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The STRIPAK complex orchestrates cell wall integrity signalling to govern the fungal development and virulence of *Fusarium graminearum*

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Abstract

Striatin-interacting phosphatases and kinases (STRIPAKs) are evolutionarily conserved supramolecular complexes that control various important cellular processes such as signal transduction and development. However, the role of the STRIPAK complex in pathogenic fungi remains elusive. In this study, the components and function of the STRIPAK complex were investigated in *Fusarium graminearum*, an important plant-pathogenic fungus. The results obtained from bioinformatic analyses and the protein–protein interactome suggested that the fungal STRIPAK complex consisted of six proteins: Ham2, Ham3, Ham4, PP2Aa, Ppg1, and Mob3. Deletion mutations of individual components of the STRIPAK complex were created, and observed to cause a significant reduction in fungal vegetative growth and sexual development, and dramatically attenuae virulence, excluding the essential gene *PP2Aa*. Further results revealed that the STRIPAK complex interacted with the mitogen-activated protein kinase Mgv1, a key component in the cell wall integrity pathway, subsequently regulating the phosphorylation level and nuclear accumulation of Mgv1 to control the fungal stress response and virulence. Our results also suggested that the STRIPAK complex was interconnected with the target of rapamycin pathway through Tap42-PP2A cascade. Taken together, our findings revealed that the STRIPAK complex orchestrates cell wall integrity signalling to govern the fungal development and virulence of *F. graminearum* and highlighted the importance of the STRIPAK complex in fungal virulence.

KEYWORDS
cell wall integrity signalling, *Fusarium graminearum*, STRIPAK complex, target of rapamycin (TOR) pathway, virulence

1 | INTRODUCTION

The striatin (STRN)-interacting phosphatase and kinase (STRIPAK) complex was first characterized in mammalian cells and has received increased attention over the last decade (Goudreault et al., 2009; Shi et al., 2016). The mammalian STRIPAK complex consists of the following core components: (i) striatins (STRN1, STRN3 and STRN4); (ii) STRN-interacting protein 1 or 2 (STRIP1 or STRIP2); (iii) monopolar spindle-one-binder 4 (Mob4, also named Mob3); and (iv) PP2Aa and PP2Ac, the scaffolding and catalytic subunits of serine/threonine protein phosphatase 2A (PP2A), respectively (Jeong et al., 2021; Shi et al., 2016; Tang et al., 2019). In addition to these core components, multiple associated constituents of the STRIPAK complex have also been identified, such as several germinal centre kinases (Kean et al., 2011; Tang et al., 2019). STRIPAK can form diverse complexes with variable-associated constituents in response to extracellular stimuli (Jeong et al., 2021). The components of the STRIPAK complex have also been detected in fungi, although the fungal STRIPAK is less complex than its mammalian counterpart. The *Neurospora crassa* STRIPAK complex is composed of Ham2, Ham3, Ham4, PP2Aa, Ppg1, and Mob3, while in *Sordaria macrospora*, the STRIPAK complex consists of Pro22, Pro11, Pro45, PP2Aa, PP2Ac, and Mob3 (Bloemendal et al., 2012; Dettmann et al., 2013). In *Saccharomyces cerevisiae*, subunits of the factor arrest (FAR) complex share significant sequence similarities with mammalian STRIPAK proteins (Kemp & Sprague Jr., 2003). However, it should be noted that the FAR complex only displays a basic STRIPAK assembly including Far11, Far8, Far9/10, Tpd3, and Pph21/22 while Mob4/Mob3 does not exist in yeast (Kück et al., 2016, 2019). In addition, Far8 lacks the large WD (tryptophan-aspartic acid) domain at its C-terminus that is critical for the STRIPAK complex functions in mammalian cells (Gordon et al., 2011; Kean et al., 2011).

The functions of STRIPAK complexes have been characterized in diverse organisms ranging from yeasts to humans. Increasing evidence has revealed that the mammalian STRIPAK complex regulates several vital signalling pathways, including cytoskeleton remodelling, mitogen-activated protein kinase (MAPK), and Hippo. In addition, a dysfunctional STRIPAK complex is associated with several human diseases, such as seizures and strokes, autism, diabetes, and cancer (Hwang & Pallas, 2014; Kück et al., 2019; Shi et al., 2016). Given the biomedical importance of the STRIPAK complex, the activity of STRIPAK components could be potential targets for drug development (Shi et al., 2016). The yeast FAR complex is implicated in mitophagy regulation, vacuolar protein sorting, and pheromone-induced cell cycle arrest (Bloemendal et al., 2012; Furukawa et al., 2021; Kemp & Sprague Jr., 2003). For example, the FAR complex negatively regulates mitophagy by mediating the phosphorylation of the mitophagy receptor Atg32 (Furukawa et al., 2021). The STRIPAK complex is involved in several different developmental processes in other fungi (Bloemendal et al., 2012; Dettmann et al., 2013; Elramli et al., 2019; Kück & Stein, 2021; Stein et al., 2020). The *N. crassa* STRIPAK complex is important for fruiting body formation, and this complex is also necessary in *S. macrospora* for sexual development by regulating the RNA-binding protein Gul1 (Beier et al., 2016; Bloemendal et al., 2012; Dettmann et al., 2013; Nordzieke et al., 2015; Stein et al., 2020). The STRIPAK complex also plays a regulatory role in secondary metabolism, such as the production of the mycotoxin sterigmatocystin in *Aspergillus nidulans* (Elramli et al., 2019). The complex is also involved in fungal virulence. Stp1 (STRIP homologue) and Fsr1 (STRN homologue) have been demonstrated to be important for virulence in the maize pathogen *Fusarium verticillioides* (Shim et al., 2006; Zhang et al., 2018). Likewise, the *Colletotrichum graminicola* striatin homologue Str1 and *Magnaporthe oryzae* homologue of Ppg1 are also important for virulence (Du et al., 2013; Wang et al., 2016). Although the functions of the individual STRIPAK subunits have been elucidated in several fungal species (Kück & Stein, 2021), systematic characterization of this complex is less clear. More importantly, other signalling pathways associated with the STRIPAK complex remain largely obscure.

Fusarium head blight (FHB) severely affects small-grain cereal crops worldwide and is mainly caused by *Fusarium graminearum* (Goswami & Kistler, 2004; Starkey et al., 2007). In addition to yield losses caused by FHB, mycotoxins produced by *F. graminearum*, including deoxynivalenol (DON) and zearalenone, are hazardous to animals and humans (Desjardins, 2003; Pestka & Smolinski, 2005). A better understanding of the molecular mechanisms associated with *F. graminearum* virulence should provide new potential targets and result in the development of new antifungals. Herein, interaction networks of the STRIPAK complex were clarified and the biological functions of the STRIPAK complex were systematically characterized in *F. graminearum*. Our results showed that all STRIPAK proteins were important for the fungal development and virulence of *F. graminearum*. The regulatory mechanisms of the STRIPAK complex in virulence were further explored and we found that the STRIPAK complex regulated the phosphorylation status and nuclear accumulation of MAPK Mgv1 in the cell wall integrity (CWI) pathway. In addition, the complex further modulates the stress response and DON production, which are necessary for *F. graminearum* virulence. The association between the STRIPAK complex and the target of rapamycin (TOR) pathway was also demonstrated in this study. Our results link the STRIPAK complex with the MAPK and TOR pathways, suggesting that an interconnected signalling network controls fungal virulence in pathogenic fungi.

2 | RESULTS

2.1 | The STRIPAK complex is conserved in fungi

To identify the STRIPAK complex in *F. graminearum*, the amino acid sequences of reported STRIPAK components in the filamentous ascomycetes *S. macrospora* and *N. crassa* (Bloemendal et al., 2012)

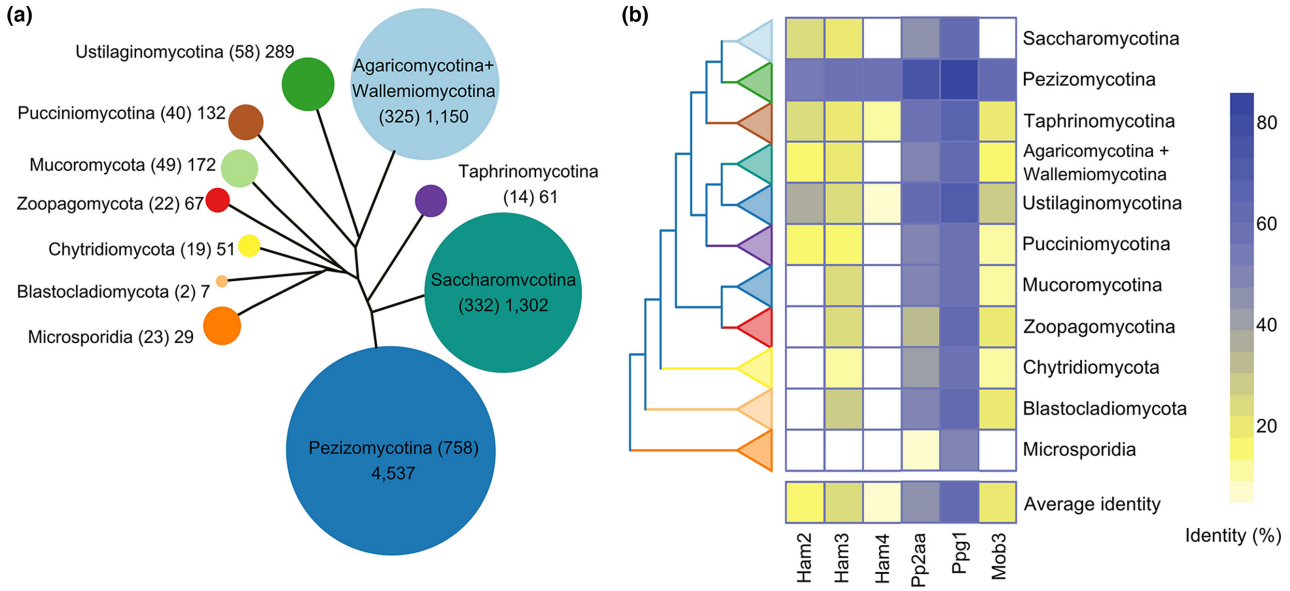


FIGURE 1 Genome-wide identification and conservation analysis of the STRIPAK proteins in fungi. (a) Number of STRIPAK proteins at the subphylum or phylum level. The evolutionary tree shows the relationships among the various fungal groups. The area of a circle is proportional to the total amount of STRIPAK proteins identified in each group. The number of species is indicated in parentheses. (b) Conservation of the STRIPAK components in fungi. The STRIPAK proteins in *Fusarium graminearum* were used as the query sequences for analysis.

were used as queries to search the *F. graminearum* genome database (https://fungidb.org/fungidb/) using BLASTp, and six proteins were identified and designated as Ham2, Ham3, Ham4, PP2Aa, Ppg1, and Mob3 (Table S1). To investigate the distribution of STRIPAK proteins throughout the fungal kingdom, 1642 published fungal genomes were screened using these six *F. graminearum* proteins as queries (Li et al., 2021) and a total of 7800 candidate proteins were identified in 11 major clades (Figure 1a and Table S2). The statistical analysis showed that each fungal species contained 4.8 STRIPAK proteins on average, but STRIPAK proteins were distributed differently among fungi. The homologues of the six STRIPAK proteins in *F. graminearum* are only complete in Pezizomycotina but not in the remaining 10 fungal branches, and PP2Aa, Ppg1, and Ham3 have homologues in all fungal genomes, but homologues of Ham4 are almost absent in branches other than Pezizomycotina (Figure S1 and Table S2).

To investigate the conservation of STRIPAK proteins in fungi, the identities among six STRIPAK proteins in *F. graminearum* and the most homologous sequences in 1642 fungi were compared. As shown in Figure 1b and Table S2, the average conservation values of these six STRIPAK proteins varied widely in fungi, ranging from 7.25% to 62.98%. Among them, Ppg1, PP2Aa, and Ham3 were the top three conserved proteins in all fungi, with average identities of 62.98%, 46.85%, and 22.01% (Figures 1b and S1 and Table S2), respectively. Notably, the similarity of these three proteins reached 85.46%, 74.67%, and 57.76% in Pezizomycotina, respectively (Table S2). Collectively, Ppg1, PP2Aa, and Ham3 are highly conserved in fungi, especially in Pezizomycotina, implying that these three proteins may be the core components of the STRIPAK complex and play important roles in fungi.

2.2 | Protein–protein interactome of the STRIPAK complex in *F. graminearum*

To reveal the interaction patterns of the abovementioned STRIPAK components in *F. graminearum*, yeast two-hybrid (Y2H) and co-immunoprecipitation (Co-IP) assays were conducted. The complete cDNA of individual STRIPAK subunits was cloned into the vector pGADT7 (AD) or pGBKT7 (BD). Pairs from different combinations of the constructs were co-transformed into *S. cerevisiae* AH109 for Y2H assays. As shown in Figure 2a, Ppg1 and Ham3 were capable of interacting with themselves while the homodimerization for the other four proteins was not detected. PP2Aa interacted with all the other five STRIPAK components, while Ppg1 and Ham3 could interact with the other four elements except for Ham4. However, yeast transformants expressing pairs of Ham2–Mob3, Ham2–Ham4, or Mob3–Ham4 did not grow on the selection media, indicating that they did not directly interact with each other. These interactions were therefore determined by Co-IP assay, including Ppg1–Ham4, Ham3–Ham4, Ham2–Mob3, Ham2–Ham4, and Mob3–Ham4. In Co-IP assay with a strain expressing GFP-Ham4 and Ppg1-FLAG constructs, Ppg1-FLAG fusion proteins were detected in proteins co-purified with GFP-Ham4 using anti-GFP beads (Figure 2b), suggesting that Ppg1 associated with Ham4. The indirect interactions between Ham3–Ham4, Ham2–Mob3, Ham2–Ham4, and Mob3–Ham4 were also determined (Figure 2c–f). Taken together, our results indicated that Ham2, Ham3, Ham4, PP2Aa, Mob3, and Ppg1 form the STRIPAK complex by interacting directly or indirectly with each other (Figure 2g). Among them, Ppg1, PP2Aa, and Ham3 may be the core components of the STRIPAK complex in *F. graminearum* according to their conservation in fungi and interaction patterns.

2.3 | The STRIPAK complex is required for fungal development and virulence

To explore the biological functions of the STRIPAK complex in *F. graminearum*, gene deletion mutants for each STRIPAK subunit were generated in this study (Figure S2). However, we could not obtain gene deletion mutants for *PP2Aa* after screening over 120 transformants from three different transformations, suggesting that PP2Aa may be essential for the high gene knockout efficiency in *F. graminearum* (Wang et al., 2011; Yun et al., 2015). To determine the roles of the STRIPAK complex in mycelial growth, all obtained deletion mutants along with the wild-type strain PH-1 were cultured on wheat-head medium. We found that the deletion of Ham2, Ham3, Ham4, Mob3, or Ppg1 led to a reduced growth when compared to that of PH-1 (Figure 3a,e). The open reading frame (ORF) of *HAM2* driven by its own promoter was fused in-frame with green fluorescent protein (*GFP*) and the resulting construct was transformed into Δ*ham2* to generate the complementation strain Δ*ham2*-C. A similar strategy was used to generate complementation strains for the other four mutants. Our data showed that the

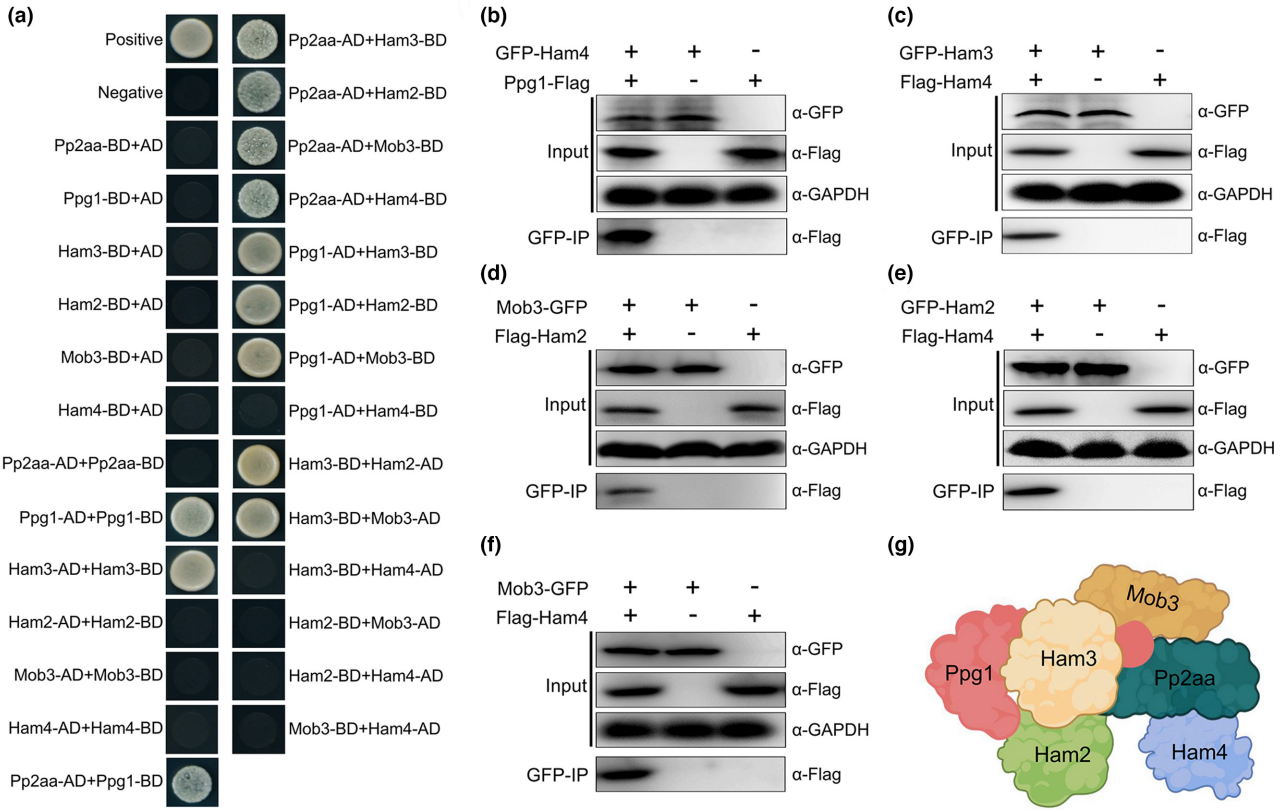
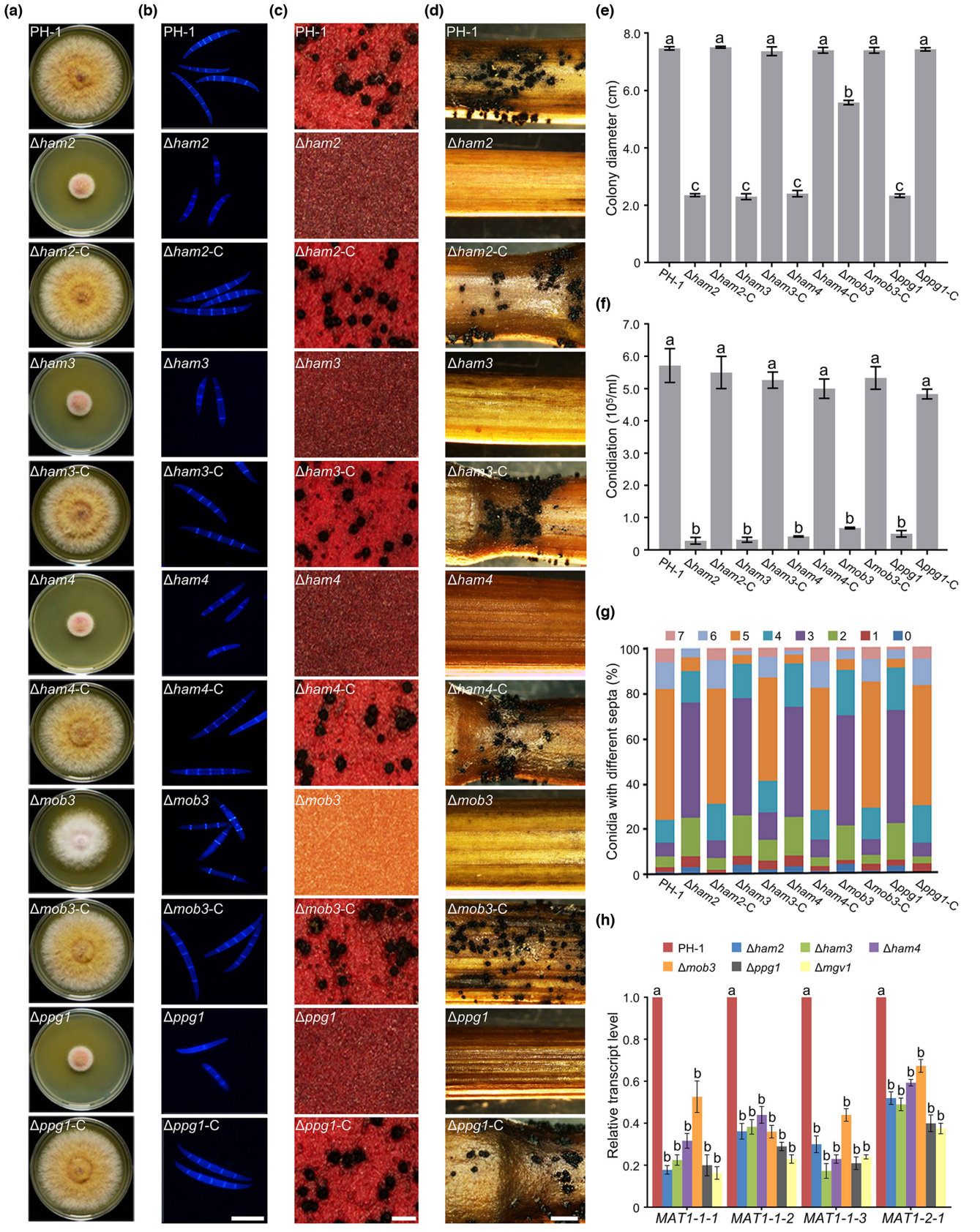


FIGURE 2 Protein–protein interactome of the STRIPAK complex in *Fusarium graminearum*. (a) Interactions between STRIPAK subcomplexes. The interactions of pGBKT7-53/pGADT7-T and pGBKT7-Lam/pGADT7-T were used as the positive and negative controls, respectively. Yeast transformants carrying the indicated constructs were plated onto selective plates supplemented without Leu/Trp/His/Ade to assay growth. (b) Western blot showing the interaction between Ppg1 and Ham4 using a co-immunoprecipitation assay. Total proteins isolated from the strains bearing GFP-Ham4 and/or Ppg1-FLAG (input) and the proteins eluted from the anti-GFP beads (elution) were detected using anti-FLAG antibody. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. (c) Western blot showing the interaction between Ham3 and Ham4. (d) Western blot showing the interaction between Mob3 and Ham2. (e) Western blot showing the interaction between Ham2 and Ham4. (f) Western blot showing the interaction between Mob3 and Ham4. (g) A model depicting the protein–protein interactions of STRIPAK subcomplexes.

FIGURE 3 The STRIPAK complex is involved in fungal development of *Fusarium graminearum*. (a) Colony of the wild type (PH-1) and each mutant strain grown on wheat-head medium at 25°C for 3 days. (b) Micrographs showing the conidial morphologies of the indicated strains. Calcofluor white (CFW) staining was used to visualize septa under a fluorescence microscope. Bar = 20 μm. (c) Micrographs showing the perithecium formation of the indicated strains after growth on carrot agar for 2 weeks. Bar = 1 mm. (d) Perithecium formation of the tested strains was photographed after growing on wheat straw for 4 weeks. Bar = 1 mm. (e) Bar charts showing the colony diameters of the indicated strains. (f) Bar charts showing the number of conidia produced by the tested strains in liquid carboxymethyl cellulose medium. (g) Percentages of conidia with different septa in tested strains. (h) Bar charts showing the relative transcriptional abundance of mating-type genes in wild type or indicated mutants using reverse transcription-quantitative PCR. Line bars denote the standard errors of three experiments. Bars with the same letter are not significantly different at $\alpha = 0.05$.


(a)
(b)
(c)
(d)
PH-1
Δham2
Δham2-C
Δham3
Δham3-C
Δham4
Δham4-C
Δmob3
Δmob3-C
Δppg1
Δppg1-C
(e)
Colony diameter (cm)
(f)
Conidiation (10⁵/ml)
(g)
Conidia with different septa (%)
7 6 5 4 3 2 1 0
(h)
PH-1 Δham2 Δham3 Δham4
Δmob3 Δppg1 Δmgv1
Relative transcript level
MAT1-1-1 MAT1-1-2 MAT1-1-3 MAT1-2-1

growth defects of the deletion mutants were rescued in all the complementation strains (Figure 3a,e), further confirming that the defects in *F. graminearum* growth were directly caused by the loss of the STRIPAK components.

The roles of the STRIPAK complex in asexual and sexual development were also determined in this study. As shown in Figure 3b, typical sickle-shaped conidia were produced by all STRIPAK mutants and the wild type. However, the number of conidia produced in five STRIPAK mutants was significantly reduced compared to that of PH-1 (Figure 3f), suggesting that the STRIPAK complex plays an important role in regulating the conidiation of *F. graminearum*. Moreover, the loss of STRIPAK led to a reduced number of septa in fungal conidia (Figure 3b,g). In addition, the wild type produced abundant perithecia after incubation on carrot medium for 4 weeks whereas STRIPAK mutants were sterile and no perithecia was observed under the identical conditions (Figure 3c) and similar results were obtained with wheat straw (Figure 3d). To further confirm the effect of the STRIPAK complex on sexual development, the mRNA levels of four key mating-type genes (*MAT1-1-1*, *MAT1-1-2*, *MAT1-1-3*, and *MAT1-2-1*) were determined in either the wild-type or mutant background. Reverse transcription-quantitative PCR (RT-qPCR) analysis showed that the transcriptional abundances of these four genes were significantly decreased in STRIPAK mutants compared to those in the wild type (Figure 3h). Collectively, we conclude that the STRIPAK complex is critical for asexual and sexual development in *F. graminearum*.

The effects of the dysfunctional STRIPAK complex on *F. graminearum* virulence were tested on flowering wheat heads. As shown in Figure 4a,b, PH-1 caused severe and typical scab symptoms while only the inoculated but not the nearby spikelet was infected by the STRIPAK mutants. Similar results were obtained for maize silks (Figure 4c,d). To track the growth and spread of fungal hyphae within the host tissue, the red fluorescent protein reporter gene *RFP* driven by the promoter of the *F. graminearum actin* gene was expressed in either the wild-type or mutant background. As shown in Figure 4e, the RFP fluorescence signals of PH-1::Actin-RFP were detected in the inoculated and nearby spikelets, whereas the signals in the mutant backgrounds were restricted only within the inoculated spikelet. Collectively, these data suggest that the STRIPAK complex is critical for *F. graminearum* development and virulence.

2.4 | The STRIPAK complex affects the phosphorylation status and nuclear accumulation of Mgv1

To explore the underlying mechanisms of the STRIPAK complex in the regulation of *F. graminearum* virulence, the STRIPAK complex-interacting proteins were identified by affinity capture-mass spectrometry (AC-MS) analysis using two core components as baits, Ham3-GFP and Ppg1-GFP. Interestingly, Mgv1, a key MAPK component in the CWI pathway, was identified in these two independent AC-MS analyses (Table S3). To further confirm the interaction between Mgv1 and the STRIPAK complex, a Y2H assay was performed and we found that Mgv1 could interact directly with Ham2, Ham3, Ppg1, and PP2Aa (Figure 5a). In addition, the associations of Mgv1–Ham4 and Mgv1–Mob3 were also confirmed in this study according to the Co-IP analysis (Figure 5b,c), suggesting that the STRIPAK complex may be involved in *F. graminearum* virulence by regulating Mgv1.

Because the association between the STRIPAK complex and Mgv1 was demonstrated, we next wondered whether the loss of the STRIPAK complex would exert an effect on the phosphorylation status and protein activity of Mgv1. To this end, phosphorylation profiling of Mgv1 was performed in either the wild-type or mutant background. According to Figure 5d,e, the phosphorylation level of Mgv1 was dramatically reduced in the STRIPAK mutants compared with that in PH-1, suggesting that Mgv1 activity was impaired on the loss of the STRIPAK complex. Previous studies have shown that after phosphorylation several MAPKs are translocated into the nucleus to phosphorylate their downstream targets on activation (Bruno et al., 2004; Raviv et al., 2004; Zheng et al., 2012). Because the phosphorylation level of Mgv1 was reduced in the STRIPAK mutants, we wondered whether the localization pattern of Mgv1 was affected therein. To this end, we first generated a strain expressing Mgv1-GFP and nuclear marker histone 1 (H1)-RFP, and the localization of Mgv1 was examined under confocal microscopy. As shown in Figure 5f, Mgv1-GFP was partially co-localized with H1-RFP, and certain fluorescence signals were also observed in the cytoplasm, suggesting that Mgv1 localized in both the cytoplasm and nucleus in the wild type. To determine the effect of the STRIPAK complex on the localization of Mgv1, a construct containing Mgv1-GFP was also transformed into the STRIPAK mutants and we found that the depletion of the STRIPAK complex reduced nuclear accumulation of Mgv1 compared to that in the wild type (Figure 5g). Quantification of the relative levels of Mgv1 in the nucleus by western blotting further confirmed these results (Figure S3). Collectively, these findings suggest that the STRIPAK complex is required for the proper phosphorylation of Mgv1 and further mediates its nuclear accumulation in *F. graminearum*.

2.5 | The STRIPAK complex regulates various stress responses

The phosphorylation and nuclear accumulation of Mgv1 was impaired in the STRIPAK mutants, implying that the response of the abovementioned mutants to cell wall stress may be affected. To test this hypothesis, PH-1 and individual mutants were inoculated on minimal medium (MM) supplemented with the cell wall stress agent calcofluor white (CFW). According to Figure 6a,b, the STRIPAK mutants were more sensitive toward cell wall stress triggered by 0.1 mg/mL CFW than PH-1. These results indicate that the STRIPAK complex might modulate the CWI pathway by affecting the phosphorylation status and nuclear accumulation of Mgv1.

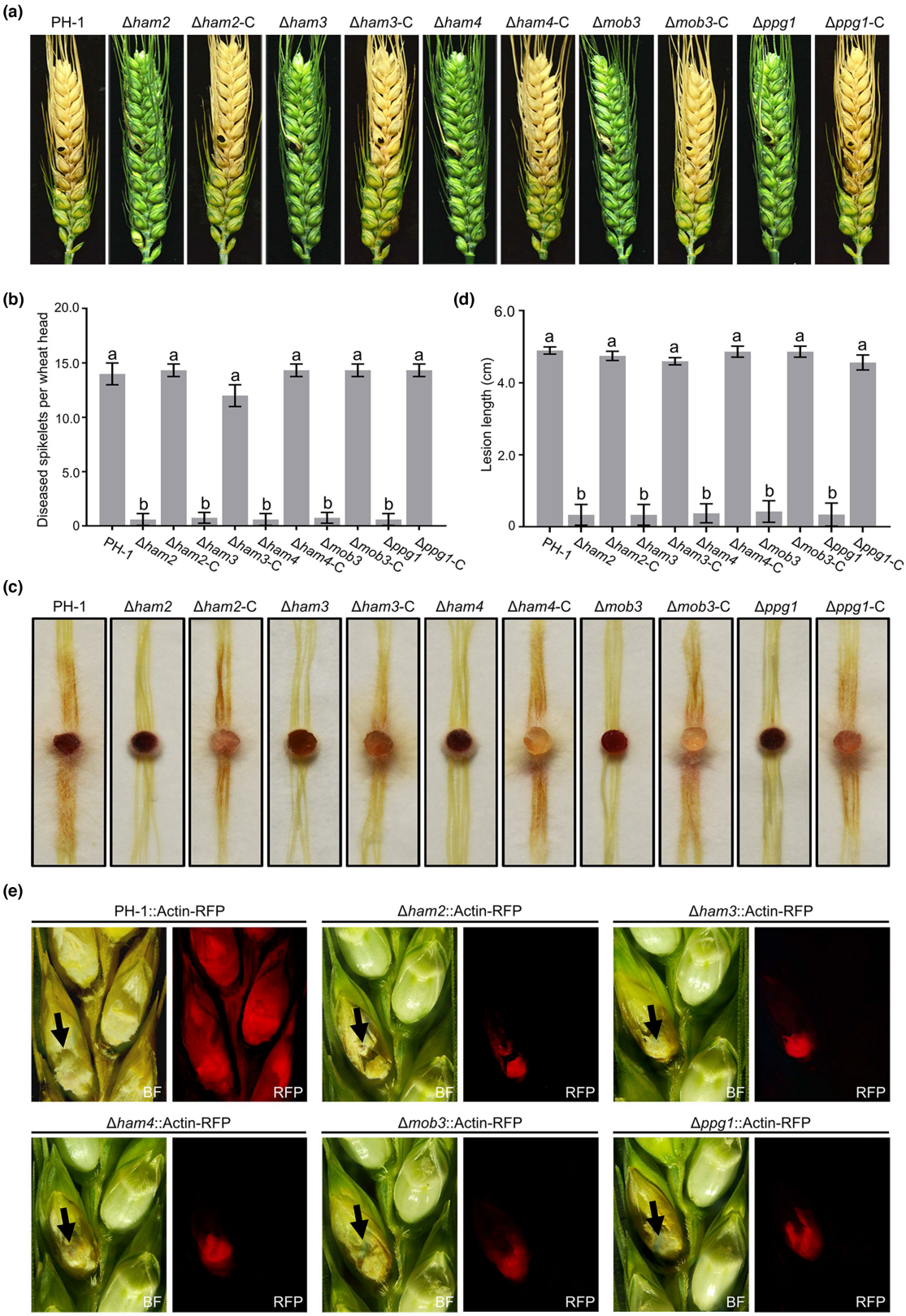


FIGURE 4 The STRIPAK complex is required for *Fusarium graminearum* virulence. (a) Flowering wheat heads were inoculated with mycelial plugs from the wild type (PH-1) and indicated mutant strains and the number of diseased spikelets per wheat head was measured at 14 days postinoculation (dpi) (b). (c) The mycelial plugs of the indicated strains were used to inoculate maize silks and the lesion lengths were examined at 7 dpi (d). (e) Cross-sections of inoculated and adjacent wheat spikelets. Spikelets were infected with the indicated strains and the photographs were taken at 7 dpi. Inoculated spikelets are indicated with black arrows. BF, bright field; RFP, red fluorescent protein. Line bars denote the standard errors of three experiments. Bars with the same letter are not significantly different at $\alpha = 0.05$.

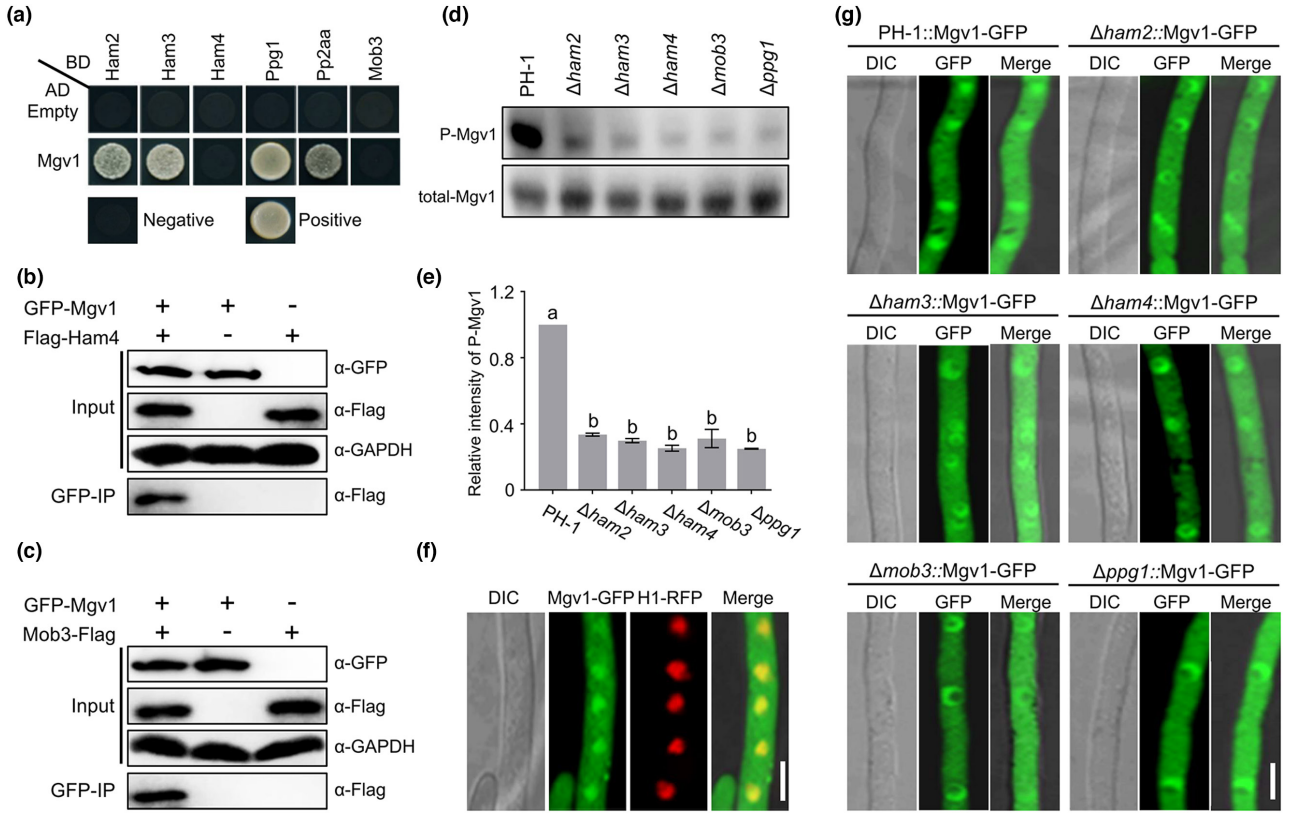


FIGURE 5 The STRIPAK complex controls the phosphorylation and nuclear accumulation of Mgv1. (a) Interactions between STRIPAK and Mgv1 based on the yeast two-hybrid assay. Yeast transformants carrying the indicated constructs were plated onto the selective plates supplemented without Leu/Trp/His/Ade to assay growth. (b) Western blot showing the interaction between Mgv1 and Ham4 using a co-immunoprecipitation assay. Total proteins isolated from the strains bearing GFP-Mgv1 and/or FLAG-Ham4 (input) and the proteins eluted from the anti-GFP beads (elution) were detected using anti-FLAG antibody. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. (c) Western blot showing the interaction between Mgv1 and Mob3. (d) Western blot showing the phosphorylation levels of Mgv1 in the indicated strains compared to the wild type (PH-1). (e) Bar charts showing the relative phosphorylation levels of Mgv1 in the indicated strains. Line bars denote the standard errors of three experiments. Bars with the same letter are not significantly different at $\alpha = 0.05$. (f) Micrographs showing the localization of Mgv1-GFP in hyphae of *Fusarium graminearum*. H1-RFP was used to visualize the nuclei. Bar = 5 μm. (g) The nuclear accumulation of Mgv1-GFP in the indicated strains. Bar = 5 μm. DIC, differential interference contrast.

In addition to the cell wall stress response, the links between Mgv1 and other stress responses such as oxidative stress and antifungal agents have also been reported (Pujol-Carrion & Torre-Ruiz, 2017; Rui & Hahn, 2007; Zhang et al., 2017). Therefore, the sensitivities of STRIPAK mutants toward oxidative stress mediated by 2 mM paraquat were determined, and we found that the STRIPAK mutants also exhibited enhanced sensitivity toward paraquat compared to that of the wild type (Figure 6a,b). Oxidative stress response is closely related to the production of endogenous reactive oxygen species (ROS) (Schieber & Chandel, 2014), therefore nitroblue tetrazolium staining was conducted to detect ROS production in the wild type and STRIPAK mutants. The dark colour at the edge of the colony indicates the relative production of ROS and we found that the mycelia of the mutants displayed a darker colour than that of the wild type (Figure 6c). These findings indicate that dysfunction of the STRIPAK complex led to the accumulation of ROS in the mycelia of *F. graminearum*, resulting in increased sensitivity to oxidative stress. We also found that STRIPAK mutants were more sensitive to two phytoalexins, benzoxazolinone (BOA) or 2-aminopyridine (2-AP), compared with PH-1 (Figure 6d,e). Collectively, the STRIPAK complex plays a critical role in response to various stresses in *F. graminearum*.

2.6 | The STRIPAK complex regulates DON production

One of the defects in the Δ*mgv1* mutant is a reduction in DON production (Hou et al., 2002). Mgv1 activity was impaired in the STRIPAK mutants, suggesting that DON production may also be down-regulated in the STRIPAK mutants. As expected, DON production was significantly reduced in the STRIPAK mutants in comparison to that of the wild type (Figure 7a). To further confirm the effect of the STRIPAK complex on DON production, the mRNA levels of four key genes encoding DON synthases (*TRI1*, *TRI5*, *TRI6*, and *TRI10*) were determined in either the wild-type or mutant

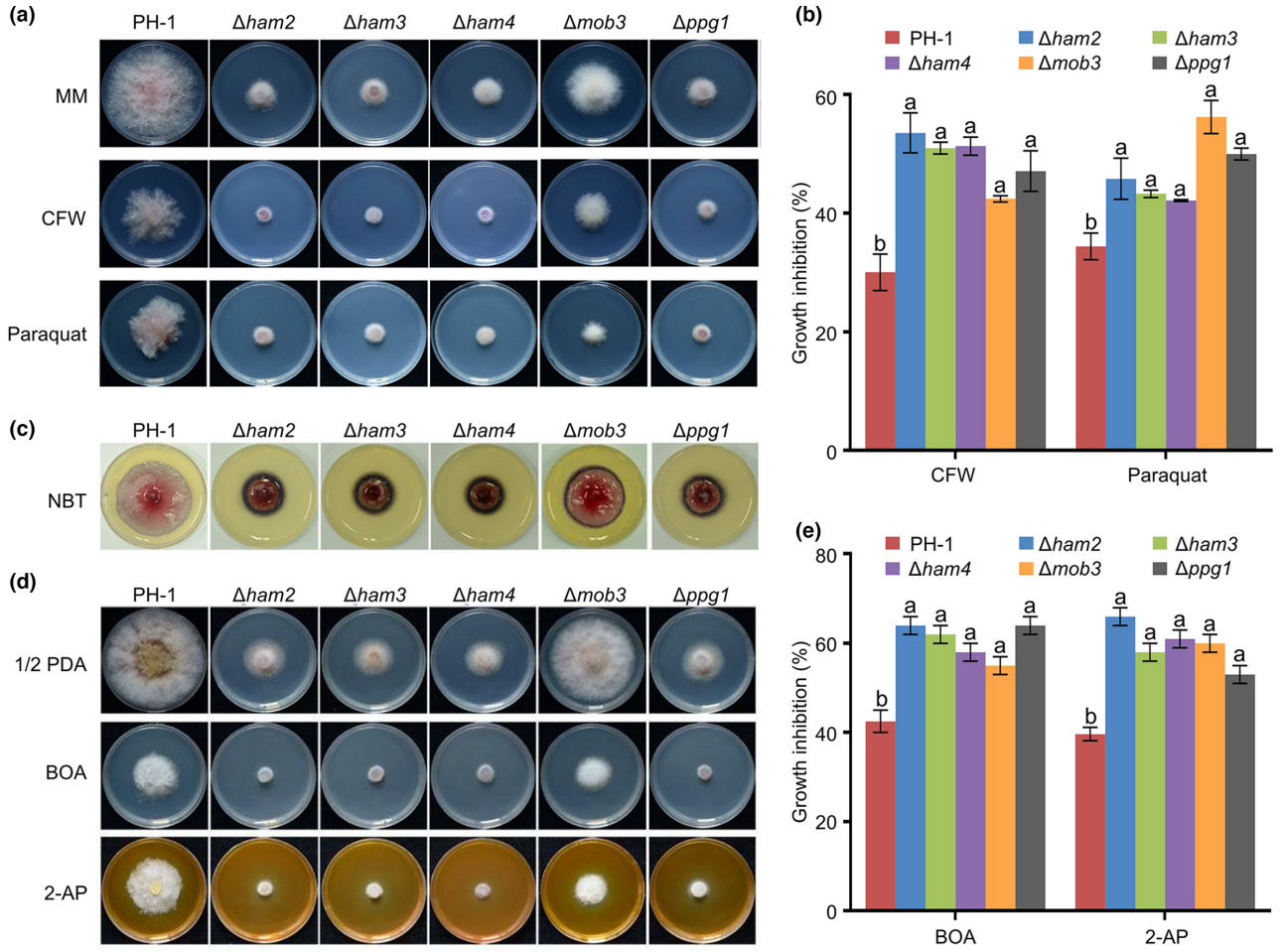


FIGURE 6 Control of cell-wall stress and oxidative responses by the STRIPAK complex. (a) Morphologies of the indicated strains after incubation on minimal medium (MM) supplemented with 0.1 mg/mL calcofluor white (CFW) or 2 mM paraquat at 25°C for 3 days compared to the wild type (PH-1). (b) The inhibition of the mycelial growth rate was examined after each strain was incubated for 3 days on MM supplemented with CFW and paraquat. (c) Indicated strains were stained with nitroblue tetrazolium (NBT) for reactive oxygen species (ROS) production. The dark colour at the edge of the colony indicates ROS production. (d) Morphologies of the indicated strains after incubation on ½ × potato dextrose agar (PDA) supplemented with 0.5 mg/mL benzoxazolinone (BOA) or 0.2 mg/mL 2-aminopyridine (2-AP). (e) The inhibition of the mycelial growth rate was examined after each strain was incubated for 3 days on ½ × PDA supplemented with BOA or 2-AP. Line bars denote the standard errors of three experiments. Bars with the same letter are not significantly different at $\alpha = 0.05$.

background. The RT-qPCR analysis showed that the relative transcriptional levels of these four genes were significantly reduced in STRIPAK mutants compared to those in the wild type (Figure 7b). The mycotoxin DON is synthesized in a specific cellular compartment known as the DON-toxisome. To detect the impact of the STRIPAK complex on DON-toxisome formation, Tri1, a calonectrin oxygenase that functions downstream of the DON biosynthesis pathway (McCormick et al., 2004; Tang et al., 2018), was fused with GFP and transformed into the wild type and individual mutants of the complex. Microscopic observations showed that strong fluorescence signals were detected in spherical- or crescent-shaped toxisomes in the wild type but not in the mutants (Figure 7c). Consistent with the microscopic analysis, an immunoblot analysis showed that the protein level of Tri1-GFP was undetectable in the mutants (Figure 7d). Collectively, these results indicate that the STRIPAK complex is essential for *F. graminearum* mycotoxin production.

2.7 | The STRIPAK complex is interconnected with the TOR signalling pathway

We previously found that Ppg1, the catalytic subunit of PP2A, physically associates with the TOR effector Tap42 and subsequently regulates the vegetative development and virulence in *F. graminearum* (Yu et al., 2014). Here, Ppg1 and PP2Aa, the scaffolding subunit of PP2A, were identified as critical components in the STRIPAK complex. This implies that the STRIPAK complex may be interconnected with the TOR signalling pathway via Tap42. To test this hypothesis, we first determined the interaction patterns between Tap42 and the components of the STRIPAK complex. Y2H and Co-IP analysis demonstrated that Tap42 physically interacted with Ham2, Ham3, and Ppg1 (Figure 8a), and associated with other three components in the STRIPAK complex (Figure 8b–d). Next, we wondered whether the PP2A phosphatase activity affects the functions of the STRIPAK

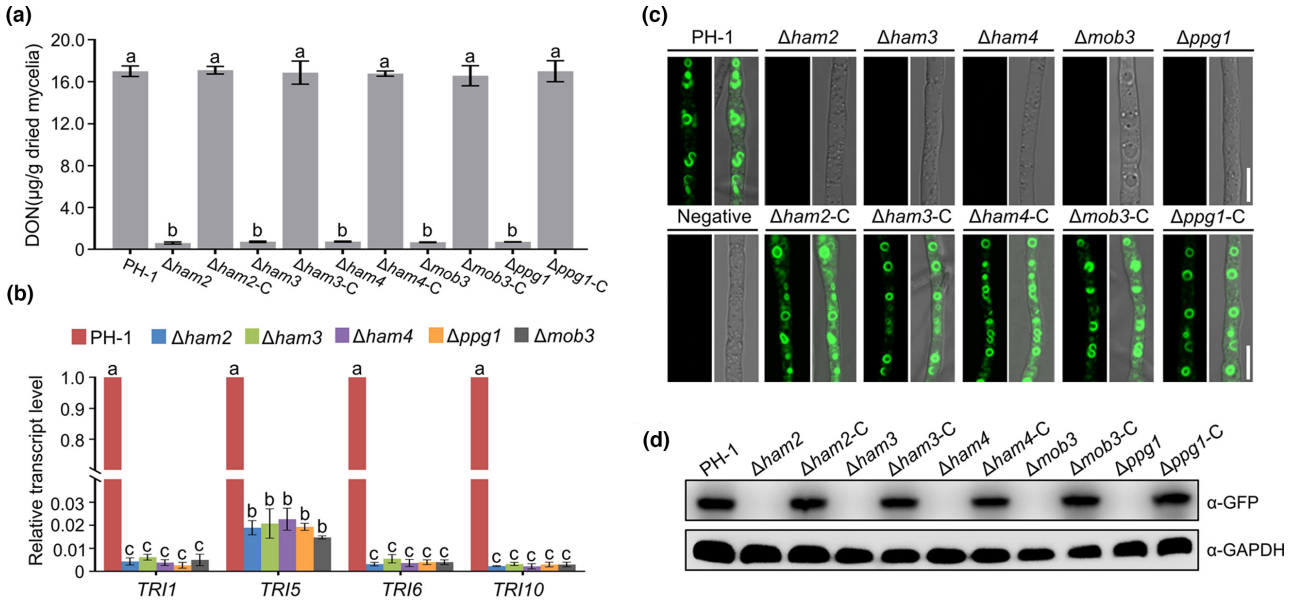


FIGURE 7 Regulation of deoxynivalenol (DON) production by the STRIPAK complex. (a) Bar charts showing DON production in 3-day-old trichothecene biosynthesis induction cultures of the indicated strains. (b) Bar charts showing the relative transcriptional abundance of *TRI* genes in wild-type (PH-1) or indicated mutant strains using reverse transcription-quantitative PCR. (c) Fluorescence signals of Tri1-GFP were detected in the tested strains. Bar = 10 μm. (d) Western blot analysis of Tri1 protein levels in tested strains expressing Tri1-GFP with the anti-GFP antibody. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Line bars denote the standard errors of three experiments. Bars with the same letter are not significantly different at $\alpha = 0.05$.

complex. The histidine (H) at position 59 of Ppg1 is highly conserved, and its substitution (histidine to glutamine) significantly impaired phosphatase activity and further blocked sexual development in *S. macrospora* (Beier et al., 2016), therefore a Ppg1^{H59Q} point mutation was generated and its effect on the formation of perithecia was assessed. The results indicated that Δ*ppg1*::Ppg1^{H59Q} was sterile, as observed with Δ*ppg1* and other STRIPAK mutants (Figures 3c and 8e), suggesting that phosphatase activity of PP2A in the STRIPAK complex is important for sexual development. Furthermore, we found that phospholipase activity of PP2A was elevated on rapamycin treatment and subsequently increased perithecium formation in *F. graminearum* (Figure 8e–g). Taken together, these results suggest that the STRIPAK complex is interconnected with the TOR signalling pathway through the Tap42-PP2A cascade.

3 | DISCUSSION

The composition and functions of the fungal STRIPAK complex have so far been elusive. In this study, the composition of the STRIPAK complex and conservation of proteins in the complex in the fungal kingdom was first analysed. Then, the function of the fungal STRIPAK complex was comprehensively investigated by phenotypic, genetic, live cell imaging, and biochemical approaches using the important plant-pathogenic fungus *F. graminearum* as a model. The results indicated that the STRIPAK complex consisted of six proteins, Ham2, Ham3, Ham4, PP2Aa, Ppg1, and Mob3, in *F. graminearum*. Except for Mob3, homologues of the other five proteins were also identified in *S. cerevisiae* (Kück et al., 2016, 2019). In addition to these six common components, the similar to human suppressor of IKK-ε (SIKE)-like protein SipB was also identified as a subunit of the STRIPAK complex in *Aspergillus nidulans* (Elramli et al., 2019). Combining bioinformatic analyses with experimental results suggested that the components of the STRIPAK complex can vary in different fungal species, while PP2Aa, Ppg1, and Ham3 homologues are highly conserved in the fungal kingdom. Protein interactions within fungal STRIPAK complexes are mainly validated by AC-MS experiments (Kück et al., 2016). However, the direct interactions among the STRIPAK components are rarely studied, except for a few physical interactions that were verified in *S. cerevisiae* and *S. macrospora* (Kück et al., 2016). Here, the interaction patterns of the STRIPAK components were systematically investigated in *F. graminearum*. We found that several interactions, such as Ppg1–PP2Aa, PP2Aa–STRIP, and STRIP–STRN, were conserved and could be detected in *S. cerevisiae* and *S. macrospora* as well as *F. graminearum* (Beier et al., 2016; Kück et al., 2016). However, there are some differences in the interacting pairs of the STRIPAK complex between *F. graminearum* and the other two fungi. For example, Far9/10 was able to interact with Far11 and Far8 in *S. cerevisiae* (Kück et al., 2016) while these interactions were not detected in *F. graminearum*. Mob3–PP2Aa interaction could be detected in *F. graminearum* but not in *S. macrospora* (Beier et al., 2016; Kück et al., 2016). Taken together, the composition and the interaction patterns of fungal STRIPAK components are species-specific.

In *S. macrospora*, the STRIPAK complex is involved in cell fusion and the formation of mature fruiting bodies (Beier et al., 2016; Bernhards & Poggeler, 2011; Bloemendal et al., 2010; Poggeler & Kück, 2004). Similarly, the *N. crassa* homologues Mob3, Ham3, and

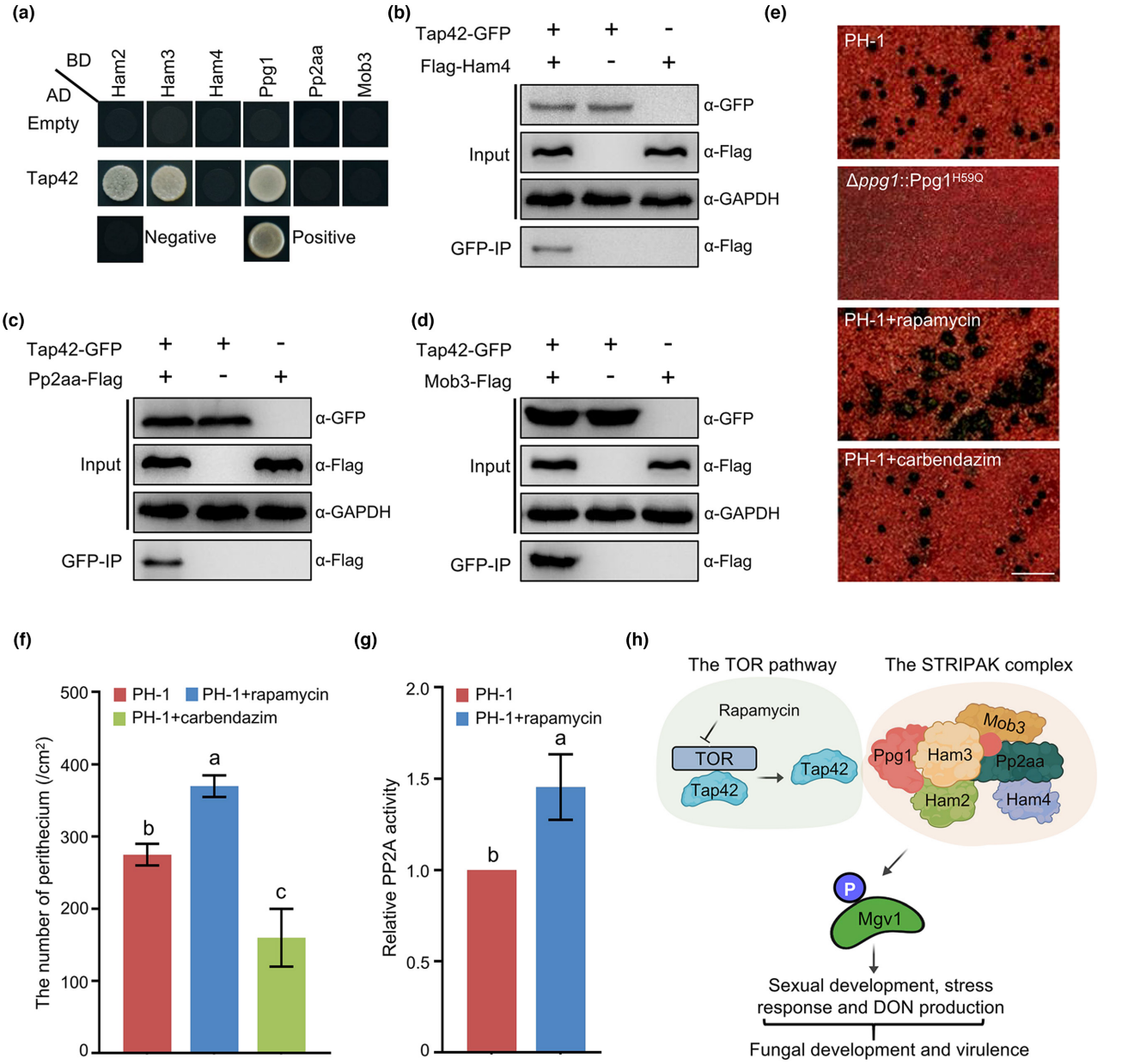


FIGURE 8 The STRIPAK complex interacts with Tap42 in the target of rapamycin (TOR) pathway. (a) Interactions between STRIPAK and Tap42 based on the yeast two-hybrid assay. Yeast transformants carrying the indicated constructs were plated onto the selective plates supplemented without Leu/Trp/His/Ade to assay growth. (b) Western blot showing the interaction between Tap42 and Ham4 using a co-immunoprecipitation assay. Total proteins isolated from the strains bearing Tap42-GFP and/or FLAG-Ham4 (input) and the proteins eluted from the anti-GFP beads (elution) were detected using anti-FLAG antibody. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. (c) Western blot showing the interaction between Tap42 and PP2Aa. (d) Western blot showing the interaction between Tap42 and Mob3. (e) Self-crossing plates showing perithecium production in the Δ*ppg1*::Ppg1^{H59Q} and the wild type (PH-1) treated with or without 0.025 μg/mL rapamycin. The fungicide carbendazim (0.5 μg/mL) was used as a fungicide control treatment. Bar = 1 mm. (f) Quantification of perithecia produced by the indicated strains or treatment. Line bars denote the standard errors of three experiments. Bars with the same letter are not significantly different at $\alpha = 0.05$. (g) Bar charts showing the relative PP2A activity in the wild-type strain treated with or without rapamycin (0.025 μg/mL). The PP2A activity of the wild-type strain without rapamycin treatment was set to 1.0. Line bars denote the standard errors of three experiments. Bars with the same letter are not significantly different at $\alpha = 0.05$. (h) Proposed model for the regulation of fungal development and virulence by the STRIPAK complex in *Fusarium graminearum*. The TOR signalling pathway is interconnected with the STRIPAK complex through the Tap42–PP2A cascade. The STRIPAK complex interacts with the mitogen-activated protein kinase Mgv1 in the cell wall integrity pathway and regulates its phosphorylation and nuclear accumulation, further modulating sexual development, the stress response, and deoxynivalenol (DON) production. Solid arrows and solid with perpendicular lines indicate the positive and negative regulation patterns, respectively.

Ham2 are also involved in cell fusion events (Dettmann et al., 2013). Dysfunction of Mgv1 deprived *F. graminearum* of the ability to produce any perithecia (Hou et al., 2002). Here, we found that the STRIPAK complex was also critical for sexual development by interacting with Mgv1 and regulating its phosphorylation status and nuclear accumulation. This implies that the function of the fungal STRIPAK complex in regulating sexual development may be conserved.

One of the remarkable defects in the STRIPAK mutants was attenuated virulence of *F. graminearum*. In *F. verticillioides*, Stp1 and Fsr1 have also been shown to be important for virulence (Shim et al., 2006; Zhang et al., 2018). In addition, the loss of striatin orthologue Str1 impairs viruelnce of *C. graminicola* in host plants (Wang et al., 2016). The mutant Δ*ppg1* fails to cause any disease lesions on rice leaves in *M. oryzae* (Du et al., 2013), suggesting a conserved function of the STRIPAK complex in fungal virulence. In *F. graminearum*, the STRIPAK mutants exhibited a severe reduction in virulence, which may result from defects in several regulatory mechanisms. First, the CWI pathway is important for *F. graminearum* virulence (Hou et al., 2002; Wang et al., 2011; Yun et al., 2014). Here, we found that Ham2, Ham3, Ppg1, and PP2Aa directly interacted with Mgv1 in the CWI pathway and the phosphorylation level of Mgv1 was significantly reduced in STRIPAK mutants, resulting in increased sensitivity to cell wall-perturbing agents; therefore, abnormal CWI signalling is among the reasons for the virulence defects displayed in the STRIPAK mutants. Secondly, the ROS produced by plant cells are known to participate in defence against fungal infections (Kachroo et al., 2003; Lehmann et al., 2015). We found that higher levels of ROS accumulated in the hyphae of STRIPAK mutants, resulting in increased sensitivity to oxidative stress, which may also impair *F. graminearum* virulence on the host plant. The deletion of the STRIPAK components impairs secondary metabolite production in *A. nidulans* (Elramli et al., 2019). In contrast, Cpp1 (Ppg1 homologue) is negatively associated with the production of the mycotoxin fumonisin B1 (FB1) in *F. verticillioides* and the Δ*cpp1* mutant produces higher levels of FB1 than the wild type (Choi & Shim, 2008), suggesting that the STRIPAK complex plays different roles in the regulation of secondary metabolism in different fungal species. Here, we found that the production of the critical virulence factor DON was significantly reduced in mutants, which largely prevented the fungus from spreading within wheat spikes. Furthermore, a previous study showed that *F. graminearum* colonization of wheat spikes is initiated by hyphal penetration (Pritsch et al., 2000) and that the MAPK module Ste11–Ste7–Gpmk1 is important for the formation of the penetration structure (Chen et al., 2019). In addition to Mgv1, another MAPK called Gpmk1 was identified as a potential interacting protein of Ham3 (Table S3), and we found that depletion of the STRIPAK complex resulted in a significant reduction in Gpmk1 phosphorylation (Figure S4). The mechanism is yet to be established, but it is likely that in addition to Mgv1, the STRIPAK complex can regulate the phosphorylation status of Gpmk1, and further modulate the penetration process, which may also partially contribute to the attenuated virulence in STRIPAK mutants. These results present new insights into the function of the STRIPAK complex in regulating fungal virulence.

Although accumulating evidence has shown that the STRIPAK complex is involved in multiple cellular processes, the factors that affect functions related to this complex are not yet fully understood. The key MAPK Mgv1 in the CWI pathway was identified as a target of the STRIPAK complex in this study. Furthermore, the association between the STRIPAK complex and the TOR downstream effector Tap42 was demonstrated. We previously showed that Ppg1 acts downstream of Tap42 (Yu et al., 2014). Here, the associations between Tap42 and other STRIPAK components were also demonstrated. In *S. cerevisiae*, rapamycin inhibits the function of TOR kinase (Heitman et al., 1991). Rapamycin abrogates the association between the Tap42–PP2A phosphatase complex and TOR complex 1. The Tap42–PP2A phosphatase complex is in turn activated and disassembled subsequently (Yan et al., 2006). We found that rapamycin can activate PP2A activity and stimulate sexual development, linking the STRIPAK complex with the TOR signalling pathway. Given the critical roles of the TOR signalling pathway in vegetative growth, DON production, sexual development, and virulence (Yu et al., 2014), which is similar to the STRIPAK complex, we propose that the STRIPAK complex associates with the TOR signalling pathway in *F. graminearum*. Taken together, an interconnected signalling network including the TOR signalling pathway, the STRIPAK complex, and the CWI pathway was found (Figure 8h), which will provide a better framework for understanding the true breadth of STRIPAK functions across diverse eukaryotic species.

4 | EXPERIMENTAL PROCEDURES

4.1 | Strains and culture conditions

The wild-type strain of *F. graminearum* PH-1 was used as the parental strain to generate different STRIPAK mutants in this study. The wheat-head medium and trichothecene biosynthesis induction (TBI) medium were used for the analysis of growth rate and DON production, respectively (Tang et al., 2018; Yin et al., 2018). Carrot medium (20% carrot and 2% agar) and carboxymethyl cellulose liquid medium were used for the analysis of perithecium formation and the conidiation assay, respectively (Chen et al., 2020). For stress response, the tested strains were grown on MM or ½ × potato dextrose agar supplemented with different stress agents and the growth inhibition rates were calculated after incubation at 25°C for 3 days.

4.2 | Y2H assay

The Y2H assay was performed as described previously (Chen et al., 2020). Briefly, STRIPAK cDNAs were cloned into pGADT7

(AD) containing GAL4 activation domain and pGBKT7 (BD) containing GAL4-binding domain to generate prey and bait constructs, respectively. After transformation into *S. cerevisiae* AH109, the growth of transformant was determined on synthetic (SD)-Trp-Leu-His-Ade medium. The interaction between BD-Lam and AD-T was used as a negative control, while the interaction between BD-53 and AD-T was served as a positive control.

4.3 | Co-IP and western blotting assays

Co-IP assay was performed as previously described (Chen et al., 2020). For western blotting assay, the fresh hyphae of the tested strain were collected and total protein was extracted using protein isolation buffer. Protein samples were separated by SDS-PAGE and transferred to nitrocellulose membranes. An anti-GFP (Abcam) antibody and anti-FLAG (Sigma) antibody were used to detect GFP- and FLAG-fused proteins, respectively. A phospho-p44/42 MAPK antibody (Cell Signalling Technology) was used to detect phosphorylated Gpmk1 and Mgv1 while the p44/42 MAPK antibody (Cell Signalling Technology) was used to detect total Gpmk1 and Mgv1. As a reference, the tested samples were detected with the anti-GAPDH antibody (HuaAn). Cytoplasmic/nuclear fractionation assay was performed as described previously (Wang et al., 2021). An anti-H3 antibody (Abcam) and an anti-GAPDH antibody were used to distinguish cytoplasmic and nuclear proteins, respectively. ImageJ software was used to quantify the intensity of the immunoblot bands. Three biological replicates were performed for all experiments.

4.4 | Generation of gene deletion mutants

The deletion of targeted genes was performed as described previously (Yu et al., 2004). Briefly, the knockout cassettes were generated using the double-joint PCR and transformed into the wild type (Hou et al., 2002; Yun et al., 2014). Positive transformants where the ORF of the targeted gene was replaced by the selection marker hygromycin phosphotransferase (*HPH*) were obtained after PCR screening with the primers listed in Table S4. The ORF of the target gene driven by its promoter, together with another selection marker, such as neomycin (*NEO*), were transformed into the corresponding mutants to generate the complementation strain.

4.5 | Pathogenicity and DON production assays

Virulence assays were carried out on wheat spikelets and maize silk as described previously (Chen et al., 2020). DON produced by tested strains after incubation in liquid TBI medium for 3 days were detected by using a DON detection kit (Wiseste Biotech Co. Ltd). To detect the mRNA levels of *TRI* genes, RT-qPCR was performed using ChamQ SYBR qPCR Master Mix (Vazyme). The primers used in RT-qPCR assay are listed in Table S4.

4.6 | Microscopic examinations

An LSM780 confocal microscope (Zeiss) was used to detect fluorescence signals and Zeiss ZEN 2010 software was used to process the images. Fresh conidia were stained with 0.1 mg/mL CFW (Sigma-Aldrich) for 30 s to visualize septa. To examine the effect of the STRIPAK complex on the expression of Tri1-GFP, the wild-type and STRIPAK mutants expressing Tri1-GFP were cultured in TBI medium for 48 h before examination.

4.7 | PP2A activity assay

The wild type was grown in complete medium supplemented with or without 0.025 μg/mL rapamycin (EC_{50}, a concentration that results in 50% mycelial growth inhibition toward *F. graminearum*). After 14 h of incubation, mycelia were harvested and subjected to PP2A activity detection. The phosphatase kit (Promega) was used to detect PP2A activity according to the manufacturer's procedure.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data supporting the findings of this study are available within the paper and its supplementary data.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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