CHARACTERIZATION OF BIOFILM FORMATION AND SILICA RESPONSE BY THE FILAMENTOUS FUNGUS *FUSARIUM GRAMINEARUM*, A PLANT PATHOGEN

By

Rebecca Shay

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Plant Biology – Doctor of Philosophy

2022

PUBLIC ABSTRACT

Plant diseases can have devastating impacts on food security. One important disease on cereal crops is Fusarium head blight (FHB), caused by the fungus Fusarium graminearum. This disease reduces the quantity and quality of crop yields, and managing disease in the field is critical to food production. Control of disease starts before the fungus has even entered the plant, and understanding the biology of the plant-pathogen interactions that happen before infection can provide new insights into control methods. To that end, I have studied two different aspects of these early plant-pathogen interactions. I characterized aquaporins in F. graminearum. Aquaporins are channels in the cells responsible for taking in water and other small molecules. I found that aquaporins are critical in fungal growth and development, and are involved in the response of the fungus to silica. Silica is an important component of plant tissues, especially the cereal crops, and F. graminearum infects at silica-rich cells. Aquaporins are involved in the fungal response to silica, which is important in understanding how infection occurs. In addition, I investigated the formation of biofilms in F. graminearum. Biofilms are three-dimensional protective structures used by microbes to survive in harsh environments. Most biofilms are studied in bacteria and yeast, not filamentous fungi. I characterized the formation of biofilms in F. graminearum, from initial adhesion to a surface, to the formation of the matrix around the cell mass, and the dissolving of mature formations. Genes identified in the work impact the formation of biofilms, including the matrix surrounding cells. Together, my work aids in the understanding of how the pathogen can successfully cause infection and disease.

ABSTRACT

Fusarium graminearum is a filamentous fungus that is the primary causal agent of the disease Fusarium head blight (FHB) on cereal crops. Understanding details of pathogen biology, especially those that are directly related to infection, is vital to control of disease. While much is known about how *F. graminearum* initiates disease into its host plants, there are still knowledge gaps related to plant signals sensed by the fungus, and mechanisms that increase success in infection. To better understand early infection, I have focused on two aspects of the plant-pathogen interaction. Aquaporins are channel proteins that bring small molecules dissolved in water into cells. In *F. graminearum,* aquaporins are important in growth, development, and spore formation, and are related to how silica is utilized by cells. Silica is an important component of cereal crops, and silica-rich cells provide infection points for *F. graminearum*. Biofilms are three-dimensional formations important to many microbes for protection from adverse environmental conditions. Biofilms have primarily been studied in single-celled organisms, but there is a growing body of work on filamentous fungal biofilms. I found that biofilm formation is initiated *in vitro* with the adhesion of propagules to a surface, followed by growth of the structures and development of an extracellular matrix, then dispersal of propagules and senescence of biofilms. I have profiled the transcriptome of biofilm formation over time, and characterized genes significant to this process. Knockouts of these genes produced altered biofilm formations, especially in matrix composition. My work has identified novel early infection characteristics in *F. graminearum,* which will provide new targets for control.

ACKNOWLEDGEMENTS

I would like to thank my committee members, Drs. Frances Trail, Martin Chilvers, George Sundin, Chris Waters, and the late Jonathan Walton for helping to guide my project, even through the difficult parts.

I also could not have done this without past and present members of the Trail lab. I'd also like to thank my PLB cohort for being there for all these years.

This work could not have been done without the financial support from the USWBSI, A.L. Rodger's Scholarship, Everett Beneke Endowment Award, and the College of Natural Science Dissertation Completion Fellowship. Many thanks to them for believing in me and my science.

Thank you to the folks who took time to read my chapters and give me feedback on them. I appreciate you all more than you know.

This dissertation would not have been possible without Chris, both his support and his cooking. Without you and Peanut, I wouldn't have made it through these last few years <3

And lastly, I'd like to thank my family for your support, understanding, and ability to not ask too many questions about my timeline.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

3D	three-dimensional
5-AZ	5-azacytidine
ABC	ATP-binding cassette
AQP	Aquaporin
AS	Artificially selected
BAM	Binary Alignment Map
BLAST	Basic local alignment search tool
BP	Base pairs
CBE	Chlorazol black E
CFW	Calcofluor white
CMC	Carboxymethyl cellulose medium
COBALT	Constraint-based multiple alignment tool
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
Dpi	Days post-inoculation
ECM	Extracellular matrix
FAA	Formalin-acetic acid
FDA	Food and Drug Administration
FGSC	Fungal Genetics Stock Center
FHB	Fusarium head blight
FPKM	Fragments Per Kilobase Million
Hph1	Hygromycin resistance cassette

Hpi	Hours post-inoculation
ICP-AES	Inductively coupled plasma atomic emission spectroscopy
KCl	Potassium chloride
Nat	Nouseothricin resistance cassette
NCBI	National Center for Biotechnology Information
NIL	Near-isogenic line
NIP	Nodulin 26-like intrinsic protein
Nit+/-	Nitrate-utilizing/non-nitrate-utilizing
NPA	Asparagine-proline-alanine
NR	Nile red
NRRL	Agricultural Research Service Culture Center
PCR	Polymerase chain reaction
PI	Propidium iodide
QoI	Quinone outside inhibitor
qPCR	Quantitative polymerase chain reactions
QTL	Quantitative trait loci
RNA	Ribonucleic acid
RT	Room temperature
RTA	Real Time Analysis
SAM	Sequence Alignment Map
SDHI	Succinate dehydrogenase inhibitor
SIP	Small basic intrinsic protein
Spp.	Species

TF	Transcription factors
TIP	Tonoplastic intrinsic protein
VeA	Velvet A
WT	Wild-type

CHAPTER 1

LITERATURE REVIEW

Part 1: Fusarium graminearum

Cereal crops including wheat, barley, and corn are important food sources for humans and livestock, and diseases of these crops can have a large impact on food security. The filamentous fungus *Fusarium graminearum* is the primary causal agent of Fusarium head blight (FHB), a disease causing significant yield losses and mycotoxin contamination in crops. In 2010, parts of Ohio reported 60% incidence of *F. graminearum* in wheat fields, which is a level of disease that occurs worldwide when environmental conditions are conducive to disease (McMullen et al. 2012). *F. graminearum* commonly initiates infection through the developing florets during anthesis. After successful infection, developing grains become filled with fungal hyphae (Figure 1.1). In the spring, fruiting bodies (perithecia) form on colonized crop residues and sexual spores (ascospores) are forcibly discharged, forming the primary inoculum of FHB. The fungus will spread to additional florets, internally through the rachis and stalk (Guenther and Trail 2005; Boenisch and Schäfer 2011; Harris et al. 2016; Jin et al. 2021). Ascospores are airborne long distances whereas asexual spores (conidia) are locally dispersed primarily by rain splash (Maldonado-Ramirez et al. 2005; Keller, Bergstrom, and Shields 2014). In order to become airborne, ascospores are forcibly discharged from perithecia and travel until spores land or rain forces spores down (Maldonado-Ramirez et al. 2005; Keller, Bergstrom, and Shields 2014). At the end of the growing season, *F. graminearum* overwinters in colonized crop residues, where during spring temperatures perithecia will develop, and the cycle continues (Andries, Jaroz, and Trail 2000; Bai and Shaner 2004; Osborne and Stein 2007; Leplat et al. 2013).

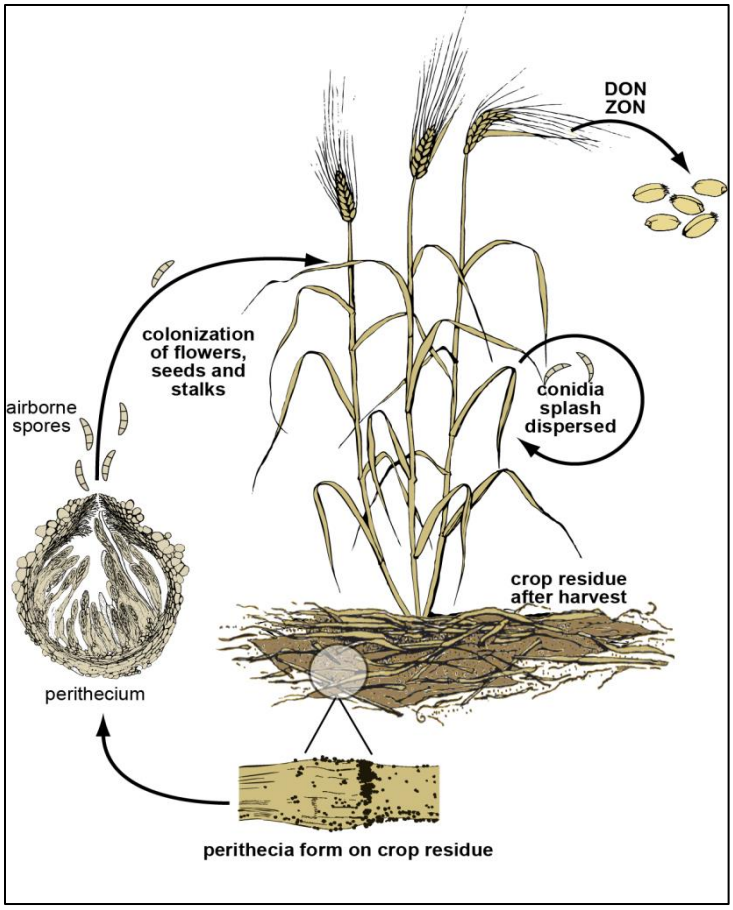


Figure 1.1: Disease cycle of *Fusarium graminearum* on wheat. Spores infect cereal crops during flowering, which leads to grains filled with fungal hyphae and production of mycotoxins. Conidia and ascospores then infect other plants in the area, perpetuating the disease. The fungus overwinters in debris left on the field. (Figure from Trail 2009).

Mycotoxins in Fusarium graminearum

Mycotoxins produced during plant infection can help the fungus evade plant defenses and colonize cells. These mycotoxins are of concern, as many are toxic to mammals, with a range of effects from intestinal distress to cancer (Bottalico and Perrone 2002; Pestka and Smolinski 2005; Escrivá, Font, and Manyes 2015). *Fusarium* species produce three classes of mycotoxins: trichothecenes, fumonisins, and zearalenones (Trail 2009; Summerell and Leslie 2011; Escrivá, Font, and Manyes 2015). The trichothecene class of mycotoxins includes deoxynivalenol (DON), the main mycotoxin contaminant found in grain in the United States (Bottalico and Perrone

2002; Cumagun et al. 2004). The US Food and Drug Administration (FDA) suggests advisory level recommendations, while industries follow DON contamination limits of ≤ 0.5 to 1.0 mg/kg (Jin et al. 2021). In Europe, both DON and zearalenones are commonly found in harvested grain, and therefore have regulatory levels imposed (Summerell and Leslie 2011; Escrivá, Font, and Manyes 2015). Mycotoxins are not just important in FHB as a risk to mammalian health, but they are also important in the infection process and life cycle of the fungus. DON has been shown to be a virulence factor important for the infection of plants (Leonard and Bushnell 2003; Kazan, Gardiner, and Manners 2012).

Current methods for managing FHB

Approaches for integrated disease management combine fungicide applications with the use of tolerant crop varieties and appropriate cultural practices (McMullen et al. 2012; Willyerd et al. 2012; Amarasinghe et al. 2013; Shah et al. 2018; A. Chen, Islam, and Ma 2022). Environmental conditions greatly impact disease progression, where high humidity is a major driving force for disease, leading to severe incidence of FHB in the field, and greater potential for DON contamination (Miedaner, Cumagun, and Chakraborty 2008). Although fungicides will provide some protection for susceptible varieties, high disease pressure in fields can result in greater than 60% disease incidence even with fungicide applications (Haidukowski et al. 2005). In wheat, fungicides are applied during a short window at flowering, up to 11 days post-anthesis, when fungal infection is highest (Freije and Wise 2015; Caldwell et al. 2017). In barley, fungicide timing is more dependent on whether the cultivar is open-flowering or closed-flowering, where closed-flowering cultivars benefit from a later application (Yoshida et al. 2012). Additionally, multiple fungicide sprays during the growing season control FHB most effectively for the full season, although different fungicides come with different timing recommendations for disease

control (Haidukowski et al. 2005; Tateishi et al. 2014; Caldwell et al. 2017).

Chemical control

The most commonly used fungicides in the United States to control FHB are from the azole class. Azoles target the ergosterol biosynthetic pathway, specifically the cytochrome P450 sterol 14α-demethylase (CYP51), leading to instability in cell membranes (Ragsdale and Sisler 1972; Y. Chen and Zhou 2009; Amarasinghe et al. 2013; Freije and Wise 2015; Caldwell et al. 2017; Paul et al. 2018; Anderson et al. 2020). When using metconazole, double applications of fungicides decrease mycotoxin contamination (Tateishi et al. 2014). For a single application protocol, a combination of tebuconazole and prothioconazole applied around flowering, along with growing resistant cultivars, provides good protection and integrated disease management (Willyerd et al. 2012). Recently, a fungicide in the succinate dehydrogenase inhibitor (SDHI) class was registered to control FHB. SDHIs inhibit the electron transport chain and reduce energy production in fungi (Avenot and Michailides 2010; Hou et al. 2017; Breunig and Chilvers 2021; Sun et al. 2021). Other classes of fungicides can have negative impacts on FHB control, where the use of quinone outside inhibitor (QoI) fungicides, which target the quinol outer binding site of the cytochrome bc_1 complex in the electron transport chain, can increase DON production, even if disease incidence in the field is reduced (Anke et al. 1977; Caldwell et al. 2017; Paul et al. 2018).

Cultural practices

Proper crop rotation to reduce the pathogen load is important in management. For example, FHB incidence is reduced when wheat is followed by soybeans in a rotation, instead of corn (Dill-Macky and Jones 2000). Additionally, DON levels were significantly reduced in wheat grown in a field that grew soybeans the previous year, while levels were increased in wheat following corn

plantings (Dill-Macky and Jones 2000). Beyond crop rotation, tillage has been shown to reduce the survival of *F. graminearum* on crop residue, where after two years, the colonization on buried crop residue was halved compared to the residue left on the surface (Pereyra, Dill-Macky, and Sims 2004). Furthermore, the kind of tillage used impacts FHB incidence, where moldboard-plowed plots had lower FHB than chisel-plowed or no-till plots (Dill-Macky and Jones 2000). Across the Midwest, no-till has been adopted to reduce water runoff and soil erosion (Silva and Delate 2017), which in turn leaves an increased amount of crop residue to contribute to FHB pathogen load (Dill-Macky and Jones 2000; Wegulo et al. 2015). It has also been shown that moisture increases FHB, therefore, irrigation impacts disease development as well (Lemmens et al. 2004; Cowger et al. 2009). However, DON may be reduced by post-anthesis irrigation, although it is likely not a significant factor to consider in management practices due to the impracticality (Lemmens et al. 2004). Applications of fertilizers have mixed impacts of FHB, dependent on conditions and forms of the fertilizers used. For an overview on the contradictory nature of fertilizers and FHB management, I recommend the review by Champeil, Doré, and Fourbet (2004).

Plant resistance

Within wheat breeding programs, screening for FHB resistance occurs during variety development, and the fine mapping of the known resistance quantitative trait loci (QTLs) has helped increase the speed and precision of early screening. Resistance to FHB is classified into categories based on the method of conferring resistance. Type I is defined by resistance to initial fungal infection, while Type II is the suppression of the spread of FHB within the host plant (Pritsch et al. 2000). Seven QTLs have been identified to specific gene regions which confer Types I and II resistance, which have been introduced to wheat and used in breeding programs to

increase crop tolerance to FHB (Brar et al. 2019). The QTLs *Fhb1* and *Fhb2* were identified in the wheat variety Sumai 3, and confer Type II resistance, and have been mapped to chromosomes 3BS and 6 BS, respectively (Cuthbert et al. 2006; Cuthbert, Somers, and Brulé-Babel 2007). *Fhb3* was identified in a screen of wheat land races and wild relatives, from the tetraploid wheat *Leymus racemosus* (Qi et al. 2008). *Fhb4* and *Fhb5* exhibit Type I resistance and were identified from the cultivated wheat variety Wanshuibai (Xue et al. 2010, 2011). *Fhb6* was identified in the perennial grass *Elymus tsukushiensis*, which exhibits resistance to FHB naturally, and was crossed into cultivated wheat varieties (Cainong et al. 2015). Lastly, *Fhb7* was identified from *Thinopyrum ponticum,* that can confer resistance by detoxifying DON (Guo et al. 2015). *Fhb7* has been incorporated into breeding programs, as it can easily pyramid with *Fhb1* to increase resistance (Guo et al. 2015). Interestingly, *Fhb7* does not have any homology to other genes in any plant species, but instead was shown to be homologous to a gene found in the endophytic fungal genus *Epichloë,* which suggests that the gene was horizontally transferred from the fungus to its plant host at some point evolutionarily, possibly to combat wild *Fusarium* infections (Wang et al. 2020). In addition to the Type I and Type II resistances that are typically utilized in breeding programs, other resistance types include resistance to kernel infection (Type III), tolerance to fungal infection (Type IV), and resistance to mycotoxin accumulation (Type V) (Pritsch et al. 2000; Shah et al. 2018). These resistance types exist but are rarely incorporated into current breeding programs due to a lack of knowledge of the mechanisms behind them. As is evidenced by the types of resistance, preventing fungal infection and internal spread is key to controlling disease.

In barley, natural Type II resistance occurs, where *F. graminearum* cannot spread internally (Bai and Shaner 2004; Boddu et al. 2006; Harris et al. 2016). However, barley tends to be very

susceptible to initial infection, with 2-row barley being typically more susceptible than 6-row barley (Bai and Shaner 2004; Boddu et al. 2006; Harris et al. 2016). Wild relatives of barley have been screened for resistance, in effort to provide a reservoir of resistance genes that would be easy to breed into barley (Bai and Shaner 2004). Wild *Hordeum* species, however, are not more resistant to FHB than the cultivated varieties, which increases the difficulty of introducing resistance to cultivated barley varieties. QTLs from cultivated barley that exhibit more resistance have been traced, with most FHB resistance-related QTLs mapping with other traits of interest, such as heading time (Bai and Shaner 2004). Since much of the barley grown in the United States is used for malting in the brewing industry, there is much need to increase work on introducing resistance to barley lines, as malting barley contaminated with FHB leads to production issues (He et al. 2015; Piacentini et al. 2015).

Part 2: Plant-pathogen interactions in early infection

Early infection is crucial in disease development

As described above, the time period for the successful control of disease by fungicides during the growing season is narrow, with only a few days around anthesis as the ideal time to manage disease (Willyerd et al. 2012; Tateishi et al. 2014). This highlights the importance of arresting initial infection by *F. graminearum*, since once the pathogen has penetrated the plant, it is less accessible for control by integrated disease management. Importantly, *F. graminearum* colonizes the plant surface before penetration, which makes this early colonization an ideal first infection time point for overall disease control (Boenisch and Schäfer 2011). The initial penetration of *F. graminearum* into the plant is well studied, and it has been shown that initial colonization and infection occurs primarily through natural openings (Pritsch et al. 2000; Leonard and Bushnell 2003; Boenisch and Schäfer 2011). The conidia of *F. graminearum* can become trapped at the

juncture of trichomes, where they germinate and the hyphae wrap around the trichome, before entering the plant at the base of the trichome (X. Chen et al. 2006; Imboden, Afton, and Trail 2018). During surface colonization, changes in transcriptional regulation of fungal genes lead to the production of secreted proteins, especially plant cell wall degrading enzymes (Paper et al. 2007; Mentges et al. 2020; Miguel-Rojas et al. *in press*). With the currently available management strategies for FHB, examining early colonization of *F. graminearum* provides new opportunities for controlling disease, with the potential to stop disease before infection occurs.

Silica and the relationship to fungal diseases

Silicon is a non-essential element that is important in plant growth and development, which is actively taken up by plants in the form of silicic acid, before being polymerized into silica gels in plant tissues (J. Ma, Miyake, and Takahashi 2001; E. Epstein 2009). In grass crops, silica, the oxide form of the element silicon, is an important component of plant structure and can make up to 10% of the dry weight (E. Epstein 2009). Polymerized silica gel, also known as amorphous silica, is deposited in the trichomes, stomata, along the vascular bundle, and in epidermal cells (E. Epstein 1994). The broad term for these deposits is phytoliths, however, that term can also specifically refer to the deposits in non-specialized epidermal cells (O'Reagain and Mentis 1989; Schilmiller, Last, and Pichersky 2008; E. Epstein 2009). The deposition of silica in trichomes has been shown to be a defense mechanism against herbivory, where silicified trichomes jut out from the plant as hardened spikes, making chewing on a plant difficult (O'Reagain and Mentis 1989; Schilmiller, Last, and Pichersky 2008; E. Epstein 2009; Kim 2019). Interestingly, this deposition of silica led to the evolution of large grinding molars in herbivorous animals (Vicari and Bazely 1993; Schilmiller, Last, and Pichersky 2008; Kim 2019).

In addition to protection from herbivory, silica has been implicated in the protection of plants

from fungal pathogens (J. F. Ma 2003; Schilmiller, Last, and Pichersky 2008; E. Epstein 2009; Hauser 2014). External application of silica has been shown to reduce severity of a variety of fungal diseases, including rice blast, sheath blight of rice, and powdery mildew in wheat, barley, and cucurbits (J. Ma, Miyake, and Takahashi 2001; J. F. Ma 2003; Sakr 2016). Silica bolsters plant cell walls, by adding a strong layer of silica below the plant surface that decreases the ability of fungi to force their way through plant cells (J. Ma, Miyake, and Takahashi 2001; Sakr 2016). Strikingly, many of the cells *F. graminearum* interacts with during the disease cycle are silica-rich cell types, such as the trichomes, which as previously discussed are common points of plant infection as well as stomates. Additionally, disease progresses through the plant with the movement of fungal hyphae through the xylem, which is part of the silica-rich vascular system (Langevin, Eudes, and Comeau 2004; Guenther and Trail 2005; Jansen et al. 2005; Trail 2009). Recent work has shown that the external application of silica can reduce Fusarium stem blight, and FHB severity in the field, especially in combination with fungicide treatments (Pazdiora et al. 2022; Sakr 2022). However, Pazdiora et al. (2022) noted that although disease severity may be reduced when plants are treated with silica, there is an increase in damage to the grains themselves. Sakr (2022) observed extensive hyphal colonization of the surface of inoculated stems, regardless of silica application. This suggests a relationship between silica and the transition of *F. graminearum* from initial colonizing hyphae to infection hyphae. Understanding whether silica is an important protective mechanism for host plants or a necessary cue for the initiation of fungal infection would allow for better disease management.

Part 3: Biofilms as an important infection and survival structure

Biofilms: characteristics and importance

Biofilms are three-dimensional structures that consist of complexes of living and dead microbes

adhering to their surroundings and to each other by an extracellular matrix (Fanning and Mitchell 2012). Microbes will form a biofilm as a collaborative community to improve survival especially in a changing environment (e.g. shifts in salinity, pH, desiccation, etc.; Harding et al. 2009). The most recognized microbial biofilm is likely dental plaque, though biofilms have been found in many fluctuating environments in food production, medical, and industrial settings (Ahmad and Husain 2017). There structures are more resistant to changes in environments than free-living cells due to their ability to resist being washed away, and the protection provided by both the extracellular matrix and the complex structure of the biofilm (Davies 2003; De Souza et al. 2004; Imamura et al. 2008; Harding et al. 2009; Nobile et al. 2009; Singh, Shivaprakash, and Chakrabarti 2011; Koczan et al. 2011; Ramage et al. 2012; Fanning and Mitchell 2012; Peiqian et al. 2014; Calvo et al. 2016; L. Epstein and Nicholson 2016; Pompilio and Di Bonaventura 2018; Kischkel et al. 2019; Kowalski et al. 2019). In general, bacterial biofilms are 10-1,000 fold less susceptible to antimicrobial agents than free-living cells (Davies 2003), and *Fusarium oxysporum* biofilms are less susceptible to UV radiation, cold, heat, and fungicides than free-living cells (Peiqian et al. 2014). In medical settings, *Candida* spp. often form biofilms in equipment and on joint replacements, which then cause infections in humans (Finkel and Mitchell 2011). If infections occur, treatment is difficult due to biofilm cells being more resistant to external stressors, as well as the difficulties posed by fungal pathogens in humans, requiring higher doses of antifungal treatments, which can increase side effects in patients (Finkel and Mitchell 2011; Ramage et al. 2012).

The ability of biofilms to resist external environmental and biological pressures works to help protect plant pathogens during infection (Harding et al. 2010; Motaung et al. 2020). Bacterial and fungal biofilms are more resistant to some plant host defenses than free-living cells (Ramey

et al. 2004; Ahmad and Husain 2017; Pandin et al. 2017). Plant pathogenic biofilms can protect the cells from host defense responses and environmental conditions. For example, xylem-dwelling microbes survive the flow of xylem sap by adhering to surfaces to avoid being washed out of the system (Almeida et al. 2001; Koczan et al. 2011; Moleleki et al. 2017). Biofilms have been hypothesized as a reason plant fungal infections are difficult to control, as fungicides are tested on free-living cells, without taking into account the added resistance of biofilm communities (Ahmad and Husain 2017). In contrast, biofilms can also trigger plant defenses. In some species, such as the bacterial plant pathogen *Pseudomonas syringae*, quorum sensing signaling molecules help initiate biofilm production, which in turn trigger plant defenses in *Arabidopsis thaliana*, showing how closely connected plant defenses are to microbial processes (Walker et al. 2004). Furthermore, plant defenses triggered by quorum sensing signals will in turn elicit an increase in salicylic acid production, a plant defense compound that is effective in limiting biofilm adhesion and formation (Prithiviraj et al. 2005). Unfortunately, the triggered plant defenses are often not sufficient to stop infection, as biofilms are stable structures that will survive defense responses, even if some cells die. This increased tolerance of biofilms over planktonic cells to traditional methods of control needs to be considered in both medical and agricultural settings.

Biofilm growth and development

Biofilm development in yeasts and bacteria is depicted in a four-stage model: (1) single free-living cells attach to a surface; (2) the attached cells recruit additional single cells from the surrounding area; (3) colonies form an extracellular matrix (ECM) and internal structures differentiate; (4) cells from the mature biofilm detach and disperse (Harding et al. 2009; Finkel and Mitchell 2011; Pandin et al. 2017; Córdova-Alcántara et al. 2019). While this model was

first developed for bacterial biofilms, it was also possible to apply to single-celled fungi such as *Candida albicans* (Reynolds and Fink 2001; Finkel and Mitchell 2011; Fanning and Mitchell 2012). For biofilm formation, adhered cells will recruit others to develop a complex structure that includes formations of water channels, and ultimately propagules, which will be dispersed from the mature biofilm. Bacterial biofilms have been most studied, due to the ease of differentiating between single free-living bacterial cells and a community of many bacterial cells. Progress in understanding bacterial biofilms has been applied to single-celled fungi such as yeasts which exhibit the same behavior under certain environmental conditions (Reynolds and Fink 2001; Fanning and Mitchell 2012). However, there is increasing interest in biofilms produced by filamentous fungi and their role in ecology and pathogenicity.

Methods for studying biofilms

Historically, biofilms have been studied in single-celled organisms, and indeed, the requirements for a structure to be called a biofilm are based on organisms with this characteristic. The traditional way of determining if an organism produces a biofilm *in vivo* is an adhesion assay, since adhesion is a first step in biofilm development. This usually involves growing cells in liquid culture in an appropriate culture plate for the species, washing off any planktonic cells with water, and quantifying the size of the biofilm, often by staining to measure quantities of adhered cells (O'Toole et al. 1999; Reynolds and Fink 2001; O'Toole 2011). Stains (often crystal violet) must be able to infiltrate both living and dead cells to fully stain the biofilm complex, and be easily recovered from cells for quantification without interference of cellular debris. High throughput versions of adhesion assays utilize 96-well plates to quickly assay multiple conditions or strains, with easy quantification from the absorbance of the stain (O'Toole 2011). In addition to adhesion assays, microscopy has allowed for the visualization of biofilms *in situ*. Laser

scanning confocal microscopy is one useful technique, as it is particularly useful for three-dimensional biofilms (Azeredo et al. 2017). Staining combined with confocal microscopy can elucidate important physical and physiological attributes of biofilms. This combination has been used to visualize the juxtaposition of both living and dead cells, as well as how the composition changes over time as the biofilm develops (Peiqian et al. 2014). Additionally, staining, together with confocal microscopy, has been used to determine that some species of fungi change the pH of their environment as they develop biofilms (Schlafer, Kamp, and Garcia 2018).

One limitation for studying biofilms in filamentous fungi is the lack of definitive methods to use. Studies in filamentous fungi have focused primarily on the initial adhesion of cells to a surface (Merritt, Kadouri, and O'Toole 2005; Harding et al. 2010; O'Toole 2011; Theodorakopoulos et al. 2011; Peiqian et al. 2014; P. Li et al. 2015; Kischkel et al. 2019). These studies were performed using traditional biofilm assays, often not changed from the methods used for single-celled organisms. However, unlike single-celled organisms, filamentous fungi produce a mycelial mat, which can obfuscate identification of biofilm structures. Adhesion assays have been adapted for filamentous fungi, where instead of adhering to the edges of a plate well, cells adhere to a wooden surface suspended in liquid medium to attempt to mimic more natural surfaces than polystyrene (Harding et al. 2010). Additionally, to view adhesion in a more "natural" environment, Harding et al. (2010) utilized balsa wood blocks, suspended in microtiter plate wells to provide a surface for adhesion, which were then viewed under a microscope, allowing for closer examination. However, the lack of common methods for studying filamentous fungal biofilms leads to the inability to compare across species, and a lack of resources available to initiate studies of biofilm formation in a species of interest. Moving away from the traditional adhesion methods has likely broadened the definition of biofilms, and

expanded our understanding of them to filamentous fungi, instead of exclusively single-celled organisms.

Genetics of biofilms

Much of the understanding of the genetics of fungal biofilm formations comes from clinically important human pathogens, where the majority of the work was done in the pseudo-hyphal *Candida* spp. The following summarizes much of what is currently known about fungal biofilm genetics. For more information, I recommend the reviews by Finkel and Mitchell (2010), Ramage et al. (2009), and Ramage et al. (2012).

Cell surface-related genes

Cell surface proteins are key to biofilm formation in yeasts, since the ability of cells to adhere to a surface and to each other is a critical step in biofilm formation (Reynolds and Fink 2001). Early studies in *Saccharomyces cerevisiae* identified *Flo11* (*Flo*culation), which encodes a cell surface glycoprotein, that when missing causes reduced biofilm adhesion to polystyrene surfaces (Reynolds and Fink 2001). In *C. albicans*, the adherence gene *ALS1* is upregulated when the fungus is in a biofilm, and is similar to the reported difference in adhesin expression seen in *A. fumigatus* biofilms (García-Sánchez et al. 2004; Fanning and Mitchell 2012; Gibbons et al. 2012). *Eap1* regulates a cell wall protein in *C. albicans* that functions directly in biofilm adherence (F. Li et al. 2007). Many glycosidases and glycosylated mannoproteins have been identified as important in the adhesion of *Candida* spp. biofilms, and can both positively and negatively regulate biofilm formation (Finkel and Mitchell 2011; Ramage et al. 2012). Single proteins that have been shown to be involved in the formation of biofilms are localized in the cell wall, or directly influence cell wall processes, which emphasizes the importance of the cell wall in biofilm formations.

Transcription factors

Not surprisingly, transcription factors (TF) have been identified as playing a role in biofilm formation, as they often regulate multiple gene clusters or cellular pathways. One of the first identified TF in biofilm formation is *Flo8*, a regulatory gene that controls the expression of *Flo11* in *S. cerevisiae* (Reynolds and Fink 2001). Numerous TF have been identified in *C. albicans* to play a role in biofilm formation and regulation, in all stages of the formation of biofilms, including six (Bcr1, Tec1, Efg1, Ndt80, Rob1, and Brg1) that have been identified as a core group of biofilm regulators (Finkel and Mitchell 2011; Nobile et al. 2012; Alim, Sircaik, and Panwar 2018). Bcr1 is a transcription factor that activates cell-surface proteins and adhesin genes, which are critical to biofilm formation (Nobile et al. 2012). Tec1, Efg1, and Ndt80 are involved in the transition from yeasts to pseudohyphal cells during early biofilm formation, and have important roles managing morphogenesis, with Tec1 and Efg1 being involved in the regulation of cell adhesion as well (Ramage et al. 2002, 2009; Nobile et al. 2012; Inglis and Sherlock 2013). Brg1 recruits the histone deacetylase Hda1 during hyphal cell elongation to help with cellular growth (Lu, Su, and Liu 2012). Interestingly, 5 of these 6 TF are involved in the Ras signal transduction pathway, which is used by *C. albicans* to respond to environmental stimuli, indicating how intertwined many of these processes are (Inglis and Sherlock 2013). In addition to the six core TF in *C. albicans*, the Zap1/Csr1 TF negatively regulates the production of biofilm matrix, specifically the carbohydrates that comprise the majority of the biofilm (Nobile et al. 2009). In filamentous fungi, the Velvet A (VeA) TF has been shown to play a role in biofilm formation in *Fusarium* spp. and in the response to external stressors in *Aspergillus* spp. (Calvo 2008; Jiang et al. 2011; P. Li et al. 2015; Calvo et al. 2016). Gene knockouts of *VeA* in *F. oxysporum* and *A. nidulans* exhibited reduced adhesion, and weaker resistance to external

stresses (Calvo 2008; Jiang et al. 2011; P. Li et al. 2015; Calvo et al. 2016). While TF impact the biofilm process, it is not always clear what else is being regulated at the same time with each TF, especially in filamentous fungi, and continuing to study the pathways involved is crucial.

Matrix formation

The ECM that surrounds biofilms is critical to the increased resistance to external stressors and is used as a protective layer as well as for cell-to-cell communication within the biofilm (Allison 2003). The ECM is a complex of molecules, including carbohydrates, proteins, extracellular nucleic acids, lipids, and more, with a major component being water (Allison 2003; Finkel and Mitchell 2011). As with other processes, the genes that regulate *C. albicans* matrix formation are relatively well-studied, with the TF Zap1 being involved in regulating multiple genes that are involved in matrix accumulation (Finkel and Mitchell 2011). Glucan, one of the major components of *Candida* spp. ECM, is positively regulated by the TF Rlm1 (Nett et al. 2010). Genes important to ECM formation in true filamentous fungi are not well-characterized, as the matrices of filamentous fungi are not well characterized. Although *Candida* spp. provide insight into genes that may be involved in ECM formation in all fungi, much more work is needed in all aspects of regulation of fungal ECM.

Quorum sensing, signaling, and influencing the surrounding environment

Quorum sensing, or the ability of microbes to sense how many like microbes are in the area, is another critical component of biofilm formation. Interestingly, farnesol is a molecule used for quorum sensing by multiple species, including both bacteria and fungi, for quorum sensing, and can aid in mixed-species biofilm formation (Finkel and Mitchell 2011). The *C. albicans* alcohol dehydrogenases CaADH5, CaCSH1 and CaLFD6 have been suggested to be involved in quorum sensing by regulating the formation of biofilms and ECM (Ramage et al. 2012). Additionally,

genetic screens of biofilms identified a large number of alcohol dehydrogenases, which are used to sense the external environment and send signals from fungal cells, processes which are important for substrate recognition and quorum sensing (Finkel and Mitchell 2011; Ramage et al. 2012). Other dehydrogenases are also likely to influence the external environment, such as changing the pH around the cells, to provide a conducive environment for nutrient uptake (Schlafer, Kamp, and Garcia 2018). Genes that confer tolerance to the surrounding environment are also important in biofilm formation. Multi-drug efflux pumps were found to be upregulated in both *Candida* and *Aspergillus* biofilms, influenced by the ATP-binding cassette (ABC) transporters *CDR1*, *CDR2*, and the major facilitator *MDR1* in *C. albicans* (Prasad et al. 1995; White 1997; Ramage et al. 2012). *CDR1* and *CDR2*, along with the ergosterol biosynthesis gene *ERG11*, have been implied in resistance to azole fungicides in *C. albicans*, leading to the increased resistance of biofilms to fungicides (Ramage et al. 2012). Similar characteristics have been shown in *Aspergillus* spp., where biofilms are more resistant to external stressors, although the underlying genes regulating this phenotype are not as well-studied as *C. albicans* (Mowat et al. 2008; Ramage et al. 2009; Zheng et al. 2015).

Part 4: Conclusions

Although much is known about the filamentous fungal plant pathogen *F. graminearum*, there are still gaps in the knowledge of the processes occurring during early infection and colonization. As shown, there is a lack of information about filamentous fungal biofilms, especially the underlying genes involved in the process, and *F. graminearum* provides an excellent study system of biofilm formation in filamentous fungi. My work on early infection and biofilm formation provides insight into infection and survival of F. graminearum. Understanding the importance of silica in infection sites may lead to different plant breeding strategies, to utilize the

natural silica in plants better. Additionally, information on how filamentous fungal plant pathogens form biofilms can be used in many pathosystems, and help to adapt treatment strategies to the more stable formations that are formed.

REFERENCES

Ahmad, Iqbal, and Fohad Mabood Husain, eds. 2017. *Biofilms in Plant and Soil Health*. Wiley.

Alim, Darakshan, Shabnam Sircaik, and Sneh Lata Panwar. 2018. "The Significance of Lipids to Biofilm Formation in *Candida albicans*: An Emerging Perspective." *Journal of Fungi* 4 (4). https://doi.org/10.3390/jof4040140.

Allison, David G. 2003. "The Biofilm Matrix." *Biofouling* 19 (SUPPL.): 139–49. https://doi.org/10.1080/0892701031000072190.

Almeida, R. P.P., E. F. Pereira, A. H. Purcell, and J. R.S. Lopes. 2001. "Multiplication and Movement of a Citrus Strain of *Xylella fastidiosa* within Sweet Orange." *Plant Disease* 85 (4): 382–86. https://doi.org/10.1094/PDIS.2001.85.4.382.

Amarasinghe, Chami C., Lily Tamburic-Ilincic, Jeannie Gilbert, Anita L. Brûlé-Babel, and W. G. Dilantha Fernando. 2013. "Evaluation of Different Fungicides for Control of Fusarium Head Blight in Wheat Inoculated with 3ADON and 15ADON Chemotypes of *Fusarium graminearum* in Canada." *Canadian Journal of Plant Pathology* 35 (2): 200–208. https://doi.org/10.1080/07060661.2013.773942.

Anderson, Nolan R., Anna N. Freije, Gary C. Bergstrom, Carl A. Bradley, Christina Cowger, Travis Faske, Clayton Hollier, et al. 2020. " Sensitivity of *Fusarium graminearum* to Metconazole and Tebuconazole Fungicides Before and After Widespread Use in Wheat in the United States ." *Plant Health Progress* 21 (2): 85–90. https://doi.org/10.1094/php-11-19-0083-rs.

Andries, Corrie, Andrew Jaroz, and Frances Trail. 2000. "Effects of Rainfall and Temperature on Production of Perithecia by *Gibberella zeae* in Field Debris." *2000 National Fusarium Head Blight Forum*, 118–22.

Anke, T, F Oberwinkler, W. Steglich, and G. Schramm. 1977. "The Strobilurins - New Antifungal Antibiotics from the Basidiomycete *Strobilurus tenacellus*." *The Journal of Antibiotics* 30 (10): 806–10. https://doi.org/10.7164/antibiotics.30.806.

Avenot, Hervé F., and Themis J. Michailides. 2010. "Progress in Understanding Molecular Mechanisms and Evolution of Resistance to Succinate Dehydrogenase Inhibiting (SDHI) Fungicides in Phytopathogenic Fungi." *Crop Protection* 29 (7): 643–51. https://doi.org/10.1016/j.cropro.2010.02.019.

Azeredo, Joana, Nuno F. Azevedo, Romain Briandet, Nuno Cerca, Tom Coenye, Ana Rita Costa, Mickaël Desvaux, et al. 2017. "Critical Review on Biofilm Methods." *Critical Reviews in Microbiology* 43 (3): 313–51. https://doi.org/10.1080/1040841X.2016.1208146.

Bai, Guihua, and Gregory Shaner. 2004. "Management and Resistance in Wheat and Barley to Fusarium Head Blight." *Annual Review of Phytopathology* 42 (1): 135–61. https://doi.org/10.1146/annurev.phyto.42.040803.140340.

Boddu, Jayanand, Seungho Cho, Warren M. Kruger, and Gary J. Muehlbauer. 2006. "Transcriptome Analysis of the Barley - *Fusarium graminearum* Interaction." *MPMI* 19 (4): 407–17.

Boenisch, Marike J., and Wilhelm Schäfer. 2011. "*Fusarium graminearum* Forms Mycotoxin Producing Infection Structures on Wheat." *BMC Plant Biology* 11 (July). https://doi.org/10.1186/1471-2229-11-110.

Bottalico, Antonio, and Giancarlo Perrone. 2002. "Toxigenic Fusarium Species and Mycotoxins Associated with Head Blight in Small-Grain Cereals in Europe." *European Journal of Plant Pathology* 108 (7): 611–24. https://doi.org/10.1023/A:1020635214971.

Brar, Gurcharn Singh, Anita L. Brûlé-Babel, Yuefeng Ruan, Maria Antonia Henriquez, Curtis Jerry Pozniak, Hadley Randal Kutcher, and Pierre Jan Hucl. 2019. "Genetic Factors Affecting Fusarium Head Blight Resistance Improvement from Introgression of Exotic Sumai 3 Alleles (Including Fhb1, Fhb2, and Fhb5) in Hard Red Spring Wheat." *BMC Plant Biology* 19 (1): 1–19. https://doi.org/10.1186/s12870-019-1782-2.

Breunig, Mikaela, and Martin I Chilvers. 2021. "Baseline Sensitivity of *Fusarium graminearum* from Wheat , Corn , Dry Bean and Soybean to Pydiflumetofen in Michigan , USA." *Crop Protection* 140 (September 2020): 105419. https://doi.org/10.1016/j.cropro.2020.105419.

Cainong, Joey C., William W. Bockus, Yigao Feng, Peidu Chen, Lili Qi, Sunish K. Sehgal, Tatiana V. Danilova, Dal Hoe Koo, Bernd Friebe, and Bikram S. Gill. 2015. "Chromosome Engineering, Mapping, and Transferring of Resistance to Fusarium Head Blight Disease from *Elymus tsukushiensis* into Wheat." *Theoretical and Applied Genetics* 128 (6): 1019–27. https://doi.org/10.1007/s00122-015-2485-1.

Caldwell, Claude D., D. Macdonald, Y. Jiang, M. A. Cheema, and J. Li. 2017. "Effect of Fungicide Combinations for Fusarium Head Blight Control on Disease Incidence, Grain Yield, and Quality of Winter Wheat, Spring Wheat, and Barley." *Canadian Journal of Plant Science* 97 (6): 1036–45. https://doi.org/10.1139/cjps-2017-0001.

Calvo, Ana M. 2008. "The VeA Regulatory System and Its Role in Morphological and Chemical Development in Fungi." *Fungal Genetics and Biology* 45 (7): 1053–61. https://doi.org/10.1016/j.fgb.2008.03.014.

Calvo, Ana M., Jessica M. Lohmar, Beatriz Ibarra, and Timothy Satterlee. 2016. "Velvet Regulation of Fungal Development." *The Mycota*, no. January: 475–97. https://doi.org/10.1007/978-3-319-25844-7_5.

Champeil, A., T. Doré, and J. F. Fourbet. 2004. "Fusarium Head Blight: Epidemiological Origin of the Effects of Cultural Practices on Head Blight Attacks and the Production of Mycotoxins by Fusarium in Wheat Grains." *Plant Science* 166 (6): 1389–1415. https://doi.org/10.1016/j.plantsci.2004.02.004.

Chen, A-hai, Md-tofazzal Islam, and Zhong-hua Ma. 2022. “An Integrated Pest Management Program for Managing Fusarium Head Blight Disease in Cereals.” *Journal of Integrative Agriculture*, 1–18. https://doi.org/10.1016/j.jia.2022.08.053.

Chen, Xinwei, Andrew Steed, Clare Harden, and Paul Nicholson. 2006. “Characterization of *Arabidopsis thaliana-Fusarium graminearum* Interactions and Identification of Variation in Resistance among Ecotypes.” *Molecular Plant Pathology* 7 (5): 391–403. https://doi.org/10.1111/j.1364-3703.2006.00349.x.

Chen, Yu, and Ming Guo Zhou. 2009. “Characterization of *Fusarium graminearum* Isolates Resistant to Both Carbendazim and a New Fungicide JS399-19.” *Phytopathology* 99 (4): 441–46. https://doi.org/10.1094/PHYTO-99-4-0441.

Córdova-Alcántara, Itzel Margarita, Diana Laura Venegas-Cortés, María Ángeles Martínez-Rivera, Néstor Octavio Pérez, and Aida Verónica Rodriguez-Tovar. 2019. “Biofilm Characterization of *Fusarium solani* Keratitis Isolate: Increased Resistance to Antifungals and UV Light.” *Journal of Microbiology* 57 (6): 485–97. https://doi.org/10.1007/s12275-019-8637-2.

Cowger, Christina, Jennifer Patton-Özkurt, Gina Brown-Guedira, and Leandro Perugini. 2009. “Post-Anthesis Moisture Increased Fusarium Head Blight and Deoxynivalenol Levels in North Carolina Winter Wheat.” *Phytopathology* 99 (4): 320–27. https://doi.org/10.1094/PHYTO-99-4-0320.

Cumagun, Christian Joseph R, Robert L Bowden, James E Jurgenson, John F Leslie, and Thomas Miedaner. 2004. “Genetic Mapping of Pathogenicity and Aggressiveness of *Gibberella zeae* (*Fusarium graminearum*) Toward Wheat.” *Phytopathology* 94 (720): 520–26. https://doi.org/10.1094/PHYTO.2004.94.5.520.

Cuthbert, Patricia A., Daryl J. Somers, and Anita Brulé-Babel. 2007. “Mapping of Fhb2 on Chromosome 6BS: A Gene Controlling Fusarium Head Blight Field Resistance in Bread Wheat (*Triticum aestivum* L.).” *Theoretical and Applied Genetics* 114 (3): 429–37. https://doi.org/10.1007/s00122-006-0439-3.

Cuthbert, Patricia A., Daryl J. Somers, Julian Thomas, Sylvie Cloutier, and Anita Brulé-Babel. 2006. “Fine Mapping Fhb1, a Major Gene Controlling Fusarium Head Blight Resistance in Bread Wheat (*Triticum aestivum* L.).” *Theoretical and Applied Genetics* 112 (8): 1465–72. https://doi.org/10.1007/s00122-006-0249-7.

Davies, David. 2003. “Understanding Biofilm Resistance to Antibacterial Agents.” *Nature Reviews Drug Discovery* 2 (2): 114–22. https://doi.org/10.1038/nrd1008.

Dill-Macky, R., and R. K. Jones. 2000. “The Effect of Previous Crop Residues and Tillage on Fusarium Head Blight of Wheat.” *Plant Disease* 84 (1): 71–76. https://doi.org/10.1094/PDIS.2000.84.1.71.

Epstein, E. 2009. “Silicon: Its Manifold Roles in Plants.” *Annals of Applied Biology* 155 (2): 155–60. https://doi.org/10.1111/j.1744-7348.2009.00343.x.

Epstein, E. 1994. “The Anomaly of Silicon in Plant Biology.” *Proceedings of the National Academy of Sciences of the United States of America* 91 (1): 11–17. https://doi.org/10.1073/pnas.91.1.11.

Epstein, Lynn, and Ralph Nicholson. 2016. “Adhesion and Adhesives of Fungi and Oomycetes.” In *Biological Adhesives*, 25–55. https://doi.org/10.1007/978-3-319-46082-6.

Escrivá, L., G. Font, and L. Manyes. 2015. “In Vivo Toxicity Studies of Fusarium Mycotoxins in the Last Decade: A Review.” *Food and Chemical Toxicology* 78: 185–206. https://doi.org/10.1016/j.fct.2015.02.005.

Fanning, Saranna, and Aaron P. Mitchell. 2012. “Fungal Biofilms.” *PLoS Pathogens* 8 (4): 1–4. https://doi.org/10.1371/journal.ppat.1002585.

Finkel, Jonathan S., and Aaron P. Mitchell. 2011. “Genetic Control of *Candida albicans* Biofilm Development.”

Freije, Anna N., and Kiersten A. Wise. 2015. “Impact of *Fusarium graminearum* Inoculum Availability and Fungicide Application Timing on Fusarium Head Blight in Wheat.” *Crop Protection* 77: 139–47. https://doi.org/10.1016/j.cropro.2015.07.016.

García-Sánchez, Susana, Sylvie Aubert, Ismaïl Iraqui, Guilhem Janbon, Jean Marc Ghigo, and Christophe D’Enfert. 2004. “*Candida albicans* Biofilms: A Developmental State Associated with Specific and Stable Gene Expression Patterns.” *Eukaryotic Cell* 3 (2): 536–45. https://doi.org/10.1128/EC.3.2.536-545.2004.

Gibbons, John G., Anne Beauvais, Remi Beau, Kriston L. McGary, Jean Paul Latgé, and Antonis Rokas. 2012. “Global Transcriptome Changes Underlying Colony Growth in the Opportunistic Human Pathogen *Aspergillus fumigatus*.” *Eukaryotic Cell* 11 (1): 68–78. https://doi.org/10.1128/EC.05102-11.

Guenther, John C, and Frances Trail. 2005. “The Development and Differentiation of *Gibberella zeae* (Anamorph: *Fusarium graminearum*) during Colonization of Wheat.” *Mycologia* 97 (1): 229–37. https://doi.org/10.3852/mycologia.97.1.229.

Guo, Jun, Xiuli Zhang, Yanlin Hou, Jinjin Cai, Xiaorong Shen, Tingting Zhou, Huihui Xu, et al. 2015. “High-Density Mapping of the Major FHB Resistance Gene Fhb7 Derived from *Thinopyrum ponticum* and Its Pyramiding with Fhb1 by Marker-Assisted Selection.” *Theoretical and Applied Genetics* 128 (11): 2301–16. https://doi.org/10.1007/s00122-015-2586-x.

Haidukowski, Miriam, Michelangelo Pascale, Giancarlo Perrone, Davide Pancaldi, Claudio Campagna, and Angelo Visconti. 2005. “Effect of Fungicides on the Development of

Fusarium Head Blight, Yield and Deoxynivalenol Accumulation in Wheat Inoculated under Field Conditions with *Fusarium graminearum* and *Fusarium culmorum*." *Journal of the Science of Food and Agriculture* 85 (2): 191–98. https://doi.org/10.1002/jsfa.1965.

Harding, Michael W., Lyriam L R Marques, Ronald J. Howard, and Merle E. Olson. 2009. "Can Filamentous Fungi Form Biofilms?" *Trends in Microbiology* 17 (11): 475–80. https://doi.org/10.1016/j.tim.2009.08.007.

Harding, Michael W, Lyriam L R Marques, Ronald J Howard, and Merle E Olson. 2010. "Biofilm Morphologies of Plant Pathogenic Fungi." *The Americas Journal of Plant Science and Biotechnology* 4 (Special Issue 2): 43–47.

Harris, Linda J., Margaret Balcerzak, Anne Johnston, Danielle Schneiderman, and Thérèse Ouellet. 2016. "Host-Preferential *Fusarium graminearum* Gene Expression during Infection of Wheat, Barley, and Maize." *Fungal Biology* 120 (1): 111–23. https://doi.org/10.1016/j.funbio.2015.10.010.

Hauser, Marie-Theres. 2014. "Molecular Basis of Natural Variation and Environmental Control of Trichome Patterning." *Frontiers in Plant Science* 5 (July): 320. https://doi.org/10.3389/fpls.2014.00320.

He, Xinyao, Mohamed Osman, James Helm, Flavio Capettini, and Pawan K. Singh. 2015. "Evaluation of Canadian Barley Breeding Lines for Fusarium Head Blight Resistance." *Canadian Journal of Plant Science* 95 (5): 923–29. https://doi.org/10.4141/CJPS-2015-062.

Hou, Yi Ping, Xue Wei Mao, Jian Xin Wang, Su Wen Zhan, and Ming Guo Zhou. 2017. "Sensitivity of *Fusarium asiaticum* to a Novel Succinate Dehydrogenase Inhibitor Fungicide Pydiflumetofen." *Crop Protection* 96: 237–44. https://doi.org/10.1016/j.cropro.2017.02.011.

Imamura, Yoshifumi, Jyotsna Chandra, Pranab K. Mukherjee, Ali Abdul Lattif, Loretta B. Szczotka-Flynn, Eric Pearlman, Jonathan H. Lass, Kerry O'Donnell, and Mahmoud A. Ghannoum. 2008. "*Fusarium* and *Candida albicans* Biofilms on Soft Contact Lenses: Model Development, Influence of Lens Type, and Susceptibility to Lens Care Solutions." *Antimicrobial Agents and Chemotherapy* 52 (1): 171–82. https://doi.org/10.1128/AAC.00387-07.

Imboden, Lori, Drew Afton, and Frances Trail. 2018. "Surface Interactions of *Fusarium graminearum* on Barley." *Molecular Plant Pathology* 19 (6): 1332–42. https://doi.org/10.1111/mpp.12616.

Inglis, Diane O., and Gavin Sherlock. 2013. "Ras Signaling Gets Fine-Tuned: Regulation of Multiple Pathogenic Traits of *Candida albicans*." *Eukaryotic Cell* 12 (10): 1316–25. https://doi.org/10.1128/EC.00094-13.

Jansen, Carin, Diter Von Wettstein, Wilhelm Schäfer, Karl Heinz Kogel, Angelika Felk, and

Frank J. Maier. 2005. “Infection Pattern in Barley and Wheat Spikes Inoculated with Wild-Type and Trichodiene Synthase Gene Disrupted *Fusarium graminearum*.” *Proceedings of the National Academy of Sciences of the United States of America* 102 (46): 16892–97. https://doi.org/10.1073/pnas.0508467102.

Jiang, Jinhua, Xin Liu, Yanni Yin, and Zhonghua Ma. 2011. “Involvement of a Velvet Protein FgVeA in the Regulation of Asexual Development, Lipid and Secondary Metabolisms and Virulence in *Fusarium graminearum*.” *PLoS ONE* 6 (11). https://doi.org/10.1371/journal.pone.0028291.

Jin, Zhao, Shyam Solanki, Gazala Ameen, Thomas Gross, Roshan Sharma Poudel, Pawel Borowicz, Robert S. Brueggeman, and Paul Schwarz. 2021. “Expansion of Internal Hyphal Growth in Fusarium Head Blight-Infected Grains Contributes to the Elevated Mycotoxin Production during the Malting Process.” *Molecular Plant-Microbe Interactions* 34 (7): 793–802. https://doi.org/10.1094/MPMI-01-21-0024-R.

Kazan, Kemal, Donald M. Gardiner, and John M. Manners. 2012. “On the Trail of a Cereal Killer: Recent Advances in *Fusarium graminearum* Pathogenomics and Host Resistance.” *Molecular Plant Pathology* 13 (4): 399–413. https://doi.org/10.1111/j.1364-3703.2011.00762.x.

Keller, Melissa D., Gary C. Bergstrom, and Elson J. Shields. 2014. “The Aerobiology of *Fusarium graminearum*.” *Aerobiologia* 30 (2): 123–36. https://doi.org/10.1007/s10453-013-9321-3.

Kim, Ki Woo. 2019. “Plant Trichomes as Microbial Habitats and Infection Sites.” *European Journal of Plant Pathology* 154 (2): 157–69. https://doi.org/10.1007/s10658-018-01656-0.

Kischkel, Brenda, Gredson Keiff Souza, Lucas Ulisses, Rovigatti Chiavelli, Terezinha Inez, Estivalet Svidzinski, and Melyssa Negri. 2019. “The Ability of Farnesol to Prevent Adhesion and Disrupt *Fusarium keratoplasticum* Biofilm.” *Applied Microbial and Cell Physiology*.

Koczan, Jessica M., Bryan R. Lenneman, Molly J. McGrath, and George W. Sundin. 2011. “Cell Surface Attachment Structures Contribute to Biofilm Formation and Xylem Colonization by *Erwinia amylovora*.” *Applied and Environmental Microbiology* 77 (19): 7031–39. https://doi.org/10.1128/AEM.05138-11.

Kowalski, Caitlin H, Joshua D Kerkaert, Ko-wei Liu, Matthew C Bond, Raimo Hartmann, Carey D Nadell, Jason E Stajich, and Robert A Cramer. 2019. “Fungal Biofilm Morphology Impacts Hypoxia Fitness and Disease Progression.” *Nature Microbiology*. https://doi.org/10.1038/s41564-019-0558-7.

Langevin, François, François Eudes, and André Comeau. 2004. “Effect of Trichothecenes Produced by *Fusarium graminearum* during Fusarium Head Blight Development in Six Cereal Species.” *European Journal of Plant Pathology* 110 (7): 735–46.

https://doi.org/10.1023/B:EJPP.0000041568.31778.ad.

Lemmens, Marc, Hermann Buerstmayr, Rudolf Krska, Rainer Schuhmacher, Heinrich Grausgruber, and Peter Ruckenbauer. 2004. “The Effect of Inoculation Treatment and Long-Term Application of Moisture on Fusarium Head Blight Symptoms and Deoxynivalenol Contamination in Wheat Grains.” *European Journal of Plant Pathology* 110 (3): 299–308. https://doi.org/10.1023/B:EJPP.0000019801.89902.2a.

Leonard, Kurt J, and William R. Bushnell, eds. 2003. *Fusarium Head Blight of Wheat and Barley*. St. Paul, Minnesota: The American Phytopathological Society.

Leplat, Johann, Hanna Friberg, Muhammad Abid, and Christian Steinberg. 2013. “Survival of *Fusarium graminearum*, the Causal Agent of Fusarium Head Blight. A Review.” *Agronomy for Sustainable Development* 33 (1): 97–111. https://doi.org/10.1007/s13593-012-0098-5.

Li, Fang, Michael J. Svarovsky, Amy J. Karlsson, Joel P. Wagner, Karen Marchillo, Philip Oshel, David Andes, and Sean P. Palecek. 2007. “Eap1p, an Adhesin That Mediates *Candida albicans* Biofilm Formation in Vitro and in Vivo.” *Eukaryotic Cell* 6 (6): 931–39. https://doi.org/10.1128/EC.00049-07.

Li, Peiqian, Xiaoming Pu, Baozhen Feng, Qiyun Yang, Huifang Shen, Jingxin Zhang, and Birun Lin. 2015. “FocVel1 Influences Asexual Production, Filamentous Growth, Biofilm Formation, and Virulence in *Fusarium oxysporum f. Sp. cucumerinum*.” *Frontiers in Plant Science* 6 (May): 1–10. https://doi.org/10.3389/fpls.2015.00312.

Lu, Yang, Chang Su, and Haoping Liu. 2012. “A GATA Transcription Factor Recruits Hda1 in Response to Reduced Tor1 Signaling to Establish a Hyphal Chromatin State in *Candida albicans*.” *PLoS Pathogens* 8 (4). https://doi.org/10.1371/journal.ppat.1002663.

Ma, JF, Y Miyake, and E Takahashi. 2001. “Silicon as a Beneficial Element for Crop Plants.” In *Silicon in Agriculture*, 17–39.

Ma, Jian Feng. 2003. “Beneficial Effects of Silicon in Different Plant Species.” *Progress in Molecular and Subcellular Biology* 33.

Maldonado-Ramirez, Sandra Lee, David G. Schmale, Elson J. Shields, and Gary C. Bergstrom. 2005. “The Relative Abundance of Viable Spores of *Gibberella zeae* in the Planetary Boundary Layer Suggests the Role of Long-Distance Transport in Regional Epidemics of Fusarium Head Blight.” *Agricultural and Forest Meteorology* 132 (1–2): 20–27. https://doi.org/10.1016/j.agrformet.2005.06.007.

McMullen, Marcia P., Gary C. Bergstrom, Erick De Wolf, Ruth Dill-Macky, Donald E Hershman, Gregory Shaner, and David A Van Sanford. 2012. “A Unified Effort to Fight an Enemy of Wheat and Barley: Fusarium Head Blight.” *Plant Disease* 96 (12): 1712–28. https://doi.org/10.1094/PDIS-03-12-0291-FE.

Mentges, Michael, Anika Glasenapp, Marike Boenisch, Sascha Malz, Bernard Henrissat, Rasmus J.N. Frandsen, Ulrich Güldener, et al. 2020. “Infection Cushions of *Fusarium graminearum* Are Fungal Arsenals for Wheat Infection.” *Molecular Plant Pathology* 21 (8): 1070–87. https://doi.org/10.1111/mpp.12960.

Merritt, Judith H., Daniel E. Kadouri, and George A. O’Toole. 2005. “Growing and Analyzing Static Biofilms.” *Current Protocols in Microbiology*, 1–29. https://doi.org/10.1002/9780471729259.mc01b01s00.Growing.

Miedaner, T, C J R Cumagun, and S Chakraborty. 2008. “Population Genetics of Three Important Head Blight Pathogens *Fusarium graminearum, F. pseudograminearum and F. culmorum*.” *Journal of Phytopathology* 156 (3): 129–39. https://doi.org/10.1111/j.1439-0434.2007.01394.x.

Miguel-Rojas, Cristina, Brad Cavinder, Zheng Wang, Jeffry P. Townsend, and Frances Trail. n.d. “Comparative Transcriptomics of *Fusarium graminearum* and *Magnaporthe oryzae* of Spor Germination Leading up to Infection.”

Moleleki, Lucy Novungayo, Rudolph Gustav Pretorius, Collins Kipngetich Tanui, Gabolwelwe Mosina, and Jacques Theron. 2017. “A Quorum Sensing-Defective Mutant of *Pectobacterium carotovorum Ssp. brasiliense* 1692 Is Attenuated in Virulence and Unable to Occlude Xylem Tissue of Susceptible Potato Plant Stems.” *Molecular Plant Pathology* 18 (1): 32–44. https://doi.org/10.1111/mpp.12372.

Motaung, Thabiso E, Chizne Peremore, Brenda Wingfield, and Emma Steenkamp. 2020. “Plant-Associated Fungal Biofilms - Knowns and Unknowns.” *FEMS Microbial Ecology* 5: 1–25.

Mowat, Eilidh, Sue Lang, Craig Williams, Elaine McCulloch, Brian Jones, and Gordon Ramage. 2008. “Phase-Dependent Antifungal Activity against *Aspergillus fumigatus* Developing Multicellular Filamentous Biofilms.” *Journal of Antimicrobial Chemotherapy* 62 (6): 1281–84. https://doi.org/10.1093/jac/dkn402.

Nett, Jeniel E., Hiram Sanchez, Michael T. Cain, and David R. Andes. 2010. “Genetic Basis of *Candida* Biofilm Resistance Due to Drug-Sequestering Matrix Glucan.” *Journal of Infectious Diseases* 202 (1): 171–75. https://doi.org/10.1086/651200.

Nobile, Clarissa J., Emily P. Fox, Jeniel E. Nett, Trevor R. Sorrells, Quinn M. Mitrovich, Aaron D. Hernday, Brian B. Tuch, David R. Andes, and Alexander D. Johnson. 2012. “A Recently Evolved Transcriptional Network Controls Biofilm Development in *Candida albicans*.” *Cell* 148 (1–2): 126–38. https://doi.org/10.1016/j.cell.2011.10.048.

Nobile, Clarissa J., Jeniel E. Nett, Aaron D. Hernday, Oliver R. Homann, Jean Sebastien Deneault, Andre Nantel, David R. Andes, Alexander D. Johnson, and Aaron P. Mitchell. 2009. “Biofilm Matrix Regulation by *Candida albicans* Zap1.” *PLoS Biology* 7 (6). https://doi.org/10.1371/journal.pbio.1000133.

O'Reagain, P. J., and M. T. Mentis. 1989. "Leaf Silicification in Grasses - A Review." *Journal of the Grassland Society of Southern Africa* 6 (1): 37–43. https://doi.org/10.1080/02566702.1989.9648158.

O'Toole, George A. 2011. "Microtiter Dish Biofilm Formation Assay." *Journal of Visualized Experiments*, no. 47: 3–5. https://doi.org/10.3791/2437.

O'Toole, George A, Leslie A. Pratt, Paula I. Watnick, Dianne K. Newman, Valerie B. Weaver, and Roberto Kolter. 1999. "[6] Genetic Approaches to Study of Biofilms." *Methods in Enzymology* 310 (1996): 91–109. https://doi.org/10.1016/S0076-6879(99)10008-9.

Osborne, Lawrence E., and Jeffrey M. Stein. 2007. "Epidemiology of Fusarium Head Blight on Small-Grain Cereals." *International Journal of Food Microbiology* 119 (1–2): 103–8. https://doi.org/10.1016/j.ijfoodmicro.2007.07.032.

Pandin, Caroline, Dominique Le Coq, Alexis Canette, Stéphane Aymerich, and Romain Briandet. 2017. "Should the Biofilm Mode of Life Be Taken into Consideration for Microbial Biocontrol Agents?" *Microbial Biotechnology* 00. https://doi.org/10.1111/1751-7915.12693.

Paper, Janet M., John S. Scott-Craig, Neil D. Adhikari, Christina A. Cuomo, and Jonathan D. Walton. 2007. "Comparative Proteomics of Extracellular Proteins in Vitro and in Planta from the Pathogenic Fungus *Fusarium graminearum*." *Proteomics* 7 (17): 3171–83. https://doi.org/10.1002/pmic.200700184.

Paul, P. A., C. A. Bradley, L. V. Madden, F. Dalla Lana, G. C. Bergstrom, R. Dill-Macky, P. D. Esker, et al. 2018. "Meta-Analysis of the Effects of QoI and DMI Fungicide Combinations on Fusarium Head Blight and Deoxynivalenol in Wheat." *Plant Disease* 102 (12): 2602–15. https://doi.org/10.1094/pdis-02-18-0211-re.

Pazdiora, Paulo Cesar, Rosane Lopes Crizel, Keilor Rosa Dorneles, Sabrina Monks, Thomas Natali Morello, Fabio Clasen Chaves, and Leandro José Dallagnol. 2022. "Silicon Amendment Improves Wheat Defense against *Fusarium graminearum* and Complements the Control by Fungicide of Fusarium Head Blight," 0–2. https://doi.org/10.1111/ppa.13648.

Peiqian, Li, Pu Xiaoming, Shen Huifang, Zhang Jingxin, Huang Ning, and Lin Birun. 2014. "Biofilm Formation by *Fusarium oxysporum f. Sp. cucumerinum* and Susceptibility to Environmental Stress." *FEMS Microbiology Letters* 350 (2): 138–45. https://doi.org/10.1111/1574-6968.12310.

Pereyra, S. A., R. Dill-Macky, and A. L. Sims. 2004. "Survival and Inoculum Production of *Gibberella zeae* in Wheat Residue." *Plant Disease* 88 (7): 724–30. https://doi.org/10.1094/PDIS.2004.88.7.724.

Pestka, James J., and Alexa T. Smolinski. 2005. "Deoxynivalenol: Toxicology and Potential

Effects on Humans." *Journal of Toxicology and Environmental Health - Part B: Critical Reviews* 8 (1): 39–69. https://doi.org/10.1080/10937400590889458.

Piacentini, Karim C., Geovana D. Savi, Maria E V Pereira, and Vildes M. Scussel. 2015. "Fungi and the Natural Occurrence of Deoxynivalenol and Fumonisins in Malting Barley (*Hordeum vulgare* L.)." *Food Chemistry* 187: 204–9. https://doi.org/10.1016/j.foodchem.2015.04.101.

Pompilio, Arianna, and Giovanni Di Bonaventura. 2018. "Microbial Biofilm: A 'Sticky' Problem." *Microbiologia Medica* 33 (1). https://doi.org/10.4081/mm.2018.7851.

Prasad, Rajendra, Philippe De Wergifosse, Andre Goffeau, and Elisabetta Balzi. 1995. "Molecular Cloning and Characterization of a Novel Gene of *Candida albicans*, CDR1, Conferring Multiple Resistance to Drugs and Antifungals." *Current Genetics* 27 (4): 320–29. https://doi.org/10.1007/BF00352101.

Prithiviraj, B, H P Bais, T Weir, B Suresh, E H Najarro, B V Dayakar, H P Schweizer, and J M Vivanco. 2005. "Down Regulation of Virulence Factors of *Pseudomonas aeruginosa* by Salicylic Acid Attenuates Its Virulence on *Arabidopsis thaliana* and *Caenorhabditis elegans*." *Society* 73 (9): 5319–28. https://doi.org/10.1128/IAI.73.9.5319.

Pritsch, Clara, Gary J. Muehlbauer, William R. Bushnell, David A. Somers, and Carroll P. Vance. 2000. "Fungal Development and Induction of Defense Response Genes during Early Infection of Wheat Spikes by *Fusarium graminearum*." *Molecular Plant-Microbe Interactions* 13 (2): 159–69. https://doi.org/10.1094/MPMI.2000.13.2.159.

Qi, L. L., M. O. Pumphrey, Bernd Friebe, P. D. Chen, and B. S. Gill. 2008. "Molecular Cytogenetic Characterization of Alien Introgressions with Gene Fhb3 for Resistance to Fusarium Head Blight Disease of Wheat." *Theoretical and Applied Genetics* 117 (7): 1155–66. https://doi.org/10.1007/s00122-008-0853-9.

Ragsdale, Nancy N., and Hugh D. Sisler. 1972. "Inhibition of Ergosterol Synthesis in *Usetilago maydis* by the Fungicide Triarimol." *Biochemical And Biophysical Research Communications* 46 (6): 2048–53.

Ramage, Gordon, Eilidh Mowat, Brian Jones, Craig Williams, and Jose Lopez-Ribot. 2009. "Our Current Understanding of Fungal Biofilms." *Critical Reviews in Microbiology* 35 (4): 340–55. https://doi.org/10.3109/10408410903241436.

Ramage, Gordon, Ranjith Rajendran, Leighann Sherry, and Craig Williams. 2012. "Fungal Biofilm Resistance." *International Journal of Microbiology* 2012. https://doi.org/10.1155/2012/528521.

Ramage, Gordon, Kacy VandeWalle, José L. López-Ribot, and Brian L. Wickes. 2002. "The Filamentation Pathway Controlled by the Efg1 Regulator Protein Is Required for Normal Biofilm Formation and Development in *Candida albicans*." *FEMS Microbiology Letters*

214 (1): 95–100. https://doi.org/10.1016/S0378-1097(02)00853-4.

Ramey, Bronwyn E., Maria Koutsoudis, Susanne B.Von Bodman, and Clay Fuqua. 2004. "Biofilm Formation in Plant-Microbe Associations." *Current Opinion in Microbiology* 7 (6): 602–9. https://doi.org/10.1016/j.mib.2004.10.014.

Reynolds, Todd B., and Gerald R. Fink. 2001. "Bakers' Yeast, a Model for Fungal Biofilm Formation" 54 (4): 421–31.

Sakr, Nachaat. 2016. "The Role of Silicon (Si) in Increasing Plant Resistance against Fungal Diseases." *Hellenic Plant Protection Journal* 9 (1): 1–15. https://doi.org/10.1515/hppj-2016-0001.

———. 2022. "Silicon Reduces the Severity of *Fusarium* Infection on Young Wheat Parts In Vitro." *The Open Agriculture Journal* 16: 1–10. https://doi.org/10.2174/18743315-v16-e2207260.

Schilmiller, Anthony L., Robert L. Last, and Eran Pichersky. 2008. "Harnessing Plant Trichome Biochemistry for the Production of Useful Compounds." *Plant Journal* 54 (4): 702–11. https://doi.org/10.1111/j.1365-313X.2008.03432.x.

Schlafer, Sebastian, Anja Kamp, and Javier E Garcia. 2018. "A Confocal Microscopy Based Method to Monitor Extracellular pH Biofilms," no. April. https://doi.org/10.1093/femsyr/foy049/4978430.

Shah, L., A. Ali, M. Yahya, Y. Zhu, S. Wang, H. Si, H. Rahman, and C. Ma. 2018. "Integrated Control of Fusarium Head Blight and Deoxynivalenol Mycotoxin in Wheat." *Plant Pathology* 67 (3): 532–48. https://doi.org/10.1111/ppa.12785.

Silva, Erin M., and Kathleen Delate. 2017. "A Decade of Progress in Organic Cover Crop-Based Reduced Tillage Practices in the Upper Midwestern USA." *Agriculture (Switzerland)* 7 (5). https://doi.org/10.3390/agriculture7050044.

Singh, Rachna, M. R. Shivaprakash, and Arunaloke Chakrabarti. 2011. "Biofilm Formation by Zygomycetes: Quantification, Structure and Matrix Composition." *Microbiology* 157 (9): 2611–18. https://doi.org/10.1099/mic.0.048504-0.

Souza, Alessandra A. De, Marco A. Takita, Helvécio D. Coletta-Filho, Camila Caldana, Giane M. Yanai, Nair H. Muto, Regina C. De Oliveira, Luiz R. Nunes, and Marcos A. Machado. 2004. "Gene Expression Profile of the Plant Pathogen *Xylella fastidiosa* during Biofilm Formation in Vitro." *FEMS Microbiology Letters* 237 (2): 341–53. https://doi.org/10.1016/j.femsle.2004.06.055.

Summerell, Brett Anthony, and John F. Leslie. 2011. "Fifty Years of Fusarium: How Could Nine Species Have Ever Been Enough?" *Fungal Diversity* 50: 135–44. https://doi.org/10.1007/s13225-011-0132-y.

Sun, Yanan, Haiping Shi, Chenxin Mao, Jianyan Wu, and Chuanqing Zhang. 2021. "Activity of a SDHI Fungicide Penflufen and the Characterization of Natural-Resistance in *Fusarium fujikuroi*." *Pesticide Biochemistry and Physiology* 179 (June): 104960. https://doi.org/10.1016/j.pestbp.2021.104960.

Tateishi, Hideaki, Taiji Miyake, Masaru Mori, Yoneko Sakuma, and Toshihide Saishoji. 2014. "Effect of Application Timing of Metconazole on Fusarium Head Blight Development and Mycotoxin Contamination in Wheat and Barley." *Journal of Pesticide Science* 39 (1–2): 1–6. https://doi.org/10.1584/jpestics.D12-077.

Theodorakopoulos, Nicolas, Benjamin Govetto, Benoit Industri, Lionel Massi, Marc Gaysinski, Emeline Deleury, Catherine Mura, et al. 2011. "Biology and Ecology of Biofilms Formed by a Plant Pathogen *Phytophthora parasitica*: From Biochemical Ecology to Ecological Engineering." *Procedia Environmental Sciences* 9 (0): 178–82. https://doi.org/10.1016/j.proenv.2011.11.027.

Trail, Frances. 2009. "For Blighted Waves of Grain: *Fusarium graminearum* in the Postgenomics Era." *Plant Physiology* 149 (1): 103–10. https://doi.org/10.1104/pp.108.129684.

Vicari, Mark, and Dawn R. Bazely. 1993. "Do Grasses Fight Back? The Case for Antiherbivore Defences." *Trends in Ecology and Evolution* 8 (4): 137–41. https://doi.org/10.1016/0169-5347(93)90026-L.

Walker, Travis S., Harsh Pal Bais, Eric Déziel, Herbert P. Schweizer, Laurence G. Rahme, Ray Fall, and Jorge M. Vivanco. 2004. "*Pseudomonas aeruginosa*-Plant Root Interactions. Pathogenicity, Biofilm Formation, and Root Exudation." *Plant Physiology* 134 (1): 320–31. https://doi.org/10.1104/pp.103.027888.

Wang, Hongwei, Silong Sun, Wenyang Ge, Lanfei Zhao, Bingqian Hou, Kai Wang, Zhongfan Lyu, et al. 2020. "Horizontal Gene Transfer of Fhb7 from Fungus Underlies Fusarium Head Blight Resistance in Wheat." *Science* 21 (1): 1–9.

Wegulo, Stephen N, P Stephen Baenziger, John Hernandez Nopsa, William W Bockus, and Heather Hallen-Adams. 2015. "Management of Fusarium Head Blight of Wheat and Barley." *Crop Protection* 73: 100–107. https://doi.org/10.1016/j.cropro.2015.02.025.

White, Theodore C. 1997. "Increased MRNA Levels of ERG16, CDR, and MDR1 Correlate, with Increases in Azole Resistance in *Candida albicans* Isolates from a Patient Infected with Human Immunodeficiency Virus." *Antimicrobial Agents and Chemotherapy* 41 (7): 1482–87. https://doi.org/10.1128/aac.41.7.1482.

Willyerd, K. T., C. Li, L. V. Madden, C. A. Bradley, G. C. Bergstrom, L. E. Sweets, M. McMullen, et al. 2012. "Efficacy and Stability of Integrating Fungicide and Cultivar Resistance to Manage Fusarium Head Blight and Deoxynivalenol in Wheat." *Plant Disease* 96 (July): 957–67. https://doi.org/10.1094/PDIS-09-11-0763.

Xue, Shulin, Guoqiang Li, Haiyan Jia, Feng Xu, Feng Lin, Mingzhi Tang, Yao Wang, et al. 2010. “Fine Mapping Fhb4, a Major QTL Conditioning Resistance to Fusarium Infection in Bread Wheat (*Triticum aestivum* L.).” *Theoretical and Applied Genetics* 121 (1): 147–56. https://doi.org/10.1007/s00122-010-1298-5.

Xue, Shulin, Feng Xu, Mingzhi Tang, Yan Zhou, Guoqiang Li, Xia An, Feng Lin, et al. 2011. “Precise Mapping Fhb5, a Major QTL Conditioning Resistance to Fusarium Infection in Bread Wheat (*Triticum aestivum* L.).” *Theoretical and Applied Genetics* 123 (6): 1055–63. https://doi.org/10.1007/s00122-011-1647-z.

Yoshida, Megumi, Takashi Nakajima, Kenta Tomimura, Fumihiko Suzuki, Michiyoshi Arai, and Atsushi Miyasaka. 2012. “Effect of the Timing of Fungicide Application on Fusarium Head Blight and Mycotoxin Contamination in Wheat.” *Plant Disease* 96 (6): 845–51. https://doi.org/10.1094/PDIS-10-11-0819.

Zheng, He, Jaekuk Kim, Mathew Liew, John K. Yan, Oscar Herrera, Jin Woo Bok, Neil L. Kelleher, Nancy P. Keller, and Yun Wang. 2015. “Redox Metabolites Signal Polymicrobial Biofilm Development via the Napa Oxidative Stress Cascade in *Aspergillus*.” *Current Biology* 25 (1): 29–37. https://doi.org/10.1016/j.cub.2014.11.018.

CHAPTER 2

BIOFILM FORMATION AND STRUCTURE IN THE FILAMENTOUS FUNGUS *FUSARIUM GRAMINEARUM*, A PLANT PATHOGEN

Source

Shay, R., Wiegand, A. A. & Trail, F. Biofilm Formation and Structure in the Filamentous Fungus *Fusarium graminearum*, a Plant Pathogen. *Microbiol. Spectr.* **10**, (2022). DOI: https://doi.org/10.1128/spectrum.00171-22

Summary

Biofilms are important protective structures of microbes, where individual cells work as a community to attach to a surface, and protect the structure from external stressors. The majority of the work on biofilms has been done in bacteria and single-celled fungi. Filamentous fungi are a large group of organisms that impact all aspects of life, and biofilms of these fungi are an important, but understudied formation. We identified biofilm formations in the plant pathogen *Fusarium graminearum in vitro*, and characterized the formation of the extracellular matrix that forms around the biofilm. The matrix of *F. graminearum* consists of extracellular nucleic acids, polysaccharides, and lipids, all of which form sequentially during the development of the whole biofilm. Additionally, we identified pellicle formations produced by these biofilms, which were the first filamentous fungal pellicles reported. Lastly, we have identified a response to oxidative stress exhibited by *F. graminearum*, which occurs in response to an absence of electron acceptors. Oxidative stress is a common trigger of biofilm formation in other fungal species, and understanding how oxidative stress responses and biofilm formation occur in *F. graminearum* is important to help advance disease control.

CHAPTER 3

TRANSCRIPTOME ANALYSIS AND CHARACTERIZATION OF GENES FROM *FUSARIUM GRAMINEARUM* BIOFILMS

Abstract

The formation of biofilms in the filamentous fungus Fusarium graminearum, an important plant pathogen, has been previously characterized. We demonstrate the first known instance of *F. graminearum* biofilms on host plant tissues. The identification of genes involved in biofilm formation is essential to determining the role of biofilms in pathogenicity, and will provide targets for control of disease. Here we apply comparative transcriptomics to identify genes important to each stage of biofilm formation, and characterize candidate genes identified in that analysis. We demonstrate that genes involved in the formation of biofilms impact the ability of the structures to adhere to a surface, and also change the composition of the extracellular matrix of the biofilm. Additionally, we show that adhesion is easily manipulated through artificial selection and that the changes are due, at least in part, to shifts in DNA methylation. Lastly, this work provides both a basis for the study of biofilms which have been understudied compared to those of bacterial and yeast.

Introduction

Biofilms are collaborative communities of microbes, often adhered to a surface, that increase survival by protecting cells against external stressors (Harding et al. 2009; Fanning and Mitchell 2012). The majority of work on biofilms has been done in single-celled organisms, with filamentous fungal biofilms being comparatively understudied (Motaung et al. 2020). Biofilms of filamentous fungi have been reported in *Aspergillus* spp, *Fusarium* spp, *Botrytis* spp, and *Verticillium* spp, as well as some filamentous oomycete plant pathogens (Harding et al. 2010;

Singh, Shivaprakash, and Chakrabarti 2011; Theodorakopoulos et al. 2011; Peiqian et al. 2014; P. Li et al. 2015; Zheng et al. 2015; Córdova-Alcántara et al. 2019; Kowalski et al. 2019; Shay, Wiegand, and Trail 2022). Beyond the lack of detailed studies of filamentous fungal biofilms, there is even less known about the genetics of biofilm formation outside of bacteria and yeasts. Much of the understanding of the genetics of fungal biofilm formation comes from clinically important human pathogens, particularly in the pseudo-hyphal *Candida* spp (Nobile et al. 2009; Ramage et al. 2009; Martins et al. 2010; Finkel and Mitchell 2011; Fanning and Mitchell 2012; Ramage et al. 2012; Alim, Sircaik, and Panwar 2018).

We have recently demonstrated that *F. graminearum*, an important plant pathogen, forms biofilms in culture. Development of biofilms in *F. graminearum* can be separated into four continuous stages: (1) adhesion of conidia to a surface, (2) formation of an extracellular matrix (ECM), (3) development of conidial propagules, and (4) mature biofilms, followed by senescence (Shay, Wiegand, and Trail 2022). Identification of genes involved in this process can lead to novel management strategies to control disease in the field. To identify candidate genes involved in each stage of development, we used a comparative transcriptomics approach, where expression of genes was compared between stages. This guided approach to studying genes allows for a more targeted understanding than a traditional forward genetics approach. Furthermore, we investigated conditions that lead to increased adhesion of biofilms to substrates.

Methods

Strains and culture conditions

F. graminearum wild type (WT) strain PH-1 (FGSC 9075, NRRL 31084, Trail and Common 2000) was maintained in long-term storage as colonized carrot agar (Klittich and Leslie 1988) blocks in 35% glycerol at -80^{o}C. Conidia were generated in liquid carboxymethyl cellulose

medium (CMC; Cappellini and Peterson 1965) with incubation at room temperature (RT; 22-25^{o}C), shaking at 225 RPM, for five days. Conidia were collected by centrifugation and adjusted to 10^{6} conidia/mL in sterile distilled water before being used in experiments. Bird medium, a defined growth medium developed for enhanced conidial germination (Metzenberg 2004) was used to induce biofilms in experiments.

RNA extraction, sequencing, and analysis

Adhesion cultures were prepared as described in Shay, Wiegand, and Trail (2022) to generate tissue for RNA extraction. In short, tissue (100 mg) was collected at selected time points and ground in liquid nitrogen. The time points selected were 4, 12, 20, and 44 hours post-inoculation (hpi). Time points that were selected as stages of biofilm formation where new characteristics developed. RNA was extracted from material collected at each time point and using the Trizol extraction method (Simms, Cizdziel, and Chomczynski 1993) for the 4 hour time point, due to low yield, and all other time points were extracted using RNEasy Plant Mini Kit (Qiagen, Hilden, Germany). Extracted RNA was DNase-treated in solution with the Qiagen DNase Set (Qiagen, Hilden, Germany) for one hour, followed by cleanup with RNA Clean & Concentrator (Zymo, Irvine, CA). Libraries were prepared using the Illumina Stranded mRNA Ligation Kit with IDT for Illumina RNA Unique Dual Indexes (Illumina, San Diego, CA) following manufacturer's recommendations, except that half recommended reaction volumes were used. Completed libraries were quality controlled and quantified using a combination of Qubit dsDNA HS (Thermo Fisher Scientific, Waltham, MA) and Agilent 4200 TapeStation HS DNA1000 (Agilent, Santa Clara, CA). The libraries were pooled in equimolar quantities and the pool was quantified using the Invitrogen Collibri Quantification qPCR kit (Invitrogen, Waltham, MA). The pool was loaded onto one lane of an Illumina NovaSeq SP (Illumina, San Diego, CA) flow

cell using the Xp loading manifold and workflow.

Sequencing was performed on the NovaSeq 6000 instrument (Illumina, San Diego, CA) using v1.5 reagent kits. Base calling was done by Illumina Real Time Analysis (RTA) v3.4.4 and output of RTA was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v2.20.0. Reads were trimmed by Trimmomatic/0.39-Java-11 (Bolger, Lohse, and Usadel 2014) for adapter content with NexteraPE adapters and low quality reads. The trimmed files were quality checked through FastQC/0.11.7-Java-1.8.0_162 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) before further processing. Reads were indexed and aligned with Hisat2 2.1.0 (Kim, Langmead, and Salzberg 2015) before being aligned to the reference genome (King et al. 2015) using Hisat2. Files were converted from SAM to BAM, sorted, and indexed using SamTools 1.11 (H. Li et al. 2009), before being converted to Ballgown (Frazee et al. 2015) objects in Stringtie 2.1.3 (Pertea et al. 2015). Ballgown objects were analyzed in R 4.0.5/RStudio 1.4.1106 (https://www.r-project.org) for differential expression, where sequential time points were compared. Figures were generated in ggplot2 (Wickham 2016).

Candidate gene list, DNA constructs and genetic transformation

The output files from Ballgown were used to generate the candidate gene list (Table 3.1). Genes were selected from the candidate gene list from the top ten most significant from each Ballgown output, with consideration of annotation and used for further study. Genes selected for further study that were annotated as “hypothetical protein” or “unnamed protein product” were run through BlastN (NCBI, Bethesda, MD) against the fungi database (taxid:4751), and the top-ranked annotated hit was used for assigning annotation. In addition, *FGSG_11955* and *FGSG_08844* were selected based on homology to genes identified as important in biofilms from

previous publications in other species (P. Li et al. 2015; Epstein and Nicholson 2016). An additional gene identified by Ballgown, *FGSG_03680*, annotated as an aquaporin, was previously knocked out as part of another project (Shay et al, in prep). Selected genes were submitted to heatmapper.ca (Babicki et al. 2016) as the log2 of Fragments Per Kilobase Million (FPKM) values to generate heatmaps for visualization, using average linkage and Euclidean distances.

DNA was isolated from wild type *F. graminearum*. Primer design for knockout experiments was performed as previously described (Hallen-Adams, Cavinder, and Trail 2011; Harrison et al. 2013). All primers are listed in Table 3.2. Transformation was performed with modifications to the protocol of Hallen-Adams, Cavinder, and Trail (2011). The protoplasting buffer consisted of 30 mL 1.2 M KCl (J.T. Baker, Phillipsburg, NJ), 100 mg VinoTaste (Novozymes, Franklinton, NC), and 60 mg Yatalase (TaKaRa Bio, San Jose, CA), was stirred for 30 minutes without heat, and sterilized through a 0.45 µm filter before use.

Confirmed transformants were individually crossed with a non-nitrate-utilizing, *nit1* mutant (Bowden and Leslie 1999; Hou et al. 2002; Cavinder et al. 2011) to determine segregation of antibiotic resistance and the phenotype of interest (Chinnici et al. 2014). Crosses were performed and progeny were selected as previously described (Cavinder et al. 2012). Cirrhi from single perithecia formed at the joining of the two strains as they grew together, were collected to analyze segregation of the nit- phenotype from hygromycin resistance, and the knockout phenotypes. The presence of *hph1* was confirmed by PCR with hygromycin-specific primers (Table 3.2). For each of the four genes, at least 20 progeny (10 nit+ and 10 nit−) were examined.

Characterization of gene knockout strains

To determine the impact of each gene knockout, all generated strains were characterized for

adhesion, pathogenicity on barley, and matrix development. Adhesion of strains was performed using the method described in Shay, Wiegand, and Trail (2022) where crystal violet staining was used to quantify the amount of tissue adhered to a polystyrene surface. T-tests were used to compare the adhesion of WT to each strain. Comparisons were done within independent experiments, to avoid differences in adhesion seen between replicates obfuscating statistical differences. For plant pathogenicity, detached floret assays were used as described (Imboden, Afton, and Trail 2018), and quantified by the percentage of florets exhibiting symptoms of discoloration and/or surface hyphal growth 5 days post inoculation (dpi). Matrix formation was determined following the protocol in Shay, Wiegand, and Trail (2022) with the exclusion of Rhodamine B, as proteins were shown to not be involved in matrix formation.

Selection and characterization of high-adhesion isolates

High-adhesion isolates were selected from the WT as follows. Polystyrene coverslips (VWR, Radnor, PA) were submerged in 7 mL liquid Bird medium in 60 mm Petri dishes, inoculated with 1 mL conidial suspension, and incubated without shaking for 24 hours at RT. The coverslips were removed, gently washed with sterile distilled water three times, and submerged in fresh Bird medium in a Petri dish for 24 hr. The rinse and transfer were repeated daily for 18 consecutive days, before the final coverslip was placed in liquid CMC medium to produce conidia of the selected isolate, which will be referred to as the artificially selected (AS) isolates from here on. Three independent isolates were generated by this method.

To determine whether the selection of high-adhesion isolates was due to changes in methylation patterns, the isolates were grown in CMC supplemented with 1 mM 5-azacytidine (5-AZ; Sigma-Aldrich, St. Louis, MO), a DNA methyltransferase inhibitor (Hu and Bidochka 2020). WT and each AS isolate were grown under standard conditions with the addition of 5-AZ, before the

cultures were analyzed for adhesion to a polystyrene surface and floret infection, as described above.

Imaging of biofilms from colonized plants

To determine whether the colonization on the surface of infected florets was a biofilm, detached barley florets were inoculated as described above. WT hyphae were gently removed from the inoculation point on colonized florets at 5 dpi, and stained for matrix components as described in Shay, Wiegand, and Trail (2022), using calcofluor white, propidium iodide, and nile red.

Results

Transcriptomic analysis of biofilm formation

Transcriptomic analysis was performed to reveal the genes that underlie biofilm formation at key time points of biofilm development. As previously described (Shay, Wiegand, and Trail 2022), conidia began adhering to the polystyrene surface at 4 hpi, which we defined as the start of a new biofilm. At 12 hpi, the ECM began to form around the developing biofilm, and was mainly composed of extracellular nucleic acids. The ECM is a vital protective structure of biofilms, and the beginning of the formation of it was of interest to us. A mature biofilm, where new conidia are starting to disperse, was formed at 20 hpi. The last stage chosen for transcriptomic analysis was 44 hpi, which is the last time point identified where biofilms still adhered to the surface, before dispersal. This final biofilm stage is characterized by a polysaccharide-rich matrix and a hydrophobic surface, with lipid-rich hyphae (Shay, Wiegand, and Trail 2022). RNA-sequencing resulted in an average of >25,000,000 reads, 36-100 base pairs per read per sample after trimming low quality reads. Each developmental time-point was individually compared to the subsequent stage, as this provided a more detailed look at development over time than comparing across all stages simultaneously. Differentially expressed genes were identified between stages,

with >250 genes significantly downregulated and >350 genes significantly upregulated when compared to their subsequent stage (Figure 3.1A). We examined the top 10 most significant genes from each time point comparison, and selected candidates for knockout with some consideration for their annotation (Table 3.1). Expression levels of the selected genes varied across the time course, with some highly expressed early, and others highly expressed in mature biofilms (Figure 3.1B).

Table 3.1: Genes selected for characterization. Genes were assigned annotations based on Blast analysis of the sequence. Fold change and P values were determined by transcriptomic analysis. For genes selected from the literature, fold change and p value are not applicable.

Gene designation	Annotation	Fold Change	P value	Reference
FGSG_03680	Aquaporin 3	4.9188891	0.00011	This paper; Shay et al. in prep
FGSG_03951	Phospholipid synthase	2.4244756	0.000222	This paper
FGSG_07168	Tuberous sclerosis	1.5921713	4.84E-05	This paper
FGSG_08844	Mad1	N/A	N/A	Epstein and Nicholson, 2016
FGSG_11205	Precursor to snodprot1	281.63879	0.000391	This paper
FGSG_11955	VeA	N/A	N/A	Li, et al. 2015; Jiang, et al. 2011
FGSG_13052	Endonuclease	0.3380854	6.55E-06	This paper

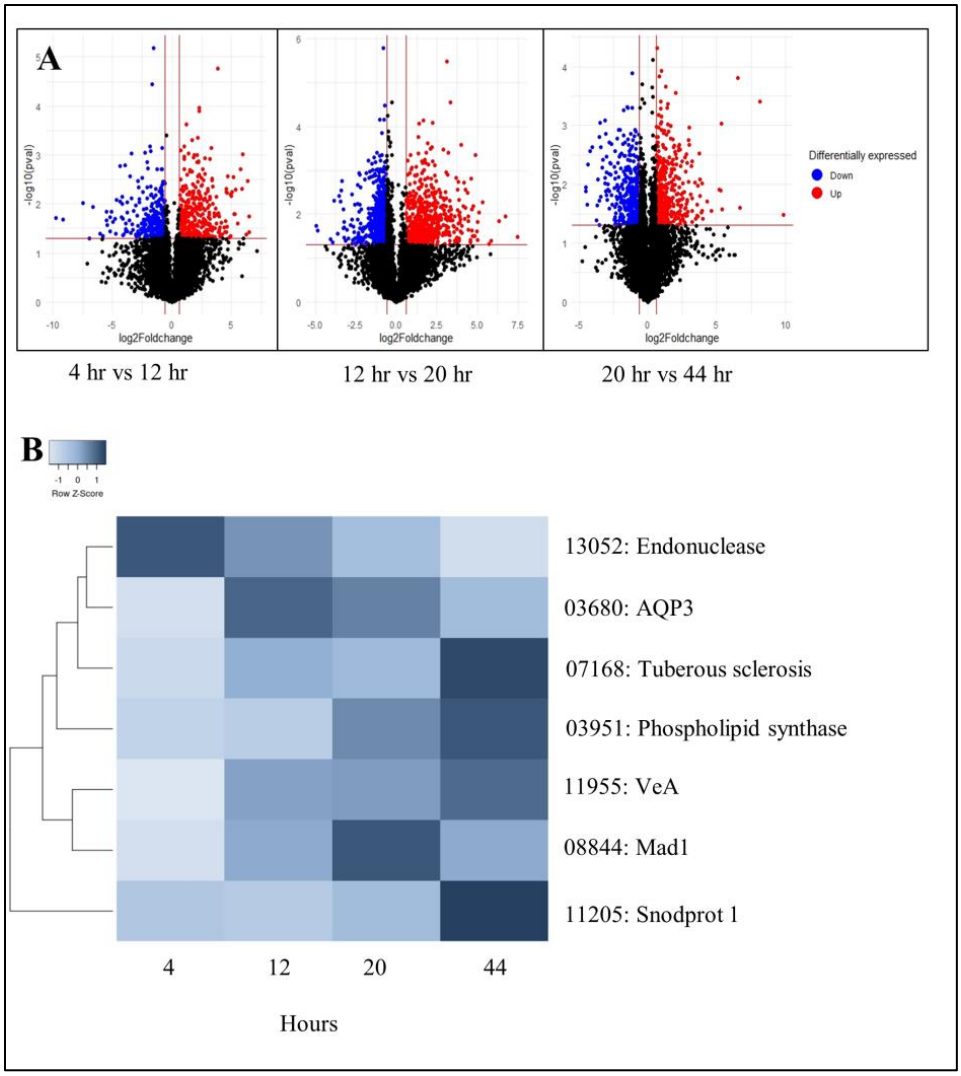


Figure 3.1: Transcriptomics results. (A) Volcano plots of differentially expressed genes between time points. Blue dots are transcripts that were downregulated from the first time point to the second, and red dots are transcripts that were upregulated from the first time point to the second. (B) Relative expression (Row Z score) of log2 FPKM of genes of interest across time points (hours).

Characterization of selected genes

To determine how the selected genes impacted characteristics of biofilm formations, all strains were compared to WT for adhesion, pathogenicity, and ECM composition. Adhesion assays were used to determine how genes impacted adhesion. Gene knockout strains of *FGSG_03680*, *FGSG_03951*, *FGSG_07168*, and *FGSG_11205* showed increased adhesion 24 hours post-

inoculation ($p<0.1$) compared to WT (Figure 3.2A).

Pathogenicity on detached barley florets was exhibited by all strains by 5 dpi (Figure 3.2B). Knockout strains were not significantly different than the WT in the percentage of florets showing symptoms. However, external hyphae were reduced in *Δ03951* and *Δ08844*, where florets had few hyphae growing on the outside of florets (Figure 3.2B). Pathogenicity data from similar analyses for FGSG_03680 will be published elsewhere (Shay et al. in prep).

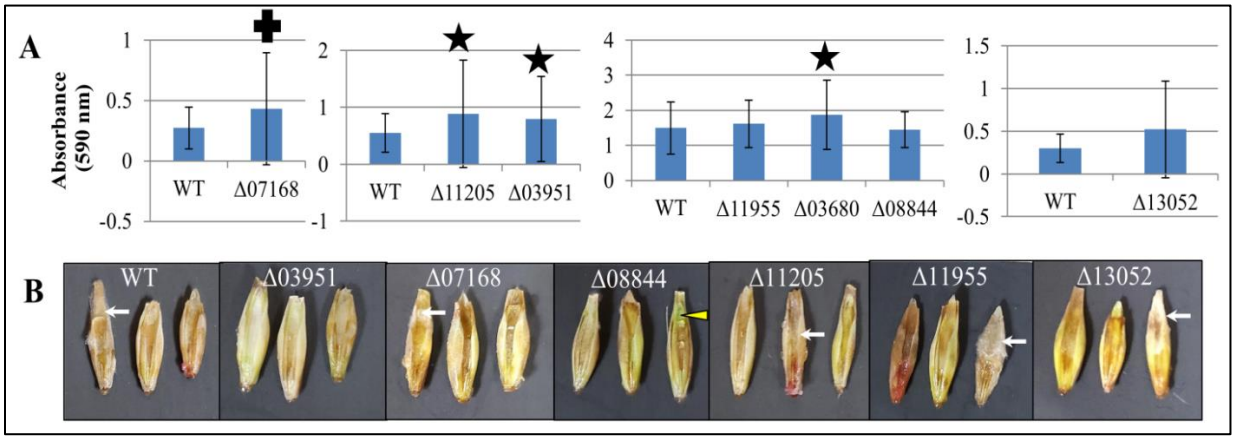


Figure 3.2: Characterization of gene knockouts. (A) Adhesion to a polystyrene surface, as measured by the absorbance of crystal violet at 590 nm. Stars indicate an increase ($P<0.1$), plus symbol indicates a significant increase ($p<0.05$). Adhesion varied between experiments, and each grouping represents three replicates of those strains, done together. (B) Representative detached florets 5 days post-inoculation. White arrows indicate areas of visible hyphae, while yellow arrowheads indicate areas without discoloration. The star indicates a significant reduction in infection (data not shown, $p<0.05$).

Gene knockout strains were screened for the development of matrix components. The formation of extracellular nucleic acids was impacted in strains *Δ07168*, *Δ08844*, *Δ11205*, *Δ11955*, and *Δ13052*. Hyphae stained with propidium iodide exhibited reduced stain retention when compared to WT, with many exhibiting no visible staining coalesced into the hyphae or matrix (Figure 3.3A). *Δ07168* exhibited no visible staining in all time points. *Δ08844* and *Δ11205* had the most prominent staining at 12 hpi, although it was reduced when compared to WT. Staining in

Δ07168, *Δ11205, Δ11955*, and *Δ13052* did not localize in the hyphae or matrix at 44 hpi (Figure 3.3A).

The formation of lipids was observed by staining with nile red. Strains *Δ03680* and *Δ07168* exhibited an increase in lipid staining in the ECM, which was not exhibited in WT (Figure 3.3B). *Δ08844* had levels of lipids similar to WT, but also exhibited lipids localizing in the matrix of mature biofilms. *Δ03951* and *Δ11955* showed reduced staining with nile red, where the stain did not localize to any distinguishable structures in either at 20 hpi, and few visible hyphae were apparent in *Δ03951* at 44 hpi (Figure 3.3B).

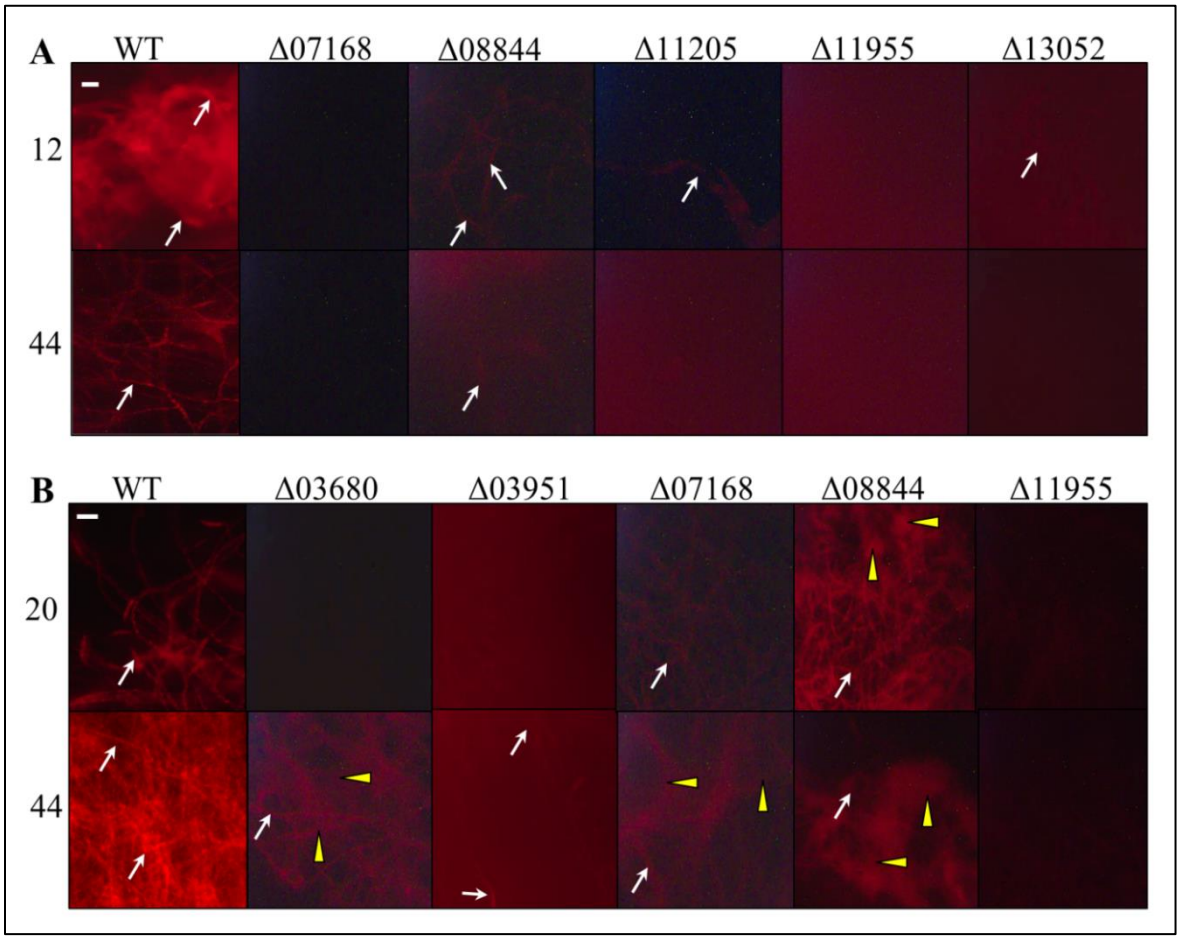


Figure 3.3: Matrix composition of gene knockouts.

Figure 3.3 (cont'd)

(A) Propidium iodide staining of extracellular nucleic acids of selected time points (hpi) and mutants. Arrows indicate hyphae with accumulated staining. Images with no arrows lacked staining, indicating no extracellular nucleic acids. (B) Nile red staining for lipids of selected mutants and time points (hpi). White arrows indicate hyphae with accumulated staining, and yellow arrowheads indicate accumulation of lipids in the matrix. Images without arrows did not have visible staining, indicating reduced lipids. Scale = 20 μm, representative of all micrographs.

Progeny arising from crosses between the gene knockouts and a *nit1* mutant segregated to exhibit the nit- or nit+ phenotype, and hygromycin resistance or susceptibility. Hygromycin resistant cultures arising from these ascospores exhibited the phenotype of the knockout parent (nit+), indicating that the observed phenotype was linked to hygromycin resistance as a result of deletion of the target gene (Table 3.3).

Generation of a high-adhesion strain

The WT strain was sequentially cultured and selected for increased adhesion. The artificially selected isolates (ASs) were significantly higher in adhesion ($p<0.05$) compared to WT (Figure 3.4A). Florets inoculated with the ASs exhibited more severe symptoms ($p<0.1$) during early infection than WT, along with an increase in visible external hyphae (Figure 3.4B). To elucidate whether there is a component of the matrix that may contribute to the increase in adhesion, the matrix was stained as previously described. Extracellular nucleic acids developed later in the ASs than WT, where the ASs exhibited less staining at 12 hpi and greater stain retention at 44 hpi when compared to WT (Figure 3.4C).

5-azacytidine (5-AZ), a DNA methyltransferase inhibitor, was added to the cultures to elucidate whether the increased adhesion and pathogenicity seen in ASs were due to epigenetic regulation. ASs grown in culture with 5-AZ were reduced to WT levels of adhesion (Figure 3.4A). Inhibiting methylation in WT and ASs did not significantly change the percentage of symptomatic florets from the WT in normal conditions, but interestingly, methylation-inhibited

isolates showed a reduction in external hyphae on symptomatic florets (Figure 3.4B).

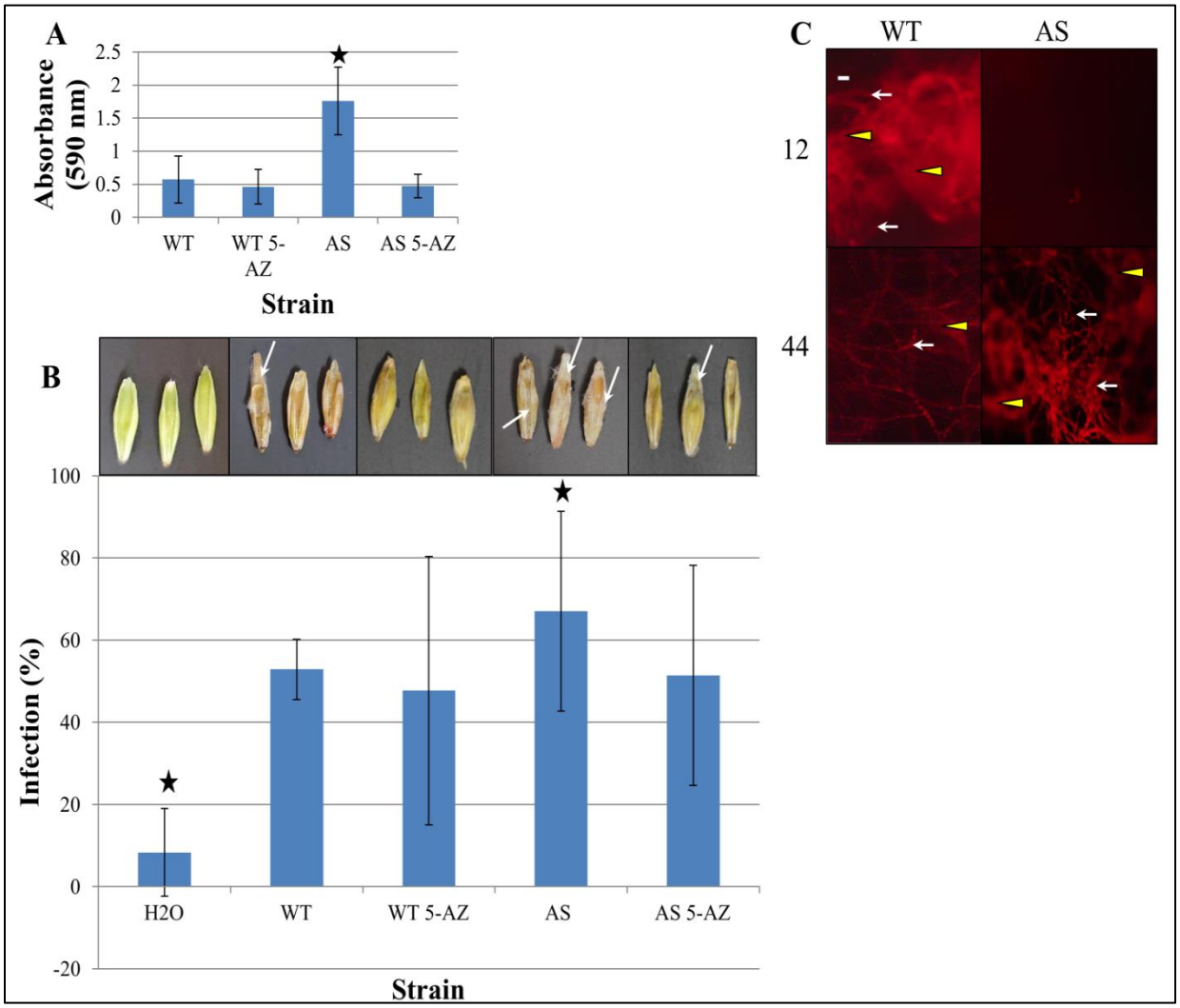


Figure 3.4: Artificial selection for adhesion. (A) adhesion of strains to a polystyrene surface, grown in cultures without or with 5-azacytidine (5-AZ), a methylation inhibitor. Star indicates a significant increase ($p < 0.05$). (B) Percent infection of detached barley florets with representative florets. White arrows indicate external hyphal growth. Stars indicate a significant difference in infection compared to WT ($p<0.1$). (C) Propidium iodide staining on matrix to visualize extracellular nucleic acids from WT and AS matrices. White arrows point to hyphae, and yellow arrowheads indicate areas of matrix. Scale bar = 20 μm, representative of all micrographs.

Surface colonization on florets have biofilm characteristics

Hyphae growing on the surface of barley florets were stained to view the hallmarks of biofilm formation: polysaccharides, extracellular nucleic acids, and lipids. Hyphae observed growing on

the surface were polysaccharide- and lipid-rich (Figure 3.5), a result similar to late stage biofilms seen *in vitro* (Shay, Wiegand, and Trail 2022). Propidium iodide stained extracellular nucleic acids on and around the hyphae, indicating their presence in the matrix (Figure 3.5).

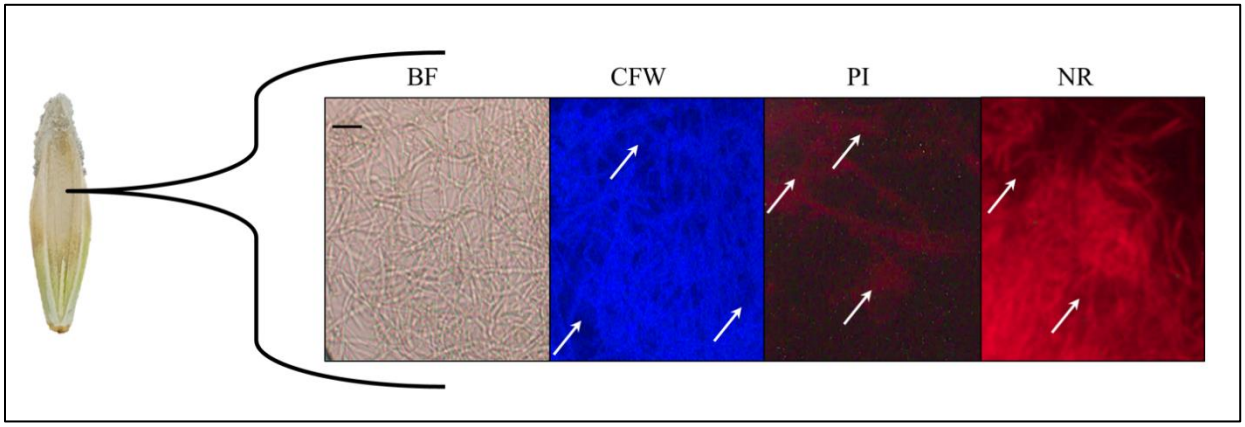


Figure 3.5: Biofilm development on plant tissue. Hyphae were taken from the inoculation point on a colonized barley floret (left; 5 dpi), and stained biofilm components. Images were recorded in brightfield (BF), calcofluor white (CFW) which stains polysaccharides, propidium iodide (PI) which stains extracellular nucleic acids, and nile red (NR) which stains lipids. White arrows indicate areas of matrix formation around hyphae. Scale bar = 20 μm, representative for all images.

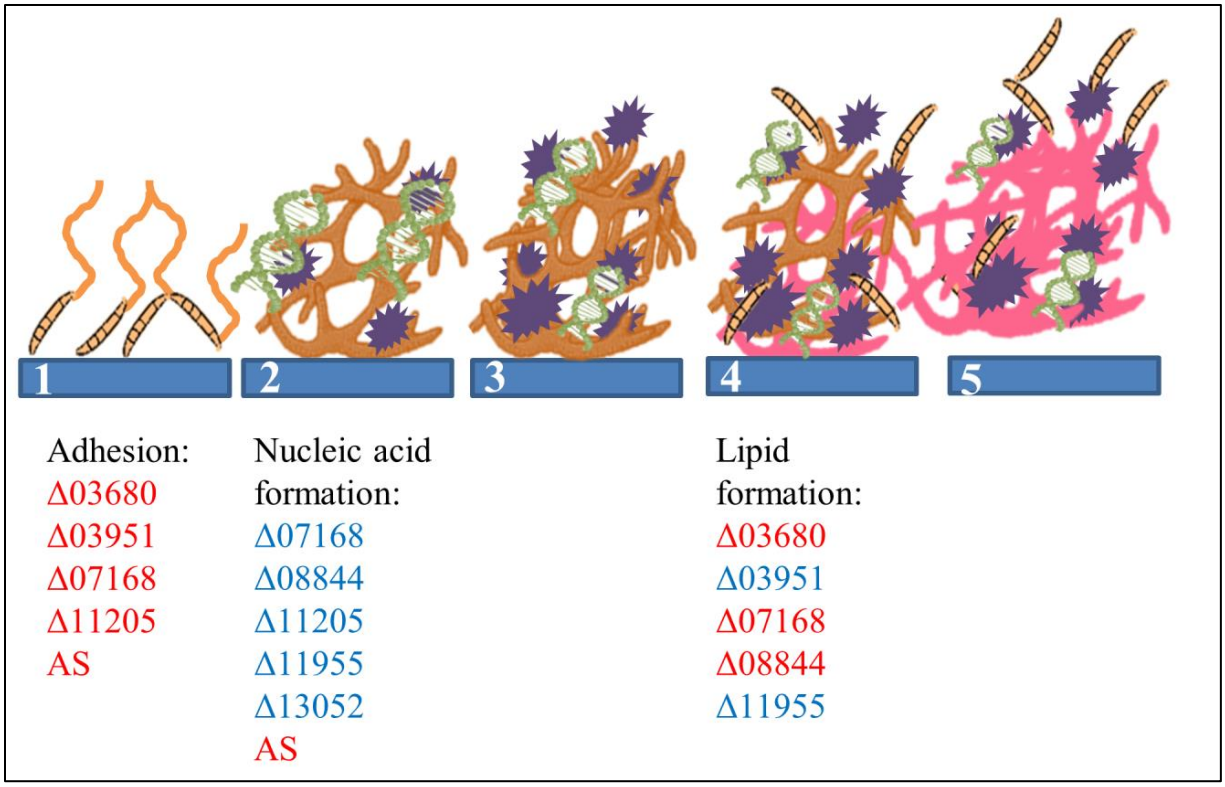


Figure 3.6: Involvement of genes in biofilm formation in *Fusarium graminearum*. Adapted from Shay, Wiegand, and Trail 2022. Biofilms form *in vitro* by conidia adhering to a surface (1), followed by proliferation (2-5) and matrix development (2-5). The matrix (purple) is nucleic acid-rich early (2; green), and more carbohydrate-rich later in development. Hyphae become lipid-rich (pink) as the biofilm develops over time. The whole biofilm stops adhering to a surface (5) at maturity. Strains were observed to either reduce (blue) matrix components, or increase development (red). Numbers indicate gene designations, and AS indicates the artificially selected isolates.

Discussion

Here we have performed transcriptomic analysis of biofilm development in the plant pathogenic fungus *Fusarium graminearum.* We performed expression studies at specific stages, and by comparing sequential steps in biofilm formation, we closely examined the specific genes that are involved at each stage. We previously developed a model of biofilm formation in *F. graminearum* that incorporates matrix formation (Shay, Wiegand, and Trail 2022). We have adapted this model to include more details on the development of the matrix, and identified

genes that impact formation of matrix components (Figure 3.6).

The transcriptomic analysis identified genes expressed in each of the stages that were consistent with the physiological activities characteristic of that stage. Genes involved in DNA cutting and replication were differentially expressed between 4 hpi and 12 hpi, where the matrix is beginning to form, and extracellular nucleic acids are a primary component. Five of the seven genes studied through knockout analysis reduced the formation of extracellular nucleic acids in biofilm ECMs. The deletion mutants of the putative endonuclease *FGSG_13052* showed a reduction in staining of extracellular nucleic acids, early in biofilm development. The other genes from this study that impacted the extracellular nucleic acids may either be the source of the DNA found in the matrix, or contribute to the signaling pathways involved in moving DNA to the ECM. In biofilms of bacteria and fungi, the DNA found in the matrix is genome-derived and regulated by quorum sensing (Allesen-Holm et al. 2006; Martins et al. 2010; Mann and Wozniak 2012; Rajendran et al. 2013; Kischkel et al. 2019). Multiple pathways are used to deposit DNA in *Pseudomonas aeruginosa* biofilms (Allesen-Holm et al. 2006), which would explain why many genes are involved in this process in *F. graminearum* biofilms. Further work on the mechanisms used to deposit DNA in the matrix of *F. graminearum* biofilms is necessary to elucidate the function of all the genes involved.

Surprisingly, knocking out genes involved in biofilm formation increased the adhesion of cells to polystyrene surfaces. *FGSG_03680,* an aquaporin, *FGSG_03951,* a phospholipid synthase, and *FGSG_07168,* which helps regulate the cell cycle, are all involved in cellular growth. *FGSG_11205* is a precursor to snodprot1, a secreted protein involved in infection (Hall, Keon, and Hargreaves 1999; Jeong, Mitchell, and Dean 2007), indicating that biofilm adhesion is linked to infection. By knocking out genes involved in the process of biofilm formation, there is

a delay in growth and development, which results in an increase in adhesion by 24 hpi. Traditional assays use adhesion at a single time point to quantify biofilm formation, which is impacted by any delays in growth from gene knockouts. It may be that adhesion assays at a single time point do not capture the nuances of biofilm formation. Expanded characterization, as done in this study, will provide a more holistic picture of the process, and should be included in all future biofilm studies across species.

At 44 hpi, hyphae in WT biofilms are lipid-rich (Shay, Wiegand, and Trail 2022), which aligns with the upregulated expression of genes involved in lipid biosynthesis between 20 and 44 hpi. The knockout of the phospholipid synthase gene *FGSG_03951* impacted lipid formation, where lipids did not coalesce in hyphae. Interestingly, *Δ03680*, *Δ07168*, and *Δ08844* exhibited lipid localization in the ECM, instead of exclusively in the hyphae, as seen in WT. The ability of *F. graminearum* to switch the localization of lipids in mature biofilms is worth further study, as lipids have been shown to be crucial in *C. albicans* biofilm matrices (Alim, Sircaik, and Panwar 2018). Lipids may be conditionally localized, depending on signals the fungus receives from the external environment. *FGSG_03680* encodes an aquaporin, potentially a transmitter of some kind of signal (Verkman 2011; G. Li et al. 2020). *FGSG_08844* encodes a cell surface adhesin, and, together with *FGSG_03680*, may be involved in sensing the signals that determine where lipids are deposited in biofilms. Lipids in the *C. albicans* ECM are involved in matrix structure, signaling, and drug resistance (Alim, Sircaik, and Panwar 2018). Further work on how lipids are localized is necessary for understanding the role of lipids in *F. graminearum* biofilms.

We show that the ability of *F. graminearum* to adhere to a surface increases in artificially selected isolates (ASs). Florets inoculated with the ASs produced symptoms more severe than those inoculated with the WT, indicating that adhesion to the floret surface, which is the initial

stage of biofilm formation, is important in the plant colonization. Staining the matrix of the ASs showed that extracellular nucleic acids develop later than in WT biofilms. The ECM of WT is nucleic acid-rich at 12 hpi, while the ASs ECM is nucleic acid-rich at 44 hpi. Adhesion data was taken at 24 hpi, which is after the concentration of nucleic acids in the ECM of the WT decreases, but before the ASs enrich ECM nucleic acids. These findings indicate that the extracellular nucleic acids in the matrix have a significant contribution to the adhesion of biofilms to the surface in filamentous fungi. Adhesion is apparently easily regulated, which was demonstrated by the speed with which increased adhesion was seen.

Methylation in *F. graminearum* is signaled by the environment, and is involved in growth and virulence (Bonner et al. 2021; Tini et al. 2021). Introducing the DNA methyltransferase inhibitor to the ASs decreased adhesion to WT levels, and decreased the presence of external hyphae in the detached floret assays. The ASs increased adhesion, through altered methylation patterns. Although the exact change in methylation is not known, the implication is that epigenetic regulation can alter biofilm formation in a very short time period. Thus the formation of a biofilm is an important adaptation used by *F. graminearum* to better survive.

Here we have reported the first, to our knowledge, filamentous fungal biofilm on the surface of plant tissues. The discovery that biofilms are an integral part of the infection process of *F. graminearum* is a novel finding in a model fungus. It may be that this phenomenon is more widely used by other fungal pathogens, but not recognized. Methylation appears to regulate adhesion of biofilms, increasing adhesion when the pathogen is exposed to environments where cells can wash away. Further work is needed to better understand the pathways the genes are involved in, and how methylation regulates biofilm formation.

Acknowledgements

We'd like to thank Spotless Amponsah for her help with data collection. We'd like to thank Soumya Moonjely and Mitch Roth for their feedback on this manuscript. This work was supported by the US Wheat and Barley Scab Initiative, A.L. Rodger's Scholarship, and Everett Beneke Endowment Award.

Supplemental Tables

Table 3.2: Primers and PCR confirmation pictures for gene knockouts.

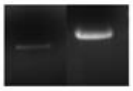
Gene	L5	L3	R5	R3	Band size (base pairs)	PCR confirmation
FGSG_03951	CAGCGTCGTAGT TGGATCG	TGGGTCAGGTGC TTTCTCC	CCGTGGAGATG GGAGAATGG	TGACGAGAGA AACAGGTGCC	KO(left) = 3417 WT (right) = 3876	
FGSG_07168	GCCTCTTGTACCT TACCGCC	CATCGGTAAGGC AAGGCAGA	GTGGAAGGCGT CTATCTCCG	GCAGAACGTC CATCTCCCAT	KO (left, arrow) = 3790 WT (right) = 7462	
FGSG_08844	TTTTGCTGTCTCA GTTGTTAC	GGTGAATGATTT GTTGGG	AAATTGGTCCCT TCTCTATG	AGGCAAAGAA GTTACCGAG	KO (left) = 2169 WT (right) = 4100	
FGSG_11205	TCTTACTGCCTAC GGACCCA	TTCGTATCGGGGC TGCTTTT	CATTCAGTTGTG CGACGGTT	GCAGCCGAGT ATTAGCCTG	KO (left) = 3313 WT (right) = 2961	
FGSG_11955	CGCGTTCTTTCAA CTGTCCG	GGCAGAGAGGAA CCCAGTTG	AACAAAGGCGT GAAGCGTCT	GACCCAGAAA CTGAACCGGA	KO (left) = 3104 WT (right; arrow) = 4177	
FGSG_13052	AGCGGCAGAACT TTCAAGGT	TCGAAACACCCA CCGCTAA	ACAACGGCATG GATCAGGTT	CATCCTTCTTC GTCCCTGCC	KO (right) = 2422 WT (left) 2020	
Overlap primers						
L overhang	CGTCAGATCGAT GGTAGTTGTCGTC GACT					
R overhang	ACACTGGTGACG GCTAACCAGAAC TGTCA					

Table 3.3: Segregation analysis of gene knockout lines, crossed with a Nit- mutant.

Segregation analysis of knockouts				
	Number of isolates showing phenotype[a]			
Gene	NIT^- Hyg^r	NIT^- Hyg^s	NIT^+ Hyg^r	NIT^+ Hyg^s
Δ3680	2	8	10	0
Δ3951	5	10	10	0
Δ7168	3	7	9	1
Δ8844	1	9	9	1
Δ11205	1	9	10	0
Δ11955	1	9	10	0
Δ13052	4	6	10	0

[a]Colonies were analyzed for segregation of two phenotypes: NIT^- (nitrate non-utilizing), and Hyg^r or Hyg^s (hygromycin resistance or hygromycin sensitivity, respectively)

REFERENCES

Alim, Darakshan, Shabnam Sircaik, and Sneh Lata Panwar. 2018. “The Significance of Lipids to Biofilm Formation in *Candida albicans*: An Emerging Perspective.” *Journal of Fungi* 4 (4). https://doi.org/10.3390/jof4040140.

Allesen-Holm, Marie, Kim Bundvig Barken, Liang Yang, Mikkel Klausen, Jeremy S. Webb, Staffan Kjelleberg, Søren Molin, Michael Givskov, and Tim Tolker-Nielsen. 2006. “A Characterization of DNA Release in *Pseudomonas aeruginosa* Cultures and Biofilms.” *Molecular Microbiology* 59 (4): 1114–28. https://doi.org/10.1111/j.1365-2958.2005.05008.x.

Babicki, Sasha, David Arndt, Ana Marcu, Yongjie Liang, Jason R. Grant, Adam Maciejewski, and David S. Wishart. 2016. “Heatmapper: Web-Enabled Heat Mapping for All.” *Nucleic Acids Research* 44 (1): W147–53. https://doi.org/10.1093/NAR/GKW419.

Bolger, Anthony M., Marc Lohse, and Bjoern Usadel. 2014. “Trimmomatic: A Flexible Trimmer for Illumina Sequence Data.” *Bioinformatics* 30 (15): 2114–20. https://doi.org/10.1093/bioinformatics/btu170.

Bonner, Christopher, Amanda Sproule, Owen Rowland, David Overy, and Rajagopal Subramaniam. 2021. “DNA Methylation Is Responsive to the Environment and Regulates the Expression of Biosynthetic Gene Clusters, Metabolite Production, and Virulence in *Fusarium graminearum*.” *Frontiers in Fungal Biology* 1 (January). https://doi.org/10.3389/ffunb.2020.614633.

Bowden, Robert L., and John F. Leslie. 1999. “Sexual Recombination in *Gibberella zeae*.” *Phytopathology* 89 (2): 182–88. https://doi.org/10.1094/PHYTO.1999.89.2.182.

Cappellini, R. A., and J. L. Peterson. 1965. “Macroconidium Formation in Submerged Cultures by a Nonsporulating Strain of *Gibberella zeae*.” *Mycologia* 57 (6): 962–66. https://doi.org/10.1080/00275514.1965.12018285.

Cavinder, Brad, Ahmed Hamam, Roger R. Lew, and Frances Trail. 2011. “Mid1, a Mechanosensitive Calcium Ion Channel, Affects Growth, Development, and Ascospore Discharge in the Filamentous Fungus *Gibberella zeae*.” *Eukaryotic Cell* 10 (6): 832–41. https://doi.org/10.1128/EC.00235-10.

Cavinder, Brad, Usha Sikhakolli, Kayla M. Fellows, and Frances Trail. 2012. “Sexual Development and Ascospore Discharge in *Fusarium graminearum*.” *Journal of Visualized Experiments : JoVE*, no. 61: 1–5. https://doi.org/10.3791/3895.

Chinnici, Jennifer L., Ci Fu, Lauren M. Caccamise, Jason W. Arnold, and Stephen J. Free. 2014. “*Neurospora crassa* Female Development Requires the Pacc and Other Signal Transduction Pathways, Transcription Factors, Chromatin Remodeling, Cell-to-Cell Fusion, and Autophagy.” *PLoS ONE* 9 (10). https://doi.org/10.1371/journal.pone.0110603.

Córdova-Alcántara, Itzel Margarita, Diana Laura Venegas-Cortés, María Ángeles Martínez-Rivera, Néstor Octavio Pérez, and Aida Verónica Rodriguez-Tovar. 2019. "Biofilm Characterization of *Fusarium solani* Keratitis Isolate: Increased Resistance to Antifungals and UV Light." *Journal of Microbiology* 57 (6): 485–97. https://doi.org/10.1007/s12275-019-8637-2.

Epstein, Lynn, and Ralph Nicholson. 2016. "Adhesion and Adhesives of Fungi and Oomycetes." In *Biological Adhesives*, 25–55. https://doi.org/10.1007/978-3-319-46082-6.

Fanning, Saranna, and Aaron P. Mitchell. 2012. "Fungal Biofilms." *PLoS Pathogens* 8 (4): 1–4. https://doi.org/10.1371/journal.ppat.1002585.

Finkel, Jonathan S., and Aaron P. Mitchell. 2011. "Genetic Control of *Candida albicans* Biofilm Development."

Frazee, Alyssa C., Geo Pertea, Andrew E. Jaffe, Ben Langmead, Steven L. Salzberg, and Jeffrey T. Leek. 2015. "Ballgown Bridges the Gap between Transcriptome Assembly and Expression Analysis." *Nature Biotechnology* 33 (3): 243–46. https://doi.org/10.1038/nbt.3172.

Hall, N., J. P.R. Keon, and J. A. Hargreaves. 1999. "A Homologue of a Gene Implicated in the Virulence of Human Fungal Diseases Is Present in a Plant Fungal Pathogen and Is Expressed during Infection." *Physiological and Molecular Plant Pathology* 55 (1): 69–73. https://doi.org/10.1006/pmpp.1999.0203.

Hallen-Adams, Heather E., Brad L. Cavinder, and Frances Trail. 2011. "*Fusarium graminearum* from Expression Analysis to Functional Assays." *Methods in Molecular Biology (Clifton, N.J.)* 722 (1): 79–101. https://doi.org/10.1007/978-1-61779-040-9_6.

Harding, Michael W., Lyriam L R Marques, Ronald J. Howard, and Merle E. Olson. 2009. "Can Filamentous Fungi Form Biofilms?" *Trends in Microbiology* 17 (11): 475–80. https://doi.org/10.1016/j.tim.2009.08.007.

Harding, Michael W, Lyriam L R Marques, Ronald J Howard, and Merle E Olson. 2010. "Biofilm Morphologies of Plant Pathogenic Fungi." *The Americas Journal of Plant Science and Biotechnology* 4 (Special Issue 2): 43–47.

Harrison, Nicholas, Brad Cavinder, Jeffrey P. Townsend, and Frances Trail. 2013. "Optimized Primers and Other Critical Conditions for Efficient Fusion PCR to Generate Knockout Vectors in Filamentous Fungi." *Fungal Genetics Reports*, no. C: 1–10.

Hou, Zhanming, Chaoyang Xue, Youliang Peng, Talma Katan, H. Corby Kistler, and Jin Rong Xu. 2002. "A Mitogen-Activated Protein Kinase Gene (MGV1) in *Fusarium graminearum* Is Required for Female Fertility, Heterokaryon Formation, and Plant Infection." *Molecular Plant-Microbe Interactions* 15 (11): 1119–27. https://doi.org/10.1094/MPMI.2002.15.11.1119.

Hu, Shasha, and Michael J. Bidochka. 2020. “DNA Methyltransferase Implicated in the Recovery of Conidiation, through Successive Plant Passages, in Phenotypically Degenerated *Metarhizium*.” *Applied Microbiology and Biotechnology* 104 (12): 5371–83. https://doi.org/10.1007/s00253-020-10628-6.

Imboden, Lori, Drew Afton, and Frances Trail. 2018. “Surface Interactions of *Fusarium graminearum* on Barley.” *Molecular Plant Pathology* 19 (6): 1332–42. https://doi.org/10.1111/mpp.12616.

Jeong, Jun Seop, Thomas K. Mitchell, and Ralph A. Dean. 2007. “The *Magnaporthe grisea* Snodprot1 Homolog, MSP1, Is Required for Virulence.” *FEMS Microbiology Letters* 273 (2): 157–65. https://doi.org/10.1111/j.1574-6968.2007.00796.x.

Kim, Daehwan, Ben Langmead, and Steven L Salzberg. 2015. “HISAT: A Fast Spliced Aligner with Low Memory Requirements.” *Nature Methods* 12 (4): 357–60. https://doi.org/10.1038/nmeth.3317.HISAT.

King, Robert, Martin Urban, Michael C.U. Hammond-Kosack, Keywan Hassani-Pak, and Kim E. Hammond-Kosack. 2015. “The Completed Genome Sequence of the Pathogenic Ascomycete Fungus *Fusarium graminearum*.” *BMC Genomics* 16 (1): 1–21. https://doi.org/10.1186/s12864-015-1756-1.

Kischkel, Brenda, Gredson Keiff Souza, Lucas Ulisses, Rovigatti Chiavelli, Terezinha Inez, Estivalet Svidzinski, and Melyssa Negri. 2019. “The Ability of Farnesol to Prevent Adhesion and Disrupt *Fusarium keratoplasticum* Biofilm.” *Applied Microbial and Cell Physiology*.

Klittich, C, and J F Leslie. 1988. “Nitrate Reduction Mutants of *Fusarium moniliforme* (*Gibberella fujikuroi*).” *Genetics* 118 (3): 417–23. https://doi.org/10.1093/genetics/118.3.417.

Kowalski, Caitlin H, Joshua D Kerkaert, Ko-wei Liu, Matthew C Bond, Raimo Hartmann, Carey D Nadell, Jason E Stajich, and Robert A Cramer. 2019. “Fungal Biofilm Morphology Impacts Hypoxia Fitness and Disease Progression.” *Nature Microbiology*. https://doi.org/10.1038/s41564-019-0558-7.

Li, Guangjin, Tong Chen, Zhanquan Zhang, Boqiang Li, and Shiping Tian. 2020. “Roles of Aquaporins in Plant-Pathogen Interaction.” *Plants* 9 (9): 1–9. https://doi.org/10.3390/plants9091134.

Li, Heng, Bob Handsaker, Alec Wysoker, Tim Fennell, Jue Ruan, Nils Homer, Gabor Marth, Goncalo Abecasis, and Richard Durbin. 2009. “The Sequence Alignment / Map (SAM) Format and SAMtools 1000 Genome Project Data Processing Subgroup.” *Bioinformatics (Oxford, England)* 25 (16): 1–2. http://www.ncbi.nlm.nih.gov/pubmed/19505943.

Li, Peiqian, Xiaoming Pu, Baozhen Feng, Qiyun Yang, Huifang Shen, Jingxin Zhang, and Birun

Lin. 2015. “FocVel1 Influences Asexual Production, Filamentous Growth, Biofilm Formation, and Virulence in *Fusarium oxysporum f. Sp. cucumerinum*.” *Frontiers in Plant Science* 6 (May): 1–10. https://doi.org/10.3389/fpls.2015.00312.

Mann, Ethan E., and Daniel J. Wozniak. 2012. “*Pseudomonas* Biofilm Matrix Composition and Niche Biology.” *FEMS Microbiology Reviews* 36 (4): 893–916. https://doi.org/10.1111/j.1574-6976.2011.00322.x.

Martins, Margarida, Priya Uppuluri, Derek P. Thomas, Ian A. Cleary, Mariana Henriques, José L. Lopez-Ribot, and Rosário Oliveira. 2010. “Presence of Extracellular DNA in the *Candida albicans* Biofilm Matrix and Its Contribution to Biofilms.” *Mycopathologia* 169 (5): 323–31. https://doi.org/10.1007/s11046-009-9264-y.

Metzenberg, Robert L. 2004. “Bird Medium: An Alternative to Vogel Medium.” *Fungal Genetics Reports* 51 (1): 19–20. https://doi.org/10.4148/1941-4765.1138.

Motaung, Thabiso E, Chizne Peremore, Brenda Wingfield, and Emma Steenkamp. 2020. “Plant-Associated Fungal Biofilms - Knowns and Unknowns.” *FEMS Microbial Ecology* 5: 1–25.

Nobile, Clarissa J., Jeniel E. Nett, Aaron D. Hernday, Oliver R. Homann, Jean Sebastien Deneault, Andre Nantel, David R. Andes, Alexander D. Johnson, and Aaron P. Mitchell. 2009. “Biofilm Matrix Regulation by *Candida albicans* Zap1.” *PLoS Biology* 7 (6). https://doi.org/10.1371/journal.pbio.1000133.

Peiqian, Li, Pu Xiaoming, Shen Huifang, Zhang Jingxin, Huang Ning, and Lin Birun. 2014. “Biofilm Formation by *Fusarium oxysporum f. Sp. cucumerinum* and Susceptibility to Environmental Stress.” *FEMS Microbiology Letters* 350 (2): 138–45. https://doi.org/10.1111/1574-6968.12310.

Pertea, Mihaela, Geo M. Pertea, Corina M. Antonescu, Tsung Cheng Chang, Joshua T. Mendell, and Steven L. Salzberg. 2015. “StringTie Enables Improved Reconstruction of a Transcriptome from RNA-Seq Reads.” *Nature Biotechnology* 33 (3): 290–95. https://doi.org/10.1038/nbt.3122.

Rajendran, Ranjith, Craig Williams, David F. Lappin, Owain Millington, Margarida Martins, and Gordon Ramage. 2013. “Extracellular DNA Release Acts as an Antifungal Resistance Mechanism in Mature *Aspergillus fumigatus* Biofilms.” *Eukaryotic Cell* 12 (3): 420–29. https://doi.org/10.1128/EC.00287-12.

Ramage, Gordon, Eilidh Mowat, Brian Jones, Craig Williams, and Jose Lopez-Ribot. 2009. “Our Current Understanding of Fungal Biofilms.” *Critical Reviews in Microbiology* 35 (4): 340–55. https://doi.org/10.3109/10408410903241436.

Ramage, Gordon, Ranjith Rajendran, Leighann Sherry, and Craig Williams. 2012. “Fungal Biofilm Resistance.” *International Journal of Microbiology* 2012. https://doi.org/10.1155/2012/528521.

Shay, Rebecca, Aaron A Wiegand, and Frances Trail. 2022. “Biofilm Formation and Structure in the Filamentous Fungus *Fusarium graminearum*, a Plant Pathogen.” *Microbiology Spectrum* 10 (4). https://doi.org/10.1128/spectrum.00171-22.

Simms, D, P Cizdziel, and P Chomczynski. 1993. “TRIzol: A New Reagent for Optimal Single-Step Isolation of RNA.” *Focus*, 99–102.

Singh, Rachna, M. R. Shivaprakash, and Arunaloke Chakrabarti. 2011. “Biofilm Formation by Zygomycetes: Quantification, Structure and Matrix Composition.” *Microbiology* 157 (9): 2611–18. https://doi.org/10.1099/mic.0.048504-0.

Theodorakopoulos, Nicolas, Benjamin Govetto, Benoit Industri, Lionel Massi, Marc Gaysinski, Emeline Deleury, Catherine Mura, et al. 2011. “Biology and Ecology of Biofilms Formed by a Plant Pathogen *Phytophthora parasitica*: From Biochemical Ecology to Ecological Engineering.” *Procedia Environmental Sciences* 9 (0): 178–82. https://doi.org/10.1016/j.proenv.2011.11.027.

Tini, Francesco, Giovanni Beccari, Gianpiero Marconi, Andrea Porceddu, Micheal Sulyok, Donald M. Gardiner, Emidio Albertini, and Lorenzo Covarelli. 2021. “Identification of Putative Virulence Genes by DNA Methylation Studies in the Cereal Pathogen *Fusarium graminearum.*” *Cells* 10 (5). https://doi.org/10.3390/cells10051192.

Trail, Frances, and Ralph Common. 2000. “ Perithecial Development by *Gibberella zeae* : A Light Microscopy Study .” *Mycologia* 92 (1): 130–38. https://doi.org/10.1080/00275514.2000.12061137.

Verkman, Alan S. 2011. “Aquaporins at a Glance.” *Journal of Cell Science* 124 (13): 2107–12. https://doi.org/10.1242/jcs.079467.

Wickham, Hadley. 2016. *Ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York. Media*. Vol. 35. http://link.springer.com/10.1007/978-0-387-98141-3.

Zheng, He, Jaekuk Kim, Mathew Liew, John K. Yan, Oscar Herrera, Jin Woo Bok, Neil L. Kelleher, Nancy P. Keller, and Yun Wang. 2015. “Redox Metabolites Signal Polymicrobial Biofilm Development via the Napa Oxidative Stress Cascade in *Aspergillus*.” *Current Biology* 25 (1): 29–37. https://doi.org/10.1016/j.cub.2014.11.018.

CHAPTER 4

THE ROLE OF AQUAPORINS IN THE PATHOGENIC RESPONSE OF *FUSARIUM GRAMINEARUM* TO PLANT SILICA

Abstract

Fusarium graminearum is the causal agent of Fusarium head blight (FHB), an economically important disease of cereal crops. *F. graminearum* has five aquaporins (AQPs), which are channel proteins involved in the transport of water and small molecules. AQP1 is associated with development, stress response, and secondary metabolism, while the other four AQPs remain largely unstudied. Our results show that AQP1, AQP3, and AQP5 play a role in the development, growth, and pathogenicity of *F. graminearum*, and the response of the fungus to silica. Silica is an important component of grasses, and interestingly, the typical infection points for *F. graminearum* are silica-rich cells. We have shown that *F. graminearum* takes silica up from its environment, and incorporates it into fungal cells. The distribution of silica in hyphae is dependent on AQPs, where silica is sequestered by membrane bound organelles when AQPs are present. Understanding the relationship between *F. graminearum* and silica contributes to early plant-pathogen interactions and provides insight into the initiation of infection.

Introduction

Aquaporins (AQPs) are a group of membrane channel proteins that function in water and small molecule transport, that are involved in a myriad of cellular processes in plants and animals, from cellular proliferation, to signaling, to defense responses (Verkman 2011; Li et al. 2020). In plants, numerous AQPs have been classified into four subfamilies based on localization and function: tonoplastic intrinsic proteins (TIPs), nodulin 26-like intrinsic proteins (NIPs), small basic intrinsic proteins (SIPs), and plasma membrane intrinsic proteins (PIPs; Kaldenhoff and

Fischer 2006). Some AQPs have high selectivity for uptake of molecules from the environment. Research has focused on AQPs that uptake silica, an important element in plant development associated with growth, and resistance to some plant pathogens. Lsi1 (Low silicon rice 1) was the first identified silica-specific AQP, and since its discovery, silica-specific AQPs have been identified across the plant kingdom (J. F. Ma et al. 2006; Exley, Guerriero, and Lopez 2020; Guerriero et al. 2019). Silica channels are especially important in grass crops, which are silica-accumulating plants with high concentrations in specific cells including trichomes, stomata, and vascular bundles (O'Reagain and Mentis 1989; Ball, Gardner, and Anderson 1999; Rudall, Prychid, and Gregory 2014). The external application of silica has been shown to increase plant defenses against fungal pathogens (J. Ma, Miyake, and Takahashi 2001; J. F. Ma 2003; Sakr 2016, 2022), and understanding this relationship may lead to better disease control.

In plant-pathogen interactions, AQPs are involved in the activation of plant defense responses, where they mediate transport of plant defense compounds across membranes, or trigger the closure of stomata (Li et al. 2020). On the pathogen side of the interaction, a few AQPs have been found to be important in the infection process. In *Botrytis cinerea*, an AQP participates in reactive oxygen species generation and diffusion across membranes, as well as cellular growth and development (Bienert and Chaumont 2014). *Fusarium graminearum* AQP1 is important in disease progression, where mycotoxin production was found to be reduced in the absence of AQP1 (Ding et al. 2018). Five predicted AQP genes have been identified in the genome of *Fusarium graminearum* (Ding et al. 2018), an economically important plant pathogen that causes the disease Fusarium head blight (FHB) on cereal crops (McMullen, Jones, and Gallenberg 1997; McMullen et al. 2012; Shah et al. 2018). With this study, we provide evidence that AQPs are taking up silica from the environment, and the relationship to plant pathogenicity. We

demonstrate the importance of AQPs in plant-pathogen interactions, particularly the infection processes and disease progression, and we discuss the implications for disease management.

Methods

Strains and culture conditions

F. graminearum wild-type (WT) strain PH-1 (FGSC 9075, NRRL 31084, Trail and Common 2000) was used for all experiments. WT and knockout strains were maintained in long-term storage as colonized blocks of carrot agar (Klittich and Leslie 1988) in 35% glycerol at -80^{o}C. Conidia were generated in liquid carboxymethyl cellulose medium (CMC; Cappellini and Peterson 1965), incubated at room temperature (RT; 22-25^{o}C), with shaking at 225 RPM, for five days. Conidia were freshly harvested and collected in sterile distilled water before adjusting to 10^6 conidia/mL with a hemocytometer (Sigma-Aldrich, St. Louis, MO) for all experiments.

DNA constructs and genetic transformation

WT was used to generate gene knockout mutants of three aquaporin genes - *FGSG_00811* (FgAQP1), *FGSG_03680* (FgAQP3), and *FGSG_10816* (FgAQP5). FgAQP2 and FgAQP4 align to glycerol-specific AQPs (Ding et al, 2018), and were therefore excluded from this study. DNA was isolated from wild type *F. graminearum* following the protocol in Hallen-Adams, Cavinder, and Trail (2011). Primer design for split-marker transformation followed the protocol in Harrison et al. (2013). All primers are listed in Table 3.2. Transformation was performed as described in Hallen-Adams, Cavinder, and Trail (2011). Three independent, confirmed knockout strains for each AQP gene were used for subsequent experiments.

To generate complementations of the AQP knockouts, the wild type gene coding region , along with ~1000 bp upstream and ~1000 bp downstream were amplified by PCR using primers L5 and R3 (Table 4.1). The nourseothricin resistance cassette Nat and its promoter were amplified

using primers Nat5 and Nat3 (Table 4.1) from the plasmid pDS23 (Schindler and Nowrousian 2014). Each amplified AQP gene was co-transformed into protoplasts of the mutant deficient in that gene, along with the resistance cassette. Complemented strains were confirmed via PCR, with two generated for each AQP knockout. Complements of *ΔAQP1*, *ΔAQP3*, and *ΔAQP5* will be referred to as AQP1-C, AQP3-C, and AQP5-C, respectively.

Homology of FgAQPs

To determine function of domains in the three FgAQPs included in this study, BLAST (Basic Local Alignment Search Tool) searches were applied to compare protein domains. The AQPs in *Oryza sativa*, of which the silica-specific AQPs are well-characterized, were used for alignment. The silica-specific rice aquaporin Lsi1 (Ma et al, 2006) protein sequence was compared to the genomes of *O. sativa* (NCBI:txid39947) and *F. graminearum* (NCBI:txid229533). Results included hits from the alignment between *F. graminearum*, and the aquaporins annotated from the NIP, PIP, and TIP families in rice (Kaldenhoff and Fischer 2006). The three FgAQPs of interest were aligned with the rice Lsi1 gene using NCBI's COBALT function (Papadopoulos and Agarwala 2007).

Characterization of hyphal growth and spore formation

To determine conidial production in the AQP mutants, conidia were produced as described above, and counted instead of diluted to working stocks. Three independent experiments were performed. T-tests were performed to determine any differences in mean conidiation rate.

To assess the ability of AQP mutants to form perithecia, strains were inoculated (10 μL) onto carrot agar in a 60 mm Petri dish, and grown for 5 days under 24 hour light before images of hyphae were taken with a Nikon DX camera (Nikon, Tokyo, Japan). Perithecial formation was then induced as follows: aerial hyphae were removed by gently scraping the surface of the Petri

plate with a sterile toothpick and 1.0 mL sterile 2.5% Tween 60 (Sigma, St. Louis, MO) was applied to the surface of the plate with a hockey stick (Cavinder et al. 2012). To determine if the availability of silica impacts development of perithecia, carrot agar was supplemented with 2 mM silicic acid and perithecia were induced as described above. Perithecial formation was quantified seven days after the application of the Tween solution. Images were captured on a Nikon DS-Ri2 camera (Nikon, Tokyo, Japan) at 10x magnification. Perithecia on three 1-cm-square images per replicate were counted, taken from a sampling across the plate. T-tests were performed to determine significance between biologically relevant pairs of conditions.

Disease assays

Stander barley seeds were sown in Suremix potting medium (Michigan Grower Products, Inc., Galesburg, MI) and grown in a greenhouse under supplemental lighting with a 16 hour day at approximately 22°C. Detached barley florets were inoculated as described in Imboden, Afton, and Trail (2018). Briefly, florets at Zadoks' stage 47 (Zadoks, Chang, and Konzak 1974) were removed from the head, placed upright into 1% water agar in petri dishes, and inoculated with 5 µL of conidial suspension (1×10^5 conidia/mL). Early symptoms were evaluated at 5 days post inoculation (dpi), and at 12 dpi for perithecial formation. Florets were deemed symptomatic if they exhibited discoloration and/or mycelial growth over the surface, and the percentage of symptomatic florets was recorded. T-tests were used to compare the percent infection for each mutant to the WT. To observe biofilm formation on the surface of florets, barley florets were inoculated as described above. Surface hyphae did not develop, and could not be removed to stain as described in Shay et al. (in progress).

Focal accumulation quantification

To quantify the number of interactions with trichomes, focal accumulations of plant defense compounds were used as a proxy. Foci are localized accumulations of cellulose and lignin produced by barley as the result of fungal penetration. WT and the AQP mutants were inoculated onto >5 independent Stander barley florets each per replicate, with three individual experiments performed. Paleae were then dissected from the floret, and foci on the palea were stained as described by Imboden, Afton, and Trail (2018). Accumulations of plant defense compounds at barley trichomes appear as a response to fungal hyphae (Imboden, Afton, and Trail 2018). T-tests were performed to compare the mean number of foci from each strain to WT.

Silica accumulation in hyphae

To determine if *F. graminearum* internalizes silica from the environment, cells were grown with and without supplemental silica. 100 μL of WT and the three AQP mutants were independently inoculated into 100 mL of Czapek-Dox medium (Zain et al. 2009), or Czapek-Dox medium supplemented with 2 mM silicic acid (Sigma-Aldrich, St. Louis, MO). Media were prepared with Milli-Q (Barnstead, Lake Balboa, CA) water for consistency in silica content, and all experiments were performed exclusively in plastic labware to avoid silica contamination from glass. After two weeks of growth with shaking (225 rpm) at RT, mycelia were collected by filtering through sterile Miracloth, and rinsed three times with sterile Milli-Q water. Tissue was lyophilized in a FreeZone 4.5 lyophilizer (Labconco, Kansas City, MO) prior to analysis. Lyophilized tissue (100 mg) was digested in a MARS6 microwave digester (CEM, Charlotte, NC) with 9 mL Trace Metal Grade nitric acid (Fisher Scientific, Waltham, MA), 1 mL Trace Metal Grade hydrofluoric acid (Fisher Scientific, Waltham, MA), using the CEM plant tissue microwave program (https://cem.com/en/digestion-of-plant-tissue-mars-6). Fully digested

samples were diluted to a final volume of 50 mL. Samples were analyzed by Agilent 5110 inductively coupled plasma atomic emission spectroscopy **(**ICP-AES; Santa Clara, CA) for quantification of silica. The calibration curve was validated using a second source quality control, and all Si standards are National Institutes of Standards and Technology traceable. Digestion and ICP-AES analysis were performed at Howmet Research Center (Whitehall, MI). T-tests were performed to compare samples for statistical differences.

To track the accumulation of silica in mycelia, fungal strains were inoculated into 50 mL of Bird medium broth (Metzenberg 2004) or the same supplemented with 2 mM silicic acid. The fluorochrome Lysosensor Yellow/Blue DND-160 (Invitrogen, Waltham, MA) was added at a concentration of 10 mM to all samples (Shimizu et al, 2001). The media were inoculated with 100 µL of conidia. Cultures were grown for seven days with shaking (100 RPM) at RT, before mycelia were collected by filtering through sterile Miracloth, followed by rinsing three times with sterile Milli-Q water. Images were taken on a FluoView FV1000 confocal laser scanning microscope (Olympus, Center Valley, PA). Fluorescent and brightfield images were taken simultaneously, with a fluorescence excitation of 425-475 nm, and emission detection of 500-520 nm for Lysosensor. Kalman averaging was used to decrease background noise. To examine the localization of silica in hyphae in three-dimensions, z-stacks of images were imported into NIS-Elements Confocal (Nikon, Tokyo, Japan) to generate three-dimensional (3D) renderings.

Expression of F. graminearum AQPs.

Gene expression levels during spore germination were measured by Miguel-Rojas et al. (*in press*) during four stages: (1) isotropic expansion of spores, (2) germ tube emergence, (3) doubling of germ tube long axis, and (4) appressorium formation. Expression levels of the three AQP genes of interest were selected from the transcriptomics data on barley, as the AQP genes

were not expressed in common culture medium. Logarithms of the Fragments Per Kilobase Million (FPKM) values of each AQP gene across the four conidia germination stages were graphed to map expression across time.

Results

Alignment to Si-specific aquaporin

To determine if any *F. graminearum* AQP sequences indicate functionality, the protein sequence for the known silica-specific rice aquaporin Lsi1 (J. F. Ma et al. 2006) was compared to aquaporins in *F. graminearum* and other aquaporin proteins in rice. The three aquaporins used in this study were similar to the silica-specific aquaporin Lsi1, with AQP3 being the most similar in sequence (Figure 4.1A). The Lsi1 shares 71% of the protein sequence with AQP3, with a significant E-value indicating sequence similarity (data not shown). FgAQP1 and FgAQP5 show 69% and 58% sequence similarity when compared to Lsi1, respectively. In plant silica-specific AQPs, there is a conserved spacing between asparagine–proline–alanine (NPA) domains of 108 amino acids (AA; Deshmukh et al. 2015). In FgAQP1 and FgAQP5, two NPA domains are present, but have 109 AA between them. FgAQP3 has one NPA domain, followed 110 AA later by the protein sequence NPV, which has a valine instead of an alanine. (Figure 4.1B)

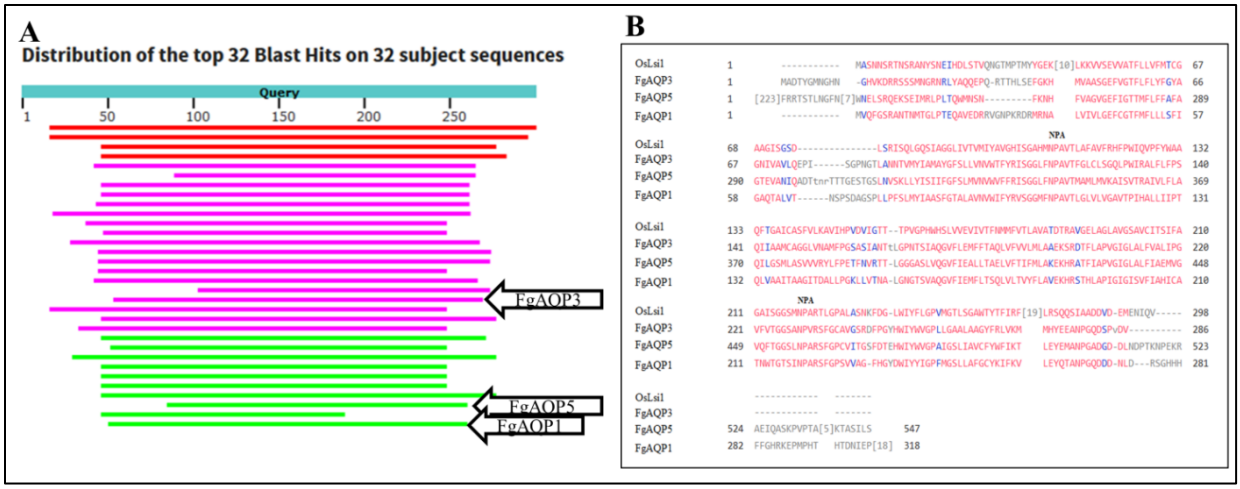


Figure 4.1: Gene homology. (A) Gene alignments of the top 32 Blast results, as compared to the query sequence of the rice aquaporin Lsi1. Arrows indicate where the *F. graminearum* aquaporins are located in the results. All other lines are rice aquaporin genes. Red lines have alignment scores of >200, pink lines have alignment scores of 80-200, and green lines have alignment scores of 50-80. (B) COBALT alignment of the silica-specific rice aquaporin Lsi1 with three *F. graminearum* aquaporins. Red letters indicate highly conserved residues, while blue are less conserved. Gray letters are where there is no alignment between sequences. The NPA domain of interest is labeled by the letters above the alignments.

Aquaporin mutants are deficient in aerial hyphae, conidiation, and pathogenicity

In culture, the aquaporin mutants exhibited reduced aerial hyphae as compared to WT. Rubrafusarin, a dark red pigment, is secreted into the agar, and the red is seen in greater amounts in the cultures of the AQP mutants than the WT due to reduced aerial hyphae (Figure 4.2A). Conidiation was significantly reduced in all AQP mutants when compared to the WT (Figure 4.2B). To study early infection, detached floret assays were inoculated with AQP mutants. All AQP mutants caused disease symptoms on florets, although there was a significant reduction in symptoms at 5 dpi ($p \leq 0.05$; Figure 4.3). The symptoms exhibited by the AQP mutants were primarily the browning in spots of the florets, whereas WT developed symptoms and numerous external hyphae that grew across the floret surface (Figure 4.3A). The complements of all AQP mutants were not significantly different than WT or their respective knockouts in percentage of symptomatic florets (Figure 4.3B). Instead, the complements exhibited more discoloration than

the AQP mutants, but not WT levels of hyphal growth (Figure 4.3A). External hyphae develop later in ΔAQP3 and ΔAQP5, but external hyphae are still minimal in ΔAQP1 at 12 dpi (Figure 4.4A). External surface colonization has been shown to be biofilm formations (Shay et al, in progress), and the AQP mutants all lack external hyphae to observe characteristics, indicating they do not form biofilms on the floret surfaces (data not shown).

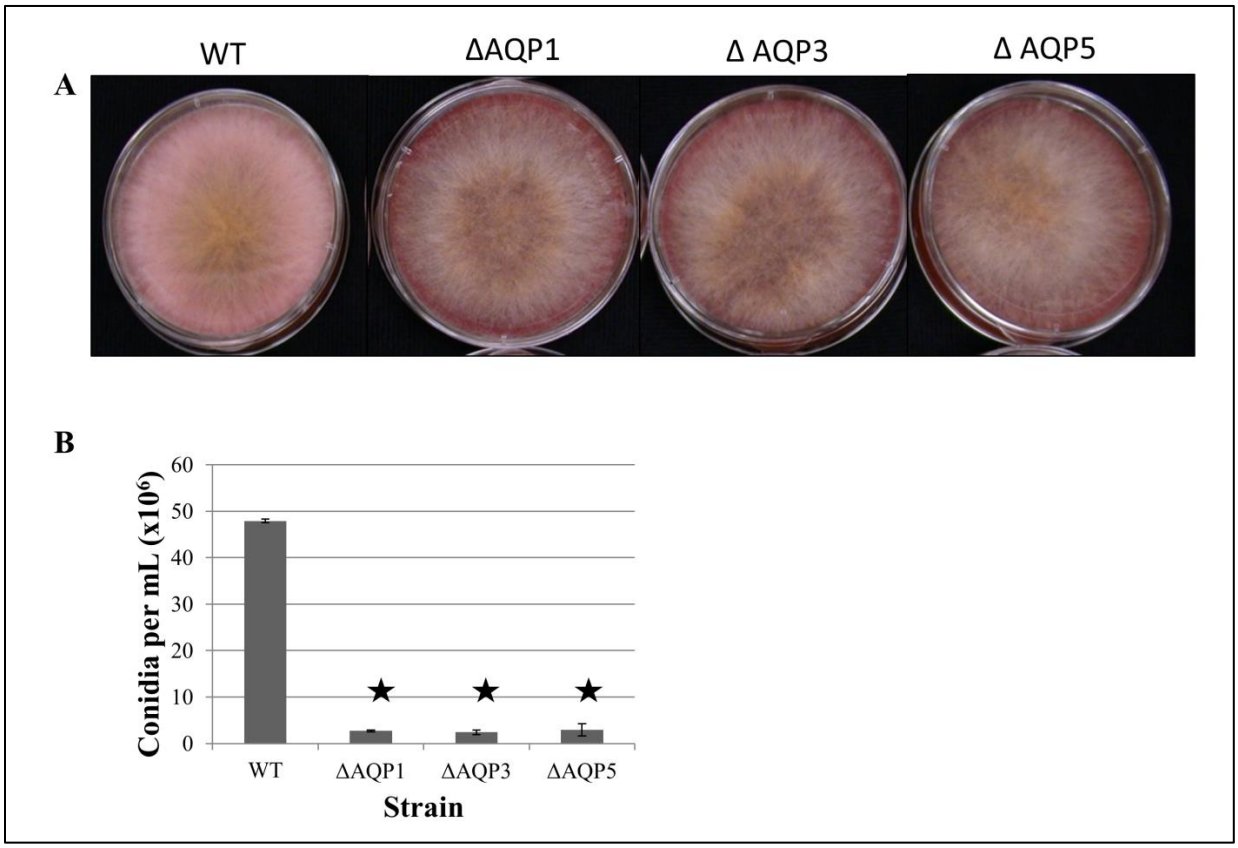


Figure 4.2: Growth and conidiation of aquaporin mutants. (A) Hyphal growth on carrot agar. Photos after 5 days. (B) Conidiation in CMC medium after 5 days of growth. Stars indicate significant differences from WT ($p<0.05$).

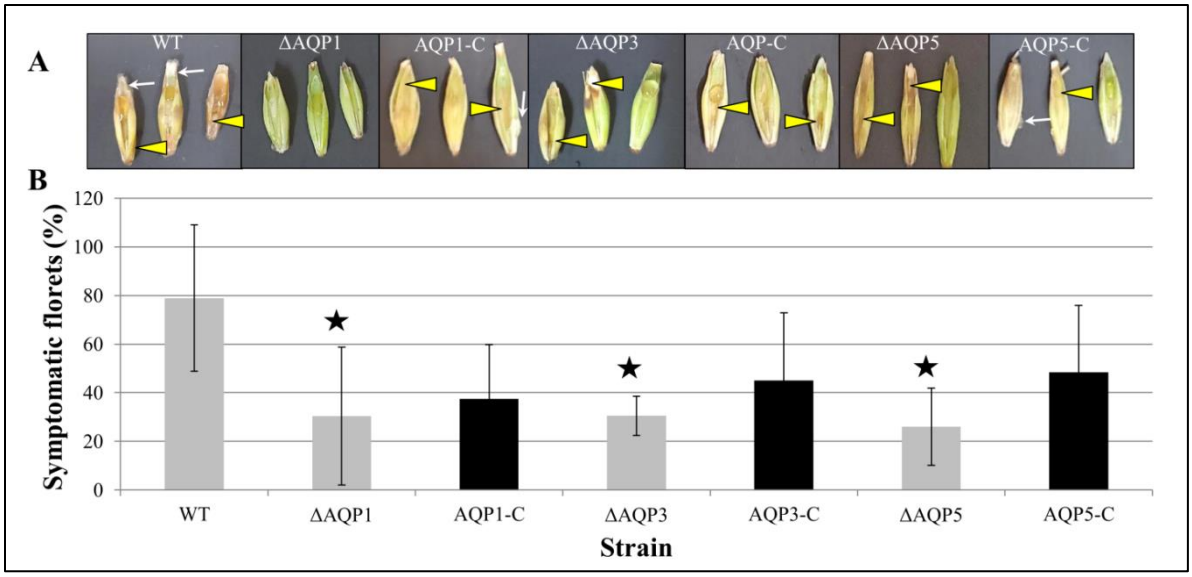


Figure 4.3: Pathogenicity of aquaporin mutants. (A) Representative florets showing the symptoms of each fungal strain at 5 dpi. -C indicates complementation of knockout. White arrows point to areas of visible hyphal growth, while yellow arrowheads indicate areas of discoloration. (B) Percent of detached florets exhibiting symptoms of FHB 5 days post-inoculation for the WT and knockout strains (gray) and their complements (black). Bars indicate standard deviation. Stars indicate significance ($p \leq 0.05$) compared to WT. There was no significant difference between the AQP mutants and each respective complement, and complements were not significantly different than the WT.

Perithecial formation is condition-dependent for AQP mutants

Interestingly, when inoculated onto detached florets, the AQP mutants all form perithecia 12 dpi, while the WT exhibits only hyphal growth (Figure 4.4A). In culture, all AQP mutants form perithecia, but the perithecia are significantly reduced in number compared to WT (Figure 2.4B, C). The size of perithecia was reduced in the AQP mutants, but not consistently across all replicates, and therefore not included in these data. Complementing the AQP genes increased the number of perithecia formed, whereas AQP3-C and AQP5-C were restored to WT levels, while AQP1-C was still significantly reduced in the number of perithecia formed compared to WT (Figure 4.4C). To determine if the type of silica accessible to the fungus plays a role in perithecia formation, silicic acid, the most biologically accessible form of silica, was added for perithecia

formation. The addition of silicic acid to cultures significantly increased the number of perithecia produced by all AQP mutants (Figure 4.4B, C). AQP1-C and AQP3-C did not have a differential response to the addition of silicic acid, although surprisingly, the addition of silicic acid significantly reduced the number of perithecia produced by AQP5-C.

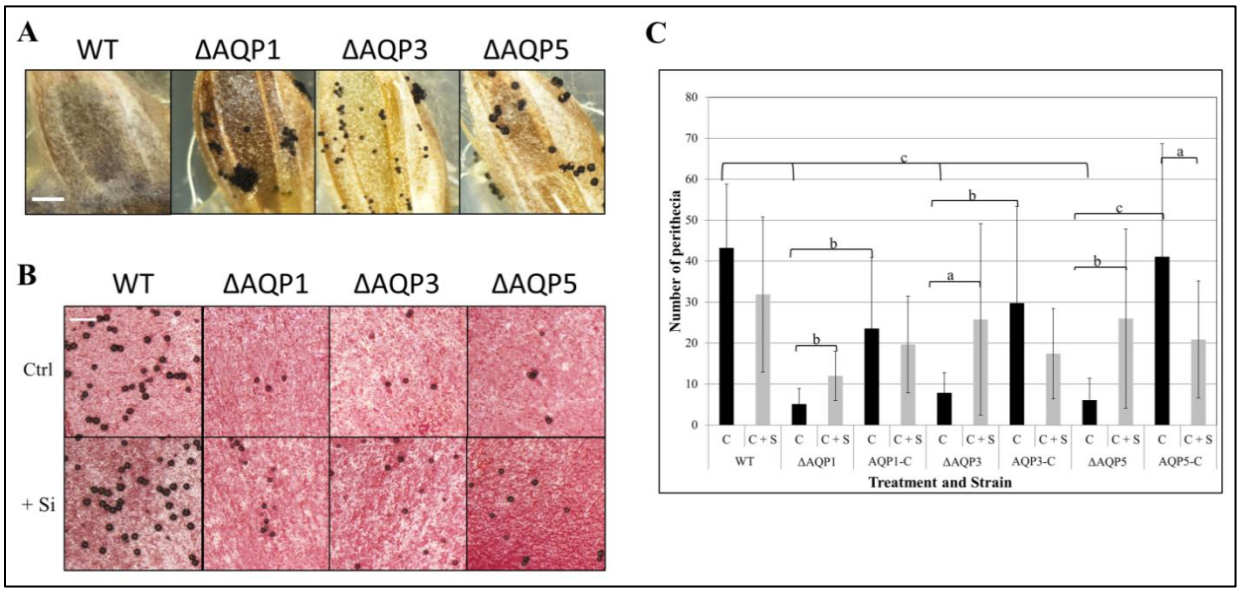


Figure 4.4: Perithecial development by AQP mutants. (A) Formation on florets at 12 dpi. Scale = 1 mm, representative of all observations for each strain. (B) Development in culture, standard lab conditions (ctrl; top row) and added silicic acid (+Si; bottom row). Scale = 0.2 mm, representative of all micrographs. (C) Counts of perithecia produced on carrot agar (C; black) or carrot agar amended with silicic acid (C + S; gray). Error bars indicate standard deviation. Letters indicate significance between treatments indicated by each bracket, with the significance determined by t-tests between the samples at each end of the bracket. a, $p<0.05$; b, $p<0.01$; c, $p<0.001$.

Involvement of FgAQP with silica

Focal accumulations were used to quantify the frequency of interactions each fungal strain has with trichomes, which are silica-rich cells. As described in Imboden, Afton, and Trail (2018), foci are accumulations of defense compounds produced by plants when fungal hyphae penetrate at trichomes. The number of foci per palea varied widely for all samples (Figure 4.5). ΔAQP1 and ΔAQP3 had significantly lower mean foci/palea than WT ($p<0.05$), indicating fewer

interactions with trichomes. All AQP complements exhibited WT-levels of foci (Figure 4.5).

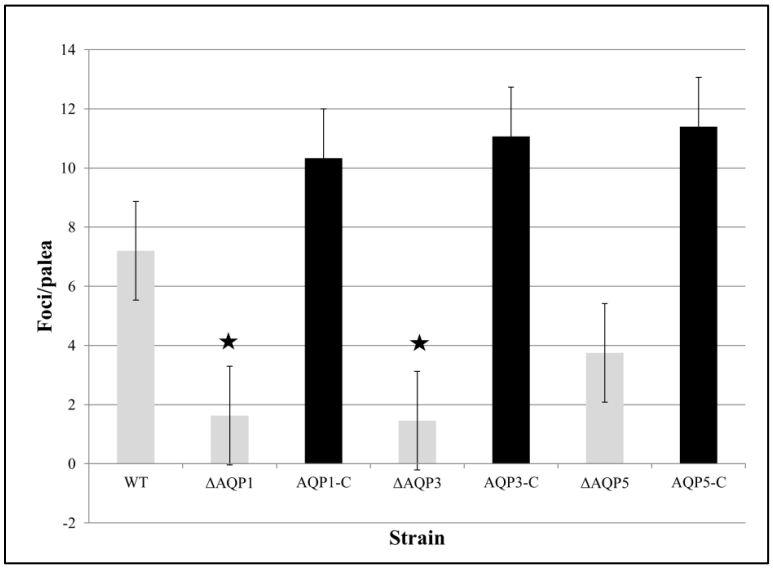


Figure 4.5: Focal accumulations on barley paleae. Bars indicate the mean number of paleae across three replicates Mutants and WT bars are gray; the complements are black. Error bars indicate standard deviation. Stars indicate significant difference from WT ($p<0.05$).

To determine if F. graminearum can incorporate silica into its cells, ICP-AES analysis was performed on WT and the AQP mutants, measuring the silica content of hyphae grown in culture without and with supplemental silica. Control samples overall had a very low silica content, which provided a baseline for the silica that is naturally present in the environment and samples, as a true silica-free environment is difficult to achieve. All strains, including the AQP mutants, can uptake silica from their surrounding environment and incorporate it into their cells. ΔAQP3 showed the lowest overall levels of silica, while the percent silica content varied the most in AQP1 (Figure 4.6A).

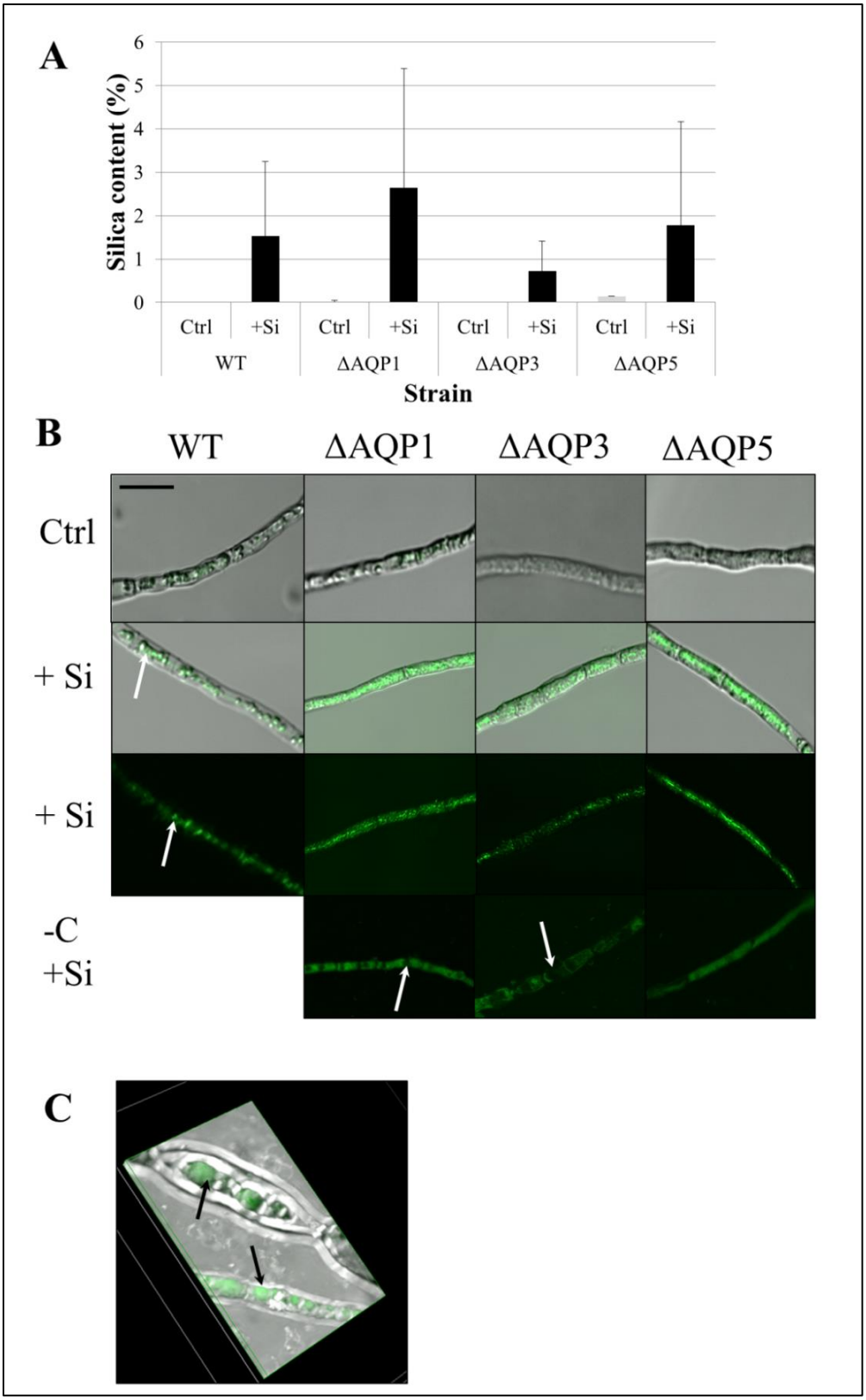


Figure 4.6: Silica accumulation within hyphae.

Figure 4.6 (cont'd)

(A) Percent silica content of hyphae grown in medium without (ctrl; gray) or with (+ Si; black) the amended silicic acid. (B) Localization of silica in fungal strains by Lysosensor in cultures with no added silica (Ctrl; top row) and silicic acid (+Si; bottom 3 rows), shown as an overlay of brightfield and fluorescence, or just fluorescence (third row), and complements the AQP mutants with added silicic acid (-C +Si; bottom row). Arrows indicate vesicles visible, either with accumulated silica or not. Scale = 10 μm, representative of all images. (C) Three-dimensional rendering of WT cells with Lysosensor fluorescence (green) contained in membrane-bound vesicles (black arrows). Membranes are white in the rendering, and white solid lines on the edge of the image are part of the three-dimensional rendering framework.

To determine where the silica is localized in cells, a fluorescent tracer (Lysosensor) was added to cultures, where it is incorporated into cells with silica. Little or no fluorescence was observed in control samples (no silicic acid amendment; Figure 4.6B). Fluorescence was localized in the cytoplasm of AQP mutant cells, and not sequestered into specific organelles (Figure 4.6B). In WT samples, fluorescence is sequestered in small clusters (Figure 4.6B), which 3D rendering revealed to be membrane-bound vesicles (Figure 4.6C). AQP1-C and AQP3-C exhibit fluorescence around internal hyphal structures (Figure 4.6B). AQP5-C shows even distribution of Lysosensor fluorescence throughout the hyphae (Figure 4.6B).

Expression of AQP genes on host tissue

Miguel-Rojas et al. (in press) studied expression of genes in F. graminearum over the course of four stages of spore germination. Interestingly, none of the AQP genes were expressed in the common-medium environment of the study, so all expression data originated from plant-host samples. AQP5 had low or no expression across all stages of spore germination. AQP1 was only expressed during growth stages 1 and 3, but not stages 2 or 4. AQP3 was expressed in all stages of spore germination on plant hosts, with stage 2 having lower overall expression than the other

stages, which are similar expression levels to each other (Figure 4.7).

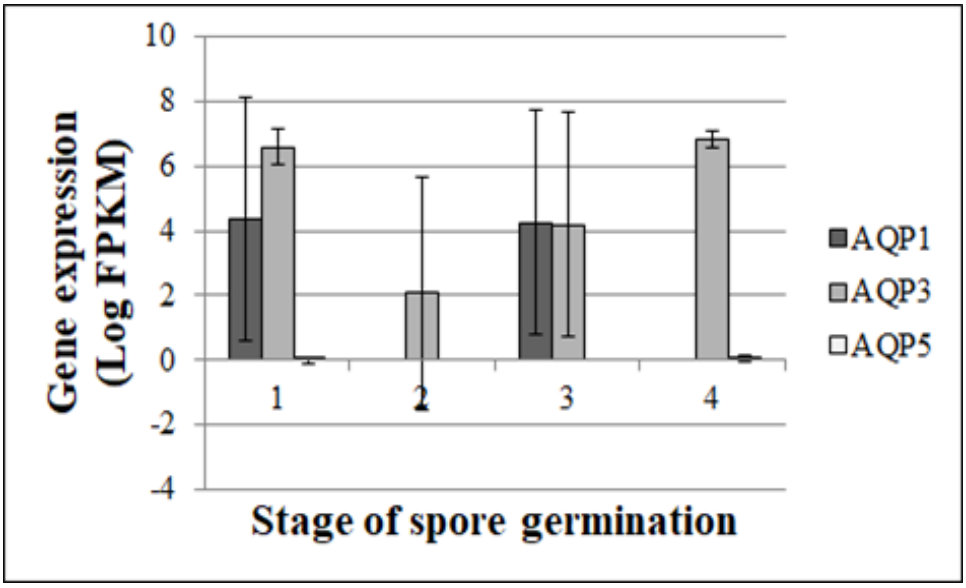


Figure 4.7: Expression levels of aquaporin genes during spore germination. Expression levels were determined by Miguel-Rojas et al (*in press*) from germinating spores on host plant tissue. Stages of germination were (1) isotropic expansion of spores; (2) germ tube emergence; (3) doubling of germ tube long axis; and (4) appressorium formation. Error bars represent standard deviations.

Discussion

Grasses have many silica-rich cell-types, including stomates, trichomes, xylem, and epidermal cells which form phytoliths (Epstein 1994). In studies of cereal crops, silica has been shown to help prevent fungal disease, usually by bolstering the cell wall with a layer of deposited silica that blocks fungal penetration (Epstein 2009; Kim 2019; Schilmiller, Last, and Pichersky 2008; Hauser 2014; J. F. Ma 2003). To our knowledge we describe the first demonstration of silica uptake by a filamentous fungus. Silica is biologically interesting in the relationship between F. graminearum and its host cereal crops, as infection, colonization and sporulation occur in association with silica-rich cells (Langevin, Eudes, and Comeau 2004; Jansen et al. 2005; Guenther and Trail 2005; Trail 2009; Imboden, Afton, and Trail 2018). We asked if an AQP was responsible for sensing silica from the plant environment to trigger the infection process. We

characterized the function of three aquaporin genes, AQP1, AQP3 and AQP5, demonstrating through knockouts a role in the life cycle and disease cycle of F. graminearum. Most importantly, we present evidence that F. graminearum takes up silica from the environment and sequesters it in vesicles within the hyphae. Furthermore, AQP3 is the primary AQP involved in the response to silica. Although we demonstrate the association of AQP activity with pathogenicity, and the accumulation of silica taken up by the AQPs, we have not identified the mechanism whereby silica affects pathogenicity.

FgAQPs are involved in growth and spore development, both sexual and asexual, of the fungus. In contrast to the findings of Ding et al (2018), we show that AQP1 mutants form perithecia in vitro as do AQP 3, and AQP5 mutants, but the number is severely reduced compared to WT in vitro. The surprising result that all AQP mutants demonstrate robust perithecium formation on inoculated florets, while WT only exhibits hyphal growth, may be due to the presence of silica available in plants. When we added silicic acid to carrot agar cultures, the numbers of perithecia increased for ΔAQP3 and ΔAQP5 to WT levels, suggesting that silicic acid influences the sexual development pathway by these two AQPs. Florets of barley are high in polymerized silicic acid (Epstein 2009), while carrots, which are used for the in vitro perithecia-inducing conditions, are low in silicic acid content (Pennington 1991). The level of silicic acid available has an impact on perithecia formation, and the mechanism by which the formation is impacted in the presence of silica is worthy of further study.

In F. graminearum, we have shown that AQP1, AQP3, and AQP5 have roles in early colonization of barley, as the AQP knockouts had reduced signs and symptoms of disease on detached florets. Surface hyphae were minimal in these assays, with little external hyphal colonization apparent. During the infection process of F. graminearum, there is a period of time

where hyphae colonize the surface of the plant without infection (Boenisch and Schäfer 2011; Osborne and Stein 2007; Imboden, Afton, and Trail 2018), and we suggest that without the signals sensed by AQPs, the initial external colonization step is greatly reduced. Complements of the mutants did not restore pathogenicity entirely, as they are not significantly different in infection levels when compared to the WT or the associated knockout. This may be due to the method of complementation, where the gene of interest is randomly inserted into the genome. The formation of foci is a native defense response in barley and has been used to quantify interactions with trichomes (Imboden et al. 2018). Here, ΔAQP1 and ΔAQP3 strains were reduced in focal density when inoculated onto barley florets, while ΔAQP5 also had reduced symptoms, but no observable reduction in foci compared to WT. This finding indicates that while some of the reduction in pathogenicity observed in the AQP mutants is due to reduced infection at trichomes, the relationship is more complex. AQP1 and AQP3 may be involved in sensing trichomes as infection points, but there are other mechanisms for fungal penetration into host tissues. The reduced interactions of the AQP mutants with trichomes has led us to hypothesize that AQPs are involved in silica sensing and import of silica into fungal cells.

We hypothesized that silica is a signal used by F. graminearum to find infection points, such as the silica-rich trichomes. Bulk analysis of silica content in the hyphae of AQP mutants and WT using ICP-AES analysis demonstrated that differences were not significant, although a small sample size and large ranges may have hindered accurate assessment of differences. Control samples were lower in silica content than hyphae grown with silica amendments, indicating that all strains can uptake silica from the environment. Microscopic visualization of accumulating silica in hyphae indicates that silica in AQP mutants is sequestered in irregular deposits in the cytoplasm, while in WT it is sequestered by membranes into vesicles. In contrast to plants, where

silica-specific AQPs have been identified (J. F. Ma et al. 2006; Gaur et al. 2020; Kaldenhoff and Fischer 2006; Li et al. 2020; Exley, Guerriero, and Lopez 2020), our data indicates that AQP1, 3, and 5 can import silica into fungal tissues, and that there is not one specialized AQP. *ΔAQP3* was reduced overall in silica content compared to WT, and with the other data presented here; this suggests it is the main AQP involved in the response to silica. The form of silica available may be important in this response as well, as added silicic acid increased perithecia formation in *ΔAQP1* and *ΔAQP3*, indicating that silicic acid is entering cells through alternative pathways, or by redundancy between AQPs. AQPs may be involved not just in the import of silica, but in the localization of silica internally. In AQP1-C and AQP3-C, silica was distributed around internal vesicles, while AQP5-C maintained an even distribution throughout the hyphae, indicating that AQP1 and AQP3 are involved in the localization of silica in cells. When aligned to the rice silica-specific AQP Lsi1, FgAQP3 had the most similarity, although the alignment with other non-silica-specific rice AQPs was also high. A conserved sequence of 108 amino acids between NPA protein domains is found across many plant silica-specific AQPs (Deshmukh et al. 2015). However, in all three F. graminearum AQPs studied, the distance between NPA domains is larger: 109 amino acids in AQP1 and AQP5, 110 AA in AQP3. In AQP3, the location of the second NPA domain occurs after the initial NPA domain, and has the alternate protein sequence of NPV. All AQPs studied show a variation of the silica-specific domain associated with the AQPs, indicating that there could be redundancy of function, and all may be related to silica transport in cells. Spacing between NPA domains has been shown to be too small for silicic acid molecules to enter (Guerriero et al. 2019), and a meta-analysis of plant silica-specific AQP suggests that these channels may function differently than previously suggested (Exley, Guerriero, and Lopez 2020). This supports our findings that, while FgAQPs interact with silica,

it may not be as specific sites of silica entry into cells. The channels previously thought to be silica-specific may instead use silicic acid as an extracellular signaling molecule, and there may be an alternative mode of entry for silica beyond the AQPs. More work into the molecular dynamics of AQPs and forms of silica needs to be done. Although it is clear that FgAQPs are involved in silica uptake and localization, double and triple mutants of the AQPs would likely tease apart this relationship further, but strains with multiple AQPs deleted were not generated in crosses despite numerous tries. Knocking out multiple AQPs may be a lethal mutation, as these AQPs are clearly involved in growth and development. Gene silencing techniques may offer an alternative approach to answering this question.

AQPs in F. graminearum are clearly important in the growth, development, and pathogenicity of the fungus. Recent work has shown that applications of silica can reduce the severity of FHB and Fusarium stem blight, although it has been noted that hyphal growth is not reduced, and that mycotoxin contamination in kernels is more severe (Pazdiora et al. 2022; Sakr 2022). Although there is similarity in sequences and domains between known silica-specific AQPs, recent work has highlighted the complexities of these channels and assigning function based on domains (Exley, Guerriero, and Lopez 2020). We have shown a relationship between the AQPs and silica, however, the mechanisms involved in these relationships still need to be elucidated, with consideration to developing a better understanding of the specific interactions between AQPs and silica and their implications for FHB disease.

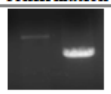
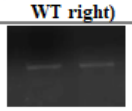
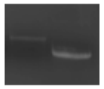
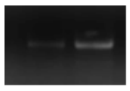
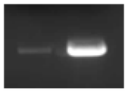
Acknowledgements

We thank Drew Afton, Lori Imboden, Ben Smith, and Molly Cavanaugh for their initial observations on the aquaporin mutants and the significance of silica. Additionally, thank you to Kristi Gdanetz MacCready and Amanda Koenig for their feedback on this manuscript. This work

was supported by the US Wheat and Barley Scab Initiative, A.L. Rodger's Scholarship, and Everett Beneke Endowment Award

Supplemental Table

Table 4.1: Primers and PCR confirmations.

Gene	L5	L3	R5	R3	Band size (base pairs)	PCR confirmation	Complement confirmation (complement left, WT right)
FGSG_03680	GTCAGACATGGCGAGCTTTATATGGAC	CGAACTGATGGAGAAATCTGGTGGGAG	GAGCACCCATCAGATAGCATTCAAGC	CATCAACAATCTCAAGAGCCGTCGTG	Mutant (left): 2598 WT (right): 2242		
FGSG_00811	CATGGCAACAGTCGCATTCTTCTCAG	GTAGATGGATGCTAGGCAATGATGAGG	CGTTCCTCGCAATCGGGTCTGGATC	CCTTTGGTTCCTATGTGTTTTGTGGTGG	Mutant (left): 2595 WT (right): 2242		
FGSG_10816	GTATTTTCCCGTCAGCCCATCCAAC	GACTTCTCACCAGCGAGACCAAATCC	CAGGAAACAGAATGCGATAAGTGCCATG	CAGATAGGGCTGGAAACCTTGAAGAG	Mutant (left): 1431 WT (right): 1700		
L overhang	CGTCAGATCGATGGTAGTTGTCGTCGACT						
R overhang	ACACTGGTGACGGCTAACCAGAACTGTCA						
Nat5	CTTCGGAARAGGAACTTCAAAGCG						
Nat3	GGATGAGACTAATCCAATTGCTGC						

REFERENCES

Ball, T. B., J. S. Gardner, and N. Anderson. 1999. "Identifying Inflorescence Phytoliths from Selected Species of Wheat (*Triticum monococcum, T. dicoccon, T. dicoccoides, and T. aestivum*) and Barley (*Hordeum vulgare* and *H. spontaneum*) (Gramineae)." *American Journal of Botany* 86 (11): 1615–23. https://doi.org/10.2307/2656798.

Bienert, Gerd P., and François Chaumont. 2014. "Aquaporin-Facilitated Transmembrane Diffusion of Hydrogen Peroxide." *Biochimica et Biophysica Acta - General Subjects* 1840 (5): 1596–1604. https://doi.org/10.1016/j.bbagen.2013.09.017.

Boenisch, Marike J, and Wilhelm Schäfer. 2011. "*Fusarium graminearum* Forms Mycotoxin Producing Infection Structures on Wheat." *BMC Plant Biology* 11 (1): 110. https://doi.org/10.1186/1471-2229-11-110.

Cappellini, R. A., and J. L. Peterson. 1965. "Macroconidium Formation in Submerged Cultures by a Nonsporulating Strain of *Gibberella zeae*." *Mycologia* 57 (6): 962–66. https://doi.org/10.1080/00275514.1965.12018285.

Cavinder, Brad, Usha Sikhakolli, Kayla M. Fellows, and Frances Trail. 2012. "Sexual Development and Ascospore Discharge in *Fusarium graminearum*." *Journal of Visualized Experiments : JoVE*, no. 61: 1–5. https://doi.org/10.3791/3895.

Deshmukh, Rupesh Kailasrao, Julien Vivancos, Gowsica Ramakrishnan, Valérie Guérin, Gabriel Carpentier, Humira Sonah, Caroline Labbé, Paul Isenring, Francois J Belzile, and Richard R. Bélanger. 2015. "A Precise Spacing between the NPA Domains of Aquaporins Is Essential for Silicon Permeability in Plants." *Plant Journal* 83 (3): 489–500. https://doi.org/10.1111/tpj.12904.

Ding, Mingyu, Jing Li, Xinyue Fan, Fang He, Xiaoyang Yu, Lei Chen, Shenshen Zou, Yuancun Liang, and Jinfeng Yu. 2018. "Aquaporin1 Regulates Development, Secondary Metabolism and Stress Responses in *Fusarium graminearum*." *Current Genetics* 64 (5): 1057–69. https://doi.org/10.1007/s00294-018-0818-8.

Epstein, E. 2009. "Silicon: Its Manifold Roles in Plants." *Annals of Applied Biology* 155 (2): 155–60. https://doi.org/10.1111/j.1744-7348.2009.00343.x.

Epstein, E. 1994. "The Anomaly of Silicon in Plant Biology." *Proceedings of the National Academy of Sciences of the United States of America* 91 (1): 11–17. https://doi.org/10.1073/pnas.91.1.11.

Exley, Christopher, Gea Guerriero, and Xabier Lopez. 2020. "How Is Silicic Acid Transported in Plants?" *Silicon* 12 (11): 2641–45. https://doi.org/10.1007/s12633-019-00360-w.

Gaur, Shweta, Jitendra Kumar, Dharmendra Kumar, Devendra Kumar Chauhan, Sheo Mohan Prasad, and Prabhat Kumar Srivastava. 2020. "Fascinating Impact of Silicon and Silicon Transporters in Plants: A Review." *Ecotoxicology and Environmental Safety* 202 (June):

110885. https://doi.org/10.1016/j.ecoenv.2020.110885.

Guenther, John C, and Frances Trail. 2005. “The Development and Differentiation of *Gibberella zeae* (Anamorph: *Fusarium graminearum*) during Colonization of Wheat.” *Mycologia* 97 (1): 229–37. https://doi.org/10.3852/mycologia.97.1.229.

Guerriero, Gea, Rupesh Deshmukh, Humira Sonah, Kjell Sergeant, Jean Francois Hausman, Esther Lentzen, Nathalie Valle, Khawar Sohail Siddiqui, and Christopher Exley. 2019. “Identification of the Aquaporin Gene Family in *Cannabis sativa* and Evidence for the Accumulation of Silicon in Its Tissues.” *Plant Science* 287 (June): 110167. https://doi.org/10.1016/j.plantsci.2019.110167.

Hallen-Adams, Heather E., Brad L. Cavinder, and Frances Trail. 2011. “*Fusarium graminearum* from Expression Analysis to Functional Assays.” *Methods in Molecular Biology (Clifton, N.J.)* 722 (1): 79–101. https://doi.org/10.1007/978-1-61779-040-9_6.

Harrison, Nicholas, Brad Cavinder, Jeffrey P. Townsend, and Frances Trail. 2013. “Optimized Primers and Other Critical Conditions for Efficient Fusion PCR to Generate Knockout Vectors in Filamentous Fungi.” *Fungal Genetics Reports*, no. C: 1–10.

Hauser, Marie-Theres. 2014. “Molecular Basis of Natural Variation and Environmental Control of Trichome Patterning.” *Frontiers in Plant Science* 5 (July): 320. https://doi.org/10.3389/fpls.2014.00320.

Imboden, Lori, Drew Afton, and Frances Trail. 2018. “Surface Interactions of *Fusarium graminearum* on Barley.” *Molecular Plant Pathology* 19 (6): 1332–42. https://doi.org/10.1111/mpp.12616.

Jansen, Carin, Diter von Wettstein, Wilhelm Schäfer, Karl-Heinz Kogel, Angelika Felk, and Frank J Maier. 2005. “Infection Patterns in Barley and Wheat Spikes Inoculated with Wild-Type and Trichodiene Synthase Gene Disrupted *Fusarium graminearum*.” *Proceedings of the National Academy of Sciences of the United States of America* 102 (46): 16892–97. https://doi.org/10.1073/pnas.0508467102.

Kaldenhoff, R., and M. Fischer. 2006. “Aquaporins in Plants.” *Acta Physiologica* 187 (1–2): 169–76. https://doi.org/10.1111/j.1748-1716.2006.01563.x.

Kim, Ki Woo. 2019. “Plant Trichomes as Microbial Habitats and Infection Sites.” *European Journal of Plant Pathology* 154 (2): 157–69. https://doi.org/10.1007/s10658-018-01656-0.

Klittich, C, and J F Leslie. 1988. “Nitrate Reduction Mutants of *Fusarium moniliforme* (*Gibberella fujikuroi*).” *Genetics* 118 (3): 417–23. https://doi.org/10.1093/genetics/118.3.417.

Langevin, François, François Eudes, and André Comeau. 2004. “Effect of Trichothecenes Produced by *Fusarium graminearum* during Fusarium Head Blight Development in Six

Cereal Species." *European Journal of Plant Pathology* 110 (7): 735–46. https://doi.org/10.1023/B:EJPP.0000041568.31778.ad.

Li, Guangjin, Tong Chen, Zhanquan Zhang, Boqiang Li, and Shiping Tian. 2020. "Roles of Aquaporins in Plant-Pathogen Interaction." *Plants* 9 (9): 1–9. https://doi.org/10.3390/plants9091134.

Ma, JF, Y Miyake, and E Takahashi. 2001. "Silicon as a Beneficial Element for Crop Plants." In *Silicon in Agriculture*, 17–39.

Ma, Jian Feng. 2003. "Beneficial Effects of Silicon in Different Plant Species." *Progress in Molecular and Subcellular Biology* 33.

Ma, Jian Feng, Kazunori Tamai, Naoki Yamaji, Namiki Mitani, Saeko Konishi, Maki Katsuhara, Masaji Ishiguro, Yoshiko Murata, and Masahiro Yano. 2006. "A Silicon Transporter in Rice." *Nature* 440 (7084): 688–91. https://doi.org/10.1038/nature04590.

Mcmullen, Marcia, Roger Jones, and Dale Gallenberg. 1997. "Scab of Wheat and Barley: A Re-Emerging Disease of Devastating Impact." *Plant Disease* 81 (12).

McMullen, Marcia P., Gary C. Bergstrom, Erick De Wolf, Ruth Dill-Macky, Donald E Hershman, Gregory Shaner, and David A Van Sanford. 2012. "A Unified Effort to Fight an Enemy of Wheat and Barley: Fusarium Head Blight." *Plant Disease* 96 (12): 1712–28. https://doi.org/10.1094/PDIS-03-12-0291-FE.

Metzenberg, Robert L. 2004. "Bird Medium: An Alternative to Vogel Medium." *Fungal Genetics Reports* 51 (1): 19–20. https://doi.org/10.4148/1941-4765.1138.

Miguel-Rojas, Cristina, Brad Cavinder, Zheng Wang, Jeffry P. Townsend, and Frances Trail. n.d. "Comparative Transcriptomics of *Fusarium graminearum* and *Magnaporthe oryzae* of Spor Germination Leading up to Infection."

O'Reagain, P. J., and M. T. Mentis. 1989. "Leaf Silicification in Grasses - A Review." *Journal of the Grassland Society of Southern Africa* 6 (1): 37–43. https://doi.org/10.1080/02566702.1989.9648158.

Osborne, Lawrence E., and Jeffrey M. Stein. 2007. "Epidemiology of Fusarium Head Blight on Small-Grain Cereals." *International Journal of Food Microbiology* 119 (1–2): 103–8. https://doi.org/10.1016/j.ijfoodmicro.2007.07.032.

Papadopoulos, Jason S., and Richa Agarwala. 2007. "COBALT: Constraint-Based Alignment Tool for Multiple Protein Sequences." *Bioinformatics* 23 (9): 1073–79. https://doi.org/10.1093/bioinformatics/btm076.

Pazdiora, Paulo Cesar, Rosane Lopes Crizel, Keilor Rosa Dorneles, Sabrina Monks, Thomas Natali Morello, Fabio Clasen Chaves, and Leandro José Dallagnol. 2022. "Silicon

Amendment Improves Wheat Defense against *Fusarium graminearum* and Complements the Control by Fungicide of Fusarium Head Blight," 0–2. https://doi.org/10.1111/ppa.13648.

Pennington, J. A.T. 1991. "Silicon in Foods and Diets." *Food Additives and Contaminants* 8 (1): 97–118. https://doi.org/10.1080/02652039109373959.

Rudall, Paula J., Christina J. Prychid, and Thomas Gregory. 2014. "Epidermal Patterning and Silica Phytoliths in Grasses: An Evolutionary History." *Botanical Review* 80 (1): 59–71. https://doi.org/10.1007/s12229-014-9133-3.

Sakr, Nachaat. 2016. "The Role of Silicon (Si) in Increasing Plant Resistance against Fungal Diseases." *Hellenic Plant Protection Journal* 9 (1): 1–15. https://doi.org/10.1515/hppj-2016-0001.

———. 2022. "Silicon Reduces the Severity of Fusarium Infection on Young Wheat Parts In Vitro." *The Open Agriculture Journal* 16: 1–10. https://doi.org/10.2174/18743315-v16-e2207260.

Schilmiller, Anthony L., Robert L. Last, and Eran Pichersky. 2008. "Harnessing Plant Trichome Biochemistry for the Production of Useful Compounds." *Plant Journal* 54 (4): 702–11. https://doi.org/10.1111/j.1365-313X.2008.03432.x.

Schindler, Daniel, and Minou Nowrousian. 2014. "The Polyketide Synthase Gene Pks4 Is Essential for Sexual Development and Regulates Fruiting Body Morphology in *Sordaria macrospora*." *Fungal Genetics and Biology* 68: 48–59. https://doi.org/10.1016/j.fgb.2014.04.008.

Shah, L., A. Ali, M. Yahya, Y. Zhu, S. Wang, H. Si, H. Rahman, and C. Ma. 2018. "Integrated Control of Fusarium Head Blight and Deoxynivalenol Mycotoxin in Wheat." *Plant Pathology* 67 (3): 532–48. https://doi.org/10.1111/ppa.12785.

Shimizu, Katsuhiko, Yolanda Del Amo, Mark A. Brzezinski, Galen D. Stucky, and Daniel E. Morse. 2001. "A Novel Fluorescent Silica Tracer for Biological Silicification Studies." *Chemistry and Biology* 8 (11): 1051–60. https://doi.org/10.1016/S1074-5521(01)00072-2.

Trail, Frances. 2009. "For Blighted Waves of Grain: *Fusarium graminearum* in the Postgenomics Era." *Plant Physiology* 149 (1): 103–10. https://doi.org/10.1104/pp.108.129684.

Trail, Frances, and Ralph Common. 2000. " Perithecial Development by *Gibberella zeae* : A Light Microscopy Study ." *Mycologia* 92 (1): 130–38. https://doi.org/10.1080/00275514.2000.12061137.

Verkman, Alan S. 2011. "Aquaporins at a Glance." *Journal of Cell Science* 124 (13): 2107–12. https://doi.org/10.1242/jcs.079467.

Zadoks, J C, T.T. Chang, and C.F. Konzak. 1974. “A Decimal Code for the Growth Stages of Cereals.” *Weed Research* 14 (6): 415–21. https://doi.org/0.1111/j.1365-3180.1974.tb01084.x.

Zain, M E, A A Razak, H H El-Sheikh, H G Soliman, and A M Khalil. 2009. “Influence of Growth Medium on Diagnostic Characters of *Aspergillus* and *Penicillium* Species.” *African Journal of Microbiology Research* 3 (5): 280–86.

CHAPTER 5

CONCLUSIONS

Fusarium graminearum is an important plant pathogen of global concern that causes the disease Fusarium head blight. Understanding the infection process of the disease is important to providing future targets for control. Although much is known about how *F. graminearum* infects, this dissertation presents new aspects of the infection process: the novel formation of biofilms, and the response to plant silica by the fungus.

Biofilms are protective structures produced by some microbes, where cells work together as a community to survive harsh environments. While biofilms are well-studied in bacteria and single-celled yeasts, filamentous fungal biofilms have not been researched extensively. In my work, I have provided, to our knowledge, the first report of biofilm formation by *F. graminearum in vitro (*Shay, Weigand and Trail, 2022). Through this work, I have shown the developmental stages of biofilm formation, and have dissected the composition of the protective matrix that forms around the biofilm. Importantly, the matrix surrounding the biofilm is composed of multiple macromolecules, including polysaccharides and nucleic acids. Lipids are also involved in the process of biofilm formation in *F. graminearum*, primarily in the hyphae in wild type (WT) biofilm formations. Further work is necessary for determining which forms of each macromolecule are present in the matrix, and the functionality of each macromolecule in the biofilm matrix.

Transcriptomic analysis of biofilm formation in *F. graminearum* identified candidate genes predicted to be involved in biofilms. Gene knockouts of these candidate genes provided phenotypic differences in biofilm formation, both in the matrix formation and adhesion to a surface. Gene knockouts impacted the formation of nucleic acids in the matrix, with a reduction

in staining of nucleic acids as compared to WT. It may be that the genes identified are either directly depositing nucleic acids into the matrix, or are the source of the nucleic acids themselves. DNA found in the matrix of other species is genome-derived, and multiple pathways have been found to be involved in the deposition of DNA (Allesen-Holm et al. 2006; Martins et al. 2010; Mann and Wozniak 2012; Rajendran et al. 2013; Kischkel et al. 2019), and the exact mechanisms utilized by *F. graminearum* are still to be determined. Additionally, gene knockouts impacted the formation of lipids in the biofilm, where a reduction in lipid formation was observed with some knockouts, and a relocalization of lipids to the matrix was observed in other knockouts. The function of the macromolecules in the matrix is still unclear, but having genes identified that impact the formation of individual matrix components allows for finer-resolution studies of the matrix composition. From these results, I have provided a basis of work for the further study of biofilms in *F. graminearum*, where more work is needed. Further studies on the candidate genes knocked out would help determine which cellular pathways are involved during biofilm formation, and how these genes are involved.

I have additionally shown that adhesion to a surface, which is the first step in biofilm formation, is easily altered in *F. graminearum*. Methylation is likely the regulatory mechanism involved in increasing the adhesion of cells to a polystyrene surface, as adding a methylation inhibitor reduces adhesion of the isolates increased in adhesion back to WT levels. From this, work on what methylation regulation was altered would identify genes involved in adhesion to a surface, which could be studied further for their involvement across the process of biofilm formation. Adhesion is an important step in the process, and as shown in this work, increasing the adhesion of the fungus increased early disease symptoms. Understanding how this process is regulated and involved in the infection process will likely be important to developing future control of the

pathogen.

Biofilm formation in *F. graminearum* is likely critical to parts of the disease cycle. Through this dissertation, I have identified the process of biofilm formation, and identified structures that have previously not been reported in filamentous fungal biofilms, including pellicles. More details on the ecological impact of biofilms can be determined, especially how biofilms impact the disease cycle of Fusarium head blight. This dissertation contains the first report, to our knowledge, of biofilm formations on the surface of host plant tissues. Moving beyond this, studies on how these biofilms are involved across the disease cycle and what role they play in the survival and success of the fungus is necessary. Although in this work biofilms were only identified *in vitro* and on detached florets in culture, the knowledge provided by this can help to identify biofilm formations in other stages of disease.

This dissertation contains the first known report of a fungus actively uptaking the element silicon, in the form of silicic acid, into its cells. I have shown that silicic acid is sequestered in unidentified membrane-bound organelles in the hyphae. The mechanism for the uptake is still ununclear, but the results presented here indicate that aquaporins are involved in the process. As I have shown through use of knockout mutants, aquaporins impact the uptake and localization of silicic acid, as well as the growth and development of *F. graminearum*. Further work on how aquaporins are involved in the movement of silicic acid, as well as what the function of silicic acid is in fungal cells is necessary to further our understanding of this process. Many of the cells *F. graminearum* interacts with during the disease cycle are silica-rich, including stomates, trichomes, silica-cells, and xylem, which indicates that the uptake of silicic acid into fungal cells is important to the disease process. The importance of this finding is illustrated by the recent push to apply silica to plants to decrease fungal disease (J. F. Ma 2003; Schilmiller, Last, and

Pichersky 2008; E. Epstein 2009; Hauser 2014), including Fusarium head blight, although these results also indicate that mycotoxins may increase with the application of silica (Pazdiora et al. 2022; Sakr 2022). Understanding the relationship between silica and the infection process of *F. graminearum* will be vital to determining whether silica can be used as a component of chemical control.

In summary, my work on how biofilms form is one of the most complete studies of a filamentous fungal biofilm to date, including both the developmental process and matrix composition. Additionally, pellicles have been reported here for the first time in filamentous fungi. Adhesion, a trait of interest in the study of biofilms, was also shown to be regulated by methylation, which could be selected for *in vitro.* This work will both impact the future control of Fusarium head blight, and the study of biofilms in other filamentous fungi. Additionally, the work presented here about the relationship between silica and aquaporins in *F. graminearum* is the first report of its kind in a plant pathogen, where I have shown that silicic acid can be incorporated into fungal cells. This has implications for the disease cycle of Fusarium head blight, as silica-rich cells are involved in this process. The data provided here introduce novel components of the early infection process of *F. graminearum*, which in time will help to determine new methods of disease control.

REFERENCES

Allesen-Holm, Marie, Kim Bundvig Barken, Liang Yang, Mikkel Klausen, Jeremy S. Webb, Staffan Kjelleberg, Søren Molin, Michael Givskov, and Tim Tolker-Nielsen. 2006. "A Characterization of DNA Release in *Pseudomonas aeruginosa* Cultures and Biofilms." *Molecular Microbiology* 59 (4): 1114–28. https://doi.org/10.1111/j.1365-2958.2005.05008.x.

Epstein, E. 2009. "Silicon: Its Manifold Roles in Plants." *Annals of Applied Biology* 155 (2): 155–60. https://doi.org/10.1111/j.1744-7348.2009.00343.x.

Hauser, Marie-Theres. 2014. "Molecular Basis of Natural Variation and Environmental Control of Trichome Patterning." *Frontiers in Plant Science* 5 (July): 320. https://doi.org/10.3389/fpls.2014.00320.

Kischkel, Brenda, Gredson Keiff Souza, Lucas Ulisses, Rovigatti Chiavelli, Terezinha Inez, Estivalet Svidzinski, and Melyssa Negri. 2019. "The Ability of Farnesol to Prevent Adhesion and Disrupt *Fusarium keratoplasticum* Biofilm." *Applied Microbial and Cell Physiology*.

Ma, Jian Feng. 2003. "Beneficial Effects of Silicon in Different Plant Species." *Progress in Molecular and Subcellular Biology* 33.

Mann, Ethan E., and Daniel J. Wozniak. 2012. "*Pseudomonas* Biofilm Matrix Composition and Niche Biology." *FEMS Microbiology Reviews* 36 (4): 893–916. https://doi.org/10.1111/j.1574-6976.2011.00322.x.

Martins, Margarida, Priya Uppuluri, Derek P. Thomas, Ian A. Cleary, Mariana Henriques, José L. Lopez-Ribot, and Rosário Oliveira. 2010. "Presence of Extracellular DNA in the *Candida albicans* Biofilm Matrix and Its Contribution to Biofilms." *Mycopathologia* 169 (5): 323–31. https://doi.org/10.1007/s11046-009-9264-y.

Pazdiora, Paulo Cesar, Rosane Lopes Crizel, Keilor Rosa Dorneles, Sabrina Monks, Thomas Natali Morello, Fabio Clasen Chaves, and Leandro José Dallagnol. 2022. "Silicon Amendment Improves Wheat Defense against *Fusarium graminearum* and Complements the Control by Fungicide of Fusarium Head Blight," 0–2. https://doi.org/10.1111/ppa.13648.

Rajendran, Ranjith, Craig Williams, David F. Lappin, Owain Millington, Margarida Martins, and Gordon Ramage. 2013. "Extracellular DNA Release Acts as an Antifungal Resistance Mechanism in Mature *Aspergillus fumigatus* Biofilms." *Eukaryotic Cell* 12 (3): 420–29. https://doi.org/10.1128/EC.00287-12.

Sakr, Nachaat. 2022. "Silicon Reduces the Severity of *Fusarium* Infection on Young Wheat Parts In Vitro." *The Open Agriculture Journal* 16: 1–10. https://doi.org/10.2174/18743315-v16-e2207260.

Schilmiller, Anthony L., Robert L. Last, and Eran Pichersky. 2008. "Harnessing Plant Trichome

Biochemistry for the Production of Useful Compounds." *Plant Journal* 54 (4): 702–11. https://doi.org/10.1111/j.1365-313X.2008.03432.x.

Shay, Rebecca, Aaron A Wiegand, and Frances Trail. 2022. "Biofilm Formation and Structure in the Filamentous Fungus *Fusarium graminearum*, a Plant Pathogen." *Microbiology Spectrum* 10 (4): 1–8.

APPENDIX

IMPACT OF BARLEY GENETICS ON DEFENSE RESPONSE

Introduction

Fusarium graminearum is a fungal plant pathogen and the primary causal agent of Fusarium head blight (FHB) on wheat and barley, which reduces quality and quantity of grain yields (Trail 2009; McMullen et al. 2012; Shah et al. 2018). FHB is partially controlled by fungicides and tolerant cultivars, but integrated pest management is necessary to maximize disease control. Barley has a natural, moderate resistance response where *F. graminearum* does not spread from individual infection sites internally in the plant (Bai and Shaner 2004; Boddu et al. 2006; Harris et al. 2016). Additionally, barley has been shown to accumulate cellulose and lignin in association with trichomes, a typical infection site, in response to *F. graminearum* infection (Imboden, Afton, and Trail 2018). The shape of trichomes impacts the accumulation of defense compounds, although it is unknown what causes the differential defense response. Understanding what genes are involved in this response is important for incorporating the phenomenon in future breeding efforts.

Barley varieties with resistance (partial or complete) to the biotrophic pathogen powdery mildew have been generated and are available for commercial use (Hall et al. 1998; Freialdenhoven et al. 1996; Hückelhoven, Trujillo, and Kogel 2000). Partial resistance is conferred through the recessive *mlo-5* allele, which increases the hypersensitive response to fungal penetration. Complete resistance relies on the recessive *ror2* allele, which regulates the *mlo-5* allele, and both alleles must be recessive for complete resistance (Freialdenhoven et al. 1996). In numerous studies, there has been a reported trade-off in resistance with the powdery mildew resistant lines of barley (Jarosch, Kogel, and Schaffrath 1999; Kumar et al. 2001; Brown 2002; Jansen et al.

2005; Acevedo-Garcia, Kusch, and Panstruga 2014). This trade-off is associated with the difference in pathogen biology between biotrophic and necrotrophic pathogens, where the powdery mildew-resistant lines protect against the biotroph powdery mildew, but show enhanced susceptibility to *Fusarium graminearum*, *Bipolaris sorokiniana*, *Magnaporthe grisea*, and *Magnaporthe oryzae* (Jarosch, Kogel, and Schaffrath 1999; Kumar et al. 2001; Jansen et al. 2005; Acevedo-Garcia, Kusch, and Panstruga 2014). Plant cells with *mlo* alleles that confer powdery mildew resistance do so primarily through a burst of hydrogen peroxide at the start of fungal penetration, which leads to cell death in both the pathogen and the host plant (Piffanelli et al. 2002; Peterhansel et al. 1997; Kumar et al. 2001). This controlled cell death response provides an opportunity for necrotrophic pathogens to infect the dead cells. However, despite the reports that a trade-off of resistance exists, it may be condition-dependent, as Hofer et al. (2014) found no increased susceptibility to necrotrophic or hemi-biotrophic pathogens, indicating that this relationship may not be as definitive as previously thought.

To determine if the defense compound accumulation seen in trichomes is decreased in the powdery mildew resistant lines, allowing *F. graminearum* to penetrate barley florets more readily, we examined the accumulation of defense compounds in the partially and completely resistant powdery mildew lines. Additionally, we employed a selection of barley near-isogenic lines (NILs) with variations in trichome morphology to determine if any allele studied was involved in the differential response between two-row and six-row barley noted by Imboden, Afton, and Trail (2018). This study aims to elucidate some of the genetic basis of the differential defense response seen in trichome morphologies. The information generated will contribute to future breeding efforts to produce more resistant wheat and barley cultivars.

Methods

Barley lines

Two powdery mildew resistance lines were included in this study, A44 and *mlo-5*. A44 is a line that is moderately susceptible to powdery mildew, and contains the alleles *mlo-5 Ror1 ror2* (Freialdenhoven et al. 1996). The second line studied contains dominant Ror alleles, and the recessive *mlo-5* allele, which confers partial resistance to powdery mildew.

Barley varieties (Table A.1) were generously provided by Drs. Jerome D Franckowiak and Kevin Smith (University of Minnesota). The vrs1 locus provided morphologically distinct lines, while the int-c locus was selected due to a suggested susceptibility to FHB (Youssef, Koppolu, and Schnurbusch 2012; Franckowiak, personal communication). The gth1.a lacks lemma vein barbs, which are extra protrusions off the lemma awn, which was suggested to be of interest while studying the morphological distinctions between domed and prickle-like trichomes (Franckowiak, personal communication).

Table A.1: Barley varieties used in this study, phenotypes and trichome types.

NIL variety	**Phenotype**	**Trichome type**
Bowman	**two-row** wild-type, susceptible	Domed

Table A.1 (cont'd)

Stander	**six-row** wild-type, susceptible	Prickle-like
int-c.5	Gene involved in switch from two-row ancestor to six-row, **two-row allele**	Domed
Vrs1.t	Central floret of six row arrangement never develops, **two-row allele**	Domed
vrs1.a	Determines arrangement of florets on spike, **six-row allele**	Prickle-like
vrs1.c	Reduced lateral spikelet appendage on the lemma, **six-row allele**	Prickle-like
gth1.a	Toothed lemma, **two-row allele**	Domed

Barley inoculation

Florets were collected at Zadoks' stage 47 (Zadoks, Chang, and Konzak 1974), when the head is emerging from the boot. The bottom 3-4 florets from each head (only the center floret on 6-row barley) were removed, the awns were cut off, and the florets were placed upright into 1% water agar. Each floret was inoculated with conidia (5 μL x 10^6 conidia/ml in water), and incubated at room temperature (22-25°C) under light conditions for 5 days before analysis.

Preparation of palea dermal slides

Paleae were removed from florets, and superficial fungal hyphae were scraped off. Paleae were collected in 10 ml FAA (50 ml ethanol, 5 ml glacial acetic acid, 10 mL formalin, 35 mL water) and placed under vacuum for 30 minutes, then stored in the dark for at least one day to ensure clearing. FAA-preserved paleae were placed in water for one hour to remove the FAA before being placed in 0.1% Chlorazol Black E solution (CBE; Sigma-Aldrich, St Louis, MO). Samples were left in stain overnight (12-16 hours). To destain, an ethanol dehydration series was used. Florets were placed in 50% ethanol, followed by 100% ethanol for 30 minutes each, before being moved to xylene for 20 minutes prior to mounting on slides. Paleae were placed epidermal side up on a slide and suspended in 2-3 drops of Cytoseal 60 (Richard Allen Scientific, Kalamazoo, MI) before application of a cover slip weighted with a fishing sinker. The slides were left to dry for two days before viewing.

Counting focal accumulations

Foci are concentrations of defense-associated compounds at discrete sites on the surface of the paleae. The foci counted had a black-blue center, with a pink halo around some. Foci are predominantly, but not exclusively located at trichomes, and the number of foci per palea were

recorded for each line of barley, with 10 palea per line per replicate counted, and 3 replicates. T-tests were used to determine significance.

Cross sectioning of barley florets

Whole florets were fixed then stained with CBE as described above. After following the destaining protocol, florets were hand-sectioned under a dissecting microscope at trichomes, before being placed on slides for viewing. Cross-sections were viewed on a Nikon SMZ800N dissecting microscope and documented with a Nikon DS-Ri2 camera (Nikon, Tokyo, Japan) at 20x magnification.

Lipid accumulations during early infection

Florets were inoculated as described above, and the paleae were sectioned out without a fixing step. Palea were gently placed on a microscope slide to not disturb any fungal hyphae, and stained with nile red (500 μg/mL; Sigma-Aldrich, St. Louis, MS) for five minutes in the dark, before destaining by rinsing once with sterile distilled water.

Results

Focal accumulations in powdery mildew resistant lines

Foci were significantly increased ($p<0.05$) in both powdery mildew resistant lines, as compared to the control susceptible barley variety Stander. There was no significant difference between A44 and the *mlo-5* barley lines (Figure A.1).

Focal accumulations across NILs

Foci varied widely in all NILs studied and the two-row wild-type barley Bowman. The six-row wild-type susceptible control Stander was significantly reduced ($p<0.05$) in foci as compared to Bowman (Figure A.2). The locus *vrs1.c* had the most differential impact on foci, although there was no significant difference compared to Bowman, as both exhibited wide ranges of foci.

Fungal penetration at focal accumulations

Inoculated palea were cross sectioned at trichomes that did not exhibit any focal accumulations, and at ones that had the distinct staining associated with the burst of cellulose and lignin. At trichomes with no visible foci, fungal penetration across the cross-section was seen (Figure A.3). At locations with foci, fungal penetration was not observed (Figure A.3). Fungal penetration was observed at trichomes frequently (Figure A.4). To determine if the penetration of fungi was associated with other defense compounds, nile red was used to stain plant cuticle. At points of fungal penetration, a higher concentration of nile red was observed (Figure A.4).

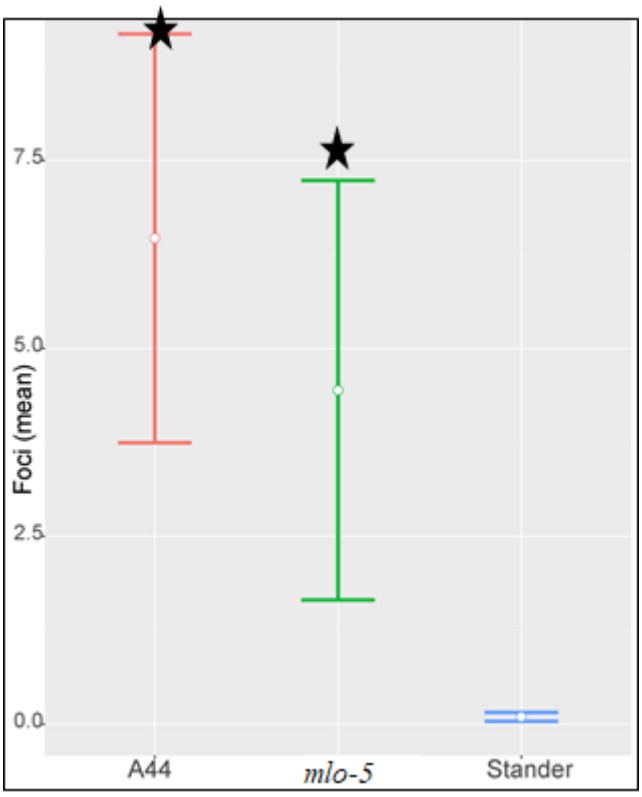


Figure A.1: Focal accumulations in powdery mildew resistant lines. Stars indicate a significant difference from the wild-type Stander ($p<0.05$). Circles indicate the average number of foci, and bars represent standard deviations.

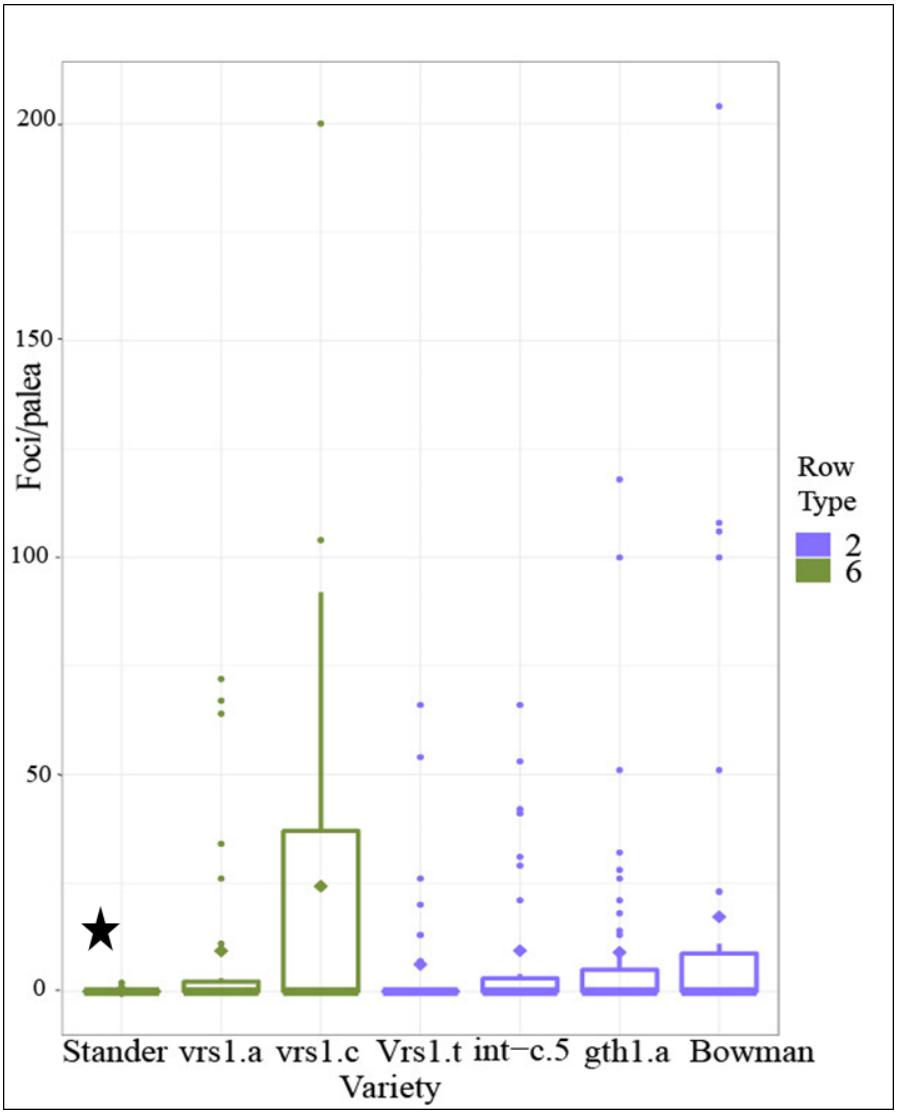


Figure A.2: Foci per palea in barley near-isogenic lines. Mean numbers of foci are indicated by diamonds. Ranges of foci per palea varied widely across most samples. The star indicates a significant reduction in foci per palea, as compared to Bowman ($p<0.05$). Row types indicate the source type of each allele, not the plant overall. All NILs were in a Bowman background.

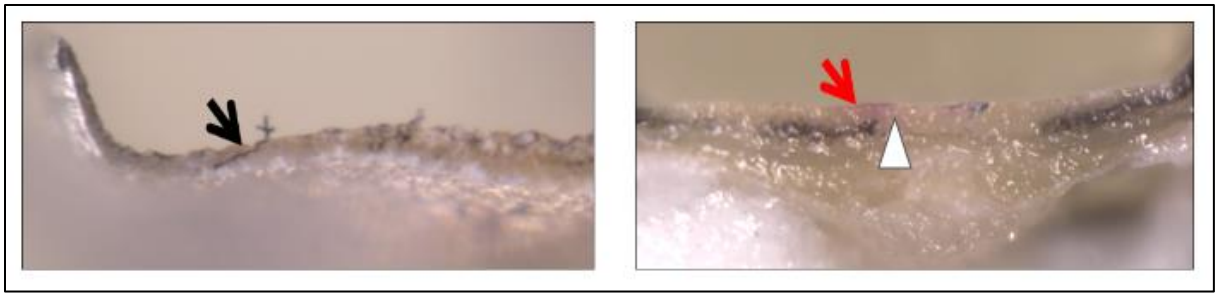


Figure A.3: Cross sections of inoculated barley palea. Sections were done at trichomes not exhibiting the focal accumulation of defense compounds (left), and at foci with defense compounds (right). Fungal hyphae are stained black with Chlorazol black E (arrows), either penetrating the plant tissue (black arrow), or unable to penetrate (red arrow). White arrowhead points to focal accumulation staining.

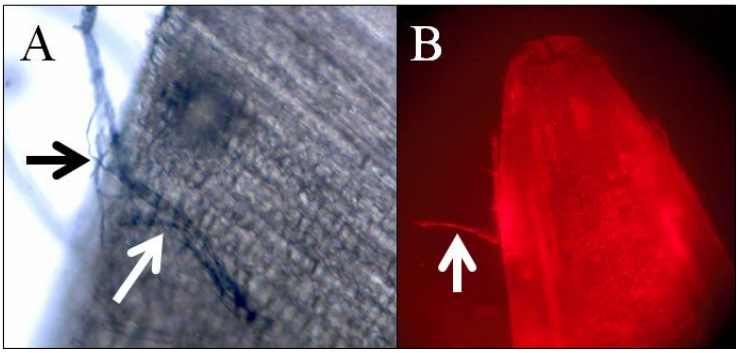


Figure A.4: Fungal penetration into Stander palea. On all micrographs, white arrows indicate *F. graminearum* hyphae, and black arrows indicate trichomes. (A) Cross section stained with Chlorazol black E, where no foci are present. (B) Palea stained with the lipid stain nile red. A higher concentration of nile red is visible next to the site of fungal entry.

Discussion

Trichomes play an important role in the defense response of barley to FHB. Previous data has shown that varieties with small, domed trichomes, typically present on two-row barley, have significantly more foci than varieties with prickle-like trichomes (Imboden, Afton, and Trail 2018). However, six-row barley is generally more resistant to FHB than two-row, which contradicts this finding. This indicates that the relationship between the ability to generate

cellular defenses against fungal penetration is more complicated than just the shape of the trichome, and there is underlying barley genetics that influence this reaction and is separate from trichome shape. The near isogenic line containing the Vrs1.c locus produced the most foci. This locus is from the six-row parent in the initial cross, and produces prickle-like trichomes. This indicates that although domed trichomes typically produce more foci, this is not due to the shape of the trichome, but rather the underlying genetics.

Cross sectioning shows that at foci, no fungal penetration has occurred. Fungal penetration can occur at areas without foci, at trichomes that do not produce the defense response, or other natural openings. In addition to the known accumulation of cellulose and lignin, we observed an increase in lipid staining at points of plant penetration, indicating that plants accumulate more cuticle at the location of fungal penetration, which likely contributes to the cessation of fungal penetration.

Interestingly, in powdery mildew resistant varieties, we showed an increase in the number of foci, indicating that the plant is producing compounds to stop fungal penetration. This contrasts with the idea that powdery mildew resistant lines are more susceptible to FHB, as the lines are able to produce defense compounds in response to *F. graminearum*. However, further work is necessary to determine if this difference is due to the genetic background of the varieties. A44 and *mo-5* barley lines are in the background of the variety Ingrid (Freiadenhoven et al. 1996), which to our knowledge, has not been studied in focal accumulations as a plant defense response. However, this does provide evidence that powdery mildew resistant lines can generate defense responses to *F. graminearum*, which may be worth further study to develop barley varieties with resistance to multiple fungal diseases with different infection styles.

REFERENCES

Acevedo-Garcia, Johanna, Stefan Kusch, and Ralph Panstruga. 2014. "Magical Mystery Tour: MLO Proteins in Plant Immunity and Beyond." *Journal of Physiology* 204 (2): 273–81. https://doi.org/10.1111/nph.12889.

Bai, Guihua, and Gregory Shaner. 2004. "Management and Resistance in Wheat and Barley to Fusarium Head Blight." *Annual Review of Phytopathology* 42 (1): 135–61. https://doi.org/10.1146/annurev.phyto.42.040803.140340.

Boddu, Jayanand, Seungho Cho, Warren M. Kruger, and Gary J. Muehlbauer. 2006. "Transcriptome Analysis of the Barley - *Fusarium graminearum* Interaction." *MPMI* 19 (4): 407–17.

Brown, James K M. 2002. "Yield Penalties of Disease Resistance in Crops." *Current Opinion in Plant Biology* 5 (4): 339–44. https://doi.org/10.1016/S1369-5266(02)00270-4.

Freialdenhoven, A, C. Peterhansel, J Kurth, F Kreuzaler, and P Schulze-Lefert. 1996. "Identification of Genes Required for the Function of Non-Race-Specific." *The Plant Cell* 8 (January): 5–14.

Hall, 1, A Rho, L Luo, Y J Liao, L Y Jan, Y N Jan, R Kozma, et al. 1998. "Role of a New Rho Family Member in Cell Migration and Axon Guidance In." *Science Genes Dev. Mol. Cell. Biol. Cell Development Nature Rev. Neurosci. Dickson, B. J. Curr. Opin. Neurobiol* 279 (8): 509–14. https://doi.org/10.1038/416447a.

Harris, Linda J., Margaret Balcerzak, Anne Johnston, Danielle Schneiderman, and Thérèse Ouellet. 2016. "Host-Preferential *Fusarium graminearum* Gene Expression during Infection of Wheat, Barley, and Maize." *Fungal Biology* 120 (1): 111–23. https://doi.org/10.1016/j.funbio.2015.10.010.

Hofer, Katharina, Andrea Linkmeyer, Katharina Textor, Ralph Huckelhoven, and Michael Hess. 2014. "MILDEW LOCUS O Mutation Does Not Affect Resistance to Grain Infections with *Fusarium* Spp. and *Ramularia collo-Cygni*." *Igarss 2014*, no. 1: 1–5. https://doi.org/10.1007/s13398-014-0173-7.2.

Hückelhoven, R, M Trujillo, and K H Kogel. 2000. "Mutations in Ror1 and Ror2 Genes Cause Modification of Hydrogen Peroxide Accumulation in Mlo-Barley under Attack from the Powdery Mildew Fungus." *Molecular Plant Pathology* 1: 287–92. https://doi.org/10.1046/j.1364-3703.2000.00032.x.

Imboden, Lori, Drew Afton, and Frances Trail. 2018. "Surface Interactions of *Fusarium graminearum* on Barley." *Molecular Plant Pathology* 19 (6): 1332–42. https://doi.org/10.1111/mpp.12616.

Jansen, Carin, Diter von Wettstein, Wilhelm Schäfer, Karl-Heinz Kogel, Angelika Felk, and

Frank J Maier. 2005. “Infection Patterns in Barley and Wheat Spikes Inoculated with Wild-Type and Trichodiene Synthase Gene Disrupted *Fusarium graminearum*.” *Proceedings of the National Academy of Sciences of the United States of America* 102 (46): 16892–97. https://doi.org/10.1073/pnas.0508467102.

Jarosch, Birgit, Karl-heinz Kogel, and Ulrich Schaffrath. 1999. “The Ambivalence of the Barley Mlo Locus : Mutations Conferring Resistance Against Powdery Mildew (*Blumeria graminis f . Sp . Hordei*) Enhance Susceptibility to the Rice Blast Fungus *Magnaporthe grisea*.” *Molecular Plant-Microbe Interactions : MPMI* 12 (6): 508–14. https://doi.org/10.1094/mpmi.1999.12.6.508.

Kumar, J, R Hückelhoven, U Beckhove, S Nagarajan, and K H Kogel. 2001. “A Compromised Mlo Pathway Affects the Response of Barley to the Necrotrophic Fungus *Bipolaris sorokiniana* (Teleomorph: *Cochliobolus sativus*) and Its Toxins.” *Phytopathology* 91 (2): 127–33. https://doi.org/10.1094/PHYTO.2001.91.2.127.

McMullen, Marcia P., Gary C. Bergstrom, Erick De Wolf, Ruth Dill-Macky, Donald E Hershman, Gregory Shaner, and David A Van Sanford. 2012. “A Unified Effort to Fight an Enemy of Wheat and Barley: Fusarium Head Blight.” *Plant Disease* 96 (12): 1712–28. https://doi.org/10.1094/PDIS-03-12-0291-FE.

Peterhansel, C., A Freialdenhoven, J Kurth, R Kolsch, and P Schulze-Lefert. 1997. “Lnteraction Analyses of Genes Required for Resistance Responses to Powdery Mildew in Barley Reveal Distinct Pathways Leading to Leaf Cell Death.” *The Plant Cell* 9 (August): 1397–1409.

Piffanelli, Pietro, Fasong Zhou, Catarina Casais, James Orme, Birgit Jarosch, Ulrich Schaffrath, Nicholas C Collins, et al. 2002. “The Barley MLO Modulator of Defense and Cell Death Is Responsive to Biotic and Abiotic Stress Stimuli 1” 129 (July): 1076–85. https://doi.org/10.1104/pp.010954.ror2.

Shah, L., A. Ali, M. Yahya, Y. Zhu, S. Wang, H. Si, H. Rahman, and C. Ma. 2018. “Integrated Control of Fusarium Head Blight and Deoxynivalenol Mycotoxin in Wheat.” *Plant Pathology* 67 (3): 532–48. https://doi.org/10.1111/ppa.12785.

Trail, Frances. 2009. “For Blighted Waves of Grain: *Fusarium graminearum* in the Postgenomics Era.” *Plant Physiology* 149 (1): 103–10. https://doi.org/10.1104/pp.108.129684.

Youssef, Helmy M., Ravi Koppolu, and Thorsten Schnurbusch. 2012. “Re-Sequencing of Vrs1 and Int-c Loci Shows That Labile Barleys (*Hordeum vulgare* Convar. Labile) Have a Six-Rowed Genetic Background.” *Genetic Resources and Crop Evolution* 59 (7): 1319–28. https://doi.org/10.1007/s10722-011-9759-5.

Zadoks, J C, T.T. Chang, and C.F. Konzak. 1974. “A Decimal Code for the Growth Stages of Cereals.” *Weed Research* 14 (6): 415–21. https://doi.org/0.1111/j.1365-3180.1974.tb01084.x.