

**FgPal1 regulates morphogenesis and pathogenesis in *Fusarium graminearum***

Jinrong Yin<sup>1†</sup>, Chaofeng Hao<sup>1†</sup>, Gang Niu<sup>1</sup>, Wei Wang<sup>1</sup>, Guanghui Wang<sup>1</sup>, Ping Xiang<sup>1</sup>, Jin-Rong Xu<sup>2</sup>, Xue Zhang<sup>1\*</sup>

<sup>1</sup> State Key Laboratory of Crop Stress Biology for Arid Areas, College of Plant Protection, Northwest A&F University, Yangling, Shaanxi, China 712100

<sup>2</sup> Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907, USA.

<sup>†</sup>These authors have contributed equally to this work.

**\*Corresponding author:** Xue Zhang

State Key Laboratory of Crop Stress Biology for Arid Areas, College of Plant Protection

Northwest A&F University, Yangling, Shaanxi 712100, China;

Tel: 13201577924, email: xuezhang@nwafu.edu

## ABSTRACT

Ascospores are the primary inoculum in *Fusarium graminearum*, a causal agent of wheat head blight. In a previous study, *FgPAL1* was found to be up-regulated in the *Fgamal* mutant and important for ascosporeogenesis. However, the biological function of this well-conserved gene in filamentous ascomycetes is not clear. In this study, we characterized its functions in growth, differentiation, and pathogenesis. The *Fgpall* mutant had severe growth defects and often displayed abnormal hyphal tips. It was defective in infectious growth in rachis tissues and spreading in wheat heads. The *Fgpall* mutant produced conidia with fewer septa and more nuclei per compartment than the wild type. In actively growing hyphal tips, FgPal1-GFP mainly localized to the subapical collar and septa. The FgPal1 and LifeAct partially co-localized at the subapical region in the interdependent manner. The *Fgpall* mutant was normal in meiosis with eight nuclei in developing asci but most asci were aborted. Taken together, our results showed that FgPal1 plays a role in maintaining polarized tip growth and coordination between nuclear division and cytokinesis, and it is also important for infectious growth and developments of ascospores by the free cell formation process.

**Short title:** FgPal1 for fungal morphogenesis and pathogenesis

**Keywords:** Fusarium head blight, polarized tip growth, cytokinesis, ascosporeogenesis, infectious growth

## INTRODUCTION

The homothallic filamentous ascomycete *Fusarium graminearum* is a causative agent of Fusarium head blight (FHB) or scab of wheat worldwide (Bai and Shaner, 2004; Zhang et al., 2012). It also infects barley, corn, and other grain crops. Ascospores forcibly released from perithecia formed on plant debris are the primary inoculum of this important pathogen (Markell and Francl, 2003). Plant infection is initiated when airborne ascospores land on flowering wheat heads, germinate, and penetrate plant cells directly or with specialized infection structures including infection cushions and lobate appressoria (Boenisch and Schäfer, 2011; Jiang et al., 2019). Infectious growth is then spread from infected kernels to other spikelets on the same wheat head via the rachis and cause head blight symptoms. Under favorable environmental conditions, *F. graminearum* can cause severe yield losses and contaminate infested grains with mycotoxins such as deoxynivalenol (DON) and zearalenone (Trail et al., 2002; Goswami and Kistler, 2004). The trichothecene mycotoxin DON is a potent protein synthesis inhibitor in eukaryotic organisms and an important virulence factor in *F. graminearum* (Van de Walle et al., 2010).

Because of the role of ascospores as the primary inoculum, sexual reproduction is a critical step in the infection cycle of *F. graminearum*. As a homothallic fungus, it produces

abundant perithecia by selfing on mating cultures in the laboratory or infected plant debris in the field (Miedaner et al., 2008; Kim et al., 2015). Mature perithecia are melanized and contain asci with eight 4-celled ascospores. In each developing ascus, the diploid nucleus formed after karyogamy goes through meiosis and one round of post-meiotic mitosis before the delimitation of eight ascospores (Nakayashiki, 2005; Hao et al., 2019). The nucleus in each developing ascospore then undergoes two more rounds of mitosis and cytokinesis before the formation of 4-celled ascospores with one nucleus per compartment. Repeat-induced point (RIP) mutation, meiotic silencing of unpaired DNA (MSUD), and A-to-I RNA editing are known to specifically occur in *F. graminearum* during sexual reproduction (Son et al., 2011; Hane et al., 2015; Liu et al., 2016). Whereas RIP is a premeiotic event, MSUD occurs during meiosis or post-meiotic mitosis. Genome wide A-to-I RNA editing has been shown to occur in mating cultures as early as 1 day post-fertilization (dpf) and increases during ascus development and ascospore formation (Liu et al., 2016; Teichert et al., 2017; Bian et al., 2019). Interestingly, 70 genes have premature stop codons (PSC) that require A-to-I editing to encode full-length functional proteins in *F. graminearum*. Several genes with PSC editing events are known to be important for ascospore formation or release, including *PUK1*, *AMD1*, and *FgAMA1* (Liu et al., 2016; Cao et al., 2017; Hao et al., 2019).

Unlike other mutants with defects in ascospore formation, the *Fgama1* mutant produced

Accepted Article

rounded ascospores that may grow by budding inside asci before being released from perithecia (Hao et al., 2019). *AMA1* is a meiosis-specific co-activator of the anaphase promoting complex/cyclosome (APC/C) in the budding yeast *Saccharomyces cerevisiae* (Cooper et al., 2000). The *Fgama1* mutant is normal in meiosis and still produces eight ascospores in each ascus although it has abnormal ascospore morphology and ascospore budding defects. The *Fgama1* ascospores are one-celled but have two nuclei in each ascospore, indicating that *FgAMA1* is essential for cytokinesis and second round of mitosis in developing ascospores. RNA-seq analysis showed that one of the genes affected by *FgAMA1* deletion is the *FgPAL1* gene, which also has one PSC RNA editing event (Hao et al., 2019). Interestingly, the *Fgpall* deletion mutant also has the ascospore budding defect and forms ascospores with abnormal morphology. However, unlike the *Fgama1* mutant, the *Fgpall* mutant had severe defects in ascosporeogenesis and rarely formed ascospores (Hao et al., 2019).

*FgPal1* is orthologous to *Pal1* of *S. cerevisiae* and *Schizosaccharomyces pombe* (Ge et al., 2005; Carroll et al., 2012). *Pal1* is a membrane-associated protein that is involved in the maintenance of cylindrical cellular morphology although its exact function is not clear in the budding and fission yeasts. It localizes to the sites of active growth in *S. cerevisiae* and *S. pombe* (Ge et al., 2005). In *S. cerevisiae*, the *pall* mutant (pears and lemons) forms yeast cells with morphology defects. *Pal1* is an early-arriving endocytic coat protein recruited by Ede1

Accepted Article

and the intermediate coat module protein Sla2, and it disassembles from endocytic sites before actin polymerization (Carroll et al., 2012). In *S. pombe*, Pal1 also physically interacts and partially co-localizes with Sla2, Ede1, and Syp1 at the endocytic site (Ge et al., 2005). Protein kinase Pom1, a member of the dual specificity DYRK kinase family, directly phosphorylates Pal1 and other cell polarity targets to regulate cell polarity and cell cycle signaling (Kettenbach et al., 2015). Pal1 is also directly phosphorylated at distinct residues by the MARK/PAR-1 protein kinase Kin1, which affects the localization of Pal1 to the growing cell ends of *S. pombe* (Lee et al., 2018).

Although there are only limited studies on the localization and functions of Pal1 in the budding and fission yeasts, its orthologs are well conserved in filamentous ascomycetes. However, the biological functions of Pal1 orthologs have not been studied in filamentous or plant pathogenic fungi. In this study, we further characterized its functions in hyphal growth, conidiation, sexual reproduction, and pathogenesis in *F. graminearum*. The *Fgpall* mutant was significantly reduced in growth rate and formed apical or subapical swollen bodies in growing hyphae. It also produced conidia with morphological defects and was defective in infectious growth in rachis tissues for disease spreading. In comparison with the wild type, the number of nuclei in each compartment was increased in the *Fgpall* mutant. FgPal1-GFP mainly localized to the subapical collar and septa in an F-actin dependent manner and deletion of *FgPAL1* affected

Accepted Article

the localization of F-actin at the subapical region. FgPal1 played a role in both general endocytosis and clathrin-mediated endocytosis by disturbing the localization of FgEde1. During sexual reproduction, the *Fgpall* mutant was normal in meiosis and formation of eight nuclei in developing asci but most of mutant asci were aborted in ascospore formation and rarely formed ascospores. Overall, our results showed that FgPal1 plays a role in maintaining normal hyphal growth, tip morphology, and coordination between nuclear division and cytokinesis, and it is also important for infectious growth and developments of ascospores by the free cell formation process. The function of FgPal1 orthologs in the control of polarized growth and spatial regulation of cytokinesis may be conserved in other filamentous ascomycetes.

## RESULTS

**FgPal1 is important for normal tip growth:** In a previous study, *FgPAL1* (FGSG\_08726) was found to be up-regulated in the *Fgama1* mutant and important for ascosporeogenesis (Hao et al., 2019). *FgPAL1* is orthologous to *PAL1* of *S. cerevisiae* and encodes a 521 amino acid (aa) protein with a conserved PAL1 domain at the C-terminal region. However, FgPal1 has an FtsK domain (55–172 aa) that is absent in its orthologs in *S. cerevisiae* and *S. pombe* (Fig. 1A). The

FtsK domain first identified in bacteria is related to the translocation of DNA prior to the completion of cytokinesis and cell division (Steiner et al., 1999). Interestingly, the FtsK domain is well conserved in the FgPal1 orthologs in filamentous ascomycetes although none of them have been functionally characterized. Because the previous study only identified one mutant strain and lacked complementation assays (Hao et al., 2019), in this study we first generated two additional *Fgpall* mutants M8 and M9 (Table 1). All the *Fgpall* mutant strains had similar defects described below although only data related to mutant M8 were presented.

The *Fgpall* mutant was significantly reduced in growth rate in comparison with the wild-type strain PH-1 on CM and 5×YEG (Fig. 1B; Table 2). It formed whitish, compact colonies that had more fluffy aerial hyphae than PH-1. Close examination of hyphal tips revealed that the *Fgpall* mutant had defects in maintaining polarized tip growth and often had apical or subapical swollen bodies. Hyphae of the *Fgpall* mutant appeared to have shorter distances between two branching sites, which might be related to its reduced growth rate (Fig. 1C). On average, the distance between two branching sites on growing hyphae was  $79.5 \pm 44.7$   $\mu\text{m}$  in the *Fgpall* mutant, which was significantly shorter than that of the wild type ( $180.4 \pm 78.5$   $\mu\text{m}$ ). When the *FgPAL1*-GFP construct was transformed into the *Fgpall* mutant, the resulting *Fgpall*/*FgPAL1*-GFP transformants (Table 1) were normal in growth rate (Fig. 1B) and hyphal branching (Fig. 1C), indicating that the growth defects of *Fgpall* mutant were directly related to



the gene replacement event. Therefore, *FgPAL1* is important for hyphal growth, possibly by playing a role in maintaining normal polarized growth at the tip.

Because the *Fgpal1* and *Fgama1* mutants had similar defects in ascospore budding and *FgPAL1* expression was affected in *Fgama1*, we assayed their interaction by yeast two-hybrid assays. Yeast cells expressing the FgAma1 bait and FgPal1 prey constructs failed to grow on SD-Trp plates and had no LacZ activities (Fig. S1), indicating that FgPal1 and FgAma1 do not directly interact with each other.

To determine the effect of *FgPAL1* deletion on the polarisome or vesicle supply center at hyphal tips, we transformed the *FgSPA2*-GFP construct (Zhang et al., 2019) into PH-1 and the *Fgpal1* mutant M8. FgSpa2, a marker for the polarisome, is orthologous to Spa2, a cell polarity protein in *S. cerevisiae* (Takeshita and Fischer, 2019). In comparison with the wild type, the localization of FgSpa2-GFP was not affected in the *Fgpal1* mutant, indicating that FgPal1 is not involved in the localization of FgSpa2 to the hyphal tip (Fig. S2).

**The *Fgpal1* mutant is defective in conidiation and sexual reproduction:** Similar to its reduced growth rate, the *Fgpal1* mutant was reduced in conidiation compared to the wild type in 5-day-old CMC cultures (Table 2). Furthermore, conidia of the *Fgpal1* mutant ( $39.6 \pm 6.4 \mu\text{m}$  in length) were shorter than those of the wild type ( $45.2 \pm 7.9 \mu\text{m}$  in length) (Fig. 2A). Instead of

forming typical 4-6 celled macroconidia, approximately 90% of *Fgpal1* conidia had three or fewer unevenly distributed septa. On average, conidia of the *Fgpal1* mutant had  $3.1 \pm 0.2$  septa but the wild type had  $4.3 \pm 0.5$  septa per conidium. However, conidium germination was normal in the *Fgpal1* mutant.

On mating cultures, the *Fgpal1* mutant still formed melanized perithecia but ascospore cirrhi were never observed (Fig. 2B) at 14 days post-fertilization (dpf) or even longer. In ascospore ejection assays, masses of ascospores were physically released from wild-type perithecia but not from *Fgpal1* perithecia (Fig. 2C). When examined for asci and ascospores inside perithecia, most of the wild-type asci were fertile and had 8 ascospores per ascus (Fig. 2D). However, majority of the *Fgpal1* asci appeared to be aborted in ascospore formation and had granular cytoplasm. For the *Fgpal1* asci with ascospores, most of them had fewer than 4 ascospores per ascus and displayed ascospore morphology defects (Fig. 2D). These results further indicate that *FgPAL1* is dispensable for ascus development but important for ascospore formation and essential for ascospore release in *F. graminearum*.

**FgPal1 is important for plant infection:** In infection assays with flowering wheat heads of susceptible wheat cultivar Xiaoyan 22, the *Fgpal1* mutant only caused scab symptoms on the inoculated florets at 14 days post-inoculation (dpi) and failed to infect neighboring spikelets on

the same head. Under the same condition, the wild-type strain PH-1 developed typical head blight symptoms on the inoculated and neighboring spikelets (Fig. 3A). In infection assays with corn silks, the *Fgpall* mutant caused only limited discoloration at the inoculation site (Fig. 3B), confirming that *FgPALI* is important for virulence in *F. graminearum*.

The *Fgpall* mutant was similar to the wild type in the penetration of wheat lemma and development of invasive hyphae in infected lemma tissues at 48 hpi (Fig. 3C). Because the *Fgpall* mutant was defective in spreading to neighboring spikelets, we examined infectious growth in the rachis tissues. At 120 hpi, PH-1 produced extensive infectious hyphae in rachis tissues adjacent to the inoculated spikelet. However, in wheat heads inoculated with the *Fgpall* mutant, we failed to observe invasive hyphae in rachis tissues below or above the inoculated spikelet (Fig. 3D). Therefore, *FgPALI* must be essential for infectious hyphae to spread from inoculated florets through the rachis to other spikelets.

Because DON is an important virulence factor in *F. graminearum*, we also assayed DON production in infected wheat kernels (Li et al., 2015). In comparison with the wild type (3564.2±312.0 ppm), the *Fgpall* mutant was significantly reduced in DON production (1212.9±193.0 ppm). These results suggest that deletion of *FgPALI* affects DON biosynthesis during infection.

**FgPal1 localizes to the subapical collar region:** In both *S. cerevisiae* and *S. pombe*, Pal1 localizes to the growing cell end at the interphase and affects cell morphology (Ge et al., 2005; Carroll et al., 2012). When hyphal tips or germ tube tips of the *Fgpal1/FgPAL1*-GFP transformant were examined, FgPal1-GFP signals were mainly observed at the subapical collar (Fig. 4A). Nevertheless, a few spots with bright GFP signals with dynamic locations also were observed in the cytoplasm in the region behind the hyphal tip (Fig. 4A), which may be related to the localization of FgPal1-GFP to developing septa.

To further characterize its subcellular localization, we co-transformed the *FgPAL1*-GFP construct with the LifeAct-RFP construct (Zhang et al., 2019) into the *Fgpal1* mutant M8. In the resulting transformants, LifeAct, a F-actin marker, localized to the tip apex (the polarisome) and collar region as being reported in other fungi (Delgado-Álvarez et al., 2010; Zhou et al., 2012). Unlike LifeAct-RFP signals, FgPal1-GFP signals were never observed at the tip apex. However, partial colocalization of FgPal1-GFP with LifeAct-RFP was observed in the sub-apical collar region (Fig. 4B). Besides the overlapping zone, FgPal1-GFP signals were on the side towards the tip and LifeAct-RFP signals were on the other side towards the rear end (Fig. 4B). The distribution of LifeAct-RFP and FgPal1-GFP signals may reflect their dynamic spatio-temporal association at the sub-apical collar.

### **The localization of FgPal1 to the collar region is dependent on F-actin and *vice versa***

Because of the partial co-localization of FgPal1 with F-actin, we treated the *Fgpal1/FgPAL1*-GFP transformant with Latrunculin A (Lat-A), an inhibitor of F-actin (Ge et al., 2005). After treating vegetative hyphae of the *Fgpal1/FgPAL1*-GFP transformant with 0.1  $\mu$ g/ml Lat-A dissolved in DMSO (dimethyl sulfoxide) for 10 min, FgPal1-GFP signals localized to the apex of hyphal tips (Fig. 4C). In the control treated with DMSO along, FgPal1-GFP signals were still mainly at the sub-apical collar region. These results suggested that the localization of FgPal1-GFP to hyphal tips is F-actin dependent.

We then transformed the LifeAct-GFP construct (Delgado-Álvarez et al., 2010; Zhou et al., 2012) into the *Fgpal1* mutant. In the resulting *Fgpal1* LifeAct-GFP transformant, the LifeAct-GFP signals were observed only at the sub-apical collar region and some spots in the back where septation may initiate (Fig. 4D). In the wild type, LifeAct-GFP localized to the tip apex (the polarisome) and collar region (Fig. 4D). These results indicated that FgPal1 also played a role in the localization of LifeAct at hyphal tips, which further proved the dynamic association of FgPal1 and LifeAct at the sub-apical collar.

**Deletion of *FgPAL1* affects the general endocytosis and clathrin-mediated endocytosis:** In *A. nidulans* and *N. crassa*, actin patches in the subapical collar are sites for the occurrence of

Accepted Article

endocytosis, a process that plays an important role in polarized tip growth in filamentous fungi (Fischer-Parton et al., 2000; Araujo-Bazán et al., 2008). To test whether FgPal1 plays a role in endocytosis in *F. graminearum*, we compared the efficiency of the wild type (PH-1) and *Fgpall* mutant in the uptake of FM4-64, a fluorescent dye commonly used as an endocytic marker or a tracer for endocytosis (Fischer-Parton et al., 2000). In the wild type, the plasma membrane and cytoplasm were quickly stained by FM4-64 in 10 min and vacuoles became visible by 30 min when FM4-64 was gradually internalized. In the *Fgpall* mutant, FM4-64 appeared at the plasma membrane at 10 min but only weak fluorescent signals were visible in the cytoplasm by 30 min (Fig. 5A). These results suggested that FgPal1 is involved in both the uptake and general endocytosis of the fluorescent dye FM4-64.

Ede1 is an endocytic scaffolding protein involved in clathrin-mediated endocytosis (CME) and it is important for endocytic site initiation and stabilization (Brach et al., 2014) (Lu and Drubin, 2017). To further characterize the role of *FgPAL1* in endocytosis, we generated the *FgEDE1*-GFP construct and transformed it into the *Fgpall* mutant and PH-1. In the wild type background, FgEde1-GFP signals localized to the sub-apical regions at hyphal tips (Fig. 5B), which was similar to the localization of FgPal1-GFP. In the *Fgpall*/*FgEDE1*-GFP transformant, however, the enrichment of FgEde1-GFP signals in the sub-apical region was not observed although faint, diffused FgEde1-GFP signals were observed in the rear region of hyphal tips (Fig.

5B). These results suggested that FgPal1 may play a role in the localization of FgEde1 via the clathrin-mediated endocytosis.

**FgPal1 localizes to the septum during cytokinesis:** Fungal cytokinesis requires the formation of the septum cell wall, which is tightly coordinated with the contraction of cytokinetic actomyosin ring (CAR) (Berepiki et al., 2011). In *F. graminearum*, FgPal1 also localized to the septum. In the transformants expressing both LifeAct-RFP and *FgPAL1*-GFP constructs, partial co-localization of LifeAct and FgPal1 was observed (Fig. 6A). However, in comparison with LifeAct-RFP signals, FgPal1-GFP signals were relatively weak at septa (Fig. 6A).

To characterize the role of FgPal1 in septation, we analyzed the spatio-temporal association of FgPal1-GFP and LifeAct-RFP during septum formation. LifeAct-RFP signals representing F-actin cables became visible as the septal actin tangle (SAT) at the septation site (Seiler and Heilig, 2019) (Fig. 6B). However, weak GFP signals were observed only after the contraction of the actin ring and the formation of septa (Fig. 6B). These results suggest that localization of FgPal1-GFP to the septum only occurred at late stages of septum formation. Therefore, FgPal1 is not important for the localization of SAT and constriction of CAR in *F. graminearum*, which is different from its ortholog in *S. cerevisiae* (Ge et al., 2005; Carroll et al., 2012).

Accepted Article

To further determine the role of F-actin in the maintenance of FgPal1 at the division site, the *Fgpal1/FgPAL1*-GFP transformant were treated with Latrunculin A (Lat-A) and DMSO as a control for 10 min. In LatA-treated hyphae, FgPal1-GFP signals were still observed at septa (Fig. 6C), indicating that localization of FgPal1 to the septum is independent of F-actin (Fig. 6C). In the *Fgpal1* LifeAct-GFP transformant, the localization of LifeAct-GFP at the septum also was not affected by deletion of *FgPAL1* (Fig. 6D). These results indicated that the assembly of F-actin at the septum was independent of FgPal1. Thus, the localization of FgPal1 and LifeAct to the septum is not interdependent, which is different from their dynamic association at the sub-apical collar of hyphal tips.

**The localization of FgPal1 in hyphae is independent of FgKin1 and FgPom1 kinases:** In the fission yeast, protein kinases Kin1 and Pom1 phosphorylate Pal1 at distinct sites to promote its localization to the tip region of the growing end (Koyano et al., 2010; Lee et al., 2018). The *Fgkin1* and *Fgpom1* mutants generated in previous studies have defects in hyphal growth, pathogenesis, and sexual reproduction in *F. graminearum* (Wang et al., 2011; Luo et al., 2014). We also transformed the *FgPAL1*-GFP construct into the *Fgkin1* and *Fgpom1* mutants. In the resulting transformants, FgPal1-GFP still localized to the collar region at hyphal tips (Fig. S3A). Localization of FgPal1-GFP to the septum also was observed in the *Fgkin1* and *Fgpom1* mutants



(Fig. S3B). These results indicate that the localization of FgPal1 to the hyphal tip collar or septum is not affected by deletion of *FgKIN1* or *FgPOM1* in *F. graminearum*. Therefore, whether FgPal1 is phosphorylated by FgKin1 and FgPom1 or not, its localization is independent of these two protein kinases.

**Deletion of *FgPAL1* affects the distribution of nuclei in conidia and vegetative hyphae:** To determine whether deletion of *FgPAL1* affects cytokinesis and nuclear distribution, we transformed a H1-GFP fusion construct (Chen et al., 2018) into PH-1 and the *Fgpal1* mutant M8. When the resulting transformants were examined by epifluorescence microscopy, most of the wild-type conidia ( $76.3 \pm 25.4\%$ ) had one nucleus in each compartment (Fig. 7A). However, the majority ( $52.5 \pm 24.3\%$ ) of the *Fgpal1* mutant conidia had two or more nuclei per compartment. Similarly, the *Fgpal1* mutant had more nuclei ( $3.2 \pm 0.1$ ) per compartment than the wild type in vegetative hyphae ( $2.5 \pm 0.2$ ) (Fig. 7B). Calcofluor white (CFW) staining showed that the *Fgpal1* mutant still formed septa in conidia (Fig. 7A) and vegetative hyphae (Fig. 7B) but mutant conidia had fewer septa and mutant hyphae had irregular distance between septa. These results indicated that the *Fgpal1* mutant may be defective in the coordination between nuclear division and cytokinesis or suppression of nuclear division after septation during asexual reproduction or vegetative growth.

***FgPAL1* is dispensable for meiosis but important for ascospore formation:** We then examined nuclear behaviors during ascus and ascospore development in perithecia formed by the *Fgpall* H1-GFP transformant. At 5 dpf, the wild type developed asci with eight nuclei but only croziers with two nuclei or developing asci with one nucleus were observed in the *Fgpall* H1-GFP transformant (Fig. 8A). Nevertheless, many developing asci with eight nuclei were observed in the *Fgpall* mutant at 7 dpf (Fig. 8A), indicating that meiosis was delayed, likely due to the reduced growth rate associated with deletion of *FgPAL1* in *F. graminearum*.

As described above, majority of *Fgpall* asci were aborted and lacked ascospores. When examined by epifluorescence microscopy, no nuclei were observed in the aborted asci with granular cytoplasm at 12 dpf. For the few ascospores formed by the *Fgpall* mutant, they varied significantly in morphology and the number of nuclei in each ascospore (Fig. 8B; 8C). Most of the *Fgpall* ascospores had less than four nuclei and fewer than four compartments. These results indicate that *FgPAL1* plays an important role in nuclear distribution in developing asci during ascospore formation. Ascospore formation was defective in the *Fgpall* mutant, probably because of the important role played by *FgPAL1* during ascospore delimitation (cell wall formation during the free cell formation process) and the coordination between cytokinesis and post-meiotic mitosis in developing asci.

**Both FtsK and PAL1 domains are essential for the function of FgPal1:** To determine the function of the FtsK and PAL1 domains, we generated the *FgPAL1*<sup>ΔFtsK</sup> and *FgPAL1*<sup>ΔPAL1</sup> constructs and transformed them into the *Fgpal1* mutant M8. The resulting *FgPAL1*<sup>ΔFtsK</sup> and *FgPAL1*<sup>ΔPAL1</sup> transformants (Table 1), similar to the original *Fgpal1* mutant, were reduced in growth rate (Fig. 9A). They also were defective in ascospore releasing (Fig. 9B) and had defects in ascospore budding inside asci (Fig. 9C). These results indicated that both FtsK and PAL1 domains are essential for the function of FgPal1 in *F. graminearum*.

## DISCUSSION

In both budding and fission yeasts, Pal1 is related to endocytosis and cell polarity although its exact function is not clear. The Pal1 orthologs with a characteristic PAL1 domain are well conserved in fungi. To date, no proteins with the PAL1 domain have been identified in plants and metazoans. Because the PAL1 domain is unique to fungi, FgPal1 orthologs likely have fungal-specific biological functions. Furthermore, the FgPal1 protein and its orthologs from other filamentous ascomycetes also contain an FtsK domain towards the N-terminal region. The FtsK domain has a putative ATP binding P-loop motif. It is first identified in the cell

Accepted Article

division gene *ftsK* of *Escherichia coli* and assumed to be involved in DNA movement coupling with ATP hydrolysis in bacteria (Steiner et al., 1999; Stouf et al., 2013). In eukaryotic organisms, no proteins with the FtsK domain have been functionally characterized. In this study, we showed that both FtsK and PAL1 domains are essential for the functions of FgPal1 in *F. graminearum*. With both a fungal-specific PAL1 domain and a bacterial FtsK domain, FgPal1 may play a unique role in regulating hyphal growth, nuclear distribution, and differentiation in filamentous fungi.

In *F. graminearum*, the *Fgpal1* deletion mutant was defective in maintaining polarized growth at the tip and often had apical or subapical swollen bodies. In *S. cerevisiae*, the *pall* mutant forms pearl- and lemon-shaped yeast cells (Ge et al., 2005). Deletion of its ortholog in *S. pombe* also resulted in the formation of yeast cells with abnormal morphology (Ge et al., 2005). Nevertheless, the *pall* mutants still produce some normal yeast cells in both *S. cerevisiae* and *S. pombe*. Similarly, the *Fgpal1* mutant still produce cylindrical hyphae although many of them have deformed hyphal tips. Therefore, the Pal1 orthologs may have a conserved but non-essential function in the maintenance of cell shape and polarity in filamentous fungi. Nevertheless, besides the defect in hyphal tip morphology, the *Fgpal1* mutant was significantly reduced in growth rate, which is different from the *pall* mutants in *S. pombe* (Kettenbach et al., 2015). Previous studies have showed that endocytosis is related to hyphal

Accepted Article

growth in other fungi (Penalva, 2010; Steinberg, 2014). The *Fgpal1* mutant was not only defective in general endocytosis as assayed by the uptake of the lipophilic dye FM4-64 but also defective in the sub-apical localization of endocytic scaffolding protein FgEde1.

In actively growing hyphae of *F. graminearum*, FgPal1-GFP mainly localized to the collar region of hyphal tips. Treatments with Latrunculin A (Lat-A), an F-actin inhibitor that depolymerizes actin filaments, resulted in the re-localization of FgPal1-GFP to hyphal tips, suggesting that the localization of FgPal1 to the subapical collar depends on F-actins. In *S. pombe*, the localization of Pal1 to the growing end and division site is independent of F-actin (Ge et al., 2005). F-actin is required to capture the secretory vesicles (SVs) and transported them to the site of exocytosis and endocytosis for tip expansion (Brand and Gow, 2009). In transformants expressing both LifeAct-RFP and FgPal1-GFP, partial colocalization of LifeAct and FgPal1 was observed in the collar region. However, only LifeAct was observed in the very apex of hyphal tips that overlaps with the polarisome, which supports actin-filament elongation (Guo et al., 2015). Deletion of *FgPAL1* affected the apex localization of LifeAct in the *Fgpal1* LifeAct-GFP transformant. Thus, FgPal1 may affect the actin cable dynamics and transportation of vesicles to the hyphal tip in *F. graminearum*.

Ede1 is one of the early phase proteins in CME that arrive at the endocytic site and plays a key role in endocytic site initiation and maturation (Brach et al., 2014; Han et al., 2020). In

our study, deletion of *FgPAL1* affected the sub-apical localization of endocytic scaffolding protein FgEde1. The *Fgpal1* mutant was defective in general endocytosis of FM4-64. Thus, FgPal1 may affect the transportation of vesicles to the hyphal tip by both general endocytosis and clathrin-mediated endocytosis. However, *FgPAL1* is not essential for polarity establishment or maintenance because the localization of FgSpa2 to hyphal tips was not affected in the *Fgpal1* mutant. Recently, components of the FgAP-2 complex that functions as a cargo-specific adaptor for endocytosis have been reported to localize to the subapical collar in *F. graminearum* (Zhang et al., 2019). Therefore, it is important to determine the co-localization and possible functional relationship between FgPal1 and the FgAP-2 complex in endocytosis at the collar region of hyphal tips.

In eukaryotic cells, cytokinesis is the final step in the cell cycle that is regulated by septation initiation network (SIN), which triggers the contraction of the actomyosin ring and formation of the division septum. Deficiencies in SIN signaling result in cytokinesis failures and the formation of multinucleated cells (Simanis, 2015). In *F. graminearum*, the *Fgpal1* mutants were defective in the distribution of nuclei in hyphae and tended to have more nuclei per compartment than the wild type. In comparison with normal conidia with one nucleus per compartment, *Fgpal1* conidia had fewer compartments or septa but most of the compartments had more than one nucleus, likely due to its defects in septation. Therefore, *FgPAL1* likely

Accepted Article

plays a role in normal cytokinesis during growth and asexual reproduction. However, septation and formation of F-actin rings were still observed in the *Fgpall* mutant. Furthermore, FgPal1-GFP signals only became visible at the septation site in hyphae after the constriction of the actin ring and septum formation, which is different from the presence of Pal1 at the division site before actin being assembled in *S. cerevisiae* and *S. pombe* (Ge et al., 2005; Kettenbach et al., 2015).

During sexual reproduction, the *Fgpall* mutant was able to form asci with eight nuclei, indicating that FgPal1 is not essential for meiosis and the post-meiotic mitosis before the delimitation of ascospores. However, most of the developing asci became aborted and only a few of them formed ascospores in the *Fgpall* mutant, suggesting the importance of *FgPAL1* in ascospore formation or delimitation. The defect of *Fgpall* in forming fewer than 8 ascospores per ascus may be also related to its functions in the nuclear distribution and cytokinesis in developing asci. Although components of the FgAP-2 complex had similar localization with FgPal1, their mutants were normal in ascospore development (Zhang et al., 2019), suggesting that FgPal1 and the FgAP-2 complex differ significantly in functions during sexual reproduction. After meiosis, the formation of ascospore wall for each haploid nucleus or the delimitation of ascospores inside asci involves the free cell formation process that has not been well characterized at the molecular level in filamentous ascomycetes. FgPal1 and its orthologs may

play an important role in the free cell formation process for ascospore formation.

## MATERIALS AND METHODS

**Strains and culture conditions:** The wild-type strain PH-1 (Cuomo et al., 2007) and mutant strains of *F. graminearum* generated in this study were routinely cultured on complete medium (CM), SYM (1% starch, 0.6% yeast extract, 0.3% sucrose, 2% agar) (Yang et al., 2017), and 5×YEG plates at 25°C. The growth rate on CM and 5×YEG plates, conidiation in liquid carboxymethyl cellulose (CMC) cultures, and mating on carrot agar plates were assayed as previously described (Hou et al., 2002; Zhou et al., 2010; Cavinder et al., 2012; Cao et al., 2016). Protoplast preparation and polyethylene glycol (PEG)-mediated transformation were performed as described (Wang et al., 2011). Hygromycin B (CalBiochem, La Jolla, CA, USA) and geneticin (Sigma-Aldrich, St. Louis, MO, USA) were added to the final concentration of 300 and 400 µg/ml, respectively, for transformant selection (Wang et al., 2011). DON production in infected wheat kernels was assayed as described previously (Jiang et al., 2016). Conidia harvested from 5 day-old CMC cultures were used to measure the length and number of



Accepted Article

septation or nuclei in each compartment as described (Chen et al., 2014). The number of nuclei per hyphal compartment and branching distance were measured with hyphae from 24 YEPD cultures (Li et al., 2015). Mean and standard deviation were calculated with data from three independent replicates, with at least 100 conidia, 100 hyphal branches, or 50 hyphal compartments examined per replicate.

**Generation of the *Fgpall* deletion mutant and complemented transformant:** The same primers used in the previous study (Hao et al., 2019) were used to generate additional *Fgpall* gene replacement mutants with the split-marker approach. For complementation assays, the *FgPALI* gene with its full length promoter region was amplified with primer pairs *FgPALINC/1F- FgPALINC/2R* and co-transformed with *XhoI*-digested pDL2 into yeast strain XK1-25 to generate the *FgPALI*-GFP construct by yeast gap repair as described (Bruno et al., 2004; Zhou et al., 2010). After verification by sequencing analysis, the *FgPALI*-GFP construct was transformed into the *Fgpall*, *Fgkin1* (Luo et al., 2014) and *Fgpom1* (Wang et al., 2011) mutants. Transformants expressing the *FgPALI*-GFP construct were identified by PCR and examined for GFP signals by epifluorescence microscopy. The same yeast gap repair approach (Bruno et al., 2004; Zhou et al., 2010) was used to generate the *FgPALI*<sup>ΔFtsK</sup> and *FgPALI*<sup>ΔPAL1</sup> alleles deleted of the FtsK and PAL1 domains with primers listed in Table S1. The resulting

*FgPALI*<sup>ΔPALI</sup> and *FgPALI*<sup>ΔFtsK</sup> alleles were verified by sequencing analysis and transformed into the *Fgpall* mutant M8.

The H1-GFP construct (Luo et al., 2014) was transformed into the wild-type strain PH-1 and the *Fgpall* mutant to observe nuclei. The FgSpa2-GFP construct was generated by yeast gap repair as described (Bruno et al., 2004, Zhou et al., 2010) and transformed into the wild-type strain PH-1 and the *Fgpall* mutant to observe the polarisome (Zhang et al., 2019). Cell wall and septa were visualized by staining conidia or hyphae with 10 μg ml<sup>-1</sup> CFW (Calcofluor White) for 2 min and observed with an Olympus BX-53 microscope as described (Luo et al., 2014; Li et al., 2015)

**Generation of the LifeAct-RFP *FgPALI*-GFP transformant:** The LifeAct fragment consisting of the first 17 amino acid residues of yeast actin-binding protein Abp140 is a F-actin marker in eukaryotes (Lopata et al., 2018). The LifeAct-RFP construct (Zhang et al., 2019) was co-transformed with *FgPALI*-GFP into the wild-type strain PH-1. Transformants resistant to geneticin were isolated and screened by PCR. Transformants expressing both LifeAct-RFP and *FgPALI*-GFP were further confirmed by examination for RFP and GFP signals. The co-localization of LifeAct-RFP and FgPal1-GFP was observed with a Nikon A1 confocal microscope. The excitation and emission wavelengths for GFP were 488 nm and 500-550 nm.

Accepted Article

For RFP, the excitation and emission wavelengths were 561 nm and 570-620 nm (Petre et al., 2015; Boenisch et al., 2019). For assaying the role of actin on the localization of FgPal1-GFP, vegetative hyphae harvested from SYM cultures were treated with 0.1  $\mu\text{g/ml}$  Lat-A (Sigma-Aldrich, USA) in DMSO for 10-30 min (Ge et al., 2005) before examination by epifluorescence microscopy.

**Assays for virulence and infectious growth:** Conidia were harvested from 5-day-old CMC cultures and resuspended to  $2.0 \times 10^5$  conidia per ml in sterile distilled water (Hou et al., 2002). Wheat heads of cultivar Xiaoyan 22 were drop-inoculated and examined for FHB symptoms as described (Zhang et al., 2017). Spikelets with typical head blight symptoms in each head were examined at 14 dpi to estimate the disease index (Ding et al., 2009). Infection assays with corn silks were conducted as described (Hou et al., 2002). All the infection assays were repeated at least three times.

For assaying infectious growth, wheat lemmas and rachis tissues were collected from inoculated wheat heads and fixed with 4% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer (pH 6.8) overnight at 4°C, dehydrated, and embedded in Spurr resin as described (Kang et al., 2008). Thick sections (1  $\mu\text{m}$ ) were stained with 0.5% (wt/vol) toluidine blue and examined with an Olympus BX-53 microscope as described (Zheng et al., 2012). At least three

independent biologic replicates were examined for the wild-type and mutant strains.

**Yeast two-hybrid assays:** The HybriZAP bait and prey system (Delgado-Álvarez et al., 2010; Zhou et al., 2012) was used to detect the interaction of FgPal1 with FgAma1. In brief, the bait construct of *FgPAL1* was generated by cloning the full-length *FgPAL1* ORF with primers Pal1AD/F and Pal1AD/R (Table S1) into pAD-GAL4. The full-length FgAma1 was amplified with primers Ama1BD/F and Ama1BD/R (Table S1) and cloned into pBD-GAL4-2 as the prey construct. The resulting bait and prey constructs were co-transformed into the yeast strain YRG2 as described (Delgado-Álvarez et al., 2010; Zhou et al., 2012). The resulting Leu<sup>+</sup> and Trp<sup>+</sup> transformants were assayed for growth on SD-Trp-Leu-His medium and the expression of the LacZ reporter as described (Li et al., 2017).

#### ACKNOWLEDGEMENTS

We thank Dr. Cong Jiang and Dr. Huiquan Liu for fruitful discussion and Dr. Larry Dunkle for critical reading of this manuscript. We also thank Dr. Hua Zhao (State Key Laboratory of Crop Stress Biology for Arid Areas, Northwest A&F University, Yangling, China) for microscope experimental assistance. This work was supported by a grant to Guanghui Wang from the National Natural Science Foundation of China (No. 31801684), a grant to Ping Xiang from Key

Research and Invention Program in Shaanxi Province of China (2018NY-107), and a grant to  
JRX from USWBSI.

Accepted Article

## TABLES AND FIGURES

**Table 1. Wild-type and mutant strains of *Fusarium graminearum* used in this study**

Strains	Brief descriptions	Reference
PH-1	Wild-type	(Cuomo et al., 2007)
P8	<i>Fgpal1</i> deletion mutant of PH-1	(Hao et al., 2019)
K1	<i>Fgkin1</i> deletion mutant of PH-1	(Luo et al., 2014)
P1	<i>Fgpom1</i> deletion mutant of PH-1	(Wang et al., 2011)
M8	<i>Fgpal1</i> deletion mutant of PH-1	This study
M9	<i>Fgpal1</i> deletion mutant of PH-1	This study
C6	<i>Fgpal1/FgPAL1</i> -GFP transformant of M8 *	This study
C16	<i>Fgpal1/FgPAL1</i> -GFP transformant of M8	This study
PK1	<i>FgPAL1</i> -GFP transformant of K1	This study
PP1	<i>FgPAL1</i> -GFP transformant of P1	This study
PP4	<i>FgPAL1</i> -GFP transformant of P1	This study
PA1	LifeAct-RFP transformant of PH-1	This study
MA1	LifeAct-RFP transformant of M8	This study
PL6	<i>FgPAL1</i> -GFP transformant of PA1	This study
PL21	<i>FgPAL1</i> -GFP transformant of PA-1	This study
PH1-H	H1-GFP transformant of PH-1	This study
MH1-H	H1-GFP transformant of M8	This study
PS1	<i>FgSPA2</i> -GFP transformant of PH-1	This study
MS1	<i>FgSPA2</i> -GFP transformant of M8	This study
PAG1	LifeAct-GFP transformant of PH-1	This study
MAG1	LifeAct-GFP transformant of M8	This study
F7	<i>FgPAL1</i> <sup>ΔFtsK</sup> in M8	This study
F8	<i>FgPAL1</i> <sup>ΔFtsK</sup> in M8	This study
P3	<i>FgPAL1</i> <sup>ΔPAL1</sup> in M8	This study
P5	<i>FgPAL1</i> <sup>ΔPAL</sup> in M8	This study

PE1	<i>FgEDE1</i> -GFP transformant of PH-1	This study
ME1	<i>FgEDE1</i> -GFP transformant of M8	This study

\* All the GFP and RFP fusion constructs were integrated ectopically in the genome.

**Table 2. Defects of the *Fgpal1* mutant in growth, conidiation, plant infection**

Strain	Growth rate (mm/d) <sup>a</sup>		Conidiation(10 <sup>5</sup> spores/ml) <sup>b</sup>	Disease index <sup>c</sup>
	CM	5×YEG		
PH-1	8.9±0.2 <sup>A</sup>	7.4±0.4 <sup>A</sup>	15.2±1.6 <sup>A</sup>	8.7±1.7 <sup>A</sup>
M8	3.4±0.0 <sup>B</sup>	3.2±0.1 <sup>B</sup>	11.3±1.7 <sup>B</sup>	1.0±0.0 <sup>B</sup>
C16	8.7±0.0 <sup>A</sup>	6.9±0.1 <sup>A</sup>	14.5±1.8 <sup>A</sup>	8.4±1.5 <sup>A</sup>

<sup>a</sup> Growth rate was measured as the average daily expansion of colony radius after incubation on CM for 3 days.

<sup>b</sup> Number of conidia per ml in 5-day-old CMC cultures.

<sup>c</sup> Disease index was rated as the number of diseased spikelets per head at 14 dpi.

Means ± SE were calculated from the results of three independent experiments. At least 10 wheat heads were examined in each repeat.

**Table S1. The primers used in this study**



**Fig. 1. Domain structure of FgPal1 and growth defects of the *Fgpall1* mutant.**

(A) Schematic drawing of the conserved domains in FgPal1. The FtsK and Pal1 domains are shaded in green and blue, respectively. The red circle marks the premature stop codon subjected to A-to-I editing. Sequence alignment of FgPal1 and its orthologs from *Fusarium verticillioides* (Fv), *Neurospora crassa* (Nc), *Botrytis cinerea* (Bc), Clustal consensus (Cc) with the identical (\*) or similar (·) amino acid residues marked. (B) Three-day-old CM and 5×YEG cultures of the wild type (PH-1), *Fgpall1* mutant (M8), and complemented transformant (C16). (C) CM cultures of PH-1, M8, and C16 were examined for hyphal tip growth and branching. Arrows point to apical or subapical swelling. Bar = 20 μm.

**Fig. 2. Defects of the *Fgpall1* mutant in conidium morphology and sexual reproduction.**

(A) Conidia produced by PH-1 (WT), *Fgpall1* mutants (M8), and *Fgpall1/FgPAL1-GFP* complemented transformant (C16) in CMC cultures. Bar = 10 μm. (B) Two-week-old mating cultures were examined for perithecia formation and ascospore cirrhi (marked with arrows). (C) Ascospore discharge was assayed with perithecia of the same set of strains collected at 7 dpf. (D) Asci and ascospores formed by PH-1, mutant M8, and complemented strain C16. The inset is a close-up view of the budding ascospore in mutant M8. Bar = 10 μm.

**Fig. 3. Defects of the *Fgpall* mutant in plant infection.**

(A) Flowering wheat heads inoculated with the wild type (PH-1), *Fgpall* mutants (M8), and complemented transformant (C16) were photographed at 14 dpi. Black dots marked the inoculation sites. (B) Corn silks inoculated with the same set of strains were photographed at 5 dpi. (C) Thick-sections of wheat lemma inoculated with PH-1 and the *Fgpall* mutant were stained with 0.5% (wt/vol) toluidine blue and examined for infectious growth at 48 hpi. (D) Thick-sections of the rachis tissues right below the inoculated spikelets were examined at 120 hpi. For C and D, invasive hyphae were marked with arrows. Bar = 20  $\mu$ m.

**Fig. 4. Localization of FgPal1-GFP to the subapical region.**

(A) Hyphal tips of the *Fgpall*/*FgPALI*-GFP transformant was examined for GFP signals after incubation in SYM medium for 12 h. Bar = 10  $\mu$ m. (B) Germ tubes of the *FgPALI*-GFP LifeAct-RFP transformant were examined by co-focal microscopy. Partial co-localization of FgPal1 and LifeAct was observed at the sub-apical collar region of actively growing hyphae. Bar = 20  $\mu$ m. (C) Vegetative hyphae of the *Fgpall*/*FgPALI*-GFP transformant were treated with Lat-A for 10 min and examined by epifluorescence microscopy. Bar = 20  $\mu$ m. (D) Hyphae of the *Fgpall*/LifeAct-RFP and PH-1/LifeAct-RFP transformant were examined by epifluorescence microscopy. Bar = 20  $\mu$ m.

**Fig. 5 Defects of the *Fgpall* mutant in endocytosis**

(A) Hyphae of the wild type (PH-1) and *Fgpall* mutants (M8) were stained with FM4-64 for 10, 20, or 30 min before being examined by epifluorescence microscopy. Bar = 10  $\mu$ m. (B) Hyphal tips of the *Fgpall*/Ede1-GFP and PH-1/Ede1-GFP transformants were examined by epifluorescence microscopy. Bar = 10  $\mu$ m.

**Fig. 6. Co-localization of FgPal1-GFP and LifeAct-RFP to the septum.**

(A) Hyphae of the *FgPAL1*-GFP and LifeAct-RFP transformant PL1 were examined by epifluorescence microscopy. Bar = 20  $\mu$ m. (B) A time course (0 - 132 seconds) to show the co-localization of LifeAct and FgPal1 during septum formation. Bar = 20  $\mu$ m. (C) Vegetative hyphae of the *Fgpall*/*FgPAL1*-GFP transformant were treated with Lat-A for 10 min and examined by epifluorescence microscopy. Bar = 20  $\mu$ m. (D) Hyphae of the *Fgpall*/LifeAct-RFP and PH-1/LifeAct-RFP transformant were examined by epifluorescence microscopy. Bar = 20  $\mu$ m.

**Fig. 7. Defects of the *Fgpall* mutant in the distribution of nuclei and septa.**

(A) Conidia and (B) vegetative hyphae of transformants of PH-1 and the *Fgpall* mutant (M8)

expressing the H1-GFP construct were stained with Calcofluor white (CFW) and examined by DIC and epifluorescence microscopy. Arrows point to the septum. Bar = 20  $\mu$ m.

**Fig. 8. Defects of the *Fgpall* mutant in the distribution of nuclei during sexual reproduction.**

(A) Perithecia formed by H1-GFP transformants of PH-1 and the *Fgpall* mutant were examined for ascogenous tissues at 5 or 7 dpf. Bar = 20  $\mu$ m. (B) The percentage of wild-type or *Fgpall* asci with the indicated number of ascospores per ascus in perithecia at 12-dpf. Vast majority of *Fgpall* asci had only one ascospore. (C) Asci and ascospores formed by H1-GFP transformants of PH-1 and the *Fgpall* mutant were examined for GFP signals at 12-dpf.

**Fig. 9. Defects of the *FgPALI* <sup>$\Delta$ PAL1</sup> and *FgPALI* <sup>$\Delta$ FtsK</sup> mutants in vegetative growth, conidium morphology and sexual reproduction.**

(A) Three-day-old PDA cultures of the wild-type (PH-1), *Fgpall* (M8), *FgPALI* <sup>$\Delta$ PAL1</sup> (F7), and *FgPALI* <sup>$\Delta$ FtsK</sup> (P3) mutant strains. (B) Perithecia of the same set of strains were assayed for ascospore discharge (upper row) and cirrhous formation (lower row). (D) Asci and ascospores formed by the marked strains. Similar to the *Fgall* mutant, the *FgPALI* <sup>$\Delta$ PAL1</sup> and *FgPALI* <sup>$\Delta$ FtsK</sup> mutants had ascospore budding defects. Bar = 20  $\mu$ m.

## Supplemental Figures

### **Fig. S1. Yeast two hybrid assays for the interaction of FgAma1 with FgPal1.**

Yeast cells of transformants expressing the FgPal1 prey and/or FgAma1 bait constructs were assayed for growth on control SD-Leu-Trp-His (left) and  $\beta$ -galactosidase activities (right). The positive and negative controls were from the HybriZAP kit.

### **Fig. S2. Localization of FgSpa2-GFP to the hyphal tip.**

Hyphal tips of transformants of the wild type (PH-1) and *Fgpal1* mutant expressing the *FgSPA2*-GFP fusion construct were examined by epifluorescence microscopy. Bar = 20  $\mu$ m

### **Fig. S3. Localization of FgPal1 to the hyphal tips and septa in the *Fgpom1* and *Fgkin1* mutant.**

Freshly harvested germlings of the *Fgkin1*/*FgPAL1*-GFP and *Fgpom1*/*FgPAL1*-GFP transformants were examined by DIC and epifluorescence microscopy. Bar = 20  $\mu$ m.

## REFERENCES

- Araujo-Bazán, L., Peñalva, M. A. and Espeso, E. A. (2008) Preferential localization of the endocytic internalization machinery to hyphal tips underlies polarization of the actin cytoskeleton in *Aspergillus nidulans*. *Mol. Microbiol.*, **67**, 891-905.
- Bai, G. H. and Shaner, G. (2004) Management and resistance in wheat and barley to Fusarium head blight. *Annu. Rev. Phytopathol.*, **42**, 135-161.
- Berepiki, A., Lichius, A. and Read, N. D. (2011) Actin organization and dynamics in filamentous fungi. *Nat. Rev. Microbiol.*, **9**, 876-887.
- Bian, Z., Ni, Y., Xu, J.-R. and Liu, H. (2019) A-to-I mRNA editing in fungi: occurrence, function, and evolution. *Cell. Mol. Life Sci.*, **76**, 329-340.
- Boenisch, M. J., Blum, A., Broz, K. L., Gardiner, D. M. and Kistler, H. C. (2019) Nanoscale enrichment of the cytosolic enzyme trichodiene synthase near reorganized endoplasmic reticulum in *Fusarium graminearum*. *Fungal Genet. Biol.*, **124**, 73-77.
- Boenisch, M. J. and Schäfer, W. (2011) *Fusarium graminearum* forms mycotoxin producing infection structures on wheat. *BMC Plant Biol.*, **11**, 110-110.
- Brach, T., Godlee, C., Moeller-Hansen, I., Boeke, D., and Kaksonen, M. (2014) The initiation of clathrin-mediated endocytosis is mechanistically highly flexible. *Curr Biol* **24**: 548-554.
- Brand, A. and Gow, N. A. R. (2009) Mechanisms of hypha orientation of fungi. *Curr. Opin. Microbiol.*, **12**, 350-357.
- Bruno, K. S., Tenjo, F., Li, L., Hamer, J. E. and Xu, J. R. (2004) Cellular localization and role of kinase activity of PMK1 in *Magnaporthe grisea*. *Eukaryotic cell*, **3**, 1525-1532.
- Cao, S., He, Y., Hao, C., Xu, Y., Zhang, H., Wang, C., *et al.* (2017) RNA editing of the *AMD1* gene is important for ascus maturation and ascospore discharge in *Fusarium graminearum*. *Sci. Rep.*, **7**, 4617.
- Cao, S. L., Zhang, S. J., Hao, C. F., Liu, H. Q., Xu, J. R. and Jin, Q. J. (2016) FgSsn3 kinase, a component of the mediator complex, is important for sexual reproduction and pathogenesis in *Fusarium graminearum*. *Sci. Rep.*, **6**, 22333.
- Carroll, S. Y., Stimpson, H. E., Weinberg, J., Toret, C. P., Sun, Y. and Drubin, D. G. (2012) Analysis of yeast endocytic site formation and maturation through a regulatory transition point. *Mol. Biol. Cell*, **23**, 657-668.
- Cavinder, B., Sikhakolli, U., Fellows, K. M. and Trail, F. (2012) Sexual Development and Ascospore Discharge in *Fusarium graminearum*. *Jove-J Vis Exp*, doi: 10.3791/3895.
- Chen, D., Wang, Y., Zhou, X., Wang, Y., and Xu, J.-R. (2014) The Sch9 kinase regulates conidium size, stress responses, and pathogenesis in *Fusarium graminearum*. *PLoS one* **9**: e105811-e105811.
- Chen, D., Wu, C., Hao, C., Huang, P., Liu, H., Bian, Z., *et al.* (2018) Sexual specific functions of

Tub1 beta-tubulins require stage-specific RNA processing and expression in *Fusarium graminearum*. *Environ. Microbiol.*, **20**, 4009-4021.

- Cooper, K. F., Mallory, M. J., Egeland, D. B., Jarnik, M. and Strich, R. (2000) Ama1p is a meiosis-specific regulator of the anaphase promoting complex/cyclosome in yeast. *Proc. Natl. Acad. Sci. U. S. A.*, **97**, 14548-14553.
- Cuomo, C. A., Gueldener, U., Xu, J. R., Trail, F., Turgeon, B. G., Di Pietro, A., *et al.* (2007) The *Fusarium graminearum* genome reveals a link between localized polymorphism and pathogen specialization. *Science*, **317**, 1400-1402.
- Delgado-Álvarez, D. L., Callejas-Negrete, O. A., Gómez, N., Freitag, M., Roberson, R. W., Smith, L. G., *et al.* (2010) Visualization of F-actin localization and dynamics with live cell markers in *Neurospora crassa*. *Fungal Genet. Biol.*, **47**, 573-586.
- Ding, S. L., Mehrabi, R., Koten, C., Kang, Z. S., Wei, Y. D., Seong, K. Y., *et al.* (2009) Transducin Beta-Like Gene *FTL1* Is Essential for Pathogenesis in *Fusarium graminearum*. *Eukaryotic cell*, **8**, 867-876.
- Fischer-Parton, S., Parton, R. M., Hickey, P. C., Dijksterhuis, J., Atkinson, H. A. and Read, N. D. (2000) Confocal microscopy of FM4-64 as a tool for analysing endocytosis and vesicle trafficking in living fungal hyphae. *J. Microsc.*, **198**, 246-259.
- Ge, W. Z., Chew, T. G., Wachtler, V., Naqvi, S. N. and Balasubramanian, M. K. (2005) The novel fission yeast protein Pal1p interacts with Hip1-related Sla2p/End4p and is involved in cellular morphogenesis. *Mol. Biol. Cell*, **16**, 4124-4138.
- Goswami, R. S. and Kistler, H. C. (2004) Heading for disaster: *Fusarium graminearum* on cereal crops. *Mol. Plant Pathol.*, **5**, 515-525.
- Guo, M., Kilaru, S., Schuster, M., Latz, M. and Steinberg, G. (2015) Fluorescent markers for the Spitzenkörper and exocytosis in *Zygozooporia tritici*. *Fungal Genet Biol*, **79**, 158-165.
- Han, X., Chen, L., Li, W., Zhang, L., Zhang, L., Zou, S. *et al.* (2020) Endocytic FgEde1 regulates virulence and autophagy in *Fusarium graminearum*. *Fungal Genet Biol* **141**: 103400.
- Hane, J. K., Williams, A. H., Taranto, A. P., Solomon, P. S. and Oliver, R. P. (2015) Repeat-Induced Point Mutation: a fungal-specific, endogenous mutagenesis process. In: *Genetic Transformation Systems in Fungi, Volume 2*. (van den Berg, M. A. and Maruthachalam, K., eds.). Cham: Springer International Publishing, pp. 55-68.
- Hao, C., Yin, J., Sun, M., Wang, Q., Liang, J., Bian, Z., *et al.* (2019) The meiosis-specific APC activator *FgAMA1* is dispensable for meiosis but important for ascosporeogenesis in *Fusarium graminearum*. *Mol. Microbiol.*, **111**, 1245-1262.
- Hou, Z. M., Xue, C. Y., Peng, Y. L., Katan, T., Kistler, H. C. and Xu, J. R. (2002) A mitogen-activated protein kinase gene (*MGVI*) in *Fusarium graminearum* is required for female fertility, heterokaryon formation, and plant infection. *Mol. Plant-Microbe Interact.*,

15, 1119-1127.

- Jiang, C., Zhang, C., Wu, C., Sun, P., Hou, R., Liu, H. et al. (2016) *TRI6* and *TRI10* play different roles in the regulation of deoxynivalenol (DON) production by cAMP signalling in *Fusarium graminearum*. *Environ Microbiol* **18**: 3689-3701.
- Jiang, C., Cao, S., Wang, Z., Xu, H., Liang, J., Liu, H., et al. (2019) An expanded subfamily of G-protein-coupled receptor genes in *Fusarium graminearum* required for wheat infection. *Nat. Microbiol.*, **4**, 1582-1591.
- Kang, Z. S., Buchenauer, H., Huang, L. L., Han, Q. M. and Zhang, H. C. (2008) Cytological and immunocytochemical studies on responses of wheat spikes of the resistant Chinese cv. Sumai 3 and the susceptible cv. Xiaoyan 22 to infection by *Fusarium graminearum*. *Eur J Plant Pathol*, **120**, 383-396.
- Kettenbach, A. N., Deng, L., Wu, Y. J., Baldissard, S., Adamo, M. E., Gerber, S. A., et al. (2015) Quantitative phosphoproteomics reveals pathways for coordination of cell growth and division by the conserved fission yeast kinase Pom1. *Mol. Cell. Proteomics*, **14**, 1275-1287.
- Kim, H.-K., Jo, S.-M., Kim, G.-Y., Kim, D.-W., Kim, Y.-K. and Yun, S.-H. (2015) A Large-scale functional analysis of putative target genes of mating-type loci provides insight into the regulation of sexual development of the cereal pathogen *Fusarium graminearum*. *PLoS Genet*, **11**, e1005486.
- Koyano, T., Kume, K., Konishi, M., Toda, T. and Hirata, D. (2010) Search for kinases related to transition of growth polarity in fission yeast. *Biosci Biotech Bioch*, **74**, 1129-1133.
- Lee, M. E., Rusin, S. F., Jenkins, N., Kettenbach, A. N. and Moseley, J. B. (2018) Mechanisms connecting the conserved protein kinases Ssp1, Kin1, and Pom1 in fission yeast cell polarity and division. *Current Biology*, **28**, 84-92.
- Li, C., Melesse, M., Zhang, S., Hao, C., Wang, C., Zhang, H., et al. (2015) *FgCDC14* regulates cytokinesis, morphogenesis, and pathogenesis in *Fusarium graminearum*. *Mol. Microbiol.*, **98**, 770-786.
- Li, G., Zhang, X., Tian, H., Choi, Y.E., Tao, W.A., and Xu, J.R. (2017) *MST50* is involved in multiple MAP kinase signaling pathways in *Magnaporthe oryzae*. *Environ Microbiol* **19**: 1959-1974.
- Liu, H., Wang, Q., He, Y., Chen, L., Hao, C., Jiang, C., et al. (2016) Genome-wide A-to-I RNA editing in fungi independent of ADAR enzymes. *Genome Res.*, **26**, 499-509.
- Lopata, A., Hughes, R., Tiede, C., Heissler, S. M., Sellers, J. R., Knight, P. J., et al. (2018) Affimer proteins for F-actin: novel affinity reagents that label F-actin in live and fixed cells. *Sci. Rep.*, **8**, 6572.
- Lu, R., and Drubin, D.G. (2017) Selection and stabilization of endocytic sites by Ede1, a yeast functional homologue of human Eps15. *Molecular biology of the cell* **28**: 567-575.



- Luo, Y. P., Zhang, H. C., Qi, L. L., Zhang, S. J., Zhou, X. Y., Zhang, Y. M., *et al.* (2014) FgKin1 kinase localizes to the septal pore and plays a role in hyphal growth, ascospore germination, pathogenesis, and localization of Tub1 beta-tubulins in *Fusarium graminearum*. *New Phytol.*, **204**, 943-954.
- Markell, S. G. and Francl, L. J. (2003) *Fusarium* head blight inoculum: Species prevalence and *Gibberella zeae* spore type. *Plant Dis.*, **87**, 814-820.
- Miedaner, T., Cumagun, C. J. R. and Chakraborty, S. (2008) Population genetics of three important head blight pathogens *Fusarium graminearum*, *F. pseudograminearum* and *F. culmorum*. *J. Phytopathol.*, **156**, 129-139.
- Nakayashiki, H. (2005) RNA silencing in fungi: mechanisms and applications. *FEBS Letters*, **579**, 5950-5957.
- Penalva, M. A. (2010) Endocytosis in filamentous fungi: Cinderella gets her reward. *Curr. Opin. Microbiol.*, **13**, 684-692.
- Petre, B., Saunders, D. G. O., Sklenar, J., Lorrain, C., Win, J., Duplessis, S., *et al.* (2015) Candidate effector proteins of the rust pathogen *Melampsora larici-populina* Target Diverse Plant Cell Compartments. *Mol. Plant-Microbe Interact.*, **28**, 689-700.
- Seiler, S. and Heilig, Y. (2019) Septum formation and cytokinesis in ascomycete fungi. In: *Biology of the Fungal Cell*. (Hoffmeister, D. and Gressler, M., eds.). Cham: Springer International Publishing, pp. 15-42.
- Simanis, V. (2015) Pombe's thirteen - control of fission yeast cell division by the septation initiation network. *J. Cell Sci.*, **128**, 1465-1474.
- Son, H., Min, K., Lee, J., Raju, N. B. and Lee, Y.-W. (2011) Meiotic silencing in the homothallic fungus *Gibberella zeae*. *Fungal Biol.*, **115**, 1290-1302.
- Steinberg, G. (2014) Endocytosis and early endosome motility in filamentous fungi. *Curr. Opin. Microbiol.*, **20**, 10-18.
- Steiner, W., Liu, G., Donachie, W. D. and Kuempel, P. (1999) The cytoplasmic domain of FtsK protein is required for resolution of chromosome dimers. *Mol. Microbiol.*, **31**, 579-583.
- Stouf, M., Meile, J.-C. and Cornet, F. (2013) FtsK actively segregates sister chromosomes in *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.*, **110**, (27) 11157-11162.
- Takeshita, N. and Fischer, R. (2019) The cytoskeleton and polarity markers during polarized growth of filamentous fungi. In: *Biology of the Fungal Cell*. (Hoffmeister, D. and Gressler, M., eds.). Cham: Springer International Publishing, pp. 43-62.
- Teichert, I., Dahlmann, T. A., Kück, U. and Nowrousian, M. (2017) RNA editing during sexual development occurs in distantly related filamentous ascomycetes. *Genome Biol. Evol.*, **9**, 855-868.
- Trail, F., Xu, H. X., Loranger, R. and Gadoury, D. (2002) Physiological and environmental

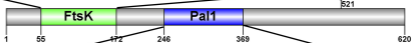
aspects of ascospore discharge in *Gibberella zeae* (anamorph *Fusarium graminearum*). *Mycologia*, **94**, 181-189.

- Van de Walle, J., Sargent, T., Piront, N., Toussaint, O., Schneider, Y. J. and Larondelle, Y. (2010) Deoxynivalenol affects *in vitro* intestinal epithelial cell barrier integrity through inhibition of protein synthesis. *Toxicol. Appl. Pharmacol.*, **245**, 291-298.
- Wang, C. F., Zhang, S. J., Hou, R., Zhao, Z. T., Zheng, Q., Xu, Q. J., *et al.* (2011) Functional analysis of the kinome of the wheat scab fungus *Fusarium graminearum*. *PLoS pathogens*, **7** (12): e1002460.
- Yang, C. D., Dang, X., Zheng, H. W., Chen, X. F., Lin, X. L., Zhang, D. M., *et al.* (2017) Two Rab5 homologs are essential for the development and pathogenicity of the rice blast fungus *Magnaporthe oryzae*. *Frontiers in Plant Science*, **8**. DOI: 0.3389/fpls.2017.00620
- Zhang, H., Van der Lee, T., Waalwijk, C., Chen, W. Q., Xu, J., Xu, J. S., *et al.* (2012) Population analysis of the *Fusarium graminearum* species complex from wheat in China show a shift to more aggressive isolates. *PLoS one*, **7** (2): e31722.
- Zhang, J., Yun, Y., Lou, Y., Abubakar, Y. S., Guo, P., Wang, S., *et al.* (2019) FgAP-2 complex is essential for pathogenicity and polarised growth and regulates the apical localisation of membrane lipid flippases in *Fusarium graminearum*. *Cell. Microbiol.*, **21**, e13041.
- Zhang, Y. M., Gao, X. L., Sun, M. L., Liu, H. Q. and Xu, J. R. (2017) The *FgSRP1* SR-protein gene is important for plant infection and pre-mRNA processing in *Fusarium graminearum*. *Environ. Microbiol.*, **19**, 4065-4079.
- Zheng, D. W., Zhang, S. J., Zhou, X. Y., Wang, C. F., Xiang, P., Zheng, Q., *et al.* (2012) The *FgHOG1* pathway regulates hyphal growth, stress responses, and plant infection in *Fusarium graminearum*. *PLoS one*, **7** (11): e49495.
- Zhou, X., Zhang, H., Li, G., Shaw, B. and Xu, J.-R. (2012) The Cyclase-associated protein Cap1 is important for proper regulation of infection-related morphogenesis in *Magnaporthe oryzae*. *PLoS Pathog*, **8**, e1002911.
- Zhou, X. Y., Heyer, C., Choi, Y. E., Mehrabi, R. and Xu, J. R. (2010) The *CID1* cyclin C-like gene is important for plant infection in *Fusarium graminearum*. *Fungal Genet. Biol.*, **47**, 143-151.

**A**

60 70 80 90 100 110 120 130 140 150 160 170

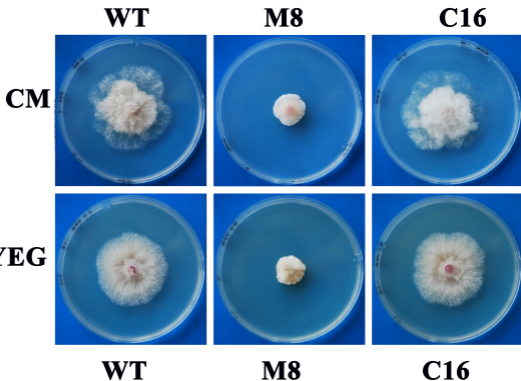
Fv DDPFRLSRNPFLLDPPVLLSQPLRS---PGAMSSHDSKSLAEETIINSMFLDDNHNDAKCCARRRNCILDPGGCFLDGLRFRPERSQEEALRARNMG--GIGPF  
 Fg DDPFPRLSRNPFLLDPPVLLSQPLRS---PGAMSSHDSKSLAEETIINSMFLDDNHNDAKCCARRRNCILDPGGCFLDGLRFRPERSQEEALRARNMG--GIGPF  
 Nc DDPFPRLSRNPFLLDPPVLLSQPLRS---PGAMSSHDSKSLAEETIINSMFLDDNHNDAKCCARRRNCILDPGGCFLDGLRFRPERSQEEALRARNMG--GIGPF  
 Bc DDPFPRLSRNPFLLDPPVLLSQPLRS---PGAMSSHDSKSLAEETIINSMFLDDNHNDAKCCARRRNCILDPGGCFLDGLRFRPERSQEEALRARNMG--GIGPF  
 Cc DDPFPRLSRNPFLLDPPVLLSQPLRS---PGAMSSHDSKSLAEETIINSMFLDDNHNDAKCCARRRNCILDPGGCFLDGLRFRPERSQEEALRARNMG--GIGPF

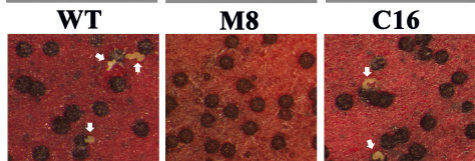
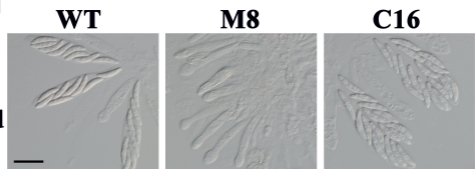
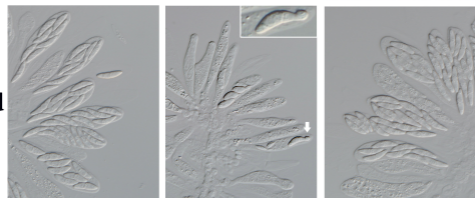
**FgPal1**

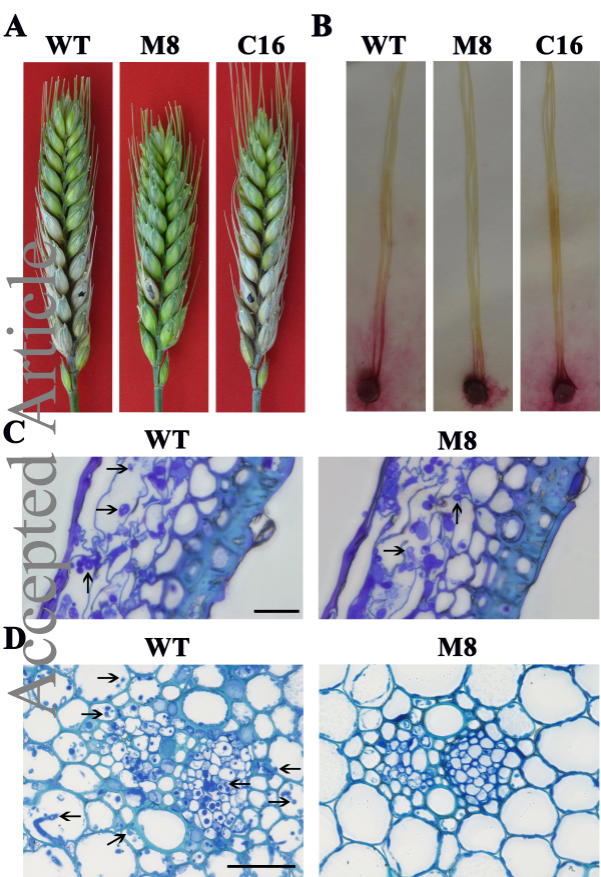
250 260 270 280 290 300 310 320 330 340 350 360 370

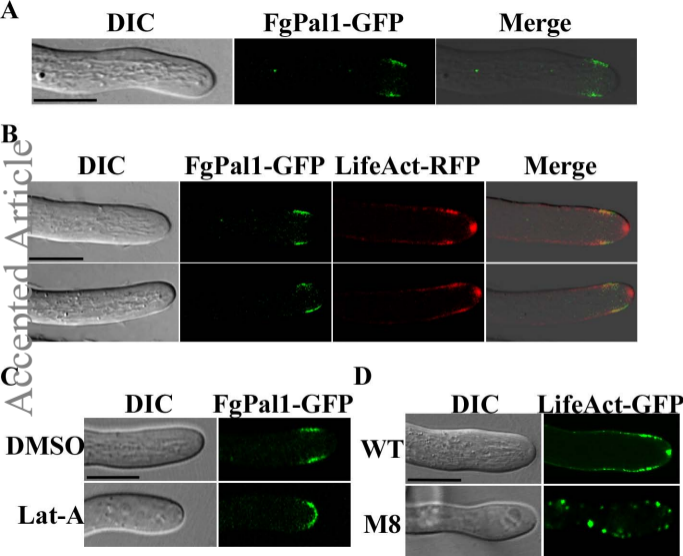
Fv RRRKQSEKRRPSKMDIIDQLDQATSIYVCTFPHDGGFDALNPERNNARRAFMQAFPKDLSNNSLGGAGPLNARVHSLVMGNATDRAVDPFSAT---SRRSHKFAIF  
 Fg RRRKQSEKRRPSKMDIIDQLDQATSIYVCTFPHDGGFDALNPERNNARRAFMQAFPKDLSNNSLGGAGPLNARVHSLVMGNATDRAVDPFSAT---SRRSHKFAIF  
 Nc RRRKQSEKRRPSKMDIIDQLDQATSIYVCTFPHDGGFDALNPERNNARRAFMQAFPKDLSNNSLGGAGPLNARVHSLVMGNATDRAVDPFSAT---SRRSHKFAIF  
 Bc RRRKQSEKRRPSKMDIIDQLDQATSIYVCTFPHDGGFDALNPERNNARRAFMQAFPKDLSNNSLGGAGPLNARVHSLVMGNATDRAVDPFSAT---SRRSHKFAIF  
 Cc RRRKQSEKRRPSKMDIIDQLDQATSIYVCTFPHDGGFDALNPERNNARRAFMQAFPKDLSNNSLGGAGPLNARVHSLVMGNATDRAVDPFSAT---SRRSHKFAIF

Accepted Article



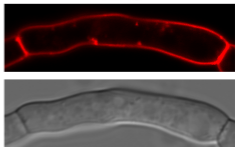
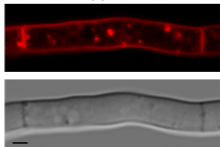
**A****B****C****D****10d**



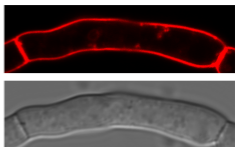
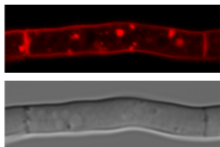


**A****WT****M8**

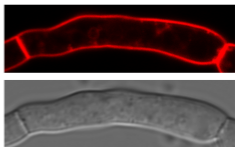
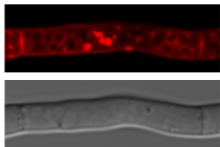
10min



20min



30min



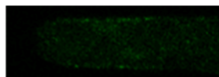
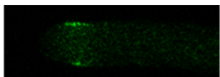
Accepted Article

**B****FgEde1-GFP/PH-1****FgEde1-GFP/*Fgpall1***

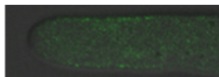
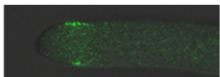
DIC

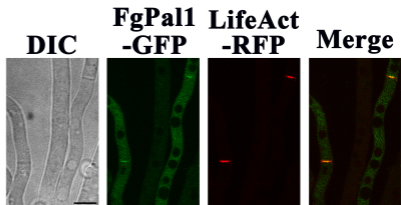


GFP

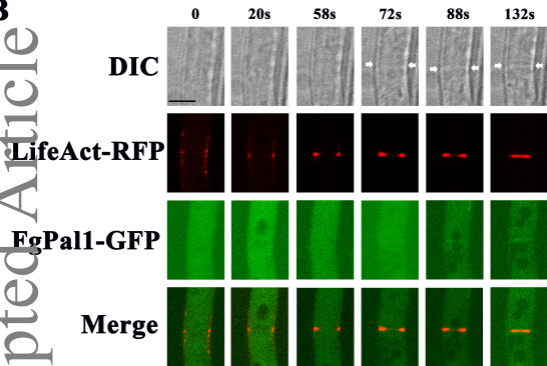
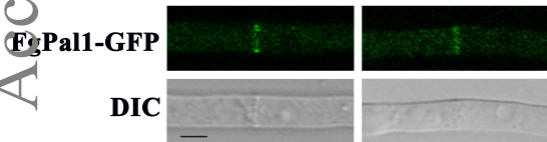
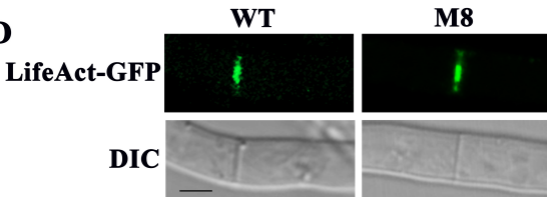


Merge

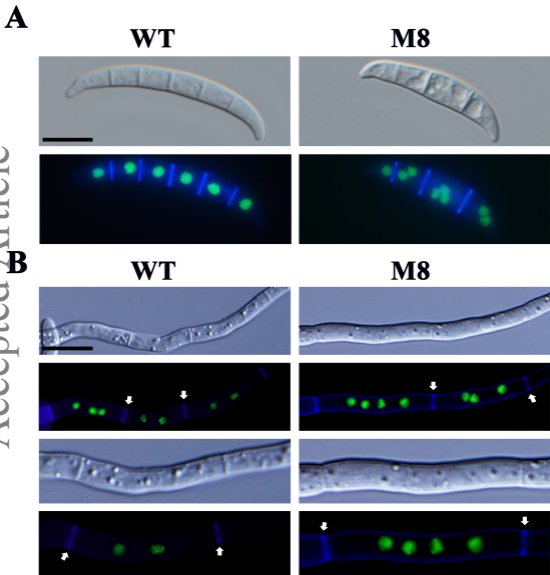


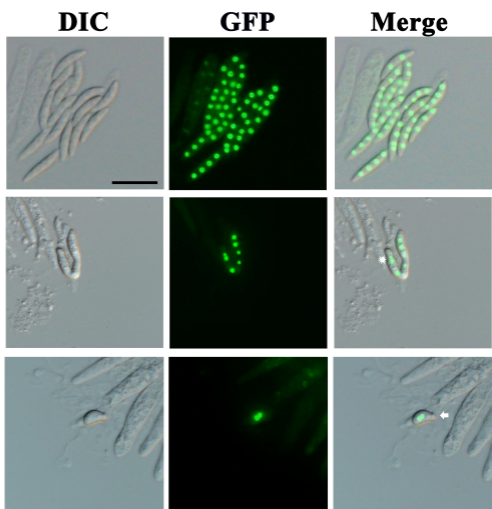
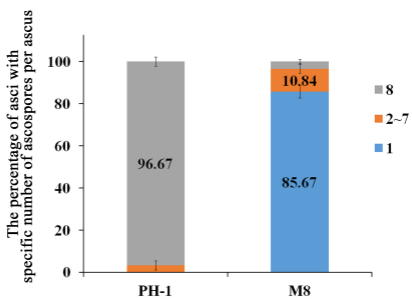
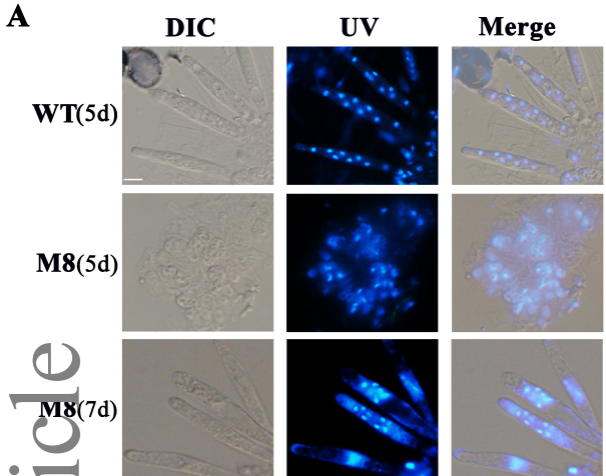
**A****B**

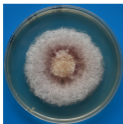
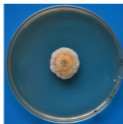
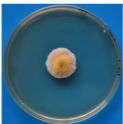
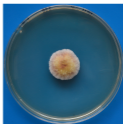
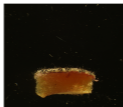
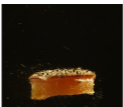
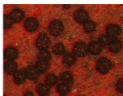
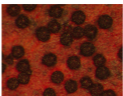
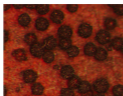
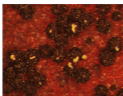
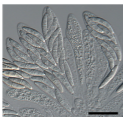
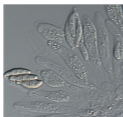
Accepted Article

**DMSO****LatA****D**







**A** **PH-1****M8****F7****P3****B****PH-1****M8****F7****P3****C****PH-1****M8****F7****P3**