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# An *in vitro* method for the analysis of infection-related morphogenesis in *Fusarium graminearum*

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## SUMMARY

*Fusarium graminearum* is a significant pathogen of many cereal crops. With its genetic tractability, ease of culture, genome sequence availability and economic significance, *F. graminearum* has become the subject of intensive molecular research. Although molecular tools have been developed to enhance research into virulence determinants of *F. graminearum*, simple assays for infection-related development are lacking. As such, the objective of this study was to develop an *in vitro* protocol for the analysis of infection-related morphogenesis in *F. graminearum*. We demonstrate that two morphologically distinct hyphal structures are produced by *F. graminearum* during the invasion of detached wheat glumes: subcuticular hyphae and bulbous infection hyphae. Specialized wheat epidermal cells (papillae) appear to act as sites of invasion by *F. graminearum* on the adaxial side of detached wheat glumes. In addition, the development of bulbous infection hyphae is dependent on the pathogenicity mitogen-activated protein kinase Gpmk1, further supporting the infection-related nature of these structures. This relatively simple assay will contribute to the tractability of the *F. graminearum* system and help to uncover molecular requirements for infection-related development.

## INTRODUCTION

*Fusarium graminearum* (teleomorph *Gibberella zeae*) is a significant pathogen to a number of plant species, but it is most notorious as the main causal agent of *Fusarium* head blight (FHB) of wheat and barley (Goswami and Kistler, 2004; McMullen *et al*., 1997). Heads infected by *F. graminearum* produce less grain, and the grain that is produced usually harbours the mycotoxin deoxynivalenol (DON). This mycotoxin causes adverse effects in humans and animals, and contaminated grain is often unmarketable (Dexter and Nowicki, 2003). The relatively recent re-emergence of this disease in the USA has prompted a renewed interest in the epidemiology and molecular biology of *F. graminearum* (McMullen *et al*., 1997).

Unlike many plant pathogenic fungi, *F. graminearum* invades host tissue without generating an appressorium (dome-shaped infection structure used to penetrate host tissues). Instead, this pathogen tends to rely on natural openings (mainly stomata) and direct penetration for access to internal tissues. The abaxial side of glumes contains stomatal rows that are exploited by *F. graminearum* and closely related *F. culmorum* (Kang and Buchenauer, 2000; Pritsch *et al*., 2000). Interestingly, the fungus also produces subcuticular coral-like hyphal mats on both glumes and ovarian tissue, although the role and significance of these structures are unknown (Jansen *et al*., 2005; Kang and Buchenauer, 2000; Pritsch *et al*., 2000). Perhaps most importantly, *F. graminearum* can readily invade young anthers and ramify through the filament to the ovary. The ovary is then invaded and provides access, through vascular tissue, to the rachis (Pugh *et al*., 1933). From the rachis, *F. graminearum* then spreads to other florets, thereby exacerbating the disease. Once the fungus gains access to internal tissue, it appears to exploit pits for intracellular travel (Guenther and Trail, 2005; Jansen *et al*., 2005). At these junctions, the hyphae of *F. graminearum* become uncharacteristically narrow in order to achieve passage. Despite the eloquent microscopy studies that have been conducted, there are still some unanswered questions regarding host invasion by *F. graminearum*. For example, over 20 genes have been implicated in the virulence/pathogenicity of *F. graminearum*; however, with a few exceptions (Ding *et al*., 2009; Jansen *et al*., 2005), microscopic observations of such mutants are often absent.

As part of the renewed interest in *F. graminearum*, several genomic tools have recently become available to investigate virulence on a molecular scale. The genome has been sequenced and is publicly available (http://www.broad.mit.edu; Cuomo *et al*., 2007). Also, an Affymetrix chip has been generated and used to study gene expression under several growth conditions (Ding *et al*., 2009; Guldener *et al*., 2006; Hallen *et al*., 2007; Seong *et al*., 2008). However, an *in vitro* protocol for the induction of infectious-related development is lacking. Such methods

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have been used to determine the molecular requirements of pathogenicity in several other plant pathogenic fungi, particularly in the rice blast fungus *Magnaporthe oryzae* (Takano *et al*., 2003). As such, the objective of this study was to generate a protocol that induces *F. graminearum* infectious-related development *in vitro*. We describe a relatively quick and easy method for the induction of both subcuticular and bulbous infection hyphae on detached wheat glumes. This method will complement many of the molecular tools that are currently available, and help to advance our knowledge of *F. graminearum* infection.

## RESULTS

Initially, several plant tissues were assessed for *F. graminearum* infection, including onion epidermis, wheat lemmas, paleas and glumes. Each tissue was inoculated with *F. graminearum* macroconidia and incubated for a given amount of time. Samples were then stained with various compounds to highlight fungal hyphae. Of the tissues tested, macroconidia inoculated on wheat glumes consistently developed morphologically distinct structures (see below). Accordingly, subsequent analyses focused on inoculated glumes.

### Morphologically distinct hyphal structures

At the earliest time points (24 h), wide, coral-shaped hyphae were observed, particularly when glumes were inoculated on the adaxial side (Fig. 1b,c). On the abaxial side, stomata were regularly invaded by hyphae, but subcuticular hyphae were not as prevalent compared with glumes inoculated on the adaxial side (Fig. 1a). At later time points (48 and 72 h), hyphae were observed on focal planes lower than those of surface hyphae (Fig. 1d–f). Interestingly, these hyphae appeared to be morphologically distinct from surface hyphae, as they were wider and more bulbous in appearance (Fig. 1e,f). These wide hyphae probably represent the dikaryotic state described by Guenther and Trail (2005) in wheat stem tissue. In addition, these hyphae appeared to modify their bulbous morphology to travel through

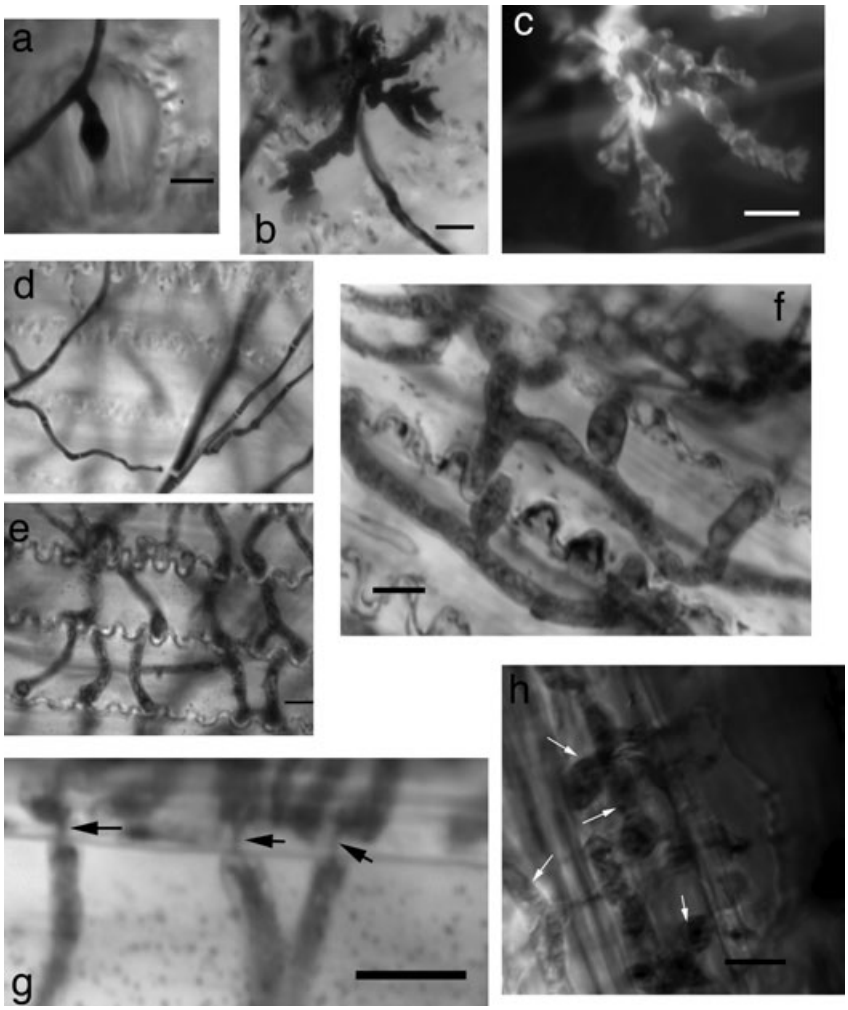


**Fig. 1** Morphologically distinct hyphal structures produced *in vitro* on wheat glumes. (a) Hyphae entering through stomata when inoculated on the abaxial side of a glume; 24 h post-inoculation (hpi). (b, c) Coral-like hyphae stained with lactophenol blue (b) or calcofluor white (c). Inoculated on adaxial side; 24 hpi. (d) Hyphae on the adaxial surface of glumes. (e) Same field and scale as (d) but lower focal plane showing bulbous infection hyphae that appear morphologically distinct from surface hyphae; 72 hpi. (f) Wide hyphae growing within epidermal cells. Inoculated on abaxial side; 48 hpi. (g) Narrowing of hyphae travelling between cells through pits (arrows); 72 hpi. (h) Wide hyphae growing within rachis tissue, 2 weeks after inoculation with wild-type strain PH-1. Scale bar, 10 μm in all panels.

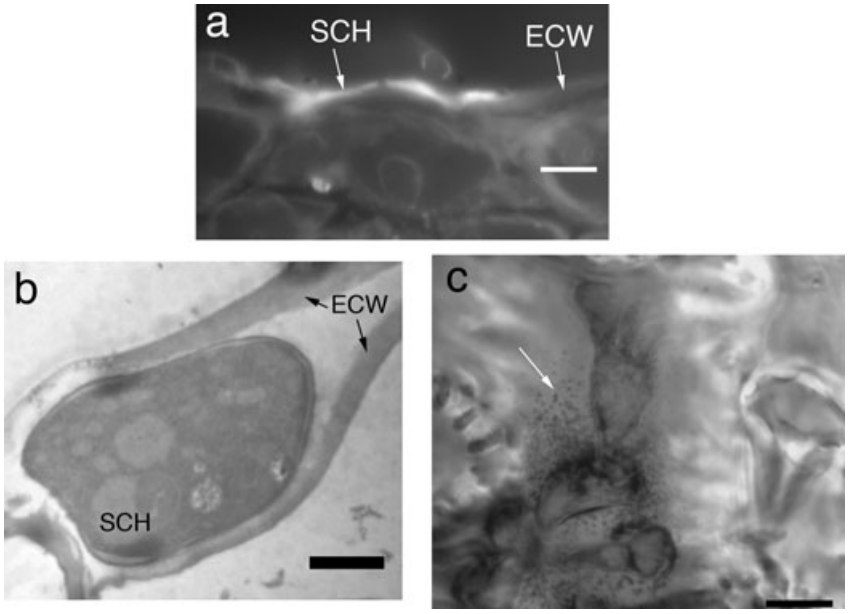


**Fig. 2** Subcuticular location of hyphae. (a) Cross-section of glume on adaxial side at 24 h post-inoculation (hpi). Hyphae stained with calcofluor white. (b) Transmission electron microscopy image of subcuticular hyphae. Scale bar, 2 μm. (c) Deteriorating subcuticular hyphae at 48 hpi. Note the heavy vacuolization and cell wall fragmentation (white arrow). Hyphae in (c) stained with trypan blue. Scale bar, 10 μm for (a, c). ECW, epidermal cell wall; SCH, subcuticular hyphae.

pits between epidermal cells, which has previously been documented *in planta* (Fig. 1g; Guenther and Trail, 2005; Jansen *et al*., 2005). In order to further confirm the *in planta* relevance of these wide hyphae, we inoculated wheat plants and observed the hyphae within the rachis (Fig. 1h). These data suggest that *F. graminearum* produces morphologically distinct hyphal structures when invading detached wheat glumes, and that these hyphal structures resemble those previously reported *in planta*.

### Subcuticular hyphae

The coral-like hyphal structures observed (Fig. 1b,c) have been reported as subcuticular hyphae (Pritsch *et al*., 2000). In order to assess whether or not these structures are growing in the subcuticular space (i.e. in the apoplastic space above the epidermal cell wall), cross-sections were taken and stained with calcofluor white. In addition, transmission electron microscopy (TEM) was used to assess subcuticular growth. Both methods provided evidence that subcuticular growth occurs on detached wheat glumes (Fig. 2a,b). At later time points [48 and 72 h post-inoculation (hpi)], the integrity of many subcuticular hyphae appeared to be compromised (Fig. 2c). Accordingly, deteriorating subcuticular hyphae were observed in 0/10, 6/10 and 10/10 inoculated glumes at 24, 48 and 72 hpi, respectively.

### Papillae-associated invasion

Whilst observing the adaxial side of inoculated glumes, most of the subcuticular and invasive bulbous hyphae appeared to be associated with circular structures surrounded by pits. On further investigation, these were identified as 'papillae', also referred to as short basal hair cells/prickle hairs (Bushnell *et al*., 2003; Tubb *et al*., 1993; Urban *et al*., 2003). Both light and electron microscopy showed that *F. graminearum* readily invaded these epidermal cells (Fig. 3). In addition, hyphae appeared to ramify through the pits at the bases of the papillae, providing an entryway to internal cells (Fig. 3b). Papillae-associated invasion was also seen on the glumes of living wheat heads, although the occurrence was drastically reduced compared with detached wheat glumes (Fig. 3e; data not shown). These data suggest that papillae act as an infection court for *F. graminearum* on wheat glumes.

### Analysis of *F. graminearum* mutants for their ability to differentiate infectious hyphae

Different *F. graminearum* pathogenicity mutants were tested for their ability to form the morphological structures observed in this study. These included the mitogen-activated protein kinase (MAPK) mutants *Δgpmk1* (Jenczmionka *et al*., 2003; Urban *et al*., 2003) and *Δmgv1* (Hou *et al*., 2002), and also the GTPase *Δras2* (Bluhm *et al*., 2007). The *Δmgv1* and *Δras2* mutants were able to form both subcuticular hyphae and bulbous invasive hyphae that were indistinguishable from those of the wild-type strain PH-1 (Table 1; Fig. 4). This similarity in glume invasion was evident despite the fact that the growth of these two mutants on artificial media was greatly reduced (Bluhm *et al*., 2007; Hou *et al*., 2002).

In contrast with the *Δras2* and *Δmgv1* mutants, *Δgpmk1* mutants formed large hyphal masses at the surface of the glume as opposed to invasive growth. The subcuticular hyphae were not as abundant on *Δgpmk1*-inoculated glumes, although they

 

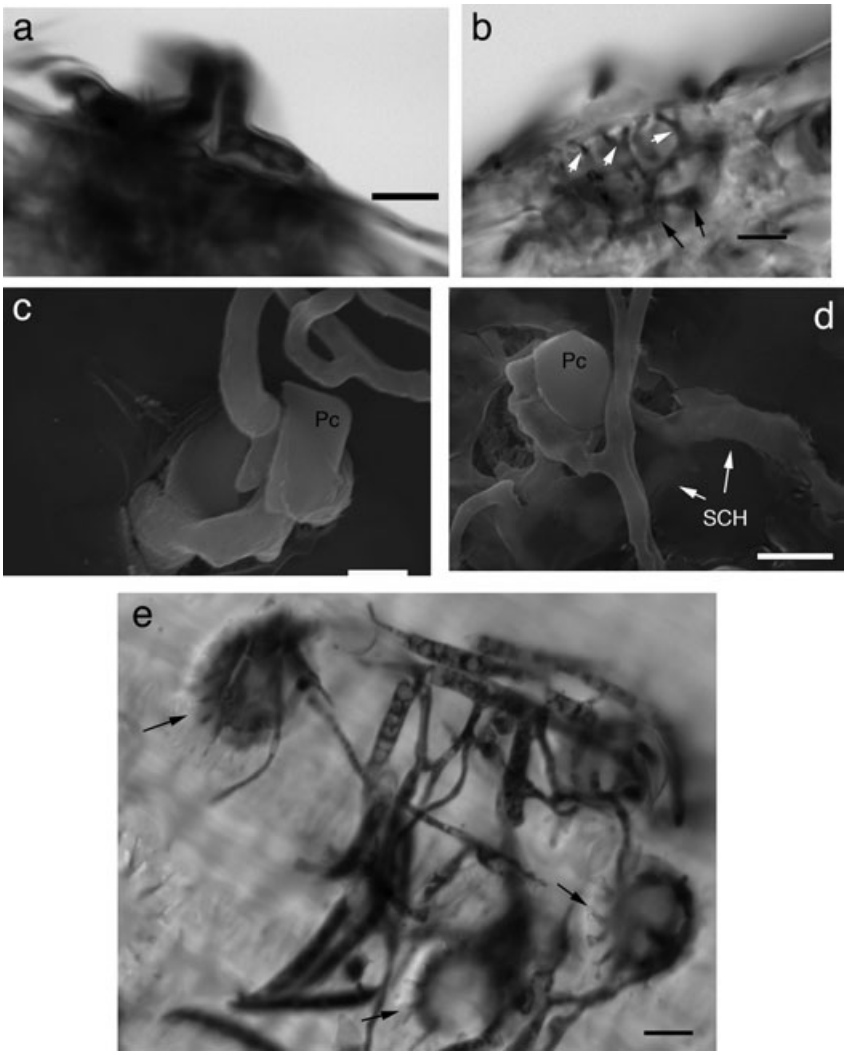


**Fig. 3** Hyphae invading through papillae. (a) Hyphae invading through a papilla cell. (b) Hyphae ramifying through pits at the base of a papilla (white arrows) and forming bulbous infection hyphae in epidermal cells (black arrows). (c, d) Scanning electron microscopy images of hyphae invading through papilla cells. (e) Papillae (arrows) of glumes on living wheat plants being invaded by *Fusarium graminearum*. Hyphae stained with trypan blue in (a, b, e). Pc, papilla cell; SCH, subcuticular hyphae. Scale bars: (a, b, d, e), 10 μm; (c), 5 μm.

**Table 1** Number of glumes with either subcuticular hyphae (SCH) or bulbous infection hyphae (BIH).*

| | SCH† | | | BIH‡ | | |
|---|---|---|---|---|---|---|
| Strain | 24 hpi | 48 hpi | 72 hpi | 24 hpi | 48 hpi | 72 hpi |
| PH-1 | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 |
| *Δras2* | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 |
| *Δmgv1* | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 |
| *Δgpmk1* | 9/10 | 10/10 | 7/10 | 0/10 | 0/10 | 0/10 |

*Values show the number of glumes across two separate experiments (five glumes per experiment).
‡Number of glumes with coral-like subcuticular hyphae at *x* hours post-inoculation (hpi).
†Number of glumes with bulbous infection hyphae at *x* hpi.

were present (Table 1; Fig. 4f). None of the glumes inoculated with the Δ*gpmk1* mutant displayed bulbous invasive hyphae, even after 72 hpi (Table 1). However, at 72 hpi, Δ*gpmk1* grew internally as narrow hyphae, probably representing saprophytic growth (Fig. 4j). In addition, when observed in cross-section, intracellular hyphae were scarce in glumes inoculated with the Δ*gpmk1* mutant (Fig. 4n). These data suggest that bulbous infection hyphae are morphologically distinct structures whose formation is genetically controlled by the pathogenicity MAPK Gpmk1.

## DISCUSSION

The goal of this study was to develop an *in vitro* protocol that can be used to easily assess infection-related morphogenesis in *F. graminearum*. Using detached wheat glumes, we were able to induce two morphologically distinct hyphal structures during glume invasion: subcuticular hyphae and bulbous infection hyphae. Furthermore, we demonstrated that the MAPK Gpmk1, previously implicated in the pathogenicity of *F. graminearum*, is essential for the induction of bulbous infection hyphae. Our results, coupled with previous studies, suggest that the bulbous

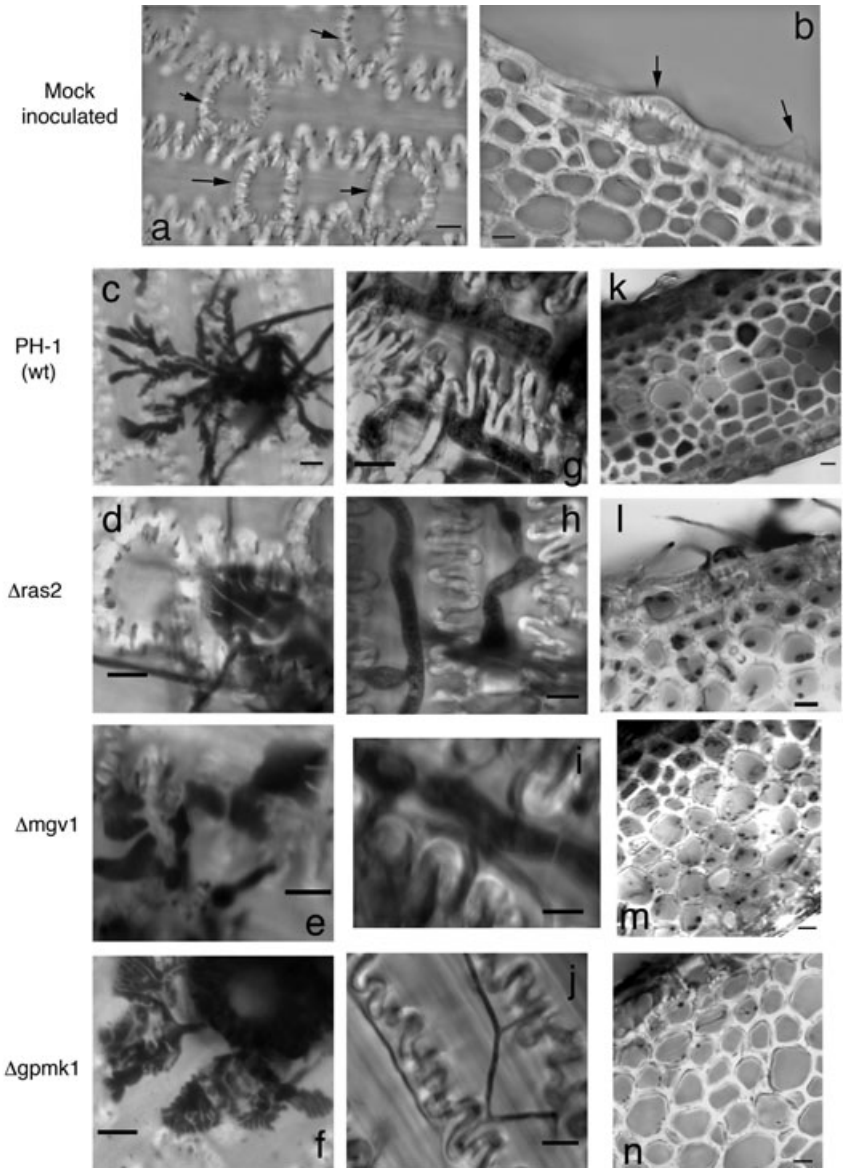


**Fig. 4** Mutants tested on detached wheat glumes for infectious-related development. (a, b) Mock-inoculated controls 72 h after inoculation. Black arrows indicate papillae. (c–f) Subcuticular hyphae produced after 24 h. (g–j) Intracellular hyphae observed after either 48 h (g–i) or 72 h (j). The thin hyphae illustrated in (j) were the only type of intracellular hyphae observed for the Δ*gpmk1* mutant. (k–n) Cross-sectional view of inoculated glumes after either 48 h (k–m) or 72 h (n). Note the decreased hyphal ramification of the Δ*gpmk1* mutant (n). Hyphae in all panels stained with trypan blue. Scale bar, 10 μm for all panels.

infection hyphae represent a morphological state that may be specific to the infection of wheat (and possibly other plant) tissue (Guenther and Trail, 2005). Indeed, the bulbous infection hyphae seen in this study are similar in appearance to the dikaryotic hyphae observed in stem tissue (Guenther and Trail, 2005) and wide hyphae observed in ovarian tissue (Jansen *et al*., 2005). In addition, we developed a relatively quick and easy protocol that can be used to assay different strains/mutants of *F. graminearum* for their ability to differentiate infection-related hyphal structures. Future work should focus on exploiting this protocol to monitor transcriptional changes during infectious development. Given that the host tissue was previously frozen, interference from host RNA may be less of an obstacle than in *in planta* studies. Transcriptional profiling of 'artificially' induced infection structures uncovered several virulence genes in other pathogenic fungi (Odenbach *et al*., 2007; Takano *et al*., 2003).

Members of the *kss1/fus3/pmk1* MAPK family mediate changes in hyphal morphogenesis in many different fungi (reviewed by Zhao *et al*., 2007). In the rice blast fungus (*M. oryzae*), and the southern corn blight pathogen (*Cochliobolus heterostrophus*), *pmk1* is absolutely required for the development of appressoria (Lev *et al*., 1999; Xu and Hamer, 1996). In addition, *pmk1* homologues are needed for the pathogenicity of fungi that do not form appressoria, including *F. graminearum* and *F. oxysporum* (Di Pietro *et al*., 2001; Jenczmionka *et al*., 2003; Mey *et al*., 2002; Urban *et al*., 2003). However, infection-related morphogenesis has not been studied in Δ*gpmk1 F. graminearum* mutants. Our data illustrate that this MAPK is absolutely neces-

 

sary for the development of bulbous infection hyphae, which is consistent with the role of Pmk1 homologues in mediating infection-related morphogenesis. In the dimorphic corn pathogen *Ustilago maydis*, the Pmk1 orthologue Ubc3 is necessary for dikaryon formation and filamentous growth (Mayorga and Gold, 1999; Muller *et al*., 1999). Given that Guenther and Trail (2005) have demonstrated previously that the wide hyphae are dikaryotic, it is possible that Gpmk1 may serve a similar role in dikaryon formation in *F. graminearum*. Interestingly, Ras2 appears to be dispensable for the formation of bulbous infection hyphae, despite the fact that Ras2 contributes to the activation of Gpmk1 (Bluhm *et al*., 2007). However, Δ*ras2* mutants still exhibit a basal level of Gpmk1 activation, which is apparently sufficient for the formation of bulbous infection hyphae.

When intracellular hyphae were present in glumes inoculated with Δ*gpmk1*, they were thin in appearance and restricted to the top two to three layers of cells in wheat glumes (Fig. 4n). This is in contrast with glumes inoculated with wild-type strain PH-1, in which bulbous hyphae proliferated to lower cell layers of the glume, even after 48 h (Fig. 4k). As the deletion of *gpmk1* has little to no effect on saprophytic growth in culture (Jenczmionka *et al*., 2003; Urban *et al*., 2003), the defect in hyphal proliferation could be caused by the decreased expression of certain virulence factors. Given that over 300 genes are differentially regulated in the Δ*gpmk1* mutant (Ding *et al*., 2007), the identification of which genes contribute specifically to infectious-related development in *F. graminearum* may prove to be a daunting task. However, this protocol can be used as a tool to help unravel the genetic requirements for the formation of bulbous infection hyphae.

Despite its role in the development of bulbous hyphae, Gpmk1 was not absolutely required for the formation of subcuticular hyphae. However, the subcuticular hyphae produced by the Δ*gpmk1* strain were not as prolific as those in the wild-type. As subcuticular hyphae grow in the apoplastic space above epidermal cells, one could predict that pectinases, xylanases and other hydrolytic enzymes would be crucial for their growth. Unfortunately, each class of hydrolytic enzyme contains multiple representatives in the genome of *F. graminearum*, making gene redundancy a barrier when considering single-gene deletions. Gpmk1 simultaneously regulates the expression of several hydrolytic enzymes, including cellulases and xylanases, but not pectinase (Jenczmionka and Shafer, 2005). The decreased ramification of Δ*gpmk1* subcuticular hyphae may represent a defect in the degradation of host cell wall materials. In order to test the role of cell wall-degrading enzymes in subcuticular growth, other mutants that affect the overall expression of such genes should be explored. For example, Gzsnf1 is a putative protein kinase that controls the expression of genes encoding cell wall-degrading enzymes and represents an excellent candidate for further study (Lee *et al*., 2009).

Subcuticular hyphae were readily observed after 24 h when glumes were inoculated on the adaxial side. Although the significance of this hyphal morphology is unknown, it has been postulated that it might provide a suitable niche for plant pathogenic fungi during the early stages of host invasion (Bushnell *et al*., 2003). Considering this hypothesis, once host infection is firmly established, subcuticular hyphae would presumably become obsolete. Consistent with this notion, many subcuticular hyphae appeared to be degraded after bulbous infection hyphae proliferated within host cells. Other plant pathogenic fungi undergo programmed cell death in older hyphal structures and transport the recycled cellular reserves to the invasion front (Asakura *et al*., 2009; Veneault-Fourry *et al*., 2006). Further investigation is needed to determine the cause of degradation of subcuticular hyphae and the role it may play in plant infection. In addition, the genetic requirements of subcuticular growth remain unknown. A recent study using the apple scab pathogen *Venturia inaequalis* demonstrated that two genes, *cin1* and *cin3*, are significantly up-regulated during subcuticular growth on cellophane sheets (Kucheryava *et al*., 2008). However, there are no *cin1* or *cin3* orthologues present in the *F. graminearum* genome. Our protocol allows for similar transcriptional studies in *F. graminearum* and represents the first step to the identification of the genetic contributions to subcuticular growth.

When inoculated onto the adaxial side of glumes, *F. graminearum* appeared to exploit papillae as invasion sites. It is possible that the elevated topography of papilla cells induces invasive development, but this is unlikely as hyphae often grow over the top of papilla cells without invading them (data not shown). A more likely possibility is that the junction between papillae and neighbouring epidermal cells contains 'weak spots' where lacerations occur. Given that the glumes were stored at −20 °C, the freezing process may have exacerbated existing weak spots and caused these lacerations. *Fusarium graminearum* also invaded through papillae on living wheat plants (Fig. 4e), although this was not as readily observed as on detached wheat glumes. Nevertheless, the inherent features of papillae make them interesting candidates for infection courts. Papillae are dome-shaped epidermal cells whose precise function is unknown, but they are surrounded by 9–13 pits that adjoin them to nearby cells (Tubb *et al*., 1993). As *F. graminearum* often invades 'passively' through stomata and ramifies internally through pits (Jansen *et al*., 2005; Kang and Buchenauer, 2000; Pritsch *et al*., 2000), invasion through papillae would be advantageous in that it allows simultaneous access to several nearby cells. Indeed, this appears to be the case on detached glumes (Fig. 3b).

Although anthers and ovaries are considered to be the primary sites of infection by *F. graminearum*, the adaxial sides of glumes represent more easily accessible infection sites that could act as inoculum reservoirs for further spread. Several

studies have demonstrated that *F. graminearum* readily invades the stomata on the abaxial side of wheat glumes (Kang and Buchenauer, 2000; Pritsch *et al*., 2000). However, the dry microenvironment on the abaxial side is not conducive to fungal growth or survival. Nevertheless, the cavity on the adaxial side, which is partially covered by the lemma, is more humid and favourable for infection. Consistent with this notion, macroconidia inoculated onto the abaxial side often grow to the edge of the glume and gain access to the adaxial side (Kang and Buchenauer, 2000). Kang and Buchenauer (2000) postulated several routes for *F. graminearum* to reach the vascular tissue of the rachis, one of which is via glume invasion. However, direct evidence of this route is lacking. Further microscopic and epidemiological investigations are necessary to determine the significance of glume invasion on both scales. Regardless of their importance *in planta*, we have demonstrated that they can be used to 'artificially' induce infectious-related development of *F. graminearum*.

## EXPERIMENTAL PROCEDURES

### Strains and culture conditions

*Fusarium graminearum* strain PH-1 (NRRL 31084) was used as the wild-type strain for glume inoculations. Strains Δ*ras2*, Δ*mgv1* and Δ*gpmk1* were kindly provided by Dr Jin-Rong Xu (Purdue University, West Lafayette, IN, USA). All strains were stored as mycelial debris in 30% glycerol at −80 °C. Strains were revived on V8 agar and transferred to yeast malt agar (YMA) (Harris, 2005) for conidiation. Carboxymethyl cellulose (CMC) medium (Cappellini and Peterson, 1965) was used for macroconidia production of Δ*mgv1*, as it did not conidiate well on YMA. Macroconidia were harvested in sterile distilled water from YMA or CMC plates with a bent glass rod and filtered through one layer of miracloth (Calbiochem, San Diego, CA, USA) to remove mycelial debris. Macroconidia were resuspended in 0.05% Tween-20 to a concentration of $5 \times 10^4$ macroconidia/mL for inoculation.

### Plant material and inoculation

Wheat heads (variety 'Norm') were removed from plants at anthesis and stored at −20 °C before inoculation. Heads were removed 1 h prior to inoculation and allowed to thaw at room temperature. After thawing, glumes were removed and placed on 3% water agar in Petri dishes. Glumes were then irradiated for 30 s with ultraviolet light for sanitation. The cavities of glumes (adaxial side) were filled with approximately 20 μL of a suspension of $5 \times 10^4$ macroconidia/mL in 0.05% Tween-20. Suspensions were allowed to stand for 1 min before being removed. For inoculation of the abaxial side of glumes, a 100-μL droplet of macroconidia was placed on a polystyrene Petri dish, and the abaxial side of the glume was held in this droplet for 1 min. The glume was then removed and placed on 3% water agar, abaxial side up. Glumes were then incubated at room temperature until processed for microscopy.

For the inoculation of living wheat plants, 20 mL of a suspension of $5 \times 10^4$ macroconidia/mL in 0.05% Tween-20 were placed on the adaxial side of the glume. The droplet was removed 1 min later. The wheat heads were then covered with a plastic bag to promote a humid environment. Glumes were removed and processed for microscopy as described above. A total of 12 glumes was observed from two experiments.

### Processing glumes for microscopy

For light microscopy, inoculated glumes were placed in clearing/fixing solution (3 : 1 ethanol–acetic acid) and incubated at room temperature on a rotary shaker at 75 r.p.m. for 16 h. Glumes were further fixed in a 5 : 1 : 1 solution (ethanol–acetic acid–glycerol) for at least 4 h under the same conditions as above. The glumes were then stained in a lactophenol blue solution (33% lactic acid, 33% phenol, 0.01% trypan blue) for at least 16 h under the conditions described above. After staining, glumes were washed in 60% glycerol for at least 3 h. Glumes were then sectioned under a dissecting scope, mounted in 60% glycerol, and observed in a bright field with an Olympus (Hitschfel Instruments, St. Louis, MO, USA) BX51 microscope. For the quantitative analysis of mutants, five glumes were inoculated for each time point (15 glumes in total). This experiment was performed twice for a total of 10 glumes per time point per strain.

In addition, calcofluor white (fluorescent brightener 28; Sigma, St. Louis, MO, USA) was used to stain some samples. For calcofluor staining, glumes were cleared/fixed in the solutions described above, and then washed in water before incubating in 0.3% calcofluor white–30% glycerol solution for 5 min in the dark. The samples were then washed twice in water, and once in 30% glycerol. Samples were then mounted in 60% glycerol and observed with fluorescent settings on an Olympus BX51 microscope.

For scanning electron microscopy, glumes were removed from Petri dishes at the given time points and fixed in 2.5% glutaraldehyde overnight at 4 °C prior to processing. The samples were then fixed for 1 h in 1% $OsO_4$ at room temperature. The samples were then washed with water and serially dehydrated with ethanol (25%–100%) and dried with $CO_2$. The samples were then affixed to aluminium stubs and coated with gold–palladium alloy before observation. The samples were then observed with a Hitachi (Pleasanton, CA, USA) S4700 field-emission scanning electron microscope. Glumes from three replicates were observed.

 

For transmission electron microscopy, the glumes were sectioned and fixed as described above for scanning electron microscopy. The samples were then embedded in LR-white and polymerized for 10 days. Ultrathin sections were cut and embedded on 200-mesh copper grids. Samples were stained with uranyl acetate and lead citrate and observed with a Hitachi H7500 microscope. Glumes from three replicates were observed.

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