 29. FgFCO1 is highlighted in red, and FoFCO1 is highlighted in blue.

Chapter 4: Gene disruption of secreted proteins in the plant pathogenic fungus *Fusarium graminearum*

Introduction

Fusarium graminearum is a necrotrophic pathogen that causes the disease known as head blight or wheat scab on cereal crops including wheat, barley, and maize. It has been postulated that *F. graminearum* is the limiting factor of wheat production worldwide (Goswami and Kistler, 2004; Trail, 2009; Windels, 2000). The infection not only causes loss of grain weight and quality, but also produces mycotoxins, whose levels are regulated in the US and Europe. An excess of these toxins can render grain unusable for human and animal consumption. Although some wheat varieties tend to be more resistant to infection, there are no known resistance genes available for breeding (Goswami and Kistler, 2004; McMullen et al., 1997; Trail, 2009; Windels, 2000). Since this fungus, like many other fungi, acquires nutrition, perceives, and responds to the environment by secreting hydrolytic enzymes and other proteins, study of these proteins may provide useful information about the mechanisms of pathogenesis by *F. graminearum.*

The role of hydrolytic enzymes in disease have been studied in many plant pathosystems (Apel-Birkhold and Walton, 1996; Apel et al., 1993; Brito et al., 2006; Schaeffer et al., 1994; Scott-Craig et al., 1998; Scott-Craig et al., 1990). In order for host tissue invasion to occur, the plant cell wall must be degraded or penetrated. Although some glycosidases are important for virulence in some host/pathogen combinations, there is no universal hydrolytic enzyme required for all fungal infections. For example, polygalacturonase is a virulence factor in the *Claviceps purpurea*/rye interaction, but it is not required in the *Cochliobolus carbonum* infection of maize (Brito

et al., 2006; Scott-Craig et al., 1990; ten Have et al., 1998). An endo β-1,4 xylanase from *Botrytis cinerea* is required for virulence on tomato leaves and grapefruit, but other xylanases are not required (Apel-Birkhold and Walton, 1996; Apel et al., 1993; Brito et al., 2006; Gomez-Gomez et al., 2002).

A secreted lipase (FGL1) of *F. graminearum* functions as a virulence factor on wheat and maize. An FGL1-deficient mutant created by gene replacement is not able to spread beyond the initially infected and, in some cases, neighboring spikelet; disease being halted at the rachis. It has been hypothesized that this gene either directly enhances cell-wall degradation, facilitating entry into the plant, or releases cutin monomers that activate transcription of other cell-wall degrading enzymes. Regardless of its exact mode of action, in the absence of the lipase the infection process is delayed long enough for the host immune response to limit growth of the pathogen (Voigt et al., 2005).

Non-enzymatic proteins may also be important for virulence. *Fusarium oxysporum* and *Magnaporthe grisea* secrete many small cysteine-rich proteins that are involved in pathogenesis. Several of those from *F. oxysporum* act as effectors and are recognized by host resistance proteins (Houterman et al., 2008; Ma et al., 2010; Michielse and Rep, 2009; Rep and Kistler, 2010; Rep et al., 2004). Similar proteins were found in the genome of *M. grisea*. These proteins are not found in the saprophytic fungus *Neurospora crassa* (Dean et al., 2005; Rep, 2005; Rep et al., 2004).

Proteomics has been used to identify other secreted proteins that may be important in pathogenesis by *F. graminearum*. Phalip and colleagues analyzed the

proteins that are secreted by *F. graminearum* when grown on glucose or hop cell wall media. Eighty-four proteins were secreted in the cell-wall medium that were not secreted in glucose. Most of these were enzymes related to cell wall degradation (Phalip et al., 2005; Phalip et al., 2009). Proteomics has also been performed on culture filtrates from *F. graminearum* grown in a DON-inducing medium (Taylor et al., 2008). These studies as well as the *in vitro* and *in planta* proteomics results described in chapter 2 have helped determine some of the proteins *F. graminearum* utilizes to infect plants (Paper et al., 2007; Phalip et al., 2005; Taylor et al., 2008).

Here we investigated the role of some of the proteins identified by the proteomics experiments in chapter 2 by creating mutants in corresponding genes. We focused on proteins secreted specifically *in planta*, those with no or few paralogs, and unknowns and assayed the mutants for changes in virulence.

Materials and Methods

Growth of Fusarium

Fusarium graminarum strain PH-1 was grown on V8 agar plates at room temperature. Tissue for DNA isolation and protoplast preparation was obtained by placing a piece of inoculated V8 agar in a 250-mL flask containing 50 mL yeast extract sucrose (YES) medium (previously described) and shaken overnight at 250 rpm at room temperature. For protoplast preparation, one mL of this first culture was placed in a fresh 250 mL flask containing 50 mL of YES and again shaken overnight at 250 rpm.

Isolation of DNA

The first culture as described above was collected in 50 mL tubes and separated by centrifugation at 1500XG in a table top centrifuge. The supernatant was discarded and the tissue was then washed with sterilized water three times before being lyophilized. The lyophilized tissue was ground to a powder by shaking with 4 mm sterilized glass beads. When necessary, liquid nitrogen was used to facilitate pulverization. DNA was extracted using the Qiagen Genomic DNA (previously Gentra Puregene -158267) isolation kit following the mouse tail protocol.

Generation of mutants

Knockout mutants were created by homologous recombination. Gene knock-out constructs were made using primers listed in Appendix A (Hallen and Trail, 2008). Fungal tissue for protoplasts was collected on Whatman #1 filter using a vacuum filter apparatus. The tissue was washed on the filter with sterile water. Protoplast preparation and transformation was done as previously described for *Cochliobolus carbonum* using approximately two square centimeters of *Fusarium* fungal tissue as described above (Scott-Craig et al., 1990). Transformants that showed resistance to hygromycin-B (Invitrogen, 10687010) at an initial concentration of150 µg/mL were confirmed by placing on V8 agar plates containing 450 µg/mL hygromycin. Conidia for single spore isolation from those transformants that were able to grow on the higher concentration of hygromycin were obtained by growth on Bilay's agar plates described in (Hallen and Trail, 2008). Replacement of the targeted gene by a hygromycin resistance cassette was confirmed by PCR using primers 5' and 3' of the targeted

insertion site with corresponding primers within the resistance gene. Primers for each gene are listed in Appendix A. Southern analysis was done as a secondary confirmation on some of the mutants according to standard protocols.

Preparation of F. graminearum conidia for inoculation

Conidia from each mutant along with the wild type strain (PH-1) were induced by inoculating 100 mL carboxymethylcellulose medium (CMC) (Sigma, C5678) with a piece of V8 agar containing hyphae. Cultures were grown at room temperature with shaking at approximately 100 rpm for 3 d. Conidia were harvested by pouring culture through sterile Miracloth® into a sterile 50 mL culture tube. Conidia were spun at 1500xG and rinsed in sterile water three times. A final solution of 1 x 10^5 conidia/mL in 0.01% Tween-80 was used for inoculation.

Inoculation and virulence testing on wheat

Wheat (*Triticum avestium*) cultivar Norm was grown using previous methods (Paper et al., 2007). At the time when the anthers began to extrude from the floret, 10 µL of conidial suspension was injected between the palea and lemma. High humidity was obtained by placing the plants in a mist chamber for three days. The plants were then kept in the greenhouse and watered as need. Initially, plants were visually screened for virulence. At a later date, virulence was assayed using a double blind scoring system. After being removed from the mist chamber, the pots were randomized and both pots and plants were assigned numbers that corresponded to the treatment.

Each plant was then scored for virulence at approximately 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, and 25 d post- inoculation (dpi) with day 3 corresponding to when the plants were removed from the mist chamber. The scoring system was based on a scale of 1 to 5 in increments of 0.5, with 1 showing no signs of disease and 5 corresponding to all of the florets on the wheat head showing disease symptoms. The person randomizing and assigning the treatment type numbers did not take part in scoring the plants. A depiction of the scale can be seen in Fig. 4.1. After the last day of scoring, the analyst was given access to the key that matched pot number with treatment so that statistical analysis could be completed.

For some experiments, seed weight was also measured. After the virulence scoring study was complete, the plants were allowed to dry in the greenhouse. When all of the heads were completely dry, they were cut from the plant and grouped according to inoculation treatment. All seeds from each treatment were collected and pooled. Twenty seeds from each treatment were randomly selected and weighed. This process was replicated 3 times.

Data analysis and statistics

Statistical analysis of scored virulence assays was done with two-way repeated measures ANOVA using IBM SPSS Statistics version 19. All virulence recordings for each set of gene transformants and corresponding wild type (scored at the same time) were compared as groups. Mock inoculation was excluded. Groups were analyzed by the change over time (a positive control since virulence ratings of all mutants and wild

type inoculated increased over time) and change between treatments over time. Those groups showing differences between treatments over time at the 95% confidence level were then analyzed individually with the Student t-test to determine which treatment and time points were significant. In those cases where transformants of the same gene were scored in separate assays, each time point was kept separate; resulting in multiple repeated measured data for the gene. Statistical analysis of seed weight was done by standard deviation with +/- one standard deviation reported as significant and reported as significant (yes) or not (no).

Results

Deletion mutants of 38 genes identified in the proteomics experiments described in chapter 3 were made, confirmed by PCR, and tested for virulence on wheat. These genes encoded glycosyl hydrolases, proteases, and a large number of hypothetical proteins with no predicted functions. Most are predicted to have secretion signals. The genes that were mutated and the virulence results are listed in Table 4.1. For those mutants for which seed weight and scored disease data were not collected, the table shows "-" and "observation only" respectively. Confirmed transformants for one gene replacement (FGSG_4074) were never achieved. Mutants listed as ID-57G, ID-119G and FGSG_5906 were assayed as controls. ID_57G and 119G were obtained in a mutant screen from Frances Trail as possible virulence mutants (Baldwin et al., 2010a). FGSG_5906 (FGL1) a secreted lipase gene previously reported to contribute to virulence was deleted as described above (Voigt et al., 2005). Only one of the three

control mutants (ID-57G) showed significant virulence reduction in both symptom scoring and seed weight (Table 4.1).

With the exception of FGSG_3003 and FGSG_4848, the virulence of the mutants was not statistically significantly different from the wild type by repeated measure ANOVA or seed weight difference, so further analysis was not performed. Since both mutants of FGSG_3003 and FGSG_4848 showed a difference between groups with repeated measures ANOVA at or above the 95% confidence level, further analysis was done on them. Results of Student's t-test, used to compare mutant to wild type (PH-1) to each transformant at each time point, are summarized in Table 4.2.

Along with a slightly lower (though significant) disease score at some time points, seeds from wheat inoculated with mutants of FGSG_3003 were also slightly heavier than those inoculated with wild type, indicating lower virulence (Fig. 4.2). Due to this subtle phenotype, virulence testing was done twice, with similar results. FGSG_3003 is annotated as related to an alpha-arabinosidase in MIPS FGDB (http://mips.helmholtz-muenchen.de/genre/proj/FGDB/) and as a hypothetical protein by the Fusarium Comparative Sequencing Project (http://www.broadinstitute.org/). It is predicted to contain 335 amino acids and be a member of CAZy glycosyl hydrolase family 43, which includes enzymes with beta-xylosidase, alpha-arabinofuranosidase, arabinanase, and xylanase activity (Cantarel et al., 2009).

One of three mutants of FGSG_4848 had slightly (although significantly) more severe disease symptoms from 13 to 25 d (Fig.4.3). Although the other mutants with the same gene replaced also caused slightly higher disease scores, these differences

were not significant. FGSG_4848 is annotated as a probable rhamnogalacturonan acetylesterase (http://mips.helmholtz-muenchen.de/genre/proj/FGDB/).

Discussion

Although the proteins products of all of the genes studied here are expressed *in planta*, the mutants had no severe virulence phenotype. This might be due to several reasons. One is that fungi, including *F. graminearum*, have a high degree of redundancy in their genomes. A number of the proteins identified during the *in planta* proteomic study had predicted paralogs within the genome and in many cases those paralogs were also identified by proteomics (Paper et al., 2007). The presence of this redundancy could explain the lack of phenotypes. That is, if there is more than one protein that acts to degrade the same polymer, the virulence of the fungus would not be affected by deleting just one of the encoding genes. Although we tried to target genes that did not appear to have a high level of redundancy, many of the genes targeted for replacement were of unknown function, and therefore only sequence similarity, not predicted function, can be used to estimate redundancy.

Although three independent mutants of FGSG_3003 (encoding a putative alpha-arabinofuranosidase) did have significantly reduced virulence at some time points, this phenotype was weak. Infection by the mutants lacking the predicted alpha-arabinofuranosidase was slightly lower during the first days of the infection. The corresponding seed data seem to suggest that this delay did have an effect on seed development. Perhaps lack of this enzyme slowed growth of the fungus just enough to allow the seeds to develop further. There are eight other genes in the genome of *F.*

graminearum that are annotated as probable arabinofuranosidases. This redundancy may explain the weak phenotype.

Although enzymatic activity on the protein product was not assayed, gene disruption of a predicted arabinofuranosidase/xylosidase gene in Sclerotinia *sclerotiorum* decreased virulence on canola (Yajima et al., 2009). Additionally, unpublished results from our lab have shown that triple mutants in three arabinofuranosidase genes of *Cochliobolus carbonum* result in a reduced virulence phenotype on maize (unpublished results). Further analysis,(i.e. making double and triple gene knock-outs along with demonstrating the enzymatic activity of these predicted genes) will be needed to more accurately define the role of these enzymes in virulence.

One mutant (out of three) of FGSG_4848, a predicted rhamnogalacturonan acetylesterase, showed a slight but significant increase in disease symptoms. Since only one of the three biological replicates showed this phenotype, we cannot exclude that this is due to chance. However, although not statistically significant, the other transformants also show increased disease symptoms. Further analysis, including Southern analysis to confirm the gene replacement in all three transformants as well as repeated virulence assays, should be performed. Although PCR confirmations were positive for all three mutants, false positives can occur, so there is a possibility that only the mutant showing the phenotype is a true knock out. A possible explanation for why mutation of this gene causes increased disease is that the enzyme or its reaction products are elicitors of host defense responses, and that in its absence the plant mounts a weaker or delayed defense response.

The virulence assays were performed on a variety of wheat (Norm) that is more susceptible than standard field varieties and other lab varieties now used for virulence testing. This could explain why phenotypes were not seen for some of the controls as well as most of the gene deletion mutants. The secreted lipase, previously reported to cause a significant virulence reduction was done on a different variety of wheat (Nandu)(Phalip et al., 2005) . It was hypothesized that lack of this lipase could be reducing the rate of infection just enough for the plant's defenses to have time to prevent spread by forming a papilla and increased cell wall fortification at the rachis. This type of host defense, specifically the speed at which it occurs, has been reported to be a difference between more susceptible and more resistant varieties of wheat (Ribichich et al., 2000). A delayed salicylic signaling cascade has also been reported in wheat cultivars that are less resistant to infection. It has not been determined if this delay is related to differences in cell wall fortification or whether it involves a separate pathway (Ding et al., 2011). It is possible that some of the mutants made in this chapter would show more dramatic reductions in virulence if we had used different cultivars of wheat.

Finally, in order to determine significant small changes in virulence, a more statistically robust study with a greater number of replicates need to be performed. Virulence assays were conducted as each mutant was made over the course of several years and the assay procedures, as well as the personnel scoring the mutants, changed during the study. There were also changes in environmental conditions (temperature, other diseases or pests present in the greenhouse, etc.) and some data collection was missed during the course of the study.

Figure 4.1
Representation of the disease rating scale used to score virulence

Table 4.1. Gene replacement virulence results. Each gene knock-out is listed along with its FGDB annotation, number of independent transformants assayed for virulence, the ANOVA repeated measures probability and indication of the statistical significance of seed weight.

Gene# or ID	Gene Annotation[a]	# of Transformants assayed	Scored virulence Repeated measures probability[d]	Seed Weight difference[b]
ID 57G	Control 1	1	0.000	yes
ID 119G	Control 2	1	0.245	no
FGSG_0028*	probable metallo-protease MEP1	2	0.249	no
FGSG_0060-0062*[c]	Related to KP4 killer toxin	2	0.197	no
FGSG_0114*	Conserved protein	2	no difference observed	-
FGSG_0346	Probable sacch-aropine reductase	2	0.105	no
FGSG_571	Probable cellulase	2	0.894	no
FGSG_2560	Conserved hypothetical protein	3	no difference observed	-
FGSG_2897	unknown	3	0.341	no
FGSG_3003*	Related to arabino-furanosidase	3	0.054	yes
FGSG_3124*	Conserved hypothetical protein	1	no difference observed	-
FGSG_3188	Conserved hypothetical protein	1	0.766	-

Table 4.1 cont'd

Gene# or ID	Gene Annotation[a]	# of Transformants assayed	Scored virulence Repeated measures probability[d]		Seed Weight difference[b]
FGSG_3211	Conserved hypothetical protein	2	no difference observed		-
FGSG_3483*	Probable pectin lyase precusor	2	no difference observed		-
FGSG_3526*	Unknown trichothecene gene cluster	2	no difference observed		-
FGSG_3628*	Probable celluase	1	0.153		no
FGSG_3632	Related to cellulose binding protein	2	0.320		no
FGSG_3842	Related to alpha amylase A	2	0.417		no
FGSG_4213	Conserved hypothetical protein	3	0.860	0.771	no
FGSG_4074	Conserved hypothetical protein	0-possible lethal	na		-
FGSG_4583*	Conserved hypothetical protein	3	no difference observed		-
FGSG_4848	probable rhamnogalacturonan acetylesterase	3	0.035		-
FGSG_5236	Related to metalloproteinase	3	0.167		no
FGSG_5906*	FGL1 – secreted lipase	2	0.368	0.831	no

Table 4.1 cont'd

Gene# or ID	Gene Annotation[a]	# of Transformants assayed	Scored virulence Repeated measures probability[d]	Seed Weight difference[b]
FGSG_6469	Conserved hypothetical protein	1	0.149	no
FGSG_7439	Probable cyclophilin	2	no difference observed	-
FGSG_7558	Conserved hypothetical protein	2	no difference observed	-
FGSG_7822	Conserved hypothetical protein	2	no difference observed	-
FGSG_8037	Conserved hypothetical protein	2	no difference observed	-
FGSG_9366	Related to 1,3 beta glucosidase	2	0.742	no
FGSG_10675	Related to lactonohydrolase	1	0.467	no
FGSG_10676	Conserved hypothetical protein	2	0.848	no
FGSG_10782	probable aspartic proteinase	2	no difference observed	-
FGSG_11036	Related to esterase D	2	0.658	no
FGSG_11037	Probable endoglucanase	3	0.164	no
FGSG_11176	Conserved hypothetical protein	1	0.221	no
FGSG_11254	FgFCO1 – alpha-fucosidase	2	0.251	no
FGSG_13094	Conserved hypothetical protein	1	0.247	-

Table 4.1 cont'd

Gene# or ID	Gene Annotation[a]	# of Transformants assayed	Scored virulence Repeated measures probability[d]	Seed Weight difference[b]
FGSG_13095	Hypothetical protein	2	0.183	-

***** - These gene replacement mutants were also confirmed by Southern analysis. The others were confirmed by PCR only.
a – Annotations based on updated MIPS FGDB Fg3 assembly (Wong et al., 2011).
b – Seed weight significance was determined by standard deviation and indicated if significant. A "–" indicates that seed weight data was not collected.
c – Three genes, FGSG_0060, FGSG_0061 and FGSG_0062, were replaced in this mutant in a single recombination.
d – ANOVA repeated measures probability indicates if disease symptoms of any mutant or wildtype grouping differed significantly during the course of the study. A result of $\leq$ 0.05% indicates that the disease symptoms of one or more groups developed differently at a 95% confidence interval. This analysis does not specifically compare mutants to wildtype groups.

Table 4.2 Student t-test P-values for individual time points of mutants

		Days Post Inoculation									
Gene/ trans-formant	**n**	**3**	**5**	**7**	**11**	**13**	**15**	**19**	**21**	**23**	**25**
FGSG 3003-1	12	ns	1.00	0.50	**0.00**	**0.01**	**0.00**	0.07	0.50	0.81	ns
FGSG 3003-2	11	ns	0.08	**0.02**	**0.01**	0.09	0.07	0.14	0.30	0.81	ns
FGSG 3003-4	9	ns	0.33	**0.01**	**0.04**	0.43	0.18	0.69	1.00	0.66	ns
FGSG 4848-2	14	ns	0.34	1.00	-	0.58	0.29	0.43	0.40	0.38	0.12
FGSG 4848-3	16	ns	0.0	0.34	-	0.67	0.99	0.95	0.79	0.78	0.65
FGSG 4848-5	15	ns	0.0	0.34	-	**0.02**	**0.03**	**0.02**	**0.02**	**0.03**	**0.04**

Results shown in bold are significant at the 95% confidence interval ($p < 0.05$)
- Data not collected
ns – not significant - t-test could not be performed due to no variance between sample groups
n- number of plants assayed

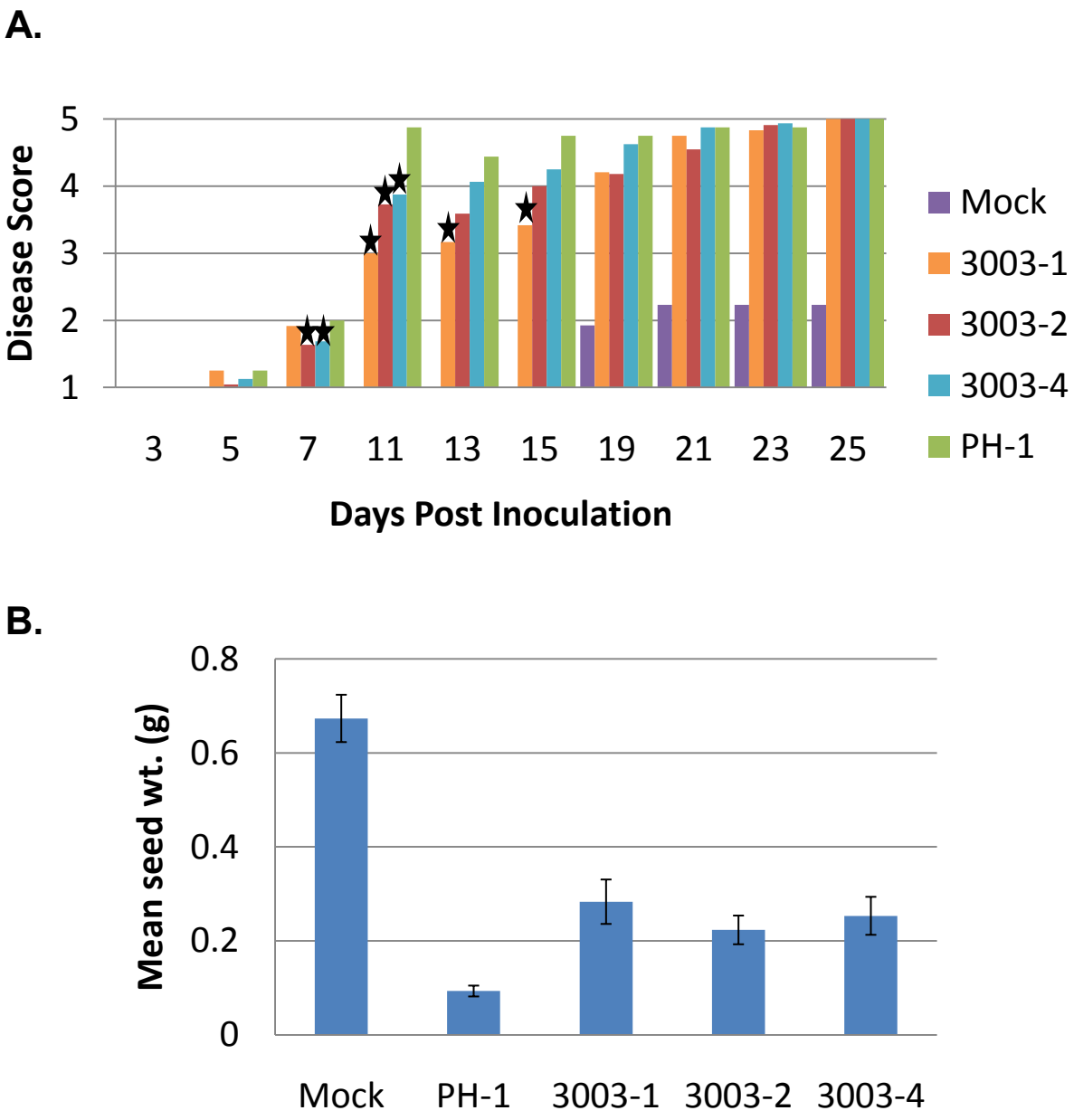

Figure 4.2
Virulence scores (A) and mean seed weight (B) for FGSG_3003 gene replacement mutant inoculated on wheat. Stars represent that the mutants were statistically significant from wildtype (PH-1). Refer to Table 4.2 for p and n values.

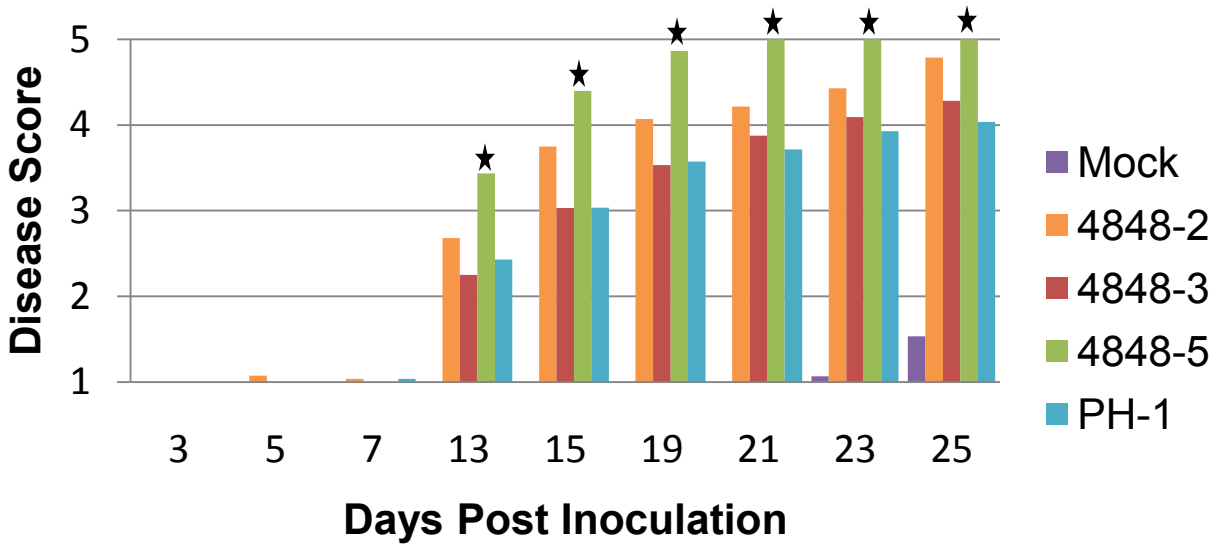

Figure 4.3

Virulence scores for FGSG_4848 mutant on wheat. Stars indicate that the mutant was statistically different from wildtype (PH-1) at a 95% confidence interval. Refer to Table 4.2 for p and n values.

Chapter 5: *Fusarium graminearum* secreted proteins and enolase as triggers of innate immunity

Introduction

Plants have multiple mechanisms to identify and respond to pathogens. The most basic of these is innate immunity, in which the plant cells recognize a distinctive molecular signature from its environment. Although plants and mammals may have evolved the ability to perceive these signals independently, the activation of an innate immune response is common to both (Ausubel, 2005; Zipfel and Felix, 2005). When recognition occurs, a signal cascade activates a basal immune response commonly referred to PAMP-triggered (pathogen associated molecular pattern) immunity or PTI (Bittel and Robatzek, 2007; Chisholm et al., 2006; Zipfel, 2008). This immune response can include but is not limited to callose deposition, a burst of reactive oxygen species (ROS), alkalinization of the surrounding medium,and closure of stomata (Bittel and Robatzek, 2007; Felix et al., 1999; Zipfel and Felix, 2005).

Some of the molecular triggers of PTI have been identified. They consist of specific parts of macromolecules, sometimes referred to as molecular patterns, that are specifically recognized through surface receptors on the plant cell (Bauer et al., 2001; Chinchilla et al., 2006; Robatzek et al., 2007). Usually these molecular patterns come from conserved proteins or molecules that are necessary for the pathogen's survival. These PAMPs are also present on non-pathogen associated microbes and are referred to as microbial associated molecular patterns (MAMPs) (Ausubel, 2005; Boller and Felix, 2009; Mackey and McFall, 2006). The initial recognition and response to PAMPs and MAMPs prevents most organisms from being pathogenic (Boller and Felix, 2009). Pathogenic organisms have evolved ways to avoid this initial defense with molecules called effectors. Both plants and pathogens have evolved multiple layers of defense

responses and reactions to these responses, amounting to what has been referred to as a molecular arms race (Chisholm et al., 2006; Jones and Dangl, 2006).

Flg22 and elf from the proteins flagellin and elongation factor Tu, respectively, are two examples of MAMPS that have been described in *Pseudomonas syringae* (Gomez-Gomez et al., 1999; Kunze et al., 2004). Receptors for both of these proteins are leucine rich repeat – receptor-like kinases (LRR-RLK), and downstream signaling responses are currently being characterized (Boller, 2008; Chinchilla et al., 2007; Jeworutzki et al., 2010). There are examples of MAMPs in other organisms, such as cell wall transglutaminase GP42 in *Phytophthora sojae,* which contains a 13-amino sequence (Pep13) that elicits a response in parsley and potato (Fellbrich et al., 2002).

Fungal PAMPs include chitin, ergosterol, and β-glucan from fungal cell walls. Ethylene-inducing xylanase (EIX) from *Trichoderma viride* is well- studied; xylanase activity is not necessary for its PAMP activity; only a five amino acid sequence on the surface of the protein is necessary (Furman-Matarasso et al., 1999; Rotblat et al., 2002). Recognition of this peptide leads to a hypersensitive response, which is not a typical PTI response. HR is usually seen when pathogen effectors are recognized by a plant (Bailey et al., 1990; Desaki et al., 2006; Granado et al., 1995; Kaku et al., 2006). . The binding sites of both EIX and chitin have been characterized as receptor-like proteins, containing extracellular domains, transmembrane spanning regions, and cytoplasmic tails, but no kinase domains (Kaku et al., 2006; Ron and Avni, 2004).

Arabidopsis and rice each have a large family of predicted RLKs and receptor like proteins (RLPs), few of which have been characterized (Shiu et al., 2004). If plants

are indeed using MAMPs as a “first alert” system to detect the presence of possible pathogens, one would expect that this would not be isolated to only a few peptide sequences. There may be many more MAMPs and PAMPs then we are currently aware of. Proteins that are secreted during infection are good candidates as MAMPs or PAMPs because they are present at the plant/pathogen interface. Housekeeping proteins are also likely MAMP candidates because they are ubiquitous, highly conserved and necessary for survival (Pancholi and Chhatwal, 2003). In this regard, they resemble flagellin and EFTu of bacteria. In fact, several possible candidate MAMPs are present in the apoplast during infection of wheat florets by *F. graminearum* (Paper et al., 2007). These include the common housekeeping proteins enolase and elongation factor *alpha*. These proteins were not found in culture filtrates or vacuum infiltrated fungal tissue (Paper et al., 2007).

Enolase is a ubiquitous metabolic enzyme responsible for the conversion of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate (PEP). Since this enzyme is necessary for survival, it is a good candidate for recognition by plants for PTI. Enolase is a major fungal allergen in humans. It is secreted by a non-classical system into the cell walls of *Saccharomyces cerevisiae* (Benndorf et al., 2008; Edwards et al., 1999; Sharma et al., 2006; Verma et al., 2003).

The *F. graminearum* enolase identified *in planta* (FGSG_01346) was expressed in *E. coli* and tested on Arabidopsis seedlings for its ability to induce PTI. We also investigated the potential of *F. graminearum* culture filtrates to induce PTI or other responses in *Arabidopsis* seedlings and cell cultures, in order to identify any other possible PAMPs or MAMPs.

Materials and Methods

Growth of F. graminearum and collection of culture filtrates

F. graminearum was grown on corn cell wall medium (Paper et al., 2007). The culture filtrate was concentrated 100-fold by lyophilization, desalted on a size exclusion columns with a 6000 Da cut-off (BioRad #732-2010), and sterilized using 0.22 µm PVDF (low protein binding) filters (Millipore, Millex®-GV).

HPLC separation of F. graminearum culture filtrates

Cation exchange chromatography was performed using a buffer containing 30 mM sodium acetate, pH 4.5, for buffer A, and with buffer B containing 1 M NaCl. One mL of culture filtrate prepared as described above was added to a 2X buffer A, and injected into a 5 mL injection loop and loaded onto a 200 mm X 4.6 cm poly sulfoethyl A column with a particle size of 5 µm and pore size of 300-Å (PolyLC 2045E0503). Run conditions were flow rate of 1 mL/min and a gradient of 0 – 5 min 0% B, 5 – 35 min to 50% B, and 35 – 45 min to 100% B.

Anion exchange chromatography was performed using a buffer system consisting of 25 mM Tris, pH 8.0 (buffer A) and 25 mM Tris, pH 8, + 0.6 M NaCl (buffer B). One mL of unsterilized culture filtrate was mixed with 1 mL of 2X buffer A and injected into a 5 mL injection loop and loaded onto a TOSOH Bioscience (18386) 8.0 mm X 7.5 cm TSK gel Super Q – 5PW column, with 10 µm particle size. A TOSOH Bioscience (08803) 8.0 mm X 7.5 cm TSK gel SP-5PW with a 10 µm particle size was

also used in some experiments. The gradient consisted of 0 – 10 min at 0% B, 10 – 45 min to 100% B with a flow rate of 1.0 mL/min. Three mL fractions were collected.

Cloning and expression of F. graminearum enolase

A *F. graminearum* cDNA library was made by reverse transcription of RNA collected at 48 hr after induction of sexual development (Hallen et al., 2007). FG_ 01346 was amplified using the primers specific for the start and stop codons containing restriction sites for BamHI and NotI, respectively (5'CCCCGGATCCATGGCTATCAAGAAGGTC and 5'TTTTGCGGCCGCCAGGTTAACAGA). The resulting product was sequenced and cloned into pET-21+ vector (Novagen, 69770-3) using standard molecular techniques with a C-terminal His tag . The resulting plasmid was transformed into One Shot® BL21(DE3) pLysE chemically competent *E. coli*, selected by ampicillin resistance, and confirmed to have the correct insert by colony PCR and restriction digestion. Several colonies along with an empty vector colony were picked and used to inoculate 3 mL of LB containing 100 µg/mL Ampicillin (LB/Amp). Cultures were grown overnight shaking at 250 rpm at 37° C and added to 250 mL sterile baffle flasks containing 100 mL LB/Amp. Cultures were grown with shaking at 250 rpm at 37°C until the OD400 reached 0.6, at which time they were split (for induced and uninduced control samples) into two 250-mL sterile baffle flasks. Samples were induced with 500 µL of 100 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) and growth continued for 2 hr. The cells were collected by centrifugation, frozen overnight, and lysed using Bugbuster® Protein

Extraction Reagent (Novagen 70584-3). Both supernatant and pellet were saved. The tagged protein in the supernatant was separated using 12 x 1.5 mL Ni-NTA Superflow Columns according to the protocol provided (Qiagen, 30622). Briefly, the soluble supernatant was applied to the column and allowed to drip through. Two 2-mL 20 mM imidazole washes were then performed followed by three 2-mL elution steps containing 250 mM imidazole. All fractions were kept. SDS-PAGE was done using Bio-Rad 4-20% Tris-HCl Ready Gels (161-1159) with a running buffer of 25 mM Tris, 192 mM glycine and 0.01% SDS buffer. Gels were run at 15 amps constant current. Western analysis was done using a 6xHis antibody/HRP conjugate from Clontech (631210) following the protocol provided. Chemiluminscent detection of the conjugate was done with Pierce® ECL Western Blotting Substrate following the protocol provided. The supernatant containing the eluted tagged protein was desalted using BioRad columns described above and stored at -20°C in 25 mM potassium phosphate buffer, pH 7.0 so that it could be directly used for seedling assays.

Growth of Arabidopsis seedlings

Seeds of *Arabidopsis thaliana* ecotype Columbia were sterilized and sown on Murashige and Skoog Basal Salt Mixture (MS) (Sigma, M5524) with 1.5% sucrose (J.T. Baker, 4072-05) and 1% agar (Accumedia, 7558A). Plates were placed in 12 hr day/night conditions under grow lights at room temperature. At the time the first true leaves emerged (approximately 10 d), the seedlings were carefully pulled from the agar

with sterile tweezers and transferred to 1-mL liquid MS medium in 24-well culture plates, two plants per well.

Assay of culture filtrates and expressed proteins on Arabidopsis

At the time that the *Arabidopsis* seedlings were transferred to liquid medium, the enolase or the culture filtrate samples were also added to the liquid medium. Volumes varying from 5 µL – 30 µL of whole culture filtrate or 5 – 100 µL of enolase solution were used. Uninoculated corn cell wall culture filtrate, processed as described above for *F. graminearum*, flg22, and non-treated blanks were also included where indicated. After the initial experiments, 10 µL became the standard volume of culture filtrate used. When the culture filtrate had been fractionated by HPLC, each fraction was desalted, lyophilized, and reconstituted so that the theoretical concentration of the active component/s would remain the same between the whole and fractionated samples. For practical purposes, the volume of water used for reconstitution and the volume applied to the plant were both doubled, so that efficient reconstitution of the lyophilized samples would occur. Volumes applied to seedlings did not exceed 50 µL. Unfractionated culture filtrate was included as a positive control. Seedlings were allowed to grow for approximately two weeks, or until plants had grown large enough to distinguish between stunted and non-stunted plants. Photographs of plants in wells and separated on a glass plate were taken.

ROS luminescence assay

ROS was measured in *Arabidopsis* leaf tissue by luminescence (Felix et al., 1999). *Arabidopsis* leaves were diced into 1-2 mm slices and submerged in water overnight. A 20 mM stock of luminol (Sigma, A8511) was made by dissolving 35 mg luminol in 1 mL of 0.2 M NaOH and then diluting with 50 mM KH_2PO_4 buffer, pH 7. Assays were done in 96-well plates. Approximately five leaf slices were placed in each well containing 200 ul of water plus 2 µL of 1 µg/mL horseradish peroxidase (Sigma, P6782), 20 µL of luminol solution, and the appropriate amount of each elicitor. Luminescence was measured immediately using a SpectraMax L from Molecular Devices (Sunnyvale, CA) using a kinetic assay for 25 min. Luminescence was measured in photon counting mode.

Results

Expression of F. graminearum enolase in E. coli

A cDNA copy of *F. graminearum* FGSG_01346 was obtained by reverse transcription of an RNA population and amplification of the specific gene and sequenced. The predicted amino acid sequence agreed with the annotation in the DataBase FGDB (http://mips.helmholtz-muenchen.de/genre/proj/FGDB/). The 1492-bp gene contains three introns and encodes a 438 amino acid protein with a molecular weight of 47.4 kDa. It has no predicted signal peptide. Although expression of the protein was present in the both the soluble and non-soluble fractions after 2 hr

induction, a sufficient amount was purified from the soluble fraction using metal chelate chromatography. After the wash step, a clear reduction of background proteins was observed and a band just under 50 kD was visible in elutions 1 and 2 corresponding to the expected size of 47.4 kD (Fig. 5.1). Although there were other bands present in the elution steps, Western analysis with an anti-His tag antibody confirmed the presence of the tagged protein in elutions 1 – 3. Western analysis also showed presence of tagged protein in the insoluble fraction (Fig. 5.1).

Assay of enolase on Arabidopsis seedlings

Enolase was applied in volumes of 20 µL and 40 µL to four wells containing *Arabidopsis* seedlings. No response was observed for either volume (Fig. 5.2). Seedlings were also treated with 1 µM flg22, which is the 22 amino acid peptide of flagellin known to trigger PTI and cause seedling stunting (Felix et al., 1999). Seedlings treated with flg22 showed stunted growth and some chlorosis, as expected (Fig. 5.2).

ROS activity was also measured in Arabidopsis leaves exposed to *F. graminearum* culture filtrates (Fig. 5.6). An increase in ROS was observed in flg22-treated samples but not in the *F. graminearum* culture filtrate-treated samples.

Arabidopsis seedling assays using culture filtrates from F. graminearum

F. graminearum culture filtrates were tested for an ability to cause stunting of Arabidopsis seedlings. After 10 -14 d, 10 µL of culture filtrate from inoculated medium caused stunting of *Arabidopsis* seedlings, whereas the culture filtrate from the

uninoculated medium did not (Fig. 5.3). If the culture filtrates were boiled prior to seedling application, stunting was no longer observed (Fig. 5.3). Therefore, the factor is heat-sensitive, perhaps a protein. When the culture filtrate was fractionated by cation exchange chromatography, the factor responsible for the stunting was present in the void volume as indicated (Figs. 5.4 and 5.5). Anion exchange chromatography was performed on the same culture filtrate. Although there was apparent separation of proteins, seedling assay results were inconsistent and often the stunting factor was lost, or all fractions appeared to have some activity.

Discussion

Enolase as a possible MAMP

One of the characteristic responses of PTI is stunted seedling growth. In this study, no stunted seedling response was seen in *Arabidopsis* ecotype Columbia in response to *F.graminearum* enolase FGSG_01346. While seedling stunting has been frequently used to investigate MAMPs, other assays such as alkalinization and ROS may also be useful for investigating PTI (Gomez-Gomez et al., 1999). Activity of the probable enolase used in this study has not been confirmed, nor has the activity of the tagged version made in this study. However, for other PAMPs, enzyme activity is not required, a small portion of the protein alone being sufficient to trigger PTI. For example, the 22-amino acid peptide from flg22 and the 18-amino acid peptide from elf18 are sufficient to trigger PTI. The concentration of the expressed enolase was not

measured so it is not known how much protein was applied to the seedlings. However, molecules that elicit PTI are usually active at nanomolar concentrations (Felix et al., 1999). Since a band is clearly visible on the gel (stained with a colloidal Coomasie solution with a detection limit of ~1 µg) when 10 µL of the elution was loaded, there should have been ample protein to elicit a response.

To initiate PTI, the plant cell must have a receptor specific to the MAMP (Bauer et al., 2001). These receptors tend to be highly conserved within plant families, although the Arabidopsis ecotype Ws-0 does not respond to flg22 because it has a mutated receptor gene (Gomez-Gomez et al., 1999). In this study, only one ecotype (Columbia) was tested. It may be useful to look at different ecotypes, although maybe more importantly, other plants. Different plant families perceive and respond to different MAMPs (Boller and Felix, 2009). *F. graminearum* has been reported to infect *Arabidopsis* floral tissue in some circumstances, but it is more commonly pathogenic on cereal crops (Trail, 2009; Urban et al., 2002). If enolase is indeed a MAMP, a specific receptor may only be found in cereal crop plants. This is the case with many characterized MAMPs including bacterial EF-Tu, which is only detected by members of the *Brassicaceae* family (Bittel and Robatzek, 2007; Kunze et al., 2004).

F. graminearum culture filtrates induce Arabidopsis seedling stunting

While *F. graminearum* enolase did not cause stunting in seedlings, the sterilized F.g. inoculated culture filtrate did show consistent stunting. Because the molecule(s) are larger than 6000 Da and were sensitive to boiling, the stunting factor is probably a protein. In order to investigate the response further, ROS activity was tested using a luminescence assay. Typically, in response to a MAMP such as Flg22 being added to

tomato or *Arabidopsis* cultures or leaf tissue, ROS is detected within 4 min of application, rises to a peak at approximately 15 to 20 min, and then gradually subsides (Felix et al., 1999). However, *F.graminearum* culture filtrates did not induce any luminescence in Arabidopsis leaves, and therefore were not acting like a classic MAMP. This conclusion is consistent with other reported results. Boiling flagellin before application to cell cultures actually increases the response (Gomez-Gomez et al., 1999).

Although *F.graminearum* culture filtrates may not contain a typical MAMP-like molecule, the stunting response caused by the filtrate may represent an important molecule in pathogenesis. We attempted to separate the active component/s from the culture filtrate by cation and anion exchange chromatography. The active component did not bind to the cation exchange column (Fig.5.5). Anion exchange chromatography did not reliably fractionate the active component(s) triggering stunting. This could be due to several reasons. First, enzyme activity may be required for the response. If the enzyme is denatured by the chromatography and desalting steps, the response would not be consistently present. Second, there may be more than one component needed to cause the response. If these components are separated and applied to the plants separately, a response might not be seen. Further analysis should employ other types of separation such as hydrophobic interaction, size exclusion, or reverse phase. Third, there may be a problem with the seedling assay. Although controls were always included in each assay, the assay is inherently difficult to repeat. In order to facilitate distribution of the culture filtrate, liquid media was used to grow the *Arabidopsis* seedlings. Because plants generally do not grow well in liquid, the seeds were germinated on agar plates and then transferred. Damage to the seedlings can occur

during this process and once in the liquid medium plants tend to be stressed. Agar growth medium was tried for the assay (just placing the culture filtrate on the medium by the plant), but repeatability was not improved.

In conclusion, *F. graminearum* secretes a molecule, likely a protein that causes seedling stunting of Arabidosis. It does not appear to act as a classic MAMP, as it does not induce the accumulation of ROS. Likewise, *F. graminearum* enolase does not appear to induce PTI in *Arabidopsis*, though it was not tested on other plants. Further experimentation will be required to separate the component(s) responsible for the seedling response, possibly including a better way to assay for the response. Although *Arabidopsis* was chosen partly for its genomic resources, the culture filtrate, the expressed enolase, and other MAMP candidates should be assayed on cereal crops, as they are more commonly infected by *F. graminearum*.

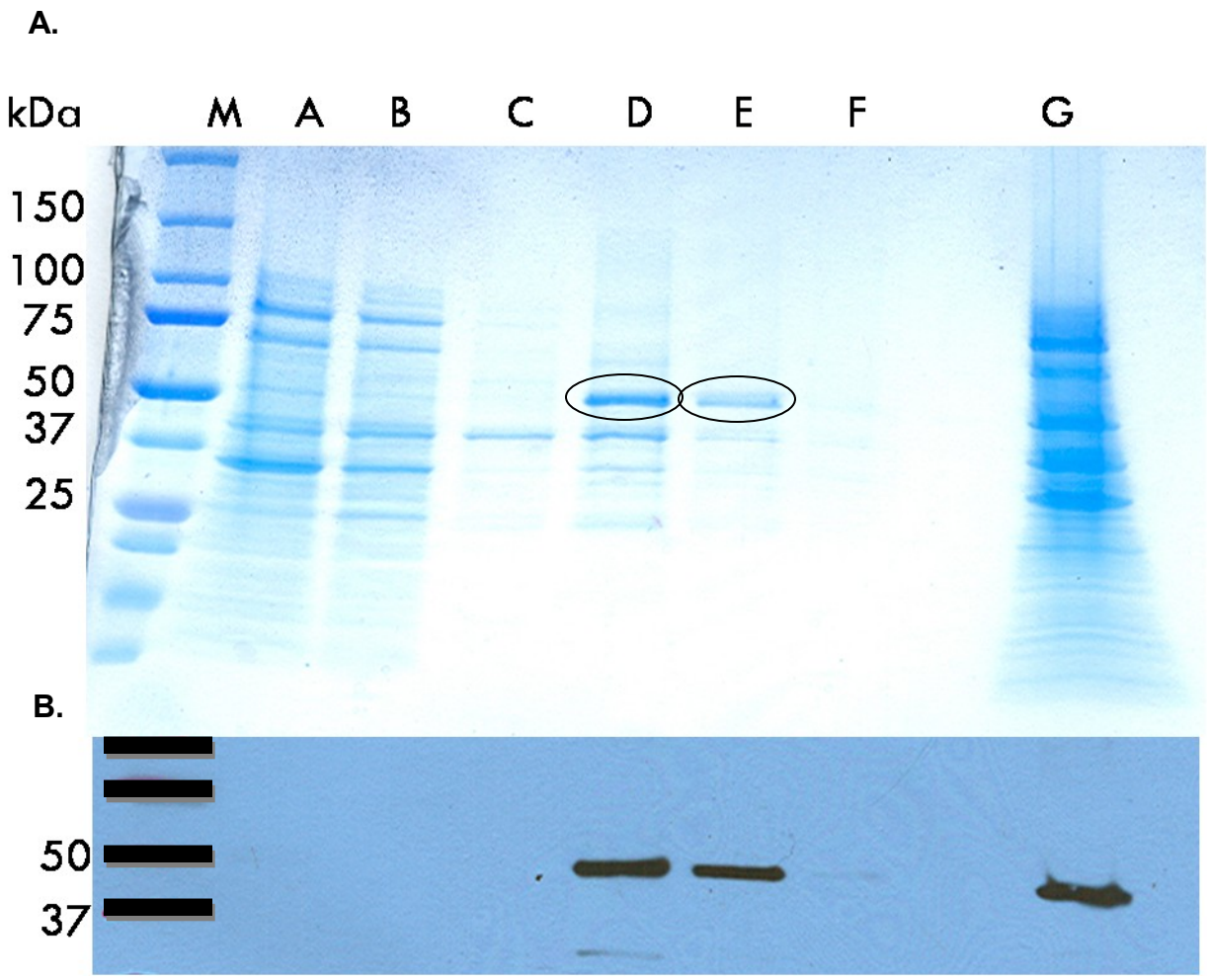

Figure 5.1

A. expression of *F. graminearum* enolase in *E. coli*. Soluble portion of the cell lysate 2 hr after induction with IPTG was purified on a metal affinity column. M – Markers, A – column flow through, B – First Wash (20 mM imidazole), C – Second Wash (20 mM imidazole) D – First elution (250 mM imidazole), E – Second elution (250 mM imidazole,) F – Third elution (250 mM imidazole), G – Insoluble fraction. **B.** Western analysis of same gel indicating the tagged enolase protein (50 kDa) in the elution steps and in the insoluble fraction. Circles indicate the eluted tagged enolase protein.

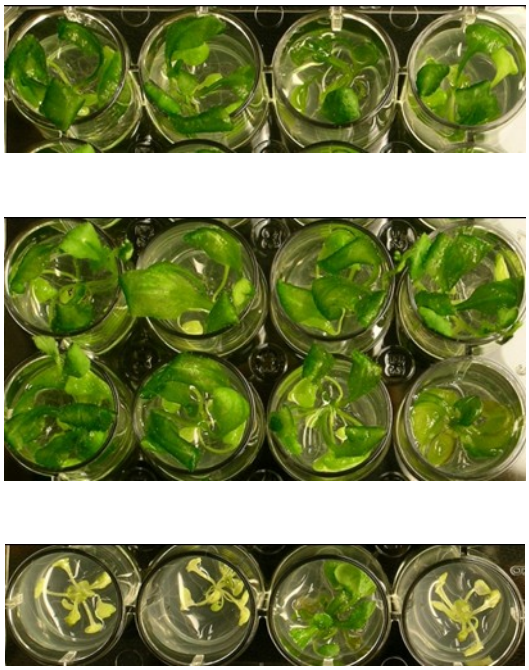

Figure 5.2

Arabidopsis seedling stunting caused by enolase and flg22 . **A.** No treatment **B.** 20 µL purified enolase from the preparation shown in lane C of Fig. 5.1. **C.** 40 µL of purified enolase. **D.** 10 µL of 10µM flg 22.

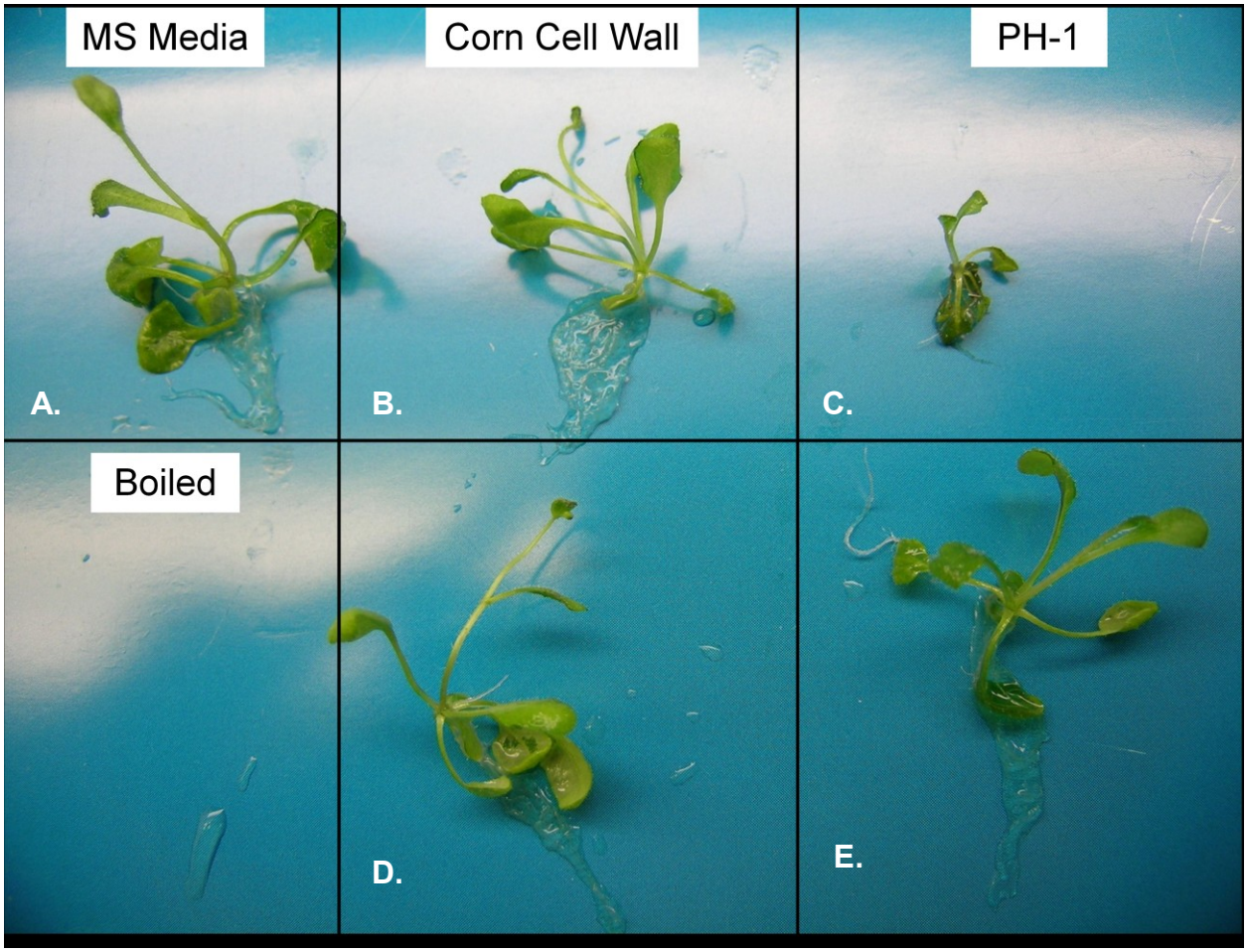

Figure 5.3

Stunting of Arabidopsis seedlings with culture filtrates from *F. graminearum* strain PH-1 A. No treatment B. Treated with 10 µL uninoculated corn cell wall culture filtrate. C. Treated with 10 µL inoculated corn cell wall culture filtrate. D. and E. Treated with 10 µL boiled culture filtrates.

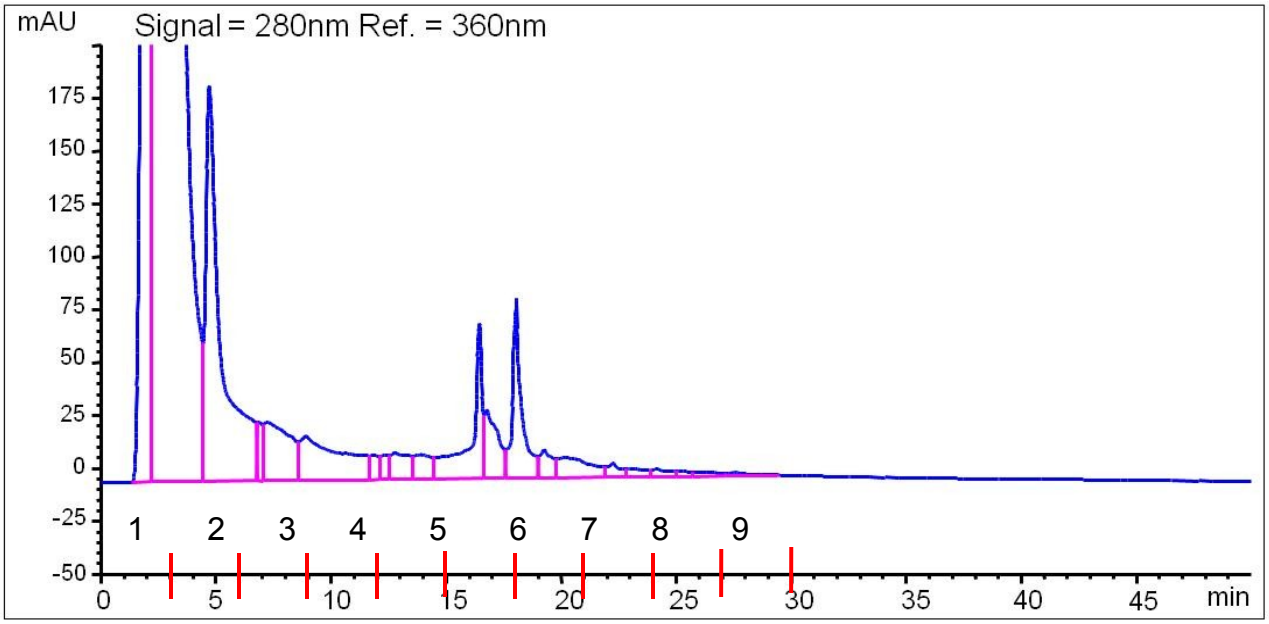

Figure 5.4

Representative cation exchange chromatogram of culture filtrate of *F. graminearum* . Buffer A = 25 mM sodium acetate, pH 4.5. Buffer B = Buffer A + 0.6 M NaCl. Gradient = 0 – 5 min 0% B, 5 – 35 min to 50% B, 35 – 45 min to 100% B with a flow rate of 1 mL/min. One mL desalted culture filtrate was added to 1 mL 2x Buffer A and then injected into a 5 mL for column loading. Red hash marks above the corresponding number indicate the fractions collected during the run.

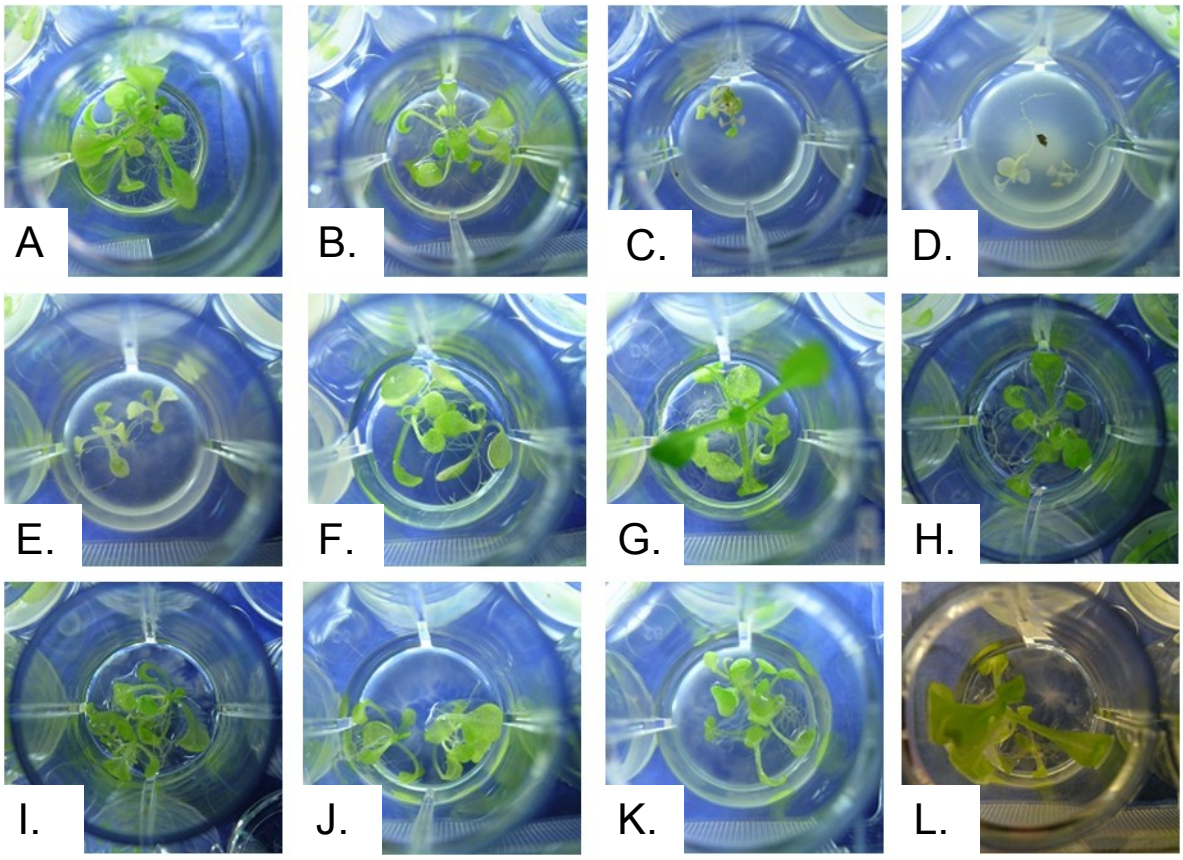

Figure 5.5

Arabidopsis seedling assays of fractions 1 – 9 from the cation exchange run shown in Fig. 4.4). Pictures are representative of multiple wells. Protein loading was equalized to 10 µL whole protein for each sample. Fraction numbers correspond to fractions indicated in Fig. 4.4. A. No treatment. B. uninoculated culture filtrate. C. *F. graminearum* culture filtrate. Lanes D through L, fractions 1-9.

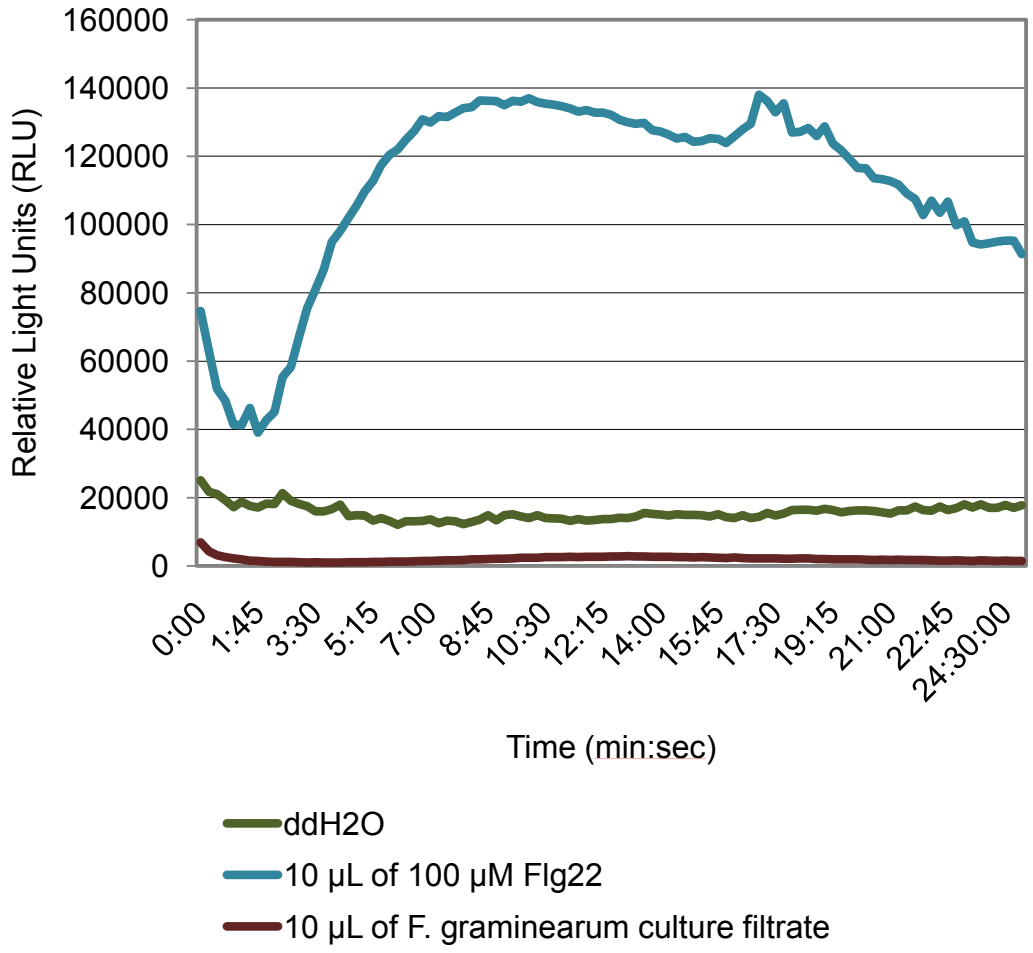

Figure 5.6

ROS response of *Arabidopsis* leaves to *F. graminearum* culture filtrates. H_2O and 100 µM Flg22 were used as negative and positive controls, respectively.

Chapter 6: Future Directions

F. graminearum pathogenesis

Research on *F. graminearum* has exploded since the genome sequence was completed (Cuomo et al., 2007). With more genomic sequences from other *Fusarium* species and other necrotrophic pathogens, this trend will continue. Genome comparison studies have already resulted in the discovery of hypervariable regions within chromosome and supernumery chromosomes that contain an overrepresentation of pathogenecity related genes (Ma et al., 2010). The future is sure to bring a deeper understanding of the evolution and virulence mechanisms of this important genus of plant pathogens.

The sequences of several hosts (maize, wheat, and barley) are also in process or available, making study of the plant/pathogen interaction even more feasible. For example, transcription and proteomic studies on both the host and the pathogen during the infection process have recently been done (Ding et al., 2011). Proteomic studies at the interface of the host/pathogen reaction (i.e the apoplast) have determined what proteins are present from the fungus. It is now possible to mine the same information from the host. In the course of our proteomics studies (Chapter 2), we collected extensive data on the host proteins as well as the fungal proteins. This data could now be re-searched using the predicted proteome of wheat to identify host proteins that are up regulated during infection. The *F. graminearum* sequence annotation has also been improved since our initial searches were done. Re-searching this data with the updated database may result in identification of new proteins, or allow us to identify some of the proteins that originally could not be matched with high confidence.

Although none of our gene deletion mutants showed significant virulence reductions, these mutants represent a valuable resource for future studies in several ways. Since different cultivars of wheat contain ranges of susceptibility, these mutants may have a phenotype on another cultivar. Deletion mutants have been used in metabolomic profiling experiments and could be tested for phenotypes under other environmental conditions (Lowe et al., 2010). A considerable number of mutants like these exist in the labs of other researchers and the *F. graminearum* community plans to maintain a master list of deletion mutants for future work (disscused at F. graminearum Workshop, 2001).

Experiments in this dissertation identified a seedling response in *Arabidops*is to culture filtrates from *F. graminearum*. Although the factor, possibly a protein, could not be isolated and identified, further work on this factor or factors should be pursued. The development of better seedling assays should result in more consistent results. Also, different methods of chromatography such as hydrophobic interaction should be tried.

Fucosidase characterization

There are several important experiments that will be completed on the two FCO1 genes and their expressed proteins in the near future. Specific activity and kinetic studies must be done in order to fully characterize both proteins. Although the FoFCO1 expressed in Pichia does not show activity on pea xyloglucan, activity of the native protein purified from culture filtrates should be examined to make sure the expression process did not alter activity. The native substrate of FoFCO1 is still not known. Other

fucosylated substrates, such as glycoproteins, arabinogalactan proteins, and rhamnogalacturonan II should also be tried. FgFCO1 and FgFCO1 will be tested to see if fucosidase can improve degradation of xyloglucan for enhanced production of biofuels.

Further analysis on the two GH29 fucosidase subfamilies identified by sequence analysis would also be interesting. *F. verticillioides* and *F. oxysporum* each contain at least one gene representing each family. Confirming the activity of at least one from each group in the same organism would provide more insight in determining whether these two groups represent different substrate specificities, as suggested by our results with FgFCO1 and FoFCO1.

Our demonstration that FoFCO1 has activity on a model pNP substrate but not a natural substrates indicates that model substrates, although convenient, do not always reflect real enzyme activity. Clearly, the activity of hydrolytic enzymes selected for possible commercial use should be confirmed on natural substrates. The lesson from our studies on fucosidases should always be considered when doing enzyme bioprospecting.

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Appendix

Table 7.1 Primers used for creating gene deletion mutants described in chapter 4.

Gene	Dir.	Upstream Megaprimers (5' → 3')	Downstream Megaprimers (5' → 3')
FGSG_0028	Fwd	GCATCGGTAGTTAGAAAT	tcctgtgtgaaattgttatccgct TTCTGCGAGCAACCTAAT
	Rev	gtcgtgactgggaaaaccctggcg GAGTTGATGAAGAGATGG	CCCTGGGGATCACTAACT
FGSG_0060-0062	Fwd	AACGGTAGCATTCTGACG	acttattcaggcgtagcaaccaggcgt ggccgtttgtccccatta
	Rev	tcagcatcttttactttcaccagcgttgat ggcgactgttactgc	CAGCTCTGATACCTACGA
FGSG_0346	Fwd	CATGTTGGCGAGTGAGAC	tcctgtgtgaaattgttatccgct CGCCGAGTTGACGATGAC
	Rev	gtcgtgactgggaaaaccctggcg GAGGGGCAGCAATAATAC	GCGGCAATGGTGGTAAGC
FGSG_0571	Fwd	CGCGAGTACAGCCTTTTC	tcctgtgtgaaattgttatccgct TTGCCCTTCTTTTGTATC
	Rev	gtcgtgactgggaaaaccctggcg AACCGGTAGATAGATGAG	TTTCCACGCTCATTTAGT
FGSG_2560	Fwd	TTCCCCAACGGTAAAGAC	tcctgtgtgaaattgttatccgct GAGCCCGAAAATGGAGAT
	Rev	gtcgtgactgggaaaaccctggcg AGCGTTGAGACTGTGGAG	TACCGGTCGATGAACTGG
FGSG_2897	Fwd	TCCTAATTACCTGACTAC	tcctgtgtgaaattgttatccgct TCGAAGCGCCTGTGTCTC
	Rev	gtcgtgactgggaaaaccctggcg TTCGACTGCGTTTCTTTG	AGGGGGTTGATGCTCGTG
FGSG_3003	Fwd	GCTGGCCTGTTGCTTACT	tcctgtgtgaaattgttatccgct GTGGCCTCGGGACTGTAA
	Rev	gtcgtgactgggaaaaccctggcg ATCGCGAGGTGGAGAATA	TCGCGTTTGGTGATTCTC
FGSG_3003	Fwd	GCTGGCCTGTTGCTTACT	tcctgtgtgaaattgttatccgct GTGGCCTCGGGACTGTAA
	Rev	gtcgtgactgggaaaaccctggcg ATCGCGAGGTGGAGAATA	TCGCGTTTGGTGATTCTC
FGSG_3124	Fwd	aacccgccccacgaataa	
	Rev		gttagggtagacaggcaa
FGSG_3211	Fwd	CAAAAGGTGATACGAGAT	tcctgtgtgaaattgttatccgct CCATTTTACTTCGTCTTC
	Rev	gtcgtgactgggaaaaccctggcg GGACAATGTTAGAAGTGG	TGGTTTTCTTGTGTTTCT

Table 7.1 cont'd

Gene	Dir.	Upstream Megaprimers (5' → 3')	Downstream Megaprimers (5' → 3')
FGSG_3483	Fwd	TAGGCGATCGCAAAATGA	TCAGCATCTTTTACTTTCA CCAGCGTTAGTGCCGGTA AGGTTCTA
	Rev	acttattcaggcgtagcaaccaggcgt caaaagccacggagagga	AAGTTGGCCGTTGCTGAT
FGSG_3526	Fwd	AACCCTGCCAAAAGACAA	NA
	Rev	NA	TCCCAAGGTAGCCAGTTT
FGSG_3628	Fwd	GAGCCGAAGCATAAGAAC	tcctgtgtgaaattgttatccgct TCATTTCTGGACCTTCAA
	Rev	gtcgtgactgggaaaaccctggcg TGAACCCTACAGCAAAGT	TGGCCCAGCGACTTTCTT
FGSG_3632	Fwd	GCCATGCCCAACCTTAGT	tcctgtgtgaaattgttatccgct ACGGAGGACCAAGAAGTG
	Rev	gtcgtgactgggaaaaccctggcg GGACACGGCAAACGAGAT	TCCAATCCGGGTTAGTGT
FGSG_3842	Fwd	CTTAGATTAGCGGTTCAG	tcctgtgtgaaattgttatccgct GGATACGGTCTTTGGTGA
	Rev	gtcgtgactgggaaaaccctggcg TTGCGGGGTCATACTCAT	CTCTGCGCATGTCTTGTT
FGSG_4213	Fwd	CCGCGTCCTCCACTGTAA	tcctgtgtgaaattgttatccgct CCTGCGGTGACTGGTGAC
	Rev	gtcgtgactgggaaaaccctggcg TAACTCGAGGGCGGACTA	ATGCCGAGGTATGTGAAA
FGSG_4074	Fwd	TAAGCCAGGACTATTCTA	tcctgtgtgaaattgttatccgct CTTCTACCTACCTTCTTG
	Rev	gtcgtgactgggaaaaccctggcg TCTTTCTGCCATCGTCAT	TTTTGCCCATCTTGTAAC
FGSG_4583	Fwd	GTGAATTGCGGGGTGTCC	acttattcaggcgtagcaaccaggcgt tgatcggccgaggttttg
	Rev	tcagcatcttttactttcaccagcgttag cggcgatgaaggtgaa	TATCGGCAGAAGAAGTGG
FGSG_4848	Fwd	GAGGGCATGATTTTGAGA	tcctgtgtgaaattgttatccgct CTACGCGGCAATCATAAA
	Rev	gtcgtgactgggaaaaccctggcg TGTTTGGGGGCTTTAGTA	CACTGGCACCAAGAAGAG
FGSG_5236	Fwd	ATGGGCGGGTTAGGTTAT	tcctgtgtgaaattgttatccgct TGATGACTGCGGATTATT
	Rev	gtcgtgactgggaaaaccctggcg TTAGCCGATTGGTTTGAA	TTGGCAGTGGTTTGATTC
FGSG_5906	Fwd	CGAACCCCTACAAACTCA	tcctgtgtgaaattgttatccgct CGCCATGCAATAGGTAAG

Table 7.1 cont'd

Gene	Dir.	Upstream Megaprimers (5' → 3')	Downstream Megaprimers (5' → 3')
	Rev	gtcgtgactgggaaaaccctggcg GACGGGATCCAAGGGTAT	AAGTGCCGACAGGTGGAC
FGSG_6469	Fwd	GATCAAAAGCGGCAGAAC	tcctgtgtgaaattgttatccgct ATACCGCGCATTTCCACA
	Rev	gtcgtgactgggaaaaccctggcg CGACGGTGACGGCATTTT	TTCGGGTTACTTTATTCC
FGSG_7439	Fwd	AGCATTCGGTGGTGATTG	tcctgtgtgaaattgttatccgct TGTCCTCTTTGTTGGTCT
	Rev	gtcgtgactgggaaaaccctggcg TGCCCTGCTCTTTGTTGA	ATCATGTCAGGGTTCAGC
FGSG_7558	Fwd	TTTGTGGCTGGCTGAGAC	tcctgtgtgaaattgttatccgct CAATATGGGATGGAGAGG
	Rev	gtcgtgactgggaaaaccctggcg CCAATATCGCACGGACAT	TTCGCAGTTGGGTATGAG
FGSG_8037	Fwd	TTTGTCCCACGAGGTATT	tcctgtgtgaaattgttatccgct AGCGAGTTTTTGGACAGG
	Rev	gtcgtgactgggaaaaccctggcg TCCGGCGTCTATCAACTT	CGGAAGACGAGGTAGCAC
FGSG_9366	Fwd	AGCGAATACCACAAAGTT	tcctgtgtgaaattgttatccgct GCCCATTAGCGTGTCTTT
	Rev	gtcgtgactgggaaaaccctggcg TGCCGGGTTCATCAGGTA	CGCCACGATTAGAGAAGT
FGSG_10675	Fwd	TTGCTGTTGATAGGATTT	tcctgtgtgaaattgttatccgct ATCAGCCACAAGTTCAGT
	Rev	gtcgtgactgggaaaaccctggcg CTTGTGCTGTCTTCGTAG	CACGCCATTCCGAGTTTT
FGSG_10676	Fwd	ATGCCTACAATGCCTATC	tcctgtgtgaaattgttatccgct TATGGCGCTAAGACTGGA
	Rev	gtcgtgactgggaaaaccctggcg GAGAGTCTTGCGGGTTTA	CCCCGAAAGCTCACATCA
FGSG_10782	Fwd	TCGCACGTCCCCATAAAA	tcctgtgtgaaattgttatccgct CGCCAAGTAAGAAATGAA
	Rev	Gtcgtgactgggaaaaccctggcg TCCCCGTCTCCACTCTCC	TACCCGACCCTTACTTGT
FGSG_11036	Fwd	GATCGACGCTTCTCTTTT	tcctgtgtgaaattgttatccgct TGCATTGCGTGTTCTTGA
	Rev	gtcgtgactgggaaaaccctggcg ATCCGTCATATTCAGGTG	CCTCCTCCACGAACTACT
FGSG_11037	Fwd	CCGTTATACCGTCATTGG	tcctgtgtgaaattgttatccgct ACCAACTTCAACGCTCAC
	Rev	gtcgtgactgggaaaaccctggcg TCGGTCCGTTTACATAGG	TCCCCGAGGTTATGCTAT

Table 7.1 cont'd

Gene	Dir.	Upstream Megaprimers (5' → 3')	Downstream Megaprimers (5' → 3')
FGSG_11176	Fwd	TCAATGGCAAGGTAGTGT	tcctgtgtgaaattgttatccgct GGAGCTGAAAAGAATGAC
	Rev	gtcgtgactgggaaaaccctggcg TTCTCCGTTCAGGTTTAT	CCGATACCTCCTCCAACT
FGSG_13094	Fwd	GGATCCCAATGAGAGTTT	tcctgtgtgaaattgttatccgct GTCCGTCGCGTCACTCTG
	Rev	gtcgtgactgggaaaaccctggcg ATTTGAGGCCGCTTAGAC	AACGCGAAATCCGAAATA
FGSG_13095	Fwd	TTGCTTACGTGGGGACTA	tcctgtgtgaaattgttatccgct GCGGCTGGGGTAGTATTG
	Rev	gtcgtgactgggaaaaccctggcg ACACGGGACACAGGACAC	GCAGTGCCTCGTGAAGAC

Table 7.2 Primers used for confirmation of gene deletions described in chapter 4.

Gene	Orientation	Confirmation Primer (5' → 3')	Hygro micin primer (5' → 3')	Missing piece primer (5' → 3')
FGSG_0028	Upstream	ATCGCGATATGTAGTGTCA	3' out #2	GCACTGAAGCTCTCGGTTACTGTT
	Downstream	GAAGTGGCGGGTATAATG	5' out #1	CATGCGGGTCTGCTGTCC
FGSG_0060-0062	Upstream	AAGAGCCAATCATACACCAACACT	3' out #1	TTGATCGGCAGTGTGGTGGGTAAG
	Downstream	ACCACGTCGGAGATAGATTGTTC	5' out #1	CATAGTGGCGGTCTCGTGGTTGAA
FGSG_0114	Upstream	GTCCTCCACCTTTTCTCGCATCAT	3' out #1	GACTCCCACGTTCCACTCAA
	Downstream	AATCGTCTTTCTCCTCTTACTTCC	5' out #1	TCATCACCCTTTCCACCTTATTCT
FGSG_0346	Upstream	ACCGCAAGGCTTTCAGTATC	3' out #1	CCGTCATCAAGGCCGCTATCA
	Downstream	GTCATCCTGTCCACAAGCCCATT	5' out #1	AGGGGGTTGTTGGAGTCTATG
FGSG_0571	Upstream	GAAGGTCTCCGCAGTAGG	3' out #1	TGTGATGCCCAGTGCCCTCGTGA
	Downstream	TAATAAAGTGGCAGAATC	5' out #1	TTGCTGCCCTTGTGGAACTGA
FGSG_2560	Upstream	CGTTGATCGCCTCGTAT	3' out #1	NA
	Downstream	AAGAAGATACAGACCGTCACAAAA	5' out #1	NA
FGSG_2897	Upstream	GGGGGCCATGTCCTAAT	3' out #1	AATTCTTCCTATCTCGGTCACAGC
	Downstream	TGAAAGGGAATATGAGCC	5' out #2	CGCGTTCCCCATCTTCGTTA
FGSG_3003	Upstream	TACGATAACGCTGGAACT	3' out #2	GACAAAGGGCGAGAAACC
	Downstream	CTCCAGTCTCAATACCGC	5' out #1	TGTCCTGTGCCCAAGATT
FGSG_3124	Upstream	CCTGTCACCAAAGTACCCATCACG	3' out #1	NA
	Downstream	TAATACAGCATCAATAACAGACCT	5' out #1	NA
FGSG_3188	Upstream	ATCGCGATGCAAGAACAA	3' out #2	CAAGACGGCGAGGAGACG
	Downstream	GAGTGATGCCAGATGTTC	5' out #1	AACCAGCGGCAGAAGATG
FGSG_3211	Upstream	GCCAAGACGCTCATAACC	3' out #1	CAGCGACTTCAGCCCAACCAACA
	Downstream	TTTTCTTGTGTTTCTGTATCTTGT	5' out #2	CACAAAACCCAGCCGAAATCAACA

Table 7.2 cont'd

Gene	Orientation	Confirmation Primer (5' → 3')	Hygromicin primer (5' → 3')	Missing piece primer (5' → 3')
FGSG_3483	Upstream	TCTGCCGATCTAAACCAACCT	3' out #1	CGCCGCCGGTGTGACTGGTA
	Downstream	TGGGCGAGAGCAACTTCACA	5' out #1	TGGATGACATGAAGGCGAATAAGG
FGSG_3526	Upstream	CGTACCGGAAGGATCGTCAAAT	3' out #1	CGTACCGGAAGGATCGTCAAAT
	Downstream	GGGTGACCAAGTTAGATGAAGC	5' out #1	GGGCCTTGACATAGATACC
FGSG_3628	Upstream	TACCGTCTTGGCACAGCATTAG	3' out #1	CTGGTGGCAACTACGCTGGTCA
	Downstream	CAATCACCCCTACAGAAT	5' out #1	GCTTGCCGGCATCCTTGTAGAG
FGSG_3632	Upstream	TTAACGTTTTGAATAGAGAAGTAACGATT	3' out #1	GCCGGTAGCGATGTCAAGTC
	Downstream	GTTGTCTCACTGTGAATCAAAACTT	5' out #1	AGTGGAAGCGGGGGCATCGTT
FGSG_3842	Upstream	GCAGATGTCATGGCAATAG	3' out #2	CCGCAAAACAGGGCATCCATCAT
	Downstream	ATTGATATGGGCATTGCTTGACTC	5' out #2	TTGCCACCGCTATCACTATCACTG
FGSG_4074	Upstream	NA	NA	NA
	Downstream	NA	NA	NA
FGSG_4213	Upstream	TCACTAAAAAGAAGATCAGACTCA	3' out #1	CAAGGCCCCAGCACCAACAT
	Downstream	CGATCCATAGCTGAGTAATAGACA	5' out #2	AAGGCTGCCGCAAAAGAAAAGA
FGSG_4583	Upstream	AGGTCTGGATCTGGGGAGTT	3' out #1	NA
	Downstream	CTTGGATGTGGACTTGGATAGACC	5' out #1	NA
FGSG_4848	Upstream	GCGTGGAGAAATAAATGG	3' out #2	GATGGCTGGGGAAACTAC
	Downstream	ATGTCGGCAGTAATTGTC	5' out #1	GGTCGCGAGGATACAAAG
FGSG_5236	Upstream	TGTGGCCAGAACCCTACT	3' out #1	ACTCGAGATTCAGGGCCAGACCAA
	Downstream	GGATGATGCCACTTTCTAATAATT	5' out #1	GGCCGCGTTCCTCGTAATCCTTC
FGSG_5906	Upstream	CACAGTGACACCAAAGTAGC	3' out #2	CACGGCGCCGCAGCATACT
	Downstream	TTTTGAGTGAGGAGAAGTGGCATGA	5' out #2	ACCTACGGAGACGACCTTGAACGAA
FGSG_6469	Upstream	AGTCGCGTGCTTTTGATA	3' out #2	TCAGCCGCGAGACGACAAGA
	Downstream	TATGCTATGTCAAATTCG	5' out #1	CCACCACCGGAAAGGCTATCAT

Table 7.2 cont'd

Gene	Orientation	Confirmation Primer (5' → 3')	Hygromicin primer (5' → 3')	Missing piece primer (5' → 3')
FGSG_7439	Upstream	TCCTTGTTCGCTGACCA	3' out #1	GACCGCTTCAAGGACGAGA
	Downstream	TAAAGATTCAACGCCAAACTCCG	5' out #1	CGGCAGCGAATTCAGATGTG
FGSG_7558	Upstream	GGGAAAATCGTGCGAACAATGA	3' out #2	CGCAAACGGTAGCCAGTG
	Downstream	TGAGTGCTGGCGTACATGAAATA	5' out #1	TCCCTCCTTAACCTTGTA
FGSG_8037	Upstream	TCTCGCCGAATCTATCAC	3' out #1	CAAGGCTCGAGGTGTGAC
	Downstream	ATTTACCCTGGCCAACAA	5' out #1	AGTGCGGCCGGTGTAGTG
FGSG_9366	Upstream	ACTGATTGGCGTGTTTTG	3' out #2	GAGAACGGCAAGGGCAATAGCATC
	Downstream	TGGAGTGAAACATACAAGCTT	5' out #2	GTGGCAGAGGCAGTGGCAGTGAC
FGSG_10675	Upstream	GAGAATTCCAGAAACCAAAGA	3' out #2	NA
	Downstream	ACCATCGGGCATTCTTATAT	5' out #2	NA
FGSG_10676	Upstream	ATCTTAAACGTAGGTCTT	3' out #1	AGCCCTGGAACGGTGATT
	Downstream	CACAAGGGACAAGACAGA	5' out #1	TATATGGCTGCGGTCTGA
FGSG_10782	Upstream	GAGATGCGTGGGCGTTGAGT	3' out #2	AGGCCGGCGTCCACAAGAT
	Downstream	CGTGGCAGCGACAGTGG	5' out #2	GCGATGCTGCCACACTCCTG
FGSG_11036	Upstream	ATCACCATCCCCTCTCC	3' out #1	GCCCCCAACGGTCTCAAC
	Downstream	CCTGTCAAGTTCCCTCTGCT	5' out #1	CGGCTGCAGGTGTAGGTA
FGSG_11037	Upstream	GCGGCATTTGATCATTAGACT	3' out #1	CTCTGGCTCTGGTTCTCA
	Downstream	TTATCAACGAGGGTCTGC	5' out #1	GGTGTTGGCAAGGTAGTT
FGSG_11176	Upstream	TTATGTCGCTGTCCTATT	3' out #1	NA
	Downstream	ATGCCGATACCTCCTCCAACT	5' out #2	NA
FGSG_13094	Upstream	CGGAATCGCGGAAATGG	3' out #2	ACCGTGGTGTAGCCCCTGATGC
	Downstream	CCATTTCCGCGATTCCG	5' out #1	GTCGCGAGATTGCCGTTGCTATT
FGSG_13095	Upstream	TTGCTCAAAATGGTATGTGG	5' out #2	CAGCTTCTTTCGCATCACTTCTAT
	Downstream	TTAGAAGCAAACCATGACAGAAGT	3' out #2	GACCATCACGGACATTACCAG