The cyclase-associated protein FgCap1 has both PKA-dependent and -independent functions during DON production and plant infection in *Fusarium graminearum*

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ABSTRACT

Fusarium graminearum is a causal agent of wheat scab and a producer of trichothecene mycotoxin deoxynivalenol (DON). The expression of trichothecene biosynthesis (*TRI*) genes and DON production are mainly regulated by the cAMP-PKA pathway and two pathway-specific transcription factors (*TRI6* and *TRI10*). Interestingly, mutants deleted of *TRI6* had reduced expression of several components of cAMP signaling, including the *FgCAP1* adenylate binding protein gene that has not been functionally characterized in *F. graminearum*. In this study, we showed that FgCap1 interacted with Fac1 adenylate cyclase and deletion of *FgCAP1* reduced the intracellular cAMP level and PKA activities. The *Fgcap1* deletion mutant was defective in vegetative growth, conidiogenesis, and plant infection. It also was significantly reduced in DON production and *TRI* gene expression, which could be suppressed by exogenous cAMP, indicating that a PKA-dependent regulation of DON biosynthesis by FgCap1. The wild type but not *tri6* mutant had increased levels of intracellular cAMP and *FgCAP1* expression under DON producing conditions. Furthermore, the promoter of *FgCAP1* has one putative Tri6-binding site that was important for its function during DON biosynthesis but dispensable for hyphal growth, conidiogenesis, and pathogenesis. In addition, FgCap1 has an actin-like localization to the cortical patches at the apical region of hyphal tips. Phosphorylation of FgCap1 at S353 was identified by phosphoproteomics analysis. The S353A mutation in *FgCAP1* had no effects on its functions during vegetative growth, conidiation, and DON production. However, expression of the *FgCAP1*^{S353A} allele failed to complement the defects of *Fgcap1* mutant in plant infection, indicating the importance of phosphorylation of FgCap1 at S353 during pathogenesis. Taken together, our results suggested that *FgCAP1* is involved in the regulation of DON production via cAMP signaling and subjected to a feedback regulation by *TRI6*, but phosphorylation of FgCap1 at S353 is likely unrelated to the cAMP-PKA pathway because S353A mutation only affected plant infection.

INTRODUCTION

Fusarium head blight (FHB) caused by *Fusarium graminearum* is one of the most destructive diseases of wheat and barley in many wheat growing regions (Goswami & Kistler, 2004). In addition to causing severe yield losses, *F. graminearum* produces trichothecene mycotoxin deoxynivalenol (DON) in infested grains. As a potent inhibitor of protein synthesis in eukaryotic organisms, DON is harmful to human and animal health (Van de Walle *et al.*, 2010, Audenaert *et al.*, 2014). DON is also an important virulence factor during plant infection. Although it is not required for *F. graminearum* to establish initial infection, DON plays a critical role in spreading from the infected kernels to neighboring spikelets (Bai *et al.*, 2002). Mutants deleted of the *TRI5* gene encoding the trichodiene synthase that catalyzes the initial step for DON biosynthesis can cause diseases on the inoculated kernels but fail to spread infection to other spikelets on the same wheat heads. The expression of *TRI5* was induced in initial infection structures, further indicating the importance of DON in plant infection (Boenisch & Schafer, 2011).

In *F. graminearum*, most of the genes involved in trichothecene biosynthesis and transport (*TRI* genes), including *TRI4, TRI5, TRI6, TRI10,* and *TRI12*, are located in the main *TRI* gene cluster (Brown *et al.*, 2004). *TRI4*, like *TRI1*, encodes a cytochrome P-450 oxygenase that localizes to toxiosomes, distinct vesicles specific for DON biosynthesis (Menke *et al.*, 2013). The Tri12 MFS transporter interacts with toxiosomes for the efflux of trichothecene mycotoxins (Menke *et al.*, 2012). For the two transcriptional factor genes in the cluster, both *TRI6* and *TRI10*, are important for regulating *TRI* gene expression (Seong *et al.*, 2009). The *tri6* and *tri10* mutants are defective in DON production and the expression of *TRI* genes under DON-producing conditions (Seong et al., 2009). Whereas the Tri10-binding site has not been identified, the putative Tri6-binding sites identified in three different studies are different, likely due to different approaches used (Sieber *et al.*, 2014, Nasmith *et al.*, 2011, Seong et al., 2009, Hohn *et al.*, 1999). Two studies were based on bioinformatics analyses with microarray data (Seong et al., 2009, Sieber et al., 2014) but the other one used the ChIP-seq and gel-mobility shift assays (Nasmith et al., 2011). It is also possible that Tri6 and Tri10 can form homo- and hetero-dimers that differ in the recognition of DNA sequences.

Various environmental factors or physiological conditions are known to affect DON biosynthesis in the wheat scab fungus, possibly by somehow activating Tri6 and/or Tri10 to regulate *TRI* gene expression (Merhej *et al.*, 2011, Jiang *et al.*, 2015, Hou *et al.*, 2015). Like in many other mycotoxin producing fungi (Roze *et al.*, 2004, Schmidt-Heydt *et al.*, 2015), the cAMP-PKA signal transduction pathway plays a regulatory role in DON biosynthesis in *F. graminearum* (Hu *et al.*, 2014, Bormann *et al.*, 2014, Jiang *et al.*, 2016b, Guo *et al.*, 2016). Exogenous cAMP (cyclic adenosine monophosphate) treatment stimulated *TRI* gene expression and cellular differentiation associated with DON production (Jiang et al., 2016b). Functional characterizations of the two genes encoding the catalytic subunits of PKA showed that *CPK1* plays a major role in regulating DON production although *CPK2* also contributes (Hu et al., 2014). The *cpk1 cpk2* double mutant, but not the *cpk1* or *cpk2* mutant, was completely blocked in DON biosynthesis. Whereas the Pde1 and Pde2 cAMP phosphodiesterases negatively regulates DON production in *F. graminearum* (Jiang et al., 2016b), the *FAC1* adenylate cyclase responsible for the synthesis of intracellular cAMP is essential for *TRI* gene expression and trichothecene biosynthesis (Bormann et al., 2014, Hu et al., 2014). In addition, a gain-of-function mutation in the adenylyl cyclase gene results in the over-production of DON under repressive conditions in *F. graminearum* (Blum *et al.*, 2016).

In the budding yeast *Saccharomyces cerevisiae*, the *SRV2* adenylate cyclase-associated protein (CAP) gene was identified as a suppressor of a constitutive active *RAS2*^{G19V} mutation (Fedor-Chaiken *et al.*, 1990). *SRV2* was shown to be required for RAS-mediated activation of adenylate cyclase and null mutations in *SRV2* was lethal in yeast (Yu *et al.*, 1999, Fedor-Chaiken et al., 1990). The N-terminal region of Srv2 has an adenylate cyclase-binding domain that is responsible for its functional relationship with RAS (Shi *et al.*, 1997). Srv2 also has a C-terminal actin-binding domain that is important for its functions in actin turnover and polarized growth (Bertling *et al.*, 2004, Quintero-Monzon *et al.*, 2009, Zhou *et al.*, 2012). These two domains are well conserved in *SRV2* and its orthologs from other fungi. Studies in a few fungal pathogens have showed that CAP proteins are generally important for the activation of adenylate cyclase and cAMP signaling (Fedor-Chaiken et al., 1990, Bahn & Sundstrom, 2001, Zhou et al., 2012). *ACA1*, the *SRV2* ortholog in the human pathogen *Cryptococcus neoformans*, is important for glucose-induced cAMP accumulation, mating, and capsule production (Bahn *et al.*, 2004). In another human pathogen *Candida albicans*, *CAP1*-mediated cAMP signaling is required for the transition of yeast cells to hyphal growth (Bahn & Sundstrom, 2001). In plant pathogenic fungi *Ustilago maydis* and *Magnaporthe oryzae*, CAP proteins are important for infection-related morphogenesis and pathogenesis (Takach & Gold, 2010, Zhou et al., 2012). Like in *S. cerevisiae*, deletion of the *CAP1* gene suppressed the effects of a dominant *RAS2* mutation in *M. oryzae* (Zhou et al., 2012).

In *F. graminearum*, the Tri6 transcription factor has been shown to play a critical role in the regulation of DON biosynthesis by the cAMP-PKA pathway (Jiang et al., 2016b, Hu et al., 2014). Interestingly, microarray analysis with the *tri6* mutant (Seong et al., 2009) showed that deletion of *TRI6* reduced the expression of several key components of PKA pathway under DON inducing conditions, including *FAC1*, *CPK1*, *CPK2*, and FGSG_01923 (an ortholog of yeast *SRV2* and *M. oryzae CAP1*), indicating a possible feedback regulation of cAMP signaling by Tri6 during DON production. Unlike other genes related to cAMP signaling, FGSG_01923 (named *FgCAP1* in this study) has not been functionally characterized in *F. graminearum*. In this study, we showed that *FgCAP1* was important for vegetative growth, conidiogenesis, plant infection, and DON production. As a Fac1-interacting protein, FgCap1 was required for maintaining a normal intracellular cAMP level to properly regulate PKA activities. However, phosphorylation of FgCap1 at S353 was important for plant infection but dispensable for DON biosynthesis. Overall, our results indicated that *FgCAP1* is involved in the regulation of DON production via cAMP signaling and subjected to a feedback regulation by *TRI6* but phosphorylation-dependent functions of FgCap1 during plant infection may be not related to cAMP signaling.

MATERIALS AND METHODS

Strains and culture conditions: All the wild-type and mutant strains of *F. graminearum* used in this study were listed in Table 1. Potato dextrose agar (PDA) cultures grown at 25^{o}C for 3 days were used for assaying growth rate and colony morphology. Conidiation and conidium morphology were assayed with conidia harvested from 5-day-old CMC cultures as described (Jiang et al., 2016b). For mating assays, aerial hyphae of 7-day-old carrot agar cultures were pressed down with 0.1% Tween 20 and further incubated under black light to induce sexual

reproduction (Luo *et al.*, 2014).

Generation of the *Fgcap1* deletion mutant: To generate the gene replacement construct for *FgCAP1* with the split-marker approach, the 0.9-kb upstream and 0.9-kb downstream flanking sequences were amplified by PCR from genomic DNA of PH-1. The resulting PCR products were connected to the neomycin resistance gene cassette (Itaya *et al.*, 1989) by overlapping PCR and transformed into protoplasts of PH-1 as described (Hou *et al.*, 2002). For transformant selection, geneticin (Sigma-Aldrich, USA) was added to the final concentration at 400 μg/ml to both top and bottom agar. Geneticin-resistant transformants were screened by PCR and confirmed by Southern blot analysis.

Generation of the *FgCAP1*-GFP, *FgCAP1*-RFP, *FgCAP1*$^{\Delta Tri6B}$, and *FgCAP1*^{S353A}-GFP transformants: For complementation assays, the *FgCAP1*-GFP fusion construct was generated with the gap repair approach (Zhou *et al.*, 2011) by co-transformation of the *FgCAP1* gene fragment and *Xho*I-digested pDL2 into yeast strain XK1-25 as described (Zhou et al., 2011). The *FgCAP1*-RFP fusion construct was generated with pXY201 (Zhou et al., 2011) using the same approach. The S353A mutation was introduced into *FgCAP1* by overlapping PCR with fragments amplified with primer pairs Cap1-NF/S353-SA-R and S353-SA-F/Cap1-GFP-R. *FgCAP1*^{S353A} was cloned into pFL2 (Zhou et al., 2011) by gap repair to generate the *FgCAP1*^{S353A}-GFP fusion construct. Deletion of the putative Tri6-binding site in the *FgCAP1* promoter was created by overlapping PCR with fragments amplified with primer pairs Cap1-NF/S353-SA-R and S353-SA-F/Cap1-R. All the resulting fusion constructs and *FgCAP1*$^{\Delta Tri6B}$ allele were recovered from Trp^{+} yeast transformants, confirmed by sequencing analysis, and transformed into protoplasts of the *Fgcap1* mutants. Transformants resistant to both hygromycin and bleomycin were screened by PCR and examined for GFP or RFP signals as described (Jiang et al., 2016b). All of the primers used in the construction of these mutant alleles are listed in Supplementary Table S2.

qRT-PCR assays: RNA samples of the wild type, *tri6*, and *Fgcap1* mutant were isolated with the TRIzol reagent (Invitrogen, USA) from hyphae harvested from 3-day-old YEPD and LTB cultures. For assaying the expression levels of *FgCAP1* and *TRI* genes, cDNA was synthesized with the Fermentas First cDNA synthesis kit (Hanover, USA) following the instructions provided by the manufacturer. The iTaq™ Universal SYBR® Green Supermix (Bio-RAD, USA) was used for qRT-PCR assays with the CFX96 Real-Time System as described (Bio-RAD, USA) (Jiang et al., 2016b). The *TUB2* beta-tubulin gene of *F. graminearum* was used as the internal control (Bluhm *et al.*, 2007). Relative expression levels of each gene were calculated with the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

Plant infection and DON production assays: Conidia harvested from 5-day-old CMC cultures were re-suspended to 10^5 spores/ml in sterile distilled water. Wheat heads of cultivar Norm or Xiaoyan 22 were drop-inoculated with 10 μl of conidium suspensions. Scab symptoms were examined 14 days post-inoculation (dpi) and infected wheat kernels were harvested and assayed for DON production as described (Bluhm et al., 2007). Thick sections of infected rachis tissues that were fixed, dehydrated, and embedded in Spurr resin as described (Kang *et al.*, 2008) were examined for infectious growth in wheat heads. Infection assays with corn silks and wheat coleoptiles were conducted as described (Hou et al., 2002, Liu *et al.*, 2015). Infectious hyphae

developed in wheat coleoptile cells were examined as described (Liu et al., 2015) 48 h post-inoculation.

DON production in LTB cultures (Jiang et al., 2016b) was assayed with a competitive ELISA based DON detection plate kit (Beacon Analytical Systems, USA) after incubation at 25°C for 1, 3, or 5 days as described (Gardiner *et al.*, 2009). To stimulate DON production, cAMP (Sigma-Aldrich, USA) was added to the final concentration of 4 mM into LTB culture as described (Jiang et al., 2016b). DON production assays with rice grain cultures were conducted as described (Jiang et al., 2015).

Co-IP assays for the interaction between FgCap1 and Fac1: The *FgCAP1*-FLAG and *FAC1*^{CT}-GFP fusion constructs were generated by the yeast gap repair approach (Zhou et al., 2011) and co-transformed into the wild-type strain PH-1. Total proteins were isolated from the resulting transformants as described (Hou et al., 2015) and the expression of both transforming constructs was verified by western blot analysis with an anti-GFP (Roche, USA) and an anti-FLAG (Sigma-Aldrich, USA) antibodies. Total proteins were isolated from transformants expressing both *FgCAP1*-FLAG and *FAC1*^{CT}-GFP constructs and incubated with anti-Flag M2 beads (Sigma-Aldrich, St. Louis, MO). Western blots of total proteins and proteins eluted from anti-Flag M2 beads were detected with the anti-GFP and anti-FLAG antibodies as described (Liu et al., 2015).

Assays for intracellular cAMP levels and PKA activities: Hyphae of the wild type and *Fgcap1* mutant were harvested from 3-day-old YEPD and LTB cultures for cAMP assays. The cAMP levels were quantified using the cAMP Biotrak Immuno-assay System (Amersham Biosciences, USA) as described (Ramanujam & Naqvi, 2010). Hyphae of the wild type and *Fgcap1* mutant cultured in LTB cultures for 3 days were used to extract protein for PKA activity assays. PKA activities were assayed with the PepTag nonradioactive PKA assay kit (Promega, USA) as described (Godson *et al.*, 2000).

RESULTS

***FgCAP1* is important for vegetative growth and conidium morphology:** FGSG_01923 (named *FgCAP1* in this study) is the distinct ortholog of *S. cerevisiae SRV2* and *M. oryzae CAP1*. It has the typical structural components of CAP proteins, including an adenylyl cyclase binding domain (ACB) and an actin binding domain (AB). To determine its function in DON production and cAMP signaling, we generated the *Fgcap1* mutant M1 in the wild-type strain PH-1 with the split-marker approach (Catlett *et al.*, 2003). In comparison with PH-1, the *Fgcap1* mutant was reduced in growth rate (Fig. 1A; Table 2). Mutant colonies tended to have irregular edges and produced more compact aerial hyphae than the wild type. When cultured at an elevated temperature (30°C), the wild type formed smaller colonies in comparison with cultures incubated at 25°C. For the *Fgcap1* mutant, the growth rate was similar between cultures grown at 30 or 25°C (Fig. 1A), indicating that the *Fgcap1* mutant had increased tolerance to high temperature.

In 5-day old CMC cultures, conidiation appeared to be normal (Table 2) but conidia produced by the *Fgcap1* mutant were shorter and had fewer septa than those of PH-1 (Fig. 1B). Approximately 69.6% of the *Fgcap1* conidia had only three septa, whereas only 4.8% of the

wild-type conidia had fewer than four septa (Fig. 1C). Nevertheless, *Fgcap1* conidia with fewer septa were normal in germination although germ tube growth was significantly reduced (Fig. 1D). On selfing plates, the *Fgcap1* mutant produced abundant perithecia with normal morphology and size. No obvious defects in perithecium development and ascospore development were observed (Fig. 1E). These results indicated that *FgCAP1* is dispensable for sexual reproduction although it is important for hyphal growth and conidiogenesis.

The *Fgcap1* mutant is defective in plant infection: In infection assays with wheat heads, unlike the wild type that spread from the inoculation site to other spikelets, the *Fgcap1* mutant only caused FHB symptoms on the inoculated spikelets(Fig. 2A). On average, the disease index of the *Fgcap1* mutant was 0.7 (Table 2). The average disease index was 13.2 for the wild type (Table 2). Similarly, the *Fgcap1* mutant caused only limited symptoms on wheat coleoptiles (Fig. 2B). In thick sections of the rachis beneath the spikelets inoculated with PH-1, extensive infectious hyphae were observed in vascular tissues at 5 dpi. Under the same conditions, the *Fgcap1* mutant had only limited infectious hyphal growth in vascular tissues of the rachis (Fig. 2C). These results suggest that the *Fgcap1* mutant is defective in spreading from the inoculation site to nearby spikelets via the rachis and *FgCAP1* is important for virulence in *F. graminearum*.

***FgCAP1* plays a critical role in regulating DON biosynthesis:** Because DON is an important virulence factor, we assayed DON production in infested wheat kernels. The *Fgcap1* mutant was significantly reduced in DON production in infested wheat kernels (Table 2). We also assayed DON production with rice grain cultures. In comparison with the wild type, DON production was reduced over 10-folds in the *Fgcap1* mutant (Table 1). When assayed by qRT-PCR with RNA isolated from hyphae collected from 3 day-old LTB (liquid trichothecene biosynthesis) cultures, the expression levels of *TRI5*, *TRI6*, and *TRI10* were 7.1-, 2.4-, 11.1- and 6.7-folds, respectively, higher in the wild type than in the *Fgcap1* mutant (Fig. 3A). Therefore, *FgCAP1* regulates *TRI* gene expression and DON production in *F. graminearum*.

Because DON production is known to be associated with special cellular differentiation(Menke et al., 2013, Jonkers *et al.*, 2012), the morphology of hyphae harvested from 3-day-old LTB cultures was examined. In comparison with PH-1, no obvious difference was observed in the formation of intercalary, bulbous hyphal structures in the *Fgcap1* mutant (Fig. 3B). Therefore, although it is important for DON production, *FgCAP1* appears to be dispensable for cellular differentiation associated with DON biosynthesis.

Regulation of DON production by FgCap1 is related to cAMP signaling: Due to the conserved functions of CAP proteins in cAMP signaling, we assayed the intracellular cAMP level and PKA activity. In vegetative hyphae grown in LTB medium, the intracellular cAMP level in the *Fgcap1* mutant was reduced approximately 70% in comparison with that of the wild-type strain PH-1 (Fig. 4A). The *Fgcap1* mutant also was reduced in PKA activities (Fig. 4B). These data indicate that *FgCAP1* plays an important role in the cAMP-PKA pathway.

Because the intracellular cAMP level was reduced in the *Fgcap1* mutant, we assayed the effect of exogenous cAMP on DON production. As expected, DON production in the wild type was significantly increased in the presence of 4 mM cAMP (Fig. 4C). Although the *Fgcap1* mutant was reduced in DON production, it still responded to exogenous cAMP and produced similar amounts of DON with the wild type in the presence of 4 mM cAMP (Fig. 4C). These results indicated that exogenous cAMP could suppress the defects of the *Fgcap1* mutant in DON

production. Therefore, the function of *FgCAP1* in DON regulation is dependent on the cAMP signaling pathway.

FgCap1 interacts with Fac1 in co-immunoprecipitation assays: To determine the relationship between FgCap1 and cAMP signaling in *F. graminearum,* the intracellular region of Fac1 was fused with GFP (*FAC1*^{CT}-GFP) and co-transformed into PH-1 with *FgCAP1*-FLAG fusion construct. In western blot analysis with total proteins isolated from the resulting transformant CF1 (Table 1), a 61-kDa band and a 77-kDA band were detected with the anti-FLAG and anti-GFP antibodies, respectively (Fig. 5). In the elution from anti-FLAG M2 beads, the 77-kDa Fac1^{CT}-GFP band was detected with an anti-GFP antibody in the *FgCAP1*-FLAG *FAC1*^{CT}-GFP transformant CF1 but not in the transformant expressing only the *CAP1*-FLAG construct CC2 (Fig. 5). Therefore, FgCap1 interacts with the C-terminal region of Fac1 in *F. graminearum*, further indicating the role of FgCap1 in cAMP signaling.

***FgCAP1* and *TRI6* mediates a feedback regulation of cAMP signaling in DON producing stage:** Because exogenous cAMP induced DON production, we measured the intracellular cAMP level in hyphae of PH-1 harvested from 3-day-old YEPD or LTB (DON-inducing) cultures. The intracellular cAMP level was significantly increased when DON production was induced in LTB cultures in comparison with YEPD cultures (Fig. 6A). When assayed by qRT-PCR, *FgCAP1* expression also was up-regulated during DON production (Fig. 6B), indicating an association between the intracellular cAMP level and *FgCAP1* expression. Interestingly, the *tri6* mutant had no obvious changes in the expression level of *FgCAP1* between YEPD and LTB cultures (Fig. 6B). Therefore, as a key regulator of DON biosynthesis, Tri6 may regulate *FgCAP1* expression to modulate the intracellular cAMP level as a feedback regulation.

The promoter of *FgCAP1* has one putative Tri6-binding site TCACTTCAC (-360 to -351) (Nasmith et al., 2011). To determine its role in *FgCAP1* function, we generated a mutant allele of *FgCAP1* deleted of this putative Tri6-binding site and transformed it into the *Fgcap1* mutant. The resulting transformant D1 showed similar growth rate with the wild type (Fig. 6C). It also produced normal conidia (Fig. 6D) and was as virulent as the wild type (Fig. 6D). However, deletion of the Tri6-binding site in *FgCAP1* significantly reduced DON production (Fig. 6F), indicating that Tri6 specially regulates *FgCAP1* during DON production.

FgCap1 localizes to the apical cortical patches in germ tubes: For complementation assays, we generated the *FgCAP1*-GFP construct and transformed it into the *Fgcap1* mutant M1. The resulting *Fgcap1*/*FgCAP1*-GFP transformant CG5 was normal in vegetative growth, conidiation, plant infection, and DON production, suggesting that the phenotypes of *Fgcap1* mutant could be fully complemented by *FgCAP1*-GFP (Fig. 7). When examined by epifluorescence microscopy, GFP signals was observed mainly at the apical region of hyphal tips (Fig. 8A). This localization pattern was similar to that of actin patches in *F. graminearum* (Li *et al.*, 2015). When *FgCAP1*-GFP transformant CG5 was treated by cytochalasin A (CytA), an inhibitor of actin elongation, GFP signals were observed throughout the cytoplasm instead of mainly at the apical cortical patches (Fig. 8A). These results indicated that FgCap1 is likely associated with F-actin cytoskeleton for localization to the cortical regions of hyphal tips.

Because of the localization pattern of FgCap1 and its actin-binding domain, we generated the *FgCAP1*-RFP construct and co-transformed it into PH-1 with the LifeAct-GFP construct (Riedl *et al.*, 2008). In the resulting transformant RG2, both FgCap1-RFP and LifeAct-GFP

mainly localized to the cortical regions of hyphae tips although they did not have identical localization patterns (Fig. 8B).

Phosphorylation of FgCap1 at S353 may play important roles in hyphal growth and pathogenesis: In mouse Cap1, Three phosphorylation sites have been identified by mass spectrometry assays (Zhou *et al.*, 2014). To identify possible phosphorylation sites of FgCap1, we isolated proteins from vegetative hyphae harvested from 12-hour-old cultures as described (Pandey *et al.*, 2004). After trypsin digestion, phosphorylated peptides were subjected to mass spectrometry analysis (Gao *et al.*, 2016). In total, we identified 2,103 phosphopeptides derived from 1,116 *F. graminearum* proteins from three independent assays (Table S1). For FgCap1, one phosphopeptide ^{350}RGKSPAPGKK^{359} with S353 as the phosphorylation site was identified. This S353 residue is in the second proline-rich region (P2) that is adjacent to the C-terminal actin-binding (AB) domain (Fig. 7A). Sequence alignment showed that this serine residue is well conserved in Cap1 orthologs from the budding yeast and other filamentous fungi (Fig.7A).

To determine the role of S353 phosphorylation, we generated the *FgCAP1*^{S353A}-GFP allele and transformed it into the *Fgcap1* deletion mutant. The resulting *Fgcap1*/*FgCAP1*^{S353A} transformant SG2 grew faster than the original *Fgcap1* mutant but still slightly slower than the *Fgcap1*/*FgCAP1*^{WT} complemented transformant CG5 (Fig. 7B). In infection assays with flowering wheat heads, similar to the *Fgcap1* mutant, the *Fgcap1*/*FgCAP1*^{S353A} transformant caused symptoms only on the inoculated kernels (Fig. 7C). Interestingly, unlike the *Fgcap1* mutant, conidia produced by the *Fgcap1*/*FgCAP1*^{S353A} transformant were normal in size and morphology, which was similar to that of the *Fgcap1*/*FgCAP1*^{WT} (Fig. 7D). The *Fgcap1*/*FgCAP1*^{S353A} transformant also was similar to the wild type and complemented transformant C5 in DON production (Fig. 7E). These data indicate that phosphorylation of FgCap1 at S353 is important for vegetative growth and plant infection but dispensable for asexual reproduction and DON biosynthesis.

S353 phosphorylation is dispensable for cAMP signaling but important for infectious growth: Because DON production was not affected by S353A mutation and *FgCAP1* is involved in regulating DON production via cAMP signaling, we assayed the intracellular cAMP level. In comparison with the original *Fgcap1* mutant, the *Fgcap1*/*FgCAP1*^{S353A} transformant S2 was increased in intracellular cAMP in vegetative hyphae. The intracellular cAMP level was similar between the *Fgcap1*/*FgCAP1*^{S353A} transformant and the wild type or complemented transformant C5 (Fig. 9A). These results indicate that phosphorylation of FgCap1 at S353 is not important for cAMP signaling in vegetative hyphae of *F. graminearum*.

The *Fgcap1*/*FgCAP1*^{S353A} transformant was defective in spreading in wheat heads but normal in DON production. To further characterize its defects in plant infection, we conducted infection assays with wheat coleoptiles as described (Liu et al., 2015). The wild type and complemented transformant could colonize and developed extensive infectious hyphae 2 dpi. Under the same conditions, the *Fgcap1*/*FgCAP1*^{S353A} transformant, similar to the original *Fgcap1* mutant, had only limited growth of infectious hyphae (Fig. 9B). These results indicate that phosphorylation at S353 plays a critical role in normal infectious growth. Besides DON production, FgCap1 likely regulates other important pathogenicity factors during plant infection.

DISCUSSION

The cAMP-PKA signaling pathway is well conserved in eukaryotic organisms and several of its key components, including *RAS1, FAC1, PDE1, PDE2, CPK1,* and *CPK2,* have been functionally characterized in *F. graminearum* (Hu et al., 2014, Jiang et al., 2016b, Bluhm et al., 2007). In the budding yeast, RAS-dependent activation of adenylate cyclase Cyr1 is required for cAMP synthesis and the interaction of Cyr1 with the Srv2 CAP protein stimulates its activation (Zou *et al.*, 2010, Zhou et al., 2012, Shima *et al.*, 1997). FgCap1 is orthologous to yeast Srv2 and it interacted with Fac1 in *F. graminearum*. Unlike the *fac1* mutant, the *Fgcap1* mutant still produced detectable amount of intracellular cAMP and PKA activities although at significantly reduced levels in comparison with the wild type. Thus, FgCap1 plays an important but not essential role in cAMP signaling in *F. graminearum*. Consistent with this observation, the reduction in growth rate in the *Fgcap1* mutant was to a less degree in comparison with that of the *fac1* or *cpk1 cpk2* mutant (Hu et al., 2014). Nevertheless, similar to the *cpk1* mutant (Hu et al., 2014), the *Fgcap1* mutant had increased tolerance to elevated temperatures during vegetative growth in *F. graminearum*. The cAMP-PKA pathway is known to mediate tolerance to elevated temperatures in *F. verticillioides* and *N. crassa* (Choi & Xu, 2010, Banno *et al.*, 2005).

In *F. graminearum*, cAMP signaling plays a critical role in regulating DON biosynthesis (Hu et al., 2014, Jiang et al., 2016b). As an activator of Fac1, we found that FgCap1 was important for the expression of *TRI* genes and exogenous cAMP fully complemented the defects of the *Fgcap1* mutant in DON production. However, FgCap1 was dispensable for the formation of bulbous, swelling hyphal structures that have been observed in DON producing cultures (Jonkers et al., 2012). These results suggest that cellular differentiation associated with DON biosynthesis and the expression of *TRI* genes may be co-regulated by different mechanisms during DON production. It is likely that *FgCAP1* is involved in the regulation of *TRI* gene expression via cAMP signaling but not in the cellular differentiation associated with DON production.

The expression of *FgCAP1* and intracellular cAMP level were increased in DON-inducing cultures of the wild type but not the *tri6* mutant. Because of conserved functions of CAP proteins in cAMP signaling (Zhou et al., 2012), increased expression of *FgCAP1* may be responsible for increased cAMP accumulation during DON biosynthesis. The promoter region of *FgCAP1* contains one putative Tri6-binding site TCACTTCAC that matches with the TCAC N1 TCAC site identified in genes regulated by Tri6 by ChIP-seq analysis (Nasmith et al., 2011). Deletion of this Tri6-binding site in *FgCAP1* led to significant reduction in DON production and virulence but had no effect on vegetative growth. In addition to *TRI* genes, *TRI6* is known to regulate the transcription of a number of genes involved in plant infection, branched-chain amino acid (BCAA) metabolism, and isoprenoid biosynthetic pathway (Subramaniam *et al.*, 2015, Seong et al., 2009). Because it is in the main *TRI* gene cluster, *TRI6* must have evolved to regulate other genes related to DON biosynthesis and plant infection, including *FgCAP1*, in *F. graminearum*. In fact, the *MAC1, CPK1,* and *CPK2* genes related to cAMP signaling all had reduced expression levels in the *tri6* mutant based on published microarray data (Seong et al., 2009) (www.plexdb.org/). Because cAMP treatment stimulates DON biosynthesis, transcriptional regulation of *FgCAP1* by *TRI6* may have a feedback effects on *TRI* gene expression by cAMP signaling in *F. graminearum*.

FgCap1 mainly localized to the apical cortical patches at hyphal tips. This actin-like subcellular localization of FgCap1 was disrupted by CytA treatment, indicating the relationship

between Cap1 and actin localization. The localization pattern of FgCap1 suggests that it may be functionally related to endocytosis. Apical actin patches may mediate endocytosis in fungi (Berepiki *et al.*, 2011). It has been reported that CAP protein interacted directly with V-ATPase and acted as a general regulator of endocytosis in Dictyostelium (Sultana *et al.*, 2005). A recently study showed Srv2/Cap1 is responsible for efficient endocytosis by aiding initial vesicle invagination and movement (Toshima *et al.*, 2016). Some of the defects observed in the *Fgcap1* mutant may be independent of cAMP signaling but related to the role of *FgCAP1* in endocytosis in *F. graminearum*.

Three phosphorylation sites, S36, S307 and S309, have been identified in the mouse CAP1 protein (Zhou et al., 2014). Although these three phosphorylation sites are conserved in mammalian CAP proteins (Zhou et al., 2014), none of them are conserved among Cap1 orthologs in filamentous fungi. Nevertheless, the phosphorylation site identified in this study, S353, is conserved in FgCap1 and its orthologs from filamentous ascomycetes. The S353 residue of FgCap1 is in the second proline-rich domain P2, which is important for the subcellular localization of Cap1 in *M. oryzae* (Zhou et al., 2012). Interestingly, although phosphorylation of S353 was identified in proteins isolated from vegetative hyphae, the S353A mutation had only a minor effect the function of FgCap1 during vegetative growth. The *Fgcap1*/*FgCAP1*^{S353A} transformant was only slightly reduced in growth rate and had similar intracellular cAMP levels with the wild type in vegetative hyphae. Expression of *FgCAP1*^{S353A} in the *Fgcap1* mutant also resulted in the formation of normal conidia and wild-type level of DON production. Therefore, phosphorylation of FgCap1 at S353 must be dispensable for its function during conidiogenesis and DON production. However, expression of *FgCAP1*^{S353A} in the *Fgcap1* mutant failed to suppress its defects in plant infection. The *Fgcap1*/*FgCAP1*^{S353A} transformant was similar to the original *Fgcap1* mutant in virulence and it was defective in infectious hyphal growth in plant tissues. These results indicated a distinct role of FgCap1 in plant infection, which may be related to actin cytoskeleton because the intracellular cAMP level was normal in the *Fgcap1*/*FgCAP1*^{S353A} transformant. Nevertheless, it remains possible that the phosphorylation of FgCap1 is associated with proteins other than Fac1 to regulate the cAMP-PKA pathway.

It is likely that the function of FgCap1 and its phosphorylation at S353 in cAMP signaling and cytoskeleton reorganization are different between vegetative hyphae and infectious hyphae. In *F. graminearum* and several other plant pathogenic fungi, infectious hyphae have distinct morphology from vegetative hyphae (Jiang *et al.*, 2016a, Liu et al., 2015, Zhao *et al.*, 2005), which may be related to differences in the functions of FgCap1 in cAMP signaling and cytoskeleton reorganization.

For the three phosphorylation sites of mouse CAP1, the GSK3 kinase is responsible for phosphorylation at S309 but the kinases responsible for phosphorylation of S36 and S307 are not clear (Zhou et al., 2014). Because phosphorylation of FgCap1 at S353 is important for infectious growth, it will be important to identify protein kinases responsible for its phosphorylation. In *F. graminearum*, targeted deletion mutants of all the non-essential protein kinase genes have been generated (Wang *et al.*, 2011). Phosphoproteomics analysis with these kinase mutants may lead to the identification of protein kinases responsible for FgCap1 phosphorylation. Prediction with program KinasePhos showed that S353 matches the consensus phosphorylation site of CDK kinases (Wong *et al.*, 2007). In *F. graminearum*, there were two Cdc2 orthologs named Cdc2A and Cdc2B (Liu et al., 2015). Although these two *CDC2* orthologs have redundant functions during vegetative growth, *CDC2A* plays stage-specific roles in cell cycle regulation during infectious growth (Liu et al., 2015). It is possible that *CDC2A* is involved in the phosphorylation

of FgCap1 in *F. graminearum*. Nevertheless, production of conidia with reduced septation (fewer conidium compartments) is a common phenotype between the *Fgcap1* and *Fggsk3* mutants (Qin *et al.*, 2015). The *Fggsk3* also share similar defects in growth, DON production, and plant infection with the Fgcap1 mutant. Therefore, it remains possible that *FgGSK3* is involved in the activation of *FgCAP1* in *F. graminearum*.

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

Strain	Genotype description	Reference
PH-1	Wild-type	(Cuomo *et al.*, 2007)
PH1Δtri6	*tri6* deletion mutant of PH-1	(Seong et al., 2009)
M1	*Fgcap1* deletion mutant of PH-1	This study
C3	*FgCAP1* complemented transformant of M1	This study
D1	Complemented transformant of M1 expressed $FgCAP1^{\Delta Tri6B}$ allele	This study
CC2	Transformant of PH-1 expressed FgCap1-FLAG	This study
CF1	Transformant of PH-1 expressed FgCap1-FLAG and Fac^{CT}-GFP	This study
RG2	Transformant of PH-1 expressed *FgCAP1*-RFP and LifeAct-GFP	This study
SG2	Complemented transformant of M1 expressed $FgCAP1^{S353A}$-GFP	This study
CG5	*FgCAP1*-GFP complemented transformant of M1	This study

Table 2. Defects of *Fgcap1* mutant in growth, conidiation, DON production, and virulence

Strains	Growth rate (mm/day) [a]	Conidiation (10^5 conidia/ml) [b]	DON production (ppm) [c]		Disease Index [d]
			Rice grains	Wheat kernels	
PH-1	11.8±0.3^{A}	10.5±0.3^{A}	922.3±55.0^{A}	506.2±42.2^{A}	13.2±2.6^{A}
M1	6.1±0.1^{B}	10.6±0.3^{A}	75.9±16.4^{B}	22.9±18.1^{B}	0.7±0.5^{B}
C3	12.0±0.1^{A}	10.3±0.6^{A}	874.6±107.1^{A}	525.0±26.4^{A}	12.9±1.8^{A}

[a] Average daily extension in colony radius on PDA plates.
[b] Conidiation in 5-day-old CMC cultures.
[c] DON production assayed with rice grain cultures and diseased wheat kernels from symptomatic spikelets.
[d] Disease index was estimated as the number of diseased spikelets on each inoculated wheat heads 14 days post-inoculation (dpi).
[*] Mean and standard deviation were calculated with results from three replicates for growth rate and conidiation assays and five replicates for DON and infection assays. Data were analyzed with Duncan's pair wise comparison. Different letters mark statistically significant differences (P = 0.05).

FIGURE LEGENDS

Fig. 1. Phenotypes of the *Fgcap1* mutant in vegetative growth, conidiogenesis, germination, and sexual reproduction.
A. Three-day-old PDA cultures of the wild type (PH-1) and *Fgcap1* mutant (M1) strains. **B**. Conidium morphology of PH-1 and M1. Bar = 20 μm. **C**. The average number of septa in conidia of PH-1 and mutant M1 harvested from 5-day-old CMC cultures. **D**. Germlings of PH-1 and M1 after incubation in YEPD medium for 10 h. Bar = 20 μm. **E**. Perithecia and asci of PH-1 and M1 formed on selfing cultures two-weeks after induction. Bar = 20μm.

Fig. 2. Defects of the *Fgcap1* mutant in plant infection.
A. Flowering wheat heads of cultivar Xiaoyan 22 were drop-inoculated with conidia from the wild type (PH-1) and *Fgcap1* mutant (M1). Spikelets with typical symptoms were photographed 14 days post-inoculation (dpi). Black dots mark the inoculated spikelets. **B**. Wheat coleoptiles inoculated with PH-1 and M1 were photographed 7 dpi. **C**. The rachis directly beneath the inoculated spikelets was examined 5 dpi. Hyphae growth (marked with arrows) was abundant in plant tissues inoculated with PH-1 and but not in samples inoculated with the *Fgcap1* mutant M1. Bar = 20μm.

Fig. 3. Defects of the *Fgcap1* mutant in DON biosynthesis.
A. The expression levels of *TRI5*, *TRI6*, *TRI10*, and *TRI101* in the wild type PH-1 and *Fgcap1* mutant M1. The relative expression level of each gene in PH-1 was arbitrarily set to 1. Mean and standard error were calculated with data from three independent biological replicates. **B**. LTB cultures of PH-1 and mutant M1 were examined for bulbous structures after incubation for 3 days. Bar = 20 μm.

Fig. 4. *FgCAP1* is involved in the cAMP signaling for regulating DON production.
A. The intracellular cAMP level in PH-1 and *Fgcap1* mutant M1. Mean and standard error were calculated with results from three independent biological replicates. **B**. PKA activities were assayed with proteins isolated from hyphae of PH-1 and M1 with the PepTag A1 PKA substrate peptide. Whereas phosphorylated peptides migrated toward the anode (+), un-phosphorylated peptides migrated toward the cathode (-) on a 0.8% agarose gel. N: non-phosphorylated sample control; P: phosphorylated sample control. **C**. DON production in 7-day-old LTB cultures of PH-1 and M1 with or without 4 mM cAMP. Different letters indicated statistically significant differences (P=0.05).

Fig. 5. FgCap1 interacts with Fac1 in co-immunoprecipitation assays.
Assays for the interaction of FgCap1 with Fac1. Western blots of total proteins and proteins eluted from anti-FLAG M2 beads of transformant CC2 (FgCap1-FLAG) and transformant CF1 (FgCap1-FLAG and Fac^{CT}-GFP) were detected with the anti-FLAG or anti-GFP antibody.

Fig. 6. The putative Tri6-binding site in the *FgCAP1* promoter is important for its function during DON production.
A. The intracellular cAMP level was assayed with hyphae of the wild-type strain PH-1 harvested from 3-day-old YEPD and LTB cultures. **B**. Expression levels of *FgCAP1* in 3-day-old YEPD or LTB cultures of PH-1 and *tri6* mutant. The relative expression level of *FgCAP1* in YEPD

cultures of PH-1 was arbitrarily set to 1. Mean and standard error were calculated with results from three independent biological replicates. **C**. Three-day-old PDA cultures of PH-1, *Fgcap1* mutant M1, and transformants of M1 expressing the wild-type *FgCAP1*$^{\Delta WT}$ (C3) or mutant allele of *FgCAP1* $^{\Delta Tri6B}$ (D1) deleted of the Tri6-binding site. **D**. Conidia of the same set of strains harvested from 5-day-old CMC cultures. Bar = 20 μm. **E**. Typical infected corn silks were photographed 6 dpi. **F**. DON production in 7-day-old LTB cultures of the same set of strains.

Fig. 7. Phosphorylation of FgCap1 at S353 is important for hyphal growth and pathogenesis but not for conidiogenesis and DON production.
A. Schematic drawing of FgCap1 and sequence alignment with its orthologs from other fungi surrounding the S353 residue (marked with an asterisk). ACB, Adenylyl cyclase binding domain; AB, actin binding domain; P1 and P2, two proline-rich regions. **B**. Three-day-old PDA cultures of the wild type (PH-1), *Fgcap1* mutant (M1), *Fgcap1*/*FgCAP1*^{S353A}-GFP (SG2), and *Fgcap1*/*FgCAP1*-GFP (CG5) strains. **C**. Flowering wheat heads of cultivar Norm were drop-inoculated with conidia from the same set of strains. Black dots mark the inoculated spikeletes. **D**. Conidia harvested from 5-day-old CMC cultures. Bar = 20μm. **E**. DON production in 7-day-old LTB cultures of the same set of strains.

Fig. 8 The subcellular localization of FgCap1 and its association with actin.
A. Germlings of transformant CG5 expressing the *FgCAP1*-GFP construct treated with or without cytochalasin A (CytA) were examined by DIC and epifluorescence microscopy. Bar = 5 μm. **B**. Germlings of transformant RG2 expressing the *FgCAP1*-RFP and LifeAct-GFP constructs were examined by DIC and epifluorescence microscopy. Bar = 5 μm.

Fig. 9. The S353A mutation in FgCap1 affect infectious growth but not intracellular cAMP levels.
A. The intracellular cAMP level in the wild type (PH-1), *Fgcap1* mutant (M1), *Fgcap1*/*FgCAP1*^{S353A}-GFP (SG2), and *Fgcap1*/*FgCAP1*-GFP (CG5). **B**. Infectious hyphae formed by the same set of strains inside wheat coleoptile cells at 48 hpi. Bar = 20 μm.

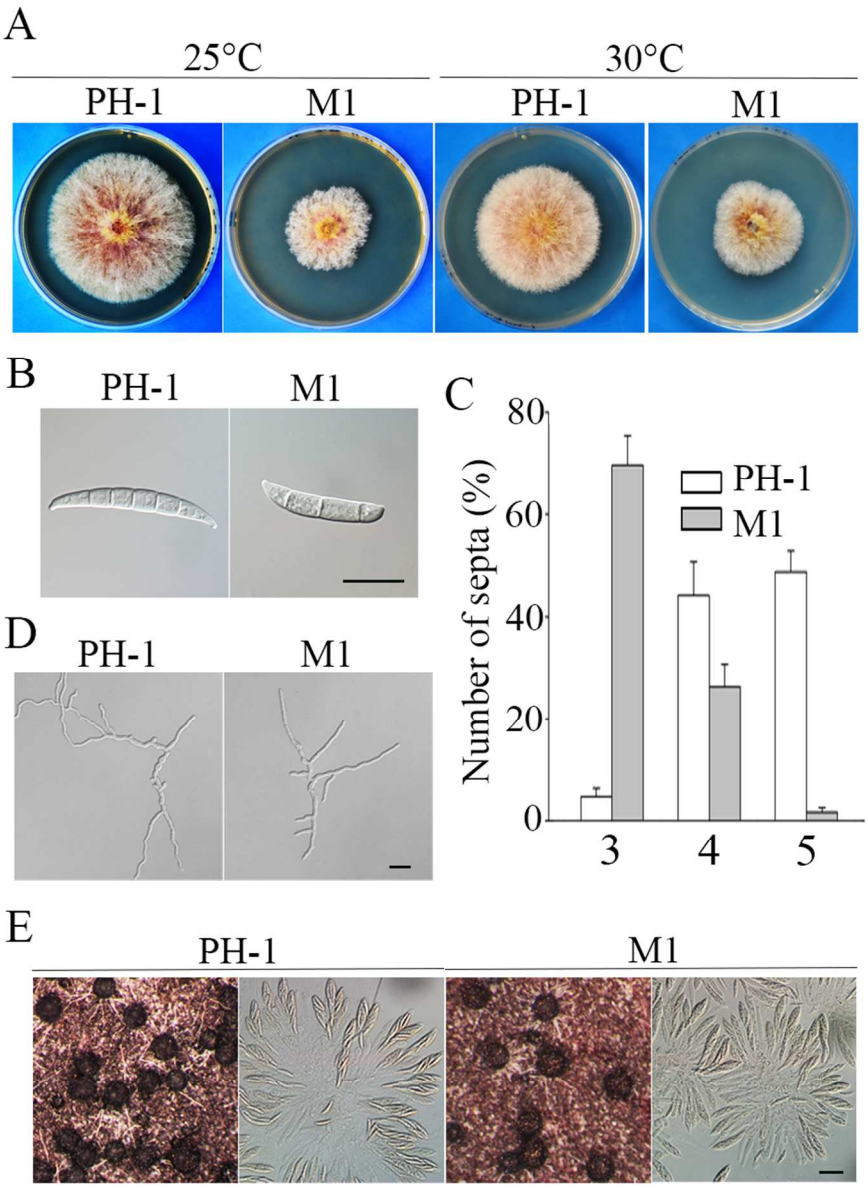

Fig. 1. Phenotypes of the Fgcap1 mutant in vegetative growth, conidiogenesis, germination, and sexual reproduction.
A. Three-day-old PDA cultures of the wild type (PH-1) and Fgcap1 mutant (M1) strains. B. Conidium morphology of PH-1 and M1. Bar = 20 μm. C. The average number of septa in conidia of PH-1 and mutant M1 harvested from 5-day-old CMC cultures. D. Germlings of PH-1 and M1 after incubation in YEPD medium for 10 h. Bar = 20 μm. E. Perithecia and asci of PH-1 and M1 formed on selfing cultures two-weeks after induction. Bar = 20μm.

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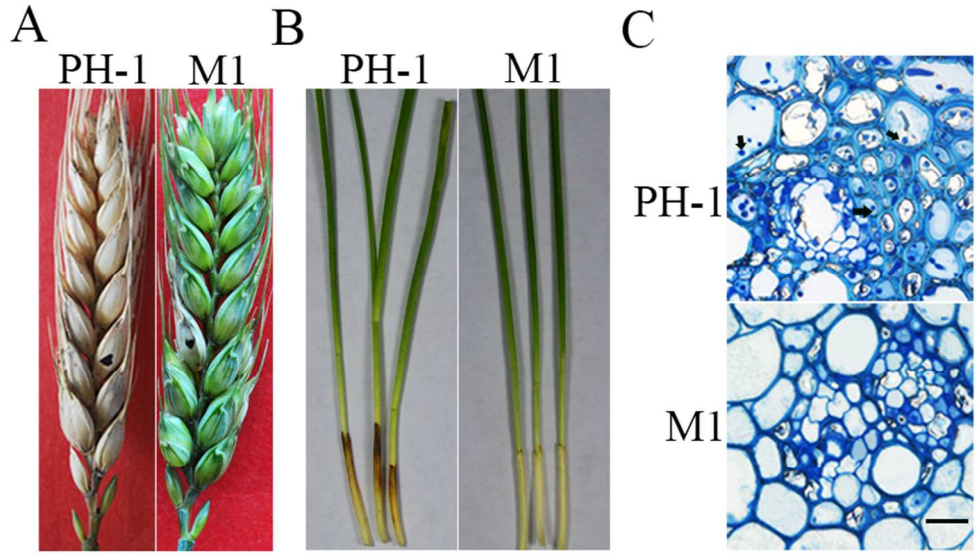

Fig. 2. Defects of the Fgcap1 mutant in plant infection.
A. Flowering wheat heads of cultivar Xiaoyan 22 were drop-inoculated with conidia from the wild type (PH-1) and Fgcap1 mutant (M1). Spikelets with typical symptoms were photographed 14 days post-inoculation (dpi). Black dots mark the inoculated spikelets. B. Wheat coleoptiles inoculated with PH-1 and M1 were photographed 7 dpi. C. The rachis directly beneath the inoculated spikelets was examined 5 dpi. Hyphae growth (marked with arrows) was abundant in plant tissues inoculated with PH-1 and but not in samples inoculated with the Fgcap1 mutant M1. Bar = 20μm.

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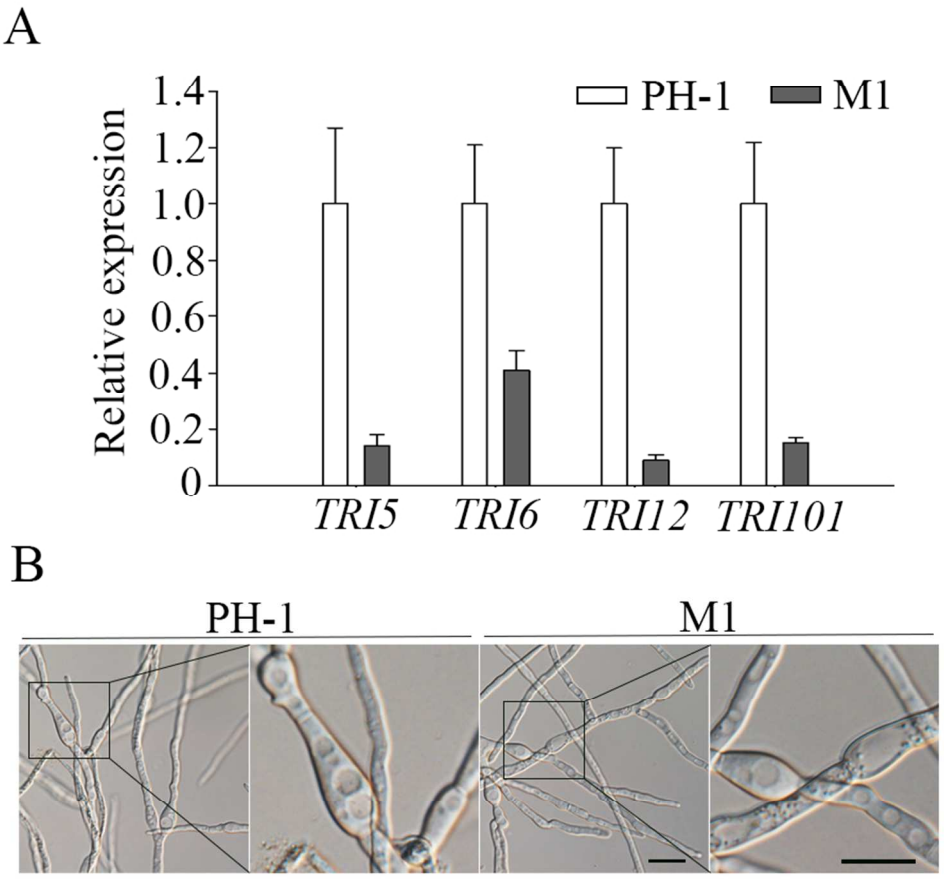

Fig. 3. Defects of the Fgcap1 mutant in DON biosynthesis.
A. The expression levels of TRI5, TRI6, TRI10, and TRI101 in the wild type PH-1 and Fgcap1 mutant M1. The relative expression level of each gene in PH-1 was arbitrarily set to 1. Mean and standard error were calculated with data from three independent biological replicates. B. LTB cultures of PH-1 and mutant M1 were examined for bulbous structures after incubation for 3 days. Bar = 20 μm.

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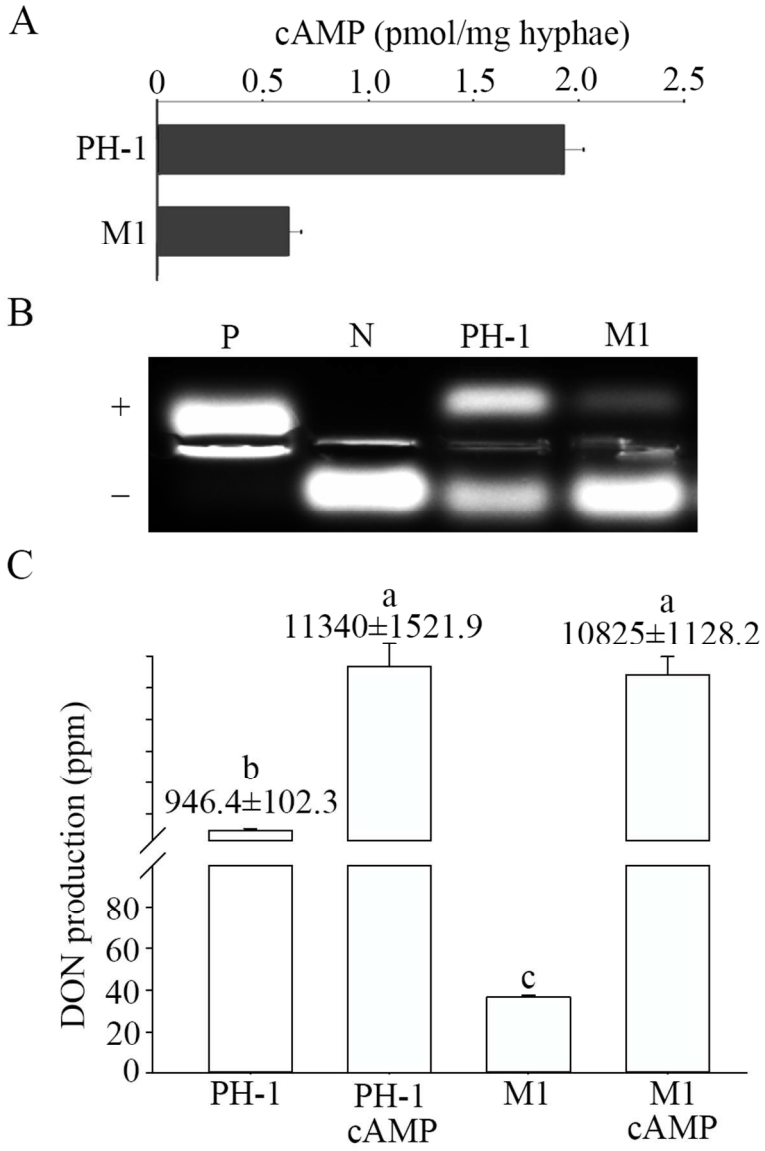

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A. The intracellular cAMP level in PH-1 and Fgcap1 mutant M1. Mean and standard error were calculated with results from three independent biological replicates. B. PKA activities were assayed with proteins isolated from hyphae of PH-1 and M1 with the PepTag A1 PKA substrate peptide. Whereas phosphorylated peptides migrated toward the anode (+), un-phosphorylated peptides migrated toward the cathode (-) on a 0.8% agarose gel. N: non-phosphorylated sample control; P: phosphorylated sample control. C. DON production in 7-day-old LTB cultures of PH-1 and M1 with or without 4 mM cAMP. Different letters indicated statistically significant differences (P=0.05).

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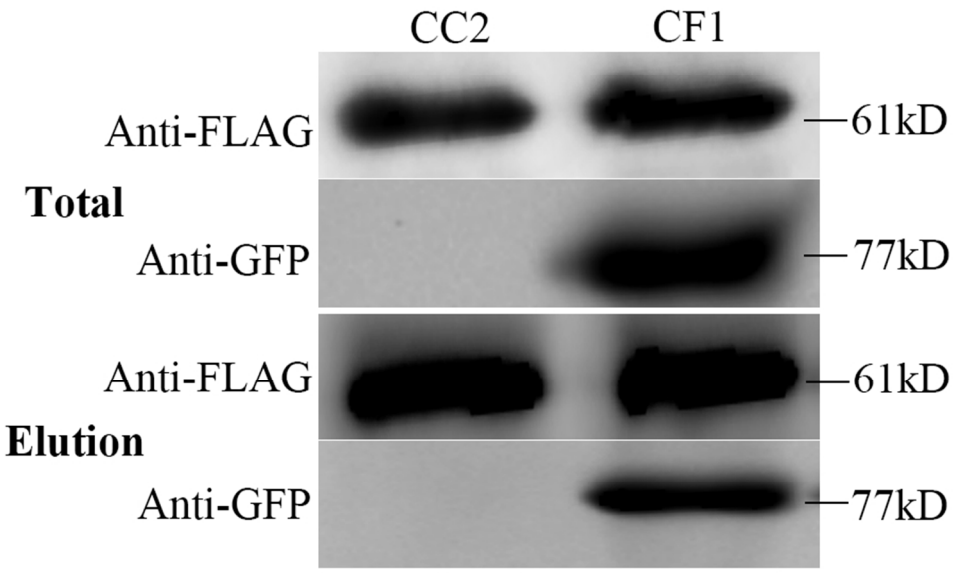

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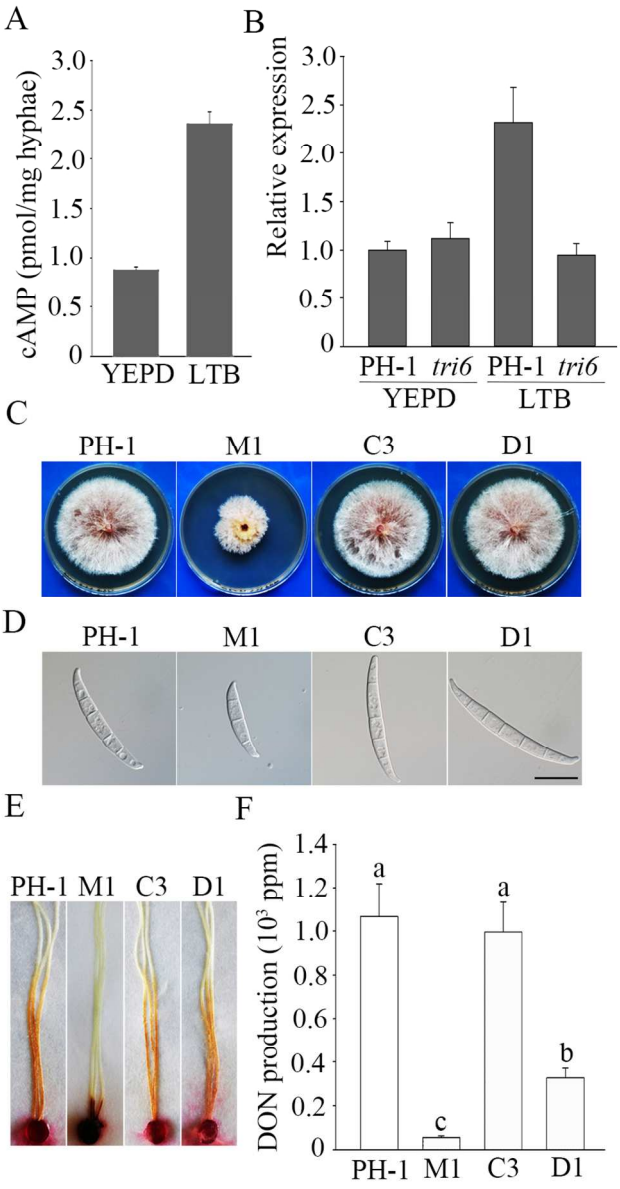

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A. The intracellular cAMP level was assayed with hyphae of the wild-type strain PH-1 harvested from 3-day-old YEPD and LTB cultures. B. Expression levels of FgCAP1 in 3-day-old YEPD or LTB cultures of PH-1 and tri6 mutant. The relative expression level of FgCAP1 in YEPD cultures of PH-1 was arbitrarily set to 1. Mean and standard error were calculated with results from three independent biological replicates. C. Three-day-old PDA cultures of PH-1, Fgcap1 mutant M1, and transformants of M1 expressing the wild-type FgCAP1ΔWT (C3) or mutant allele of FgCAP1 ΔTri6B (D1) deleted of the Tri6-binding site. D. Conidia of the same set of strains harvested from 5-day-old CMC cultures. Bar = 20 μm. E. Typical infected corn silks were photographed 6 dpi. F. DON production in 7-day-old LTB cultures of the same set of strains.

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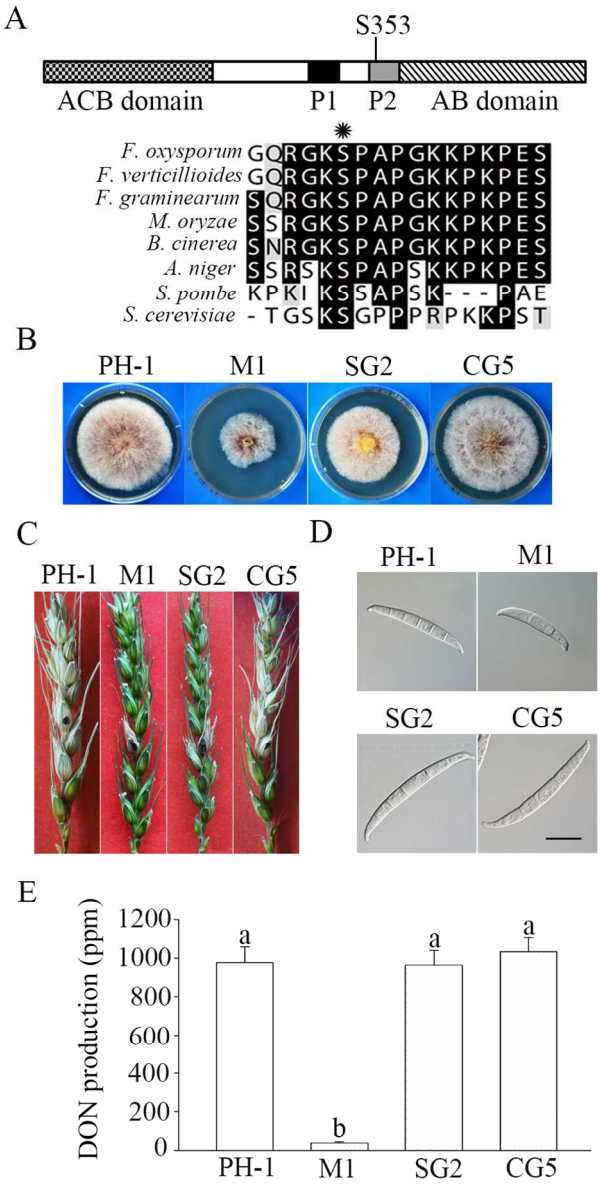

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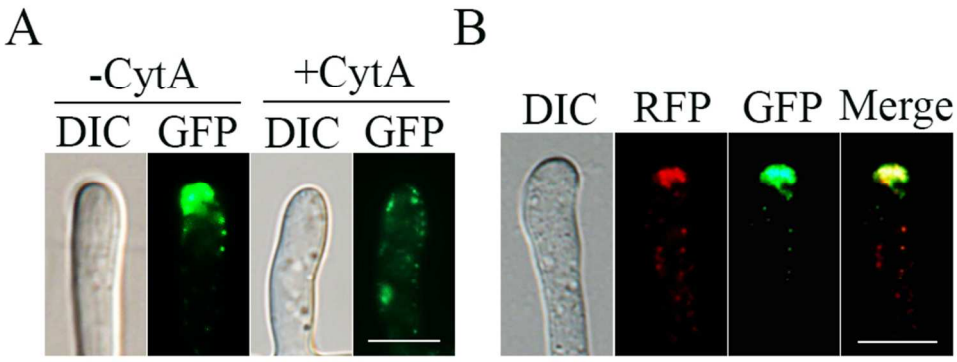

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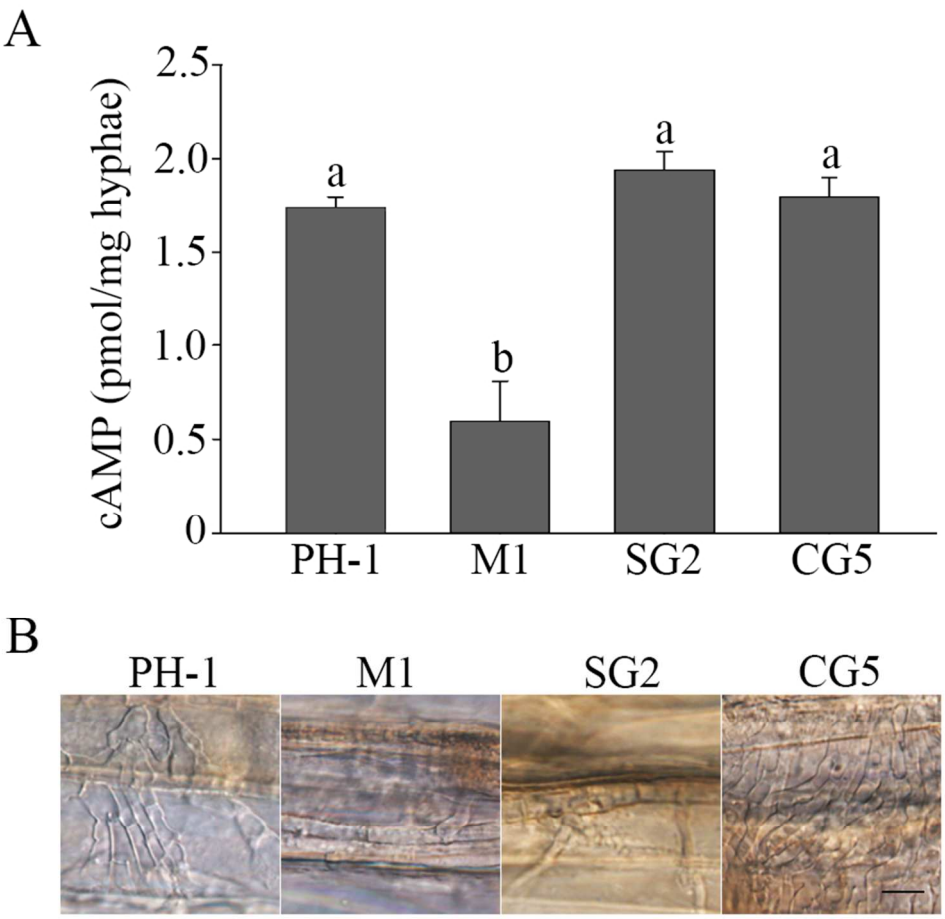

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