Capping proteins regulate fungal development, DON-toxisome formation and virulence in *Fusarium graminearum*

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SUMMARY

Deoxynivalenol (DON) is an important trichothecene mycotoxin produced by the cereal pathogen *Fusarium graminearum*. DON is synthesized in organized endoplasmic reticulum structures called toxisomes. However, the mechanism for toxisome formation and the components of toxisomes are not yet fully understood. In a previous study, we found that myosin I (FgMyo1)-actin cytoskeleton participated in toxisome formation. In the current study, we identified two new components of toxisomes, the actin capping proteins (CAPs) FgCapA and FgCapB. These two CAPs form a heterodimer in *F. graminearum*, and physically interact with FgMyo1 and Tri1. The deletion mutants ΔΔFgcapA and ΔΔFgcapB and the double deletion mutant ΔΔFgcapA/B dramatically reduced hyphal growth, asexual and sexual reproduction and endocytosis. More importantly, the deletion mutants markedly disrupted toxisome formation and DON production, and attenuated virulence in planta. Collectively, these results suggest that the actin CAPs are associated with toxisome formation and contribute to the virulence and development of *F. graminearum*.

Keywords: actin cytoskeleton, capping protein, deoxynivalenol (DON), *Fusarium graminearum*, toxisome, virulence.

INTRODUCTION

The actin cytoskeleton is crucial for various eukaryotic cellular processes, such as cell division, cytokinesis, endocytosis, vesicle trafficking and motility (Pollard and Borisy, 2003; Pollard and Cooper, 2009). For the progression of these cellular processes, cells must rapidly regulate the turnover between the monomeric form of actin and its filamentous form, F-actin, in response to environmental stimuli (Cooper and Schafer, 2000; Rohatgi et al., 1999). A number of actin-interacting or related proteins are involved in modulating actin dynamics, including the actin-depolymerizing factor (ADF)/cofilin family, Arp2/3 complex and capping proteins (CAPs) (Cooper and Schafer, 2000).

Actin filaments are polar double-helical polymers of globular subunits and have two ends, referred to as the barbed and pointed ends. The barbed end, compared with the pointed end, plays a major role in filamentous dynamics, as it has higher association and dissociation rate constants for actin subunits (Wear and Cooper, 2004). Specialized proteins bind to the ends of actin to regulate the assembly and disassembly of actin filaments (Cooper and Schafer, 2000; dos Remedios et al., 2003). CAPs are important for the assembly of various actin structures by tightly capping the barbed end of actin filaments to prevent the addition or loss of actin subunits (Cooper and Schafer, 2000; Shekhar et al., 2016; Wear and Cooper, 2004). CAPs are ubiquitous and highly conserved in all eukaryotic organisms from yeasts to mammals. CAPs exist in cells in a stable heterodimeric form consisting of alpha (α) and beta (β) subunits that have similar secondary structures but lack sequence similarity (Cooper and Sept, 2008; Wear and Cooper, 2004). Studies have demonstrated that deletion mutants or loss-of-function of CAPs cause defects in several cellular and developmental processes in various organisms. CAPs participate in stereocilia widening by preventing newly elongated actin filaments from depolymerizing in mice (Avenarius et al., 2017). Silencing CAPs in both cultured mammalian B16F10 cells and neurons of developing neocortices impairs cell migration (Sinnar et al., 2014). In the apicomplexan parasite *Plasmodium*, the β subunit of the CAP is an essential regulator of sporozoite motility and malaria transmission (Ganter et al., 2009). In *Arabidopsis*, CAP mutants have an abnormal cell morphology and have 10–20% longer hypocotyls than wild type (Li et al., 2012). In *Saccharomyces cerevisiae*, deletion of either the CAP1 or CAP2 gene leads to an abnormal actin distribution with fewer actin cables and an increased number of actin patches. The mutant cells appear round and enlarged and exhibit growth defects with a heterogeneous size distribution (Amatruda et al., 1990; Kovar et al., 2005). In the plant pathogenic fungus *Magnaporthe oryzae*,...
the CAP homologues MoCapA and MoCapB are important for endocytosis and actin dynamics and are directly linked to fungal growth, conidiation and pathogenicity (Li et al., 2017b).

Fusarium head blight (FHB), predominately caused by Fusarium graminearum, is one of the most devastating diseases of wheat worldwide (Xu and Nicholson, 2009). FHB epidemics cause yield losses in FHB-prone regions of the world (Nganje et al., 2004). In addition to severe yield losses, FHB leads to harmful mycotoxin contamination in infested grains, such as deoxynivalenol (DON), nivalenol and zearalenone (Chen et al., 2019). Among them, DON biosynthesis enzymes and regulators encoded by 15 TRI genes have been well characterized (Alexander et al., 2018). The biosynthetic enzymes and regulators encoded by 15 TRI genes have been well characterized (Alexander et al., 2018). The biosynthetic enzymes and regulators encoded by 15 TRI genes have been well characterized.

RESULTS
Capping proteins interact with Tri1 and FgMyo1 in F. graminearum

The toxisome is considered the DON biosynthesis compartment in F. graminearum, while proteins that participate in toxisome formation are still under investigation (Boenisch et al., 2017). DON biosynthetic enzymes such as Tri1, Tri4 and the class I myosin FgMyo1 have been identified as components of the toxisome (Tang et al., 2018). To identify other proteins involved in toxisome formation, we previously performed affinity capture-mass spectrometry (AC-MS) assays using Tri1 and FgMyo1 as baits (Tang et al., 2018). Two proteins (loci FGSG_08621 and FGSG_01226) encoding homologues to fungal F-actin CAP subunits were captured in both AC-MS assays. A phylogenetic analysis of the putative CAPs, including F. graminearum and seven other tested fungi, demonstrated that the fungal CAP α and β subunits were highly conserved. Interestingly, the CAP subunit homologues in filamentous fungi and yeasts were notably divided into two groups. Based on the phylogenetic tree, we named FGSG_08621 FgCapA (α subunit of CAP) and FGSG_01226 FgCapB (β subunit of CAP) in F. graminearum (Fig. S1).

To further determine the interaction patterns between FgCAPs and FgMyo1 or FgTri1, we conducted coimmunoprecipitation (Co-IP), colocalization and bimolecular fluorescence complementation (BiFC) assays under DON-inducing conditions. As shown in Fig. 1A,B, FgCapA interacted with FgMyo1 and Tri1 in the Co-IP assay. A strain bearing FgCapA-mCherry (red fluorescent protein) and Tri1-GFP (green fluorescent protein) was constructed in the wild-type background and cultured in liquid trichothecene biosynthesis induction (TBI) medium to observe colocalization. The red fluorescence signals (FgCapA-mCherry) were diffuse throughout the cytoplasm and partially colocalized with Tri1-GFP at the toxisome (Fig. 1C). Moreover, the direct interaction between FgCapA and Tri1 was verified by BiFC (Fig. 1D). Similar to FgCapA, FgCapB also interacted with FgMyo1 and Tri1 (Fig. S2). Combining the AC-MS, Co-IP, colocalization and BiFC assays, these results indicate that CAPs interact with Tri1 and FgMyo1 in F. graminearum.

FgCapA and FgCapB form a heterodimer

FgCapA and FgCapB are predicted to encode proteins of 316 and 282 amino acids and share 37% and 45% sequence identity, respectively, with counterparts in S. cerevisiae. However, FgCapA and FgCapB share only 10% amino acid sequence identity.

It has been shown that CAPs exist in a more stable heterodimer form consisting of α and β subunits in cells. To test whether FgCapA and FgCapB also interact with each other to form a heterodimer in F. graminearum, a yeast two-hybrid assay was performed. The results indicated that FgCapA and FgCapB indeed interact with each other (Fig. 2A). Subsequently, the localization patterns of FgCapA and FgCapB were visualized by confocal microscopy. The FgCAPA-GFP and FgCAPB-mCherry fusion constructs were co-transformed into the wild-type strain. As shown in Fig. 2B, the FgCapA-GFP and FgCapB-mCherry fused proteins were mainly distributed in a similar patch pattern in hyphae grown in potato dextrose broth (PDB) medium. Importantly, the colocalization of FgCapA-GFP and FgCapB-mCherry was clearly observed. Additionally, a Co-IP assay showed that FgCapA and FgCapB interact with each other (Fig. 2C). A homology model of the F. graminearum CAP, based on the structure of the chicken CAP (Yamashita et al., 2003), yielded a mushroom-like structure (Fig. 2D). The stalk was formed by a six α-helices from N-terminal regions of each subunit). The mushroom
**Fig. 1**  FgCapA interacts with FgMyo1 and Tri1. (A) The interaction of FgCapA-GFP and FgMyo1-Flag was verified by the co-immunoprecipitation (Co-IP) assay. Total protein (Input) extracted from the strain bearing FgCapA-GFP and FgMyo1-Flag constructs or a single construct (FgCapA-GFP or FgMyo1-Flag) were subjected to SDS-PAGE, and immunoblots were incubated with anti-FLAG and anti-GFP antibodies, as indicated (Input panel). Each protein sample was pulled down using anti-FLAG agarose and further detected with anti-GFP antibody (Flag pull-down panel). Protein samples were also detected with anti-GAPDH antibody as a reference. (B) The interaction of FgCapA-mCherry and Tri1-GFP was verified by the Co-IP assay. Protein samples were pulled down using anti-GFP agarose and further detected with an anti-mCherry antibody. Protein samples were also detected with anti-GAPDH antibody as a reference. (C) FgCapA-mCherry was partially colocalized with Tri1-GFP on DON-toxisomes at 48 h of incubation in trichothecene biosynthesis induction (TBI) medium. Localization is indicated with yellow arrows. Bar = 10 μm. (D) The interaction of FgCapA with Tri1 was confirmed by bimolecular fluorescence complementation (BiFC) assay. The constructs of YFP-N-Tri1 and pFgCapA-YFPC were co-transformed into Fusarium graminearum PH-1 to generate the strain YFPN-Tri1 + FgCapA-YFPC. The strains bearing a single construct (YFPN-Tri1 or FgCapA-YFPC) were used as negative controls. The yellow fluorescent protein (YFP) signals in hyphae of each strain grown in the TBI medium were examined under a confocal microscope. DIC, differential interference contrast. Bar = 10 μm.

**Fig. 2**  FgCapA and FgCapB form a heterodimer. (A) The CAPs interacted with each other in the yeast-two-hybrid assay. Serial concentrations of yeast cells transferred with the bait and prey constructs indicated in the figure were assayed for growth on SD-Leu-Trp-His-Ade plates. pGBKT7-Lam and pGADT7 were used as positive controls. Another pair of plasmids, pGBKTK7 and pGADT7, were used as negative controls. Images were taken after 3 days of incubation at 30 °C. pGADT7 and pGBKTK7 are abbreviated to AD and BD, respectively. (B) FgCapA-GFP colocalized with FgCapB-mCherry. Vegetative hyphae of dual-labelled strains were observed under a confocal microscope after incubation in PDB medium for 24 h. DIC, differential interference contrast. Bar = 10 μm. (C) The interaction of FgCapA-GFP and FgCapB-mCherry was verified by the Co-IP assay. Protein samples were pulled down using anti-GFP agarose and further detected with an anti-mCherry antibody. Protein samples were also detected with anti-GAPDH antibody as a reference. (D) A three-dimensional homology model of the Fusarium graminearum CAP heterodimer based on the structure of chicken CAPs (Protein Data Bank, accession code 1IZN).
cap consisted of a ten-stranded anti-parallel β-sheet (five strands from each subunit), on top of which was two long C-terminal α-helices (one helix from each subunit) running perpendicular to the β-sheet strands (Fig. 2D).

**CAPs interact with actin and participate in actin organization**

CAPs bind the ends of actin filaments and play a critical role in regulating the addition and dissociation of actin subunits (Rao *et al.*, 2014). Therefore, we determined whether the FgCAPs also interacted with actin. First, the colocalization patterns were observed in the wild-type strain dual-labelled with FgCapA-GFP or FgCapB-GFP and the actin reporter, Lifeact-RFP. As shown in Fig. 3A,B, signals of FgCap-GFP mostly colocalized with the red fluorescence signals of actin patches, especially in the cells of hyphal tips, suggesting that the FgCaps potentially interacted with actin. Next, we conducted Co-IP assays and confirmed that the CAPs interacted with actin in *F. graminearum* (Fig. 3C,D).

To investigate whether or not CAPs are involved in the actin organization, we constructed deletion mutants ΔFgcapA, ΔFgcapB and double-mutant ΔFgcapA/B in the wild-type strain expressing the actin reporter, Lifeact-RFP, and observed the actin patterns in these strains. Most of the wild-type strain generally showed several long actin cables in the hyphae, while fewer and shorter cables were formed in the hyphae of mutants (Fig. 3E). Meanwhile, the actin patches were reduced at the top of the hyphae.
of the mutant hyphae compared with wild type (Fig. 3E). Taken together, FgCapA and FgCapB physically form a heterodimer, interact with actin and participate in actin organization in *F. graminearum*.

**FgCaps are important for hyphal growth and fungal reproduction**

Our in-house RNA-sequencing (RNA-Seq) data indicated that *FgCAPA* and *FgCAPB* had similar transcriptional patterns in all five tested conditions, including on conidiation medium (carboxymethyl cellulose, CMC), carrot agar (sexual reproduction), PDB, TBI and during plant infection. Notably, their expression was increased under DON-inducing conditions and *in planta* (Fig. 4A).

To characterize the function of FgCapA and FgCapB, we constructed single and double deletion mutants, Δ*FgcapA*, Δ*FgcapB* and ΔΔ*FgcapA/B* in the wild-type strain using homologous recombination. The deletion mutants were identified by polymerase chain reaction (PCR) amplification and Southern blot analysis (Fig. S3). At least three independent transformants of the Δ*FgcapA*, Δ*FgcapB* and ΔΔ*FgcapA/B* mutants were obtained, and all transformants showed similar defective phenotypes under the tested conditions. Therefore, the phenotypes of one representative transformant for each mutant are shown in the following experiments. To confirm that phenotypic defects of mutants were directly related to the deletion, we complemented single deletion mutants with the corresponding open reading frame (ORF) fused with *gfp*, encoding green fluorescent protein (GFP), at the carboxyl terminus under the native promoter and generated the complementation strains. Thus, our results indicate that both FgCapA and FgCapB are critical for hyphal growth, asexual and sexual reproduction in this fungus.

**FgCaps are required for endocytosis and adaptation to abiotic stress**

In a previous study, we found that deletion mutants of the actin cytoskeleton-related genes *FgPRK1* and *FgEND3* caused endocytic defects in *F. graminearum* (Tang et al., 2018). Given that the FgCaps interacted with actin, we tested whether deletion of the CAP genes also affected the endocytosis process using the FM4-64 staining assay. The plasma membrane and septa were quickly stained with FM4-64 in both the conidia and mycelia of all tested strains. The FM4-64 dye was endocytosed and generated clear fluorescence signals on the membrane of intracellular organelles, such as vacuoles and endosomes, after a 10 min staining of the wild-type conidia and mycelia. However, the endocytosis of the fluorescent dye was dramatically hindered in all mutants under the same conditions (Fig. 5). These results suggest that the FgCap proteins are important for the endocytosis process in *F. graminearum*.

Eukaryotic cells rapidly regulate the turnover of actin forms in response to environmental stimuli, which require actin CAPs (Cooper and Schafer, 2000; Rohatgi et al., 1999). Therefore, we were interested in determining the sensitivities of the FgCAP gene mutants to various abiotic stresses, including cell membrane stress, cell wall and oxidative stresses generated by sodium dodecyl sulphate (SDS), Congo red and H₂O₂, respectively. The sensitivity assays showed that the CAP mutants demonstrated significantly decreased sensitivity to all three tested abiotic agents compared to the wild-type strain PH-1 (Fig. 6). The complementation strains Δ*FgcapA-C* and Δ*FgcapB-C* exhibited similar sensitivities to that of the wild-type strain (Fig. 6), indicating that the complementation strains successfully rescued the defects of the corresponding mutant to abiotic stresses.

**F-actin-capping motifs are essential for the function of FgCapA but not FgCapB**

Sequence analysis indicated that FgCapA contains two conserved F-actin-capping motifs and FgCapB contains one F-actin-capping motif (Fig. 7A). To analyse the role of the F-actin-capping motifs in FgCapA and FgCapB, we constructed the Δ*FgcapA-C-DΔA1*, Δ*FgcapA-C-DΔA2* and Δ*FgcapB-C-DΔB* strains. The native promoter and ORF of *FgCAPA* lacking the F-actin-capping A1 motif was fused with a GFP fragment, and the resulting cassette was...
transformed into a ΔFgcapA strain background. The resulting transformant was identified and designated as ΔFgcapA-C-ΔA1. Using a similar procedure, ΔFgcapA-C-ΔA2 expressing FgCAPA-GFP and lacking the F-actin-capping A2 motif in the ΔFgcapA strain and ΔFgcapB-C-ΔB expressing FgCAPB-GFP and lacking the F-actin-capping B motif in the ΔFgcapB strain were generated.

As shown in Fig. 7B, ΔFgcapA-C-ΔA1 and ΔFgcapA-C-ΔA2 failed to complement the growth defects of ΔFgcapA, indicating that both
motifs A1 and A2 are important for the function of FgCapA on vegetative growth. In these two strains, the GFP signals diffused evenly in the cytoplasm of mycelia without an actin localization pattern (Fig. 7C). Further investigation indicated that the complementation strains lacking motif A1 or A2 showed similar defects in conidiation, sexual reproduction and endocytosis to those of ΔFgcapA strain (Fig. 7D–G). However, FgCapB-GFP lacking F-actin-capping motif B still exhibited actin localization in the ΔFgcapB-C-ΔB strain (Fig. 7C). Moreover, the ΔFgcapB-C-ΔB strain demonstrated similar mycelial growth rates and all tested phenotypes to those of ΔFgcapB-C and the wild-type strain PH-1 (Fig. 7B,D–G).

To further verify whether the F-actin-capping motifs are essential for interaction between CAPs and actin in vivo, the actin-RFP fusion construct was individually introduced into the ΔFgcapA-C-ΔA1, ΔFgcapA-C-ΔA2 and ΔFgcapB-C-ΔB. Total protein was isolated from the positive transformants and Co-IP analysis was performed. As shown in Fig. 8A,B, FgCapA lacking either the F-actin-capping A1 or A2 motif was unable to interact with actin protein. In consistence with phenotypes of ΔFgcapB-C-ΔB, FgCapB lacking F-actin-capping B motif did not affect the interaction between FgCapB and actin (Fig. 8C). Taken together, these results indicated that the F-actin-capping motifs were essential for the functions of FgCapA but not for FgCapB in F. graminearum.

**FgCAP deletion mutants have attenuated DON production and virulence**

FgCapA and FgCapB interacted with Tri1 and FgMyo1 and were localized partially to the toxisome (Figs 1 and S2). Additionally, both FgCapA and FgCapB showed relatively high transcriptional levels in the DON-inducing TBI medium and during the infection process in planta (Fig. 4A). Thus, we were interested in investigating the function of FgCapA and FgCapB in toxisome formation, DON production and virulence.

As described previously, Tri1-GFP can be used as an indicator for DON-toxisome formation (Tang et al., 2018), thus Tri1-GFP was transformed into the wild-type and CAP mutant strains. As shown in Fig. 9A, expression of Tri1-GFP was highly induced, and typical spherical toxisomes were formed in the mycelia of the wild-type strain after 2 days of incubation in TBI. In contrast, the Tri1-GFP signals in ΔFgcapA and ΔFgcapB mutants dramatically decreased, and very faint toxisomes were observed (Fig. 9A). Western blot analysis confirmed that the amounts of the Tri1-GFP protein in the CAP mutants were also considerably lower than that in the wild-type strain (Fig. 9B). Meanwhile, the transcriptional levels of the TRI genes in the deletion mutants were assayed by RT-qPCR after incubation in TBI medium. Three selected TRI genes (TRI1, TRI5 and TRI6) were dramatically down-regulated in the ΔFgcapA and ΔFgcapB mutant, compared to those in the wild-type strain (Fig. 9C). Consistent with toxisome formation and expression of TRI genes, CAP mutants produced less DON than the wild-type strain after 7 days of incubation in TBI (Fig. 9D). These results suggest that FgCaps participated in expression of TRI gene and toxisome formation, and were important for DON biosynthesis in F. graminearum.

DON is a key virulence factor in F. graminearum, thus the virulence of the CAP mutants was evaluated by point-inoculating conidial suspensions in flowering wheat heads and mycelial plugs
on the leaves of wheat seedlings. On wheat heads inoculated with the wild-type strain PH-1, scab symptoms first developed on the inoculated spikelets and rapidly spread to the neighbouring wheat head. Fifteen days after inoculation, severe and typical scab symptoms were caused by the wild-type and complementation strains. In contrast, scab symptoms caused by mutants were restricted to the inoculated spikelets and failed to spread from the inoculated floret to the rachis (Fig. 9E). Additionally, all mutants demonstrated attenuated virulence on seedling leaves (Fig. 9F). The complementation strains $\Delta FgcapA-C$ and $\Delta FgcapB-C$ exhibited wild-type levels of DON production and virulence (Fig. 9D–F). Moreover, F-actin-capping motif truncated mutant $\Delta FgcapA$-$\Delta A1$ and $\Delta FgcapA$-$\Delta A2$ demonstrated similar reduction of DON production and virulence as $\Delta FgcapA$ mutant. The pathogenicity and DON biosynthesis of $\Delta FgcapB$-$\Delta B$ mutant was the same as that of wild type, as other tested phenotypes (Fig. 9C–F). Collectively, our results suggest that CAPs are important for toxisome formation, DON production and virulence in *F. graminearum*.

**DISCUSSION**

The actin cytoskeleton plays an important yet poorly understood role during cellular development in eukaryotes. Remodelling of the actin cytoskeleton in response to internal signals or environmental stimuli is controlled by a plethora of actin-binding proteins, including the CAP proteins (Cooper and Sept, 2008). Although CAPs have been investigated in yeast, *Arabidopsis* and mammalian cells (Amatruda et al., 1990; Huang et al., 2003; Kim et al., 2004; Sinnar et al., 2014), the function of CAPs in filamentous fungi is still largely unknown. Here, we identified and genetically characterized two CAPs, FgCapA and FgCapB, in the plant pathogenic fungus *F. graminearum*. FgCapA interacted with FgCapB. Deletion of FgCAPA and FgCAPB resulted in various defects, including a reduction in hyphal growth and conidiation,
Capping proteins contribute DON-toxisome formation

sexual reproduction and decreased endocytosis, DON biosynthesis and virulence in *F. graminearum* (Figs 4–7 and 9). The double gene deletion mutant exhibited similar phenotypes to the single gene deletion mutants, supporting the hypothesis that the two CAP proteins form a heterodimer. These results were consistent with studies of other eukaryotes (Amatruda et al., 1990; Blanchin et al., 2014; Li et al., 2017b). In yeast and *M. oryzae*, the F-actin-capping motifs of CapA were required to interact with actin (Amatruda et al., 1990; Li et al., 2017b). Similarly, in the current study, we found that FgCapA interacted with actin, and the interaction depended on its F-actin-capping motifs (Figs 3 and 8). Unexpectedly, although FgCapB also interacted with actin, ΔFgcapB-C-ΔB completely rescued the defects of the ΔFgcapB mutant, indicating that the F-actin-capping motif B was not required for the function of FgCapB, which was different from the results of previous studies in other fungi (Amatruda et al., 1990).
et al., 1990; Li et al., 2017b). Other unidentified motif(s) in FgCapB may be responsible for its interaction with actin. Taken together, these results indicate that CAPs may have species-specific interaction patterns with actin in different organisms.

The DON biosynthetic organelle, the toxisome, is a remodelled perinuclear endoplasmic reticulum in F. graminearum (Boenisch et al., 2017, 2019; Flynn et al., 2019; Tang et al., 2018). The assembly of toxisomes is mediated by the myosin-actin cytoskeleton (Tang et al., 2018). Inactivation of FgMyo1 by the fungicide phenamacril or disruption of F-actin formation using latrunculin A dramatically inhibited toxisome formation and DON biosynthesis (Tang et al., 2018; Zhang et al., 2015; Zheng et al., 2015). Additionally, deletion of the actin-associated protein genes FgPRK1 or FgEND3 also hindered toxisome formation and reduced the concentration of trichothecenes that accumulate in cultures (Tang et al., 2018). In this study, we further found that actin CAPs interact with actin (Fig. 3) and the deletion of FgCAPA or FgCAPB leads to defects in toxisome formation and subsequent DON production (Fig. 9), indicating that FgCaps are required for toxisome formation. Moreover, previous studies have shown that the trichothecene efflux pump Tri12 interacts with toxisomes and may participate in DON transport through vesicles and vacuoles. The motility of vesicles containing Tri12 was largely dependent on filamentous actin (Menke et al., 2012, 2013; Roze et al., 2011), suggesting that the actin cytoskeleton plays an important role in DON export. Thus, it is likely that the actin cytoskeleton can be used as a potential target for the development of new fungicides to inhibit DON biosynthesis.

Emerging evidence has demonstrated that the actin cytoskeleton plays an important role during plant–microbe interactions. In Arabidopsis epidermal cells, cortical actin was remodelled within minutes for defence responses in response to pathogenic and nonpathogenic microbes and diverse microbe-associated molecular patterns (MAMPs) (Henty-Ridilla et al., 2013, 2014). Capping proteins integrate multiple MAMP signalling pathways to modulate actin dynamics for plant resistance against bacterial and fungal phytopathogens (Li et al., 2012, 2015, 2017a). Moreover, the actin cytoskeleton is critical for virulence in plant pathogens. For example, assembly of an F-actin network was required for initiation of the infection process in M. oryzae (Kankanala et al., 2007). CAP mutants in M. oryzae dramatically reduced fungal virulence in planta (Li et al., 2017b). In Botrytis cinerea, deletion of the capping gene BcCAP1 impaired the ability of the pathogen to penetrate host tissue and its subsequent invasive growth in the leaves of Phaseolus vulgaris (Gonzalez-Rodriguez et al., 2016). Here, we found that the deletion mutants of CAP genes attenuate the disease symptoms, although they could successfully penetrate the hosts, indicating that CAPs play an important role in F. graminearum growth during invasion (Fig. 9E,F). DON has been well characterized as a critical virulence factor for the spread of F. graminearum within wheat spikelets (Desjardins et al., 1996), therefore the reduced spread of the capping gene mutants during invasion could primarily result from the defect in DON production (Fig. 9D). Additionally, the growth defect of the mutants may affect the development of disease symptoms in planta (Fig. 4B). Taken together, the defects of hyphal growth and DON biosynthesis contributed to the attenuated virulence of the F. graminearum actin capping mutants on the host plants.

**EXPERIMENTAL PROCEDURES**

**Fungal strains and culture conditions**

As a parental strain, the F. graminearum strain PH-1 was used in this study. The wild-type and mutant strains were grown at 25 °C on potato dextrose agar (PDA) (200 g potato, 20 g glucose, 10 g
agar and 1 L water), minimal medium (MM) (10 mM K$_2$HPO$_4$, 10 mM KH$_2$PO$_4$, 4 mM (NH$_4$)$_2$SO$_4$, 2.5 mM NaCl, 2 mM MgSO$_4$, 0.45 mM CaCl$_2$, 9 mM FeSO$_4$, 10 mM glucose, 1% agar and 1 L water, pH 6.9) and complete medium (CM) (1% glucose, 0.2% peptone, 0.1% yeast extract, 0.1% casamino acids, nitrate salts, trace elements, 0.01% vitamins and 1 L water, pH 6.5) to determine the growth rate and colony morphology.

For conidiation assays, ten mycelial plugs (5 mm in diameter) of each strain were taken from the periphery of a 3-day-old colony and inoculated in a 50-mL flask containing 20 mL of CMC liquid and incubated in trichothecene biosynthesis induction (TBI) medium for 48 h before imaging. Bar = 10 μm. (B) The accumulation of the Tri1-GFP protein in each strain was further determined by western blot assay using the anti-GFP antibody. The protein samples were also detected with anti-GAPDH antibody as a reference. (C) Relative mRNA expression level of TRI1, TRI5 and TRI6 in the strains tested. After culturing in TBI for 2 days, mycelia of each strain were harvested for mRNA extraction. The Actin was used as a reference gene. (D) DON production in the wild-type, mutant and various complementation strains after 7 days of incubation in TBI. Bars denote standard deviations from three experiments. Columns labelled with the same letter are not significantly different according to the LSD test at P = 0.05. (E) Virulence of the wild-type, mutant and various complementation strains on wheat heads. Infected wheat heads were examined at 15 days after inoculation with a conidial suspension of each strain. The inoculation sites are indicated as black dots. (F) Disease symptoms on young wheat leaves infected by the wild-type, mutant and various complementation strains. The images were taken at 6 days post-inoculation.

Fig. 9 Capping gene deletion mutants attenuated toxisome formation, DON production and virulence in planta. (A) Toxisome formation in Fusarium graminearum wild-type PH-1, ΔFgcapA, ΔFgcapB and F-actin-capping motif truncated mutants. All strains were labelled with Tri1-GFP as a toxisome indicator and incubated in trichothecene biosynthesis induction (TBI) medium for 48 h before imaging. Bar = 10 μm. (B) The accumulation of the Tri1-GFP protein in each strain was further determined by western blot assay using the anti-GFP antibody. The protein samples were also detected with anti-GAPDH antibody as a reference. (C) Relative mRNA expression level of TRI1, TRI5 and TRI6 in the strains tested. After culturing in TBI for 2 days, mycelia of each strain were harvested for mRNA extraction. The Actin was used as a reference gene. (D) DON production in the wild-type, mutant and various complementation strains after 7 days of incubation in TBI. Bars denote standard deviations from three experiments. Columns labelled with the same letter are not significantly different according to the LSD test at P = 0.05. (E) Virulence of the wild-type, mutant and various complementation strains on wheat heads. Infected wheat heads were examined at 15 days after inoculation with a conidial suspension of each strain. The inoculation sites are indicated as black dots. (F) Disease symptoms on young wheat leaves infected by the wild-type, mutant and various complementation strains. The images were taken at 6 days post-inoculation.
medium (15 g carboxymethyl cellulose, 1 g yeast extract, 0.5 g MgSO₄, 1 g NH₄NO₃, 1 g KH₂PO₄ and 1 L water) (Cappelini and Peterson, 1965). The flasks were incubated at 25 °C for 4 days in a shaker (180 rpm), and the resulting conidia were germinated in distilled water with 2% sucrose. Perithecium formation was assayed on carrot agar medium (CA) (200 g carrot, 20 g agar and 1 L water) at 25 °C under a 12/12 h light/dark cycle. Mycelia were grown on CA for 7 days and then rubbed with a glass spreader after applying 0.1% sterilized Tween 20 solution to induce sexual reproduction. Liquid triothecene biosynthesis induction (TBI) after applying 0.1% sterilized Tween 20 solution to induce sexual growth on CA for 7 days and then rubbed with a glass spreader could not be achieved on the control without treatment and N is that with treatment.

Deletion mutants were identified by PCR with relevant primers (Table S1). The deletion mutants FgcapA/C and FgcapB were generated using a previously described protocol (Yun et al., 2013). To generate the double mutant of FgCAPA and FgCAPB, FgCAPA was knocked out in the ΔFgcapB mutant and the resulting double mutant was designated ΔΔFgcapA/B. The primers used to amplify the flanking sequences for each gene are listed in Table S1. Deletion mutants were identified by PCR with relevant primers and Southern blot assay (Fig. S3).

To construct the FgCAPA-GFP fusion cassette, the FgCAPA fragment containing the native promoter and ORF (without the stop codon) was amplified with primers P17 and P18 (Table S1). The resulting PCR products were co-transformed with XhoI-digested pHZ65 vector harbouring YFP₅ and the hygromycin B resistance cassette, and pHZ68 vector that carries YFP₂ and the zeocin resistance cassette. The final plasmid constructs of pYFP₅-TrI1 and pFgCapA-YFP₂ were verified by sequencing and then co-transformed into the protoplasts of PH-1 in pairs. Transformants resistant to both hygromycin and zeocin were isolated and confirmed by PCR. The recombination plasmid pYFP₅-TrI1 or pFgCapA-YFP₂ was individually transformed into PH-1, and resultant transformants were used as negative controls. YFP signals in the mycelia grown in TBI for 48 h were examined under a confocal microscope.

**Construction of gene deletion mutants and complementation strains**

The deletion mutants ΔFgcapA and ΔFgcapB were generated using a previously described protocol (Yun et al., 2013). To generate the double mutant of FgCAPA and FgCAPB, FgCAPA was knocked out in the ΔFgcapB mutant and the resulting double mutant was designated ΔΔFgcapA/B. The primers used to amplify the flanking sequences for each gene are listed in Table S1. Deletion mutants were identified by PCR with relevant primers and a Southern blot assay (Fig. S3).

**Microscopic examinations**

For toxisome observation, each strain labelled with Tri1-GFP was grown in liquid TBI medium at 28 °C in darkness for 2 days prior to examination under a Zeiss LSM780 confocal microscope (Göttingen, Niedersachsen, Germany). The following confocal microscopy settings were used for GFP observation: laser 488 nm at 50% power, pinhole 90 μm, master gain 580. Conidial germination was stained with CFW at a concentration of 0.1 mg/mL for 30 s before confocal observation. To observe endocytosis, fresh conidia or mycelia were collected and strained with FM4-64 at a concentration of 7.5 μM for 1, 5 or 10 min in the dark at room temperature (Li et al., 2017b). The laser excitation wavelength was set at 561 nm for FM4-64, mCherry and RFP (red fluorescence).

**Bimolecular fluorescence complementation assay**

For BIFC strain construction, the ORF fragments of TRI1 and FgCAPA were respectively fused into Xhol-digested pHZ65 vector harbouring YFP₅ and the hygromycin B resistance cassette, and pHZ68 vector that carries YFP₂ and the zeocin resistance cassette. The final plasmid constructs of pYFP₅-TrI1 and pFgCapA-YFP₂ were verified by sequencing and then co-transformed into the protoplasts of PH-1 in pairs. Transformants resistant to both hygromycin and zeocin were isolated and confirmed by PCR. The recombinant plasmid pYFP₅-TrI1 or pFgCapA-YFP₂ was individually transformed into PH-1, and resultant transformants were used as negative controls. YFP signals in the mycelia grown in TBI for 48 h were examined under a confocal microscope.

**Western blot assay**

Protein isolation was performed as previously described (Yun et al., 2015). The resulting proteins were separated by 10% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto an Immobilon-P transfer membrane (Millipore, Billerica, MA, USA). Monoclonal anti-GFP (ab32146, Abcam, Cambridge, UK), anti-mCherry (ab125096, Sigma, St Louis, MO, USA), anti-RFP (ab65856, Sigma) and anti-Flag (A9044, Sigma, St Louis, MO, USA) antibodies were used at a 1:10 000 dilution for immunoblot analyses. The samples were also detected with monoclonal anti-GAPDH antibody (EM1101, HuaAn Biotechnology Co., Ltd, Hangzhou, Zhejiang, China) as a control.

**Coimmunoprecipitation assay**

The GFP, RFP, 3 × Flag or mCherry fusion constructs were verified by DNA sequencing and transformed in pairs into PH-1. Transformants expressing pairs of fusion constructs were confirmed by western blot analysis. In addition, the transformants expressing a single fusion construct were used as references. For Co-IP assays, total proteins were extracted and incubated with
anti-GFP or anti-Flag agarose. Proteins eluted from agarose were analysed by western blot detection with a polyclonal anti-Flag or an anti-GFP antibody. The protein samples were also detected with monoclonal anti-GAPDH antibody as a reference. Each experiment was repeated twice.

**Yeast two-hybrid assay**

To construct plasmids for the yeast two-hybrid analysis, the ORFs of the FgCAPA and FgCAPB genes were amplified using PH-1 cDNA as a template. The PCR product was inserted into the yeast GAL4-binding domain vector pGBKT7 or GAL4 activation domain vector pGADT7 (Clontech, Mountain View, CA, USA). The pairs of yeast two-hybrid plasmids were co-transformed into the S. cerevisiae strain AH109 following the LiAc/ss-DNA/PEG (lithium acetate/single-stranded DNA/polyethylene glycol) transformation protocol (Yu et al., 2014). In addition, the plasmids pGBK7T-53 and pGADT7 served as positive controls, while the plasmids pGBK7T-Lam and pGADT7 served as negative controls. Transformants were grown at 30 °C for 3 days on synthetic medium (SD) lacking Leu and Trp and then transferred to SD lacking His, Leu, Trp and Ade to assess interaction. Three independent experiments were performed to confirm the results.

**Plant infection, TRI gene expression and DON production assays**

The conidia of each strain formed in CMC medium were collected and suspended in sterile distilled water to a final concentration of 10^5 conidia/mL. A 10 μL suspension of fresh conidia of each strain was injected into a floret in the central section spikelet of the susceptible cultivar Zimai22. At 15 days after inoculation, the infected spikelets in each inoculated wheat head were recorded. For wheat leaf infection, fresh mycelial plugs of each strain were inoculated in the middle of the leaves and incubated in a growth chamber at 25 °C. Images were taken 6 days after inoculation. There were 15 replicates for each strain in each experiment, and these experiments were repeated three times.

To compare the TRI genes expression, the wild-type and CAP mutant strains were inoculated into TBI medium and cultured at 28 °C in a shaker (150 rpm). After 2 days of incubation, mycelia of each sample were harvested and the total RNA was extracted. The mRNA expression of TRI1, TRI5 and TRI6 in PH-1 and the mutants was determined using a quantitative reverse transcription PCR (RT-qPCR) method as described previously (Liu et al., 2015). The experiment was repeated three times. To quantify DON production, each strain was grown shaking (150 rpm) in TBI medium at 28 °C for 7 days in darkness. The supernatant was collected by filtration through three layers of gauze and then purified and quantified using the LC-MS/MS system (Tang et al., 2018). The experiment was repeated three times.

**ACKNOWLEDGEMENTS**

The research was supported by the National Natural Science Foundation of China (31672064), the International Science & Technology Cooperation Program of China (2016YFE0112900), the Natural Science Foundation of Zhejiang Province for Distinguished Young Scholar (LR17C140001), the China Agriculture Research System (CARS-3-29) and the Young Elite Scientist Sponsorship Program (2017QNRC001). The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher’s web site:

Fig. S1 Phylogenetic analysis of the putative CAPs from Fusarium graminearum, two yeasts and five filamentous fungi. Amino acid sequences of CapA and CapB orthologues were aligned using CLUSTALW, and a neighbour-joining tree was generated by MEGA 5.0. The names or loci of proteins are indicated in the figure.

Fig. S2 FgCapB interacts with FgMyo1 and Tr1. (A) The interaction of FgCapB-GFP and FgMyo1-Flag was verified by the co-immunoprecipitation (Co-IP) assay. Total protein (Input) extracted from the strain bearing FgCapB-GFP and FgMyo1-Flag constructs or a single construct (FgCapB-GFP or FgMyo1-Flag) were subjected to SDS-PAGE and immunoblots were incubated with anti-FLAG and anti-GFP antibodies, as indicated (Input panel). Each protein sample was pulled down using anti-Flag agarose and further detected with anti-GFP antibody (Flag pull-down panel). Protein samples
Capping proteins contribute DON-toxisome formation

were also detected with anti-GAPDH antibody as a reference. (B) The interaction of FgCapB-mCherry and Tri1-GFP was verified by the Co-IP assay. Protein samples were pulled down using anti-GFP agarose and further detected with an anti-mCherry antibody. Protein samples were also detected with anti-GAPDH antibody as a reference. (C) FgCapB-mCherry was partially colocalized with Tri1-GFP on DON-toxisomes at 48 h of incubation in trichothecene biosynthesis induction (TBI) medium. Bar = 10 µm.

Fig. S3 Identification of deletion mutants. (A) PCR identification of deletion mutants ΔFgcapA and ΔFgcapB. (B) Southern blot analysis of the deletion mutants of ΔFgcapA and ΔFgcapB using the hygromycin fragment as the probe. ΔFgcapA had an anticipated 4539 bp band, but lacked the 4539 bp band presented in Fusarium graminearum wild-type PH-1. ΔFgcapB had an anticipated 5172 bp band, but lacked the 5172 bp band presented in the wild-type PH-1.

Fig. S4 Deletion mutants of FgCAP genes reduced the rate of hyphal growth and altered the morphologies of conidia. (A) Colony diameter of Fusarium graminearum wild-type PH-1, ΔFgcapA, ΔFgcapB and ΔΔFgcapA/B grown on potato dextrose agar (PDA), complete medium (CM) and minimal medium (MM) agar plates for 3 days at 25 °C. Bars denote standard deviations from three experiments. Columns labelled with the same letter are not significantly different according to the least significant difference (LSD) test at P = 0.05. (B) Ratio of the different number of conidial septa in PH-1, mutants and complemented strains harvested from 4-day-old carboxymethyl cellulose (CMC) cultures. (C) The representative conidial morphology of the wild-type PH-1 and mutants. The septa were stained with calcofluor white and imaged with a fluorescence microscope. Bar = 20 µm.

Table S1 A list of primers used in this study.