Identification of candidate effector proteins from *Fusarium
graminearum* during infection of *Arabidopsis thaliana*
using proximity-dependant biotin identification (BioID)

by

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Abstract

Fusarium graminearum is a fungal pathogen that causes Fusarium head blight (FHB) in cereal crops. Identifying proteins that are secreted from pathogens to overcome plant defenses and cause disease, collectively known as effectors, can reveal new targets for fungicides or other control measures.

Proximity-dependent biotin identification (BioID) was used to identify potential effector proteins secreted *in planta* by *F. graminearum* during the infection of *Arabidopsis* seedlings. BioID analysis revealed over 300 proteins from *F. graminearum*, of which 99 were considered to be candidate effector proteins (CEPs).

A subset of CEPs was functionally characterized in wheat. Assays examining the ability of a CEP to induce cell death or affect the growth of a bacterial pathogen were performed to determine their role in plant defenses. The expression of four CEPs in wheat were found to alter bacterial growth, supporting their putative role as effector proteins promoting infection by *F. graminearum*.

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List of Abbreviations

Avr - avirulence

BioID – proximity-dependant biotin identification

CEP – candidate effector protein

CFEM – common in fungal extracellular membrane

CFU – colony forming units

CM – complete medium

CMC – carboxymethylcellulose

CRN – crinkler

CWDE – cell-wall degrading enzyme

DEX – dexamethasone

DCM – double complete medium

DON – deoxynivalenol

Dpi – days post infection

ECV – extracellular vesicles

ETI – effector-triggered immunity

ETS – effector-triggered susceptibility

EV – empty vector

FDR – false discovery rate

FHB – Fusarium head blight

FTIR – Fourier Transform Infrared

FunCat – functional catalogue

FuNTAP – Fusarium Network of Trichothecene Associated Proteins

GFP – green fluorescent protein

GO – gene ontology

HR – hypersensitive response

HRC – histidine-rich calcium-binding protein

HRP – horseradish peroxidase

Hpi – hours post-infection

HSP – heat shock protein

KEGG – Kyoto Encyclopedia of Genes and Genomes

LC-MS/MS – liquid chromatography-tandem mass spectrometry
LFQ – label-free quantification
LysM – lysin motif
MAMP – microbe-associated molecular pattern
MAPK – mitogen-activated protein kinase
MES – 2-(N-morpholino) ethanesulfonic acid
MIPS – Munich Information Centre for Protein Sequences
NB-LRR – nucleotide-binding and leucine rich repeat
PAMP – pathogen-associated molecular pattern
PCI – phenol:chloroform:isoamyl
PDA – potato dextrose agar
PFT – pore-forming toxin-like
PRR – pattern recognition receptor
PTI – pattern-triggered immunity
QTL – quantitative trait loci
R - resistance
ROS – reactive oxygen species
S - susceptibility
SCS – sodium citrate sorbitol buffer
SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM – standard error of the mean
SNARE – soluble n-ethylmaleimide-sensitive factor attachment protein receptor
SNP – single nucleotide polymorphism
TRI - trichothecene

Chapter 1: Introduction

1.1 *Fusarium graminearum* and Fusarium Head Blight

Fusarium graminearum is a fungal pathogen that infects cereal crops. It is the primary cause of Fusarium head blight (FHB) disease that affects wheat, barley, and corn grown across Canada (Goswami & Kistler, 2004). FHB causes bleaching and premature ripening of grains, leading to a reduction in grain size (Gilbert & Tekauz, 2000; Wegulo et al., 2015). This damage results in low yields and poor grain quality. Infection by FHB can also result in the production of trichothecene (TRI) toxins, including deoxynivalenol (DON). When present at high levels in grains, DON can result in sickness in livestock and humans (Goswami & Kistler, 2004). FHB infection can downgrade the quality of grain or make it unsuitable for sale, leading to lower profits for farmers. The impacts of FHB are of great concern for Canadian farmers, especially as Canada grows 30 million tonnes of wheat (Statistics Canada, 2018) and exports over 9 million tonnes per year (Canadian Grain Commission, 2018). Economic losses due to FHB have ranged from \$1 million to \$9 million yearly in Alberta between 2004 and 2012 (Government of Alberta, 2015) and losses are likely similar across other prairie provinces where wheat is grown.

Due to the impacts of climate change, FHB outbreaks are likely to become more common and severe. *F. graminearum* thrives in warm, humid conditions, meaning that epidemics of FHB are likely to increase as seasonal temperatures and rainfall levels rise (Shah et al., 2014). There is a strong need to prevent the spread of FHB and reduce the damage it causes when infection occurs.

There are several methods used to control the spread and severity of FHB, but often these have limited success. Limiting the growth of *F. graminearum* with fungicide

application tends to be inconsistent because environmental factors such as temperature and moisture make predicting the effect of fungicide application more difficult.

Although *F. graminearum* is the primary contributor of FHB, multiple *Fusarium* species including *F. culmorum*, *F. poae*, *F. avenaceum*, and *F. sporotrichoides* can also contribute, rendering fungicide application less effective (Gilbert & Tekauz, 2000; Osborne & Stein, 2007; Pirgozliev et al., 2003). Crop rotation and tillage are another measure that can reduce the amount of *F. graminearum* overwintering on crop debris, but this generally needs to be combined with other control methods as it is not effective enough on its own (Wegulo et al., 2015). The most effective method of FHB control is through the breeding of resistance traits in wheat.

There has been significant progress in wheat breeding programs to improve resistance, particularly in the identification of quantitative trait loci (QTL) that confer resistance to FHB. QTL are regions of the genome that contribute to quantitative variation in a trait. Currently there are over 100 QTL which have been shown to play a role in resistance to *F. graminearum* (Dweba et al., 2017), meaning that many genes contribute to the resistance phenotype and variation in resistance can occur throughout a population. Breeding for *F. graminearum* resistance is focused primarily on the prevention of initial infection and spread of the fungus. However, there is still no wheat cultivar available with complete resistance to *F. graminearum* or FHB (Dweba et al., 2017). Therefore, it is imperative to understand the nature of the interactions between *F. graminearum* and wheat rather than to focus exclusively on the host side of the interaction. Research into the infection mechanisms of *F. graminearum* can help to prevent its spread and reduce damages. Gaining a better understanding of this interaction

can also lead to the development of novel methods of control that can be deployed in a much more specific manner to directly combat *F. graminearum*.

1.2 Life Cycle and Pathogenesis of *F. graminearum*

F. graminearum colonizes cereal monocots including barley (*Hordeum vulgare*), oats (*Avena sativa*), and corn (*Zea mays*), with its primary host being wheat (*Triticum aestivum*) (Goswami & Kistler, 2004). It is mainly spread through the release of sexual spores (ascospores) from sac-like structures known as perithecia, which form on crop debris in fields. During early spring, ascospores are forcibly discharged from perithecia, landing on wheat heads, where they can germinate and initiate infection during flowering (Figure 1) (L.-J. Ma et al., 2013; Trail, 2009; Trail et al., 2002). A secondary mode of spore dispersal is through asexual spores (conidia), which can be spread by wind or rain, resulting in greater potential of infection in nearby plants (Figure 1) (Goswami & Kistler, 2004). After spores have landed and germinated on wheat heads, *F. graminearum* then infiltrates the plant through natural openings such as stoma or the base of the outer hull (lemma), but can also directly penetrate cells (Trail, 2009; Walter et al., 2010).

F. graminearum is considered a hemibiotrophic pathogen. Hemibiotrophs have two growth stages: a biotrophic phase (where the pathogen co-exists with the host), and a necrotrophic phase (where infection results in the death of host cells). Hemibiotrophs depend on keeping the host plant alive during the biotrophic phase, in part to obtain nutrients from the host, but then switch to a necrotrophic phase, which results in toxin production and death of host cells (Koeck et al., 2011; L.-J. Ma et al., 2013).

The biotrophic phase of *F. graminearum* is symptomless and during this phase, the pathogen rapidly grows between cells (Walter et al., 2010). The necrotrophic phase

Figure 1: Life cycle of *F. graminearum*. The fungus overwinters on crop residue, on which perithecia develop. Perithecia then release spores which land on nearby plants. Spores germinate on wheat heads during flowering and infect grain, resulting in poor yields and grain contaminated with toxin.

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begins when *F. graminearum* penetrates the vascular structures, which blocks the transport of water and nutrients, resulting in the symptoms of FHB such as water soaking and bleaching of colonized tissue (Goswami & Kistler, 2004; Trail, 2009).

F. graminearum forms specific structures as infection progresses, including what are known as infection cushions: structures made up of grouped, branching hyphae (Boenisch & Schäfer, 2011). Necrosis is observed at this stage, particularly in host cells surrounding the infection cushions. Although trichothecene biosynthesis occurs in infection cushions, their formation is not required or affected by trichothecene production, nor does it affect cell penetration (Boenisch & Schäfer, 2011). This suggests that there may be other factors at play during these stages of early infection, such as the secretion of proteins that facilitate the infection process.

1.3 Plant-Pathogen Interactions

Plants are constantly defending themselves against attack from herbivores, insects, and fungal or bacterial pathogens. As immobile organisms, plants have developed sophisticated methods of defense against predators and pathogens. It is generally recognized that plants have two stages of defense: briefly, the first stage can be considered a form of innate immunity, known as pattern-triggered immunity (PTI), which protects the plant against a broad range of attackers. The second stage, known as effector-triggered immunity (ETI), can be considered a more directed form of pathogen-specific defense. The zig-zag model of plant defense, originally proposed by J. D. G. Jones & Dangl (2006), illustrates these two stages (Figure 2). Plants are able to recognize certain signals from bacteria or fungi that grow in proximity or in contact with

Figure 2: Zig-zag model of plant defense. PTI is first induced by pathogen PAMPs, resulting in a mild defense response. This can be overcome by pathogen effectors, which may then trigger a stronger ETI response if they are recognized by *R* genes. If an effector protein can avoid detection, ETS results allowing successful pathogen colonization. *R* proteins and effectors (*Avr* proteins) are constantly evolving in an arms race to overcome each other.

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the plant, which are known as pathogen-associated molecular patterns (PAMPs) (Bigeard et al., 2015).

However, because non-pathogens can also be recognized, these signals are also known as microbe-associated molecular patterns (MAMPs). PAMPs or MAMPs include such molecules as bacterial flagellin or chitin from fungi, a component of fungal cell walls. These molecules are usually recognized by plant transmembrane pattern recognition receptors (PRRs), which results in the first stage of plant defense, or PTI (Figure 2). PTI involves the activation of a variety of defense mechanisms to prevent initial infection by microbes (Dodds & Rathjen, 2010). These include callose deposition to strengthen cell walls, production of reactive oxygen species (ROS) for defense signaling or antimicrobial properties, stomatal closure to prevent pathogen infiltration, and mitogen-activated protein kinase (MAPK) cascades to amplify signalling and activate defense-related genes (Ramirez-Prado et al., 2018; Selin et al., 2016; Torres, 2010).

These initial defenses deter the majority of microbes and many non-host pathogens; however, other pathogens have a specialized arsenal to circumvent these defenses: proteins known as effectors. Selin et al. (2016) defines effectors as “molecules that alter host cell structure and function, facilitate infection, and/or trigger defense responses.” Effectors can be considered any molecule (including toxins, antigens, carbohydrates, or lipids) that contributes to virulence, such as by promoting infection, protecting the pathogen against host defenses, or assisting in nutrient acquisition or entry into the host cell.

Effectors are secreted by pathogens to suppress the initial PTI stage of plant defense and allow the pathogen to successfully colonize the host, in what is termed

effector triggered susceptibility (ETS; Figure 2) (J. D. G. Jones & Dangl, 2006; Lo Presti et al., 2015). Effectors perform their function in several ways: some bacterial effectors mimic host proteins to disrupt cellular function, such as the AvrPtoB protein from *Pseudomonas syringae*, which mimics the host ubiquitin ligase to suppress plant defenses (Janjusevic et al., 2006). Ubiquitin ligases have a role in plant cell death during infection by initiating apoptosis pathways to signal target proteins for degradation (Zeng et al., 2004). AvrPtoB works to redirect ubiquitin to suppress host programmed cell death. Other effectors may modify host proteins, such as the phosphothreonine lyase OspF effector from *Shigella* species (H. Li et al., 2007). OspF removes phosphate groups from MAPKs, resulting in their inactivation and subsequent suppression of MAPK-associated PTI responses (H. Li et al., 2007).

In a host-specific resistance response, plants can recognize pathogen effectors, and this recognition elicits the second stage of plant defense, known as effector-triggered immunity (ETI). ETI is triggered by either direct or indirect recognition of effectors by Resistance (*R*) genes, which results in a more robust defense response compared to PTI (J. D. G. Jones & Dangl, 2006). This defense response can be considered an amplified PTI, as many of the same signaling pathways are involved. Additionally, ETI can also result in the programmed death of cells at the site of infection, known as a hypersensitive response (HR) that prevents the spread of a pathogen (Cui et al., 2014; J. D. G. Jones & Dangl, 2006). *R* genes often code for nucleotide-binding and leucine rich repeat (NB-LRR) proteins, which recognize effectors and initiate ETI (Eitas & Dangl, 2010). Effectors that are recognized by NB-LRR proteins are termed Avirulence (Avr) proteins (J. D. G. Jones & Dangl, 2006; Khan et al., 2016). It is from this specific interaction that

the gene-for-gene model arose, where the specific interactions between *R* and *Avr* genes determines resistance (Cui et al., 2014; Flor, 1942). Examples of a direct interaction between *Avr*-*R* proteins include the NB-LRR resistance proteins RGA4 and RGA5 from rice that directly recognize the Avr1-CO39 effector from *Magnaporthe oryzae* and activate ETI to confer resistance against this pathogen (Cesari et al., 2013). In contrast, the *Arabidopsis* NB-LRR protein RPM1 does not recognize effectors directly; instead, the effector AvrRPM1 from the pathogen *Pseudomonas syringae* DC3000 targets another protein, RIN4, and modifies it by phosphorylation (Mackey et al., 2002). The post-translational modification of RIN4 enables it to interact with RPM1, leading to its activation and inducing an ETI response (Mackey et al., 2002). There are numerous examples where NB-LRR proteins have diversified their function with respect to recognizing effectors, including changes in cellular homeostasis or regulating transcription (Cui et al., 2014).

In summary, PTI can be considered a broad defense response against a wide range of pathogens and is integral to non-host resistance. Non-host resistance is the ability of a plant to resist infection to pathogens that lack the mechanisms to overcome host defenses. ETI can be considered a pathogen-specific response and results in a stronger defense response when compared to PTI (Dodds & Rathjen, 2010; J. D. G. Jones & Dangl, 2006).

Correspondingly, the study of effector function is of similar importance during plant-pathogen interactions because of the ability of effectors to evolve and adapt to different hosts. If the effector repertoire of a pathogen can be established, a great amount of information on how a pathogen infects and colonizes a host can be revealed. Moreover, characterization of these effectors and their targets in the host can be used by

plant breeders as markers and accelerate their breeding programs to develop novel resistance varieties.

1.4 Fungal Effector Proteins

Fungal pathogens have varied lifestyles and infection mechanisms. They can be biotrophic, necrotrophic, or hemibiotrophic (Selin et al., 2016). Fungal pathogens have specialized infection structures used for plant colonization, such as appressoria that form at the end of germinating hyphal tubes and can be as simple as swelling of the fungal tissue or that can differentiate into more complex dome-shaped structures (Ryder & Talbot, 2015). Appressoria can generate sufficient turgor pressure to penetrate a host cell (Ryder & Talbot, 2015). Some pathogens, such as *F. graminearum* or *Cladosporium fulvum*, are also able to passively enter the host through stomata. After cell penetration, some pathogens such as *Phytophthora infestans* or *Blumeria graminis* form specialized feeding structures known as haustoria, which allow the pathogen to take up nutrients from inside the cell (Perfect & Green, 2001). Other pathogens such as *Magnaporthe oryzae* produce invasive hyphae that grow within cells, while others only grow in the plant apoplast (Giraldo & Valent, 2013). Effectors can be secreted into the cell apoplast or cytoplasm by these infection structures, by either direct penetration into the cell, or by other yet unknown mechanisms (Selin et al., 2016).

Fungal effectors are understudied compared to bacterial effectors and appear to be much more varied in their structure and function. Fungal effectors generally do not have any broadly conserved protein motifs or features that are seen in bacterial or oomycete pathogens, making their prediction difficult when based on either primary amino acid

sequences or tertiary structures. (Sperschneider et al., 2015). Identification of fungal effectors often utilizes the characteristics identified in bacterial effectors, and many of the currently identified fungal effectors consist of the following properties: they are small proteins (<300 amino acids), possess secretion signals, are cysteine-rich (2-20% cysteine residues), and lack orthologues outside of a genus (D. A. Jones et al., 2018; Lo Presti et al., 2015). As well, effectors tend to be present in genomic regions that are repeat-rich with high recombination activity (Raffaele & Kamoun, 2012). The high recombination rate benefits effectors, allowing them to evolve constantly to overcome potential plant defenses (Raffaele & Kamoun, 2012). When defining the effector repertoire of a fungal pathogen, the properties described above are commonly used to filter predicted effectors from other secreted proteins, or to further validate predicted effectors (D. A. Jones et al., 2018).

One of the major caveats with current prediction algorithms is that most of the predicted effectors have not been functionally characterized, and more importantly, they may inadvertently exclude potential effectors (Selin et al., 2016). For example, the effector AvrM from the flax rust *Melampsora lini* contains over 300 amino acids and has a single cysteine residue (Catanzariti et al., 2010). The effector chorismate mutase from the corn smut *Ustilago maydis* is also larger than 300 amino acids and has conserved domains outside of the *Ustilago* genus (Djamei et al., 2011). Furthermore, isochorismatases from the oomycete *Phytophthora sojae* and the fungal pathogen *Verticillium dahliae* have been identified as effectors but do not have signal peptides (T. Liu et al., 2014). Clearly, defining fungal effectors as small, secreted, and cysteine-rich does not apply in all cases, and exceptions must be considered.

Several reviews of fungal effectors have been published: Selin et al. (2016), Sharpee & Dean (2016), and Rovenich et al. (2014) provide summaries of currently known effectors from both fungi and oomycetes. Novel effectors are constantly being identified and characterized, and a summary of recently characterized fungal effectors is described in Table 1. The list showcases the diversity of effectors and their function. The effectors described in Table 1 have sizes ranging from fewer than 70 to larger than 600 amino acids (Sanz-Martín et al., 2016; Zhong et al., 2017). Some have very few cysteines, while others are considered cysteine-rich (L. Ma et al., 2018; Qi et al., 2016a). Most do not have any corresponding *R* genes in the host, and instead function to suppress host defenses in a variety of ways, such as targeting plant chloroplast function, interfering with signaling pathways, or blocking antifungal activity (Fan et al., 2019; L.-S. Ma et al., 2018; L. Zhang et al., 2017). Interestingly, some of the described effectors have roles in RNA degradation or silencing, while others interact with DNA to regulate transcription (Ahmed et al., 2018; Yin et al., 2019).

Currently, there are several fungal species with well-defined effector repertoires. *Fusarium oxysporum* f. sp. *lycopersici* (causal agent of Fusarium tomato wilt), has effectors known as Six (secreted in xylem) proteins which are recognized by the tomato *I* resistance genes (Selin et al., 2016; Takken & Rep, 2010). Avr2 is another effector from *F. oxysporum* which is recognized by the resistance protein I-2 in the host, resulting in HR (Houterman et al., 2009). More than 40 *Avr* genes have been identified in the rice pathogen *Magnaporthe oryzae* and are classed into different families based on sequence similarity and function (S. Zhang & Xu, 2014). Effectors from *M. oryzae* include Avr-Piz-t which suppresses PTI by inhibiting ubiquitin ligase, while Avr-Pita is a zinc

Table 1: Recently characterized effector proteins from fungal pathogens

Pathogen	Effector Protein	Type/Function	Role in Virulence	Size	Signal peptide; location	Cysteine Content	Reference
<i>Blumeria graminis</i>	CSEP0064/BEC1054	RNase-like protein	Binds to host ribosomes to inhibit cell death; increases host susceptibility to pathogen	118 aa	Cytoplasm	Not stated	Pennington et al., 2019
<i>Colletotrichum falcatum</i>	CfPDIP1	Unknown	Triggers HR; upregulates defense-related genes	132 aa	Yes; unknown	4.5%	Ashwin et al., 2018
<i>Colletotrichum graminicola</i>	CgEP1	dsDNA binding protein	Necessary for anthracnose development; targets host DNA to prevent host defenses	328 aa	Nucleus	Not stated	Vargas et al., 2016
	Cgfl	Fungalysin metalloprotease	Involved in chitinase degradation; required for virulence	639 aa	Yes; unknown	Not stated	Sanz-Martín et al., 2016
<i>Colletotrichum orbiculare</i> , <i>M. oryzae</i>	NIS1	Necrosis-inducing secreted protein	Suppresses HR and ROS production; interacts with host BIK1 BAK1 to inhibit kinase activity	162 aa, 145 aa	Cytoplasm	Not stated	Irieda et al., 2019
<i>Leptosphaeria maculans</i>	AvrLm1	Unknown	Interacts with MAPK9 causing increased phosphorylation and triggers cell death	206 aa	Apoplast	0.5%	L. Ma et al., 2018
<i>Melampsora larici-populina</i>	Mlp124478	Unknown	Represses plant defense genes by putatively binding to DNA promoters	96 aa	Nucleus	6.25%	Ahmed et al., 2018
<i>Phakopsora pachyrhizi</i>	PpEC23	Unknown	Interacts with host transcription factor to negatively	267 aa	Yes; cytoplasm	7.9%	Qi et al., 2016

			regulate host defenses				
<i>Puccinia graminis</i>	PgtSR1	RNA silencing suppressor	Suppresses RNA silencing by changing levels of small RNAs that act in defense roles	146 aa	Unknown	Not stated	Yin et al., 2019
<i>Ustilaginoidea virens</i>	UV_1261	Unknown	Targets plant chloroplasts; suppresses ROS production and defense genes	130 aa	Yes; cytoplasm and chloroplast	3%	Fan et al., 2019
<i>Ustilago maydis</i>	Rsp3	Repetitive secreted protein	Blocks antifungal activity by interacting with maize AFP proteins	789 aa to 869 aa	Associated with hyphal surfaces	1%	L.-S. Ma et al., 2018
<i>Valsa mali</i>	VmPxEl	Unknown	Suppresses Bax-induced cell death; KO results in reduced virulence; targets apple peroxidase	213 aa	Yes	Not stated	M. Zhang et al., 2018
<i>Verticillium dahliae</i>	VdSCP7	Unknown	Activates salicylic and jasmonate signaling and modulates plant immunity	201 aa	Yes; nucleus	1.5%	L. Zhang et al., 2017
<i>Zymoseptoria tritici</i>	Zt6	Ribonuclease	Cleaves rRNA; toxin to plant cells	137 aa	Yes; cytoplasm	1.5%	Kettles et al., 2018
	AvrStb6	Unknown	Recognized by Stb6 in wheat	63 aa	Yes	14%	Zhong et al., 2017
	Avr3D1	Unknown	Recognized by wheat Stb7, causing an incomplete, quantitative resistance that impairs infection but allows pathogen to complete its life cycle	92 aa	Yes; unknown	11.3%	Meile et al., 2018

metalloprotease that is recognized by the Pi-ta protein in rice to confer resistance (Khang et al., 2008; W. Li et al., 2009). *M. oryzae* also has effector proteins with no corresponding R proteins, such as Slp1 which is a secreted protein containing a Lysin motif (LysM). LysM proteins are present across a range of fungal species as well as bacteria, and are a class of effectors with a single LysM domain (de Jonge & Thomma, 2009). They act by binding to chitin that is released during infection, thus pre-empting recognition by the host immune system and the activation of PTI (de Jonge & Thomma, 2009).

The chorismate mutase Cmu1 effector from *Ustilago maydis* (maize smut) disrupts host metabolism by reducing salicylic acid levels during infection (Djamei et al., 2011). This is an interesting example of a fungal effector targeting a metabolic pathway, by working in tandem with an orthologous host protein (i.e., the maize chorismate mutase) to redistribute metabolites to aid in infection. Other effectors from *U. maydis* include Pep1, which interacts with maize peroxidase POX12 to suppress ROS (Hemetsberger et al., 2012). The effector Tin2 interacts with TTK1 in maize to induce anthocyanin biosynthesis (Tanaka et al., 2019). Thus far, no gene for gene interaction has been uncovered in *U. maydis* and its maize host, suggesting that resistance to this fungus may operate similarly to wheat against *F. graminearum* (Brefort et al., 2009).

Oomycetes, or “water moulds”, are a distinct group of eukaryotic pathogens with fungal-like characteristics and include *Phytophthora* species and downy mildews. Many of these secrete effectors with a specific protein motif: RXLR, which is a domain involved in translocation of effectors into the host cells (Schornack et al., 2009). Other motifs found among a number of fungal or oomycete species include the RGD motif,

found in ToxA from *P. tritici-repentis* and IpiO from *P. infestans* (Manning et al., 2008; Senchou et al., 2004). Crinkler (CRN) motifs are also found in several oomycete species, which can invoke necrosis (Kale & Tyler, 2011; Torto et al., 2003).

1.5 Current Knowledge of *F. graminearum* Effector Proteins

F. graminearum has a poorly defined effector repertoire. Research has primarily been focused on the trichothecene mycotoxins produced by *Fusarium* species. One of these well-studied metabolic effectors in *F. graminearum* is the secreted mycotoxin deoxynivalenol (DON). This secondary metabolite binds to the A-site in the peptidyltransferase center in ribosomes to prevent polypeptide synthesis (de Loubresse et al., 2014). This is further supported by evidence that DON interacts with magnesium in the A-site, changing the ribosomal conformation to confer toxicity (Foroud et al., 2016). Interestingly, DON is not required for initial infection by *F. graminearum*, but is necessary for the spread of *F. graminearum* within wheat (Bai et al., 2002). This implies that there are likely other secreted proteins or effectors that aid in initial infection that have not yet been identified.

The hemibiotrophic lifestyle of *F. graminearum* implies that effectors could be secreted during either its biotrophic or necrotrophic phase of host colonization. As well, because *F. graminearum* primarily infects inflorescence (floral) tissues, secretion of effectors may be tissue specific. Thus, the time point of infection and type of tissue may greatly impact the number and type of effectors being secreted into the host and must be taken into consideration when studying *F. graminearum*.

Cell-wall degrading enzymes (CWDEs) are secreted proteins that play a role in breaking down of plant cell walls, allowing for easier penetration and entry by the fungi. Several studies examining the proteome or secretome of *F. graminearum* have identified a number of CWDEs, including lipases or pectinases (Kikot et al., 2009; Paper et al., 2007; Phalip et al., 2005). CWDEs are typically secreted and can be considered to promote virulence as they allow for successful entry of the pathogen. They technically fall within the effector definition introduced in section 1.3 because they modify host cell structure but are generally considered their own class of virulent proteins. Their specific role is in the breaking down of host cell walls and they are likely to be identified when examining fungal secretomes.

Several CWDEs have been identified and characterized from *F. graminearum*. One of these is a secreted lipase, FGL1, which was confirmed to be involved in virulence by the inability of a *fgl1*-deletion strain to infect wheat spikelets (Voigt et al., 2005). Although the function of FGL1 was not validated, it was suggested that it plays a role in cell-wall degradation or in the activation and expression of enzymes responsible for hyphal growth rates (Voigt et al., 2005).

Recently, a secreted arabinanase from *F. graminearum*, Arb93B (FGSG_03598), was found to reduce ROS production in *N. benthamiana*, and was able to suppress host-induced cell death and promote colonization of the fungus (Hao et al., 2019). While this protein could be considered a CWDE, it has a clear function in disrupting plant defenses more so than degrading the cell wall, and so could be considered as an effector. Four KP4 killer toxin proteins (FGSG_00060, FGSG_00061, FGSG_00062, and FGSG_10551) have also recently been identified in *F. graminearum* (Lu & Faris, 2019).

Killer toxins are secreted polypeptides that were initially identified in viruses to disrupt ion flow by creating pores in host cells (Park et al., 1994). All four KP4 proteins identified in *F. graminearum* contain a signal peptide, however, the proteins were not confirmed to be secreted or localized *in planta*. One of the proteins (FGSG_00061) was shown to inhibit the growth of wheat roots but did not cause cell death on wheat leaves or spikelets (Lu & Faris, 2019). Although these KP4 proteins were examined in the context of seedling rot caused by *F. graminearum* and not head blight, they could be considered effectors as they putatively function *in planta* to promote virulence.

While the known effector repertoire of *F. graminearum* is lacking, several secretome and proteome studies of *F. graminearum* have been published that have identified proteins with a putative role in virulence. A summary of these is provided in Table 2.

Brown et al. (2012) compiled a list of secreted proteins and identified 574 proteins based on SignalP, TargetP, and other *in silico* predictive software that utilizes signal peptide or location predictors. Besides this *in silico* study, other secretomes have been compiled through proteomic methods such as mass spectrometry and identified proteins secreted in the presence of wheat flour (Yang et al. 2012) or secreted proteins present both *in planta* and *in vitro* (Fabre et al., 2019). Paper et al. (2007) identified *F. graminearum* proteins *in planta* by submerging wheat heads in water and performing vacuum-infiltration to isolate proteins without cell lysis. This methodology provides one of the more accurate secretomes as the proteins are identified *in planta*. Even with their limitations, these secretome studies provide valuable information on proteins that could be involved in the *F. graminearum* infection process.

Effectors can be expected to be expressed during early stages of the infection process. A number of transcriptomic datasets from *F. graminearum* during infection or infection-like conditions have been published, and a subset are listed in Table 3. These datasets include genes which are expressed over several infection time points or stages of infection (Brown et al., 2017; Lysøe et al., 2011). Transcriptomic data can also reveal valuable information about genes that are expressed during infection and when consolidated with secretome data, can be used to identify potential effector proteins. However, considerable challenges remain in characterizing these secreted proteins with respect to their function in *F. graminearum* pathogenesis.

1.6 *F. graminearum* and Wheat Interactions

One of the major challenges in identifying *F. graminearum* effectors is the fact that there are currently no known *R* genes in wheat that have been identified in response to *F. graminearum* infection. Instead, resistance is conferred through many quantitative trait loci (QTL) and is therefore considered as multi-genic (Buerstmayr et al., 2009; Gunnaiah et al., 2012). Resistance conferred by a single QTL is partial and it is inferred that genes responsible are involved in basal PTI defense responses (Poland et al., 2009).

Fhb1 is a well-studied QTL in wheat known to confer resistance to *F. graminearum*. One of the genes at this loci is a pore-forming toxin-like (PFT) gene that encodes a chimeric lectin with an ETX/MTX2 toxin domain, and confers partial resistance to *F. graminearum* (Rawat et al., 2016). Interestingly, lectin domains can resemble PRR proteins that function to recognize PAMPs at the cell membrane (Lannoo & Van Damme, 2014). A more recent paper has revealed another gene at the same Fhb1

Table 2: Published secretomes and proteomes from *F. graminearum*

Reference	Type of '-ome'	Conditions	<i>F. graminearum</i> Strain	Num of Identified Proteins
Rampitsch et al., 2013	In vitro secretome	Liquid culture strained from mycelia grown in N-limiting conditions	DAOM 233423	58
F. Yang et al., 2012	In vitro secretome	Liquid culture strained from mycelia, grown in presence of ground barley/wheat flour	PH1	69
Subramaniam Exoproteome (unpublished)	In vitro secretome	Liquid culture supernatant from mycelia grown in presence of wheat head	DAOM 233423	512
Brown et al., 2012	Predicted secretome	Various predictive tools	PH1	574
Phalip et al., 2005	In vitro secretome	Liquid culture supernatant grown in presence of glucose or hop cell wall preparation	Unknown; isolated from hop plants	84
Paper et al., 2007	In planta secretome	Vacuum-infiltration of wheat heads submerged in water	PH1	120
Lu & Edwards, 2016	In vitro secretome/ Predicted	Liquid culture supernatant grown in minimal media	PH1	190
Ji et al., 2013	In vitro secretome	Supernatant of mycelia grown in minimal media	Not mentioned	87
Lysenko et al., 2013	Predicted proteome	Bioinformatics to predict virulence proteins	PH1	215
Taylor et al., 2008	In vitro proteome	Whole tissue grown in minimal media, examined change in protein expression in minimal conditions	DAOM 180378	435
W. Zhou et al., 2006	In planta secretome	Protein extracted from wheat spikelets inoculated with <i>F. graminearum</i> harvested at 1, 2, or 3 dpi	N2	8
Fabre et al., 2019	In planta/in vitro proteome	Protein extracted from wheat spikelets at 1, 2, 3, or 4 dpi; and from <i>F. graminearum</i> spores or germinating spores	MDC_Fg1	1132

Table 3: Published transcriptomes from *F. graminearum*

Reference	Method for Obtaining Transcriptome	Conditions	<i>F. graminearum</i> Strain
Qinhu Wang et al., 2017	RNA-Seq	Vegetative vs infective stage	HN9-1
Lysøe et al., 2011	Microarray	Time series of <i>F. graminearum</i> infection from wheat head point inoculation from 0 hpi to 24, 48, 96, 144, 192 hpi	PH-1
Brown et al., 2017	Microarray	Symptomless vs symptomatic infected wheat tissues	PH-1
Pan et al., 2018	RNA-Seq	Infected wheat heads at 2dpi or 4dpi vs water control	DAOM 233423

loci that confers resistance (Su et al., 2019). This gene encodes a histidine-rich calcium-binding protein (HRC) and is a susceptibility (S) gene containing a mutation that results in FHB resistance (Su et al., 2019).

Given the fact that resistance to *F. graminearum* is quantitative, a non-host system such as *Arabidopsis thaliana* with its immense genetic resources may be a suitable system to study *F. graminearum* pathogenesis. The *F. graminearum*-*Arabidopsis* pathosystem has been used in several studies to better understand *F. graminearum* infection mechanisms. *F. graminearum* is able to colonize young seedlings and floral tissues of *Arabidopsis* (Brewer & Hammond-Kosack, 2015; Cuzick et al., 2008; Schreiber et al., 2011; Urban et al., 2002). *F. graminearum* can infect both wheat and *Arabidopsis*, implying that effectors are secreted in both systems to overcome the initial plant defenses (PTI) and allow successful colonization of the respective host tissues. Unlike wheat, which has a large genome size and long growing time, *Arabidopsis* is amenable to genetic transformations and can be grown quickly. These factors, in combination with the ability of *F. graminearum* to infect *Arabidopsis*, make the *F. graminearum*-*Arabidopsis* pathosystem an ideal platform to perform molecular characterizations of *F. graminearum* infection and identify potential effectors.

1.7 Overview of BioID and its Application for Identifying Candidate Effectors

As previously described, numerous proteome, transcriptome, and bioinformatic studies have been undertaken to identify the effector repertoire of *F. graminearum*. Each of these methods have their drawbacks; predominantly, they lack *in planta* context and technically, proteins are subjected to extraction and purification conditions that make it

impossible to identify proteins that interact transiently during the initial stages of plant-pathogen interactions. As mentioned, predicting effectors based on pre-determined effector properties may exclude those lacking these properties, but that still have effector function.

Proximity-dependent biotin identification, also known as BioID, is a relatively recent method that can be used to identify protein interactions *in vivo* or as a proteome mapping tool (Gingras et al., 2019; Kim & Roux, 2016; Roux et al., 2012). BioID involves the use of a mutated biotin ligase isolated from *E. coli*, BirA*, to promiscuously biotinylate proximal proteins within a 10 nm range that contain a primary amine (Roux et al., 2013). The mutated ligase has a lower affinity for its substrate (bioAMP) than the non-mutated ligase, allowing it to biotinylate proximal proteins (Figure 3) (Roux et al., 2013). Once proteins are biotinylated, they can be extracted under denaturing conditions, isolated using affinity purification, and identified by mass spectrometry. This technique can reveal novel protein interactions, and more importantly, allows a ‘real-time’ glimpse of protein interactions as the technique is performed using *in vivo* conditions and over a period of several hours. More broadly, BioID can capture an image of protein populations within specific cellular compartments or of protein complexes under specific environmental conditions

BioID is particularly useful in identifying transient or low-affinity interactions in an *in vivo* system, which overcomes the drawbacks of other protein identification methods described above. BioID was first used for identification of interacting proteins with lamin-A (LaA) in mammalian cells (Roux et al., 2012). It has since been used in a number of systems and characterized novel components of cellular regions, such as

Figure 3: Proximity-dependant biotin identification, recently developed for use in *Arabidopsis*. BirA* is expressed with a fusion protein *in vivo*. After exogenous biotin (B) is added to the system, BirA* will biotinylate proximal proteins containing a primary amine within a 10 nm radius (black circle). These tagged proteins can then be isolated via affinity purification using streptavidin antibodies.

mapping protein interactions between the centrosome and cilium in mammalian cells (Gupta et al., 2015), or examining the proteome of vacuolar compartments in red blood cells where malaria parasites develop (Khosh-Naucke et al., 2018).

BioID has been successfully used in plants. BioID used in rice protoplasts identified proteins involved in flowering or vegetative growth (Lin et al., 2017). This study revealed interactors with OsFD2, a transcription factor involved in vegetative growth, and identified twelve putative neighbouring proteins (Lin et al., 2017). BioID in *N. benthamiana* examined plant targets of the bacterial effector AvrPto, by fusing BirA* with AvrPto and expressing the fusion construct in *N. benthamiana* to biotinylate plant interactors (Conlan et al., 2018). Five interacting proteins were identified, with putative roles in plant immunity. Our group deployed BioID in *Arabidopsis* to identify targets of the effector HopF2 from *P. syringae* (Khan et al., 2018). Two transgenic *Arabidopsis* (Col-0) were engineered: one expressing a cytoplasmic BirA*, and one expressing BirA* fused to the membrane-associated effector HopF2 (Khan et al., 2018). Nineteen proteins were identified that associated with HopF2, revealing both known immunity-related proteins and several novel ones (Khan et al., 2018).

The use of BioID to identify proteomes, in combination with the *F. graminearum*-*Arabidopsis* pathosystem, provides a unique setting to analyze the secretome of a pathogen *in planta*. My thesis uses BioID to identify proteins from *F. graminearum* that are secreted *in planta* that could potentially contribute to virulence.

1.8 Thesis Outline

The overall aim of this thesis was to identify and characterize candidate effector proteins (CEPs) from *F. graminearum* using BioID. In Chapter 2, BioID is used to identify potential effectors secreted from *F. graminearum* during infection of *Arabidopsis* seedlings. Transgenic *Arabidopsis* plants expressing either cytoplasmic or membrane (HopF2) BirA* were inoculated with *F. graminearum* and proteins were isolated by affinity chromatography and subjected to mass spectrometry. In Chapter 3, a subset of the candidate effectors identified from Chapter 2 were characterized via functional analysis to determine their role in virulence using phenotypic assays developed in wheat. Overall, this thesis describes almost 100 proteins secreted by *F. graminearum* identified *in planta* that may play a role in virulence.

Chapter 2: Identification of Candidate Effector Proteins from *F. graminearum* using BioID

2.1 Summary

BioID was originally developed to identify novel protein-protein interactions with lamin-A in mammalian cells by fusing the bait protein lamin-A to the biotin ligase BirA* (Roux et al., 2012). BioID and its ability to identify protein interactors (stable or transient) *in vivo* and in denaturing conditions provides a powerful tool in molecular biology. In my thesis, I used BioID to characterize secreted proteins from *F. graminearum* in *Arabidopsis*. Specifically, the proteins from the cytoplasmic and membrane fractions of *Arabidopsis* were identified after infection with *F. graminearum*. A total of 351 *F. graminearum* proteins were identified via affinity purification and mass spectrometry across all infection time points and cellular fractions. Of these, 99 were considered as candidate effector proteins (CEPs). A detailed characterization of the CEPs is the subject of Chapter 2.

2.2 Materials and Methods

2.2.1 Plant Material and Growth Conditions

The two transgenic *Arabidopsis* (*Col-0*) BioID lines, pBD::BirA*-FLAG-HA (*BirA**) and pBD::HopF2-BirA*-FLAG-HA (*HopF2-BirA**) were generated as described in Khan et al. (2018). Seeds were generously donated by Dr. Madiha Khan. The plants were grown in a vermiculite and soil mix, covered with a propagation dome, and stratified in the dark for 2 days at 4°C. Trays were then moved to growth cabinets at 22°C under long days (16 hr light / 8 hr dark), and after approximately a week, each pot

was thinned to contain only 2 plants. Plants were grown until inflorescences were mature (approximately 6 weeks), and seeds were harvested.

2.2.2 Extraction of Genomic DNA from *Arabidopsis* and Genotyping to Confirm Presence of BioID Inserts

From each *Col-0*, *BirA**, or *HopF2-BirA** line, a young rosette leaf was harvested for genotyping. DNA was extracted using a modified Edwards DNA prep (Edwards et al., 1991). Leaves were flash frozen in liquid nitrogen and 750 μ L of EDM buffer (200 mM Tris pH 7.5, 250 mM NaCl, 25 mM EDTA pH 8.0, and 0.5% SDS) was added to each leaf, incubated at 95°C for 5 minutes, and left overnight at room temperature. An aliquot of 750 μ L of isopropanol was added and mixed by inverting. Samples were left to sit at room temperature for 5 minutes, then centrifuged at 14,000 rpm. DNA pellets were washed with 1 mL of 70% ethanol, mixed by inversion, and spun at 14,000 rpm. The pellet was left to dry at room temperature, and then resuspended in 100 μ L of 1X TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA pH 8.0).

Primers used for genotyping are listed in Appendix A, Table 1. PCR reactions were performed using a standard 30 μ L Phusion DNA Polymerase (Thermo Scientific, USA) reaction with 5 μ L of DNA template, 0.2 μ M primers, 0.2 μ M dNTPs, 1X buffer, and 0.013 U of Taq polymerase. PCR conditions were as follows: denaturation for 15 seconds at 95°C, elongation for 20 seconds at 60°C, and extension for 90 seconds at 72°C, for 35 cycles. Plants lacking *BirA** or *HopF2-BirA** were discarded.

2.2.3 *F. graminearum* Strains, Propagation, and Storage

F. graminearum wild-type strain DAOM 233423 was obtained from the Canadian Collection of Fungal Cultures, Agriculture and Agri-Food Canada, Ottawa, Canada. The *F. graminearum*-GFP (*Fg*-GFP) strain was obtained from R. Proctor, National Center for Agricultural Utilization Research, Agricultural Research Service, United States Department of Agriculture, and Peoria, IL U.S.A.

Mycelia were grown on potato dextrose agar (PDA) at 28°C for approximately 5 days. To generate spores, 3-mm plugs of agar/mycelia were taken from the plate and transferred to 50 mL of carboxymethylcellulose (CMC) media (Cappellini & Peterson, 1965). Cultures were grown at 28°C for 3-5 days in a shaking incubator to generate spores, which were then isolated from mycelia by filtration through cheesecloth, pelleted by centrifugation at 5000 rpm, and rinsed twice with sterile water. Spores were counted with a haemocytometer and stored at 4°C until use.

2.2.4 *F. graminearum* - *Arabidopsis* Infection Assay and Infection Imaging

Arabidopsis-*F. graminearum* infection assays were developed by Schreiber et al. (2011). *Col-0*, *BirA**, or *HopF2-BirA** *Arabidopsis* seeds were surface-sterilized for 10 minutes in 30% bleach and 0.01% Tween-20, and then rinsed with sterile water. Five to seven seeds were placed in each well of a 96-well plate tissue culture plate (Sarstedt, Germany), excluding the outermost wells. A 200 µL aliquot of 0.5X MS (Murashige and Skoog basal media (Murashige & Skoog, 1962), 2.5 mM 2-(N-morpholino) ethanesulfonic acid (MES)) was added to each well, including outermost wells to prevent evaporation. Plates were stratified in the dark for 2 days at 4°C on a Heidolph Titramax 1000 vibrating shaker at 600 rpm. Plates were then moved into a growth chamber at

22°C under long days (16 hr light, 8 hr dark) for 5 days, at which point the liquid media was removed, and 200 µL of *F. graminearum* spores were added at a concentration of 800 spores/mL in 0.5X MS. Plates were incubated at 22°C under long days for another 24, 48, or 60 hours, with shaking at 600 rpm. D-biotin (1 mM) (BioBasic Canada Inc., Canada) and 30 µM of dexamethasone (DEX) (Sigma-Aldrich) were added to each well 24 hours prior to collecting seedlings.

Lactophenol-trypan blue staining (Koch & Slusarenko, 1990) was used to visualize cell death and fungal growth on infected seedlings. Briefly, infected seedlings were collected and boiled in lactophenol-trypan blue solution (500 mL phenol, 500 mL lactic acid, 500 mL glycerol, 1 g trypan blue dye in 2 L) at 95°C for approximately 10 minutes. Seedlings were then incubated at room temperature for approximately an hour and destained with 1.25 g/mL chloral hydrate solution overnight on a rocking shaker. Seedlings were mounted in 100% glycerol on glass slides and imaged with a Leica microscope and Google Pixel 2 phone camera.

For brightfield and fluorescent images, seedlings were imaged within wells or mounted on agar plates using an Olympus SZX16 stereo microscope and GFP filter. Images were captured by an Infinity 3 camera and Infinity Analyze and Capture software v6.2.0 (Lumenera, Canada).

2.2.5 Optimization of Biotin Levels for Infection Assay and Confirmation of BirA* Expression by Immunoblot Analysis

To optimize biotin levels, concentrations ranging from 0 mM to 2 mM of biotin were added to *BirA** seedlings and BirA* was induced with 30 µM dexamethasone

(DEX). Twenty-four hours after the addition of biotin and DEX, seedlings from each biotin concentration were pooled, ground to a powder in liquid nitrogen, and resuspended in protein extraction buffer (20 mM Tris pH 8.0, 100 mM NaCl, 0.4% SDS, 0.1% TritonX-100, protease inhibitor cocktail (Sigma P9599), 1 mM DTT). Samples were incubated on ice for 30-40 minutes with occasional vortexing, then spun down at 16,000 g for 15 minutes at 4°C. The protein supernatant was quantified with Fourier Transform Infrared (FTIR) spectroscopy (Direct Detect Infrared Spectrometer, Millipore Sigma, USA).

An aliquot (20-30 µg) of protein extract in 1X sample buffer (2% SDS, 10% glycerol, 0.002% bromophenol blue dye, 62.5 mM Tris pH 6.8, 5% DTT) was run on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto a nitrocellulose membrane using a semi-dry method at a constant voltage of 8 V for 45 minutes. Protein transfer was confirmed using Ponceau S (1% acetic acid, 0.5% Ponceau S dye) stain. Membranes were blocked overnight in 3% ECL blocking buffer (Amersham BioSciences, UK) and then incubated for 1 hour with either streptavidin-HRP antibodies (1:5000) (GE Healthcare, USA) or HA antibodies (1:10,000) (Roche, Switzerland). Subsequently, membranes were rinsed with 1X TBST (50 mM Tris pH 8.0, 150 mM NaCl, 0.1% Tween-20). HA-incubated membranes were incubated with secondary goat anti-rat IgG-HRP antibodies (1:30,000) (Santa Cruz Biotechnologies, USA) for another hour and rinsed with 1X TBST. Membranes were developed with Lumigen ECL Ultra (TMA-6) solutions (Lumigen Inc, USA) and imaged with Bio-Rad ChemiDoc and Image Lab.

2.2.6 Affinity Purification of Biotinylated Proteins

Three biological replicates were used for affinity purification and mass spectrometry analysis. Each replicate consisted of *Arabidopsis* seedlings pooled from two 96-well plates (~400 mg total). Seedlings were ground into a powder in liquid nitrogen, and protein was extracted and quantified as described in Section 2.2.5. An aliquot of the supernatant was used as the input fraction for immunoblot analysis. Protein was concentrated and excess biotin was removed by centrifugation by an Amicon Ultra-4 (Ultracel-10) filter column (Millipore Sigma, USA) at 3000 g for 5 minutes. The filter columns were rinsed with 3 mL of extraction buffer (with no detergents) and centrifuged at 4000 g for 15 minutes. The rinse step was repeated 3-4 times, and protein extract was concentrated to a final volume of 500 μ L.

Approximately 1 mg of protein extract was incubated with 50 μ L prewashed streptavidin-sepharose high performance beads (GE Healthcare, USA) for 1 hour at 4°C. Beads were spun down for 2 minutes at 500 g and the supernatant (unbound fraction) was saved for immunoblot analysis. Beads were rinsed once with 2% SDS buffer (2% SDS, 200 mM Tris pH 8.0), and three times with NaCl buffer (20 mM Tris pH 8.0, 100 mM NaCl). A 50 μ L aliquot of resuspended beads was set aside as the bound fraction for immunoblot analysis. After the final rinse, all supernatant was removed, and dry beads were sent for mass spectrometry. Immunoblot analyses were performed as described in Section 2.2.5 using input, bound, and unbound fractions. A 2 mM aliquot of biotin was added to the bound fraction before boiling to prevent rebinding of protein to the beads.

2.2.7 LC-MS/MS Analysis to Identify *Arabidopsis* and *F. graminearum* Peptides

Dried beads obtained from affinity purification were sent to the Morden Research Centre of Agriculture and Agri-Food Canada where liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed by Dr. Christoph Rampitsch.

Proteins were digested on-bead with trypsin as described in Hesketh et al. (2017). A description of each treatment or control sent for mass spectrometry analysis is described in Appendix A, Table 2.

To prevent cross-contamination, control samples were injected first into the mass spectrometer. Tryptic peptides were separated through a C18 column (12-cm fused silica column, 75 µm ID, packed with Vydac C18, 5 µm beads, 300 Å pores) coupled to an Orbitrap Q Exactive mass spectrometer (ThermoFisher, USA) via a nanoelectrospray ionization source. An acetonitrile gradient of 2% (v/v) acetonitrile and 0.1% (v/v) formic acid was delivered at 300 nl/min over 60 minutes, with a total run of 120 minutes. A survey scan was acquired over an m/z range of 300-2000, followed by 12 MS₂ scans of the most intense ions.

Mascot (v2.5.1; MatrixScience, USA) was used for protein identification. Raw mass spectrometry data files were converted to MGF using Mascot Distiller and queried against the SwissProt *Arabidopsis* database (<https://www.uniprot.org/>) (535,248 sequences) and the MIPS *F. graminearum* database (13,826 sequences) (Güldener et al., 2006), including our annotations from the DAOM 233423 isolate. Mascot searches used to identify proteins were set with the following parameters: monoisotopic mass accuracy of +/- 5ppm, up to two missed cleavages, peptide charge up to +5, fixed modification of carbamidomethyl and variable modifications of oxidation (M) and biotin (K, N-term).

The false-discovery rate (FDR) set to 1% was validated against a target-decoy database. Proteins were considered correctly identified if hits contained two or more peptides with a significant ion score, based on the probability of the match being a random event (Perkins et al., 1999).

2.2.8 Identification of Candidate *F. graminearum* Effector Proteins

F. graminearum proteins identified in Mascot searches were filtered from *Arabidopsis* proteins and other contaminants. A series of filters were applied to generate a list of candidate effector proteins (Figure 4). Each protein identified by Mascot was assigned a protein score (derived from the ion score) to represent significant (95% confidence) identity and homology to the reference protein based on the size of the reference database and the number of peptides found from mass spectrometry analysis. When using the *F. graminearum* MIPS database, peptides with a score above 20 were considered significant (95% confidence) with a strong identity and homology to the matching protein. *F. graminearum* proteins with a score above 20, present in 2 of 3 biological replicates, and absent in any control treatment were considered to be reliably identified *F. graminearum* proteins, hereon referred to as candidate effector proteins (CEPs).

Figure 4: Filters applied to identify *F. graminearum* proteins identified by BioID. A total of 351 *F. graminearum* proteins were identified via mass spectrometry across all cellular locations and time points. To generate a list of candidate proteins, any protein with a Mascot score of less than 20, only present in 1 of 3 biological replicates, or present in any controls was removed. This resulted in a list of 99 candidate proteins.

2.2.9 Analysis of Candidate Effector Proteins – Software and Published Datasets

Secretion signals for CEPs were assessed based on SignalP 5.0 scores (Almagro Armenteros et al., 2019). Localization for CEPs was assessed based on TargetP 1.1 (Emanuelsson et al., 2000). CEPs were also analyzed with EffectorP 1.0 and EffectorP 2.0 (probability >0.55 = effector, <0.55 = non-effector) (Sperschneider et al., 2018, 2016). The number of cysteine residues was determined with ExPasy ProtParam (Gasteiger et al., 2005).

Functional categorization analysis of CEPs was performed by FungiFun2 (Priebe et al., 2015). Protein domains were identified with Pfam 32.0 from EMBL-EBI (El-Gebali et al., 2019). CEPs were mapped to the *F. graminearum* genome (from MIPS) using Fgra3Map and aligned with regions of high recombination and high SNP density (Antoniw et al., 2011). CEPs were screened through a set of previously published secretome, proteome, and transcriptomic datasets from *F. graminearum* and assigned scores based on the number of datasets they were present in (i.e., score of 5 = present in 5 datasets). The datasets used are listed in Table 2 and Table 3.

2.3 Results

2.3.1 BirA* and HopF2-BirA* are Expressed in *Arabidopsis*

Arabidopsis BioID lines were obtained from Dr. M. Khan and were first bulked for seeds and genotyped to confirm the presence of the BioID vectors. The vector pBD::BirA*-FLAG-HA (*BirA**) is localized to the cytoplasm, and pBD::HopF2-BirA*-FLAG-HA (*HopF2-BirA**) is membrane-localized (Appendix A, Figure 1A) (Khan et al., 2018). Both are under the control of a DEX-inducible promoter and are expressed with a C-terminal FLAG and HA epitope tags (Appendix A, Figure 1A). *Arabidopsis* plants expressing *BirA** or *HopF2-BirA** were genotyped for the BioID plasmids by PCR. Ninety percent of plants grown from the *BirA** line contained the *BirA** gene, which is representative of a homozygous line (Appendix A, Figure 1B). However, for the two *HopF2-BirA** lines, only 56% and 63% of plants were confirmed positive, indicating heterozygosity (Appendix A, Figure 1C). Individual progenies of the original *HopF2-BirA** lines were subsequently genotyped to identify and confirm homozygosity. No amplification for either gene was seen in *Col-0* controls (Appendix A, Figure 1D).

2.3.2 Optimization of *F. graminearum* Infection in *Arabidopsis* Seedlings

The *Arabidopsis*-*F. graminearum* 96-well plate infection assay developed by Schreiber et al. (2011) was used in our BioID experiments with modifications. The modifications included the addition of biotin and dexamethasone (DEX) to the growth medium. To ensure that seedling infection by *F. graminearum* was not altered with modifications, *Arabidopsis* seedlings inoculated with *F. graminearum* spores were examined for visual phenotypes associated with infection. Infected *Arabidopsis*

seedlings were grown for 24 hours in the presence of biotin and DEX. The samples were collected after 24, 48, or 72 hours and stained with Trypan Blue. Trypan Blue allows for visualization of dead cells (Shipton & Brown, 1962). After three days of infection (72 hpi), dark spots representing dead tissue were clearly seen in cotyledon leaves, indicating the breach of host cells (Figure 5A). After 2 days (48 hpi), some cell death was also visible at the tips of cotyledons (Figure 5A). As cell death is clearly occurring after 72 hours of infection, a final time-point of 60 hpi (2.5 dpi) for tissue collection was chosen in order to limit BirA* contamination of samples from lysed *Arabidopsis* and *F. graminearum* cells.

Infection was also visualized with fluorescence (Figure 5B) or brightfield (Figure 5C) microscopy. *F. graminearum* mycelia expressing the green fluorescent protein (GFP) can be clearly seen in abundance at later infection time-points (48 and 72 hpi; Figure 5B). Brightfield images showed brown lesions and bleaching on cotyledons at 72 hpi (Figure 5C), confirming the infection pattern seen in Trypan Blue-stained seedlings. Earlier time-points showed no visible signs of infection (Figure 5C).

Figure 5: Infection progress of 5-day old *Arabidopsis* seedlings grown in liquid culture, inoculated with *F. graminearum* spores and grown in the presence of 1 mM biotin and 30 μ M of DEX. **A:** Trypan Blue staining of cotyledons revealed cell death due to *F. graminearum* infection around 48 hpi, while by 72 hpi cell death was visible and mycelia were seen entering the cotyledon via stomata. **B:** Fluorescent microscopy images of *F. graminearum*-GFP displayed fungal growth in cultures across 3 days of infection; abundant mycelial growth was seen at 72 hpi. **C:** Brightfield microscopy showed symptoms of infection at 72 hpi as faint brown lesions visible on the cotyledons. No symptoms were visible at earlier time points.

2.3.3 Optimization of Biotin Concentrations for BioID in *Arabidopsis* Seedlings

Since biotin is required for the optimal activity of BirA* to biotinylate proximal proteins in BioID, we optimized biotin concentrations for the *Arabidopsis-F. graminearum* 96-well plate infection assay. *Arabidopsis* seedlings were grown in a range (0 – 2 mM) of biotin concentrations and biotinylated proteins from total protein lysates from both cytoplasmic (BirA*) and membrane (HopF2) fractions were detected with streptavidin-conjugated horseradish peroxidase (HRP) antibodies. Biotinylation levels saturated at 1 mM, and no additional biotinylated proteins were detected at higher concentrations (Figure 6, 1-2 mM biotin). Only endogenous biotin labelled proteins were detected when *Arabidopsis* seedlings were grown with no exogenous biotin (Figure 6, 0 mM biotin). BirA* expression across protein extracts was confirmed via immunoblot with HA antibodies (Figure 6). Similar patterns were observed with the membrane fractions (data not shown). No visible differences in biotinylation levels were observed between infected (pFg) or non-infected (mFg) treatments, nor between the two time points 24 and 48 hpi (Figure 6, pFg 48 hpi, pFg 24 hpi, mFg 24 hpi).

Figure 6: Biotin optimization for BioID. Immunoblot of protein lysates from *Arabidopsis* seedlings expressing BirA* (cytoplasmic) grown in a biotin concentration gradient. Biotin concentrations from 0 mM to 2 mM were tested across three treatments for the cytoplasmic (BirA*) fraction (infected (pFg) seedlings at 48 hpi, infected seedlings at 24 hpi, and non-infected (mFg) seedlings at 24 hpi). Biotinylated proteins were detected with streptavidin-HRP antibodies (top panel). Biotin levels were saturated at 1 mM, and only endogenously biotinylated proteins were detected when no exogenous biotin was added (0 mM). HA antibodies were used to confirm BirA* expression (middle panel). BirA* expression was constitutive across all biotin concentrations and treatments. Ponceau S stain was used to confirm protein transfer (bottom panel).

2.3.4 Affinity Purification of Biotinylated Proteins from Non-Infected and Infected *Arabidopsis* Seedlings

After biotin concentrations were optimized for the *F. graminearum*-*Arabidopsis* 96-well plate infection assay, affinity purification with streptavidin beads was performed to purify biotinylated proteins from *F. graminearum* non-infected (mFg) and infected (pFg) seedlings. Total protein (input) from *Arabidopsis* seedlings expressing cytoplasmic BirA* (Figure 7A) or membrane-bound BirA* (HopF2-BirA*; Figure 7B) was extracted in denaturing conditions and purified by affinity purification with streptavidin beads. The ability of streptavidin beads to purify and isolate biotinylated proteins from total protein lysates was confirmed by immunoblot analysis with streptavidin-HRP antibodies. Immunoblot analysis confirmed the lack of BirA* in control treatments (*Col-0* and transgenic *Arabidopsis* grown without DEX; Appendix A, Figure 2).

Affinity purification successfully enriched biotinylated proteins in cytoplasmic (BirA*; Figure 7A) and membrane (HopF2-BirA*; Figure 7B) fractions. Biotinylated proteins were detected in both total protein lysate (input) and affinity purified protein (bound), with the highest biotinylation levels seen in the bound fraction (Figure 7). No biotinylated proteins were detected in the unbound fraction (supernatant from affinity purification; Figure 7), confirming that the majority of biotinylated proteins were captured in the bound fraction. BirA* expression was confirmed for all fractions by immunoblot with HA antibodies (Figure 7).

These results show that affinity purification successfully enriched biotinylated proteins from *Arabidopsis* seedlings in both cellular fractions, for *F. graminearum*-infected and non-infected treatments (Figure 7).

Figure 7: Confirmation of affinity purification enrichment of biotinylated proteins by immunoblot analysis for (A) cytoplasmic (BirA*) or (B) membrane (HopF2) fractions. Biotinylated proteins from 60 hpi non-infected (mFg) and infected (pFg) *Arabidopsis* seedlings grown in 1 mM of exogenous biotin were detected via streptavidin-HRP antibodies (top panel). Biotinylated proteins were detected in total protein extract (input) and at higher levels in purified biotinylated proteins (bound). No biotinylated proteins were detected in the affinity purification supernatant fraction (unbound), indicating affinity purification successfully captured the majority of biotinylated proteins. BirA* activity was confirmed by immunoblot with HA antibodies for all fractions (middle panel) and is similar across all three affinity purification fractions. Ponceau S stain (bottom panel) was used to confirm protein transfer. Protein in the bound fraction was not detected with Ponceau S stain.

2.3.5 Mass Spectrometry Analysis of Affinity Purified Biotinylated Proteins by BioID Reveals Putative Secreted *F. graminearum* Proteins

Biotinylated proteins from non-infected and infected *Arabidopsis* seedlings isolated by affinity purification were sampled at three time-points (24 hpi, 48 hpi, and 60 hpi) and analyzed by mass spectrometry analysis (Morden Research and Development Centre, AAFC). Analyses were performed for three replicates per treatment, and Mascot outputs for all treatments can be found in External Files Tables S3-S10. All experimental treatments and controls are described in Appendix A, Table 2.

In total, 351 *F. graminearum* proteins were identified from mass spectrometry analysis (External Files, Table S1). After applying the filters outlined in Figure 4, 99 *F. graminearum* proteins were considered to be candidate effector proteins (CEPs) and potentially secreted proteins with a role in virulence (Table 4).

2.3.6 Structural Properties of Candidate Effector Proteins

The 99 CEPs were further analyzed for structural properties with SignalP 5.0, TargetP 1.1, and EffectorP 1.0 and 2.0 to determine presence of a signal peptide, their cellular localization, and predicted as a fungal effector. In addition, the size and cysteine content of CEPs was also examined. Our analyses revealed that only four of the ninety-nine CEPs were predicted to have a signal peptide by SignalP 5.0: FGSG_02077, FGSG_04527, FGSG_09366, and FGSG_03569 (Table 4). Four proteins were predicted by TargetP 1.1 to be in the secretory pathway, and three of these overlapped with SignalP predictions: FGSG_04527, FGSG_02077, and FGSG_03569 (Table 4). The other protein predicted to be in the secretory pathway by TargetP is FGSG_01111 (Table 4). No

Table 4: Candidate effector proteins (CEPs) identified by BioID and LC-MS/MS

Accession	Function	Pfam Domain	Infection Time Point	Cellular Fraction	Effector P 1.0	Effector P 2.0	Signal P 5.0	Target P 1.1	Protein Size (aa)	Cysteine Residues (%)
FGSG_00330	Acetyl-coenzyme A synthetase	AMP-binding	48 Hrs, 60 Hrs	Cytoplasm and membrane	No	No	No	Other location	712	0.8
FGSG_00421	S-adenosylmethionine synthetase	S-adenosylmethionine synthetase, C-term domain	60 Hrs	Cytoplasm	No	No	No	Other location	402	1
FGSG_00496	Inorganic pyrophosphatase	Inorganic pyrophosphatase	60 Hrs	Cytoplasm and membrane	No	No	No	Other location	364	1.4
FGSG_00631	40S ribosomal protein S8-B	Ribosomal protein S8e	60 Hrs	Cytoplasm	No	No	No	Other location	206	0
FGSG_00777	Peptidyl-prolyl cis-trans isomerase, mitochondrial (cyclophilin B)	Cyclophilin type peptidyl-prolyl cis-trans isomerase/CLD	60 Hrs	Cytoplasm and membrane	Yes	Yes	No	Other location	228	0.9
FGSG_00838	Heat shock protein 70	Hsp70 protein	60 Hrs	Cytoplasm and membrane	No	No	No	Other location	653	0.6
FGSG_00921	Heat shock protein SSB1	Hsp 70	48 Hrs, 60 Hrs	Cytoplasm and membrane	No	No	No	Other location	614	1
FGSG_00979	Aldehyde dehydrogenase	Aldehyde dehydrogenase family	48 Hrs, 60 Hrs	Cytoplasm and membrane	No	No	No	Other location	492	1.6
FGSG_01016	Probable ribosomal protein L6.e.B, cytosolic	Ribosomal protein L6e	60 Hrs	Cytoplasm and membrane	No	No	No	Other location	222	0
FGSG_01081	60S ribosomal protein L11	ribosomal L5P family C-terminus	60 Hrs	Cytoplasm	No	No	No	Other location	173	1.2

FGSG_01111	6-phosphogluconate dehydrogenase	6-phosphogluconate dehydrogenase, C-term domain	48 Hrs, 60 Hrs	Cytoplasm and membrane	No	No	No	Secretory	492	1.2
FGSG_01154	60S ribosomal protein l9-B	Ribosomal protein L6e	60 Hrs	Cytoplasm	Yes	No	No	Other location	250	1.2
FGSG_01238	Probable 60S ribosomal protein L36	Met-10+ like-protein	60 Hrs	Cytoplasm	Yes	No	No	Other location	456	1.1
FGSG_01346	Enolase	Enolase, C-term TIM barrel domain	60 Hrs	Cytoplasm	No	No	No	Other location	352	0.9
FGSG_01422	Citrate synthase	Citrate synthase, C-term domain	48 Hrs, 60 Hrs	Cytoplasm and membrane	No	No	No	Mitochondria	470	0.6
FGSG_01425	Plasma membrane ATPase	E1-E2 ATPase	48 Hrs, 60 Hrs	Cytoplasm and membrane	No	No	No	Other location	922	1.4
FGSG_01433	Glutamate synthase precursor	Glutamine amidotransferases class-II	60 Hrs	Cytoplasm and membrane	No	No	No	Other location	2113	1.8
FGSG_01504	60S ribosomal protein L31	Ribosomal protein L31e	60 Hrs	Cytoplasm	Yes	Yes	No	Other location	123	0
FGSG_01516	60S ribosomal protein L20	Ribosomal proteins 50S-L18Ae/60S-L20/60S-L18A	60 Hrs	Cytoplasm	No	No	No	Other location	176	0
FGSG_01591	Phosphate carrier protein	mitochondrial carrier protein	60 Hrs	Cytoplasm	No	No	No	Other location	369	1.1
FGSG_01871	60S ribosomal protein L17	Ribosomal protein L22p/L173	60 Hrs	Cytoplasm	Yes	No	No	Mitochondrial	185	0.5
FGSG_01962	Argininosuccinate synthase	Argininosuccinate synthase	60 Hrs	Membrane	No	No	No	Other location	406	2.2
FGSG_02077	Hypothetical protein	CFEM domain	60 Hrs	Membrane	No	No	Yes	Secretory	184	4.3
FGSG_02461	Malate dehydrogenase	Lactate/malate dehydrogenase, alpha/beta C-term domain	60 Hrs	Cytoplasm	No	Yes	No	Mitochondrial	336	0

FGSG_02473	Probable mitochondrial ATP synthase subunit f	Mitochondrial F1-F0 ATP synthase subunit F of fungi	60 Hrs	Cytoplasm	Yes	No	No	Mitochondrial	96	0
FGSG_02523	Probable IgE-dependant histamine-releasing factor	Translationally controlled tumour protein	60 Hrs	Cytoplasm	No	No	No	Other location	170	0.6
FGSG_02541	40S ribosomal protein S14		60 Hrs	Cytoplasm and membrane	No	No	No	Other location	150	0.7
FGSG_02542	Probable 60S ribosomal protein L7 subunit	Ribosomal L30 N-term domain	60 Hrs	Cytoplasm and membrane	No	No	No	Other location	258	0.4
FGSG_02937	Hypothetical protein	Alpha/beta hydrolase fold	60 Hrs	Membrane	No	No	No	Other location	574	1.9
FGSG_03569	Galactose oxidase precursor	Domain of unknown function	60 Hrs	Cytoplasm	No	No	Yes	Secretory	679	1.2
FGSG_04181	Elongation factor 3	ABC transporter	48 Hrs, 60 Hrs	Cytoplasm and membrane	No	No	No	Other location	1055	1.4
FGSG_04312	Probable H ⁺ -transporting ATP synthase beta chain	ATP synthase alpha/beta family, nucleotide-binding domain	48 Hrs, 60 Hrs	Cytoplasm and membrane	No	No	No	Mitochondria	649	1.8
FGSG_04458	Nitric oxide dioxygenase	Oxidoreductase NAD-binding domain	60 Hrs	Cytoplasm and membrane	No	No	No	Other location	415	0.7
FGSG_04476	Probable 40S ribosomal protein S24	Ribosomal protein S24e	60 Hrs	Cytoplasm	No	No	No	Other location	136	0
FGSG_04527	Carboxypeptidase S1	Serine carboxypeptidase	60 Hrs	Cytoplasm and membrane	No	No	Yes	Secretory	473	1.9
FGSG_04826	L-xylulose reductase	Enoyl-(Acyl carrier protein) reductase	60 Hrs	Cytoplasm	Yes	Yes	No	Other location	266	1.1

FGSG_05035	Pyridoxine biosynthesis protein PDX1	SOR/SNZ family	60 Hrs	Cytoplasm	No	No	No	Other location	311	1.9
FGSG_05433	40S ribosomal protein S3	Ribosomal protein S3, C-term domain	60 Hrs	Cytoplasm and membrane	Yes	No	No	Other location	260	1.2
FGSG_05493	40S ribosomal protein S20	Ribosomal protein	60 Hrs	Membrane	Yes	No	No	Other location	116	0.9
FGSG_05615	Adenosylhomocysteinase	S-adenosyl-L-homocysteine hydrolase	48 Hrs, 60 Hrs	Cytoplasm	No	No	No	Other location	449	2.4
FGSG_05972	Nucleoside diphosphate kinase	Nucleoside diphosphate kinase	48 Hrs, 60 Hrs	Cytoplasm and membrane	No	Yes	No	Other location	238	1.3
FGSG_05997	Udp-galactopyranose mutase	Flavin containing amine oxidoreductase	60 Hrs	Membrane	No	No	No	Other location	516	1.2
FGSG_06019	40S ribosomal protein S5-B	Ribosomal protein S7p/S5e	48 Hrs, 60 Hrs	Cytoplasm and membrane	Yes	Yes	No	Other location	213	1.4
FGSG_06020	Probable ribosomal protein S25.e.c7	S25 ribosomal protein	48 Hrs, 60 Hrs	Cytoplasm and membrane	Yes	Yes	No	Other location	94	1.1
FGSG_06021	ADP, ATP carrier protein	mitochondrial carrier protein	48 Hrs, 60 Hrs	Cytoplasm and membrane	No	No	No	Other location	311	0.3
FGSG_06246	Heat shock protein 60	TCP-1/cpn60 chaperonin family	60 Hrs	Cytoplasm	No	No	No	Mitochondrial	587	0.7
FGSG_06270	Probable 40S ribosomal protein s10-b	plectin/S10 domain	60 Hrs	Cytoplasm	Yes	No	No	Other location	169	0
FGSG_06289	60S ribosomal protein L3	Ribosomal protein L3	48 Hrs	Cytoplasm	No	No	No	Mitochondria	391	0.5
FGSG_06290	Serine hydroxymethyltransferase	Serine hydroxymethyltransferase	48 Hrs, 60 Hrs	Cytoplasm and membrane	No	No	No	Other location	484	1.4

FGSG_06384	Probable COPII coated vesicle component SEC24	Sec23/Sec24 trunk domain	60 Hrs	Membrane	No	No	No	Other location	943	1.5
FGSG_06407	Probable 40S ribosomal protein S27-2		60 Hrs	Cytoplasm	Yes	Yes	No	Other location	82	7.3
FGSG_06566	Succinate/fumarate transporter	mitochondrial carrier protein	60 Hrs	Cytoplasm and membrane	No	No	No	Other location	322	0.9
FGSG_06893	Probable RPS18A-ribosomal protein S18.e.c4	Ribosomal protein S13/S18	60 Hrs	Cytoplasm and membrane	No	No	No	Other location	156	0.6
FGSG_06924	60S ribosomal protein		60 Hrs	Membrane	Yes	Yes	No	Other location	217	0.9
FGSG_07048	40S ribosomal protein S13-1	Ribosomal S13/S15 N-term domain	60 Hrs	Cytoplasm	Yes	No	No	Mitochondrial	151	0
FGSG_07186	60S ribosomal protein L4-A	Ribosomal protein L4/L1 family	60 Hrs	Cytoplasm and membrane	No	No	No	Other location	352	0.9
FGSG_07401	Elongation factor 1-gamma 1	Elongation factor 1 gamma, conserved domain	60 Hrs	Cytoplasm and membrane	No	No	No	Other location	425	0.7
FGSG_07457	Related to rRNA processing protein EBP2	Eukaryotic rRNA processing protein EBP2	60 Hrs	Membrane	No	No	No	Other location	418	0
FGSG_07480	40S ribosomal protein S3aE	Ribosomal S3Ae family	60 Hrs	Cytoplasm	No	No	No	Other location	256	0.8
FGSG_07856	60S ribosomal protein L28	Ribosomal proteins 50S-L15, 50S-L18e, 60S-L27A	60 Hrs	Cytoplasm	No	No	No	Other location	149	0
FGSG_07953	Aconitate hydratase	Aconitase family (aconitate hydratase)	48 Hrs, 60 Hrs	Cytoplasm and membrane	No	No	No	Mitochondria	788	1.3
FGSG_08335	Probable ribosomal protein L13B	Ribosomal protein L13e	60 Hrs	Membrane	No	No	No	Other location	212	0.5

FGSG_08478	60S ribosomal protein L8	Ribosomal protein L7Ae/L30e/S12e/Gadd45 family	60 Hrs	Cytoplasm	No	No	No	Other location	262	0.4
FGSG_08561	60S ribosomal protein L14-B	Ribosomal protein L14	60 Hrs	Cytoplasm	Yes	No	No	Other location	147	0
FGSG_08601	Phosphoenolpyruvate carboxykinase	Phosphoenolpyruvate carboxykinase	48 Hrs, 60 Hrs	Cytoplasm and membrane	No	No	No	Other location	439	1.5
FGSG_08676	60S ribosomal protein L24	Ribosomal protein L24e	60 Hrs	Cytoplasm	No	No	No	Other location	160	0
FGSG_08700	Malate synthase	Malate synthase	48 Hrs, 60 Hrs	Cytoplasm and membrane	No	No	No	Other location	542	0.6
FGSG_08714	40S ribosomal protein S4	Ribosomal family S4e	60 Hrs	Cytoplasm	No	No	No	Other location	261	0.4
FGSG_08737	Probable woronin body major protein precursor	None	48 Hrs, 60 Hrs	Cytoplasm	No	No	No	Other location	588	0.7
FGSG_08811	Elongation factor alpha	Elongation factor Tu GTP binding domain	48 Hrs, 60 Hrs	Cytoplasm and membrane	No	No	No	Other location	460	1.3
FGSG_08895	Probable ribosomal protein L21.e.A, cytosolic	Ribosomal protein	60 Hrs	Cytoplasm	Yes	Yes	No	Other location	160	0
FGSG_08896	40S ribosomal protein S9	S4 domain	60 Hrs	Cytoplasm and membrane	No	No	No	Mitochondrial	191	0.5
FGSG_09366	Glucan 1,3-beta-glucosidase	Glycosyl hydrolases family 17	60 Hrs	Membrane	No	No	Yes	Other location	461	0.4
FGSG_09454	probable kap95 protein	HEAT repeat	60 Hrs	Membrane	No	No	No	Other location	875	1.4
FGSG_09574	Elongation factor 2	Elongation factor Tu GTP binding domain	60 Hrs	Cytoplasm and membrane	No	No	No	Other location	844	0.6

FGSG_09690	Probable peptidylprolyl isomerase (FK506-binding protein homolog)	FKBP-type peptidyl-prolyl cis-trans isomerase	60 Hrs	Cytoplasm and membrane	Yes	Yes	No	Mitochondrial	146	0
FGSG_09866	Probable ribosomal protein L18, cytosolic	Ribosomal protein 60S L18 and 50S L18e	60 Hrs	Cytoplasm	No	No	No	Other location	184	0.5
FGSG_09870	Probable CPC2 protein	WD domain, G-beta repeat	60 Hrs	Cytoplasm and membrane	No	No	No	Other location	333	3.3
FGSG_09896	Isocitrate lyase	Isocitrate lyase family	48 Hrs, 60 Hrs	Cytoplasm	No	No	No	Other location	546	1.1
FGSG_09933	Probable mitochondrial porin	Eukaryotic porin	60 Hrs	Cytoplasm and membrane	No	No	No	Other location	352	0
FGSG_10010	60S ribosomal protein L5	Ribosomal large subunit proteins 60S L5, and 50S L18	60 Hrs	Cytoplasm	No	No	No	Other location	301	0.3
FGSG_10118	Ketol-acid reductoisomerase	Acetohydroxy acid isomeroreductase, NADPH-binding domain	60 Hrs	Cytoplasm and membrane	No	No	No	Mitochondrial	405	0.7
FGSG_10246	60S ribosomal protein L10-B	Ribosomal protein L16p/L10e	60 Hrs	Cytoplasm	No	No	No	Other location	221	2.3
FGSG_10267	C-1-tetrahydrofolate synthase	Formate-tetrahydrofolate ligase	60 Hrs	Membrane	No	No	No	Other location	923	0.8
FGSG_10549	Hypothetical protein	None	60 Hrs	Cytoplasm	Yes	Yes	No	Other location	178	4.5
FGSG_10746	Aspartate aminotransferase	Aminotransferase class I and II	48 Hrs, 60 Hrs	Cytoplasm	No	No	No	Mitochondria	422	0.7
FGSG_10825	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	Cobalamin-independent synthase, catalytic domain	48 Hrs, 60 Hrs	Cytoplasm and membrane	No	No	No	Other location	766	0.5

FGSG_10855	Alcohol dehydrogenase 1	Alcohol dehydrogenase GroES-like domain	48 Hrs, 60 Hrs	Cytoplasm and membrane	No	No	No	Other location	353	2.5
FGSG_10905	40S ribosomal protein S0	Ribosomal protein S2	60 Hrs	Cytoplasm	No	No	No	Other location	294	0.7
FGSG_10942	Hypothetical protein	Ribosomal protein S26e	60 Hrs	Cytoplasm	Yes	Yes	No	Other location	142	5.6
FGSG_10974	Hypothetical protein	Domain of unknown function	60 Hrs	Membrane	Yes	Yes	No	Other location	171	0.6
FGSG_11627	Probable HTA2-histone H2A.2	C-terminus of histone H2A	60 Hrs	Cytoplasm	Yes	Yes	No	Other location	134	0
FGSG_12582	Related to sterol glucosyltransferase	SnoaL-like domain	48 Hrs	Cytoplasm	No	No	No	Mitochondria	437	0.5
FGSG_12851	Allantoicase		60 Hrs	Cytoplasm	No	No	No	Other location	359	2.5
FGSG_15824	ATP synthase subunit alpha	ATP synthase alpha/beta family, nucleotide-binding domain	48 Hrs, 60 Hrs	Cytoplasm and membrane	No	No	No	Mitochondria	552	0.4
FGSG_16615	Hypothetical protein	PPR repeat family	60 Hrs	Membrane	No	No	No	Other location	1207	0.3
FGSG_16627	Glyceraldehyde-3-phosphate dehydrogenase	Glyceraldehyde 3-phosphate dehydrogenase, C-term domain	60 Hrs	Cytoplasm and membrane	No	Yes	No	Other location	338	0.6
FGSG_16707	Hypothetical protein	None	60 Hrs	Membrane	Yes	No	No	Other location	487	1.2
FGSG_17593	Probable translation activator GCN1	Generalcontrol nonderepressible 1 (Gcn1) N-term	60 Hrs	Cytoplasm	Yes	No	No	Other location	2858	0.8

proteins were predicted to be targeted to either the nucleus or chloroplasts.

EffectorP is a machine-learning algorithm used for fungal effector prediction (Sperschneider et al., 2018, 2016). Twenty-four proteins were predicted as effectors by EffectorP 1.0, while 16 proteins were predicted to be effectors by EffectorP 2.0 (probability > 0.55) (Table 4). Thirteen are predicted by both EffectorP 1.0 and 2.0, which provides a higher prediction confidence (Table 4; Sperschneider et al., 2018). None of the proteins predicted by SignalP or TargetP overlapped with those predicted by EffectorP.

A size less than 300 amino acids and high cysteine content are other indicators of effectors. Proteins are considered as cysteine-rich proteins when they contain 2-20% cysteine residues, and typically have an even number of cysteines that correlates to the formation of disulphide bridges. Just under half (44%) of the CEPs were smaller than 300 amino acids (Table 4). The smallest protein identified was 82 aa in size, while the largest was over 2800 aa (Table 4). Ten CEPs have a cysteine content of greater than 2%, with the highest cysteine content at 7.3%. Of these, the top 3 CEPs with the highest cysteine content were also predicted by both EffectorP 1.0 and 2.0: FGSG_06407, FGSG_10942, and FGSG_10549 (Table 4). FGSG_02077 has the fourth-highest cysteine content, with a predicted signal peptide, and is targeted to the secretory pathway (Table 4).

In summary, we did not find a single CEP that comprised all of the examined structural properties. However, some of the CEPs did contain a number of structural properties. FGSG_10942, FGSG_10549, and FGSG_06407 were predicted by both versions of EffectorP, have a cysteine content above 2%, and are smaller than 300 aa.

Interestingly, two of these proteins were annotated as ribosomal, while FGSG_10549 is a hypothetical protein. Only one of the proteins predicted to have a signal peptide was small and cysteine-rich: FGSG_02077, with a size of 184 aa and a cysteine content of 4.3% (Table 4).

2.3.7 Identities and Functional Categories of Candidate Effector Proteins

In order to further characterize CEPs, their functions and domains were analyzed through FungiDB (<https://fungidb.org/fungidb/>) or Pfam (<https://pfam.xfam.org/>), respectively (Table 4). The majority of CEPs had an annotation or a predicted domain. Only three CEPs FGSG_10549, FGSG_10974, and FGSG_16707 were hypothetical with no predicted Pfam domain or annotated function. Thirty-six proteins were predicted to be ribosomal proteins, and one protein was predicted to be a histone protein (Table 4). Three CEPs were annotated as heat shock proteins (Table 4). Over 30 CEPs had metabolic function, including proteins annotated as hydratases, synthases, transferases, and dehydrogenases (Table 4).

The 99 CEPs were analyzed for enrichment of functional categories through FungiFun2 (<https://sbi.hki-jena.de/fungifun/>; Priebe et al., 2015). FungiFun2 assigns functional categories to fungal proteins based on either Functional Catalogue (FunCat; Ruepp et al., 2004), Gene Ontology (GO; Ashburner et al., 2000), or the Kyoto Encyclopedia of Genes and Genomes (KEGG; Kanehisa & Goto, 2000) and presents significantly enriched functional categories from an input of proteins. Analysis via FunCat showed that 14 distinct functional categories were significantly enriched (Figure 8A). The majority of CEPs (over 75%) were assigned to categories involving translation, ribosomal proteins, and ribosome biogenesis (Figure 8A). When ribosomal proteins were

A

B

Figure 8: FunCat analysis of CEPs via FungiFun2. **A:** Functional categories of all 99 CEPs. 14 categories were significantly enriched, of which the top three categories are translation, ribosomal proteins, and ribosome biogenesis. **B:** functional categories of CEPs with ribosomal proteins removed, leaving 63 proteins. The top three categories are C-1 compound anabolism, C-2 compound and organic acid catabolism, and translation elongation.

Removed from the analysis, the majority of remaining CEPs were assigned to the categories of C-1 compound (single carbon molecules) anabolism, C-2 (two carbon molecules) compound and organic acid catabolism, and translation elongation (Figure 8B). Overall, analysis showed enrichment of translation (Figure 8A) and primary metabolism (Figure 8B).

2.3.8 Comparative Analysis of Candidate Effector Proteins with Published Literature

CEPs were compared with previously published literature on *F. graminearum* secretomes/proteomes (Brown et al., 2012; Fabre et al., 2019; Ji et al., 2013; Lu & Edwards, 2016; Lysenko et al., 2013; Paper et al., 2007; Phalip et al., 2005; Rampitsch et al., 2013; Taylor et al., 2008; Yang et al., 2012; W. Zhou et al., 2006) and transcriptomes (Brown et al., 2017; Lysøe et al., 2011; Pan et al., 2018; Qinhu Wang et al., 2017). In total, twelve proteome datasets and four transcriptome datasets were used for comparative analysis. Details on the literature used can be found in Table 2 for proteome datasets and Table 3 for transcriptome datasets. Each CEP was tabulated a score based on their presence in a dataset. If a CEP was present in a single dataset it was assigned a score of 1. Table 5 describes a summary of the top proteins identified across all datasets (Table 5A), top CEPs in proteome datasets (Table 5B), and top proteins in transcriptome datasets (Table 5C). CEP presence across all datasets can be found in External Files, Table S2.

The examination of all 16 secretome, proteome, and transcriptome datasets revealed that all CEPs were identified in at least one protein or transcript dataset.

Specifically, FGSG_09366 (glycosyl hydrolase domain) was present in the most datasets (10 out of 16; Table 5A), followed by FGSG_01346, FGSG_08811, FGSG_09690, and FGSG_04826, present in 9 of the 16 datasets (Table 5A).

Analyses of the proteome datasets alone showed that FGSG_09366 was identified in most of them (8 out of 12; Table 5B), and FGSG_04826 (L-xylulose reductase) was present in 6 of 12 proteome data sets (Table 5B). Six CEPs were not identified in any of the proteome datasets, and no CEPs were present in all twelve proteome datasets (External Files Table S2). The proteome dataset from Fabre et al. (2019) and the exoproteome generated by the Subramaniam lab had the largest overlaps with the identified CEPs, at 83% and 88% respectively (External Files Table S2). No other protein datasets had coverage of the CEPs above 40%; 34% of CEPs were found in the proteome by Taylor et al. (2008) and 12% of CEPs were found in the secretome by Paper et al. (2007) (External Files Table S2).

The four transcriptome datasets examined differentially expressed genes (>2-fold change) and were compared to the list of CEPs (External Files Table S2). This comparison would indicate CEPs that may be differentially regulated during infection and may contribute to pathogenicity. The examination of transcriptome datasets identified 27 CEPs in all four transcriptomic datasets (Table 5C).

Specifically, 89% of CEPs were identified in the transcriptome data from Pan et al. (2018), while 75% of CEPs were identified in the dataset from Lysøe et al. (2011) (External Files, Table S2). Only one CEP, FGSG_10267, was not identified in any of the transcriptome dataset, but was found in one proteome dataset (External Files, Table S2).

Table 5: Top candidate effector proteins present in previously published datasets

A: Top CEPs in All Datasets		B: Top CEPs in Proteomic Datasets			C: Top CEPs in Transcriptomic Datasets		
Accession	Score (out of 16)	Accession	Score (out of 12)	References	Accession	Score (out of 4)	References
FGSG_09366	10	FGSG_09366	8	Fabre et al., 2019; Ji et al., 2013; Paper et al., 2007; Phalip et al., 2005; Rampitsch et al., 2013; Taylor et al., 2008; F. Yang et al., 2012; exoproteome	FGSG_01346	4	Brown et al., 2017; Lysøe et al., 2011; Pan et al., 2018; Qinhua Wang et al., 2017
FGSG_01346	9	FGSG_04826	6	Fabre et al., 2019; Paper et al., 2007; Rampitsch et al., 2013; Taylor et al., 2008; F. Yang et al., 2012; exoproteome	FGSG_08811	4	
FGSG_04826	9	FGSG_01346	5	Fabre et al., 2019; Paper et al., 2007; Rampitsch et al., 2013; Taylor et al., 2008; exoproteome	FGSG_09690	4	
FGSG_08811	9	FGSG_08811	5	Fabre et al., 2019; Lysenko et al., 2013; Rampitsch et al., 2013; Taylor et al., 2008; exoproteome	FGSG_10825	4	
FGSG_09690	9	FGSG_09690	5	Fabre et al., 2019; Lysenko et al., 2013; F. Yang et al., 2012; W. Zhou et al., 2006b; exoproteome	FGSG_01111	4	
FGSG_02077	8	FGSG_02077	5	Brown et al., 2012; Ji et al., 2013; Lu & Edwards, 2016; Rampitsch et al., 2013; exoproteome	FGSG_02523	4	

FGSG_10825	8	FGSG_00777	5	Fabre et al., 2019; Paper et al., 2007; Taylor et al., 2008; F. Yang et al., 2012; exoproteome	FGSG_07048	4	
FGSG_00777	7	FGSG_04527	5	Brown et al., 2012; Fabre et al., 2019; Ji et al., 2013; F. Yang et al., 2012; exoproteome	FGSG_07401	4	
FGSG_01111	7	FGSG_08737	5	Fabre et al., 2019; Lysenko et al., 2013; Taylor et al., 2008; W. Zhou et al., 2006b; exoproteome	FGSG_09870	4	
FGSG_02461	7	FGSG_10825	4	Fabre et al., 2019; Lysenko et al., 2013; Paper et al., 2007; exoproteome	FGSG_01433	4	
FGSG_02523	7	FGSG_02461	4	Fabre et al., 2019; Paper et al., 2007; Taylor et al., 2008; exoproteome	FGSG_01516	4	
FGSG_04527	7	FGSG_00979	4		FGSG_02542	4	
FGSG_07048	7	FGSG_05972	4		FGSG_04476	4	
FGSG_07401	7	FGSG_00496	4	Fabre et al., 2019; Lysenko et al., 2013; Taylor et al., 2008; exoproteome	FGSG_05433	4	
FGSG_09870	7	FGSG_06021	4		FGSG_05493	4	
		FGSG_11627	4		FGSG_06924	4	
		FGSG_00838	4	Fabre et al., 2019; Lysenko et al., 2013; Rampitsch et al., 2013; exoproteome	FGSG_07186	4	
					FGSG_08561	4	
					FGSG_08676	4	
					FGSG_08700	4	
					FGSG_08714	4	
					FGSG_08895	4	
					FGSG_09574	4	
					FGSG_10010	4	
					FGSG_17593	4	
					FGSG_08601	4	
					FGSG_09896	4	

When we combined the structural analysis of CEPs with their presence in the published literature, FGSG_09366 was present in the most datasets (10 of 16), and has a predicted signal peptide (Table 4 and Table 5A). FGSG_04826 was present in 9 of 16 datasets, was predicted by both EffectorP 1.0 and 2.0, and was smaller than 300 aa (Table 4 and Table 5A). Overall, we did not observe clear parallels between structural properties and the presence of CEPs in the proteome/transcriptome datasets.

The Subramaniam lab recently developed a Fusarium Network of Trichothecene Associated Proteins, also known as the FuNTAP (Mirmiran, 2018; Mirmiran et al., 2018). The FuNTAP is a network that shows interactions among proteins associated with the trichothecene cluster in *F. graminearum*, which is involved in biosynthesis of the mycotoxin DON (Figure 9A). Proteins in the FuNTAP can be scored by their degree, which represents the number of interactors they have in the network. For example, large hubs (represented by larger circles) have higher numbers of interactors and thus a larger degree (Figure 9A). The protein with the highest degree in the FuNTAP is FGSG_01511 with a degree of 55, meaning it has 55 interactors. It is also one of the largest hubs, (pink circle) indicating it plays a crucial role in the network (Mirmiran, 2018). Five of the CEPs were present in the FuNTAP (Figure 9B). None of the CEPs have a high degree, however two CEPs, FGSG_06924 and FGSG_09870, interact with a larger hub. The three other proteins FGSG_04826, FGSG_08811, and FGSG_10855 are on the periphery of the network with few interactions to large hubs. FGSG_04826 and FGSG_06924 were predicted by both EffectorP 1.0 and 2.0 (Table 4), while FGSG_04826 and FGSG_08811 were both present in 9 of 16 published datasets (Table 5A).

B

Accession	Function	Degree
FGSG_04826	L-xylulose reductase	4
FGSG_06924	60S ribosomal protein L10a	5
FGSG_08811	Elongation factor 1-alpha	3
FGSG_09870	Guanine nucleotide-binding subunit	4
FGSG_10855	Alcohol dehydrogenase 1	7

Figure 9: Five CEPs were present in the Fusarium Network of Trichothecene Associated Proteins (FuNTAP). **A:** FuNTAP network map, with arrows notating the locations of the five CEPs within the network. **B:** Five CEPs identified in FuNTAP, including their degree that represents the number of interactions.

2.3.9 Temporal Patterns of Candidate Effector Proteins during *F. graminearum* Infection in *Arabidopsis* Seedlings

The presence of CEPs in a certain cellular fraction and their temporal expression can imply their potential function. The occurrence of CEPs in the membrane or cytoplasmic fractions is represented as a Venn diagram in Figure 10A, while the occurrence of CEPs at different points of infection is represented as a Venn diagram in Figure 10B.

Among the 99 CEPs identified, 44 proteins were identified only in the cytoplasmic fraction (BirA*) and 14 proteins were unique to the membrane-bound fraction (HopF2-BirA*) (Figure 10A, Table 4). Less than half (41%) of the CEPs were found in both cellular fractions (Figure 10A, Table 4).

With respect to infection time points, the majority (73%) of CEPs were identified at only the 60 hpi time-point, with 24 proteins identified in both the 60 hpi and the 48 hpi fractions (Figure 10B, Table 4). Only 2 proteins were identified as unique to the 48 hpi fraction (Figure 10B, Table 4). No CEPs were identified in the 24 hpi (Figure 10B, Table 4). Although *F. graminearum* proteins were identified at this time point (External Files Table S3), they did not pass the filters applied as described in Figure 4.

Figure 10: Time points and cellular locations distributions of 99 CEPs identified by mass spectrometry. **A:** Proteins identified in two cellular locations: CEPs present in the cytoplasmic fraction alone (44), in the membrane fraction alone (14), or detected in both (41). **B:** Proteins identified at three infection time-points: 2 proteins were only identified at 48 hpi, while 73 were only detected at 60 hpi. No proteins were detected at all three time points, while 24 proteins were detected at both 48 hpi and 60 hpi. Numbers in brackets represent total proteins in each group.

2.3.10 Localization of Candidate Effector Proteins on *F. graminearum*

Chromosomes

Effectors are typically located in genomic regions with high recombination frequency and single nucleotide polymorphism (SNP) occurrence (Raffaele & Kamoun, 2012). CEPs were mapped to the *F. graminearum* genome using the Fgra3Map software (<http://www.omnimapfree.org/downloads/fgramap/>). CEPs are located on all four chromosomes and are distributed evenly across the genome (Figure 11, CEPs).

Regions of high recombination and SNP-dense regions were aligned with the location of each CEP on each chromosome. Regions of high recombination (Figure 11, HR) and SNPs (Figure 11, SNPs) are located primarily on chromosome ends, while some are found in centromeric regions. Only a small number of CEPs were present in regions of high recombination and high SNP density. This included FGSG_10549 on chromosome 1, FGSG_02937, FGSG_03569, FGSG_04476, and FGSG_04527 on chromosome 2, FGSG_12582 and FGSG_04826 on chromosome 3, and FGSG_06566 on chromosome 4 (Figure 11, CEPs).

FGSG_10549 and FGSG_04826 were identified by EffectorP 1.0 and 2.0 and are both smaller than 300 amino acids (Table 4). FGSG_04826 is also in 9 of 16 datasets (Table 5A). FGSG_03569 and FGSG_04527 were predicted to be secreted but are both larger than 300 aa and have a low cysteine content. FGSG_03569 was present in few datasets, but FGSG_04527 was present in 7 of 16 datasets.

In summary, these results showcase several CEPs that, based on their structural properties, occurrence in published datasets, and genomic location, may point to their identity as true effector proteins.

Figure 11: Genomic location of 99 CEPs on the four *F. graminearum* chromosomes, indicated by green boxes (CEPs). Chromosomes were aligned with regions of high recombination (HR), from low recombination (blue) to high recombination (red). Chromosomes were also aligned with SNP density (SNPs), from low SNP density (pink/yellow) to high density (orange/red).

Chapter 3: Functional Characterization of Candidate Effector Proteins from *F. graminearum*

3.1 Summary

In order to verify that the *F. graminearum* proteins identified *in planta* using BioID are effector proteins, their localization and function in the host must be confirmed. If the CEPs are shown to have a role in virulence and are confirmed to associate with the host through secretion, then this further supports their identity as effector proteins.

Several methods can be used to elucidate effector function, including identifying protein interactors, localization, gene expression assays, knock-out studies, enzymatic assays, or determining protein structure via crystallography. Since many CEPs were identified in Chapter 2, we were interested to develop a high-throughput assay to determine the function of CEPs.

Effectors modulate all aspects of plant defense and interfere with both PTI and ETI pathways. As such, changes to these pathways can be monitored by various assays. Cell death-based assays are often used to determine the role of an effector. For example, a Bax-induced cell death system is often used in plants to determine the effect of protein expression on induced cell death (Abramovitch et al., 2003; Z. Li et al., 2015; Qunqing Wang et al., 2011). Briefly, an effector can be determined to have a role in virulence if its expression suppresses Bax-induced cell death. However, this assay is usually performed in heterologous systems, and therefore does not truly reflect the role of effectors in their native host. As well, the assay design may not be sensitive enough to allow detection of minor phenotypic changes caused by effectors.

In my thesis, we used an assay recently developed by Dr. Guus Bakkeren (Unpublished; AAFC-Summerland) to test CEP function in wheat, a native host of *F. graminearum*. The assay was used to functionally characterize a subset of the CEPs identified in Chapter 2. The assay uses the barley smut fungus *Ustilago hordei* transformed with a protein expression vector that delivers CEPs into wheat leaves. *U. hordei* is a fungal pathogen of barley, but a non-host pathogen on wheat (Gaudet et al., 2010). The assays used will test: 1) the ability of a CEP expressed in wheat leaves to induce visible cell death and 2) the ability of a CEP expressed in wheat leaves to suppress or activate defense responses.

The first assay (CEP cell death assay) was used to monitor cell death phenotypes caused by either a disease- or defense-related pathway triggered in wheat. Since resistance to *F. graminearum* is not based on gene for gene interactions, an HR response is unlikely, and so any phenotype would likely be the result of necrosis caused by a disease response or defense response related to other uncharacterized pathways.

The second assay (CEP-pathogen overlap assay) utilizes the ability of a non-pathogen such as *U. hordei* to induce plant defenses that then renders a pathogen to reduce its disease potential. Iterations of the overlap assay have been shown to function effectively in several systems. For example, in *N. benthamiana*, the non-pathogen *P. fluorescens* induced PTI and protected the plant against HR-induced cell death caused by the pathogen *P. syringae* DC3000 (Chakravarthy et al., 2010). In *N. tabacum*, *P. fluorescens* induced PTI to protect against cell death caused by a bacterial effector (Crabill et al., 2010). In wheat leaves, *P. fluorescens* induced PTI to prevent disease by *P. syringae* DC3000 (C. Liu et al., 2016). Since *P. syringae* DC3000 growth can be

quantitatively measured with this assay, an increase or decrease of bacterial growth compared to a control will indicate whether a given effector candidate suppresses or activates levels of plant defense.

Here, a subset of the 99 CEPs identified in Chapter 2 were chosen for functional analyses. Both cell death and overlap assays were used to determine function of 20 CEPs. The candidate CEPs were cloned and expressed in *U. hordei* for delivery into wheat leaves.

3.2 Methods

3.2.1 Selection of Candidate Effector Proteins for Functional Analysis

CEPs were prioritized for cloning and functional analysis as outlined in Figure 12. CEPs were chosen based on their presence in the exoproteome and predicted by the following *in silico* prediction software: SignalP 5.0 (Almagro Armenteros et al., 2019), TargetP 1.1 (Emanuelsson et al., 2000), and EffectorP 1.0 or 2.0 (probability >0.55 = effector, <0.55 = non-effector) (Sperschneider et al., 2018, 2016). Any ribosomal and histone proteins were excluded. In addition to the prioritized CEPs, all other CEPs from the 48 hpi time point, excluding ribosomal proteins, were selected for cloning.

3.2.2 Fungal and Bacterial Strains and Growth Conditions

Ustilago hordei haploid Uh364 strain (Uh364) (Linning et al., 2004) was used as a parental strain and was donated by Dr. Guus Bakkeren (AAFC-Summerland). The Uh364 strain was grown in YEPS (1% yeast extract, 2% peptone, 2% sucrose) at 22°C, and transformed Uh364 strains were grown in complete medium (CM) (2.5 g casamino

Figure 12: Filters applied to prioritize CEPs for functional analysis. A subset of the 99 CEPs identified in Chapter 2 was chosen, including CEPs present in the exoproteome and with hits from three prediction softwares (EffectorP, TargetP, and SignalP). Ribosomal and histone proteins were excluded.

acids, 1.5 g ammonium nitrate, 10 g yeast extract, 60 mL *Ustilago* macro salts (16 g KH_2PO_4 , 4 g Na_2SO_4 , 8 g KCl , 2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 8 mL trace elements solution (30 mg boric acid, 70 mg MnCl_2 , 200 mg ZnCl_2 , 20 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 50 mg FeCl_3 , 200 mg CuSO_4 in 1 L) in 1 L), 1% glucose in 1 L) with 5 $\mu\text{g/mL}$ of the fungicide carboxin (Sigma-Aldrich, USA) at 22°C.

P. syringae pv *tomato* DC3000 (DC3000) was grown on King's B media (1% peptone, 1% tryptone, 1% glycerol, 0.1% K_2HPO_4 , 0.1% MgSO_4) containing 50 $\mu\text{g/mL}$ rifampicin and 30 $\mu\text{g/mL}$ kanamycin at 28°C.

3.2.3 Generation of *F. graminearum* cDNA Library

F. graminearum DAOM 233423 was grown in GYEP medium (3 g NH_4Cl , 2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 2 g KH_2PO_4 , 2 g peptone, 2 g yeast extract, 2 g malt extract, 20 g glucose in 1 L) for 24 hours. The medium was removed, then mycelia was transferred to nitrogen-poor medium (1 g $(\text{NH}_4)_2\text{HPO}_4$, 3 g KH_2PO_4 , 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5 g NaCl , 40 g sucrose, 10 g glycerol in 1 L; pH 4.0) and grown for additional 6 hours or 24 hours. Mycelia cultures were harvested and rinsed with water, then frozen in liquid nitrogen. RNA was extracted using the TRIzol method (Rio et al., 2010). RNA was further purified using Invitrap Spin Cell RNA MiniKit (Strattec Molecular, Germany) following the manufacturer's instructions, and the RNA concentration was measured using a NanoDrop Spectrometer. The cDNA library was generated using HighCapacity cDNA Reverse Transcription Kit following the provided protocol (Applied Biosystems, USA).

3.2.4 Gateway Cloning of Candidate Effector Proteins

The cDNA library was used to clone CEPs with primers designed to amplify the coding sequence of CEPs. The reverse primers were designed both with and without the stop codon. The coding sequences were amplified by touchdown PCR using Phusion High-Fidelity DNA polymerase (ThermoScientific, USA). Briefly, the cDNA library (100 ng) was amplified in a 20 µL PCR reaction for 10 cycles from 68-58°C (touchdown step), then for 20 cycles at 58°C. All primers used for cloning CEPs are listed in Appendix B, Table 1. The purified PCR products were cloned into pENTR/D-TOPO according to the Gateway cloning protocol (Invitrogen, USA), and transformed into Stellar™ *E. coli* (ClonTech, USA). Plasmids were extracted using GeneJet Plasmid Miniprep Kit (ThermoFisher, USA) and confirmed by sequencing (Eurofin, Toronto, Ontario). CEPs were cloned into pUHES destination vector (unpublished) (Appendix B, Figure 1A) from pENTR/D-TOPO entry clones using the Gateway LR Clonase II reaction (Invitrogen, USA). Plasmids were transformed into Stellar™ *E. coli*.

3.2.5 Generation of *U. hordei* Spheroplasts

Spheroplasts were prepared based on the protocol by Tsukuda et al. (1988). *U. hordei* cultures were grown on PDA plates at room temperature. A single colony was isolated and cultured to an OD₆₀₀ of 0.5-0.8. Cells were harvested by centrifugation and washed with SCS buffer (20 mM sodium citrate pH 5.8, 1 M sorbitol). Cells were resuspended in SCS buffer with 200 µL of Vinoflow enzyme (400 mg/mL in SCS) (Gusmer Enterprises, USA) and gently rotated at room temperature. After 40-50 minutes (80% spheroplasting), ice-cold 1 M sorbitol solution was added to cells and centrifuged at 2500 rpm for 10 mins. Cells were washed twice with sorbitol, then resuspended in

sorbitol to an approximate final concentration of 2×10^9 spheroplasts/mL and stored at minus-80°C until use.

3.2.6 Transformation of *U. hordei* Spheroplasts

Candidate effector genes in pUHES were transformed into *U. hordei* spheroplasts using PEG-mediated transformation according to Ali et al. (2014). An aliquot of 1 µg of plasmid was mixed with 1 µL of 15 mg/mL heparin, and added to 50 µL of 10^6 spheroplasts, then mixed with 500 µL each of PEG-4000 and STC buffer (10 mM Tris pH 7.5, 100 mM CaCl₂, 1 M sorbitol). Spheroplasts were centrifuged for 15 minutes at 1000 g in a swinging bucket rotor, gently resuspended in STC, then plated on double complete medium (DCM) with 1 M sorbitol and 5 µg/µL of carboxin. Plates were incubated at 22°C for 7-10 days at room temperature until colonies appeared, which were then transferred to CM plates supplemented with 5 µg/µL carboxin.

3.2.7 Genotyping of Transformed *U. hordei* by PCR

Genomic DNA extraction from *U. hordei* was adapted from Elder et al. (1983). A volume of 1 mL of transformed *U. hordei* culture grown in liquid CM with 5 µg/µL carboxin was harvested by centrifugation at 14,000 rpm for 1 minute. Supernatant was removed, and 0.2 g of acid-wash glass beads, 500 µL of lysis buffer (0.5 M NaCl, 0.2 M Tris pH 8.0, 0.01 M EDTA pH 8.0, 1% SDS) and 250 µL of phenol:chloroform:isoamyl alcohol (PCI) 25:24:1 were added to the cell pellet, and vortexed for 3 minutes. Samples were centrifuged for 3 minutes at 14,000 rpm. To the 450 µL of aqueous phase, 250 µL of PCI was added, briefly vortexed, and spun for 30 seconds at 14,000 rpm. Isopropanol (250 µL) was added to the aqueous phase, vortexed briefly, and incubated on

ice for 15 minutes. Samples were centrifuged for 5-10 minutes at 14,000 rpm and supernatant was removed. Pellets were washed with 1 mL of 70% ethanol, air-dried, then resuspended in 100 µL of 1X TE buffer (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0). PCR reactions (as described in Section 3.2.4) were performed with 150 ng of gDNA as template and pUHES- (Appendix A, Table 1) and gene-specific primers (Appendix B, Table 1).

3.2.8 Detection of Candidate Effector Proteins in *U. hordei* Cultures

Transformed *U. hordei* cultures were grown in liquid CM with 5 µg/µL carboxin until dense. Protein was extracted from ground, frozen *U. hordei* cells according to Laurie et al. (2008). Two volumes of extraction buffer (10 mM KCl, 5 mM MgCl₂, 400 mM sucrose, 100 mM Tris pH 8.0, 10% glycerol, 10 mM DTT) was added to ground cells, then centrifuged at 14,000 rpm for 5 minutes at 4°C. Proteins from the supernatant were quantified by Bradford assay, and 30 µg of protein was dissolved in 1X sample buffer (2% SDS, 10% glycerol, 0.002% bromophenol blue dye, 62.5 mM Tris pH 6.8, 5% DTT) and separated by 12% SDS-PAGE gels. Proteins were transferred to nitrocellulose membranes and were detected by immunoblot analysis with HA antibodies and imaged as described in section 2.2.5.

3.2.9 Candidate Effector Protein Functional Assays in Wheat

Wheat (*Triticum aestivum* L.) cultivars ‘Roblin’ (susceptible to *F. graminearum*) or ‘Sumai 3’ (resistant to *F. graminearum*) were used for all wheat infiltration assays. Wheat seeds were sterilized in 50% bleach for 15 minutes and rinsed with water. Seeds were planted in regular potting soil and stratified for 2 days at 4°C, then moved to a

growth chamber under long days at 22°C for two to three weeks until the first and second leaves were fully developed.

For the CEP cell death assay, *U. hordei* expressing the empty vector pUHES, the CEPs, and the positive control SnTox3 (Z. Liu et al., 2009) were cultured and washed twice with 10 mM MgCl₂ and resuspended in 10 mM MgCl₂ to an OD₆₀₀ of 1.0. A sterile needle was used to puncture a small hole in the center of wheat leaves and approximately 100-200 µL of culture was infiltrated using a 1 mL needleless syringe. Leaves were imaged between 5 and 7 days after inoculation.

For the CEP-pathogen overlap assay, *U. hordei* cultures expressing CEPs or the empty vector were infiltrated into wheat leaves as above. *P. syringae* DC3000 was cultured and resuspended in 10 mM MgCl₂ to an OD₆₀₀ of 0.2, then infiltrated 48 hours after *U. hordei* infiltration into an area overlapping *U. hordei* on the same wheat leaves (Figure 13). Leaves were imaged 3 days after DC3000 infiltration.

3.2.10 Quantification of *P. syringae* DC3000 from CEP-Pathogen Overlap Assay

The growth of DC3000 infiltrated into wheat leaves for the CEP-pathogen overlap assay was quantified based on the protocol by X. Liu et al. (2015). Immediately after DC3000 infiltration (0 dpi) and at 3 dpi, the overlap area (Figure 13) was cut from all leaves, which were then pooled, divided into four replicates, and weighed. An aliquot of 500 µL of 10 mM MgCl₂ and a one quarter-inch ceramic bead was added to samples, which were then disrupted at 5000 rpm for 15 seconds using a Precellys 24 tissue homogenizer (Bertin Technologies, France). Six serial dilutions for each of the four biological replicates was prepared (from 10⁻¹ to 10⁻⁶), and 5 µL of each dilution and replicate were plated on King's B media. Plates were incubated for 48 hours and

colonies were counted. Colony-forming units (CFU)/gram of leaf tissue were calculated using the following formula: (colonies from one replicate $\times 10^{\text{dilution factor}} / 5 \mu\text{L}$) $\times 500 \mu\text{L} / \text{g of tissue}$). A one-tailed, unpaired students t-test assuming unequal variance was used to determine if the growth of DC3000 in wheat leaves infiltrated with CEPs was significantly different ($p\text{-value} < 0.1$) than the empty vector control.

Figure 13: Schematic of CEP-pathogen overlap assay of wheat leaves. *U. hordei* expressing pUHES-EV or pUHES-CEP are inoculated into a wheat leaf (blue arrow), and edges of infiltration area are marked (blue lines). Forty-eight hours later, DC3000 is infiltrated (red arrow) in an overlapping area (black bracket) and infiltration areas are marked (red lines).

3.3 Results

3.3.1 Cloning Candidate Effector Proteins for Functional Analysis in Wheat

Seventeen CEPs were prioritized for functional analysis based on the criteria outlined in Figure 12. The 17 CEPs present in the exoproteome (Table 2) were positively predicted by EffectorP, SignalP, or TargetP, and did not include ribosomal and histone proteins (Table 6, highlighted in bold). In addition to the 17 prioritized CEPs, 16 CEPs identified in the 48 hpi time point were also cloned, as well as three proteins previously identified as effectors through genomic analysis (Table 6). Twelve of the CEPs selected for cloning are present in seven or more transcriptome or proteome datasets, and 25 of the CEPs are present in six or more datasets.

Of the 36 CEPs selected for cloning and functional analysis, 20 were successfully cloned into the pUHES vector (Appendix B, Figure 1A) and transformed into *U. hordei* as indicated in Table 6 (“Successfully Cloned” column). Each candidate effector gene was cloned both with and without a stop codon to allow expression with or without a C-terminus 3XHA tag; 16 genes were successfully cloned and transformed excluding a stop codon, while nine were successfully cloned and transformed with a stop codon (five genes were successfully cloned both with and without a stop codon). All candidate effector genes cloned with or without the stop codon were confirmed to be transformed into *U. hordei* (Appendix B, Figure 1B). Expression of the 16 CEPs cloned without a stop codon and expressing the C-terminal 3X HA tag in *U. hordei* cultures was also confirmed via immunoblot analysis using HA antibodies (Appendix B, Figure 2).

Table 6: Candidate effector proteins chosen for cloning and functional analysis

Accession*	Function	EffectorP 1.0	Effector P 2.0	SignalP 5.0	TargetP 1.1	Successfully Cloned
48 hpi						
FGSG_00330	Acetyl-coenzyme A synthetase	No	No	No	Other location	No
FGSG_00921	Heat shock protein SSB1	No	No	No	Other location	No
FGSG_00979	Aldehyde dehydrogenase	No	No	No	Other location	Yes
FGSG_01111	6-phosphogluconate dehydrogenase	No	No	No	Secretory	Yes
FGSG_01422	Citrate synthase	No	No	No	Mitochondria	Yes
FGSG_01425	Plasma membrane ATPase	No	No	No	Other location	No
FGSG_04181	Elongation factor 3	No	No	No	Other location	No
FGSG_04312	Probable H ⁺ -transporting ATP synthase beta chain	No	No	No	Mitochondria	No
FGSG_05615	Adenosylhomocysteinase	No	No	No	Other location	Yes
FGSG_05972	Nucleoside diphosphate kinase	No	Yes	No	Other location	No
FGSG_06021	ADP, ATP carrier protein	No	No	No	Other location	Yes
FGSG_06290	Serine hydroxymethyltransferase	No	No	No	Other location	No
FGSG_07953	Aconitate hydratase	No	No	No	Mitochondria	No
FGSG_08601	Phosphoenolpyruvate carboxykinase	No	No	No	Other location	No
FGSG_08700	Malate synthase	No	No	No	Other location	No
FGSG_08737	Probable woronin body major protein precursor	No	No	No	Other location	No
FGSG_10746	Aspartate aminotransferase	No	No	No	Mitochondria	Yes
FGSG_10825	5-methyltetrahydropteroyltri glutamate-homocysteine methyltransferase	No	No	No	Other location	No
FGSG_10855	Alcohol dehydrogenase 1	No	Unlikely	No	Other location	Yes

FGSG_15824	ATP synthase subunit alpha	No	No	No	Mitochondria	Yes
60 hpi CEPS						
FGSG_00777	Probable peptidylprolyl isomerase precursor, mitochondrial (cyclophilin B)	Yes	Yes	No	Other location	Yes
FGSG_02077	Hypothetical protein (CFEM domain)	No	No	Yes	Secretory	Yes
FGSG_02461	Malate dehydrogenase	No	Yes	No	Mitochondrial	No
FGSG_03569	Galactose oxidase precursor	No	No	Yes	Secretory	No
FGSG_04527	Carboxypeptidase S1	No	No	Yes	Secretory	Yes
FGSG_04826	L-xylulose reductase	Yes	Yes	No	Other location	Yes
FGSG_09690	Probable peptidylprolyl isomerase (FK506-binding protein homolog)	Yes	Yes	No	Mitochondrial	Yes
FGSG_10549	Hypothetical protein	Yes	Yes	No	Other location	Yes
FGSG_10942	Hypothetical protein	Yes	Yes	No	Other location	Yes
FGSG_10974	Hypothetical protein	Yes	Yes	No	Other location	Yes
FGSG_16627	Glyceraldehyde-3-phosphate dehydrogenase	No	Yes	No	Other location	No
FGSG_17593	Hypothetical protein	Yes	No	No	Other location	No
Previously Cloned						
FGSG_01346	Enolase	No	No	No	Other location	Yes
FGSG_08811	Elongation factor alpha	No	No	No	Other location	Yes
FGSG_09366	related to glucan 1,3-beta-glucosidase	No	No	Yes	Other location	Yes
FGSG_09870	Probable CPC2 protein	No	No	No	Other location	Yes

*Bolded CEPs represent those filtered by screening criteria for functional analysis as outlined in Figure 12.

3.3.2 Optimization of Candidate Effector Protein Cell Death Assay

The *U. hordei*-wheat cell death assay was originally developed by Dr. Guus Bakkeren (AAFC-Summerland, British Columbia) to test the ability of effectors from rust fungi to generate a cell death response in wheat. Before testing the CEPs from *F. graminearum*, we confirmed that pUHES could successfully deliver the effector and cause a phenotype in wheat. To this end, the effector protein Tox3 from the necrotrophic wheat fungus *Stagonospora nodorum* (SnTox3) was used as a positive control. The SnTox3 protein, expressed and delivered by *U. hordei*, targets the host resistance protein Snn3 in the wheat cultivar ‘Sumai 3’ causing cell necrosis (Appendix B, Figure 3) (Z. Liu et al., 2009; Z. Zhang et al., 2011). In my thesis, I adapted this delivery system to determine function of *F. graminearum* CEPs in wheat. As a negative control, we used the wheat cultivar ‘Roblin’, which does not harbor an intact *Snn3* gene (Appendix B, Figure 3) (personal communication, Laroche, AAFC-Lethbridge).

To determine the optimal levels of culture for inoculation, wheat leaves of both ‘Roblin’ and ‘Sumai 3’ cultivars were infiltrated with an OD₆₀₀ of 0.2, 0.5, or 1.0 of *U. hordei* expressing SnTox3 or the empty vector and phenotypes were imaged after 6 days. The expression of SnTox3 causes necrosis in ‘Sumai 3’ wheat leaves when inoculated at an OD₆₀₀ of 0.2, 0.5 or 1.0 after 5 days, however much weaker symptoms in ‘Roblin’ were observed (Figure 14). Expression of the empty pUHES vector exhibited no phenotype in either wheat cultivars (Figure 14). An OD₆₀₀ of 1.0 was used for all further inoculations.

Figure 14: Optimization of the CEP cell death assay. *U. hordei* expressing either the pUHES empty vector (left) or positive control SnTox3 (right) were inoculated into 2-week old wheat leaves of cultivars ‘Sumai 3’ (top) and ‘Roblin’ (bottom) at three different OD₆₀₀. Symptoms were undetectable in the empty vector in both cultivars. ‘Sumai 3’ inoculated with SnTox3 showed visible symptoms of disease at all three OD₆₀₀, with symptoms the most intense at OD₆₀₀=1.0. Expression of SnTox3 in ‘Roblin’ displayed little to no phenotype.

3.3.3 Characterization of Candidate Effector Protein Expression in Wheat Leaves

U. hordei cultures expressing the CEPs were inoculated into 2-week old wheat leaves of cultivars ‘Roblin’ and ‘Sumai 3’, and phenotypes were observed after 5 days. Both MgCl₂ and the empty pUHES vector served as negative controls. SnTox3 was expressed as the positive control. No cell-death phenotype was observed for any of the CEPs expressed in either ‘Roblin’ or ‘Sumai 3’ wheat leaves (Figure 15). As expected, no phenotype is seen in the MgCl₂ or empty vector control in either wheat cultivar (Figure 15). SnTox3 showed a cell death phenotype in ‘Sumai 3’ but not ‘Roblin’ (Figure 15). These results suggest that CEPs expressed alone are either non-functional, or functional but unable to cause a visible phenotype. Therefore, we developed another assay based on the role of effectors in plant disease.

3.3.4 Optimization of CEP-Pathogen Overlap Assay

This assay tests for the ability of CEPs to suppress or activate *U. hordei*-induced defense responses. Briefly, a non-pathogen such as *U. hordei* (Figure 13, blue arrow) is first inoculated to induce a PTI defense response in wheat. This is followed by inoculation with the virulent pathogen *Pseudomonas syringae* DC3000 at a site that partly overlaps with the area inoculated with *U. hordei* (Figure 13, red arrow). The inoculation with DC3000 results in disease, as bacterial growth increases over 3 days and results in a necrosis phenotype (Appendix B, Figure 4). However, PTI induced by *U. hordei* protects wheat from DC3000 infection. This is observed in the overlapping infiltration region, resulting in the suppression of necrosis. These phenotypes can then be monitored visually or quantified by the growth of DC3000.

Figure 15: CEP cell death assay. *U. hordei* cultures expressing CEPs were infiltrated into 2-week old wheat leaves of cultivars ‘Sumai 3’ (left) and ‘Roblin’ (right) at an OD₆₀₀ = 1.0. *U. hordei* cultures were infiltrated into 2 leaves per plant, representing one biological replicate (n=10). Leaf images are representative of all biological replicates. No phenotypes were observed in any of the CEPs tested in ‘Sumai 3’ or ‘Roblin’ 5 days after infiltration. SnTox3 expression resulted in a cell death phenotype in ‘Sumai 3’ but not ‘Roblin’. MgCl₂ and EV controls showed no phenotype in ‘Sumai 3’ or ‘Roblin’.

For a given CEP, there are four possible outcomes, as outlined in Figure 16: A) MgCl_2 is used as a control and is first infiltrated into wheat leaves (blue lines). Forty-eight hours later the pathogen *P. syringae* DC3000 (DC3000) is infiltrated (red lines). MgCl_2 does not induce defenses, resulting in disease caused by DC3000 in the entire infiltration area. B) *U. hordei* expressing the pUHES empty vector (EV) is infiltrated and induces a defense response, resulting in suppression of disease-induced cell death by DC3000 in the overlapping area. C) If the expression of a CEP can suppress defenses, then DC3000 infiltration results in disease in the entire infiltration area and increased bacterial growth as compared to the EV. D) If a CEP activates plant defenses, no disease is caused by DC3000 infiltration, and bacterial growth is greatly reduced as compared to the EV. Lastly, a CEP may have no function in altering plant defenses, resulting in the same phenotype as B, with the same level of bacterial growth

As *U. hordei* has previously been shown to display no phenotype in wheat leaves (Appendix B, Figure 3), we used this non-pathogen to both deliver CEPs and induce plant defenses in the overlap cell death assay. *P. syringae* DC3000 was inoculated at either 24 hours or 48 hours after infiltration of *U. hordei* expressing the EV to determine at which time point plant defenses could still be induced and prevent disease. *U. hordei* expressing the EV was able to induce defenses and suppress disease at both 24 hours and 48 hours after infiltration (Figure 17). The time point of 48 hours was chosen for subsequent assays to allow for adequate expression of the CEP from the pUHES vector. Only the ‘Roblin’ cultivar was used for subsequent assays as it is susceptible to *F. graminearum*.

Figure 16: Schematic representing the CEP-pathogen overlap assay used to determine the ability of CEPs to suppress or activate wheat defense responses in wheat leaves. The following phenotypes can be expected: **A:** MgCl₂ serves as a control. It does not induce PTI, resulting in disease-induced cell death by DC3000 across the entire infiltration area. **B:** *U. hordei* expressing the empty vector induces defenses, resulting in a reduction of DC3000 disease in the overlapping area (black bracket). Bacterial growth quantified here serves as the control. **C:** If the CEP suppresses plant defenses, DC3000 can cause disease in the entire infiltration area. Bacterial growth is expected to increase as compared to the EV. **D:** If the CEP activates plant defenses, DC3000 is unable to grow, and therefore disease symptoms are reduced. Bacterial growth can be expected to be reduced as compared to bacterial growth in the EV control. If CEP expression has no effect, the phenotype will be the same as the empty vector and bacterial growth will be at similar levels.

Figure 17: Optimization of infiltration time-points of the CEP-pathogen overlap assay. MgCl_2 or *U. hordei* expressing the empty pUHES vector were first infiltrated (blue lines), then DC3000 (red lines) was infiltrated either **A**: 24 hours or **B**: 48 hours later in an overlapping area (black bracket, indicated by white bars on images). Phenotypes were imaged after 3-4 days. PTI was induced by *U. hordei* expressing the EV at both 24 and 48 hours, as there was a reduction in cell death in the overlapping area (black bracket) as compared to MgCl_2 . No differences in phenotype were seen between the 24 or 48 hpi time points in either the MgCl_2 control or the EV. *U. hordei* cultures were infiltrated at an $\text{OD}_{600} = 1.0$ and DC3000 infiltrated at an $\text{OD}_{600} = 0.2$.

3.3.5 Characterization of Candidate Effector Proteins in Wheat Defense

Responses

Cultures of *U. hordei* expressing CEPs were infiltrated into 2-week old wheat leaves ('Roblin') at an OD₆₀₀ of 1.0 (Figure 18). DC3000 cultures were then infiltrated 48 hours later into an overlapping area at an OD₆₀₀ = 0.2. We first qualitatively assessed disease symptoms in the overlapping area. Disease symptoms in the overlapping area were recorded and represented as the percentage of number of leaves exhibiting symptoms on the left-hand column in Figure 18, where 'n' represents the number of leaves infiltrated. Visually, all CEPs showed a variation in disease levels, ranging from 0% to 33% of infiltrated leaves showing disease symptoms (Figure 18). The MgCl₂ control resulted in disease from DC3000 in the overlapping area, as expected because no defenses were induced (Figure 18). Expression of the empty vector displayed no disease phenotype (Figure 18).

Next, we were interested in quantifying bacterial growth in the overlapping area of the CEP-pathogen overlap assay. DC3000 growth in the overlapping area of wheat leaves was quantified at 0 dpi and 3 dpi for eight CEPs and is displayed in Figure 19.

After the 3 dpi time point, bacterial growth was significantly attenuated ($p < 0.05$) in the empty vector control as compared to the MgCl₂ control, indicated by a double asterisk, suggesting that *U. hordei* induced PTI and reduced the virulence of DC3000. The expression of FGSG_02077 resulted in significantly increased bacterial growth as compared to the EV ($p < 0.1$, single asterisk). Supporting this, disease symptoms in the overlap region from FGSG_02077 also displayed the highest level, with 33% of leaves showing disease in the overlap region (Figure 18). This indicates FGSG_02077 may

Figure 18: CEP-pathogen overlap assay. *U. hordei* cultures expressing CEPs were infiltrated into 2-week old leaves from Roblin cultivars (blue line). Each CEP was infiltrated into 4-6 plants, with one plant representing one biological replicate. Two leaves were infiltrated per plant. Forty-eight hours later, DC3000 (red line) was infiltrated into an overlapping area (black bracket, white bar on images). Images were taken 3 days after DC3000 infiltration. Disease symptoms in the overlapping area is represented as the percentages of leaves showing disease (left column, n = number of infiltrated leaves). Phenotypes shown here are representative of all replicates. There was little to no disease in the overlapping areas for any CEP; phenotypes were similar to the empty vector control. *U. hordei* cultures were infiltrated at an OD₆₀₀ = 1.0 and DC3000 at an OD₆₀₀ = 0.2.

have a role in virulence.

The expression of FGSG_00777, FGSG_01346, and FGSG_01422 resulted in significantly less growth of DC3000, compared to the EV ($p < 0.1$, single asterisk).

These results suggest that these three CEPs may activate plant defenses, preventing bacterial growth. Other CEPs that were tested showed no significant differences in growth compared to the EV (Figure 19).

Figure 19: Quantification of DC3000 growth from overlapping area of infiltrated wheat leaves. Bars represent log₁₀ of colony forming units (CFU) per gram of leaf tissue. One-tailed student's t-test was used to identify bacterial growth that was significantly different ($p < 0.05$ ** and $p < 0.1$ *) from the EV control. Error bars represent the standard error of the mean ($\pm$ SEM). Bacterial growth was sampled from 10 wheat leaves from five plants, pooled, and divided into four replicates ($n = 4$).

Chapter 4: Discussion

4.1 Summary

The effector repertoire of *F. graminearum* is poorly understood. Identifying effectors and characterizing their function will provide a better understanding of the infection mechanism of *F. graminearum* and potentially lead to novel methods of control. Here, a proximity-dependent labeling technique known as BioID was used to identify putative secreted proteins during *F. graminearum* infection in *Arabidopsis* seedlings. The use of the *Arabidopsis*-*F. graminearum* pathosystem combined with BioID provides an ideal platform to study the infection mechanisms of *F. graminearum*. After optimization of BioID for use in the *F. graminearum*-*Arabidopsis* 96-well plate infection assay, over 350 *F. graminearum* proteins were identified and 99 of those considered with confidence to be candidate effector proteins or CEPs.

The BirA* protein was expressed cytoplasmically or localized to the plasma membrane. Several infection time points were sampled for protein analysis to examine patterns of secreted proteins throughout the course of *F. graminearum* infection. At the 24 hpi time point, there were no *F. graminearum* proteins detected with high confidence (Figure 10B). At this time point, infection likely had not progressed enough for *F. graminearum* proteins to be secreted into the plant. Indeed, imaging of *Arabidopsis* coleoptiles during infection showed no mycelial growth (Figure 5), and even during later infection no *F. graminearum* proteins were confidently detected in the growth media (External Files Table S9). Alternatively, proteins may be secreted but in low abundance and may not be identified via BioID. Newer iterations of BioID have been generated that

could overcome some of the drawbacks with the version used in my thesis and are described further in section 4.4.

More CEPs were detected in the cytoplasmic fraction than in the membrane fraction (Figure 10A). This may indicate that *F. graminearum* is secreting effectors into the cytoplasm rather than the apoplast, corresponding to its necrotrophic growth phase. This may also explain why less CEPs were identified in the membrane fraction. However, effectors that are involved in the early infection process, for example in the penetration of host cells, likely localize to plasma membranes and are therefore identified by HopF2-BirA*. Currently, no temporal or spatial patterns of effectors have been characterized in *F. graminearum*, but the 99 CEPs identified here provide a road map and insight into the workings of the *F. graminearum* infection process. The 99 CEPs were closely examined for their role as effectors.

4.2 Characterization of Candidate Effector Proteins in *F. graminearum*

Proteins defined as effectors typically contain a signal peptide, are small in size, and have unique domains (Lo Presti et al., 2015; Selin et al., 2016; Stergiopoulos & De Wit, 2009). However, as effectors from more fungal species are identified, it is clear that effectors cannot be explicitly defined by these characteristics. As described in Chapter 1, there are several fungal effectors that have been characterized that fall exception to the rules (Catanzariti et al., 2010; Djamei et al., 2011; Yin et al., 2019). In particular, *in silico* computational analyses are routinely used to identify effectors from a pathogen proteome or transcriptome, but genuine effectors may be excluded based on the filtering criteria. Thus, identifying *in planta* secreted proteins through proteomic techniques

provides a more accurate picture of a pathogen's effector repertoire without the bias of predictive software. The use of BioID to identify secreted proteins from a fungal pathogen *in planta* provides a unique opportunity to identify secreted proteins involved in virulence. Although many of the CEPs identified via BioID in Chapter 2 lack classic effector characteristics, their presence *in planta* during infection implies they may have a role in promoting fungal infection, and each must be examined for their potential to act as an effector protein.

4.2.1 Effector Secretion: Alternate Pathways

Evidence of secretion, often indicated by the presence of a signal peptide, is usually the first criteria used to determine whether a protein may classify as an effector. Only a small fraction of the CEPs identified in Chapter 2 were predicted for secretion or for effector function by *in silico* prediction softwares, with the majority of CEPs lacking a signal peptide (Table 4). However, lack of a signal peptide does not immediately exclude a protein from being secreted.

In fungi and other eukaryotes, proteins are normally secreted through the endoplasmic reticulum and Golgi pathways, where an N-terminal signal peptide is recognized and then cleaved after transport of the protein across a membrane (Shoji et al., 2014). There are examples of secreted effector proteins from fungi that do not contain a signal peptide. For example, two Avr proteins from *Blumeria graminis*, Avr-a10 and Avr-k1, induce cell death when expressed in a host and are recognized by the host *R* genes, but lack secretion signals (Ridout et al., 2006). As described in Chapter 1, two isochorismatase effectors Pslsc1 and Vdlsc1 from the oomycete *Phytophthora sojae* and

the fungal pathogen *Verticillium dahlia* respectively, also lack signal peptides but were identified in the culture supernatant, indicating they are secreted proteins (T. Liu et al., 2014). Both of these effectors disrupt salicylate metabolism function in the host and compromise host defense signalling pathways (T. Liu et al., 2014). These examples suggest that non-canonical secretion pathways exist for effector proteins.

Extracellular vesicles (ECVs) are an alternate secretion method that were first characterized in *Cryptococcus neoformans* (Rodrigues et al., 2007), and recently described in other fungal species (Oliveira et al., 2013; Rodrigues et al., 2014). ECV secretion has been shown for use in the transport of virulence-associated proteins into host cells (Rodrigues et al., 2008; Vallejo et al., 2011), which would allow efficient delivery of multiple virulence factors or effectors. Proteins that have been identified in ECVs from *Cryptococcus neoformans* include heat shock proteins and other known virulence proteins, as well as ribosomal proteins and proteins involved in primary metabolism (Rodrigues et al., 2008).

Effectors from the closely related ascomycete fungi *M. oryzae* have been shown to be secreted via an exocyst complex, which are secreted vesicles that deliver effectors to the host cell (Giraldo et al., 2013). While there is currently no evidence of ECVs in *F. graminearum*, these examples suggest that *F. graminearum* may also have similar non-classical secretion pathways for effector delivery.

However, the lack of signal peptide of many CEPs may suggest that these are abundant proteins extracted from lysed *F. graminearum* cells in plants that bind non-specifically to the streptavidin-sepharose beads. We used several approaches to minimize this problem. First, we used internal controls including WT *Col-0* and uninduced BirA*

treated similarly to other samples. Any *F. graminearum* proteins originating from these control samples were subtracted from experimental samples and excluded from further analysis. Second, we harvested tissues during early infection when fungal hyphae are actively growing, minimizing fungal lysis. Lastly, the majority (90%) of the CEPs are present in the exoproteome generated by our lab, where no fungal cell lysis was observed (External Files Table S2, 'Exoproteome' column).

4.2.2 Candidate Effector Proteins with a Role in Virulence in *F. graminearum* and in Other Pathogens

Several of the CEPs identified here have been previously characterized in *F. graminearum* with roles in virulence. One of the hypothetical proteins identified, FGSG_10974, is a putative interactor of FgSnx41, a protein associated with the early endosome membrane and coordinates the sorting and trafficking of vesicles between donor and recipient membranes (Zheng et al., 2018). The deletion of *FgSnx41* results in a reduction in virulence, suggesting interacting proteins of FgSnx41 such as FGSG_10974 may have similar functions in virulence.

FGSG_02077 is a predicted small secreted protein with high cysteine content and contains a CFEM (common in fungal extracellular membrane) domain (Table 4). Mutants of FGSG_02077 show reduced virulence on wheat coleoptiles and decreased hyphal growth (X.-W. Zhang et al., 2012). FGSG_02077 mutants also displayed reduction in visible disease symptoms on wheat spikelets (Dufresne et al., 2008). The FGSG_02077 protein is localized to the fungal cell surface (X.-W. Zhang et al., 2012), which supports our observation; FGSG_02077 was identified in the membrane fraction.

FGSG_01346 has been annotated as an enolase, present in 9 of 16 published datasets (Table 5) and was identified as a secreted protein in both wild-type *F. graminearum* strain PH-1 cultures and in the deletion mutants of *Vam7*, a SNARE homolog (soluble n-ethylmaleimide-sensitive factor attachment protein receptor) (H. Zhang et al., 2016). SNARE proteins are involved in vesicle transport and membrane fusion, and have established roles in virulence (H. Zhang et al., 2016). FGSG_01346 was found to be expressed at low levels in *Vam7* mutants compared to the WT. Interestingly, FGSG_01346 had decreased abundance in the *tri6* mutant strain in *F. graminearum* (Rampitsch et al., 2013). *TRI6* is a regulatory gene involved in the production of the trichothecene 15-ADON, implying FGSG_01346 likely has a role in virulence (Rampitsch et al., 2013).

A number of CEP homologues have been shown to function in virulence in other pathogenic microbes. The CEPs FGSG_00777 and FGSG_09690 have peptidylprolyl isomerase (PPI) domains, which are found in cyclophilins. Cyclophilins catalyze peptide bonds in proline residues and facilitate protein folding (P. Wang & Heitman, 2005). A deletion of cyclophilin homolog CYP1 in *M. grisea* showed a reduction in virulence (Viaud et al., 2002). In the parasite *Trypanosoma cruzi*, a FK506 binding protein with a PPI domain was shown to be secreted and involved in cell infection (Moro et al., 1995).

Heat shock proteins (HSPs) have known functions in infection, and several are classified as virulence proteins (Gophna & Ron, 2003). Heat shock proteins have been documented in the bacterial pathogen *Helicobacter pylori* as being surface-associated proteins that contribute to infection (Kamiya et al., 1998). As well, HSPs in the parasite *Toxoplasma gondii* help protect the pathogen against host defenses (Dobbin et al., 2002).

We identified three HSPs (FGSG_00921, FGSG_06246, and FGSG_00838) as CEPs, and mutant analysis would elaborate their function.

Several metabolic enzymes have functions in virulence. Malate dehydrogenase (FGSG_02461) has a proposed function in the necrotrophic crop pathogen *Botrytis cinerea* which is to create an acidic environment more suitable for the pathogen (Fernández-Acero et al., 2006, 2007). Another enzyme, alcohol dehydrogenase (FGSG_10855), influences infection by the fungal pathogen *Aspergillus fumigatus* (Grahl et al., 2011). Deletion analysis showed reduced fungal growth and resulted in an increase of host immune cells in mice (Grahl et al., 2011).

Thus, evidence showing that several CEPs have previously established roles in virulence validates the BioID technique, and ongoing work to functionally characterize other CEPs will establish BioID as viable method to identify and characterize effectors.

4.2.3 Function of Candidate Effector Proteins and their Roles in Infection

The majority of the CEPs we identified were grouped into functional categories involved in translation (primarily ribosomal proteins) or in primary metabolism (Figure 8). Although these functional categories may not immediately indicate a role in pathogen virulence, known fungal effectors display a wide range of function.

Taylor et al. (2008) performed proteomic analysis of *F. graminearum* grown in mycotoxin-inducing culture conditions and found that most down-regulated proteins were involved in primary metabolism and protein regulation and synthesis, and that most up-regulated proteins were also involved in primary metabolism or were unclassified (Taylor et al., 2008). A large proportion of the CEPs identified in Chapter 2 are also involved in

primary metabolism (Figure 8; Table 4), supporting these findings in previous studies. The *F. graminearum* secretome analyzed by Rampitsch et al. (2013) also found that approximately 40% of secreted proteins were involved in metabolism. Fungal proteins involved in primary metabolism have been shown to act as effectors, although often these are exceptions to the rule. For example, as described in Chapter 1, the chorismate mutase from *U. maydis* acts as an effector by redirecting chorismate from plasmid to cytosol, and also lowers the amount available to the plant to synthesize salicylic acid (Djamei et al., 2011).

Cell-wall degrading enzymes (CWDEs) are part of the arsenals of plant pathogens, allowing the pathogen to enter host cells and colonize the plant, and to break down host cells during later stages of colonization. CWDEs are upregulated during infection, however their disruption often does not result in a loss of virulence (Kubicek et al., 2014). As described in Chapter 1, FGL1 is a well-characterized CWDE in *F. graminearum*, which encodes a secreted lipase (Voigt et al., 2005). Several other CWDEs have also been characterized in *F. graminearum*, including cellulases, pectinases, and xylanases (Kikot et al., 2009; Phalip et al., 2009). Glycosyl hydrolases are proteins which function to break down saccharides and are considered CWDEs (Kubicek et al., 2014). One CEP (FGSG_09366) has a glycosyl hydrolase domain, while another (FGSG_02937) has a hydrolase fold (Table 4). FGSG_03569 is a galactose oxidase precursor that may also function in the breakdown of starches in the cell wall. These three CEPs could be considered potential CWDEs, which is a very small fraction of the 99 CEPs identified. Literature on previous *F. graminearum* secretomes has found that over half of the proteins were classified as CWDEs (Paper et al., 2007; Phalip et al.,

2005; Yang et al., 2012). The lack of CWDEs identified here via BioID may imply that the infection mechanism of *F. graminearum* varies between wheat and *Arabidopsis*, or that the presence of CWDEs depends on the time point of infection. As well, the conditions and nutrient sources used for secretome analysis will contribute to differences in protein profiles.

Many of the CEPs can be classified as housekeeping enzymes. Housekeeping enzymes are proteins that are expressed constitutively in almost all living organisms and function in necessary cellular pathways (Pancholi & Chhatwal, 2003). Housekeeping enzymes identified in Chapter 2 include GAPDH, glycolytic enzymes such as malate dehydrogenase and enolase, and elongation factors. Housekeeping enzymes may have roles as effectors to disrupt normal host cell function. For example, GAPDH (FGSG_16627) was identified as an adhesin from the pathogenic fungus *Paracoccidioides brasiliensis* where it promotes the interaction between the pathogen and host cells (Barbosa et al., 2006). Subsequently, it was shown to function at the cell surface and is required for virulence (Jin et al., 2011). Enolase (FGSG_01346) can function as a surface receptor in bacterial pathogens that binds plasminogen to promote infection (Pancholi, 2001; Sha et al., 2009). It has also been identified as a cell wall component in yeast (*Candida albicans*) and may act as an antigen (Angiolella et al., 1996). Other glycolytic enzymes have been identified as cell-surface proteins in bacteria to promote infection, suggesting some CEPs identified here may have similar functions (Pancholi & Chhatwal, 2003).

A large percentage of the CEPs are annotated as ribosomal proteins (Table 4). At first, this may be suggestive of fungal lysis occurring *in planta*, resulting in the

contamination of the secretome with cytoplasmic proteins. No ribosomal proteins from *F. graminearum* were found during the 24 hpi time point (External Files, Table S3), and only 3 of 26 (12%) CEPs from the 48 hpi time point were annotated as ribosomal (Table 4). The majority of ribosomal proteins were identified at 60 hpi, which may indicate that fungal cell lysis is only occurring at later infection time points. However, very few ribosomal proteins are found in controls that were not induced for BirA* by DEX at 60 hpi (External Files Tables S7 and S8). Ribosomal proteins are known to bind non-specifically to sepharose beads (Trinkle-Mulcahy et al., 2008), and so an enrichment of ribosomal proteins in these controls would be expected if fungal lysis is occurring. Moreover, all the ribosomal proteins identified in Chapter 2 through BioID were also identified in the exoproteome (External Files Table S2), providing further support that these proteins are secreted. If *F. graminearum* secretes proteins through ECVs, this may explain the high number of ribosomal proteins identified. For example, proteome analysis of ECVs from *C. neoformans* identified several ribosomal proteins (Rodrigues et al., 2008). Ribosomal proteins have been documented to have specific function in cancer development, immune signaling, or function in DNA repair and expression – they are not strictly involved in protein translation (Bhavsar et al., 2010; X. Zhou et al., 2015). This may be the case for a number of the ribosomal proteins identified in our BioID experiments.

4.3 Functional Characterization of Candidate Effector Proteins in Wheat

Currently, there are no methods available to characterize effector function of *F. graminearum* in the native host, wheat. Deletion of CEPs in *F. graminearum* will inform

us about their role in the fungus and potentially their role in virulence through pathogenicity assays on wheat. However, their role as effectors requires characterization of their function in the host.

A subset of the CEPs identified in Chapter 2 were further examined for their host function. This was performed by protein expression assays in wheat using a *U. hordei* protein-delivery system. Expression of the twenty CEPs assayed did not result in any visible phenotype such as necrosis of host cells by disease, exemplified by the SnTox3-Snn3 interaction (Figure 14). This may be for several reasons. Firstly, the CEPs may not be properly delivered by *U. hordei* into wheat leaves. Although CEPs are expressed in *U. hordei* (Appendix B, Figure 2), repeated immunoblot analyses of CEPs in wheat leaves were unsuccessful. It is therefore likely that CEPs are present in small amounts and need to be enriched by immunoprecipitation for detection. Secondly, CEP function may be masked by host defense activation of *U. hordei*. As shown in Figure 17, *U. hordei* expressing pUHES-EV dramatically reduced disease progression of DC3000. This implies *U. hordei* itself activates some manner of plant defenses, likely by host recognition of PAMPs, and thus may interfere with the effector function of CEPs.

A more plausible explanation of the lack of visible phenotype is that CEPs may require the expression of multiple effectors to confer disease. Expression of single effectors at a time may not result in a detectable phenotype (Munkvold & Martin, 2009). Co-expression of several CEPs may instead be required to induce a visible phenotype.

4.3.1 A Pathogen Overlap Assay System to Decipher Candidate Effector Protein Function in Wheat

Monitoring growth of a bacterial pathogen after effector infiltration would reveal the effector function on plant immune responses (X. Liu et al., 2015). In this regard, we measured bacterial growth to quantify the effect of expression of a subset of CEPs on plant defenses. The effect of CEP expression on bacterial growth by the overlap assay is validated by the expression of FGSG_02077 (CFEM1). As described in section 4.2.2, FGSG_02077 has previously been characterized to have a role in virulence, as knockouts of this gene result in reduced virulence on wheat coleoptiles (X.-W. Zhang et al., 2012). Expression of FGSG_02077 results in increased bacterial growth ($p < 0.1$) as compared to the EV (Figure 19). This indicates that this CEP suppresses plant defenses, supporting its role as a putative effector. Moreover, the result imparts confidence to the CEP-pathogen overlap assay system.

The expression of FGSG_00777, FGSG_01346, and FGSG_01422 resulted in significantly lower bacterial growth as compared to the empty vector control (Figure 19). The decrease in bacterial growth indicates that these proteins may have activated a defense response in plants, preventing successful growth of the bacteria. However, no HR symptoms were observed (Figure 15), which are typically seen in classic *Avr-R* gene interactions. These CEPs may be inducing plant defenses such as PTI or ETI, however it is difficult to provide a clear mechanism. As *U. hordei* is also inducing plant defenses in some manner, the interaction between *U. hordei* and wheat may confound the activity of CEPs in plant defense activation, making it difficult to draw conclusions on the possible effect of these CEPs.

While quantification showed significant differences between the EV and MgCl₂ ($p < 0.05$), the other CEPs tested showed varying levels of bacterial growth (Figure 19). The variation seen is likely due to the “resistant” qualities of the ‘Roblin’ genotype to *U. hordei* that interfere with DC3000 virulence, which can be seen in Figure 17A as ‘Sumai 3’ phenotypes of disease appear stronger than ‘Roblin’ phenotypes. It will be more fitting to perform a screen with other wheat varieties that show lower resistance to *U. hordei*. Alternatively, a weaker pathogen other than DC3000 could also be used. A combination of these approaches will show more consistent phenotypes with respect to the effect of CEP expression on plant defenses.

4.4 Perspectives and Future Directions

BioID provides an ideal system to identify novel protein interactions and populations. Although BioID has distinct advantages over other protein identification and isolation methods in that it can be done *in vivo* and in denaturing conditions, like any scientific method, it does not come without its drawbacks.

BioID requires the expression of BirA*, which generally involves the genetic transformation of an organism. The adaptation of BioID for wheat would be the most ideal system in which to identify novel effectors from *F. graminearum*. However, not all wheat varieties are amenable to transformation, especially those that are used in *Fusarium* studies. Although the *F. graminearum*-*Arabidopsis* infection assay is a suitable system, there may be factors in wheat that influence secretion of *F. graminearum* proteins that are not present in *Arabidopsis*, and thereby not detected by BioID. Moreover, *F. graminearum* primarily infects floral tissues of mature wheat. Here, BioID

is utilized in the vegetative tissue of very young *Arabidopsis* seedlings. The infection mechanism of *F. graminearum* in these two instances is most likely different, which would again result in differences in protein expression and secretion. *F. graminearum* is able to infect *Arabidopsis* floral tissues (Urban et al., 2002), and so utilizing BioID in *Arabidopsis* floral tissues rather than vegetative tissues would provide a more representative picture of effector populations that would be present in a wheat infection system.

BirA* biotinylates primary amines of proximal proteins (Roux et al., 2012). Any proteins lacking a primary amine or with no accessible primary amine in its structure will not be identified by BioID, resulting in false negatives. Low abundant proteins may not be consistently identified across replicates, or at all, resulting in more false negatives and their exclusion from analysis.

The mass spectrometry data collected here also includes several thousand *Arabidopsis* proteins. Further examination of these proteins could reveal the effect of *F. graminearum* infection on host proteins. Determining the changes in protein abundance or absence/presence of host proteins between infected and non-infected samples may indicate host proteins affected by pathogen infection or which play a role in defense responses. It must be kept in mind that because BirA* is the cytoplasmic form with no bait protein, there is no specific interactions between host proteins, and protein biotinylation may be inconsistent between treatments. It would be more accurate and effective to apply a bait protein, such as a known defense-related protein, in order to obtain more valuable information about the nature of *Arabidopsis* responses to infection.

Effector proteins can be targeted to multiple cellular areas, as evidenced by apoplastic and cytoplasmic targets of *M. oryzae* effectors (Giraldo et al., 2013). BioID can be applied to identify protein populations of specific cellular regions. In addition to the cytoplasmic and membrane fractions analysed here, BirA* could be targeted to the nucleus or chloroplast. These are cellular regions where effectors are known to be targeted to (G. Li et al., 2014; Vargas et al., 2016), and using BioID in these organelles would reveal more potential effectors.

As well, several new adaptations of BioID have recently been developed. TurboID and miniTurbo are variation of BirA* enzyme that allows much faster biotinylation times, as well as provide higher efficiency in biotinylation (Branon et al., 2018). These techniques could provide more detail on effector populations in *F. graminearum* and could easily be applied to other plant-pathogen systems to identify novel proteins involved in infection.

There are several limitations to examining protein function in monocots such as wheat. Traditional protein expression techniques, such as via *Agrobacterium*, have generally not been successful in wheat, and success has largely depended on the genotype. Particle bombardment is the most common method use for transformation but is not suited to high-throughput screens. The *U. hordei* protein-delivery assay provides a fungal secretion system to deliver candidate fungal effectors in wheat in a high-throughput manner.

Future work to validate the secretion of these proteins would be to deliver through *F. graminearum*. CEPs could be tagged with a fluorescence protein such as mCherry and delivered by a GFP-labelled *F. graminearum*. Protein secretion could be visualized via

microscopy *in planta* and would confirm the secretion of proteins in the native environment. Furthermore, studies examining the fate of host defenses after the expression of CEPs could also be conducted. BioID in plants has revealed novel host defense proteins that interact with bacterial effectors (Khan et al., 2018), and therefore similar studies may be performed to identify interacting host proteins with *F. graminearum* effectors. Expressing a CEP as a fusion protein with BirA* *in planta* and determining interactions with host proteins would both confirm the CEP role as an effector in the host and reveal novel host targets.

In conclusion, the results collected here reveal the potential function of CEPs from *F. graminearum* during infection. Characterization of their function will reveal how plant defenses against this pathogen are deployed. This knowledge will open new venues in mitigation strategies such as developing novel chemicals or other biocontrol methods to combat the devastation that *F. graminearum* inflicts on agricultural crops.

Appendices

Appendix A

Appendix A Figure 1: Confirmation of *BirA in *Arabidopsis*.** **A:** Schematic of BioID vectors constructed by Dr. M. Khan. Both constructs are present in a pBD backbone, under the control of a DEX-inducible promoter, with a C-terminal FLAG and HA tag. *HopF2* is N-terminally fused to *BirA** to allow for localization of HopF2 to the membrane, while BirA* and tags are expressed on the internal side of the membrane. **B.** PCR confirmation of one *BirA** line. Each lane (numbered) represents a rosette sampled from one plant. The expected product size of *BirA** is 489 bp. 35 of 39 of samples were positive for *BirA**. Arrows show faint but present products. **C.** PCR confirmation of two *HopF2-BirA** lines (top gel represents 18 rosettes from one line, bottom gel represents 19 rosettes from a second line). The expected product size of *HopF2-BirA** is 611 bp. 10 of 18 samples amplified for one *HopF2-BirA** line (top gel), while 12 of 19 samples amplified for the second *HopF2-BirA** line (bottom gel). **D.** WT *Col-0* was sampled as a negative control. No amplification was observed for *BirA** (lane 1) nor *HopF2-BirA** (lane 2).

Appendix A Figure 2: Immunoblot confirming BirA* inactivity in control treatments.

Biotinylated proteins from total protein lysates (input) from Col-0, the cytoplasmic fraction (BirA*) without DEX (mDEX), the membrane fraction (BirA*) without DEX (mDEX), and a positive control of the cytoplasmic fraction with DEX (pDEX) were detected via streptavidin-HRP antibodies (top panel). Endogenously biotinylated proteins were seen in input fractions for Col-0 and mDEX fractions, with much higher biotinylation levels occurring in the cytoplasmic fraction with DEX. Expression of BirA* was confirmed via HA antibodies (middle panel). No HA signal was seen for Col-0 and cytoplasmic (BirA*) or membrane (HopF2) fractions without DEX (mDEX) but was detected in the cytoplasmic fraction with DEX (pDEX). This confirms BirA* inactivity in control treatments. Ponceau S stain was used to confirm protein transfer (bottom panel).

Appendix A Table 1: Primers for genotyping of BirA* lines and *U. hordei* transformants

Primer Name	Sequence	T _m (°C)	Notes
BirA-F	GGA CAA CAC CGT GCC CCT GAA G	62.7	Genotyping BirA* lines with BirA-R
BirA-R	CCC AGC TTC CGC AGC ACC	62.3	
HopF2-F	GAG GCG CGC CAA GGA TGG GTA ATA TTT GCG	66	Genotyping HopF2- BirA* lines with BirA-R
pUHESDest-F	TACATAAGCATGAGTCTGTC CTGATTTCCACCC	62.1	anneals 168 bp upstream of gateway cassette
pUHESDest-R	CAGATAATCAACGTAAGCCG AACAGTGCATG	60.9	anneals 172 bp downstream of gateway cassette

Appendix A Table 2: BioID treatments and controls analyzed by LC-MS/MS

Treatment Name ¹	Cellular Fraction of <i>BirA</i> *	Time point	Infected/Non-infected	DEX-induced	Notes
Experimental Treatments					
BirA* pFg pDEX	Cytoplasmic	24 hpi, 48 hpi, 60 hpi	Infected	Yes	
BirA* mFg pDEX	Cytoplasmic	24 hpi, 48 hpi, 60 hpi	Non-infected	Yes	
HopF2-BirA* pFg pDEX	Membrane	60 hpi	Infected	Yes	
HopF2-BirA* mFg pDEX	Membrane	60 hpi	Non-infected	Yes	
Control Treatments					
Col-0 pFg pDEX	Not applicable	24 hpi	Infected	No	
Col-0 mFg pDEX	Not applicable	24 hpi	Non-infected	No	
BirA* pFg mDEX	Cytoplasmic	60 hpi	Infected	No	No BirA* activity; Col-0 phenotype
BirA* mFg mDEX	Cytoplasmic	60 hpi	Non-infected	No	No BirA* activity; Col-0 phenotype
HopF2-BirA* pFg mDEX	Membrane	60 hpi	Infected	No	No BirA* activity; Col-0 phenotype
HopF2-BirA* mFg mDEX	Membrane	60 hpi	Non-infected	No	No BirA* activity; Col-0 phenotype
Growth media	Not applicable	60 hpi	Infected	Yes	Growth media taken from 96-well plates at time of Arabidopsis seedling harvest; media serves as a negative control for <i>F. graminearum</i> secreted proteins that are not tissue-specific.
Growth media	Not applicable	60 hpi	Non-infected	Yes	Growth media taken from 96-well plates at time of Arabidopsis seedling harvest; media serves as a negative control for <i>F. graminearum</i> secreted proteins that are not tissue-specific.

¹ pFg/mFg = plus/minus infection with *F. graminearum*; pDEX/mDEX = plus/minus addition of DEX to media

Appendix B

Appendix B Figure 1: Confirmation of CEPs in pUHES destination vector. **A:**

Schematic of pUHES destination vector. The vector is an episomal construct with a pCM60 backbone and contains an *Ustilago* autonomously replicating sequence (UARS) for increased transformation frequency. A carboxin-resistance gene (*cbxR*) allows for selection in *U. hordei*. Candidate vectors are cloned by replacing the GatewayTM Reading Frame A cassette (GW-RFA), from which they are expressed from the Hsp70 promoter of *U. maydis* (UmHsp70), in frame with the UhAVR1 signal peptide (SP) and with a C-term 3X HA tag if the stop codon is excluded. **B:** PCR genotyping of successful transformation of CEPs into *U. hordei*. Genes were amplified from genomic DNA using a pUHES-specific forward primer and gene-specific reverse primers. Lanes are described below with the gene name and expected product size:

Top gel (genes without stop codon):

Lane 1: FGSG_00777: 854 bp
 Lane 2: FGSG_00979: 1647 bp
 Lane 3: FGSG_01346: 1227 bp

Lane 4: FGSG_01422: 1581 bp
Lane 5: FGSG_04527: 1590 bp
Lane 6: FGSG_06021: 1104 bp
Lane 7: FGSG_09690: 606 bp
Lane 8: FGSG_10549: 702 bp
Lane 9: FGSG_09870: 1170 bp
Lane 10: FGSG_10746: 1437 bp
Lane 11: FGSG_10942: 594 bp
Lane 12: FGSG_10974: 681 bp
Lane 13: FGSG_15824: 1827 bp
Lane 14: FGSG_01111: 1647 bp
Lane 15: FGSG_05615: 1518 bp
Lane 16: FGSG_10855: 1230 bp
Lane 17: water control

Bottom gel (genes with stop codon):

Lane 1: FGSG_01346: 1227 bp
Lane 2: FGSG_01422: 1581 bp
Lane 3: FGSG_02077: 723 bp
Lane 4: FGSG_04826: 966 bp
Lane 5: FGSG_08811: 1551 bp
Lane 6: FGSG_09366: 1554 bp
Lane 7: FGSG_09690: 606 bp
Lane 8: FGSG_09870: 1170 bp
Lane 9: FGSG_10855: 1230 bp
Lane 10: water control

Appendix B Figure 2: Confirmation of CEP expression in *U. hordei* cultures by detection of N-terminal HA tag. Total protein was extracted from *U. hordei* cultures expressing CEPs cloned without their stop codon (allowing N-terminal HA-tag expression), separated on SDS-PAGE, and blotted with HA antibodies (top blot). Red asterisks indicate expected molecular weight. Expected protein sizes are listed:

FGSG_00777: 21 kDa
 FGSG_00979: 59 kDa
 FGSG_01111: 59 kDa
 FGSG_01346: 42 kDa
 FGSG_01422: 56 kDa
 FGSG_04527: 57 kDa
 FGSG_05615: 54 kDa
 FGSG_06021: 37 kDa
 FGSG_09690: 17 kDa
 FGSG_09870: 40 kDa
 FGSG_10549: 21 kDa
 FGSG_10746: 50 kDa
 FGSG_10855: 42 kDa
 FGSG_10942: 17 kDa
 FGSG_10974: 20 kDa
 FGSG_15824: 66 kDa

Appendix B Figure 3: Confirmation of phenotypes in CEP cell death assay in 2-week old wheat leaves. MgCl_2 (right) caused no phenotype in either ‘Sumai 3’ (top) or ‘Roblin’ (bottom). The WT *U. hordei* caused a very mild phenotype in ‘Sumai 3’ (middle, top) and a very mild phenotype in ‘Roblin’ (middle, bottom). *U. hordei* expressing SnTox3 (left) showed an extremely robust phenotype in ‘Sumai 3’ (top) and a milder response in ‘Roblin’ (bottom). Phenotypes were imaged after 5 days.

Appendix B Figure 4: Interaction between wheat ‘Roblin’ and *P. syringae* DC3000.

A: *P. syringae* infiltrated into ‘Roblin’ wheat leaves resulted in disease symptoms after 3 days. Cultures were infiltrated at an $OD_{600} = 0.2$. **B:** *P. syringae* DC3000 grew within wheat leaves over the course of three days, indicating the phenotype seen in **A** is a result of disease. Error bars represent standard error (n=3).

Appendix B Table 1: Primers for generating destination vectors of CEPs and for genotyping *U. hordei* transformants

Accession	Forward Primer for pENTR/D-TOPO (5'-3')	T _m (°C)	Reverse Primer for pENTR/D-TOPO (5'-3') (no stop codon)	T _m (°C)	Reverse Primer for pENTR/D-TOPO (with stop codon)	T _m (°C)	Amplicon Size (bp)
FGSG_00330	CACCATGGCAGCCGCT GTTGAACACG	66.2	CTTGCGCTTGGCATC ATGAACAGCCT	63.9	TCACTTGCCTTGGCAT CATGAACAGCCT	65.7	2139
FGSG_00777	CACCATGAGACCCTCC CTCTTCAAGCC	64.1	CAGCTCACCGGACTT CTCGATCTTGG	62.5	TTACAGCTCACCGGAC TTCTCGATCTTGG	62.7	686
FGSG_00921	CACCATGGCGGACGAA GTTTACGACGG	64.7	TCGGGAGGACATGG CCTTGGTG	63.7	TTATCGGGAGGACATG GCCTTGGTG	62.6	1845
FGSG_00979	CACCATGGCTCTCACCG TCGAGCT	64.8	GTACAGCTTGATGGC AACCGACT	59.5	TTAGTACAGCTTGATG GCAACCGACT	60	1479
FGSG_01111	CACCATGTCTGGCGCTG TTGCGG	65.3	AGCCTGGTAAGTAG AAGCGGAGACG	61.8	TTAAGCCTGGTAAGTA GAAGCGGAGACG	61.2	1479
FGSG_01346	CACCATGGCTATCAAG AAGGTCTTCGCCCCGCTC	67.1	GTCAATCTGGTTGAC CTTGAGAAGGAGAG CGTT	63.8	TCAGTCAATCTGGTTG ACCTTGAGAAGGAGAG CGTT	65.2	1059
FGSG_01422	CACCATGGCTTCAGTCG CTCGTCTCAGC	65.9	AAACAGCTTCTTAAC AAGCTCAGCCAC	61.2	TTACAGCTTCTTAACAA GCTCAGCCAC	60.6	1413
FGSG_01425	CACCATGGCCGAAGAG AAGGCGGTC	65.6	CTGAGACTTCTCGTG CTGGGTCG	61.3	TTACTGAGACTTCTCGT GCTGGGTCG	61.6	2769
FGSG_02077	CAC CAT GAA GTA CTC CGT CGC TTT CGT C	62.3	CAG AGC AAG GGC AGC GAA AGC AG	63	TTA CAG AGC AAG GGC AGC GAA AGC AG	63.2	
FGSG_02461	CACCATGTTGCGCGCTT CTCGCATCCAG	66.5	TTTGGGAGGGTTGGA GGCAACGAAG	63.3	TTATTTGGGAGGGTTG GAGGCAACGAAG	62.4	1010
FGSG_04181	CACCATGCCTACCGAG AACGAGCAGTCG	65.7	CAAGTCATCCTCGTC ACTGAACACCTCC	62	No primer ordered		3168
FGSG_04312	CACCATGTTCAAGAGC GGCATTTCTCCTTCG	65.2	CCCAGTAAGAGCGA CACGAGCAC	61.7	TTACCCAGTAAGAGCG ACACGAGCAC	62	810

FGSG_04527	CAC CAT GAA GGC GGC TTT GTT GAA TGT CAT C	63.1	TGT TGA TGA AAT AGG CTT CTT GGA AAG TGT GG	60.5	TCA TGT TGA TGA AATAGGCTTCTTGGA AGTGTGG	61.5	
FGSG_04826	CACCATGGTTCAACAA ATCCCCAAGGCC	63.7	TCGGCAAGTGTATCC ACCATCAATGACG	62.2	CTATCGGCAAGTGTAT CCACCATCAATGACG	61.7	798
FGSG_05615	CACCATGTCTGCTCCCG CCCACAAATTCAAG	65.7	GTAGCGGTAGATGTC GGCCTTGAAGGGA	64.7	TTAGTAGCGGTAGATG TCGGCCTTGAAGGGA	64.6	1350
FGSG_05972	CACCATGGTTGCTGAA GAGCCCCG	64.3	AGCCTTCTCGTAGAC CCAGTTGAAC	60.2	No primer ordered		717
FGSG_06021	CACCATGTCTTCCGCTG AACCCCAAGG	65.6	CTTGAAGGCCTTGCC GAAGAGAAGGACC	61.7	TTACTTGAAGGCCTTGC CGAAGAGAAGGACC	64.4	936
FGSG_06290	CACCATGTCTCCACAA ACGCCGAGTATGCGC	65.9	GATGCTGGCGTCCAG GCGCCAA	67.2	TTAGATGCTGGCGTCC AGGCGCCAA	66.7	1455
FGSG_07953	CACCATGCTCGCTACCC GCCAGGTTCT	64.8	GTTCTTGGTAGCCTT CGCCATGGTGTG	63.2	TTAGTTCTTGGTAGCCT TCGCCATGGTGTG	63.3	2367
FGSG_08601	CACCATGAATGACCCC GTCTTCAAACTGCTTC TC	61.9	GACAACGGGGCCGG CCTTGACGACATCTT	68.7	No primer ordered		1773
FGSG_08700	CACCATGGCTTCTCTCG ATTCCGTCCTCC	62	TAGCTTCGAAGCAG GGCTAGGGG	58.8	TCATAGCTTCGAAGCA GGGCTAGG	60.4	1629
FGSG_08737	CACCATGCAGGCCCCC CTGGCATA	67.7	CAGGCGAGAGCCGT GGATGACC	64.3	TTACAGGCGAGAGCCG TGGATGACC	64.3	1767
FGSG_08811	CACCATGGGTAAGGAG GAGAAGACTCACC	59.5	TTTCTTACCGGCCTT GGCAGC	60.5	TTATTTCTTACCGGCCT TGGCAGC	59.8	1383
FGSG_09366	CAC CAT GAA GGT TTC ATC CGC TGC CG	63.9	CAG AGC AAG CAT GGC GAA GAA GGC	62.7	TTA CAG AGC AAG CAT GGC GAA GAA GGC	63	
FGSG_09690	CACCATGCGGCCTTTCT CTCTTGATC	63.5	AAAACCGATCTTCTT GAGCTCGACGTCG	62	TTAACCGATCTTCTTGA GCTCGACGTCG	61.4	438
FGSG_09870	CACCATGGCCGAACAA TTGATCTTGAAGGG	62.9	TGC CCT CGA CAT GAC ACC CCA G	63.6	TTATGC CCT CGA CAT GAC ACC CCA G	62.5	1002
FGSG_10549	CACCATGCGGGATACT GAGGAGGTATC	61.8	GTGCAAGTCTCCCTT GCCACCAGAATC	63.2	CTAGTGCAAGTCTCCCT TGCCACCAGAATC	63.5	534

FGSG_10746	CACCATGCTGTCCACCC TCCGAGTCGCC	63.2	ACCCTTGACCTTGTA GATGGCCTCAGC	63.8	TTAACCCTTGACCTTGT AGATGGCCTCAGC	63	1269
FGSG_10825	CACCATGGTTCAGTCCG CTATTCTCGGTTTCC	64.6	TTTGGCGTACTTGTT TCGGAAGTACTTGGC G	63.6	TTATTTGGCGTACTTGT TTCGGAAGTACTTGGC G	63	2301
FGSG_10855	CACCATGGCCGCTCCCC AGATTCCTTC	66.5	TCGGCTGGTGTCAAC GACATAGCGA	63.9	TTATCGGCTGGTGTCA ACGACATAGCGA	62.9	1062
FGSG_10942	CACCATGGTCAAGAAG AGAAAGAACAACGG	60.9	AGCCTTAGGGGCAG TAGGGACGATC	63.6	CTAAGCCTTAGGGGCA GTAGGGACGATC	63	426
FGSG_10974	CAC CAT GAG CTA CAC TCT CTA CGA CGC	61.9	CTGGAGGTACTGGCC AATGAAAGGACC	62.6	TTACTGGAGGTACTGG CCAATGAAAGGACC	62.8	513
FGSG_15824	CACCATGTTCCGGAAC GCCCTTCGACAG	66.1	ACCGAGGAAGCTCTT GACGAAGGAC	62.2	TTAACCGAGGAAGCTC TTGACGAAGGAC	61.5	1659
FGSG_16627	CAC CAT GGC TCC CAT CAA GGT CGG CAT C	66.3	CTTGGAGGCATCGAC CTTGGCAACGTGG	66.5	TTACTTGGAGGCATCG ACCTTGGCAACGTGG	66.3	1014

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