

MECHANISM OF SURFACE ESTABLISHMENT AND COLONIZATION OF BARLEY FLORETS BY
FUSARIUM GRAMINEARUM

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ABSTRACT

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Despite increased research into head blight disease caused by *Fusarium graminearum*, little is known about the surface interactions between the fungus and its host, barley (*Hordeum vulgare*). Trichomes on barley florets were characterized as macro- and micro-trichomes according to previous descriptions from other grasses. These studies have identified differential interactions between macro- trichomes, which predominate vascular bundles and bract margins, and micro- trichomes, which cover the remainder of the bract surface, and *F. graminearum*.

In infections initiated at early grain developmental stages, attachment of germinated conidia to trichomes was accompanied by growth to bract margins. In infections initiated at later developmental stages, aerial hyphae and surface colonization were reduced and infections were only observed in conjunction with trichomes. Macro-trichomes on vascular bundles provided pathways for the fungus to the vasculature below, allowing the infection to spread. Similar trichome interactions in a *tri5* knockout mutant indicated that the mycotoxin deoxynivalenol does not play a role in establishment. Furthermore, perithecia emergence from inoculated florets indicated the importance of grain developmental stage in pathogen spread, and of the vascular tissue in sexual development in later infections.

Silica accumulates in grasses, commonly in the xylem, trichomes, and epidermal cells. Biogenic silica, in the form of mono-silicic acid, was shown to reduce aerial hyphae and stimulate mycotoxin production *in-vitro*. These results strongly implicate silica in the interactions between *F. graminearum* and barley.

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INTRODUCTION

Economic impact of head blight caused by *Fusarium graminearum* Schwabe.

F. graminearum causes several diseases on wheat, corn and barley. Wheat can succumb to crown rot as a seedling, to head blight in stages from anthesis to grain filling, and continued colonization of the grain post harvest. Barley also suffers from seedling and head blight. Corn is susceptible to stem or stalk rot, ear rot, and can suffer from severe post harvest rot if stored improperly (Trail 2009). For all grains, even a minor presence of *F. graminearum* can result in mycotoxin contamination. Devastation by *F. graminearum* of grains in major production areas worldwide occurs almost annually. The susceptibility of wheat, corn, and barley links this pathogen tightly with world health in both first and third world nations. Losses in yield are largely associated with shrinkage of the kernels (Nicholson 2009). This, coupled with the production of mycotoxins such as the trichothecene deoxynivalenol (DON), make *F. graminearum* an important factor in public health, food shortages, food quality, land use and sustainability, international trade, and economics.

Life cycle and general characteristics of *Fusarium graminearum*.

F. graminearum is a haploid homothallic fungi in the Phylum Ascomycota and the Class Sordariomycetes. *F. graminearum* produces half-moon or “canoe” shaped asexual conidia ranging in size from 20 to 50µm. Conidia contain 3-7 septate cells and are produced on doliiform phialides, branched conidiophores, or sporodochia (Domsch *et al.* 1971). Originating from infected plants, conidia can serve as primary inoculum for the disease, being dispersed by rain and wind although the sexually produced ascospores are the initial source of inoculum (Trail 2009). Ascospores, which are 20-25µm long and contain 3 septate cells, originate from asci within ovoid perithecia. Perithecia usually contain many asci and each asci contains 4-8

ascospores (Domsch *et al.* 1971). Growth in culture is characterized by white aerial hyphae when young. Older cultures may become yellow at the center especially when nutrients are not limited. Some carmine red, which can appear pink or purple, may appear in the media below the culture especially when grown on Potato Dextrose or Carrot Agar (Leslie and Summerell 2006).

Gibberella zeae Schwabe is the teleomorph of *F. graminearum*. Ascospores, which are the sexual spores, are discharged from perithecia forming on crop residue and are most often thought of as the primary inoculum for head blight (Trail 2009). Being homothallic, or self-compatible, *F. graminearum* can produce large amounts of primary inoculum without the need to outcross. Although the ascospores are forcibly discharged from fruiting bodies and spread further by wind and rain, conidia are only transmitted passively in wet or windy conditions. Canoe shaped conidiospores can adhere to small droplets of water and carried be on the wind to other plants within the field or even greater distances to other fields and regions (Deacon 2006). Conidia, which originate from crop tissue, can further spread the pathogen during the growing season and increase disease severity and contamination of grain (Lewandowski *et al.* 2006).

Successful infection of a host plant by *F. graminearum* can take place at almost any time in the host's life cycle by either sexual or asexual spores, although infection of the leaves and stems exclusively cannot lead to severe economic impacts without colonization of the grain as well (Nicholson 2009). The success of the disease, and its severity, relies heavily on weather conditions at the time that inoculum is spread. Humidity and rain are essential to the development, and germination of the sexual and asexual spores of *F. graminearum* (Beyer *et al.* 2005). **Mycotoxins produced by *F. graminearum*.**

Mycotoxins produced by *F. graminearum* include DON, nivalenol, aurofusarin, rubrofusarin, zearalanone, and fusarin C (Trail 2009). DON, a protein biosynthesis inhibitor, is

the most prevalent mycotoxin of concern in the United States, although the others also have detrimental impacts as well. Aurofusarin is responsible for the yellow pigmentation of *F. graminearum* hyphae in culture and in contaminated grain and is especially toxic to poultry (Kim *et al.* 2005). Rubrofusarin, an intermediate in the aurofusarin pathway (Frandsen *et al.* 2006) which is the pink or reddish pigment, has antimycobacterial properties and is phytotoxic to some weeds (Kim *et al.* 2005). Zearalenone binds to estrogen receptors and can disrupt pregnancy and affect fertility (Zinedine *et al.* 2007). Head blight pathogens isolated from different grain growing regions of the world have been shown to produce different levels of these toxins with unique patterns of toxin production, strains with these unique patterns are referred to as chemotypes (Maier *et al.* 2006). Other head blight pathogens, such as *F. verticillioides* and *F. culmorum* or isolates within the *F. graminearum* species complex, have varying toxin signatures. Aside from those that produce DON there are chemotypes that predominately produce nivalenol or 3-acetyldeoxynivalenol (Starky *et al.* 2007).

Mycotoxins play varied and debated roles as virulence factors in the infection of wheat, barley, and corn by *F. graminearum*. DON was clearly implicated as a virulence factor in wheat (Desjardins *et al.* 1996) yet studies have suggested different mechanisms for resistance to spread between wheat and barley (Jansen *et al.* 2005). Other surveys have shown no difference in overall disease in infections of wild type and strains that do not produce DON in barley (Maier *et al.* 2006). Similar results were observed for wheat with other chemotypes (Maier *et al.* 2006). However, barley expresses specific genes in the presence of trichothecenes, implicating those genes in barley's type VI resistance and suggesting overlapping roles between trichothecenes and other virulence factors in barley (Boddu *et al.* 2007).

Grain development and host resistance to head blight.

Resistance mechanisms of wheat and barley are categorized as either morphological or physiological. Morphological resistance can include head characteristics that limit humidity around the florets, such as absence of awns (Rudd *et al.* 2001). Physiological mechanisms for resistance are categorized into five “Types”: Type I exhibits resistance to initial infection; Type II, resistance to spread within the rachis; Type III, kernel size and retention of normal kernel number; Type IV, yield tolerance; and Type V, decomposition or non-accumulation of mycotoxins (Rudd *et al.* 2001, Schroeder and Christensen 1963). In general barley is thought to have a higher type II resistance, and wheat a higher Type I resistance, although Type I resistance is difficult to assess without the pre-existence of Type II (Jansen *et al.* 2005).

Grain development in wheat and barley differ and these differences affect disease development during infection by *F. graminearum*. In covered barley, the hull (the outer floral bracts) fuses to the grain after harvest and is thus maintained through processing for products such as beer and most flours. The fusion of these bracts occurs at their respective margins and takes place as the grain fills. In hull-less barley and wheat the bracts do not fuse with the grain. Removal of the hull can result in shedding of *F. graminearum* infections that do not reach beyond the floral bract tissue (Legzdina and Buerstmayr 2004). However, hull-less barley is not as widely used as covered barley due to other grain characteristics. The deactivation of mycotoxins, especially DON, is also thought to play a role in Type II resistance of some barley varieties, implicating it as virulence a factor, although this role is debated (Jansen *et al.* 2005).

Infection pathways in barley.

Pathways for infection by *F. graminearum* differ between grains (Maier *et al.* 2006). Elucidation of the infection pathway of *F. graminearum* in these grains has been a major point of

interest in searching for targets for resistance breeding programs. *F. graminearum* has been shown to penetrate cuticles and cell margins, grow through plant tissue both through cells and through intercellular spaces, produce infection structures specific to mycotoxin production, penetration, and colonization, and sense the cell wall components of its host (Boenisch and Schafer 2011, Jansen *et al.* 2005, Lewandowski *et al.* 2006, Maier *et al.* 2006, Rittenour and Harris 2010).

Colonization of the bract vasculature is important for infection in wheat and barley and gives the fungus access to both the endosperm and to the rachis (Jansen *et al.* 2005). Hyphae travel through the rachis, leading to colonization of stem tissue in which the fungus overwinters as lipid rich hyphae and emerges as perithecia during warmer temperatures (Guenther and Trail 2005). Recently, infection hyphae on wheat have been shown to form either independent bulbous hypha with infecting arms, also referred to as lobate appresoria, or highly branched coral like infections structures that resemble infection cushions (Rittenour and Harris 2010). The highly branched structures have been associated with mycotoxin production and lesion formation. These can occur in most locations on the host surface but appear to prefer silicified epidermal cells (Boenisch and Schafer 2011).

In barley and wheat infection, surface interactions appear similar, but differ according to the differences in anatomy of the two hosts. A previous report on barley shows, after spore germination, hyphae growing across the surface of the floret to the bract margins where growth through the margin leads to colonization of the endosperm (Lewandowski *et al.* 2006). Spores which land near or within the floret have access to susceptible brush hairs at the apex of the endosperm, but barley closes sooner and more completely than wheat and so is more resistant to this sort of infection in later stages of grain development (Skadsen and Hohn 2004). Direct

penetration leading to colonization of the vasculature is thought to occur, however mechanisms have not been determined (Lewandowski *et al.* 2006). Recently *Brachypodium distachyon* was characterized as a model system for analyzing *F. graminearum* cereal interactions. In this system, surface establishment was associated with trichomes and papillae. Trichomes over the vascular bundles were suggested as a link to the vasculature (Peraldi *et al.* 2011). In *F. graminearum* specific responses to host surfaces have been suggested (Boenisch and Schafer 2011, Lewandowski *et al.* 2006, Peraldi *et al.* 2011, Rittenour and Harris 2010), however mechanisms for induction of these responses remain largely elusive.

Silica in grasses.

Silica, in the form of mono-silicic acid, is a vital plant nutrient in grasses, with conserved roles in plant physiology. Accumulation of silica occurs mainly in the panicle, in trichomes and papilla cells on the florets as spikelets emerge. Other cells on the spikelet known to accumulate silica in grasses are, bulliform cells, and vascular bundle tissue (Parry *et al.* 1984, Ma and Yamaji 2006). Uptake of silica in rice has been tied to genes encoding a plasma-membrane transport protein (Ma and Yamaji 2006). Silica-containing cells and vascular tissues have been implicated in the infection pathways of *F. graminearum* in grains (Boenisch and Schafer 2011, Guenther and Trail 2005, Jansen *et al.* 2005, Lewandowski *et al.* 2006, Maier *et al.* 2006, Nicholson 2009, Peraldi *et al.* 2011, Rittenour and Harris 2010, and Walter *et al.* 2009).

Accumulation of silica occurs along microtubules in cells that cause the mono-silicic acid to form aggregates (Sangster *et al.* 1983). During aggregate formation the outer edge of the silica aggregate contains active hydroxyl groups that cause additional reactions with more mono-silicic acid or other biologically active compounds (Piperno 2006). These aggregates are referred to as phytoliths (“plant-rocks”) by the paleo-botanists who study them. Phytolith

structure has been shown to be species specific and conserved through long periods of evolutionary history (Ball *et al.*2009). Their presence in the fossil record has given great insight into the locations in which various grass species evolved (Ball *et al.*2009). Although much of the physiological work on silica in plants has been done in rice, mechanisms of silica accumulation are thought to be similar in other grasses as the timing and location of accumulation has already been shown to be similar (Ma and Yamaji 2006). The presence of mono-silicic acid in and on trichomes and papillae cells and in the vasculature of grasses (Hayward *et al.*1972, Sangster *et al.*1982) poses interesting questions for its potential roles in the infection pathway of *F. graminearum*.

CHAPTER 1

INFECTION PATHWAY OF *FUSARIUM GRAMINEARUM* IN BARLEY

INTRODUCTION

Fusarium graminearum is one of the most economically important pathogens of grains worldwide, being the causal agent of stalk and ear rot of corn, and head blight of wheat and barley. Ascospores originate from perithecia on crop residues in which the fungus overwinters in the form of lipid rich hyphae (Guenther and Trail 2005). With few resistant crops available for effective rotation (Dill-Macky and Jones 2000), and difficulties associated with practical fungicide applications, plant breeders are left to focus on host resistance as the most effective means of achieving a reduction of this disease (Chain *et al.* 2009, McMullen 1997, and Rudd *et al.* 2001).

F. graminearum causes reduced yield and mycotoxin contamination, imposing economic hardship on growers. Deoxynivalenol (DON), the most important mycotoxin associated with *Fusarium* head blight disease of wheat and barley, inhibits protein biosynthesis. DON accumulates in all grains susceptible to the disease, although its role as a virulence factor is thought to vary both between different stages of infection and different hosts (Boenisch and Schafer 2011, Ilgen *et al.* 2008, Jansen *et al.* 2005, Yoshida *et al.* 2006). The genetics of the DON biosynthesis pathway has been well described (Kimura *et al.* 2007). Deletion of *TRI5*, the gene encoding trichodiene synthase, resulted in significant reduction in virulence in *F. graminearum* on wheat (Desjardins *et al.* 1996).

Barley (*Hordeum vulgare*) has naturally high resistance to spread of *F. graminearum* (type II resistance) beyond the floret into the rachis (Schroeder and Christensen 1963). For this reason a primary target for reducing head blight severity in barley is to reduce the establishment

of the fungus on the plant host. While DON is associated with successful infection in wheat and thought to be responsible for overcoming type II resistance in that crop, this is not so in barley (Ilgen *et al.* 2008). The different roles of DON in these two crops suggest different mechanisms for type II resistance between these grains (Jansen *et al.* 2005, Maier *et al.* 2006).

Recently, an investigation into the *Brachypodium distachyon*—*F. graminearum* interaction demonstrated that trichomes and papillae (analogous to small underdeveloped trichomes) were important infection sites (Peraldi *et al.* 2011). Bulbous hyphae formed in association with both epidermal cell-types. In addition, penetration of the trichome cuticle by the infection hyphae, which moved through the cell to its base, was also observed. Papillae of frozen wheat glumes have previously been associated with hyphal branching and bulbous hyphae by *F. graminearum* (Rittenour and Harris 2010). Interactions with trichomes were also described for *Brachypodium distachyon* (Peraldi *et al.* 2011). On floral bracts, macro-trichomes densely cover the bract margins, the vascular bundles (Fig. 1), and the rachilla. Micro-trichomes cover the surface of the lemma and the palea, which, collectively, are the major floral bracts of barley. Micro-trichomes are markedly less dense, but occur across a larger surface area than macro-trichomes and increase in density towards the floral opening

Trichomes and papillae both accumulate silica as phytoliths, which are aggregates of silicon dioxide, via the translocation of mono-silicic acid from xylem vessels (Ma and Yamaji 2006). This silica, often referred to as biogenic silica, accumulates in the floral bracts of grasses in higher concentrations than almost any other plant (Sangster *et al.* 1983). Mono-silicic acid accumulates within cells by adhering to microtubules and becomes gel-like when concentrations approach 2 mM (Ma and Yamaji 2006). The gel hardens to form the phytoliths. The surface of the developing aggregate has active hydroxyl groups, which can form strong bonds to one

another and to other molecules such as lipids (Piperno 2006). This process occurs not only in the trichomes and

papillae of the epidermis, but also within the vascular bundles, bulliform cells, some intercellular spaces, and other locations depending upon the species of grass (Piperno 2006).

We examined the process of colonization of *F. graminearum* through trichomes on the surface of barley florets and followed the pattern of ingress. In addition, we examined the role of mono-silicic acid in the infection pathway into barley.

MATERIALS AND METHODS

Strains and culture conditions.

The strains of *F. graminearum* used in this investigation were PH-1 (NRRL 31084 FGSG 9075), a wild-type strain originating from Michigan (Trail and Common 2000) and a *TRI5* knock-out mutant generated from PH-1. Strains were stored as mycelia-colonized agar in 35% glycerol at -80°C. Conidial suspensions (2.5×10^5) for inoculation were generated as previously described (Guenther and Trail, 2005) and stored at -80°C prior to use.

Plant cultivation and inoculation.

The barley varieties Quest (M122; resistant to FHB) and Stander (susceptible to FHB) were grown in the greenhouse 6-inch clay pots as previously described for wheat (Guenther and Trail, 2005). Spray inoculations were performed at full head emergence from the flag leaf sheath (Zadok stage 60; Anderson *et al.* 2008). Prior to inoculation, plants were misted with sterile distilled water to imitate the wet plant surface that spores would likely encounter in the field. Greenhouse inoculations were accomplished using a spray bottle to mist barley florets with a conidial suspension in sterile water (5×10^3 spores/mL). Following inoculation, heads were covered for 72 hrs with a waxed paper bag, closed at the base, to allow the infection to initiate.

Whole heads were harvested from 8 separate experiments of each Quest and Stander. Tissue was fixed and prepared for microscopy as described below. The number of heads examined from each spray inoculation experiment depended upon lesion development but included a minimum of 5 florets from each of 3 heads per collection.

A detached floret assay was adapted from Lewandowski et al. (2006) as a convenient and consistent inoculation method for analysis of *F. graminearum* establishment and spread on florets in Stander, and Quest. A 5 µl drop of conidial suspension (2.5×10^5 spores/mL) of PH-1 was placed within the furrow of the palea of detached florets, which were mounted on water agar (15%; Fig. 2). Control florets were similarly treated with sterile distilled water. All florets were incubated at room temperature (24-26 °C) 18 inches below two 34 watt fluorescent light bulbs (Phillips F34T12/ADV835/EW). Inoculated and control florets were harvested every 12 hrs for the first three days post inoculation (dpi) and every 24 hrs for 4-6 dpi. Five florets were harvested from each of the two varieties at each time-point, and two florets exhibiting symptoms representative of the majority from each set of five were fixed and dermal peels prepared as described below. The entire experiment was replicated six times with PH-1. Similar experiments were performed for the *TRI5* mutant with collections at 24 hour intervals through 3 dpi.

The detached floret assay was also used for investigations into the location of perithecia emergence. Florets were inoculated as above with PH-1 and incubated until perithecia formed on the surface of the bracts. Florets were inoculated at four stages of development early (Zadok's 5.5-5.7; partial head emergence from the flag leaf sheath), middle (Zadok's 5.9-6.1; full head emergence through the initiation of flowering); late (Zadok's 7-7.3; early milk stage in kernel); and very late (Zadok's 7.7-7.8; late milk stage to early dough stage). For each of the four developmental stages 34 florets were inoculated in each of the two separate

experiments. Lesion observations were made at 3 dpi (MacCallum and Tekauz 2002). Numbers of perithecia were tallied at 12 dpi.

Statistical analyses.

The location of perithecium emergence data was subjected to analysis of variance (ANOVA Alpha=.05). The dependent variable was the location of the emergence of perithecia and the independent variable was the developmental stage of the grain at inoculation. The location was divided into four categories: perithecia emerging from vascular bundles at the point of inoculation; perithecia emerging from the palea surface at the point of inoculation; perithecia emerging from the vascular bundles beyond the point of inoculation; and perithecia emerging on the palea surface beyond the point of inoculation. The developmental stage of the grain at inoculation was divided into two categories for statistical analyses: Zadok's 5.5-6.1; partial head emergence from the flag leaf sheath through the initiation of flowering and Zadok's 7-7.8; early milk stage in kernel to early dough stage. These two categories represent developmental stages of the grain prior to and after fusion of the bract margins respectively.

Silica bioassay.

Czapek-Dox's agar medium (Difco, Detroit, MI) amended with silicon dioxide or silicic acid was used to determine the response of *F. graminearum* to silica *in vitro*. For silicon dioxide assays, powdered silicon dioxide was added to media to concentrations of 1 or 2 mM in the final media. For mono-silicic acid concentrations of 0.5, 1, and 2 mM and a water control were added as liquid to the surface at equidistant points on the edge of 100 mm Czapek-Dox agar plates. Three volumes, 50, 100, and 200 μ l, were used and each was replicated three times both under light and dark conditions to total 6 replicates. Each plate consisted of four treatments, one of each concentration and the control, which were all of the same volume. Mono-silicic acid

treatments were applied beyond the outer edge of growing hyphae after two days of growth. Cultures were incubated at room temperature (24-26°C) 14 days under both continuous light and continuous dark. Plates were center-inoculated with conidia and hyphal growth response was recorded. An additional experiment with the same replicates was completed to control for the impacts of pH on hyphal growth with mono-silicic acid buffered in 50mM HEPES buffer. Previously, 2 mM silicic acid was shown to be the concentration at which polymerization occurs *in planta* (Ma 2006), so concentrations above 2 mM were not used.

Fixation, embedding, sectioning, staining and microscopy.

Fixation and clearing of infected tissue was initiated by submerging freshly harvested florets and heads into cold 10% Formal Acetic Acid (FAA; 50% ethanol, 10% formaldehyde, 5% acetic acid, and 35% water) solution under vacuum in a dessicator for 30 minutes or until the tissue sank to the bottom of the vial. For tissue that was not immediately processed further, the FAA solution was changed, and tissue was stored in the dark at RT. Fixed tissue was carried through a tertbutyl alcohol (TBA)-ethanol dehydration series (ethanol:TBA:Water-50:10:40, 50:20:30, 50:35:15 at 4°C for 2 hrs each and ethanol :TBA--50:50, 25:75, and 0:100 at room temperature overnight (12-15hrs) for each followed by infiltration and embedding in Tissue Prep paraffin wax (Fisher Scientific Fair Lawn, NJ; Cat#T555).

Dehydrated tissue in TBA was incubated at 68°C and wax was added as the TBA evaporated until a final change to fresh molten wax was done immediately prior to embedding. Bracts of mature barley florets were punctured with an insect pin at the base of the bract to allow infiltration of the wax. Paraffin-embedded tissue was sectioned to 12-15 µm thick with a rotary microtome (American Optical Spencer). Sections were placed on glass slides, dewaxed with xylene, and rehydrated through an ethanol series (100% xylene for 5 minutes and 100, 95, 80,

60, and 30 percent ethanol for 3 minutes each). Sections were stained in 1% aqueous solution of Toluidine blue for 3 hours for differentiation of plant cell types and fungal hyphae. Sections were de-stained in 30% ethanol for 1-3 hours, mounted in Cytoseal 60 permanent mount mixture (Richard Allen Scientific, Kalamazoo MI) and allowed to dry overnight in a chemical fume hood.

Florets used for dermal peels were fixed whole in cold 10% FAA under vacuum in a dessicator. Following fixation, the bracts were removed and the adaxial and abaxial portions of each bract separated, forming the dermal peels. Peels were dehydrated as above, and stained prior to manual processing for permanent mounts. Dermal peels were stained in a 0.1% aqueous solution of chlorazol black E overnight. Permanent mounts in Cytoseal 60 were generated immediately. Tissue was observed on a Leica DMLB microscope equipped with an AxioCam HRc camera by Zeiss with AxioVision Rel 4.8 image capture software. Images were processed and annotated using Adobe Photoshop CS2, Version 9.0.2.

Gene disruption and characterization.

Generation of a *TRI5* mutant was accomplished by the split marker method of gene replacement (Catlett *et. al* 2003). Two 500-700 base pair regions flanking the *TRI5* locus (one upstream region and one downstream region) were amplified from PH-1 genomic DNA. *F. graminearum* gene sequence was downloaded from the Munich information Center for Protein Sequences (mips) website at <http://mips.helmholtz-muenchen.de/genre/proj/fusarium/>. Overlapping 5' and 3' segments of the hygromycin resistance cassette *hph* were amplified from the plasmid pCB1004 with an attached trpC promoter and terminator, respectively (Carroll *et al.* 1994). The reverse primer of the upstream flanking region and the forward primer of the 5' *hph* amplicon both contained the novel overlap sequence

5'-GTCGACGACAACTACCATCGATCTGACG-3'. Likewise, the forward primer of the downstream flanking region and the reverse primer of the 3' *hph* amplicon contained the novel overlap sequence 5'-ACACTGGTGACGGCTAACCAGAACTGTCA-3'. These overlapping sequences permitted fusion of the upstream flanking region with the 5' *hph* amplicon and the downstream flanking region with the 3' *hph* amplicon by overlap extension PCR (also known as fusion PCR). This produced the two split-marker constructs necessary for gene replacement of the *TRI5* locus with *hph* and its *trpC* promoter and terminator. Protoplasting and PEG-mediated transformation were carried out as previously described (Hallen-Adams *et al.* 2011) with hygromycin selection of putative transformants. Putative transformants were transferred to V8 agar containing hygromycin B at a concentration of 3.183g/L. Single spore isolates of transformants able to readily colonize the hygromycin containing plates were checked by PCR with the forward and reverse primers, 5'-GGT CTC TCT TCA CGA CTG TCT GGT TG-3' and 5'-CCT GAC TGC AAA GCT GTG GAC CAT C-3' respectively, in order to confirm loss of the *TRI5* sequence.

RESULTS

Interactions of *F. graminearum* with trichomes.

Conidia of *F. graminearum* accumulated on macro- and micro-trichomes of spray-inoculated plants and inoculated detached florets (Fig. 3 A). However, there were some differences between the two treatments in the placement of the accumulated conidia. Sprayed drops rolled across the surface of the bracts of spray inoculated plants, causing the conidia to become trapped by the trichomes. Spray inoculations were previously stated as best representing field conditions (MacCallum and Tekauz 2002). In the detached floret assay conidia settled to the bottom of the single droplet placed on the floret surface. In the detached floret assay conidia came into contact with trichomes and interactions were observed, but conidia were not observed to accumulate in association with trichomes.

Germinating conidia came into contact with micro-trichomes on the bract surface, and the larger more densely clustered macro-trichomes covering the vascular bundles of the floret (Fig. 1). Hyphal penetration into trichome cells occurred by two mechanisms. First, hyphae formed bulbous cells on the surface of the trichomes, and then formed “arms” that wrapped around the trichome. Penetration hyphae emerged from the arms and penetrated the cell margin at the base of the trichome (Fig. 3B; Fig. 4), growing into the intercellular spaces. Conidia in direct contact with trichomes also germinated to form bulbous hyphae and arms (Fig 3 B and C). Infecting hyphae grew between trichomes and adjoining cells (Fig. 4A and D) or directly through the trichome cell wall (Fig. 4 C). *F. graminearum* responded to macro-trichomes on the vascular bundles and to micro-trichomes similarly, however, hyper-branching of hyphae occurred exclusively on the macro-trichomes above vascular bundles where hyphae completely enveloped trichomes by 5dpi (Fig.3 C). Hyphae that had interacted with either type of trichome also

continued to grow across the bract surface, beyond the trichome to interact with additional trichomes (Fig. 3 B) or to penetrate the bract margin. In several instances, attachment to one macro-trichome was followed by hyphal branching and penetration of a neighboring macro-trichome (Figs. 3B). Interactions of *F. graminearum* with barley varieties Stander and Quest were indistinguishable.

Penetrating hyphae initiated the colonization of internal tissue (Fig. 4 A, B and D). Subcuticular hyphae grew through the vascular bundles and developed sporodochia in the sub-stomatal cavities (Fig. 5). Colonization of the hypodermis, the first layer of cells beneath the epidermis was possible through the bottom of trichome cells. Once within the hypodermis the hyphae grew through intercellular spaces and colonized the vasculature (Fig. 4C). Surface interactions continued to center around trichomes with the accumulation of hyphae at the tips of both infected macro and micro-trichomes which also served as the location of lesion initiation (Fig 3C).

Hyphal interactions with trichomes were first observed at 2 dpi and at the leading edge of the infection front. Penetration of the trichomes was not observed until 3 dpi, although growth to the bract margin between trichomes and their neighboring cells was observed at 2 dpi. Hyphal branching on trichomes and growth towards the bract margins was observed at 4 dpi. Bulbous infecting hyphae were observed on trichomes from 2-4 dpi with most occurring from 3-4 dpi which coincided with lesion formation at trichomes. Lesion formation was first observed on trichomes as a change from their usual transparent appearance to a reddish-brown discoloration. Sporodochia began to emerge from stomates at 5 dpi, however, conidia were not mature until 6 dpi. A difference in the amount of hyphae colonizing the surface of Quest and Stander was observed. The resistant variety, Quest, was more mature at emergence from the flagleaf sheath

than the susceptible variety Stander. Although this observation was not quantified, as it was not an intended component of the original investigation, the observation led to the subsequent investigation into the impact of the developmental stage of the grain on the colonization of the floret post establishment.

For generation of the *tri5* mutant three putative transformants were selected for normal mycelial growth in the presence of hygromycin. One of those was confirmed to lack a functional *TRI5* gene PCR. Interactions of the *tri5* mutant with Stander barley showed no difference in surface interactions and early colonization as compared to the wild-type, however, as the infection developed, lesions did not form in the barley inoculated with the *tri5* mutant by 5 dpi, as was characteristic of the wild-type (results not shown). Growth to trichome tips and hyphal branching with growth towards trichome cell margins was observed by 3 dpi.

Location of perithecia emergence on barley florets.

The detached floret assay (Fig. 2) was also used to observe disease spread and perithecium formation across a time-course of floret developmental. Inoculation of florets at the earlier developmental stage when the bract margins are open resulted in mycelial growth on the surface of the lemma and palea, and growth to the margins, in contrast to inoculation of florets at later developmental stages with closed margins where surface mycelial growth was not observed. Lesions did not form at 3 dpi on 48 of 136 (35%) of florets with margins closed. All of the 136 florets with margins open exhibited lesions by 3 dpi. By 3 dpi, tissue necrosis on florets with margins closed was much less severe than in florets with open margins and many of the seeds from the florets with margins closed remained viable at maturity, as indicated by green shoots emerging from the florets in the days immediately following the end of the assay. Due to the loss of green color on florets lesions were no longer evident at perithecia emergence. For this

reason the asymptomatic florets were marked with a pen and the subsequent emergence of perithecia confirmed successful infection of those florets. Observations using microscopy showed that the trichome interactions were present on plants inoculated at all stages investigated and no difference was observed in the nature of these interactions.

In florets with margins closed perithecia emerged in rows along the vascular bundles, and intermittently on the rest of the palea surface (Fig. 6). These results indicated that the fungus spread from the inoculation site. Conversely, in florets with margins open, infections leading to perithecia formation did not spread beyond the location of the inoculation droplet (Fig. 6). Quantification of the difference between the location of perithecia emergence on the palea of florets inoculated at early and late developmental stages revealed significant differences in the distribution when subjected to ANOVA (Fig. 7). Although total perithecia did not differ between the two treatments, there was a significant reduction in the number of perithecia emerging from the palea surface ($F=108.97$, $p = 4.92 \times 10^{-9}$) (Fig. 7 A) and an increase in the proportion emerging away from the point of inoculation in the florets with margins closed ($F=26.04$, $p= 1.12 \times 10^{-6}$) (Fig. 8). Perithecia emerging away from the inoculation point did so almost exclusively on vascular bundle tissue in late stage inoculations ($F=51.89$, $p=2.26 \times 10^{-11}$) (Fig. 8). In comparing the number of perithecia emerging on the palea surface as opposed to the vascular bundles there was no correction for the difference in surface area that exists between the two regions on the palea. Although perithecia emerged only from or near stomates on the vascular bundles perithecia emergence on remainder of the palea surface could be linked to any cell-type. In cross-section perithecial initials were observed in aleurone cells and mature perithecia emerged directly from this tissue.

Role of silica in aurofusarin production and changes in hyphal morphology.

Specific interactions of hyphae with trichomes, which contain silica, and previous reports of sporulation in association with silica cells (Guenther and Trail, 2005) prompted exploration of the effect of mono-silicic acid on hyphal growth and development. Silica-specific methyl red staining of sectioned florets confirmed the location of tissues previously described in other grasses for their silicon dioxide content. Tissues that stained the darkest red (indicating the highest silica concentrations) were the hypodermis and the xylem vessels (results not shown). Trichomes and cells in the testa near the aleurone also stained red, but were much lighter (results not shown). The epidermis and cuticle were destroyed in the sulfuric acid treatment required for the silica specific staining and thus could not be observed in sections. Stained dermal peels, however, revealed pink colored trichomes with an epidermis that remained unstained.

The effect of silicic acid and silicon dioxide on growth of *F. graminearum* in culture on Czapek-Dox agar was examined to determine its potential role in growth morphology. Accumulation of rubrofusarin (Fig. 9A and B) and the production of aurofusarin (Fig. 9C) was induced and aerial hyphae were reduced (Fig. 9D) when hyphae came into contact with silicic acid. Although rubrofusarin was induced by all concentrations aurofusarin was only induced by the 2mM mono-silicic acid treatment at the 100 μ l volume. The reduction of aerial hypha occurred immediately upon contact of the advancing hyphal front with the 100 μ l volume of the 2mM silicic acid applied to the surface of the agar, and was accompanied by a reduction in the directionality of the outward growth of hyphae. No responses were induced by the silicon dioxide amended cultures. Higher levels of rubrofusarin, as indicated by deeper red, were observed in cultures grown in the dark with 200 μ l drops of 0.5 and 1 mM silicic acid than with

other treatments. Treatments buffered with 50 mM HEPES showed similar responses to those without the buffer (Fig. 9D).

FIGURES

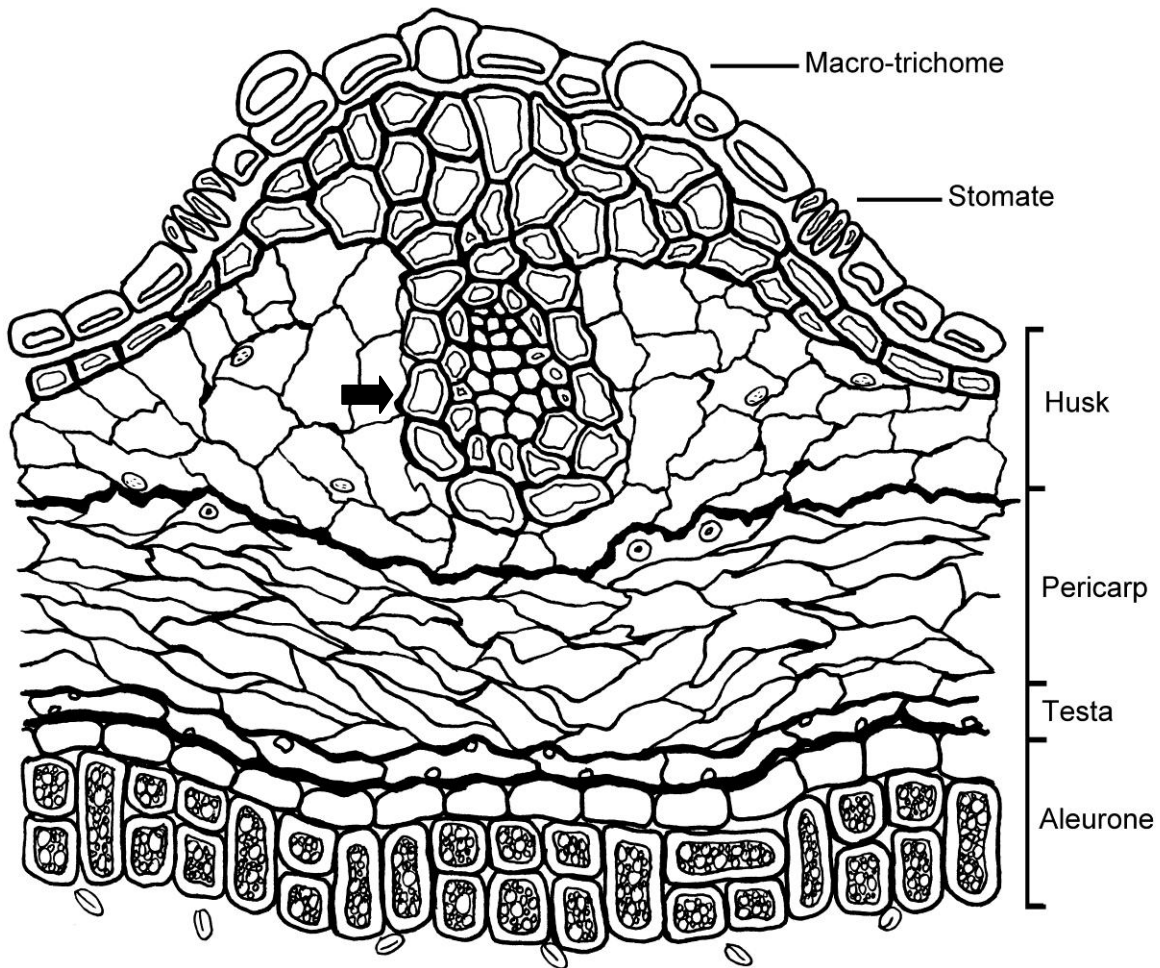


Figure 1. Anatomical drawing of cross-section showing layers of cells surrounding barley floret vascular bundle (arrow). (Top down) Husk (epidermis, stomata, and trichomes (only macro-trichomes shown), hypodermis, cross cells, and vascular bundle cells), pericarp (tube cells), seed coat (testa), nuclear tissue, and aleurone (cells filled with starch). The endosperm is internal to the aleurone and is not shown.

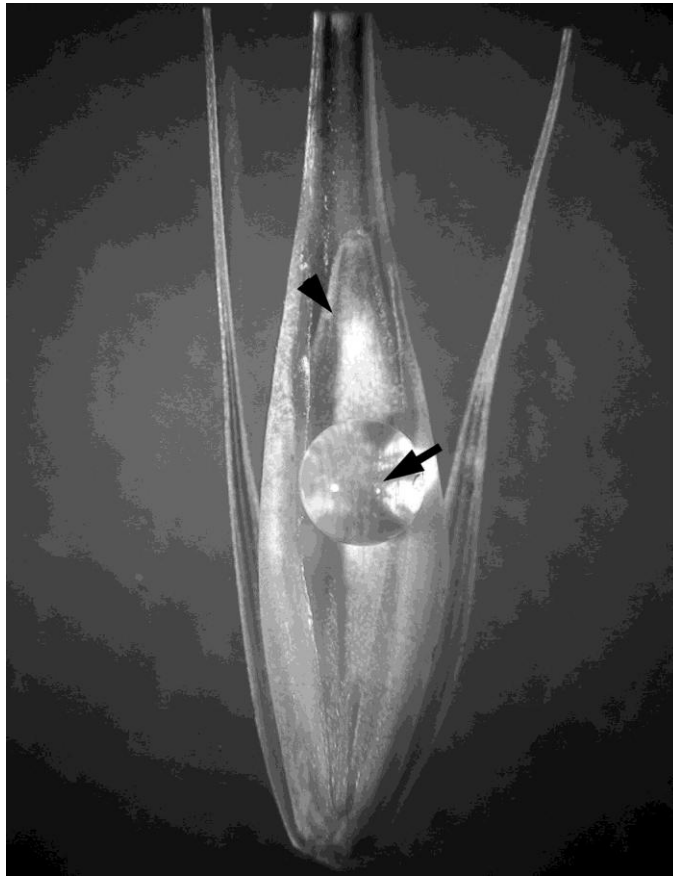


Figure 2. Detached floret assay: Adaxial view of barley floret. Drop of conidial suspension which served as inoculation point for the detached floret assay (arrow) and palea furrow delineated on each side by vascular bundles (arrow).

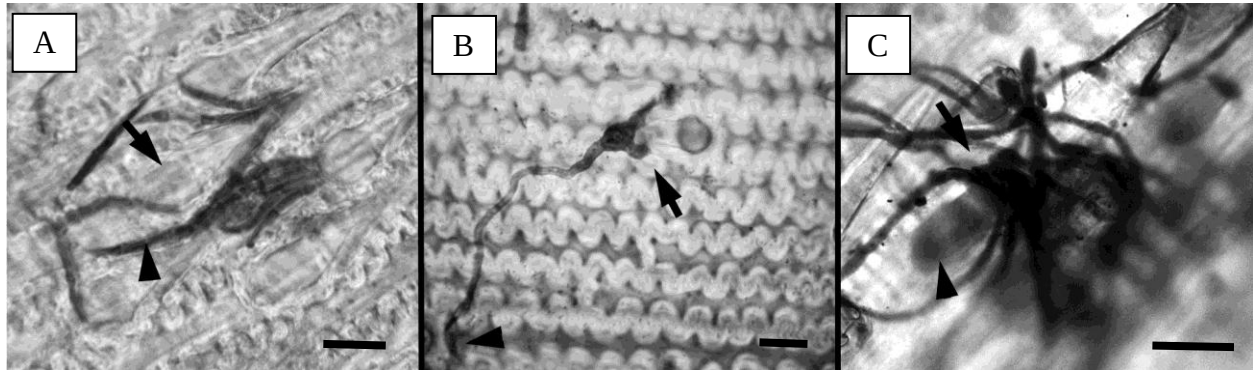


Figure 3. Early interactions of *F. graminearum* with trichomes in establishment and infection. A) Conidia trapped on micro-trichomes at 1 dpi. B) Germinating conidium on a micro-trichome with infection arm (arrow) and terminal end of hypha penetrating base of neighboring trichome (arrowhead) at 3 dpi. C) Bulbous hyphae on macro-trichome at 5dpi (arrow) and darkened trichome due to lesion initiation(arrowhead). Scale bars 20μm.

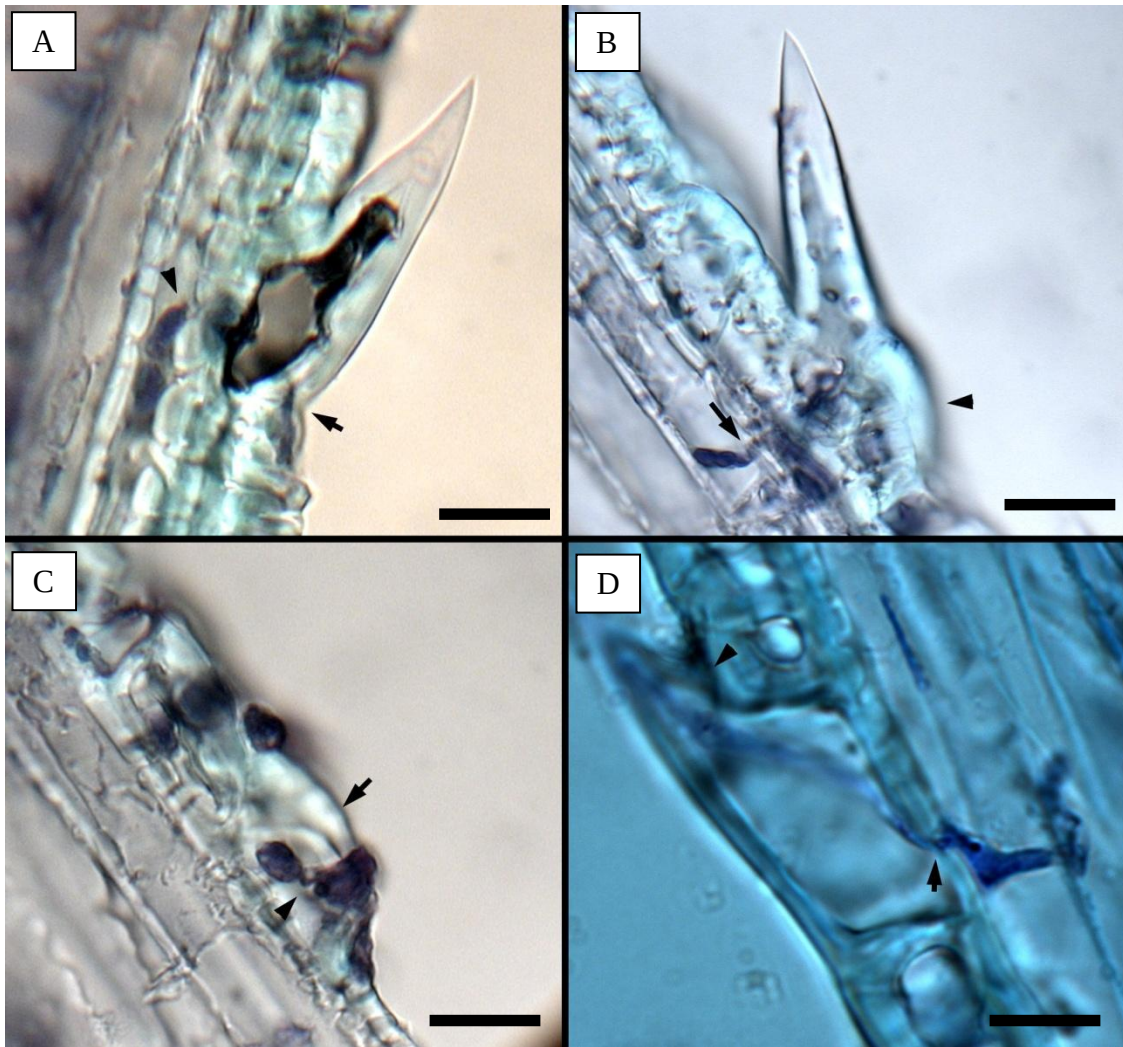


Figure 4. Penetration of trichomes by *F. graminearum*. A) Penetration of base of micro-trichome (arrow) and growth into hypodermis (arrowhead). B) Macro-trichome near bract margin with fungal growth into hypodermis (arrow). Note companion cell (arrowhead). C) Infection of companion cell from B (arrow) showing thin penetration hypha (arrowhead). D) Thin hyphae penetrating base of micro-trichome (arrow) and upper cell margin (arrowhead). Scale bar 20μm.

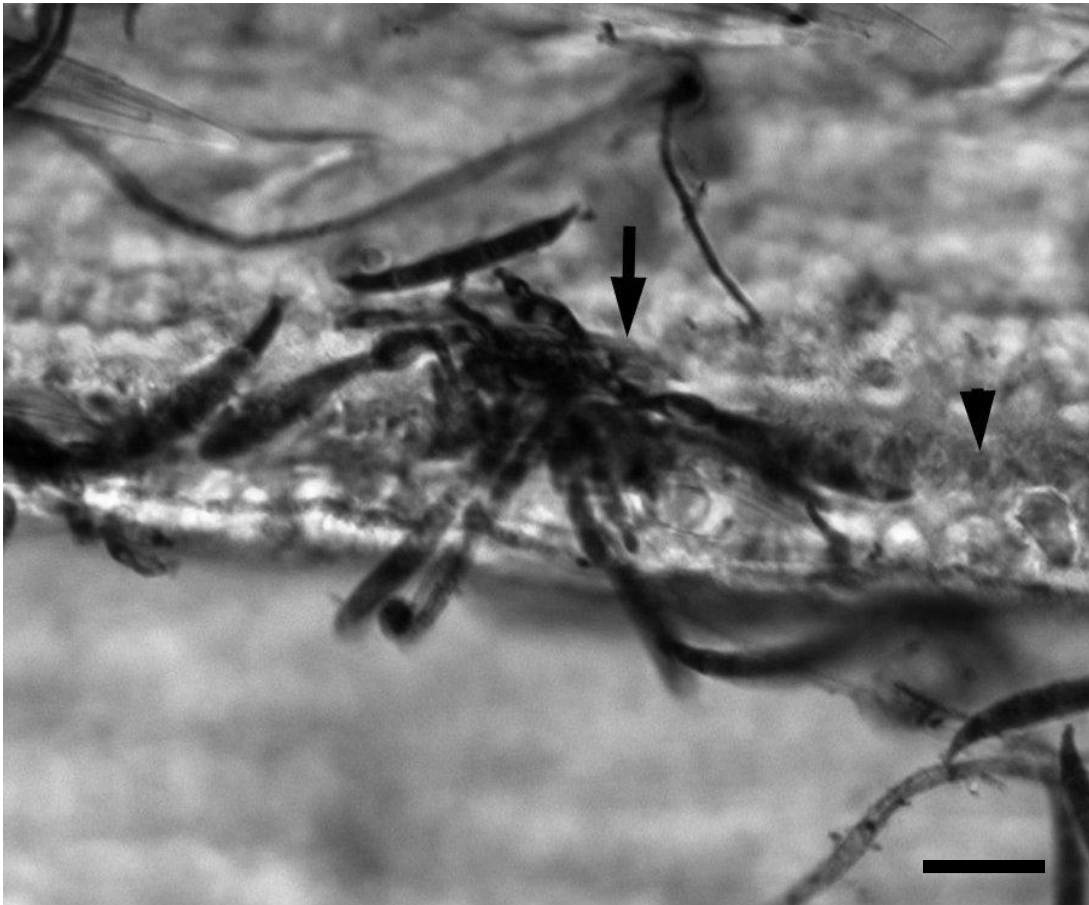


Figure 5. Sporodochia emerging from stomate on vascular bundle. Sporodochia (arrow) emerged from stomate at 6 dpi. Note hyphae in vascular bundle (arrowhead). Scale bar 20 μ m.

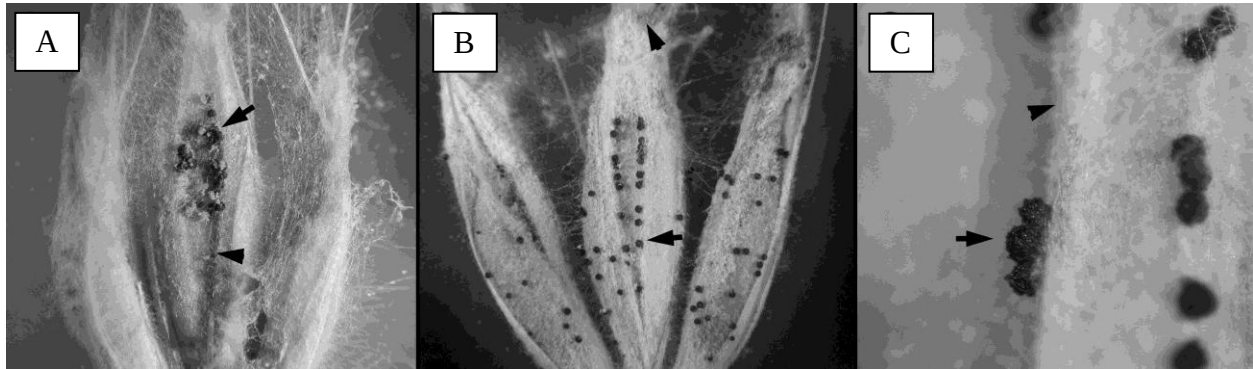


Figure 6. Perithecia emerging from surface of barley floret. A) Perithecia emerging from the location of infection on the pale of a floret infected early in development (arrow). Note vascular bundle (arrowhead). B) Perithecia emerging from vascular bundle beyond the location of infection in floret inoculated later in development (arrow) note germination of the barley kernel (arrowhead). C) Detail of perithecia (arrow) emerging from edge of vascular bundle (arrowhead). For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis.

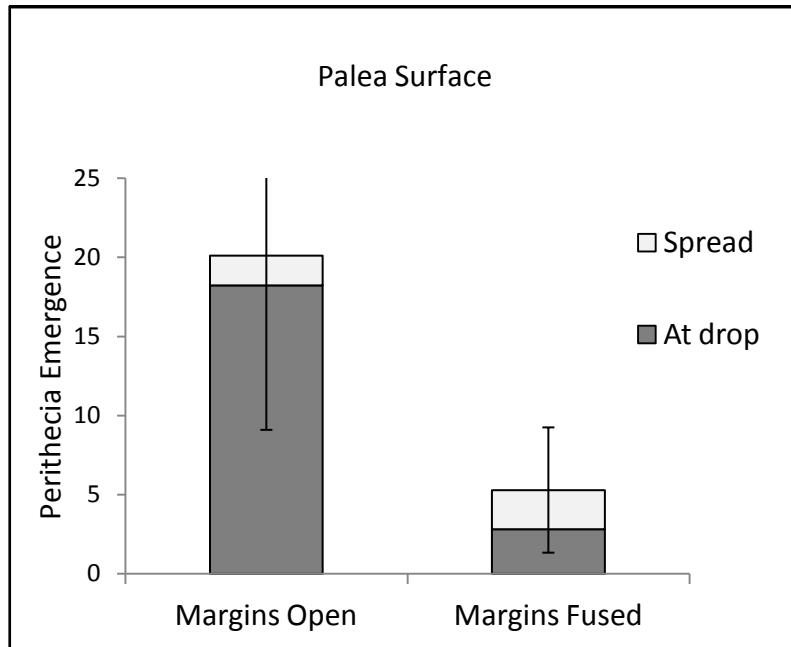


Figure 7. Effect of developmental stage at inoculation on location of perithecia emergence. Mean perithecia emergence from locations on the palea surface away from vascular bundles. The decrease in the number of perithecia emerging in this location after bract fusion was significant ($F=108.97$, $p = 4.92 \times 10^{-9}$) Error bars indicate standard deviation.

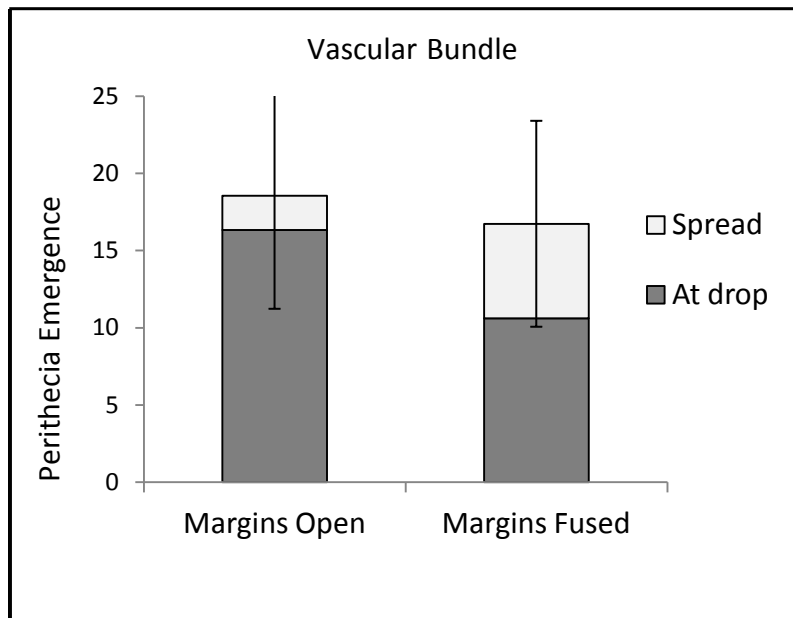


Figure 8. Effect of developmental stage at inoculation on location of perithecia emergence. Mean perithecia emergence from vascular bundle of the palea when inoculated at early and late developmental stages. The increase in the number of perithecia spread beyond the inoculation point was significant ($F=26.04$, $p= 1.12 \times 10^{-6}$) as well as the difference between the total perithecia on the palea surface vs. the vascular bundle after bract margin fusion ($F=51.89$, $p=2.26 \times 10^{-11}$). Error bars indicate standard deviation.

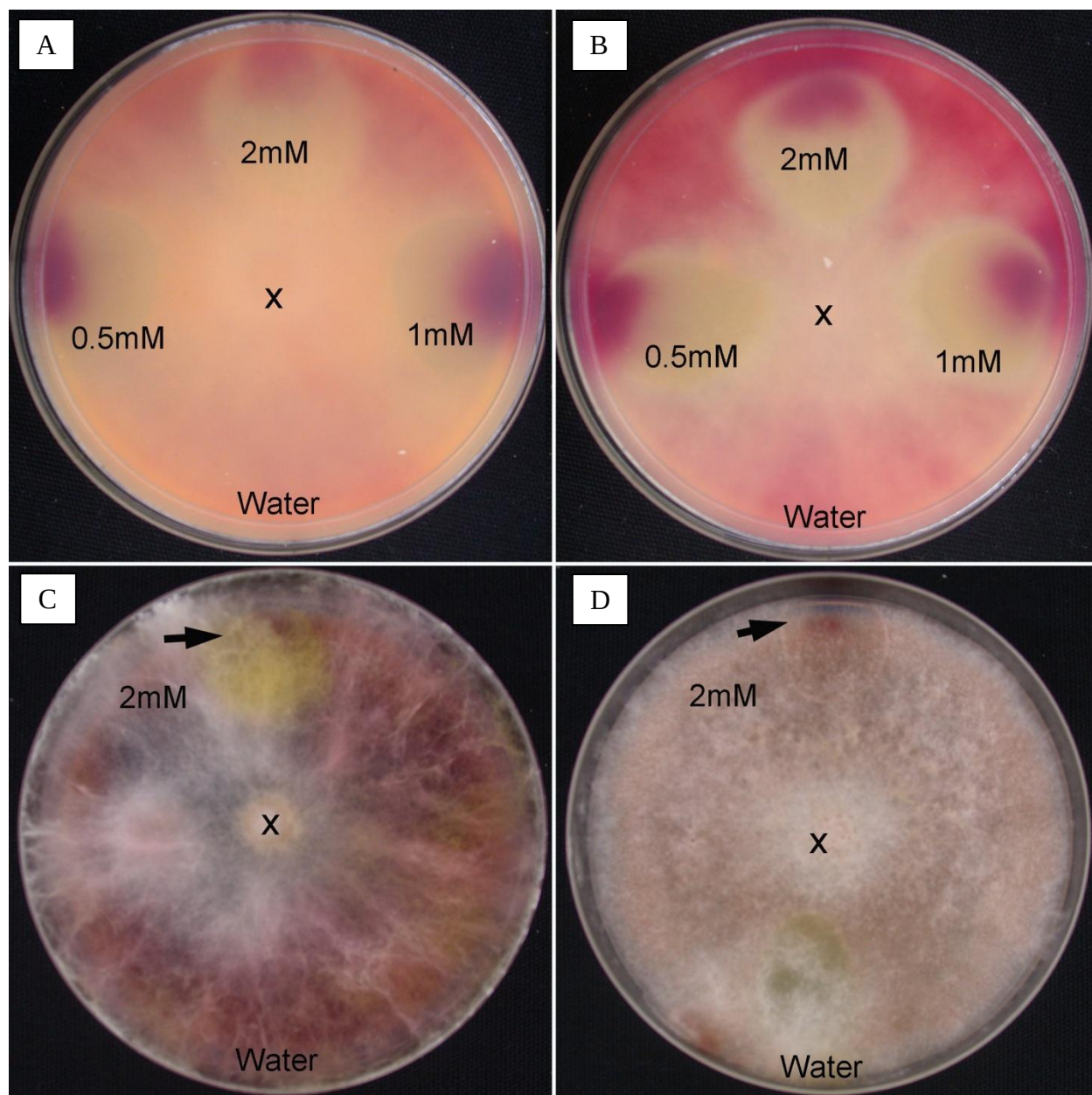


Figure 9. Impact of mono-silicic acid on *in-vitro* growth of *F. graminearum*. Mono-silicic acid emended Czapek-Dox medium induced production of rubrofusarin at the far edge of the silicic acid droplet (indicated by treatment concentration) under both light (A) and dark (B) conditions. C) Aurofusarin at location of 2mM treatment under dark conditions (arrow). D) Replicate of A buffered with 50mM HEPES. X: point of inoculation. Water indicates location of control. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis.

DISCUSSION

Our results demonstrate that the stage of grain development of barley at inoculation dictates the mode of infection by *F. graminearum*. Infection at early stages of grain development, prior to bract fusion, resulted in penetration of micro-trichomes that assisted in growth towards bract margins. These infections led to formation of perithecia localized to the initial point of infection. At later stages of grain development, after bract fusion, direct penetration of macro-trichomes led to colonization of the vasculature, and the region of perithecium development extended beyond the point of penetration. Thus, trichomes played a vital and different role in facilitating fungal ingress in early and in late stages of grain development. Previous work using *Brachypodium distachyon* showed strong evidence supporting the importance of trichomes of *F. graminearum* infections of cereal grains and suggested access to the vasculature as an outcome of those infections (Peraldi *et al.* 2011). Our study confirms this interaction in barley, and extends it by linking access to the vasculature with stages of development in the fungus important to sexual and asexual sporulation. Lewandowski *et al.* (2006) showed that infection of barley occurred predominantly via bract margins, but their study was based on early stage infections and gave no mechanism for establishment of those infections. Trichomes provide direct routes for penetration of the floral bracts at developmental stages that occur after fusion of the bract margins. Here we show a role for trichomes in establishment of conidia in those infections and suggest that hyphal interactions with trichomes could facilitate hyphal growth across the surface of the floret to the bract margins. In addition, we present evidence that silica has an inducing effect on *F. graminearum* during infection.

Vascular bundles on the lemma and palea run parallel to margins. The lemma, which is thicker than the palea, contains three vascular bundles, while the palea contains two (Fig. 1). The vascular bundles create “furrows” on the surface of the bracts, especially the palea, and these furrows, along with the bract margins, are often the location of lesion initiation in the field (Lewandowski *et al.* 2006). Trichomes located over vascular bundles are important as hyphal conduits to the endosperm (Kirby and Rymer 1974). An increase in the number of perithecia emerging in conjunction with vascular bundles in late stage infections illustrates the use of vascular bundles as conduits for spread (Fig 1).

Several reports, including this one, demonstrate specific interactions between *F. graminearum* and trichomes (this work; Jansen *et al.* 2005, Peraldi *et al.* 2011, Stephens *et al.* 2008). Interactions of trichomes with conidia begin with the trapping of conidia. The lunate conidial shape promotes trapping and the hydrophilic conidial surface facilitates their adherence to water droplets (Deacon 2006). Their shape also allows them to be trapped as water droplets roll down the plant surface and trichomes rake them from the surface of the water droplet. The formation of infection arms and hyphal branching in association with trichomes in both the wild-type and the *tri5* mutant demonstrated that surface establishment and the early stages of infection are not dependent upon DON production. For these reasons we began to investigate components of the host which could be triggering the formation of structures for the infection of trichomes.

In barley, high silica accumulation correlates with developmental stages that occur during grain maturation after fusion of the bract margins (Hayward *et al.* 1972, Kirby and Rymer 1974). In rice, vascular bundles are the conduit bringing the silicic acid to the trichomes and contain the highest concentration of non-polymerized silicic acid (Ma and Yamaji 2006). In wheat and barley, vascular bundles are an important link to the endosperm (Rittenour and Harris 2010,

Kirby and Rymer 1974) and to the rachis and colonization spreads outward to the epidermis from the vascular system (Guenther and Trail 2005). In the present study, the perithecia emergence spread further from the point of inoculation in more mature florets in both vascular bundles and palea surface although the increase was more dramatic in the vascular bundles. The differences that we found in perithecia emergence between the developmentally dependent infection pathways relate directly to the timing and location of increases in silica content of the floral bracts (Hayward *et al.* 1972). Previous work showed that perithecia emergence occurred preferentially from silica cells on stem nodes in wheat, and suggested a link between the two, which our work substantiates (Guenther and Trail 2005). All of this strongly indicates silicic acid as playing a role in infection which leads to sexual development.

Trichomes are one cell type among many that are sinks of mono-silicic acid and accumulate silicon dioxide in the form of biogenic silica in grasses. This also occurs in macro-trichomes of the vascular bundle, micro-trichomes of the epidermal surface, papillae, bulliform cells, xylem vessels, and certain intercellular locations (Ma and Yamaji 2006, Hayward *et al.* 1972, Kaufman *et al.* 1985, Parry *et al.* 1984, and Sangster *et al.* 1983). These cell types all have been implicated in *Fusarium graminearum* infection pathways in graminaceous species (Guenther and Trail 2005, Boenisch and Schafer 2011, Jansen *et al.* 2005, Maier *et al.* 2006, Peraldi *et al.* 2011, Rittenour and Harris 2010, Walter *et al.* 2009, Lewandowski *et al.* 2006, Ma and Yamaji 2006, and Nicholson 2009). More specifically, papillae were implicated as sites of hyphal differentiation, penetration and formation of toxin producing infection structures in wheat (Boenisch and Schafer 2011; Rittenour and Harris 2010).

Guenther and Trail (2005) reported that perithecia initials formed beneath silica cells and stomates on the wheat stalk. Since perithecia require light to develop, it was suggested that light

passing through the stomates and silica cells triggered the initials to form. However, in one study, the amount of light transmitted through cells containing silica aggregates was found to be less than the amount of light transmitted through cells, which do not contain silica aggregates (Kaufman *et al.* 1985). Our results showing a morphological effect of silicic acid on *F. graminearum* amended media that included secretion of rubrofusarin and morphological changes in the hyphae. These observations support the idea of chemical sensing of silica by the fungus. A buffered silicic acid solution elicited the same response indicating that mono-silicic acid and not changes in pH were responsible for this response. The initiation of the sexual cycle is accompanied by secretion of rubrofusarin in culture (Trail and Common 2000). Thus, it is possible that the presence of silicic acid in the stomates and silica cells trigger sexual differentiation. Investigation of this possibility is now in progress.

We demonstrated the importance of trichomes in later stages of plant and disease development in the ability of *F. graminearum* to overcome type II resistance in barley. This infection route occurs on trichomes of more mature florets, which would occur later in the season. Other work has suggested infection of trichomes of the rachis (Jansen *et al.* 2005). Trichomes in this location would also serve as a route for *F. graminearum* to overcome type II resistance in barley. Asymptomatic infections late in grain development could be a source of mycotoxin and fungal contamination nearly impossible to visually assess in harvested grains. Late stage infections can be caused by conidia from sporodochia that emerge from stomata at ~6 dpi on the vascular bundle of infected florets (Lewandowski *et al.* 2006, this work) or from airborne inoculum. If field conditions are conducive, a single symptomatic floret could be the source of inoculum for several neighboring asymptomatic kernels, yet have significant fungal colonization of the bracts. We show that 35% of florets did not exhibit symptoms at 3 dpi and

whose endosperm was not damaged substantially enough to prevent germination, despite perithecia formation. Since the fungus cannot spread through the rachis node of barley (Jansen *et al.* 2005), trichomes remain a significant entry route to late stage asymptomatic infections. This infection pathway could lead to low visual estimations of disease in the field when humidity and wind conditions are conducive to the spread and establishment of inoculum both early and late in the season. This demonstrates the importance of testing for mycotoxin accumulation in barley even when visual disease estimates are low.

CONCLUSIONS AND FUTURE DIRECTIONS

Plant pathogens develop elaborate sensing mechanisms to respond to their host. *F. graminearum* exhibits highly specific responses to its host and its widespread success as a pathogen reflect these adaptations. The results presented here demonstrate trichome-specific interactions between the fungus and the host, the presence of these interactions in a DON mutant, the potential role of mono-silicic acid in these interactions, and the impact of developmental stage at inoculation on the spread of *F. graminearum* and the emergence of perithecia. Silica deposits are important components of the cell walls of grasses. Characterization of other cell-wall components of barley epidermal cells may lead to identification of other specific interactions with *F. graminearum*, including other triggers for sexual and asexual sporulation, and mycotoxin production.

Future work elucidating the nature of *F. graminearum* host sensing and its potential role in sexual development would greatly enhance our understanding of the epidemiology of this disease. A better understanding of infection and colonization mechanisms leading to sporulation would enhance our ability to prevent the establishment of the pathogen and thus dramatically reduce mycotoxin levels in the finished grain.

APPENDIX A

Introduction

Surface interactions between plant pathogens and their hosts can be difficult to visualize. Attempting to correlate the expression of specific genes with these events can prove even more difficult. To visualize the expression of *TRI5*, encoding the trichodiene synthase that catalyzes the first step in the deoxynivalenol (DON) biosynthesis pathway, in the infection of barley by *F. graminearum*, the coding sequence for Yellow-Fluorescent-Protein (YFP; Hastings 1996) was attached to the 3' end of *TRI5*. The goal of this tagging was to have expression of *YFP* coincide with expression of *TRI5*, so that the role of DON could be elucidated at various time-points in the infection pathway.

Methods

Primers were generated for the amplification of *TRI5*. The forward primer 5'-CTA GAA CTA GTG GAT CCC CCG GGC T-3' and reverse 5'- GCC TCC GCC TCC GCC TCC GCC GCC TCC GCC CTC CAC TAG CTC AAT TGA ACT TAG GAT GG -3', with overlap sequence for the 10x glycine linker, resulted in successful amplification. Primers used amplified the entire gene and the reverse contained sequence overlap with sequence overlap of a YFP-*hph* (YFP-HYG) amplified from a previously generated strain in the trail lab labeled M1YFP with the forward primer 5'-- GGC GGA GGC GGC GGA GGC GGA GGC GGA GGC ATG GTG AGC AAG GGC GAG GAG C --3' and a reverse primer previously generated, 5'-GAT CTT CAG CAT CTT TTA CTT TCA CCA GCG TT-3'HYG R. *Hph* encodes the hygromycin phosphotransferase gene from *E. coli*, conferring hygromycin resistance to transformed strain. The YFP-HYG sequence fragment was amplified with primers to generate sequence overlap with the end of a 10X glycine linker sequence and sequence downstream of *TRI5*. The 10X glycine

linker overlap sequences between the *TRI5* and YFP-HYG sequence were designed to fully overlap one another. The downstream sequence of the gene was amplified with a forward primer with overlap with the hygromycin resistance gene, 5'-CAC CAG CGT TTG GTA CGG ATA CTC GTT-3'', and the reverse primer 5'-CAT CAA CAG GCT CGT CTC TCG GAC-3'.

A merge reaction between the YFP-HYG and the *TRI5* downstream sequence was performed and the merged product was purified using agarose gel electrophoresis. A yeast transformation using the *TRI5* upstream sequence and the merged YFP-HYG-*TRI5* downstream sequence allowed them to combine via homologous recombination within competent yeast cells produced following the protocol for the Frozen-EZ Yeast Transformation II (www.zymoresearch.com). The final construct was ordered as -*TRI5*-10XGLY linker-YFP-HYG-*TRI5*. The 10XGLY linker was designed to physically separate the proteins so that each would fold and function properly. Transformants were confirmed by PCR amplification using the forward primer from the original *TRI5* amplification and the reverse primer from the sequence downstream of *TRI5*. DNA amplified from the yeast transformants was ligated into *E. coli* plasmid pCB1004 (Carroll *et al.* 1994) and transformed into *E. coli*. *E. coli* transformants were selected using carbenicillin on Luria Broth solidified with 1.5% agar. The resulting plasmid, containing the *TRI5* gene fused at the 3' end with YFP, was used to transform *F. graminearum*. Protoplasting and PEG-mediated transformation were carried out as previously described (Hallen-Adams *et al.* 2011) with hygromycin selection of putative transformants on V8 agar containing hygromycin B at a concentration of 3.183g/L.

From two transformation experiments, 7 transformants were obtained and 2 were confirmed with PCR amplification and check primers from outside of the previously amplified *TRI5* region, and the transformants were labeled T1 and T5. The forward and reverse check

primers used were 5'-GGT CTC TCT TCA CGA CTG TCT GGT TG-3' and 5'-CCT GAC TGC AAA GCT GTG GAC CAT C-3' respectively. T1 and PH-1 were grown in a modified Czapek's DON inducing medium (Jaio *et al.* 2008). Expression of YFP was examined using a Leica 339 DMR fluorescent microscope and T1 showed higher fluorescence with the standard peaks for YFP of 527nm for emission and 515nm for excitation. Fluorescence of vesicles within growing hyphal tips 5 days after inoculation of the medium confirmed function of the tag although the strength varied between the strains. No fluorescence was observed during examination of similarly grown PH-1 hyphae observed in the same manner. Attempts at observing surface interactions with barley were not successful due to auto-fluorescence of the plant material. A GFP-tagged strain may work for detection of expression in surface interactions, however, based on the confirmation of the surface interactions with trichomes in the *tri5* mutant from this work. The expression of *TRI5* is likely not important at this stage of infection so that would not be a useful as more advanced stages of infection would run into the auto-fluorescence and the small size of the expressing vesicles as problems. However, the use of more advanced microscopic techniques able to differentiate the fluorescent signals of the fungus from those of the plant may be able to overcome those difficulties.

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